



**PROCEEDINGS OF  
IV. INTERNATIONAL  
AGRICULTURAL, BIOLOGICAL  
& LIFE SCIENCE CONFERENCE  
AGBIOL 2022**

**29-31 AUGUST, 2022**

**EDIRNE, TURKEY**



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## WELCOME NOTES

You are welcome to our IV. AGBIOL Conference that is organized by Trakya University. The aim of our conference is to present scientific subjects of a broad interest to the scientific community, by providing an opportunity to present their work as oral or poster presentations that can be of great value for global science arena. Our goal was to bring three communities, namely science, research and private investment together in a friendly environment of Edirne, Turkey in order to share their interests and ideas and to get benefit from the interaction with each other.

In September 2018, we organized the first AGBIOL Conference with more than 700 scientists and researchers from all over the world with over 800 scientific papers. Due to COVID-19 situation, II. AGBIOL 2020 has organized fully on-line event which was one of the biggest online conferences in recent years in the world with 499 papers and 1133 authors with 333 oral and 166 e-poster presentations from 55 countries. Due to COVID-19 situation, AGBIOL 2021 was organized on-line again. There is a worldwide participation from 44 countries with 422 papers by contributing 1066 authors with 288 oral, 134 e-poster presentations.

The AGBIOL 2022 is organized with normal participation in controlled conditions as well as with online participation in Trakya University Balkan Congress Center in Edirne, Turkey on August 28-31, 2022. There is a worldwide participation from 44 countries with 522 papers by contributing over 1300 authors.

The participants with paid conference fee will be able to access all the normal and virtual presentation talks in each session, as well as to visit the virtual poster hall via preliminary provided participant ID and codes. The selected ABSTRACTs will be published in the Conference ABSTRACT and Proceedings Book. Participants might send us their full papers, which based on their preferences will be published either in our Conference ABSTRACT and Proceedings Book or in selected International Indexed Scientific Journals.

### **Conference Topics:**

Agriculture, Forestry, Life Sciences, Agricultural Engineering, Aquaculture and Biosystems, Animal Science, Biomedical science, Biochemistry and Molecular Biology, Biology, Bioengineering, Biomaterials, Biomechanics, Biophysics, Bioscience, Biotechnology, Botany, Chemistry, Chemical Engineering, Earth Sciences, Environmental Science, Food Science, Genetics and Human Genetics, Medical Science, Machinery, Pharmaceutical Sciences, Physics, Soil Science.

We would like to thank all of you for joining this conference and we would like to give also special thanks to our sponsors and collaborators for giving us a big support to organize this event.

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## CONTENTS

|   |     |
|---|-----|
| ORGANIZING COMMITTEE.....   | 4   |
| SCIENTIFIC COMMITTEE.....   | 5   |
| SOME DATA OF THE TRUE BUGS MIRIDAE IN THE DIFFERENT ECOSYSTEMS IN KRUJA REGION.....   | 12  |
| SOME DATA OF SEED BUGS ( <i>LYGAEIDAE</i> ) IN ECOSYSTEMS OF KRUJA (ALBANIA)..  | 18  |
| DETERMINATION OF BIOLOGICAL POTENTIAL OF <i>TILIA CORDATA</i> FLOWER EXTRACTS .....   | 22  |
| SEED GERMINATION TEST OF <i>Maerua siamensis</i> (Kurz) Pax .....   | 30  |
| <i>COLCHICUM TRIPHYLLUM</i> AND <i>HYACINTHELLA LEUCOPHAEA</i> SSP. <i>ATCHLEYI</i> , SHEBENIK NATIONAL PARK, ALBANIA .....                   | 39  |
| FLORISTIC COMPOSITION AND STRUCTURE OF THE ZOUAGHA FOREST (EASTERN ALGERIA) .....   | 46  |
| THE FLORISTIC DIVERSITY OF KRASTA PARK, ELBASAN, ALBANIA.....   | 56  |
| VINEYARDS WATER STRESS ESTIMATION IN SOUTHERN ROMANIA TO INTEGRATE IRRIGATION MANAGEMENT STRATEGIES IN THE CONTEXT OF CLIMATE CHANGE .....    | 64  |
| EVALUATION OF THE VIABILITY OF <i>QUERCUS CANARIENSIS</i> IN THE FOREST OF OULED BECHIH (SOUK AHRAS).....                                     | 79  |
| COMPARATIVE STUDY OF THE VEGETATIVE GROWTH OF SEEDLINGS OF <i>Q. SUBER</i> ET <i>Q. CANARIENSIS</i> (OULED BECHIH FOREST-SOUK AHRAS).....     | 85  |
| STRUCTURE DEMOGRAPHIQUE ET REPARTITION SPATIALE DE CHENE VERT DANS LA FORET DE CHETTABA.....  | 90  |
| THE EFFECT OF PERICARPS ON GERMINATION OF HOLM OAK ( <i>QUERCUS ILEX</i> )...   | 96  |
| ANTIMICROBIAL AND ANTIOXIDANT PROPERTIES OF POMEGRANATE ( <i>Punica granatum</i> L) PEEL BIOACTIVE COMPOUNDS ( <i>In-vitro</i> ) .....        | 101 |
| ANTIMICROBIAL AND ANTIOXIDANT PROPERTIES OF CHESTNUT ( <i>Castanea sativa</i> ) FLOWER EXTRACT AND ITS POTENCY AS POULTRY FEED ADDITIVES..... | 108 |
| DIVERSITY AND ENHANCEMENT OF NTFP FROM PLANT GROUPS IN THE FOREST OF TESSER MRAMET OF THE MOUNTAINS OF TLEMCEN (ALGERIA).....                 | 117 |
| INSIGHTS INTO PHYLOGENETIC UTILITY OF <i>RPD2</i> GENE: A CASE STUDY OF <i>SILENE</i> L. ( <i>CARYOPHYLLACEAE</i> ).....                      | 123 |
| EVALUATION OF POLLUTION STATUS OF STREAMS IN THRACE REGION .....  | 129 |
| FOREST, FORESTS FIRES AND ECOSYSTEM .....   | 142 |
| EXPLORING <i>STREPTOMYCES</i> BIOAGENTS FOR THE MANAGEMENT OF BACTERIAL FIRE BLIGHT UNDER IN VITRO AND IN VIVO CONDITIONS.....                | 153 |
| IN VITRO AND IN VIVO SCREENING OF <i>BEAUVERIA BASSIANA</i> STRAINS FOR ENDOPHYTIC AND INSECTICIDE ACTIVITY .....                             | 166 |
| STATUS OF PLANT GENETIC RESOURCES FOR FOOD AND AGRICULTURE IN BULGARIA: FROM GENOTYPE COLLECTING TO INFORMATION ACCESS .....                  | 178 |

|   |            |
|---|------------|
| <b>THE INDUSTRY 4.0 ASSESSMENTS OF VALUE-ADDED FOOD PRODUCTS DURING 2020-22 PERIOD .....</b>  | <b>188</b> |
| <b>ZINC AND VITAMIN C CAN PREVENT OXIDATIVE STRESS INDUCED BY LEAD NITRATE IN MOUSE LEYDIG CELLS.....</b>                                 | <b>191</b> |
| <b>HIGH HYDROSTATIC PRESSURE (HHP) PROCESSING STRATEGIES ON ANTIOXIDANT PHENOLIC BIOACTIVE COMPONENTS IN FOODS AND BEVERAGES .....</b>    | <b>209</b> |
| <b>INFLUENCE OF THE STALLION’S ORIGIN ON SPERM MOBILITY AND MORPHOLOGICAL ABNORMALITIES IN THAWED SEMEN.....</b>                          | <b>214</b> |
| <b>CIELAB COLOR PARAMETERS AND PIGMENT CONTENTS IN VIRGIN OLIVE OIL FROM DIFFERENT AREAS OF PRODUCTION IN ALBANIA.....</b>                | <b>217</b> |
| <b>CHARACTERIZATION OF RABBIT PRODUCTION SYSTEMS IN TUNISIA .....</b>   | <b>224</b> |
| <b>NETWORK FORMATION AND INTERFACE BEHAVIOR OF PU COATINGS WITH DUAL HYDROPHILIC/HYDROPHOBIC DANGLING CHAINS.....</b>                     | <b>228</b> |
| <b>RECENT DISSIPATIVE PARTICLE DYNAMICS PARAMETERIZATION MIMICS EXPERIMENTAL STRUCTURE AND PROPERTIES OF WATER AND ALCOHOLS.....</b>      | <b>232</b> |
| <b>BORON AND SELENIUM ACCUMULATIONS IN DRINKING WATER OF IPSALA DISTRICT (EDIRNE, TÜRKIYE).....</b>                                       | <b>239</b> |
| <b>PHENO-MORPHOLOGICAL STUDY BETWEEN FIVE VARIETIES OF QUINOA (<i>CHENOPODIUM QUINOA</i> WILLD.) IN SEMI ARID REGION OF ALGERIA .....</b> | <b>247</b> |
| <b>EFFECTS OF VITAMIN C AND ZINC ON FURAN INDUCED TOXICITY IN TM4 SERTOLI CELLS.....</b>  | <b>251</b> |
| <b>FUNCTIONAL FOOD - PRODUCTS FROM TOMATO WITH LYCOPENE IN PREVENTION OF CARDIOVASCULAR DISEASES .....</b>                                | <b>262</b> |
| <b>HEALTH RISK ASSESSMENT OF NITRATE IN DRINKING WATER OF IPSALA DISTRICT (THRACE REGION OF TÜRKİYE).....</b>                             | <b>268</b> |
| <b>MONITORING, INTRODUCTION, AND USEFUL TRAITS OF <i>AGRIOPHYLLUM SQUARROSUM</i> L.....</b>   | <b>275</b> |
| <b>THE EFFECT OF DIFFERENT WHEAT CULTIVAR AND TURMERIC/GINGER POWDER USAGE ON PHYSICAL AND SENSORY PROPERTIES OF BULGUR.....</b>          | <b>287</b> |
| <b>EFFECT OF PERICARP ON THE GERMINATION OF GREEN OAK ACORNS (<i>Quercus ilex</i>).....</b>   | <b>293</b> |
| <b>DEMOGRAPHIC STRUCTURE AND SPATIAL DISTRIBUTION OF HOLM OAK IN THE CHETTABA FOREST.....</b>   | <b>298</b> |
| <b>PRELIMINARY STUDY ON SOURCES AND CAUSES OF MILK LOSSES IN DAIRY FARMS IN NORTH-EAST OF TUNISIA (BIZERTE) .....</b>                     | <b>305</b> |
| <b>DETERMINATION OF DYE REMOVE CAPABILITIES OF BACTERIAL ISOLATES .....</b>   | <b>311</b> |
| <b>COMPARATIVE ANATOMICAL INVESTIGATIONS ON FOUR <i>VIOLA</i> L. (<i>VIOLACEAE</i>) TAXA FROM EUROPEAN TURKEY .....</b>                   | <b>321</b> |
| <b>BACTERIAL EXOTOXINS IN FOOD AS CAUSES OF ALIMENTARY INTOXICATIONS OF HUMANS AND UTILIZATION OF EXOTOXINS.....</b>                      | <b>337</b> |
| <b>PROBABLE HEALTH RISK ASSESSMENT OF ESSENTIAL ELEMENTS IN WATER OF NATURAL – DAM LAKES OF THRACE REGION (TÜRKIYE) .....</b>             | <b>359</b> |

|   |            |
|---|------------|
| <b>ASSOCIATION OF DIET WITH THE OCCURRENCE OF TYPE 2 DIABETES, POLYCYSTIC OVARY SYNDROME, VAGINAL TRACT DYSBIOSIS AND NEOPLASTIC DISEASES IN WOMEN AND CARNIVORES .....</b> | <b>366</b> |
| <b>EFFECTIVENESS OF <i>ALOE VERA</i> LEAF EXTRACT AS A BIO-COAGULANT FOR THE TREATMENT OF INDUSTRIAL EFFLUENT BY COAGULATION-FLOCCULATION ...</b>                           | <b>385</b> |
| <b>QUALITY CHANGES IN DIFFERENT PARTS OF FRUITS OF SOME MORPHOLOGICALLY DIFFERENT WATERMELON HYBRIDS .....</b>  | <b>391</b> |
| <b>VIRTUAL SCREENING FOR IDENTIFICATION OF HUMAN BLEOMYCIN HYDROLASE INHIBITORS.....</b>  | <b>400</b> |
| <b>ANTIMICROBIAL WOUND DRESSING MATERIALS.....</b>  | <b>412</b> |
| <b>BIOCHEMICAL CHARACTERISTICS OF WINES FROM AUTOCHTHONOUS GRAPE CULTIVARS IN VARIOUS AREAS OF ALBANIA .....</b>  | <b>431</b> |
| <b>PRELIMINARY SURVEY FOR MAPPING THE DISTRIBUTION OF LOCAL VARIETIES BY USING THE NATIONAL REGISTER OF PLANT GENETIC RESOURCES IN BULGARIA .....</b>                       | <b>442</b> |
| <b>THE INFLUENCE OF CLIMATIC CONDITIONS ON THE CHEMICAL AND BIOACTIVE COMPONENTS OF SOME APPLE VARIETIES CULTIVATED IN THE AREA OF KORÇA .....</b>                          | <b>449</b> |
| <b>EFFECTS OF SOME CHEMICAL TREATMENTS ON POLLEN GERMINATION AND POLLEN TUBE GROWTH IN ‘RED LAKE’ and ‘ROSENTHALL’ CURRANT CULTIVAR (<i>Ribes Rubrum</i>) .....</b>         | <b>459</b> |
| <b>EVALUATION OF CELLULASE ACTIVITY OF <i>bcsE</i> MUTANT <i>SALMONELLA</i> STRAINS .....</b>   | <b>464</b> |
| <b>CRISPR/CAS9 GENOME EDITING TECHNIQUE FOR ABIOTIC STRESS RESPONSES IN HORTICULTURE .....</b>  | <b>471</b> |
| <b>DETERMINATION OF CORRELATION VARIATION OF MORPHOLOGICAL AND SOME MOLECULAR MARKER TECHNIQUES IN WATERMELON .....</b>   | <b>479</b> |
| <b>MORPHOLOGICAL AND BIOCHEMICAL RESPONSES OF GRAND NAIN AND DWARF CAVENDISH (<i>Musa spp.</i>) BANANA SEEDLINGS TO DROUGHT STRESS SIMULATED BY PEG-6000 .....</b>          | <b>483</b> |
| <b>FACILE SYNTHESIS OF SnO<sub>2</sub> NANOPARTICLES FOR PHOTOCATALYTIC DECOLORIZATION OF AZO DYE SOLUTION.....</b>   | <b>491</b> |
| <b>EVALUATION OF DEVELOPMENTAL TOXICITY OF EVERZOL BLUE EDG ON ZEBRAFISH (<i>DANIO RERIO</i>) EMBRYOS.....</b>  | <b>498</b> |
| <b>EFFECTS OF HAWTHORN (<i>Crataegus spp</i>) ROOTSTOCK ON VEGETATIVE GROWTH IN LOQUAT (<i>Eriobotrya japonica</i> Lindl).....</b>  | <b>503</b> |
| <b>EXTENDING SHELF LIFE OF MINI BREADS BY DEVELOPMENT AND APPLICATION OF EDIBLE CELLULOSE-BASED FILMS.....</b>  | <b>507</b> |
| <b>PRELIMINARY RESULTS OF INFLUENCES OF SOME QUINCE ROOTSTOCKS ON YIELD AND FRUIT QUALITY IN LOQUAT.....</b>  | <b>515</b> |
| <b>THE EFFECT OF EXTERNALLY APPLIED SALICYLIC ACID ON SOME PHYSIOLOGICAL PROPERTIES OF LETTUCE (<i>Lactuca sativa</i> L.) UNDER DROUGHT STRESS CONDITIONS .....</b>         | <b>521</b> |



|  |     |
|--|-----|
| <b>THE CHARACTERIZATION AND BIOTECHNOLOGICAL APPLICATION OF THERMOPHILIC ALPHA-L-ARABINOFURANOSIDASE FROM <i>GEOBACILLUS</i> SPECIES</b> .....   | 532 |
| <b>THE IMPACT OF DILL LEAVES ON THE SENSORY QUALITY OF TUNA PATE</b> .....   | 539 |
| <b>FROM INVASION TO CONSUMER TABLE: AS A MODEL: LIONFISH</b> .....   | 544 |
| <b>FUNCTIONS OF TYPE V SECRETION SYSTEM AND AUTOTRANSPORTER PROTEINS IN <i>SALMONELLA</i></b> .....  | 547 |
| <b>ARTISANAL FRESH CHEESE QUALITY IMPROVEMENT USING DIFFERENT HURDLE TECHNOLOGIES COMBINATION</b> .....  | 554 |
| <b>EFFECT OF LACTOPEROXIDASE SYSTEM ACTIVATION AND HEAT TREATMENTS OF REFRIGERATED COW'S MILK ON THE QUALITY OF UNCOOKED PRESSED CHEESE SAINT-PAULIN TYPE DURING RIPENING</b> .....                                | 562 |
| <b>RELATIONSHIP BETWEEN SRNA AND BIOFILM FORMATION</b> .....   | 569 |
| <b>VARIATION OF BIOAEROSOL CONCENTRATIONS DURING THE WILDFIRES BROKE OUT IN 2021 IN TURKEY</b> .....   | 576 |
| <b>THE ROLE OF ENZYMES IN THE IMPROVEMENT OF DOUGH PROPERTIES PREPARED WITH WHEAT FLOUR</b> .....  | 583 |
| <b><i>pagN</i>: A NOVEL ADHESIN/INVASIN GENE IN <i>SALMONELLA</i></b> .....  | 595 |
| <b>INVESTIGATION OF THE EFFECT OF CAPSAICIN WITH SODIUM SELENITE ON MDA-MB-231 CELL LINE</b> .....   | 601 |
| <b>EFFECTS OF <i>csgD</i>, <i>fliZ</i>, <i>stjC</i>, <i>yaiC</i> AND <i>rmbA</i> GENES ON BIOFILM STRUCTURES FORMED AT SOLID-AIR AND LIQUID-AIR INTERFACES IN <i>SALMONELLA TYPHIMURIUM</i> 14028 STRAIN</b> ..... | 608 |
| <b>CLIMATE CHANGE AND FOOD SECURITY</b> .....  | 619 |
| <b>HURDLE EFFECT APPROACH IN DRYING OF DAIRY PRODUCTS</b> .....  | 624 |
| <b>PROPERTIES OF FOOD POWDERS, PRODUCTION METHODS AND CHARACTERIZATION</b> .....   | 632 |
| <b>EVALUATION OF ULTRAVIOLET (UV) ABSORPTION ABILITY OF MENENĞİÇ AND OLIVE OILS FROM TURKEY</b> .....  | 642 |
| <b>INVESTIGATION OF OLIVE AS AN ALTERNATIVE TO SYNTHETIC FOOD PRESERVATIVES</b> .....  | 648 |
| <b>DETERMINATION OF USEGA POTENTIAL OF RED PITAHAYA EXTRACTS AGAINST <i>Escherichia coli</i> AND <i>Listeria monocytogenes</i> FOR THE FOOD INDUSTRY</b> .....   | 653 |
| <b>WHITE PITAHAYA IN AQUACULTURE: INVESTIGATION OF THE POTENTIAL USAGE OF FRUIT AND PEEL</b> .....   | 659 |
| <b>EFFECTS OF FERMENTED OLIVE LEAF AND ITS WASTEWATER ON PERFORMANCE PARAMETERS OF BROILER CHICKENS</b> .....  | 665 |
| <b>DETERMINATION OF CHANGES IN SOME NUTRIENT CONTENT OF <i>Agaricus bisporus</i> CAP PART FERMENTED WITH RUMEN LIQUID IN DIFFERENT ENVIRONMENTAL CONDITIONS</b> .....  | 669 |
| <b>SUN PROTECTION FACTORS OF COMMERCIAL OILS</b> .....   | 673 |
| <b>DETERMINATION OF THE POTENTIAL FOR USE IN THE COSMETIC INDUSTRY OF <i>Ziziphus Jujuba</i> BRANCH WATER EXTRACTS</b> .....   | 679 |

|  |            |
|--|------------|
| <b>YIELD AND SOME PROPERTIES OF SILAGE CORN AS INFLUENCED BY DIFFERENT NITROGEN AND VERMICOMPOST RATES .....</b>   | <b>685</b> |
| <b>HEAVY METALS IN FISH EGGS OF RED MULLET AND EUROPEAN HAKE .....</b>   | <b>692</b> |
| <b>DETERMINATION OF FORAGE YIELD, QUALITY AND SOME CHARACTERISTICS OF VEGETATION IN GÜNALAN VILLAGE PASTURE IN GÖLBAŞI DISTRICT OF ANKARA PROVINCE.....</b>                          | <b>697</b> |
| <b>EFFECTS OF VARIOUS CLIPPING FREQUENCY AND HEIGHTS ON THE HERBAGE YIELD AND ROOT DEVELOPMENT OF TUBEROUS CANARY GRASS (<i>PHALARIS TUBEROSA</i> L.).....</b>                       | <b>707</b> |
| <b>EFFECTS OF DIFFERENT TPMS LATTICE STRUCTURES ON THE MECHANICAL BEHAVIOUR OF HIP IMPLANT .....</b>   | <b>714</b> |
| <b>EFFECT OF TREATMENT WITH HERBICIDES IN SUNFLOWER HYBRIDS ON WEED INFESTATION .....</b>  | <b>718</b> |
| <b>PALYNOLOGICAL, ANATOMICAL AND MICROMORPHOLOGICAL FEATURES OF ENDEMIC <i>COUSINIA WORONOWII</i> .....</b>  | <b>723</b> |
| <b>DEVELOPING ORGANIC AGRICULTURE PRODUCTION IN TUYEN QUANG PROVINCE, VIET NAM .....</b>   | <b>732</b> |
| <b>EFFECTS OF DIFFERENT DRIP IRRIGATION REGIMES ON POTATO TUBER MINERAL CONTENTS.....</b>  | <b>741</b> |
| <b>EFFECT OF ARTICHOKE BRACT POWDER ON BREAD QUALITY .....</b>   | <b>746</b> |
| <b>THE USES OF THE ELECTROPOLYMERIZED FILMS AS IMMOBILIZATION MEDIUM FOR CHOLESTROL BIOSENSOR DESIGN .....</b>   | <b>755</b> |
| <b><i>Bacillus subtilis</i> IMPROVES THE NUTRITIONAL VALUE OF GRAPE SEEDS THROUGH SOLID-STATE FERMENTATION .....</b>   | <b>764</b> |
| <b>EFFECT OF LIQUID STATE FERMENTATION USING <i>Lactobacillus</i> spp. ON THE NUTRITIONAL COMPOSITION OF THE STALK PARTS OF <i>Agaricus bisporus</i> IN DIFFERENT PH LEVELS.....</b> | <b>767</b> |
| <b>THE PLACE OF FOOD INSPECTIONS IN THE LEGISLATION AND THE EXAMINATION OF FOOD INSPECTION ACTIVITIES PERFORMED IN KASTAMONU PROVINCE.....</b>                                       | <b>771</b> |
| <b>FUNCTIONAL FOOD - PRODUCTS FROM TOMATO WITH LYCOPENE IN PREVENTION OF CARDIOVASCULAR DISEASES .....</b>   | <b>787</b> |
| <b>PRELIMINARY REVIEW AND EVALUATION PROCESS OF ENVIRONMENTAL IMPACT ASSESSMENT REGULATION .....</b>   | <b>794</b> |
| <b>POSSIBILITIES OF USING WIND TURBINE ENERGY SYSTEM IN VERMICOMPOST FERTILIZER PRODUCTION FACILITIES .....</b>  | <b>798</b> |
| <b>THE METALLOTHIONEIN AND GLUTATHIONE LEVELS OF MEDITERRANEAN MUSSEL (<i>Mytilus galloprovincialis</i>) IN THE ÇANAKKALE STRAIT .....</b>   | <b>806</b> |
| <b>CRISPR/CAS TECHNOLOGY FOR WHEAT BREEDING .....</b>  | <b>812</b> |
| <b>METHODS OF MECHANOCHEMICAL ACTIVATION OF INDUSTRIAL CATALYSTS OF CARBON OXIDES HYDROGENATION.....</b>   | <b>818</b> |
| <b>THE REFLECTANCE INDEX AS INDICATORS OF DROUGHT TOLERANCE OF SOME DURUM WHEAT (<i>TRITICUM DURUM</i> DESF.) GENOTYPES GROWING UNDER SEMI-ARID CONDITIONS -SETIF- ALGERIA .....</b> | <b>833</b> |

|  |             |
|--|-------------|
| <b>BIOACTIVE AND ANTIOXIDANTS COMPOUNDS: TRENDS AND CHALLENGES FOR FOOD PRESERVATION.....</b>  | <b>843</b>  |
| <b>INHERITANCE OF LINT PERCENTAGE IN F1 DIALLEL COTTON CROSSES (GOSSYPIUM HIRSUTUM L.) .....</b>   | <b>849</b>  |
| <b>DIRECTIONS AND ACHIEVEMENTS OF COTTON BREEDING IN BULGARIA .....</b>  | <b>865</b>  |
| <b>INFLUENCE OF OSMOTIC STRESS ON THE GROWTH OF YOUNG COTTON SEEDLINGS .....</b>   | <b>883</b>  |
| <b>COMPARATIVE TESTING OF FORAGE PEAS VARIETY "MIR" IN THE CONDITIONS OF ORGANIC AND CONVENTIONAL AGRICULTURE.....</b>   | <b>889</b>  |
| <b>HARMFUL ENTOMOFAUNA BY CHICKPEAS-CICER ARIETINUM L. (FABALES, FABACEAE).....</b>  | <b>893</b>  |
| <b>PRODUCTION OF BIODIESEL FROM WASTE VEGETABLE OILS .....</b>   | <b>902</b>  |
| <b>CURRENT APPROACHES TO BIOREMEDIATION OF LANDFILL LEACHATE.....</b>  | <b>909</b>  |
| <b>THE FACTORS THAT EFFECTS MICROORGANISMS IN THE RECOVERY OF RARE EARTH ELEMENTS.....</b>   | <b>916</b>  |
| <b>DETECTION AND GENETIC INVESTIGATION OF POTATO LEAFROLL VIRUS IN KAZAKHSTAN.....</b>   | <b>923</b>  |
| <b>EVALUATION OF RASPBERRY VIRUSES' SPREADING IN KAZAKHSTAN .....</b>  | <b>927</b>  |
| <b>DETERMINATION OF LAND USE AND LAND COVER CHANGES USING REMOTE SENSING: GEBZE-IZMIR HIGHWAY KARACABEY ROUTE .....</b>  | <b>931</b>  |
| <b>MONITORING OF URBAN AREA AND AGRICULTURAL LAND CHANGES AFTER GOLCUK EARTHQUAKE (17 AUGUST, 1999) .....</b>  | <b>940</b>  |
| <b>VISUAL BASIC STUDY BASED ON AUTOMATION PROGRAM FOR THE MAPPING OF THE SPATIAL DISTRIBUTION OF SOIL PARAMETERS USING GIS AND KRIGING INTERPOLATION METHOD OF THE SURFER SOFTWARE .....</b> | <b>947</b>  |
| <b>CONTRIBUTION TO THE DIACHRONIC STUDY OF THE SPATIOTEMPORAL EVOLUTION OF THE VEGETATION COVER OF BELEZMA NATIONAL PARK (BATNA, ALGERIA) .....</b>  | <b>957</b>  |
| <b>RECOVERY OF RARE EARTH ELEMENTS FROM INDUSTRIAL WASTE .....</b>   | <b>965</b>  |
| <b>FOOD WASTAGE IN A TUNISIAN UNIVERSITY CANTEEN: IMPACT OF AN EDUCATION AND AWARENESS CAMPAIGN ON STUDENTS' ATTITUDES AND BEHAVIORS .....</b>   | <b>972</b>  |
| <b>MICROALGAE CULTURE FOR FOOD SUPPLEMENTATION: AN INSIGHT INTO LIPID FRACTION.....</b>  | <b>979</b>  |
| <b>INVESTIGATION OF FIN DESIGNS FOR ENHANCING THERMAL EFFICIENCY OF PV .....</b>   | <b>987</b>  |
| <b>THE EFFECT OF FRONT WING MODELS ON AERODYNAMICS OF F1 CARS .....</b>  | <b>994</b>  |
| <b>EFFECT OF TOBACCO WASTE ON YIELD AND MINERAL NUTRITION OF BARLEY .....</b>  | <b>1001</b> |
| <b>EFFECT OF CULTIVATION ON SPATIAL VARIABILITY OF SOIL ORGANIC CARBON CONTENT IN A SMALL SCALE FIELD .....</b>  | <b>1007</b> |
| <b>THE IMPORTANCE OF MOLECULAR METHODS USED IN DETERMINING HERITAGE DISEASES IN CATTLE.....</b>  | <b>1014</b> |

|  |             |
|--|-------------|
| <b>COMPARISON OF TWO APPS BUILT WITH UNITY AND VUFORIA .....</b>   | <b>1028</b> |
| <b>STUDY ON METHANE COMBUSTION UNDER OXYGEN DILUTED CASES IN A TUBE BURNER.....</b>  | <b>1037</b> |
| <b>PRODUCTION OF STARCH-BASED BIOPLASTIC AND ITS USE IN 3D PRINTERS .....</b>  | <b>1043</b> |
| <b>EFFECT OF WELDING CHAMBER GEOMETRY ON THE PRODUCT QUALITY FOR HOLLOW ALUMINUM PROFILES .....</b>  | <b>1053</b> |
| <b>MANUFACTURING STRATEGIES ON FOOD SUPPLEMENT EFFERVESCENTS AND BIOACTIVE COMPONENT RETENTION.....</b>                                    | <b>1062</b> |
| <b>IMPACT OF COVID-19 PANDEMIC ON MENSTRUAL CYCLE IN YOUNG WOMEN IN EDİRNE PROVINCE, TURKEY .....</b>                                      | <b>1066</b> |
| <b>GENDER INEQUALITY DURING THE COVID-19 PANDEMIC; FROM THE WINDOW OF UNIVERSITY STUDENTS.....</b>   | <b>1070</b> |
| <b>VAGINISMUS - IS IT POSSIBLE TO SCREEN YOUNG WOMEN? .....</b>  | <b>1075</b> |
| <b>COMPARATIVE RESISTANCE OF STORED CEREALS AND PULSE TO <i>Sitophilus zeamais</i> MOTSCHULSKY (<i>COLEOPTERA: CURCULIONIDAE</i>).....</b> | <b>1080</b> |
| <b>WEED MANAGEMENT PRACTICES OF THE BAYINDIR DISTRICT OUTDOOR ORNAMENTAL PLANTS' PRODUCERS.....</b>  | <b>1088</b> |
| <b>SIMULATION STUDY OF LECITHIN BASED NANOLIPOSOME ENCAPSULATING COENZYME Q10 .....</b>  | <b>1098</b> |
| <b>DISSIPATIVE PARTICLE DYNAMICS SIMULATIONS OF MRNA DELIVERY WITH IONIZABLE CATIONIC LIPID DOTAP.....</b>                                 | <b>1102</b> |
| <b>SEXUAL AND REPRODUCTIVE HEALTH EDUCATIONAL NEEDS AND RELATED FACTORS AMONG UNIVERSITY STUDENTS IN EDİRNE, EASTERN THRACE .....</b>      | <b>1109</b> |
| <b>RELATIONSHIP BETWEEN STRESS LEVEL AND VAGINITIS SYMPTOMS; A STUDY WITH TRAKYA UNIVERSITY HEALTH SCIENCES UNDERGRADUATE STUDENTS</b>     | <b>1114</b> |
| <b>EVALUATION OF SUBTERRANEAN CLOVER FROM A POINT OF VIEW POSSIBILITY TO USE IN BULGARIAN ENVIRONMENTAL CONDITIONS.....</b>                | <b>1118</b> |
| <b>EXPERIMENTAL INVESTIGATION OF OUTPUT POWER USING PHASE CHANGE MATERIAL (RT-44HC) IN POLYCRYSTALLINE SOLAR PANELS.....</b>               | <b>1132</b> |
| <b>PHENOLOGICAL DEVELOPMENT AND GRAIN YIELD PRODUCTIVITY OF WINTER PEA GENOTYPES IN TWO LOCATIONS .....</b>                                | <b>1142</b> |
| <b>AQUATIC FLORA OF VJOSA RIVER.....</b>   | <b>1151</b> |
| <b>PARTICIPANT LIST .....</b>  | <b>1156</b> |

## SOME DATA OF THE TRUE BUGS MIRIDAE IN THE DIFFERENT ECOSYSTEMS IN KRUJA REGION

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### ABSTRACT

This study aims to present a systematic and ecological analysis to the family *Miridae* the true bugs, in the different ecosystems of Kruja region, Albania. The collection of biological material is performed during the period 2019- 2021. The study represents 19 genus and 26 species. By analyzing the collected material, the genera *Deraeocoris* is the most represented with 4 species and a frequency of 15.38%. Habitats of Mali Kruja station are represented by more species than the other stations, with 14 species and a frequency of 53.85%, with less species Boje station with 5 species and a frequency of 19.23%. Based on the “*Jaccard index of similarity coefficient*”, Mali Kruje with Boje, have a higher similarity coefficient than the other stations, of 18.75%, with the lowest coefficient Droja and Boje with 7.69%, showing a similarity of the ecological factors between these stations, which means a similarity between these habitats.

**Key words:** *Hemiptera*, *Miridae*, ekosystems, Kruja

### INTRODUCTION

The family of *Miridae* Hahn, 1831 (plant bugs), presents a considerable number of species on Hemiptera, approx. 9800 species (Schuh, 1995), but this number can reach up to 20000 (Henry *et al*, 198). Members of this family possess a number of consistent characteristics. The body length ranges from 2–11 mm. Distinguishing features include an oval, extended, ellipsoidal body, which is often colourful, from dark to bright vermilion (Mc Gavin, 2000). One useful feature in identifying species of the family is the presence of a cuneus; it is the triangular tip of the corium, the firm, horny part of the forewing, the hemelytron. The cuneus is visible in nearly all *Miridae*, and only in a few other *Hemiptera*. The tarsi almost always have three segments (Richards, 1977). These insects can cause substantial damage to agricultural crops (Silvestri, 1939; Servadei, 1972). The family is dominated by phytophagous species, though some predators also are present (Carvalho *et al*, 1968; Tremblay, 1981; 1990). Due to some of their features they have been described as integrated biological weapons (Miller, 1971; Gennaro, 1977; Tremblay, 1990; Pollini, 2002).

In this study we present some data for family *Miridae* collected in coastal habitats in Kruja region.

### MATERIAL AND METHODS

Biological material was collected from field trips undertaken between 2017 and 2019 in different habitats near Droja, Brret, Mali Kruje, Boje stations. In the present investigation, entomological mowing nets of diameter 80cm, aspirators and Pitt's traps were employed. Mowing with entomological nets was undertaken using diagonals across surfaces of 100m<sup>2</sup> (10m x 10m), passing five times across each square's diagonal (Colas, 1969). Once collected, the individuals were placed in plastic bottles and labelled with the date and name of station.

The fine biological materials are placed in plastic flacons 150-200 cc. they were sent to the scientific laboratory and preserved in bottles of ethanol solution 95%, acetic acid, distilled water in v:v:v (80:5:20 ml) and some ether drops (Colas, 1969; Chapman, 1985). Taxonomic determination is done with stereomicroscope Trinocular Stereo Microscope (*with still camera model 50240003 n/s C88794*) in the MSN lab employing standard classification keys for this family (Drake, 1965; Servadei, 1967; Dolling, 1991; Aukema et al., 1999; Misja, 1973; Halimi et al, 2018). *Jaccard similarity* coefficient (Jaccard, 1901) was used to assess the species similarity at the different stations. In the present investigation, efforts were made to record the characteristics of the different sites and thus to assess any impact the different habitats have on the distribution of the species.

### RESULT AND DISCUSSIONS

In this study are determined species of the *Miridae* family, by listing them in the table according to the encountered species in the ecosystem in Kruja region, accompanied by the number of individuals and the sites for every station where they encountered Droja, Brret, Mali Kruje, Boje. (Anex).

The scientific determination of the collected materials in this study presents individuals of the *Miridae* family to the ecosystem in Kruja region, represented by 19 genera and 26 species (Table 1, Figure 1).

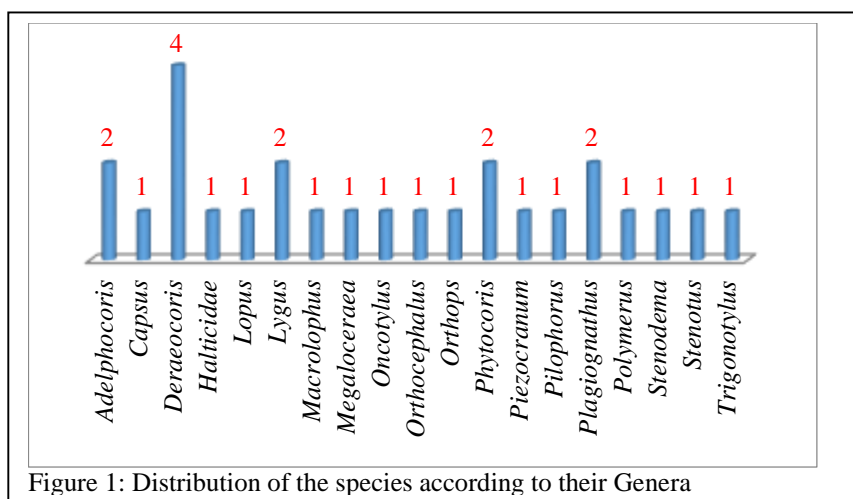


Figure 1: Distribution of the species according to their Genera

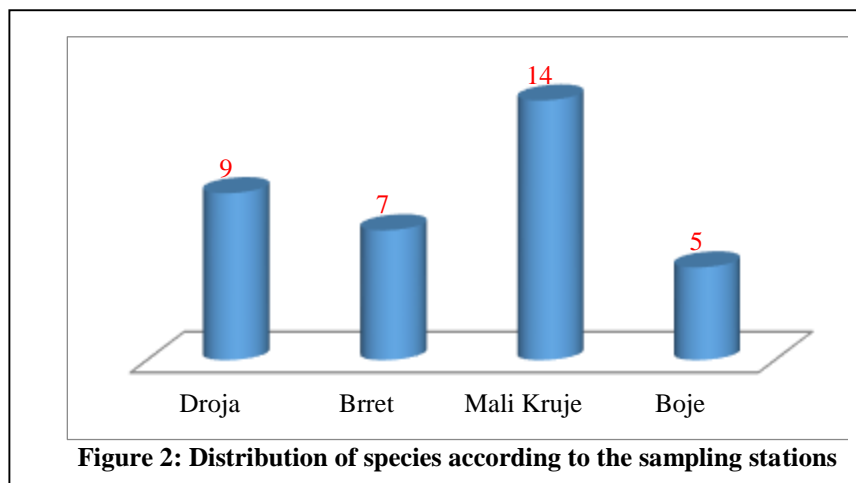


Figure 2: Distribution of species according to the sampling stations

**Table 1: Number of species according to their genera**

| Nr | Scientific name      | Number of species | Species frequency (%) |
|----|----------------------|-------------------|-----------------------|
| 1  | <i>Adelphocoris</i>  | 2                 | 7.69%                 |
| 2  | <i>Capsus</i>        | 1                 | 3.85%                 |
| 3  | <i>Deraeocoris</i>   | 4                 | 15.38%                |
| 4  | Halticidae           | 1                 | 3.85%                 |
| 5  | <i>Lopus</i>         | 1                 | 3.85%                 |
| 6  | <i>Lygus</i>         | 2                 | 7.69%                 |
| 7  | <i>Macrolophus</i>   | 1                 | 3.85%                 |
| 8  | <i>Megaloceraea</i>  | 1                 | 3.85%                 |
| 9  | <i>Oncotylus</i>     | 1                 | 3.85%                 |
| 10 | <i>Orthocephalus</i> | 1                 | 3.85%                 |
| 11 | <i>Orthops</i>       | 1                 | 3.85%                 |
| 12 | <i>Phytocoris</i>    | 2                 | 7.69%                 |
| 13 | <i>Piezocranum</i>   | 1                 | 3.85%                 |
| 14 | <i>Pilophorus</i>    | 1                 | 3.85%                 |
| 15 | <i>Plagiognathus</i> | 2                 | 7.69%                 |
| 16 | <i>Polymerus</i>     | 1                 | 3.85%                 |
| 17 | <i>Stenodema</i>     | 1                 | 3.85%                 |
| 18 | <i>Stenotus</i>      | 1                 | 3.85%                 |
| 19 | <i>Trigonotylus</i>  | 1                 | 3.85%                 |

Analysis of the data give evidences that based on the diversity, the genera *Deraeocoris* are represented by higher number of species, 4 species or by 15.38 % of the overall species; the Genera *Adelphocoris*, *Lygus*, *Phytocoris* and *Plagiognathus*, are represented by 2 species or 7.69%; the Genera *Capsus*, *Halticidae*, *Lopus*, *Macrolophus*, *Megaloceraea*, *Oncotylus*, *Orthocephalus*, *Orthops*, *Piezocranum*, *Pilophorus*, *Polymerus*, *Stenodema*, *Stenotus*, and *Trigonotylus* by 1 specie or 3.85%.

Analysis of the diversity of habitats where this study is conducted, base on the number of the species for each station, the station of Mali Kruje is presented by 14 species or 53.85%, followed by Droj with 9 species or 34.62%, Brret station by 7 species or 26.92%, and finally stations of Vora by 5 species or 19.23% (Table 2, Figure: 2).

**Table 1: Number of species according to sampling stations**

| Station    | Species number | Species frequency |
|------------|----------------|-------------------|
| Droja      | 9              | 34.62%            |
| Brret      | 7              | 26.92%            |
| Mali Kruje | 14             | 53.85%            |
| Boje       | 5              | 19.23%            |

Calculation of the coefficient of similarity Jaccard gave an indication on the species similarity among the stations (Jaccard, 1901). In the table are presented the numbers of common species C, according to the stations, and the coefficient of similarity  $C_j$  for each station (Table 3). The stations represent according to their geographical spreading, a high level of diversity in ecological conditions and habitats. Based on that situation, another effort is addressed to the constructing of the correlation among the species to put in light the influence of these conditions in the spreading of the species, by keeping in mind that even the species own their ecological valence.

**Table 2:** Number of species and similarity coefficient according to the sampling stations

|            | Brret | Mali Kruje                       | Boje                             |
|------------|-------|----------------------------------|----------------------------------|
| Droja      | C = 0 | C = 3<br>C <sub>J</sub> = 15%    | C = 1<br>C <sub>J</sub> = 7.69%  |
| Brret      |       | C = 2<br>C <sub>J</sub> = 10.52% | C = 0                            |
| Mali Kruje |       |                                  | C = 3<br>C <sub>J</sub> = 18.75% |

Analysis of the data give indication that the highest coefficient of species similarity belongs to the Mali Kruja stations and Boje by 18.75%, followed by similarity among the Mali Kruja and Droja by 15%, between Mali Kruja and Brret by 10.52%, finally between Droja and Boje by 7.69%. Analysis of the similarity based on the species distribution; give indication on the affinity of species distribution between the studied stations, and impact of the ecological factors in overall, but specially the anthropogenic factor impact.

## CONCLUSIONS

This study presents that analysis of exemplars for the *Miridae* Family, in the habitats of Kruja region, distributed in 19 genera and 26 species.

It was concluded that a higher diversity belongs to the *Deraeocoris* genera by 4 species, or 15.38 % of the overall species encountered.

The Mali Kruje station dominates related to the diversity of species by 14 species, or 53.85%, while the Boje station was presented by the smallest number of species, only 5, or 19.23%. The maximum value of the species similarity belongs to the Mali Kruje and Boje by 18.75%, while the minimum value of the similarity belongs to the Droja and Boje by 7.69%.

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**Anex: List of *Miridae* specie in Kruja**

| No | Scientific name                                      | Droja | Birret | Mali Kruje | Boje |
|----|--|-------|--------|------------|------|
| 1  | Genus <b>Adelphocoris</b>                            |       |        |            |      |
| 1  | <i>Adelphocoris lineolatus</i> Goeze, 1778           | +     |        | +          |      |
| 2  | <i>Adelphocoris vandalicus</i> Rossi, 1790           | +     |        | +          |      |
| 2  | Genus <b>Capsus</b>                                  |       |        |            |      |
| 3  | <i>Capsus wagneri</i> Remane, 1950                   | +     |        |            |      |
| 3  | Genus <b>Deraeocoris</b>                             |       |        |            |      |
| 4  | <i>Deraeocoris lutescens</i> Schilling, 1837         |       |        |            | +    |
| 5  | <i>Deraeocoris schach</i> Fabricius, 1781.           |       | +      | +          |      |
| 6  | <i>Deraeocoris serenus</i> Douglas & Scott, 1868     |       | +      |            |      |
| 7  | <i>Deraeocoris rutilus</i> Herrich-Schäffer, 1839    |       |        | +          | +    |
| 4  | Genus <b>Halticidae</b>                              |       |        |            |      |
| 8  | <i>Halticidae punctulata</i> Reuter, 1091            |       | +      |            |      |
| 5  | Genus <b>Lopus</b>                                   |       |        |            |      |
| 9  | <i>Lopus decolor</i> Fallén, 1807                    |       |        | +          |      |
| 6  | Genus <b>Lygus</b>                                   |       |        |            |      |
| 10 | <i>Lygus pratensis</i> Linnaeus, 1758                | +     |        | +          |      |
| 11 | <i>Lygus rugulipennis</i> Poppius, 1911              | +     |        |            |      |
| 7  | Genus <b>Macrolophus</b>                             |       |        |            |      |
| 12 | <i>Macrolophus pygmaeus</i> Herrich – Schäffer, 1835 |       |        | +          |      |
| 8  | Genus <b>Megaloceraea</b>                            |       |        |            |      |
| 13 | <i>Megaloceraea recticornis</i> Geoffroy, 1787       |       |        | +          |      |
| 9  | Genus <b>Oncotylus</b>                               |       |        |            |      |
| 14 | <i>Oncotylus punctipes</i> Reuter, 1875              |       | +      |            |      |
| 10 | Genus <b>Orthocephalus</b>                           |       |        |            |      |
| 15 | <i>Orthocephalus saltator</i> Hanh, 1835             |       |        | +          | +    |
| 11 | Genus <b>Orthops</b>                                 |       |        |            |      |
| 16 | <i>Orthops kalmi</i> Linnaeus, 1758                  |       |        | +          |      |
| 12 | Genus <b>Phytocoris</b>                              |       |        |            |      |
| 17 | <i>Phytocoris insignis</i> Reuter, 1876              |       | +      |            |      |
| 18 | <i>Phytocoris ustulatus</i> Herrich-Schaffer, 1835   |       | +      | +          |      |
| 13 | Genus <b>Piezocranum</b>                             |       |        |            |      |
| 19 | <i>Piezocranum medvedevi</i> V.G. Putshkov, 1961     | +     |        |            |      |
| 14 | Genus <b>Pilophorus</b>                              |       |        |            |      |
| 20 | <i>Pilophorus cinnamopterus</i> Kirschbaum, 1856     | +     |        |            |      |
| 15 | Genus <b>Plagiognathu</b>                            |       |        |            |      |
| 21 | <i>Plagiognathus fulsipens</i> Kirschbaum, 1856      |       |        | +          |      |
| 22 | <i>Plagiognathus cunctator</i> Horvath, 1887         | +     |        |            | +    |
| 16 | Genus <b>Polymerus</b>                               |       |        |            |      |
| 23 | <i>Polymerus cognatus</i> Fieber, 1858               | +     |        |            |      |
| 17 | Genus <b>Stenodema</b>                               |       |        |            |      |
| 24 | <i>Stenodema holstatum</i> Fabricius, 1787           |       |        | +          |      |
| 18 | Genus <b>Stenotus</b>                                |       |        |            |      |
| 25 | <i>Stenotus binotatus</i> Fabricius, 1794            |       | +      |            |      |
| 19 | Genus <b>Trigonotylus</b>                            |       |        |            |      |
| 26 | <i>Trigonotylus ruficornis</i> Geoffroy, 1785        |       |        | +          | +    |

## SOME DATA OF SEED BUGS (*LYGAEIDAE*) IN ECOSYSTEMS OF KRUJA (ALBANIA)

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### ABSTRACT

Study of the ecological and systematical aspects for the families *Lygaeidae* in ecosystems Kruja Region is presented in that paper. The biological material was collected during the period of time 2019-2021. The family *Lygaeidae* was presented by 11 genera and 15 species. The systematical analysis to the *Lygaeidae* resulted that the genera represented by the highest number of species was *Lygaeus* by 3 species, and frequency 20 %. By analyzing the material the station with more species, is Brret, with 9 species or frequency 60 %, while with less species, is Droja and Mali Kruje or 5 species or 33.33%.

**Key words:** Hemiptera, *Lygaeidae*, ecosystems, Kruja

### INTRODUCTION

The *Lygaeidae* family includes species of small to middle dimensions, or too small. Some of them show bright colors, red, black and yellow, but mainly are dominated by the red and black color (Dolling, 1991; Péricart, 1988). They are distinguished from two simple eyes and two composed eyes, and the head is very small. They have one trumpet with four segments, and their antennae are composed of four segments. Scutellum contains a swelling like Y letter, their legs have composed tarsi from 3 segments (Slater, 1975). They are phytophagous species, which feed by seed, but some species can feed also with the vegetative parts, like flowers. In that family are identified some predators (Servadei, 1967; Miller, 1971; Servadei et al. 1972; Silvestri, 1939). This paper presents our study on the species belonging to this family for the ecosystems of Kruja region attempting to give a thorough analysis of the species from this family. This publication is in memory of our professor Kastriot Misja, who died from Covid19 in February 2022, when we were finishing our work.

### MATERIALS AND METHODS

The biological material is collected during the expeditions of 2008-2010 in the ecosystem habitats of Tirana, stations of Droja, Brret, Mali Kruja and Boja. Samplings of the biological material were realized randomly in the May-September period, respectively during the 09<sup>00</sup>-15<sup>00</sup> day hours. Entomological mowing nets of 80cm diameter, aspirators and Pitt's traps were employed. Mowing with Entomological nets is achieved according to the diagonals for surfaces of 100 m<sup>2</sup> (10m x 10m), passing five times across each square's diagonal (Colas, 1969; Chapman, 1988). After collection, the individuals are placed in plastic bottles, labelled with the date and station. The fine biological materials are placed in plastic flacons 150-200 cc. they were sent to the scientific laboratory and preserved in bottles of ethanol solution 95%, acetic

acid, distilled water in 80:5:20 ml, and some ether drops (Colas, 1969; Chapman, 1988). Determination of the collected material was analyzed by observing with stereomicroscope ZEISS (*Carl Zeiss*), and use of determination keys to this family, previous collections, and other article for this family (Aukema et al. 1999; Çağatay A. 1989; Misja, 1973; Halimi et al, 2016).

## RESULTS AND DISCUSSION

In this study are determined species of the *Lygaeidae* family by listing them in the table 1 according to the encountered species in the ecosystem of Kruja for Droja, Brret, Mali Kruje, and Boja stations.

**Table 3: List of species for *Lygaeidae* family**

| Nr. | Scientific name  | Droje | Brret | Boja | Mali Kruje |
|-----|--|-------|-------|------|------------|
| 1   | Genus <i>Beosus</i>  |       |       |      |            |
| 1   | <i>Beosus quadripunctatus</i> O.F. Müller, 1766              | +     | +     | +    |            |
| 2   | Genus <i>Geocoris</i>  |       |       |      |            |
| 2   | <i>Geocoris erythrocephalus</i> Le Peletier – Serville, 1825 |       | +     |      | +          |
| 3   | Genus <i>Graptopeltus</i>                                    |       |       |      |            |
| 3   | <i>Graptopeltus lynceus</i> Fabricius, 1775                  | +     |       | +    |            |
| 4   | Genus <i>Heterogaster</i>                                    |       |       |      |            |
| 4   | <i>Heterogaster urticae</i> Fabricius, 1775                  |       | +     |      |            |
| 5   | Genus <i>Ischnodemus</i>                                     |       |       |      |            |
| 5   | <i>Ischnodemus sabuleti</i> Fallén, 1826                     | +     | +     |      | +          |
| 6   | <i>Ischnopeza hirticornis</i> Herrich-Schäffer, 1850         |       |       | +    |            |
| 6   | Genus <i>Lygaeus</i>   |       |       |      |            |
| 7   | <i>Lygaeus equestris</i> Linnaeus, 1758                      | +     |       | +    |            |
| 8   | <i>Lygaeus pandurus</i> Scopoli, 1763                        |       | +     |      | +          |
| 9   | <i>Lygaeus saxatilis</i> Scopoli, 1763                       | +     |       |      |            |
| 7   | Genus <i>Metopoplax</i>                                      |       |       |      |            |
| 10  | <i>Metopoplax origani</i> Kolenati, 1845                     |       | +     |      |            |
| 8   | Genus <i>Nysius</i>  |       |       |      |            |
| 11  | <i>Nysius graminicola</i> Kolenati, 1846                     |       |       |      | +          |
| 12  | <i>Nysius senecionis</i> Schilling, 1829                     |       | +     | +    |            |
| 9   | Genus <i>Orsillus</i>  |       |       |      |            |
| 13  | <i>Orsillus maculatus</i> Fieber, 1861                       |       |       | +    |            |
| 10  | Genus <i>Pezocoris</i>                                       |       |       |      |            |
| 14  | <i>Pezocoris apicimacula</i> Costa, 1853                     |       | +     |      | +          |
| 11  | Genus <i>Platyplax</i>                                       |       |       |      |            |
| 15  | <i>Platyplax salviae</i> Schilling, 1829                     |       | +     | +    |            |

From analyzing of the scientific material collected in the area under study, are present 11 genera and 15 species to *Lygaeidae* family (Table 2, Figure 1).

Analysis of the results based on the diversity for the *Lygaeidae* family, *Lygaeus* is the most represented by 3 species or by 20%, followed by *Ischnodemus* and *Nysius* by 2 species or 13.33%. Other genera: *Beosus*, *Geocoris*, *Graptopeltus*, *Heterogaste*, *Metopoplax*, *Orsillus*, *Pezocoris* *Platyplax* are represented by one specie or by 6.67%.

Table 4: Species numbers according to the genera for the *Lygaeidae* family

| No | Scientific name     | No. of Species | Species frequency |
|----|---------------------|----------------|-------------------|
| 1  | <i>Beosus</i>       | 1              | 6.67%             |
| 2  | <i>Geocoris</i>     | 1              | 6.67%             |
| 3  | <i>Graptopeltus</i> | 1              | 6.67%             |
| 4  | <i>Heterogaster</i> | 1              | 6.67%             |
| 5  | <i>Ischnodemus</i>  | 2              | 13.33%            |
| 6  | <i>Lygaeus</i>      | 3              | 20.00%            |
| 7  | <i>Metopoplax</i>   | 1              | 6.67%             |
| 8  | <i>Nysius</i>       | 2              | 13.33%            |
| 9  | <i>Orsillus</i>     | 1              | 6.67%             |
| 10 | <i>Pezocoris</i>    | 1              | 6.67%             |
| 11 | <i>Platyplax</i>    | 1              | 6.67%             |

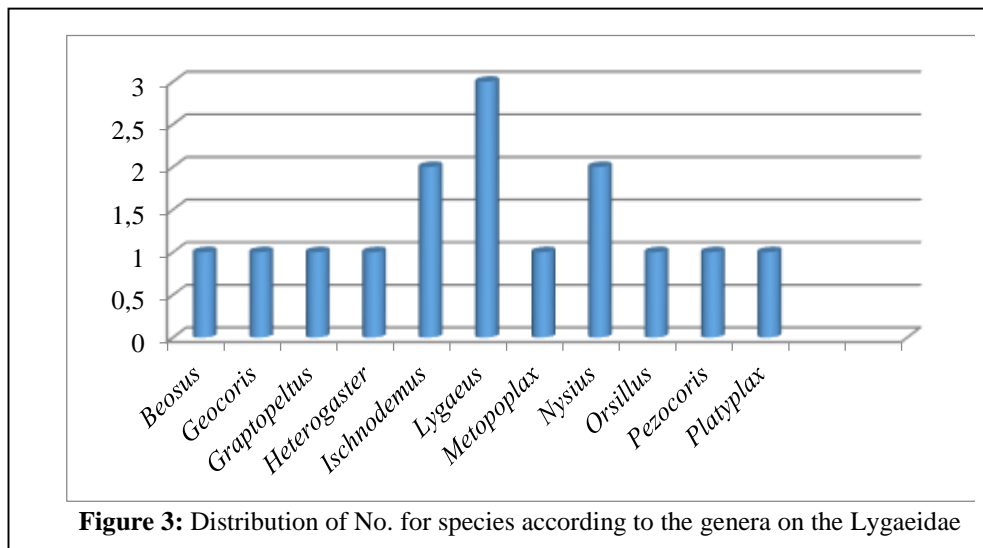
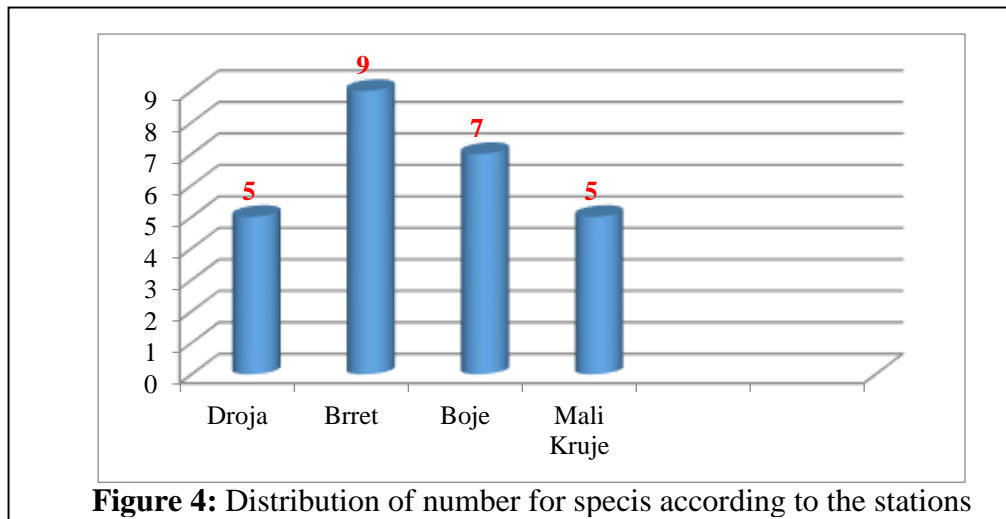


Figure 3: Distribution of No. for species according to the genera on the Lygaeidae

Analyzing of the diversity to the different stations, indicates that most represented regarding to the *Lygaeidae* family, is the Brret stations by 9 species, or 60%, followed by Boja station with 7 species, or 46.67%, Droja and Mali Kruje by 5 species or 33.33%. (Table 3, Figure 3).

Table 5: The number of species according to the stations

| Station    | Species number | Species frequency |
|------------|----------------|-------------------|
| Droja      | 5              | 33.33%            |
| Brret      | 9              | 60.00%            |
| Boje       | 7              | 46.67%            |
| Mali Kruje | 5              | 33.33%            |



## CONCLUSIONS

This study presents results for Lygaeidae in the ecosystems of the Kruja region. In total are encountered 11 genera and 15 species. Highest diversity to the *Lygaeidae* family is *Lygaeus* genera by 3 species or 20 %. To the *Lygaeidae* family, most represented is the Brret station by 9 species or 60%, while with less species, is Droja and Mali Kruje with with 5 species or frequency 33.33%.

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## DETERMINATION OF BIOLOGICAL POTENTIAL OF *TILIA CORDATA* FLOWER EXTRACTS

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### ABSTRACT

Extraction is a very important stage in the isolation, as well as the identification of different bioactive compounds in the plants. The aim of this research was to produce aqueous and ethanolic extracts from *Tilia cordata* flowers, as well as to determine its antioxidant and antimicrobial potential. Ethanolic extract was characterized with higher ( $p < 0.05$ ) ability to capture free DPPH radicals compared to the aqueous extract. From the point of ability to chelate iron ions can be proved that ethanolic extract was characterized with slightly higher ( $p < 0.05$ ) values compared to the aqueous one, whereas at the highest tested concentration both of the extracts (51.57%, i.e. 49.03%, respectively) had higher ( $p < 0.05$ ) antioxidant potential compared to the citric acid (12.66%). These values were followed by IC<sub>50</sub> values. Furthermore, ethanolic linden extract had higher ( $p < 0.05$ ) antimicrobial potential against most of the tested strains compared to the aqueous linden extract. Even that ethanolic extract showed the highest activity against *Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes*, *Escherichia coli*, *Yersinia enterocolitica*, *Proteus vulgaris* and *Pseudomonas aeruginosa* only in one case, against *Listeria monocytogenes* (15.9 mm), showed higher ( $p < 0.05$ ) activity compared to the tetracycline. According to that, ethanolic linden flower extract showed good antioxidant and antimicrobial potential while it can be used in the food industry for producing functional food with increased biological value.

**Keywords:** *Tilia cordata*, extracts, antioxidant potential, antimicrobial potential.

### INTRODUCTION

Plants, their extracts or pure components isolated from them are used in various industries, such as pharmaceutical, cosmetic, food, etc. Consumption of the plants and its products have been constantly growing. Studies showed that several classes of compounds present in these plants are responsible for biological activity (Mitic et al., 2021).

Among the four species of the genus *Tilia* that grow naturally in Europe, the small-leaved lime (*T. cordata* Mill.) is the most widespread in temperate woodlands. Although it is a relatively rare and scattered species, it was very abundant in the past. Indeed, its relatively good shade-tolerance and its mid- to late-successional character in forest dynamics made it originally a co-dominant species of temperate primeval woodlands of central and Eastern Europe (Jaegere et al., 2016).

*Tilia cordata* is a minor, broadleaved species with wide, but scattered distribution in Europe, characterized as a species with wide ecological tolerance and numerous ecosystem services (Jaegere et al. 2016). Besides its natural distribution, *T. cordata* is a common species in parks, or other urban green areas in the Eastern Balkan region (Zorić et al., 2020). As flowers of *Tilia* species are widely used in the traditional medicine as herbal tea for cough treatment or restlessness, most of the previous research have put its focus on the organic composition of the dried inflorescence (Zorić et al., 2020). These plants contain a number of derivatives such as hydrocarbons, esters, terpenoids, quercetin, kampferol, phenolic compounds, condensed tannins and scopoletin (Wissam et al., 2017). Linden flowers have been used to treat several illnesses like bacterial infections as well as their effects in reducing tension. Alcoholic extracts have antibacterial properties while flower infusion is used to treat diseases of respiratory tract (Özbucak et al., 2013). These effects could be attributed to the presence of flavonoids and phenolic compounds.

Extraction is a very important stage in the isolation, as well as the identification, of phenolic compounds. However, the compositions of natural sources of phenolic compounds and the structure and physicochemical properties make a universal extraction protocol not conceivable. A definite extraction procedure must be designed and optimized for each phenolic source, compounds that are correlated to the antioxidant activity of the extracts (Mitic et al., 2021).

The aim of this research was to produce aqueous and ethanolic extracts from *Tilia cordata* flowers, as well as to determine its antioxidant and antimicrobial potential.

## **MATERIAL AND METHODS**

### **Collection, preparation and drying of linden flowers**

As a work material *Tilia cordata* flowers were used, that are located in Ohrid, the southwestern part of North Macedonia, on the shore of Lake Ohrid, at an elevation of 695 m above sea level.

In order to remove dust particles and other impurities, fresh flowers were washed with distilled water. After that, the fresh linden flowers were dried in a laboratory dryer (60 °C, 4 to 5h) to a constant mass (Stojanova, 2019). After drying linden flowers were grounded to a fine powder and were stored in a refrigerator until the analysis.

### **Preparation of aqueous extract**

Aqueous extract was prepared according to Sławińska et al. (2013) and Ribeiro et al. (2015) method. 10g of dried and powdered linden flowers was poured with 200 mL of distilled water, and after that was extracted on a boiling water bath for 1h. The extract was strained through filter paper, then rinsed once more with boiling water and the sample was filtered again. The resulting supernatant was combined and evaporated on a vacuum evaporator. For each sample, the extraction procedure was done in triplicates.

### **Preparation of ethanolic extract**

Ethanolic extract was prepared according to Vidović et al. (2011) method. 10 g of dried and finely powdered flower samples was poured with 100 mL of 70% ethanol and extract was covered for 40 minutes on an ultrasonic bath at 45°C. The sample was filtered through filter paper. The resulting supernatant extract was evaporated at 60°C to constant mass. For each sample, the extraction procedure was done in triplicates.



### **Determination of antioxidant potential of linden extracts**

#### ***Ability to capture DPPH radicals***

The ability to capture DPPH radicals was determined by Brand-Williams et al. (1995) method.

$$I\% = [(A_{\text{blank}} - A_{\text{sample}})/A_{\text{blank}}] \times 100\%$$

The radical scavenging capacity of the samples was calculated as IC<sub>50</sub> values (inhibitory concentration of extract reducing the absorbance of DPPH solution by 50%) by regression analysis:

$$IC_{50} \text{ (mg/mL)} = (50 - b)/a^* \text{ (*a - slope; b - intercept)}$$

BHT was used as positive control.

The results are expressed as the mean of the three measurements.

#### ***Ability to chelate iron ions***

The chelating ability of iron was determined by Dinis et al. (1994) method. The chelating ability of iron ions is calculated by the formula:

$$\text{Ability to chelate iron \%} = [(A_{\text{blank}} - A_{\text{sample}})/A_{\text{blank}}] \times 100\%$$

The ability to chelate iron ions of the samples was calculated as IC<sub>50</sub> values by regression analysis:

$$IC_{50} \text{ (mg/mL)} = (50 - b)/a^* \text{ (*a - slope; b - intercept)}$$

Citric acid was used as positive control. The results are expressed as the mean of the three measurements

### **Determination of antimicrobial potential of linden extracts**

Antimicrobial potential was determined by disk-diffusion method. 9 pathogenic bacteria were used: *Staphylococcus aureus* ATCC 25923; *Bacillus cereus* ATCC 10876; *Listeria monocytogenes* ATCC 19115; *Enterococcus faecalis* ATCC 29212; *Escherichia coli* ATCC 11230; *Yersinia enterocolitica* ATCC 27729; *Shigella sonnei* ATCC 29930; *Proteus vulgaris* ATCC 8427; *Pseudomonas aeruginosa* ATCC 35554. The tested bacteria were stored on suitable oblique agar at +4 °C.

#### ***Disc-diffusion method***

Disc diffusion analysis was performed by Klaus et al. (2015) method. Tested microorganisms were prepared in the appropriate broth, sieved 2 times for 24 h, whereby the concentrations were about 1·10<sup>6</sup> to 1·10<sup>8</sup> CFU/mL. Then the suspension of each culture of microorganisms (100 µL) was seeded on appropriate agar. Three sterile filter discs (6 mm) were placed on the agar surface and then soaked with 50 µL of suspension of each of the extracts. After standing for 2 hours 25 °C, petri dishes were incubated for 24 h at 37 °C. After incubation, the zone of inhibition (mm) was measured.

## Statistical analysis

The obtained results were statistically processed using the software package SPSS 20. To determine the statistical significant differences of the obtained values the Independent Sample T-test ( $p = 0.05$ ) as well as ANOVA post hoc Tukey's test ( $p = 0.05$ ) was performed.

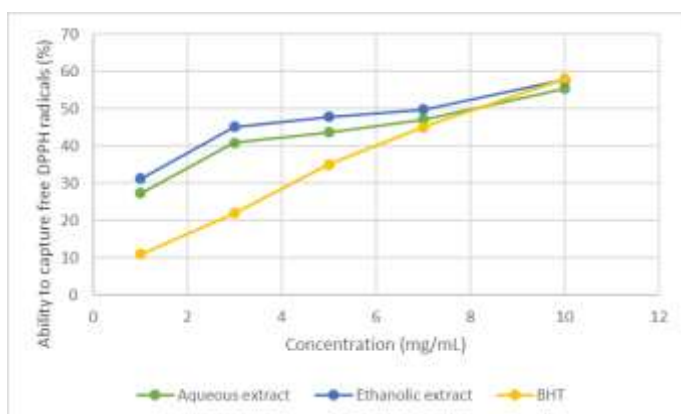
## RESULTS AND DISCUSSION

The healing properties of different plant species are numerous, and many plants are used in folk medicine as a good source of different biologically active compounds (Stojanova et al., 2022). The importance of antioxidant components of natural origin has been increasing lately, since some of the frequently used synthetic antioxidants, especially in the food industry (butylated hydroxyanisole [BHA] and butylated hydroxytoluene [BHT]), have been found to possess certain toxic properties (Vidović et al., 2011). Resistance to available antibiotics in pathogenic bacteria is currently a global challenge since the number of strains that are resistant to multiple types of antibiotics has increased dramatically each year, and the strains have spread worldwide (Stojanova et al., 2022).

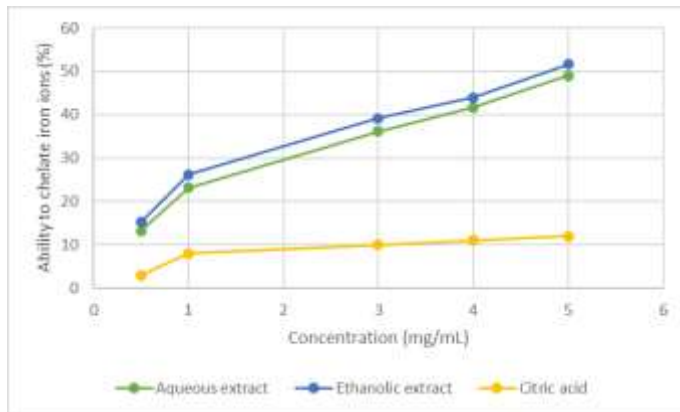
### Antioxidant potential of linden extracts

From the data presented in Figure 1, can be seen that ethanolic extract was characterized with higher ( $p < 0.05$ ) ability to capture free DPPH radicals compared to the aqueous extract at all of the tested concentrations. At the highest tested concentration (10 mg/mL) can be highlighted that aqueous (55.39%) as well as ethanolic (57.66%) extracts were competitive with the BHT as a positive control (58.10%).

From the point of ability to chelate iron ions (Figure 2), once again can be proved that ethanolic extract was characterized with slightly higher ( $p < 0.05$ ) values compared to the aqueous one, whereas at the highest tested concentration (5 mg/mL) both of the extracts (51.57%, i.e. 49.03%, respectively) had much higher ( $p < 0.05$ ) antioxidant potential compared to the citric acid (12.66%).



**Figure 1:** Ability of linden extracts to capture DPPH radicals



**Figure 2:** Ability of linden extracts to chelate iron ions

**Table 1:** IC<sub>50</sub> values of tested linden extracts

| Linden flower extract    | n | IC <sub>50</sub> (mg/mL)  |                                 |
|--------------------------|---|---------------------------|---------------------------------|
|                          |   | DPPH                      | Chelating Fe <sup>3+</sup> ions |
|                          |   | $\bar{x} \pm SD$          | $\bar{x} \pm SD$                |
| <b>Aqueous extract</b>   | 3 | 3.12 ± 0.12 <sup>aA</sup> | 2.65 ± 0.16 <sup>aB</sup>       |
| <b>Ethanolic extract</b> | 3 | 2.01 ± 0.05 <sup>bA</sup> | 1.37 ± 0.10 <sup>bB</sup>       |

a, b - values of the different extract and the same test marked with different letters, have a statistically significant difference ( $p < 0.05$ ), T-test.

A, B - values of same extract and the different test, marked with different letters, have a statistically significant difference ( $p < 0.05$ ), T-test.

These values were followed by those for the IC<sub>50</sub> values (Table 1). In this case, significantly lower ( $p < 0.05$ ) values were determined for the ethanolic extract, compared to the aqueous extract for both antioxidant tests. Furthermore, can be seen that both of the extracts had higher ( $p < 0.05$ ) ability to chelate iron ions, compared to its ability for capturing free DPPH radicals.

Antioxidants, on interaction with DPPH, transfer electron or hydrogen atoms to DPPH, and thus neutralize its free-radical character (Naik et al., 2003). Meanwhile, Akyuz et al. (2014) pointed that the DPPH scavenging activity of linden extracts and fractions, expressed in the term of SC<sub>50</sub>, was in the range of 0.106–0.231 mg/mL, with the strongest antioxidant potency for phenolic extracts of tilia leaves.

According to Wissam et al. (2017) the ethanolic extracts of *Tilia cordata* leaves is a rich source of polyphenols and exhibit high antioxidant activity (IC<sub>50</sub>=0.3303±0.0896 mg/ml calculated as DPPH scavenging activity).

### Antimicrobial potential of linden extracts

According to data presented in Table 2, can be seen that ethanolic linden extract was characterized with higher ( $p < 0.05$ ) antimicrobial potential against most of the tested strains compared to the aqueous linden extract. Even that ethanolic extract showed the highest activity against *Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes*, *Escherichia coli*, *Yersinia enterocolitica*, *Proteus vulgaris* and *Pseudomonas aeruginosa* only in one case, against *Listeria monocytogenes* (15.9 mm), showed higher ( $p < 0.05$ ) activity compared to the

tetracycline. Aqueous linden extract had better ( $p<0.05$ ) activity only against *Enterococcus faecalis* and *Shigella sonnei* compared to the ethanolic one.

In accordance, Pavlovic et al. (2020) in their study for antimicrobial activity of linden extracts found that yeast strains of *C. albicans* and Gram-negative pathogens such as *K. pneumoniae*, *E. coli*, *R. nepotum*, *P. aeruginosa*, *P. syringe* pv. *tomato*, and *E. persicina* have been shown highest resistance to the tested linden extracts. Slightly higher susceptibility has been observed against *C. glabrata*, *E. coli* and *P. aeruginosa*. Authors found that the most sensitive strains, were Gram-positive isolates of *S. mutans*, *S. pyogenes*, *E. faecalis* and *S. aureus* with clear zones of inhibition in range from 12 to 15 mm. On the other hand, Yıldırım et al. (2000) and El-Farmawi et al. (2014) have reported the absence of antimicrobial activity of linden tea extracts against *C. albicans*, *P. aeruginosa* and *K. pneumoniae*.

**Table 2:** Antimicrobial activity of tested extracts (mm)

| Microorganism                                | n | Linden flower extract       |                             | Tetracycline<br>30 µg/disc  | Chloramphenicol<br>30 µg/disc |
|--|---|-----------------------------|-----------------------------|-----------------------------|-------------------------------|
|  |   | Aq*                         | EtOH**                      |                             |                               |
|  |   | $\bar{x} \pm SD$            | $\bar{x} \pm SD$            |                             |                               |
| <i>Staphylococcus aureus</i><br>ATCC 25923   | 3 | 10.5 ±<br>0,01 <sup>a</sup> | 17.1 ±<br>0,02 <sup>b</sup> | 29,0 ±<br>0,01 <sup>c</sup> | 21,2 ±<br>0,03 <sup>d</sup>   |
| <i>Bacillus cereus</i><br>ATCC 10876         | 3 | 1.0 ±<br>0,07 <sup>a</sup>  | 5.3 ±<br>0,05 <sup>b</sup>  | 11,5 ±<br>0,02 <sup>c</sup> | 19,3 ±<br>0,02 <sup>d</sup>   |
| <i>Listeria monocytogenes</i><br>ATCC 19115  | 3 | 13.7 ±<br>0,05 <sup>a</sup> | 15.9 ±<br>0,02 <sup>b</sup> | 15,3 ±<br>0,03 <sup>c</sup> | 14,5 ±<br>0,04 <sup>d</sup>   |
| <i>Enterococcus faecalis</i><br>ATCC 29212   | 3 | 10.9 ±<br>0,03 <sup>a</sup> | 7.2 ±<br>0,06 <sup>b</sup>  | 15.8 ±<br>0,01 <sup>c</sup> | 17,6 ±<br>0,03 <sup>d</sup>   |
| <i>Escherichia coli</i><br>ATCC 11230        | 3 | 6.6 ±<br>0,03 <sup>a</sup>  | 9.5 ±<br>0,04 <sup>b</sup>  | 11,2 ±<br>0,02 <sup>c</sup> | 12,1 ±<br>0,02 <sup>d</sup>   |
| <i>Yersinia enterocolitica</i><br>ATCC 27729 | 3 | 18.1 ±<br>0,01 <sup>a</sup> | 21.4 ±<br>0,07 <sup>b</sup> | 27,0 ±<br>0,02 <sup>c</sup> | 26,3 ±<br>0,01 <sup>d</sup>   |
| <i>Shigella sonnei</i><br>ATCC 29930         | 3 | 9.7 ±<br>0,03 <sup>a</sup>  | 6.8 ±<br>0,02 <sup>b</sup>  | 11,5 ±<br>0,01 <sup>c</sup> | 13,7 ±<br>0,02 <sup>d</sup>   |
| <i>Proteus vulgaris</i><br>ATCC 8427         | 3 | 14.5 ±<br>0,09 <sup>a</sup> | 15.0 ±<br>0,05 <sup>a</sup> | 18,2 ±<br>0,01 <sup>b</sup> | 16,6 ±<br>0,01 <sup>c</sup>   |
| <i>Pseudomonas aeruginosa</i><br>ATCC 35554  | 3 | 11.2 ±<br>0,02 <sup>a</sup> | 13.7 ±<br>0,03 <sup>b</sup> | 15.4 ±<br>0,01 <sup>c</sup> | 13.9 ±<br>0,01 <sup>b</sup>   |

a, b, c, d – values marked with different letters have statistically significant difference ( $p<0.05$ ), ANOVA, post hoc Tukey's test.

\* Aqueous extract

\*\* Ethanolic extract

## CONCLUSION

Based on the results, it can be concluded that water and ethyl alcohol are suitable for producing linden flower extracts. Ethanolic extract was characterized with higher ( $p < 0.05$ ) ability to capture free DPPH radicals as well as ability to chelate iron ions, compared to the aqueous one, that is proved by the appropriate  $IC_{50}$  values. Moreover, both can be competitive with BHT and citric acid at the tested concentrations. However, ethanolic extract showed higher ( $p < 0.05$ ) activity against *Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes*, *Escherichia coli*, *Yersinia enterocolitica*, *Proteus vulgaris* and *Pseudomonas aeruginosa* compared to the aqueous linden extract.

According to that, ethanolic linden flower extract showed good antioxidant and antimicrobial potential while it can be used in the food industry for producing functional food with increased biological value.

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## SEED GERMINATION TEST OF *Maerua siamensis* (Kurz) Pax

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### ABSTRACT

*Maerua siamensis* (Kurz) Pax belongs to Capparaceae distributed in mainland southeast Asia. Its populations have been threatened by forest destruction and over collecting for garden landscape use. To conserve this species, the reproductive biology study is crucially needed. The propagation via seeds is one of the productivity protocols for population conservation. This study was conducted to test the germination rate of *M. siamensis* and find out the suitable method for species conservation. Five pretreatments including control were designed which are (1) untreated seeds as control, (2) soaking in water 24 hours, (3) soaking in hot water 80°C for 5 mins, (4) soaking in 98% sulfuric acid for 5 mins, and (5) scarification with nail clip. The experiment was conducted in Completely Randomized Design (CRD) with 3 replicates containing 90 seeds in each treatment. The results showed that the mechanical scarification with nail clip gave the highest germination rate. However, untreated seeds treatment is an option for producing seedlings due to cost saving. This species is non-dormant seeds due to germination occurs within few days after sowing. The fruit ripening time fit with the beginning of the rainy season. It seems to be an orthodox seed because seeds kept in refrigerator for one year gave high germination rate. This species can be applied to seed banking, but it needs to monitor long term survival rate after seed banking.

**Keywords:** Pretreatment, seed germination, *Maerua siamensis*, Capparaceae

### INTRODUCTION

*Maerua siamensis* (Kurz) Pax is a slow growth tree species occurring in Thailand, Cambodia, Vietnam and Myanmar, classed in Capparaceae. In Thailand it usually grows in rather dry tropical forest such as dry evergreen forest, mixed deciduous forest, dry dipterocarp forest, open scrub jungle and on limestone hill at alt. 0-400 m (Chayamarit, 1991). It is an edible plant, eaten with chili paste and used for the medicinal products such as toothache medicine, day or night blindness, malaria disease, compression for muscle paralysis (Chadchen, 2010).

*M. siamensis* is a small to medium tree 5-10 m high, sometimes a shrub, with smooth bark, branches glabrous. Leaves are spiral, 3-5 palmately compound leaves, Leafless is obovate, oblong or linear, 2-12 cm long, 1-3 cm wide. Petiole is slender, 1.5-6.5 cm long. Flowers are in terminal or lateral corymb or raceme inflorescence. Pedicels are 1.5-5.5 cm long. Bracts are linear, small. Sepals are ovate, 7-10 cm long, 2-3 mm wide. Sepal apex is acuminate, glabrous on both sides. Stamens present 9-12. Filaments are robust, 10-15 mm long. Anthers are oblong, 1.5-2 mm long, Anther apex is mucronate. Gynophore is robust, 1.5-2 cm long, and glabrous. Ovary is cylindrical, 1.5-2 mm long and ca. 1 mm wide, glabrous. Fruits are ellipsoidal or rounded, 2-2.5 cm long and 1.3 -1.5 cm wide. Fruit stripes are 4.5 - 7.5 cm, slender. Seeds are reniform (Figure 1) (Chayamarit, 1991).

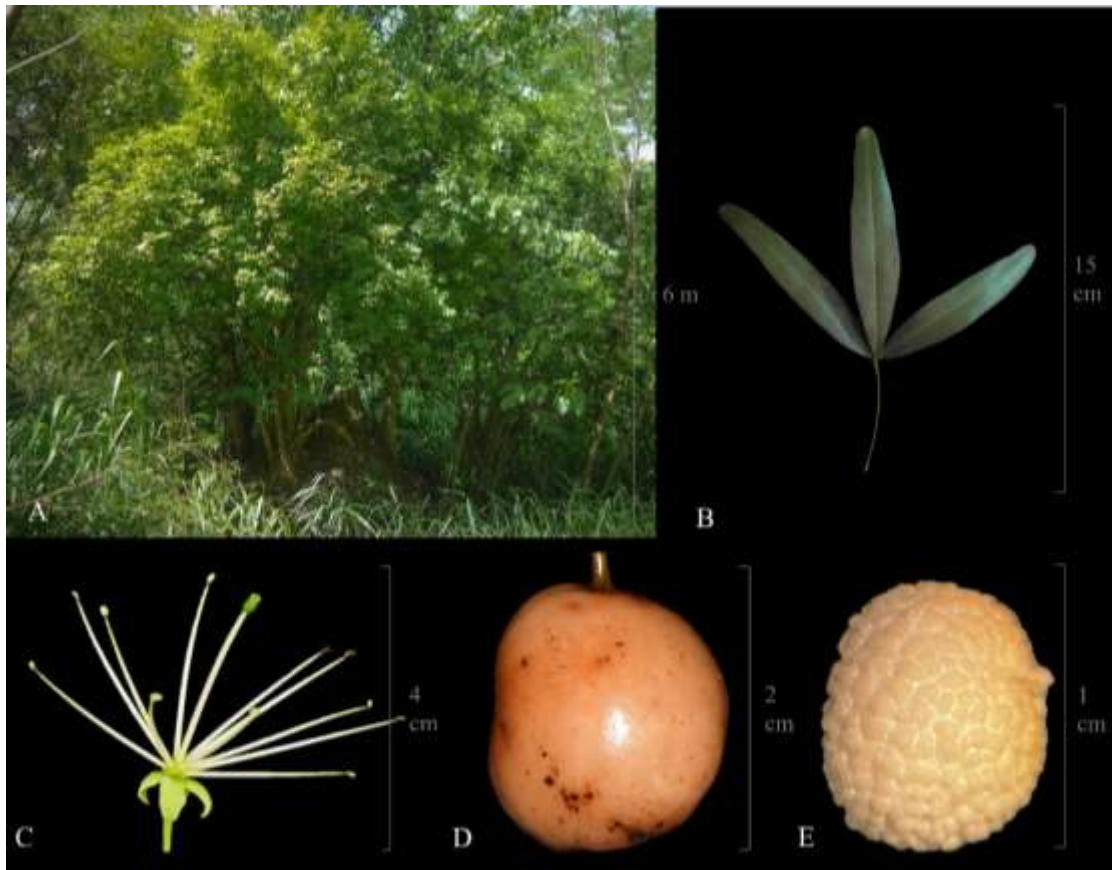


Figure 1. Botanical characteristics of *M. siamensis*. A—Tree, B—leaves, C—Floret, D—Fruit, E—seed.

Recently, *M. siamensis* is probably a threatened species due to deforestation and over exploitation. The large trees of this species have been removed from natural habitat to ornamental gardens with high prices in Thailand. However, it has never been evaluated for conservation status. Seed germination rate is one of key results for species or population survival (Donohue et al., 2010).

There is no information on reproductive biology *M. siamensis*, especially seed germination and dormancy. Thus, this study was conducted to determine the seed germination and dormancy of *M. siamensis* after various seed pre-treatments that might be applied to re-introduction and conservation in the near future.

## MATERIAL AND METHOD

Three individuals of the *M. siamensis* from the forested fragment in Suranaree University of Technology (SUT), Nakhon Ratchasima Province, Thailand was selected for fruit collection. About 300 ripened fruits were collected. Fruits were immediately washed with tap water and then dried at room temperature. Sarcotesta was removed from the seeds by scrubbing with a towel. Uncompleted seeds were excluded for the experiment. All seeds are sealed in polyethylene bags and transferred to storage in the refrigerator (5°C) before starting the seed germination experiment in 7-14 days. One reason for using seeds shortly after storage is that they may change their germination responses during long-term dry storage at room temperature (Baskin and Baskin, 2014). Before doing the pre-treatment of seed germination test, all seeds were cleaned for reducing the bacteria and fungi by soaking with Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) 10% v/v for 10 minutes, followed by soaking in Clorox 10% v/v for 10 minutes. Then we used



Phthalimide (Captacide 50) 1 g/l for 5 minutes to prevent fungi growth. The pre-treatment of seed germination test was conducted in Completely Randomized Design (CRD) with 3 replicates containing 30 seeds, total 90 seeds in each treatment. 450 seeds were sampled for seed germination with 5 treatments i.e. (1) untreated seeds as control, (2) soaking in water 24 hours, (3) soaking in hot water 80°C for 5 minutes, (4) soaking in 98% sulfuric acid for 5 minutes, and (5) scarification with nail clip. Thirty seeds were placed in plastic boxes (19x28x5.5 cm) with four layers of tissue paper, moistened with distilled water. All experiments were kept at a temperature of 28 – 34°C during April - June 2020. We collected data daily at the same time starting from 4-6 PM for 28 days. Seed germination was recorded if radicle length is >1 mm. The germination indices were calculated using the formula reported by Czabator (1962), Ellis and Roberts (1981), and Rusdy (2017).

1) Germination percentage

$$GP = \frac{\text{total number of germinated seeds}}{\text{total seeds}} \times 100$$

2) Mean daily germination (MDG) (calculated by Gordon, 1973)

$$MDG = \frac{\text{Final germination (\%)}}{\text{total number of days of test}}$$

3) Germination speed (GS)

$$GS = n1/d1+n2/d2+n3/d3+...+n28/d28$$

where: n is number of germinated seeds

d is number of days

4) Mean germination time (MGT)

$$MGT = \frac{\sum nTi}{\sum n}$$

where Ti is the number of days from the beginning of the experiment

n is the number of newly germinated seeds on day Ti.

Collected data was input to Microsoft Excel with careful checking. Then, we analyzed the effect of different treatments on germination by one-way analysis of variance (ANOVA) along with the Least Significant Difference (LSD) test at  $p < 0.05$ . The assumptions of ANOVA were used in the Duncan Post Hoc test and Turkey. Means and standard errors of germination percentages and viability tests were calculated. All statistical analyses were carried out using SPSS (version 20).

## RESULTS AND DISCUSSION

Seed germination of *M. siamensis* showed the highest number of germination percentage in mechanical scarification (96.67%) as shown in Figure 2 and soaking in water 24 hours' treatment (75.56%) respectively, while the control showed 58.89% of seed germination. It was hardly germinated when conducting the pre-treatment with hot water (2.22%) and no germination found in sulfuric acid treatment (0.00%). Besides giving the highest seed germination percentage, the mechanical scarification method also promoted the mean daily germination, germination speed and mean germination time significantly different from other methods (Table 1).

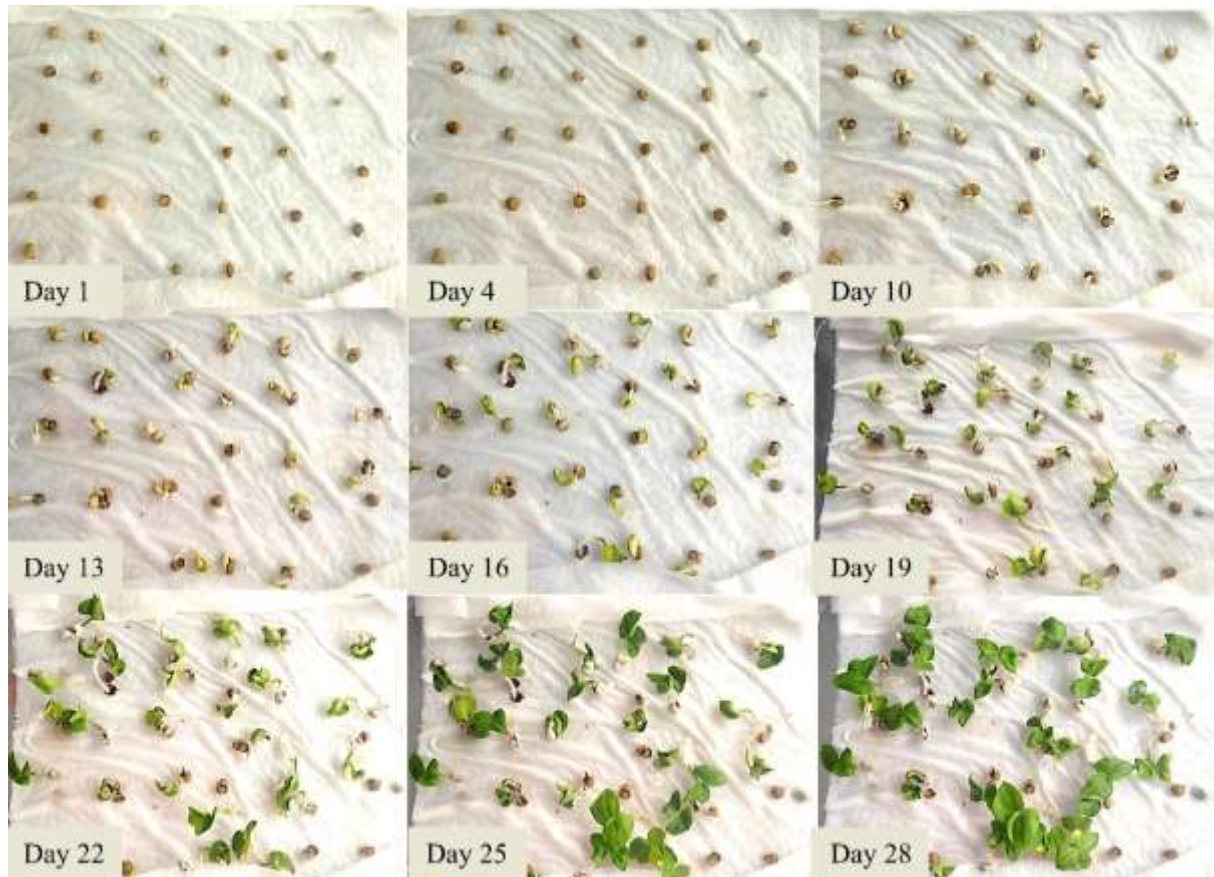


Figure 2. Seed germination within 28 days with mechanical scarification treatments.

The result from this study showed that this species has the highest germination rate in scarification with nail clip treatment. It is the similar result of *Leucaena leucocephala* (Tadros et al., 2012), *Spondias mombin* (Oyebamiji et al., 2014). Seed scarification can break hard structures around seeds, making it permeable for absorbing more water and exchanging gas during the stratification period (Rostami and Shasavar, 2009; Voyiatzis and Porlingis, 1987).

Soaking of *M. siamensis* in cold water increased a bit of seed germination when compared with the control treatment. The germination speed was significantly different from the control treatment. Compare to other species, soaking in cold water (25°C) can have both negative and positive effects, depending on species and seed characteristics. This pre-treatment for seed germination decreased physical seed dormancy of tomato (*Lycopersicon esculentum* Mill), yielding more seed germination (Sabongari and Aliero, 2004). It can also enhance seed germination of *Azadirachta indica* (Owonubi et al., 2005). In contrast, *Pouteria campachina* had poor germination rate when soaking in cold water, due to thick seed coat, but it had higher germination by mechanical scarification (Amoakoh et al., 2017). However, the over-soaking seeds in water may reduce germination rate through oxygen deficiency (Amoakoh et al., 2017). Ibrahim and Otegbeye (2004) had also reported that the efficiency of seed germination rate by soaking in water highly depends on the testa characteristics.

High water temperature (80°C) can damage the seed embryo (Oyebamiji et al., 2014) but it could promote seed germination in some species such as golden shower (*Cassia fistula* L.). Soaking its seeds in hot water at 100 °C for 6 minutes increased the germination percentage to 96% (Soliman and Abbas, 2013). Using this treatment to apply with *Maerua siamensis* seeds revealed a very low seed germination rate.

Sulfuric acid stopped *M. siamensis* seed germination. However, the previous studies showed that sulfuric acid can enhance seed germination rate such as *Leucaena leucocephala* (Koobonye et al., 2018), *Cassia fistula* L. (Soliman and Abbas, 2013), *Psoralea corylifolia* (Arya and Gothwal, 2017) and *Hippophae rhamnoides* (Olmez, 2011). Time for soaking with sulfuric acid to promote higher rate of seed germination varies by species and depends on seed coat characteristics.

In *M. siamensis*, the first seed germination appeared on day 5, in mechanical scarification pre-treatment. It took 10 days for germination without any pre-treatment (Figure 3). This indicated that the physical breaking of the seed coat promoted more germination, due to faster imbibition by means of water uptake into the seeds. However, soaking with water for 24 hours also gave a better germination than without any treatment but a bit slower than mechanical scarification (day 6). Thus, the seed coat of this species does not prohibit the water diffusion into the seed. The control treatment of this species showed more than 50% seed germination less than 4 weeks (mean germination time, 17.52), indicating that they are non-dormant seeds according to Baskin and Baskin (2014), as same as *Capparis* sp. which is in the same family.

Considering the phenology of this species, the fruits are ripened during May-June, the late dry season begins to the beginning of rainy season in the upper part of Thailand. It seems that these species have been adapted to the environment, met with moisture condition, for seedling survival. Thus, there is no dormancy to protect the embryo anymore.

Table 2 showed that seeds of *M. siamensis* can germinate at a rather high rate (84.44%) after being kept in the refrigerator about 5°C in the plastic bag after drying in room condition. It indicated that it is an orthodox seed (Elliott et al., 2003). Thus, this species can be conserved by means of the seed banking. However, it needs to do further long-term monitoring on survival rate after keeping in seed bank condition, low temperature and moisture content.

Table 1. Effect of treatments on various parameters related to germination of *M. siamensis*.

| Treatments                                 | Germination (%)           | Mean Daily Germination (%/day) | Germination Speed (seed/day) | Mean Germination Time (days) |
|--|---------------------------|--------------------------------|------------------------------|------------------------------|
| Control                                    | 58.89±14.19 <sup>b</sup>  | 2.10±0.50 <sup>b</sup>         | 1.05±0.12 <sup>c</sup>       | 17.52±1.92 <sup>ab</sup>     |
| Soaking in water 24 hours                  | 75.56± 6.76 <sup>ab</sup> | 2.70±0.24 <sup>ab</sup>        | 1.80±0.32 <sup>b</sup>       | 14.78±1.58 <sup>bc</sup>     |
| Soaking in hot water 80°C for 5 minutes    | 2.22±1.11 <sup>c</sup>    | 0.08±0.04 <sup>c</sup>         | 0.03±0.02 <sup>d</sup>       | 22.00±4.00 <sup>a</sup>      |
| Soaking in 98% sulfuric acid for 5 minutes | 0.00±0.00 <sup>c</sup>    | 0.00±0.00 <sup>c</sup>         | 0.00±0.00 <sup>d</sup>       | Undefined                    |
| Mechanical scarification                   | 96.67±1.92 <sup>a</sup>   | 3.45±0.12 <sup>a</sup>         | 3.13±0.40 <sup>a</sup>       | 10.17±0.96 <sup>c</sup>      |

Means in the same column with different letters are significantly different at  $p < 0.05$ .

Table 2. Germination percentage (GP), Mean Daily Germination (MDG), Germination Speed

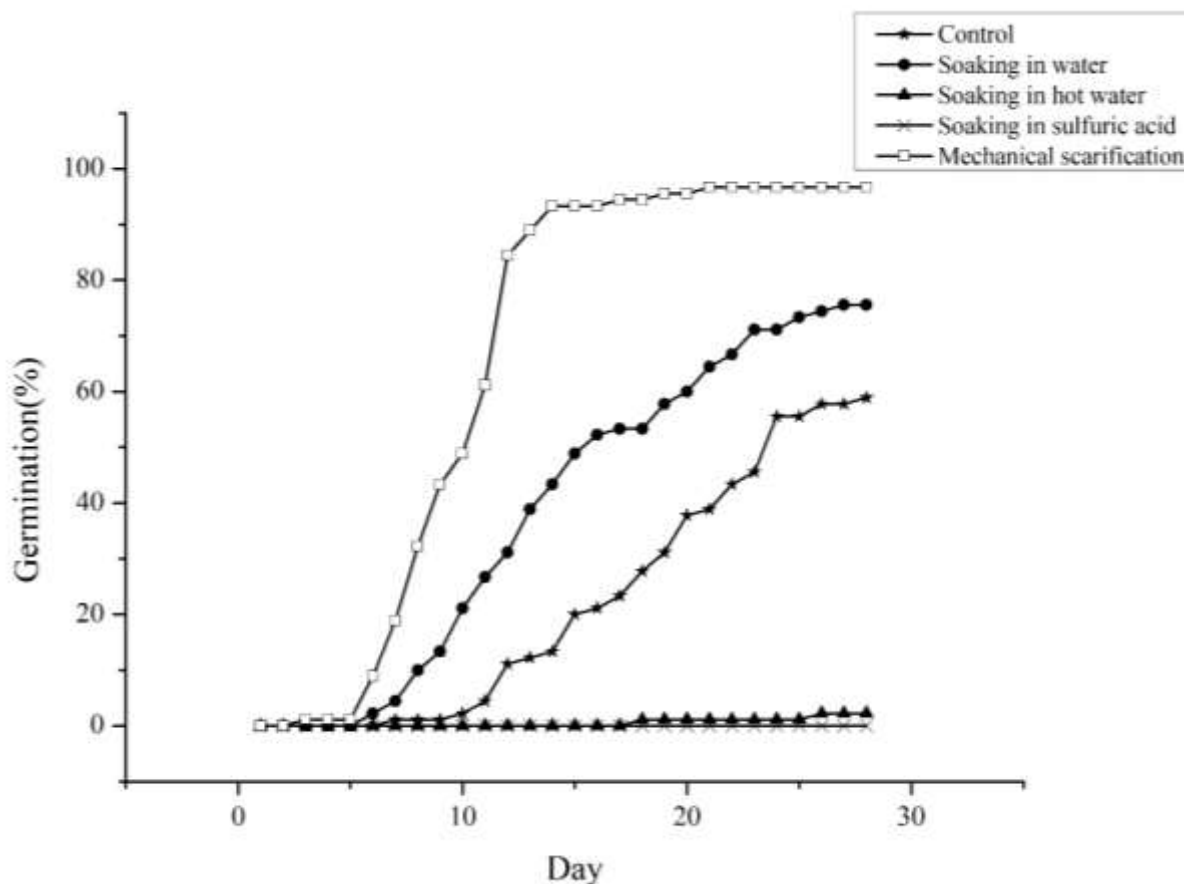
| Treatments               | Storage time | Germination (%)          | Mean Daily Germination (%/day) | Germination Speed (seed/day) | Mean Germination Time (days) |
|--------------------------|--------------|--------------------------|--------------------------------|------------------------------|------------------------------|
| Control                  | one week     | 58.89±14.19 <sup>b</sup> | 2.10±0.50 <sup>b</sup>         | 1.05±0.12 <sup>b</sup>       | 17.52±1.93 <sup>c</sup>      |
|                          | one year     | 7.78±2.22 <sup>c</sup>   | 0.28±0.07 <sup>c</sup>         | 0.12±0.04 <sup>c</sup>       | 19.67±3.67 <sup>ab</sup>     |
| Mechanical scarification | one week     | 96.67±1.92 <sup>a</sup>  | 3.45±0.07 <sup>a</sup>         | 3.13±0.40 <sup>a</sup>       | 10.17±0.97 <sup>ab</sup>     |
|                          | one year     | 84.44±4.44 <sup>a</sup>  | 3.01±0.16 <sup>a</sup>         | 1.70±0.15 <sup>b</sup>       | 16.28±1.13 <sup>a</sup>      |
| F-test                   |              | *                        | *                              | *                            | -                            |

(GS) and Mean Germination Time (MGT) values of *M. Siamensis* seeds stored for one week compared with one year. \* significant  $p < 0.05$

Table 3 Analysis of variance (Two-way ANOVA) with interaction calculation

| Germination index | Source of variation | Sum of squares | Df | Mean square | F      | Sig. |
|-------------------|---------------------|----------------|----|-------------|--------|------|
| GP                | Storage period (S)  | 3008.333       | 1  | 3008.333    | 17.468 | .003 |
|                   | Treatment (T)       | 9823.148       | 1  | 9823.148    | 57.038 | .000 |
|                   | Interaction (S*T)   | 1134.259       | 1  | 1134.259    | 6.586  | .033 |
|                   | Error               | 1377.778       | 8  | 172.222     |        |      |
|                   | Total               | 61388.889      | 12 |             |        |      |
| MDG               | Storage period (S)  | 3.837          | 1  | 3.837       | 17.468 | .003 |
|                   | Treatment (T)       | 12.530         | 1  | 12.530      | 57.038 | .000 |
|                   | Interaction (S*T)   | 1.447          | 1  | 1.447       | 6.586  | .033 |
|                   | Error               | 1.757          | 8  | .220        |        |      |
|                   | Total               | 78.302         | 12 |             |        |      |
| GS                | Storage period (S)  | 4.180          | 1  | 4.180       | 27.958 | .001 |
|                   | Treatment (T)       | 10.058         | 1  | 10.058      | 67.273 | .000 |
|                   | Interaction (S*T)   | .197           | 1  | .197        | 1.319  | .284 |
|                   | Error               | 1.196          | 8  | .150        |        |      |
|                   | Total               | 42.604         | 12 |             |        |      |
| MGT               | Storage period (S)  | 51.049         | 1  | 51.049      | 3.513  | .098 |
|                   | Treatment (T)       | 86.506         | 1  | 86.506      | 5.954  | .041 |
|                   | Interaction (S*T)   | 11.820         | 1  | 11.820      | .813   | .393 |
|                   | Error               | 116.240        | 8  | 14.530      |        |      |
|                   | Total               | 3303.786       | 12 |             |        |      |

The analysis of variance was calculated by Two-way ANOVA between storage period (S) and treatments (T). The result of this calculation is presented in Table 3. Based on this calculation, the results showed that storage period (S) affected all germination indexes except MGT, and all germination index depended on treatments (T). The interaction of storage period(S) and treatments(T) are significant in GP and MDG.



**Figure 3.** Germination of *M. siamensis* seeds stored for one week within 28 days.

For conservation implementation, due to the reduction of *M. siamensis* number in natural habitat, the reproduction of its individual is required. The seed propagation is a cheaper method. Moreover, it can be re-introduced the number of the individual via direct seeding which is easier and cheaper (Elliot et al., 2013). The best treatment for germination is scarification by nail clip, but scarification process cost more time. The second choice of germination of *M. siamensis* is soaking in water and untreated seeds respectively. It saves more time than scarification by nail clip. Hot water and sulfuric acid shall not be used for germination test of this species.

### CONCLUSIONS

This species is non-dormant seeds. They seem to adapt to the environment. Time of fruit and seed ripened in the late of dry season in Thailand. Seeds are ready to germinate at the beginning of the rainy season. Thus, they do not need to be dormant waiting for moisture.

The germination test from this study revealed that mechanical scarification with nail clip gave the highest germination rate in this species. For *M. siamensis*, untreated seeds treatment is an option for producing seedlings due to cost saving. *M. siamensis* seems to be an orthodox seed This species can be applied for seed banking, but it needs to monitor long term survival rate after seed banking.

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**COLCHICUM TRIPHYLLUM AND HYACINTHELLA LEUCOPHAEA SSP. ATCHLEYI, SHEBENIK NATIONAL PARK, ALBANIA**

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**ABSTRACT**

Shebenik National Park is situated in the eastern part of Albania and has a rich flora. Ruen area is located in the south-eastern part of this national park, next to the border with the North Macedonia. During the field trips compiled in this area, we found a population of *Colchicum triphyllum* Kunze, that is the second location of this species in Albania and *Hyacinthella leucophaea subsp. atchleyi* (A. K. Jacks. & Turrill) K. Perss & Jim Perss., as a new subspecies for the country flora and a new location to the species as well. The Albanian flora has present five taxa of *Colchicum* genus, without including here the recently founded. The first location of *C. triphyllum* is reported and ecologically described as present from the southern part of Albania. For the genus *Hyacinthella* Albanian flora comprises only two species, *H. leucophaea* and *H. dalmatica* (Baker) Chouard. In this article are given data on the distribution and ecology for the two above mentioned species. Their occurrences and extended distributions in Albanian territory contributes in enriching the floristic diversity and values of this country.

**Keywords:** Flora, national park, location, distribution, ecology, Shebenik, Albania

**INTRODUCTION**

The mountaineous area of Ruen is located over Rrajca village, at the most south-eastern part of Shebenik National Park, next to the border with the North Macedonia. It is on limestone mother rocks and has different types of soils, vegetated by alpine grasslands and meadow, *Fagus sylvatica* forests, mixed beech and oak forest (*Quercus sp.*), shrublands etc. (Gjeta, 2014; Xiong et al., 2015; Teqja et al. 2017), representing a richness of floristic composition. All the water streams and sources or flows are collected by the Bushtrica river that constitutes the main river of the hydrological network in Rrajca. The location of *Colchicum triphyllum* Kunze is found on limestone rocks outcrops and the second location that of *Hyacinthella leucophaea subsp. atchleyi* (A. K. Jacks. & Turrill) K. Perss & Jim Perss., a new subspecies present for Albanian flora, was on siliceous substrata, near some parcels used for aromatic and medicinal plant cultivation by a private company.

For Albanian flora the *Colchicum* genus comprises 5 taxa five taxa in Vangjeli at al., 2000. Barina et al., 2011 reported one new taxon for this genus for the country flora, while two other new taxa were reported in Malo & Shuka, 2013, where was described for the first time



the presence of *Colchicum triphyllum* from the southern part of Albania. According to EDIT, 2022 there are listed 10 taxa as present in Albanian territory. Referring Barina et al., 2017, there are listed 8 taxa, of which 7 taxa have their distribution maps and *C. triphyllum* distribution map is presented with only one location in south Albania, the same location reported by Malo & Shuka, 2013.

The genus *Hyacinthella* is not included in Flora of Albania (Vangjeli et al. 2000) and EDIT, 2022. The two known species for Albania of this genus are reported by Barina et al., 2009, 2017. According to these literatures *H. dalmatica* (Baker) Chouard has a distribution between Shkodra and Tamara, on limestone substrata, whereas *H. leucophaea* has a wider distribution and occurrence only on serpentine.

## MATERIAL AND METHOD

In Shebenik National Park (Figure 1) are conducted several expedition in aim of evaluating its floristic diversity and values. From the Ruen areas are collected several plant exemplars, among them *Colchicum* and *Hyacinthella* species. The plant identification follows: Flora e Shqipërisë (Paparisto et al., 1988; Qosja et al., 1992, 1996; Vangjeli et al., 2000); Flora d'Italia (Pignatti, 1982); Flora e Europaea (Tutin et al., 1964, 1968, 1972, 1976, 1980) and consultations with National Herbarium. The data collections on their distribution localities and habitat ecology is based on Barina et al., 2010; Barina et al., 2013; Rakaj et al., 2013; Gjeta, 2014; Barina et al., 2017; EDIT, 2022; POWO, 2022; etc. Plant specimens were deposited at University of Elbasan.

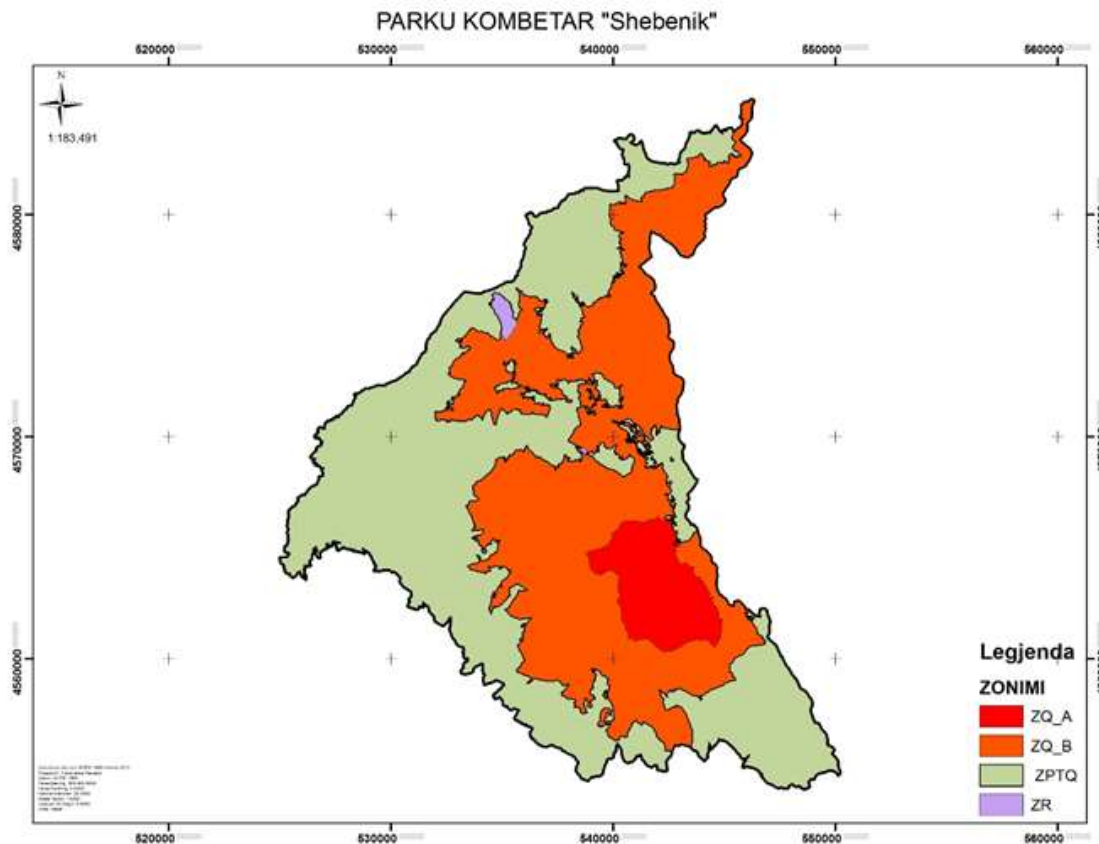


Figure 1. Shebenik National Park zonation map

## RESULTS AND DISCUSSION

During several field trips carried out in Ruen area, we found a population of *Colchicum triphyllum* Kunze and of *Hyacinthella leucophaea* subsp. *atchleyi* (A. K. Jacks. & Turrill) K. Perss & Jim Perss. 5 taxa belonging to the genus *Colchicum* are present in the Albanian Flora (Vangjeli et al., 2000). 3 other new species were added to the flora country list to this genus by: Barina et al., 2011 that reported *Colchicum bivonae* Guss from Saranda district as a new species to Albanian flora and Malo & Shuka, 2013 recorded and reported the occurrence of the two other new species, that of *C. doerfleri* Halácsy found in Dry Mountain in the east-southern Albanian part and of *C. triphyllum* in Çajupi Mountain in the southern part of Albania. *C. triphyllum* has a distribution from Mediterranean to NW Iran (EDIT, 2022; POWO, 2022; Alexiou, 2013).

### Family *Colchicaceae* DC.

#### Genus *Colchicum* L.

Species *Colchicum triphyllum* Kunze is herbaceous perennial plant, geophytes with flowering period February-April. It grows up to 15 cm, in dry pastures, calcareous soils and stony slopes, the bulb is spherical to ellipsoidal, 1.5-2.5cm, covered by a thin red-brown membrane, leaves 3(-4), 3-4(-7) cm at anthesis, 12-15 cm x 4-8(-10) mm at maturity, linear to lanceolate, obtuse to subacute, directed upwards at the flowering time, margins scabrid or glabrous, flowers 1-3, rarely 6, purplish-pink with perianth segments 15-30 x 6-12 mm, elliptical, obtuse, filament 5-7 mm, glabrous, anthers 2-5 mm, purplish-black or purplish green, pollen yellow, styles straight, stigmas punctiform. (Tutin et al. 1980, Malo & Shuka, 2013) (Figure 2, 3)



Figure 2. *Colchicum triphyllum*, Ruen, Shebenik National Park, Albania

In Ruen was found a small population in size of *C. triphyllum*, on limestone rocky substrate, in open areas near beech forest, at coordinates 41° 7'41.73"N and 20°34'49.74"E

(Figure 3, A), in a disturbed place, on a pathway that is frequented by tourists, residents of the village, herbal collectors or by livestock that graze in the upper pastures of Ruen. According to Malo & Shuka, 2013 the population of *C. triphyllum* reported and described from Çajupi Mountain (Figure 3, B greenmarked icon) found near a water sources close to the road, has a few individuals number.

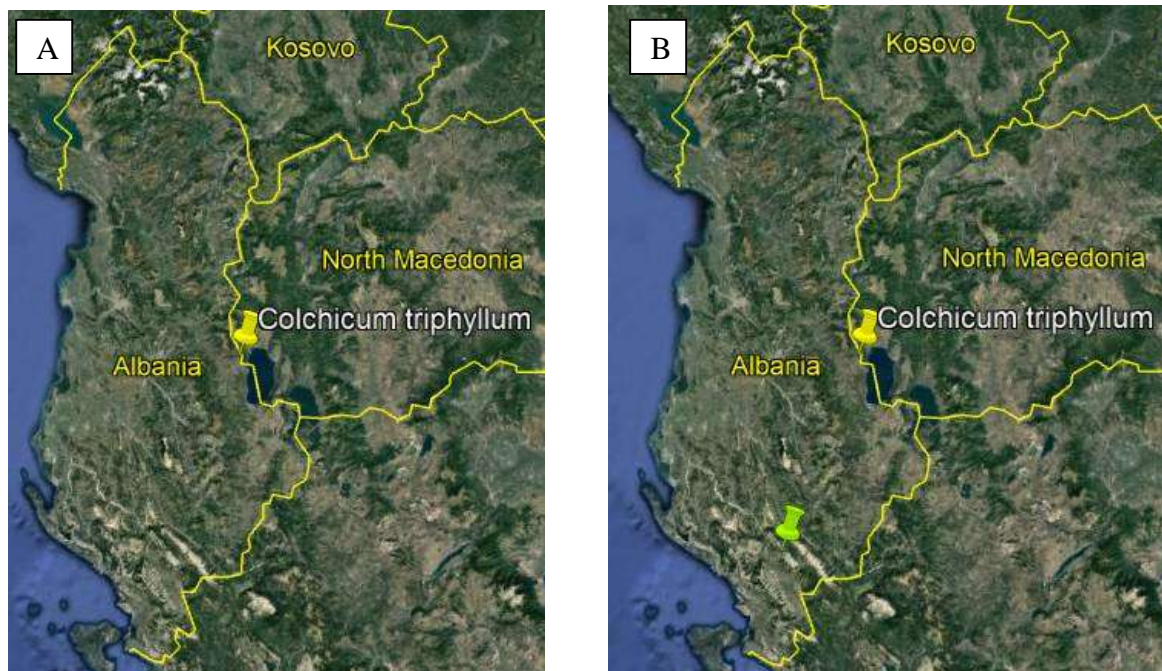


Figure 3. Distribution and location of *Colchicum triphyllum* in Ruen, Shebenik National Park (A) and in Albania (B) (Imagery from Google Earth)

*C. triphyllum*, as other plants belonging to *Colchicaceae* family, is a herb with mainly uses in pharmaceutical application because of the presence of colchinoids (alkaloids), such as colchicine and demecolcine. This species can produce rich extracts, largely contributing to antioxidants and other pharmaceutical properties, making this species a source for food, pharmaceutical and cosmetic industries. (Senizza et al., 2020)

A new taxon for Albanian flora, *H. l. subsp. atchleyi* (Figure 4, 5) was found in Ruen, located at 41° 8'33.41"N and 20°35'18.96"E, in an open area and siliceous substrata (serpentinite outcrops). Probably the substrate/soil where the population of this taxon was found, could have been discarded at that surface, as all the area around is on carbonate platform (Xiong et al., 2015) and serpentines with limestones rocks are divided between them at Bushtrica river.

**Family Asparagaceae Juss**

**Genus Hyacinthella Schur**

**Species Hyacinthella leucophaea (K. Koch) Schur**

Subspecies *Hyacinthella leucophaea subsp. atchleyi* (A. K. Jacks. & Turrill) K. Perss & Jim Perss. is herbaceous glabrous, bulbous, perennial plant, flowering period March – April, leaves linear lanceolate, basal, erect, (4)5-15(17) mm wide, with prominently raised veins, glabrous to scaberulous in margins, raceme oblong to ellipsoid, pedicels erecto-patent to less or more patent, usually 2-5 mm, periant erecto-patent to patent (to nodding), lively sky-blu to deep blu, 4.5-6(7) mm long, tubular-campanulate with spreading lobes. (Tutin et al. 1980; Persson & Persson, 2001; Tzonev & Panovska, 2017) (Figure 4)



Figure 4. *Hyacinthella leucophaea subsp. atchleyi*, Ruen, Shebenik National Park, Albania



Figure 5. Occurrence of *H. l. subsp. atchleyi* in Ruen, Shebenik National Park, Albania (Imagery from Google Earth)

The genus *Hyacinthella*, for Albanian flora, is presented by only two species (Barina et al. 2017), one of them is the *H. leucophaea*, which was firstly reported by Barina et al., 2009 from north Albania, in Shkodra district near Mjedë village and the road to Pukë and in other localities (Barina et al., 2010, 2017) (Figure 6).

According EDIT, 2022 the *H. l. subsp. atchleyi* occurs in all Albanian neighboring countries, in Greece, Bulgaria, Former Yugoslavia and Romania, but not in Albania (Vangjeli et al., 2000; Barina et al., 2017). While POWO, 2022, refers and lists this subspecies present in Albania with the literature source cited Barina et al., 2010, but in this publication are reported the occurrence and the locations only of the species *H. leucophaea* present for Albanian country, with localities in Mirditë, Pukë and Shkodër districts (Figure 6) and no occurrence neither locality of the subspecies *H. l. subsp. atchleyi*.



Figure 6. Occurrence of *H. leucophaea* in Albania reported by Barina et al., 2009, 2010, 2017 (A) and (B) its occurrences near Ruen, between Rrajca & Sutaj village (Google Earth)

## CONCLUSIONS

The presence in the area of Ruen, Shebenik National Park of *C. triphyllum* and *H. l. subsp. atchleyi* enlarge the distribution areas and increase the number of the floristic diversity not only for the Shebenik National Park, but for Albanian too. *C. triphyllum* is used as a medicinal herb, due to the presence of colchinoids and its presence, its distribution map, with only two localities, a few population of individuals, exposed to the impacted environments, indicate the needs for further protection and conservation considerations for this species in Albania. As well as the *H. l. subsp. atchleyi* as a new subspecies and a new location for the country flora needs further evaluation of its status in Albania.

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## **FLORISTIC COMPOSITION AND STRUCTURE OF THE ZOUAGHA FOREST (EASTERN ALGERIA)**

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### **ABSTRACT**

The general objective of this study is the rational management of forest ecosystems for the sustainable conservation of floristic biodiversity. Two types of analysis are carried out: structural analysis and analysis of the principal tree species. The dominant woody species are *Quercus suber*, *Quercus canariensis* and *Quercus afares*. The cork oak is the principal species in the Zouagha forest (East Algeria). The average density of this species in this forest is 375 individuals/ha. The structure shows that the trees in the height classes between 2 and 6 m have a very high density, which is evidence of natural regeneration. The total structure indicates that smaller trees are more abundant than larger ones ( $17.5 < d \leq 27.5$  cm). In effect, this work provides a database for the forest, but it represents only one facet to be considered for its protection and restoration.

**Key words:** Cork oak, Zouagha, Structure, Principal species

### **INTRODUCTION**

The Mediterranean forests represent a fragile natural environment and deeply disturbed by the action of the man. Barbero (1990), emphasizes that these ecosystems are characterized by two types of criteria: their spatial heterogeneity and their vulnerability due to their irregular exploitation by man. In Algeria, the oaks (green, cork, zeen, kermes and afarès) represent a forest capital, they cover large areas especially in the North and Northeast, or about 40% of the Algerian forest. However, the Algerian forest cover has been in recent years the scene of a great destruction. Its estimated at 1.3 million hectares of real natural forests is experiencing a regression quasi exponential and is today in a dismaying state (Alatou, 1984).

Given the policies and programs, the fate of Algerian forests remains tragic pernicious and hopeless. The estimation of the major disadvantage of mediterranean species, notably the oaks, lies in fact in a lack of knowledge of the ignorance of the forestry techniques which would be applicable to them in order to obtain the most satisfactory results. However, the valorisation of oaks requires the promotion and application of a silviculture based on a perfect knowledge of their growth. Taking into account these findings, we aim in this contribution, the description of the stand of the forest of Zouagha. This contribution, the description of the stand of the forest

of Zouagha, the latter is essentially made up of stands of *Quercus suber*, in order to establish a state of translated.

## MATERIAL AND METHOD

The forest of Zouagha is located in the north of the wilaya of Mila (north-east Algeria). The forest of Zouagha has been definitively classified in the state domain and subjected to the forestry regime by the government order of December 19, 1995 (DGF, 2018). It extends over an area of 3915.52 ha; it is characterized by an altitude that rises from south to center with a ridge line between Djbel Boughara and Kef Bouhamara. It reaches 1334m, then the altitudinal levels decrease to 600 m at the level of Oued El Mechta in the north, the altitudes then evolve by increasing to 1000 m in the extreme north.

### *Choice of study plots*

Two plots of cork oak are randomly selected with a rectangular shape and equivalent area of 2500 m<sup>2</sup> (50m x 50m), within each plot all individuals are inventoried (Rached-Kanouni *et al.*, 2020) all individuals are inventoried (Rached-Kanouni *et al.*, 2019). The parameters taken into consideration are:

- The diameter of the trees is measured on bark at chest height (1.30m) above the ground. The measurement can be made with a forestry compass.
- The total height (Ht) defined by the length of the straight line joining the foot of the tree at ground level to the end of the terminal bud of the stem.
- The geographical coordinates (longitude and latitude) of each plot were taken using a GPS taken with a GPS (Global Positioning System, satellite geolocation system).

### *Data processing and analysis*

All measurements and observations collected on the sampled trees during field trips (vegetation survey, GPS coordinates, etc.) are field trips (vegetation survey, GPS coordinates, etc...) are copied to the Excel spreadsheet to facilitate on the Excel spreadsheet in order to facilitate their treatment.

The stability of *Quercus suber* stands is measured using the height/diameter (H/D) ratio. This ratio is used at the stand level and is sometimes referred to "stability factor" to quantify the risk of significant windthrow (Oswald, 1984). In the second case, it is called the "slenderness factor", It is not only a form coefficient but also allows us to characterize the stability of a tree. characterize the stability of a stand and gives an idea on the dynamics of its growth (Rajoelison *et al.*, 2008). When: H/D < 100 represents a regular and stable stand with a complete and dense canopy. H/D > 100 means that the regular stand is unstable.

Abundance (A) is an important parameter for the description of a stand (Ramade, 2009). It provides an estimate of the stand density (number of stems per hectare stand (number of stems per hectare: N/ha) of a forest type (Rakotomalala, 2008).

n: number of trees inventoried in the plot;

s: plot area (in ha); s = 0.04 ha.

The dominance (G) evaluates the basal area of a stand. It is the total area of stem sections at 1.30m height for a given forest area. The dominance reflects the degree of filling of the forest (Razanatsimba, 2005). The dominance is given by the formula:

$$G = \sum gi = \pi / 4 \sum di^2 \text{ (m}^2 \text{ /ha)}$$

di: diameter at 1.30m from the ground of each stem (in m).



The natural regeneration corresponds to the seedling stage when the young plants have not yet grown beyond the herbaceous stratum and are 20 to 30 cm high, individuals of this stratum were in height, the individuals of this stratum were counted in the two study plots. Natural regeneration refers to the ability of an ecosystem (generally forest) to spontaneously reconstitute it self after the removal of all or part of the forest cover .

It is one of the modes of renewal of the forest, this one being able to be made also by clear cutting followed by replanting. This last solution, simple and proven, also allows a cultural rotation, to choose a species and/or an origin better adapted to the site (improved variety), or even according to the landscape desired landscape. In addition, natural regeneration can lead to a regular or irregular forest. The regeneration rate (%) indicates the ratio between the number of regenerated individuals (n) and the number of seed individuals (N) (Robisoa, 2008).

## RESULTS AND DISCUSSION

In forestry practice, understanding the structure of trees and forests is essential to management and development activities. All human interventions in forest management and nature conservation are essentially changes in the structure of forests for a specific purpose, by removing and adding individual trees. Therefore, a good understanding of forest characteristics helps to predict the likely outcomes of forest management. According to Pranjić and Lukić (1996), the term stand structure implies the distribution of species, the number of trees and the distribution of tree sizes per unit area (Indir *et al.*, 2013).

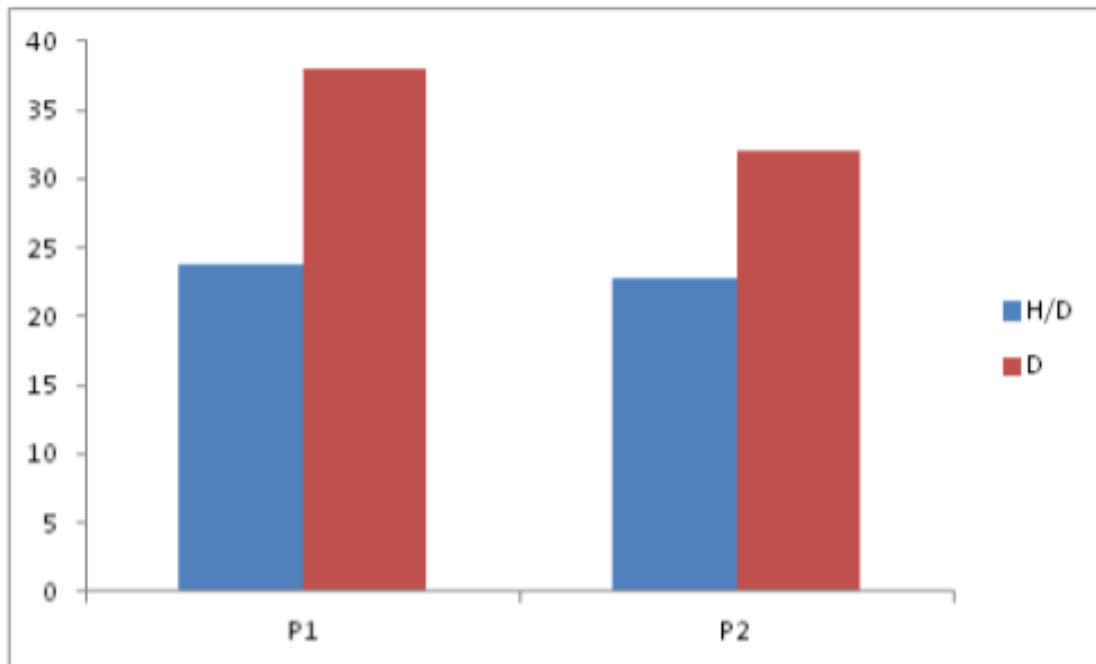
The horizontal structure of a species gathers the distribution of stems and basal area by diameter class. Since the density (abundance), basal area (dominance) and the developmental state of stands are strongly linked, the study of one cannot be done without the introduction of another (Rajoelison *et al.*, 2008) .This study will be done by taking into account at least two of the factors studied. This analysis consists in studying the spatial structure of the stands in terms of abundance and dominance. The results obtained are shown in table 1.

Table 1. Dendrometric characteristics of cork oak.

| Plot | D (m) | H (m) | H/D   | N (ha) | G (m <sup>2</sup> /ha) |
|------|-------|-------|-------|--------|------------------------|
| P1   | 0.38  | 6.11  | 23.73 | 275    | 60                     |
| P2   | 0.32  | 7.41  | 22.82 | 450    | 36                     |

The density of the stand is high in plot P2 (450 individuals/ha), while it is low in plot P1 (275 individuals/ha). The basal area of cork oak is significantly different in the two plots; it varies from 60 m<sup>2</sup> /ha in P1 to 36 m<sup>2</sup> /ha in P2. The youngest of all is the one in plot 2 with an average diameter at 0.32 cm on average; this stand is at the stage of a young forest stage. High diameters are observed on the trees of plot 1 with a maximum of 0.88, the trees have reached the stage of mature forest with diameters (Andriamahazo, 2003). The maximum values of the heights are also recorded in plot 1 (13.5 m).

For the two plots, having an average diameter between 0.38 and 0.32 m, have low slenderness coefficients between 23.73 and 22.82%). This observation allows us to assume that the slenderness coefficient is a function of the average diameter and therefore the age of the stand. The slenderness coefficients are less than 100 means that the stand is stable with a complete and dense canopy (Erlbeck, 2002). The figure 1 shows that the slenderness coefficient as a function of the average diameter of the of the stand.



**Figure 1.** Slenderness coefficient as a function of average diameter.

Natural regeneration is presented by young wood of less than 5 cm in diameter at human height, therefore diameter at man's height, therefore of basal area not yet measurable. It corresponds precisely to the state of thicket. As in the previous studies, the study natural regeneration of cork oak will be studied in ascending order of average stand diameter of the stand. The stand of cork oak is characterized by a low rate of regeneration and mortality. In general, the natural regeneration in the forest massif of Zouagha is high, it is 2200 seedlings/ha for the stands of the plot 1 and 1765 seedlings/ha in the 1 and 1765 seedlings/ha in plot 2. To the east and south, it is respectively 980 plants/ha and 820 plants/ha; while it is satisfactory for the stands exposed to the North with 215 feet/ha. It is satisfactory for the viability of this forest.

## CONCLUSION

Monitoring a forest allows for the detection of changes over time. All living environment is in perpetual change. The study of the characteristics dendrometric characteristics of cork oak following the study plots showed that this stable and regular stand with a complete and dense canopy, characterized by young and mature young and adult stage trees with a high rate of regeneration. By extending our study to the entire Zouagha forest, the results can serve as a baseline for serve as a reference condition for forest managers.

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## CONTRIBUTION TO THE STUDY OF THE VIABILITY OF THE ZOUAGHA FOREST (EAST ALGERIA)

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### ABSTRACT

The Zouagha forest is used for forestry and ecotourism. This massif is essentially formed of *Quercus suber*, *Quercus canariensis* and *Quercus afares*. It covers an area of 3915.52 ha. The quality of the stands can be given by the PHF index, a three-digit index that gives a judgement of the position of the tree in relation to the others and thus indicates the dominance and the stage of competition or exposure to the dominant stage. The objective of this work is to assess the viability of this forest by the PHF index in order to predict the future of this stand. The results obtained indicate that the majority of the trees observed have a state of health that varies between average and good, indicating that the forest remains viable despite being subject to several degradation factors. These results can also be used by forest managers in the management of this forest.

**Keywords:** PHF, cork oak, vitality, forest management

### INTRODUCTION

The Algerian forest, with a primeval surface area of 7318000 ha, is currently 2910000 ha. The rate of afforestation has therefore fallen from 27.17% to 11%, and despite its exploitation, it has never claimed to be a forest of high silvicultural production (Djallil, 1994). The forest wealth of the Zouagha forest is made up of a heritage of oaks (*Quercus suber*, *Quercus canariensis*, *Quercus afares*) and other trees of different species. However, this wealth is subject to latent degradation that may in the long term have harmful consequences both ecologically and socio-economically.

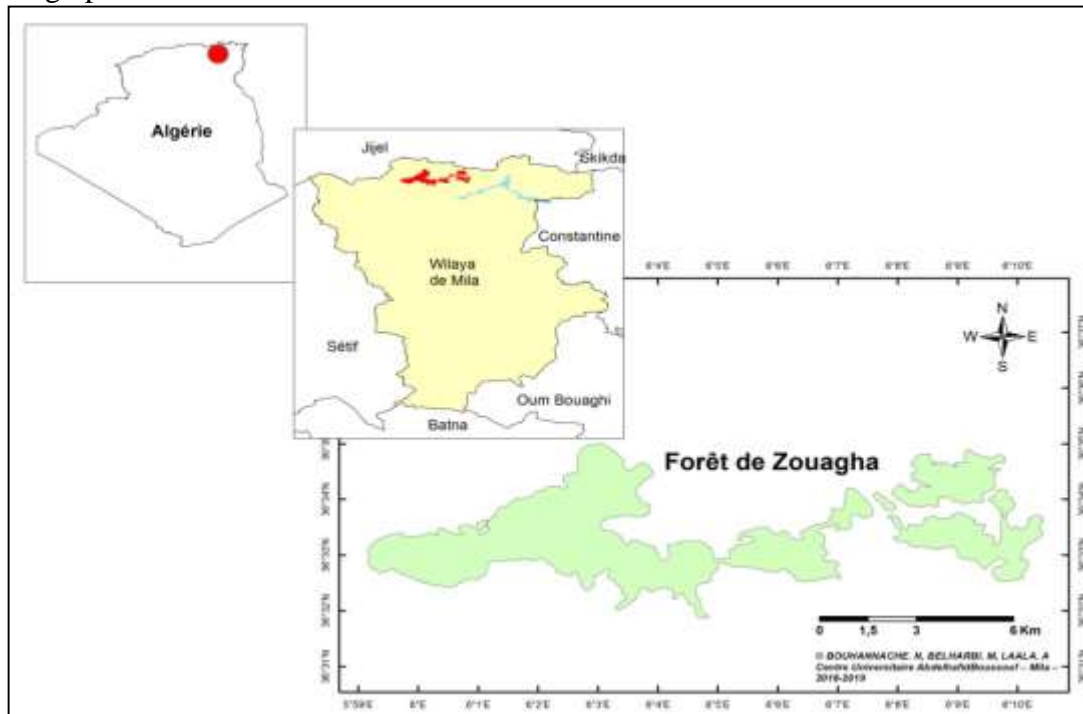
In the interests of sustainability, our study focuses on the Zouagha forest, which covers an area of 3106 ha and contains numerous forest species (broadleaved and coniferous). The forest thus has a scientific, educational, cultural and recreational function.

Sustainability monitoring is crucial to give credibility, validation and value to the options implemented, and deserves to be taken into account very early on, during the preliminary reflections. To achieve this, reliable indicators need to be developed. It will also be necessary to define a reference state to be able to constitute these indicators. This is why we thought it would be useful to carry out this study in order to know the viability of this forest massif, which could serve as a decision-making tool for future management work.

## MATERIAL AND METHOD

### *Presentation of the study area*

The Zouagha forest is located in the north of the wilaya of Mila (north-east Algeria). The Zouagha forest was definitively classified in the state domain and subjected to the forestry regime by the governmental decree of 19 December 1995 (DGF, 2018). It covers an area of 3915.52 ha; it is characterised by an altitude that rises from the south to the centre with a ridge line between Djbel Boughara and Kef Bouhamara, reaching 1334m, then the altitudinal levels decrease to 600 m at the level of Oued El Mechta in the north, the altitudes then evolve by increasing up to 1000 m at the extreme north.



**Figure 1.** Geographic location of the Zouagha forest (Belharbi and Benhannache, 2019).

Tree plots are randomly selected with a rectangular shape and an equivalent area of 400m<sup>2</sup> (20m x 20m); within each plot all individuals are surveyed (Rached-Kanouni *et al.*, 2019).

### *Estimation of the vitality status of cork oak by the PHF Index*

Stand quality can be given by the PHF index, a three-digit index that is a judgement of tree position (relative to others and thus indicating dominance and stage of competition or exposure to the dominant storey), general crown shape and bole shape (Robisoa, 2008).

- P represents the position of the crown and P can be assigned values from 100 to 500 depending on whether the crown is completely free and well exposed to the sun or not;

- H gives the shape of the crown and can take values from 10 to 50 which correspond respectively to the shape of the crown which is well symmetrical, poor or degraded; the crown of a dead tree is given the value 0.

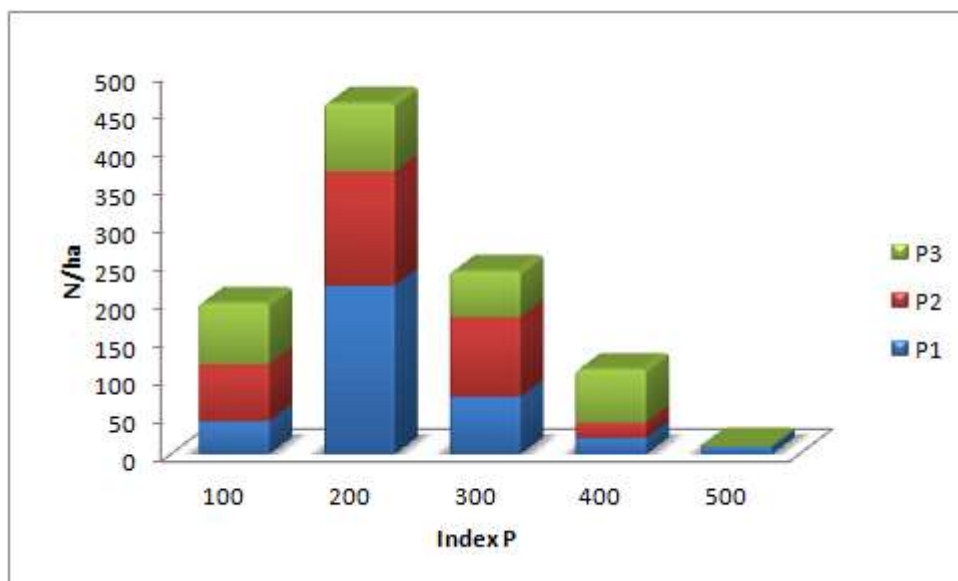
- Finally, the F index indicates the shape of the bole (with values from 1 to 6), i.e. a well-cylindrical and straight trunk up to a tortuous and or humped trunk (Andriamihariso, 2003).

## RESULTS AND DISCUSSION

Stand quality can be given by the PHF index, a three-digit index that gives a judgment on the position of the tree (P) in relation to others and thus indicates dominance and stage of competition or exposure to the dominant storey in general crown shape (H) and bole shape (F) (Robisoa *et al.*, 2008). It allows a more detailed silvicultural interpretation to predict the future of the stands (Blaser, 1990) and ultimately their viability (Rajoelison *et al.*, 2008).

The P-index gives an idea about the position of the crown of a tree under consideration and that of neighboring trees. It indicates the dominance, the stage of competition or the exposure towards the dominant state of the crown (Blaser, 1990). It is expressed by the values 100, 200, 300, 400 and 500 (Rasatatsihoarana, 2009). The results obtained are shown in Figure 2 where:

- 199 individuals/ha of three plots characterised by a P-index equal to 100, are in full light from above and laterally. These are generally dominant trees or trees in gaps.
- 462 individuals/ha in plots P1, P2 and P3, which have a P-index equal to 200, have full light crowns from above but lateral cover.
- 241 individuals/ha of the stand in three plots have a P-index of 300; they have a crown partially in full light from above.
- 112 individuals/ha have a covered crown (P=400), without top light and partially lit from the side, these are dominated overstorey trees or small trees at the edge of a gap.
- Only 13 individuals/ha (P1) have a fully covered crown, without direct light, these are mainly understorey trees (P=500).

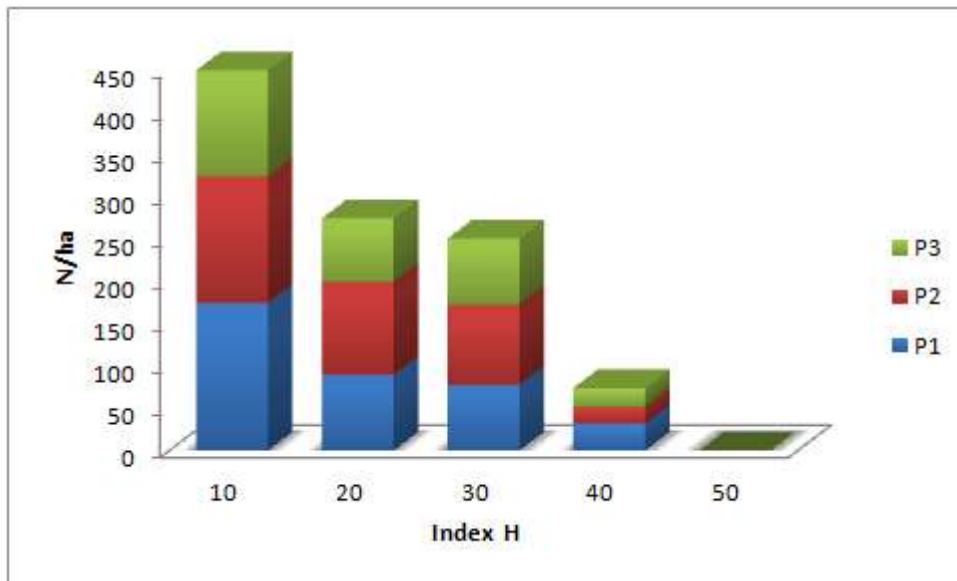


**Figure 2.** Index P of study plots.

In relation to the size and stage of development of a tree, the appearance of the crown quality will determine increment. The shape of the crown indicates qualitatively the previous development of a tree and probably its future potential (Blaser, 1990). The H-index varies from 10 to 50. From figure 3 it can be deduced that (Figure 3):

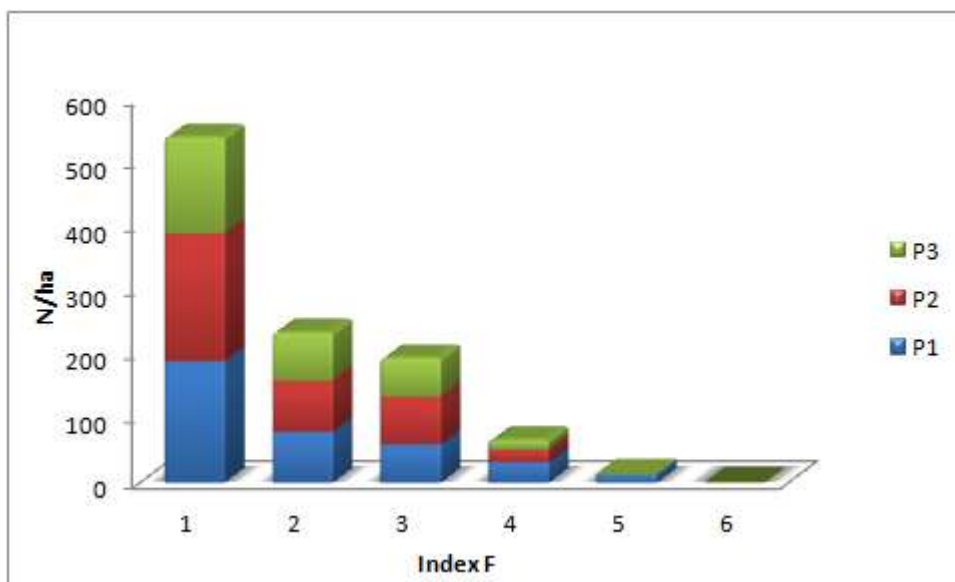
- 450 individuals/ha that have their H-index equal to 10 are the trees with a perfect crown (i.e. circular crown in plan, symmetrical, dense, extensive); these are the dominant trees in this stand.
- 275 individuals/ha with an H-index of 20 are trees with a more or less circular crown with some symmetry deficiencies.

- 251 individuals/ha with an H-index of 30 are trees with tolerable crowns (partially asymmetrical, open crowns).
- 74 individuals/ha with an H-index of 40 are trees with a strongly asymmetric crown.
- Trees with degraded crowns are totally absent (H = 50).



**Figure 3.** Index H of study plots.

The shape of the bowl is an index of wood quality. The shape of the bowl is not related to increment, but it certainly influences future silvicultural practices (Blaser 1990). Figure 4 shows that :



**Figure 4.** Index F of study plots.

- 540 individuals/ha have a straight, round and full, cylindrical bole, without defects, without branches.
- 275 individuals/ha have a straight, cylindrical, slightly rounded bole, without defects, without branches.

- 241 individuals/ha have a partially straight, partly cylindrical, generally conical and flawless bole.
- 112 individuals/ha have a straight bowl over a few meters, conical, without serious defects.
- 31 of the trees have an irregular, tortuous, strongly conical bole with partly defective forks.

## CONCLUSION

The Zouagha forest is made up of cork oak, the main species, occupying 50% of its area (6582 ha). The results obtained from the silvicultural analysis parameters showed a good viability level for this forest. This level of viability is generated by a stable and good stand quality (PHF=123) and almost zero mortality.

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## THE FLORISTIC DIVERSITY OF KRASTA PARK, ELBASAN, ALBANIA

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### ABSTRACT

Krasta park is in a pine forest hill located at the eastern part of Elbasan. Over 5.6 km of its area around Krasta peak is used for many years as a leisure park, which is under the administration of Elbasan municipality. Nearly fifty years before some of its surfaces were used for *Morus sp.* and pine or other coniferous species plantation, as well as some areas were used as military places, where now are found the remains of bunkers and other places used for military training. This park is very interesting for its floristic composition and vegetation, as it has a diversity of habitats and ecological areas, where can be found wild rare floristic species and ruderal, invasive, nonnative species, as well as new species for Albanian flora. In different areas such as calcareous outcrops, wet places, scrubs, meadows etc. we have found and identified 294 plant taxa, such as: *Fritillaria messanensis ssp. gracilis* (Ebel) Rix, *Sternbergia lutea* (L.) Ker Gawl. ex Spreng., *Ornithogalum sp.*, *Carex sp.*, *Allium chamaemoly* L. etc. We have analyzed the family abundance, their life form and chorological spectrum, as well we have recorded data on their ecological distribution.

**Keywords:** Flora, park, floristic diversity, chorology, life form,

### INTRODUCTION

Krasta park is a hilly forested park at the eastern part of Elbasan, with altitudes up to 270 m a.s.l. at Krasta peak. It is located on calcareous mother rock and has a surface which is mostly dominated by *Pinus halepensis* Mill. forest, but also can be found other ecological areas with a diverse flora and vegetation, such as cliffs, rocks with chasmophytic vegetation, wet meadows, scrubs, limestones outcrops etc. The park is used as a leisure place for family picnics, hiking, religious and historical sites, and is the most nearest green vegetated area for Elbasan city.

Also some areas of the park, for more than 50 years before have been used for *Morus sp.* and pine or other coniferous species plantation, as well as some areas were used as military places, where now can be found the remains of bunkers and other places used before 1990 for military training.

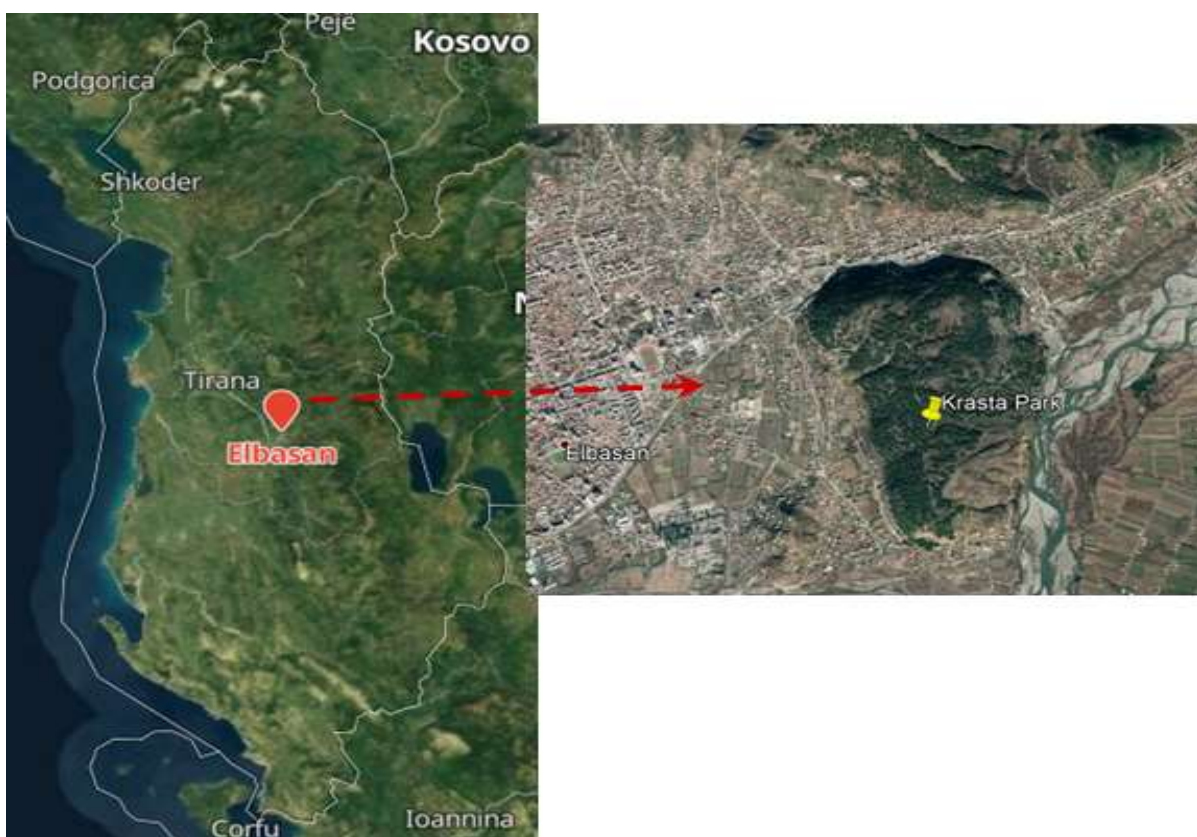
The park has a multitude of uses of the resources including grazing goats and cows, woodcutting and plant gathering, that increase the influence and the impact of the anthropogenic factor in this environment. Although this area is a disturbed place, it seemed that there are new species and a different plant community awaited around every bend.

In Krasta park we observed and identified 294 plant taxa, two of which were previously undocumented in Albania and quite a few more were rare, and compiled their floristic list. Some of the most interesting plant species, that we found and identified, include *Asyneuma anthericoides* (Janka) Bornm. generally considered an endemic of serpentine habitats, *Colchium autumnale* L. a beautiful plant of higher elevations, *Sisyrinchium rosulatum* E. P. Bicknell. a non-native species not known to occur in Albania (Gjeta et al., 2020), *Fritillaria messanensis ssp. gracilis* (Ebel) Rix a very rare lily and *Galium setaceum* Lam. (Gjeta et al., 2020) a species new to the Albanian flora.

The floristic diversity of the Krasta park reveals the dominance of the Mediterranean influence in this hill, as well as presence of the natural areas with rare and different species and habitats.

## MATERIAL AND METHOD

During several field trips in different areas of Krasta hill (Figure 1 and 2), we have worked on floristic observations, identification and data collections on the plant species and their habitat. We used for the plant identification, floristic list compilation and elaboration the following literature: Flora e Shqipërisë (Paparisto et al., 1988; Qosja et al., 1992, 1996; Vangjeli et al., 2000); Flora d'Italia (Pignatti, 1982); Flora e Europaea (Tutin et al., 1964, 1968, 1972, 1976, 1980); FNA, 2002; Barina et al., 2013; Goranova et al., 2013; Rakaj et al., 2013; Gjeta, 2014; Vangjeli, 2016; Barina et al., 2017; EDIT, 2022; etc.



**Figure 1.** Krasta park location, view from Google Earth

We surveyed different ecological areas around Krasta park such as rock outcrops, places around bunkers, pine forest areas, meadows, rock slopes, wet places, etc. for which we identified and recorded their floristic diversity and composition for further analyzes. For each

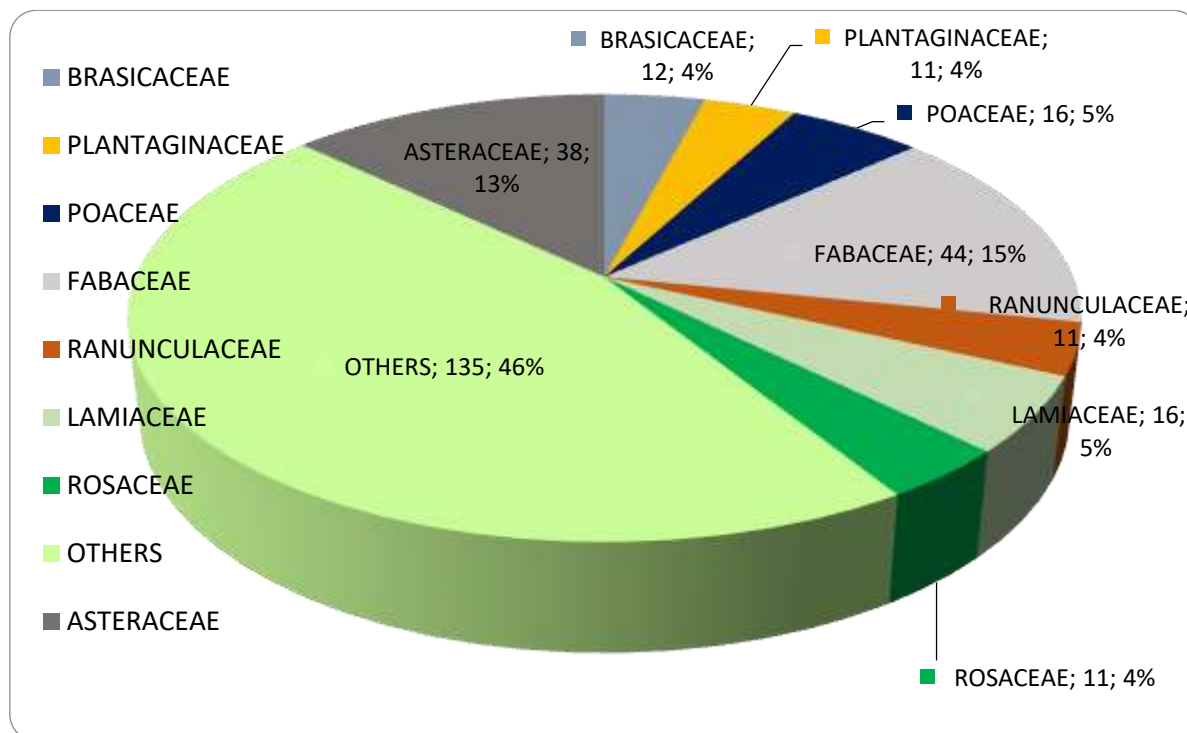
inventoried plant, we determined the species, compiled the floristic list for further analyzes and all the specimens collected were deposited at University of Elbasan “Aleksandër Xhuvani”.



**Figure 2.** Krasta park areas dominated by *Pinus halepensis* Mill. forest

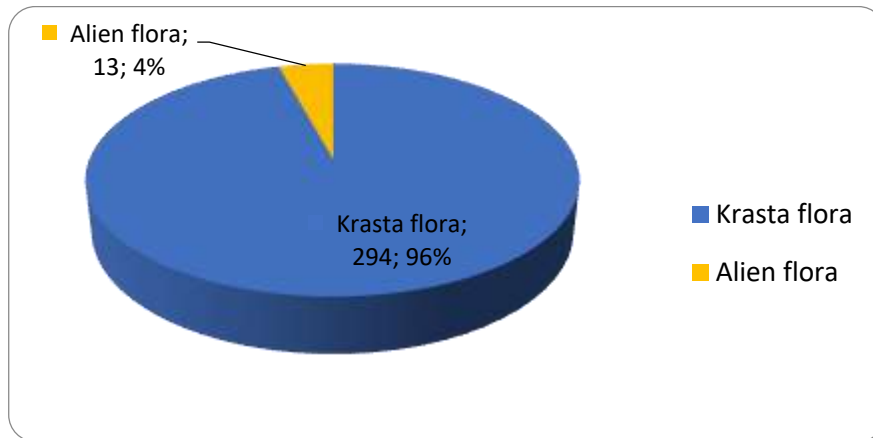
### RESULTS AND DISCUSSION

In the studied and explored areas of the Krasta park, we found and identified 294 plant taxa that belongs to 60 families. From them 52 families are presented up to 10 taxa, so we grouped them as “others”. The most representative family is the *Fabaceae* family with 44 plant taxa or 15% of the total flora number (Figure 3), with the most common genres that are *Lathyrus*, *Trifolium* and *Vicia*. The second family with a large number of plant species is *Asteraceae* family with 38 taxa or 13% of the total flora number. This family has a diversity number of genres, such as *Centaurea*, *Galatella*, *Xeranthemum*, *Artemisia*, *Crepis*, *Filago*, *Cota*, *Crupina*, *Hedypnois* etc.



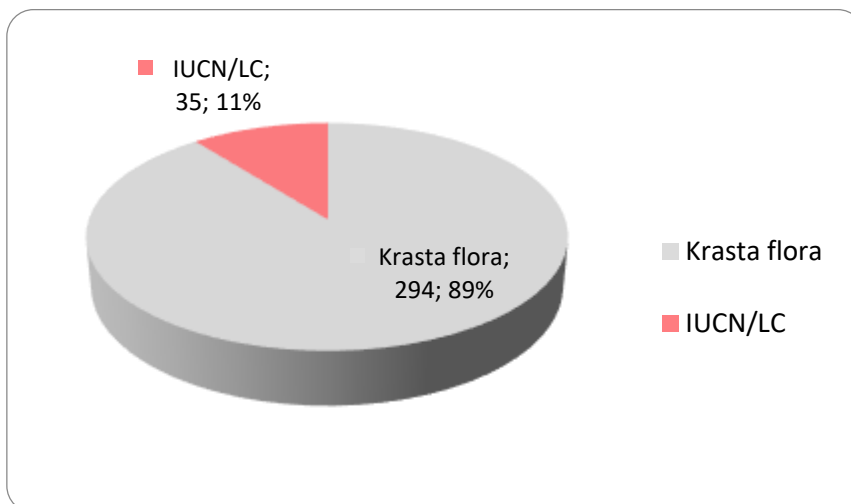
**Figure 3.** The plant family diversity of Krasta park

The 4% (Figure 4) of the floristic composition of Krasta park is manifested by alien flora species such as *Ailanthus altissima* (Mill.) Swingle (Figure 9, a.), *Phytolacca americana* L., *Oenothera speciosa* Nutt., *Morus alba* L. etc. that almost all them are natyralised species and populate the areas roasides and those that are common to human activities and presence. As well there are found new speciesto Albanian flora *Galium setaceum* (Figure 8, c) and *Sisyrinchium rossulatum* (Figure 9, b) (Gjeta et al. 2020).



**Figure 4.** The alien flora of Krasta park

The evaluated flora from IUCN categories compiles the 11% of the Krasta floristic diversity number within LC threaten status (Figure 5) and representative species such as: *Pinus halepensis* Mill., *Juniperus oxycedrus* L., *Lathyrus bauhinii* P. A. Genty, *Sesleria autumnalis* (Scop.) F. W. Schultz etc.



**Figure 5.** Evaluated flora of Krasta park by IUCN

The biological type (Figure 6) is represented almostly in an equal taxa number between two life forms, that of Therophytes 39 % and Hemicryptophytes 36%. The difference between them is of 10 taxa. The life forms presence reflects the adaptions and the relationships of the plants with the climate conditions.

The large number of therophytes is typical for areas with Mediterranean influence and that of hemicryptophytes is typical by temperate-continental influence (Goranova et al., 2013).

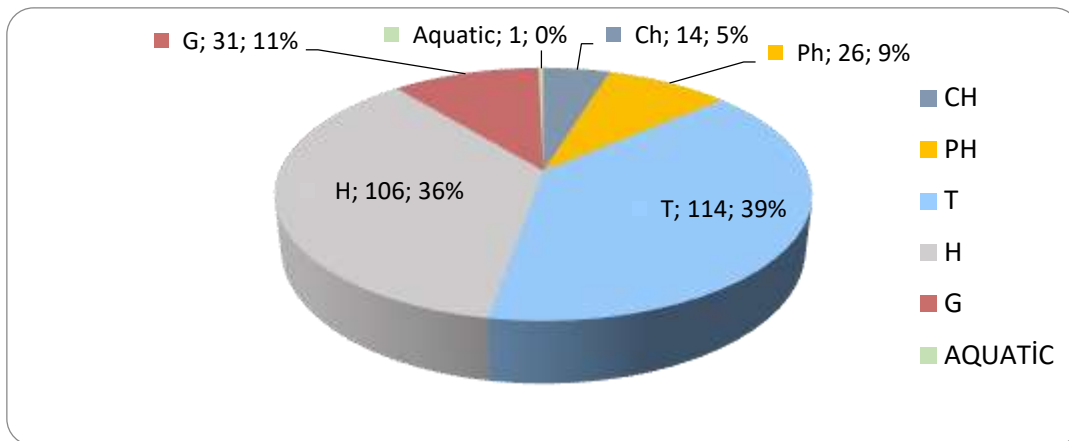


Figure 6. The biological spectrum of floristic diversity in Krasta park

Chorological spectrum (Figure 7) is represented by 29 chorological forms, which were grouped according their affinities into 8, for simplifying the graphical presentation and analyzation. Mediterranean element has the highest percentage with 125 plant species or 43% of total flora, which is followed by European element with 97 plant species or 33% present in the park. The dominance of Mediterranean element supports and reflects the Mediterranean climateric conditions of this area over the continental ones. Some Mediterranean species are *Anemone hortensis* L. (Figure 10, a), *Thymus longicaulis* C. Presl, *Hymenocarpos circinnatus* (L.) Savi etc. With a great interes is the presence of the Balkan species, which represent the 5% of the flora *Scabiosa tenuis* Boiss., *Crocus cancellatus* Herb., *Sideritis romana* L. *subsp. purpurea* (Talbot ex Benth), *Vincetoxicum hirundinaria ssp. nivale* (Boiss. & Heldr.) Markgr. (Figure 10, b) etc.

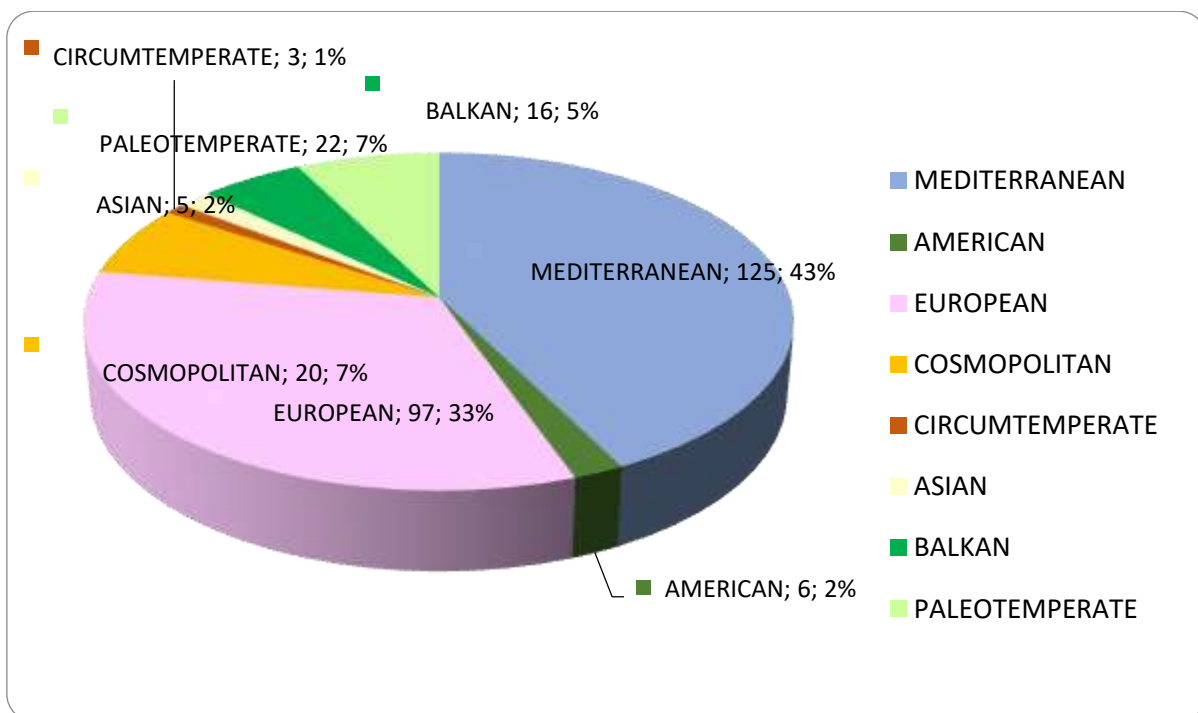


Figure 7. The chorological spectrum of floristic diversity in Krasta park

## CONCLUSIONS

The area of Krasta park has a floristic diversity of 294 plant taxa in which is distinguished the *Fabaceae* family dominance, with the most common genres that are *Lathyrus*, *Trifolium* and *Vicia*. The following most common family is *Asteraceae* with a diversity number of genres, such as *Centaurea*, *Galatella*, *Xeranthemum*, *Artemisia*, *Crepis*, *Filago*, *Cota*, *Crupina*, *Hedypnois* etc.

The plant species occupy different interesting ecological areas in Krasta park and their presence reflects and face the condition of natural habitats and the disturbance of the human pressure showed by the presence of the alien mostly natyralised flora, which is manifested by species of *Phytolacca americana* L., *Ailanthus altissima* (Mill.) Swingle *Oenothera speciosa* Nutt., *Morus alba* L. etc. Population and vegetation with rare plant species such as *Fritillaria messanensis ssp. gracilis* (Figure 8, a), *Galatella linosyris* (Figure 8, b), *Viola reichenbachiana*, *Asyneuma anthericoides* can be found too. The presence in this area of *Pinus halepensis* Mill., *Juniperus oxycedrus* L., *Lathyrus bauhinii* P. A. Genty, *Sesleria autumnalis* (Scop.) F. W. Schultz that are some of the plant species under the evaluation of IUCN categories and of two new species to Albania flora *Galium setaceum* and *Sisyrinchium rosulatum* increases the importance and the ecological values of this park.

With a major interest is the presence of the Balkan element with species such as *Scabiosa tenuis*, *Crocus cancellatus*, *Sideritis romana subsp. purpurea*, *Vincetoxicum hirundinaria ssp. nivale*. The dominance of Mediterranean element, reflected by mediterranean plant species *Anemone hortensis* L., *Thymus longicaulis* C. Presl, *Hymenocarpos circinnatus* (L.) Savi etc. that are adapted with the ecological condition in the park, is an indicator of the influence of Mediterranean over the continental climate.

Understanding and evaluating the plant distribution and presence in the areas of this park, serve as an indicator for a good management, protection and conservation of the biodiversity, natural habitats and ecological values.

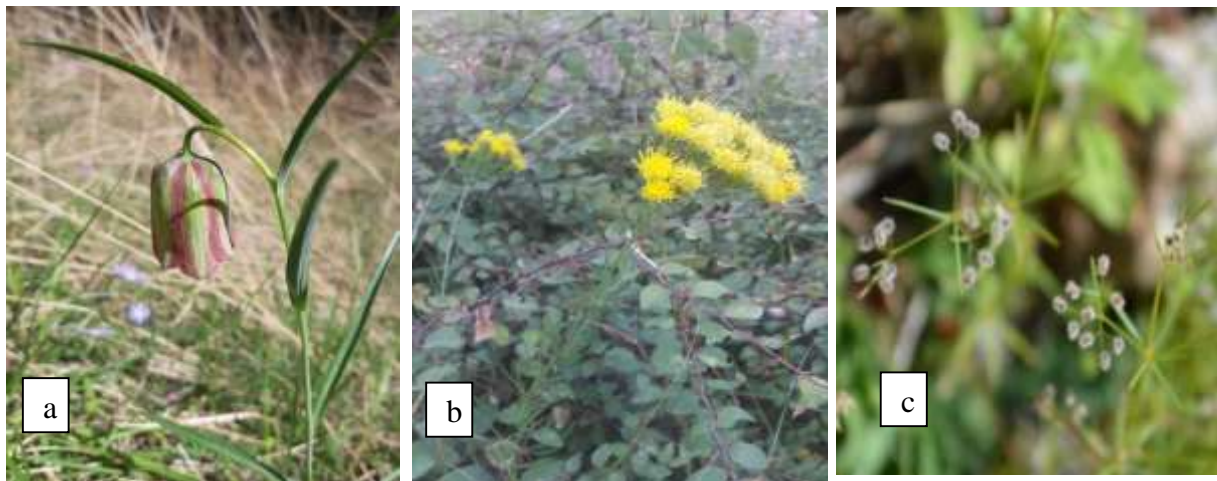


Figure 8. *F. messanensis ssp. gracilis* (a), *Galatella linosyris* (b) dhe *Galium setaceum* (c)



Figure 9. *Ailanthus altissima* (a) and *Sisyrrinchium rosulatum* (b) alien species

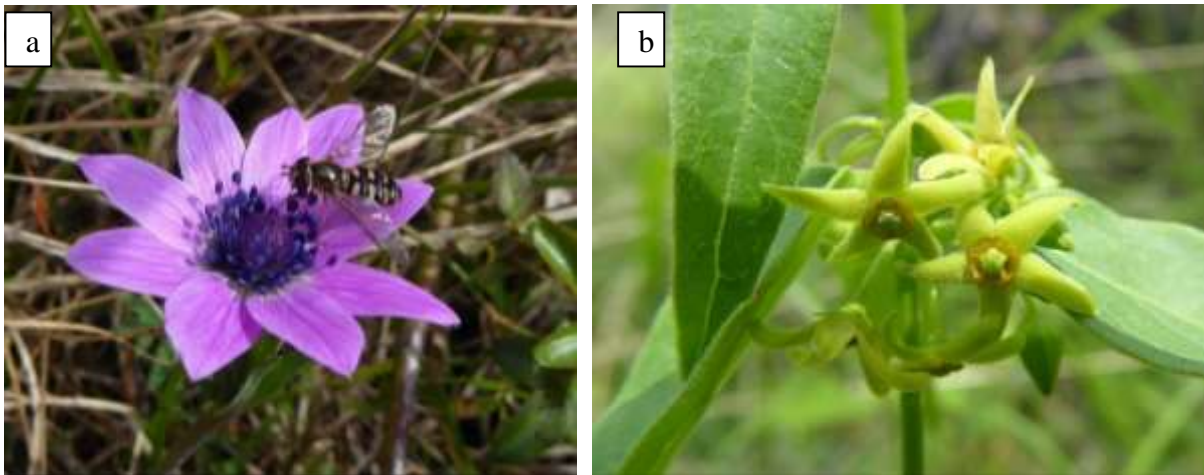


Figure 10. *Anemone hortensis* (a) and *Vincetoxicum hirundinaria ssp. nivale* (b)

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## VINEYARDS WATER STRESS ESTIMATION IN SOUTHERN ROMANIA TO INTEGRATE IRRIGATION MANAGEMENT STRATEGIES IN THE CONTEXT OF CLIMATE CHANGE

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### ABSTRACT

Determining the timing of water stress in vineyards, at the plot level, is of significant importance for supporting crop management decisions and applying precision farming practices. In this study, we will present an overview of the main meteorological parameters, average values of temperature, precipitation, and potential evapotranspiration Penman-Monteith (Eto) from the last 42 years representing the normal or average multiannual state that will be compared with the climate diagram of the agricultural year 2021, in the area where the research was carried out, in southern Romania. Based on these indicators were determined both the pluviometrical deficit and the surplus during an agricultural year. CWSI was determined using an easy-to-use portable device, crop water stress. Our results show that, from a thermal point of view, the average temperature of the agricultural year was 11.6°C, so with 0.8°C higher than normal. By the largest deviations of the monthly average values from the normal one, the month of February stands out, which had 5.5°C above the normal one, the situation being general for the whole country. According to this regression, the practical recommendation in irrigation scheduling is that CWSI values of 0.20 to 0.30 are suitable for the irrigation application of more than 250 to 500 m<sup>3</sup> ha<sup>-1</sup>. CWSI 0 indicates that the plant has no water stress and a value of 1 represents the maximum water stress. The values of the plants water stress index that signal the need for irrigation are crop-specific and should take into account factors such as the decrease in yield at the occurrence of water stress, probably the value of production, and the cost of irrigation water.

**Keywords:** climate change, drought, irrigation, crop water stress, *Vitis vinifera*

### INTRODUCTION

Water is a basic requirement for plant growth and performance. Both oversupply and shortage of water result in unbalanced vine growth and poor production. Irrigation is a powerful management tool for improving vine performance, as it allows us to manipulate an environmental variable (water). Not surprisingly, grapevine has one of the longest histories of irrigation of any agricultural crop. In the south of Romania, in the area where the research was done, the average annual rainfall in the last 42 years was 637 mm. However, the amount of rainfall can vary greatly from season to season, which may drastically impair vine performance and the economics of viticulture in some years. Moreover, the use of irrigation depends not

only on how much rainfall a vineyard receives but also on when the rain falls, and on how rapidly it evaporates. In addition, the amount of available water varies for different soil types; for example, fine loam soil has up to six times more available water than does coarse sand. Therefore, variation in soil moisture due to differences in water holding capacity and effective rootzone also has a pronounced impact on vine performance both between and within vineyards. Irrigation can be used as a supplement to compensate for the climatic shortcomings; i.e., to provide adequate moisture when nature does not supply it during the critical stages of the seasonal growth cycle. Climate change adaptation is a key to the future of agriculture, a particularly vulnerable economic sector that depends heavily on weather and climatic conditions. Climate change adaptation can be broadly defined as “the set of actions and processes that societies must take to limit the negative impacts of the changes and maximize their beneficial effect” (Carter, 1996). In the past three decades, abundant literature has been published on the impacts of climate change in viticulture. The major trends identified are a 50% increase in biomass production in an elevated CO<sub>2</sub> environment (Bindi et al., 1996); a 3 to 4 days per decade advancement of the vegetative and reproductive cycle due to higher temperatures, and a higher risk of water stress impacting yield in quantity and quality (Jones et al., 2005; Schultz, 2010; Mosedale et al., 2016; Van Leeuwen and Darriet, 2016, Deloire, 2020).

Climate change scenarios predict that Central Europe, including Romania, will be affected by water constraints, especially in southern Europe, changing rainfall patterns, and will suffer from summer drought (Serra, et al., 2014). Drought indicates the state of a biological system in which the water requirement is below the optimal values and works in terms of absorbing a considerable variable power depending on the growth phase and the stage of development. This phenomenon can be considered meteorological, hydrological, agricultural and economic. Many scientists report a rise in temperature in Europe, e.g. Guedon & Legave (2008) with about 1.1-1.3 ° C in France, Blanke & Kunz (2017) with 0.6 ° C and Waldau & Chmielewski (2018) with 1.9 ° C in different regions of Germany.

Adaptation of fruit crops grown in temperate areas in many places will be endangered in the future due to climate change, with warmer winters and earlier springs (Wenden et al., 2017, Florea et al., 2020). Climate data for the investigated region were first reported in Romania about two decades ago (Păltineanu et al., 2000, Florea et al., 2020), and the warming trend was observed by Păltineanu et al. (2011, 2012), later by Florea et al. (2020). Studies reported by Busuioc et al. (2015) highlight a trend of increasing air temperature in the period 1962-2010 in Romania, while Chitu et al. (2015) and Florea et al. (2020) highlighted the increased variability of seasonal and annual extreme temperature trends over the last three decades in the study region. Chmielewski (2004) mentions that the phenological stages of German crops have shown an advanced trend due to heating

In other parts of Europe, such as France, an advance in flowering tree species has been reported for some fruit tree species due to rising temperatures from January to April. Also in Germany, Chmielewski et al. (2011) showed an advance of the beginning of apple blossoming from 1989 to 2011, caused by climate change in the studied areas, and Rivero et al. (2017) in Scandinavia. Similar studies have been performed by Slavko Bernáth et al. (2021) which analyze the period 1985 to 2018 for the vine and report an earlier budding by five to seven days, the earlier onset of flowering by 7 to 10 days, the earlier softening of the berries by 18 days and dates of advanced harvesting with 8 to 10 days on average.

In the many Mediterranean and south-eastern areas of Europe, with low rainfall, often below 250 mm per year (Williams & Matthews, 1990), and the negative effects of climate change could be exacerbated, water scarcity has become a problem. Recent studies show that limiting water availability in vine cultivation can affect productivity (Chai et al., 2015; Pérez-Pastor et. al., 2014, 2016), moderate water scarcity can reduce yield, but with benefits some

aspects of fruit quality; Severe water shortages lead to low yields and lower fruit quality, while the absence of water exacerbates these negative aspects, thus harming the proper production of crops. Consequently, water storage has become a major environmental challenge to limit the expansion of irrigated agriculture (Williams et al., 2003). Some drought tolerance studies of cultivated vine genotypes have focused on key agronomic indicators, such as grape yield and physico-chemical composition (fruit quality indicators), while others have focused on the physiology of grapes to the finer scale of plants, such as stomatal regulation, carbon assimilation, etc. However, it remains an open and critical question for water management in vineyards and how different varieties respond to drought and water needs, fruit growth, yield, and quality. Although the yield is affected by drought, a recent meta-analysis reported by Dayer et al. (2020) suggests that this decrease may be specific to the variety.

Recent work on plant physiological indicators suggests that all genotypes can regulate the use of vine water (ie stomatal conductance) to protect against more severe damage, through physiological indicators provided by the petiole cavity or leaf, even fall (Hochberg et al., 2017; Dayer et al., 2020). Among these three main factors (biomass increase, cycle advancement, and water stress), the latter is the most preoccupying, as water resources are particularly vulnerable in most grape-producing areas in Europe.

Climate change effects on soil water balance and plant transpiration can impact crop productivity and quality significantly. Both quality and yield of crops can be enhanced by adequate and timely irrigation based on real-time monitoring of water status without increasing the level of agricultural water used. Such improvements in water use efficiency include changing irrigation frequency to match the crop water requirement that maximizes yield and quality. As an efficient indicator of crop water status, the crop water stress index (CWSI) has been introduced based on the difference between foliage and air temperature (Idso et al. 1981; Jackson et al., 1981), and it has been commonly used for computing CWSI (Siyu, 2021, Han et al, 2018; DeJonge et al., 2015; Irmak et al., 2000).

The water stress index (CWSI) is an indicator of the relative transpiration rate of a plant, which depends on the difference between the temperature of the leaf and the air and the pressure deficit of water vapor in the atmosphere. When the water level in the plant decreases, the stomata close and the intensity of perspiration decreases, while the temperature of the foliar apparatus exposed to direct solar radiation increases. When a plant is well supplied with water, it perspires with high intensity, the lower the temperature of the leaf, the lower the saturation deficit of water vapor in the atmosphere (1-2°C), in this case, the CWSI value is approaching 0. When the intensity of perspiration decreases, the temperature of the leaves exposed to direct solar radiation increases and can exceed by 4-6 ° C the air temperature. When the plant reduces its transpiration, CWSI tends to 1 (Păltineanu, 2009).

The transpiration is amplified by the deficit of water vapor saturation of the atmosphere. The use of crop water stress index (CWSI) is preferred in agriculture, being one of the best indicators for irrigation programming and management (Costa et. al., 2020; Jackson et al., 1981; Payero and Irmak, 2006; Reginato and Howe, 1985; Wanjura and Upchurch, 1991; Siyu 2021), for estimating production (Idso et al., 1981; Smith et al., 1992) and can also be used to assess grapevine drought stress conditions during the flowering-veraison phase (Yang et. al., 2021).

This paper evaluated the main meteorological parameters and their evolution over the last 42 years and the Water Stress Index (CWSI) from vineyards for three grape varieties for wine grapes. All these results can be used to take action and integrate on time the strategies of rational management of irrigation in vineyards.

## **MATERIAL AND METHODS**

### **Materials**

The study has been carried out in a grapevine plantation with genotypes for wine located at the National Research and Development for Biotechnology in Horticulture Ștefănești Argeș county, southern Romania. The region is characterized by a humid temperate-continental climate, with a mean annual temperature ( $T_{\text{mean}}$ ) of 9.6°C and a precipitation amount of 671,8 mm for the 1979-2020 period not uniformly distributed across the year.

The weather data were collected in the NRDBH Ștefănești meteorological platform, at 44,86° North, 24,96° East, and altitude of 278 m a.s.l., during 1979-2020. From 1979 to 2010 there was a continuous recording of weather data with instruments read by a meteorologist four times a day. Starting with 2010, there were weather measurements by meteorologists in parallel with direct readings by an automatic weather station IMETOS from Spectrum. The weather station is equipped with Watermark Soil Moisture Sensors 6450WD sensors, at two profile depths (30 cm depth and 60 cm), and the interpretation of how the rain was distributed throughout the growing season was made with the Spectrum Pro9 program.

Technologies Inc., USA. The soil from the experimental plots from NRDBH Ștefănești is loamy-textured, with a clay content of 30 – 37 %  $\text{g g}^{-1}$  throughout the 0 – 0,7 m depth profile (loamy-clay texture). Soil moisture content (SMC, %  $\text{cm}^3\text{cm}^{-3}$ ) throughout the experiment was generally measured weekly, using Delta - T Devices ThetaProbe PR1 moisture. The measurements were performed at intervals of 10 cm depth over a total depth of 0,5 m. The data were averaged over a soil depth of 0.5 m because grapevine roots in a young vineyard in such soils in the region explore the depth of about 0.5 m - 0,7 m.

The SMC data were subsequently converted to deficits of soil moisture (mm) as differences between field capacity (FC) and SMC. Deficits are equivalent to required irrigation depths (ID). The experiments for assessing the water stress of the vine (CWSI determination) were performed in August 2021, on three grapevine genotypes for wine 12 years old: Fetească neagră, Fetească albă, and Fetească regală.

The temperature of the plant leaf was measured with a high-precision infrared thermometer (Extech HD500, Extech Instruments). All determinations were made in a high-density young plantation, planting distance of 2.20 x 1 m, 4,545 hubs/ha; the genotypes are grafted on the rootstock SO 4-4, provided with the irrigation system consisting of 16 mm diameter tubes with drips arranged at 50 cm and a flow rate of 1.2 l / hour. The form of leadership is Multiple Guyot (low leading form). The vineyard maintenance and operation technology for high productive performance was applied.

### **Methods**

We had at our disposal for this analysis, daily climatological databases collected from the meteorological platform of the National Research-Development Institute for Biotechnologies in Horticulture Ștefănești Argeș, starting with 1979 (42 years of uninterrupted observations). From the daily data, the monthly averages of the following meteorological elements were calculated: thermal amplitude of the air (maximum daily temperature minus minimum daily temperature), average, minimum, and maximum temperature measured at the surface of the soil maintained as "black" field, relative air humidity (average, maximum and minimum), amount of precipitation, wind speed 2 m above ground level, potential reference evapotranspiration (grass), calculated using the Penman-Monteith equations from InStat + v3.38 (Statistical Services Center of the University) from Reading, UK and rainfall deficit, representing the difference between  $ET_o - PM$  and the amount of rainfall. Crop Water Stress Index (CWSI) was developed in response to the need to quantify plant water stress beyond the individual leaf.

The temperature of the plant leaf was measured with a high-precision infrared thermometer psychrometer (Extech HD500, Extech Instruments), oriented when making determinations in direct sunlight. The electronic psychrometer with an infrared thermometer, HD500, at the time of the determinations was oriented so that the sun was behind the operator and the measurement direction is as close as possible to the direction below in which the sun's rays fall on the measured areas (crowns of vines).

To establish the lower baseline, which expresses the lack of water stress of the plants, the temperature of the foliage was determined on plants very well supplied with water by irrigation, from 8:00 to 16:00, from one meter away from the foliar apparatus of plants, always on the sunny side of the canopy, in a measuring spot with a diameter of about 3-5 cm that included both the sunny side of the leaves and the shaded one. Air temperature and relative humidity were measured with a 64 suction Assmann psychrometer and the Extech HD500 infrared thermometer psychrometer.

All measurements were downloaded via a serial interface to a notebook in the field. These data were used to determine the differences between the mentioned temperatures (leaf temperature - air temperature), the saturation deficit of water vapor in the atmosphere (VPD, kPa), the lower baseline (LBL), the upper baseline (UBL), and, in finally, the crop water stress index (CWSI). The lower limit of CWSI will change as a function of the saturation deficit of atmospheric water vapor pressure (VPD) because at lower VPD values the leaf perspiration occurs at a lower rate, so the magnitude of the process of cooling the leaves compared to air temperature in the vicinity is low. Idso (1982) demonstrated that the lower limit of CWSI is a linear function of VPD for several crops, especially depending on the species, but also the particularities of the variety. For the CWSI calculation, the lower baseline (LBL) and upper baseline (UBL) were first calculated, after Idso et al., 1981 and Jackson et al., 1981. The difference between leaf temperature ( $T_c$ ) and air temperature ( $T_a$ ), respectively  $T_c - T_a$ , was then plotted against the atmospheric water vapor voltage (VPD), and the baseline was obtained by linear regression ( $Y = a + bx$ ) of  $T_c - T_a$  ( $Y$ ) versus of VPD ( $x$  used), least-squares method.

The water stress index of the plants was calculated by determining the relative distance between the lower baseline, which conditions the water nonstress, and the upper baseline which conditions the lack of perspiration. Then, the CWSI was correlated with both soil moisture content (SMC) and irrigation water depth (ID) in a loamy-clay texture soil in southern Romania, for use in irrigation scheduling.

## RESULTS AND DISCUSSION

To analyze the behavior and critical periods for the water of the vine in the growing season 2020-2022, we compared the monthly average values of meteorological parameters in the agricultural year October 1, 2020 - September 30, 2021, with their multiannual average values (1979 - 2020). We rely on this assumption that plants adapt and can withstand the values of meteorological factors that occur more frequently in the vine-growing area and are all the more vulnerable to extreme values, as they are recorded with a lower frequency. The growth and fruiting of perennial crops are inhibited much more often by unfavorable environmental conditions than by the particularities of the genotype, so the real productivity is far below the potential, genetically determined (Van Leeuwen et. al, 2009).

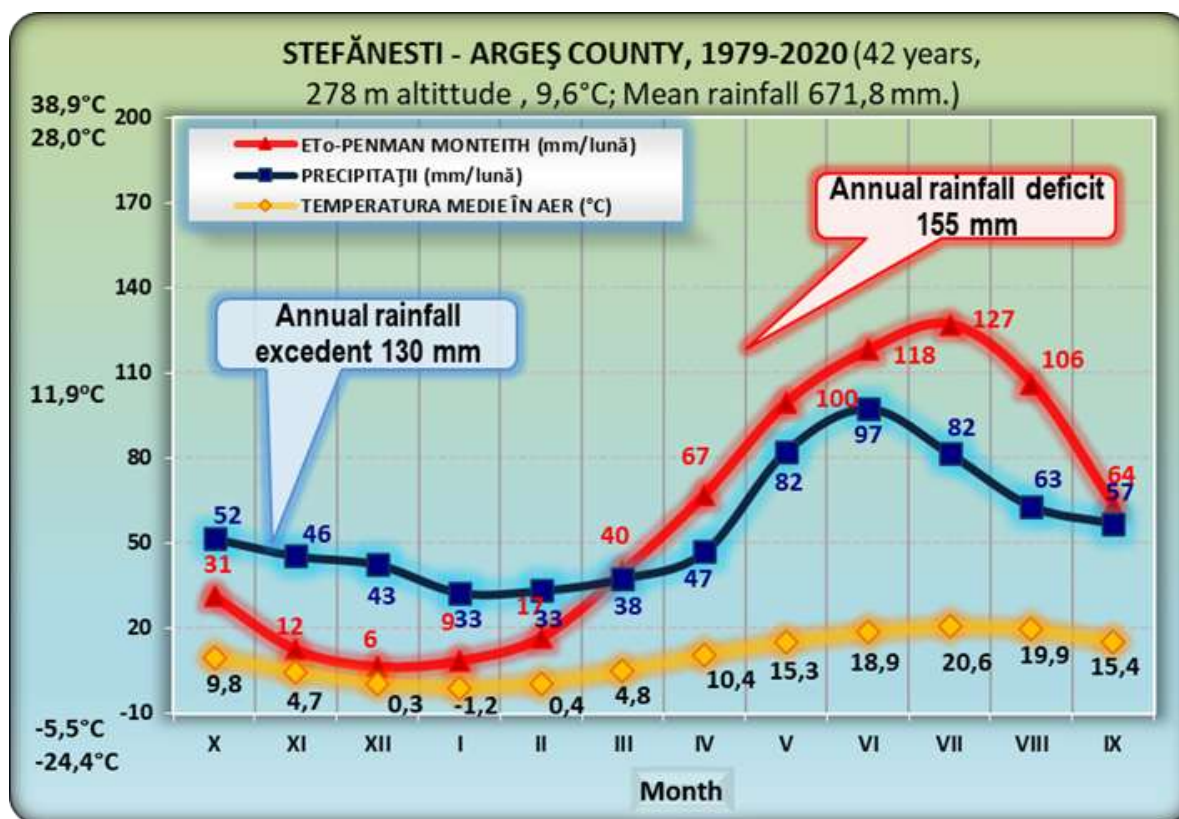
The multiannual, average state is presented in a climate diagram (Figure 1) adapted from Walter (1975), which shows the monthly evolution over the last 42 years of average air temperatures, precipitation, and potential reference evapotranspiration. Penman-Monteith (ET<sub>o</sub>-PM), is in the area where the research is being carried out. It can be seen at the top of the

climate diagram that the average air temperature, over the period for which there are climatological data, was 9.6°C, and the sum of the annual precipitation of 671.8 mm.

Figure 1 also shows the thermal extremes as follows (left side of the graph, from top to bottom): the absolute maximum temperature recorded in the air was 38.9°C (July 4, 2001), and the average of the maximums of the warmest month (July) of 28.0°C, the average daily thermal amplitude of 11.9°C, the average of the minimums of the coldest month (January) of -5.5°C and the absolute minimum temperature -24.4°C (January 1985).

Regarding the hydrological regime, characterized here by the difference between the monthly values of ETo-PM and precipitation, there are two distinct seasons: one from autumn to winter (5 months, from October to February) with a rainfall surplus of 130 mm and another longer one, from spring to summer (7 months, from March to September) with a significant deficit of precipitation (155 mm).

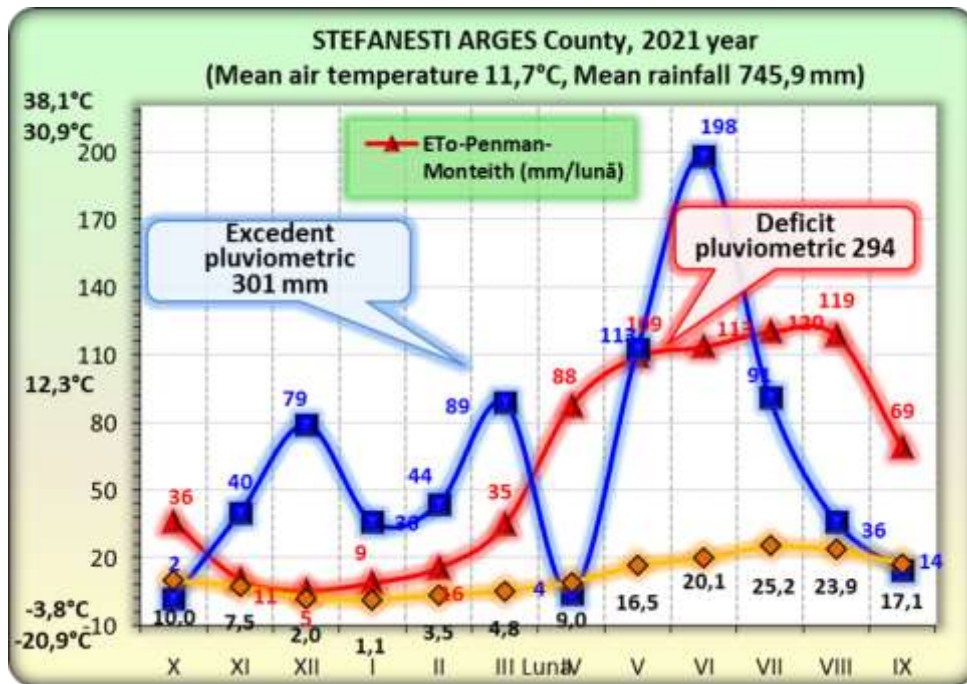
The driest months are July with a deficit of 45.5 mm and August with 43.2 mm (about 60% of the annual deficit concentrated in just two months), followed by June with 20.8 mm, more with 17.2 mm, and April with only 10.5 mm.



**Figure 1** Evolution of monthly values of temperature, precipitation and evapotranspiration Penman-Monteith potentials, 1979-2020

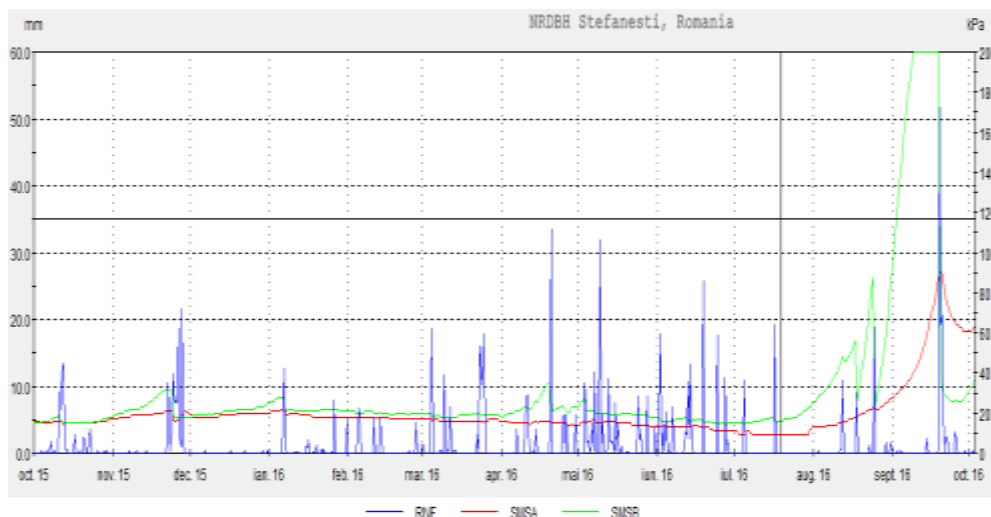
At the same time, the climatic conditions of 2020-2021, and the level of monthly average indicators were analyzed (Figure 2). From the thermal point of view, the whole agricultural year follows the trend manifested in the last 30 years of warming weather: the average temperature of the agricultural year was 11.7°C, so 2.1°C higher than normal.

By the largest deviations of the monthly average values from normal, February stands out, which had 3.2°C above normal. Also, the amount of precipitation during the agricultural year was 74.1 mm higher than normal (745.9 mm compared to 671.8 mm as normal).



**Figure 2.** Evolution of monthly values of temperature, precipitation, and evapotranspiration Penman-Monteith potential in the agricultural year 2020-2021

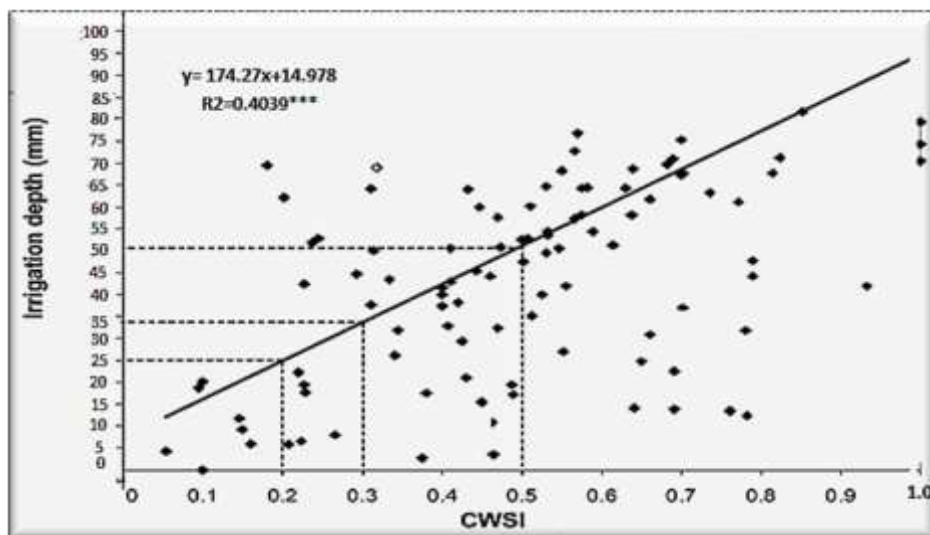
The dynamics of groundwater potential at 2 depths per profile (30 cm depth and 60 cm), shown in Figure 3, (measured with Watermark Soil Moisture Sensors 6450WD sensors) provided at the weather station, demonstrates how rainfall was distributed throughout the growing season. It can be seen that the values of soil water potential did not exceed 20 kPa (approximately the capacity of the field for water) throughout the growing season, requiring additional irrigation, depending on the needs of the plant for a particular phase of vegetation



**Figure 3.** Dynamics of precipitation and soil water potential at 30 cm depth (SMSB) and 60 cm depth (SMSA), on the meteorological platform from NRDBH Stefanesti - Argeş (The data in figure 3 were interpreted using Spectrum Pro9 software)

The analysis of the main meteorological indicators conducted in this study shows that the calculation of a periodic CWSI by monitoring the temperature of the vine canopy and the

environmental conditions of the vineyards can be used to schedule irrigation. To save water resources, irrigation under water stress conditions is recommended both for the current situation and especially for global warming scenarios, provided that soil moisture does not reach the level of the wilting coefficient on all sub-horizons of the root system. Irrigation brings substantial increases in production (Han et al., 2018), as well as larger fruit sizes, but in the context of global warming, when the use of water resources is increasingly restrictive, the optimal application of water no longer seems a unique solution; on the contrary, the most rational use of irrigation water by irrigation under water stress, even if no maximum harvest is obtained, is a recommended option (Luan et al., 2021, Han et al., 2018, O’Shaughnessy et al., 2017, Păltineanu et al., 2009.). Adaptation to climate change in recent years presupposes the appreciation of plant water stress for adequate and timely irrigation based on real-time monitoring of soil water status, but also the main meteorological parameters. Lack of water in globally irrigated agricultural areas puts more pressure on irrigators to maximize their productive use (Araus, 2004). Extensive irrigation research on vines has shown that soil water deficits leading to vine water stress can lead to reduced leaf gas exchange, accelerated ripening, loss of yield by decreasing berry weight and may have a negative impact on grape composition and wine quality (Chapman et al., 2005; Chaves et al., 2010; Dry, et. al., 1998; McCarthy, 1997; Ojeda, 2002; Van Leeuwen, et. al. 2009). These responses are genotype-dependent (cultivar) within *Vitis vinifera* L.; for example, the “Grenache” vine has regulated its hydraulic path more strongly than the “Chardonnay” in response to water stress (Vandeleur, et. al, 2009). In another study, the vine "Syrah" ("Shiraz") had a similar response to "Chardonnay" when subjected to water stress in the field (Schultz, 2003). These intra-species differences, called “isohydric” and “anizohydric” (Pagay and Kidman, 2019), are related to differences in the stomatal behavior of varieties in response to the availability of soil moisture. The isohydric behavior is characterized by stomatal closure under decreasing soil moisture to maintain homeostasis of leaf water potential. Under the same conditions of decreasing soil moisture, the anizohydric stomatal behavior results in a decrease in the water potential of the leaves, as the stomata remain relatively open compared to isohydric plants (Schultz, 2003; Costa et. al., 2012).



**Figure 4.** Correlation between CWSI and irrigation depth over the 0 – 50 cm soil depth vine plantation for wine grapes, Feteasca regală, Feteasca albă, and Feteasca neagră, summer 2021 at NDIBH Stefanesti, Romania

The linear correlation between the CWSI of the vine and the irrigation depth over 0 - 50 cm calculated by the least squares method has a very significant regression equation, although



with a relatively low value of  $R^2$  (0.414 \*\*\*). According to fig. 4, an ID value of approximately 35 mm of irrigation corresponds to a CWSI value of 0.2, and 50mm ID to 0.50 CWSI. This regression equation could be used in scheduling irrigation in the vineyards under the environmental and cultivation conditions described here, on the soils of southern Romania. However, due to the relatively low value of  $R^2$ , more replicates should be taken to determine the CWSI for each vineyard plot. At the same time, further studies are needed to increase the number of determinations and thus the  $R^2$  value. The water potential of the soil should also be considered in the future. According to the regression equation, the practical recommendation in irrigation scheduling is to apply irrigation for CWSI values of 0.20 to 0.30, 250 to over 500 m<sup>3</sup> ha<sup>-1</sup> (or over 25 and over 50 mm, respectively). This amount of irrigation depth is common for such soils under experimental conditions, and a CWSI of 0.20 for the onset of irrigation is also consistent with the findings of Ben-Asher et al. (1989) and Ben-Asher and Phene (1992). Therefore, it is recommended that CWSI values greater than 0.5 be exceeded before irrigation is applied to avoid any leakage and deep leakage. For the CWSI calculation, the intensity of the correlation between the temperature difference between leaf and air and the water vapor pressure deficit for each researched genotype was plotted (graphs were drawn with Vpd as horizontal axis and Tc-Ta difference as vertical axis), (Figures 4,5, 6). The space between the lower basal lines LBL and the upper basal line UBL is the domain of this index. The slope of the straight lines corresponding to the lower baselines (non-stress) - an indicator of adaptability to water stress, for the three varieties, is slightly different from one variety to another but is particularly pronounced. The higher the slope, the more intensely the plant perspires in the area of higher water vapor pressure deficits. The lower the slope of the straight, the less the plants sweat and are better adapted to high temperatures. The temperatures determined by us with the Extech HD500 infrared thermometer psychrometer represent average values of shady and sunny areas, obtained by continuously changing the position of the measuring spot on the leaf surface of the entire plant. In the Fetească alba, the surface of the leaves since the morning, recorded values between 24.2°C in shady areas and 35.8°C in sunny areas of leaves, while the leaf surface was perpendicular to the direction of the sun's rays and the plants were well supplied with water from the soil. For the Fetească regală cultivar, the surface of the leaves, at noon, registered values between 25.2°C and 35.6°C in the shaded areas, and the Fetească neagră cultivar recorded a temperature of 24.0 °C and 35.1 °C. Figure 5 shows that, at values of water vapor deficit in the atmosphere of 3.5 kPa, the Fetească regală variety has a leaf about 6°C colder, suggesting that it is less adapted to water stress than the Fetească neagră variety (figure ...., only 4.2°C colder than air at the same pressure deficit of water vapor in the atmosphere. In the case of the Feteasca albă variety, it is also observed that the slope of the straight lines corresponding to the lower basal lines (non-stress) is less accentuated than that of the Feteasca regală variety, which indicates its better adaptability to water stress. CWSI remains an easy method of field determination. When a plant is well supplied with water, it sweats with great intensity, the lower the temperature of the leaf, the lower the saturation deficit of water vapor in the atmosphere (1-2°C), Godson-Amamoo (2022), Păltineanu (2009), in this study the CWSI value is approaching 0. When the intensity of perspiration decreases, the temperature of the leaves exposed to direct solar radiation increases and can exceed by 4-6°C the air temperature. When the plant reduces its transpiration, CWSI tends to 1 (Luan et al., 2021, Păltineanu, 2009). The transpiration is amplified by the deficit of water vapor saturation of the atmosphere.

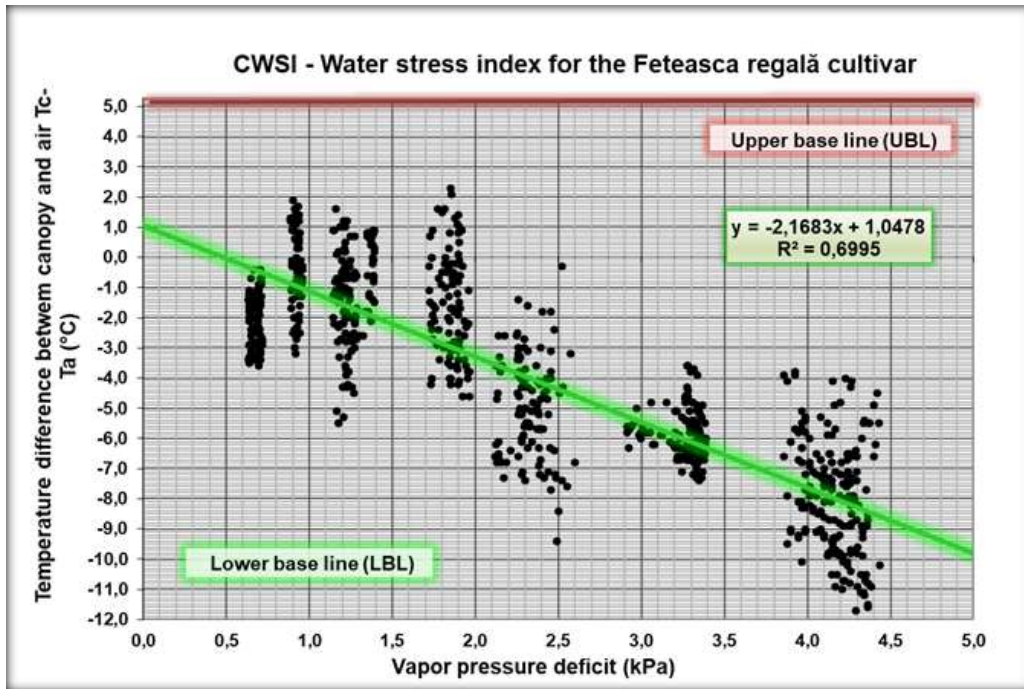


Figure 5. Lower and upper baselines are required to calculate the stress index for the Fetească regală variety

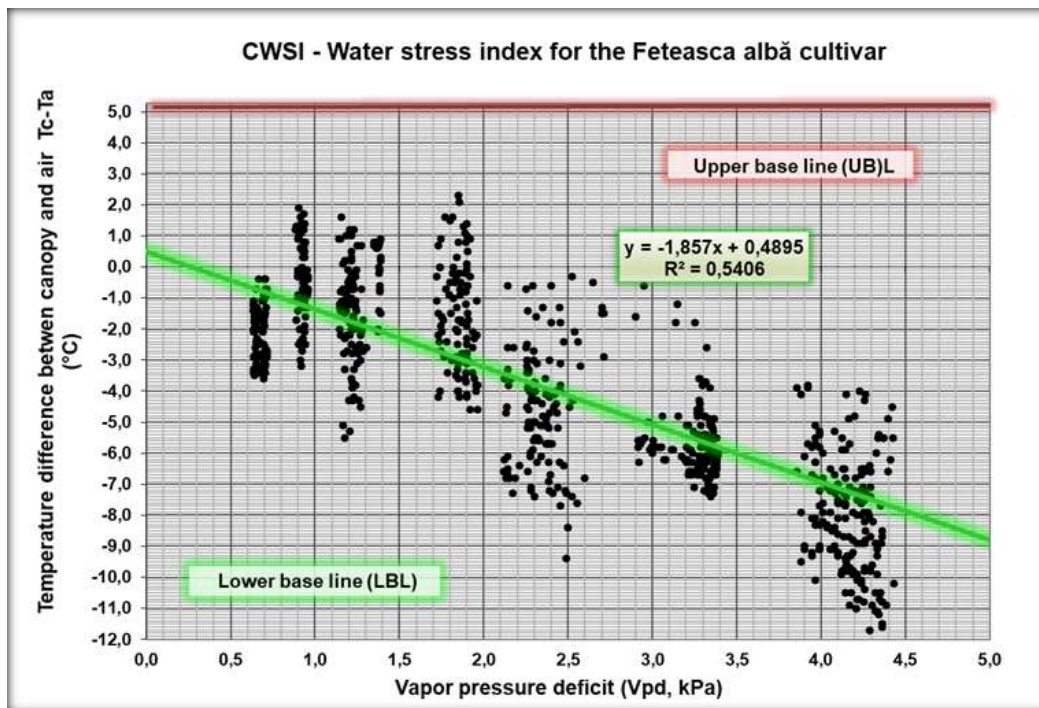
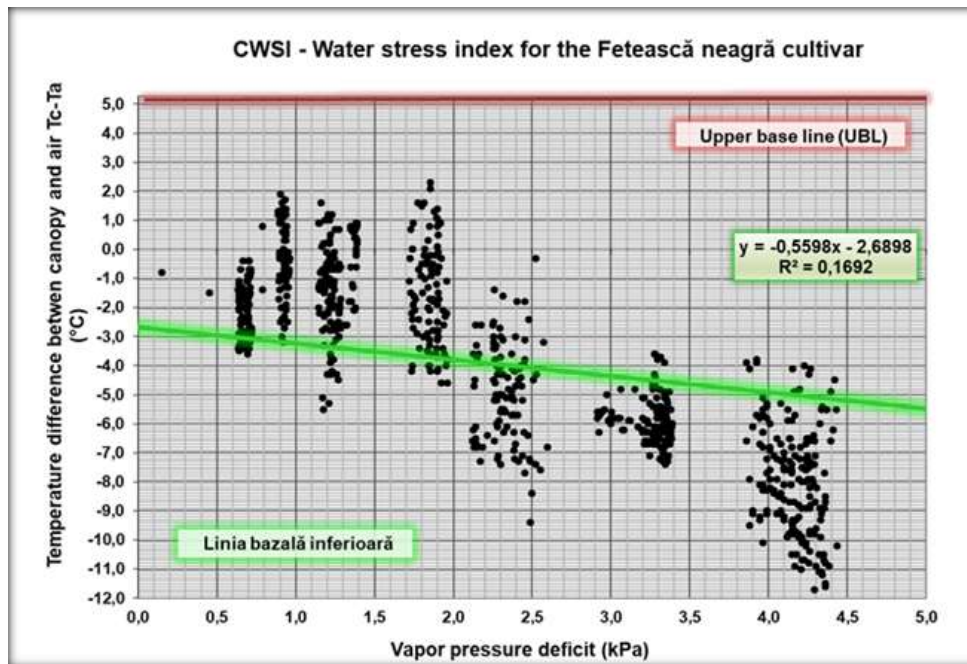


Figure 6. Lower and upper baselines are required to calculate the stress index for the Fetească albă variety



**Figure 7.** Lower and upper baseline are required to calculate the stress index for the Fetească neagră variety

## CONCLUSIONS

The cultivation of vines for wine grapes is increasingly affected by the summer heat, by the stress of the heat wave. The cultivation of vines for wine grapes is increasingly affected by the summer heat, of the heat waves stress. It is necessary to choose and adapt optimized technological sequences such as irrigation and their impact on plant growth and development processes, and the quality of the resulting products. It should be noted that ID depends mainly on the physical properties of the soil. This soil of the experimental plot has a clayey clay texture that is deep and well ventilated. For more highly textured soils, e.g. clayey or sandy, the ID values will be considerably different. Another aspect is the size of the culture canopy. Reliable measurements can only be obtained for mature, well-developed plants that form a continuous canopy along the rows. The values of the water vapor deficit in the atmosphere of 3.5 kPa, in the Fetească regală variety, register foliage with approximately 6°C colder, suggesting that it is less adapted to water stress than the variety Feteasca neagră, with only 4.2°C colder than air at the same deficit of water vapor pressure in the atmosphere. The correlation between CWSI of wine varieties and irrigation depth (ID) is represented by a very significant direct linear regression equation, but with a relatively low R<sup>2</sup> value (0.414 \*\*\*). Soil variability and agronomic factors may be responsible for this. According to this regression, the practical recommendation in irrigation scheduling is that CWSI values of 0.20 to 0.30 are suitable for the application of irrigation of more than 250 to 500 m<sup>3</sup> ha<sup>-1</sup>. From an economic point of view, the method of early estimation of the stress state of plants by using CWSI is much more efficient, both from a scientific point of view, by determining a much more accurate indicator, of the plant, and from an economic point of view of the use of humidity sensors placed in the ground in fixed positions, which is much more expensive.

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## EVALUATION OF THE VIABILITY OF QUERCUS CANARIENSIS IN THE FOREST OF OULED BECHIH (SOUK AHRAS)

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### ABSTRACT

The forest of Ouled Bechih is for forestry and ecotourism. Managed by the Conservation of Forests of Souk Ahras; this massif is formed specially by the cork oak (*Quercus suber*) and the zeen oak (*Quercus canariensis*) spreading over an area of 6582 ha. The quality of the stands can be given by the PHF index, a three-digit index that gives a judgment of the position of the tree in relation to the others and thus indicating the dominance and the stage of competition or exposure to the dominant stage. The objective of this work is to analyze the PHF index to assess the state of viability of zeen oak in the forest of Ouled Bechih to predict the future of this stand. The results obtained can be used for the management of this forest.

**Keywords:** PHF, zeen oak, vitality, management.

### INTRODUCTION

Mediterranean forests represent a fragile natural environment that is deeply disturbed by human activity. Barbero (1990), emphasizes that these forest ecosystems are characterized by two types of criteria: their spatial heterogeneity and their vulnerability due to their irregular exploitation by man.

Belonging to the Mediterranean forests, the Algerian forest with its biological diversity, presents an essential element of the ecological, climatic and socio-economic balance of different regions of the country. Its current situation is one of the most critical in the mediterranean region (Ikermoud, 2000).

In Algeria, the oaks (holm, cork, zeen, kermes and afarès) represent a forest capital, they cover large areas especially in the North and North-East, that is to say approximately 40 % of the Algerian forest (Alatou, 1994). However, the Algerian forest cover was these last fifty years the scene of a great destruction. Its surface estimated at 1.3 million hectares of real natural forests (Ferka Zazou, 2006) knows an almost exponential regression, and is today in an appalling state.

Given the policies and programs, the fate of Algerian forests remains tragic, pernicious and hopeless. Rabhi, 2011, consider that the major disadvantage of the Mediterranean species, in particular the oaks, resides in fact in a lack of knowledge of the forestry techniques which would be applicable to them in order to obtain the most satisfactory results. However, the development of oaks requires the promotion and application of silviculture based on a perfect knowledge of their growth.



Few research works have been carried out so far on oaks in Algeria, however, oak forests are the only forests in the country that are of great interest from the ecological, biological, aesthetic, socioeconomic, landscape and hunting points of view.

The present study focuses on the zeen oak of the Ouled Bechih forest, located in eastern Algeria; it is characterized by a very important biodiversity. The objective of this study is to identify ecological monitoring indicators to measure the state of viability of zeen oak and propose a simplified management plan based on the current ecological status and future trend of the ecosystem.

## MATERIAL AND METHODS

### *Presentation of the study area*

Ouled Bechih forest located in the highlands of eastern Algeria, north of the Souk-Ahras region, near the Tunisian-Algerian border; the geographical coordinates are 36° 21' 26" N, 7° 50' 08" E. This forest covers an area of 6582 ha and consists mainly of cork oak and zeen oak. It is characterized by a mountainous relief, being part of the Tellienne chain, with very steep slopes ranging from 15% to over 20%. The region has a subhumid climate, the extreme altitudes of the forest are varied between 790-1050m, an average annual temperature of 16 ° C, average annual precipitation of 625 mm, and a significant atmospheric humidity of 68% [6] (Ganaoui et al., 2019).

### *Choice of the study plots*

4 plots were randomly selected with a rectangular shape and an equivalent area of 900m<sup>2</sup> (30m x 30m); within each plot all individuals were inventoried [7] (Rached-Kanouni et al., 2019). Table 1 summarizes the geographic characteristics of these plots.

**Table 1. Geographical characteristics of the study plots**

| Plot | The geographical positions |              |          |
|------|----------------------------|--------------|----------|
|      | Latitude                   | Longitude    | Altitude |
| 1    | 36°23'10.264"              | 7°51'39.737" | 989      |
| 2    | 36°24'02.521"              | 7°55'23.811" | 882      |
| 3    | 36°23'01.244"              | 7°51'34.498" | 1001     |
| 4    | 36°22'52.414"              | 7°52'13.099" | 1038     |

### *Estimating the vitality status of Zeen oak by the PHF Index*

The quality of the stands can be given by the PHF index (three-digit index) which gives a judgment of the position of the tree in relation to the others and thus indicates the dominance and the stage of competition or the exposure to the dominant stage, the general shape of the crowns, and the shape of the shafts (Robisoa et al., 2008). It allows a more detailed silvicultural interpretation to predict the future of the stand (Blaser, 1984) and ultimately deduce the viability of the stands (Robisoa et al., 2008).

### *Position of the crown (P-index)*

The P-index is between 100 and 500 and gives an idea about the position of the tree crown and that of the neighboring trees, and describes the insolation intensity. It indicates the dominance, the stage of competition or the exposure to the dominant stage of the crown (Blaser, 1984).

*Crown shape (H index)*

In relation to the size and stage of development of a tree, the appearance of the crown quality will determine the growth (Blaser, 1984). The shape of the crown qualitatively indicates the previous development of a tree and probably its future potential. Values of H vary between 10 and 50.

*Shape of the bole (F-index)*

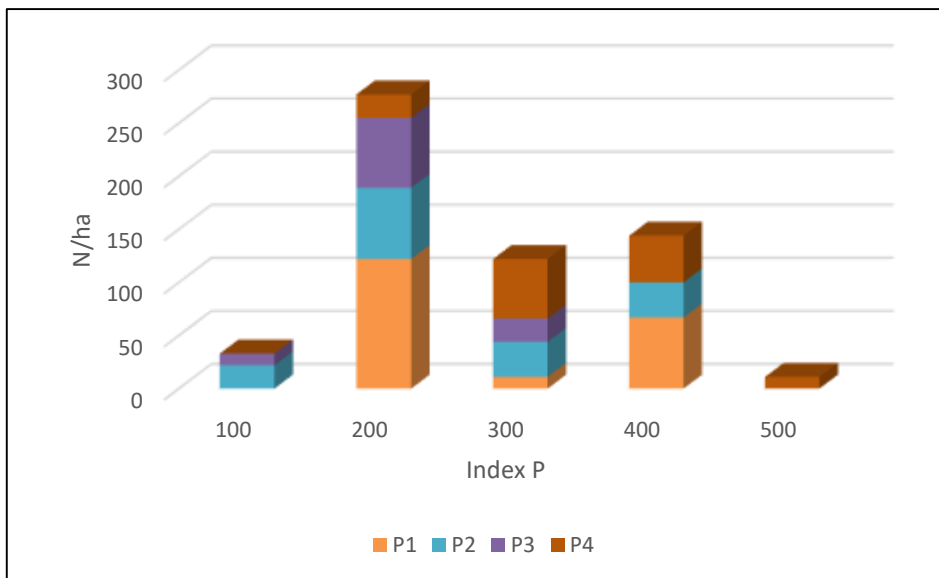
The bole shape is an index of wood quality. The bole shape is not related to increment, but it certainly influences future silvicultural practices (Blaser, 1984). It varies from 1 to 6.

**RESULTS AND DISCUSSION**

The PHF index indicates the quality of the stand. The PHF value given for the Zeen oak stratum is X (average of the PHF index of the 4 plots: Y for P1, Z for P2).

The results obtained for the P index show that 22 individuals/ha (P2) and 11 individuals/ha (P3), have a crown in full light, completely free from above and laterally, they are mostly dominant trees. Half of the Zeen oak stands have a crown in full light from above but covered laterally (47.27%); the maximum value is obtained in plot 1 (122 individuals/ha) while the minimum value is 22 individuals/ha is encountered in plot 4.

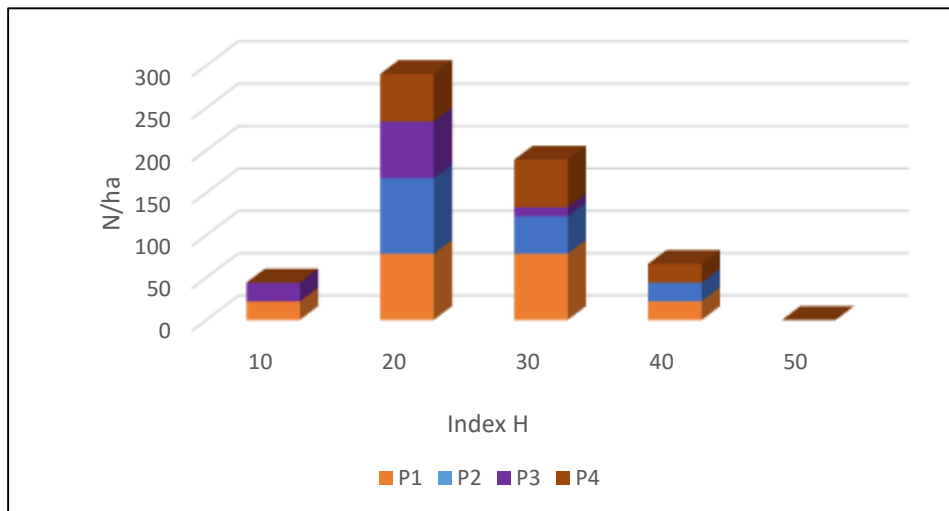
For trees with a partially free crown and full light from above (intermediate or over story trees) and trees with a covered crown, without light from above and partially illuminated laterally (over story trees), their percentages are respectively 20.74 and 24.48%; while trees with a completely covered crown, without direct light (understory trees), present the lowest percentage (1.87%).



**Figure 1.** Proportion of the number of stems according to the P index.

The results of the H-index indicate that the trees with a perfect, circular, symmetrical, dense and extensive crown have a low percentage of about 7.48%; these are trees of the dominant stage with a very good exposure to the sun. Half of the Zeen oak trees have a more or less circular crown with some symmetry deficiencies or with some dead branches and their percentage is 49.15% (these are the codominant trees of the main storey); the maximum value is obtained in plot 2 (122 individuals/ha) while the minimum value is 55 individuals/ha is encountered in plot 4. For individuals have a tolerable, partially asymmetrical and open crown and trees have a strongly asymmetrical crown with only a few green and dense branches, but

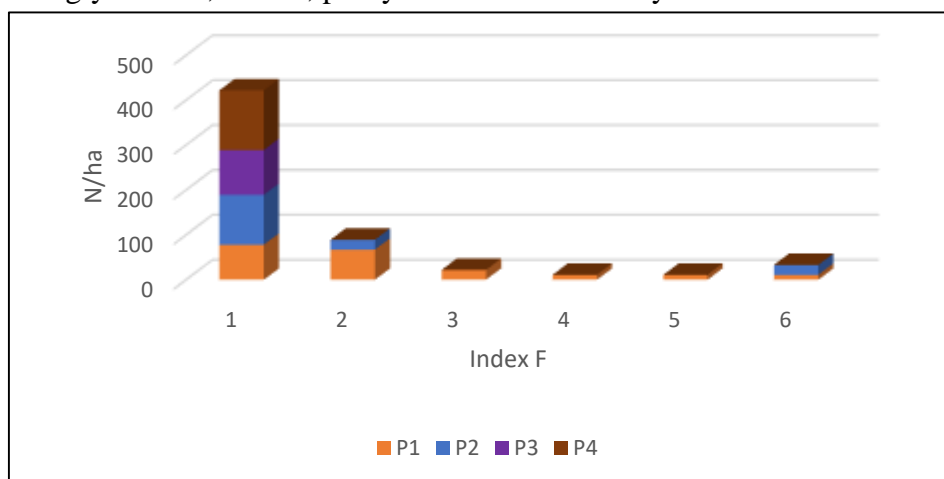
still having the appearance of a tree that can survive; These two types of the H index are dominated and their percentages are 32.14% and 11.22% respectively. These crown types are



likely to respond positively

**Figure 2.** Proportion of the number of stems according to the H Index.

The results obtained from the F-index show that the Zeen oak trees have a straight, round and full bole; cylindrical, without defects and without branching represent the majority of the trees in the studied stand (71.77%). 15.14% of trees have a straight, cylindrical, slightly domed, full bole for sectional division, without defects and without branches. This type of bole provides partly veneer wood. For trees have a partially straight, domed bole up to 2 meters high, partly cylindrical, generally conical and without defects and have good sawing wood, have a low rate and which is 3.74%. The trees have a very irregular bole, very forked and twisted, conical, with clearly visible defects; they are mostly used as energy wood and have a rather low percentage (5.61%). The lowest percentage of the F-index is 1.87% (an irregular, tortuous, strongly conical, forked, partly defective bole. They can be used as construction wood.



**Figure 3.** Proportion of the number of stems according to the F Index.

Table 2 summarizes the quality of the stands studied according to the PHF index, this quality is quite variable between the 4 plots studied. Plots 1, 2 and 3 have a good quality (PHF = 221) compared with plot 4 has a medium quality (PHF = 331), which allows us to deduce

their good viability (Rajoelison et al., 2008) and their great competitive aspect especially towards the dominant stage.

The cumulative PHF shows that the highest number of stems for the 4 plots is 278 individuals/ha for the P index, 289 individuals/ha for the H index and 89 individuals/ha for the F index, which gives an average of 219 individuals/ha for a good PHF index (222), 166 individuals/ha for a Perfect PHF (111), 111 individuals/ha for a Medium PHF (333), 74 individuals/ha for a Tolerable PHF (444), 7 individuals/ha for a Poor PHF (555) and 11 individuals/ha for a Very Poor PHF (556).

The average PHF score was **221** for the 4 plots studied, from which a good quality of the Zeen oak stand was inferred.

**Table 2. Quality of zeen oak stands according to the PHF index.**

| Plot       | PHF        | Quality | PHF Cumulative |      |            |      |            |      |            |     |          |
|------------|------------|---------|----------------|------|------------|------|------------|------|------------|-----|----------|
|            |            |         | P              | N/ha | H          | N/ha | F          | N/ha | PHF        | Moy | Qualit y |
| <b>P1</b>  | 221        | G       | <b>100</b>     | 33   | <b>10</b>  | 44   | <b>1</b>   | 422  | <b>111</b> | 166 | P        |
| <b>P2</b>  | 221        | G       | <b>200</b>     | 278  | <b>20</b>  | 289  | <b>2</b>   | 89   | <b>222</b> | 219 | G        |
| <b>P3</b>  | 221        | G       | <b>300</b>     | 122  | <b>30</b>  | 189  | <b>3</b>   | 22   | <b>333</b> | 111 | M        |
| <b>P4</b>  | 331        | M       | <b>400</b>     | 144  | <b>40</b>  | 66   | <b>4</b>   | 11   | <b>444</b> | 74  | T        |
| <b>TOT</b> | <b>221</b> | G       | <b>500</b>     | 11   | <b>50</b>  | 0    | <b>5</b>   | 11   | <b>555</b> | 7   | P        |
|            |            |         | <b>0</b>       | 0    | <b>0</b>   | 0    | <b>6</b>   | 33   | <b>556</b> | 11  | VP       |
|            |            |         | <b>TOT</b>     | 588  | <b>TOT</b> | 588  | <b>TOT</b> | 588  | <b>TOT</b> | 588 |          |

VG: Very good; G: Good; FG: Fairly good; M: Medium; P: Perfect; T: Tolerable; P: Poor; VP: Very poor

## CONCLUSION

The forest of Ouled Bechih is for forestry and ecotourism. Managed by the Conservation of Forests of Souk Ahras. This massif is formed especially by the cork oak and the zeen oak spreading over an area of 6582 ha. This study carried out only on the stands of zeen oak of the forest of Ouled Bechih from the analysis of the PHF index. The results obtained from this index, showed that the forest of zeen oak are of good quality in which the value of PHF = **221**. These results can help the conservation of Souk Ahras to use them in silvicultural analysis and to identify ecological monitoring indicators to measure the state of viability of zeen oak and propose a simplified management plan based on the current ecological status and future trend of the ecosystem.

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## COMPARATIVE STUDY OF THE VEGETATIVE GROWTH OF SEEDLINGS OF *Q. SUBER* ET *Q. CANARIENSIS* (OULED BECHIH FOREST-SOUK AHRAS)

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### ABSTRACT

In order to compare the emergence and growth of seedlings of two species of the genus *Quercus* (*Q. suber* and *Q. canariensis*), experimental trials were conducted. The following assessment parameters were used: emergence and mortality rates, height growth, crown diameter growth and leaf production. The results obtained showed that the emergence rate of cork oak seedlings was close to the emergence rate of zeen oak seedlings with an average mortality rate not exceeding 38%. The results of the development of young seedlings showed that the cork oak has a better growth performance compared to the zeen oak, in this context this study is therefore necessary

**Keywords:** *Q. suber*, *Q. canariensis*, seedling, growth, regeneration.

### INTRODUCTION

Mediterranean oak forests are characterized by the presence of deciduous oaks in humid bioclimatic stages and evergreen oaks in humid, subhumid and even semi-arid bioclimates (Hasnaoui, 1992). The oak forest provides very important forest income and contains a very high genetic diversity. Despite this economic and ecological importance, some oaks remain little known or threatened species, particularly the rare and endemic hybrid oaks.

The genus (*Quercus spp.*) is one of the most species-rich forest genera. It includes several hundred woody species from temperate and Mediterranean zones, America, Europe, and also Asia, among which are some species of high economic importance (Sarir and Benmahioul 2017).

In Algeria, the oaks (holm, cork, zeen, kermes and afarès) represent a forest capital, they cover large areas especially in the North and North-East, that is to say approximately 40 % of the Algerian forest (Alatou, 1994). These oaks play an undeniable role on the ecological, economic and social level.

The multiple aggressions of overgrazing, fires, attacks and insects, aging, poor silvicultural practices, as well as climate change, lead to the decay and weakening of oak groves (Becker and Levy, 1983; Delatour, 1980). The recourse to artificial regeneration is of great necessity to rehabilitate these degraded ecosystems and to develop the Algerian oak forest. However, several reforestation failures have been recorded in recent years (Bensaid et al., 1998; Bouhraoua et al., 2014). In this context is carried this study which has as main objective to study and compare the vegetative growth of the two species of oak: cork oak and oak zeen for

the evaluation of the success of the artificial regeneration of these two species to have a good continuity of the forest of Ouled Bechih.

## **MATERIAL AND METHODS**

### *Origin of acorns*

The acorns of cork oak and zeen oak come from the state forest of Ouled Bechih which is part of the willaya of Souk Ahras (Eastern Algeria). It is located north of the region, near the Tunisian border. Occupying an area of 6582 ha and consists mainly of cork oak and oak zeen. Its altitude varies from 790 to 1050m. From a climatic point of view, the forest of Ouled Bechih is characterized by a humid climate. The development of plants is not simply due to the absolute amount of water available but more precisely to the way it is distributed during its vegetative cycle (Boudy, 1948).

### *Acorn preparation*

Acorns were randomly harvested using two harvesting methods: collection of fallen acorns and direct harvesting by sapling in November 2021. After harvest, 75 acorns of each species were sorted and selected. Germination was monitored on acorns placed in pots containing peat for one month. The germinated acorns were then moved to polyethylene bags filled with topsoil at the end of January. The bags were placed in natural conditions and watered regularly twice a week.

### *Measurements and Observations*

#### *Seedling emergence*

Acorns were considered germinated when the radicle appeared. Each time a seedling appeared it was counted until the last emergence. The rate of development of the seedlings was measured twice a week for 4 months.

#### *Morphological parameters*

In order to evaluate the vigor of the seedlings of the three treatments for the two oak species (cork oak and zeen oak) studied, the following parameters were measured twice a week for four months:

- Growth in length: The length of the stems
- Growth in diameter: The diameter of the seedlings at the crown.
- Leaf production: The number of leaves per plant was monitored and recorded for each measurement. Indeed, the estimation of the number of leaves is a good indicator of the assimilative capacities of the plant and its production in biomass.

## **RESULTS AND DISCUSSION**

The emergence rates obtained were 69.33% for zeen oak acorns and 60% for cork oak acorns (Tab. 1). The rate of emergence was faster for zeen oak acorns than for cork oak acorns. The average mortality rate recorded after emergence for cork oak and zeen oak seedlings was 35.33%. The highest mortality rate was recorded for cork oak seedlings (40%).

Despite these satisfactory emergence rates, a difficult problem to solve is that of maintaining the moisture content at a relatively high level. However, poor emergence is due to several causes, namely the depth of seeding (deep or shallow), drought or pests. These results are consistent with those obtained by M'sadak et al. (2013). These authors showed that no-till okra plants manifested the best growth performance compared to transplanted plants. In contrast, Koumiche (2016) noted in holm oak, high mortality rates.

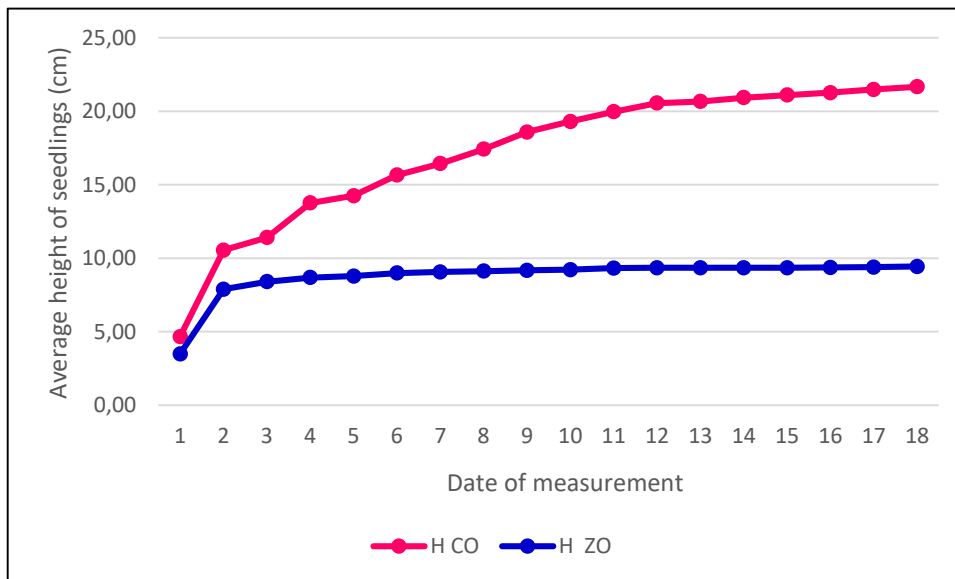
Table 1. Seedling emergence and mortality rates in the two oaks (cork oak and zeen oak).

|                        | Zeen oak | Cork oak |
|------------------------|----------|----------|
| Seedling emergence (%) | 69,33    | 60       |
| Mortality (%)          | 30,67    | 40       |

*Height measurement*

Comparative analysis of the heights of cork oak and zeen oak seedlings shows that the average lengths recorded are different (Fig. 1). Indeed, the average height of cork oak seedlings is higher than that of zeen oak. The analysis of the heights of the two oak species shows that the average heights recorded are different after the second measurement date. After this date, the average height of zeen oak grows slowly, and stabilizes at 9.35cm from the twelfth measurement date. On the other hand, the cork oak seedlings grew rapidly and reached 21.66cm for the same previous date.

These results show that the two oaks studied follow the same growth pattern with differences in their average height where the cork oak and the zeen oak have an average length of 17.20 and 8.76 cm respectively.



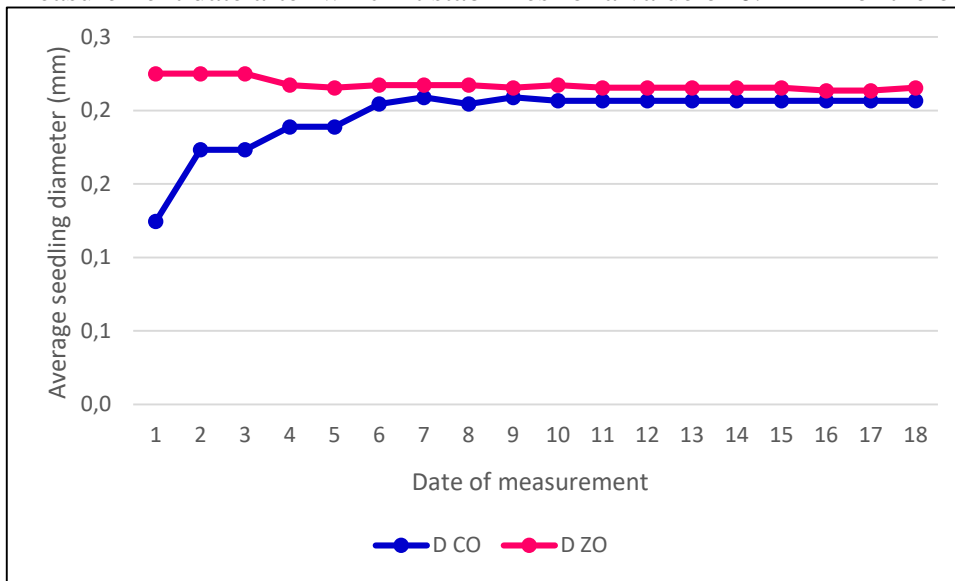
**Figure 1.** Growth in height (cm) of cork oak and zeen oak seedlings.

*Diameter measurement*

Diameter at the crown is an important morphological character that can best predict seedling performance after planting. The results of the evolution in diameter are shown in figure 2 for the two oak species studied. The comparison of the average values after 4 months of measurement, shows that the average diameter of cork oak is higher and stable than that of zeen oak.

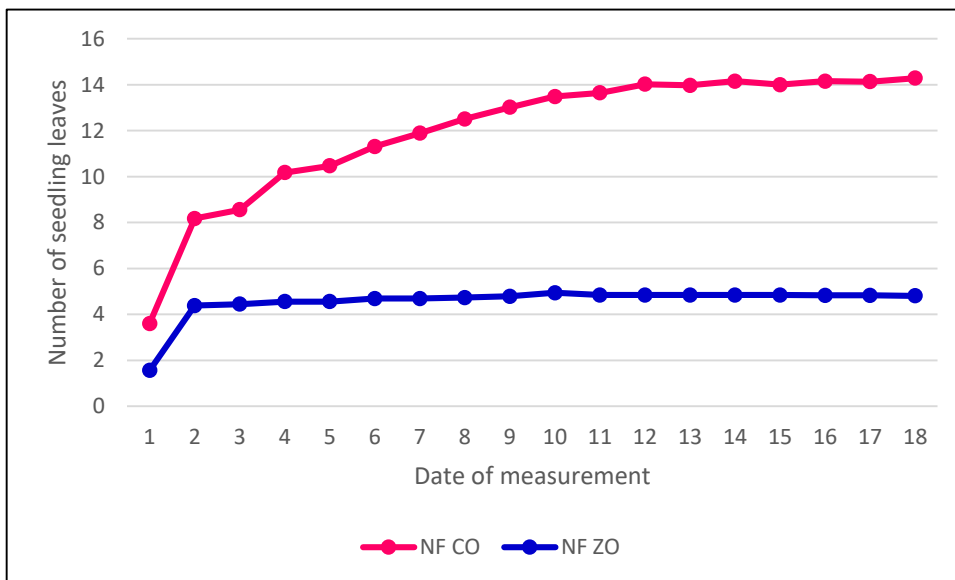


For the Zeen oak seedlings the radial stem growth does not exceed 0.1 mm for the first measurement date after which it stabilizes for a value of 0.2 mm for the other measurements.



**Figure 2.** Diametric growth (mm) of cork oak and zeen oak seedlings.  
*Average number of leaves*

Leaf organogenesis is an indication of good water and mineral nutrition of the plant. The results of the evolution of the average number of leaves are given in figure 3. The analysis of this figure shows that leaf organogenesis is a function of the oak species studied. Indeed, the average number of leaves is high in the cork oak, where we recorded an average of 12 leaves per seedling; whereas it is low in the zeen oak with an average of 5 leaves per seedling.



**Figure 3.** Average number of leaves of cork oak and zeen oak seedlings.

According to these experiments, we note that the germination of seedlings differs according to the origin, this is due to the qualitative and quantitative characteristics of acorns which vary according to the origin of the species and even between the acorns.

## CONCLUSION

This study conducted on the growth of seedlings of two oak species (cork oak and zeen oak) demonstrated the effectiveness of direct seeding of acorns compared to transplanting. The direct seeding process resulted in emergence rates ranging from 60 to 69%. The monitoring of different growth parameters (height, collar diameter and leaf organogenesis) showed that the cork oak had a better growth performance compared to the zeen oak in the Ouled Bechih forest.

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## **STRUCTURE DEMOGRAPHIQUE ET REPARTITION SPATIALE DE CHENE VERT DANS LA FORET DE CHETTABA**

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### **ABSTRACT**

The holm oak is the main species of the state forest of Chettaba which covers an area of 2398ha where it occupies 1127ha. However, during the last decade, a particular attention and a scientific and forestry interest have been expressed for this species. Plantations of holm oak were studied in the Chettaba forest, the diameter at 1,30 m from the ground, the total height of holm oak individuals were measured on 4 plots according to an altitudinal gradient. The diameter and height structures were fitted to the theoretical Weibull distribution. According to this test of fit, the holm oak stand is characterized by a relative predominance of young and small diameter individuals, which suggests a good regeneration of the species. These results contribute to the improvement of the knowledge on the indicators of the current state of the holm oak stands which can be used as a basis in the management of the Chettaba forest.

**Keywords:** holm oak, Weibull distribution, demographic structure, spatial structure.

### **INTRODUCTION**

Sclerophyllous oaks participate in, or even constitute practically by themselves, various types of landscape highly characteristic of the Mediterranean world. The holm oak is the typical species of the Mediterranean forest, where it currently occupies between 354,000 and 433,000 ha, part of which is in the form of coppice. It is adapted to continental and altitudinal conditions between semi-arid and subhumid, it can climb in altitude up to the limit of 1600 m where it is in direct competition with the cedar, nevertheless it overflows on semi-arid stations in the most degraded stations (Haichour, 2009; Nasrallah, 2014).

In Algeria, oaks represent a forest capital where they cover nearly 40% of the Algerian forest (Alatou, 1994). These oaks play an undeniable role on the ecological, economic and social levels. However, in Algeria, the holm oak forest is threatened by multiple factors, such as the extension of agriculture, infrastructure (settlements and roads), overgrazing and fires (Nasrallah, 2007). The management of forest stands requires the control of the structure of trees (Van Laar and Akça, 2007). These structures are indicative of events related to stand life, site conditions and to define better management options (Rondeux, 1999; Feeley et al., 2007). To represent the theoretical structure of a stand, several types of distributions can be used (normal distribution, lognormal distribution, exponential distribution, Weibull distribution, etc.).

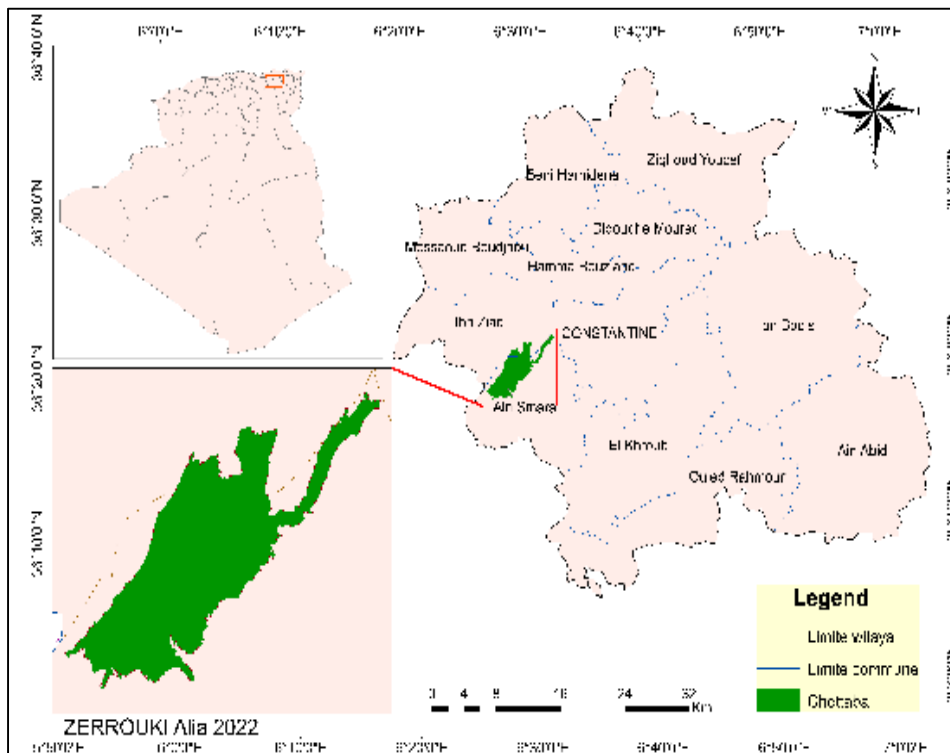
Parameters are estimated from observed data (Husch et al. 2003). However, the Weibull distribution is more appropriate because it is characterized by a great flexibility of use and presents a great variability of forms according to the values taken by its theoretical parameters (Bullock Burkhardt, 2005)

The main objective of this study is to analyze the demographic structure and spatial distribution of holm oak populations in the Chettaba forest located southwest of Constantine (Algeria) and their effects on natural regeneration for future development and sustainable management of the forest.

## MATERIAL AND METHODS

### Presentation of the study area

Forest of Chettabah is located southwest of Constantine (Algeria). The estimate terrain elevation above sea level is 865 meters. The study area is located on the map topographic Constantine Scale 1/200 000 sheet N° 17 and located between the coordinates 36°19'4" north latitude and 6°28'36" East longitude.



**Figure 1.** Location of the study area.

### Methodology

#### Data collection

The forest inventories were carried out in plant formations dominated by holm oak. Four plots of 0,09 ha (30 x 30 m) were set up in the Chettaba forest. The dendrometric parameters measured are the diameters at 1.30 m from the ground (dbh: diameter at breast height) and the heights of trees.

### Data processing

The collected data are entered into Microsoft Excel spreadsheet (2019), which was used to determine the ecological characteristics and natural population structure. In these plots, demographic structures were modeled by Weibull distributions by Minitab 16 software. The Weibull distribution is well suited to model the observed diametric structures (Husch et al. 2002). From the parameters (a, b and c: see below) conclusions are drawn about the life conditions of the trees. The established structure models then allow for the definition of appropriate management options for the studied stands. The Weibull distribution is based on the probability density function defined as follows:

$$F_{(x)} = \frac{c}{b} \left( x - \frac{a}{b} \right)^{c-1} \exp \left[ - \left( \frac{x - a}{b} \right)^c \right]$$

where x is the diameter (respectively height) of the trees; a is the origin or position parameter. It corresponds to the pre-count diameter and height of the adult population; b is the scale parameter related to the median value of the diameters and heights of the trees; c is the shape parameter related to the structure considered (Table 1). The values of "c" are related to well-established distribution forms (Husch et al., 2002).

Table 1. Shape of the Weibull distribution according to the values of the parameter c.

|             |   |
|-------------|---|
| c < 1       | Inverted "J" distribution, characteristic of multi-species or uneven-aged stands.   |
| c = 1       | Exponentially decreasing distribution, characteristic of populations in extinction.   |
| 1 < c < 3,6 | Positive asymmetric or right asymmetric distribution, characteristic of monospecific stands with a predominance of young or small diameter individuals. |
| c = 3,6     | Symmetrical distribution; normal structure, characteristic of even-aged or monospecific stands even-aged or monospecific stands of the same cohort.     |
| c > 3,6     | Negative or left-skewed distribution, characteristic of monospecific stands with a predominance of older individuals.                                   |

Stand demographic structures were also analyzed by calculating skewness coefficients. Skewness coefficients (g) were calculated to determine trends in population structures using the following formula:

$$g = \frac{n \sum_i (x_i - \bar{x})^3}{(n - 1)(n - 2)\sigma^3}$$

where n is the number of stems, xi is the diameter at breast height, the mean diameter, and σ is the standard deviation of xi. The skewness coefficient is a measure of the relative proportion of small stems to large stems within a population. It describes the uniformity of truncated distributions: g > 0 for diametric distributions with relatively few small stems and many large stems; g < 0 for diametric distributions with relatively few large stems and many more small stems (Feeley et al., 2007).

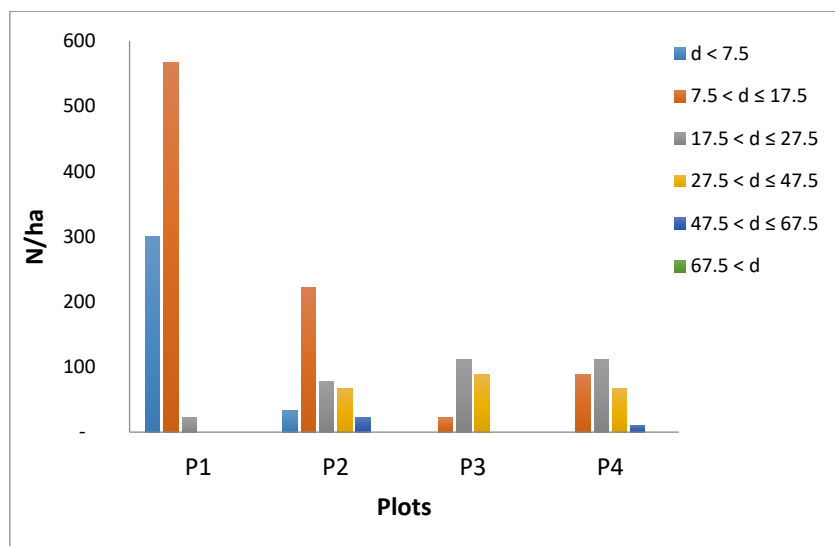
## RESULTS AND DISCUSSION

The average density of woody at the level of the forest of Chettaba is varied between 889 and 222 individuals/ha. The average diameter is between 10,33 and 26,31cm, these stands are in the state of thicket-gallery to mature forest. Plot 3 is characterized by the lowest density (222 individuals/ha). Plot 1 had the highest density at 889 individuals/ha (Table 2).

**Table 2.** Dendrometric characteristics

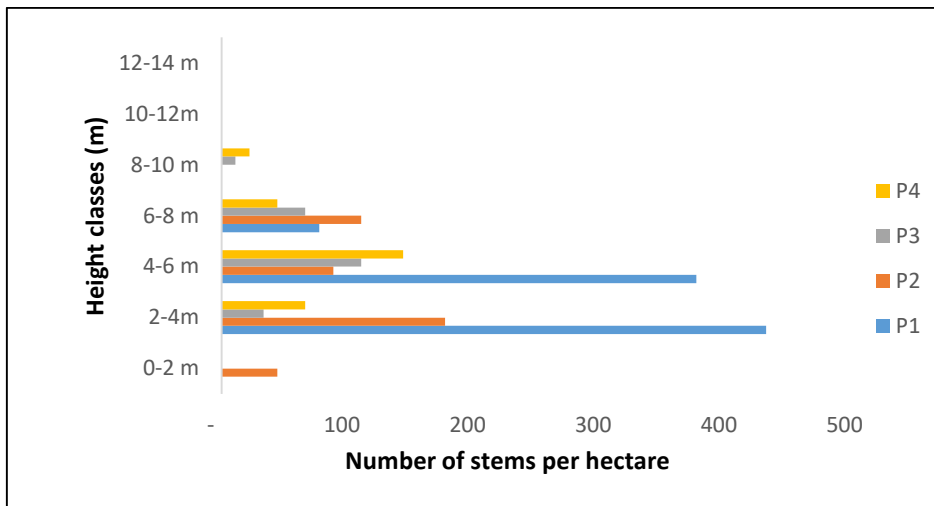
| parcelles | N/ha | D (cm) | H (m) |
|-----------|------|--------|-------|
| P1        | 889  | 10,29  | 4,17  |
| P2        | 422  | 12,51  | 4,12  |
| P3        | 222  | 26,31  | 5,34  |
| P4        | 278  | 18,77  | 4,96  |

The diameter class ( $d < 7,5\text{cm}$ ) is present in plots 1, 2, and 4 and characterizes good generation of the species (Figure 1). The 17,5 to 27,5cm diameter class has a high rate in plots 3 and 4. In addition, many individuals of this species are only at a young life stage. On the other hand, the large diameter stems are almost completely weak (P2 and P4).



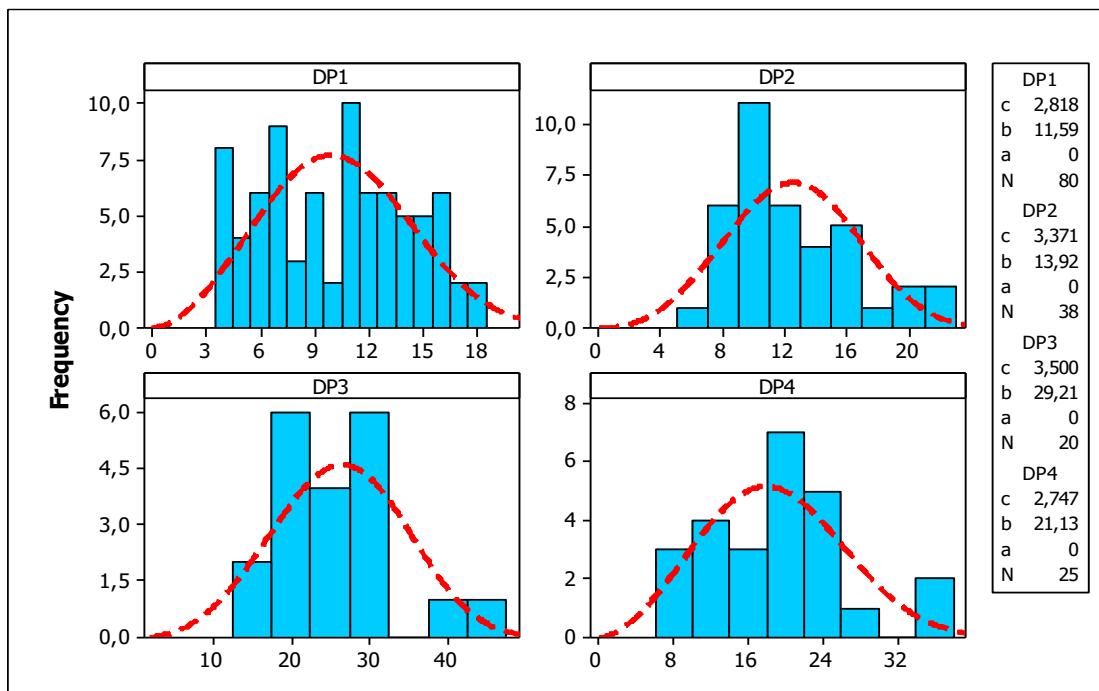
**Figure 2.** Density by diameter class.

The height structure is given by the distribution of the number of stems in all plots per hectare for each class and provides information on the vertical stratification of the stand. A large proportion of the trees are between 2 and 8 m high (low pole saplings) with 1733 individuals; this class has a large number of individuals because of regeneration, which remains fairly average, while the 0 to 2 m and 8 to 10 m classes are characterized by a low number of trees (Figure 2). There is an almost total absence of trees over 10 m.



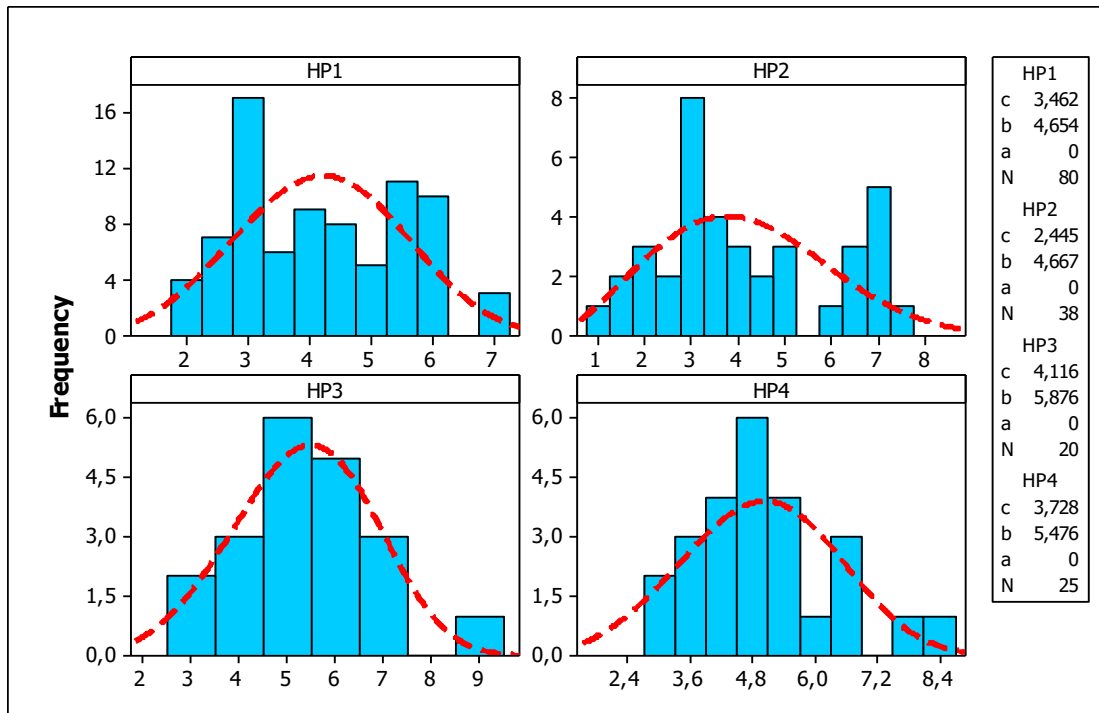
**Figure 3.** Density by height class

Diameter structures of adult holm oak populations are similar and have a bell-shaped appearance in all four study plots (Figure 3). This characterizes low proportions of stems with diameters near the pre-count diameter (dbh = 5 cm). Indeed, holm oak stems are concentrated in the 7,5 to 17,5 and 17,5 to 27,5 cm diameter classes. All distributions have a "c" value between 1 and 3,6. These "c" values indicate positive skewed distributions for all diameter structures and a predominance of young stands (Husch et al., 2003).



**Figure 3.** diameter structure of holm oak according to Weibull.

The values of the shape coefficient  $c$  of the Weibull distribution of the height structure of all woody plants between 2,83 and 3,37 for plots P1 and P2 respectively. This value indicates a positive asymmetric distribution (right) with a predominance of low height individuals (Figure 4). On the other hand, the distribution of individuals in plots P3 and P4 in diameter classes shows a negative asymmetrical or left asymmetrical distribution, characteristic of monospecific stands with a predominance of old individuals.



**Figure 2.** Tall structure of holm oak.

## CONCLUSIONS

The establishment and interpretation of diameter and height structures are essential for forest management decisions. The characteristics of the plant formations studied show that the structure of the identified plant groups is still dominated by stems of small diameter classes. The study of the dendrometric characteristics of the holm oak of some plots of the Chettaba forest (East Algerian) showed that the individuals of big diameter are totally absent. Finally, this diagnostic state will constitute a reference for the next monitoring results and decision for the foresters in the framework of a management plan.

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## **THE EFFECT OF PERICARPS ON GERMINATION OF HOLM OAK (*QUERCUS ILEX*)**

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### **ABSTRACT**

The knowledge of the germinative properties of acorns allows to define with precision the conditions of use of acorns according to the desired objectives. The objective of this work is to analyze the effect of certain physical treatments on the germination of holm oak acorns. The results obtained show that the acorns tested show a variation in biometry. These variations have no effect on germination. The removal of the acorn skins of two varieties (Tebessa and Batna) gave the highest germination rates (100%). Untreated acorns gave a germination rate of 40%. The results obtained during these experiments clearly show that the pericarp constitutes a significant obstacle to the rapid and homogeneous germination of holm oak acorns.

**Keywords :** holm oak, acorns, germination, treatment

### **INTRODUCTION**

Mediterranean forests cover about 81 million hectares, i.e., a global forest area of 9.4%. They are made up of a mosaic of forest species, particularly hardwoods which represent about 60% (Mugnossa et al. 2000).

In Algeria, the oaks represent a forest capital of about 40% of the Algerian forest. The holm oak appears from 400 m above sea level and rises to 1700 m in the Aures (Alatou, 2007; Boukhelkhal, 2017).

Germination is a complex phenomenon involving several factors such as growth regulators and hydrolytic enzymes that interact to trigger the process and subsequent growth (Côme, 1970; Roberts et al., 1988). In natural ecosystems, germination may be limited by factors such as predation or seed infestation. In addition, the maintenance of forest species through their seeds poses the problem of germination in the first place.

Faced with this situation, the need to assist natural regeneration is obvious. Although certain methods of vegetative propagation of the holm oak seem to be usable, such as cuttings, the principal mode of propagation of the species is sowing.

It is thus essential, for a good diffusion of the holm oak, to control the techniques and the conditions of breeding of the seedlings and, in particular, those of the germination of acorns. It is in this context that the main objective of this work is to study the germinative behavior of *Quercus ilex* acorns subjected to several treatments. In addition to the optimization and

homogenization of its germination, the knowledge of the germinative strategy adopted by the holm oak allows to better understand its natural dynamics.

## **MATERIAL AND METHODS**

### **Plant material**

The acorns of holm oak harvested in late October and early November 2021, come from the regions of Batna and Tebessa. The plant material consists of young plants of two different varieties of this species, resulting from the germination of acorns.

In the laboratory, the acorns were first separated by variety and by treatment. They were cleaned and sorted by a densimetry test with ordinary water. The supernatants, considered non-viable, were eliminated (Dupouey and Le Boulter, 1989). Healthy acorns were selected from each variety to study morphological variability.

### **Determination of moisture content**

The water content is one of the limiting factors of germination, for that a minimal threshold must be preserved: threshold below which germination does not take place. This minimum quantity of water makes it possible to maintain in life the embryo, thus its germinative faculty (Alatou, 1984).

The water content is the difference between the weight of the fresh matter (FP) and the weight of the dry weight (DW), obtained after a passage in an oven at 96°C for 48 hours. It was determined for 10 acorns of different holm oak varieties. The determination of the water content is done according to the formula of Vilain (1987):

$$WC = \frac{FP - DW}{FP} \times 100$$

**WC** : Water content

**FP** : Fresh weight

**DW** : Dry weight

### **Treatments applied to acorns**

Germination is defined as the transitional phase between the dry seed stage and the appearance of the radicle (Gimeno-Gilles, 2009). This phase requires first of all the imbibition of the tissues. If the physiological conditions are favorable (absence of primary dormancy) as well as the environmental conditions (availability of oxygen, adequate temperature), germination is possible. The treatments applied are the following:

T1: Intact acorns (Batna variety)

T2: Acorns without pericarp (Batna variety)

T3: Intact acorns (Tebessa variety)

T4: Acorns without pericarp (Tébessa variety)

Ten acorns of each treatment are placed in germination pots containing moist topsoil (watering done every two days). The pots were placed in natural conditions.

Germination was monitored for 30 days, with twice-daily counts of germinated acorns. Acorns that showed positive geotropism with an elongated radicle of at least 2 mm were considered germinated (Côme and Corbineau, 1998)

The germination rate is calculated by the formula:

$$GR(\%) = \frac{NGG}{NTGS} \times 100$$

GR = Germination Rate;

NGG = Number of Acorns Germinated;

NTGS = Total Number of Acorns Sown.

## RESULTS AND DISCUSSION

The average length of the acorns varies between 3,68 and 3,88 cm for the two varieties of holm oak and Tébessa respectively. The width varies between 1,31 and 1,95 cm (acorns of the two varieties of Tébessa intact and without pericarp). The weight varies between 2 g (Batna acorns without pericarp) and 6 g (intact Tébessa acorns) (Table 1).

The difference between the minimum and the maximum of the acorns can go from 3,4 to 4,3 cm for length and from 1,1 to 2,1 cm for width (Figure 1). For weight, the difference between the minimum and maximum is important: it varies from 2,7 to 7,5 g.

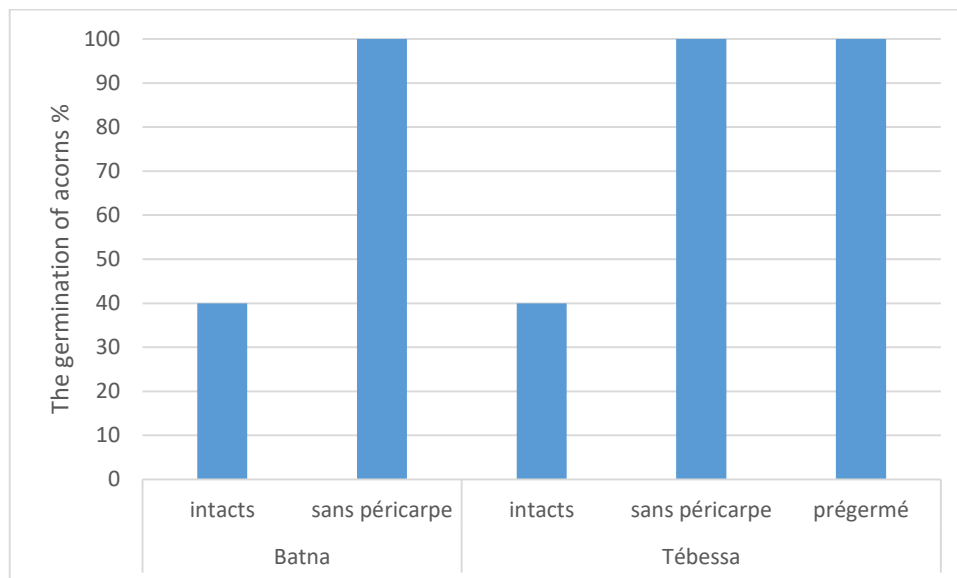


**Figure 1:** Morphology of different acorns (a: Batna; b: Tébessa).

**Table 1: Acorn biometric characteristics**

| characteristics |                    | Batna   |                  | Tébessa |                  |
|-----------------|--------------------|---------|------------------|---------|------------------|
|                 |                    | Intacts | without pericarp | Intacts | without pericarp |
| <b>Longueur</b> | Average            | 3,83    | 3,68             | 3,87    | 3,88             |
|                 | Max                | 4,2     | 4,1              | 4       | 4,3              |
|                 | Min                | 3,5     | 3,5              | 3,7     | 3,4              |
|                 | Standard deviation | 0,23    | 0,20             | 0,13    | 0,26             |
| <b>Largeur</b>  | Average            | 1,31    | 1,44             | 1,95    | 1,95             |
|                 | Max                | 1,6     | 1,7              | 2,1     | 2,1              |
|                 | Min                | 1,1     | 1,1              | 1,7     | 1,7              |
|                 | Standard deviation | 0,14    | 1,16             | 0,13    | 0,12             |
| <b>Poids</b>    | Average            | 2,2     | 2                | 6       | 3,4              |
|                 | Max                | 4,5     | 3,2              | 7,5     | 6,8              |
|                 | Min                | 3,25    | 2,7              | 6,66    | 5,44             |
|                 | Standard deviation | 0,75    | 0,39             | 0,61    | 0,84             |

### The germination of acorns



**Figure. 2:** Acorn germination rate of holm oak by variety

The analysis of figure 2, shows that the germination rate is very high, reaching a maximum of 100% for the acorns without pericarp of two varieties. As for the intact acorns, the germination rate is 40%. Acorns from Tébessa have a germination rate equal to 100%. It is noted that the germination rate of the region of Tébessa is higher than that of the region of Batna with rates of 80% and 70% respectively.

The results obtained during these tests let appear that the teguments constitute a not negligible obstacle to the homogeneous and fast germination of the acorns of green oak. Indeed, it is shown the beneficial action of the ablation of the seminal envelopes on the kinetics of germination. (Lamond, 1978) has already reported the influence of tegument removal on the improvement of the germination power of acorns of pedunculate oak. Benmahioul et al, (2010) also report the effect of seed coats on the germination of *Pistacia vera* L. seeds. The best germination rates were recorded with the seed lot without seed coats. Levert (1977) noted that the bare embryo imbibed much better and faster than the whole acorn of pedunculate oak. The same author points out that the poor germination of the whole acorn could be explained at least in part, by inadequate rehydration of the embryos.

### CONCLUSIONS

A corn abundance, plant health, morphological and physiological maturity, and acorn size play an important role in the natural regeneration of holm oak. The results obtained in these experiments clearly show that the pericarp and/or the seed coats constitute a significant obstacle to the rapid and homogeneous germination of holm oak acorns. Unfortunately, we have not yet been able to pinpoint the mechanism(s) responsible for this depressive effect of the seminal envelopes on germination of this seed.

Intact holm oak acorns have a lower germination rate than acorns without pericarp. This difficulty can be overcome by total or partial removal of the seminal envelopes. The removal of the seminal envelopes is easy to perform manually and does not pose any problem at the laboratory scale. On a forest scale, however, this operation requires the prior development of a mechanical process for decorticating the acorns, which is likely to pose technical problems.

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## **ANTIMICROBIAL AND ANTIOXIDANT PROPERTIES OF POMEGRANATE (*Punica granatum* L) PEEL BIOACTIVE COMPOUNDS (*In-vitro*)**

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### **ABSTRACT**

This review aimed to discuss the pomegranate peel extraction methods and the ability of its bioactive compound as an antimicrobial and antioxidant agent. Currently, extracts of plant bioactive compounds are commonly used as medicinal properties. Pomegranate planting is predicted to reach approximately 300 thousand hectares worldwide, with a production of 3 million metric tons of which about 76% of production comes from Turkey, India, Iran, and the USA. The antimicrobial and antioxidant activity of pomegranate peel extract (PPE) is related to its phytochemical compounds including punicalagin, ellagic acid ellagitannin anthocyanins, catechol, catechin, hesperidin, quercetin, and glucosides. Because of their ability to protect sensitive bioactive components from thermal degradation and oxidation, non-conventional pomegranate peel extraction methods such as supercritical fluid extraction, ultrasound-assisted extraction, and microwave-assisted extraction yielded total phenolics more efficiently than soxhlet extraction and maceration methods. Previous studies reported that methanol solvent has the highest yield of that other polar solvent in extracting the pomegranate peel. PPE increased DPPH activity and showed antioxidant activity similar to synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). PPE has a broad antimicrobial spectrum which has an inhibitor against both gram-negative and gram-positive bacteria including *Salmonella*, *E. coli*, *S. aureus*, *P. aeruginosa*, *B. subtilis*, and *S. typhimurium*. Furthermore, PPE extracted with methanol and ethanol is reported to have great antimicrobial properties among the other solvents.

**Keywords:** Antimicrobial, antioxidant, extract, pomegranate peel

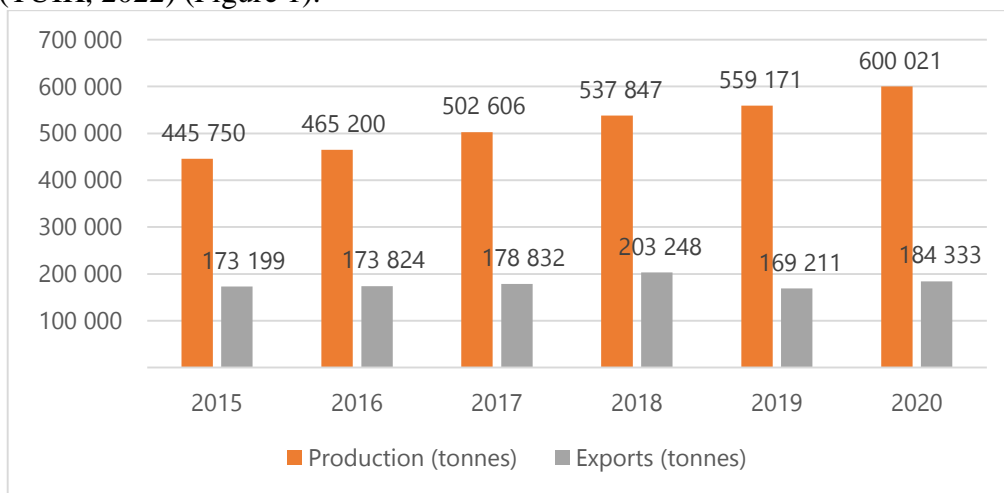
### **INTRODUCTION**

Plants are known as natural sources of many bioactive compounds that provide health benefits and are commonly used as alternative medicine. *Punicagranatum* is a fruit with many properties that have been known since ancient times. It is widely cultivated in several subtropical and tropical countries and is estimated to be around 300 thousand hectares worldwide, with a production of 3 million metric tons (Sharma et al., 2014). The secondary metabolite components in pomegranate peels have antimicrobial and antioxidant activity related to their high flavonoid and phenolic content including pyrogallol, anthocyanins, ellagic acid, catechol, catechin, hesperidin, quercetin, and glucosides (El-Hadary and Taha, 2020). However, the extraction method, extractor solvent, temperature, and extraction duration are important factors in the yield of these compounds. Soxhlet extraction and maceration are

common conventional methods. Meanwhile, non-conventional extraction methods such as microwave-assisted extraction, ultrasound-assisted extraction, and supercritical fluid extraction yield different quantitative and qualitative results (Latiff, 2021). High concentrations of PPE can increase 2,2-diphenyl-1-picrylhydrazyl (DPPH) activity whereas it is higher than that of pomegranate seed and arils extract (Benchagra et al., 2021; Sabraoui et al., 2022). PPE has a broad antimicrobial spectrum which has an inhibitor against both gram-negative and gram-positive bacteria including *Salmonella*, *E. coli*, *S. aureus*, *P. aeruginosa*, *B. subtilis*, and *S. typhimurium* (Hamady et al., 2015; Abdealsiede et al., 2020; Yassin et al., 2021). In addition, several studies revealed that phenolic content and its antimicrobial and antioxidant ability differ with extractor solvent polarities, therefore this review aimed to discuss the ability of pomegranate peel bioactive compound as an antimicrobial and antioxidant agent and its extraction methods.

### Pomegranate Fruit Production

*Punica granatum*, a member of the *Punicaceae* family, is a fruit with many properties that have been known since ancient times. It is widely cultivated in several subtropical and tropical countries, including Turkey, Afghanistan, Egypt, China, India, Thailand, Malaysia, the United States, Spain, and Italy (Sharma et al., 2014). Pomegranate planting is predicted to reach approximately 300 thousand hectares worldwide, with a production of 3 million metric tons of which about 76% of production comes from Turkey, India, Iran, and the USA. Except in very cold regions, pomegranate is grown in Turkey from Southeast Anatolia to the Eastern Black Sea region (Bozkurt and Ergun, 2021). Furthermore, pomegranate production has increased over the last five years in Turkey, with production reaching 559.171 tonnes and 600.021 tonnes in 2019 and 2020, respectively. Meanwhile, the average export for five years reached 180.441 tonnes (TUIK, 2022) (Figure 1).



**Figure 1.** Production and export of pomegranates fruit in Turkey (TUIK, 2022)

Pomegranate fruit consists of several parts, including peel, seeds, and arils. The peel accounts for about 43% of the fruit's weight, while the arils account for about 46% of the fruit's weight and are commonly used to produce pomegranate juice. Meanwhile, pomegranate seeds account for about 11% of the fruit's weight and are a source of oil with therapeutic potential (Ko et al., 2021). Pomegranate peel is commonly regarded as agro-industrial waste in pomegranate juice processing, and it is a potential source of a variety of nutrients and secondary metabolites. Previous research has found that pomegranate peel contains 3.3 % of protein, 1.2 % of fat, and 17.9 % of crude fiber, as well as vitamins and minerals (Ranjha et al., 2020).

Generally, processing one tonne of pomegranates yields approximately 669 kg (66.9%) of pomegranate by-products including peel, rinds, and pith (Abdel-salam et al., 2018).

### **Pomegranate Peel Extraction Method and Its Bioactive Compounds**

The secondary metabolite components in pomegranates peels have antimicrobial and antioxidant activity related to their high flavonoid and phenolic content including pyrogallol, anthocyanins, ellagic acid, catechol, catechin, hesperidin, quercetin, and glucosides with the main phenolic fraction of pyrogallol (453.58 mg/kg) and ellagic (125.61 mg/kg) and also main flavonoid fraction of hesperidin (50.47 mg/kg) and quercetin (35.19 mg/kg) (El-Hadary and Taha, 2020). Indeed the extraction method, type of solvent, and temperature are important factors in the yield of these compounds. According to Sharmin et al. (2016), increasing the temperature can improve extraction efficiency since it causes the cell wall more permeable, increases the solubility and diffusion into the compound, and decreases the solvent viscosity. However, temperatures higher than 40 °C reduce the yield of flavonoids and polyphenols due to degradation. Other studies also found that methanol had the highest yield of that other polar solvent in extracting the pomegranate peel (Sharmin et al., 2016; Ranjha et al., 2020). They explained that phenolics and flavonoids are polar compounds, therefore a more polar solvent (methanol) is a better extractor for these compounds. Regarding the extraction method, conventional extraction methods (eg. maceration, soxhlet) are characterized by low cell wall degradation and solvent diffusion. Meanwhile, non-conventional extraction methods (eg. supercritical fluid extraction, ultrasound-assisted extraction, microwave-assisted extraction) increase extraction efficiency and protect bioactive components against thermal degradation and oxidation (Latiff al, 2021). Table 1 shows that non-conventional extraction methods of pomegranate peel yield total phenolics more efficiently than conventional methods.

Maceration is a traditional extraction method that is suitable for thermolabile materials but requires a long time and a lot of solvents compared to the soxhlet method. In contrast to the soxhlet method, which cannot be applied for thermolabile compounds because prolonged heating (6-24 hours) can degrade the compounds (Muhamad et al., 2017). Meanwhile, the microwave-assisted extraction method has several advantages, including increased performance, low solvent consumption, and energy savings when combined with high automation. It uses high-frequency electromagnetic waves (0.3 -300 GHz). The principle of microwave heating is based on the direct effect of microwaves on molecules through ion conduction when a magnetic field is applied. The solution's resistance to ion flow causes friction, which causes heating (Lampakis et al., 2021; Calderón-Oliver and Ponce-Alquicira, 2021).

In the ultrasound-assisted extraction method, ultrasonic waves are employed with a frequency range of 20-2000 kHz to increase the permeability of plant cells. The mechanism is the concentration and acceleration of the solvent in accessing the active ingredient compounds in plant cells due to cell wall destruction. The bioactive components are released by the solvent through the diffusion process in the plant cell wall. However, ultrasound energy application higher than 20 kHz can affect the active phytochemicals through free radicals formation (Lampakis et al, 2021).

In the supercritical fluid method, carbon dioxide which has low pressure and critical temperature (73.8 bar; 30.9°C) is used as a supercritical fluid, where it is in a single phase (not gas or liquid) and cannot be dissolved and liquefied or evaporated by increasing pressure or temperature. It has faster reaction kinetics than liquids because of its higher solubility and



dispersion. The extraction requires 10-20 mL of solvent with extraction time ranging from 20-60 minutes, therefore is suitable for thermally labile analytes (Buyüktuncel, 2012).

**Table 1. Total Phenolics of pomegranate peel from different duration, solvents, and extraction methods**

| Extraction methods                    | Duration (minute) | solvent  | Total Phenolics (mg GAE/g) | Reference               |
|---------------------------------------|-------------------|----------|----------------------------|-------------------------|
| Maceration                            | 240               | acetone  | 24.50                      | Kupnik et al. (2022)    |
|                                       |                   | methanol | 25.92                      |                         |
|                                       |                   | ethanol  | 25.41                      |                         |
| Soxhlet                               | 300               | acetone  | 39.26                      |                         |
|                                       | 420               | methanol | 40.55                      |                         |
|                                       | 360               | ethanol  | 40.33                      |                         |
| Ultrasound-assisted extraction        | 20                | water    | 188.1                      | Hayder et al. (2020)    |
|                                       | 10                | methanol | 177.54                     | More and Arya, (2021)   |
|                                       |                   | water    | 119.82                     | Kaderides et al. (2019) |
| Enzyme-supercritical fluid extraction | 80                | -        | 301.53                     | Mushtaq et al. (2015)   |
| Microwave-assisted extraction         | 4                 | ethanol  | 199.4                      | Kaderides et al. (2019) |

### Antioxidant and Antimicrobial Activity of PPE (*In-vitro*)

Based on in vitro studies, Hamady et al. (2015) reported that PPE displayed the activity of antioxidants equivalent to butylated hydroxytoluene and greater than butylated hydroxyanisole at high concentrations (25, 50, and 100 ppm). Furthermore, due to its higher phenolic content, high concentrations of PPE can increase DPPH (2,2-diphenyl-1-picrylhydrazyl) activity whereas it is higher in extracts of relatively polar solvents (eg. ethanol, acetone, ethyl acetate) than non-polar solvents (eg. petroleum ether, chloroform) (Abdealsiede et al., 2020). For the 50% radical scavenger, PPE had an EC<sub>50</sub> of 0.04 mg/ml and pomegranate seeds extract had an EC<sub>50</sub> of 0.88 mg/ml. Also, using ABTS, pomegranate peel antioxidant activity was found to be 6.5-fold higher than that of pomegranate seed extract (Sabraoui et al., 2022). Moreover, pomegranate peel antioxidant activity was 1.73-fold higher than that of arils extract, as demonstrated by IC<sub>50</sub> values of 12.49 g/mL and 21.58 g/mL, respectively (Benchagra et al., 2021).

PPE has a broad antimicrobial spectrum which has an inhibitor against both Gram-negative and Gram-positive bacteria. Regarding the solvent type, Akarca and Başpınar (2019) reported that the highest antimicrobial activity was observed in the methanol solvent, as evidenced by the largest zone diameter. They also reported that PPE had a lower antimicrobial effect in water solvents against gram-negative bacteria (*Salmonella* and *E. coli*) than other solvents. The minimum inhibitory concentration and minimum bactericidal concentration of PPE against *S. aureus* extracted with methanol were 0.125 and 0.250 mg/mL, respectively (Yassin et al., 2021). Another study reported that PPE by ethanol solvent has the same inhibitory effect as the antibiotic Ampicillin against *E. coli* and *Salmonella*, and it has a better inhibitory effect against *S. aureus*, *S. typhimurium*, *P. aeruginosa*, and *B. subtilis* (Hamady et al., 2015; Abdealsiede et al., 2020; Yassin et al., 2021). The great antimicrobial property of PPE by methanol and ethanol solvent is due to its polarity, which can attract all phenolic compounds (Mehmood et al., 2018). Meanwhile, water is the most polar solvent compared to methanol and

ethanol which may be able to dissolve all polar components such as carbohydrates and lead to low total phenolics per sample weight while gram-negative bacteria's outer membrane has a strong barrier that prevents phenolic compounds from diffusing through the lipopolysaccharide layer (Borges et al., 2020).

The antioxidant and antimicrobial capacity of pomegranate is closely related to its two main polyphenolic tannins, namely punicalagin, ellagic acid, and ellagitannin (Shaygannia et al., 2016; Jafari, 2020). According to Hamady et al. (2015), because of their redox properties as hydrogen donors, phenolic compounds demonstrate antioxidant activity through reactions with free radicals. Furthermore, several mechanisms mediating the antioxidant activity of plant bioactive compounds include lipid peroxidation inhibition, reactive oxygen species (ROS) scavenging, several signaling pathways inhibition, and gene expression modulation (Hussain et al., 2016). Meanwhile, several antimicrobial mechanisms of polyphenolic compounds are based on their ability to precipitate bacterial cell membrane proteins and cause a decrease in membrane fluidity which causes bacterial cell lysis (Akhtar et al., 2015). Polyphenols have the ability to inhibit microbial enzymes by reacting with the sulfhydryl groups of proteins resulting in protein inactivation and microbial growth inhibition. Polyphenols are also able to limit NADH oxidation by reducing microbial oxygen consumption (Han et al., 2007). The hydroxyl group (-OH) can interfere with the metabolism of microorganisms by binding to the bacterial cell membrane and interacting with the active site of the enzyme (Pereira et al., 2006).

## CONCLUSION

Non-conventional extraction methods (supercritical fluid extraction, ultrasound-assisted extraction, microwave-assisted extraction) improve extraction efficiency by protecting sensitive bioactive components from thermal degradation and oxidation. Furthermore, because of its polarity, methanol is an excellent solvent for extracting pomegranate peels, which yields the highest concentration of phenolic compounds. Likewise, its strong antibacterial properties are demonstrated in methanol and ethanol solvents. PPE has a broad antimicrobial spectrum which has an inhibitor against both gram-negative and gram-positive bacteria including *Salmonella*, *E. coli*, *S. aureus*, *P. aeruginosa*, *B. subtilis*, and *S. typhimurium*. The antimicrobial and antioxidant activity of PPE is related to its phytochemical compounds including punicalagin, ellagic acid, ellagitannin, anthocyanins, catechol, catechin, hesperidin, quercetin, and glucosides.

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## **ANTIMICROBIAL AND ANTIOXIDANT PROPERTIES OF CHESTNUT (*Castanea sativa*) FLOWER EXTRACT AND ITS POTENCY AS POULTRY FEED ADDITIVES**

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### **ABSTRACT**

Antimicrobial misuse due to overuse or lack of dosage in animal production and healthcare practices is the main factor contributing to the Antimicrobial Resistance (AMR) problem. Plant bioactive compounds have been reported extensively to be a feasible substitute with equivalent efficacy to antibiotic growth promoters. Therefore, this review aimed to discuss the antimicrobial properties of chestnut flower extract and the ability of its bioactive compound as a poultry feed additive. The potential of chestnut flowers is high since Turkey is the world's third-largest producer of chestnuts with 72.655 tons produced in 2019. *In vitro* studies have reported that extracts of chestnut flowers contain high levels of antioxidants and antimicrobial compounds such as hydrolyzable tannins (gallic and ellagic acid), and flavonoids (catechin, myricetin, and kaempferol). The antimicrobial characteristics of the chestnut flower extract are excellent for both gram-positive and gram-negative such as *E. coli*, *E. cloacae*, *S. typhimurium*, and *B. cereus*. Although the information is very limited regarding *in vivo* studies of chestnut flower extracts in poultry, several studies regarding the effect of chestnut hydrolyzable tannin (HCT) extracts from chestnut wood have been carried out. HCT reduced the number of coliform bacteria and *E. coli* and increased the largest population of *Lactobacillus* in the small intestine. HCT significantly increased final weight, body weight gain, and feed efficiency, and reduced blood and egg cholesterol. Chestnut flower extract may have potential as a poultry feed additive since it contains polyphenols similar to its wood extract which have been evaluated to have a positive impact on broilers and laying hens, therefore further *in vivo* study on poultry is needed

**Keywords:** Antioxidant, antimicrobial, chestnut flower, extract, tannin

### **INTRODUCTION**

Poultry products are one of the most widely consumed products in the world due to their low production costs and lack of cultural and religious restrictions on consumption. Misuse of antimicrobials due to overuse in poultry production and health care is a major contributing factor to the problem of antimicrobial resistance (AMR). AMR in bacteria especially zoonotic bacteria represents a risk to human health as well as the presence of antimicrobial residue in meats and eggs. As a result, it is critical to identify natural feed additives that are safe for both animals and consumers, one of which does not cause AMR.

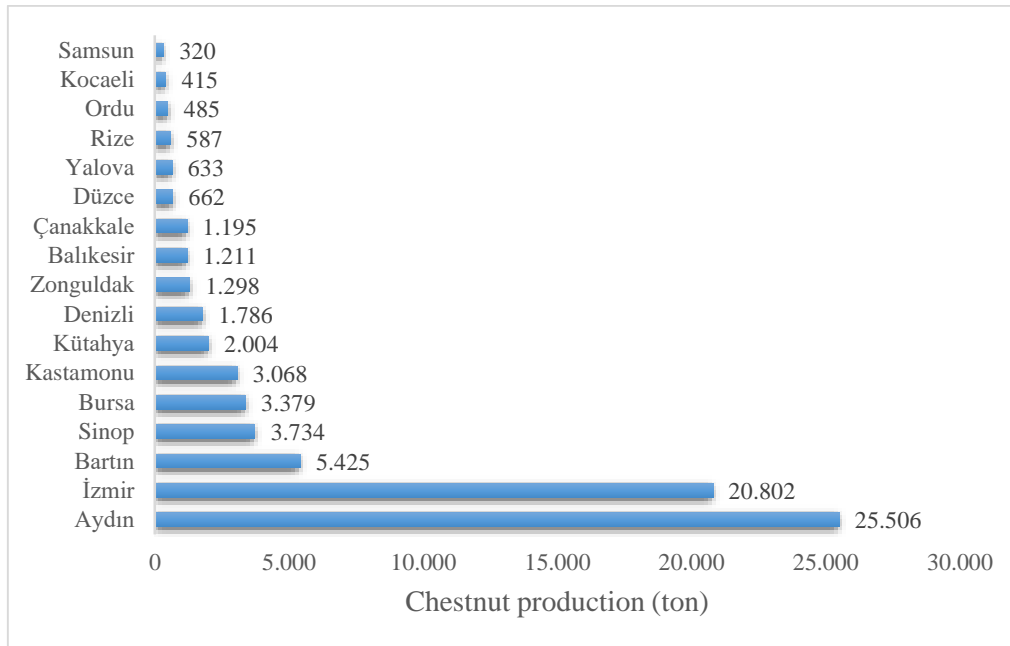
In Turkey, there are many potential plants with high bioactive compounds (essential oils, flavones, phenolics) as antimicrobial and antioxidant sources. Bioactive compounds or secondary metabolites are a group of different organic compounds produced by plants in response to biotic and abiotic factors to be used as defense mechanisms (Isah, 2019). Chestnut

(*Castanea sativa*) is one of the potential plants as a natural feed additive source. This potential is high since Turkey is the world's third-largest producer of chestnuts with 72.655 tons in 2019 (Table 1) and Aydin province is the largest producer of chestnuts (44.36%) in Turkey (Figure 1). Flowers and burs are plant wastes that can encourage the growth of insect larvae that can damage crops after the harvest season (Costa-Trigo et al., 2021). For this reason, some farmers prefer to collect and burn them, which has a negative impact on the environment.

In a previous study, with infusion and boiling methods, chestnut flower extract is the strongest of the antioxidants with its antitumor activity, anticancer and antibacterial properties (Carocho et al., 2014a, 2014b). However, the total extract yield is determined by various extraction properties, especially solvent type, extract methods, and plant varieties (Chemat et al., 2017; Carocho et al (2014a). The antimicrobial properties of chestnut flower extract by ultrasound-assisted extraction are excellent for both gram-positive and gram-negative bacteria, and can also inhibit lipid peroxidation with low IC50 and TBARS values (Silva et al., 2020; Alaya et al., 2021). There is limited information on the use of chestnut flower extract as an antioxidant and antimicrobial and antioxidants in poultry. However, 0.2% tannin obtained from chestnut wood extract resulted in higher body weight and it was noted that it could be used as an antibacterial and as a substitute for antibiotic growth promoters (Mannelli et al., 2019). Therefore, this review aimed to discuss the antioxidants and antimicrobial properties of chestnut flower extract and its possibility as a natural feed additive in poultry.

**Table 1. The largest producer of chestnuts in the world (FAOSTAT,2019)**

| Country     | Production (ton) |
|-------------|------------------|
| Chinese     | 1.849.137        |
| Spain       | 188.930          |
| Turkey      | 72.655           |
| South Korea | 54.708           |
| Italy       | 39.980           |
| Portugal    | 35.830           |
| Greece      | 28.980           |
| Japan       | 15.700           |
| North Korea | 12.872           |
| France      | 7.350            |
| Total       | 2.306.142        |



**Figure 1.** Production of chestnut in Turkey (TUİK,2020)

### **Chestnut Flower Bioactive Compounds**

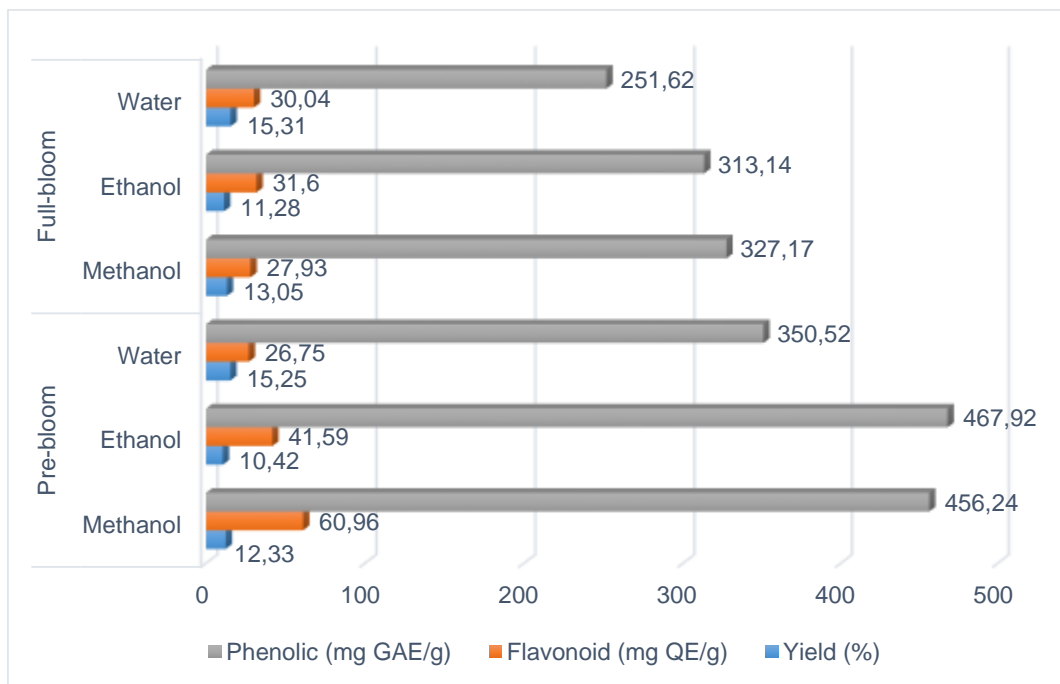
The chestnut flower blooms from late June to July where it consists of male and female flowers that appear on catkins. Chestnut flowers consist of two types of catkins, namely unisexual catkin (male flower), and bisexual which have one or more female flowers at the base and male flowers at the ends of the catkin (Figure 2). Male catkins are found in abundance and can produce pollen and are not used after fertilization and bur development (Miller et al., 2013). Previous studies reported that the male chestnut flower contains high bioactive compounds. Bioactive compounds or secondary metabolites are a group of different organic compounds produced by plants in response to biotic and abiotic factors to be used as defense mechanisms (Isah, 2019). In animals, they work to maintain a healthy gut bacterial population, reduce the number of pathogenic bacteria in the digestive tract, and stimulate an immune response. A healthy digestive system provides benefits for their growth, increased stress tolerance, and disease susceptibility. Phenolic compounds have the property of being antibacterial by diffusion to the outer membrane of gram-negative and positive bacteria through the lipopolysaccharide layer (Borges et al., 2020). Phenolic compounds have antioxidant activity through reactions with free radicals as hydrogen donors (Hamady et al., 2015).



**Figure 2.** Chestnut flower

Previous studies have shown that extracts of chestnut by-products contain many antioxidants and antimicrobial phenolic substances (Carocho et al., 2014a, 2014b; Silva et al., 2020; Alaya et al., 2021). However, there is a variation in yield among the studies which may be due to differences in extract methods, solvents, and varieties, Chemat et al., 2017; Carocho et al (2014). The male chestnut flower is one of the by-products that contain the highest bioactive compounds. Tuyen et al. (2017) reported that Japanese chestnut (*Castanea crenata*) flowers had the highest total flavonoids compared to the inner skins and burs. The phenolic content of chestnut flowers ranges from 48.6 mg/g to 72.20 mg/g (De Vasconcelos et al., 2010; Carocho et al., 2014a). Chestnut flowers contain 4.26 mg/g of total flavonoids together with main compounds such as catechins, myricetin, and kaempferol (Carocho et al., 2014a). A recent study by Ghellam et al (2021), which used 16 cultivars spread across the Samsun province, Turkey, showed that the Albayrak cultivar had the highest total phenolic content (231 GAE/g dry flower) followed by SAS-1, Erfelek, and Marigoule cultivars, where the major phenolic compounds found were rutin, chlorogenic acid, and gallic acid. Furthermore, Barros et al (2013) reported that the hydrolyzed tannin content of chestnut flowers was 14873 g/g. Methanol extraction has also produced promising results, as methanol can attract hydrophobic antioxidant compounds. Regarding flavonoids extracted by methanol, flavonol derivatives were the main compounds found including catechins, myricetin 3-O-glucoside, quercetin 3-O-rutinoside, quercetin 3-O-glucoside, kaempferol 3-O-rutinoside, and kaempferol 3-O-glucoside (Barros et al 2013). Furthermore, based on the chestnut cultivar, Carocho et al (2014b) reported that the *Judia* cultivar had higher antioxidant potential than *Longal*. In addition, they discovered that *Judia* cultivar samples contained higher flavonoid compounds, while *Longal* cultivar samples contained higher hydrolyzed tannins. Further studies on pre and full-bloom stages with various solvents were reported by Sapkota et al. (2010), where the highest total phenolic content was obtained at the pre-bloom stage using ethanol and methanol of 467.92 GAE mg/g and 456.24 mg GAE/g respectively. They reported that the largest total flavonoid content was also produced at the pre-bloom stage using methanol and ethanol of 60.96 mg QE/g and 41.59 mg QE/g, respectively (Figure 2). Figure 2 also shows that the water solvent had the highest yield compared to methanol and ethanol. It may be due to the fact that water is a universal solvent with the ability to dissolve both polar and nonpolar molecules and some impurity compounds such as proteins and carbohydrates (Mujtaba et al., 2016).





**Figure 2.** Yields, total phenolic, and flavonoid with different solvents in pre and full-bloom chestnut flower (Sapkota et al., 2010)

### Antioxidant and Antimicrobial Properties of Chestnut Flower Extract

The measurement of antioxidant activity of plant bioactive compounds extracts can be conducted by several methods. Caleja et al (2020) used  $\beta$ -carotene bleaching and TBARS on chestnut *Judia* cultivar flower using ethanol: water: (67:33) where it showed excellent results in inhibiting  $\beta$ -carotene bleaching and TBARS formation with EC50 values of 32  $\mu$ g /mL and 13  $\mu$ g /mL extract, respectively. This EC50 value obtained using the water: ethanol solvent is lower which indicates a higher antioxidant activity than that obtained with the water solvent. They stated that the detected bioactivity may be related to the phenolic profile of chestnut male flowers, including hydrolyzed tannins and flavonoids where trigalloyl-HHDP-glucoside is the main hydrolyzed tannin and myricetin-3-O-glucoside as the main flavonoid. A recent study by Ghellam et al (2021), which used DPPH and FRAP on 16 cultivars showed that high antioxidant activity was shown in Erfelek, Marigoule, Albayrak, and J17 cultivars with DPPH and FRAP values reaching 1339 mM TE/g and 1550 mM TE/g, respectively. This high antioxidant activity may be related to its phenolic content. This argument is supported by Carocho et al (2014b) that the *Judia* cultivar infusion with the highest total polyphenol concentration has the highest antioxidant activity. Also, Barreira et al (2008) found a significant negative linear correlation between polyphenol content and antioxidant activity EC50. It means that the high polyphenol content results in a lower EC50 value which proves that phenolics and flavonoids contribute to antioxidant activity. Although, they explained that flavonoids have a poor correlation with EC50 values for scavenging capacity. In particular, the tannin content has a very positive correlation with the antioxidant activity of the chestnut flower extract (Tuyen et al.,2017)

Regarding antimicrobial properties, Carocho et al (2014b) reported that *Castanea sativa* flower infusion and decoction were effective as antimicrobials where decoctions proved a better antibacterial effect than infusions with lower MIC values. In terms of MBC, the chestnut extract had a lower value than streptomycin and ampicillin against *Salmonella typhimurium*, *Escherichia coli*, and *Enterobacter cloacae* bacteria. Similar results were reported by Caleja et al (2020), that chestnut flower extract showed significant antibacterial activity with lower MBC

and MIC values compared to the antibiotic ampicillin against gram-negative bacteria including *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, and *Enterobacter cloacae* as well as gram-positive bacteria such as *Bacillus cereus*. According to Alaya et al. (2021), the great value of MIC and MBC has been associated with high amounts of hydrolyzed tannins such as pentagalloyl-glucoside and trigalloyl-hexahydroxydiphenoyl-glucoside and also flavonoids such as quercetin-3-O-glucuronide, myricetin-3-O-glucoside, quercetin-3-O-glucoside, and kaempferol-3-O-rutinoside. According to Horne et al. (2020), gram-negative bacteria have a double layer in the form of a thin layer of peptidoglycan (inner lipid bilayer) and an outer asymmetric lipid bilayer in the form of lipopolysaccharide while gram-positive bacteria only have a single lipid bilayer. However, based on an *in vitro* study, chestnut flower extract had higher inhibitory and bactericidal activity against gram-negative bacteria than antibiotics, indicating that chestnut flower polyphenol compounds may be able to destabilize and penetrate bacterial membranes.

### **The Potency of Chestnut Flower Extract as Poultry Feed Additives**

The highest bioactive compound content in chestnut flowers is hydrolyzable tannins and flavonoids. The presence of tannins in monogastric feeds has long been considered an antinutrient that has negative effects on monogastric animals. In particular, condensed tannins are able to inhibit digestive enzymes such as proteases, amylase, lipase, cellulase, and pectinase (Bhat et al., 2013) thus it reduces the digestibility of proteins, carbohydrates, and energy. However, based on numerous *in vitro* studies demonstrate that tannins have antimicrobial and antioxidant properties. Similarly, *in vivo* studies on poultry have shown that the proper dosage of tannins improves poultry performance and can replace growth promoter antibiotics.

Although the information is very limited regarding *in vivo* studies of chestnut flower extracts in poultry, several studies regarding the effect of chestnut hydrolyzable tannin (HCT) extracts from chestnut wood have been carried out. The positive effect of chestnut tannin extract on broilers challenged with necrotic enteritis was reported by Tosi et al. (2013) where HCT was able to control colonization of pathogens in the intestine without microbial resistance. The authors reported that HCT at a level of 250 mg/kg reduced the number of coliform bacteria and *E. coli* in the small intestine and increased the largest population of *Lactobacillus* at a dose of 1000 mg/kg. Furthermore, Jamroz et al. (2009) found that HCT supplementation at 500 mg/kg of feed had no effect on the histology of jejunal walls, body weight, or feed conversion of broilers, but HCT supplementation at 1000 mg/kg reduced final body weight and blood cholesterol. However, contrary to the results of the study by Dialoke et al. (2020) who reported that HCT supplementation at 1000 mg/kg significantly increased final weight, body weight gain, and feed efficiency for the starter and finisher periods, and Schiavone et al. (2008) who stated that administration HCT at the level of 2000 mg/kg had a positive effect on growth performance, especially in young birds and significantly increased final body weight. Also, the administration of 2000 mg/kg commercial HCT (750 g of tannic acid equivalents/kg product) in laying hens' diet had no effect on egg weight, egg production, eggshell thickness, but it reduced egg cholesterol. It is interesting since high doses of tannins have been proven to lower blood and egg yolk cholesterol without adverse egg production and egg quality. This mechanism was explained by (Zhao et al., 2014) that tannins may be able to limit fat solubility and fat absorption in the intestine.

Several studies reported on the effect of plant extracts on poultry, which may be due to these bioactive compounds entering the tissue through the circulatory system and being stored in the tissue and then acting as antioxidants. The presence of flavonoids can prevent cell damage due to free radicals through several mechanisms including scavenging reactive oxygen species,

activating antioxidant enzymes, reducing alpha-tocopheryl radicals, and increasing low molecular antioxidant properties (Procházková et al., 2011). Meanwhile, mechanisms of tannins in improving poultry performance include tannins at low doses increasing feed consumption and improving intestinal ecological health (Huang et al., 2018), thus it has a positive effect on nutrient absorption and poultry growth. Moreover, Min et al., (2008) explained that the possible mechanisms of inhibiting bacteria are through destabilization and permeabilization of cytoplasmic membranes, inhibiting enzymes in microbial metabolism, and absorbing substrates (e.g. iron and zinc) for their growth. Tannins also can bind to microbial enzymes and proteins as well as inhibit DNA replication through the binding of ions (Pathakoti et al., 2017). In addition, tannin can also prevent the formation of biofilms on the intestinal epithelium where it plays an important role in bacterial colonization since it can inactivate antibiotics and penetrate the microbial membrane (Girard and Bee, 2020).

## CONCLUSION

Based on *in vitro* studies, chestnut flower extract has high inhibitory and bactericidal activity against gram-negative and gram-positive bacteria and has high antioxidant properties which are indicated by a low EC<sub>50</sub>, TBARS, and DPPH value. Chestnut flowers have a high content of polyphenolic compounds, especially hydrolyzed tannins and flavonoids which are positively correlated with their antimicrobial and antioxidant properties. Chestnut flower extract may have potential as a poultry feed additive since it contains polyphenols similar to its wood extract which have been evaluated to have a positive impact on broilers and laying hens. As far as we know, no study has been conducted on the use of chestnut flower extract as a poultry feed additive, therefore further *in vivo* study on poultry is needed. We consider tannin to be studied in detail due to its diverse chemical structures and biological properties, especially as a poultry feed additive application where tannin has a positive effect at the right dose, on the other hand, it will reduce the poultry performance because of its ability to inhibit digestive enzymes thereby reducing the digestibility of protein, fat, carbohydrates, and energy.

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## **DIVERSITY AND ENHANCEMENT OF NTFP FROM PLANT GROUPS IN THE FOREST OF TESSER MRAMET OF THE MOUNTAINS OF TLEMCCEN (ALGERIA)**

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### **ABSTRACT**

The general objective of this study is the sustainable management of the forest ecosystems of the Tlemccen Mountains with a view to lasting conservation of floristic biodiversity, to promote these non-timber forest products (N T F P). A random inventory was carried out and dendrometric measurements focused on height and diameter at 1.30m (DBH). In this study, diversity indices were calculated. The results obtained made it possible to note that the average values of specific richness, of the diversity index of Shannon ( $H'$ ) and of equitability of Pielou (E) are respectively of  $7 \pm 2$  species /plot,  $H' = 0.5$  et  $E = 0.2$ . The average density of woody plants is 1100 individuals/ha with an average basal area of  $1.8 \pm 0.5$  m<sup>2</sup> /ha. These results contribute to evaluating the floristic diversity of the forest and the improvement of knowledge on the state indicators of the natural woody populations that can serve as a basis for the management of NTFP of forest species in the different economic and social domains.

**Keywords:** diversity, NTFP, sustainable management.

### **INTRODUCTION**

The forests in Algeria occupy an area of 4.1 million hectares of which 1.3 million are natural forests (FAO, 2000). The region of Tlemccen covers a forest area of 225,000 ha made up of forest, maquis and scrub (BNEDER, 2008). The center of Tlemccen is occupied from east to west by a mountainous area; which constitutes an important forest reserve of cork oak, zeens oak and Aleppo pine forests.

According to studies, the forest stands are composed as follows (Aleppo pine 83,000 ha, Holm oak 82,000 ha, Thuja 16,500 ha, Juniper 13,000 ha, Cork oak 4,800 ha), others 25,700 ha (brush, lentisks, doum, oleaster).

The Mediterranean studies have been approached by several authors such as Quézel (1964), Guinochet (1980), Alcaraz (1982), Hadjadj Aoul (1995) and Benabdellah et al. (2010). In our study, we were interested in the mountains of Tlemccen which has a flora rich in biodiversity which has been the subject of numerous works relating to various research themes. Our research consists in inventorying the ligneous species of the forest of Tesser Mramet to promote tree co-products.

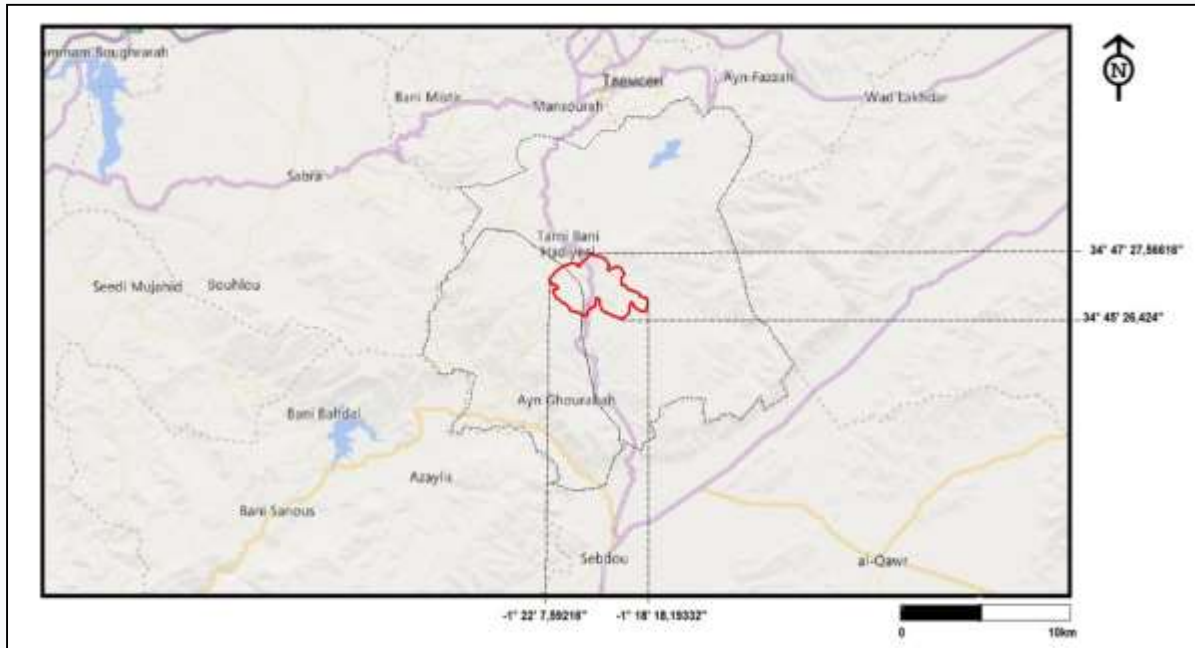
In the first part of the work, we made an inventory of ligneous species, calculated some parameters of diversity and dendrometry to see the appropriate strategies for the sustainable development and management of these co-products.

## MATERIAL AND METHODS

### 1. Presentation of the study area

The Tesser Mramet national forest, which covers an area of 1379 ha, is made up entirely of maquis, of which 647 ha are dense and 732 ha are clear.

The forest formations cover an area of 238 820 ha, representing an afforestation rate estimated at 26%. The tree stratum which corresponds to natural forests and reforestation occupies an area of 28 856 ha or 12% of the wooded areas of the Tlemcen (Figure 1).



**Figure 1.** Map of study area.

### 2. Data collection

The method adopted for data collection is the inventory foot by foot in the study plots. This method is an elementary and essential tool for any forest management, because it allows a description of the state of the resource. Characterizations of stands in terms of structure are linked to floristic composition. Within each plot, all individuals were counted. For each tree, the traditional diameter or circumference at 1.30 m from the ground (DBH, breast height) was measured.

### 3. Data processing

One of the first indices of diversity is species richness (SR). This index assesses the number of tree species in the stand. Although it allows distinguishing diversity based on the number of species, it gives no information about the weight of each species in the mixture.

Shannon's Index (Shannon, 1948) is an example of a time-independent algorithm distance. This index is undoubtedly most used to describe the diversity of species. The Shannon index ( $H'$ ), makes it possible to express the diversity by taking into account the number of species and the abundance of individuals within each of these species. Thus, a community dominated by a single species will have a lower coefficient than a community of which all species are codominant. If only one species is recorded in the plot, the Shannon index  $H'$  is equal to zero. For  $k$  species with equal proportions,  $H'$  corresponds to  $\ln(P_j)$  (Keren et al.,

2020). It derives from the theory of information and measures the entropy of a sample, or the "saturation" of the community.

The index is given by the formula:

$$H' = \sum_{i=1}^{RS} P_i \ln P_i$$

Pi: ni/N

RS: total number of species

Ni: number of individuals of a species in the sample

N: total number of individuals of all species in the sample.

The evenness index (E) is the ratio between the calculated diversity H' and the maximum theoretical diversity H'max which is represented by the log2 of the total richness S (Blondel, 1979). This index varies from zero to 1 (Barbero et al., 1987). An equitability equal to 1 corresponds to a community whose numbers are perfectly evenly distributed between the species, i.e. where all the species have the same number of individuals. Evenness is 0 when a single species dominates. Thus, equitability takes into account the potential absolute diversity of the community represented by H'max, thus reflecting the capacity of the system to support S species represented with equivalent proportions. This index measures equitability in relation to a theoretical equal distribution for all species:

$$E = H'/H'max$$

H'max: log S (number of species)

#### *Horizontal analysis*

In our case, it studies two dendrometric characteristics:

- Tree density (or stand density) which is given by the number of stems per hectare (N/ha).

- The basal area G, expressed in m<sup>2</sup>/ha, is the sum of the cross sections at a height of 1.30 m from the ground, of all the trees (Rabiou et al., 2015). It is determined by the following formula:

$$G_i = \sum g = \sum (\pi * D_i^2 / 4)$$

g: Basal area of individual i

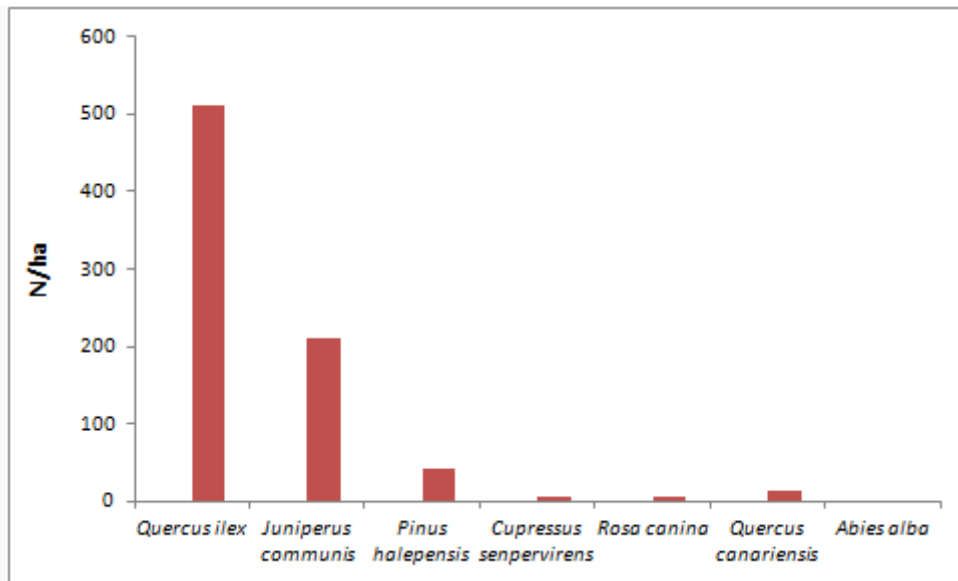
Gi: basal area of the stand

Di: Diameter at chest height of individual i

## **RESULTS AND DISCUSSION**

The most represented species are *Quercus ilex* by 500 individuals and *Juniperus* by 200 individuals followed by other species. Specific richness population studied is illustrated in figure 2.





**Figure 2.** Different species of the study area.

The Shannon index makes it possible to express the specific diversity of a studied stand. As a reminder, specific diversity characterizes the greater or lesser number of species present in a stand. If it is homogeneous (consisting of one and the same species), then the index  $H'=0$ . Shannon's diversity indices on the eight plots studied are variable, ranging from 0.02 to 0.17 so this index is low and indicates that our study area is less diversified, the number of species of which is 7 genders and in total  $H'=0.57$  (Table 1).

A weak equitability represents a great importance of some dominant species. In our study this index is variable between 0.01 and 0.09 on the eight plots whose tattle  $E=0.3$ . These variations in the equitability index are essentially linked to the pressures exerted by man and grazing (Sonke, 1998).

**Table 1. Diversity indices**

| Plot | P1   | P2   | P3   | P4   | P5   | P6   | P7   | P8   | Total |
|------|------|------|------|------|------|------|------|------|-------|
| (SR) | 43   | 81   | 50   | 194  | 141  | 108  | 66   | 111  | 794   |
| H'   | 0.08 | 0.17 | 0.14 | 0.03 | 0.08 | 0.03 | 0.02 | 0.02 | 0.57  |
| E    | 0.05 | 0.09 | 0.08 | 0.01 | 0.04 | 0.01 | 0.01 | 0.01 | 0.3   |

SR: Number of species, H': Shannon index, E: Equity index

This is the distribution of vegetation along a horizontal plane, and corresponds to the different types of tree distribution according to their distribution per unit area (Indir et al., 2013). The forest inventory carried out in the study area gave the results illustrated in Table 2. Generally speaking, the density of species encountered varies according to the plots studied. It varies from 477 individuals/ha in plot 1 to 2155 individuals/ha in plot 4. The average density of woody species in this forest is around 1102 individuals/ha; it is higher than that of the Sidi R'Ghies forest, which is  $735 \pm 368$  individuals/ha (Rached-Kanouni et al., 2019), despite the fact that these two forests are located in the same semi-arid bioclimatic zone.

**Table 2. Dendrometric parameters**

| Plots     | P1   | P2   | P3   | P4   | P5   | P6   | P7   | P8   | Mean |
|-----------|------|------|------|------|------|------|------|------|------|
| <b>D</b>  | 477  | 900  | 555  | 2155 | 1566 | 1200 | 733  | 1233 | 1102 |
| <b>Gi</b> | 0,82 | 0,78 | 0,52 | 1,58 | 1,83 | 1,1  | 0,51 | 0,12 | 0,91 |

Dendrometric characteristics are important indicators for measuring the qualitative and quantitative evolution of forest stands (Rached-Kanouni et al., 2000). Basal area is a criterion to judge the condition of a species in a stand. For all stands it is 0.91 m<sup>2</sup>/ha. Since basal area is closely related to diameter, land-use units with many small-diameter trees have low basal areas. Thus, the largest basal areas are observed in the stands of plot 5 (1.83 m<sup>2</sup>/ha), followed by the stands of plot 4 (1.52 m<sup>2</sup>/ha). The lowest value is observed in plot 8 (0.12 m<sup>2</sup>/ha).

### CONCLUSION

The sustainability of a forest ecosystem depends closely on its health. The Tesser Mramet forest in the Tlemcen Mountains (western Algeria), is a natural complex characterised by a plant cover, and one of the most important areas where floristic biological diversity is found, consisting essentially of *Quercus ilex*, the main species. The study of the dendrometric characteristics of this forest showed that this stand is stable and regular with a dense canopy, characterised by young trees. By extending our study to the entire forest, the results can serve as a reference for forest managers.

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## INSIGHTS INTO PHYLOGENETIC UTILITY OF *RPD2* GENE: A CASE STUDY OF *SILENE* L. (*CARYOPHYLLACEAE*)

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### ABSTRACT

Increasing availability of DNA sequence data have led to better understanding of the phylogenetic relationships in many difficult organismal groups more than ever. Genome scale data are now accessible on affordable cost, however due to the various limitations of the analyzing tools, genome based phylogenetic inference is challenging. Low-copy nuclear genes have been successively used for phylogenetic reconstruction in complex groups such as genus *Silene* L., where evolutionary events (e.g., incomplete lineage sorting, gene duplications etc.) lead to reticulate interspecific relationships. Nevertheless, there is not only a shortage of low-copy nuclear markers but also, the phylogenetic usefulness of such markers is an issue. In this study, using sequence data of *RPD2* region from the samples representing some major lineages of genus *Silene* we have attempted to evaluate phylogenetic usefulness of this marker. Our results are in agreement with the studies indicate duplicate copies of *RPD2* locus in *Silene*.

**Keywords:** Paralogues, Phylogenetics, Polymerases, *Silene*, Species complex.

### INTRODUCTION

Advances in sequencing technologies have made the presence of genome scale sequence data at much reasonable costs while increasing the need for powerful analyzing tools that can handle such amount of information (Christe et al., 2021). The associated problems for phylogenetic estimation from large scale data makes the multiple, unlinked, low-copy genes a good alternative for resolving complex situations where evolutionary relationships do not suit to bifurcated trees due to several biological reasons including hybridizations, paralogy, gene/genome duplications etc (Petri and Oxelman, 2011; Bertrand et al., 2018). Using of multiple low-copy nuclear markers jointly can disentangle reticulated phylogenetic relationships with higher accuracy, nevertheless the number of such markers that has been proved for their phylogenetic utility, is somehow limited. A number of RNA polymerases (RNAP) genes have been used for phylogenetic inference of complicated plant groups including genus *Silene* L (Oxelman et al. 2004; Popp and Oxelman, 2007).

RNAP are the group of enzymes that are responsible for gene expression. In eukaryotes, typically there are three forms of RNA polymerase (Pol I, II, III) whereas they are present as five variants (Pol I, II, III, IV and V) in angiosperms. The RNAP have about 12 subunits (Luo

and Hall, 2007). The largest subunits of Pol I, II, III, IV and V are reported as *Nrpa1*, *Nrpb1*, *Nrpc1*, *Nrpd1* and *Nrpe1* and the genes encoding these subunits follow as *NRPA1*, *NRPB1*, *NRPC1*, *NRPD1* and *NRPE1* (Marcussen et al., 2010). In the same way, the genes encoding the second-largest subunits of the five polymerases are called as *NRPA2*, *NRPB2*, *NRPC2*, *NRPD2* and *NRPE2* (Marcussen et al., 2010). Pol IV exists in all plants. The *NRPD1* and *NRPD2* genes, encoding the largest and second-largest subunits of Pol IV, are functionally different and earlier studies have stated the independent duplication of their Pol II homologs *NRPB1* and *NRPB2*, as their origin (Popp and Oxelman 2004). The duplication for *NRPD1* and *NRPE1* genes is noted as widespread in angiosperms while *NRPD2* and *NRPE2* are reported as rare in comparison, and therefore stands as single copies in monocots and some families of eudicots. On the other hand, several families (e.g., Brassicaceae, Euphorbiaceae, Salicaceae, Caryophyllaceae, Violaceae) of eudicots carry duplicate copies of *NRPD2/E2* (The Arabidopsis Genome Initiative, 2000; Marcussen et al., 2010). Two distinguishable paralogs of *RPD2* are reported (Popp and Oxelman, 2004; 2007) in *Silene*. Yet, not all the genes encoding the 12 subunits of RNAP have been fully checked for phylogenetic purposes, however *RPA2* and *RPB2* are largely studied in *Silene* (e.g., Popp and Oxelman, 2004; Toprak et al., 2016; Naciri et al., 2017). Comparing to the former two markers, *RPD2* gene have been used in a lesser extent (Grundt et al., 2004; Petri and Oxelman, 2011) within *Silene*.

*Silene* is the largest genus of the Caryophyllaceae family with about 870 reported species (Greuter, 1995; Jafari et al., 2020). The genus has a world-wide distribution excepting Australia and Antarctica. In the latest global revision of the genus by Jafari et al. (2020), *Silene* is divided into three subgenera; *S.* subgenus *Silene*, *S.* subgenus *Lychnis* (L.) Greuter, and *S.* subgenus *Behenantha* (Otth) Endl (Greuter, 1995; Oxelman et al., 2001; Jafari et al., 2020). The delimitation of the first two subgenera is well supported (Oxelman et al., 2001; Petri and Oxelman, 2011; Aydin et al., 2014). However at lower taxonomic levels there are uncertainties for many section, subsection, group and series. The majority of the *Silene* species are diploid with  $x = 12$ , but there are also, a considerable number of polyploid species mainly occur in North America and Northern Asia (Petri and Oxelman, 2011). Normally, diploid *Silene* species are expected to possess a single copy of *RPD2* gene, however several earlier studies (e.g., Popp and Oxelman, 2007; Petri and Oxelman 2011) have reported two paralogue copies *RPD2* gene. Here, using sequence of *RPD2* gene from 55 taxa of *Sileneae* tribe, we check for the paralogous of *RPD2* and evaluate the phylogenetic utility of this marker.

## MATERIAL AND METHOD

The sequences used in the study were selected to represent several major lineages of *Silene* [e.g., *S. cryptoneura* Stapf, (subgenus *Behenantha* section *Cryptoneurae*), *S. uralensis* (subgenus *Behenantha* section *Physolychnis*), *S. latifolia* Poir. (subgenus *Behenantha* section *Melandrium*), *S. schafta* (subgenus *Silene* section *Auriculatae*), *S. nivalis* (subgenus *Silene* section *Siphonomorpha*) *S. aegyptiaca* L.f (subgenus *Behenantha* section *Atocion*)] as well the two close genera (*Petrocoptis* A.Braun ex Endl., and *Atocion* Adans.). In total, 55 sequences of *RPD2* marker were used. About 30% of the sequences were newly generated from the members of *Silene* sections *Atocion*, *Cryptoneurae*, and *Sordidae*. Rest of the sequences (70%) were already been published elsewhere, and downloaded from BoxTax Database (<http://www.sileneae.info/>). These sequences are also available on genbank and can be accessed via their corresponding genbank number.

Genomic DNA was extracted by a modified CTAB protocol (Yoon et al., 1991) from the dried plant material followed by a purification step made with custom purification kits (Qiagen). PCR reactions were performed using the primer pairs “*RPD2FP/RPD2RP*” developed by Popp

and Oxelman (2004), and having the following sequences “5’ GCATGTGGTGGYACD TTGAGATATGCT 3’ ”/ “5’ CTTTCAYTYCCCCATCGACAGAATCCAG 3’ ” respectively. Amplified products were sequenced with the same primer pairs using Sanger method by MacroGen Inc. (Seoul, South Korea). Details of the applied protocols can be accessed from BoxTax Database. Alignments were done via Geneious Prime version 2022.1.1., Biomatters, <http://www.geneious.com>. The parsimony informativeness of the data, and the choice of the substitution model were estimated using PAUP\* Version 4.0a169 (Swofford, 2002).

The relationships among the 55 RPD2 sequences were estimated with maximum likelihood (ML) inference method using PhyML 3.0 (Guindon et al. 2010), available on [www.phylogeny.fr](http://www.phylogeny.fr). The data file was specified for the GTR+G as substitution model with 4 rate categories. The analysis was performed on a complete random starting tree without specification of any out group, and using 100 bootstrap replicates. Rest of the other parameters were accepted as the defaults implemented by [www.phylogeny.fr](http://www.phylogeny.fr). The estimated ML tree visualized in FigTree v1.4.4 <http://tree.bio.ed.ac.uk/>.

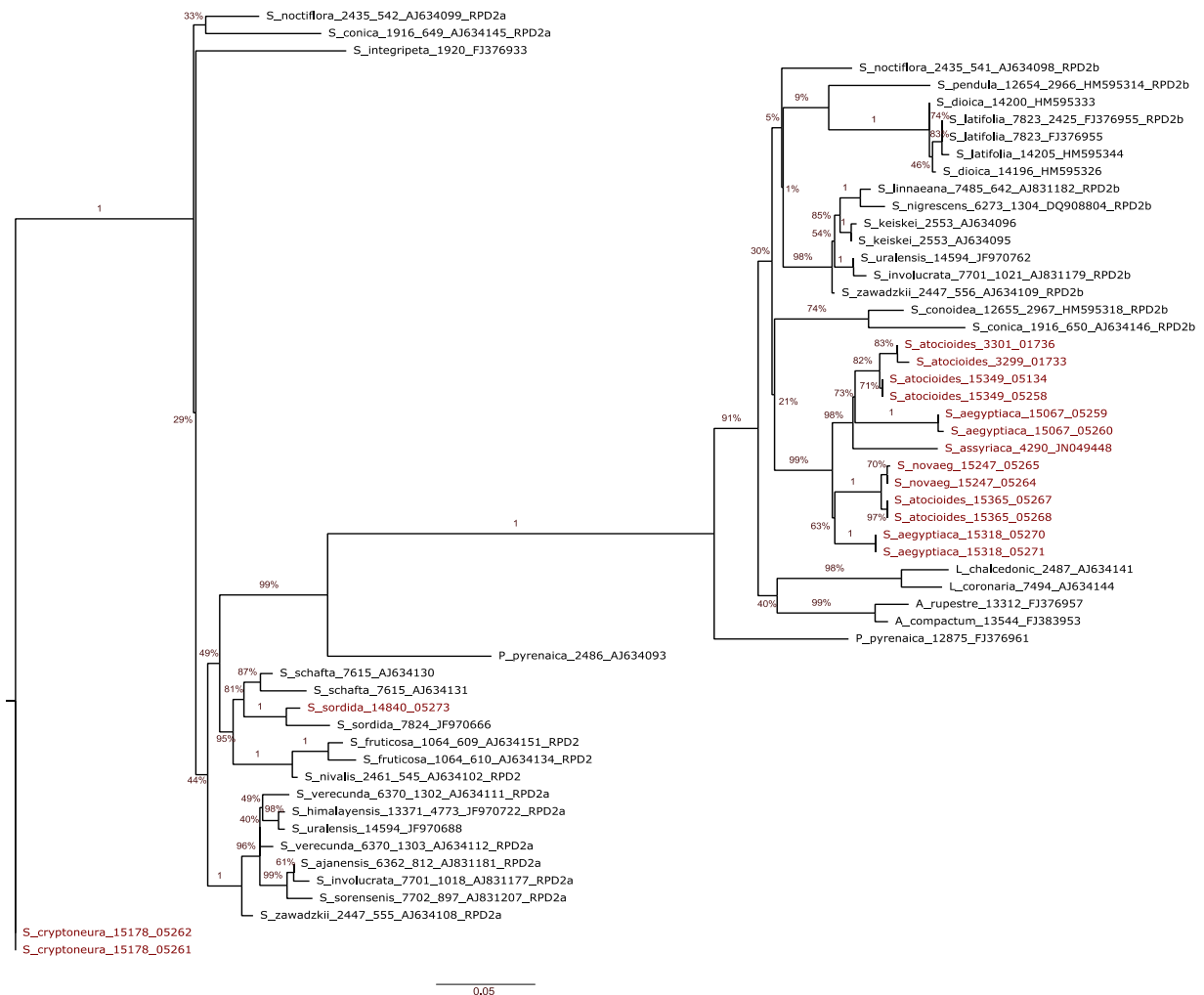
## RESULTS AND DISCUSSION

The information and parsimony statistics of the RPD2 data set is presented in Table 1. The observed ML tree (Fig. 1) produces a rather conflicting pattern regarding the position of several taxa (e.g., *S. latifolia*, *S. dioica*) whose phylogenetic position is well known within *Silene*. The relationships among the major lineages are poorly resolved. The representatives of the three subgenera of *Silene* (including *Lychnis coronaria* and *L. chalcedonica*) were recovered as intermingled across the tree without a clear subgeneric delimitation. Overlapping with previous studies (Petri and Oxelman 2011), the majority of the sequences are placed as forming two separate clades no matter the sequence is from the same species or not, which well matches to a clear phylogenetic pattern of the paralogy case. Each allele for diploid species such as *S. uralensis*, *S. zawadzki*, are shown in two divergent clades. That was the case for several other species (e.g., *S. involuclrata*, *S. noctiflora*, *S. conica*) belonging to different sections. The two sequences of *S. verecunda* known as a polyploid species, are displayed in the same clade but as non sister. These outcomes indicate that the shown relationships are rather based on the copy of the RPD2 recovered for that particular species rather than been reflecting the common ancestry. All the sequences of the section *Atocion* fall into the same clade indicating that the same copy of the RPD2 paralogues being captured in this group. However, the sequences *S. sordida* are located in the other clade, suggesting that the other copy (than the copy found in samples of section *Atocion*) being captured for this species. This observation strongly support the hypothesis states the duplicate copies of RPD2 locus within *Silene* (Popp and Oxelman, 2004; 2007). The samples of *S. cryptoneura* are placed on a highly divergent position than the rest of the sequences, which prevent the clear understanding of which copy is been caught within this group.

To summarise, the nuclear RPD2 locus seems under ongoing evolutionary process compatible with a recent duplication event, at least in genus *Silene*. Therefore, it bears conflicting phylogenetic signals and should be used with extra caution. Nevertheless, the data set used in this study is in a rather small scale and analyzed in a very simple framework, especially considering how vast could be the topic of phylogenetic inference. More sophisticated analysing methods run on more comprehensive data may reveal further knowledge of RPD2 marker contradicting outcomes of this study.

**Table 1. Statistics of parsimony analysis for the RPD2 locus.**

|                                    | RPD2  |
|------------------------------------|-------|
| Number of samples                  | 55    |
| Aligned matrix length [bp]         | 970   |
| Constant characters [bp]           | 441   |
| Variable characters [bp]           | 117   |
| Parsimony-informative charact [bp] | 412   |
| Consistency index (CI)             | 0.725 |
| Retention index (RI)               | 0.921 |
| Homoplasy index (HI)               | 0.275 |
| Rescaled consistency index (RC)    | 0.668 |



**Figure 1.** Maximum likelihood tree of the *RPD2* locus. Numbers on the branches of the tree are the Bootstrap values (Bs). Numbers following the sample names are the specimen & genbank IDs, respectively, as they are stored in BoxTax Database.

## CONCLUSIONS

*RPD2* locus is in duplicated copies which may cause inconsistent inference of phylogenetic relationships in genus *Silene*. The phylogenetic tree obtained from this study is well suits with a tree topology that would be expected from a typical paralogues loci. Therefore, phylogenetic inference solely based on *RPD2* locus may be insufficient.

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## EVALUATION OF POLLUTION STATUS OF STREAMS IN THRACE REGION

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### ABSTRACT

Thrace Region is a region where environmental pollution is intense due to industrialization. The most important environmental problem of the Thrace Region, especially in terms of agricultural land, is that it is close to the Ergene River and has a negative impact on it. As a result of the growth and decentralization of industrialization in İstanbul that started after the 1980s, the Thrace Region was faced with intense industrialization. Previously, there was an economy dependent on agriculture and industrial facilities using agricultural inputs such as factories producing flour, dairy products, vegetable oil and feed in the region. Afterwards, industrial establishments such as textile, leather, metal goods and chemistry, which did not use local resources, came to the region quickly with the incentives. Thanks to this process, today's social, economic and ecological picture has emerged. The waters in the Ergene Basin, which was a region rich in groundwater and surface waters in the past, were polluted by domestic, industrial and agricultural pollution, and water withdrawals from unauthorized wells caused an extreme decrease in groundwater amounts. Groundwater and surface waters are polluted, especially as a result of untreated domestic and industrial wastewater, unconscious agricultural spraying and fertilization. The issue of protection of water basins, which is important around the world, is very critical for the Ergene River. Although the rapid industrialization in the region contributes a lot to employment, the economy of the region and the country, it should be sustainable and care should be taken not to create pressure on natural resources. The concept of environmental protection and environmental right is a problem of democracy and democratic participation in the realization of human rights. No economy or economic system can be considered successful if the level of civilization and achievements that humanity has reached today have been created at the expense of future generations and the number of poor people has gradually increased. In this study, it is aimed to deal with the pollution status of water resources in the Thrace Region, located on the European side of Turkey, and the problems experienced in the use of water in the region.

**Keywords:** Thrace Region, Ergene Basin, Water pollution, Agricultural situation

### INTRODUCTION

Environmental pollution is one of the most important problems of our age. Since the beginning of civilizations, the effects of human beings on the environment have necessarily started. The resources in the world have been used as if they will not be exhausted for centuries and today, these resources are faced with the danger of extinction. Moreover, the amount of consumption continues to increase exponentially with the effect of rapid population growth. The depletion or damage of natural resources poses a vital threat not only to human beings but

also to all living things. Among these natural resources, water is one of the sine qua non of life (Yılmaz, 2013).

### **Thrace Region Water Collection Basins**

There are two water collection basins in the Thrace region. One of these is the Meriç River, which forms the Turkey-Greece border, the Tunca River, the Ergene River and the Meriç-Ergene basin formed by its branches. The basin covers most of the region and is a major river system. Many streams combine to form the Ergene River, and the Ergene River merges with the Meriç River and flows into the Aegean Sea. The other is the Marmara Basin, which is formed by many coastal streams flowing into the Black Sea and Marmara Sea (İstanbulluoğlu et al., 2006).

The Thrace Region is a very rich region in terms of surface and underground water resources. The main surface water resources of the region are the Meriç, Tunca and Ergene Rivers. The Meriç River, which forms the border of Turkey with Greece and Bulgaria, is the most important stream of the Thrace peninsula. The Tunca River, which is the tributary of the river, originates from Bulgaria, the Arda River from Greece and the Ergene River from Turkey. The Meriç Delta, formed in the area where the Meriç River empties into the Saros Gulf, is in the protection status of a National Park and Natural Protected Area. The Meriç River originates in Bulgaria and flows south-east, drawing the Greece-Bulgaria and Turkey-Greece border. Within the borders of Edirne province, it first joins the Arda River and then the Tunca River. After drawing the border between Edirne and Greece, it merges with Ergene around Balabancık village. From now on, it flows towards the south-west and pours into the Aegean Sea from the town of Enez. It has a length of 185 km along the Turkey-Greece border. Its depth varies between 60 cm and 520 cm (Anonymous, 2005; Hallı, 2013).

### **Meriç-Ergene Basin Water Pollution**

Today, Turkey is a country whose water resources are rapidly depleted and polluted. In order for a country or province to be considered water-rich, it must have more than 10,000 m<sup>3</sup> of water per capita. Countries with water resources between 1000-2000 m<sup>3</sup> per capita per year are defined as 'facing water shortages' and countries with less than 1000 m<sup>3</sup> are defined as 'water scarce countries'. Countries in this situation have serious problems in food production, economic development and protection of natural systems (Falkenmark, 1989; URL 2). The amount of water per capita in Turkey is 1,400 m<sup>3</sup>, and in Thrace it is approximately 400 m<sup>3</sup>. This situation requires not only to develop water resources, but also to seek solutions that can use existing resources more wisely (Anonymous, 2007).

With the increase in population in the world and the development of industry, the natural structure of water on the earth is deteriorated in an undesirable way. Water pollution arises when spring water or any natural water is affected by physical, chemical, biological or radioactive additives. The water becomes polluted by adding sewage water, wastes from industrial and agricultural activities, and other harmful and undesirable substances to the water in amounts or concentrations that may worsen the quality of the water. As a threat to human and animal health and plant growth, the property of water deteriorates. Everything that will make it difficult for living things to live and disrupt the ecosystem balance is directly or indirectly 'water pollution' (Tan, 2006).

The increasing population rate and industrial pressure in İstanbul, Turkey's largest city, has shifted towards the settlement cities in its immediate vicinity, and an intense industrialization has occurred around the Ergene Basin, especially in the Tekirdağ province within the Thrace Region. This situation has led to various levels of pollution around the Ergene

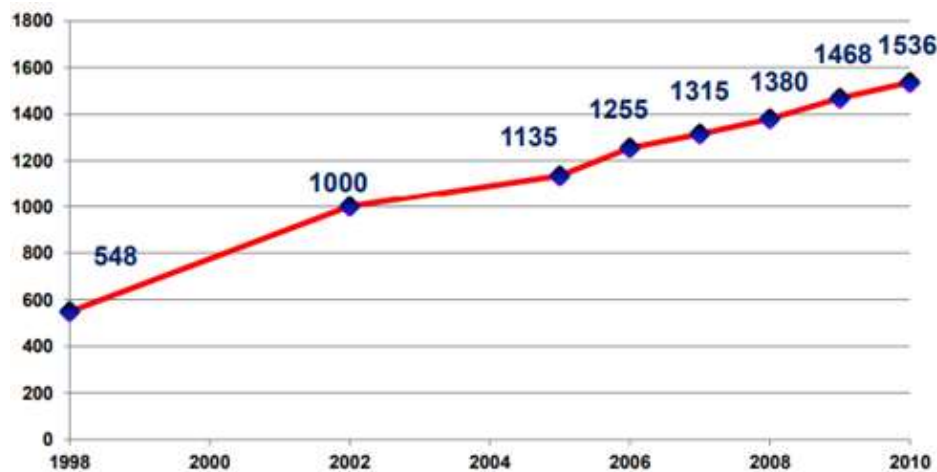
Basin caused by industrialization. In settlements along the river, odor and visual pollution are unbearable, especially during the summer season (Sezen, 2013).

The Meriç River, which enters our country by collecting wastewater from Bulgaria and Greece, is firstly polluted by the wastewater of the textile factories in the north of Edirne. Afterwards, it is observed that the pollution load increases with the mixing of the Tunca River, which carries the domestic wastes of the city of Edirne. This pollution load increases even more from the point where it joins the Ergene River. The Ergene River is polluted by the domestic and industrial wastewater of settlements such as Çerkezköy in the east, Uzunköprü in the west, Kırklareli in the north and Kozyörük in the south, as well as the streams and brooks flowing into it.

In the fertile lands of the Thrace Region, agriculture is carried out almost all year round, and industrial activities have a very important place. There is pollution in the river due to the industrial facilities concentrated around the Ergene River and various pollutants mixed into the stream from the settlements located there (Özuluğ, 2018). Urbanization activities have accelerated in Çorlu and Çerkezköy districts, which are close to İstanbul, as a result of intense industrial activities and internal migration, which has increased especially in Tekirdağ province since the beginning of the 1990s (Altürk, 2017).

Figure 1 shows the number of industrial establishments in Tekirdağ province. The number of industrial establishments updated in 2011 and registered as of the end of 2010 is 1536 (Anonymous, 2011).

#### NUMBER OF INDUSTRIAL ORGANIZATIONS IN TEKIRDAG



**Figure 1.** Number of Industrial Establishments in Tekirdağ Province by Years (Anonymous, 2011)

The pollution caused by the industrial wastewater discharges in the basin in the Ergene River is much higher than the other polluting factors. With the discharge of wastewater from industrial facilities to the receiving environment without treatment, both the ecological balance in the Ergene River has deteriorated and the water drawn from the river for irrigation has caused the pollution of agricultural lands. 82% of the industrial areas are located in Tekirdağ, 10% in Kırklareli and 8% in Edirne (Anonymous, 2017, URL 2). Most of the facilities in the basin are gathered around Çorlu, Çerkezköy, Muratlı and Lüleburgaz Districts.

The amounts of industrial water originating from the businesses operating in the Thrace Region are shown in Table 1. The most important reason for the pollution of the Ergene River,

which is the most important problem of the region, is the industrial wastewater originating from the industrial facilities (Anonymous 2012; Sezen, 2013).

**Table 1.** Thrace Region (Tekirdağ-Edirne-Kırklareli) Industrial Establishments Total Industrial Wastewater Amounts (Including OIZ) (Anonymous 2012).

| Province     | Wastewater amount m <sup>3</sup> /day |
|--------------|---------------------------------------|
| Tekirdağ     | 283.780                               |
| Kırklareli   | 47.090                                |
| Edirne       | 1.183                                 |
| <b>TOTAL</b> | <b>332.053</b>                        |

The water pollution in the streams of the Ergene River, especially in the Çorlu, Çerkezköy and Muratlı regions, has affected the living life and caused the extinction of the aquatic organisms. It is not possible for any living thing to live in the river, which was once used for fishing and harbored various kinds of creatures (Dede, 2010). Agricultural and livestock activities in the region were also affected by the water pollution in the river. The transformation of the Ergene River into a wastewater channel as a result of industrial wastes and the increase in heavy metal load in the water and soil as a result of rain waters and floods have terminated the animal husbandry activities in this region (Sezen, 2013).

Amount of wastewater discharged into Ergene River (%) is given in Figure 2. Accordingly, the highest amount of wastewater discharged to the Ergene River originates from the industrial establishments of Tekirdağ with a share of 85% (Anonymous, 2012).

#### Distribution of Wastewater Discharged into the Ergene River



**Figure 2.** Amount of Wastewater Discharged into the Ergene River (%) (Anonymous, 2012)

When the distribution of wastewater on the basis of provinces is examined, Tekirdağ province discharges 84% of industrial waste and 40% of domestic waste. Edirne ranks second and Kırklareli third in domestic waste discharge. In industrial waste discharge, Edirne only makes 3% of the total discharge with 10,000 m<sup>3</sup>/day. Tekirdağ makes 66% of all wastewater discharge. Two thirds of this is industrial waste discharge.

#### Agriculture and Heavy Metal Pollution in the Meriç-Ergene Basin

The geography of Thrace is 2.3% of the country's surface area and 82% of these lands are arable. Although it is 8% of the agricultural areas, 10% of wheat production, 63% of sunflower and 44% of rice are grown in these agricultural lands. The Ergene River, which originates from

the Yıldız Mountains, is the jugular vein of these lands and gives life to these lands. Ergene Basin, on the other hand, forms the heart of Thrace. These highly fertile agricultural lands have been irreversibly contaminated with heavy metals and chemicals. There are also toxic substances in groundwater (Hallı, 2013).

Ergene River pollution has become a symbol of river pollution in Turkey. In the research report of the pollution and environmental problems in the Ergene River, the data entered the parliamentary records as documents. Industrial wastes are primarily responsible for river pollution. The water-based industry, which came to the region with the 1st decentralization of İstanbul, soon spread out of the Çerkezköy Organized Industrial Zone. As an illegal industrial establishment, the Ergene River and its branches began to be built around it without permission, no treatment plants were established or the compulsory ones were not operated, the waste waters containing chemicals and heavy metals were discharged into the river and its branches, making Ergene an 'industrial sewer'. Agricultural lands, where the river is used for irrigation purposes, have started to be polluted permanently. It is documented by scientific reports that this water cannot be used for any purpose (İnci et al., 2011).

Germination and emergence times of seeds sown in soils irrigated with Ergene water were prolonged. Negative effects on plant vegetative development such as height and number of leaves were observed. It has been revealed that the polluted Ergene water should be used for irrigation purposes in agriculture in a very, very controlled manner (Konukcu et al., 2004) (Figure 3). However, it is possible to use the water of the Ergene River, especially in the irrigation of paddy fields, after mixing with this river, downstream from the point where it pours into the Meriç River in the region forming the northwest and western border of the region. In addition, there is no problem in terms of irrigation water in some branches of the Ergene River and in many small streams flowing into the Black Sea and Marmara Sea (İstanbuluoğlu et al., 2006; Tok et al., 2005).



**Figure 3.** Pollution status of Çorlu Stream ([URL 3](#))

Living things in the water column are most affected by chemical pollution. In general, all metals that are toxic and causes environmental pollution are called heavy metals. Heavy metal definition is used for metals with a density higher than 5 g/cm<sup>3</sup> in terms of physical properties.

This group includes more than 60 metals, including lead, cadmium, chromium, iron, cobalt, copper, nickel, mercury and zinc. Heavy metals pass into water resources when industrial wastes or acid rain dissolve the soil and therefore heavy metals in its composition, and the dissolved heavy metals reach rivers, lakes and groundwater (Kahvecioğlu et al., 2004).

The extent to which industrial and domestic wastes in the Ergene River basin affect the metal accumulation in the current sediments and the effect of possible pollution on the Aegean Sea were investigated with the Al, Fe, Mn, As, Cr, Cu, Ni, Pb, V and Zn contents in the surface sediment sample. Interval of metals in grab samples; Al 4% - 9.6%, Fe 1.4% - 4.2%, Mn 133-865, As 11 - 52 ppm, Cr 95 - 304 ppm, Cu 23-203 ppm, Ni 19-155 ppm, Pb 77 - 145 ppm, V 258 - 966 ppm and Zn 74 - 388 ppm. The average values are respectively; 7.5% (Al), 2.8% (Fe), 352 ppm (Mn), 25 ppm (As), 162 ppm (Cr), 65 ppm (Cu), 70 ppm (Ni), 100 ppm (Pb), 483 ppm (V) and 189 ppm (Zn). When the averages of all these metals and the shale averages are compared; It was determined that Al, Fe, Mn and Ni values were low, while As, Cr, Cu, Pb, V and Zn values were high. It is inevitable that these high metal values are transported to the Aegean Sea with dissolved or suspended particles in water.

Erbay (2010) calculated that 5.8 tons of As, 8.5 tons of Cu and 11.2 tons of Pb, dissolved in the water of the Meriç River, are transported to the Aegean Sea annually. He also determined that the As, Cu and Pb values were higher than the river waters joining the Mediterranean from Greece and Italy. The pesticides and fertilizers contained in the widespread agricultural activities in the Meriç Basin caused the increase of As, in particular, and the increase of other metals in the industrial residues. As the Meriç River has carried high amounts of As, Pb, V and Zn to the Aegean Sea, it is inevitable that the sediments in the coastal areas of the Aegean Sea will be enriched with these metals (Hallı, 2013).

The river has been heavily polluted by the discharge of industrial and domestic wastewater into the river and its tributaries without being treated, as well as the discharge of domestic wastewater into the river without treatment in the settlements around the river (Özkan, 1998). According to the Water Pollution Control Regulation, the Ergene River IV. class water (very polluted water). The Ergene River is widely used for agricultural irrigation. In this case, industrial wastes spread to agricultural lands and pass on to agricultural products. Heavy metal levels exceeding the Turkish food codex limits were detected in various agricultural products grown in the region. Heavy metals pass through the food chain and accumulate in their tissues and organs, causing various diseases, including cancer (Yolal, 2014). Fe was significantly lower and Cd level was significantly higher in the adolescent group. In the normal tissue comparison, Fe was significantly lower in the Ergene group, and Zn, Cd and Pb were significantly higher. In general, surface water quality classes and heavy metal pollution are classified as IV class in Çerkezköy, Çorlu, Muratlı, Lüleburgaz, Pehlivan köyü, Uzunköprü in the Ergene Basin environmental plan surface water quality classes' map, and III after mixing with the Meriç River is the class. Surface water quality classes and heavy metal pollution are generally IV class in Çerkezköy, Çorlu, Muratlı, Lüleburgaz, Pehlivan köyü, and Uzunköprü in the Ergene basin environmental plan surface water quality class's map. After mixing with the Meriç River, III. is the class. In recent years, toxic substances have also been observed in groundwater (TCYEHCDP, 2007).

It is widely used in agricultural irrigation in both rivers. Heavy metal level determination studies have been carried out for years in the paddy plant, the most important agricultural product of Thrace. In the study in the central part of Thrace, where agriculture is done with Ergene water; Fe and Mn were found in every part of the plant, Zn, Pb and Ni were found only in the root, and Cu was found in the product (rice grain) at toxic levels (Tok et al., 2005). Cd, Co, Cr, Ni, Fe and Cu were found to be toxic levels in rice after irrigation with water after Meriç and Ergene water were mixed (Filiz, 2011). In the study conducted to determine the heavy metal

concentration in plants grown in Corlu and its vicinity, it is stated that 'Lead values exceeding the Turkish food codex limits were detected in all tuberous and leafy plants' (Çalışkan, 2007).

According to the Water Pollution Control Regulation enacted within the framework of the Environmental Law No. 2872, the Ergene River has the characteristics of 'much polluted water'. It should not be used at all, especially in agricultural production. Chemical oxygen demand value (COD), which is an important parameter in determining pollution; It has been determined by the analysis reports that the Ergene River is 18 mg/l close to the source, the Çerkezköy outlet is 163 mg/l, the Çorlu water is 734 mg/l, the Muratlı town İnanlı is 270 mg/l and the Edirne outlet is 120 mg/l. As a result, the Ergene River receives a large pollution load within the borders of Tekirdağ province (Anonymous, 2004, Apak and Süzen, 2005).

Different levels of heavy metals (Cd, Pb, Cr and Cu) were determined according to the results of the analysis made with the rice samples collected from the rice producers irrigating from the Ergene River in Uzunköprü district of Edirne province (Arıcı et al., 2000). The rice farming in the region has been affected by pollution and has caused yield-quality losses in this product (Anbarcı, 2010; Çalışkan, 2007; Filiz, 2011; Hurma, 2007; Kubaş and Hurma, 2005; Sungur, 2013).

Güneş et al (2001) performed Pb, Fe, Cu and Zn analyzes of water samples taken from 8 selected sampling points on Çorlu Stream, one of the tributaries of Ergene River. The researchers, respectively, the values of these elements; 0.096- 0.352; 0.896-3.68; they determined it between 0.244- 1.63 and 0.169- 0.349 ppm. They found that there is significant Pb and Fe pollution in the water of Çorlu Stream.

According to a study conducted in the Meriç River (DSİ, 2003; 2009; 2011), significant amounts of heavy metals were detected in the river. The concentrations of Pb and Cu in the water samples taken from the Kapıkule station of the river are well above the allowable limit values. The water quality class of the Meriç River was determined as 4th class in terms of Pb and Cu.

Tok et al (2005) determined that Fe and Mn toxicity were found in the above-ground and root parts of the plants in a study they conducted in the paddy fields of Edirne province Uzunköprü and Meriç districts of the Thrace Region, where rice cultivation is intense. It was determined that lead, Zn and Ni were at toxic levels in the roots. Researchers have revealed that this pollution originates from the Ergene River. They determined that the heavy metal pollution in the river was directly reflected on the rice plant, and the heavy metal pollution reached the grain.

Bayrak (2004), investigating the dynamics of heavy metal concentration in and around Lake Gala, found significant heavy metal pollution in the lake. According to the researcher, the lake water is significantly polluted in terms of Pb, Cd, Cu and Co. III. and IV. it has a class irrigation water feature.

Gala Lake is the best example on the effect of pesticides on water pollution in the Thrace Region. The medicated waters discharged from the paddy fields around Gala Lake into the lake environment adversely affect the living things in the lake. Pesticides reach the irrigation water resources in the region through precipitation, surface erosion, washing and spraying of agricultural lands (Tok, 1997).

### **Studies Related to Water Pollution**

The first comprehensive study on the prevention of water pollution in the Ergene Basin was conducted by T.C. It is the 'Ergene Basin Protection Action Plan' put forward by the Ministry of Forestry and Water Affairs in 2011. In 2013, the Thrace Development Project (TRAGEP), which aims at a holistic environmental management in the basin and consists of 15 basic actions, was implemented. One of the scopes of these actions; the plan is to build 12



treatment plants in 13 settlements with a population of over 10000. The other is the target of constructing 5 joint treatment plants in the OIZs around Çorlu, Çerkezköy, Muratlı and Lüleburgaz.

Untreated wastewater from industrial facilities to the Ergene River is purified by the Ergene Deep Sea Discharge Project and is aimed to be discharged into the Marmara Sea. In the project, the wastewater coming from the industrial facilities will be treated in 5 treatment plants and discharged to a depth of 47.5 m, 4.5 km from the seaside, with an 82 km collector line (URL 5).

Improvement OIZs were established in order to discharge the wastewater of the industrial enterprises in the Thrace Region; but later on, the name improvement was abolished and turned into OIZs. Especially in these OIZs, which are established in areas where the industry is concentrated and clustered, the wastewater of industrial enterprises will be collected and after physical, chemical and biological treatment in the common treatment facilities, deep sea discharge will be carried out into the Marmara Sea. Construction works regarding the deep sea discharge to the Marmara Sea between Tekirdağ province Süleymanpaşa district and Marmara Ereğlisi are ongoing (Kubaş, 2017).

Clustering areas of industrial enterprises in Tekirdağ have been determined as organized industrial zones and are given in the table below (Table 2). As can be seen from here, 13 OIZs are located in Tekirdağ. When we add the European Free Zone in Ergene district of Tekirdağ province to this, it is seen that there are 14 clustered industrial zones in total.

**Table 2.** Organized Industrial Zones in Thrace Region

| The Name of the Organized Industrial Zone                  | Province | District  | Foundation Year |
|--|----------|-----------|-----------------|
| Tekirdağ   |          |           |                 |
| Çerkezköy Organized Industrial Zone                        | Tekirdağ | Çerkezköy | 1976            |
| Hayrabolu Organized Industrial Zone                        | Tekirdağ | Hayrabolu | 1994            |
| Malkara Organized Industrial Zone                          | Tekirdağ | Malkara   | 1994            |
| Enterprising Organization Presidency                       |          |           |                 |
| Çorlu Leather Specialization and Organized Industrial Zone | Mixed    | Çorlu     | 1997            |
| Çorlu 1 Organized Industrial Zone                          | Tekirdağ | Çorlu     | 2012            |
| Ergene 2 Organized Industrial Zone                         | Tekirdağ | Çorlu     | 2012            |
| Ergene 1 Organized Industrial Zone                         | Tekirdağ | Çorlu     | 2012            |
| Kapaklı Organized Industrial Zone                          | Tekirdağ | Çerkezköy | 2012            |
| Muratlı Organized Industrial Zone                          | Tekirdağ | Muratlı   | 2012            |
| Veliköy Organized Industrial Zone                          | Tekirdağ | Çerkezköy | 2012            |
| Velimeşe Organized Industrial Zone                         | Tekirdağ | Çorlu     | 2012            |
| Yalıboyu Organized Industrial Zone                         | Tekirdağ | Çerkezköy | 2013            |
| Tekirdağ Organized Industrial Zone                         | Tekirdağ | Centre    | 2014            |

Source: [URL 4](#)

The enterprises located in the OIZ will send their industrial wastewater to these treatment plants and ensure that they are purified, thanks to the common treatment facilities. These facilities will cover the costs of the treatment facilities by charging according to the pollution loads of the wastewater coming from the enterprises (Kubaş, 2017).

In order to eliminate the negative externalities caused by industrial enterprises, the common waste water treatment facilities to be established within the OIZ must be completed and put into operation immediately. For this reason, the amount of wastewater from each

enterprise and the pollution load in it reveal the treatment cost per liter. Businesses have to contribute to the common treatment plant expenses to the extent they pollute. Sustainability of the production processes in the industry will reduce the pressure on the natural resources in the region (Kubaş, 2017).

## **DISCUSSION AND CONCLUSION**

Turkey is a country with water shortage. All this aside, we unconsciously pollute the water. By the way, not only the polluted Ergene River, but also some of our streams, for example; Gediz, Yeşilırmak and Kızılırmak etc. We pollute all of our water resources in the Aegean, Black Sea and other regions. This pollution occurs with household waste, agricultural pesticides and industrial wastes (Aysu, 2011).

Thrace region with the effect of industrialization; It has faced a rapid population growth with internal and external migrations. These developments cause the region to experience various environmental problems. In order to eliminate the pollution load in the Ergene River and to ensure that domestic waste water is treated and discharged, the municipalities in the Thrace region have been obliged to establish a treatment plant, and there will be no municipalities without a treatment plant in the coming years. However, with these studies, local governments should quickly complete their work on the treatment of domestic waste water and giving it to the receiving environment. In the removal of industrial surface water pollution, those who cause pollution should be included in the cost of removing the pollution. For this, the operation of the treatment plant, which requires expertise, by OIZs with the appropriate technology will contribute to the efficiency, productivity, various quality practices and relations with the bureaucracy of the enterprises. In the treatment process to be carried out by the treatment plants to be established by the local governments for domestic waste water, it would be appropriate to meet the waste water expense share within the water cost collected depending on the amount of water used by the families (Kubaş, 2017).

Non-governmental organizations, municipalities and some organizations take actions in order to warn the authorities against river pollution and to provide solutions. Speeches are made by going down to the streets and expressing their legal rights within the limits of the law. The meeting held in Karamusul on April 10, 2011 was very crowded and well organized. It is inevitable for agricultural products in the Ergene River basin to reach people through the food chain. Studies examining the presence, accumulation and possible consequences of heavy metals in the human body are being carried out. Heavy metals reach fish and humans from water. Heavy metals reach the soil, agricultural products (plants, vegetables, fruits, grains, etc.), animals and ultimately humans through air and water. The carcinogenic effects of chromium, cadmium, nickel, mercury and lead have been proven.

The most important factor affecting the determination of environmental sensitivity in the Thrace Region is the education and information factor. This result shows that environmental awareness in the region is formed through education and information and reaches the masses. The high level of literacy in the Thrace Region reveals that people are interested in mass media, magazines, articles, newspapers and publications related to the environment and that information is transferred in this way. Education and information will enable societies to have more knowledge about the environment, increase environmental awareness, create a positive effect in solving problems, and make decisions with objective evaluation. It is extremely important to increase the awareness and importance of the environment, to have a high level of education and knowledge of both administrators, non-governmental organizations, local people and all individuals, in the formation of a sustainable environment (Sezen, 2013; Sezen and Kubaş, 2015).

While immediate action is required, the situation is being overlooked. In the end, the river was killed together. Agriculture in the Ergene Basin, which benefits from the river, is dying. There is no oxygen and living life in the river. There are bacteria that can live in an oxygen-free environment. The smell of the river disturbs the settlements around it. In summer, the smell becomes unbearable. From time to time, the river flows yellow, blue, red depending on the color of the chemical contained in the waste water given from the factories, but its constant color is gray-black. Some toxic substances that come with industrial and domestic waste affect human health, food and environmental health. The higher the toxic substances and heavy metals in the water, the higher these substances are in the products in the agricultural areas. Pollution, toxic substances and heavy metals in the Ergene River vary according to the seasons of the year and the years. It changes depending on the seasons, annual precipitation, the release of wastes and the intensity of domestic waste. In Muratlı district, pollution and odor affect life in the district center negatively. Because of the smell, people feel uncomfortable in their homes and do not want to open their windows.

There are 2537 industrial enterprises in Tekirdag (Bal and Özil, 2016). The rapid industrialization in the region makes very important contributions to employment and the economy of the region and the country. However, these positive developments in the industry should be sustainable and care should be taken not to create pressure on natural resources.

Work continues on the Ergene Basin Protection Action Plan, which will save the rivers, especially the Ergene River, which is on the agenda with its pollution in Thrace, from pollution and give life to the region. President Recep Tayyip Erdoğan said that with the project, which she stated to be one of the biggest environmental projects in Europe, pollution will be eliminated in Thrace. When this work, which is the largest deep discharge project in Turkey and Europe, is completed, Turkey's first watershed protection project will be completed and the discharge of domestic and industrial wastewater into the Ergene River will be prevented. After a while, the Ergene River will have the quality of irrigation water. The Ergene Basin Protection Action Plan, which will improve the water quality of the Ergene River, which has come to the fore with its pollution in Thrace, transform its color into its natural state and change the face of the region, is advancing rapidly.

While the natural flow rate of the Ergene River is 3 m<sup>3</sup>/sec per day, this amount rises to approximately 11 m<sup>3</sup>/sec with domestic and industrial wastewater. In other words, approximately 700 thousand m<sup>3</sup> of wastewater is discharged into the Ergene River daily in the basin. While approximately 240 thousand m<sup>3</sup> of this is domestic wastewater, 460 thousand m<sup>3</sup> is industrial wastewater. These conditions have made the water quality in the basin a 4th class, highly polluted and unusable water source. It adversely affects industrial production and agriculture, especially human health. Within the scope of the Ergene Basin Protection Action Plan, when all treatment plants for Organized Industrial Zones are commissioned, domestic and industrial waste water will be discharged to the Marmara Sea with a deep discharge line after being treated in treatment plants with completely closed pipes. When this work, which is the largest deep discharge project in Turkey and Europe, is completed, Turkey's first watershed protection project will be completed and the discharge of domestic and industrial wastewater into the Ergene River will be prevented. After a while, the Ergene River will have the quality of irrigation water (URL 1).

With the EU Water Framework Directive, it is obligatory to make planning at the basin scale in the use and protection of water and soil resources. For this purpose, the Ergene Basin management planning was made in the region. Necessary studies have been initiated for the establishment and operation of solid and hazardous waste processing, recycling and disposal facilities, and environmental services unions have been established in the region. The solid waste landfill facility in Tekirdağ has been designed in accordance with EU standards. Studies

for the establishment of biogas facilities in the evaluation of organic wastes have started (Anonymous, 2016; Sezen, 2013).

Pollution of clean water resources has ended irrigated agriculture in the areas opened to industry in the region and has caused product losses in non-industrial areas. In determining the main lines of the EU water policy, it is aimed to protect and improve all water resources (drinking and bathing water, etc.), not a single water source. In order to improve the ecological structure of water and ensure its continuity, water monitoring should be provided through a database, hazardous material discharges, emissions and losses should be stopped gradually, and the protection and improvement area should be expanded through specific measures.

In EU countries, especially biodiversity and the protection of natural areas are environmental issues that have a significant impact on the decisions taken by policy makers. The Thrace region, with its natural vegetation, agricultural structure and land situation, has a structure that can be considered as a natural protected area. However; the only place in the region declared as a special environmental protection zone is the Gulf of Saros. In modern agricultural practices in the region, organic agriculture should gain importance and use less harmful resources to nature, improve irrigation systems, expand rural and wetlands with international investments in agricultural mechanization (Sezen, 2013).

As in Turkey and all countries, the environment has a tremendous importance in terms of its protection, development and management in line with sustainable development goals. In the formation of environmental policies, a holistic understanding should be adopted, knowing that natural resources constitute the limit of economic resources, and environmental values should be considered at national and international level, believing that individual contribution and participation in the protection and development of the environment is necessary. It should not be forgotten that both the administrators and all segments of the society have great responsibilities in the solution of environmental pollution, which is increasing and creating a complex structure. In the absence of an environment to live in, first of all, the right to life and health cannot be mentioned. Protection of the livable environment should be considered as one of the basic human rights and should have international sanctions.

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## FOREST, FORESTS FIRES AND ECOSYSTEM

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### ABSTRACT

The ability of forests to perform their duties is ensured by protecting them from abiotic and biotic damages. The most important abiotic factor that damages forests is fire. If fires, which are important in the evolution of natural systems, occur at certain intervals and degrees within the regeneration limits of forests, they provide a new beginning opportunity and cause biodiversity and rejuvenation. When it comes to fire damage, the first thing that comes to mind is the damage caused by the fire to the trees in the forest. However, forest fires affect the entire forest ecosystem as well as the trees. Apart from the increase in the number of insectivorous birds and predatory species after the fire, wildlife is adversely affected. The way in which wildlife is affected by fire varies considerably according to the animal species and the nature of the fire. Abiotic (nesting, hiding and wintering places, etc.) and biotic factors (food sources, predators, disease factors, etc.) that reoccur in the region after the fire are important for the fauna. Large forest fires are rare, but are of devastating importance to living things in the affected area. The aim of this study is to emphasize the importance of forest fires, which increase with global climate change and human factor, especially in summer, on the ecosystem.

**Keywords:** Forest, Forests fires, Ecosystem, Abiotic and biotic factors, Biodiversity

### INTRODUCTION

Forests cover approximately 1/3 of the world's land surface. A healthy forest ecosystem is the main source of biodiversity. This rich biodiversity; It contributes directly and indirectly to agriculture, tourism, urban and village life, construction, medicine and pharmacy, in short, ecological, economic and social life. For these reasons, forests have an important place in terms of environmental values that need to be protected (Anonymous, 2016).

As the world's population has grown exponentially in the last 100 years, the pressure on forests has reached an unprecedented level. This pressure is most intense on forests; Opening takes place in the form of cuttings and forest fires. Forest fires not only cause damage to forest areas, but also pose a threat to settlements in or around forested areas (Ayberk et al., 2010). At the same time, non-human creatures that use forests as their homes both lose their habitats and suffer from forest fires (Yılmaz, 2012; Şahan and Kaya, 2022).

In addition to the material damage caused by forest fires, the decrease in forests also causes the benefits they provide to society to disappear. Forest fires cause some negative changes in the physical and chemical properties of the soil, depriving the soil of its protective cover, and as a result, it causes erosion (Ayberk et al., 2009). It also causes negative effects on flora and fauna, climate change as a result of greenhouse effect and global warming, and disasters such as desertification, landslide, mud flow, siltation, avalanche and drought with atmospheric pollution and deterioration of water order (Tolunay, 2017).

Forest areas, which are vulnerable to environmental factors, are damaged significantly due to fires that occur for different reasons. In addition to the decrease in biological diversity, forest fires destroy the living environment, which is extremely important for plants and animals, and often cause invasive species to invade the environment (Harper, 2011).

### **Forest Compositions and Ecological Dynamics**

Forests are a vast set of living and non-living things. Healthy natural forests consist of extensive and multi-layered elements from plants to animals. Forests are a collection of layers containing a wide variety of microorganisms from soil to tall trees, large and small species from the animal kingdom, herbaceous plants, ground cover and climbing plants, shrubs, low and high trees. The trees at the highest level and consisting of more than one species are the crown of the forests. Forests mature in stages at varying times depending on regional and current conditions. Forest succession is the development of forests by starting with pioneer species and developing with gradual formations, after the previous formation and by developing on it with the opportunities provided by it and paving the way for the next compositions. Some species in the stages of succession are withdrawn from the environment in advanced stages with the completion of their pioneering duties, and some species may continue to remain in the forest composition despite the termination of their pioneering duties (Dalling, 2008; Kemer, 2022).

The forest absorbs most of the sun's rays with its roof. Some of them are used for photosynthesis and some for transpiration. According to a study conducted in California, the solar energy absorbed by the needle leaves is  $1.3 \text{ g Cal/cm}^2/\text{min}$ . It was determined that 61% of it was given to the air above it as a temperature. Thus, the forest prevents all of the solar energy from passing to the soil and the air above the ground, ensuring that its air and soil are cooler in summer, especially during the daytime, compared to bare land. It softens the average and extreme temperature values by preventing excessive energy loss by radiation at night and in winter. Thus, the temperature of the forest air and soil is lower in summer and higher in winter than in bare land.

The forest lowers the maximum temperatures of its air and soil and raises the minimums. With the removal of the forest cover, a local climate prevailing in the opposite direction is created. This change in temperature is not only caused by excessive insulation (heating by the sun's rays hitting a certain surface) and the removal of plant layers that prevent radiation, but also by the destruction of litter. Because with the partial or complete disappearance of the forest dead cover, a layer that isolated the soil surface has disappeared. As a result of this, an excessive heating of the upper layers of the soil may occur with the direct sun rays, and therefore the temperature may rise so much on some well-exposed slopes that newly germinated seedlings may die.

Biodiversity is defined as the multi-layered compositional richness created by the mutual interaction of a wide variety of biotic species living together in natural and healthy forests, ecological systems and habitats. Different ecological systems with biological diversity interact with each other and contribute greatly to the global vitality on earth with regional and wider effects. Biodiversity is of great importance for the development of resilient and sustainable healthy forests based on the efficient use of resources and energy. The concept of biological diversity was first brought to the agenda at the United Nations World Environment Summit, which was held in Rio in 1992, based on sustainability. As one of the important achievements of this summit, it was decided to protect the global biodiversity with an international agreement. In natural and semi-natural forest formations where a single species is dominant and named after that prime tree, there are other tree species besides the dominant species. For example, there are tree and shrub species such as oak, maple, pine tree, mountain ash, carob, ash in the red pine-dominated forests. Even in homogeneous monotone woodlands for industrial



production, there is a very rich biochemistry laboratory on the ground that feeds and supports those trees.

### **Types of Forest Fire and Ground Structure Characteristics**

Forest fires occur in three ways as ground, surface and crown fires (Küçükosmanoğlu, 1994; Halter, 2013). The fires caused by the burning of the organic matter layer on the forest soil are ground fires and are the type of fire with a low probability of breaking out. If the soil is covered with a thick organic material, it spontaneously ignites and burns without flame, and such fires can occur even if the organic material is moist. While a ground fire can kill the fine roots of plants in the upper part of the soil, it does not cause much damage to strong and thick roots and thick stem plants. Surface fire is a type of fire that occurs on the ground and destroys herbaceous plants and shrubs, and can damage trees by burning the trunks. Such fires can grow with the effect of the wind and turn into crown fires. With grazing, it is possible to reduce the risks of ground and surface fires (Strand et al., 2014; Bilgili et al., 2017). While removing grasses, which can accumulate in sub-forest vegetation and create a fire risk due to drying, by grazing reduces surface fires (Harper, 2011), the risk of ground fire is also reduced by preventing such plants from constantly drying out and accumulating. It can be an important application to reduce the surface fires caused by the ignition of flammable materials in the forest, which is one of the causes of hill fires, and to reduce the under-forest herbaceous and shrubby species by grazing. Surface fires, which may occur depending on the density of bush species, especially close to the surface, can turn into the ignition of the bushes and then into crown fires.



**Figure 1.** Crown fires in the forest ([URL 1](#))

The temperature differences that occur during the fire force the cones to open in coniferous forests, causing the seeds to fall to the ground, and as a result, the dead cover layer in the forest causes the fire to continue and increase in intensity due to the low moisture content. The ash formed after the fire replaces the natural fertilizer for the soil and supports the germination and fruit formation in many plants (Arslantürk, 2007; Ayanoğlu et al., 2017).

Forest fires have different effects on different animal species and habitats, and wild animals are greatly affected by these fires. Fires not only have a lethal effect on wild animals, but also cause destruction of their habitats and migration. In addition, the arrival of other species in the burned areas after the fire is an undesirable situation in terms of wildlife (Özkazanç and Ertuğrul, 2011).



**Figure 2.** Surface fire in the forest (URL 2)

It is almost impossible to prevent natural fires. In particular, forest fires that spread over large areas can reach threatening dimensions for settlements in cities, and many countries may be insufficient in extinguishing efforts. In addition to the loss of natural wealth, such fires can also cause significant economic losses for countries due to the loss of forest trees and fire extinguishing costs (Altın et al., 2005; Bilgili et al., 2017).

In order to reduce the risk of fire, grazing practices are carried out with cattle and sheep in forest areas around the world (Gold and Hanover, 1987). With grazing, the amount of combustible materials is reduced and the spread of the fire is reduced (Davies et al., 2010), and the decay of dry grass accelerates with the animals' wandering (Strand et al., 2014). Thus, animals are also provided with feed (Charles and Taylor, 2015; Bilgili et al., 2017).

In many parts of the world, animal grazing is practiced in order to reduce the risk of forest fires, especially by reducing the grassy and shrubby vegetation under the forest. As a matter of fact, it has been stated that as a result of the removal or reduction of combustibles that can ignite well by grazing livestock, the frequency of fire occurrence decreases and the fire regime may change (Baisan and Swetnam, 1990). Controlled grazing in forest areas will both reduce unnecessary competition and allow the growth of young seedlings in the forest.

### **Fires and their Role in Biodiversity**

Fire is a reality that is not alien to nature. The vast majority of the world's forests, including the Mediterranean belt forests, are a collection of fire-evolved ecological systems. As a result of human activities or various natural processes, some foreign and exotic invasive plant species spread in natural environments or forests, causing deterioration of forest compositions, reduction or extinction of native species. In time, the system will not be able to renew itself naturally when some species, albeit native, dominate the environment and then age as the dominant species. Due to both invasive species and the dominance of a single species, the decrease in endemic and native species in the environment and the deterioration of natural compositions weakens biological diversity. Fires remove such unhealthy communities (populations) with weak ecosystems that lack biodiversity, allowing natural environments to rejuvenate by renewing themselves with rich and diverse species. By ensuring the participation of a wide variety of living species with equal chances, conditions are prepared for the beginning of new and rich ecosystems (Keeley, 2012; Kemer, 2022).

The severity and destructive effects of fires can be very different. However, fires that progress rapidly and superficially, burning only the top cover without reaching high temperatures, open very suitable and equitable areas for new vital formations in the rear. Tavşanoğlu et al. (2015) observed in their study that the seeds of herbaceous-leaved species exposed to smoke and heat germinate relatively quickly. On the other hand, depending on the amount of accumulated combustible material, vegetation and climatic conditions, some fires can continue for a long time in certain areas and reach very high temperatures, destroying all life in the environment. Such areas should be carefully identified and the remaining soil should be protected, organic matter additives, seeds and microorganisms and mycorrhizal fungi should be inoculated. Planting tree saplings on a ground where the soil is almost cooked due to high temperatures, the edaphone is destroyed and every living organism such as embryos and roots dies in the seeds will not provide the expected benefit anyway. For all forests of the world that have evolved by fire, the base soil is invaluable vitality reserves. It will be sufficient to believe that the seeds waiting in the soil will germinate again, and that the roots will sprout again in surface fires and to protect the soil. In this way, the formation or regeneration of real forests and natural structures with biological diversity and richness will be provided naturally, working for the benefit of fires, forests and natural environments.

Although moderate disturbances by fire appear to increase species diversity, major and minor disturbances have significant effects on community structure. Small-scale disturbances can create distinct patches of habitat throughout the area that help maintain species diversity within the community. Large-scale disturbances are also a natural occurrence in communities. For example, the dominant species of Yellowstone National Park is the maritime pine (*Pinus contorta*), which needs the regenerating effects of periodic fires. The cones of this species remain closed until exposed to intense heat. As the trees burn in the forest fire, the cones open and the seeds are scattered around. The new generation of pine trees thrive on nutritive minerals left by burnt trees and sunlight, where taller trees no longer cast shade (Campbell, 2017).

In the summer of 1988, numerous lightning-induced fires in the forested areas of Yellowstone National Park in the United States combined into a major disaster that lasted for months. Previously, excessive amounts of combustible material had accumulated on forest floors, as US National Parks and Forestry management policies had run a conservation program to extinguish every small fire as soon as it started. Therefore, this great fire, which lasted for months without being controlled, reached very high temperatures and destroyed 320 000 hectares of land. It took years for some forest lands, which lost their seed reserves to be sterilized and deprived of microorganism vitality, to revive together with their flora and fauna. Success was achieved by completely closing the burned areas, limiting human activities,

protecting them, and patiently waiting for nature to repair itself. At the point reached today, it can be observed that the forest ecosystem is revitalized with all its flora and fauna richness, and the habitats and forests are rejuvenated and renewed (Kemer, 2022).

Forests fulfill the services they provide for humans, similarly to wild animals. There are many biotic and abiotic factors in the interruption or change of these services provided by forests. Among these factors, fires are among the fastest, destructive and regenerative in forest ecosystems. The greatest impact occurs in living and non-living vegetation. In particular, the different degrees of exposure of the living vegetation from the fire reveal a pattern within the fire area. The repetition of the fire, its size, and their coincidences with each other in temporal and spatial scales are effective in the formation of a mosaic structure throughout the ecosystem, depending on this pattern revealed by each fire. This structure, which emerges as a result of the effects of fire for a certain time and place, is generally expressed as the fire regime (Bilgili and Baysal, 2012). After different fire regimes, extremely important habitats can emerge for wild animals that are shaped and ordered according to their vital activities such as feeding, sheltering and reproduction, pattern and mosaic structure seen in forest areas.

The fact that birds can easily escape from fires, use fire as a means of feeding, and benefit from post-fire environments for nutrition, shelter and reproduction provide important clues to researchers and planners about the functioning and processes in ecosystems (Furness and Greenwood, 1993). Fires threaten the future of forests in Mediterranean countries and in some regions of Australia and the USA (Avcı and Korkmaz, 2021).

It has been determined that the birds take advantage of the sudden increase in food sources during the fire, and after the fire they are in the area to use these habitats, which are generally more suitable. Fires cause many effects on the living and non-living environment in forest ecosystems. Birds are one of the important living groups that are directly affected by fires or indirectly due to changes in their habitats. Factors such as juvenile or adult status, feeding habits, habitat use, time, size and type of fire play a critical role in determining the benefit and harm that birds will suffer from fires.

Swallow, Hawk, and Kestrel species fly in very close distances to the fire line to catch escaping reptiles such as grasshoppers, insects, lizards, and snakes, as well as small mammals and birds (Pons, 2002). Some studies have shown that especially birds of prey benefit from fires in catching reptiles and small mammals that escape from fires (Whelan, 1995). There are similar findings regarding this behavior of birds of prey in fires and newly burned areas (Komarek, 1969; Tewes, 1984; Dodd, 1988; Baysal et al., 2017).

A large number of Storks (*Ciconia ciconia*) were detected on the fire area and in the burned area in the fire, which had a medium intensity effect on approximately 6 hectares of degraded oak forest and stubble area on 27 July 2007 in Edirne province, Keşan district, Çavuşlu Village, Ocak Location. While the fire was still in progress, it was determined that groups of different sizes, ranging from 5 to 40, flew over the fire area and fed by landing on the burning area. It is stated that this behavior of storks by collecting insects, reptiles and small mammals in the fire area is frequently encountered in savannah and grassland areas where fires of low intensity dominate, with frequent intervals such as 2-3 years (Goldammer and Ronde, 2004; Baysal et al., 2017).

With 1300 species, bats make up about a quarter of the total mammals. Bats can use a wide variety of habitats and ecosystems to feed, roost, and reproduce. One of the most important of these areas is forests. Forests are sometimes roosting and sheltering places for bats, and sometimes they are feeding areas. It is known that bats prefer old trees, loose bark backs, some bird nests, fallen tree stumps, tree crevices, cavities and cavities formed by various natural processes in forests. In forest areas where bats are likely to live, they can be identified using light traps, detectors and nets. In addition, with the monitoring devices attached to the bats, it

is possible to monitor which areas in the forest they prefer for roosting and feeding, and how their seasonal activities in the forest are. Human activities such as unconscious and uncontrolled tree cutting in forest areas, habitat fragmentation due to various reasons, forest fires, and chemical control against insects, mining activities and the establishment of wind turbines threaten bats using the forest (Yorulmaz et al., 2018).

In addition to being a country with approximately 27% of its surface area covered with forest areas, Turkey is home to a total of 39 bat species, 38 of which feed on insects and 1 on fruit. However, it is known that approximately 77% of Turkey's bats are in the forest to feed and/or roost (Yorulmaz et al., 2016; Yorulmaz and Arslan, 2016a; 2016b).

### **What Happened After the Forest Fire?**

Minerals, organic materials and seeds in the soil of the burning areas are the sources of renewal of the forests, but they are also very susceptible to loss by erosion. As a vital resource, the soil should be kept in place and carefully protected. Intensive activities for cleaning, evacuation and restoration in the field after fires can cause the loss of this precious resource by wind and surface flow erosion. Therefore, the regeneration of the forest may be much delayed.

All restoration works should be carried out within the framework of biological diversity principles, with the aim of natural forest ecosystem restoration. Just as no tree saplings can be planted almost anywhere, the same recipe cannot be applied to every burned or destroyed forest area. Even if the saplings to be planted with innocent and well-intentioned attempts and hastily are successful, these formations will remain as tree fields for many years and a holistic forest formation will only be possible in very long years.

In today's life, where economic and cultural exchange has increased in the globalizing world and material access has gained speed and ease, plant and animal species invade local habitats by easily reaching almost everywhere thanks to a wide variety of bridges and canals. In particular, fire-affected areas remain open and vulnerable to the invasion of foreign plant species (Keeley and Brennan, 2012). After fires, forest lands should be left in a way that allows them to restore themselves with native species from their own resources, and protection measures should be taken against the invasion of foreign species. Alien species should be removed from the land by regular controls and mechanical methods and destroyed. Otherwise, invasive alien and exotic species will develop rapidly, prevent a natural healthy succession and dominate the land as a dominant species.

Ash and other organic residues from fire-stricken lands contain rich and precious minerals. The ash and plant nutrients in the soil, as well as the remaining organic materials depending on the severity of the fire, adhere to each other with moisture and form a protective layer. The preservation of this layer is more important than planting new saplings on burned lands. With the support of micro-organisms such as fungi, bacteria and compositions consisting of small living things such as lichens and mosses, seeds and spores waiting in the soil under the protection provided by this layer will rapidly produce and support new plant communities. In particular, it is of great importance to support the symbiotic formation between mycorrhizal fungi and plants for the rapid healthy development of all plants from herbaceous plants to low shrubs and tall trees in the repaired forest areas. It is very important to preserve the existing organic soil layer containing fungal spores, which are the building blocks of this partnership.

### **Causes of Forest Fires**

It can be said that the most dangerous creature for the forests of Turkey is human. Because the damages done by people in the forest in the country include forest fires, grazing of their animals in the forest, trenching, smuggling, environmental pollution, etc. considerations are

available. When the statistics of forest fires in the forests of Turkey are examined, it is understood that most of these fires (99%) were started by humans (Çanakçıoğlu, 1985; Küçükosmanoğlu, 1994).

The fact that a large population of Turkey lives in or near the forest and people go to forests to meet their various recreational needs cause's people to be alone with the forest very often. This close relationship inevitably develops to the detriment of the forest, and in the end, the forest suffers greatly from this relationship. As long as man and forest live together and the love of man for the forest does not reach the desired level, it will always be the forest that suffers.

The negative effects of forest fires for the world's forests and forestry will be seen today and in the future, as in the past. Forest fires occur more frequently both in number and area in fire seasons, which vary according to geographical regions and are between 2-8 months in length.

In order to minimize the harmful effects of fires, the General Directorate of Forestry should take and implement all the measures regarding protection, prevention and extinguishing of fires in the protection of forests from fire. In this, all planned investments on forest and forest fires should be realized. Regardless of the type, these investments and activities should not be interrupted for a number of reasons. Delaying or neglecting activities for both protecting the forest from fire and fighting forest fires will cause great material and moral damages for the country's forests in the future as in the past (Küçükosmanoğlu, 1994).

Monitoring and protection of forest-dependent bat species in cases such as forestry activities, forest fires, activities for the use of natural resources for the forest, the use of chemicals in the fight against harmful insects in the forest, mining activities in the forest, and the establishment of Wind Power Plants (WPP) in forest areas, which has been increasing work becomes more important in recent years. In order to reduce the possible and direct effects of the related activities on the bats using the forests, before starting such activities, it is necessary to determine the forest-dependent bat species, and to reveal the species with protection priority and their relations with the forest (Yorulmaz et al., 2018).

## **DISCUSSION AND CONCLUSION**

Informing the public about healthy forests, forest ecology and the basic building blocks of forests will ensure effective participation of the public and relevant institutions in the works of fire protection, protection and rehabilitation of burned areas. Rehabilitation efforts should focus on the production of healthy, balanced, rich ecological systems rather than a wooded facility. It development of natural habitats (habitats) with biological diversity such as meadows, grasslands, maquis, wetlands and forests should be supported (Kemer, 2022).

Forest fires pose various dangers not only for forests, but also for almost all other ecosystems in the immediate vicinity of the fire. For this reason, forest fires are also a factor that directly threatens bats, but also indirectly threatens their habitats. In order to be able to fight forest fires, first of all, the places where the fire risk and fires are high should be re-determined, and in these areas, climate elements such as temperature, precipitation, relative humidity and wind should be constantly monitored, and much more care should be taken during fire seasons. New regulations and restrictions should be imposed on touristic activities to be carried out in forest areas in fire-sensitive areas, especially during fire seasons. In order to extinguish the fires before they grow, emergency response teams should be placed in the necessary places of the forest areas. Awareness and awareness-raising activities should be increased by increasing the number of warning signs (Perry, 2011; Yorulmaz et al., 2018).

It is very important to contribute to the preservation of ecological balance by preserving forests, which are one of the most important elements of the ecosystem. Although the countries of the world have taken special measures to protect these valuable treasures, even in many developed countries, serious destructive forest fires cannot be prevented. Measures to prevent forest fires that may occur intentionally or as a result of accident, such as security measures and the existence of serious criminal sanctions in addition to training activities, have significantly reduced forest fires caused by such factors.

Fire protection corridors can be created in the forest. In this way, the fire will be limited to the island where it first started and will be prevented from spreading and growing. These corridors should be used and expanded to prevent the spread of fires, especially in areas where there are sensitive and endemic species that require absolute protection, which need to be protected and where there is a fire risk. Corridors will also make very important contributions to fire control and extinguishing works, especially in areas with difficulty in transportation, as openings that provide ease of transportation.

In the fight against forest fires, besides the existing precautions and intervention techniques, different approaches should be taken into account and additional strategies should be developed and tested. One of these different approaches can be animal grazing, which can be done to remove under-forest herbaceous and shrubby plants that can cause cover fires and subsequently crown fires. It can be thought that controlled grazing practices to be carried out at the appropriate time and amount in areas suitable for grazing where there is no risk of grazing can play an active role in the cleaning of superficial combustibles (low vegetation, woody fuel, bush layer) which are flammable substances under the forest. Reducing the biomass density of the forest, especially before seasonal fires, will both reduce the risk of forest fires and cause changes in the forest fire regime.

Since trees are the largest members of forest ecosystems that can be seen from the outside, the perception that forests consist of only trees is dominant. By the same illusion, erosion is thought to be largely prevented by trees. Since well-intentioned efforts to restore the destroyed forest areas mainly focus on planting saplings, unhealthy tree fields are created instead of holistic forests. By planting just any tree sapling anywhere, not a forest, but a kind of agricultural production area or industrial forest-like woodlands can be established. Real, natural forests are much more than trees.

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## EXPLORING *STREPTOMYCES* BIOAGENTS FOR THE MANAGEMENT OF BACTERIAL FIRE BLIGHT UNDER IN VITRO AND IN VIVO CONDITIONS

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### ABSTRACT

*Erwinia amylovora* is the causal agent of fire blight disease affecting rosaceous plants. The present study focuses on the potential of *Streptomyces* strains as a promising biological control agent toward fire blight pathogen. Native *Streptomyces* strains were screened for antibiotic compounds active against *E. amylovora* isolates in vitro antibiosis test over a further 96 h and were evaluated by the resultant antagonistic and hyper parasitic effects. The inhibition zones and stability of the zones were determined. Secondary screening of the natural actinomyces isolates for antimicrobial activity was conducted in a liquid medium (MPB). Both microorganisms—the bioagent and the pathogen—were co-cultured for 72 h, and the contents of each incubated tube observed under a microscope, followed by inoculation on MPA and SCN media result was considered positive for antimicrobial activity when the antagonistic microorganism completely suppressed the growth of the pathogen. It was found that two isolates (Tal-44-18 and Tal-44-21) were active against *E. amylovora*. RNA sequencing was conducted to identify the isolates. The ability of the *Streptomyces* sp. to protect seedlings against fire blight was then assessed under in vivo conditions in a quarantine greenhouse. The results indicated that both protective and curative treatments with antagonistic bacterium effectively reduce the disease progression in apple seedlings. It was found that the higher the resistance variety (Red Chief), the better the preventive bactericidal effect of the *Streptomyces* sp., and the disease progression was stopped entirely. The disease exhibited more intensive development in the more sensitive Starkrimson variety than in the more resistant variety. Due to their demonstrated preventive and healing effects, the *Streptomyces* bioproducts described here have potential as early-stage fire blight inhibitors. In the future, tree coating technology based on this bioproduct should be developed to protect plants from the fire blight pathogen.

**Keywords:** *Erwinia amylovora*, *Streptomyces* strains, biocontrol

### INTRODUCTION

Fruit growing is one of the leading branches of agriculture in Kyrgyzstan, where more than 70 species of apple and pear are grown. Local species of apple and pear trees are a vital genetic resource; they must be protected from possible hybridization with apple cultivars and from the invasion of dangerous pests and diseases (First national forest inventory of the Kyrgyz Republic, 2000; Wilson et al., 2019).

Bacterial blight caused by the bacterium *Erwinia amylovora* (Enterobacterales; Erwiniaceae) is the most devastating disease affecting pome fruit production worldwide (Van der Zwet et al., 2012; Thompson, 2000). Since fire blight was first reported in Kyrgyzstan in 2009, it has steadily spread from its original epicentre, the northern part, to the southern and

eastern parts of the country. From 2011-2019, fire blight caused significant damage to orchards in the north and eastern regions of the country (Doolotkeldieva et al., 2019).

Due to the lack of adequate protective measures against the causative agent, *Erwinia amylovora*, farmers are forced to uproot diseased trees. Applying copper compounds during blooming has been recommended for disease control, but these treatments usually cause phytotoxicity effects on blossoms and fruits. Additionally, antibiotics such as streptomycin are the most effective compounds, often leading to antibiotic resistance in causal endophytic bacteria. The world health organization has prohibited antibiotic application in plant protection.

Last decade bacterial biocontrol agents were found to identification of their role in fire blight pathogen management. Suppression of *E. amylovora* on blossoms and shoots with antagonistic bacteria has been considered a viable alternative method for control of fire blight disease (Firouzian and Rahimian, 2016; Mirzaie et al., 2008; Medhioub et al., 2022; Racheal et al., 2022).

Many studies report that these biocontrol agents can protect plants from pathogens through various mechanisms. The primary means of action are direct interaction with the pathogen through hyperparasitism or antagonism; they compete with the pathogen for nutrients and habitat. These agents can increase plant resistance to pathogens without direct opposition to the phytopathogen (Köhl et al., 2019. Ghorbanpour et al., 2018; Raaijmakers and Mazzola, 2012).

Endophytic *Streptomyces* bacteria are of increasing interest because of their potential for biocontrol activity and their contributions to healthy organic qualities of crops. *Streptomyces* are one of the most attractive sources of biologically active substances such as vitamins, alkaloids, plant growth factors, enzymes and enzyme inhibitor. They can exhibit beneficial plant effects, including promotion and enhanced resistance to fungi and bacterial diseases (Olanrewaju and Babalola, 2019; Shams and Shahnavaz, 2017).

The present study focuses on the potential of endophytic *Streptomyces* species as a promising biological control agent for *Erwinia amylovora* bacteria isolated from different pome fruit trees grown in wild forests and orchards of the country.

## MATERIAL AND METHOD

*Isolation of E. amylovora into pure culture.* Diseased plants were collected according to the protocol EPPO (European and Mediterranean Plant Protection Organization). Infected apple (*Malus domestica*) and pear (*Pyrus communis*) tissues (leaves, stems with cankers), as well as twig pieces with well-developed symptoms, were used for isolation of *E. amylovora*. Samples taken from the internal tissues of the plant were macerated with 3 - 5 ml of PBS buffer solution in a sterile mortar. A loop full of the resulting suspension was streaked on the Levan medium. Single colonies were observed after 48h of incubation at 25°C. For confirmation, the colonies were grown on King B and Miller- Schroth agar.

*Virulence tests of E. amylovora isolates.* Immature fruits of *Pyrus communis* were used to study the pathogenicity and virulence of obtained *E. amylovora* isolates. Fruits surface were disinfected with 70% ethanol (v/v), punctured with a sterile needle, and inoculated at the incision site with 10 µL of bacterial suspension in 1× PBS or with 10 µL of 1× PBS for the negative control, respectively. Five fruits per isolate, as well as for the negative control, were used. After injection, fruits were kept in a sterile, airtight plastic container at 18 °C/ 24 °C for 6 d. The Horizontal (H) and vertical (V) length of the lesion produced by each isolate was recorded six days after injection. Koch's postulates were fulfilled by re-isolating the bacterium on a nutrient agar (NA) medium and confirming conventional PCR.

*Isolation of DNA.* Genomic DNA was extracted from bacterial cultures grown overnight at 27 °C in liquid LB medium using the DNeasy Blood and Tissue kit (Qiagen) according to the

standard protocol provided by the manufacturer. The samples were kept on ice to proceed immediately with subsequent experiments or stored at 4°C for later use. The quality of the extracted DNA was checked by running 5 µl of the obtained eluate in an electrophoresis on a 1% agarose gel.

*To determine the genotype of E. amylovora PCR test was doing according to the following protocol:* PCR amplifications of the three CRISPR regions were performed with the Phusion Green Hot Start II High-Fidelity PCR Master Mix (Thermo Fisher) in a total volume of 40 µl using 2 µl of a 1:20 dilution in water of overnight bacterial cultures in LB as template. From single colony was taken and resuspended in 10 mL LB medium, then incubated overnight at 27°C. The well-grown suspension was diluted at 1:20 in distill. water and lysate at 95° C for 10 min. Then were made master mix and the samples were incubated in a Thermocycler. Primers pairs C1f01/C1r1, C2f01/C2r01 and C4f01/C4r1 [Rezzonico et al, 2011) were used at a final concentration of 0.4 mM for each primer. The end program PCR products were mixed with 4 µL Loading Dye, put on a 1.5% agarose gel, and run at 60 V for 2h.

**Table 1.** Used 4 different primers

|       |                       |  |
|-------|-----------------------|--|
| C1f01 | CRR1, complete region | 5' - TG AGT AGC AAA TCC GTG CGT GCT - 3' |
| C1r01 | CRR1, complete region | 5' - AA TCA GTC CCC CCA TGC TGT GAC - 3' |
| C1f04 | CRR1, Spacer 1030     | 5' - CGATCAACCTGTTTTTCAGTAGGT - 3'       |
| C1r09 | CRR1, Spacer 1027     | 5' - CCGCCGAGACAACCGGCTATCC - 3'         |
| C1f03 | CRR1, Spacer 1023     | 5' - GAGACTTAAAGATCGTCTGCTAGT - 3'       |
| C1r11 | CRR1, Spacer 1002     | 5' - ATGCCCTCACCGTTGTGTGTG - 3'          |
| C2f03 | CRR2, Spacer 2020     | 5' - GATGGTGGCGCTGGTTGCGCTGGC - 3'       |
| C2r02 | CRR2, Spacer 2010     | 5' - CTGAGTCTGGAATGTACACACTGG - 3'       |

#### *Determination of antibiotic activity of Streptomyces strains against fire blight pathogen*

Thus, we first performed an in vitro antibiosis test. Native *Streptomyces* strains were screened for antibiotic compounds active against *E. amylovora* isolates. The *Streptomyces* strains were cultured in media for 7 days until antibiotic substances were produced. Then, 48 h culture suspensions of *E. amylovora* samples isolated from an apple tree (*M. domestica*) were sprayed onto the *Streptomyces* colonies. The antibacterial activity of the *Streptomyces* strains was tested over a further 96 h and was evaluated by the resultant antagonistic and hyper parasitic effects. The inhibition zones and stability of the zones were determined.

Secondary screening of the natural actinomyces isolates for antimicrobial activity was conducted in a liquid medium (MPB). Both microorganisms—the bioagent and the pathogen—were co-cultured for 72 h, and the contents of each incubated tube observed under a microscope, followed by inoculation on MPA and SCN media result was considered positive for antimicrobial activity when the antagonistic microorganism completely suppressed the growth of the pathogen, that is, when only the *Streptomyces* mycelium and no pathogen cells remained in the tube. It was found that two isolates (Tal-44-18 and Tal-44-21) were active against *E. amylovora*.

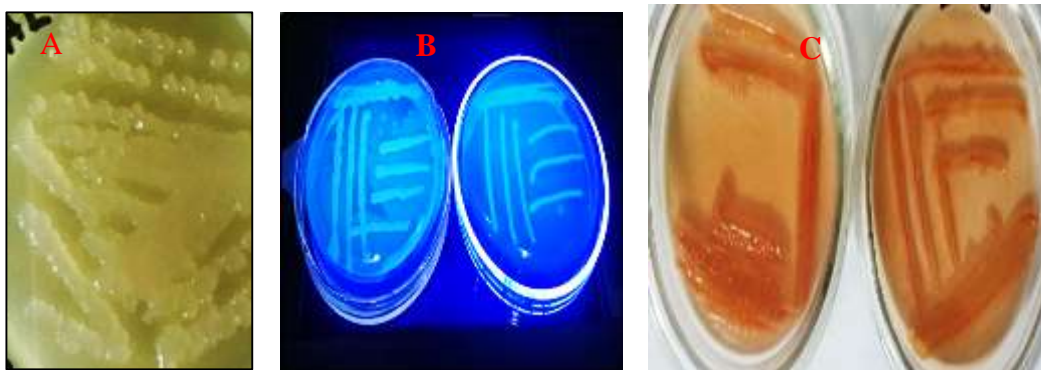
#### *Molecular identification of active Streptomyces isolates against Erwinia amylovora*

The purified bacteria were incubated in meat peptone medium (MPM) for 2days at 25°C. Cells were harvested at the early exponential growth phase, and their DNA was then extracted by the alternative protocol of the Mo Bio Laboratories. Successful DNA extraction was determined by agarose gel electrophoresis (1.0% agarose). Amplification was performed with a Multi Gene Thermal Cycler (TC9600-G/TC, Labnet International, Edison, New Jersey, USA), using a 25 µL

mixture containing 15  $\mu$ L of PCR MasterMix (Taq DNA polymerase, MgCl<sub>2</sub>, deoxyribonucleotide triphosphate, and reaction buffer), 2  $\mu$ L of each primer, 1  $\mu$ L of template DNA, and 1  $\mu$ L of H<sub>2</sub>O. The amplification program was used as the follows: 94°C for 5 minutes, 35 cycles at 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 60 seconds, and 72°C for 7 minutes. PCR products were electrophoresed in a 1.0% agarose gel and visualized using the BioDoc-It Imaging Systems (Ultra-Violet Products Ltd) after ethidium bromide staining. To control contamination, we used a negative control reaction, and sterile water was added as a matrix. Almost full-length fragments of 16S rRNA genes were amplified using the primers 16S-27F and 16S-907R. Sequence analysis was performed by the Macrogen Company (10F World Meridian Center, Seoul, Korea), and sequences were edited with Applied Biosystems 3730XL sequencers. Only sequences with more than 900 nucleotides were used for BLAST analyzing.

## RESULTS

*Isolation of E. amylovora into pure culture.* The single colonies with *E. amylovora* characteristics were tested on different media separately for confirmation (Fig.1).



**Figure 1.** Colonies of *E. amylovora* on different solid growth media.

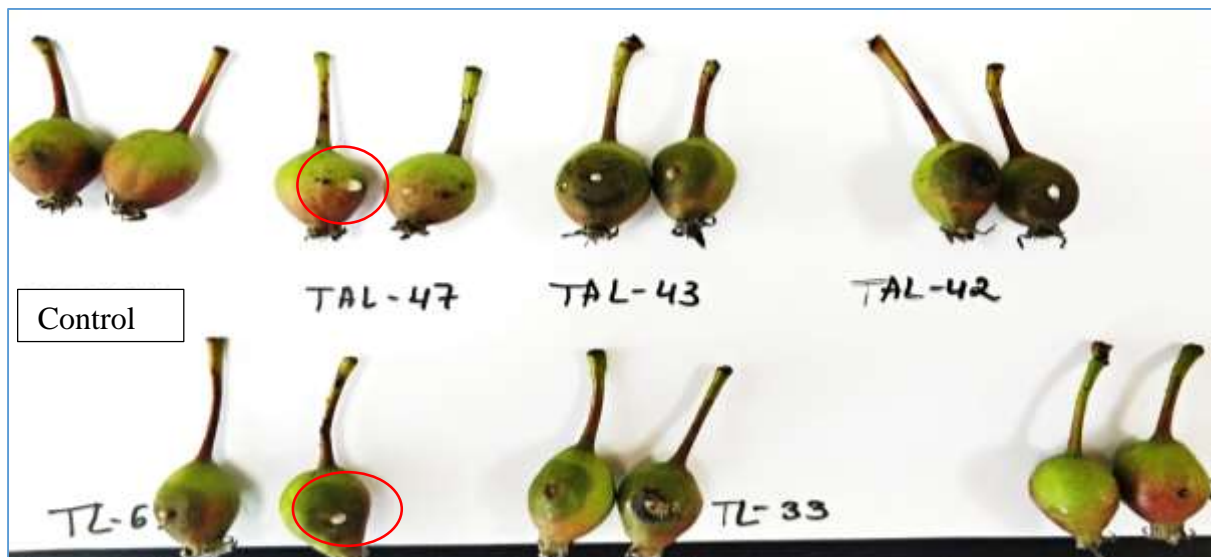
On Levan medium (A), *E. amylovora* formed milky or light yellow, circular, domed, smooth, mucoid colonies after 48 h of incubation at 28 °C. Under the same growing conditions on KB agar (B), the colonies had a typical white mucoid appearance and were non-fluorescent under UV light at 366 nm. After five days on Miller-Schroth agar (C), the colonies were reddish-orange and approximately 1 mm in diameter and had a round, smooth and domed appearance with entire margins. The substrate colour changed from blue-greenish to reddish. Other plant-associated bacteria usually do not produce the same colony morphology on these three media, thus allowing the discrimination of *E. amylovora*.

*Virulence tests of E. amylovora isolates.* Pathogenicity and virulence of the pathogen *E. amylovora* depend on different factors. Besides the production of the siderophore desferrioxamine for acquiring iron molecules from the host tissue [Smits and Duffy, 2011] and other virulence factors such as metalloproteases (Zhang et al., 1999) are also essential factors in pathogenesis. All strains that were positively identified as *E. amylovora* were pathogenic in the immature pear fruit assay, producing a visible bacterial ooze 48 h after injection (Fig. 2).

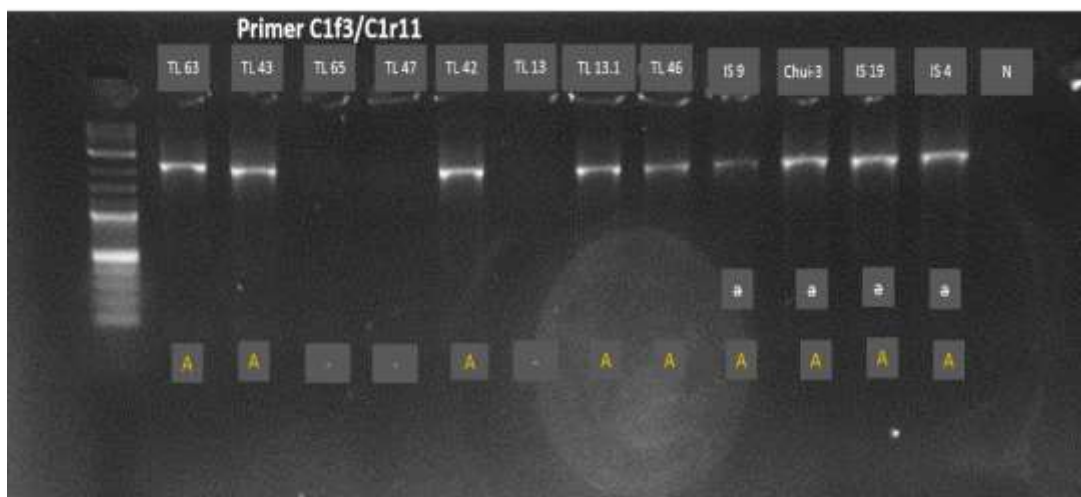
*Analysis and PCR genotyping of E. amylovora CRISPR regions.*

The genetic variability of *E. amylovora* obtained from different plant hosts, and geographic locations in 2020-2021 were assessed by amplifying potentially variable parts of their CRRs using the primers deduced with the approach described above (Table 1). In total,

fifteen different *E. amylovora* isolates collected during 2020-2021 in other regions of Kyrgyzstan were positively analyzed. The isolates from the locations (Tal-13, Tal-42, Tal-43, Tal-46, Tal-63, Table 2) in the Talas region and the isolates from the sites ( IS-4, IS-9, IS-19) surrounding lake Issyk-Kul, and one isolate ( Chui-3) from the Chui region displayed PCR results compatible with genotype A by amplifying with primers C1f03 and C1r11 ( Figure 3).

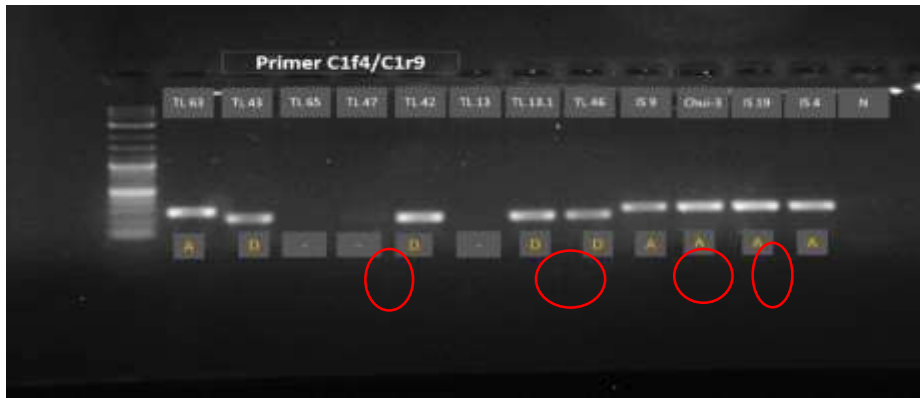


**Figure2.** A visible bacterial ooze in the immature pear fruits 48 h after injection by *E. amylovora*.



**Figure 3.** Electrophoretic profiles of new *E. amylovora* isolates, obtained in Talas, Ussyk-Kul, and Chui regions in 2020 by amplifying with primers C1f3/C1r11.

First time genotype D was detected in *E. amylovora* species isolated from Talas region by amplifying with primers C1f4/C1r9 (Figure 4).



**Figure 4.** Electrophoretic profiles of new *E. amylovora* isolates, obtained in Talas, Ussyk-Kul and Chui regions in 2020 by amplifying with primers C1f4/C1r9.

**Primary screening of natural *Streptomyces* isolates for antimicrobial activity on solid medium (SCN).** As shown in Table 2, natural *Streptomyces* isolates have shown different inhibitory activity against *Erwinia amylovora*. The isolates Tal -33.7, Tal-44.18, IK-7.10, and IK -32.25 have shown the highest activity. Their zone of inhibition of the growth of *E. amylovora* colonies ranged from 17.8 to 19.8± 0, 32 mm. The rest of the natural *Streptomyces* isolates have manifested a moderate or low activity towards to pathogen (Figures 5 and 6).



**Figure 5.** The photos show the hyper parasitic effect of *Streptomyces* isolates, i.e., its mycelium covers and grows on the surface of pathogenic bacteria colonies.



**Figure 6.** The photos show low activity of *Streptomyces* isolates to pathogen bacteria

***Secondary screening of natural actinomyces isolates for antimicrobial activity in the liquid medium***

**Table 2.** Results of biotests for antibiotic activity of natural *Streptomyces* isolates against *Erwinia amylovora* culture

| #   | Lab. collection designation of Streptomyces isolates | Inhibition zone of <i>E.amylovora</i> colonies growth on medium, mm | Preliminary assessment of the shown activity of Streptomyces sp. |
|-----|--|---|--|
| 1.  | TAL-31.2   | 9,1 ± 0,18  | moderate activity  |
| 2.  | TAL-31.3   | 16,3 ± 0,23   | high activity  |
| 3.  | TAL-33.7   | 18,3 ± 0,31   | high activity  |
| 4.  | TAL-42.22  | 11,3 ± 0,51   | moderate activity  |
| 5.  | TAL-44.8   | 0,3 ± 0,34  | low activity   |
| 6.  | TAL-44.15  | 10,3 ± 0,64   | moderate activity  |
| 7.  | TAL-44.16  | 1,7 ± 0,52  | low activity   |
| 8.  | TAL-44.17  | 17,8 ± 0,43   | high activity  |
| 9.  | TAL-44.18  | 19,4 ± 0,32   | high activity  |
| 10. | TAL-44.19  | 11,3 ± 0,53   | moderate activity  |
| 11. | TAL-44.21  | 16,3 ± 0,37   | high activity  |
| 12. | TAL-44.22  | 16,9 ± 0,41   | high activity  |
| 13. | TAL-44.23  | 16,4 ± 0,32   | high activity  |
| 14. | TAL-44.24  | 0,7 ± 0,18  | low activity   |
| 15. | TAL-44.25  | 2,8 ± 0,24  | low activity   |
| 16. | TAL-70.26  | 1,7 ± 0,35  | low activity   |
| 17. | TAL-70.27  | 1,3 ± 0,46  | low activity   |
| 18. | TAL-70.28  | 0,7 ± 0,63  | low activity   |
| 19. | TAL-78.32  | 0,6 ± 0,21  | low activity   |

After the primary screening, the most highly active isolates were selected in a liquid MPB medium. At the second stage of screening in a liquid medium, both microorganisms: the bioagent, and the pathogen, were cocultured for 72 hours, and the contents of each incubated tube were checked under a microscope, followed by inoculation on MPA and SCN medium. A positive and highly active result was considered where the antagonist microorganism completely suppresses the growth of pathogen cells, i.e., only the streptomycetes mycelium and the absence of pathogen cells remain in the incubating tubes—complete inhibition. When inoculated from the contents onto nutrient agar media, the presence of pathogen colonies or biocontrol colonies growth also confirms the activity of the biocontrol agent. As can be seen

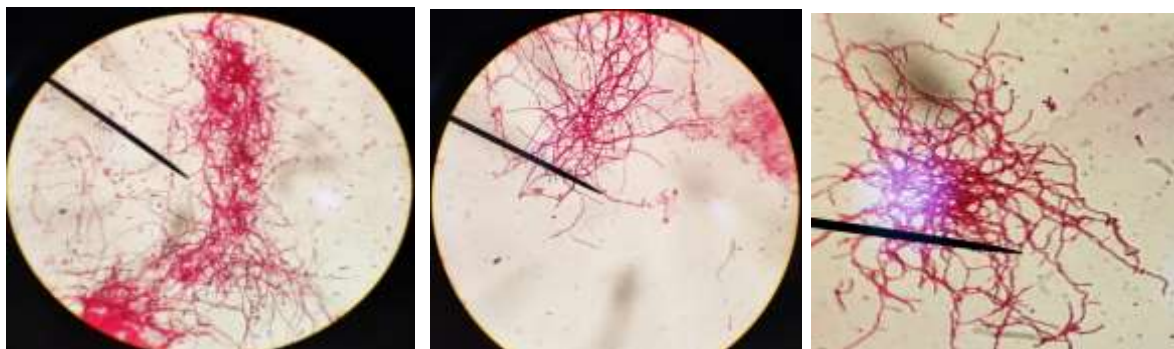


from table 3, the active isolates of *Streptomyces* at the first screening were taken for the second screening against *E. amylovora* bacteria. During the second screening, out of 10 isolates tested in a liquid medium, only two isolates, Tal- 44-18 and Tal- 44-21, have shown complete inhibition of the *E. amylovora* cells growth during co-cultivation. Three isolates - Ik-7.10, Tal -44-22 and Tal- 44-17- showed moderate activity, while the rest of the isolates had a weak inhibitory activity; in such test tubes, the rapid growth of pathogen cells and weak growth of the bioagent were noted. Figures 7 and 8 show some microscopic pictures showing solid and soft effects of *Streptomyces* isolates against *E. amylovora* 48 hours after co-cultivation.

**Table 3.** The results of co –incubation of *Streptomyces* isolates and *E. amylovora* bacteria in liquid medium for 72 h.

| Co-incubation in liquid medium of biocontrol and pathogen cells | <i>Erw. amylovora</i> diluted culture                          | <i>Streptomyces</i> mycelium growth in liquid medium |
|---|--|--|
| <i>Strep.</i> Tal-31.3 + <i>Erw. amylovora</i>                  | 1x10 <sup>-1</sup><br>1x10 <sup>-2</sup><br>1x10 <sup>-3</sup> | +  |
| <i>Strep.</i> Tal-33.7+ <i>Erw. amylovora</i>                   | 1x10 <sup>-1</sup><br>1x10 <sup>-2</sup><br>1x10 <sup>-3</sup> | +  |
| <i>Strep.</i> Tal -44.17+ <i>Erw. amylovora</i>                 | 1x10 <sup>-1</sup><br>1x10 <sup>-2</sup><br>1x10 <sup>-3</sup> | ++   |
| <i>Strep.</i> Tal -44.18+ <i>Erw. amylovora</i>                 | 1x10 <sup>-1</sup><br>1x10 <sup>-2</sup><br>1x10 <sup>-3</sup> | +++  |
| <i>Strep.</i> Tal -44.21+ <i>Erw. amylovora</i>                 | 1x10 <sup>-1</sup><br>1x10 <sup>-2</sup><br>1x10 <sup>-3</sup> | +++  |
| <i>Strep.</i> Tal -44.22+ <i>Erw. amylovora</i>                 | 1x10 <sup>-1</sup><br>1x10 <sup>-2</sup><br>1x10 <sup>-3</sup> | ++   |
| <i>Strep.</i> Tal -44.23+ <i>Erw. amylovora</i>                 | 1x10 <sup>-1</sup> , 1x10 <sup>-2</sup> , 1x10 <sup>-3</sup>   | +  |
| <i>Strep.</i> UK-7.10+ <i>Erw. amylovora</i>                    | 1x10 <sup>-1</sup> , 1x10 <sup>-2</sup> , 1x10 <sup>-3</sup>   | ++   |
| <i>Strep.</i> IK-32.25+ <i>Erw. amylovora</i>                   | 1x10 <sup>-1</sup> , 1x10 <sup>-2</sup> , 1x10 <sup>-3</sup>   | +  |

Footnote : + - weak growth of antagonist mycelium; ++ - moderate growth of antagonist mycelium; +++- strong and active growth of antagonist mycelium.



**Figure 7.** A- active growth of *Strep.* Tal-44.21 isolates mycelium in the tubes with *E. amylovora* in 48 h co-incubation; B- active growth of *Strep.* Tal-44.18 isolates mycelium in the tubes with *E. amylovora* in 48 h co-incubation; C- moderate growth of *Strep.* Uk-7.10 isolates mycelium in the tubes with *E. amylovora* in 48 h co-incubation.



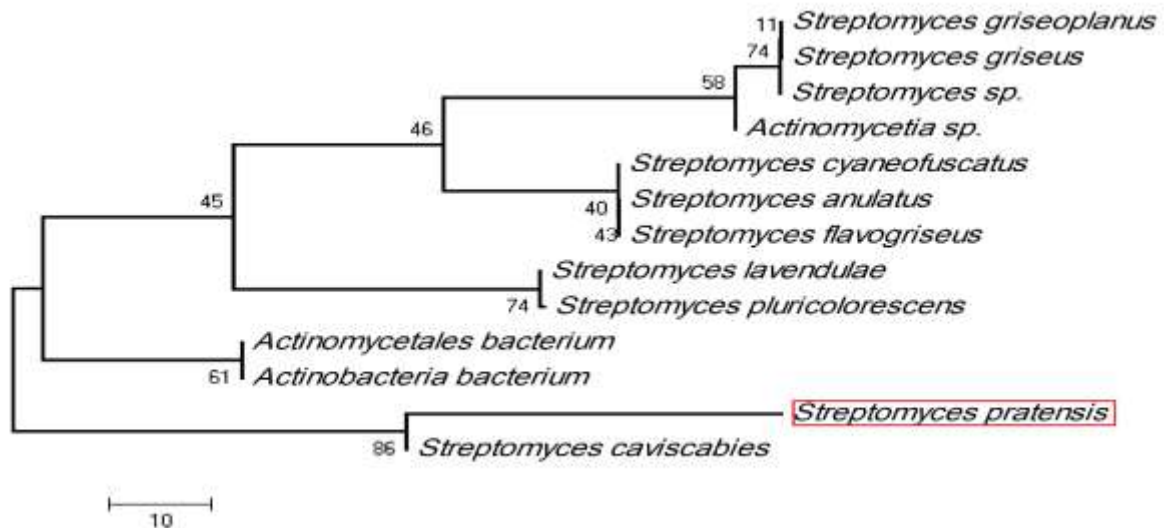
**Figure 8.** A- weak growth of *Strep.* Tal- 31.1 isolates mycelium in the tubes with *E. amylovora* in 48 h co-incubation; B- weak growth of *Strep.* UK-32.25 isolates mycelium in the pipes with *E. amylovora* in 48 h co-incubation; C- weak growth of *Strep.* Tal-33.7 isolates mycelium in the tubes with *E. amylovora* in 48 h co-incubation.

In this experiment, at the initial, the 54 isolates obtained from 38 soil samples were **screened against *Erwinia amylovora* pathogen**. 46.6% of actinomycetes have weak activity, 31.1% moderate exercise, and 28.1% high activity.

As a result of the second screening, only two isolates (Tal- 44-18 and Tal- 44-21) were active against the *Erwinia amylovora* bacteria. Both isolates were isolated from the soils of the Talas region. Thus, the grounds of the Talas region are an essential source for studying antibiotic-producing actinomycetes.

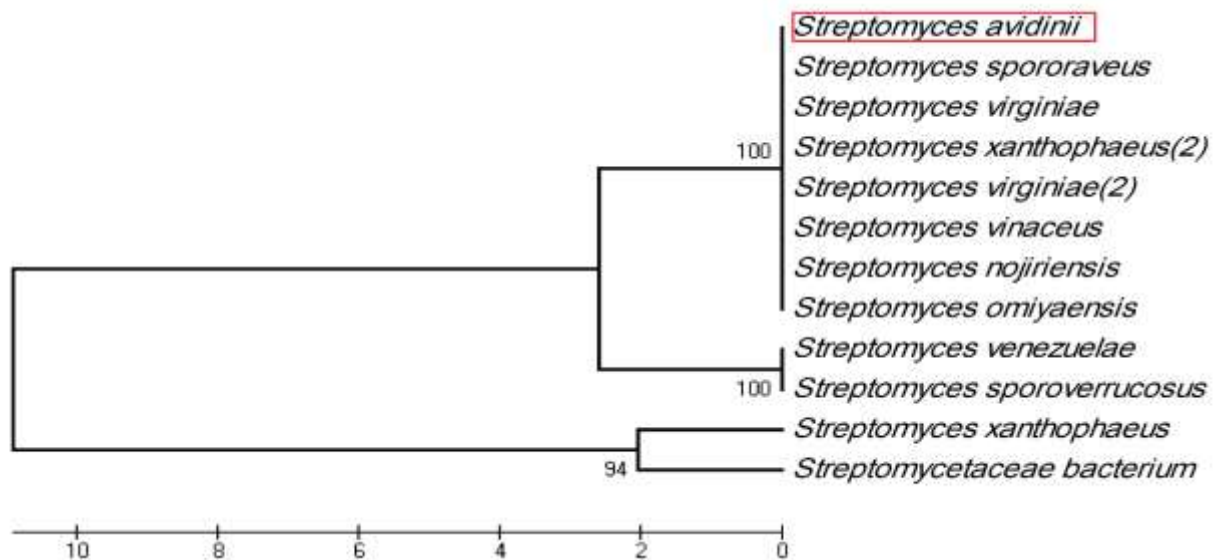
#### *Molecular identification of active Streptomyces isolates against Erwinia amylovora.*

Almost full-length fragments of 16S rRNA genes of two active *Streptomyces* strains (Tal-44.21 and Tal-44.18) were amplified using 16S-27F and 16S-907R. Sequences were edited with Applied Biosystems 3730XL sequencers. Only rows with more than 900 nucleotides were used by a BLAST search based on the sequences. When compared with those existing in the sequence database, the sequences of our strains were 99.9% identical to the registered strains- *Streptomyces pratensis* (Tal-44.21) and *Streptomyces avidini* (Tal- 44-18) (Figures 9 and 10).



**Figure 9.** Neighbor-joining tree based on 16S rRNA gene sequences showing the relationships between *Streptomyces pratensis* (Tal-44.21) and other *Streptomyces spp.*

Numbers on branches indicate confidence limits estimated from bootstrap analysis of 1000 replicates.



**Figure 10.** Neighbor-joining tree based on 16S rRNA gene sequences showing the relationships between *Streptomyces avidinii* (Tal-44.18) and other *Streptomyces spp.* Numbers on branches indicate confidence limits estimated from bootstrap analysis of 1000 replicates.

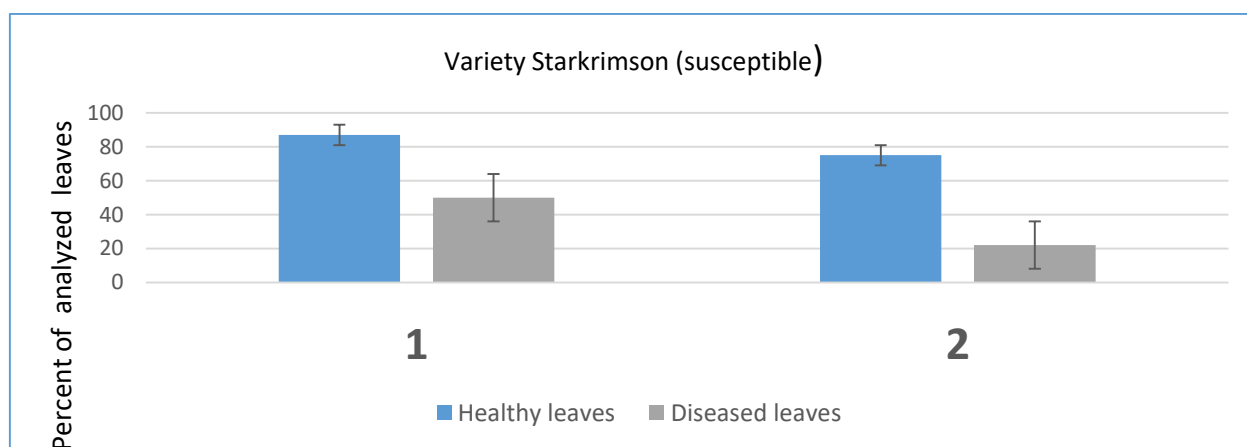
According to studies, *Streptomyces pratensis* produced angucycline antibiotics like streptomycin A (1) and B (2) that exhibited antimicrobial activities against *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Klebsiella pneumoniae*, and *Escherichia coli* with equal minimum inhibitory concentration (MIC) values of 16 µg/mL, while these antibiotics showed inhibition against *Bacillus subtilis* at MIC value of approximately 8–16 µg/mL, respectively (Akhter et al., 2018).

And *Streptomyces avidinii* is well known as the producer of the antibiotic [Streptavidin](#). It has an extraordinarily high affinity for [biotin](#). It is used extensively in molecular biology and

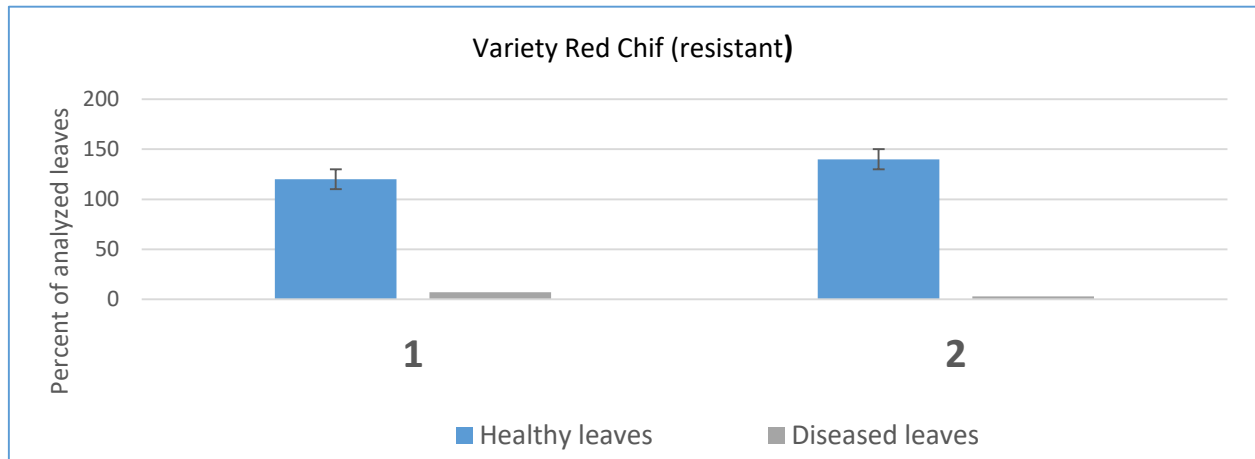
bionanotechnology as a high-affinity biotin-binding agent resistant to extreme pH, temperature, [organic solvents](#), denaturants, detergents, and [proteolytic enzymes](#) ( Lu and Chen, 2011). Thus, from the soils of the Talas region, we have isolated *Streptomyces* strains, which are sources of antibiotics, exhibiting antimicrobial activities against *E. amylovora*.

The ability of the *Streptomyces* sp. to protect seedlings against fire blight was then assessed under in vivo conditions in a quarantine greenhouse. Two-year-old apple seedlings were planted in plastic pots and grown for 5–6 weeks to develop sufficient leaf biomass. Two experiments were then conducted at the same time. In the first experiment, which was designed to test the preventive effect of the *Streptomyces* sp., Red Chief (4 pieces) and Starkrimson (4 pieces) apple varieties and their leaves and shoots were abundantly sprayed with solutions containing  $5.2 \times 10^7$  cells/mL of *Streptomyces* sp. strains TAL-44.18 and Tal-44.21. On Day 10, the treated seedlings were artificially infected with *E. amylovora* to determine whether the *Streptomyces* sp. had a prophylactic effect. In the second experiment, seedlings were artificially infected by cutting the leaf blade with scissors dipped in an *E. amylovora* suspension containing  $1 \times 10^9$  cells/mL. Ten days later, the infected seedlings were sprayed with a *Streptomyces* solution to determine whether the *Streptomyces* sp. had a therapeutic effect. In both experiments, disease development was monitored for 30 days, and the size of the necrotic lesions on the leaves and shoots were measured every other day.

During the month of observation, the results of the two experiments differed depending on the resistance of the apple variety. It was found that the higher the resistance of the variety, the better the preventive effect of the *Streptomyces* sp. In the first experiment, in the resistant Red Chief variety, the disease began on the tips of only two upper leaves as necrotic blackening and did not progress further. Hence, the *Streptomyces* strains had a bacterial effect. When the bioactive product was introduced in the second experiment, the disease progress was completely stopped. The leaves that were affected during the artificial infection remained hanging on the crown, but other leaves were no longer involved. Whereas in the more sensitive Starkrimson variety, in the first experiment, the disease exhibited more intensive development than in the more resistant variety (Figures 11 and 12).



**Figure 11.** 1 - Therapeutic treatment (infection with the bacterium *Erwinia amylovora* and in 10 days treatment with *Streptomyces*); 2 - Prophylactic treatment with *Streptomyces* and in 10 days infection with the bacterium *Erwinia amylovora*



**Figure 12. 1** – Therapeutic treatment (infection with the bacterium *Erwinia amylovora* and in 10 days treatment with *Streptomyces*); 2 - Prophylactic treatment with *Streptomyces* and in 10 days infection with the bacterium *Erwinia Amylovora*

## CONCLUSION

Therefore, in this study, we identified two potential fire blight antagonists: *S. pratensis* (Tal-44.21) and *S. avidini* (Tal-44.18). This can be considered a new finding in the field of biological control research in Kyrgyzstan. The *Streptomyces* bioproducts described here have potential as early-stage fire blight inhibitors due to their demonstrated preventive and healing effects. In the future, tree coating technology based on this bioproduct should be developed to protect plants from the fire blight pathogen.

## ACKNOWLEDGEMENTS

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## IN VITRO AND IN VIVO SCREENING OF *BEAUVERIA BASSIANA* STRAINS FOR ENDOPHYTIC AND INSECTICIDE ACTIVITY

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### ABSTRACT

*Beauveria bassiana* (Bb); Ascomycota: Clavicipitaceae) is an entomopathogenic fungus used as an eco-friendly insecticide due to its ability to infect and kill arthropods. In addition, Bb possesses endophytic activity that may be able to contribute to plant growth. This study aimed to evaluate the potential insecticidal and endophytic activities of local native Bb isolates. The larval and adult stages of the apple tree aphid (*Aphis pomi*) were used as the target in the bioinsecticidal experiments, and crops (i.e. beans, tomatoes and cucumbers) were used as the targets in the biostimulant experiment. The first step involved developing a cheap nutrient media suitable for cultivating Bb isolates and obtaining a high biomass. The Col-2, VT and 12K strains were found to produce high-density mycelia and conidia on the surface of media consisting of wheat and barley groats. The endophytic activity of the tested Bb strains was strongly manifested by bioinoculation through the soil. For example, watering around the root system three times with a suspension of the Col-2 strain caused the tomato roots to lengthen by  $5.2\text{--}5.5 \pm 0.01$  cm more than the control plant roots, the bean roots to lengthen by  $25.0\text{--}25.7 \pm 0.01$  cm more, and the cucumber roots to lengthen by  $4.2\text{--}4.3 \pm 0.01$  cm more. The bioactive Bb products were found to have high insecticidal activity against aphids. After 3 days, the cuticle and outer covers of the larvae and adults were covered with fungal mycelium. On the seventh day, the mortality rate resulting from treatment with the Col-2 and VT strains was  $90 \pm 1.27\%$ , and the mortality rate resulting from treatment with the 12K strain was  $80 \pm 1.23\%$  ( $P \leq 0.05$ ) in the tested pest population.

**Keywords:** Entomopathogenic fungus, bioinsecticide, bioinoculant, biocontrol.

### INTRODUCTION

The excessive use of chemical fertilisers has led to several ecological and environmental problems, such as soil pollution and degradation, reduction of beneficial soil organisms. In recent years, there has been a growing trend of using biological agents as an alternative to chemically synthesized pesticides in global markets. Currently, many microorganisms are used as biopesticides because they have several advantages. From this group, entomopathogenic fungi play an important role and are widely studied (Pan and Zhang, 2019; Zélé et al., 2020).

*Beauveria bassiana* (Bb); Ascomycota: Clavicipitaceae) is an entomopathogenic fungus that is used as an eco-friendly insecticide due to its ability to infect and kill arthropods. It causes mycosis in about 700 species of harmful insects from various orders, including Lepidoptera, Hemiptera, Hymenoptera, Coleoptera and Diptera (Alagesan et al., 2019; Zimmermann, 2007; De Faria and Wraight, 2007; Wright and Lax, 2016).

Like other entomopathogenic fungi, Bb produces conidia that penetrate the cuticle of host insects and mites (Pedrini, et al., 2007, Pedrini et al., 2013). Hence, Bb may have an advantage over other biological agents (i.e. bacteria and viruses) for some applications as a contact biopesticide. Because of its ability to rapidly produce infectious conidia when incubated on inexpensive nutrient media, there is significant interest in the commercial mass production of highly effective bioproducts with broad ranges based on Bb (Gabriel and Jaronski, 2016).

In addition, Bb possesses endophytic activity; that is may be able to contribute to plant growth. Endophytic Bb has been engineered, although its application as a bioinoculant in modern agricultural systems is relatively under explored (Vega, 2018; Jaber and Ownley, 2021). However, it is often necessary to obtain and use local isolates to meet regulatory and environmental requirements for field applications of such bioagents.

Therefore, the identification of Bb strains that possess both high bioinsecticidal and growth-stimulating activities is an urgent issue in the development of biological products based on these fungi. The aim of this study was to evaluate the potential insecticidal and endophytic activities of local native Bb isolates. The larval and adult stages of the apple tree aphid (*Aphis pomi*) were used as the target in the bioinsecticidal experiments, and crops (i.e. beans, tomatoes and cucumbers) were used as the targets in the biostimulant experiments.

## MATERIAL AND METHOD

The local native Bb strains tested were the Col-2, VT and 12K strains, which were isolated from soil and dead insects, and kept in the laboratory collection of the Plant Protection Department, KTU Manas (Figure 1). The cultures of the above strains were stored at a temperature of 4 °C on Czapek's agar medium (Sucrose-20.0 g; NaNO<sub>3</sub> -2 g; KH<sub>2</sub>PO<sub>4</sub> -1 g; MgSO<sub>4</sub>·7H<sub>2</sub>O-0.5 g; KCl-0.5 g; FeSO<sub>4</sub> -0.01 g; agar -20g; distilled water-1000 ml).



Figure 1. Colonies on Czapek's medium and microscopic picture (x100) of conidiophores and conidia of *Beauveria bassiana* isolates

To grow biomass of Bb strains for determining its entomopathogenic activity, low-cost nutrient solid media: barley, wheat, millet and rice groats were used.

These food wastes were sterilized in an autoclave and dried in an oven at 120-140°C for additional sterilization. Ready food media were distributed into sterile dishes with a 5–10 cm layer. To inoculate the solid medium, an inoculant was prepared from Bb strains grown previously in liquid modified Czapek's medium (fodder yeast – 15 g; sucrose – 3.0 g; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> – 0, 3g; KH<sub>2</sub>PO<sub>4</sub> - 0.1g; MgSO<sub>4</sub> - 0.05g; KCl - 0.05g; ZnSO<sub>4</sub> - 0.002g; KI - 0.003g; FeSO<sub>4</sub> - traces; water - 100 ml) on a shaker (Shaker-incubator ES-20/8 , BIOSAN, USA) within 48 hours. After incubation the Bb spores number in 1 ml of liquid was measured using a



spectrophotometer (UV/VIS, Jenway, Stone, and UK) at 600 nm. Ready-made inoculants containing  $1 \times 10^9$  spores/ml were added to prepared media from food waste, mixed well for uniform distribution of Bb spores on all layers of the nutrient medium and grown for 15 days.

The spore solution of each strain was prepared from dried biomass that had been produced for 15 days in food waste media prepared. 10 ml distilled water containing 1% Tween 80 was added to 1g of obtained biomass. Then the conidia suspension of each strain was transferred into 50 ml volume tubes and mixed for 3 minutes at 1500 rpm to separate the conidia and mycelium from each other. Conidial concentration in suspension was detected under phase contrast microscope using Neubauer chamber by counting the conidia in/mL by the formula:  $N = a \cdot 1000K/h \cdot S$ , (N - the number of conidia in 1g of biomass; a - the average number of spores in one cell of chamber; K - the dilution number; h - the depth of the chamber; S - the area of one cell of chamber) (Masoudi et al., 2018).

For biotesting of Bb isolates for entomopathogenic activity in vitro conditions, 1 ml of the conidial suspension of each isolate was applied to filter paper discs (diameter 9 cm) in Petri dishes. Apple leaves with infested aphid (*Aphis pomi*) larvae and adults (10 pieces) were placed in a Petri dish and sprayed with another 1 ml of biological product suspension. Thus, 2 ml of a suspension containing  $1 \times 10^9$  conidia/ml of an entomopathogenic fungus was added to each dish. The experiments were carried out in triplicate. In control, experimental insects were sprayed with warm sterile water. The development of mycosis on experimental insects and the humidity in Petri dishes were monitored daily. To create moist conditions for the germination of fungal conidia, the filter paper was sprayed with sterile water as the humidity decreased. The infected and died pests were observed under a microscope every day and the mortality of insects has relieved within five days.

The endophytic activity of the Bb strains was evaluated by bioinoculating crops in three ways: through the seeds (soaking vegetable seeds for 2 h in a  $1 \times 10^6$  spores/mL suspension of Bb strains); through the soil (watering with a  $1 \times 10^6$  spores/mL suspension of Bb around the root system); and through the leaves (spraying the surface of the leaves and stems with a  $1 \times 10^6$  spores/mL suspension of Bb strains). The plants were bioinoculated and left for 15 days before the endophytic activity of the Bb strains was assessed.

## RESULTS

To grow the biomass of the fungus Bb in laboratory conditions and to determine its entomopathogenic and endophytic activities, it was necessary to choose low-cost nutrient media that meet the physiological requirements of these fungus isolates. We used solid low-cost nutrient media for their cultivation: barley, wheat, and millet and rice groats. The colonization of solid media with fungi mycelium to be seen from the third day. All tested strains have grown well on wheat and barley, completely covering the medium and forming dense mycelium. The millet and rice groats media were not completely covered with fungi mycelium. For example, Figure 2 has shown the growth of the Bb Col-2 strain on the cereal groats in 10 days of incubation.

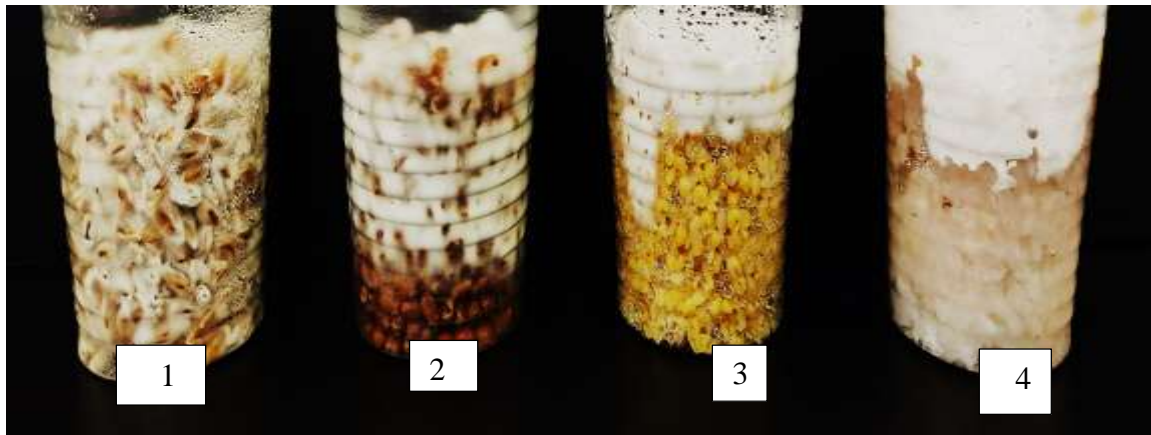


Figure 2. The mycelium forming of the Bb Col-2 strain on the cereal groats: 1- on barley; 2- on wheat; 3- on millet; 4- on rice during ten days of incubation.

To determine the titre of conidia in the biomass grown on various food wastes, to choose the most suitable composition for creating inexpensive formulas of bioproducts based on Bb fungi, a calculation was made in 1 g of the resulting product. As the calculation results showed, the tested strains produced a high conidia titre on wheat groats, so the VT strain produced  $25 \times 10^9$  spores/g, and the Col-2 strain produced  $23 \times 10^9$  spores/g. On barley groats, the VT strain produced  $23 \times 10^9$  spores/g; 12K –  $24 \times 10^9$  spores / g; on millet groats the VT strain formed  $17 \times 10^9$  spores / g; Col-2 –  $18 \times 10^9$  spores / g; 12K -  $14 \times 10^9$  spores / g. The lowest titre of conidia was produced on rice groats in strain Col-2 –  $8 \times 10^9$  spores/g and in strain 12K –  $10 \times 10^9$  spores/g. Thus, these results allowed us to select two compositions: wheat and barley groats, to create samples of bio formulas based on Bb strains.

The resulting biomasses were dried at a temperature of 30-31°C in a drying oven and packed in crave paper bags indicating the product's weight.

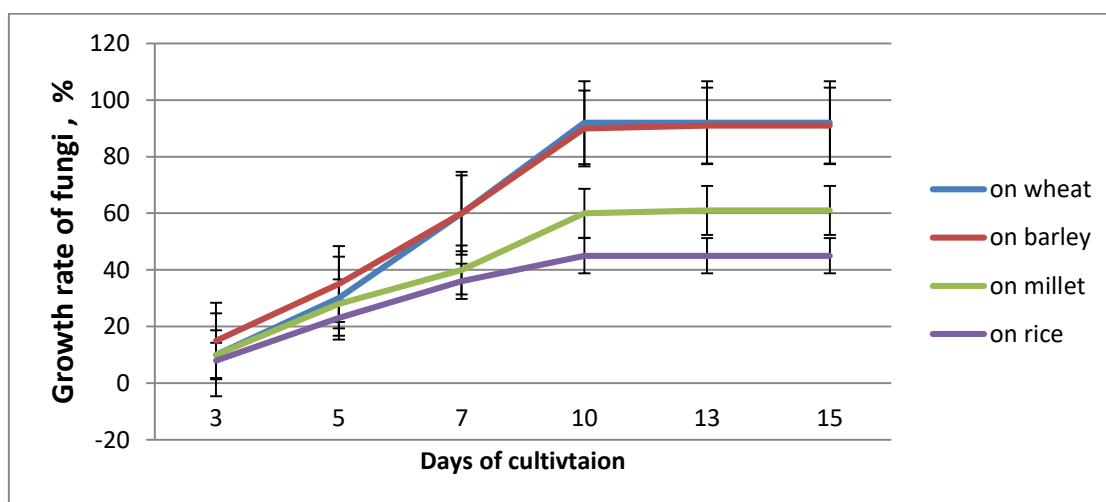


Figure 3. Growth intensity of Bb VT strain on food waste media during 15 days of surface cultivation. Values are given as mean  $\pm$  SD, n = 3, significantly different at  $P \leq 0.05$ .

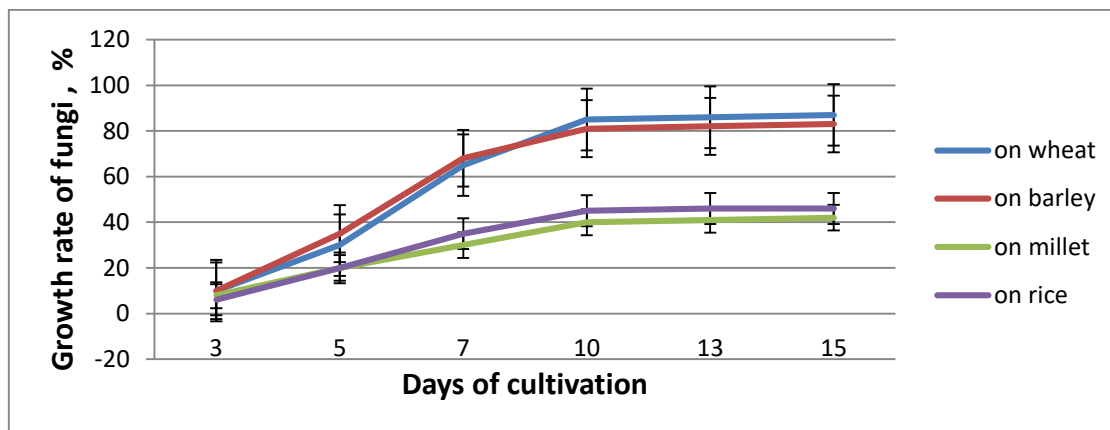


Figure 4. Growth intensity of Bb 12K strain on food waste media during 15 days of surface cultivation. Values are given as mean  $\pm$  SD, n = 3, significantly different at  $P \leq 0.05$ .

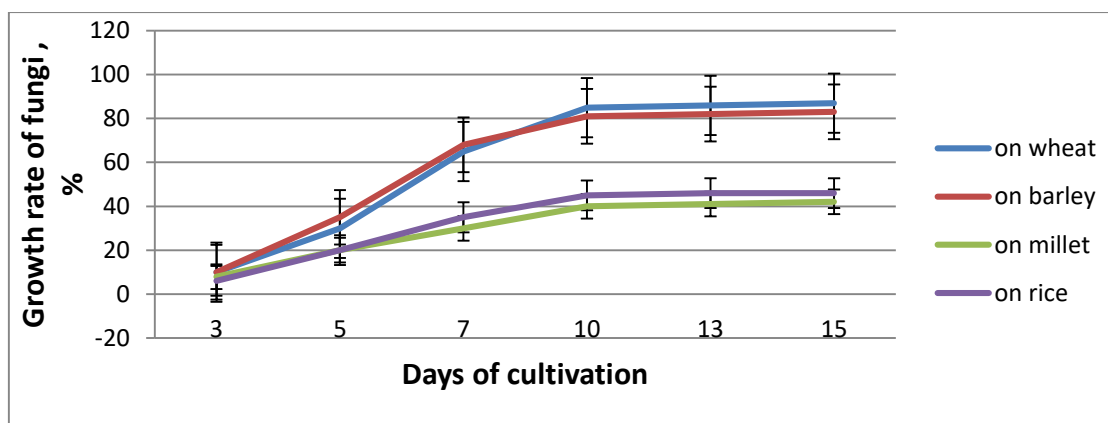


Figure 4. Growth intensity of Bb Col-2 strain on food waste media during 15 days of surface cultivation. Values are given as mean  $\pm$  SD, n = 3, significantly different at  $P \leq 0.05$ .

It was found that the growth-stimulating effect of the Bb strains on the different plants varied with the bioinoculation method.

*The results of using the Bb strains bioproducts as a bioinoculant by spraying the leaves of vegetable crops.*

The VT strain had the highest effect on the growth and development of tomatoes; the experimental plants were ahead of the control ones in stem growth by  $4.0 - 4.5 \pm 0.71$  cm. When using the strains Col-2 and 12K products, the experimental plants were  $2.5 - 3.0 \pm 0.45$  cm higher than the control ones (Figures 5).

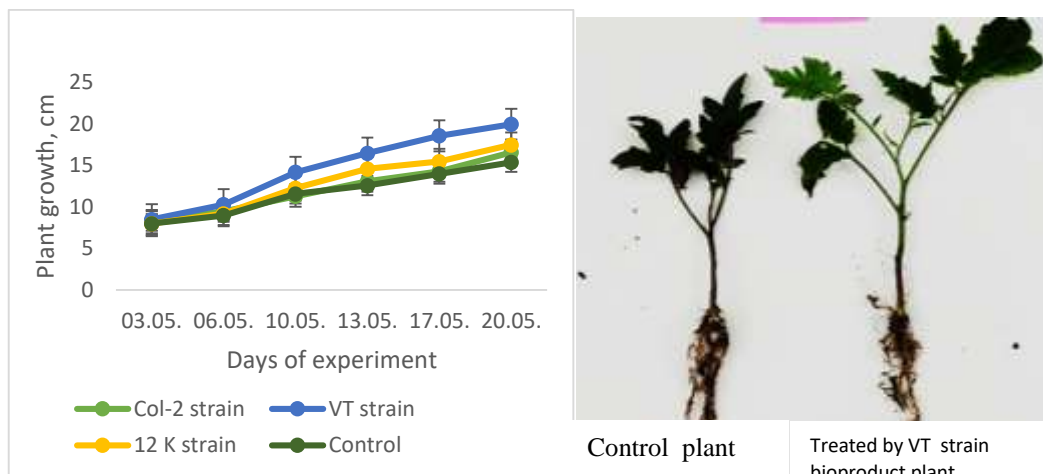


Figure.5. Growth improving effects of Bb strains bioproducts on tomatoes' seedlings by spaying inoculation.

Values are given as mean  $\pm$  SD, n = 3, significantly different at  $P \leq 0.05$ .

On cucumber, the efficiency of the strains bioproducts was lower than on tomato; here, the 12K strains bioproduct had a more active growth stimulating effect; the difference with control plants was  $2.0 \pm 0.31$ cm, while in the other two strains, Col-2 and VT, the difference was only  $1.0 \pm 0.15$  cm ( Figure 6).

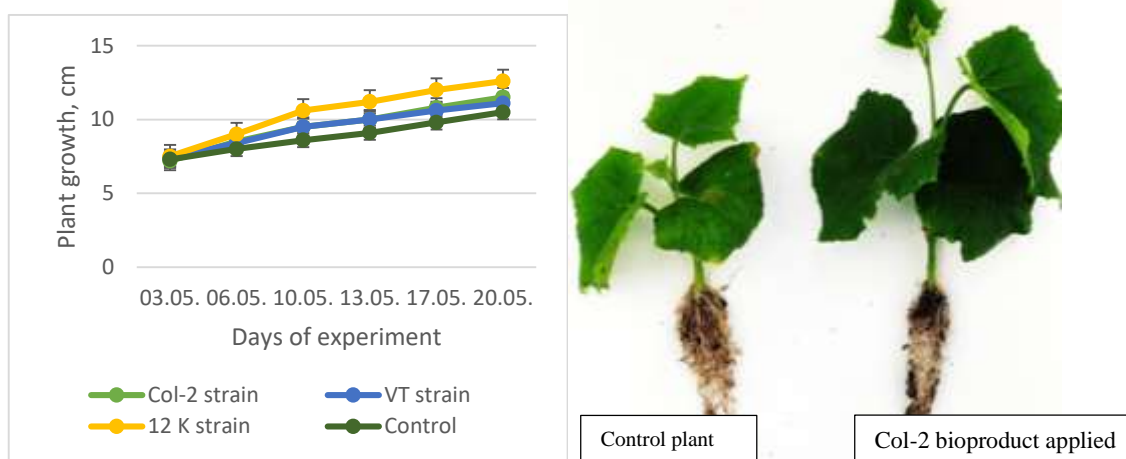


Figure.6. Growth improving effects of Bb strains bioproducts on cucumber s' seedlings by spaying inoculation.

Values are given as mean  $\pm$  SD, n = 3, significantly different at  $P \leq 0.05$ .

The most potent growth stimulating effect of these bioinoculant Bb strains has been seen in beans. Three days after spraying, the experimental plants were ahead of the control ones by  $5.5-7.0 \pm 0.90$  cm, and after seven days by  $10.0 \pm 0.95$  cm. When spraying with VT product suspension by  $9.0 \pm 0.73$  cm and using a 12K strain product by  $5.5 \pm 0.71$  cm difference with control plants (Figure 7).

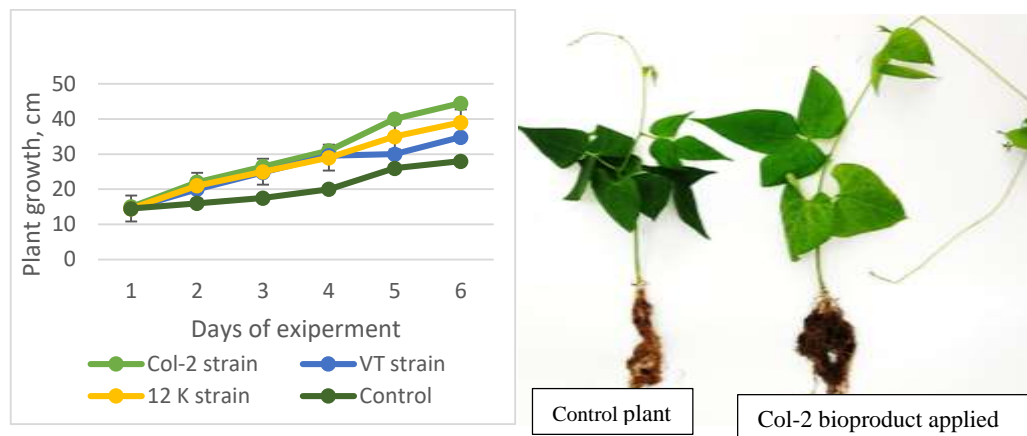


Figure.7. Growth improving effects of Bb strains bioproducts on beans seedlings by spraying inoculation.

Values are given as mean  $\pm$  SD, n = 3, significantly different at  $P \leq 0.05$ .

*Results of using Bb strains as a bioinoculant when applying a suspension around the root system of experimental plants*

With three single waterings of the soil around the root system of the tomato, bioproduct based on strain VT has shown the best result; at the end of the experiment, the plants were  $5.0 \pm 0.63$ cm ahead of the growth of the control ones. The growth-stimulating effect of strain 12K bioproduct appeared only after the second inoculation; at the end of the experiment, the difference between the experimental and control plants was  $4-4.5 \pm 0.65$ cm in stem growth. Whereas the bioproduct based on Col-2 did not show any stimulating effect on tomato within one week, only the second application began to show its impact. At the end of the experiment, it was  $3.5-4.0 \pm 0.42$  cm ahead of the control plants.

On cucumber, the bioproduct based on the Col-2 strain showed more significant efficiency compared to others after the first injection. At the end of the experiment, the experimental plants were  $3.0-3.5 \pm 0.37$  cm ahead of the control ones. Whereas the strains VT and 12K at the beginning of the investigation did not show any noticeable effects on the growth of experimental plants, only after the second inoculation began to show a growth different from control plants, so the difference with the control was  $2-2.5 \pm 0.31$  cm for the VT strain, and  $2-2.5 \pm 0.27$  cm; for the strain 12K product only  $0.8-1.1 \pm 0.17$  cm.

The action of bioinoculum strains on beans was not observed during the first injection of the suspension into the soil. On the seventh day, the Col-2 strain's product showed its growth-stimulating effect, and after the second injection, its effect increased; at the end of the experiment, the difference with the controls was  $6.5-7 \pm 0.47$  cm growth in the experimental version. Strain VT after the second injection began to show its growth-enhancing effect, and at the end of the experiment, the plants were  $4.5-5 \pm 0.41$ cm longer than in control. Strain 12K product did not show any strengthening effect on plants.

*The results of using Bb strains as a bioinoculant by soaking plant seeds in a bioproduct suspension.*

Seeds of tomato, cucumber and beans were soaked for 2 hours in a suspension of a bioproduct based on the tested strains Bb, in control in sterile water.

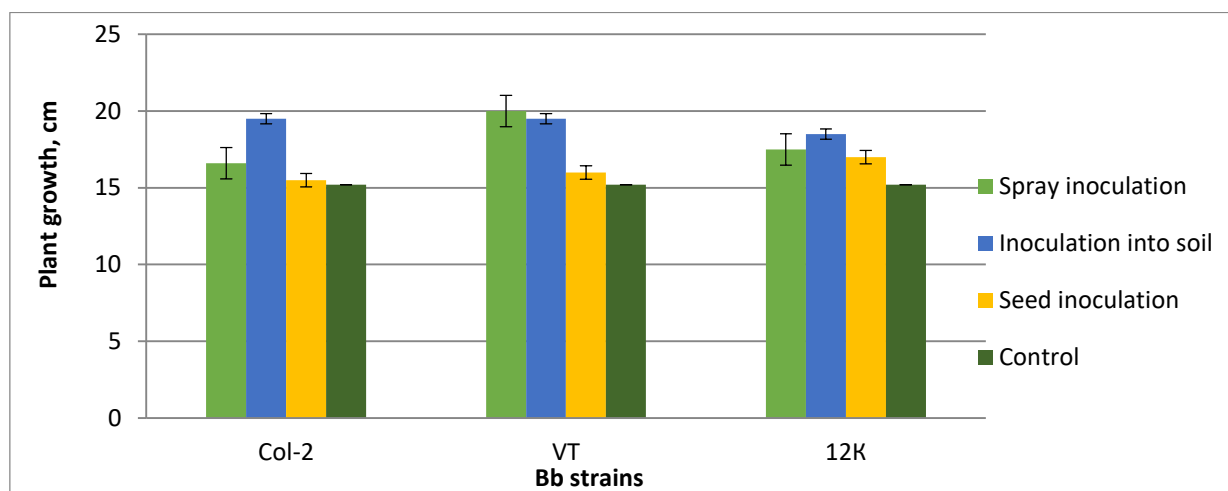
On tomatoes, the growth-stimulating effect of bioproducts was manifested only after the appearance of several true leaves, seven days after the start of the experiment. Unlike other strains, the 12K strain product has shown promising results, while the experimental plants were

3 ± 0.21 cm longer than the control ones. The length of shoot growth in the experimental group was minimal, by 1.5 ± 0.11cm, when using strains VT and Col-2.

Slight differences from the control group were also noted in the cucumber; for example, when seeds were soaked in a suspension of Col-2 and 12 K strains bioproduct, the experimental plants were 1.8-2.5 ± 0.12 cm longer; while using the VT strain, they were 1.5 ± 0.09 cm longer.

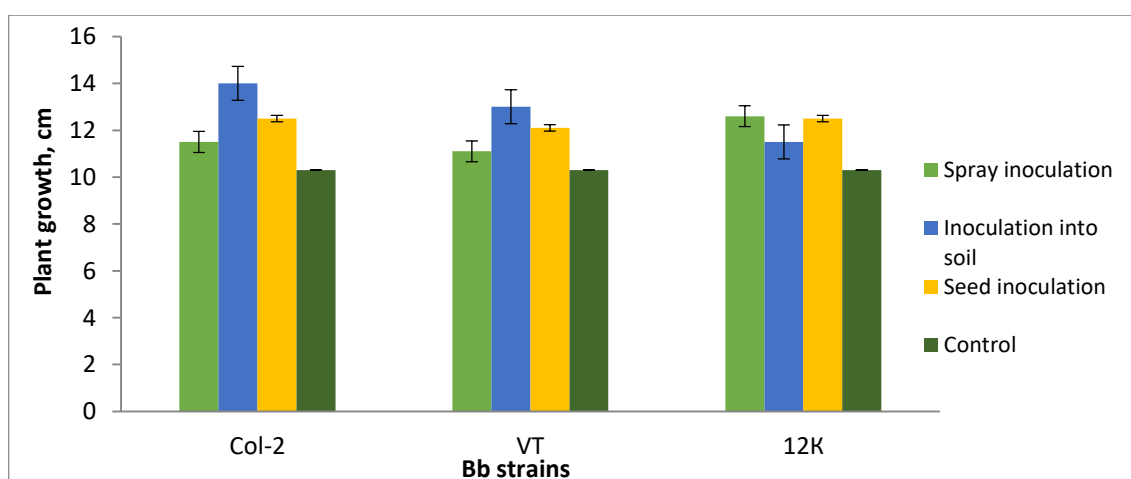
A sound effect was observed on beans when seeds were soaked in a suspension of all tested strains products. The difference in height between control and experimental was almost 2.0 ± 0.19 cm.

Hence, it was concluded that Bb has endophytic activity and can affect plant growth. In this study, the results were dependent on the exposure method. The best results were obtained for tomatoes and cucumbers using soil bioinoculation, whereas the best results for beans were obtained using all the tested types of bioinoculation (Figures 7,8 and 9).



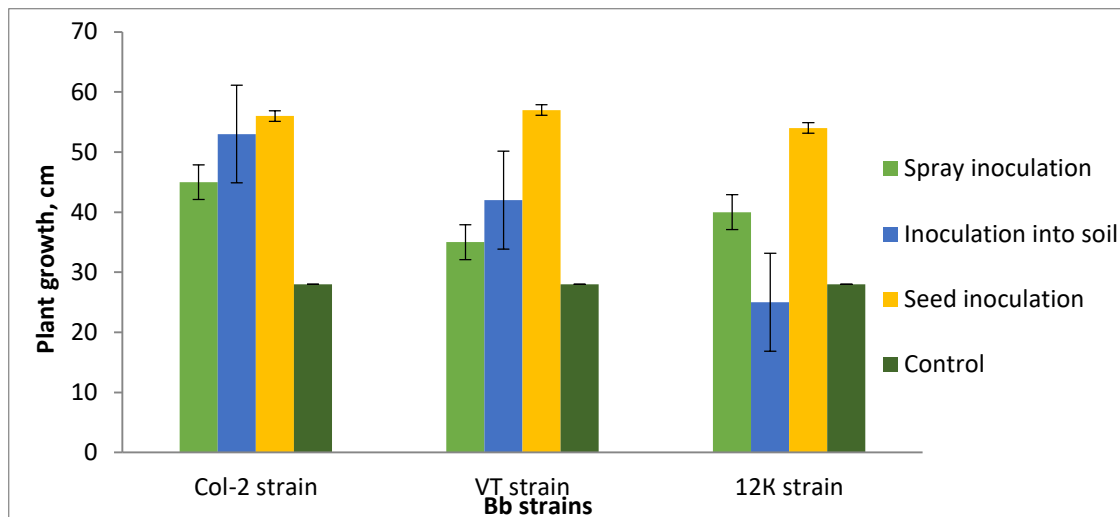
**Figure 7.** The effects of Bb bioinoculants on tomato seedlings' growth by different way applications.

Values are given as mean ± SD, n = 3, significantly different at P ≤ 0.05.



**Figure 8.** The effects of Bb bioinoculants on cucumber seedlings' growth by different way applications.

Values are given as mean ± SD, n = 3, significantly different at P ≤ 0.05.



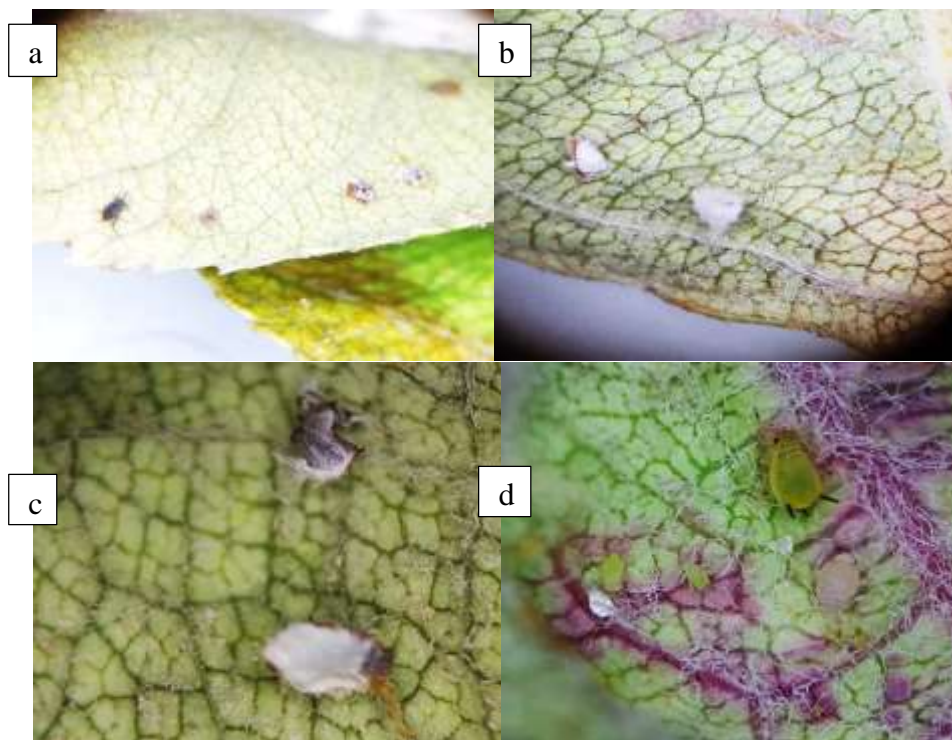
**Figure 9.** The effects of Bb bioinoculants on bean seedlings' growth by different way applications.

Values are given as mean  $\pm$  SD, n = 3, significantly different at  $P \leq 0.05$ .

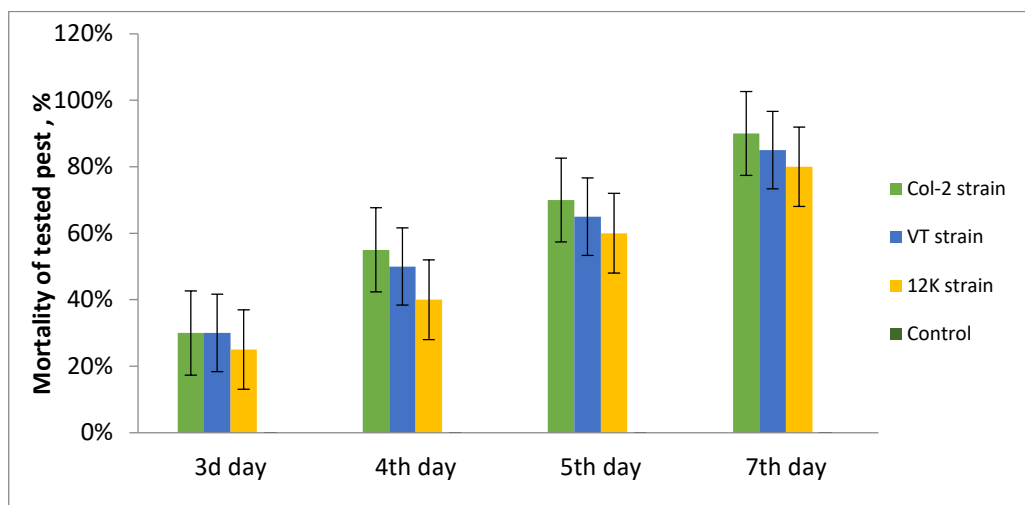
*Insecticidal activity of Bb strains of bioproducts against apple aphids (Aphis pomi)*

When testing a suspension of biological products of Bb strains at a dose of  $1 \times 10^9$  conidia/ml in triplicate at the larval and adult stages of aphids (*Aphis pomi*), the number of dead and weakened individuals was checked under a microscope for seven days. Maintaining humidity up to 80% allowed to increase the effectiveness of fungus products. The mycosis covering the body of insects by mycelium began in 48 hours (Figure 10).

Mortality after 72 hours was  $50-55 \pm 1.01\%$  using all three strains. On the seventh day, the mortality rate resulting from treatment with the Col-2 and VT strains was  $90 \pm 1.27\%$ , and the mortality rate resulting from treatment with the 12K strain was  $80 \pm 1.23\%$  ( $P \leq 0.05$ ) in the tested pest population (Figure 11).



**Figure 10.** Mycosis of aphid's larvae and adults using the Bb products: a- Col-2; b – 12K, c – B.VT and d – Control insects (x 40 stereo microscopy)

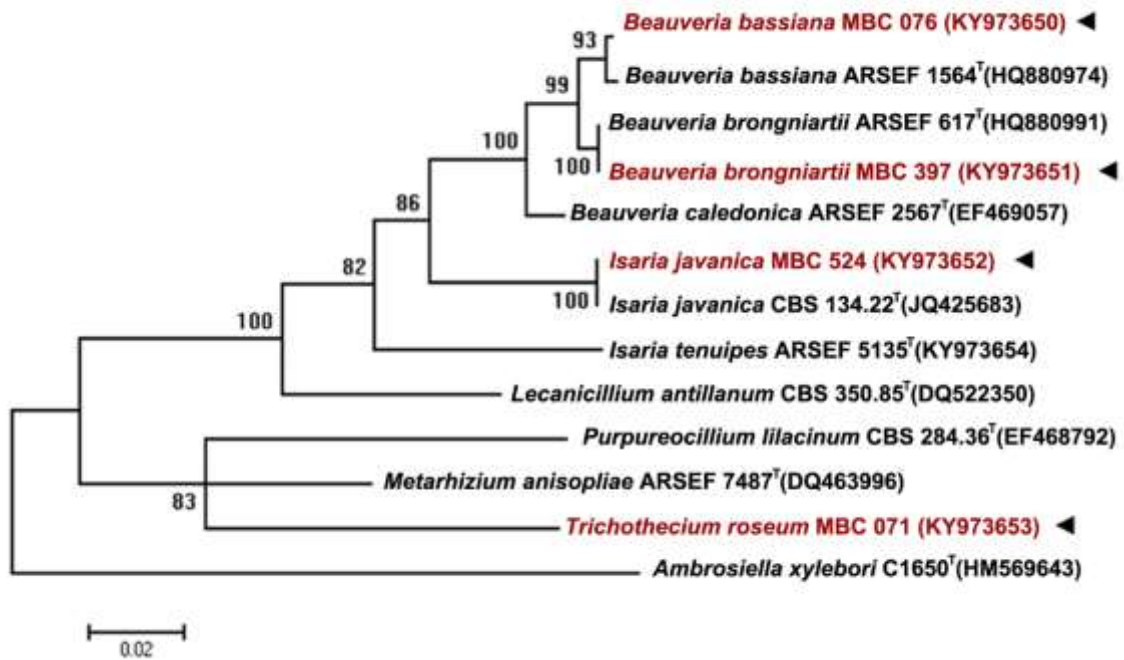


**Figure 11.** The insecticidal activity of Bb strain bioproducts on the *Aphis pomi* larvae and adults by contact infection ( $1 \times 10^9$  conidia/ml).

Values are given as mean  $\pm$  SD, n = 3, significantly different at  $P \leq 0.05$ .

Selected fungal isolates were subjected to DNA sequencing for identification. The ITS1 and ITS4 DNA fragments of select fungal isolates were amplified by PCR using the primer pairs ITS1 (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (CAGGAGACTTGTACACGGTCCAG) and genomic DNA as the template. Based on their morphological characteristics, including the production of ellipsoidal conidia, and molecular characteristics (ITS, partial 18S [SSU rDNA] and EF1- $\alpha$  sequences), the isolates were identified as *B. bassiana* belonging to Clade E from Asia (Figure 12).





**Figure 12.** Neighbors-joining phylogenetic tree based on 16SrRNA gene sequences showing the position of Bb Col-2 and VT strains (KY973650) among other closely related *Beauveria bassiana* species from the database.

## CONCLUSIONS

The findings of this study confirm the potential of these Bb strains as biological pest control agents and bioinoculants that increase the growth and development of plant seedlings. These findings can be used to develop bioagents that will reduce the indiscriminate use of chemical insecticides on crops and thereby protect the environment and its vital natural components from excessive pesticide pollution.

## ACKNOWLEDGEMENTS

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## STATUS OF PLANT GENETIC RESOURCES FOR FOOD AND AGRICULTURE IN BULGARIA: FROM GENOTYPE COLLECTING TO INFORMATION ACCESS

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### ABSTRACT

One of the main priorities of the National Program of Plant Genetic Resources in Bulgaria is through international cooperation and establishment of collaborative networks to increase the utilization of conserved genotypes and scientific knowledge by free access and exchange of information. During the period 1982-2021 the fund of National Genebank in the Institute of Plant Genetic Resources – Sadovo is enriched with 53,648 accessions. The status of the stored in the genebank collection belongs to 122 botanical families and is characterized by 3,702 taxonomic descriptions. There are 36,754 accessions, introduced from foreign genebanks or botanical gardens by international free exchange. 10,817 accessions are acquisitioned through expeditions, conducted by national and international projects. A great diversity of cereal, grain legume and vegetable local varieties from home gardens and crop wild relatives from natural habitats are collected. 6,077 advanced cultivars and lines from breeding institutes in Bulgaria are preserved. The electronic register includes passport information according to FAO/Biodiversity descriptor. The standard allows Bulgarian *ex situ* collection to be published on European Search Catalogue for Plant Genetic Resources – EURISCO. From 2020 intelligent documentation system with specialized software, functional ontologies for free access to plant genetic resources for all stakeholders and assured security of records through blockchain technologies starts to be established. The study aims to present the status and the free access to the Bulgarian genebank in connection with the Second Global Plan of Action for the Conservation and Sustainable Utilization of Plant Genetic Resources for Food and Agriculture. This work is supported by Bulgarian National Science Fund under the project BGPlantNet “Establishment of National Information Network GenBank – Plant genetic resources”, and obtained results will be applied in the National Research Program “Smart Crop Production”.

**Keywords:** plant diversity, seed genebank, botanical garden, data base, international networks.

### INTRODUCTION

The EU has attached great importance to the conservation and utilization of plant genetic resources for food and agriculture. The Second Global Plan of Action for the Conservation and Sustainable Utilization of Plant Genetic Resources for Food and Agriculture address new challenges, such as climate change and food insecurity, as well as novel opportunities, including information activities, free access to the genebanks, development of international networks, etc. According to 18 priority activities it is focused in four main groups: *in situ* conservation and management; *ex situ* conservation; sustainable use; and building sustainable institutional and human capacities (FAO, 2011). The Global Plan has formulated and perfected a series of regulations and has strengthened the management of plant genetic resources. Through training

and popularization of scientific knowledge related to genetic resources, the public awareness has been promoted. Through the international cooperation and establishment of collaborative networks, it has promoted the exchanges of information and germplasm. Through the implementation of various European programmes and projects, the conservation system for plant genetic resources has been established and improved gradually to achieve the objectives of safe conservation and sustainable use of plant genetic resources, which has played a great role in plant breeding and food security.

Plant genetic resources are cultivated and wild forms, local populations, old, forgotten and improved varieties, carriers of a functional unit of heredity and possessing real or potential value for science and practice (ITPGRFA, 2009; Mattana et al., 2021). The main goal of their collection is the creation of well-documented, preserved and studied collections that represent the widest possible diversity of the gene pool (Engels and Visser, 2008; Engels and Ebert, 2021).

Maintaining plant genetic diversity through seeds of cultivated species and their wild relatives at national, regional and international levels, and promoting access to and equitable sharing of benefits arising from the use of these resources and associated traditional knowledge is one of the objectives in the Sustainable Development Goals of FAO (<https://www.fao.org/sustainable-development-goals/indicators/251a/en/>).

Due to its specific geographical location, diverse relief and climatic features, our country is distinguished by a rich botanical diversity, which has been used for millennia in traditional Bulgarian cuisine and medicine. With its role as a national coordinator of plant genetic resources, IPGR-Sadovo follows international documents establishing obligations such as conducting measures for public awareness of the importance of plant genetic resources, cooperating with European partners to conduct scientific research for transfer of new technologies, contributing to information exchange and increasing access and use of the gene pool (ECPGR, 2021).

The Nagoya Protocol (CBD, 2011) recognizes that countries have sovereign rights over their genetic resources and provide a framework for domestic legislations on Access and Benefit-Sharing. Although the ITPGRFA's Multilateral System of Access and Benefit-Sharing provides opportunities for easier access to plant genetic resources for food and agriculture (ITPGRFA, 2009), genebanks face increasing complexity in their operation. Adding material to genebank collections has become more difficult, not only because collecting missions need to be negotiated with national and local authorities, but also because acquiring material from other collections is possible if the origin of the material is properly documented and is done in compliance with regulations (Halewood et al., 2018, Brink and Hintum, 2020, Pilling et al., 2020; Weise et al., 2020; Weise, 2021).

The study aims to present the status and the free access to the Bulgarian genebank in connection with the Second Global Plan of Action for the Conservation and Sustainable Utilization of Plant Genetic Resources for Food and Agriculture.

## **MATERIAL AND METHODS**

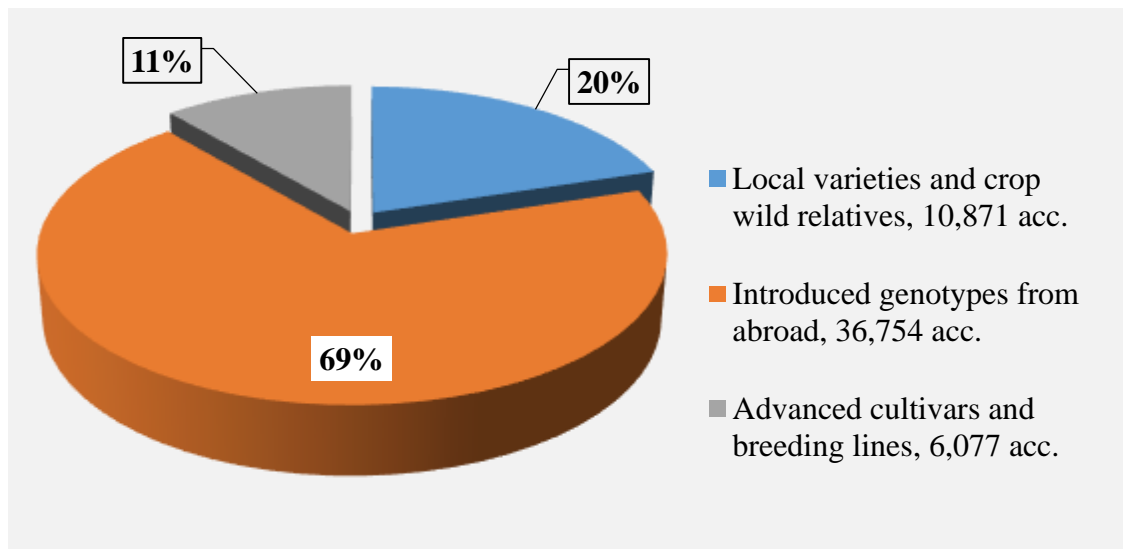
The Center for information and documentation of plant genetic resources at IPGR-Sadovo has been established in 1982 and completely renovated in 2021 under a project BGPlantNet, financed by Bulgarian National Science Fund of the Ministry of Education and Science (Grant KP-06H36/2/13.12.2019). It works according to the international standard of FAO/Bioversity (2017).

The National genebank of Bulgaria, situated at IPGR-Sadovo, was built in 1984 and carries out a scientific program for the long-term preservation of germplasm with seeds under controlled

conditions in accordance with the standards developed by FAO (2013). The genebank facilities are designed both for long-term storage and medium-term storage. The Botanical Garden at IPGR-Sadovo was created in 2002 to protect wild and native species through living conservation. Annually, accessions from foreign genebanks are introduced, registered in IPGR database, studied and maintained. Expeditions in different geographical regions of the country are conducted and valuable local varieties and crop wild relatives are collected. In the fund of genebank are stored new improved varieties with Bulgarian origin from the specialized breeding institutes. The electronic database contains the following passport information: taxonomy, catalogue number of accessions, acquisition date, country of origin, donor of the sample, collection site, ecology-geographical characterization, biological status, type of storage: base collection (long-term), exchange collection (medium-term), work collection (short-term), *in vitro* and/or field collection, botanical garden, etc. The taxonomic description of the crops is under the nomenclature of USDA (GRIN, 2015). The National Genebank is nominated as a focal point in the European Search Catalogue for Plant Genetic Resources – EURISCO (<http://eurisco.ecpgr.org>). EURISCO is an open access network, providing information about *ex situ* plant collections in Europe (Hintum and Knüpffer, 2010; Dias et al., 2012; Weise et al., 2017; Kreide et al., 2019). It provides information at accession level about more than 2 million samples from 6,736 genera and 45,171 species, conserved in European genebanks. Numerous of ECPGR Central Crop Databases are *on line* available, as well as knowledge on their potential for use (Gass et al., 1997). Through EURISCO the information about the Bulgarian National Inventory is accessible in other international databases, such as A European Genebank Integrated System – AEGIS (Engels and Maggioni, 2012; Hintum et al., 2021), World Information and Early Warning System – WIEWS (FAO, 2020) and electronic platform for Plant Genetic Resources for Food and Agriculture, conserved in genebanks worldwide - GENESYS (2015). From 2020 intelligent documentation system with specialized software, functional ontologies for free access to plant genetic resources for all stakeholders and assured security of records through blockchain technologies starts to be established. Starting with the use of field books, a gradual development of electronic data base as national register, nowadays the intelligent data management system aims to improve the availability of conserved seed accessions in genebank to users (Doychev et al., 2020; Stoyanova-Doycheva et al., 2020; Doukovska, 2021).

## RESULTS AND DISCUSSION

Collections of plant genetic resources and a set of technologies for their conservation, study and practical use are now the basis of bioeconomy, biosafety and food security. It is the foundation underpinning production chains, leading from basic research to various technological areas and industries. There are 53,648 preserved seed accessions in IPGR-Sadovo from 69,768 totally registered plant germplasm in Bulgaria (<http://eurisco.ecpgr.org>), where 43,147 accessions are under long-term storage conditions. The National collection of plant genetic resources consists of cultivated species and their wild relatives, local and foreign varieties, populations, breeding lines and samples, as well as threatened and endangered species. During the period 1982-2021, the fund of the National genebank in IPGR-Sadovo was enriched with accessions, distributed according to their status and acquisition source (**Fig. 1**). Through conducted expeditions, 10,871 accessions - great diversity of cereals, grain legumes and vegetable local varieties (called also farmers' varieties or landraces) from home gardens and crop wild relatives from natural habitats, were collected. Local plant genetic resources comprise 20% of the genebank enrichment.



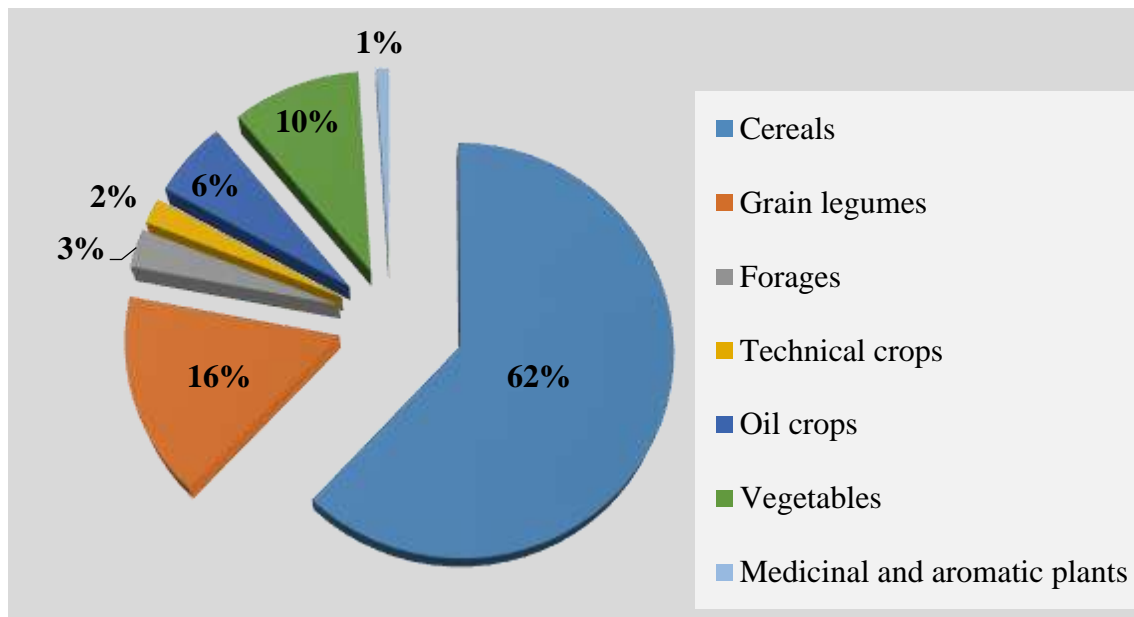
**Fig. 1.** Enrichment of conserved plant diversity in Bulgarian seed genebank (1982-2021)

The first direction of expeditions is collection of cultural forms from field and vegetable crops. Traditional accessions from tomato, pepper, cucumber, pumpkin, melon, watermelon, onion, lettuce, adapted to the specific agro-ecological conditions, characterized with valuable traits such as early maturity, resistance to biotic and abiotic stress, high biological content, were found in home gardens. For cereals and grain legumes the attention is directed to collecting of primitive wheat accessions, old and local populations of corn, bean, cowpea, lentil, chickpea, fava bean, etc. Of particular interest is the species diversity of forgotten spices and medicinal plants, which are being rediscovered for the purposes of dietary, healthy nutrition and applied in therapies for the alternative treatment of a number of diseases.

Another direction of expeditions is the preservation of wild, semi-natural diversity and crop wild relatives. The high urbanization, developed transport infrastructure and ecological threats put a large number of wild species of different botanical families at great risk.

The described ecologi-geographical characteristic of the collected accessions makes it possible to return the local varieties to the regions of origin through the seed samples, stored in the *ex situ* collections. The foreign germplasm comprises 69% of the genebank enrichment. 36,754 accessions from advanced varieties and breeding lines of diverse geographical origin were introduced for evaluation in our conditions and for use as donors of valuable economic traits in crop breeding. The germplasm free exchange significantly contributes to the enrichment of the plant diversity and expands the possibilities for accelerating the breeding process of peanut and sesame. Active international contacts with centers of plant genetic resources from all over the world are maintained. The main partners in free germplasm exchange of the National genebank are: USDA (USA), ICARDA (Syria), VIR (Russia), NordGen (Sweden), IPK (Germany), INRA (France), John Innes Center (Great Britain). The institute maintains collective membership in the international organizations ECPGR (European Cooperative Program for Plant Genetic Resources) and EUCARPIA (European Association for Research on Plant Breeding). There are working contacts, joint research and development of international projects with scientific teams of foreign scientific organizations as Swedish Biodiversity Center (SEEDNet) - Sweden; CIMMYT-FAWWON (Facultative and Winter Wheat Observation Nursery) – Mexico/Turkey; Svalbard Global Seed Vault – Norway; Global Crop Diversity Trust; Heilongjiang Agricultural Academy - Harbin, China; Center for Plant Genetic Resources - Norway; International Biodiversity Institute, Rome, Italy (Bioversity International);

Norwegian Center for Plant Genetic Resources - Ashe; Institute of Plant Breeding, Chinese Academy of Agricultural Sciences - Beijing, China; Hokkaido Agricultural Research Center, Japan; Polytechnic Institute - Beja, Portugal; Research Center for Plant Breeding at Trakya University - Edirne, Turkey; Institute of Field and Vegetable Crops - Novi Sad, Serbia, have been created. Analyzing the status of newly registered accessions during the period, a relatively low percentage (11%) of the enrichment with new Bulgarian varieties and breeding lines was found. 6,077 advanced cultivars and breeding lines from breeding institutes in Bulgaria are conserved in IPGR-Sadovo. The research work of the IPGR-Sadovo team is aimed at breeding-genetic, immunity and biotechnological studies to create new varieties of common wheat, rice, peanuts and sesame, through the methods of classical breeding and plant biotechnology; develops methods of variety maintenance and production of pre-basic and basic seeds; develops and improves methods and means for increasing the productivity of plants and the quality of production. The Wheat breeding started in 1902 as a part of the first program of the Agricultural experimental station in Sadovo. During its long history 48 varieties and 195 breeding lines have been created. The directions of the research of the program in common winter wheat are creation of new varieties of common winter wheat with a high productivity potential, with high grain quality, possessing great ecological plasticity, drought resistance, cold resistance, resistance to economically important diseases and suitable for cultivation at reduced levels of inputs and development of agrotechnological solutions related to the influence of predecessors, optimal seeding density and fertilizer rates to increase the economic efficiency of varieties (Uhr et al., 2020). The institute is unique in Bulgarian state system with complex scientific research in the field of breeding-genetics and agrotechnics with peanut, sesame and rice crops. The main areas of research are introduction and evaluation of new varieties of peanuts and sesame for the purposes of genetic improvement and crop production; creation of early-ripening, high-yielding, disease-resistant, with good economic qualities, suitable for mechanized inspection and harvesting; development and optimization of individual units of the technology for growing and harvesting peanuts and sesame; improvement of methods for assessing the strength of the attached placenta in sesame (Stamatov et al., 2017; Stamatov et al., 2020). The status of Bulgarian peanut collection covers 1237 accessions, including Bulgarian (435) and foreign origin (802), consisting of varieties (54%) and breeding lines (46%). The Bulgarian sesame collection consist 495 accessions, including Bulgarian (204) and foreign origin (291), consisting of varieties (30%) and breeding lines (70%). The directions of research and applied activity in rice breeding are collection, study, enrichment, storage and reproduction of rice accessions, creation and implementation of new high-yielding with high grain quality and short vegetation period varieties resistant to abiotic and biotic stress factors (Tosheva, 2013). The Bulgarian rice collection consist 495 accessions, including 34 Bulgarian breeding lines and varieties and 799 introduced from abroad with foreign origin. In carrying out the research work over enrichment of *ex situ* collections, IPGR-Sadovo maintains partnerships with all bioresource institutes through the country as Crop Research Institutes in the Agricultural Academy, Institute of Plant Genetics and Physiology, Institute of Biodiversity and Ecosystem Research at the Bulgarian Academ of Science, University Botanical Garden – Sofia; Agricultural University - Plovdiv, Faculty of Agriculture at Trakya University – Stara Zagora and Forestry University - Sofia; Primary and secondary schools; Agricultural cooperatives; Associations of agricultural producers; farmers, trading companies, etc. The enrichment of the *ex situ* collections is divided into eight crop groups: cereals, grain legumes, forages, technical crops, oil crops, vegetables, medicinal and aromatic plants (Fig. 2).

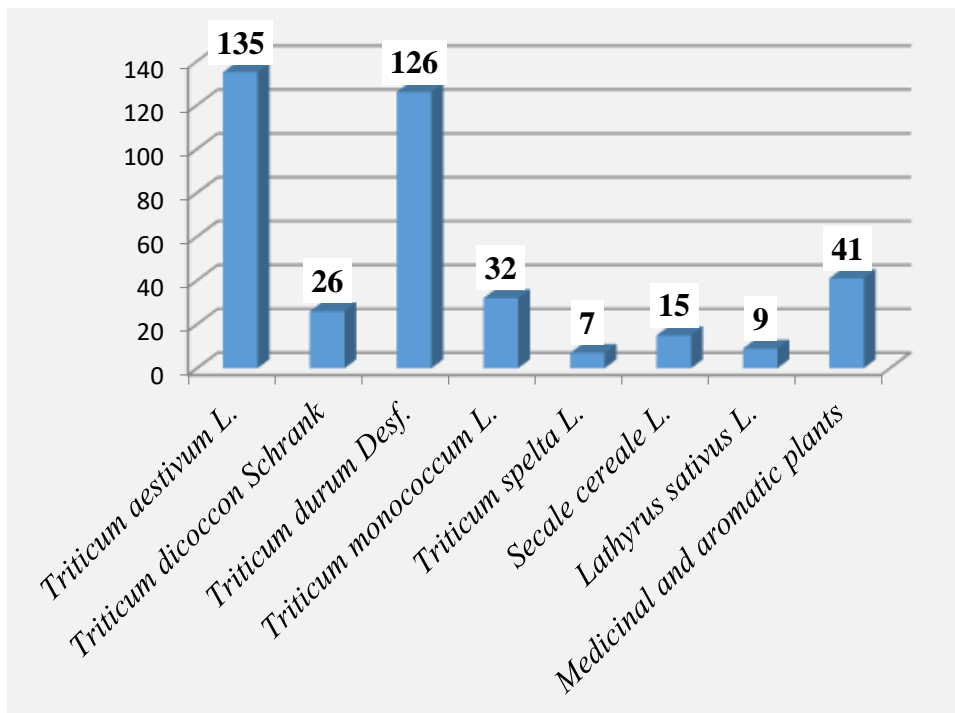


**Fig. 2.** Distribution of accessions by the main crop groups

A database of marked and surveyed localities for *in situ* maintenance of wild species in Strandja, South Dobrudja, the North Black Sea, part of the East and West Rhodopes regions has been created. In the botanical garden of IPGR-Sadovo local forms are preserved through *in garden / in vivo* conservation. 450 plant species from 60 botanical families are maintained as a permanent living collection. Plant species are divided according to the potential of use: crop wild relatives, food plants, medicinal species, herbs, spices, forages, oil and ornamental crops. The protection of this valuable genetic diversity is of great importance as breeding source material for development of novel varieties and for educational purpose of various students. Development of *ex situ* collections, focused on wild flora, need to be coupled with reintroduction measures for threatened taxa into wild populations. Systematic investigation of the genetic diversity may contribute effectively to the long-term conservation of endemic, rare and threatened plant species in Bulgaria and may help preserve the natural genetic patterns of species in the wild when restoration measures are carried out. Especially due to the vulnerability status of these plant taxa, extensive studies on vegetative multiplication, development of optimal *in vitro* propagation protocols and strategic conservation programs are required (Kovács et al., 2021). The documentation system optimizes the data management of collection activities in the process of registration, storage, study, reproduction and distribution. The National Register is characterized by 3,701 taxonomic descriptions, distributed in 122 botanical families. The number of addresses of foreign partners in germplasm exchange is 203. According to the open access catalogue EURISCO (data check – August, 2022) Bulgaria maintains the richest plant genetic resources collection in Southeast European region. The national collection is the 7th biggest in Europe and has a share of 3,4%, after Great Britain, Russia, Germany, Ukraine, Poland and Spain. In connection to its taxonomic composition, the preserved accessions belong to 532 genera and 1,927 plant species. The highest number of accessions is from the genera *Triticum* (16,624 acc.), *Hordeum* (6,451 acc.), *Zea* (4,828 acc.), *Phaseolus* (3,952 acc.), *Avena* (2,674 acc.), *Capsicum* (2,160 acc.), *Pisum* (1,749 acc.), *Linum* (1,482 acc.), *Arachis* (1,373 acc.).

Bulgarian National Inventory is a part from a "virtual" European Genebank Integrated System – AEGIS. The status of the Bulgarian collection amounts to 391 local accessions and it is presented on figure 3.





**Fig. 3.** Distribution of Bulgarian local accessions in AEGIS database by crop species

The new national documentation intelligent system improve the management of all processes related to conservation, study and access to the stored plant gene pool. Basic elements of the ontology are the taxonomic description and EURISCO descriptors. It is possible to supplement the passport information with characterization and evaluation data and photos. The built server infrastructure "GenBank - plant genetic resources" will be used for the purposes of the National Research Program "Smart Crop Production". The overall goal of the program is to conduct fundamental and applied scientific research to create models for diagnosis and forecasting through digital methods for managing agricultural holdings in crop production and ensuring a sustainable and efficient food system (Doukovska, 2021).

## CONCLUSIONS

Although Bulgaria has gained remarkable achievements in conservation and utilization of plant genetic resources, there still are a number of challenges. The study presents an analysis of the current state and an assessment of the prospects for the development of germplasm collections in Bulgaria. In the National genebank there is an important number of conserved plant genetic resources with high degree of diversity and wide represented in European networks. A great diversity of farmers' varieties and landraces from home gardens and crop wild relatives from natural habitats were collected, but further work on evaluation and characterization of these valuable accessions continues. The intelligent documentation system is a 'knowledge bank' and a platform for collaboration between researchers, plant breeders and all users of conserved plant genetic resources. The improvement of management of plant genetic resources: from genotype collecting to information access and sharing the germplasm accessions in the country and expanding the exchanges with foreign partners, is greatly contributing to food security, rapid development of National economy and increase of the farmers' income.

## ACKNOWLEDGEMENTS

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## **THE INDUSTRY 4.0 ASSESSMENTS OF VALUE-ADDED FOOD PRODUCTS DURING 2020-22 PERIOD**

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### **ABSTRACT**

Food and beverage industries encounter manifold challenges from recalls regarding serving customer demands. It is predicted that Industry 4.0 will turn manufacturers into predictors instead of reactors. The sustainability of the food systems in the pandemic duration is another issue that this sector should address in order to restrain the possible forthcoming pandemic times. In COVID-19 duration, there is need the sustainability of bioactive constituent applications for ``value-added food and supplement products`` and ``functional foods`` and need the pursuing and monitoring of food plant design studies, production strategies through Industry 4.0. implementations. In this proceeding content, value-added food products in food science in terms of Industry 4.0 procedures in COVID-19 duration.

**Keywords:** Value-added products, food science, Industry 4.0, COVID-19

### **INTRODUCTION**

Food and beverage industries encounter manifold challenges from recalls regarding serving customer demands. It is predicted that Industry 4.0 will turn manufacturers into predictors instead of reactors; this fact will give easier collecting of data, time-saving / money-saving for investors and who utilize and apply in the food science and technologies (Tokusoglu,2020;2018).

Industry 4.0 Systems and transition equipment preparations require a considerable investment in new equipment and employees. Various manufacturers feel extempore for replacing their equipments that currently working well, or for replacing less-talented labour with technical professionals. In numerous places, there is a deficiency of talented labour with the necessary qualifications. As another efficiency defect and its solving prior to real manufacturing, production matching must be controlled. When manufacturing model does not match demand, overproduction occurs; owing to the fact that food and beverage manufacturers must gather data regarding the demand for their products prior to altering production levels.

Food and beverage industries encounter manifold challenges from recalls regarding serving customer demands. It is predicted that Industry 4.0 will turn manufacturers into predictors instead of reactors. The sustainability of the food systems in the pandemic duration is another issue that this sector should address in order to restrain the possible forthcoming pandemic times. On the other hand, it is stated that there are three category engage in value-

added productions but their differences are substantial in attempting to figure out their decisions (Tokusoglu,2020; 2018).

The first category (1) food sector is concerning demand driven; companies are located in exceedingly populated areas and metropolitan locations aim for developing innovative and value-added products. The second category (2) of food industry is concerning agriculturally related sectors owing to principal food ingredients are bulky or perishable products and also farmers are their customers; these food companies are located in prominent agricultural production and husbandry zones. The third category (3) of food industry named as footloose industry can generally satisfy demand from a wide geographic era with one plant; these type of sector typically produces foods and beverages with high values such as functional foods enhanced with bioactive components, prepared flour mixes, frozen foods, canned specialties, functional beverages enhanced vitamins and minerals.

The sustainability of the food systems in the pandemic duration is another issue that this sector should address in order to restrain the possible forthcoming pandemic times. In 2020-2022 duration, there is need the sustainability of bioactive constituent applications for ``value-added food and supplement products`` and ``functional foods`` and need the pursuing and monitoring of food plant design studies, production strategies through Industry 4.0. implementations (Anonymous 2020).

In the food industry, the diverse types of by-products can be evaluated by various branches of industry due to their selected desired properties of food by-products. The pulps, dregs and wastes in food processing depends on the quality of by-product management, while ensuring the environmental protection and sustainability (Tokusoglu,2018). Food by-product in the food industry is characterized by a high ratio of product specific waste not only does this mean that the generation of this waste is unavoidable, but also that the level and the kind of by-product which consists primarily of the organic residue of processed raw materials, can scarcely be changed if the finished product quality is to remain consistent (Tokusoglu,2020;2018).

In food science, recent trends are ``value-added`` foods and ``value-added`` supplements and ``functional foods``; especially bioactives from fruits, vegetables, from spices, from by-products, also from alternative sources (like algae) are hot topic. Epidemiological studies have pointed out that fruits and vegetable consumption imparts health benefits including certain types of cancer, reduced risk of coronary heart diseases. The health benefits of fruits and vegetables are majorly attributed to bioactive nutrients as phytochemicals, phenolic compounds, carotenoids, vitamins (ascorbic acid, tocopherol etc.), also to dietary fiber of these products (Tokusoglu, 2020; 2018).

For sustainability assesment, general flow can be given as inputs, supply chain step and outputs parts (Yakovleva and Flynn, 2004). It is expressed that the advanced set of sustainability indicators should be contemplated in conjunction with the inputs and outputs model of the food consumption and production system outlined (Tokusoglu,2020,2018; Yakovleva and Flynn, 2004). The proposed indicators mplicate environmental criteria that could include impacts on the system, such as energy utilising as well as outputs such as waste.

The sustainability assessment is broadened from a potentially narrow economic focus on the supply chain to a broader analysis. Even though a sectoral-based assessment to sustainability appraisal is advantageous for improving sustainability indicators for the food supply chain, it can be utilized only for the processing stage. In this context, in COVID-19, there is a requirement on pursuing of input /output strategies of other steps in supply chain in terns of Industry 4.0 implementations.

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## ZINC AND VITAMIN C CAN PREVENT OXIDATIVE STRESS INDUCED BY LEAD NITRATE IN MOUSE LEYDIG CELLS

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### ABSTRACT

Lead and lead compounds, known to be serious environmental pollutants, are abundant in the earth's crust. Water-soluble lead nitrate, one of the most common inorganic lead compounds, is taken into the body through the mouth and skin. It has been shown in studies that lead nitrate, which is known to have various toxic effects on humans and animals, causes Leydig cell damage, a decrease in sperm motility and sperm count, and disorders in testosterone hormone levels and sperm quality in the male reproductive system. Leydig cells were exposed to lead nitrate at a concentration of 300  $\mu\text{M}$  and vitamin C (VitC) and/or zinc (Zn) at a concentration of 50  $\mu\text{M}$  for 24 h. Cell viability, lipid peroxidation, and levels of antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase) were analyzed in the samples obtained when the experiment period was completed. The results showed that lead decreased cell viability and antioxidant enzyme activity and increased lipid peroxidation in Leydig cells. Furthermore, it was determined that VitC and Zn supplementation prevented oxidative damage and improved the activity of decreased enzymatic antioxidants in Leydig cells exposed to lead. In conclusion, it was found in this study that VitC and Zn may have a preventive effect against lead nitrate toxicity on TM3 Leydig cells due to their anti-oxidative abilities.

**Keywords:** Lead nitrate, Leydig cells, Zinc, Vitamin C, Oxidative damage.

### INTRODUCTION

Heavy metals are common in the environment, and these metals can cause toxic effects on all living organisms (Fu and Wang, 2011). Lead is one of the heavy metals with known toxic effects and poses a great risk to the ecosystem due to its long biological half-life. (Hernberg, 2000). The most common sources of lead exposure include the mining profession, lead-contaminated soils, battery manufacturing, and the use of lead-based paints (Shukla et al., 2018). Studies have shown that lead enters the body through inhalation, ingestion, or skin (Alissa and Ferns, 2011). Therefore, lead inorganic compounds have been classified as "possible human carcinogens (Group 2A)" by the International Agency for Research on Cancer (IARC, 2016). According to guidelines published by the Center for Disease Control and Prevention, blood lead levels above 10  $\mu\text{g/dL}$  are dangerous to human health and require treatment (CDC, 2005). Studies in many toxicology fields have shown that even low lead concentrations negatively affect various biochemical, physiological, and behavioral functions in experimental animals and humans (Assi et al., 2016). Studies have proven that this heavy metal damages body systems such as endocrine, skeletal, nervous, excretory, and reproductive systems (ATSDR, 2007). Although there is a lot of research on lead, the mechanism of its toxicity is not clear. However, some studies have shown that this metal can cause the formation of reactive oxygen species and inhibit the activity of antioxidant enzymes in the tissue. There is also evidence to suggest that lead toxicity causes lipid peroxidation, DNA damage, and



apoptosis. (Fu and Xi, 2020; Rebelo and Caldas, 2016; Massanyi et al., 2020; Charkiewicz and Backstrand, 2020). It has been discovered that lead toxicity causes cellular damage in two main ways by increasing free radical production. The first is to increase the production of reactive oxygen species, including superoxide, hydrogen peroxide, and peroxy radical, and the second is to cause a direct reduction of antioxidant reserves (Ercal et al., 2001). Therefore, this damage induced by lead may also cause adverse effects on testicular tissue (Huang et al., 2021). There are studies proving that lead has negative effects on the male reproductive system, such as decreased sperm motility and number, impaired testosterone hormone levels, and sperm quality (Hasanein et al., 2017; Huang et al., 2021).

Compounds with antioxidant characteristics, such as VitC and Zn, can be utilized alone or in combination to prevent oxidative stress in conditions where antioxidant enzymes are insufficient. VitC is a hydrophilic free radical scavenger that captures radicals in the aqueous phase and protects biomembranes from peroxidative damage. It reduces the formation of free radicals in many cellular processes caused by oxidative damage and the resulting lipid peroxidation (Hassan and Meligi, 2021). The protective effects of VitC on genotoxicity and cytotoxicity have been confirmed by *in vivo* studies with mice (Surjyo and Rahman, 2004; Song et al., 2006). In particular, VitC has proven to be of critical importance in semen content. Therefore, it has been found to increase sperm quality, prevent sperm agglutination, and increase sperm motility (Hajjar et al., 2020). Zn is one of the most important and vital trace elements needed by all living organisms for various physiological processes and has three basic biological roles: catalytic, structural, and regulatory (Hassan and Meligi, 2021). In the male reproductive system, Zn is essential for hormone metabolism and sperm production (Hajjar et al., 2020). Studies have shown that hormonal and anticancer drugs significantly reduce Zn levels in serum and testicular tissue (Maremanda et al., 2014). On the other hand, since Zn is an essential element required for maintenance of germ cells, progression of spermatogenesis, and regulation of sperm motility, supplementation with this element has been found to protect against ROS-induced testicular damage (Yamaguchi et al., 2009).

In this study, it is aimed to reveal the effect of vitamin C and/or zinc, which have an anti-oxidative effect against the toxicity of lead nitrate on Leydig cells, by examining cell viability, oxidative damage and antioxidant defense system parameters.

## **MATERIAL AND METHODS**

### **Cell Culture**

The TM3 Leydig cells used in the experiment are non-tumorigenic cells obtained from the testicular tissues of 11–13-day old mice. These cells were obtained from the American Type Culture Collection (Manassas, VA, USA). For Leydig cells, cell culture medium containing 50:50 DMEM-F12 supplemented with 5% horse serum and 2.5% fetal bovine serum was used. In an incubator, cells were incubated in sterile conditions at 37 °C with 5% CO<sub>2</sub> and 95% air. Different concentrations of lead nitrate (0.01-1.5 mM) were applied to TM3 Leydig cells for 24 h, and a 300 µM lead nitrate concentration, which reduced cell viability by up to 60%, was selected for use in the experiment. In addition, the concentration of VitC and Zn to be used for this study was determined as 50 µM, which is the concentration proven by *in vitro* studies to have antioxidant properties. In the last case, eight groups were designed in the experiment: control, lead, Zn, Zn+lead, VitC, Vitc+lead, Zn+VitC, Zn+VitC+lead.

### **Cell Viability**

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) kit (Roche, Germany) was used to determine the effects of lead, VitC, and Zn on Leydig cell viability. Cells were seeded at  $5 \times 10^3$  cells/well in 100  $\mu$ L of assay medium in a 96-well plate. After the incubation period, 10  $\mu$ l of MTT I solution was added to each well and the plates were incubated for 4 hours at 37°C in a CO<sub>2</sub> incubator. Then, 100  $\mu$ l of MTT II solution (SDS) was added to each well and incubated overnight in a CO<sub>2</sub> incubator. Absorbances were read using the ELISA instrument at a wavelength of 540 nm. The viability of the control cells untreated with the test substance was considered 100% and the viability of the lead, VitC, and Zn-treated groups was expressed as a percentage relative to the control, and the experiments were repeated three times.

#### **Preparation of Cells for Biochemical Analysis**

For biochemical analyses,  $1 \times 10^6$  cells were seeded in 6-well culture plates, and Leydig cells harvested after 24 h of exposure were transferred to cold tris-HCl buffer (pH:7.2). The cells were then sonicated in an ultrasonicator, and the cell membranes were disrupted. The resulting cell suspension was centrifuged to remove the supernatant. Obtained supernatants were used to measure total protein determination, lipid peroxidation, antioxidant enzymes activities (superoxide dismutase, catalase, glutathione peroxidase).

#### **Total Protein Determination**

A total protein determination was performed to determine the amount of protein obtained from the samples. The SMART™ protein assay kit was used for this procedure. The SMART™ protein assay is a bicinchoninic acid (BCA)-based method for the colorimetric detection and quantification of total protein. The results obtained were used in the calculation of biochemical analyzes.

#### **Determination of Membrane Lipid Peroxidation (MDA)**

Lipid peroxidation was measured with respect to malondialdehyde (MDA) content as in Heath and Packer's (1968) method. According to this method, MDA, one of the products of polyunsaturated fatty acid peroxidation in cells, reacts with thiobarbituric acid (TBA) for 20 min at a high temperature and acidic pH. At the end of this reaction, a pink compound is formed, and the amount of this compound is measured in the spectrophotometer at a wavelength of 532 nm.

#### **Determination of Superoxide Dismutase (SOD)**

The method of Marklund and Marklund (1974) was used to demonstrate the SOD enzyme. The aim of the experiment was based on the amount of SOD enzyme required for inhibition of pyrogallol autoxidation. The change in absorbance was recorded at 420 nm for 3 min at 30 s intervals on a spectrophotometer.

#### **Determination of Catalase (CAT)**

The method determined by Sinha (1972) was used for catalase activity. The aim of the experiment is based on the reduction of H<sub>2</sub>O<sub>2</sub> to chromic acetate by heating with dichromate/acetic acid reagent and the dark blue-violet precipitate in the assay mixture turning into a light green color with boiling. The obtained absorbances were measured calorimetrically at a wavelength of 570 nm.

#### **Determination of Glutathione peroxidase (GPx)**

The experimental method of Hafeman et al. (1974) was used for the determination of glutathione peroxidase. GPx catalyzes the decomposition of hydrogen peroxide in the presence of oxidized GSH and reduced GSH, forming water. The final absorbance of the test tubes and standard was read at 412 nm in a blank versus spectrophotometer containing only phosphate solution and DTNB reagent.

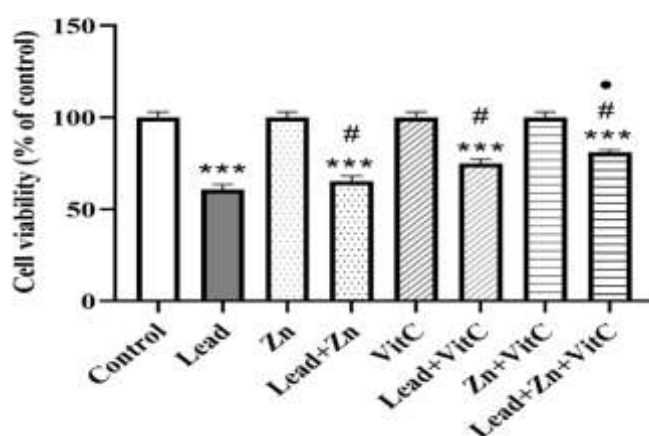
### Statistical Analysis

Statistical analysis was carried out using GraphPad Prism 6 software (GraphPad Software, San Diego, California, USA) and analysis of variance (ANOVA) was used for comparisons among all groups. Data distribution was tested for normality using the Shapiro-Wilk test. All data was calculated by a one-way ANOVA with Tukey's multiple comparisons test. The results were expressed as mean ± standard error and value of  $p < 0.05$  was considered statistically significant.

## RESULTS

### Cell Viability

The effect of lead on cell viability was investigated using the MTT kit after 24 h of administration of lead to TM3 Leydig cells with VitC and Zn separately and together. Obtained cell viability rates (%) are shown in figure 1. When the control and lead groups were compared in terms of MTT values, a significant decrease was observed in the group exposed to lead alone at the end of 24 h compared to the control group. A significant increase is observed in the lead+Zn, lead+VitC, and lead+Zn+VitC groups when the group exposed to lead alone is compared in terms of MTT values of Zn and/or VitC administered with lead. At the same time, a significant increase was observed when the groups in which lead and Zn were administered together and the Lead+Zn+VitC group were compared in terms of cell viability. Based on the cell viability data we obtained, it was understood that lead has a cytotoxic effect on Leydig cells. However, it has been determined that Zn and VitC can be used as effective antioxidants to improve this negative effect of lead on Leydig cells.

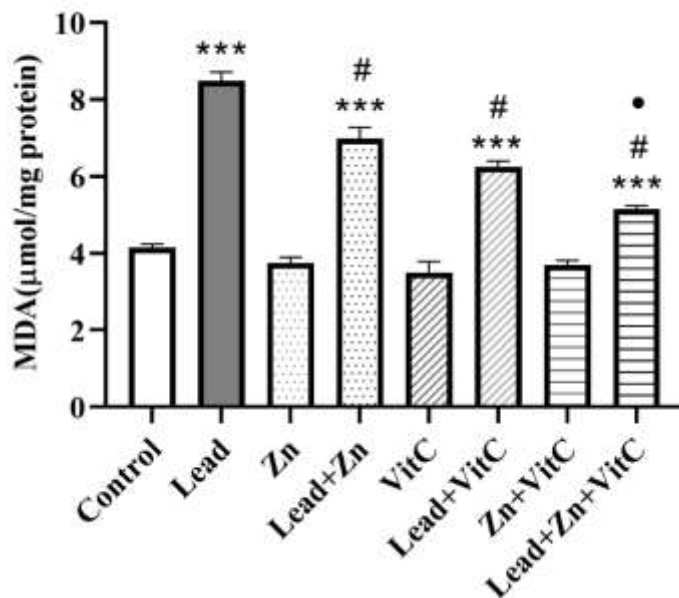


**Figure 1.** Effects of lead, VitC, and Zn on the viability rate in TM3 Leydig cells. (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*: compared with controls; #: compared with lead; •: compared with lead+Zn group).

### Lipid Peroxidation

The amounts of MDA calculated using the ELISA method after TM3 Leydig cells were exposed separately and together with lead, Zn, and VitC for 24 h are shown in figure 2. In terms of MDA amount, a significant increase was found in the lipid peroxidation rate when the control

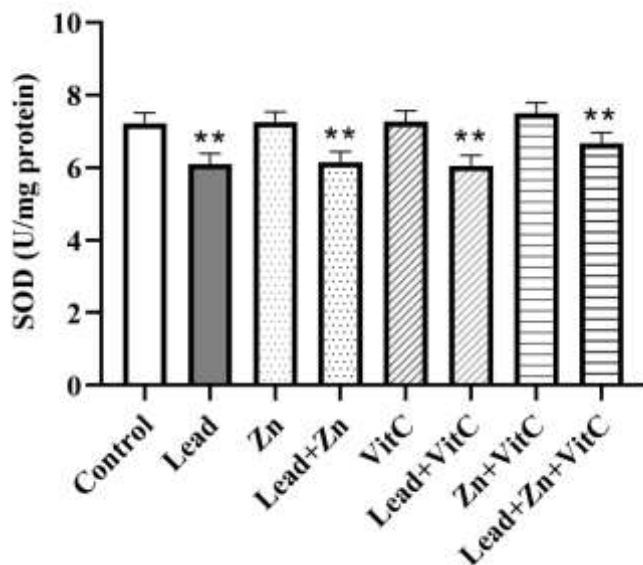
group and the group exposed only to lead were compared. A significant decrease was observed when the group exposed only to lead was compared in terms of Zn and/or VitC MDA values administered with lead. However, when the lead+Zn group and the lead+Zn+VitC group were compared in terms of MDA values, a significant decrease was detected. According to the lipid peroxidation data we obtained, it has been determined that the use of Zn and VitC together is more effective than the use of them separately in improving lead-induced lipid peroxidation in Leydig cells.



**Figure 2.** Effects of lead, VitC, and Zn on lipid peroxidation in TM3 Leydig cells. (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; #: compared with controls; #: compared with lead; •: compared with lead+Zn group).

### SOD Activity

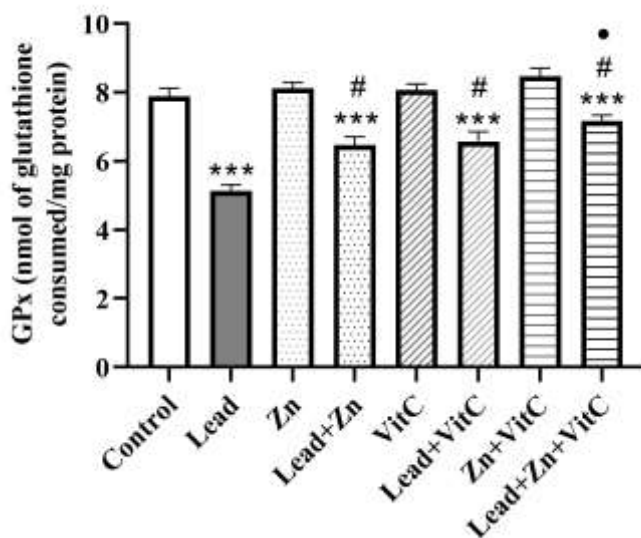
The changes in the amount of SOD enzyme calculated as a result of the administration of lead, VitC, and Zn separately and together for 24 h to TM3 Leydig cells are presented in figure 3. With regard to the amount of SOD enzyme, when the control groups and the groups exposed to lead were compared, a significant decrease was found in the lead-administered groups. When the lead group and the groups administered VitC and/or Zn in addition to lead were compared in terms of the amount of SOD enzyme, no significant difference was found.



**Figure 3.** Effects of lead, VitC, and Zn on SOD activities in TM3 Leydig cells. (\*\* $p < 0.01$ ; \* compared with controls).

#### GPx Activity

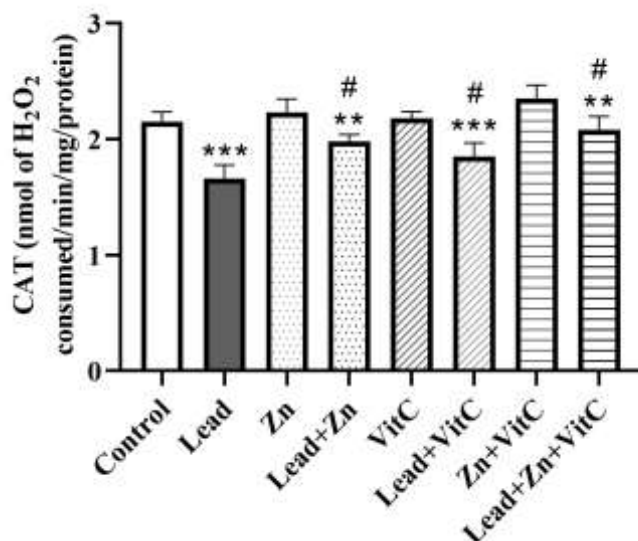
The amounts of GPx enzyme evaluated based on the amount of glutathione consumed in TM3 Leydig cells, which were treated with lead, VitC, and Zn for 24 h, are shown in Figure 4. According to the results, when the control group and lead groups were compared in terms of the amount of GPx enzyme, a significant decrease was observed in all lead groups. In addition, when the lead-administered group and the lead+Zn and/or lead+VitC administered groups were compared in terms of GPx enzyme amount, a significant increase was found. When the lead+Zn group and the lead+Zn+VitC group were compared in terms of GPx enzyme amount, it was determined that the combined administration of Zn and VitC caused a significant increase.



**Figure 4.** Effects of lead, VitC, and Zn on GPx activities in TM3 Leydig cells. (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; #: compared with controls; #: compared with lead; •: compared with lead+Zn group).

### CAT Activity

Figure 5 shows the CAT levels determined by administering lead, VitC, and Zn to TM3 Leydig cells for 24 h, either independently or together. The level of CAT enzyme was found to be significantly lower in all lead-administered groups when compared to the control groups. A considerable increase was observed in all experimental groups when the quantities of catalase enzymes in the lead group and the Zn and/or VitC groups provided in addition to lead were compared.



**Figure 5.** Effects of lead, VitC, and Zn on CAT activities in TM3 Leydig cells. (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; #: compared with lead).

### DISCUSSION

Many studies have been conducted to examine the harmful effects of lead and lead derivatives on the male reproductive system. When the *in vivo* studies are examined, it has been observed that lead reduces the amount of testosterone produced, causes a decrease in sperm count, viability and motility, and degeneration in the seminiferous tubules in the male reproductive system (Huang et al., 2021; Abdel-Emam and Ahmed, 2021; Hasanein et al., 2017). The protective effects of VitC and Zn, which are known to have antioxidant activity, on the male reproductive system have been confirmed by many *in vivo* studies. In a study conducted by Akorede et al. (2020) on mice, it was stated that VitC has a protective effect on lead toxicity. According to a study by Anjum et al. (2016), Zn supplementation significantly reduced lead-induced toxicity in male Wistar rats. In this study, the combined effect of VitC and Zn against the toxicity of lead on Leydig cells, which have an important role in the male reproductive system, was investigated for the first time.

There have been many studies investigating the effects of lead exposure on various cell lines. According to a study, it was determined that the administration of lead to human leukemia cell line at concentrations of 3.12  $\mu\text{g/mL}$  and higher for 24 hours significantly reduced cell viability (Yedjou et al., 2003). In a study by Liu et al. (2021), it was determined that lead administration at 10, 15, 20, 25, 30, 35  $\mu\text{M}$  concentrations to cultured kidney cells significantly reduced cell viability according to MTT findings. According to this study, it was reported that

VitC supplementation applied to cells with lead at concentrations of 60, 80, 100, 120, and 140  $\mu\text{M}$  reduced the negative effect of lead on cell viability. In our study, it was observed that lead at a concentration of 300  $\mu\text{M}$  caused a significant decrease in cell viability in Leydig cells, similar to the studies performed with other cell lines. However, it was determined that VitC and Zn in groups administered together were more effective in suppressing the cytotoxic effect of lead exposure.

Lead can induce oxidative stress by causing excessive production of reactive oxygen species (ROS) at the mitochondrial level, which causes damage to cellular components. The increase of reactive oxygen species such as superoxide anion and hydroxyl radical leads to the oxidation of membrane lipids. As a result of this oxidation, the level of MDA, which is the end product of lipid peroxidation, increases and cell membrane integrity is disrupted (Sani and Amanabo, 2021). According to an *in vivo* study in male rats, it was reported that lead exposure significantly increased the MDA level in testicular tissue (Abdel-Emam and Ahmed, 2021). In an *in vivo* study with chickens, a significant increase in oxidative stress and lipid peroxidation levels was observed in testicular tissue in lead-administered groups (Huang et al., 2021). In another *in vivo* study on the testicular tissue of male mice, it was determined that exposure to lead significantly increased the level of MDA. According to the same study, Zn supplementation applied to testicular tissue significantly reduced the damage caused by lead on testicular tissue (Zhang et al., 2021). In our *in vitro* study, it is paralleled with *in vivo* studies that lead applied at 300  $\mu\text{M}$  concentration causes lipid peroxidation in Leydig cells. At the same time, according to the data obtained, the use of VitC and Zn together is more effective than using them separately in protecting biological membranes from peroxidation and improving lipid peroxidation caused by lead exposure.

Many antioxidant enzymes play a role in protecting cells from the harmful effects of reactive oxygen species. Antioxidant enzymes such as SOD, CAT, and GPx are the main enzymes that play a role in providing oxidant/antioxidant balance. As a result of a decrease in the levels of these enzymes, oxidative stress occurs in cells (Walczak-Jedrzejowska et al., 2013). According to an *in vivo* study, it was determined that SOD levels were significantly reduced after 1000 ppm/kg oral lead administration to male mice (Soleimanzadeh et al., 2018). In another *in vivo* study, lead was administered to male Albino Wistar rats at 750 mg/kg, 1500 mg/kg and 2250 mg/kg concentrations. Lead administration significantly reduced testicular antioxidant enzyme activities (Ezejiolor et al., 2019). According to the study of Anjum et al. (2016), male Wistar rats were exposed to lead at a concentration of 819 mg/l and Zn at a concentration of 71 mg/l. There was a significant reduction in SOD and CAT activity levels in the testicles and epididymis of lead-exposed rats. However, no significant difference was observed in SOD and CAT levels in the Zn group (Anjum et al., 2016). In a study by Zhang et al. (2021), a 200 mg/l concentration of lead and a 15 mg/l concentration of Zn were administered orally to mice, either alone or together. As a result of the experiment, it was determined that SOD activity significantly decreased in lead groups and increased in Zn groups. In another *in vivo* study, rats were treated with lead alone at a concentration of 100 mg/kg or with 227 mg/kg of Zn in combination with lead. At the end of 8 weeks, a significant decrease in GPx and CAT levels was observed in the groups that were applied only to the lead. In addition, Zn was found to increase the levels of CAT and GPx enzyme activity (Babdhhu et al., 2006). According to the results of our study, lead decreased the SOD, CAT, and GPx enzyme activities in Leydig cells, while Zn and VitC significantly increased the activities of these enzymes and assumed a protective role in lead toxicity. When the group in which VitC and Zn were applied together, it was revealed that the combined effects of the mentioned antioxidant enzymes only on GPx were more effective in eliminating the harmful effects of lead.

In conclusion, Leydig cells, one of the main cells of the male reproductive system, were used as an *in vitro* model in this study. According to the findings of the study, lead exposure decreased cell viability, caused lipid peroxidation, and suppressed the activities of antioxidant enzymes in Leydig cells. Also, it is thought that the use of Zn and VitC separately, as well as their use together, may play a more active role in the improvement of oxidative damage caused by lead.

## FUNDING

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## DYNAMICS OF THE APHID POPULATION ON TOBACCO IN PRILEP REGION

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### ABSTRACT

Leaf aphids are among the most important pests on tobacco. They directly affect tobacco yield and quality. Investigation was carried out in 2017-2021 on tobacco plants in Prilep, by application of Method of survey of 20 randomly selected tobacco stalks infested with aphids. During the summer 2017-2021, aphids developed many parthenogenic generations of apterous aphids on tobacco, which depends primarily on temperature fluctuations and precipitation, as well as on the physiological state of the plant and soil nitrogen fertilization. Aphid infestations were often found first along the field margins nearest the direction of prevailing winds. Aphid colonization begins with the movement of few winged females into tobacco fields that give birth to live nymphs. These offspring will become mature, wingless aphids that in turn will deposit more live nymphs and make colonies on top tobacco leaves and flowers. *M. persicae* was present on tobacco plants from the beginning of July until the end of October. Following the dynamics of the aphid population in Prilep region in 2017-2021, the most intensive attack of aphids on tobacco occurs in August. The maximum incidence of aphids was on the 10th of August 2017, on the 20th of August 2018-2020 and on the 1st of August 2021, when aphids form large, dense colonies at the growing points. On the examined stalks, in 2017 were observed 70.707 aphids, 48.527 in 2018, 54.036 in 2019, 59369 in 2020 and 20738 aphids in 2021.

**Keywords:** Sunflower, Sustainable production, Drought tolerance, Hybrid, Yield traits, Yield performance,

### INTRODUCTION

The green peach aphid, *M. persicae*, is a highly polyphagous species, colonizing over 500 species of host plants from at least 40 different families (Blackman and Eastop, 2000, cit. Srigiriraju, 2008; Grigorov, 1979).

The holocycle of *M. persicae*, with sexual reproduction and overwintering of eggs on *Prunus*, occurs in the temperate regions of every continent, and although anholocycle is widespread in warm climates there are indications that the potential for sexual reproduction may be retained throughout the whole range of the species (Blackman, 2009).

During each annual cycle, cyclically parthenogenetic *M. persicae* aphids reproduce asexually several times on herbaceous plants (secondary hosts) and once sexually on peach trees -*Prunus persica* L. (Guillemaud et al. 2003).

In field conditions of Macedonia, it has a holocyclic life cycle where the sexual phase is completed on a peach and asexual phase occurs on tobacco and other secondary host species (Janusevska, 2001; Krsteska, 2007).

In addition to damaging the field, it easily attacks vegetables and ornamental plants grown in greenhouses (Krsteska, 2016).

Aphid diet causes damages on tobacco leaves and reduction of carbohydrates, soluble sugars and glucoses. They may also cause water stress and reduced growth rate of tobacco plant (Todoroski, 1965; Todoroski and Maceljki, 1983; Srigiriraju et al., 2010; Maric and Camprag, 1982).

The main goal of the investigations was to perform analysis of population dynamics of aphids in tobacco fields.

## MATERIAL AND METHOD

Investigations were carried out during 2017-2021, on tobacco plants in Prilep. The observations were made with application of Method of survey of 20 randomly selected tobacco stalks infested with aphids.

Tobacco stalks were sampled from the whole area of the trial at 10-days interval, starting from June 1, up to the beginning of October. The investigations were performed on parts of tobacco (leaves, tobacco flowers, seed capsules).

**Table 1.** Observation of tobacco leaves 2017-2021  
Method of survey of 20 tobacco stalks

| Date of control | N <sup>o</sup> of tobacco leaves/year |             |             |             |             |
|-----------------|---------------------------------------|-------------|-------------|-------------|-------------|
|                 | 2017                                  | 2018        | 2019        | 2020        | 2021        |
| 01.07.          | 326                                   | 287         | 257         | 221         | 247         |
| 10.07.          | 362                                   | 351         | 362         | 376         | 356         |
| 20.07.          | 517                                   | 501         | 505         | 498         | 508         |
| 01.08.          | 598                                   | 536         | 566         | 562         | 561         |
| 10.08.          | 642                                   | 611         | 602         | 623         | 622         |
| 20.08.          | 699                                   | 678         | 692         | 687         | 658         |
| 01.09.          | 724                                   | 713         | 717         | 705         | 679         |
| 10.09.          | 655                                   | 619         | 609         | 613         | 601         |
| 20.09.          | 617                                   | 596         | 587         | 520         | 515         |
| 01.10.          | 587                                   | 567         | 569         | 548         | 534         |
| <b>Total</b>    | <b>5727</b>                           | <b>5459</b> | <b>5466</b> | <b>5353</b> | <b>5281</b> |

10 checks were made by this method in each of the years of investigations, i.e. 200 stalks per year, or 1000 stalks in total.

The investigation included a total of tobacco leaves (5727 in 2017, 5459 in 2018, 5466 in 2019, 5353 in 2020, 5218 in 2021).

## RESULTS AND DISCUSSION

The aphid attack commercial varieties of *Nicotiana tabacum* L. and they appear in all tobacco producing regions in Macedonia.

During investigation of the species of Aphididae family, tobacco was attacked only by *Myzus persicae* Sulzer (Figure 1).



**Figure 1.** Aphid colonies on top tobacco leaves and flowers

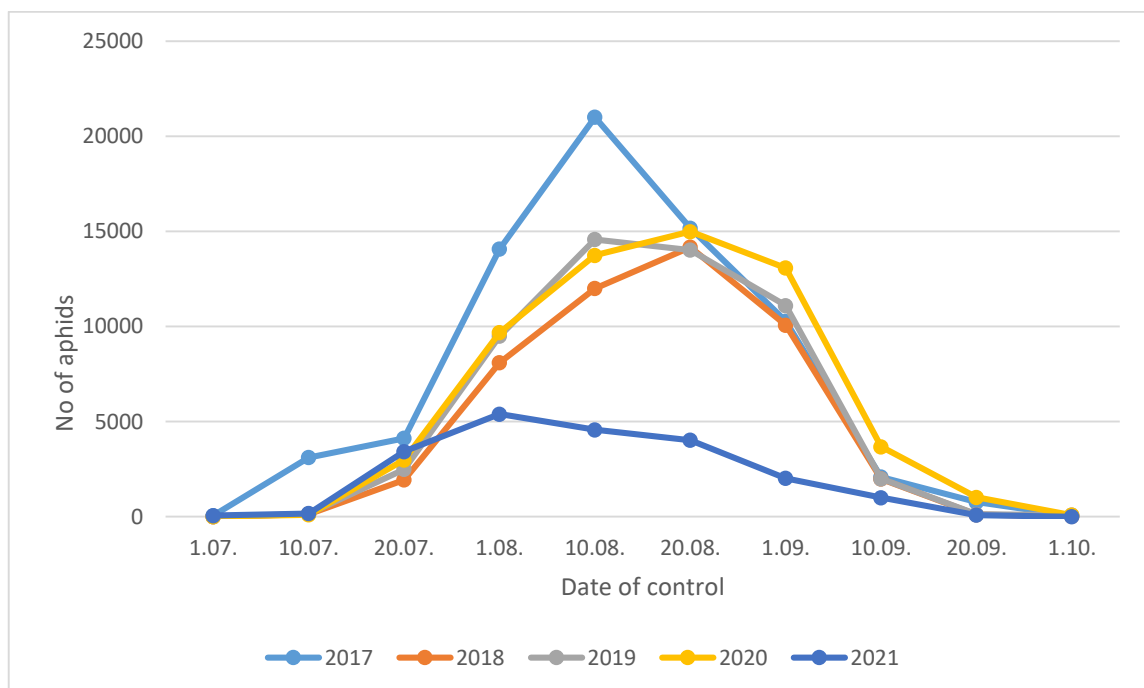
*M. persicae* developed many parthenogenic generations of apterous aphids on tobacco, which depends primarily on temperature fluctuations and precipitation, as well as the physiological state of the plant and soil nitrogen fertilization.

Aphid infestations were often found first along the field margins nearest the direction of prevailing winds. Aphid colonization begins with the movement of few winged females into tobacco fields that give birth to live nymphs. These offspring will become mature, wingless aphids that in turn will deposit more live nymphs and make colonies on top tobacco leaves (Figure 2) and flowers.



**Figure 2.** Aphid collonies on tobacco leaves

Following the dynamics of the aphid population in Prilep tobacco producing region during 2017-2021, the most intensive attack of aphids on tobacco occurs in August (Graph. 1). *M. persicae* was present on tobacco plants from the beginning of July to the beginning of October.



**Graph. 1** Dynamics od aphid population on tobacco

Climate conditions lower the plant defense system against insect pests, and cause tobacco plant to be more vulnerable to pests attack.

Aphids were identified in large quantitative representation in 2017, 70.707 aphids were found on the examined stalks (Table 1). In 2018 aphid population decreased and 48.527 aphids were observed. In 2019 a slightly higher population of aphids was determined compared to the previous year i.e. 54.036 aphids. This increasing trend continued in the following year and 59369 aphids were found in 2020. Unfavorable climatic conditions caused the development of a smaller population of aphids in 2021 when a total of 20738 aphids were determined.

The maximum incidence of aphids was on the 10th of August 2017, on the 20th of August 2018-2020 and on the 1st of August 2021, when aphids form large, dense colonies at the growing points on tobacco plants.

*M. persicae* make colonies on young tobacco leaves, buds and flowers and in strong attack, they are dried and covered with an abundance of honeydew, which is populated with black sooty mold. Infected tobacco plants lag behind in growth and are susceptible to attack by other plant pests and pathogens.

On tobacco leaves 2017-2021 the apterous (wingless) aphids have various shades of green, orange, red or yellow color with oval body, approximately 2.15 mm long (Figure 3).



**Figure 3.** Wingless aphids on tobacco

This color morphism in *M. persicae* results from the presence of a series of glycosides in the aphid hemolymph (Blackman, 1974).

According to Capinera (2020) the wingless (apterous) aphids are yellowish or greenish in color. They measure about 1.7 to 2.0 mm in length. A medial and lateral green stripes may be present. The cornicles are moderately long, unevenly swollen along their length, and match the body in color. The appendages are pale.

According to Blackman, Eastop (2017) adult apterous parthenogenetic female are small to medium sized, pale greenish-yellow or various shades of green, pink red or almost black.

On tobacco leaves nymphs resemble parthenogenetic, apterous aphids and their color is green, yellow or red (Figure 4).



**Figure 4.** Nymphs on tobacco leaves

According to Capinera (2020) nymphs initially are greenish, but soon turn yellowish, greatly resembling viviparous (parthenogenetic, nymph-producing) adults. The nymphs that give rise to winged females (alatae) may be pinkish.

According to Blackman, Eastop (2017) immature female alatae are often red or pink, and immature males are always some shade of yellow or yellow-green.

As aphid densities on tobacco increase 2017-2021, winged forms are produced to aid dispersal. Alate aphids have a black head and black-redish thorax, and a yellowish green abdomen with a large dark patch dorsally. Their body is oval and although they looked bigger than apterous aphids (because of the wings) they measure approximately 2.05 mm in length.

Capinera (2020) winged (alate) aphids have a black head and thorax, and a yellowish green abdomen with a large dark patch dorsally. They measure 1.8 to 2.1 mm in length.

**Table 2.** Quantitative representation of aphid population on tobacco

| Date of control | N <sup>o</sup> of aphids/year |              |              |              |              |
|-----------------|-------------------------------|--------------|--------------|--------------|--------------|
|                 | 2017                          | 2018         | 2019         | 2020         | 2021         |
| 01.07.          | 54                            | -            | -            | 7            | 56           |
| 10.07.          | 3111                          | 132          | 154          | 98           | 167          |
| 20.07.          | 4120                          | 1938         | 2488         | 2987         | 3421         |
| 01.08.          | 14072                         | 8098         | 9492         | 9678         | 5397         |
| 10.08.          | 21005                         | 12004        | 14574        | 13745        | 4567         |
| 20.08.          | 15176                         | 14176        | 14023        | 14990        | 4021         |
| 01.09.          | 10272                         | 10073        | 11098        | 13082        | 2021         |
| 10.09.          | 2090                          | 1982         | 1995         | 3678         | 1001         |
| 20.09.          | 766                           | 107          | 127          | 1021         | 87           |
| 01.10.          | 41                            | 17           | 85           | 83           | -            |
| <b>Total</b>    | <b>70707</b>                  | <b>48527</b> | <b>54036</b> | <b>59369</b> | <b>20738</b> |

## CONCLUSIONS

During investigation of the species of Aphididae family, tobacco was attacked only by *M. persicae*. It has high potential for reproduction and development.

*M. persicae* was present on tobacco plants from the beginning of July until the beginning of October. The most intensive attack of aphids on tobacco occurs in August when aphids form large, dense colonies at the growing points of tobacco plants.

In tobacco biocenosis in the region of Prilep 2017-2019, *M. persicae* developed many generations with high quantitative representations of aphids. Due to the unsuitable climate conditions in 2021, the number of its generations on tobacco was reduced.

On the examined stalks, in 2017 were observed 70.707 aphids, 48.527 in 2018, 54.036 in 2019, 59369 in 2020 and 20738 aphids in 2021.



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## **HIGH HYDROSTATIC PRESSURE (HHP) PROCESSING STRATEGIES ON ANTIOXIDANT PHENOLIC BIOACTIVE COMPONENTS IN FOODS AND BEVERAGES**

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### **ABSTRACT**

Phenolic compounds, especially flavonoids have health-promoting benefits that play some important roles in foods as visual appearance, taste, aroma and represent an abundant antioxidant component of the human and animal diet. High hydrostatic pressure processing (HHPP) conditions (300–700 MPa) at moderate initial temperatures (around ambient) are generally sufficient to inactivate vegetative pathogens for pasteurization processes, some enzymes, or spoilage organisms to extend the shelf-life. The aim of the review is to reveal the effect of high hydrostatic pressure processing strategies on the retention of antioxidant phenolic bioactives in foods and beverages. HHPP can increase extraction capacity of phenolic constituents, and ensure higher levels of preserved bioactive constituents. High pressure extraction (HPE) can shorten processing times, provide higher extraction yields while having less negative effects on the structure and antioxidant activity of bioactive constituents. HPE enhances mass transfer rates, increases cell permeability, increases diffusion of phenolics and retains higher levels of bioactive compounds. Total phenolics in HHPP-treated foods were either unaffected or actually increased in concentration and/or extractability following treatment with high pressure.

**Keywords:** HHP, Plant Foods, Plant Beverages, Phenolics, Bioactives

### **INTRODUCTION**

Emerging non-thermal processing technologies are becoming widespread in the food industry, mainly as post-packaging interventions for food safety assurance and stabilization of all-natural, preservative-free propositions. Techniques such as HPP prove to be effective and economically feasible, showing now consistent double-digit growths. HP in comparison to sterilization can give an energy saving of around 20% and can be applied in packaged foods, eliminating possible recontamination after the treatment. Various studies have shown that HP treatment can alter meat structure, color, and lipid oxidation levels (Tokusoglu and Swanson, 2015).

High-pressure processing (HPP) is a nonthermal processing method that holds promise for retaining wholesomeness and freshness of the processed food products. HPP is an emerging technology that can be used instead of thermal process for pasteurization and sterilization. Recent work provides studies to illustrate the ability of this nonthermal food preservation

technology, regarding the preservation of the phenolic bioactives of plant foods and health-related compounds (Tokusoglu and Swanson, 2015).

## **PHENOLICS AS BIOACTIVE COMPOUNDS**

Phenolic compounds occur as plant secondary metabolites. Their ubiquitous presence in plants and plant foods, favors animal consumption and accumulation in tissues. Polyphenols are widely distributed in the plant kingdom and represent an abundant antioxidant component of the human diet. Interest in the possible health benefits of polyphenols has increased due to the corresponding antioxidant capacities (Tokusoglu and Hall, 2011; Tokusoglu 2001).

Recent evidence shows that there is a great interest to anticarcinogenic effects of polyphenolic compounds, as well as the potential to prevent cardiovascular and cerebrovascular diseases. As the name suggests, phytochemicals working together with chemical nutrients found in fruits, cereals, and nuts may help slow the aging process and reduce the risk of many diseases, including cancer, heart disease, stroke, high blood pressure, cataracts, osteoporosis, and urinary tract infections (Cheynier, 2005; Meskin et al., 2003; Tokusoglu and Hall, 2011).

Polyphenols divide into several subgroups including flavonoids, hydroxybenzoic and hydroxycinnamic acids, lignans, stilbenes, tannins, and coumarins that have specific physiological and biological effects (Andersen and Markham, 2006; Meskin et al., 2003; Tokusoglu 2001);

Flavonoids are a major group of polyphenols that include flavan-3-ols, flavonols, flavones, flavanones, isoflavones, anthocyanidins, anthocyanins, flavonols, and chalcones as subgroups which are distributed in plants and foods of plant origin. Recently, there has been an increasing interest for nonthermal technologies as HPP to preserve fruits, vegetables, daily foods, and beverages (Barbosa-Cánovas et al., 1998). Great technological and research efforts have been made to obtain foods and beverages by HPP without the quality and nutritional damage caused by heat treatments. HPP or ultra-HPP or HPP is one technology that has begun to fulfill its potential to satisfy both consumer and scientific requirements, and is a leading alternative in replacing thermal processing in some food applications in the drive to meet increasing consumer demand for foods featuring improved organoleptic qualities and higher acceptance (Patterson et al., 2008; Bevilacqua et al., 2010; Tokusoglu and Doona, 2011a).

HPP can be used to obtain a high-quality food/beverage and increases its shelf life while maintaining its physicochemical, nutritional characteristics, and bioactive profiles (Tokusoglu, 2011; Tokusoglu, 2012a,b; Tokusoglu and Doona, 2011a,b; Tokusoglu et al., 2010).

The technology is especially beneficial for heat-sensitive products (Barbosa-Cánovas et al., 2005; Tokusoglu and Doona, 2011). HPP can be conducted at ambient or moderate temperatures, thereby eliminating thermally induced cooked off-flavors. Compared to thermal processing, the HPP of foods results in products with a fresher taste, better appearance, and texture. Foods are processed in batch (for solid products) or continuous and semicontinuous systems (for liquid products) in a pressure range of 50–1000 MPa; process temperature during

pressure treatment can be from below 0°C to above 100°C, while exposure time usually ranges from seconds to 20 min (Bevilacqua et al., 2010; Corbo et al., 2009; Patterson et al., 2008).

HPP technology has been successfully applied in several industrial sectors such as meat, seafood, dairy food, fruit juices, fruit, and vegetable products (Figure 2.4). HPP has been found to inactivate several microorganisms and enzymes. However, it has less effect on low-molecular-weight food components such as vitamins, pigments, flavoring agents, and other nutritional compounds. HPP conditions in the range of 300–700 MPa at moderate initial temperatures (around ambient) are generally sufficient to inactivate vegetative pathogens for pasteurization processes, some enzymes, or spoilage organisms to extend shelf life. HPP can also increase the extraction capacity of phenolic constituents, and higher levels of bioactive compounds and phytochemicals are preserved in HPP-treated samples (Tokusoglu and Doona, 2011).

Consumer perception of food quality depends not only on microbial quality but also on other food factors such as biochemical and enzymatic reactions and structural changes. In this context, HPP can have an effect on food yield and on sensory qualities such as food color and texture. High pressures (HPs) can also be used to enhance extraction of compounds from foods. Recent studies have shown that high-pressure extraction (HPE) can shorten processing times, and provide higher extraction yields while having less negative effects on the structure and antioxidant activity of bioactive constituents. The use of HPE enhances mass transfer rates, increases cell permeability, and increases diffusion of secondary metabolites (Cheftel, 1995; Tokusoglu and Doona, 2011).

HHP increases the dissolution rate of the bioactives. A rapid permeation is observed under HPE owing to the large differential pressure between the cell interior and the exterior of cell membranes (Zhang et al., 2005). This situation increases the solvent penetration through the broken membranes into cells or increases the mass transfer rate due to increased permeability (Shouqin et al., 2005). This means the higher the hydrostatic pressure is, the more solvent can enter into the cell. More compounds can permeate the cell membrane that could cause the higher yield of extraction (Tokusoglu et al., 2010; Tokusoglu and Doona, 2011; Zhang et al., 2005; Shouqin et al., 2005).

## CONCLUSION

As a result, the extraction capacity of phenolic constituents has been increased by HHP and HPP-treated samples that retain higher levels of bioactive compounds. Studies on HPP effects on total phenolics determined that these compounds were either unaffected or actually increased in concentration and/or extractability, following treatment with HPP. HPP is an excellent food-processing technology that has the potential to retain the bioactive constituents with health properties in plant foods. HPP-treated foods retain more of their fresh-like features and can be marketed at a premium over their thermally processed counterparts.

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## INFLUENCE OF THE STALLION'S ORIGIN ON SPERM MOBILITY AND MORPHOLOGICAL ABNORMALITIES IN THAWED SEMEN

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### ABSTRACT

The objective of the study was to evaluate the post-thawed semen quality of oriental and occidental Arabian stallions raised in Tunisia. A total of 11 Arabian stallions were assigned into 2 groups according to their origin (5 oriental and 6 occidental). The stallion's semen was collected and frozen in the centre of equine semen production of the National Foundation for the Improvement of the Horse Breed (2 ejaculates /stallion, n=22). A sample of 4 straws per ejaculate was chosen randomly to study the quality of semen after thawing. For this purpose, the percentages of mobile and abnormal spermatozoa were determined. ANOVA was carried out using the software SAS (SAS Institute Inc.<sup>®</sup>). Results showed that the percentage of mobile spermatozoa in thawed semen was higher in the occidental stallions compared to the oriental ones (64% vs 40%, p<0.05). However, the percentage of abnormal spermatozoa was higher in oriental stallions compared to the occidental ones (27% vs 15%, p<0.05). Moreover, the percentages of mobile and abnormal spermatozoa also varied according to stallions (p<0.05). But, statistical analysis didn't show any variation between ejaculates from the same stallion and even between straws from the same ejaculate (p>0.05). In conclusion, our results suggest that the quality of thawed semen is better in the occidental stallions. Based on these findings, it seems that the semen of occidental Arabian stallions endures the effects of the freezing – thawing process.

**Keywords:** semen, quality, thawed, oriental, occidental, Arabian stallions

### INTRODUCTION

Progress in the process of frozen – thawed stallion semen was reached regarding the improvement of the composition of extenders and types of cryoprotectants, the use of new artificial techniques such as the deep one which was associated with the use of small number of spermatozoa. This progress led interest results in fertility in horse breed (Alvarenga et al., 2016). Despite the advances in the freezing –thawing processes and sperm technology, an important proportion of stallions still not suitable for artificial insemination using frozen – thawed semen. In addition, other factors could affect the quality and thus the fertility of post – thawed semen stallion. The most cited ones were the considerable variation between individual stallions (Katila, 2001) and breed (Greiser et al., 2020). In this study, semen frozen – thawed was evaluated for sperm mobility and morphology of oriental and occidental Arabian stallions.

## MATERIAL AND METHODS

Semen of 11 Arabian stallions (oriental, n=5; Occidental, n=6) were collected in the centre of equine semen production of the National Foundation for the Improvement of the Horse Breed (FNARC). Two ejaculates /stallion (n=22) were frozen according to the method of Haras Nationaux (2004). For semen evaluation, 4 straws /ejaculate (total: 88) were chosen randomly and thawed in a water bath at 37°C during 30 sec. After thawing, semen was diluted in the INRA Freeze extender. Then, the percentage of mobile sperm was determined using a contrast microscope (x40). The abnormal sperm was studied using an eosin-nigrosin stain and counting a sample of 150 spermatozoa over the slide. ANOVA was carried out using the software SAS (SAS Institute Inc.®). The GLM procedure was used to study the stallion's origin and individual effects on mobile spermatozoa and abnormal sperm.

## RESULTS AND DISCUSSION

The analysis of variance showed stallions' origin and individual effects on the percentages of mobile and abnormal sperm (Table 1).

**Table 1.** Results of the analysis of variance of qualitative semen parameters

|           | ddl | Percentage of mobile sperm (%) | Percentage of abnormal sperm (%) |
|-----------|-----|--------------------------------|----------------------------------|
| Stallion  | 10  | *                              | *                                |
| Origin    | 1   | *                              | *                                |
| Ejaculate | 1   | <i>ns</i>                      | <i>ns</i>                        |
| Straw     | 3   | <i>ns</i>                      | <i>ns</i>                        |

\* :  $p < 0.05$  *ns*: not significant

The percentages of mobile and abnormal spermatozoa varied according to stallions ( $p < 0.05$ , Table 2). But, statistical analysis didn't show any variation between ejaculates from the same stallion and even between straws from the same ejaculate ( $p > 0.05$ ). Indeed, individual differences were showed in previous studies (Kuisma et al., 2006; Najjar et al., 2009). Vidament et al. (2005) reported that there are stallions who their semen can not handle the freezing-thawing process.

**Table 2.** Results of the sperm mobility and morphological abnormalities according to stallions (Means±SD)

| Origin     | Stallion    | Percentage of mobile sperm (%) | Percentage of abnormal sperm (%) |
|------------|-------------|--------------------------------|----------------------------------|
| Oriental   | Stallion 1  | 73.7±7.5                       | 18.0±0.8                         |
|            | Stallion 2  | 28.7±2.5                       | 27.0±12.4                        |
|            | Stallion 3  | 41.2±13.1                      | 25.2±6.8                         |
|            | Stallion 4  | 25.0±12.9                      | 26.0±7.3                         |
|            | Stallion 5  | 30.0±8.1                       | 40.0±9.5                         |
| Occidental | Stallion 6  | 58.7±6.2                       | 12.2±2.5                         |
|            | Stallion 7  | 77.5±3.5                       | 25.5±0.7                         |
|            | Stallion 8  | 62.5±9.5                       | 21.5±3.3                         |
|            | Stallion 9  | 57.5±9.5                       | 11.2±0.9                         |
|            | Stallion 10 | 77.5±5.0                       | 11.5±1.9                         |
|            | Stallion 11 | 42.5±3.5                       | 14±4.2                           |



The percentage of mobile spermatozoa in thawed semen was higher in the occidental stallions compared to the oriental ones (64% vs 40%,  $p < 0.05$ , Table 3). The percentage of abnormal spermatozoa was higher in the oriental stallions compared to the occidental ones (15% vs 27%,  $p < 0.05$ , Table 2). Despite the poor quality found in oriental stallions, the results showed that the semen quality of oriental stallions is acceptable because the percentages of mobile and abnormal sperm were more than 30% and less than 50% respectively (Najjar et al., 2010).

**Table 3.** Results of the sperm mobility and morphological abnormalities according to stallion's origin (Means±SD)

|                                  | Oriental stallions     | Occidental stallions   |
|----------------------------------|------------------------|------------------------|
| Percentage of mobile sperm (%)   | 40.0±18.2 <sup>a</sup> | 64.0±12.9 <sup>b</sup> |
| Percentage of abnormal sperm (%) | 27.0±10.6 <sup>a</sup> | 15.5±5.7 <sup>b</sup>  |

*a, b : p < 0.05*

### CONCLUSIONS

The study suggested that the quality of thawed semen is better in the occidental stallions. Based on these results, it seems that the semen of occidental Arabian stallions endures the effects of the freezing – thawing process.

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## **CIELAB COLOR PARAMETERS AND PIGMENT CONTENTS IN VIRGIN OLIVE OIL FROM DIFFERENT AREAS OF PRODUCTION IN ALBANIA**

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### **ABSTRACT**

Color makes a significant impression on the consumer and has a major effect on the acceptance of the oil due to the current association of green color with high quality oils, and pale-yellow color with refined oils. The color of olive oil is due to two types of pigments, chlorophylls and carotenoids, which draw the attention of the scientist due to the probable health benefits that they can provide. The chlorophyllic and carotenoids compositions of virgin olive oil are parameters of authenticity and quality. The aim of this paper is to characterize quality parameters (acidity value, peroxide index and specific extinction coefficient of  $K_{232}$  and  $K_{270}$ ), pigments content, and CIELAB color parameter that contribute to the composition of virgin olive oil. The material used for this experiment consisted of 13 samples of virgin olive oil, collected from different areas of cultivation in Albania, during the crop season of 2020-2021. Olive oils from coastal region (V, H1, H2 and S) were the richest in chlorophylls and carotenoids, ranging from 6.79 – 8.65 mg/kg and 8.12 – 9.38 mg/kg, respectively. Color analysis showed luminosity values  $L^*$  are close to each other, with a range from 29.99 to 32.27. The  $-a^*$  describing the greenness of the oil fluctuated from 0.7 – 1.31, meaning that olive oil sample B2 is greener than the others. The  $b^*$  value, describing the yellowness of the oil, ranged from – 0.07 to 4.54, meaning that olive oil sample V is more yellow than the others. Geographical location is a prevailing factor that in conjunction with other factors impacts both the color parameter and pigment content in virgin olive oil.

**Keywords:** CIELAB parameters, Chlorophylls, Carotenoids, Virgin Olive Oil

### **INTRODUCTION**

Color and appearance constitute the first contact we have with food, by determining our preferences and influencing our choices. Concerning the olive fruit, its color changes from deep green to yellow-green and then purple and black as it grows, ripens, and matures. The green color of the tissue is caused by chlorophyllic and carotenoids pigments, and as their concentrations fall progressively during ripening, it gives way to the synthesis of anthocyanins compounds (Aparicio R. and Harwood J. 2013). The color pigment plays an important role in oxidative stability during storage and in the preservation of oil quality, as a result of its antioxidant characteristics and pro-oxidant roles under light. The pigments are used as indicators of olive oil freshness and/or storing conditions; for example, a low amount of pheophytins indicates a fresh, well-stored olive oil, whereas a high amount indicates old bad-stored oil (Giuliani A. et al., 2011; Cayuela J.A. et al., 2014).

Pigments are divided into two main groups in food, namely carotenoids and chlorophylls. Chlorophylls are responsible for the greenish color of extra virgin olive oil. On the other side,  $\beta$ -carotene, lutein, zeaxanthin and xanthophylls called carotenoids are natural antioxidants found in various amounts in olive oil. They have beneficial properties for human

health. For example,  $\beta$ -carotene appears to lower the risk of various heart diseases, while lutein protects the eye retina from oxidative damage. Some studies have shown that carotenoids exhibit bioactivities in photosynthesis, possess special antioxidant properties and produce improvements in cognitive function as well as cardiovascular health (Tapiero H. et al., 2004; Fiero J. and Bra K. 2014; Eggersdorfer M. and Wyss A. 2018).

The primary feature of chlorophylls is derived from their chromophoric capacity, which is responsible for the highly valued green color that the consumer often associates with something being freshly prepared. In both in vitro tests and in vivo tests using animal models, chlorophyllic compounds have demonstrated antioxidant (Ferruzzi et al., 2002) and antimutagenic activities (Dashwood et al., 1998), modulating action of the xenobiotic enzymes (Fahey et al., 2005) and induction of apoptic events in cancer cell lines (Chan et al., 2006). During food conservation, chlorophyll degradation may continue until oxidation leading to a colorless products, a process that may involve different oxidative enzymatic systems (Gross 1991; Holden 1965; Minguez-Mosquera et al., 1990). In light conditions, a series of radicalary reactions are unleashed, leading to the destruction of the chlorophyll (Gutierrez-Rosales et al., 1992).

It is well known that the methodological bases set out by the Commission International d'Eclairage (International Commission on Illumination) (CIE 1978) state that all the colors can be represented on the CIE chromatic diagram.  $L^*$  is an estimation of the luminosity, which allows any given color to be viewed as equivalent to a member of the gray scale, between black ( $L^* = 0$ ) and white ( $L^* = 100$ ) on the y-axis, where  $a^*$  is positive for reddish colors and negative for the greenish ones on the x-axis and  $b^*$  is positive for yellowish colors and negative for the bluish ones on the z-axis. The  $a^*$  value correlates with the green pigment content, while the  $b^*$  with that of the carotenoids, so that the  $a^*/b^*$  ratio correlates with the chlorophyll/carotenoid ratio. From  $a^*$  and  $b^*$ , the psychological parameters chroma ( $C^*$ ) and hue ( $h^*$ ) are also defined, where  $C^*$  allows the user to determine for each individual hue its corresponding degree of difference in comparison to a gray color with the same lightness. Chroma is considered to be the quantitative attribute of colorfulness. The brightness  $L^*$  decreases with the decrease in chlorophyll pigments in the oil (Escolar et al., 1994; Cerretani et al., 2008).

The aim of this paper is to characterize quality parameters (acidity value, peroxide index and specific extinction coefficient of  $K_{232}$  and  $K_{270}$ ), pigments content, and CIELAB color parameter that contributes to the composition of virgin olive oil, collected from major areas of olive oil production in Albania.

## **MATERIAL AND METHOD**

This study was performed on 12 samples of olive oil, collected during the crop season 2020-2021 from the following areas of cultivation and production: Tirana, Elbasan, Fier, Vlore, Himare, and Saranda.

### *Analytical Determination of the Quality Indices*

Free acidity, peroxide value (PV), and UV spectrophotometric indices ( $K_{232}$  and  $K_{270}$ ) were determined according to the analytical methods described in EEC Regulation 2568/91 and the following amendments. Free acidity was given as a percentage of oleic acid and PV was expressed in milliequivalents of active oxygen per kilogram of oil (mequiv  $O_2/kg$ ). Spectrophotometric determinations were made using a spectrophotometer UV-vis (Biochrom Libra S22). The  $K_{232}$  and  $K_{270}$  extinction coefficients were calculated from absorption at 232 and 270nm, respectively. Oil samples were previously diluted (1:10 v/v) in cyclohexane when

the  $K_{232}$  and  $K_{270}$  extinction coefficients were analyzed. All parameters were determined in triplicate for each sample.

#### *Spectrophotometric estimation of total pigments*

The total chlorophyll and carotenoids contents were calculated using the spectrophotometric method, at 670 and 470nm respectively. The absorption at 670nm is usually considered to be related to the chlorophyll fraction (pheophytin 'a' as its major component) and 470nm to the absorption of the carotenoid fraction (lutein). Samples of olive oil (7.5 g) were accurately weighted and dissolved in 25mL of cyclohexane, and their concentrations were calculated using the absorbance value and specific extinction coefficients (613 for pheophytin'a' and 2000 for lutein) according to Minguez-Mosquera et al. All the absorbance measurements were performed in a spectrophotometer UV-vis (Biochrom Libra S22). Three replicates were prepared and analyzed for each individual sample.

#### *CIELAB color parameters*

A high quality colorimeter (NH 310, 3NH Technology Co.Ltd.) was used to assess the oil color and the CIELAB colorimetric system was applied. The oil samples were examined without dilution to avoid color variation. Color was expressed using the chromatic coordinates  $L^*$ ,  $a^*$ ,  $b^*$ ,  $C^*$ ,  $h^*$ , adopted by the CIE in 1976 where  $L^*$  is the brightness varying from 0 to 100, while  $a^*$  from green to red and  $b^*$  from blue to yellow are two chromatic components ranging between -120 and 120.

## **RESULTS AND DISCUSSION**

In this work, olive oil samples, collected from main regions where olive trees are cultivated, including central areas (Tirana, Elbasan, Fier and Berat) and coastal areas (Vlore, Himare dhe Sarande) in Albania during the crop season 2020-2021, were investigated in order to determine physicochemical parameters, pigment contents and their color parameters, through the use of the CIELAB system.

The results depicted in Tab.1 showed the free acidity, peroxide value and UV Spectrophotometric indices  $K_{232}$  and  $K_{270}$  of mono-cultivar olive oils. Free acidity content is one of the most important parameters featuring the quality of an olive oil and it is often determined to classify and/or evaluate oil (Mariotti et al., 2001). It is a measurement of hydrolytic breakdown of the fatty acid chains from triglycerides into diglycerides and monoglycerides liberating free fatty acids (Vossen 2007). Acidity values range from 0.23% oleic acid (E1) to 0.78% oleic acid for samples (T1) and (F). The acidity values presented in Table 1 indicated that the acidity levels of olive oil in the different studied regions are in accordance with the standards established by International Olive Council (IOC) and the European Regulation (0.80%) for oils to be considered as extra virgin olive oil.

The values for the determination of the UV absorption coefficients ( $K_{232}$  and  $K_{270}$ ) of conjugated bonds provide information on the presence or absence of precursors or on the onset of oxidation and thus on the prediction of the oil stability (Hadj et al., 2018). The results in table 1 showed that  $K_{232}$  fluctuated from 0.659 (B1) to 1.2 (S) and  $K_{270}$  from 0.042 (T2) to 0.101 (S) and all the samples analyzed are characterized by  $K_{270} \leq 0.22$  and  $K_{232} \leq 2.50$  respecting the standard established by the IOC for category of 'extra virgin' olive oil.

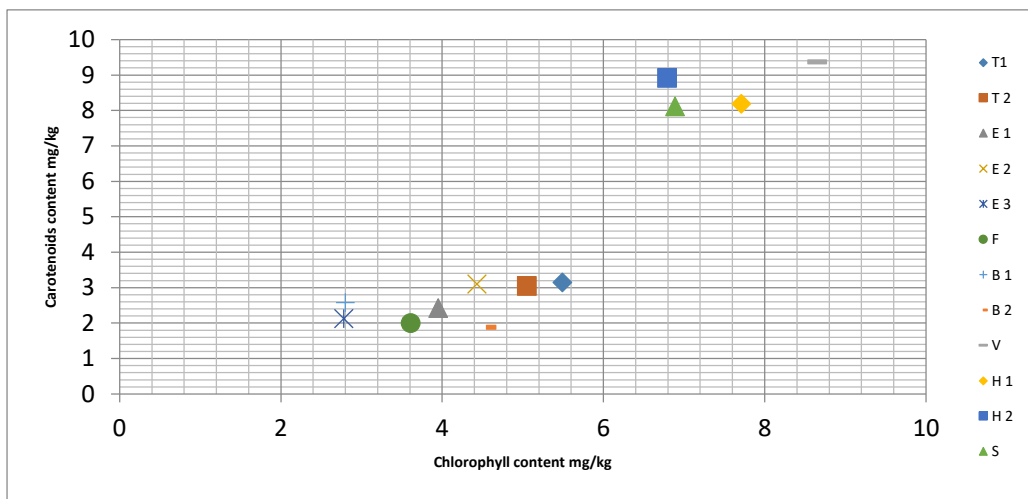
**Table 1.** Physical-chemical parameters of olive oils. Free acidity is expressed as % of oleic acid (mass/mass); peroxide value is given in meq O<sub>2</sub>/kg of olive oil.

| Sample           | Total acidity | K 232        | K 270        | Index peroxide |
|------------------|---------------|--------------|--------------|----------------|
| <b>Tirana 1</b>  | 0.78 ± 0.03   | 0.817± 0.005 | 0.070± 0.002 | 17.36 ± 0.87   |
| <b>Tirana 2</b>  | 0.61± 0.04    | 0.685± 0.006 | 0.042± 0.004 | 13.02 ± 0.25   |
| <b>Elbasan 1</b> | 0.42 ± 0.04   | 0.903± 0.019 | 0.057± 0.006 | 19.08 ± 0.35   |
| <b>Elbasan 2</b> | 0.23 ± 0.00   | 0.862± 0.004 | 0.053± 0.002 | 18.62 ± 0.25   |
| <b>Elbasan 3</b> | 0.37 ± 0.04   | 0.934± 0.025 | 0.045± 0.002 | 19.02 ± 0.28   |
| <b>Fier</b>      | 0.78 ± 0.01   | 0.896± 0.014 | 0.071± 0.012 | 15.79 ± 0.28   |
| <b>Berat 1</b>   | 0.5 ± 0.00    | 0.659± 0.006 | 0.056± 0.005 | 10.19 ± 0.84   |
| <b>Berat 2</b>   | 0.73 ± 0.01   | 1.046± 0.004 | 0.074± 0.003 | 13.98 ± 0.23   |
| <b>Vlore</b>     | 0.65 ± 0.04   | 1.009± 0.004 | 0.099± 0.004 | 15.18 ± 0.57   |
| <b>Himare 1</b>  | 0.36 ± 0.04   | 1.121± 0.006 | 0.076± 0.005 | 22.57 ± 0.27   |
| <b>Himare 2</b>  | 0.53 ± 0.04   | 1.080± 0.001 | 0.105± 0.003 | 22.0 ± 0.00    |
| <b>Sarande</b>   | 0.51 ± 0.02   | 1.200± 0.000 | 0.101± 0.003 | 22.99 ± 0.26   |

PV is a measure of the active oxygen bound by the oil which reflects the degree of oxidation of oils, accelerated by the presence of oxygen, temperature, and certain catalysts; these factors act on the double bonds of unsaturated fatty acids to form peroxides and hydroperoxides (Cimato, 1990). According to the standards of the IOC 2019, a peroxide value must be less than or equal to 20 meqO<sub>2</sub>/kg. Indeed, the PV for our samples of olive oil from coastal area H1 (22.57 meqO<sub>2</sub>/kg), H2 (22.0 meqO<sub>2</sub>/kg), and S (22.99 meqO<sub>2</sub>/kg) marks a slight exceedance of the standard of IOC 2019. These values indicate an oxidation process that these oils have passed, could be due to several conditions during the stages proceeding the extraction of the oil (harvesting), and inadequate or prolonged storage is also one of the causes of increase of this parameter (Meftah et al., 2014; Tanouti et al., 2011). Nevertheless, the other samples showed consistent peroxide values ranging from 10.19 meqO<sub>2</sub>/kg (B1) to 19.08 meqO<sub>2</sub>/kg (E1) that are within the limit determined by IOC for category of 'extra virgin' olive oil.

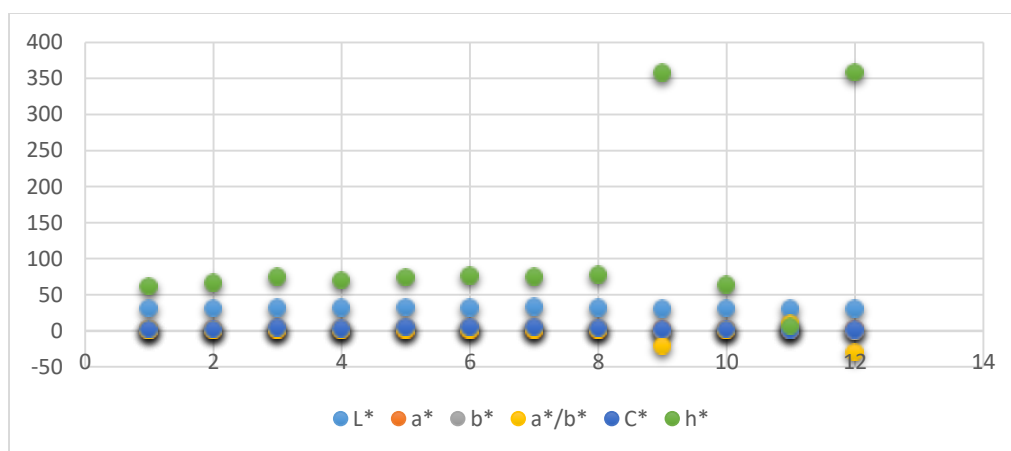
The results of average content of chlorophyll and carotenoid of olive oil samples from different studied regions, expressed in (mg/kg) by applying the spectroscopic method proposed by Minguez-Mosquera et al., are summarized in Figure 1. Olive oils from coastal region (V, H1, H2 and S) were the richest in chlorophylls and carotenoids, ranging from 6.79 – 8.65 mg/kg and 8.12 – 9.38 mg/kg, respectively. The presence of carotenoids in sufficient quantities in the oil allows the delay of the photooxidation and the preservation of the quality parameters of the oil during its storage (Lazzer et al., 2006).

The chlorophyll and carotenoid contents of other samples were inferior to 5.49 mg/kg and 3.11 mg/kg, respectively (figure 1). The final concentration of each pigment in the final VOO relies on the physicochemical characteristics of the fruit, the geographic origin, climate and irrigation conditions, and the mechanic extraction process used. Additionally, storage conditions and final packaging play a role in pigment concentration and type (Lazzerini et al., 2016; Lazzerini et al., 2017; Gandul-Rojas et al., 2016). The results showed that pigments content in olive oil depend on the geographical origin of cultivation, where the coastal area of cultivation produces oils with much higher pigments content.



**Figure 1.** Chlorophyll and carotenoid contents of olive oil samples from crop season 2020 expressed in (mg/kg) applying the spectroscopic method proposed by Minguez-Mosquera et al., 1992.

The color of oils depends on the wavelengths of transmitted visible light, and is primarily influenced by two groups of minor oil constituents, carotenoids and chlorophylls. The degradation of these constituents during storage obviously affects the oil color. To evaluate such changes quantitatively, we have measured the CIELAB color parameters. For color characterization, coordinates based on the Hunter solid are widely used:  $L^*$  (lightness),  $a^*$  and  $b^*$ , where + a represents red; -a, green; + b, yellow; and -b blue.



**Figure 2.** Color of olive oil expressed as chromatic coordinates  $L^*$ ,  $a^*$ ,  $b^*$ ,  $C^*$  and  $h^*$ .

Color analysis showed luminosity values  $L^*$  are close to each other and range from 29.99 to 32.27. Usually this coordinate increases with the reduction in the pigment content of the oils, as the pigments would capture part of the light, instead of transmitting it. This trend is in accordance with the chlorophylls and carotenoids content in olive oil samples. The  $-a^*$  describing the greenness of the oil fluctuates from 0.7 – 1.31, which means that olive oil sample B2 is greener than the other. The  $b^*$  value describing the yellowness of the oil range from – 0.07 to 4.54, meaning that olive oil sample V is more yellow than the others. The ratio  $a/b$  is usually computed. It is negative for green plant material, approximately 0 for yellow material, such as virgin olive oil, and positive for orange or red material (Criado et al. 2008). In our case, there were two olive oil samples that have a negative value (S and V) exhibiting a greener color compared to other samples.

## CONCLUSIONS

Color is perhaps a very important sensory characteristic of olive oil, especially as it is related to some quality parameters. The color of virgin olive oil ranges from light yellow to deep green depending on the content of liposoluble pigments (chlorophylls and carotenoids), naturally occurring in the fruits. The level of these pigments is influenced by climatic conditions and the geographical area of cultivation, where the coastal area creates virgin olive oil with more extinguished green color than the central area of Albania. These results prove that the measurement of oil color can provide valuable information regarding numerous important analytical parameters related to quality. Although, at the moment, the profile of chlorophyllic and carotenoid pigments present in olive oil is not included in the regulated quality standards (IOC 2009), a recent regulation has included the color of oils as one of the obligatory quality parameters (USDA 2010).

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## CHARACTERIZATION OF RABBIT PRODUCTION SYSTEMS IN TUNISIA

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### ABSTRACT

The study aimed to determinate the breeding practices of rabbit production systems in Tunisia. A survey was carried out to collect data from a sample of rabbit breeding (n=60). The breeders were chosen randomly from the North Est, the Center Est-West and South West of Tunisia. The survey was interested to identify the practices of housing and reproduction in rabbit rearing to characterize the production systems. Then, the breeding were classified into the different production systems according to the number of does in production and the reproduction techniques. Preliminary results showed that rabbit rearing belongs to 3 production system classes: traditional (35%) and industrial (65%) systems. The latter is composed of the conventional industrial system (33%) using the natural mate and the modern industrial system (32%) using the artificial insemination. The traditional system is characterized of small farms with a number of does <50. The industrial system is characterized by large farms with a number of does >50. Besides, the rabbit rearing is considered as a principle activity in 53% of breeders. However, 47% of breeders practice others types of rearing than the rabbit one. Multiple activities of livestock are noted in most farmers of the traditional system due to the few numbers of does. The livestock housing deferred according to the systems. In fact, the average area of the rearing building was 55m<sup>2</sup> in the traditional system, 236m<sup>2</sup> in conventional industrial system and 425m<sup>2</sup> in the modern industrial system. The breeding in the traditional system is carried out outdoor or in and old and not converted building. However, the ones of the conventional and modern industrial systems were carried out in converted building with a separation between the mother and fattening cells using airlocks. In this system, the management of the breeding is based on the concept «all in all out». The environmental conditions are not respected in the traditional system. The farrowing rate varied between the traditional and the industrial systems (p<0.05). However, reproduction technique didn't affect the farrowing rate in the systems. Regarding rabbit's growth, the average daily gain varied between the systems (p<0.05). The results showed that 20% of breeders are about to abandon their rearing because of constraints of health (36%), feed supply (23%), commercialization (23%) and covid-19 situation (18%). The survey suggests that most of breeding belong to the industrial rabbit production system. This one provides a sustainable productivity for farmers. Nevertheless, the sustainability of rabbit's breeding is threatened by constraints including feed supply, covid-19 situation, commercialization and health.

**Keywords:** breeding practices, rabbits, production systems, reproduction techniques, constraints.

## INTRODUCTION

Rabbit farming has developed in Tunisia since the 80's and 90's by some farmers who reported either the benefits on the consumer health or its contribution to the national meat production, especially to fill the red meat deficit. This development has gradually led to the installation of a rabbit industry in the country (Bargaoui and Kriaa, 2001). Then, small rabbit farming has been promoted to fight poverty and contribute to food security (Oseni and Lukefahr, 2014; Medenou et al., 2020). In Tunisia, the rabbit farming was present in the North, littoral, center and south regions. It is practiced around the big cities. Since the year 2001, the farming rabbit has been divided into 2 categories: the traditional (family) and the modern farming rabbit (Bargaoui et Kriaa, 2001). Traditional family farms are often oriented towards self-consumption, and have low-intensity practices. Modern large farms rear rabbits in cages with wire floors, practice band management and artificial insemination, and have a very short production cycle that allows them to be very productive. However, a few data was recorded regarding the rabbit production system. Moreover, the typology of rabbit system production has been changing over these 10 last years because of political and economic crisis in the one hand, and climate change in the other hand. Therefore, performances of farming rabbit in different production systems were affected. In this context, it is important to carry out further studies on production systems to determinate the most sustainable and economically viable system (Pothin et al., 2017). The objective of this study was to describe the main rabbit production systems and their influences on performances of the farming, and to find how to improve productivity to award sustainability in rabbit farming.

## MATERIAL AND METHODS

### *Study location and animal sampling*

The study was taken on a group of rabbit breeders (n=60) from the North Est, Center Est-West and South West of Tunisia. A total of 6341 reproductive does has been noted during the survey. The study was conducted from the 1<sup>st</sup> February to 15<sup>th</sup> March 2020. The visits to rabbit farmer were supported by the socio-economic organism, the Livestock and Pasture Office as a partner in this study.

### *Survey*

The survey was based on 3 parts: the characteristics of the production system, performance of the farm, and constraints of rabbit breeding. The production system part was interested to identify the structure of the rabbit farm (housing, animal demographic variables), the reproduction technique (artificial insemination or natural mate). This part was used to characterize the production system. The performance part included information on maternity livestock (reproductive does, renewal rate, farrowing rate) and fattening livestock (body weight at weaning, average daily gain and fattening duration). The rabbit breeders were also asked about constraints on breeding (health, marketing,...) .

### *Statistical analysis*

Data were analysed with SAS software (SAS Institute Inc., USA). The ANOVA was carried out using the GLM procedure. The variable means were compared with DUNCAN test. The significance level was set for  $p < 0.05$ .

## RESULTS AND DISCUSSION

### *Rabbit production systems*

The results showed that rabbit rearing belongs to 3 production system classes: traditional (35%) and industrial (65%) systems. The latter is composed of the conventional industrial system (33%) using the natural mate and the modern industrial system (32%) using the artificial insemination. The traditional system is characterized of small farms with a number of does <50. The industrial system is characterized by large farms with a number of does >50; and the number of reproductive does was higher in the industrial system. The rabbit rearing is considered as a principle activity in 53% of breeders. However, 47% of breeders practice others types of rearing than the rabbit one. Multiple activities of livestock are noted in most farmers of the traditional system due to the few numbers of does.

The rabbit housing deferred according to the systems. In fact, the average area of the rearing building was 55m<sup>2</sup> in the traditional system, 236m<sup>2</sup> in conventional industrial system and 425m<sup>2</sup> in the modern industrial system (Table 1).

**Table 1.** Characteristics of the rabbit production systems.

| Production system                                      | Traditional | Industrial Conventional | Industrial Modern |
|--|-------------|-------------------------|-------------------|
| Average area of the rearing building (m <sup>2</sup> ) | 55±10       | 236±54                  | 424±95            |
| Reproduction technique                                 | NM          | NM                      | AI                |

*NM: natural mate; AI: artificial insemination*

The breeding in the traditional system is carried out outdoor or in an old and not converted building. However, the ones of the conventional and modern industrial systems were carried out in converted building with a separation between the mother and fattening cells using airlocks. In this system, the management of the breeding is based on the concept «all in all out». The environmental conditions are not respected in the traditional system. The farrowing and the renewal rates (Table 2) varied between the traditional and the industrial systems ( $p<0.05$ ). However, reproduction technique didn't affect the farrowing rate in the production systems. Regarding rabbit's growth (Table 2), the body weight, the average daily gain and the fattening duration varied between the systems ( $p<0.05$ ).

**Table 2.** Rabbit's performances according to the production systems.

| Production system           | Traditional            | Industrial Conventional | Industrial Modern        |
|-----------------------------|------------------------|-------------------------|--------------------------|
| Number of reproductive does | 357                    | 2276                    | 3758                     |
| Farrowing rate (%)          | 85±13 <sup>a</sup>     | 77±14 <sup>b</sup>      | 79±8 <sup>b</sup>        |
| Renewal rate (%)            | 3.5±1.2 <sup>a</sup>   | 10.5±3 <sup>b</sup>     | 12±4 <sup>a</sup>        |
| Body weight at weaning (g)  | 0.5±0.09 <sup>a</sup>  | 0.6±0.1 <sup>a</sup>    | 0.8±0.1 <sup>b</sup>     |
| Average daily gain (g/d)    | 0.02±0.01 <sup>a</sup> | 0.04±0.01 <sup>b</sup>  | 0.03±0.006 <sup>ab</sup> |
| Fattening duration (d)      | 54±12 <sup>a</sup>     | 43±10 <sup>b</sup>      | 42±2 <sup>b</sup>        |

*a, b: p<0.05*

The results showed that 20% of breeders are about to abandon their rearing because of constraints of health (36%), feed supply (23%), commercialization (23%) and covid-19 situation (18%). The identified constraints are related to the lack of technical information and veterinary support mainly the increase of the mortality rate in farming (Gono et al., 2013; Hungo et al., 2013; Baruwa, 2014). Low incomes discourage farmers to maintain productivity of their rearing (Ramalakshmi and Thanasundari, 2017). Consequently, these constraints hinder, rabbit farming production and then its profitability and sustainability. The survey showed that in addition to known constraints as diet, marketing and viral diseases, the Covid-19 pandemic is added to plague the rabbit farming sector. Based on this finding, it would be wise to think of innovative alternatives to market and manage the production in crisis period. Including technology and digital marketing is one of the best solutions that contributes to the maintain farmer incomes.

## CONCLUSION

The survey suggests that most of rabbit farms belong to the industrial rabbit production system. This one provides a sustainable productivity for farmers. Nevertheless, the sustainability of rabbit's breeding is threatened by constraints including feed supply, covid-19 situation, commercialization and health.

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## NETWORK FORMATION AND INTERFACE BEHAVIOR OF PU COATINGS WITH DUAL HYDROPHILIC/HYDROPHOBIC DANGLING CHAINS

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### ABSTRACT

In this work, we present the results of coarse-grained (CG) simulation study on a PU coating consisting dual hydrophobic/hydrophilic dangling chains. We employed Dissipative Particle Dynamics (DPD) simulation to create the network structures through a two-step reaction route. The dual nature of dangling chains gives a smart behavior to the coatings in response to the environmental polarity. The effect of solvent evaporation on the final structure of the coatings and the smart response of the coatings to water (polar) and air/oil (apolar) environments has been extensively studied. This study paves the way of formulating stimuli-responsive PU coatings for anti-biofouling applications.

**Keywords:** Dynamics, coarse-grained

### INTRODUCTION

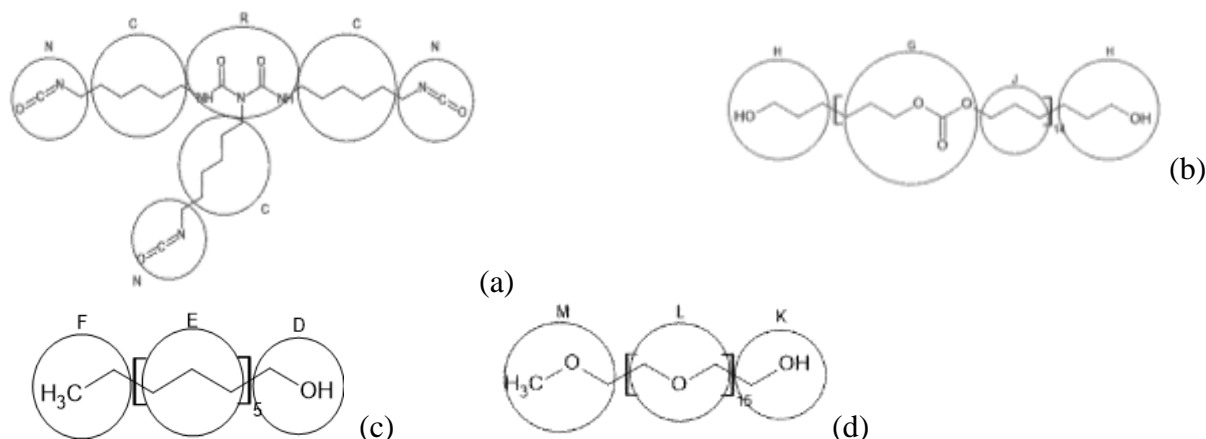
Polyurethane (PU) smart coatings are industrially relevant polymeric materials that are widely used in aviation, automotive, and medical device industries. These coatings are generally designed for many different purposes such as self-cleaning, anti-corrosion, anti-bacterial, anti-fouling, low friction, biocompatibility [1]. PU coatings with dual functionality, namely involving hydrophobic and hydrophilic functionalities, can serve as materials with superior properties compared to conventional ones. To that purpose, smart amphiphilic PU coatings can be designed by introducing hydrophilic and hydrophobic dangling chains to the network during polymerization. By incorporating the hydrophilic and hydrophobic chains, the material becomes responsive to the change of the environment by the segregation of the dangling chains towards the interface [2]. In this work, we synthesized the network formation of a particular PU coating containing 1-octadecanol (oDEC) and methoxy polyethylene glycol (mPEG) dangling chains and characterized its smart behavior in response to the environment by employing CG molecular dynamic simulation method, DPD. This method is quite useful for modeling and studying cross-linked polymer network structures [2,3]. We model the network creation process of the PU systems contained the dual hydrophobic/hydrophilic dangling chains by initially forming the cross-link reactions in the system in the presence of the solvent. Later, we evaporate the solvent from the system by gradually deleting the solvent molecules, which is followed by a further relaxation at the constant pressure condition. We employed a solvent evaporation procedure which to mimic the real reaction conditions and to form a surface.

In the current work, two types of monofunctional dangling chains, one hydrophilic (mPEG) and the other hydrophobic (oDEC), were initially introduced to the system forming covalent bonds

with tris(isocyanatohexyl)biuret (HDI-BT) in simulation. Afterwards, poly(hexamethylene carbonate)diol (PC) was introduced to the reactor with the additional cross-linker to create the final polymer network structure. We studied the cross-linking processes and the smart and responsive behavior of the coatings under polar (water) and apolar (air and oil) environments. We found out that solvent evaporation from the surface leads to the migration of mPEG dangling chains towards the surface. We also proved the smart and responsive behavior of the coatings by simulating the polymer/water polymer/air and polymer/oil interfaces.

## MATERIALS

The chemical structure and coarse graining procedure are schematically shown in Fig. 1.



**Figure 1:** Schematic representation of the coarse-graining and the beads of (a) HDI-BT, (b) PC with  $n = 14$ , (c) 1-octadecanol with  $n = 5$  and (d) mPEG with  $n = 15$ .

PC ( $M_w = 2134$  g/mol) as the macrodiol, mPEG ( $M_w = 736$  g/mol) as the hydrophilic dangling chain, oDEC ( $M_w = 270$  g/mol) as the hydrophobic dangling chain, HDI-BT as the cross-linker, n-butyl acetate (nBAC) and methyl ethyl ketone (MEK) as the solvent are used as materials in this work. The mass ratio in experimental synthesis and the total number of molecules used in our simulations are shown in Table 1.

**Table 1:** Materials and number of molecules used in the study.

| System details |        | HDI-BT | PC   | mPEG | 1-octadecanol | nBAC  | MEK   |
|----------------|--------|--------|------|------|---------------|-------|-------|
| <i>Mix 15%</i> | Step 1 | 1040   | –    | 470  | 350           | 31406 | 9923  |
|                | Step 2 | –      | 1150 | –    | –             | 6168  | 70237 |

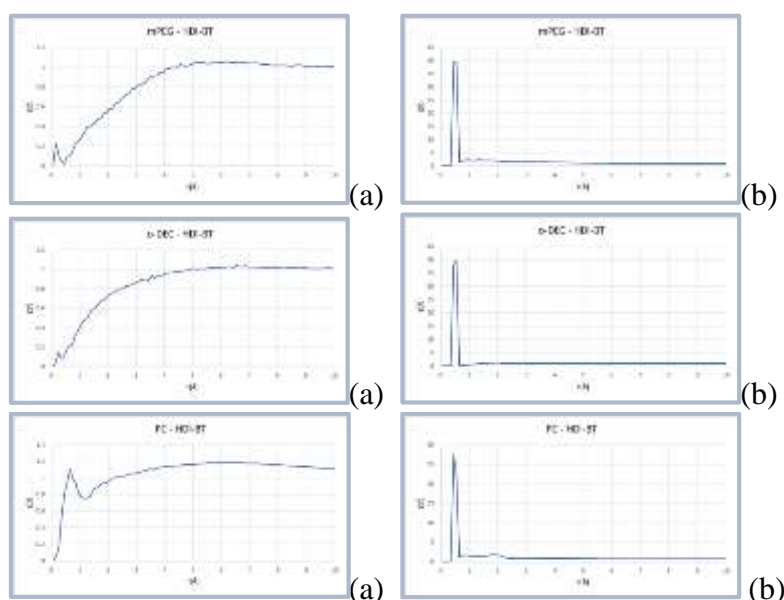
## METHODS

The simulation methods we employed in our study is coarse-grained DPD. The idea of coarse-graining is dividing the molecules into molecular sub-units, which are called as the coarse-grained beads [4]. In DPD, the non-bonded interactions between these beads are completely repulsive. Performing this coarse-graining and identifying the repulsive interactions between beads allows achieving longer time and length scales compared to the atomistic scale molecular simulations. This is especially important for the cross-linked polymer systems, which have significantly high relaxation times. In our simulations, beads have variable volumes. Therefore, instead of conventional parameterization, we use the alternative DPD

parameterization, where the volumes of beads are dictated by their pure-liquid densities [5]. We carried out simulations in three steps: polymerization at the *NVE* condition, polymer relaxation and solvent evaporation at the *NPT* condition.

## RESULTS

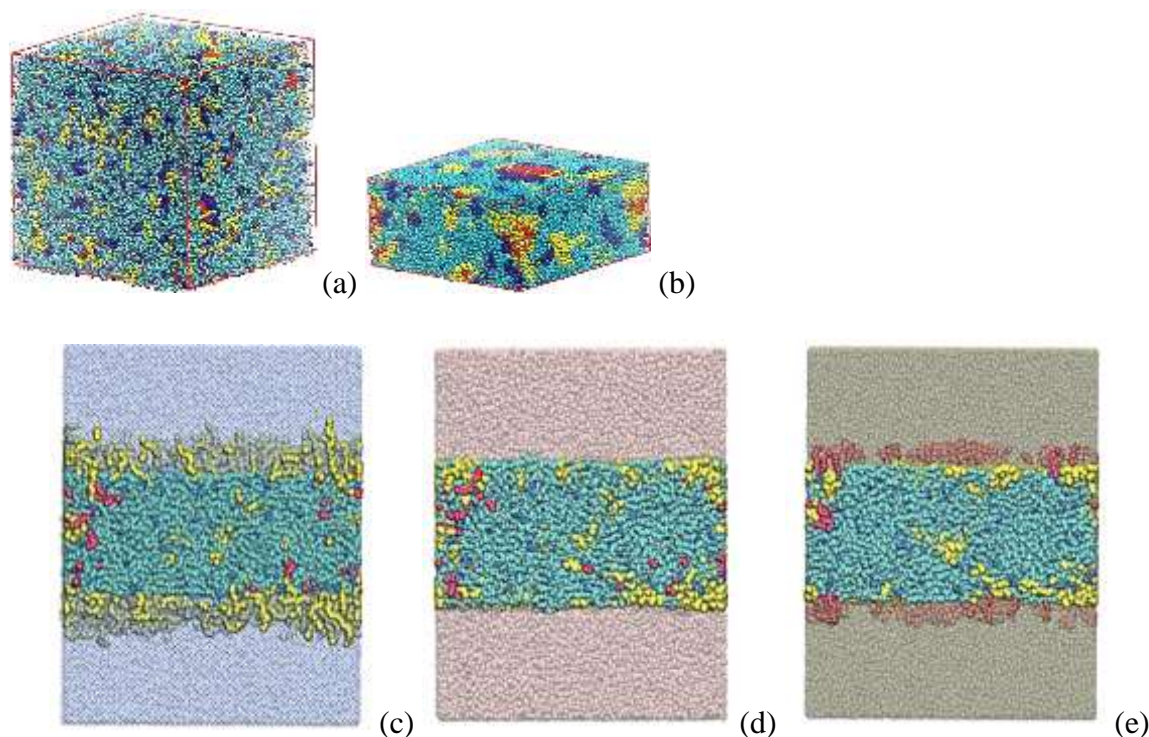
Throughout the cross-link simulations, the beads supposed to cross-link turn into new bead types as a result of polymerization at the coarse-grained level. The cross-link conversion is obtained by dividing the number of cross-links that a particular bead makes to the total number of cross-links that HDI-BT molecule is supposed to make. In the simulations, where two types of dangling chains are present, the total conversion equals to 94.77%. The individual cross-link conversions of mPEG, 1-octadecanol and PC with HDI-BT read as 15%, 11.18% and 68.59%, respectively. This means that 0.28% mPEG, 0.42% 1-octadecanol and 6.95% PC beads remain unreacted. To comment on the molecular structure of the coating, we plot the radial distribution function (RDF) between the reactive ends prior to and after cross-linking takes place. The RDFs are shown in Fig. 2.



**Figure 2:** RDF plots between reactive ends before (a), and after (b) the cross-linking.

Following the cross-link and polymer relaxation simulations (Fig. 3a), we perform the solvent evaporation simulation as shrinking in only  $z$ -dimension (Fig. 3b). The idea in shrinking the simulation box only in  $z$ -dimension is to observe the effect of the dimensionality in the polymer film formation.

The final structure of the coating was placed in contact with a polar (water) and apolar (air and oil) environments. A responsive behavior of the coating with dangling chains is illustrated in Fig. 3c,d,e respectively.



**Figure 3:** Simulation snapshots of the solvent evaporation to form the surface before (a), and after (b) evaporation takes place, and the polymer/water (c), polymer/air (d), polymer/oil (e) interfaces. Colors navy blue, yellow, red, cyan and dark green are assigned to cross-linked HDI-BT, mPEG, 1-octadecanol, PC, nBAc and MEK, respectively.

## CONCLUSIONS

In this work, we modeled and simulated a hydrophobic/hydrophilic smart coating for studying its network formation and molecular structure as well as its interface with polar (water) and apolar (air and oil) environments. We reached a high cross-link conversion is obtained at the end and we observed that solvent evaporation can lead to a dangling-chains-rich interfacial layer when a mixture of hydrophilic/hydrophobic dangling chain strategy is applied. We evaluated the surface-stimuli response of the coatings to the environment, in which a smart surface rearrangement was observed. The next steps in our work involve incorporating different ratios of dangling chains and estimating the material properties *via* atomistic molecular dynamics simulations with the reverse-mapped coordinates.

## ACKNOWLEDGEMENTS

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## RECENT DISSIPATIVE PARTICLE DYNAMICS PARAMETERIZATION MIMICS EXPERIMENTAL STRUCTURE AND PROPERTIES OF WATER AND ALCOHOLS

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### ABSTRACT

Mimicking experimental properties of water and simple hydrogen bonding liquids (methanol, ethanol and 1-propanol) *via* simulations has been one of the major challenges in the modeling community. So far, atomistic molecular dynamics simulations have been employed to study the structure and thermodynamic properties of such systems at their pure-liquid states or in liquid mixtures. Recently, attention has been given at the coarse-grained scale to characterize these properties due to the presence of water in complex environments such as physiological conditions and interacting with polymers. One of the challenges in parameterizing the coarse-grained interactions is the absence of atomistic detail, which leads to poor definition of interactions. Moreover, poorly defining interactions would possibly lead to unrealistic mesoscopic structure and properties. With this motivation, we aim at building a parameterization scheme for a coarse-grained method, namely dissipative particle dynamics (DPD) to achieve a realistic modeling of hydrogen bonding liquids. Our parameterization scheme involves the contribution of hydrogen bond interactions as computed from a statistical mechanic's approach combined with a fine-tuning of interactions based on experimental radial distribution functions (RDF). The results describe the experimental RDF and some physical properties of water and low molecular weight alcohols (i.e., methanol, ethanol and 1-propanol) such as viscosity, angle distribution and isothermal compressibility reasonably well. With the proposed parameterization, we hope to extend the current parameterization practice of DPD to cover a wider range of applications, where hydrogen bonding interactions are dominant.

**Keywords:** Dynamics, DPD

### INTRODUCTION

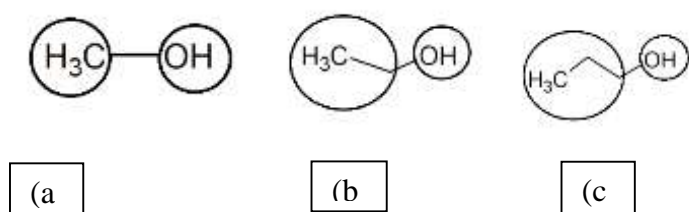
We aim at developing a DPD parameterization scheme to examine the anomalies in the structural and physical properties of water, alcohol and their mixtures in a simulation environment with the Dissipative Particle Dynamics (DPD) method, which is a coarse-grained simulation method. The reason for using DPD is that coarse-grained methods are convenient tools to obtain the structural properties of complex molecular systems, which characteristically require long simulation times. In other words, long-term structural evolution of polymers or biological molecules are not suitable for studying with classical molecular dynamics methods (Kacar and Peters 2013). Since the DPD method covers a wide range of length and time scales and accurately reveals the hydrodynamic properties of liquids, it has become frequently used in the modeling of complex systems (Groot and Warren 1997). DPD interactions consist of only

repulsive interactions, they are insufficient to model attractive interactions such as hydrogen bonds. For this reason, in order to model attractive hydrogen bonds, attractive interactions formed as Morse potential were defined in addition to the repulsive interactions defined in the classical DPD method. In order to obtain the numerical values of the attractive interactions, the computational procedure were carried out for the adaptation of the partition function of water, which was previously developed as the Mercedes-Benz water model (Urbic and Dill 2018). In our approximation to model pure water and alcohols, the Morse potential is used as the attractive contribution, which is added to the DPD potential.

The results that are obtained during this work will add to the current literature by; defining attractive interactions for water-water interactions in DPD, developing statistical mechanics techniques to compute interactions to be used in DPD simulations, study of structural anomalies via DPD simulations, analysis of currently available materials or developing procedures to design new materials that are relevant to biological systems (DNA, RNA, cell membrane) and nanomaterials and biotechnological applications (hydrophilic polymers, hydrogels, drug delivery nanostructures).

## MATERIALS

The chemical structure and coarse graining procedure are schematically shown in Fig. 1.



**Figure 1:** Schematic representation of coarse modeling of molecules a: methanol, b: ethanol, c: 1-propanol

The Morse potential was calculated for the water-water interaction. The reason is that our coarse-graining of alcohols include an -OH group, which is practically a water molecule in the proper bead representation. Since the beads formed after the coarse-graining contain hydrogen bonds, the alcohols are modeled as two particles such as methanol (methane + water), ethanol (ethane + water) and 1-propanol (propane + water). The composition of the simulated systems are given in Table 1.

**Table 1:** Number of beads used in the study

| Case             | System details |          |         |             |
|------------------|----------------|----------|---------|-------------|
|                  | Water          | Methanol | Ethanol | 1-Prophanol |
| <i>Pure 100%</i> | 24000          | 24000    | 24000   | 24000       |

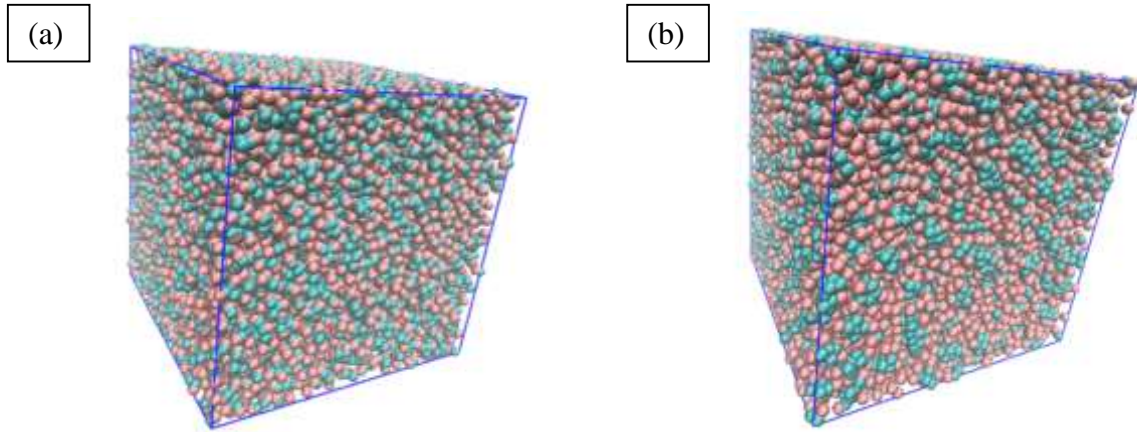
## METHODS

In our studies, DPD potential is used for repulsive interactions and Morse potential was used for the attractive interactions. Equilibrium and cut-off distances of Morse potential were estimated from the OH-OH RDF graph as a result of the molecular dynamics simulations, which we performed as a separate study. Since the systems we use are very small molecules, the parameters we will calculate are very sensitive to the proper selection of the bond length and

bond stiffness. Molecular dynamics simulations were used for the bond length calculations in DPD. Our alcohols consist of two particles and the bond length between the centers of mass (CoM) of the two particles is used. The results are given in Table 2. With the Morse potential, the bond length can vary greatly in small systems, which can cause the system to freeze and solidify. To prevent this, the Shake algorithm is used in the DPD simulations, which resulted in a fluid the system. The comparison of the simulations with and without the Shake algorithm is shown in Figure 2.

**Table 2:** Bond lengths connecting two beads and the scale factor to convert DPD units to the physical units.

| System     | Bond length | $r_{\text{DPD}}$ [Å] |
|------------|-------------|----------------------|
| Methanol   | 0.30        | 4.483                |
| Ethanol    | 0.35        | 5.063                |
| 1-Propanol | 0.45        | 5.519                |



**Figure 2.** *a* and *b* show the simulation results with and without the shake algorithm, respectively.

In our parameterization, the DPD repulsive interaction is computed as

$$a_{ij} = \hat{a}_{ij} + \frac{p}{0.0454(a_{ii}\rho_{i,pure} + a_{jj}\rho_{j,pure})} k_B T \quad (1)$$

where,  $\chi_{ij}$  is a function of solubility parameters for each molecule and varies with temperature. To calculate the attractive strength of the hydrogen bond, the partition function obtained by Urbic *et al.* (Urbic and Dill 2018) is used as shown in equation 2.

$$\Delta_{HB} = 4\pi^2 c(T) v_{ef}^{HB} \exp\left(\frac{\epsilon_{HB} + \epsilon_{LJ} - p v_{HB}/2}{k_B T}\right) \sqrt{\frac{4k_B T \pi}{k_s}} \operatorname{erf}\left(\sqrt{\frac{k_s}{4k_B T}}\right) \quad (2)$$

In this equation,  $c(T)$  is the kinetic energy contribution,  $v$  is the molecular volume,  $T$  and  $p$  are the temperature and pressure, respectively.

The viscosity is computed at *NVE* conditions by using the Green-Kubo relation (Viscardy, Servantie *et al.* 2007). The results are presented given both in DPD units and in real units in Table 3. The viscosity  $\eta$  is,

$$\eta = \frac{V}{k_B T} \int_0^{\infty} dt \langle P_{xy}(0) P_{xy}(t) \rangle \quad (3)$$

In this expression,  $V$  is the volume of the system,  $T$  is the temperature,  $k_B$  is the Boltzman constant,  $\langle \dots \rangle$  is the ensemble mean, and  $P_{xy}$  is the off-diagonal element of the pressure tensor.  $\tau$  indicates the unit DPD time in real units. To compute  $\tau$ , the expression in the work of Groot and Rabone (Groot and Warren 1997) is used:

$$\tau = \frac{N_m D_{sim} r_{DPD}^2}{D_{su}} \quad (4)$$

In Eq. 4,  $N_m$  is the coarse-grained particle number density,  $D_{sim}$  is the diffusion coefficient obtained from the DPD simulations,  $r_{DPD}$  is the DPD unit length in Ångström, and  $D_{water}$  is the experimental diffusion coefficient for pure water. Eq. 5 is used to calculate the  $D_{sim}$  parameter as in (Partington, Hudson et al. 1952).

$$D = \lim_{t \rightarrow \infty} \frac{\langle [r(t) - r(0)]^2 \rangle}{6t} \quad (5)$$

To convert the viscosity result obtained from DPD units to real units,

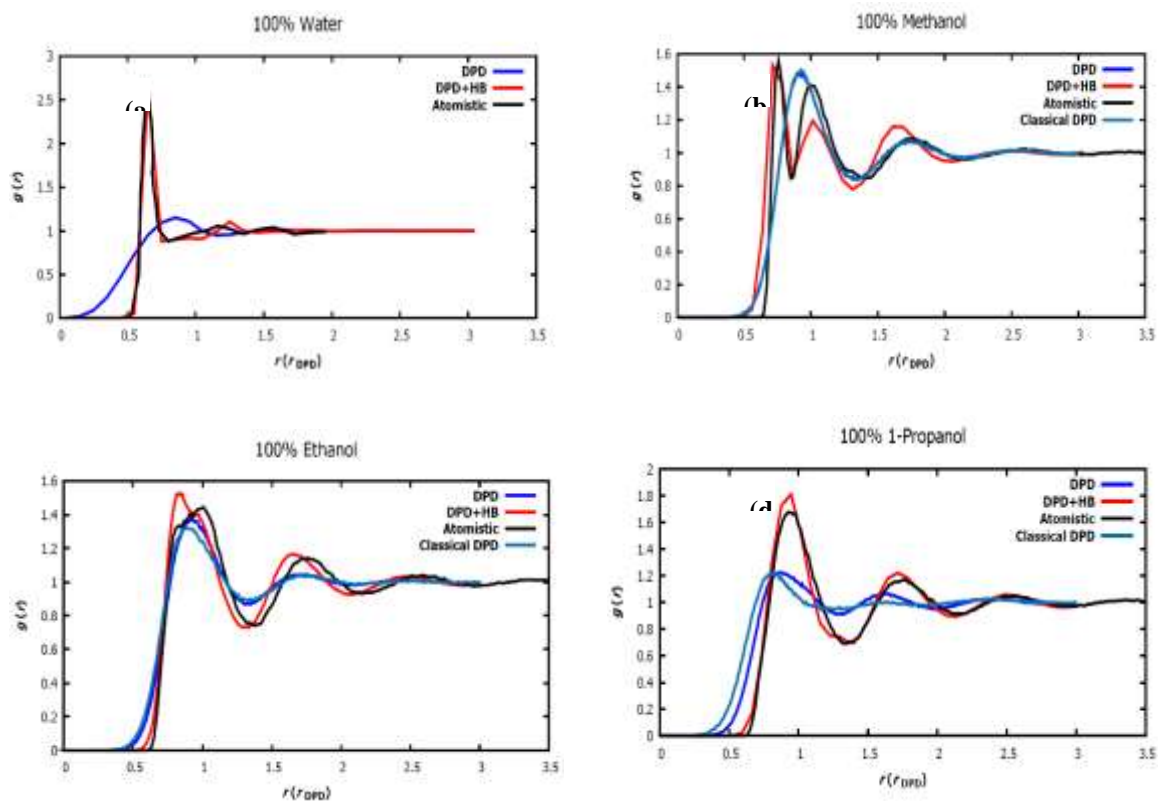
$$H = \eta \tau k_B T / r_c^3 \quad (6)$$

is used as in (Boromand, Jamali 2015).  $H$  and  $\eta$  are viscosity in real units and in DPD units, respectively.

## SIMULATION DETAILS

DPD simulations are performed for 24000 beads and  $20^3 r_{DPD}^3$  box sizes. The systems are first equilibrated at 10000 steps after energy minimization, and then simulated for 200000 steps under  $NVE$  conditions. The time step used for each system is  $0.001 t_{DPD}$ . Data to analyze are collected in the last 50000 steps of the simulations and used for calculations. Bond lengths in alcohols are fixed with shake algorithm and used in radial distribution plots. Simulations for viscosity calculations are run for another  $10^6$  steps under  $NVE$  conditions. In MD simulations, firstly, 20000 step energy minimization is performed with the steepest-descent algorithm, and then the systems are simulated for 1 nanoseconds at  $NPT$  conditions to maintain the experimental density. Finally, 1 nanosecond  $NVE$  simulations are performed and data are collected in the last 200 picoseconds. Berendsen thermostat is used for pressure and temperature control. The force field used in the simulations is OPLS-AA and TIP4P and SPCE water models are used for water. The cut-off distance was determined as 1.1 nm for electrostatic and van der Walls interactions in alcohols and 0.9 nm for water.

## RESULTS



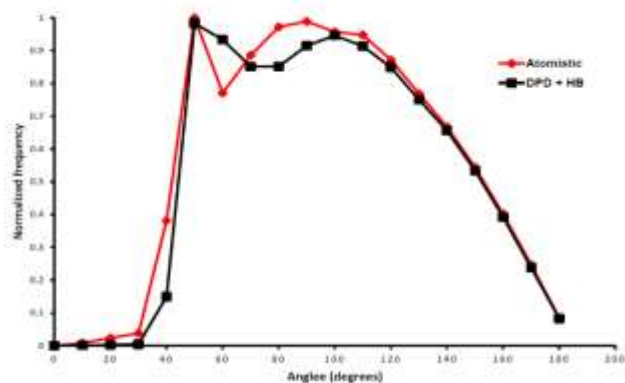
**Figure 3.** The Computed CoM RDF results for pure systems. **DPD** shows the classical DPD technique, **DPD+HB** is the DPD with the Morse potential, **Atomistic** is the Molecular Dynamics simulation. *a, b, c, d* are water, methanol, ethanol and 1-propanol, respectively.

It can be observed in Figure 3 that the classical DPD is insufficient to model structures that is a result of the hydrogen bond interactions. In other words, the Morse potential and the hydrogen bonds lead to more realistic results.

Viscosity results obtained as a result of DPD simulations are very close to the experimental results. To comment more on the structure, we compute the threebody angular distribution calculations. This property will lead to an estimation of the tetrahedral structure of molecules, that is present in the experiments. For the calculations, an in house written script is used and angles are calculated between beads that are present within a cut-off distance. The results for both DPD and MD simulations and compared. In line with the results obtained, the DPD simulation for pure water successfully models the known tetrahedral lattice structure formed by water as shown in Figure 4.

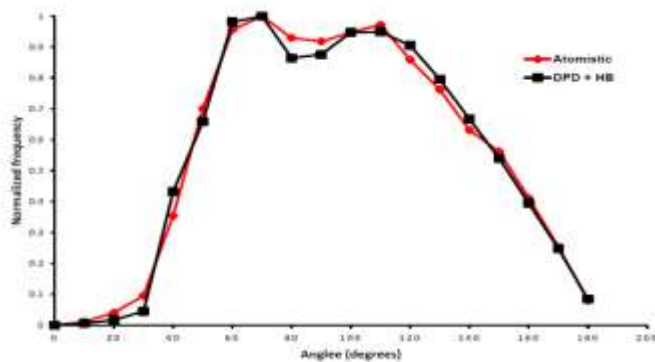
**Table 3.** The viscosity values calculated for pure alcohol and water at 298.15 K.

| System   | Viscosity ( $t_{DPD}$ ) | Viscosity (cP) | Experimental (cP)         |
|----------|-------------------------|----------------|---------------------------|
| Su       | 3,13                    | 0,865          | 0,888 (Lide 2002)         |
| Metanol  | 5,27                    | 0,50           | 0,544 (Pal and Gaba 2008) |
| Etanol   | 6,68                    | 0,98           | 1,040 (Center. 2007)      |
| Propanol | 15,40                   | 1,20           | 1,959 (Pal and Gaba 2008) |

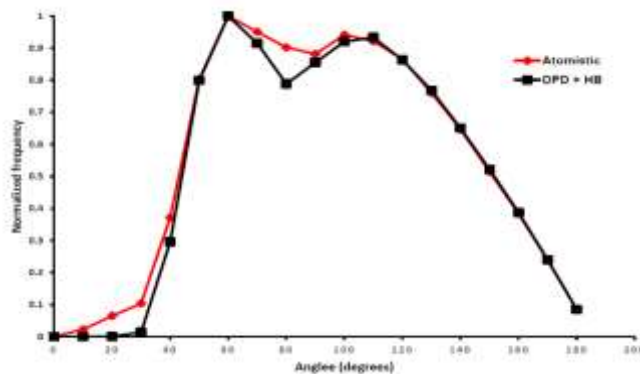


**Figure 4.** Angle frequency plot for pure water. The atomic data was obtained from the MD simulation we performed. The center of mass (CoM) of the DPD simulation was used.

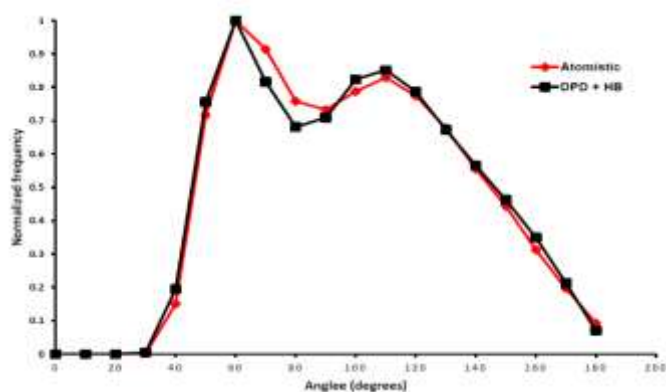
In figures 5-7 the presence of tetrahedral structure is noted. In all, we achieve the proper atomistic structural representation of water and alcohols *via* DPD simulations.



**Figure 5.** Pure methanol angle frequency plot. The atomic data was obtained from the MD simulation we performed. The center of mass (CoM) of the DPD simulation was **used**.



**Figure 6.** Pure ethanol angle frequency plot. The atomistic data was obtained from the MD simulation we performed and the center of mass of the DPD particle was used.



**Figure 7.** Graph of pure 1-propanol angle frequency. The atomistic data was obtained from the MD simulation I performed and the center of mass of the DPD particle was used.

## CONCLUSION

Hydrogen bond modeling in DPD simulations can properly model attractive interactions that are lacking in DPD. The hydrogen bond attractor interaction calculated by the partition function can be adapted to other systems and more complex structures can be simulated in DPD with more accurate results. Since coarse grained method gives faster results than MD in simulating large systems such as DNA, protein, polymer systems and drug transport systems, this study can be integrated into future studies.

## ACKNOWLEDGEMENTS

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## **BORON AND SELENIUM ACCUMULATIONS IN DRINKING WATER OF İPSALA DISTRICT (EDİRNE, TÜRKİYE)**

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### **ABSTRACT**

The aim of this research was to determine the boron and selenium accumulations in drinking water of İpsala District located in the west of Thrace Region and to assess the non-carcinogenic health risks of these elements via daily human intake. Drinking water samples were taken during the summer season of 2020 from İpsala District and 22 villages. The element levels were measured by using an ICP-MS device and Estimated Daily Intake (EDI) and Hazard Quotient (HQ) of boron and selenium were calculated for all the investigated locations. Boron concentrations were varied from 3.539 – 25.366 ppb and selenium concentrations were varied from 0.080 – 0.805 ppb. All the calculated HQ values for all the investigated locations in terms of both boron and selenium for all the investigated age groups were recorded as less than the critical limit of 1.

**Keywords:** İpsala District, Boron, Selenium, Health risk assessment

### **INTRODUCTION**

Thrace Region has a great agricultural potential. Therefore, about 80% of the region consists of agricultural lands. İpsala District is located in the Meriç Plain in the west part of Thrace Region. The district is very suitable for wet agriculture applications due to its rich groundwater and surface water resources. Accordingly, surface and groundwater resources of Thrace Region, Meriç Plain and İpsala District have been facing various pollution problems due to intensive agricultural applications and runoff from these lands (Anonymous, 2018; Tokatlı, 2019; Onur and Tokatlı, 2020; Tokatlı and Ustaoglu, 2020; Tokatlı and Varol, 2021; Tokatlı, 2021; 2022; Tokatlı et al., 2022; Varol and Tokatlı, 2022).

Boron and selenium are widely occurring elements found in the earth's crust. They are also trace elements and essential nutrients for humans. However, they can be harmful when regularly taken in amounts higher than those needed for good health. It is well documented that significant amounts of boron and selenium can be released from agricultural applications (ATSDR, 2003; 2010).

Health risk assessment techniques are being widely used all over the world in especially recent years. They are very useful and effective tools in estimating carcinogenic and non-carcinogenic hazards, which may occur when people are exposed to certain toxicants (Ustaoglu and Aydın, 2020; Tokatlı and Ustaoglu, 2020; Tokatlı and Varol, 2021; Varol and Tokatlı, 2022). The aim of this research was to determine the levels of boron and selenium in drinking water of İpsala District and connected villages and to evaluate the non-carcinogenic health risks of these essential elements via daily human intake.



## MATERIALS AND METHODS

### Water Collection

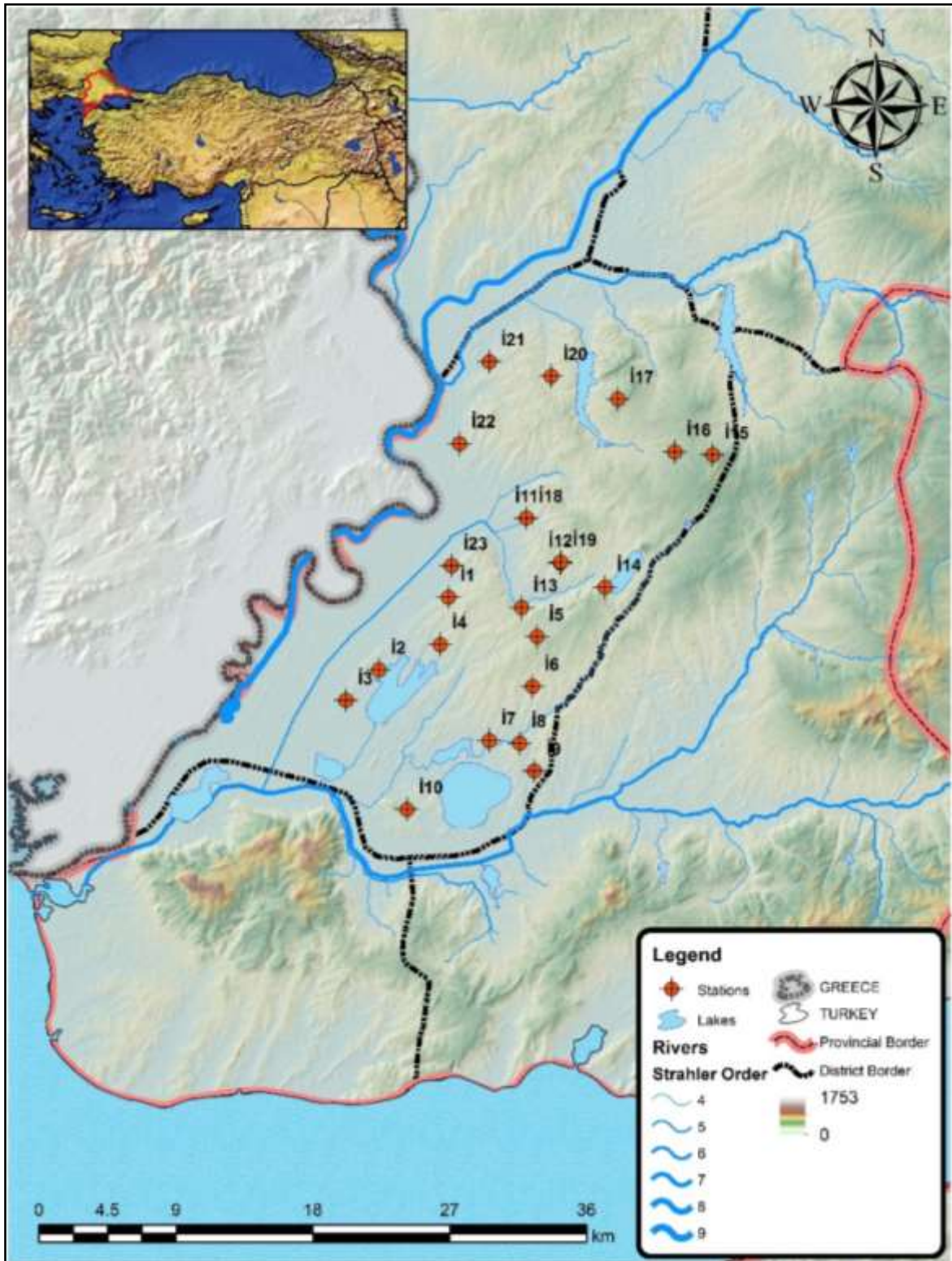
In this research, 23 sampling points were selected in the district for the tap – drinking waters. The topographic map of study area and selected stations (İ1 – İ23) are given in Figure 1. The coordinates with the names of locations are given in Table 1. Water samples were taken from the tap waters of the villages located in the İpsala District in summer season of 2020 by using polyethylene bottles.

### Element Analysis

pH values of water samples were decreased to 2 by adding about 2 ml of nitric acid per 1 L into each. The samples were filtered by using a 0.45 µm – cellulose nitrate filter. The volumes of samples were made up to 50 ml with ultrapure water. In the acidic – filtered water samples, a total of 3 metal(loid)s (B and Se) were determined with an inductively coupled plasma – mass spectrometry (ICP – MS) in the laboratory of Technology Research Development Application and Research Centre of Trakya University – an international accreditation certificated institution. All the element analyses were determined as means of triplicate reads (TS EN / ISO IEC 17025) (EPA, 2001; APHA, 2005). The accuracy of analytical method was controlled by using a certified reference material (CRM) (CPAchem – Ref Num: 110580.L1).

**Table 1.** Coordinate information and the locations of the investigated stations

| Station Code | Location              | GPS – North | GPS – East |
|--------------|-----------------------|-------------|------------|
| İ1           | Ahırköy Village       | 40.894      | 26.374     |
| İ2           | Paşaköy Village       | 40.850      | 26.320     |
| İ3           | Yenikarpuzlu Town     | 40.832      | 26.295     |
| İ4           | Kumdere Village       | 40.866      | 26.368     |
| İ5           | Esetçe Town           | 40.871      | 26.443     |
| İ6           | Aliçopehlivan Village | 40.841      | 26.440     |
| İ7           | Kocahıdır Village     | 40.809      | 26.407     |
| İ8           | Küçük Doğanca Village | 40.808      | 26.430     |
| İ9           | Yapıldak Village      | 40.791      | 26.442     |
| İ10          | Koyuntepe Village     | 40.768      | 26.343     |
| İ11          | Turpçular Village     | 40.941      | 26.434     |
| İ12          | Hıdırköy Village      | 40.914      | 26.461     |
| İ13          | Sarpdere Village      | 40.888      | 26.431     |
| İ14          | Korucu Village        | 40.900      | 26.496     |
| İ15          | Pazardere Village     | 40.979      | 26.580     |
| İ16          | Hacıköy Town          | 40.981      | 26.550     |
| İ17          | İbriktepe Town        | 41.012      | 26.505     |
| İ18          | Karaağaç Village      | 40.941      | 26.434     |
| İ19          | Hıdırköy Village      | 40.915      | 26.461     |
| İ20          | Sultanköy Town        | 41.025      | 26.453     |
| İ21          | Balabancık Village    | 41.033      | 26.404     |
| İ22          | Sarıcaali Village     | 40.985      | 26.382     |
| İ23          | İpsala District       | 40.913      | 26.376     |



**Figure 1.** Topographic map of Ipsala District and selected lentic habitats

## Health Risk Assessment

In the current research, population was divided into four age groups based on their physiological and behavioural differences as follow: infants (< 2 years old), children (2 – 6 years old), teenagers (6 – 16 years old) and adults (> 16 years old). The daily exposure to boron and selenium were calculated in these four different groups by using the equation 1. Hazard Quotient (HQ) describes the non-carcinogenic risk of b, Se and Mn and it was calculated by using the equation 2 (Liu et al., 2018; Zango et al., 2019; Ghosh et al., 2020). A lower value of HQ than 1 reflects a negligible risk of non-carcinogenic effects and a higher value of HQ than one reflects an important health risk.

$$EDI = \frac{C_{sample} \times C_d}{B_w} \quad (1)$$

$$HQ = \frac{EDI}{RfD} \quad (2)$$

EDI: Estimation of fluoride consumption – daily (mg/kg/day)

C<sub>sample</sub>: B and Se levels in the investigated water samples (mg/L)

C<sub>d</sub>: Mean daily water intake (L/day) (the mean water consumption rates in infants, children, teenagers and adults were 0.1, 0.85, 2 and 2.5 L/day, respectively)

B<sub>w</sub>: Body weight (kg) (body weights of investigated groups were considered 5, 10, 50 and 75 kg, respectively)

RfD: Reference dose (mg/kg/day) (0.13 and 0.005 mg/kg/day for B and Se respectively) (EPA, 1992)

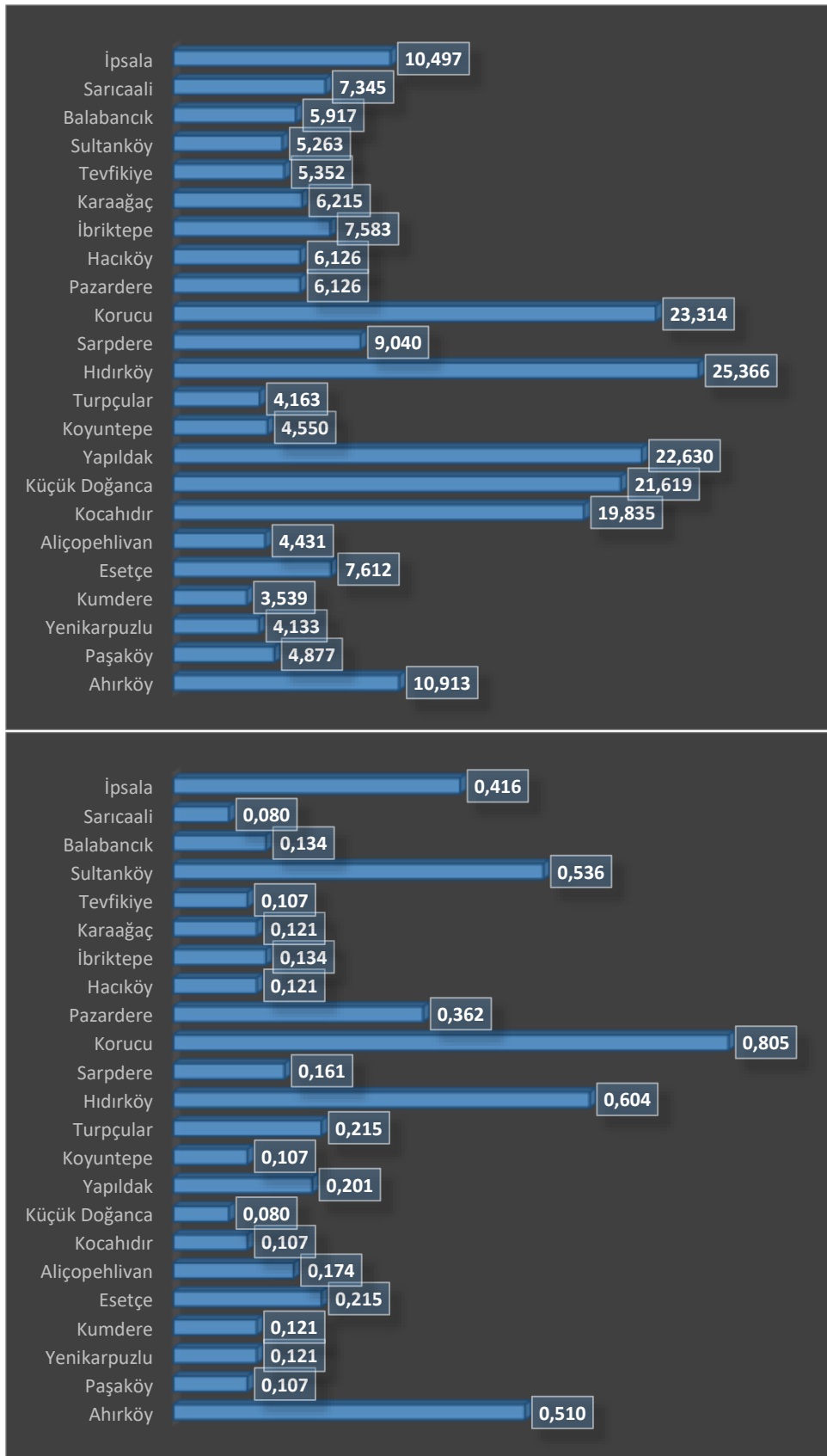
## RESULTS AND DISCUSSION

Boron and selenium accumulation levels in tap – drinking water of investigated villages are given in Figure 2 and detected EDI and HQ coefficients in the tap water samples are given in Table 2.

Boron concentrations were varied from 3.539 (Kumdere Village) – 25.366 (Hıdırköy Village) ppb with an average of 9.845 ppb; and selenium concentrations were varied from 0.080 (Küçük Doğanca Village) – 0.805 (Korucu Village) ppm with an average of 0.241 ppb.

All the calculated HQ values in all the investigated locations in terms of all the investigated age groups were recorded as less than the critical limit of 1 with a spatial mean of 0.00151 and 0.00004 for infants; 0.00644 and 0.00016 for children; 0.00303 and 0.00007 for teenagers; and 0.00252 and 0.00006 for adults in terms of boron and selenium respectively.

The EDI and HQ risk rankings among the investigated different age groups were found as follows: children > teenagers > adults > infants in all the investigated stagnant water bodies including natural and dam lakes, in general. In many of researches on the assessment of probable health risks associated with boron, selenium and manganese in water performed in many different countries confirmed that the probable chronic health risks in children due to the intake of these elements are more possible than the other age groups (Liu et al., 2018; Zango et al., 2019; Ghosh et al., 2020). In this research, as similar with the literature data, children were found as the riskiest age group for the probable health risk due to the intake of boron and selenium in tap – drinking water İpsala District.



**Figure 2.** Boron (up) and selenium (down) levels (ppb) in drinking water samples

**Table 2.** EDI – HQ values of boron

|             | EDI     |          |           |         | HQ      |          |           |         |
|-------------|---------|----------|-----------|---------|---------|----------|-----------|---------|
|             | Infants | Children | Teenagers | Adults  | Infants | Children | Teenagers | Adults  |
| <b>İ-1</b>  | 0.00022 | 0.00093  | 0.00044   | 0.00036 | 0.00168 | 0.00714  | 0.00336   | 0.00280 |
| <b>İ-2</b>  | 0.00010 | 0.00041  | 0.00020   | 0.00016 | 0.00075 | 0.00319  | 0.00150   | 0.00125 |
| <b>İ-3</b>  | 0.00008 | 0.00035  | 0.00017   | 0.00014 | 0.00064 | 0.00270  | 0.00127   | 0.00106 |
| <b>İ-4</b>  | 0.00007 | 0.00030  | 0.00014   | 0.00012 | 0.00054 | 0.00231  | 0.00109   | 0.00091 |
| <b>İ-5</b>  | 0.00015 | 0.00065  | 0.00030   | 0.00025 | 0.00117 | 0.00498  | 0.00234   | 0.00195 |
| <b>İ-6</b>  | 0.00009 | 0.00038  | 0.00018   | 0.00015 | 0.00068 | 0.00290  | 0.00136   | 0.00114 |
| <b>İ-7</b>  | 0.00040 | 0.00169  | 0.00079   | 0.00066 | 0.00305 | 0.01297  | 0.00610   | 0.00509 |
| <b>İ-8</b>  | 0.00043 | 0.00184  | 0.00086   | 0.00072 | 0.00333 | 0.01414  | 0.00665   | 0.00554 |
| <b>İ-9</b>  | 0.00045 | 0.00192  | 0.00091   | 0.00075 | 0.00348 | 0.01480  | 0.00696   | 0.00580 |
| <b>İ-10</b> | 0.00009 | 0.00039  | 0.00018   | 0.00015 | 0.00070 | 0.00297  | 0.00140   | 0.00117 |
| <b>İ-11</b> | 0.00008 | 0.00035  | 0.00017   | 0.00014 | 0.00064 | 0.00272  | 0.00128   | 0.00107 |
| <b>İ-12</b> | 0.00051 | 0.00216  | 0.00101   | 0.00085 | 0.00390 | 0.01659  | 0.00780   | 0.00650 |
| <b>İ-13</b> | 0.00018 | 0.00077  | 0.00036   | 0.00030 | 0.00139 | 0.00591  | 0.00278   | 0.00232 |
| <b>İ-14</b> | 0.00047 | 0.00198  | 0.00093   | 0.00078 | 0.00359 | 0.01524  | 0.00717   | 0.00598 |
| <b>İ-15</b> | 0.00012 | 0.00052  | 0.00025   | 0.00020 | 0.00094 | 0.00401  | 0.00188   | 0.00157 |
| <b>İ-16</b> | 0.00012 | 0.00052  | 0.00025   | 0.00020 | 0.00094 | 0.00401  | 0.00188   | 0.00157 |
| <b>İ-17</b> | 0.00015 | 0.00064  | 0.00030   | 0.00025 | 0.00117 | 0.00496  | 0.00233   | 0.00194 |
| <b>İ-18</b> | 0.00012 | 0.00053  | 0.00025   | 0.00021 | 0.00096 | 0.00406  | 0.00191   | 0.00159 |
| <b>İ-19</b> | 0.00011 | 0.00045  | 0.00021   | 0.00018 | 0.00082 | 0.00350  | 0.00165   | 0.00137 |
| <b>İ-20</b> | 0.00011 | 0.00045  | 0.00021   | 0.00018 | 0.00081 | 0.00344  | 0.00162   | 0.00135 |
| <b>İ-21</b> | 0.00012 | 0.00050  | 0.00024   | 0.00020 | 0.00091 | 0.00387  | 0.00182   | 0.00152 |
| <b>İ-22</b> | 0.00015 | 0.00062  | 0.00029   | 0.00024 | 0.00113 | 0.00480  | 0.00226   | 0.00188 |
| <b>İ-23</b> | 0.00021 | 0.00089  | 0.00042   | 0.00035 | 0.00161 | 0.00686  | 0.00323   | 0.00269 |

**Table 3.** EDI – HQ values of selenium

|             | EDI     |          |           |         | HQ      |          |           |         |
|-------------|---------|----------|-----------|---------|---------|----------|-----------|---------|
|             | Infants | Children | Teenagers | Adults  | Infants | Children | Teenagers | Adults  |
| <b>İ-1</b>  | 0.00001 | 0.00004  | 0.00002   | 0.00002 | 0.00008 | 0.00033  | 0.00016   | 0.00013 |
| <b>İ-2</b>  | 0.00000 | 0.00001  | 0.00000   | 0.00000 | 0.00002 | 0.00007  | 0.00003   | 0.00003 |
| <b>İ-3</b>  | 0.00000 | 0.00001  | 0.00000   | 0.00000 | 0.00002 | 0.00008  | 0.00004   | 0.00003 |
| <b>İ-4</b>  | 0.00000 | 0.00001  | 0.00000   | 0.00000 | 0.00002 | 0.00008  | 0.00004   | 0.00003 |
| <b>İ-5</b>  | 0.00000 | 0.00002  | 0.00001   | 0.00001 | 0.00003 | 0.00014  | 0.00007   | 0.00006 |
| <b>İ-6</b>  | 0.00000 | 0.00001  | 0.00001   | 0.00001 | 0.00003 | 0.00011  | 0.00005   | 0.00004 |
| <b>İ-7</b>  | 0.00000 | 0.00001  | 0.00000   | 0.00000 | 0.00002 | 0.00007  | 0.00003   | 0.00003 |
| <b>İ-8</b>  | 0.00000 | 0.00001  | 0.00000   | 0.00000 | 0.00001 | 0.00005  | 0.00002   | 0.00002 |
| <b>İ-9</b>  | 0.00000 | 0.00002  | 0.00001   | 0.00001 | 0.00003 | 0.00013  | 0.00006   | 0.00005 |
| <b>İ-10</b> | 0.00000 | 0.00001  | 0.00000   | 0.00000 | 0.00002 | 0.00007  | 0.00003   | 0.00003 |
| <b>İ-11</b> | 0.00000 | 0.00002  | 0.00001   | 0.00001 | 0.00003 | 0.00014  | 0.00007   | 0.00006 |
| <b>İ-12</b> | 0.00001 | 0.00005  | 0.00002   | 0.00002 | 0.00009 | 0.00039  | 0.00019   | 0.00015 |
| <b>İ-13</b> | 0.00000 | 0.00001  | 0.00001   | 0.00001 | 0.00002 | 0.00011  | 0.00005   | 0.00004 |
| <b>İ-14</b> | 0.00002 | 0.00007  | 0.00003   | 0.00003 | 0.00012 | 0.00053  | 0.00025   | 0.00021 |
| <b>İ-15</b> | 0.00001 | 0.00003  | 0.00001   | 0.00001 | 0.00006 | 0.00024  | 0.00011   | 0.00009 |
| <b>İ-16</b> | 0.00000 | 0.00001  | 0.00000   | 0.00000 | 0.00002 | 0.00008  | 0.00004   | 0.00003 |

|             |         |         |         |         |         |         |         |         |
|-------------|---------|---------|---------|---------|---------|---------|---------|---------|
| <b>İ-17</b> | 0.00000 | 0.00001 | 0.00001 | 0.00000 | 0.00002 | 0.00009 | 0.00004 | 0.00003 |
| <b>İ-18</b> | 0.00000 | 0.00001 | 0.00000 | 0.00000 | 0.00002 | 0.00008 | 0.00004 | 0.00003 |
| <b>İ-19</b> | 0.00000 | 0.00001 | 0.00000 | 0.00000 | 0.00002 | 0.00007 | 0.00003 | 0.00003 |
| <b>İ-20</b> | 0.00001 | 0.00005 | 0.00002 | 0.00002 | 0.00008 | 0.00035 | 0.00017 | 0.00014 |
| <b>İ-21</b> | 0.00000 | 0.00001 | 0.00001 | 0.00000 | 0.00002 | 0.00009 | 0.00004 | 0.00003 |
| <b>İ-22</b> | 0.00000 | 0.00001 | 0.00000 | 0.00000 | 0.00001 | 0.00005 | 0.00002 | 0.00002 |
| <b>İ-23</b> | 0.00001 | 0.00004 | 0.00002 | 0.00001 | 0.00006 | 0.00027 | 0.00013 | 0.00011 |

## CONCLUSIONS

In the current research, spatial variations of boron and selenium concentrations and the probable non-carcinogenic health risks of these elements via daily human intake in tap – drinking water of 23 selected locations – villages located in the İpsala District in the Meriç Plain of Thrace Region. As a result of this study, the detected HQ values of all age groups were found as less than the critical limit of 1 and the risk rankings among the investigated different age groups were found as follows: children > teenagers > adults > infants, in general.

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## **PHENO-MORPHOLOGICAL STUDY BETWEEN FIVE VARIETIES OF QUINOA (*CHENOPODIUM QUINOA* WILLD.) IN SEMI ARID REGION OF ALGERIA**

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### **ABSTRACT**

Quinoa (*Chenopodium quinoa* Willd.) has been cultivated for several thousand years in South America, It is a highly nutritious food product, with a high mineral content. Quinoa is now considered an alternative to traditional crops in a climate change scenario, given its more stability to adapt to marginal soils, droughts and frosts. Despite the interesting agronomic and nutritional characteristics of this crop, quinoa is characterized by individual attempts to define its phenological stages. This work consists of evaluating the phenological, and morphological behavior of five quinoa varieties in arid region. The research demonstrated considerable variation across genotypes in a range of morphological characters, which are useful as indicators in characterizing quinoa plants, including height in (cm) and color of the stem, morphology of the leaves (size, serration), and color of the seeds. Genotypic differences were noted. The varieties have more favorable characteristics.

**Keywords:** *Chenopodium quinoa* Willd., Adaptation, Genetic diversity, Morphology, Phenological stages, Drylands.

### **INTRODUCTION**

Quinoa (*Chenopodium quinoa* Willd.), is a pseudocereal native from the Andean region of South America that has increased in importance worldwide. Quinoa is now considered an alternative to traditional crops in a climate change scenario, considering its ability to adapt to marginal soils, droughts and frosts. Quinoa cultivars present a high genetic variability in many regions of the world, to be adapted to their climatic properties, including drought and high salinity. Our study is devoted to a comparison between five varieties of quinoa by evaluating the pheno-morphological behavior of these varieties in arid condition by drip irrigation.

### **MATERIALS AND METHODS**

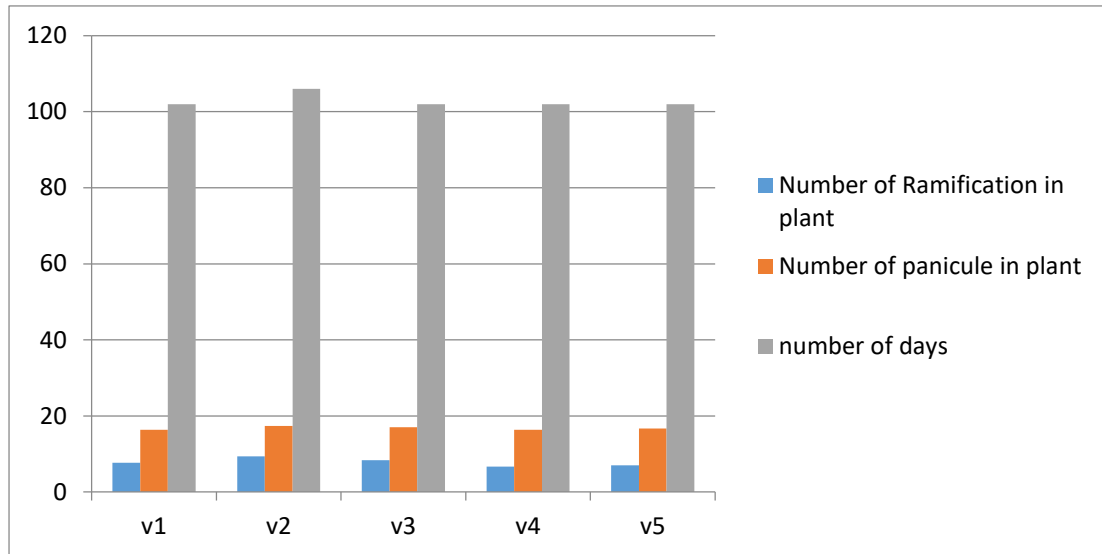
#### **Pheno-morphological study**

This work deals with the study of five varieties of quinoa (*Chenopodium quinoa* willd.). These varieties are V1: Q2, V2: Q12, V3: Q29, V4: Q18, V5: Q26. The experimentation is carried out in the field at arid conditions By drip irrigation between February and mid April where the temperature varies between 20-30c°. The studied characters that correspond to the number of days from sowing until panicle formation are the height in (cm) and the color of the stem, the morphology of the leaves (size and serration), and the color of the seeds



## RESULTS

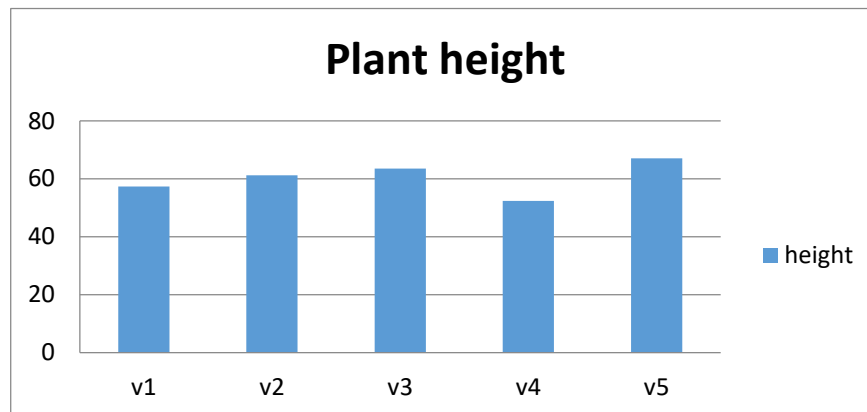
### Phenological parameters



**Fig.1.** Phenological phases of five genotypes of *chenopodium quinoa* willd.

### 3.2. Morphological parameters

**Plant height:** Our results showed that the height of the plant was variable between genotypes:



**Fig.2.** The height of five genotypes of *Chenopodium quinoa* willd.

## Morphological characters

**Table 1.** Study of the morphological characters of the plant *Chenopodium quinoa* willd.

|                          | V1           | V2                         | V3     | V4                  | V5         |
|--------------------------|--------------|----------------------------|--------|---------------------|------------|
| <b>Color of the seed</b> | Creamy white | White, raspberry and brown | Ochre  | Beige and raspberry | Beige      |
| <b>Color of the stem</b> | Green        | Green                      | purple | purple              | purple     |
| <b>Leafcolor</b>         | medium green | dark green                 | purple | dark green          | dark green |
| <b>Leaf size</b>         | large        | medium                     | large  | medium              | large      |
| <b>Leafserration</b>     | weak         | strong                     | weak   | medium              | medium     |

## DISCUSSION

### Phenological characteristics

The number of days is used as an indicator of earliness. The length of the cycle of the tested varieties varies between 106 days and 102 days. We distinguish among the tested varieties:

- early genotypes v1,v3,v4,v5
- late genotype is v2

### Morphological characteristics

**The height of the stem:** presents a significant variability, the variety v5 had the greatest height (67 cm), while the stem varieties from 63.5 cm (v3) to v4 (with short stem (52.3 cm).

## CONCLUSIONS

The different varieties studied behave differently By drip irrigation in arid conditions, especially in height and number of days, the number of favorable characteristics for drought tolerance, taking into account only the varieties ranked first and second V5:Q26 and V3:Q29 compared to genotypes V1:Q2,V2:Q12 V4:Q18, .In order to escape periods of drought, the plant resorts to a lengthening of the cycle during the first stages of growth while it follows other strategies to tolerate stress, mainly through the elasticity of its tissues, its potential low osmotic and the formation of special vesicular glands and small cells with a thick wall. This plant is characterized by a very spreading root system on the surface and which can be deep in the ground.

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## EFFECTS OF VITAMIN C AND ZINC ON FURAN INDUCED TOXICITY IN TM4 SERTOLI CELLS

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### ABSTRACT

Furan (Fu) or furfuran is a colorless, volatile heterocyclic aromatic organic compound formed during heat treatment and storage techniques of food products. Fu is also used industrially as an intermediate in the production of certain compounds such as resins, varnishes, pesticides, and drugs. It has been determined that Fu, which is inevitably taken into the body, negatively affects human health and causes toxicity. Fu has potential harmful effects on the skin, liver, kidney, immune system, nervous system and adipose tissue. In addition to its effects that disrupt the endocrine system, it also affects the reproductive system and causes infertility. However, there are not enough studies on the toxicological effects of Fu on the male reproductive system especially Sertoli cells. Free radicals damage the cell and the organism by entering into rapid oxidation reactions with food components. Vitamin C (VitC) and zinc (Zn) contribute to minimizing the effect of free radicals and protects the cell from oxidative damage. In this study, Fu at a concentration of 3 mM and in addition to Fu, 50 µM VitC and 50 µM Zn were applied to TM4 Sertoli cells for 24 hours. After the applications, cell viability, lipid peroxidation amount, antioxidant enzyme activities such as superoxide dismutase, catalase, glutathione peroxidase and apoptotic cell ratios were examined in Sertoli cells. The findings revealed that Fu decreased cell viability and antioxidant enzyme activities, while increasing lipid peroxidation and apoptotic cell rates. In addition to Fu, it has been determined that VitC and Zn applications minimize this damage caused by Fu in conditions where VitC and Zn are applied together. These findings revealed the toxicity of Fu on the TM4 Sertoli cell, which plays an important role in the male reproductive system, and the protective role of antioxidant VitC and Zn against this toxicity.

**Keywords:** Furan, Sertoli cells, Zinc, Vitamin C, Oxidative damage, Apoptosis.

### INTRODUCTION

Furan (Fu) is a liquid, colorless, and volatile compound formed during processing and storage techniques, including heat treatment of food products. Fu, which is found in high amounts in heat-treated food products such as coffee, baby food, fruit juices, jars and canned foods, is inevitable to be taken into the body (Seok and Lee, 2019). Also, the use of Fu in the chemical industry is also common. According to studies, it has been determined that Fu negatively affects human health and causes toxicity. As a result of these studies, it has been classified as a "possible human carcinogen (Group 2B)" by the International Agency for Research on Cancer (Seok et al., 2015). For this reason, it is extremely important to study the effects of Fu on the body. Despite the widespread use of Fu and the studies of various

international organizations to set limits for Fu exposure, there are very few studies on Fu toxicity in the literature.

Fu can be rapidly and intensely absorbed from the lungs and small intestine, passing through the biological membrane and entering various organs (Alizadeh et al., 2018). Fu can cause harmful effects on adipose, kidney, and liver tissue. If the critical value of dibenzofurans accumulating in human adipose tissue is exceeded, water enters the cells, causing an increase in reactive oxygen radicals. These radicals cause oxidative stress in cells, causing mutations in DNA and protein structures, apoptosis, disruption of cellular structure and lipid peroxidation in cellular membranes (Seok et al., 2015; Rehman et al., 2020; Alizadeh et al., 2018). If these effects are considered in terms of the male reproductive system, oxidative stress is high in testicular tissue due to the low amount of oxygen in the testicles. Because the testis has the potential to generate large amounts of unsaturated fatty acids and reactive oxygen species. As a result, increased oxidative stress can have very harmful effects on spermatogenesis and steroidogenesis (Asadi et al., 2017). In addition, studies have shown that Fu causes disorders in the testis, epididymis, and prostate gland, has harmful effects on sperm count and morphology, and significantly increases apoptotic cells in the testis (Rehman et al., 2019). These results indicate that exposure to Fu induces toxicity of the male reproductive system.

It is known that VitC, which has water-soluble and chain-breaking antioxidant properties, is an important micronutrient in plants and animals (Kökoğlu and Alturfan, 2010). It shows its antioxidant effect by increasing the activity of enzymes. VitC reduces the formation of free radicals and protects the thiols of proteins that form antioxidant compounds and is also a powerful antioxidant both in the plasma and inside the cell (Halliwell and Whiteman, 1998). Studies have shown that VitC inhibits lipid peroxidation, reduces endothelial dysfunction, and regenerates vitamin E in lipoproteins and membranes (Evans, 2000). These results showed that VitC has a protective effect on the male reproductive system, DNA methylation, and central nervous system (Rizk et al., 2020; Huff et al., 2021). Oxidative stress is a metabolic dysfunction that causes oxidative damage to cells and tissues and causes many chronic diseases. Zn, in particular, is one of the essential minerals for human health and a trace element that cannot be stored in the body, as it acts as a cofactor for more than 300 enzymes and 2000 transcription factors due to its antioxidant properties (Bhowmik et al., 2010). Studies have determined that Zn, an anti-inflammatory agent, is an important part of cellular signaling because it provides structural stability to cell membranes and is also a regulator of gene expression. Also, this element can be replaced at certain binding sites as a redox active metal and can alleviate cellular site-specific oxidative damage (Yamaguchi et al., 2009). In studies, it has been discovered that due to the antioxidant effect of Zn, it increases the sperm quality and fertilization potential in men. In addition, Zn has been found to help hormones such as testosterone in the male reproductive system as a hormone balancer. In many studies, it has been emphasized that Zn deficiency inhibits spermatogenesis, causes sperm abnormalities, and has a negative effect on serum testosterone concentration (Prasad, 2008; Boran and Özkan, 2004).

In this study, it was aimed to reveal the possible protective roles of VitC and Zn, which are known to have strong antioxidant properties against the toxicity that Fu may cause in Sertoli cells, by measuring cell viability, lipid peroxidation, antioxidant enzymes, and apoptosis.

## **MATERIAL AND METHODS**

### **Cell Culture and Experimental Grouping**

In this study, the TM4 cell line used in cell culture experiments was the Sertoli cell line isolated from the testes of 11–13-day old mice. This cell line was supplied to our laboratory

from the ATCC (American Type Culture Collection): Global Biological Resource Center and was grown with regular passages once or twice a week. Sertoli cells were grown in 50:50 DMEM/F12 medium in flasks supplemented with 5% horse serum, 2.5% fetal bovine serum, and 1% penicillin-streptomycin-amphotericin in an incubator containing 5% CO<sub>2</sub> and 95% air.

First, a stock solution of Fu was prepared by dissolving it in 100 % ethanol before being applied at various concentrations to the TM4 Sertoli cell line. Then, Fu concentrations of 50, 100, 250, 500, 1000, 2500, 3000, and 6000 µM were applied to the cells for 24 h by dilution from the stock solution with 0.1% ethanol. A Fu concentration of 3000 µM, which reduced cell viability to 80%, was chosen for use in other experiments. A medium containing 0.1% ethanol was applied to the control group. The concentration of Zn and VitC to be administered was determined as 50 µM, which was used as the average antioxidant concentration in *in vitro* studies with various cell lines (Orta and Erkan, 2014; Gürbay, 2012). Zn and VitC dissolved in the medium were prepared freshly before each experiment. The experiment was divided into nine groups: Control, Fu, VitC, Fu+VitC, Zn, Fu+Zn, VitC+Zn and Fu+VitC+Zn.

### **Assessment of Cell Viability**

MTT assay was performed to determine the changes in cell viability due to exposure of TM4 Sertoli cells proliferating under appropriate conditions to concentrations of Fu, VitC, and Zn prepared alone or together. The obtained cells were seeded in 96-well culture dishes at  $5 \times 10^3$  cells per well and incubated overnight at 37°C in an incubator containing 5% CO<sub>2</sub>. The next day, the prepared Fu, VitC, and Zn concentrations were applied to the cells and incubated for 24 hours. At the end of the exposure period, 10 µl of MTT I solution was added to each well and incubated at 37°C for 4 hours. Then, 100 µl of MTT II solution (SDS) was added to each well and incubated overnight. The optical densities of cells were measured in a spectrophotometer at a wavelength of 540 nm. The cell viability of the control group was accepted as 100% and the cell viability of the other experimental groups was calculated as a percentage compared to the control. This test was designed as three independent experiments, each with three replicates.

### **Biochemical Experiments**

Sertoli cells ( $5 \times 10^5$ ) were seeded in six-well culture plates to perform biochemical analyses. For 24 h, Fu, Zn, and VitC were applied to the cells at the determined concentrations, either alone or in combination. Then, the membranes of the cells transferred to cold tris buffer (Tris-HCl, pH:7.2) were disrupted by the ultrasonicator. The suspension of lysed cells was centrifuged at 14,000 g in a refrigerated centrifuge (Nüve). Supernatants were collected and stored at -80°C for analysis of total protein, lipid peroxidation, catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) activity.

### **Determination of Membrane Lipid Peroxidation**

Lipid peroxidation was determined by the assay of malondialdehyde (MDA) according to the method of Heath and Packer (1968). The principle of the method is based on measuring the amount of the pink-colored compound formed as a result of the reaction of MDA, a product of polyunsaturated fatty acid peroxidation in cells, with thiobarbituric acid (TBA) in a hot and acidic pH, at a wavelength of 532 nm in a spectrophotometer.

### **Measurement of Total Protein**

Protein concentrations of the samples were determined by a bicinchoninic acid (BCA) assay. The Smart BCA Protein Assay Kit was used for the BCA assay. The principle of this test is based on the colorimetric detection of the purple-colored reaction product formed as a result of the binding of a copper ion to two BCA molecules, thus determining the amount of protein. This purple-colored reaction product gives a linearly strong absorbance with increasing protein amount at 562 nm.

### **Determination of SOD Activity**

Total SOD enzyme activity was evaluated using the method of Marklund and Marklund (1974). The experimental principle is based on the inhibition of pyrogallol autoxidation by the SOD enzyme at alkaline pH. One unit of SOD activity is defined as the amount of enzyme required to reduce pyrogallol autoxidation by 50%. After the applied method, the varying absorbance of the test solution was read in a spectrophotometer at 420 nm at seven time points in total, at 30 second intervals for 3 min.

### **Determination of CAT Activity**

CAT enzyme activity was determined according to the method of Sinha (1972). The enzyme CAT, one of the antioxidants, catalyzes the decomposition of hydrogen peroxide, which causes damage to the cell, into water and molecular oxygen, as it is a very stable reactive oxygen species. The principle of this method is based on the reduction of dichromate in acetic acid to chromic acetate by heating in the presence of H<sub>2</sub>O<sub>2</sub>, and the transformation of the dark blue-purple color formed by dichromate acetic acid with H<sub>2</sub>O<sub>2</sub> into light green with heat. The resulting test mixtures were read in the spectrophotometer at 570 nm against a blank.

### **Determination of GPx Activity**

The GPx enzyme catalyzes the decomposition reaction of H<sub>2</sub>O<sub>2</sub>, which forms oxidized glutathione and water in the presence of reduced glutathione. In the determination of GPx enzyme level, the method of Hafeman (1974) using H<sub>2</sub>O<sub>2</sub> as a substrate was applied. The absorbance values of the test mixtures were read in the spectrophotometer at 412 nm against a blank.

### **Determination of Apoptosis by Double Fluorescent Staining (Propidium Iodide/Hoechst) Method**

In the determination of apoptosis, simultaneous double fluorescent staining was used to mark apoptotic cells using Hoechst 33342 (Ho342) and to mark dead cells using Propidium iodide (PI) dyes. Accordingly, cells were seeded at 10<sup>4</sup> cells per well in 24-well culture plates and incubated overnight. The next day, the assay concentrations were applied to the cells and incubated again for 24 h. At the end of the exposure time, the cells were first washed with PBS solution. Then, the experimental mixture prepared with 1 mg/ml PI, 1 mg/ml Ho342 and 1M PBS (pH 7.4) was added to each well as 0.2 ml and incubated at 37°C for 15–30 min. During the incubation, the Ho342 fluorescent dye in the assay mix marks the apoptotic cells in dark-blue, while the PI fluorescent dye marks the dead cells in red. At the end of this period, the cells were rinsed again with PBS and examined under a fluorescent microscope. The Olympus IX71 was examined with a UVB filter on an inverted microscope with fluorescent attachment and

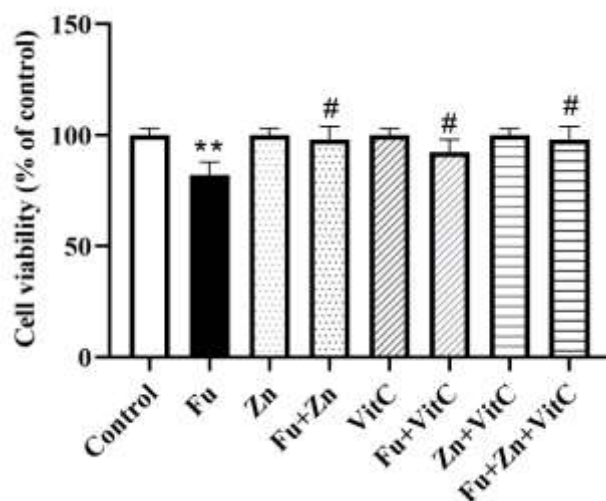
photographed in series with an Olympus DP72 video camera at equal intervals. Live, apoptotic, and dead cells were counted by scanning 10-15 fields in 12 wells and 1000 cells, per well in three different experiments for each group. For each group, the ratio of live, apoptotic, and dead cells to all cells was calculated as percentiles.

### Statistical Analysis

The GraphPad Prism 9.0 program was used for the statistical evaluation of the changing data of cell viability, cell cytotoxicity, total protein amount, malondialdehyde amount, CAT enzyme, SOD enzyme, and glutathione peroxide enzyme levels depending on the application of Fu, VitC and Zn in determined concentrations alone or in combination (GraphPad Prism Software, San Diego, California, USA). The obtained data were statistically analyzed and expressed as mean standard deviation using Tukey's multiple comparison test and one-way ANOVA. Results were evaluated with reference to the significance levels of  $p < 0.001$ ,  $p < 0.01$ ,  $p < 0.05$ .

## RESULTS AND DISCUSSION

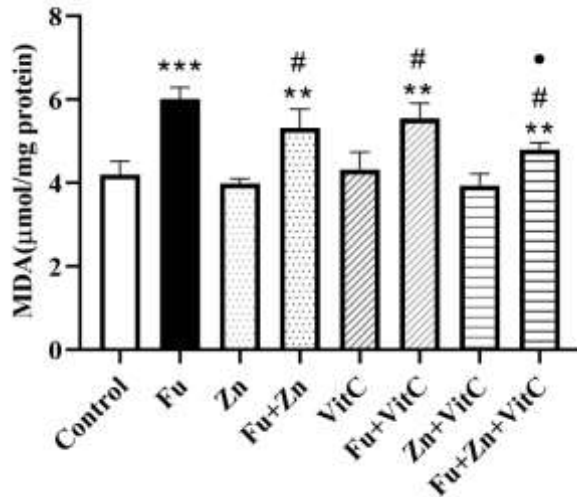
There are very limited studies on cell viability at the cellular level to determine Fu toxicity (Barros et al., 2019; Karacaoğlu et al., 2010). This *in vitro* study with Sertoli cells found that 3 mM Fu significantly reduced cell viability, which is consistent with a small number of studies in the literature that found significant reductions in cell viability as a result of Fu administration (Figure 1). Additionally, it was found in our study that applying Zn and VitC to Fu as antioxidants increased cell viability significantly. These findings suggest that using Zn and VitC together in Fu toxicity may improve cell viability.



**Figure 1.** Effects of Fu, VitC, and Zn on the viability rate in TM4 Sertoli cells. (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; #: compared with Fu).

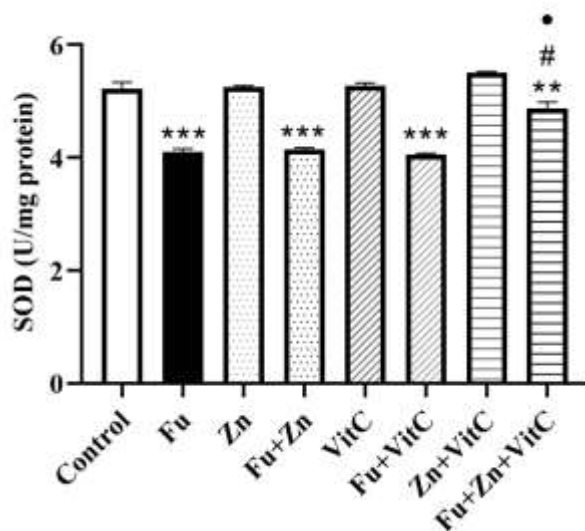
The effects of Fu on lipid peroxidation have been the subject of numerous *in vivo* investigations (Karacaoğlu et al., 2010; Wang et al., 2014; El-Hakim et al., 2018). In this study, we found that Fu significantly increased lipid peroxidation in Sertoli cells, similar to *in vivo* studies investigating Fu toxicity (Figure 2). In addition, Zn and VitC such as lycopene, spirulina platensis extract, garlic oil, and quercetin, which have been found to have curative roles in Fu toxicity in the literature, can also be used singly or in combination to treat lipid peroxidation (El-Hakim et al., 2018; Kara et al., 2016; El Akabawy and El-Sherif, 2016; Alam et al., 2017).



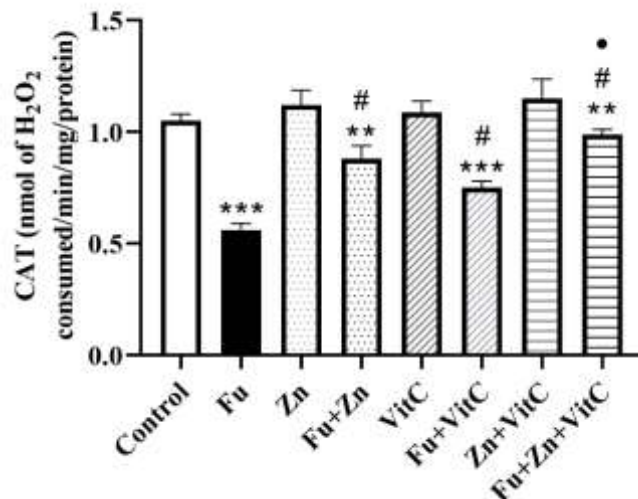


**Figure 2.** Effects of Fu, VitC, and Zn on lipid peroxidation in TM4 Sertoli cells. (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; #: compared with Fu; •: compared with Fu+Zn group).

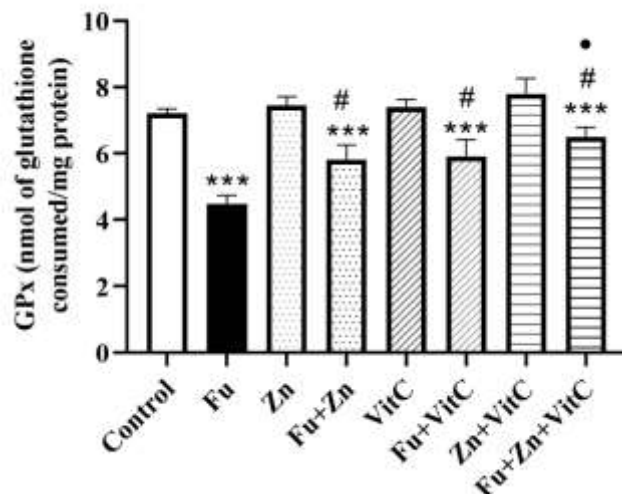
In biological systems under oxidative stress, levels of intracellular enzymes including CAT, SOD, glutathione peroxidase, and glutathione rise, and the antioxidant defense system is triggered (Peltola et al., 1992). According to studies (Wang et al., 2014; Bař and Pandır, 2016; El Akabawy and El-Sherif, 2016; Rehman et al., 2019), Fu suppresses the antioxidant defense system. In Sertoli cells, it was observed that Fu significantly decreased the activity of the enzymes SOD, CAT, and GPx. When the effects of Zn and VitC supplied as antioxidants on this Fu damage were evaluated, it was shown that they did not have substantial antioxidant qualities like garlic oil and lycopene, but they improved the effects of antioxidant enzymes when applied jointly (Figure 3-5).



**Figure 3.** Effects of Fu, VitC, and Zn on SOD activities in TM4 Sertoli cells. (\*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; #: compared with Fu; •: compared with Fu+Zn group).



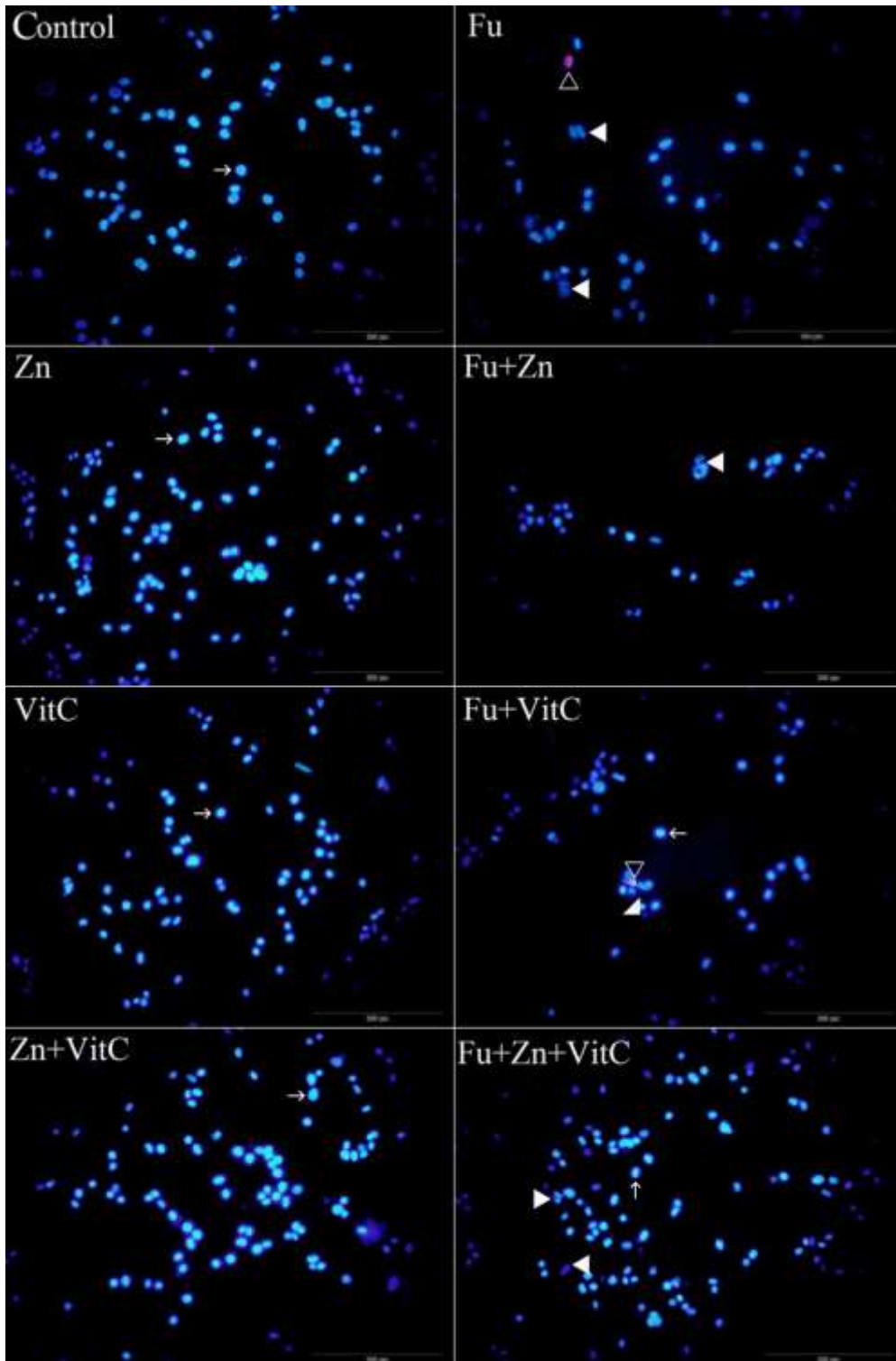
**Figure 4.** Effects of Fu, VitC, and Zn on CAT activities in TM4 Sertoli cells. (\*\*p<0.01; \*\*\*p<0.001; \*: compared with controls; #: compared with Fu; •: compared with Fu+Zn group).



**Figure 5.** Effects of Fu, VitC, and Zn on GPx activities in TM4 Sertoli cells. (\*\*\*p<0.001; \*: compared with controls; #: compared with Fu; •: compared with Fu+Zn group).

Cell homeostasis and energy-dependent biochemical pathways are both involved in the complex process of apoptosis, also referred to as programmed cell death. Oxidative stress, which occurs as a result of exposure to substances like environmental pollutants and hormone-disrupting chemicals, causes cells to undergo apoptosis. Oxidative stress damages the mitochondrial membrane, which results in apoptosis (Jin and El Deiry, 2005). Various apoptotic processes have been implicated by numerous investigations as the mechanism of action of Fu (Chen et al., 2010; Zhang et al., 2015; El-Hakim et al., 2018). The findings of this work using TM4 Sertoli cells support previous *in vivo* and *in vitro* investigations on this topic, showing that Fu induces apoptosis by raising the proportion of apoptotic cells in Sertoli cells. The findings of this study using TM4 Sertoli cells support previous *in vivo* and *in vitro* investigations on this topic since Fu causes apoptosis by increasing the number of apoptotic cells in Sertoli cells. According to a review of the literature, this study is the first one to address the utilization of different antioxidant molecules in the reduction of Fu-induced apoptosis. The administration of

Zn, VitC, and their combination all considerably reduce Fu-induced apoptosis, according to the findings of this study (Figure 6 and Table 1).



**Figure 6.** Effects of Fu on viable, apoptotic and dead cells in Sertoli cells after 24 hours in control, Fu, vitC and Zn groups (▲: viable cells, □: Apoptotic cells, ▽: Dead cells).

**Table 1:** Effects of Fu, VitC and Zn on viable, apoptotic and dead cells in TM4 Sertoli cells after 24 h (\*: compared to controls, #: compared to Fu, •: compared to Fu+Zn group; \*p<0.05, #p<0.05, •p<0.05).

| Groups     | Viable Cells          | Apoptotic Cells        | Dead Cells           |
|------------|-----------------------|------------------------|----------------------|
| Control    | 98.1±0.9              | 0.9±0.0                | 1.0±0.1              |
| Fu         | 81.5±0.5**            | 16.2±0.3***            | 2.3±0.2**            |
| Zn         | 99.5±0.8              | 0.4±0.0                | 0.1±0.0              |
| Fu+Zn      | 96.3±0.3 <sup>#</sup> | 3.3±0.1** <sup>#</sup> | 0.4±0.0 <sup>#</sup> |
| VitC       | 98.3±1.1              | 0.8±0.0                | 0.9±0.1              |
| Fu+VitC    | 91.4±0.6 <sup>#</sup> | 6.4±0.5** <sup>#</sup> | 2.2±0.1**            |
| Zn+VitC    | 99.8±1.2              | 0.1±0.0                | 0.1±0.0              |
| Fu+Zn+VitC | 96.7±0.8 <sup>#</sup> | 3.2±0.2** <sup>#</sup> | 0.1±0.0 <sup>#</sup> |

## CONCLUSIONS

In conclusion, it was discovered in our study that Fu decreased cell viability and enhanced lipid peroxidation, which is one of the toxicity markers. Fu has also been shown to reduce the activity of CAT, SOD, and GPx, enzymes involved in the intracellular antioxidant defense system, in Sertoli cells, leading to oxidative damage. In addition, Fu has been shown to induce apoptosis in Sertoli cells. VitC and Zn treatment showed that they had a favorable impact on cell viability, lipid peroxidation, antioxidant enzymes including CAT and GPX, and apoptosis. When the SOD enzyme activity was examined, it was determined that it was curative only in the groups in which VitC and Zn were applied together against the damage caused by Fu. Despite the toxicity of Fu, these findings led researchers to the conclusion that using Zn and VitC together more effectively mitigates the harm produced by Fu. Results indicate that consuming foods high in antioxidants, such VitC and Zn, or taking supplements to guard against compounds like Fu, which we cannot avoid due to our eating and drinking habits in our daily routines, play a significant role in reducing the damage.

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## **FUNCTIONAL FOOD - PRODUCTS FROM TOMATO WITH LYCOPENE IN PREVENTION OF CARDIOVASCULAR DISEASES**

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### **ABSTRACT**

Tomato, as a functional food product, includes a source of lycopene that serves to prevent cardiovascular disease. This study aims to perform a comparative analysis of the microbiological and chemical characteristics of different tomato products available to buy in the area of Mostar, Bosnia, and Herzegovina. Tomato products for analysis were randomly sampled and purchased from shoppicentersres in the Mostar area from three different producers: Sample no. “: “Passata” - sterilized tomato paste, producer “Podravka d.d.” Country of origin: Croatia, Sample no. 2: “Russo” - tomato paste, producer “AR Industire Alimentari S.p.A”, country of origin: Italy and Sample no. 3: “Sava Semberija” - tomato juice producer “Sava Bijeljina” country of origin: Bosnia and Herzegovina. The following parameters were included in the microbiological analysis: Salmonella, coagul. pos.staphylococci, sulph. red. Clostridia, Proteus species, Escherichia coli, and total bacterial count. Chemical analyses were based on the determination of lycopene content and artificial colours. The results were compared with the provisions of the Rulebook on the conditions regarding chemical and microbiological safety, which food products must meet in transport, Official Gazette of the Republic of BiH 2/92, Rulebook on microbiological criteria for food (Official Gazette of BiH No. 11/13), as well as with literature sources. The obtained results indicate the significant nutritional value of the samples in our diet, especially regarding human health and the prevention of cardiovascular diseases. For instance, lycopene is an antioxidant that cannot be created naturally by the human body, as it is exclusively of plant origin.

**Keywords:** tomato, lycopene, cardiovascular disease, prevention

### **INTRODUCTION**

Due to the humanization and modernization of work, the physical activity of man is reduced, which enables disorders of the overall physiology and circulation of substances in the body that results in various diseases. Genetic factors certainly have an impact on cardiovascular diseases. Many researches today prove that it is a disease that can be predicted accurately and therefore prevented. One of the essential steps in prevention is proper nutrition. It is not unknown that regular consumption of fruits and vegetables during the day has a very beneficial effect on health, so consuming at least five portions of fruits and vegetables per day is recommended. By the new lifestyles, the “Mediterranean diet,” which is char,”terized by a significant intake of fruits and vegetables, monounsaturated and fatty acids, primarily of olive and olive oil origin, is being increasingly promoted in the world today. One of the crucial components of the Mediterranean diet is the tomato, both fresh and processed, and it is an indispensable part of traditional dishes. Tomatoes have a low energy value (only 18 kcal per 100 g), given that they contain 94% water, 2.63% carbohydrates, 0.88% protein, 0.2% fat, and

1.2% dietary fiber. It is one of the richest natural sources of vitamin C. It also contains significant amounts of vitamin B, E, and K. It is rich in potassium, sodium, magnesium, calcium, iron, and trace elements. Tomatoes are the richest in copper and contain more iron than chicken, fish, and milk. Tomatoes and tomato products are an important source of carotenoids in the daily diet (600 µg/100 g). Of about 600 different carotenoids, alpha-carotene, beta-carotene, lutein, zeaxanthin, and lycopene stand out as one of the most important representatives.

**Table 1.** Lycopene content in fresh tomato fruits by the degree of maturity

| Samples                          | Lycopene<br>(mg/100g of fresh sample) |
|----------------------------------|---------------------------------------|
| Fresh tomato (October - March)   | 2,60-3,10                             |
| Fresh Tomato (June - August)     | 3,80-6,60                             |
| Green tomato juice               | 0,17                                  |
| Juice of partially ripe tomatoes | 0,24                                  |
| Ripe tomato juice                | 3,71                                  |

(Heinonen i sar.,1989., Beerh i Siddappa, 1959.)

Lycopene is the dominant carotenoid in human plasma after consumption of tomatoes or its products (Agarwal and Rao, 2000). Many clinical studies have linked high tomato intake and high tissue lycopene levels to a reduced risk of several types of cancer, especially prostate cancer (Giovannucci et al., 2002; Canene-Adams et al., 2005). The antiproliferative activity of tomatoes has been proven in several in vitro tests on different tumor cell lines, such as prostate, lung, breast, and cervical tumors (Boivin et al., 2009; Choi et al., 2011). Several epidemiological studies have shown that increased levels of lycopene in the blood plasma are associated with a reduced risk of developing cardiovascular diseases, especially with reduced levels of LDL cholesterol in the blood (Ried and Fakler, 2011; Böhm, 2012). However, consumption of lycopene preparations alone did not show the same beneficial effects as consumption of fresh tomatoes or tomato products (Basu and Imhran, 2007). Lycopene and phenolic compounds are more resistant to heat treatments, representing the primary antioxidants in tomato products (Navarro-González et al., 2011). Many studies have shown that the intake of lycopene from thermally processed tomato products is higher, which is explained by the increased bioavailability of lycopene due to the breakdown of cell walls, the presence of fat in food, as well as isomerization from the all-trans to the cis conformation under the influence of heat (Agarwal and Rao, 2000). This diet is associated with a positive effect on human health, first of all, reducing the risk of chronic diseases and, first of all, cardiovascular systems (Goñi and HervertHernández, 2011; Pinela et al., 2012; Di Lecce et al., 2013). The work aimed to examine the microbiological and chemical characteristics with particular reference to the content of lycopene in three different types of ready-made tomato products, compare the results with the parameters of the current Rulebook and literature extracts, and evaluate the acceptability of the product for potential future consumers on the free market, with the tabular presentation of analysis results.

## MATERIAL AND METHOD

The complete work was done in terms of microbiological and chemical analysis at the Agromediterranean Faculty in Mostar and the Institute for Public Health of the Federation of Bosnia and Herzegovina, the Health Ecology Service in Sarajevo. The tomato products that



were analyzed were bought in shopping centers in a retail store in the area of the city of Mostar, where three samples from different producers were selected.

Sample 1: "Passata" - sterilized puréed tomato, country of origin Croatia, Manufacturer "Podravka d.d."

Sample 2: "Russo" - puréed tomato, country of origin: Italy, Manufacturer "AR Industrie Alimentari S.p.A"

Sample 3: "Sava Semberija" - tomato juice, Country of origin: Bosnia and Herzegovina, Manufacturer "Sava Bijeljina"



Sample 1.



Sample 2.



Sample 3.

Sampling was done by random selection.

The samples were subjected to chemical analyzes that included the following parameters:

1. Determination of lycopene content expressed in g,
2. The presence of artificial colors

Sampling was done by random selection.

The samples were subjected to chemical analyzes that included the following parameters:

1. Determination of lycopene content expressed in g,
2. The presence of artificial colors. To meet the goal of the research within the framework of chemical analysis, the following methods were used:

1. Determination of lycopene content mg/100g - spectrophotometrically at 468 nm.
2. Presence of artificial colors - color identification by the chromatographic method.

The microbiological analysis of all three samples included research on Salmonella and Escherichia coli, Coagul. pos. staphylococci, sulph. red. Clostridia, Proteus species, and a total number of bacteria were made per food safety criteria for cut fruits and vegetables.

## RESULTS AND DISCUSSION

The research was conducted on tomato products purchased in various stores in the city of Mostar. Analyzes included: chemical and microbiological, and the obtained results were compared with existing legal regulations, on the basis of which we can talk about quality and recommendations for use without danger to the health of the population.

In table 2, the obtained results of the chemical analysis of the lycopene content in tomato products indicate that the lycopene content in sample number 1 was recorded in the highest presence of 12.52 mg/100g, and the lowest in sample number 3 in 6.56 mg/100g. The obtained results were compared with parameters from literature sources. It can be concluded that sample 1 and sample 3 are in accordance with the literature excerpts of Tavares and Rodriguez-Amaya, 1994 and Lindner et al., 1984, while sample number 2 shows somewhat lower values for 0.16 mg/100g compared to the literature extract of Tavares and Rodriguez-Amaya, 1994. Compared with the content of lycopene in fresh tomato fruits (table 1), the chemical analysis determined that all three analyzed samples of ready-made tomato products have a significantly higher lycopene content. Lycopene is a powerful antioxidant, ten times more potent than vitamin E.

Studies have shown that adding lycopene, 7 mg per day, for two months, can normalize the function of the endothelium in patients with cardiovascular disease. The endothelium is a thin layer of cells located on the inner surface of blood vessels. Endothelial dysfunction is associated with atherosclerosis, which can increase the risk of heart attack and stroke. Based on the above, it can be concluded that all three analyzed samples are significant in terms of lycopene content, which can provide potential consumers with an appropriate level of prevention in cardiovascular diseases. Many studies have proven that the concentration of lycopene in the plasma is higher when food is ingested into the body based on thermally processed tomatoes, in contrast to the concentration after the intake of unprocessed tomatoes (Böhm and Bitsch 1999). It can be concluded that the analyzed finished products from tomatoes are a rich source of lycopene for the human body; from availability, its protective role in the prevention of many cardiovascular and other diseases will also depend.

**Table 2.** Lycopene content in tomato products

| Analyzed samples | Unit of measure<br>mg/100g | Results of literature<br>extracts | Authors                         |
|------------------|----------------------------|-----------------------------------|---------------------------------|
| Sample 1         | 12,51                      | 8,93-19,37                        | Tavares i Rodriguez-Amaya, 1994 |
| Sample 2         | 8,77                       | 8,93-19,37                        | Tavares i Rodriguez-Amaya, 1994 |
| Sample 3         | 6,56                       | 5,8-9,0                           | Tavares i Rodriguez-Amaya, 1994 |

**Table 3.** Results of chemical analyzes of the presence of artificial colors in tomato products

| Analyzed product samples | Presence of artificial colors |
|--------------------------|-------------------------------|
| Sample no.1              | /                             |
| Sample no.2              | /                             |
| Sample no.3              | /                             |

The obtained results of the chemical analysis of the presence of artificial colors in tomato products indicate that the presence of artificial colors was not proven in any of the analyzed samples, per the current Rulebook.

**Table 4.** Results of microbiological analysis of SAMPLES 1, 2 and 3

| Microorganism                    | Unit of measure | Test result | MDK |
|----------------------------------|-----------------|-------------|-----|
| Salmonella                       | CFU / 25g ( ml) | 0           | /   |
| Coagulase-positive staphylococci | CFU / g ( ml)   | 0           | /   |
| Sulfite-reducing Clostridia      | CFU / g ( ml)   | 0           | /   |
| Proteus species                  | CFU / g ( ml)   | 0           | /   |
| Escherichia coli                 | CFU / g ( ml)   | 0           | /   |
| Total number of bacteria         | CFU / g ( ml)   | 0           | /   |

The obtained results of microbiological analysis on the presence of Salmonella species, coagulase-positive staphylococci, sulfite-reducing Clostridia, Proteus species, Escherichia coli, and the total number of bacteria show that not a single analyzed species were isolated on any of the analyzed samples, which indicates that all analyzed parameters are in accordance with

the valid Rulebook on microbiological criteria for food Official Gazette of BiH No. 11/13. and from the aspect of hygienic and sanitary correctness, there is no apprehension about the consumption of the same by potential consumers.

## CONCLUSIONS

The task of this scientific research work was to examine the quality of three different types of ready-made tomato products in terms of lycopene content, the presence of artificial colors, as well as microbiological correctness, and to compare the obtained results with valid Rulebooks and literature sources. All analyzes were done at the Agromediterranean Faculty in Mostar and the Institute for Public Health of the Federation of Bosnia and Herzegovina, Service for Health Ecology in Sarajevo. The results of the chemical analysis of the lycopene content in tomato products indicate that the lycopene content in sample number 1 was recorded at the highest level of 12.52 mg/100g and the lowest in sample number 3 at 6.56 mg/100g. The obtained results were compared with parameters from literature sources, and it can be concluded that samples 1 and sample 3 follow the literature excerpts of Tavares and Rodriguez-Amaya, 1994 and Lindner et al., 1984, while sample number 2 shows somewhat lower values of 0.16 mg/100g compared to the literature extract of Tavares and Rodriguez-Amaya, 1994. In comparison with the content of lycopene in fresh tomato fruits (table 1), the chemical analysis determined that all three analyzed samples of ready-made tomato products have a significantly higher content of lycopene. The results of the chemical analysis of the presence of artificial colors in tomato products indicate that the presence of artificial colors was not proven in any of the analyzed samples, which is in accordance with the current Rulebook. The results of the microbiological analysis show that not a single analyzed species was isolated on any of the samples, indicating that all analyzed parameters are in accordance with the valid Rulebook on microbiological criteria for food Official Gazette of BiH number 11/13. and from the aspect of hygienic and sanitary correctness, there is no apprehension about the consumption of the same by potential consumers. Results indicate that all three analyzed samples have a significant nutritional value in terms of lycopene content; as such, they should have an essential place in our diet and the prevention of cardiovascular diseases. Lycopene is less available from the raw material (Gärtner et al., 1997).

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## HEALTH RISK ASSESSMENT OF NITRATE IN DRINKING WATER OF İPSALA DISTRICT (THRACE REGION OF TÜRKİYE)

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### ABSTRACT

In this study, nitrate accumulations in drinking water of İpsala District and connected villages, an important agricultural stressed area, were investigated in a 2-year period (2021 – 2022). The potential health hazards due to nitrate intake through drinking water were also assessed for all the investigated villages by calculating Chronic Daily Intake (CDI) and Hazard Quotient (HQ). As a result of this research, nitrate concentrations in drinking water samples were ranged from 1.31 ppm to 26.70 ppm (with mean of 12.43 ppm) in 2021 and from 1.80 ppm to 53.10 ppm (with mean of 12.67 ppm) in 2022. According to the results of non – carcinogenic health risk assessment of nitrate, although the HQ values in drinking water of all the investigated villages of İpsala District were found as below the limit coefficient of 1 both for adults and children in general, the HQ values calculated for nitrate were found above 1 in the drinking water of Pazardere and Hacıköy Villages in 2022.

**Keywords:** İpsala District, Drinking water quality, Health Risk Assessment

### INTRODUCTION

Water, which is among the most significant natural resources, is one of the most significant components for life to continue. However, despite its critical importance, it is one of the most poorly managed resources in the world. Water pollution is among the most significant problems of the mankind in especially recent years. Though only about 30% of the global freshwater resources are groundwater, it is one of the most widely used freshwater components as drinking water. But unfortunately, drinking water resources of the world are significantly being affected by developing technology and population growth and no environmental awareness. Nitrate contamination in drinking water in especially rural lands is an increasing problem threatening both human health and ecosystems (Chen et al., 2012; Çiçek et al., 2013; Wang et al., 2014; Onur and Tokatlı, 2020; Köse et al., 2020; Tokatlı, 2021; 2022; Yüksel et al., 2022; Varol et al., 2022).

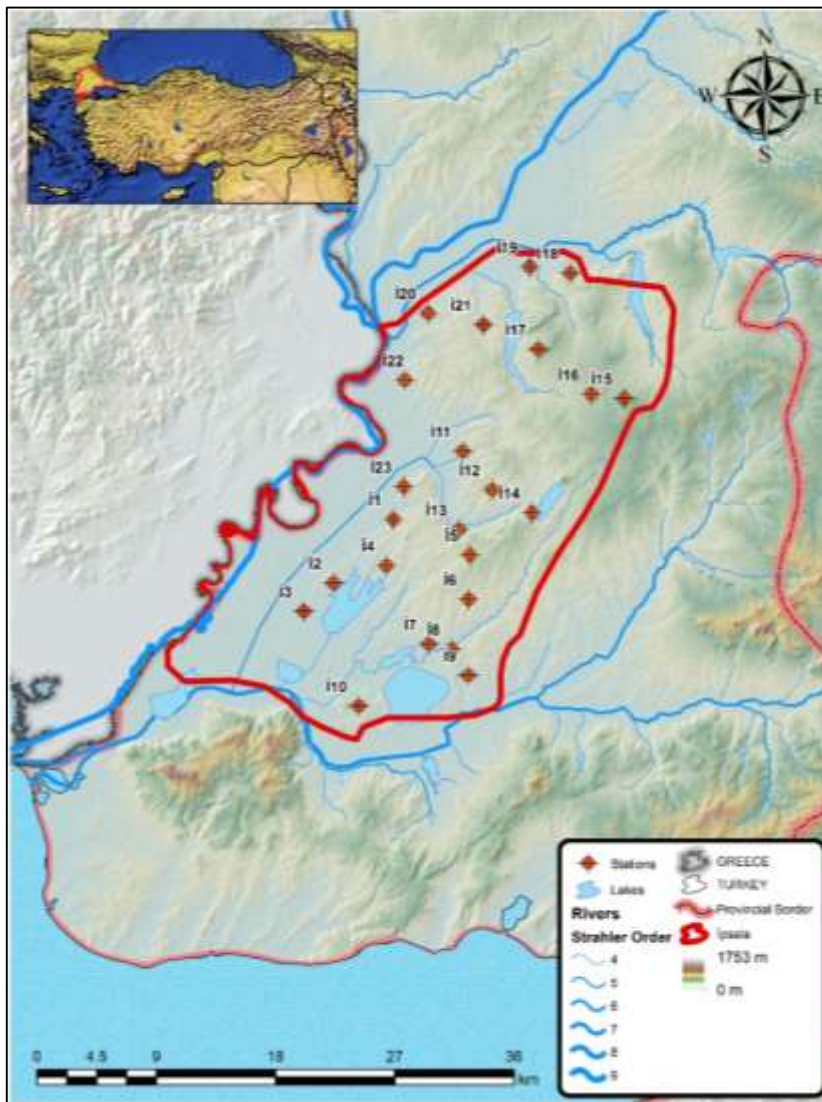
Thrace Region is located in the north – west part of Marmara Region of Türkiye and it has a great agricultural potential due to its fertile clayey soil. Therefore, approximately 80% of the Thrace Region occurs agricultural lands for crop production. İpsala District, which is very suitable for wet agriculture applications due to its rich groundwater and surface water resources, is located in the Meriç Plain in the west part of Thrace Region (Tokatlı and Ustaoglu, 2020; Tokatlı and Varol, 2021; Tokatlı et al., 2022; Varol and Tokatlı, 2022). The aim of this research was to determine the nitrate levels in drinking water resources of İpsala District and evaluated the water quality in terms of probable non – carcinogenic health risks of nitrate.

## MATERIAL AND METHODS

### *Sample Collection and Chemical Analysis*

Within the scope of this research, 23 locations were selected in the region including İpsala District and connected villages. Location map together with the topography for the selected stations (İ1 - İ23) are given in Figure 1. The coordinates with the names of locations are also given in Table 1.

Drinking water samples were taken from the tap waters of the villages, which have a direct impact on the health of the local people, in January 2021 and January 2022 by using polyethylene bottles. Nitrate ( $\text{NO}_3$ ) accumulations in drinking water samples were determined by using a Hach Lange branded DR890 Colorimeter device during the laboratory studies.



**Figure 1.** İpsala District and selected stations

**Table 1.** Locations of sampling stations

| Station Code | Location              | GPS - North | GPS - East |
|--------------|-----------------------|-------------|------------|
| İ1           | Ahırköy Village       | 40.894      | 26.374     |
| İ2           | Paşaköy Village       | 40.850      | 26.320     |
| İ3           | Yenikarpuzlu Town     | 40.832      | 26.295     |
| İ4           | Kumdere Village       | 40.866      | 26.368     |
| İ5           | Esetçe Town           | 40.871      | 26.443     |
| İ6           | Aliçopehlivan Village | 40.841      | 26.440     |
| İ7           | Kocahıdır Village     | 40.809      | 26.407     |
| İ8           | Küçük Doğanca Village | 40.808      | 26.430     |
| İ9           | Yapıldak Village      | 40.791      | 26.442     |
| İ10          | Koyuntepe Village     | 40.768      | 26.343     |
| İ11          | Turpçular Village     | 40.941      | 26.434     |
| İ12          | Hıdırköy Village      | 40.914      | 26.461     |
| İ13          | Sarpdere Village      | 40.888      | 26.431     |
| İ14          | Korucu Village        | 40.900      | 26.496     |
| İ15          | Pazardere Village     | 40.979      | 26.580     |
| İ16          | Hacıköy Town          | 40.981      | 26.550     |
| İ17          | İbriktepe Town        | 41.012      | 26.505     |
| İ18          | Karaağaç Village      | 40.941      | 26.434     |
| İ19          | Hıdırköy              | 40.915      | 26.461     |
| İ20          | Sultanköy Town        | 41.025      | 26.453     |
| İ21          | Balabancık Village    | 41.033      | 26.404     |
| İ22          | Sarıcaali Village     | 40.985      | 26.382     |
| İ23          | İpsala District       | 40.913      | 26.376     |

**Health Risk Assessment**

In this research, potential health risks due to nitrate exposure was estimated using the model recommended by the USEPA (EPA, 1989);

$$CDI = C_{water} \times \frac{(IR \times EF \times ED)}{(BW \times AT)}$$

$$HQ = \frac{CDI}{RfD}$$

CDI: Chronic Daily Intake (mg/kg/day)

C: Nitrate levels in water (mg/L)

IR: Ingestion rate (2.2 and 0.95 L/day for adults and children, respectively)

ED: Exposure duration (6 years for children and 30 years for adults)

EF: Exposure frequency (it is assigned 365 days per year)

AW: Average body weight (67.5 kg for adults and 16.5 kg for children, respectively)

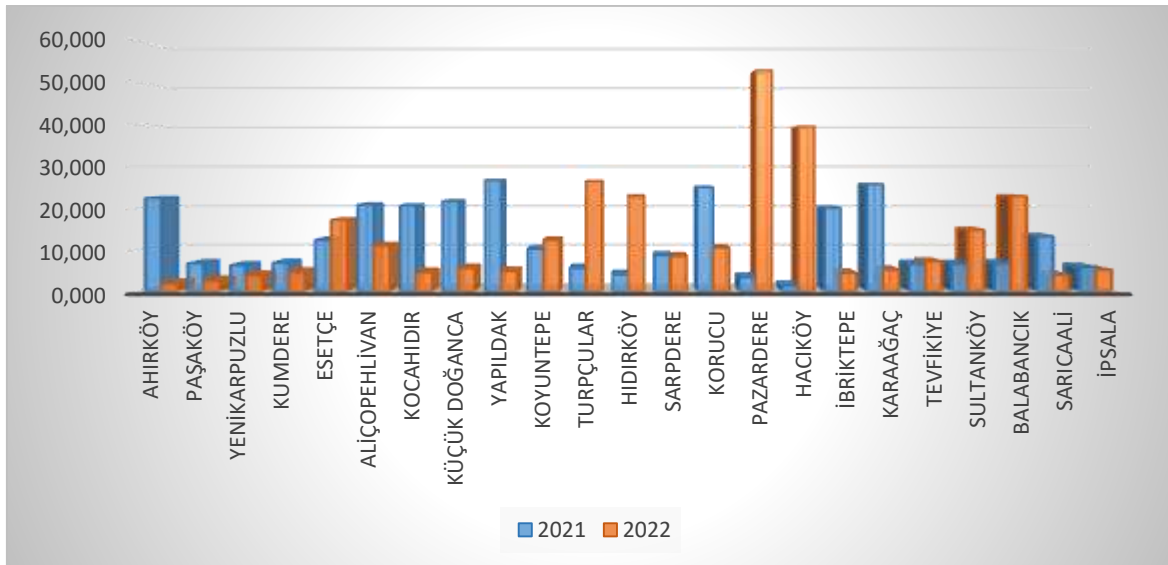
AT: Average time (10950 and 2190 for adults and children, respectively)

RfD: Reference dose of nitrate (1.6 mg/kg/d)

HQ: Hazard quotient (if HQ is higher than 1, the risk is unacceptable)

## RESULTS AND DISCUSSION

Nitrate accumulations in drinking water of İpsala District are given in Figure 2 and the results of applied non – carcinogenic health risk assessment indices are given in Table 2.



**Figure 2.** Nitrate levels in drinking water (ppm)

**Table 2.** CDI and HQ coefficient

|     | 2021    |         |         |         | 2022    |         |         |         |
|-----|---------|---------|---------|---------|---------|---------|---------|---------|
|     | CDI     |         | HQ      |         | CDI     |         | HQ      |         |
|     | Ch      | Ad      | Ch      | Ad      | Ch      | Ad      | Ch      | Ad      |
| İ1  | 1.3E+00 | 7.3E-01 | 8.1E-01 | 4.6E-01 | 1.0E-01 | 5.9E-02 | 6.5E-02 | 3.7E-02 |
| İ2  | 3.8E-01 | 2.2E-01 | 2.4E-01 | 1.3E-01 | 1.4E-01 | 8.1E-02 | 9.0E-02 | 5.1E-02 |
| İ3  | 3.5E-01 | 2.0E-01 | 2.2E-01 | 1.3E-01 | 2.2E-01 | 1.2E-01 | 1.4E-01 | 7.7E-02 |
| İ4  | 3.9E-01 | 2.2E-01 | 2.4E-01 | 1.4E-01 | 2.6E-01 | 1.5E-01 | 1.7E-01 | 9.4E-02 |
| İ5  | 7.1E-01 | 4.0E-01 | 4.4E-01 | 2.5E-01 | 9.8E-01 | 5.6E-01 | 6.2E-01 | 3.5E-01 |
| İ6  | 1.2E+00 | 6.8E-01 | 7.5E-01 | 4.2E-01 | 6.3E-01 | 3.6E-01 | 3.9E-01 | 2.2E-01 |
| İ7  | 1.2E+00 | 6.7E-01 | 7.4E-01 | 4.2E-01 | 2.6E-01 | 1.5E-01 | 1.6E-01 | 9.2E-02 |
| İ8  | 1.2E+00 | 7.0E-01 | 7.8E-01 | 4.4E-01 | 3.2E-01 | 1.8E-01 | 2.0E-01 | 1.1E-01 |
| İ9  | 1.5E+00 | 8.7E-01 | 9.6E-01 | 5.4E-01 | 2.8E-01 | 1.6E-01 | 1.7E-01 | 9.8E-02 |
| İ10 | 5.8E-01 | 3.3E-01 | 3.6E-01 | 2.1E-01 | 7.1E-01 | 4.0E-01 | 4.4E-01 | 2.5E-01 |
| İ11 | 3.2E-01 | 1.8E-01 | 2.0E-01 | 1.1E-01 | 1.5E+00 | 8.6E-01 | 9.5E-01 | 5.4E-01 |
| İ12 | 2.3E-01 | 1.3E-01 | 1.4E-01 | 8.0E-02 | 1.3E+00 | 7.4E-01 | 8.2E-01 | 4.6E-01 |
| İ13 | 5.0E-01 | 2.8E-01 | 3.1E-01 | 1.8E-01 | 4.8E-01 | 2.7E-01 | 3.0E-01 | 1.7E-01 |
| İ14 | 1.4E+00 | 8.1E-01 | 9.0E-01 | 5.1E-01 | 6.0E-01 | 3.4E-01 | 3.7E-01 | 2.1E-01 |
| İ15 | 1.9E-01 | 1.1E-01 | 1.2E-01 | 6.6E-02 | 3.1E+00 | 1.7E+00 | 1.9E+00 | 1.1E+00 |
| İ16 | 7.5E-02 | 4.3E-02 | 4.7E-02 | 2.7E-02 | 2.3E+00 | 1.3E+00 | 1.4E+00 | 8.1E-01 |
| İ17 | 1.2E+00 | 6.5E-01 | 7.2E-01 | 4.1E-01 | 2.5E-01 | 1.4E-01 | 1.5E-01 | 8.8E-02 |
| İ18 | 1.5E+00 | 8.4E-01 | 9.2E-01 | 5.2E-01 | 2.9E-01 | 1.7E-01 | 1.8E-01 | 1.0E-01 |
| İ19 | 3.8E-01 | 2.1E-01 | 2.3E-01 | 1.3E-01 | 4.1E-01 | 2.3E-01 | 2.6E-01 | 1.4E-01 |
| İ20 | 3.7E-01 | 2.1E-01 | 2.3E-01 | 1.3E-01 | 8.5E-01 | 4.8E-01 | 5.3E-01 | 3.0E-01 |
| İ21 | 3.8E-01 | 2.2E-01 | 2.4E-01 | 1.4E-01 | 1.3E+00 | 7.4E-01 | 8.2E-01 | 4.6E-01 |
| İ22 | 7.6E-01 | 4.3E-01 | 4.8E-01 | 2.7E-01 | 2.2E-01 | 1.2E-01 | 1.4E-01 | 7.7E-02 |
| İ23 | 3.3E-01 | 1.9E-01 | 2.1E-01 | 1.2E-01 | 2.9E-01 | 1.7E-01 | 1.8E-01 | 1.0E-01 |



In 2021, the lowest nitrate value was measured in Hacıköy Village as 1.31 ppm, while the highest nitrate value was measured in Yapıldak Village as 26.70 ppm. In 2022, the lowest nitrate value was measured in Ahır Village as 1.80 ppm, while the highest nitrate value was measured in Pazardere Village as 53.10 ppm. Although significant changes were recorded in the maximum nitrate accumulation levels detected in the drinking water of the region, no significant change have been determined in the average nitrate accumulation levels of the region in 2021 and 2022.

The results of this study demonstrate that the CDI values are in the ranges of 0,04270 – 0.87022 and 0.07542 – 1.53727 mg/kg/day for adults and children, respectively, in 2021; and 0.05867 – 1.73067 and 0.10364 – 3.05727 mg/kg/day for adults and children, respectively, in 2022 at different sampling locations of the study area. The average CDI values for adults and children are 0.40523 and 0.715841 mg/kg/day, respectively, in 2021; and 0,41293 and 0,72946 mg/kg/day, respectively, in 2022 (Table 2). HQ values in drinking water of all the investigated villages of İpsala District were found as below the limit coefficient of 1 both for adults and children both in 2021 and 2022 in general, while the HQ coefficients in drinking water of Pazardere and Hacıköy Villages were found above 1 in 2022.

Nitrogen is an essential element for the human body to synthesise proteins and nucleic acids. Nitrate ingested to human body through the foods and drinking water (Lundberg et al., 2018). Nitrate is a significant organic pollutant, and nitrate values in drinking water may reach quite high concentrations in especially rural areas, where intensive agricultural, fertilization and livestock activities are carried out (Çiçek et al., 2013; Peiyue et al., 2019). In addition, it may reach quite high levels in groundwater, especially in areas such as the present study area, where there is no sewage system (Tokatlı, 2014; 2019). It is thought that the quite high nitrate values detected in the drinking water of the İpsala District and connected villages may be caused by the agricultural activities carried out in the region and the widely used septic tank systems in the region.

## CONCLUSIONS

Drinking water quality studies are critical in especially arid and semiarid regions to facilitate the sustainable economic - social development. In the present research, the spatial - temporal distribution of nitrate accumulations in drinking water of İpsala District were investigated and additionally, the probable non - carcinogenic health risks due to exposure to nitrate through the drinking water intake were assessed for children and adults. As a result of this research, the following conclusions can be summarized; (1) nitrate levels in drinking water of İpsala District were ranged from 1.31 ppm to 26.70 ppm in 2021, while they were ranged from 1.80 ppm to 53.10 ppm in 2022; (2) although the HQ values in drinking water of İpsala District were found as below the limit coefficient of 1 both for adults and children in general, the HQ values were found above 1 in Pazardere and Hacıköy Villages in 2022, which means possible non - carcinogenic health risk of nitrate in these regions.

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## **MONITORING, INTRODUCTION, AND USEFUL TRAITS OF *AGRYOPHYLLUM SQUARROSUM* L.**

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### **ABSTRACT**

The aim of this work is to determine the distribution areas of *Agryophyllum squarrosum* (L.) plant in the Western Kazakhstan region and the Aktobe region of the Republic of Kazakhstan. Scope of the work included the following activities: monitoring and introduction of species composition, studying the economically useful potential, phenological observations, biochemical analysis in terms of distribution areas with description of coordinates of germination sites, introduction, studying the biological features of *Agryophyllum squarrosum* (L.) in culture. The fodder productivity of the wild *Agryophyllum squarrosum* (L.) in the monitoring points of Zhanakala area of the West Kazakhstan region was 6–8 c / ha of green mass or 2–2.5 c / ha of hay, in the Shalkar area of the Aktobe region - 4–5 c / ha; 1.0-1.5 c / ha.

**Keywords:** *Agryophyllum squarrosum* (L.), plant, forage productivity, green mass, hay, yield, distribution area, expedition, monitoring, species, biochemical analysis, productivity.

### **INTRODUCTION**

The world's population is growing, therefore in order to avoid global hunger, it is necessary to increase agricultural production. On our planet more than 8 billion people are expected to live, which will increase the nutritional needs. The increase in food production should take place not only because of increasing the yield of existing crops, but also because of attracting new, long-forgotten plant resources. Therefore, the conservation of biological diversity, monitoring and reproduction of previously unstudied plant genetic resources for food self-sufficiency is relevant.

Existing range of host plants do not currently meet the requirements of the radical transformation of low-yield pasture vegetation in desert, semi-desert of western Kazakhstan.

Irregular grazing animals has led to the impoverishment of grass and thinning of vegetation, and in the future to fail and display of wind erosion, desertification. Despite the fact that Kazakhstan has taken a number of organizational and economic measures to prevent further deterioration of pastures, yet the rate of reduction of areas susceptible to soil and vegetation degradation still weak. Therefore, it is necessary to engage in the culture some new species of plants. One of them is *Agryophyllum squarrosum* (L.), which has valuable fodder properties.

Wild *Agryophyllum squarrosum* (L.) is of great interest for the introduction to the culture in order to create autumn pastures and hay fields, where in autumn can be harvested considerable amount of valuable hay containing a significant amount of seed that is readily eaten by sheep in winter .

*Agryophyllum squarrosum* (L.) develops in sandy desert, which belongs to the dry Salsola. These kinds of thistles begin to develop in the spring and complete the development in August and September, the autumn gives a good forage and pasture herbage.

In the south of Aktobe and the south-east of the West Kazakhstan regions large areas are occupied by shifting sands, which will cause huge damage to agriculture. As the main reasons for the formation of new areas of mobile sand is unconscionable grazing or plowing overgrown sand and sandy loam soils, they should be secured. *Agryophyllum squarrosum* (L.) - A plant indicator. If the crop has taken roots- then sands fixing started.

All kinds of the young plants of *Agryophyllum squarrosum* (L.) are good as fodder for grazing sheep, cattle and camels. 100 kg of green mass contains about 25 k.ed. and 3.7 kg of digestible protein. Yields are up to 20 quintals of green mass and 30 kg of seeds per 1 hectare.

Composition of the plant at the time of fruiting is: water 9.9%, fiber 22,5-25%, crude protein 5.5-6.2% pure protein 5,3-5,9%, 2,1-2 phat - 3% of nitrogen-free extractives 50-55,5% and ash 9,9-11,0%. Starch equivalent of the plant is quite high: 34-37,6.

Sandy desert species is widely distributed in western Kazakhstan. Kazakhs have long used the seeds of edible plants as a delicacy in the form of lightly toasted and for making flour tortillas. It is not difficult to judge the economic importance of species, because in good years seeds of plants were collected in an amount not less than 500 000-700 000.kg. In such years, raw, unprocessed seeds *Agryophyllum squarrosum* (L.) valued at 30% more than millet and 15-20% more expensive rye and wheat flour.

Dry seeds of *Agryophyllum squarrosum* (L.) contain: 16-17% protein, 6-10% protein fat, edible oil and semidrying and 60% carbohydrates, mainly starch, in an amount up to 87% of the substances perfectly absorbed. In 100 g of the substances used as food, is 343 more calories, which brings *Agryophyllum squarrosum* (L.) to wheat flour, has 344 calories. The oil obtained by pressing the seeds - a semi-liquid, to taste it resembles a sunflower, and the composition is close to sesame. The nutritional value of *Agryophyllum squarrosum* (L.) exceeds kinoa, it is greater than this culture by the content of linoleic acid, but unlike it, the introduction of *Agryophyllum squarrosum* (L.) was not carried out in full. It should be noted that polyunsaturated fatty acids, such as linoleic acid, reduce the risk of cardiovascular disease and improve insulin sensitivity, and they are recommended as a substitute for saturated animal fats.

In China, they tried to grow *Agryophyllum squarrosum* (L.) as an agricultural crop in Lanzhou. The yield of dry matter (hay) was 1.75 t / ha, and the yield of seeds is about 39 kg / ha .

In the Kazakhstani history yields of *Agryophyllum squarrosum* (L.) in the best years reached 30-32 kg per 1 ha and about 15-20 c plant mass. *Agryophyllum squarrosum* (L.) seeds were harvested in September, after careful distribution of the plots occupied by them in auls, families and consumers, and not spontaneously, wherever they want. The time of harvest was considered to be a time when the seeds are not ripe, and the stems are still soft. The plant is squinted or curled with a sickle, tumbles down and is trampled down in flat grassy cakes, 1 m in diameter, called "baspa" or "ber-baspa". On the sandy hillocks, the "baspa's" are placed in mounds, on the edge, for drying, the last one lasts about a month and during its seed ripens. Then the baspa is grinded on the special woolen cloth. The broken heap is collected from the fabric and raked into mounds called "tuban", which goes for secondary, spring grinding. The grinded seeds are removed, sifted and poured into sacks-"kapy", getting a selling product.

Plant seeds are used for therapeutic purpose. Saponins were found in the above-ground part. In Mongolian medicine, decoction of seeds is traditionally used as an antipyretic and analgesic agent. According to recent studies by Mongolian and Chinese scientists, *Agryophyllum squarrosum* (L.) and its extracts have significant lipid-lowering activity. The effect of *Agryophyllum squarrosum* (L.) and its extracts on lowering the level of total

cholesterol, triglyceride and low-density lipoprotein cholesterol was equal to the indicators of simvastatin, which is a drug for the treatment of hyperlipidemia.

In the traditional Chinese medicine, *Agropyllum squarrosus* (L.) is used at high temperature for colds treatment; with inflammation of the kidneys they suggest to take a decoction of grass. In China, because of edible seeds, the *Agropyllum squarrosus* (L.) was called "shami", which translates as sandy rice. According to archaeological records dated 688 AD. it is established that the ancient Chinese warriors collected seeds of *Agropyllum squarrosus* (L.) as provisions for military campaigns. The local population of western China still retains the tradition of using this plant for food purposes to prepare various culinary dishes. The most popular is a cold dish of crushed *Agropyllum squarrosus* (L.) seeds, prepared in the form of jelly, seasoned with spices. The research work on the study, preservation of the genetic potential of wild *Agropyllum squarrosus* (L.) and its introduction into cultivation in the context of Western Kazakhstan was held for the first time. Introduction wild *Agropyllum squarrosus* (L.) in cultivation carried out in several stages: the collection of seed and planting material; study of the biological characteristics; conducting experimental cultivation and identification of optimum range of accommodation; selection of commercially valuable populations; develop effective ways of cultivation. In Kazakhstan, the idea of introducing wild *Agropyllum squarrosus* (L.) in culture has no analogues.

In addition, the subsequent introduction of the results of scientific development will also have a social value, as given crops of *Agropyllum squarrosus* (L.) not only improve the productivity of pastures, but also will contribute to the consolidation of sandy soils. The project is focused on the socio-economic development of regionalization, which means improving of pastures productivity, which contributes to the development of forage and livestock increasing for various livestock industries. Number of livestock in western Kazakhstan is increasing every year and the management, conservation and improvement of pasture resources become relevant. Social benefits in the implementation of the subject will be concluded in creation of additional jobs, prestige of the livestock industry, influx of young people in the village.

## **MATERIAL AND METHOD**

Scientific research work has been conducted by the expeditionary surveys and collecting the promising species, populations and forms to create a gene pool. The description of the biological characteristics of plant growth in nature has been made; maps of accumulation of taxa of studied species were created.

The collection nursery of promising plants was established on the basis of the collected expeditionary material.

Following surveys and observations were held in the nursery:

- Phenological observations during the growing season of plants from seed to harvest (shoots, tillering or branching plants, flowering, full maturation);
- Biometric measurements (visual estimates the nature and intensity of regrowth, and the power of growth, in% leafy, plant height);
- Evaluation of phyto-and ento-damage indicating pathogens and pests;
- Determination of hardiness and drought resistance of the species studied;

The study of some elements of technology of cultivation: planting dates (spring, autumn).

Plant density is determined after overwintering plants at the end of the growing season and after each harvest.

Determination of soil moisture for the 0-10 cm layer at a depth of 0-100 cm performed thermogravimetric-gravimetric method. Time of determination background - before laying experiments, the beginning and the end of the growing season.

Biometric counts are carried out by measuring the length and width of the leaf blade, the determination of the total surface area of the sheet.

Consideration of the harvest is carried out by weighing the biomass of leaves and seeds.

Accounting for crop productivity with the definition of *Agryophyllum squarrosum* (L.) (structure of the harvest, biometrics) is carried out according to the procedure of the State of crop variety trials (Almaty, 2002).

- The analysis of sheaves is revealed:
- plant density at the time of harvesting, pc. / m<sup>2</sup>;
- plant height (from 25 plants) cm;
- biological mass of leaves
- weight of 1000 seeds, g;
- biological seed yield is determining by calculating method, t / ha

## RESULTS AND DISCUSSION

Experimental seedlings from seeds collected in the Western Kazakhstan area and seeded on sandy soils of the Aktobe area (Shalkar region) showed that the seeding made in the autumn had better germination compared to the seeding in the spring. Apparently, the seeds must undergo stratification in natural conditions, which was noted during the autumn sowing. To develop the best adapted techniques for private agricultural techniques of *Agryophyllum squarrosum* (L.) cultivation, crops were planted in autumn - October 15, spring - April 30, depth of seeding 2-3 cm. Seeding rates: 20,40,60 pcs /l. m. Phenological observations showed that shoots in spring appeared on 10-12 May, field germination - 30-50%.

At fixed sites, the phases of development of the *Agryophyllum squarrosum* (L.) plant were recorded. It was noted that rapid growth of the root system contributes to its survival in conditions of hot, mobile sands. With the growth of the root system, the aboveground mass increases, the plant forms many branches (branched from the base) with integral, regular leaves. The number of plants per 1m<sup>2</sup> was about 7-9 pcs / m<sup>2</sup>. In spring time young sandy *Agryophyllum squarrosum* (L.) plants are well eaten by cattle, sheep and camels. Phenological and biometric observations of the growth and development of the *Agriophyllum* L. plant under cultivation are made in the collection nurseries. Seedlings were recorded on May 12-15, the number of plants for 1m<sup>2</sup> was 11-15 pcs / m<sup>2</sup>.

The *Agryophyllum squarrosum* (L.) plant forms long, strong thread-like processes reaching the horizon of constant moisture in the sands. With the growth of the root system, the aboveground mass increases, the plant forms many branches (branched from the base) with integral, regular leaves. At the beginning of the growing season, a rapid growth of the root system was observed-the formation of long, strong thread-like processes reaching the horizon of constant moisture in the sands. The first pair of lateral roots develops immediately after emergence.

With the growth of the root system, the aerial mass increases, the leaves are lanceolate, with strongly protruding veins, and pointed at the end. The length of the leaf blade was 3.0 - 5 cm, the width of the plate was 2-8 mm. At the time of investigation of the *Agryophyllum squarrosum* (L.) plant, the following phases were recorded: seedling start, flowering and seed ripening (Figure 1).



**Figure 1** – *Agryophyllum squarrosum* (L.) seed ripening phase

All the observations were made by means of expeditions conducted along the route "Aktobe - Alga - Emba - Shalkar-Shalkar region - Aktobe". The length of the route is 1000 km. The expedition examined the sands of the Great Badgers, the Lesser Badgers, the Zhylytyr and Togyz points, where the places of growth of the *Agryophyllum squarrosum* (L.) are found in the wild state on sandy soils.

Sands Big Badgers, sands Small Badgers, sand deserts, elongated in the form of two long and narrow stripes are mostly in the Aktobe region. They are mainly composed of Paleogene sands and only the sands of the northwestern part of the Big Badgers consist of alluvial deposits.

The sands are scattered in places, forming hillocks, ridges and sand-dune. Xerophytic shrubs, wormwood, saltwort and ephemera predominate on the leveled spaces; on the slopes of the hillocks and ridges - thickets of dzhuzgun, sandy acacia, shrub astragalus, kandym and *Agryophyllum squarrosum* (L.).

The vegetation of sand deserts is very specific and has a high percentage of endemics. For the development of plants there are two features of the substrate of great importance: its mobility and relatively good, in comparison with other types of deserts, moisture availability. Due to the failed moisture capacity atmospheric precipitation is almost completely accumulated in the sand, soaking it by 1-1.5 m, and due to small capillarity the stored moisture is consumed almost entirely by transpiration of plants.

At large diurnal temperature fluctuations in the sand, water vapor condenses from the air. All this leads to the occurrence in the sands of a constantly moist horizon at a depth of 100-140 cm. Above it, the so-called suspended moisture horizon is formed, which in spring is near the surface itself, and later moves to a depth of 40-120 cm, coinciding in the driest time with the horizon constant humidity.

The desert territory is used for pasture livestock breeding, especially in summer due to good water availability (a relatively high level of standing of groundwater).

As a result of summer expeditions on fixed sites, the phases of development of the *Agryophyllum squarrosum* (L.) plant were recorded. It was noted that rapid growth of the root system contributes to its survival in conditions of hot, mobile sands. The results of phenological observations of wild growing *Agryophyllum squarrosum* (L.) in the growing places (The Big Badgers sands, Shalkar region of the Aktobe area) are presented in Table 1.



**Table 1** - Results of phenological observations

| Name of points on the map | Marked coordinates          | Seedlings of plants | Flowering of plants | Seed ripening |
|---------------------------|-----------------------------|---------------------|---------------------|---------------|
| Zhyltyr 1                 | N 47053'12'<br>E 59 0 48'48 | 07.05-17.05         | 26.07-26.08         | 17.09-19.10.  |
| Zhyltyr 2                 | N 47051'37'<br>E 59052.58   | 03.05-12.05         | 21.07-24.08         | 15.09-25.09   |
| Togyz 1                   | N 47048'44'<br>E 59 0 49'10 | 07.05-18.05         | 26.07-27.08         | 17.09-25.10   |
| Togyz 2                   | N 47046'13'<br>E 59 0 42'60 | 09.05-17.05         | 26.07-26.08         | 16.09-25.10.  |
| Togyz 3                   | N 47044'36'<br>E 59 0 37'36 | 03.05-12.05         | 21.07-24.08         | 17.09-25.09   |

Flowering was observed from the middle of summer (late July), the flowering period was 30-35 days. The first pair of lateral roots develops, immediately after emergence. The second pair and the main root grow later, forming long, strong threadlike processes reaching a horizon of constant humidity (40-120 cm) in the sands. With the growth of the root system, the aboveground mass increases, the plant forms many branches (branched from the base) with integral, regular leaves.

Vegetation period: seeding - shoots, shoots - branching, budding - flowering, flowering - ripening. Seed ripening period was 30-40 days, possibly this period may be longer, since when collecting the material, the seeds were slightly ripe, and the stems were still soft and green. Observations of the growth and development of plants have shown that in young plants near the settlements (Zhyltyr 1, Togyz 1 and Togyz 2) young *Agriophyllum* L. plants are well eaten by cattle, sheep and camels. The number of preserved plants per 1 square meter was less than in the points (Zhyltyr 2, Togzhy 3) remote from settlements. By the time of the flowering of the *Agryophyllum squarrosum* (L.), the consumption of it by livestock is falling, and by the time of ripening of the seeds they have become so prickly that they were not eaten at all. The largest distribution area was recorded at the Zhyltyr 2 point, the *Agryophyllum squarrosum* (L.) is growing mainly at the foot of the dunes, forming landscape groups. During the maturation phase, the maximum height of plants was 80 cm, the smallest 40 cm. The length of the leaf blade is 3.6-5 cm, the width of the plate is 2-4 mm

The results of phenological observations of the wild growing *Agryophyllum squarrosum* (L.) in the growing places - the sands of the Zhanakalinsky region of the Western Kazakhstan area are presented in Table 2.

**Table 2** - The results of phenological observations in Western Kazakhstan area

| Name of points on the map | Marked coordinates                                       | Seedlings of plants | Flowering of plants | Seed ripening |
|---------------------------|--|---------------------|---------------------|---------------|
| Kuspan                    | N 48 <sup>0</sup> 51'09.53<br>E 49 <sup>o</sup> 33'45.09 | 01-10.05            | 15.08-30.08         | 1.09 - 30.09  |
| Bulanay                   | N 48 <sup>0</sup> 48'396<br>E 49 <sup>0</sup> 42'289     | 01-10.05            | 15.08-30.08         | 01.09- 30.09  |
| Ali                       | N 48 <sup>0</sup> 50'038<br>E 49 <sup>o</sup> 28'559     | 05-12.05            | 14.08-30.08         | 01.09 - 29.09 |
| Zhanaoryn                 | N 48 <sup>0</sup> 47'335<br>E 49 <sup>o</sup> 33'296     | 03-11.05            | 15.08-30.08         | 05.09- 10.10. |
| Kalmakshagyl              | N 48 <sup>0</sup> 47'586<br>E 49 <sup>o</sup> 25' 932    | 02-10.05            | 12.08 – 28.08       | 30.08 - 28.09 |
| Zhumbay kurgan            | N 48 <sup>0</sup> 41'797<br>E 49 <sup>o</sup> 20'970     | 01-10.05            | 15.08- 30.08        | 01.09 - 02.10 |
| Abil                      | N 48 <sup>0</sup> 46'808<br>E 49 <sup>o</sup> 13'553     | 03-11.05            | 15.08– 30.08        | 01.09 - 28.09 |
| Sarsek                    | N 48 <sup>0</sup> 36'206<br>E 49 <sup>o</sup> 32'487     | 01-11.05            | 15.08- 29.08        | 30.08 - 28.09 |
| Beketay                   | N 48 <sup>0</sup> 16'047<br>E 49 <sup>o</sup> 28'579     | 03-11.05            | 15.08- 30.08        | 01.09 - 28.09 |

During the maturation phase, the maximum height of plants was 60 cm, the smallest 35 cm. The length of the leaf blade was 3.0-3.6 cm, the width of the plate was 4-8 mm (Table 3).

Table 3 - Results of biometric measurements of *Agryophyllum squarrosum* (L.) in Western Kazakhstan area

| Name of points on the map | Number of plants, pieces/m <sup>2</sup> |        | Height of plants, cm | Leaf plate |           |
|---------------------------|---|--------|----------------------|------------|-----------|
|                           | Spring                                  | Autumn |                      | length, cm | width, mm |
| Kuspan                    | 5-6                                     | 3-4    | 30-40                | 3,0-3,2    | 4-6       |
| Bulanay                   | 7-8                                     | 7-8    | 50-60                | 3,0-3,5    | 6-8       |
| Ali                       | 6-8                                     | 3-4    | 35-40                | 3,0-3,2    | 6-7       |
| Zhanaoryn                 | 5-6                                     | 3-4    | 35-40                | 3,1-3,6    | 6-7       |
| Kalmakshagyl              | 6-7                                     | 6-7    | 35-38                | 3,0-3,3    | 5-6       |
| Zhumbay kurgan            | 5-6                                     | 3-4    | 35-40                | 3,0-3,2    | 6-8       |
| Abil                      | 5-6                                     | 3-4    | 35-38                | 3,0-3,5    | 6-8       |
| Sarsek                    | 7-8                                     | 7-8    | 50-60                | 3,0-3,5    | 6-8       |
| Beketay                   | 7-8                                     | 7-8    | 50-60                | 3,0-3,6    | 6-8       |

In the spring, counting the number of plants per one square meter showed that there were more plant on remote points than in nearby ones. At remote points of study (35-75 km), the

number of preserved plants per one square meter was more by 3-4 pcs, than at the points located from Novaya Kazanka in a radius of 15-20 km away.

To develop the elements of cultivation technology of the *Agryophyllum squarrosum* (L.) plant in the region, two features of the soil substrate are of great importance: its mobility and relatively good moisture content compared to other types of deserts. It is caused by water - physical properties of sand. Due to the failed moisture capacity atmospheric precipitation is almost completely accumulated in the sand, soaking it by 1-1.5 m, and the stored moisture is consumed almost entirely by transpiration of plants due to small capillarity. In addition, at large diurnal temperature fluctuations in the sand, water vapor condenses from the air. All this leads to the occurrence in the sands of a constantly moist horizon at a depth of 100-140 cm.

To develop the optimal adapted methods of private agricultural techniques of *Agryophyllum squarrosum* (L.) cultivation on sandy soils of the Aktobe region (Shalkar region), experimental crops were grown from seeds harvested in 2015 (Figure 2)



**Figure 2-**Experimental cultivation of *Agriophyllum* L. with a seed rate of 40 pcs/m<sup>2</sup>

To develop the best adapted techniques for private agricultural techniques of cultivation of *Agryophyllum squarrosum* (L.), crops were planted in autumn - October 15, in spring - April 30, depth of seeding 2-3 cm. The seeding rates were: 20,40,60 pcs /line meter. Phenological observations showed the following: shoots appeared on 10-12 May, field germination - 30-50%. With the emergence of shoots, intensive development of the first pair of lateral roots, from which the long, strong filamentary processes that penetrate into the moist horizon of sandy soils are formed. This helps the *Agriophyllum* plant survive in conditions of hot, mobile sands.

Harvesting was carried out in a phase of full ripeness by weighing the biological mass of leaves and seeds. The estimated yield of *Agriophyllum* L. (*Agryophyllum squarrosum* (L.)) in the Shalkar region of the Aktobe area seeded on October 15, 2015, amounted to a green mass of 4-5 c / ha or 1.0-1.5 c / ha of hay. The estimated yield of seeds in the Shalkar region was 5-6 kg / ha, Zhanakalinsky region- 10-12 kg of seeds from 1 hectare. The seeds are 3-3.5 mm in size, rounded glabrous on the edge are wide-winged, with gnawed - serrated margins and 2 horns on the top in accordance with Figure 3.



**Figure 3** – Seeds of *Agriophyllum squarrosum*(L.)

The optimum seeding rate is 60 pcs / m., The seeding dates belong to autumn (October). The crops performed in autumn had better germination compared to the crops given out in the spring. Apparently, the seeds must undergo stratification in natural conditions, which was noted during the autumn seeding.

The harvesting, threshing and cleaning of seeds of collection samples of *Agryophyllum squarrosum* (L.) were carried out. The *Agryophyllum squarrosum* (L.) is thawed manually, which is due to the high prickliness and rudeness of the dry ripe plants. Samples of *Agryophyllum squarrosum* (L.) studied in the collection nursery. This plant is abundant in places with soil prone to erosion (around populated areas, wells, wintering grounds, along an oil pipeline route crossing sands, etc.) in the sandy massifs of the Northern Aral Sea region.

During the full ripeness phase, the harvest was carried out manually, by weighing the biological mass of the leaves and seeds. In the Shalkar region, the estimated yield of *Agryophyllum squarrosum* (L.) was 4-5 c / ha or 1.0-1.5 c / ha of hay. The fact that the seeds of this culture had extremely low germination was mentioned above. Stratified seeds yielded seedlings 9-10 days after seeding which was carried out on 11 April. The onset of the next phase - the onset of branching occurred on May 24-26. Thus, 34-36 days after the emergence of shoots, branching and shoot formation begins on the wild samples of sandy *Agryophyllum squarrosum* (L.). By this time 25-30 narrow-lanceolate leaves are formed on the plant, and the root system reaches 30-40 cm.

The duration of the sprouting period - the beginning of budding in most of the collection samples of sandy *Agryophyllum squarrosum* (L.) was an average of 76 days and occurred approximately 6-7 August (Fig. 4). The individual *Agryophyllum squarrosum* (L.) plants within the samples have therefore deviated, therefore, we tend to attribute them to modifications, since the process of seedling survival depended to a great extent on several factors, including the diligence of the assistants. The beginning of flowering started in 8-9 days on August 14-15.

Buds of *Agryophyllum squarrosum* (L.) are thorny clusters formed one by one almost in all the axils of the leaves. The buds are prickly due to the fact that the bracts are turned into spines up to 5-7 mm in length. In the axils of the branches of plants from the main stem, two lateral plus one central (adherent) bud are formed. The beginning of flowering is very difficult to determine due to the fact that the flowers are very small, formed from a single-trilobate dentate incised perianth. Stamens, 2-3 are weakly protruding from the perianth, preferably to contemplate with a magnifying glass. Maturation of collection samples of *Agryophyllum squarrosum* (L.) occurred in the period October 13-21. The shortest vegetation period was 113 days. The longest vegetation period was 121 days. The remaining wild-growing samples of the *Agryophyllum squarrosum* (L.) collection had an interphase sprouting period - a maturation of 116-118 days. As a result of the expeditions, the seed material was collected.

Biochemical analysis of fodder values of the plants and determination of quality of the seeds. Sandy *Agryophyllum squarrosum* (L.) widespread in the Western Kazakhstan, often meets and forms abundant landscape groups, especially in Bukeyevsky sands, between the Volga and the Ural rivers. According to literary sources, all kinds of *Agryophyllum squarrosum* (L.) in their young form serve as pasture for sheep, cattle and camels. 100 kg of green mass contains about 25 fodder units and 3.7 kg of digestible protein. Productivity is up to 20 quintals of green mass and 30 kg of seeds from 1 hectare. Fodder productivity of green plant mass and hay were defined. Laboratory analyzes of trial sheaves of *Agryophyllum squarrosum* (L.) were carried out. Sheaves were sheared after drying in an air-dry state to determine the yield of dry matter (Table 4).

**Table 4** - Results of the tests to determine fodder merits in Zhanakalinsky region, Western Kazakhstan area

| Lab. No | Product name                 | Humidity at % | Crude protein % | Crude cellulose % | Crude fat % | Crude ash % | Nutrition in fodder units |
|---------|------------------------------|---------------|-----------------|-------------------|-------------|-------------|---------------------------|
| 112     | Agriophyllum squarrosum (L.) | 13,11         | 11,24           | 40,3              | 4,0         | 9,26        | 0,49                      |

As a result of the analysis of sheaves, the protein content was found to be 11.24%, crude fat 4.0%, crude ash 9.26%, crude fiber 40.3%, nutritional value in fodder units - 0.49. The *Agryophyllum squarrosum* (L.) is eaten in green and dry form (after rains since it is very hard and prickly in dry form), especially by sheep and camels. In the phase of fruiting, the *Agryophyllum squarrosum* (L.) feedstuff resembles meadow hay. The most valuable and nutritious are fruits.

Small seeds of the plant are considered edible. Dry seeds of *Agryophyllum squarrosum* (L.) contain: 16-17% protein, 6-10% fat, food and semi-drying oil and 60% carbohydrates, mainly starch, up to 87% of substances, with high level of digestibility. In 100 g of these substances used as food contains 343 kcal, which brings *Agryophyllum squarrosum* (L.) closer to wheat flour, which is 344 kcal. The oil obtained by pressing the seeds of *Agryophyllum squarrosum* (L.), semi-liquid, its taste resembles sunflower, and by composition it approaches sesame. The seeds are 3-3.5 mm in size, rounded, glabrous, wide-winged along the margin, with notched teeth-jagged edges and 2 crowns.

Germination of seeds. Wild species from the family of goosefoot have traditionally low laboratory germination of seeds. The biological peculiarity of these plants is that they have evolved adaptive mechanisms that allow the genus to survive in deserts, salinization, frequent thaws and recurrent frosts, wind blowing the seeds with abrasive sand grains of quartz,

engulfing plants and seeds with sand and vice versa denuding the roots, transplanting seeds located in the surface layer of the soil, etc. In the literature, cases of very low laboratory germination of seeds of *Agryophyllum squarrosum* (L.) are also described. It was partially succeeded in carrying out stratification of seeds and, even despite these measures, two out of five species of *Agryophyllum squarrosum* (L.), prevalent in Kyzylkum desert, failed to obtain field shoots. In our experiments, the determination of the laboratory germination of seeds in the spring showed a very low seed germination of only 1%.

The estimated yield of wild growing *Agryophyllum squarrosum* (L.) in the monitoring points of Zhanakalinsky region was 6-8 c / ha of green mass or 2-2.5 c / ha of hay, in the Shalkar region 4-5 c / ha; 1.0-1.5 c / ha accordingly (Table 4).

The plant, at the time of fructification, contains:

water 9.9%, fiber 22.5-25%, crude protein 5.5-6.2%, pure protein 5.3-5.9%, fat-2.1-2.3%, nitrogen-free extractives 50-55.5% and ash 9.9-11.0%. The starch equivalent of the plant is quite high: 34-37.6%. In the autumn-winter period, after precipitation, the dry stems of *Agryophyllum squarrosum* (L.) become soft and therefore are well eaten by animals.

**Table 5** – Results of the tests to determine fodder merits in Shalkar region, Western Kazakhstan area

| Product name                        | Humidity at % | Crude protein in% | Crude cellulose in% | Crude fat In % | Crude ash in % | Nutrition in fodder units |
|-------------------------------------|---------------|-------------------|---------------------|----------------|----------------|---------------------------|
| <i>Agryophyllum squarrosum</i> (L.) | 9,9           | 5,5-6,2           | 22,5-25,0           | 5,3-5,9        | 9,9-11         | 50-55,5                   |

## CONCLUSIONS

As a result of the expeditions carried out in order to survey the distribution areas of Western Kazakhstan, monitoring was conducted to determine composition and distribution area of the *Agryophyllum squarrosum* (L.); description of individual plant species; phenological observations; biometric analysis with the establishment of their productivity from a unit area in the context of the distribution area, with the establishment of the coordinates of the sites of germination. Maps of distribution areas in Western Kazakhstan area and Aktobe area have been drawn up. The collection nursery revealed that the shortest vegetation period was 113-115 days. The longest vegetation period was 121-125 days. The introduction and study of biological features of growth of the *Agryophyllum squarrosum* (L.) in culture has been studied by means of experimental sowing made in autumn and spring. It appeared that autumn sowing had better germination since it passed stratification in natural conditions. Biochemical analysis of hay feed in the Western Kazakhstan area was revealed: the protein content was 11.24%, crude fat 4.0%, crude ash 9.26%, crude fiber 40.3%, nutritional value 0, 49. According to the result of the research maps of accumulation of taxa of *Agryophyllum squarrosum* (L.) have been drawn up; a herbarium collected; the seed material has been collected.

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## THE EFFECT OF DIFFERENT WHEAT CULTIVAR AND TURMERIC/GINGER POWDER USAGE ON PHYSICAL AND SENSORY PROPERTIES OF BULGUR

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### ABSTRACT

In this study, two different wheat cultivar (*Tr durum* and *Tr aestivum*) were used in bulgur production with and without turmeric and ginger powder addition. Turmeric and ginger powder were used during cooking stage of bulgur process. The effect of wheat cultivar and turmeric/ginger powder addition on physical and sensory properties of bulgur were determined. L\*, a\*, b\*, SI and Hue values of the bulgur samples changed between 41.95-52.23, 3.31-5.46, 13.86-26.86, 14.54-27.24 and 71.47-82.09, respectively. Bulgur produced from *Tr.durum* wheat showed higher lightness and yellowness and lower redness than *Tr. aestivum* bulgur. The use of turmeric powder in both bulgur varieties decreased the lightness and increased the yellowness value. According to cooking test results, bulgur produced from *Tr. aestivum* wheat revealed higher weight and volume increase than that of *Tr.durum* bulgur. Utilization of turmeric and ginger powder in bulgur production process did not change significantly weight and volume increase values. While the use of turmeric powder in both wheat cultivars affected the sensory properties positively, ginger powder decreased the taste and general appreciation score.

**Keywords:** Bulgur, Wheat cultivar, Turmeric powder, Ginger powder, Durum, Aestivum,

### INTRODUCTION

Bulgur is one of the world's first whole wheat-based products. It has been produced since the beginning of wheat production in Anatolia and the Middle East (Evlice and Özkaya, 2020). Turmeric is traditionally used as a spice and coloring in foods. It is an important ingredient in curry and gives curry powder its characteristic yellow color (Esatbeyoglu et al., 2012). Ginger is an herbal plant and is widely distributed in tropical and subtropical regions. It has been used worldwide as a spice, seasoning, juice, and wine as well as in disease prevention and treatment (Han et al., 2013). Ginger is abundant in active constituents, such as phenolic and terpene compounds (Mao et al., 2019). In this study, it was aimed to investigate the effects of different wheat varieties and the addition of turmeric/ginger powder on the color, cooking properties and sensory attributes of bulgur.

### MATERIAL AND METHOD

*Tr. durum* (Çeşit 1252) and *Tr. aestivum* (Esperia) wheat were obtained from commercial flour mill from Konya, Turkey. Turmeric and ginger roots were obtained from local market of



Konya, Turkey. Turmeric and ginger roots were dried and grinded in a laboratory mill to obtain turmeric and ginger powder (<500 µm).

### ***Bulgur production***

Bulgur samples were produced according to Ertaş (2017). Wheat samples were soaked in distilled water and then cooked in the autoclave until the starch was entirely gelatinized. Cooked wheat samples were dried at 50±5 °C to 10% moisture content in the oven (Nüve FN-500, Ankara, Turkey). The dried wheat seeds were tempered with 2% additional water by mixing for 10 min, and then milled into coarse grist with disk mill (Inovamer, Mersin, Turkey). All the cracked material was passed through a 3.55 mm sieve and over 1.6 mm sieve, and aspirated to remove bran material.

### **Laboratory analyses**

#### *Color measurement*

Color values were measured using Hunter Lab Chroma Meter (Minolta CR-400, Osaka, Japan). The instrument was calibrated by a white reference tile before the measurements. The color of the samples was evaluated by measuring the L\* (100 = white; 0 = black), a\* (+, red; -, green) and b\* (+, yellow; -, blue) values.

#### *Cooking test*

Cooking properties (weight increase, volume increase and cooking loss) were determined according to Evlice (2016).

#### *Sensory analysis*

Bulgur samples (500 g) were boiled in water (1000 ml). After all the boiling water was absorbed, bulgur sample was served to the panelists. Sensory analyses of bulgur samples were conducted by seven panelists. Sensory properties were evaluated in terms of color, taste, odor, mouthfeel and overall acceptability. Bulgur characteristics were rated on a 1–9 scale: 1 – dislike extremely and 9 – like extremely.

## **RESULTS AND DISCUSSION**

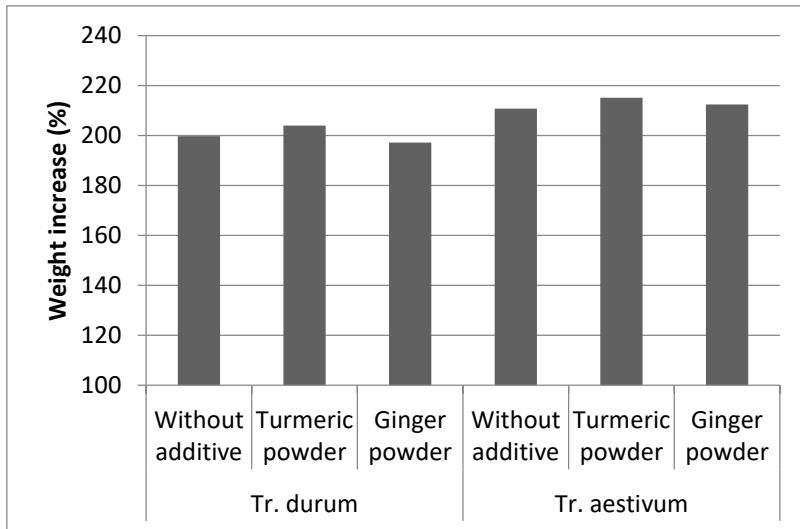
Color values of bulgur samples are given in Table 1. L\* values of the bulgur changed between 41.95 and 52.23. Bulgur produced from *Tr.durum* wheat showed higher lightness than *Tr. aestivum* bulgur. The use of turmeric powder in both bulgur varieties decreased the brightness value. As expected, a\* values of bulgur produced from *Tr. aestivum* were higher than those produced from *Tr. durum* wheat. The characteristic red color of *Tr aestivum* wheat had an effect on this result. On the other hand, the use of turmeric and ginger powder increased the redness in bulgur produced from *Tr. durum* wheat but decreased the redness in bulgur produced from *Tr aestivum*. While b\* values of bulgur produced from *Tr.durum* wheat varied between 22.61 and 26.86, this value was found between 13.86 and 18.46 in those produced from *Tr aestivum* wheat. The distinctive yellow color of durum wheat is also reflected in its bulgur. Utilization of turmeric powder caused an increase in yellowness in both *Tr.durum* and *Tr aestivum* bulgur. The SI values of bulgur showed a parallel trend with the b\* values. Among the bulgur samples, the highest Hue value was obtained in *Tr.durum* bulgur without additives,

and the lowest value was obtained in *Tr aestivum* bulgur without additives. Ertaş (2017) reported L\*, a\* and b\* color values of 12 different industrial bulgur samples between 52.48-75.16, -0.52-4.59 and 21.81-29.97, respectively. Evlice (2016) found L\*, a\* and b\* color values of bulgur which produced using seven durum wheat and two bread wheat between 78.0-80.6, 1.63-3.05 and 13.3-18.6, respectively. Bulgur produced from bread wheat exhibited higher redness and lower yellowness values than bulgur produced from durum wheat. In the literature, there are many studies on the use of turmeric in cereal products to increase the value of yellowness (Biancolillo et al., 2020; Wahanik et al., 2018; Seo et al., 2010; Levent et al., 2021; Nguyen and Le, 2018 ).

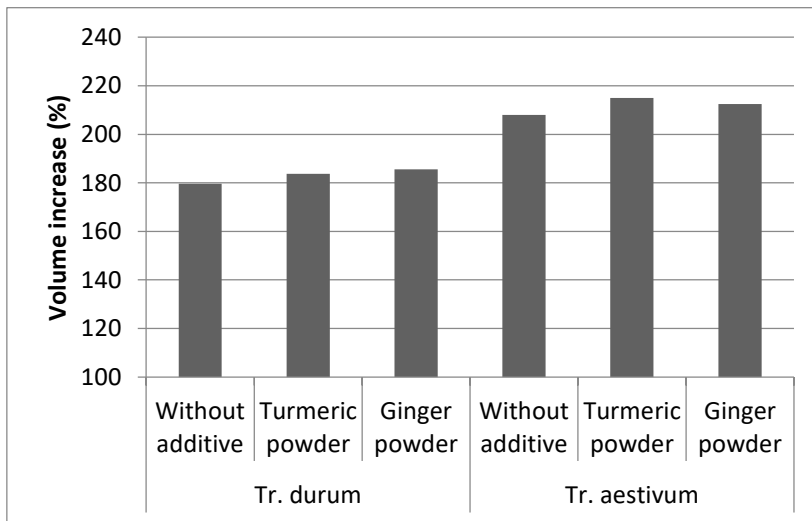
**Table 1.** Color values of bulgur samples

| Wheat cultivar      | Additive         | L*    | a*   | b*    | SI    | Hue   |
|---------------------|------------------|-------|------|-------|-------|-------|
| <i>Tr. durum</i>    | Without additive | 52.23 | 3.31 | 23.79 | 24.02 | 82.09 |
|                     | Turmeric powder  | 48.08 | 4.55 | 26.86 | 27.24 | 80.38 |
|                     | Ginger powder    | 51.14 | 4.08 | 22.61 | 22.97 | 79.77 |
| <i>Tr. aestivum</i> | Without additive | 46.26 | 5.46 | 16.31 | 17.20 | 71.47 |
|                     | Turmeric powder  | 41.95 | 5.17 | 18.46 | 19.17 | 74.35 |
|                     | Ginger powder    | 43.04 | 4.38 | 13.86 | 14.54 | 72.48 |

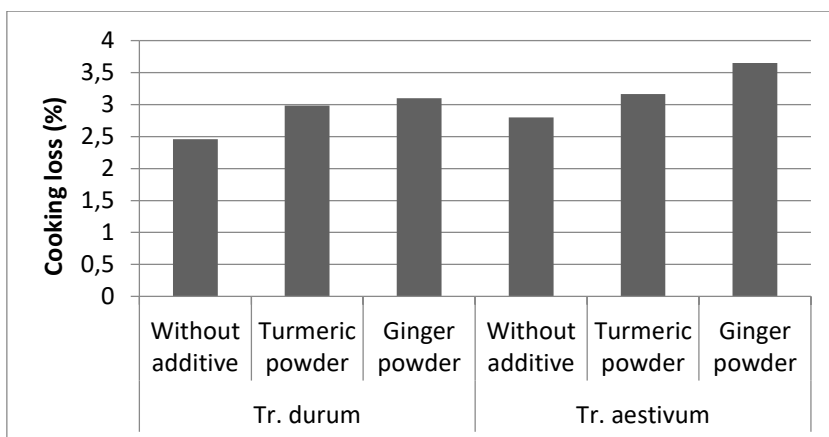
Cooking properties of bulgur samples are presented in Fig 1-3. While weight increase values of bulgur produced from *Tr.durum* wheat varied between 197.20% and 203.93% this value was found between 210.75% and 215.11%, in those produced from *Tr aestivum* wheat. Bulgur produced from *Tr. aestivum* wheat has higher weight increase than *Tr.durum* bulgur (Fig 1). Utilization of turmeric and ginger powder in bulgur production did not changed weight increase significantly. The lowest volume increase value for bulgur samples was obtained with utilization of *Tr.durum* without additive, and the highest was found as *Tr aestivum* with turmeric powder usage (Fig 2). The use of turmeric and ginger powder did not cause a significant change in the volume increase values of bulgur, as in the weight increase values. Cooking loss values of bulgur produced from *Tr.durum* wheat varied between 2.46% and 3.10, and this value was found between 2.80 and 3.65% in those produced from *Tr aestivum* wheat (Fig 3). As expected, bulgur produced from bread wheat gave more cooking loss than durum wheat, and also the use of turmeric and ginger powder increased the cooking loss. This increase reached higher values with the use of ginger powder. Certel (1990) also reported that bulgur produced from bread wheat absorbs more water than that produced from durum wheat. Evlice (2016) reported water absorption, volume increase and total organic matter values of bulgur between 219-245%, 187-207% and 2.92-3.80, respectively. Bulgur produced from bread wheat exhibited higher water absorption, volume increase and total organic matter values than bulgur produced from durum wheat. Hayta (2002) investigated the effects of different drying techniques on the quality characteristics of bulgur, and determined the weight increase values of bulgur samples between 2.33 and 2.56 (g su /g bulgur). Tağar (2021) determined cooking loss values between 3.40 and 5.77 in bulgur produced from durum wheat and between 3.78 and 5.56 in bread wheat.



**Figure 1.** Weight increase of the bulgur samples

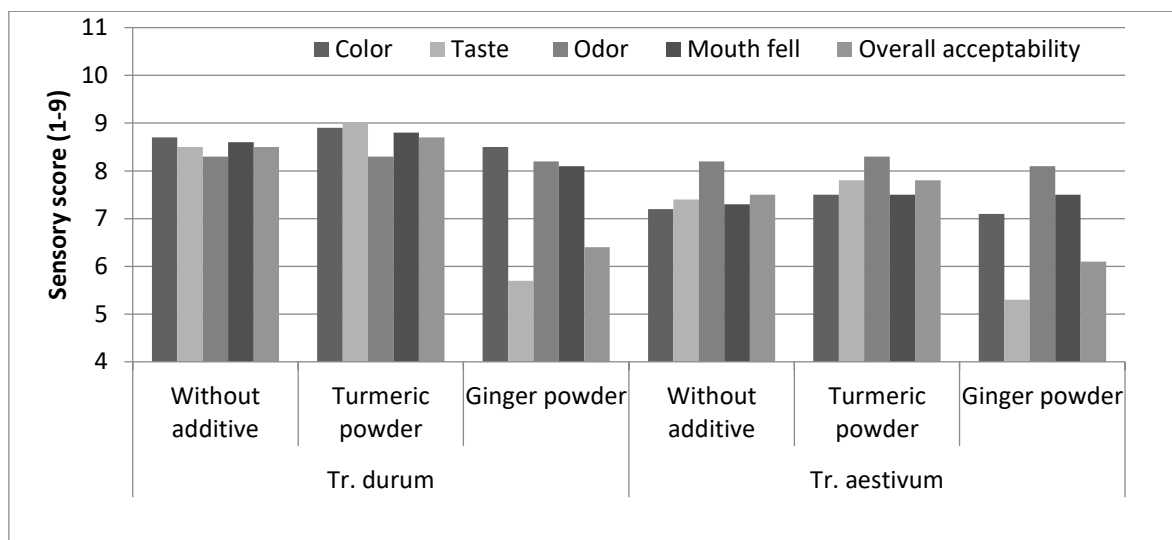


**Figure 2.** Volume increase of the bulgur samples



**Figure 3.** Cooking loss of the bulgur samples

Sensory properties of bulgur samples are presented in Fig 4. *Tr. durum* bulgur samples had higher color values than *Tr. aestivum* bulgur. The highest color value was determined in *Tr. durum* bulgur with turmeric powder addition, the lowest value was determined in *Tr. aestivum* bulgur with ginger powder addition. Turmeric powder has improved the color value of both bulgur varieties. In *Tr. durum* and *Tr. aestivum* bulgur samples, the use of turmeric powder increased the taste score, while ginger powder negatively affected the taste score. The use of ginger powder caused a bitter taste and decreased the taste score in both bulgur varieties. Mouth fell score was higher in *Tr. durum* bulgur than *Tr. aestivum* bulgur. The use of turmeric or ginger powder in *Tr. aestivum* bulgur did not cause any change in mouth fell scores. Bulgur produced from durum wheat had higher overall acceptability values than those produced from bread wheat. The use of ginger powder in both wheat cultivars decreased the general appreciation score. Among all samples, bulgur produced with the addition of turmeric powder from *Tr. durum* wheat has the highest overall acceptability score.



**Figure 4.** Sensory properties of bulgur samples

## CONCLUSIONS

In this study, the effect of the turmeric and ginger powder used in bulgur production from two different wheat varieties on some physical and sensory properties of bulgur was investigated. The use of turmeric powder decreased the lightness of the bulgur and improved the yellowness value in both types of bulgur. Bulgur produced from *Tr. aestivum* wheat has higher weight increase, volume increase and cooking loss values than *Tr. durum* bulgur. Cooking loss values also increased with the turmeric and ginger powder usage in bulgur production. According to the sensory analysis results, the use of ginger powder decreased the taste and overall acceptability scores of both bulgur varieties. Bulgur produced with the addition of turmeric powder from *Tr. durum* wheat was the most appreciated by panelist.

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## **EFFECT OF PERICARP ON THE GERMINATION OF GREEN OAK ACORNS (*Quercus ilex*)**

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### **ABSTRACT**

The knowledge of the germinative properties of acorns allows to define with precision the conditions of use of acorns according to the desired objectives. The objective of this work is to analyze the effect of certain physical treatments on the germination of holm oak acorns. The results obtained show that the acorns tested show a variation in biometry. These variations have no effect on germination. The removal of the acorn skins of two varieties (Tebessa and Batna) gave the highest germination rates (100%). Untreated acorns gave a germination rate of 40%. The results obtained during these experiments clearly show that the pericarp constitutes a significant obstacle to the rapid and homogeneous germination of holm oak acorns.

**Keywords:** holm oak, acorns, germination, treatment.

### **INTRODUCTION**

Mediterranean forests cover about 81 million hectares, i.e., a global forest area of 9.4%. They are made up of a mosaic of forest species, particularly hardwoods which represent about 60% (Mugnossa et al. 2000).

In Algeria, the oaks represent a forest capital of about 40% of the Algerian forest. The holm oak appears from 400 m above sea level and rises to 1700 m in the Aures (Alatou, 2007; Boukhelkhal, 2017).

Germination is a complex phenomenon involving several factors such as growth regulators and hydrolytic enzymes that interact to trigger the process and subsequent growth (Côme, 1970; Roberts et al., 1988). In natural ecosystems, germination may be limited by factors such as predation or seed infestation. In addition, the maintenance of forest species through their seeds poses the problem of germination in the first place.

Faced with this situation, the need to assist natural regeneration is obvious. Although certain methods of vegetative propagation of the holm oak seem to be usable, such as cuttings, the principal mode of propagation of the species is sowing.

It is thus essential, for a good diffusion of the holm oak, to control the techniques and the conditions of breeding of the seedlings and, in particular, those of the germination of acorns. It is in this context that the main objective of this work is to study the germinative behavior of *Quercus ilex* acorns subjected to several treatments. In addition to the optimization and

homogenization of its germination, the knowledge of the germinative strategy adopted by the holm oak allows to better understand its natural dynamics.

## **MATERIAL AND METHODS**

### **Plant material**

The acorns of holm oak harvested in late October and early November 2021, come from the regions of Batna and Tebessa. The plant material consists of young plants of two different varieties of this species, resulting from the germination of acorns.

In the laboratory, the acorns were first separated by variety and by treatment. They were cleaned and sorted by a densimetry test with ordinary water. The supernatants, considered non-viable, were eliminated (Dupouey and Le Boulter, 1989). Healthy acorns were selected from each variety to study morphological variability.

### **Determination of moisture content**

The water content is one of the limiting factors of germination, for that a minimal threshold must be preserved: threshold below which germination does not take place. This minimum quantity of water makes it possible to maintain in life the embryo, thus its germinative faculty (Alatou, 1984).

The water content is the difference between the weight of the fresh matter (FP) and the weight of the dry weight (DW), obtained after a passage in an oven at 96°C for 48 hours. It was determined for 10 acorns of different holm oak varieties. The determination of the water content is done according to the formula of Vilain (1987):

$$WC = \frac{FP - DW}{FP} \times 100$$

**WC** : Water content

**FP** : Fresh weight

**DW** : Dry weight

### **Treatments applied to acorns**

Germination is defined as the transitional phase between the dry seed stage and the appearance of the radicle (Gimeno-Gilles, 2009). This phase requires first of all the imbibition of the tissues. If the physiological conditions are favorable (absence of primary dormancy) as well as the environmental conditions (availability of oxygen, adequate temperature), germination is possible. The treatments applied are the following:

T1: Intact acorns (Batna variety)

T2: Acorns without pericarp (Batna variety)

T3: Intact acorns (Tebessa variety)

T4: Acorns without pericarp (Tébessa variety)

Ten acorns of each treatment are placed in germination pots containing moist topsoil (watering done every two days). The pots were placed in natural conditions. Germination was monitored for 30 days, with twice-daily counts of germinated acorns. Acorns that showed positive geotropism with an elongated radicle of at least 2 mm were considered germinated (Côme and Corbineau, 1998)

The germination rate is calculated by the formula:

$$GR(\%) = \frac{NGG}{NTGS} \times 100$$

GR = Germination Rate;

NGG = Number of Acorns Germinated;

NTGS = Total Number of Acorns Sown.

## RESULTS AND DISCUSSION

The average length of the acorns varies between 3,68 and 3,88 cm for the two varieties of holm oak and Tebessa respectively. The width varies between 1,31 and 1,95 cm (acorns of the two varieties of Tebessa intact and without pericarp). The weight varies between 2 g (Batna acorns without pericarp) and 6 g (intact Tébéssa acorns) (Table 1).

The difference between the minimum and the maximum of the acorns can go from 3,4 to 4,3 cm for length and from 1,1 to 2,1 cm for width (Figure 1). For weight, the difference between the minimum and maximum is important: it varies from 2,7 to 7,5 g.



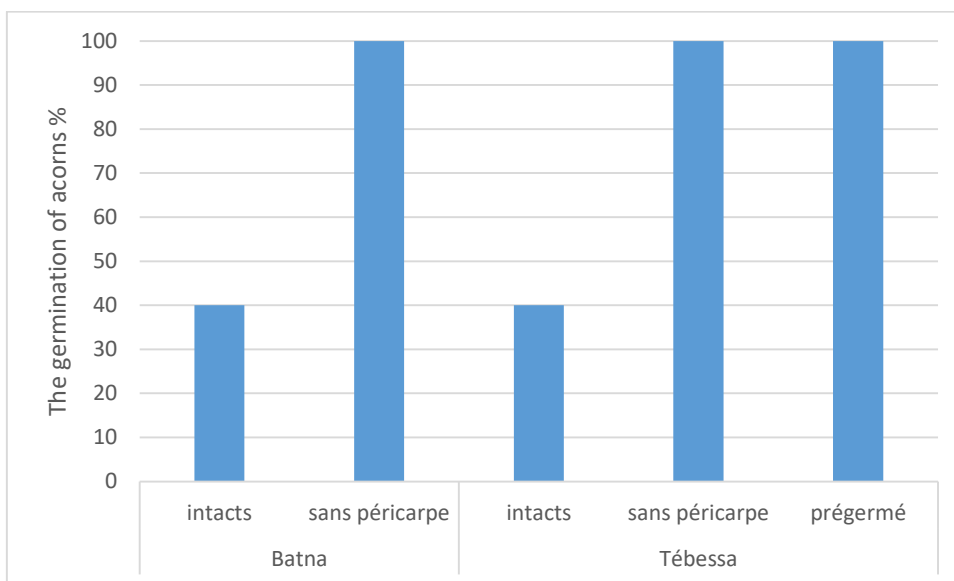
**Figure 1:** Morphology of different acorns (a: Batna; b: Tébéssa).



**Table 1:** Acorn biometric characteristics

| characteristics |                    | Batna   |                  | Tébessa |                  |
|-----------------|--------------------|---------|------------------|---------|------------------|
|                 |                    | Intacts | without pericarp | Intacts | without pericarp |
| <b>Longueur</b> | Average            | 3,83    | 3,68             | 3,87    | 3,88             |
|                 | Max                | 4,2     | 4,1              | 4       | 4,3              |
|                 | Min                | 3,5     | 3,5              | 3,7     | 3,4              |
|                 | Standard deviation | 0,23    | 0,20             | 0,13    | 0,26             |
| <b>Largeur</b>  | Average            | 1,31    | 1,44             | 1,95    | 1,95             |
|                 | Max                | 1,6     | 1,7              | 2,1     | 2,1              |
|                 | Min                | 1,1     | 1,1              | 1,7     | 1,7              |
|                 | Standard deviation | 0,14    | 1,16             | 0,13    | 0,12             |
| <b>Poids</b>    | Average            | 2,2     | 2                | 6       | 3,4              |
|                 | Max                | 4,5     | 3,2              | 7,5     | 6,8              |
|                 | Min                | 3,25    | 2,7              | 6,66    | 5,44             |
|                 | Standard deviation | 0,75    | 0,39             | 0,61    | 0,84             |

**The germination of acorns**



**Figure. 2:** Acorn germination rate of holm oak by variety

The analysis of figure 2, shows that the germination rate is very high, reaching a maximum of 100% for the acorns without pericarp of two varieties. As for the intact acorns, the germination rate is 40%. Acorns from Tébessa have a germination rate equal to 100%. It is noted that the germination rate of the region of Tébessa is higher than that of the region of Batna with rates of 80% and 70% respectively.

The results obtained during these tests let appear that the teguments constitute a not negligible obstacle to the homogeneous and fast germination of the acorns of green oak. Indeed, it is shown the beneficial action of the ablation of the seminal envelopes on the kinetics of germination. (Lamond, 1978) has already reported the influence of tegument removal on the improvement of the germination power of acorns of pedunculate oak. Benmahioul et al, (2010) also report the effect of seed coats on the germination of *Pistacia vera* L. seeds. The best

germination rates were recorded with the seed lot without seed coats. Levert (1977) noted that the bare embryo imbibed much better and faster than the whole acorn of pedunculate oak. The same author points out that the poor germination of the whole acorn could be explained at least in part, by inadequate rehydration of the embryos.

## CONCLUSIONS

Acorn abundance, plant health, morphological and physiological maturity, and acorn size play an important role in the natural regeneration of holm oak.

The results obtained in these experiments clearly show that the pericarp and/or the seed coats constitute a significant obstacle to the rapid and homogeneous germination of holm oak acorns. Unfortunately, we have not yet been able to pinpoint the mechanism(s) responsible for this depressive effect of the seminal envelopes on germination of this seed.

Intact holm oak acorns have a lower germination rate than acorns without pericarp. This difficulty can be overcome by total or partial removal of the seminal envelopes. The removal of the seminal envelopes is easy to perform manually and does not pose any problem at the laboratory scale. On a forest scale, however, this operation requires the prior development of a mechanical process for decorticating the acorns, which is likely to pose technical problems.

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## DEMOGRAPHIC STRUCTURE AND SPATIAL DISTRIBUTION OF HOLM OAK IN THE CHETTABA FOREST

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### ABSTRACT

The holm oak is the main species of the state forest of Chettaba which covers an area of 2398ha where it occupies 1127ha. However, during the last decade, a particular attention and a scientific and forestry interest have been expressed for this species. Plantations of holm oak were studied in the Chettaba forest, the diameter at 1,30 m from the ground, the total height of holm oak individuals were measured on 4 plots according to an altitudinal gradient. The diameter and height structures were fitted to the theoretical Weibull distribution. According to this test of fit, the holm oak stand is characterized by a relative predominance of young and small diameter individuals, which suggests a good regeneration of the species. These results contribute to the improvement of the knowledge on the indicators of the current state of the holm oak stands which can be used as a basis in the management of the Chettaba forest.

**Keywords:** holm oak, Weibull distribution, demographic structure, spatial structure.

### INTRODUCTION

Sclerophyllous oaks participate in, or even constitute practically by themselves, various types of landscape highly characteristic of the Mediterranean world. The holm oak is the typical species of the Mediterranean forest, where it currently occupies between 354,000 and 433,000 ha, part of which is in the form of coppice. It is adapted to continental and altitudinal conditions between semi-arid and subhumid, it can climb in altitude up to the limit of 1600 m where it is in direct competition with the cedar, nevertheless it overflows on semi-arid stations in the most degraded stations (Haichour, 2009; Nasrallah, 2014).

In Algeria, oaks represent a forest capital where they cover nearly 40% of the Algerian forest (Alatou, 1994). These oaks play an undeniable role on the ecological, economic and social levels. However, in Algeria, the holm oak forest is threatened by multiple factors, such as the extension of agriculture, infrastructure (settlements and roads), overgrazing and fires (Nasrallah, 2007). The management of forest stands requires the control of the structure of trees (Van Laar and Akça, 2007). These structures are indicative of events related to stand life, site conditions and to define better management options (Rondeux, 1999; Feeley et al., 2007). To represent the theoretical structure of a stand, several types of distributions can be used (normal

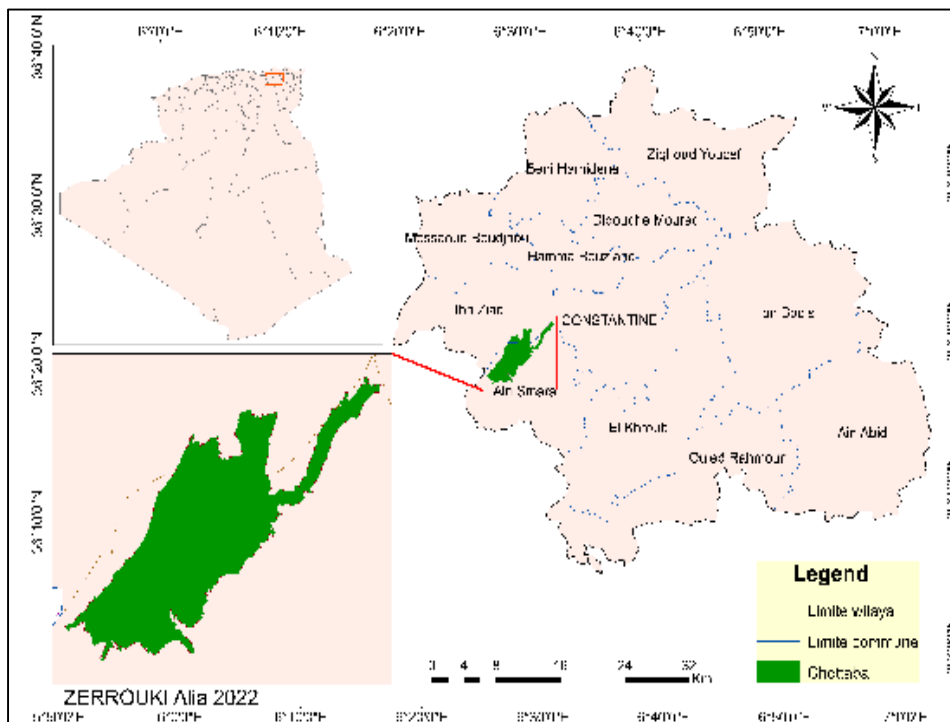
distribution, lognormal distribution, exponential distribution, Weibull distribution, etc.). Parameters are estimated from observed data (Husch et al. 2003). However, the Weibull distribution is more appropriate because it is characterized by a great flexibility of use and presents a great variability of forms according to the values taken by its theoretical parameters (Bullock Burkhart, 2005)

The main objective of this study is to analyze the demographic structure and spatial distribution of holm oak populations in the Chettaba forest located southwest of Constantine (Algeria) and their effects on natural regeneration for future development and sustainable management of the forest.

## MATERIAL AND METHOD

### Presentation of the study area

Forest of Chettabah is located southwest of Constantine (Algeria). The estimate terrain elevation above sea level is 865 meters. The study area is located on the map topographic Constantine Scale 1/200 000 sheet N° 17 and located between the coordinates 36°19'4" north latitude and 6°28'36" East longitude.



**Figure 1.** Location of the study area.

### Methodology

#### Data collection

The forest inventories were carried out in plant formations dominated by holm oak. Four plots of 0,09 ha (30 x 30 m) were set up in the Chettaba forest. The dendrometric parameters measured are the diameters at 1.30 m from the ground (dbh: diameter at breast height) and the heights of trees.

### Data processing

The collected data are entered into Microsoft Excel spreadsheet (2019), which was used to determine the ecological characteristics and natural population structure. In these plots, demographic structures were modeled by Weibull distributions by Minitab 16 software. The Weibull distribution is well suited to model the observed diametric structures (Husch et al. 2002). From the parameters (a, b and c: see below) conclusions are drawn about the life conditions of the trees. The established structure models then allow for the definition of appropriate management options for the studied stands. The Weibull distribution is based on the probability density function defined as follows:

$$F_{(x)} = \frac{c}{b} \left( x - \frac{a}{b} \right)^{c-1} \exp \left[ - \left( \frac{x - a}{b} \right)^c \right]$$

where x is the diameter (respectively height) of the trees; a is the origin or position parameter. It corresponds to the pre-count diameter and height of the adult population; b is the scale parameter related to the median value of the diameters and heights of the trees; c is the shape parameter related to the structure considered (Table 1). The values of "c" are related to well-established distribution forms (Husch et al., 2002).

Table 1. Shape of the Weibull distribution according to the values of the parameter c.

|             |   |
|-------------|---|
| c < 1       | Inverted "J" distribution, characteristic of multi-species or uneven-aged stands.   |
| c = 1       | Exponentially decreasing distribution, characteristic of populations in extinction.   |
| 1 < c < 3,6 | Positive asymmetric or right asymmetric distribution, characteristic of monospecific stands with a predominance of young or small diameter individuals. |
| c = 3,6     | Symmetrical distribution; normal structure, characteristic of even-aged or monospecific stands even-aged or monospecific stands of the same cohort.     |
| c > 3,6     | Negative or left-skewed distribution, characteristic of monospecific stands with a predominance of older individuals.                                   |

Stand demographic structures were also analyzed by calculating skewness coefficients. Skewness coefficients (g) were calculated to determine trends in population structures using the following formula:

$$g = \frac{n \sum_i (x_i - \bar{x})^3}{(n - 1)(n - 2)\sigma^3}$$

where n is the number of stems, xi is the diameter at breast height, the mean diameter, and σ is the standard deviation of xi. The skewness coefficient is a measure of the relative proportion of small stems to large stems within a population. It describes the uniformity of truncated distributions: g > 0 for diametric distributions with relatively few small stems and many large stems; g < 0 for diametric distributions with relatively few large stems and many smaller stems (Feeley et al., 2007).

### RESULTS AND DISCUSSION

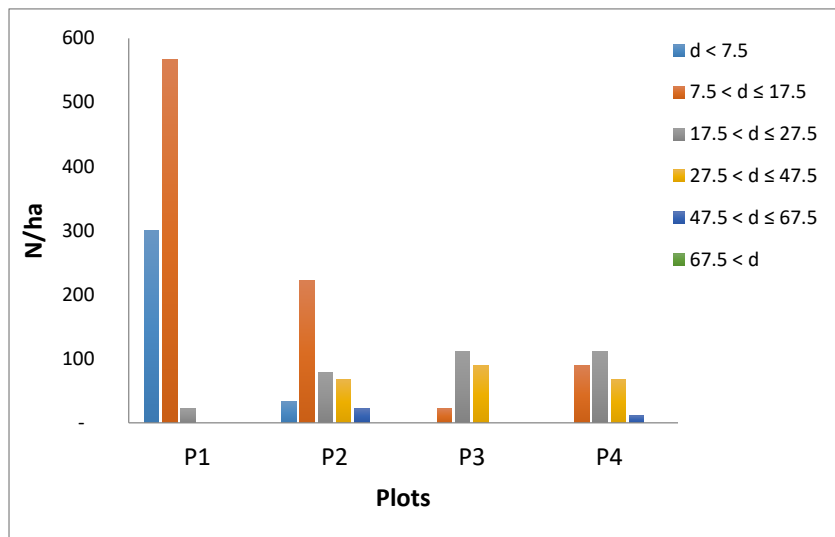
The average density of woody at the level of the forest of Chettaba is varied between 889 and 222 individuals/ha. The average diameter is between 10,33 and 26,31cm, these stands are

in the state of thicket-gallery to mature forest. Plot 3 is characterized by the lowest density (222 individuals/ha). Plot 1 had the highest density at 889 individuals/ha (Table 2).

**Table 2.** Dendrometric characteristics

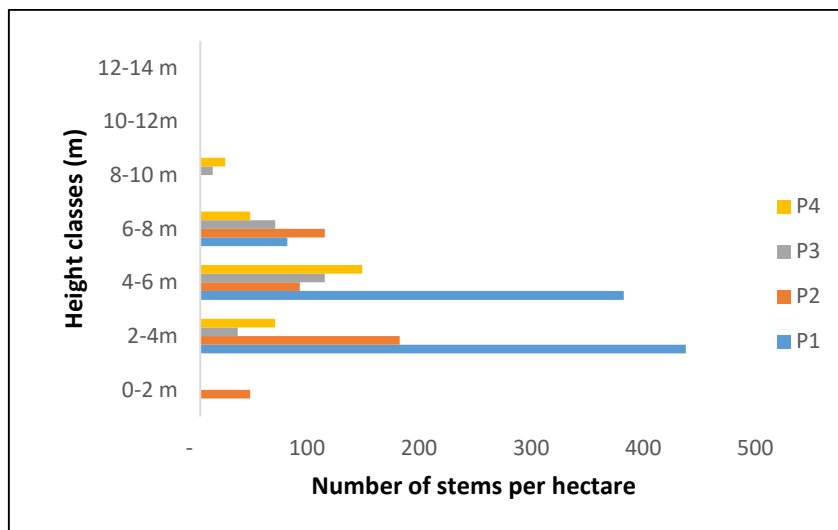
| parcelles | N/ha | D (cm) | H (m) |
|-----------|------|--------|-------|
| P1        | 889  | 10,29  | 4,17  |
| P2        | 422  | 12,51  | 4,12  |
| P3        | 222  | 26,31  | 5,34  |
| P4        | 278  | 18,77  | 4,96  |

The diameter class ( $d < 7,5\text{cm}$ ) is present in plots 1, 2, and 4 and characterizes good generation of the species (Figure 1). The 17,5 to 27,5cm diameter class has a high rate in plots 3 and 4. In addition, many individuals of this species are only at a young life stage. On the other hand, the large diameter stems are almost completely weak (P2 and P4).



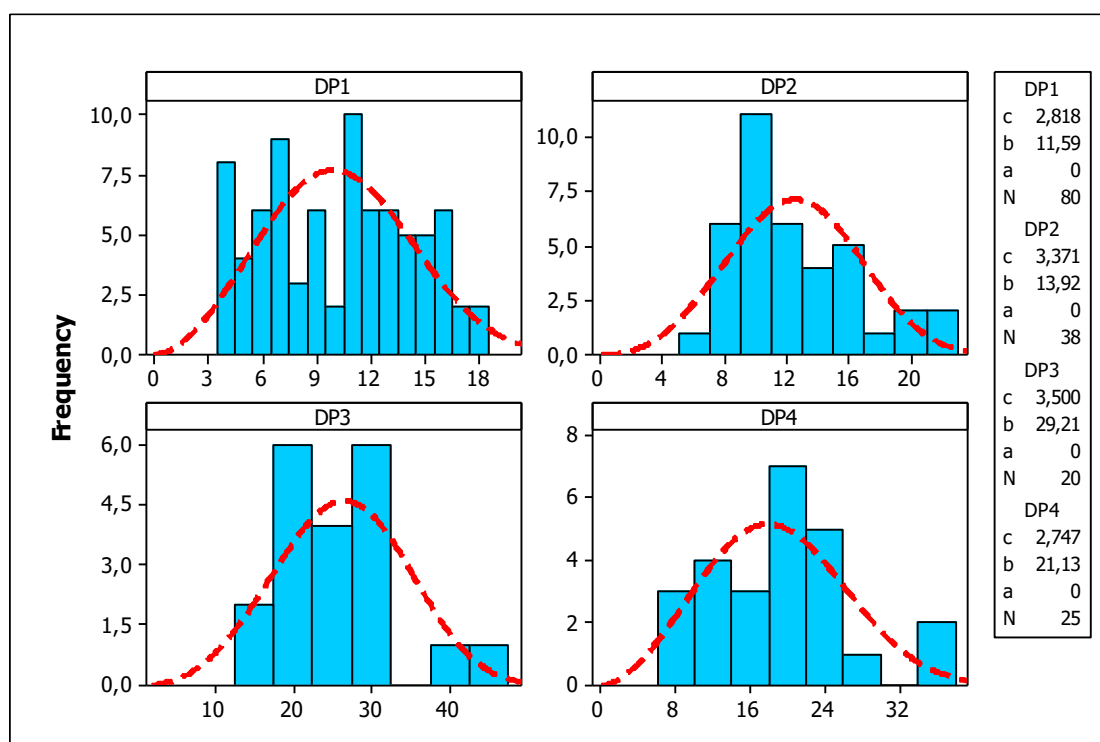
**Figure 2.** Density by diameter class.

The height structure is given by the distribution of the number of stems in all plots per hectare for each class and provides information on the vertical stratification of the stand. A large proportion of the trees are between 2 and 8 m high (low pole saplings) with 1733 individuals; this class has a large number of individuals because of regeneration, which remains fairly average, while the 0 to 2 m and 8 to 10 m classes are characterized by a low number of trees (Figure 2). There is an almost total absence of trees over 10 m.



**Figure 3.** Density by height class.

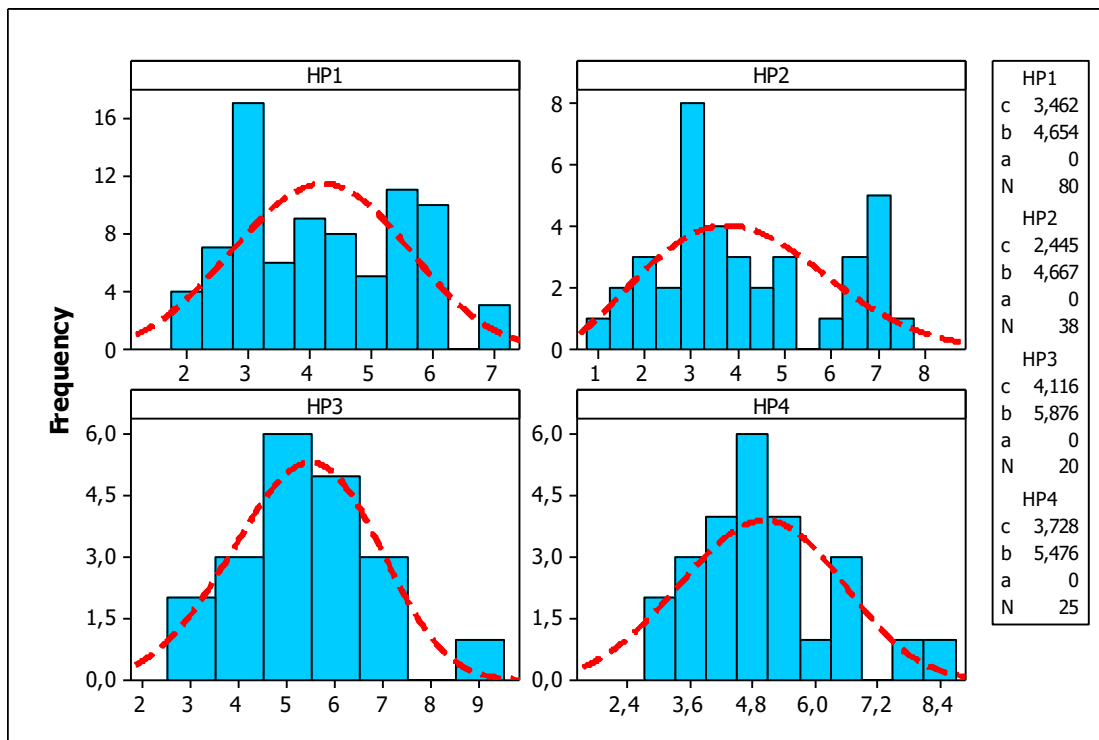
Diameter structures of adult holm oak populations are similar and have a bell-shaped appearance in all four study plots (Figure 3). This characterizes low proportions of stems with diameters near the pre-count diameter (dbh = 5 cm). Indeed, holm oak stems are concentrated in the 7,5 to 17,5 and 17,5 to 27,5 cm diameter classes. All distributions have a "c" value between 1 and 3,6. These "c" values indicate positive skewed distributions for all diameter structures and a predominance of young stands (Husch et al., 2003).



**Figure 4.** diameter structure of holm oak according to Weibull.

The values of the shape coefficient c of the Weibull distribution of the height structure of all woody plants between 2,83 and 3,37 for plots P1 and P2 respectively. This value indicates

a positive asymmetric distribution (right) with a predominance of low height individuals (Figure 4). On the other hand, the distribution of individuals in plots P3 and P4 in diameter classes shows a negative asymmetrical or left asymmetrical distribution, characteristic of monospecific stands with a predominance of old individuals.



**Figure 5.** Tall structure of holm oak.

## CONCLUSIONS

The establishment and interpretation of diameter and height structures are essential for forest management decisions. The characteristics of the plant formations studied show that the structure of the identified plant groups is still dominated by stems of small diameter classes. The study of the dendrometric characteristics of the holm oak of some plots of the Chettaba forest (East Algerian) showed that the individuals of big diameter are totally absent. Finally, this diagnostic state will constitute a reference for the next monitoring results and decision for the foresters in the framework of a management plan.

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## **PRELIMINARY STUDY ON SOURCES AND CAUSES OF MILK LOSSES IN DAIRY FARMS IN NORTH-EAST OF TUNISIA (BIZERTE)**

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### **ABSTRACT**

Food waste is a growing global issue, posing a challenge to food security, nutrition and environmental sustainability. In MENA region, most of food waste take place at early stages of the food value chain. For instance, Tunisian dairy value chain is characterized by loss rates averaging 9% in Bizerte and 6.5% in Mahdia, according to Food and Agriculture Organization (FAO) in 2017. Methodology: The present study aimed to investigate the association between raw milk loss in primary production and on-farm management practices, as well as farmers' characteristics. A survey was conducted in 25 dairy farms in the region of Bizerte, (North of Tunisia), through face-to-face interviews to farmers, during two months. Information about socio-demographic characteristics of the farmers, general farm demographics and management, on-farm milk loss, as well as milking procedures and storage, were collected. Pareto analysis was performed and for all contributing causes, 80:20 rule was applied. Results: Respondents' farming systems were characterized mainly by integrated production systems (92%). Only 35% of respondents used milking parlors and 32% had refrigerated milk tanks. About 60% of respondents declared to have milk losses. Self reported milk losses were estimated to 573 L/year/farmer, representing an average loss of about 176 euros/year/farmer. On a nutritional point of view, this represented per year and per farmer, about 36 000 Calories and 19 Kg of proteins, not available for human consumption. Interestingly these losses were significantly associated with socio-demographic characteristics of the farmers and farms: significant negative effects in milk losses were observed within non-educated and older farmers (above 50 years old), as well as in small sizes (less than 20 lactating cows) and low efficiency (14-17L/cow) of the dairy farms. Milking management practices also influenced self-reported milk losses: the use of milking parlor and refrigerated milk tanks significantly decreased the level of milk losses by respectively -86% and -86%. Moreover, Pareto chart analysis has revealed five main causes responsible of 80% milk losses : in descending order of critically, poor milking conditions (hygiene, equipments), poor quality of milk, poor technical support, long time between milking and collecting, and presence of antibiotic residues. Conclusion: The present study indicated a significant milk loss in primary production in Bizerte region: reducing loss of nutritious foods such as milk can help not only fight hunger and malnutrition, but also strengthen economies and protect the environment. Measures and actions to reduce these losses should strengthen, especially to improve hygiene, infrastructure and training.

**Key words :** SDG12, Milk loss reduction, refrigerated milk tank, farmers, Pareto diagram

## **INTRODUCTION**

The world population is constantly increasing from about 7.96 billion in 2021 to a projected 8.5 billion in 2030 and 9.7 billion in 2050, according to latest United Nations (UN) projections. Together with increased incomes and thus an improvement of living standards, this will lead to an increase in the demand for highly nutritious products, especially animal-source food products, such as milk and dairy products (OECD-FAO, 2022). The OECD-FAO (2022) predict global consumption (per capita consumption) of dairy products to increase 0.4% p.a. to 21.9 kg (milk solids equivalent) by 2031 in high-income countries compared to 2.0% p.a. (21.2 kg) and 1.5% p.a. (5.4 kg) in low-middle income and low-income countries, respectively. This implies a growth in world milk production of 1.8% p.a. (projection) over the next decade (to 1 060 Mt in 2031), faster than most other main agricultural commodities (OECD-FAO, 2022). In a climate change context, this increase in milk production should be based on sustainability, as well as environmental, nutritional, and socioeconomic empowerment, and meet 2030 Agenda for Sustainable Development. By protecting natural resources, dairy farmers will be able to not only grow their businesses but also ensures the land's preservation for future generations.

Tunisian dairy sector plays a strategic role in economic growth, enhancing food security, reducing poverty and promoting rural development (ONAGRI, 2018). However, it has to face major challenges related to climate change, as well as mishandling and mismanagement, leading in particular to milk losses at the production and post-harvest levels (Sraïri et al. 2013). In fact, Tunisian dairy value chain is characterized by loss rates averaging 9% in Bizerte and 6.5% in Mahdia, according to Food and Agriculture Organization (FAO) in 2017 (FAO, 2018). These losses at the primary production and post-harvest levels are mainly due to poor handling practices (FAO, 2018). On-farm spoilage occurs during harvesting and storage of milk at the farm (Abdelhafidh et al., 2018). Therefore it is important to control on-farm practices in order to guarantee milk safety and quality and to decrease losses at production and post-harvest levels. GIVLait program with FAO has promoted implementation of the cold in dairy farms (FAO, 2018). In order to improve on-farm practices, it is necessary as a first step, to quantify and analyze causes of milk losses at this level. This represents an opportunity to enhance profitability and to promote sustainable development, and in particular meet UN sustainable developments goal SDG 12 sustainable production and consumption.

The objective of this preliminary work was to assess raw milk losses in primary production, associated with farmers' characteristics and on-farm management practices, including the cold at the farm, in the North-East of Tunisia.

## **MATERIAL AND METHOD**

This preliminary study was carried out in Bizerte Region (North-East of Tunisia), where dairy farming occupies second place nationally, behind the Center-East (ONAGRI, 2018). A survey was conducted in 25 dairy farms (as probabilistic sample), through direct interviews and an observation checklist, during June and July 2020. Information about farmers' socio-demographic characteristics, general farm demographics and management, on-farm milk loss, as well as milking procedures and storage, were collected.

Primary data analysis consisted in frequency counts and percentages, using Excel software. Chi-square tests of independence were performed to test for associations with demographic and farm variables, using GraphPad Prism (version 8, 2019). Pareto analysis based on frequency was used for the identification of causes that produced significant overall effect. For all contributing causes, 80:20 rule was applied.

## RESULTS AND DISCUSSION

Table 1 indicates socio-demographics of survey respondents.

**Table 1.** Survey respondents' socio demographic profile (n=25 farmers)

|                           |            | % respondents |
|---------------------------|------------|---------------|
| <b>Gender</b>             | Men        | 96            |
|                           | Women      | 4             |
| <b>Age (years old)</b>    | 20-40      | 4             |
|                           | 41-50      | 20            |
|                           | 51-60      | 48            |
|                           | Above 60   | 28            |
| <b>Level of Education</b> | None       | 12            |
|                           | Primary    | 40            |
|                           | Secondary  | 32            |
|                           | University | 16            |

Socio-economic characteristics indicate that most respondents were male (96%) and above 51 years old (68%). This advanced age is associated to the difficult working conditions these past decades, leading to a shortage of farm workers and to an increase in urban migration. 48% of farmers were educated up to primary level, indicating a lack of professionalization (Abdelhafidh et al., 2018). Only 16% have a University degree.

Of the surveyed farmers, only 12% owned a land area larger than 10 ha, whereas 40% owned between 3 and 5.99 ha and 12% owned less than 1 ha. In all surveyed farms, cow genetic resources consisted of the Holstein high-yielding dairy breed. In terms of number of cows, 24% of the farmers had fewer than 5 cows, 44% had 11–20 cows and 20% had more than 20 cows. In Tunisia, smallholders represent about 80% of the total number of dairy farmers in the country (ONAGRI, 2018). Respondents' farming systems were mainly integrated (92%). These small-scale farms were showing low efficiency in milk production (14-17L/cow). Only 35% of respondents used milking parlors and the milking process was mainly mechanical. On the surveyed farms, 32% had refrigerated milk tanks.

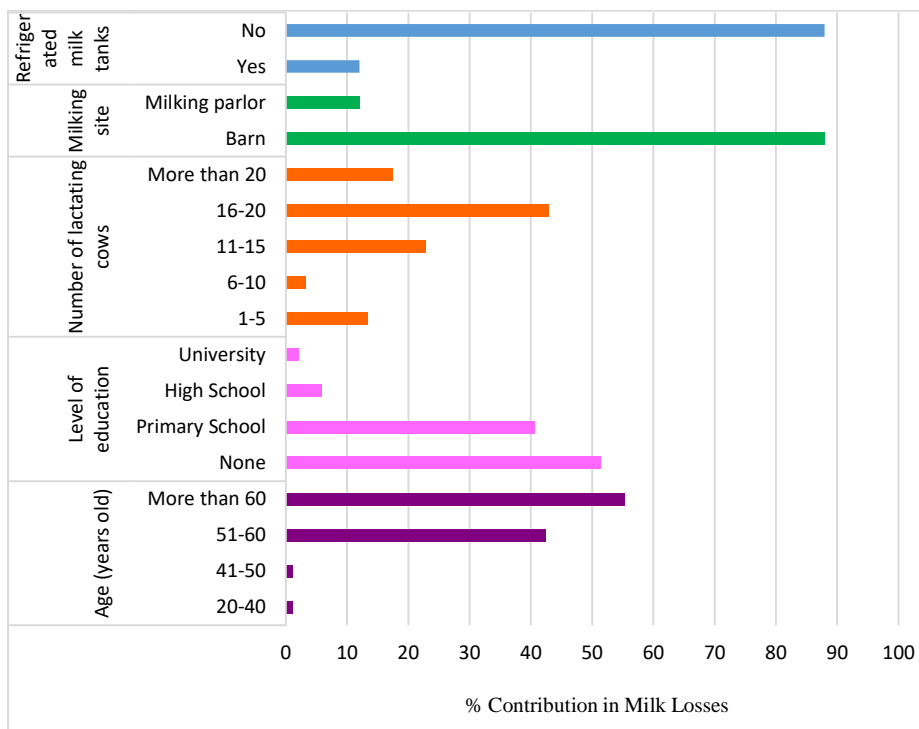
About milk losses, 60% of respondents stated to have observed some in their farms (Table 2). Self-reported milk losses were evaluated to be about 573 L/year/farmer. This represented per year and per farmer, an average economic loss of about 176 euros/year/farmer and, on a nutritional point of view, a loss of about 36 000 Calories and 19 Kg of proteins (FAO, 2022), not available for human consumption. In contrast, FAO (2021) has reported a loss of 681 L/year/farmer, this difference was related to the size of sample, and the method of quantification. Survey respondents also tend to substantially under-report their waste levels (Jribi et al., 2020).

Table 2 summarized typology of dairy farming systems of survey respondents.

Figure 1 showed the impact of milk harvest management practices and farmers' socio demographics on self reported on-farm milk losses. Statistical tests have shown significant negative effects ( $p < 0.0001$ ) on self-reported milk losses of farmers' older age ( $R^2 = 0,8796$ ) and low education ( $R^2 = 0,9121$ ). Higher milk losses were observed in dairies with 16-20 lactating cows ( $R^2 = 0,6856$ ;  $p < 0.0001$ ), whereas the use of milking parlors and refrigerated milk tanks reduced significantly ( $p < 0.0001$ ) milk losses by respectively  $-86\%$ , and  $-86\%$  (Figure 1).

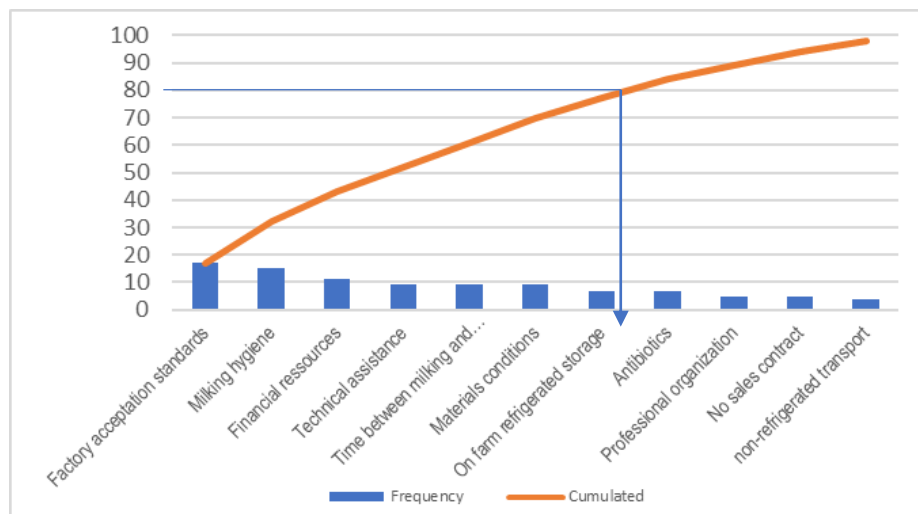
**Table 2.** Dairy farm management practices of survey respondents and self reported milk losses (n=25 farmers)

|                                       |                 | % respondents |
|---------------------------------------|-----------------|---------------|
| <b>Farm Surface (Ha)</b>              | <1              | 12            |
|                                       | 1-2.99          | 16            |
|                                       | 3-5.99          | 40            |
|                                       | 6-10            | 12            |
|                                       | >10             | 20            |
| <b>Number of Dairy Cows (Unit)</b>    | 1-5             | 24            |
|                                       | 6-10            | 12            |
|                                       | 11-15           | 24            |
|                                       | 16-20           | 20            |
|                                       | >20             | 20            |
| <b>Farming System</b>                 | Integrated      | 92            |
|                                       | Semi-integrated | 4             |
|                                       | Above ground    | 4             |
| <b>Milking Site</b>                   | Milking parlor  | 35            |
|                                       | Barn            | 65            |
| <b>On Farm Refrigerated Milk Tank</b> | Yes             | 32            |
|                                       | No              | 68            |
| <b>Reported On-Farm Milk Loss</b>     | Yes             | 60            |
|                                       | No              | 40            |



**Figure 1.** Association between milk losses at the production/post-harvest levels and on-farm management practices, as well as farmers’ characteristics (n=25 farmers)

As shown in Figure 2, Pareto chart analysis has pointed out five main causes responsible of 80% milk losses: in descending order of critically, poor milking conditions (hygiene, equipments), poor quality of milk, poor technical support, long time between milking and collecting, and presence of antibiotic residues, corroborating findings of FAO (2021).



**Figure 2.** Pareto diagram of main causes of milk losses at dairy farm level (n=25 farmers)

Indeed, poor hygienic conditions of the cows and of the farm environment as well as technical inefficiency and mismanagement impaired milk quality (Zucali et al., 2011; Sharma, 2016; Dhraief et al., 2019; FAO, 2021), leading to losses. The time between milking and the milk collection by the transporter is probably linked to the number of farmers per transporter, about 67 in the Bizerte region (FAO, 2021). Optimizing the milk collection circuit would reduce this delay.

These problems could be partially solved by technical support: farmers need guidance to improve hygiene through cow cleanliness, milking environment, milking per-person and milk harvesting and storage containers. Farmers' age and educational level of dairy farmers affect knowledge and adoption of various recommended practices (Sharma et al., 2016), and of livestock-related improved technologies (Dhraief et al., 2019). Thus, efforts should focus on trainings, demonstrations, practical skills etc. particularly for low-educated farmers.

Limitations of this study were mainly based on the low number of respondents impairing representativeness as well as on the survey method. Semi-structured interviews are known to be biased by the interviewee as well as on interviewer factors, since their personal opinion on the topic, the mood on the day or perception on the other person could interfere. This could introduce subjectivity in the collected data making them less reliable than quantitative analytical data.

## CONCLUSIONS

Based on the study, these preliminary results contribute in developing mitigation measures to reduce on-farm milk losses, estimated at 573 L/year/farmer. Measures and actions to reduce these losses should strengthen, especially to improve hygiene, infrastructure (milking parlors), and farmers' training. In this context, GIVlait with FAO, has implemented refrigerated milk tanks in farms, and organized training on good practices for reducing milk loss. Progress made to reduce milk loss in primary production should help preservation of natural resources, decrease production of green house gas emissions, and should improve food security.

## ACKNOWLEDGMENTS

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## DETERMINATION OF DYE REMOVE CAPABILITIES OF BACTERIAL ISOLATES

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### ABSTRACT

Difficulties are encountered in the treatment of dyestuff-containing wastewater from the textile and dyestuff production industries, due to the synthetic origin and aromatic molecular structure of dyestuffs. Recent studies have shown that biological methods used in the removal of dyestuffs are the most advantageous and economical methods. For this purpose, it was aimed to remove dyestuff by using alternative bacterial isolates in this study. Bacterial isolates used in the study were isolated from plant roots of *Alyssum pinifolium* (Nyar, T.R. Dudley) growing on the serpentine slopes 6 km north of Ezine district of Çanakkale province and were identified by 16S rDNA analysis. As a result of the 16S rDNA analysis, it was determined that 5 isolates belonged to the genus *Bacillus* and the removal abilities of the isolates against Orange G and Methylene Blue dyestuffs were investigated. Samples taken from dyestuff and bacteria mixtures at certain periods (1./24./96./120./144./168. hours) were measured at the maximum absorbance values of dyestuffs using a spectrophotometer device. When the results obtained are evaluated, the percentage of dyestuff removal varies between 8.05% and 84.18% for Orange G dyestuff, and between 0.40% and 60.14% for Methylene Blue dyestuff. The period in which the maximum removal was observed for both dyestuffs was determined as the 120th hour. *Bacillus toyonensis* NMCC-157 bacteria with the code of 1N13 for Orange G dyestuff was determined as the bacteria with the highest removal rate, while *Bacillus toyonensis* NMCC-157 bacteria with the code of 1TB14 for Methylene Blue dyestuff was determined. In the light of the findings obtained in the study, the high removal values of Orange G and Methylene Blue dyestuffs of *Bacillus* genus bacteria used in dye removal experiments indicate that these microorganisms can be used in industrial applications. In this context, we think that our study will lead to elucidation of the mechanisms related to the removal abilities of *Bacillus* species and various dyes.

**Keywords:** Dyestuff removal, Decolorization, Textile dyes, Industrial waste, *Bacillus* sp.

### INTRODUCTION

It is known that the wastes released as a result of production in the textile industry are mixtures containing many pollutants such as heavy metals, chlorinated compounds and dye pigments (Saraswathi and Balakumar, 2009). About 15% of waste dyestuffs are lost during treatment and processing steps, and the remaining 85% is released into the environment without degradation (Khaled et al., 2009). While the presence of textile dye in wastewater is undesirable even at very low concentrations, over 700 thousand tons are produced commercially per year. Synthetic dyes used in the textile industry are divided into three main groups according to their



chemical structures: azo, triphenylmethane and polymeric. Synthetic dyes are designed as long-lasting colorants. These dyestuffs, which can maintain their stability as textile dyestuffs, create negative effects on the ecosystem when released into water resources without treatment. The azo group (N=N) found in dyestuffs transforms into aromatic amines, which are absolute human carcinogens, and harms living organisms (Cheunbarn et al., 2008, Banat et al., 1996). Some dyes and their compounds resulting from the degradation of these dyes cause mutagenic and toxic effects on organisms (Erysipelothrix, 2012).

The release of textile dyestuffs into the nature without treatment causes biological accumulations that can affect human health by being included in the food chain. The dyestuffs involved in the food chain suppress the activity of tyrosinase enzyme, which leads to inhibition of melanin pigment synthesis in humans (Dubey et al., 2007, Pinherio et al., 2004).

Physicochemical methods are generally used for the removal of dyestuffs in water sources. These methods cause the problem of concentrated sludge accumulation, as well as the process steps are long and costly (Mahbub et al., 2011). On the contrary, biological treatment based on the purification of dyestuffs using microbial methods provides a low-cost and more efficient waste treatment compared to other methods. In studies on this subject, it has been determined that *Pseudomonas* (Zimmermann et al., 1982), *Aeromonas* (Chen et al., 2008), *Proteus mirabilis*, *Micrococcus luteus*, *Staphylococcus aureus* (Mahbub et al., 2011) bacteria are effective in the decolorization of textile dyestuffs.

In this study, it was aimed to remove Methylene blue and Orange G dyestuffs, which are frequently used in the field of textile, by bacteria isolated from plant roots of *Alyssum pinifolium* (Nyar, T.R. Dudley), which is distributed on the serpentine slopes 6 km north of Ezine district of Çanakkale province.

## **MATERIAL AND METHOD**

### **Bacterial Isolation**

Soil samples taken from the roots of *A. pinifolium* plants growing on serpentine slopes located 6 km north of Ezine town of Çanakkale province (39.840.276 N, 26.320.107 E) were used for bacterial isolation. The soil sample was suspended in 0.9% isotonic saline and serial dilutions were made. 100 µl of each dilution sample was taken and planted on Nutrient Agar (NA) and Tryptic Soy Agar (TSA) media by the spread plate method and incubated at 30±1°C for 24 hours. After incubation, single and clean-looking colonies with different morphological appearances were determined at 10<sup>-4</sup> and 10<sup>-5</sup> dilutions, and pure cultures of the isolates were obtained by replanting them on the growing media. To check the purity of the isolates, Gram stains were made and the samples were examined under a light microscope. The pure isolates obtained were stored at +4°C to be used in the study (Bozkurt, 2016, Karakaş et al., 2022).

### **Bacterial Identification**

In order to determine the biochemical properties of the pure isolates; oxidase, catalase, indole, citrate, methyl red, Voges-Proskauer (VP) and hydrogen sulfide tests were performed (Bozkurt, 2016). When the cell density suitable for DNA isolation of the isolates incubated in Nutrient Broth (NB) medium was achieved, DNA isolation stage was started. DNA isolation was performed using the GeneJET Genomic DNA Purification Kit (K0721, Thermo Scientific). For the analysis of the 16S rDNA gene, the synthesis of the primer pair, which was thought to have the highest efficiency as a result of the literature review, was made with the Sentegen Primer 50 nM (Table 1).

**Table 1.** Primer sequences of 16S Rdna

| Gene     | Primers | Sequence (5'→3')    | Reference               |
|----------|---------|---------------------|-------------------------|
| 16S rDNA | 0341f   | CCTACGGGGGGCGCAG    | Klindworth et al., 2013 |
|          | 0785r   | GACTACGGGTATCTAATCC |                         |

The PCR components used in the amplification of the 16S rDNA gene region to be used for species-level identification were determined as a result of the optimization study performed in our laboratory (Table 2).

**Table 2.** PCR components of 16S Rdna

| PCR Components                            | $\mu\text{l}/\text{Tube}$ | Final Concentration           |
|---|---------------------------|-------------------------------|
| Sterile bidistilled H <sub>2</sub> O      | 17.0                      | -                             |
| 10 X Buffer                               | 2.5                       | 1X                            |
| MgCl <sub>2</sub> (25 mM)                 | 2.0                       | 2 mM                          |
| dNTP (25 mM)                              | 0.3                       | 0.3 mM                        |
| Primer 341F (10 pmol $\mu\text{l}^{-1}$ ) | 0.5                       | 10 pmol 25 $\mu\text{l}^{-1}$ |
| Primer 785R (10 pmol $\mu\text{l}^{-1}$ ) | 0.5                       | 10 pmol 25 $\mu\text{l}^{-1}$ |
| Taq polymerase (5U $\mu\text{l}^{-1}$ )   | 0.2                       | 1 U 25 $\mu\text{l}^{-1}$     |
| DNA (150 ng $\mu\text{l}^{-1}$ )          | 2.0                       | 300 ng 25 $\mu\text{l}^{-1}$  |
| Total volume                              | 25.0                      |                               |

The amplification of the 16S rDNA gene region of all isolates was carried out with these determined conditions. The presence of PCR products was checked and verified on a 2% agarose gel. The PCR products of the 16S rDNA gene, which were used for species-level identification, were sent for sequence analysis. The products of the 16S rDNA gene belonging to all isolates are provided by Medsantek Laboratory Materials Industry and Trade Ltd. through service procurement. Sti. DNA sequencing analysis was performed by the company. Sequencing and Fragment Analysis Bidirectional sequencing analyzes were performed with the help of Applied Biosystems brand, 8-capillary 3500 device. Sequencing Analysis Software and Sequencing – SeqScape® Software v2.7 programs were used for sequence analysis. “Reference sequences” to be used for comparison of sequence analysis data were obtained through NCBI (National Center for Biotechnology Information) databases. 16S rDNA gene sequence analyzes of all isolates were performed with NCBI, BLASTn application, and genetic identification of each isolate was performed by looking at the sequence similarity rates in the databases.

### Properties of Methylene Blue and Orange G Dyes

Methylene blue dyestuff is a blue textile dye and has a high water-holding property. This dye, which has the ability to dissolve with various solvents such as ethanol and chloroform, is widely used in areas such as health and food, apart from the textile industry. Although it has antiseptic properties, mixing it with other antiseptic products is known to cause harmful effects. Due to the widespread use of methylene blue dyestuff and the lack of proper treatment, this dyestuff is frequently encountered in the ecosystem (Yaşar and Özcan, 2004).

Orange G dyestuff, like Methylene blue dyestuff, is a dye that is commonly found in textile waste waters in orange color and powder form. It is also used in various fields such as food, cosmetics, palynology and leather dyeing. Apart from this, Orange G dyestuff is used as an electrophoretic color marker to follow the DNA molecule. Although it has two different ionizable groups, it gives an orange color in aqueous solutions at neutral and acidic pH, while it gives a red color at pH 9 and above (Carson and Hladik, 2009).

## **Decolorization Studies**

### **Detection of Decolorization in Solid Media**

Media containing dyestuffs of bacterial strains; Starch 10 g L<sup>-1</sup>, nutrient broth 8 g L<sup>-1</sup>, agar 20 g L<sup>-1</sup>, dyestuff 0.15 g L<sup>-1</sup> and it was prepared at a pH of 7, sterilized at 121 °C for 15 minutes with an autoclave device and poured into petri dishes. The cooled petri dishes were incubated for 24 hours at 37 °C after the bacteria were inoculated. This process was applied separately for methylene blue and orange G dyestuff and incubated. After incubation, the presence of dyestuff removal was evaluated depending on the lightening of the color of the medium around the colonies and the color of the colony taking the color of the dyestuff (Çitekçi, 2020).

### **Generating Standard Curve Graphs of Methylene Blue and Orange G Dyes**

Since the concentration of the dyestuffs in the medium could not be determined directly, the dyestuffs were diluted depending on the concentrations in the concentration-absorbance value. The absorbance values of the medium containing methylene blue and Orange G were determined by measuring at 665 nm and 492 nm wavelengths, respectively. Firstly, dilution was made by adding 0.0154 mg ml<sup>-1</sup> dyestuffs into tubes containing distilled water. The standard curve was obtained by measuring the dyestuff concentrations in a UV spectrophotometer (Nemr et al., 2014).

### **Determination of Decolorization Percentage**

The content of Luria bertani (LB) broth medium to be used in decolorization experiments in our study; Tryptone was prepared as 5 g L<sup>-1</sup>, yeast extract 10 g L<sup>-1</sup>, NaCl 10 g L<sup>-1</sup> and dyestuff 0.15 g L<sup>-1</sup>. Each bacterial strain was inoculated separately as 10 ml (0.5 McFarland (1.5x10<sup>8</sup> cells/mL)) into 90 ml LB broth and incubated for 7 days at 37 °C in a shaking oven at 150 rpm. Samples taken at certain intervals (1./24./96./120./144./168. hours) were centrifuged at 3000 rpm for 10 minutes and the supernatants were taken and read at the maximum absorbance value of each dye in the spectrophotometer device. According to the measurement results, the percentage of decolorization was calculated according to the formula below (Asad et al., 2007; Karim et al., 2018).

$$\text{Percent decolorization \%} = (A_0 - A) / A_0 \times 100$$

A<sub>0</sub> = Initial absorbance

A = Absorbance after decolorization

## RESULTS AND DISCUSSION

### Biochemical Characteristics of Isolates

The values of the biochemical properties of the isolates used in our study are given in Table 3. In line with the data obtained, it was determined that 5 isolates had endospore bacillus showing gram positive characteristics. While all isolates were oxidase positive, 2 isolates were catalase negative. In addition, all isolates were citrate negative, 2 isolates were methyl red negative and 1 isolate was VP negative.

**Table 3.** Biochemical properties of isolates

| Izolate codes | Gram reaction | Morphology | Endospore | Oxidase | Catalase | Indole | Citrate | H <sub>2</sub> S |        |     | Methyl red | VP Test |
|---------------|---------------|------------|-----------|---------|----------|--------|---------|------------------|--------|-----|------------|---------|
|               |               |            |           |         |          |        |         | S<br>C           | B<br>C | Gas |            |         |
| 1T 23         | +             | Bacil      | +         | +       | -        | -      | -       | P                | P      | -   | +          | +       |
| 1N 15         | +             | Bacil      | +         | +       | +        | -      | -       | P                | P      | -   | -          | +       |
| 1N 13         | +             | Bacil      | +         | +       | -        | -      | -       | Y                | Y      | -   | +          | +       |
| 1TB 14        | +             | Bacil      | +         | +       | +        | -      | -       | P                | Y      | -   | -          | -       |
| 1N 14         | +             | Bacil      | +         | +       | +        | -      | -       | P                | Y      | -   | +          | +       |

### 16S rDNA Sequencing Analysis

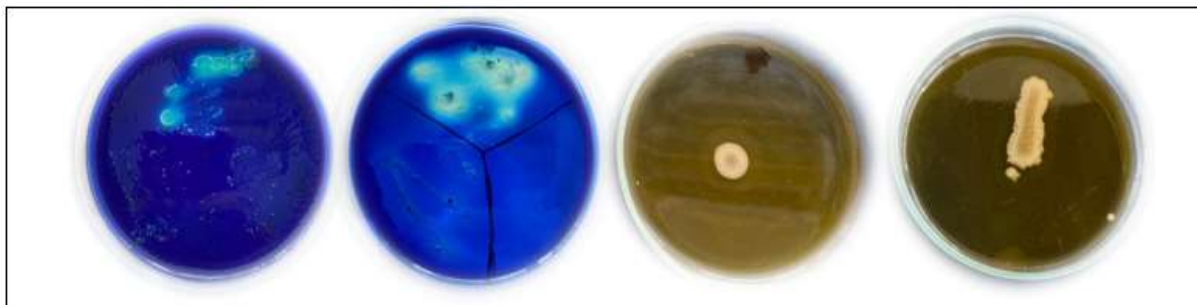
As a result of the evaluation of sequence analysis results using bioinformatic tools, 5 isolates were identified at the species level. The obtained species-level definitions are given in Table 4.

**Table 4.** Bioinformatic analysis results of isolates

| Izolate codes | Similar type                        | Similarity rate (%) |
|---------------|-------------------------------------|---------------------|
| 1T 23         | <i>Bacillus toyonensis</i> NMCC-157 | 100                 |
| 1N 15         | <i>Bacillus toyonensis</i> NMCC-157 | 100                 |
| 1N 13         | <i>Bacillus toyonensis</i> NMCC-157 | 100                 |
| 1TB 14        | <i>Bacillus toyonensis</i> NMCC-157 | 100                 |
| 1N 14         | <i>Bacillus toyonensis</i> NMCC-157 | 100                 |

### Detection of Decolorization in Solid Media

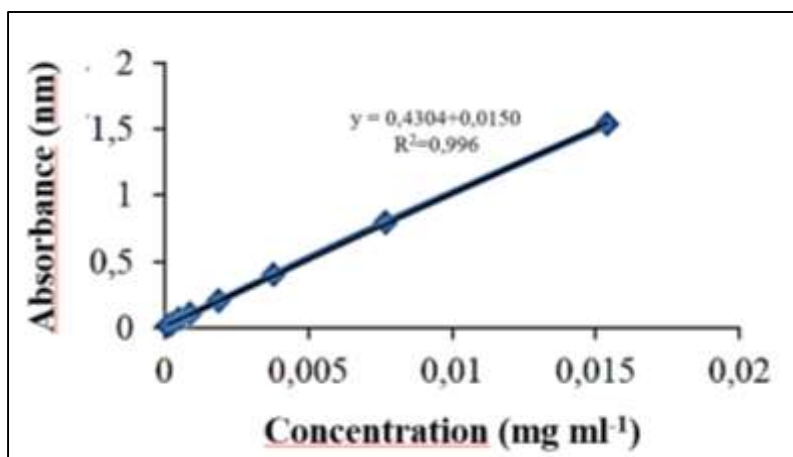
As a result of inoculation on solid media containing methylene blue and Orange G dyestuff, it was determined that 5 isolates formed a transparent zone by absorbing the dye in the solid medium. In addition, color change was observed in colonies grown on solid media in direct proportion to the color of the dye. The images of the media containing methylene blue and Orange G dyestuffs are given in Figure 1.



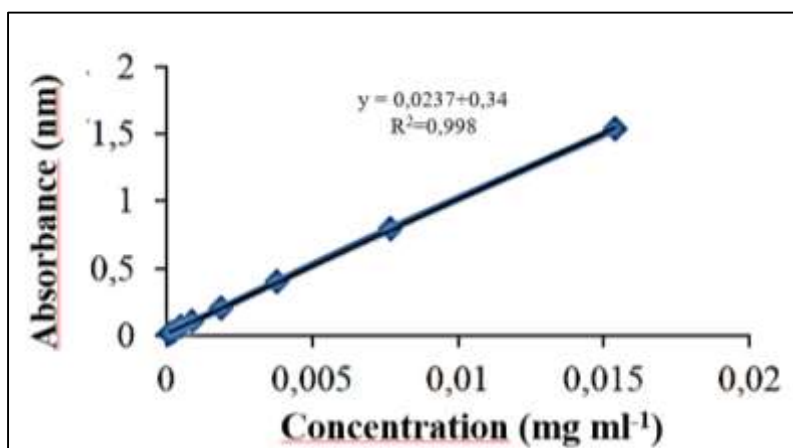
**Figure 1.** Bacterial growth on solid media containing Methylene blue and Orange G

### Generating Standard Curve Graphs of Methylene Blue and Orange G Dyes

The maximum peak value of methylene blue dyestuff in UV spectrophotometer was determined as 665 nm, and the maximum peak value of Orange G dyestuff in UV spectrophotometer was determined as 492 nm. The measured values were read at different dye concentrations to form a standard curve. The standard curves obtained from the measurement results are given in Figure 2 and Figure 3.



**Figure 2.** Absorbance-concentration graph of Methylene blue dyestuff



**Figure 3.** Absorbance-concentration graph of Orange G dyestuff

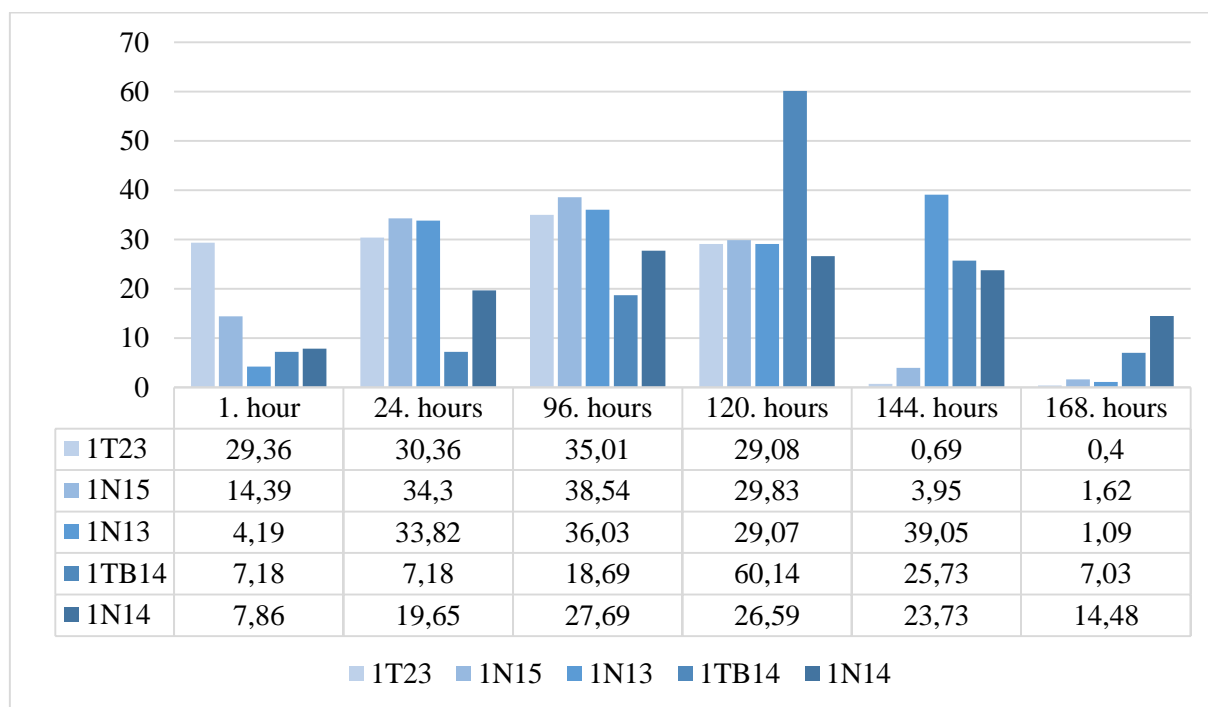
### Determination of Decolorization Percentage

In our study, the ability of 5 different isolates to remove Methylene blue and Orange G dyestuffs was investigated. Removal percentages obtained from methylene blue dyestuff are given in Table 5, and removal percentages obtained from Orange G dyestuff are given in Table 6.

Within the scope of the results obtained in the study, the removal rates of methylene blue dyestuff by bacteria were determined between 0.4% and 60.14%. The highest removal rate of 60.14% was obtained from *Bacillus toyonensis* NMCC-157 bacteria with the code 1TB14. In addition, the interval with the highest removal was determined as the 120th hour.

Removal percentages of Orange G dyestuff by bacteria varied between 8.05% and 84.18%. The highest removal of Orange G dyestuff was obtained from 1N13 coded *Bacillus toyonensis* NMCC-157 bacteria. The intervals with the highest dye decolorization were determined as 168th hour for 1T23 and 1N14 coded isolates, 144th hour for 1N15 and 1TB14 coded isolates, and 120th hour for 1N13 coded isolates.

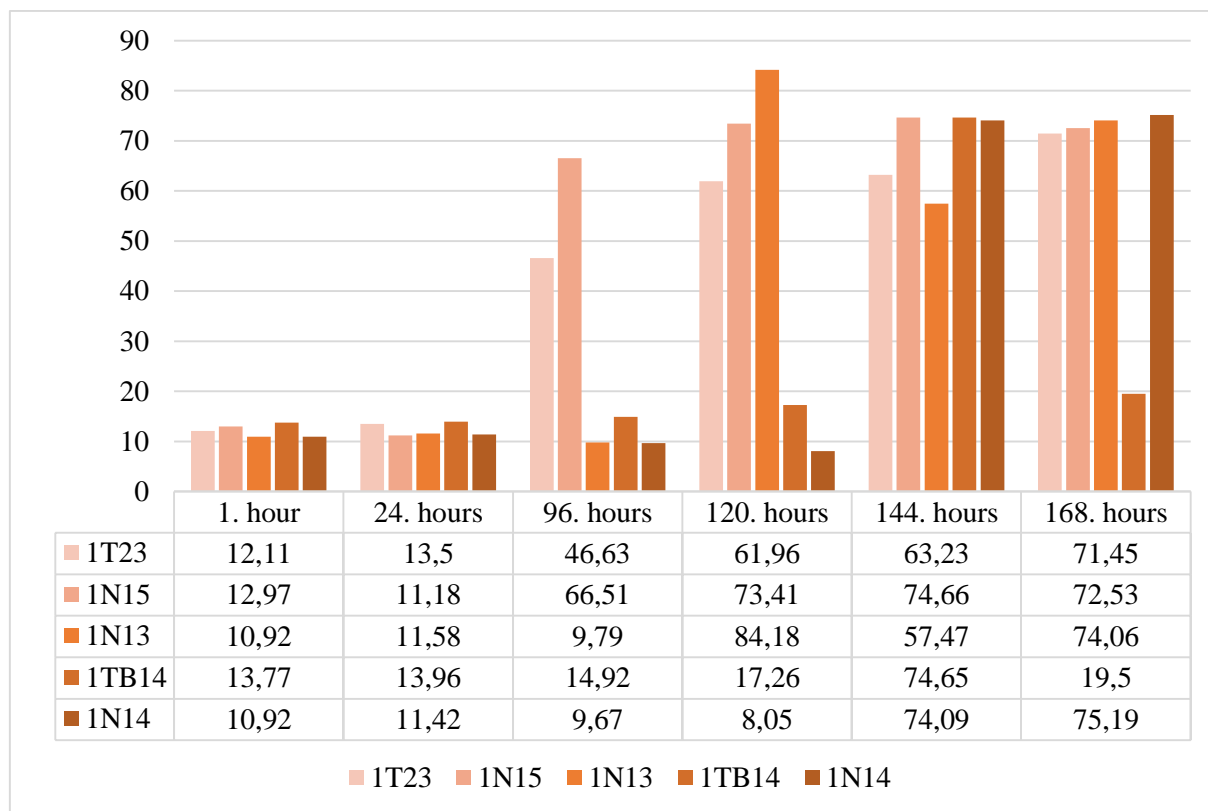
**Table 5.** Removal percentages obtained from Methylene blue dyestuff



In our study, it was aimed to biologically remove *Bacillus* bacteria isolated from soil and two different dyestuffs commonly used in textiles. As a result of the 16S rDNA sequence analysis, it was determined that the 5 bacteria we used in our study were 100% similar to *Bacillus toyonensis* NMCC-157, but their biochemical properties were different from each other. In line with the data obtained, it was determined that all of the isolates used had dyestuff decolorization ability. The highest removal percentages for methylene blue dyestuff were determined as 60.14%, 39.05% and 38.54%, respectively, while the highest removal percentages for Orange G dyestuff were determined as 84.18%, 75.19% and 74.66%, respectively detected. When the time-dependent removal results were examined, it was determined that the 1N15 coded bacterium removed the methylene blue dyestuff at a rate of

34.3% in the 24th hour. In addition, it was determined that the same bacteria removed 66.51% of Orange G dye at the 96th hour.

**Table 6.** Removal percentages obtained from Orange G dyestuff



In the studies, it has been determined that the azo reductase enzyme and gene, which is found in the content of azo group dyes used in the field of textiles and breaks the azo bond, are found in *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Mycoplasma pneumonia* bacteria (Suzuki et al., 2001).

In a study done, 3 different gram-negative bacteria strains *Aeromonas* sp., *Pseudomonas luteola* and *Escherichia coli*, and two different gram-positive bacteria strains *B. subtilis* and *Staphylococcus aureus* were selected to compare biosorption and biodegradation in the removal of reactive dyes. costs are compared. According to this study, it was determined that dye removal by biosorption in gram-negative bacteria was higher than biodegradation (Hu, 1998).

*Bacillus* sp. are effective microorganisms for removal of different dyes or textile effluent. Although, different microorganisms are useful for dye decolorization such as *Corynebacterium* sp. or *Pseudomonas* sp. (Gül, 2018).

*Bacillus* sp. was used for removal of Acid Red 2, Acid Orange 7, Remazol Black B and Congo Red and the removal rate were found as 90-100% (Jaiswal and Gomashe, 2017, Shah et al., 2013). However, the *B. subtilis* was removed the textile effluent at 63% decolorization rate (Sivaraj et al., 2011).

In a study conducted in 2011, the decolorization abilities of 4 different isolates of *Bacillus* species against maxilon red, methyl red and doracryl blue dyestuffs were investigated. Among the four *Bacillus* species, *Bacillus megaterium* was found to be the most effective in decolorization experiments of all three dyes, with a rate of 64.99% (Canpolat et al., 2021).

In the study conducted by Park et al., the percentage of decolorization for black B was found to be 48.68% for *B. subtilis*. However, the percentages of decolorization in the presence of *Bacillus cereus* and *Bacillus licheniformis* and Congo red starch were determined as 72% and 80.32%, respectively (Park et al., 2019).

In a study in 2007, it was reported that rapid results were obtained in the decolorization of eight different sulfonated azo dyes, especially in the decolorization of Red, aerobically using 16S rDNA sequencing of the bacterium identified as *B. subtilis* HM (Kalyani et al., 2007).

In a recent study, as a result of incubation in LB medium at 37 °C in an oven with shaking for 24-196 hours, 47.08% color reduction was determined for *B. licheniformis* bacteria in Acid Blue dye and 69.74% for *Bacillus proteolyticus* isolate in Acid Red dyestuff (Çitekçi et al., 2020).

## CONCLUSIONS

In the light of the findings obtained in the study, the high removal values of Orange G and Methylene Blue dyestuffs of *Bacillus* genus bacteria used in dye removal experiments indicate that these microorganisms can be used in industrial applications. In this context, we think that our study will lead to elucidation of the mechanisms related to the removal abilities of *Bacillus* species and various dyes.

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## COMPARATIVE ANATOMICAL INVESTIGATIONS ON FOUR *VIOLA* L. (*VIOLACEAE*) TAXA FROM EUROPEAN TURKEY

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### ABSTRACT

Comparative anatomy of 4 taxa of *Viola* distributed in Edirne province of European Turkey was studied. The investigated species are *Viola kitaibeliana* Roem. & Schultes, *Viola tricolor* L., *Viola arvensis* Murray (sect. *Melanium*; annual, biennial and caulescent), *V. odorata* (sect. *Viola*; perennial and acaulescent). General anatomical features of examined *Viola* species are discussed. Cross sections of root, stem, leaves and petiole (in *V. odorata*) were observed under light microscope for various anatomical features. The following characters were found to be taxonomically informative: cross-section shapes of the aerial stem, as well as the number of vascular bundles in the aerial stem. The results showed that, root included periderm in the outer. There were parenchymatous cortex, endodermis and pericycle under the periderm respectively. Pericycle contains primary phloem and xylem and pith was filled with xylem elements. In taxa of sect. *Melanium*, stem cross-sections are elliptic with two wings, but they are semi-circular with two wings in taxon of sect. *Viola*. The number of vascular bundles in the stem can be used to delimit the species. Sclerenchymatic ring was not seen in stem of *V. odorata*. While it was the same thickness in the *V. tricolor* and *V. kitaibeliana*, it had thicker in *V. arvensis*. Calcium oxalate crystals were observed in all vegetative organs of taxa examined *Viola*. The root all examined taxa had a secondary structure. The amphistomatic leaves had a single layered epidermis with usually hairs and bifacial mesophyll. Stomata are anisocytic. The cell shapes of the epidermis are different in superficial sections. Our results show that anatomical characters are useful for delimiting species within *Viola*.

**Key words:** *Anatomy; Violaceae; Viola, European Turkey*

### INTRODUCTION

*Viola* L. Comprises 525-620 species and is the largest genus of the Violaceae family. Mostly constituted by shrubs or annual, biennial or perennial herbs. It is distributed in mainly temperate and subtropical regions or tropical high mountains throughout the World (Ballard et al. 1999, 2004, Wahlert et al. 2014). The genus *Viola* is variable from the ecological viewpoint. Its species spread out over various habitats including fields, highways, rock cliff, roadsides, waste places habitats and woodland communities (Davis et al., 1988). Approximately 500 species of *Viola* members are distributed in Subtropical North America, South America, the

mountainous regions of South America (Andes), Australia and New Zealand (Heywood, V.H. 1978). It is widespread throughout the temperate and warm regions with 92 species in Europe (Valentine et al. 1968). There are 58 species of this genus in the Balkans, 24 of which are endemic (Polunin, 1987). Turkey shows the distribution of 21 species of the genus *Viola* L. (tubives, <http://www.weski.tubitak.gov.tr/tubives/>). According to Coode and Cullen (1965) 9 species are present within European Turkey. Recently, 11 new species of *Viola* have been added to the Flora of Turkey and the number of *Viola* taxa in the flora of Turkey has risen to 32 (Coode & Cullen 1965; Davis et al. 1988; Yıldırım 1994, 200; Dinc et al. 2001; Dinc & Yıldırım 2002; Yıldırım & Dinc 2002; Dinc et al. 2003; Blaxland 2004). Nowadays, a new species from Thailand; *Viola umphangensis* S. Nansai, Srisanga & Suwanph. is described and illustrated (Nansai et al., 2020). Morphometric studies of the European *Viola* species (Marcussen & Borgen 2000; Marcussen et al. 2001; Hodálová et al. 2008) and Phylogenetic studies of the section *Melanium* of the genus *Viola* (Ballard, H. E. et al. (1999, Yockteng et al., 2003, Wahlert et al., 2014) have also been carried out. Numerous anatomical studies have been published on the genus *Viola*. There are several anatomical studies covering certain geographical areas of the genus *Viola* (Metcalf & Chalk 1950; Rubin & Paolillo 1978; Akarsu, 1989 a, b; Colombo et al., 2007, Dinç et al. 2007; (Bağcı et al. 2008; Dinç 2009; Toiu, et al., 2010, Yousefi et al., 2012; Mehrvarz et al., 2013; Shahrestani et al., 2014; Mareacre et al., 2014, Pilberg et al., 2016).

In Turkey, The anatomic structure of *Viola* species has been little investigated until now. In Anatolia, on *Viola odorata* and *Viola reichenbachiana* Jord. ex Bor. that showing natural distribution in Western Anatolia (Akarsu, 1989a,b) and on Turkish endemics *Viola yildirimlii* M. Dinç & Y. Bağcı. (Bağcı et al. 2008), *Viola kizildaghensis* M. Dinç and S. Yıldırım (Dinç et al. 2007) and *Viola sandrasea* Melchior (Dinç 2009) have been studied. Anatomical studies have not been reported on *Viola* taxa that showing natural distribution in European Turkey although several anatomical studies have been carried out Anatolia.

In the present study, we systematically examined the anatomy of root, stem and leaves in 3 species of *Viola* sect. *Melanium* and *V. odorata* (sect. *Viola*) that showing natural distribution in Edirne (European Turkey), in order to find useful characters for the taxonomy of the genus.

## MATERIAL AND METHODS

### Plant material

During the master thesis in 2012-2014 years, titled (The Morphological, Anatomical and Palynological Investigation on *Viola* L. (Violaceae) in Edirne, (Müge Türkoğlu Koç, 2014), (in Turkey, unpubl.) in Thrace University, *Viola* specimens were collected from their natural habitats in Edirne province (European Turkey). This specimen was determined to according Coode and Cullen (1965) as *Viola odorata* L., *Viola kitaibeliana* Roem. & Schultes, *Viola arvensis* Murray and *Viola tricolor* L. The specimens were prepared as herbarium materials and voucher specimens deposited in the Herbarium of Trakya University, Edirne (EDTU). The collection data of the examined specimens for anatomy are given in Table 1.

### Anatomical studies

Selected plants materials were fixed in ethyl alcohol and glacial acetic acid (3:1 v/v) at room temperature for performing anatomical studies. Five central leaflets from each plant were clarified according to Dizeo de Strittmatter (1984). Hand-sections were taken from fixed root, stems, and leaves. Cross-sections of stems, roots and petiol were dyed with a mixture of 1% safranin-alcian blue 8GX at a ratio of 4:6 (Davis & Barnett. 1997). The sections allowed to absorb a drop of dyed and left for 5 min. Surface section from the leaf (the upper and the

lower) were dyed with lugol. The stained and unstained sections were mounted in glycerine-gelatine to make permanent preparations (Jensen, 1962). Also, in the anatomical section, the existence of calcium oxalate (CaOx) crystals was investigated. The samples were treated with 2.5% commercial bleach (2.5% sodium hypochlorite) for 4 h. After washing in a 96% ethanol for 10 min., the samples were infiltrated with xylene for 10 min., mounted in entellan on slides. Histochemistry of calcium oxalate crystals were determined according to Yasue (1969). Crystals in the cleaned tissues were examined using a light microscope. Well-staining sections were photographed with an Olympus BH2 research microscope from permanent slides. Photographs were taken with an Olympus BH-2 microscope under 10x, 100x and 400x magnifications. The parameters of stomata density and tissue size were analyzed. The surface sections of the leaves were studied under a light microscope. Stomatal density was calculated as the average number of stomata infive randomly chosen microscopic fields on the abaxial and adaxial surfaces of the leaf. The stomatal index was calculated as described by Meidner & Mansfield (1968). [Stoma index= Number of stomata in mm<sup>2</sup> / (Number of stomata in mm<sup>2</sup> + number of epidermis cells in mm<sup>2</sup>) X 100]. All measurements and observations were made three or four times from several sections taken from at least two selected specimens.

## RESULTS

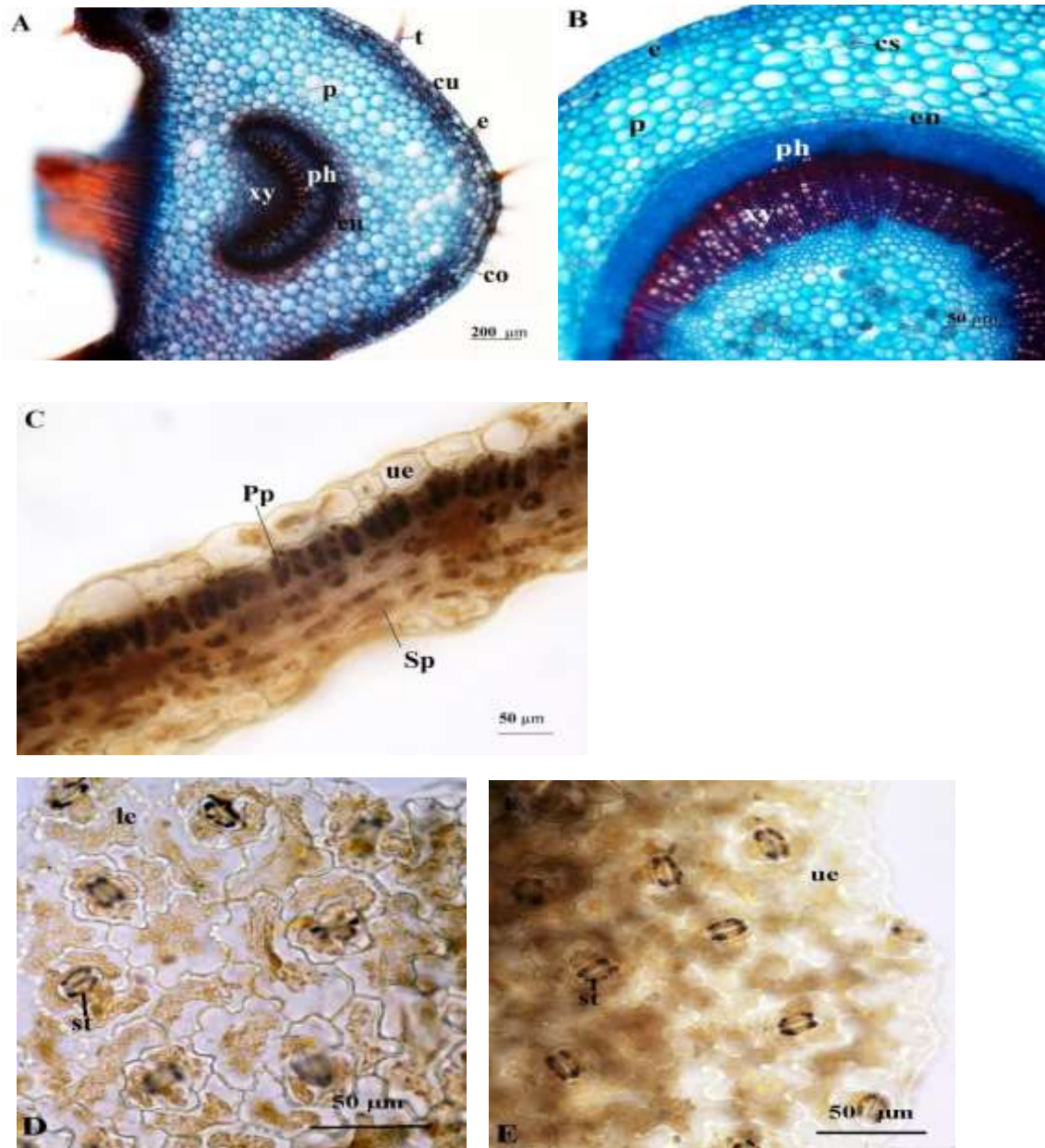
### **Anatomical Results of the *Viola odorata* L.**

**Stem;** general shape is triangular and the core region is not empty. There is a thick cuticle surrounding the epidermis at the outermost. Just below the cuticle is a layer of epiderma consisting of a single row of round cells. Corner collenchyma is located just below the epidermis. Collencyhma in these regions consist of 2-3 rows. After the collenchyma, there is a cortex parenchyma layer consisting of 4-9 rows of round thin-walled cells. A single row of epidermis lies below the cortex parenchyma. Vascular bundle is lined up between a 1-2 rows sclerenchyma layer. There is an open collateral type vascular bundle. The cambium between xylem and phloem is 1-2 rows and thin. There is an open collateral type conduction bundle (Figure 1a). There is cover hair on the stem epidermis cells. Numerical values of stem anatomy are given below (Table 2).

**Root;** Generally, a root anatomy that has passed into a secondary structure is seen. As the protective tissue, the epidermis and exodermis are fragmented and not distinct, instead periderma has begun to form. Cortex: The exodermis has 3-4 rows and contains cells that are very well shaped. Cells are thin-walled, broad, generally smooth. Druse crystals are randomly distributed in the parenchyma. Numerical values of root anatomy are given on the (Table 3). Vascular tissue: In secondary structure, there are approximately 8-9 rows of flattened secondary phloem externally and a circular secondary xylem internally. Secondary xylem has small cell, primary xylem is wide trachea. The central part of the vascular cylinder contains the non-ligninized parenchyma and primary xylem elements. Parenchymatic cells in the central part are relatively large with thick walls and there is a space between them. Primary xylem elements are embedded in this parenchyma (Figure 1b).

**Leaf;** The lower and upper epiderma are covered with a cuticle layer. There are parenchyma cells on the surfaces facing the lower and upper epidermis of the vascular bundles. It consists of palisade and spongy parenchyma (Figure 1c). Stomata in the upper and lower epidermis. Epidermis cells do not vary in size on the upper and lower surfaces. In the cross section, there is a middle vein region in the middle of the leaf. In the center there are open colleteral type vascular bundles surrounded by parenchymal and oval shaped cells on all

sides. There is a large vascular bundle in the center of the mid-vein, and between these side vessels there are small vascular bundles. Leaf anatomy numerical values are given below (Table 3). When looking at superficial sections, the stomata were observed on the lower and upper surface of the leaf, so there was an amphistomatic leaf condition (Figure 1d) and (Figure 1e). Stomas are of anisocytic type. Upper surface stoma index is 0.78, lower surface stoma index 0.76 (Table 4).



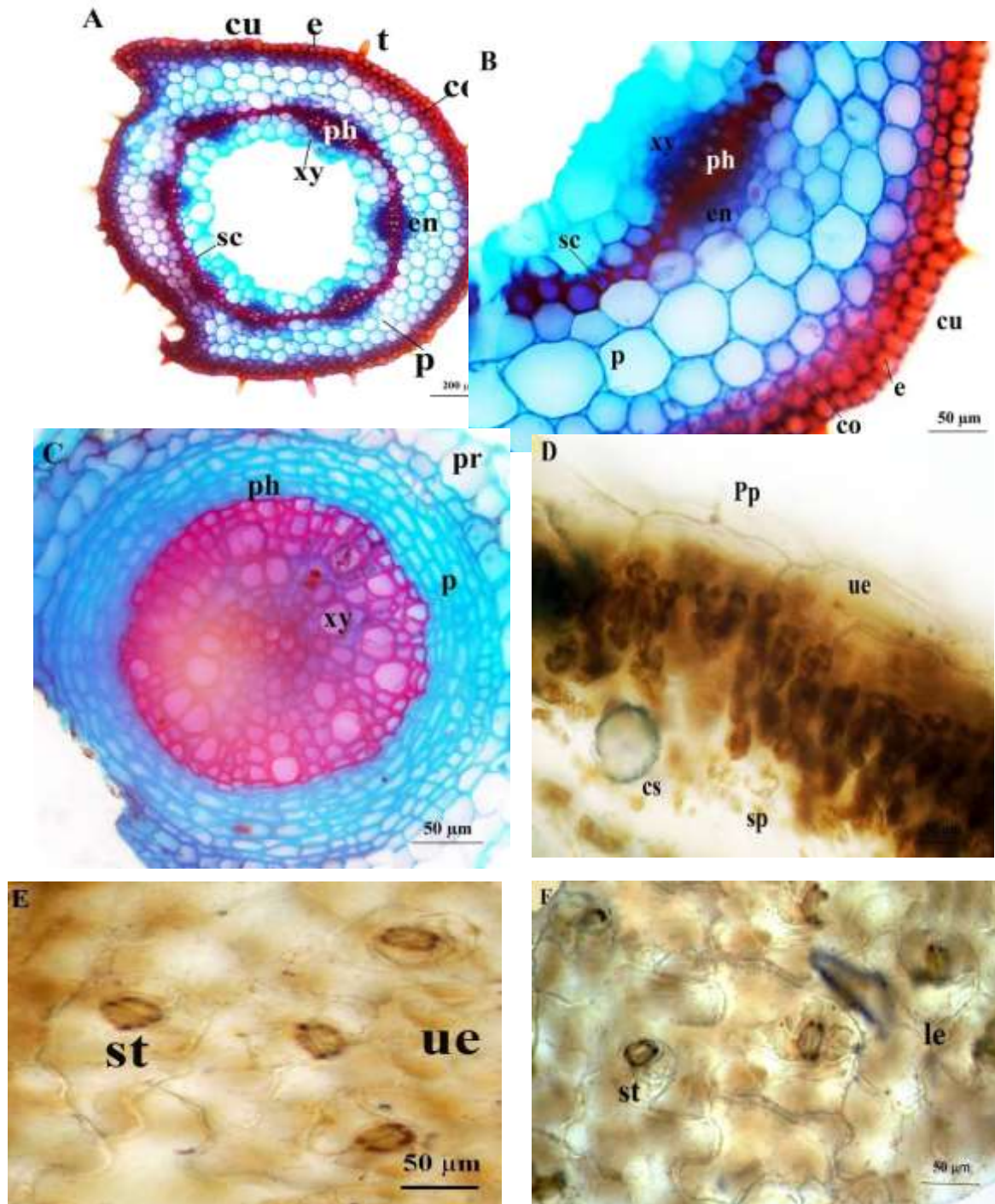
**Figure 1.** (A) cross-section of petiole in *Viola odorata*, stained with safranin-alcien blue (cu: cuticle, e: epidermis, co: collenchyma, en: endodermis, ph: phloem, xy: xylem, t: trichome, p: parenchyma), (B) root cross section (cs:crystal), (C) leaf cross section, staining with lugol (Pp: palisade parenchyma, Sp: spongy parenchyma, ue: upper epidermis), (D) leaf lower epiderma surface section (le: lower epidermis, st:stomata), (E) leaf upper epiderma surface section (ue: upper epidermis)

### **Anatomical Results of the *Viola tricolor* L.**

**Stem;** general shape is round and the pith region is not empty. There is a thick cuticle surrounding the epidermis at the outermost. Just below the cuticle is an epiderma layer consisting of a single row of oval cells. The corner collende is located below the epidermis. Below the collenchyme is the cortex parenchyma layer consisting of 2-4 rows of oval-round thin-walled cells. Vascular bundles are arranged between a 1-2 rows sclerenchyma layer. There are open collateral type of vascular bundles lying along the sclerenchymatous ring. The cambium between the xylem and phloem has 1-2 rows and is thin. Trachea with xylem are in radial rows. The core parenchyma cells are located close to the vascular bundles. These cells are oval-round in shape and sometimes larger than the cortex parenchyma. There is cover hair on the body epidermis cells (Figure 2a and 2b). Numerical values of the anatomy of the stem are given below (Table 2).

**Root;** generally, a root anatomy that has passed into a secondary structure is seen. As the protective tissue, the epidermis and exodermis are fragmented and not distinct. Cortex: contains 2-3 rows of highly malformed cells. The cells are thin-walled, broad, with anticline walls generally indented or lobed. Numerical values of root anatomy are given below (Table 3). Vascular tissue: secondary structure, approximately 6-7 rows of flattened secondary phloem on the outside and circular secondary xylem on the inside. Cambium tissue is in 2-3 rows. The central part of the vascular cylinder contains the lignified parenchyma and primary xylem elements. Lignification is intense in xylem. Parenchymatic cells in the central part have relatively large, thick walls and there is no space between them. Primary xylem elements are embedded in this parenchyma (Figure 2c).

**Leaf;** The upper and lower epidemas are covered with a cuticle layer. Parenchyma cells are located on the surface facing the lower and upper epidermis of the vascular bundles. In the mesophyll tissue there are smaller side veins than the middle vein. It consists of palisade and spongy parenchyma. There are lower and upper epidermis stomata. Epidermis cell sizes are almost the same on the upper and lower surfaces. There is druse crystal in mesophyll. In the cross section, there is a middle vein region in the middle of the leaf. It is surrounded on four sides by parenchymal and oval shaped cells in the center. There are open collateral-type conduction bundles. There is a large vascular bundle in the middle of central vein. Leaf anatomy numerical values are given on (Table 2.) When looking at superficial sections, there is an amphystomatic leaf condition as the stomata are observed on the lower and upper surface of the leaf (Figure 2d and 2e). Stomas are of anisocytic type. Upper surface stoma index is 0.46, lower surface stoma index 0.44 (Table 4).



**Figure 2.** (A) and (B) Cross-section of stem in *Viola tricolor*, stained with safranin-alcien blue (cu: cuticle, e: epidermis, co: collenchyma, en: endodermis, ph: phloem, xy: xylem, p: parenchyma, t: trichome, sc: sclerenchymatous ring), (C) root cross section (pr: periderm), (D) leaf cross section, staining with lugol (Pp: palisade parenchyma, Sp: spongy parenchyma, ue: upper epidermis, cs: crystal), (E) leaf upper epidermis surface section (st: stomata), (F) leaf lower epidermis surface section (le: lower epidermis)

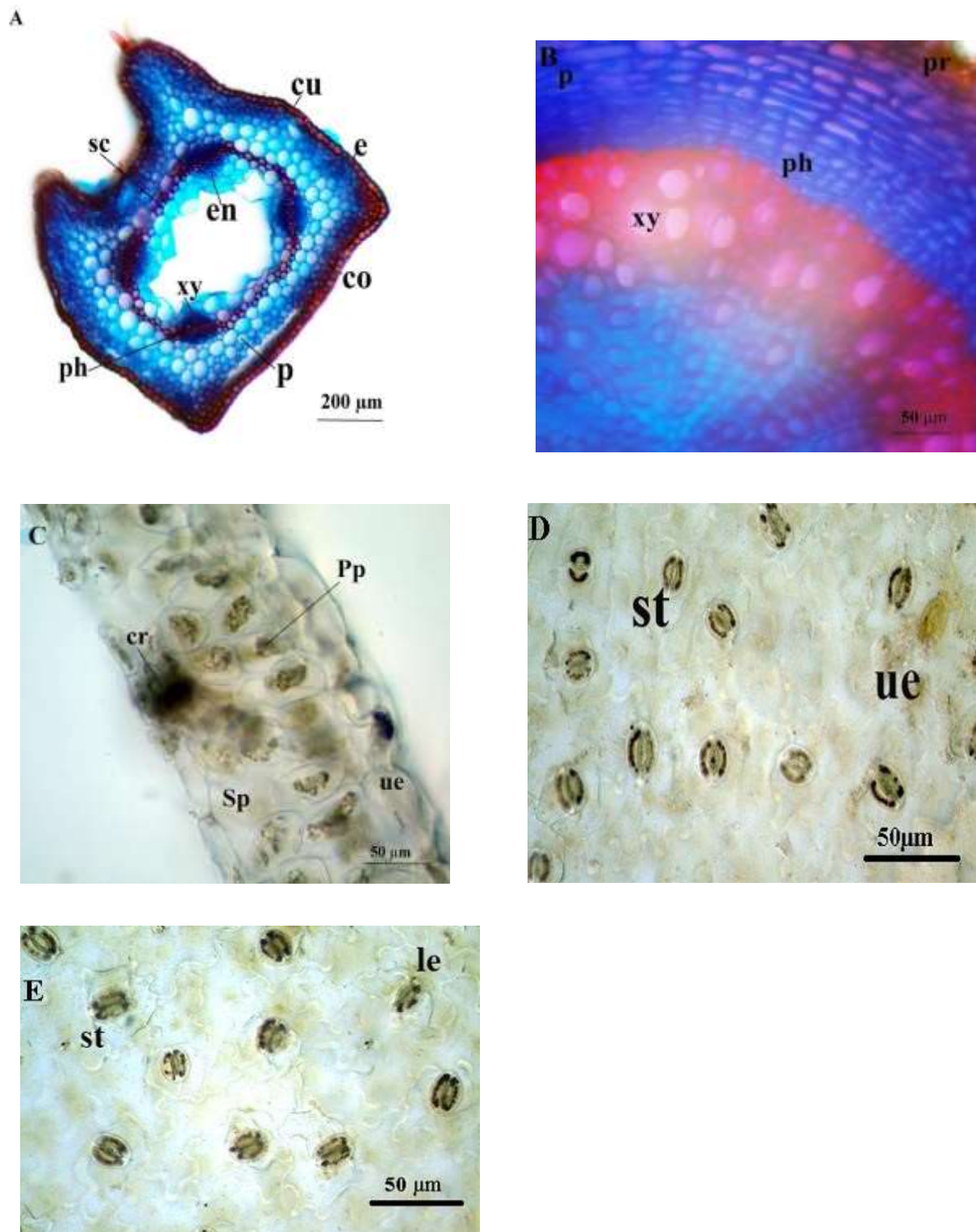
### **Anatomical Result of the *Viola kitaibeliana* Roem. & Schultes**

**Stem;** general shape is rectangular and its core region is not empty. There is a thick cuticle surrounding the epidermis at the outermost. There are hair on the body epidermis cells. Just below the cuticle there is an epiderma layer consisting of a single row of oval cells. The corner column is located below the epidermis. Collenchyma is densely accumulated at the corners opposite the transmission beams. Collenchyma consist of 2-3 rows in these regions. Below the collenchyma there is the cortex parenchyma layer consisting of 2-6 rows of oval-round thin-walled cells. The vascular bundles are arranged between a 1-2 rows of sclerenchyma layer. There are open collateral-type vascular bundles running along the sclerenchymatous ring. Tracheae are in radial rows in xylem. The core parenchyma cells are located close to the conduction bundles. These cells are oval-round in shape and sometimes larger than the cortex parenchyma (Figure 3a). Numerical values of the anatomy of the stem are given below (Table 2).

**Root;** generally, a root anatomy that has passed into a secondary structure is seen. As the protective tissue, the epidermis and exodermis are fragmented, instead periderma is formed. Cortex: It contains 2-4 rows of well-shaped parenchyma cells. Cells are thinwalled, wide, and generally smooth or lobed. Numerical values of root anatomy are given below (Table 3). Vascular tissue: secondary structure, approximately 7-8 rows of flattened secondary phloem on the outside, and a circular secondary xylem on the inside. Secondary xylem is small cell, primary xylem is broad trachea. Cambium tissue is 3-4 rows. The central part of the vascular cylinder contains the non-ligninized parenchyma and primary xylem elements. The parenchymal cells in the central part are large and thinwalled. There is no space between them. The primary xylem elements are embedded in the parenchyma (Figure 3b).

**Leaf;** upper and lower epidermis covered by a cuticle layer. Parenchyma cells are located on the surface facing the lower and upper epidermis of the vascular bundles. Sizes of epidermal cells are almost the same on the upper and lower surfaces. In the mesophyll tissue there are smaller side veins than the middle vein. It consists of palisade and spongy parenchyma. There is a druse crystal in mesophyll. There are stomata in the lower and upper epidermis. In cross sections, a central vein region is located in the middle of the leaf. It is surrounded on four sides by parenchymal and oval shaped cells in the center(Figure 3c). There is a large vascular bundle in the middle vein center. There are open colleteral type transmission bundles. Leaf anatomy numerical values are given below (Table 2). When looking at superficial sections, there is an amphystomatic leaf condition as stomata are observed on the lower and upper surface of the leaf (Figure 3d and 3e). Stomas are of anisocytic type. Upper surface stoma index is 0.58, lower surface stoma index 0.55 (Table 4).





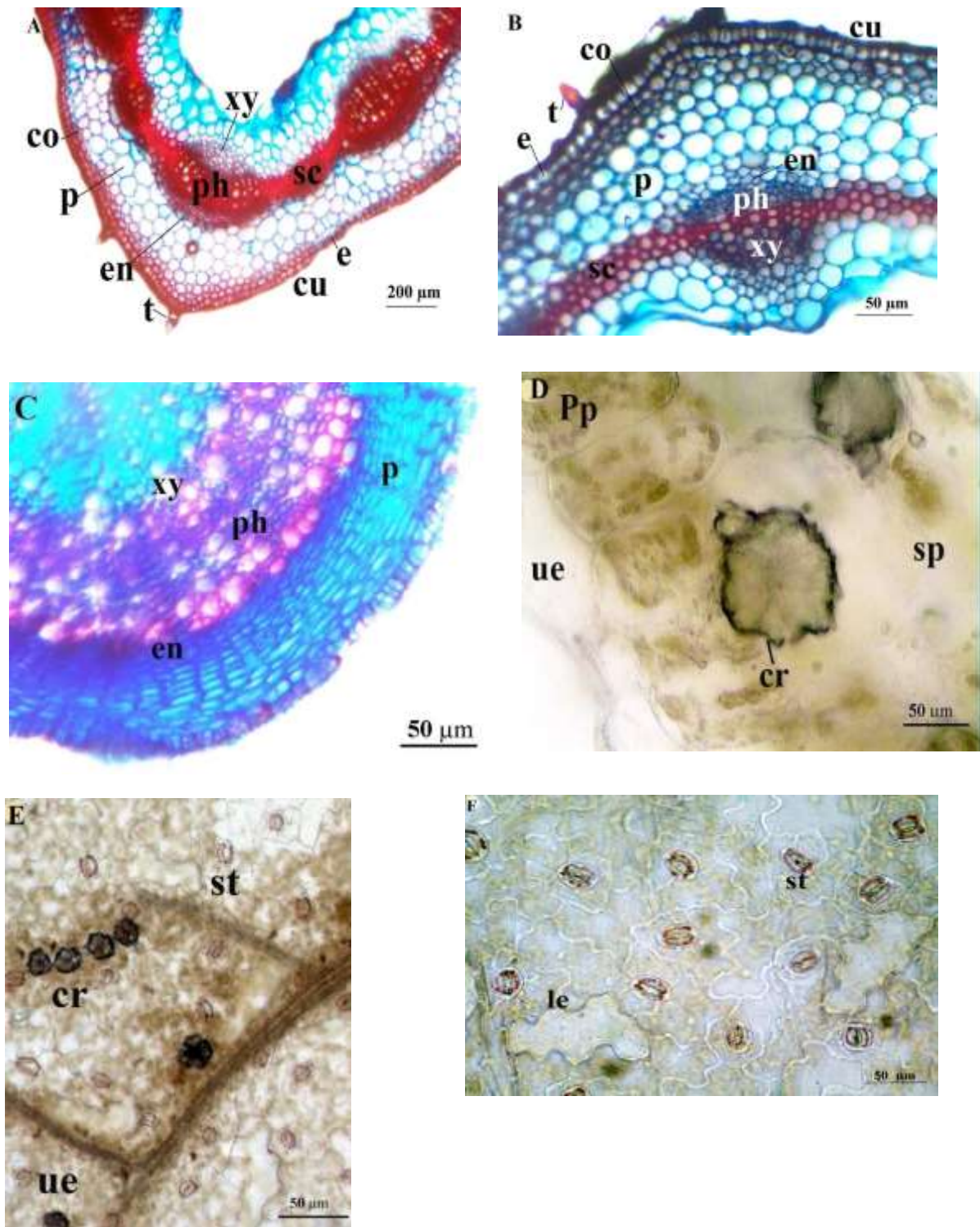
**Figure 3.** (A) Cross-section of stem in *Viola kitaibeliana*, stained with safranin-alcien blue (cu: cuticle, e: epidermis, co: collenchyma, en: endodermis, ph: phloem, xy: xylem, p: parenchyma, t: trichome, sc: sclerenchymatous ring), (B) root cross section (pr: periderm), (C) leaf cross section, staining with lugol (Pp: palisade parenchyma, Sp: spongy parenchyma, ue: upper epidermis, cr: crystal), (D) leaf upper epiderma surface section (ue: upper epidermis, st: stomata), (E) leaf lower epiderma surface section (le: lower epidermis)

### **Anatomical Results of the *Viola arvensis* Murray**

**Stem;** general shape is roundish and the core region is empty. It is covered with a thick cuticle layer surrounding the epidermis at the outermost. There is hair on the body epidermis. Just below the cuticle are parenchyma cells consisting of a single row of roundish-round cells. There is a corner column under the epidermis. The number of rows of collective cells varies according to the regions opposite the conduction beams. Collenchyma tissue continues along the entire line and consists of 2-3 rows. Below the collenchyma there is a cortex parenchyma layer consisting of 3-8 rows of oval and thinwalled cells. Below the 2-row cortex layer is a single-row epidermis (Figure 4a). Transmission bundles are arranged between a 2-3 rows sclerenchyma layer. There are open colleteral type vascular bundles along the sclerenchymatous ring. Cambium is located between xylem and phloem is in 1-2 rows. Tracheae are in radial rows in xylem. Self paransima cells are located inside the body. These cells are oval in shape and are almost in the same size as the cortex parenchyma. Numerical values of body anatomy are given below (Table 2).

**Root;** generally, a root anatomy that has passed into a secondary structure is seen. As the protective tissue, the epidermis and exodermis are fragmented and are not distinct. Cortex: It contains 2-3 rows of well-shaped cells. The cells are thin-walled, broad, mostly periclinal, with generally smooth or lobed walls. Numerical values of root anatomy are given below (Table 3). Vascular tissue: In secondary structure, there are approximately 10-12 rows of flattened secondary phloem on the outside and secondary xylem in circular form on the inside. Secondary xylem is small celled, primary xylem has broad trachea. The cambium has 3-4 rows. The central part of the vascular cylinder contains the nonligninized parenchyma and primary xylem elements. Parenchymal cells in the central part have relatively large with thick walls and there is no space between them. The primary xylem elements are embedded in the parenchyma (Figure 4b).

**Leaf;** The lower and upper epiderma are covered with a cuticle layer. There are parenchyma cells on the surfaces facing the lower and upper epidermis of the vascular bundles. There are stomata in the lower and upper epidermis cells. Epidermis cells do not vary much in size on the upper and lower surfaces. Immediately after the parenchyma cells, mesophyll tissue begins. It consists of palisade and spongy parenchyma. There is druse crystal in mesophyll. In the mesophyll tissue, the lateral veins are smaller than the middle vein (Figure 4c). In cross section of the leaf, there is a middle vein region. There is a large vascular bundle in the center of the middle vein. At its center is an open colleteral type vascular bundle surrounded by parenchymal and circular shaped cells on all four sides. Leaf anatomy numerical values are given below (Table 2). When superficial sections viewed, the stomata there are observed on the lower and upper surface of the leaf, so there is an amphystomatic leaf condition(Figure 4d) and (Figure 4e). Stomas are of anisocytic type. Upper surface stoma index is 0.57, lower surface stoma index 0.56 (Table 4).



**Figure 4.** (A) and (B) Cross-section of stem in *Viola arvensis*, stained with safranin-alcian blue (cu: cuticle, e: epidermis, co: collenchyma, en: endodermis, ph: phloem, xy: xylem, p: parenchyma, t: trichome, sc: sclerenchymatous ring), (C) root cross section (p: periderm), (D) leaf cross section, staining with lugol (Pp: palisade parenchyma, Sp: spongy parenchyma, ue: upper epidermis, cr: crystal), (E) leaf upper epidermis surface section (ue: upper epidermis, st: stomata), (F) leaf lower epidermis surface section (le: lower epidermis)

**Table 1.** Collection data of investigated *Viola* tax

| <b>Taxon</b>                            | <b>Locality</b>   | <b>Collector and plant number of collector (EDTU)</b> |
|---|---|---|
| <i>V. odorata</i> L.                    | A1E Edirne, Karaağaç, Söğütlük Forest, 41°39'28"N, 26°31'25"E, 13.03.2012       | M. Türkoğlu Koç, 13165, 13166, 13167, 13168, 13170    |
| <i>V. arvensis</i> Murray               | A1 (E) Edirne: Keşan, Mecidiye, 41°40'28"N, 26°33'39"E, 07.03.1999              | G. Dalgıç, 5431                                       |
| <i>V. kitaibeliana</i> Roem. & Schultes | A1(E) Edirne: Center, Balkan Campus, 41°40'28"N, 26°33'39"E, 18.03.2012         | M. Türkoğlu Koç, 13162, 13163, 13164, 13173, 13176    |
| <i>V. tricolor</i> L.                   | A1E, Edirne, Center, Binevler, 41° 40' 37.6752" ve 26° 33' 20.5776", 10.03.2012 | F. Dane, 13499  |

**Table 2.** Numerical values of the stem anatomy of taxa

| <b>Taxa</b>            | <b>Parenchymal cortex<br/>Min- Max (<math>\mu\text{m}</math>)</b> | <b>Sclerenchyma layer<br/>Min- Max (<math>\mu\text{m}</math>)</b> | <b>Body transmission bundles (diameter / length)<br/>Min- Max (<math>\mu\text{m}</math>)</b> |
|------------------------|---|---|--|
| <i>V. odorata</i>      | 35-117  | 25-36   | 71-125   |
| <i>V. tricolor</i>     | 150-200   | 100-200   | 44-48  |
| <i>V. kitaibeliana</i> | 250-400   | -   | 300-350  |
| <i>V. arvensis</i>     | 100-150   | 6-13  | 50-100   |

**Table 3.** Numerical values of leaf and root anatomy of taxa

| <b>Taxa</b>            | <b>Upper epidermis<br/>Min- Max<br/>(<math>\mu\text{m}</math>)</b> | <b>Lower epidermis<br/>Min- Max<br/>(<math>\mu\text{m}</math>)</b> | <b>Mesophyll<br/>Min- Max<br/>(<math>\mu\text{m}</math>)</b> | <b>Root<br/>Min- Max<br/>(<math>\mu\text{m}</math>)</b> |
|------------------------|--|--|--|---|
| <i>V. odorata</i>      | 12.5-25  | 12.5-25  | 81-88  | 71.42   |
| <i>V. tricolor</i>     | 12.5-18.75   | 12.5-18.75   | 75-81.25   | 62.5  |
| <i>V. kitaibeliana</i> | 12.5-16.25   | 12.5-16.25   | 25-62.5  | 25-62.5   |
| <i>V. arvensis</i>     | 12.5-20  | 12.5-20  | 40-50  | 47-60   |

**Table 4.** Stomata index of taxa

| <b>Taxa</b>            | <b>Upper Surface Stoma Index</b> | <b>Lower Surface Stoma Index</b> |
|------------------------|----------------------------------|----------------------------------|
| <i>V. odorata</i>      | 0.78                             | 0.76                             |
| <i>V. tricolor</i>     | 0.46                             | 0.44                             |
| <i>V. kitaibeliana</i> | 0.58                             | 0.55                             |
| <i>V. arvensis</i>     | 0.57                             | 0.56                             |

## DISCUSSION

In this study, anatomical features of four *Viola* L. species: *Viola kitaibeliana* Roem. & Schultes, *Viola tricolor* L., *Viola arvensis* Murray (sect. Melanium; annual, biennial and caulescent), *V. odorata* (sect. *Viola*; perennial and acaulescent) were investigated for the first time from Turkish materials. Anatomically, cross sections from the stem and roots, surface sections from the leaf (top and bottom) were examined and presented with their microphotographs. Leaf anatomy common features of the studied taxa; bifacial leaf variety, amphistomatic leaf, stomata are anisocytic. The stoma index is lower in *Viola kitaibeliana*, *Viola arvensis*, *Viola tricolor* species in the Melanium section compared to the *Viola odorata* species in the *Viola* section. Vascular tissue is open collateral and thinwalled in all taxa. The important distinguishing features we see in taxa depending on the leaf anatomy is that all except *Viola odorata* have druse crystals in the mesophyll layer. Among these three taxa, druse crystal density was mostly observed in *Viola arvensis*. The presence of dense crystals in the root anatomy of *Viola odorata* is remarkable. Collenchyma row number was 4-5 rows in *Viola odorata*, 3-4 rows in *Viola tricolor*, 6-7 rows in *Viola kitaibeliana* and 5-6 rows in *Viola arvensis*. It was observed that the lower and upper epiderma surface walls of *Viola odorata*, *Viola kitaibeliana*, *Viola arvensis* taxones were wavy, whereas this fluctuation was less in *Viola tricolor*. The common features of the stem anatomy of the studied taxa are single-row epidermis cells and hairy cover. The cortex parenchyma is round, thin-walled, and the corner collenchyma are opposite the transmission beams. Vascular bundles are sclerenchymatous in 1-2 rows. The vascular tissue; open colleteral and cambium 1-2 rows. The important distinguishing features of the studied taxa depending on the body anatomy are; epidermis cell shape round in *Viola odorata*; It is oval in *Viola tricolor* and *Viola kitaibeliana*, and in *Viola arvensis* it is oblong-round. Parenchyma numbers are 4-9 rows in *Viola odorata*, 2-4 rows in *Viola tricolor*, 2-6 rows in *Viola kitaibeliana* and 3-8 rows in *Viola arvensis*. The number of transmission bundles is; There are 1 in *Viola odorata*, 7 in *Viola tricolor*, 4 in *Viola kitaibeliana*, and 5 in *Viola arvensis*. The sclerenchymatic ring is absent in the *Viola odorata* species, while it has the same thickness in *Viola tricolor* and *Viola kitaibeliana* taxons, it has a thicker appearance in *Viola arvensis*. The common features of the root anatomy of the studied taxa are thin-walled cortex. Vascular cylinder secondary structure, flattened secondary phloema, inner circular secondary xylem. The important distinguishing features of the studied taxa depending on the root anatomy of the cortex; 1-2 rows for *Viola odorata*, periclinal, smooth or lobed and druse crystal, 2-3 rows for *Viola tricolor*, anticline, slightly indented-protruding, for *Viola kitaibeliana* and *Viola arvensis*, 2 to 4 rows, periclinal. It was observed that the part called self parenchyma was not found in *Viola odorata*, parenchyma cells in *Viola tricolor* and *Viola kitaibeliana* species were lined up regularly in this region, and in *Viola arvensis* the parenchyma clusters in this part were broken and there were gaps between them.

Anatomy of taxon *Viola yildirimli* which is endemic to Turkey was analyzed through the morphological and ecological studies (Bagci Yavuz et al., 2008). In the anatomical study, it was observed that the upper epidermis cells were flat in the superficial section of leaf, and in this study, the upper and lower epidermis were wavy in the leaf superficial sections in the taxon we examined. In the article published by Yousefi et al., 2012; it has been reported that root, stem, petiole and peduncle cross sections (sect. *Viola* subsect. *Viola*), *Viola caspia*, *Viola reichenbachiana* and *Viola rupestris* (sect. *Viola* subsect. *Rostratae*) were examined for the taxa *Viola kitaibeliana*, *Viola arvensis*, *Viola occulta*, *Viola tricolor* (sect. Melanium), *Viola somchetica* (sect. *Plagiostigma*), *Viola spathulata* (sect. *Spathulidium* ined.), *Viola alba*, *Viola odorata*. It was determined that the cross section of the external body and its vascular tissues,

petiole and peduncle anatomy are very important anatomical characters, the body cross section is elliptical and there are 2 rings on the sides. In the other section, the body is semi-circular and it is stated that it has 2 ring-shaped protrusions in its structure. It has been explained that the number of vascular bands in the body is determinant in some subsections. Crystals have been found in all *Viola* types. As a result, they stated that anatomical characters are very important in distinguishing taxa (Yousefi et al., 2012). When our results of the taxa were compared with the taxa in Yousefi et al., 2012 article, the same anatomical results were encountered. In the melanium sections, our stem cross-section is elliptical, with 2 ringshaped protrusions on the sides and the presence of crystals in all *Viola* taxa examined in the same way. The collenchyma, consisting of two to four cell layers, is located between the epidermis and the parenchymatous cortex around the vascular bundle. The number of the collenchyma differs in the upper and lower surfaces of leaves. All species are characterised by the presence of crystal cells. Unicellular trichomes on the adaxial side of leaves are restricted only to the midrib region of *Viola alba* subsp. *alba* and *Viola odorata* (Mehrvarz et al. 2013).

Previous reports show that there has not been extensive research on the anatomical field of *Viola* taxa. We believe that the results of this study in the field of anatomy related to *Viola* taxa in the Thrace region will make great contributions to all future studies on *Viola*.

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## BACTERIAL EXOTOXINS IN FOOD AS CAUSES OF ALIMENTARY INTOXICATIONS OF HUMANS AND UTILIZATION OF EXOTOXINS

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### ABSTRACT

Narrative review paper describes *Clostridium tetani*, *Clostridium botulinum*, *Vibrio cholerae*, *Escherichia coli*, *Campylobacter jejuni*, *Corynebacterium diphtheriae*, *Shigella dysenteriae*, *Streptococcus pyogenes*, *Clostridium perfringens*, *Staphylococcus aureus* and bacteriotoxins. Bacterial toxins in humans can cause food intoxication. Symptoms range from mild gastrointestinal to life-threatening symptoms. Bacteria secrete exotoxins during life and endotoxins after death. The purpose of this paper is to point out the danger posed by food contaminated with bacteria that produce exotoxins causing food poisoning in humans, but also to point out the possibility of using bacterial toxins for medical purposes. Alimentary intoxications in humans are most often caused by consuming food contaminated with toxin-producing bacteria, and exotoxins that act as superantigens can also cause autoimmune diseases. Some toxins can be used as preservatives in vaccines, and some toxins can also be used in the treatment of neoplasms.

**Key words:** food bacteria, exotoxins, alimentary intoxications, utilization of bacterial toxins

### INTRODUCTION

In the spring-summer season there is the greatest danger of food spoilage. High temperatures favor the development of various microorganisms, including bacteria that release exotoxins. Therefore, in the summer, you should be especially careful when handling food, due to the rapid multiplication of bacteria and food should be kept in the refrigerator until use (Lake, 2017; Ndraha *et al.*, 2018). Bacteria are all around us, in the environment and in the air, so contamination is always possible (Kuzman, 2012). In summer, the human body is particularly sensitive to food intoxications, because during high heat, a large amount of fluid is consumed, which alkalizes the body and reduces the amount of hydrochloric acid secreted by the stomach, which aims to eliminate a certain amount of pathogens from food (Hennekinne *et al.*, 2012).

Bacterial exotoxins (specific toxins) are the secretions of pathogenic bacteria, most often gram positive and gram negative bacteria, which are formed as a product of their metabolism, during bacterial life. It is a chemical substance of protein nature, more precisely of polypeptide composition (Chakraborty *et al.*, 2012; Kumar, 2017). In most cases, it is characterized by thermolability (>60°C), high toxicity, but it can be detoxified (Nakayama & Ezoe, 2014).

The mechanism of action of bacterial exotoxin is based on damage to the metabolism of the target cell, which can be a nerve cell susceptible to bacterial neurotoxin, causing disorders of the CNS and peripheral nervous system, enterocyte (enterotoxin), damages intestinal mucosa, blood cell, without selectivity of action on cell type (cytotoxin) and necrotoxin, an exotoxin that causes tissue death (El-Masry & Abou-Donia, 2015).

Bacterial exotoxins, based on the structure of action, are divided into AB toxins that enter the cytoplasm of one of the mentioned target cells, act through protein AB components, A (enzymatic, penetrating), B (binding), causing damage through enzymatic reactions within the cell (Hu & Nakane, 2014; Gaytán *et al.*, 2016; Sessler *et al.*, 2022). Membrane toxins cause lysis (bursting) of the membrane of the target cell of the macroorganism. Superantigenic exotoxins act as a "*foreign substance*" causing an autoimmune reaction of the attacked organism (Spaulding *et al.*, 2013; Cavaillon, 2018).

## **BACTERIAL EXOTOXINS**

Bacterial exotoxin is the strongest known poison, whose strength is expressed in the form of LD 50 and it represents the smallest amount of bacterial exotoxin that would cause death in 50% of laboratory animals. The toxicity of the same is so great that it would cause damage to the bacteria themselves, so that the bacteria does not secrete it (Chakraborty *et al.*, 2012). Bacterial exotoxins directly damage the host organism with their pathogenicity. In addition to the direct impact, given the toxicity of the toxin, the host organism is also damaged by the body's immune response (cellular immunity and T cells) due to the presence of the pathogen and its products, trying to get rid of the toxin (Spaulding *et al.*, 2013; Cavaillon, 2018). The body reacts with an inflammatory reaction to the entry of pathogens, by releasing inflammatory cells such as phagocytes, which in order to defend the body from pathogens release enzymes and their own toxic products that are without selectivity of action, and can harm the host (El-radhi, 2018). In case the organism manages to defend itself from pathogenic bacteria, the inflammatory cells withdraw. In addition to the described immune response of the macroorganism, the entry of pathogenic bacteria into the host organism is a present "*foreign substance*" or antigen, to which the organism responds by producing antibodies (humoral immune response) (Bitto & Kaparakis-Liaskos, 2017). This bacterial phenomenon represents its antigenicity. Pathogenic bacteria colonize the skin, mucous membranes, and pass through the epithelium (Tizard, 2017). Infection occurs when the number of bacteria increases, when the bacteria multiply to such an extent that the number causes a disease state of the organism, the production of toxins or activation of the immune response due to the presence of pathogens (Greaney *et al.*, 2015; Cavaillon, 2018).

Bacterial exotoxin can also be ingested parenterally, through food of protein origin that contains pathogenic (toxinogenic) bacteria that produce exotoxin in food (Krakauer & Stiles,

2013). The exotoxin thus produced is subject to denaturation within the digestive tract by HCl (hydrochloric acid) (Kristlova & Vytasova, 2012). This exotoxin is non-functional, but it is still an antigen for the host organism, ie its individual parts activate the immune system as a result of defense against a "*foreign body*", in the reaction already described (Kumar, 2017). Some bacterial exotoxins are resistant to the acidic medium of the stomach and small intestines, and this ability allows them to have a harmful effect in the digestive tract, as is the case with AB enterotoxins of bacteria, introduced through contaminated food, which will be discussed below (Asadpoor *et al.*, 2021).

Exotoxins are very strong poisons, that they can cause damage to the host organism before the organism reacts with an immune reaction. Immunization against diseases that cause pathogenic (toxinogenic) bacteria such as tetanus, diphtheria, etc., is possible with the help of inactivated exotoxin, with the help of iodine, formaldehyde and other inactivators or by giving ready-made antibodies created in another infected host (passive immunity) (Nash *et al.*, 2015). For some toxinogenic bacteria such as e.g. *Vibrio cholerae*, which secretes the so-called cholera toxin (AB enterotoxin), world scientists have not yet found a vaccine (O'Ryan *et al.*, 2015).

### **Bacterial exotoxins ranked by structure**

Bacterial exotoxins, based on structure, are divided into AB toxins, membrane toxins and superantigen, and this classification is the most commonly used. Some bacterial exotoxins are without the possibility of categorization, causing symptoms related to the reaction of the immune system to their presence and action (Chakraborty *et al.*, 2012).

#### **A – B toxin**

AB types of exotoxins are among the most powerful bacterial, protein toxins produced by gram positive bacteria. Prominent in this group of exotoxins are the poison gram positive *bacillus*, *Bacillus Anthracis*, the causative agent of anthrax or anthrax (soil disease), sporogenic bacillus. Then AB clostridial toxins, anaerobic, sporogenic, gram positive bacteria that cause severe human and animal diseases, and both types of bacteria and their toxins, can be misused as biological weapons (Benz *et al.*, 2017). They are built of two subunits A (active), B (binding) (Zuverink & Barbieri, 2017). The role of component B is reflected in its binding to specific receptors of the target cell (glycolipids), while component A has an active role in entering the cytoplasm of the target cell and performing toxic enzymatic reactions, causing harmful effects on organisms (Thompson, 2018).

#### **A – B toxin (Neurotoxins)**

This group includes bacterial exotoxins that act on the central nervous system (CNS) and peripheral nervous system (PNS). Neurotoxin inhibits neurotransmission at synapses, blocking the release of acetylcholine (Thompson, 2018).

### ***Clostridium tetani* (Tetanolyzine, Tetanospasmin)**

*Clostridium tetani* is an anaerobic, sporogenic, Gram positive bacterium that builds its spores within soil, dust, plants, the digestive tract of humans and animals (Dürre, 2014). Bacteria most often enter the macroorganism through damaged skin, injuries, burns (Thompson, 2018).

It achieves its harmful effects directly, by producing exotoxins (Cohen *et al.*, 2017). One is tetanolyzine, with no known role in pathology, and tetanospasmin (Enany, 2014). Tetanospasmin has two peptide chains, with a B component (binding), which binds to specific receptors located on motor nerve cells, allowing endocytosis of the lighter chain of the A component (enzymatic), via the enzyme metalloprotease. A component breaks down proteins that help the secretion of neurotransmitters and that have an inhibitory function in the anterior part of the spinal cord. This mechanism of action causes spasm and spasms of the transverse striated muscles. By blocking the action of inhibitory neurons, uncontrolled contraction of the mentioned muscle type occurs (Enany, 2014; Thompson, 2018).

In case of the appearance of the disease, the patient is hospitalized, wound debridement is necessary, constant supervision and certainly the use of an appropriate antibiotic. The use of antitoxic serum is effective only if the exotoxin has not reached the central nervous system. Vaccination is recommended every two to three days, ie vaccine therapy with the mentioned serum. Symptoms primarily include a local reaction at the site of entry (Thompson, 2018).

Only when the bacteria enters the bloodstream, through the wound, do symptoms appear, which are related to the innervation disorder, since the neurotoxin that causes tetanus is pathogenic for nerve cells, which is its direct pathogenicity to host cells. Local muscle spasm occurs (Enany, 2014). First there is a spasm, then a permanent spasm. General spasm with hypersensitivity is a clinically pathognomonic symptom. The favorable course of the disease includes the relaxation of muscle spasms, while the unfavorable course of the same is associated with sudden symptoms, sudden general spasm of the muscles. Without treatment, mortality from tetanus toxin ranges up to 100% (Enany, 2014).

### ***Clostridium botulinum* (Botulin)**

*Clostridium botulinum* is an anaerobic, Gram positive bacterium, whose spores can be found in canned food, canned vegetables (usually prepared at home), rotting food and in sausages, fish, wastewater, digestive tract, animal feces, most often rodents, birds, cats (Martinović *et al.*, 2016; Bhunia, 2018).

In humans, in the case of mass intoxication, it is a parenteral intake of bacterial exotoxin through canned food containing spores of the mentioned bacteria. Spores in food germinate into a vegetative form and they secrete exotoxins that are resistant to enzymes of the digestive tract (Taylor *et al.*, 2013; Bhunia, 2018).

Some species of these bacteria have a pronounced thermal stability at 121°C for three minutes, while other strains are mostly thermolabile, and their inactivation is achieved at 80°C within 15 minutes. After heat treatment of food, if 1% of slow bacteria remains, a new vegetative form will germinate from it (Carter, 2014; Shen *et al.*, 2018).

This bacterium present in food secretes the exotoxin botulin which is so strong that 500 mg of the said toxin is enough to kill one nation and is considered to be the strongest exotoxin ever discovered (Chen *et al.*, 2021).

Different strains of this bacterium synthesize one of seven antigenically different toxins A, B, C, D, E, F and G, of which A, B, E and F are responsible for most cases of botulism in humans and C and D cause botulism in animals, most toxic to birds and fish (Bhunias, 2018; Plöbl *et al.*, 2021).

Exotoxin botulin consists of A and B components. Specific receptors are located on the motor nerves to which the B component binds, and the enzymatically active part A becomes an active peptidase that breaks down protein from the membrane of synaptic vesicles, ie synaptobrevin, and prevents exocytosis of acetylcholine neurotransmitters, preventing acetylcholine release. In this way, muscles contract and lead to paralysis (Popoff, 2016).

Botulinum can cause a sudden lethal reaction in the body (Bhunias, 2018). In the period of 18-36 hours from the intake of the bacterial exotoxin botulinum in the digestive tract of an animal or human, there is a general weakness, dryness of the oral mucosa, digestive tract disorders that otherwise occur in poisoning. Because toxins are nerve toxins, they attack *n. hypoglossus*, *n. glosopharyngeus* and medulla oblongata nerves. Speech dysfunctions, reasoning disorders and balance center disorders occur. Death occurs due to depression of the respiratory and cardiac centers (Popoff, 2016). Therapy includes the use of laxatives, homologous serums, but the prognosis is not favorable (Lovato *et al.*, 2017).

#### **A – B toxins (Enterotoxins)**

Bacterial exotoxins (AB toxins) that act on enterocytes are enterotoxins. Enterotoxins damage the digestive tract, with consequences in the form of osmotic diarrhea and vomit. Components A and B of enterotoxins act on epithelial cells by binding the B component of the exotoxin to specific receptors located on the microvilli of small intestinal epithelial cells (Simon *et al.*, 2014). The active, enzymatic A component of the exotoxin transfers adenosine diphosphate ribose from nicotinamide adenine nucleotide to G-protein which becomes reversibly activated. The amount of it created exceeds physiological limits, and G-protein accumulates in intestinal cells, within cyclic-adenosine monophosphate. The described phenomenon results in constant secretion of fluid inside the intestine, rich in electrolytes and chlorine ions. Under normal conditions, a small amount of intestinal fluid is easily resorbed, so it is not a problem. Under the above conditions, the intestines are unable to absorb large amounts of fluid in the intestines due per unit time and severe osmotic diarrhea and acid-base imbalance occur in the body (Thompson, 2018; Dupont *et al.*, 2019).

#### ***Vibrio cholerae* (toxin Cholerae)**

*Vibrio cholerae* is aerobic, facultatively anaerobic, Gram positive, a rod-shaped bacterium that does not build spores. It is the etiological cause of cholera. It enters the body through contaminated water and food and is transmitted within the community by the feco-oral route (Wang *et al.*, 2016; Yoon & Waters, 2019).

It also adapts to the acidic environment of the digestive tract, and inhabits the small intestine under conditions with pH 5. In general, bacteria of the genus *Vibrio* are sensitive to low pH and often one of the prerequisites for this type of bacteria is hypochloridia or achloridia, acidity of the organism (Asadpoor *et al.*, 2021).

It is a primarily non-pathogenic bacterium that turns into a pathogenic bacterium through lysogenic conversion. The described phenomenon occurs when a bacterial (bacteriophage) virus or called a phage attaches to a bacterial prokaryotic cell, more precisely to its chickens (which allow it to adhere to the intestinal wall) and injects its DNA into it (transformation), "kidnaps, infects bacteria" and use its components, to form new parts of the phage. In some cases, the reproductive cycle of the phage kills the bacterium, and in other cases the bacterium survives. Then the virus incorporates its DNA into the DNA of the bacteria. New phage depends on bacteria, for replication of new parts of phage. When a new phage leaves the host by a process of lysis (cell breakdown), some also carry parts of bacterial DNA. If such a bacteriophage is attached to a "new bacterium" by the same process originally described and such created DNA is incorporated into the DNA of a new bacterium, a new genetic type of bacterium can be formed (Zajdowicz, 2022).

When phage affects the genetic recombination of bacteria, the process is called transduction. All mechanisms of gene recombination are: transformation, conjugation and transduction. The bacterium becomes pathogenic when the bacterial virus enters the lysogenic cycle and incorporates its genome within the bacterial chromosome, and it acquires the toxic properties of the virus (Pavlica, 2012; Zajdowicz, 2022).

Symptoms caused by the exotoxin of the mentioned bacteria are related to the appearance of severe watery diarrhea, where a person can lose several liters of fluid in just one hour (Pérez-Reytor *et al.*, 2018). If rehydration of the organism is started on time with adequate antibiotic therapy, the cure rate is high. In untreated cases, the outcome is often quick and fatal. The oral vaccine exists but does not affect everyone (Wang *et al.*, 2016; Van Alst & DiRita, 2020).

### **Enterotoxinogen *Escherichia coli* (LT and ST Enterotoxin)**

The bacterium *Escherichia coli* is a gram negative bacillus, an aerobic, facultative anaerobic bacterium that does not build spores. There are strains of *Escherichia coli* that secrete enterotoxins. They differ from other strains of the same bacterium that are commensals of the intestinal tract, in terms of genetic material (Gonzales-Siles & Sjöling, 2016; Thompson, 2018).

The enterotoxigenic strain of *E. coli* (ETEC), a toxin strain, contains a toxin gene. *Escherichia coli* possesses adherence, the ability to attach to the intestinal wall. In adhesion they have the help of the enzyme adhesin. The same enzyme, together with the released enterotoxin, has the ability to encode genes on plasmids that this bacterium can transfer from one to another by conjugation (Roussel *et al.*, 2018).

Enterotoxigenic strains of *Escherichia coli* cause diarrhea in pigs, goats, sheep, horses, dogs and humans. Enterotoxin causes the movement of water from the tissues into the intestinal lumen and leads to the appearance of osmotic diarrhea, the so-called. traveler's diarrhea. There are two exotoxins released by *E. coli*. One is thermolabile LT and the other is thermostable ST and it is not destroyed even at a temperature of 100°C for 30 minutes (Wang *et al.*, 2019). These

toxins cause the activity of adenyl cyclase, ie guanyl cyclase, under the action of which cAMP and cGMP are created, which stimulate secretion and at the same time block the resorption of ions from the intestinal lumen, and watery diarrhea occurs (Vansofla *et al.*, 2021). Thermolabile LT non-toxic toxins *Escherichia coli* can be used as potent preservatives in vaccine preparation (Ma, 2016).

Infection and the disease itself, which cause the mentioned toxin, results in diarrhea as in the case of cholera toxin, with one toxin of the mentioned bacterium being identical to the enterotoxin that causes cholera (Sahl *et al.*, 2015; Hrustemović *et al.*, 2021a).

### ***Campylobacter jejuni* (Enterotoxin, similar to Cholera Enterotoxin)**

*Campylobacter jejuni* is a gram negative bacterium of zoonotic potential that causes campylobacteriosis (Smajlović & Tanković, 2016). The most common causative agent of campylobacteriosis in humans is *C. jejuni* (95%) (Wieczorek & Osek, 2013). Campylobacteriosis is a seasonal zoonosis with a site of action of bacteria on the epithelium of the intestinal and reproductive tract (Chukwu *et al.*, 2019). Warm-blooded animals are considered reservoirs of infection, especially poultry. Thermophilic *Campylobacter jejuni* is found in the digestive tract of seemingly healthy poultry, often with a percentage of 100% and are also found on feathers and are a source of infection for humans. Cross-contamination with raw chicken meat is a high risk factor for getting campylobacteriosis (Alagić *et al.*, 2016). Food should be handled with care to prevent possible food poisoning, the symptoms of which range from mild symptoms (gastroenterocolitis) to possible death (Hrustemović *et al.*, 2021b). Contamination of raw chicken meat largely depends on slaughter processing. Hazards represent different stages of primary production (fattening, slaughter and primary processing of chicken meat), so it is important to respect sanitary standards in different stages of primary production “farm to table” (Crim *et al.*, 2014; Facciola *et al.*, 2017). *Campylobacter jejuni* is transmitted to humans through contaminated food and causes alimentary intoxications through exotoxins released during life and endotoxins after death. These are most often chicken meat that can be contaminated with fecal bacteria during processing of carcasses in primary production or cross-contamination during meat handling (Markey *et al.*, 2013). The most significant enterotoxin that is similar to the cholera exotoxin activates inflammation in the gut. *Campylobacter jejuni* also produces a cytotoxin CDT that breaks the DNA chain (He *et al.*, 2019). The humoral response is more important in disease control (Hrustemović *et al.*, 2021b).

### **A – B toxins (Cytotoxins)**

Bacteria that secrete exotoxins of AB structure, and they have no affinity for certain host cells, belong to cytotoxins (Sessler *et al.*, 2022).

### ***Corynebacterium diphtheriae* (Diphtheria toxin DT)**

*Corynebacterium diphtheriae* is an aerobic, Gram positive *bacillus* that does not build spores. It is the etiological cause of diphtheria (Domachowske & Suryadevara, 2020). The



harmful effect is formed through the synthesized diphtheria toxin (DT). The strength of the produced toxin depends on the lysogenic conversion, ie the created bacteriophage that carries the gene for that toxin (Mansfield *et al.*, 2019). The repressor of the toxin gene is active only if it binds to iron, if it is inactive, the toxin gene is expressed. The bacteriophage produces a strong encoded protein of the exotoxin DT that causes the symptoms of diphtheria. Diphtheria is a potentially fatal disease (Gower *et al.*, 2020). The route of transmission is usually direct, via aerosols. Man is a reservoir of infection. The bacterium is not invasive. Through the toxin it acts locally at the point of entry, pharynx, tonsils, cervical lymph nodes, causing localized inflammation with the creation of pseudomembranous changes from organic detritus. Pseudomembranes bleed a lot by tearing. The bacterium can break into the bloodstream and result in degenerative changes in the heart muscle, liver, kidneys and nervous system (Martin, 2015). The mechanism of action of the mentioned exotoxin is based on blocking the protein synthesis of the attacked cell, which leads to cell death. Bacteria secrete an inactive form of AB toxin (cytotoxin) which is further cleaved into A and B components. B binds to specific receptors, while A, an enzymatic component catalyzes the chemical reaction ADP-ribosylation, inactivates elongation factor 2 (EF-2). It is required for the movement of ribosomes along the mRNA molecule, and the protein synthesis of the attacked cell is stopped and the cells of the attacked macroorganism die (Domachowske & Suryadevara, 2020; Zhao *et al.*, 2020). Recently, the possibilities of using DT toxins to destroy brain cancer cells have been investigated. Animal studies show solid results on killing cancer cells, but poor brain penetration has been reported (Li *et al.*, 2013).

#### ***Shigella dysenteriae* (Shiga toxin)**

*Shigella dysenteriae* is an aerobic, asporogenic, Gram positive bacterium. It produces a very strong shiga toxin that attacks host cells in which it blocks protein synthesis and causes its death. A very similar mechanism of action in causing host cell death has a toxin secreted by the enterohemorrhagic strain of *E. coli*, also called "Shiga like" toxin or Verotoxin (Amani *et al.*, 2015). The virulent strain has a plasmid through which it enters the target cell, below the host epithelial cells, where the B component binds to specific receptors and enters the epithelial cells which are in the phagosome. Then they come out and multiply in the cytoplasm of the cell and thus cause its death. It is structurally similar to Cholera toxin. It causes purulent, bloody dysentery and diarrhea. There is currently no treatment for alimentary intoxication caused by Shiga-like exotoxins. These exotoxins can be used in the treatment of certain neoplasms (Bergan *et al.*, 2012).

#### **Enterohemorrhagic strain of *Escherichia coli* („Shiga like“ or Verotoxin)**

Enterohemorrhagic *Escherichia coli* (EHEC), anaerobic, asporogenic, Gram positive bacillus coli, through verotoxin secreted in large quantities, causes severe mucosal damage and causes bloody colitis of calves, goats, sheep and humans. It is an alimentary intoxication (Korotkov & Hol, 2013). The most famous strain of pathogenic bacteria belongs to serovar O157: H7 (Amani *et al.*, 2019). In the target macroorganism, with a weakened immune system,

it can lead to hemolytic ammonia syndrome, which includes hemolytic anemia, thrombocytopenia purpura (fatal outcome 50%), renal failure, and if it reaches the CNS, it causes neurotic symptoms (Nguyen & Sperandio, 2012). Enterohemorrhagic *Escherichia coli* is introduced into the macroorganism by feco-oral route, most often through contaminated raw beef, chicken, although any food can be exposed to feces (Persad & Lejeune, 2015; Heredia & García, 2018). In diagnostics, it is important that the enteropathogenicity of Enterohemorrhagic *E. coli* isolated from faeces is demonstrated by culture tests, serological and molecular tests that are also available (Amani *et al.*, 2019). In its genome, verotoxin has a DNA sequence that is not present in non-pathogenic strains, rather than embedded phage, and the strength of the toxin depends on lysogenic conversion with the bacterial virus, which contains the gene for the poison (Hrustemović *et al.*, 2022). Toxins of this strain of *E. coli* are the cause of severe disease with bloody diarrhea, but the disease is rare, although in some parts of the United States it is the second alimentary intoxication, after salmonella infection (Pacheco & Sperandio, 2012; Ayoade *et al.*, 2021). Enterohemorrhagic *Escherichia coli* produces Shiga-like toxin that can be used in the treatment of neoplasms (Weidle *et al.*, 2014).

### **Membrane toxins**

This group includes exotoxins that base their mechanism of action on the lysis of the membrane of the attacked cell of the macroorganism. We distinguish between hemolysin toxins that cause hemolysis of erythrocytes, ie cytolysins. Hemolysins induce lysis of erythrocytes by leaking hemoglobin, a dye and protein component, iron and ions (Otto, 2014). Then there are leukocidins that have the ability to cause lysis of the membrane of phagocytic leukocytes, reducing the immune system of the macroorganism (Spaan *et al.*, 2017). Leukocidins and hemolysins form pores (channels) on the membranes of the attacked cells, binding to the cholesterol of the same membranes, with consequent leakage of nutrients from the cytoplasm of the cell, while water enters the osmosis process, the cell swells and bursts. There are also bacterial phospholipases, enzymes that break the polar part of the head of the membrane of the attacked cell, which is built of phospholipids (Otto, 2014; Spaan *et al.*, 2017).

***Streptococcus pyogenes* (enzymes: Streptokinase, Hyaluronidase, Hemolysins: Streptolysin S, Streptolysin O, exotoxins: Streptococcal pyrogens, Erythrogenic toxin)**

*Streptococcus pyogenes* is aerobic, facultatively anaerobic, asporogenic, Gram positive coccus, serological group A (SGA 1), beta hemolytic, extremely invasive (Watanabe *et al.*, 2016). Initially causes pharyngitis, angina, otitis media, sinusitis, peritonsillar abscesses, skin inflammation, erysipelas, impetigo, phlegmon cellulitis, and rarely sepsis, rheumatic fever, acute glomerulonephritis, meningitis, as a complication, often in untreated targets, on the presence of hemolysins (Streptolysin O) which are antigenic and Streptococcal pyrogens exotoxins that can lead to toxic shock and act as a superantigen (Sumitomo, 2019). Streptococci possess Streptokinases and Hyaluronidases (antigenic), Hemolysins, more precisely Streptolysins (Streptolysins O and S) which act by lysing the membrane of target cells of the macroorganism, which include leukocytes, platelets, macrophages and streptolysin S is

cytotoxic and will be cytotoxic to cells (Hynes & Sloan, 2016). Beta hemolytic streptococci differ from alpha and gamma hemolytic streptococci in that Streptolysin O, produced by beta hemolytic streptococci, on a culture medium, blood agar, creates a zone of clean space, because it causes complete hemolysis of erythrocytes, under anaerobic conditions, because oxygen inactivates it, while Streptolysin S is stable under aerobic conditions (Gera & McIver, 2013). Streptolysin O acts as a protein antigen and the body responds by making antibodies. The production of antibodies begins after infection, at the earliest after 1 week and at the latest within 6 weeks and with proper therapy after treatment, they withdraw within a year and are only evidence of the presence of antigens, ie the presence of the bacteria with antigenic enzymes hemolysins and exotoxins, unless they lead to the complication mentioned above (O'Seaghda & Wessels, 2013). *Streptococcus piogenes* secretes erythrogenic exotoxins that cause rash in scarlet fever and are responsible for toxic shock syndrome (septic streptococcal fever), which leads to hyperthermia and acts as a superantigen. In addition to the symptoms of shock, there is also a failure of the function of some of the organs. Scarlet fever can occur during and after angina as a type of complication caused by bacteria, exotoxins and the body's reaction to exotoxins (Sumitomo, 2017).

### ***Clostridium perfringens* (Alpha toxin)**

*Clostridium perfringens* is an anaerobic, Gram positive, sporogenic bacterium (Brocca *et al.*, 2022). *Clostridium perfringens* spread in "dead soil", non-oxygenated, in anaerobic conditions, intestines of animals, humans. According to some authors, the spread in the soil may be related to vultures and other animals that are considered reservoirs of infection (Meng *et al.*, 2017). Of the animals, lambs from dairy sheep lying on dirty litter contaminated with feces are most often affected. They are infected through the mother's udder, mostly lambs with a good appetite that have "full stomachs" in which favorable anaerobic conditions for the survival of the bacterium prevail. The bacterium multiplies rapidly and can cause fatal enterotoxemia via Alpha toxin (Li *et al.*, 2013). The mechanism of action of Alpha toxin is based on the action of lecithin on the host cell membrane, which is an important component of the membrane. It acts as a phospholipase enzyme. By diffusing into the bloodstream, the toxin kills leukocytes, erythrocytes, damages capillaries, other structures. It produces the enzymes Collagenase and Hyaluronidase, which degrade in dead tissue and swell. *Clostridium perfringens* ferments glycogen and amino acids within the muscle and during growth it produces hydrogen and carbon dioxide and develops gas gangrene. The Alpha exotoxin produced by *Clostridium perfringens* plays an important role in the formation of gas gangrene (Oda *et al.*, 2015).

### **Super antigen**

Exotoxins as superantigens act directly on T lymphocytes, the antigen presenting the cells of the immune system, non-specifically activating them. The toxins bind directly to the MCH II 1 antibody molecule (the major component of tissue tolerance) major histocompatibility complex, but to specific, unusual sites for peptides. At the same time, they bind to specific receptors on T lymphocytes, leading to their massive proliferation and the consequent release of

lymphocytes, monocytes, cytokines (responsible for signaling to other cells). The described phenomenon leads to toxic shock, which is characterized by hypotension, fever and sometimes rash (Krakauer, 2013).

### ***Staphylococcus aureus* (TSST 1 – Toxic shock syndrome toxin - 1, enterotoxin)**

*Staphylococcus aureus* is an aerobic, Gram positive coccoid, conditionally pathogenic bacterium. It synthesizes one or more exotoxins that act as a superantigen in the macroorganism. Strains of bacteria that cause toxic shock secrete TSST 1 toxin and strains that release exotoxins in contaminated food belong to enterotoxins and lead to alimentary intoxication. *Staphylococcus aureus* is a common contaminant in cow's cheese, but adhering to the HACCP concept reduces the chances of infection in consumers (Silva *et al.*, 2021). Strains, which cause toxic shock, since they act as a superantigen (SAg) the organism reacts by releasing cytokines that lead to a drop in blood pressure and kidney failure (Krakauer *et al.*, 2019; Peng *et al.*, 2021).

### **Other poisons (exfoliatin)**

The toxin exfoliatin is encoded on the plasmid, and the other on the bacterial chromosome. It is produced by some strains of *Staphylococcus aureus*. Exotoxin causes exfoliation of the skin, which reaches the bloodstream. The skin appears burnt and, without a defensive role, is exposed to secondary bacterial infections. Bacterial enzymes: hydrolytic enzymes (proteases, lipases, collagenases), break down parts of tissues, and help spread the infection (Del Giudice, 2020).

## **BACTERIAL EXOTOXINS**

### **Bacterial exotoxins from food, health problems and possible usefulness for medical purposes**

*Clostridium tetani*, *Clostridium botulinum*, *Vibrio cholerae*, *Escherichia coli*, *Campylobacter jejuni*, *Corynebacterium diphtheriae*, *Shigella dysenteriae*, *Streptococcus pyogenes*, *Clostridium perfringens*, *Staphylococcus aureus* and other similar bacteria are bacteria that contaminate various types of food and produce exotoxins that can cause various diseases in consumers of infected food, even those that can be fatal (Thompson, 2018). Diseases caused by exotoxins of these bacteria are: tetanus, botulism, cholera, colibacillosis, campylobacteriosis, diphtheria, shigellosis, streptococcus, clostridiosis, (Table 1) (Li *et al.*, 2013; Martin, 2015; Sumitomo, 2019; Caruso *et al.*, 2020; Popoff, 2020; Van Alst & DiRita, 2020; Hrustemović *et al.*, 2021; Plöbl *et al.*, 2021). Symptoms of alimentary intoxications vary from gastrointestinal problems to life-threatening (Hrustemović *et al.*, 2021; Ličková *et al.*, 2021). Frequent food poisoning in consumers can have consequences in the form of chronic diseases of the gastrointestinal tract and certain immune disorders, autoimmune diseases and various allergic reactions that represent diseases of the "new age" (Hrustemović *et al.*, 2021b).

Exotoxins cause disease in consumers of contaminated food, but due to their specific characteristics and their immunogenicity, they can also be used for medical purposes (Mazor & Pastan, 2020). Thus, the notorious exotoxins can be preservatives in the preparation of vaccines, because they will increase the immunogenicity of the vaccine (Samaras *et al.*, 2021). They can also be used in the treatment of certain neoplastic diseases, because they activate a strong immune response of the organism (Table 2) (Hashimi *et al.*, 2013).

**Table 1.** Food contaminants and the diseases they cause

| Food contaminants                  | Alimentary intoxications      |   |                                     |
|------------------------------------|-------------------------------|---|-------------------------------------|
|                                    | Exotoxins                     | Disease and symptoms  | Source                              |
| <i>Clostridium tetani</i>          | Neurotoxin                    | Tetanus. Muscle spasm   | (Popoff, 2020)                      |
| <i>Clostridium botulinum</i>       | Neurotoxin                    | Botulism. Muscle paralysis  | (Plöbbl <i>et al.</i> , 2021)       |
| <i>Vibrio cholerae</i>             | Enterotoxin                   | Cholera. Predominantly gastrointestinal ailments (severe diarrhea vomiting)   | (Van Alst & DiRita, 2020)           |
| <i>Escherichia coli</i>            | Enterotoxin                   | Colibacillosis. Predominantly gastrointestinal disorders (bloody diarrhea dependent on soy, vomiting)   | (Hrustemović <i>et al.</i> , 2021a) |
| <i>Campylobacter jejuni</i>        | Enterotoksin                  | Campylobacteriosis. Mostly gastrointestinal problems similar to cholera, possible neurological disorders  | (Hrustemović <i>et al.</i> , 2021b) |
| <i>Corynebacterium diphtheriae</i> | Cytotoxin                     | Diphtheria. Hyperthermia, bad breath, heart, kidney and peripheral nervous system symptoms  | (Martin, 2015)                      |
| <i>Shigella dysenteriae</i>        | Cytotoxin<br>Shiga toxin      | Shigellosis. Purulent, bloody dysentery, diarrhea   | (Caruso <i>et al.</i> , 2020)       |
| <i>Streptococcus pyogenes</i>      | Membrane toxin                | Streptococcus. Asymptomatic, pharyngitis, angina, otitis media, sinusitis, peritonsillar abscesses, dermatitis, erysipelas, impetigo, phlegmon cellulitis, rare complications such as sepsis, rheumatic fever, acute glomerulonephritis, meningitis | (Sumitomo, 2019)                    |
| <i>Clostridium perfringens</i>     | Membrane toxin<br>Alpha toxin | Clostridium. Predominantly gastrointestinal disorders (diarrhea and vomiting).<br>Complications are fatal enterotoxemia   | (Li <i>et al.</i> , 2013)           |
| <i>Staphylococcus aureus</i>       | Membrane<br>Enterotoxin       | Staphylococcus. Various infections, serious complications are sepsis, toxic shock syndrome (super antigen)  | (Peng <i>et al.</i> , 2021)         |

**Table 2.** Utilization of exotoxins for medical purposes

| Food contaminants                  | Utilization of exotoxins for medical purposes                       |  |                                    |
|------------------------------------|---|--|------------------------------------|
|                                    | Exotoxins   | Vaccine preparation and inhibitory activity on tumor cells   | Source                             |
| <i>Clostridium tetani</i>          | Neurotoxin  | Due to their protein structure, exotoxins are suitable for making vaccines. Detoxified exotoxins (toxoids) are used, which are non-toxic but retain immunogenicity.  | (Dürre, 2014)                      |
| <i>Clostridium botulinum</i>       | Neurotoxin  | The most well-known exotoxin vaccine is the diphtheria, tetanus and pertussis vaccine.   |                                    |
| <i>Clostridium perfringens</i>     | Membrane toxin<br>Alpha toxin                                       | Exotoxins have a targeted and immunomodulatory effect on tumor cells. Proteolytic enzymes have a selective ability to degrade tumor tissues causing lysis.<br>Differentiated endospores have the ability to kill solid tumor cells, because anaerobes administered intravenously selectively grow only in hypoxic/tumorous tissue. |                                    |
| <i>Vibrio cholera</i>              | Enterotoxin   | They target the inhibition of protein synthesis, reduce the growth of tumor cells, participate in the regulation of the cell cycle and various processes in tumor cells.   | (Sharma <i>et al.</i> , 2021)      |
| <i>Escherichia coli</i>            | Enterotoxin   | Induction of apoptosis and necrosis of tumor cells. They express guanylate cyclase and inhibit the growth of tumor cells. Consideration of tumor support therapy.  | (Liu <i>et al.</i> , 2010)         |
| <i>Campylobacter jejuni</i>        | Enterotoxin<br>Genotoxin,<br>cytotoxic<br>distending<br>toxin (CDT) | The mechanism of action of the CdtA subunit on the disruption of the double-stranded DNA resulting in cell cycle disruption and apoptosis. Cytotoxic toxin is used in radioresistant prostate cancer (PCa).  | (He <i>et al.</i> , 2019)          |
| <i>Corynebacterium diphtheriae</i> | Cytotoxin   | The cytotoxic activity of the protein on radiation-resistant tumors is considered.   | (Voltà-Durán <i>et al.</i> , 2021) |
| <i>Shigella dysenteriae</i>        | Cytotoxin<br>Shiga toxin  | T-lymphocyte activation. Induction of tumor cell apoptosis. Proven inhibition of cervical tumor growth as well as successful immunization against high-risk HPV-16 virus type in mice providing encouraging data.<br>Production of vaccines (toxoids) and killing of tumor cells.  | (Sadraei <i>et al.</i> , 2013)     |
| <i>Streptococcus pyogenes</i>      | Membrane toxin  | The use of non-detoxified toxins activates the immune response and releases inflammatory mediators including tumor necrosis factor $\alpha$ , but the treatment is toxic and the efficiency is 30-50%. Killing tumor cells.  | (Chandrasekaran & Caparon, 2016)   |
| <i>Staphylococcus aureus</i>       | Super antigen<br>Enterotoxin  | Induction of tumor cell apoptosis.   | (Zhang <i>et al.</i> , 2017)       |

Bacterial exotoxins, AB neurotoxins produced by *Clostridium tetani* and *Clostridium botulinum* due to their protein structure are suitable for making vaccines. Detoxified toxoids that retain immunogenicity are used. The most famous vaccines are against diphtheria, tetanus and pertussis. Also, exotoxins, membrane (Alpha toxin) from *Clostridium perfringens* injected into tumor tissue causes selective lysis of tumor cell membrane, because endospores selectively grow in hypoxic tumor tissue (Dürre, 2014). Bacterial exotoxin, AB enterotoxin of *Vibrio cholerae* (Cholerae toxin), reduce the growth of tumor cells, inhibit protein synthesis and participate in the cell cycle of tumor cells, and their use is considered as a form of supportive therapy in the treatment of tumors (Sharma *et al.*, 2021). AB enterotoxin of *Escherichia coli* (LT and ST Enterotoxin) causes apoptosis and necrosis of tumor cells. It expresses guanylate cyclase and inhibits the growth of tumor cells (Liu *et al.*, 2010). AB enterotoxin from *Campylobacter jejuni* (Enterotoxin Genotoxin, cytolethal distending toxin, CDT) causes tumor cell apoptosis and is used in radioresistant prostate carcinoma (PCa) (He *et al.*, 2019). AB cytotoxin from *Corynebacterium diphtheriae* (Diphtheria toxin DT), its cytotoxicity is considered in the treatment of tumors resistant to radiation (Voltà-Durán *et al.*, 2021). AB cytotoxin of *Shigella dysenteriae* (Shiga toxin) induces tumor cell apoptosis. It has been proven to inhibit the growth of cervical tumors. In mice, the success of a vaccine made from toxoid against the high-risk type of the HPV-16 virus has been proven, which is encouraging data (Sadraeian *et al.*, 2013). Membrane exotoxin from *Streptococcus pyogenes* (Streptococcal pyrogens, Erythrogenic toxin) which is used undetoxified, activates a strong immune response in the host, inflammatory mediators are released, including tumor necrosis  $\alpha$ . The success rate of tumor treatment is 30-50%, but the treatment is toxic, because it has no selectivity of action (Chandrasekaran & Caparon, 2016). Super antigen exotoxin from *Staphylococcus aureus* (TSST 1 – which causes Toxic shock syndrome toxin - 1, enterotoxin and exfoliatin) causes apoptosis of tumor cells (Zhang *et al.*, 2017).

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## CONCLUSION

Many exotoxins are pathogenic to both animals and humans, but many diseases are not transmitted directly, often by food poisoning. Exotoxins are thermostable and survive even after heat treatment of food. Of particular importance is the ability of exotoxins to act as a super antigen, and they are thought to be the cause of autoimmune diseases. Bacterial toxins can be detoxified with formalin, iodine, pepsin, ascorbic acid, ketones and the like. After detoxification, they retain their immunogenicity and vaccines can be made. Also, some exotoxins can be used to treat neoplasms.

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## **PROBABLE HEALTH RISK ASSESSMENT OF ESSENTIAL ELEMENTS IN WATER OF NATURAL – DAM LAKES OF THRACE REGION (TÜRKIYE)**

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### **ABSTRACT**

The aim of this research was to determine the boron, selenium and manganese accumulations in water of natural – dam lakes located in the Thrace Region of Türkiye and to assess the non-carcinogenic health risks of these elements via daily human intake. 13 stagnant water bodies were selected in the region and water samples were collected during the dry season of 2020 (summer). The element levels were measured by using an ICP-MS device and Estimated Daily Intake (EDI) and Hazard Quotient (HQ) of boron, selenium and manganese were calculated separately. Boron concentrations were varied from 291.084 – 322.599 ppb for the natural lakes and 3.063 – 287.241 ppb for the reservoirs; selenium concentrations were varied from 0.704 – 0.932 ppb for the natural lakes and 0.067 – 1.291 ppb for the reservoirs; and manganese concentrations were varied from 5.990 – 2652.970 ppb for the natural lakes and 0.214 – 48.440 ppb for the reservoirs. All the calculated HQ values in all the investigated locations in terms of all the investigated age groups were recorded as less than the critical limit of 1.

**Keywords:** Thrace Region, Natural – dam lakes, Boron, Selenium, Manganese, Health risk assessment

### **INTRODUCTION**

Boron is a widely occurring element in minerals found in the earth's crust. It is the 51st most common element found in the earth's crust and is found at an average concentration of 8 mg/kg (approximately 0.0008%). Selenium and manganese, which are commonly found in rocks and soil, are naturally occurring elements. They are all trace elements and essential nutrients for humans and necessary for good health. However, they can be harmful when regularly taken in amounts higher than those needed for good health. Significant amounts of boron can be released from agricultural fertilizer and pesticide usage. Also, manganese is known as being used in a wide variety of products including fertilizer. However, the principal release of selenium into the environment from anthropogenic sources is reported as coal combustion (ATSDR, 2003; 2010; 2012).

Thrace Region a major agricultural zone of Türkiye is located in the north – west part of Marmara Region. The region has a great agricultural potential due to its fertile clayey soil. Therefore, intensive agricultural activities in the region have a significant impact on freshwater and soil quality. There are 2 significant natural lakes and 11 significant dam lakes in the region. Drinking waters of regional cities and many districts are obtained from dam lakes constructed in the region (Anonymous, 2018; Onur and Tokatlı, 2020; Tokatlı and Ustaoglu, 2020; Tokatlı and Varol, 2021a; Tokatlı, 2021).



Health risk assessment techniques are being widely used all over the world in especially recent years. They are very useful and effective tools in estimating carcinogenic and non-carcinogenic hazards, which may occur when people are exposed to certain toxicants (Ustaoğlu and Aydın, 2020; Tokatlı and Ustaoğlu, 2020; Tokatlı and Varol, 2021a; 2021b; Varol and Tokatlı, 2022).

The aim of this research was to determine the levels of boron, selenium and manganese in water of 2 natural lakes and 11 dam lakes located in the Thrace Region and to evaluate the non-carcinogenic health risks of these essential elements via daily human intake.

## MATERIALS AND METHODS

### Water Collection

In this research, total of 13 stagnant water bodies (2 natural lakes and 11 dam lakes) located in the Meriç – Ergene River Basin in the Thrace Region were selected. Coordinate information and station codes are given in Table 1. The topographic map of Meriç – Ergene River Basin and selected sampling stations are given in Figure 1. Water samples were collected in the summer of 2020 (dry season) with a telescopic water sampling device approximately 3 meters from the shore into the pre – cleaned polyethylene bottles.

Table 1. Coordinate information of selected stagnant water bodies

| Name of Habitat | Station Code | GPS – North | GPS – East |
|-----------------|--------------|-------------|------------|
| Gala            | L1           | 40.765      | 26.165     |
| Pamuklu         | L2           | 40.793      | 26.276     |
| Kırklareli      | R1           | 41.740      | 27.277     |
| Kayalıköy       | R2           | 41.789      | 27.135     |
| Süloğlu         | R3           | 41.790      | 26.918     |
| Çakmak          | R4           | 41.378      | 26.683     |
| Çerkezmüsellim  | R5           | 41.257      | 27.038     |
| Karaidemir      | R6           | 40.955      | 27.008     |
| Kadıköy         | R7           | 40.794      | 26.773     |
| Altinyazı       | R8           | 41.081      | 26.588     |
| Sultanköy       | R9           | 41.037      | 26.479     |
| Sığircı         | R10          | 40.817      | 26.314     |
| Hamzadere       | R11          | 40.793      | 26.394     |

### Element Analysis

pH values of water samples were decreased to 2 by adding about 2 ml of nitric acid per 1 L into each. The samples were filtered by using a 0.45 µm – cellulose nitrate filter. The volumes of samples were made up to 50 ml with ultrapure water. In the acidic – filtered water samples, a total of 3 metal(loid)s (B, Se and Mn) were determined with an inductively coupled plasma – mass spectrometry (ICP – MS) in the laboratory of Technology Research Development Application and Research Centre of Trakya University – an international accreditation certificated institution. All the element analyses were determined as means of triplicate reads (TS EN / ISO IEC 17025) (EPA, 2001; APHA, 2005). The accuracy of analytical method was controlled by using a certified reference material (CRM) (CPAchem – Ref Num: 110580.L1).

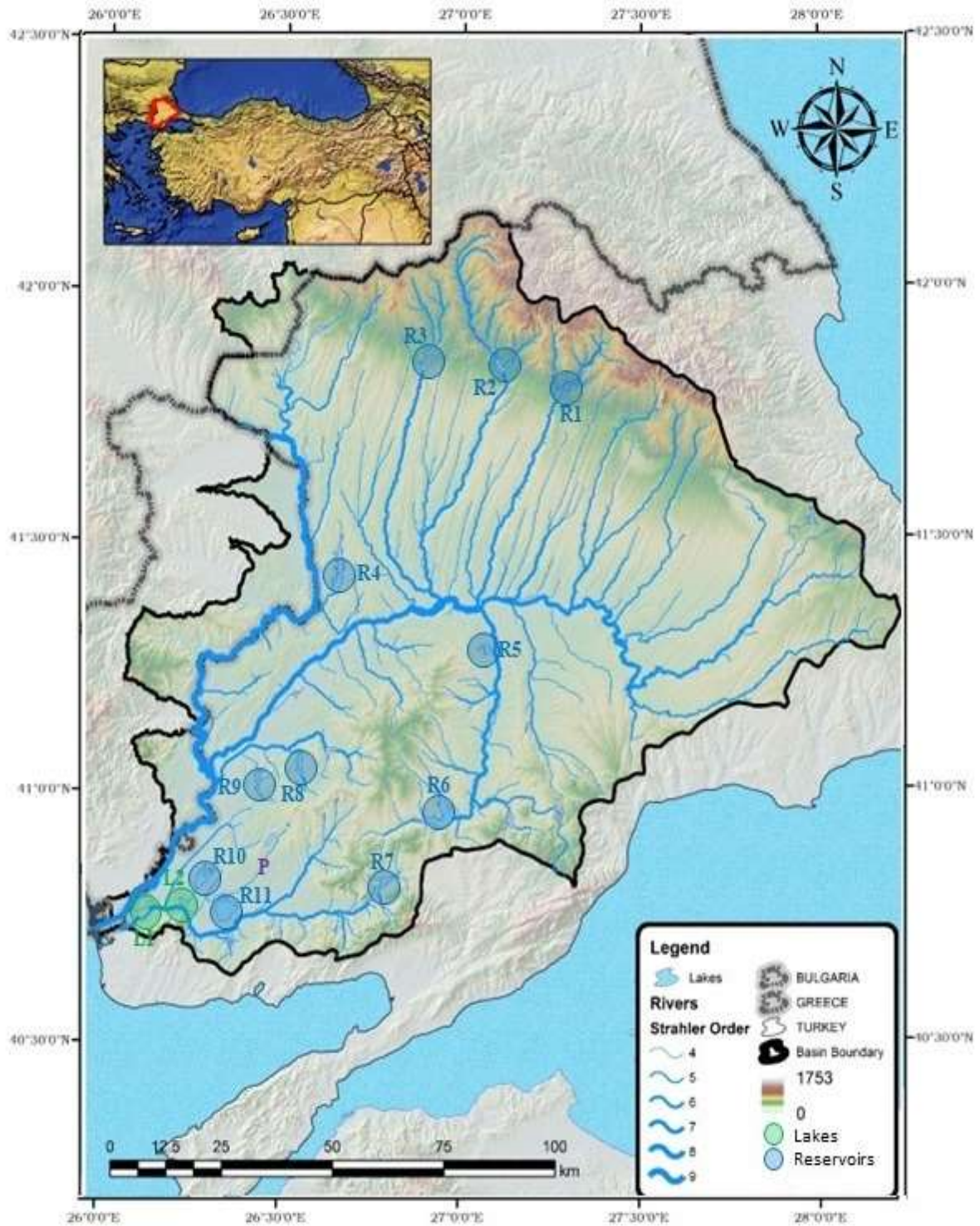


Figure 1. Topographic map of Meriç – Ergene River Basin and selected lentic habitats

### Health Risk Assessment

In the current research, population was divided into four age groups based on their physiological and behavioural differences as follow: infants (< 2 years old), children (2 – 6 years old), teenagers (6 – 16 years old) and adults (> 16 years old). The daily exposure to boron, selenium and manganese were calculated in these four different groups by using the equation 1. Hazard Quotient (HQ) describes the non-carcinogenic risk of b, Se and Mn and it was calculated by using the equation 2 (Liu et al., 2018; Zango et al., 2019; Ghosh et al., 2020). A lower value of HQ than one reflects a negligible risk of non-carcinogenic effects and a higher value of HQ than one reflects an important health risk.

$$EDI = \frac{C_{sample} \times C_d}{B_w} \quad (1)$$

$$HQ = \frac{EDI}{RfD} \quad (2)$$

EDI: Estimation of fluoride consumption – daily (mg/kg/day)

C<sub>sample</sub>: B, Se and Mn levels in the investigated water samples (mg/L)

C<sub>d</sub>: Mean daily water intake (L/day) (the mean water consumption rates in infants, children, teenagers and adults were 0.1, 0.85, 2 and 2.5 L/day, respectively)

B<sub>w</sub>: Body weight (kg) (body weights of investigated groups were considered 5, 10, 50 and 75 kg, respectively)

RfD: Reference dose (mg/kg/day) (it is 0.13, 0.005 and 0.14 mg/kg/day for B, Se and Mn respectively) (EPA, 1992)

### RESULTS AND DISCUSSION

Boron, selenium and manganese accumulations in water of investigated natural and dam lakes located in the Thrace Region are given in Figure 2 and detected EDI and HQ coefficients in investigated stagnant water bodies are given in Table 2.

Boron concentrations were varied from 291.084 – 322.599 ppb with an average of 306.841 ppb for the natural lakes and 3.063 – 287.241 ppb with an average of 55.338 ppb for the reservoirs; selenium concentrations were varied from 0.704 – 0.932 ppm with an average of 0.818 ppb for the natural lakes and 0.067 – 1.291 ppm with an average of 0.554 ppb for the reservoirs; and manganese concentrations were varied from 5.990 – 2652.970 ppm with an average of 1329.480 ppb for the natural lakes and 0.214 – 48.440 ppm with an average of 7.708 ppb for the reservoirs.

All the calculated HQ values in all the investigated locations in terms of all the investigated age groups were recorded as less than the critical limit of 1 with a spatial mean of 0.01447, 0.00238 and 0.03015 for infants; 0.06148, 0.01011 and 0.12814 for children; 0.02893, 0.00476 and 0.06030 for teenagers; and 0.02411, 0.00397 and 0.05025 for adults in terms of boron, selenium and manganese respectively.

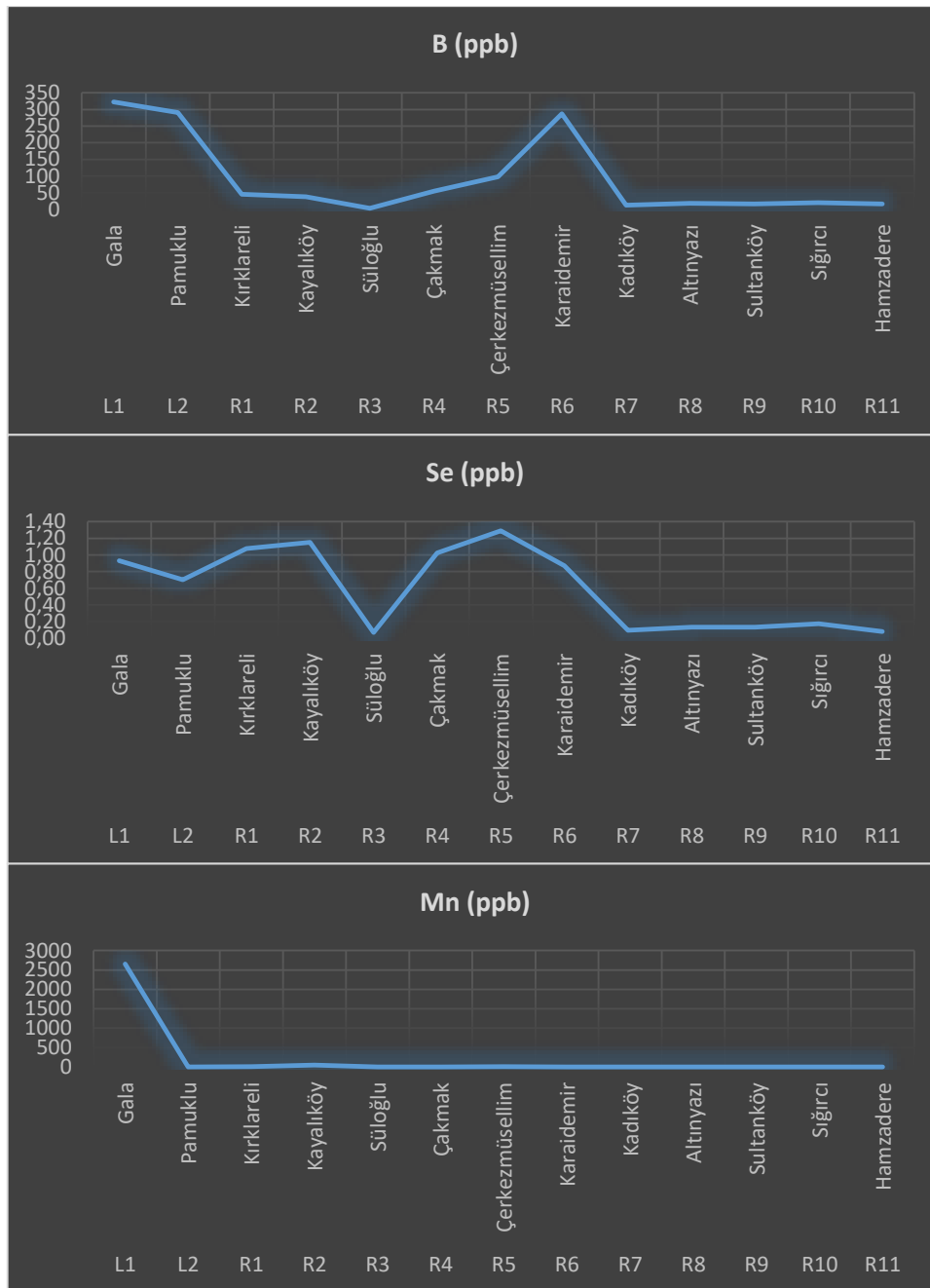


Figure 2. Boron (up), selenium (middle) and manganese (down) levels in water samples

The EDI and HQ risk rankings among the investigated different age groups were found as follows: children > teenagers > adults > infants in all the investigated stagnant water bodies including natural and dam lakes, in general. In many of researches on the assessment of probable health risks associated with boron, selenium and manganese in water performed in many different countries confirmed that the probable chronic health risks in children due to the intake of these elements are more possible than the other age groups (Liu et al., 2018; Zango et al., 2019; Ghosh et al., 2020). In this research, as similar with the literature data, children were found as the riskiest age group for the probable health risk due to the intake of boron, selenium and manganese in water of natural – dam lakes of Meriç – Ege River Basin.

Table 2. EDI and HQ values

|           |         | EDI     |          |           |         | HQ      |          |           |         |
|-----------|---------|---------|----------|-----------|---------|---------|----------|-----------|---------|
|           |         | Infants | Children | Teenagers | Adults  | Infants | Children | Teenagers | Adults  |
| Boron     | L1      | 0.00645 | 0.02742  | 0.01290   | 0.01075 | 0.04963 | 0.21093  | 0.09926   | 0.08272 |
|           | L2      | 0.00582 | 0.02474  | 0.01164   | 0.00970 | 0.04478 | 0.19032  | 0.08956   | 0.07464 |
|           | R1      | 0.00091 | 0.00388  | 0.00183   | 0.00152 | 0.00703 | 0.02987  | 0.01406   | 0.01171 |
|           | R2      | 0.00075 | 0.00320  | 0.00151   | 0.00126 | 0.00580 | 0.02465  | 0.01160   | 0.00967 |
|           | R3      | 0.00006 | 0.00026  | 0.00012   | 0.00010 | 0.00047 | 0.00200  | 0.00094   | 0.00079 |
|           | R4      | 0.00109 | 0.00463  | 0.00218   | 0.00182 | 0.00838 | 0.03564  | 0.01677   | 0.01397 |
|           | R5      | 0.00196 | 0.00832  | 0.00391   | 0.00326 | 0.01506 | 0.06399  | 0.03011   | 0.02509 |
|           | R6      | 0.00574 | 0.02442  | 0.01149   | 0.00957 | 0.04419 | 0.18781  | 0.08838   | 0.07365 |
|           | R7      | 0.00024 | 0.00103  | 0.00048   | 0.00040 | 0.00186 | 0.00791  | 0.00372   | 0.00310 |
|           | R8      | 0.00036 | 0.00154  | 0.00072   | 0.00060 | 0.00278 | 0.01182  | 0.00556   | 0.00464 |
|           | R9      | 0.00032 | 0.00137  | 0.00065   | 0.00054 | 0.00248 | 0.01056  | 0.00497   | 0.00414 |
| R10       | 0.00040 | 0.00170 | 0.00080  | 0.00067   | 0.00308 | 0.01310 | 0.00617  | 0.00514   |         |
| R11       | 0.00033 | 0.00139 | 0.00065  | 0.00054   | 0.00251 | 0.01065 | 0.00501  | 0.00418   |         |
| Selenium  | L1      | 0.00002 | 0.00008  | 0.00004   | 0.00003 | 0.00373 | 0.01585  | 0.00746   | 0.00622 |
|           | L2      | 0.00001 | 0.00006  | 0.00003   | 0.00002 | 0.00281 | 0.01196  | 0.00563   | 0.00469 |
|           | R1      | 0.00002 | 0.00009  | 0.00004   | 0.00004 | 0.00431 | 0.01832  | 0.00862   | 0.00718 |
|           | R2      | 0.00002 | 0.00010  | 0.00005   | 0.00004 | 0.00461 | 0.01958  | 0.00921   | 0.00768 |
|           | R3      | 0.00000 | 0.00001  | 0.00000   | 0.00000 | 0.00027 | 0.00114  | 0.00054   | 0.00045 |
|           | R4      | 0.00002 | 0.00009  | 0.00004   | 0.00003 | 0.00409 | 0.01740  | 0.00819   | 0.00682 |
|           | R5      | 0.00003 | 0.00011  | 0.00005   | 0.00004 | 0.00516 | 0.02195  | 0.01033   | 0.00861 |
|           | R6      | 0.00002 | 0.00007  | 0.00003   | 0.00003 | 0.00348 | 0.01481  | 0.00697   | 0.00581 |
|           | R7      | 0.00000 | 0.00001  | 0.00000   | 0.00000 | 0.00038 | 0.00160  | 0.00075   | 0.00063 |
|           | R8      | 0.00000 | 0.00001  | 0.00001   | 0.00000 | 0.00054 | 0.00228  | 0.00107   | 0.00089 |
|           | R9      | 0.00000 | 0.00001  | 0.00001   | 0.00000 | 0.00054 | 0.00228  | 0.00107   | 0.00089 |
| R10       | 0.00000 | 0.00001 | 0.00001  | 0.00001   | 0.00070 | 0.00296 | 0.00139  | 0.00116   |         |
| R11       | 0.00000 | 0.00001 | 0.00000  | 0.00000   | 0.00032 | 0.00137 | 0.00064  | 0.00054   |         |
| Manganese | L1      | 0.05306 | 0.22550  | 0.10612   | 0.08843 | 0.37900 | 1.61073  | 0.75799   | 0.63166 |
|           | L2      | 0.00012 | 0.00051  | 0.00024   | 0.00020 | 0.00086 | 0.00364  | 0.00171   | 0.00143 |
|           | R1      | 0.00025 | 0.00104  | 0.00049   | 0.00041 | 0.00175 | 0.00745  | 0.00351   | 0.00292 |
|           | R2      | 0.00097 | 0.00412  | 0.00194   | 0.00161 | 0.00692 | 0.02941  | 0.01384   | 0.01153 |
|           | R3      | 0.00002 | 0.00009  | 0.00004   | 0.00004 | 0.00016 | 0.00068  | 0.00032   | 0.00027 |
|           | R4      | 0.00008 | 0.00033  | 0.00016   | 0.00013 | 0.00055 | 0.00236  | 0.00111   | 0.00092 |
|           | R5      | 0.00014 | 0.00058  | 0.00027   | 0.00023 | 0.00098 | 0.00414  | 0.00195   | 0.00163 |
|           | R6      | 0.00007 | 0.00029  | 0.00014   | 0.00011 | 0.00049 | 0.00208  | 0.00098   | 0.00082 |
|           | R7      | 0.00001 | 0.00005  | 0.00002   | 0.00002 | 0.00009 | 0.00037  | 0.00017   | 0.00014 |
|           | R8      | 0.00006 | 0.00024  | 0.00011   | 0.00009 | 0.00041 | 0.00172  | 0.00081   | 0.00068 |
|           | R9      | 0.00001 | 0.00004  | 0.00002   | 0.00001 | 0.00006 | 0.00025  | 0.00012   | 0.00010 |
| R10       | 0.00010 | 0.00040 | 0.00019  | 0.00016   | 0.00068 | 0.00289 | 0.00136  | 0.00113   |         |
| R11       | 0.00000 | 0.00002 | 0.00001  | 0.00001   | 0.00003 | 0.00013 | 0.00006  | 0.00005   |         |

## CONCLUSIONS

In the current research, spatial variations of boron, selenium and manganese concentrations and the probable non-carcinogenic health risks of these elements via daily human intake in water of 2 natural lakes and 11 dam lakes located in the Meriç – Ergene River Basin in the Thrace Region of Türkiye were investigated. As a result of this study, the detected HQ values of all age groups were found as less than the critical limit of 1 and the risk rankings among the investigated different age groups were found as follows: children > teenagers > adults > infants, in general.

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## ASSOCIATION OF DIET WITH THE OCCURRENCE OF TYPE 2 DIABETES, POLYCYSTIC OVARY SYNDROME, VAGINAL TRACT DYSBIOSIS AND NEOPLASTIC DISEASES IN WOMEN AND CARNIVORES

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### ABSTRACT

Improper diet causes hyperglycemia, which leads to hyperstimulation of the pancreas, which secretes insulin in large quantities, causing hyperstimulation of the ovaries, which leads to polycystic ovary syndrome in women. Hyperproduction of androgens and estrogens is one of the risk factors for the development of neoplastic diseases of the reproductive tract in women. The end result is insulin resistance and type 2 diabetes. Carnivorous females are naturally insulin resistant. In carnivores of reproductive age, polycystic ovary syndrome and reproductive tract cancer are rare. They occur in the generative age, which can be related to the exclusively meat diet of carnivores, which results in a large consumption of anticancer trypsin, which is used to denature meat proteins. The purpose of this paper is to point out the importance of a balanced diet in women that can prevent type 2 diabetes, improve reproductive health, maintain a healthy genital microbiota and to some extent prevent neoplastic diseases of the reproductive tract. A balanced diet improves reproductive health, and intravaginal administration of lactobacilli prevents and treats recurrent and resistant vaginosis and vaginal dysbiosis in women.

**Key words:** Nutrition, type 2 diabetes, polycystic ovary syndrome, neoplastic diseases of the reproductive tract, lactobacilli

### INTRODUCTION

The cause of the Polycystic Ovary Syndrome is multiple, but the main reasons for its occurrence in many literatures are genetic etiological factors (together with the appearance of diabetes), consequent hormonal imbalance, unbalanced diet, obesity, various etiologies of occurrence (Bednarska & Siejka, 2017; Djedjibegovic *et al.*, 2020). Proper nutrition with physical activity contributes to health and is the basis for the treatment of diabetes and Polycystic Ovary Syndrome in accordance with international guidelines, although in Bosnia and Herzegovina the profession of dietitian is not legally recognized, prevention is carried out

by medical experts (Djedjibegovic *et al.*, 2020). Diabetes is a disease that manifests itself in various clinical symptoms and damage to many organ systems caused by an increase in blood glucose. Type 2 diabetes (insulin-independent type) is associated with excessive consumption of foods rich in fats and carbohydrates, unbalanced diet, consequent reduction of probiotic flora, reduced physical activity and genetic predisposition to get this type of disease (Otto-Buczowska *et al.*, 2018; Barrea *et al.*, 2019; Osibogun *et al.*, 2020). In humans, glucose is a source of energy for cells, tissues and organs. If there is no insulin production, the body draws energy from the fat depot and depletes proteins, in this regard, the body is in crisis (Pathak, 2014). In the phase of hyperinsulinemia, when the body struggles with an excessive influx of refined carbohydrates and fats, there is also an overproduction of hormones that cause polycystic ovaries in women (Paoli *et al.*, 2020; Osibogun *et al.*, 2020). Also, an unbalanced diet in women reduces the number of probiotics in the genital tract which has the effect of increasing the incidence of reproductive tract cancer (Nené *et al.*, 2019). Carnivores get their energy from animal fats, fatty tissue depots, and partly from deposited liver glycogen. Carnivores are naturally insulin resistant, which is considered a natural evolutionary adaptation to diet (Schermerhorn, 2013). Carnivores of reproductive age also have a diverse vaginal flora (Maksimović *et al.*, 2012). In healthy women, the amount of lactobacilli is proportional to a woman's reproductive health (Kovachev, 2018).

## THE ROLE OF PANCRAS

The pancreas is a gland with endocrine and exocrine secretion. Endocrine function of the pancreas is the secretion of hormones in the blood, the opposite effect: Glucagon, whose function is to break down glycogen, or the breakdown of "stored passive glucose" in the liver and activation of stored glucose (glycogen) in terms of releasing glucose into the bloodstream energy of all cells, tissues and organs (Abdalla *et al.*, 2021). Then the secretion of insulin whose function is to increase the entry of glucose into the cell, tissues, organs, in order to supply the energy necessary for life (Atkinson *et al.*, 2020). The exocrine part of the pancreas secretes the juices needed to process food in the small intestine, which mixes with salts of bile acids from the liver (Campbell-Thompson *et al.*, 2015). The basic ingredients of pancreatic juice are digestive enzymes, the most important of which are trypsin, chymotrypsin, lipase and amylase. It also secretes phospholipase, elastase and ribonuclease (Yuan *et al.*, 2021). Some authors believe that type 1 diabetes (complete cessation of insulin production) is associated with diseases of the endocrine, but also exocrine pancreas, which is often neglected. It is clinically manifested by mass loss of pancreatic  $\beta$ -cells (Campbell-Thompson *et al.*, 2015). Hyperfunction of the pancreas causes increased secretion of hormones, which results in increased stimulation of the organs of action and the possible occurrence of neoplastic diseases in synergy with other etiological agents (Wittenberg *et al.*, 2016). There is also a special type of diabetes associated with exocrine pancreatic disease (Radlinger *et al.*, 2020). In diagnosis, it is often misdiagnosed as type 2 diabetes. This type of diabetes requires a rapid transition to injectable, insulin treatment (Wynne *et al.*, 2019).



## **RELATIONSHIP OF DIET WITH TYPE 2 DIABETES IN HUMANS**

Type 2 diabetes is a modern disease and the ninth cause of death in the world, and the onset of the disease is related to lifestyle (lack of physical activity, smoking), unbalanced diet, and intestinal dysbiosis (Zheng *et al.*, 2015). Prevention of type 2 diabetes is actually its best treatment. It is possible to prevent type 2 diabetes with appropriate physical activity and proper nutrition. It is necessary to find a balance in the diet and that the basic diet should be based on fruits and vegetables that are rich in fiber, healthy simple sugars bound to fibers, complex carbohydrates or polysaccharides with the addition of healthy sources of protein from legumes, nuts, white meat, chicken, turkey, blue fish, low-fat dairy products, and avoiding saturated fats (Ley *et al.*, 2014). Excellent dietary regimens for the prevention of type 2 diabetes are Mediterranean diet, vegetarian diet, preference for foods with low glycemic index and low level of carbohydrates (Table 1) (Ley *et al.*, 2014). What should be avoided when it comes to protein sources are refined grains, red meat, meat products, drinks with sugar sweeteners, because they increase the risk of type 2 diabetes (Ley *et al.*, 2014; Rahati *et al.*, 2014; McMacken & Shah, 2017). Carbohydrates are the main source of energy needed by the body, which from food are broken down into glucose that enters the bloodstream, but depending on the length of the process of breaking down carbohydrates in the blood, we have a normal level of glucose or a sudden increase in the level of glucose, so in the prevention of type 2 diabetes, you should choose and foods with a low glycemic index (Wang *et al.*, 2015; Sami *et al.*, 2017). Well-known foods with a low glycemic index are avocados, olives, nuts, soybeans, sauerkraut, while those with a high glycemic index are potatoes, rice, honey, various glucose syrups, etc. (Viguiliouk *et al.*, 2014; Bozzetto *et al.*, 2016; Tabeshpour *et al.*, 2017). Glucose is the main source of energy for all cells in the body and the body needs it as fuel, but if we have it in excess, it turns into fat, which is not the case with the simple sugar fructose from fruits and vegetables (Qi & Tester, 2019). The excess glucose created is stored for future needs, so over time weight gain and the appearance of type 2 diabetes occur. Type 2 diabetes occurs because excess fat accumulates in the intercellular space, around the blood vessels, and in order for glucose to enter the cell, insulin is needed. as a carrier, and that's why with excessive intake of unhealthy simple sugars, we have the appearance of hyperinsulinemia, which is not the case with simple sugars from fruits and vegetables, because fructose diffuses through the cell wall without an insulin carrier (Qi & Tester, 2019). To prevent type 2 diabetes, it is necessary to reduce the intake of unhealthy simple sugars as much as possible, and increase the intake of healthy simple sugars, then complex polysaccharides which take much longer to break down into glucose, we remember being full longer, that glucose enters the bloodstream more slowly per unit of time and we do not have a sudden increase in glucose as is the case with unhealthy simple sugars from various sweets, glucose drinks that can only be used in hypoglycemia when we want a sudden increase in glucose without high energy consumption, but in general they are unhealthy and should be avoided just like white bread and various starchy foods whose excess is also converted into fat (Imamura *et al.*, 2015). White bread should be replaced with wholemeal bread, which is not harmful even for diabetics (Ley *et al.*, 2014). If we want to suddenly increase blood glucose, it is best to reach for healthier variants of simple sugars from fruits, vegetables and grains, which are an excellent source of magnesium, which in some studies has been shown

to be good in the prevention of type 2 diabetes (Rahati *et al.*, 2014). In the prevention of type 2 diabetes, in addition to consuming healthy foods and a balanced diet, it is necessary to take care of the amount of foods that increase blood sugar levels. It is necessary to consume food with a low fat content, reduce the consumption of carbohydrates, and increase food rich in fiber (Sami *et al.*, 2017).

Mediterranean and vegetarian diets reduce inflammation in the body (Ley *et al.*, 2014). Increasing inflammation in the body leads to blood clotting and various chronic diseases. The Mediterranean diet prevents and treats many chronic diseases, including type 2 diabetes, and prevents complications in the form of cardiovascular disorders, which cause death in 50% of diabetics (Sami *et al.*, 2017; Galicia-Garcia *et al.*, 2020). Physical activity with proper nutrition is important, because it reduces the accumulation of fat around the stomach, which is associated with an increased risk of insulin resistance, which is one of the prediabetic stages (Sami *et al.*, 2017).

### **Epidemiology of type 2 diabetes**

Type 2 diabetes, if we look at global reports, is the leading disease in the world, and the leading cause of death in Asian countries, and the most affected are China and India, which is related to the traditional way of eating (Zheng *et al.*, 2015; Sami *et al.*, 2017). Rates of increase in the prevalence of type 2 diabetes have been recorded in developed countries, in Western Europe, but the increase in underdeveloped countries is particularly worrying, and the search for prevention measures is very important (Khan *et al.*, 2020). Based on reporting from 2011, it was estimated that 8.3 % of people worldwide have type 2 diabetes, and this is expected to increase to 9.9 % in 2030. In Saudi Arabia, a high prevalence of type 2 diabetes is noticeable, especially because Saudis too often consume various sweet drinks (Sami *et al.*, 2017). Death occurs as a result of cardiovascular complications where the risk of thromboembolism is quite high due to increased blood coagulability (Galicia-Garcia *et al.*, 2020).

**Table 1.** Mediterranean diet in the prevention of type 2 diabetes

| Type of food  | Mediterranean diet, type of food, macronutrients and benefits          |  |   |
|---|--|--|---|
|   | Macronutrients   | Benefits   | Source  |
| Olive oil<br>(extra virgin)   | The main source of fat in the form of monosaturated fatty acids.       | Polyphenols from olive oil have an anti-inflammatory, antioxidant effect and are a prebiotic (food) for bifidobacteria from the intestine, preventing dysbiosis that is associated with type 2 diabetes. Olive oil also improves the sensitivity of cells to insulin.  | Hidalgo-Mora <i>et al.</i> (2020)<br>Mirabelli <i>et al.</i> (2020)<br>Ley <i>et al.</i> 2014 |
| Fruits and vegetables   | Main source of fiber and complex carbohydrates, minerals and vitamins. | Fruits and vegetables are also rich in polyphenols. Purple fruits and vegetables are also rich in anthocyanins, and grapes and resveratrol, and animal studies show that they increase the sensitivity of cells to insulin. Berries, dates, grapes, oranges, peppers and broccoli are particularly good antioxidants, and green vegetables are a particularly good source of fiber.  | Calcaterra <i>et al.</i> (2021)   |
| Low-fat dairy products  | Main source of protein.  |  | Calcaterra <i>et al.</i> (2021)   |
| Whole grains  | A major source of protein and fiber.                                   | In addition to being a good source of protein, whole grains are also a good source of fiber that feeds probiotic bacteria in the intestines and lowers blood glucose. Barley is rich in fiber, oats are an excellent source of soluble fiber, flax is also, and they also reduce blood glucose.  |   |
| Fish, salmon, tuna and poultry/week   | Source of protein.   |  |   |
| Red meat/monthly  | Source of protein.   |  |   |
| High intake of dietary fiber from white beans, cereals, especially barley, oats, magnesium, varying intake of macronutrients. |  | A keto diet with 30% of energy from protein and fat contributes to weight loss and prevention of type 2 diabetes. It primarily reduces hyperinsulinemia, the prediabetic phase and has a beneficial effect on the prevention of polycystic ovary syndrome.<br><br>The high proportion of dietary fiber from white beans and cereals is rich in polyphenols. Minerals, especially selenium, zinc, magnesium, calcium, as well as vitamins, especially vitamin D, are considered to have a preventive effect on the occurrence of type 2 diabetes and polycystic ovary syndrome. |   |

## **MECHANISM OF POLYCYSTIC OVARIAN SYNDROME IN CORRELATION WITH TYPE 2 DIABETES (related to excessive consumption of carbohydrates and fats)**

### **Incidence of Polycystic Ovary Syndrome in female and female carnivores**

Polycystic Ovary Syndrome (PCOS) is a common occurrence in women with diabetes and hormonal imbalance (Nikokavoura *et al.*, 2015; Dabravolski *et al.*, 2021).

Diabetes is not a disease that occurs in nature. Diabetes in carnivores occurs in obese, inactive pets that consume large amounts of food, which does not happen in nature. In the case of feline disease, type 2 diabetes occurs in humans (Nelson & Reusch, 2014). The occurrence of PCOS in carnivores is rare. Polycystic ovary syndrome in bitches can be associated with previous corticosteroid use in the treatment of certain conditions (Ghasemzadeh-nava & Malmasi, 2020).

Neoplastic diseases of the reproductive tract of carnivores often occur in the generative age of unsterilized individuals (Salazar & Perales, 2017). Type 1 diabetes (insulin dependent type) - the pancreas produces little or no insulin. It is a primary autoimmune disease of the B cells of the pancreas. The disease is sudden associated with young age and occurs in 15% of cases in humans (Chellappan *et al.*, 2018).

Dogs do not develop type 2 diabetes, but similar forms of type 1 diabetes occur and require the urgent use of insulin injections (Nelson & Reusch, 2014; Gilor *et al.*, 2016).

Also, in female carnivores, bitches develop gestational diabetes, which is very similar to gestational diabetes in women (Niaz *et al.*, 2018).

### **Type 2 diabetes and insulin resistance**

Type 2 diabetes (non-insulin dependent type) - the mechanism of development is closely related to the occurrence of insulin resistance. Insulin resistance represents the resistance of target tissues to the pancreatic hormone insulin (Polak *et al.*, 2017).

Excessive use of fats and carbohydrates has been linked to hyperglycemia and type 2 diabetes, which has been known since ancient times, but each patient requires a special approach and today the etiology is being investigated (Cena *et al.*, 2020; Reusch, 2011).

Hyperglycemia causes hyperstimulation of the pancreas (hyperinsulinemia). Hyperinsulinemia enhances the work of the organs of action (Rosenfield & Ehrmann, 2016).

Ovarian hypertimulation leads to polycystic ovary syndrome (Baptiste *et al.*, 2010; Zhang *et al.*, 2019). Over time, insulin resistance occurs, tissue resistance to insulin: "*Cells swim in sugar, and stay hungry.*" The end result of hyperinsulinemia leads to pancreatic damage, resulting in hypoinsulinemia or complete cessation of insulin secretion (Legro, 2012).

### **Gestational type of diabetes**

Gestational type diabetes occurs during pregnancy (Alejandro *et al.*, 2020). Gestational diabetes is actually one of the complications of pregnancy that occurs due to improper diet, ie

excessive consumption of refined sugars and fats during pregnancy with a lack of adequate physical activity. This type of diabetes is treated with the help of insulin only if the therapy with a proper and balanced diet with physical activity does not give results. A balanced diet with enough physical activity can prevent gestational diabetes and can prevent all the consequences that this disease brings with it and which can be dangerous for both mother and fetus, because they increase the risk of various metabolic, hormonal, cardiovascular and other disorders (McIntyre *et al.*, 2019).

### **PROBLEMS OF POLYCYSTIC OVARY SYNDROME**

Polycystic Ovary Syndrome (PCOS) is directly related to excessive intake of foods rich in carbohydrates saturated fats, trans fats (Szczuko *et al.*, 2021).

Polycystic Ovary Syndrome, as well as improper diet, along with other risk factors, can contribute to the development of neoplastic diseases of the reproductive tract in women (Soave *et al.*, 2020).

Carnivores are more resistant to consuming large amounts of saturated fat from meat, which they deposit in the form of adipose tissue, partly glycogen and used as an energy source (Schermerhorn, 2013).

The occurrence of obesity in women is closely related to the occurrence of type 2 diabetes, but the occurrence of obesity in female carnivores is also related to the occurrence of type 1 diabetes (Schermerhorn, 2013; Forslund *et al.*, 2020).

In nature, the disease rarely occurs. The occurrence of type 2 diabetes, as well as PCOS in female carnivores, is rare, which proves the connection between PCOS and excessive carbohydrate consumption (Osibogun *et al.*, 2020). Since carnivores have the ability to store large amounts of fat, the use of large amounts of saturated fat as a factor in the development of type 1 diabetes is not excluded, since according to many authors, type 1 diabetes is closely related to obese carnivores (Schermerhorn, 2013).

The incidence of reproductive tract cancer in female carnivores is rare, proving the causal relationship of ovarian tumors with PCOS and hormonal imbalance. The frequency of oncological diseases in female carnivores of childbearing age is evident, which indicates that meat consumption carries with it the risk of developing tumorous diseases. Trypsin is a pancreatic enzyme whose main role is in protein denaturation, but it also has anticancer properties (Bhatia & Bhatia, 2018). The influx of large amounts of protein in the normal diet of carnivores, uses large amounts of the enzyme trypsin, the percentage of which to protect cells from cancer decreases in a unit of time. The thesis can be explained by the frequency of occurrence of tumorous diseases in carnivores aged 5 to 10 years, regardless of the occurrence of type 1 diabetes, due to the natural diet (Ghasemzadeh-nava & Malmasi, 2020).

### **INFLUENCE OF NUTRITION ON THE INTESTINAL MICROBIOTA**

Depending on the type of food we eat in the microbiological composition of the intestinal microbiota we will have more pathogenic bacteria or more good probiotic bacteria that protect

the body from overgrowth of pathogenic bacteria through bacteriocins (bactericides, fungicides, virucides) released in their constant fight against various microorganisms create dominance and lead to infection, various diseases. The food we eat should act as a prebiotic, ie food with probiotics that will strengthen and multiply with the help of such food in order to be in greater numbers than pathogenic microorganisms and to win in this constant struggle in the intestines for dominance. Foods that have a positive effect on the pH of the gut and act as a prebiotic are plant foods that are rich in fiber, and foods that act as a probiotic contain probiotic cultures, which are usually fermented products. Probiotic bacteria in the intestines release free fatty acids, maintain optimal pH and thus prevent colonization by pathogens. Also, it is very important that everything we eat and absorb from food, that the body uses all the nutrients, nutrients from food, and this will only happen if we have a healthy microbiota that is very sensitive to change. The use of antibiotics, stress, poor diet, very easily changes the composition of the microbiota and pathogenic microorganisms predominate. Probiotic culture is responsible for maintaining homeostasis in the gut, for reducing pathogens, neutralizing toxins, metabolic products and deactivating carcinogenic substances (Hrustemović *et al.*, 2021).

In recent times, the intestinal microbiota has been called the endocrine system by many authors, because it greatly influences hormonal status, so it also plays a role in the development of Polycystic Ovary Syndrome and neoplastic diseases (Kawa *et al.*, 2021; Qi *et al.*, 2021).

Food affects the composition of the intestinal microbiota, and Polycystic Ovary Syndromes are often present in women and neoplastic diseases are possible in older and, more recently, younger, but the mechanisms are still poorly understood. What has been investigated is that improper diet causes hyperglycemia leading to hyperinsulinemia acting on the ovaries that stimulate androgens and estrogen and all this can be linked to the development of Polycystic Ovary Syndrome and neoplastic diseases (Kawa *et al.*, 2021; Qi *et al.*, 2021).

Many more studies are needed on the mechanism of these diseases that is associated with disturbed intestinal microbiota, which will contribute to better, more effective prophylaxis of diseases that are very dangerous for the health of the reproductive tract of women.

#### **FOOD THAT INCREASES THE NUMBER OF *LACTOBACILLUS* SPECIES IN WOMEN'S VAGINAL TRACT**

Food that naturally contains probiotic cultures and prebiotics (food for probiotics) is fermented food, which is: yogurt, kefir, various other fermented milk products: cheese, cream, butter, mileram, then various sour salads (Terzić-Vidojević *et al.*, 2020).

In addition to probiotics, less researched prebiotics are used to protect our natural microbiota (Lavefve *et al.*, 2019). Prebiotics are used in the diet of probiotics and thus our probiotic culture multiplies, grows, because without food there is no growth or life of probiotics, and increasing probiotic flora, reduces the number of pathogenic microorganisms and dominates the probiotic flora (Dimidi *et al.*, 2019). Prebiotics are glucans, fructans and other substances, polyphenols, etc. (Sakkas *et al.*, 2020; Bandyopadhyay *et al.*, 2021).

Probiotics and other microorganisms are in a constant struggle for dominance and that is why the urogenital flora as well as the intestinal flora are very unstable and can be easily disrupted by unhealthy lifestyles, improper diet, use of antibiotics, etc. (Goldstein *et al.*, 2015).

The food presented as a prebiotic is food of plant origin, fruits, vegetables, cereals, various legumes, beans (protein foods) that are rich in polyphenols (Sanders *et al.*, 2019; Sakkas *et al.*, 2020).

Foods that reduce the concentration of lactobacilli are fast food and foods that are rich in refined sugar and fatty foods that are rich in saturated fats of animal origin and trans fatty acids that are present in fried foods, meat products, chips, french fries, etc. (Ros *et al.*, 2015).

It should be emphasized that animal fats are used moderately in a proper and healthy human diet, as they are part of a proper diet (Yung *et al.*, 2016).

## **MICROBIOLOGICAL COMPOSITION OF THE VAGINS OF BITCHES AND WOMEN IN THE REPRODUCTIVE AGE**

In the vagina of bitches of reproductive age are most often isolated: coagulase-negative *Staphylococcus* spp., *S. pseudintermedius*, *Staphylococcus aureus*,  $\beta$  hemolytic *Streptococcus* spp., A hemolytic *Streptococcus* spp., non-hemolytic *Streptococcus* spp., *Escherichia coli*, *Pasteurella* spp., *Enterococcus* spp. and *Neisseria* spp., in different concentrations (Maksimović *et al.*, 2012).

In women of reproductive age, under the influence of the hormone estrogen, different types of lactobacilli use glycogen as a diet and produce lactic acid, which is responsible for the acidic pH of the vagina and resistance to infections (Javed *et al.*, 2019). Lactobacilli produce lactic acid, lower pH (acidic pH), produce hydrogen peroxide that destroys pathogenic bacteria and produce substances, bacteriocin enzymes that condition the cleanliness of the vagina and reduce the chance of infection. A generally healthy vagina contains an abundance of lactobacilli that condition a woman's urogenital health (Kovachev, 2018).

They are the dominant microbiome of the vagina in healthy women of reproductive age, and the number decreases with aging (hormonal imbalance, decreased estrogen), pregnancy (hormonal imbalance of estrogen and progesterone), improper diet (high refined sugar, saturated fats, trans fats), poor sexual hygiene, sexual habits (promiscuity, unfaithful partner), improper lifestyle, insufficient exercise, smoking (exposure to tobacco smoke, so-called passive smoking), use of antibiotics (first die gram + bacteria, in this case gram positive lactobacilli), various stress situations that impair immunity, etc. (Javed *et al.*, 2019; Di Simone *et al.*, 2020).

The following types of lactobacilli are most common in the vagina: *Lactobacillus crispatus*, which is the most dominant, *Lactobacillus gasseri*, *Lactobacillus iners* (increases in dysbiosis) and *Lactobacillus jensenii* (Abdelmaksoud *et al.*, 2016; Alonzo-Martínez *et al.*, 2021; Zheng *et al.*, 2020).

The vagina can be colonized by other microorganisms that can multiply in the event of a decrease in the number of lactobacilli and an increase in pH that occur for various reasons. Colonizers in the reproductive age of women are usually various aerobes, anaerobes, intestinal bacteria inhabiting the perineum, environmental bacteria (Borges *et al.*, 2014). In the case of vaginal dysbiosis, bacterial vaginosis, the predominance of anaerobic *Gardnerella vaginalis* is usually mentioned, which can be found in small amounts in the vagina, and women of reproductive age are most susceptible to infection (Machado & Cerca, 2015). Anaerobes are not generally considered dangerous inside the vagina, however, anaerobic infection can damage

the epithelium and increase susceptibility to sexually transmitted diseases, including high-risk HPV virus that has high oncological potential (Ilhan *et al.*, 2019; Coudray & Madhivanan, 2020).

In the case of vulvovaginal dysbiosis, fungal vaginosis, vulvovaginal candidiasis caused by *Candida albicans*, which is found in small concentrations inside the vagina, is most commonly mentioned, and older women are most susceptible to infection (d'Enfert *et al.*, 2021). More recently, scientists have linked dysbiosis to an increased risk of developing reproductive cancer (Łaniewski *et al.*, 2020). In the absence of sexually transmitted diseases, the cause of infection in reproductive age is dysbiosis caused by a decrease in lactobacilli, and the dominance of aerobes, anaerobes and colonizers (Amabebe & Anumba, 2018).

As a form of prevention of urogenital dysbiosis, many authors recommend the use of lactobacilli: *Lactobacillus fermentum* and *Lactobacillus rhamnosus*, and the best prevention would be the use of lactobacilli in the vagina, or the use of probiotics *Lactobacillus crispatus* (Ngugi, 2011; Javed *et al.*, 2019).

Susceptibility to dysbiosis is individual in women and in addition to lifestyle depends on genetics, race, ethnicity (Borgdorff *et al.*, 2017; Alonzo-Martínez *et al.*, 2021). Black women are more susceptible to dysbiosis (Javed *et al.*, 2019). It is considered that the use of intravaginal lactobacilli would be useful in frequent bacterial infections that have shown resistance to antibiotics (Valenti *et al.*, 2018).

#### **SIGNIFICANCE OF *LACTOBACILLUS* SPECIES DOMINANCE IN THE VAGINAL MICROBIOM OF WOMEN AND FEMALE CARNIVORES**

*Lactobacillus* species dominate the vaginal microbiome of healthy European women, accounting for >70% of the vaginal microbiota, while in the healthy female carnivore population it is a significantly lower percentage <1%, which can be attributed to diet (Miller *et al.*, 2016). Probiotic vaginal culture consists of *Lactobacillus* spp. which are present to protect against various infections that can be caused by various pathogens that are part of the vaginal microbiome that are present in different concentrations depending on the health status of the individual, lifestyle, sexual activity, hygiene, diet, etc. (Lewis *et al.*, 2017; Peric *et al.*, 2019).

The composition of the vaginal microbiome is quite dynamic and changes depending on the age of the individual, hormonal status, especially estrogen levels. In healthy, younger individuals that are in puberty, *Lactobacillus* spp. due to the hormone estrogen, which increases glycogen and lowers pH, while in older individuals the level of estrogen decreases with age, which affects the number of good bacterial species and increases susceptibility to infections. Reproduction and dominance of facultatively pathogenic and anaerobic bacteria over *Lactobacillus* spp. leads to bacterial vaginosis, which increases the chance of getting even more dangerous infections that pose a threat to the health of the reproductive tract of women and female carnivores (Heil *et al.*, 2019).

From all the above, it is possible to understand the importance of maintaining the dominance of *Lactobacillus* spp. in the vaginal microbiome which is very sensitive and subject to changes depending on changes in hormonal status, pregnancy, menstruation, changes in pH



of the vaginal tract for any reason, sexual activity, diet that reduces the number of *Lactobacillus* spp. (sugars, fats) etc. (Dong & Gupta, 2019; Saraf *et al.*, 2021).

It is important to mention that in the period of estrus the largest number of all microorganisms is present in the vaginal tract of women and when testing the vaginal microbiome it is best to do it in the period of estrus, and the worst is to examine the vaginal microbiome in the period of diestrus when the number of microorganisms is smallest. A resistant vaginal microbiome is present in carnivores (Lyman *et al.*, 2019).

Recent research suggests that a reduction in *Lactobacillus* spp. and that they have frequent bacterial vaginosis. Researchers emphasize that the use of probiotics has proven good in the treatment of bacterial vaginosis without harmful effects, and that the presence of *Lactobacillus crispatus* is most important for the health of women's vaginal tract and dominance with these species is highly desirable within the vaginal microbiome (Lewis *et al.*, 2017; Saraf *et al.*, 2021).

## **MECHANISM OF NEOPLASTIC DISEASES OF THE REPRODUCTIVE TRACT**

Long-term excessive intake of foods rich in fats and sugars affects the excessive release of insulin into the bloodstream. Hyperstimulation of the pancreas as a form of compensatory mechanism, results in a number of changes, primarily to increase the production of hormones, enzymes, hyperstimulation of organs and the appearance of neoplastic diseases (Szczyko *et al.*, 2021).

In carnivores, the occurrence of cancer, in addition to genetic factors, may be conditioned by the diet (high protein diet) (Rosta, 2011). Excessive consumption of the anticancer enzyme trypsin for protein denaturation. A cell is a basic building block, a tissue is a set of cells of similar structure and function, an organ is a set of tissues, and the organism is a set of organs that need energy for life and therefore we can conclude the severity of diseases changes in organs with possible neoplastic changes caused by hormonal imbalance (Wojciechowska *et al.*, 2016). If we consider that the cell is the basic building block, tissue is a set of cells of similar structure and function, organ is a set of tissues, and the organism is a set of organs that need energy for life, we can conclude the severity of the disease the occurrence of changes in the organs and the consequent possible neoplastic changes on the same, caused by hormonal imbalance (Naz *et al.*, 2020).

High levels of fat, carbohydrates, and lack of physical activity lead to hyperglycemia, increased blood sugar that causes hyperstimulation, overactive pancreas, and the production of enzymes, including insulin, that aims to reduce blood glucose levels. The amount of glucose reached, including fat that is converted into sugar, is proportional to the amount of insulin released, so it can be concluded that hyperglycemia leads to hyperinsulinemia over time, and such abnormal hyperproduction of insulin abnormally affects the ovaries (Qi *et al.*, 2021).

Prolonged ovarian hyperstimulation causes Polycystic Ovary Syndrome (PCOS) in both female and female carnivores, but this is quite rare in carnivores because they are naturally insulin resistant which in turn can be brought about by the natural diet of carnivores that have evolutionarily acquired this insulin resistance (Qi *et al.*, 2021).

Furthermore, the occurrence of Polycystic Ovary Syndrome causes hyperproduction or excessive production of androgens/estrogens, which have long been known to be produced in large quantities due to their known mechanisms of action may contribute to the development of various tumor diseases and overproduction of these hormones can cause neoplastic disease (Qi *et al.*, 2021).

Based on all the above, it can be concluded how important proper nutrition is, which affects first of all the microbiological composition of intestinal microorganisms, and then all other organs. Therefore, it can be said that the intestines are actually an endocrine organ, because their microbiological composition greatly affects the hormonal status on which the formation of both polycystic ovaries and neoplastic diseases of the reproductive tract depends. Research on the mechanism of occurrence of these diseases is still scarce and the mechanism of occurrence is still in principle unresolved (Kawa *et al.*, 2021; Qi *et al.*, 2021).

## CONCLUSION

In humans, a balanced diet rich in fruits and vegetables with the addition of healthy proteins from legumes, nuts and white meat are very important, as they contribute to the prevention and treatment of type 2 diabetes, Polycystic Ovary Syndrome, neoplastic diseases and the preservation of the microbiota of the intestinal and genital tract. Proper nutrition can be a supportive therapy in the treatment of Polycystic Ovary Syndrome, genital infections and cancer prevention. Previously known benefits of intravaginal use of probiotics *Lactobacillus fermentum* and *Lactobacillus rhamnosus* used in the prevention and treatment of vaginal infections, but the best prevention and treatment of recurrent bacterial vaginosis and those resistant to antibiotics would be the use of *Lactobacillus crispatus*, because it is found naturally in the vaginal tract of women.

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## **EFFECTIVENESS OF *ALOE VERA* LEAF EXTRACT AS A BIO-COAGULANT FOR THE TREATMENT OF INDUSTRIAL EFFLUENT BY COAGULATION-FLOCCULATION**

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### **ABSTRACT**

The substantial amounts of industrial wastewater involving organic contaminants are generated worldwide depending on the population and the development of industry. Due to these activities, water pollution is becoming a serious environmental problem as it can harm ecosystem, humankind and other living organism's life. The contaminants such as heavy metals, dyes, pesticides, and pharmaceutical products in the wastewater have been a major environmental issue owing to their toxicity, carcinogenic and non-biodegradability. Consequently, the physicochemical technologies must be advanced for the influential treatment of wastewaters. Coagulation-flocculation is one of the conventional wastewater treatment utilized to uptake contaminants from aqueous media. This technology is regarded an economical and practical process for removing pollutants from wastewater. The present work focused on a green coagulant of *Aloe vera* leaf extract for treat organic pollutants from water bodies. *Aloe vera* leaf extract was obtained using distilled water at feed-to-solvent ratio of 1:5. The experiments were performed at various pHs (2–8), coagulant dosages (1–7 mL/L), and organic pollutant concentrations (50–110 mg/L). The highest treatment was obtained at pH of 6, coagulant dosage of 3 mL/L, and concentration of 50 mg/L. The results indicate about 80% of removal of organic contaminants existing in wastewater by coagulation-flocculation.

**Keywords:** *Aloe vera* leaf, bio-coagulant, coagulation-flocculation, extract, water treatment.

### **INTRODUCTION**

The development of many industries to meet global demand has produced large volumes of wastewater in the environment. Wastewater arises from urban, domestic, agriculture, and other industries (Ahmad et al., 2022). Among these industries, the textile industry is a sector that needs large volumes of water consumption and therefore produces large amounts of wastewater (El Gaayda et al., 2022). Considering the various contaminants existed in industrial wastewater, dyes cause major environmental issues because dyes block the passage of light, leave toxic substances to organisms in the food chain, and damage the water quality of receiving streams (Tan et al., 2015). The dyestuffs contained in wastewater can have critical adverse effects on aqueous media and can also affect humans as well as living things (El Gaayda et al., 2022). For this reason, an effective process is needed for the removal of dyestuffs in aqueous systems.

There are several removal techniques available to eliminate dyes from aquatic environments, including membrane technology, electrocoagulation, advanced oxidation

processes, and coagulation-flocculation methods (El Gaayda et al., 2022). Coagulation and flocculation is widely utilized for treating water. This process is a simple and economical method capable of eliminating inorganic-organic substances and colloidal particles relying on the coagulant type, process condition, and wastewater characteristics (Ahmad et al., 2022). Lim et al. (2022) reported that suspended matter in wastewaters could not readily precipitate without the coagulants. On the other hand, Hoa and Hue (2018) stated that common coagulants like iron salt and aluminum sulphate affect human health and are generally expensive. They emphasized that unnecessary aluminum may be linked to Alzheimer's disease. Therefore, bio-coagulant could be proposed as an alternative to traditional chemical coagulants. Since bio-coagulants are highly biodegradable environmentally friendly materials, they have gained great importance in terms of environment and human health. Bio-coagulants involve a significant number of active compounds, like water-soluble protein, which have positive charges. This protein can bind to negatively charged ions that cause turbidity in wastewater (Ahmad et al., 2022). *Aloe vera* leaves, which are by-products of the agricultural industry, contain numerous bioactive compounds. *Aloe vera* has the potential to be used as a bio-coagulant for wastewater treatment.

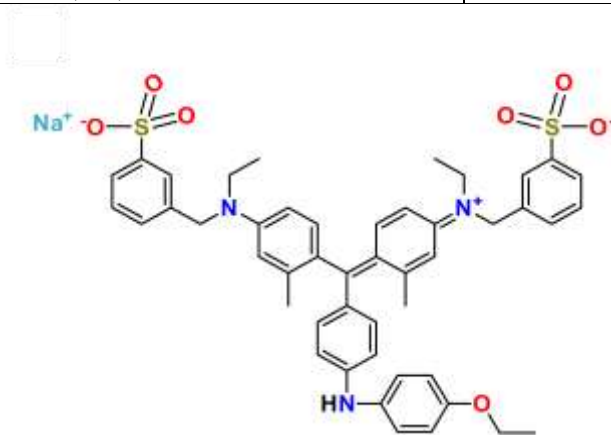
The purpose of this study is to evaluate *Aloe vera* leaf as a bio-coagulant for the treatment of dyestuffs and investigate the effects of the independent parameters such as pH, coagulant dosage, and organic pollutant concentration on the treatment of dye wastewater.

## MATERIAL AND METHOD

*Aloe vera* leaves were obtained locally from a vendor. They were used to evaluate the coagulation performance as a bio-coagulant. Coomassie brilliant blue G-250 (CBB) provided by Sigma–Aldrich, Germany was selected as the target contaminant and utilized without further purification. The chemical structure and characteristic of CBB is presented in Figure 1 and Table 1. Sodium hydroxide (NaOH) and nitric acid (HNO<sub>3</sub>) were purchased from Merck, Germany.

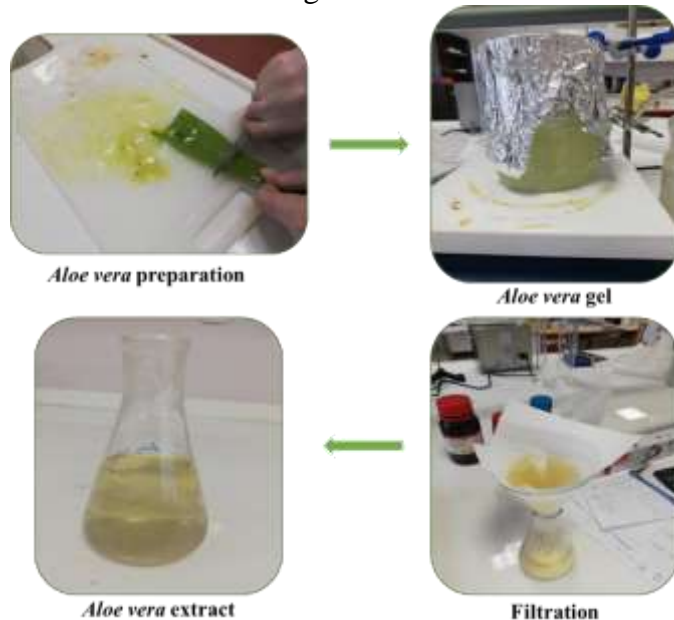
**Table 1.** Chemical structure and characteristic of CBB

|                       |   |
|-----------------------|---|
| Generic name          | Coomassie brilliant blue G-250                                  |
| Molecular weight      | 854.02 g/mol  |
| Solubility in water   | Soluble   |
| Chemical formula      | C <sub>47</sub> H <sub>48</sub> NaO <sub>7</sub> S <sub>2</sub> |
| λ <sub>max</sub> (nm) | 590   |



**Figure 1.** Chemical structure of CBB

In order to prepare *Aloe vera* as a bio-coagulant, firstly, *Aloe vera* leaves were washed with ultra-pure water for several times to eliminate the adhered particles and impurities on the surface. *Aloe vera* gel was separated by cutting the *Aloe vera* leaf. 250 mL ultra-pure water was poured in a 500 mL beaker and about 50 g of the separated gel were added into the beaker (1:5), followed by magnetic stirring at 60 °C for 3 h. Then, the prepared extract was kept at ambient temperature. After that, the mixture was filtered using by a filter paper (Whatman 4, UK). The final solution was then kept at 4 °C until further use. Figure 2 shows the preparation of *Aloe vera* leaf extract as a bio-coagulant.



**Figure 2.** The preparation of *Aloe vera* leaf extract

The coagulation–flocculation process was performed using a Jar Test equipped with six agitators to investigate the performance of the bio-coagulant (Figure 3). The range of parameters affecting coagulation–flocculation process including pH 2-8, coagulant dosage 1 to 7 mL/L, and organic pollutant concentration 50 to 110 mg/L were selected in this work. About 250 mL of CBB in different concentrations was taken in a 500 L beaker, and the desired bio-coagulant dosages were added for different independent parameters. For each experiment, the adjustment of pH was performed by 1 M NaOH and HNO<sub>3</sub> via a pH-meter. The coagulation and flocculation stage were carried out by rapid mixing followed by slow mixing. In the coagulation stage, the solutions were mixed rapidly at 160 rpm for 5 min. After rapid mixing, the CBB samples were slowly agitated at 35 rpm for 30 min (flocculation). At the end of the coagulation–flocculation process, the supernatant was taken and measured in terms of dye removal. The CBB concentrations in solutions were measured using a UV-vis spectrophotometer (Genesys 10S, Thermo Scientific, USA) at 590 nm. Performance of *Aloe vera* leaf extract in removing CBB was evaluated by determining the dye removal percentage based on following equation.

$$CBB \text{ removal } (\%) = \frac{(Absorbance)_{initial} - (Absorbance)_{final}}{(Absorbance)_{initial}} \times 100 \quad (1)$$

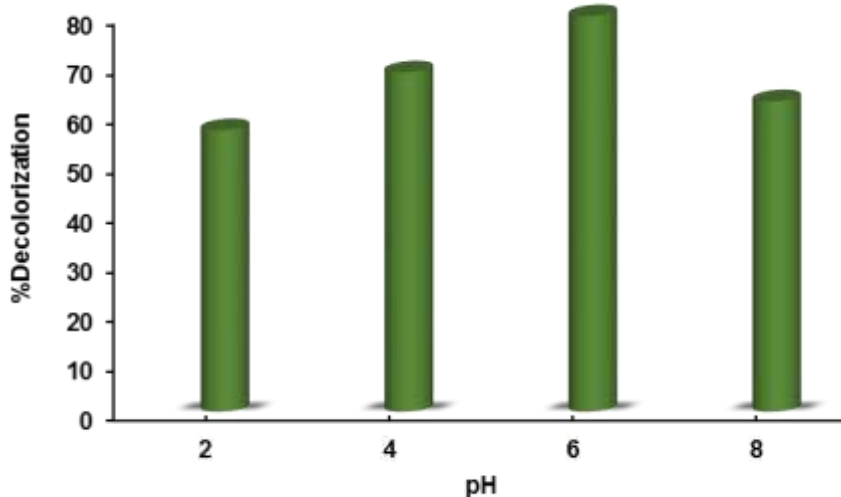
where (Absorbance)<sub>initial</sub> and (Absorbance)<sub>final</sub> are the initial and final absorbance values, respectively.



**Figure 3.** Jar test set-up

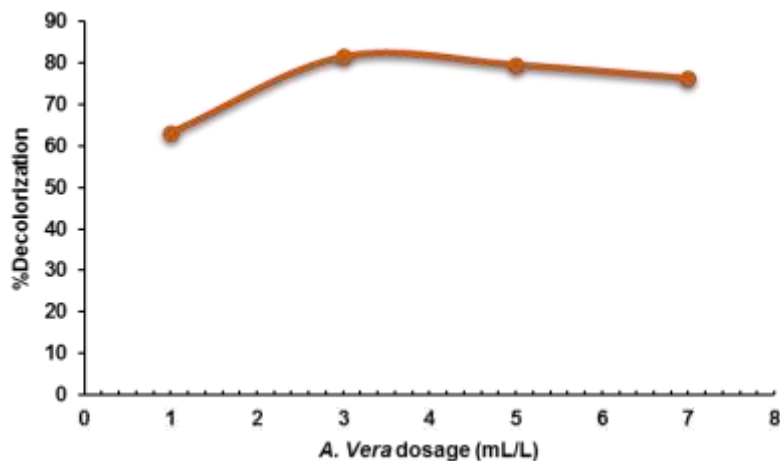
## **RESULTS AND DISCUSSION**

Figure 4 shows the removal percentage in CBB solutions after variation of pH during coagulation-flocculation process. The elimination in dye color increases with increase pH up to 6 value and after that the dye removal percentage decreases. The results indicated that at pH 6, removed approximately 80% of removal percentage, after coagulation-flocculation process. Hence, the optimal pH to have maximum removal of CBB is pH 6. At low pH values, CBB can compete with the active compounds of the bio-coagulant by forming a bond with *Aloe vera*. At high pH values, however, excess hydroxide ions in the system can compete with negatively charged that decrease the concentration of effective ligands that aid flocculation (Benalia et al., 2021). Therefore, optimum pH value was selected as 6 and used to evaluate the effects of coagulant dosage and organic pollutant concentration.



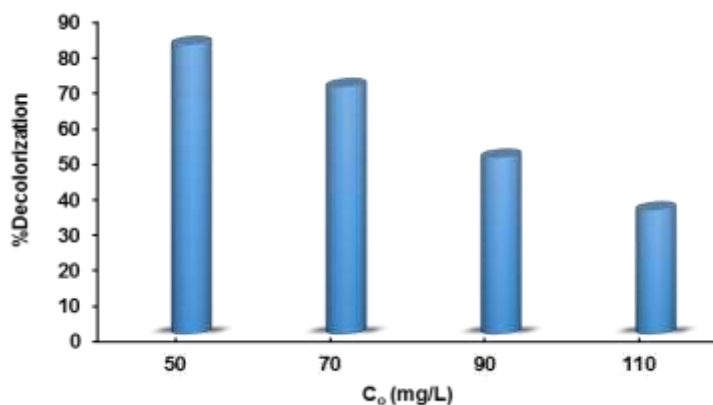
**Figure 4.** Effect of pH on CBB removal

The effect of coagulant dosage on removing CBB was investigated by varying the coagulant dosage from 1 mL/L to 7 mL/L while maintaining the both pH and organic pollutant concentration at 6 and 50 mg/L, respectively. The evolution of CBB removal percentage as a function of coagulant dosage is given in Figure 5. The results indicated that the CBB decolorization yield increases as the coagulant dosage increases from 1 to about 3 mL/L after that slightly decreased at higher levels. It can be stated that in high amounts, decreases in color removal efficiency may occur by causing turbidity of water based on the particles do not interact with the oppositely charged colloidal particles (Muruganandam et al., 2017; Ayekoe et al., 2017).



**Figure 5.** Effect of coagulant dosage on CBB removal

To evaluate the effect of organic pollutant concentration on CBB removal percentage, it was varied from 50 to 110 mg/L. Optimal pH 6 and coagulant dosage of 3 mL/L were utilized to evaluate the effect of organic pollutant concentration. The effect of organic pollutant concentration on CBB removal percentage is shown in Figure 6. As can be seen, a decrease in CBB removal occurs when organic pollutant concentration exceeds 50 mg/L. When organic pollutant concentration was prolonged from 50 to 110 mg/L, removal percentage declined from about 81% to 35%. Organic pollutant concentration of 50 mg/L appears to be the optimal condition. The reductions at higher concentrations are likely due to repulsive interactions between the unremoved anionic dye and the coagulant complex (El Gaayda et al., 2022).



**Figure 6.** Effect of organic pollutant concentration on CBB removal

## CONCLUSIONS

This work showed the effectiveness of *Aloe vera* leaf extract as a potential bio-coagulant for the removal percentage of CBB from aquatic media using the coagulation-flocculation procedure. In present study, the optimization of coagulation-flocculation process was investigating to determine optimum pH, coagulant dose, and organic pollutant concentration to eliminate maximal CBB of aqueous environments. Bio-coagulant was demonstrated to have excellent potential in wastewater treatment. The optimum conditions were determined as pH of 6, coagulant dose of 3 mL/L, and organic pollutant concentration of 50 mg/L. It achieved removal percentages of CBB of 79.88%, 81.5%, and 81.47% at optimal pH, coagulant dose, and organic pollutant concentration, respectively. As a result, the results showed that *Aloe vera* leaf extract, as a bio-coagulant, can play a significant role in the treatment of colored wastewater from aqueous environments.

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## QUALITY CHANGES IN DIFFERENT PARTS OF FRUITS OF SOME MORPHOLOGICALLY DIFFERENT WATERMELON HYBRIDS

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### ABSTRACT

The study was carried out at Alata Horticultural Research Institute. In this study, the fruits of watermelon hybrids with different morphological characteristics were divided into six. The part where the fruits touch the ground was divided into 3 parts and the sun-exposed part was divided into 3 parts and examined in 6 parts in total. In terms of L\* value, it can be said that part 5 has the highest values and part number 1 has the lowest values. In terms of ho value, it can be stated that the 2<sup>nd</sup> part is the highest value and the lowest value is the 3<sup>rd</sup> part in general. In terms of C\* value, it can be said that part 6 has the highest values in general, and the lowest values are generally taken from part number 2. In terms of total soluble solid content, it can be stated that the highest value is taken from the 3<sup>rd</sup> part and the lowest value from the 2<sup>nd</sup> part. In terms of titratable acidity, the highest value is taken from part 6 and the lowest value from part 2. In terms of pH, the highest values are taken from the parts 5 and 6, and the lowest value is taken from the part number 2.

**Keywords:** Watermelon, Hybrid, Different Parts, Quality Changes,

### INTRODUCTION

There are 101,620,420 tons of watermelons produced worldwide. After China (59.19%), Turkey (3.43%) is the second-largest producer. Turkey produces 3.49 million tons of watermelon on 78,179 acres of land (Faostat 2020).

Watermelon fruit is a vegetable with low protein, fat and starch content in terms of nutritional value. European consumers have started to be health conscious. This attitude has also started to be supported by governments with high investments to encourage fresh fruit and vegetable consumption. Watermelon provides a wide range of antioxidants such as carotenoids (lycopene and beta-carotene), phenols, vitamins (A, B, C and E) and certain amino acids (citrulline and arginine) in the diet (Perkins-Veazie, 2002; Perkins-Veazie et al., 2007), which is thought to play a protective role in reducing the risk of some types of cancer, cardiovascular diseases and age-related degenerative pathologies (Giovannucci, 1999; Rao, 2006). Watermelons are often prepared for the market in the field, and skin death, skin, pre-chilling, cold storage, and cold transportation are generally disregarded in watermelons that are being delivered to domestic and international markets. During marketing, watermelon fruit is subjected to high temperatures because its ripening period falls during the sweltering summer months. In particular when it comes to export, skin death, skin, cold storage, and transportation are crucial for extending the shelf life. In recent years, our nation has seen a sharp rise in the production of grafted watermelons. According to parameters like the auricles and leech on the fruit stem drying, hair shedding on the stem, and the fruit growing to the specified size of the variety, skin death of watermelon fruits is monitored (Özdemir et al., 2014; Aras et al., 2015).



In this study, the fruits of watermelon hybrids with different morphological characteristics were divided into six and the changes in total soluble solid content, titratable acidity, pH, L and h° values in each part of the fruits were examined.

## MATERIAL AND METHOD

The study was carried out on an open field at 36° 37' 08.03" N and 34° 21' 00.5" E that belonged to the Ministry of Agriculture and Forestry's Alata Horticultural Research Institute (Erdemli, Mersin, Türkiye). In the study, 36 hybrids from striped hybrids and 10 hybrids from varieties with dark green back ground color were used as material. The area where the study was conducted contains loamy soil with a pH value of 7,71, according to soil testing. Table 1 contains the results of the soil analysis.

**Table 1.** Soil analysis results of the parcel where the study was carried out

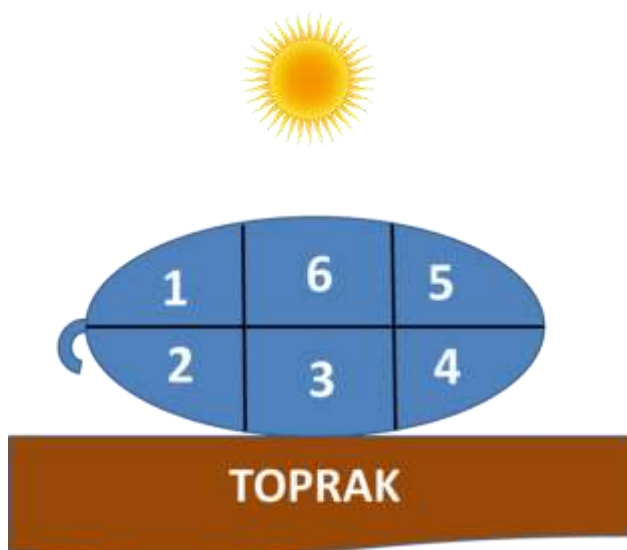
| Analyzes                             | Limit Values | Analysis Results (0-30 cm) |
|--------------------------------------|--------------|----------------------------|
| Texture (100 g/ml)                   | 30-50        | 48,00 (loamy)              |
| Total Calcitic (CaCO <sub>3</sub> %) | 5-15         | 40,00 (high calcareous)    |
| Salinity E.C. ds/m (25 °C)           | 0-0.8        | 0,32 (slightly salty)      |
| Organic matter (%)                   | 3-4          | 2,20 (deficient)           |
| pH 1: 2,5                            | 6.0-7.0      | 7,71 (slightly high)       |
| Available potassium (mg/kg)          | 244-300      | 70,60 (very low)           |
| Receivable phosphorus (mg/kg)        | 20-40        | 21,30 (optimum)            |

Seed sowing was carried out on 4 March 2017, land preparation was carried out on 6 April 2017, and seedlings were planted on 11 April 2017. On the prepared banks with a width of 70 cm and a height of 40 cm, the seedlings were planted with 2,5 x 0,7 m spacings and distances, covered in black mulch. Using a drip irrigation system, irrigation was carried out. Gudemir (2012) claims that fertilizations were carried out following soil analysis. Each irrigation included a drip irrigation system for fertilization. Spider mites and other pests were sprayed as soon as they were discovered. Mechanical and manual methods were used to control weeds. Harvests on 4 July 2017, as soon as the tendril and auricles were dry, the fruits were harvested and brought to the cold storage. The climate values of the months in which the research was carried out are given in Table 2.

In this study, the fruits of watermelon hybrids with different morphological characteristics were divided into six. The part where the fruits touch the ground was divided into 3 parts and the sun-exposed part was divided into 3 parts and examined in 6 parts in total. The changes in total soluble solid content, titratable acidity, pH, C\*, L and h° values in each part of the fruit were examined.

**Table 2.** Monthly climate data of the months in which the experiment was conducted.

| March  | April | May  | June | July |
|--|-------|------|------|------|
| Maximum Temperature Values (°C)                      |       |      |      |      |
| 23,5   | 28,2  | 30,0 | 34,2 | 40,5 |
| Minimum Temperature Values (°C)                      |       |      |      |      |
| 2,6  | 6,1   | 2,0  | 14,7 | 19,3 |
| Average Temperature Values (°C)                      |       |      |      |      |
| 13,4   | 16,8  | 20,0 | 24,6 | 28,8 |
| Maximum Humidity Values (%)                          |       |      |      |      |
| 90,5   | 88,8  | 84,0 | 81,3 | 80,4 |
| Minimum Humidity Values (%)                          |       |      |      |      |
| 39,6   | 41,4  | 59,0 | 61,5 | 43,5 |
| Average Humidity Values (%)                          |       |      |      |      |
| 69,9   | 67,2  | 75,8 | 75,0 | 71,4 |
| Average Precipitation Values (mm=kg÷m <sup>2</sup> ) |       |      |      |      |
| 211,6  | 76,4  | 12,8 | 0,2  | 0,0  |



**Figure 1.** Locations of samples taken from different parts of the fruit.

In the study, 36 striped and 10 hybrids with dark green back ground color were used. For the purpose of analyzing watermelon hybrids, fruit samples from six different fruit-related areas were collected. Figure 1 shows the chronological order of the fruit samples. Total amount of titratable acid (TEA, potentiometric method), % determined by the Atago ATC-1E Model hand refractometer (Atago Co. Ltd., Tokyo, Japan) from fruit juice derived from fruits, and total amount of total soluble solid content (fruit obtained) With the help of the 0.1 N NaOH amount used as a result of the titration carried out until the pH reached 8.1, 5 ml of water was obtained and it was completed to 100 ml with pure water to determine the acidity value (Sadler, 2008).

Three replications of the experiment were set up using a randomized plot design. In each cycle, 3 fruits were employed. JMP statistical software (JMP®, Version 7, SAS Institute Inc.; Cary, NC, 1989-2007, NC 27513-2414, USA) was used to statistically analyze the data. T-Student was used to assess the differences between pairwise comparisons, Tukey test, and

differences between multiple means ( $P < 0.05$ ). Statistical analyses were performed after applying angle transformation to percentage values.

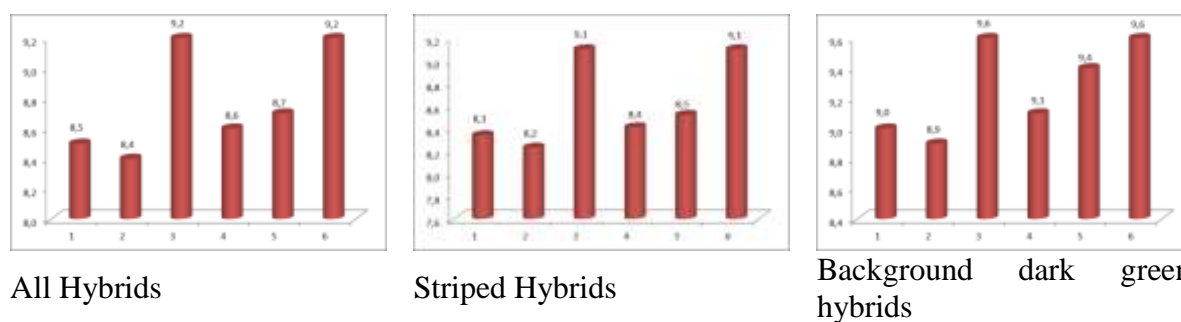
## RESULTS AND DISCUSSION

### *Total Soluble Solid Content (%)*

When all hybrids were examined in terms of SÇKM, the highest value was obtained from part 6 (9.2), and the lowest value was obtained from part number 2 (8.4). When the striped hybrids were evaluated within themselves, the highest values were obtained from the 6 (9.1) and 3 (9.1) numbers, and the lowest values were obtained from the 5 (8.5), 4 (8.4), 1 (8.3), 2 (8.2) parts. There was no statistical difference between the parts in hybrids with dark green ground color (Table 3, Figure 2). Aras et al. (2021) reported that soluble solid content ranged from 7,38 to 13,23 in their study.

**Table 3.** Changes of all hybrids, striped and background dark green in total soluble solid content according to parts.

| Parts | All Hybrids | Striped Hybrids | Background dark green hybrids |
|-------|-------------|-----------------|-------------------------------|
| 1     | 8,5 BC      | 8,3 B           | 9,0                           |
| 2     | 8,4 C       | 8,2 B           | 8,9                           |
| 3     | 9,2 A       | 9,1 A           | 9,6                           |
| 4     | 8,6 BC      | 8,4 B           | 9,1                           |
| 5     | 8,7 B       | 8,5 B           | 9,4                           |
| 6     | 9,2 A       | 9,1 A           | 9,6                           |
| CV    | 5,6         | 5,7             | 5,5                           |



**Figure 2.** Total soluble solid content changes of all hybrids according, striped and dark green ground color to parts.

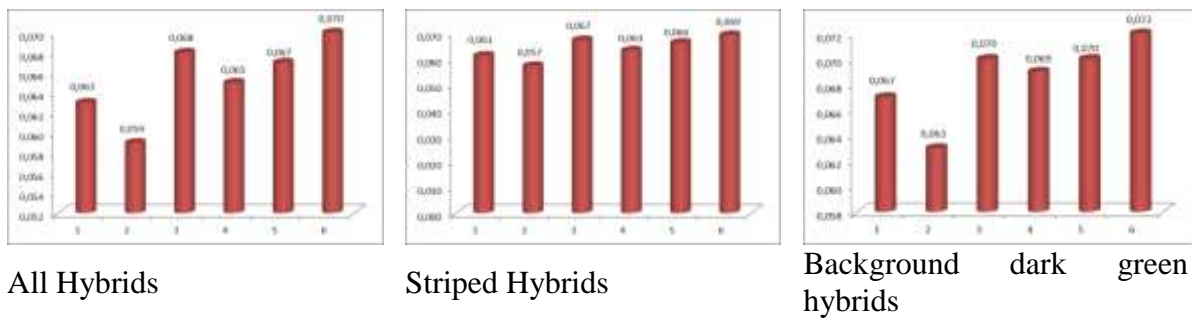
### *Titrateable Acidity (%)*

When all hybrids were examined in terms of titrateable acid, the highest value was obtained from part 6 (0.070), and the lowest value was obtained from part number 2 (0.059). When the striped hybrids were evaluated within themselves, the highest value was obtained from the parts numbered 6 (0.069), and the lowest value was obtained from the part numbered 2 (0.057). In the hybrids with dark green ground color, the highest values were obtained from parts numbered 6 (0.072), 5 (0.070) and 3 (0.070), while the lowest value was obtained from parts numbered 2

(0.063) (Table 4, Figure 3). Aras et al. (2021) reported that pH values ranged from 0,20 to 0,49 in their study.

**Table 4.** Changes of all hybrids, striped and background dark green in titratable acidity according to parts.

| Parts | All Hybrids | Striped Hybrids | Background dark green hybrids |
|-------|-------------|-----------------|-------------------------------|
| 1     | 0,063 D     | 0,061 D         | 0,067 B                       |
| 2     | 0,059 E     | 0,057 E         | 0,063 C                       |
| 3     | 0,068 B     | 0,067 B         | 0,070 A                       |
| 4     | 0,065 C     | 0,063 C         | 0,069 AB                      |
| 5     | 0,067 B     | 0,066 B         | 0,070 A                       |
| 6     | 0,070 A     | 0,069 A         | 0,072 A                       |
| CV    | 5,70        | 5,74            | 5,58                          |



**Figure 3.** Titratable acidity changes of all hybrids according, striped and dark green ground color to parts.

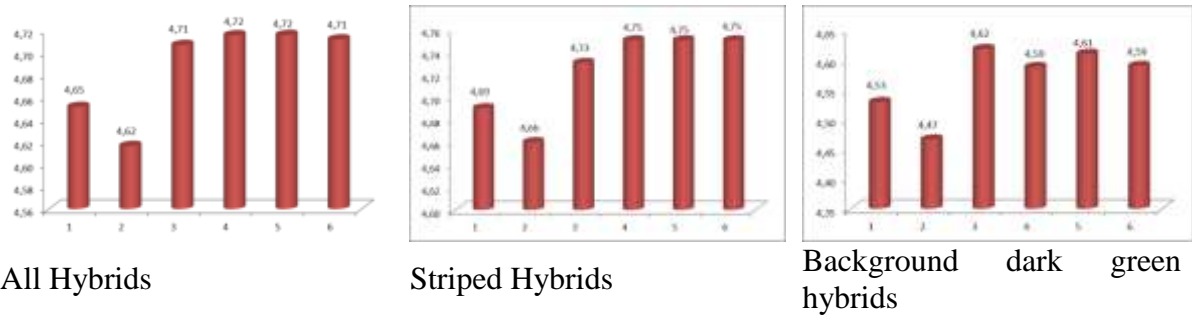
### *Fruit Juice pH*

When all hybrids were examined in terms of pH, the highest values were obtained from parts 4 (4.72), 5 (4.72) and 6 (4.71), and the lowest value was obtained from part number 2 (4.62). When the striped hybrids were evaluated within themselves, there was no statistical difference between the sections. There was no statistical difference between hybrids with dark green ground color (Table 5 , Figure 4).

Aras et al. (2021) reported that pH values ranged from 4.20 to 6.44 in their study.

**Table 5.** Changes of all hybrids, striped and background dark green in fruit juice pH according to parts.

| Parts | All Hybrids | Striped Hybrids | Background dark green hybrids |
|-------|-------------|-----------------|-------------------------------|
| 1     | 4,65 AB     | 4,69            | 4,53                          |
| 2     | 4,62 B      | 4,66            | 4,47                          |
| 3     | 4,71 AB     | 4,73            | 4,62                          |
| 4     | 4,72 A      | 4,75            | 4,59                          |
| 5     | 4,72 A      | 4,75            | 4,61                          |
| 6     | 4,71 A      | 4,75            | 4,59                          |
| CV    | 5,53        | 5,53            | 5,53                          |



**Figure 4.** Fruit juice pH changes of all hybrids according, striped and dark green ground color to parts.

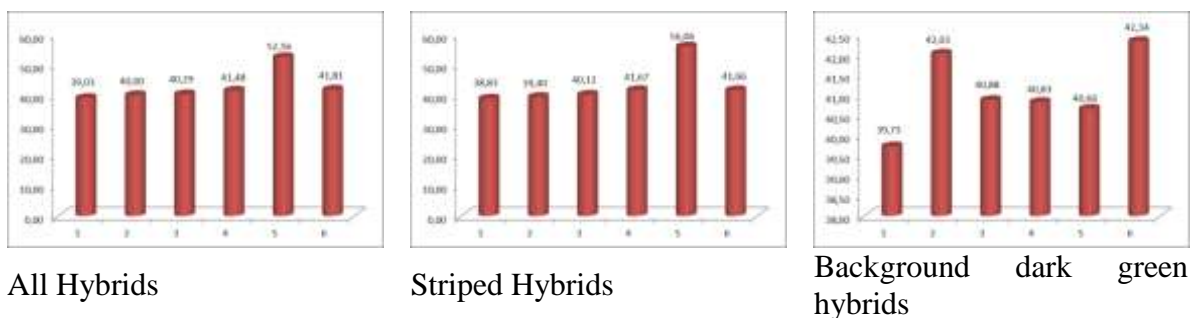
*Fruit flesh color L\* value*

When all hybrids were examined in terms of fruit flesh L\* value, the highest value was obtained from part 5 (52,56), and the lowest value was obtained from part number 1 (39,03). When the striped hybrids were evaluated within themselves, the highest value was obtained from part 5 (56,06), and the lowest value was obtained from part number 1 (38,83). In hybrids with dark green ground color, the highest values were obtained from parts 6 (42,34) and 2 (42,03), while the lowest value was obtained from part 1 (39,73) (Table 6, Figure 5).

Tokgöz et al. (2015) in his study, L\* color value varied between 35,26 and 37,17. Aras et al. (2021) reported that L\* color value ranged from 15,21 to 41,23 in their study.

**Table 6.** Changes of all hybrids, striped and background dark green in fruit flesh color L\* value according to parts.

| Parts | All Hybrids | Striped Hybrids | Background dark green hybrids |
|-------|-------------|-----------------|-------------------------------|
| 1     | 39,03 D     | 38,83 D         | 39,73 B                       |
| 2     | 40,00 C     | 39,40 CD        | 42,03 A                       |
| 3     | 40,29 C     | 40,12 C         | 40,88 AB                      |
| 4     | 41,48 B     | 41,67 B         | 40,83 AB                      |
| 5     | 52,56 A     | 56,06 A         | 40,66 AB                      |
| 6     | 41,81 B     | 41,66 B         | 42,34 A                       |
| CV    | 6,32        | 6,51            | 5,58                          |



**Figure 5.** Fruit flesh color L\* value changes of all hybrids according, striped and dark green ground color to parts.

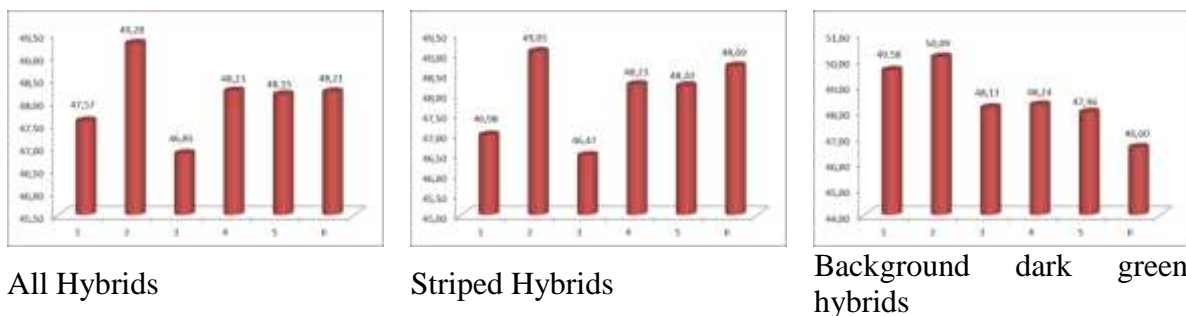
*Fruit flesh color h° value*

When all hybrids were examined in terms of fruit pulp h° value, the highest value was obtained from 2 (49.28) and the lowest value was obtained from 3 (46.85). When the striped hybrids are evaluated within themselves, the highest values are 2 (49.05), 6 (48.69), 4 (48.23) and (48.20), the lowest value is 1 (46.98) and 3 It was obtained from sections (46.47). In hybrids with dark green ground color, the highest value was obtained from part 2 (50.09), while the lowest value was obtained from part 6 (46.60) (Table 7, Figure 6).

Tokgöz et al. (2015) in his study, the h value ranged between 26.67 and 33.28 on average. Karaca et al. (2012), on the other hand, the h° values ranged between 35.2-42.3, respectively. Aras et al. (2021) reported that h° value ranged from 36,21 to 69,12 in their study.

**Table 7.** Changes of all hybrids, striped and background dark green in fruit flesh color h° value according to parts.

| Parts | All Hybrids | Striped Hybrids | Background dark green hybrids |
|-------|-------------|-----------------|-------------------------------|
| 1     | 47,57 BC    | 46,98 B         | 49,58 AB                      |
| 2     | 49,28 A     | 49,05 A         | 50,09 A                       |
| 3     | 46,85 C     | 46,47 B         | 48,13 ABC                     |
| 4     | 48,23 B     | 48,23 A         | 48,24 ABC                     |
| 5     | 48,15 B     | 48,20 A         | 47,96 BC                      |
| 6     | 48,21 B     | 48,69 A         | 46,60 C                       |
| CV    | 5,55        | 5,55            | 5,56                          |



**Figure 6.** Fruit flesh color h° value changes of all hybrids according, striped and dark green ground color to parts.

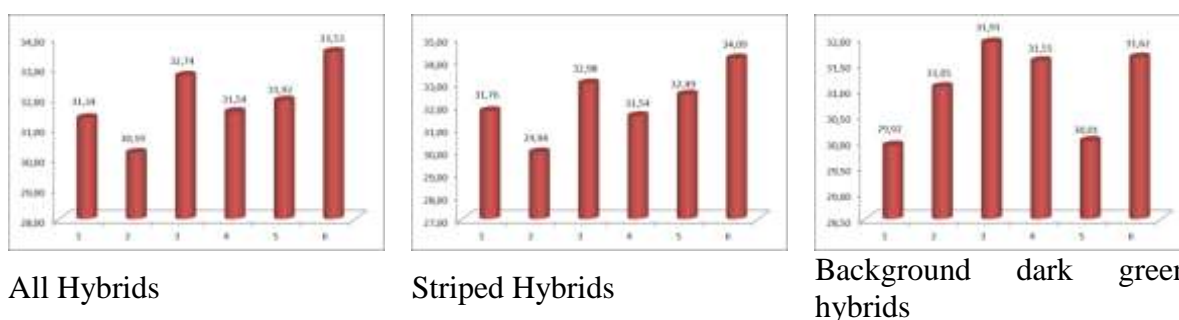
*Fruit flesh color C\* value*

When all hybrids were examined in terms of fruit pulp C\* value, the highest value was obtained from part 6 (33.53) and the lowest values were obtained from part 1 (31.34), 4 (31.54) and 5 (31.92). When the striped hybrids were evaluated within themselves, the highest value was obtained from the 6 (34.09) parts, and the lowest values were obtained from the 4 (31.54) and 1 (31.76) parts. In hybrids with dark green ground color, the highest values are obtained from 3 (31.91), 6 (31.62) and 4 (31.55) numbers, while the lowest values are 1 (29.92) and 5 (30.01). It was obtained from part numbered (Table 8, Figure 7).

Tokgöz et al. (2015) determined the C\* value between 26.72 and 33.25. Karaca et al. (2012), on the other hand, C\* values ranged between 28.1-35.9. Aras et al. (2021) reported that C\* value ranged from 14,88 to 47,09 in their study.

**Table 8.** Changes of all hybrids, striped and background dark green in fruit flesh color C\* value according to parts.

| Parts | All Hybrids | Striped Hybrids | Background dark green hybrids |
|-------|-------------|-----------------|-------------------------------|
| 1     | 31,34 C     | 31,76 C         | 29,92 B                       |
| 2     | 30,19 D     | 29,94 D         | 31,05 AB                      |
| 3     | 32,74 B     | 32,98 B         | 31,91 A                       |
| 4     | 31,54 C     | 31,54 C         | 31,55 A                       |
| 5     | 31,92 C     | 32,49 B         | 30,01 B                       |
| 6     | 33,53 A     | 34,09 A         | 31,62 A                       |
| CV    | 5,62        | 5,61            | 5,66                          |



**Figure 7.** Fruit flesh color C\* value changes of all hybrids according, striped and dark green ground color to parts.

## CONCLUSIONS

In determining reported health levels, bioactive compounds and antioxidant levels of genotypes of fruits and vegetables are strongly affected by agrotechnical processes, environmental conditions, ripening stage, harvest and postharvest changes as well as external factors (Waterman and Mole, 1994; Abushita et al., 2000; Dumas et al. et al., 2003; Lenucci et al., 2009). In terms of L\* value, it can be said that part 5 has the highest values and part number 1 has the lowest values. In terms of ho value, it can be stated that the 2<sup>nd</sup> part is the highest value and the lowest value is the 3<sup>rd</sup> part in general. In terms of C\* value, it can be said that part 6 has the highest values in general, and the lowest values are generally taken from part number 2. In terms of total soluble solid content, it can be stated that the highest value is taken from the 3<sup>rd</sup> part and the lowest value from the 2<sup>nd</sup> part. In terms of titratable acidity, the highest value is taken from part 6 and the lowest value from part 2. In terms of pH, the highest values are taken from the parts 5 and 6, and the lowest value is taken from the part number 2.

In practice, researchers divide the fruit into four and use the middle of any ¼ slice. It is necessary to take a sample from the same place, from the whole watermelon fruit or by dividing it into 6 parts.

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## VIRTUAL SCREENING FOR IDENTIFICATION OF HUMAN BLEOMYCIN HYDROLASE INHIBITORS

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### ABSTRACT

Knowledge of accurate binding poses of small-molecule ligands to the rapidly increasing number of proteins with known three-dimensional structures is key to understanding the mechanisms of function of these proteins. Protein activity is often modulated by binding of a small molecule to specific sites on the protein, and the disturbance of such regulation can cause diseases. Hence, many drugs work by inhibiting the function of excessively active proteins. Also, the proper activity of malfunctioning or non-functioning proteins causing a disease can be restored by the binding a specific chemical. Human bleomycin hydrolase is a papain superfamily cysteine protease that has the signature active site residues of this family and acts as an aminopeptidase. The main physiological role of bleomycin hydrolase is not yet known, but it catalyzes the inactivation of the antitumor drug bleomycin. However, its presence in dystrophic neurites of amyloid plaques suggests that it plays a role in processing of the amyloid precursor protein, which is the source of  $\beta$ -amyloid peptides that aggregate creating amyloid plaques of sporadic and familial cases of Alzheimer's Disease. Therefore, in this study we suggest that inhibiting human bleomycin hydrolase would prevent the degradation of the amyloid precursor protein to  $\beta$ -amyloid peptides. In order to find potential inhibitors, we developed a computational workflow for virtual screening and applied it to human bleomycin hydrolase. The workflow includes i) pharmacophore creation from either the target protein or the known active ligand or from both, ii) pharmacophore filtering to reduce the size of the library, iii) molecular docking of the filtered compounds to the target's binding site, iv) scoring the bound poses with different scoring functions, and v) running two sets of molecular dynamics simulations on a smaller selected subset of compounds to predict their binding free energy to the target protein by the LIE method. As a result of the study, four molecules were shown to micromolar inhibition activity.

**Keywords:** computer-aided drug design, docking, pharmacophore, bioinformatics, molecular dynamics simulations

### INTRODUCTION

The rapid increase in the number of proteins with known three-dimensional structures has brought the need to discover the accurate binding poses of the small molecules to these proteins since this knowledge has the key towards understanding the protein function mechanisms. Specific interactions of small molecules with proteins lie at the center of many biological processes and metabolic pathways. Protein activity is often modulated by binding of a small molecule to the specific sites on the protein and the disturbance of such regulation can cause

diseases (Gholke and Klebe, 2002). A malfunctioning or non-functioning protein causing a disease can be restored by the binding of a specific molecule to it.

However, synthesizing and testing all probable ligand candidates for one specific protein is still not very easy experimentally; a manageable number of candidates should be passed to the experimental stage. Therefore, a method reducing the large space of small molecules to an optimal size would solve the problem for the experimentalists (Bajorath, 2002; McInnes, 2007).

Virtual screening is a method to scan large numbers of small molecules to see whether they bind to a target protein and function in the desired manner (Shoichet, 2004). It is a complementary approach to experimental screening that aims to enhance the lead discovery process. Drug discovery researches for both hit identification and lead optimization has shifted towards computational methodologies, which are able to handle millions of molecules in a much shorter time compared to experimental time. The increase in the number of known protein structures and enormous chemical space of conceivable small molecules has drawn the attention more to virtual screening techniques (Walters et al., 1998; Muegge and Olof, 2006).

In this study, we developed a combined virtual screening procedure that aims to bring out strengths and compensate the weaknesses of individual methods. The computational workflow includes i) selection of a tolerant pharmacophore model without too many features ii) application of this pharmacophore model as a filter to reduce the size of the small molecule library, iii) molecular docking of the filtered library to the target's binding site, iv) scoring the docked poses with 5 different scoring functions and combining their outcomes with a consensus rule, and v) running two sets of molecular dynamics simulations on a subset of compounds selected according to the consensus score to predict their binding free energies to the target protein with the LIE method. This procedure was applied to human bleomycin hydrolase with PDB id 1CB5 (O'Farrell et al., 1999).

Human bleomycin hydrolase is a papain superfamily cysteine protease that has the signature active site residues of this family and acts as an aminopeptidase with broad substrate specificity (Sebti et al., 1988; Sebti et al., 1989; Enenkel and Wolf, 1993; Berti and Storer, 1995; Bromme et al., 1996). Even though bleomycin hydrolase was found out primarily for its ability to detoxify the cancer therapeutic bleomycin, the abundance of its homologs in different tissues of different organisms and the evolutionary conservation of the active residues propose a currently undiscovered cellular function (O'Farrell et al., 1999). Since it deactivates bleomycin, bleomycin hydrolase is thought to be the major cause of tumor cell resistance to bleomycin chemotherapy (Sebti et al., 1988). Human bleomycin hydrolase has also been shown to interact with human ribosomal proteins (Koldamova et al., 1999), ubiquitin-conjugating enzyme 9 (Koldamova et al., 1999) and amyloid precursor protein (Lefterov et al., 2000). Amyloid precursor protein is the source of  $\beta$ -amyloid peptides that aggregate creating amyloid plaques of sporadic and familial cases of Alzheimer's Disease (Selkoe, 1996). Various studies have indicated the presence of bleomycin hydrolase in dystrophic neurites of amyloid plaques and proven its presence in the processing of the amyloid precursor protein (Montoya et al., 1998; Namba et al., 1999). Human bleomycin hydrolase doesn't have a particular identified inhibitor, however it is inhibited irreversibly with covalent binding by a specific cysteine protease inhibitor trans-epoxysuccinyl-L-leucylamido(4-guanidino) butane (E64) (Enenkel and Wolf, 1993).

## MATERIAL AND METHOD

### Preparation of small molecule libraries and the target proteins:

#### The small molecule libraries:

The small-molecule library used in this work are compilations of compounds commercially available from several vendors. For the library, molecular configurations (states) and three-dimensional conformations of the compounds in the larger set have been generated with LigPrep (LigPrep, Schrödinger, LLC, New York, NY, 2021), enumerating tautomers, ionization states and, when the chirality is not specified, enantiomers. Finally, the molecules were minimized using the OPLS force-field (Cornell et al., 1995). A subset of compounds was selected for virtual screening by applying filters such as the Lipinski Rules (Lipinski et al., 2001), Veber Rules (Veber et al., 2002), and by requiring that compounds do not have reactive moieties and do not encompass more than 4 states (tautomeric, ionization and enantiomeric). The filtered virtual screening library was stored in SD format and contains 2157575 compounds.

#### The target protein:

Only one chain of the homohexamer human bleomycin hydrolase was used for the study. The protein was pre-processed with the Biopolymer module of SYBYL-X by Tripos (SYBYL-X, Tripos, USA) before docking; water molecules were removed, hydrogens and charges (AMBER7\_F99 charge set) were added and bound ligands were also removed. A small minimization using the AMBER7\_F99 force field with Powell method and termination after 500 iterations was done after the addition of hydrogens. This pre-processed structure was used as input for pharmacophore modeling with Unity module of SYBYL-X, docking with AutoDock Vina (Trott and Olson, 2010) and consensus scoring with CSCORE module of SYBYL-X. However, for AutoDock Vina's file format PDBQT, the charges were replaced with Gasteiger charges<sup>97</sup> and non-polar hydrogens were merged.

#### Pharmacophore creation and filtering:

In this study, Unity tool from SYBYL-X was used to create pharmacophore queries and

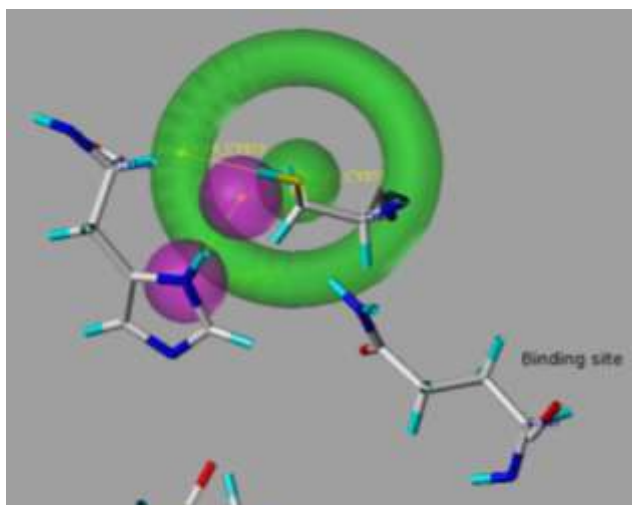


Figure 5. The pharmacophore filter for human bleomycin hydrolase (excluded volumes not shown)

3D flexible database search. Unlike static 3D search, 3D flexible search doesn't restrict the search to the 3D conformations as stored in the database, it takes the single conformation of each compound stored in the database, generates all feasible conformations giving out only the relevant ones to the query.

Human bleomycin hydrolase is a cysteine protease with a ring barrel structure that has the active residues Cys73, His372 and Asn396 embedded in the central cavity (O'Farrell et al., 1999). Cysteine protease mechanism of peptide bond cleavage is well defined and involves a series of steps that starts with the deprotonation of the thiol of Cys by the His side chain, which is oriented by Asn to allow this protonation. Anionic Cys sulphur makes a nucleophilic attack on the substrate carbonyl atom, releasing the amino terminus of the substrate and changing the His back to its deprotonated form. The intermediary bond linking the the carboxy terminus to Cys is broken by hydrolysis to release the carboxy terminus of the substrate and to restore the enzyme back to its free form. Preventing the deprotonation of Cys by His that starts further mechanism was the approach used for designing the pharmacophore filter. Therefore, we defined a hydrogen bond donor site feature on Cys73 that would need a torus shaped hydrogen bond acceptor atom feature in the pharmacophore filter. Then a hydrogen bond acceptor site feature on His372 that would need a hydrogen bond acceptor atom feature in the ligand was added to the filter (Figure 1). The directionality of the hydrogen between His372 and the candidate ligand was adjusted to be towards Cys73. Finally, the excluded volume feature was added from the binding site residues. The Van der Waals scales were 1 for all hydrogen bond acceptor and donor features, and 0.25 for the excluded volume features. Since the cysteine protease inhibitor E64 makes a covalent bond with the thiol of Cys, this information wasn't used for pharmacophore design, making the process strictly structure-based.

### **Docking the pharmacophore-filtered libraries to the targets:**

Molecular docking was done to predict the optimum non-covalent binding of the ligands in the receptor binding sites and their corresponding binding affinities. For high throughput docking of the library to each protein target, AutoDock Vina was used. Even though AutoDock Vina allows flexibility on protein residue side chains, we left the target binding sites rigid in our study.

Since the number of molecules that passed the pharmacophore filter for human bleomycin hydrolase was quite large, and because the binding site of human bleomycin hydrolase is a large surface exposed area, a small grid was used to restrain the molecules to the binding site and to prevent molecules from being docked to irrelevant regions. The docking experiment was done in an 18 x 18 x 18 Å grid with 1 Å spacing and placed on the active site residues Cys73, His372 and Asn396. Vina docking experiments were performed with an exhaustiveness parameter of 8, generating 9 different poses per compound with a maximum energy difference between the best and worst displayed binding modes of 3 kcal/mol. The pose with the lowest calculated binding free energy was kept for each compound for the next step of consensus scoring.

### **Consensus Scoring**

In this study, a given ligand-receptor complex produced by AutoDock Vina was rescored using D-Score, PMFScore, ChemScore and G-Score with CSCORE of SYBYL-X. The four scores produced by CSCORE were combined with the score obtained with AutoDock Vina. However, instead of CSCORE's rank-by-vote consensus scoring approach, a modified rank-by-number approach was used to combine these five scoring functions. For each scoring function,

all scores were normalized to values between 0 and 1 (0 representing the most favorable compound, 1 representing the least favorable by each scoring function), putting the scores from different scoring functions on the same scale and therefore making them comparable. Two different normalization procedures were implemented. First normalization procedure was applied to all scores of all docked ligands. However, the second normalization was done after truncating the 0.5% of the poorly scoring molecules for each scoring function by directly assigning 1 as their normalized scores. In other words, 99.5% of the scores were normalized after the deletion of the poorly scoring 0.5% part for the second normalization procedure.

### **Estimation of binding free energies by LIE**

Binding affinities were calculated using the LIE method, described in detail elsewhere (Åqvist and Medina, 1994; Hansson and Åqvist, 1998). Basically, this approach estimates the ligand free energy of binding from the difference in the ligand-surrounding interaction energies in both its bound and free state. The relationship between the ligand intermolecular interaction energies and the free energy of binding is given by the Lennard-Jones and electrostatic interactions between the ligand and its surroundings. These interactions are evaluated as energy averages from separate MD simulations of the free (*f*) and bound (*b*) states of the ligand (solvated in water and bound to the solvated protein, respectively).

All required MD simulations have been performed with the program Q (Marelius et al., 2004) and the OPLS force field implemented therein (Jorgensen and Rives, 1988). Since many of the parameters needed for the ligands were not present in the original version of the force field, an automated parameterization protocol was designed. MD simulations were performed using spherical boundary conditions, thus a definition of a sphere of simulation around the ligand was required. The center of the sphere was determined manually, making little changes to the docking grid center used. The sphere radius were calculated according to the length of largest compound as docked into the protein binding site. Non-bonded interaction energies were calculated up to a 10 Å cutoff, except for the ligand atoms for which no cutoff was used. Protein atoms outside the simulation sphere were restrained to their initial positions, and only interacted with the system through bonds, angles and torsions. The ionization states of titratable residues inside the simulation sphere was manually assessed, in order to obtain neutral simulation systems in the protein simulation, which is needed in order to compare the ligand-surrounding energies between bound and free states at least for charged ligands. In the human bleomycin hydrolase system, the charged residues were: Lys68, Lys107, Lys162, Lys309, Lys330, Lys335, Lys359, Lys405, Arg72, Arg83, Arg110, Arg175, Arg176, Arg362, Arg393, Asp35, Asp38, Asp106, Asp143, Asp179, Asp327, Asp401, Glu60, Glu96, Glu167, Glu172, Glu367, Glu395, Glu400 and Glu421 in a sphere of 26 Å radius. In addition, any titratable residues closer than 3-5 Å to the boundary, which together with those outside the solvent sphere should be modeled as neutral because of the lack of dielectric screening.

For both the protein-bound and the protein-free simulations, an initial heating and equilibration MD simulation was carried out before the data collection phase, starting with a very short time step of 0.1 fs, a strong coupling to a temperature bath of 1 K and positional restraints of 25 kcal/(mol·Å<sup>2</sup>) on all non-hydrogen protein and ligand atoms in the case of the protein-ligand complex simulation. The system was then gradually heated up to 310 K during 95.5 and 65.25 ps for the protein-bound and the protein-free simulations, respectively, in which the bath coupling was relaxed to a final value of 100 fs, the timestep was increased to 1 fs and the force constant of the positional restraints was gradually lowered to 0.

A production-run molecular dynamics simulation then followed for 500 ps at 310 K (100 fs coupling time) with a time step of 1 fs. In the case of the protein-free simulation, the center

of the ligand was restrained to the center of the solvation sphere with a force constant of 5 kcal mol<sup>-1</sup> Å<sup>-2</sup>. Energies were collected at regular intervals of 25 fs. Energy averaging was performed on this collection period, and stability was addressed by an estimation of the convergence errors of the potential energies of the ligand with its surroundings.

For the estimation of the binding free energies, only the period 100-500 ps of the production-run simulations were considered. Convergence of the simulations was assessed by calculating the difference between the LIE energy calculated over the periods 100-300 ps and 300–500 ps of the production-run simulations. We refer to this difference as the LIE error.

## RESULTS AND DISCUSSION

Small molecule library pharmacophore filtering was not very efficient for human bleomycin hydrolase. The binding site of bleomycin hydrolase is part of a large solvent-exposed cavity. This may cause human bleomycin hydrolase to have little substrate specificity, similar to its yeast homolog (O'Farrell et al., 1999). Therefore, the pharmacophore filter created for bleomycin hydrolase was rather tolerant with few excluded volume features. As a result, 471198 out of the 2157575 molecules of the library passed the filter. The solvent-exposed binding site of bleomycin hydrolase also brought extra challenges for determining the parameters for the grid size used for docking. Using a big grid covering the whole cavity could have caused docking to other regions than the binding site region. Therefore, the grid chosen was as big as possible to cover the binding site residues and the docked ligands while preventing irrelevant binding.

After the docking calculations were completed, we selected the binding pose with the lowest energy calculated by AutoDock Vina for each ligand. These poses were subsequently rescored with the four additional scoring functions implemented in CSCORE, and the scores were normalized.

Normalizing the scores produced by CSCORE and AutoDock Vina to values between 0 and 1 without produced similarly shaped sigmoidal curves for all scoring functions. However, the individual scoring functions showed trails of high values to largely different extents, most

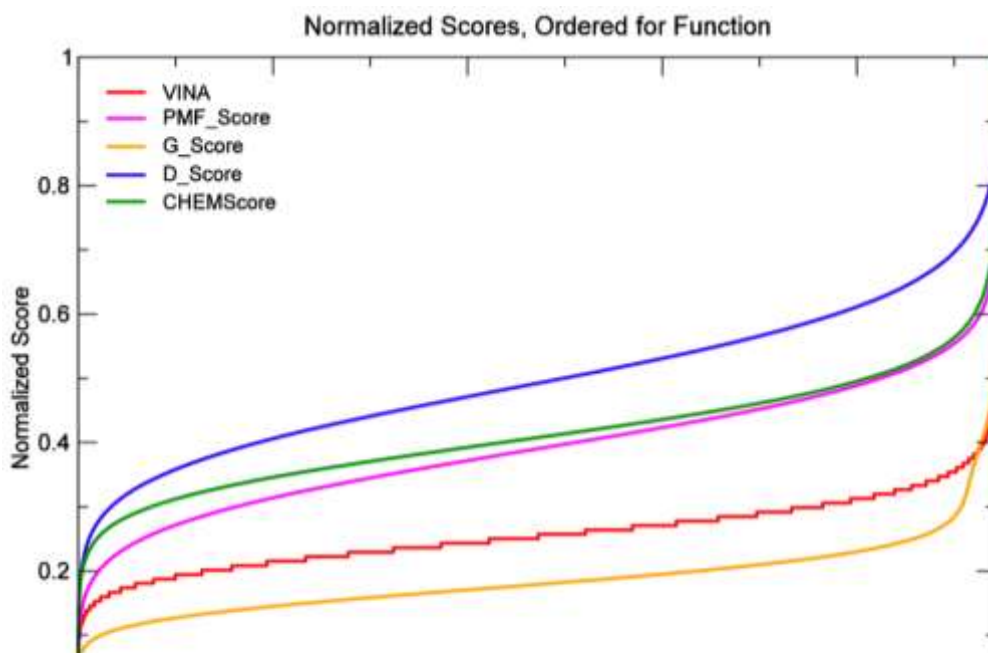


Figure 6. Normalized scores calculated without truncation with the five scoring functions Vina, PMFScore, G-Score, D-Score and ChemScore for human bleomycin hydrolase.

pronounced in the case of G-Score. Figure 2 shows that the ability of the normalized AutoDock Vina score and G-Score to distinguish “well docked” molecules from “poorly docked” ones is strongly affected by few compounds receiving very high scores.

Therefore, an adjustment excluding poorly scoring compounds for each energy function was done with our normalization procedure using a truncation cut-off of 99.5%. As Figure 3 shows, the discrepancy between scoring functions has become less pronounced after this adjustment. In addition, the slopes of the curves for intermediate ranks have increased, improving the distinction between well-scored and poorly scored molecules. Hence, the sum of normalized scores calculated with the 99.5% truncation cut-off normalization procedure was used as the final normalized consensus score, *NCS*, for each compound. *NCS* values ranges between 0 and 5, the values closer to 0 representing well-scored compounds and the values closer to 5 representing poorly scored compounds.

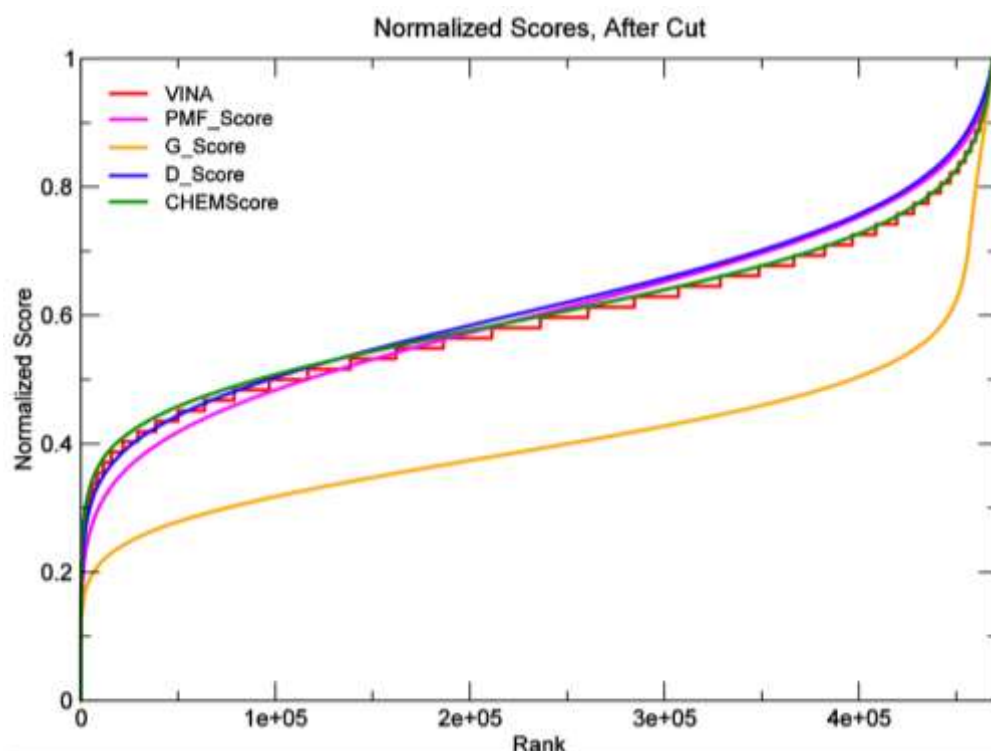


Figure 7. Normalized scores calculated with the second normalization procedure against compound rank (after the 0.5% worst-scoring compounds were excluded) for human bleomycin hydrolase.

Even though the normalized scores were correlated with individual scoring functions, binding free energies calculated with LIE only showed minor correlation with the AutoDock Vina score (Figure 4). Also, there is hardly significant correlation between different scoring functions (Table 1). However, this is not unexpected considering the approximations applied by scoring functions. The other reason for the lack of correlation lies in the different characteristics of the scoring functions used. The scoring functions of AutoDock Vina and ChemScore are empirical and PMF Score is a knowledge-based scoring function, which means that they were trained against a set of protein-ligand complexes and their performances are distinctly dependent on these training sets. On the other hand, it is known that force-field based scoring functions like D-Score and G-Score usually overestimate the binding affinities.

For human bleomycin hydrolase, we selected 40 molecules. The selection process was done in two parts. In the first part, the 4960 molecules evaluated with LIE were visually analyzed according to their docked conformations. A set of 100 compounds was selected based on their hydrogen bonding patterns with the binding site residues and the abundance of other noncovalent interactions. Then these compounds were ordered according to their binding free energy calculated with LIE. Compounds with a binding free energy smaller than -10 kcal/mol made it to the final selection set. The final selection set has 14 compounds chosen with this criterion. The second part of the selection process was done taking only into account the binding free energies calculated with LIE. The 4960 molecules were ordered based on their binding free energy and compounds with a value smaller than -15 kcal/mol were included in the final selection set. This second criterion contributed 26 compounds to the final selection. From this set of 40 molecules, 9 were available from a single vendor and they were purchased for experimental testing. The selection criteria and binding free energies calculated with the LIE method are summarized in Table 2.

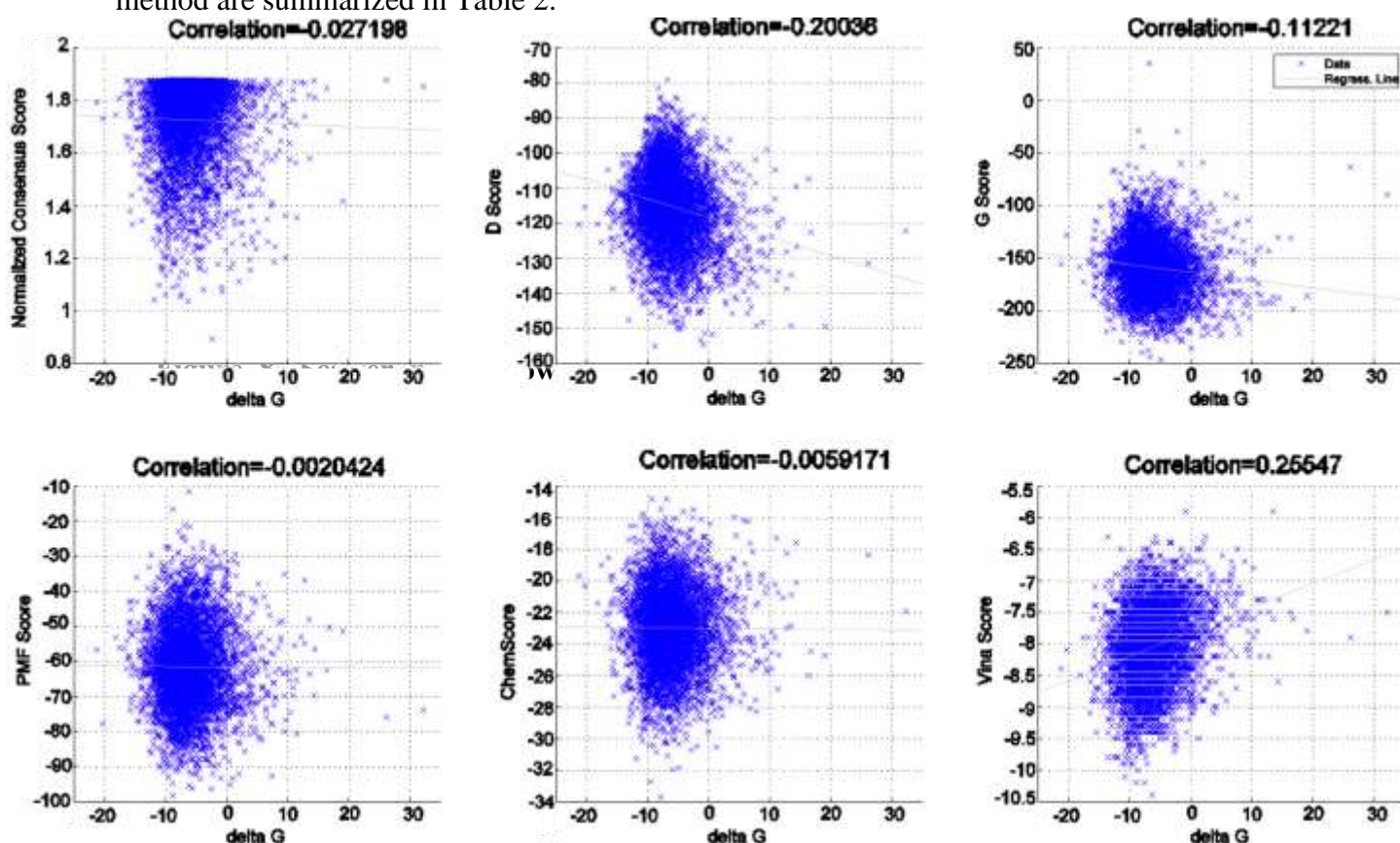


Table 6. Pearson's Correlations of scoring functions with each other and normalized consensus score of the selected molecules for human bleomycin hydrolase.

|           | NCS  | D-Score | G-Score | PMFScore | ChemScore | VinaScore |
|-----------|------|---------|---------|----------|-----------|-----------|
| NCS       | 1    | 0.32    | 0.31    | 0.25     | 0.4       | 0.28      |
| D-Score   | 0.32 | 1       | 0.31    | -0.31    | -0.19     | -0.24     |
| G-Score   | 0.31 | 0.31    | 1       | -0.28    | -0.17     | -0.11     |
| PMFScore  | 0.2  | -0.31   | -0.28   | 1        | -0.03     | -0.26     |
| ChemScore | 0.4  | -0.19   | 0.17    | -0.03    | 1         | 0.05      |
| VinaScore | 0.28 | -0.24   | -0.11   | -0.26    | 0.07      | 1         |



Table 7. The list of selection criteria fulfilled by the chosen molecules for testing against human bleomycin hydrolase.

| Compound no. | Selection criteria | LIE Energy (kcal/mol) | Rmsd between docking and LIE (Å) |
|--------------|--------------------|-----------------------|----------------------------------|
| 1            | LIE                | -16                   | 5.2                              |
| 2            | LIE                | -17                   | 5.4                              |
| 3            | Visual             | -11                   | 2.3                              |
| 4            | LIE                | -16                   | 1,6                              |
| 5            | LIE                | -16                   | 3,5                              |
| 6            | LIE                | -16                   | 5.7                              |
| 7            | LIE                | -16                   | 2.3                              |
| 8            | LIE                | -15                   | 2.8                              |
| 9            | LIE                | -16                   | 4.4                              |

Out of nine compounds, Compounds **3**, **6** and **8** did not inhibit the hydrolysis by human bleomycin hydrolase and Compound **4** was not soluble at a concentration sufficient to be tested. Values of  $K_m$  in presence of inhibitor were similar to that of human bleomycin hydrolase alone, except for compound **7**, which had the highest determined  $K_i$ . The compounds showed different modes of inhibition: competitive, noncompetitive or mixed (**Hata! Başvuru kaynağı bulunamadı.** 3). The derived inhibition constants  $K_i$  ranged from 30 to 356  $\mu\text{M}$ .

Table 8. Observed activities of the candidate compounds for human bleomycin hydrolase.

| No      | Mode of inhibition | $K_m$ [ $\mu\text{M}$ ] | $K_i$ [ $\mu\text{M}$ ] |
|---------|--------------------|-------------------------|-------------------------|
| Control | n.a.               | 182 $\pm$ 12            | n.a.                    |
| 1       | Noncompetitive     | 190 $\pm$ 10            | 30 $\pm$ 2              |
| 2       | Mixed              | 195 $\pm$ 14            | 89 $\pm$ 11             |
| 3       | No inhibition      | 163 $\pm$ 9             | n.a.                    |
| 4       | n.a.               | n.a.                    | n.a.                    |
| 5       | Noncompetitive     | 212 $\pm$ 16            | 263 $\pm$ 24            |
| 6       | No inhibition      | 176 $\pm$ 16            | n.a.                    |
| 7       | Competitive        | 138 $\pm$ 7             | 356 $\pm$ 155           |
| 8       | No inhibition      | 199 $\pm$ 10            | n.a.                    |
| 9       | Mixed              | 221 $\pm$ 14            | 127 $\pm$ 48            |

When the position predicted by docking of Compound **1** in the binding site is examined, it is seen that the compound is stretched along the binding site contacting the catalytic residues and making hydrogen bonds with residues Gln67, Ser334, Thr371 and His372 (Figure 5). There is also a  $\pi$ - $\pi$  interaction between Compound **1** and the indole ring of Trp398.

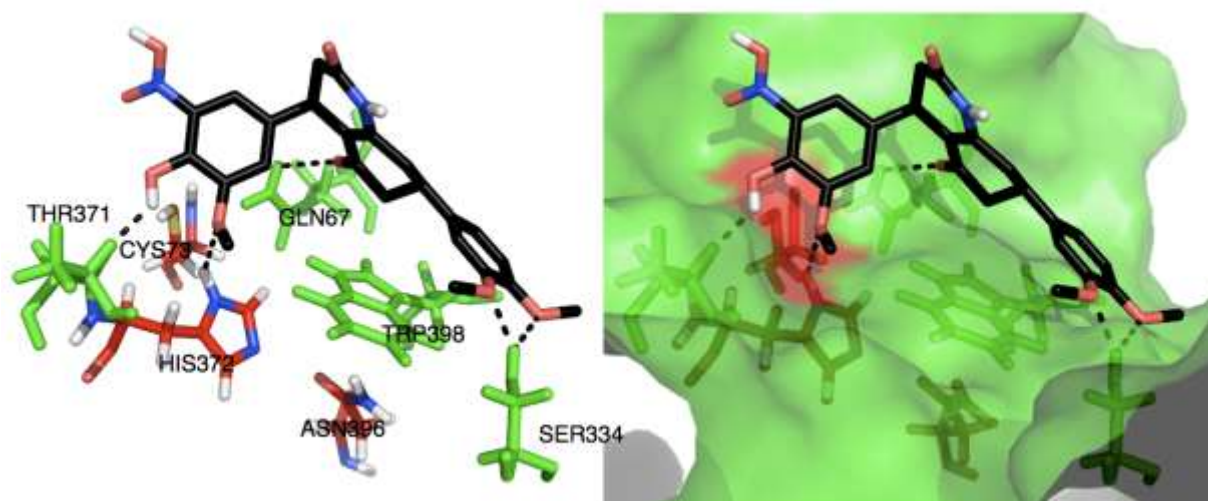


Figure 9. Binding mode of Compound 1 as predicted by docking. Compound 1 is shown in black, catalytic residues Cys73, His372 and Asn396 are shown in red while other residues contacting the compound are shown in green.

## CONCLUSIONS

In this study, we proposed a virtual screening procedure that combines pharmacophore design, high-throughput molecular docking, consensus scoring and evaluation of binding free energy by LIE method. A large library of small molecules was screened to find potential active binders to human bleomycin hydrolase that is suggested to take part in Alzheimer's Disease. Even though this protein was not primarily discovered due to their involvement in Alzheimer's Disease, its proposed role may be important in the pathological pathway of the disease, therefore they are appropriate drug targets. For the screening experiment, pharmacophore filtering and molecular docking was employed to reduce the library size and to find possible hits. The evaluation of binding modes of molecules docked in the protein binding site was done with a modified rank-by-number consensus scoring method. Afterwards, the compounds with the best normalized consensus scores were subjected to two molecular dynamics simulations for binding free energy estimation by LIE. However, predicted binding free energies were not significantly correlated with either the individual scoring functions or the normalized consensus score for the three screening experiments. Nine compounds were selected for experimental testing based on abundance of interactions with the target protein and calculated LIE scores. Four of these molecules showed different modes of inhibition with inhibition constants  $K_i$  varying between 30 and 365  $\mu\text{M}$ .

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## ANTIMICROBIAL WOUND DRESSING MATERIALS

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### ABSTARCT

Wound management consists of a process that includes several factors and different procedures which can lead to complex clinical problems. Determining an efficient approach to enhance the healing process is necessary using certain materials and methods. One of these approaches is using wound dressing materials. The purpose of wound dressing is to support the local wound environment and absorb moisture to facilitate wound healing. Recently, thanks to development achieved in biotechnology and nanotechnology, especially advanced wound dressing materials with different structures and forms have attracted attention and have been researched because of fascinating functions such as enhanced mechanical strength, antimicrobial activity, high surface area to volume ratio, and special functionalities shown in the wound surface triggered by the nanoscale dimensions. Here in, focused on reviewing various antimicrobial agents, biomaterials, and their recent advances used in designing wound dressing. Moreover, wound dressing materials synthesized by using green chemistry, nanomaterials, and antimicrobial activity, their future perspective, main strategies, and challenges were emphasized.

**Keywords:** Nanotechnology; nanoparticles; wound healing; wound dressing; antimicrobial activity

### INTRODUCTION

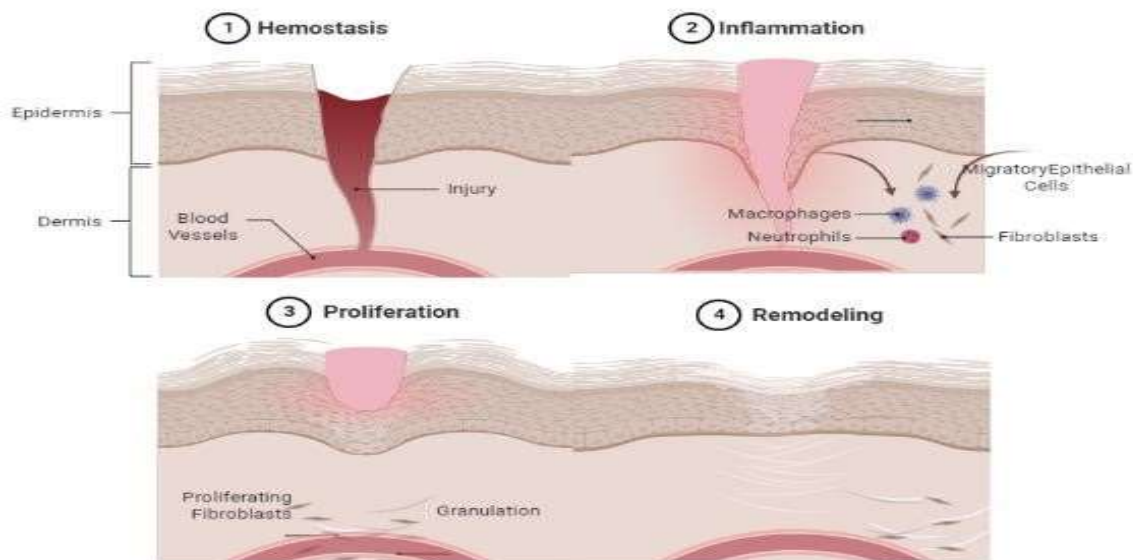
Wound dressings are materials which have a great importance in the wound care industry and have commercial value in all around the world. According to Global Market data, acute wounds (such as minor burns) and chronic wounds (such as diabetic ulcers) are crucial for both patients and the health care system. In Europe, there are nearly 55 million diabetics. As a result of insufficient treatment, 8 million of them develop diabetic ulcers and amputation is performed in approximately 450,000 patients and this costs € 2-2.5 million. Similarly, for America, this cost is \$ 25 million for 6.5 million patients. In acute wounds, this cost reaches \$ 670 million for 41 million patients. The cost of wound care, which was \$ 17 million in 2016, reached \$ 20.4 million in 2021 due to the increase in chronic diseases and population. To overcome such costs and numbers, a widespread work schedule is adopted. In these studies, advanced wound dressing materials are examined by using natural polymeric nanofibers, antimicrobial nanoparticles, and functional biological agents. This creates a potential for advanced dressing materials to replace conventional dressing materials. In addition, since bacteria that develop antibiotic resistance raise concerns, the use of classical drug treatment is replaced by different materials that will not cause resistance. The main purpose of the development of this technology is to provide fruitful and cost-effective treatment shortly. An appropriate treatment strategy consists of wound healing in a short time, coping with infection, cost reduction and prevention of undesirable effects (Homaieghar & Boccaccini, 2020).

## What Is the Wound

Skin is the structure that completely covers the body and constitutes 15% of the total body weight. In daily life, the skin needs to be protected because it is exposed to many physical, chemical or microbial factors (Xu et al., 2020a). A wound is an anatomical formation of any structure or function on the skin (Okur et al., 2020). Acute or chronic wound formation occurs when skin is exposed to a factor. For example; burns and postoperative cuts are called acute wounds and most of them heal in about three weeks. But in some cases, such as uncontrollable infections, acute wounds can turn into chronic wounds. In addition, chronic wounds can occur with the increase of chronic diseases such as obesity and diabetes (Wang et al., 2021).

## Wound Healing

Wound healing involves several components. In case of any injury or tissue damage, successive processes occur to heal tissue for returns to its normal state. Different types of cells, enzymes, cytokines, proteins and hormones involves to this process. Wound healing consists of four phases called (i)hemostasis, (ii)inflammation, (iii)proliferation and (iv)maturation (remodelling) (Figure 1). In addition, according to the healing time, the wounds are divided into two which are acute (normal) wounds and chronic wounds (Rajendran et al., 2018).



**Figure 1.** Wound healing phases

### Acute Wound Healing Hemostasis

When the injury occurs, it is aimed to stop the bleeding by forming a blood clot. In the area where the wound is formed, platelets are stimulated by thrombin and growth factors are produced. Growth factors trigger fibroblast formation and migration, as well as vascular endothelial cell migration. With the secretion of serotonin and histamine by mast cells, endothelial cells are opened and neutrophil and monocyte releases occur. In the meantime, vasodilation and vascular permeability are triggered, thus cause the passage of inflammatory cells to the extracellular matrix. In turn, the leukocyte is released into the injured area with activated components. Thus, it is aimed to prevent infection in the region (Okur et al., 2020; Rajendran et al., 2018).

### **Inflammation**

The inflammation phase first begins with neutrophil migration. In the inflammatory response, neutrophils and macrophages are activated. Neutrophils, on the other hand, initiate the release of pro-inflammatory cytokines and active antimicrobial components. Active neutrophil release strengthens the inflammatory response by providing platelet degranulation and bacterial degradation. Dead cell residues and bacteria are removed by macrophages and triggered the normal wound healing process (Okur et al., 2020).

### **Proliferation**

The proliferation phase consists of angiogenesis, extracellular matrix formation and epithelialization. Extracellular matrix formation begins with platelet deregulation. It is supported by proteoglycan and collagen formation. In the proliferative phase, the damaged tissue area is closed with a compact connective structure. This process is called "Tissue granulation". In the next stage, angiogenesis begins with the help of the formation of a hemostatic plug under the influence of growth factors released by platelets. With the help of other cytokines, damaged blood vessels are repaired as a result of triggering neovascularization of endothelial cells. A single layer is formed on the wound by the epithelial cells surrounding the wound. Then, with the proliferation of cells, cell migration stops and basement membrane formation begins (Okur et al., 2020).

### **Maturation (Remodeling)**

The maturation phase, which is the final stage of wound healing, begins with granulation replacing the fibrin formation. In addition, with the accumulation of white blood cells in the wound area, the enzyme necessary for collagen synthesis is secreted by increasing the lactate level. Reshaping of the wound occurs with the accumulation of collagen in the wound area. Thus, collagen integration occurs on the wound again. Cellular proliferation is triggered by growth factors and new cell migrations occur in the wound. During the maturation phase, extracellular matrix proteins are synthesized and stored with the help of fibronectin, glycosaminoglycans, polysaccharides and proteoglycans (Okur et al., 2020; Rajendran et al., 2018).

### **Delayed Wound Healing**

A normal wound healing process takes about 2-3 weeks. But if healing does not follow the normal wound phases, imbalances occur in the physiological mechanism. If any of the healing phases are not met, the wound has chronic or non-healing wound properties. If there is no healing within 6-8 weeks, the wound is called a chronic wound. At this stage, infection develops in the wound, pathological conditions are present and even tissue loss may occur (Rajendran et al., 2018).

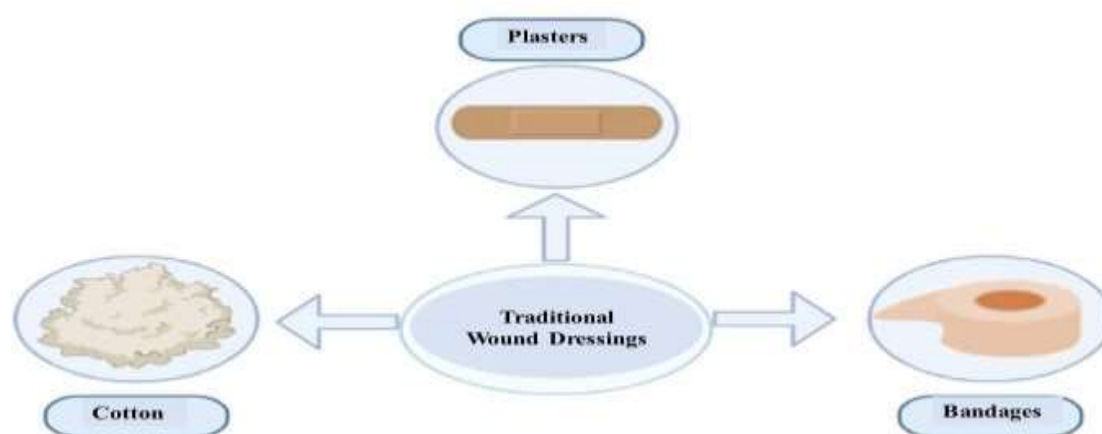
### **Wound Dressings**

Wound dressing materials are divided into various groups according to the type of material used, the purpose of use and its activity. Especially by using natural polymers, bioactive and advanced wound dressing materials are obtained. In addition, with the addition of nanomaterials, these structures are supported and various properties are gained. For example, wound dressing materials with antimicrobial activity are developed using bioactive materials

and nanomaterials. In line with the existence of various wound types and developments in the biomedical field, many wound care regimes emerged and various solutions are developed for effective wound healing. Although basically two types are used to classify wounds, when examined in more detail, it is seen that there are many classification criterias. From this point of view, it turns out that the most important part of the treatment is the appropriate covering material. Unlike the wound dressing materials currently used, it is important to use a dressing material that is suitable and characterized by the nature of the wound. Although bandages, cottons or gauzes, which are traditional covering materials, perform the basic protection task for the wound, parameters such as antimicrobial activity, sufficient moisture and gas permeability that contribute to healing cannot be provided by these materials. In order to overcome such negative properties, various studies are carried out and more advanced wound dressing materials with appropriate properties attract attention (Niculescu & Grumezescu, 2022). Advanced wound dressings have advantages in terms of patient comfort compared to traditional wound dressings and faster healing of the wound without extra damage. Wounds have their unique features, which leads to the use of wound specific covering material. At this point, it is necessary to use different materials in the wound dressing. Herein, polymers attract attention. The functions of polymers can be altered by various chemical modifications, thus improving their physical characteristics and wound-specific use. In addition, especially natural polymers are already present in the structure of living organisms. Some of these form structural components can be used in the wound healing process. In addition, the features required in a suitable wound dressing, such as rapid healing, having the least side effects and costeffectiveness are also met (Rahimi et al., 2020).

### Limitations of Common Wound Dressings

Since ancient times, various materials have been used to treat wounds. These are; bandages, cotton and gauzes (Figure 2) (*Gallery*, n.d.; Liu et al., 2021). The main purpose of the use of these materials is to keep the materials expelled during wound healing and to ensure the healing of the patient. For healing to occur in the wound area, various cell types must migrate to the wound. However, for an effective treatment, various parameters such as environmental conditions and properties of the cover should be evaluated. Considering the various complications of each wound, it is not possible to achieve healing with a single product. At this point, it is especially important to choose the wound dressing depending on the type of wound, wound size, amount of substance expelled, risk of infection and other factors (Okur et al., 2020).



**Figure 2.** Traditional wound dressings



### Advanced Wound Dressings

Advanced wound dressings consist of hydrogels, films, alginates, hydrocolloids and foams (Figure 3)



Figure 3. Advanced wound dressing materials

### Biomaterials Used in Advanced Wound Dressings

Biopolymers, or natural polymers, are obtained from natural sources such as plants, animals, bacteria or fungi. These materials are mainly obtained by transforming components such as amino acids and monosaccharides into forms such as peptides and polysaccharides. There are commonly used polymers in wound healing (Figure 4) (Ferreira et al., 2006; Laurienzo, 2010; Nwodo et al., 2012; A. M. Smith et al., 2016; Vashi et al., 2006). Some plant based polymers are; cellulose, starch and rubber. Examples of animal derived polymers are collagen, hyaluronic acid and chitosan. Fungus derived polymer is chitin. Alginate is an algae derived polymer and bacterial cellulose is an example of a bacteria derived polymer. These samples are preferred because of their more biocompatible, biodegradable and low antigenic properties compared to synthetic polymers (Osmani et al., 2021b).



Figure 4. Biomaterials used in wound dressings

Since most biopolymers have antibacterial or anti-inflammatory properties, they can be targeted to specific cell types, therefore preferred in wound healing. In addition, thanks to the developments in nanotechnology, new generation nanotechnology products dressing materials are obtained by using these polymers. In addition, hydrogels, films or tissue scaffolds can be easily obtained by using biopolymers, or more effective wound dressing materials can be synthesized by combining with other polymers (Table 1) (Osmani et al., 2021a).

**Table 1.** Comparison of polymers which used in wound dressings

| Biopolymer  | Wound Type   | Biological Feature                                 | Disadvantage  |
|-------------|--|--|---|
| Cellulose   | Burns, chronic wounds, some post operative wounds                          | Antibacterial                                      | Not suitable for use in severe burns                        |
| Collagen    | Decubitus sores, minor burns, foot ulcers, chronic wounds, infected wounds | Low cost, high water holding capacity, flexibility | Not suitable for use in patients allergic to animal sources |
| Alginate    | Pressure ulcers, infected wounds, postoperative wounds                     | Hemostatic effect, high water holding capacity     | Not suitable for use in dry wounds and third degree burns   |
| Chitosan    | Acute wounds, pressure ulcers  | Antimicrobial                                      | -   |
| Hyaluronate | Chronic wounds   | Elastic, bacteriostatics                           | -   |

### Hydrogels

Hydrogels are usually transparent. They offer the opportunity to observe without separating from the wound. It is mostly used to provide a moist environment for the wound, but it also provides some absorption of discarded materials. It is used in postoperative wounds, oncological burns and necrotic wounds. It is generally available in three forms; (i) amorphous gels, (ii) gauze impregnated dressings and (iii) paper hydrogels. Hydrogels also have positive effects on the wound, such as reducing pain (Vowden & Vowden, 2017). Release platforms can also be created by combining hydrogel covering materials with hyaluronic acid, antimicrobials or antibiotics. For example; a hydrogel dress containing oxime releases both oxygen and iodine, contributing to the healing of the wound without infection (Vowden & Vowden, 2017).

### Films

Films are covering materials made of thin and elastic polymer materials which are not semipermeable or permeable and protect the wound from external factors. Film covers can be shaped according to wounds in different anatomical regions. While providing permeability mostly in terms of oxygen and moisture, it has no bacterial permeability property. Since it has

a transparent structure, the wound can be observed without separating the covering material. Another advantage is can be produced with multifunctional biomaterials and bioactive agents such as antibiotics and nanoparticles (Liang et al., 2022).

### **Alginates**

Alginates are natural polymers derived from brown seaweed that can often be used as paper or cotton-like coverings. In addition, it retains liquid materials that are thrown out of the wound due to its gelling property. Due to its high water retention capacity, it is especially notable for its use as a blood stopper. It can also be used for infected and non infected wounds. However, alginate dressings are not suitable for use for dry wounds due to their high absorption capacity. In addition, it is required to be used in combination with a secondary cover (Vowden & Vowden, 2017).

### **Hydrocolloids**

Hydrocolloids are usually synthesized using materials such as gelatin, pectin or carboxymethylcellulose and have absorption capacity. Similar to hydrogels, they can hold small amounts of expelled material, therefore can be used in acute and chronic wounds. By completely covering the wound surface, they do not allow the passage of water, oxygen or bacteria. Due to this property, it supports angiogenesis and granulation. In addition, hydrocolloids affect the pH level of the wound and provide an acidic pH and support the destruction of bacteria. Although hydrocolloids have positive properties in promoting epithelialization and retaining necrotic tissues, they are not suitable for use in infectious wounds such as diabetic wounds. Because they are not permeable (Vowden & Vowden, 2017).

### **Hydrofibers**

Hydrofibers are type of hydrocolloid coverings. They have high absorption capacity and releases from the wound surface when they reaches capacity. Such dressings are synthesized using sodium carboxymethylcellulose. For this reason, it prevents the tissue from moisturize. A secondary covering is needed at the point of use of such covering materials (Vowden & Vowden, 2017).

### **Foams**

Foam wound dressings have especially semi-permeable property, can be synthesized in hydrophilic or hydrophobic properties and are used as bacterial barriers. Due to the type of polymer used, it has a high absorption capacity and therefore the capacity to hold the materials thrown out of the wound. It provides thermal insulation, it provides moisture to the wound and also separates easily because it does not stick to the wound. This type of cover can be used with a secondary cover and antimicrobial properties can be gained. However, if it is synthesized with adhesive properties, a secondary cover is not needed. In addition, agents that can be released with foam dressings such as antimicrobial, moisturizer or anti-inflammatory can be used (Vowden & Vowden, 2017).

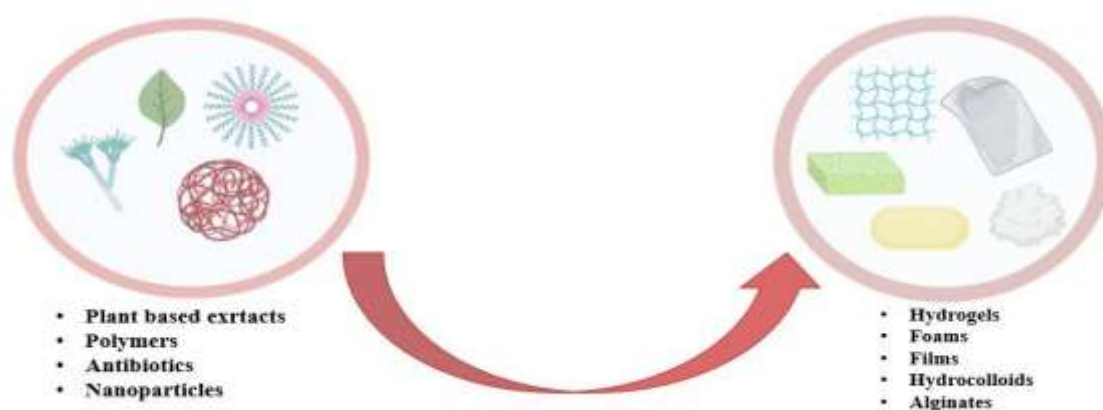
### **Ideal Wound Dressing Materials**

Compared to the traditional wound dressing materials currently used, the properties that should be found in an ideal wound dressing are listed in the form of substances (Selig et al., 2012; Vowden & Vowden, 2017).

- According to the wound characteristics, the dressing that adheres to the wound or does not stick should be preferred
- Wound dressing material should have pain-reducing effect.
- During wound healing, it is necessary to absorb substances expelled.
- Depending on the type of wound, a dress should be used which has different sizes and thicknesses
- The dress should be easily separated from the wound after healing and not cause any tissue loss.
- Dressing material should have antimicrobial activity singly.
- The dressing should be designed for minimal drape during the wound healing process.
- Dress should protect the wound from negative external factors.
- Following the wound, dress should have moisture and oxygen permeability.
- Dress should provide the appropriate moist environment for wound healing.
- It should promote recovery in the fastest way.
- It should not have side effects similar to drugs.
- Dress should be designed ergonomically for the anatomical region to be used and daily life.
- Dress should overcome aesthetic problems caused by wound healing
- Dress must be synthesized from a biocompatible material, biodegradable and cost-effective.
- It must be synthesized from biocompatible material, it must be biodegradable and cost-effective.
- Dress should be easy for the patient to apply without the need for any health client.

### **Wound Dressings with Antimicrobial Activity**

With the use of antimicrobials, favourable conditions are provided for rapid wound healing. In the use of antimicrobial agents in wound dressing materials, it is important to use non-cytotoxic, non-allergenic agents that are specific to the microorganism causing the wound and do not act on normal cells. Commonly used topical agents contain antiseptics such as iodine, silver, polyhexamine, and natural ingredients such as honey (Vowden & Vowden, 2017). Wound dressing materials with antimicrobial activity are synthesized in combination with antibiotics, nanoparticles or biological extracts (Figure 5) (Liang et al., 2022).



**Figure 5.** Antimicrobial wound dressings

### **Antibiotics**

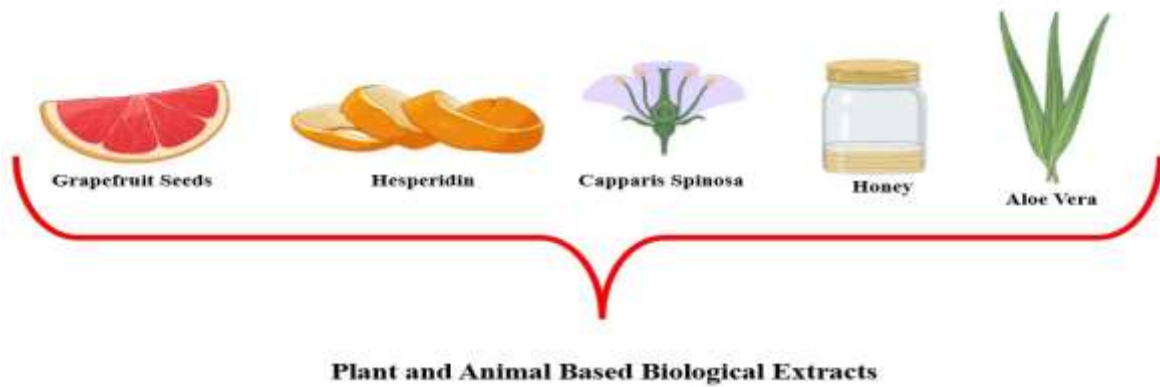
It is known from past to present that antibiotics are the most common and effective antimicrobial agents. Antimicrobial activity is provided by interacting antibiotics with covering materials. For example; by adding amoxicillin to the precursor solution prepared to obtain the hydrogel, the antibiotics are trapped in the hydrogel and controlled release is made (Abbasi et al., 2020). In another example, amikacin was similarly loaded into the hydrogel and reported to affect *S. aureus* and *P. aregnosa* (Choudhury et al., 2020).

### **Biological Extracts**

Some biological extracts of vegetable and animal origin are known to exert antimicrobial action (Figure 6) (Gallery, n.d.; Okur et al., 2020; Zhang et al., 2007). For instance; fumaric acid is a natural acid found in honey, it has been used as an antibacterial, anti-inflammatory and painkiller for a very long time. In the studies carried out, positive results were obtained in about 80 types of bacteria and yeast species after treatment with honey. With a small amount of honey, the growth of many types of microorganisms is inhibited. For this reason, there are various studies on the use of honey with covering materials (Rafati et al., 2020).

Hesperidin is a type of flavonoid found in fruits and vegetables. It has the potential to be used as a therapeutic agent in skin diseases because it has anti-inflammatory, antibacterial, antioxidant and angiogenic effects. In a study by Majid Salehi et al. (2020), a hydrogel was obtained by adding hesperidin to chitosan/alginate solution and this combination had a synergistic effect in strengthening the antibacterial effect (Bagher et al., 2020).

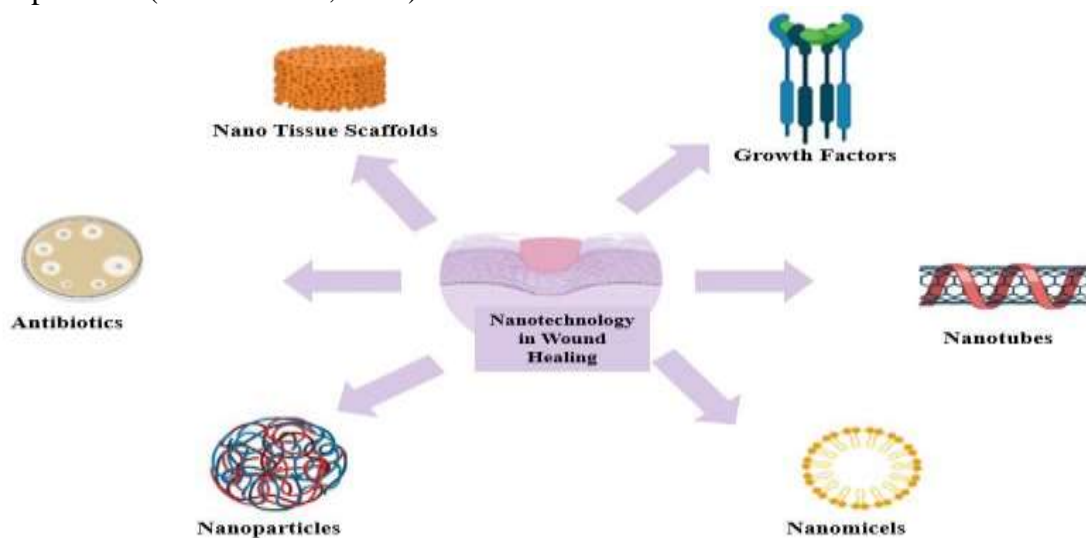
It has been observed that the extract obtained from grapefruit seeds forms an inhibition zone in bacterial colonies with sodium carboxymethylcellulose/hydroxymethyl cellulose hydrogel films (Koneru et al., 2020).



**Figure 6.** Biological extracts which used wound dressings

### Nanotechnology in Wound Healing

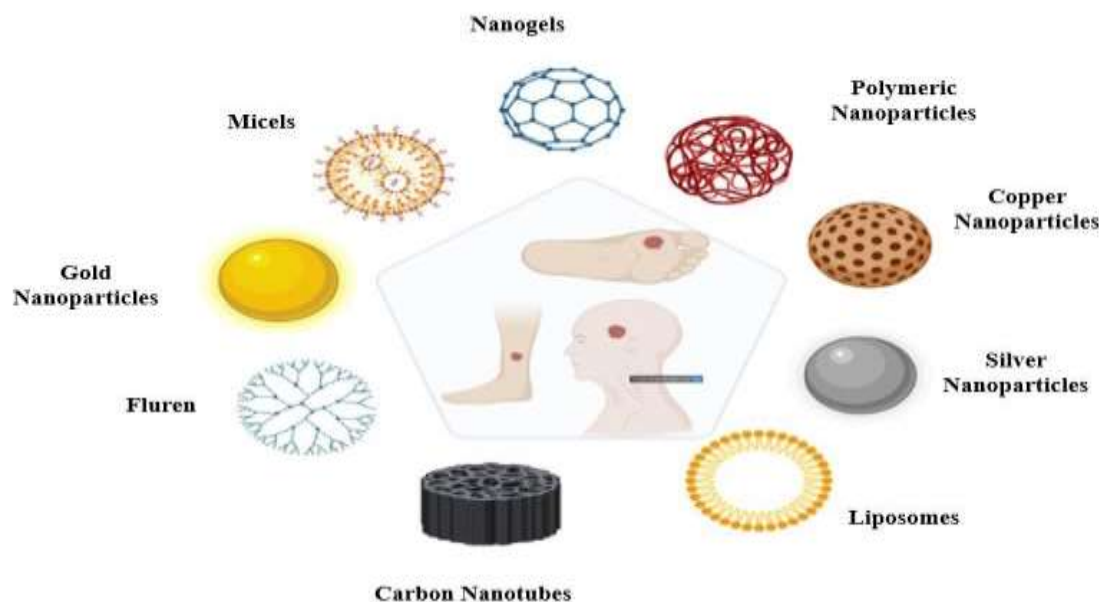
With nanotechnology, the direct release of gas molecules, natural products, bioactive plant components and growth factors is achieved (Figure 7) (Kant et al., 2014; Osmani et al., 2021a; Rajendran et al., 2018). With nano tissue scaffolds, appropriate wound dressing materials can be produced reduce infection and provide rapid healing (Rajendran et al., 2018). Nanotechnology allows the use of bioactive plant components as antimicrobials and wound healers. For example; Turmeric is a plant variety which knows to have antimicrobial, antiinflammatory and antioxidant activity. Studies have shown that reepithelization improves and fibroblast proliferation increases as a result of topical application of turmeric, as well as storing collagen and various growth factors. Besides, turmeric has a very short half-life and can be easily absorbed. For this reason, since it has a disadvantage, its use with nanoparticles as a solution is noteworthy (Kant et al., 2014; Kiran et al., 2013). Nanoparticles are used to deliver various components to the wound site. For example; nitric oxide (NO), which is one of the important endogenous molecules, is found in very low amounts especially in chronic wounds, so giving NO to the wound area from the outside attracts attention as a promising type of therapy. However, there are limited number of studies on this subject in terms of application. A study by Blencher et al. (2012) showed that blood vessels and fibroblast formation increased and collagen formation was triggered in mice treated with topical application of NO containing nanoparticles (Blecher et al., 2012).



**Figure 7.** Nanotechnology in wound healing

### The Use of Nanoparticles in Wound Healing

Since wound healing is a complex process, there are still various problems. For this reason, different strategies need to be developed. The use of nanotechnology and nanomaterials in modern medicine, which offers the opportunity to work at nanometer size, has gained great speed in recent years. The combined use of biomaterials with nanoparticles have a positive potential in wound dressing materials. At this point, the metal nanoparticles silver, gold and zinc are particularly noteworthy ( Figure 8) (Blanco-Fernandez et al., 2021).



**Figure 8.** The use of nanoparticles in wound healing

Metal nanoparticles have properties such as low *in vivo* toxicity, bacteriostatic effect and bactericidal activity. When the material size drops to nano size, the surface area and surface area/volume ratio increase, which gives the material positive properties and offers the possibility of active use in wound healing (Table 2) (Rajendran et al., 2018; Kalashnikova et al., 2015).

**Table 2.** Nanoparticles and biological features in wound dressings

| Nanoparticle Species    | Biological Feature  |
|-------------------------|---|
| Silver Nanoparticles    | Antibacterial activity, anti-inflammatory effect, wound healing, disrupting the DNA and electron transfer system in the microorganism |
| Gold Nanoparticles      | Acquiring antimicrobial properties, accelerating wound healing  |
| Nanogels                | Activation of fibroblasts   |
| Polymeric Nanoparticles | Accelerating angiogenesis, accelerating cell proliferation, hemostasis  |

|                               |   |
|-------------------------------|---|
| <b>Titanium Nanoparticles</b> | Accelerating the release of ROS to the microorganism, disrupting the cell membrane  |
| <b>Zinc Nanoparticles</b>     | Antimicrobial property, anti-keratinase, antiinflammatory, anti-elastase, disrupting the cell membrane in the microorganism |

### **Silver Nanoparticles (AgNPs)**

For many decades silver has been used for wound healing. Among the wounds, the use of silver especially in burns and diabetic wounds is noteworthy. Although the first use of silver nitrate began with ulcer treatment in the 17th-18th century, its antimicrobial activity has been discovered in the 19th century. It was used for the treatment of soldiers during the First World War but lost its popularity with the discovery and synthesis of antibiotics afterward. More recently, it has been particularly notable for its nanoparticle form (Rahimi et al., 2020). Silver nanoparticles have a size of 1-100 nm and exhibit chemical stability, antibacterial properties, and anti-inflammatory activity. In biomedical applications, silver or silver nanoparticles have antimicrobial activity on approximately 650 microorganisms by adding to covering materials, creams or antiseptic sprays. Although silver normally has a toxic effect in the form of silver nitrate, silver nanoparticles used as a nanotechnology product have an antibacterial effect on bacteria and have lower toxicity due to their large surface area and volume ratio (Rahimi et al., 2020).

Recently, there are wound dressing materials containing silver in different forms, which play an active role in the process of healing chronic wounds. The potential effect of silver as a nanoparticle is increased by the combined use of AgNPs with biomaterials. AgNPs are known to have antimicrobial activity against bacteria, fungi, viruses and protozoa. AgNPs prevent biofilm formation and detoxify bacterial toxins by causing damage to the quorum-sensing mechanism in the microorganism. *In vivo* conditions, AgNPs oxidize in the acidic environment and release silver ions into the environment. While silver nanoparticles do not have a biological function alone, silver ions formed as a result of oxidation show antimicrobial activity, causing damage to the cell wall and DNA, inhibiting ATP production and causing ROS products (Rajendran et al., 2018).

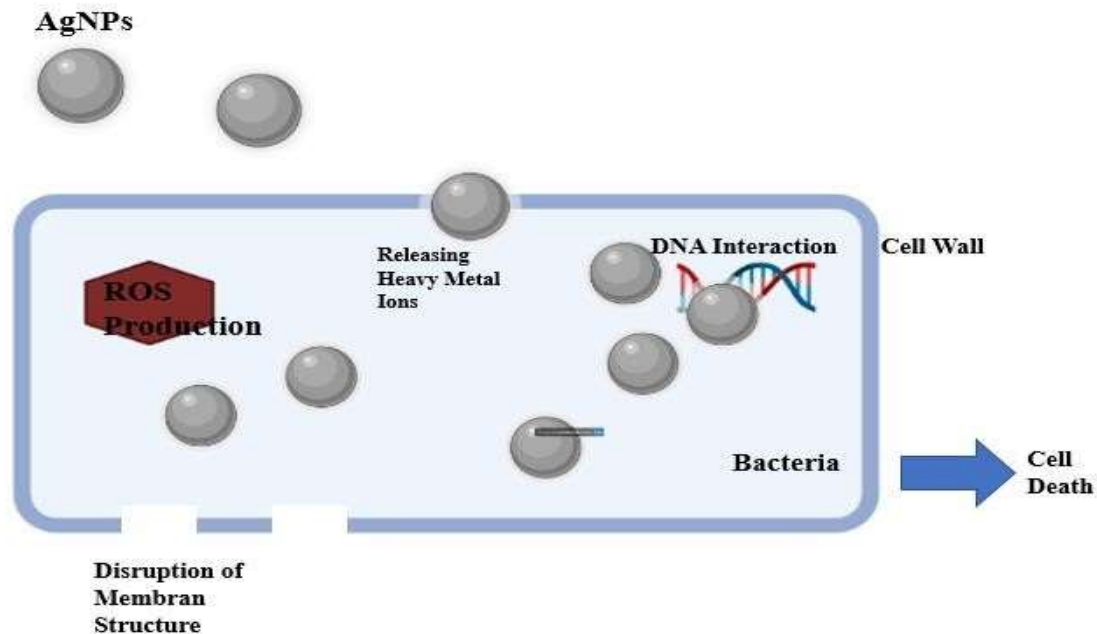
Due to their antimicrobial properties, AgNPs support normal wound healing phases by providing bacterial inhibition. In a recent *in vitro* study, the addition of AgNPs in human keratinocytes and dermal fibroblasts resulted in lower levels of inflammatory cytokines, reduced oxidative stress, and increased wound healing. Similarly, the combination of a covering material made of biocompatible bacterial cellulose with AgNPs has shown increased keratinocyte proliferation at the wound site (GhavamiNejad et al., 2015).

### **Antimicrobial Activity of AgNPs**

Silver nanoparticles are used as antimicrobial agents, especially in the medical field. In aqueous solutions, Ag<sup>+</sup> ions are formed by the decomposition of silver nanoparticles into ions and which interact electrostatically when the microorganism encounters it. Ag<sup>+</sup> groups that interact with sulfur and phosphorus groups in the bacterial cell wall, cause damage to the structural proteins of the bacterium and the cell wall. Damaged proteins directly endanger the viability of the microorganism. In addition, Ag<sup>+</sup> ions bind to negatively charged units on the membrane, creating an opening and disrupting the proton gradient, causing cell death. In



addition, Ag<sup>+</sup> ions that interact with bacterial DNA and RNA also inhibit cell division (Figure 9) (Rahimi et al., 2020; Rajendran et al., 2018).



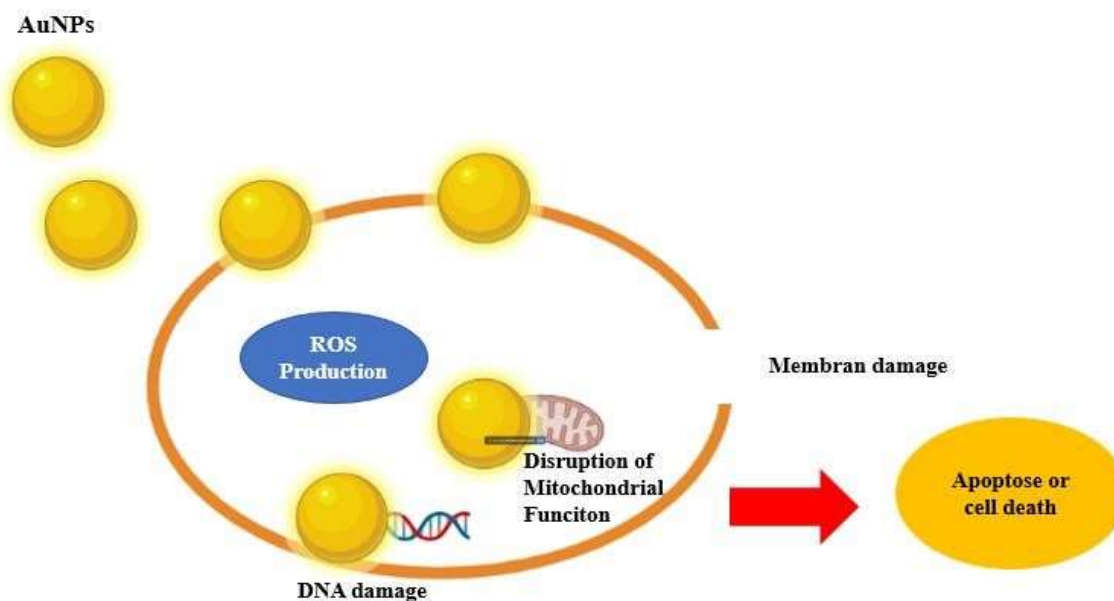
**Figure 9.** Antimicrobial mechanism of AgNPs

### **Gold Nanoparticles (AuNPs)**

Gold nanoparticles exhibit biocompatibility properties and are widely used in tissue regeneration, targeted drug delivery and wound healing. Unlike silver, gold nanomaterials alone do not have antimicrobial activity. For this reason, AuNPs need to be used in combination with a biomolecule. For example; when AuNPs are crosslinked with collagen, they can be combined with another polysaccharide, growth factor, peptide or molecule. Thus, it can be used in wound healing by gaining properties such as biocompatibility, and biodegradability. In addition, similar to collagen, biomaterials such as gelatin and chitosan can be combined to obtain materials with reliable and positive properties and these can be used in wound healing (Akturk et al., 2016; Jayakumar et al., 2011). Moreover, gold can be conjugated with antimicrobial drugs or other nanoparticles, thus, can be increased its antimicrobial potential. For example; AuNPs conjugated with vancomycin; increase the activity of vancomycin and show activity against vancomycin resistance. In addition, AuNPs are used in photothermal therapy by conjugating pathogen-specific antibodies (Jayakumar et al., 2011). AuNPs are combined with polymers or stem cells to give wound healing properties. For example; biocompatibility is supported by using chitosan-AuNPs and free radicals are eliminated (Volkova et al., 2016).

### **Antimicrobial Activity of AuNPs**

Antimicrobial activity in gold nanoparticles occurs when they enter the cell and change the membrane potential. Thereby inhibiting the enzyme ATP synthase. Cell death occurs with the consumption of ATP in the cell and the disruption of energy metabolism (Figure10) (Akturk et al., 2016). In addition, AuNPs support wound healing by triggering anti-inflammatory cytokines and angiogenesis (Rajendran et al., 2018).



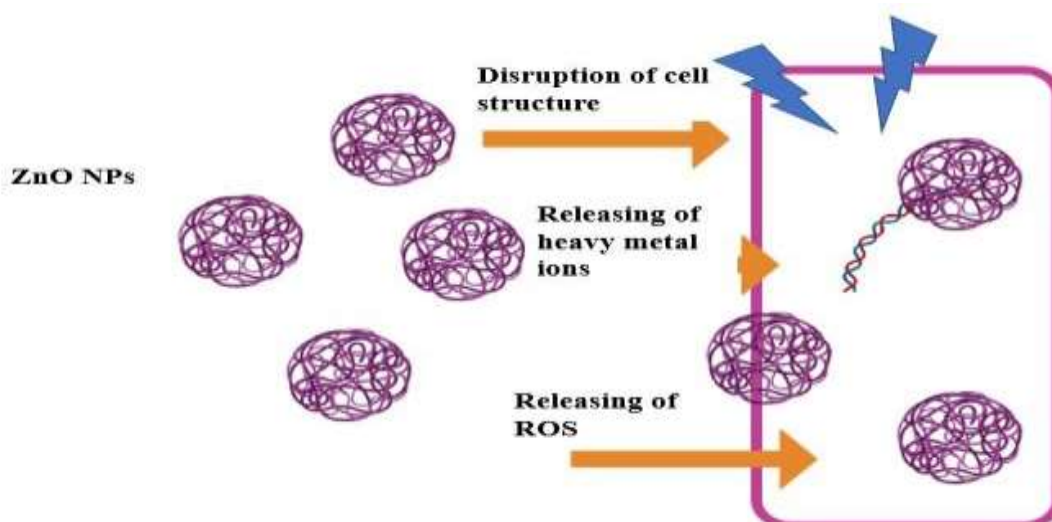
**Figure 10.** Antimicrobial mechanism of AuNPs

### **Zinc Oxide Nanoparticles (ZnONPs)**

ZnONPs are widely used in the medical and cosmetic industry due to their biocompatibility, biodegradability and bactericidal activity. In addition, as shown in previous studies, it shows inhibitory properties against many pathogenic bacteria. For example; 1 mM 8 nm ZnONPs have an inhibitive effect of approximately 95% on *S. aureus* growth. However, the inhibition effect decreases as the nanoparticle size increases. For this reason, the antimicrobial effect in zinc oxide nanoparticles varies depending on particle size (Xu et al., 2020b).

### **Antimicrobial Activity of ZnONPs**

When ZnONPs interact with the bacterial cell wall or are taken into the cytoplasm, cell death occurs as a result of interacting with the cell membrane (Figure 11) (Dimapilis et al., 2018). In addition, oxidative stress is triggered because zinc oxide nanoparticles cause high amounts of ROS production in aqueous media (Xu et al., 2020b).



**Figure 11.** Antimicrobial mechanism of ZnONPs

### **Polymeric Nanoparticles**

Polymers are long-chain organic molecules consisting of repeating units and can also be found in the nano dimension. Polymers can have natural or synthetic origins and can be shaped for specific needs and biological applications. Most biopolymers are widely used in biomedical equipment, coating of medical products and vascular grafts. The biggest advantage of polymers is that they are easy to use, low cost and suitable for use in various covering materials. Nanostructured polymeric materials also allow use in tissue scaffold construction, biological carriers and vascular grafts. For example; coating materials synthesized using natural polymeric hydrogels show better results in tissue regeneration compared to synthetic polymers. Coating materials obtained by the combined use of hydrogel tissue scaffolds and various growth factors provide infiltration of waste materials which are released during wound healing, contribute to the support of new blood vessels and rapid healing of the wound without allowing it to become chronic. Therefore, with the effective use of polymeric compositions in wound healing, positive results can be obtained in both normal wounds and chronic wounds (Rajendran et al., 2018).

### **DISCUSSION**

Today, almost every individual is exposed to wound formation due to cuts, burns, any disease or an operation. In some cases, these wounds heal quickly, while in others they are contaminated by various pathogens and undergo microfloral changes. Contamination occurs due to gram-positive and gram-negative pathogenic bacteria and infections are triggered. At this point, the use of wound dressing material with antimicrobial activity for preventing or eliminating infection is noteworthy. As a result of the use of various materials with different surface combinations or antimicrobial agents, antibacterial, antifungal wound dressing materials are developed. It is thought that such therapeutic solutions will have positive results soon (Simões et al., 2018). It is important to determine the appropriate material in wound dressing applications. At this point, polysaccharide-based materials have many biological and healing properties that are desired to be in the wound dressing due to their high biocompatibility, biodegradability, low toxicity and carrying various functional groups. In addition, the fact that it can carry many bioactive components brings with it different healing and antimicrobial properties. Silver nanoparticles are widely used in polysaccharide based wound dressing

materials. Thus, antibacterial and antifungal properties are gained. Although there are many developments in wound dressing materials, there are still points to be improved and disadvantages that need to be worked on to improve them. For this reason, modifications should be developed to minimize the disadvantages on the subject, and the wound healing process should be made more economical and effective by using different polymer types and techniques (Rahimi et al., 2020). Although various detection methods have been found to prevent antimicrobial resistance, the development of multidrug resistance has led to the idea that new therapeutic agents should be developed. At this point, nanotechnology based metallic nanoparticles come to mind first. Although nanoparticles are considered alternatives to antibiotics due to their high biological activity, there are still number of problems with their toxic effect. To solve this problem, studies are being carried out to biocompatible metal based nanoparticles with various materials. Thus, it is aimed to prevent drug resistance by using nano structures alone or combined with various platforms (Shaikh et al., 2019).

Each wound has its own characteristics and requires complex treatment in terms of time and effectiveness. The treatment of an infected wound is a long term process and it can causes various problems such as biofilm formation, multi drug resistance or heterogeneous wound nature from a clinical point of view. Although there are more than 3000 different wound dressing materials on the market, there is still no covering material suitable for use in chronic wounds. Besides, the method of treatment with antibiotics is widely used today but there are drug resistance problems. It is thought that all of these problems can be overcome with topically appropriate antimicrobial application. Treatment methods are expanded through the use of enhanced wound dressings, nanomaterials and natural materials, resulting in better drug release, degradation profile and adhesion (Rezvani Ghomi et al., 2019 ;R. Smith et al., 2020).

## CONCLUSIONS

Wound healing is not a process that proceeds in the same direction for every wound. For this reason, it is necessary to fulfil the appropriate requirements in each phase and to be treated with an appropriate covering material. There are various limitations to the use of traditional wound dressings. In recent years, with the developments in the medical field especially advanced wound dressing materials are widely studied and these materials replace traditional dressing materials. Advanced wound dressing materials, which have a wide range of species, are synthesized using bioactive materials and supported by nanoparticles. Thus, various properties such as antimicrobial activity, biocompatibility, biodegradability and cost-effectiveness are given to the products. Wound dressing materials with antimicrobial activity attract attention with their easy use, fast healing feature and ideal dressing properties. Soon, the use of nanoparticle based and multifunctional wound dressing materials in wound treatment is promising. (Niculescu & Grumezescu, 2022; Wang et al., 2021; Zhang et al., 2007).

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## **BIOCHEMICAL CHARACTERISTICS OF WINES FROM AUTOCHTHONOUS GRAPE CULTIVARS IN VARIOUS AREAS OF ALBANIA**

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### **ABSTRACT**

The aim of this study is to investigate the biochemical components of Albanian wine produced from autochthonous grape varieties from different growing regions. Alcoholic fermentation is a biological process carried out by yeast. Maceration is a physic-chemical process that allows the extraction of anthocyanin and tannins to obtain the typical colour and structure of red wine. The control parameters of both processes are different, so it is necessary to optimise the conditions to control each of them. Temperature, mechanical processes and aeration are useful to promote extraction and improve the final balance of phenolic compounds in the wine. Phenolic compounds are natural bioactive known for their numerous health benefits. In the case of wines in particular, polyphenols and quality are closely linked. These components are very important for the quality of the wine as they contribute to the sensory characteristics: colour, taste, mouth feel, aroma, astringency and bitterness. For this study, several autochthonous varieties of black and white grapes (*Vitisvinifera* L.) were considered, such as: SheshiZi, (from the areas of Lundra, Berat, Paskuqan, Durrës), Kallmet (from Lezha, Kolplik, Rrëshen), Sheshi i Bardhë (from Paskuqni, Berat) and the last variety Pulës (from Berat) and are compared with the respective variety from the "Agricultural Technology Transfer Centre", Vlora. The trials and analyses are carried out in the laboratories of the Food Research Centre of the Faculty of Biotechnology and Food.

**Key Words:** Wine, Biochemical Components, Phenolic Compounds; Black and White grape

### **INTRODUCION**

The development of viticulture and oenology in Albania dates back to ancient times, due to the soil and favourable climatic conditions. The combination of natural resources of the regions of the country and possible varieties will make possible the production of typical wines (Yoncheva and Gülcü, et al, 2017). Both grapes and wine have a complex and diverse chemical content. Various technical and agricultural aspects influence it. Winemakers in Europe have found that the type of soil when growing grapes can create the difference between a great wine and a poor wine (Cheng. et al, 2015). The phenolic compounds are important components of wines. They have a significant effect on their organoleptic profile, especially in terms of colour intensity and taste characteristics such as density, tartness, and bitterness. Most of them pass from the grapes, so their content in wines is determined by the variety, and its potential and phenolic reserves (Ghiselli et al., 1998; Lee and Koh, 2001). These components are extracted from the grapes during the alcoholic fermentation process thanks to the influence of various technological parameters. place. (Lamce, et al, 2020; Yoncheva, et al, 2018; Niculescu, et al, 2018). In recent years, due to the interest in the production of quality wines in addition to the work done, with the selection and cultivation of varieties suitable for the area, there has been a



tendency to change or improve fermentation schemes (Lamce, et al, 2018;). This interest has also appeared due to the limited knowledge of grape varieties of specific areas with destination wine production (Mitrevska. et al, 2020). Based on this fact, the purpose of this study is to evaluate the chemical composition of wine produced from autochthonous grape varieties grown in some areas of the country.

## **MATERIALS AND METHODS**

The varieties of black and white grapes (*Vitisvinifera* L.) used for this study, such as: Shesh i Zi, from the area of Lundra, Berat, Paskuqani, Durrës, and QTTB of Vlora, Kallmet from the area of Lezha, Kolplik. Rrësheni and Vlora QTTB, Sheshi i Bardhë from the area of Paskuqni, Berat and Vlora QTTB, and the last variety Pules from the area of Berat and Vlora QTTB. The samples, was harvest in optimal ripping and were transported to the Research Center of the Faculty of Biotechnology and Food. This study was carried out according to the classic fermentation scheme. Thus, the quantity of grapes obtained was divided from 100 kg for each variety. In order to control the fermentation process, the wines were treated with the same dose of 5 g/hl of SO<sub>2</sub> and inoculated with a dose of 20 g/hl of commercial yeast. Fermentation took place at a controlled temperature of 14-18 °C for 10 days. The progress of fermentation was checked daily (% sugar and temperature). To observe the progress of the colour change of the wine, samples were taken for analysis every two days during fermentation and every six months. Before analysis, the samples were centrifuged to remove solid suspensions.

*Determination of free and bound sulphur dioxide.*

For the determination of sulfuric anhydride, the method according to OIV (method OIV-MA - AS323-04B) was used.

*Determination of total acidity.* This determination is carried out according to the OIV (method OIV-MA -AS313-01).

*pH determination.* The pH value is determined according to the method of the OIV (method OIV-MA -AS313-15).

*Determination of alcohol content.* The alcohol content is determined according to the OIV method - MA - AS312 - 01A.

*Determination of volatile acidity.* The determination of volatile acidity is carried out according to (OIV-MA -AS313-17 method).

*Determination of total polyphenols.* The determination of total polyphenols was carried out according to the method described in Cetó et al, 2012.

*Determination of tannins.* Tannins are determined according to the method described by Porter et al. 1985.

*Determination of the total amount of anthocyanins in wine.* Anthocyanins are chemical compounds found in the skin of grapes whose color changes with the acidity of the environment in which they are found. Their quantitative determination is carried out according to the method described by Puissant and Leon, 1967.

*Color Intensity.* Color intensity is done according to Glories, 1984. For red wines, the influence of both red color (from anthocyanins, which are responsible for red color in wines) and yellow color (from tannins) must be considered.

*Statistical analysis.* Analyzes were performed with at least three replicates, and all results were statistically analyzed with Statistix 9 software using analysis of variance (ANOVA). Differences were considered significant when  $p < 0.05$  and PC principal component using Cambo the Unscramble 10 software.

## RESULTS AND DISCUSSIONS

During fermentation, a series of biochemical processes take place, that affect the extraction of the components responsible for the aromas and flavors in wine (Añón. et al. 2014). Based on different bibliographies, the study was carried out to investigate the performance of the chemical compositions of wine produced by native cultivars in different growing areas.

### Chemical content in red wine.

#### a. Total polyphenols

Polyphenols are important constituents present in considerable amounts in white and red wines. Their content is influenced by many factors, such as genetic (Fang et al., 2008), agronomic (Di Majo et al., 2008), technological (Olejar et al., 2015), etc. However, different fermentation schemes directly affect the extraction of these components in wine (Olejar et al., 2015). The results obtained in this study on the total content of polyphenols are shown in Table 1.

**Table 1:** Average content of total polyphenols of Shesh i Zi and Kallmet cultivars grown in different areas.

| Cultivars  | Area     | Total Polyphenols (mg/L gallic acid) |
|------------|----------|--------------------------------------|
| Shesh i Zi | Paskuqan | 507.58 ± 8.5 <sup>a</sup>            |
|            | Lundër   | 2284.2 ± 4.7                         |
|            | Berat    | 2199.9 ± 79.6                        |
|            | Vlorë    | 1177.3 ± 0.87                        |
|            | Durrës   | 529.94 ± 0.81                        |
| Kallmet    | Lezhe    | 1013.4 ± 21.6                        |
|            | Vlorë    | 1431.4 ± 5.4                         |
|            | Koplik   | 632.3 ± 2.0                          |
|            | Rrëshen  | 440.49 ± 1.02                        |

a – Means ± SD

As shown in Table 1, the total polyphenol content of Sheshi i Zi wine had higher average values of this component in the Lundra and Berat area than in the Durrës, Vlora and Paskuqan area. In addition, the behavior of this variety contributed lower amounts of total polyphenols in the areas of origin, Paskuqan and Durrës. The average content of total polyphenols in the wine of the Kallmet variety is shown in Table 1. According to this Table it can be seen that the area of origin of the cultivation of this variety presented the lowest values for this parameter, while

in the Vlora area, the wine produced by the Kallmet variety has obtained the highest average values in polyphenols. total about 1431 mg/kg of gallic acid. These differences in the amount of total polyphenols between the area of origin and other cultivation areas of the two varieties studied are due to climatic and agronomic conditions, as well as the composition of the soil, which directly affect the amount of total polyphenols in wine. Several studies have shown the impact of climatic parameters on the final concentration of phenols in the grape, also in the results obtained by Van Leeuwen et al. 2004 have shown the influence of climatic conditions, the soil and the variety on the chemical composition of the grape.

### *b. Total tannins*

Tannins play an important role in winemaking, as they are used to improve various wine characteristics (mainly color stability) or to supplement low tannin levels. Table 2 show the average values of total tannins in red wine from two autochthonous varieties grown in different areas of the country.

**Table 2.** The average content of total tannins varies. Shesh i Zi and Kallmet grown in different areas.

| Cultivars  | Area     | Total Tannins<br>(mg/L epicatechin) |
|------------|----------|-------------------------------------|
| Shesh i Zi | Paskuqan | 2.37 ± 0.01 <sup>a</sup>            |
|            | Lundër   | 3.82 ± 0.01                         |
|            | Berat    | 3.46 ± 0.03                         |
|            | Vlorë    | 1.66 ± 0.02                         |
|            | Durrës   | 2.44 ± 0.01                         |
| Kallmet    | Lezhe    | 2.51 ± 0.04                         |
|            | Vlorë    | 1.86 ± 0.07                         |
|            | Koplik   | 2.54 ± 0.07                         |
|            | Rrëshen  | 3.13 ± 0.03                         |

a – Means ± SD

Table 2 shows the total tannin content of red wine from two autochthonous varieties grown in different areas. Table 2 shows the average values of total tannins of the red wine of the Shesh i Zi variety, where it can be seen that the wine of this variety grown in the Lundra area has higher values, while the wines of this variety cultivated in the Vlora area have shown lower mean values (1.66 mg/L). Table 2 shows the mean values of the total tannins of the wines produced by the Kallmet variety, as it can be seen that the wine produced by this variety in the Lezha and Koplik areas of origin has the highest values. The wine produced by the Kallmet variety, cultivated in the Vlora area, presented the lowest average values of these compounds, with an average value of 1.85 mg/L of epicatechin. As can be seen, the content of total tannins in the wine was higher in the area of origin in the two varieties studied. The differences in this component in other cultivation areas is possibly due to the concentration of tannins in the fruit, which can be reduced by environmental factors such as the water status of the vine, heat, solar radiation and the energy of the vine. (Kennedy et al. 2000, 2002, Cortell et al. 2005). In addition, its content can be affected by winemaking techniques, since tannins are extracted from the skins and seeds into the wine during alcoholic fermentation; its final concentration in the wine can increase or decrease depending on the winemaking style and technique (Sacchi et al. 2005, Lamce et al., 2020). Since in this study the technological scheme for vinification is the same,

we conclude that the influence of climatic and environmental conditions could have affected the total content of tannins in the wine.

### *c. Total anthocyanins.*

Anthocyanins are chemical compounds found in the skin of grapes and that change colour depending on the acidity of the environment in which they are found. As the wine matures, they form easily soluble aggregates in the wine and precipitate, so the wine loses colour over time. The purpose of adding oenological tannins is to keep the anthocyanins bound in the wine, preventing their precipitation. The results of the total amount of anthocyanins in the wine produced from two native varieties of red wine grown in Albania are shown in Table 3.

**Table 3.** Average total anthocyanin content of Shesh i zi and Kallmet cultivars grown in different areas.

| Cultivars  | Area     | Total anthocyanins (mg/L gallic acid) |
|------------|----------|---------------------------------------|
| Shesh i Zi | Paskuqan | 418.10 ± 0.39 <sup>a</sup>            |
|            | Lundër   | 235.73 ± 1.06                         |
|            | Berat    | 145.21 ± 0.39                         |
|            | Vlorë    | 234.20 ± 5.41                         |
|            | Durrës   | 416.73 ± 8.00                         |
| Kallmet    | Lezhe    | 390.78 ± 3.43                         |
|            | Vlorë    | 164.10 ± 1.57                         |
|            | Koplik   | 306.35 ± 5.12                         |
|            | Rrëshen  | 329.34 ± 9.59                         |

a – Means ± SD

As shown in Table 3, the wine produced by the Shesh i zi variety in the Durrës area had the highest average values of total anthocyanins (416.73 mg/L), while the red wine produced by the same variety but in the Berat area it had the lowest values for these compounds. In the wines made by the Kallmet variety (Table 3), the total anthocyanin content in the Lezha area was around 390.78 ± 3.43 mg/l, a mean value higher than that obtained in other studied areas. As can be seen from the results, it is noted that these compounds are related to the content of tannins. Well, the wines that have shown low total tannin content for the two varieties studied, have high total anthocyanin content. According to the ANOVA statistical analysis, it is observed that the influence of the cultivation area has a significant influence with a value of  $p \leq 0.05$ . However, it is mentioned in the literature that the presence and concentration of each group of anthocyanins is specific to the variety, but the content of this component varies due to the influence of environmental conditions and viticultural practices. These changes are reflected in the colour and consistency of the wine (Košmerl et al., 2013).

### *d. Colour Intensity.*

The colour intensity is an important parameter and is related to the evaluation of the oxidation of the red wine as a result of various changes that occur during the fermentation and storage of this product. The oxidation of the wines is shown by the darkening of the colours caused by the increase in the value of the optical density, the oxidation of the phenolic components. The results of evaluation of colour of wines from Shesh i Zi and Kallmet varieties in some growing areas are presented in Table 4.

**Table 4.** Values of intensity, index and tonality of wine colour of two autochthonous varieties in different growing areas.

| Cultivar   | Area     | Colour Intensity         | Colour Index | Colour Tonality |
|------------|----------|--------------------------|--------------|-----------------|
| Shesh i Zi | Vlorë    | 60.57 ± 0.5 <sup>a</sup> | 1.59 ± 0.01  | 0.62 ± 0.00     |
|            | Paskuqan | 8.74 ± 0.04              | 0.40 ± 0.01  | 2.44 ± 0.01     |
|            | Durrës   | 10.20 ± 0.1              | 1.79 ± 0.01  | 0.56 ± 0.00     |
|            | Berat    | 7.43 ± 0.06              | 1.80 ± 0.01  | 0.55 ± 0.00     |
|            | Lundër   | 13.68 ± 0.04             | 8.02 ± 0.02  | 2.15 ± 0.01     |
| Kallmet    | Vlorë    | 5.04 ± 0.09              | 1.56 ± 0.02  | 0.64 ± 0.01     |
|            | Lezhe    | 22.64 ± 0.09             | 0.75 ± 0.00  | 1.33 ± 0.00     |
|            | Koplik   | 7.17 ± 0.10              | 1.67 ± 0.02  | 0.60 ± 0.01     |
|            | Rrëshen  | 8.73 ± 0.09              | 1.49 ± 0.02  | 3.14 ± 0.04     |

a, Mean ± SD.

As can be seen in this table, the colour intensity values for the Shesh i zi variety are higher when the wine is produced in the Vlora area (60.57 ± 0.5). The statistical analysis ANOVA shows that the area has a significant influence with a value of  $p \leq 0.05$ . The wine obtain from Kallmet variety, has shown a high intense colour when this variety is grown in the Lezha area. The wine produced in the Vlora area has a lower colour intensity with a value of around 5.04 ± 0.9.

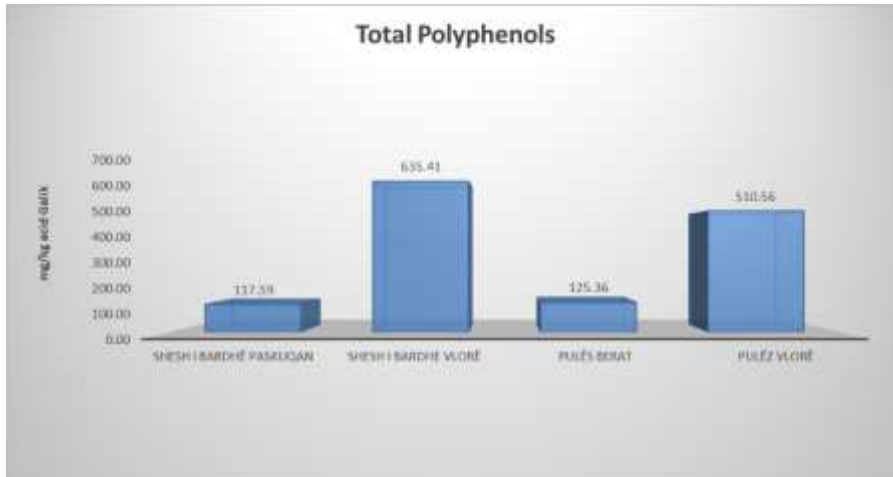
### *Chemical content in white wine*

#### *a. Total polyphenol*

In the literature, different classifications of wines are made by variety, vintage and geographical origin based on different criteria such as phenolic compounds (Jaitz et al., 2010, Soto Vázquez et al., 2011), combination of polyphenols and antioxidant activity. (Hosu et al., 2014), isotopic ratios (Adami et al., 2010, Avram et al., 2015), volatile aromatic compounds (Rodríguez-Nogales et al., 2009) and amino acids (Bouloumpasi, et al., 2002). Polyphenols control colour, aroma, flavour and bitterness, act as photoprotective pigments and antioxidants, and play a fundamental role in wine quality (Cáceres-Mella et al., 2014). The phenolic composition of wines also affects colour stability and browning responses, and higher polyphenolic content contributes to wine stabilization against deleterious effects of temperature (Czibulya et al., 2012). Phenolic compounds can be successfully used to assess wine

authenticity, as they are characteristic of the wine variety and can provide information on geographic origin (Pavloušek et al., 2013).

In this study, two types of white wine were investigated, namely Pules and Shesh i Bardhë. The selected wines are produced in two different regions. The results of total polyphenols of white wine are shown in Figure 1.



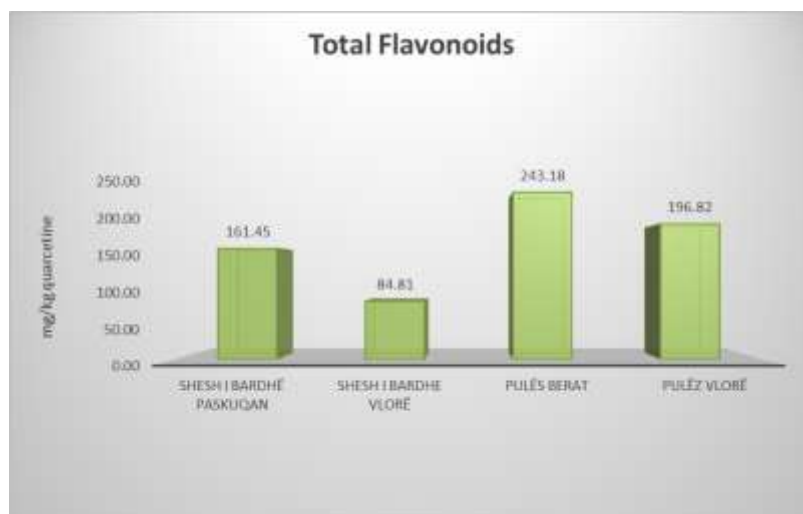
**Figure 1.** Average content of total polyphenols of the varieties Shesh i Bardhë and Pules grown in different areas.

In the Shesh I Bardh wines, there is a difference between the two areas. The content of total polyphenols in the Paskuqan area is 117.59 mg/l, while in the Vlora area it is 635.41 mg/ml. This difference can be attributed to the different winemaking conditions in these wineries. Polyphenols are found in the solid part of the grapes, and their extraction depends on the winemaking conditions, such as the pressing process, pH and temperature. Among the wines from Pules variety, the wines from the Berat region have the highest total polyphenol content (125.36 mg/L), while the wines from the Vlora region have a total polyphenol content of 510.56 mg/ml. The total polyphenol content of the two wines grown in specific areas showed different values. Thus, the wine from White Square, grown in Vlora, had almost twice the content of the wine from the Paskuqan area of origin. The same tendency was observed in Pulësi, the area of origin of the variety that produced the wine with the lowest total polyphenol content. The analysis of statistical data proves that the growing area has a significant influence on the content of total polyphenols, while the grape varieties did not have a significant influence on the amount of these compounds. These results suggest that polyphenol content depends mainly on the vineyard and winemaking technology, which is consistent with other reported results (Garrido and Borges, 2013).

#### ***b. Total flavonoid content in white wine.***

Polyphenolic compounds are divided into flavonoids (flavanols, flavanols, dihydroflavonols, and anthocyanins) and non-flavonoids (hydroxybenzoic acid and hydroxycinnamic acid, stilbenes, and phenolic alcohols), with the latter accounting for the majority of polyphenolic compounds in white wines. However, flavonoids have a greater influence on the structure and colour of wine than non-flavonoids. Flavonoids are found in the skins, seeds, and stems of white wine grapes and account for about 25% of the total phenolic content of white wines (Kennedy et al., 2006). In European white wines grown under cold

climatic conditions, the presence of flavonoids is considered undesirable because it reduces the typical aroma of the grape variety (Schneider, 1998). The results of the average values of total flavonoid content obtained in this study for the two varieties studied are shown in Figure 2.

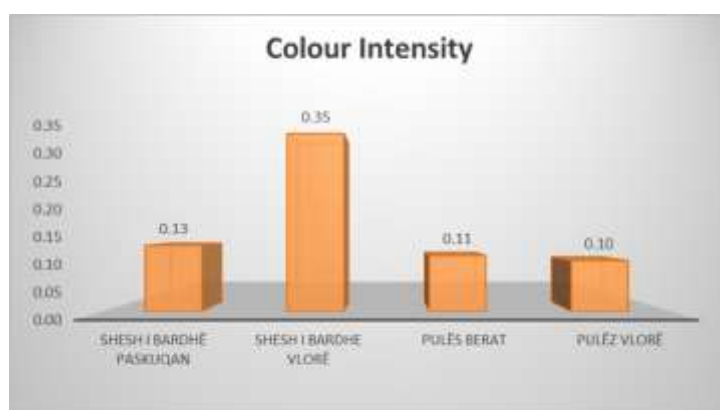


**Figure 2.** Total flavonoid content in wines from Shesh i Bardhë and Pulës varieties grown in different areas.

The experimental values for total flavonoid content ranged from 84.84 mg/ml to 243.18 mg/ml, values that show that they are significantly influenced by the variety and the growing region. In the white wines of Shesh i Bardhe the average values of total flavonoid content are different in the different growing areas, with the greatest differences in the Paskuqani area. The highest content of total flavonoids was found in the wines of the Pules variety from the Berat growing region (243.18 mg/ml). From these results, differences between the two areas can be seen, with the lowest values of total flavonoid content in wines from the Pules variety in the Vlora area.

### *c. Colour intensity*

Colour is considered one of the parameters for evaluating the oxidative stability of wine (Beccheti, 1999). According to the results of this study, the wines produced in 2015 were more intense than those obtained in 2016; these values were within the established limits (Beccheti, 1999). The increase in colour intensity is due to the extraction of pigments present in the skin of the fruit during skin fermentation. The results of the colour intensity of wines from two autochthonous white grape varieties grown in two different areas are shown in Figure 3.



**Figure 3.** Colour intensity of wines from Sheshi Bardhë and Pulës varieties grown in different areas.

From this figure it can be seen that the intensity of the colour had a greater influence on the area of cultivation in the case of the Sheshi i Bardhe variety. The wine from this variety, grown in Vlora, has higher values around 0.35 nm. In the case of the Pules variety, it can be noted that the growing area, climatic and agronomic conditions, had no influence on this parameter. From these results it can also be seen that the influence of the genetic factor, the variety, had no significant influence. This means that this parameter can be influenced more by the growing area than by the variety.

## CONCLUSIONS

From this study we conclude that; the variety and the growing area have no influence on the quality of the wines. The total polyphenol content of the red wine from the area of Lundra for Sheshi zi and Vlorë Kallmet grapes was higher than that of the area of origin of these grape varieties. The red wines from the area of Lundra for Sheshin e zi and Rrëshen for Kallmet had higher total tannin contents than the wines from other areas. The wines of the studied grape varieties have higher total anthocyanin content in the areas of Durrës (Sheshi zi) and Lezhë (Kallmet). From this study we conclude that white wines from autochthonous varieties show significant influences of the varietal factor and the growing area for the measured parameters total polyphenols, total flavonoids, while for color intensity only the wines from Sheshi Bardhë of Vlora showed influences of the growing area. However, other influences, especially technological, on the extraction of these components should be observed and analyzed in the following years.

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## **PRELIMINARY SURVEY FOR MAPPING THE DISTRIBUTION OF LOCAL VARIETIES BY USING THE NATIONAL REGISTER OF PLANT GENETIC RESOURCES IN BULGARIA**

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### **ABSTRACT**

The intensive agriculture production and the climate changes continue to be the major factors affecting genetic erosion in rural areas. Collection of locally adapted traditional crop varieties is carried out by implementing the National Program of Plant Genetic Resources for conservation and management with the focus on their sustainable preservation and use. During the recent years expeditions in rural areas of Bulgaria were conducted. The exploration, inventorying and collecting local genetic resources in these areas was performed using a GPS system. The *ex situ* collection was enriched with 10,817 local accessions from cereals, vegetables, pulses, medicinal and aromatic plants. Collected materials are listed in the National register of Bulgaria, according to international descriptor of EURISCO. The accessions are stored in genebank and included in survey for characterization and evaluation. The purpose of the study is to determine the distribution of local varieties in Bulgaria based on information for agro-ecological origin of conserved accessions by collecting missions. The results from mapping show that the country is rich in genetic biodiversity of vegetable crops and pulses, which requires community support initiatives to preserve them also *in situ/on farm*, through the distribution of knowledge, publicity and cooperation. This work is supported by Bulgarian National Science Fund under the project BGPlantNet “Establishment of National Information Network GenBank – Plant genetic resources”, and obtained results will be applied in the National Research Program “Smart Crop Production”.

**Key words:** plant genetic resources, local origin, expeditions, passport descriptor, documentation

### **INTRODUCTION**

Bulgaria is characterized by one of the richest countries with plant diversity in the Balkans. The food security of the population highly depends on the diversity and adaptability of the species providing clean and healthy foods. Global climate changes affect the environmental factors, which put crops in unfavorable conditions for cultivation and development and this leads to ecosystem destruction (FAO, 2008; Stoilova et al., 2014).

The intensive agriculture production and the climate changes continue to be the major factors affecting genetic erosion in rural areas. The trend of continuing changes in the global climate in our country and the dynamics of local meteorological phenomena raise the question of growing appropriate, well-adapted to different regions crops and optimal combination of different varieties on farms. Collection of locally adapted traditional crop varieties is carried out by implementing the National Program for Conservation and Management of Plant Genetic

Resources (PGRs) with the focus on their sustainable preservation and use, according to Plant Genetic Resources Strategy for Europe (ECPGR, 2021). The main activities of the National Program of PGRs are: (1) Mapping and collecting genetic resource – organizing expeditions to map the genetic resources of agricultural crops and wild crop relatives, to collect information and to supplement *ex situ* collections; (2) Preservation and management of PGRs in genebank collections; (3) Documentation and transfer data to National and International PGRs networks; (4) Phenotypic and genotypic characterization; (5) Making the PGRs available for research and development, teaching, crop breeding – exchange in accordance with ITPGRFA (2009) and Nagoya Protocol (CBD, 2011); (6) Expanding the use of PGRs – cooperation with research institutions, international collaboration, return the local and traditional varieties to hobby gardeners, promoting exhibitions, publications, media coverage, etc.

Home gardens contribute to the conservation of biodiversity at the ecosystem, species and within species levels. They provide complex, multi-layered environments in which farmers can maintain large numbers of useful plant species managed in a sustainable manner over decades or even centuries. They may also provide a basis for the maintenance *in situ / on farm* of significant amounts of intra-specific (genetic) diversity of useful plant species (Hodgkin, 2002; Kehlenbeck et al., 2007; Krasteva et al., 2009; Galluzzi et al., 2010; Knüpffer, 2016).

The preservation of the plant biodiversity from the wild and cultivated flora is the main priority of the IPGR Sadovo as a National Coordinator in the European Programme for Plant Genetic Resources (ECPGR).

The aim of the study is to determine the distribution of local varieties in Bulgaria based on information for agro-ecological origin of conserved accessions in the National Genebank by conducted collecting missions in rural areas.

## MATERIAL AND METHODS

The genetic diversity of Bulgaria is maintained in 19 research institutes at the Agricultural Academy. A total number of over 160,000 plant forms are maintained in various collections – over 100,000 field crops, over 5,000 vegetable crops, over 40,000 perennials and over 130 essential oil crops.

The Center for information and documentation of plant genetic resources at IPGR-Sadovo has been established in 1982 and completely renovated in 2021 under a project BGPLANTNET, financed by Bulgarian National Science Fund of the Ministry of Education and Science (Grant KP-06H36/2/13.12.2019). It works according to the international documentation standard of EURISCO (FAO/Bioversity, 2017).

The National genebank of Bulgaria, situated at IPGR-Sadovo, was built in 1984 and carries out a scientific program for the long-term preservation of germplasm with seeds under controlled conditions in accordance with the standards developed by FAO (2013). The genebank facilities are designed both for long-term storage and medium-term storage.

The electronic database contains the following passport information: taxonomy, catalogue number of accessions, acquisition date, country of origin, donor of the sample, collection site, ecology-geographical characterization, biological status, type of storage: base collection (long-term), exchange collection (medium-term), work collection (short-term), *in vitro* and/or field collection, botanical garden, etc.

The taxonomic description of the crops is under the nomenclature of USDA (GRIN, 2015).

The collecting mission activities are conducted under the methodology of Guarino et al. (2011).

## RESULTS AND DISCUSSION

The territory of Bulgaria is located in the transition zone of the temperate climate between three vast bioclimatic regions - the Central European continental, the Eurasian steppe and the Mediterranean, which overlap. Apart from the change in the values of the climatic indicators in the north-south and east-west directions, there is a great variety in the climatic conditions due to the influence of the heavily rugged terrain of the mountains and the large water masses of the Black Sea and Aegean Sea. The geographical location of the country on the border between the temperate and subtropical physical-geographical zone determines its diverse natural conditions - climate, water regime and soil and vegetation cover.

The physico-geographical conditions and the historical development of Bulgaria have been a prerequisite for the establishment of a wide variety of PGRs that are used for commercial and non-commercial purposes. The autochthonous economic valuable species (*Triticum durum*, *Triticum aestivum*, *Secale cereale*, *Avena sativa*, *Hordeum vulgare*, *Sorghum*, *Zea mays*, *Solanum lycopersicum*, *Capsicum annuum*, *Allium cepa*, *Brassica oleracea*, *Cucumis sativus*), horticultural and field, cereals and fodder, annual and perennial legumes and 250 species of traditional medicinal plants) provide products for local consumption, domestic trade and export.

The rich natural diversity of plant genetic resources as sources of genes to increase the resistance to abiotic and biotic stress in cultivated varieties in the last few decades has been exposed to anthropogenic pressure in the form of overuse, urban sprawl, pollution and successive changes in the land management regime.

Currently, accessions with local origin comprise 20% of the *ex situ* collection, conserved at the genebank in IPGR-Sadovo as the main priority in the enrichment in accordance with the National Program of PGRs. The accessions acquisitioned by expeditions are 10,871 – species and varietal diversity of landraces, including local varieties from home gardens and crop wild relatives from their natural habitats.

During the period 1982-2021 expeditions for collecting local PGRs were organized in various regions of the country, funded by national and international projects (**Fig. 1**). The routes of the collecting missions were determined on the basis of prior awareness of the specifics of the respective production areas or local habitats.



**Fig. 1.** Main routes of conducted expeditions for local PGRs in Bulgaria

Villages, sufficiently distant and with different ecological and geographical characteristics were marked. The selection of home gardens and small farms to be visited from researchers was made with the kind assistance of a representative agent from the relevant mayorality or municipality.

The priorities of collecting missions are: to collect variability in a particular crop or crop group; to collect tolerant forms to biotic and abiotic stress; to collect crop wild relatives, weedy types and related taxa of agri-horticultural relevance.

Sustainable conservation of PGRs includes documentation activities as well as preservation of traditional knowledge and good practices of farmers who cultivate the local varieties.

Collected local accessions of cereals, grain legumes, vegetables and medicinal crops are a significant resource as a source material for crop breeding and for the utilization of their biological potential for healthy nutrition. These genotypes, formed as a result of the natural or artificial selection in population from farmers and adapted to a special regions in the country, get used very well to the unfavorable agri-environmental conditions.

The number of local cereal landraces and old varieties from durum wheat (118), common winter wheat (736), einkorn (66), oat (17), rye (42) and corn (250) were collected.

Grain legumes are a major contributor to global food production worldwide and are a major source of protein. They occupy about 15% of the local accessions, stored in the genebank, represented by 45 species and the following main crops: chickpea (48), common vetch (29), latyrus (222), lupin (10), pea (29), originating from Sofia, Plovdiv, Stara Zagora, Strandzha and General Toshevo regions. The bean (2113) accessions are collected from the villages around Smolyan, Velingrad, Kazanlak, Haskovo, Dimitrovgrad and Svilengrad.

The Cucurbits have been grown from thousands of years in our lands. They are of great economic importance in our country and worldwide. The fruits and seeds are used for

consumption, medical purposes, as forage, as well as for decoration. The group includes *C. maxima* (65), *C. moschata* (40) and *C. pepo* (290), collected from Plovdiv and Harmanli regions. Traditional vegetable varieties of tomato (433), pepper (1826), eggplant (56), onion crops (547), cabbages (91) and leafy vegetables (106) and spices.

Landraces of medicinal and aromatic plants, used in bio and home gardens for medicinal purposes, herbal teas or decoration, were collected.

The inventory of the conserved gene fund identifies areas in the country with a concentration of local PGRs, where collecting missions are urgently needed to prevent the loss of valuable for crop breeding and agricultural practice diversity (**Table 1**).

**Table 1.** Local varieties distribution by regions of Bulgaria

| Crop groups                   | Regions in Bulgaria   |
|-------------------------------|---|
| Cereals                       | Regions with extensive agriculture, including mountainous and semi-mountainous – south-east and south-west parts of the country, closed border regions, monastery lands, etc. |
| Grain legumes                 | The regions of Blagoevgrad, Kyustendil, Strandzha-Sakar, Rodopi Mountains, Ludogorie, etc.  |
| Vegetable crops               | All home garden regions near Gorna Oryahovitsa, Veliko Tarnovo, Svishtov, Vidin, Plovdiv, Pazardzhik, Haskovo, Dimitrovgrad, Popovo, Petrich, Sandanski, etc.                 |
| Cucurbits                     | All home garden regions near Pleven, Vidin, Razgrad, Shumen, Yambol, Lyubimets, Svilengrad, Ivailovgrad, etc.   |
| Medicinal and aromatic plants | Home gardens, monastery lands, mountainous and semi-mountainous regions.  |

In the recent years, the local accessions became associated with more complete passport information, because of the use of a GPS system during expeditions. For older germplasm samples the lack of eco-geographical characteristics could be partially restored through the modern satellite systems. Ethnobotanic data and other information of interest regarding aspects related to the cultivation, utilization and genetic erosion process were also recorded.

Economic development in rural areas is linked to the development of markets and participation of local farmers in them. Development and increasing reach of modern value chains may make traditional value chains linked to niche markets to become uncompetitive, leading to less commercial opportunities for marketing diverse varieties or products derived from them. In local markets, we can see a trend for increased demand for quality products with traditional flavors and a trend that is expanding in agro tourism areas. Some of local farmers already sell the production of vegetables and beans, as well as their products for supply guesthouses and tourist resorts with added value. Home gardens should be considered as a model for sustainable agro-food systems, integrating both economic and ecological advantages.

Preliminary survey for mapping the distribution of local varieties provides critical information on PGRs to assess their current preservation status and prioritize areas for conservation. They have also proven useful for effective genebank management, such as the definition of main collections and identification of collection gaps.

Bulgaria is a traditional producer of quality agricultural products. There are conditions for environmentally sustainable agriculture and producers of food and beverages unique for the country by authentic methods. The added value of the activities of small and medium-sized enterprises using local resources are socio-economic and environmental benefits for the regions. Organic production is still weak in the country, but the market for organic products is

developing rapidly. The prerequisites for the development of this type of production and the factors motivating farmers in this direction are the natural resources, ecologically preserved areas, the perceived benefits for rural development, the growing demand for healthy food from consumers and the existence of a legal framework that makes Bulgarian organic products legitimate on the common market of the EU.

## CONCLUSIONS

In the period 1982-2021 the Bulgarian genebank was enriched with 10,817 local varieties from cereals, grain legumes, vegetables, medicinal and aromatic plants.

Collected materials are listed in the National register of PGRs, according to the descriptor of FAO/Bioversity.

The accessions are stored in genebank and included in survey for characterization and evaluation.

Based on information for agro-ecological origin the distribution of local varieties from the main crop groups in Bulgaria are determined.

The result from mapping shows that the country is rich in genetic biodiversity of grain legumes and vegetables, which requires community support initiatives to preserve them also on farm, through the distribution of knowledge, publicity and cooperation.

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## **THE INFLUENCE OF CLIMATIC CONDITIONS ON THE CHEMICAL AND BIOACTIVE COMPONENTS OF SOME APPLE VARIETIES CULTIVATED IN THE AREA OF KORÇA**

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### **ABSTRACT**

Apple (*Malus domestica*) is a valuable part of fruit culture in our country. Apple cultivation has been known since ancient times and is still widely cultivated today. In recent years, apple cultivation has experienced increasing development, so that the consumption of apples is growing more and more. This growth is due to the favorable climate for cultivation, but also to consumer demand and their preference for new products that can be made from the apple fruit. Apple is a fruit with high nutritional value, necessary for the human body, as it contains vitamins A and C, sugars, acids, minerals and so on. The content of these components varies depending on the variety, climate, soil and climatic conditions. This fruit is consumed not only as fresh fruit, but also in the form of unfermented and fermented juices such as apple juice, cider, vinegar, etc. The aim of this study is to characterize apple cultivars grown in Korça region through a two-year study of physicochemical parameters and antioxidant activity of apples. This study was conducted on four apple variety Idared, Starking, Gold and Pink Lady. The analyzes performed are physicochemical analyzes, vitamin C, total polyphenols, flavonoids, antioxidant activity, etc. The results of the two-year study show that the values vary not only from year to year, but also from variety to variety. It is assumed that these changes are due to the climatic changes and the varietal characteristics of the apple variety in the Korça area.

**Keywords:** Apple , Climatic Condition , Bioactive Components, Variety

### **INTRODUCTION**

The apple is a valuable asset of the fruit culture in Albania. Apple cultivation has been known since ancient times. This is a fruit with high nutritional value and necessary for the human body because it contains vitamins A and C, sugars, acids, minerals, etc. (Jan, 2012; UIE, 2013). The content of these components varies according to variety, climate, soil and climatic services.

Albania has favorable climatic and soil conditions for the large-scale development of the apple fruit (Ferraj & Thomaj, 2014). The development of apple fruit cultivation in Albania is

concentrated more in the northern area, Kukës, Peshkopi and in the area of Korça. In the south of Albania, the spread of its cultivation is few, since these areas do not have favorable climatic conditions for large plantations. In southern areas, apple fruit is severely damaged by diseases and insects.

The apple is thought to be native to the area between the Caspian Sea and Europe. This assumption is based on the fact that in these areas apples are found in the form of forests, where the first man used them for his food. The apple is thought to be over 4000 years old in Europe (Fideghelli, 2008.)

Botanically, the apple is classified in the class Dicotyledons and subclass Coripetale, order Rosali, family Rosaceae, subfamily Pomoidae and genus Malus. As a species of the genus Malus, the apple includes about 30 species that have great similarity between varieties, so that most of them can be grafted with each other. (Ferraj & Thomaj, 2014).

The production of apples in the country, in recent years, has had a growing development, causing its consumption to appear in ascending order (Skreli, E., & Imami, D. (2012). This order is due to the favorable climate for its cultivation, but also thanks to the demands of the consumer and their preferences for new products that can be offered from the apple fruit (Imami *et al.*, 2017). In terms of consumption, the focus is mainly on fresh apple fruit, but also on unfermented and fermented juices such as apple juice, cider, vinegar, etc. Based on these consumer demands, this study was presented, which aims to characterize the apple varieties cultivated in the Korça area, through a two-year study of the physical-chemical parameters and antioxidant activity of apples.

## **MATERIALS AND METHODS**

For the realization of this study, four apple varieties were taken into consideration: Idared, Starking, Gold and Pink Lady in two consecutive years of production. These samples after being placed in optimal ripening conditions, were transported to the Food Research Center of the Biotechnology Food Faculty.

Immediately after arriving on the laboratory, those samples underwent physical-chemical analyzes.

### **The determination of acidity of apple fruit.**

This parameter is performed according to the AOAC method (1990), and is based on the titration of acid-based fruit juice in the presence of phenolphthalein as an indicator. The calculation is done using the formula:

$$\text{Total acidity (gr/L)} = 0.67 \times n/\text{Gr}$$

### **Determination of total soluble solid (TSS)**

The liquid is extracted, at room temperature ( $20 \pm 1$  °C) and filtered. The TSS of the filtrate was measured three times using a hand-held digital refractometer.

### **Determination of vitamin C.**

The determination of vitamin C was done according to the method of Mussa and El Sharaa, 2014. Vitamin C (ascorbic acid) is an important antioxidant in the human diet. In a 250

mL Erlenmeyer flask, add 25 mL of fruit juice, 10 drops of 1% starch indicator solution. The solution is titrated until a blue-black color is reached, a color that remains for at least 20 seconds. The calculation is done using the formula:

$$\text{Vitamina C (mg/L)} = \frac{25 \times a}{b}$$

Where: a – iodine solution used for sample titration,

b - iodine solution used for titration of standard vitamin solution.

#### **Determination of total polyphenol content (TPC).**

Total polyphenol content (TPC) of fresh fruit and cider was determined spectrophotometrically according to FC method (Singelton & Rossi Jr. 1965).

The measurements were compared to a standard curve of prepared gallic acid solutions (25–500 mg/l) and expressed as mg of gallic acid equivalents (GAE) per 1 l ± SD of apple juice. All measurements were performed in triplicate.

#### **Determination of Antioxidants by DPPH Radical Scavenging Activity**

The analysis was performed according to the modified method of Blois, (1958). 1 mL of 0.1 mM solution of DPPH in methanol was mixed with 2 mL of extract at different concentrations (0.5-5.0 mg/mL). The mixture was then incubated at room temperature for 30 min in the dark. Absorbance measurement was performed at 517 nm. Low absorbance value indicates higher DPPH free radical activity. Ascorbic acid was used as a standard. The percentage of activity of each extract on the DPPH radical was calculated as % DPPH inhibition (I%) using the following equation:

$$I \% = \frac{A_0 - A_s}{A_0} \times 100$$

Where: A<sub>0</sub> – absorbance value of the control, A<sub>s</sub> – absorbance value of the analyzed extracts.

## **RESULTS AND DISCUSSIONS**

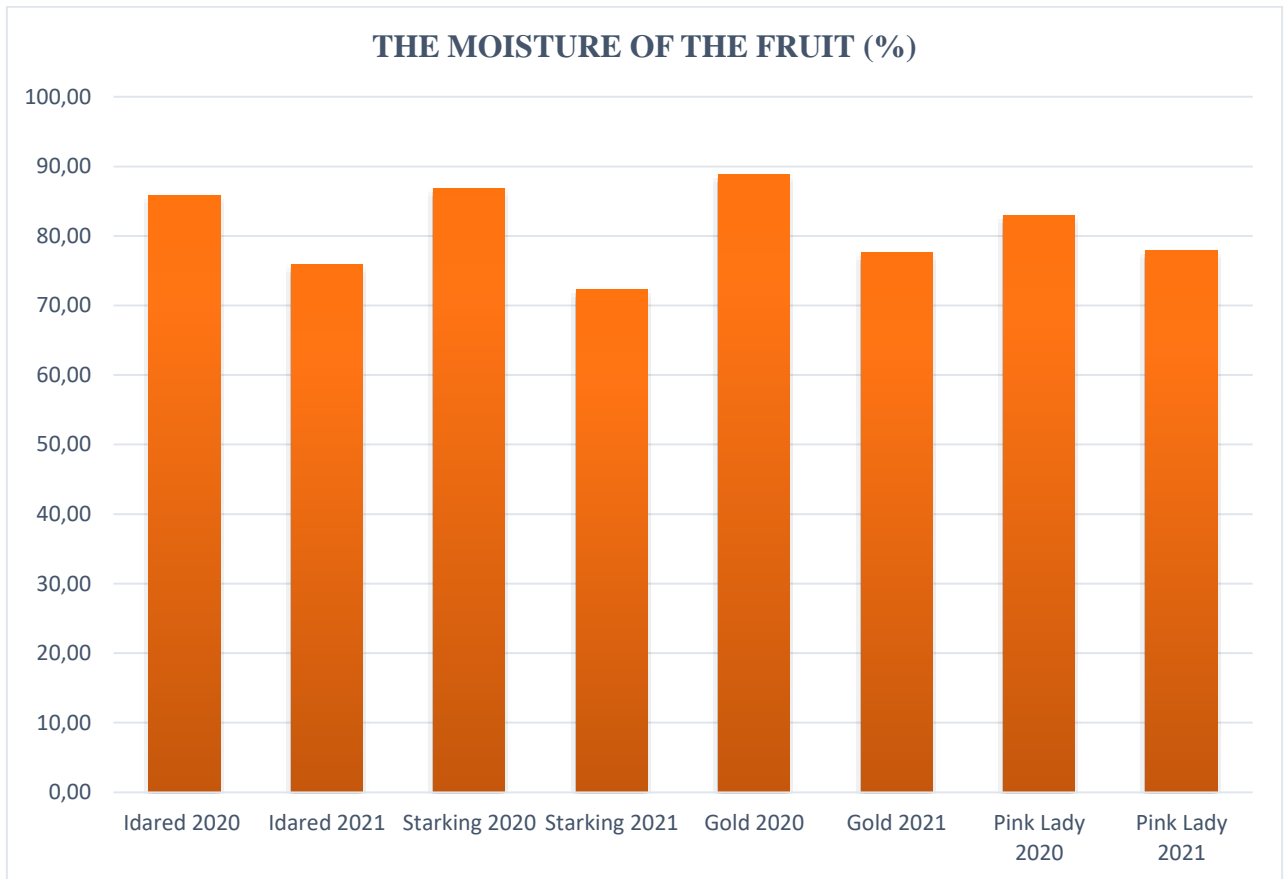
The apple fruit is one of the fruits that has great importance in terms of nutritional value, therefore this fruit has become part of the food diet, but it is also used as a raw material for many processed products (Mukhtar *et al.*, 2010). In Albania, the apple is a fruit that stands out for fresh consumption, but in recent years a large amount of it, is being used for processing to obtain new products such as cider, apple juice, dried apples, etc.

#### **Pomological parameters of apple fruit.**

The quality of the apple fruit (*Malus domestica*) has a high influence from its stage of maturity, this influence is observed during the harvesting process as well as especially during storage. Early harvest, like late harvest, provides non-optimal parameters especially for fruits that will be stored for a long time (Plotto *et al.*, 1995, Tanger *et al.*, 2013, Scolaro *et al.*, 2015).

**The moisture of the fruit.**

In this study, several important parameters were evaluated in determining the quality of apple fruit. Figure 1., shows the graph of average fruit moisture values for apple varieties cultivated in the Korca area, in two years of production (2020 and 2021).



**Figure 1.** The average moisture content of apple fruits obtained in the area of Korça, in the years 2020 and 2021.

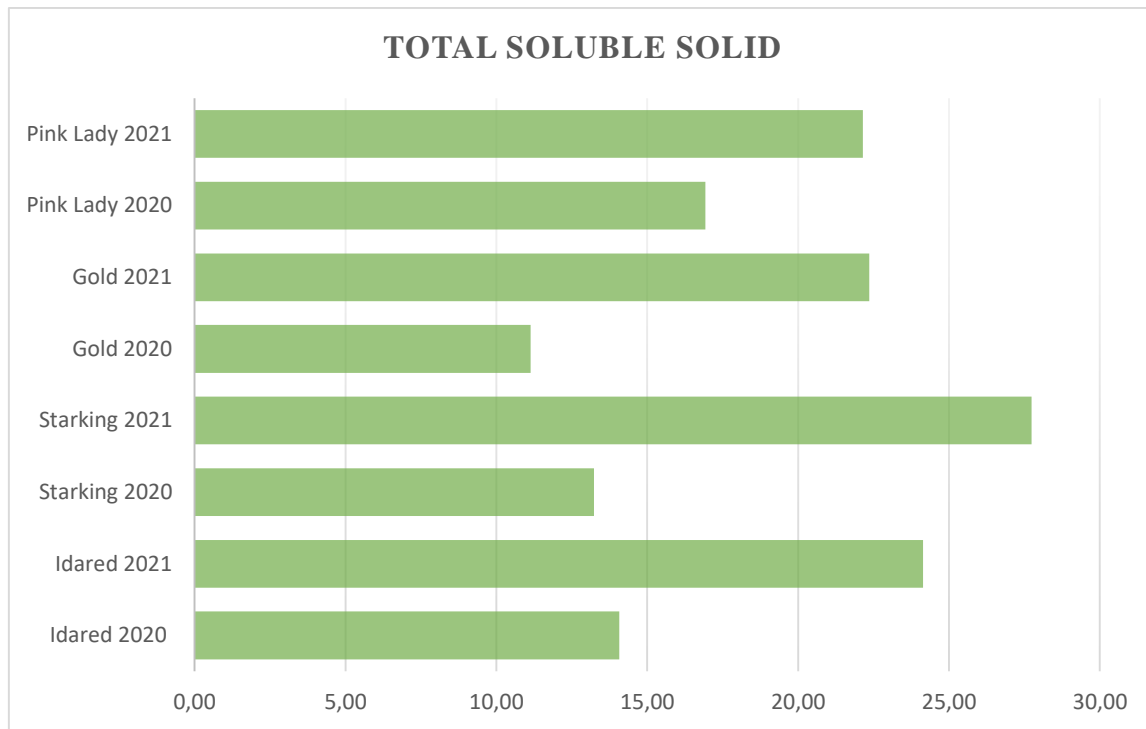
From Figure 1., it can be seen that the average moisture content comes to lower values in 2021 compared to 2020. The average moisture content for 2021 in all varieties of apples taken in the study fluctuates in the values of 72-78%, although the apple is a fruit that stands out for its high moisture content (Sharabiani *et al.*, 2021).

In 2021, the fruits of the variety Starking presented a lower average value of moisture (72.26 gr) compared to the fruits of other varieties, while the varieties Pink Lady and Gold had the highest moisture values of 77.85 gr and 77.64 gr, respectively.

In 2020, the variety with the highest moisture value was Gold with 88.8 g and the variety with the lowest moisture value was Pink Lady with 82.9 g. As can be seen, the Gold variety, in both years of the study, stands out for high moisture values, an indicator that is very important as it is also related to the shelf life of the fruit.

### Total Soluble Solid (TSS)

Moisture is an indicator which is inversely dependent on total soluble solids. In the figure below, the TSS data for the apple varieties under study in the years 2020-2021 are presented.

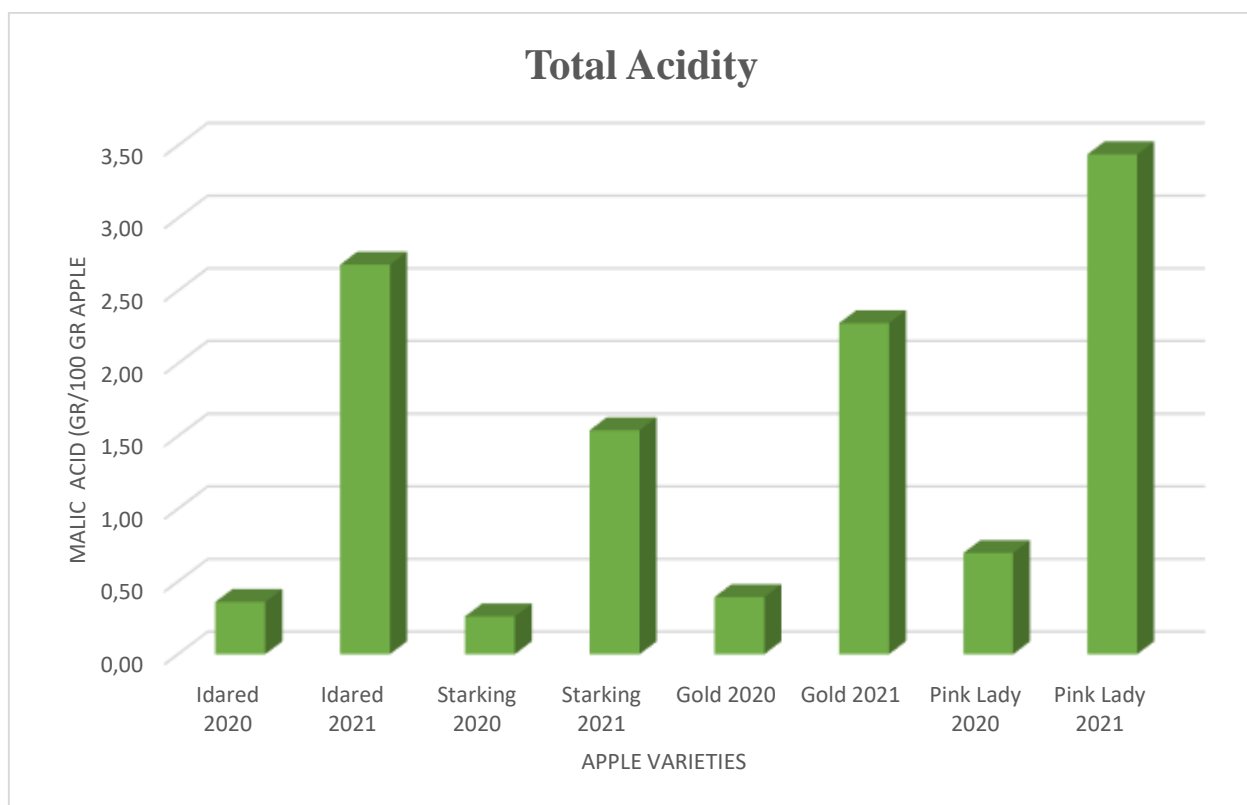


**Figure 2.** The average content of total soluble solids of apple fruits obtained in the area of Korça, in the years 2020 and 2021.

As can be seen from the graph, the variety that presented the lowest total soluble solid value for 2020 is Pink Lady (13.2 g), while for 2021 it is the cultivar Starking (27.74 g).

### Total acidity

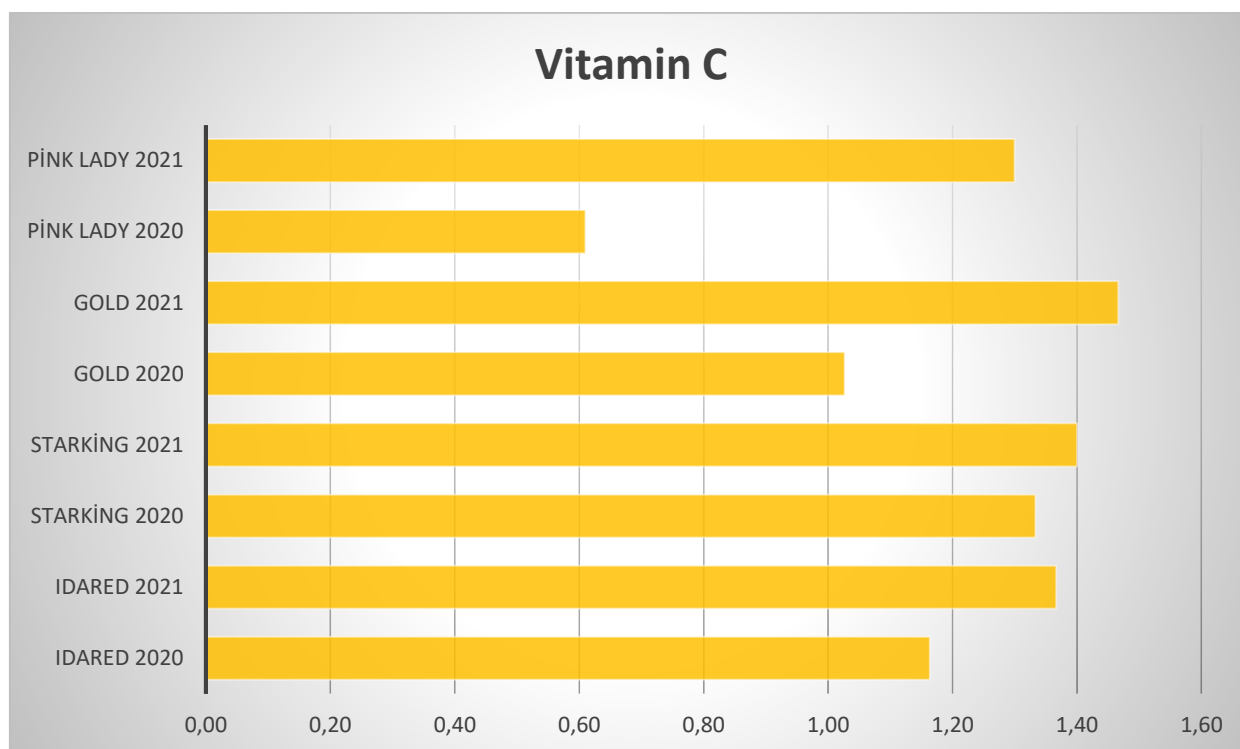
Fruit acidity is an indicator which is different depending on some external factors such as cultivation areas, climatic conditions, etc. (Ali *et al.*, 2004). Figure 3 shows the results obtained on total acidity. The Pink Lady variety in both years of the study showed higher average values compared to the other varieties. While the Starking variety presented the lowest total acidity values in the two years of the study, respectively 0.26 gr/100 gr of apples in 2020 and 1.54 gr/100 gr of apples in 2021.



**Figure 3.** Average titratable acidity values of apples obtained in two years of study, 2020 and 2021.

### **Vitamin C**

The content of vitamin C in different fruits is different, this happens because its concentration changes according to the season, place of cultivation, cultivar, storage time, etc. (Murneek *et al.*, 1954).



**Figure 4.** Vitamin C content in apples obtained in two years of study, 2020 and 2021.

The figure above shows the average values of vitamin C of apple fruits in the two years of the study. In 2021, it is noticed that the cultivars stand out for higher values of vitamin C, compared to the production year 2020. The most dominant varieties in terms of vitamin C are the Gold variety for 2021 and the Starking variety for 2020.

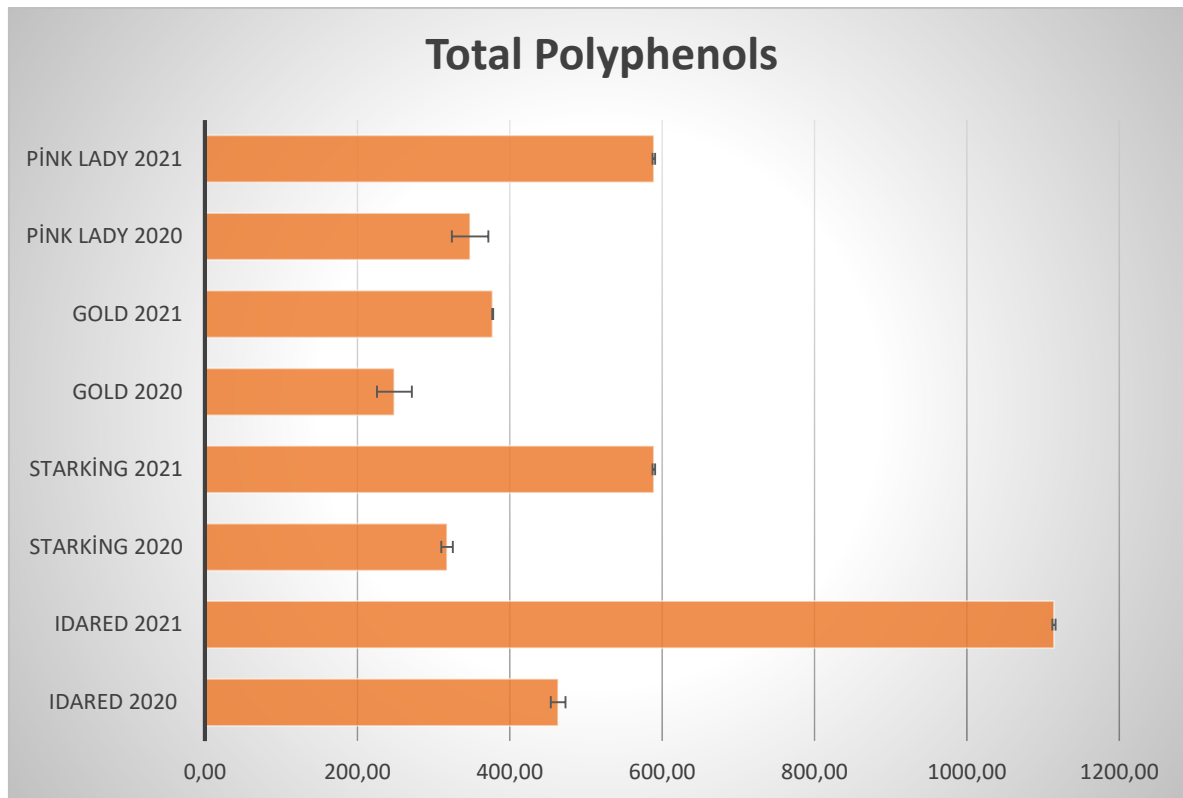
### **Total polyphenols**

Polyphenols in apples are dependent on a number of internal (genus, species, cultivars) and external (agronomic, environmental, handling and storage) factors (Tomás-Barberán and Espín, 2001). Phenolic content in apples is also affected by physiological disturbances in fruits such as water (Zupan *et al.*, 2016).

The diversity of varieties, agrotechnical conditions, climatic conditions, etc. have a great influence on the total amount of polyphenols (Nadulski *et al.*, 2019). Also, different parts of the fruit are distinguished by different contents of polyphenols, and their composition is variable in different parts (Awad *et al.*, 2000). Numerous studies have shown that the peel is distinguished by a high content of polyphenols compared to the pulp of the apple fruit.

The content of total polyphenols of the varieties under study is presented in Figure 5. From the results obtained, it can be seen that the polyphenolic content is the highest in the second year of the study, in 2021. The Idared variety stood out for the highest average values for both years of the study compared to other varieties. The lowest average values for both years were found in the Gold variety.





**Figure 5.** The content of total polyphenols in apples obtained in two years of study, 2020 and 2021.

Other researchers have found similar variations in phytochemical content among different apple varieties. Van der Sluis *et al.*, (2001) analyzed several apple varieties and found that Jonagold stood out for the highest concentration of quercetin glycosides, catechins and chlorogenic acid when compared to other varieties.

### **Antioxidant Activity of Apple**

The importance of antioxidant activity in apple fruits is very high. From the studies conducted, there is a direct relationship between the phenolic content of apples and antioxidant activity, so varieties with high polyphenol content are also distinguished for high antioxidant activity (Jeanelle Boyer and Rui Hai Liu 2004).

### **CONCLUSIONS**

The study of physical-chemical parameters of apple varieties in two production years 2020-2021 in the area of Korça showed that the changes are in accordance with the literature and that they are mainly influenced by the variety. Based on the obtained data, we can say that the apple varieties obtained in the Korça area during the two years of study (Idared, Starking, Gold and Pink lady) present good values of their physical and chemical characteristics.

The highest average values for moisture content were distinguished by the Gold variety, being thus qualified as a cultivar that can be stored for a long time. The Starking variety stood out for its high dry matter content. Antioxidant activity of apple for all studied varieties are

relatively high regardless of the method used for their determination. The variety 'Idared' presented higher antioxidant values in both years of the study, this high amount of antioxidant activity comes as a result of the high content of polyphenols in the fruit.

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**EFFECTS OF SOME CHEMICAL TREATMENTS ON POLLEN GERMINATION AND POLLEN TUBE GROWTH IN ‘RED LAKE’ and ‘ROSENTHALL’ CURRANT CULTIVAR (*Ribes Rubrum*)**

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**ABSTRACT**

Fertilization biology is very important criteria for fruitful. Pollen germination and pollen tube growth are major components of fertilization success in fruit trees. A mature pollen is used for the germination of pollen *in vitro* and *in vivo* in its nutrient storage and nutrient content, just like the seed. However, the nutrients contained in the pollen are often not enough for the pollen tubes to reach the seed drafts. For this reason, the development of pollen tubes after a certain stage is realized through the use of nutrients in the stigma. In this study, pollens belonging to ‘Red Lake’ and Rosenthal varieties were used to examine the effects of certain growth regulators and mineral substances on pollen germination and pollen tube growth. “Agar in plate” method was used the for germination tests which was contain 0.5 g agar-agar +15g sucrose + 5ppm boric acid, potassium nitrate (50 ppm), thioure (50 ppm), benzyladenine (5ppm), gibberellic acid (10 ppm) and indole butyric acid (10 ppm) solutions were added germination medium (0.5 g agar- agar +15g sucrose + 5ppm boric acid and incubated at 21 °C Pollen tube growth was measured by ocular micrometer after 24 hours later germination. Statistical analyses were performed by SPSS 22.0 version. The effects of chemicals on pollen germination and tube growth were found as statistically different. according to minerals and plant growth regulators. Potassium nitrate and gibberellic acid were determined as promoter while thioure and benzyladenine effected as inhibitory in pollen germination and tube growth.

**Keywords:** Pollen germination, black currant, pollen tube growth, plant growth regülatör

**INTRODUCTION**

The crops within the “berries” including *Fragaria*, *Rubus*, *Ribes*, *Morus*, *Vaccinium* etc. have been the subject of increased interest by both industry and individuals worldwide, mainly due to the perceived health benefits of consumption of anthocyanins, phenolics, vitamins, minerals, sugars, organic acids etc. associated with these fruits (Henriques et al., 2004). *From among these berries, Ribes* is usually regarded as a member of the Saxifragaceae, and as such it has few related crop genera. However, a more recent study by Sinnott (1985) places the genus in the Grossulariaceae because of its floral morphology. All of the species in the family are diploids, and considerable debate has been generated over the past century as to whether the

currants and gooseberries form a single genus, *Ribes*, or two, *Ribes* and *Grossularia*. A single genus is now the most common consensus, based on morphology, cross compatibility and molecular grounds (Senters and Soltis, 2003). The genus *Ribes* is native to the high latitudes of the northern hemisphere. Europe, Asia, and North America all have native species. Most commercial production is concentrated in Europe and the Russia Federation, and most cultivars have been derived from species native to these areas. Currant cultivation has been practiced at least since the 1500's in Europe, and the late 1700's in N. America when the first colonists arrived. Production of blackcurrant in Europe is on large plantations with a fully mechanized harvest, and much of the fruit is now used for juice production and other processing applications. However, there is a small but expanding fresh market sector, including some protected cropping in some countries of Europe such as Belgium. The cultivars used for processing are generally different from fresh market types, with widely divergent characteristics and requirements. Poland is the largest world producer of blackcurrant in recent years, followed by Russia, the United Kingdom and Scandinavia, with some production also taking place in New Zealand (Brennan, 2008).

Pollen germination and pollen tube growth are important components of fertilization success in fruit trees. Optimal germination of pollen levels can vary plant species and variety, environment nutrient content, humidity, pressure, pH status and ecology. A mature pollen is in the same seed as it stores nutrients in its contents and nutrients both *in vitro* and in germination of pollen *in vivo* used for. However, pollens contained in pollen tube to reach the germ stubs often not enough. For this reason, pollen tube growth from a certain stage its subsequent development occurs in the stigma through the use of nutrients is taking place. Boron, calcium, potassium and some mineral substances such as magnesium and growth regulators required for pollen germination and tube growth. Among inorganic substances boron; in the form of boric acid and borate very important in pollen germination and tube growth has an effect.

The aim of this study is determine the effects of some chemicals and plant growth regulators on pollen germination and tube growth in "Rosental" and "Red Lake" currant cultivars.

## MATERIAL AND METHOD

Plants of *Ribes nigrum* L. 'Rosenthal' and *Ribes rubrum* L. 'Red Lake' growing at the experimental field of the Sivashlı district of Usak province were used for the experiment. Pollens were obtained from flowers at balloon stage. The flowers were transferred to the laboratory immediately. Anthers were removed and placed into the dark-colored bottle to promote dehiscence at room temperature.

"Agar in plate" method (Güçlü et al., 2019) was used the for germination tests which was contain 0.5 g agar-agar +15g sucrose + 5ppm boric acid, potassium nitrate ( $KNO_3$ ) (50 ppm), thioure (50 ppm), benzyladenine (BA) (5ppm), gibberellic acid ( $GA_3$ ) (10 ppm) and indole butyric acid (IBA) (10 ppm) solutions were added germination medium (0.5 g agar- agar +15g sucrose + 5ppm boric acid) and incubated at 21 °C.

Pollen germination mediums:

- I. Control: 0.5 g agar-agar +15g sucrose + 5ppm boric acid
- II. 0.5 g agar-agar +15g sucrose + 5ppm boric acid+**50 ppm  $KNO_3$**
- III. 0.5 g agar-agar +15g sucrose + 5ppm boric acid+**50ppm Thioure**
- IV. 0.5 g agar-agar +15g sucrose + 5ppm boric acid+**5 ppm BA**
- V. 0.5 g agar-agar +15g sucrose + 5ppm boric acid+**10 ppm  $GA_3$**
- VI. 0.5 g agar-agar +15g sucrose + 5ppm boric acid+**10 ppm IBA**

Pollen tube long at least as its diameter was considered to be “germinated” (Güçlü et al., 2018). An ocular micrometer was used to measure pollen tube length, under a light microscope, at magnification 40X. Four Petri dishes were used for germination and pollen tube experiments. Counts were made from 4 different microscope fields (100-150 pollen grains per field) in each petri dish. The differences among means were analyzed by Duncan’s multiple range test at the 0.05 level of significance. Statistical analyses were performed by SPSS 22.0 version.

## RESULTS AND DISCUSSION

Different chemical treatments (potassium nitrate (50 ppm), thiourea (50 ppm), benzyladenine (5ppm), gibberellic acid (10 ppm) and indole butyric acid (10 ppm) were evaluated on the pollen germination and expressed as the percentage of germinated pollen (Table 1). As seen as Table 1, the effects of different chemical treatments on pollen germination were statistically significant ( $p < 0.05$ ).

Different chemicals and plant growth regulator effects were found similar at for both cultivars. The highest pollen germination was determined in Rosenthal cultivar with 87.55% medium which was added 10 ppm GA<sub>3</sub> like Red Lake cultivar (96.27%). Also higher results were obtained from the medium to which 50 ppm potassium nitrate was added, compared to the control group (Rosenthal; 81.26%, Red Lake; 93.51%). The lowest pollen germination rate was obtained from at the medium which was added 50 ppm thiourea (81.26; Rosenthal, 93.51; Red lake).

**Table 1.** Effects of different chemicals and plant growth regulators *in vitro* pollen germination (%) of Rosenthal and Red Lake Currant Cultivars.

|                  | Control             | KNO <sub>3</sub> | Thiourea | BA     | IBA    | GA <sub>3</sub> |
|------------------|---------------------|------------------|----------|--------|--------|-----------------|
| <b>Rosenthal</b> | 76.64c <sup>x</sup> | 81.26b           | 68.93e   | 72.45d | 75.27c | <b>87.55a</b>   |
| <b>Red Lake</b>  | 89.37c              | 93.51b           | 80.21e   | 82.60d | 91.43c | <b>96.27a</b>   |

<sup>x</sup>Values within a column followed by different letters are significantly different ( $p < 0.05$ ).

The *in vitro* elongation of pollen tubes was affected by different chemicals and plant growth regulators (Table 2). Different chemicals and plant hormones on pollen tube growth were statistically significant ( $p < 0.05$ ). The highest pollen tubes length was measured as 172.39 µm for Rosenthal and 178.51 µm for Red Lake at the medium contains 10 ppm GA<sub>3</sub>. Pollen tube length also high at added 50 ppm potassium nitrate (KNO<sub>3</sub>) germination medium with 168.25 µm; Rosenthal, 174.54 µm; Red Lake. The shortest pollen tube length was measured at germination medium which was added thiourea for Rosenthal; 150.23 µm, 165.31 µm.

Different growth regulator and mineral substances, pollen germination and tube effects on growth, the variety used, change according to the dose of use. Substances such as boron, gibberellic acid polyamine have been reported to be effective on pollen germination and tube growth (Voyiatzsis and Paraskevopoulou-Paroussi, 2005; Çetinbaş-Genç et al., 2020)

**Table 2.** Effects of different chemicals and plant growth regulators *in vitro* pollen tube length ( $\mu\text{m}$ ) of Rosenthal and Red Lake Currant Cultivars.

|                  | <b>Control</b>       | <b>KNO<sub>3</sub></b> | <b>Thioure</b> | <b>BA</b> | <b>IBA</b> | <b>GA<sub>3</sub></b> |
|------------------|----------------------|------------------------|----------------|-----------|------------|-----------------------|
| <b>Rosenthal</b> | 154.27c <sup>x</sup> | 168.25b                | 150.23d        | 151.31d   | 155.29c    | <b>172.39a</b>        |
| <b>Red Lake</b>  | 169.48c              | 174.54b                | 165.31d        | 164.27d   | 170.26c    | <b>178.51a</b>        |

<sup>x</sup>Values within a column followed by different letters are significantly different ( $p < 0.05$ ).

Pollen grains are rich in hormones, vitamins and amino acids. They generally do not require exogenous supply of these substances for germination and tube growth. There are a few reports in which auxins, gibberellins and cytokinins promote germination and tube growth (Singh, et al., 2002). Absence of calcium in the medium results in an increase in the membrane permeability leading to the loss of internal metabolites. In fruit crops, once pollination and fertilization of the ovules within the flowers have taken place, fruit set occurs, generally with development of a fruit from an ovary. Developmental processes in seeds and fruit are principally controlled by growth regulators (GRs). Gibberellins (GAs) induce germination via promotion of amylase activity. GAs plays a crucial role in several plant growth and developmental processes, particularly flower induction, leaf and stem elongation and fruit and seed development (Sun, 2004). It is also reported to induce pollen tube growth under *in vitro* conditions in *Pistacia vera* (Acar et al., 2010). GAs plays a role in pollen viability and pollen tube growth in *Arabidopsis* and rice (Singh et al., 2002; Chhun et al., 2007). IBA and kinetin were found to enhance pollen tube growth in *Calotropis procera*, as these hormones play an important role in activating the catalytic activity of peroxidase, an enzyme essential for pollen germination and pollen tube growth (Parui et al., 1998).

Fertilization success in plants is the result of processes that take place during the progamic phase (Thompson, 2004). Pollen germination and pollen tube growth are important research materials for morphological, physiological, biotechnological, ecological, evolutionary, biochemical, systematic and molecular studies. Additionally, testing pollen performance could be helpful for a fruit cultivation of genetic progeny for breeding purpose, and especially for selecting which cultivars should be used by researchers and growers. *In vitro* pollen germination is of practical importance as it can unravel the physiological and biochemical conditions required for the successful pollen germination and pollen tube development. Pollen conditions such as reserved food material, conditions of membrane and rate of conversion could be judged by *in vitro* pollen germination. *In vitro* germination studies are powerful tools for genetic, physiological, biochemical and cytochemical studies for a wide range of plant species belonging to different families (Radičević et al., 2013). These studies are also a good predictor of *in vivo* pollen behavior but only for autotrophic phase of pollen growth where the initial steps of pollen germination and pollen tube growth are independent of style nutrients, sugars and plant growth regulators. They help with selections for breeding programmers, *in vitro* assessments can also help to predict possible problems of sterility of that particular genotype in commercial orchards (Fotirić Akšić et al., 2017).

## CONCLUSIONS

As a result, 10 ppm gibberellic acid and 50 ppm potassium nitrate were promoter for pollen germination and pollen tube growth for both cultivars. Thioure and benzyladenine

effected as inhibitory pollen germination and pollen tube growth according to the control germination medium. Fertilization biology studies should be continued at *in vivo* conditions. *In vitro* pollen germination would be a precisely accurate and comparatively easy protocol if optimum conditions were standardized. We hope these results will be useful for researchers and growers for future breeding studies.

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## EVALUATION OF CELLULASE ACTIVITY OF *bcsE* MUTANT *SALMONELLA* STRAINS

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### ABSTRACT

*Salmonella* infections lead to serious public health problems worldwide. They also cause major economic losses, especially in the food industry. *Salmonella* strains have an elasticity in their genetic structure that occurs in response to a variety of situations and environments, including human, animal, and non-animal hosts. One of the most typical mechanisms of this adaptation and persistent infectivity of these bacteria is their ability to form biofilms on biotic and abiotic surfaces. Since the pathogenicity of *Salmonella* is not clearly understood, the control of infections caused by these bacteria is challenging. In this study, the effects of BcsE, the c-di-GMP-binding protein expressed by the *bcsE* gene, on the cellulase activity of *Salmonella* Typhimurium (DMC4) and *Salmonella* Group C1 (DMC2) strains were investigated. Compared to wild-type strains, a significant decrease in cellulase activity was observed in the *bcsE* mutant strains on agar media. While no cellulase activity was observed in the *bcsE* mutant strains, cellulase activity was 1.28 cm in the  $\Delta$ DMC2 strain and 1.68 cm in the  $\Delta$ DMC4 strain. These results indicate that the *bcsE* gene is essential for *Salmonella* physiology and adaptation to its environment.

**Keywords:** *Salmonella*, biofilm, cellulase, *bcsE*

### INTRODUCTION

*Salmonella* is a Gram-negative, facultative anaerobic rod-shaped bacterium in the *Enterobacteriaceae* family that can adapt to a wide range of hosts, including humans and animals (Barlow and Hall 2002, Allerberger et al. 2003). Poultry, eggs, and dairy products are the most common sources of *Salmonella*. (Silva et al. 2011). Other food products that contribute to *Salmonella* transmission include fresh fruits and vegetables. (Pui et al. 2011). *Salmonella* infections are most common in pigs, poultry, and cattle. Animal trade and raw animal products are the main sources of pathogenic bacteria transmission. *Salmonella* is the most common source of contamination in organs and carcasses from slaughterhouses (Gillespie et al. 2005).

In *Salmonella* pathogenicity, cellulose plays a key role in modulating the virulence of many human and plant pathogens and is one of the major structural components of the biofilm matrix (Spiers et al. 2003, Römling et al. 2013). Two different operons involved in cellulose biosynthesis have been identified in *Salmonella* (*bcsABZC* and *bcsEFG*). In studies performed with the *bcsA* gene, which is located in these operons and forms the catalytic subunit of the enzyme cellulose synthase, it was found that biofilm formation on solid surfaces and pellicle formation in liquid media decreased in mutants in which the aforementioned gene was inactivated (Zogaj et al. 2001, Prigent-Combaret et al. 2012). In bacteria, cellulose is one of the basic structural components that enable cell surface and cell-cell interaction and protects them

from chlorine treatments (Solano et al. 2002, Zogaj et al. 2003, Grantcharova et al. 2010). Cellulose is the main component of the rdar biofilm structure of *S. Typhimurium*. CsgD, the major biofilm activator, positively regulates the production of amyloid-folded fimbriae and cellulose in the dominant "rdar" morphotype (Simm et al. 2014).

Cellulose biosynthesis is positively regulated by CsgD. AdrA activates cellulose production at the post-transcriptional level. This activation is achieved by direct interaction with *bcs* operons or indirect interaction with c-di-GMP (Garcia et al. 2004, Römling 2005, Simm et al. 2005).

Little is known about the regulation of cellulose biosynthesis, and it is generally assumed that the cellulose biosynthesis operon is constitutively transcribed (Saxena et al. 1995). In *Enterobacteriaceae*, cellulose biosynthesis is regulated by the *bcsABZC* operon. *bcsA* encodes cytoplasmic beta-glycosyltransferase 2, which is the catalytic subunit of cellulose synthase and binds the substrate UDP-glucose (Morgan et al. 2014). BcsB is required for catalytic activity and is consistently localized with *bcsA*. This leads to the formation of the BcsAB fusion protein in some strains (Saxena et al. 1994, Morgan et al. 2013). BcsC is thought to form the outer membrane pore. BcsE is essential for optimal cellulose biosynthesis. (Fang et al. 2014). BcsZ, on the other hand, encodes a cellulase from the glycoside hydrolase family, whose biological function in bacterial cellulose biosynthesis has not yet been elucidated (Mazur and Zimmer 2011).

## **MATERIAL AND METHODS**

### **Bacterial Strains and Growth Conditions**

All strains used in this study were cultured in Luria-Bertani broth (LB) and incubated at 37 °C. Chloramphenicol (25 µg mL<sup>-1</sup>), ampicillin (100 µg mL<sup>-1</sup>), or L-arabinose (10%) were added as needed to LB broth and LB agar plates. Strains *S. Typhimurium* DMC4, *S. Grup* C1 DMC2 ve *E. coli* DH5α, and *E. coli* MZ2997 stored at -80 °C in LB broth containing 60% glycerol were obtained from the Prokaryotic Genetics Laboratory of the Biology Department Faculty of Science, Ankara University.

### **Construction of *bcsE* Mutants in Target *Salmonella* Strains**

Homologous region recombination was carried out by using PCR products and synthetic oligonucleotides as substrates. Plasmid pKD46 is a temperature-sensitive vector and contains lambda red recombinase enzyme and ampicillin resistance genes. This plasmid, induced by L-arabinose, was transferred to *Salmonella* strains by electroporation. To obtain PCR products containing homologous regions specific to the *bcsE* gene region, target regions were selected and specific primers were designed. After the preparation of the primers, they were prepared for use by binding to the 5' end of the chloramphenicol gene cassette (Helix Biotek). Touchdown polymerase chain reaction (PCR) was used to generate the chloramphenicol gene cassette, which has homologous arms with the *bcsE* gene. The obtained PCR products were purified according to the protocol of the "Roche High Pure PCR Product" purification kit. The purified PCR products were subjected to enzyme digestion with DpnI for 4 hours at 37 °C. The chloramphenicol functional cassette containing homologous regions was transformed by electroporation into *Salmonella* strains containing the pKD46 plasmid, and the gene mutation was confirmed by colony PCR (Özdemir et. al 2021).

### Cellulase activity

Cellulase activity of bacteria was determined by agar well diffusion assay. Under shaking conditions, *bcsE* mutant and wild-type strain cultures were incubated for 18 hours in LB<sup>-NaCl</sup> broth containing 5% carboxymethylcellulose. After this time, bacterial cultures were maintained in PBS with an OD<sub>600</sub> of 5. In LB<sup>-NaCl</sup> solid media containing 5% carboxymethylcellulose, 100  $\mu$ L of the obtained culture suspension was transferred to the wells. After 48 h of incubation at 20 °C, a PBS solution containing 0.1% Congo red was spread on the agar surface and incubated for 30 min. The agar plates were washed three times with PBS at the end of incubation (15 minutes). After staining the agar with Congo red solution, the diameters of the degradation zones formed around the wells were measured as an indication of cellulase activity (Ahmad et al. 2016).

The cellulolytic index is calculated using the yellow halo (clear zone) data (Bradner et al. 1999). The higher the cellulolytic index (CI), the more bacterial cellulase enzyme is produced. The cellulolytic index (CI) was classified as low when it was less than one, medium when it was between one and two, and high when it was greater than two (Choi et. al. 2005). The following equation (Bradner et al. 1999) was used to determine the cellulolytic index:

$$\text{Cellulolytic index} = \frac{\text{clear zone diameter (cm)} - \text{colony diameter (cm)}}{\text{colony diameter (cm)}}$$

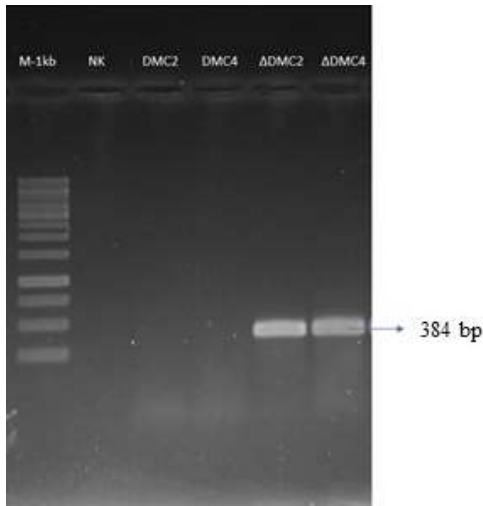
### Statistical analysis

Statistical analyses were performed using GraphPad Prism 8 software. The one-way test ANOVA was used to determine whether differences between groups were statistically significant based on the F value of the experimental results.

## RESULTS

### Obtaining *bcsE* mutants

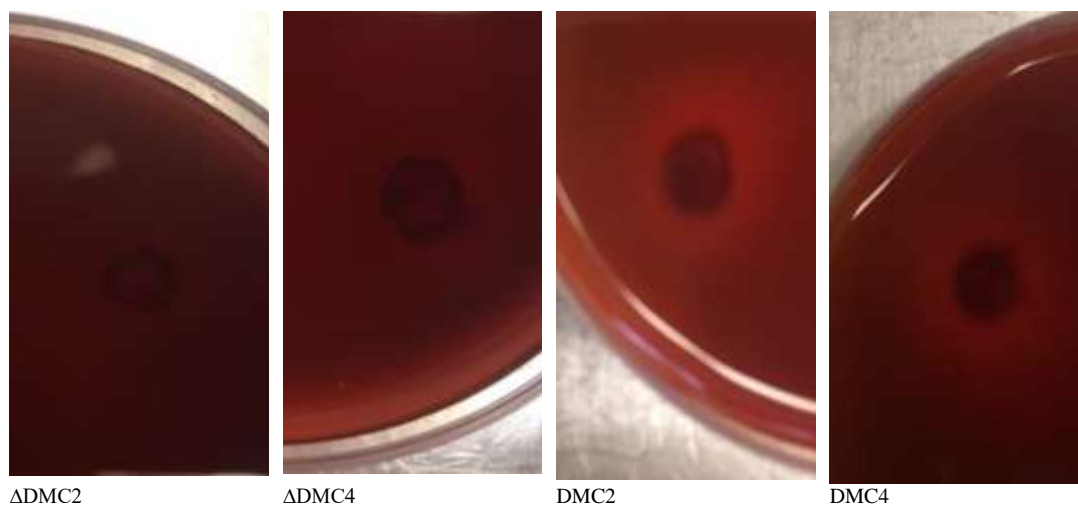
In this study, red recombinase-based mutation of the *bcsE* gene, which is a c-di-GMP-binding protein involved in cellulose biosynthesis in *Salmonella* biofilms, was performed. In this method, which relies on the disruption of chromosomal genes that have homology to the target gene, the temperature-sensitive low copy number pKD46 plasmid for recombination was isolated from *E. coli* DH5 $\alpha$  cells. The phage red recombinase system is contained in this plasmid and can be synthesized under the control of an inducible promoter. The isolated pKD46 plasmid was transferred into DMC2 and DMC4 strains in which the *bcsE* mutation was to be performed, and the presence of the plasmid in the strains was confirmed. The chloramphenicol resistance plasmid (pKD3) associated with the inactivation regions of the gene FRT (FLP recognition target) was isolated from *E. coli* MZ2997 cells. The pKD3 plasmid was used as a template to obtain the chloramphenicol functional cassette containing homologous regions. Primer base sequences 50 nucleotides long homologous to the *bcsE* gene region were inserted into the 5' ends of the chloramphenicol cassette by PCR. After this step, the functional chloramphenicol cassette with homologous regions was transformed into *Salmonella* strains containing the pKD46 plasmid. Using primers that amplify the chloramphenicol gene cassette, colony PCR was performed with mutant strains growing on agar media containing chloramphenicol, and the mutation was verified by amplification of the gene cassette (Figure 1).



**Figure 1.** Gel image obtained after colony PCR with chloramphenicol gene cassette primers designed to generate mutations in the *bcsE* gene

### Determination of cellulase activity

c-di-GMP is involved in cellulose biosynthesis in *Salmonella* biofilms and controls several aspects of bacterial physiology. To evaluate cellulase activity, *bcsE* mutant and wild-type *Salmonella* cultures were incubated in a medium containing 5% carboxymethylcellulose, and the agar well diffusion assay was performed. Cellulase activity was assessed by the presence or absence of the halo that formed around the well on the agar plates stained with Congo red. The yellow halo formed around the well in the DMC2 and DMC4 wild-type strains proved the existence of carboxymethylcellulase degradation and extracellular cellulase activity. No cellulase activity was detected on the agar surface in the  $\Delta$ DMC2 and  $\Delta$ DMC4 strains (Figure 2). Cellulolytic index was determined high ( $>2$ ) in the wild-type *Salmonella* strains (Table 1).



**Figure 2.** Cellulase activity in wild-type and *bcsE* mutant strains used in the experiment

**Table 1.** Cellulolytic index *Salmonella* strains (P<0,05)

| <i>Salmonella</i> strains | Yellow halo diameter (cm) | Cellulolytic index |
|---------------------------|---------------------------|--------------------|
| DMC2                      | 1,28±0,02                 | 2,56               |
| DMC4                      | 1,68±0,07                 | 3,36               |
| ΔDMC2                     | 0                         | ND                 |
| ΔDMC4                     | 0                         | ND                 |

## DISCUSSION AND CONCLUSION

The fact that cellulase enzyme activity detected in wild-type strains is not observed in *bcsE* mutants in our study indicates that *bcsE* gene is involved in the synthesis of cellulase enzyme as a regulator. Cellulase activity is critical for *Salmonella* virulence as well as many other cellular processes. As previously stated, cellulose is of great importance for *Salmonella* biofilms since it is one of the two major components of the biofilm matrix. However, when *Salmonella* enters macrophages during the infection process, the continuation of cellulose production significantly reduces the virulence of this bacterium (Pontes et al. 2015). For this reason, cellulose production in *Salmonella* strains inside macrophages is suppressed, or the cellulose produced and released from the cell is degraded by the cellulase enzyme. The decrease in cellulase enzyme production due to the inactivation of the *bcsE* gene identified in our study, which does not respond to the cellulase assay, suggests that there is an interaction between cellulase activity and the protein product of the *bcsE* gene. The cellulase BcsZ, which is periplasmically localized in *S. Typhimurium*, is a negative regulator of cellulose biosynthesis. The BcsZ enzyme was also found to regulate cellulose-related phenotypes such as the "rdar" biofilm morphotype, cell aggregation, biofilm formation, pellicle formation, and flagella-dependent motility in a non-polar mutant strain. (Ahmad et al. 2016, Ahmad et al. 2017). Combining these literature data with the results of our study, we can conclude that the *bcsE* gene plays a key role in phase variation of *Salmonella* to adapt to host systems. The discovery of the mechanism by which the *bcsE* gene regulates cellulase enzyme activity will contribute significantly to a better understanding of the molecular mechanisms of *Salmonella* pathogenicity.

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## **CRISPR/CAS9 GENOME EDITING TECHNIQUE FOR ABIOTIC STRESS RESPONSES IN HORTICULTURE**

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### **ABSTRACT**

Factors such as the pressure of world population growth on ecology and climate change negatively affect the sustainability of agriculture. Improvement in quality and yield, as well as resistance and tolerance to stress factors, are among the breeding goals in agricultural production. Garden plant species such as fruit, vegetables and ornamental plants are the first group to be affected by adverse conditions in plant cultivation. With the developed genome editing technologies, tolerance to abiotic stress conditions controlled by multiple genes makes an important contribution to breeding studies. The CRISPR/Cas9 editing module can be used to target genes linked to abiotic stress factors. Successful studies have been carried out in some horticultural plant species against some abiotic stress factors. As a result, it has been evaluated that genome editing tools can be significantly effective in developing abiotic stress tolerant varieties in horticultural plant species.

**Keywords:** Genom Editing, Abiotic Stress, Horticulture, CRISPR/Cas9

### **INTRODUCTIONS**

Climate change is increasing agricultural and food security problems (Piao et al., 2010). Ensuring sufficient and quality food production for the human population is the first condition of sustainability in agriculture. Agricultural areas around the world are gradually decreasing due to urbanization and erosion. The fact that people's diet has changed also increases the demand for different food products. With the increase in population, the necessity of obtaining higher yield and quality products from the unit area with less cost by using the modern agricultural system has increased. Environmental changes with the increasing world population endanger global food security (Bangira, 2018). The current agricultural product cultivation amounts are not sufficient to feed the growing population. In order to overcome the climate change and other stress conditions that limit yield, it is necessary to develop varieties that can tolerate stress.

It is imperative to develop product varieties that better adapt to biotic and abiotic stress conditions. Although the conventional breeding approach provides an increase in production, it may cause problems such as loss of genetic diversity. The dependence on natural allelic variations with this type of breeding program makes it insufficient to obtain a desired trait in production (Wang et al., 2019). In addition, while classical breeding results in gain in one character, it may cause loss of another character. For this reason, new methods are being



researched to overcome the limitations of traditional breeding approaches to eliminate the factors causing crop losses or to improve desired traits. One such approach is to precisely and accurately edit plant genomes. In recent years, some researchers have started to use the transgenic breeding system to create the desired trait through external gene transfer instead of classical breeding (Anwar and Kim, 2020). Genetically modified (GM) products are obtained by transferring genes or gene elements with known functions to elite varieties. However, some ethical problems have emerged with genetic modification studies (Robinson, 1999). There are strict regulations regarding the use, import and export of these products.

## **GENE EDITING**

With the genome editing technologies that have emerged in recent years, this ethical problem has been tried to be eliminated. Because it is stated that the genome-edited varieties are not different from the varieties obtained by traditional breeding technologies (Songstad et al., 2017). By using this technology, fast and efficient new varieties of agricultural products can be developed. Because this technology allows genetic deletions or alterations without adding foreign DNA, the end product may not be considered a genetically modified organism. However, genome editing also leads to genetic modifications like genetically modified organisms. This situation has led to the evaluation of agricultural products using genome editing methods as GMOs in some countries.

Biotechnological methods are used to perform genome editing in living organisms with high accuracy (Zhang et al., 2018). By using these technologies, genetic material can be added, removed or changed at certain points in the genome. Among the genome editing tools, the most important are: Zinc Finger Nucleases (ZFN), Transcription Activator-Like Effector Nucleases (TALEN), CRISPR/Prevotella and Francisella 1 (CRISPR/Cpf1), CRISPR/Cas9. CRISPR-Cas9 allows various parts of the genome to be inserted, removed or altered in the DNA sequence. It is described as the simplest and cheapest genetic manipulation method currently used. With the CRISPR/Cas9 genome editing tool, plant genomes can be edited more efficiently and precisely. The CRISPR/Cas9 editing module has opened new avenues for developing cultivars that are resistant to different stresses. This technique has been used successfully in many horticultural crops.

## **MODIFICATIONS THAT CAN BE OBTAINED IN HORTICULTURE BY CRISPR-CAS9**

### **1.1. Development of Growing Traits**

Among the features obtained for this purpose are dwarf, early flowering, parthenocarpy, increasing the seed germination rate, monoecious flowers. Studies were carried out on horticultural species, tomato (Xu et al., 2016; Chen et al., 2017; Hu et al., 2018; Chen et al., 2018; Jung et al., 2020), cucumber (Hu et al., 2017), watermelon (Wang et al., 2021), apple (Charrier et al., 2019), and pear (Charrier et al., 2019).

## **1.2.Improve Fruit Quality**

Among the properties obtained for this purpose, there are lycopene increase, bitterness reduction, GABA increase, shelf life extension. Studies were carried out on horticultural species, tomato (Ulusik et al., 2016; Rodriguez et al., 2017; Yu et al., 2017; Li et al., 2018a; Wang et al., 2019), potato (Nakayasu et al., 2018; Gonzalez et al., 2020) and eggplant (Maioli et al., 2020).

## **1.3.Enhance Resistance Biotic Stress**

Studies have been carried out to provide resistance to fire blight, mildew and ZYMV. Studies were carried out on horticultural species, cucumber (Chandrasekaran et al., 2016), citrus fruit (Jia et al., 2017), tomato (Nekrasov et al., 2017), banana (Tripathi et al., 2019), apple (Malnoy et al., 2016) and grape (Malnoy et al., 2019).

## **1.4.Enhance Resistance Abiotic Stress**

The CRISPR/Cas9 editing module can be used efficiently to target genes linked to abiotic stress factors. High temperature (tomato, lettuce), low temperature (tomato), drought (tomato), salt (tomato) studies were carried out under abiotic stress conditions.

Many physical, chemical and biological factors cause yield and quality losses on horticultural crops. Any increase in the frequency of abiotic stresses negatively affects the plant (Waqas et al., 2019). Abiotic stress factors include physical and chemical factors such as drought, salinity, temperature (high or low), ultraviolet (UV) radiation, heavy metals, etc. Although abiotic stresses have shaped and continue to shape the evolution of plants, disruption of physiological, biochemical and molecular processes results in severe consequences on the growth and development of plants and an overall reduction in yield. In the last decade, a significant decrease has occurred in field and horticultural crops in terms of yield due to abiotic stress conditions.

### **1.4.1. High Temperature Stress**

Plants grow at the optimum temperature and therefore any increase or decrease in this temperature can seriously inhibit their growth and yield. As the earth's temperature is constantly rising due to global climate change, developing new strategies to combat the effects of extreme temperatures on plants is challenge. Scientists are trying to improve the survival of different plants in response to heat stress using genome editing. Despite several reports, the potential for genome editing to confer heat stress resistance in plants has not been fully explored. It has been reported that NCED4 gene inactivated mutants of lettuce plants produced using CRISPR/Cas9 show better tolerance at the germination during (Bertier et al., 2018). Silencing of SIMAPK3 in tomato using the CRISPR/Cas9 technique was achieved better heat tolerance by reducing ROS accumulation (Yu et al., 2019).

### 1.4.2. Low Temperature Stress

Horticulture crops are sensitive to chills. CBF1 mutants produced by CRISPR/Cas protect the plant from cold damage (Li et al., 2018b). In a study, it was determined that mutants can accumulate higher levels of indole acetic acid and provide cold tolerance in tomato plants (Li et al., 2018b).

### 1.4.3. Drought Stress

Drought is another multidisciplinary stress that affects plant growth at various levels. The impact of this stress is expected to increase further in the coming years due to climate change. Genome editing has been used successfully to improve drought tolerance of different agricultural crops. The OST2 gene encodes an H<sup>+</sup>-ATPase and is involved in establishing proton gradients in plant cells. It has been reported that modification of this gene via CRISPR/Cas9 modulates stomatal closure in response to water-deficient conditions, thereby conferring drought stress tolerance. The CRISPR/Cas9 system was used to develop a drought resistant tomato plants mutant for the SLNPR1 gene (Li et al., 2019). Using the CRISPR-Cas9 technique in tomato, TEY (Thr-GluThy) motifs in the SIMAPK3 gene were found to play an active role in the response to abiotic stress. It was determined that the lines in which this gene was inactivated in tomato gave resistance to drought.

### 1.4.4. Salinity Stress

Salinity stress is one of the important abiotic stress factors limiting cultivation. Plants respond to salt stress through morphological and physiological adaptations that are the result of major changes in gene expression and signaling pathways (Julkowska and Testerink, 2015).

**Table.1.** Genome Editing Studies in Horticulture

|                                     | Fruit  | Vegetable  | Grapes        |
|-------------------------------------|--|--|---------------|
| Improvement of Cultivation Traits   | Apple (2019), Pear (2019), Kiwi (2019) Banana (2020) | Tomatoes (2016, 2017, 2017, 2018, 2018, 2019, 2020, Cucumber (2017), Watermelon (2021)   | -             |
| Improving Fruit Quality             |  | Tomatoes (2016, 2017, 2017, 2017, 2018, 2019), Potatoes (2018, 2020), Eggplant (2020)  | -             |
| Enhancing Resistance Biotic Stress  | Citrus (2017, 2020), Banana (2019), Apple (2020)     | -  | Grapes (2020) |
| Enhancing Resistance Abiotic Stress | -  | High Temperature (Tomatoes-2018, Lettuce-2018)<br>Low Temperature (Tomatoes-2018)<br>Drought (Tomatoes-2019)<br>Salinity (Tomatoes-2017) | -             |

Several genes have been validated with CRISPR/Cas editing to increase resistance to salt stress. It has been reported that CRISPR-mediated loss of function mutations of the SAPK-1 and-2 genes of SnRK2 and osmotic stress/ABA-activated protein kinases in rice confer salinity resistance (Lou et al., 2017). In addition, OsSPL10 genes were found to increase salt tolerance in rice (Lan et al., 2019). It has been reported that loss of function of the OsRR22 gene can significantly inhibit the cytokinin pathway and thus provide salt stress resistance in plants. In a study conducted in my tomato plant, it was determined that inactivation of the SIMAP3 gene caused a decrease in SIOX, SIGST and SIDREB gene expressions, and this situation provided salt tolerance in tomatoes (Wang et al., 2017).

## CONCLUSION AND FUTURE PERSPECTIVES

Abiotic stress factors caused by climate change have serious consequences on the growth and development of plants. Under these conditions, it becomes necessary to adopt strategic policies aimed at ensuring agricultural sustainability in order to meet the food needs of the growing population. These problems have pushed people to seek new alternatives. Editing the genome for desirable traits that can provide sustainability in agriculture is the latest finding in these pursuits. By using gene editing techniques such as CRISPR/Cas9, the production, quality and nutritional properties of agricultural products can be improved. A significant advantage is that CRISPR has protocols for generating trans-gene-free plants (Ma et al., 2015). This advantage provides scientists with the opportunity to produce agricultural products similar to classically bred plants (Khan et al., 2019). This also facilitates the use and public acceptance of CRISPR/Cas9 systems in horticultural crops (Kim and Kim, 2016; Gao, 2018). Public acceptance and valuation play an important role in the adoption of CRISPR technology in food production applications (Gao, 2018; Wolter and Puchta, 2017). Consumption of CRISPR products is more reliable than GMO products. This is important because it shows an opportunity to reduce skepticism about agricultural biotechnology. The practices of some countries where CRISPR gene-edited agricultural products must be labeled and regulated in the same way as transgenic GMOs negatively affect the commercial viability of CRISPR gene-editing in agricultural production (Stokstad, 2018). A global scientific consensus among countries will contribute to the usefulness of gene editing technology.

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## **DETERMINATION OF CORRELATION VARIATION OF MORPHOLOGICAL AND SOME MOLECULAR MARKER TECHNIQUES IN WATERMELON**

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### **ABSTRACT**

Different marker techniques are used for molecular marker assisted selection. It is important to identify molecular marker techniques that are highly correlated with morphological data. The correlation between the clustering results obtained using morphological and genetic data and the distance matrix can be determined. In this study, correlation coefficient values between morphological data of different watermelon genotypes and data of different molecular marker techniques (SSR, ISSR and iPBS) were obtained. This indicates that iPBS primers are more distantly related to morphological data. It can be concluded that the reason for the difference is the differences in the number of loci obtained from different techniques.

**Keywords:** Watermelon, molecular marker, correlation, Mantel test

### **INTRODUCTION**

Characters whose inheritance can be traced at different levels are called genetic markers. Markers are divided into three classes as morphological, biochemical and molecular (Mondini et al., 2009). Different marker systems have advantages and disadvantages compared to each other. The most important advantage of morphological markers is that they are cheap and easy. However, the number of these markers is limited and they are affected by environmental factors. In addition, these markers are dominant, so heterozygous phenotypes cannot be distinguished (Bretting and Widrechner, 1995).

Molecular markers are used in many areas of biotechnology (Parveen et al., 2016). DNA markers are the best tool for selection of plant materials in breeding studies (Ovesna et al., 2002). SSR is a codominant marker system that gives repeatable bands (Sun et al., 2013). The ISSR technique examines nucleotide sequences between simple sequence repeats (Zietkiewicz et al., 1994). Retrotransposons constitute the largest portion of transposons and are the units with the largest gene numbers in eukaryotic genomes (Feschotte et al., 2002; Sabot and Schulman 2006).

Genetic distance matrices are used to determine relationships between populations. It is common to use clustering (such as UPGMA or Neighbor-Joining) and coordination techniques (Basic Coordinates Analysis) to visualize these matrix results (Felsenstein, 2004). The Mantel



test is the most widely used method to evaluate the relationship between geographic distance and genetic deviation (Mantel, 1967; Manly, 1997). It is important to identify molecular marker techniques that are highly correlated with morphological data. In this study, it was aimed to determine the correlation between morphological data and different molecular marker techniques.

## **MATERIAL AND METHOD**

In the study, 96 watermelon (*Citrullus lanatus*) genotypes were used. 29 morphological data were used for watermelon genotypes. CTAP method was used for DNA isolation and the quantity/quality of the isolated DNAs were determined by running on an agarose gel. Primers that gave reliable bands were determined by performing the primer test. A total of 62 SSR, 36 iPBS and 12 ISSR primers were used in the study. PCR processes were performed for different marker techniques. Agarose and polyacrylamide gel electrophoresis processes were performed and scoring was done. As a result of molecular studies, 1, 0 and 9 data were obtained. These data were analyzed using NTSYS (Numerical Taxonomy Multivariate Analysis System, NTSYS-pc version 2.1, Exeter Software, Setauket, N.Y., USA) package program (Rohlf, 2000).

## **RESULTS AND DISCUSSION**

Cluster analyzes based on similarity index were performed on 96 watermelon genotypes using morphological data. In the molecular study, iPBS, ISSR and SSR techniques were analyzed separately and combined (iPBS+ISSR+SSR). As a result of the iPBS primer study, the mantel correlation value of the morphological and iPBS DNA matrix data was low ( $r=0.006$ ). An ultrametric similarity matrix was created from the dendrogram obtained by clustering analysis and this matrix was subjected to the Mantel test with DICE similarity matrix. In the iPBS studies, the 'r value' of this test was determined as 0.994. Mohammadi and Prasanna (2003) stated that the cophenetic correlation coefficient between the similarity indices and the dendrogram is equal to or greater than 0.9, there is a very high correlation between the dendrogram and the similarity indices, and the dendrogram represents the similarity index very well.

As a result of ISSR primer study, the mantel correlation value of morphological and ISSR DNA matrix data was found low ( $r=0.033$ ). An ultrametric similarity matrix was created from the dendrogram obtained by clustering analysis and this matrix was subjected to the Mantel test with DICE similarity matrix. In ISSR studies, the 'r value' of this test was determined as 0.992. As a result of the SSR primer study, the mantel correlation value of the morphological and SSR DNA matrix data was found to be low ( $r=0.029$ ). An ultrametric similarity matrix was created from the dendrogram obtained by clustering analysis and this matrix was subjected to the Mantel test with DICE similarity matrix. In SSR studies, the 'r value' of this test was determined as 0.968.

The mantel correlation value of morphological and combined (iPBS+ISSR+SSR) DNA matrix data was low ( $r=0.025$ ). An ultrametric similarity matrix was created from the dendrogram obtained by clustering analysis and this matrix was subjected to the Mantel test with DICE similarity matrix. In the combined data, the result of this test was determined as 'r value' 0.863.

**Table 1.** Mantel correlation values between different marker techniques

|               | Morphological | iPBS | ISSR | SSR  | Combine |
|---------------|---------------|------|------|------|---------|
| Morphological | 1             |      |      |      |         |
| iPBS          | 0.006         | 1    |      |      |         |
| ISSR          | 0.03          | 0.44 | 1    |      |         |
| SSR           | 0.03          | 0.47 | 0.71 | 1    |         |
| Combine       | 0.025         | 0.81 | 0.78 | 0.85 | 1       |

Low r values were obtained between morphological data and molecular data. Despite the low values, it can be said that ISSR and SSR values show a higher correlation with morphological data than iPBS data. This indicates that iPBS primers are more distantly related to morphological data. Correlation coefficients of 0.44-0.71 were determined between three different molecular marker techniques. The lowest was determined between iPBS and ISSR (0.44), and the highest was determined between ISSR and SSR techniques (0.71). In the analyzes made with the combined data, it was determined that the data showed a correlation of 0.85 with SSR primers, 0.81 with iPBS primers, and 0.78 with ISSR primers. It can be concluded that the reason for the difference here is the differences in the number of loci obtained from different techniques.

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## **MORPHOLOGICAL AND BIOCHEMICAL RESPONSES OF GRAND NAIN AND DWARF CAVENDISH (*Musa* spp.) BANANA SEEDLINGS TO DROUGHT STRESS SIMULATED BY PEG-6000**

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### **ABSTRACT**

Banana (*Musa acuminata* L.), an indispensable fruit of countries with tropical and subtropical climates, has come face to face with the effects of global climate change. In the present study, drought stress was stimulated by using polyethylene glycol (PEG-6000) *in vitro* conditions and tolerance levels of Grand Nain (GN) and Dwarf Cavendish (DC) banana cultivars were determined. Seedlings of the cultivars were placed in the MS nutrient medium combined with 5 mg L<sup>-1</sup> BA and 1 mg L<sup>-1</sup> NAA, 5%, 15%, 25%, and 35% PEG to be observed for forty-five days. In the study, cultivar, treatment, and treatment x cultivar interactions were significant in all parameters. Statistically, the worst affected plants in terms of plant height were 15%-DC, 25%-GN, 25%-DC, 35%-DC, and 35%-GN treatments while 35% PEG were the most effective treatments in terms of plant diameter with the GN cultivar (2.71 mm). According to the visual scale results, DC-control had a minimum injury, whereas maximum injury was recorded at 35%-GN. The least visual effect was observed at 5% and 15% PEG levels of DC with a score value of 1.5. While DC produced a tighter tissue pseudostem structure, GN produced a more diffuse and drooping pseudostem. In addition, while the GN root structure was more developed, the DC root structure was shorter and made few lateral roots. While there was no difference in RWC % between control plants of both cultivars and DC cultivar with 5% PEG, similarly, there was no difference between 5%-GN cultivar and 15%-DC cultivar. However, it appears that the DC cultivar had the highest RWC % content at the highest PEG ratio. Total chlorophyll content and SPAD values supported each other and 35%-GN had the lowest value in both parameters with 2.73 and 6.82 mg g<sup>-1</sup> Fw, respectively. The highest MDA and proline content were also determined in the 35%-GN with 0.71 nmol g<sup>-1</sup> and 17.71 nmol mg<sup>-1</sup> Fw, respectively. The results showed that the DC variety which is grown mostly in open fields in Turkey will show higher tolerance than the GN cultivar in many respects when water stress occurs. Therefore, the DC cultivar is a preferable cultivar under drought conditions.

**Keywords:** Banana, Drought stress, PEG, Proline

### **INTRODUCTION**

Banana (*Musa acuminata* L.) is a monocotyledon plant that is widely distributed throughout tropical and subtropical countries, mainly in the warm and humid regions of the world. As a result of climate change, adverse effects of drought have become more pronounced in the tropics and subtropics (Ravi et al., 2013).

Banana plants are extremely sensitive to stress conditions induced by drought, osmotic, salt, cold, and other environmental stressors. Mahouachi (2007) and Uma et al. (2002) reported

that the drought-sensitive banana plants showed characteristic symptoms internally and externally during stress. Physiological and biochemical changes at the cell level create internal changes while burning and drying of leaves are the most common external symptoms (Surendar et al., 2013b).

Field studies for drought resistance create some difficulties in terms of the need for large areas, long vegetation periods, and other variable factors (temperature, humidity, wind, transpiration, etc.). Thus, *in vitro* studies are advantageous because it is faster and less plant material is required, allowing for the rapid multiplication of selected genotypes (Rai et al., 2011), control of conditions, and the detection of differences in reduced growth (Vanhove et al., 2012). Polyethylene glycol (PEG) has been commonly used *in vitro* culture to induce drought or osmotic stress because, it modifies the osmotic potential of the medium and induces plant water deficit in a relatively controlled manner (Bidabadi et al., 2012; Putnik-Delic et al., 2013). There are some scientific reports on using polyethylene glycol (PEG) as water stress inducers of various banana cultivars (Saeedavi et al., 2017; Mohsen et al., 2006; Mahmood et al., 2012; Xiong et al., 2013). They showed that water stress adversely affected fresh weight increase, shoot vigor, and multiplication rate. However, debate continues about the best strategies for improving the efficiency of the tissue culture propagation methods for this highly valued crop.

Cavendish (*Musa cavendish*), a worldwide cultivated commercial subgroup banana grown mainly for the export market, is a pure triploid *M. acuminata* (AAA) and its cultivars include Grand Nain and Dwarf Cavendish (Anyasi et al., 2013). In the present study, drought stress was stimulated by using PEG-6000 *in vitro* conditions and tolerance levels of Grand Nain (GN) and Dwarf Cavendish (DC) banana cultivars were determined.

## MATERIAL AND METHOD

*Plant Material:* Two cultivars of *Musa* spp. obtained from the Alata Horticultural Research Institute located in the Mersin city (34°E 36°N, sea level, average annual temperature: 23.3/14.7°C, mean relative humidity: 70%, mean annual precipitation: 138 mm), Erdemli, in Southern Türkiye (one of the most important areas of banana cultivation in the country). The cultivars were Dwarf Cavendish and Grand Nain which are of triploid *Acuminata* type.

*Preparation of Explants:* The suckers are cut to expose the shoot tip of 10 cm<sup>3</sup> and cut further to about 3 cm diameter and 3-5 cm length. After being kept under pressurized water for 1 hour to remove the phenolic compounds in the plant, they were incubated in 96 % alcohol for 1 minute. After surface sterilization for 30 minutes with 10 % hypochlorite, they were passed through sterile distilled water 3 times and kept in distilled water. At the laminator flow cabin, the explants were trimmed using a surgical blade to bring the final size to about in 3-4 cm length and in 1-2 cm diameter. After this stage, the isolated shoot tips were transferred directly to the basic nutrient medium. Shoot tips were cultured for four weeks on Murashige-Skoog basal medium (Murashige and Skoog, 1962) supplemented with benzyl adenine (3 mg/l) and agar (6 g/l). The growing explants were re-cultured on fresh media until the proliferation at four week intervals. To obtain a sufficient number of explants, the produced shoots were subcultured four times on solid Murashige Skoog basal media supplemented with benzyl adenine (5 mg/l) in a so-called multiplication phase. After a sufficient number of materials was obtained, the plantlets were taken into media containing PEG-6000.

*Incubation with polyethylene glycol:* The nutrition media is cooled until 60-70°C after autoclave, different ratio of PEG-6000 is passed through filters with 22 µm nanopores and cold sterilization. 0, 5%, 15%, 25% and 35% PEG 6000 has been added into MS basal media. Media content for rooting was 5 mg/l benzyladenine and 1 mg/l naphthalene acetic acid. The seedlings

were first placed in a PEG-6000-free medium for four weeks to promote rooting. They were then placed in a medium containing PEG-6000 for 45 days.

*Visual Scale:* The scale created by Baysal (2022) was used to visually determine the degree of stress created by PEG-6000.

*The pseudostem length (mm), and diameter (mm):* The area was measured from the root throat to the shoot tip by meters as mm ( $\pm 0.5$ ). Pseudostem diameter was determined as mm ( $\pm 0.1$ ) with the help of a digital caliper.

*Quantification of leaf relative water content (RWC %):* The relative water content (RWC) of the leaves was measured according to the method suggested by Barrs and Weatherley (1962). The leaf RWC was calculated with the formula;

$$\text{RWC \%} = (\text{leaf fresh weight} - \text{leaf dry weight}) \times (\text{leaf turgid weight} - \text{leaf dry weight}) \times 100$$

*Chlorophyll fluorescence:* SPAD values were recorded using a SPAD-502 meter (Konica-Minolta, Japan).

*Total Chlorophyll Content:* Total chlorophyll (TC) analysis was performed according to the protocol of Arnon (1949). The TC content in the samples was measured at 652 nm by spectrophotometer and calculated as mg per fresh weight.

*Lipid peroxidation:* The malondialdehyde (MDA) content was estimated according to Sun et al. (2006). The supernatant was analyzed with a spectrophotometer and calculated from A535-A600 using the coefficient of absorbance of  $155 \text{ mM}^{-1}\text{cm}^{-1}$  (Lei et al., 2010).

*Proline Content:* Proline was determined spectrophotometrically using the ninhydrin method described by Bates et al. (1973). Absorbance at 520 nm was determined in a BioMate spectrophotometer (Thermo Spectronic).

#### *Statistical analysis*

Data were presented as mean $\pm$ SDA and subjected to two-way ANOVA with a randomized plot design with fifteen replications for each parameter using JPM 5.0.1. software (SAS Institute, Cary, NC, 1989) followed by the LSD test ( $p < 0.05$ ).

## **RESULTS AND DISCUSSION**

Drought, which is one of the most important stress factors that threaten crop production in our world today, not only decreases nutrient uptake in general but also reduces the transport of nutrients from the roots to the stem, because of the reduction in the rate of transpiration is caused by the damage suffered by active transport and membrane permeability (Viets, 1972; Alam, 1999).

The effects of PEG-6000 were determined by measuring some morphological traits of banana cultivars. When Table 1 is examined, the highest plant height was determined in control of the GN cultivar. DC results of 15% PEG and 35% and 25% PEG treatments of both cultivars showed that there was no significant difference between plant height. In addition, The plant height was higher in GN variety in each treatment. This may be related to the variety feature. Singh et al. (2021) reported that the longest shoot length ( $3.7 \pm 0.14$ ) was recorded on a medium without PEG supplementation and the shortest shoot length ( $2.9 \pm 0.04$ ) was recorded in 3% (w/v) PEG concentration. Similarly, Kayongo et al. (2016) in their drought stress study in the field, reported that plant heights decreased from 67-86 and 64-93 cm to 60-83 and 46-64 cm, respectively. In conclusion, higher ratios of PEG-6000 were used in this study and decreased plant height as the ratio increased.

**Table 1.** Variation of some morphological indices under drought stress of banana seedlings

| Treatments | Cultivars | Plant height (mm)        | Plant diameter (mm)      | R. water content (%)    |
|------------|-----------|--------------------------|--------------------------|-------------------------|
| Control    | DC        | 20.45±1.57 <sup>a</sup>  | 4.60±0.28 <sup>a</sup>   | 98.52±0.55 <sup>a</sup> |
|            | GN        | 35.23±6.96 <sup>b</sup>  | 3.57±0.45 <sup>c</sup>   | 96.33±1.53 <sup>a</sup> |
| 5% PEG     | DC        | 19.17±0.09 <sup>bc</sup> | 4.07±0.13 <sup>b</sup>   | 96.19±0.51 <sup>a</sup> |
|            | GN        | 21.70±0.38 <sup>b</sup>  | 3.64±0.19 <sup>bc</sup>  | 86.82±0.74 <sup>b</sup> |
| 15% PEG    | DC        | 15.20±0.17 <sup>d</sup>  | 2.96±0.43 <sup>de</sup>  | 87.50±1.91 <sup>b</sup> |
|            | GN        | 16.37±0.29 <sup>cd</sup> | 3.39±0.24 <sup>cd</sup>  | 66.60±1.71 <sup>d</sup> |
| 25% PEG    | DC        | 14.49±0.95 <sup>d</sup>  | 3.23±0.02 <sup>cd</sup>  | 76.93±2.09 <sup>c</sup> |
|            | GN        | 15.19±0.13 <sup>d</sup>  | 3.50±0.41 <sup>c</sup>   | 54.64±1.79 <sup>f</sup> |
| 35% PEG    | DC        | 14.07±0.43 <sup>d</sup>  | 3.17±0.15 <sup>cde</sup> | 61.82±3.49 <sup>e</sup> |
|            | GN        | 15.06±0.46 <sup>d</sup>  | 2.71±0.13 <sup>de</sup>  | 42.50±0.71 <sup>g</sup> |

Mean ± SD; Mean of 15 replicates; within the same column, values followed by different letters are significantly different at  $p \leq 0.05$  according to two-way analysis of variance (ANOVA) statistical procedure (student *t*-test).

In control and 5% PEG treatment, DC had the highest value in terms of plant diameter, while control plants of GN variety took third place. As seen in Fig. 1, there are visually distinct morphological differences between the two cultivars. According to the visual scale results, DC-control had a minimum injury, whereas maximum injury was recorded at GN-35% PEG-6000 (Fig. 1). The mean values of the scale varied between 0.0 and 7.00. The least visual effect was observed at 5% and 15% PEG levels of DC with a score value of 1.5. While DC produced a tighter tissue pseudostem structure, GN produced a more diffuse and drooping pseudostem. In addition, while the GN root structure was more developed, the DC root structure was shorter and made few lateral roots. This may be due to the different effects of the MS medium used on the cultivars.

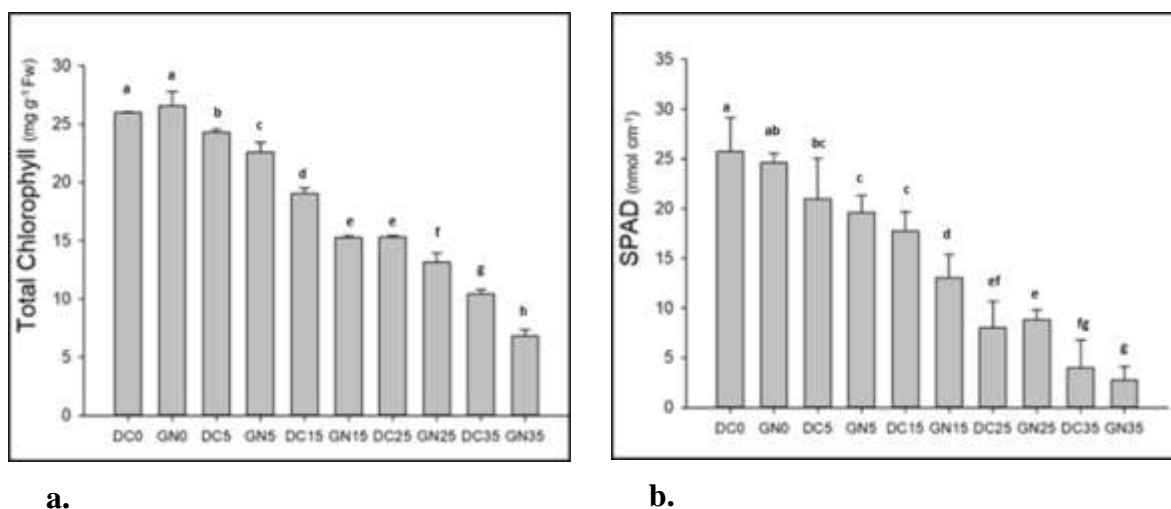


**Figure 1.** Morphological damage of cultivars after stress temperatures (Images represent replicates and visual scale. a. DC, b. GN. from left to right control, 5%, 15%, 25%, and 35% PEG-6000, respectively).

The relative leaf water content is a relevant physiological component for assessing the leaf osmotic potential. It is a variable for estimation of the current water content of the leaf tissue, relative to the maximal water content it can hold at full turgidity, in a situation of moisture deficits. While there was no difference in RWC % between control plants of both cultivars and DC cultivar with 5% PEG, similarly, there was no difference between 5% PEG

treatment of GN cultivar and 15% PEG treatment of DC cultivar. However, it appears that the DC cultivar had the highest RWC % content at the highest PEG ratio (Table 1). Our finding is in agreement with Saeedavi et al. (2017) and Putnik-Delic et al. (2013) findings which showed the reduction of tissue water content.

Water stress results in reduced production and increased breakdown of chlorophyll in leaves, which is manifested as leaf senescing or chlorosis (Dekov et al., 2000). The total chlorophyll content was decreased when responding to all PEG concentrations (Figure 2a). The activation of chlorophyllase enzyme might be a significant reason for the depletion of chlorophyll content as reported by Mihailovic et al. (1997). In terms of total chlorophyll values, there was no difference in both cultivars in the control group plants, while DC took the highest value in SPAD values (Figure 2b). When 35% of PEG treatments were evaluated, the highest SPAD and TC values were determined in the DC cultivar.

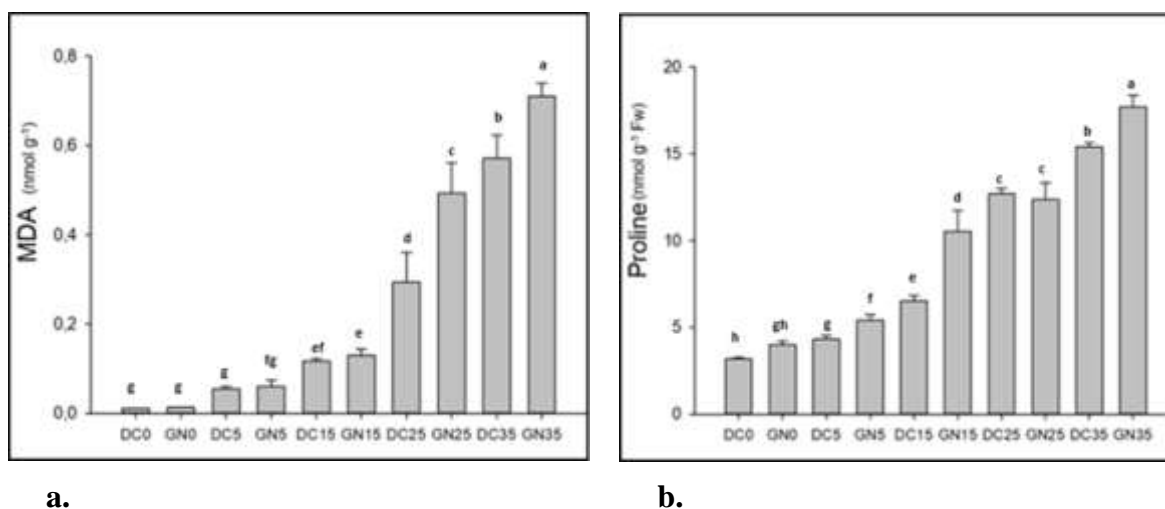


**Figure 2.** Changes in photosynthetic in the leaves of banana seedlings. a. total chlorophyll content, b. chlorophyll fluorescence (SPAD). The different letters on the top of the error bars show statistically different means at  $p \leq 0.05$  according to the two-way analysis of variance (ANOVA) statistical procedure (student *t*-test)

Lipid peroxidation that arises due to osmotic stress can induce chlorophyll degradation (Ali et al., 2019; Sadak et al., 2020). MDA content, which is an important criterion in determining the stress level, was found in the highest GN variety and 35% PEG treatment. At the level of coping with stress, when the proline content, which is another parameter, was evaluated, the GN variety had the highest value. A significant difference was observed between MDA levels in 25% PEG treatment of both cultivars (Figure 3a). According to Bidabadi et al. (2012) reported that MDA content decreased when PEG and salicylic acid content were applied together in their study on the Breangan banana cultivar.

Proline is an amino acid, which is known to involve in cell osmotic adjustment and cell component protection during drought stress. In terms of proline content, DC variety had the highest value up to 25% PEG, while GN variety had the highest value in 35% PEG (Figure 3b). While Mohsen et al. determined the highest proline value as 6 mg/g in 20 g/l PEG-6000 treatment, close values were obtained in 15% PEG treatment in this study. Badruzaman et al. (2021) reported that the total proline content was significantly decreased in seedlings treated with 5% of PEG ( $6.44 \pm 0.78 \mu\text{mole/g FW}$ ) and 4% of PEG ( $4.81 \pm 0.04 \mu\text{mole/g FW}$ ) compared to control of Lang banana (a cooking banana). The reason why the study results are so different is thought to vary according to the PEG ratio, stress duration, and genotype used.





**Figure 3.** The MDA (a) and proline content (b) in the leaves of banana seedlings. The different letters on the top of the error bars show statistically different means at  $p \leq 0.05$  according to the two-way analysis of variance (ANOVA) statistical procedure (student *t*-test).

Several studies have reported banana genotypes with the “B” genome such as AAB and ABB to be more drought-tolerant than those entirely based on the “A” genome, for example, ‘Cavendish’ AAA (Robinson, 1996; Robinson and Sauco, 2010). This drought tolerance has been imputed to the belief that the *Musa balbisiana* originated from drier parts of South Asia including the lower Himalayan ranges (Tenkouano, 2006) unlike *Musa acuminata*, whose origin is the humid forest regions of South-East Asia (Kissel et al., 2015). In this study, cultivars with AAA genome were used. However, the Dwarf cavendish variety has been grown in the open field for many years in Turkey. This may be the reason behind its good tolerance to drought stress.

## CONCLUSION

Drought tolerance is a complex trait whose expression is controlled by many genes and environmentally varies over location and time, which complicates the development of a standard for drought. Polyethylene glycol has been commonly used in plant tissue culture to induce controlled drought/osmotic stress because it modifies the osmotic potential of the medium and induces plant water deficit in a relatively controlled manner. In this study, the effects of stress were evaluated *in vitro* cultures with different ratios of PEG-6000, two commercially important banana cultivars for Turkey. As a result of the study, it was understood that the Dwarf Cavendish variety showed a little more tolerance to drought stress. However, other internal factors that cause this need to be investigated in more detail.

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## FACILE SYNTHESIS OF SnO<sub>2</sub> NANOPARTICLES FOR PHOTOCATALYTIC DECOLORIZATION OF AZO DYE SOLUTION

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### ABSTRACT

Azo dyes constitute one of the largest groups of synthetic colorants due to their ease of application, brilliant colors, and high adsorption affinity properties. These dyes are widely used in various industries, including textiles, paper, and leather. Improper discharge of effluents containing azo dyes leads to severe environmental problems. Consequently, dye removal from water and wastewater is a significant concern worldwide. Among advanced oxidation processes, heterogeneous photocatalysis is a favorable treatment method using a semiconductor and UV light simultaneously. SnO<sub>2</sub> is an n-type semiconductor that has high chemical stability and is environmentally friendly and inexpensive. The present work was focused on preparing SnO<sub>2</sub> nanoparticles with a precipitation method. The structural and morphological analysis of SnO<sub>2</sub> nanoparticles was performed by XRD, FTIR, ESEM, and Raman spectroscopy. The obtained results stated that SnO<sub>2</sub> nanoparticles consisted of agglomerated particles. The photocatalytic activity of SnO<sub>2</sub> nanoparticles was also tested in the degradation of Reactive Red 194 (RR-194) under UV irradiation. RR-194 was used as a mono azo model dye compound. A pseudo-first order kinetics model was used to describe the photocatalytic degradation kinetics of RR-194.

**Keywords:** Azo dye, decolorization, heterogeneous photocatalysis, Reactive Red 194, SnO<sub>2</sub>.

### INTRODUCTION

The textile industry provides a crucial benefit to the economy, but this industry also generates huge amounts of industrial wastewater associated with dyeing and printing processes. The discharge of untreated wastewater, including dyes, chemicals, and organic and inorganic compounds, can cause a vital threat to the environment and human health (Al-Mamun et al., 2019; Lum et al., 2020). Therefore, it is important to develop new water treatment technologies to overcome this global environmental pollution issue. In this regard, semiconductor photocatalysis is a promising method with advantages such as simplicity, high efficiency, and low cost (Jabbar and Graimed. 2022). In recent years, SnO<sub>2</sub>, an n-type semiconductor, has been found to have a potential application in the photodegradation of dyes with chemical inertness as well as non-toxicity (Chen et al., 2015; do Nascimento et al., 2022; Gnanamoorthy et al., 2021; Habte et al., 2020; Shanmugam et al., 2016; Sun et al., 2022; Tammina et al., 2018; Yousefi et al., 2021).

In this study, SnO<sub>2</sub> nanoparticles were synthesized via a precipitation method. The synthesized SnO<sub>2</sub> nanoparticles were well characterized by using XRD, FTIR, ESEM, and Raman spectroscopy. Reactive Red 194 (RR-194) was chosen as a representative mono azo dye

model used in cotton dyeing. The photocatalytic behavior of SnO<sub>2</sub> nanoparticles was tested through the degradation of RR-194 under UVA irradiation.

## MATERIALS AND METHOD

All chemical reagents were analytical grade and used without further treatment. Tin(II) chloride dihydrate (SnCl<sub>2</sub>·2H<sub>2</sub>O), 25% ammonia solution (NH<sub>3</sub>) were purchased from Merck. SnO<sub>2</sub> nanoparticles were prepared by precipitation method with reference to the methodology reported by Yousefi and colleagues with minor modifications (Yousefi et al., 2021). Briefly, NH<sub>3</sub> (8 mL) was added dropwise into a 0.1 M SnCl<sub>2</sub>·2H<sub>2</sub>O (100 mL) solution in a flat bottomed flask with vigorous stirring by a magnetic stirrer. Afterwards, the stirring was continued for another 40 min. The suspension was kept at room temperature for 18 h to obtain a white residue. Finally, the precipitate was filtered and thoroughly washed with first distilled water, then dried in an air oven at 80 °C for 24 h, and finally calcined at ethanol, and finally calcined at 500 °C for 3h.

FT-IR measurements were performed in the range of 4000–400 cm<sup>-1</sup> using Thermo Scientific Nicolet 6700. Raman spectroscopy was acquired by a Thermo Scientific DXR Raman Microscope using Ar<sup>+</sup> laser excitation at λ=532 nm. Cu Kα radiation (λ=1.54 Å) was used to generate on a Rigaku-D/MAX-Ultima diffractometer. ESEM analysis was recorded on a FEI-Philips XL30 instrument.

Photocatalytic activity experiments were conducted in a cylindrical Pyrex reaction vessel containing 50 mL of a solution that was illuminated from the top with a 125 W black light fluorescent lamp (λ<sub>max</sub>=365 nm). The experiments were performed at a concentration of 5 mg/L RR-194 solutions, keeping the photocatalyst dose at 0.25 g/L constant without pH adjustment. Following each treatment period (0 min-240 min), the photocatalysts were immediately removed through 0.45 μm membrane filters. The concentration changes of RR-194 were monitored by examining the alterations at λ<sub>max</sub>=535 nm on a Thermo Scientific Genesys 10S double beam UV-vis spectrophotometer.

## RESULTS AND DISCUSSION

The structural and morphological analysis of the synthesized SnO<sub>2</sub> nanoparticles were investigated. FTIR spectroscopy was used to identify the functional groups of the synthesized SnO<sub>2</sub> nanoparticles, and the spectrum of SnO<sub>2</sub> was shown in Figure 1. The weak bands at 3445 cm<sup>-1</sup> and 1650 cm<sup>-1</sup> were assigned to the OH stretching of adsorbed water molecules. The observed intense bands at 600 cm<sup>-1</sup> and 516 cm<sup>-1</sup> were related to the Sn-O and O-Sn-O stretching and bending modes of SnO<sub>2</sub> nanoparticles, respectively (Gnanamoorthy et al., 2021; Zhang et al., 2011).

The XRD pattern of the synthesized SnO<sub>2</sub> nanoparticles was revealed in Figure 2. The observed diffraction peak positions were well matched with JCPDS No. 41-1445 and verified the tetragonal rutile structure of SnO<sub>2</sub>. The diffraction peaks at 26.58°, 33.88°, 37.90°, 51.80°, 54.80°, 57.98°, 62.00°, 66.08°, 71.40°, and 78.80° corresponded to the (110), (101), (200), (211), (220), (002), (310), (301), (202), and (321) planes (Tammina et al., 2018).

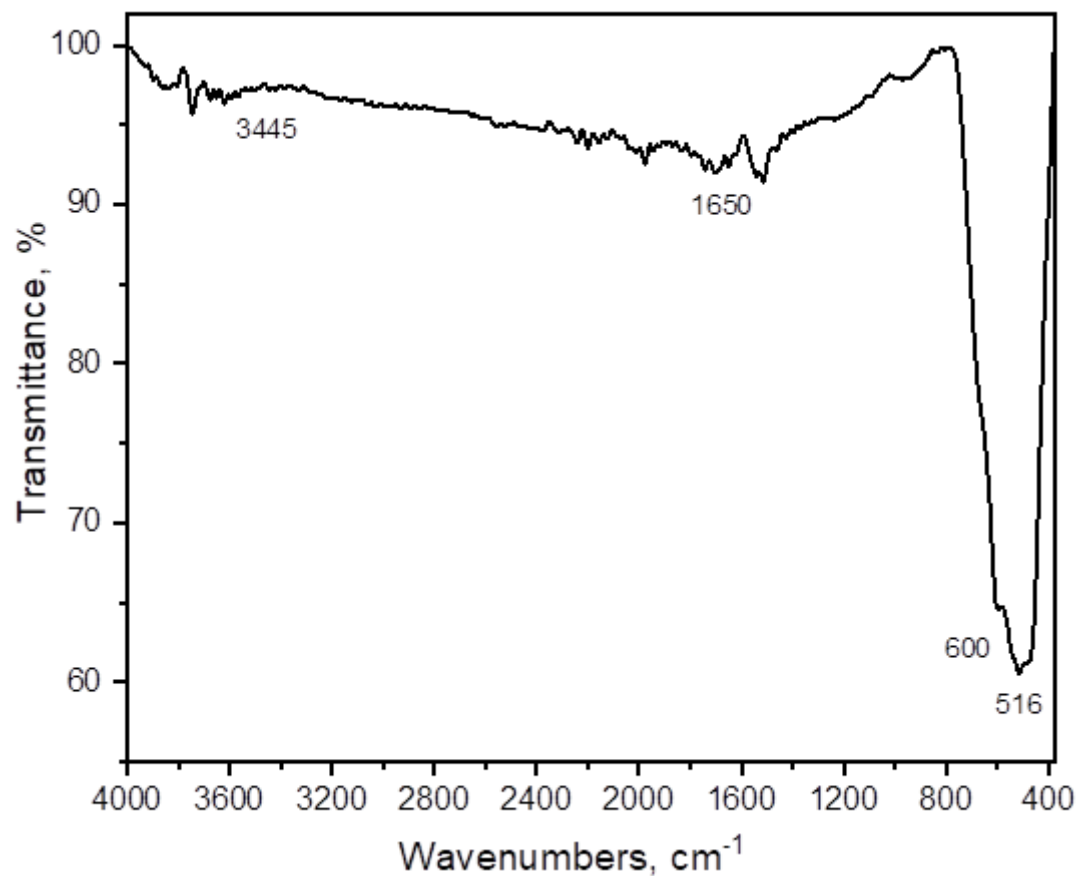


Figure 1. FTIR spectrum of SnO<sub>2</sub> nanoparticles.

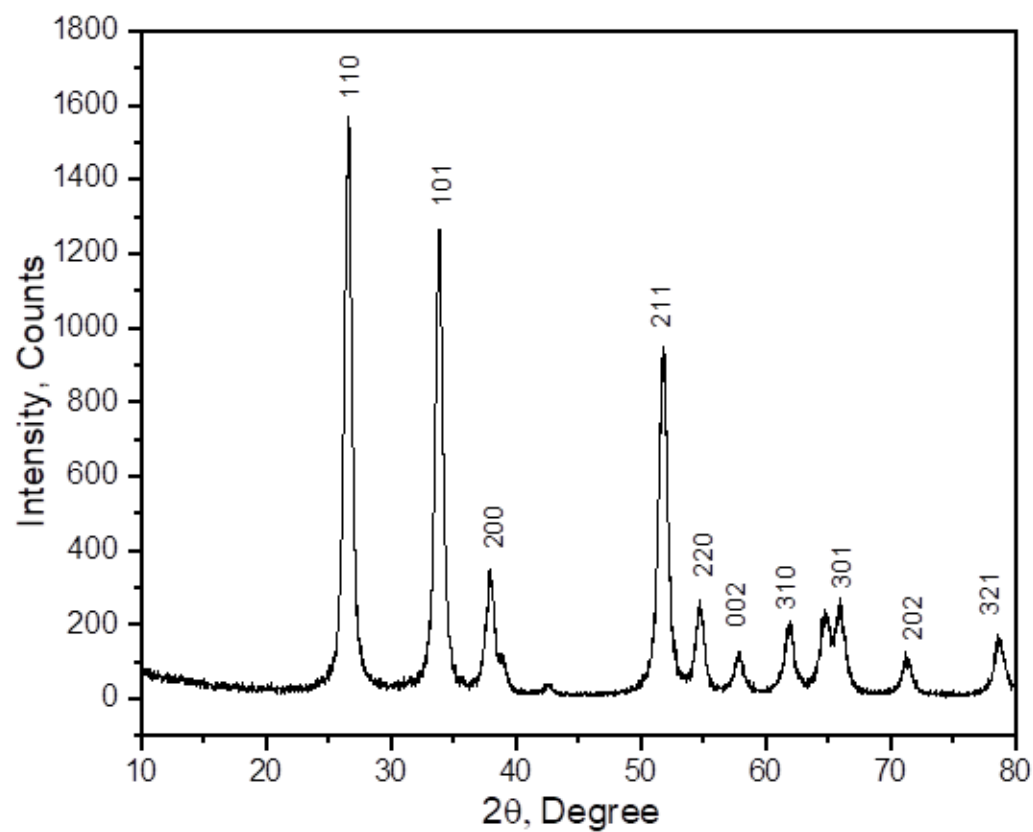
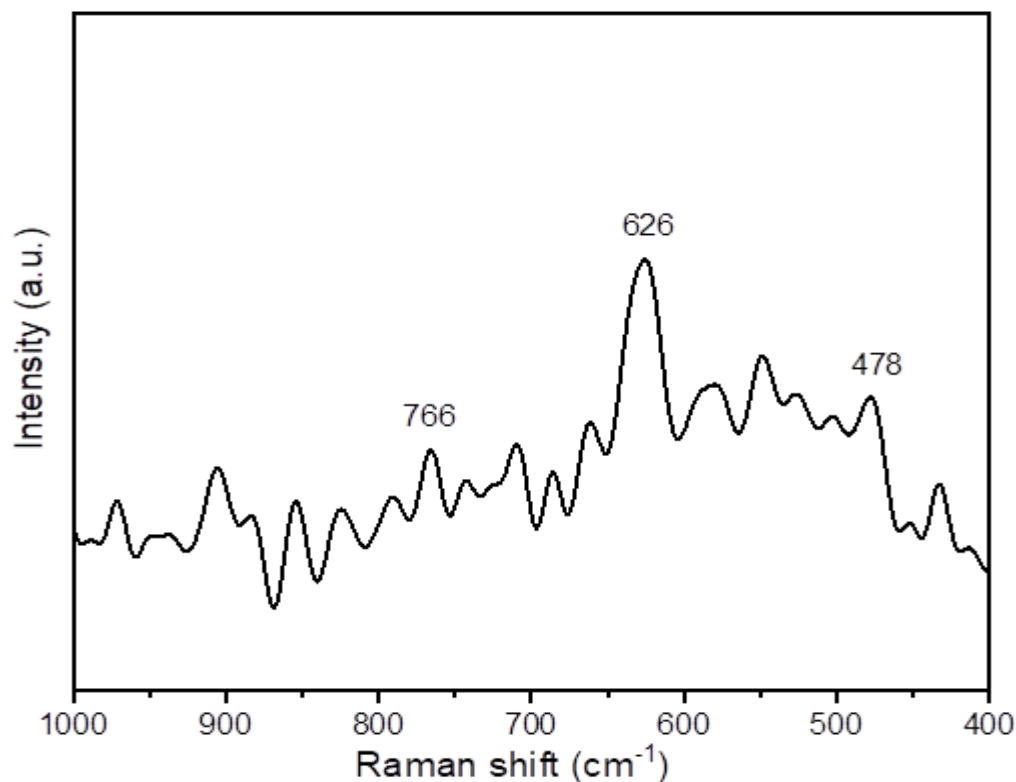


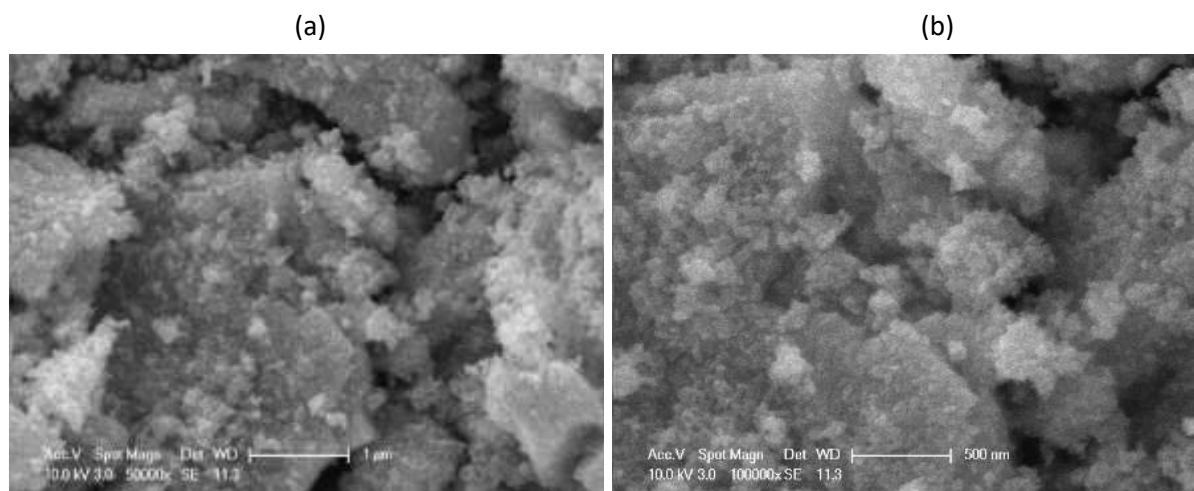
Figure 2. XRD pattern of SnO<sub>2</sub> nanoparticles.

The Raman spectrum of SnO<sub>2</sub> nanoparticles was displayed in Figure 3. The spectrum exhibited three Raman bands at 766 cm<sup>-1</sup>, 626 cm<sup>-1</sup>, and 478 cm<sup>-1</sup> corresponding to the tetragonal rutile structure SnO<sub>2</sub> active modes, B<sub>2g</sub>, A<sub>1g</sub>, and E<sub>g</sub>, respectively (Sangeetha et al., 2011). Raman results were consistent with XRD.



**Figure 3.** Raman spectrum of SnO<sub>2</sub> nanoparticles.

The ESEM images of SnO<sub>2</sub> nanoparticles with different magnifications were presented in Figure 4. The particles were almost spherical in shape with slight aggregations. The particle size and distribution were not homogeneous due to the presence of agglomeration (Habte et al., 2020).



**Figure 4.** ESEM images of SnO<sub>2</sub> nanoparticles (a) x50000 and (b) x100000.

The kinetic constant of SnO<sub>2</sub> nanoparticles was calculated by the following pseudo first-order kinetic model expressed by the equation (1):

$$\text{Rate (R)} = -dA / dt = kA \quad (1)$$

where,

R: pseudo-first order rate ( $\text{cm}^{-1}\text{min}^{-1}$ ),

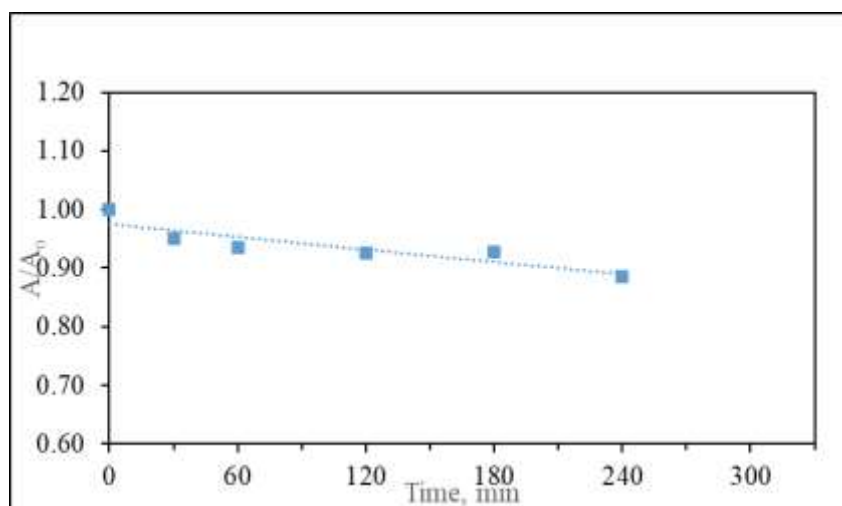
A<sub>0</sub>: initial absorbance of RR-194,

A: absorbance of RR-194 at time t,

t: irradiation time, min,

k: pseudo-first order reaction rate constant,  $\text{min}^{-1}$ .

The kinetics of photocatalytic degradation of RR-194 in the presence of SnO<sub>2</sub> nanoparticles was presented in Figure 5. Kinetics analysis indicated that the photocatalytic degradation rate of RR-194 could be approximated by a pseudo-first-order model with a [correlation coefficient](#) value ( $R^2 > 0.79$ ). The rate constant of RR-194 was calculated as  $k=3.82 \times 10^{-4} \text{ min}^{-1}$  and rate as  $R=4.00 \times 10^{-5} \text{ cm}^{-1} \text{ min}^{-1}$ .



**Figure 5.** Photocatalytic degradation of RR-194 in the presence of SnO<sub>2</sub> nanoparticles.

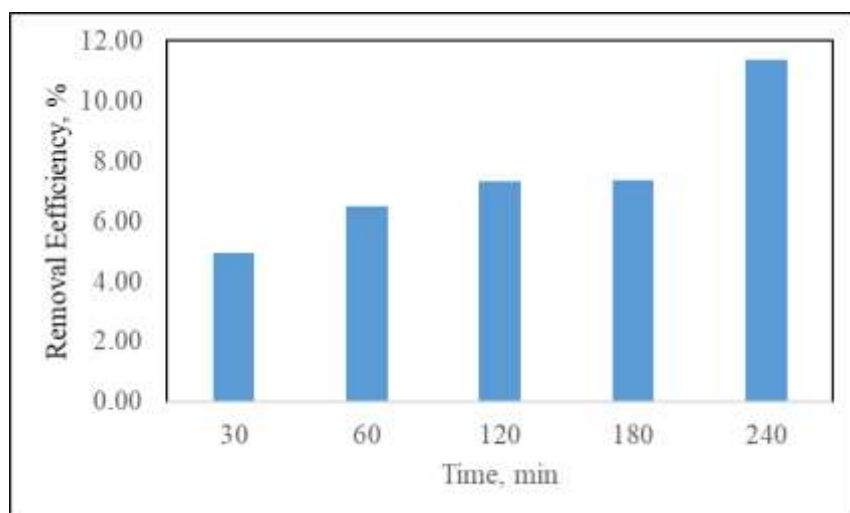
Moreover, the degree of RR-194 decolorization by using SnO<sub>2</sub> (Figure 6) was also calculated by the following equation (2).

$$\text{Decolorization, \%} = ((A_o - A)/A_o) \times 100 \quad (2)$$

where,

A<sub>o</sub>=initial absorbance of RR-194 and A=absorbance of RR-194 at irradiation time t.





**Figure 6.** Photocatalytic degradation of RR-194 upon use of SnO<sub>2</sub> nanoparticles.

A slight decolorization percentage of RR-194 was observed (ca.12%) in the presence of SnO<sub>2</sub> under 240 min UVA irradiation. Low decolorization could be attributed to the surface charges of SnO<sub>2</sub> nanoparticles and RR-194 dye. The isoelectric point (IEP) of SnO<sub>2</sub> was reported in the range of  $3.5 < \text{pH}_{\text{IEP}} < 5$  (Najjar et al., 2021; Qian et al., 2009). The pH of the suspension was  $\text{pH}=6$ . This value was higher than the IEP of SnO<sub>2</sub> ( $\text{pH} > \text{pH}_{\text{IEP}}$ ) and the surface of SnO<sub>2</sub> was negatively charged. An electrostatic repulsion could be formed between the negatively charged SnO<sub>2</sub> surface and the anionic reactive dye resulting in a considerable decrease in decolorization efficiency (Mera et al., 2017; Pouretedal et al., 2012). To overcome the low decolorization efficiency, a cationic dye could be used as a model dye pollutant.

## CONCLUSIONS

SnO<sub>2</sub> nanoparticles were successfully synthesized by a facile precipitation method. XRD and Raman spectroscopy results revealed evidence of tetragonal rutile structure SnO<sub>2</sub>. Besides, the FTIR spectrum confirmed the presence of functional groups of SnO<sub>2</sub> vibrational bands. The surface morphology of SnO<sub>2</sub> consisted of almost spherical particles with agglomeration. The photocatalytic degradation kinetics of RR-194 followed a pseudo-first-order kinetics model. The rate constant of RR-194 was calculated as  $k=3.82 \times 10^{-4} \text{ min}^{-1}$ .

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## EVALUATION OF DEVELOPMENTAL TOXICITY OF EVERZOL BLUE EDG ON ZEBRAFISH (*DANIO RERIO*) EMBRYOS

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### ABSTRACT

Textile manufacturing is one of the most polluting industrial sectors due to the release of potentially toxic compounds such as synthetic dyes into the environment. The most widely used group of these synthetic dyes is azo dyes. The aim of this study was to evaluate the toxic effects of Everzol Blue EDG (EB EDG) in zebrafish embryos. Embryos were exposed to 19.51-750 mg L<sup>-1</sup> EB EDG for 96 hours and the survival rates, heart rates and embryonic growth rates of these individuals were determined. According to the results of the study, the 96-hour LC<sub>50</sub> value of the textile dye was determined as 604.7 (423.2-1069.3) mg L<sup>-1</sup>. Heart rates of *Danio rerio* embryos exposed to the dye were significantly reduced at concentrations of 220-750 mg L<sup>-1</sup>. In addition, EB EDG at a concentration of 750 mg L<sup>-1</sup> caused significant inhibition of embryonic growth in *D. rerio* embryos.

**Keywords:** Synthetic dye, zebrafish, Everzol Blue EDG, embryotoxicity

### INTRODUCTION

The textile industry is one of the major sources of pollution problems worldwide (Ayadi et al., 2016). This industry greatly contributes to environmental pollution due to the generation of large quantities of dye-laden wastewater. Textile dyes are synthetic chemical compounds that have an aromatic structure and are resistant to biodegradation (Gita et al., 2017).

It is thought that there are about 10,000 different synthetic dyes on the market today. (Ayadi et al., 2016). The most widely used group of these synthetic dyes is azo dyes. Azo dyes are water-soluble even at very low concentrations, and it is stated that their biotransformation products are toxic and in some cases these compounds can be carcinogenic and mutagenic (Bae et al. 2006). Moreover, textile dyes in water bodies hinder the oxygenation potential of water received, so hinders biological activity in aquatic organisms (Mehra et al. 2021).

In the literature, *Danio rerio* has been used to determine the toxicity of some dyes to aquatic organisms. *D. rerio* is one of the widely used model organisms in aquatic toxicology. *D. rerio* are often used in experiments because of their low cost, small size and easy laboratory care (Pruvot et al., 2012).

The aim of this study, different biological responses such as lethality, growth and development, heart rate, *D. rerio* embryos were determined after 96 hours of exposure Everzol Blue EDG (EB EDG) according to the Fish Embryo Acute Toxicity Test (OECD, 2013).

## **MATERIAL AND METHOD**

### **Testing organisms**

Embryos used in the study were produced from adult zebrafish grown in the zebrafish production system (ZebTec Active Blue, Tecniplast, Italy) in the Zebra Fish Unit, Inonu University Faculty of Arts and Sciences, Aquatic Vertebrate Experimental Animals Unit. In the zebrafish system with continuous water circulation, pH 7.30, conductivity 720  $\mu\text{S}/\text{cm}$ , temperature 28.2 °C and photoperiod 14 hours' light and 10 hours' dark. Zebrafish embryos were obtained with the same aquatic characteristics as the parent system and a filtered rearing system (iSpawn, Tecniplast, Italy) connected directly to the water circulation fed by the parent system. Fertilized eggs were collected within 3 hours and stored in an oven at 28.5 °C in standard embryo broth.

### **Chemicals and exposure**

For fish embryo-toxicity testing, zebrafish embryos 6-8 hours after fertilization were exposed to EB EDG at 10 different concentrations (19.51-750  $\text{mg L}^{-1}$ ) for 96 hours in 96-well microplates. 250  $\mu\text{l}$  of EB EDG solution prepared at different concentrations and a zebrafish embryo were added to each well. A total of 24 embryos were used for each concentration. Mortality rates of individuals examined with a stereomicroscope every 24 hours for a 96-hour period were recorded. At the 48th hour, the heart beats per minute of the embryos were determined. At the end of the 96th hour, the malformation rates and malformation types of the surviving individuals were determined with a stereomicroscope, while their heights were measured using Euromex Image Focus 4.0 software.

### **Statistical Analysis**

The statistical analysis of the collected data was conducted with SPSS and GraphPad Prism 5 (SPSS Inc., USA). The results that did not comply with the required hypotheses for parametric tests were processed with the Mann-Whitney U and Kruskal Wallis test.

## **RESULT AND DISCUSSION**

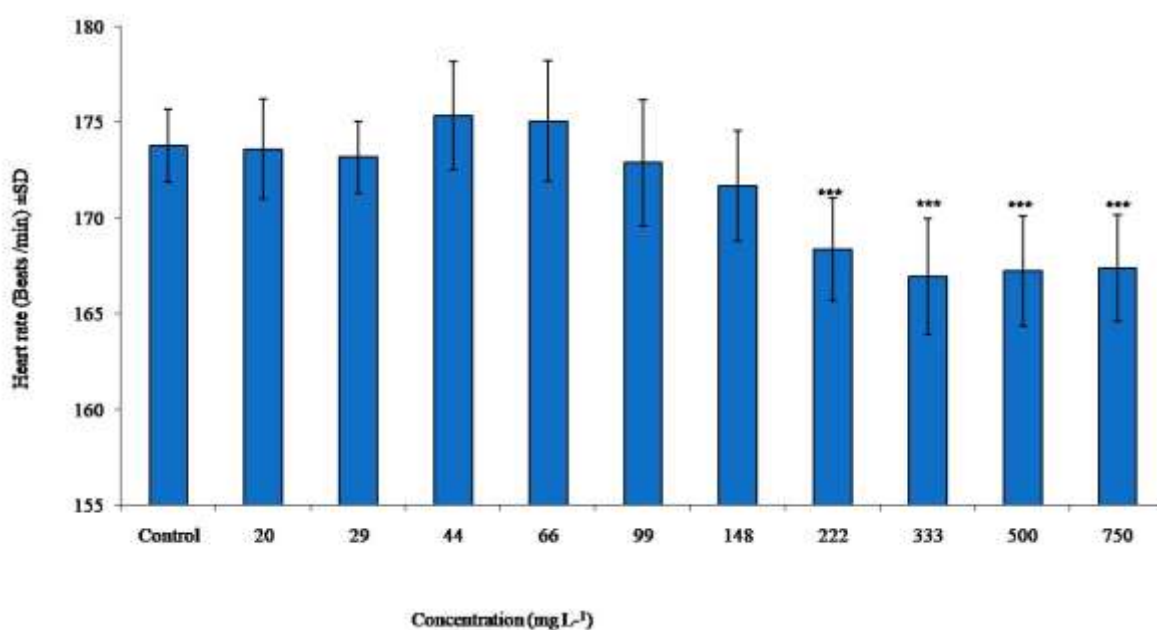
The effects of dyes produced and used in large quantities should be determined by considering the possibility of their introduction and accumulation in the environment over time. In this study, changes in embryo survival rate, and body length were analyzed to evaluate the possible toxicity on embryonic development of zebrafish exposed to EB EDG at concentrations between 19.51-750  $\text{mg L}^{-1}$ .

48, 72 and 96 hour  $\text{LC}_{50}$  values in *D. rerio* embryos exposed to EB EDG were calculated as 785, 660, and 604.7  $\text{mg L}^{-1}$ , respectively. In a previous study, Everzol Red LFB (ERL) and Everzol Yellow CGL (EYC) 96 hour  $\text{LC}_{50}$  values were determined as 292 and 127  $\text{mg L}^{-1}$ , respectively (Turhan, 2022). It was observed that the  $\text{LC}_{50}$  values of these dyes were lower than those of EB EDG. No mortality was observed at concentrations between 19.5-43.9  $\text{mg L}^{-1}$ . At concentrations of 65.8  $\text{mg L}^{-1}$  and above, an increase in the mortality rate was observed due to the increase in concentration (Table 1).

**Table 1.** Time-dependent mortality levels for *D. rerio* embryos exposed to different concentrations of EB EDG

| Consantrasyon<br>(mg L <sup>-1</sup> ) | n  | Σ (Mortalite) |     |     |     |
|--|----|---------------|-----|-----|-----|
|  |    | 24h           | 48h | 72h | 96h |
| Control                                | 24 | 0             | 0   | 0   | 0   |
| 19.5                                   | 24 | 0             | 0   | 0   | 0   |
| 29.2                                   | 24 | 0             | 0   | 0   | 0   |
| 43.9                                   | 24 | 0             | 0   | 0   | 0   |
| 65.8                                   | 24 | 2             | 2   | 2   | 3   |
| 98.8                                   | 24 | 0             | 0   | 0   | 2   |
| 148.2                                  | 24 | 3             | 4   | 6   | 6   |
| 222.2                                  | 24 | 6             | 7   | 7   | 7   |
| 333.3                                  | 24 | 6             | 6   | 7   | 7   |
| 500                                    | 24 | 5             | 8   | 9   | 10  |
| 750                                    | 24 | 11            | 11  | 12  | 13  |

The heart is the first functional organ developed in zebrafish, and the change in heart rate due to the effects of pollutants is an important marker used in embryonic testing (Mu et al., 2013). In this study, it was shown that zebrafish embryos exposed to EB EDG caused a significant dose-dependent (222-750 mg L<sup>-1</sup>) decrease in heart rate at 48 hours (Figure 1). There are many studies in the literature showing that pollutants cause inhibition of heart rate in zebrafish embryos (Pruvot et al., 2012; Mu et al., 2013).

**Figure 1.** Heart rate per minute at 48 hours in *D. rerio* embryos exposed to different concentrations of EB EDG

\*\*\* Differential indicators from control (p < 0.001)

Determining the change in body size is an important parameter in evaluating the potential effects of pollutants on fish (Lu et al., 2022). Because the change in the length of the fish significantly reflects many molecular and cellular responses that may occur in the individual depending on the effect of pollutants (Cook et al. 2005). In our study, concentration EB EDG 750 mg L<sup>-1</sup> resulted in significant inhibition of embryonic growth (Table 2). Similar to the results of this study, Turhan (2022) reported that Everzol Red LFB and Everzol Yellow CGL dyes cause growth inhibition depending on the concentration in zebrafish.

**Table 2.** Length of *D. rerio* larvae exposed to different concentrations of EB EDG for 96 hours

| Concentrations | Everzol Blue EDG |             |   |      |    |
|----------------|------------------|-------------|---|------|----|
|                | <i>n</i>         | Length (mm) |   |      |    |
| Control        | 24               | 3.72        | ± | 0.04 |    |
| 20             | 24               | 3.70        | ± | 0.05 |    |
| 29             | 24               | 3.71        | ± | 0.04 |    |
| 44             | 24               | 3.71        | ± | 0.06 |    |
| 66             | 21               | 3.71        | ± | 0.05 |    |
| 99             | 22               | 3.71        | ± | 0.08 |    |
| 148            | 18               | 3.69        | ± | 0.06 |    |
| 222            | 17               | 3.68        | ± | 0.05 |    |
| 333            | 17               | 3.67        | ± | 0.08 |    |
| 500            | 14               | 3.68        | ± | 0.05 |    |
| 750            | 11               | 3.62        | ± | 0.07 | ** |

24 individuals were exposed for each concentration. Lengths are expressed as mean ± standard errors.

These values are derived from the lengths of surviving individuals.

\*\* Indicates groups that differ significantly from control (p< 0.01)

## CONCLUSIONS

This study showed that DKFS caused dose-dependent developmental toxicity in zebrafish embryos. The effects that DKFS can cause on aquatic organisms have been demonstrated in various aspects in this study. However, this dye should be evaluated at the biochemical and molecular level with new studies.

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## EFFECTS OF HAWTHORN (*Crataegus spp*) ROOTSTOCK ON VEGETATIVE GROWTH IN LOQUAT (*Eriobotrya japonica* Lindl)

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### ABSTRACT

The aim of this study is to investigate the effects of hawthorn rootstock on vegetative growth of 'Hafif Çukurgöbek (HCG)' loquat cultivar. In the study, loquat seedling rootstock was also used as a control. Vegetative growth parameters such as annual shoot length, trunk diameter of stock and scion and also structure canopy of the cultivar/rootstock combinations were investigated. Hawthorn rootstock gave significantly lower values than loquat seedling rootstock in terms of annual shoot length, scion and rootstock diameter. In terms of all other parameters examined, it was determined that hawthorn rootstock showed weaker vegetative growth than loquat seedlings. According to these first data obtained, hawthorn rootstock provides approximately 60% dwarfing in grafted scion compared to loquat seedling rootstock.

**Keywords:** Dwarf rootstock, *Eriobotrya japonica*, Flowering, Hawthorn

### INTRODUCTION

Loquat is a large tree which restricts the number of trees that can be planted per unit area and makes it necessary to harvest fruit with ladders. Most loquats are grown on loquat seedlings and the genetic variability of rootstocks probably contributes to variability of performance in grafted trees (Janick, 2011). The use of dwarfing rootstocks using quince (*Cydonia oblonga*) and hawthorn (*Crataegus spp.* L.) is one method to reduce tree size, facilitate harvest, and increase early yield.

Considering horticultural cultures, it is seen that the hawthorn has the potential to be used as rootstock for some important pome fruit species, but this potential has not been sufficiently evaluated yet. Hawthorns that grow naturally in different regions of Turkey are often transformed into pears and sometimes apples by turning grafts. Hawthorn is a good rootstock for pears to be grown in shallow, dry, sandy and stony soils. Pears grafted on hawthorn rootstock remain stunted and do not grow much (Özbek 1978). Hawthorn also has the potential to be used as rootstock for apples. On the other hand, although it is not very common, it is reported that the rootstock is also used for quince.

There is some information in the literature that hawthorn can be used as rootstocks for loquat (Polat and Kaşka, 1992a; Polat, 1995; Polat, 2018); however, there is not enough research on this subject. Only three studies (Jamil et al., 2012; Polat, 2020, Polat, 2021) on the use of hawthorn rootstocks in loquat cultivation have been found. However, all three literatures are studies to determine the success of the grafting. Therefore, with this planned study, it was aimed to determine the vegetative growth status of loquat grafted on hawthorn rootstock.



It can be said that our findings have guiding features for both loquat researchers in their new research and loquat growers in the selection of rootstocks in their new orchard plant.

## **MATERIAL AND METHOD**

### ***Material***

This study was carried out in the loquat orchard in the research area of Hatay Mustafa Kemal University, Faculty of Agriculture, Department of Horticulture, Hatay, Turkey in 2020. The research area is at 36°12' east and 36°52' north latitudes and its altitude is 80 meters from the sea.

In the study, a loquat orchard established with a 2-year-old HÇG loquat cultivar grafted on loquat and hawthorn seedling rootstocks was used. In the study, the spacing of plants with hawthorn rootstock was 1.0 m x 0.5 m, while the spacing of plants with loquat seedling rootstock was 1x1 m. Trial material plants were trained with the Goble system and irrigated with a drip irrigation system. In addition, annual technical and cultural maintenance operations were carried out regularly.

### ***Method***

Vegetative growth of trial plants was measured at three month intervals starting from February 2020.

*Annual shoot length (cm)*: Four shoots from each plant were measured from 4 sides of the plants.

*Trunk diameter (mm)*: Scion and rootstock trunk diameters (5 cm below and above the bud union) were measured in all plants with a digital caliper sensitive to 0.01 mm.

*Bud union-first branching (cm)*: The distance between the bud union and the first branching on the scion trunk was measured.

*First branching - longest shoot (cm)*: The distance between the first branching and the top of the longest shoot on the scion trunk was measured.

*Bud union-longest shoot (cm)*: The distance between the bud union and the top of the longest shoot on the scion trunk was measured.

### ***Data Analysis and Statistics***

The variance analyzes of the data obtained from the experiment were performed in the SPSS computer package program, according to the "Completely Randomised Design" (Bek and Efe, 1987). The differences between the rootstock means were compared with the LSD test.

## **RESULTS AND DISCUSSION**

The data on the effects of rootstocks on the vegetative growth of the HÇG loquat variety are given in Table 1.

**Table 1.** Average annual vegetative parameters of ‘HÇG’ loquat budded on hawthorn rootstocks.

| Rootstock         | Annual shoot length, (cm) | Scion trunk diameter (mm) | Rootstock trunk diameters (mm) | Bud union-first branching (cm) | First branching-longest shoot (cm) | Bud union - longest shoot (cm) |
|-------------------|---------------------------|---------------------------|--------------------------------|--------------------------------|------------------------------------|--------------------------------|
| Hawthorn          | 11.78 b <sup>(x)</sup>    | 5.45 b                    | 15.36 b                        | 26.50 a                        | 39.95 b                            | 66.45 b                        |
| Loquat seedling   | 65.02 a                   | 41.09 a                   | 43.18 a                        | 11.00 b                        | 148.30 a                           | 159.30 a                       |
| LSD <sub>5%</sub> | *                         | *                         | *                              | *                              | *                                  | *                              |

<sup>(x)</sup>: Means followed by different lowercase letters indicate significant difference by Tukey’s test at  $p < 0.05$  level.

Plants on loquat seedling rootstock (65.02 cm) produced significantly longer shoots than those on hawthorn rootstock. In the study of Polat and Çalışkan (2011), the annual shoot length was found to be 23.1 cm, while in the study of Akkuş and Polat (2022), the lowest was 39.64 cm (Quince-A) and the highest was 74.13 cm (BA-29). It is thought that these differences may be due to the suppression of vegetative growth by annual maintenance procedures and fruit load.

In terms of rootstock and scion diameter, loquat seedling rootstock gave significantly higher values than hawthorn rootstock. Lower values were obtained from hawthorn rootstock than loquat seedling rootstock in terms of length values between first branching-shoot top and grafting point-major axis top. The differences between rootstocks in terms of all parameters examined were found to be statistically significant. This shows that hawthorn rootstock suppresses vegetative growth of grafted scion and has a dwarfing effect similar to quince rootstocks. According to these first data, the dwarfing effect of hawthorn rootstock (about 60%) is much more pronounced than quince rootstocks. As a matter of fact, in studies performed on HÇG loquat cultivars grafted on three quince clone rootstocks (Akkuş and Polat, 2022; Sezer and Polat, 2022), it is seen that the values measured in terms of all vegetative parameters examined are higher than the values determined in the hawthorn rootstock in our study. In addition, in some studies (Polat and Kaşka 1992 a,b; Polat, 1995) on loquat varieties grafted on MA, MC and loquat seedling rootstocks, it was reported that quince rootstocks provide 20-25% dwarfing compared to loquat seedling rootstocks. This shows that hawthorn rootstock provides a much more dwarfing effect. On the other hand, in some studies conducted in Iran, it was determined that hawthorn rootstock provided significant dwarfing in different quince and pear varieties grafted on it (Abdollahi et al. 2012; Ghasemi et al. 2013; Rahmati et al. 2016; Abdollahi et al. 2018; Tataria et al., 2020). The dwarfing effect of hawthorn rootstock is of great importance in terms of dense planting in loquats and shows that it can be used as a dwarf rootstock in loquat culture.

## CONCLUSION

Although some researchers have stated that hawthorn can be used as rootstock for loquats, there are no data on the use of hawthorn rootstock both in Turkey and in other countries. In this study, the effects of hawthorn rootstock on the vegetative growth of the grafted HÇG variety were studied for the first time. For this purpose, hawthorn rootstock was compared with loquat seedling rootstock. The findings of our study are very important and valuable in terms of being the first findings regarding the use of hawthorn rootstock as rootstock in loquat. It was found to be remarkable that the hawthorn rootstock provided a significant dwarfing by suppressing the vegetative growth of the scion. It is expected that this significant dwarfing effect seen in

two-year-old trees will reach much higher values at later ages. However, in order to obtain more precise results in such studies, it is necessary to continue the studies for a while and determine the effects of rootstock on fruit yield and quality characteristics.

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## **EXTENDING SHELF LIFE OF MINI BREADS BY DEVELOPMENT AND APPLICATION OF EDIBLE CELLULOSE-BASED FILMS**

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### **ABSTRACT**

The application of essential oils to edible films has the potential to increase the microbiological safety of foods. Edible coatings are a recent application in bakery products to extend shelf life and incorporate functional properties such as antimicrobial and antioxidant compounds, where the coating is an important step. In this study, essential oils found in laurel, clove, and oregano were applied to an edible film surface and used to treat preservatives and salt-free breads. Salt and preservative-free breads were prepared and baked according to the specified formulations. Ethanolic extractions of laurel, clove, and oregano were carried out using the ultrasonic-assisted extraction method. The edible film mixture was prepared by adding microcrystalline cellulose, NaOH, urea and distilled water. The synthesized films were stored in a polyethylene bag for further analysis. Just before the breads coating application, laurel, clove, thyme oils and a commercial preservative (10 mg/g bread) were sprayed onto the surface of the sterilized films. Bread samples were coated one by one with the films with different contents. The coated breads taken into sterile petri dishes were kept at room temperature for 15 days. Uncoating breads were also prepared as a control group. Yeast and mold count analyses were performed at 5-day intervals. Laurel oils slowed mold growth on the bread for 15 days longer than a commercial preservative and oregano oils, which lost their effectiveness after 5 days. Antimicrobial films containing laurel oils have the potential to improve the safety and quality of breads, thereby meeting the expectations of both food manufacturers and consumers who purchase them.

**Keywords:** Edible Cellulose-Based Films, Coating, Anti-microbial effect, Laurel oil, Bread.

### **INTRODUCTION**

Bread, which has been the most produced and consumed food product by human beings throughout history, is one of the primary food sources. An average of 50 percent of daily energy is met from bread and grain products. Bread is obtained by mixing wheat flour, yeast, salt and water, kneading and fermenting the dough for a certain period of time and cooking it. It is an inexpensive, readily available, nutritious and satisfying food. Bread production includes kneading, fermentation and baking the dough, respectively. The kneading process is where all components are mixed and a visco-elastic structure is provided for fermentation. This process is continued until the surface of the dough is smooth. During the fermentation stage, microbial and biochemical activities occur in the dough and flavor elements such as taste and aroma develop in bread. During the baking phase, maillard and caramelization events occur, the bread is browned and the remaining flavor and flavor elements are formed (Ertugay, et al., 1994).

Salt, which is one of the components of bread, is used as a preservative or flavor. The amount of salt consumed daily is significant for health. As a result of studies, it has been found that clinical complaints increase when excessive salt is consumed in chronic kidney patients, congestive heart patients and hypertension patients. These patients should follow a salt-free diet. Considering this situation, health needs to consume bread products, which are our most basic food source, with reduced salt content or without salt. However, when salt, which is one of the components of bread, is removed, a decrease in the shelf life of the bread and an increase in mold formation are observed. The shelf life of bread is only 3-7 days at room temperature. Proper packaging, storage, and refrigerator or freezer can help prevent mold and increase shelf life. Bread keeps fresh for only a short time before it starts to spoil. Therefore, many applications to keep the bread fresh as long as possible without spoiling (Gómez-Estaca et al., 2010). Edible films and coatings are one of these applications developed (Dursun, Erhan, 2009; Güral et al., 2012).

Edible films and coatings are substances obtained from non-synthetic natural sources, formed on the surface of the food or between food components, in a thin layer, edible with food, to prevent quality losses in food and to extend shelf life. In producing these films, proteins, carbohydrates, polysaccharides and lipid-based compounds can be used individually or in combination. Since edible films and coatings have a certain water permeability or gas (oxygen, carbon dioxide) permeability, they prevent the water loss of the food they are used in, as well as reduce the oxygen permeability and protect the food against microbiological and chemical spoilage (Oğuzhan, Yangılar, 2016).

The application of essential oils to edible films has the potential to increase the microbiological safety of foods. In this study, it was aimed to reduce the shelf life of unsalted breads and prevent mold formation by applying essential oils from laurel, clove and thyme to an edible film surface. Laurel, a plant of the Lauraceae family (Erkmen and Özcan, 2008), is an evergreen bush native to the Mediterranean region and its essential oil is used as a flavoring additive in the culinary and food industry (Tural, Turhan, 2017). The laurel plant has antimicrobial and antioxidant effects (El et al., 2014; Ramos et al., 2012). Clove (*Syzygium aromaticum*), which is frequently used with its antioxidant and antimicrobial properties, is a valuable plant belonging to the Myrtaceae family (Torlak, Nizamlioğlu, 2009). Clove contains intense amounts of phenolic acids and flavonoids (Cortés-Rojas et al., 2014). Thyme (*Thymus vulgaris*) plant, which is in the Lamiaceae family, grows on grassy field shores, forest edges, rocky and mountainous areas (Ertürk et al., 2010). Thyme, which has a unique smell, contains active substances called thymol and carvacrol. These substances are phenolic compounds that give thyme its distinctive aroma and give it antimicrobial, antifungal and antioxidant properties (Ložienė et al., 2007). The application of essential oils to edible films has the potential to increase the microbiological safety of foods. Edible coatings are a recent application in bakery products to extend shelf life and incorporate functional properties such as antimicrobial and antioxidant compounds, where the coating is an important step.

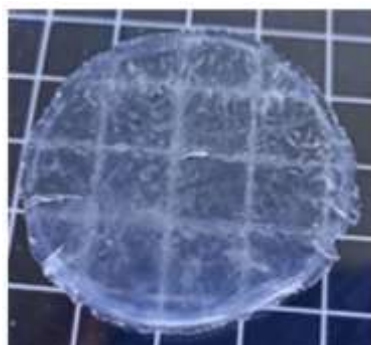
The purpose of this study is to increase the shelf life of unsalted breads and prevent mold formation by applying laurel, clove and thyme essential oils to renewable cellulose films.

## **MATERIAL AND METHOD**

### **Preparation of edible films**

The edible film mixture was prepared by adding microcrystalline cellulose (500 g/L), NaOH (570 g/L), urea (380 g/L) and distilled water to a final volume of 1L (Yang et al., 2011). The mixture was stirred at 0-5°C for 15 min on a magnetic stirrer and kept at -8°C for 6 h. At

the end of the incubation period, a clear homogeneous solution was obtained. This mixture was poured into sterile plates to form thin layers. Coagulation was achieved by adding 1 M CH<sub>3</sub>COOH dropwise to the mixture. The synthesized films were washed with distilled water after 15 min and left to dry at room temperature.



**Figure 1.** The image of edible cellulose based film.

Each film was carefully peeled from the plate and stored in a polyethylene bag for further analysis. To sterilize, films were kept under UV light 2 min before applications. Laurel, clove and oregano oil were sprayed onto the surface of the sterilized films before bread coating by the experimental design (Table 1).

**Table 1.** Composition of cellulose-based edible films with different antimicrobial agents.

| <b>Treatment</b> | <b>Composition</b>                                   |
|------------------|--|
| CBE film (CBEF)  | Microcrystalline cellulose <sup>1</sup> , NaOH, urea |
| F1               | CBEF + L <sup>a</sup>                                |
| F2               | CBEF + C <sup>b</sup>                                |
| F3               | CBEF + O <sup>c</sup>                                |
| F4               | CBEF + PS <sup>d</sup> (10 mg/g bread)               |

<sup>a</sup> L: Laurel

<sup>b</sup> C: Cover

<sup>c</sup> O: Oregano

<sup>d</sup> O: Potassium sorbate

<sup>1</sup> Concentration were measured as dry-bases of cellulose

#### **Extraction of Laurel, Clove and Oregano Oil**

Laurel, clove and oregano were ground in the grinder. Ethanolic extractions of laurel, clove, and oregano were carried out using the ultrasonic-assisted extraction method.

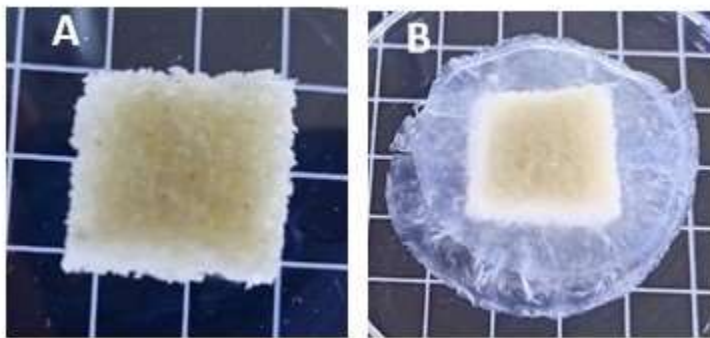
#### **Bread coating**

Salt and preservative-free breads were prepared and baked according to the specified formulations (Figure 1).



**Figure 2.** Image of prepared bread.

Thin sections (1-3 mm) of  $1 \times 1$  cm in size and approximately 1 g in weight were taken from three different points of each piece of bread. Each sample was numbered and placed in sterile petri dishes (Figure 3A). Breads were covered with films on the inner surface of which various antimicrobial agents were sprayed (Figure B). Also, bread not covered with the film was used as a control.



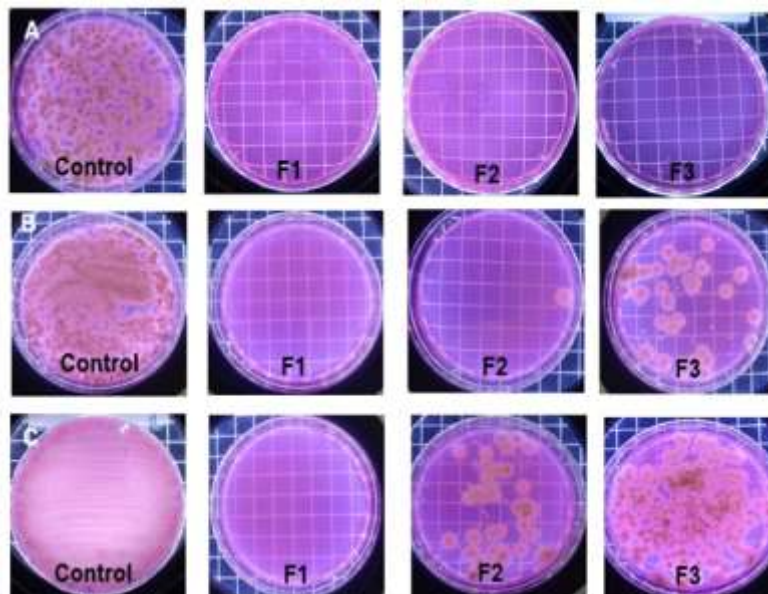
**Figure 3.** The images of bread samples (A) and the coating of bread samples with cellulose-based antimicrobial edible film (B).

#### **Homogenization of bread and live cell counting**

Bread samples were taken into sterile stomacher bags for homogenization processes. In sterile stomacher bags, the sample was homogenized with PBS buffer (phosphate buffer, pH; 7.4). The homogenate liquid was diluted and inoculated onto DRBC agar plates with the spread plate method.

### **RESULTS AND DISCUSSION**

As a result of this study, intense mold growth was observed in the control group, which increased depending on the days from the 5th day onwards. In the positive control group, mold formations were detected from the 10th day. In the negative control group, mold formation was observed, similar to the growth seen in the bread samples that were not covered with film from the 5th day onwards, but at a lower density. Differ from the literature among the essential oils used in the study, the highest antifungal effect was obtained with laurel oil (Gümüş, at all., 2010). The antifungal activity of cellulose-based composite films notably increased with containing laurel oil films, with growth only observed after 15 days (Figure 4).



**Figure 4.** Antifungal effects of edible cellulose-based films containing Laurel (F1), clove (F2) and oregano (F3) in the 5<sup>th</sup> (A), 10<sup>th</sup> (B) and 15<sup>th</sup> (C) days of the storage process.

While no mold formation was observed in the first 10 days in the clove oil applied samples, growth was detected after the 10th day. The least antifungal effect was found in thyme oil, and visible fungal growth appeared on the surface of the containing thyme oil sample after 5 days of storage at room temperature. Similar studies with the thyme and clove oil applied films show parallelism and reported antifungal effects. (Suhr and Nielsen, 2003; Hassiotis, 2013).

**Table 2.** The number of fungi naturally present in (log CFU/g) in bread coated with CBE films containing antimicrobial agents during storage at 25°C.

| Treatment | Storage Day (15 <sup>th</sup> ) |
|-----------|---------------------------------|
| Control   | 10.74± 0.66                     |
| F1        | -----                           |
| F2        | 3.95±0.80                       |
| F3        | 5.67±0.13                       |
| F4        | 6.87±0.39                       |

CBEF: Cellulose-based edible film

F1: CBEF + L

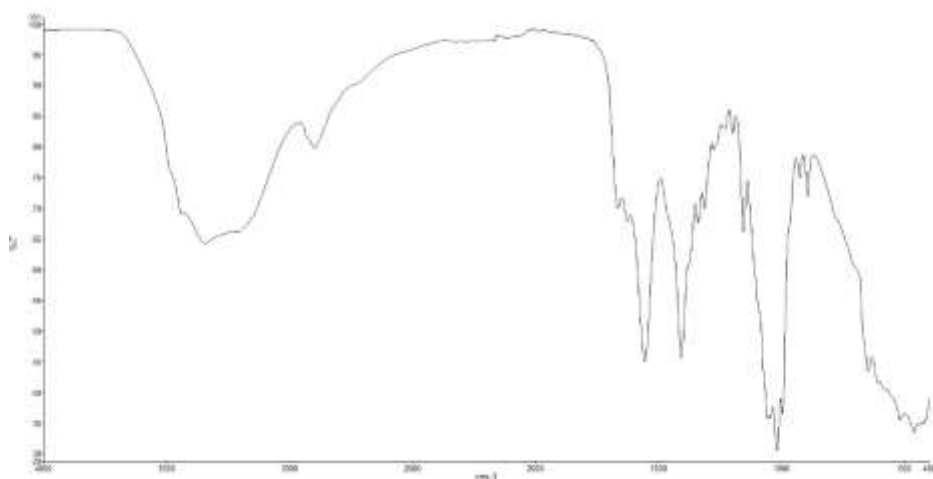
F2: CBEF + C

F3: CBEF + O

F4: CBEF + PS (10 mg/g cheese)

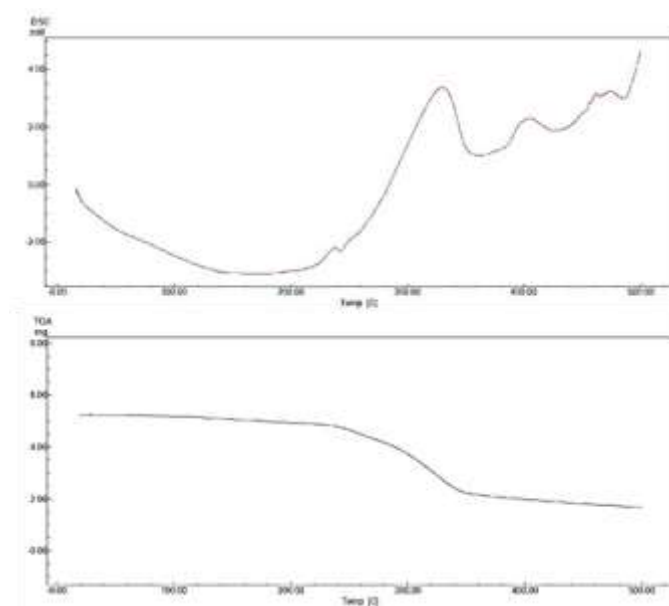
On the 15<sup>th</sup> day of storage, while the amount of live mold in the control group was 10.74 CFU/g, no mold formation was found in the breads covered with films containing laurel essential oil (F1) (Table 2). The amount of mold in the breads covered with clove-containing films (F2) was also reduced by 63% compared to the control group.





**Figure 5.** Spectrum measurement (FTIR) of cellulose-based edible film.

According to the FTIR (Figure 5), TGA and DSC (Figure 6) analysis results, it is seen that the edible cellulose film has been produced successfully.



**Figure 6.** Temperature analysis of cellulose-based edible film; Differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA).

The result from the antifungal activity evaluation indicated that the antimicrobial edible cellulose-based films improved the antifungal properties of the film and significantly retarded the proliferation of fungal bread samples.

## CONCLUSIONS

The result of the antifungal activity evaluation showed that essential oil-treated antimicrobial edible cellulose-based films improved their anti-fungal properties and significantly delayed fungal growth in bread samples. Therefore, edible cellulose-based films with the incorporation of essential oils, having good antifungal properties is promising to be used as an edible cellulose-based packaging material, to enhance food safety and extend the shelf-life of packaged food in the area of food technology. Thereby meeting the expectations of both food manufacturers and consumers who purchase them.

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## PRELIMINARY RESULTS OF INFLUENCES OF SOME QUINCE ROOTSTOCKS ON YIELD AND FRUIT QUALITY IN LOQUAT

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### ABSTRACT

The aim of this study was to investigate the effect of different quince clone rootstocks on yield, and fruit quality of loquat cv. 'Hafif Çukurgöbek(HÇG)'. In the trial, HÇG loquat cultivar grafted on Quince-A(MA), Quince-C(MC) and BA-29 rootstocks was used. In cultivar/rootstock combinations, yield and fruit pomological properties were determined. In order to determine the fruit quality, a total of 50 fruits were taken from each cultivar/rootstock combination and physical and chemical measurements and analyzes were carried out including fruit weight (g), fruit dimensions (mm), seed weight (g), flesh/seed ratio (%), total soluble solids (TSS) (%), titratable total acidity (TA) (%), pH. It was determined that the highest values in terms of fruit weight and fruit sizes were obtained from the plants of MC rootstock and followed by the plants of BA-29 rootstock; and the lowest values were obtained from the plants of MA rootstock. MC rootstock yielded higher values in terms of seed number, seed weight and flesh/seed ratio than the other two rootstocks. Differences between the rootstocks in terms of these characteristics were found to be statistically significant. BA-29 and MA rootstocks had similar values in terms of total soluble solids, while Quince-C rootstock had the lowest value. In terms of yield per plant, MC gave the highest value, followed by BA-29 rootstock. The lowest yield value was obtained from MA. These differences between rootstocks were found to be statistically significant at 1% level. The data obtained from this study show that it is possible to use quince rootstocks as dwarf rootstocks in loquat cultivation.

**Keywords:** Loquat, rootstock, dwarfing, fruit quality, yield

### INTRODUCTION

Since the ripening time of the loquat fruit coincides with a period when the harvest of many other fruit species has not yet begun, the demand for this fruit is high and it can find buyers at high prices. For this reason, loquat is an important fruit species for both producers and consumers (Polat and Kaşka, 1991; Özçağiran et al., 2011).

Loquat (*Eriobotrya japonica* Lindl.) is propagated mainly by budding method, and when it is budded mostly loquat seedling is used as rootstock. Loquat trees on the seedling rootstocks are very tall and large crowned. There are several difficulties of establishing an orchard with such large trees. First of all, the number of trees that can be planted per unit area is limited, and also, operations such as pruning, spraying and fruit picking are difficult (Polat, 1995; Crane and Caldeira, 2006).

Researchers conducted various experiments to solve these difficulties in loquat cultivation. The most important of these is the applications that reduce the tree's vegetative growth. The best solution for this is to use dwarf rootstocks (Polat and Kaşka, 1992 a, b). As a

matter of fact, quince rootstocks are used as a dwarf rootstock in loquat cultivation (Lin, 2007; Hueso et al., 2007), although it is very limited.

There is some information in the literature that Quince can be used as rootstocks for loquat (Polat, 1995); however, there is not enough research on this subject. Although quince rootstocks have dwarfing effect on loquat, the effects of quince rootstocks on the fruit yield and quality have not been evaluated on Turkish cultivars. The present study evaluated yield and quality of 'HÇG' loquat budded on MA, MC and BA-29 rootstocks. Here, the first results were presented.

## **MATERIAL AND METHOD**

### ***Plant materials and experimental design***

The experiment was conducted in Hatay, Turkey, located at 36°52' N latitude, 36°12'E longitude, with an elevation of 80 m. Hatay is located on the Mediterranean coast of Turkey and is completely under the influence of a Mediterranean climate. Long-term average temperature for Hatay is 18.3°C and total annual precipitation is 1168 mm. Two-year old 'HÇG' loquat saplings budded on BA-29, MA and MC quince rootstocks were planted at spacings of 1.0 x 0.5 m (2000 trees/da) in January 2017, drip irrigated, with standard cultural practices. The experiment was arranged according to a completely randomized design with 5 replications and 6 plants were used in each replicate.

### ***Fruit quality assessments***

To determine fruit quality, 50 fruits (10 fruits from each replicate) were randomly sampled from each cultivar/rootstock combination and physical and chemical measurements and analyzes were carried out including fruit weight (g), fruit dimensions (mm), seed weight (g), number of seeds (pieces), flesh seed<sup>-1</sup> ratio (%), total soluble solids (TSS) (%), titratable total acidity (TA) (%), pH.

### ***Yield***

To assess yield efficiency, the total yield per tree (kg fruit per tree), the total yield per plot (tone fruit per plot), and the total yield per trunk cross-sectional area (TCSA) (g fruit per TCSA) were calculated.

To calculate fruit yield per trunk cross-sectional area (g fruit per mm<sup>2</sup> TCSA), the total fruit yield per tree was divided by TCSA measured at 5 cm above the graft union at harvesting time. Considering the planting distances in the experiment, the yield for area-basis was calculated by multiplying yield per tree by the number of plants.

### ***Statistical analysis***

The experiment was arranged based on the completely randomized design with six single-trees per each replicated and five replications. After checking for normality, data were analyzed for statistical significance, using SAS software (version 9.4; SAS Inc., Cary, NC). Tukey's HSD multiple comparison test (p<.01) (Steel and Torrie, 1980) was used for mean separation.

## **RESULTS AND DISCUSSION**

### ***Fruit Quality***

Year effect was found to be statistically significant in terms of all parameters examined

except the pH, and generally, higher values were obtained in 2019 compared to those in 2018.

There were statistically significant differences among rootstocks ( $p > 0.01$ ) in terms of average fruit weight and fruit size of 'HÇG' loquat, and the highest values in terms of both parameters were obtained from plants with rootstock Quince-C, followed by BA-29, and the lowest one was obtained from MA (Table 1).

Quince-C rootstock also gave higher values in terms of seed number and seed weight ratio than the other two rootstocks. Differences between the rootstocks were statistically significant. The highest value in terms of flesh seed<sup>-1</sup> ratio was determined in Quince-C rootstock however the difference between the rootstocks was not significant. Soluble Solid Content (SSC), Titratable Acidity (TA) and pH were significantly affected by rootstocks ( $p < 0.01$ ). Fruit SSC and pH were the highest on MA rootstock, whereas fruit TA concentration was the highest on BA 29 rootstock (Table 1).

Year x rootstock interaction effects were found to be statistically significant in terms of all other parameters examined except the seed number per fruit.

Fruit size is an important criterion that can vary depending on tree age and fruit set (Durgaç et al., 2006). In the previous studies, the fruit weight, fruit width and fruit length of 'HÇG' on loquat seedling were between 20.45 g and 39.70 g, between 36.79 mm and 40.10 mm, and between 37.3 mm and 42.40 mm, respectively (Paydaş et al., 1992; Polat et al., 2004, 2005 and 2010; Polat and Caliskan, 2011). Fruit weights and fruit widths determined in our study are lower than the values found in previous studies. This difference may have resulted from the fact that the plants in this experiment were younger than the plants in other studies. In terms of fruit length, it was partially similar.

In this study, the number of seeds and seed weight were determined to be between 3.36-3.82, and 4.46-5.71 g, respectively. In the previous studies, seed weight and number of seeds were found to be between 4.0 and 7.73 g, and between 2.4 and 4.16, respectively (Polat et al., 2004, 2005; Durgac et al., 2006; Polat et al., 2010; Polat and Caliskan, 2011). Our seed weight values (between 4.46-5.71 g) were found to be lower than those of researchers except for Durgac et al. (2006) and Polat and Çalışkan (2011). The data of the combinations examined show similarities with the data determined in other studies.

While the flesh/seed ratio was determined to be between 3.08-2.89 in the study, this value was measured as between 3.43 and 5.42 in previous studies (Paydaş et al., 1992; Polat et al., 2004, 2005 and 2010; Durgac et al., 2006). In previous studies, researchers measured TTS as between 8.05-12.80%. The values determined in our study are generally similar to those of previous studies. In this study, titratable acid was determined to be between 0.62-0.75 and pH as 3.42-3.78, while in previous studies, acidity was 0.63-9.92 and pH was measured to be between 3.29 and 4.5 (Polat et al., 2004, 2005; Durgac et al., 2006; Polat et al., 2010; Polat and Caliskan, 2011). Our findings are lower than the values of Polat et al. (2010) and higher than the values of Polat et al. (2005). TTS, acidity and pH are affected by such factors as time of picking, climatic conditions, cultivars, etc.

According to the two-year averages, the highest yield value in terms of yield per plant was obtained from the MC and BA-29 rootstocks (787.98 g plant<sup>-1</sup> and 723.02 g plant<sup>-1</sup>, respectively) while Quince-A rootstock gave the lowest value. This difference between the rootstocks was found to be statistically significant at 1% level (Table 2).

Table 1. The effects of quince rootstocks on fruit quality parameters of the HÇG loquat cultivar (2018-2019 years)

| Rootstock          | Years                        | Fruit weight (g) | Fruit width (mm) | Fruit length (mm) | Seed weight (g) | Seed number per fruit | Flesh/seed ratio | Soluble solids (%) | Acidity (%) | pH                |
|--------------------|------------------------------|------------------|------------------|-------------------|-----------------|-----------------------|------------------|--------------------|-------------|-------------------|
| MA                 | 2018                         | 13,26            | 14,44            | 15,00             | 3,86            | 3,54                  | ,44              | 12,47              | 0,27        | 4,03              |
|                    | 2019                         | 22,60            | 33,96            | 39,10             | 5,07            | 3,17                  | ,59              | 10,84              | 0,97        | 3,54              |
| MC                 | 2018                         | 23,71            | 34,58            | 36,98             | 5,59            | 4,04                  | ,16              | 11,52              | 0,84        | 3,30              |
|                    | 2019                         | 22,93            | 33,84            | 37,48             | 5,82            | 3,60                  | ,01              | 9,12               | 0,63        | 3,76              |
| BA-29              | 2018                         | 19,78            | 32,00            | 35,56             | 5,23            | 3,86                  | ,84              | 11,76              | 0,80        | 3,32              |
|                    | 2019                         | 20,66            | 33,41            | 36,36             | 5,30            | 3,30                  | ,93              | 12,96              | 0,70        | 3,52              |
| Average. Rootstock | MA                           | 17,93 c<br>(x)   | 24,20 c          | 27,05 c           | 4,46 c          | 3,36 c                | ,02 a            | 11,65 b            | 0,62 b      | 3,78 a            |
|                    | MC                           | 23,32 a          | 34,21 a          | 37,23 a           | 5,71 a          | 3,82 a                | ,08 a            | 10,32 c            | 0,73 a      | 3,53 b            |
|                    | BA-29                        | 20,22 b          | 32,71 b          | 35,96 b           | 5,27 b          | 3,58 b                | ,89 a            | 12,36 a            | 0,75 a      | 3,42 c            |
| Average Year       | 2018                         | 18,91 b          | 27,00 b          | 29,18 b           | 4,89 b          | 3,81 a                | ,81 b            | 11,92 a            | 0,64 b      | 3,55 a            |
|                    | 2019                         | 22,06 a          | 33,74 a          | 37,65 a           | 5,40 a          | 3,36 b                | ,18 a            | 10,97 b            | 0,77 a      | 3,61 a            |
|                    | LSD(year <sup>a</sup> )      | **               | **               | **                | **              | **                    | **               | **                 | **          | NS <sup>(y)</sup> |
|                    | LSD(rootstock <sup>b</sup> ) | **               | **               | **                | **              | **                    | NS               | **                 | **          | **                |
|                    | LSD(a x b)                   | **               | **               | **                | **              | NS                    | **               | **                 | **          | **                |

<sup>(x)</sup>: Mean separation by LSD at p < 0.01 (\*\*) level.

<sup>(y)</sup>NS: Not significant

### Yield

The yields of loquat cv HÇG on different quince rootstocks are given in Table 2.

**Table 2.** The effects of quince rootstocks on fruit yield of HÇG loquat (2018-2019 years)

| Rootstock               | Years                        | Yield (g plant <sup>-1</sup> ) |
|-------------------------|------------------------------|--------------------------------|
| MA                      | 2018                         | 75.68                          |
|                         | 2019                         | 681.52                         |
| MC                      | 2018                         | 445.06                         |
|                         | 2019                         | 1130.90                        |
| BA-29                   | 2018                         | 274.09                         |
|                         | 2019                         | 1171.94                        |
| Average.<br>(Rootstock) | MA                           | 378.60 b                       |
|                         | MC                           | 787.98 a                       |
|                         | BA-29                        | 723.02 a                       |
| Average<br>(Year)       | 2018                         | 264.94 b                       |
|                         | 2019                         | 994.79 a                       |
|                         | LSD(year <sup>a</sup> )      | **                             |
|                         | LSD(rootstock <sup>b</sup> ) | **                             |
|                         | LSD(a x b)                   | **                             |

(x): Means followed by different lowercase letters within a column indicate significant difference by LSD test at  $p < 0.01$ (\*\*) level.

### CONCLUSION

In this study, the effects of three quince rootstocks (MA, MC, and BA-29) on fruit yield and quality of 'HÇG' loquat were compared in 2018 and 2019. The preliminary data indicate that dwarfing quince rootstocks can be used in intensive plantings of loquat. In this study, BA-29 and MC rootstocks performed better than MA. Considering yield and fruit quality based on total soluble solid, BA-29 rootstock seems the best choice.

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## **THE EFFECT OF EXTERNALLY APPLIED SALICYLIC ACID ON SOME PHYSIOLOGICAL PROPERTIES OF LETTUCE (*Lactuca sativa* L.) UNDER DROUGHT STRESS CONDITIONS**

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### **ABSTRACT**

The most limiting factor for plant production is the lack of water in arid and semi-arid climates. Therefore, agricultural irrigation is inevitable for profitable crop production. However, in conditions where water is really limited, developing drought resistance mechanisms in plants; are among the measures that can be taken against drought. It is possible to provide resistance to drought in plants with the use of various chemicals. Salicylic Acid, a plant growth regulator that increases drought tolerance, is used in many plants for this purpose. Within the scope of this research, the physiological responses of the plant were investigated by applying different doses of salicylic acid to the lettuce irrigated with different amounts of irrigation water. For this purpose, three different amounts of irrigation water and four different concentrations of salicylic acid were applied to the lettuce plant. In this research, three different amounts of irrigation water and four different concentrations of salicylic acid were applied to the lettuce plant. Despite the application of irrigation water in deficient conditions, the heaviest above-ground green parts were obtained from the subject to which salicylic acid was applied at a dose of 0.5 mM. Under the same under irrigation conditions and 0.5 mM Salicylic acid application, root wet weight was also higher when compared to full irrigation conditions. Considering the dry weights of the green parts and roots, the salicylic acid applied increased the weights despite the lack of irrigation conditions. Regardless of the amount of irrigation water applied, increasing salicylic acid doses also increased root lengths. When the root collar diameter values were examined, it was observed that the amount of irrigation water was applied completely and the increasing levels of salicylic doses gave the highest values. According to these results, increasing salicylic acid application doses had positive results in a sensitive plant such as lettuce in terms of drought tolerance.

**Keywords:** Lettuce, Drought Stress, Irrigation, Adaptation

### **INTRODUCTION**

Due to the rapidly increasing population and industrialization; global warming, excessive use of pesticides and fertilizers, incompatibility with the environment (Akin and Akin, 2007), excessive irrigation without knowing the amount causes pollution, and insufficiency of clean water resources. It is known that the most restrictive factor for vegetative growth in arid and semi-arid climates is the lack of useful water held in the root zone (Falkenmark and Rockström, 1993; Lal, 1991). Today, lack of water and drought are the main problems that need to be taken care of, especially in food production. Considering the increasing water demand of agriculture, domestic and industrial sectors due to the advancement of time and technology, it is predicted that the excessive amount of water used for agricultural irrigation will also put pressure on it

and the frequency of drought will increase in the future. As a result, some cultural practices such as the breeding of drought-resistant plant varieties and the use of some chemicals that provide resistance, limited irrigation programs, the reuse of marginal water resources of different quality in irrigation, the use of water-saving methods and systems should be developed as a precaution. Plants respond to stress conditions that affect them by re-regulating their cellular metabolism and activating their defense mechanisms. One of these mechanisms is the accumulation of high amounts of salicylic acid (SA). According to the studies carried out on the subject, it has been determined that either an increase in the amount of SA synthesis is observed in plants that can tolerate stress factors or an increase in the concentration of SA in the plant with external applications to the plant, and it has been determined that the tolerance mechanisms against stress are activated ( Klessing and Malamy 1994, Hayat et al. 2010). ).

Senerata et al. (2000) investigated the effects of salicylic acid (SA) and acetylsalicylic acid (ASA) on heat, frost and drought stress in tomatoes and beans. In the study, it was observed that the tolerance of the plants obtained from the seeds treated with SA or ASA to heat, frost and drought stress increased. In addition, the resistance of plants sprayed with foliar SA or ASA to heat, frost and drought stress was examined, and it was determined that foliar application did not have the same effects as seed application. However, it has been reported that SA and ASA can survive longer in seedlings exposed to cold and drought stress. Kang and Saltveit (2002) studied the effects of SA on cold stress in maize, cucumber, and, rice seedlings.

Lettuce plants are also extremely sensitive to drought due to their high water content. Therefore, try; In order to minimize the product losses that may occur due to the sensitivity of lettuce to water, by applying appropriate doses of salicylic acid, which is known as an easy and economical plant growth regulator, increasing the resistance of plants against biotic and abiotic stresses, and different doses of plant water consumption ( ETc ) in the form of limited irrigation; The study was carried out to determine the plant's tolerance to drought and other yield and yield parameters.

## **MATERIAL AND METHOD**

This study was carried out for one year in the trial area of the Faculty of Agriculture of Dicle University. The soils of the trial area are in the alluvial large soil class and generally have a clayey texture. Diyarbakir, where the experiment was conducted, has a hot and dry climate in summer and a warm and rainy climate in winter. In the study, Yedikule type (cos) plain lettuce variety was used. It is a delicious lettuce variety with its high-quality head and leaf structure and is suitable for cratering and thus storage and transportation. The maturity period is 75-90 days depending on climatic conditions. It is resistant to various races of lettuce mildew (Anonymous, 2019). The irrigation water used in the research was obtained from the mains water in the Faculty of Agriculture of Dicle University. Since the lettuce row spacing was 30 cm (narrow row spacing), irrigation was carried out using a lateral drip irrigation method between the two plant rows. The experiment was carried out according to the randomized blocks experimental design in the split-plot design with 3 replications. Its main subjects are 3 (three) different amounts of irrigation water (100%, 75%, and 50% of the amount of accumulated water evaporated from A class evaporation pot) and its sub-topics are 4 different Salicylic Acid (SA) doses (0, 0.25, 0.5 and 0.75 mM ). Salicylic acid was applied by spraying on the leaves. The total number of parcels in the experiment was 36 ( thirty-six ).

Experimental plots were planned in 4 rows, with 30 cm x 20 cm row spacing and spacing between rows, each parcel 1.8 m x 0.9 m (1.62 m<sup>2</sup>). In order to prevent side effects, a gap of 1 m was left between the parcels and 2 m between the blocks. The observations and measurements

given below were made during the physiological maturity period when plant development completely ceased.

**Root length (cm):** Root length was determined by measuring from the root collar after carefully removing and washing the roots so as not to damage the roots during harvest.

**Root collar diameter (mm):** The root collar diameter of the harvested plants was measured with a ruler.

**Root fresh weight (g plant<sup>-1</sup>):** After the harvest of the plants, the roots were cut from the part where they were attached to the stem and the root length was measured with the help of a ruler. Above-ground parts and roots were weighed separately.

**Root dry weight (g plant<sup>-1</sup>):** It is aimed to dry the green parts and roots by keeping them at 70 °C for 72 hours (until the weight is stabilized). Then, the green parts and roots were weighed separately from each other and their dry weights were determined. Wet and dry weight measurements were made with a scale with an accuracy of 0.01 g.

**SA Acid Applications:** When the lettuce seedlings became 3-4 leaves, salicylic acid doses were applied to the seedlings by foliar spraying, while spraying was not applied to the control groups. The application was made 2 times with an interval of 2 weeks. SA doses were prepared in vitro. The prepared solutions were placed in cleaned bottles and applied to the plants.

The data from the experiment were evaluated using the MSTAT-c statistical package program. The data obtained in the study, which was carried out in three replications according to the randomized blocks experimental design of the divided plots experimental design (Yıldız and Bircan, 2003), were subjected to analysis of variance.

## RESULTS AND DISCUSSION

### Effects of Irrigation Water Levels and SA Doses Applied in Different Amounts on Wet Weight of Lettuce Above Ground Parts

For each plot, the plants were cut from the root stems and weighed instantly on precision scales without loss of time (without moisture loss). Accordingly, ten plants were taken from each experimental plot, excluding the edge effects, and evaluated. In the table below (Table 1), the average yield values of the upper part of the lettuce plants obtained from three replications and ten plants, and the wet weight results of the above-ground part of the lettuce plant per plant are given. The different amounts of irrigation water used in the experiment and the different doses of SA, the fresh weight of the above-ground part of the lettuce plant varied between 349.62 – 592.68 g plant<sup>-1</sup>. The highest wet weight of the lettuce plant was obtained from 592.68 g plant<sup>-1</sup> and from I<sub>3</sub>SA<sub>3</sub> subject where 50% of the evaporation amount of irrigation water from Class A evaporation pot was applied and the SA dose was 0.50 mM. Variance on the obtained data, the effects of different amounts of irrigation water and different SA doses on the wet weight of the above-ground part of the lettuce plant were not found statistically significant. In addition, no statistically significant interaction (interaction) was obtained between different amounts of irrigation water and SA levels. When the wet weight results of the above-ground parts are examined, it will be seen that the weight has decreased with the decreasing amount of irrigation water. At the same time, the wet weight of the above-ground part of the lettuce plant increased due to the increase in the amount of irrigation water and SA doses. Based on the wet weight of the above-ground part of the highest lettuce plant, the wet weight of the above-ground

part of the lettuce plant obtained from other applications was compared and the proportional above-ground wet weight values were found. These values are shown in Table 2.

When the subjects are examined separately, in the subject  $I_1$ , in which 100% of the evaporation from the A class evaporation container is given, and in the  $SA_4$  subject, where the SA is applied the highest (0.75 mM), the wet weight of the above-ground part of the lettuce plant is the highest, on the other hand, the class A evaporation It was observed that the lowest wet weight of the above-ground part of the lettuce plant was obtained in the subject  $I_3$ , in which 50% of the evaporation from the container was given, and in the subject of  $SA_1$ , in which no SA application was made (0 mM). Mendoza et al. (2002) examined. 0.1 mM SA and 0.1 mM SSA applied to the seeds caused an increase in plant fresh and dry weights and increased tolerance to cold stress in pepper seedlings. As a result, it was determined that the decreasing irrigation water application and SA doses applied in the experiment decreased the wet weight of the above-ground parts of the lettuce plant.

### **Effects of Irrigation Water Levels and SA Doses Applied in Different Amounts on Dry Weight of Lettuce Above Ground Parts**

Ten plants, whose wet weights were taken, were cut, and the upper parts and roots were weighed separately, and then dried in an oven at 65 °C for one day. The dry weight of the dried aerial parts was calculated. In the relevant table (Table 7), the dry weight of the above-ground part of the lettuce plant per plant is given by taking the average of the dry weight values of the above-ground parts of the lettuce plants obtained from three replications and ten plants.

The dry weight of the aboveground part of the lettuce plant was obtained depending on the different amounts of irrigation water used in the experiment and the different doses of SA varied between 20.72 – 31.47 g plant<sup>-1</sup>. The highest dry weight of the lettuce plant was obtained from  $I_3SA_3$  subject with 31.47 g plant<sup>-1</sup> and 50% of the evaporation amount of irrigation water from the Class A evaporation pot and the SA dose of 0.50 mM was applied. The lowest dry weight of the lettuce plant was obtained from 20.72 g plant<sup>-1</sup> and from  $I_3SA_1$  subject where 50% of the evaporation amount of irrigation water from Class A evaporation pot was applied and SA was not applied (0 mM).

Variance analysis of the obtained data, the effects of different amounts of irrigation water on the root dry weight of the lettuce plant were found to be statistically significant at 5%, and the effects of different SA doses on the root dry weight of the lettuce plant were not statistically significant. Again, the effect of replications on the root dry weight of whole lettuce plants was not statistically significant. In addition, no statistically significant interaction ( interaction ) was obtained between different amounts of irrigation water and SA levels. When the root dry weight results of the lettuce plant are examined, it will be seen that there is a decrease in the root dry weight with the decreasing amount of irrigation water. Likewise, the root dry weight of the lettuce plant increased due to the increase in SA doses.

When the subjects and the repetition averages are examined separately, in subject  $I_1$ , where 100% of the evaporation from the A class evaporation vessel is given, and in  $SA_4$ , where the SA is applied at a dose of 0.75 mM, the dry weight of the above-ground part of the lettuce plant is the highest, on the other hand, the class A evaporation It was observed that the dry weight of the above-ground part of the lettuce plant was the lowest in the subject  $I_3$ , in which 50% of the evaporation from the container was given, and in the subject of  $SA_1$ , in which no SA application was made (0 mM) (Table 7).

### **The Effects of Irrigation Water Levels and SA Doses Applied in Different Amounts on the Root Wet Weight of Lettuce Plant**

With the different amounts of irrigation water used in the experiment and the different doses of SA, the fresh root weight of the lettuce plant varied between 42.92 – 95.59 g plant<sup>-1</sup>. The highest root wet weight of lettuce plant was obtained from I<sub>1</sub>SA<sub>3</sub> subject with 95.59 g plant<sup>-1</sup> and 100% of the evaporation amount of irrigation water from Class A evaporation pot and the SA dose were applied as 0.50 mM. The lowest root wet weight of lettuce plant was obtained from I<sub>3</sub>SA<sub>3</sub> subject with 42.92 g plant<sup>-1</sup> and 50% of the evaporation amount of irrigation water from A class evaporation pot and the SA dose was 0.50 mM.

Variance analysis of the obtained data, the effects of different amounts of irrigation water on the root-wet weight of the lettuce plant were statistically significant at the level of 1%, and the effects of different SA doses on the root-wet weight of the lettuce plant were statistically significant at the 5% level (Table 5). When the root wet weight results are examined, it will be seen that there is a decrease in root weights with the decreasing amount of irrigation water. Likewise, the root fresh weight of the lettuce plant increased due to the increase in SA doses (Table 6).

When the subjects are examined separately, in the subject I<sub>1</sub>, where 100% of the evaporation from the A class evaporation pot is given, and in the SA<sub>4</sub>, in which the SA is applied the highest (0.75 mM), the root fresh weight of the lettuce plant is the highest, on the other hand, the ones from the A class evaporation pot. It was observed that the root-wet weight of the lettuce plant was the lowest in I<sub>3</sub> subject where 50% of the evaporation was given and SA<sub>1</sub> without SA application (0 mM). (2003) reported that foliar application of salicylic acid had a positive effect on plant growth (root length, root, and stem fresh and dry weight) and nitrogen metabolism depending on the applied dose. Uzunlu (2006), on the other hand, examined the effects of aspirin applied from seeds and leaves on melon seedlings exposed to different stress factors (drought, cold, and salt). Aspirin-treated plants generally had lower visual damage and higher chlorophyll, stomatal conductivity, leaf, and root fresh and dry weight, and carbohydrate content compared to control plants. In our study, which gave similar results, a decrease in plant fresh weight was observed in plants with low doses and incomplete irrigation. As a result, it was determined that decreasing irrigation water amounts and SA doses decreased root fresh weight in lettuce plants.

### **The Effects of Irrigation Water Levels and SA Doses applied in Different Amounts on the Root Dry Weight of Lettuce Plant**

After ten plants were cut with fresh weights, the upper parts and roots were weighed separately, the tare weights were numbered on the paper bags and dried in an oven at 65 °C for one day. In the relevant table (Table 8) below, the average of the dry root weight values of the lettuce plants obtained from three replications and ten plants, and the results of the dry root weight of the lettuce plant per plant are given.

With the different amounts of irrigation water used in the experiment and the different doses of SA, the root dry weight of the lettuce plant varied between 10.7 – 19.1 g plant<sup>-1</sup>. The highest root dry weight was obtained from I<sub>1</sub>SA<sub>3</sub> with 19.1 g plant<sup>-1</sup>, where 100% of the evaporation amount of irrigation water from the Class A evaporation pot was applied and the SA dose was 0.50 mM. The lowest root dry weight was obtained from the I<sub>2</sub>SA<sub>3</sub> subject, where 10.7 g plant<sup>-1</sup> and 75% of the evaporation amount of irrigation water from the Class A evaporation pot were applied and the SA dose was 0.50 mM. Variance analysis of the obtained data, the effects of different amounts of irrigation water on the root dry weight of the lettuce

plant were found to be statistically significant at 5%, and the effects of different SA doses on the root dry weight of the lettuce plant were not statistically significant. Yildirim et al. (2006) investigated the effects of foliar salicylic acid application on mineral substance content, chlorophyll content, and plant growth of cucumber plants grown under salt stress. The study was carried out in pots under greenhouse conditions. Different doses of salicylic acid and two (0.6 and 1.2 mM) different salt (NaCl) solutions were applied to the cucumber seeds (0, 0.25, 0.50, and 1 mM). Salicylic acid applications had a positive effect on plant growth, plant fresh and dry weight, and root fresh and dry weight. When the root dry weight results of the lettuce plant are examined, it will be seen that there is a decrease in the root dry weight with the decreasing amount of irrigation water. Likewise, the root dry weight of the lettuce plant increased due to the increase in SA doses (Table 8). When the subjects and the repetition averages are examined separately, subject I<sub>1</sub> in which 100% of the evaporation from the A class evaporation pot, and SA<sub>2</sub> in which SA is applied at a dose of 0.25 mM, the root dry weight of the lettuce plant is the highest, on the other hand, the ones from the A class evaporation pot. It was observed that the lowest dry root weight was obtained in subject I<sub>3</sub>, in which 50% of the evaporation was given, and in subject SA<sub>1</sub>, in which no SA application was made (0 mM).

#### **The Effects of Irrigation Water Levels and SA Doses Applied in Different Amounts on Root Collar Diameter in Lettuce**

The root collar part, which is the part where ten plants were randomly taken from each plot, and rise above the soil, was measured with a digital caliper and the values were recorded. The root collar diameter values of the lettuce plant obtained depending on the different amounts of irrigation water and different doses of SA used in the experiment varied between 19.15 and 26.20 cm. The highest root collar diameter value of subject I<sub>1</sub> in which 100% of the evaporation from the Class A evaporation pot is given and the subject SA<sub>3</sub> in which the SA is applied as 0.50 mM I<sub>1</sub>SA<sub>3</sub> obtained from the interaction. The lowest root collar diameter results are again in subject I<sub>1</sub>, in which 100% of the evaporation from the Class A evaporation pan, and the subject SA<sub>2</sub>, in which the SA is applied as 0.25 mM, I<sub>3</sub>SA<sub>2</sub> obtained from the interaction. When we look at the sub-topic in general, increases in the diameter of the root neck are also observed depending on the increases in SA application doses. When the results are analyzed independently of the analysis of variance in the main subjects, it is seen that the highest root collar diameter is obtained from the I<sub>1</sub> subject, but the I<sub>2</sub> and I<sub>3</sub> subjects also give close results (Table 9)

#### **Effects of Irrigation Water Levels and SA Doses applied in Different Amounts on Root Length of Lettuce**

The root lengths of ten plants taken randomly from each plot were measured one by one with a ruler and average values were given (Table 10). Khan et al. (2003) 's study on beans, foliar application of salicylic acid had a root lengthening effect in the plant. In maize and soybean, foliar application of salicylic acid increased pore density and transpiration, as well as leaf area and plant dry weight, but did not affect plant height and root length Khan et al. (2003) reported. Root length values of the lettuce plant obtained depending on the different amounts of irrigation water and different doses of SA used in the experiment varied between 9.53 and 14.30 cm. The highest root length value was obtained from I<sub>1</sub>SA<sub>3</sub> with 14.30 cm, and the lowest root length value was obtained from I<sub>3</sub>SA<sub>1</sub> subject with the least level of irrigation water and no SA application. When we look at the main subject in general, the highest root length values are observed in the subject where 100% of the evaporation amount of the irrigation water from the class A evaporation pot is applied, while I<sub>2</sub> where 75% of the evaporation amount of the irrigation water from the class A evaporation pot is applied and I<sub>3</sub> where 50% is applied. root

length values were obtained equal to each other (Table 10). variance analysis of the obtained data, the effects of different amounts of irrigation water on root length were not found to be statistically significant, while the effects of different SA doses on root length values of lettuce plants were statistically significant at the 1% level. When the root length values of the lettuce plant were examined, it was observed that there was an insignificant increase in root length with the increasing amount of irrigation water. Considering the sub-topics, it will be seen that there is a noticeable increase in root length despite an increase in the SA application dose in the lettuce plant (Table 11 ). When the subjects and the replication averages are examined separately, it is seen that the irrigation water levels alone do not affect the root lengths much, however, an increase in root length has occurred at all irrigation water levels, especially at 0.50 mM (SA<sub>3</sub>) and 0.75 mM (SA<sub>4</sub>) doses of SA. It will be seen that the highest root length values were recorded when compared with other SA levels in all irrigation water levels, especially for S<sub>4</sub>, the highest SA dose of 0.75 mM.

### CONCLUSIONS AND RECOMMENDATIONS

This study is especially important for regions that have semi-arid climate characteristics such as the Southeastern Anatolia Region and receive almost no precipitation, especially in the summer and autumn periods. Because irrigation has the power to increase the yield at different rates in arid and semi-arid regions. In our study, an increase was observed in all physical parameters examined in parallel with the increase in the amount of irrigation water. In addition, decreases were observed in the investigated values depending on the decrease in irrigation water. However, with the increase in the dose of foliar Salicylic Acid, the negative effects of drought could be reduced to a certain extent. In lettuce cultivation in the spring months, we do not encounter the problem of drought because the rainfall is sufficient and the soil moisture remaining from the winter is high. For this reason, the use of such chemicals in certain doses in autumn lettuce cultivation in Diyarbakır, which has a semi-arid climate, will increase the tolerance of the plant against drought.



**Figure 1.** Preparing the land for planting by removing the worshiper in the trial land



**Figure 2.** Laying mulch covers on the field after plowing and leveling



**Table 1.** Average yields of fresh weight of lettuce plant aboveground parts of the trial subjects

| Main Topics    | Sub Topics      | Above-ground wet weight (g plant <sup>-1</sup> ) |
|----------------|-----------------|--|
| I <sub>1</sub> | SA <sub>1</sub> | 490,08   |
|                | SA <sub>2</sub> | 510,59   |
|                | SA <sub>3</sub> | 529,46   |
|                | SA <sub>4</sub> | 525,99   |
| I <sub>2</sub> | SA <sub>1</sub> | 483,97   |
|                | SA <sub>2</sub> | 474,04   |
|                | SA <sub>3</sub> | 482,97   |
|                | SA <sub>4</sub> | 488,52   |
| I <sub>3</sub> | SA <sub>1</sub> | 451,85   |
|                | SA <sub>2</sub> | 482,15   |
|                | SA <sub>3</sub> | 482,40   |
|                | SA <sub>4</sub> | 487,30   |

**Table 2.** The effects of different irrigation water amounts and different SA doses on the wet weight of the lettuce plant

| Main Topics    | Above-ground wet weight (g plant <sup>-1</sup> ) | Relative yield decrease (%) | Sub Topics      | Above-ground wet weight (g plant <sup>-1</sup> ) | Relative yield decrease (%) |
|----------------|--|-----------------------------|-----------------|--|-----------------------------|
| I <sub>1</sub> | 514,03   | 6,16                        | SA <sub>1</sub> | 475,30   | 0,46                        |
| I <sub>2</sub> | 482,38   | 1,34                        | SA <sub>2</sub> | 488,93   | 1,88                        |
| I <sub>3</sub> | 475,92   | -                           | SA <sub>3</sub> | 498,28   | 2,79                        |
|                |  |                             | SA <sub>4</sub> | 500,60   |                             |

**Table 3.** Average of repetitions of dry weights of above-ground parts of lettuce plant according to trial subjects

| Main Topics    | Sub Topics      | Dry weight of the aboveground part of the plant (g plant <sup>-1</sup> ) |
|----------------|-----------------|--|
| I <sub>1</sub> | SA <sub>1</sub> | 25,37  |
|                | SA <sub>2</sub> | 26,60  |
|                | SA <sub>3</sub> | 27,31  |
|                | SA <sub>4</sub> | 28,45  |
| I <sub>2</sub> | SA <sub>1</sub> | 24,06  |
|                | SA <sub>2</sub> | 24,90  |
|                | SA <sub>3</sub> | 27,09  |
|                | SA <sub>4</sub> | 27,35  |
| I <sub>3</sub> | SA <sub>1</sub> | 22,81  |
|                | SA <sub>2</sub> | 24,29  |
|                | SA <sub>3</sub> | 27,37  |
|                | SA <sub>4</sub> | 27,92  |

**Table 4.** The effects of different irrigation water amounts and different SA doses on the dry weight (g plant<sup>-1</sup>) of lettuce plant aerial parts for the trial year

| Main Topics    | Dry weight of the aboveground part of the plant (g plant <sup>-1</sup> ) | Sub Topics      | Dry weight of the aboveground part of the plant (g plant <sup>-1</sup> ) |
|----------------|--|-----------------|--|
| I <sub>1</sub> | 26,93  | SA <sub>1</sub> | 24,08  |
| I <sub>2</sub> | 25,85  | SA <sub>2</sub> | 25,27  |
| I <sub>3</sub> | 25,60  | SA <sub>3</sub> | 27,26  |
|                |  | SA <sub>4</sub> | 27,91  |

**Table 5.** Variance analysis results of different irrigation water amounts and different salicylic acid levels on root-wet weights of lettuce plant

| Variation Sources        | DF     | Squares average | F value calculated | materiality | F value (table) |       |
|--------------------------|--------|-----------------|--------------------|-------------|-----------------|-------|
|                          |        |                 |                    |             | 0,001           | 0,005 |
| Block                    | 2      | 773.664         | 20.2832            | 0.0081      | 6.94            | 18.00 |
| Main topic               | 2      | 867.582         | 22.7454            | 0.0065**    | 6.94            | 18.00 |
| Error                    | 4      | 38.143          |                    |             |                 |       |
| Sub topic                | 3      | 433.577         | 3.2579             | 0.045*      | 3.16            | 5.09  |
| main topic x subtopic    | 6      | 89.001          | 0.6688             |             | 2.66            | 4.01  |
| Error                    | 18     | 133.084         |                    |             |                 |       |
| Correction Coefficient % | 17.88% |                 |                    |             |                 |       |

**Table 6.** The effects of different irrigation water amounts and different SA doses on root wet weight of lettuce plant for the trial year

| Main Topics    | Lettuce root wet weight (g plant <sup>-1</sup> ) | Sub Topics      | Lettuce root wet weight (g plant <sup>-1</sup> ) |
|----------------|--|-----------------|--|
| I <sub>1</sub> | 71,98  | SA <sub>1</sub> | 54,87  |
| I <sub>2</sub> | 66,29  | SA <sub>2</sub> | 65,27  |
| I <sub>3</sub> | 55,26  | SA <sub>3</sub> | 66,57  |
|                |  | SA <sub>4</sub> | 71,35  |

**Table 7.** Lettuce root dry weights average of repetitions according to trial subjects

| Main Topics    | Sub Topics      | Plant root dry weight (g plant <sup>-1</sup> ) |
|----------------|-----------------|--|
| I <sub>1</sub> | SA <sub>1</sub> | 16,08  |
|                | SA <sub>2</sub> | 17,37  |
|                | SA <sub>3</sub> | 18,80  |
|                | SA <sub>4</sub> | 17,54  |
| I <sub>2</sub> | SA <sub>1</sub> | 16,61  |
|                | SA <sub>2</sub> | 16,10  |
|                | SA <sub>3</sub> | 14,85  |
|                | SA <sub>4</sub> | 16,09  |
| I <sub>3</sub> | SA <sub>1</sub> | 15,44  |
|                | SA <sub>2</sub> | 16,41  |
|                | SA <sub>3</sub> | 14,86  |
|                | SA <sub>4</sub> | 14,91  |

**Table 8.** Effects of different irrigation water amounts and different SA doses on dry weight of lettuce plant

| Main Topics    | Lettuce root dry weight (g plant-1) | Sub Topics      | Lettuce root dry weight (g plant-1) |
|----------------|-------------------------------------|-----------------|-------------------------------------|
| I <sub>1</sub> | 17,45                               | SA <sub>1</sub> | 16,04                               |
| I <sub>2</sub> | 15,91                               | SA <sub>2</sub> | 16,63                               |
| I <sub>3</sub> | 15,41                               | SA <sub>3</sub> | 16,17                               |
|                |                                     | SA <sub>4</sub> | 16,18                               |

**Table 9.** The effects of different irrigation water amounts and different SA doses on the root collar diameter (cm)

| Main Topics    | Root collar diameter (cm) | Sub Topics      | Root collar diameter (cm) |
|----------------|---------------------------|-----------------|---------------------------|
| I <sub>1</sub> | 23,04                     | SA <sub>1</sub> | 22,53                     |
| I <sub>2</sub> | 22,50                     | SA <sub>2</sub> | 22,25                     |
| I <sub>3</sub> | 22,65                     | SA <sub>3</sub> | 23,04                     |
|                |                           | SA <sub>4</sub> | 23,11                     |

**Table 10.** Root length repetition averages in lettuce according to the trial subjects

| Main Topics    | Sub Topics      | Root length (cm) |
|----------------|-----------------|------------------|
| I <sub>1</sub> | SA <sub>1</sub> | 10,75            |
|                | SA <sub>2</sub> | 10,79            |
|                | SA <sub>3</sub> | 12,27            |
|                | SA <sub>4</sub> | 12,47            |
| I <sub>2</sub> | SA <sub>1</sub> | 10,60            |
|                | SA <sub>2</sub> | 11,44            |
|                | SA <sub>3</sub> | 11,82            |
|                | SA <sub>4</sub> | 11,70            |
| I <sub>3</sub> | SA <sub>1</sub> | 10,81            |
|                | SA <sub>2</sub> | 10,85            |
|                | SA <sub>3</sub> | 12,00            |
|                | SA <sub>4</sub> | 11,89            |

**Table 11.** The effects of different irrigation water amounts and different SA doses on root length (cm) in lettuce plant

| Main Topics    | Root length (cm) | Sub Topics      | Root length (cm) |
|----------------|------------------|-----------------|------------------|
| I <sub>1</sub> | 11,57            | SA <sub>1</sub> | 10,72            |
| I <sub>2</sub> | 11,39            | SA <sub>2</sub> | 11,03            |
| I <sub>3</sub> | 11,39            | SA <sub>3</sub> | 12,03            |
|                |                  | SA <sub>4</sub> | 12,02            |

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## THE CHARACTERIZATION AND BIOTECHNOLOGICAL APPLICATION OF THERMOPHILIC ALPHA-L-ARABINOFURANOSIDASE FROM *GEOBACILLUS* SPECIES

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### ABSTRACT

Industrial processes usually take place at high temperatures since high temperatures are important to solve contamination problems and provide soluble substrate. For this reason, the use of thermophilic enzymes, which have high stability at high temperatures, is generally preferred in industrial processes. Microorganisms of the genus *Geobacillus* are important microbial sources used for thermophilic enzymes and they live in regions with very high temperatures such as geothermal waters and hot springs. The genus *Geobacillus* includes 16 species that have been systematically made today. Recently, *Geobacillus* species are of great attraction because they are important sources of thermophilic enzymes such as hemicellulases, proteases, amylases, lipases and pullanases. Among these enzymes, hemicellulases are a group of enzymes, which can degrade the lignocellulosic material, the most abundant undiscovered carbon source in nature. Alpha-L-arabinofuranosidase (Abf), a type of hemicellulase enzyme, catalyzes the hydrolysis of terminal and non-reducing  $\alpha$ -1,2-,  $\alpha$ -1,3- and/or  $\alpha$ -1,5-L-arabinofuranoside by breaking down the side chains of the polymer called xylan, which is in the main structure of hemicellulose. Abf enzymes synergistically play a role with other glycoside hydrolases such as arabinanases and xylanases in a variety of agro-industrial processes, including improving animal feed digestibility, delignification of paper pulp, enhancing flavor during winemaking, and clarification of juices. Due to the wide range of industrial use of abf enzymes, they have been the subject of many researches. In this study, characterization and biotechnological applications of Abf enzymes from thermophilic *Geobacillus* have been considered.

**Keywords:** *Geobacillus*, Alpha-L-arabinofuranosidase, thermophilic enzyme

### INTRODUCTION

Lignocelluloses in plant cell walls consist of cellulose, hemicellulose and lignin. Hemicellulose, the second most common polysaccharide in plants, represents about 20-35% of lignocellulosic biomass (Saha 2003). Microbial degradation of hemicellulose material requires a wide variety of enzymes including alpha-L-arabinofuranosidase (Madeira et al. 2017). The hemicellulases containing Alpha-L-arabinofuranosidase enzymes have received much attention in recent years for their potential industrial use in the biobleaching of pulp, the bioconversion of lignocellulose into fermentative products, and the improvement of digestion in animals (Bhattacharya et al. 2020; Cozannet et al. 2017; Parab and Khandeparker 2021).

The genus *Geobacillus* are one of the most important sources of thermophilic alpha-L-arabinofuranosidase enzymes, and they can live especially in geothermal soils, waters, oil fields and hot springs with high temperatures. The ability of these species to survive at high

temperatures makes them attractive as a source of thermostable enzymes, including Alpha-L-arabinofuranosidase enzymes (Khaswal et al. 2022).

In this study, sources, production, characterization and biotechnological applications of thermophilic Alpha-L-arabinofuranosidase enzymes from *Geobacillus* species have been considered.

### **THERMOPHILIC ALPHA-L-ARABINOFURANOSIDASES FROM GEOBACILLUS SPECIES**

Alpha-L-arabinofuranosidase (Abf) (EC 3.2.1.5) catalyzes the hydrolysis of terminal and non-reducing  $\alpha$ -1,2-,  $\alpha$ -1,3- and/or  $\alpha$ -1,5-L-arabinofuranoside in  $\alpha$ -L-arabinosides such as arabinan, arabinoxylan and other polysaccharides containing arabinose. These enzymes break down the side chains of the polymer called xylan, which is the main structure of hemicellulose. These enzymes work synergistically with other hemicellulases for complete hydrolysis of hemicellulose (Kaji 1984). Abf enzymes are divided into six families of glycoside hydrolases (GH) on the basis of amino acid sequence similarities and the three-dimensional structure of the active site. These classes are GH3, GH10, GH43, GH51, GH54 and GH62 (Hoffman et al., 2013) (<http://www.cazy.org/>).

Abf enzymes are commonly found in bacteria, fungi and plants (Hashimoto and Nakata 2003; Lee et al. 2003; Rahman et al. 2003). Although several plants for the production of Abf enzymes have been reported (Chavez Montes et al., 2008), large-scale production of these enzymes requires sophisticated microbial strains with high production capabilities. As a matter of fact, microorganisms play an important role in the biological transformation of lignocellulosic materials, including cellulose, hemicellulose and pectin, by producing and secreting different hydrolytic enzymes, including Abf enzymes. In this respect, it has been reported that microorganisms secrete Abf enzymes along with other lignocellulosic enzymes (Maheswari and Chandra 2000; Wilson 2011).

Abf enzymes work synergistically with other glycoside hydrolases such as arabinanases (EC 3.2.1.99) and xylanases (EC 3.2.1.8) in various agro-industrial processes. These processes can be listed as improving the digestibility of animal raw materials, bleaching the pulp, enhancing the aroma during winemaking, biofuel production, prebiotic production and clarification of fruit juices (Saha 2000). In industrial processes, fermentation conditions usually require a high temperature, such as a minimum of 60 °C because high temperatures eliminate contamination problems and provide not only fluid media but also soluble substrates (Morozkina et al., 2010). Therefore, enzymes from thermophilic microorganisms (especially bacteria and fungi) are primarily preferred in industrial processes. Recently, the identification of Abf enzymes from thermophilic microorganisms has been extensively carried out (Geng et al. 2019; İlgü et al. 2018; Saleh et al. 2022; Shi et al. 2022)

One of the most important sources of thermophilic Abf enzymes is *Geobacillus* species. The genus *Geobacillus* has a wide range of habitats, including geothermal soils, waters, oil fields and hot springs. They are rod-shaped, aerobic or facultatively anaerobic, endospore-forming microorganisms. *Geobacillus* species are obligate thermophiles, and they have the ability to live in a wide temperature range (35-80°C) (Khaswal et al. 2022). However, growth temperatures of about 45°C to 70°C are required for many strains (Nazina et al. 2001). The ability of these species to survive at high temperatures makes them attractive as a source of thermostable enzymes including Abfs (Khaswal et al. 2022).

## **PRODUCTION AND CHARACTERIZATION OF THERMOPHILIC ALPHA-L-ARABINOFURANOSIDASES FROM *GEOBACILLUS* SPECIES**

There have been various studies on the production and characterization of thermophilic Abf from *Geobacillus* species isolated from different regions. Some of these studies have been on heterologous expression of the Abf enzyme in a different host. For the heterologous expression of the Abf enzyme, *E. coli* BL21(DE3) and pET28a (+) have been used as the host and the expression vector system, respectively (Canakci et al. 2007; Huang et al., 2017; İlgü et al. 2018). The pure protein is obtained via Nickel affinity chromatography by adding the histidine tags to the N-terminal end of the heterologous expressed protein through this vector system (Sürmeli, 2013). On the other hand, one study has been reported on the production of Abf enzyme in its native strain. In this study, the Abf enzyme of the native strain *Geobacillus* sp. DC3 isolated from gold mines has been produced by adding xylan substrate directly to the growth medium and it has been purified from the supernatant as an extracellular enzyme (Bergdale et al. 2014). When enzyme is produced by the native strain it generally possesses low specific activity. For example, the specific activity of the Abf enzyme produced by native *Geobacillus* sp. strain DC3 was found as 0.230 U/mg (Bergdale et al. 2014). Recombinant Abf enzyme production has led to a higher specific activity (Canakci et al. 2008; Saleh et al. 2022; Shi et al. 2022). However, extracellular secretion of the enzymes is generally preferred in protein production because it is an easy way to recover the enzyme product. There have been some studies on the construction of recombinant enzyme production system with extracellular secretion. For example, Wirajana and colleagues (2016) have succeeded in the extracellular secretion of recombinant *Geobacillus thermoleovorans* IT-08 Abf enzyme in *Saccharomyces cerevisiae* host by fusing a signal peptide to the enzyme (Wirajana et al., 2016).

Optimum pH of Abf enzymes from *Geobacillus* species was found to be in the range of 5.0-7.0, which is weakly acidic to neutral (Table 1) and these enzymes are active in a variety of pH ranges. For example, İlgü and colleagues (2018) have shown that the relative activity of alpha-L-arabinofuranosidase (GvAbf) enzyme of *Geobacillus vulcani* GS90 isolated from İzmir Balçova geothermal hot springs did not decrease below 60% in a wide range of pH (3.0-10.0) (İlgü et al. 2018). In contrast, the alpha-L-arabinofuranosidase (AbfA) enzyme from *Geobacillus thermodenitrificans* NG80-2 exhibited more than 60% activity in a narrower pH range (pH 5.0-8.0) (Huang et al. 2017). In addition, optimum temperatures of Abf enzymes from the genus *Geobacillus* are generally in the range of 60-70°C (Bergdale et al. 2014; Huang et al. 2017; İlgü et al. 2018). Different from these Abf enzymes, the optimum temperature of AbfATK4c enzyme of *Geobacillus caldoxylolyticus* TK4 isolated from Çanakkale Kestanbol thermal springs was found as 75-80°C (Canakci et al. 2007). The temperature ranges, where the Abf enzymes are active, are also comparable to each other. For example, GvAbf and AbfA enzymes are active in broad range of 30-90°C. Nevertheless, AbfA activity decreased to 20%, whereas GvAbf exhibited a minimum of 94% activity in this temperature range (Huang et al. 2017; İlgü et al. 2018). According to the thermal stability analyses of Abf enzymes, to the best of our knowledge, the enzyme with the highest stability has been AbfATK4c, which preserved approximately 60% of its activity even after 50 hours at 75°C (Canakci et al., 2007). On the other hand, AbfA decreased to a relative activity of 60% after only 3 hours (Huang et al. 2017). GvAbf completely lost its activity after 15 minutes at 75°C (İlgü et al., 2018). In literature, comparable results on kinetic parameters of Abf enzymes from *Geobacillus* species have been obtained (Canakci et al., 2007; İlgü et al., 2018).

**Table 1.** Abf enzymes, their characteristics and Abf-producer *Geobacillus* species

| <b>Abf-producer<br/><i>Geobacillus</i><br/>species</b> | <b>The habitat of<br/><i>Geobacillus</i><br/>species</b>  | <b>Vmax<br/>(U/mg)<br/>Km (mM)</b> | <b>Optimum pH and<br/>temperatures</b> | <b>Reference</b>     |
|--|---|------------------------------------|--|----------------------|
| <i>Geobacillus vulcani</i> GS90                        | Balçova geothermal region, İzmir, Turkey                  | Vmax: 200<br>Km: 0.2               | pH 5.0, 70°C                           | İlgü et al. 2018     |
| <i>Geobacillus</i> sp. DC3                             | the Former homestake gold mine in Lead, South Dakota, USA |                                    | pH 7.0, 70°C                           | Bergdale et al. 2014 |
| <i>Geobacillus thermodenitrificans</i> NG80-2          | A deep oil reservoir in northern China                    |                                    | pH:6.5, 60°C                           | Huang et al. 2017    |
| <i>Geobacillus caldxylolyticus</i> TK4                 | the Kestanbol hot spring, Çanakkale, Turkey               | Vmax:151.<br>5 Km: 1.57            | pH:6.0, 75-80°C                        | Canakci et al. 2007  |

### BIOTECHNOLOGICAL APPLICATIONS OF THERMOPHILIC ALPHA-L-ARABINOFURANOSIDASES FROM *GEOBACILLUS* SPECIES

Abf enzymes work synergistically with other xylanolytic enzymes in different agro-industrial processes. These agro-industrial processes are mostly the production of short xylooligosaccharides used as prebiotics (Bhattacharya et al. 2020), the increase of animal feed digestibility and feed utilization in monogastric animals as an animal feed additive (Cozannet et al. 2017), the production of biofuels such as bioethanol (Tu et al. 2019), juice clarification to increase the nutritional value of fruit juices (İlgü et al. 2018) and biological bleaching of pulp as a biobleaching agent (Parab and Khandeparker 2021). In literature, Abf enzymes from *Geobacillus*, to the best of our knowledge, have only been applied for biofuel production and juice enrichment.

It is known that *Geobacillus* species, which can produce the xylanolytic enzymes including Abf, possess a versatile catabolism in the degradation of hemicellulose and starch, and they have a rising profile in terms of the potential to produce second generation biofuels (Hussein, et al., 2015). Regarding this, Huang and colleagues (2017) have achieved the complete conversion of oat-spelt xylan, beechwood xylan and birchwood xylan into xylose and arabinofuranose monosaccharides using different combination of cocktails including *Geobacillus thermodenitrificans* Abf, xylanase and  $\beta$ -xylosidase enzymes. As a result of the study, it was concluded that the use of cocktails consisting of *Geobacillus thermodenitrificans* Abf, xylanase and  $\beta$ -xylosidase enzymes has a great potential in the conversion of plant biomass to biofuels (Huang et al., 2017).

Polysaccharides containing hemicellulose reduce juice quality in beverages and chemicals are widely used to solve this problem. However, enzymes containing Abfs are more preferred for obtaining high quality products due to their non-toxic properties and substrate specificity (Cakmak & Saglam Ertunga 2016; Shahrestani et al. 2016). A recent work has applied *Geobacillus vulcani* Abf enzyme and a commercial xylanase enzyme separately and simultaneously to enrich the apple, grape, orange and peach juices. As a result, it has been



emphasized that the simultaneous synergistic behavior of GvAbf and xylanase enzymes has a higher potential for application in fruit juice processing (İlgü et al., 2018).

## CONCLUSION

To conclude, Abf enzymes are accessory enzymes that are more suitable to be used together with other xylanolytic enzymes. It is known that these enzymes have potential to be used in many agro-industrial processes such as animal feed additive, pulp bleaching agent, lignocellulose decomposition agent in second generation biofuel production, fruit juice clarification agent. The fact that *Geobacillus* species have a wide range of habitats including geothermal soils, waters, oil fields and hot springs and they are obligatory thermophiles make these bacteria and their enzymes attractive to be used in agro-industrial processes requiring high temperatures. Supporting this, Abf enzymes obtained from *Geobacillus* species work optimally at high temperatures (60-80°C) and have a very high thermal stability. The suitability of Abf enzymes to many agro-industry process conditions will make them more attractive for the next investigations.

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## THE IMPACT OF DILL LEAVES ON THE SENSORY QUALITY OF TUNA PATE

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### ABSTRACT

Canned tuna is one of the most traded food products around the world due to its availability in processing and consumption. Similar to other seafood processing methods, many by-products are generated during the tuna canning process and utilization of these by-products in the alternative value-added products production is a promising approach for circular economy and the environment. In this research, tuna pate was produced with the usage of chunks and trimming of canned tuna products. For these aims, two tuna pate groups were produced from without or dill leaf addition. Then each pate group was separated into three sub-groups in terms of packaging material; flint glass (F), amber glass (A), and plastic container (P). Packaged tuna pate groups are stored in the refrigerator during six months. Sensory attributes such as colour, odour, taste, texture, and general acceptance were also determined. The results showed that the sensory quality of dill leaf enriched tuna pate groups had higher sensory scores than control tuna pate groups. The results has also revealed that the tuna pate groups in the glass jar, especially in amber glass groups compared to plastic container groups had been evaluated more appropriate by panelists.(P<0.05). These results revealed that the usage of dill leaf prolonged the shelf life of tuna pate within sensory quality of tuna pate. The structure and colour of packaging materials also impacted the quality of tuna pate sensory quality.

**Keywords:** Seafood, quality, sensory attributes, dill leaves, tuna, spreadable pate

### INTRODUCTION

With increasing demand of healthy food products globally, alternative products have become popular in the food industry. In particularly, ready-to-eat food items are most commonly preferred by consumer due to can be easily consumed and do not required any process. Pate is one of the ready to eat food products containing meat, spice, additional oil and other food additives. Traditionally, pate prepared with goose liver which is known as "foie- grass", the increasing demand to this product and the price had to tended to producer usage to alternative sources. From pork livers and chicken livers to different animals' offal have been used for pate production (Estévez et al., 2007; Polak et al., 2011). The market size of pate was more than a billion USD in 2018, and it is estimated to increase total size by 1.3 % up to 2025 (Grand View Research, 2019). Recently fatty fish species have used in pate production due to their functional characteristics resulted by fat content and health benefits. Higher bioavailability rate and containing 20% more protein than meat pate makes fish pate a popular seafood product

(Branciari et al., 2019). Especially, fatty fish species such as tuna and anchovy are used for pate production owing to their desirable taste and other sensory quality parameters (Aksun Tumerkan et al., 2021)

Tuna species accepted as one of the biggest group of significant fishes that belong to the Scombridae family, are classified into three genera, *Katsuwonus*, *Sarda*, and *Euthynnus* and one of the most consumed species globally (Abdullah and Rehbein, 2016). Especially canned tuna has been accepted as one of the traded food products owing to its efficiency to processing, easy availability, and sensory attributes to consumer preference (Dantas et al., 2021). Same as other seafood processing methods, a widerange by-products such as tuna trimming and tuna chunk generated during canning process (Malaweera and Wijesundara, 2014). Valorisation of these by-products in value-added novel food products offers economic and eco-friendly production with contributing to sustainable development goals and sustainable production. Since, pate products are highly susceptible to oxidation as a result of containing external high lipid rate and commonly fatty species preferred. Different processing techniques, using of natural antioxidants, packaging materials are robust approaches to avoiding chemical quality deterioration and improving sensory quality. Utilization of several plants directly using leaves or indirectly using extracts leads to delaying to oxidation in addition to improvement of the sensory attributes of food products. Thus, this research aimed to investigate dill leaf usage as taste enhancer and sensory quality improver of refrigerated tuna pate in different packaging materials during 6 months.

## **MATERIAL AND METHOD**

### **Materials**

Tuna (*Katsuwonus pelamis*) by-products (chunks and trimmings obtained after canned tuna processing) in pre-cooked form used as raw material for tuna pate production. The chunks and trimmings were obtained from seafood processing plant (Sasu Ltd., Adana, Turkey) the other ingredients (milk powder, spices, starch, and lemon juice) were obtained from local market located in Adana, Turkey in fresh form. Dill leaves (*Anethum graveolens*) were also obtained from the local market as fresh form and then washed, dried and grinded until further usage.

### **Tuna pate preparation and storage**

For the production of the tuna pate, tuna chunks and other ingredients were blended in two different formulations. Frozen tuna trimmings and tuna chunks were thawed and chopped then mixed with mush potato, milk powder, salt, starch, and sunflower oil, spices and other ingredients in a dough mixer at 4°C until obtaining a homogeneous tuna trimmings dough (10 min). Mixed pate was divided into 2 groups: Control group (C) without any other ingredient, and for dill leaf enriched (DE) group, dried dill leaves and lemon juice were added to control groups while mixing slowly. Then, each pate group was each pate group was separated equally into three groups for different packaging material (F: flint glass jar, A: amber glass jar and P: plastic container). Different tuna pate groups (C and DE) in different packaging materials classified as CF, CA, CP, DEF, DEA, DEP. The control and dill leaves enriched pate sample were manually full filled into the glass jars and plastic container as around 100 g. Then, the packaged tuna pate pasteurized at 80 °C for 45 min laboratory-type oven with slight modification the method described by Estévez, and Cava, (2004). All the pate samples were refrigerated storage at 2 ± 1 °C through 6 months.

## Sensory attributes of tuna pate during storage

The sensory evaluation of the tuna pate (CF, CA, CP, DEF, DEA, DEF) was conducted with 10 experienced panelists (5 women, 5 men). Panelists assessed flavor, odour, taste texture and general acceptance of pate groups. Sensory assessment performed with nine-point descriptive scale described by UNE 87-020-93 (UNE, 1993). According to scale, higher scores were evaluated as “good quality”, and lower scores evaluated as deterioration of quality.

## RESULTS AND DISCUSSION

### Sensory attributes of tuna pate

The descriptive sensory attributes of tuna pates during storage period shown in Figure 1. Significant differences were detected related to sensory quality in terms of taste, odour, texture, general acceptance ( $P < 0.05$ ). Dill leaf enrichment lead to improvement of sensory attributions of tuna pate regardless of packaging material especially for odour and taste parameters. Control groups reached the rejection limit at the 22th week of storage and the dill leaves enriched groups reached the same limit at the end of storage (24th week).

Dill leaves enrichment extended the shelf life of tuna pate stored in refrigerated storage by 2 weeks compared to the control tuna pate groups. Similar results highlighted by Özoğul et al., (2017) who declared the usage of plant based material on the sensory quality of fish products. Packaging material also impacts the sensory quality of tuna pate. While, tuna pate stored in plastic packaging material reached at the rejection limit at the 22th week, the tuna group stored in the glass jar achieved at the 24 th week of storage.

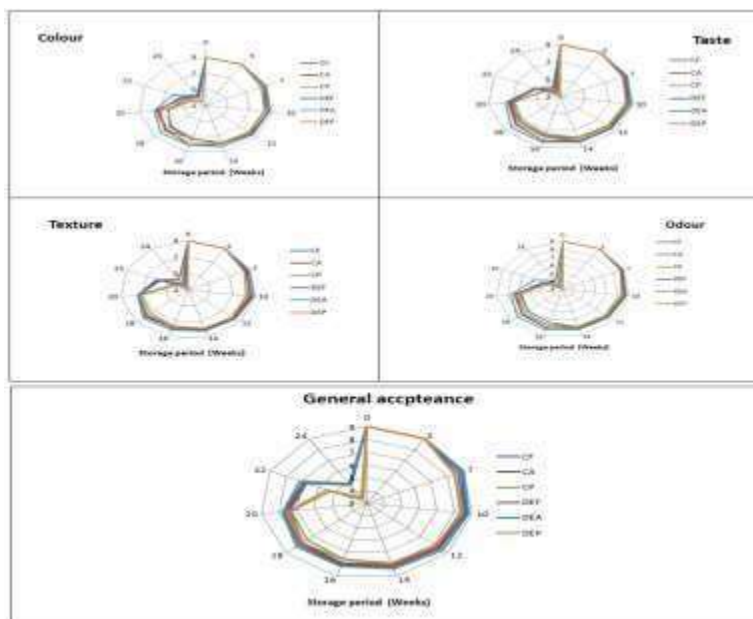


Figure.1. Sensory attributions of tuna pate during refrigerated storage

Groups: CF: Control tuna pate stored in flint glass jar, CA: Control tuna pate stored in amber glass jar, CP: Control tuna pate stored in plastic container, DEF: Dill leaf enriched tuna pate stored in flint glass jar, DEA: Dill leaf enriched tuna pate stored in amber glass jar, DEP: Dill leaf enriched tuna pate stored in plastic container

## CONCLUSIONS

Due to sensory quality impacts on the product acceptability and the feasibility of processing of any food products, this quality is so important. Tuna pate enriched with dill leaf proved the sensory quality which could be related to lower oxidation capacity. In terms of packaging material, stored in glass jar especially in amber glass jar lead to higher sensorial acceptance. In terms of sensory attributes, texture, color, odor and general acceptance among tuna pate groups, dill leaf enrichment protect the sensory quality. These findings recommended that dill leaf could be a practical taste and odor enhancer for other food items have high fat content such as pate products. Glass jar could be a more suitable packaging material for pate type products, especially during refrigerated storage. The findings of research revealed that usage of plant allows better quality of seafood in addition to improving of taste and the packaging material impact on the quality of food product, especially high lipid contained food.

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## FROM INVASION TO CONSUMER TABLE: AS A MODEL: LIONFISH

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### ABSTRACT

While the importance of marine sources for not only food and feeding but also for biomaterial and sustainability of fisheries has become more essential for the public, decision-makers and researchers; the threat of invasive species on the marine sources has still been an important problem. According to the research, the main effects that threaten the coastal system are invasive species, tourism, destruction of the coasts and the lack of awareness of people. Alien species, which are one of these effects, face very different ecological factors in the geography where they have just moved. Some of these alien species spread rapidly, using the environment's available resources better than other species that already existed in the environment, and over time, they become invasive species, replacing natural species in the environment and invading new ecosystems to which they are moved. The lionfish, which is naturally found in the Pacific Ocean, has attracted the attention of researchers in recent years with its spread in different seas of the Earth. This invasive species has become prevalent in Turkey from Eastern Mediterranean to the Aegean Sea. Using and valorization of natural sources for maximum benefits are one of the important parts of sustainability goals globally. Therefore, the usage of invasive marine species as an alternative for food and feed sources is a promising approach for converting a threat into benefits. This review will evaluate the potential of utilization of lionfish as a food or feed sources model for invasive species in Turkey. The proximate composition, nutritional benefits and potential risks such as bioaccumulation of heavy metals or other environmental pollutants will be criticized. The economical feasibility will also determine within previous research. The results could be useful for the food industry and fisheries science.

**Keywords:** Lionfish, seafood, invasive fish species, valorization, and nutritional value

### INTRODUCTION

Invasive species that settle in the area where they have just moved, cause damage by competing with native species, change the balance in the ecosystem, and have negative effects on human health and economy are classified as IS. Circular Bioeconomy is defined as "the conversion of biological residues (residual) into bio-based products that can be shared/reused/reproduced, recycled or safely released into the biosphere through organic and nutrient cycles". Depending on Circular Economy and bioeconomy: some of the main aims accepted as ;prevention and reduction of urban biological waste, conversion of inevitable urban biological waste into valuable resource management, Climate change mitigation: Reducing greenhouse gas emissions by avoiding landfill and capturing carbon in bio-waste-based products, Industrial symbiosis : Linking the bio-waste and wastewater sectors with other sectors

such as bio-based, chemicals, plastics, food and feed, etc., Resource efficiency and safety: Conversion of municipal biological waste into valuable substances such as Nitrogen (N), Phosphorus (P) and Potassium (K), Sustainable food and nutrition security: Improving local food systems through bio-based solutions such as the production of feed (and ultimately food) components from urban biological waste (European Commission, 2018). All species of Pterois, which belong to the family of scurvyes (Scorpaenidae), have poisonous dorsal, anal and pelvic spines (Froese and Pauly, 2019). The poison of these spines is effective enough to cause injury and death for fishermen and divers (Schofield, 2009). Due to its poisonous spines, it has few natural predators. *P. miles* of Indo-Pacific origin live in hard ground structures, longoses, seagrass meadows, coral reefs, artificial reefs, cliffs, muddy bottoms and sandy habitats in temperate waters deep between 1-300 feet (Khalaf and Kochzius, 2002; Albins and Hixon, 2008; Ferrieira et al., 2015). They hide on rocks and in hollows (Hare and Whitfiel, 2003). The lesepian species entering the Mediterranean ecosystem, the evaluation of ecological niches that are not evaluated by native species, their easy population formation and the dominance on native species in bilateral competition are interpreted as an indication that this migration will be continuous (Ergüden and Doğdu, 2020). While the process is still experienced in the form of pressure of foreign species on native species, it is expected that new foreign species will replace the foreign species that have become dominant in areas where native species are found. Studies on biodiversity in the region show that this change can only be experienced in a few years (Gürlek et al., 2016). For these reasons, the importance of combating the species that valorization of invasive species in circular economy is so important since the easiest and most functional way to combat these species is to bring the species into the economy (Zenetos et al., 2012).

### **VALUE-ADDED PRODUCTS FROM LIONFISH**

The lionfish, whose spiny fins are poisonous, but whose meat does not contain poison, was taken to the menu of social facilities in order to spread its consumption. Lionfish are generally served after their spines are cleaned and processed into soup, grilled, steamed and fried. For the processing and consumption of an animal source as safe food; Physicochemical suitability (Nutrient composition, fat protein ratios), textural properties Heavy metal, Pesticide, Pcb content should be tested to be safe in terms of Cytokoxydede and in terms of allergen. Purification of protein isolate or other biomaterial from lionfish is another alternative for processing of this species.

### **CONCLUSIONS**

It is important to investigate the availability of fish species that show invasive characteristics in the waters of our country as functional products together with their nutritional composition, suitability for processing techniques and other food matrices. It affects the feasibility of processing during the use of invasive species as raw materials as food products, and the composition of nutrients, which differ from species to species and even from individual to individual in a bear species, leads to differences in the accumulation of aquatic pollutants in fish muscle. The processing, packaging and introduction of invasive aquatic species, which are one of the leading environmental risk factors and which cannot have economic importance, to the aquaculture processing sector with different methods will give a new perspective to the aquaculture circular economy, which is one of the leading adequate and balanced nutrition, which has become increasingly important recently. With this application, the AIM is to hunt the existing invaders in an environment, process them and bring them to the economy and turn

them into high value-added products. The risk of invasive species moving to a new location and showing their effect there is the most important part of feasibility studies.

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## FUNCTIONS OF TYPE V SECRETION SYSTEM AND AUTOTRANSPORTER PROTEINS IN *SALMONELLA*

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### ABSTRACT

*Salmonella*, a Gram-negative foodborne pathogen, is known as a serious infectious agent all over the world. There are various mechanisms that play a role in *Salmonella* pathogenicity and secretion systems are one of them. These secretion systems play a key role in the formation of biofilms, which are known as forms of resistance to all adverse environmental conditions, as well as adhesion to host tissues, colonization, and host cell invasion, which are the main elements that manage *Salmonella* pathogenicity. Autotransporter proteins are one of the important pathogen-associated molecular patterns (PAMP) of *Salmonella* in attachment to host tissues, colonization, and therefore host cell invasion. These autotransporter proteins called MisL, ShdA and SadA are secreted through the Type V secretion system (T5SS). This review aims to display the importance of T5SS and autotransporters secreted by this system for *Salmonella* virulence and summarize their functions on *Salmonella* adhesion, invasion, and biofilm formation.

**Keywords:** *Salmonella*, T5SS, autotransporters, MisL, ShdA, SadA, biofilm, adhesion

### INTRODUCTION

*Salmonella* is a Gram-negative, rod-shaped bacteria that is identified as a foodborne pathogen. This pathogen is facultatively anaerobic and it does not have the spore-forming ability (Fàbrega and Vila, 2013). *Salmonella* is one of the most commonly detected organisms as the cause of foodborne diseases, and it causes serious health problems among individuals. Therefore, it is regarded as a serious threat across the world. *Salmonella* pathogenicity has been intensively studied in order to reduce disease and death caused by this bacteria and to better understand it. As a consequence of these studies, a lot of mechanisms that contribute to *Salmonella* virulence are discovered. These mechanisms include various secretion systems and agents that are secreted through these systems. Various factors such as substrates, proteins or toxins that are secreted via these secretion systems play a critical role in the attachment of *Salmonella* to host cells, entrance into the host cell as well as survival and proliferation of *Salmonella* inside the host (Bao et al., 2020). There are at least six secretion systems identified in *Salmonella* which are type I, II, III, IV, V and VI secretion systems. Type V secretion system secretes three significant proteins which contribute to *Salmonella* pathogenicity. These proteins are defined as MisL, ShdA and SadA proteins. The proteins secreted by the type V secretion system are known as "autotransporter proteins" since they can transport proteins out of the cell without needing any energy source. In addition, these proteins are also described as autotransporter adhesins. Although there is not enough knowledge regarding the specific roles of these proteins in *Salmonella* pathogenicity, studies have demonstrated that these proteins

play a role in *Salmonella* adhesion, invasion, colonization, and biofilm formation (Wagner and Hensel, 2011).

### **Type V Secretion System (T5SS)**

Type V secretion system (T5SS), also known as the autotransporter system is one of the essential secretion systems in *Salmonella*. This secretion system differs from the other secretion systems in terms of complexity, size, and the area that it is spread. T5SS is much smaller compared to the other secretion systems found in Gram-negative bacteria and it localizes only in the outer membrane (Saier, 2006). T5SS is regarded to be a simpler secretion system than the other secretion systems since it only consists of a single polypeptide chain. In type V secretion, the passenger domain that is secreted to the cell surface and secretion pore (translocation domain) localize at the same polypeptide chain (Fan et al., 2016). This system contributes to *Salmonella* adherence to host cells and is in charge of secreting the virulence factors involved in *Salmonella* pathogenicity. (Leo et al., 2012). Type V secretion system does not have an evident energy source for the transportation of the proteins since there is no chemical energy as ATP found in the periplasm and stable protons or other ion gradients are absent throughout the outer membrane where T5SS is localized (Meuskens, 2019). Many studies have found that multiple factors might play a role in the secretion of the autotransporter proteins, however, the energy sources that are utilized by the autotransporter systems are still being investigated and discussed among researchers (Thanassi et al., 2005; Drobnak et al., 2015).

Autotransporter proteins that are secreted by T5SS consist of three functional motifs. One of these motifs is an N-terminal signal peptide that facilitates protein transport from the inner membrane to the periplasm. The other one is the passenger domain which is transported across the bacterial surface and the third one is the C-terminal translocation domain (translocator) which allows the passenger domain to be exported through the outer membrane (Yen and Stathopoulos, 2007). Autotransporter proteins follow a conserved pathway in their biogenesis, like the majority of outer membrane proteins (Meuskens et al., 2019). Since T5SS is only found in the outer membrane, proteins that are to be secreted are transported in an unfolded state from the inner membrane into the periplasm by the Sec apparatus (Desvaux et al., 2004; Green and Meccas, 2016). Therefore, autotransporter proteins have an N-terminal signal sequence that is recognized by the Sec mechanism (Green and Meccas, 2016). In addition to this mechanism, the Bam complex can also contribute to the transportation of proteins. Bam complex facilitates the folding of the outer membrane proteins to the  $\beta$ -barrel conformation and enables their insertion into the outer membrane (Van Ulsen et al., 2014).

Type V secretion systems are classified into subgroups based on structural characteristics and domain organization and these groups include Va, Vb, Vc, Vd and Ve secretion systems (Meuskens et al., 2019). Some researchers claim that there might be another potential subgroup (Vf), however, this consideration needs further investigation (Grijpstra et al., 2013).

Type Va class is commonly considered as the classical autotransporters. IgA protease from *Neisseria meningitidis* and a lipase named EstA from *Pseudomonas aeruginosa* are the best-known members of this secretion pathway (Henderson et al., 2004). A 12-stranded  $\beta$ -barrel domain which is embedded in the outer membrane serves as a C-terminal anchor and is required for the transportation of the N-terminal passenger domain to the extracellular environment in type Va autotransporters (Meuskens et al., 2019). This makes it easier for the passenger domain to get transported to the outer surface. After the translocation process, the passenger domain might be cleaved off and secreted to the outside of the cell. In some circumstances, the cleavage of the passenger domain might not take place and be left at the cell surface via the translocation domain (Fan et al., 2016). Most of the passenger domains of the autotransporter proteins have

enzymatic activities such as lipase or protease and these passengers are mostly involved in the group of cleaved passengers. However, the cleavage process can also be observed in some of the adhesins (Leo et al., 2012; Fan et al., 2016).

Unlike other subclasses of type V secretion systems, type Vb secretion systems are made up of two different polypeptide chains which are encoded in a single operon (Jacob-Dubuisson et al., 2013). Because of this feature, these systems are also called two-partner secretion systems and they consist of two proteins which are the translocator (TpsB) and the passenger (TpsA). Since the translocator and the passenger domain are separated into two distinct polypeptide chains, the passenger domain can be released to the outer cell without any cleavage process (Kajava et al., 2001).

Type Vc systems are the most complicated secretion systems among other type V classes. The architecture of type Vc secretion systems is similar to that of type Va systems, but they form heavily entangled trimeric complexes. Because of these characteristics, they are also called trimeric autotransporter adhesins (Meuskens et al., 2019). The proteins belonging to the type Vc systems are made up of three identical polypeptide chains. Their eventual folded form consists of 12 stranded  $\beta$ -barrel and a trimeric passenger domain that folds into a structure similar to the lollipop-like shape (Linke et al., 2006; Meuskens et al., 2019).

Type Vd secretion systems show similarity to both type Va and type Vb autotransporters. Type Vd autotransporters resemble type Vb translocators (TpsB) in terms of the C-terminal domain as they both have 16  $\beta$ -strands (Meuskens et al., 2019). Type Vb translocator proteins (TpsB), on the other hand, contain two POTRA (polypeptide transport-associated) domains, whereas type Vd autotransporters have only one. (Leo et al., 2012).

Type Ve autotransporter proteins are also known as inverse autotransporters. These autotransporters belonging to the type Ve class have similar characteristics to type Va autotransporters. The autotransporters proteins in both systems consist of 12 stranded  $\beta$ -barrel domain and a monomeric passenger domain which is left at the surface without getting cleaved after the translocation process. The primary distinction between type Ve autotransporters and type Va autotransporters is that while in type Va autotransporters, the  $\beta$ -barrel is localized at the C-terminal end and the passenger at the N-terminus, type Ve autotransporters have the inverted domain order (Oberhettinger et al., 2015, Meuskens et al., 2019).

### **Autotransporter Proteins and Their Functions in *Salmonella***

Autotransporter proteins are one of the important pathogen-associated molecular patterns (PAMP) of *Salmonella* in attachment to host tissues, colonization, and host cell invasion. There are three autotransporter proteins that are secreted by the type V secretion system and are thought to play a role in *Salmonella* pathogenicity. These autotransporter proteins are MisL, ShdA and SadA proteins. According to the studies that are performed up to date, it is claimed these proteins might play a role in biofilm formation, which is required for *Salmonella* resistance to adverse environmental conditions, adhesion to host tissues, or colonization and invasion of host cells.

#### **MisL Autotransporter Protein**

MisL, which stands for membrane insertion and secretion, autotransporter protein which is encoded by the *misL* gene, is localized at the *Salmonella* Pathogenicity Island 3 (SPI-3) and secreted via the type V secretion system. MisL protein belongs to the class of monomeric adhesins and it consists of 955 amino acids (Blanc-Potard et al., 1999). MisL autotransporter protein shows resemblance to the immunoglobulin A1 protease family. Moreover, VirG protein from *S. flexneri* and AIDA-I protein from *E. coli* exhibit similarities to MisL protein, however, these similarities are only restricted to the C-terminal domain (Blanc-Potard et al., 1999). The

C-terminal domain of MisL protein exhibits homology with the C-terminal domain of *E. coli* AIDA-I protein at the rate of 43% (Benz and Schmidt, 1992) and the C-terminal domain of VirG protein of *S. flexneri* at the rate of 38% (Goldberg et al., 1993). Studies have shown that the VirG protein of *S. flexneri* participates in adhesion to HeLa cells and the AIDA-I protein from *E. coli* plays a role in bacterial spreading through epithelial cells. The homology between these proteins and MisL autotransporter protein indicates that these proteins exhibit similar autotransporter functions (Blanc-Potard et al., 1999). Furthermore, both AIDA-I protein and MisL are lacking cysteine residues that are required for membrane translocation (Jose, 1995).

MarT (membrane-associated regulator) protein, which is another protein encoded by SPI-3, shows similarity in its N-terminal region with the transmembrane regulatory protein, ToxR, which takes part in the synthesis of cholera toxin in *Vibrio cholerae* and also has homology with a protein from *E. coli* K-12 (Blanc-Potard et al., 1999). Like the ToxR protein, MarT protein consists of a transmembrane domain in its core region. It has been suggested that *marT* gene encodes a regulatory protein, since MarT has sequence similarity with the DNA binding domain of the CadC transcriptional activator of *E. coli* K-12 at the rate of 41% and also exhibits homology with another member of the OmpR family (Watson et al., 1992, Blanc-Potard et al., 1999). This suggestion is verified in research performed by Tükel and her colleagues (2007) by detecting that MarT is a positive regulator of MisL autotransporter protein. In this research, the T-POP transposon method is used and the activation of *misL* expression as a consequence of MarT binding to the promoter region of *misL* gene is observed with gel shift analysis (Tükel et al., 2007).

In a study conducted by Dorsey and colleagues (2005), it has been demonstrated that MisL autotransporter protein binds fibronectin and plays a role in intestinal colonization as an extracellular matrix adhesin. Also, it is observed that *misL* expression is increased in mice that are infected by *S. Typhimurium* *in vivo* (Dorsey et al., 2005). In the studies about *in vivo* infection models, it is determined that MisL autotransporter protein does not contribute to lethal infection in mice, however, it is required for the intestinal colonization and persistence of *Salmonella* (Blanc-Potard et al., 1999). In addition to these findings, it is suggested that MisL autotransporter might contribute to the *Salmonella* biofilm formation. In research conducted by Wang and his colleagues (2018), a decrease was observed in the biofilm formation of *S. Typhimurium* *misL* gene mutant strain compared to the wild-type strain. In the same study, the effect of MisL on *Salmonella* adhesion and invasion to host cells was also investigated. According to the bacterial adhesion and invasion assays, mutant strains in terms of *misL* gene displayed significantly reduced adhesion and invasion capacities to HeLa and Caco-2 cells compared to the wild-type strains. In addition to biofilm formation, adhesion, and invasion, it is suggested that MisL can also mediate bacterial aggregation and settling (Wang et al., 2018).

### **ShdA Autotransporter Protein**

ShdA is an autotransporter protein that belongs to the class of monomeric adhesins similar to the MisL protein and is known to be encoded from the type V secretion system. It is encoded by *shdA* gene which is localized at the CS54 pathogenicity island (Wagner and Hensel, 2011). Database studies indicated that the passenger domains of various autotransporter adhesins, such as AIDA of *E. coli*, MisL of *S. Typhimurium*, and VirG which is involved in intracellular motility of *Shigella flexneri*, shared 92% sequence similarity with ShdA protein (Kingsley et al., 2000; Wagner and Hensel, 2011). ShdA has only been detected in *Salmonella enterica* subspecies I, which includes mammalian pathogenic serovars while it was lacking in subspecies II, IIIa, IIIb, IV, and VI, which contains the cold-blooded vertebrates pathogenic serovars and *S. bongori* (Kingsley et al., 2000).

It has been observed that ShdA is localized on the bacterial surface and, in addition, the GST-fusion proteins of the passenger domain of ShdA can bind fibronectin as well as the MisL

protein (Kingsley et al., 2002). In research performed by Kingsley and colleagues (2004), it is found that this interaction between ShdA protein and fibronectin can be inhibited by heparin (Wagner and Hensel, 2011). When investigating the cause of this situation, it was discovered that the binding sites of ShdA and heparin to fibronectin are the same, and this region is known as the Hep-2 domain. Therefore, when heparin binds fibronectin, the region that ShdA uses to bind fibronectin becomes unavailable and the binding of ShdA is inhibited by heparin (Kingsley et al., 2004). In another study conducted by Kingsley et al. (2002), it is observed that ShdA expression is induced in the caecum of mice that are exposed to *S. Typhimurium*. Furthermore, *Salmonella* colonization is reduced in mice that are infected by *S. Typhimurium* mutant strain in terms of ShdA gene. These pieces of evidence demonstrate that the ShdA autotransporter protein participates in the colonization and persistence of *Salmonella* in the murine intestine (Kingsley et al., 2003). On the other hand, a study performed on pigs shows that there is no significant change in colonization and persistence in pigs infected by ShdA mutant strain suggesting that ShdA does not contribute to colonization or persistence of *Salmonella* in infected pigs. This evidence proves that these *Salmonella* adhesins have host-specific activities (Boyen et al., 2006).

### **SadA Autotransporter Protein**

SadA protein, which is another autotransporter produced in *Salmonella*, is secreted through type V secretion system like MisL and ShdA autotransporters. However, this protein is classified as a trimeric autotransporter adhesin (TAA), in contrast to MisL and ShdA autotransporters which are involved in the class of monomeric adhesins. SadA autotransporter protein exhibits similarities with YadA which is a trimeric autotransporter protein found in *Yersinia enterocolitica* (Wagner and Hensel, 2011).

The exact role of SadA protein in *Salmonella* pathogenicity is not yet known. However, based on the findings of the investigations, SadA is considered to have a role in host cell adhesion and invasion. According to the research performed by Raghunathan and colleagues (2011), there was no significant change observed in adhesion to host tissues and invasion of the cells in *sadA* mutant strains of *Salmonella*. However, when SadA protein is expressed in *E. coli* K-12, an increase was detected in *Salmonella* adhesion and invasion to host cells. This evidence suggests that SadA might play a role in *Salmonella* adhesion as well as invasion of the host cells and other factors may compensate when SadA is not functioning properly (Raghunathan et al., 2011).

### **FUTURE PERSPECTIVES**

*Salmonella* is identified as a dangerous infectious pathogen and it leads to serious health problems or even deaths among individuals. Since this pathogen is transmitted by contaminated foods or water, it also causes significant harm to the food industry and hence the economy. In order to cope with these adverse effects of *Salmonella*, it is crucial to have a better understanding of this pathogen and its mechanisms. The studies in this field that are performed up to date have demonstrated that there are numerous mechanisms and elements which *Salmonella* utilizes to infect its host. Many researchers are still conducting research on other potential mechanisms of *Salmonella* and the exact role of proteins on *Salmonella* pathogenicity. When the autotransporter proteins are taken into account, the exact role of ShdA and SadA proteins on *Salmonella* biofilm formation is still unclear. Since biofilm formation is an important feature of *Salmonella* virulence, it is critical to investigate the effects of these proteins on *Salmonella* biofilms. Furthermore, there hasn't been enough study done on the impact of these proteins on *Salmonella* infection in various hosts, such as plants. These studies on this subject might be beneficial to gain a better insight into *Salmonella* infections and to develop



powerful strategies to prevent this dangerous pathogen from causing further damage in various fields.

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## ARTISANAL FRESH CHEESE QUALITY IMPROVEMENT USING DIFFERENT HURDLE TECHNOLOGIES COMBINATION

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### ABSTRACT

The aim of this work was to compare the effect of different hurdle techniques combinations, namely ionizing radiation by electron beam combined with LPS and incorporation of thyme essential oil encapsulated with LPS on the quality of artisanal fresh cheese and during storage at +4°C. Both combinations had a significant impact on studied physico-chemical parameters throughout the shelf life of the cheese. However, no significant effects were observed on the texture. The use of ebeam combined with LPS had a significant effect on the microbiological quality (aerobic mesophilic germs, yeasts and moulds and coliforms) compared to control cheeses and those activated only by LPS throughout the shelf life. The combination of encapsulated thyme essential oil with LPS demonstrated that on the first day the different concentrations (0.5, 1 and 1.5 ml/Kg) had a significant effect on the microbiological quality compared to the controls and LPS, but after nine days it was noted that only 1.5 and 1ml/kg had a significant impact on the microbiological quality of the cheese compared to the other samples. On the other hand, the activation of LPS alone had no significant impact on the evolution of these germs. The determination of the expiry date showed that the use of these combinations allowed an extension of the latter since it was noticed that the control cheese had an expiry date of 3.63 (4 days) days and that of LPS only 3.09 days (3 days). On the other hand, the combination of essential oil of thyme encapsulated with LPS gave different results depending on the concentration since the lowest concentration of 0.5 ml / kg with LPS resulted in 4.19 days, the average concentration of 1 ml /kg with LPS gave 8.01 days and the higher concentration of 1.5 ml/kg with LPS allowed an extension of the consumption date up to 10.40 days. Electron beam irradiation with LPS extended the expiration date of cheese up to 6.82, 10.92, 16.93 days respectively for the low dose (0.5 kGy), medium dose (1kGy) and the highest one (1.5kGy).

**Keywords:** cheese, microbiological quality, essential oil encapsulation, electron accelerator, quality, shelf life.

### INTRODUCTION

Cheese is an ancient food product that can be prepared from different types of milk. The consumption of cheese has increased, in consequence, cheese industry has now evolved into a global business where research has an important role on the increase of shelf-life and promotion of cheese products quality and safety (Costaa *et al.*, 2018). Activation of the LP system is amongst the most cost effective approaches to extend the stability of pasteurised and raw milk (Ndambi *et al.* 2008). Other alternative processes are therefore needed to solve this problem, mainly the use of combination technology (Peng *et al.*, 2015). At the crossroads of important economic, environmental and public health issues, food preservation techniques deserve today

a mobilization of all actors to make technologies evolve towards solutions more respectful of both the environment and the consumer (Gontard et al., 2017). This makes the application of barrier technology very interesting since it can extend the shelf life while preserving the nutritional and sensory value (Predrag Putnik et al., 2020). Indeed the combination technique consists in the preservation of food by several agents/methods applied simultaneously or sequentially (Peleg et al., 2020). Non-thermal processes are implemented aiming at inactivating spoilage microorganisms and improving the nutritional, sensory and microbial characteristics of the product; a good opportunity that arises from the use of irradiation during cheese processing (Huo et al., 2013). Food irradiation has been identified as a safe technology on the food process and used to disinfect and preserve food, including extending shelf life (Huo et al., 2013). Encapsulation is one of the commonly used techniques. It allows immobilizing the volatile compounds of essential oils, stabilizing the latter and protecting it from light, oxygen and temperature as well as modulating its release by prolonging its kinetic profile (Kerdudo et al., 2015). Therefore, this process tends to protect and preserve the biological activities of these oils. To this end, the general problem of this research topic concerns the study of the effect of the addition of encapsulated essential oils to the activation of LPS and compared with the irradiation by electron beams with an activation of this enzyme (LPS).

## **MATERIAL AND METHODS**

### **Cheese manufacture**

The cheese was produced from raw cow's milk (Barukcic et al., 2020). The sample was subjected to refrigeration combined with activation of the LP system by addition of sodium thiocyanate (NaSCN) as a thiocyanate (SCN) source at a final concentration of 14 mg/L (Boulares et al., 2011). Encapsulation is a potentially beneficial procedure for the protection and proper preservation of essential oils against degradation processes (oxidation and hydrolysis), as well as for stabilizing the release of high-value compounds extracted from fruits, vegetables, and waste products (Didi et al., 2021). The sodium alginate gel is poured very gradually to form the essential oil bead, and each bead remains in the calcium chloride solution for about 30min (Didi et al., 2021). The cheeses were irradiated in the National Center for Nuclear Science and Technology (CNSTN) of Sidi Thabet of Tunisia using a gas pedal 10 MeV electron beam at doses of 0.5; 1; 1.5 kGy respectively (Huo et al., 2013).

### **Physicochemical analysis**

The determination of pH, total dry extract, fat, moisture and acidity titration has been carried out according to the method described by Fedala et al. (2020). Hardness (N), of cheese was determined by texturometer analyzer type TVT 6700 (Barukcic et al., 2020). Once the production is finished, a hedonic test is carried out for the two cheeses. The purpose of this test is to compare the overall hedonic appreciation of the different cheeses by focusing on the individual feelings related to the pleasure or displeasure caused by the food (Fedala et al., 2020).

### **Microbiological analysis**

The numbers of total coliforms, total aerobic mesophilic flora, yeasts and molds were monitored on consecutive days during storage (Fedala et al., 2020).

### **The determination of shelf life**

The general equation that describes the loss of quality of a food is applicable for any factor A is as follows:

$$r = d [A] / dt = K [A]^n \quad (1)$$

r : rate of the degradation reaction = rate of formation of A ; 0

K : reaction rate constant or apparent rate ;

A : concentration of the factor to be followed;

n : order of the degradation reaction.

### **Statistical analysis**

All experiments were repeated at least three times. The results were subjected to a one-factor analysis of variance with a significance level of 95% using Excel for the radar presented for the sensory analysis and by SPSS software using the one-factor ANOVA test.

## **RESULTS AND DISCUSSION**

### **Effect of hurdle technologies on the evolution of the microbiological quality of cheese**

We recorded a slight non-significant decrease of the microflora in the LPS activated cheese compared to the untreated control cheese for all flora. The application of e-beam and LPS activation allowed to decrease the concentration of total aerobic mesophilic flora, coliforms and yeast and mold. We obtained a total absence for the LPS combination with the highest ionization dose of 1.5kGy. These same evolutions were recorded with the LPS/HET combination with an almost total absence of germs for the 1.5 mg HET/kg dose (figure 1).

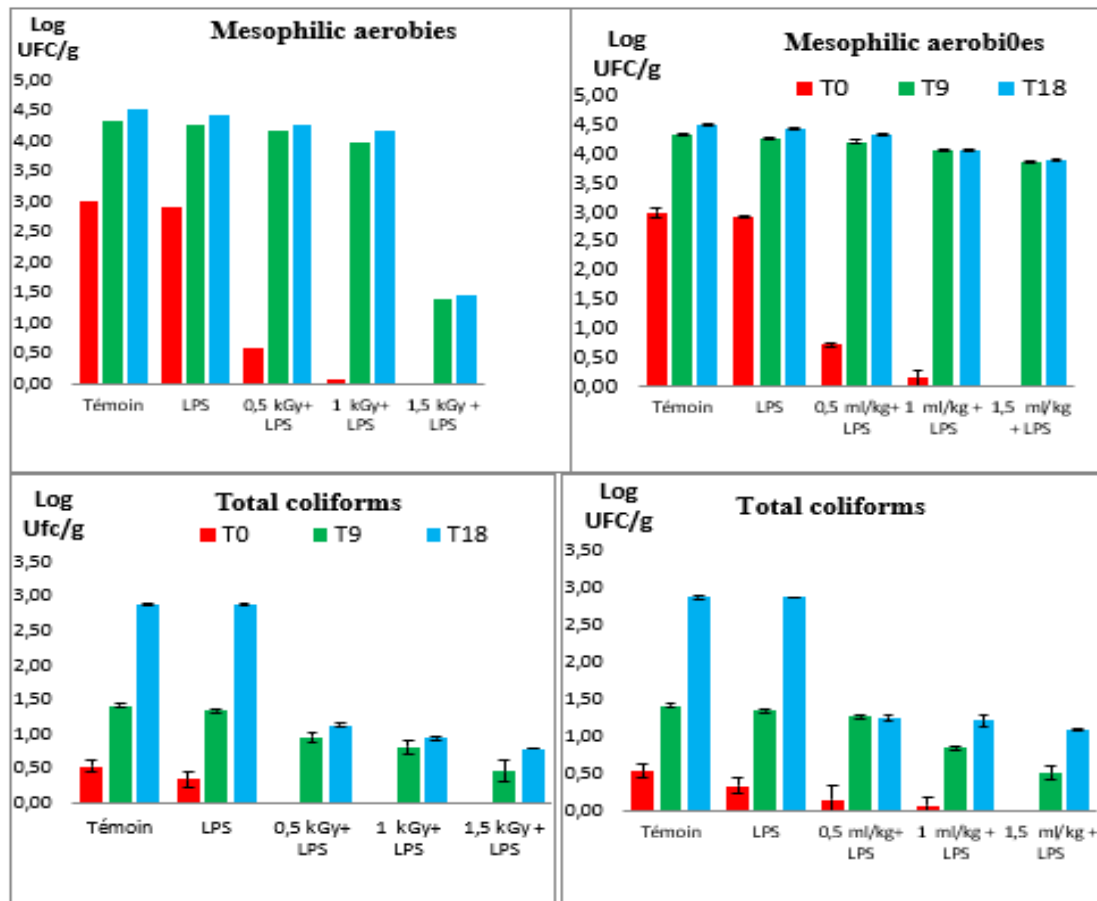


Figure 1: Effect of hurdle technology on the microflora of fresh cheese (Témoin; LPS; E-beam: 0.5kGy +LPS; 1 kGy+LPS; 1.5kGy+LPS; Het: 0.5ml/kg +LPS; 1 ml/kg +LPS; 1.5ml/kg+LPS)

### Effect of hurdle technologies on the evolution of the physicochemical characteristics of cheese

No difference ( $P > 0.05$ ) in pH was observed between the control and the LPS-activated cheese. The pH of the control and LPS-activated cheeses decreased significantly during the +4°C storage. This significant decrease can be attributed to the microbial flora and in particular the lactic bacteria responsible for the acidification of the cheese. Synergistic effect of the combination E-beam/LPS and HET/LPS on the inhibition of flora and in particular FML allowing a stability of the pH during its conservation. A significant increase in acidity in control and LPS-activated cheeses during storage. Stabilization of acidity in cheeses irradiated /by E-beam and HET combined with LPS during their conservation. Synergistic effect of E-beam/LPS and HET/LPS combination (Figure 2).

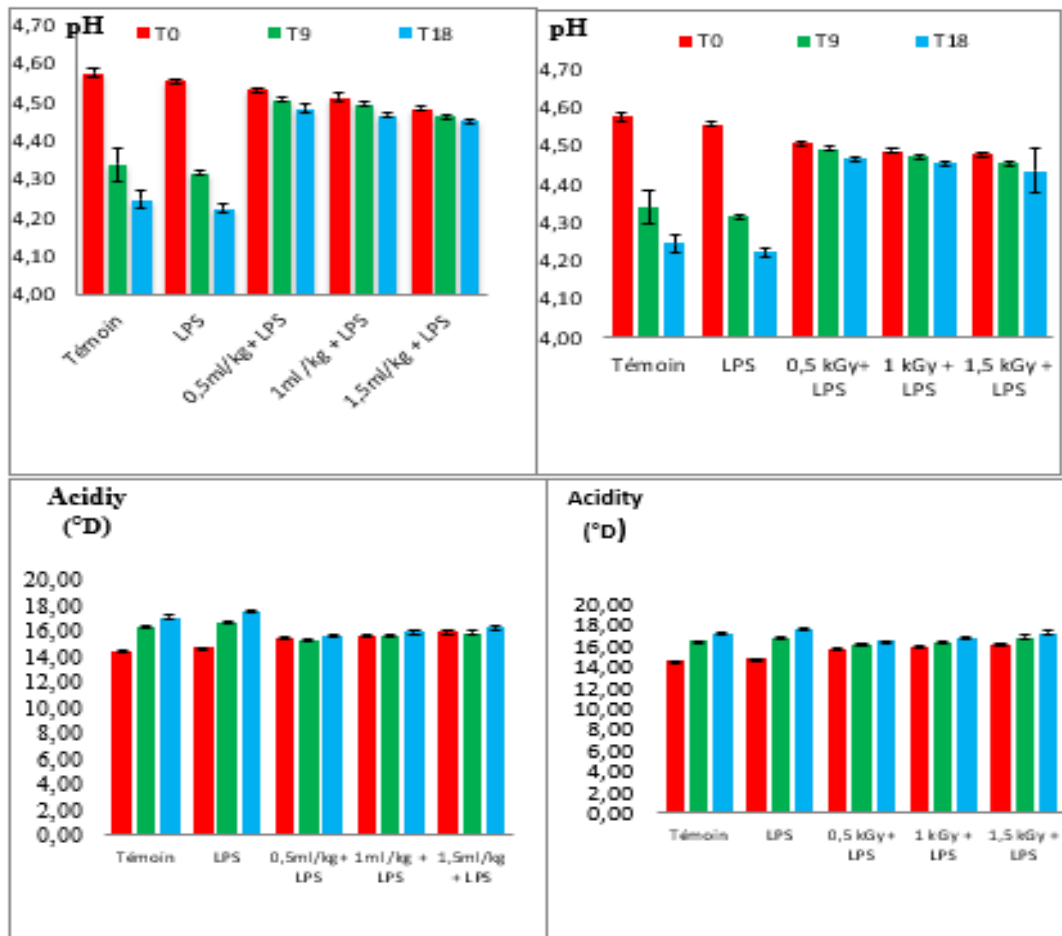


Figure 2: Effect of hurdle technology on the pH and the titratable acidity of fresh cheese (Témoin; LPS; E-beam: 0.5kGy +LPS; 1 kGy+LPS; 1.5kGy+LPS; Het (encapsulated thyme essential oil): 0.5ml/kg +LPS; 1 ml/kg +LPS; 1.5ml/kg+LPS)

The total dry extract of the control and LPS-activated cheeses decreased significantly during the +4°C storage (figure 3). A significant increase of the total dry extract was observed with the LPS ebeam/ or HEt combination compared to the control and LPS activated cheeses. Synergistic effect of the E-beam/LPS and HEt/LPS combination on the increase and stability of total dry extract during storage. Significant decrease of moisture in cheeses with the highest ionization dose combined with LPS compared to all other samples. The increase in HEt concentration combined with LPS, resulted in a significant decrease in the water content of the cheese compared to the control and LPS samples throughout the storage period. The three doses of ionization applied in combination with LPS do not have a significant effect on cheese firmness at t<sub>0</sub>, t<sub>9</sub> and t<sub>18</sub>.

The incorporation of the essential oil with LPS has no significant variation in firmness compared to the controls and those activated by LPS (figure 4). The average firmness value of the cheeses decreased directly with time. No significant effects on the sensory parameters of the cheeses preserved by Hurdles Technologies compared to the control cheeses.

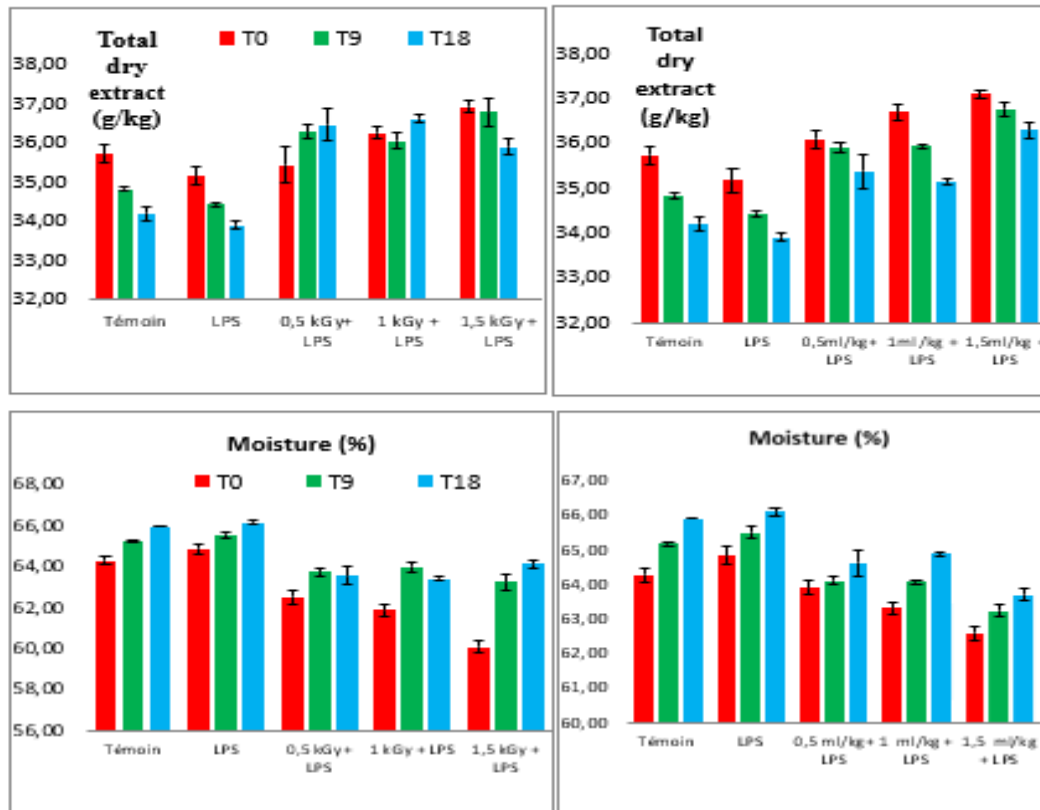


Figure3: Effect of hurdle technology on the total dry extract of fresh cheese and the moisture of fresh cheese (Témoin; LPS; E-beam: 0.5kGy +LPS; 1 kGy+LPS; 1.5kGy+LPS; Het (encapsulated thyme essential oil: 0.5ml/kg +LPS; 1 ml/kg +LPS; 1.5ml/kg+LPS).

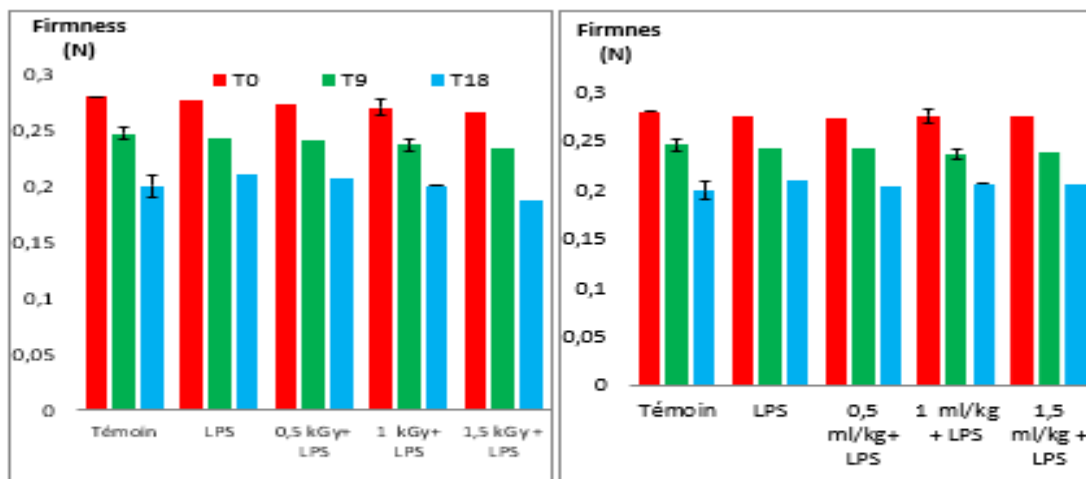


Figure4: Effect of hurdle technology on the firmness of fresh cheese (Témoin; LPS; E-beam: 0.5kGy +LPS; 1 kGy+LPS; 1.5kGy+LPS; Het (encapsulated thyme essential oil: 0.5ml/kg +LPS; 1 ml/kg +LPS; 1.5ml/kg+LPS).

### Effect of hurdle technologies on the evolution of the the shelf life of cheese

The spoilage kinetics of a foodstuff is a representation of the deterioration of a parameter A as a function of time and temperature. This kinetics are generally of order zero or of order one. The determination of the order of the spoilage reaction is obtained by comparing the



coefficient of determination  $R^2$  of the linear regression of the three kinetic models related to a quality criterion A by drawing the graphs:

Order zero:  $A = f(t)$

Order 1:  $\ln(A) = f(t)$

Order 2:  $(1/H) = f(t)$

Determination of the use-by date by monitoring the evolution of yeasts and molds, indeed the microbial limit of acceptability was estimated by fitting the experimental data to the Gompertz equation modified by Corbo (Zantar *et al.*, 2013)

A concentration  $\geq 10^5$  CFU / g of yeasts and molds marks the end of the useful life of fresh cheese. This level of contamination corresponds to the appearance of defects, abnormal colors and odors (Zantar *et al.*, 2013). We noticed that both combinations resulted in an extension of the shelf life of fresh cheese compared to control and LPS-activated cheeses only. In contrast, to Zantar *et al.* (2013) who showed that the addition of bulk thyme (0.5 or 1 ml/kg) to fresh goat cheese did not show a significant effect on the extension of the shelf life of fresh cheese. On the other hand, we recorded an increase in the use-by date of 4.19; 8.01 and 10.40 days for the different concentrations of encapsulated thyme respectively 0.5;1 and 1.5 ml/kg with LPS. This highlighted the importance of thyme essential oil encapsulation and the synergistic effect with the activation of the LPS system of the raw milk of manufacture.

On the other hand, we noticed that the combination of ebeam with LPS allowed a more important extension compared to the control cheeses, activated only with LPS and also combined with the essential oil. This shows that the irradiation has an important bacteriostatic or bacteriocidal action. It also confirms the synergic effect between ebeam and LPS, since we registered an increase of the shelf life to 6,82, 10,92 and 16,93 days respectively for 0,5, 1 and 1,5 kGy combined with the activation of the lactoperoxidase system (figure 5).

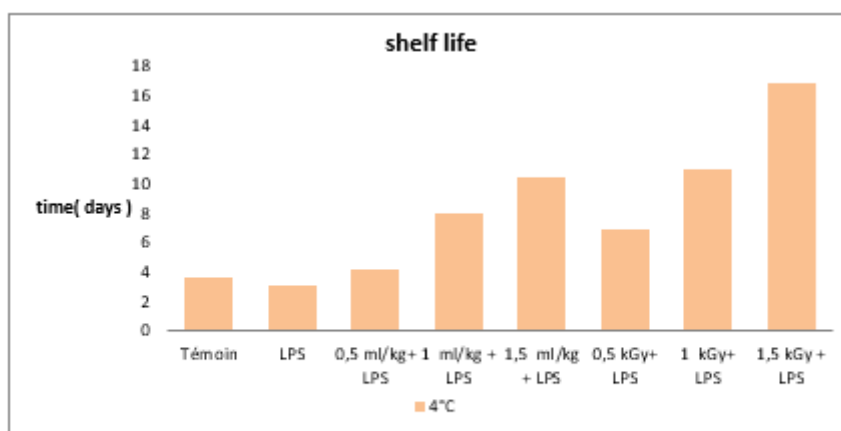


Figure5: Effect of hurdle technology on the shelf life of fresh cheese (Témoin; LPS; E-beam: 0.5kGy +LPS; 1 kGy+LPS; 1.5kGy+LPS; Het (encapsulated thyme essential oil: 0.5ml/kg +LPS; 1 ml/kg +LPS; 1.5ml/kg+LPS.

## CONCLUSION

The two storage combinations did not have significant effects on texture throughout the duration of refrigerated storage. The study of the microbiological quality of the cheese showed that the hurdle treatment had a significant effect on the microbiological quality of the refrigerated fresh cheese. Both combinations had a significant positive impact on all physicochemical parameters and allowed an extension of the shelf life of fresh cheese E-beam irradiation (<2 kGy) combined with LPS activation seems to be an optimal process to guarantee the safety and quality of refrigerated fresh cheese.

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## EFFECT OF LACTOPEROXIDASE SYSTEM ACTIVATION AND HEAT TREATMENTS OF REFRIGERATED COW'S MILK ON THE QUALITY OF UNCOOKED PRESSED CHEESE SAINT-PAULIN TYPE DURING RIPENING

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### ABSTRACT

Saint-Paulin cheese was made from cow's milk refrigerated at 4°C for 72h and preserved by the combined effect of the lactoperoxidase (LPA) system and different heat treatments (55 °C/15s and 72°C/15s). The effect of combination treatment on the physicochemical, microbiological and biochemical properties of cheese over a ripening period of 23 days was investigated, using a control (C0), refrigerated LP-inactivation cow's milk (C1) and refrigerated LP-activated cow's milk (LPA). The LPA treatment showed the least contamination in flora count and the sample cheese made from milk refrigerated at 4 °C/72h, activated by LP and heat-treated at 55 °C for 15s (P<sub>55a</sub>) is comparable to the sample cheese made from milk refrigerated at 4 °C/72h and heat treated at 72 °C for 15s (P<sub>72</sub>). LP cheese had significantly lower coliform, yeast and mould counts (P <0.05) than the other cheeses; this confirmed the bacteriostatic effect of the LP system. Lipolysis did not reveal significant dissimilarities between samples. By contrast, the activation of LP caused a 20% decrease in the index of proteolysis of cheeses for the same heat treatment, with indices close enough for the cheeses P<sub>72</sub> and P<sub>55a</sub>. These results were confirmed by the azocasein method measuring the change in optical density of soluble nitrogen and non-protein nitrogen, during the ripening period of the four cheese samples studied. LP activation remedied to lower cheese yield. A gain of approximately 2,41 kg in the cheese produced using milk treated at 55 °C/15s after LP activation was perceived, against 1Kg of the amount of cheese per 100 L of milk with the activation of LPA cheese treated at 72°C/15s. Finally, sensory evaluation showed a preference of the cheeses P<sub>72</sub> and P<sub>55a</sub>.

**Keywords:** Refrigerated milk, Semi-hard uncooked cheese, Lactoperoxidase system, Antibacterial, Proteolysis, Lipolysis, Sensory analysis.

### INTRODUCTION

Cheese is a complex ecosystem that is in continuous flux in terms of both external factors, such as cheese making techniques and ripening conditions, and intrinsic factors, such as physicochemical composition and interactions amongst the different microbial communities (Da Costa et al., 2018). The wide range of biochemical reactions and microbial interactions that take place during cheese ripening is the basis of desirable product characteristics such as good taste and aroma, spoilage prevention, inhibition of food-borne pathogens and, more recently, modulation of health (Desmasures et Irlinger, 2018).

During milk storage by refrigeration, psychrotrophs can proliferate, these bacteria hydrolyse milk proteins and lipids through secretion of a variety of heat stable extracellular proteases and lipases (Mankai et al.2003), which cause different defects in dairy products as various off-flavours (Mankai et al., 2012), abnormal texture and reduced cheese yields (Boulares et al., 2011). The use of such cooled milk for producing cheese results in greater losses of fat and curd

finds into the whey, lower cheese yields, and difficulties in the way of draining (Aydemir, 2018).). For this reason, alternative solutions to inhibit psychrotrophs have been considered e.g. use of lactic acid bacteria (LAB), activation of the lactoperoxidase (LP) system that needs the presence of two factors, hydrogen peroxide and thiocyanate to develop its antimicrobial function (Ay & Boston, 2017). Lactoperoxidase is one of the most heat stable enzymes in milk. Its destruction has been used as an index of pasteurisation efficiency of milk. LP retains its activity during normal pasteurization of cow milk (63 °C for 30 min or 72 °C for 15 s) but - destroyed at 80 °C in 4 s (Seifu et al. 2004, Ben moussa et al., 2013, Zhu et al., 2020). The objective of the experiment was, therefore, to compare the effect of activating the LP system in cow's milk at different heat treatment 72°C/15 s and 55°C/15s on the microbiological, physicochemical and biochemical properties of Saint-Paulin cheese over a ripening period of 23 days.

## **MATERIAL AND METHODS**

### **Cheese manufacture**

Experimental design Saint-Paulin cheese making trials were undertaken in duplicate with four treatments: LP-inactivated cow's milk cheese refrigerated at 4 °C for 72 h and heated at 55 °C for 15s (P55) and LP-activated and refrigerated at 4 °C for 72 h and heated at 55 °C for 15s (P55a), and LP-inactivated milk refrigerated at 4 °C for 72 h and heated at 75 °C for 15s (P72) and LP-activated and refrigerated at 4 °C for 72 h and heated at 75 °C for 15s (P72a). The two first samples received a refrigerated at 4 °C for 72 h before transformation to cheese. The two other sample was subjected to the refrigeration combined with the activation of the LP system by addition of sodium thiocyanate (NaSCN) as a source of thiocyanate (SCN)) to a final concentration of 14 mg/L. After 1 min of thorough mixing of the milk, 30 mg /L of sodium percarbonate (2Na<sub>2</sub>CO<sub>3</sub>·3H<sub>2</sub>O) was added as a source of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) as recommended by the International Dairy Federation (CAC, 1991). General cheese making procedure Saint-Paulin cheeses were manufactured from cow's milk at semi-industrial plant, using the method described by Mankai et al. (2012). After manufacturing, cheeses were ripened in a warm room at 13 °C and 95% relative humidity for 23 days.

### **Physicochemical analyses**

Cheese yield was the mathematical expression of the quantity of cheese obtained from 100 L (or 100 kg) of milk; it was measured by weighing the cheese blocks on an analytical balance and was expressed as kg dry matter per 100 litres of milk (Mankai et al., 20012). Total nitrogen (TN), water-soluble nitrogen (WSN) were determined by Kjeldahl method (Dimitrellou et al. 2010; Moatsou et al. 2002) at 0, 2, 9, 14, 19 and 23 days of ripening. The ripening coefficient (or proteolysis level) =  $100 \cdot [\text{WSN (g/100 g)}/\text{TN (g/100 g)}]$  as described by others (Mankai et al., 2003; EL-Ahwal et al., 2018).

### **Microbiological analyses**

Coliforms were enumerated using desoxycholate gelose 1% and incubated at 30 °C for 24 h. For psychrotrophs germs Standard Milk Agar (SMA, Oxoid) were used, and plates were incubated at 7 °C for 10 days (Mankai et al., 2005).

## **RESULTS AND DISCUSSION**

### **Effect of the lactoperoxidase system and heat treatment on the microflora of cheese**

Large numbers of psychrotrophics germs and coliforms in cheese milk were observed (6.86 and 5.41 log cfu/mL, respectively) resulting in many defects throughout the body of the cheese. From the current study, activation of the LP system in cow's milk had a significant effect ( $P < 0.05$ ) on the growth of APC and coliforms.

For total psychrotrophs germs, the counts decreased in all cheese samples and the highest regression was observed in the LPA cheese. Therefore, this study showed that the activation of the LP system combined with the pasteurisation of milk used for producing cheeses, had a bacteriostatic effect against psychrotrophic flora, for all the investigated cheeses (Mankai et al., 2003).

### **Effect of the lactoperoxidase system on the yield and biochemical composition of cheese**

It should be noted that the period of ripening seems to be an important factor affecting the indices of proteolysis of cheese. During ripening and until the end of ripening, the proteolysis index increased significantly ( $p < 0.05$ ) in the four studied samples and in particular for P<sub>55</sub> cheese. Significant increase rates of about 19.2%; 19.89%; 32.88% and 23.05% for P<sub>72</sub>; P<sub>72a</sub>; P<sub>55</sub> and P<sub>55a</sub> sample productions were noted, respectively. It is important to note that this index of proteolysis is the lowest for cheese from the production P<sub>72a</sub> (16.22%), and the highest for cheese from the production P<sub>55</sub> (26, 86%), the samples from productions P<sub>72</sub> and P<sub>55a</sub> are comparable although the difference was significant with respective averages of 20.97 and 23.04% on the five samples considered during the ripening. The proteolysis index of P<sub>55a</sub> cheese is slightly higher than that recorded for cheese refrigerated for 72h, nevertheless a clear improvement is noted compared to the P<sub>55</sub> sample (Table 1). As a result, the activation of the lactoperoxidase system in the manufacturing milk seems to have a considerable effect on the proteolysis of cheeses, this is due to its effect against the psychrotrophic flora of the milk and thus on the production of extracellular proteases likely to degrade the caseins of milk.

The Azocasein method measures the absorbance of the enzymatic activity of proteases found in the medium degrading an artificial substrate which is azocasein. These results show that the internal composition of protease enzymes in soluble nitrogen varies, but does not change significantly, depending on the stage of ripening and the type of treatment: activation of the LPS system and heat treatment. The smallest increase in proteolytic activity is recorded in the cheese sample in which the lactoperoxidase system has been activated. Indeed, at the beginning of the ripening process, the curd presents an optical density of about 0.491; 0.312; 0.534 and 0.46 with low standard deviations for the cheeses P<sub>72</sub>, P<sub>72a</sub>, P<sub>55</sub> and P<sub>55a</sub>, respectively. At the end of ripening, proteolytic activity increases non-significantly ( $p < 0.05$ ) for all four cheese samples while remaining higher for the cheese sample from P<sub>55</sub> production (Table 2).

**Table 1: Evolution of nitrogen fractions during ripening**

| Nitrogen forms (g/100g) | Samples          | ripening (Days) |             |             |           |            |
|-------------------------|------------------|-----------------|-------------|-------------|-----------|------------|
|                         |                  | 0               | 2           | 9           | 16        | 23         |
| NT                      | P <sub>72</sub>  | 2,15±0,21       | 2,31 ± 0,19 | 2,54 ± 0,06 | 2,7± 0,28 | 3,43± 0,04 |
|                         | P <sub>72a</sub> | 2,46± 0,014     | 2,56±0,08   | 3,04±0,28   | 3,13±0,61 | 3,66±0,14  |
|                         | P <sub>55</sub>  | 2,53±0,04       | 2,58±0,05   | 2,67±0,13   | 2,7±0,09  | 2,82±0,18  |
|                         | P <sub>55a</sub> | 2,59±0,09       | 2,8±0       | 3,1±0,07    | 3,3±0,23  | 3,4±0,11   |
| NS                      | P <sub>72</sub>  | 0,39±0,01       | 0,49±0,14   | 0,51±0,18   | 0,62±0,04 | 0,77±0     |
|                         | P <sub>72a</sub> | 0,35±0,21       | 0,42±0,28   | 0,48±0,01   | 0,53±0,17 | 0,65±0,13  |
|                         | P <sub>55</sub>  | 0,56±0,1        | 0,64±0,07   | 0,68±0,03   | 0,78±0,16 | 0,93±0,01  |
|                         | P <sub>55a</sub> | 0,51±0,04       | 0,63±0,11   | 0,69±0,03   | 0,83±0,14 | 0,87±0,06  |

- NT = total nitrogen, NS= soluble nitrogen, NNP=non-proteinic nitrogen, Significance level<5%.

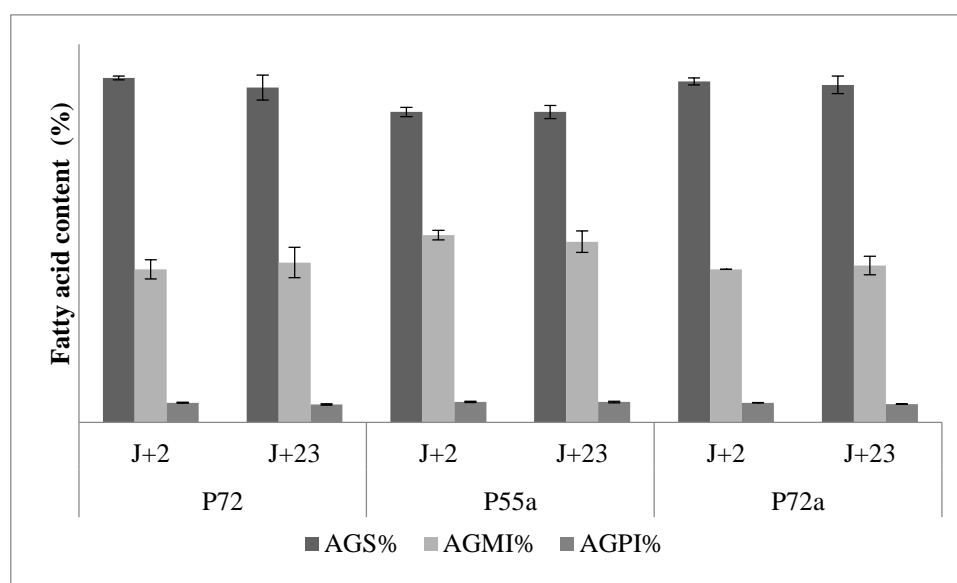
**Table 2: Evolution of proteolytic activity in soluble nitrogen during ripening**

| DO NS at 345nm   | Maturing stage (Days) |            |            |
|------------------|-----------------------|------------|------------|
|                  | 0                     | 9          | 23         |
| P <sub>72</sub>  | 0,491±0,005           | 0,525±0,02 | 0,51±0,035 |
| P <sub>72a</sub> | 0,312±0,003           | 0,462±0,01 | 0,453±0,02 |
| P <sub>55</sub>  | 0,534±0               | 0,613±0,04 | 0,582±0,01 |
| P <sub>55a</sub> | 0,46±0,001            | 0,554±0    | 0,573±0,01 |

- NS= soluble nitrogen; Significance level<5%.

•

Nine fatty acids were identified in the total lipids of cheese samples P<sub>72</sub>, P<sub>72a</sub> and P<sub>55a</sub> at the beginning (d+2) and at the end (d+23) of ripening. Saturated fatty acids constitute the most important proportion of the total fatty acids of the three cheeses studied, both at the beginning and at the end of the maturation process. They are represented by myristic acid (C14:0), pentadecanoic acid (C15:0), palmitic acid (C16:0) and stearic acid (C18:0), followed by monounsaturated fatty acids, which are mostly oleic acid (C18:1) and palmitoleic acid (C16:1) in smaller proportions. Polyunsaturated fatty acids represent the lowest proportion (about 2.5% of total fatty acids) for the three cheeses studied. These are essentially the fatty acids of the w3 and w6 series. These are the fatty acids (C18:2 w6), (C18:3 w3) and (C18:4 w3). The proportion of saturated fatty acids is similar for both samples P<sub>72</sub> and P<sub>72a</sub>, while the cheese sample P<sub>55a</sub>, contains an average of 57.7% SFA during ripening. Regarding MUFA, the cheese sample P<sub>55a</sub> contains the highest content: an average of 34.072%, against 28.348% for the lot P<sub>72</sub> and 28.903% for P<sub>72a</sub>. The difference is mainly in the C18:1w9 fatty acid, which is present in higher proportions in the P<sub>55a</sub> sample. Many authors have reported that activation of the LP system in cow's milk inhibited lipoprotein lipase activity and reduced free fatty acid levels in milk. Thus, less lipolysis is observed in LPS-activated milk-based Gouda cheeses.



**Figure 1 : Evolution of fatty acid content (%) during ripening**

Examination of the data on the evolution of cheese yield as a function of the activation of the lactoperoxidase system and the heat treatments (Table 3), shows a gain of about 1 kg of cheese per 100 liters of milk, with the activation of LPS in cheeses treated at 72°C/15s. A much higher gain was observed in cheeses made from milk treated at 55°C/15s after LP activation is 2.41 Kg. It can be concluded that the activation of the LPS system, which has an inhibitory power on the proliferation of psychrotrophs responsible for the degradation of caseins through the action of their proteases, improved the cheese yield. On the other hand, the increase in yield was more marked at 55°C, which is in agreement with the results found in the previous sections of the increases in dry extract and the proportion of soluble nitrogen preponderant. The samples P<sub>55a</sub> and P<sub>72</sub>, which showed clear similarities in the proteolysis index and the proportion of soluble nitrogen, as well as in the evolution of the dry extract during the ripening period, have the same cheese yields.

**Table 3 : Variation in cheese yield during ripening**

|                         | Cheese samples  |                  |                 |                  |
|-------------------------|-----------------|------------------|-----------------|------------------|
|                         | P <sub>72</sub> | P <sub>72a</sub> | P <sub>55</sub> | P <sub>55a</sub> |
| <b>Cheese yield (%)</b> | 9,53            | 10,61            | 6,82            | 9,23             |

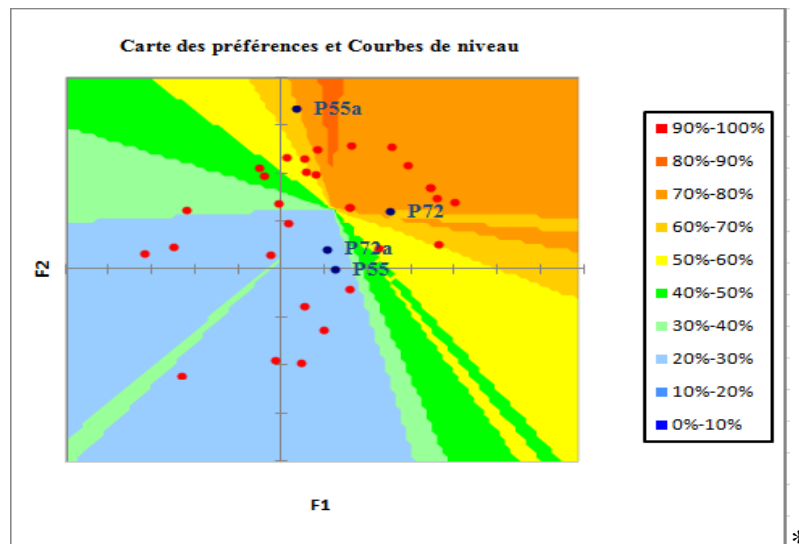
Finally, it would be interesting to relate the tasters' preferences to the sensory characteristics of the cheeses and to visualize their relationships. To do this, we used preference mapping superimposed on a level map. Indeed, Figure 2 below reflects the mathematical correlations between the variables. The proximity on the graph of a taster point (the red points in the figure) with a cheese point (the blue points in the figure) indicates that this taster appreciated this cheese. The analysis of the density of the "taster" points on the map shows that there are three main groups. A first group located around the cheeses P<sub>72</sub> and P<sub>55a</sub> representing about 57% of the panel (red dots) appreciates these two cheeses at a level >50% (light and dark orange parts of the level curve). A second group, clearly in the minority with a minimum of judges, in fact 13% of the panel (in the extreme left part of the figure), with an appreciation level of the four cheeses estimated between 20 and 30% (light blue part of the level curve). The

last group, closer to the cheeses P<sub>72a</sub> and P<sub>55</sub>, formed by 30% of the panel, have an appreciation level between 20 and 50% (figure 2).

In conclusion, according to the results of this part of the sensory evaluation, there seems to be a consensus on the appreciation of the cheeses P<sub>72</sub> and P<sub>55a</sub>, which can be largely attributed to the degrees of proteolysis of these cheeses which are higher compared to P<sub>72a</sub> and lower compared to P<sub>55</sub>. In addition, lipolysis, even if it does not occur to a significant extent, participates in the formation of the flavors and organoleptic characteristics particularly recognized in these cheeses.

## CONCLUSION

We were able to highlight during this study, the synergistic effect of the heat treatment "low" (55 ° C / 15s) with a "low" cost with the activation of the lactoperoxidase system giving comparable results in terms of index of proteolysis, lypolysis cheese yield and overall appreciation of the taster similar to those found for an ordinary pasteurization treatment (72 ° C / 15s). Hence the interest of this combination on the industrial level and in terms of sustainable development.



**Figure 2: Preference mapping and contour lines. Plan determined by principal components 1 and 2**

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## RELATIONSHIP BETWEEN sRNA AND BIOFILM FORMATION

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### ABSTRACT

Biofilm is described as a multicellular microorganism community which binds the surfaces such as substrate, interfaces and each other with an extracellular matrix produced by themselves. Biofilms are regulated by various factors such as genetic elements, signal molecules and environmental factors. One of these genetic factors is the small RNAs (sRNAs). sRNAs play an important role in accelerating the biofilm formation process. Bacteria use a range of RNA regulators which are named as sRNAs that aid to respond to the environmental alterations. This review gives an overview on the association between sRNA and biofilm formation and regulation along with its importance for the bacterial virulence.

**Keywords:** sRNA, Biofilm, Virulence, *Salmonella*, *Escherichia coli*

### INTRODUCTION

Small RNAs (sRNAs), which typically have a size of 18–30 nucleotides, are noncoding RNAs that are involved in a variety of biological processes. sRNAs are divided into two main classes: siRNA (small interfering RNA), which has a double-stranded RNA predecessor, and hpRNA (hairpin RNA), which has a single-stranded RNA predecessor (Yao et al., 2020). In both gram-negative and gram-positive bacteria, sRNAs have become a significant, distinct class of virulence factors (Diallo and Provost, 2020). sRNAs transcribed from free-standing sRNA genes located in intergenic regions (IGRs) (Chao et al., 2012). Contrary to mRNA, sRNAs are transcribed from DNA. Untranslated sRNAs often control translation by interacting with mRNA (Fu et al., 2019). sRNAs are a significant class of post-transcriptional regulators that regulate the expression of genes involved in a variety of processes, including membrane homeostasis, carbon metabolism, and quorum sensing. They have both activating and inhibiting activity through interactions with proteins or mRNA. While some sRNAs move by matching with target-mRNAs, others carry out their regulatory function by titrating a protein and thereby changing its availability. Basepairing is dependent on short and flawed duplexes when sRNAs target mRNAs transcribed at a separate locus. A conserved bacterial RNA-binding protein is called Hfq. These sRNAs are required for the RNA chaperone Hfq to function in *Salmonella* and *Escherichia coli*. This protein plays a complicated role in post-transcriptional gene regulation (Vogel and Luisi, 2011; Mandin and Guillier, 2013).

More than one sRNA may regulate a given mRNA, and a given sRNA may have multiple mRNA targets, including those that encode regulatory genes (Van Puyvelde et al., 2013). Short bacterial npcRNAs are frequently referred to as small RNAs (sRNAs). sRNAs that regulate the activity of proteins, cis- and trans-acting sRNAs, and CRISPR sRNA can all be classified according to the regulation mechanism (Ghaz-Jahanian et al., 2013). The term "cis-encoded" refers to sRNAs that are synthesized from the DNA sequence immediately opposite to mRNA targets and have strong complementarity to these targets. On the other hand, trans-

acting sRNAs are found elsewhere in the genome and have limited complementarity (Ghaz-Jahanian et al., 2013; Ryan et al., 2017). sRNAs often regulate the genes that have critical roles in response to particular environmental stress conditions. When stressful changes in the environment of the cell occur, sRNAs frequently serve as environmental signal transmitters. These RNAs modulate alterations in cellular metabolism caused by factors such as cell density, iron deprivation, oxygen tension, temperature fluctuation and carbon source stress by down-regulating gene expression. As a result, the usage of available nutrients is optimized, improving the likelihood that will survive (Ghaz-Jahanian et al., 2013; Bak et al., 2015)

#### Base-pairing of sRNA with the target mRNA

In response to environmental stress conditions, sRNAs employ a variety of ways to balance the expression of their target genes. Base-pairing-based sRNAs frequently change the expression of their target genes at the post-transcriptional stage. The location of sRNAs in the bacterial genome varies depending on their targets. Bacterial sRNAs are a significant class of post-transcriptional regulators. They can be further classified into the *cis*-encoded sRNA and *trans*-encoded sRNA groups (Ghaz-Jahannian et al., 2013; Dutta and Srivastava, 2018; Brantl and Müller, 2021).

#### a. *cis*-encoded sRNAs and *trans*-encoded sRNAs

Most sRNAs alternate mRNA translation via base pairing with mRNAs. These sRNAs can be grouped into two main groups: those with high complementarity and higher complementarity with their target mRNAs, according to their base complementation properties (Waterz and Storz, 2009). *Cis*-encoded sRNAs on bacterial chromosomes are extensively complementary to their targets and are produced from the DNA strand across another gene. Beyond all of these, *trans*-encoded sRNAs are also located far from the mRNAs they regulate in the genome. These RNAs only have a little amount of complementarity to their targets because of their placement (Thomason and Storz, 2010). In addition, *trans*-encoded sRNAs are also called as small regulatory RNAs (sRNAs), while *cis*-encoded sRNAs are also called as antisense RNAs (asRNAs) (Zhao et al., 2018). *Trans*-encoded sRNAs usually have short structure, encoded in intergenic regions. These sRNAs affect protein expression by binding to their target mRNAs through base pairing or modulate their activity by binding to regulatory proteins (Gripenland et al., 2010). Due to the restricted complementarity between the target mRNA and sRNA, RNA chaperone Hfq is required to facilitate RNA-RNA interactions (Waters and Storz, 2009).

#### b. sRNA chaperone Hfq

Hfq affects sRNA levels, which helps to regulate sRNA. Without base-pairing with mRNAs, Hfq, shields sRNA from degradation. Many well-known sRNA-mRNA pairings are consequently subject to RNase E destruction after base-pairing with target mRNAs. Furthermore, one of the elements regulating sRNA activity *in vivo* may be competition between sRNAs for binding to Hfq (Waters and Storz, 2009). RNase E, which breaks down single-stranded RNA, is a part of the multiprotein complex known as the degradosome, which impacts mRNA stability globally and is known to interact with Hfq (Thomason and Storz, 2010). In as mentioned earlier, sRNAs are classified in two major groups as hpRNA and siRNA. miRNA (microRNA) is a subclass of hpRNAs (Yao et al., 2020). The differing features of the *trans*-encoded and *cis*-encoded base pairing sRNAs may lead to mechanistic discrepancies, just like with eukaryotic miRNAs and siRNAs. Hfq frequently interacts with *trans*-encoded sRNAs, which, like miRNAs, have poor base pairing with their targets. Contrarily, *cis*-encoded sRNAs

have extensive complementarity with targets like siRNAs; hfq may not seem necessary for this sort of sRNA, but it is possible that it is more organized and may use other factors to aid in base-pairing (Waters and Storz, 2009).

### Role of the sRNA on Biofilm Formation

In the 17th century, a scientist named Antonie van Leeuwenhoek was the first to record the existence of microbial biofilms in his study on dental plaque (Percival et al., 2011). Biofilm is described as a multicellular microorganism community which binds the surfaces such as substrate, interfaces and each other with an extracellular matrix produced by themselves (O'Toole et al., 2000; Donlan, 2002; Percival et al., 2011; Jamal et al., 2018). Biofilm formation helps bacteria build resistance to their host defense mechanisms. Bacteria need to have gotten close enough to a surface in order for biofilm formation. If bacteria approach a surface, both attractive and repulsive forces then come into play. When the negative charges on the surface structures of the bacteria encounter the negative charges of the environmental abiotic surfaces, a repulsive force occurs. However, there is a chance that the bacteria can use fimbriae or flagella structures to attach to the target surface. In addition, the repulsion between the surface and the bacteria and the van der Waals forces between the bacterial cells and the surface can be reversed (Rabin et al., 2015). Process of biofilm formation occurs in five major stages (Figure 1). In the first stage, free planktonic cells attach to a surface. This attachment is reversible, so planktonic cells can be easily removed from the surface. These cells then begin to be coated with an extracellular polymeric material (EPS) that they produce. In the second stage, attached cells secrete extracellular polymeric substance (EPS), and after this stage, these cells attach to the surface irreversibly and obtain the biofilm structure. In the third stage, biofilm cells form microcolonies and water channel architecture. In the fourth stage, a fully mature biofilm reaches its maximum cell density and acquires its mushroom-like three-dimensional structure. In the final stage, mature biofilms release microcolonies into the environment for all these stages to be repeated over and over (Verderosa et al., 2019).

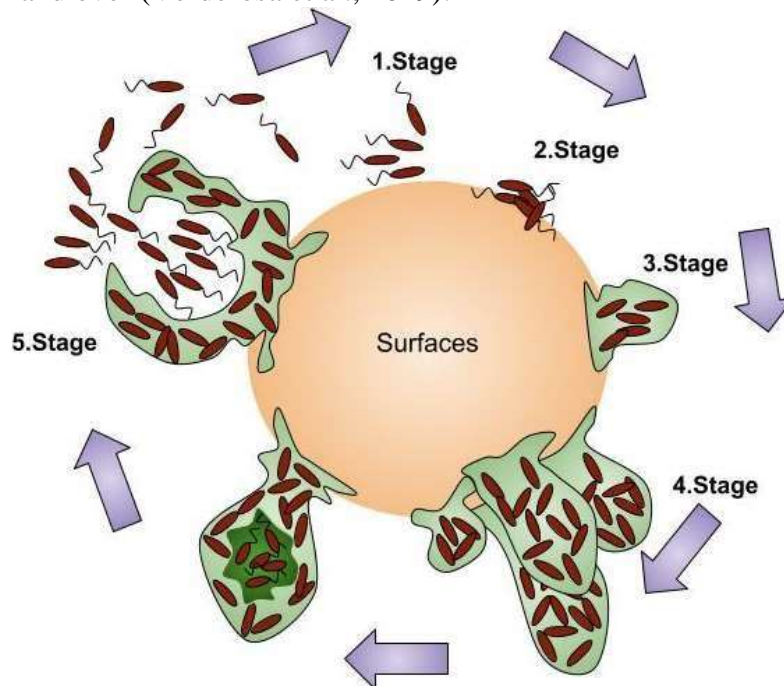


Figure 1. Diagram of biofilm formation

When considering the studies carried out so far, the effect of sRNAs on the biofilm formation of *Escherichia coli* and *Salmonella* has been relatively resolved. Well, what results obtained as a result of these studies?

Lamas et al. (2018a) identified six different sRNAs related to biofilm formation in their experiments on a stainless steel surface in *Salmonella* Typhimurium to define the role of sRNA in biofilm formation in different media (TSB, Milk, CTLB) and different oxygen levels in the atmosphere (aerobiosis, micro-aerobiosis and anaerobiosis). Six sRNAs that were found to affect biofilm formation (*arcZ*, *sroC*, *arcZ*, *dsrA*, *csrB*, *oxyS*, and *rprA*) were evaluated under two classes such as activators and repressors. Activators of biofilm production such as *arcZ*, *sroC*, and *csrB* are sRNAs that get down-regulated on all three growth mediums. The sRNAs for the genes such as *oxyS*, *dsrA*, and *rprA* are inhibitors of biofilm formation. These sRNAs revealed variations in transcription profiles according on growth medium and atmospheric oxygen concentrations. While *dsrA* was considerably elevated in CTLB in the same two environments, TSB was significantly downregulated in all anaerobic conditions. Except for *oxyS* in micro-aerobiosis and TSB, both *rprA* and *oxyS* were downregulated in all media tested. Some food preservatives are used to reduce the persistence of foodborne pathogens in food products. Lamas et al. (2018b), in their study to describe the role of preservatives that commonly used in food industry, on biofilm formation, stated that food preservatives affect the transcription of genes related to biofilm formation. The *adrA* gene was found to be upregulated in all three growth mediums. When it came to the biofilm inducer sRNA *csrB*, SN and SS did not result in any differences in fold change, while SA significantly downregulated *csrB*. In contrast to those findings, the various growth mediums employed showed increased levels of the biofilm repression sRNA *oxyS*. In particular, *oxyS* expression was markedly increased in SN and SA. Considering all strains, vast differences in transcription of *arcZ* were observed. Fuentes et al. (2015) investigated the effect of *sroC* which is a flagellar gene and sRNA target, on biofilm formation. It was determined that biofilm formation decreased in the *sroC* mutant compared to the wild type strain, and that the *sroC* mutant increased the expression of flagellar genes such as *flhBAE* and *fliE*, thus modulating flagellar synthesis. In the electron microscopy study to determine the phenotype of wild-type and mutant strains, it was stated that the wild-type strain had a non-flagellar phenotype. In swimming and swarming experiments to determine whether *SroC* also plays a role in motility, it was determined that the *sroC* mutant increased motility compared to the wild-type strain.

In their study to better understand the role of CsgD, the main regulator of biofilm formation, Ogasawara et al. (2011) identified a total of 20 new regulation target genes. CsgD targets are divided into two classes as Group I and Group II genes. CsgD inhibits the expression of *fliE*, which is included in group I genes, and activates the expression of *adrA*, which is included in group II genes.

Various compounds found in meat residues cause changes in the physiology of bacteria. Based on this event, Hu et al. (2020) investigated the transcriptional profiles and expression of new sRNAs involved in biofilm formation of *S. Enteritidis* strain exposed to MTLB medium. A total of nineteen sRNAs involved in the regulation of biofilm formation were detected. Four deletion mutants ( $\Delta$ sRNA73,  $\Delta$ sRNA109,  $\Delta$ sRNA237, and  $\Delta$ sRNA290) were generated that caused increased swimming, swarming and autoaggregation abilities. As a result of biofilm experiments on stainless steel and polystyrene surfaces, it was determined that new sRNAs reduced biofilm formation.

A communication system known as quorum sensing controls biofilm formation in some bacterial species. The quorum sensing synthase LuxS produces the AI-2 (Autoinducer-2) signaling molecules (Hardie and Heurlier, 2008). Kint et al. (2010), in their study to determine the role of the *luxS* gene region in biofilm formation in *S. Typhimurium*, determined that the

LuxS enzyme is dependent on MicA, an sRNA molecule encoded in the adjacent genomic region of *luxS*. As a result of the deletion of the *luxS* gene, they determined that the LuxS enzyme is not a priority for the mature biofilm formation of the wild-type strain that overexpresses *luxS*. However, as a result of transcriptomic analysis, it was determined that MicA expression level has a decisive role in the formation of mature *Salmonella* biofilm. Trials of mutant *hfq* and *rpoE* genes proved that sRNA molecules play an indirect role in the regulation of biofilm formation. Sigma E is encoded by *rpoE* and is involved in the transcription of this transcription factor MicA. According to the data obtained as a result of the peg biofilm experiment, both *hfq* and *rpoE* mutants did not form biofilms.

In another study to understand the role of sRNAs in biofilm formation, Bak et al. (2015) constructed a collection of plasmids that overexpress different sRNAs in *E. coli* strains. 33 of these 99 distinct sRNAs were shown to strongly inhibit type 1 fimbriae, curli fimbriae, biofilm formation, swimming and swarming motility. It was shown that 5 sRNAs that decrease biofilm formation also lessen the creation of type 1 fimbriae. MicA, which increased swimming motility, slightly inhibited biofilm development, but dramatically inhibited the production of type 1 fimbriae and swarming motility. Biofilm production is inhibited when RpoS, a differentially expressed protein, is absent or expressed at high levels. ArcZ and DsrA, which belong to the group of *rpoS* activating sRNAs, inhibited biofilm formation to a high extent, while RprA, another sRNA belonging to this group, showed lower inhibition than these two sRNAs. Overexpression of ArcZ has been identified to decrease the biofilm formation as well as swimming and swarming motility.

Thomason et al. (2012) investigated the function of McaS, an sRNA, in biofilm formation of *E. coli*. McaS has three distinct single-stranded regions. These regions are where mRNA targets involved in biofilm formation are regulated. Inhibiting *csgD*, the transcription regulator of curli fimbria biogenesis, McaS activates *flhD*, the main transcription regulator of flagella synthesis, resulting in increased motility.

## FUTURE PROSPECTS

Today, there are many studies at the gene level, especially in pathogens with a wide host spectrum such as *Salmonella* and *E. coli*. However, post-transcriptional regulations and that take in charge here sRNAs are as important as the transcriptional regulation. The virulence, persistence and biofilm formation capacity of the microorganisms cause harm to human health and industrial systems. Traditional methods, such as antibiotics and disinfectants, are no more effective for minimizing this damage. Because as time passes, the resistance of these pathogens to antibiotics increases and thus it becomes difficult to remove the pathogen from the environment with traditional methods. For this reason, it has become a necessity to focus on studies on the eradication of these pathogens with studies at the gene level. Investigation of the effects of sRNAs especially on biofilm and compiling the data obtained in this field is a promising research topic in the intervention against pathogens such as *Salmonella* and *E. coli*.

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## VARIATION OF BIOAEROSOL CONCENTRATIONS DURING THE WILDFIRES BROKE OUT IN 2021 IN TURKEY

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### ABSTRACT

Over two hundred wildfires burnt more than 1,500 square kilometres of forest area in Turkey between July 28 and August 12, 2021. This period was recorded as the worst-ever wildfire season in the country's history, and therefore poor air quality was observed over the southwestern regions. It is possible that bioaerosols could be transported tens or hundreds of kilometres in the smoke of large fires. This study investigated the temporal variation of outdoor airborne mold and bacteria concentrations during the wildfire period in the atmosphere of Izmir city, the third most populated city of Turkey and located a couple of hundred kilometres from the northwest of the wildfire region. In this study, 44 airborne mold and 7 bacteria samples were collected with Anderson single-stage sampler between July 23 and August 20, 2021. Enumeration and genus-based identification were made for airborne mold samples collected on two culture media simultaneously during wildfires, namely Potato Dextrose Agar (PDA) and Malt-Extract Agar (MEA). Besides, bacterial samples were collected on Columbia blood agar (CBA) a week before the start of the fires, during the fires, and a week after the fire extinguishment. Some meteorological parameters, such as air temperature and humidity, as well as particulate matter (PM<sub>10</sub>) concentrations, were recorded during the study period. The smoke mainly concentrated around the Aegean Region between August 5 and 7, and thus more frequent samples were collected on those days in Izmir. The airborne mold concentrations on PDA and MEA ranged from 63 to 742 CFU/m<sup>3</sup> and 56 to 2338 CFU/m<sup>3</sup>, respectively. The peak airborne mold level was observed on August 6 at 11:30. Additionally, the airborne bacteria concentrations ranged from 112 to 5376 CFU/m<sup>3</sup>, and the peak level was observed on August 7, during the fires simultaneously in multiple locations around Antalya-Manavgat, Isparta, Muğla-Yatağan, Aydın-Nebiler. The peak value was 48 times greater than the previous week's mean concentration (112 CFU/m<sup>3</sup>) on the sampling station. A correlation was found between total airborne mold concentrations ( $p < 0.01$ ) and PM<sub>10</sub> concentrations observed in the city during the wildfires. Consequently, this study demonstrated the significance of considering wildfire influences when conducting bioaerosol monitoring.

**Keywords:** Bioaerosol monitoring, wildfire, airborne mold, fire ecology

## INTRODUCTION

Mold, bacteria and archaea inhabit various forest habitats such as foliage, the wood of living trees, the bark surface, ground vegetation, roots and the rhizosphere, litter, soil, deadwood, rock surfaces, invertebrates, wetlands or the atmosphere (Baldrian, 2017). Specific features, such as nutrient availability or temporal dynamics and specific drivers that affect the microbial abundance, the level of dominance of bacteria or mold, as well as the composition of their communities, affect the bioaerosol concentrations in the atmosphere (Baldrian, 2017). According to a previous study, Eukarya (29%–72%) and Bacteria (13–26%) dominated the Amazonian bioaerosol population and variability, whereas Archaea (0.5–5%) played numerically only a minor role. On average, ~70% of the coarse mode particles (i.e., 0.7–10 µm) were attributed to presumably intact cells. In contrast, under the given conditions, the remaining ~30% can be regarded as an upper limit estimate for biological fragments and degenerated biological material in this size fraction (Prass et al., 2021). In another study which investigated airborne mold spores at the Belgrad forest near the city of Istanbul (Turkey); 13 genera (*Mucor*, *Rhizopus*, *Absidia*, *Aspergillus*, *Penicillium*, *Trichoderma*, *Trichothecium*, *Stemphylium*, *Cladosporium*, *Alternaria*, *Ulocladium*, *Aureobasidium* and *Fusarium*), 25 species were determined (Colakoğlu, 2003). In a recent study (Chandelier et al., 2021), a large number of fungal pathogens were detected, with most of them being forest pathogens (e.g., *Apiognomonina errabunda*, *Bjerkandera adusta*, *Fomes fomentarius*, *Fomitopsis pinicola*, *Gymnopus fusipes*, *Laetiporus sulphureus*, *Lophodermium piceae*, *Melampsora laricis-populina*, *Meria laricis*, *Nothophaeocryptopusgaeumannii*, *Taphrina carpini*, and *Trametes versicolor*). Fungal pathogens of cultivated plants, notably *Blumeria graminis*, *Claviceps purpurea*, *Microdochium nivale*, *Fusarium graminea-rum*, *Sclerotinia sclerotiorum*, and *Ustilago striiformis*, were also detected.

Bioaerosols could be transported in smoke from large fires more than 1500 km distant (Mims and Mims, 2004). However, few studies have examined the effect of fire on airborne bacteria and mold (Mims and Mims, 2004; Camacho et al., 2018). Societal impacts of smoke-transported living bioaerosols could be indirect (e.g., ecosystem services) and direct (human health). The viability and composition of bioaerosols transported by smoke may also have significant implications for forest health. For example, the pathogenic mold, *Cronartium ribicola* (J.C. Fisch.), which causes white pine blister rust and threatens the endangered whitebark pine (*Pinus albicaulis* Engelm.), was spread to new hosts in the western United States via long-distance dispersal by atmospheric transport (Kobziar et al., 2018; Frank et al., 2008).

Over two hundred wildfires burnt in Turkey between July 28 and August 12, 2021. This period was recorded as the worst-ever wildfire season in the country's history, and therefore poor air quality was observed over the southwestern regions. This study investigated the temporal variation of outdoor airborne mold and bacteria concentrations during the wildfire period in the atmosphere of Izmir city, which is the third most populated city of Turkey and located a couple of hundred kilometres away from the northwest of the wildfire region.

## MATERIAL AND METHOD

44 airborne mold and 7 bacteria samples were collected with Anderson single-stage sampler (SKC Inc.) between July 23 and August 20, 2021. Enumeration and genus-based identification were made for airborne mold samples collected on two culture media simultaneously during wildfires, namely Potato Dextrose Agar (PDA) and Malt-Extract Agar (MEA). Besides, bacterial samples were collected on Columbia blood agar (CBA) a week before the start of the fires, during the fires, and a week after the fire extinguishment. Figure 1

shows the sampling area on NASA Fire Information for Resource Management System Map between July 30 and August 12, 2021. The smoke mainly concentrated around the Aegean Region between August 5 and 7, and thus more frequent samples were collected on those days in Izmir.



**Figure 1.** NASA Fire Information for Resource Management System (FIRMS) map dated between July 30 and August 12 on the study area (NASA FIRMS uses satellite observations from the MODIS and VIIRS instruments to detect active fires and thermal anomalies)

Some meteorological parameters, such as air temperature and humidity, as well as particulate matter (PM<sub>10</sub>) concentrations ( $\mu\text{g}/\text{m}^3$ ), were recorded during the study period. Bioaerosol samples were transported to the laboratory, and airborne mold samples were kept at room temperature (25°C) in the dark 3–7 days until colonies had developed. Airborne bacteria samples were incubated at 37°C for 1-2 days.

## RESULTS AND DISCUSSION

Table 1 shows the airborne bacteria concentrations ranging from 112 to 5376 CFU/m<sup>3</sup>. The peak level was observed on August 7, during the fires occurred simultaneously in multiple locations around Antalya-Manavgat, Isparta, Muğla-Yatağan, Aydın-Nebiler. The peak value was 48 times greater than the previous week's mean (112 CFU/m<sup>3</sup>) on the sampling station.

**Table 1.** Sampling program for airborne bacteria before, during and after the wildfires

| DATE       | WILDFIRES  | RH (%) | T (°C) | TBC on CBA (CFU/m <sup>3</sup> ) | PM <sub>10</sub> (µg/m <sup>3</sup> ) |
|------------|--|--------|--------|----------------------------------|---------------------------------------|
| 23/07/2021 | A week before wildfires  | 38.2   | 25.7   | 413                              | 51.57                                 |
| 30/07/2021 | Start of wildfires   | 41.0   | 28.5   | 112                              | 83.31                                 |
| 06/08/2021 | During Manavgat, Isparta, and Yatağan fires                                      | 35.5   | 28.4   | <b>1372</b>                      | 91.06                                 |
| 07/08/2021 |  | 40.0   | 26.3   | <b>5376</b>                      | 61.07                                 |
| 08/08/2021 | Manavgat and Yatağan fires were controlled<br>Aydın Nebiler fires were continued | 40.7   | 27.2   | 602                              | 44.66                                 |
| 13/08/2021 | End of fires   | 32.9   | 26     | 231                              | 53.99                                 |
| 20/08/2021 | A week after all wildfires   | 40.7   | 28     | 336                              | 72.65                                 |

The airborne mold concentrations on PDA and MEA ranged from 63 to 742 CFU/m<sup>3</sup> and from 56 to 2338 CFU/m<sup>3</sup>, respectively (Table 2). The peak airborne mold level was observed on August 6 at 11:30 (the 9<sup>th</sup> day of the wildfire event). The peak value was 10 times greater than the previous week's mean (224 CFU/m<sup>3</sup>) on the sampling station. A recent study also found that after a fire occurrence, the concentration of mold spores tends to increase in the air, peaking on the 10<sup>th</sup> day after a fire event. The retention of mold spores in the atmosphere observed 1–2 weeks after wildfires are hypothesized because the meteorological conditions were stagnant, unfavourable to the reduction in spore counts by transport or dilution by wind (Camacho et al., 2018).

**Table 2.** Sampling program for airborne mold before, during and after the wildfires

| DATE             | WILDFIRES  | RH (%)   | T (°C) | TMC on PDA (CFU/m <sup>3</sup> ) | TMC on MEA (CFU/m <sup>3</sup> ) |        |
|------------------|--|--|--------|----------------------------------|----------------------------------|--------|
| 30/07/2021 11:30 | Start of wildfires   | 41   | 28.5   | 112.00                           | 63.00                            |        |
| 30/07/2021 11:40 |  | 41   | 28.5   | 469.00                           | 224.00                           |        |
| 06/08/2021 11:30 | During Manavgat, Isparta, and Yatağan fires                | 35.50  | 28.40  | 252.00                           | <b>2338.00</b>                   |        |
| 06/08/2021 11:40 |  | 35.50  | 28.40  | 231.00                           | 91.00                            |        |
| 07/08/2021 11:30 | During Manavgat, Isparta, Yatağan, and Aydın-Nebiler fires | 40.00  | 26.30  | 77.00                            | 329.00                           |        |
| 07/08/2021 11:40 |  | 40.00  | 26.30  | 196.00                           | 294.00                           |        |
| 07/08/2021 13:30 |  | 31.90  | 29.50  | 70.00                            | 210.00                           |        |
| 07/08/2021 13:40 |  | 31.90  | 29.50  | 112.00                           | 273.00                           |        |
| 07/08/2021 15:30 |  | 34.20  | 30.80  | 126.00                           | 77.00                            |        |
| 07/08/2021 15:40 |  | 34.20  | 30.80  | 98.00                            | 175.00                           |        |
| 07/08/2021 17:30 |  | 25.70  | 30.20  | 301.00                           | 105.00                           |        |
| 07/08/2021 17:40 |  | 25.70  | 30.20  | 133.00                           | 427.00                           |        |
| 07/08/2021 19:30 |  | 28.80  | 29.40  | 224.00                           | 259.00                           |        |
| 07/08/2021 19:40 |  | 28.80  | 29.40  | 350.00                           | 203.00                           |        |
| 07/08/2021 21:30 |  | 32.40  | 29.00  | 196.00                           | 462.00                           |        |
| 07/08/2021 21:40 |  | 32.40  | 29.00  | 119.00                           | 553.00                           |        |
| 08/08/2021 11:30 |  | Manavgat and Yatağan fires were controlled<br>Aydın Nebiler fires were continued | 40.70  | 27.20                            | 273.00                           | 294.00 |
| 08/08/2021 11:40 |  |  | 40.70  | 27.20                            | 259.00                           | 259.00 |
| 08/08/2021 13:30 |  |  | 33.80  | 28.70                            | 217.00                           | 399.00 |
| 08/08/2021 13:40 |  |  | 33.80  | 28.70                            | 336.00                           | 231.00 |
| 08/08/2021 15:30 | 35.30  |  | 29.30  | 434.00                           | 798.00                           |        |
| 08/08/2021 15:40 | 35.30  |  | 29.30  | 742.00                           | 784.00                           |        |
| 08/08/2021 17:30 | 33.40  |  | 30.00  | 511.00                           | 511.00                           |        |
| 08/08/2021 17:40 | 33.40  |  | 30.00  | 357.00                           | 553.00                           |        |
| 08/08/2021 19:30 | 37.40  |  | 29.30  | 385.00                           | 406.00                           |        |
| 08/08/2021 19:40 | 37.40  |  | 29.30  | 371.00                           | 385.00                           |        |
| 08/08/2021 21:30 | 29.90  |  | 28.50  | 245.00                           | 441.00                           |        |
| 08/08/2021 21:40 | 29.90  |  | 28.50  | 413.00                           | 357.00                           |        |
| 09/08/2021 11:30 |  | 24.10  | 29.90  | 168.00                           | 224.00                           |        |
| 09/08/2021 11:40 |  | 24.10  | 29.90  | 161.00                           | 126.00                           |        |
| 09/08/2021 19:30 |  | 38.20  | 29.70  | 84.00                            | 203.00                           |        |

|                  |                                      |       |       |        |        |
|------------------|--------------------------------------|-------|-------|--------|--------|
| 09/08/2021 19:40 | During Bodrum, Milas, Köyceğiz fires | 38.20 | 29.70 | 273.00 | 259.00 |
| 10/08/2021 11:30 |                                      | 27.40 | 29.20 | 112.00 | 105.00 |
| 10/08/2021 11:40 |                                      | 27.40 | 29.20 | 329.00 | 196.00 |
| 10/08/2021 19:30 |                                      | 33.50 | 29.20 | 154.00 | 133.00 |
| 10/08/2021 19:40 |                                      | 33.50 | 29.20 | 63.00  | 119.00 |
| 11/08/2021 11:30 |                                      | 44.70 | 28.30 | 98.00  | 336.00 |
| 11/08/2021 11:40 |                                      | 44.70 | 28.30 | 364.00 | 259.00 |
| 11/08/2021 19:30 |                                      | 27.30 | 33.10 | 301.00 | 147.00 |
| 11/08/2021 19:40 |                                      | 27.30 | 33.10 | 287.00 | 175.00 |
| 12/08/2021 11:30 |                                      | 27.80 | 28.90 | 259.00 | 196.00 |
| 12/08/2021 11:40 |                                      | 27.80 | 28.90 | 259.00 | 56.00  |
| 12/08/2021 19:30 |                                      | 27.70 | 30.60 | 322.00 | 560.00 |
| 12/08/2021 19:40 |                                      | 27.70 | 30.60 | 497.00 | 511.00 |

Table 3 reports a correlation between total airborne mold concentrations on PDA ( $p<0.01$ ) and MEA ( $p<0.05$ ) and PM<sub>10</sub> concentrations observed in the city during the wildfires. Mims and Mims (2004) observed a strong correlation ( $R^2 = 0.78$ ) between airborne mold and aerosolized PM (assessed microscopically through visual counts of particles) deposited in Texas, USA, by smoke originating from wildfires on the Yucatan Peninsula, Mexico.

Table 3. Spearman rank correlations among the measured parameters

|                                       | RH (%)         | T (°C) | TMC on PDA (CFU/m <sup>3</sup> ) | TMC on MEA (CFU/m <sup>3</sup> ) | PM <sub>10</sub> (µg/m <sup>3</sup> ) | Ws (m/s) |
|---------------------------------------|----------------|--------|----------------------------------|----------------------------------|---------------------------------------|----------|
| RH (%)                                | 1.000          |        |                                  |                                  |                                       |          |
| T (°C)                                | <b>-0.60**</b> | 1.000  |                                  |                                  |                                       |          |
| TMC on PDA (CFU/m <sup>3</sup> )      | -0.02          | 0.08   | 1.000                            |                                  |                                       |          |
| TMC on MEA (CFU/m <sup>3</sup> )      | 0.14           | -0.06  | <b>0.39**</b>                    | 1.000                            |                                       |          |
| PM <sub>10</sub> (µg/m <sup>3</sup> ) | 0.03           | -0.04  | <b>0.54**</b>                    | <b>0.42*</b>                     | 1.000                                 |          |
| Ws (m/s)                              | 0.10           | -0.15  | 0.19                             | 0.06                             | 0.17                                  | 1.000    |

\*\*  $p<0.01$  \* $p<0.05$

RH: Relative humidity (%)

T: Ambient temperature (°C)

Ws: Wind speed (m/s)

A total of 19 mold genera, including yeast and sterile hyphae were observed over the study period, independent from the culture media (Table 4). Total colony counts (CFU/m<sup>3</sup>) of each mold genus on culture media of PDA, and MEA were variable. The most abundant mold was *Cladosporium* spp. on all culture media ( $>5 \times 10^3$  CFU/m<sup>3</sup>), where the highest colony counts were found on MEA. *Cladosporium* spp. is a genus common in bioaerosols and was the most frequently observed genus shared between ambient and smoke air (found in 80% of samples) (Kobziar et al., 2022). *Cladosporium* spp. and *Alternaria* spp. include phytopathogenic species, sources that increase their abundance in the phyllosphere following high-severity fire could have relevance to post-fire aspen recovery (Kobziar et al., 2022; Dove et al., 2021).

Table 4. Total numbers of colony (CFU/m<sup>3</sup>) of each mold genus collected simultaneously by PDA and MEA from all samples<sup>a</sup>

| Genus                               | Total colony counts (CFU/m <sup>3</sup> ) |              |
|-------------------------------------|---|--------------|
|                                     | PDA                                       | MEA          |
| <i>Cladosporium</i> spp.            | <b>5593</b>                               | <b>10367</b> |
| Yeast                               | 1428                                      | 791          |
| <i>Alternaria</i> spp.              | 1260                                      | 1400         |
| <i>Aspergillus</i> spp.             | 882                                       | 980          |
| <i>Mucor</i> spp.                   | 427                                       | 350          |
| <i>Penicillium</i> spp.             | 252                                       | 518          |
| <i>Chrysosporium</i> spp.           | 231                                       | 98           |
| <i>Fusarium</i> spp.                | 105                                       | 77           |
| <i>Acremonium</i> spp.              | 91  | 7            |
| <i>Bipolaris</i> spp.               | 84  | 336          |
| Sterile hyphae                      | 79  | 112          |
| <i>Microsporium</i> spp.            | 70  | 56           |
| <i>Nigrospora</i> spp.              | 45  | <7           |
| <i>Botrytis</i> spp.                | 35  | 133          |
| <i>Geotrichum</i> spp.              | <7  | 98           |
| <i>Pleurostomophora richardsiae</i> | <7  | 84           |
| <i>Chrysonilia</i> spp.             | <7  | 42           |
| <i>Ulocladium</i> spp.              | <7  | 14           |
| <i>Trichoderma</i> spp.             | <7  | 7            |

<sup>a</sup> n =44 for each culture media; the maximum colony counts of the predominant mold genera were shown in bold.

*Pleurostomophora richardsiae* is found in soil, decaying wood and vegetation (Levenstadt et al., 2012). It was only observed in the atmosphere on August 8 at 19:30.

## CONCLUSIONS

Airborne mold and bacteria concentrations during 9<sup>th</sup> and 10<sup>th</sup> days of wildfires were 10 and 48 times greater than the concentrations when the wildfires started, respectively. During the wildfire, the dominant mold genus was *Cladosporium* spp., yeast and *Alternaria* spp. This study demonstrated the importance of considering fire episodes in future aero-microbiological surveys.

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## THE ROLE OF ENZYMES IN THE IMPROVEMENT OF DOUGH PROPERTIES PREPARED WITH WHEAT FLOUR

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### ABSTRACT

The use of enzymes in the food industries has grown in the past few years due to their natural origin of them. They are used to improve the quality of the product, to make diversity and new products. The enzymes are extracted from the living cells and they are very active when the right substrate to interact with. In the baking industry, different types of enzymes are added to improve the texture and quality of the dough and the final products. They can be combined with ascorbic acid to develop and to give their contribution in the process. This study aims to determine the effect of enzymes: hemicellulose, xylanase, lipase, cysteine, and ascorbic acid in the dough properties. The analyzes were done in the same sample of wheat flour, at a different rate, and for two hemicelluloses, cysteine and ascorbic acid a combination was done to see how they can interact with each other. The result has shown that cysteine has a soft effect on the dough properties affecting the energy and the resistance of the dough. The addition of the cysteine from 0 to 40 ppm has effect the  $R_{max}$  from 387 to 226 BU in 45 min, elasticity from 149 to 153 mm, and energy of the dough from 81 to 51 cm<sup>2</sup>. The  $K_{max}$  was also reduced from 2.6 to 1.48 in 45 min of the proofing time. The use of hemicellulose from 0-50 ppm has increased the  $R_{max}$  from 387 to 400 BU in 45 minutes of proofing, elasticity from 149 to 153 mm energy of the dough from 81 to 86 cm<sup>2</sup> and  $K_{max}$  hasn't changed. But the combination of the two enzymes in 20-20 ppm has put the value of the energy to 66 cm<sup>2</sup>, decrease the  $R_{max}$  to 322 BU, and has less effect on the elasticity. Adding ascorbic acid to the dough till 40 ppm as an oxidizer agent has increased the value of  $R_{max}$  to 20% BU, elasticity to 7%, and energy of the dough to 12 %. The resting time affects the dough's properties. In mostly all the samples the 90 min of proofing helps the dough to express all its properties.

**Keywords:** enzymes, dough, wheat flour, ascorbic acid,  $R_{max}$ ,  $K_{max}$ .

### INTRODUCTION

Enzymes are protein with catalytic properties due to its power of specific activation. (Dixon et al,1979). They have been used in the food industries for centuries, even when no one new them, but they used their properties. Enzymes can increase shelf life, change the texture, flavors, aroma, and which is most important reduce the quantity of chemical additives used. The first one was discovered and isolated from the jack bean in 1926 (*Sumner, 1926*). They are natural component that have high activity when find the right substrate to interact with. The structure of enzymes is organizing by a main structure which mostly is a protein. Most enzymes, but not all, require a small molecule to perform their activity as a catalyst. These molecules are known as cofactors or coenzymes. Cofactors are non-proteinaceous chemical compounds that are bound to an inactive protein part of enzyme in order to increase the biological activity of the enzyme required for its function. (*Kuddus, 2019*). In order for a reaction to take place, the



reactant molecules (substrate) require some amount of energy to cross the transition state of the reaction and become products. Enzymes reduce the activation energy of enzymes catalyzed in chemical reactions and therefore increases the rate of reaction ranging from 10<sup>6</sup> to 10<sup>24</sup>-fold in comparison to nonenzymatic reactions (*Illanes, 1999*). In the baked industry a lot of enzymes are used in order to improve the quality of the dough and to the baked products. Some of them and their effect are listed below: *α-Amylase*. hydrolyze complex starch molecules into simple monomer units of glucose. Sources of *α-amylase* are plants, animals, and microorganisms, but commercially viable amylases are produced from microorganisms, especially bacterial and fungal species. Thermostable *α amylase* is produced by some potential bacterial species like *Bacillus licheniformis* and *Bacillus stearothermophilus*, *Pseudomonas*, and the *Clostridium* family. (*Singh et al., 2019*) *α-Amylase* also degrades the starch in wheat flour into small dextrins, thus allowing yeast to work continuously during dough fermentation, proofing, and the early stages of the baking process. *α-Amylases* are also employed in many other aspects of the food industry like clarification of beer, fruit juices, and pretreatment of animal feed to improve the digestibility of fiber (*Ziegler, 1999*). *Lipase*, has recently been recognised as a strong dough-conditioning enzyme. It shows excellent effects on bread performance. (*Qi Si. et al, 2002*) Addition of a fungal lipase to a flour dough does not significantly change the rheological properties of the dough measured by both farinograph and extensograph (*Si, 1996*) with optimum water absorption. This is a good characteristic for bread making, because most bakers do not like major changes in the dough system. Lipase does not make the dough sticky and significantly improves dough stability and crumb structure, synergistic effects between xylanase, amylase and lipase could improve bread quality. Due to its effect on gluten strengthening, lipase improves dough stability against over-fermentation. Possible new combinations with improved functionality are continuously arising from new enzymes through product development in the enzyme industry (*Si, 1996*). *Cysteine*, is an amino acid constituent of all proteins and produced by hydrolysis of extremely cysteine-rich proteins such as those from feathers or hair and complex purification procedures or by synthetic means. Also, L-cysteine occurs naturally in wheat flours (*Joye et al., 2009*). Cysteine supplementation results in dough weakening, with decreases in the elastic and viscous properties, mixing time and tolerance to mixing. In contrast, adhesiveness, extensibility and machinability are increased (*Angioloni et al., 2007, Miller et al., 1999*) The using of L-cysteine is indicated in technologies that provide short fermentation and processing times, because lead to increases in volume (up to 10%), porosity and elasticity of the products (*Stoica et al, 2010*)

This study aims to evaluate some properties of the dough when enzymes are added as additive. The role of hemicellulose, xylanase, lipase, cysteine, is determined and also the role of ascorbic acid as an functional agent that is used a lot in the milling industry to improve the properties of the dough. The enzymes were added in different quantity, solo or combined in order to evaluate the interaction with each other.

## **MATERIAL AND METHODS**

In order to see the effect of additives, mainly enzymes, in improving the properties of flour, we made additions of several enzymes with different percentages and ascorbic acid to our samples. The data in the table below give in detail the whole object of our study.

**Table 1.** Sample preparation

| No | Additive                 | Quantity (ppm)                               |
|----|--------------------------|--|
| 1  | Cysteine                 | 10,15,20,25,30,35,40                         |
| 2  | HCE                      | 10,30,40                                     |
| 3  | Ascorbic Acid            | 5,10,15,20,30,40,50, 100                     |
| 4  | Xylanase                 | 5,10,15                                      |
| 5  | Lipase                   | 10, 20, 30, 40                               |
| 6  | Ascorbic Acid - Cisteine | 10-20, 20-20, 50-20, 100-20, 40-15 and 80-15 |
| 7  | HCE-Cisteine             | 10-20, 20-20, 40-20, 40-10 and 50-10         |

- **Determination of moisture content**

Moisture content was determined using Method 44-15 A, a method approved by the AACC. In this method, a small amount of flour (2-3 g) is weighed and placed in a moisture pan, and then the sample is heated in an air oven at 130°C for 1 hour. Then the sample is cooled and weighed (AACC Approved Method, 44-15.02).

- **Determination of gluten**

As described in Method 38-12 of the AACC, a 10-gramme flour sample is weighed and placed on the polyester sieve in the glutomatic wash chamber. The sample is mixed and washed with a 2% salt solution for 5 minutes. The wet gluten is removed from the wash chamber, placed in the centrifuge holder and centrifuged. The residue retained on top of and through the sieve is weighed (AACC Approved Method, 38-12.02).

- **Estensograph measurements**

The estensograph device is used to determine the energy of the dough, the extensibility, maximum resistance ( $R_{max}$ ), the ratio ( $R/E$ ), the resistance of constant deformation and stretching characteristics in a different time and we can see the changes that happen in this parameters as the time pass. In this way, we can determine the correct time where the parameters have reached their optimum value. For the estensograph test, the dough is prepared from flour, water and salt in the farinograph as we mention above. For the preparation of the dough, firstly prepare the sodium chloride solution and then add to the flour in the chamber mix. The procedure is done according to ISO 5530\_2\_2012 part 2.

- **Farinograph measurements**

The Farinograph device is used for the determination of water absorption, degree of softening, development time, the stability of the dough and the Farinograph quality number. For the Farinograph test, for each sample 300 gr of flour wheat, 6 gr of NaCl were pre-mixed for one minute and then with water to measure the parameters mentioned above. All the measurements were done according to ISO 5530\_2\_2012 part 1.

## RESULT AND DISCUSSIONS

- *Effect of cysteine on dough*

Cysteine is an enzyme that is present in wheat flour and mainly has reducing properties, which helps to soften the structure of the dough. For this reason, reports of various cysteine supplements were taken into analysis. Its effect on the dough is better shown in the figure below.

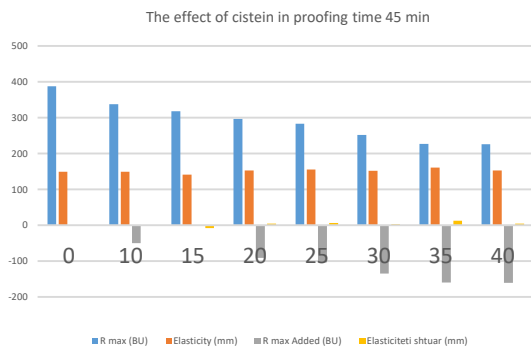


Figure 1. Effect of cysteine on dough for 45 min

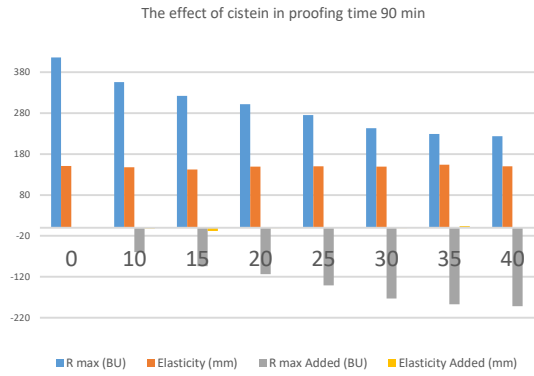


Figure 2. Effect of cysteine on dough for 90 min

As can be seen from the figure 1 and 2, with the addition of cysteine at values from 5 to 40 ppm, a decrease in dough strength and strength was observed, which is related to the softening of the dough, the decrease of S-S bonds and the increase of S-H end groups. Regarding elasticity, the addition of cysteine did not affect this parameter much. And in the case of leaving the dough at rest for 90 min, it was seen that we have a significant difference in the resistance and strength of the dough, where the values decrease with the increase of the addition of cysteine. And in this case there is no significant effect on elasticity.

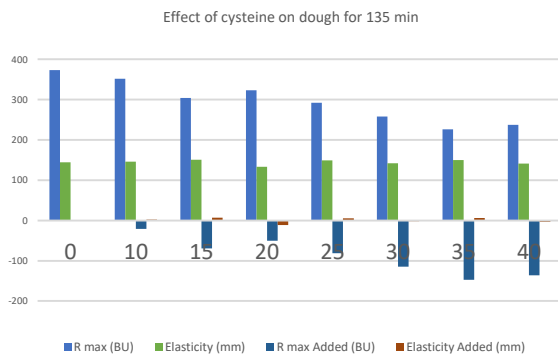


Figure 3. Effect of cysteine on dough for 135 min

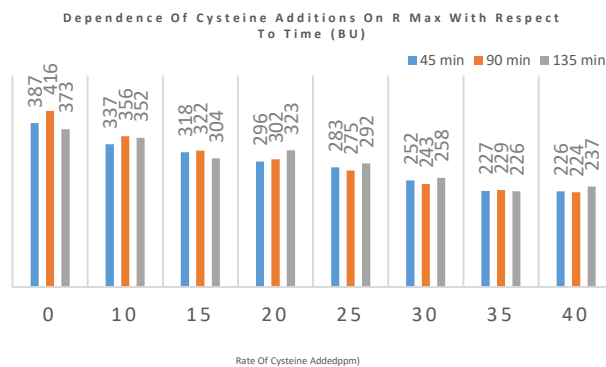


Figure 3.1 Dependence of cysteine additions on dough strength with respect to time (cm<sup>2</sup>)

In the last case, proofing for 135 min at constant temperature gave a slightly smaller effect compared to the two times above. The same thing is observed in sample 0 taken as a reference, where the extension of the residence time over 90 min decreases the strength and resistance of the dough. If we compare the three figures above, we will see that the greatest effect of the addition of cysteine was realized at a proofing time of about 90 min and the softening of the dough was realized with the addition of cysteine at 40 ppm. Regarding the elasticity, no change was observed in the new residence times and in all realized additions of cysteine.

*Effect of hemilulase on dough.*

Hemicelluloses have only a limited effect on the *Falling Number*, but their activity can sometimes be very clearly recognized in the amylogram (lower gelatinization temperature and maximum viscosity) and also in the alveogram where some hemicelluloses cause a change in the curve similar to that produced from cysteine but without any protein breakdown. It should be mentioned that the softening effect is the sum of the loosening of the pentosan-protein network and the release of water from the pentosan gel, which makes water available for further hydration of the gluten and thus it's softening

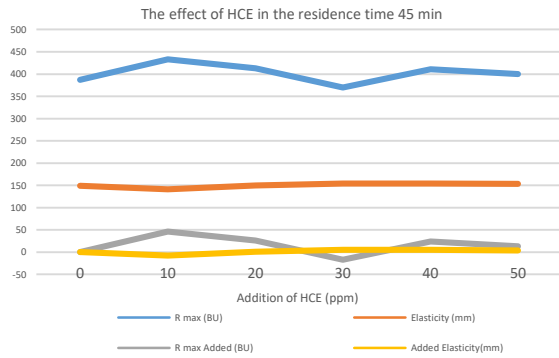


Figure 4. Effect of hemicellulose in the dough in 45 min

The figure 4 shows that the changes with the addition of hemicellulose increase slightly. So the addition of hemicellulose causes an increase in the strength and energy of the dough. The impact on elasticity and in this case is in very small values.

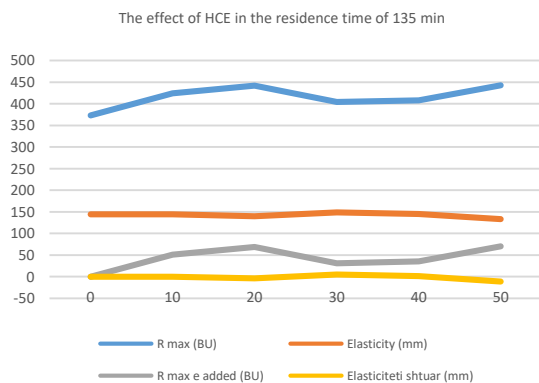
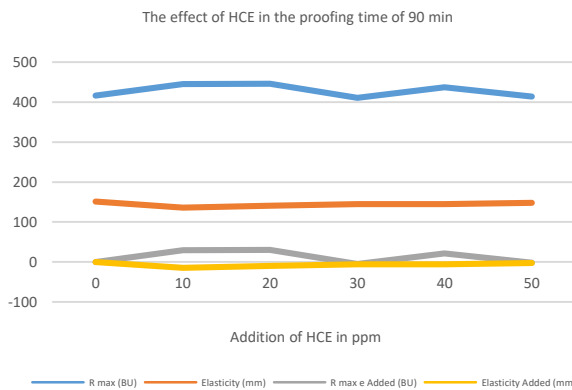


Figure 5. Effect of hemicellulose in the dough in 90 min Figure 6. Effect of hemicellulose in the dough at 135 min

Similar results were observed during the evaluation of parameters on resistance, power and elasticity during the proofing time at 90 min. We have an increase in strength and almost negligible impact on elasticity. In the last case, it can be seen from the graph (figure 6) that we are dealing with a relatively significant change in resistance and strength, which shows an

increase of about 70 units, which indicates a hardening of the dough during the time of proofing at 135 min and shot of HCE at 50 ppm. Meanwhile, we have a decrease in elasticity by 11 mm in this case. This helps technologists and manufacturing companies evaluate different situations when it comes to products that need a long proofing time before going into the baking process.

*Analysis of the effect of ascorbic acid on dough.*

Seeing the wide use of ascorbic acid in the flour industry, in this study we investigated the effect of ascorbic acid alone and combined with enzyme. In this way, we see how these combined additives will affect the quality of the dough.

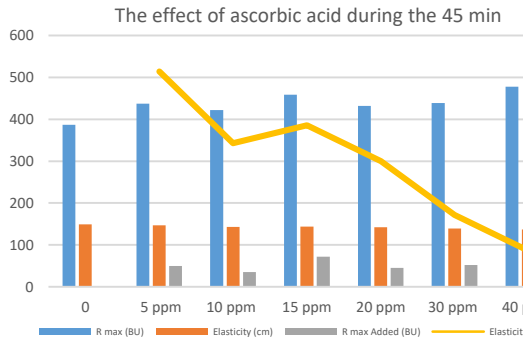


Figure 7. The effect of ascorbic acid on the dough in 45 min

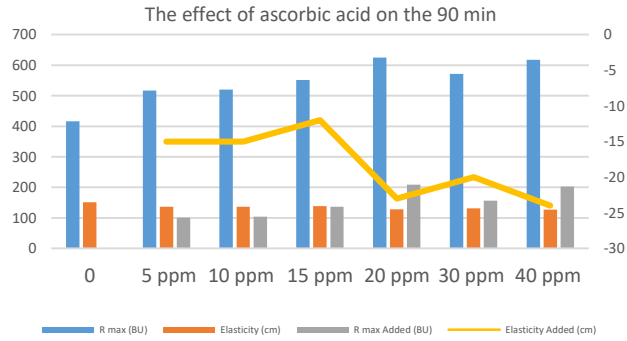


Figure 8. The effect of ascorbic acid on the dough in 90 min

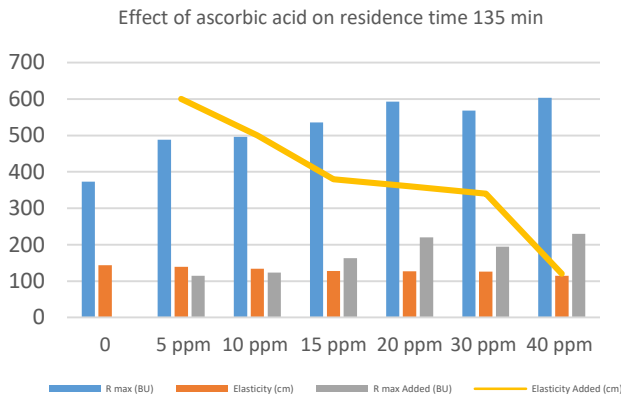


Figure 9. The effect of ascorbic acid on dough in 135 min

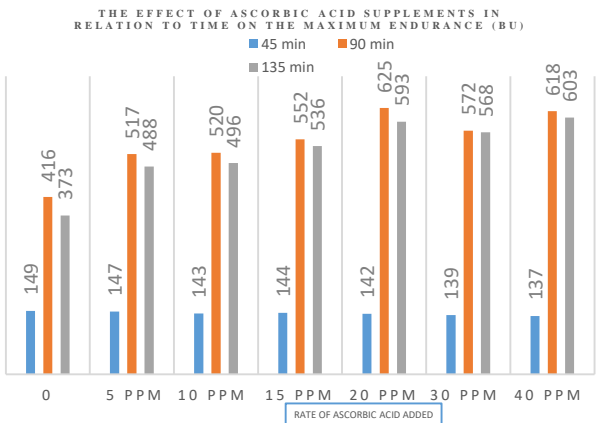


Figure 10. The effect of ascorbic acid additions on dough strength as a function of time

The graphs in figures 7, 8 and 9 present in detail the effect of ascorbic acid on dough resistance, strength and elasticity.

In figure 7, it is shown that the addition of ascorbic acid from 0-40 ppm shows an improvement in the resistance and strength of the dough by increasing its hardness. This is also shown in the effects of ascorbic acid. Tests carried out up to 100 ppm showed an increase in

$R_{max}$  from 387 BU to 478 BU. The greatest effect can be observed with increasing proofing time and increasing the amount of ascorbic acid, where the values almost doubled going from 373 BU in sample 0 taken as a reference, in 633 BU in the last sample.

What is noticed in this case is again the fact that the proofing time is 90 min at a constant temperature, it is seen that we have the maximum resistance and power concentrated in the dough, with the extension of time we have a reduction in power but not less than the initial one.

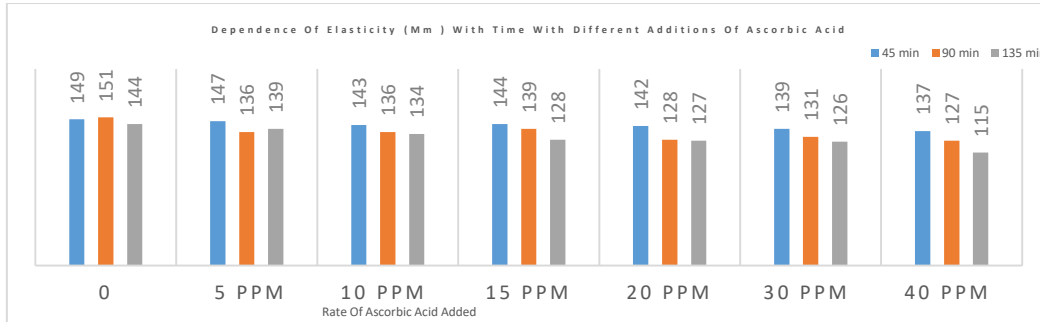


Figure 10.1. Dependence of elasticity (cm) in relation to time and additions of ascorbic acid

Figure 10.1 shows the effect of ascorbic acid additions on elasticity with respect to time. If we look more carefully, we will notice that we have an opposite effect in relation to resistance, power on a smaller scale. We have a decrease in the development of the elasticity of the dough with the addition of ascorbic acid and also with the extension of the residence time.

*Effect of Hemicellulase-cysteine combination on dough.*

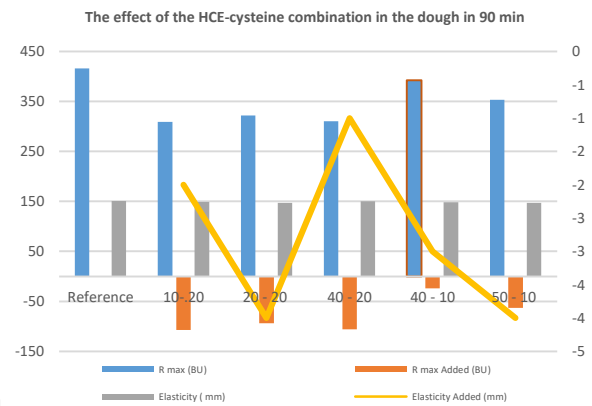
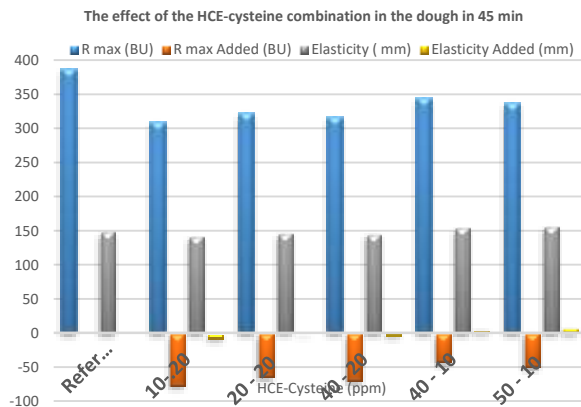


Figure 11. The effect of the combination on the dough at the time 45 min Figure 11. The effect of the combination on the dough at the time 90 min

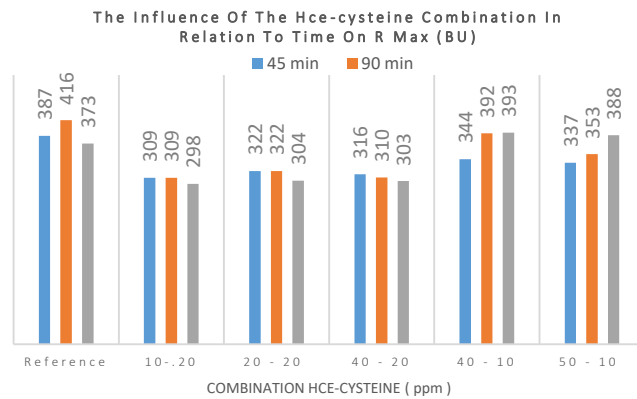
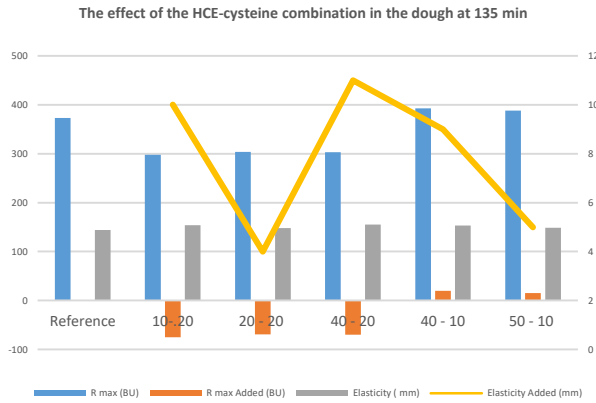


Figure 13. The effect of the combination on the dough at 135 min      Figure 14. The influence of the HCE-cysteine combination in relation to time

Observing the figures clearly shows us that the HCE-cysteine combination does not allow the effect of cysteine alone to appear, which caused a significant decrease in dough resistance and strength (figure 1-3.1), while the combination of these two enzymes improves the properties of dough and in turn has a small effect on reducing power. Almost the same conclusion can be said in this case, that increasing the time of proofing at a constant temperature causes a significant decrease in the strength of the dough, thus affecting its quality.

*Effect of ascorbic acid-cysteine combination on dough.*

If we remember the special effects of ascorbic acid and cysteine, one has an oxidizing effect, hardening the dough, increasing its resistance and strength, the other has the opposite effect. Their addition in certain ratios will affect the improvement of the structure properties of the dough, making it possible to have a continuous improvement of the ratio between sulfur bridges and hydrogen bonds.

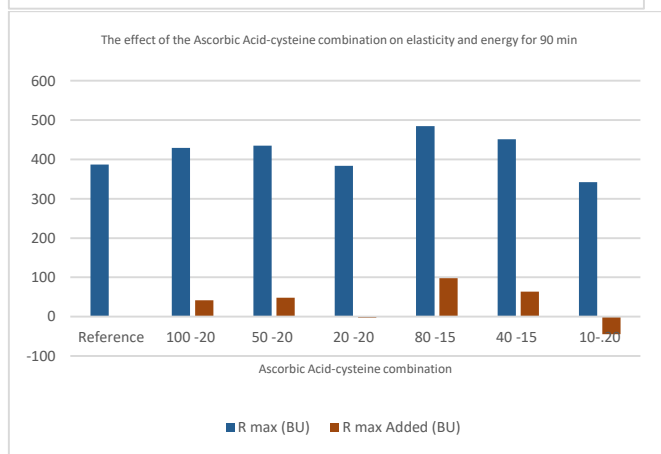
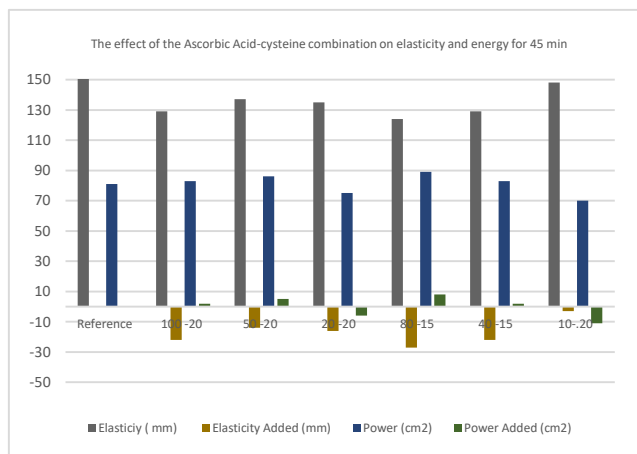


Figure 15. Effect of ascorbic acid-cysteine combination on dough

Figure 16. Effect of ascorbic acid-cysteine combination on dough

From the graphs presented in figure 15 and 16, we can see that by keeping cysteine constant (20 ppm) and adding ascorbic acid at 10, 20, 50 and 100 ppm, it was observed to have an increase in strength and resistance and a fluctuation of elasticity parameters. With the reduction of the addition of cysteine, we have a higher degree of values growth and  $R_{max}$  since ascorbic acid is not hindered by cysteine to act on the gluten structures and make a change to it. What can be noticed if we compare the data obtained from the addition of only ascorbic acid and the combined one, the inhibitory effect of cysteine can be distinguished even at low values since, in the tests carried out up to 100 ppm of ascorbic acid, showed an increase in  $R_{max}$  from 387 BU to 478 BU. The greatest effect can be observed with increasing residence time and adding only the amount of ascorbic acid, where the values almost doubled, going from 373 BU in sample 0, taken as a reference, to 633 BU in the last sample.



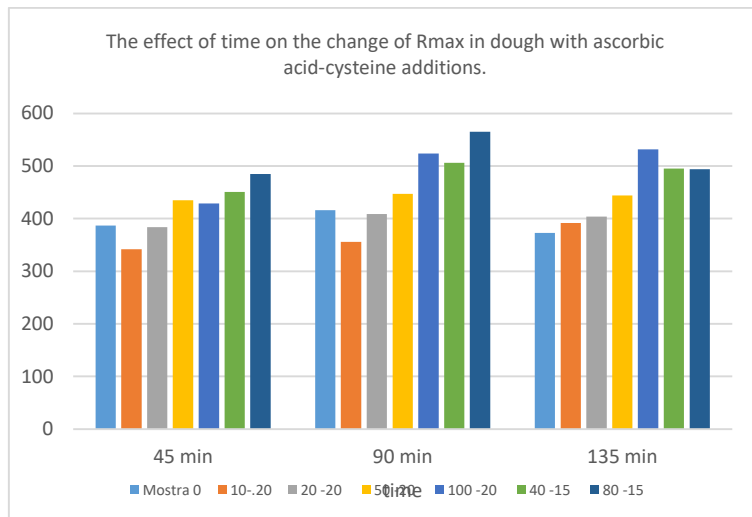


Figure 17. Effect of time on the change of  $R_{max}$  in dough with ascorbic acid-cysteine additions

If we notice figure 17, this effect is much more restrained but still with the increase of the proofing time, we have a very slight increase in  $R_{max}$  with the addition of ascorbic acid from 10 to 100 ppm and keeping the cysteine values constant, 20 ppm.

## CONCLUSIONS

The purpose of this study was to analyze and evaluate some of the main parameters in the rheology of doughs, which will change in cases where additions, mainly enzymes, are made to the flour. The role of enzymes is different depending on the substance where they will act. For the study, not only enzymes were considered, but also ascorbic acid as one of the additives that has a wide use in the industry. First, sample 0 (reference) was analyzed, which had no additives in its composition. Some of the parameters measured, that impact the study were, moisture which was about 14.7%, gluten 27.5%, absorption capacity 53.4 %. In the estensograph the maximum resistance of the dough  $R_{max}$  was measured, which ranged from 387 BU in 45 min, 416 BU in 90 min and 373 BU in 135 min. In terms of elasticity and extensibility which are again parameters that can be determined on the estensograph, the values of sample 0 ranged from 149 mm at 45 min, 151 mm at 90 min and 144 mm at 135 min.  $K_{max}$  given by the ratio of  $R_{max}/E$ , gave values ranging from 2.6 to 2.59. The additives were made separately and combined, to see in this way the effect they will have not only on the dough but also on each other. The addition of cysteine at values from 5 to 40 ppm showed a decrease in dough resistance and strength, which is related to dough softening, decreasing SS bonds and increasing SH end groups. Regarding elasticity, the addition of cysteine did not affect this parameter much. The use of lipase and xylanase gave suspicion result and this is the reason we didn't put in this article. They need further evaluation. Additions of hemicellulose showed an increase in dough strength but did not affect elasticity. The combination of hemicellulose and cysteine in certain ratios showed that the HCE-cysteine combination does not allow the effect that cysteine alone had, which caused a significant decrease in dough resistance and strength (figure 1- 3.1), while the combination of these two enzymes improves dough properties and on the other hand has a small effect on reducing strength.

Almost the same conclusion can be said in this case, that increasing the proofing time at a constant temperature causes a significant decrease in the strength of the dough, thus affecting

its quality. Better changes can also appear in  $K$  (Max Resistance / Elasticity), where the combination of enzymes has behavior and combination of values making it more suitable to use.

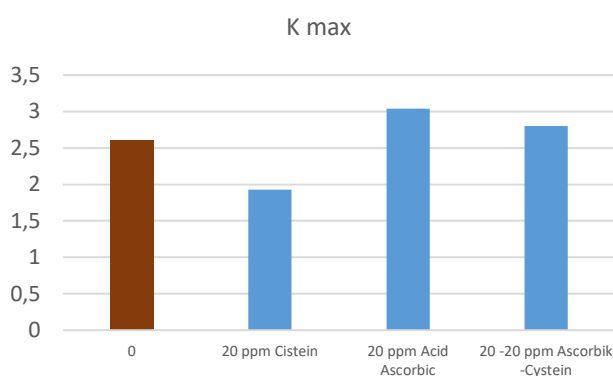
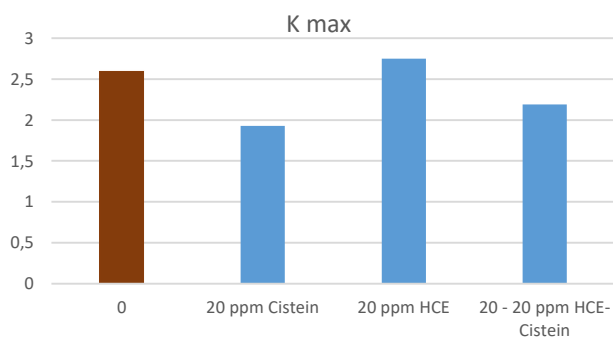


Figure 18. Change of  $K_{max}$  depending on enzymatic additions

Figure 19. Change of  $K_{max}$  depending on enzyme additions and ascorbic acid

The addition of ascorbic acid gives very good results in the quality of the dough as it affects the hardness of the dough, which was also observed in our study. Additions from 5-100 ppm showed increased resistance and dough strength.

Conjugation with cysteine reduced the potency of ascorbic acid but did not block it as it again resulted in dough firming, but to a lesser degree.

Regarding  $K_{max}$ , the graph below shows that for the same added values, when combining ascorbic acid with cysteine, we have an improvement of the latter, bringing the value of  $K_{max}$  to 2.8.

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## ***pagN*: A NOVEL ADHESIN/INVASIN GENE IN *SALMONELLA***

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### **ABSTRACT**

*Salmonella* genus is able to live in a wide range of environments and it is especially described as a facultative intracellular organism. Various adhesins and invasins play a role in this life form. One of them PagN is an outer membrane protein which is known to act as a part of invasion. This review summarizes the current advances about the main characteristics of *pagN* gene. Clarifying the PagN functions on *Salmonella* invasion and understanding its effects on *Salmonella* dispersion and foodborne outbreak has an importance for novel medical and industrial approaches.

**Keywords:** *Salmonella*, *pagN*, outer membrane protein, invasion, pathogenicity

### **INTRODUCTION**

*Salmonella* is a Gram-negative, facultative anaerobe and motile bacteria belonging to the *Enterobacteriaceae* family. It also fulfils the definition of *Enterobacteriaceae* with 0.7-1.5 x 2.0-5.0 µm rod form (Popoff and Le Minor, 2015). Due to the fact that it has numerous virulence factors, it is evaluated as a model for bacterial pathogenicity.

*Salmonella* genus is divided into two species as *Salmonella bongori* and *Salmonella enterica* which consists of *indica*, *diarizonae*, *arizonae*, *salamae*, *houtenae* and *enterica* subspecies. *Salmonella enterica* subsp. *enterica* is classified as more than 2600 serovars (Roy et al., 2021). Approximately 99% of *Salmonella* strains belonging to *Salmonella enterica* species lead to infections in humans and warm-blooded animals (Wu, 2015; Kurtz et al., 2017). On account of contaminated poultry meat and eggs, *Salmonella* causes food borne diseases in addition to zoonotic disease. *Salmonella* causes non-invasive non-typhoidal salmonellosis, invasive non-typhoidal salmonellosis, and typhoid fever in humans (Kurtz et al., 2017). *Salmonella* is a facultative intracellular bacterium hence, an essential feature of the *Salmonella* pathogenicity is its ability to move across host cell barriers (Wu, 2015). In order for bacteria to bind, enter and eventually invade the host cells, a number of adhesins and invasins are required. It had previously been presumed that *Salmonella* enters the host cells only via the trigger mechanism which is coordinated by the Type 3 Secretion System (T3SS), however it has been shown that *Salmonella* is able to invade eukaryotic cells via zipper mechanism as well, which is mediated by outer membrane proteins (Boumart et al., 2014).

In this review, it has been assessed that PagN which is an outer membrane protein of *Salmonella* plays a role in the bacterial entry and acts as an adhesin and invasin.

The *pagN* gene is widely distributed within *Salmonella* genus and this situation has been demonstrated in a research performed by Barilleau et al. (2021). In this study which is performed in a wide range of data set consisting of *S. bongori*, *S. enterica* and *S. enterica* subspecies, it has been observed that 99.6% of the genomes were tested positive in terms of *pagN* gene. Lambert and Smith (2008) indicated that the protein which has the same name with *pagN* gene, and also encoded by this gene is an adhesin and invasin for *Salmonella*.

The *pagN* gene is located on *Salmonella* centisome 7 genomic island (SCI) and this island shows a region as a mosaic structure. The *pagN* is near the *saf* (*Salmonella* Atypical Fimbria) operon, and it is positioned at 336 bp downstream of the *sinR* gene which is thought as a transcription regulator, and it is encoded backwards (Gunn et al., 1998; Folkesson et al., 1999). G+C content of this region is lower than *Salmonella* genome (Conner et al., 1998; Folkelsson et al., 1999), therefore it indicates that this region may be acquired by horizontal gene transfer.

SCI island of *S. Typhimurium* is 47 kb long and contains 37 ORFs that possibly consist of Shine-Dalgarno sequence (Folkesson et al., 2002). This genomic island encodes proteins that are predicted to be located on the outer side of the cytoplasm. These proteins are similar to the other proteins that are expressed by this island, which is found in most Gram-negative bacteria that can closely interact with eukaryotic cells, manipulate the cell response and also can be a secretion form or biosynthesis system (Folkesson et al., 2002). The mutant which is created by deletion of *Salmonella* Centisome 7 Genomic Island alters the ability to enter the eukaryotic cells. Therefore, this island has an impact on *Salmonella* ecology and survival (Folkesson et al., 1999). Likewise, *pagN* (*iviVI-A*) gene region deleted mutant and wild type strains were compared in a study and it is displayed that survival, adaptation and competitive capacities were reduced in mutant strains (Conner et al., 1998). Similar results have been observed in other experiments conducted with *pagN* mutants. For example, in the research performed by Yang and colleagues (2013) the mice were infected by wild type *Salmonella* and  $\Delta$ *pagN* *Salmonella* strain and the results revealed that 80% of mice recovered from the illness and their infection period is shorter than mice that are infected by wild type strain. It demonstrates that the *pagN* gene is an important factor for *Salmonella* virulence. In the same way, Lambert and Smith (2008) reported that *pagN* gene deletion of *S. Typhimurium* caused a decreased invasion and adhesion to the enterocytes. This situation implies that *pagN* gene is one of the essential genes for *Salmonella* entry to epithelial cells and beginning its intracellular life.

*pagN* gene is one of the PhoP activated genes (*pags*). It means that *pagN* activation depends on the PhoP/PhoQ two component regulation system. When the bacterium is in its intracellular phase, the *pagN* gene has been observed at its highest expression (Conner et al., 1998, Heithoff et al., 1999). When bacteria is in *Salmonella* containing vacuole, T3SS is downregulated because of environmental conditions. In this circumstance, bacteria can be thought to use optimally expressed PagN protein instead of T3SS to start the invasion again after they leave macrophages and epithelial cells.

### **PhoP/Q Regulation System**

The PhoP/PhoQ two component regulation system is an environmental response regulator which controls the transcription of *Salmonella* virulence genes. It is predicted that the PhoP/PhoQ system regulates the expression of 3% percent of *Salmonella* genome (Kato and Groisman, 2008). Additionally, it is presumed that it mediates at least 40 protein expressions (Groisman, 2001).

The *phoP* locus has an operon which harbours two genes *phoP* and *phoQ* that encode 224 and 487 amino acid length polypeptides (Miller 1991; Vescovi et al., 1994). This locus is prevalent among the enteric bacteria (Kato and Groisman, 2008). *Salmonella* PhoP protein is a member of the OmpR response regulator family and functions as a phosphorylation-dependent response regulator. On the other hand, PhoQ is a bifunctional protein that acts as both a kinase

and a phosphatase (Kato and Groisman, 2008). The major three starter variables for activation in the PhoPQ system have been considered to be a medium acidic pH, antimicrobial peptides, and a low magnesium content. (Lambert and Smith, 2008). Environmental activation of inner membrane PhoQ sensor kinase with one of these factors increases phosphorylation of response regulator PhoP. Phosphorylated PhoP activates the genes that encode outer membrane proteins, regulators, intracellular T3SS components, inner membrane transporters who act as buffers for cytosolic pH and enzymes that covalently modify outer membrane barrier components while it represses the genes that encode effectors of T3SS related to flagella and invasion (Dalebroux and Miller, 2014).

In general, transcriptional regulators of the two component systems family are thought to attach to enhancer regions in order to activate or repress the genes under their control (Vescovi et al., 1994). The genes activated by PhoP/PhoQ involve the pho box which has (T/G)GTTTA repeat on their promoter and thuswise, PhoP procures the gene activation (Vescovi et al., 1994; Groisman, 2001; Ktao and Groisman, 2008). Meanwhile, the PhoP/Q system positively regulates its own transcription and it is shown that this situation is critical for *Salmonella* pathogenicity.

### **PagN Protein**

PagN is encoded by the *pagN* gene of *Salmonella* which includes 239 amino acids and it is a 25,708 Da outer membrane protein whose synonym is iViVI-A, also this protein of *S. Typhi* is named as T2942 (Hume et al., 2017). This protein is mostly conserved in *Salmonella* species, subspecies and serovar levels and it exhibits allelic specificity in these status (Barilleau et al., 2021). This degree of conservation suggests that PagN protein is significant especially for *Salmonella* pathogenicity. Moreover, it may include allelic differences which are unique to the host.

This protein has 54% similarity with Hek and Tia that are the adhesin and invasin of *E. coli* (Lambert and Smith, 2008). PagN is thought to be an adhesin and an invasin based on this homology. Lambert and Smith (2008) had displayed that 60% of bacteria expressing PagN invade host cells and 27 fold more invasive than control. Furthermore, they discovered that invasion did not occur in Cytochalasin D-treated cell culture studies. Consequently, the PagN protein has been proven to be an invasin for *Salmonella*. What's more it has been shown that the PagN is masked by LPS and LPS inhibits functional receptor binding during hemagglutination (Lambert and Smith, 2008).

The host proteoglycans are used as a binding receptor by the majority of bacterial pathogens. Accordingly, Lambert and Smith (2009) have detected that PagN-mediated invasion seriously decreased in the glycosylation-deficient cell culture. This situation indicates that glycosylated proteoglycans are required for PagN-mediated invasion. Along with it, they have shown that the use of heparin reduces the rate of invasion by 90%. As a result, heparin sulphate proteoglycan (HSPG) acts as a binding receptor for PagN protein. According to Barilleau and colleagues (2021), HSPG may act as a co-receptor with  $\beta$ 1 integrin for PagN binding and they are thought to activate the internalization. Furthermore, syndecan-1, the primary protein on epithelial cell surfaces, is considered to have a function in binding. (Barilleau et al., 2021, Lambert and Smith, 2009).

The PagN protein has four loops and eight  $\beta$  strands in its secondary structure, and these strands turn to create the protein's main structure, which is the  $\beta$  barrel conformation. Deletion of the first three loops has decreased the invasion 98% rate, while deletion of the fourth loop has reduced the invasion 94% rate (Lambert and Smith, 2009). Thereby, it shows that the four loops are necessary for *Salmonella* PagN-mediated invasion. It is substantiated by Wu and colleagues (2021) that one amino acid difference in distinct loop regions of the PagN sequence

among *S. Typhi*, *S. Typhimurium* and *S. diarizonae* led to the alteration of invasion and adhesion degree.

It has been confirmed that signal transmission which led to PagN-mediated invasion needs Class I PI3 kinases (Barilleau et al., 2021). Additionally, invasion induced by PagN has been considerably decreased in cells treated with the inhibitor genistein, suggesting that tyrosine phosphorylation is essential for PagN-mediated invasion (Barilleau et al., 2021). As a result, whole evidence indicates that PagN-mediated invasion is provided by a zipper-like entrance mechanism similar to that used by Rck. The first step in the zipper mechanism is direct interaction with receptors (EGFR for Rck or proteoglycan/ $\beta$ 1 integrin for PagN) located on the surface of cells that *Salmonella* targets. This interaction results in actin polymerization, membrane reorganization, and internalization of bacteria within a vacuole. Despite all this, the intracellular behaviour of *Salmonella* still remains unclear (Ménard et al. 2022; Figure 1).

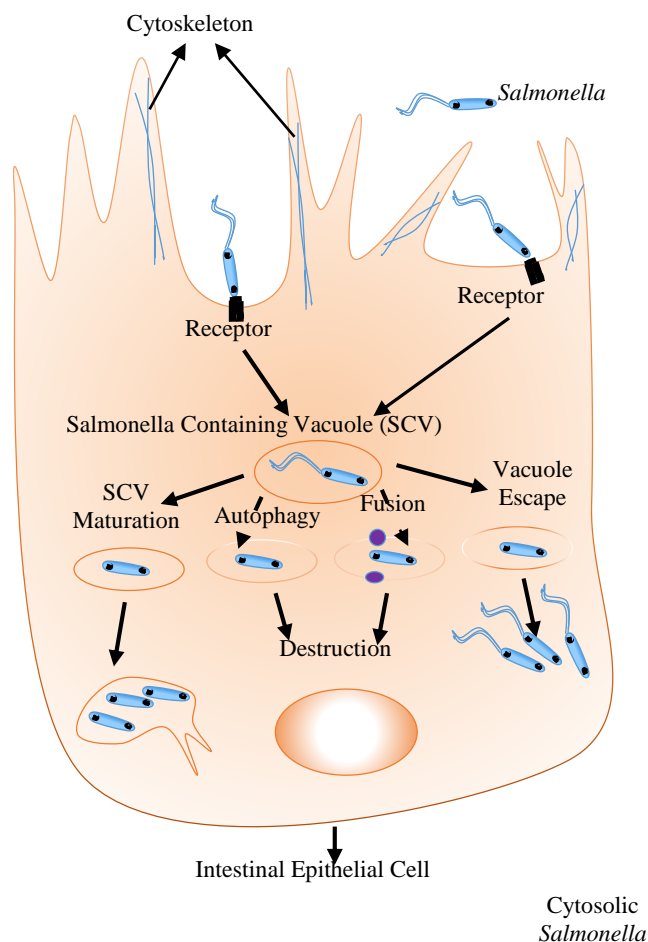


Figure 1. *pagN* mediated invasion mechanism (modified from Ménard et al. 2022)

## FUTURE PERSPECTIVES

As previously stated, the PhoP/PhoQ two-component regulatory mechanism activates certain genes while repressing others. T3SS is significantly downregulated within cells, whereas PagN is expressed at maximum level intracellularly. Therefore, it has been considered that PagN can be supplanted in the absence of a functionally T3SS to initiate the invasion again (Lambert and Smith, 2008). Although the PagN's structure and function as an adhesin and invasin have been thoroughly defined, there is still some confusion concerning this protein. It was mentioned here that PagN uses heparin sulphate proteoglycans as binding receptor,

however HSPG can't transduce a signalling cascade (Wu, 2015) thus it raises a co-receptor requirement. The exact mechanism of PagN entrance and the activated pathways that are connected with PagN invasion are still unclear. Additionally, it is unknown which secretion mechanism is employed for the translocation of the outer membrane protein PagN. Accurate receptors for PagN attachment and its invasion mechanism should be depicted in the future along with the definition of the secretion system which secretes PagN. Furthermore, whether PagN protein plays a role as an adhesin in *Salmonella* biofilm or not has not been examined. It should be investigated in the next researches.

Understanding them entirely gives a chance for learning intracellular behaviours of a bacterial pathogen in addition to reducing harsh effects on public health and economical loss caused by *Salmonella*.

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## INVESTIGATION OF THE EFFECT OF CAPSAICIN WITH SODIUM SELENITE ON MDA-MB-231 CELL LINE

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### ABSTRACT

The determination of the in vitro cytotoxic effects of active substances in foods has gained importance in recent years. There are many methods for determining cytotoxicity or cell viability. In our study, apoptosis test was performed to determine cell viability. Apoptosis is a physiological event known as cell suicide. After the cell receives a signal for apoptosis, it shows biochemical and morphological changes. It shrinks and begins to condense, the cytoskeleton disintegrates, the nuclear membrane dissolves from place to place, and the nuclear DNA fragments. The cytoplasm of a cell undergoing apoptosis shrinks. As the cell shrinks and shrinks, it breaks up into small membrane-enclosed fragments. These fragments are called apoptotic bodies. The fluorescent microscopy method we used in our study is a form of staining using fluorescent substances to determine apoptosis. Since fluorescent dyes can bind to DNA, they make the cell's chromatin, or nuclear region, visible. Apoptotic cells were visualized by using dyes that bind to DNA to detect changes in capsaicin and sodium selenite treated cells. In this way, the separation of dead and living cells was made. In our study, acridine orange:ethidium bromide 1:1 ratio was used to separate live and dead cells. MDA-MB-231 cells were seeded in 6 flasks with  $2 \times 10^5$  cells in each well, and after overnight incubation, the control was incubated with 50  $\mu\text{M}$  and 100  $\mu\text{M}$  capsaicin. At the end of 24 hours, cells were stained with 3  $\mu\text{L}$  of acridine orange (0.1 mg/ml) and 3  $\mu\text{L}$  of ethidium bromide (0.1 mg/ml) and evaluated for apoptosis under fluorescence microscope. In addition to the cells prepared in the same way, sodium selenite solution prepared in  $10^{-1}$  nM,  $10^{-2}$  nM and  $10^{-3}$  nM doses was given to the cells. The results were compared with respect to the control groups. Since acridine orange only binds to the DNA of living cells, viable cells appeared green, while ethidium bromide caused a red color in necrotic cells. However, when the two dyes were used together, they stained apoptotic cells in a yellow-orange color and reflected the characteristic apoptotic nucleus appearance, allowing the detection of apoptosis. As a result; After the application of increasing doses of capsaicin and sodium selenite, it caused a significant increase in the number of apoptotic cells, while sodium selenite positively affected this effect. The highest apoptosis rate was reached at 100  $\mu\text{M}$  capsaicin and  $10^{-1}$  nM sodium selenite concentration. The highest number of necrotic cells was observed in the concentration of 100  $\mu\text{M}$  capsaicin and  $10^{-1}$  nM sodium selenite, parallel to the number of apoptotic cells. The number of necrotic cells was determined less than the number of apoptotic cells.

**Keywords:** Capcaicin, Apoptosis, MDA-MB-231

### INTRODUCTION

Cancer is a disease that is formed by the uncontrolled growth of cells and can be found and spread in almost every organ or tissue of the body. It is an important public health problem

worldwide (Siegel et.al., 2018,2021). According to the latest data, it caused 9.6 million deaths in 2018. It is the second leading cause of death worldwide, with one in six deaths. Lung, prostate, colorectal, stomach and liver cancers are the most common cancer types in men, while breast, colorectal, lung, cervix and thyroid cancers are the most common cancers in women. According to the World Health Organization, 1.2 million people worldwide are diagnosed with breast cancer every year, and about 500 000 die from this disease (Ries et al., 2007). In cancer treatment; surgery, radiotherapy, hormone therapy, adjuvant therapy and chemotherapy are used. Among them, chemotherapy is one of the most widely used methods (Saip,2011). In this method, different drugs or drug combinations are used to kill cancer cells or stop tumor growth. Chemotherapy is the mainstay of treatment in breast cancer. Usually, a combination of more than one drug is used, where the use of multiple drugs is more effective in chemotherapy (Portakal et.al. 2000, Sener 2010). Studies are carried out on plants and their active ingredients in order to find drugs that stop or slow down the progression of cancer. One of these studies was Spyridopoulou et al. (2021) Selenium (Se) was used in the study. Selenium is preferred because it is abundant in sedimentary rocks and can be easily made bioavailable by erosion of the soil or reduction by microorganisms. Since Se has been reported as a promising compound in the prevention and treatment of colorectal, lung and prostate cancers, it is used in combination to prevent the side effects of chemotherapy drugs. In addition to this study, Büyüksulu et al. (2015) stated that selenium inhibits the energy generation pathway of cancer cells by slightly blocking glucose uptake. They noticed that the MCF7 cell line used in breast cancer studies was sensitive to selenium, while the MDA-MB-231 cell line was resistant to selenium. It is emphasized that this new approach may have great potential to increase drug sensitivity in breast and colon cancer cells (Kempaiah 2004, Karabulut 2008).

Capsaicin, which is used in our study, is an alkaloid substance that is called *Capsicum annuum*, which is the capsicum genus of the solanaceae family, which gives the hot pepper its bitterness. Capsaicin was first isolated from red hot pepper in 1846 and named capsaicin (Amantini et al., 2007). In addition to its use as a spice, hot pepper has also had an important place in the field of medicine for many years and has been used. In addition, it has been observed that capsaicin prevents pain signals from reaching the brain by acting on unmyelinated nerve fibers that are involved in the transfer of pain sensations from the periphery to the center (Başak et.al. 2005). It has been used a lot in alternative medicine creams due to its analgesic properties. As a result of the studies, the effects of capsaicin vary depending on the dose and duration of use (Surh 1995,2002). The most important feature of capsaicin is that it is an antioxidant. Thanks to this feature, it acts as a cancer suppressing agent, blocks several signal transmission pathways and shows its anti-cancer effect. It shows this effect differently at low and high doses. It has been revealed by studies that the application of low doses stimulates the immune system, while the high dose suppresses the immune response. While capsaicin is known to be effective in both cell proliferation and apoptosis (Kim et.al. 1997), its mechanism at the molecular level is still not fully elucidated. As a result of the studies, the most common diagnosis is that capsaicin inhibits the growth of cancer cells by affecting apoptosis (Jang 1989, Park 1997, Czaja 2002, Tanaka 2002, Mori 2006). Apoptosis; It is a genetically controlled programmed cell death that ensures the safe destruction of cells that are not needed as a requirement of intercellular relations in advanced organisms, whose functions are impaired, which are overproduced, aged, irregularly developed or whose DNA is damaged, without harming the environment (Thompson 1999). Cells in the tissue undergoing apoptosis dissociate from their junctional sites, lose specialized surface organelles and shrink significantly, lose 1/3 of their volume in a few minutes, budding at the plasma membrane, and the cell disintegrates into apoptotic bodies composed of fragments of chromatin surrounded by cytoplasm (Huppertz et al., 1999, Willingham 1999, Barisic et al., 2003, Brouckaert et al., 2004). In order for the cell

to undergo apoptosis, it must first encounter a signal that will activate the genetic mechanism related to apoptosis. This signal can come from inside the cell (DNA damage, etc.) or outside the cell (hypoxia, heat, anticancer drugs, etc.). Signals coming from outside the cell are transmitted by death receptors and adapter proteins, and internal signals are transmitted via mitochondria. Apoptosis begins in the cell that receives the signal (Chinnaiyan et al., 1995). Apoptotic signals such as DNA damages, radiation and growth factor deficiency caused by chemotherapeutic agents cause an increase in the permeability of the outer membrane of the mitochondria. This is the most important factor initiating apoptosis (Korsmeyer 1999, White and McCubrey 2001). A wide variety of methods have been developed to detect apoptosis. The fluorescent microscopy we used in our study is one of these methods. In this study, the effect of synthetic capsaicin and selenium molecule on MDA-MB-231 breast cancer cell line was evaluated in terms of apoptosis.

## **MATERIAL METHOD**

### **Preparation of Stock Solutions**

#### **1. Preparation of Sodium Selenite Solution**

A standard selenium solution was prepared by taking 2.39 mg of powdered sodium selenate ( $\text{Na}_2\text{SeO}_4$ ) and dissolving it in 100 ml of water. Stored at  $-20\text{ }^\circ\text{C}$ . The prepared main stock solution was diluted with the medium and stored at the desired concentration.

#### **2. Preparation of Capsaicin Solution**

Capsaicin (305.41 gr/mol) dissolves in dimethyl sulfoxide (DMSO). A stock solution was formed by dissolving 0.061 g of capsaicin in 20 mL of dimethyl sulfoxide (DMSO). This created solution was stored at  $-20\text{ }^\circ\text{C}$ .

### **Characteristics and Preparation of the Cell Line**

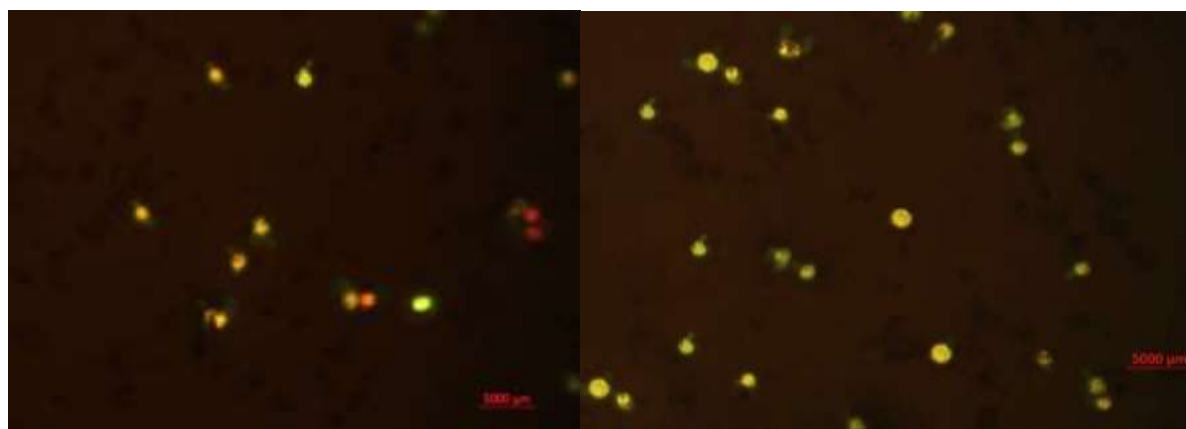
**MDA-MB-231 Cell Line:** It is a human breast cancer cell line. Estrogen receptor and E-cadherin are absent. It is active for Caspase 2 and 3. It can spread widely. The MDA-MB-231 breast cancer cell line used in our study was obtained from the American Type Culture Collection (ATCC). Frozen cells of the MDA-MB-231 cell line are removed from the nitrogen tank and incubated at  $37\text{ }^\circ\text{C}$  for 5 minutes. Cells were grown in DMEM-F12 Raw medium (Dulbecco's Modified Eagle Medium, nutrient mixture F12 Raw medium) containing inactivated 10% fetal bovine serum (FCS), 0.2 mM glutamine, 100  $\mu\text{g/ml}$  streptomycin and 100 IU/ml penicillin are cultured at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$  and 1 atmosphere pressure. 10 mL of cell culture medium is added to it and shaken gently. The prepared culture medium is checked under a light microscope and incubated at  $37^\circ\text{C}$  in an oven containing 5%  $\text{CO}_2$ . At the 24th hour of cell culture cultivation, the viability of the cells is checked under the light microscope. Then, it is first washed with 10 mL of phosphate buffered saline solution (PBS) and 10 mL of the prepared cell culture medium is added. This process is repeated every 48 hours and the cell culture is incubated at  $37^\circ\text{C}$  in an oven containing 5%  $\text{CO}_2$ . When the growing cells fill approximately 70-80% of the flask floor, after the cell culture is washed 3 times with 5 mL of PBS in a laminar cabinet, 2 mL of Trypsin-EDTA is added. After 3 min, 10 mL of cell culture medium is added to the cells. Cells separated from the flask are taken into a 15 mL polypropylene tube and centrifuged at  $4^\circ\text{C}$  2100 rotation speed/min for 10 min. Cells that settled to the bottom after centrifugation are diluted to  $4 \times 10^5$  cells per milliliter. Then, cells are seeded into 24-well flasks,  $2 \times 10^5$  in each well. When the cells cover the floor of the wells in the flash by 70-80%, groups are formed and the other stages of the study are started.

### Apoptosis Staining Method

Fluorescence microscopy method was used to determine apoptosis. In this method, using fluorescent substances, the cell's chromatin and thus its nucleus becomes visible. In our study, acridine orange: ethidium bromide 1:1 ratio was used to separate live and dead cells using BAB image analysis systems microscope. MDA-MB-231 cells were seeded in 6 flasks with  $2 \times 10^5$  cells in each well and after overnight incubation, the control was incubated with 50  $\mu\text{M}$  and 100  $\mu\text{M}$  capsaicin. After 24 hours, cells were stained with 3  $\mu\text{L}$  of acridine orange (0.1 mg/ml) and 3  $\mu\text{L}$  of ethidium bromide (0.1 mg/ml) and evaluated for apoptosis under fluorescence microscope. In addition to the cells prepared in the same way, sodium selenite solution prepared in  $10^{-1}$  nM,  $10^{-2}$  nM and  $10^{-3}$  nM doses was given to the cells. The results were compared with respect to the control groups. Since acridine orange only binds to the DNA of living cells, viable cells appeared green, while ethidium bromide caused a red color in necrotic cells. However, when the two dyes were used together, they stained apoptotic cells in a yellow-orange color and reflected the characteristic apoptotic nucleus appearance, allowing the detection of apoptosis.

### CONCLUSIONS AND DISCUSSION

In this study, it was aimed to observe the effect of synthetic capsaicin and sodium selenite on MDA-MB-231 breast cancer cell line. Based on the findings obtained as a result of the analyzes carried out for this purpose, it has been determined that capsaicin does not have a reducing effect on cell growth at low doses, it supports apoptosis when we exceed the LD50 dose, and it causes a significant increase in the number of apoptotic cells after the application of capsaicin and sodium selenite at increasing doses. It has been determined that sodium selenite protects cells at low doses and has toxic effects at high doses. The highest apoptosis rate was reached at 100  $\mu\text{M}$  capsaicin and  $10^{-1}$  nM sodium selenite concentration. The highest number of necrotic cells was observed in the concentration of 100  $\mu\text{M}$  capsaicin and  $10^{-1}$  nM sodium selenite, parallel to the number of apoptotic cells. The number of necrotic cells was determined less than the number of apoptotic cells.



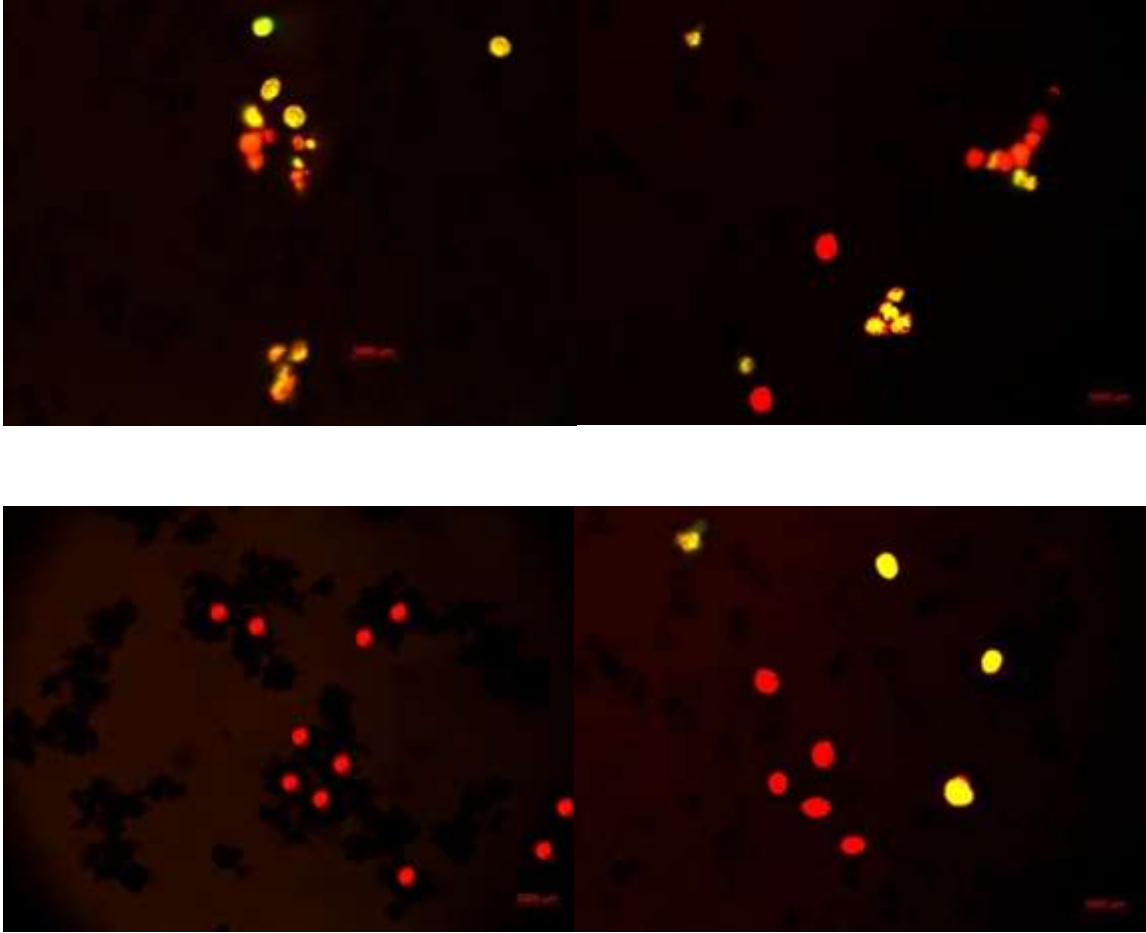


Figure 1: Microscope images of apoptosis test of capsaicin and sodium selenite administration at 24 hours in MDA-MB-231 cells ( a: Control , b:  $10^{-1}$  nM sodium selenite, c 50  $\mu$ M capsaicin, d : 50  $\mu$ M capsaicin and  $10^{-1}$  nM sodium selenite , e : 100  $\mu$ M capsaicin and  $10^{-1}$  nM sodium selenite f : 100  $\mu$ M capsaicin)

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**EFFECTS OF *csgD*, *fliZ*, *stjC*, *yaiC* AND *rmbA* GENES ON BIOFILM STRUCTURES FORMED AT SOLID-AIR AND LIQUID-AIR INTERFACES IN *SALMONELLA TYPHIMURIUM* 14028 STRAIN**

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**ABSTRACT**

In this study, comparative phenotypic analyzes were performed using *S. typhimurium* 14028 strain and its mutants of *csgD*, *fliZ*, *stjC*, *yaiC* and *rmbA* genes deleted by homologous site recombination technique. As a result of the analyzes carried out, it was determined that the biofilms formed in both solid-air and liquid-air phases in all mutant strains were statistically significantly reduced compared to the wild-type strain. The gene with the highest effect on biofilm structures was determined as *csgD* gene. Because, in the mutant in which this gene is deleted, biofilm formation decreased by 97-99%. Biofilm morphotyping studies; It has been determined that the morphotype (saw) that cannot produce biofilm due to the blockage of cellulose and curli fimbriae production in the *csgD* gene mutant, and intermediate morphotypes that differ from the wild-type strain due to the decrease in the production of cellulose and curli fimbriae in other mutants. Planktonic motility (swimming) and community motility (swarming) were decreased at the highest level in *csgD* mutant especially due to the abolition of cellulose production. Since cellulose production continued, albeit at a reduced rate, in other mutants, significant reductions were observed in both movements compared to the wild-type strain.

**Key Words:** *S. Typhimurium*, *csgD*, *fliZ*, *stjC*, *yaiC*, *rmbA*, biofilm

**INTRODUCTION**

*Salmonella* is a foodborne pathogen that causes many cases of typhoid fever, gastroenteritis, and related deaths worldwide (Ruby et al., 2012). The ability of these bacteria to adhere to biotic and abiotic surfaces and to form biofilms on these surfaces is important in all areas of life, especially in food production and medical disinfection. Dirt and food residues on surfaces that are not constantly cleaned properly allow pathogens to adhere to these surfaces. In this way, pathogenic microorganisms become permanent by forming a biofilm structure on the surfaces they attach to. Microbial biofilms are the most important factors triggering foodborne disease outbreaks by causing contamination of food products. Although *Salmonella* is mainly detected in meat, milk and egg products, the frequency of *Salmonella* outbreaks originating from vegetables and vegetable products has been increasing in recent years. Especially fresh products such as lettuce, cucumber, watermelon, melon, parsley, and fruit juices are risky foods for salmonellosis cases. The starting point to minimize this risk is to take control measures at the relevant stages of food production, processing, and distribution (Shen and Fang, 2012). The most important problem here is which life form will be developed on the basis of *Salmonella* control strategies. Because classical sanitation strategies targeting

planktonic forms of *Salmonella* are ineffective due to the high resistance properties of these bacteria in biofilm forms (Le Guyon et al., 2014).

Planktonic forms of *Salmonella* strains also have resistance systems against environmental stress conditions such as temperature, antibiotic supply, heavy metal ion concentration and salinity, but the resistance in biofilm forms is much higher than planktonic forms. The main reasons for this situation are the ability of the extracellular polymeric matrix (EPS) forming the biofilm community to compensate for adverse external environmental conditions, as well as the global genetic expression differences that occur in the transition from the planktonic form to the multicellular biofilm form in these bacteria. The most important genetic expression differences in terms of biofilm structures are the changes in the genes that control the production of cellulose, curli fimbriae and other fimbriae and flagella (Shen and Fang, 2012; Spector and Kenyon, 2012). Determining the changes in these genes will allow to understand the regulation of the biofilm and, accordingly, to develop effective agents for the control of biofilm structures.

Curli fimbriae and cellulose are the main components of the biofilm matrix. These structures are the most effective factors in the environmental persistence and resistance of *Salmonella* biofilms to stress conditions. Based on the presence of these two elements in the extracellular polymeric matrix structure, *Salmonella* biofilms are classified as rdar (red, dry, and rough morphotype containing curli fimbriae and cellulose), pdar (containing only cellulose, no curli fimbriae, brown, dry and rough morphotype), bdar (only curli fimbriae, free of cellulose, pink, dry, and rough) and saw (free of cellulose and curli fimbriae, smooth and white). Among them, the most resistant biofilm form to environmental conditions was determined as radr (Solano et al., 2002). Therefore, in addition to these two structural elements of the EPS matrix, it is of great importance to define the structural and regulatory characteristics of the fimbriae that control the adhesion of biofilm cells to each other and to surfaces, and the flagellar genes important for cellular movement in biofilm structures.

In this study, it is known that *csgD*, one of the genes whose activities were investigated on the phenotypic properties of biofilm structures, positively regulates the transcription of the operon responsible for the production of curli fimbriae (*csgBAC*) (Hammar et al., 1995) and also contributes to cellulose production by increasing the expression of *adrA* (Gerstel and Romling, 2003). It is suggested that *adrA* (*yaiC*), which encodes a diguanylate cyclase enzyme, directly participates in cellulose production by activating the cellulose synthase (Simm et al., 2004; Cotter and Stibitz, 2007; Ahmad et al., 2011; Le Guyon et al., 2014). On the other hand, there is evidence that flagellar genes are differentially expressed at different phases of biofilm formation and modulate biofilm formation (Iyoda et al., 2001; Crawford et al., 2010). However, there is no information in the literature about their role in biofilm formation of RmbA which acts as a porin in the translocation of MisL autotransporter protein (Tükel et al., 2007; Hamilton et al., 2009) and Stj fimbria, which has been defined as the pathogen-related molecular pattern (PAMP) of *Salmonella* (Akçelik and Akçelik, 2021).

In this study, it was aimed to define the direct effects of *csgD*, *fliZ*, *stjC*, *yaiC* and *rmbA* genes selected in line with the literature data summarized above, on *Salmonella* biofilms by performing comparative phenotypic tests.

## **MATERIAL AND METHOD**

### **Bacterial strains and culture conditions**

*Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028 was obtained from the American Type Culture Collection (ATCC) and *csgD*, *fliZ*, *stjC*, *yaiC* and *rmbA* mutants, obtained from the culture collection of Ankara University, Faculty of Science, Department of Biology, Prokaryote Genetics Laboratory by using the lambda red recombinase

method. Strains were grown in Luria Bertani (LB) broth (chloramphenicol (20 µg/mL) added to the medium for mutant strains) at 37 °C at 200 rpm for 18 h in a shaking incubator.

### **Quantification of biofilm formation and determination of biofilm morphotypes**

To determine the optimum biofilm production capacities of wild-type and all mutant strains, a 5-day trial was set up on a polystyrene substrate. Since the biofilm structure is the protection mechanism of bacteria under stress condition, heat and salt stress were applied to the strains to produce biofilm. For heat stress to strains with optimum growth temperatures of 37 °C, the incubation temperature was kept at 20 °C throughout the experiment and the experiment was carried out in salt-free LB Broth (LB<sup>-NaCl</sup>) medium. On the first day of the experiment, the optical density of the overnight cultures was adjusted to 0.2 ABS at OD<sub>595</sub> and 30 µL of these cultures were inoculated into 100 µL of pre-added LB<sup>-NaCl</sup> in a 96-well microtiter plate. Only 100 µL of LB<sup>-NaCl</sup> was added to the negative control wells. After loading in 3 parallel wells every day and for each strain, they were incubated at 20 °C. After every 24 hours, the wells of that day were washed 3 times with 140 µL 1X PBS to remove planktonic cells and medium arcs, and after fixation of the biofilm cells attached to the surface with 140 µL of 95% methanol, incubation was continued at 20 degrees for the following days. At the end of the 5th day, 140 µL of 1% crystal violet was added to all wells, incubated for 40 minutes at room temperature and excess dye was removed with distilled water. To dissolve the dye that only binds to the biofilm cells, 140 µL of 33% glacial acetic acid was added to the wells and kept at room temperature for 45 minutes. At the end of the incubation period, the biofilm production capacities of all strains on all days were determined by measuring the dye density in OD<sub>595</sub> with an ELISA reader (Stepanovic et al., 2000; Woodward et al., 2000).

To determine biofilm morphotypes, LB<sup>-NaCl</sup> agar supplemented with Congo red (40 mg/L, Sigma-Aldrich Steinheim, Germany) and controlled LB<sup>-NaCl</sup> agar supplemented with Calcofluor and LB<sup>-NaCl</sup> water were incubated at 20 degrees for 8 days (20 mg/L, Sigma-Aldrich Steinheim, Germany) (Marin et al., 2009). The study was conducted in two parallel and two repetitions.

### **Swimming and swarming capability**

To evaluate the motility characteristics of *S. Typhimurium* wild-type and mutant strains, overnight cultures were inoculated on LB agar plates and incubated overnight at 37 °C. At the end of the incubation period, a single colony was taken from the agar surface and inoculated into the center of the 0.3% LB agar plate for 'swimming' motility. To determine the 'swarming' motility; A single colony was removed from the agar surface and inoculated into 0.5% LB agar containing 0.5% glucose. Agar plates were incubated for 9 hours at 20 °C, 28 °C and 37 °C separately each other. The zone from the center to the edge of the swimming and swarming motility zone was evaluated by measuring its diameter (Ahmad et al., 2016). This trial was performed in 3 parallel and 2 repetitions (p<0.05).

## **RESULTS AND DISCUSSION**

Biofilm production capacities of *S. Typhimurium* ATCC 14028 wild-type strain and its *csgD*, *fliZ*, *stjC*, *yaiC* and *rmbA* mutants were observed for 20, 24, 48, 72, 96 and 120 hours. In our study, 20 °C was used as the optimum biofilm production temperature, as determined by both other researchers (Romling et al., 1998) and our study group (Uğur et al., 2018) (Figure 1). Biofilm production capacity was significantly reduced in all mutant strains compared to wild type (P<0.05). The most significant reduction (97-99%) for all time was detected in the

*csgD* mutant. After wild-type strains and mutants reached their maximum biofilm production capacity at 72 hours, biofilm production continued to decrease in the following periods.

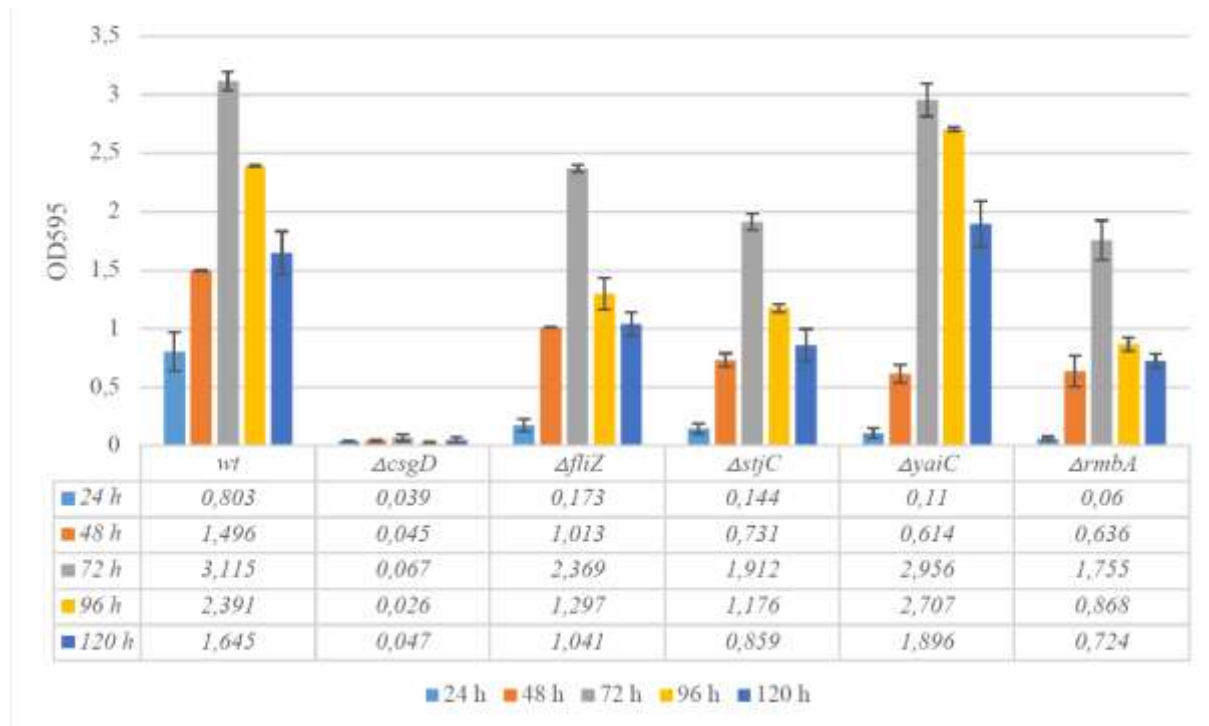


Figure 1: Biofilm production capacities of *S. Typhimurium* ATCC 14028 wild-type strain and its *csgD*, *fliZ*, *stjC*, *yaiC* and *rmbA* mutants

There are many studies on the time-dependent variation of biofilm production in *S. enterica*. In these articles, it has been suggested that the expression of major extracellular matrix components (curli fimbriae and cellulose) that stabilize the biofilm structure is associated with CsgD, a transcriptional regulator (Gerstel and Römling, 2001, 2003; Gerstel et al., 2003; Römling, 2005; Kim and Wei, 2009; White et al., 2010; Steenackers et al., 2012). In this study, it is important to determine that the effect of *csgD* mutant on biofilm production capacity is higher than all other genes, in terms of phenotypic evidence of the literature data summarized above.

In the second phase of the study, biofilm morphology determination studies were performed in *S. Typhimurium* ATCC 14028 wild-type strain and its *csgD*, *fliZ*, *stjC*, *yaiC* and *rmbA* mutants. The saw (smooth and white) morphotype was detected in the *csgD* mutant, in which the expression of cellulose and curli fimbriae did not occur. In the *yaiC* mutant responsible for cellulose production, the bdar (brown, dry and rough) morphotype, in which only curli fimbriae is expressed, was determined. In *fliZ*, *stjC* and *rmbA* mutants, intermediate morphotypes very close to the bdar morphotype were defined due to the fact that curli fimbriae synthesis was not affected, but cellulose production was decreased at a high level. Strains cultured on LB<sup>-NaCl</sup> agar containing the cellulose indicator chalcoflour, and colonies examined under 366 nm UV light in *csgD* and *yaiC* mutants were negative for cellulose production, while cellulose production was found to be highly decreased in other mutants. In tests based on the evaluation of pellicle forming properties in the liquid-air interface, it was determined that pellicle production was significantly reduced in all mutant strains compared to the wild-type strain (Figure 2).

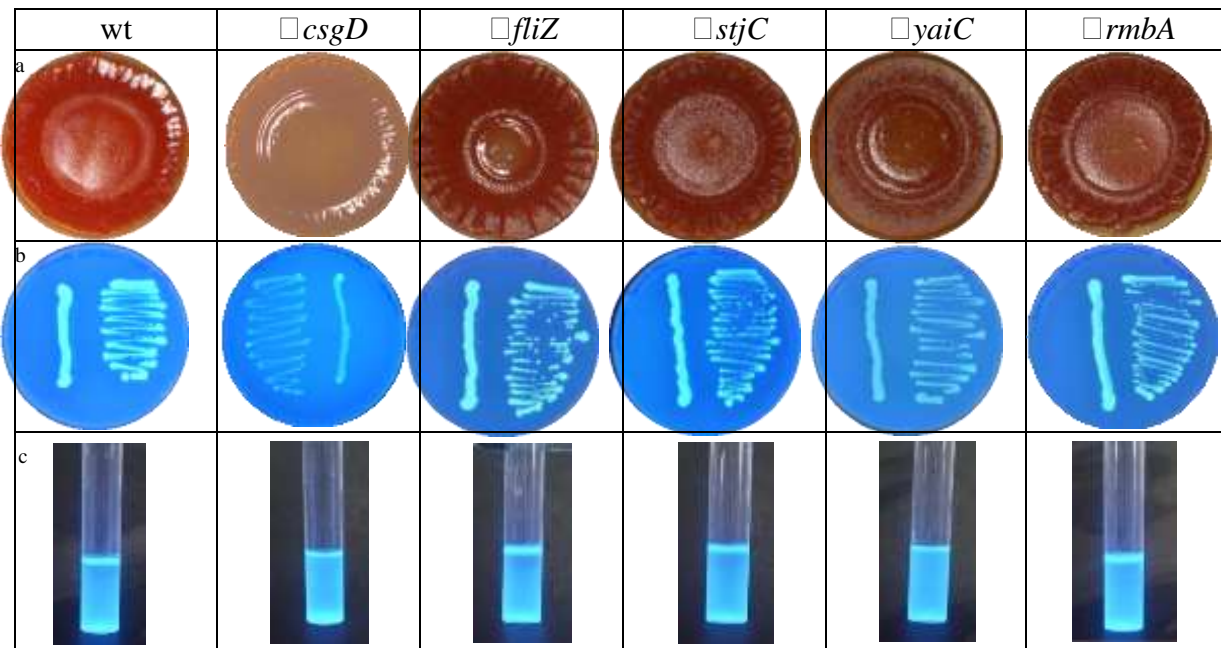


Figure 2: Determination of biofilm morphotypes of *S. Typhimurium* ATCC 14028 wild-type strain and its *csgD*, *fliZ*, *stjC*, *yaiC* and *rmbA* mutants a) Morphotypes of wild-type and mutant strains on LB<sup>-NaCl</sup> agar containing Congo red. b) Determination of cellulose production capacity of wild type and mutant strains on LB<sup>-NaCl</sup> agar containing calcofluor. c) Liquid- interphase pellicle production capacities of wild type and mutant strains in LB<sup>-NaCl</sup> broth containing calcofluor.

These findings from our study provide additional phenotypic evidence that the *csgD* transcriptional regulator is responsible for the formation of the rdar morphotype, which is known to be the most resistant biofilm morphotype to environmental stress conditions in *S. Typhimurium*. These findings, as in the literature data (Kader et al., 2006; Kim and Wei, 2009; White et al., 2010; Van Puyvelde et al., 2013; Bordeau and Felden, 2014), show that the "rdar" morphotype is the main target in the fight against biofilm forms. These findings are also important in that they indicate that the molecular target can be chosen as *CsgD* in antibiofilm agent development studies. On the other hand, it is important in this respect that no cellulose production was detected in *yaiC* mutants. Because hard biofilm structures that have lost their elasticity due to the production of curli fimbriae in strains that do not produce cellulose become very sensitive to physical effects (Scher et al., 2005; Vestby et al. 2009; Chia et al., 2011). It is possible to say that the other gene mutants used in our experiment are less important in this respect. Swimming motility in wild-type strain and the mutants of *S. Typhimurium* decreased in direct proportion to the change in incubation temperature. At all incubation temperatures attempted, motility was decreased the most in the *csgD* mutant (90%), followed by *fliZ* (77%). While the motility of *stjC*, *yaiC* and *rmbA* mutants decreased by 30% at 37 °C, this rate was determined as 66-88% at 28 °C and 20 °C, respectively (Figure 3, Figure 4).



















| Swimming motility | 37 °C  | 28 °C  | 20 °C   |
|-------------------|--|--|---|
| Wild-type         | 42 mm<br>   | 30 mm<br>   | 9 mm<br>   |
| <i>ΔcsgD</i>      | 6 mm<br>    | 3 mm<br>    | 1 mm<br>   |
| <i>ΔfliZ</i>      | 11 mm<br>   | 7 mm<br>    | 3 mm<br>   |
| <i>ΔstjC</i>      | 28 mm<br> | 10 mm<br> | 3 mm<br> |
| <i>ΔyaiC</i>      | 32 mm<br> | 10 mm<br> | 2 mm<br> |
| <i>ΔrmbA</i>      | 30 mm<br> | 12 mm<br> | 5 mm<br> |

Figure 3: Flagella-dependent swimming motility of *S. Typhimurium* ATCC 14028 wild-type and its *csgD*, *fliZ*, *stjC*, *yaiC* and *rmbA* mutants

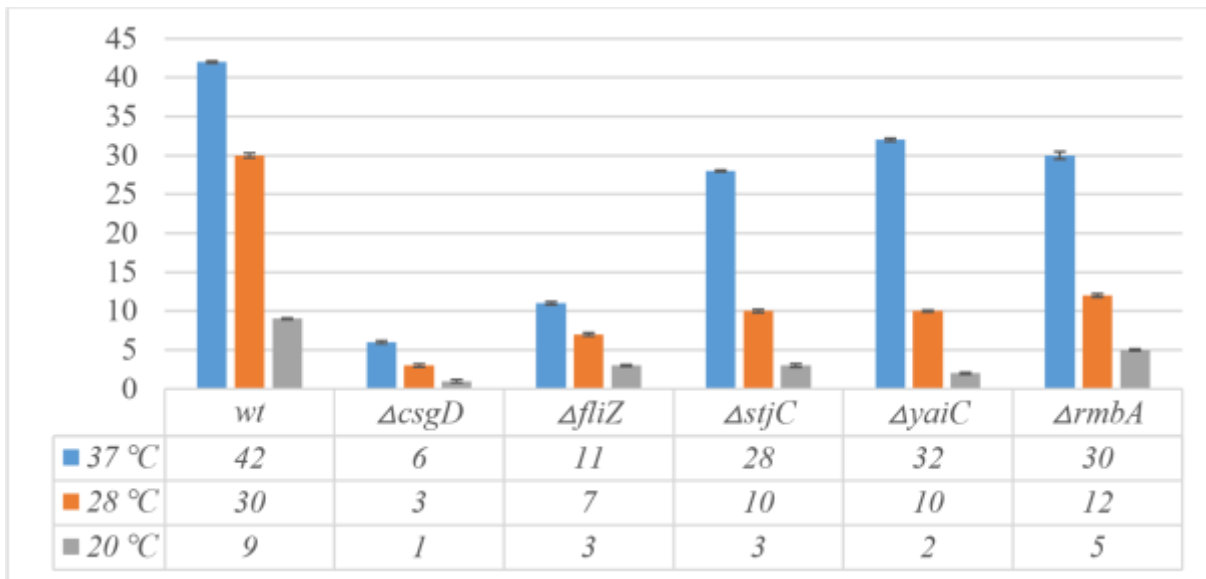


Figure 4: Swimming motility of *S. Typhimurium* wild-type strain and its *csgD*, *fliZ*, *stjC*, *yaiC* and *rmbA* mutants

In the study in which the swarming motility of *S. Typhimurium* wild-type strain and its mutants were determined, the highest motility of all strains was at 37 °C, while the motility of mutant strains was significantly reduced compared to wild-type at all tested temperatures (37 °C, 28 °C and 20 °C). No motility was observed in *csgD* and *fliZ* mutants at 20 °C (Figure 5, Figure 6).

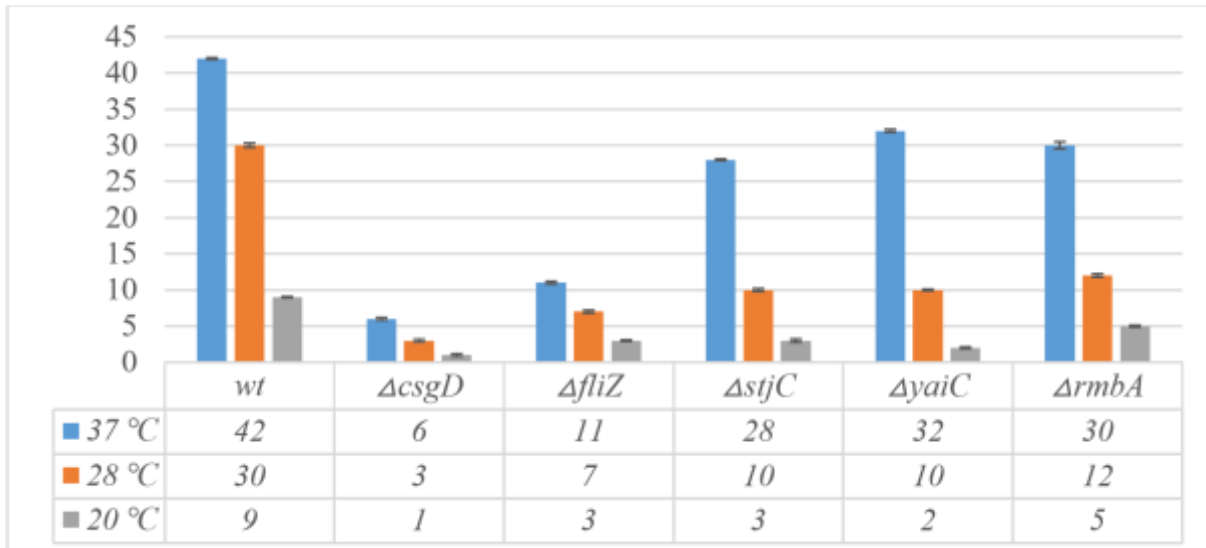


Figure 5: Swarming motility of *S. Typhimurium* wild-type strain and its *csgD*, *fliZ*, *stjC*, *yaiC* and *rmbA* mutants

Bacterial swimming (individual) and swarming (community) motility are directly related to pathogenicity and virulence. *S. Typhimurium* is an enteric bacterium adapted to the human body (Tan et al., 2014). In our study, the highest motility in *S. Typhimurium* and all its mutants occurred at 37 °C, which is a clear sign of this adaptation. On the other hand, the fact that these bacteria suppress their motility-related genes and tend to a more sessile form as a result of

various cellular regulations at low temperatures is proof that they protect themselves by forming a biofilm life form.



















| Swarming motility | 37 °C  | 28 °C  | 20 °C  |
|-------------------|--|--|--|
| Wild-type         | 40 mm<br>   | 15 mm<br>   | 8 mm<br>         |
| <i>ΔcsgD</i>      | 10 mm<br>   | 2 mm<br>    | No movement<br>  |
| <i>ΔfliZ</i>      | 17 mm<br>  | 9 mm<br>   | No movement<br> |
| <i>ΔstjC</i>      | 30 mm<br> | 10 mm<br> | 2 mm<br>       |
| <i>ΔyaiC</i>      | 13 mm<br> | 9 mm<br>  | 3 mm<br>       |
| <i>ΔrmbA</i>      | 16 mm<br> | 9 mm<br>  | 3 mm<br>       |

Figure 6: Flagella-dependent swarming movements of *S. Typhimurium* ATCC 14028 wildtype strain and its *csgD*, *fliZ*, *stjC*, *yaiC* and *rmbA* mutants.



## CONCLUSIONS

In our study, which is based on explaining the environmental stress adaptation and survival mechanisms of *Salmonella* with phenotypic and genetic evidence, it was determined that these bacteria mainly modulate the mechanisms controlling cellular and community motility, as well as the synthesis of curli fimbriae and cellulose in the transition from the planktonic form to the biofilm form. These findings are of critical value in understanding *Salmonella*'s development of resistance to different environmental stress conditions and, more importantly, in identifying molecular targets to be used in fighting against biofilm structures.

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## CLIMATE CHANGE AND FOOD SECURITY

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### ABSTRACT

Food security, which means dependable access to safe, affordable, and nutritious food is completely linked with climatic conditions of ecosystems. Weather describes the behavior of the atmosphere in the short term in a particular region, while the term climate describes the long-term more general weather conditions in larger areas. While a significant part of the sun's rays reaching the earth is reflected from the earth, our atmosphere sends some of the sun rays reflected from the earth back to the earth with the help of gases such as carbon dioxide, methane and water vapor, which are also described as greenhouse gases. With the greenhouse gases that act as insulation in this way, the average temperature on earth reaches 15 °C levels, allowing humans, animals and plants to continue their lives. This natural effect of greenhouse gases is called the “greenhouse gas effect”. The amount of greenhouse gases in the composition of the atmosphere started to increase after the industrial revolution, and the rate of carbon dioxide in the atmosphere increased by 40% in this process. The burning of fossil fuels, especially coal, is the main responsible for the increase in the carbon dioxide ratio in the atmosphere, and due to this proportional increase, the earth's temperature has warmed over 1 °C nowadays. Climate change negatively impacts conditions that have devastating effects on food security, livelihoods and human health. While the effects of climate change on agricultural production are increasingly felt all over the world, most of the food production systems are pushed to the breaking point at the point of adequate and balanced nutrition for people. In addition to all these, climate change also threatens global food security through reduced availability of local agricultural products, price increases, logistics problems and production cuts due to raw materials. In short, climate change puts adequate and quality food production at risk. In this context, in order to avoid the worst climate change scenarios in the future, it is imperative that we must reduce the carbon emission rate and create a world where the average global temperature rise does not exceed 2 °C.

**Keywords:** Food security, climate change, carbon emission, food safety, greenhouse gases.

### INTRODUCTION

In the 21st century, human health is under threat of climate change. Since human and animal are ecosystem's participants, climate change will affect overall food accessibility (Rocha et al., 2022). Direct and indirect effects of climate change can be observed on agriculture as decline in crop production. As a consequence, food prices and markets are affected by the extreme climatic events. Global food security is threatened by climate change, any attempt

contributing to the occurrence of climate change should be prevented by humankind and with policies accepted by global authorities.

### **Climate Change and Extreme Weather Conditions**

Weather describes the behavior of the atmosphere in the short term in a particular region, while the term climate describes the long-term more general weather conditions in larger areas. Climate change is characterized by temperature and rainfall events (Purwaningsih et al., 2022). Internationally and domestically observed effects of climate change are irregular weather conditions as extended periods of increased temperature and precipitation trends, droughts, floods, elevated sea level, retreating of global ice sheets (Abbass et al., 2022). In climate change usually changes are observed over 30 years (Birgani et al., 2022).

Climate change and global warming are important global changes of past 65 years and the effects are widely observed in the world (Abbass et al., 2022). The impact of climate change on agriculture is evidenced by decrease in crop production and directly appeared as higher food prices (Birgani et al., 2022).

### **Food Security and Climate Change Relation**

Food security, which means dependable access to safe, affordable, and nutritious food is completely linked with climatic conditions of ecosystems. According to FAO (2002), "Food security requires that 'all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life'".

Climate change affects food security in 4 dimensions (Barokatuminalloh et al., 2021).

- 1) Food availability
- 2) Food Access
- 3) Food utilization
- 4) Food system stability

Globalization affected food chain. Food system is under pressure of climate change, increased population, environmental challenges and political economic difficulties (Alexandratos & Bruinsma, 2012). Climate change threatens global food security through reduced availability of local agricultural products, price increases, logistics problems and production cuts due to raw materials (Badgley&Perfecto, 2007).

Food access to agricultural products is declining due to losses in incomes, and people in low-income countries have to spend more money on food supply. This is an indication that food safety is in danger (Barokatuminalloh et al., 2021). While the effects of climate change on agricultural production are increasingly felt all over the world, most of the food production systems are pushed to the breaking point at the point of adequate and balanced nutrition for people (Challinor et al., 2015).

By the effect of climate change, food consumption will be decreased. Food prices would be increased one and a half fold as it is food prices would increase as 35% in low income countries and 23% in high-income countries. The difference in high-income and low-income countries is based on the fact that presence of better fundamental conditions in high-income countries and policies followed against climate change. Drought is the most common climatic event that is responsible for higher food prices (Birgani et al., 2022).

Climate change related weather extremes stimulates crop deterioration. Falling, discoloration etc. The integrity of the ecosystem will be spoiled by climate change. Species that can tolerate environmental stress can survive. The extinction of species is also pronounced related to climate change.

**Factors affecting crop production:**

- Temperature
- Solar radiation
- Precipitation
- CO<sub>2</sub>

**Impact of Greenhouses Gases on Climate Change and Food Security**

The increasing world population leads to demand for food. Food production and consumption should be in balance for continuous supply of food (Manteghi et al., 2022). In 2021, there exist a drastic change in global foods sector and also energy demand in the world. Since world population is increasing, the demand for food also increases which brings higher energy consumption (Malliaroudaki et al., 2022).

CO<sub>2</sub>, CH<sub>4</sub>, N<sub>2</sub>O and H<sub>2</sub>O are greenhouse gases exist in the atmosphere and released to the atmosphere by volcanoes, forest fires and seismic activities before industrialization. Agricultural production is the fundamental sector account for 30-40% of all greenhouse emissions so have an important effect on global warming and climate change. It is announced that the world could see a temperature rise from 1-3.7°C at the end of this century meaning that very adverse effects will be observed in crop production (Abbass et al., 2022).

Greenhouse gases are the indicators of environmental challenges faced due to climate change and global warming.

- Pollution
- Global warming
- Environmental degradation
- Loss of biodiversity
- Depletion of natural resources are the environmental challenges.

While a significant part of the sun's rays reaching the earth is reflected from the earth, our atmosphere sends some of the sun rays reflected from the earth back to the earth with the help of gases such as carbon dioxide, methane and water vapor, which are also described as greenhouse gases. (Lindsey, 2009). With the greenhouse gases that act as insulation in this way, the average temperature on earth reaches 15°C levels, allowing humans, animals and plants to continue their lives. This natural effect of greenhouse gases is called the “**greenhouse gas effect**”. (IPCC, 2007).

The amount of greenhouse gases in the composition of the atmosphere started to increase after the industrial revolution, and the rate of carbon dioxide in the atmosphere increased by 40% in this process (United Nations Report, 2020). The burning of fossil fuels, especially coal, is the main responsible for the increase in the carbon dioxide ratio in the atmosphere, and due to this proportional increase, the earth's temperature has warmed over 1°C nowadays (United Nations Report, 2020). The main goal should be keeping temperature increase in 1.5°C levels that is well-below 2°C the limits of pre-industrialization (Abbass et al., 2022).

## CONCLUSIONS

Extreme weather conditions as heatwaves, drought, floods and storms are increasing and expected to continue. Changing food sources and changes in conventional food production practices lead people to become unhealthy. Environmental changes result in infectious diseases (Rocha et al., 2022). Wheat is the most common cultivated plant in the world. Researchers stated that the decrease in wheat production is related to higher temperatures; that the plant expands faster with a decrease in photosynthetic period resulting in disruption of reproductive reactions (Abbass et al., 2022).

The effects of climate change should be minimized by taking preventive actions, removal of circumstances contributing the climate change. People will have to adapt to climate change by reducing consumption of crops, by changing dietary patterns and by reducing the amounts and the quality of foods that they eat (Birgani et al., 2022). In short, climate change puts adequate and quality food production at risk. In this context, in order to avoid the worst climate change scenarios in the future, it is imperative that we must reduce the carbon emission rate and create a world where the average global temperature rise does not exceed 2°C.

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## HURDLE EFFECT APPROACH IN DRYING OF DAIRY PRODUCTS

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### ABSTRACT

Control of microbial growth in foods by various methods as cooling, pasteurization, acidification, fermentation and water activity reduction etc. is the major object of both consumer and food industry. Minimally processing of foods is a target of today's consumer and hurdle technology is an approach that was developed for precisely these reasons. Hurdle effect is a term used to define using different acting preservation methods for maintaining microbiological stability, safety together with protection of sensory and nutritional quality. Combination of preservation factors can be more effective in maintaining microbial stability and safety. In product point of view, use of two preservation methods together in a mild manner could be more effective microbiologically and more satisfactory in terms of product quality. The selection of coupling methods is crucial, it could be product-dependent and process dependent. The initial material of dairy products is raw milk. Raw milk is highly perishable due to its nutritious composition so requires rapid cooling below 6°C after milking under suitable sanitary conditions. Dairy products can deteriorate due to physical conditions that the product is exposed to and also chemical, enzymatic and microbiological means starting from milking to processing, packaging, distribution, storage and during consumption. The rate of these deterioration reactions depends mainly the state of the product and the conditions maintained. Refrigeration is mandatory for dairy products except for dried dairy products. Dried dairy products need dry, cool, odorless environment and low relative humidity conditions. Milk powder (skimmed or full-fat), whey powder, caseinates, yogurt powder and cheese powder are dairy powders having potential use in food industry. Dried cheeses, dried yogurts known as Kurut or Keş are produced for supplying extended shelf-life compared to their fresh-state and snacks of dairy origin are also produced by drying for novelty. In drying, water activity ( $a_w$ ) of the product is reduced by removal of water that slows down the possibility of microbial growth in the product, but not a complete inactivation is supplied. The  $a_w$  tolerance of bacteria changes according their nature either being spoilage or pathogenic bacteria. Since pseudomonads are the major spoilage microorganisms in chilled stored products especially threats microbiological quality of dairy products with high water activity and pH near to neutral. Lowered  $a_w$  (~0.97) by drying prevents their growth. There are various applications used in  $a_w$  reduction both related and applied to food itself (hypertonic medium) and/or use of technologies (vacuum effect etc.) coupled with drying. In this study, use of hurdle technologies in drying of dairy products is covered.

**Keywords:** Drying, dairy products, hurdle effect, minimally processing, osmotic drying, packaging.

## **INTRODUCTION**

Food preservation is a crucial concern for supply of continuous, safe and high-quality food required for survival of humankind. Hurdles are tools used in food preservation for maintaining nutritional, sensorial and microbiological quality of foods by physical, physicochemical and microbiological means. In food processing “hurdle technologies” use combined preservation methods together in a mild manner than conventional methods and more satisfactory solutions are obtained in terms of food safety and total food quality (Bharti et al. 2017). Foods can be spoiled physically, chemically or microbiologically (Garnier et al., 2017). Dairy products are highly perishable as other animal origin foods (poultry, egg, fish, meat etc.) (Pat al.et. 2017). High nutritious nature, neutral pH and high water activity makes raw milk a good medium for growth of different microorganisms (Claeys et al., 2013). Drying is one of the oldest method applied for preservation of foods by removal of water in food thus the growth of spoilage microorganisms and deteriorative reactions are slowed down or inhibited. Shelf life of dairy products can be extended by various drying methods. This study covers the principle of hurdle technology and its possible alternative uses in drying of dairy products.

### **Food Preservation and Hurdles**

Heating, chilling, use of preservatives and irradiation are conventional methods used in food preservation. In conventional methods single parameter is focused on that results in changes in sensory and nutritional quality of foods (Pal et al., 2017). In principle of food preservation, environmental conditions are rendered unfavorable for the growth of microorganisms. Since microorganisms were kept in a hostile environment they give some physiological responses like homeostasis, metabolic exhaustion and stress reactions according to selected food preservation method (Leistner, 2000). These reactions could result in death or sub-lethal injury of microorganisms. Homeostasis is the effort of microorganism to maintain its own balance in regulation of internal balance against adverse conditions (Anonymous, 2022a). Mainly in food preservation the main mechanism used is the disturbance of homeostasis of microorganisms (Singh and Shalini, 2016). Metabolic exhaustion of microorganisms is defined as the auto sterilization of food. Stress reaction of some bacteria could result in problems in use of hurdle technology. Some bacteria can become more virulent and more resistant due to stress by generating shock protein. In order to overcome this and supply microbial stability and safety in foods multi-target preservation methods should be required (Leistner, 2000).

Hurdle is generally a term used to define a factor, a condition or a processing step that is used for the aim of preserving overall quality of food product but firstly by prevention of microbial growth. In food preservation temperature (either high or low), acidity (pH), water activity ( $a_w$ ), redox potential (Eh) and competitive microorganisms as lactic acid bacteria etc. are used as hurdles (Leistner, 2000). Hurdles are the preservation factors ensuring microbial safety and stability. Hurdle technology is the application of different preservation factors (hurdles) together (Pat al.et. 2017). At least two or three hurdles are combined together for inhibition or elimination of the growth of microorganisms that could cause food spoilage and food poisoning (Pal et al. 2017). In hurdle technology microorganisms are destroyed in an enhanced lethality degree in a hostile environment by application of preservative factor at an optimum level so that sensorial quality is protected in a better way (Bharti et al., 2017; Singh and Shalini, 2016).

### Advantages of Hurdle Technology

Hurdle technology provides many advantageous solutions (Bharti et al., 2017; Pal et al., 2017)

- Use of mild treatments in processing by using combined preservative factors together (heating, chilling, drying, curing etc.)
- Hurdles used can act as preservative and at the same time as flavor enhancing component
- Use of less additives or alternatively natural/green preservatives
- Serving stable and ready-to-eat food alternatives required for harsh conditions (space activities, mountaineers etc.)
- Getting enhanced organoleptic quality and safety in foods and prolonged shelf-life

Hurdles used can act as preservative and at the same time as flavor enhancing component (Leistner, 2000). The intensive use of a hurdle could adversely affect the product depending on the product's characteristics and also compatibility of the hurdle used and the product is very crucial (Badola et al., 2022; Leistner, 2000). In the selection of hurdles, the goal should be keeping total quality of the product in optimum by considering safety and quality together (Leistner, 2000). Leistner (1978), introduced the "hurdle technology" for preservation of products that are susceptible to degradation reactions such as high or intermediate moisture foods and foods with less salt etc. (Leistner, 2000).

Hurdles used in food preservation are categorized as physical, physico-chemical and microbial and summarized in general in Table 1 (Pal et al., 2017).

Table 1. Hurdles used in food preservation

| Hurdle Type      |  |
|------------------|--|
| Physical         | <ul style="list-style-type: none"> <li>- Packaging alternatives: Aseptic packaging, Modified atmosphere packaging (MAP), active packaging etc.</li> <li>- Thermal treatments: Pasteurization, sterilization, evaporation, baking, frying, drying etc.</li> <li>- Non-thermal preservation methods: Pulsed electric field (PEF), high hydrostatic pressure (HHP) etc.</li> <li>- Electromagnetic energy: Microwave energy, Radio frequency energy etc.</li> </ul> |
| Physico-chemical | Carbon dioxide, organic acids, lactic acid, lactoperoxidase, low pH, low redox potential, low water activity, maillard reaction products, oxygen, ozone, spices and herbs, salt, sodium nitrite/nitrate  |
| Microbiological  | Competitive microorganisms, protective cultures, bacteriocins etc.   |

## **Hurdle Technologies Applied in Dairy Processing**

Milk is regarded as one of the potentially hazardous foods that is highly and rapidly perishable by the action of microbial growth (Bharti et al. 2017). The bacterial growth depends on the storage temperature and presence of competitive microorganisms and their metabolites. Protecting the raw milk's initial quality depends mainly on the use of hygienic conditions during milking, manufacturing, handling and also packaging (Claeys et al., 2013).

Raw milk can be kept at most 48 hours under refrigerated conditions without heat treatment. Raw milk is rapidly cooled to temperatures below 6°C prior to heat treatment for microbial safety concerns. Chilling temperatures minimize growth of microorganisms present in raw milk and provides extension of shelf-life in dairy products that are non-sterile (Garnier et al., 2017). Raw milk used is generally heat-treated prior to production unless the product is raw milk originated. Heat treatments as thermization, pasteurization and UHT treatment are applied for partial or complete reduction of microorganisms in milk. Temperature control of raw milk and also dairy products is critical for nutritional, sensorial and microbiological quality (Claeys et al., 2013). Non-sterile dairy products generally have 1-3 weeks of shelf-life mostly depending on initial microbial quality of raw milk, processing conditions and post-process operations. UHT treated dairy products can be stored for 3 months to 1 year without refrigeration (Singh et al., 2011).

Heat treatments, chilling, modified atmosphere packaging (MAP), use of preservatives, fermentation, competitive microorganisms and drying are some of food preservation applications used in dairy processing (Garnier et al., 2017). These processing steps are preservation factors applied for ensuring microbial safety and stability together with nutritional and sensorial quality of dairy products (Pat et al. 2017).

Industrially dairy products are produced by thermal pasteurization of raw milk except raw milk cheeses. Pasteurization could cause degradation of nutritional components and also sensory attributes of milk. Minimally processed food products get attention of today's consumer. Based on that, researchers investigated alternative inactivation methods to conventional thermal processes. Combined use of pulsed electric field (PEF) and microfiltration (MF) as a hurdle technology called as "cold pasteurization of milk" was reported as more effective than conventional thermal pasteurization. Similarly, combined use of antimicrobial compounds (nisin) with another non-thermal preservation technique "high pressure, HP" enhanced inactivation effect of individual use of HP (Anonymous 2022b). Reported studies exist about combining bacteriocin use with milk thermal treatments or non-thermal technologies as hurdle technology against microbial inactivation (Sobrino-López and Belloso, 2008).

Use of bio-preservatives that is competitive flora such as in microbial fermentation is one of the hurdles used in food preservation (Pal et al., 2017). From this point fermentation in milk is stimulated by the use of starter cultures as competitive microflora. Decrease in pH meaning development of acidic environment and presence of organic acids are concerned as preservative factors (Garnier et al., 2017). In raw milk cheese production, fermentative bacteria (lactic acid bacteria) can inhibit or kill pathogenic bacteria by nutritional competition and/or production of some metabolites as lactic acid, bacteriocins (Dupas et al., 2020).

In probiotic dairy foods, another example of microbial fermentation, the stability of probiotic strains is an important issue (Gomes da Cruz et al. 2009). Probiotics are live microorganisms that are members of *Lactobacillus* and *Bifidobacterium* used in fermented products for their therapeutic effects. Enhancement of human gut flora, suppression of pathogens and protecting against intestinal diseases are some of health benefits. Survival

degree of probiotic bacteria depends on many factors as, strain, pH, dissolved oxygen, storage atmosphere, presence of organic acids that are metabolites of competitive microflora, buffers as whey proteins etc. In yogurt manufacturing presence of oxygen both during processing and also storage adversely affects the survival of *L. acidophilus* and *Bifidobacterium spp.* (Talwalkar and Kailasapathy, 2004).

Gomes da Cruz et al. (2009) reported possible hurdle technologies that could be used in probiotic dairy products as:

- Plastic films used in cheese packaging are permeable to oxygen and probiotics are oxygen sensitive due to aerobic metabolism. Low oxygen-permeability plastic films can be chosen, use of vacuum packaging can be applied in probiotic cheeses.
- Use of oxygen tolerant, acid tolerant and bile resistant strains of probiotic bacteria, addition of amino acids, peptides for supplementing the growth of probiotic bacteria in cheese.
- Use of probiotic strains with other starter strains and providing their bio-preservative effect.
- Probiotic bacteria are sensitive to salt concentrations above 4%. Using salt concentrations less than % 4.
- Protecting probiotic microorganisms from hostile environment (cheese environment) by microencapsulation in probiotic cheese manufacturing.
- Use of high oxygen consuming *S. thermophilus* in combination with *Bifidobacteria* for protecting against oxygen stress.

Investigation of paneer cheese was studied by Thippeswamy et al., (2011), water activity, ( $a_w$ ) and pH were selected as hurdles and coupled with modified atmosphere packaging (MAP). These preservation factors were varied by using different concentrations of NaCl solution (%) and citric acid solutions (%) for  $a_w$  and pH, respectively

In raw milk cheese production, water activity can be reduced by salting that inhibits some pathogenic bacteria (Dupas et al., 2020). The effects of plant extracts can be enhanced by other preservation technologies as a hurdle approach.

Hurdle technology is a product-dependent application and should be selected accordingly. Application of salt in proper amounts in fresh cheeses, use of natural preservatives as herbs, spices and some plant itself or its extracts on cheese surfaces or directly adding, active packaging solutions as use of oxygen scavengers in probiotic products, antioxidant compounds embedded in active packages for high-fat dairy products etc. some examples of hurdle technologies can be used in combination in dairy products (Dupas et al., 2020).

### **Hurdles Technologies in Drying of Dairy Products**

In principle drying is the removal of water in food either by circulating air or any other mechanism. Water present in food is converted from liquid to gas phase (Indiarto et al., 2021). Drying, curing and conserving are inference of water activity reduction in foods (Bharti et al., 2017). The growth of bacteria, yeasts and molds is retarded by drying. Enzymatic and chemical reaction rates are slowed down by drying depending on  $a_w$  reached. Reaction rates depending on  $a_w$  is summarized in Table 2 (Perera, 2005). Water activity ( $a_w$ ) is the major parameter determining food stability since it prevents or limits the microbial growth. The limiting values factor in microbial growth is reported as:

Table 2. Microbiological and chemical reaction rates

| Water activity ( $a_w$ ) |  |
|--------------------------|--|
| $a_w < 0.85$             | No growth of bacteria                        |
| $a_w < 0.70$             | No growth of yeast                           |
| $a_w < 0.65$             | No growth of molds                           |
| $a_w = 0.4-0.65$         | Incidence of non-enzymatic browning reaction |

Milk powder (skimmed or full-fat), buttermilk, yogurt powder, cheese powder, dried casein and whey proteins are some of dairy powders. All dairy-originated powders have technological potential as ingredients in food industry. Whole milk powder has a shelf-life lower than 6 months and usually susceptible to fat oxidation. Dried dairy products requires special attention since it is directly consumed after reconstitution without additional heat treatment (Singh et al., 2011). Dairy products are dried by various drying methods to a moisture content below %5 for storage stability. Storage stability of dried dairy products depends on product characteristics as moisture content (%), fat (%) content, relative humidity and oxygen content of storage environment, storage temperature, characteristics of packaging material, packaging method used (air, vacuum or a definite gas composition etc.). Salar et al., 2021 studied the effect of gentle pasteurization (two different milk pasteurization temperature and time combination) and drying (spray and freeze drying) combination as hurdle technology in determining the quality attributes of cow and buffalo colostrum. Spray drying is known with being simple, serving high-quality powders and easy scale-up drying method used in drying of dairy powders. However, bioactive proteins of colostrum are denatured thermally during spray drying. In considering quality of colostrum components, milder pasteurization conditions and dehydration method were investigated.

Modified atmosphere packaging (MAP) can be applied in different gas compositions and enhance shelf-life by changing respiration rate, microbial growth and oxidation reactions (Thippeswamy et al., 2011). In packaging of dried dairy products,  $O_2$  concentration in package medium should be reduced due to possible auto-oxidation reaction. For long term storage modified atmosphere packaging (MAP) with a suitable gas composition (mixture of  $CO_2$  and  $N_2$ , 100%  $N_2$  or reduced  $O_2$  atmosphere etc.) is required. Storage temperature also plays an important role in shelf-life of dried dairy products. Additionally, gas flushing and use of  $O_2$  absorbers in packages are the other packaging solutions (Singh et al., 2011).

Commercially carbon dioxide is added either by MAP or as gas flushing for extension of shelf life of dairy products in a simple and economic manner. Carbon dioxide is a natural antimicrobial agent and regarded as generally recognized as safe (GRAS) component, effective on psychrotrophic microorganisms and specifically aerobic bacteria limiting oxidative rancidity. (Singh et al., 2011). Since  $CO_2$  is naturally exist in freshly drawn milk it can be directly flushed to raw milk and/or also the package headspace (Singh et al., 2011).

## CONCLUSIONS

Food-borne diseases are concern of public health for both developed and developing countries. Today's consumer demand "clean label" foods with fresh-like, healthy, minimally processed properties. There is worldwide attempt to use minimal processing methods with less chemical additives in getting microbiologically safe food products. Use of hurdle technologies serve good alternative ways of approaching these demands when compared with conventional methods used in food processing. Hurdle technology is a method using combined preservative factors together for maintenance of microbial, sensorial and nutritional quality of foods. At least

two or three preservation factors are combined as hurdles for serving promising solutions in food preservation.

Drying is the oldest of food preservation methods used. Sun drying, hot air blowing by the wind are the natural mechanisms used in drying of foods. However, the product can be exposed to longer drying times under these natural conditions which could cause quality losses in chemical composition and also brings microbiological risks (Indiarto et al., 2021). Therefore, in recent years rapid developments occurred in drying technology supplying more efficient drying and high-quality products. Hot-air drying, spray drying, osmotic drying, microwave drying, vacuum drying and freeze drying are the novel technologies studied recently (Kutlu, 2021). However, the characteristics of the product should be considered in selecting the drying process. Osmotic dehydration (Kutlu, 2021), mild pasteurization and /or non-thermal processes prior to drying (Salar et al. 2021; Aaliya et al., 2021) are some of pretreatments of drying. Combining vacuum with other drying techniques as vacuum microwave drying, vacuum- oven drying etc. for preserving quality of product and drying in a short time (Anli, 2020; Chudy et al. 2019), use of ohmic heating for osmotic dehydration before microwave drying etc. (Kutlu, 2021), converting the food to a stable foam by using foaming agent and stabilizer prior to drying (Krasaekoopt and Bhatia, 2012) are some of combined treatments (hurdle technologies) applied with drying.

As given in above sections there are various alternative hurdle combinations that can be applied in dairy products. Also in drying, there are also a wide ranges of hurdle application subjects and researches have been continuing. Therefore, it is thought to be promising to combine vacuum effect, osmotic effect, foaming effect, packaging techniques and/or any other minimal processing alternatives with known and novel drying technology are promising topics of hurdle technology alternatives of drying.

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## PROPERTIES OF FOOD POWDERS, PRODUCTION METHODS AND CHARACTERIZATION

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### ABSTRACT

Food powders are one of the most common forms of food materials. Day by day the volume and types of production of powders are increasing as a result of it being the most stable form of food that is also easy to use, handle, package and distribute. It has a longer shelf life compared to the liquid form of the products. Food powders are particulate solids that are produced from granular or liquid solids materials. One of the most common formate of food materials is food powder. Currently, many of the food are available in the market as powder form. Drying and crystallization are the two main methods of converting a liquid into a powder form and size reduction processes such as grinding and milling contribute to the production of the powder. The distribution, particle size, shape and surface properties depending on both properties of the raw materials and the processing conditions and methods used during their formation. Food powders are types from dry foods. Many new product formulations are developed by mixing several powders and the final product is obtained by water rehydration and further processing. Conditions that may cause physical and chemical changes leads to changes in functional properties or deterioration of sensory quality should be avoided during the production, packaging and storage of food powders. This study explained the characteristics and methods of producing food powders. Consumers tend to use strong foods that do not cause sticking and clumping during storage. The shelf life of foods is generally affected by physical changes and chemical reactions. All conditions that cause food powders to change must be controlled so that these changes do not occurs.

**Keywords:** Food Powders, granulation, drying, crystallization, rehydration, storage.

### INTRODUCTION

Food powders play a major role in the conversion of agricultural raw materials into food Powders are particulate solid-state materials containing discrete particles ranging in size from nanometers to millimeters (Cuq, Rondet, and Abecassis 2011). Bulk powder properties are the combined effect of particulate properties, as well as solid or liquid food products being converted into powder form for ease of use, processing and preservation of quality (Fitzpatrick, 2007). In addition, many food products are currently available in powder form on the market. This powder has many benefits and economic advantages in light of the reduced volume/weight of foods, ease of packaging, handling and transportation, improved bioaccessibility and higher nutritional value (Dag, Singh, and Kong 2020). Bulk solid particles such as powder, granules and flour have variable structure so it depends on the size or source of the materials (Figure 1.1) shows some examples of granules, powders and flour (Bhandari, 2013). Food powders can be

crystalline, amorphous or mixed (semi-crystalline) in their molecular structure. Depending on the process applied, powders can be produced in any of these forms (Bhandari, 2013).

**Crystalline structure** Salts, sugars and organic acids Common powders in crystalline states. Crystalline powders are non-hygroscopic, stable and easy to use. The crystal form is characterized by a tightly packed molecular arrangement; Therefore, only external substances can interact with molecules at the interface of air crystals (Hartel, 2002). The crystalline form is in thermodynamically stable equilibrium. The process of solute concentration above normal saturation by solvent removal by evaporation and/or cooling is the crystallization process (Metin and Hartel 1996).

**Amorphous structure** Many important food powders, such as high or low molecular weight carbohydrates and proteins, have an amorphous structure. Many food products are normally amorphous, such as milk powders, juice powders, honey powders and hydrolyzed protein powders (Sharma, Jana, and Chavan 2012). Molecules in the amorphous state are irregular, more open and porous. Therefore, a single molecule has more space for external interactions enabling them to absorb volatiles, for example an amorphous structure can easily absorb water (Yu, 2001).

**Mixed structure** In mixed powders, there is a combination of amorphous and crystalline regions in the powder particles. When the system is pre-crystallized and dried with the mother liquor, two structures will form in the dried product, as the mother liquor will turn into an amorphous state during drying. For example when drying the whey and the molasses dried (Bhandari , 2013 and Fitzpatrick , 2007) .



Figure 1.1 Some examples of granules, powders and flour (Bhandari , 2013).

### **Manufacturing and Processing Methods of Food Powder**

All powders are generally free flowing, can be used in very small quantities (in milligrams) and cannot be conveyed pneumatically in pipes. Powder forms of foods are involved in many processes such as mixing and dissolving. Particles are formed from bulk liquid or solid materials by drying, grinding, crushing, etching, pulverizing, precipitation or crystallization.

The two main methods of converting liquid into powder form are drying and crystallization. Size reduction processes such as grinding also contribute to dust production (Bhandari et al ., 2013).

### **Spray drying for food powder production**

Spray drying is one of the commonly applied low-cost food drying methods for converting liquid foods into powder. Spray drying is the convective dehydration of atomized droplets. Figure 2.1. is a simplified step-by-step drawing showing each droplet being dehydrated in a spray dryer. The mechanism that makes up the entire dehydration process is the convective heat and mass transfer mechanism. The convective transport mechanism is induced by the movement of particles in the chamber. Moisture is lost from a droplet, the solute in the material solidifies, resulting in a solid particle (Kamal et al ., 2019).

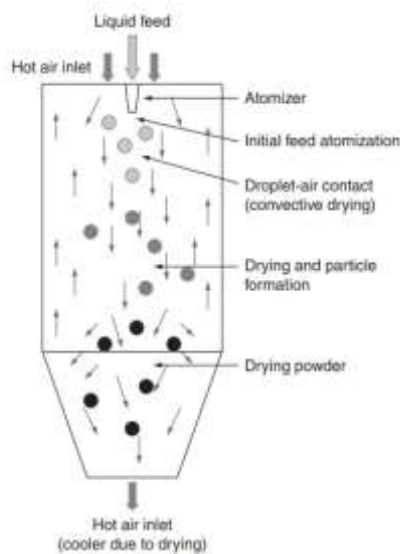


Figure 2.1 Overall illustration of the spray drying process (Bhandari et al ., 2013).

### **Freeze drying for food powder production**

The freeze drying process is also known as Lyophilization. Lyophilization is the process of removing frozen water (free water) before the product (sublimation) and then removing unfrozen water (bound water) (desorption) in a vacuumed environment (Moraga, Talens, and Moraga 2011). The most important part of the lyophilization process is sublimation. Sublimation is the process by which frozen (free) water in a product passes directly into the gas phase without melting. Under certain conditions, there are three phases of matter. The point where these phases coexist is the triple point. Figure 2.2 (Çelen, 2018, Taylor et al ., 2007).

Substances pass from the solid state to the vapor phase in two different ways. The first of these is the transition of the substance to the liquid form and then to the gas phase. The second is the direct conversion of solid into gas. The equilibrium phase diagram of water can be used to determine the pressure and temperature values required in a sublimation event (Acar et al ., 2019). There are four main stages of freeze drying. These are freezing, vacuum, sublimation and condensation. Each of these processes shares the total energy consumption and sublimation takes half of the total energy of the process, while the freezing stage does not consume much energy (Huang et al ., 2009).

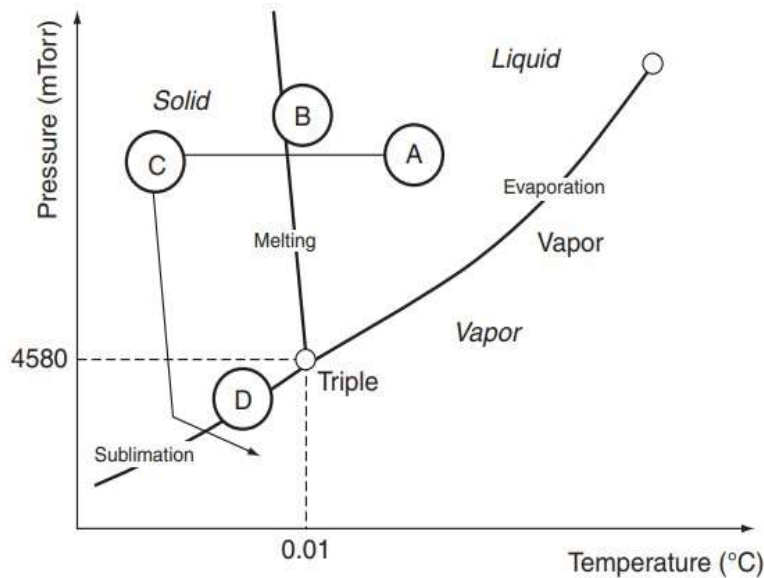


Figure 2.2. Equilibrium phase diagram (triple point) (Çelen , 2018).

### Drum Drying for Food Powder Production

Drum dryers used for drying solutions and suspensions are made of stainless steel. It is deposited on the outer surface of a rotating, hollow cylinder filled with continuously superheated steam. Inside the cylinder, steam condenses and heat is transmitted to the product along the conductive walls Figure 2.3 (İlyas and Korkmaz 2021, Korucu , 2009)

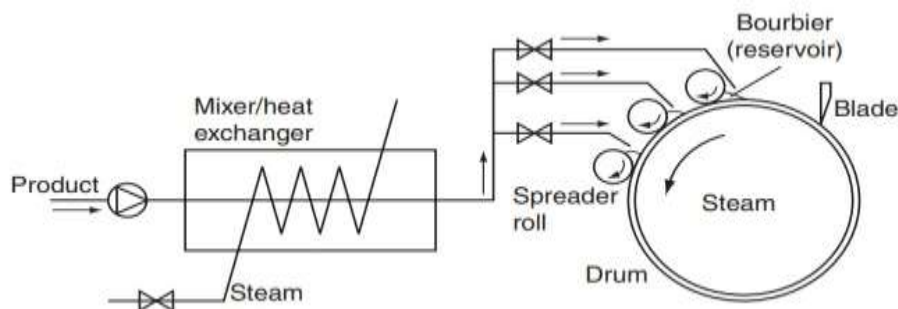


Figure 2.3 Schematic of a drum drying process (İlyas and Korkmaz 2021)

### Grinding for food powder production

Grinding is a widely used process in the food industry. It is an important process for reducing the size of materials, giving a form and separating their components (Djantou and Moses , 2007). Grinding is a complex process that depends on several parameters such as the nature of the material to be ground, the type of equipment and technology used, and the operating parameters (Canbay et al ., 2021). The result of the grinding process depends both on the properties of the food material and on the nature, distribution and intensity of the pressure exerted by the grinding tool. The size reduction process known as grinding can be done in hammer mills, balls, knives, disc, rolling devices (Tarhan , 2007, Hac , 2009).

## **Powder mixing in the production of food powders**

Powder mixing is one of the most common processes in powders related industries (Dikmen ve Ergün, 2004).. Various food powders (for example, flour, sugar, salt, dry additives, dry ingredients, dried milk, dried vegetables, dried fruits, cereal grains, baking mixes, soup powders, spice mixes, juice powders, etc.) in the food processing industry. The main purpose of mixing in the food industry is to create product homogeneity (Bridgwater , 2010). Mixing quality is mainly affected by mixer type, mixer design, mixing time, as well as the types of powder mixed. The degree of uniformity required may vary from one application to another (MayerLaigle et al ., 2011).. The homogeneity of the final powder depends on the nature of the mixing procedure used (Sert and Altun 2021).

## **Properties of Food Powders**

The physical properties of powder products are examined under two terms as particle properties and bulk properties. Particle shape and size, particle size distribution and particle density of the powder product are examined within the "particle properties", which express the properties of a single particle. Among the "bulk properties", which represent a whole powder product, compacted density, bulk density, porosity, caking and hygroscopic properties of the powder product come to the fore (Koç et al ., 2011).

### **Moisture content**

Moisture content plays an important role in many ways in the processing of most powders (Fitzpatrick, Barringer, and Iqbal 2004). For example, it is critical for powders as it affects the stickiness of the powder and can cause unwanted caking and sticking to surfaces. The moisture content is also noteworthy for its beneficial effect in the agglomeration and granulation processes (Opaliński, Chutkowski, and Stasiak 2012).

### **Particle properties**

#### **Particle size**

Particle size is one of the most important particle properties, as the size of particles in a powder affects many aspects of the bulk behavior of powders (Mani, Tabil, and Sokhansanj 2008). Particle size affects the surface area of the particle in a powder, which is crucial in determining the degree of interaction between the particles themselves and between the particles and the surrounding liquid. Particles in a powder often do not have the same particle size (Benkovi and Igor 2013).

#### **Particle shape**

The shape of the particles can affect the properties of the bulk powder and the performance of the particle unit operations. For example, the shape of the particles can determine the packing behavior of powders, and hence their overall density and storage capacity (Einhornstoll , 2018). Particle surfaces in contact with each other are important for powder fluidity and performance in powder handling processes. Assessing the shape of real dust particles can be very difficult because dust particles are 3-dimensional objects that can have very complex random shapes (Lore'n , 2006).

### **Particle strength**

The strength of food powder particles is of most interest in the context of particle fracture, so particle fracture susceptibility is affected by the strength of the particles. Particle breakage is generally undesirable except in size reduction operations such as grinding. Particle fracture mechanisms can be classified according to whether the applied force is normal to the particle surface and whether this force is low or high (Ermiş , 2015).

### **Powder densities and particle density**

Density is defined as mass divided by volume, and bulk powders can have a range of different densities, such as solid, particle and bulk densities. Many powders are porous, meaning the particle itself has voids or pores (C' anovas, 2005).

### **Bulk properties of powders**

#### **Bulk density**

Bulk density is an important quality criterion during the packaging and transport of powder products from one place to another (Ganesan et al ., 2008). Bulk density also gives information about the drying of the final product by grinding to the desired moisture content. The bulk density of a powder is the mass of the powder divided by the bulk volume and is based on the compactness of the powder (Soottitantawat et al ., 2005).

#### **Powder flowability**

Powder fluidity is all about overcoming flow resistance and allowing powder particles to move over each other. The determination of the flow properties of the powder, which is a quantitative and qualitative description, gives information about the design and performance of the equipment. The flow properties of powder products are of great importance for bulk transportation and storage. The main forces affecting the flowability of the powder are gravity, friction, cohesion and adhesion (C' anovas, 2005, Stoklosa et al ., 2012). Environmental humidity can cause moisture to agglomerate in crystalline components, which affects the fluidity of the powder (Mathlouthi and Roge 2003). It is expressed by the Carr index as a function of bulk and graded density. If the Carr index value is less than 15, the flowability of the particle is considered very good, between 15 and 20 good, between 20 and 35 poor, between 35 and 45 bad, and above 45 very bad. Powder flow can also be explained by the concept of compressibility. For this, the Hausner ratio expression is used. Hausner (1967) stated that the Hausner ratio (HR) can be calculated by dividing the compacted density of the powder to the bulk density. When the HR value is less than 1.2, the stickiness of the powder product is low; 1.2–1.4 medium; when it is larger, it is defined as high stickiness (Koç et al ., 2012).

#### **Powder clumping and stickiness**

Moisture is often cited as the main factor or main cause in cases where caking occurs. Therefore, it is very important to know the water sensitivity and hygroscopicity of the main powder components to predict the caking kinetics (Ermiş, 2015).

## **Food powder rehydration**

Powder rehydration capability is an important feature as the industrial user or home consumer does not want to face problems during rehydration. Some food powders can be easily rehydrated naturally, while others can be problematic (Felix et al ., 2022).. Powder rehydration is often broken down into a number of sub-processes that may overlap. Examples are wetting, sinking, dispersion, swelling, disintegration, and dissolution. Wetting refers to the process that comes into contact with the liquid and surrounds the powder particles with the liquid. Immersion refers to the powder immersed below the surface of the liquid. Dispersion is the dispersion of wetted powder material throughout the bulk liquid. Dissolving, on the other hand, involves dissolving powder particles in a liquid to form a solution (Mitchell et al ., 2015).

## **Shelf-life of food powders**

Dry foods are produced by mixing dried food ingredients or using various drying methods such as roller drying, spray drying and freeze drying of liquid foods. Food powders constitute an important type of dry food or food ingredient. For this, they must be homogeneous and have good flow properties. During the manufacture, packaging and storage of food powders it is necessary to avoid conditions that can cause visible Physicochemical changes resulting in changes in functional properties of sensory quality (Sezer and Ayhan, 2017). For powdered ingredients, the stability of each ingredient should be evaluated individually and the product should be assigned a shelf-life appropriate to the shelf-life of the least stable ingredient under actual conditions (Hashemi et al ., 2017). External factors such as heat, humidity, oxygen and light exposure are important in determining the shelf life of dry foods (Rao et al ., 2012).

## **CONCLUSION**

In recent years, the increasing health awareness of food consumers has developed an awareness of the quality, food safety and nutritional value of the products they consume. Food powders are the most common form of food materials. Since it is the most stable form of food that is easy to use, package, distribute and process, the volume of powder production is increasing day by day. Many new product formulations are now developed by mixing several powders and the final product is then obtained by water rehydration and further processing.

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## EVALUATION OF ULTRAVIOLET (UV) ABSORPTION ABILITY OF MENENĞİÇ AND OLIVE OILS FROM TURKEY

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### ABSTRACT

Ultraviolet (UV) rays from the sun cause negative effects on humans and protective products should be used to protect against harmful rays. People prefer to use natural preservatives instead of chemical containing preservatives. Oily substances are very effective in forming a long-lasting sunscreen film on the skin. In addition, the emollient properties of oily substances protect the skin from drying effects such as wind and sun exposure. In our study, the sun protection factor (SPF) of Menengiç and olive oils from Turkey was investigated to obtain usage potential as natural additive in cosmetic industry. SPF values of oil samples were measured spectrophotometrically at the wavelength of UV-B light (290-320 nm) in vitro. SPF values of Menengic and olive oils were determined as 1.11 and 3.12. The percentage of UV blocked was found to be 27% for menengiç oil and 65% for olive oil. Menengiç and olive oils have the potential to be used as herbal sunscreen agents in cosmetic industry as natural additives.

**Keywords:** Menengiç oil, olive oil, sun protection factor (SPF), Ultraviolet (UV) rays

### INTRODUCTION

People are exposed to the sun while performing some of their social and vital activities. Ultraviolet (UV) rays from the sun cause the release of toxic radicals for skin cells (Matsumura and Ananthaswamy, 2004). All rays of different wavelengths emitted from the sun are called the "electromagnetic spectrum of the sun" (Petrazzuoli, 2000). UV radiation forms a specific part of the electromagnetic spectrum that has a shorter wavelength and higher energy than visible light. There are 3 types of UV of sunlight falling on the earth's surface; UVC (200-290 nm), UVB (290-320 nm), and UVA (320-400 nm). UV rays constitute 5% of the rays reaching the earth. UVA wavelength is in the range of 320-400 nm and it constitutes about 90% of UV rays. UVA rays can damage human cells by penetrating up to 100 µm deep to the skin surface. It causes sagging and wrinkles by causing loss of elasticity in human skin. Thus, it causes premature aging. UVA rays cause enzyme inactivation, protein denaturation, cell organelles and cell membrane damage (Azevedo et al., 1999). UVB is one of the ultraviolet rays reaching the earth's surface, although the majority of it is filtered by the atmosphere, with a wavelength of 290-320 nm. UVB rays are the type of rays that have the most effect on the formation of melanin pigment in the skin and sunburns (Azevedo et al., 1999). UVC is UV rays with the highest energy and lowest wavelength (200-290 nm) (Dutra et al., 2004). Sunscreen products should be used to protect against the harmful effects of UVA and UVB. Sunscreen agents have a sun protection factor (SPF) value. This value is defined as the ratio of the minimal erythema dose over the sunscreen agent (Algaba et al., 2002). Today, the importance given to sunscreen

agents is increasing. The use of synthetic preservatives and their prolonged exposure to the skin can lead to potentially harmful xenobiotic and allergenic effects (Boehm, 1995). The use of natural phenols in the skin has been suggested in the literature (Fang and Bhandari 2010). Research on the use of herbal sunscreens without side effects by using naturally produced herbal bioactive substances in sunscreens is increasing day by day (Ahmady, 2020 and Turhan, 2019). Plant extracts obtained by various methods can prevent the acceleration of transcription factors in skin cells caused by UV rays (Rodrigues et al., 2014). Sunscreens with many natural ingredients have been reported to have UV-filtering activity, including coconut, almond, olive, avocado, cottonseed, sesame, peanut, and soybean oils (Kaur and Saraf, 2010).

Menengiç (*Pistacia terebinthus L.*) is known with different names such as çedene, chitlenbik, çıtlık, chitemik and bittim, depending on the regions in Turkey. The aroma and medicinal properties of the Menengiç plant, known since primitive times, are due to the fact that it is a plant rich in tannin (polyphenolic compounds) and resin substances (Davis, 1970). In ancient times, Menengiç was used in the treatment of rheumatism, cough and stomachache, as well as in the treatment of stomach by boiling the leaves (Matthaus and Özcan 2006). Menengiç fruit has 35-47% fat content, is rich in macro-micro elements, essential fatty acids and fat-soluble vitamins (Alçiçek et al., 2004). The composition of *P. terebinthus* oil, which grows wild in Turkey, was determined as essential fatty acids oleic (52.3%), palmitic (21.3%) and linoleic (19.7%) acids. Saturated fatty acids constituted 23.6% of total fatty acids (Özcan, 2004).

The olive tree (*Olea europaea L.*) is one of the most important fruit trees of the Mediterranean countries, where it covers an area of 8 million hectares, constituting approximately 98% of the world's crop (Guinda et al., 2004). Mankind has always been interested in olives for thousands of years because of its long life, healing fruit and oil. It is known that olive oil obtained by appropriate methods is more beneficial than the effect of many drugs. In some countries, olive oil is of great interest as a medicinal product (Bakır, 2021). The main antioxidants of olive oil are carotenes and phenolic substances including hydrophilic and lipophilic phenols (Boskou, 2006).

Plants have been used in the past and today in the pharmaceutical and cosmetic areas. The sun protection factor (SPF) of the oils obtained from Menengiç and olives were investigated in vitro and their potential for use as a natural ingredient in the cosmetics industry was determined.

## **MATERIAL AND METHOD**

### **Plant Material**

Menengiç oil samples were from Adıyaman, olive oil samples were from Hatay.

### **Determination of SPF**

The SPF value of Menengiç oil and olive oil (0.006 g) was weighed. The weighed oils were mixed with 3 ml of ethanol (96%) with a vortex until homogenized. The homogeneous mixture was measured in 3 repetitions in a spectrophotometer (Beckman Coulter) at 5 nm intervals in the wavelength range of 290-320 nm. The obtained values were calculated using the Mansur equation (Mansur et al., 1986).

The following equation proposed by Mansur et al. (1986) was used to determine the sun protection factor (SPF) of the oils.

$$\text{SPF} = \text{CF} \times \sum_{290}^{320} \text{EE}(\lambda) \times I(\lambda) \times \text{Abs}(\lambda) \quad (1)$$

CF = Correction factor (= 10);

**EE(λ)** = Erythemetogenic effect radiation wavelength (λ);

**I (λ)** = Intensity of sunlight at wavelength (λ);

**Abs (λ)** = Absorbance of extracts at wavelength (λ).

## RESULTS AND DISCUSSION

The relationship between the radiation intensity at each wavelength and the erythemetogenic effect is given in Table 1. SPF values of menengic oil and olive oil samples were recorded as 1.11 and 3.12 (Table 2-3). According to Table 4 (Imam et al., 2015), the percentage of UV blocked was found to be 27% for menengiç oil and 65% for olive oil.

**Table 1.** Relationship between radiation intensity at each wavelength and erythemetogenic effect

| λ (nm)       | EE (λ)x I(λ)  |
|--------------|---------------|
| 290          | 0.0150        |
| 295          | 0.0817        |
| 300          | 0.2874        |
| 305          | 0.3278        |
| 310          | 0.1864        |
| 315          | 0.0839        |
| 320          | 0.0180        |
| <b>Total</b> | <b>1.0000</b> |

**Table 2.** SPF values of Menengiç oil sample.

| λ (nm) | Ab1   | Ab2   | Ab3                    | CFx<br>EE(λ)xI(λ)x<br>Ab (λ)1 | CFx<br>EE(λ)xI(λ)x<br>Ab (λ)2 | CFx<br>EE(λ)xI(λ)x<br>Ab (λ)3 |                |             |
|--------|-------|-------|------------------------|-------------------------------|-------------------------------|-------------------------------|----------------|-------------|
| 290    | 0.211 | 0.205 | 0.218                  | 0.03165                       | 0.03075                       | 0.0327                        |                |             |
| 295    | 0.163 | 0.16  | 0.164                  | 0.133171                      | 0.13072                       | 0.133988                      |                |             |
| 300    | 0.131 | 0.13  | 0.133                  | 0.376494                      | 0.37362                       | 0.382242                      |                |             |
| 305    | 0.104 | 0.103 | 0.104                  | 0.340912                      | 0.337634                      | 0.340912                      |                |             |
| 310    | 0.083 | 0.084 | 0.084                  | 0.154712                      | 0.156576                      | 0.156576                      |                |             |
| 315    | 0.07  | 0.07  | 0.071                  | 0.05859                       | 0.05859                       | 0.059427                      |                |             |
| 320    | 0.056 | 0.056 | 0.055                  | 0.01008                       | 0.01008                       | 0.0099                        | <b>Average</b> | <b>SD</b>   |
|        |       |       | <b>Total<br/>(SPF)</b> | <b>1.11</b>                   | <b>1.10</b>                   | <b>1.12</b>                   | <b>1.11</b>    | <b>0.01</b> |

**Table 3.** SPF values of Olive oil sample.

| $\lambda$ (nm) | Ab1   | Ab2   | Ab3                    | CF <sub>x</sub><br>EE( $\lambda$ )xI( $\lambda$ )x<br>Ab ( $\lambda$ )1 | CF <sub>x</sub><br>EE( $\lambda$ )xI( $\lambda$ )x<br>Ab ( $\lambda$ )2 | CF <sub>x</sub><br>EE( $\lambda$ )xI( $\lambda$ )x<br>Ab ( $\lambda$ )3 |                |             |
|----------------|-------|-------|------------------------|---|---|---|----------------|-------------|
| 290            | 0.541 | 0.54  | 0.039                  | 0.08115   | 0.081   | 0.00585   |                |             |
| 295            | 0.447 | 0.447 | 0.447                  | 0.365199  | 0.365199  | 0.365199  |                |             |
| 300            | 0.38  | 0.378 | 0.379                  | 1.09212   | 1.086372  | 1.089246  |                |             |
| 305            | 0.292 | 0.295 | 0.291                  | 0.957176  | 0.96701   | 0.953898  |                |             |
| 310            | 0.241 | 0.239 | 0.241                  | 0.449224  | 0.445496  | 0.449224  |                |             |
| 315            | 0.205 | 0.203 | 0.202                  | 0.171585  | 0.169911  | 0.169074  |                |             |
| 320            | 0.173 | 0.178 | 0.173                  | 0.03114   | 0.03204   | 0.03114   | <b>Average</b> | <b>SD</b>   |
|                |       |       | <b>Total<br/>(SPF)</b> | <b>3.15</b>   | <b>3.15</b>   | <b>3.06</b>   | <b>3.12</b>    | <b>0.05</b> |

**Table 4.** Percentage of UV blocked by SPF value (Imam et al., 2015).

| SPF | Percentage of UV Blocked |
|-----|--------------------------|
| 2   | 50                       |
| 4   | 75                       |
| 5   | 80                       |
| 10  | 90                       |
| 15  | 93                       |
| 25  | 96                       |

In a study by Galanakis et al. (2018), the SPF values of olive wastewater and water and ethanol olive fruit extracts were evaluated in the spectrum ranges of 220-400 nm, and the obtained SPF values were in the range of 4.3-5.6. In a study to evaluate the ultraviolet absorption ability of essential oils and herbal oils used in cosmetics and the sun protection factor was determined. Olive oil showed the highest sun protection value with 7.549 among the 16 tested oils in their study (Kaur and Saraf, 2010). The difference between our data and these studies may be due to differences such as the region where these plants are grown, soil conditions, and climatic conditions. Because the differences in these conditions will cause different phenolic contents in the fruits, which will also affect the SPF value. There is no study in the literature with the sun protection factor of Menengiç oil. Phenols, which have a high antioxidant capacity in plants, appear to take place in many cosmetic products, especially in UV protection and anti-aging products (Ustündağ et al., 2020). Fang and Bhandari (2010) suggested the importance of natural phenols found in plants and should be considered as sunscreen agents. It has been reported that flavonols found in plants can prevent UV-induced damage to the skin and the flavonols kaempferol and galangin can be used as the most promising topical skin photoprotectants (Maini et al., 2015). Menengiç oil and olive oils have rich phenols (Dalgıç et al., 2011; Armutçu et al., 2013). Vegetable oils with high commercial power are used in cosmetics as essences, moisturizers and sunscreen agents. They are alternative products with sun protection potential with various phenols in their content and possibilities for use in cosmetics.

## CONCLUSION

SPF values of menengiç oil and olive oil samples were investigated in vitro. It was determined that the samples had ultraviolet (UV) absorption ability. The rich phenol content of menengiç oil and olive oil samples has the potential to be used as natural sunscreen agents in pharmaceutical and cosmetic fields.

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## INVESTIGATION OF OLIVE AS AN ALTERNATIVE TO SYNTHETIC FOOD PRESERVATIVES

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### ABSTRACT

Olive (*Olea europaea L.*) and olive extracts have been used as a natural medicine from past to present. Olive extracts have many biological activities, especially antimicrobial effect. Today, the trend towards natural foods has increased and synthetic food preservatives are being replaced by herbal products. In our study, the antimicrobial activity of olive fruit water extract from Ayvalık Yağlık Olive was investigated against food-borne and clinical pathogens (*Salmonella enteritidis* ATCC 171, *Bacillus cereus* RSKK 863, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 11229, *Pseudomonas aeruginosa* ATCC 27853 and *Candida glabrata* RSKK 04019) to determine its potential use as a natural resource. The disc diffusion assay was used to obtain the antimicrobial activity. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extract were also determined. The olive water extract showed the highest inhibition effect (19.46 mm) against *E. coli* ATCC 11229. The inhibition zone diameters ranged from 19.46 mm to 12.35 mm. The extract has the lowest MIC and MBC values (10 mg/ml) against *S. aureus* ATCC 25923. MIC and MBC values of the extract against the tested microorganisms varied from 10 mg/ml to 80 mg/ml. Ayvalık Yağlık olive water extract with good bioactivity can be used as a natural substance in the food and pharmaceutical industries alternative to chemical preservatives. The extract also may extend the shelf life of foods.

**Keywords:** Antimicrobial; extract; natural product, Ayvalık Yağlık.

### INTRODUCTION

Olive and olive extract products are marketed as a natural medicine that is beneficial to health. Olives have been popularly used for medicinal purposes in the field of skin diseases, external disease, orthopedics and gastroenterology (Bakır, 2021). Olive and its by-products contain phenolic substances at a rate of 1-3% of its volume. The main phenolics substances of olive are phenolic acids, phenolic alcohols, flavonoids and sequoiridoids (Servili, 2002). In the literature, it has been reported that the phenolic substances found in olives and their products are important for human health on cardiovascular diseases and cancer (Boskou, 2006). The homeland of olive is Anatolia. Egyptian, Lydian, Ionian, Ancient Greek, Roman, Seljuk, Ottoman civilizations cultivated olives. The Phoenicians and especially the Romans spread olives from the Eastern Mediterranean to Spain. Located in the Mediterranean Basin, Turkey is one of the world's important olive and olive oil producing countries (Bakır, 2021).

*Salmonella* spp., which has high morbidity and mortality in the world, is among the important pathogens that cause problems in animal health, human health and food safety (Atasever, M., 2017). *Salmonella enteritidis* is one of the most common serotypes of *Salmonella* worldwide, especially in developed countries (Patrick, M., et al. 2004). *Bacillus cereus* is a Gram-positive and endospore-forming facultative anaerobic bacterium. The

production of toxins leads to foodborne diseases. Spore forms of *B. cereus* cause serious problems for food and consumer health (Griffiths, M. W., and Schraft, H., 2017). *Staphylococcus aureus* is a highly dangerous pathogen that causes a variety of serious diseases. *S. aureus* causes general infections in humans, postoperative wound infections, ailments such as osteomyelitis (Smeltzer and Gillaspay, 2000). *Escherichia coli* is the most dominant pathogenic bacterium of the human intestine. *E. coli* has the ability to cause diseases of the gastrointestinal, urinary or central nervous system in humans. *E. coli* is the most common cause of pediatric diarrhea worldwide (Nataro, J. P., and Kaper, J. B., 1998). *Candida glabrata* is a type of yeast-like fungus that can be found in the flora of healthy individuals and cause various infections in humans. In addition to local infections, *C. glabrata* also causes systemic infections that are fatal. *C. glabrata* infections are difficult to treat and are often resistant to many azole-group antifungal drugs, especially fluconazole (Uçak and Şatana, 2021). *Pseudomonas aeruginosa* is a gram-negative, rod-shaped, obligate anaerobe in the family of *Pseudomonaceae*, which may also include opportunistic species, which act with polar flagella and are the last electron acceptor of oxygen. Some of the *Pseudomonas* species, many of which are common in nature, soil and waters, are pathogenic in plants or animals (Nickerson et al. 1977). In this study, we aimed to determine the antimicrobial activity of the water extract obtained from Ayvalık Yağlık olive fruit against food and clinical pathogens, and to reveal the potential use of this extract as a natural additive for various industries.

## MATERIAL AND METHOD

### Plant Material

Ayvalık Yağlık variety olive fruit samples were obtained from Izmir Olive Research Institute (Turkey) in August 2019 (Figure 1A).

### Preparation of Extracts

Ayvalık Yağlık olive fruit samples were washed and then dried. The dried sample was grounded with the Waring blender. The powdered sample (10 g) was extracted for 3 hours in a hot water bath with 30 ml of water (Figure 1B). After extraction, the solvents were evaporated (Figure 1C). Then, the extracts were stored at 4°C in dry conditions until they were used.



**Figure 1.** Ayvalık Yağlık Olive (A) and Preparation of Extracts (B-C)

### Test Microorganisms

In the study, *Salmonella enteritidis* ATCC 171, *Bacillus cereus* RSKK 863, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 11229, *Pseudomonas aeruginosa* ATCC 27853 and *Candida glabrata* RSKK 04019 were used as food-borne and clinical test microorganisms.

## Determination of Antimicrobial Activity

### *Disc diffusion assay*

*S. enteritidis* ATCC 171, *B. cereus* RSKK 863, *S. aureus* ATCC 25923, *E. coli* ATCC 11229 and *P. aeruginosa* ATCC 27853 as test microorganisms were cultured in Nutrient broth (NB) medium. Yeast Extract-Peptone-Dextrose (YPD) was used as growth medium for *C. glabrata* RSKK 04019. The test microorganisms were adjusted to a concentration of 0.5 McFarland and 100 µl bacterial suspensions was spread over the solid medium. Sterile discs were placed on agar medium in 2 replications. 20 µl (4000µg/disc) of olive water extract was dropped onto the discs. *C. glabrata* RSKK 04019 at 30°C and the test bacteria at 37°C for 24 hours were incubated. Inhibition zone diameters around the discs were measured and recorded at the end of incubation.

### *Determination of Minimal Inhibition Concentration (MIC) and Minimal Bactericidal Concentration (MBC)*

The medium and olive fruit extract were added to the tubes and diluted to determine the minimum inhibition concentration (MIC). The microbial suspension (adjusted to 0.5 McFarland) was added to the tubes and then incubated for 24 hours at the appropriate temperature. The concentration in the tubes where no growth was observed after incubation was recorded as the MIC value of the olive fruit extract. Then, the spotting cultivation from the test tubes was performed onto the solid medium. The inoculated petri dishes were left to incubate for 24 hours at appropriate temperatures. At the end of incubation, the concentration at which growth was not observed was determined as the MBC value.

## RESULT AND DISCUSSION

The antimicrobial activity of Ayvalık Yağlık olive water extract against food-borne and clinical microorganisms was determined by disc diffusion and micro-dilution method. The disc diffusion assay results of olive water extract are given in Table 1. The inhibition zone diameters ranged from 19.46 mm to 12.35mm. The highest antibacterial effect of olive water extract was determined against *E. coli* ATCC 11229 (19.46 mm). The antifungal activity against *C. glabrata* RSKK 04019 was obtained as 14.12 mm of inhibition zone diameter. The MIC and MBC or MFC results of the olive water extract on the test microorganisms are given in Table 2. *S. aureus* ATCC 25923 has the lowest MIC and MBC values of 10 mg/ml. MIC values changed from 10 mg/ml to 20 mg/ml. MBC or MFC values were determined as 10 mg/ml-80 mg/ml.

**Table 1.** The disc diffusion assay results of Ayvalık Yağlık olive water extract

| Test Microorganisms             | Inhibition zone diameter (mm) |
|---------------------------------|-------------------------------|
| <i>S. enteritidis</i> ATCC 171  | 12.35±0.45                    |
| <i>B. cereus</i> RSKK 863       | 12.75±0.17                    |
| <i>S. aureus</i> ATCC 25923     | 18.37±0.01                    |
| <i>E. coli</i> ATCC 11229       | 19.46±0.05                    |
| <i>P. aeruginosa</i> ATCC 27853 | 14.32±0.01                    |
| <i>C. glabrata</i> RSKK 04019   | 14.12±0.17                    |

**Table 2.** The MIC and MBC or MFC values of Ayvalık Yağlık olive water extract

| Test Microorganisms             | MIC ( $\mu\text{g}/\mu\text{l}$ ) | MBC/MFC ( $\mu\text{g}/\mu\text{l}$ ) |
|---------------------------------|-----------------------------------|---------------------------------------|
| <i>S. enteritidis</i> ATCC 171  | 20                                | 40                                    |
| <i>B. cereus</i> RSKK 863       | 20                                | 80                                    |
| <i>S. aureus</i> ATCC 25923     | 10                                | 10                                    |
| <i>E. coli</i> ATCC 11229       | 20                                | 40                                    |
| <i>P. aeruginosa</i> ATCC 27853 | 20                                | 40                                    |
| <i>C. glabrata</i> RSKK 04019   | 20                                | 80                                    |

The tested extract is considered bactericidal if the MBC/MIC ratio is  $\leq 4$ , and bacteriostatic if the MBC/MIC or MFC/MIC ratio is  $>4$  (Krishnan, N. et al. 2010; Hazen, K. 1998). The MBC/MIC or MFC/MIC ratio of the olive extract was found to be equal to or below 4. Olive water extracts have a bactericidal or fungicidal effect against all the tested microorganisms (Table 3).

**Table 3.** MBC/MIC or MFC/MIC values of Ayvalık Yağlık olive water extract.

| Test Microorganisms             | MBC/MIC or MFC/MIC |
|---------------------------------|--------------------|
| <i>S. enteritidis</i> ATCC 171  | 2                  |
| <i>B. cereus</i> RSKK 863       | 4                  |
| <i>S. aureus</i> ATCC 25923     | 1                  |
| <i>E. coli</i> ATCC 11229       | 2                  |
| <i>P. aeruginosa</i> ATCC 27853 | 2                  |
| <i>C. glabrata</i> RSKK 04019   | 4                  |

In a study conducted with the fruit of alcaparra olive, olive methanol extract was tested on bacteria (*B. cereus*, *Bacillus subtilis*, *S. aureus*, *P. aeruginosa*, *E. coli*, *Klebsiella pneumoniae*, *Candida albicans* ve *Cryptococcus neoformans*) related human gastrointestinal and respiratory tract infection. *C. albicans* and *C. neoformans* were found to be resistant to the olive extract at a concentration of 50 mg/ml. The other test microorganisms were found to be sensitive to the extract (Sousa, A. et al., 2006). In a study conducted by Markin et al. (2003), they investigated the antimicrobial effect of an water extract obtained from olive leaves on pathogenic bacteria (*E. coli*, *P. aeruginosa*, *B. subtilis*, *S. aureus*, *K. pneumoniae* and *C. albicans*). The olive leaf extract at a concentration of 20% inhibited *B. subtilis*, and 0.6% of concentration for other bacteria within 3 hours.

As a result of the study, it was found that olive fruit water extract has high antimicrobial activity. Olive water extract has functional properties in foods with its bioactivity and has the ability to extend the shelf life of foods. In addition, it is possible to use the flavoring feature as well as the service of the food service of herbal antimicrobials (Liu et al. 2017).

## CONCLUSION

The antimicrobial activity of olive fruit water extract against food and clinical pathogens has been investigated. It has been established that it has a high antimicrobial activity against test microorganisms. It has been determined that olive fruit extract may have the potential to be used as an antibacterial or antifungal additive in the food industry and as an alternative herbal source in pharmacology.

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## **DETERMINATION OF USEGA POTENTIAL OF RED PITAHAYA EXTRACTS AGAINST *Escherichia coli* AND *Listeria monocytogenes* FOR THE FOOD INDUSTRY**

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### **ABSTRACT**

The increasing population of the world also increases the need in the food industry. The preservation and prolongation of the shelf life of foods provides advantages in the food industry. Food-borne pathogens can be controlled with synthetic and natural protective substances. Pitahaya has started to gain popularity in Turkey as well as in all countries. It is known as a fruit that provides benefits in many areas thanks to its rich bioactive compounds. In the current study, antimicrobial activity of methanol and water extracts from red pitahaya fruit and its peel prepared by using sonication device against food-borne pathogens was determined. Disc diffusion and micro-dilution assays were used to obtain antimicrobial activity. The highest inhibition zone diameter was determined as 10.62 mm in fruit methanol extract against *Listeria monocytogenes* ATCC 7644. The lowest MIC value was determined as 10 µg/µl against *Escherichia coli* O157:H7 in fruit water extract. The MBC value was determined as 80 µg/µl for all extracts in *E. coli* O157:H7. The results of the study indicated that red pitahaya extracts can be used as a natural protective substance in the food industry.

**Keywords:** Red pitahaya, Food-borne pathogens, Natural food protective, Antimicrobial activity,

\* The study is produced from master thesis of the first author.

### **INTRODUCTION**

In the food industry, it is of great importance to preserve and extend the shelf life of foods for the purpose of cope with the increasing demand for food. Various chemicals are used to increase the shelf life of foods. However, studies have reported that the chemicals used have many negative effects, such as creating antibiotic resistance and contributing to cancer formation, especially in people who consume it regularly, especially in children. (Bearth et al., 2014; Shim et al., 2011). Controlling food-borne pathogens can be achieved by using chemical and natural antimicrobial food protective. Herbal antimicrobial food protective compared to chemical food protective, are considered more dependable and can benefit human health (Seow et al., 2014). In addition, herbal antimicrobial protective can add flavor and flavor to foods.

Pitahaya (*Hylocereus* spp.) is the fruit of the tropical cactus, known as the tropical and subtropical regions of Central and South America with its homeland Mexico. Pitahaya, which has spread as an ornamental plant, has been consumed as fresh fruit in recent years (Gunasena et al., 2007). Pitahaya is a phytochemical storehouse with bioactivities such as betacyanin, phenolic compounds, polysaccharides and terpenoids. It has been reported that pitahaya fruit helps to cope with metabolic syndromes such as obesity, cancer, type 2 diabetes (Joshi and

Prabhakar, 2020; Ramli et al., 2014; Song et al., 2016). It has also been reported that Pitahaya has the potential to be used as a colorant in foods because it contains high phytochemical compounds (Manihuruk et al., 2017).

Food-borne microorganisms are among the important problems affecting food safety. It causes infections in humans after consumption of food products contaminated with these microorganisms or their toxins (Heredia and Garcia, 2018). Since there is usually an incubation period for food-borne infections, the time from ingestion to the appearance of symptoms is much longer than for food-borne intoxications (Bintsis, 2017). *E. coli* and *L. monocytogenes* are among the most known pathogens (Bintsis, 2017).

*E. coli* is a rod-shaped, Gram-negative bacterium belonging to the family of *Enterobacteriaceae* (Assefa and Bihon, 2018). *E. coli* contains a large and diverse group of bacteria, and most strains are harmless, but some strains have properties that make them pathogenic to humans, such as toxin production (Garcia et al., 2010). *E. coli* is among the many pathogenic microorganisms that can access food of animal origin and is considered an indicator of contamination with fertilizers, soil, contaminated water (Disassa et al., 2017).

*L. monocytogenes* is among the important food pathogens commonly found in nature (O'Grady et al., 2009). It is an important cause of diseases in both animals and humans (Derra et al., 2013). *L. monocytogenes* causes the disease Listeriosis that is a serious infection. It usually occurs by consuming foods contaminated with *L. monocytogenes* and has a high mortality rate of 20-30% (Jemmi and Stephan, 2006). In our study, the usage potential of methanol and water extracts from red pitahaya fruit and peel as natural additives in the food industry was investigated.

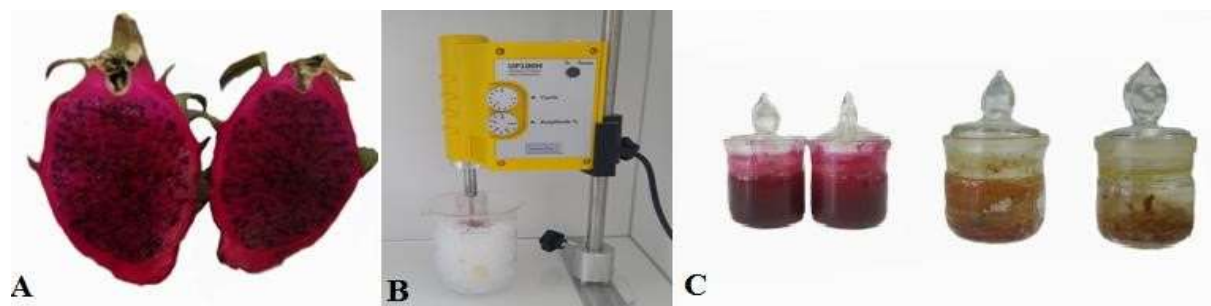
## MATERIAL-METHOD

### Plant Material

The red pitahaya fruit was purchased from Antalya-Turkey in October 2021.

### Preparation of Extracts

The pitahaya fruit samples were washed with distilled water. Then, the fruits and their peels were air-dried separately at room temperature. Dried fruit samples were ground with Waring blender. In extraction, 10 g powder from red pitahaya fruit and peel were extracted with 30 ml of methanol (96%) and water using a sonication device (Hielscher) on ice in 2 repetitions in 10 minutes every day (2 days) each. red pitahaya fruit and peel extracts were dissolved in dimethyl sulfoxide (DMSO) and then sterilized with a 0.45 µm filter. The extracts were stored at 4°C until used.



**Figure 1.** A: Red pitahaya, B: Preparation of the extracts, C: Red pitahaya fruit-peel methanol and water extracts

### Determination of Antimicrobial Activity Disc Diffusion Assay

Disc diffusion method was used to determine the antimicrobial activity of methanol and water extracts from red pitahaya fruit and peel. Test microorganisms including food-borne bacteria (*L. monocytogenes* ATCC 7644 and *E. coli* O157:H7) was used. Food-borne pathogens were cultured at 37°C in Tryptic Soy Broth (TSB) and Nutrient Broth (NB) media for 24 hours. The test microorganisms were washed twice with saline solution and their concentration was adjusted to 0.5 Mcfarland. 100 µl of prepared microbial suspension (0.5 Mcfarland) was spread onto agar media. Sterile discs (diameter of 6 mm) were placed on the solid agar and 20 µl (4000 µg/disc) of red pitahaya fruit and peel extracts were then dropped onto the discs in triplicates. The petri dishes were incubated at appropriate temperatures for 24 hours. After incubation, the inhibition zones around the disc were measured with a caliper and recorded.

### Minimum Inhibition Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts were determined using the micro-dilution assay. The red pitahaya extracts were added to each growth medium to obtain a final concentration of 80 µg/µl and diluted to 40, 20, 10 and 5 µg/µl in tubes. The tested microorganisms adjusted to 0.5 McFarland were inoculated into each tube. The tubes containing the mixture were incubated at 37°C for 24 h. After incubation, the concentration of the extract in the non-growth tube was recorded as MIC values. Then, the samples from tubes were inoculated onto solid media using spotdropping method and the plates were incubated at 37°C for 24 h. At the end of the incubation, the extract concentrations that prevent bacterial growth on the solid media were evaluated as MBC values. The results are expressed as µg/µl.

## RESULTS AND DISCUSSION

Pathogen microorganisms are the main cause of a wide variety of diseases. Antimicrobial activities of red pitahaya fruit and peel extracts were investigated on food-borne test microorganisms by disc diffusion and micro-dilution experiments. Disc diffusion assay results are given in Table 1. The highest inhibition zone diameter in methanol extracts were found to be 10.62 mm on *L. monocytogenes* ATCC 7644 for fruit extract. The highest inhibition zone diameter in water extracts were found to be 9.35 mm on *E. coli* O157:H7 for fruit extract. The peel water extract has shown static effect on *L. monocytogenes* ATCC 7644.

**Table 1.** Inhibition zone diameter extracts of red pitahaya (mm ± SD)

| Test microorganisms                     | Extract Inhibition zone diameter (mm) |           |           |           | Antibiotics inhibition zone diameter (mm) |
|---|---------------------------------------|-----------|-----------|-----------|---|
|   | RPFM                                  | RPPM      | RPFW      | RPPW      | Ampicillin(10µg)                          |
| <i>Listeria monocytogenes</i> ATCC 7644 | 10.62±0.30                            | 9.09±0.65 | 7.16±0.37 | 6.95±0.32 | 17.76±0                                   |
| <i>Escherichia coli</i> ATCC O157:H7    | 7.85±1.11                             | 9.6±0.34  | 9.35±1.75 | 8.06±0.51 | 16.81±0.2                                 |

\* RPFM: Red Pitahaya Fruit Methanol extract, RPPM: Red Pitahaya Peel Methanol extract, RPFW: Red Pitahaya Fruit Water extract, RPPW: Red Pitahaya Peel Water extract



In a study, the antimicrobial activity of red pitahaya fruit peel extract at concentrations in the range of 100-1000 µg/ml on *Neisseria meningitidis*, *Streptococcus pneumoniae* and *L. monocytogenes* test microorganisms was investigated. The highest inhibition zone diameter was determined as 25.64 mm at 1000 µg/ml concentration on *S. pneumoniae* (Ritarwan and Nerdy,2018). In another study conducted on red pitahaya fruit peel, the antimicrobial activity of extracts prepared at various concentrations against the test microorganism *Pseudomonas* sp. was tested. It was determined that as the concentration increased, the diameter of the inhibition zones increased (Setiani, 2020).

MIC values of red pitahaya water-methanol fruit and peel extracts on all tested pathogens were determined between 10 µg/µl and 40 µg/µl and the results are given in Table 2. Among the red pitahaya extracts tested, the lowest MIC value was found to be 10 µg/µl in the fruit water extract against *E. coli* ATCC O157:H7.

**Table 2.** MIC values of red pitahaya extracts

| Test microorganisms                     | MIC (µg/µl) |      |      |      |
|---|-------------|------|------|------|
|   | RPFM        | RPPM | RPFW | RPPW |
| <i>Listeria monocytogenes</i> ATCC 7644 | 40          | 40   | 40   | 40   |
| <i>Escherichia coli</i> ATCC O157:H7    | 20          | 40   | 10   | 20   |

\* RPFM: Red Pitahaya Fruit Methanol extract, RPPM: Red Pitahaya Peel Methanol extract, RPFW: Red Pitahaya Fruit Water extract, RPPW: Red Pitahaya Peel Water extract

In the study, MIC value of red pitahaya peel extract was determined. Five Gram-negative bacteria (*E. coli*, *Klebsiella pneumoniae*, *Morganella morganii*, *Pseudomonas aeruginosa* and *Proteus mirabilis*) and three Gram-positive bacteria (methicillin-resistant *Staphylococcus aureus* (MRSA), *L. monocytogenes* and *Enterococcus faecalis*) were tested. The lowest inhibition concentration was determined as 5 mg/ml against *E. faecalis* (Roriz et al., 2022). The MBC values of methanol and water extracts obtained from red pitahaya fruit and peel are given in Table 3. The MBC value of all the extracts on *E. coli* ATCC O157:H7 was determined as 80 µg/µl. The lowest MBC value against *L. monocytogenes* ATCC 7644 was found in the peel methanol extract as 80 µg/µl.

**Table 3.** MBC values of red pitahaya extracts

| Test microorganisms                     | MBC (µg/µl) |      |      |      |
|---|-------------|------|------|------|
|   | RPFM        | RPPM | RPFW | RPPW |
| <i>Listeria monocytogenes</i> ATCC 7644 | >80         | 80   | >80  | >80  |
| <i>Escherichia coli</i> ATCC O157:H7    | 80          | 80   | 80   | 80   |

\* RPFM: Red Pitahaya Fruit Methanol extract, RPPM: Red Pitahaya Peel Methanol extract, RPFW: Red Pitahaya Fruit Water extract, RPPW: Red Pitahaya Peel Water extract

In the study, determined the MBC value of red pitahaya fruit peel methanol extracts against various test microorganisms (*Staphylococcus epidermidis* ATCC 12228, *S. aureus* ATCC25179, *E. faecalis* ATCC 10100, *L. monocytogenes* ATCC 13932, *Salmonella enterica typhi* ATCC 10749, *Serratia marcescens* ATCC 8100, *Shigella flexneri* ATCC 25923, *Klebsiella* ATCC 10273, *P. aeruginosa* ATCC 14149 and *E. coli* ATCC 85218). MBC values of fruit and peel extracts were given in the range of 0.39-100 mg/ml. The lowest MBC value against all test microorganisms was 0.39 mg/ml (Rohin et al., 2012).

## CONCLUSIONS

Biological activities of red pitahaya fruit and peel extracts were investigated to determine usage potential of pitahaya fruit as natural antimicrobial additive in food industry. The extracts exhibited antibacterial activities against the tested microorganisms. The extracts have the potential to be used as a natural additive and colorant in the food industry. Thus, pitahaya fruit extracts can be an alternative solution to the prevention of various problems by reducing the use of chemical additives in the food industry.

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## WHITE PITAHAYA IN AQUACULTURE: INVESTIGATION OF THE POTENTIAL USAGE OF FRUIT AND PEEL

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### ABSTRACT

Fish and seafood are widely consumed as protein sources that are valuable for balanced nutrition and health. One of the problems of fish farms is infectious diseases. These problems cause serious economic losses to the manufacturer. Plants have the potential to be used in the treatment of diseases in animals. The white pitahaya fruit has recently become a fruit of interest. In the present study, antimicrobial activity of methanol extracts of fruit and peel of white pitahaya was determined by disc diffusion and micro-dilution assays. The results of the disc diffusion test indicated that the highest inhibition zone diameter was found to be 12.93 mm in peel methanol extract and 11.54 mm in fruit methanol extract against *Aeromonas hydrophila* ATCC 19570. MIC value was found to be 40 mg/ml in fruit and peel methanol extracts against the microorganisms of *A. hydrophila* ATCC 19570, *Lactococcus garvieae* and *Yersinia ruckeri*. The lowest MBC value was determined as 40 mg/ml against *A. hydrophila* ATCC 19570, *L. garvieae* in fruit extract, against *A. hydrophila* and *Y. ruckeri* in peel extract. MIC and MBC values of white pitahaya extracts were determined as 80 mg / ml against *Vibrio anguillarum* A4. As a result of our study, it was reported that white pitahaya extracts have the potential to be used as a feed additives or natural antimicrobial substances in aquaculture.

**Keywords:** White pitahaya, Extract, Antimicrobial activity, Fish pathogens,

### INTRODUCTION

Pitahaya is a species of hanging cactus fruit belonging to the family *Cactaceae* (Patwary et al., 2013). Its fruit is of interest due to its color and exotic appearance (Liaotrakoon, 2013). Pitahaya fruits are gaining popularity due to the nutritional values and medicinal properties contained in them (Sonawane, 2017). Since human existence, plants have been used primarily to meet nutritional needs and find solutions to health problems (Nohutçu et al., 2019). The active molecules in plants (saponins, alkaloids, flavonoids and terpenoids) believed, often without revealing or stop the growth of pathogenic microorganisms, bacterial resistance to antibiotic use-can provide inhibiting activity (Chandra et al., 2017).

Although the increasing need for protein throughout the world every day makes it necessary to focus on various foods in different countries, another source that comes to mind after terrestrial protein sources in our country, which is surrounded by seas on three sides, is aquatic protein sources (Kocatepe, 2018). It is estimated that the world's population will be around 9 billion by 2050. For this reason, aquaculture will have an important role in meeting the increasing demand for protein foods (Godfray et al., 2010). To meet this growing demand and remain economically profitable, fish farms need to implement intensive production conditions. This process, in aquaculture, promotes the emergence of pathogenic bacteria and

creates a favorable environment for the spread of the disease (Pulkkinen et al., 2010; Sundberg et al., 2016). In aquaculture, pathogens that cause the disease include *A. hydrophila*, *L. garvieae*, *Y. ruckeri*, *V. anguillarum*, *Streptococcus agalactiae*, *Vibrio alginolyticus*, as well as aeromonads, mycobacteria (Acha and Szyfres, 2003; Lipp and Rose, 1997; Zlotkin et al., 1998). The increase of aquaculture in the world has been associated with the use of antibiotics, which are widely used in treatment due to their negative impact on human and animal health (Cabello et al., 2013).

The disadvantage of antibiotic use is resistance genes. Resistant bacteria or resistance genes can spread among aquatic communities, which may enter the human food chain (Chen et al., 2015). Therefore, alternatives that will not harm the environment or minimize this harm for prophylactic and therapeutic purposes have priority in solving the health problems of aquatic creatures. In this regard, the use of plants can provide an environmentally friendly solution for the development of sustainable health management in aquaculture. There is increasing scientific evidence on the beneficial effects of diets enriched with natural compounds or plant extracts on fish health and the prevention of disease outbreaks (Reverter et al., 2014). Potential antimicrobial activity of plant extracts in addition to the digestibility and availability of nutrients can increase immunity in fish as well as various culture, thus can cause an increase in feed conversion efficiency and protein synthesis (Talpur and Ikhwanuddin, 2013).

In our study, the antimicrobial activities of the extracts were evaluated to develop white pitahaya extracts products as a natural additive in the aquaculture feed industry.

## MATERIAL-METHOD

### Plant Material

The white pitahaya fruit was purchased from Antalya-Turkey in September 2021.



**Figure 1.** Pitahaya greenhouse in Antalya (A), Pitahaya fruit (B)

### Preparation of Extracts

The Pitahaya fruit samples were washed with distilled water. Then, the fruits and their peels were dried separately in an airy condition. Dried fruit samples were ground with Waring blender. For extraction, 10 g of plant material was weighed and then 30 ml of methanol (96%) added. Fruit samples were extracted every day for 6 hours (3 days) in a hot water bath (Figure 2). White pitahaya fruit and peel extracts were dissolved in dimethyl

sulfoxide (DMSO) and then sterilized with a 0.45  $\mu\text{m}$  filter. The extracts were stored at 4°C until used.



**Figure 2.** Hot water bath extraction method

### **Determination of Antimicrobial Activity Disc Diffusion Assay**

Disc diffusion method was used to determine the antimicrobial activity of methanol extracts obtained from white pitahaya fruit and peel against fish pathogens. Fish pathogens (*A. hydrophila* ATCC 19570, *L. garvieae*, *Y. ruckeri*, *V. anguillarum* A4) were used. *A. hydrophila* ATCC 19570 was cultured at 37°C in Nutrient Broth (NB) media for 24 hours. *L. garvieae*, *Y. ruckeri* and *V. anguillarum* A4 were cultured at 25°C in Tryptic Soy Broth (TSB) and Tryptic Soy Broth/Sodium Chloride (TSB/NaCl) media for 24 hours. The test microorganisms were washed twice with saline solution and their concentration was adjusted to 0.5 Mcfarland. 100  $\mu\text{l}$  of prepared microbial suspension (0.5 Mcfarland) was spread onto agar media. Sterile discs (diameter of 6 mm) were placed on the solid agar and 20  $\mu\text{l}$  (4000  $\mu\text{g}/\text{disc}$ ) of white pitahaya fruit and peel extracts were then dropped onto the discs in triplicates. The petri dishes were incubated at appropriate temperatures for 24 hours. After incubation, the inhibition zones around the disc were measured with a caliper and recorded.

### **Micro-dilution Assay**

The minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts were determined using the micro-dilution assay. Test microorganisms adjusted to 0.5 McFarland were added to each tube containing extract and media. The tubes containing the mixture were incubated at appropriate temperatures for 24 h. After incubation, the concentration of the extract in the non-growth tube was recorded as MIC values. Then, the samples from tubes were inoculated onto solid media using spot-dropping method and the plates were incubated at appropriate temperatures for 24 h. At the end of the incubation, the extract concentrations that prevent bacterial growth on the solid media were evaluated as MBC values.

## **RESULTS AND DISCUSSION**

The antimicrobial activity of methanol extracts of fruit and peel of white pitahaya was determined by disc diffusion method against fish pathogens and the results are given in Table 1. The results of the disc diffusion test indicated that the highest inhibition zone diameter was found to be 12.93 mm in peel methanol extract and 11.54 mm in fruit methanol extract against *A. hydrophila* ATCC 19570. The methanol extract from white pitahaya fruit showed the lowest

inhibitory effect against *L. garvieae* and *Y. ruckeri* (6.99 mm and 6.76 mm). The inhibition zone diameters against the tested fish pathogens varied from 6.76 mm to 12.93 mm. The fruit methanol extracts showed a static effect on *L. garvieae* and *Y. ruckeri*. In general, the peel methanol extract showed higher inhibition zone diameters than fruit extract against all test microorganisms.

**Table 1.** Inhibition zone diameters of extracts from white pitahaya

| Test microorganisms                    | Extracts                     |            |
|--|------------------------------|------------|
|  | Inhibition zone diameter(mm) |            |
|  | WPFM                         | WPPM       |
| <i>Aeromonas hydrophila</i> ATCC 19570 | 11.54±1.77                   | 12.93±1.59 |
| <i>Lactococcus garvieae</i>            | 6.99±0.43                    | 10.84±1.13 |
| <i>Yersinia ruckeri</i>                | 6.76±0.53                    | 9.67±0.62  |
| <i>Vibrio anguillarum</i> A4           | 11.33±2.38                   | 11.43±0.39 |

\*WPFM: White Pitahaya Fruit Methanol extract, \*WPPM: White Pitahaya Peel Methanol extract

The micro-dilution test results of the extracts are presented in Table 2. MIC value was found to be 40 mg/ml in fruit and peel methanol extracts against the microorganisms of *A. hydrophila*, *L. garvieae* and *Y. ruckeri*. The extracts had 80 mg/ml of MIC value against *V. anguillarum* A4. The lowest MBC value was determined as 40 mg/ml against *A. hydrophila*, *L. garvieae* in fruit extract, against *A. hydrophila* and *Y. ruckeri* in peel extract.

**Table 2.** Micro-dilution Assay Results of the White Pitahaya Extracts

| Test microorganisms                    | Extracts    |      |             |      |
|--|-------------|------|-------------|------|
|  | MIC (mg/ml) |      | MBC (mg/ml) |      |
|  | WPFM        | WPPM | WPFM        | WPPM |
| <i>Aeromonas hydrophila</i> ATCC 19570 | 40          | 40   | 40          | 40   |
| <i>Lactococcus garvieae</i>            | 40          | 40   | 40          | >80  |
| <i>Yersinia ruckeri</i>                | 40          | 40   | >80         | 40   |
| <i>Vibrio anguillarum</i> A4           | 80          | 80   | 80          | 80   |

\*WPFM: White Pitahaya Fruit Methanol extract, \*WPPM: White Pitahaya Peel Methanol extract

The tested extract is considered bactericidal if the MBC/MIC ratio is  $\leq 4$ , and bacteriostatic if the MBC/MIC ratio is  $>4$  (Gatsing et. al., 2009). MBC/MIC ratios of white pitahaya fruit and peel extracts were found to be 1. It has been determined that the extracts have antibacterial activity against the tested microorganisms except not determined (ND) group in the study (Table 3).

**Table 3.** MBC/MIC ratio of white pitahaya extracts

| Test microorganisms                    | MBC/MIC |      |
|--|---------|------|
|  | WPFM    | WPPM |
| <i>Aeromonas hydrophila</i> ATCC 19570 | 1       | 1    |
| <i>Lactococcus garvieae</i>            | 1       | ND   |
| <i>Yersinia ruckeri</i>                | ND      | 1    |
| <i>Vibrio anguillarum</i> A4           | 1       | 1    |

\*WPFM: White Pitahaya Fruit Methanol extract, \*WPPM: White Pitahaya Peel Methanol extract, \*ND: Not Determined

## CONCLUSION

The increasing need for protein throughout the world every day leads to the fact that this need is provided from different sources. One of the first sources that comes to mind is aquaculture. Bacterial diseases that occur on fish farms can cause large crop losses and material losses. Due to the disadvantages of the antibiotics used in the treatment at the same time, the orientation to naturalness has begun. Pitahaya fruit has recently gained great popularity in our country as well. In particular, the possibility of using peel extracts as additives can also result in great commercial gains. In our study, it was determined that methanol extracts of white pitahaya fruit and peel have antimicrobial activity. There are no studies investigating the antimicrobial activity of white pitahaya extracts on fish pathogens in the literature. It has been reported that methanol extracts of fruit and peel of white pitahaya have the potential to be used as a feed additive or natural antimicrobial additive in aquaculture.

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## EFFECTS OF FERMENTED OLIVE LEAF AND ITS WASTEWATER ON PERFORMANCE PARAMETERS OF BROILER CHICKENS

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### ABSTRACT

The present study was conducted to determine the effects of olive leaf fermented with sheep rumen liquid and its wastewater on the performance parameters in broilers. In fermentation, 500 g of olive leaves, 400 ml of distilled water, and 8.4 g of urea (46%) as a nitrogen source was used. Fermentation starting pH value was arranged with 1N HCl. Then sterilization was applied at 120 °C for 15 minutes, and the inoculation (%2) was done in a sterile cabin using microorganisms from the rumen liquid (OD=0,8). Fermentation was carried out in an oxygen-free environment and at 38 °C temperature. For the animal experiment, there were four treatment groups (five replications, seven birds in each replication), and a total of 140 one-day-old Ross 308 male broiler chickens were used. Birds were fed with a control diet (without olive leaf and wastewater) (**CON**), non-fermented olive leaf (**OL**) (7.5 g/kg feed), fermented olive leaf (7.5 g/kg feed) (**FOL**), and leaf wastewater (7.5 ml /kg feed) (**FW**) at 42 days. Diets were presented to birds as *ad-libitum*. At the end of the study, there were no differences in the final weight (FW), weight gain (WG), feed conversion ratio (FCR), and feed intake (FI) between treatment at 42 days ( $P>0.05$ ). In the finishing period (24-42 d), olive leaf wastewater had a negative effect on WG compared to the other groups, and the difference between the other groups was statistically significant ( $P<0.05$ ). In conclusion, the diets fed with olive leaves and wastewater fermented with rumen liquid could not negatively affect broiler performance.

**Key Words:** Olive leaves, Fermentation, Rumen liquid, Broiler, Performance

### INTRODUCTION

Olive (*Olea europaea*) is a shrub native to the Mediterranean climate, belonging to the olive family (Oleaceae) family, or a tree up to 10 meters tall, with dense branches and broad branches, crowns, and evergreen leaves. Approximately 17 million tons of olives are cultivated worldwide. Spain, Italy, Greece, Turkey, Tunisia, and Morocco are critical olive-producing countries (T.C. Ticaret Bakanlığı, <https://ticaret.gov.tr/data/5d41e59913b87639ac9e02e8/3acedb62acea083bd15a9f1dfa551bcc.pdf>). The total olive production of Turkey was announced as 1,316,626 tons in 2020 (TUIK, 2020). Although the number of olive leaves obtained during the olive harvest varies according to the tree's age and the pruning method, it is reported as approximately 12-30 kg/tree (Nefzaoui, 1983). On the other hand, these leaves are separated during olive processing and

have antimicrobial and antioxidant properties thanks to their various phenolic compounds. They have significant potential in animal nutrition.

The olive leaf's nutrient content contains approximately 12.92% crude protein, 2.39% crude fat, 6.41% crude ash, and 27.83% crude cellulose in dry matter (Altop et al., 2018). However, it is rich in valine, arginine, and leucine but poor in cystine and tyrosine (Martin-Garcia et al., 2006). Apart from this, it is known that olive leaf contains more than 30 phenolic compounds.

The fermentation method increases feed raw materials' nutrient content and eliminate anti-nutritional factors (Cao, 2012; Zhang et al., 2013; Xie et al., 2016; Altop et al., 2017). Studies have reported that fermented products' nutrient composition is increased, and anti-nutritional factors are reduced (Adeyemi et al., 2008; Akinfemi, 2010; Ari et al., 2012; Ari & Ayanwale, 2012). Although bacteria, fungi, yeast, or various combinations of these are generally used in the fermentation method, there are studies in which rumen liquid has been used as an inoculant in recent years. However, the expected product improvements cannot be obtained in the research using rumen liquid. Because the optimized conditions of fermentation, such as the initial pH of the fermentation medium, nutritional salt addition, humidity, and inoculant level, could not be determined, the desired results could not be obtained. On the other hand, in a study conducted by optimizing the fermentation conditions, it was determined that the anti-nutritional factor cellulose decreased and the protein content increased by fermenting cottonseed meal with rumen liquid (Koç et al., 2021).

The present study was conducted to determine the effects of olive leaf fermented with sheep rumen liquid and its wastewater on the performance parameters in broilers.

## **MATERIAL AND METHOD**

The experiment consists of the fermentation of the olive leaf (in vitro) and the use of the unfermented olive leaf in broilers (in vivo) together with the fermented olive leaf and wastewater obtained at the end of the fermentation.

The olives brought to the laboratory in a dry form were ground and made ready for fermentation. In fermentation, 500 g of olive leaves, 400 ml of distilled water, and 8.4 g of urea (46%) as a nitrogen source was used. Fermentation starting pH value was arranged with 1N HCl. Then sterilization was applied at 120 °C for 15 minutes, and the inoculation (%2) was done in a sterile cabin using microorganisms from the rumen liquid (OD=0,8). Fermentation was carried out in an oxygen-free environment and at 38 °C temperature.

For the animal experiment, there were four treatment groups (five replications, seven birds in each replication), and a total of 140 one-d-old Ross 308 male broiler chickens were used. Birds were fed with a control diet (without olive leaf and wastewater) (**CON**), non-fermented olive leaf (**OL**) (7.5 g/kg feed), fermented olive leaf (7.5 g/kg feed) (**FOL**), and leaf wastewater (7.5 ml /kg feed) (**FW**) at 42 days. Diets were presented to birds as *ad-libitum*.

## RESULTS AND DISCUSSION

At the end of the study, there were no differences in the final weight (FW), weight gain (WG), feed conversion ratio (FCR), and feed intake (FI) between treatment at 42 days ( $P>0.05$ ). In the finishing period (24-42 d), olive leaf wastewater had a negative effect on WG compared to the other groups, and the difference between the other groups was statistically significant ( $P<0.05$ ).

Fermentation is a method that allows the use of various feedstuffs in diets. It does this by reducing feedstuff's antinutritional factors (Altop, 2019) and improving the nutrient composition (Altop et al., 2018b). The fermentation method reduces tannin compounds and high cellulose content in olive leaves (Altop et al., 2018). A study showed that using fermented olive leaves at the rate of 10% in the diet positively affected performance parameters (Xie et al., 2016). It was reported that the use of 5% also had a positive effect, but the highest live weight gain was 10%. These results are not similar to the research because the inoculants used in fermentation are different; accordingly, the changes in the composition of the fermented product differ. In addition, it is thought that the dose used is insufficient to achieve the expected effect from the olive leaf. Therefore, it is recommended to use higher doses in future studies.

## CONCLUSION

In conclusion, the diets fed with olive leaves and wastewater fermented with rumen liquid could not negatively affect broiler performance.

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## **DETERMINATION OF CHANGES IN SOME NUTRIENT CONTENT OF *Agaricus bisporus* CAP PART FERMENTED WITH RUMEN LIQUID IN DIFFERENT ENVIRONMENTAL CONDITIONS**

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### **ABSTRACT**

In this study, the changes in nutrient composition were investigated by fermenting *Agaricus bisporus* cap part with liquid rumen study was carried out on a total of 36 samples, with three different fermentation times (3, 5, and 7 days), two different initial pH (6 and 7), and two different inoculations (Yes or no) in a 3x2x2 factorial experiment design and three replications in each group. The cap and stem part of the mushrooms brought to the laboratory were separated, and the cap part was sliced into small pieces for fermentation. The rumen content used in fermentation was obtained from Bafra sheep on a farm in Samsun. The rumen liquid brought to the laboratory was centrifuged at 1000 rpm for 10 minutes in pre-sterilized falcon tubes. Then it was made ready for inoculation. The fermentation medium was decontaminated by the autoclave method before adding rumen liquid, and then 1000 µl of rumen liquid was added to each medium. Fermentation was carried out in incubators set at 37 °C. The samples, which completed the fermentation period, were dried and analyzed for crude protein, cellulose, ash, acid detergent fiber, and neutral detergent fiber. At the end of the research, it was determined that the *A. bisporus* cap part fermented with rumen liquid positively affects the nutrient content. According to the results obtained, the most effective fermentation time was seven days, regardless of the initial pH value.

**Key Words:** Fermentation, rumen liquid, nutrient composition, *Agaricus bisporus*.

### **INTRODUCTION**

*Agaricus bisporus* is a plant that has been used in human nutrition for many years in the world. It has a wide production range worldwide, and the Asian region is among the most important producers (Sonnenberg et al., 2011). This production is followed by Italy and the USA (Atila et al., 2017). According to FAO (2019) data, it is reported that annual production of 10 million tons is made in the world. However, Turkey has an annual production of 55 thousand tons (TUIK 2021).

*A. bisporus* is a valuable plant with its nutrients and various biochemical substances. It contains incredibly high levels of polysaccharides, fiber compounds, antioxidants, and vitamins C, B12, and D. In addition, folate, ergothioneine, and polyphenol compounds in its structure show that mushrooms have potential anti-inflammatory, hypoglycemic and

hypocholesterolemic effects (Fukushima et al. 2000, Mattila et al. 2001, Koyyalamudi et al. 2009 (a), Koyyalamudi et al. 2009 (b)).

The fermentation method using bacteria, yeast, or fungal inoculants improves the nutrient composition of the feedstuffs. In this way, it increases the use of feedstuffs in poultry diets (Altop et al., 2018). There are studies in which rumen liquid is used as an inoculant in fermentation. For example, it has been reported that when cottonseed meal is fermented with rumen liquid when suitable conditions are provided, the crude cellulose content decreases, the crude protein level increases, and as a result, the nutrient composition improves (Koç et al., 2021). However, studies in which feedstuffs are fermented with rumen liquid are limited.

The present study it is aimed to reduce the cellulose content and improve the nutritional composition of the cap of *A. bisporus* fermented using sheep rumen liquid. In addition, at the end of the study, it is aimed to determine optimal the fermentation time and pH conditions of fermented *A. bisporus*.

## **MATERIAL AND METHOD**

The cap and stem part of the mushrooms brought to the laboratory were separated, and the cap part was sliced into small pieces for fermentation. The rumen content used in fermentation was obtained from Bafra sheep on a farm in Samsun. The rumen liquid brought to the laboratory was centrifuged at 1000 rpm for 10 minutes in pre-sterilized falcon tubes. Then it was made ready for inoculation.

The study was carried out on a total of 36 samples, with three different fermentation times (3, 5, and 7 days), two different initial pH (6 and 7), and two different inoculation (Yes or no) in a 3x2x2 factorial experiment design and three replications in each group.

Before the study, 100 g of caps, 400 ml of distilled water, and 8.4 g of urea (46%) as nitrogen sources were combined into each fermentation medium. The appropriate pH value of the fermentation medium was provided by using 1N HCl. The samples were then sterilized in an autoclave. Sterilized samples were inoculated around the burner flame using microorganisms obtained from rumen liquid. The study was carried out in an anaerobic environment at 37 °C.

The pH values of the samples and fermentation medium that completed the fermentation process were determined. Then after samples were dried in the air, dry matter, crude ash, and crude protein analyses were determined according to AOAC (2000).

The data were analyzed using the Windows version of SPSS 21.0 (SPSS Inc., NY, and the USA) statistical package program. The Duncan test was used to compare the differences between groups after the ANOVA test for the data variance. Results were considered significantly different at  $P < 0.05$ .

## **RESULTS AND DISCUSSION**

At the end of the study, the initial pH value did not affect the nutrient composition of *A. bisporus* ( $P > 0.05$ ). The inoculation effect was found to be effective on the fermentation medium pH ( $P < 0.01$ ), fermented product pH ( $P < 0.01$ ), and crude protein ( $P < 0.01$ ) values. Inoculation decreased the pH values in the medium and product. This result shows that fermentation is affected positively. Because the cellulolytic bacteria in the rumen liquid reduce the pH value of

the fermentation medium. (See article on working pH of cellulosic bacteria) Additionally, inoculation has been found to increase crude protein levels ( $P < 0.05$ ). This result is similar to Koç et al. (2021). Microorganisms in the rumen liquid positively contribute to the protein level by using urea nitrogen. Also, there was no effect of inoculation on dry matter ( $P > 0.05$ ) and crude ash ( $P > 0.05$ ) parameters.

When the day-based results were examined, it was found that the effect on the pH of the fermentation medium and the pH of the fermented product was significant ( $P < 0.01$ ). The lowest pH levels were determined, especially on the third and seventh days, and the difference between the fifth and fifth days was statistically significant. The effects of fermentation on dry matter ( $P < 0.01$ ), crude ash ( $P < 0.01$ ), and crude protein ( $P < 0.01$ ) values were found to be statistically significant. The highest crude protein level was determined on the seventh day, then on the third and fifth days. In addition, the lowest dry matter level was found on the seventh day and the fifth and third day, respectively.

## CONCLUSION

It has been determined that the *A. bisporus* cap part fermented with rumen liquid positively affects the nutrient content. According to the results obtained, the most effective fermentation time was seven days, regardless of the initial pH value.

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## SUN PROTECTION FACTORS OF COMMERCIAL OILS

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### ABSTRACT

Oils are used in the cosmetic industry for skin care and protection from the harmful effects of the sun. The possibilities of using commercially available (rosemary (*Rosmarinus officinalis*), tea tree (*Melaleuca alternifolia*), St. john's wort (*Hypericum perforatum*), ant egg, garlic (*Allium sativum*), almond (*Prunus amygdalus dulcis*) and peppermint (*Mentha piperita*)) oils in the cosmetic industry have been determined as in-vitro. Sun protection factor (SPF) of oils and commercially cream and oil mixtures were determined by spectrophotometric measurements between 290-320 nm wavelength. SPF values of oils varied between 0.21 and 3.15. The highest SPF value determined (3.15) has St. john's wort oil and its UV blocking percentage is over 50%. SPF values of commercial oils and cream mixtures were investigated at 2.5 ml, 5 ml, and 10 ml concentrations. SPF value of cream-oil mixtures in 10 ml concentration is between 4.41 and 24.20. Almond oil has the highest SPF value (24.20) of cream and oil mixtures, and its UV blocking percentage is over 96%. Commercially available oils and cream oil mixtures have potential to be used as natural ingredients for sunscreen in the cosmetic industry.

**Keywords:** Sun Protection Factor, Rosemary Oil, Tea Tree Oil, St. John's Wort Oil, Ant Egg Oil, Garlic Oil, Almond Oil, Peppermint Oil

### INTRODUCTION

Oils are used in the fields of food and cosmetics as industrial raw materials and edible resources (Hill, 2000). Oils contain many bioactive compounds used in various fields (Reichert, 2002). Some herbal oils obtained have medicinal properties and are used in nutrition and skin care products (Lin et al., 2010). The use of appropriate oils can delay the aging period of the skin (Chanhal and Swarnlata, 2008). Harmful sun rays and environmental effects cause deterioration in the skin structure. Oil-based cosmetics are used to prevent skin disorders and protect the skin from harmful effects (Kala and Palaparathi, 2017). Oils from plants usually have the property of easy penetration, moisturizing and softening when applied to the skin (Burnett et al., 2017).

UV causes chronic changes in the skin such as edema, pigmentation and photocarcinogenicity (Bauman, 2007). Sunscreens have been developed to protect against the harmful effects of UV (Reichert, 2002). Sunscreens absorb the harmful rays of the sun (Bauman, 2007). The use of herbal oils in sunscreen products increases the photoprotective effect (Montenegro and Santgati, 2019). It is known that vegetable oils obtained from plants can be used safely in commercially available sunscreens today (Acsova et al., 2021). The

beneficial effect of herbal resources on the skin becomes an advantage, allowing the demand in the cosmetic industry to increase and to be used as a natural resource (Calka and Pawel, 2019). The increase in sun protection property depends on the sun protection factor (SPF) (Comoğlu, 2012) and the effects may vary depending on the individual's skin phenotype, regular use, and whether the product used has sufficient sun protection factor (Moloney et al., 2002). Sunscreen factors prevent the skin from losing water and deteriorating its structure (Comoğlu, 2012).

Ultraviolet light is called visible light with a wavelength between 200-400 nm, and it is divided into 3 parts according to the wavelength (Donglikar and Deore, 2016). These; They are UVA, UVB and UVC. UVA with a wavelength of 320-400 nm (Kullavvanjiava and Lim, 2005) can also cause excessive melanin secretion as a result of the effect of the skin layer (Jou et al., 2012) premature aging (Kullavvanjiava and Lim, 2005) immunological damage (Longstreth et al., 1998) and darkening of the skin tone. UVB can cause sunburns at high levels in the wavelength range of 290-320 nm, as well as cause cancer formation after long-term contact (Kaimal and Abraham, 2011). It is the radiation that has more sunburn formation than UVA and has a significant effect on skin cancers (Jou et al., 2012). A UVC is a beam that is filtered in the ozone layer until it reaches the earth's surface with a wavelength of 200-290 nm and has little danger (Kullavvanjiava and Lim, 2005).

In this study, sun protection factors of various commercial oils and creams and oil mixtures were investigated to develop alternative natural additive alternatives for the cosmetic industry.

## MATERIAL AND METHOD

### Commercial Oils

Rosemary oil, tea tree oil, St. john's wort oil, ant egg oil, garlic oil, almond oil and peppermint oil were purchased commercially.



**Figure 1.** A: Rosemary Oil, B: Tea Tree Oil, C: St. john's wort Oil, D: Ant Egg Oil, E: Garlic Oil, F: Almond Oil and G: Peppermint Oil.

### Determination of Sun Protection Factor

The commercially available oil samples were weighed 0.006 g. Then, it was mixed with 3 ml of 96% ethanol and homogenized. The oils samples were measured in a spectrophotometer (Beckman Coulter) at 290-320 nm waves with 5 nm intervals. Measurements were carried out in 3 repetitions. The results were determined using the Mansur equation (Mansur et al., 1986).

Mansur's equation;

$$SPF = CF \times \sum_{290}^{320} EE(\lambda) \times I(\lambda) \times Abs(\lambda) \quad (1)$$

**CF**= Correction factor (= 10);

**EE(λ)** = Erythemogenic effect radiation wavelength (λ);

**I (λ)** = Intensity of sunlight at wavelength (λ); **Abs**

**(λ)** = Absorbance of extracts at wavelength (λ).

### Determination of Sun Protection Factor of Oil and Cream Mixtures

Sun protection factor of oil and cream mixtures was determined spectrometrically. 1 gr cream and 0.5 g oil sample is completed to 10 g with ethanol (40%). 0.1 g of the prepared mixture was taken into another tube, made up to 10 ml volume with ethanol (40%) and sonicated for 5 minutes. The mixture was filtered through Whatman No:1 filter paper and 0.5 ml of ethanol (40%) mixture was made up to 5 ml in another tube. Then 0.5 ml of the mixture was taken, and the volume was made up to 2.5 ml with ethanol (40%). Mixtures with a final volume of 2.5 ml, 5 ml and 10 ml were measured in triplicate using a spectrophotometer (Beckman Coulter) at 5 nm intervals in the wavelength range of 290 nm-320 nm. SPF values of cream and oil mixtures were calculated using the Mansur equation as above.



**Figure 2.** SPF Determination of Oil and Cream Mixtures

### RESULTS AND DISCUSSION

Spectrophotometric measurements of SPF values of commercially available oils and cream and oil mixtures were determined spectrometrically. The relationship between the radiation intensity at each wavelength and the erythemogenic effect is given in Table 1. SPF values of oils varied between 0.21 and 3.15 (Table 3). According to Imam et al. (2015) (Table 2), the UV percentages of the oils were found to be below 50% for rosemary oil, tea tree oil, garlic oil, almond oil and peppermint oil, while the St. john's wort oil and ant egg oil were above 50%.

**Table 1.** The Intensity of Radiation at Each Wavelength and the Effect of the Erythematogenic Effect

| $\lambda$ (nm) | AB (I)x I(I)  |
|----------------|---------------|
| 290            | 0.0150        |
| 295            | 0.0817        |
| 300            | 0.2874        |
| 305            | 0.3278        |
| 310            | 0.1864        |
| 315            | 0.0839        |
| 320            | 0.0180        |
| <b>Toplam</b>  | <b>1.0000</b> |

**Table 2.** Percentage of UV Blocked by SPF Value (Imam et al., 2015)

| SPF | Percentage of UV Blocked |
|-----|--------------------------|
| 2   | 50                       |
| 4   | 75                       |
| 5   | 80                       |
| 10  | 90                       |
| 15  | 93                       |
| 25  | 96                       |

**Table 3.** Sun Protection Factors of Oils

| Oils                | SPF Value |
|---------------------|-----------|
| Rosemary Oil        | 0.52±0.01 |
| Tea Tree Oil        | 0.21±0    |
| St. John's Wort Oil | 3.15±0.12 |
| Ant Egg Oil         | 2.89±2.73 |
| Garlic Oil          | 0.81±0.16 |
| Almond Oil          | 0.55±0.01 |
| Peppermint Oil      | 0.27±0.01 |

As a result of the study conducted by Kaur and Saraf (2010), SPF values of some vegetable oils were determined using the Mansur equation. It was determined that SPF values of almond oil 4.65, tea tree oil 1.70, and peppermint oil 6.66. Couteau et al. (2022) were investigated SPF value of almond oil to determine its photoprotective potential. The in vitro SPF value of almond oil was determined as 0.94. As a result, it has been revealed that almond oil contains natural compounds that can provide 50% protection against UV blocking. Therefore, determination the SPF values of various oils can create a potential for use in the cosmetics industry.

SPF values of 2.5 ml, 5 ml and 10 ml concentrations prepared with cream and oil mixtures were determined as a result of spectrophotometric measurements. The results obtained in 10 ml concentration were recorded as 13.78 for rosemary oil, 4.41 for tea tree oil, 15.38 for St. john's wort oil, 15.08 for ant egg oil, 23.76 for garlic oil, 24.20 for almond oil and 5.23 for peppermint oil (Table 4). Commercial oil and cream blends have the potential to block most of the harmful rays from the sun.

**Table 4.** SPF Values of Cream and Oil Mixtures

| Oils                | SPF Value |            |            |
|---------------------|-----------|------------|------------|
|                     | 2.5 ml    | 5 ml       | 10 ml      |
| Rosemary Oil        | 0.40±0    | 1.69±0     | 13.78±0.03 |
| Tea Tree Oil        | 0.08±0.01 | 0.37±0.01  | 4.41±0.01  |
| St. John's Wort Oil | 0.91±0.01 | 1.88±0.01  | 15.38±0.06 |
| Ant Egg Oil         | 0.38±0.02 | 1.97±0     | 15.08±0.08 |
| Garlic Oil          | 2.44±0.01 | 10.63±0.01 | 23.76±0.02 |
| Almond Oil          | 2.45±0    | 10.10±0.02 | 24.20±0.08 |
| Peppermint Oil      | 0.40±0    | 0.66±0     | 5.23±0.04  |

## CONCLUSION

In the study, the use of commercially available oils in the cosmetic industry was investigated. The results indicated that the oils have the potential to be natural ingredients as an alternative to synthetic sunscreens in cosmetic industry.

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## DETERMINATION OF THE POTENTIAL FOR USE IN THE COSMETIC INDUSTRY OF *Ziziphus Jujuba* BRANCH WATER EXTRACTS

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### ABSTRACT

Sunscreens are a cosmetic product that is widely used in many areas of our lives. It is used to protect from harmful ultraviolet (UV) rays of the sun, especially in summer. Sunscreens come into direct contact with our skin, so it is of great importance that the product used is reliable. However, chemical additives can cause side effects on our skin. Nowadays, studies on the search for natural additives are gaining importance in order to minimize the use of chemical additives in many industrial areas. *Ziziphus jujuba* is a fruit widely grown in our country. It is very popular lately due to its high nutritional content and health-enhancing effects. In the study, the solar protection factor (SPF) of *Z. jujuba*, hot (HWB) and cold (SN) branch water extracts and commercial cream mixture was investigated. The *Z. jujuba* branch extracts presented good SPF values with 25.32 for HWB extract and 25.67 for branch SN extract at a concentration of 10 ml. *Z. jujuba* branch extracts may have the potential to be used as a natural additive in the cosmetic industry.

**Keywords:** Jujube, sun cream, natural additive, solar protection factor

### INTRODUCTION

Plants are among the most important basic resources of life. People use plants widely in the treatment process of various diseases apart from basic needs such as nutrition, clothing and shelter. Plants have various compounds that they produce as secondary metabolites. Secondary metabolites produced by plants provide the potential of plants to be used in alternative medicine. *Ziziphus jujuba* belongs to the genus of the *Rhamnaceae* family and is cultivated in southern and eastern Asia, Australia, and Europe (Gao et al., 2013). The *Z. jujuba* fruit was determined as an excellent herbal medicine in *Shennong Bencao Jing* (300 BC-200 AD) and in an old Chinese book on herbal medicine, *Huangdi Neijing* (475-221 BC), the *Z. jujuba* fruit was considered one of the five most valuable fruits (Chen et al., 2014; Chen et al., 2015; Chen et al., 2013). In Chinese folk medicine, it has been shown to implement numerous health-enhancing effects, such as protecting the gastrointestinal system, antiinflammatory, antioxidant, antimicrobial, and apoptotic effects in breast cancer cells (Plastina et al., 2010; Yu et al., 2012). Flavonoids are a class of natural products found in fruits, vegetables, and beverages. They are synthesized by plants have many important effects, such as protection against pathogens and ultraviolet B (UV-B) radiation (De Cooman et al., 1998).

Sunscreens are among the most important products of the cosmetic industry. Especially in the summer months, the use of sunscreens is increasing. The basic protection principle of sunscreens is based on their ability to absorb harmful UV rays from the sun. UV rays are the invisible part of the electromagnetic spectrum reaching the earth from the sun (Joux et al., 1999; Matallana-Surget et al., 2008). Ultraviolet radiation consists of three parts: ultraviolet A (UV-



A), UV-B and ultraviolet C (UV-C). Most of UV-C (200-290 nm) is absorbed by the atmosphere (Allen and Bain, 1994). UV-B is the most damaging UV light to human skin. UVB rays can cause acute changes such as pigmentation and sunburn, suppression of the immune system and chronic changes such as photocarcinogenesis (De Buys et al., 2000). Natural antioxidants are used in sunscreens to increase ray protection, as they can prevent damage caused by free radicals produced by sun rays (Chaudhuri, 2015). In our study, the potential of using *Z. jujuba* branch extracts as natural sunscreen additives in the cosmetic industry was investigated.

## MATERIAL AND METHOD

### Preparation of the extracts

The *Z. jujuba* branch were washed and then dried (Figure 1). After grounding with a Waring blender, the powdered branch samples were extracted with water by using hot water bath (HWB) and sonication devices (SN). In the hot water bath extraction, the branch materials were extracted with water at 100°C for 36 h. In the sonication extraction method, the ground branch materials were extracted with water on ice for a total of 30 minutes at 10 minute intervals. The solvents of the extracted samples were evaporated and then the dried extract was obtained. The water branch extracts were dissolved in dimethylsulfoxide (DMSO) and then sterilized with 0.45 µm filter.



**Figure 1.** *Z. jujuba* branch picture.

### Determination of sun protection factors (SPF) of extract and sunscreen cream mixtures

The methods of Bambal et al. (2011) were modified to determine sun protection factor of the extract and sunscreen cream mixtures. 1 g of sunscreen cream and 0.5 g of *Z. jujuba* branch extract was mixed. The mixture was made up to 10 g with distilled water. 0.1 g of this prepared mixture was taken into another tube and completed to 10 ml volume with ethanol (40%). It was then sonicated for 5 minutes and this mixture was filtered through Whatman No:1 filter paper, 0.5 ml of the mixture was made up to 5 ml with ethanol in another tube. Then, 0.5 ml of the mixture was taken and the volume was completed to 2.5 ml. The mixtures adjusted as 2.5 ml, 5 ml and 10 ml were measured in 3 repetitions using the spectrophotometer (Beckman Coulter) at 5 nm intervals in the wavelength range of 290 nm-320 nm. The SPF values of the cream and

extract mixtures were calculated using the Mansur equation mentioned as above (Mansur et al., 1986).

**Mansur equation;**

$$\text{Solar protection factor (SPF)} = \text{CF} \times \sum_{290}^{320} \text{EE}(\lambda) \times \text{I}(\lambda) \times \text{Abs}(\lambda) \quad (1)$$

CF = Correction factor (= 10);

EE(λ) = Erythemogenic effect radiation wavelength (λ);

I(λ) = Solarlight intensity at wavelength (λ);

Abs(λ) = Absorbance of the extracts at wavelength (λ).

**RESULTS AND DISCUSSION**

There is increasing evidence that various herbs and plant-derived compounds have therapeutic anti-inflammatory effects with little or no side effects (Musialik et al., 2017; Oguntibeju, 2018). SPF values of commercially available sunscreen cream+extract mixtures of *Z. jujuba* branch at 10 ml, 5 ml and 2.5 ml concentrations were investigated by in-vitro assay. The results obtained are given in Table 1. The relationship between the radiation intensity at each wavelength and the erythemogenic effect is given in Table 2. The results showed that *Z. jujuba* branch extracts increased the SPF value of sunscreen cream at all tested concentrations. *Z. jujuba* HWB branch extract and sunscreen cream mixture presented SPF value as 25.32 at a concentration of 10 ml, 5.11 at a concentration of 5 ml and 1.69 at a concentration of 2.5 ml. The SPF values of *Z. jujuba* SN branch extract and sunscreen cream mixture were found to be 25.67 at 10 ml concentration, 4.81 at 5 ml concentration and 0.63 at 2.5 ml concentration. The sunscreen cream showed SPF value as 19.17 at 10 ml concentration and 1.73 at a concentration of 5 ml and 0.36 at a concentration of 2.5 ml. The results obtained in our study were evaluated according to Imam et al. (2015) (Table 3). The percentage of UV blocking of *Z. jujuba* HWB extract and sunscreen cream mixture at a concentration of 10 ml was approximately 96%, and the percentage of UV blocking at a concentration of 5 ml was found to be approximately 80%. The percentage of UV inhibition of *Z. jujuba* SN branch extract and sunscreen cream mixture in 10 ml concentration was approximately 96% and the percentage of UV inhibition in 5 ml concentration was approximately 75%. The sunscreen cream presented UV blocking over 93% at a concentration of 10 ml and below 50% at a

concentration of 5 ml. As a result of the study, it was stated that *Z. jujuba* branch extracts increased the SPF value of the sunscreen cream. Therefore, *Z. jujuba* extracts have the potential to be used as a natural additive in sunscreen creams.

**Table 1:** SPF values of *Z. jujuba* branch extracts and sunscreen cream mixture

| Extract Concentrations | Solar Protection Factors |                                     |                                    |                                 |
|------------------------|--------------------------|-------------------------------------|------------------------------------|---------------------------------|
|                        | Cream                    | HWB Branch Extract+Sunscre en Cream | SN Branch Extract+Sunscre en Cream | Branch Extract+Sunscre en Cream |
| 2.5 ml                 | 0.36                     | 1.69                                | 0.63                               |                                 |
| 5 ml                   | 1.73                     | 5.11                                | 4.81                               |                                 |
| 10 ml                  | 19.17                    | 25.32                               | 25.67                              |                                 |

**Table 2:** Relationship between radiation intensity at each wavelength and erythemalogenic effect.

| $\lambda$ (nm) | EE ( $\lambda$ )x I( $\lambda$ ) |
|----------------|----------------------------------|
| 290            | 0.0150                           |
| 295            | 0.0817                           |
| 300            | 0.2874                           |
| 305            | 0.3278                           |
| 310            | 0.1864                           |
| 315            | 0.0839                           |
| 320            | 0.0180                           |
| Total          | 1.0000                           |

**Table 3:** Percentage of UV blocked (Imam et al., 2015)

| SPF | Percentage of UV blocked |
|-----|--------------------------|
| 2   | 50                       |
| 4   | 75                       |
| 5   | 80                       |
| 10  | 90                       |
| 15  | 93                       |
| 25  | 96                       |

Cosmetic products come into direct contact with our skin. For this reason, the reliability of the products used in their content is very important. Sunscreens are among the most widely used products in cosmetic industry to protect from the harmful rays of the sun. Chemical additives are used to give sunscreens the ability to absorb sun rays. However, today, the harmful effects of chemical additives on our skin are known by consumers. Therefore, the use of natural additives is of great importance in many industries instead of the use of chemical additives. It has been determined in the current study that *Z. jujuba* branch extracts have a high sun protection factor. *Z. jujuba* fruit trees can be grown in many regions of our country and are among the fruit trees that are easy to grow with their drought resistance. Pruning residues can be evaluated in the cosmetic industry. *Z. jujuba* branch extracts have the potential to be used as an alternative sunscreen in the cosmetic industry. As a result of the literature review, no study investigating the sun protection potential of *Z. jujuba* branch extracts was found.

## CONCLUSION

Cosmetic products are frequently used in our daily life. Since cosmetic products are in direct contact with the skin, it is of great importance to use reliable additives. Chemical additives added to cosmetic products increase the cost of the product even more. Plants are widely used in cosmetic industry. In our study, the sun protection potential of *Z. jujuba* water extracts obtained by two methods was investigated. The results obtained in our study have been reported that *Z. jujuba* branch extracts have a high sun protection potential. The use of water solvent in the extraction is important for manufacturers to obtain extracts more cheaply. In addition, the fact that water solvent does not have a harmful effect on our skin increases the importance of *Z. jujuba* branch extracts. The fact that the branch samples, which are considered as waste during the pruning of *Z. jujuba* fruit trees, have the potential to be used as a natural additive in the cosmetics industry, further increases the importance of our study. The extracts of *Z. jujuba* branch have the potential to be used as natural sunscreen additives in the cosmetic industry.

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## **YIELD AND SOME PROPERTIES OF SILAGE CORN AS INFLUENCED BY DIFFERENT NITROGEN AND VERMICOMPOST RATES**

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### **ABSTRACT**

Corn (*Zea mays* L.) is the most preferred and most suitable type for silage production all over the world. However, in order to obtain high yield from corn, it is necessary to use a large amount of nitrogen fertilizer. This can lead to environmental pollution and also to some nutritional disorders in animals. Therefore, techniques for reducing the use of chemical fertilizers in silage corn farming should be identified and implemented. The aim of this study was to determine the optimum combination of vermicompost and chemical nitrogen in silage corn production under the conditions of Erzurum. The study conducted according to the Randomized Complete Blocks experimental design, 5 doses of vermicompost (0, 2.500, 5.000, 7.500 and 10.000 kg ha<sup>-1</sup>) and 4 doses of chemical nitrogen (0, 50, 100 and 150 kg N ha<sup>-1</sup>) in combination with 3 replications was applied. The increase in silage yield was determined up to 100 kg ha<sup>-1</sup> of nitrogen and 7.500 kg ha<sup>-1</sup> of vermicompost. The highest silage yield (7.997,1 kg ha<sup>-1</sup>) were taken from 100 kg N + 10.000 kg ha<sup>-1</sup> vermicompost application.

**Keywords:** Silage corn, Vermicompost, Nitrogen, Silage yield

### **INTRODUCTION**

One of the most important problems of livestock in many countries is the shortage of high quality roughage (Özkan and Şahin Demirbağ, 2016). Forage is a plant material that is fed to livestock freshly, dried or made by silage (Bahtiyarca and Cufadar, 2003). Forage has an important place in animal feed. In order to close the gap of quality roughage in animal husbandry and to make the animal feeding conscious, forage crops sowing area and production should be increased. In this context, plants such as corn with high yield and nutritional value should be spread. In Turkey, corn takes the first place in silage production. 30% of the corn produced in the world is used in human nutrition and 70% is used as animal feed. Today, corn is regarded as animal feed by making silage to a great extent in many countries of the world. It is a feed obtained by fermenting the fresh forages rich in soluble carbohydrates, dry matter and water and finely chopped and compressed. Corn plant is one of the important fresh roughages in animal nutrition both as green and as silage. Seed supply is easy and suitable for mechanization from sowing to harvest. It is easy to store and use, low loss amount. It contains high dry matter and soluble carbohydrate in its structure and makes it easy to ferment. The corn can be stored in silo without the need for any additives. It has a unique and pleasant aroma; therefore, it produces a delicious silage that is loved by animals. Because of all these, it is the most used plant as silage both in the world and in our country. Today, silage feeding has become an indispensable technique and silage maize cultivation has become widespread in Eastern Anatolia. Erzurum-Kars plateau is a disadvantageous area for silage maize cultivation due to short development period. However, yields of 74,2-91,5 ton ha<sup>-1</sup> can be obtained from silage

corn in the studies conducted in the region (Öztürk and Akkaya, 1996; Bulut et al., 2008; Güney et al., 2010). The yield of silage corn is largely dependent on soil fertility and nitrogen fertilization. Studies show that the recommended amount of N for corn farming is between 80-300 kg ha<sup>-1</sup>, but the optimum dose is concentrated around 100-200 kg N ha<sup>-1</sup> (Sağlamtimur et al., 1996; Çelebi et al., 2009; Budaklı Çarpıcı et al., 2010; Gül et al., 2020). Recommended fertilizer doses vary depending on varieties and environmental conditions. The amount of nitrogenous fertilizer used is greatly influenced by the vegetation period of the region and variety. Gül et al. (2020) reported that the most suitable nitrogen dose in silage maize is 100-150 kg N ha<sup>-1</sup> in places like Erzurum due to short development period. Nevertheless, the amount of fertilizer used in corn farming is high. Economic losses and environmental pollution increase as high nitrogen fertilizer is used.

The use of vermicompost in crop production has been a subject of great interest in recent years. Production and trade of this manure, which is obtained by the separation of farm manure and some organic wastes by vermicompost, is becoming more common day by day. In order to reduce environmental pollution and to implement sustainable agriculture, the use of organic fertilizers and compost is gaining more importance every day. In this way, the effects of vermicompost on the yield of silage maize will be tried to determine the most suitable combination of nitrogen fertilizer.

## MATERIALS AND METHODS

The research was carried out in Atatürk University Plant Production Application and Research Center in 2018 and 2019 summer growing seasons. In the study, Reserve variety suitable for silage production of corn (*Zea mays* L.) was used (Güney, 2017). This variety, belonging to Syngenta, is known to be tall, broad-leaved, resistant to lodge and disease, coming to harvest of silage in 105-110 days. Ammonium sulphate (20-21% N) and triple superphosphate (43-45% P<sub>2</sub>O<sub>5</sub>) were used as chemical fertilizers. The research was carried out in Erzurum province which is located on 39° 51<sup>1</sup> north latitude and 41°61<sup>1</sup> east longitude. The altitude of Erzurum province is 1869 m and winters are cold and snowy and summers are cool and dry (Table 1).

Table 1. Some climate data for the June-September period of Erzurum province for the years 2018, 2019 and the long-term average (LTA, 1950-2017)<sup>1</sup>

| Months            | Rainfall (mm) |      |       | Temperature (°C) |      |      | Relative Humidity (%) |      |      |
|-------------------|---------------|------|-------|------------------|------|------|-----------------------|------|------|
|                   | 2018          | 2109 | LTA   | 2018             | 2019 | LTA  | 2018                  | 2019 | LTA  |
| <b>June</b>       | 74,3          | 23,6 | 47,2  | 15,5             | 17,8 | 14,9 | 64,3                  | 57,2 | 58,7 |
| <b>July</b>       | 43,0          | 3,0  | 25,9  | 21,3             | 19,0 | 19,4 | 45,0                  | 49,4 | 52,7 |
| August            | 45,2          | 11,6 | 16,6  | 20,1             | 20,2 | 19,3 | 50,6                  | 46,5 | 50,5 |
| <b>September</b>  | 14,0          | 28,4 | 22,7  | 16,0             | 14,5 | 14,5 | 51,6                  | 51,7 | 52,4 |
| <b>Total/Mean</b> | 176,5         | 66,6 | 112,4 | 18,2             | 17,9 | 17,0 | 52,9                  | 51,2 | 53,6 |

<sup>1</sup>Taken from Erzurum Meteorology Regional Directorate data

Soil samples taken from 0-30 cm depth before sowing and fertilization of the research area were analyzed in the laboratories of Atatürk University, Faculty of Agriculture, Department of Soil and Plant Nutrition. According to these results, the soils of the experiment area are salt-free, slightly alkaline, less calcareous, poor in organic matter, insufficient phosphorus and rich in potassium (Table 2).

Table 2. Some characteristics of the soils of the study area

| Soil characteristics | Results   | Soil characteristics  | Results |
|----------------------|-----------|---|---------|
| Clay (%)             | 36,70     | pH  | 7,64    |
| Silty (%)            | 29,56     | CaCO <sub>3</sub> (%)   | 2,24    |
| Sand (%)             | 33,74     | Phosphorus (kg P <sub>2</sub> O <sub>5</sub> da <sup>-1</sup> ) | 5,32    |
| Texture class        | Clay-loam | Potassium (kg K <sub>2</sub> O da <sup>-1</sup> )               | 153,1   |
| Salt (%)             | 0,05      | Organic matter (%)  | 1,05    |
| EC (mmhos/cm)        | 2,23      |   |         |

The research was carried out with 3 replications according to factorial arrangement in the experimental design of Randomized Complete Blocks in the irrigated conditions of Erzurum. Five doses of vermicompost (0, 2.500, 5.000, 7.500 and 10.000 kg ha<sup>-1</sup>) and 4 doses of nitrogen (0, 50, 100 and 150 kg N ha<sup>-1</sup>) were repeated in combination in each block. When the soil pan in early May, the seed bed was made ready for planting. After parceling before planting, vermicompost and nitrogen fertilizer were weighed to each parcel in the planned amount and mixed with rake. In the same period, phosphorus fertilizer was applied to all parcels as standard (75 kg P<sub>2</sub>O<sub>5</sub> ha<sup>-1</sup>). All of the nitrogen applied to the parcels is not given with sowing, half of it is applied before planting and the other half is applied when the plants are 40-50 cm tall (Tan, 2018). Silage corn sowing period for Erzurum is the middle of May (Güney, 2017). Planting was done by hand to the seed bed which was 70 cm row spacing and 15 cm row spaced (Tan, 2018). After the exits were completed, the first weed control was carried out in the form of hoeing. The second hoeing was made in the form of throat filling when the plants were 40-50 cm tall and the other half of the nitrogen fertilizer was applied in this order. Considering the precipitation and morphological structures of the plants, irrigation was made 4 times every year.

The silage maize harvest was carried out on September, which is the beginning of the period of milk-dough of the plants. During the harvest, 10 plants were taken from each plot and used to determine the plant characteristics. After these measurements, the plants were kept in the open air for 1 week and then kept in a 70 °C drying oven until they reached constant weight and weighed.

In this research, plant height (cm), stem thickness (mm), corncob ratio (%) and silage yield (kg ha<sup>-1</sup>) were investigated to determine the effects of nitrogen fertilizer and vermicompost. In the article, the results are given as a two-year average. The obtained data were subjected to variance analysis in MSTAT-C package program. The differences between the applications were identified and written according to the LSD Multiple Comparison Test.

## RESULTS AND DISCUSSIONS

**Plant Height:** Plant height of silage corn was significantly affected by both nitrogen fertilizer and vermicompost application, and plant height increased as nitrogen and vermicompost doses increased (Table 3). According to the two-year average results, the longest plant height (178,0 cm) was obtained from 150 kg ha<sup>-1</sup> nitrogen + 5.000 kg ha<sup>-1</sup> vermicompost application.



Table 3. Plant height of silage corn with nitrogen and vermicompost in different doses (cm)<sup>1</sup>

| Nitrogen<br>(kg N ha <sup>-1</sup> ) | Vermicompost (kg ha <sup>-1</sup> ) |          |          |          |         | Average  |
|--------------------------------------|-------------------------------------|----------|----------|----------|---------|----------|
|                                      | 0                                   | 2.500    | 5.000    | 7.500    | 10.000  |          |
| 0                                    | 139,1                               | 163,9    | 167,4    | 173,1    | 171,4   | 162,9 b  |
| 50                                   | 160,7                               | 161,6    | 166,7    | 161,6    | 174,4   | 165,0 b  |
| 100                                  | 163,5                               | 167,6    | 168,0    | 168,1    | 177,3   | 168,9 ab |
| 150                                  | 177,3                               | 167,6    | 178,0    | 175,4    | 173,6   | 174,4 a  |
| Average                              | 160,2 c                             | 165,2 bc | 170,0 ab | 169,6 ab | 174,2 a | 167,8    |

LSD Vermicompost: 6,1, Nitrogen: 7,4, Vermicompost x Nitrogen: 10,6

<sup>1</sup>Different letters show significance within 0,05 probability limits.

**Stem thickness:** There was no significant effect of nitrogen and vermicompost applications on stem thickness of silage corn (Table 4). However, the nitrogen x vermicompost interaction was found to be statistically significant ( $P < 0,05$ ). In general, high doses of nitrogen and vermicompost increased stem thickness. The highest stem thickness (28,2 mm) was determined at the highest doses of nitrogen and vermicompost.

Table 4. Stem thickness of silage corn with nitrogen and vermicompost in different doses (mm)

| Nitrogen<br>(kg N ha <sup>-1</sup> ) | Vermicompost (kg ha <sup>-1</sup> ) |       |       |       |        | Average |
|--------------------------------------|-------------------------------------|-------|-------|-------|--------|---------|
|                                      | 0                                   | 2.500 | 5.000 | 7.500 | 10.000 |         |
| 0                                    | 22,5                                | 25,0  | 27,5  | 26,8  | 26,0   | 25,6    |
| 50                                   | 24,6                                | 25,0  | 25,8  | 26,4  | 25,6   | 25,5    |
| 100                                  | 26,2                                | 26,7  | 26,2  | 26,2  | 27,2   | 26,5    |
| 150                                  | 27,6                                | 26,1  | 25,6  | 26,4  | 28,2   | 26,8    |
| Average                              | 25,2                                | 25,7  | 26,2  | 26,5  | 26,8   | 26,1    |

LSD Vermicompost: non-significant, Nitrogen: non-significant, Vermicompost x Nitrogen: 3,1

**Corn cob ratio:** The corn cob ratio of silage maize was significantly affected by both treatments. As the doses of the applications increased, the corn cob ratio increased. In vermicompost application, this increase was significant up to 7.500 kg ha<sup>-1</sup>, and the subsequent increase was found to be statistically insignificant. The corn cob ratio increased from 30,0% to 35,5% depending on nitrogen doses, and from 28,6% to 34,8% depending on vermicompost doses. When both applications are considered together, it is seen that the highest corn cob ratio (39,4%) is determined at the highest application doses (Table 5).

Table 5. Corn cob ratio of silage corn with nitrogen and vermicompost in different doses (%)

| Nitrogen<br>(kg N ha <sup>-1</sup> ) | Vermicompost (kg ha <sup>-1</sup> ) |         |         |        |        | Average |
|--------------------------------------|-------------------------------------|---------|---------|--------|--------|---------|
|                                      | 0                                   | 2.500   | 5.000   | 7.500  | 10.000 |         |
| 0                                    | 25,3                                | 29,4    | 32,0    | 32,1   | 31,3   | 30,0 c  |
| 50                                   | 27,0                                | 29,5    | 30,1    | 31,8   | 32,0   | 30,1 c  |
| 100                                  | 29,3                                | 29,4    | 33,8    | 35,8   | 36,4   | 32,9 b  |
| 150                                  | 32,7                                | 32,4    | 34,7    | 38,2   | 39,4   | 35,5 a  |
| Average                              | 28,6 c                              | 30,2 bc | 32,7 ab | 34,5 a | 34,8 a | 32,1    |

LSD Vermicompost: 3,1 Nitrogen: 2,4 significant, Vermicompost x Nitrogen: 4,9

<sup>1</sup> Different letters show significance within 0,05 probability limits.

**Silage yield:** In the research, it was determined that both nitrogen and vermicompost applications and their interaction are important on silage yield. Nitrogen fertilizer increased silage yield up to 100 kg ha<sup>-1</sup> dose, and vermicompost increased up to 7.500 kg ha<sup>-1</sup> dose. When nitrogen and vermicompost are considered together, it is seen that the highest yield (7.997,1 kg ha<sup>-1</sup>) is obtained from 100 kg ha<sup>-1</sup> nitrogen + 10.000 kg ha<sup>-1</sup> vermicompost application (Table 6).

Table 6. Silage yield of corn with nitrogen and vermicompost in different doses (kg ha<sup>-1</sup>)

| Nitrogen<br>(kg N ha <sup>-1</sup> ) | Vermicompost (kg ha <sup>-1</sup> ) |            |           |          |          | Average   |
|--------------------------------------|-------------------------------------|------------|-----------|----------|----------|-----------|
|                                      | 0                                   | 2.500      | 5.000     | 7.500    | 10.000   |           |
| 0                                    | 5675,3                              | 6808,0     | 7260,5    | 7701,2   | 7236,5   | 6936,3 b  |
| 50                                   | 6878,5                              | 7259,7     | 7057,5    | 7450,6   | 7645,5   | 7258,4 ab |
| 100                                  | 7332,0                              | 7983,5     | 7828,1    | 7868,9   | 7997,1   | 7801,9 a  |
| 150                                  | 7345,3                              | 7690,2     | 7711,2    | 7894,1   | 7990,4   | 7726,3 ab |
| Average                              | 6807,8 b                            | 7,435,4 ab | 7464,3 ab | 7728,7 a | 7717,4 a | 7430,7    |

LSD Vermicompost: 778,2, Nitrogen: 805,7, Vermicompost x Nitrogen: 942,9

<sup>1</sup> Different letters show significance within 0,05 probability limits.

Corn is the most used plant for silage production in the world. However, the productivity of corn depends on soil fertility. In order to obtain high silage yields in poor soils, high amounts of nitrogen are needed. In soils poor in organic matter, such as the soils where this experiment was established (Table 2), nitrogen should be applied to the soil in the form of fertilizer. As a matter of fact, in this study, it was observed that plant height, corn cob ratio and silage yield increased significantly with nitrogen fertilizer application. Corn is a plant that removes large amounts of nutrients from the soil. Although some studies suggest nitrogen up to 300 kg ha<sup>-1</sup> dose for silage maize (Karasu et al., 2009), higher yields were obtained from lower nitrogen dose applications in this study. It is seen that the highest yield was obtained from the nitrogen dose of 100 kg ha<sup>-1</sup> (Table 6). The reason for this is that Erzurum, located at a high altitude, has a short development period.

Vermicompost both improves the soil structure and adds nutrients to the soil. Therefore, in general, vermicompost application caused an increase in yield and plant characteristics. The interaction of vermicompost and nitrogen was found to be important both in silage yield and plant height and ear ratio. It was also determined in studies with other plants that vermicompost increased yield (Jahan et al., 2014; Moraditochae et al., 2011). In this study, high silage yields were obtained in 100-150 kg N ha<sup>-1</sup> and 7.500-10.000 kg ha<sup>-1</sup> vermicompost applications.

## CONCLUSION

Reducing the use of high amounts of chemical fertilizers, which has become an important problem nowadays, replacing them with organic fertilizers such as vermicompost is very important for sustainable agriculture. Nitrogenous fertilizer and vermicompost fertilizer were used together and in combination in this study it was determined that both materials had significant effects on plant properties and silage yield. According to the two years results, yield is very low when corn is grown without nitrogen application. There is an increase in yield up to 100 kg ha<sup>-1</sup> of nitrogen fertilizer and 7.500 kg ha<sup>-1</sup> of vermicompost.

## ACKNOWLEDGMENTS

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## HEAVY METALS IN FISH EGGS OF RED MULLET AND EUROPEAN HAKE

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### ABSTRACT

This study determined the content of heavy metals in eggs of Red mullet (*Mullus barbatus*) and European hake (*Merluccius merluccius*) with different weight. The content of heavy metals in fish eggs was performed by using atomic absorption spectrophotometer (AAS). The analysis showed statistically significant differences on mercury (Hg) concentration in fish species with different weight (Red mullet:  $p < 0.001$ , European hake:  $p = 0.028$ ), while lead, cadmium and chrome were not detected. According to the results the concentration levels of mercury resulted to be within permissible levels for human consumption by EC legislation.

**Keywords:** *Heavy metals, fish eggs, Red Mullet, European hake*

### INTRODUCTION

Aquatic pollution by industrial waste discharges is a matter of concern. Chemical wastes have a negative effect not only to aquatic organisms and fish but also to consumers. Pollution of the aquatic environment by chemicals as heavy metals and pesticides may lead to fish accumulation of theirs as well. Fish have the ability and tendency to accumulate and bioaccumulate in their tissues chemicals over the time.

Heavy metals as mercury, lead and cadmium are a special category of chemical pollutants that do not play any biological function and can be harmful to human health. They persist over the time and they accumulate at different quantities in tissues of fish which are consumed by humans.

Fish are valuable species, they are rich on proteins, polyunsaturated fatty acids, minerals and vitamins (Storelli, 2008). Red mullet and European hake are typical benthic fish species of Adriatic Sea. The local production according to Instat (Instat, 2022) is decreased over the years by overfishing and pollution of aquatic environment. These local species are very appreciated by Albanian consumers. They have an excellent white meat and a low fat content.

Many studies (Yilmaz, 2005; Storelli et al., 2008; Copat et al., 2012; Ozuni et al., 2010; Ozuni et al., 2021) on heavy metals content of these species are focused mainly on muscle tissue or internal organs avoiding eggs. This study aims to determine the heavy metal content of eggs in two commercially valuable fish species of Adriatic Sea and then compare the results with the EC legislation.

## **MATERIAL AND METHOD**

The fish species named Red mullet (*Mullus barbatus*) and European hake (*Merluccius merluccius*) was collected and purchased during summer 2018. Fish eggs samples of red mullet and European hake originated from Adriatic Sea, were purchased directly from the fisherman of Durres. The study included 40 samples of eggs (20 samples for each species). The fish samples were first, identified, weighed, catalogued and conserved at - 18°C and then they were sent for further investigation to the Laboratory of Toxicology, Institute of Veterinary and Food Safety, Tirana.

A total of 40 samples of fish eggs were evaluated for the concentration level of mercury (Hg), lead (Pb), cadmium (Cd), and chrome (Cr) by using an Atomic Absorption Spectrophotometer (AAS). Fish tissue was homogenized in a blender; and then they were dried at 100 °C. One g of sample was weighed and then treated with 10 ml of HNO<sub>3</sub> and 5 ml of concentrated H<sub>2</sub>SO<sub>4</sub> and let in overnight. The next day they were dried at 150° C for at least, 30 minutes and 50 ml of it were put into a normal flask, and filled with tap water. The heavy metals were measured by ICP-OES, Optima 2100 Dv produced by Perkin Elmer.

### **Statistical evaluation of the data**

The statistical evaluation of the data was evaluated by using SPSS (Statistical Package for Social Sciences) 25.0. The level of significance was set as ( $p \leq 5\%$ ). The comparison values between groups were performed by using student test. The statistical data on the below table comprised average, standard deviation, standard error, p value and interval of confidence.

## **RESULTS AND DISCUSSION**

The concentration level and (SD) of heavy metals (mg/kg wet weight) in fish eggs samples of Red mullet and European hake are given in the below tables (Table 1). The results of the study show that mercury are present at different concentration levels in all eggs samples of fish with different weight. Mean while lead, cadmium and chrome concentration levels in eggs samples of both fish species where not detected (nd). According to the results the concentration level (mg/kg ww) of mercury (Hg) in eggs samples of Red mullet and European hake resulted within the maximum permitted level for human consumption (EC, 2006 & 2008), set by EC legislation.

Table 1. Average mean concentration levels of heavy metals in fish eggs of Red mullet and European hake with different weight (mg/kg wet weight)

| Heavy metals<br>mg/kg ww |       | N  | Mean | SD   | t     | df | p value |
|--------------------------|-------|----|------|------|-------|----|---------|
| Hg_red_mullet            | small | 10 | .019 | .012 | 7.638 | 18 | <0.001  |
|                          | large | 10 | .085 | .025 |       |    |         |
| Hg_european_hake         | small | 10 | .048 | .043 | 2.573 | 18 | .028    |
|                          | large | 10 | .084 | .010 |       |    |         |
| Pb_red_mullet            | small | 10 | .00  |      |       |    |         |
|                          | large | 10 | .00  |      |       |    |         |
| Pb_european_hake         | small | 10 | .00  |      |       |    |         |
|                          | large | 10 | .00  |      |       |    |         |
| Cd_red_mullet            | small | 10 | .00  |      |       |    |         |
|                          | large | 10 | .00  |      |       |    |         |
| Cd_european_hake         | small | 10 | .00  |      |       |    |         |
|                          | large | 10 | .00  |      |       |    |         |
| Cr_red_mullet            | small | 10 | .00  |      |       |    |         |
|                          | large | 10 | .00  |      |       |    |         |
| Cr_european_hake         | small | 10 | .00  |      |       |    |         |
|                          | large | 10 | .00  |      |       |    |         |

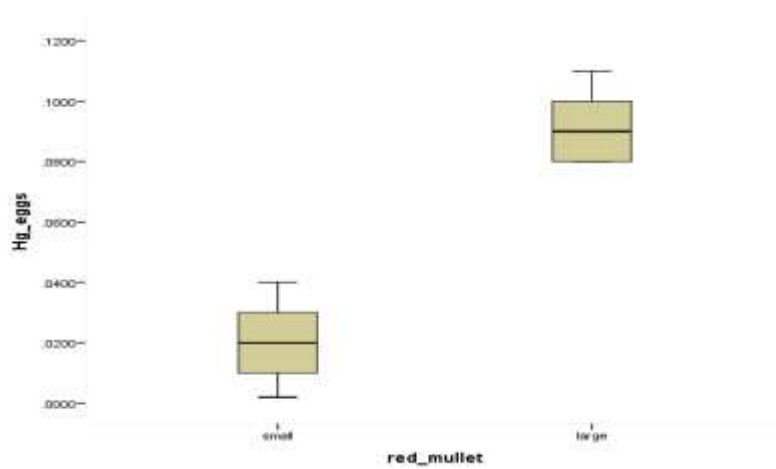


Figure 1. Mercury concentration in eggs of Red mullet (small & large fish size )

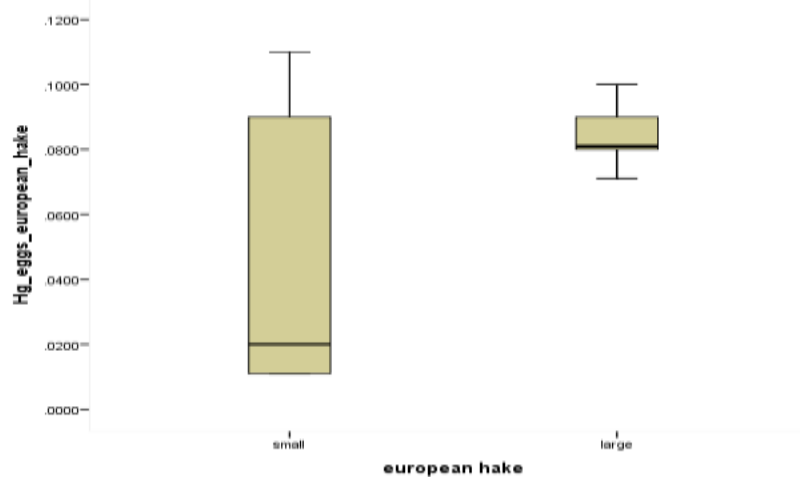


Figure 2. Mercury concentration in eggs of European hake (small & large fish size)

Mercury (Hg) occurrence in fish eggs samples of Red mullet: According to the results average means concentration level of Hg resulted  $0.019 \pm 0.012$  mg/kg ww in eggs of small fish size into  $0.085 \pm 0.025$  mg/kg ww in eggs of large sized fish.

Mercury (Hg) occurrence in fish eggs samples of European hake: According to the results the average mean concentration level of Hg resulted  $0.048 \pm 0.043$  mg/kg ww in eggs of small fish size into  $0.084 \pm 0.010$  mg/kg ww in eggs of large sized fish.

Concentration levels of Hg revealed statistically significant differences in fish eggs samples of fish species with different weight, (Red mullet:  $p < 0.001$ , European hake:  $p = 0.028$ ).

Mercury is one of the most toxic chemical compounds. Its effects vary on several factors, such as, chemical form, doze, way of ingestion, status of the organisms, sex, age, physical condition (Eisler R, 1987) and other factors related to the water environment as, salinity, pH, temperature (Romeo et al., 1999).

In our study mean concentration level of mercury in all fish eggs samples of Red mullet and European hake resulted to be lower than the maximum permitted levels for human consumption set by EC legislation (Hg – 1.0 mg/kg ww).

Beside the results it is of great concern further monitoring of heavy metal contamination of local fish species with the aim to safeguard Albanian consumers.

## CONCLUSIONS

Based on the results of the study, can be concluded that the concentration level of mercury in fish eggs samples of Red mullet and European hake are in accepted limits for human consumption set by EC regulation. Whatever further monitoring is needed to ensure human health by heavy metals contamination.

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## **DETERMINATION OF FORAGE YIELD, QUALITY AND SOME CHARACTERISTICS OF VEGETATION IN GÜNALAN VILLAGE PASTURE IN GÖLBAŞI DISTRICT OF ANKARA PROVINCE**

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### **ABSTRACT**

Meadows and pastures in Türkiye, which cover an area of 12.8 million Ha, have an important place in the feeding of livestock. However, due to the early and excessive grazing practices that have been going on for many years, the feed yields and quality of these feed sources have decreased a lot. Along with the deterioration of the vegetation cover, the pasture soil is also lost. In order to prevent these problems and to make the rangelands better and more productive, it is necessary to create improvement and management plans for these areas. Within the framework of the Pasture Law in force, rangeland improvement and management projects are carried out every year for implementation in a certain area. Before starting the improvement and management works in these areas, sufficient information should be obtained about the status of the pasture. In the light of this information, appropriate improvement and management programs related to that area are put into practice. In this study, some measurements and evaluations were made for the pasture survey in a village pasture selected from Ankara Province, which has an important pasture existence. The transect method which has been shown to be reliable for scientific studies in arid and semi-arid region pastures and is preferred in many studies, was used to reveal the status of the pasture and primarily to determine the structure and composition of the vegetation on the pasture. With this method, a pasture protected from grazing was selected on the pasture of Günalan Mahallesi, which is connected to the Göbaşı district of Ankara, and an area that is homogeneous in terms of vegetation was determined. On this area, which is homogeneous in terms of vegetation, 100 transect samples were taken at 3 different points by considering the vectors. In each sample, the 1 cm wide and 100 cm long area on the right side of the transect bar was examined and the area covered by each plant species was recorded. Later, by analyzing these records, different features of the cover such as planted area, bare area, botanical composition, recurrence rates of plant species were revealed. In addition, grass samples were taken from 3 different points on the pasture to determine the feed efficiency of the pasture. For this purpose, in 1 m<sup>2</sup> areas, the vegetation was cut from the ground, the plant species and parts that animals could not eat were discarded, and the remaining specimens were separated according to their families and dried in an oven. The samples, which were dried at 70 degrees for 48 hours, were then weighed and the dry grass ratio was determined. Raw ash was determined after the obtained dry grass was ground in the mill. The results obtained were analyzed using appropriate statistical methods.

**Keywords:** Forage yield, vegetation, botanical composition, grazing capacity

### **INTRODUCTION**

Despite the fact that Turkey has one of the largest livestock populations in the world, the livestock productivity and total livestock production fall well below the potential production. Animal feeding largely depends on grazing depleted natural range and pasture. As the portion

of the improved pastures is really small, the majority of the livestock of the country has to graze on the unimproved poor natural rangelands. In pasture most of the livestock is supported by grazing on rangeland (Kendir and Bakır 1997).

This situation is worsening day by day because the grassland area is diminishing and becoming less productive as a result of excessive and uncontrolled grazing. Most of the grazinglands in Turkey has been deteriorated and lost their productivity. Generally, the vegetation cover is insufficient to protect the soil from the wind and water erosion. It would be possible to save this natural resource and improve its productivity by implementing good management systems, including proper grazing systems and range improvement plans. These natural grazing lands have a great potential in increasing profits of the grazing sector of the national economy. These lands must also be protected for the benefit of the next generation.

It is difficult to initiate improvement and grazing plan studies at present, because of the lack of qualitative and quantitative information about our range vegetation in the various agricultural regions. It is essential to have information on the percentage cover and botanical composition of the vegetation in order to implement any range improvement measures successfully on these natural rangelands.

Plant and animal species in meadows and pastures increase the biological value of these areas and contribute to the formation, development and maturation of the soil on which they live. In this way, the lands are protected by the meadow and pasture culture and become a more favorable living environment for many species. Meadows and pastures increase soil fertility by being the source of plant nutrients, on the other hand, by playing a role in erosion control and soil improvement, and even in crop rotation. The plant species that make up the meadow and pasture vegetation are very important in soil improvement as they have different characteristics as well as being numerous.

Meadows and pastures, as a natural ecosystem, are habitats for countless plants and animals. Today, with a better understanding of the contribution of these areas to natural life, a special value has been given to meadows and pastures, especially in developed countries. The ecosystem continues its cycle as thousands of plant and animal species continue their lives by finding food and living environment in these areas.

Most of our country is located in the semi-arid climate zone. In these regions, the amount of natural precipitation is low and its distribution throughout the year is not regular. Insufficient rainfall is the biggest problem that restricts plant growth. In these regions, underground water reserves increase by infiltration of natural precipitation into the soil and stored in different layers, and the disappearance of scarce water in the form of surface flow is prevented. Since meadows and pastures, which are among the natural resources, are an important water collection basin, they take an important place in the water cycle in nature by feeding the ground waters, springs and streams throughout the year by the penetration of natural precipitation into the soil.

The plant species that make up the meadow and pasture vegetation convert the carbon dioxide gas, which is caused by the excessive use of fossil fuels and can exceed the normal amount in the atmosphere, into organic matter by using them in photosynthesis. Thus, carbon dioxide is not only converted into a form that can be used by other organisms, but also prevents global warming, which is called the greenhouse effect, which occurs in excess of this gas.

Thanks to the oxygen produced by photosynthesis in the meadows and pastures, these areas also have the feature of being a source of clean air and cleaning the polluted air.

In our country, meadows and pastures of 12.8 million ha constitute 16% of the total area. All of our pastures and most of the meadows are state-owned lands. These areas are evaluated by the people of the settlement where they are located, by grazing animals together. Natural meadows and pastures are the most important source of roughage for our animals. Most of the quality roughage consumed by pets in our country is obtained from meadows and pastures. However, since 75% of our pastures are located in arid and semi-arid regions and the necessary maintenance is not done, their yield is low. The ratio of the area covered with vegetation in the majority has decreased to 15-20%, and the dry grass yields vary between 45-100 kg/da according to the regions (Bakır and Açıkgöz 1976).

Solving the problem of overgrazing of pastures in our country only depends on the development of feed resources and increasing feed production. In the pasture areas, which are the most important of these resources, the feed production in these areas can be at least doubled by conducting the necessary studies, revealing their situation, grazing to certain rules, applying various maintenance and irrigation, fertilization, weed control and soil water conservation methods.

The first problem to be faced in a study of grazing land is the identification of plant species found in the vegetation. Therefore, floristic studies such as collecting, identifying should always be the first step in any range study. The agricultural traits of the plant species should then be investigated. Once sufficient information about the key species is available, further work to improve the vegetation through different treatment(s) can be done. Once the species present have been documented, grazing management and range improvement issues can be implemented. Any plan to improve natural feed resources in a particular area of range can be successfully implemented if it is based on a sound floristic knowledge of the area.

In this study some properties of Günalan Village pasture in Göbaşı, Ankara were investigated. This study will form the basis for any further studies on similar range in the region.

## **MATERIAL AND METHOD**

In this study, some measurements and evaluations were made for the pasture survey in a village pasture selected from Ankara Province, which has an important pasture land in our country. The transect method, which has been shown to be reliable for scientific studies in arid and semi-arid region pastures and preferred in many studies range (Bakır 1970a), was used to reveal the status of the pasture and to determine the structure and composition of the vegetation on the pasture. This method was used to find out the proportions of ground cover of these selected species, and their distribution patterns. A hundred transect units were investigated. The method described by Bakır (1970b) was used to calculate the species basal cover ratios.

With this method, a pasture protected from grazing was chosen because it is surrounded by cultivated lands on the Günalan Village pasture of the Göbaşı district of Ankara. 100 transect samples were taken at 3 different points, taking into account the directions on an area that is homogeneous in terms of vegetation and has a low slope of 10-15%. Each sample consisted of 5 strips two meters apart. 20 samples were taken on each strip. The 1 cm wide and 100 cm long

area on the right side of the transect bar was examined and the area covered by each plant species was recorded. Later, by analyzing these records, different features of the cover such as basal cover, bare area, botanical composition, recurrence rates of plant species were revealed (Kendir and Bakır 1997, Alan and Ekiz 2001).

In addition, forage samples were taken from 3 different points on the pasture to determine the forage yield and quality of the pasture. For this purpose, in 1 m<sup>2</sup> areas, the plant material was cut from the ground, the plant species and parts that animals could not eat were discarded, and the remaining specimens were separated according to their families and dried in an oven. The samples, which were dried at 70 degrees for 48 hours, were then weighed and the dry grass ratio was determined (Alan and Ekiz 2001). Crude ash was determined according to Akyıldız (1969) after the dried forage was ground in the mill.

## RESULTS AND DISCUSSION

Thirty plant species were determined in the transect lines. 10 of them were Gramineae, 7 were leguminosae and 13 were other plant families (Table 1). *Festuca ovina* was the most abundant species in the study area. It's basal cover ratio varied between 2.68%- and 2.10%. Among the legume species *Trigonella corniculata* was the most abundant species in the study area. It's basal cover was 0.44%, 0.28% and 0.30% in North, South-east and West directions respectively. Among the species from other families, the most abundant species was *Thymus sipyleus*. It showed a basal cover degree between 1.52-1.15% in three directions.

Table 1: Basal cover ratios of plant species found in the study area in three directions (%)

| Gramineae                       | Common name       | North | South-east | West |
|---------------------------------|-------------------|-------|------------|------|
| <i>Festuca ovina</i>            | Sheep fescue      | 2.68  | 2.10       | 2.3  |
| <i>Koeleria cristata</i>        | Prairie junegrass | 0.34  | 0.21       | 0.10 |
| <i>Poa bulbosa</i>              | Bulbous bluegrass | 1.18  | 0.85       | 1.14 |
| <i>Stipa pennata</i>            | Needlegrass       | 0.08  | 0.02       | 0.20 |
| <i>Bromus arvensis</i>          | Field brome       | 0.07  | 0.11       | 0.08 |
| <i>Bromus erectus</i>           | Meadow brome      | 0.08  | 0.02       | 0.03 |
| <i>Aegilops ovata</i>           | Goatgrass         | 0.12  | -          | 0.08 |
| <i>Andropogon ischaemum</i>     | Yellow bluestem   | -     | 0.28       | 0.16 |
| <i>Taeniatherum caputmedusa</i> | Medusahead        | 0.04  | 0.12       | 0.07 |
| <i>Trachynia distachya</i>      | -                 | 0.12  | 0.04       | 0.36 |
| Leguminosae                     | Legumes           |       |            |      |
| <i>Astragalus angustifolius</i> | Milkwetch         | 0.22  | 0.10       | 0.36 |
| <i>Onobrychis armena</i>        | Wild sainfoin     | 0.12  | -          | 0.14 |
| <i>Astragalus dencifolius</i>   | Milkwetch         | -     | 0.36       | 0.14 |
| <i>Astragalus vulnenaria</i>    | Milkwetch         | 1.22  | 0.65       | -    |
| <i>Trigonella corniculata</i>   | Fenugreek         | 0.44  | 0.28       | 0.30 |
| <i>Trigonella brachycarpa</i>   | Fenugreek         | 0.16  | 0.20       | -    |
| <i>Coronilla varia</i>          | Crownvetch        | 0.02  | 0.38       | 1.36 |

| <b>Other Plant Families</b>   |                 |      |      |      |
|-------------------------------|-----------------|------|------|------|
| <i>Thymus sipyleus</i>        | Thyme           | 1.52 | 1.15 | 1.36 |
| <i>Teucrium polium</i>        | Germander       | 0.70 | 0.58 | 0.90 |
| <i>Teucrium chamedrys</i>     | Germander       | 0.74 | -    | 0.52 |
| <i>Salvia cryptantha</i>      | Sage            | 0.80 | 1.10 | 0.56 |
| <i>Scabiosa ucranica</i>      | Scabious        | 0.25 | 0.30 | 0.10 |
| <i>Veronica multiflora</i>    | Veronika        | 0.16 | -    | 0.30 |
| <i>Sanguisorba minor</i>      | Garden burnet   | 1.08 | 0.20 | 0.42 |
| <i>Verbascum leptophyllum</i> | Mullein         | 0.08 | 0.05 | 0.05 |
| <i>Alyssum condensatum</i>    | Madwort         | 0.44 | 0.14 | 0.52 |
| <i>Erodium sp.</i>            | Alfilaria       | 0.08 | 0.02 | 0.24 |
| <i>Convolvulus lineatus</i>   | Bindweed        | -    | 0.34 | 0.22 |
| <i>Achillea sp.</i>           | Yarrow          | 0.08 | 0.12 | 0.30 |
| <i>Galium verum</i>           | Yellow bedstraw | 0.10 | 0.24 | -    |

Basal cover ratios changed 2.15% and 5.25% depending on plant families. Other families group was the most abundant group with 5.25% (Table 2). Plant cover ratios varied between 9.96% and 12.92% according to direction. North direction had the highest degree of plant cover among three directions and South had the lowest degree of cover with 9.96%. All the area had the 11.73% plant cover degree which means 88.27% of the soil surface was

Table 2. Cover ratios of plant families in the research area according to the directions (%)

| Family         | North | South-east | Weast | Mean  |
|----------------|-------|------------|-------|-------|
| Gramineae      | 4.71  | 3.75       | 4.52  | 4.33  |
| Leguminosae    | 2.18  | 1.97       | 2.3   | 2.15  |
| Other Families | 6.03  | 4.24       | 5.49  | 5.25  |
| Total          | 12.92 | 9.96       | 12.31 | 11.73 |

Botanical composition ratios of plant species in the study area according to directions are given in Table. As seen in the Table 3, *F. ovina* was the most abundant species in the study area. Its cover ratio was 21.08% in the South-east and 18.68% in the West direction. *Thymus* was the second most abundant species in the study area, its cover ratio was highest in the North direction with 11.76% and lowest in West with 11.05%.

Table 3. Botanical composition ratios of plant species in the study area according to directions

| <b>Gramineae</b>                | <b>Common name</b> | <b>North</b> | <b>Southeast</b> | <b>West</b> |
|---------------------------------|--------------------|--------------|------------------|-------------|
| <i>Festuca ovina</i>            | Sheep fescue       | 20,74        | 21,08            | 18,68       |
| <i>Koeleria cristata</i>        | Prairie junegrass  | 1,78         | 2,11             | 0,81        |
| <i>Poa bulbosa</i>              | Bulbous bluegrass  | 9,13         | 8,53             | 9,26        |
| <i>Stipa pennata</i>            | Needlegrass        | 0,62         | 0,20             | 1,62        |
| <i>Bromus arvensis</i>          | Field brome        | 0,54         | 1,11             | 0,61        |
| <i>Bromus erectus</i>           | Meadow brome       | 0,62         | 0,20             | 0,24        |
| <i>Aegilops ovata</i>           | Goatgrass          | 0,93         | -                | 0,61        |
| <i>Andropogon ischaemum</i>     | Yellow bluestem    | -            | 2,81             | 1,23        |
| <i>Taeniatherum caputmedusa</i> | Medusahead         | 0,31         | 1,20             | 0,57        |
| <i>Trachynia distachya</i>      | -                  | 0,93         | 0,40             | 2,92        |
| <b>Leguminosae</b>              | <b>Legumes</b>     |              |                  |             |
| <i>Astragalus angustifolius</i> | Milkwetch          | 1,70         | 1,00             | 2,92        |
| <i>Onobrychis armena</i>        | Wild sainfoin      | 0,93         | -                | 1,14        |
| <i>Astragalus dencifolius</i>   | Milkwetch          | -            | 3,61             | 1,14        |
| <i>Astragalus vulnenaria</i>    | Milkwetch          | 9,44         | 6,53             | -           |
| <i>Trigonella corniculata</i>   | Fenugreek          | 3,40         | 2,81             | 2,44        |
| <i>Trigonella brachycarpa</i>   | Fenugreek          | 1,24         | 2,00             | -           |
| <i>Coronilla varia</i>          | Crownvetch         | 0,15         | 3,82             | 11,05       |
| <b>Other Plant Families</b>     |                    |              |                  |             |
| <i>Thymus sipyleus</i>          | Thyme              | 11,76        | 11,55            | 11,05       |
| <i>Teucrium polium</i>          | Germander          | 5,42         | 5,82             | 7,31        |
| <i>Teucrium chamedrys</i>       | Germander          | 5,72         | -                | 4,22        |
| <i>Salvia cryptantha</i>        | Sage               | 6,19         | 11,00            | 4,55        |
| <i>Scabiosa ucranica</i>        | Scabious           | 1,93         | 3,01             | 0,81        |
| <i>Veronica multiflora</i>      | Veronika           | 1,24         | -                | 2,44        |
| <i>Sanguisorba minor</i>        | Garden burnet      | 8,34         | 2,00             | 3,41        |
| <i>Verbascum leptophyllum</i>   | Mullein            | 0,62         | 5,02             | 0,41        |
| <i>Alyssum condensatum</i>      | Madworth           | 3,40         | 1,41             | 4,22        |
| <i>Erodium sp</i>               | Alfilaria          | 0,62         | 2,00             | 1,95        |
| <i>Convolvulus lineatus</i>     | Bindweed           | -            | 3,41             | 1,79        |
| <i>Achillea sp.</i>             | Yarrow             | 0,62         | 1,20             | 2,44        |
| <i>Galium verum</i>             | Yellow bedstraw    | 0,77         | 2,40             | -           |

Botanical composition ratios of families in the study area according to directions are given in Table 4. Other families were the most abundant group among the families in the areas. As a mean of 3 directions it had 44.61% in botanical composition. Grass or Gramineae family had 36.87% ratio in botanical structure of the vegetation in the area. Legumes had the lowest degree in botanical composition with a portion of 18.52%.

Direction also had a slightly effect on botanical composition on the vegetation in the study area (Table 4).

Table 4. Botanical composition ratios of families in the study area (%)

| Families       | North | South-east | West  | Mean  |
|----------------|-------|------------|-------|-------|
| Gramineae      | 36,45 | 37,45      | 36,72 | 36.87 |
| Leguminosae    | 16,87 | 19,78      | 18,68 | 18.52 |
| Other Families | 46,67 | 42,57      | 44,60 | 44.61 |

*Festuca ovina* was the most repeated species in the study area. It existed 55, 52 and 56 times in North, South-east and North directions respectively, in 100 samples taken (Table 5) *Poa bulbosa* was the second most frequent species with 24, 19, and 28 times in North, South-east and West directions.

Table 5. Frequency values of plant species found in the research area

| Gramineae                       | Common name       | North | South-east | West |
|---------------------------------|-------------------|-------|------------|------|
| <i>Festuca ovina</i>            | Sheep fescue      | 55    | 52         | 56   |
| <i>Koeleria cristata</i>        | Prairie junegrass | 6     | 4          | 6    |
| <i>Poa bulbosa</i>              | Bulbous bluegrass | 24    | 19         | 28   |
| <i>Stipa pennata</i>            | Needlegrass       | 5     | 5          | 7    |
| <i>Bromus arvensis</i>          | Field brome       | 5     | 4          | 4    |
| <i>Bromus erectus</i>           | Meadow brome      | 4     | 4          | 3    |
| <i>Aegilops ovata</i>           | Goatgrass         | 5     | -          | 5    |
| <i>Andropogon ischaemum</i>     | Yellow bluestem   | -     | 2          | 2    |
| <i>Taeniatherum caputmedusa</i> | Medusahead        | 4     | 2          | 4    |
| <i>Trachynia distachya</i>      | -                 | 1     | 1          | 2    |
| <b>Leguminosae</b>              | <b>Legumes</b>    |       |            |      |
| <i>Astragalus angustifolius</i> | Milkwetch         | 2     | 2          | 4    |
| <i>Onobrychis armena</i>        | Wild sainfoin     | 4     | -          | 4    |
| <i>Astragalus dencifolius</i>   | Milkwetch         | -     | 2          | 1    |
| <i>Astragalus vulnenaria</i>    | Milkwetch         | 4     | 2          | -    |
| <i>Trigonella corniculata</i>   | Fenugreek         | 5     | 2          | 2    |
| <i>Trigonella brachycarpa</i>   | Fenugreek         | 3     | 1          | -    |
| <i>Coronilla varia</i>          | Crownvetch        | 1     | 2          | 4    |
| <b>Other Plant Families</b>     |                   |       |            |      |
| <i>Thymus sipyleus</i>          | Thyme             | 2     | 2          | 1    |
| <i>Teucrium polium</i>          | Germander         | 2     | 1          | 2    |
| <i>Teucrium chamedrys</i>       | Germander         | 3     | -          | 2    |
| <i>Salvia cryptantha</i>        | Sage              | 1     | 4          | 1    |
| <i>Scabiosa ucranica</i>        | Scabious          | 1     | 1          | 1    |
| <i>Veronica multiflora</i>      | Veronika          | 1     | -          | 2    |
| <i>Sanguisorba minor</i>        | Garden burnet     | 5     | 2          | 1    |
| <i>Verbascum leptophyllum</i>   | Mullein           | 1     | 1          | 1    |
| <i>Alyssum condensatum</i>      | Madwort           | 4     | 1          | 4    |
| <i>Erodium sp</i>               | Alfilaria         | 1     | 1          | 1    |
| <i>Convolvulus lineatus</i>     | Bindweed          | -     | 2          | 1    |
| <i>Achillea sp.</i>             | Yarrow            | 1     | 1          | 1    |
| <i>Galium verum</i>             | Yellow bedstraw   | 1     | 1          | -    |



The hay yields according to their families calculated according to the values obtained from the plants that were cut one square meters of land in three different directions are given in Table 6. As the average of the data obtained in three direction, the dry forage grass yield of the pasture was found to be 98.6 kg.

Plants from the Gramineae family made the biggest contribution to the hay yield of the pasture with 52.06 kg. This was followed by plants belonging to other families with 37.96 kg and plants belonging to the legumes family with 8.58 kg. There was statistically significant difference between the forage yields of the plant groups calculated from the samples taken.

Forage yields of Central Anatolian pastures were found between 25 kg/da and 340 kg/da dry grass in various studies (Bakır and Açıkgöz 1976, Büyükburç 1983). Alan and Ekiz (2001), working in the forest pasture of the town of Bala, found the forage yield of the pasture to be 138 kg/da dry forage. Depending on the applied grazing pressure, there is a great decrease in the feed efficiency of the arid region pastures (Bakır 1987).

Table 6. Forage yield in three direction (kg/da)

| Group          | North | South-east | West  | Means*   |
|----------------|-------|------------|-------|----------|
| Gramineae      | 45,17 | 44,88      | 66,12 | 52,06 A  |
| Legumes        | 9,73  | 6,94       | 9,08  | 8,58 C   |
| Other families | 43,30 | 29,78      | 40,8  | 37,96 AB |
| Total          | 98,2  | 81,6       | 116,0 | 98,6     |

\*There is no statistical difference between the averages with the same letter.

Botnical composition of the forage in three directions are given in Table 7. The plant families had the different portion in botanical composition of the forage. Gramineae plant made the biggest contribution to forage yield. They had the 52.75% of the forage yield produced in the grasslanf as a mean of three directions.

Table 7. Botanical composition of the forage in three directions (%)

| Group          | North | South-east | West | Means   |
|----------------|-------|------------|------|---------|
| Gramineae      | 46.0  | 55.1       | 56.9 | 52.7 A  |
| Legumes        | 9.9   | 8.5        | 7.8  | 8.7 C   |
| Other families | 44.1  | 36.4       | 35.3 | 38.6 AB |
| Total          | 100   | 100        | 100  | 100     |

\*There is no statistical difference between the averages with the same letter.

Table 8. Raw ash ratios in forage taken from different directions of the research area (%)

|               | North | South-east | West  | Mean |
|---------------|-------|------------|-------|------|
| Crude Ash (%) | 9.11  | 9.46       | 10.02 | 9.51 |

Raw ash ratios of the forage in three directions are given in Table 8. As a mean raw ash ratio was the 9.51%. It was highest in the forage taken from West direction of the pasture with 10.02%. And it was 9.11% in North direction and 9.46% in south-east direction.

## CONCLUSIONS

The area covered with vegetation was found to be 11.73% as the average of the directions. This value, which was measured as the bottom coating, varied between 9.96% (South-east) and 12.92% (North) according to the directions. Accordingly, it turns out that approximately 88.27% of the soil surface is bare area. The fact that the soil surface is covered with such a small amount of vegetation is an indication that the pasture is worn out and that the soil is not covered with enough vegetation. This is the biggest cause of erosion, especially in sloping and open areas.

Considering the botanical composition values, the dominance of the grass family plants is clearly seen. Although the proportion in the botanical composition varies according to the directions, it turns out that the most dominant species is sheep's ball. The share of Sadeve sheep ball in vegetation is around twenty percent. This plant, which is the most common type of grasshopper in the pastures of almost all Anatolian geography, is the species that bears the burden of grazing in dry and hot conditions, besides its different soil structure, especially due to its root and leaf structure, despite its low productivity.

The most recurrent or most widespread species in the pasture area are grasses. The sheep ball, which is the most common species in the botanical composition, is seen as the species with the highest frequency or the highest frequency in all directions. This species is followed by *Poa bulbosa*, which stays green for a short period of time on the pasture and provides forage especially in the early period. Another one with a high recurrence rate is *Stipa pennata*. These species are among the dominant plant species reported by many researchers working in Central Anatolian pastures.

In the sampling in the pasture area studied, 30 plant species were recorded. Of these, 10 are grasses, 7 are legumes, and 13 are other family species. Many species that are not included in the sample units were also observed during the study. The high diversity of plants in a small area, as well as the low rate of vegetation cover, can be considered as a sign of deterioration in the rangeland.

The hay yields of the pasture in different directions varied between 81.6 kg/da and 116 kg da. The highest hay yield was obtained from the area under the influence of the western direction. These hay yields match the figures given by the researchers to the people working in the rangelands of the region.

According to the directions, the rate of raw ash in the pasture hay varied between 9.11% and 10.02%. The average raw ash content was 9.51%. The high rate of raw ash is an indicator of the high quality of pasture grass. The mineral substances contained in the raw ash are known

as a quality factor that directly affects the yield, as they are important in the protection of nutrition and health of pasture animals and in breeding activities.

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## **EFFECTS OF VARIOUS CLIPPING FREQUENCY AND HEIGHTS ON THE HERBAGE YIELD AND ROOT DEVELOPMENT OF TUBEROUS CANARY GRASS (*PHALARIS TUBEROSA* L.)**

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### **ABSTRACT**

Meadows and pastures not only provide food for animals, but also prevent water and wind erosion, keeping the soil in place and improving its physical and chemical structure. In addition to these benefits, it has important contributions to human beings in many ways such as being a water collection basin, being a gene source for cultivated plants, hosting wildlife, and storing carbon. Undoubtedly, the functions expected from the pastures whose vegetation is deteriorated are also decreasing. One of the most important tools in the protection and improvement of meadow and pasture conditions is to carry out grazing under controlled conditions and in a way that does not impair the yield and quality of the climax vegetation. Grazing should improve the pasture vegetation and increase its yield and quality. Mowing or grazing time, frequency and height affect the forage yield, quality and root development of pasture plants. Tuberous canary grass (*Phalaris tuberosa* L.) is a perennial, tall, rhizomous, fast-growing, upright growing grassy forage plant. It is a species recommended to be used in artificial pasture creation studies in our country. In this study, it is aimed to reveal the effect of overgrazing, which is important for the productivity and continuity of pasture plants, on tuberous stock. The study was carried out as a pot experiment in greenhouse conditions. The plants used as material were removed from the field and divided into similar sizes and then planted in pots. Plants were expected to grow up to 20 cm in a suitable greenhouse environment. Plants are grouped into 3 groups according to their development. The experiment was carried out in randomized blocks according to the factorial design with 3 replications. The first factor was “mowing height” (when the plants were 15, 20 and 25 cm tall), and the second factor was “mowing frequency” (2.5-5.0-7.5 and 10 cm height). 36 pots were used, excluding control plants. The green forage obtained from the mowing applications were immediately weighed and recorded. Cuttings were continued until control plants reached grazing maturity. At the end of this period, the remaining plant stubbles were cut and weighed. In order to determine root development, the fresh roots obtained by washing the soil in the pots were dried in a shaded environment in the greenhouse and weighed. The usable forage, total forage and root weights obtained from the mowings in each pot were analyzed using appropriate statistical methods. According to the results of the study, it was determined that mowing height and frequency were significantly effective on feed yield, number of forms and root growth in tuberous canary grass.

**Keywords:** Cutting height, number of cutting, forage yield, root growth

### **INTRODUCTION**

Animal feeding largely depends on grazing depleted natural range and pasture. As the portion of the improved pastures is really small, the majority of the livestock of the country has to graze on the unimproved poor natural rangelands. In pasture most of the livestock is

supported by grazing on rangeland. This situation is worsening day by day because the range area is diminishing and becoming less productive as a result of excessive and uncontrolled grazing. Most of the range in Turkey has been deteriorated and lost their productivity. Generally, the vegetation cover is insufficient to protect the soil from the wind and water erosion (Kendir and Bakır, 1997).

Under the excessive grazing conditions a number of desirable plants have been disappeared because they could not tolerate uncontrolled continuous grazing practised for many years. Wolf plants have invaded the places where left by desirable plants. Some desirable ones have tolerated this heavy and early grazing but lost their vigour. As long as this grazing practise goes on, rangelands' big potential will not be transferred into the practise (Kendir, 1997).

The impact of excessive and early grazing on the range plants has been investigated inside and outside the country. Many researchers have tried to get knowledge on the level of grazing by various clipping experiments. Heavy grazing causes plants to produce less forage and less root development (Weaver 1950, Ertan 1991). Excessive grazing leads plants to develop relatively small root system. Plants with small root system are not being able to produce ample forage. Root and stem are connected so vitally that any effect on either of them affects the other one (Wagner 1952, Kacar 1983).

Under the excessive grazing condition, the spaces left by desirable plants are invaded by weeds and low-quality plants that cannot be exploited by animals or are used very little. The small amount of pasture plants, which could maintain their existence based on excessive and early grazing practices, lost their growth power to a large extent. The amount of feed production cannot be expected from these plants as long as excessive and uncontrolled grazing continues. In other words, as long as today's grazing conditions continue, it is not possible to fully benefit from the potential production opportunity created by these climatic and soil conditions.

Plants of the genus *Phalaris* are delicious, although they have coarse and hard leaves. They are grown as fodder crops due to their features such as being drought resistant, showing strong development, being consumed with love by animals and being suitable for hay production. Tuberous canary grass (*Phalaris tuberosa* L.) is a cool climate plant. It is quite resistant to drought. It reaches grazing height in early spring. It is moderately resistant to soil salinity. It shows strong improvement. It is delicious. It is an important plant of winter pastures. It is suitable for hay production and seed production is easy (Tansı 2009).

In this study, the effects of overgrazing, which is vitally important for pasture plants, on root and stem development were tried to be revealed on tuberous canary.

## **MATERIAL AND METHOD**

The research was planned as a pot experiment and carried out under greenhouse conditions. The plants used as material were removed from the trial fields of Ankara University Faculty of Agriculture in early spring, divided into similar sizes and then planted in pots. Plants were expected to grow up to 15, 20 and 25 cm in a suitable greenhouse environment. For each grazing maturity height, the plants were grouped into 3 groups according to their development and replications (blocks) were formed. The experiment was carried out in randomized blocks according to the factorial design with 3 replications. Grazing maturity periods (when the plants are 15, 20 and 25 cm tall), which means "mowing frequency", were applied as the first factor, and "mowing height" (2.5-5.0-7.5 and 10 cm height cutting) was applied as the second factor. A total of 36 pots were used, excluding control plants.

For each application, one sample was taken from the plants grouped according to their development. During the experiment, the plants were watered. The green fodders obtained from the mowing applications were weighed without waiting and their weights were recorded.

Cuttings were continued until control plants reached grazing maturity. At the end of this period, the remaining plant stubbles were cut and weighed. In order to determine the root development, the fresh roots obtained by washing the soil in the pots were dried in a shaded environment in the greenhouse and weighed. The usable forage, total forage and root weights obtained from the mowings in each pot were analyzed using appropriate statistical methods.

## RESULTS AND DISCUSSION

### Number of mowing

The number of formats obtained from each application as the average of the treated groups are given in Table 1.

Table 1. Number of cuttings in tuberous canary grass with different mowing frequency and mowing height

| Height | 2.5 cm | 5 cm | 7.5. cm | 10 cm | Mean  |
|--------|--------|------|---------|-------|-------|
| 15 cm  | 6      | 10   | 11      | 15    | 10.75 |
| 20 cm  | 5.34   | 6    | 9       | 10    | 7.58  |
| 25 cm  | 5      | 5    | 7       | 7     | 6     |
| Mean   | 5.44   | 7    | 9       | 10.6  |       |

As can be seen from the table, the number of shapes differed according to the frequency of the shapes and the height of the shapes. It was obtained from plants cut from 10 cm height when the highest number of forms reached 15 cm in length. In this group, the plants were mowed 15 times. The lowest number of forms was obtained from the plants that were cut by leaving 2.5 and 5 cm stubble heights when they reached a height of 5 to 25 cm. Frequent coming to form maturity can be evaluated as a positive response of pasture plants to grazing. The reaction that occurs according to different characteristics of plants may occur differently in different species (Ertan 1991, Tükel et al. 1996, Kendir 1997).

### Usable forage yield

In the analysis of variance on the usable feed yields obtained from tuberous canary grass applied at different mowing frequencies and mowing heights, the effect of mowing frequency on the usable forage yield was found to be insignificant, while the effect of mowing height was significant (Table 2).

Table 2. Usable feed yields (g/plant) in tuberous canary grass applied with different mowing frequency and mowing height

| Height | 2.5 cm | 5 cm   | 7.5. cm | 10 cm | Mean  |
|--------|--------|--------|---------|-------|-------|
| 15 cm  | 18.48  | 24.97  | 29.66   | 32.77 | 26.47 |
| 20 cm  | 23.18  | 30.20  | 28.05   | 34.70 | 29.03 |
| 25 cm  | 19     | 27.55  | 29.81   | 35.72 | 28.02 |
| Mean   | 20.24c | 27.58b | 29.17ab | 34.4a |       |

The highest usable green forage yield was obtained from plants cut from 10 cm height when they reached 25 cm height. 35.72 g feed yield was obtained from the plants harvested from these heights. The lowest vulnerable green fodder yield was obtained from plants cut from

2.5 cm height when they reached 15 cm height. These plants were able to produce 18.48 g of feed per pot. In terms of mowing frequency or grazing period, the highest forage yield was obtained from the plants that were cut when it reached 20 cm. Plants in this group produced 29.03 g of feed. The lowest forage yield was obtained from the plants harvested when they reached a height of 26.47 g and 15 cm. In terms of mowing height, the highest usable forage yield was obtained as 34.4 g from plants mowed from 10 cm height, while the lowest usable forage yield was obtained as 20.24 g from plants mowed from 2.5 cm height.

Various researchers (Bakır, 1969, Ertan 1991, Tükel et al. 1996) have shown that as the mowing height decreases, the usable forage yield also decreases.

### **Total forage yield**

In the analysis of variance on the total forage yields obtained from tuberous canary grass applied at different mowing frequencies and mowing heights, the effect of mowing frequency on total feed yield was found to be significant, while the effect of mowing height was found to be very important (Table 3).

Table 3. Total forage yields (g/plant) in tuberous canary grass with different mowing frequency and mowing height

| Height | 2.5 cm | 5 cm   | 7.5. cm | 10 cm  | Mean    |
|--------|--------|--------|---------|--------|---------|
| 15 cm  | 21     | 28.62  | 33.71   | 39.55  | 30.72b  |
| 20 cm  | 26.51  | 34.52  | 36.48   | 39.68  | 34.30a  |
| 25 cm  | 22.10  | 31.13  | 34.27   | 42.57  | 32.51ab |
| Mean   | 23.20c | 31.42b | 34.82ab | 40.60a |         |

At the end of the study, the highest yield in terms of total feed obtained by adding the stubble yields left in the pots to the feed obtained from the mowing was obtained from the plants in the pots mowed from 10 cm height when they reached 25 cm mowing maturity. These plants produced 42.57 g of total feed. The lowest total green forage yield was obtained from plants cut from 2.5 cm height when they reached 15 cm height. These plants produced 21 g of feed per pot.

In terms of mowing frequency or grazing period, the highest forage yield was obtained from the plants that were cut when they reached a height of 34.30 g and 20 cm. The lowest forage yield was obtained from the plants harvested when they reached a height of 30.72 g and 15 cm. In terms of mowing height, the plants cut from 10 cm height gave the highest yield. Plants in this group produced 40.60 g of feed per pot, while plants mowed from 2.5 cm height produced 23.20 g of forage. This result is consistent with studies reporting that mowing close to the soil surface reduces forage yield (Ertan 1991, Tükel et al. 1996).

### **Root development**

In the analysis of variance performed on the air dry root weights obtained from tuberous canary grass with different mowing frequencies and mowing heights, the effect of mowing frequency on root yield or development was found to be insignificant, while the effect of mowing height was found to be significant (Table 4).

Table 4. Air-dry root weights (g/plant) in tuberous canary grass with different mowing frequency and mowing height

| Height | 2.5 cm | 5 cm   | 7.5. cm | 10 cm | Mean |
|--------|--------|--------|---------|-------|------|
| 15 cm  | 2.54   | 2.25   | 3.88    | 4.88  | 3.64 |
| 20 cm  | 3.02   | 3.83   | 4.14    | 4.56  | 3.88 |
| 25 cm  | 2.49   | 3.23   | 4.34    | 5.27  | 3.83 |
| Mean   | 2.68c  | 3.44bc | 4.12ab  | 4.90a |      |

Means with same letter differ statically

The highest air dry root weight was obtained from plants cut from 10 cm height when they reached 25 cm height. 5.27 g air-dry root weight was obtained from the plants harvested from these heights. The lowest air dry root weight was obtained from plants cut from 5 cm height when they reached 25 cm height. 2.25 g air-dry root weight per pot was obtained from these plants. In terms of mowing frequency or grazing period, the highest air dry root weight was obtained from the plants that were cut when it reached 20 cm. Plants in this group produced 3.88 g air-dry root weight. The lowest air dry root weight was obtained from the plants harvested when they reached a height of 3.64 g and 15 cm.

In terms of mowing height, the highest root growth was obtained from the group of plants cut from 10 cm height, which gave a root weight of 4.9 g. Plants mowed from a height of 2.5 cm, indicating deep cutting or grazing, were the plants that showed the least development with 2.68 g root development. It has been revealed by many researchers that deep shapes limit the root development of plants (Weaver 1950, Ertan, 1991).

#### Forage yields of control plants

The total forage and air dry root weights that can be used from tuberous canary grass plants that were not mown until the end of the study and that reached the maturity stage are given in Table 5.

Table 5. Comparison of forage, total forage and root yields of control plants and mowed plants at different mowing heights

|                               |         |       |         |       |        |
|-------------------------------|---------|-------|---------|-------|--------|
| Forage efficiency             | Control | 10 cm | 7.5. cm | 5 cm  | 2.5 cm |
| Forage used                   | 74.18   | 34.40 | 29.17   | 27.58 | 20.24  |
| When the control is taken 100 | 100     | 46.37 | 39.32   | 37.17 | 27.28  |
| Total feed                    | 92.36   | 40.6  | 34.82   | 31.42 | 23.2   |
| When the control is taken 100 | 100     | 43.95 | 37.7    | 34.01 | 25.11  |
| Air dry root weight           | 12.91   | 4.9   | 4.12    | 3.44  | 2.68   |
| When the control is taken 100 | 100     | 37.95 | 31.91   | 26.64 | 20.75  |



When the usable feed yields of the control plants were compared with the plants harvested at different cutting heights, it was found that the plants harvested from 10 cm height produced 46.37% of the feed produced by the control plants. Plants cut from 2.5 cm height produced 27.28% of the control plants forage.

When this comparison was made in terms of total feed, the plants mowed from 10 cm height produced 43.95% of the control group, while the plants mowed from 2.5 cm height produced 25.11% of the total feed of the control group.

Like the above-ground parts of plants, underground root growth also showed different development depending on different mowing heights. Depending on the mowing depth, the plants formed less root systems. Compared to the control plants, the plants cut from 10 cm height showed 37.95% root growth, while the root growth obtained from the plants cut from 2.5 cm height, which refers to the deep form, was only 20.75% of the control plants.

Table 6. Comparison of forage, total forage and root yields of control plants and harvested plants at different mowing periods

| Feed efficiency               | Control | 25 cm | 20 cm | 15 cm |
|-------------------------------|---------|-------|-------|-------|
| Feed used                     | 74.18   | 28.02 | 29.03 | 26.47 |
| When the control is taken 100 | 100     | 37.77 | 39.13 | 35.68 |
| Total feed                    | 92.36   | 32.51 | 34.3  | 30.72 |
| When the control is taken 100 | 100     | 35.19 | 37.13 | 33.26 |
| Air dry root weight           | 12.91   | 3.83  | 3.88  | 3.64  |
| When the control is taken 100 | 100     | 29.66 | 30.05 | 28.19 |

When the usable forage yields of the control plants are compared with the crops mowed at different mowing periods, it is seen that the crops mowed when they reach a height of 20 cm produce 39.13% of the feed produced by the control plants. The crops cut when they reached a height of 15 cm produced 35.68% of the usable forage of the control crops. When this comparison is made in terms of total forage, the mown crops produced 37.13% of the total feed of the control group when they reached a height of 20 cm, while the mown crops produced 33.26% of the total feed of the control group when they reached a height of 15 cm. Compared to the control plants, the mowed plants showed 30.05% root growth when they reached a height of 20 cm, while the mown plants showed 28.19% root growth when they reached a height of 15 cm.

## CONCLUSIONS

The effects of mowing frequency (grazing maturity) and height on forage yield and root growth in tuberous canarian grass were investigated in a pot experiment conducted under greenhouse conditions.

The results present that the effect of mowing frequency on vulnerable forage yield and root growth was not significant. However, root development was less in plants that were cut frequently (15 cm). The effect of cutting height on all the characters examined in tuberous cane was significant. Deep (2.5 cm) forms are vulnerable and have low overall forage yield and root growth is limited.

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## EFFECTS OF DIFFERENT TPMS LATTICE STRUCTURES ON THE MECHANICAL BEHAVIOUR OF HIP IMPLANT

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### ABSTRACT

Triply periodic minimal surfaces (TPMS) porous lattice structures have remarkable properties in terms of designing bio-inspired medical implants. TPMS porous structures that repeating regular solids can be mathematically controlled and defined by functions. Characteristically qualifications of TPMS structures such as energy absorption, lightweighting, good mimic to natural bone, increasing celling on contact area of bone and implant are desired for hip implant. In this study, Hip implant is shelled and infilled with Gyroid, Diamond, Lidinoid, Schwarz and SplitP TPMS lattice structures and their mechanical behaviours are investigated. Resultant force and fixed supports are applied according to literature. Magnesium AZ91 mechanical properties are appointed to hip implant. Finite element analysis (FEA) is carried out with constant conditions for each lattice structure and results are evaluated. The results show that highest Von-Mises stress is observed on Schwarz infilled hip implant. Lidinoid infilled hip implant which has the lowest Von-Mises stress is most suitable design. Implicit body is created from CAD body. Unit cell size is defined same value for every lattice structure.

**Keywords:** Hip Implant, TPMS Lattice Structures, Finite Element Analysis (FEA), Static Analysis,

### INTRODUCTION

Hip and knee replacements improve the patient's life quality via pain relief and restored function, through the success of implants fixation to bone. Roughened and porous surfaces have been used to improve biological fixation and osseointegration. Recently, porous metals were used to improve fixation to bone for orthopedic applications (Muth et al., 2013). The possibility of applying bioinspired lattice structures are investigated in order to topologically optimize an orthopedic hip implant made of Inconel 718 superalloy. Three different bioinspired advanced lattice structures that they are Voronoi, Gyroid, Schwarz Diamond were investigated through finite element analysis (FEA) under in vivo loading. Results have shown that optimal design of hip implant geometry, in terms of stress behavior, was achieved through functionally graded Voronoi and Gyroid lattice structures (Kladovasilakis et al., 2020).

Porous gyroid Ti6Al4V structure is investigated compare to a solid stem model for human tibial-knee implantation of total knee replacement through finite element analysis (FEA). The results indicated enhanced clinical performance of the porous tibial-knee implant compared to the solid titanium implant via increasing the maximum von-Mises bone stresses and decreasing the maximum shear stress at the bone/ implant interface. ((Eltahawy et al., 2022). Rigid shell structures are infilled as lattice structure in hip implant. The lattice types reduce the implant mass and, due to higher stress distribution and deformations as compared to the rigid

implants, it brings down the stress shielding issues. The Finite Element Analysis (FEA) demonstrated that the Weaire-Phelan lattice structure exhibits the least stress and deformation among the other types at various design parameters while applying 2300 N axial force is explored numerically. (Izri et al., 2022)

## MATERIAL AND METHOD

Hip implant CAD model is drawn using in Solidworks. Real dimensions are used according to literature during drawing model. The cad model was prepared proper file format and properties for transfer to nTopology software where we will carry out FEA and create lattice structures.



Figure 1. Hip Implant CAD Model

CAD model is imported from Solidworks proper file format and created implicit body. Implicit body is shelled as 3 mm and infilled Gyroid, Diamond, Lidinoid, Schwarz and SplitP by this means Volume Lattice function of software. Finally implicit bodies ready to carry out FEA and evaluate results.

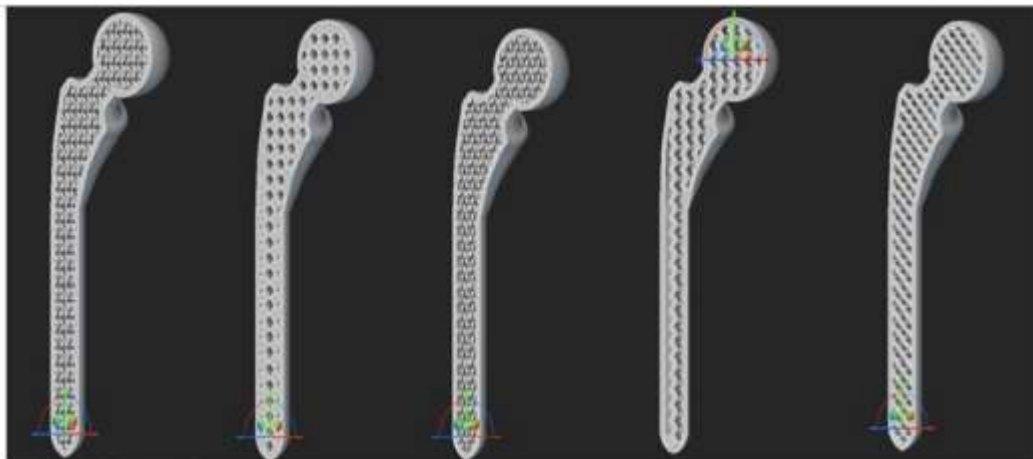


Figure 2. TPMS Lattice Structures in Hip Implant

Simulation is carried out to investigate effects of different TPMS lattice structures on the mechanical behaviour of hip implant. Numerical simulation results throughout FEA using nTopology. Simulation is accomplished in four main steps;

Creating Mesh: After standard mesh, volume mesh is applied. Tetrahedral elements are used.

Applying Boundary Conditions: 2200 N resultant force is applied on head of hip implant. Femoral stem face is fixed support.

Defining Material Properties: 45 GPa elastic modulus and 0.3 poisson ratio is determined  
Performing Simulation and Getting Results: Generating Results, graphs, charts, and animations

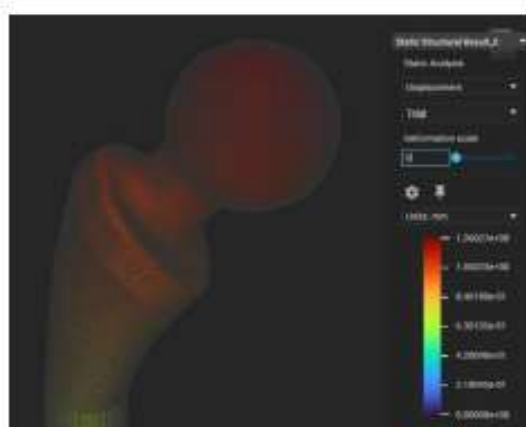


Figure 3. Static Analysis

## RESULTS AND DISCUSSION

Static analysis is performed infilled lattice structures through finite element analysis and results are investigated. Maximum Von Mises stress is seen Schwarz lattice structure. Deflection values are almost similar in every lattice structure. Stresses generally concentrate on head of hip implant that force is applied on all lattice structures. Results show that among the various lattice structures, Lidinoid has best mechanical performance. Future studies will focus on ;

- ▷ Implementation mechanical properties,
- ▷ Optimizing implant geometry,
- ▷ Adapting these lattice structures to implant.

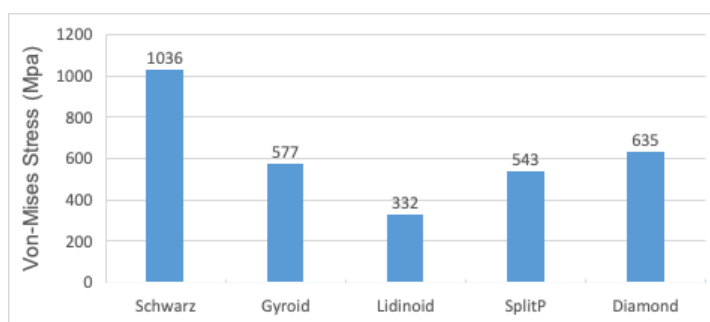


Figure 4. Stress Results

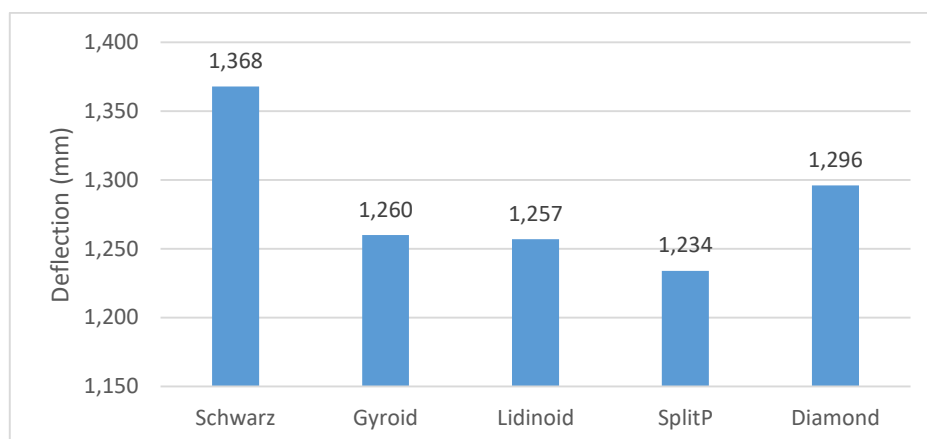


Figure 5. Deflection Results

## CONCLUSIONS

TPMS lattice structures have lots of potential to use them on hip implant. TPMS lattice structures can be used instead of solid implant if its mechanical properties are improved apart from its properties such as providing celling, compatibility with bone structure and ability to energy absorbtion.

According to the finite element analysis results Lidinoid have high benefit and mechanical performance compared to other lattice structures. Considering that Magnesium AZ91 to be used has a yield strength of 150 MP, the design that have 332 MPa Von-Mises Stress should be optimized and work with an appropriate safety factor.

Future studies will focus on;

- ▷ Implementation mechanical properties,
- ▷ Optimizing implant geometry,
- ▷ Adapting these lattice structures to implant.

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## EFFECT OF TREATMENT WITH HERBICIDES IN SUNFLOWER HYBRIDS ON WEED INFESTATION

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### ABSTRACT

The investigations were carried out during 2018–2021 at Dobrudzha Agricultural Institute – General Toshevo (DAI). The aim of this investigation was to determine the effect of the treatment with herbicides in sunflower hybrids on weed infestation. The following herbicides combinations were used: dimetenamid-P (140 ml/da)+kletodim (160 ml/da); pendimetalin (400 ml/da) + tsikloksidim (200 ml/da); dimetenamid –P (140 ml/da)+ tsikloksidim (200 ml/da); imazamoks (160 ml/da) and imazamoks (200 ml/da) . The herbicides were applied at stage 01 (BBCH), and tsikloksidim (200 ml/da) and kletodim (160 ml) at stage 12-14 (BBCH) on three conventional sunflower hybrids, Vesi, Linzi and Deveda and imazamox (160 ml/da and imazamoks 200 ml/da) on clearfield hybrids Enigma and Danaia. The herbicide effect was determined by the quantitative weight method and evaluated by the EWRS scale. Regardless of the used hybrid, the herbicide combinations dimetenamid-P+kletodim, pendimetalin + tsikloksidim and dimetenamid + tsikloksidim had highest efficiency (90-94%) on grassy weeds- *Setaria viridis* L. and *Echinochloa crus-galli* L. and lower effect on broadleaved *Sinapis arvensis* L., *Datura stramonium* L., *Xanthium strumarium* L., *Amaranthus retroflexus* L., *Polygonum convolvulus* L., *Chenopodium album* L., *Polygonum hydropiper*, *Convolvulus arvensis* L., *Cirsium arvensis* (L.) Scop and *Canabis sativa* L. (82-90%). Imazamoks (160 ml/da and 200 ml/da) had highest efficiency (90-94%) on broadleaved weeds and lower effect on grassy weeds (82-90%).

**Key words:** Sunflower, Herbicides, Weeds, Efficiency, Selectivity

### INTRODUCTION

Sunflower (*Helianthus annuus* L.) is the most important oil crop in Bulgaria. It had enormous agrotechnical importance. Weed infestation was one of the reason to obtained bad results. (Lubenov, 1987; Sabev, 2000; Sabev, 2001) The extremely fertility of weeds, the ability of seeds to kept alived in the earth and to sawed from different depth in a long period of time, showed any difficulties (Lubenov, 1987). The main method to control the weed infestation in the all kind of field was usied of herbicides. Its introduction allows to have new, more effective technologies for the production. It was established, in the sunflower fields in Bulgaria had 130 kind of weeds (Kolev, 1963).

Herbicides linuron, prosulfocarb and pethoxamid showed good selectivity for sunflower. Crop injury rate of 5-15% was recorded after application of flurochloridone and acetochlor. For flurochloridone, the phytotoxicity increased due to irrigation after herbicide application. The hightest sunflower injury rate (27-35%) was recorded after application of oxyfluorfen (Jursík et al., 2015). The use of Clearfield system proved to a great option for areas with monocotilidon weed infestation, since it allows the application of acetolactate synthase – inhibiting herbicides achieving excellent weed control, without causing visial injury and maintaining the original

stand, without affecting crop yield (Francischini et al., 2012). A high level of weed control is achieved by timely harrowing at the stage of mass weed emergence (not later than the stage of the first leaf of the grassy weed species and not later than the first to second leaf of the broad-leaved weed species), (Mitkov and Stoychev, 2014). Inclusion of sunflower in proper, scientifically – based crop rotations, in which it is sown mainly after merged surface crops, makes weed control of perennial and late spring weeds much easier (Malidža et al., 2011; Reis et al., 2014).

The weeds had mechanisms to adapt upon bad conditions in their development. The problem weeds cause arises from their wide specific variability and their high biological and ecological plasticity (Wanikorn, 1991). The herbicides will left in the future in the agriculture an effective method to control weeds as a part of integrate control, it was needed to optimized its using (Mitric and Vuckovic, 2008; Jocić et al., 2011; Knežević et al., 2011).

The aim of this investigation was to determine the effect of the treatment with herbicides in sunflower hybrids on weed infestation.

## MATERIAL AND METHODS

The investigations were carried out during 2018 – 2021 at Dobrudzha Agricultural Institute – General Toshevo. The field trial was designed according to the block method in three replications, the size of the trial area - 14 m<sup>2</sup> and crop density 5500 plants per da. Two controls are included: K<sub>1</sub> - control, without weeds manually harvested to sunflower booting stage and the K<sub>2</sub> - weeded control until the end of the crop's vegetation (Table 1).

Table 1. Variants

| Variants                   | Trade names                   | Doses ml/da |
|----------------------------|-------------------------------|-------------|
| Control-without weeds      | -                             |             |
| Dimetenamid-P+Kletodim     | Spektrum+Select super 120EC   | 140+160     |
| Pendimetalin+Tsikloksidim  | Stomp Nov 330EK+Stratos ultra | 400+200     |
| Dimetenamid-P+Tsikloksidim | Spektrum+Stratos Ultra        | 140+200     |
| Imazamoks                  | Pulsar plus                   | 160         |
| Imazamoks                  | Pulsar plus                   | 200         |
| Weeded control             | -                             |             |

The herbicides were applied at stage 01 (BBCH) and at stage 12-14 (BBCH) on conventional sunflower hybrid Vesi, Linzi, Deveda and clearfield hybrids Enigma and Danaya. Relying on natural weed infestation from grassy weeds: green hides - *Setaria viridis L.* and cockspur - *Echinochloa crus-galli L.* and broadleaved weeds: wild mustard - *Sinapis arvensis L.*, thorn-apple - *Datura stramonium L.*, rough cocklebur - *Xanthium strumarium L.*, common amaranth - *Amaranthus retroflexus L.*, black-bindweed - *Polygonum convolvulus L.*, fat-hen - *Chenopodium album L.*, water papper - *Polygonum hydropiper*, field bindweed - *Convolvulus arvensis L.*, creeping thistle - *Cirsium arvense (L.) Scop* and hemp - *Canabis sativa L.* Weed density was measured quantitatively per unit area by species using ¼ frame in four replications prior to introduction of herbicides.

The herbicide efficiency was estimated 25-30 days after the use of the preparations according to species, by amount and weight, using ¼ frame in four replications, measuring the weight of the weeds in fresh and dry condition. The effect was evaluated according to the 9-degree scale of European Weed Research Society (EWRS) for reading of the herbicide activity



and selectivity, 1 corresponding to 100 % efficiency of the preparation, without symptoms of phytotoxicity on the cultural plants; and 9 corresponding to 29.9 % - 0 % effect of the preparation and complete perishing of the plants (Table 2).

Table 2. Herbicide activity and selectivity according to 9-degree scale of EWRS

| Rank | Herbicide effect % | Damage symptoms   | General evaluation |
|------|--------------------|---|--------------------|
| 1    | 100                | No symptoms – healthy plants  | Excellent          |
| 2    | 99,9-98            | Very weak symptoms – slight stunt effect                              | Very good          |
| 3    | 97,9-95            | Weak but discernable symptoms   | Good               |
| 4    | 94,9-90            | Better expressed symptoms (eg. chlorosis) which do not affect yield   | Satisfactory       |
| 5    | 89,9-82            | Thinning of the crop, strong chlorosis or stunt. Lower yield expected | Indefinitely       |
| 6    | 81,9-70            | Heavy damage or perishing of plants                                   | Unsatisfactory     |
| 7    | 69,9-55            | Heavy damage or perishing of plants                                   | Poor               |
| 8    | 54,9-30            | Heavy damage or perishing of plants                                   | Very poor          |
| 9    | 29,9-0             | Heavy damage or perishing of plants                                   | Extremely poor     |

## RESULTS AND DISCUSSION

During 2018-2021, after treatment at „stage 12-14” of hybrid Vesi, Linzi and Deveda the weed composition was read using the quantitative weight method. 25-30 days after the application of herbicides, partial and full action of the used products was observed. Single species from the above were determined. In some variants newly emerging plants of field bindweed were found. The dry matter weight of the weeds was minimal in individual variants of the trial as a result from the very good herbicide effect. Herbicide combinations: dimetenamid-P+kletodim; pendimetalin+tsikloksidim; dimetenamid-P+tsikloksidim had highest effect (90%) on green habit and cockspur and on broadleaved weeds - 82%.

At the same investigated period it was determined dry matter amount from green hides varied from 3,1 to 7,2 g/m<sup>2</sup> comparison 29,3 g/m<sup>2</sup> in weeded control after treatment with dimetenamid-P+kletodim, after pendimetalin+tsikloksidim – from 3,4 to 9,0 g/m<sup>2</sup> and after dimetenamid-P+tsikloksidim – from 4,0 to 9,2 g/m<sup>2</sup>. The herbicide combinations had efficiency on cockspur - 90%. The dry matter weight of the weeds at the combinations varied as followed: after treatment with dimetenamid-P+kletodim from 2,0 to 5,6 g/m<sup>2</sup>, with pendimetalin+tsikloksidim from 2,9 to 8,1 g/m<sup>2</sup>, with dimetenamid-P+tsikloksidim – from 2,7 to 6,8 g/m<sup>2</sup> comparison 29,0 g/m<sup>2</sup> in weeded control. The weed infestation with broadleaved weeds varied insignificantly for the investigation period. The efficiency of the introduced conventional herbicides was very high. The effect of used herbicides was lower in all broadleaved weeds, regardless of treated hybrid – 82% (Table 3). Did not observed visible symptoms of phytotoxicity on tested conventional hybrids.

During the study period on clearfield hybrids Enigma and Danaia, active substance imazamoks (160 and 200 ml) had 82% effect (Table 4). It was investigated dry matter weight of green habit varied from 2,8 to 4,7 g/m<sup>2</sup> comparison 38,6 g/m<sup>2</sup> in weeded control after treatment with imazamoks (160 ml), after imazamoks (200 ml) – from 5,8 to 9,1 g/m<sup>2</sup> comparison 33,7 g/m<sup>2</sup> in weeded control. Imazamoks, introduced in first and second dose had lower herbicide effect on cockspur (82%). Dry matter weight at these combinations varied as followed: after treatment with imazamoks (160 ml) from 2,5 to 7,6 g/m<sup>2</sup>, with imazamoks (200

ml) from 5,3 to 9,7 g/m<sup>2</sup> in weeded control. The infestation of broadleaved weeds varied insignificantly for the period of investigation on Enigma and Deveda. The effect of tested set of herbicides was higher in all broadleaved weeds, regardless of the used clearfield hybrid – 90%. Did not observed visible symptoms of phytotoxicity on tested clearfield hybrids.

Table 3. Efficiency and selectivity of some herbicides against grassy and broadleaved weeds in conventional sunflower hybrids according to 100% visual scale of EWRS during 2018-2021

| Weeds                    | Variants               |                           |                            |              | Selectivity |
|--------------------------|------------------------|---------------------------|----------------------------|--------------|-------------|
|                          | Dimetenamid-P+Kletodim | Pendimetalin+Tsikloksidim | Dimetenamid-P+Tsikloksidim | Weeded check |             |
| <i>S. viridis L.</i>     | 90                     | 90                        | 90                         | 0            | 1           |
| <i>E. crus-galli L.</i>  | 90                     | 90                        | 90                         | 0            | 1           |
| <i>S. arvensis L.</i>    | 82                     | 82                        | 82                         | 0            | 1           |
| <i>D. stramonium L.</i>  | 82                     | 82                        | 82                         | 0            | 1           |
| <i>X. strumarium L.</i>  | 82                     | 82                        | 82                         | 0            | 1           |
| <i>A. retroflexus L.</i> | 82                     | 82                        | 82                         | 0            | 1           |
| <i>P. convolvulus L.</i> | 82                     | 82                        | 82                         | 0            | 1           |
| <i>Ch. album L.</i>      | 82                     | 82                        | 82                         | 0            | 1           |
| <i>P. hydropiper</i>     | 82                     | 82                        | 82                         | 0            | 1           |
| <i>C. arvensis L.</i>    | 82                     | 82                        | 82                         | 0            | 1           |
| <i>C. arvensise L.</i>   | 82                     | 82                        | 82                         | 0            | 1           |
| <i>C. sativa L.</i>      | 82                     | 82                        | 82                         | 0            | 1           |

Table 4. Efficiency and selectivity of some herbicides against grassy and broadleaved weeds in clearfield sunflower hybrids according to 100% visual scale of EWRS during 2018-2021

| Weeds                    | Variants             |                       |            | Selectivity |
|--------------------------|----------------------|-----------------------|------------|-------------|
|                          | Imazamoks(160 ml/da) | Imazamoks (200 ml/da) | Weed check |             |
| <i>S. viridis L.</i>     | 82                   | 82                    | 0          | 1           |
| <i>E. crus-galli L.</i>  | 82                   | 82                    | 0          | 1           |
| <i>S. arvensis L.</i>    | 90                   | 90                    | 0          | 1           |
| <i>D. stramonium L.</i>  | 90                   | 90                    | 0          | 1           |
| <i>X. strumarium L.</i>  | 90                   | 90                    | 0          | 1           |
| <i>A. retroflexus L.</i> | 90                   | 90                    | 0          | 1           |
| <i>P. convolvulus L.</i> | 90                   | 90                    | 0          | 1           |
| <i>Ch. album L.</i>      | 90                   | 90                    | 0          | 1           |
| <i>P. hydropiper</i>     | 90                   | 90                    | 0          | 1           |
| <i>C. arvensis L.</i>    | 90                   | 90                    | 0          | 1           |
| <i>C. arvensise L.</i>   | 90                   | 90                    | 0          | 1           |
| <i>C. sativa L.</i>      | 90                   | 90                    | 0          | 1           |

## CONCLUSIONS

Regardless of the used hybrid, the herbicide combinations dimetenamid-P+kletodim, pendimetalin + tsikloksidim and dimetenamid + tsikloksidim had highest efficiency (90-94%) on grassy weeds-*Setaria viridis* L. and *Echinochloa crus-galli* L. and lower effect on broadleaved *Sinapis arvensis* L., *Datura stramonium* L., *Xanthium strumarium* L., *Amaranthus retroflexus* L., *Polygonum convolvulus* L., *Chenopodium album* L., *Polygonum hydropiper*, *Convolvulus arvensis* L., *Cirsium arvensis* (L.) Scop and *Canabis sativa* L. (82-90%). Imazamoks (160 ml/da and 200 ml/da) had highest efficiency (90-94%) on broadleaved weeds and lower effect on grassy weeds (82-90%).

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## PALYNOLOGICAL, ANATOMICAL AND MICROMORPHOLOGICAL FEATURES OF ENDEMIC *COUSINIA WORONOWII*

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### ABSTRACT

The *Cousinia* (Asteraceae) is the third largest genus in the Asteraceae family. It contains approximately 700 species currently accepted in Southwest and Central Asia. The genus *Cousinia* has the characteristics of the typical Irano-Turanian phytogeographic region and has a high endemism rate. The *Cousinia* is represented with 40 taxa, of which 28 (70%) endemic to Turkey. The main purpose of this study is to investigate the palynological, anatomical, and achene micromorphological features of *Cousinia woronowii* to contribute to the systematics of *Cousinia*. In this study, the plant specimens were collected from their natural distribution areas. Pollen slides were prepared according to the methods described by Wodehouse (1935) and then examined under the Leica DM750 light microscope. For anatomical studies, plant specimens were preserved in bottles containing 70% alcohol. The paraffin method was used for cross sections of stems, leaf and midrib. The sections were taken by microtome and stained with safranin and fast-green (Johansen 1940). For micromorphological investigations, mature achene collected from natural populations of plants were used. In the transverse sections of the stem, as a protective tissue, the epiderm which has 1 layer was located in the outermost layer. The cortex parenchyma has rectangular-oval-shaped cells of 7-11 rows. Phloem is surrounded by sclerenchymatic cells. The mesophyll consists of elongated palisade and spongy paranchyma cells in the cross section of the leaf. The midrib shape is circular. The pollen grains of *Cousinia woronowii* are radially symmetrical, isopolar and have a tricolporate aperture. Their shape is prolate, large size.

**Keywords:** Asteraceae, Cardueae, *Cousinia*, Endemic, Micromorphology.

### INTRODUCTION

The genus *Cousinia* is the third largest genus of the Asteraceae family, after *Senecio* (Canary Grass) and *Vernonia*, and the largest genus of the Cardueae tribus. (Rechinger, 1986; Susanna and Garcia-Jacas, 2007). The genus *Cousinia* is represented by about 700 species in the world. (Rechinger 1972; Rechinger 1979; Rechinger 1986; Attar and Djavadi, 2010). The majority of the species in the genus are distributed in Southwest Asia and Central Asia. The genus *Cousinia* was first described by Cassini (1827) based on *Carduus orientalis* Adams. In the Flora of Turkey, this genus is represented by a total of 38 species in 6 sections and 26 of them are endemic (Davis, 1975). According to Turkey's Plants List, *Cousinia* genus is represented by 39 species in Turkey and the Turkish name of the genus is "Kızandikeni" and finally, it reached 40 with the addition of a recently published new one (Tugay, 2012; Tugay et al., 2019). Sect. *Cousinia* consists of 22 species, the representative section in Turkey, even more than Flora Iranica (ca. 10 species (Rechinger, 1979).

*Cousinia* sect. *Cousinia* species are perennial herbs, growing mainly on dry, limestone, volcanic and rocky slopes, fallow fields, serpentine hills, or steppes (Huber-Morath 1975). To date, the palynological, micromorphological, anatomical, and molecular studies have been performed on the genus *Cousinia* (Ahmad et al., 2011; Ulukuş, 2019; Ulukuş and Tugay, 2019a, 2019b; Tugay et al., 2019; Ulukuş and Tugay, 2020; Ulukuş et al., 2021; Atasagun et al., 2021 Susanna et al., 2003a). This study was conducted on the endemic *Cousinia woronowii* Bornm. It will contribute to revealing different studies of the genus by making palynological, micromorphological and anatomical studies of the genus.

## MATERIAL AND METHOD

In this study, the plant specimens were collected from their natural distribution areas: – A8 Erzurum; between Tortum-Artvin, steppe, 1450 m, 23.07.2022, B. Atasagun-1117 & D. Ulukuş (Figure 1).

The collected plant samples were dried according to common herbarium techniques. In anatomical studies, stem and leaf parts, which were taken into 70% etil alcohol during field work, were used. In the study, 8 and 10 µm thick sections were taken with a Leica RM2125RT microtome using the paraffin embedding method. Sectioned samples were stained with the Fastgren-safranin staining method and made into a fixed preparation using enthallan (Johansaen, 1940). Preparation pictures were taken under a Leica DM1000 binocular light microscope and a Leica DFC280 camera. For palynological studies, pollen material was taken from the collected samples and the preparation was prepared according to Woodehouse (1935). 30 measurements were made for the light microscope of the species. SEM microscope was used to determine the surface ornaments of the pollen. The pollen terminology was determined by using various source (Faegri and Iversen, 1989; Punt et al., 2007; Hesse et al., 2009). 10-20 measurements were made to determine the seed width and length. SEM microscope photographs were used to determine the seed surface ornaments. Stearn (1983) terminology was used to determine seed surface ornaments.

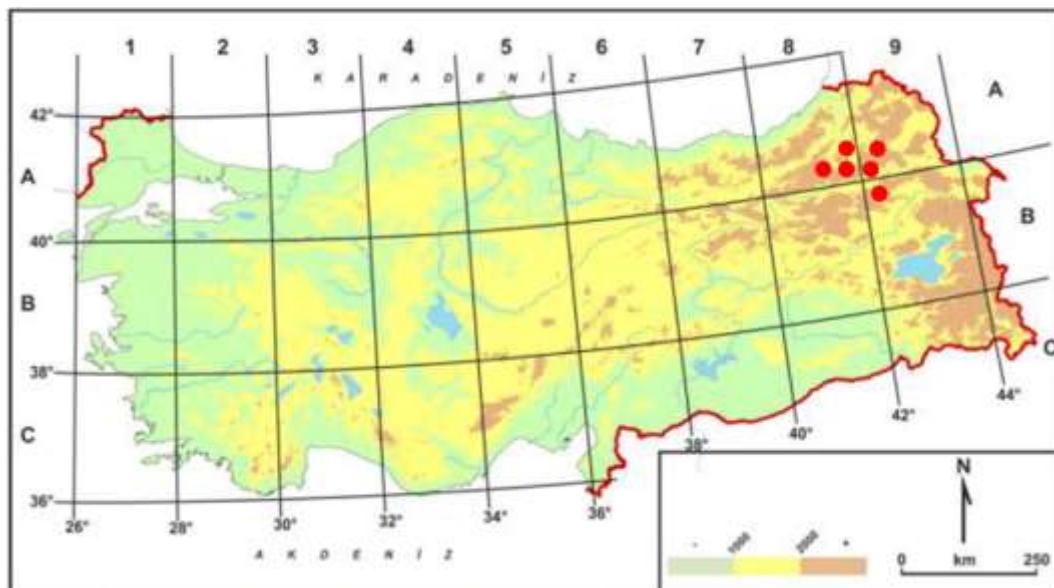


Figure 1. Distribution map of *Cousinia woronowii*

## RESULTS

### Anatomical Characteristics

*Stem:* There is a single layer of epidermis as the outermost protective tissue. The epidermis is composed of rectangular cells with cell dimensions of  $8.33 \times 23.44 \times 7.29 \times 14.58 \mu\text{m}$ . A thin cuticle layer is located on the epidermis layer. Under the epidermis layer, there is the cortex parenchyma consisting of parenchymatic cells. The cortex parenchyma has rectangular, pentagonal and oval shapes and has 7-11 rows. Under the cortex parenchyma, there is a sclerenchyma layer,  $3.91 \times 17.18 \times 4.69 \times 16.40 \mu\text{m}$  in size, consisting of 10-14 rows of densely arrayed cells. The phloem layer is composed of dense small cells and the diameter of the cells is  $3.13 \times 9.38 \mu\text{m}$ . Between the phloem and the xylem, 3-4 rows of cambium layer stand out. Tracheal cells are composed of quadrangular and oval cells with a diameter of  $3.13 \times 32.03 \mu\text{m}$ . There are 4-5 rows of pith rays between the bundles. In the center, there is a well-developed pith region composed of pentagonal cells. Cells of the core parenchyma are  $12.50 \times 45.31 \mu\text{m}$  in diameter (Figure 2).

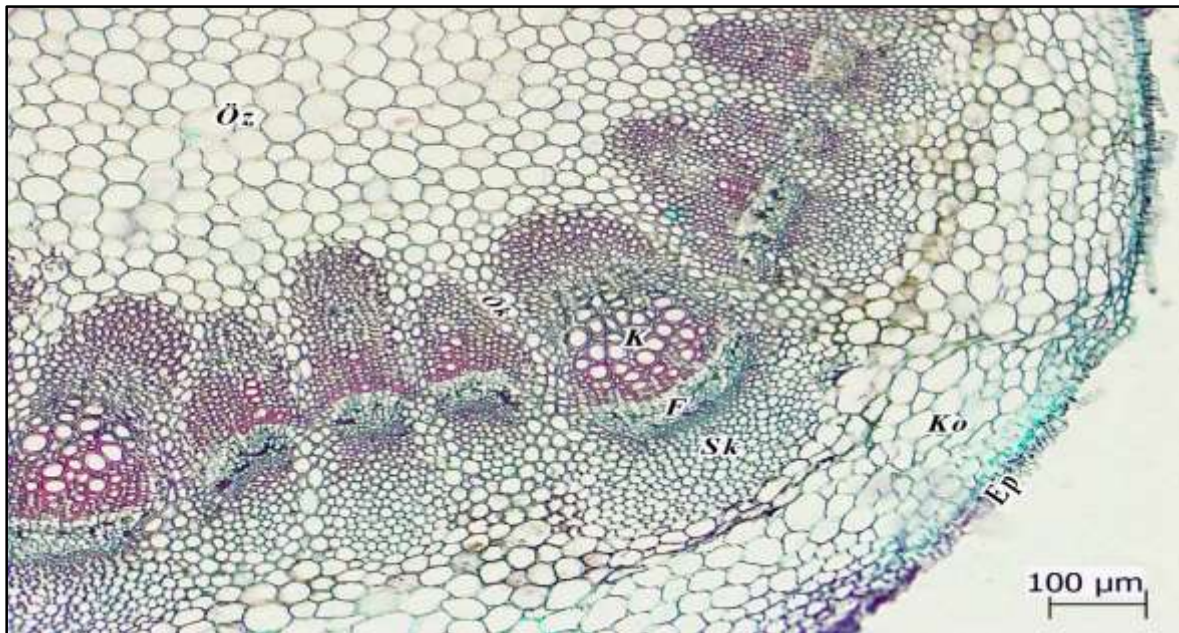


Figure 2. Cross-section of *Cousinia woronowii* stem (ep: epidermis, ko: cortex, en: sk: sclerenchyma, f: phloem, k: xylem, ök: pith rays, öz: pith).

*Leaf:* In the upper and lower parts, there is the epidermis layer consisting of rectangular cells arranged in a single row. Upper epidermis cells are larger than lower epidermis cells and their size varies between  $14.9 \times 41.56 \times 10.19 \times 25.88 \mu\text{m}$ . Cell sizes of the lower epidermis are between  $5.21 \times 26.47 \times 4.69 \times 16.47 \mu\text{m}$ . The mesophyll tissue between the upper epidermis and lower epidermis is parenchymatic and arranged in the form of palisade-sponge. The palisade layer under the upper epidermis consists of two rows, abundant chloroplasts and cylindrical shaped cells, with cell sizes between  $11.45 \times 27.08 \times 9.54 \times 101 \mu\text{m}$ . Sponge parenchyma cells are irregular in shape and range in diameter from  $9.38$  to  $26.56 \mu\text{m}$ . The leaf is of the bifacial type, with a large vascular bundle of collateral type in the middle (Figure 3).

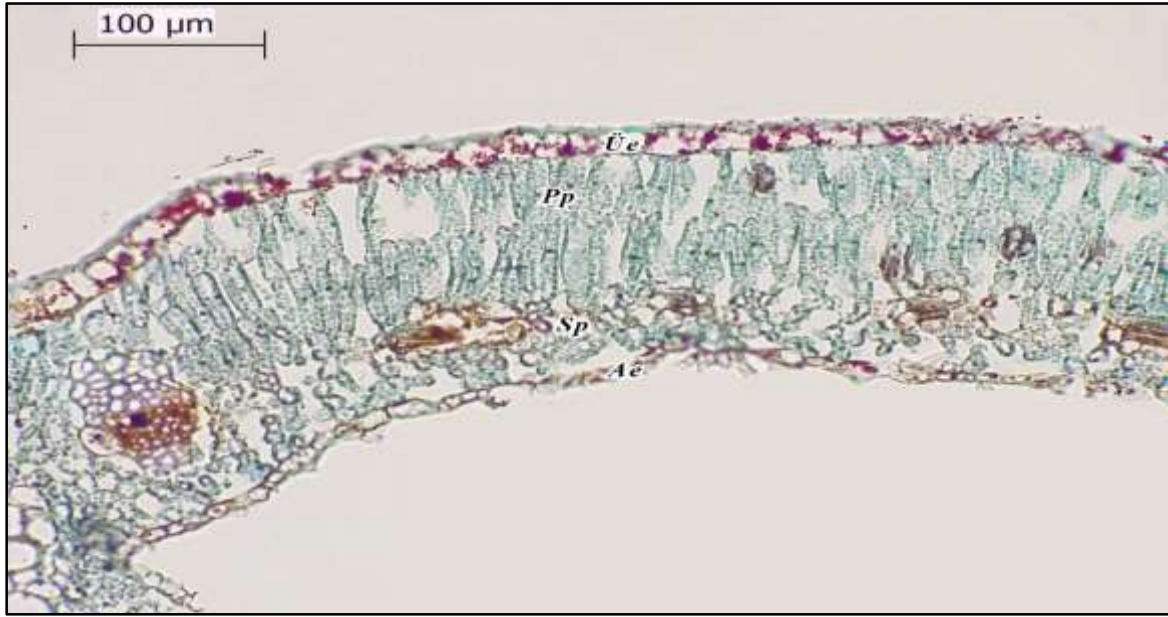


Figure 3. Cross-section of *Cousinia woronowii* leaf (üe: upper epidermis, ae: lower epidermis, pp: palisade parenchyma, sp: spongy parenchyma)

**Midrib:** In the cross-section of the leaf, it is seen that the midrib shape is orbicular. A total of 5 vascular bundles, 1 large and 4 small, were determined in the midrib. The xylem and phloem tissues are surrounded by sclerenchyma tissue. There are parenchyma cells and collenchyma from both the upper and lower parts of the vascular bundles to the epidermis. Parenchymatic cells are composed of pentagonal and hexagonal cells. The collenchyma spacing of the lower epidermis is 98.38-366.10 µm, and the collenchyma spacing of the upper epidermis is 217.60-491.30 µm. The xylem is located on the adaxial (closer to the axis) side and the phloem is located on the abaxial (off-axis) side. The xylem tissue is well developed, the tracheal elements are oval and hexagonal, and their cell diameters vary between 4.84-16.12 µm. Phloem tissue is dense, composed of small cells, and layer sizes are between 19.35-78.26 µm (Figure 4).

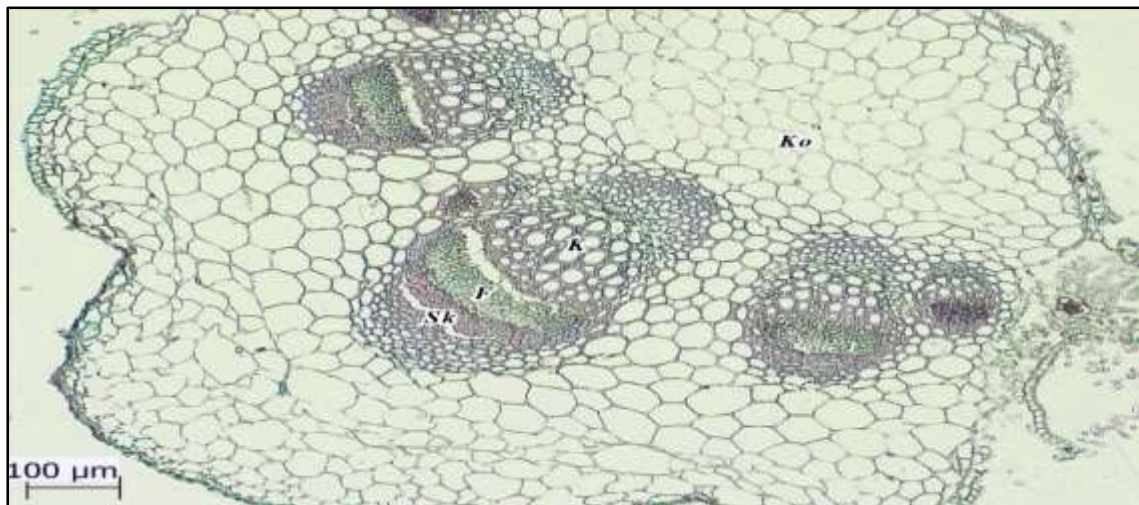


Figure 4 Cross-section of *Cousinia woronowii* midrib (ko: collenchyma, sk: sclerenchyma, f: phloem, k: xylem).

## Pollen Morphology

The pollen grains of *C. woronowii* are radially symmetrical, isopolar, tricolporate. Pollen shape prolate, in large scale, polar axis 45.71-56.23  $\mu\text{m}$ , equatorial axis 32.96-39.21  $\mu\text{m}$ . Amb 31.32-39.13  $\mu\text{m}$ , circular. Ornamentation is verrucate. Colpus length 35.13-45.24  $\mu\text{m}$ , colpus width 4.40-8.58  $\mu\text{m}$ . Pore prolate (Plg 7.54-15.36  $\mu\text{m}$ , Plt 4.48-7.52  $\mu\text{m}$ ). The exine is 4.23  $\mu\text{m}$  and the intin is 2.17  $\mu\text{m}$  (Table,1 Figure 5).

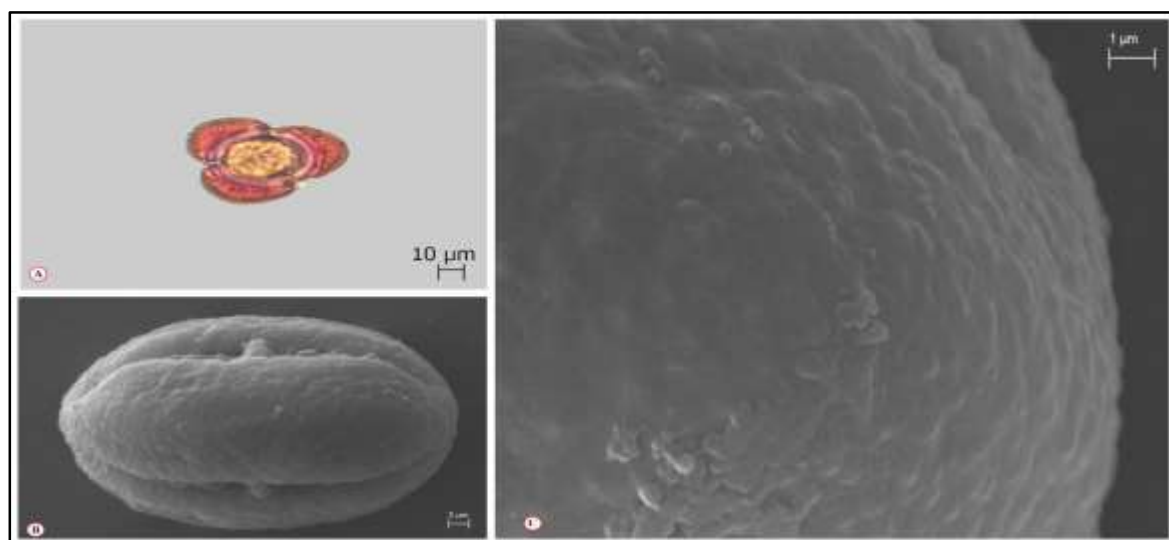


Figure 5 *C. woronowii* **A.** General view of pollen grains under LM. **B.** Equatorial view of pollen in SEM. **C.** Close-up of pollen ornamentation in SEM.

**Table 1.** Pollen morphological features of *Cousinia woronowii* (P: polar axis, E: Equatorial axis, clt: colpus width, clg: colpus length, plg: pore length, plt: pore width, t: apocolpium, L=AMB: diameter of pollen at the polar view).

|  | Min.         | Max.  | Mean  | S.D. |
|--|--------------|-------|-------|------|
| <b>P (<math>\mu\text{m}</math>)</b>      | 45.71        | 56.23 | 51.15 | 2.62 |
| <b>E (<math>\mu\text{m}</math>)</b>      | 32.96        | 39.21 | 35.99 | 1.72 |
| <b>P/E</b>                               | 1.32         | 1.65  | 1.42  | 0.07 |
| <b>clg (<math>\mu\text{m}</math>)</b>    | 35.13        | 45.24 | 40.45 | 2.77 |
| <b>clt (<math>\mu\text{m}</math>)</b>    | 4.40         | 8.58  | 6.34  | 1.15 |
| <b>plg (<math>\mu\text{m}</math>)</b>    | 7.54         | 15.36 | 10.33 | 1.77 |
| <b>plt (<math>\mu\text{m}</math>)</b>    | 4.48         | 7.52  | 5.63  | 0.65 |
| <b>plg/plt</b>                           | 1.68         | 2.21  | 1.83  | 0.11 |
| <b>t (<math>\mu\text{m}</math>)</b>      | 4.98         | 9.28  | 6.79  | 0.90 |
| <b>L=AMB (<math>\mu\text{m}</math>)</b>  | 31.32        | 39.13 | 35.13 | 2.01 |
| <b>Exine (<math>\mu\text{m}</math>)</b>  | 2.59         | 5.65  | 4.23  | 0.87 |
| <b>Intine (<math>\mu\text{m}</math>)</b> | 1.56         | 2.98  | 2.17  | 0.29 |
| <b>Pollen shape</b>                      | Prolate      |       |       |      |
| <b>Aperture</b>                          | Tricolporate |       |       |      |
| <b>Pore shape</b>                        | Prolate      |       |       |      |
| <b>Ornamentation</b>                     | Verrucate    |       |       |      |
| <b>Size</b>                              | Large        |       |       |      |



### Achene Morphology

The achenes of *C. woronowii* are brown, obovate-shaped, prominently striate, with small denticulate at apex. Its dimensions are 3.8-4.3 x 2-2.3 mm. Surface ornamentation retipilate, bristles of pappus barbellate, 2.3- 3.2 mm long (Figure 6).

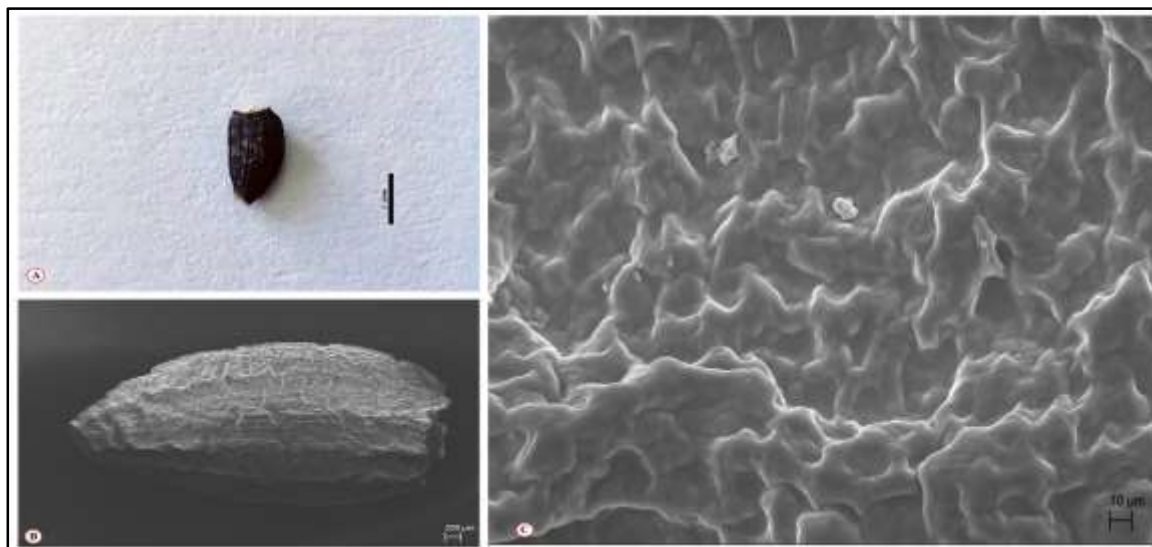


Figure 6. Achene photographs of *C. woronowii* A. Achene general view in LM. B. General view in SEM. C. Testa surface in SEM.

### DISCUSSION

There are a few anatomical studies on the genus *Cousinia*. Attar et al. (2004) performed the anatomical studies of 14 species belonging to the *Serratuloideae* section of the genus *Cousinia* and comparatively the anatomical features of the stem, leaf and midrib were examined. It was stated that the stem anatomy was generally similar between the species, and in the leaf anatomical examinations, they stated that the lamina and midrib differed between the species. It has been determined that the number of palisade parenchyma in leaf anatomy differs between species and it has been reported that the shape of the midrib, lobes and the number of vascular bundles are significant character in the differentiation of species.

Ulukuş & Tugay (2019a) studied the stem, leaf and midrib anatomy of the endemic *C. halysensis* species. They stated that the number of cortex layers, the shape of the midrib and the number of vascular bundles in the midrib are significant anatomical characters. Ulukuş and Tugay (2020) examined the anatomical, palynological and morphological features of *Cousinia eleonora* and *Cousinia humilis* and determined anatomical characters such as the number of cells in the cortex, the number of vascular bundles and the shape of the midrib as significant characters in the differentiation of species. Ulukuş and Tugay (2019b) investigated the micromorphological, anatomical and palynological features of *C. iconica*, and it was stated that the number of cortex layers, the number of vascular bundles and the shape of the midrib are significant structures.

When the anatomical features of *C. woronowii* were compared with the above species, it was determined that the stem structure was similar, the number of palisade parenchyma, the shape of the midrib, the number and arrangement of the vascular bundles.

Saber et al. (2009) examined the pollen morphology of 25 species from the *Stenocephalae* section. It was determined that the pollen grains of the examined species were prolate, isopolar, tricolporate and exine dense or loose verrucate. Ahmad-Khanbeygi et al. (2011) conducted morphological and palynological studies on 16 populations of 8 species in the *Cousinia* section. Pollen grains were determined as spherical or cylindrical, tricolpate, and exine surface ornamentation as verrucate-perforate. Jafari and Ghanbarian (2007) stated that the pollen shape of *C. eriobasis* species has subprolate and verrucose surface ornamentation. Ulukuş and Tugay (2019a) determined the pollen surface ornament of *Cousinia halysensis* as verrucose and the pollen shape as subprolate. Ulukuş and Tugay (2020b) examined the anatomy and palynology of the *C. foliosa* species, and the pollen shape was determined as subprolate and the surface ornamentation as verrucose. In our study, the pollen grains of *C. woronowii* were tricolporate, the pollen shape was prolate, and the ornamentation was verrucate, which showed that it was compatible with the pollen characters of the genus *Cousinia*.

As a result of our SEM studies, it was determined that the achenes of *C. woronowii* were obovate in shape and the surface ornamentation was retipilate. Ulukuş and Tugay (2019a) *C. halysensis* seed surface is retipilate, *C. iconica* achene surface is reticulate-sitriate (2019b), *C. eleonora* and *C. humulis* achene surface is striate-irregular reticulate (Ulukuş & Tugay, 2020a), *C. agridaghensis* and *C. urumiensis* achene surfaces were determined as striate-irregular and reticulate-faveolate (Tugay et al., 2019). Our study results were found to be compatible with the achene characteristics of the genus *Cousinia*.

## CONCLUSIONS

In this study, stem, leaf and midrib anatomy, pollen and achene micromorphological features of *C. woronowii* are given in detail for the first time. The results obtained will shed light on future palynological, micromorphological and anatomical studies on the genus *Cousinia* and will contribute to the systematic studies of the genus of the characters determined to be important as a result of the examinations.

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## **DEVELOPING ORGANIC AGRICULTURE PRODUCTION IN TUYEN QUANG PROVINCE, VIET NAM**

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### **ABSTRACT**

Organic agricultural production is a trend worldwide and in Vietnam to meet the needs of food safety, benefit human health and ensure sustainability for the natural environment. With favorable natural conditions and climate, Tuyen Quang, a mountainous province to the North of Vietnam, with great potential for developing organic agricultural production. Over the years, the locality has made many efforts to convert some crops to organic production, however it is still fragmented and has not achieved the expected results. By using descriptive statistics and consultation with experts, this study evaluated the current status of organic agricultural production and analyzed the advantages as well as difficulties in the process of converting production towards organic in Tuyen Quang province. Some solutions were proposed in order to develop local organic agricultural production in the coming time such as: enhance mechanisms and policies, planning specialized production areas, increasing technical training, raising awareness for people about organic agriculture.

**Keywords:** Organic farming, agricultural production, conversion of organic agricultural production, Tuyen Quang, Vietnam

### **INTRODUCTION**

Organic farming is a sustainable farming production system that uses ecologically based pest controls and biological fertilizers, unused growth promoters, pesticides and farm feed additives (Meemken & Qaim, 2018). Organic farming systems depend on crop rotation, green manure, legumes, animal manure, crop residues and natural waste (Seufert et al., 2017). Adoption of organic farming brings balance and better condition for the environment, prevents degradation and avoids chain reaction in the environment caused by chemical spray and dust; provide safe products and living conditions for people; create jobs for idle workers (Reganold & Wachter, 2016).

Faced with the challenges of environmental pollution and food safety issues for human health, organic agricultural production is a trend not only in Vietnam but also around the world. According to statistics, by 2020, 186/204 countries are producing following organic methods, of

which 100 have legal regulations on organic product management (Nguyen Duy Hoan, 2021). The area of organically cultivated land worldwide has reached 76.6 million hectares, accounting for about 1.7% of the total agricultural land area, with an average growth rate of 7.86%/year (FiBL and IFOAM, 2020).

In Vietnam, by 2020, there have been 33/63 provinces and cities implemented organic agricultural production and converted production towards organic farming. The total area of organic farming land has reach 237 thousand hectares (accounting for about 1.1% of the total agricultural land area), ranking 32nd in the world and second in Southeast Asia after Indonesia (Nguyen Duy Hoan, 2021). Organic agricultural production is considered an appropriate direction to help Vietnam develop the domestic agricultural product market, towards exporting agricultural products with high added value (Cao Dinh Thanh, 2019). Accordingly, the government has issued Decree 109/2018/ND-CP on organic agriculture, which specifies policies to encourage the development of organic agriculture.

Tuyen Quang is a province with many advantages in terms of land and climate for the development of agriculture and forestry, especially the potential for developing organic agricultural production. The local agricultural, forestry and fishery production value in the period 2013-2020 always achieves a growth rate of over 4%/year (Department of Agriculture and Rural Development of Tuyen Quang province, 2021). By 2021, there have been 03 production models according to organic agricultural standards in the province, including: Green Tea production, Oranges production and Grapefruit production. However, these models are still fragmented and facing many difficulties to replicate. Therefore, the survey and assessment of the current status of organic models and the process of making the transition to organic farming will guide the development and propose policies that are close to reality to promote agricultural production. sustainability is essential. On the basis of analyzing the current situation of organic agricultural production development in Tuyen Quang province, the study points out the advantages and disadvantages in the process of converting organic agricultural production, thereby proposing some solutions to develop organic agricultural production in Tuyen Quang province in the coming time.

## **MATERIAL AND METHOD**

Descriptive statistical methods were used in the study to clarify the current status of organic agricultural production in the province. The data used in the article is collected through direct survey questionnaires for 80 farmer households who have participated in converting conventional production to organic farming methods.

## RESULTS AND DISCUSSION

### Current situation of organic agricultural production development in Tuyen Quang Province

#### *Situation of organic agricultural production*

By 2021, in Tuyen Quang Province, there have been 03 crop products being produced following organic agricultural methods including: Oranges (Ham Yen, Yen Son); Green Tea (Son Duong, Na Hang); Grapefruit (Phuc Ninh) (Table 2.1). These products have all been certified organic production converted to the PGS standard (Participatory Guarantee System), which ensures that the products are produced according to the correct process as well as in compliance with the regulations of organic production.

Table 1. Organic farming models in Tuyen Quang Province in 2021

| <b>Organic farming models</b> | <b>Inorganic cultivation<br/>(ha)</b> | <b>Organic conversion<br/>(ha)</b> | <b>Ratio<br/>(%)</b> |
|-------------------------------|---------------------------------------|------------------------------------|----------------------|
| Grapefruit production         | 5,200                                 | 292.2                              | 5.6                  |
| Oranges production            | 8,648                                 | 772.5                              | 8.9                  |
| Green Tea production          | 8,468                                 | 1,324                              | 15.6                 |
| Total                         | 22,316                                | 2,388.7                            | 10.7                 |

*Source: Department of Agriculture and Rural Development, 2021*

For Phuc Ninh pomelos products (in Phuc Ninh Commune, Yen Son District): since 2017, after being trained on organic agriculture, both local authorities and some households are aware of the benefits of organic farming so that they decided to change from conventional agriculture production into organic methods. In 2021, about 292.2 hectares of pomelos in Phuc Ninh Commune, Yen Son District have been converted to organic farming, reaching 5.6% of the total area under conventional methods in the province. In the production season of 2021, the harvested organic pomelo output is 1,887,116 fruits. However, currently, not yet any households can sell pomelos at organic prices, without geographical indications and labels to certify organic pomelos. Therefore, 100% of organic growers are still selling at the normal price of pomelos from 8,000 VND to 10,000 VND/fruit (Table 2.2). This is also the reason why other households are hesitant to participate in organic pomelo.

Oranges products (in Ham Yen and Yen Son): Tuyen Quang province currently has 772.5 hectares of oranges (accounting for 8.9% of the province's total orange area) grown by organic production methods. Organic production of orange products has been implemented since 2018. In the two districts of Ham Yen and Yen Son, some cooperative production groups according to organic standards have been formed. These groups work with the purpose of sharing experiences, cross-checking the growing process, connecting the consumer market... In 2021, the yield of organic oranges has reach 7,543.8 tons, the selling price of oranges Organic

products range from VND 23,000 to VND 25,000/kg, with a value higher than the market price (from VND 8,000 to VND 10,000/kg). Organic orange products are mainly consumed by companies and enterprises trading in clean fruit in Hanoi and some southern provinces. Before each harvest, these companies come to order quantities and pay directly to households. However, the survey results of organic orange growing households showed that the consumption of organic oranges has not been stable because there is no long-term consumption contract. Besides, the production of organic oranges is still limited to meet the consumption needs of companies.

Green tea products (Shan Tuyet green tea in Hong Thai, Na Hang; Organic green tea in Son Duong): This is the organic production model with the highest percentage among others (reaching 15.6%). This model has many advantages because tea trees have been familiar with organic growing methods so far, especially Shan Tuyet tea trees in the high mountainous area in Hong Thai Commune, Na Hang District. Moreover, farming practices of the local are also completely based on nature, without the use of fertilizers and pesticides. In 2021, the output of organic tea reached 1,205 tons with a high selling price compared to conventional tea. Green tea products are divided into 3 levels: Type 1 costs 1,500,000 VND/kg; Type 2: 600,000 VND/kg; Type 3: 250,000 VND/kg. Shan Tuyet tea products have had brands, designs and packaging with full information, are known and accepted by consumers.

Table 2. Production and consumption of organic products in Tuyen Quang in 2021

| <b>Organic products</b> | <b>Unit</b> | <b>Quantity</b> | <b>Price</b>   |
|-------------------------|-------------|-----------------|--|
| Phuc Ninh Grapefruit    | Fruit       | 1.887,116       | 8,000 VND – 10,000 VND/fruit   |
| Ham Yen Orange          | Ton         | 7,543.8         | 23,000 - 25,000 VND/kg   |
| Shan Tuyet Green Tea    | Ton         | 1.205           | Type 1: 1,500,000 VND/kg<br>Type 2: 600,000 VND/kg<br>Type 3: 250,000 VND/kg |

*Source: Department of Agriculture and Rural Development, 2021 and survey data compilation, 2021*

### ***Advantages and disadvantages for the development of organic agricultural production***

#### *Advantages*

Organic farming conversion has become an agricultural production trend in the province. Local authorities have much paid attention on how to develop this type of production. The provincial government has identified the development of organic agricultural production as a priority direction and a foundation for sustainable agricultural production. In order to promote the development of organic production, the province has established the Organic Agriculture Association with the aim of guiding and supporting businesses, cooperatives and farmers to easily access and update information on organic farming, helps to promote, introduce and consume organic products. In addition, the province has also issued many policies to



support the development of commodity agricultural production such as: Resolution No. 10/2014/NQ-HDND on encouraging the development of farm economy; Resolution No. 12/2014/NQ-HDND on supporting the production of some key crops and livestock; especially Resolution No. 06/2020/NQ-HDND stipulating policies to encourage the development of organic agricultural production in Tuyen Quang province, including financial and training support, and transfer technology for households who converted to organic production. These are decisions that create favorable conditions for the development of organic production.

Table 3. Advantages in organic farming production of households

| <b>Content</b>   | <b>Frequency<br/>(n=80)</b> | <b>Ratio<br/>(%)</b> |
|--|-----------------------------|----------------------|
| Higher price   | 70                          | 87,5                 |
| Training organic knowledge and technical support                 | 80                          | 100,0                |
| Loan policies support organic farming production from government | 72                          | 90,0                 |
| Increasing organic products consumption trend                    | 74                          | 92,5                 |

Source: Summary of survey data, 2021

100% organic converted households are trained with production techniques. Households know very well the process and apply techniques to production (Table 4). This training is organized by the Provincial Organic Association in collaboration with the Department of Agriculture and Rural Development and the Fruit Service Centers of the districts. This is a favorable condition for the replication of organic models, because the households themselves will propagate and share knowledge for others.

Table 4. Technical training in organic farming

| <b>Content</b>   | <i>Unit: %</i>               |                                  |                                 |
|--|------------------------------|----------------------------------|---------------------------------|
|  | <b>Orange<br/>Production</b> | <b>Grapefruit<br/>Production</b> | <b>Green Tea<br/>Production</b> |
| Percentage of households receiving training in organic production techniques | 100.0                        | 100.0                            | 100.0                           |
| Percentage of households who understand the training content                 | 88.3                         | 87.5                             | 76.5                            |
| Percentage of households applying technology to production                   | 56.7                         | 70.6                             | 72.7                            |

Source: Summary of survey data, 2021

Through the survey, most of the households that converted to organic production came from their own perception of organic production. Households are aware that organic production will ensure the environment and protect the health of themselves and those around them.

Therefore, it is very desirable to participate in organic production. The households all wish to continue receiving training to improve production techniques.

The policies of borrowing capital and supporting production for producing in the province is very favorable. Most of the surveyed households said that they did not face any difficulties in the usual loan procedures at banks.

The demand for organic agricultural products consumption is increasing day by day and is popular in the market. Besides, organic products are also sold at higher prices than conventional agricultural products. This is the basis to encourage households to participate in organic farming.

### *Difficulties*

Besides the advantages, organic agricultural production in Tuyen Quang is also facing many difficulties and challenges in the process of conversions and development.

Currently, the rate of organic cultivation area has been still limited. Through the survey, the most reason has caused households have not yet participated in organic farming is that the households lack of knowledge about the organic production. Households, who have received technical training, believe that production processes and techniques are complicated and have many stages. It takes more labors than conventional production, so they cannot follow them due to insufficient resources.

Table 5. Difficulties in organic farming production of households

| <b>Content</b>                                   | <b>Frequency (n=80)</b> | <b>Ratio (%)</b> |
|--|-------------------------|------------------|
| Difficulty in pest control                       | 77                      | 96,3             |
| Fragmentation in organic farming conversion area | 75                      | 93,4             |
| Lower yields                                     | 80                      | 100,0            |
| Lack of knowledge about organic product          | 74                      | 92,5             |

*Source: Summary of survey data, 2021*

Difficulty in sourcing inoculants for organic production. Currently, all kinds of organic fertilizers, biological products used in organic in the district is still limited. There are not many facilities providing materials. Preparations for making organic fertilizers and biological products for organic production are not available in the area. Currently, the supplier in the area is almost exclusively, so households do not have many options. Moreover, its quality is not really guaranteed and not easy to buy.

Yields of organic farming is lower than conventional products (decreased by 40-50% of output). On the other hand, organic products do not have a beautiful design like regular products, and the yield is not high due to many pests and diseases. Households said that when converting production from inorganic to organic, due to environmental changes, plants have not been able to

absorb nutrients from fertilizers. As a result, some households transferred trees that were stunted, yield reduced and susceptible to pests and diseases.

Most of the farming households produce on a small scale and fragmented lands. The scale of organic production is still small. The organic production area is interspersed with the conventional production area, so it is inevitable that the spread of pests and diseases from the normal production area to the organic production area is inevitable. Therefore, organizing production to meet the strict regulations of organic agriculture standards is a big challenge.

Awareness about organic production of many households has been still limited. Up to 80% of the interviewed households are hesitant to continue organic production or not. Because of the high cost of organic production, it is labor-intensive. Households who prefer to finish work do not have enough resources in terms of production capital, limited supply of fertilizers and biological products, difficult techniques to apply while the selling price of organic products is currently not high.

### **Proposing effective solutions for developing organic agricultural production in Tuyen Quang Province**

First, the province needs to clearly identify potential and advantageous products to promote the conversion of organic production. On that basis, localities need to have strategies for planning and protecting land in organic production areas, ensuring area and concentrated production. At the same time, there are mechanisms and policies to encourage households to convert to organic production such as: support for production capital; preferential land lease; continue to support training on methodical knowledge on organic production techniques and post-harvest preservation.

The localities need to do a good job of managing the quality and safety of agricultural products; improve the state management capacity of organic agricultural production such as: monitoring and certifying organic agricultural products, managing the supply of materials and preparations for organic production; promote cooperation in scientific research and technology transfer; continue to implement researches and projects that evaluate organic production models to recommend replication.

Continuing to promote the dissemination of knowledge about organic agricultural products to people is necessary, and at the same time local authorities should continue to mobilize households to expand production and change into organic farming. In addition, local authorities also need to effectively support product trademark registration; stamps of origin; promote trade promotion, brand promotion and improve the competitiveness of agricultural products produced by organic methods to consumers; connect consumers with businesses to get long-term consumption contracts.

Government should encourage and create favorable mechanisms to attract domestic and foreign enterprises to invest in the production and trading of organic agricultural products such as: suppliers of pesticides and organic fertilizers; animal feed of organic origin; attractive

businesses that trade in and consume organic products. These will be the leading institutions for the application and replication of the standard organic production models in the area.

## CONCLUSIONS

Organic agricultural production plays an important role in foundation and stepping stone for Tuyen Quang's agriculture to develop and move forward with timely and appropriate orientation. The organic products in the province are gradually being accepted by consumers. Although 3 organic models have faced many difficulties and challenges such as replication, compliance with production processes and consumption of organic products, the replication of the organic production model in the near future in Tuyen Quang still has many potentials and opportunities for development. It is necessary to carry out a number of solutions synchronously: i) Planning for concentrated organic production areas; ii) Having appropriate mechanisms and policies to support the development of organic production; iii) Enhancing state management capacity for officials in organic production; iv) Raising the knowledge of producers and consumers about the production of organic agricultural products; v) Encouraging and attracting businesses to invest in organic agricultural production. The development orientation of organic agricultural production is creating opportunities for the province's agricultural products to reach beyond domestic and international markets.

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## EFFECTS OF DIFFERENT DRIP IRRIGATION REGIMES ON POTATO TUBER MINERAL CONTENTS

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### ABSTRACT

Potato is normally grown in soil under conditions using sprinkler and furrow irrigation methods during spring and fall seasons in order to respond to the high demand of potato on markets. Potato quality under irrigation applications is important to enhance water management in arid regions. A field experiment was conducted in 2021 spring growing seasons using potato (*Solanum tuberosum*) grown in Nigde region to evaluate potato response to different drip irrigation regimes. A Randomized Split-Plot Design with irrigation regimes randomly distributed. Each treatment was repeated four times. The total irrigation water applied 530.7, 632.6, 728.6 respectively S33, S66, S100. Potato tuber mineral contents were significantly affected by different irrigation application. The average content of N ranged from 1.729 to 1.971 % and the average content of P<sub>2</sub>O<sub>5</sub> ranged from 0.590 to 0.683 %. The content of K<sub>2</sub>O was not significant. Irrigation treatment implicated in SO<sub>3</sub> and MgO concentrations was significant. The average content of SO<sub>3</sub> ranged from 0.408 to 0.457 % and the average content of MgO ranged from 0.500 to 0.531 %. CaO and Fe<sub>2</sub>O<sub>3</sub> in the tuber were also affected by the change in irrigation amount. The average content of CaO ranged from 504.790 to 596.670 ppm and the average content of Fe<sub>2</sub>O<sub>3</sub> ranged from 43.635 to 60.592 %. However, Zn, Mn and Cu concentrations did not show any significant changes under different irrigation application conditions.

**Keywords:** Potato, tuber quality, mineral contents, drip irrigation

### INTRODUCTION

Potato is impressive importance in global food production, along with wheat, rice, and maize. Potato can be effective in providing organic compounds and minerals such as phosphorus (P) and potassium (K) (Camire et al., 2009). Potato is susceptible to drought stress due to shallow roots and low root length per unit area and consequent poor water conductivity. Potato plants can develop drought stress responses even under irrigated conditions, as drought causes morphological and physiological changes that cause decline in growth and yield and affect mineral accumulation in tubers (Ierna and Mauromicale, 2012).

For adequate irrigation in potato, knowledge of both soil-water interaction and the characteristics of irrigation systems is required. Potato can be grown with different irrigation methods. However, some irrigation methods provide higher quality tubers than others. Since potato is a water sensitive plant, systems that can make frequent and homogeneous irrigation should be chosen (King et al., 2020; Ayas, 2021). Excessive irrigation promotes potato diseases because potato crop has shallow roots and sensitive to water stress. Water deficits reduce tuber yields and quality. Potato tuber is highly sensitive to irrigation management deficiencies. Tuber responses to deficit irrigation make potato a challenging crop to irrigate.

As agriculture is considered the main user of water, its effective use is necessary to conserve such limited resources. In order to maintain agricultural production, it is necessary to use different rational agricultural waters, especially in regions where current irrigation practices and systems are not efficient. Therefore, irrigation management is seen as an attractive opportunity to reduce water scarcity in semi-arid regions. Since water is expensive and its supply is limited in semi-arid regions, it is important to provide better irrigation management that can increase its effective and efficient use to save water (Ierna and Mauromicale, 2012). Drip irrigation provides many agricultural and water-saving benefits for irrigated agriculture, such as increased plant feeding efficiency. Especially under drip irrigation, there is a positive relationship between total applied water and tuber yield (King et al., 2020).

Water supply and its management are considered most major limiting factors that affect the yield and quality of potato. The objective of the study the effects of three regimes of the drip irrigation (S100 = 100% FC; S66 = 66% FC; and S33 = 33% FC) on tuber mineral contents.

## MATERIAL AND METHOD

Potato cultivar Agria (*Solanum tuberosum*) was grown in 2021 spring growing seasons at Nigde Omer Halisdemir University, Faculty of Agricultural Sciences and Technologies Research and Application Area to investigate the effects of three water regimes of drip irrigation on tuber mineral composition during spring season (2021/2022). Urea was used as the nitrogen source. Potato was cultivated on the twelve of May using potato planter and harvested on fourth of October. Each plot was applied with a basal dose of phosphorus (P) and potash (K) fertilizers at the rate of 110 kg P + 110 kg K ha<sup>-1</sup> at the planting. Nitrogen was applied half at planting and half at tuber formation period. Each of the trials had standard practices of weed, pest and disease control. The province of Nigde has continental climate with hot and dry summers and cold and snowy winters. The daily climatic data were obtained from Nigde Meteorology Services General Directorate. The mean values of the climatic date are given in Table 1.

A randomized split plot design with three drip irrigation applications systematically measured along irrigation line. The drip irrigation systems were installed after the potato seed were planted. Drip tape with emitters spaced at 30 cm and emitter flow rate of 4 l/h at the operating pressure of 0.1MPa was placed on the soil surface in the center of the beds. soil water content determination is gravimetric method with oven drying. The method involves weighing a moist sample, oven drying at 105°C for 24h, reweighing, and calculating the mass of water lost as a percentage of the mass of the dried soil. The equation given below was used to determine soil moisture.

$$\text{Soil water (\%)} = \frac{\text{weight of wet soil (g)} - \text{weight of dry soil (g)}}{\text{weight of dry soil (g)}} * 100$$

Table 1. Monthly rainfall, temperature and relative humidity during the growing period of 2021

| Year | Month     | Average temperature (°C) | Precipitation (mm) | Relative humidity (%) |
|------|-----------|--------------------------|--------------------|-----------------------|
| 2021 | May       | 18.3                     | 1.6                | 41.0                  |
|      | June      | 19.5                     | 51.4               | 50.2                  |
|      | July      | 24.0                     | 0.8                | 40.3                  |
|      | August    | 22,8                     | 9.6                | 43.8                  |
|      | September | 18.6                     | 18.4               | 51.5                  |
|      | October   | 12.3                     | 0.4                | 49.0                  |

In order to determine the plant nutrient contents, the samples taken during the harvest were milled and analyzed. The samples were washed first with tap water, then with 0.1 N HCl, and finally with distilled water twice, and the excess water was dried with tissue paper and dried in an oven at 700 C for two days. The total P, K, Ca, Mg, S, Fe, Zn, Mn, and Cu determinations were made in the ICP-OES device by grinding the dried samples and burning them in a microwave system with 15 ml HNO<sub>3</sub>- 5 ml HCl=4. The total N contents of the samples were determined by the Kjeldahl method for wet-burned samples with H<sub>2</sub>SO<sub>4</sub> + H<sub>2</sub>O<sub>2</sub>.

Analysis of variance (ANOVA) was used for each measured variable, to assess the overall significance (F tests) of effects irrigation regimes. Means of interest were then compared using the least significant difference (LSD) at 5% based on the residual degrees of freedom (df) from the ANOVA.

## RESULTS AND DISCUSSION

The total effective rainfall during the growing seasons was 70 mm. The total amount of water applied to the plots varied according to the irrigation application. The total irrigation water applied 530.7, 632.6, 728.6 respectively S33, S66, S100. Nitrogen has been recognized as essential for different physiological properties and processes in plant cells (Naumann et al., 2019). The highest contents of nitrogen (1.971 %) in tubers were obtained when potato plants were grown with S66 (66 % Field Capacity), followed by S100 (100 % Field Capacity), and S33 (33 % Field Capacity) (Table 3). Irrigation regime showed that S66 ≥ S100 > S33, indicating that the lowest and the medium irrigation regime resulted in the highest nitrogen. Likewise, Eid et al. (2020), less or moderate irrigation (G2 = 100% ETc irrigation; G3 = 80% ETc irrigation) have determined that the amount of nitrogen in the tuber increases. The effect on phosphorus content was significantly varied with different irrigation regimes (Table 2). The highest phosphorus content (0.683 ppm) was obtained from plants treated with S100, while the lowest phosphorus content (0.590 ppm) was obtained from plants treated with S66. However, no difference was observed in terms of phosphorus content between S33 and S100 applications (Table 3). The amount of potassium in the tuber was not affected by irrigation applications. There was no change in potassium between the three treatments (Table 2,3). Mohamed et al. (2018), reported that as the amount of irrigation increased (75% and 100% of Et0), N, P and K levels increased. Similarly, Taha and Roshdy (2021), found that as the amount of irrigation water decreased, the nutrient content of potato tuber decreased significantly, linearly.

Table 2. Summary of analysis of variance for all studied variables

| Source of Variation | df | N      | P <sub>2</sub> O <sub>5</sub> | K <sub>2</sub> O | SO <sub>3</sub> | MgO   |
|---------------------|----|--------|-------------------------------|------------------|-----------------|-------|
| Irrigation (I)      | 2  | 9.76** | 4.19*                         | 1.64             | 6.26**          | 3.79* |
| Replication         | 2  | 4.89*  | 0.14                          | 13.78**          | 3.25            | 0.25  |
| CV (%)              | -  | 9.54   | 16.07                         | 4.98             | 9.74            | 7.03  |

| Source of Variation | df | CaO     | Fe <sub>2</sub> O <sub>3</sub> | Zn      | Mn      | Cu    |
|---------------------|----|---------|--------------------------------|---------|---------|-------|
| Irrigation (I)      | 2  | 3.40*   | 3.86*                          | 0.07    | 0.66    | 0.42  |
| Replication         | 2  | 50.30** | 122.77**                       | 12.82** | 20.36** | 1.19  |
| CV (%)              | -  | 21.48   | 35.65                          | 17.12   | 45.47   | 17.88 |

\* means p<0.05, \*\* means p<0.01



According to Anova, irrigation treatment implicated in  $\text{SO}_3$  and MgO concentrations was significant (Table 2). Different drip irrigation regimes had the following pattern  $\text{S66} \geq \text{S100} > \text{S33}$ , indicating that S66 resulted in higher tuber  $\text{SO}_3$  and MgO content than S100 and S33 (Table 3). In addition, CaO and  $\text{Fe}_2\text{O}_3$  in the tuber were also affected by the change in irrigation amount. While there was no difference between S66 and S100 applications for calcium, S33 application was lower. The highest value was found in S66 application, and the lowest value was found in S33 application for  $\text{Fe}_2\text{O}_3$  (Table 3). However, Zn Mn and Cu concentrations did not show any significant changes under different irrigation application conditions.

Table 3. Means Table of Irrigation Regimes on Tuber Nitrogen Content and Mineral Contents

| Exp. Factors              | N       | $\text{P}_2\text{O}_5$ | $\text{K}_2\text{O}$ | $\text{SO}_3$ | MgO     |
|---------------------------|---------|------------------------|----------------------|---------------|---------|
| <b>Irrigation Regimes</b> | %       |                        |                      |               |         |
| S33                       | 1.729 b | 0.668 a                | 2.937 a              | 0.408 b       | 0.500 b |
| S66                       | 1.971 a | 0.590 b                | 3.027 a              | 0.457 a       | 0.531 a |
| S100                      | 1.941 a | 0.683 a                | 2.989 a              | 0.441 a       | 0.505 b |
| LSD (5 %)                 | 0.121   | 0.070                  | 0.100                | 0.028         | 0.024   |

-Different letters indicate different grouping

| Exp. Factors              | CaO       | $\text{Fe}_2\text{O}_3$ | Zn       | Mn       | Cu       |
|---------------------------|-----------|-------------------------|----------|----------|----------|
| <b>Irrigation Regimes</b> | Ppm       |                         |          |          |          |
| S33                       | 504.790 b | 43.635 b                | 15.160 a | 11.562 a | 16.824 a |
| S66                       | 595.840 a | 60.592 a                | 15.124 a | 13.380 a | 17.690 a |
| S100                      | 596.670 a | 50.595 ab               | 15.428 a | 13.602 a | 16.956 a |
| LSD (5 %)                 | 82.340    | 12.464                  | 1.767    | 3.958    | 2.078    |

-Different letters indicate different grouping

Llin et al. (2002), in research determined the average content of K ranged from 302.07 to 306.12 mg.100 g<sup>-1</sup> and the average content of P ranged from 58.22-60.16 mg.100 g<sup>-1</sup>, respectively, in irrigated plots. Also, Ca ranged from 2.84-4.32 mg.100 g<sup>-1</sup> in irrigated plots and the content of Mg ranged from 21.41 to 24.93 mg.100 g<sup>-1</sup>, respectively. Differences in mineral element contents with values found in literature may depend on genotypes and environmental factors by Andre' et al. (2007). Abou El-Khair et al. (2011), was found that the nitrogen and potassium content of the tuber increased with the increase in the amount of irrigation, but the amount of phosphorus did not change. Also, Palta (2010), explained with continuous irrigation, water-soluble Ca is leached from the top. Therefore, the soil surrounding the tubers will contain very low soluble calcium, especially later in the season when tubers are developing.

## CONCLUSIONS

Our research has demonstrated that it is possible to save irrigation water and good nutritional composition of tubers. Also, the concentrations of mineral elements in potato tubers are influenced by both environmental and genetic factors. One of the most significant environmental factors is the phytoavailability of mineral elements in the soil. Differences fertilisation practices on the mineral levels were also analysed to evaluate the influence of external factors on the quality.

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## EFFECT OF ARTICHOKE BRACT POWDER ON BREAD QUALITY

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### ABSTRACT

Evaluation of nutritional and functional valuable by-products in the food industry is important in terms of both food enrichment and economic gains. The stem and bract parts make up about approximately 60 % of the total weight of the artichoke plant (*Cynara scolymus* L.). Discarded parts of the artichoke are rich in some nutritional compounds (dietary fiber, antioxidant contents, minerals, etc.) but do not have a usage area in the food industry. In this study, artichoke bracts were dried and ground as powder and artichoke bract powder (ABP) was added to the wheat flour at various rates (0, 2.5, 5, 7.5 and 10%) and used in bread making. Physical, textural, nutritional and quality parameters of ABP added bread samples were investigated. When the color values of the bread crumbs were measured, it was determined that redness and yellowness values increased contrary to lightness value as the ABP addition rate increased significantly ( $p<0.05$ ). It was observed that the addition of ABP significantly decreased the lightness, redness and yellowness value of bread crust compared to control sample. The highest volume and the specific volume were found in control bread and it was determined that as the amount of ABP increased, the volume and the specific volume of breads decreased significantly ( $p<0.05$ ). The highest hardness value was found in 2.5 % ABP added bread sample. It was determined that ABP addition reduced the hardness value of breads significantly ( $p<0.05$ ). When the highest cohesiveness and resilience values were found in control sample, there was no significant difference between ABP added breads ( $p>0.01$ ). Texture analysis showed that the addition of ABP reduced the chewiness values of the breads. It was observed that as the ABP addition ratio in the bread recipes increased, the protein content of the breads increased due to the high protein content of ABP. As the ratio of ABP, which is known to be a good source of antioxidants, in the bread recipe increased, the antioxidant capacity of the breads also increased. At the end of the study, it was determined that the addition of ABP affected and improved various physical, textural and nutritional properties of breads. The addition of ABP up to 2.5 % was found acceptable in terms of the physical and textural properties of the bread. **Keywords:** Artichoke bract powder, Food by-products, Fortification, Bread.

### INTRODUCTION

Artichoke (*Cynara scolymus* L.), which is a native to the Mediterranean Basin, is a plant from the *Asteraceae* family (Ceccarelli et al., 2010). The edible parts of the artichoke are the inner bracts and the capitulum. Artichokes, which can be also sold with fresh stems and bracts, are generally available in the markets as peeled and canned. The artichoke canning industry generates large amounts of by-products, consisting mainly of the inner and outer bracts (Pandino et al., 2011). The amount of these by-products can reach approximately 60 % total weight of artichoke.

The bracts of artichoke are modified leaves. They have the same function as leaves: protect the flower from dust, bugs, hungry animals etc. As a by-product, bracts are considered as food waste in canning industry. But evaluated in chemically, bracts are a natural source of phenolic acids (Claus et al., 2015); some flavonoid derivatives (Moglia et al., 2008); anthocyanins, terpenoids (Dabbou et al. 2016) and large amounts of dietary fibers such as inulin (22–47 g/100 g) and pectin (19–26 g/100 g) (Domingo et al., 2019). Polyphenols and flavonoids are related to a decrease in risk of chronic diseases, such as diabetes, cancer, cardiovascular disease (Holst and Williamson, 2008). Inulin is digested in the alimentary canal and converted by colon bacteria into short-chain fatty acids, enhancing the gastrointestinal and immune systems (Lopez-Molina et al., 2005; Morris and Morris, 2012). In addition to health benefits, inulin has been used as a fat substitute in meat (Alaei et al., 2018; Leroy et al., 2010) and low-fat dairy products (Faustino et al., 2019). Also, pectin is added as a gelling agent in the food industry (Sabater et al., 2018).

For economic and environmental concerns, adding value to agro-industrial by-products is very interesting. In the preliminary study, artichoke bracts were added to wheat flour at various rates (0, 2.5, 5, 7.5 and 10%) and the rheology and physical properties of doughs were investigated (Çevik and Ertaş, 2021). In this study, ABP added flour mixtures were used to bread making, aimed to investigate the effect of artichoke bracts on the quality and nutritional properties of bread.

## **MATERIAL AND METHOD**

### **2.1. Materials**

The analyses were carried out on artichoke (*Cynara scolymus* L.) bracts which were obtained from the plant peeled for canning. All fresh plants were grown in open-field in Urla, Izmir, Turkey by local farmers. Collected bracts were dried at 50°C for 2 days by oven (Nüve FN-500, Ankara, Turkey), and then ground in a laboratory grinder through 350 µ sieve. Wheat flour was obtained from a local mill in Konya, Turkey. It contained 14.0 % of moisture (AACC 44-12), 27.1 % of wet gluten (AACC 38-12) and 96 % gluten index indicating that the flour was characterized by high gluten quality (AACC, 1990). In the preparation of the flour mixtures, five levels of additional doses were applied. ABP was substituted for wheat flour at 0 - 2.5 - 5 - 7.5 – 10 % (w/w) amounts in bread formulation.

### **2.2. Methods**

#### **2.2.1. Bread making method**

The bread ingredients used were 3.0% yeast, 2.0 % salt, 0 – 2.5 – 5 – 7.5 – 10 % ABP (all based on percentage of flour weight). All the ingredients were mixed using a Hobart mixer (Hobart N50; Hobart, Canada Inc., North York, Ontario, Canada). A straight dough process (AACC 10-10) was used with some modification (bulk fermentation: 30 + 30 min; proofing: 60 min) (AACC, 1990). After that the dough was baked at 235 °C for 15 min in a commercial oven (Fimak, Konya, Turkey). Bread samples were packaged in polyethylene bags and stored at room conditions (22 ±2°C, 45%±10 RH) during storage.

#### **2.2.2. Color properties**

The color of bread samples was evaluated by measuring the L (100 = white; 0 = black), a (+, red; -, green) and b (+, yellow; -, blue) values using a Hunter Lab Color QUEST II Minolta

CR-300 (Minolta Camera, Co., Ltd., Osaka, Japan) with illuminate D63 as reference. Values are the mean of five determinations (Francis, 1998).

### **2.2.3. Volume and specific volume of bread samples**

Bread volumes were obtained by weighing loaves and measuring their volume by pearl rape seeds displacement. Specific volume was calculated by dividing the bread volume by the bread weight.

### **2.2.4. Texture analyses**

Hardness, springiness, cohesiveness, chewiness and resilience of bread samples were measured by a texture analyzer instrument (TA-XT plus, Stable Microsystems, UK) at room temperature, and was used an aluminum P36/R cylinder as the probe. The optimal test conditions in this study were: strain was 25%, and the pre-test, test and post-test speeds were 1.0, 1.0 and 10.0 mm/s, respectively.

### **2.2.5. Protein content**

The protein content of the samples was determined following the AACC method 46-12.01 (AACC, 1999).

### **2.2.6. DPPH Assay**

Antioxidant activity was measured using the DPPH (2,2-diphenyl-2-picrylhydrazyl) method (Beta et al., 2005; Gyamfi et al., 1999). The antioxidant activity of the samples was evaluated based on the radical scavenging effect of the stable 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH) and calculated as percent discoloration. The antioxidant activity value as inhibition percentage was calculated according to Equation 1.

$$\text{Inhibition, \%} = [(\text{Abs control} - \text{Abs sample}) / \text{Abs control}] \times 100$$

### **2.2.7. Statistical analysis**

JMP (version 5.0, Izmir) software was used to perform the statistical analyses. Differences in samples due to addition of ABP was tested for statistical significance at  $p = 0.05$  level. Multiple range tests were used to differentiate between the mean values. Standard deviations were calculated using the same software.

## **RESULTS AND DISCUSSION**

The color of the bread is one of the key criteria for customers. For generations, white bread was the preferred bread of the rich while the poor ate dark (whole-grain) bread. However, in most western societies, the connotations reversed in the late 20th century, with whole-grain bread becoming preferred as having superior nutritional value (Hupkens et al., 1997). Color properties of samples were shown in Table 1. Both bread crumb and crust were analyzed for color. When the color results were examined, despite the low addition rates, it was observed that the ABP significantly ( $p < 0.05$ ) affected the color values of the bread samples. As seen in Table 1, the decrease in ABP addition ratio to bread recipe caused an increase in all color values of bread crust. In bread crumb, the highest lightness value was found in control sample and it was observed that the addition of ABP decreased the lightness value of breads significantly ( $p < 0.05$ ) contrary to redness and yellowness value. The highest redness and yellowness values of bread crumb were found in 10 % ABP added sample while the lowest values were found in control. When the color values of flour mixtures were examined, it was seen that as the addition amount of ABP increased, the value of lightness decreased and yellowness values increased (Çevik and Ertaş, 2021). It was determined that the bread crumb color values gave parallel

results with the color values of the flour mixtures. It is thought that the differences between the color values of the bread crust and the color values of the flour mixtures were due to the Maillard and caramelization reactions due to baking.

**Table 1.** Color properties of bread samples<sup>1</sup>

| ABP level (%) | Crumb color        |                    |                    | Crust color         |                     |                    |
|---------------|--------------------|--------------------|--------------------|---------------------|---------------------|--------------------|
|               | L*                 | a*                 | b*                 | L*                  | a*                  | b*                 |
| 0             | 78.92 <sup>a</sup> | -0.30 <sup>e</sup> | 14.12 <sup>c</sup> | 56.73 <sup>a</sup>  | 13.17 <sup>a</sup>  | 36.15 <sup>a</sup> |
| 2.5           | 64.51 <sup>b</sup> | 0.79 <sup>d</sup>  | 15.72 <sup>b</sup> | 54.93 <sup>a</sup>  | 10.83 <sup>b</sup>  | 25.83 <sup>b</sup> |
| 5             | 57.46 <sup>c</sup> | 1.46 <sup>c</sup>  | 17.10 <sup>a</sup> | 49.54 <sup>b</sup>  | 10.87 <sup>b</sup>  | 22.69 <sup>c</sup> |
| 7.5           | 52.18 <sup>d</sup> | 1.95 <sup>b</sup>  | 17.36 <sup>a</sup> | 46.33 <sup>bc</sup> | 10.14 <sup>bc</sup> | 20.04 <sup>d</sup> |
| 10            | 47.72 <sup>e</sup> | 2.47 <sup>a</sup>  | 17.68 <sup>a</sup> | 43.79 <sup>c</sup>  | 9.93 <sup>c</sup>   | 17.97 <sup>e</sup> |

<sup>1</sup>Values followed by different superscript letters (series “a-e”) within each column are significantly different at  $p < 0.05$ .

Gluten is a long, molecular structure or a mesh formed by two seed storage proteins, gliadin, and glutenin that are connected by disulfide bridges, created by mechanical stress in an aqueous environment. A three-dimensional gluten mesh structure is formed, when gliadin and glutenin combine with water. In the gluten mesh, glutenin provides strength and structure, while gliadin gives the dough plasticity and elasticity. Due to the formation of these intermolecular bonds, the gluten mesh or matrix retains and traps starch granules and carbon dioxide that is produced during mixing and the yeast fermentation process, and this causes the dough to rise. The retention of gas gives the final product a doughy, airy, and highly desired texture. These are the properties that make gluten products so appealing in the food industry (Hoseney, 1991; Pendergrass, 2022). For all these reasons, the use of vital gluten is very common in the bakery industry to increase the gas holding capacity of the dough. So, there is a linear relationship between the gluten content of the dough and its gas holding capacity.

In our study, the volume of the bread samples was measured and the specific volume results were calculated. The results were shown in Table 2. The highest volume (770 ml) and specific volume (5.28) values were found in control bread and it was determined that as the amount of ABP increased, the volume values of breads decreased significantly ( $p < 0.05$ ). Since ABP does not contain any gluten, the gluten content of the flour mixtures decreased proportionally as the ABP ratio increased. Accordingly, the decrease in bread volumes and specific volumes with the increasing ABP ratio is an expected result.

**Table 2.** Volume and specific volume values of bread samples<sup>1</sup>

| ABP Level (%) | Volume (ml)       | Specific Volume    |
|---------------|-------------------|--------------------|
| 0             | 770 <sup>a</sup>  | 5.28 <sup>a</sup>  |
| 2.5           | 480 <sup>b</sup>  | 3.15 <sup>b</sup>  |
| 5             | 455 <sup>bc</sup> | 2.83 <sup>bc</sup> |
| 7.5           | 395 <sup>cd</sup> | 2.36 <sup>cd</sup> |
| 10            | 355 <sup>d</sup>  | 2.10 <sup>d</sup>  |

<sup>1</sup>Values followed by different superscript letters (series “a-e”) within each column are significantly different at  $p < 0.05$ .

Throughout history, the bread-making method has changed under the influence of different cultures and environments. So, there is no such thing as a ‘standard’ bread production method. Interest in bread texture arises because of its direct link with shelf life, eating qualities, flavor and consumer demands. Different baking methods, shapes and sizes affect the bread texture and pore structure. Gases trapped by gluten are critical in creating an expanded structure of dough and homogeneously dispersed pore structure in bread (Cauvain, 2004). Therefore, there is a correlation between the gluten content and quality of flour mixture and the textural properties of bread. A strong gluten mesh structure provides that the gas bubbles are large and dispersed homogeneously. Different substances such as dietary fiber in the gluten network cause the gluten structure to weaken and thus the gas holding capacity to decrease. The addition of ABP causes both a proportional decrease in the amount of gluten and a weakening of the gluten network due to its high dietary fiber content. When the textural properties of breads were examined (Table 3) the highest hardness value was found in 2.5 % ABP added bread sample (4320.19 g) and the lowest in control sample (350.69 g). While the ABP addition ratio reduced the hardness value of breads increased significantly ( $p < 0.05$ ). As in hardness value results, the highest chewiness value (1682.22) was found in 2.5 % ABP added bread and the lowest (244.89) in control sample and the increase in the ABP addition ratio to recipe decreased the chewiness value of samples significantly ( $p < 0.05$ ). The hardness and chewiness values of breads are highly affected by the moisture content of the bread crumb (Cauvain and Young, 2000). Bread moisture content varies depending on different parameters such as baking methods, baking time, dietary fiber content of the flour mixture. In this study, the bread baking method and the baking time were the same for all samples. When the studies in the literature were examined, it was seen that the dietary fiber content of the artichoke bract varies between 50 and 67 % (Larrauri, 1999; Ruiz-Cano et al., 2014); while dietary fiber content of refined wheat flour varies between 2-3 % (USDA, 2019). Therefore, it is known that the dietary fiber content of the control sample and ABP added samples were quite different from each other.

**Table 3.** Textural properties of ABP added bread samples<sup>1</sup>

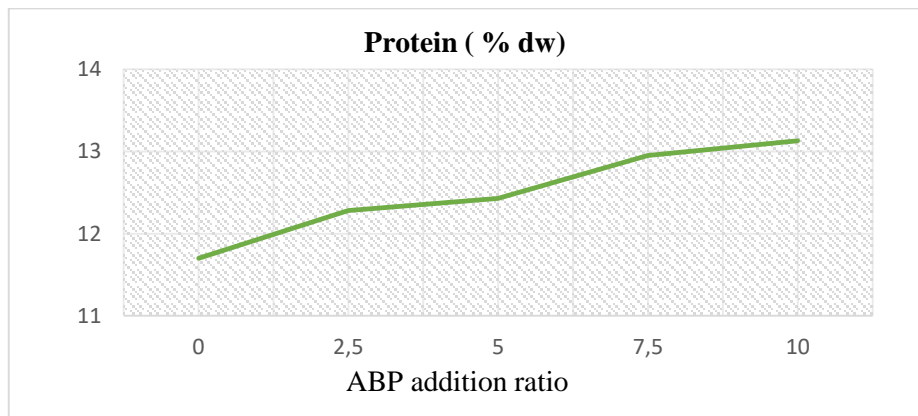
| ABP (%) | Level | Hardness (g)         | Springiness       | Cohesiveness      | Chewiness            | Resilience        |
|---------|-------|----------------------|-------------------|-------------------|----------------------|-------------------|
| 0       |       | 350.69 <sup>e</sup>  | 0.96 <sup>a</sup> | 0.73 <sup>a</sup> | 244.89 <sup>d</sup>  | 0.27 <sup>a</sup> |
| 2.5     |       | 4320.19 <sup>a</sup> | 0.91 <sup>a</sup> | 0.46 <sup>b</sup> | 1682.22 <sup>a</sup> | 0.10 <sup>b</sup> |
| 5       |       | 3529.29 <sup>b</sup> | 0.87 <sup>a</sup> | 0.43 <sup>b</sup> | 1447.02 <sup>b</sup> | 0.10 <sup>b</sup> |
| 7.5     |       | 2573.80 <sup>c</sup> | 0.84 <sup>a</sup> | 0.38 <sup>b</sup> | 538.23 <sup>c</sup>  | 0.08 <sup>b</sup> |
| 10      |       | 2012.64 <sup>d</sup> | 0.71 <sup>b</sup> | 0.37 <sup>b</sup> | 300.43 <sup>d</sup>  | 0.08 <sup>b</sup> |

<sup>1</sup>Values followed by different superscript letters (series “a-e”) within each column are significantly different at  $p < 0.05$ .

Therefore, it was thought that these differences in hardness and chewiness values were due to the difference in dietary fiber contents of flour mixtures. On the other hand, when the highest cohesiveness (0.73) and resilience (0.27) values were found in control sample, there was no significant difference between ABP added breads ( $p > 0.01$ ).

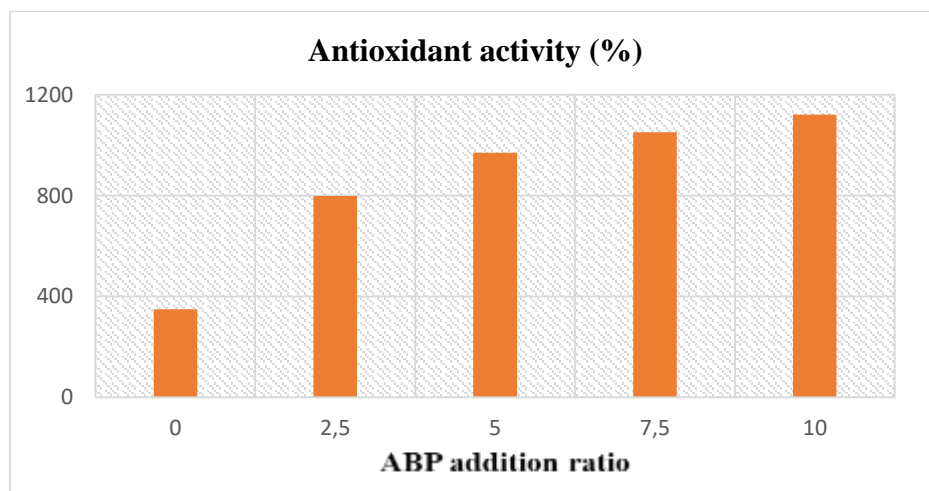
While the protein content of artichoke bract was found to range from 10.5 to 15.2 % (Ruiz-Cano et al., 2014), the protein content of refined wheat flour was between 9 and 12 % (Chu, 2004). Protein content of bread samples were shown in Figure 1. It was observed that the highest protein content (13.13 %) was found in 10 % ABP added bread sample while the lowest value was found in control. Since the higher protein content of artichoke bracts than that wheat

flour, the increase in ABP ratio caused an increase in the protein content of samples statistically ( $p < 0.05$ ).



**Figure 1.** Protein content of bread samples

Different studies about artichoke by-products, which have been used in health treatments since traditional Chinese medicine, have demonstrated their health-protective potential especially their hepatoprotective (Gebhardt, 1995; Gebhardt, 1997), anticarcinogenic (Clifford, 2000), and hypocholesterolemic (Clifford and Walker, 1987; Englisch et al., 2000) activities. Because artichoke by products, which contain large amounts of caffeic acids (Chen and Ho, 1997; Tomás-Barberán et al., 2000), are a potentially good source of antioxidant activity. In this study, antioxidant activity of bread samples was determined by DPPH assay. Results (Figure 2) showed that the addition of ABP to recipe affected the antioxidant activity of breads, significantly ( $p < 0.05$ ). Antioxidant activity increased significantly based on increased addition ratio of the ABP. In the literature, there were similar results about the antioxidant activity of artichoke bract. Llorach et al., (2002) found the antioxidant activity of raw artichoke by-products (bracts and steam) between 0.17 – 0.55 g of TEAC/100 g. Ruiz-Cano et al., (2014) investigated six fractions of artichoke by-products and found their hydrophilic antioxidant activity ranged from 100 to 300  $\mu\text{mol}$  of ascorbic acid eq.  $\text{gr}^{-1}\text{d.w}$ .



**Figure 2.** Antioxidant activity results of bread samples



## CONCLUSIONS

Evaluation of food by-products is very important and interesting for economic and environmental concerns. According to results of this study, it was seen that the addition of ABP affected and improved various physical, textural and nutritional properties of breads. The addition of ABP up to 2.5 % was found acceptable in terms of the physical and textural properties of the bread. Pimentel (2015) was also found that ABP at a 3 % substitution level can be used to obtain cookies without compromising consumer acceptability. Also, it is thought that the nutritional content of wheat flour bread will be enriched and the ratio of dietary fiber will increase.

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## THE USES OF THE ELECTROPOLYMERIZED FILMS AS IMMOBILIZATION MEDIUM FOR CHOLESTROL BIOSENSOR DESIGN

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### ABSTRACT

In this study, we aimed to use various electropolymerized films for the creation of cholesterol biosensors. Chemical and enzymatic methods can be used to measure blood cholesterol levels. The enzymatic reaction between cholesterol and cholesterol oxidase, which is measured at 0.6–0.7 V against Ag/AgCl in enzymatic methods, produces hydrogen peroxide. In this study, polyindoline and poly(4-methoxyphenol), two different polymeric materials made electrochemically, were used to immobilize cholesterol oxidase. Utilizing variables like pH, applied potential, and cycle count, the effects of the immobilization of the enzyme were investigated. We asserted that the aforementioned polymers could serve as a medium for the immobilization of cholesterol oxidase.

**Keywords:** Enzyme immobilization, cholesterol oxidase, polymer matrix, biosensor.

### INTRODUCTION

Blood cholesterol concentration is a clinically important parameter for the diagnosis and treatment of some clinical diseases such as cardiovascular disease, hypertension, and atherosclerosis (Lang, 1990; Trettnak et al., 1993; Charpentier, et al., 1995; Tan, et al., 2005) Various chemical or enzymatic methods have been used to determine the level of cholesterol in human blood. Chemical reactions for cholesterol determination are associated with certain difficulties, including lack of specificity and selectivity due to interfering reactions and the use of unstable and corrosive reagents.

Enzymatic methods for measuring total cholesterol using cholesterol ester hydrolase (CEH) and cholesterol oxidase (COx) appear to be a real improvement over the chemical method, but both techniques are still time-consuming (Pioch, et al., 1992). However, the enzymatic method has some advantages, such as high selectivity, short reaction time, small electrodes, low cost, and high reproducibility.

Immobilization of enzymes on the electrode surface is a crucial step in the construction of amperometric biosensors (Guilbault and Kauffmann, 1987; Bokoch, et al., 2004). In recent years, mediators and conducting polymers have been used as immobilization media.

The enzymatic reactions in the use of cholesterol oxidase (COx) are as follows: The enzymatic reactions in the use of cholesterol oxidase (COx) are as follows:

$\text{Cholesterol} + \text{O}_2 \rightarrow \text{Cholest-4-en-3-one} + \text{H}_2\text{O}_2$  (Brahim, et al., 2001; Kumar, et al., 2001)

$\text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2\text{H}^+ + 2\text{e}^-$  (Bongiovanni, et al, 2001; Ekinici, et al., 1996)

Amperometric detection of hydrogen peroxide is usually performed anodically (e.g., oxidation at +0.7 V with a Pt working electrode), but is strongly influenced by many readily

oxidizable interfering substances present in real samples (Ekinci, et al., 1998). We have used various polymeric materials prepared by electrochemical methods using polyindoline and poly(4-methoxyphenol).

In this study, polymeric materials were used as support materials for the immobilization of cholesterol oxidase. Immobilization was achieved by electrochemical polymerization, which served as an electron acceptor for ChOx. Electrochemical detection of cholesterol was performed using these electron mediators.

## MATERIAL AND METHOD

Cholesterol oxidase (COx) (E.C.1.1.3.6. *Pseudomonas* sp. = *E.coli*) with a specific activity of 1.5 U/mg solids was obtained from MP Biomedicals. Indoline, 4-methoxyphenol and all other chemicals such as 2-propanol, KCl, NaCl, Na<sub>2</sub>HPO<sub>4</sub> used for PBS (phosphate buffer salts) and Triton X-100 were purchased from Merck ([www.merck-chemicals.com](http://www.merck-chemicals.com)). Hexane, KH<sub>2</sub>PO<sub>4</sub> for PBS, and cholesterol as reagent were purchased from Riedel-De Haen ([www.riedeldehaen.com](http://www.riedeldehaen.com)), Carlo Erba ([www.carloerbareagenti.com](http://www.carloerbareagenti.com)), and Sigma ([www.sigmaaldrich.com](http://www.sigmaaldrich.com)), respectively.

All aqueous solutions were prepared with deionized and double distilled water. The nitrogen gas used for purging and blanketing during electropolymerization was of high purity.

Cyclic voltammetry (CV), Bulk electrolysis (BE), and steady-state amperometric response (time-based; TB) were performed with an electrochemical analyzer, BAS 100W (Bioanalytical Systems, Inc., West Lafayette, IN, USA). BAS 100W used the standard 3-electrode system consisting of a Pt disk (BAS, MF-2013, 1.98 mm<sup>2</sup>) as the working electrode and an Ag/AgCl reference electrode together with a Pt wire coil as the auxiliary electrode. The pH was measured using a Jenway 3010 pH meter.

At the beginning of the electrochemical polymerization, the Pt disc working electrode was cleaned according to the standard procedure (Ekinci, 1999) and polished to 0.05 μL with aqueous alumina slurry. The monomers were electropolymerized in an unstirred deaerated solution containing 0.10 M monomer and 0.05 M KCl. In the preparation of the biosensor, we tried two different methods. One of them was prepared with adsorption for this purpose. A different amount (1-10 μl) of cholesterol oxidase was dropped on the polymer electrode and waited for 2 hours at +4 °C for immobilization. Second, 0.10 M monomer and different amounts of cholesterol oxidase were mixed in the same solution, and the biosensor was prepared via CV or BE.

For the amperometric experiments, the PBS solution was aerated for approximately 15 minutes and kept at room temperature. A potential of +0.75 V was applied to the cell system because of the H<sub>2</sub>O<sub>2</sub> generated by the enzymatic reaction between cholesterol and cholesterol oxidase. The biosensor responses were registered as a current signal (nA-μA) by tracking the oxygen consumption at +0.75 V with respect to the Ag/AgCl electrode due to the enzymatic activity. When the constant background current value was reached, the substrate was added to the reaction cell. The buffer was refreshed after each measurement.

In the presence of surfactants, cholesterol is soluble in water. The literature (Vasudevan, et al., 1997; Li, et al., 2003; Li, et al., 2005; Srisawasdi, et al., 2005; Arya, et al., 2006; López, et al., 2006) contains numerous procedures for creating cholesterol standard solutions. In the literature, various solvents including alcohol, hexane, and triton were employed to prepare standard solutions of cholesterol. A bare platinum electrode was used to examine the effects of the solvents (Figure 1). To achieve this, steady-state amperometric behavior (TB) was assessed at +0.75 V in 0.1 M PBS. For additional research, hexane was chosen. The cholesterol standard

solution was made by combining 20 mg of cholesterol with 10 mL of hexane (Fletouris, et al., 1998).

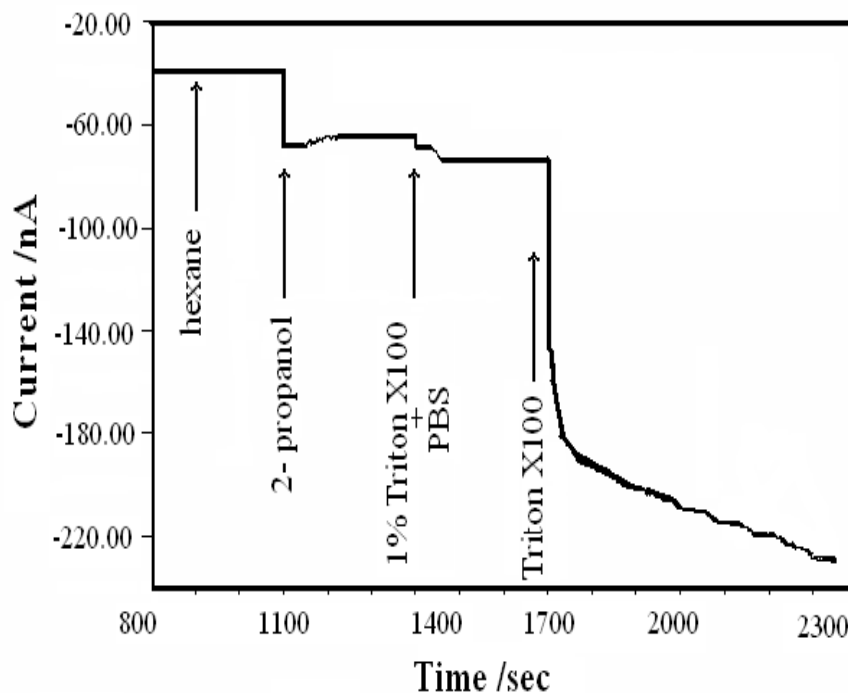


Figure 1. Effect of different solvents on bare Pt electrode: 900. s hexane; 1100.s 2-propanole; 1400.s %1 Triton X-100 + 0,1 M PBS; 1700.s Triton X-100.

## RESULTS AND DISCUSSION

Indoline, also known as 2,3-dihydroindole, was polymerized electrochemically by CV and BE methods. It was found that the films grown with CV were more homogeneous and stable. The indoline concentration in 100 mM KCl was adjusted to 100 mM, and polymer films (polyindoline) of different thicknesses were prepared at 0-1200 mV and  $10^{-5}$  sensitivity. It was decided that 24 cycles of these films were suitable. The CV for indoline is shown in Figure 2.

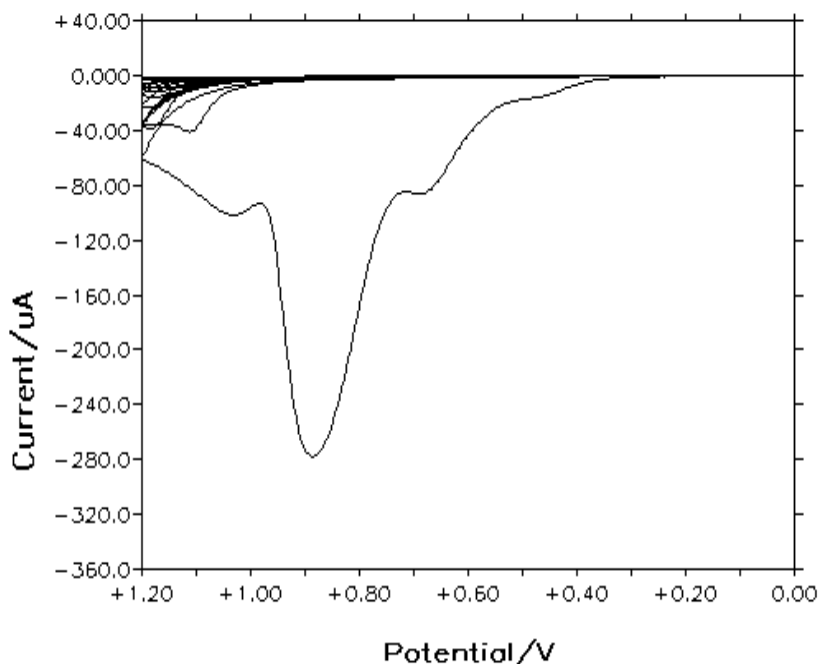


Figure 2. CV of Indolin (potential range of 0-1200 mV, scan rate of 50 mV/s, 24 cycles)

The COx (enzyme) solution was dropped onto the prepared polymer films and dried. The electrochemical behavior of the as-prepared enzyme electrode against the cholesterol solution was investigated using TB (Figure 4).

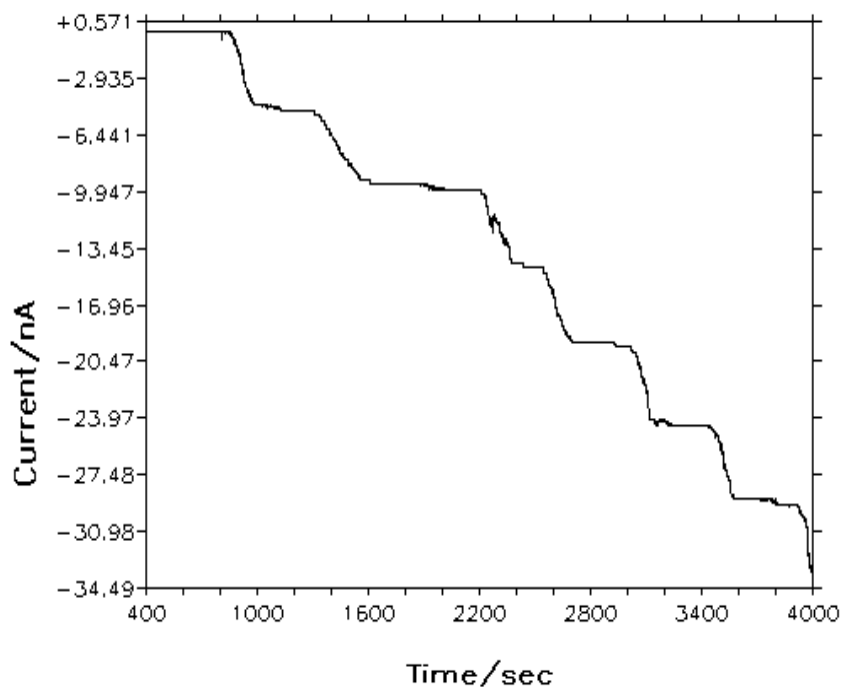


Figure 4. polyindoline COx electrode responses to cholesterol injections: 600, 1100, 1600, 2100, 2600, 3100, 3600 s (750 mV).

After detecting the expected electrochemical responses, the potential (550, 650, 750, 850 mV) and pH (5, 7, 9, 11) of the polyindoline cox electrode were determined to smooth the recorded current responses, as shown in Figures 5 and 6.

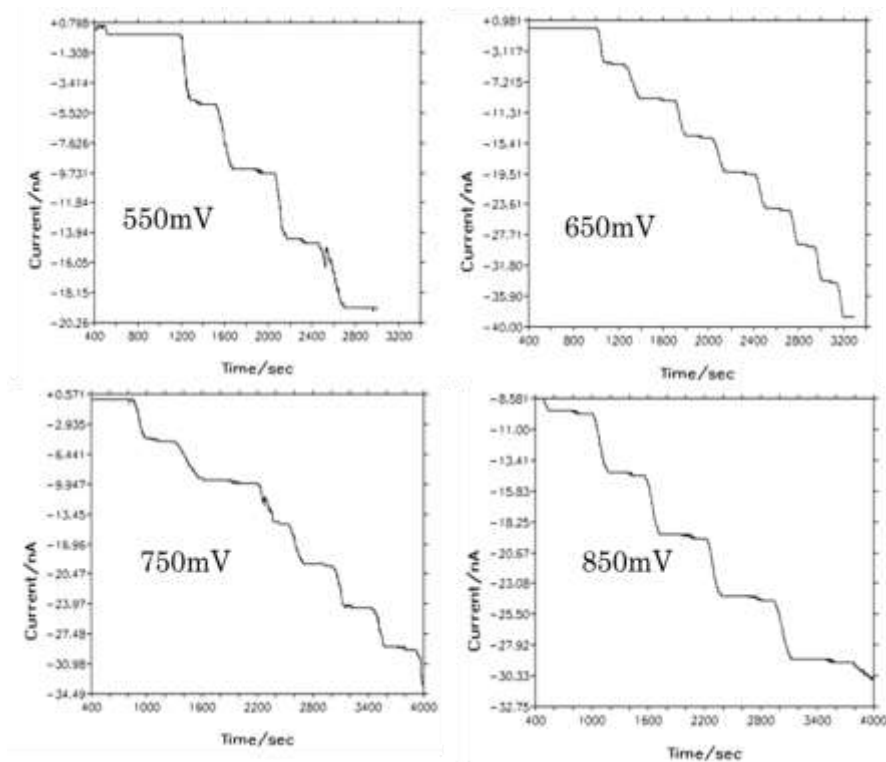


Figure 5. Responses of polyindoline COx electrodes to injections of cholesterol at 500, 1000, 1500, 2000, and 2500 s (at different potentials).

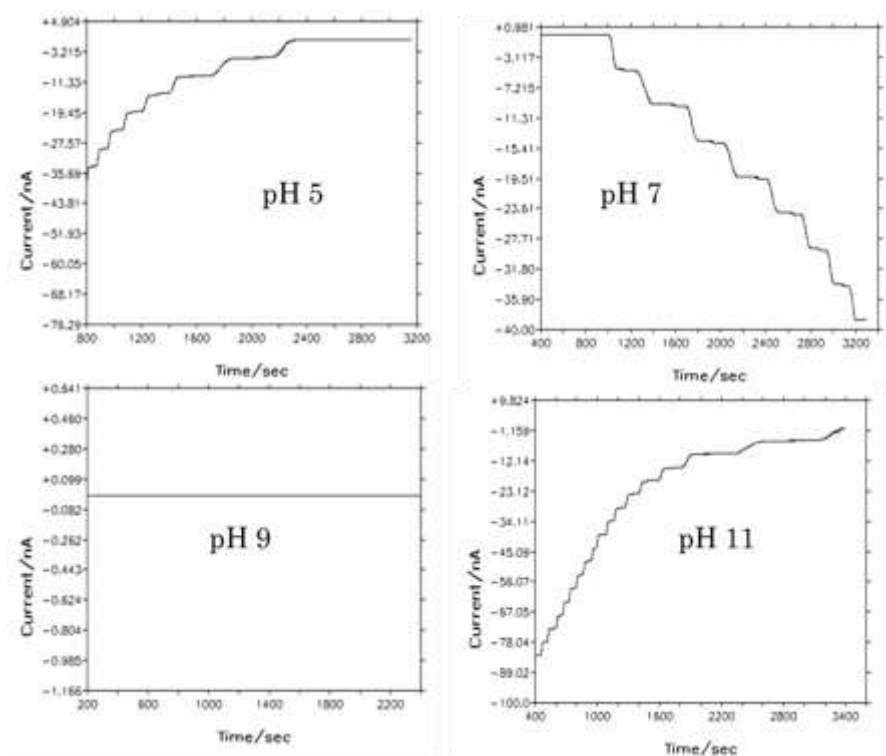


Figure 6. Responses of polyindoline COx electrodes to injections of cholesterol at 600, 1200, 1800, 2400, and 3000 seconds (at different pH values).



For poly(indolin), it was decided that 5  $\mu\text{L}$  of COx, PBS solution at neutral pH and 750 mV was more suitable for cholesterol measurement.

Poly(4-methoxyphenol) films of different thicknesses were obtained by BE at 600 mV for 4-methoxyphenol prepared at a concentration of 100 mM in a 0.1 M KCl solution. The stepwise responses are due to the long measurement time of cholesterol. Therefore, the effects of applied potential (550, 650, 750, 850, 950 mV), pH (5, 7, 9, 11), and amount of enzyme dropped (1-4  $\mu\text{L}$ ) were systematically studied to make these reactions as linear as possible. They are shown in Figures 7, 8 and 9.

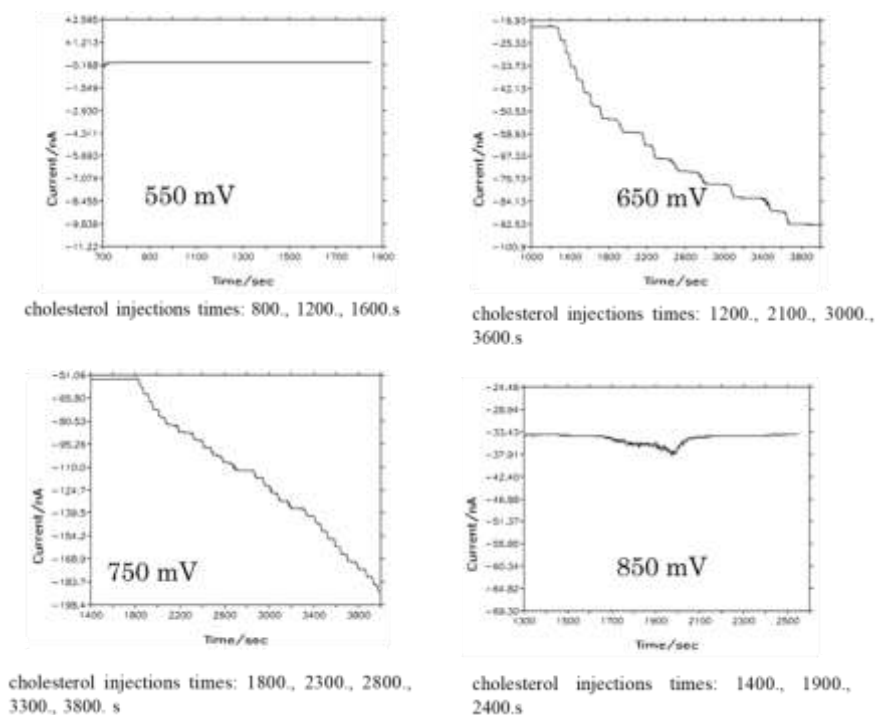


Figure 7. Responses of poly(4-methoxyphenol)-2  $\mu\text{L}$  COx electrode to cholesterol injection

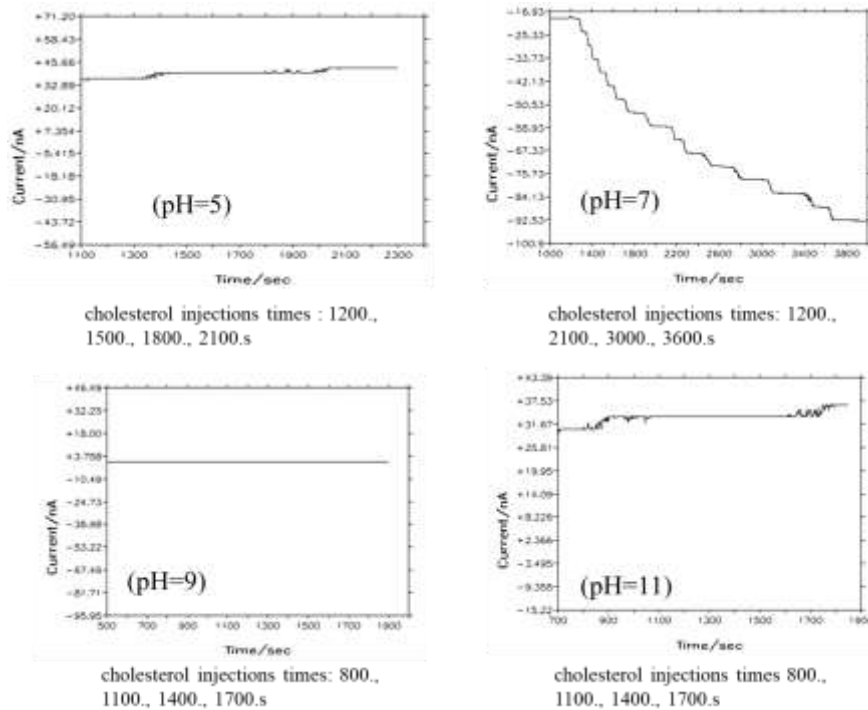


Figure 8. Responses of poly(4-methoxyphenol)-2  $\mu\text{L}$  COx electrode to cholesterol injection

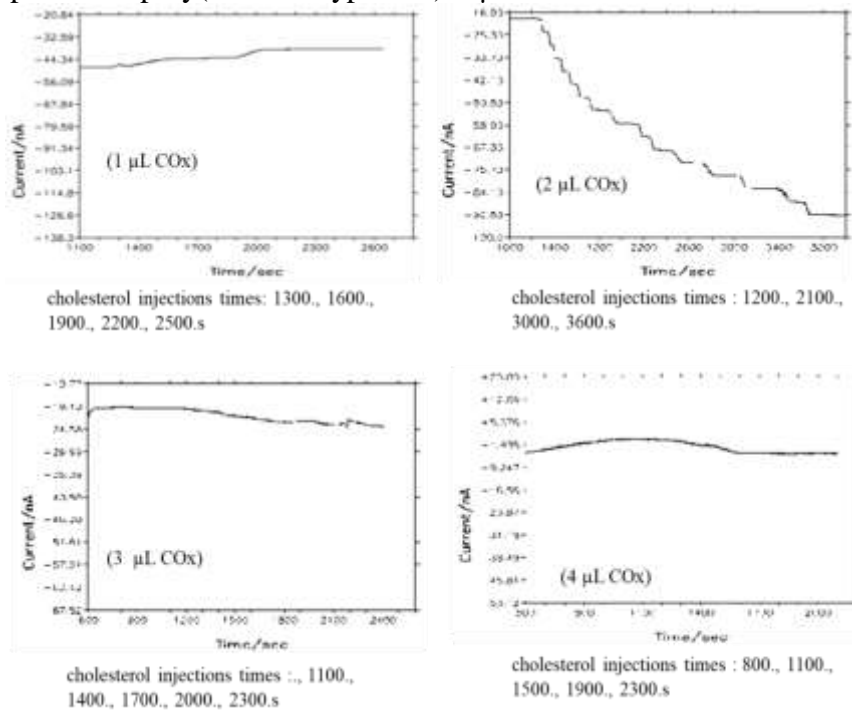


Figure 9. Responses of poly(4-methoxyphenol)-COx electrode to cholesterol injection

For poly(4-methoxyphenol), it was decided that 2  $\mu\text{L}$  of COx, PBS solution at neutral pH and 750 mV was more suitable for cholesterol measurement.

## CONCLUSIONS

Of the polymeric matrices prepared by the electropolymerization method, the enzyme immobilization studies performed with polyindoline, poly(o-toluidine), and poly(4-methoxyphenol) found that the appropriate response was obtained at neutral pH, and this finding was consistent with the literature. Charpentier and Murr chose 7 as the optimum pH in their study.

It has been shown that polyindol and poly(4-methoxyphenol) prepared by electrochemical polymerization can be used as a suitable immobilization medium for cholesterol oxidase.

## ACKNOWLEDGEMENT

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## **Bacillus subtilis IMPROVES THE NUTRITIONAL VALUE OF GRAPE SEEDS THROUGH SOLID-STATE FERMENTATION**

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### **ABSTRACT**

Solid-state fermentation has been used to improve the nutritional values of agricultural by-products. In this study, the effect of solid-state fermentation using *Bacillus subtilis* on the nutritional composition of grape seeds was investigated. Grape seeds were fermented by *B. subtilis* (ATCC 21556) for 48 hours in solid-state conditions. The unfermented and fermented grape seeds were analyzed for determination of the crude protein, ether extract, ash, crude fiber, neutral detergent fiber (NDF), and acid detergent fiber (ADF) content. Fermented grape seeds had higher crude protein ( $P<0.001$ ), ether extract ( $P<0.05$ ), and ash ( $P<0.001$ ) content than unfermented grape seeds. However, crude fiber, NDF, ADF, and nitrogen-free extract were lower ( $P<0.001$ ) in fermented grape seeds compared to unfermented grape seeds. *Bacillus subtilis* can be used to improve the nutritional composition of grape seeds.

**Keywords:** Grape seed, Solid-state fermentation, Nutritional value, *Bacillus subtilis*

### **INTRODUCTION**

Grape is one of the most produced fruits in the world. The annual production of grape has reached 78 million tons (FAOSTAT, 2020). Grape seed is a by-product occurred during processing of grapes in fruit juice and wine factories (Farahat et al., 2017). Grape seeds are a good source of protein and oil (Rodríguez and Ruiz, 2016)

Fermentation has been carried out traditionally for many years. It has received great interest from researchers for the detoxification and biotransformation of agricultural residues. Fermentation may be divided into liquid-state (submerged) and solid-state fermentation. Solid-state fermentation (SSF) refers to the development of microorganisms within moistened solid substrates without free water (Gungor and Erenner, 2020). The fermentation method can be used to improve the nutritional composition of agricultural products (Altop, 2019). Solid-state fermentation using *B. subtilis* increased the crude protein and decreased the crude fiber content in pomegranate seed (Güngör et al., 2020) and sweet cherry kernel (Altop et al., 2020). *Bacillus subtilis* is used as a probiotic in animal nutrition and is preferred for solid-state fermentation (Teng et al., 2012). This study aimed to investigate the impact of solid-state fermentation using *B. subtilis* on the nutritional composition of grape seeds.

## MATERIAL AND METHOD

Grape seeds were supplied from a fruit juice factory in Türkiye. Grape seeds were subjected to solid-state fermentation, according to Altop et al. (2020). Briefly, grape seeds were milled to pass through a 2 mm sieve and enriched with the nutritional salt (glucose:urea:(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>:peptone: KH<sub>2</sub>PO<sub>4</sub>:MgSO<sub>4</sub>.7H<sub>2</sub>O=4:2:6:1:4:1). Grape seeds were sterilized at 121°C for 15 min. *B. subtilis* were cultured in Tryptic Soy Broth and inoculated to grape seeds at 10<sup>10</sup> CFU per kg. Afterward, samples were incubated at 60 °C for 48 hours and dried at room temperature for six days till reaching %90 dry matter.

Raw and fermented grape seeds were analyzed to determine the dry matter, ash, crude protein, and ether extract content according to AOAC (2000). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) analyses were conducted according to Van Soest et al. (1991).

All experimental analyses were performed in triplicate. Data were analyzed with the Student t-test (SPSS 21.0 Statistics). Results were considered significantly different at P < 0.05.

## RESULTS AND DISCUSSION

*Bacillus subtilis* increased (P<0.001) the crude protein content of grape seed. Similar to the results of the present study, *B. subtilis* increased the crude protein content of pomegranate seed (Güngör et al., 2020) and sweet cherry kernel (Altop et al., 2020). This increase may be due to microbial protein produced by *B. subtilis* (Raimbault, 1998).

The ether extract content was increased (P<0.05) by *B. subtilis* solid-state fermentation. Similar to the results of the present study, ether extract was increased in pomegranate seed by *B. subtilis* solid-state fermentation (Güngör et al., 2020).

*Bacillus subtilis* increased (P<0.001) the ash content of grape seed. Similarly, *B. subtilis* increased the ash content of sweet cherry kernel (Altop et al., 2020) and pomegranate seed (Güngör et al., 2020). These results may be due to the relative increase of ash content because of the decrease in NFE, crude fiber, NDF and ADF content of grape seed by fermentation.

*Bacillus subtilis* decreased (P<0.001) the nitrogen-free extract of grape seed. Soluble carbohydrates are preferred by the microorganism to other nutrients for carbon sources (Papagianni, 2007). Similarly, *B. subtilis* decreased the nitrogen-free extract of sweet cherry kernel (Altop et al., 2020) and pomegranate seed (Güngör et al., 2020).

The crude fiber, neutral detergent fiber, and acid detergent fiber contents of grape seed were decreased after solid state fermentation using *B. subtilis* in this study. *Bacillus subtilis* can produce cellulase in the conditions of solid-state fermentation (Ritter et al., 2018), which can be the reason of the degradation and decrease of the structural carbohydrates in this study. Similarly, *B. subtilis* decreased the crude fiber and NDF content of sweet cherry kernel (Altop et al., 2020) and crude fiber, NDF, and ADF content of pomegranate seed (Güngör et al., 2020).

## CONCLUSIONS

The obtained results showed that *Bacillus subtilis* improved the nutritional composition of grape seed by increasing crude protein, ether extract, and ash content and decreasing crude fiber, NDF, and ADF content. Animal experiments can be conducted to investigate the effect of the fermented grape seed on farm animals.

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## **EFFECT OF LIQUID STATE FERMENTATION USING *Lactobacillus* spp. ON THE NUTRITIONAL COMPOSITION OF THE STALK PARTS OF *Agaricus bisporus* IN DIFFERENT PH LEVELS**

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### **ABSTRACT**

This study was investigated to determine the effect of the liquid state fermentation using *Lactobacillus* spp. on the nutritional composition of the stalk parts of *Agaricus bisporus* at different pH levels. Fresh *A. bisporus* was obtained, and the stalk parts were separated from the cap parts. After cutting into small pieces, each 100 grams of the mushroom stalk was mixed with 400 ml of distilled water in the fermentation flasks. The mixture was enriched with 8.4 g urea (46% N) as a nitrogen source. The fermentation flasks were allocated to two pH groups (6 and 7), and the pH of the fermentation media was adjusted to 6 and 7 by using 1 N HCl and 1 N NaOH. Mushroom stalks were sterilized at 121 °C for 15 min by autoclave and then inoculated with 1 ml *Lactobacillus* spp. ( $10^8$  CFU/ml). One uninoculated flask was also separated as a positive control for each inoculated flask. The inoculated mushrooms were incubated at 30 °C for 48 hours. At the end of the fermentation, raw, fermented, and uninoculated mushroom stalks were analyzed to determine the *Lactobacillus* spp. count, pH value, crude protein, and ash content. *Lactobacillus* spp. decreased ( $P<0.001$ ) the crude protein content of the mushroom stalk in both pH level compared with the uninoculated mushroom stalk. However, the lowest crude protein content was observed in the pH 7 group among the fermented stalks. The ash content of the mushroom stalk was also decreased ( $P<0.001$ ) after liquid fermentation at both pH levels. After all, there was no difference in ash content between the fermented stalks. *Lactobacillus* spp. decreased the pH level of mushroom and liquid in both initial pH levels compared with the uninoculated mushroom stalks. *Lactobacillus* spp. count tended to be higher ( $P=0.078$ ) in the pH 6 group than in the pH 7 group. The fermentation conditions need to be studied to investigate the usability of the liquid state fermentation for improving the nutritional composition of the mushroom stalk.

**Keywords:** Button mushroom, Stalk, Liquid state fermentation, *Agaricus bisporus*, *Lactobacillus* spp.

### **INTRODUCTION**

Mushrooms have been considered important food sources since ancient times because of their nutritional values and therapeutic properties (Atila et al., 2021). The white button mushroom (*Agaricus bisporus*) is an edible Basidiomycete fungus. *Agaricus bisporus* has important bioactive phenolic components such as cinnamic acid derivatives (cinnamic acid, p-



cou-marinic acid, ferulic acid and chlorogenic acid) and benzoic acid derivatives (p-hydroxybenzoic acid, protocatechuic acid, and gallic acid) (Ramos et al., 2019). Mushrooms also have strong antioxidant, immunostimulatory, anti-inflammatory, antibacterial, antiviral and hypocholesterolemic effects on broiler chickens (Bederska-Łojewska et al., 2017).

The bottom part of the mushroom stalks is not suitable for eating because it is contaminated with compost. Therefore, some parts of mushroom stalks are usually discarded during the packing process in mushroom production facilities. This quantity of by-products is equal to 20% of total mushroom production (Altop et al., 2021). If these by-products are not used in any field, they will cause environmental pollution (Yang et al., 2021). Therefore, the use of mushroom stalks in animal nutrition will help to reduce environmental pollution.

Fermentation can be used to improve the nutritional composition of agricultural residues (Gungor et al., 2021). Fermentation is divided into liquid-state and solid-state fermentation. Solid-state fermentation refers a microbial growth in moistened solid substances without free water (Gungor and Erener, 2020). However, liquid state (submerged) fermentation refers to microbial cultivation in the presence of plenty of free water (liquid medium, Behera and Ray, 2019).

The objective of this study was to investigate the effect of the liquid state fermentation using *Lactobacillus* spp. on the nutritional composition of the stalk parts of *Agaricus bisporus* at different pH levels.

## MATERIAL AND METHOD

Fresh *A. bisporus* was obtained from a local market at Samsun, Türkiye. The stalk parts of *A. bisporus* were separated from the cap parts. After cutting into small pieces, each 100 grams of the mushroom stalk was mixed with 400 ml of distilled water in the fermentation flasks. The mixture was enriched with 8.4 g urea (46% N) as a nitrogen source. The fermentation flasks were allocated to two pH groups (6 and 7), and the pH of the fermentation media was adjusted to 6 and 7 by using 1 N HCl and 1 N NaOH. Mushroom stalks were sterilized at 121 °C for 15 min by autoclave.

*Lactobacillus* spp. were cultivated with MRS broth in a shaking incubator at 30 °C and 120 rpm for 48 hours (Kumoro and Hidayat, 2018). Sterilized mushroom stalks were inoculated with 1 ml *Lactobacillus* spp. ( $10^8$  CFU/ml). One uninoculated flask was also separated as a positive control for each inoculated flask. The inoculated mushrooms were incubated at 30 °C for 48 hours. At the end of the fermentation, raw, fermented, and uninoculated mushroom stalks were analyzed to determine the *Lactobacillus* spp. count, pH value, crude protein, and ash content, according to AOAC (2000).

All data were analyzed by one-way ANOVA using SPSS 21.0 Statistics. The statistical differences between treatments were determined by Duncan's multiple range test. The level of statistical significance was declared at  $P \leq 0.05$ .

## RESULTS AND DISCUSSION

*Lactobacillus* spp. count tended to be higher ( $P=0.078$ ) in the pH 6 group than in the pH 7 group. Similarly, the higher productivity of *Lactobacillus* spp. was observed at pH6 level compared with 6.5 and 6.75 pH levels (Aasen et al., 2000). Similar results were also reported from the study comparing the pH6 and pH8 levels (Tang et al., 2016).

*Lactobacillus* spp. decreased ( $P<0.001$ ) the crude protein content of the mushroom stalk in both pH levels compared with the uninoculated mushroom stalk. However, the lowest crude protein content was observed in the pH 7 group among the fermented stalks. *Lactobacillus* spp. was reported to increase the crude protein content of durian seed with submerged fermentation (Kumoro and Hidayat, 2018). However, the crude protein content was decreased after liquid state fermentation in this study. The different substrates used in the studies can explain the different results.

The ash content of the mushroom stalk was also decreased ( $P<0.001$ ) after liquid fermentation at both pH levels. After all, there was no difference in ash content between the fermented stalks. Similarly, *Lactobacillus* spp. decreased the ash content of durian seed after submerged fermentation (Kumoro and Hidayat, 2018). The soluble minerals in the mushroom stalk can be dissolved in the liquid medium during fermentation. This can be a reason for the decrease in the ash content of both uninoculated and inoculated stalks.

*Lactobacillus* spp. decreased the pH level of mushroom and liquid in both initial pH levels compared with the uninoculated mushroom stalks. *Lactobacillus* spp. can produce lactic acid during submerged fermentation (Brinques et al., 2010). Decrease in the pH level can be due to the lactic acid production of *Lactobacillus* spp.

## CONCLUSIONS

Liquid state fermentation decreased the crude protein and ash content of mushroom stalk. Besides, *Lactobacillus* spp. decreased the pH level of the mushroom stalk. The fermentation conditions need to be studied to investigate the usability of the liquid state fermentation for improving the nutritional composition of the mushroom stalk.

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## **THE PLACE OF FOOD INSPECTIONS IN THE LEGISLATION AND THE EXAMINATION OF FOOD INSPECTION ACTIVITIES PERFORMED IN KASTAMONU PROVINCE**

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### **ABSTRACT**

Food safety is defined as complying with the necessary rules and taking precautions in the production, processing, storage, transport and distribution stages of foods that will not harm people's health. In ensuring food safety, effective food inspection systems developed by governments are of utmost importance in addition to food safety awareness of consumers and responsible behaviour of companies. In Turkey, the inspection of foods offered for consumption within the scope of food safety is conducted by the Food and Feed Branch Units of the Provincial/District Directorates of Agriculture and Forestry of the Republic of Turkey in the provinces as per to the legislation on food inspection. Provincial/District Directorates have been conducting key studies and inspections on food inspections in recent years. However, there are not many studies on the information of these studies and the conclusions to be drawn from these audits. However, it is important to increase the number of these studies, to share the results of these inspections and to question the effectiveness of food inspections according to these conclusions, and it is crucial to do this effectively in order for the society to have access to safe food. In this study, the place of food inspection conducted by Provincial / District Directorates of Agriculture and Forestry in the legislation is briefly explained. The functioning of the food inspection process in Kastamonu province is described. Food inspection activities conducted by Kastamonu Provincial Directorate of Agriculture and Forestry Food and Feed Branch Unit in food production places, food sales places and public consumption places between 2015-2021 in Kastamonu are presented in tables. It is attempted to ensure that the importance of food inspections in ensuring food safety of the consumer is understood. Based on the data of Kastamonu province, the importance of food inspections and suggestions to decision makers on this issue were put forward.

**Keywords:** food, food inspections, food policies

### **INTRODUCTION**

Food safety is defined as complying with the necessary rules and taking precautions in the production, processing, storage, transport and distribution stages of foods in order to ensure safe food production (Uzunöz, et al, 2008) and includes the concepts of safe, healthful and healthy food (Artık, et al, 2019). In the report published by FAO, food safety is defined as a

set of hazards that may acutely or chronically harm consumer health (FAO, 2003). Food safety consists of public opinion created by the consumer, legislation created by the state, scientific and technological knowledge used by food producers and marketers. In order to create an effective food safety, these three elements must fulfil their responsibilities (Bayrak and İlbeği, 1997).

Within the scope of food safety, a series of measures are taken to eliminate physical, chemical, biological and all kinds of dangerous risks that foods offered for consumption may encounter during their stages (Artık, et al, 2013). It is a legal requirement and the responsibility of the food business to take these measures and to produce at predetermined standards. However, since some food businesses sometimes do not comply with legal requirements and do not act responsibly, they should be inspected in the production, distribution, sale and consumption of food. Within the scope of food inspections, it is aimed to protect human health by ensuring that consumers have access to reliable food and by the work of the Republic of Turkey, Ministry of Agriculture and Forestry, General Directorate of Food and Control at the centre and by the Food and Feed Branch Unit affiliated to the Provincial / District Directorates of Agriculture and Forestry.

It is one of the basic requirements that food inspections are performed in a certain systematic manner and that consumers are assured of access to safe food. The restructuring of food safety management in Turkey, which started with the Law No. 5996, and studies such as the establishment of a risk-based inspection system, the continuation of the obligation of HACCP programmes in enterprises, more effective inspection of food exports and imports, and a thorough review of the relevant legislation are important steps towards ensuring food safety (GIDAMUHDR, 2013).

The restructuring of official food inspections is one of the issues on the agenda, especially in the process entered with the changing legislation in recent years and the projects conducted by the Republic of Turkey, Ministry of Agriculture and Forestry. In recent years, there have been significant developments in the field of food safety in Turkey compared to previous years with the exposure of businesses engaged in counterfeiting and adulteration, increased efforts to register unregistered businesses and the introduction of the ALO 174 hot line. It is important to evaluate the notifications of consumers, to enable Provincial/District Directorates of Agriculture and Forestry to carry out food inspections in the most effective way, to measure the outputs of food inspections and to give a direction to food inspection activities. Provincial/District Directorates of Agriculture and Forestry have been carrying out important activities on food inspection in recent years. However, there is a limited number of studies on the outcomes of these studies. Increasing studies in this field will increase the understanding of food inspections as well as the importance and quality of food inspections. In this study, it is aimed to contribute to the limited literature on food inspection performed in Provincial/District Directorates of Agriculture and Forestry.

## **MATERIAL AND METHOD**

In this study, firstly, the sections of the Veterinary Services, Plant Health, Food and Feed Law No. 5996, dated 13/06/2010 and numbered 27610 in Turkey, related to food safety and food inspections; By examining the regulations, procedures and guidelines prepared on the basis of Law No. 5996, the current legislation on the inspection activities to be performed in the provinces and the status of food inspections in legal regulations were examined. By explaining the legislation, it is aimed to provide an understanding of the basis of the food inspections conducted in Kastamonu. Afterwards, the process of food inspection activities, inspection information, number of inspections and statistics obtained from Kastamonu

Provincial Directorate of Agriculture and Forestry Food and Feed Branch Unit were organised. With these statistics, comprehensible tables have been arranged, all this information has been arranged and the situation of food inspection studies has been evaluated based on the example of Kastamonu province. It is important to explain the process of food inspections in Kastamonu province and to present the work done in tables as explanatory. There is no data sharing from the Provincial/District Directorates of Agriculture and Forestry on food inspections over the years in this way. In this sense, the study is valuable. Based on Kastamonu province, it is important to show the food inspection process performed in the provinces in such an explanatory way. In addition, the wide variety of data makes it easier to make comparisons and inferences. Recently, it is also important for consumers to make food notifications and to create statistics on this issue. In this study, the notifications made in Kastamonu province and the statistics related to them are also shown. Another important contribution of the study to the literature is that the food inspection legislation is briefly explained and as can be seen from this brief explanation, the functionality of food inspections in Kastamonu province can be briefly evaluated with the food inspection legislation.

## **RESULTS AND DISCUSSION**

### **Place of Food Inspections in Legislation**

Turkey's current legislation in the field of food safety started with the opening of the European Union (EU) 'Food Safety Chapter' in June 2010. The Veterinary Services, Plant Health, Food and Feed Law No. 5996 dated 11/06/2010 and published in the Official Gazette No. 27610 dated 13/06/2010 was prepared by taking into account the negotiations with the EU. The Law aims to protect and ensure food safety and public health, taking into account consumer interests and environmental protection. The Law covers all stages of production, processing and distribution of food, food contact materials and substances, official controls and sanctions related to these issues.

Registration of food enterprises is the most important factor determining the effectiveness of inspections. With the entry into force of all provisions of the Law No. 5996 on 13/12/2010, the obligation to obtain a production permit for food producing workplaces was abolished. Based on the provisions of the Regulation on the Registration and Approval Procedures of Food Enterprises published in the Official Gazette dated 17/12/2011 and numbered 28145 and the Regulation on the Amendment of the Regulation on the Registration and Approval Procedures of Food Enterprises published in the Official Gazette dated 10/01/2013 and numbered 28254 (2020), it has come to the agenda that food enterprises should be registered or subject to approval according to their subjects. After these regulations, food inspections were supervised by explaining these implementation procedures.

In food inspections, with the Provincial Annual Food Control Plan Preparation and Implementation Procedure (2022) prepared within the framework of the provisions of the Regulation on Official Controls of Food and Feed No. 28145 dated 17/12/2021 (2011) and the Regulation Determining Special Rules for Official Controls of Animal Food No. 28145 dated 17/12/2011 (2011), food production, food sales and mass consumption enterprises in provinces and districts are subject to regular inspections according to the risk-based inspection plans determined by the Republic of Turkey, Ministry of Agriculture and Forestry.

In addition, if there is a suspicion that consumers are suspected of working outside the legislation in food products, they can report this situation, and inspections are conducted within the legal period to the enterprise / enterprises requested to notify / complain / receive information. Consumers are informed in 3 different types;

- Complaints related to price increases: This is to notify the competent authorities when it is thought that there is an unfair price increase. In such a case, 'Unfair Price Increase Complaint Notification' can be made from the Services section of the Ministry of Trade via e-government. In addition, notifications can be made via the mobile application of the General Directorate of Domestic Trade of the Ministry of Trade or by telephone to the Alo 175 Consumer Information Line.
- Stockpiling or a difference between the price on the label and the price at the cash register: In food production, processing, storage or sales processes, stockpiling of food products can be performed by some companies or individuals in a way that distorts the market and gains unfair profit. In another case, consumers may think that the labelled price and the cash register price are different and that they have been deceived. In both of these cases, consumers can file a complaint to the Provincial Directorates of Trade.
- Labelling information is incorrect/inaccurate: Consumers may think that the product information on the food label is not sufficiently stated, is not legible or contains incorrect information. In such cases, a complaint can be lodged if it is considered that the labelling regulations have not been complied with. In the other case, if there is a belief that the food is not produced according to the regulation on nutrition and health claims, consumers can complain about the company/product. In such cases, the point of complaint can be the Provincial/District Directorates of Agriculture and Forestry, and with the establishment of the 174 Food Line Procedure in 2009, complaints made by consumers on labelling or other food-related issues are combined here.

ALO 174 Food Hotline is an application that became operational on 14 February 2009 after the preparation of ALO 174 Food Hotline Procedure (2009) in order to enable consumers to easily reach the relevant authority for all kinds of notifications, complaints and requests for information on food safety, to direct communication from a single centre, to return to the consumer as soon as possible and to follow up the result. In case of a request for notice/complaint/information request; a record is created if the business name and address and the product information in question are shared. The notices and complaints received are forwarded to the Republic of Turkey, Ministry of Agriculture and Forestry or to the contact points previously determined in 81 provinces for evaluation. The finalisation time of incoming applications varies depending on the risk status of the complaint and the intensity of the Provincial Directorate to which the complaint is submitted. This period is calculated as 15 days on average in Turkey.

The responsibilities of food business operators in ensuring the safety of food are regulated in the Law No. 5996. The Law states that 'In order to ensure traceability, food operators are obliged to establish a system for the tracing of the food under their responsibility, any substance to be added to the food and the animal from which the food is obtained at all stages of production, processing and distribution, and to submit this information to the Ministry of Agriculture and Forestry of the Republic of Turkey upon request' (Art. 24/1). In addition, in order to ensure the traceability of the food to be placed on the market, the food operator must ensure that the food to be placed on the market is properly labelled or properly identified with the information and documents to be determined by the Ministry (Art. 24/2). The responsibilities of the food operator are listed in the Regulation on Official Controls of Food and Feed (2017), which was prepared based on Article 22 of the Law on Responsibilities in Ensuring Food Safety (Art. 40), and detailed in the guideline of the regulation. Food establishments must fulfil the responsibilities imposed on them by the regulation.

The operator operating in the field of food, substances and materials in contact with food and feed is obliged to meet the conditions specified in the law at every stage of its field of activity (Art. 40/1).

In the event that the food and feed operator assesses that a product that it produces, processes, imports, sells or distributes does not comply with the food and feed safety requirements or has reasonable grounds for this, it is obliged to immediately initiate the necessary procedures for the collection of the product in question, starting from the stage where it leaves its control, and to inform the provincial / district directorate of the Ministry on the subject. The food and feed operator is obliged to inform the consumer or user correctly and effectively about the reason for collection when the product has to be collected and, where necessary, to call for the return of the product to the consumer or user if the measures to be taken to protect human and animal health are not sufficient (art. 40/2).

The food and feed operator is obliged to keep the following records related to its activities up to date and to submit them to the Ministry of Agriculture and Forestry of the Republic of Turkey when requested (Art. 40/3);

- a) Documents related to business approval or registration,
- b) Procedures and up-to-date records of food and feed safety systems based on the principles of hazard analysis and critical control points,
- c) Device calibration records,
- ç) Device measurement records,
- d) Records of the purchase and sale, including a mark or number and other information enabling the lot to be identified,
- e) Traceability records in food and feed,
- f) Pest and rodent control records,
- g) Records of analyses of raw materials, auxiliary materials used in production, intermediate and/or final products,
- ğ) Records of the employment of compulsory personnel who have undergraduate education on the subject according to the type of work.

The food and feed operator is responsible for preventing, reducing or eliminating the risk associated with the product and co-operates with the Ministry in taking such measures. The persons concerned may not obstruct in any way the implementation of the measures taken by the Ministry (art. 40/4).

The food and feed operator is obliged to use the products of enterprises registered or approved by the Ministry in production, preparation and sale, and approved products if the product is subject to approval (art. 40/5).

Public and private institutions and organisations, as well as workplaces and catering factories that produce food on site and offer it for mass consumption, are obliged to keep a sample taken from each type of food batch produced under suitable conditions for seventy-two hours (Art. 40/6).

In food inspections, sanctions are imposed on those who are found to act contrary to the legislation in accordance with the relevant articles of the Law No. 5996. The Law No. 7255 on Certain Regulations in the Field of Food, Agriculture and Forestry, which amended the Law No. 5996 in order to protect the health and interests of consumers at the highest level and to make the penalty clause a deterrent, entered into force after being published in the Official Gazette dated 04/11/2020 and numbered 31294. The following sentence has been added to Article 24/4 of the Law No. 5996. "Counterfeit and adulterated products cannot be processed and placed on the market. (art. 29). Article 40/1-a and 40/1-l of the Law No. 5996 are amended as follows.

"a) Foods that may endanger the life and health of persons shall be withdrawn from the market at the expense of the responsible person and destroyed by transferring ownership to the public. *Food business operators who produce, import or supply these foods to the market under their own name or trade name shall be sentenced to imprisonment from one*



*year to five years and a judicial fine from one thousand days to five thousand days. If the act is repeated within three years, the food operator who produces, imports, supplies the food to the market under its own name or trade name shall be banned from food sector activities from five to ten years."*

*"1) Food or feed that has been counterfeited or adulterated in violation of the fourth paragraph of Article 24 shall be seized. The products shall be collected from the market at the expense of the responsible person. Products that cannot be utilised for purposes other than placing on the market shall be destroyed by the operator under the supervision of the Ministry. It is decided to transfer the ownership of the products that can be utilised for purposes other than placing on the market to the public. Imitation or adulterated food or feed;*

*1) Fifty thousand Turkish Liras to the food or feed operator who produces, imports or places on the market under its own name or trade name,*

*2) An administrative fine of not less than five thousand Turkish Liras and not exceeding five hundred thousand Turkish Liras shall be imposed on the retail food or feed operator who supplies to the market without ensuring traceability, at the rate of one per cent of the annual gross revenues generated at the end of the financial year preceding the act or, if it is not possible to calculate this, at the end of the financial year closest to the date of the act. In the event that the act is repeated for the first time within three years, the lower and upper limits of the above-mentioned administrative fines shall be applied to the food or feed operator who produces, imports or places it on the market under its own name or trade name, with a one-fold increase. If the act is repeated for the second time within the same period, the food or feed operator who produces or imports the food or feed shall be imposed a judicial fine from one thousand days to three thousand days and the food or feed operator shall be banned from its activity in this sector from five to ten years. The food or feed operator who repeats the act for the second time within three years and supplies it to the market under its own name or trade name shall be imposed an administrative fine of not less than two hundred thousand Turkish Liras and not exceeding two million Turkish Liras, at the rate of one per cent of its annual gross revenues at the end of the fiscal year preceding the act or, if it is not possible to calculate this, at the end of the fiscal year closest to the date of the act."*

Based on these amended articles of the Law, the Regulation on the Act of Imitation and Adulteration in Food and Feed and Calculation of Administrative Fines (2021) was published in the Official Gazette dated 16/04/2021 and numbered 31456 and penalties were regulated in this regard.

In the relevant article of the Law No. 5996 (art. 31/6) and in the Regulation on the Official Control of Food and Feed published in the Official Gazette No. 28145, the company and product information producing/importing the foods that are confirmed to be counterfeited and adulterated with the laboratory result, and the information of the products that have deteriorated in a way that endangers the life and health of people, The Ministry of Agriculture and Forestry of the Republic of Turkey may make available to the public information on the official website of the Ministry of Agriculture and Forestry (Art. 8). As a result of official controls in this context;

1. The name, product name, brand, batch and / or serial number of the company producing / importing food and feed that is confirmed to be counterfeited or adulterated by laboratory results,

2. The name, product name, brand name, batch and/or serial number of the company that produces and / or sells spoiled or modified foods that endanger the life and health of persons are disclosed to the public.

In line with these provisions, public announcements have been made on the official website of the Ministry since 2012 regarding the companies and products in question. The announcements are made in 2 different groups as stated above.

Within the scope of the Law No. 5996 (Art. 40/1), an administrative fine is imposed on the companies producing the products in the first group, which are confirmed to be counterfeit or adulterated by laboratory results, and it is decided to take the ownership of these products to the public.

As a result of the laboratory analyses in the second group, a criminal complaint is filed with the Public Prosecutor's Office within the scope of offences against public health against those who produce or place on the market products (containing the active ingredient of the drug) that are found to be degraded and modified in a way that may endanger the health of persons, and the products are collected from the market at the expense of the responsible person and the ownership is transferred to the public.

Public information announcements are of great importance in terms of protecting the interests and health of the society, as they are a right granted to consumers. The main purpose of the public announcement practice is to protect the health and interests of consumers and to prevent unfair competition in the sector. In this way, it is aimed to establish a control mechanism on the market through consumers, apart from official controls and the self-control system of companies, and to encourage the production of safe food. Publicising product/company information is effective in the formation of consumer preferences.

### **Activities conducted within the scope of Food Inspections in Kastamonu Province**

#### **Functioning of Food Inspection Process in Kastamonu Province**

In Turkey, official controls of food enterprises are carried out by the Republic of Turkey, Ministry of Agriculture and Forestry and control officers. Food inspections in the provinces and districts are performed as stated in the Directive (2016) on the Duties of the Food and Feed Branch Directorates of the Provincial Directorate of Agriculture and Forestry (Art. 8). Accordingly, the control officer is the person authorised by the Ministry of Agriculture and Forestry of the Republic of Turkey to carry out official controls including monitoring, surveillance, verification, inspection, audit, audit, sampling and analysis on behalf of the Ministry of Agriculture and Forestry of the Republic of Turkey, without prior notification, without notice, at appropriate frequencies on the basis of risk, free from any influence and interest relations, impartially, objectively and independently, and imposes administrative sanctions stipulated in the law within the framework of the powers granted to him.

Kastamonu Provincial Directorate of Agriculture and Forestry Food and Feed Branch Directorate has 5 teams on food and feed inspections. These teams inspect all food and feed enterprises in Kastamonu Province and districts for compliance with legislation and procedures. If there is a control officer in the district, the personnel in the districts carry out the inspections and controls of the sales and mass consumption places. In districts such as Pınarbaşı, Abana and İhsangazi, where there is no control officer, inspections are conducted by personnel from the centre.

According to the risk-based inspection plan determined by the Ministry of Agriculture and Forestry of the Republic of Turkey, food production, food sales, and mass consumption enterprises in the province and district are subject to inspections at regular periods of 6-8 months. In addition, in line with the complaints received by the Ministry of Agriculture and Forestry, ALO 174 Food Hotline and the Republic of Turkey, Presidential Communication Centre (CIMER), inspections are performed to the complained business within the legal period.

'Annual Food Control Plan' of the province is prepared in order to control the compliance of the products produced in the production sites operating within the provincial borders with the legislation. 'Provincial Annual Food Control Plan' covers the sampling plan for products produced in food establishments operating within the provincial borders. The 'Provincial Annual Food Control Plan Preparation and Implementation Procedure', which is the justification for the Plan, is based on Law No. 5996 (art. 31/9) and the Regulation on the Official Control of Food and Feed published in the Official Gazette No. 28145 dated 17/12/2011 (art. 5/11). The number of production sites in the province, production capacity, product diversity, the number of consumers addressed by the production and the risk level of the product are taken into consideration in the formulation of the plan. In the selection of the enterprises from which the samples will be taken, priority is given to risky enterprises, taking into account the frequency of risk-based audits. All data are only recorded in the Food Safety Information System (FSIS). The results are not sent in writing to the General Directorate of Food and Feed Control unless requested.

In the inspections carried out for sampling within the scope of the Annual Control Plan, the inspection frequencies according to the subjects are specified in the procedure. The process is described in the Official Sampling Procedure (2022). Considering the type and intensity of food production in the province, the risk of food and the capacity of the laboratories, an annual sampling plan is prepared on a provincial basis together with the laboratory directorates in December of each year and updated as necessary during the year. In this plan to be prepared, the number of samples taken from food establishments in the province, the distribution of these samples on the basis of food groups, the negative data obtained, the risk status of food, complaints and notifications are taken into consideration, except for the annual control plan of the Republic of Turkey, Ministry of Agriculture and Forestry for the last three years. Samples are taken when necessary during official controls. The samples taken must be representative of the product batch (batch / batch and label number and code numbers are the same).

The frequency of inspection should be proportionate to the risk of the product produced or offered for consumption by the establishment and the risk arising from the technical and hygienic conditions of the establishment. In addition, the self-control programme practices of the enterprise, hygiene management and the information obtained by the provincial/district directorate from the audits conducted in the past are taken into consideration in determining the audit frequency. In determining the audit frequency, the Procedure for Determining the Risk-Based Audit Frequency of Enterprises (2011) is utilised. Audit frequency is determined according to routine audits and threats created on the basis of risk determined according to the procedure. In determining the frequency of inspection; four main criteria such as

- Sector of the food business,
- Records of the food business in previous periods,
- Reliability of autocontrols of the food business,
- hygiene management of the food business are effective.

Official control of food in the provinces and districts is performed according to the 'Procedure on Official Control of Food and Administrative Sanctions (2022). Official controls of food establishments are carried out by a control team consisting of at least 2 (two) control officers for food production sites. However, if there is only 1 (one) control officer working within the branch directorate or authorised district directorate of the Provincial Directorate of Agriculture and Forestry, then 1 (one) control officer can perform the official control. In food sales and mass consumption places, official control can be performed by 1 (one) control officer. Control officers working in the General Directorate of Food and Control can carry out official controls in provinces/districts, as well as control officers working in the food and feed branch

directorates of the provincial directorates and control officers working in the delegated districts can carry out official controls in food establishments.

Official controls should be conducted with appropriate frequency and in proportion to the risk posed by the foodstuff. In accordance with the procedure for determining the frequency of risk-based audits of enterprises, official controls are carried out without prior notice (except for HACCP audits and public institutions and organisations such as prisons that cannot be entered without prior permission). In the HACCP audit, the food business is informed in advance.

Device calibrations are performed on time, calibration documents are recorded and kept in provincial/district directorates. Continuity of the use of equipment is ensured and in case of insufficient equipment during inspections, it is provided by the provincial/district directorate of the Republic of Turkey, Ministry of Agriculture and Forestry.

It is obligatory for the provincial/district directorate and laboratory directorate to establish the necessary conditions for sending the analysis and witness samples to the authorised laboratories under appropriate conditions and for the proper preservation of the witness sample. In order to ensure that the samples are transported at the appropriate temperature during shipment to the laboratory, to ensure sample safety and integrity and to prevent sample deterioration, a contract is made with the cargo company in which these issues are clearly stated.

The results of official controls of food establishments and related evaluations are entered into the Food Safety Information System (FSIS) on a daily basis. If the detection of a non-conformity requires official controls that exceed the normal control activities of the Ministry of Agriculture and Forestry of the Republic of Turkey, e.g. if a case related to human health is detected in any enterprise; in cases where the enterprise cannot or is judged to be unable to solve the problem alone, the costs arising from additional controls or analyses performed by the Ministry of Agriculture and Forestry of the Republic of Turkey and exceeding the routine control activities are paid by the natural or legal persons holding the products.

Normal control activities are routine control activities, including those determined by the multi-annual national control plan of the Republic of Turkey, Ministry of Agriculture and Forestry, as well as routine control activities and notifications and complaints deemed necessary under Law No. 5996. For example, if deficiencies are detected in an establishment as a result of an official control, follow-up inspections to be conducted at the end of the period given for the elimination of deficiencies are also considered within this scope.

Activities exceeding normal control activities are additional inspections to determine the scope of the problem and the studies required to determine or confirm whether corrective actions are performed by the operator to eliminate the problem. These studies include all kinds of controls, including sampling and analysis, to detect or prove non-compliance with the legislation or a non-conformity.

If the enterprise subject to the notification and complaint is in another province/district, the notification or complaint letter and its annexes are sent to the province/district directorate where this enterprise is located, provided that the information of the notification and complainant remains confidential. If the establishment subject to the notice and complaint is in another province/district, the notice or complaint letter and its annexes are sent to the province/district directorate where this establishment is located. The information of the notification and complainant is never disclosed to the food business operator complained about.

In case it is deemed necessary in the inspection of all kinds of food offered for sale through media organs such as television, newspapers, magazines, internet, radio, etc., the control officer may obtain the food material based on the sample through the provincial directorate and have the necessary analyses made. Regarding these products, in cases of doubt about their compliance with the food legislation, denunciation or complaint, etc., orders are placed with the approval of the provincial director for the supply of foodstuffs. For the

foodstuffs to be delivered, measures are taken by the control officers to conceal the identity of the provincial directorate. In order to ensure the retrospective traceability of these products and to ensure that the sample can be subject to administrative sanctions, all kinds of measures are taken by the control officers at all stages from the placing of the order to the receipt of the product from the cargo and sending it to the relevant laboratory as a sample.

In case of any uncertainty in practice, the provincial/district directorate contacts universities, public institutions and organisations, research institutions, real and legal entities, professional organisations and conducts research, but in cases where the desired result cannot be reached as a result of this study, the Republic of Turkey, Ministry of Agriculture and Forestry is asked for an opinion. These opinions may sometimes be repetitive. For this purpose, opinions are published under the legislation module in FSIS. The provincial directorate utilises this module before asking for an opinion.

As a result of the official control, the administrative sanction required by the non-conformity detected is applied.

### **Number of Enterprises within the Scope of Food-Related Inspection in Kastamonu Province**

In Kastamonu provincial centre and 19 districts of the province, food establishments within the scope of the audit are inspected by the relevant teams. It is important to identify food establishments, to distinguish them as production places, sales places and places of mass consumption, to determine the inspection standards in this way and to include them within the scope of inspection.

Table 1. Distribution of the Number of Food Establishments in the Districts of Kastamonu Province between 2016-2021

| <b>Number of Enterprises</b> | <b>2016 Year</b> | <b>2017 Year</b> | <b>2018 Year</b> | <b>2019 Year</b> | <b>2020 Year</b> | <b>2021 Year</b> |
|------------------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| Manufacturing Facilities     | 268              | 302              | 339              | 470              | 492              | 485              |
| Sales Facilities             | 927              | 1003             | 1107             | 1505             | 1562             | 1552             |
| Mass Consumption Facilities  | 798              | 900              | 1030             | 1680             | 1664             | 1577             |
| <b>Total</b>                 | 1993             | 2205             | 2476             | 3655             | 3718             | 3614             |

Source: Kastamonu Provincial Directorate of Agriculture and Forestry, 2022.

The number of food establishments in Kastamonu province for 2016-2021 is provided in Table 1. According to Table 1, there has been an increase in the number of establishments in terms of production, sales and mass consumption places over the years. In Kastamonu province, 3614 places, including 485 production places, 1552 sales places and 1577 public consumption places, are within the scope of food inspection as of 2021. Comparing 2016 and 2021, there has been almost a two-fold increase in the places covered by the audit. This case increases the need for food inspections.

### Statistics of Food Inspections Carried Out in Kastamonu Province

In Kastamonu province, within the scope of food inspections, production places, sales places and places of mass consumption were inspected by control teams between 2016-2021. It is seen that food inspections continue to increase in production, sales and public consumption places from 2016 to 2021.

In Table 2, the number of inspections performed is shown separately according to food business classes. Accordingly, it is observed that audits have increased almost every year in every subject within the scope of the audit. When 2021 is analyzed, it is seen that places of production were inspected 1120 times, places of sale 2280 times and places of mass consumption 2748 times. In 2021, the total number of inspections in all food business classes is calculated as 6148.

Table 2. Number of Inspections Conducted in Kastamonu Province

| Number of Inspections       | 2016 Year | 2017 Year | 2018 Year | 2019 Year | 2020 Year | 2021 Year |
|-----------------------------|-----------|-----------|-----------|-----------|-----------|-----------|
| Manufacturing Facilities    | 945       | 972       | 1028      | 1102      | 1111      | 1120      |
| Sales Facilities            | 1809      | 2050      | 2042      | 2155      | 2414      | 2280      |
| Mass Consumption Facilities | 2216      | 2509      | 2697      | 2881      | 2775      | 2748      |
| <b>Total</b>                | 4970      | 5531      | 5767      | 6138      | 6300      | 6148      |

Source: Kastamonu Provincial Directorate of Agriculture and Forestry, 2022.

### Determination of the Frequency of Food Inspections Realized in Kastamonu Province

The frequency of food inspections is determined as a result of the criteria for routine food inspections determined according to the food business class and the procedure for determining the frequency of risk-based inspections of enterprises that determine the number of food inspections. When calculated in terms of audit frequency, it is observed that the number of audits per enterprise has decreased.

Table 3. shows the number of inspections per food business per year. Changes are seen over the years. In 2021, it is seen that production places were inspected 2.31 times on average, sales places 1.47 times on average and places of mass consumption 1.74 times on average.

Table 3. Number of Inspections per Food Establishment in Kastamonu Province

| Number of Inspections       | 2016 Year | 2017 Year | 2018 Year | 2019 Year | 2020 Year | 2021 Year |
|-----------------------------|-----------|-----------|-----------|-----------|-----------|-----------|
| Manufacturing Facilities    | 3.53      | 3.22      | 3.03      | 2.34      | 2.26      | 2.31      |
| Sales Facilities            | 1.95      | 2.04      | 1.84      | 1.43      | 1.55      | 1.47      |
| Mass Consumption Facilities | 2.78      | 2.79      | 2.62      | 1.71      | 1.67      | 1.74      |
| <b>Total</b>                | 2.49      | 2.51      | 2.33      | 1.68      | 1.69      | 1.70      |

Source: Kastamonu Provincial Directorate of Agriculture and Forestry, 2022.

### Food Inspection Activities in Kastamonu Province by Center and Districts

In Kastamonu province, food inspections are carried out by teams in the center and 19 districts. In Kastamonu Provincial Directorate of Agriculture and Forestry Food and Feed Branch Directorate, 5 teams in charge of food and feed inspections inspect all food and feed enterprises in the province and its districts for compliance with legislation and procedures. In districts with control officers, inspections are performed by these units. In districts such as Pınarbaşı, Abana and İhsangazi, there are no control officers and food inspections are performed by inspection personnel in the center.

In Table 4, the food business inspection activities conducted in Kastamonu between 2016-2021 are grouped as production, sales and public consumption inspections and shown by Kastamonu Center and districts. As seen in Table 4, the most inspected places were places of mass consumption. Food inspections were mostly conducted in the central district of Kastamonu. It is observed that inspections continue to increase year by year for each food business class.

Table 4. Food Establishment Inspection Statistics by Kastamonu Province Center and Districts

| Districts          | Number of Inspections       | 2016 Year | 2017 Year | 2018 Year | 2019 Year | 2020 Year | 2021 Year |
|--------------------|-----------------------------|-----------|-----------|-----------|-----------|-----------|-----------|
| <b>Head office</b> | Manufacturing Facilities    | 391       | 421       | 447       | 546       | 490       | 493       |
|                    | Sales Facilities            | 668       | 689       | 744       | 736       | 886       | 874       |
|                    | Mass Consumption Facilities | 826       | 888       | 1061      | 1028      | 994       | 1010      |
| <b>Abana</b>       | Manufacturing Facilities    | 7         | 10        | 10        | 7         | 9         | 8         |
|                    | Sales Facilities            | 29        | 38        | 41        | 32        | 39        | 35        |
|                    | Mass Consumption Facilities | 59        | 88        | 87        | 83        | 64        | 72        |
| <b>Ağlı</b>        | Manufacturing Facilities    | 18        | 12        | 8         | 7         | 6         | 7         |
|                    | Sales Facilities            | 18        | 17        | 19        | 23        | 32        | 20        |
|                    | Mass Consumption Facilities | 21        | 23        | 18        | 17        | 28        | 16        |
| <b>Araç</b>        | Manufacturing Facilities    | 45        | 40        | 33        | 42        | 47        | 59        |
|                    | Sales Facilities            | 109       | 106       | 79        | 103       | 144       | 124       |
|                    | Mass Consumption Facilities | 129       | 112       | 87        | 93        | 92        | 101       |
| <b>Azdavay</b>     | Manufacturing Facilities    | 17        | 18        | 25        | 16        | 24        | 21        |
|                    | Sales Facilities            | 15        | 30        | 24        | 37        | 53        | 7         |
|                    | Mass Consumption Facilities | 33        | 44        | 57        | 60        | 99        | 112       |
| <b>Bozkurt</b>     | Manufacturing Facilities    | 18        | 30        | 22        | 14        | 22        | 22        |
|                    | Sales Facilities            | 53        | 40        | 46        | 65        | 61        | 53        |
|                    | Mass Consumption Facilities | 87        | 61        | 66        | 70        | 73        | 91        |
| <b>Cide</b>        | Manufacturing Facilities    | 28        | 28        | 36        | 25        | 28        | 32        |
|                    | Sales Facilities            | 128       | 131       | 149       | 173       | 123       | 143       |
|                    | Mass Consumption Facilities | 211       | 165       | 246       | 299       | 189       | 234       |
| <b>Çatalzeytin</b> | Manufacturing Facilities    | 13        | 19        | 20        | 19        | 22        | 14        |
|                    | Sales Facilities            | 22        | 51        | 62        | 52        | 67        | 64        |
|                    | Mass Consumption Facilities | 36        | 47        | 61        | 71        | 68        | 69        |
| <b>Daday</b>       | Manufacturing Facilities    | 25        | 30        | 30        | 31        | 32        | 30        |
|                    | Sales Facilities            | 31        | 52        | 33        | 49        | 43        | 41        |
|                    | Mass Consumption Facilities | 57        | 78        | 53        | 76        | 73        | 65        |
| <b>Devrekani</b>   | Manufacturing Facilities    | 49        | 28        | 34        | 35        | 51        | 53        |
|                    | Sales Facilities            | 49        | 61        | 57        | 58        | 76        | 55        |
|                    | Mass Consumption Facilities | 63        | 86        | 75        | 83        | 107       | 73        |
| <b>Doğanyurt</b>   | Manufacturing Facilities    | 4         | 7         | 5         | 4         | 10        | 8         |

|                  |                             |     |     |     |     |     |     |
|------------------|-----------------------------|-----|-----|-----|-----|-----|-----|
|                  | Sales Facilities            | 27  | 42  | 35  | 48  | 37  | 49  |
|                  | Mass Consumption Facilities | 32  | 45  | 40  | 48  | 51  | 49  |
| <b>Hanönü</b>    | Manufacturing Facilities    | 6   | 9   | 7   | 9   | 8   | 10  |
|                  | Sales Facilities            | 14  | 22  | 22  | 28  | 34  | 23  |
|                  | Mass Consumption Facilities | 20  | 32  | 26  | 31  | 16  | 30  |
| <b>İhsangazi</b> | Manufacturing Facilities    | 24  | 18  | 27  | 30  | 23  | 25  |
|                  | Sales Facilities            | 22  | 28  | 25  | 22  | 20  | 33  |
|                  | Mass Consumption Facilities | 24  | 35  | 24  | 20  | 28  | 32  |
| <b>İnebolu</b>   | Manufacturing Facilities    | 65  | 62  | 49  | 52  | 63  | 54  |
|                  | Sales Facilities            | 140 | 177 | 114 | 129 | 144 | 127 |
|                  | Mass Consumption Facilities | 150 | 206 | 152 | 151 | 164 | 154 |
| <b>Küre</b>      | Manufacturing Facilities    | 8   | 14  | 12  | 14  | 22  | 13  |
|                  | Sales Facilities            | 20  | 30  | 23  | 25  | 27  | 41  |
|                  | Mass Consumption Facilities | 29  | 43  | 35  | 44  | 32  | 41  |
| <b>Pınarbaşı</b> | Manufacturing Facilities    | 17  | 24  | 36  | 19  | 19  | 29  |
|                  | Sales Facilities            | 20  | 18  | 17  | 23  | 27  | 20  |
|                  | Mass Consumption Facilities | 20  | 24  | 18  | 41  | 33  | 35  |
| <b>Seydiler</b>  | Manufacturing Facilities    | 15  | 12  | 25  | 22  | 29  | 25  |
|                  | Sales Facilities            | 20  | 27  | 22  | 36  | 42  | 37  |
|                  | Mass Consumption Facilities | 28  | 32  | 22  | 38  | 46  | 28  |
| <b>Şenpazar</b>  | Manufacturing Facilities    | 3   | 4   | 5   | 6   | 4   | 5   |
|                  | Sales Facilities            | 30  | 33  | 60  | 42  | 40  | 36  |
|                  | Mass Consumption Facilities | 17  | 30  | 39  | 36  | 34  | 28  |
| <b>Taşköprü</b>  | Manufacturing Facilities    | 109 | 118 | 114 | 116 | 122 | 114 |
|                  | Sales Facilities            | 123 | 170 | 168 | 178 | 199 | 166 |
|                  | Mass Consumption Facilities | 156 | 197 | 238 | 269 | 278 | 248 |
| <b>Tosya</b>     | Manufacturing Facilities    | 83  | 68  | 83  | 88  | 82  | 98  |
|                  | Sales Facilities            | 271 | 288 | 302 | 296 | 325 | 332 |
|                  | Mass Consumption Facilities | 218 | 273 | 292 | 323 | 286 | 260 |

Source: Kastamonu Provincial Directorate of Agriculture and Forestry, 2022.

### Studies on Complaints in Kastamonu Province

According to the risk-based inspection plan determined by the Ministry of Agriculture and Forestry, food production, food sales and mass consumption enterprises in the province and district are subject to inspections at regular intervals of 6-8 months. In addition, in line with the complaints received by the Ministry of Agriculture and Forestry, ALO 174 Food Hotline and the Republic of Turkey, Presidential Communication Center (CIMER), inspections are carried out to the complained enterprise(s) within the legal period.

As seen in Table 5, there are fluctuations in the complaints received by the ALO 174 Food Hotline from year to year. The number of complaints received through CIMER is low. However, the number of complaints received in this way has increased.



Table 5. Number of Complaints to 174 ALO Food Line and CIMER in Kastamonu Province by Years

|                   | <b>2016 Year</b> | <b>2017 Year</b> | <b>2018 Year</b> | <b>2019 Year</b> | <b>2020 Year</b> | <b>2021 Year</b> |
|-------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| 174 ALO Food Line | 122              | 127              | 117              | 155              | 185              | 143              |
| CIMER             | 9                | 13               | 11               | 26               | 54               | 23               |
| <b>Total</b>      | 131              | 140              | 128              | 181              | 239              | 166              |

Source: Kastamonu Provincial Directorate of Agriculture and Forestry, 2022.

### **Fines Imposed as a Result of Inspections in Kastamonu**

The results of planned inspections conducted in Kastamonu city center and districts and the results of inspections carried out upon complaints are shown in Table 6. According to Table 6, 35 administrative fines were imposed in 2016, 41 in 2018, 25 in 2019, 34 in 2020 and 27 in 2021.

Table 6. Number of Administrative Fines in Kastamonu Province by Years

| <b>2016 Year</b> | <b>2017 Year</b> | <b>2018 Year</b> | <b>2019 Year</b> | <b>2020 Year</b> | <b>2021 Year</b> |
|------------------|------------------|------------------|------------------|------------------|------------------|
| 35               | 24               | 41               | 25               | 34               | 27               |

Source: Kastamonu Provincial Directorate of Agriculture and Forestry, 2022.

### **CONCLUSIONS**

With the better understanding that the easiest way to ensure food safety of individuals and to protect public health can be realized through access to safe food, paying attention to food inspections has been one of the issues that have become more important in recent years. In addition, important legislative arrangements have recently been made in order to increase the effectiveness of food inspections. In particular, negotiations with the EU have accelerated concrete steps in this regard.

Accurate and consistent food inspections are one of the priorities in ensuring food safety of individuals and society. The work of the Ministry of Agriculture and Forestry of the Republic of Turkey, both at the central and provincial level, should include effective food inspections to ensure that consumers have access to safe food. Food inspections are performed by provincial/district Directorates of Agriculture and Forestry with limited teams but with great effort and dedication. It is seen that inspections have become more frequent with the increase in the level of awareness of consumers about food, their ability to report faulty/incorrect issues about food and their inclusion in the system.

In recent years, it is known that the scope of food inspections has been expanded with the legislative regulations and the number of enterprises included in the scope of inspections has increased. Based on the province of Kastamonu, it is seen that the number of food establishments included in the scope of inspection has increased. This case has naturally increased the number of inspections. Although there is not a proportional increase in the frequency of business inspections, the fact that inspections have been increased throughout the province/district shows that the enterprises provide better self-control, and on the other hand, it shows that the number of penalized enterprises has decreased despite the increasing number of enterprises within the scope of inspection. In a way, the increase in inspections and the

awareness that inspections will be performed act as a deterrent to counterfeiting in this field. As can be seen, the fact that inspections are expected to increase has reduced food fraud in statistics.

Another conclusion is that the food inspection process has gone through various stages in terms of shaping food inspection procedures and their functionality in practice. When statistics are calculated, it is seen that current procedures ensure that food inspection processes are performed in the most effective way today. However, the fact that the understanding of food safety is open to change and food inspection procedures may change in the future with this understanding, it should not be neglected that food inspection legislation should be renewed every period with both EU negotiations and the realities of the country.

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## **FUNCTIONAL FOOD - PRODUCTS FROM TOMATO WITH LYCOPENE IN PREVENTION OF CARDIOVASCULAR DISEASES**

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### **ABSTRACT**

Tomato, as a functional food product, includes a source of lycopene that serves to prevent cardiovascular disease. This study aims to perform a comparative analysis of the microbiological and chemical characteristics of different tomato products available to buy in the area of Mostar, Bosnia, and Herzegovina. Tomato products for analysis were randomly sampled and purchased from shopping centers in the Mostar area from three different producers: Sample no. 1: "Passata" - sterilized tomato paste, producer "Podravka d.d." Country of origin: Croatia, Sample no. 2: "Russo" - tomato paste, producer "AR Industrije Alimentari S.p.A", country of origin: Italy and Sample no. 3: "Sava Semberija" - tomato juice producer "Sava Bijeljina" country of origin: Bosnia and Herzegovina. The following parameters were included in the microbiological analysis: Salmonella, coagul. pos. staphylococci, sulph. red. Clostridia, Proteus species, Escherichia coli, and total bacterial count. Chemical analyses were based on the determination of lycopene content and artificial colours. The results were compared with the provisions of the Rulebook on the conditions regarding chemical and microbiological safety, which food products must meet in transport, Official Gazette of the Republic of BiH 2/92, Rulebook on microbiological criteria for food (Official Gazette of BiH No. 11/13), as well as with literature sources. The obtained results indicate the significant nutritional value of the samples in our diet, especially regarding human health and the prevention of cardiovascular diseases. For instance, lycopene is an antioxidant that cannot be created naturally by the human body, as it is exclusively of plant origin.

**Keywords:** tomato, lycopene, cardiovascular disease, prevention

### **INTRODUCTION**

Due to the humanization and modernization of work, the physical activity of man is reduced, which enables disorders of the overall physiology and circulation of substances in the body that results in various diseases. Genetic factors certainly have an impact on cardiovascular diseases. Many researches today prove that it is a disease that can be predicted accurately and therefore prevented. One of the essential steps in prevention is proper nutrition.

It is not unknown that regular consumption of fruits and vegetables during the day has a very beneficial effect on health, so consuming at least five portions of fruits and vegetables per day is recommended. By the new lifestyles, the "Mediterranean diet," which is characterized by a significant intake of fruits and vegetables, monounsaturated and fatty acids, primarily of olive and olive oil origin, is being increasingly promoted in the world today. One of the crucial components of the Mediterranean diet is the tomato, both fresh and processed, and it is an indispensable part of traditional dishes.

Tomatoes have a low energy value (only 18 kcal per 100 g), given that they contain 94% water, 2.63% carbohydrates, 0.88% protein, 0.2% fat, and 1.2% dietary fiber. It is one of the richest natural sources of vitamin C. It also contains significant amounts of vitamin B, E, and K. It is rich in potassium, sodium, magnesium, calcium, iron, and trace elements. Tomatoes are the richest in copper and contain more iron than chicken, fish, and milk. Tomatoes and tomato products are an important source of carotenoids in the daily diet (600 µg/100 g). Of about 600 different carotenoids, alpha-carotene, beta-carotene, lutein, zeaxanthin, and lycopene stand out as one of the most important representatives.

**Table 1.** Lycopene content in fresh tomato fruits by the degree of maturity

| Samples                          | Lycopene (mg/100g of fresh sample) |
|----------------------------------|------------------------------------|
| Fresh tomato (October - March)   | 2,60-3,10                          |
| Fresh Tomato (June - August)     | 3,80-6,60                          |
| Green tomato juice               | 0,17                               |
| Juice of partially ripe tomatoes | 0,24                               |
| Ripe tomato juice                | 3,71                               |

(Heinonen i sar.,1989., Beerh i Siddappa, 1959.)

Lycopene is the dominant carotenoid in human plasma after consumption of tomatoes or its products (Agarwal and Rao, 2000). Many clinical studies have linked high tomato intake and high tissue lycopene levels to a reduced risk of several types of cancer, especially prostate cancer (Giovannucci et al., 2002; Canene-Adams et al., 2005). The antiproliferative activity of tomatoes has been proven in several in vitro tests on different tumor cell lines, such as prostate, lung, breast, and cervical tumors (Boivin et al., 2009; Choi et al., 2011). Several epidemiological studies have shown that increased levels of lycopene in the blood plasma are associated with a reduced risk of developing cardiovascular diseases, especially with reduced levels of LDL cholesterol in the blood (Ried and Fakler, 2011; Böhm, 2012). However, consumption of lycopene preparations alone did not show the same beneficial effects as consumption of fresh tomatoes or tomato products (Basu and Imhran, 2007).

Lycopene and phenolic compounds are more resistant to heat treatments, representing the primary antioxidants in tomato products (Navarro-González et al., 2011). Many studies have shown that the intake of lycopene from thermally processed tomato products is higher, which is explained by the increased bioavailability of lycopene due to the breakdown of cell walls, the presence of fat in food, as well as isomerization from the all-trans to the cis conformation under the influence of heat (Agarwal and Rao, 2000).

This diet is associated with a positive effect on human health, first of all, reducing the risk of chronic diseases and, first of all, cardiovascular systems (Goñi and HervertHernández, 2011; Pinela et al., 2012; Di Lecce et al., 2013).

The work aimed to examine the microbiological and chemical characteristics with particular reference to the content of lycopene in three different types of ready-made tomato products, compare the results with the parameters of the current Rulebook and literature extracts, and evaluate the acceptability of the product for potential future consumers on the free market, with the tabular presentation of analysis results.

## MATERIAL AND METHOD

The complete work was done in terms of microbiological and chemical analysis at the Agromediterranean Faculty in Mostar and the Institute for Public Health of the Federation of Bosnia and Herzegovina, the Health Ecology Service in Sarajevo.

The tomato products that were analyzed were bought in shopping centers in a retail store in the area of the city of Mostar, where three samples from different producers were selected.

Sample 1: "Passata" - sterilized puréed tomato, country of origin Croatia, Manufacturer "Podravka d.d."

Sample 2: "Russo" - puréed tomato, country of origin: Italy, Manufacturer "AR Industrie Alimentari S.p.A"

Sample 3: "Sava Semberija" - tomato juice, Country of origin: Bosnia and Herzegovina, Manufacturer "Sava Bijeljina"



Sample 1.



Sample 2.



Sample 3.

Sampling was done by random selection.

The samples were subjected to chemical analyzes that included the following parameters:

1. Determination of lycopene content expressed in g,
2. The presence of artificial colors

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1. Determination of lycopene content expressed in g,
2. The presence of artificial colors.

To meet the goal of the research within the framework of chemical analysis, the following methods were used:

1. Determination of lycopene content mg/100g - spectrophotometrically at 468 nm.
2. Presence of artificial colors - color identification by the chromatographic method.

The microbiological analysis of all three samples included research on *Salmonella* and *Escherichia coli*, *Coagul. pos. staphylococci*, *sulph. red. Clostridia*, *Proteus* species, and a total number of bacteria were made per food safety criteria for cut fruits and vegetables.

## RESULTS AND DISCUSSION

The research was conducted on tomato products purchased in various stores in the city of Mostar. Analyzes included: chemical and microbiological, and the obtained results were

compared with existing legal regulations, on the basis of which we can talk about quality and recommendations for use without danger to the health of the population.

In table no.2, the obtained results of the chemical analysis of the lycopene content in tomato products indicate that the lycopene content in sample number 1 was recorded in the highest presence of 12.52 mg/100g, and the lowest in sample number 3 in 6.56 mg/100g. The obtained results were compared with parameters from literature sources. It can be concluded that sample 1 and sample 3 are in accordance with the literature excerpts of Tavares and Rodriguez-Amaya, 1994 and Lindner et al., 1984, while sample number 2 shows somewhat lower values for 0.16 mg/100g compared to the literature extract of Tavares and Rodriguez-Amaya, 1994.

Compared with the content of lycopene in fresh tomato fruits (table 1), the chemical analysis determined that all three analyzed samples of ready-made tomato products have a significantly higher lycopene content.

**Table 2.** Lycopene content in tomato products

| Analyzed samples | Unit of measure mg/100g | Results of literature extracts | Authors                         |
|------------------|-------------------------|--------------------------------|---------------------------------|
| Sample 1         | 12,51                   | 8,93-19,37                     | Tavares i Rodriguez-Amaya, 1994 |
| Sample 2         | 8,77                    | 8,93-19,37                     | Tavares i Rodriguez-Amaya, 1994 |
| Sample 3         | 6,56                    | 5,8-9,0                        | Tavares i Rodriguez-Amaya, 1994 |

Lycopene is a powerful antioxidant, ten times more potent than vitamin E. Studies have shown that adding lycopene, 7 mg per day, for two months, can normalize the function of the endothelium in patients with cardiovascular disease. The endothelium is a thin layer of cells located on the inner surface of blood vessels. Endothelial dysfunction is associated with atherosclerosis, which can increase the risk of heart attack and stroke. Based on the above, it can be concluded that all three analyzed samples are significant in terms of lycopene content, which can provide potential consumers with an appropriate level of prevention in cardiovascular diseases.

Many studies have proven that the concentration of lycopene in the plasma is higher when food is ingested into the body based on thermally processed tomatoes, in contrast to the concentration after the intake of unprocessed tomatoes (Böhm and Bitsch 1999). It can be concluded that the analyzed finished products from tomatoes are a rich source of lycopene for the human body; from availability, its protective role in the prevention of many cardiovascular and other diseases will also depend.

**Table 3.** Results of chemical analyzes of the presence of artificial colors in tomato products

| Analyzed product samples | Presence of artificial colors |
|--------------------------|-------------------------------|
| Sample no.1              | /                             |
| Sample no.2              | /                             |
| Sample no.3              | /                             |

The obtained results of the chemical analysis of the presence of artificial colors in tomato products indicate that the presence of artificial colors was not proven in any of the analyzed samples, per the current Rulebook.

**Table 4.** Results of microbiological analysis of SAMPLES 1, 2 and 3

| Microorganism                    | Unit of measure | Test result | MDK |
|----------------------------------|-----------------|-------------|-----|
| Salmonella                       | CFU / 25g ( ml) | 0           | /   |
| Coagulase-positive staphylococci | CFU / g ( ml)   | 0           | /   |
| Sulfite-reducing Clostridia      | CFU / g ( ml)   | 0           | /   |
| Proteus species                  | CFU / g ( ml)   | 0           | /   |
| Escherichia coli                 | CFU / g ( ml)   | 0           | /   |
| Total number of bacteria         | CFU / g ( ml)   | 0           | /   |

The obtained results of microbiological analysis on the presence of Salmonella species, coagulase-positive staphylococci, sulfite-reducing Clostridia, Proteus species, Escherichia coli, and the total number of bacteria show that not a single analyzed species were isolated on any of the analyzed samples, which indicates that all analyzed parameters are in accordance with the valid Rulebook on microbiological criteria for food Official Gazette of BiH No. 11/13. and from the aspect of hygienic and sanitary correctness, there is no apprehension about the consumption of the same by potential consumers.

## CONCLUSIONS

The task of this scientific research work was to examine the quality of three different types of ready-made tomato products in terms of lycopene content, the presence of artificial colors, as well as microbiological correctness, and to compare the obtained results with valid Rulebooks and literature sources.

All analyzes were done at the Agromediterranean Faculty in Mostar and the Institute for Public Health of the Federation of Bosnia and Herzegovina, Service for Health Ecology in Sarajevo. The results of the chemical analysis of the lycopene content in tomato products indicate that the lycopene content in sample number 1 was recorded at the highest level of 12.52 mg/100g and the lowest in sample number 3 at 6.56 mg/100g. The obtained results were compared with parameters from literature sources, and it can be concluded that samples 1 and sample 3 follow the literature excerpts of Tavares and Rodriguez-Amaya, 1994 and Lindner et al., 1984, while sample number 2 shows somewhat lower values of 0.16 mg/100g compared to the literature extract of Tavares and Rodriguez-Amaya, 1994.

In comparison with the content of lycopene in fresh tomato fruits (table 1), the chemical analysis determined that all three analyzed samples of ready-made tomato products have a significantly higher content of lycopene.

The results of the chemical analysis of the presence of artificial colors in tomato products indicate that the presence of artificial colors was not proven in any of the analyzed samples, which is in accordance with the current Rulebook.

The results of the microbiological analysis show that not a single analyzed species was isolated on any of the samples, indicating that all analyzed parameters are in accordance with the valid Rulebook on microbiological criteria for food Official Gazette of BiH number 11/13. and from the aspect of hygienic and sanitary correctness, there is no apprehension about the consumption of the same by potential consumers.

Results indicate that all three analyzed samples have a significant nutritional value in terms of lycopene content; as such, they should have an essential place in our diet and the prevention of cardiovascular diseases. Lycopene is less available from the raw material (Gärtner et al., 1997).



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## PRELIMINARY REVIEW AND EVALUATION PROCESS OF ENVIRONMENTAL IMPACT ASSESSMENT REGULATION

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### ABSTRACT

EIA Regulation, which was published by the Ministry of Environment, Urbanization and Climate Change on 7th of February, 1993 and had various revisions over time, was updated with the regulation numbered 31907 on 29th of July, 2022, within the scope of green development goals. With this regulation, the Environmental Impact Assessment Regulation published in the Official Gazette dated 25/11/2014 and numbered 29186 was repealed. With the context of Environmental Impact Assessment (EIA) Regulation, many plans such as Zero Waste, Greenhouse Gas Mitigation, Impacts on Climate Change, Environmental Monitoring, Environmental and Social Management are required to be included in the EIA reports under "Sustainability Plan". With the arrangements for informing the public and increasing their participation in the process (art. 9) are included in the Stakeholder Engagement Plan, the number of communication ways in participation were increased. Considering the environmental impacts of the activities/projects, EIA lists have been adjusted. In this scope, the number of activities for which it is required to prepare an EIA Report has been increased. Some of the sectors were also included in the list of projects that will be fully implemented, regardless of the threshold value. Projects that require an EIA report are listed in Annex-1 of the regulation. For the activities/projects included in the list of the projects whose environmental impacts are subject to preliminary examination and evaluation, it is obligatory to conduct a cumulative impact assessment, prepare an environmental and social action plan, and prepare a sustainability and environmental monitoring plan for a more comprehensive and detailed examination of their environmental impacts. In this study, the application and examination process of the projects whose environmental impacts are subject to preliminary examination and evaluation within the scope of the Annex-2 list of the updated EIA regulation, which has a much higher rate in Turkey, and the process of deciding whether an EIA report is required or not are explained.

**Keywords:** Environmental Impact Assessment, EIA Legislation, EIA Regulation

### INTRODUCTION

EIA Regulation, which was published by the Ministry of Environment, Urbanization and Climate Change on 7th of February, 1993 and had various revisions over time, was updated with the regulation numbered 31907 on 29th of July, 2022, within the scope of green development goals. With this regulation, the Environmental Impact Assessment Regulation published in the Official Gazette dated 25/11/2014 and numbered 29186 was repealed.

For the activities/projects included in the list of the projects whose environmental impacts are subject to preliminary examination and evaluation, it is obligatory to conduct a cumulative impact assessment, prepare an environmental and social action plan, and prepare a sustainability and environmental monitoring plan for a more comprehensive and detailed examination of their environmental impacts.

In this study, the application and examination process of the projects whose environmental impacts are subject to preliminary examination and evaluation within the scope of the Annex-2 list of the updated EIA regulation, which has a much higher rate in Turkey, and the process of deciding whether an EIA report is required or not are explained.

## **RESULTS**

Projects whose environmental impacts are subject to preliminary examination and evaluation;

- A) Projects specified in Annex-2 list of the regulation (Article 15/1a).
- B) In case capacity increase and/or expansion is planned for the projects that are considered out of scope or that are exempted from the law, the new capacity of the project together with the sum of the existing project capacity and capacity increases, the projects specified in the list in Annex-2 (article 15/1b).

are responsible for having the Project Introduction File for their Project Subject to Preliminary Review and Evaluation of Environmental Impacts, prepared by the institutions/organizations authorized by the Ministry, ensuring that it is submitted to the relevant authority and complying with the commitments they have made within the scope of the project.

## **DISCUSSION**

### **Application and review process**

In order to examine the environmental impacts of the projects defined in Article 15, by the institutions/organizations authorized by the project owner by proxy and authorized by the Ministry; Project introduction file is prepared according to the format in Annex-4. The project introduction file prepared, indicates that the information and documents in the project introduction file and its annexes are correct, and are submitted to the Ministry together with a letter of commitment approved by the project owner and a receipt showing that the application fee has been paid (Article 16/1).

Ministry examines project introduction file prepared for project in the format given in Annex-4 within 5 working days.

- A) The EIA review and evaluation process is initiated regarding the project subject to the project introduction file, which is determined to be prepared in accordance with the format (article 16/2).
- B) The project introduction file, which is determined not to be prepared in accordance with the format, is returned for completion.
  - If it is not submitted within the specified time, the EIA process is terminated.
  - The completed file is submitted to the Ministry within 15 calendar days.

In the projects determined to be prepared in specified format and regarding which EIA examination and evaluation process has started (Article 16/3);

- A) In cases where it is not necessary to take the opinion of the institution/organization and hold a meeting, the prepared file is found to be sufficient and similar cases, the EIA examination and evaluation process is completed by the Ministry within 15 working days and the decision phase is started.
- B) If deemed necessary by the Ministry, a written opinion may be requested from the relevant institutions/organizations or a meeting may be held with the relevant institutions/organizations, taking into account the information in the file. If a written opinion is requested, the opinion of the institution/organization that does not submit an opinion within 30 calendar days from the date of request for opinion, and in case of a meeting, within 30 calendar days from the date of the meeting, is accepted as positive. If additional time is needed to express an opinion, the request is submitted to the Ministry in writing by the relevant commission member. Additional time requests of institutions/ organizations are taken into consideration by the Ministry.
- C) If deemed necessary by the Ministry, the authorized institutions/organizations may be asked to provide comprehensive information about the project, to provide equipment, to carry out analysis, experiments and measurements or to have them done.

By the Ministry, the deficiencies identified in the file or the information/documents that should be included and the opinions of the institution/organization received are forwarded in writing to the authorized institution/organization. Deficiency notification can be made by the Ministry for maximum 3 times. The project introduction file prepared by the competent institution/organization in line with the opinions conveyed by the Ministry is submitted to the Ministry within 6 months. This period represents the total time to be used by authorized consultant firms.

- If the project introduction file is submitted within the specified time and the information/documents are found to be sufficient, the decision stage is started.
- EIA process is terminated if the returned project introduction file is not submitted within the period or if the correction is not deemed sufficient.

## **CONCLUSION**

The Ministry decides on whether "EIA is Required" or "EIA is Not Required" within 5 working days about the project, which has passed the decision stage after the review and evaluation process has been completed. The decision made is notified to the provincial directorate, the institutions/ organizations whose opinion is received, the project owner and the institutions/organizations that have been qualified by the Ministry are informed. This decision is announced to the public by the provincial directorate on the website for an indefinite period of 30 calendar days (Article 17/1).

- A) If the investment is not started without a force majeure within 5 years with regards to the project for which decision is taken that "EIA is not Required" decision, the "EIA Not Required" decision is deemed invalid (Article 17/2). and if the request is deemed appropriate by the Ministry, it shall be deemed invalid. Decisions deemed invalid in this way are announced on the website by the Ministry and the provincial directorate (Article 17/3).

B) For the projects for which the "EIA is Required" decision is taken, an EIA application file should be prepared and an application should be made to the Ministry. However, if the basic conditions for the "EIA is Required" decision change, a re-application can be made in accordance with the provisions of Article 15 (Article 17/4).

It monitors, controls and inspects whether the issues committed in the project introduction file, which is the basis of the decision of "EIA Not Required", are fulfilled. The Ministry checks the accuracy of the works and processes specified in the project progress reports (Article 18/1). The Ministry cooperates with relevant institutions/organizations when it deems necessary while performing these duties (Article 18/2). After taking the decision of "EIA is not Required", the project owner is obliged to notify the planned changes in the project subject to the EIA Regulation to the Ministry or the relevant provincial directorate (Article 19).

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## POSSIBILITIES OF USING WIND TURBINE ENERGY SYSTEM IN VERMICOMPOST FERTILIZER PRODUCTION FACILITIES

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### ABSTRACT

The shrinkage of agricultural areas in the world due to various reasons, the increase in population and the increase in people's living standards have increased the need for agricultural products. As a solution to this situation, studies are carried out to get more products per unit area in agriculture. The excessive use of agro-chemicals, which provide significant increases in plant production, quality and yield, has caused environmental and health problems in the following years. Achieving an increase in productivity in agricultural production is only possible with energy consumption. In the production of vermicompost, electrical energy obtained from fossil energy sources, whose price is constantly increasing, is used. The aim of this study is to show that wind energy, one of the renewable energy sources, can be used in vermicompost production facilities. This can only be feasible in regions with wind energy potential, such as the Marmara Region. The use of vermicompost (worm) fertilizer can be effective and beneficial in reducing the use of excessive inorganic fertilizers and pesticides in agriculture and in solving problems such as organic waste and residues. In a good vermicompost production facility, the highest energy required by the facility, where there are 4 of 15.9 m long fertilizer production units and a drying system of 200-m<sup>2</sup>, is 4.48 kWh. Since wind energy varies randomly depending on climatic conditions, a wind turbine system such as 6.0 kWh should be used, which is 25-30% higher than the required 4.48 kWh. The remaining electrical energy from the system is stored in batteries for later use or can be used in different places where it is needed in the production facility.

**Keywords:** Wind energy, Wind turbine, Electrical energy, Vermicompost (worm manure), Vermicompost (worm manure) production facility

### INTRODUCTION

Acreage of agricultural lands has been significantly decreasing year by year as they are used for a number of reasons other than their intended purposes. In other words, these agricultural lands cannot be used for their intended purposes since they are used for residential purposes as well as road, bridge and dam constructions and since these lands become arid after improper cultivation. On the other hand, population continues to grow and people have higher living standards and these factors increase the demand for agricultural products. The solution seems to be producing more and high-quality products per unit area. In agriculture, yield from the land

and quality have increased significantly with the cultural measures taken and use of new, quality and improved seeds as well as agrochemicals. However, intensive use of fertilizers and agricultural pesticides has a number of impacts on the environment and health. Residues of agrochemicals, which can contaminate drinking and domestic waters and accumulate on the products, emerge as a problem. Thus, use of chemicals in agricultural production must be restricted, and healthy and safe food production must be assured. In this way, we must prevent return of these toxic substances back to the humans and other living creatures through the food chain (Saber, 2001; Broun and Supkoff, 1994; Çakmakçı et al., 2005; Kitiş, 2012). Intensive use of agrochemicals in the last fifty years caused a number of environmental and health problems such as contamination of underground and surface waters. Furthermore, increase in the quantity of solid organic wastes and residues is another environmental problem caused by rapid increase in population, industrial developments and higher level of welfare. Production and use of Vermicompost (worm) manure in agriculture might help solving problems by reducing use of agricultural pesticides and inorganic fertilizers in agriculture (Yüksel-Türkboyları and Yüksel, 2021).

There are studies focusing on development of organic based alternative products to be used instead of agrochemicals, in other words instead of chemical manures and pesticides. These products are aerobic composts and, in addition to their plant nutrition properties, they are known to minimize diseases and harmful effects of soil borne plant pathogens (Hoitink et al., 1975; Hadar, 1991).

Urban wastes and residues, which have become an alarming environmental problem in parallel with the level of development and urbanization, are the other factors that contributed to the acceleration of compost practices after the 1980s. Producing compost seems to be a cost-effective, sustainable and eco-friendly option for processing such wastes and residues (Şimşek-Erşahin, 2007). Studies on compost concluded that Vermicompost -worm (mesophilic) compost method- has properties superior to aerobic compost in the field of recovering urban and industrial organic wastes, in terms of product and processing (Dominguez et al., 1997). The production process of Vermicompost is much shorter than the thermophilic compost.

When it comes to the product quality, compared to the thermophilic compost products, vermicompost has much more superior physical, chemical and biological properties and economic value (Şimşek-Erşahin, 2007).

In case of worm manure or vermicompost, composting process of organic wastes or residues is completed by worms. In this process, the organic wastes and residues are fermented by the microorganisms existing in the environment. When passing through the digestive system of worms, an accelerated humidification and detoxification process takes place. Coelom fluid in the digestive tract of worms has the capacity to transform all inorganic materials into organic forms (Tutar, 2013).

Today, vermicompost offers the highest economic benefits out of all methods supporting sustainability in agriculture. This method might be extensively used for repurposing solid organic wastes and residues which have become an alarming environmental concern due to rapid industrial development and population growth. The vermicompost manure, if produced correctly and properly, will be a bio-fertilizer and bio-pesticide offering a very high commercial value (Şimşek-Erşahin, 2007).

This study focuses on the possibility of providing energy need of the vermicompost production facilities, which have an extensive area of application, with the wind turbine systems since it is a renewable energy resource. If the wind power is used to cover energy costs at vermicompost production facilities, the products to be obtained can be more cost-effective. Furthermore, losses of production caused by the power outages in the mains supply might be avoided.



Marmara Region and Tekirdağ Province have the best wind potential in Turkey and this is why this region was chosen for the project.

As a result of its geographical location, Turkey, compared to several other countries, has more advantageous wind and solar energy potential. The initial investment cost of these systems are high. However, elimination of raw material and operating costs lowers the costs of generating energy. There are studies indicating that using renewable energy resources in agricultural businesses might be cost-effective (Küsek et al., 2016; Yüksel and Yüksel-Türkboyları, 2018; Orhan and Şahin, 2022).

### ***Wind Power Potential in Turkey***

In Turkey, cyclones and anti-cyclones are always observed between the cold Black Sea & North Asia Steppe and the hot Aegean and Mediterranean Regions. The differences between these cyclones and anti-cyclones cause strong and continuous winds along the Thrace, South Marmara, Aegean and Mediterranean shores (Koçarslan, 2010).

According to the average seasonal readings, wind speed reaches the highest levels along the shorelines and round the Marmara Sea in winter (Tunus, 2019).

Solar radiation warming up the earth surfaces at different levels causes the wind. Surfaces warmed at different levels lead to differences in air temperature, humidity and in atmospheric pressure. These differences, particularly the difference in the atmospheric pressure, enable circulation of the air and create wind. The wind moves from an anticyclone to a cyclone. Differences in local geography and non-homogenous distribution of heat on earth determine the wind characteristics. These characteristics change depending on time and location. The wind speed increases at heights and its theoretical power increases in proportion to the cube of its velocity (Anonymous, 2022a).

Winds blowing from the west and south will be more humid and hotter whereas winds blowing from the east and north will be dry and lower in temperature (Can, 2020).

The velocity of wind changes constantly and this fluctuates the energy carried by the wind. Therefore, efficiency of turbines vary with the wind power and the efficiency of energy generation will be less than the installed capacity (Can, 2020). A wind turbine can generate electric power between the wind velocities known as the cut-in and cut-out. If the wind velocity is less than the initial velocity known as the cut-in and required for operating the wind turbine, which is 2-4 ms<sup>-1</sup>, the wind turbine cannot generate electric power. If the wind velocity is more than the cut-out velocity, namely more than 25-35 ms<sup>-1</sup>, the system should stop automatically so the wind turbine will not be damaged. The nominal wind velocity, which is the optimum velocity for a wind turbine, is between 10-15 ms<sup>-1</sup> (Anonymous, 2022a).

## **MATERIALS AND METHODS**

### ***Wind Turbine and Vermicompost Production System***

The wind turbines are the primary structural elements of wind power plants; they are the machines that transform the kinetic energy of circulating air into mechanical energy at first and then to the electric power (Anonymous, 2022a).

Depending on the application, the following items might be the structural elements of a wind turbine system: wind turbine, battery, battery charge control unit (charge regulator), inverter, miscellaneous electronic circuits and operations center (Toprak, 2011; Şenel and Koç, 2015). In this system, a wind turbine having the required capacity will be used as a source of energy. The supply and demand are mismatched when it comes to wind power; in other words, energy

might not be generated when needed and therefore, batteries must be integrated into the system. The battery will provide energy to the system when there is no wind but the system needs energy. Generally, the system uses GEL (gelled electrolyte sealed lead acid) batteries. A charge regulator is added to the system for preventing overcharge or discharge of the batteries. This addition increases the lifecycle of batteries and lowers the investment costs. Depending on the battery condition, the charge regulator interrupts the current coming from the wind turbine or current coming from the load. If the system will use 220 V alternating current or if the current is to be provided to mains supply, in other words, if the system is to be used as on-grid, the system must have an inverter (Toprak, 2011; Şenel and Koç, 2015).

In addition to small scale production on soil surface and beds designed in various ways, the vermicompost production might be on a commercial and industrial scale. Compared to the production on floor beds, industrial production of worm manure will save on manpower. The products will have better quality and will be cleaner. Reproduction and production activities of the worms will increase. Loss of worm in the system is lesser. The production process will be monitored properly; feeding and harvest will be at the optimum times.

At the moment, length of a worm manure production facility might be between 2.5-meter to 20-meter for commercial production. Its width and height are around 1.30 m. After being produced by the worms on these beds, the manure will be cut from the bottom with the double-bladed knife placed 10 to 15-cm above and it will be poured on the conveyor band installed below. The knife will repeat this process a few times since the layer cut from the bottom is not very thick. Then, the cut manure will be collected in one side of the manure machine as the conveyor band is operated. An electric motor with a capacity of 2.2 kWh is required for operating the double-bladed knife and 1.1 kWh is required for operating the conveyor band in the vermicompost production facility.

Electricity generated with the wind turbine system can be used in the vermicompost production facility and this will significantly save on energy used for production activities (Figure 1). In Figure 1, the inverter, charge regulator and battery group have been purchased in a project supported by the Scientific Research Project Office of T.N.K.U. They have been used in a high tunnel that belongs to Vocational School of Technical Sciences. The picture of wind turbines has been taken at a location close to Hasköy (Edirne).



**Figure 1.** Wind Turbine and vermicompost production facility 1. Control Panel 2. Double bladed manure harvesting knife 3. Conveyor band 4. Electric motor operating the knife 5. Electric motor operating the conveyor band

### ***Drying Vermicompost***

When harvested, the agricultural produces generally have a high moisture content. The moisture ratios of these produces must be lowered, in other words these products must be dried, for extending their storage and shelf lives without impairing their qualities. The agricultural produces are produced during specific periods throughout the year and they must be consumed within a short period of time so some products are consumed when they are fresh and the remainder must be preserved using a number of methods before they are spoiled (Çakır, 2015). As a matter of fact, the agricultural produces tend to spoil very quickly during the period between its harvest and consumption due to several enzymatic, microbial and chemical processes. Drying is one of the methods that allow us to store harvested agricultural produces for a long time and this process reduces the water content in produces by using thermal energy (Akman et al., 2018; Yüksel-Türkboyları, 2021). The drying operations require a lot of energy and they are extensively used for industrial operations. Energy used for drying operations in the industrialized countries accounts for 7 to 15 % of the energy consumed for the industrial operations. The drying processes account for more than 60 % of the total energy needed for production of the agricultural produces (Güngör et al., 2014).

The moisture content of the worm manure produced in the production facility is very high and around 80 %. However, according to the vermicompost regulations, the moisture content in the manure must be reduced and the maximum moisture content should be around 35 %. Therefore, the manure must be dried. The purpose of drying manure is to reduce the product moisture content down to the last value as soon as possible and by consuming the minimum amount of energy but not by impairing the product quality in any way (Polatçı and Tarhan, 2009). Another purpose of the drying process is to reduce the product volume and thus increase efficiency in transportation and storage of the manure (Güngör et al., 2014).

The drying process should not be done outdoors under natural conditions. As a matter of fact, manure dried outdoors will be exposed to the outside weather conditions. Rains or dust, soil particles carried with the wind, contamination with plant residues and such other factors impair the product quality. Therefore, the drying process should be done in controlled and indoor environments. The drying environment must be ventilated for obtaining the desired result from the drying process. Drying the manure under controlled conditions shortens the drying time and improves the product quality as well as its other characteristics (Kara et al., 2014).

The facility to be installed will be cost effective if 3 or 4 machines are used. The monthly manure yield in a 15.9-meter long vermicompost production system with a 1.3-meter height and width is around 3.0 to 3.5 tons. The moisture ratio of the resulting product will be very high, around 80 %, and the product must be dried to lower the ratio down to 35 %. When dried, this product will weigh around 1.2 to 1.4 tons (Yüksel Türkboyları and Yüksel, 2021).

Electric power need of each production unit in a 15.9-meter-long worm manure production facility is as follows: a 2.2 kWh electric motor is used for cutting the manure with a blade. A 1.1 kWh electric motor is used for pouring the cut manure onto the conveyor band and collecting the manure by operating the conveyor band.

Location of the machine in vermicompost production facilities must be illuminated with dim light at all times. Two or three 10 W LED bulbs will be enough for this purpose. As a matter of fact, the worms head to the top of the organic substance in dark environments and the worms dry and die there. Therefore, the production facility must have electricity at all times (Yüksel Türkboyları and Yüksel, 2021).

A 150 to 200-m<sup>2</sup> indoor area is required for drying the products under controlled conditions. Greenhouses, a type of structure under protective cover, might be used for this purpose.

Calculation of the ventilation need might be made based on base area of the drying facility. Accordingly, the ventilation requirement per 1 m<sup>2</sup> of drying facility base area will be between 0.033-0.042 m<sup>3</sup>s<sup>-1</sup> or 120-150 m<sup>3</sup>h<sup>-1</sup> (Anonymous, 1993; Yüksel and Yüksel, 2012). The ventilation requirement of a drying facility with base area of 120-150 m<sup>3</sup>h<sup>-1</sup> can be calculated as follows based on the value of 120-150 m<sup>3</sup>h<sup>-1</sup>:

$$200 \times 120 = 24000 \text{ m}^3 \text{ h}^{-1}$$

$$200 \times 150 = 30000 \text{ m}^3 \text{ h}^{-1}$$

According to the results calculated, the average value is around 27000 m<sup>3</sup>h<sup>-1</sup>. The number of mono-phase, 1400 rpm (dd<sup>-1</sup>), 0.75 kWh aspirator with 60-cm diameter to be used for providing this level of ventilation in the drying facility and ensuring air exchange of 9500 m<sup>3</sup>h<sup>-1</sup> might be calculated as follows (Anonymous, 2022b):

$$27000/9500 = 2.8 \sim 3 \text{ units}$$

As seen in this equation, it is determined that the system needs 3 aspirators.

### ***Energy Requirement of System***

The harvesting knife and conveyor band motors are operated at different times in a vermicompost production facility. This system will need the maximum energy when the harvesting knife (2.2 kWh), 3 ventilation fans (0.75 kWh) and 3 lighting armatures (10 W=0.01 kWh) consume energy at the same time.

The energy need of the vermicompost production system can be calculated as follows.

$$2.2 + 0.55 \times 3 + 0.01 \times 3 = 4.48 \text{ kWh}$$

For efficiently operating the system, a wind turbine system having a capacity higher than the calculated need, approximately 6 kWh instead of 4.48 kWh, is required because the wind power has a timing problem, in other words, energy might not be generated when needed (Tunus, 2019). The wind turbine cannot generate electric power when the wind velocity is below the cut-in level and over the cut-out level (Anonymous, 2022a). On the other hand, the desired energy generation level might not be achieved if the wind velocity fluctuates all the time. For these reasons, a system greater than the need must be preferred. The excess energy in the system can be stored in batteries and used afterwards. Also, the surplus energy generated can be used in the vermicompost production facility, air pressure tanks, cleaning tools and for purposes such as lighting.

### **CONCLUSION**

Population growth all around the world and improved quality of life have increased the need for the agricultural produces. Agrochemicals are being excessively used for a very long time for increasing the quantity of produces in agriculture. Long-term use of fertilizers and pesticides leads to a number of environmental issues and health problems. Rapid increase in the quantity of organic wastes and residues emerges as the garbage problem. The increasing number of residues and wastes can be quickly transformed into a high-quality product with worms. These organic wastes and residues are transformed into manure by worms and this manure is very rich

in microbial activity as well as having very high commercial value. Also, the nutritive value of this manure is very high for the plants.

The wind power is one of the green energy resources and the cost of energy generation might be lowered by using it for vermicompost production. In the northwest regions of our country, the wind turbines can be particularly used in Marmara Region because this area is one of the windiest locations of Turkey. The lighting system in worm manure facilities must be on all the time and this suggests that using a wind turbine system for the production activities will be optimum. Covering a portion of the electricity need of a production facility with renewable energy will make the manure production more cost-effective.

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## THE METALLOTHIONEIN AND GLUTATHIONE LEVELS OF MEDITERRANEAN MUSSEL (*Mytilus galloprovincialis*) IN THE ÇANAKKALE STRAIT

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### ABSTRACT

The parameters considered as biomarkers are especially useful in detecting and evaluating the possible effects of various pollutants that aquatic organisms may be exposed to. In this study, total glutathione (GSH) and metallothionein (MT) levels, which are used as biomarkers, were evaluated in Mediterranean mussel (*Mytilus galloprovincialis*) samples at different points of the Çanakkale Strait, which are under the influence of different pollutants. In October 2020, sampling was made in 3 different regions of the Dardanelles Strait (Yenikordon-Station 1, Kepez Harbour-Station 2, Çardak Lagoon-Station 3), and a total of 30 mussels (10 samples per station) were collected. The MT level was determined in the digestive gland, while the GSH level was measured in both the gill and digestive gland tissues. MT mean values were measured as  $20.75 \pm 5.1 \text{ mg.g}^{-1}$  at Station 1,  $29.25 \pm 4.3 \text{ mg.g}^{-1}$  at Station 2, and  $45.13 \pm 5.6 \text{ mg.g}^{-1}$  at Station 3. The differences between the MT levels measured at the stations were also statistically significant ( $p < 0.05$ ). Higher levels of GSH were found in digestive gland tissue at Station 1, while higher levels were recorded in gill tissue at Station 2. In Station 3, the highest GSH levels were measured in the digestive gland tissue ( $1.23 \text{ } \mu\text{mol.mg}^{-1}$ ). The variations based on stations were statistically significant ( $p < 0.05$ ). This research is a preliminary study designed to reveal the pollutant effects on Mediterranean mussels, which use this region as a habitat, which is under the influence of various pollutants. With the results obtained, it was aimed to pave the way for more comprehensive research in the context of monitoring spatial and temporal pollution in the region.

**Keywords:** Mediterranean mussel, biomarker, Çanakkale Strait

### INTRODUCTION

Industrial, mining, farming, and storage can be counted among the activities that have the greatest role in the formation of metal pollution. At this point, the extraction and analysis of heavy metals and their various by-products gain importance in order to determine the level of existing pollution and to produce solutions to eliminate the pollutants (Ding et al., 2019; Ding and Lisak, 2019; Joon et al., 2020a, 2020b; Yuvaraj et al., 2021). Biochemical biomarkers are mostly used to assess ecological responses in aquatic and terrestrial ecosystems to the effects of pollutant/xenobiotic exposure. Biomarkers can act as a stimulus in predicting the potential negative effects of pollutants and detecting environmental damage early (Otitoju and Onwurah, 2011).

Metallothionein (MT), a biochemical biomarker, is frequently used to assess metal exposure and reveal the potential harmful effects of metal pollution (Viarengo, 1989, Roesijadi,

1992; Pavicic et al., 1987, 1993; Ivankovic et al., 2005). In addition to functioning as antioxidants in a wide variety of eukaryotes, they are also effective in eliminating the negative effects of hydroxyl free radicals (Kägi and Schäffer, 1988; Thirumoorthy et al., 2007).

Glutathione (GSH) is known as the most important hydrophilic antioxidant in protecting cells from reactive oxygen species and in addition it regulates cell proliferation, apoptosis, immune function, and fibrogenesis (Lu et al., 2013; Altın, 2016). The glutathione antioxidant system has played an important role in the detoxification and/or metabolism of prooxidant molecules. Maintaining a physiological level of oxidant forcing with minimal damage is to manage life processes through redox signaling. From this point of view, it can be thought that the GSH antioxidant system also plays an important role in redox signaling (Ren et al., 2017; Sies et al., 2017; Farina and Aschner, 2019).

This study was designed to observe MT and GSH levels in Mediterranean mussels collected from different points of the Dardanelles Strait, which are thought to be under the influence of various pollutants and heavy metals. The results of the study were aimed to be a preliminary study for more detailed studies.

## MATERIAL AND METHOD

In October 2020, sampling was made from 3 different regions of the Dardanelles Strait (Yenikordon-Station 1, Kepez Port-Station 2, Çardak Lagoon-Station 3), which are thought to be affected by anthropogenic, agricultural or industrial activities, and a total of 30 mussels (per station) 10 samples) were collected (Fig. 1). During the field studies, care was taken to select individuals in similar sizes so that morphological changes do not adversely affect the results of the study. The samples were transported to the laboratory in the cold chain.

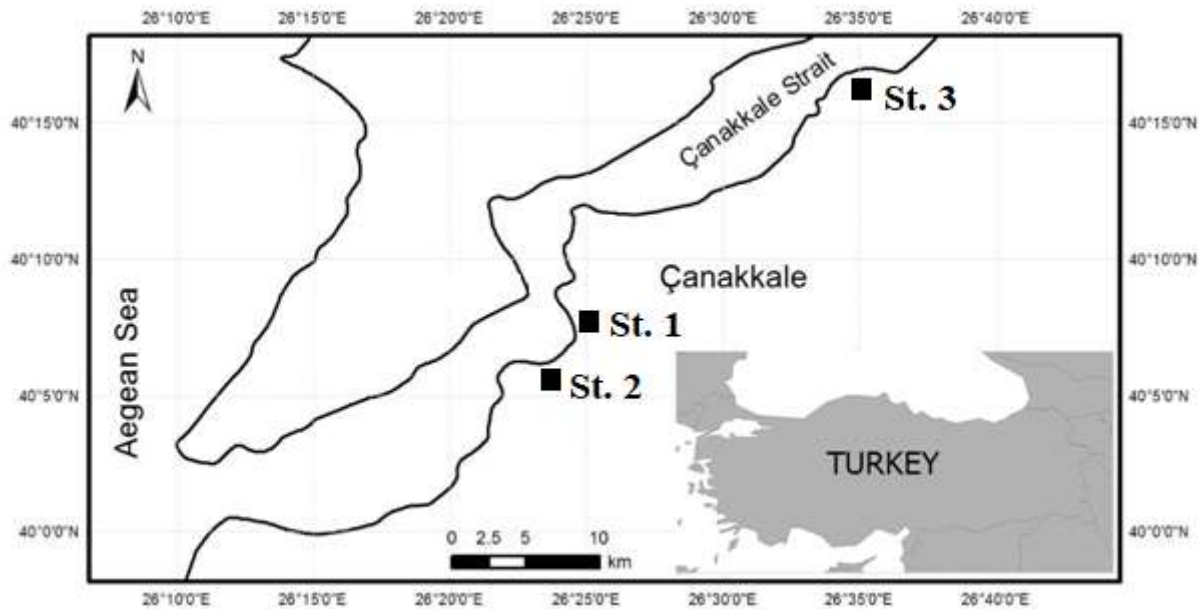


Figure 1. The sampling regions (St. 1: Yenikordon, St. 2: Kepez Harbour, St. 3: Çardak Lagoon).

After the length and weight of the samples were taken, dissected gill and digestive gland tissues were taken from the samples under appropriate conditions, and the tissues were fixed with liquid nitrogen and stored at -80 °C until the study was conducted. The MT level was



determined in the digestive gland, while the GSH level was measured in both the gill and digestive gland tissues.

Studies of MT acquisition Viarengo et al. (1997) method was used. First, homogenate was prepared from the digestive gland tissues (20mM Tris HCl, 0.006mM leupeptine, 0.5mM PMSF, 1mM DDT, and 10% glycerol) and the pellet was removed. Absolute ethanol and chloroform were added to the supernatant, and the pellet was removed by centrifuging again at 6000 xg for 10 minutes. Absolute ethanol containing 10% HCl was added to the supernatant and incubated at -20°C for 1 hour, and after incubation, the supernatant was centrifuged at 6000 xg for 10 minutes. Then, the supernatant was removed, the MT fraction obtained by adding 0.1M Kpi buffer to the pellet was stored in a deep freezer at -20°C for MT activity measurements. MT activity was determined spectrophotometrically at 412 nm after 2 minutes of incubation by adding 100 µl of MT fraction to 1 ml reaction medium containing 0.43mM DTNB dissolved in 0.1M Kpi buffer. While calculating MT activity, 0-15 µg GSH standard was used.

GSH analysis was performed in both gill and digestive gland tissues. Glutathione amount as the basic principle; measured by the enzymatic cycling procedure (Owens and Belcher, 1965). GSH; While it is oxidized by DTNB, the disulfide bonds formed by GSSG and other soluble thiol compounds in the environment and GSH are converted to GSH. The absorbance of the resulting yellow 2-nitro-5-thiobenzoic acid was measured at 412 nm.

Statistical analyses were made in SPSS 21.0 program. The normality of the data was tested using Kolmogorov Smirnov, and the homogeneity of variances was tested using Levene's tests. Results were compared using One-Way ANOVA. The  $\alpha$  significance level was determined as 0.05 for all analyses.

## RESULTS AND DISCUSSION

Based on the study results, the descriptive statistics of morphological measurements are presented in Table 1. As a result of the measurements, it was determined that the average weight of the individuals was between 21-22.5 gr. Their lengths varied between 60-6.1 mm, and their widths varied between 28-30 mm.

Table 1. Descriptive statistics of weight, length, width, and height values of mussel samples

| Stations            | Weight (mm)<br>mean±sd | Length (mm)<br>mean±sd | Width (mm)<br>mean±sd | Height (mm)<br>mean±sd |
|---------------------|------------------------|------------------------|-----------------------|------------------------|
| Station 1<br>(n=10) | 21.78±3.1              | 60.03±2.88             | 28.74±1.71            | 22.13±0.56             |
| Station 2<br>(n=10) | 22.15±2.4              | 61.24±2.56             | 30.02±1.2             | 20.39±1.01             |
| Station 3<br>(n=10) | 22.49±2.1              | 60.68±1.94             | 29.48±0.64            | 20.89±0.88             |

The MT levels in the digestive glands of the mussel samples collected from the stations were closer to each other in Station 1 and 2, while the values in Station 3 were much higher. The mean MT values were 20.75±5.1 mg.g<sup>-1</sup> in Station 1, 29.25±4.3 mg.g<sup>-1</sup> in Station 2, and 45.13±5.6 mg.g<sup>-1</sup> in Station 3 (Fig. 2). These variations on the basis of stations were statistically significant ( $F = 48.5$ ,  $df = 2$ ,  $p < 0.01$ ).

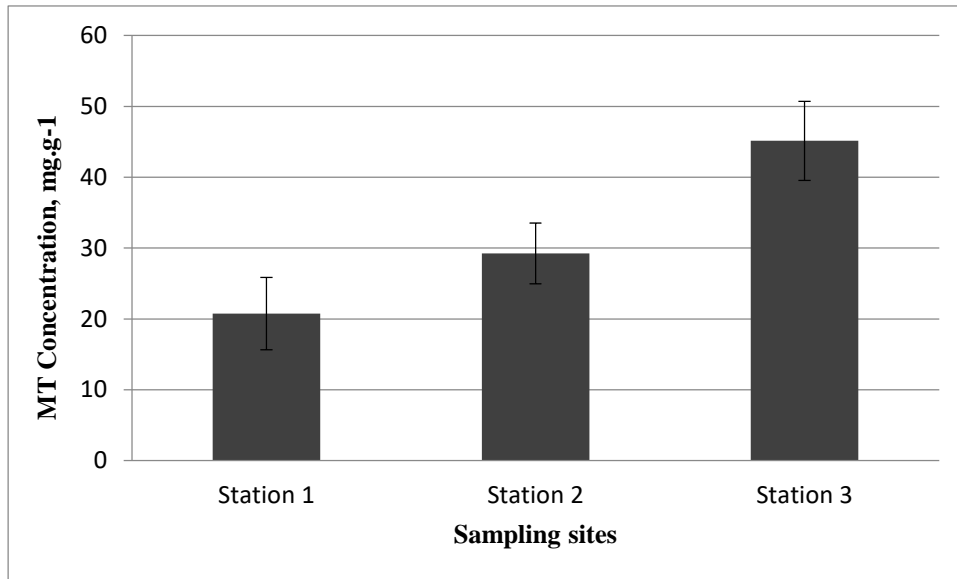


Figure 2. The MT level in the digestive gland tissues of *M. galloprovincialis*

GSH levels were measured in two different tissues of the samples, and in general (except Station 2), the levels measured in the digestive gland were higher than in the gill tissue. In the samples collected from Station 1, the mean GSH level was  $0.41 \pm 0.1$   $\mu\text{mol}/\text{mg}$  in the gill tissue and  $0.58 \pm 0.03$   $\mu\text{mol}/\text{mg}$  in the digestive gland tissue. Similarly, while the GSH level in the gill tissue was  $0.71 \pm 0.11$   $\mu\text{mol}/\text{mg}$  in Station 3, it was detected as  $1.04 \pm 0.2$   $\mu\text{mol}/\text{mg}$  in the digestive gland. In Station 2, the mean GSH level measured in the gill tissue was  $0.67 \pm 0.12$   $\mu\text{mol}/\text{mg}$ , while it was  $0.59 \pm 0.08$   $\mu\text{mol}/\text{mg}$  in the digestive gland. Differences in GSH levels between both stations ( $F = 129.9$ ,  $df = 2$ ,  $p < 0.01$ ) and tissues ( $F = 79.8$ ,  $df = 1$ ,  $p < 0.05$ ) were statistically significant.

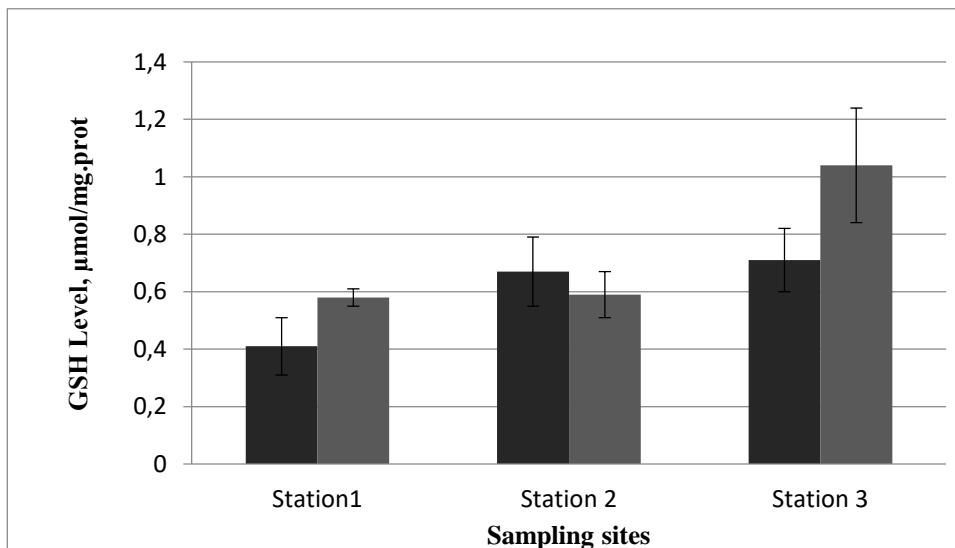


Figure 3. The GSH level in the tissues of *M. galloprovincialis* (Black bar: gill, Grey bar: digestive gland)

## CONCLUSIONS

Based on the study, Mediterranean mussel samples from different regions of the Çanakkale Strait exhibit different antioxidant status and MT values, which are considered an indicator of heavy metal accumulation. In particular, while the samples in Station 2 were both lower, these values were higher in the other station samples. This may be related to the fact that Station 1 is a port area and Station 3 is a region with very serious agricultural activities. In order to reveal this in more detail, this research may be a guide for more detailed studies.

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## CRISPR/CAS TECHNOLOGY FOR WHEAT BREEDING

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### ABSTRACT

The rapid growth of the world population and the current climate change show that genome technologies with the potential to accelerate crop breeding are in an important position. Genetic engineering techniques have various applications in agricultural crops. Advances in genome sequencing and genome editing technology have enabled more effective application of molecular breeding studies on agriculturally important characters in plants. ZFN (Zinc Finger Nucleases), TALEN (Transcription Activator-Like Effector Nuclease) and clustered regulatory interspaced short palindromic repeats associated protein 9 (CRISPR/Cas9) are significant genome editing technologies. CRISPR/Cas9 allows improvement of important agronomic traits related to crop quality, yield, nutritional value, and biotic-abiotic stress tolerance. Wheat, the basic source of cereal-based processed products, is one of the most widely produced cereals in the world. CRISPR/Cas-mediated genome editing technology was used for various purposes such as knockout of genes, base editing, prime editing, homology-directed repair (HDR) to improve wheat yields and quality. In this study, recent situation of CRISPR/Cas-mediated genome editing technology in wheat breeding and the research carried out using CRISPR/Cas technology in wheat is presented.

**Keywords:** Abiotic stress, Biotic stress, CRISPR/Cas9, Genome Editing, Wheat

### INTRODUCTION

Wheat, being the raw material for the most basic nutrients, is one of the most strategic crops due to having a high ratio in the total agricultural area in the world (Li et al., 2021). Common wheat (*Triticum aestivum* L.) is an allohexaploid species (AABBDD) that is a combination of three interrelated diploid genomes (Petersen et al., 2006; Venske et al., 2019). Common wheat global harvested area was 219,006,893 ha and production was 760,920,831 tonnes in 2020 (FAO, 2022). The top three producers of common wheat in the World are China, India, and United States of America according to average production from 1994 to 2020. Turkey is in the 10th place in this ranking (FAO, 2021).

Factors such as reductions in agricultural areas, global climate change, and increasing biotic and abiotic stress factors are seriously threatening agriculture and food production (Haque et al., 2018; Tufan and Keles, 2019). In order to secure agricultural production and food safety, crops that are efficient and resistant to various stress factors are needed. With the rapid developments in the field of biotechnology, especially in genome editing technologies, high potential can be reached in the production of crops with the desired properties. As a result of the development of high-precision genome editing tools, rapid advances are being made in crop breeding (Zhang et al., 2018a; Li et al., 2021a). Although genome editing studies in wheat are at a low level compared to other food crops due to its complex and polyploid genome and relatively low transformation efficiency (Li et al. 2021a), it is observed that studies in the literature are increasing day by day.

The most commonly used core technologies for Genome-editing can be listed as Homing Endonucleases, Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector nucleases (TALENs), and Clustered Regularly Interspaced Short Palindromic Repeats Associated Protein 9 (CRISPR/Cas9) (Gaj et al., 2016). Meganucleases, ZFNs, and Talens are known as the Method of the Year in 2011 (Li et al., 2021a). Later CRISPR/Cas was promoted as the Breakthrough of the Year at the 2015; after that Dr. E. Charpentier and Dr. Doudna won the Nobel Prize in Chemistry for their impressive studies of the CRISPR/Cas system in 2020. In this study, recent situation of CRISPR/Cas-mediated genome editing technology in wheat breeding and the research carried out using CRISPR/Cas technology in wheat is presented.

### APPLICATIONS OF CRISPR/CAS-MEDIATED GENOME EDITING IN WHEAT

Recently, CRISPR/Cas9 has become a significant tool for genome editing due to its relatively easy-to-use and cost-effectiveness compared to other methods and is considered to be a potential revolution for crop improvement strategies (Kim et al., 2018; Li et al., 2021b). CRISPR/Cas9 technology has been used to improve various traits of wheat including yields and quality. In Table 1, some scientific studies on genome editing in wheat conducted last ten years are summarized.

Shan et al., (2013) which is the first report of CRISPR/Cas9 technology for wheat, provided powdery mildew resistant wheat plants by knockout of TaMLO gene. Wang et al., (2014) reported powdery mildew resistant bread wheat genotypes by editing of three homoeoalleles by CRISPR/Cas9 system. Zhang et al. (2017) also provided powdery mildew resistant wheat plants by knockout of TaEDR1 gene.

In Su et al., (2018), *Fusarium* head blight resistant wheat plants were obtained by knockout of TaHRC gene. In similar manner, base insertion/deletion of TaNFXL1 gene performed and *Fusarium graminearum* resistant wheat plants were obtained (Brauer et al., 2020). Hahn et al., (2021) use three target genes for resistance to wheat spindle streak mosaic virus and wheat yellow mosaic virüs in the spring and winter wheat cultivars and successfully performed transformation and genome editing. Based on these studies it has been concluded that CRISPR/Cas9 technology is an efficient for improving biotic stress resistant wheat plants.

CRISPR/Cas-mediated genome editing for improvement of crop yield and quality also have been widely used. Sanchez-Leon et al., (2018) emphasized on improving quality of wheat by base deletion of  $\alpha$ -gliadin gene. Zhang et al., (2018b) and Zhang et al., (2019) aimed improving wheat yield and quality by targeting TaGW2 and TaCKX2 genes, respectively. In Jouanin et al., (2019), low gluten wheat obtained by mutations of  $\alpha$ -gliadin and  $\gamma$ -gliadin genes.

Altenbach et al., (2020) reported low gluten wheat by targeting  $\alpha$ -gliadin with CRISPR/Cas9 technology. Liu et al., (2020) increased branched starch content by editing TaQ genes. In another study, high amylose contained wheat obtained by TaSBEIIa gene in winter and spring wheat cultivars with CRISPR/Cas9 system (Li et al., 2020a). In Ouyang et al., (2016), increased phosphorus uptake and grain yield under low phosphorus conditions were reported with the mutation of the phosphate 2 gene TaPHO2-A1 in wheat. It has been concluded that there were significant researches with CRISPR/Cas9 technology for improving wheat yield and quality.

Table 1. Some CRISPR/Cas-mediated genome editing studies in wheat last 10 years

| Reference | Target Gene(s) | Agronomic Traits | Transformation |
|-----------|----------------|------------------|----------------|
|-----------|----------------|------------------|----------------|

|                           |   |  |                           |
|---------------------------|---|--|---------------------------|
| Hahn et al., 2021         | TaBAK1-2, Ta-eIF4E, Ta-eIF(iso)4E             | Wheat spindle streak mosaic virus (WSSMV) and wheat yellow mosaic virus (WYMV) | Biolistic transformation  |
| Altenbach et al., 2020    | $\alpha$ -gliadin                             | Low gluten   | Biolistic transformation  |
| Brauer et al., 2020       | TaNFXL1                                       | Resistance to <i>Fusarium graminearum</i>                                      | Protoplast transformation |
| Li JY et al., 2020        | TaSBella                                      | Amylose and resistant starch content   | Biolistic transformation  |
| Li et al., 2020b          | TaDA1/TaPDS/TaNCED1                           | -  | <i>Agrobacterium</i>      |
| Lin et al., 2020          | Ubi10, TaGW2, TaGASR7, TaDME1, TaLOX2, TaMLO, | -  | Protoplast transformation |
| Tang et al., 2020         | Ms2   | Male sterility   | <i>Agrobacterium</i>      |
| Abe et al., 2019          | Qsd1  | Seed dormancy  | <i>Agrobacterium</i>      |
| Arndell et al., 2019      | EPSPS   | Herbicide glyphosate tolerance   | Protoplast transformation |
| Jouanin et al., 2019      | $\alpha$ -gliadin and $\gamma$ -gliadin       | Low gluten   | <i>Agrobacterium</i>      |
| Zhang et al., 2019        | TaCKX2  | Increased grain number   | <i>Agrobacterium</i>      |
| Okada et al., 2018        | TaMs1   | Male sterility   | Biolistic transformation  |
| Kim et al., 2018          | TaDREB2, TaERF3                               | Abiotic Stress Resistance, Drought Tolerance                                   | Protoplast transformation |
| Sanchez-Leon et al., 2018 | $\alpha$ -gliadin                             | Low gluten   | Biolistic transformation  |
| Zhang et al., 2018b       | TaGW2   | Improved seed weight, Protein content  | -                         |
| Gil-Humanes et al., 2017  | Ubiquitin                                     | -  | Biolistic transformation  |
| Zhang et al., 2016        | TaLOX2  | Altered grain size, weight and increased storability                           | Biolistic transformation  |
| Shan et al., 2014         | TaLOX2  | Altered grain size, weight and increased storability                           | Biolistic transformation  |
| Wang et al., 2014         | TaMLO   | Powdery mildew resistance  | Biolistic transformation  |
| Shan et al., 2013         | TaMLO   | Powdery mildew resistance  | Biolistic transformation  |

## CONCLUSION

Articles reviewed in the literature using CRISPR/Cas9 genome editing have shown that CRISPR/Cas9 technology is a useful and important tool for genome editing in many model plants and crops. CRISPR/Cas9 is of significant value in crop breeding efforts, with its simplicity and higher accuracy compared to other genome editing techniques such as ZFNs and TALENs (Chaudhary et al. 2022).

In these days aimed for sustainable and productive agriculture, and also understand the importance of agriculture more with the Covid-19 pandemic, we must be aware of the need for a genome editing approach to obtain products with high nutritional value, resistant to various stress factors, and with increased yields and quality. Therefore, we can say that CRISPR/Cas9-based genome editing technology has great potential to obtain crops equipped with the desired characters to achieve the goal of sufficient food production to feed the global human population.

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## METHODS OF MECHANOCHEMICAL ACTIVATION OF INDUSTRIAL CATALYSTS OF CARBON OXIDES HYDROGENATION

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### ABSTRACT

Nowadays, one of the promising areas is the chemical utilization of carbon oxides in chemicals and fuels. This direction is extremely attractive because it not only reduces global warming caused by increasing atmospheric CO<sub>2</sub> concentrations but also offers solutions to replace exhaustible fossil fuels. However, it is well known that carbon oxides are fairly stable molecules. This is the biggest obstacle to the implementation of industrial processes with their use, as the transformation of carbon oxides requires a significant amount of energy. Hydrogen is a material with a relatively sufficient energy reserve. It is the main reagent in the conversion of carbon oxides. The development of an efficient method of extracting hydrogen from renewable sources, the conversion of a mixture of CO and CO<sub>2</sub> can open a key approach to the accumulation and conservation of energy in the form of chemicals and fuels. The effectiveness of mechanical flow during the hydrogenation process has been repeatedly proven. This is due to the local release of significant energy sufficient for the hydrogenation process in softened conditions at the point of contact of two catalytically active surfaces. Improving existing industrial catalysts in this way will increase the overall efficiency and cost-effectiveness of hydrogenation processes in general. A laboratory installation was developed and installed to study the process of hydrogenation of carbon oxides using both classical granular and mechanoactivated catalysts before the process or *in situ*. The reaction device is designed in such a way that it is possible to activate the catalyst mechanically – the creation of elastic-deformation oscillations at the points of contact of the active surfaces of the granules. Preliminary mechanical activation of industrial catalysts was carried out and their physicochemical characteristics were investigated by various methods: XRD, XRF, FTIR-ATR, SEM, AFM, TGA, DSC.

**Keywords:** Mechanochemical activation, Hydrogenation, Catalyst, Optimization, Carbon oxides

### INTRODUCTION

Mechanochemical activation (MCA) of solids has long been known to induce chemical changes which initiate or promote chemical reactions. In recent decades, the possibility of using mechanochemical methods of the technological processes intensification is considered (Hannah Schreyer, 2018; Amol P. Amrute, et al. 2021).

Physical processes of grinding are the cause of a chemical reaction or change in the reactivity of solids often. In most cases, dispersion is not an easy way to increase the free surface of a solid, but is also accompanied by changes in the physical state, chemical properties and composition of the substance to be ground. The study of physicochemical changes in dispersed substances is related to understanding the differences between the concepts of mechanochemistry and the mechanical activation of matter. In the first case –

mechanochemistry – studies the physicochemical transformations of matter under the action of mechanical forces. However, mechanical activation studies the "effects of consequence" due to the fact that the dispersed substance has "excess energy" that accumulates during grinding. The requirement to study the properties of substances in the fine state increases due to the necessity to study the methods for changing their reactivity and intensification of technological processes. Practical aspects of mechanical activation are the ability to improve many known technological processes, as well as the creation of fundamentally new ways to design technological schemes. Impact and friction are the main methods of mechanical action on a solid body during dispersion (Heinicke, 1984). They cause various physical phenomena: initiation of electromagnetic wave radiation; heat production, which causes heating of the crushed material; stimulating electron emission and creating potential differences; violation of the integrity of the material and increase the free surface of the substance; occurrence of plastic and elastic deformations – relaxation of deformations and residual stresses in solids at low temperatures is quite slow, and, as a consequence, the substance, which is exposed to mechanical force, for some time has a reserve of "excess" energy; distortion of the crystal lattice of substances that are the cause of point defects and linear dislocations with a corresponding reserve of "excess" energy; violation of the integrity of the material leads to the rupture of chemical bonds of the substance – the resulting uncompensated chemical bonds or free radicals have a supply of "excess" energy; rearrangement or change of the crystal lattice with a corresponding change in all thermodynamic characteristics of the substance and its reactivity.

It was shown that mechanochemical reactions occur for several reasons, the existence of each of which may explain the occurrence of the activation effect on one side or another. The action of heat released by impact or friction explains the increase in the rate of a chemical reaction as for most conventional processes. The electrons flow accelerates reactions which occurs when grinding matter. The increase in the free surface of the solid, during its dispersion, promotes a chemical reaction, the rate of which is described by the kinetics of grinding. Mechanochemical reactions are also due to the deformation of the substance and occur at the time of application of mechanical force, as well as in the process of relaxation of deformations and stresses, so the nature of the reactions is related to the energy state of deformed catalysts. The energy of distortions and defects of the crystal lattice formed during MCA also serves as one of the driving forces of mechanochemical transformations.

Acceleration of the reaction in heterogeneous catalysis in most cases is because the activation energy of all stages of the new reaction path resulting from surface interaction with the catalyst is lower than the activation energy of the reaction in the absence of a catalyst. Only in some cases is the acceleration of the process associated with the occurrence of a chain reaction that turns into a volume. Therefore, in the vast majority of cases, the catalytic activity of solid catalysts is proportional to the magnitude of their working surface and can be expressed as the product of the magnitude of this surface on the specific catalytic activity – activity per surface:

$$\kappa = k_{\text{spec.}} * S,$$

where  $\kappa$  – is the total catalytic activity;

$k_{\text{spec.}}$  – specific catalytic activity;

$S$  – the working surface of the catalyst.

The share of the working surface depends on the specific activity, porous structure, grain size of the catalyst, temperature, composition of the reaction mixture, and other factors that determine the reaction rate. Consider the first multiplier that determines the activity of catalysts – specific catalytic activity. This value is specific to the catalyst substance. From the chemical nature of the intermediate interaction in heterogeneous catalysis, it directly follows that the specific activity of the catalyst in a particular reaction should depend on the chemical

composition of the catalyst. It is noted that the high specific activity is inherent in the substance in the fine state, with a broken, deformed crystal lattice, while large crystals are inactive or inactive. In many sources on heterogeneous catalysis, there is an indication that due to heating at high temperatures, the catalytic activity of the catalyst decreases much faster than its surface decreases. For example, copper chemisorbs hydrogen at a noticeable rate only at high temperatures (above 400 ° C). Accordingly, the catalytic activity of copper to the hydrogen atoms exchange reaction is minimal and specific (Heinicke, 1984; Balaz, et al. 2013).

Thus, we can draw certain conclusions that the improvement of technological processes for obtaining valuable products of organic and petrochemical synthesis remains an important task of physicochemical research. It is known that heterogeneous catalytic processes (both oxidative and acid-base) play a decisive role in this area. Catalysts can be characterized at the molecular (chemical composition) and supramolecular (supramolecular structure) levels. The chemical composition of the catalyst for the methanol synthesis – an extremely valuable high-tonnage product – from synthesis gas has long been known. Therefore, improving the performance of this process or reducing energy consumption can be achieved either by creating the necessary structure (texture) of the catalyst or by technological optimization of the heterogeneous catalytic process. The research results will contribute to the development of a new direction – mechanocatalysis – in two aspects: mechanochemical activation of catalysts before and during use. Secondly, these results allow for to improvement of a specific catalytic process – the synthesis of methanol. Third, they can extend to other catalytic processes.

## **MATERIAL AND METHOD**

Industrial aluminum-cobalt-molybdenum (ACM) and catalyst for the synthesis of methanol CHM-Y were used as a catalyst in the hydrogenation of carbon oxides. Glass-ceramic balls were used as the catalyst support. For better catalyst application, they were pre-prepared. Modification of the surface of glass-ceramic balls was carried out in order to achieve a heterogeneous rough surface of the latter. To do this, a certain volume (5 ml) of balls was placed in a polypropylene cup, 20% ammonium fluoride solution in a volume of 20 ml was added so that the balls were completely covered with the solution, heated to 80 ° C in a water bath and kept for this temperature for 2 hours. The balls were then thoroughly (15-20 times) washed from ammonium fluoride residues with hot distilled water, dried, and used as intended. Granular industrial catalysts were ground in a mortar to a homogeneous state for 1 hour. A certain portion of the obtained powder with a fraction of  $\approx 100\text{-}200\ \mu\text{m}$  was applied to an inert carrier – glass-ceramic balls, original and treated, with a diameter of 1.0-1.2 mm. Before applying the catalyst powder on the surface of the carrier, first of all, the surface of the glass-ceramic balls was prepared for the adhesion of the catalyst on them. To do this, a certain volume of 1.0-1.2 mm glass balls was shaken in a laboratory sieve with a hole diameter of 0.4-0.6 mm for 30 minutes to remove non-standard impurities and grind the surface of the balls. The ground balls were placed in a glass container, a portion of the catalyst powder was added, closed with a stopper, and the powder was applied to the balls by mechanical shaking for 1 hour. The catalyst system was then removed from the flask, sieved through a sieve to remove some of the catalysts that did not adhere to the balls, and weighed to determine the weight of the catalyst applied by shaking the system.

Chemical analysis was conducted to determine the content of the catalysts using Expert 3L XRF analyser. The porous properties of the catalysts were studied using N<sub>2</sub> adsorption at – 195.8 ° C on the specific surface area and porosity analyser Nova 1200e (Quantachrome, USA). The phase identification of the catalysts was examined under X-ray diffraction (XRD) using the MiniFlex 300/600 diffractometer (Rigaku, Japan). The diffraction patterns were recorded

using Cu-K $\alpha$  radiation ( $\lambda = 0.15418$  nm), the operating voltage of 40 kV and the current of 15 mA. XRD pattern of samples was obtained in the  $2\theta$  range between  $2^\circ$  and  $80^\circ$  with a step of  $0.02^\circ$ . FTIR analysis of the catalysts was performed using IRAffinity-1S FTIR spectrometer (Shimadzu, Japan) equipped with a Quest ATR Diamond GS-10800X (Specac, UK) within the wavenumber range of  $4000$  to  $400$   $\text{cm}^{-1}$ . The scanning electron microscopy (SEM) images were taken using Zeiss Evo-10 (Carl Zeiss Microscopy, USA) microscope working at 20.0 kV. The sample was analysed three times. The surface morphology was investigated with atomic force microscope (AFM) NT-206 (Company with double liability “Microtestmachines”, Belarus) equipped with standard sonde CSC37 and rigidity of console 0.3-0.6 N/m. The scan was run in a contact static mode at 10  $\mu\text{m/s}$  with a step of 0.3 nm. Samples of catalysts were stirred in ethyl alcohol (5 ml) for 15 min. The resulting suspension (0.25 ml) was applied to quartz glass and dried at  $50^\circ\text{C}$  to constant weight.

The hydrogenation reaction of carbon oxides occurs at elevated temperatures and pressures. For the study of catalytic properties initial and activated catalysts, such basic parameters were the basis for the creation of a laboratory installation (Fig. 1). In fig. 1 shows a principle scheme of laboratory catalytic installation.

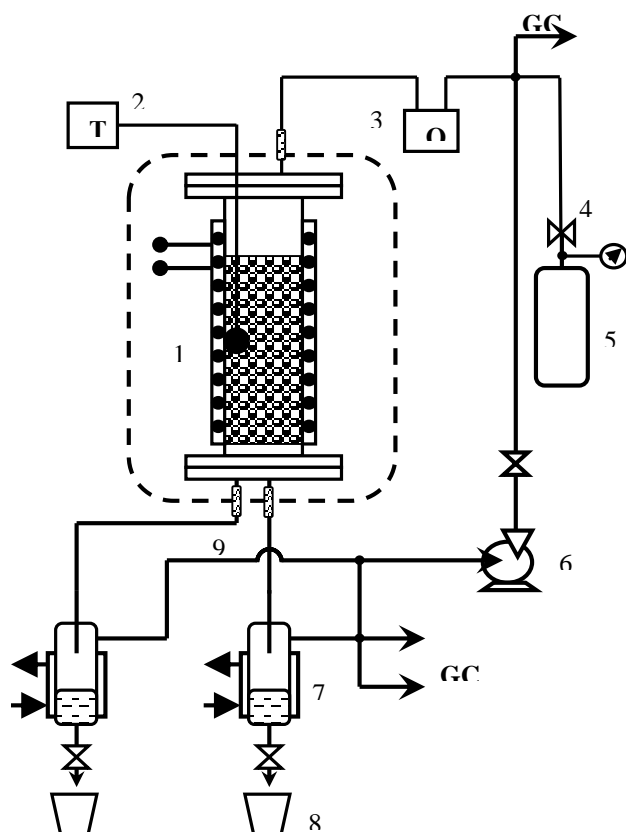


Figure 1. Principle scheme of laboratory catalytic installation of carbon oxides hydrogenation: 1 – catalytic reactor; 2 – temperature recorder with thermocouple; 3 – pumped gas meter; 4 – valve; 5 – cylinder with the initial mixture; 6 – compressor; 7 – separator with the cooling system on Peltier elements; 8 – collection container for liquid products; 9 – damper joint.

The main element of the installation is a cylindrical reactor (1) with a height of 300 mm, an outer diameter of 30 mm, a wall thickness of 3 mm, made of stainless steel brand X18H9T, equipped with an autonomous electric heater with temperature control using a thermocouple (2). A gas meter (3) is installed in the gas line at the reactor inlet to record the amount of gas (working raw material) pumped through the reaction zone for a certain period of time. The

feedstock supply system is mounted in such a way that it can operate in both flow and circulation modes (6). In the first case, the feedstock (gas mixture) is pumped from the cylinder (5) and after the reactor is fed for gas chromatographic analysis. In the case of circulation, the gas system is partially replenished with fresh raw material from the cylinder only to compensate for the reaction mixture consumed in the hydrogenation process. For maximum separation of liquid and gas phases after the reactor, separators equipped with a controlled cooling system on Peltier elements with the possibility of obtaining negative temperatures have been installed. This approach makes it possible to obtain the most "dry" gas mixture of reaction products at the inlet to the chromatograph and compressor in the circulating mode. Since the study investigated additional levers of influence on the process of hydrogenation – mechanical activation, the reaction device was mounted taking into account the above points.

In the conditions of mechanical activation due to the mobility of the reaction space, all communication systems are equipped with special dampers (Fig. 1 (9)). An electromechanical system (Fig. 2 (3)) with an eccentric gearing (Fig. 2 (2)) was installed for this mode. The speed of the eccentric gearing is regulated by of the motor one. The power and torque transfer coefficients are selected at the unit level.

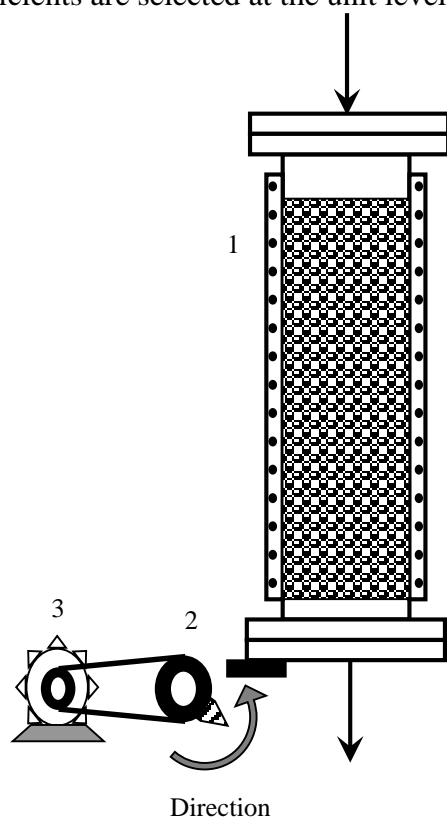


Figure 2. Scheme of reactor operation under mechanical activation mode: 1 – reaction device with a catalyst supported on the elastic carrier (glass balls); 2 – eccentric gearing; 3 – electric motor with a system for regulating and controlling the speed of the eccentric gearing.

## RESULTS AND DISCUSSION

To improve the catalytic properties of industrial catalysts for hydrogenation processes, it is necessary to know all the physicochemical properties they possess.

The composition of industrial catalysts was confirmed by the XRF method (Table 1).

Table 1. Chemical composition of the studied catalysts.

| <b>ACM catalyst</b>            |                        |
|--------------------------------|------------------------|
| <b>Element</b>                 | <b>Mass content, %</b> |
| <sup>8</sup> O                 | 42.773±1.372           |
| <sup>13</sup> Al               | 37.479±0.909           |
| <sup>14</sup> Si               | 0.311±0.037            |
| <sup>26</sup> Fe               | 0.112±0.007            |
| <sup>27</sup> Co               | 6.835±0.166            |
| <sup>42</sup> Mo               | 12.490±0.301           |
| <b>Formula</b>                 | <b>Mass content, %</b> |
| Al <sub>2</sub> O <sub>3</sub> | 70.817                 |
| CoO                            | 9.619                  |
| Fe <sub>2</sub> O <sub>3</sub> | 0.160                  |
| MoO <sub>3</sub>               | 18.739                 |
| SiO <sub>2</sub>               | 0.666                  |
| <b>CHM-Y catalyst</b>          |                        |
| <b>Element</b>                 | <b>Mass content, %</b> |
| <sup>8</sup> O                 | 21.203±0.048           |
| <sup>13</sup> Al               | 2.212±0.134            |
| <sup>14</sup> Si               | 0.139±0.022            |
| <sup>26</sup> Fe               | 0.094±0.008            |
| <sup>29</sup> Cu               | 50.056±0.086           |
| <sup>30</sup> Zn               | 26.296±0.058           |
| <b>Formula</b>                 | <b>Mass content, %</b> |
| Al <sub>2</sub> O <sub>3</sub> | 4.180                  |
| CuO                            | 62.659                 |
| Fe <sub>2</sub> O <sub>3</sub> | 0.134                  |
| SiO <sub>2</sub>               | 0.297                  |
| ZnO                            | 32.730                 |

As can be seen from the above results (Table 1) for the ACM catalyst, the basis is alumina and oxides of cobalt and molybdenum in a certain ratio, which does not contradict the literature and passport data. Regarding the CHM-Y, its composition also fully complies with the passport characteristics for this type – the ratio is 2: 1 between oxides of copper and zinc. Aluminum oxide plays the role of a binder component.

One of the important conditions in the manufacture of composite catalysts is the stability of the components. That is the invariance of their phase composition. The results of such control are shown on the corresponding radiographs (Fig. 3, 4). Figure 3 presents the comparative spectra of the phase composition of the original glass balls before and after the digestion process. The spectra (Fig. 3) show that the balls consist of silicon dioxide in the amorphous state. The etching process of the outer surface does not change their phase composition. According to the analysis results (Fig. 4), spectra were obtained, which are characterized by diffraction peaks for alumina in the gamma phase – at angles  $2\theta = 45.90; 66.90$  (Mónica Ayala-G et al. 2015; Awadallah et al. 2013; Londoño-Calderón et al. 2020). In all cases no detected XRD diffraction peaks were observed for any types of oxides Mo, Ni, Co, Cu, and Zn. Probably this is because the crystallites are too small to give XRD signals, or the particles of these oxides are well dispersed in the carrier (Mónica Ayala-G et al. 2015; Awadallah et al. 2013).



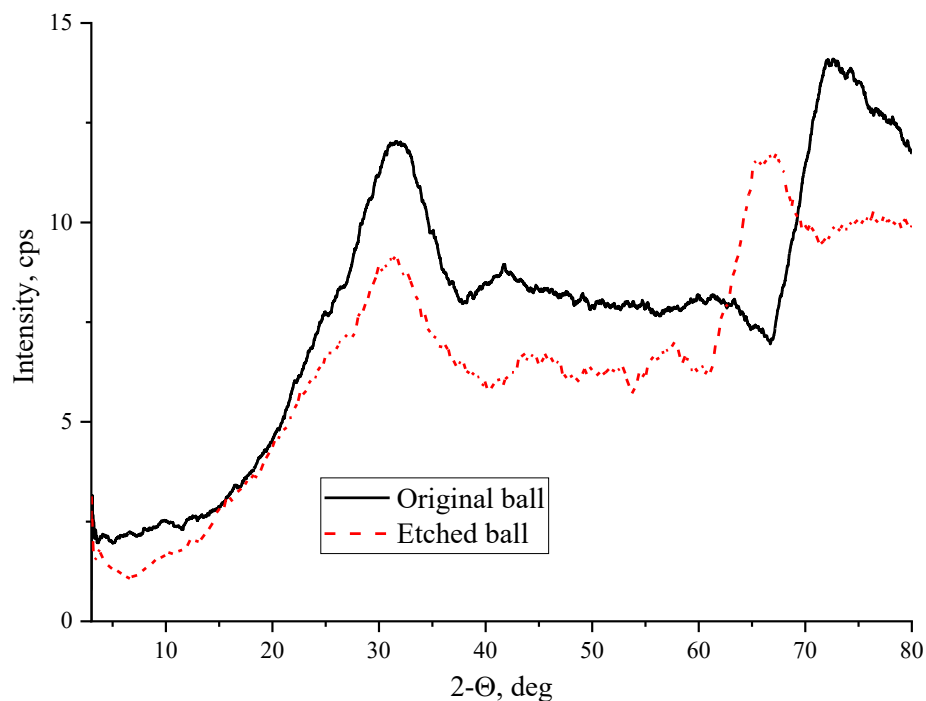


Figure 3. XRD patterns of the balls: solid line – original; dotted line – etched

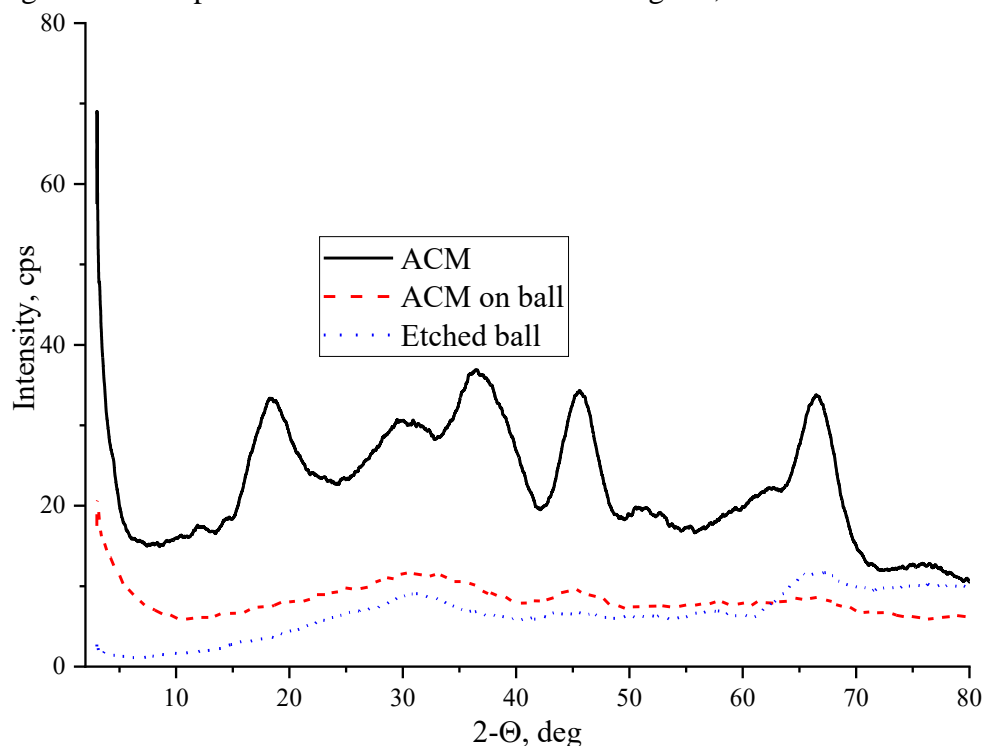


Figure 4. XRD patterns of the ACM

In the IR spectra of all catalysts, the wide absorption band at  $3600\text{--}3000\text{ cm}^{-1}$  belongs to the valence vibrations of the associated OH groups (Fig. 5, curves 2, 3). For the ACM of the catalyst, the band at  $1630\text{--}1600\text{ cm}^{-1}$  is characteristic of H-O-H strain vibrations (Mónica Ayala-G et al. 2015; Al-Fatesh et al. 2021), the absorption band at  $430\text{ cm}^{-1}$  belongs to the valence vibrations of the O-Mo-O groups (Mónica Ayala-G et al. 2015; Coates, 2000; Pletincx et al. 2017), and the band at  $1067\text{ cm}^{-1}$  is characteristic of O-Al-O valence vibrations (Fig. 5,

curve 2). For the CHM-Y catalyst, a wide band at 1475 and 1400  $\text{cm}^{-1}$  is characteristic of ZnO vibrations. The bands at 410, 550  $\text{cm}^{-1}$  correspond to  $\text{SiO}_2$  vibrations (Fig. 5, curve 1) (Thanh Nhan Tran et al. 2013; Salazar-Hernández Carmen et al. 2017). These data show that the catalyst did not change its structural characteristics during mechanical activation, which is important for further catalytic studies.

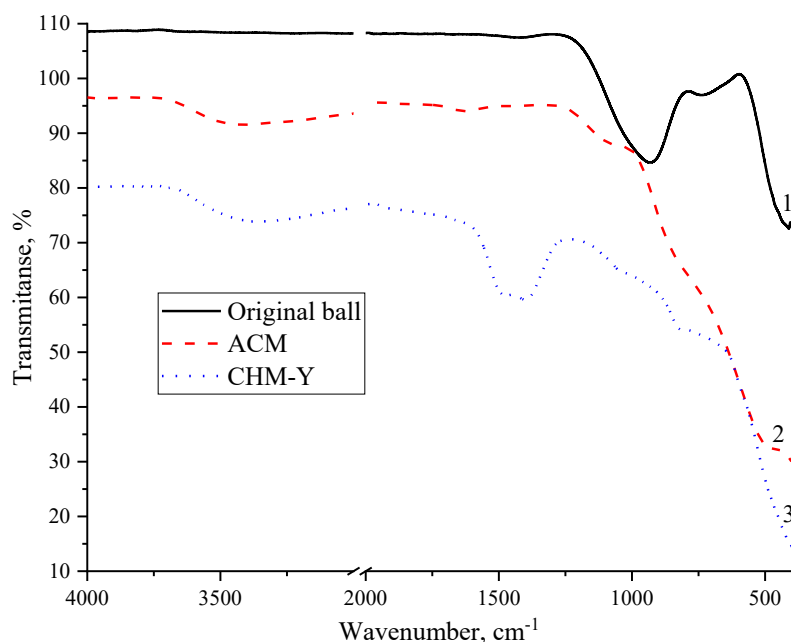


Figure 5. FT-IR spectra of: 1 – original glass balls, 2 – ACM, 3 – CHM-Y.

Thus, mechanical activation was found not to change the nature of the catalysts, as evidenced by infrared spectral analysis with Fourier transform in incomplete reflection and X-ray fluorescence spectroscopy.

Scanning microscopy was used to study the surface layer of all starting materials used in the composite catalysts manufacture. Figures 6 and 7 show images of two non-reduced industrial catalysts. Figures 6 (a, b) show that the surface is homogeneous and has an amorphous structure. Figure 7 shows a photo of the CHM-Y catalyst surface.

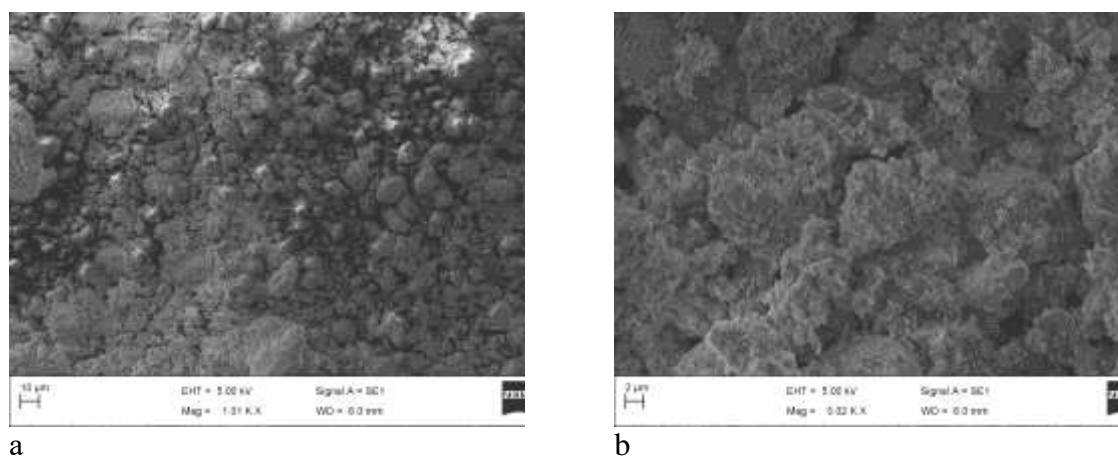


Figure 6. SEM micrographs of the surface of the non-reduced ACM catalyst at different magnifications: a – 1000 times; b – 5000 times

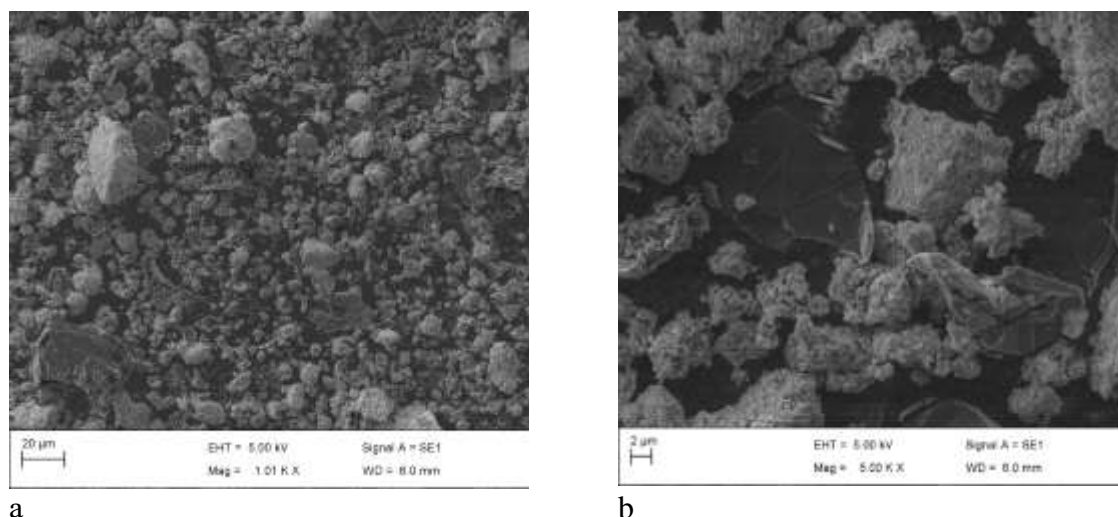


Figure 7. SEM micrographs of the surface of the non-reduced catalyst CHM-Y at different magnifications: a – 1000 times; b – 5000 times

Figure 7 shows that the catalyst consists of both amorphous and crystalline structures. The amorphous part is a mixture of copper and zinc oxides, and the crystalline part is alumina.

One of the levers of influence on the catalyst activity is mechanical activation. An important condition for its implementation is that the active particles under mechanical loads must be elastic and deformation resistant. This is because at the time of the collision the point of contact is concentrated maximum energy. This energy serves as an activator of various properties of materials, including catalytic. Glass balls with an average diameter of 1 mm were chosen as the elastic basis of the catalyst (Fig. 8). However, these balls cannot be used immediately as a catalyst carrier because they have a smooth surface. This creates problems in achieving strong contact between the catalyst and the base. Thus, the surface of the balls was modernized by treating it in a solution of ammonium fluoride. At the same time etching of a smooth surface was observed and a certain roughness sufficient for strong inoculation was reached. Figure 9 (a, b) shows a photo to compare the surface after the etching process. The detector SE1 shows that the surface has a layered structure. The BSD detector helped to obtain a contrasting photo, where the shells and depths were visible.

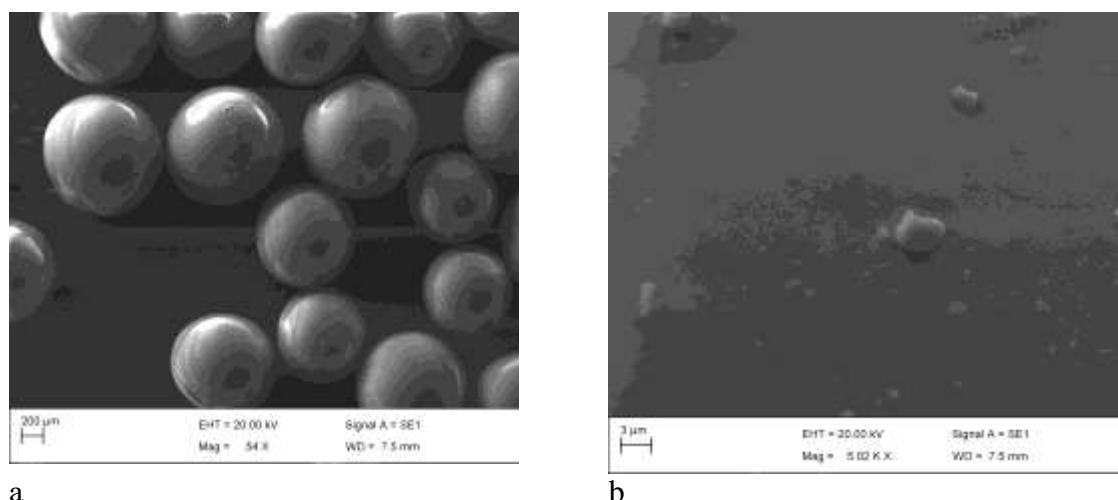


Figure 8. SEM micrographs of the surface of the original glass balls

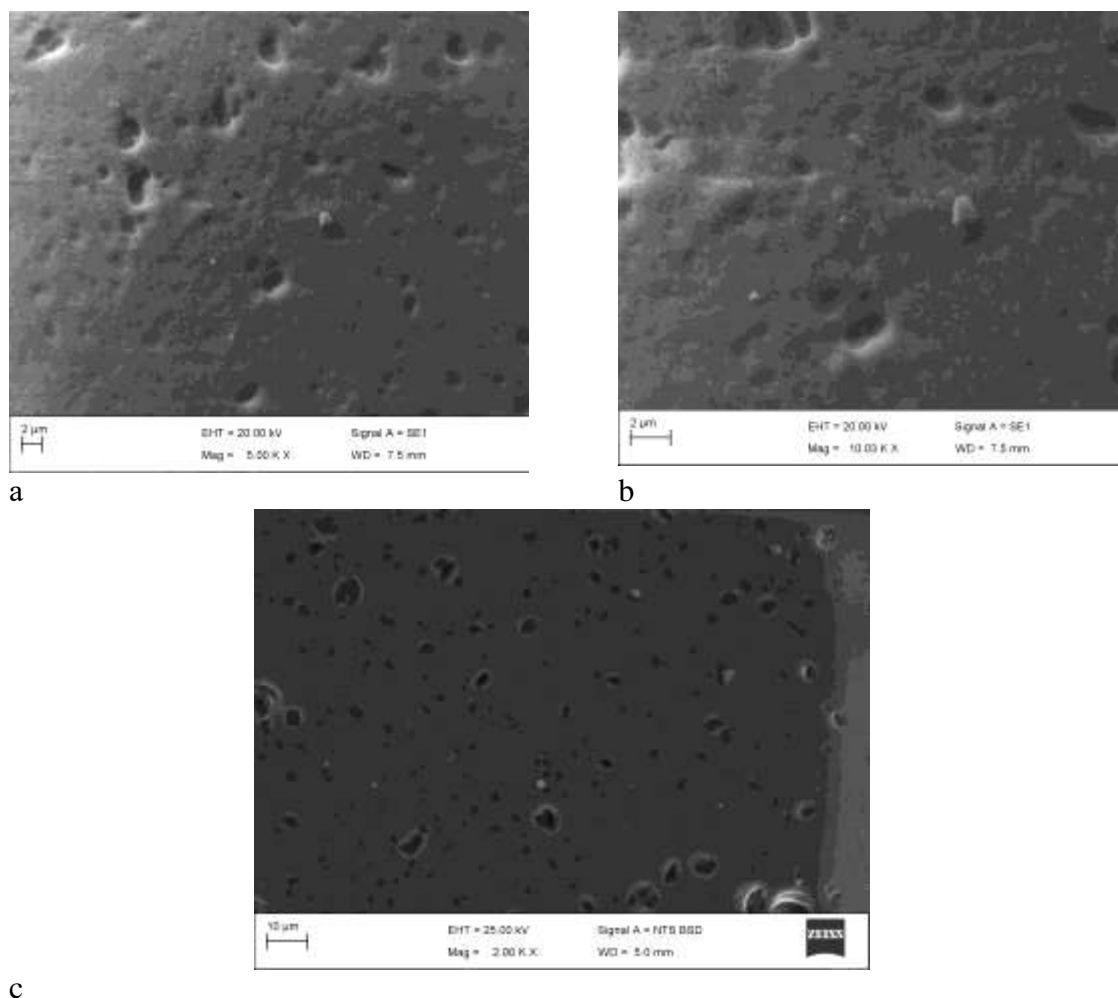


Figure 9. SEM micrographs of the surface of glass balls after the etching process: a – magnification 5000 times, detector SE1; b is a magnification of 10000 times, detector SE1; c – magnification 2000 times, BSD detector.

The quality of the catalysts applied to the surface of the etched glass balls was also performed visually. Figures 10 and 11 show ready-to-test balls with a catalyst bed.

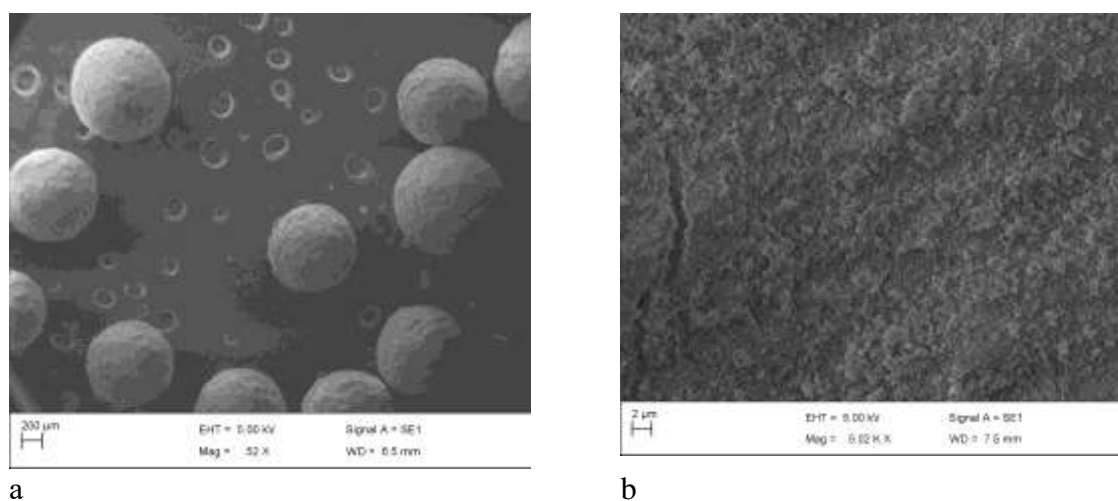


Figure 10. SEM micrographs of glass balls with ACM: a – magnification 52 times; b – increase in 5000 times

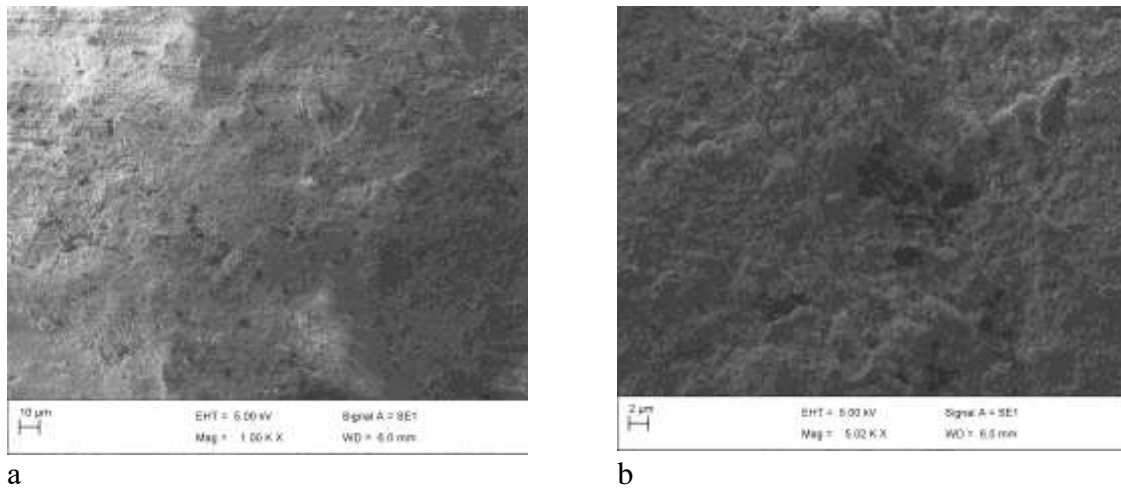


Figure 11. SEM micrographs of glass balls with CHM-Y: a – magnification of 1000 times; b – increase in 5000 times

As can be seen from Figure 10, the catalyst is applied in a uniform layer without obviously visible delamination. Figure 11 shows the qualitative application of the catalyst. The main factor, in this case, is that the catalytic layer consists of both amorphous and crystalline components.

Figure 12 shows images of the original glass balls. As shown in Figure 12, the surface of the source media is homogeneous but has some defects (dents). This is most likely due to shortcomings in the technological process of their manufacture.

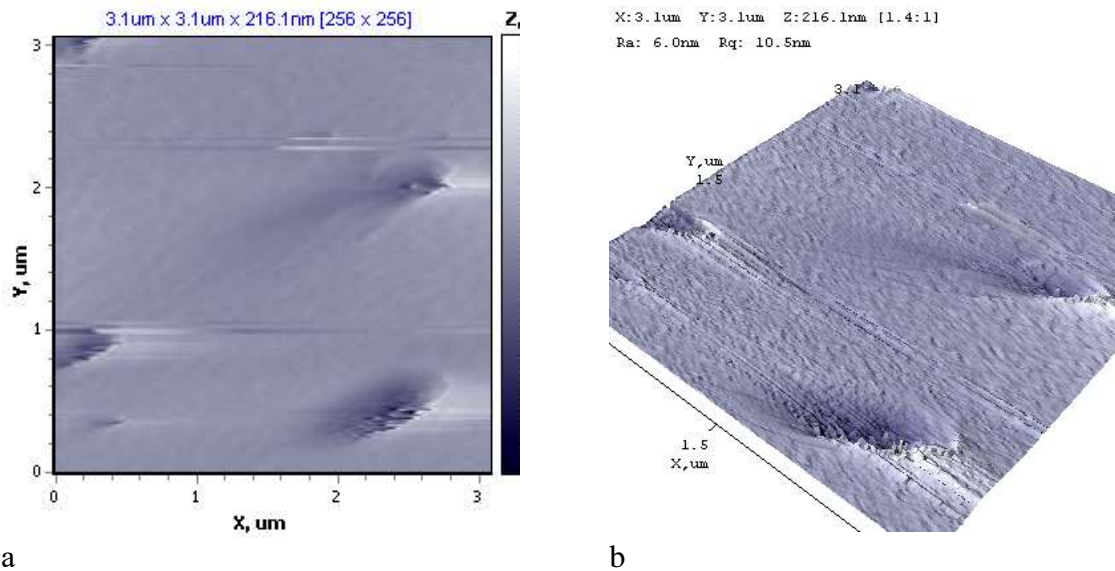
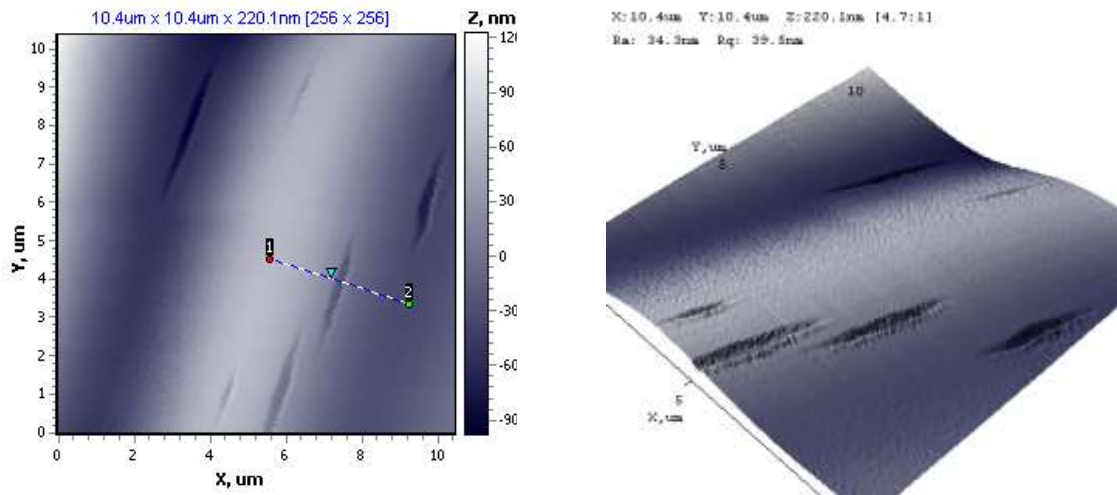
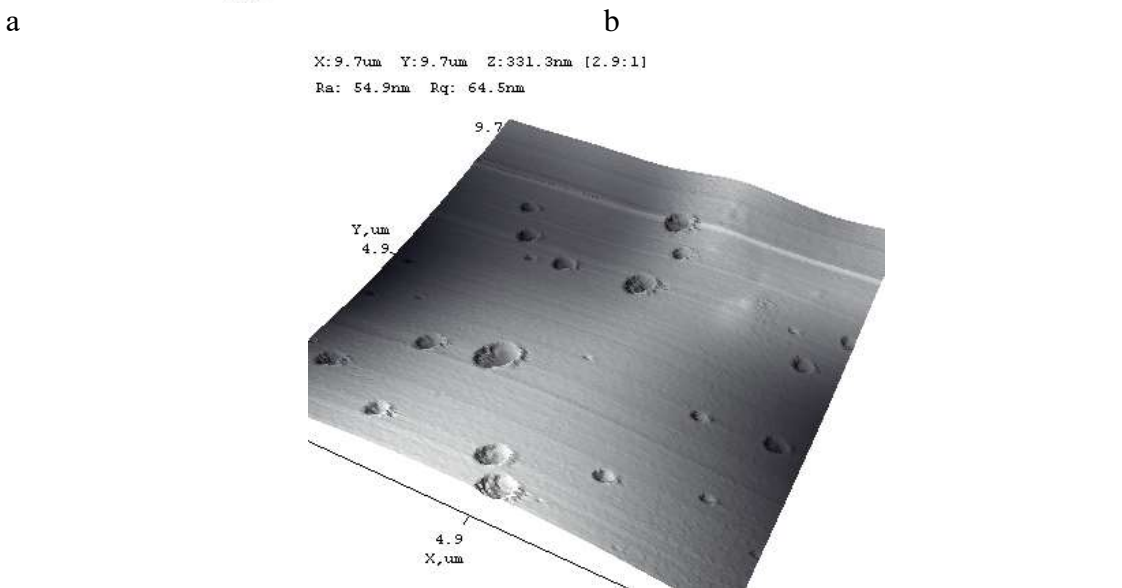
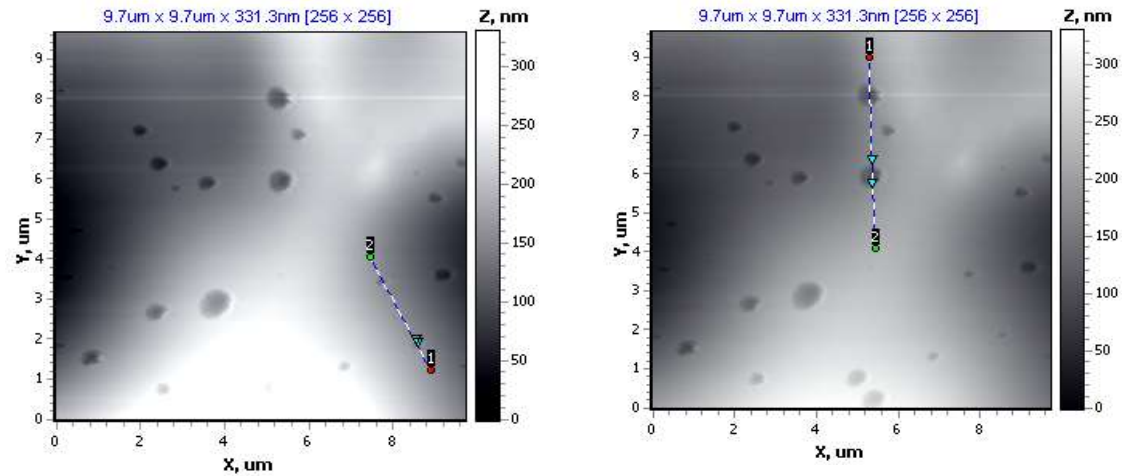


Figure 12. AFM micrographs of the original surface of glass balls: a – 2D; b – 3D image of a surface

According to the data from Figure 13 it was found that as a result of etching on the surface of the glass balls formed longitudinal grooves up to 35.7 nm deep and up to 2.3 μm wide. In addition to longitudinal rounded pores with a diameter of 215.5 nm to 691 nm and a depth of 18.9 nm to 80.7 nm are also observed, as shown in Figure 14.



a b  
Figure 13. AFM micrographs of the surface of the etched balls: a – 2D; b – 3D image of a surface



a b c  
Figure 14. AFM micrographs of the surface of the original balls after the etching process: a, b – 2D image of the surface with cross-section lines in different directions; c – 3D image of a surface

The catalyst supported on a glass balls has the shape of a solid agglomerate, which is completely consistent with the results of SEM. The scan results are shown in Figure 15. It was found that the thickness of the catalyst layer is from 150.8 nm to 395.6 nm. Also, AFM analysis of industrial catalysts after mechanical grinding showed that the CoO-MoO<sub>3</sub>-Al<sub>2</sub>O<sub>3</sub> surface is characterized by the presence of individual spherical particles and elongated aggregates, the size of which is in the nanoscale and varies from 2.8 nm to 12.6 nm. The particle sizes of CHM-Y are in the range from 1.1 nm to 2 nm. You can see both individual spherical particles and aggregates of arbitrary shape.

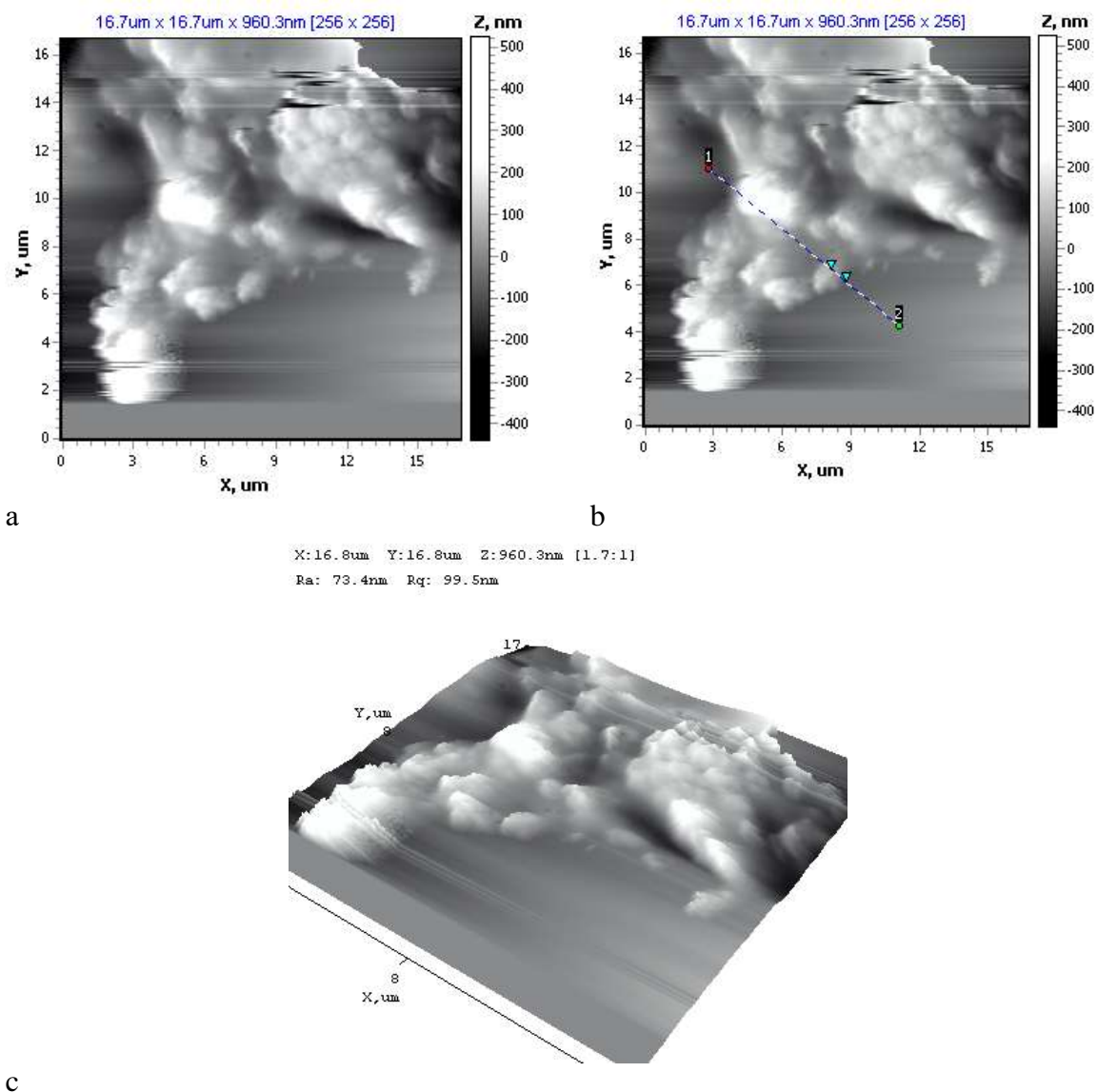


Figure 15. AFM micrographs of the surface of the balls with a layer of ACM catalyst: a – 2D image of the surface, b – 2D image of the surface with cross-section lines in different directions; c – 3D image of a surface

Isotherms of adsorption-desorption of nitrogen and curves of distribution of pores on the sizes of the investigated catalysts are given in Fig. 16, and the corresponding data (specific surface area, pore volume, and pore diameter) are presented in Table 2. All catalysts show (Fig. 16) an isotherm with a hysteresis loop of type IV, which indicates the presence of mesopores (Wen Qian Jiao et al. 2012; Liu et al. 2015). There was a significant reduction in the specific

surface area of the catalyst deposited on the balls (Fig. 16). It may be due to a significant reduction in the catalyst compared to the carrier, the specific surface area and porosity of which is zero.

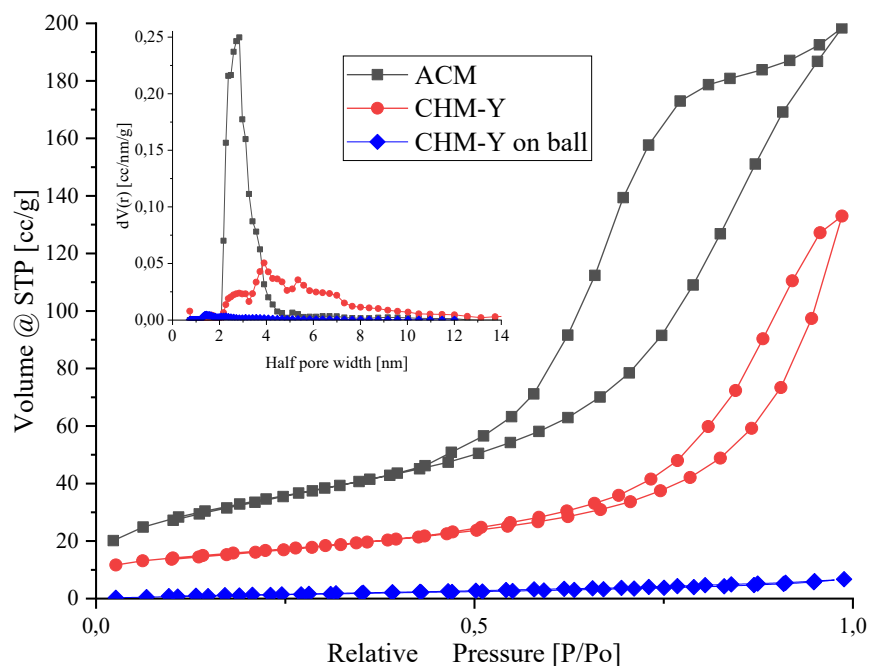


Figure 18. Nitrogen adsorption–desorption isotherm, and pore distribution (insert) curves for the investigated catalysts

Table 2 Textural properties of the investigated catalysts

| Catalyst      | Surface Area (m <sup>2</sup> /g) | Pore Volume (cm <sup>3</sup> /g) | d <sub>BJH</sub> (nm) |
|---------------|----------------------------------|----------------------------------|-----------------------|
| ACM           | 118.9                            | 0.30                             | 9.24                  |
| CHM-Y         | 55.9                             | 0.40                             | 7.80                  |
| CHM-Y on ball | 6.8                              | 0.01                             | 3.02                  |

## CONCLUSIONS

A laboratory installation was developed and installed to study the process of hydrogenation of carbon oxides using classical granular catalysts. The reaction device is designed in such a way that it is possible to carry out the catalysts activation by mechanical means - the creation of elastic-deformation oscillations at the points of contact of the active surfaces of the granules. A method of applying industrial catalysts to an inert carrier is proposed. The selected materials have been studied by modern physico-chemical methods.

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## THE REFLECTANCE INDEX AS INDICATORS OF DROUGHT TOLERANCE OF SOME DURUM WHEAT (*TRITICUM DURUM* DESF.) GENOTYPES GROWING UNDER SEMI-ARID CONDITIONS -SETIF- ALGERIA

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### ABSTRACT

Water shortage stress and the associated challenges are a major set of abiotic factors damaging and constraining the international production of durum wheat. The objectives of this study are to evaluate the efficiency of using the RGB reflectance index and chlorophyll contents as best tools to select a high yielding of 15 durum wheat genotypes growing under semi-arid conditions. The number of days to heading, grain yield, thousand kernels weight, number of spikes per meter square, and plant height were measured at maturity. RGB reflectance index by numerical images analysis and chlorophyll contents were estimated. ANOVA showed a significant effect of genotypes for all traits. The local landrace Boutaleb which was the best yielding genotype registered a low Red reflectance index. The correlations test revealed that the chlorophyll contents was significantly and negatively correlated ( $P < 0.01$ ) with reflectance index at red and blue. PCA showed that grain yield was affected by Number of spikes per meter square, the high values of RGB reflectance index contribute at the elevation of the weight of 1000 kernels and plant height, a negative relation was observed between chlorophyll contents and RGB reflectance index.

**Key words:** Grain yield, Reflectance, RGB, chlorophyll, semi-arid, Algeria

### INTRODUCTION

Durum wheat [*Triticum turgidum* L. ssp. *durum* (Desf.) Husn.] is one of the most essential cereal species and is cultivated worldwide over almost 17 million ha, with a global production of 38.1 million tonnes in 2019 (Ioannis *et al.* 2020). Canada is the largest cultivator in the world, followed by Italy and Turkey (Pastaria International, 2015). However, the largest consumers are the Mediterranean countries, where most of the production process takes place. The main environmental constraints limiting the cultivation of durum wheat in the Mediterranean Basin are drought and extreme temperatures (Nachit *et al.*, 2004) Algeria, with these topographical and bioclimatic characteristics which show a diversity of landscapes and cropping systems, cereal growing is the predominant speculation of agriculture. It covers an annual area of approximately 3.6 million hectares of the useful agricultural areas (UAA) (MADR, 2012). Solar radiation impinging on the leaf surface is either reflected, absorbed or transmitted. The nature and amounts of reflection, absorption and transmission depend on the wave length of radiation, angle of incidence, surface roughness and the differences in the optical properties and biochemical contents of the leaves. (Guendouz *et al.* 2013).

Pigments are integrally related to the physiological function of leaves. Chlorophylls absorb light energy and transfer it into the photosynthetic apparatus. Carotenoids (yellow pigments) can also contribute energy to the photosynthetic system. (Demmig-Adams and Adams, 1996). When light strikes a leaf, part of the light is reflected towards the observer. The amount energy reflected at each light frequency is named reflectance spectrum, sometimes abbreviated by spectra or by reflectance. Reflectance depends on leaf surface properties and internal structure, as well as by the concentration and distribution of biochemical components. In the visible spectrum, (VIS, between 400 and 700 nm) reflectance depends mainly on the presence of photosynthetic pigments such as chlorophyll. In the near infrared domain (NIR, between 700 and 13000 nm), where there are no strong absorption features, the magnitude of reflectance is governed by structural discontinuities encountered in the leaf. The shortwave infrared region (SWIR, between 1300 nm and 3000 nm), (Peñuelas et al.1998). This study aim to determine the effects of Red , Green , Blue reflectance index (RGB) and chlorophyll contents on yielding of 15 [*Triticum turgidum* L. ssp. *durum* (Desf.) Husn.] genotypes growing under semi-arid conditions in the eastern of Algeria.

## **MATERIAL AND METHODS**

This study was conducted during the 2020/2021 cropping season at Setif Agricultural Experimental Station (ITGC-AES, 36 ° 12'N and 05 ° 24'E and 1.081 m asl, Algeria). The genetic material used in this study consists of 11 advanced lines and 4 genotypes of which 3 are local landrace used as control to evaluate their performance (table 1). The genotypes tested were sown at November 19 with sowing density adjusted to 300 grains.m<sup>-2</sup> in a random block design with three replications, each plot consisted of 6 lines of 10 m long spaced of 0.2 m witch make 12 m<sup>2</sup> as plot area. R.G.B reflectance index (Red, Green and Blue) was evaluated by numerical image analysis (NIA) according to Guendouz and Maamri (2011); Leaves were photographed on black surface, between 11:00 and 12:00 solar time with a color digital camera (Canon, Power Shot A460, AiAF, CHINA). Images were stored in a JPEG (Joint Photographic Expert Group) prior to downloading onto a PC computer and analyzed using IPP (Image Pro Plus, Version 4, Media Cybernetics, Silver Spring, MA, USA) software. Chlorophyll contents (CC) of the flag leaf was measured using digital chlorophyll meter (CCM) with (cci) units, this device allows measuring the absorbance of light in the leaf. Agronomic traits were measured at maturity: Grain yield (GY) the cereal yield performances of the different cultivars were measured at maturity in quintals per hectare (Qs. ha<sup>-1</sup>) by measuring the grain yield in one linear meter and converting it into quintals per hectare. Thousand kernels weight (TKW) (g). Number of spikes per meter square (NSm<sup>-2</sup>). Number of days to heading (DH) (days) calculated from sown date 19/11/2020 and Plant height (PH) (cm).

All statistical analyses will be performed by Costat 6.400 (1998) and R core Team (2020) Softwares.

## **RESULTS AND DISCUSSIONS**

### **1. Analysis of Variance (ANOVA)**

Analysis of variance (ANOVA) is a statistical tool used to detect differences between experimental group means. (Sawyer, 2009).

### 1.1 Agronomic traits

ANOVA (table 2) showed that genotypic effect was significant ( $p < 0.05$ ; 0.001) with TKW,  $\text{NSm}^{-2}$ , DH and PH.GY ranged from 2.87  $\text{Q}\cdot\text{ha}^{-1}$  for G11 advanced line to 13.59  $\text{Q}\cdot\text{ha}^{-1}$  for the local landrace Boutaleb with 6.34  $\text{Q}\cdot\text{ha}^{-1}$  as genotypic mean. TKW ranged from 30.91 g for the advanced line G8 to 46.69 g for G9 with genotypic mean of 39.40 g, the high value of TKW was observed with the local landrace Boutaleb with 44.96 g.  $\text{NSm}^{-2}$  ranged from 178.33  $\text{s}\cdot\text{m}^{-2}$  for G4 advanced line to 320  $\text{s}\cdot\text{m}^{-2}$  for the local landrace Boutaleb with a genotypic mean of 255.77  $\text{s}\cdot\text{m}^{-2}$ . DH ranged from 136 days for advanced lines G1, G2, G8, G10 and Jupare C 2001 foreign race to 147 for Boutaleb local landrace with 140.6 as genotypic mean. Plant height ranged from 56.11 cm for G4 to 67.38 cm for G10 with a mean of 62.76, local landrace Boutaleb registered a high plant height (66.16 cm).

### 1.1 physiologic traits

ANOVA showed that genotype effect was high significant ( $P < 0.001$ ) for reflectance index at all bands (Red-R, Green-G, Blue-B) and chlorophyll contents (CC) (table 3). Reflectance index at red (R) ranged from 41.86 % for advanced line G6 to 48.76 % for local landrace Oued El bared with 44.67 % as genotypic mean. Reflectance index at green band (G) ranged from 38.23% for G7 advanced line to 44.13% for Oued El bared local landrace with genotypic mean of 40.65. Reflectance index at Blue (B) ranged from 28.77% for G10 advanced line to 33.04 % for the same local landrace (Oued El Bared) with 30.72 % as mean for all genotypes studied. This results were very consistent with the study of Guendouz et al (2012a) who found that the lowest reflectance was observed at the Blue band of the spectrum from 400 to 500 nm. Chlorophyll contents ranged from 20.42 for Oued El Bared to 31.01  $\text{cci}$  for G4 advanced line with genotypic mean of 26.58  $\text{cci}$ . Chlorophyll tends to decline more rapidly than carotenoids when plants are under stress or during leaf senescence (Gitelson and Merzlyak, 1994). Variations in leaf chlorophyll content detectable by spectral reflectance have also been shown to be related to leaf development and senescence (Carter and Knapp, 2001). The local landrace Boutaleb which was the best yielding genotype ( $\text{GY} = 13.59 \text{ Qs}\cdot\text{ha}^{-1}$ ) registered the low reflectance index at Red (42.59%), a Green reflectance index, blue reflectance index and chlorophyll contents close to the average (30.09 30.25 % and 24.18  $\text{cci}$  respectively) comparing to genotypic means.

## 2. Simple Linear Correlation (SLC)

A simple linear correlation was used when there is only one predictor variable, matrix of simple between grain yield and its components was computed according to the formula given by Snedecor and Cochran (1981).

$$r(x, y) = \frac{(\sum xy - (\sum x)(\sum y)/n)}{\sqrt{(\sum x^2 - (\sum x)^2/n)(\sum y^2 - (\sum y)^2/n)}}$$

**r:** correlation coefficient, **x:** first character, **y:** second character, **n:** total of number of observations.

### 2.1 Correlations among agronomic traits

The simple linear correlation (table 4) showed that GY was highly, significantly ( $P < 0.01$ ; 0.001) and positively correlated with TKW and  $\text{NSm}^{-2}$  ( $r = 0.38^{**}$ ;  $0.61^{***}$ ). A high, significant ( $P < 0.01$ ; 0.001) and positive correlation was observed between TKW on the one hand and  $\text{NSm}^{-2}$ , PH on the other hand ( $r = 0.39^{**}$ ;  $0.61^{***}$ ).  $\text{NSm}^{-2}$  was significantly ( $P < 0.05$ ) and positively correlated with PH ( $r = 0.34^*$ ). Several works have proven the high correlation

between Grain Yield and some agronomic traits (Farih et al 2021;Guendouz et al., 2012b; Aissaoui and Fenni, 2021 and Mansouri et al., 2018).

**Table 1.** Varieties and their pedigrees

| <b>Genotype</b> | <b>Pedigrees</b>  |
|-----------------|---|
| <b>G1</b>       | RASCON_37/GREEN_2/9/USDA595/3/D67.3/RABI//CRA/4/ALO/5/... |
| <b>G2</b>       | MINIMUS_6/PLATA_16//IMMER/3/SOOTHY_9/RASCON_37/9/...      |
| <b>G3</b>       | CMH77.774/CORM//SOOTHY-9/RASCON-37/3/SOMAT-4              |
| <b>G4</b>       | CNDO/PRIMADUR//HAI-OU-17/3/SNITAN/4/SOMAT-3/              |
| <b>G5</b>       | RASCON_37/GREEN_2/9/USDA595/3/D67.3/RABI//CRA/4/ALO/5/... |
| <b>G6</b>       | SILVER 14/MOEWE//BISU_1/PATKA_3/3/PORRON_4/YUAN_1/9/...   |
| <b>G7</b>       | GUANAY /HU ALITA / 10/PLATA _10/6/MQUE/4/USDA573/...      |
| <b>G8</b>       | BCRIS/BICUM//LLARETA INIA/3/DUKEM_ 12/2*RASCON 21/5/R     |
| <b>G9</b>       | Simeto/3/Sora/2*Plata_12//SRN_3/Nigris_4/5/Toska_26/...   |
| <b>G10</b>      | Ossl1/StjS5/5/Bicrderaal/4/BEZAIZSHF//SD19539/Waha/3/St   |
| <b>G11</b>      | Stj3//Bcr/Lks4/3/Ter-3/4/Mgnl3/Aghrass2                   |
| <b>G12</b>      | Jupare C 2001   |
| <b>G13</b>      | Boussellem  |
| <b>G14</b>      | Boutaleb  |
| <b>G15</b>      | Oued Bared  |

**Table 2.** Analysis of variance of agronomic traits.

| Genotypes              | Agronomic traits          |              |                  |                  |              |
|------------------------|---------------------------|--------------|------------------|------------------|--------------|
|                        | GY (Qs.ha <sup>-1</sup> ) | TKW (g)      | NSm <sup>2</sup> | DH (days)        | PH (cm)      |
| <b>G1</b>              | 7.00 (bc)                 | 41.98 (bc)   | 236.66 (bcde)    | 136 (d)          | 62.72 (cd)   |
| <b>G2</b>              | 3.96 (bc)                 | 35.62 (ef)   | 213.33 (de)      | 136 (d)          | 61.50 (d)    |
| <b>G3</b>              | 7.05 (bc)                 | 40.83 (bcd)  | 220.00 (cde)     | 142 (c)          | 61.88 (d)    |
| <b>G4</b>              | 2.88 (c)                  | 36.80 (de)   | 178.33 (e)       | 142 (c)          | 56.11 (e)    |
| <b>G5</b>              | 4.70 (bc)                 | 36.10 (e)    | 220.00 (cde)     | 142 (c)          | 61.27 (d)    |
| <b>G6</b>              | 6.16 (bc)                 | 31.88 (fg)   | 240.00 (bcde)    | 142 (c)          | 61.27 (d)    |
| <b>G7</b>              | 6.89 (bc)                 | 34.36 (efg)  | 253.33 (abcde)   | 142 (c)          | 57.66 (e)    |
| <b>G8</b>              | 4.45 (bc)                 | 30.91 (g)    | 268.33 (abcd)    | 136 (d)          | 61.16 (d)    |
| <b>G9</b>              | 6.61 (bc)                 | 46.69 (a)    | 310.00 (ab)      | 142 (c)          | 65.55 (abc)  |
| <b>G10</b>             | 8.69 (ab)                 | 41.98 (bc)   | 268.33 (abcd)    | 136 (d)          | 67.38 (a)    |
| <b>G11</b>             | 2.95 (c)                  | 44.47 (abc)  | 285.00 (abcd)    | 142 (c)          | 67.22 (a)    |
| <b>Jupare C 2001</b>   | 7.89 (bc)                 | 41.98 (bc)   | 255.00 (abcd)    | 136 (d)          | 63.61 (bcd)  |
| <b>Boussellem</b>      | 4.75 (bc)                 | 40.39 (cd)   | 291.66 (abc)     | 142 (c)          | 63.94 (bcd)  |
| <b>Boutaleb</b>        | 13.59 (a)                 | 44.96 (ab)   | 320.00 (a)       | 147 (a)          | 66.16 (ab)   |
| <b>Oued El Bared</b>   | 7.59 (bc)                 | 43.27 (abc)  | 276.66 (abcd)    | 146 (b)          | 63.88 (bcd)  |
| <b>Mean</b>            | <b>6.34</b>               | <b>39.40</b> | <b>255.77</b>    | <b>140.6</b>     | <b>62.76</b> |
| <b>Min</b>             | <b>2.87</b>               | <b>30.91</b> | <b>178.33</b>    | <b>136</b>       | <b>56.11</b> |
| <b>Max</b>             | <b>13.59</b>              | <b>46.69</b> | <b>320</b>       | <b>147</b>       | <b>67.38</b> |
| <b>Génotype effect</b> | *                         | ***          | *                | ***              | ***          |
| <b>LSD (5%)</b>        | <b>5.672</b>              | <b>4.162</b> | <b>76.53</b>     | <b>1.526</b>     | <b>3.167</b> |
| <b>CV %</b>            | <b>53.45</b>              | <b>6.30</b>  | <b>17.89</b>     | <b>6.4901e-8</b> | <b>3.017</b> |

significant (P<0.05); \*\*\* very highly significant (P<0.001).

**Table 3.** Analysis of variance of physiologic traits

| Genotypes              | Physiologic traits |              |              |              |
|------------------------|--------------------|--------------|--------------|--------------|
|                        | R (%)              | G (%)        | B (%)        | CC(cci)      |
| <b>G1</b>              | 47.20 (b)          | 42.07 (b)    | 32.31 (b)    | 24.59 (f)    |
| <b>G2</b>              | 46.75 (bc)         | 41.75 (c)    | 30.91 (e)    | 25.48 (ef)   |
| <b>G3</b>              | 46.48 (c)          | 41.86 (bc)   | 31.57 (cd)   | 26.02 (def)  |
| <b>G4</b>              | 43.86 (f)          | 40.63 (e)    | 30.33 (fg)   | 31.01 (a)    |
| <b>G5</b>              | 43.17 (g)          | 40.43 (ef)   | 30.12 (gh)   | 24.13 (f)    |
| <b>G6</b>              | 41.86 (i)          | 39.26 (gh)   | 31.30 (d)    | 27.39 (cde)  |
| <b>G7</b>              | 41.93 (i)          | 38.23 (i)    | 28.88 (i)    | 29.98 (ab)   |
| <b>G8</b>              | 44.65 (e)          | 40.17 (f)    | 30.75 (e)    | 28.29 (bcd)  |
| <b>G9</b>              | 44.23 (ef)         | 40.40 (ef)   | 30.62 (ef)   | 29.08 (abc)  |
| <b>G10</b>             | 43.92 (f)          | 40.49 (e)    | 28.77 (i)    | 25.08 (ef)   |
| <b>G11</b>             | 43.11 (g)          | 39.54 (g)    | 29.88 (h)    | 28.65 (abc)  |
| <b>Jupare C 2001</b>   | 46.31 (c)          | 41.10 (d)    | 30.27 (fg)   | 29.55 (abc)  |
| <b>Boussellem</b>      | 45.32 (d)          | 40.56 (e)    | 31.80 (c)    | 24.87 (f)    |
| <b>Boutaleb</b>        | 42.59 (h)          | 39.09 (h)    | 30.25 (g)    | 24.18 (f)    |
| <b>Oued El Bared</b>   | 48.76 (a)          | 44.13 (a)    | 33.04 (a)    | 20.42 (g)    |
| <b>Mean</b>            | <b>44.67</b>       | <b>40.65</b> | <b>30.72</b> | <b>26.58</b> |
| <b>Min</b>             | 41.86              | 38.23        | 28.77        | 20.42        |
| <b>Max</b>             | 48.76              | 44.13        | 33.04        | 31.01        |
| <b>Génotype effect</b> | ***                | ***          | ***          | ***          |
| <b>LSD (5%)</b>        | 0.645              | 0.312        | 0.358        | 2.495        |
| <b>CV %</b>            | 0.62               | 0.46         | 0.70         | 5.61         |

\*\*\* Very highly significant ( $P < 0.001$ )

### 2.2. Correlations among physiologic traits

The simple linear correlation (table 4) showed that reflectance index at Red band (R) was very highly, significantly ( $P < 0.001$ ) and positively correlated with reflectance index at Green (G) and Blue (B) bands (0.94\*\*\*; 0.70\*\*\* respectively), it is also highly, significantly ( $P < 0.01$ ) and negatively correlated with chlorophyll contents (-0.41\*\*). A very high significant ( $P < 0.001$ ) and positive correlation was observed between reflectance index at Green band (G) and reflectance index at Blue band (B) and chlorophyll contents (0.72\*\*\*; 0.49\*\*\* respectively). Chlorophyll contents was highly, significantly ( $P < 0.01$ ) and negatively correlated to reflectance index at Blue band (B) (-0.46\*\*). The negative and significant correlation between reflectance at Red and Blue and chlorophyll content suggest that the decrease in the photosynthetic capacity of the canopy increase leaf reflectance at Red and Blue due to the degradation of chlorophyll content (guendouz et al.2012a). In the Blue region, both chlorophylls and carotenoids have high absorbances (Penuelas and Filella, 1998). Red reflectance, especially when standardized by reflectance in a non-absorbing waveband is highly correlated with chlorophyll content (Everitt et al. 1985).

**Table 4.** Correlations among physiologic and agronomic traits

|                         | <b>R</b>       | <b>G</b>       | <b>B</b>       | <b>CC</b> | <b>GY</b>      | <b>TKW</b>     | <b>NSm<sup>-2</sup></b> | <b>DH</b> | <b>PH</b> |
|-------------------------|----------------|----------------|----------------|-----------|----------------|----------------|-------------------------|-----------|-----------|
| <b>R</b>                | 1              |                |                |           |                |                |                         |           |           |
| <b>G</b>                | <b>0.94***</b> | 1              |                |           |                |                |                         |           |           |
| <b>B</b>                | <b>0.70***</b> | <b>0.72***</b> | 1              |           |                |                |                         |           |           |
| <b>CC</b>               | <b>-0.41**</b> | <b>0.49***</b> | <b>-0.46**</b> | 1         |                |                |                         |           |           |
| <b>GY</b>               | 0.01           | -0.01          | -0.04          | -0.08     | 1              |                |                         |           |           |
| <b>TKW</b>              | 0.24           | 0.25           | 0.09           | -0.11     | <b>0.38**</b>  | 1              |                         |           |           |
| <b>NSm<sup>-2</sup></b> | -0.06          | -0.11          | -0.02          | -0.04     | <b>0.61***</b> | <b>0.39**</b>  | 1                       |           |           |
| <b>DH</b>               | -0.25          | -0.11          | 0.17           | -0.22     | 0.18           | 0.28           | 0.20                    | 1         |           |
| <b>PH</b>               | 0.09           | 0.08           | 0.01           | -0.17     | 0.27           | <b>0.61***</b> | <b>0.35*</b>            | 0.03      | 1         |

\* significant (P<0.05); \*\* highly significant (P<0.01); \*\*\* very highly significant (P<0.001).

**Table 5.** Correlations of the traits measured with the first 3 components of the PCA.

| <b>Components</b> | <b>% de var</b> | <b>Measured traits</b> |             |             |              |             |             |                         |             |              |
|-------------------|-----------------|------------------------|-------------|-------------|--------------|-------------|-------------|-------------------------|-------------|--------------|
|                   |                 | <b>R</b>               | <b>G</b>    | <b>B</b>    | <b>CC</b>    | <b>GY</b>   | <b>TKW</b>  | <b>NSm<sup>-2</sup></b> | <b>PH</b>   | <b>DH</b>    |
| <b>PC1</b>        | 37.03           | <b>0.69</b>            | <b>0.71</b> | <b>0.64</b> | <b>-0.78</b> | 0.45        | <b>0.69</b> | 0.46                    | <b>0.63</b> | 0.24         |
| <b>PC2</b>        | 30.22           | -0.64                  | -0.66       | -0.55       | 0.11         | <b>0.57</b> | 0.45        | <b>0.75</b>             | 0.56        | 0.39         |
| <b>PC3</b>        | 12.65           | 0.23                   | 0.08        | -0.31       | 0.25         | -0.13       | 0.20        | 0.09                    | 0.42        | <b>-0.83</b> |

**Table 6.** Coordinates of the 15 genotypes on the first 3 components of PCA.

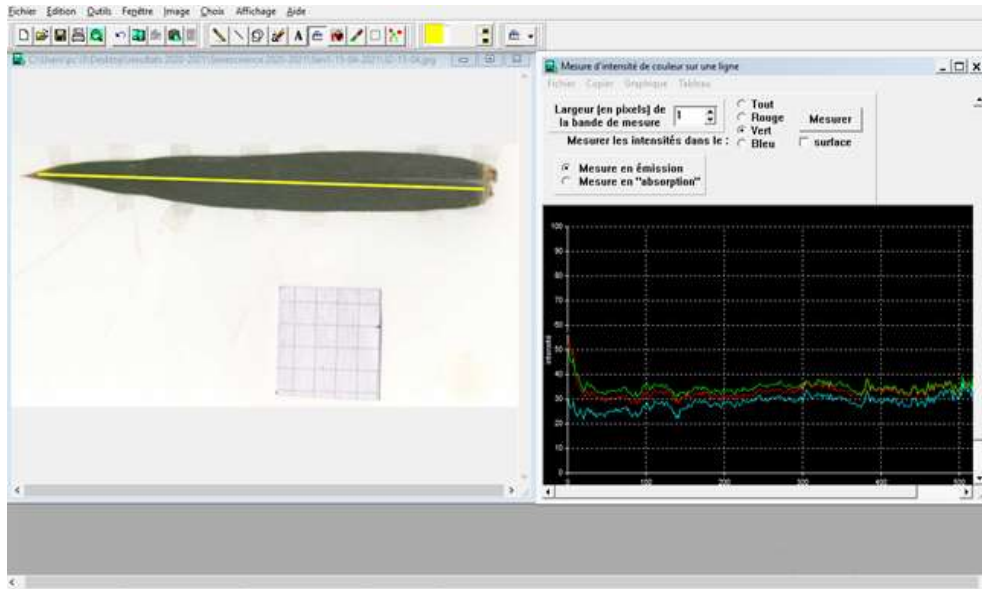
| <b>Components</b> | <b>Genotypes</b> |              |              |              |              |              |              |              |             |             |             |              |             |             |             |
|-------------------|------------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|-------------|-------------|-------------|--------------|-------------|-------------|-------------|
|                   | <b>G1</b>        | <b>G2</b>    | <b>G3</b>    | <b>G4</b>    | <b>G5</b>    | <b>G6</b>    | <b>G7</b>    | <b>G8</b>    | <b>G9</b>   | <b>G10</b>  | <b>G11</b>  | <b>J2001</b> | <b>Bous</b> | <b>Bout</b> | <b>OEB</b>  |
| <b>PC1</b>        | 1.56             | -0.19        | 0.93         | <b>-2.71</b> | <b>-0.93</b> | <b>-1.71</b> | <b>-3.18</b> | <b>-1.52</b> | 0.76        | 0.30        | -0.36       | 0.09         | <b>1.05</b> | 1.58        | <b>4.32</b> |
| <b>PC2</b>        | <b>-1.78</b>     | <b>-2.30</b> | <b>-1.24</b> | -1.81        | -0.44        | -0.08        | 1.15         | -0.88        | <b>1.77</b> | 1.56        | <b>1.72</b> | -0.12        | 0.04        | <b>3.80</b> | -1.53       |
| <b>PC3</b>        | 0.75             | 0.84         | -0.48        | -0.86        | -0.85        | -1.28        | -0.84        | 0.68         | 0.63        | <b>1.88</b> | 0.86        | <b>1.69</b>  | -0.32       | -1.32       | -1.39       |

### 3. Principal Components Analysis (PCA)

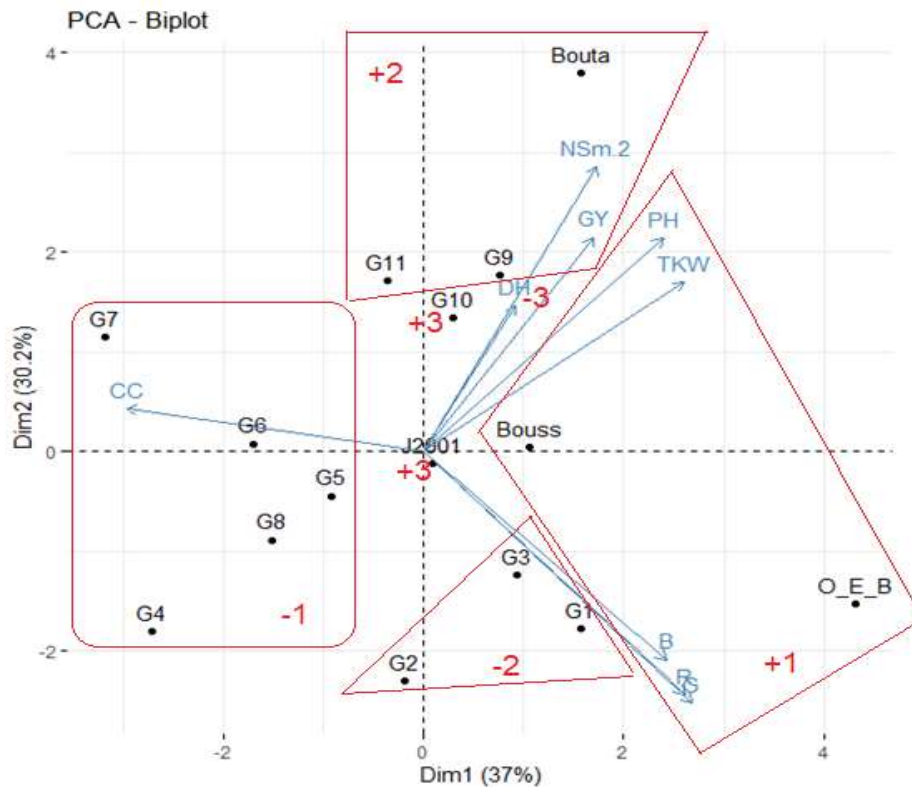
The principal component analysis PCA reflects the importance of the largest contributor to total variation at each axis of differentiation (Sharma, 1998). The data presented in the table 5 showed that the first 3 components of the PCA were the most important, they accumulate alone nearly than 80 % of the information on variability. Table 5 and 6 show that PC1 was positively correlated with reflectance index at R.G.B bands ( $r = 0.69$ ;  $0.71$ ;  $0.64$  respectively), TKW ( $r = 0.69$ ) and PH ( $r = 0.63$ ), Boussemel and Oued El Bared local landraces were the best genotypes related to this component ( $cor = 1.05$ ;  $4.23$ ). PC1 is also negatively correlated to CC ( $r = -0.78$ ) with the advanced lines G4, G5, G6, G7, G8 as best related genotypes ( $cor = -2.71$ ;  $-0.93$ ;  $-1.71$ ;  $-3.18$ ;  $-1.52$ ). PC2 was positively correlated with GY and NSm<sup>-2</sup> ( $r = 0.57$ ;  $0.75$ ) with G9, G11 advanced lines and Boutaleb as best related genotypes ( $cor = 1.77$ ;  $1.72$ ;  $3.80$ ), G1, G2, G3



advanced lines were negatively related to this component. PC3 was negatively correlated with DH ( $r = -0.83$ ), G10 advanced line and jupare C2001 foreign race were positively related to this component. The relations of measured traits and the 15 genotypes tested with the first 3 components are graphically summarized in Fig 1.



**Figure 1.** Reflectance calculating in R.G.B bands using Mesurim\_pro\_02 software.



**Figure 2.** Biplot of the relation of the 15 genotypes studied and the measured traits with the first 3 components of the PCA.

## CONCLUSION

ANOVA showed that genotype effect was significant ( $P < 0.001$ ;  $0.01$ ;  $0.05$ ) for all traits studied. The local landrace Boutaleb witch was the best yielding genotype registered a low Red reflectance index and an average Green reflectance index, blue reflectance index and chlorophyll contents. The study of the correlations revealed that chlorophyll contents was significantly ( $P < 0.01$ ) and negatively correlated with reflectance index at Red and Blue bands and very significantly ( $P < 0.001$ ) and positively correlated with reflectance index at Green band. PCA showed that grain yield was affected by Number of spike per meter square, the high values of RGB reflectance index contribute at the elevation of the weight of 1000 kernels and plant height, a negative relation was observed between chlorophyll contents and RGB reflectance index.

**Conflict of Interest:** *The authors have declared no conflict of interests exists.*

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## **BIOACTIVE AND ANTIOXIDANTS COMPOUNDS: TRENDS AND CHALLENGES FOR FOOD PRESERVATION**

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### **ABSTRACT**

The preservation of food is essential for human health and survival. Nowadays numerous strategy are used including reducing water activity, increase or decrease the temperature, modify the redox potential, preservatives, and competitive microorganisms are mostly used in the preservation of food products. Moreover, artificial preservatives (sorbate, sulfite, or nitrite) are supplemented into the food. Those chemical molecules may cause serious health hazards such as hypersensitivity, asthma, neurological damage, hyperactivity, and cancer. For this reason, scientist are investigating on the possibility to use natural compound including plant extract to preserve the nutritional and quality of the food. Plant extracts are engorged with various bioactive compounds expressing antioxidants and antimicrobials activities, such as phenolic compound. Those compounds are largely represented in numerous plants species and can be easily extracted. Moreover, phenolic compounds have witnessed of their beneficial properties to human health including in the prevention of cancer, diabetic and cardiovascular diseases, and treatment of numerous microbial infection. Those biological activities are related to their potent antioxidant and scavenging activity and antimicrobial activities. Thus, incorporation of plant extracts rich in phenolic compounds in foods can be considered in order to preserve the human health and food quality.

**Keywords:** Plant extract, antioxydants, antimicrobials, food preservation.

### **INTRODUCTION**

In response to environmental aggression such as abiotic and biotic or interspecific interactions, plants produce secondary metabolites also commonly named bioactive compounds (BA)(Álvarez-Martínez et al., 2021). These bioactive compounds are also obtained from fungi, animals, and bacteria as well as from agro-industrial residues (Shirahigue & Ceccato-Antonini, 2020). The bioactive compound can also be produced by chemical or biotechnological synthesis. However, biotechnological synthesis is more preconized than chemical synthesis due bioactive substances following biotechnology synthesis can be produced under the label of natural and organic products, such compounds are produced from renewable corps, or even they are derived from wastes or by-products (Jain & Anal, 2017). Human has used essential oils and plant extracts for a decade as folk medicines and as food preservatives (Hintz et al., 2015).

Bioactive compounds from plant material are classified as essential oils, polyphenols, triterpenes and phytosterols, terpenoids, alkaloids, saponins glucosinolates and other (Marcillo-Parra et al., 2021). These bioactive compounds account for various health benefits and help to prevent numerous diseases and metabolic dysfunction.

Their large chemical diversity and their traditional, plants are defined as a very attractive natural reservoir for research into the discovery of new antimicrobial and antioxidant compounds and use them in food and cosmetic industry as well as in pharmaceutical industry. Essential oils, flavonoids, tannins, phenolic acids, carotenoids, organosulfur compounds, phytosterols and tocopherols are used in the processing of vegetable oils, meat and seafood products, bakery products, dairy products, etc (Pai et al., 2022)

Numerous investigations revealed that we could use these bioactive compounds as antimicrobial agents to prevent food poisoning, or as antioxidant agents to reduce the oxidation in food. Besides all these scientific achievements, we keep highlighting that the importance of the extraction process is a critical part in the strategy of phytochemical processing for the discovery of bioactive constituents from plants. Because the extraction methods could affect the bioactive compounds composition. Moreover, the bioactive compounds are defined for their unstable and sensitive state and they tend to degrade quickly. Thus it is necessary to find a strategy to add the bioactive compound in the selected food and to guarantee the bioactivity at all stages of production's life and absorption during digestion in the gastrointestinal tract (Pinheiro et al., 2021). As a result, with scientists many food manufacturers are on the lookout for to make health claims for their products and launching initiatives to help address human health challenges.

In this context, many questions arise regarding to the nature and the activity of the bioactive compound supplemented in the food. The purpose of this review is highlight on the challenges and trend to use these compounds as food preservative.

### **BIOACTIVE COMPOUNDS: FROM EXTRACTION TO THE FOOD PRESERVATION**

Bioactive compounds defined all compounds able to express bioactive properties in the human health without supplementing any nutritional benefit (Câmara et al., 2020). They account for various health benefits and help to prevent numerous diseases and metabolic dysfunction (Câmara et al., 2020). Moreover, they gain scientific and industry attention to be used as an alternative to synthetic food preservative (Gavriil et al., 2021). Besides medicinal plants, bioactive compounds may be extracted from foods such as vegetables, fruits, and whole grains. They are also obtained from fungi, animals, and bacteria (Câmara et al. 2020). Moreover, a wide range of by-products coming from fruit and vegetable processing from agro-industrial including peels, seeds, flower, leaf, stem, pomace, bagasse, and extracts are enriched by a variety of bioactive compounds. Among the bioactive compounds scientists characterized including polyphenols, glucosinolates, carotenoids, terpenoids, alkaloids, saponins, and essential oils (Marcillo-Parra et al., 2021).

Phenolic compounds are ubiquitous in plants, with aromatic plants such as herbs and spices being especially rich in their phenolic content. By-products of plant origin foods and essential oil industry are also rich in phenolic compounds (Gavriil et al., 2021). They express a wide range of physiological properties, including antioxidant effects and antimicrobial activity against a broad spectrum of pathogenic and spoilage bacteria (Marín et al., 2015). Polyphenol compounds present a high chemical diversity. According to their chemical structures, they can be subdivided into families including flavonoids, stilbenes, lignans, tannins and phenolic acids, among others (Álvarez-Martínez et al., 2021). Another group of plant agents that have been extensively studied is essential oils. They are mainly composed of aromatic and volatile

compounds harvested from different parts of plants, especially the leaves and flowers. Essential oils are usually engorged with terpenes, sesquiterpenes, esters, aldehydes, phenols, ethers and peroxides. Essential oils derived from plants have expressed an interesting antimicrobial therapeutic effect, antioxidant and cytotoxic properties (Ghavam et al., 2020).

Extraction is an important step in the strategy of phytochemical processing for the discovery of bioactive constituents from plants part. The importance of the extraction process is critical part, because the extraction methods could influence the composition of bioactive compounds. The basics extraction process flows some steps including collection, pre-washing, drying or freeze-drying of plant materials. The plant materiel is grinding in order to obtain a homogenous sample, and then the extraction process can be launched. Extraction strategies can subdivide in two type: conventional methods and non-conventional methods. The conventional methods include maceration and decoction are widely used for the essential oils extraction at a household scale. On another hand, the model laboratory-scale such as Soxhlet extraction method based on the use of slightly elevated temperatures to recirculate solvent within an apparatus and to aid the extraction of compounds from samples placed in a thimble (Pai et al., 2022). Rather than these, conventional methods have limitations. They require high extraction time and bulk solvent use, lower efficiencies when relative yields and specificity of extraction compounds are taken into consideration. Often, pure, expensive and toxic solvents are necessaire in these conventional methods. Moreover, elevated temperatures are required for the conventional methods which may altered or modify the nature of the compounds. In order to overcome these limitations, new methods “non-conventional methods” are developed, some of them are named “Green extraction”. They are based on the use of new type of solvents, less toxic, cost effective and more specific. In these non-conventional methods ionic liquids (Chemat et al., 2012), deep eutectic solvents (Fomo et al., 2020) and aqueous two-phase systems have been used to increase yield and speed of extraction. Ionic liquids is an alternative organic solvent mainly in fields of food, flavors and fragrances. Even ionic liquids show theirs performance in the extraction of polyphenol, the only issues with the use of Ionic liquids is difficulty to harvest the final active compounds (Ratti, 2014). The main aim of the non-conventional extraction method is the cell wall rupture or deterioration, then the liberation of cell content. To ensure that, the non-conventional extraction method is assisted using ultrasound pressurized liquids, microwaves and pulsed electric fields. Thus increasing mass transfer and facilitating effective mixing due to the exposure of cytoplasmic content to the solvent(Fomo et al., 2020). Enzyme assisted extraction is mostly recommended to extract actives compounds associated with the cell wall rather than the cytoplasm. The main extraction process following enzyme assisted extraction method is the use of enzyme driven cell wall digestion. Aqueous based methods are promising methods because water is cheap and safe. However, following these methods in extraction reduce the extraction yield. The mass transfer rate of soluble ingredient into the solvent depend in the concentration of bioactive soluble ingredient. Thus, heating the solvent can improve mass transfer(Ivanov et al., 2022). Indeed, the active compounds are sensitive to heat. Extraction is higher wen the heating time and temperature increases. Unfortunately, for heat sensitive compound the yield may be low due to the simultaneous degradation. While some investigation shows that moderate heat can improve the antioxidant proprieties of actives compounds by changing the isomer content (Mostafa et al., 2018). Now days, scientist encourage the use of green and sustainable extraction to obtain bioactive compounds. Ultrasonic assisted combined with microporous resin enrichment method is another green technology, according to Zhang and his colleagues how used this method for the flavonoid extraction from *Acanthopanax senticosus*, they defined this method as the most effective process and recyclable strategy (Zhan et al., 2022). On another hand Yazid and his collaborators, demonstrate that the application of an enzyme such as pectinase, cellulose and

tannase for phenolic compound extraction from pistachio green hull improve their extraction rate, selectivity and yields. While the combination of enzymes such as tannase with other enzyme increased the extraction yield and the antioxidant activity 2.6 fold and 4.6 fold compared with the solvent extraction (Ghandahari Yazdi et al., 2019). However, enzyme assisted extraction method has some limitation. In general, enzymes are relatively expensive, cannot break down cell walls and their behavior has been rigidity is limited by environment conditions (Zuorro et al., 2019). Câmara and his collaborators, revealed that the ultrasonic waves can improve the ability of the enzyme extraction (Câmara et al., 2020).

Controlling microbial mainly pathogenic germ growth in food products has always been a principal thread for the different food field investors. Due to the fact that microbes responsible for infectious diseases increase the morbidity and mortality rate. Phenolic compounds may spread out the shelf life of several food products or improve food safety by promoting inactivation or inhibiting the foodborne pathogens growth (Gavriil et al., 2021). The antimicrobial action of phenolic components are considered to have different target within microbial cellular level. They may modify of the permeability of cell membranes by the formation of cytoplasmic granules and rupture of the cytoplasmic membrane; changing in various intracellular functions induced by hydrogen bonding of the phenolic compounds to enzymes through their OH groups; and modifying of fungal morphology) induced by different interactions with cell membranes (Álvarez-Martínez et al., 2021). Reyes and his collaborators revealed that *Campylobacter jejuni* and *Shewanella putrefaciens* were effectively inhibited by the *Simira ecuadorensis* phenolic extracts with minimum inhibitory concentration (MIC) of 80 mg/mL. *Bacillus cereus*, *Yersinia enterocolitica*, *Clostridium perfringens*, and *Leuconostoc mesenteroides* were also inhibited by ethanolic extract (Reyes et al., 2022) Related to their antioxidant proprieties phenolic compound can be used for food safety purposes are related to their antioxidant activity example of rosemary extract rich in phenolic compounds (Bouarab Chibane et al., 2019).

The bioactive compound are defined for their unstable state and they tend to be degraded quickly. Thus it is necessary to find a formula or structure to add functionality to the selected food and guarantee the maintain of the bioactivity at all stage of the product's life, from manufacture to incorporation and storage to consumption, ingestion and absorption during digestion in the gastrointestinal tract (Ramachandraiah et al., 2014). One of the solution proposed by the technology to solve the previous issues is the encapsulation technology. The encapsulation process based on coating the bioactive compounds with another single material or combination of materials to stabilize sensitive bioactive compounds (Marcillo-Parra et al., 2021). (Alu'datt et al., 2022).

In order to select the standard method for the extraction of bioactive compound from plants and food supplementing with bioactive compound, we have to understand the nature of the plant sample, the critical input parameters, the sensitivity of the molecules of interest and the endues.

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## INHERITANCE OF LINT PERCENTAGE IN F<sub>1</sub> DIALLEL COTTON CROSSES (*GOSSYPIUM HIRSUTUM* L.)

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### ABSTRACT

Research on the genetic control of lint percentage is of great importance to improve this cotton trait through selection. A higher lint percentage contributes to the increase of fiber yield per hectare. The aim of this research was to study the inheritance of lint percentage in two half diallel crosses each one included six *G. hirsutum* L. varieties as parental forms differing in lint percentage. Each diallel cross was tested in two consecutive years. One set of F<sub>1</sub> hybrids and their parental forms were studied. Genetic structure of studied trait, genetic components of variance, some genetic parameters and indexes of inheritance and the variability due to the year conditions, as well as general combining ability (GCA) and specific combining ability (SCA) of parental forms were studied. It was found that statistically significant were both additive and dominant gene effects. Dominant gene action predominated over additive one for both diallel crosses indicating the greater importance of dominant variance in the inheritance of lint percentage. The parental forms had different dominance (recessiveness) in the two years of study probably due to genotype-environment interaction. Because of high degree of dominance, weakly genetic variability and different expression of genes depending on environmental conditions, quick and successful selection can not be expected. Dorina and Barut 2005 (Turkish) varieties from the 1<sup>st</sup> diallel combination, Chirpan-539 and Helius from the 2<sup>nd</sup> diallel combination had the highest mean values and the highest GCA for lint percentage. These varieties proved to be the best general combinator for this trait.

**Keywords:** Cotton, *G. hirsutum* L., Diallel analysis, Lint percentage, Inheritance, Combining ability

### INTRODUCTION

Creation of cotton varieties having high lint percentage has economic and social importance. To create such varieties it is necessary to develop the theoretical basis of selection as well as to enrich and improve the selection methods and to improve the selection of parental forms. Research on the selection value of source material is very limited in our country. For its effective use in breeding programs it is necessary to determine the parameters of genetic systems determining the dispersion of lint percentage in hybrid populations, the genetic character and combining ability of parental components.

Lint percentage is a relatively stable trait, but it is also affected by environmental conditions and modification of genetic parameters for this trait is insufficiently studied.

From the specialized literature, it can be seen that various selection methods and techniques are used for the genetic improvement of traits and genotypes. Gene action and genetic variation are some of the most important criteria for any breeding program.

Application of diallel analysis helps to obtain the information necessary for selection programs. Diallel crosses and diallel analysis are widely used in the genetic and selection studies to assess the selection value of parental forms and hybrids even in the earliest segregated generations. This analysis allows parents to be crossed in all possible combinations and is mainly used to estimate genetic variance. A number of authors using various schemes of complete and incomplete diallel crosses have established the breeding value of a large number of cotton varieties and the effects of gene action on the characteristics related with yield and fiber quality (Dukre et al., 2009; Khan et al., 2009; 2011; Batool, 2011). Basal and Turgut (2005) revealed the presence of genes showing overdominance for lint percentage.

Ali et al. (2009) reported that dominant effects ( $H_1$  and  $H_2$ ) were major controlling factors for monopodia per plant, boll number, lint percentage and seed cotton yield.

Lu and Myers (2011) for diallel crosses used 10 cultivars from 16 selection programs most commonly used in crosses. Additive effects were more important than non-additive for the inheritance of number of bolls per plant, boll weight, lint percentage, seed index and lint index. Sarwar et al. (2011) in a 4×4 diallel cross reported that additive gene effects with partial dominance controlled plant height, number of sympodial branches, number of bolls, seed cotton yield, boll weight and lint percentage. In Vasconcelos' study (2018) of 20 cotton hybrids, additive gene effects controlled yield, boll weight, lint percentage and flowering. Wu et al. (2010), Karademir and Gencer (2010), Igbal et al. (2013), Raza et al. (2013) reported positive additive effects for lint percentage, while Singh et al. (2010) observed the opposite, a predominance of non-additive gene action for this trait.

The aim of this study was through diallel analysis of lint percentage in  $F_1$  cotton hybrids from two diallel crosses to determine the parameters of variance genetic components and some indicators of inheritance, needed to determine the selection strategy, as well as to determine the breeding value of parental forms in view of their effective use in breeding programs.

## MATERIAL AND METHOD

The hybrid populations of two diallel crosses were studied. In the first one (1<sup>st</sup> DC) six upland cotton varieties (four Bulgarian and two foreign) were included: Beli Iskar -  $P_1$ ; Barut 2005 (Turkish) -  $P_2$ ; Darmi -  $P_3$ ; Mytra (Greek) -  $P_4$ ; Helius -  $P_5$  and Dorina -  $P_6$ . Second diallel cross (2<sup>nd</sup> DC) included varieties: Chirpan-539 -  $P_1$ ; Helius -  $P_2$ ; Rumi -  $P_3$ ; Boyana -  $P_4$ ; Natalia -  $P_5$  and Nelina -  $P_6$  (Bulgarian selection).

An incomplete diallel scheme was used, including the parents and one set of  $F_1$  hybrids from direct crosses. The study was carried out at the Field Crops Institute in Chirpan in two consecutive years. The trials were set in three replicates, the parents and their  $F_1$  hybrids were sown in 2 rows of 2.4 m in a 60×20×1 sowing scheme. Ten plants from each replication were accounted.

Dispersion and diallel analyses, adequate of diallel scheme, were applied to the data processing (Mather and Jinks, 1982). Adequacy of data for diallel analysis was assessed by the regression coefficient  $b$  and  $t$  ( $W_r - V_r$ ) (Mather and Jinks, 1982).

The following components of genotypic variation along with their standard errors were calculated:  $D$  – additive genetic variance;  $H_1$  and  $H_2$  – dominance variances;  $F$  - shows the ratio of dominant to recessive genes in parents;  $E$  - variability due to the influence of environmental conditions.

Based on the above components the following indicators were calculated:  $(H_1/D)^{1/2}$  - average degree of dominance in each locus;  $H_2/4H_1$  - ratio of the positive and negative alleles in the loci showing dominance in the parents;  $K_D/D_R [(4DH_1)^{1/2} + F/(4DH_1)^{1/2} - F]$  - the ratio of dominant to recessive genes in parents;  $K=(h^2/H_2)$  - number of effective factors;  $H^2$  and  $h^2$  –

coefficients of heritability in a broad and in a narrow sense, calculated by Mather and Jinks (1982).

## RESULTS AND DISCUSSION

Lint percentage is a genetically stable trait and is less influenced by environmental conditions. Ranking of parents was relatively the same during the two years of study. Preliminary analyses of variances showed that crosses from both diallel combinations differed significantly in lint percentage (data are not given here).

The results obtained from the analyses carried out for adequacy of data of the additive-dominance model (Mather and Jinks, 1982) are presented in Table 1. In all analyses, with a full set of parents, the regression coefficients had lowered values suggesting non-allelic interaction effects.

In the first diallel combination in the first year of testing (1<sup>st</sup> DC 1<sup>st</sup> year) the analysis results showed a partial adequacy of the additive-dominant model even after excluding one of the parents, the regression coefficient  $b$  was low, statistically did not differ from zero and differed from unity, but the variation of parental rows on the  $W_r-V_r$  parameter was statistically insignificant, and did not indicate the presence of non-allelic interactions.

After excluding one of the parents -  $P_1$  (Beli Iskar) or  $P_6$  (Dorina) from the scheme of 1<sup>st</sup> diallel combination tested for 2<sup>nd</sup> year (1<sup>st</sup> DC 2<sup>nd</sup> year), in both cases, the resulting set of parents satisfied the requirements for diallel analysis. The scheme of 2<sup>nd</sup> diallel combination tested for 1<sup>st</sup> year and for 2<sup>nd</sup> year (2<sup>nd</sup> DC 1<sup>st</sup> year and 2<sup>nd</sup> DC 2<sup>nd</sup> year), from which  $P_2$  (Helius variety) or  $P_5$  (Natalia variety) were excluded from the analysis, also corresponded to the additive-dominant model.

Table 1. Additive-dominant model test for lint percentage in two diallel crosses carried out in two consecutive years

| Diallel cross, Year                     | Excluded parent | $b_{W_r/V_r} \pm b$ | $0 > b > 0$<br>$b \neq 0$ | $1 > b > 1$<br>$b \neq 1$ | $t_{(W_r-V_r)}$ |
|---|-----------------|---------------------|---------------------------|---------------------------|-----------------|
| 1 <sup>st</sup> DC 1 <sup>st</sup> year | $P_3$           | 0.551±0.446         | 1.238                     | 1.006                     | 0.129           |
| 1 <sup>st</sup> DC 2 <sup>nd</sup> year | -               | 0.779±0.174         | 4.481*                    | 1.275                     | 0.908           |
|   | $P_1$           | 0.827±0.235         | 3.525*                    | 0.736                     | 0.370           |
|   | $P_6$           | 1.033±0.174         | 5.926**                   | -0.193                    | 0.528           |
| 2 <sup>nd</sup> DC 1 <sup>st</sup> year | $P_2$           | 0.931±0.398         | 2.338                     | 0.177                     | 0.492           |
|   | $P_5$           | 1.172±0.365         | 3.201*                    | -0.461                    | 1.209           |
| 2 <sup>nd</sup> DC 2 <sup>nd</sup> year | -               | 0.668±0.314         | 2.124                     | 1.058                     | 0.474           |
|   | $P_2$           | 1.005±0.347         | 2.896                     | -0.016                    | 0.419           |
|   | $P_5$           | 0.965±0.310         | 3.110*                    | 0.113                     | 0.230           |

DC - Diallel combination (diallel cross)

Significant  $p \leq 0.05$  \*;  $p \leq 0.01$  \*\*

The graphical diallel analysis of first diallel combination tested in the first year (1<sup>st</sup> DC 1<sup>st</sup> year) with excluded  $P_3$  - Darmi variety, showed that the regression line intersected the  $W_r$ -axis above the origin meaning incomplete dominance (Figure 1). The relative positions of the array points on the regression line showed that Barut 2005 variety was highly dominant because of it was nearest to the origin, while Dorina variety was away and it was highly recessive. The other three varieties occupied an intermediate position. It is possible that all varieties had more dominant alleles it is not known where exactly the parabola intersected the regression line.

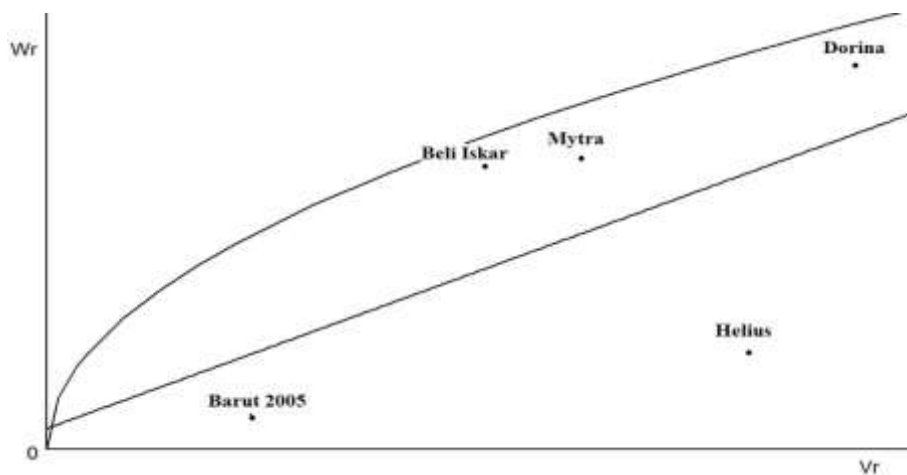
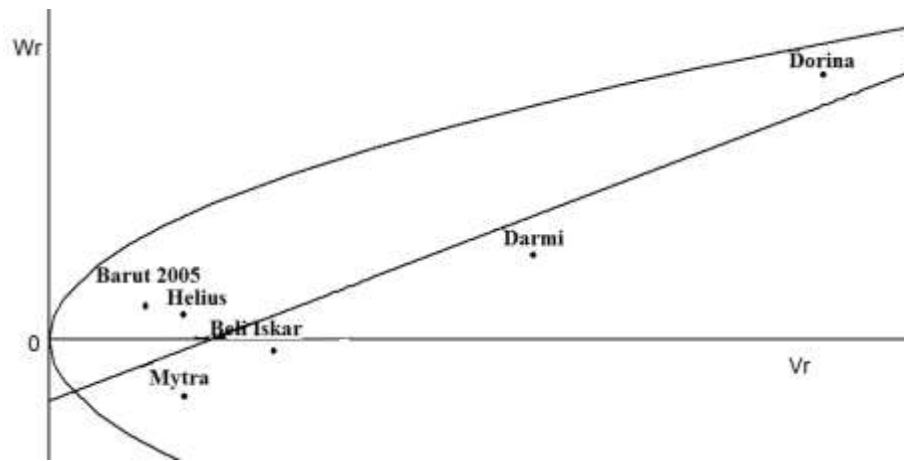


Figure 1. Graphical diallel analysis of lint percentage for the first diallel combination tested for first year (1<sup>st</sup> DC 1<sup>st</sup> year) with excluded parent P<sub>3</sub> – Darmi variety

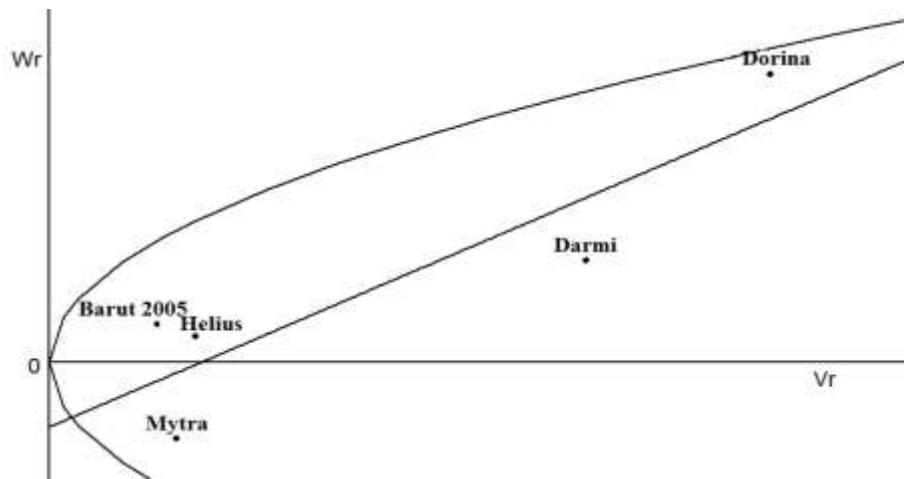
Figure 2a presents the  $W_r/V_r$  graph analysis of the first diallel combination tested in the 2<sup>nd</sup> year (1<sup>st</sup> DC 2<sup>nd</sup> year) without exclusion of parents and Figure 2b and 2c present the same diallel cross with excluded P<sub>1</sub> (Beli Iskar variety) and P<sub>6</sub> (Dorina variety) for which it was considered to cause non-allelic interactions. From the graph with full set of parents (Figure 2a) it is seen that the regression line intersected the  $W_r$ -axis slightly below the origine in its negative side showing weak over-dominance. The varieties Barut 2005 (P<sub>2</sub>), Mytra (P<sub>4</sub>), Helius (P<sub>5</sub>) and Beli Iskar (P<sub>1</sub>) located nearest to the origin had maximum dominant genes, whereas Dorina variety (P<sub>6</sub>), with the highest lint percentage, was located away from the origin and had maximum recessive genes. From the position of Dorina variety (P<sub>6</sub>), it can be seen, that this one was far from possible full recessivity it is not known where exactly the parabola cut the regression line. Darmi variety (P<sub>3</sub>), with the lowest lint percentage, occupied an intermediate position.

In the diallel graph after excluding P<sub>1</sub> - Beli Iskar variety (Figure 2b) there was no displacement of parental points, parents retained their positions. After P<sub>6</sub> (Dorina variety) was excluded a slight displacement of parental points located in the dominant part of the diallel graph was observed (Figure 2c). Barut 2005 (P<sub>2</sub>) (with the highest lint percentage) and Mytra (P<sub>4</sub>) varieties had high dominance with maximum of dominant genes, while Darmi variety (P<sub>3</sub>) (with the lowest lint percentage) had a high recessiveness, with a maximum of recessive genes. Beli Iskar (P<sub>1</sub>) and Helius (P<sub>5</sub>) varieties have moved a little to the right, but were in the dominant part of the graph, which expresses preponderance of dominant genes over recessive ones.

a)



b)



c)

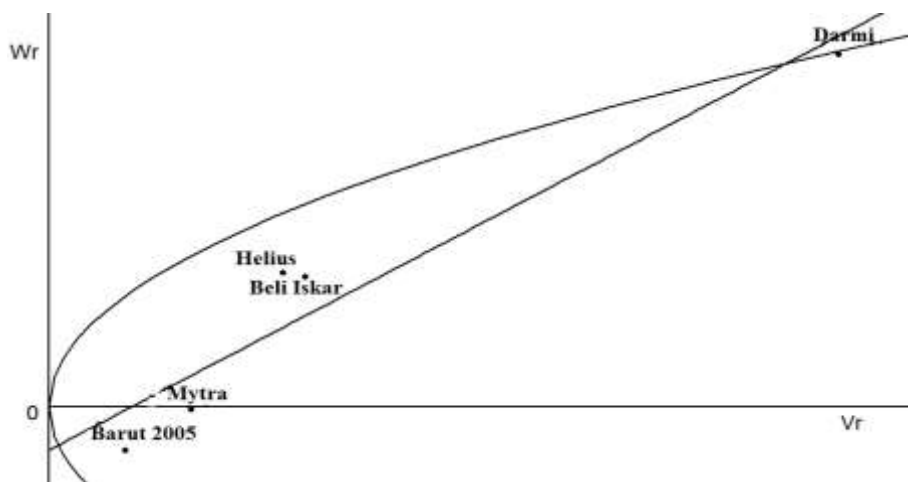


Figure 2. Graphical diallel analysis of lint percentage for the first diallel combination tested for second year (1<sup>st</sup> DC 2<sup>nd</sup> year). **a** – all parents; **b** - excluded parent P<sub>1</sub>- Beli Iskar variety; **c** - excluded parent P<sub>6</sub> – Dorina variety

Figure 3a presents a graphical diallel analysis of the 2<sup>nd</sup> diallel combination tested in the first year (2<sup>nd</sup> DC 1<sup>st</sup> year) after excluding of P<sub>2</sub> (Heliuss variety), and Figure 3b presents the

same diallel combination, but after excluding of P<sub>5</sub> (Natalia variety). From the graph with excluded P<sub>2</sub> (Helius variety) (Figure 3a) it can be seen that the regression line intersected the ordinate in its negative part indicating overdominance. The location of Rumi (P<sub>3</sub>) and Natalia (P<sub>5</sub>) varieties, with the lowest lint percentage, defined them as parents with maximum of dominant genes, and Chirpan-539 (P<sub>1</sub>) variety, with the highest lint percentage, with the most recessive genes. From its location, it can be considered to be far from the possible full recessiveness. The other two varieties occupied an intermediate position and had a different ratio of dominant and recessive genes, Boyana variety (P<sub>4</sub>) was in the more dominant part, which expresses a preponderance of dominant genes over recessive ones, and Nelina variety (P<sub>6</sub>) was in the more recessive part, which expresses a preponderance of recessive genes over dominant ones. It is possible that in Nelina variety dominant and recessive alleles had the same frequencies it is not known where exactly the intersection of regression line with the parabola was. After excluding P<sub>5</sub> (Natalia variety) from the diallel analysis Nelina and Boyana varieties have moved noticeably further to the left in the dominant part of graph. These two varieties, as well as Helius P<sub>2</sub> and Boyana P<sub>4</sub> varieties, showed high dominance (Figure 3b). Chirpan-539 variety retained its location.

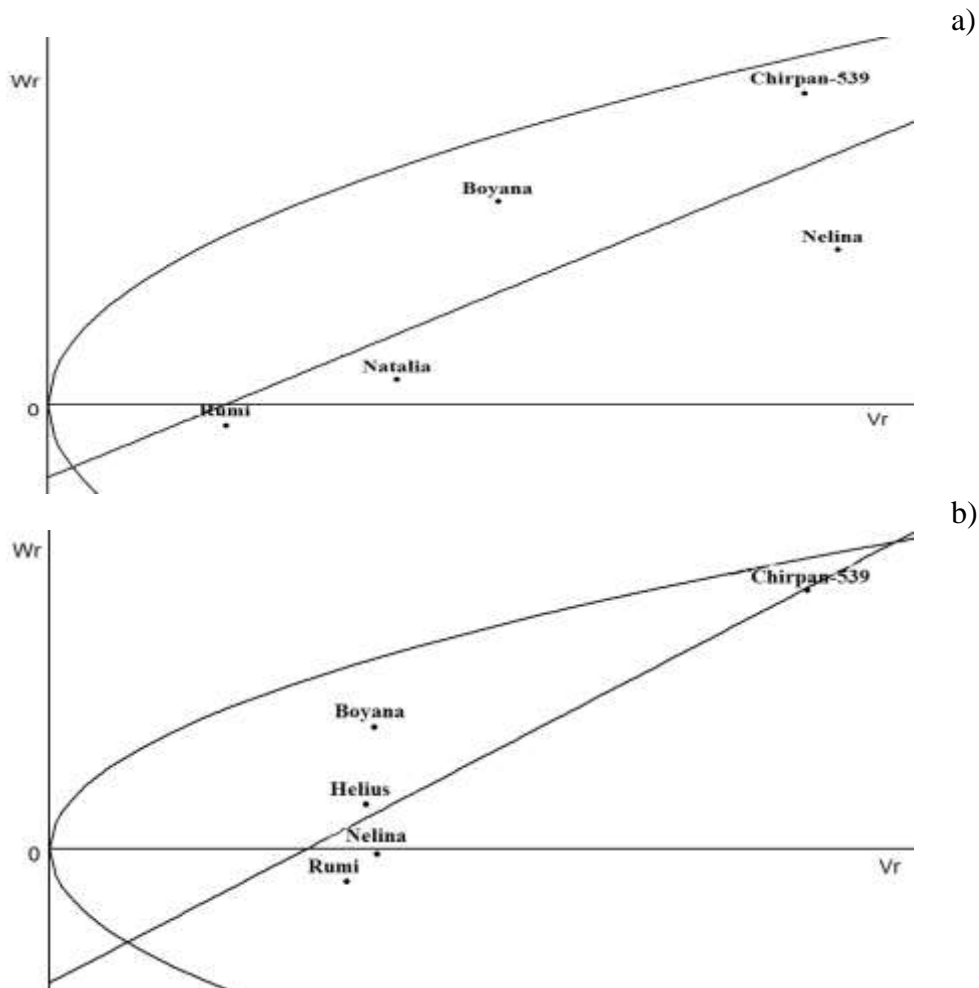


Figure 3. Graphical diallel analysis of lint percentage for the 2<sup>nd</sup> diallel combination tested in the first year (2<sup>nd</sup> DC 1<sup>st</sup> year): **a** - excluded parent P<sub>2</sub> - Helius variety; **b** - excluded parent P<sub>5</sub> - Natalia variety

There was a significant to opposite displacement in parental loci because of changes in environmental (year) conditions. In the diallel graph of 2<sup>nd</sup> diallel combination tested for 2<sup>nd</sup> year (2<sup>nd</sup> DC 2<sup>nd</sup> year) with a full set of parents the regression line intersected the ordinate in its negative part expressing overdominance (Figure 4a). Rumi (P<sub>3</sub>) and Chirpan-539 (P<sub>1</sub>) varieties, differing in lint percentage, had the highest dominance, whilst the highest recessiveness was found for Helius variety (P<sub>2</sub>). The other two varieties Boyana (P<sub>4</sub>) and Natalia (P<sub>5</sub>) were in the more recessive part of diallel graph, which means a preponderance of recessive alleles over dominant ones.

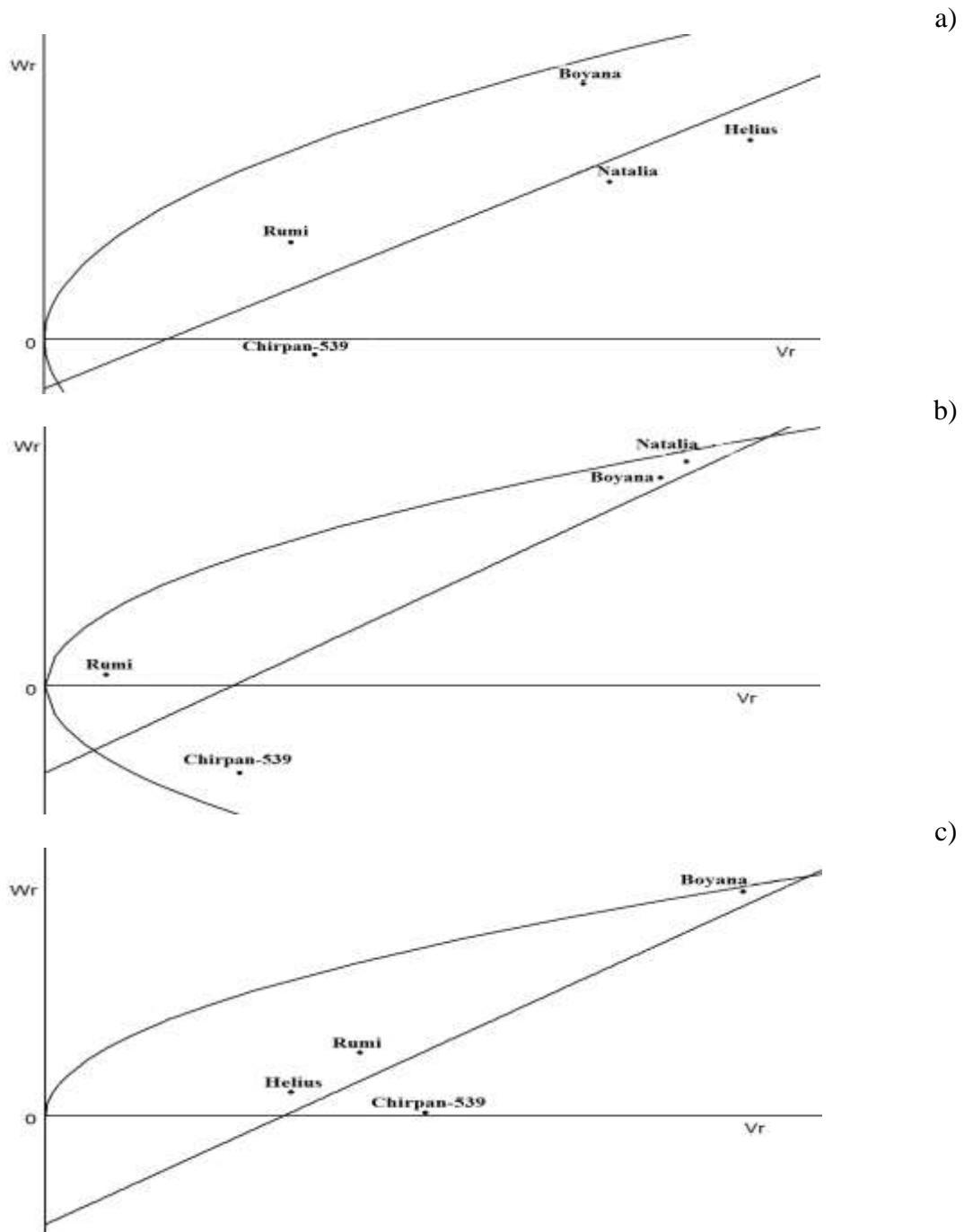


Figure 4. Graphical diallel analysis of lint percentage for the 2<sup>nd</sup> diallel combination tested for second year: **a** – all parents; **b** - excluded parent P<sub>2</sub> - Helius variety; **c** - excluded parent P<sub>5</sub> - Natalia variety



After excluding P<sub>2</sub> (Helius variety) there was a slight displacement of the parents (Figure 4b). Rumi variety (P<sub>3</sub>) shifted further to the left, in the more dominant part of diallel graph, and emerged as the parent with the most dominant genes, and Boyana and Nataliya varieties - as the parents with the most recessive genes. After exclusion of P<sub>5</sub> – Natalia variety, Helius variety (P<sub>2</sub>) showed the highest dominance and Boyana variety (P<sub>4</sub>) showed the highest complete recessiveness (Figure 4c). Rumi (P<sub>3</sub>) and Chirpan-539 varieties (P<sub>1</sub>) were also in the dominant part of diallel graph and showed a preponderance of dominant alleles over recessive ones.

In all diallel graphs, except for second diallel combination tested in the first year (2<sup>nd</sup> DC 1<sup>st</sup> year) and excluded P<sub>5</sub> (Natalia variety), the regression line crossed the  $W_r$ -axis below the origin in its negative part expressing overdominance. A deviation of the regression line down from the slope of 45° was observed (except for the second diallel combination tested for first year - 2<sup>nd</sup> DC 1<sup>st</sup> year and excluded P<sub>5</sub> - Natalia variety), which was indicative for the presence of complementary epistasis (Mather, 1967; Jinks et al., 1969). Effect of epistasis genes for all tested traits, except for the number of sympodia, was found by Singh *et al.* (2009).

The performed diallel analyses showed significant values for both additive and dominant effects (Table 2 and Table 3). The genetic parameter  $D$  has varied in ratios with the dominant parameters  $H_1$  and  $H_2$  showing a pronounced non-additive effect of genes, with the exception of first diallel combination tested for second year (1<sup>st</sup> DC 2<sup>nd</sup> year) and excluded P<sub>6</sub> - Dorina variety.

The parameter  $H_1$  indicated a high degree of dominance. The ratio  $H_1/D$  had values expressing overdominance in both diallel combinations. Mean dominance in loci, expressed as  $H_1/D^{1/2}$ , was also overdominant.  $M_{L1}-M_{L0}$  had positive values for all crosses and confirmed that the average degree of dominance was in direction to increase lint percentage. The values of  $H_2$  were close to those of  $H_1$  for the first diallel combination tested during the second year (1<sup>st</sup> DC 2<sup>nd</sup> year) without P<sub>6</sub> – Dorina variety and for the second diallel combination tested first and second year (2<sup>nd</sup> DC 1<sup>st</sup> year and 2<sup>nd</sup> year).

The  $H_2/4H_1$  ratio showed unequal distribution of positive and negative alleles in the loci exhibited dominance in the parents, except for the first diallel combination tested for first year (1<sup>st</sup> DC 1<sup>st</sup> year).

The parameter  $F$  for the first diallel combination tested in the first year (1<sup>st</sup> DC 1<sup>st</sup> year) and for the second diallel combination tested during the first and second years (2<sup>nd</sup> DC 1<sup>st</sup> year and 2<sup>nd</sup> year) was insignificant and changed its sign, which means that dominant and recessive alleles had the same frequencies. The  $F$  value for the 1<sup>st</sup> DC 2<sup>nd</sup> year showed predominance of dominant alleles, but their increasing position was confirmed by the  $h^2$  parameter only when P<sub>6</sub> (Dorina variety) was excluded.

With a full set of parents and excluded P<sub>1</sub> – Beli Iskar, the positive values of  $h^2$  were insignificant. The values of this variance component ( $h^2$ ) were positive and significant for the 2<sup>nd</sup> DC 1<sup>st</sup> and 2<sup>nd</sup> years and did not confirm the parameter  $F$ , whose insignificant values gave reason to assume that dominant and recessive alleles had close relative frequency.

The  $K_D/K_R$  ratio expressed a preponderance of dominant alleles, with the exception of 1<sup>st</sup> DC 1<sup>st</sup> year and 2<sup>nd</sup> DC 2<sup>nd</sup> year (with all parents and excluding P<sub>5</sub> – Natalia variety). In these,  $F$  had a negative sign, indicating a preponderance of recessive alleles,  $h^2$  values were positive but insignificant, and did not support an increasing importance of dominant alleles.

The correlation coefficients between the parental mean values and the sum of covariance-variance values -  $r_{xp}(W_r+V_r)$  for the 1<sup>st</sup> DC 1<sup>st</sup> and 2<sup>nd</sup> years with all parents and excluded P<sub>1</sub> – Beli Iskar were low and positive, when P<sub>6</sub> - Dorina variety was excluded the correlation coefficient was high and negative suggesting that the high lint percentage of parents having low  $W_r+V_r$  values was due to dominant genes. Parents containing maximum dominant genes were

responsible for lint percentage increasing in the 1<sup>st</sup> DC 2<sup>nd</sup> year with excluded P<sub>6</sub> - Dorina variety.

It was difficult to assess the nature of dominant determination of studied trait in the populations with a weak to medium relationship. This relationship in the 2<sup>nd</sup> DC 1<sup>st</sup> year was high and positive suggesting that the high lint percentage of parents having high  $W_R + V_R$  values was due to recessive genes. Parents containing maximum recessive genes were responsible for increasing the lint percentage in the 2<sup>nd</sup> DC 1<sup>st</sup> year. In the 2<sup>nd</sup> DC F<sub>1</sub>-2<sup>nd</sup> year, the correlation was from low negative (with full set of parents) to high negative (with excluded parents), which means that the high lint percentage of parental forms was determined by dominant genes with a unidirectional trait enhancing action.

The k values - number of effective factors indicated one or a group of genes controlling lint percentage. Stoilova and Taofic (2000) also reported one gene in interspecific cotton (*Gossypium hirsutum* L. x *G. barbadense* L.) hybrids, while Bhatade (1981) reported three genes.

Table 2. Genetic components and indicators of inheritance of lint percentage in the 1<sup>st</sup> diallel combination

| Genetic components   | 1 <sup>st</sup> DC 1 <sup>st</sup> year | 1 <sup>st</sup> DC 2 <sup>nd</sup> year |                                      |                                  |
|--|---|---|--------------------------------------|----------------------------------|
|  | Excluded P <sub>3</sub> – Darmi variety | All parents                             | Excluded P <sub>1</sub> – Beli Iskar | Excluded P <sub>6</sub> – Dorina |
| b  | 0.551 ± 0.446                           | 0.779 ± 0.174                           | 0.827 ± 0.235                        | 1.033 ± 0.174                    |
| <b>Parameters</b>  |   |   |                                      |                                  |
| D  | 1.212±0.302                             | 2.761±0.367                             | 3.404±0.473                          | 2.031±0.168                      |
| F (FR)   | -0.916±0.755                            | 2.778±0.897                             | 2.872±1.182                          | 1.013±0.420                      |
| H <sub>1</sub>   | 3.218±0.816                             | 7.287±0.932                             | 8.218±1.278                          | 2.825±0.453                      |
| H <sub>2</sub>   | 2.651±0.741                             | 4.838±0.833                             | 6.183±1.159                          | 2.392±0.412                      |
| h <sup>2</sup>   | 0.055±0.500                             | 0.112±0.561                             | 0.303±0.783                          | 3.079±0.278                      |
| E  | 0.132±0.123                             | 0.143±0.139                             | 0.134±0.193                          | 0.177±0.069                      |
| <b>Indicators</b>  |   |   |                                      |                                  |
| H <sub>1</sub> /D <sub>1</sub>   | 2.655                                   | 2.639                                   | 2.414                                | 1.391                            |
| H <sub>1</sub> /D <sub>1</sub> <sup>1/2</sup>                          | 1.629                                   | 1.250                                   | 1.554                                | 1.179                            |
| (M <sub>L1</sub> -M <sub>L0</sub> ) <sup>2</sup>                       | 0.035                                   | 0.048                                   | 0.097                                | 0.798                            |
| F <sup>2</sup> //4D (H <sub>1</sub> - H <sub>2</sub> )/ <sup>1/2</sup> | 0.618                                   | 1.484                                   | 1.550                                | 0.547                            |
| H <sub>2</sub> /4 H <sub>1</sub>                                       | 0.026                                   | 0.166                                   | 0.188                                | 0.212                            |
| Kd/kr  | 0.624                                   | 1.897                                   | 1.745                                | 1.536                            |
| r xp (W <sub>r</sub> + V <sub>r</sub> )                                | 0.143                                   | 0.305                                   | 0.218                                | -0.925                           |
| k  | 0.021                                   | 0.023                                   | 0.049                                | 0.057                            |
| <b>Heritability, %</b>   |   |   |                                      |                                  |
| H <sup>2</sup>   | 0.939                                   | 0.820                                   | 0.840                                | 0.805                            |
| h <sup>2</sup>   | 0.630                                   | 0.095                                   | 0.358                                | 0.240                            |
| <b>Prediction of the most dominant/recessive parent</b>                |   |   |                                      |                                  |
| YD   | 38.79                                   | 39.96                                   | 40.19                                | 41.47                            |
| YR   | 39.47                                   | 41.60                                   | 41.42                                | 37.89                            |

Coefficients of heritability in broad sense ( $H^2$ ) were high and in narrow sense ( $h^2$ ) were very low to moderately high. Relatively high heritability coefficient in narrow sense ( $h^2$ ) was

found for the 1<sup>st</sup> DC 1<sup>st</sup> year revealing the possibility for selection by this trait in the earlier hybrid generations - F<sub>2</sub>-F<sub>3</sub>.

Desalegn et al. (2009) reported high broad-sense heritability coefficients for lint percentage ( $h^2=97\%$ ). ChengQi et al. (2009) reported high broad- and narrow-sense heritabilities for lint percentage and fiber index, revealing that these traits were influenced by additive gene effects. Ali et al. (2009) noted that seed cotton yield and lint percentage had low heritability. Another study (Ali et al., 2010) reported high narrow-sense heritability. Aziz et al. (2014), Srinivas et al. (2014), Ahsan et al. (2015), Wagar et al. (2016), Nizamani et al. (2017) reported moderate to high heritability.

Table 3. Genetic components and indicators of inheritance of lint percentage in the 2<sup>nd</sup> diallel combination

| Genetic components  | 2 <sup>nd</sup> DC 1 <sup>st</sup> year  |   | 2 <sup>nd</sup> DC 2 <sup>nd</sup> year |  |   |
|---|--|---|---|--|---|
|   | Excluded P <sub>2</sub> – Helius variety | Excluded P <sub>5</sub> – Natalia variety | All parents                             | Excluded P <sub>2</sub> – Helius variety | Excluded P <sub>5</sub> – Natalia variety |
| <b>Parameters</b>   |  |   |   |  |   |
| D   | 1.179±0.163                              | 0.766±0.116                               | 1.921±0.361                             | 1.913±0.410                              | 1.428±0.237                               |
| F   | 0.500±0.407                              | 0.517±0.290                               | 0.802±0.903                             | 0.052±1.054                              | -0.149±0.609                              |
| H <sub>1</sub>  | 2.203±0.440                              | 2.028±0.314                               | 6.605±0.976                             | 5.523±1.193                              | 5.828±0.689                               |
| H <sub>2</sub>  | 2.101±0.399                              | 1.878±0.285                               | 6.223±0.885                             | 5.128±1.101                              | 5.512±0.636                               |
| h <sup>2</sup>  | 1.405±0.269                              | 0.738±0.192                               | 6.716±0.598                             | 6.578±0.747                              | 8.414±0.431                               |
| E   | 0.176±0.066                              | 0.162±0.047                               | 0.133±0.147                             | 0.127±0.183                              | 0.188±0.106                               |
| <b>Indicators</b>   |  |   |   |  |   |
| H <sub>1</sub> /D   | 1.869                                    | 2.648                                     | 3.438                                   | 2.887                                    | 4.081                                     |
| H <sub>1</sub> /D <sup>1/2</sup>                                    | 1.367                                    | 1.627                                     | 1.854                                   | 1.699                                    | 2.020                                     |
| M <sub>L1</sub> – M <sub>L0</sub>                                   | 0.379                                    | 0.210                                     | 1.70                                    | 1.668                                    | 2.139                                     |
| F <sup>2</sup> /4D.(H <sub>1</sub> -H <sub>2</sub> ) <sup>1/2</sup> | 0.360                                    | 0.394                                     | 0.135                                   | 0.00                                     | 0.01                                      |
| H <sub>2</sub> /4H <sub>1</sub>                                     | 0.239                                    | 0.231                                     | 0.235                                   | 0.232                                    | 0.236                                     |
| K <sub>D</sub> /K <sub>R</sub>                                      | 1.367                                    | 1.524                                     | 0.797                                   | 1.016                                    | 0.949                                     |
| rxp(W <sub>r</sub> +V <sub>r</sub> )                                | 0.960                                    | 0.883                                     | -0.380                                  | -0.891                                   | -0.866                                    |
| k   | 0.669                                    | 0.393                                     | 1.079                                   | 1.283                                    | 1.526                                     |
| <b>Heritability, %</b>  |  |   |   |  |   |
| H <sup>2</sup>  | 0.840                                    | 0.805                                     | 0.959                                   | 0.950                                    | 0.925                                     |
| h <sup>2</sup>  | 0.358                                    | 0.240                                     | 0.479                                   | 0.445                                    | 0.377                                     |
| <b>Prediction of the most dominant/recessive parent</b>             |  |   |   |  |   |
| YD  | 36.34                                    | 37.20                                     | 39.84                                   | 39.67                                    | 40.823                                    |
| YR  | 41.14                                    | 40.69                                     | 37.57                                   | 36.82                                    | 37.187                                    |

The parents were ranked by mean and by presence of dominant/recessive genes expressed by the sum of covariances and variances ( $W_r+V_r$ ) (data are not given here).

From the 1<sup>st</sup> diallel combination (1<sup>st</sup> DK 1<sup>st</sup> and 2<sup>nd</sup> years) Dorina variety (P<sub>6</sub>) had the highest lint percentage and the highest  $W_r+V_r$  values, which means that lint percentage was determined by recessive genes. Barut 2005 and Mytra varieties were ranked the next in lint percentage and had low  $W_r+V_r$  values, which means that their high lint percentage was determined by dominant genes. As for Beli Iskar and Helius varieties this trait was determined

by dominant and recessive genes, respectively with increasing (Beli Iskar variety) and decreasing (Helius variety) the trait values action.

Darmi variety had high  $W_r+V_r$  values, which means that its low lint percentage was caused by recessive genes that acted in direction of reducing this trait. Rumi and Natalia varieties (2<sup>nd</sup> DC 1<sup>st</sup> year) had lower lint percentage and low  $W_r+V_r$  values, which means that their lower values were determined by dominant genes that acted in direction to reduce lint percentage.

Chirpan-539 variety (2<sup>nd</sup> DC 2<sup>nd</sup> year) had high lint percentage and high dominance (low  $W_r+V_r$  values), dominant genes in it acted in the direction of increasing this trait. Boyana, Helius and Natalia varieties had low  $W_r+V_r$  values in the first year (2<sup>nd</sup> DC 1<sup>st</sup> year) and were highly dominant and high  $W_r+V_r$  values in second year (2<sup>nd</sup> DC 2<sup>nd</sup> year) and were highly recessive. Dominant genes responsible for lint percentage in the 1<sup>st</sup> year (2<sup>nd</sup> DC F<sub>1</sub>-1<sup>st</sup> year) had a decreasing effect on the trait values,  $rxp(W_r+V_r)$  was high and positive. The change in this indicator is an indicator of the trait variability, depending on the expressivity of genes associated with the redefinition of genetic control depending on the year conditions.

The  $F_r$  parameter (Table 4) showed that Barut variety (P<sub>2</sub>) (1<sup>st</sup> diallel combination) was highly dominant for lint percentage in both years. Mytra variety (P<sub>4</sub>) had high lint percentage and low dominance in the first year and high dominance in the second year.  $F_r$  parameter confirmed the high recessivity of Darmi (P<sub>3</sub>) and Dorina (P<sub>6</sub>) varieties.

Table 4. Mean values of the  $F_r$  parameter for each parental row for lint percentage in the 1<sup>st</sup> DC in the 1<sup>st</sup> and 2<sup>nd</sup> years

| Excluded P <sub>3</sub> – Darmi variety |        | All parents                |        | Excluded P <sub>1</sub> – Beli Iskar variety |        | Excluded P <sub>6</sub> – Dorina variety |        |
|---|--------|----------------------------|--------|--|--------|--|--------|
| b=0.551                                 |        | b=0.779                    |        | b=0.827                                      |        | b=1.033                                  |        |
| Parent                                  | Fr     | Parent                     | Fr     | Parent                                       | Fr     | Parent                                   | Fr     |
| P <sub>2</sub> - Barut 2005             | 2.314  | P <sub>4</sub> -Mytra      | 7.760  | P <sub>4</sub> -Mytra                        | 9.701  | P <sub>2</sub> - Barut 2005              | 4.810  |
| P <sub>5</sub> -Helius                  | -0.818 | P <sub>2</sub> -Barut 2005 | 5.973  | P <sub>2</sub> -Barut 2005                   | 6.924  | P <sub>4</sub> -Mytra                    | 3.767  |
| P <sub>1</sub> -Beli Iskar              | -0.920 | P <sub>5</sub> -Helius     | 5.702  | P <sub>5</sub> -Helius                       | 6.706  | P <sub>5</sub> -Helius                   | 1.198  |
| P <sub>4</sub> -Mytra                   | -1.484 | P <sub>1</sub> -Beli Iskar | 5.509  | P <sub>3</sub> -Darmi                        | -0.723 | P <sub>1</sub> -Beli Iskar               | 1.105  |
| P <sub>6</sub> -Dorina                  | -3.671 | P <sub>3</sub> -Darmi      | -0.085 | P <sub>6</sub> -Dorina                       | -8.248 | P <sub>3</sub> -Darmi                    | -5.814 |
|   |        | P <sub>6</sub> -Dorina     | -8.187 |  |        |  |        |

From the 2<sup>nd</sup> diallel combination Rumi (P<sub>3</sub>) (2<sup>nd</sup> DC 1<sup>st</sup> year) and Chirpan-539 (P<sub>1</sub>) varieties (2<sup>nd</sup> DC 2<sup>nd</sup> year) had a large number of dominant genes, in the first one acting in direction of reducing lint percentage and in the second one acting in the direction of its increase.

Many authors in cotton found a predominance of dominant genes over recessive ones for most traits. Others found opposite, more recessive genes than dominant ones or roughly equal for both types. In the studies of Basal and Turgut (2005) dominant alleles prevailed for earliness, number of bolls per plant, lint percentage and fiber strength.

Ali et al. (2009) observed superiority of dominant alleles for number of sympodia, lint percentage and seed cotton yield, and superiority of recessive alleles for plant height, boll number, staple length and fiber strength. In the studies of Basal and Turgut (2005), Ali et al. (2009) dominant alleles prevailed for lint percentage.

Individual crosses showed a different type of inheritance of this trait (data are not given here). The crosses Barut 2005 × Mytra, Barut 2005 × Helius and Barut 2005 × Dorina, first diallel combination, and Chirpan-539 × Helius, second diallel combination, showed the highest

lint percentage during the two years of study. Their both parental forms had a high lint percentage. In these crosses the inheritance of lint percentage was additive, incompletely dominant and overdominant to the better parent. Overdominance caused a weak heterosis effect, in the first diallel combination from 1.0% to 3.0% (Mytra  $\times$  Helius, 1<sup>st</sup> DC 2<sup>nd</sup> year) and in the second diallel combination from 1.3% to 5.7% (Chirpan-539  $\times$  Helius, 2<sup>nd</sup> DC 2<sup>nd</sup> year).

The GCA variances and the SCA variances were significant for both diallel combinations, in both years (Table 6).

Table 5. Mean values of the Fr parameter for each parental row for lint percentage in the second diallel combination during the first and the second years (2<sup>nd</sup> DC 1<sup>st</sup> and 2<sup>nd</sup> years)

| 2 <sup>nd</sup> DC 1 <sup>st</sup> year |  |   |   |   | 2 <sup>nd</sup> DC 2 <sup>nd</sup> years |             |   |  |   |   |   |
|---|--|---|---|---|--|-------------|---|--|---|---|---|
| Parents                                 | Excluded P <sub>2</sub> -Helius variety<br>b=0.931 |   | Excluded P <sub>5</sub> -Natalia variety<br>b=1.172 |   | Parents                                  | All parents |   | Excluded P <sub>2</sub> -Helius variety<br>b=1.000 |   | Excluded P <sub>5</sub> -Natalia variety<br>b=0.964 |   |
|   | Fr   | R | Fr  | R |  | Fr          | R | Fr   | R | Fr  | R |
| P <sub>1</sub> -Chirpan539              | -1.632   | 5 | -1.982  | 5 | P <sub>1</sub> -Chirpan539               | 3.863       | 1 | 5.780  | 1 | 1.663   | 2 |
| P <sub>2</sub> -Helius                  | -  | - | 1.050   | 3 | P <sub>2</sub> -Helius                   | -4.337      | 5 | 4.989  | 2 | 2.548   | 1 |
| P <sub>3</sub> - Rumi                   | 2.721  | 1 | 1.681   | 1 | P <sub>3</sub> -Rumi                     | 2.185       | 2 | -4.957   | 3 | 1.070   | 3 |
| P <sub>4</sub> - Boyana                 | 0.172  | 3 | 0.453   | 4 | P <sub>4</sub> -Boyana                   | -3.568      | 4 | -5.605   | 4 | -5.877  | 4 |
| P <sub>5</sub> - Natalia                | 1.820  | 2 | -   |   | P <sub>5</sub> -Natalia                  | -2.156      | 3 | -  | - | -   |   |
| P <sub>6</sub> -Nelina                  | -0.579   | 4 | 1.383   | 2 | P <sub>6</sub> - Nelina                  | -           | - | -  | - | -   |   |

Table 6. Analysis of GCA and SCA variances for lint percentage in the first and second diallel combinations, each one tested two years

| Diallel combination. year                    | Source of variation | Degrees of freedom | Mean squares | F experimentally |
|--|---------------------|--------------------|--------------|------------------|
| <b>1<sup>st</sup> DC 1<sup>st</sup> year</b> | Crosses             | 20                 | 8.564        | 24.45**          |
|  | GCA                 | 5                  | 20.171       | 57.60**/4.30**   |
|  | SCA                 | 15                 | 4.695        | 13.41**          |
|  | Errors              | 40                 | 0.350        |                  |
| <b>1<sup>st</sup> DC 2<sup>nd</sup> year</b> | Crosses             | 20                 | 6.779        | 17.11**          |
|  | GCA                 | 5                  | 12.653       | 31.93**/2.62ns   |
|  | SCA                 | 15                 | 4.821        | 12.17**          |
|  | Errors              | 40                 | 0.396        |                  |
| <b>2<sup>nd</sup> DC 1<sup>st</sup> year</b> | Crosses             | 20                 | 3.105        | 7.91**           |
|  | GCA                 | 5                  | 4.375        | 11.15*/1.63ns    |
|  | SCA                 | 15                 | 2.682        | 6.83**           |
|  | Errors              | 40                 | 0.392        |                  |
| <b>2<sup>nd</sup> DC 2<sup>nd</sup> year</b> | Crosses             | 14                 | 8.499        | 22.90**          |
|  | GCA                 | 4                  | 14.427       | 38.87**/2.35ns   |
|  | SCA                 | 10                 | 6.128        | 16.51**          |
|  | Errors              | 28                 | 0.371        |                  |

Significant:  $p \leq 0.05$  \*;  $p \leq 0.01$  \*\*; ns – non-significant

The varieties Dorina (P<sub>6</sub>) and Barut 2005 (P<sub>2</sub>) (first diallel combination) and Chirpan-539 (P<sub>1</sub>) and Helius (P<sub>2</sub>) (second diallel combination) had the highest mean values and the highest GCA effects for lint percentage and appeared to be the best general combiners for this trait (Table 7 and Table 8). Mean values of parents showed a positive relationship with their GCA. Zhang et al. (2016) reported that GCA effects were closely related to parental mean values for yield and fiber quality.

Table 7. Ranking of parents by GCA for lint percentage in the first diallel combination

| Ranking by GCA                          |      |        |   |      |        |
|---|------|--------|---|------|--------|
| 1 <sup>st</sup> DC 1 <sup>st</sup> year |      |        | 1 <sup>st</sup> DC 2 <sup>nd</sup> year |      |        |
| Parent                                  | x    | GCA    | Parent                                  | x    | GCA    |
| P <sub>6</sub> - Dorina                 | 40.6 | 1.162  | P <sub>2</sub> - Barut 2005             | 41.1 | 1.383  |
| P <sub>2</sub> - Barut 2005             | 39.9 | 1.042  | P <sub>6</sub> - Dorina                 | 41.7 | 0.020  |
| P <sub>4</sub> - Mytra                  | 39.0 | -0.137 | P <sub>4</sub> - Mytra                  | 39.8 | -0.083 |
| P <sub>1</sub> -Beli Izkar              | 39.0 | -0.225 | P <sub>5</sub> - Helius                 | 38.6 | -0.183 |
| P <sub>3</sub> - Darmi                  | 37.7 | -0.908 | P <sub>1</sub> -Beli Iskar              | 39.5 | -0.437 |
| P <sub>5</sub> - Helius                 | 37.5 | -0.933 | P <sub>3</sub> - Darmi                  | 37.9 | -0.700 |
| <i>GD. 5 %</i>                          | 0.98 |        | <i>GD. 5 %</i>                          | 1.04 |        |
| Standard error                          |      | 0.171  | Standard error                          |      | 0.181  |

Table 8. Ranking of parents by GCA for lint percentage in the second diallel combination

| Ranking by GCA                          |      |   |        |   |      |   |        |
|---|------|---|--------|---|------|---|--------|
| 2 <sup>nd</sup> DC 1 <sup>st</sup> year |      |   |        | 2 <sup>nd</sup> DC 2 <sup>nd</sup> year |      |   |        |
| Parent                                  | x    | R | GCA    | Parent                                  | x    | R | GCA    |
| P <sub>1</sub> -Chirpan-539             | 39.9 | 1 | 0.613  | P <sub>1</sub> -Chirpan-539             | 40.2 | 1 | 1.043  |
| P <sub>2</sub> - Helius                 | 38.3 | 4 | 0.433  | P <sub>2</sub> - Helius                 | 39.9 | 2 | 0.567  |
| P <sub>4</sub> - Boyana                 | 38.5 | 3 | -0.054 | P <sub>3</sub> - Rumi                   | 38.5 | 3 | -0.208 |
| P <sub>6</sub> - Nelina                 | 38.7 | 2 | -0.242 | P <sub>4</sub> - Boyana                 | 37.5 | 4 | -0.308 |
| P <sub>3</sub> - Rumi                   | 37.2 | 5 | -0.321 | P <sub>5</sub> - Natalia                | 37.0 | 5 | -1.094 |
| P <sub>5</sub> - Natalia                | 37.1 | 6 | -0.429 | P <sub>6</sub> - Nelina                 | -    | - | -      |
| GD 5 %                                  | 1.03 |   |        | GD 5 %                                  | 2.06 |   |        |
| Standard error                          |      |   | 0.181  | Standard error                          |      |   | 0.188  |

From the 1<sup>st</sup> diallel combination seven (46.7%) and eight (53.3%) crosses had positive SCA effects during the first year and second year, respectively. Two crosses Mytra × Helius and Barut 2005 × Darmi had significant positive SCA effects in both years and exhibited heterosis relative to the mean of both parents. In the second diallel combination significant positive SCA effects in both years were found for the crosses Helius × Rumi, Chirpan-539 × Boyana, Rumi × Natalia and Chirpan-539 × Helius (data are not given here).

Estimations of variance components for GCA and SCA are presented in Table. 9. Mainly non-additive gene effects were of importance for the inheritance of this trait. A non-additive type of gene action for lint percentage was noted by Singh et al. (2010), Lu and Myers (2011), Rasheed et al. (2014), Khan et al. (2017). Additive gene effects for this trait were found by ChengQi (2009), Wu et al. (2010), Karademir and Gencer (2010), Iqbal et al. (2013), Raza et al. (2013), Vasconcelos (2018).

Table 9. Variance components for lint percentage in the first and second diallel combinations tested for two years

| Sources of variation | Variance ± standard error               |   |   |   |
|----------------------|---|---|---|---|
|                      | 1 <sup>st</sup> DC 1 <sup>st</sup> year | 1 <sup>st</sup> DC 2 <sup>nd</sup> year | 2 <sup>nd</sup> DC 1 <sup>st</sup> year | 2 <sup>nd</sup> DC 2 <sup>nd</sup> year |
| Crosses              | 2.738 <sup>*</sup> ±0.903               | 2.127 <sup>**</sup> ±0.715              | 0.904 <sup>**</sup> ±0.328              | 2.709 <sup>*</sup> ±1.071               |
| GCA                  | 0.645±0.536 ns                          | 0.326 ±0.341 ns                         | 0.070±0.122 ns                          | 0.395±0.503 ns                          |
| SCA                  | 1.448 <sup>**</sup> ±0.572              | 1.475 <sup>**</sup> ±0.587              | 0.763 <sup>*</sup> ±0.328               | 1.919 <sup>*</sup> ±0.914               |
| Errors               | 0.350 <sup>***</sup> ±0.078             | 0.396 <sup>***</sup> ±0.088             | 0.392 <sup>***</sup> ±0.088             | 0.371 <sup>***</sup> ±0.099             |

p≤0.05 \*; p≤0.01 \*\*; p≤0.0001 \*\*\*; ns – non-significant

Summarized research results showed that most of genetic variation for lint percentage in both diallel combinations was non-additive and the selection for this trait should be conducted in the later hybrid generations. Varieties Barut 2005 (first diallel combination) and Chirpan-539 (second diallel combination) possessing high dominance for high lint percentage in different ecological environments could be successfully used in selection to improve this trait. The varieties Dorina, Barut 2005 (first diallel combination), Chirpan-539 (second diallel combination) and Helius (1<sup>st</sup> and 2<sup>nd</sup> diallel combinations) exhibited the highest lint percentage and the highest GCA appeared to be the best general combiners and can be used in future breeding programs to enhance this trait.

## CONCLUSIONS

Additive and non-additive gene effects were of importance for the inheritance of lint percentage. The non-additive gene action prevailed over additive one and the selection by this trait should be conducted in the later hybrid generations - F<sub>3</sub>-F<sub>4</sub>.

Barut 2005 and Chirpan-539 varieties had high dominance for high lint percentage during the two years, different environments, while Dorina variety had high recessivity for high level of this trait. These cultivars exhibited high GCA and emerged as very good general combiners to increase lint percentage.

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## DIRECTIONS AND ACHIEVEMENTS OF COTTON BREEDING IN BULGARIA

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### ABSTRACT

The main goal of cotton breeding in Bulgaria is to create new early and high-yielding varieties with improved fiber quality, resistant to abiotic and biotic stress factors of environment. In recent years, the new cotton varieties Pirin, Perun, Kristal, Aida, Anabel, Tiara and Melani have been created and approved (2019-2021). Pirin, Perun, Kristal and Annabelle varieties were obtained by inter-varietal crossing, Aida, Tiara and Melani varieties were created by interspecific hybridization and backcross technology. Pirin, Perun and Kristal varieties were new achievements in cotton breeding for earliness and productivity. In the State variety test, these varieties realized high yields, and exceeded the two standards and the average standard by 2.9% and 9.5%, respectively in fiber yield they exceeded it by 3.5% and 12%. Aida, Anabel and Melani varieties were new achievements in cotton breeding for fiber quality. These varieties had improved technological fiber properties and in some qualities they surpassed Avangard-264 variety - a standard for fiber quality, in others were equal to it. Egea and Nike varieties, confirmed in 2017, were another new achievement in the selection of colored (brown) cotton. The most valuable characteristic of these varieties was their longer fibre than that of Izabell variety approved as a standard for coloured cotton. In Upper Half Mean length Egea exceeded it by 1.13 mm, Nike - by 1.22 mm and had better spinning characteristics. Base on the obtained results of performed diallelic and line  $\times$  tester crosses and analyses, the genetic control of studied traits was established, the options for selection were clarified and the selection value of parental forms was determined. Genetic control of productivity and its elements was mainly non-additive. Additive and non-additive gene effects were important for the inheritance of fiber length and lint percentage; the non-additive gene effects were more important. In the interspecific crosses of *G. hirsutum* L.  $\times$  *G. barbadense* L. traits were also more strongly affected by non-additive gene effects, except boll weight and lint percentage. Parental forms with positive and high GCA were identified for the studied characters, suitable for heterosis and synthetic selection. Different approaches have been developed and applied to overcome the incrossability and incompatibility of *G. hirsutum* L. and the wild cotton species. Different backcrossing approaches have been used.

**Keywords:** Cotton, New varieties, Diallel crosses, Line  $\times$  tester crosses, Inheritance, Combining ability, Genetic control

### INTRODUCTION

The main goal of cotton breeding in our country is to create new germplasm and varieties with high and stable yield over the years and better fiber quality, adapted to specific growing conditions, resistant to abiotic and biotic stress. Priority is given to earliness due to the short growing season of cotton. The yield and fiber quality is improved by applying the classical methods of selection. By these methods a number of varieties have been created of which

especially valuable are Avangard-264, obtained by interspecific hybridization of *G. hirsutum* L. × *G. barbadense* L. (Koynov and Stoilova, 1996), Perla-267, Vega, Colorit, Darmi, Natalia, Rumi and IPK Nelina obtained by combining the interspecific hybridization of *G. hirsutum* L. × *G. barbadense* L. with intraspecific hybridization (Stoilova and Saldzhiev, 2000; 2005; 2008a; 2008b; 2010; Stoilova and Meluca, 2013), Helius, Trakia, obtained by experimental mutagenesis (Valkova, 2009), Boyana, Denitsa, Philipopolis, Viki, Kris, Plovdiv and Dorina, obtained by intraspecific hybridization (Valkova and Bozhinov, 2010; Valkova, 2014a; 2014b; Stoilova and Nistor, 2012; Meluca et al., 2012). Sirius and Tsvetelina varieties, obtained by experimental mutagenesis, were newer achievements in the selection of earliness and productivity (Valkova, 2017; Koleva and Valkova, 2019). The Avangard-264 variety (Koynov and Stoilova, 1996) has initiated a new generation of varieties with better fiber quality the result of the development of a new breeding direction. It has been approved as a fiber quality standard since 1996.

In 2009 the first brown cotton variety Izabell was approved. With it the beginning of a new generation of varieties, with natural colored fibers, of high ecological and economic effects was marked (Stoilova et al., 2010). In 2017 the new brown cotton varieties Egea and Nike were approved.

Pirin and Aida white cotton varieties were approved in 2019 and Perun variety was approved in 2020. In 2021 four new cotton varieties were recognized and these were Anabel, Tiara, Melani and Kristal.

The aim of this study was to evaluate the productivity and fiber technological properties of the newest cotton varieties, compared to the standard varieties; to clarify the inheritance of most important economic traits, related to the productivity and fiber quality, in connection with the choice of selection scheme and work with the hybrid progenies.

## MATERIAL AND METHOD

Diallel and line × tester crosses and their respective analyses are used for genetic analysis of quantitative traits. Classical selection schemes are applied to the initial selection units. The testing of promising breeding materials is organized in competitive variety trials using the standard and block methods in 4 repetitions and a harvest plot of 20 m<sup>2</sup>. Observations during the growing season and investigated indicators are reported according to accepted and approved methods for conducting of competition varietal trials for biological and economic qualities according to UPOV (2003) and IPGRI (1994). Experimental data are processed by applying of various statistical methods using computer programs. Multivariate methods such as hierarchical cluster analysis, PC analysis and phenotypic stability of traits and genotypes with the application of various stability measures are applied to evaluate the resulting genetic diversity. Principles and methods categorized very well by Syukov (2007) are applied to the selection of parental forms.

Genetic research are focused on: establishing the inheritance of individual traits and the manifestations of overdominance and epistasis; clarification the genetic structure of traits - additivity, dominance, epistasis, influence of the environment on their manifestation; establishing the number of effective factors (genes); determining the heritability coefficients in a broad and narrow sense for each trait; study of the general and specific combinative ability of genotypes and assessment of their perspective as parental components for hybridization; reaction of genotypes to years conditions.

Breeding research is aimed at: creation of new germplasm; research and conservation of genetic resources and their use in selection; seedproduction.

**Studies conducted:**

The hybrid populations of two diallel combinations with the participation of the parental components presented below were studied.

| 1 ST DIALLEL COMBINATION    | 2 ND DIALLEL COMBINATION     |
|-----------------------------|------------------------------|
| P <sub>1</sub> – Beli Iskar | P <sub>1</sub> – Chirpan-539 |
| P <sub>2</sub> - Barut 2002 | P <sub>2</sub> - Helius      |
| P <sub>3</sub> - Darmi      | P <sub>3</sub> - Rumi        |
| P <sub>4</sub> - Mytra      | P <sub>4</sub> Boyana        |
| P <sub>5</sub> - Helius     | P <sub>5</sub> - Natalia     |
| P <sub>6</sub> - Dorina     | P <sub>6</sub> - Nelina      |

The diallel schemes included one set of F<sub>1</sub>'s and parents. On the experimental data 22 diallel analyzes of the traits were carried out: productivity/plant; boll weight; lint percentage and fiber length. Genetic structure of traits, genetic components of variance and some genetic parameters and indexes of inheritance, as well as general combining ability (GCA) and specific combining ability (SCA) of parental forms were studied.

The hybrid populations of 55 line × tester crosses from four experimental settings (from four line × tester crosses) with different maternal and paternal components were studied. Selection value of parental forms, inheritance of some traits of great interest for cotton breeding and manifestations of heterosis were studied. The influence of the year conditions on the effects of the general and specific combinative ability, and on inheritance, was studied.

The inheritance and breeding value of parental forms was studied in the interspecific *G. hirsutum* L. × *G. barbadense* L. cross. The varieties Chirpan-539, Helius, Natalia, Darmi and Boyana (Bulgarian selection) were included from the species *G. hirsutum* L., from the species *G. barbadense* L. three Spanish varieties FR-B-201, FR-B-202 and FR-B-203 were involved. The parental forms of both species were used as maternal and as paternal components. Some of the results were included in a dissertation (Dimitrova, 2019).

**RESULTS AND DISCUSSION**

Considerable selection work has been carried out both to increase the yield and to improve the fiber quality. New cotton varieties very early, high-yielding and of improved fiber quality have been created.

**Pirin and Perun cotton varieties.** In the IASAS system, Pirin variety was tested in 2016-2017, Perun variety was tested in 2017-2018. Both varieties were certified and included in the official variety list of the Republic of Bulgaria, Pirin variety in 2018, Perun variety in 2019. These varieties were created by intervarietal crossing. Both varieties were early in maturity and were distinguished by high productivity and other valuable qualities.

In the state variety test, these two varieties realized high yields, Pirin variety - 2029 kg/ha, Perun variety - 2255 kg/ha, on average for the test period, exceeding the two standards and the average standard by 2.9% and 9.5%, respectively, in fiber yield they exceeded it by 3.5% and 12%. Perun variety showed a specific adaptation to more favorable environments. The high productivity of these varieties was combined with good technological fiber qualities, which fiber was distinguished by high whiteness C Grade 11-1.

Pirin variety in fiber technological properties was inferior in some qualities (spinning, fineness, strength, average length, uniformity in length) to Avangard-264 variety (a standard for fiber quality). Nevertheless, Pirin variety was characterized by good quality of fiber, which was a medium fine and strong, with a medium long staple, good uniformity in length and high

maturity and very good whiteness. Perun variety had fiber with better elongation, better RD difference reflection spectroscopy and better whiteness in comparison to the two standards. Its fiber was characterized by whiteness Color grade (C Grad) Upland was 11-1 in Radnevo and Burgas test stations in 2017 and 11-2 in Radnevo in 2018 surpassing both standards. It had a slightly finer fiber than Chirpan-539. Averaged over the two years, the micronaire value was 4.79 mic, with 4.87 mic for Chirpan-539 and 4.62 mic for Avangard-264. Perun variety in fiber length - 25.48 mm, average for the two years, and in degree of spinning (SCI) Index was inferior to Avangard-264 and was equal to Chirpan-539. The content of short fibers was higher compared to Avangard-264. In terms of the other technological fiber qualities - maturity, yellowness, strength and uniformity in fiber length, Perun variety was leveled with both standards.

**Aida cotton variety.** In the IASAS system Aida variety was tested in 2017-2018. It was approved as a new cotton variety in 2019 and was certified in 2020. Aida variety was created by interspecific hybridization, by crossing the allotetraploid *G. thurberi* Tod. × *G. raimondii* Ulbr. with Darmi variety - *G. hirsutum* L. and backcrossing of the triple hybrid (*G. thurberi* Tod. × *G. raimondii* Ulbr.) × Darmi with Darmi variety. Darmi variety was obtained by combining the interspecific *G. hirsutum* L. × *G. barbadense* L. hybridization with intraspecific *G. hirsutum* L.

Based on the IASAS data, in seed cotton yield of 2380 kg/ha, on average for the two years of testing, Aida variety exceeded the two standard varieties Chirpan-539 and Avangard-264 by 11.3% and 14.7%, respectively, the average standard - by 13.0% (Table 1). It also realized higher September yield and fiber (lint) yield. Aida variety in some fiber properties was superior to both standards or was leveled with Avangard-264 variety – a standard for fiber quality. Compared to the standard varieties, Aida variety had a better fiber consistency spinning (SCI) Index, greater Upper Half Mean Length (UHML), better uniformity in fiber length and a better spectroscopy with a reflection of the RD difference. The content of short fibers was very low.

Table 1. Test results of Aida variety in the IASAS system (State variety testing) in 2017-2018 (Averaged data for two years)

| Variety          | September harvest | In % to average standard | Seed cotton yield | In % to average standard | Lint yield | In % to average standard |
|------------------|-------------------|--------------------------|-------------------|--------------------------|------------|--------------------------|
|                  | kg/ha             | %                        | kg/ha             | %                        | kg/ha      | %                        |
| Average standard | 1711              | 100.0                    | 2060              | 100.0                    | 759        | 100.0                    |
| Chirpan-539      | 1752              | 102.4                    | 2091              | 101.5                    | 789        | 103.9                    |
| Avangard-264     | 1670              | 97.6                     | 2029              | 98.5                     | 731        | 96.3                     |
| Aida             | 1984              | 116.0                    | 2328              | 113.0                    | 859        | 113.2                    |

**Anabel cotton variety.** In the IASAS system Anabel variety was tested in 2017-2018. It was approved for the new cotton variety in 2021. Anabel variety was obtained by intraspecific hybridization. The averaged test results showed that in seed cotton yield of 2026 kg/ha Anabel variety was equal to Avangard-264 and it was inferior by 3.1% to Chirpan-539 (a standard for productivity). In fiber (lint) yield, it was equal to Chirpan-539 and was superior to Avangard-264, as a result of higher lint percentage. In lint percentage it exceeded both standards.

This variety possessed a number of valuable fiber technological properties and in some respects exceeded both standard cultivars or was equal to Avangard-264 – a standard for fiber quality (Table 2). Compared to the standard cultivars, Anabel variety possessed a better fiber

consistency spinning (SCI), Index longer fiber, lower microner, which means finer fiber, greater strength, and better spectroscopy with reflection of the RD difference.

Table 2. Technological fiber properties of Anabel variety according to the IASAS data, 2017-2018 (Averages for two years)

| Year  | Average standard | Chirpan-539 | Avangard-264 | Anabel |
|---|------------------|-------------|--------------|--------|
| Fiber consistency spinning (SCI), Index               | 116              | 114         | 118          | 124    |
| Micronaire (Mic)                                      | 4.75             | 4.87        | 4.62         | 4.44   |
| Maturity(Mat) Index                                   | 0.87             | 0.87        | 0.87         | 0.86   |
| Fiber mean length (UHML - Upper Half Mean Length), mm | 25.85            | 25.52       | 26.18        | 26.64  |
| Uniformity (UL) %                                     | 82.0             | 81.9        | 81.9         | 82.0   |
| Short fibers (SFL), 12.7 mm                           | 8.8              | 9.0         | 8.5          | 8.8    |
| Strength (Str), g/tex                                 | 27.9             | 27.8        | 27.9         | 29.0   |
| Elongation (Elg), %                                   | 7.1              | 7.2         | 7.1          | 7.3    |
| Spectroscopy with reflectance of the difference RD    | 81.3             | 81.2        | 81.5         | 82.2   |
| Yellowness (+b)                                       | 8.5              | 8.6         | 8.5          | 8.5    |
| Color Grade (C Grad) Upland                           |                  |             |              |        |
| Radnevo - 2017  | -                | 21-1        | 11-1         | 11-1   |
| Burgas - 2017   | -                | 11-2        | 11-2         | 11-1   |
| Radnevo - 2018  | -                | 21-1        | 21-1         | 21-1   |

**Tiara and Melani cotton varieties.** These two varieties were included for testing in the IASAS system in 2019 and 2020. Both varieties were approved in 2021. Tiara variety was created by interspecific hybridization and backcross technology, through the crossing of introgressive line 413 ( $2n=52$ ) and the wild diploid species *G. davidsonii* Kel. ( $2n=26$ ) and triple backcrossing of the amphidiploid (allohexaploid) ( $413 \times G. davidsonii Kel.) ( $2n=78$ ), as the first backcrossing was to  $C_1$  and the next two were to  $F_3BC_1$ . Line 413 was obtained by combining the interspecific *G. hirsutum* L.  $\times$  *G. barbadense* L hybridization with intraspecific *G. hirsutum* L. Melani variety was created by crossing the allotetraploid *G. thurberi* Tod.  $\times$  *G. raimondii* Ulbr. with Darmi variety (*G. hirsutum* L.) and backcrossing of the triple hybrid (*G. thurberi* Tod.  $\times$  *G. raimondii* Ulbr.  $\times$  Darmi) with Darmi variety.$

September harvest, seed cotton yield harvested until September 30, was used as main criterion to determine the earliness of varieties. According to the IASAS data, these two varieties in September yield, on average for the two years, were equal to the standard cultivar Chirpan-539, but were superior to the Avangard-264 and the average standard, Tiara variety exceeded the average standard by 3.9%. Melani variety exceeded it by 3.1%.

The results for the total seed cotton yield corresponded to the September harvest, in 2019 the new varieties exceeded the two standard cultivars and the average standard, in 2020 they were inferior to Chirpan-539 and exceeded Avangard-264 and the average standard. On average for the two years, in seed cotton yield of 2809 kg/ha for Tiara and 2784 kg/ha for Melani, these varieties were leveled with the cultivar Chirpan-539 and exceeded the cultivar Avangard-264 and the average standard, Tiara variety exceeded the average standard by 4.1%, Melani variety exceeded it by 3.1%. In terms of fiber yield (1141 kg/ha and 1066 kg/ha) Tiara variety exceeded the two standard cultivars and the average standard by 7.4%, Melani variety

was inferior to the cultivar Chirpan-539, surpassed Avangard-264 and was leveled with the average standard.

Tiara variety showed better agronomic performance than Melani variety, achieving higher fiber yield as a result of higher lint percentage. Melani variety had better fiber technological qualities than Tiara variety. Compared to the two standard cultivars, Melani variety was characterized by a better fiber consistency spinning (SCI) Index, lower micronaire, greater strength and better spectroscopy with reflection of the RD difference and it had longer fiber than Chirpan-539. This variety showed a better combination of agronomic traits and fiber technological qualities than the cultivar Avangard-264 – a standard for the fiber quality.

Melani variety has shown stable values for most fiber quality traits as length, uniformity in fiber length, fiber elongation, short fiber content, spectroscopy with reflectance of the RD difference, color grade, for the two years of testing. In this variety a more optimal combination of productivity and fiber quality has been achieved than the cultivar Avangard-264 – a standard for fiber quality.

Compared to the cultivar Avangard-264 (a standard for fiber quality) the new variety Melani had better productivity performance (higher September harvest, seed cotton yield and fiber yield), the same Upper Half Mean Length, greater fiber strength, better fiber consistency spinning (SCI) Index, lower micronaire, better uniformity in length, better spectroscopy, lower short fibers and lower trash content.

The new **brown cotton varieties Egea and Nike** were achievements in a new direction in the Bulgarian cotton breeding. They belonged to a new generation of naturally coloured cotton varieties of high ecological and economic effects. The two colored cotton varieties are natural hybrids, Egea variety was selected from the cross of Avangard-264 × Eva (Greek variety) and Nike was selected from the cross of Chirpan-539 × L. 40. Selected hybrid plants were naturally pollinated by brown cotton hybrids obtained by controlled crossing of white and brown cotton.

The most valuable characteristic of these varieties was their longer fibre than that of Izabell variety approved as a standard for the colored (brown) cotton (Stoilova and Dimitrova, 2017). According to data of the Executive Agency for Variety Testing, Approbation and Seed Control, Egea variety in fiber length (modal 23.32 mm and staple 26.83 mm) surpassed the standard variety Izabell by 1.35 mm and 1.57 mm, in Upper Half Mean Length (UHML 23.51 mm for Egea, 23.60 mm for Nike and 22.38 for Izabell) they exceeded it by 1.13 mm and 1.22 mm, and had better spinning characteristics. With these two varieties significant progress in improving the fiber quality of coloured cotton by selection has been made.

Egea and Nike varieties in productivity were close to the white cotton standards, the seed cotton yield on average for three years from three experimental stations was 1921 kg/ha for Egea variety and 1917 kg/ha for Nike variety compared to 1946 kg/ha for Chirpan-539 variety and 1933 kg/ha for Avangard-264 variety (white cotton standards, respectively for productivity and fiber quality). They were inferior to the white cotton standards in lint percentage and lint yield by 8.9% and 9.6% to Chirpan-539 and by 2.2% and 2.9% to Avangard-264.

### **Inheritance, heterosis and combining ability in diallel cotton crosses**

Based on the performed diallel analyzes of productivity it was found that there was significant dislocation of parental points in diallel graphs by years as a result of their interaction with the environment. Deviation of the regression line below the 45° slope in some diallel graphs revealed the presence of complementary epistasis (Mather, 1967; Jinks et al., 1969).

Epistatic genes action for all investigated traits, except for the number of sympodia, was found by Singh et al. (2009).

Coefficients of heritability in narrow sense  $h^2$  were from very low to moderately high, due to the great influence of dominant gene action in the inheritance of this trait. This means that effective selection of productivity should be conducted only in the later hybrid generations - F3-F4, when the dominant gene effects have decreased.

Varieties Darmi, Helius, Beli Iskar, Dorina, Rumi and Chirpan-539, showed high productivity, had low  $W_r+V_r$  values. This means that their high productivity was determined by dominant genes with a one-way productivity increasing action. Values for  $k$  (number of effective factors) indicated at least 2 to 3 genes or groups of genes. Soomro et al. (2008) also concluded that at least 3 groups of genes control raw cotton yield.

Varieties Beli Iskar (1st diallel combination) and Natalia (2nd diallel combination) showed high productivity/plant had high significant positive GCA effects. The presence of a positive GCA in some of the parental forms depended on the years conditions.

Analysis of variance components of GCA and SCA (Table 3) showed that non-additive gene effects were more important for the inheritance of productivity/plant, which confirmed the results of diallel analysis, that statistically significant were both additive and dominant effects. Dominant gene action ( $H_1$ ,  $H_2$ ) exceeded the additive ( $D$ ) one and reflected the greater significance of non-additive variance in the inheritance of productivity. Research results confirmed other authors' research that seed cotton yield was more strongly influenced by non-additive gene effects (Khan et al., 2011; Makhdoom, 2011; Khan, 2013).

Table 3. Variance components for the productivity/plant in the 1st and 2nd diallel combinations

| Sources of variation | 1 <sup>st</sup> diallel combination |                |                      |                | 2 <sup>nd</sup> diallel combinaton |                |
|----------------------|-------------------------------------|----------------|----------------------|----------------|------------------------------------|----------------|
|                      | 1 <sup>st</sup> year                |                | 2 <sup>nd</sup> year |                | 1 <sup>st</sup> year               |                |
|                      | Variance                            | Standard error | Variance             | Standard error | Variance                           | Standard error |
| Crosses              | 52.921 <sup>++</sup>                | 16.905         | 8.571 <sup>+</sup>   | 2.873          | 1.412 <sup>+</sup>                 | 0.574          |
| GCA                  | 4.660 <sup>ns</sup>                 | 6.744          | 0.378 <sup>ns</sup>  | 0.974          | 0.098 <sup>ns</sup>                | 0.202          |
| SCA                  | 43.601 <sup>++</sup>                | 16.117         | 7.815 <sup>++</sup>  | 3.041          | 1.215 <sup>+</sup>                 | 0.589          |
| Errors               | 1.607 <sup>+++</sup>                | 0.359          | 1.523 <sup>+++</sup> | 0.341          | 1.146 <sup>+++</sup>               | 0.256          |

The diallel analysis of boll weight showed significant values of additive ( $D$ ) gene effects only for the 1st diallelic cross. Dominant ( $H_1$ ,  $H_2$ ) gene effects were significant for both the 1st and the 2nd diallel crosses. Genetic control of this trait in the 2nd diallel cross was non-additive. Additive genetic variance and non-additive genetic variance controlled boll weight in the 1st diallel cross. Dominant gene action surpassed additive one, which means that dominant variance was of greater importance in the inheritance of this trait. A non-additive type of gene action for this trait was noted by Ali et al. (2011), Khan and Hassan (2011).

The diallel analysis for lint percentage showed significant values for additive ( $D$ ) and dominant gene effects ( $H_1$  and  $H_2$ ). The genetic parameter  $D$  varied in ratios with the dominant parameters  $H_1$  and  $H_2$  showing a very pronounced non-additive effect of genes, except for one of the diallel schemes with removed  $P_6$  - Dorina variety (1st diallel combination). Coefficients of heritability in wroad sense ( $H_2$ ) were high and in narrow sense ( $h_2$ ) were very low to moderately high. Varieties Dorina and Barut from the 1st diallel combination (Table 4), Chirpan-539 and Helius from the 2nd diallel combination (Table 5) had the highest values and the highest GCA for lint percentage. These varieties were the best general combinator for this trait.



Table 4. Ranking of parents based on GCA for lint percentage in the 1st diallel combination

| 1 <sup>st</sup> year       |      |        | 2 <sup>nd</sup> year       |      |        |
|----------------------------|------|--------|----------------------------|------|--------|
| Parent                     | x    | GCA    | Parent                     | x    | GCA    |
| P <sub>6</sub> -Dorina     | 40.6 | 1.162  | P <sub>2</sub> -Barut      | 41.1 | 1.383  |
| P <sub>2</sub> -Barut      | 39.9 | 1.042  | P <sub>6</sub> -Dorina     | 41.7 | 0.020  |
| P <sub>4</sub> -Mytra      | 39.0 | -0.137 | P <sub>4</sub> -Mytra      | 39.8 | -0.083 |
| P <sub>1</sub> -Beli Iskar | 39.0 | -0.225 | P <sub>5</sub> -Helius     | 38.6 | -0.183 |
| P <sub>3</sub> -Darmi      | 37.7 | -0.908 | P <sub>1</sub> -Beli Iskar | 39.5 | -0.437 |
| P <sub>5</sub> -Helius     | 37.5 | -0.933 | P <sub>3</sub> -Darmi      | 37.9 | -0.700 |
| GD 5 %                     | 0.98 |        | GD. 5 %                    | 1.04 |        |
| Standard error             |      | 0.171  | Standard error             |      | 0.181  |

Table 5. Ranking of parents based on GCA for lint percentage in the 2nd diallel combination

| 1 <sup>st</sup> year        |      |        | 2 <sup>nd</sup> year        |      |        |
|-----------------------------|------|--------|-----------------------------|------|--------|
| Parent                      | x    | GCA    | Parent                      | x    | GCA    |
| P <sub>1</sub> -Chirpan-539 | 39.9 | 0.613  | P <sub>1</sub> -Chirpan-539 | 40.2 | 1.043  |
| P <sub>2</sub> -Helius      | 38.3 | 0.433  | P <sub>2</sub> -Helius      | 39.9 | 0.567  |
| P <sub>4</sub> -Boyana      | 38.5 | -0.054 | P <sub>3</sub> -Rumi        | 38.5 | -0.208 |
| P <sub>6</sub> -Nelina      | 38.7 | -0.242 | P <sub>4</sub> -Boyana      | 37.5 | -0.308 |
| P <sub>3</sub> -Rumi        | 37.2 | -0.321 | P <sub>5</sub> -Natalia     | 37.0 | -1.094 |
| P <sub>5</sub> -Natalia     | 37.1 | -0.429 | P <sub>6</sub> -Nelina      | -    | -      |
| L.S.D. 5 %                  | 1.03 |        | L.S.D. 5 %                  | 2.06 |        |
| Standard error              |      | 0.181  | Standard error              |      | 0.188  |

Additive and dominant gene effects were also statistically significant for fiber length. Depending on the diallel combination, dominant gene action slightly surpassed additive one (D) or both components of variance had an equal contribution - when P<sub>5</sub> (Helius variety) in the 1st diallel combination was removed, or dominant gene action was much more pronounced than additive one and showed the greater importance of dominant variance.

Bölek et al. (2014), Khan et al. (2017) also reported that non-additive gene effects had a greater importance for the inheritance of fiber length.

Coefficients of heritability in a broad ( $H^2$ ) sense were in most cases high, in a narrow ( $h^2$ ) sense they were low to medium high and effective selection should be conducted in later hybrid generations – F<sub>3</sub> -F<sub>4</sub>.

There were significant differences between the parents in GCA and tested crosses in SCA. Varieties Darmi, Mytra, Dorina from the 1st diallel combination and Natalia from the 2nd diallel combination showed positive and high GCA in different environments (during the two studied years) (Table 6 and Table 7). It is known that GCA is determined by the action of additive genes and it can be considered that these varieties possessed the highest number of additive genes.

Table 6. Ranking of parents based on GCA for fiber length in 1st diallel combination

| 1 <sup>st</sup> year       |      |        | 2 <sup>nd</sup> year       |      |        |
|----------------------------|------|--------|----------------------------|------|--------|
| Parent                     | x    | GCA    | Parent                     | x    | GCA    |
| P <sub>6</sub> -Dorina     | 29.6 | 0.828  | P <sub>3</sub> -Darmi      | 27.6 | 0.436  |
| P <sub>4</sub> -Mytra      | 28.2 | 0.453  | P <sub>6</sub> -Dorina     | 28.2 | 0.303  |
| P <sub>3</sub> -Darmi      | 28.2 | 0.124  | P <sub>4</sub> -Mytra      | 27.9 | 0.303  |
| P <sub>2</sub> -Barut      | 27.9 | -0.360 | P <sub>2</sub> -Barut      | 26.9 | -0.297 |
| P <sub>1</sub> -Beli Iskar | 26.0 | -0.489 | P <sub>5</sub> -Helius     | 26.5 | -0.335 |
| P <sub>5</sub> -Helius     | 27.3 | -0.555 | P <sub>1</sub> -Beli Iskar | 26.6 | -0.410 |
| L.S.D. 5 %                 | 1.01 |        | L.S.D. 5 %                 | 1.34 |        |
| Standard error             |      | 0.179  | Standard error             |      | 0.234  |

Table 7. Ranking of parents based on GCA for fiber length in 2<sup>nd</sup> diallel combination

| 1 <sup>st</sup> year        |      |        | 2 <sup>nd</sup> year        |      |        |
|-----------------------------|------|--------|-----------------------------|------|--------|
| Parent                      | x    | GCA    | Parent                      | x    | GCA    |
| P <sub>5</sub> -Natalia     | 26.4 | 0.658  | P <sub>5</sub> -Natalia     | 27.4 | 0.408  |
| P <sub>3</sub> -Rumi        | 25.9 | 0.211  | P <sub>4</sub> -Boyana      | 27.0 | 0.032  |
| P <sub>4</sub> -Boyana      | 25.4 | 0.194  | P <sub>3</sub> -Rumi        | 27.0 | -0.096 |
| P <sub>6</sub> -Nelina      | 26.8 | 0.119  | P <sub>1</sub> -Chirpan-539 | 26.9 | -0.139 |
| P <sub>1</sub> -Chirpan-539 | 24.1 | -0.514 | P <sub>2</sub> -Helius      | 26.9 | -0.205 |
| P <sub>2</sub> -Helius      | 24.0 | -0.639 |                             |      |        |
| L.S.D. 5 %                  | 1.09 |        | L.S.D. 5 %                  | 0.7  |        |
| Standard error              |      | 0.191  | Standard error              |      | 0.138  |

### **Inheritance, heterosis and combinatorial ability in line × tester crosses**

Line × tester analysis provides information about combinability of parental forms and their F<sub>1</sub> hybrids, useful for breeding programs (Shakeel et al., 2012, Ali et al., 2018).

#### ***Line × tester crosses of 5 Bulgarian varieties used as mothers and 3 foreign ones (Nazili-954, Mytra, Korina) used as fathers***

Line × tester analysis of five Bulgarian varieties – Chirpan-539, Helius, Rumi, Boyana and IPC Nelina, used as mothers, and 3 foreign ones – Nazili-954 (Turkish), Mitra and Korina (Greek), as fathers was applied. Inheritance of most important economic characters and selection value of parental forms and hybrids were studied.

Different types of inheritance were found for the productivity, boll weight, fiber length and lint percentage. Heterosis manifestations were most pronounced in terms of productivity, where the heterosis effect reached 39.1% and transgressive segregation in F<sub>2</sub> can be expected. For all studied traits the genetic variance was mainly of non-additive type (Table 8). Of the varieties Bulgarian selection, used as mothers, Boyana and IPC Nelina emerged as good general combiners for productivity (Table 9) and lint percentage (Table 10), having high values for both characteristics and a high GCA. Their high GCA was combined with low SCA variances, which makes them very suitable for the synthetic selection.

Among the foreign varieties, used as fathers, the Turkish Nazili-954 emerged good combinator for lint percentage, the Greek Korina - for fiber length. These two varieties had high

values and high GCA for the relevant traits. Because of high SCA variances they appeared to be more suitable for the heterosis selection.

Table 8. Variance components for productivity, lint percentage and fiber length in  $F_1$  – five mothers  $\times$  3 fathers

| Sources of variation | Productivity/plant | Lint percentage | Fiber length |
|----------------------|--------------------|-----------------|--------------|
| SCA%                 | 33.8               | 44.7            | 71           |
| $\sigma^2_{GCA}$     | 0.712              | 0.201           | -0.223       |
| $\sigma^2_{SCA}$     | 9.132              | 0.594           | 0.141        |

Table 9. Evaluation of GCA and SCA effects for productivity/plant in  $F_1$  - 5 mothers  $\times$  3 fathers

| Mothers                   | GCA    | Fathers | GCA    | Fathers / SCA effects |        |        |                 |
|---------------------------|--------|---------|--------|-----------------------|--------|--------|-----------------|
|                           |        |         |        | Nazili 954            | Mytra  | Korina | $\sigma^2_{Si}$ |
| Chirpan-539               | -4.844 | Nazili  | -4.895 | 7.451                 | -4.049 | -3.402 | 40.58           |
| Helius                    | 0.500  | Mytra   | 0.671  | 3.473                 | -2.493 | -0.971 | 8.35            |
| Rumi                      | -4.178 | Korina  | 4.224  | -4.549                | -1.555 | 4.564  | 19.78           |
| Boyana                    | 2.822  |         |        | -0.449                | 4.351  | -3.02  | 16.95           |
| Nelina                    | 5.700  |         |        | -5.927                | 2.207  | 3.20   | 26.31           |
| Standard error            | 1.052  |         | 0.815  |                       |        |        |                 |
| $\sigma^2_{sj}$ – fathers |        |         |        | 30.15                 | 10.17  | 14.23  |                 |

Table 10. Evaluation of GCA effects for lint percentage in  $F_1$  - 5 mothers  $\times$  3 fathers

| 1 <sup>st</sup> year |        |            |        | 2 <sup>nd</sup> year |        |            |        |
|----------------------|--------|------------|--------|----------------------|--------|------------|--------|
| Mothers              | GCA    | Fathers    | GCA    | Mothers              | GCA    | Fathers    | GCA    |
| Chirpan-539          | -0.091 | Nazili 954 | 0.842  | Chirpan-539          | 0.100  | Nazili 954 | 0.807  |
| Helius               | -0.258 | Mytra      | -0.318 | Helius               | -1.589 | Mytra      | -0.031 |
| Rumi                 | -0.924 | Korina     | -0.524 | Rumi                 | -0.111 | Korina     | -0.077 |
| Boyana               | 0.631  |            |        | Boyana               | 0.455  |            |        |
| Nelina               | 0.642  |            |        | Nelina               | 1.145  |            |        |
| Standard error       | 0.281  |            | 0.217  | Standard error       | 0.222  |            | 0.172  |

***Line  $\times$  tester crosses of 3 Bulgarian varieties used as mothers and 2 Spanish (FR-H-1001 and FR-H-1002) used as fathers***

A line  $\times$  tester analysis of three Bulgarian varieties Cirpan-539, Helius and Darmi used as mothers and two 2 Spanish FR-H-1001 and FR-H-1002 used as fathers was conducted. The GCA effects of mothers and fathers were significant for productivity/plant and lint percentage. As for fiber length the GCA effects of mothers were significant, while the GCA effects of fathers were non-significant. SCA effects of crosses were significant for lint percentage and fiber length and non significant for productivity per plant.

The calculated components of genetic variance for lint percentage were:  $\sigma^2_{GCA}=0.256$  and  $\sigma^2_{SCA}=0.169$ ; for fiber length were:  $\sigma^2_{GCA}=0.007$  and  $\sigma^2_{SCA}=0.166$ . The comparison of  $\sigma^2_{GCA}/\sigma^2_{SCA}$  showed that the additive gene effects were more important for the inheritance of

lint percentage, while the non-additive gene effects were more important for the inheritance of fiber length.

Darmi variety, from mothers, had a positive GCA for productivity/plant and fiber length (Table 11) and low variances of SCA for both traits, which makes it very suitable for synthetic selection.

The Spanish FR-H-1001 variety showed a positive GCA for lint percentage and fiber length making it very valuable for breeding programs with a view to a more optimal combination of the two traits, which are in negative correlation, FR-H-1002 variety had positive GCA for productivity/plant.

Table 11. Evaluation of GCA effects for productivity/plant, lint percentage and fiber length in setpro crosses of 3 Bulgarian and 2 Spanish cultivars

| Productivity/plant |        |                |        |
|--------------------|--------|----------------|--------|
| Mothers            | GCA    | Fathers        | GCA    |
| Chirpan-539        | -3.544 | FR-H-1001      | -2.650 |
| Helius             | -0.611 | FR-H-1002      | 2.650  |
| Darmi              | 4.156  | -              | -      |
| Standard error     | 1.411  | Standard error | 1.152  |
| Lint percentage    |        |                |        |
| Chirpan-539        | 1.156  | FR-H-1001      | 1.328  |
| Helius             | 0.339  | FR-H-1002      | -1.328 |
| Darmi              | -1.494 |                |        |
| Standard error     | 0.326  | Standard error | 0.266  |
| Fiber length       |        |                |        |
| Chirpan-539        | -0.067 | FR-H-1001      | 0.006  |
| Helius             | -0.667 | FR-H-1002      | -0.006 |
| Darmi              | 0.733  |                |        |
| Standard error     | 0.150  | Standard error | 0.123  |

Summarized results from the analysis of line  $\times$  tester crosses showed that additive and non-additive genes controlled the variation of productivity/plant, boll weight and lint percentage and fiber length. Ashokkumar et al. (2010), Azam et al. (2013), Swami et al. (2013), Memon et al. (2017) in line  $\times$  tester analyzes reported that both additive and non-additive variances were important and involved in the control of seed cotton yield and its components.

The results of conducted studies showed that non-additive gene effects were of greater importance for the inheritance of productivity/plant, except for the line  $\times$  tester crosses involving the Spanish varieties FR-H-1001 and FR-H-1001 as fathers, and for fiber length. The presence of non-additive effects in the genetic control of many traits related to yield and fiber properties was reported by Karademir et al. (2016), Sajjad et al. (2016) and Sivia et al. (2017).

In the inheritance of lint percentage, in some cases, non-additive gene effects were more important and in others, additive ones were more important, depending on the selection of parental forms included in crosses. Manifestations of heterosis were found for all traits most strongly expressed for productivity/plant. Parents had both high mean performance and high GCA effects, suitable for synthetic selection and F<sub>1</sub> hybrid combinations suitable for heterosis selection were identified.

### **Inheritance, heterosis and combining ability in interspecific *G. hirsutum* L. × *G. barbadense* L. crosses.**

In the F<sub>1</sub> hybrids of *G. hirsutum* L. × *G. barbadense* L. the inheritance of higher productivity of *G. hirsutum* L. species was incompletely dominant, completely dominant and overdominant. Overdominance prevailed, which caused heterosis up to 36.1% in hybrids with mother *G. hirsutum* L. and up to 32.7% in hybrids with mother *G. barbadense* L. Combining ability of cultivars of both species was of importance for the inheritance and the degree of heterosis. The direction of crossing did not show a regular pattern of inheritance.

The higher productivity observed in some crosses with the mother *G. hirsutum* L. can be explained by the better adaptation of varieties of this species (used as mothers) to the local climatic conditions, which are unsuitable for growing the species *G. barbadense* L. in our country.

The longer fiber of the species *G. barbadense* L. was inherited incompletely dominantly, in some cases additively or with positive overdominance with weak heterosis. F<sub>1</sub> hybrids had fiber length of 30.1-34.5 mm, by 2.5 mm to 8.2 mm longer than that of the parental forms of the *G. hirsutum* L. species. Heterosis for fiber length was reported by Bõlek et al. (2014). Hybrids with mother *G. barbadense* L. species had a longer fiber by 0.6-1.3 mm. As for the effect of crossing direction on the inheritance of fiber length, it was of importance the genome of *G. barbadense* L. species in its own (as mother) or in foreign cytoplasm (as father) was located.

From the analysis of combining ability variance in the *G. hirsutum* L. × *G. barbadense* L. hybrids, reliable differences were found in the GCA of maternal forms and SCA for productivity/plant and lint percentage, unreliable for both traits were the GCA of paternal forms. Significant differences in the GCA of paternal forms and SCA of crosses were established for fiber length, the GCA of maternal forms was insignificant. The presence of significant variances of GCA and SCA for productivity, lint percentage and fiber length indicated that additive and non-additive gene effects were important for their inheritance.

Calculated components of genetic variance for productivity were:  $\sigma^2_{GCA}=0.185$ ;  $\sigma^2_{SCA}=1.807$ . The contribution of  $\sigma^2_{GCA}$  and  $\sigma^2_{SCA}$  to genetic variance revealed that non-additive genetic effects were of greater importance for the inheritance of productivity, which was consistent with that reported by Basal et al. (2009) and Munir et al. (2017).

Calculated genetic variance components for lint percentage were:  $\sigma^2_{GCA}=-0.013$ ,  $\sigma^2_{SCA}=0.850$ ; for fiber length were:  $\sigma^2_{OKC}=0.013$ ,  $\sigma^2_{CKC}=0.346$ . The ratio  $\sigma^2_{OKC}/\sigma^2_{CKC}$  indicated that non-additive gene effects were of greater importance for the inheritance of these two traits.

Hosseini (2014) in a complete diallel cross including 9 cotton genotypes (*G. hirsutum* L. × *G. barbadense* L.) reported that in the F<sub>1</sub> generation, the most of traits were influenced by additive gene action. Non-additive gene action in the inheritance of lint percentage and fiber length was reported by Patel et al. (2014), Bõlek et al. (2014), Munir et al. (2017).

Of the *G. hirsutum* L. species Chirpan-539 variety had a high and significant GCA for productivity, Helius variety had a positive but non-significant GCA, and from the species *G. barbadense* L. FR-B-201 variety exhibited a positive GCA (Table 12).

Parental forms (Chirpan-539 and FR-B-201) showed high GCA for productivity had high variances ( $s^2_i$ ,  $s^2_j$ ) of SCA and are recommended for heterosis selection (Table 13). Among the maternal forms, Chirpan-539 variety had significant high positive GCA for lint percentage and Darmi variety had high GCA for fiber length, among the paternal forms FR-B-201 variety had significant high positive GCA for lint percentage, FR-B-203 for fiber length.

Table 12. Evaluation of GCA effects for productivity, lint percentage and fiber length in F<sub>1</sub> interspecific hybrids of *G. hirsutum* L. × *G. barbadense* L.

| Mothers                          | GCA    | Fathers  | GCA    |
|----------------------------------|--------|----------|--------|
| <b><i>Productivity/plant</i></b> |        |          |        |
| Chirpan-539                      | 4.758  | FR-B-201 | 0.949  |
| Helius                           | 0.458  | FR-B-202 | -0.611 |
| Natalia                          | -0.398 | FR-B-203 | -0.338 |
| Darmi                            | -3.464 |          |        |
| Boyana                           | -1.353 |          |        |
| Standard error                   | 1.145  |          | 0.887  |
| <b><i>Lint percentage</i></b>    |        |          |        |
| Chirpan-539                      | 0.980  | FR-B-201 | 0.527  |
| Helius                           | 0.202  | FR-B-202 | -0.300 |
| Natalia                          | -0.309 | FR-B-203 | -0.227 |
| Darmi                            | -1.409 |          |        |
| Boyana                           | 0.536  |          |        |
| Standard error                   | 0.467  |          | 0.362  |
| <b><i>Fiber length</i></b>       |        |          |        |
| Chirpan-539                      | -0.333 | FR-B-201 | -0.847 |
| Helius                           | -0.089 | FR-B-202 | -0.327 |
| Natalia                          | -0.144 | FR-B-203 | 1.173  |
| Darmi                            | 0.600  |          |        |
| Boyana                           | -0.033 |          |        |
| Standard error                   | 0.337  |          | 0.261  |

Table 13. Evaluation of SCA ( $S_{ij}$ ) and the variances ( $\sigma^2_{si}$ ;  $\sigma^2_{sj}$ ) for productivity, lint percentage and fiber length

| Mothers/Fathers                  | FR-B-201   | FR-B-202 | FR-B-203 | $\sigma^2_{si}$ |
|----------------------------------|------------|----------|----------|-----------------|
| <b><i>Productivity/plant</i></b> |            |          |          |                 |
| Chirpan-539                      | 3.029      | -0.611   | -2.418   | 6.648           |
|                                  | MD = 1.355 |          |          |                 |
| $\sigma^2_{sj}$                  | 7.601      | 0.8015   | 3.215    |                 |
| <b><i>Lint percentage</i></b>    |            |          |          |                 |
| Chirpan-539                      | -0.827     | 0.967    | -0.140   | 0.644           |
| Boyana                           | -1.515     | -0.622   | 2.138    | 3.453           |
|                                  | MD = 0.552 |          |          |                 |
| $\sigma^2_{sj}$                  | 2.646      | 0.945    | 1.643    |                 |
| <b><i>Fiber length</i></b>       |            |          |          |                 |
| Darmi                            | 0.247      | 0.360    | -0.607   | 0.188           |
|                                  | MD = 0.399 |          |          |                 |
| $\sigma^2_{sj}$                  | 0.752      | 0.584    | 0.808    |                 |

Estimates of the SCA variances of parental forms had high GCA effects for lint percentage and fiber length showed that those of *G. hirsutum* L. species (Chirpan-539 and Darmi) had low SCA variances for both traits and their high GCA was mainly caused by

additive gene effects making them suitable for synthetic selection, and those of the *G. barbadense* L. species had high SCA variances and their high GCA due to additive and non-additive gene effects and interactions, and they were more suitable for heterosis selection. Some parents showed high GCA effects for two or more traits. Chirpan-539 variety had a high GCA for productivity and lint percentage, used as a maternal and paternal component. FR-B-201 variety had a positive GCA for productivity, boll weight and lint percentage used as mother and as father.

### Hybridization of *G. hirsutum* L. with some wild diploid cotton species

Amphidiploids (allohexaploids) *G. hirsutum* × *G. sturtii*, *G. hirsutum* × *G. thurberi* and *G. hirsutum* × *G. davidsonii*, and trispecific hybrids *G. hirsutum* - *G. arboreum* - *G. raimondii* A<sub>1</sub>D<sub>1</sub>A<sub>2</sub>D<sub>5</sub> (2n=4x=52), *G. hirsutum* - *G. arboreum* - *G. thurberi* A<sub>2</sub>A<sub>1</sub>D<sub>1</sub>D<sub>1</sub> (2n=4x=52) and *G. hirsutum* - *G. thurberi* - *G. raimondii* A<sub>1</sub>D<sub>1</sub>D<sub>1</sub>D<sub>5</sub>, (2n=4x=52) were obtained. Two-or three times backcrossing in C<sub>1</sub> and F<sub>1</sub> (at the triple hybrids) and in later backcross generations was applied. After backcrossing to the cultivated species valuable introgression forms were obtained.

Three introgression methods were used for transferring genetic material from the wild species into the genome of cultivated *G. hirsutum* species depending on the affinities between the implicated genomes: paraphylogenetic method; pseudophylogenetic introgression and aphylogenetic introgression (Ndungo *et al.*, 1988).

The affinity of subgenome A of the amphidiploid species with the genome A of diploid species is very strong. Concerning the affinity of D-subgenome of the amphidiploid species with D-genome of the diploid species there are three groups: strong (with *G. thurberi* - 2D<sub>1</sub>); not very strong (with *G. raimondii* - 2D<sub>5</sub>); causing lethality (with *G. davidsonii* - 2D<sub>3-d</sub>) (Louant, 1973; Valicek, 1978).

The paraphyletic and pseudophyletic methods based on the synthesis of natural allotetraploids with genomic formula 2(AD) led to the producing of trispecific hybrids. Our and other studies showed that trispecies hybrids were high heterozygous forms and therefore in the backcross progenies still in F<sub>1</sub> process of segregation was observed.



Figure 1. Allotetraploid C6 *G. thurberi*. × *G. raimondii* and F<sub>1</sub> plant of the trispecific hybrid (*G. thurberi*. × *G. raimondii*) × Chirpan 603

The trispecific hybrid *G. hirsutum* - *G. thurberi* - *G. raimondii*  $A_1D_1D_1D_5$  ( $2n=4x=52$ ) has two subgenomes  $A_1$  and  $D_1$  from the *G. hirsutum* species one genome of *G. thurberi* -  $D_1$ , and one genome of *G. raimondii* -  $D_5$  i.e. one A-genome and three D-genomes. Mehetre (1993) at an amphihaploid of *G. hirsutum*  $2(AD)_1 \times G. raimondii$  ( $2D_5$ ) observed 4 to 6 bivalents as a result of pairing between Ah and  $D_5$  chromosomes, respectively from *G. hirsutum* and *G. raimondii* species, as well as of Ah with Dh and Dh with  $D_5$ -chromosomes. The formation of pairs between non-homologous chromosomes showed that the chromosomes of the genome  $D_5$  - *G. raimondii* have close homology with the AhDh chromosomes of *G. hirsutum*.

The results obtained from studies with molecular markers (Reinsch et al., 1994; Lacape et al., 2003) and QTL analyses of the same characters of chromosome supplemented lines (Endrizzi et al., 1984) support the existence of a homologous connection between the Ah- and Dh-subgenomes.

In the hybridization of *G. hirsutum*  $\times G. davidsonii$  the incompatibility of two species was overcome by the use of interspecific  $F_1 G. hirsutum$  L.  $\times G. barbadense$  L. hybrids, later varieties derived from this hybridization. Amphidiploids were genetically unstable. Due to the limited number of plants in  $F_1BC_1$  backcrosses with to the *G. hirsutum* L. species were made in  $F_2BC_1$  and later backcross generations which also produced positive results. Backcrossing in the later and in backcross generations allowed for more efficient use of the resultant genetic material, which at the remote hybridization was very limited.

Effectiveness of selection process greatly depended on the genotype of parental varieties included in crosses with the wild species and the genotype of varieties used for backcrosses. When used for backcrossing varieties which genotype was different from the maternal form of hybrids, backcross generations were more vigorous as a result of their higher heterozygosity. In two- and three-times backcrossing with to the same parent, heterozygosity decreased.

To obtain forms with longer fiber the backcrossing with to introgressed varieties and lines with germplasm of the *G. barbadense* species as Avangard-264, Pearla-267, Darmi and Natalia was of greater importance. Simultaneously improvement of fiber length and lint percentage was found for the hybrids obtained from the backcrosses with Dorina variety with longer fiber and higher lint percentage.

The triple hybrids produced from the crossing of synthetic allotetraploids with the species *G. hirsutum* formed highly heterogeneous populations with low productivity in the early generations. The low productivity was due to genotypic imbalance specific for the early generations of interspecific hybrids.

The studies carried out and the results obtained showed that the use of foreign geneplasm in cotton selection is very reliable.

## CONCLUSIONS

Pirin, Perun and Tiara varieties were new achievements in the selection of earliness and productivity an increase in seed cotton yield by 7.8% (Perun variety) was achieved compared to Chirpan-539 – standard for productivity.

Aida, Anabel and Melani varieties were new achievements in selection for fiber quality. These varieties, in some fiber technological properties were superior to Avangard-264 variety – a standard for fiber quality, or were equal to it. With them, a better combination of economic and fiber technological qualities than Avangard-264 has been achieved.

Egea and Nike varieties were a new achievement in the selection of colored cotton in our country. The most valuable characteristic of these varieties was their longer fiber, for Egea by 1.35 mm and 1.57 mm (modal and staple length), than that of Izabell variety approved as a



standard for the colored cotton. With these two varieties significant progress in improving the fiber quality of coloured cotton by selection has been made.

Genetic control of productivity and its elements in the studied sets of genotypes included in diallel and line×tester crosses was mainly non-additive. Overdominant action of genes prevailed with possible manifestation of complementary epistasis.

Additive and non-additive gene effects were important for the inheritance of fiber length and lint percentage. The non-additive gene effects were more important and the selection should be conducted in the later hybrid generations - F<sub>3</sub>-F<sub>4</sub>.

In the interspecific crosses of *G. hirsutum* L. × *G. barbadense* L., additive and non-additive gene effects were important for the inheritance of studied traits. The genetic variance for boll weight was additive, suggesting faster selection, while for the other traits, the genetic variance was mainly non-additive.

Parental forms having positive and high GCA were identified for the studied characters, suitable for heterosis and synthetic selection. The best intra- and interspecific hybrid combinations were selected for breeding programs.

Allohexaploids of the *G. hirsutum* L. (2n=56) species with the wild diploid species *G. thurberi* Tod., *G. sturtii* F. Mull. and *G. davidsonii* Kell. (2n=26), and three-species hybrids *G. hirsutum* L. - *G. arboreum* L.- *G. raimondii* Ulbr., *G. hirsutum* L. - *G. thurberi* Tod. - *G. raimondii* Ulbr. and *G. hirsutum* L. - *G. thurberi* Tod. - *G. arboreum* L. were obtained.

Different approaches have been developed and applied to overcome the incrossability and incompatibility of *G. hirsutum* L. and some wild cotton species. Different backcrossing approaches have been used.

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## **INFLUENCE OF OSMOTIC STRESS ON THE GROWTH OF YOUNG COTTON SEEDLINGS**

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### **ABSTRACT**

In this study 17 cotton varieties were included. Their relative resistance to drought was determined by establishing the germ reaction to osmotic stress with the use of 1M solution of sucrose. Genetic distance was also determined by observation of characteristics related to their reaction to simulated dry conditions. Based on the overall depression in growth of cotton seedlings grown in an osmotic environment, Helius, Perla-267 and Natalia varieties were determined as water stress-tolerant varieties and FR-H-1001, FR-H-1002, Denitsa and Avangard-264 varieties were found the most sensitive.

**Keywords:** cotton, osmotic stress

### **INTRODUCTION**

Drought is one of the most limiting factors for profitable cotton production (Paytas, 2009, Molasadeghi et al., 2011). Although cotton successfully adapts to drought, its cultivation under a favorable water regime (through irrigation or in areas with abundant rainfall) is a prerequisite for obtaining high and quality yields (Hearn, 1979). When cotton is grown under optimal soil moisture, then its phenological development lasts longer, resulting in well-developed, high-yielding plants. During drought, cotton stops its morphological development and focuses on the retention of growth and generative organs (Hearn, 1994).

Osmotic regulation is one of the most important adaptive cellular mechanisms to maintain cell hydration and avoid decreased yield (Blum, 2005). Plants with better osmoregulation ability show better growth and higher yield under drought conditions. Genotypic differences in the ability to osmoregulate have been reported in different cultures (Morgan et al., 1986; Blum, 1989; Morgan, 1992).

A major goal of many breeding programs is the creation of varieties with high yield potential under adverse conditions. Researchers use various methods to assess genetic differences in drought tolerance. New reliable methods and approaches are sought in screening genotypes for water stress tolerance. There is a need to develop tests for faster identification of tolerant genotypes (Bozhanova and Stoilova, 2002).

An indirect physiological method for identifying drought-resistant genotypes is the germination of seeds and the development of seedlings in a solution with increased osmotic pressure (Bozhanova, 1997; Bozhanova et al., 2005; Blum et al., 1980). The degree of suppression of growth processes in shoots under the action of osmotic stress correlated to the greatest extent with general drought resistance (Udovenko, 1976). According to Marcheva et al. (2005) the application of osmotic stress on germinating seeds enabled the distinction of a large number of genotypes, easy and rapid differentiation of tolerant forms at early stages of the selection process.

The aim of this research was to study the response to osmotic stress of Bulgarian and foreign cotton varieties grown in water and in an osmotic solution, with a view to create a more complete characterization of drought tolerance of the different genotypes and to determine the genetic distance based on the traits related to osmotic stress, which could serve as a means of increasing the selection process.

## MATERIAL AND METHODS

The study included 17 cotton varieties: Bulgarian - Chirpan-539, Avangard-264, Perla-267, Natalia, Darmi, Colorit, Vega, Dorina, IPK Nelina, Rumi, Helius, Boyana, Viky, Denitsa; Spanish - FR-H-1001, FR-H-1002 and Nazilli variety - Turkish.

Seeds of the tested cotton varieties were placed on filter paper moistened with distilled water, which was rolled up. Two rolls were prepared from each genotype. All rolls were placed in plastic boxes with distilled water in a thermostat at 25 °C for germination. After three days, half of the rolls with the shoots were placed again in distilled water and the other half - in a 1M sucrose solution in a thermostat under the same conditions. After next three days the length of roots and the length of vegetative part of the seedlings, both of the control and those grown in sucrose solution, were measured. The experiment was performed in three replicates for each variant and genotype, and 25 seedlings (shoot and root) were measured for each replicate.

Cotton seedling growth depression was calculated as a percentage using the formula of Blum et al. (1980):

**Depression coefficient, %** =  $[(A-B)/A] \times 100$ , where:

A – mean length (mm) of shoots/roots in the control variant (H<sub>2</sub>O)

B – mean length (mm) of shoots/roots in osmotic medium (1 M sol. sucrose).

The osmotic concentration was determined based on research done by other authors (Bozhanova, 1997).

The genetic distance of studied cotton varieties was established by applying of cluster analysis. The computer program Statistica 7 was used. Genetic distance was expressed by determining the Euclidean distance between two objects in multidimensional space.

## RESULTS AND DISCUSSION

Under the influence of a simulated water deficit created by adding sucrose to the growing medium of young cotton shoots, their growth was suppressed. In general, water deficit caused a more intensive suppression in growth of the above ground parts compared to roots (Bozhanova, 2005). The average percentage of depression in the growth of shoots was 13.1% and in the roots - 12.3% (Table 1).

The percentage of shoot depression varied from 2.5% to 19.8% and for Helius, Perla-267 and Avangard-264 varieties it was below 10%. The above-ground part development was most affected for the cultivars Colorit (19.8%), FR-H-1001 (19.7%) and Viki (17.9%), while Helius variety reacted with the least suppression of shoot growth to the applied osmotic stress (Table 1). For the varieties Avangard-264, Helius, Chirpan-539, Perla-267, Vega, Boyana, Denitsa, FR-H-1002 and Nazilli the degree of root growth suppression was higher compared to the depression of shoots, as in Chirpan-539, Vega and Boyana varieties the limitation of root growth was insignificantly higher than that of the above-ground part. The percentage of root depression was the highest for Avangard-264 variety.

The varieties Helius, Perla-267 and Natalia reacted the least to the applied water stress, the total depression values were the lowest and these genotypes could be defined as drought tolerant. A high percentage of total depression was accounted for the varieties FR-H-1001 (17.1%), FR-H-1002 (16.9%), Denitsa (16.3%), and Avangard-264 (16.2%), which defined them as sensitive to water stress. The high percentage of total depression for Avangard-264 variety, regardless of the low percentage of shoot depression, is explained by a significant reduction in root growth compared to the above-ground mass, which is contrary to the generally accepted trend according to which water stress affects to a greater extent the growth of the vegetative part compared than the roots growth.

Table 1. Osmotic stress response of cotton shoots/seedlings

| Variety        | Root length, cm |                 | Shoot length, cm |                 | Depression coefficient, % |             |             |
|----------------|-----------------|-----------------|------------------|-----------------|---------------------------|-------------|-------------|
|                | Control         | 1M sol. sucrose | Control          | 1M sol. sucrose | Root                      | Shoot       | Total       |
| Chirpan-539    | 13.9            | 12.3            | 10.4             | 9.2             | 11.7                      | 11.3        | <b>11.5</b> |
| Avangard-264   | 14.2            | 10.6            | 10.7             | 9.9             | 24.9                      | 7.5         | <b>16.2</b> |
| Perla-267      | 15.5            | 13.3            | 11.6             | 11.0            | 14.2                      | 4.7         | <b>9.4</b>  |
| Natalia        | 15.8            | 14.9            | 12.6             | 11.0            | 5.7                       | 12.5        | <b>9.1</b>  |
| Darmi          | 14.9            | 13.9            | 11.7             | 9.9             | 7.0                       | 15.9        | <b>11.4</b> |
| Colorit        | 13.2            | 12.9            | 12.7             | 10.2            | 2.4                       | 19.8        | <b>11.1</b> |
| Vega           | 14.5            | 12.8            | 11.8             | 10.6            | 11.8                      | 10.0        | <b>10.9</b> |
| Dorina         | 14.8            | 13.0            | 12.5             | 10.6            | 12.2                      | 15.3        | <b>13.7</b> |
| IPK Nelina     | 15.2            | 13.2            | 12.0             | 10.0            | 13.3                      | 16.2        | <b>14.8</b> |
| Rumi           | 15.3            | 13.6            | 11.6             | 9.8             | 11.0                      | 15.1        | <b>13.0</b> |
| Helius         | 14.2            | 12.5            | 10.4             | 10.1            | 12.2                      | 2.5         | <b>7.3</b>  |
| Boyana         | 13.7            | 11.9            | 11.6             | 10.2            | 13.3                      | 11.8        | <b>12.5</b> |
| Viki           | 13.9            | 13.0            | 12.0             | 9.9             | 6.2                       | 17.9        | <b>12.1</b> |
| Denitsa        | 15.7            | 12.9            | 12.2             | 10.4            | 17.8                      | 14.8        | <b>16.3</b> |
| FR-H-1001      | 15.0            | 13.1            | 11.7             | 9.4             | 14.4                      | 19.7        | <b>17.1</b> |
| FR-H-1002      | 13.9            | 11.41           | 11.5             | 9.7             | 18.0                      | 15.7        | <b>16.9</b> |
| Nazilli 954    | 10.0            | 8.5             | 9.7              | 8.5             | 15.0                      | 12.4        | <b>13.7</b> |
| <b>Average</b> | <b>14.3</b>     | <b>12.6</b>     | <b>11.6</b>      | <b>10.0</b>     | <b>12.3</b>               | <b>13.1</b> | <b>12.8</b> |

As a result of the induced water stress the ratio between the root length and the shoot length also changed. It increased to a different extent in most studied genotypes (**Fig. 1**).

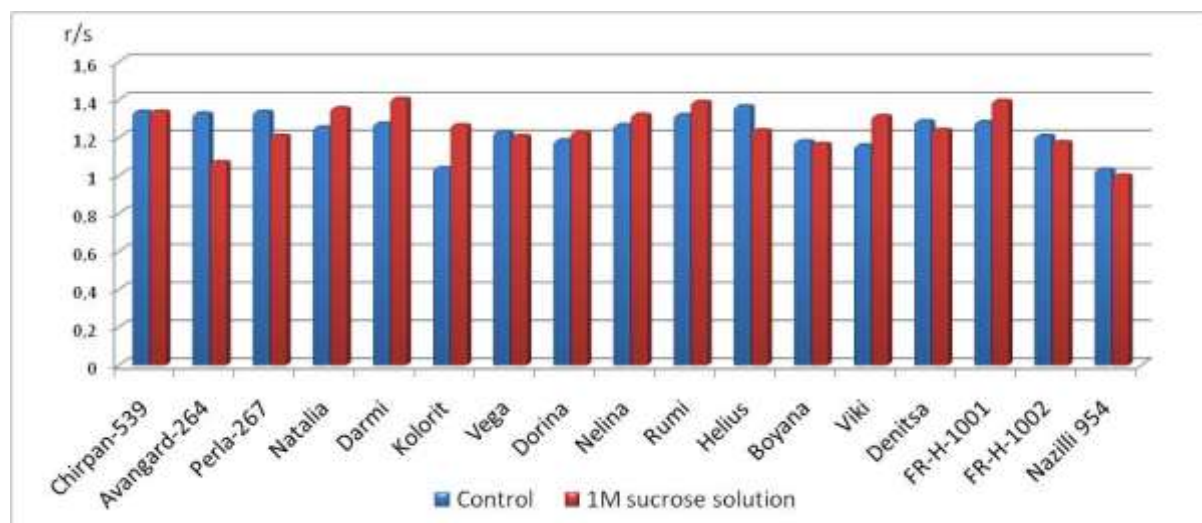


Figure 1. Ratios between root lengths and shoot lengths of cotton varieties grown to osmotic stress (1M sucrose solution)

The root/shoot average ratio for the entire set of varieties in the control variant was 1.24 and in 1M sucrose solution it was 1.27. In drought tolerant genotypes root/shoot ratio increased least (Bajji et al., 2000; Dhanda et al., 2002). The increase was the greatest for the varieties Colorit, Viki, Darmi and FR-H-1001 – i.e. they stood out as sensitive to water shortage. The varieties Helius and Perla-267 appeared to be tolerant to water stress according to this indicator, in which there was a reduction in the root/shoot ratio in the stressed option.

An increase in the ratio of root to shoot lengths when dehydration occurred at the shoot level was also reported by Bajji et al. (2000), Dhanda et al. (2002), Bojanova (2002) and could be used as an indirect and rapid screening method for drought tolerant genotypes.

From the dendrogram in **Figure 2** is visible that the varieties included in this study formed 4 groups (A, B, C, D). The varieties Helius and Perla, which were characterized by relative good drought resistance, formed cluster "B". Clusters "C" included the sensitive varieties FR-H-1001, FR-H-1002, Denitsa and Avangard-264. In cluster "D" two subgroups were formed and the first ones included the osmotic stress-sensitive varieties Nelina, Rumi and Dorina. The second subgroup included moderately drought tolerant varieties Vega, Chirpan-539, Boyana and Nazilli-954.

Genetically the most distant were found the variety Kolorit (A) and the varieties of the second subgroup of cluster "D" (Chirpan-539 and Vega).

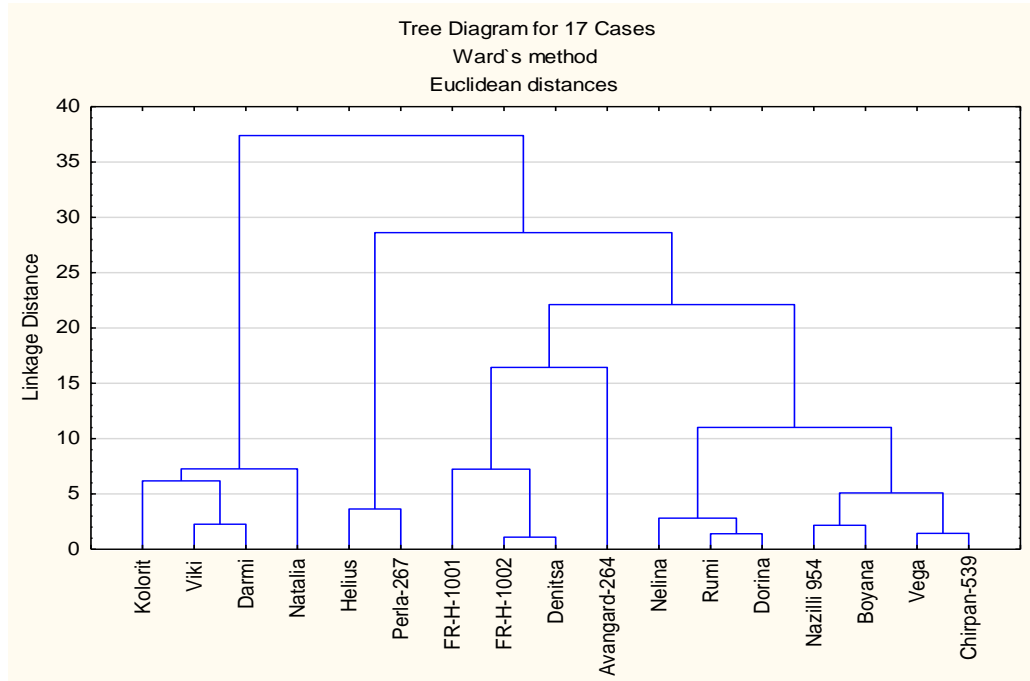


Figure 2. Dendrogram of cotton varieties grouped based on response to osmotic stress (in 1M sucrose solution)

## CONCLUSIONS

The varieties Helius, Perla-267 and Natalia reacted the least to the applied water stress, the total depression values were the lowest and these genotypes can be defined as drought tolerant.

A high percentage of total depression was accounted for the varieties FR-H-1001 (17.1%), FR-H-1002 (16.9%), Denitsa (16.3%), and Avangard-264 (16.2%), which defined them as sensitive to water stress.

As dehydration occurs at the shoot level, the ratio between root and shoot lengths increased.

Research results gives reason to recommend the varieties Helius and Perla-267 be included in the selection programs for research in order to increase the drought resistance of cotton.

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## COMPARATIVE TESTING OF FORAGE PEAS VARIETY "MIR" IN THE CONDITIONS OF ORGANIC AND CONVENTIONAL AGRICULTURE

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### ABSTRACT

During the period 2019 - 2020 on the experimental field in Field Crops Institute (FCI) - Chirpan was conducted an experiment with winter forage peas variety Mir, grown in organic and conventional agriculture. The soil in the area is leached resin. Forage peas are sown after the predecessor durum wheat variety Progress. The following phenological observations were made: beginning of stem formation, beginning of flowering, duration of flowering, full maturity and the vegetation period of peas was traced. Biometric indicators are also taken into account: betting height of the first bean, number of beans per plant, number of seeds in one bean and seed weight per plant. The yield of green mass is reported in the phase of mass flowering - the beginning of fruiting. A comparison was made between the reported indicators of peas in organic and conventional cultivation systems. There is no difference in the occurrence and duration of the individual phenological phases. During the years of research, the yield of green mass in winter forage peas variety Mir, grown conventionally, averaged 4,785 t / da, while in the biological system of cultivation was 3,675 t / da.

**Key words:** forage peas, organic farming, conventional farming, green mass yield

### INTRODUCTION

Forage peas are one of the valuable sources of plant protein and together with other legumes is important for solving the protein problem in the country (Kertikov, 2010). Winter and spring forage peas are grown in Bulgaria (Mehandjiev et al., 2006; Kertikova, 2012). The Mir variety has been adopted as a standard for winter forage peas.

Winter forage pea variety "Mir" is characterized by extremely high winter hardiness, early maturity, high productivity of green mass, hay and seeds - a typical forage variety for green. In Bulgaria, studies have been conducted on the productive potential of winter forage pea varieties (Kertikova and Kertikov, 2012b), but only in the conditions of conventional agriculture. This article presents the results of the test on biological and economic qualities of forage peas variety "Mir", grown in organic and conventional agriculture, and a comparison is made between the two systems of cultivation.

### MATERIAL AND METHODS

In the period 2018-2020, in the experimental field of the Field Crops Institute, Chirpan, a field experiment was conducted to compare between organically and conventionally grown winter forage peas "Mir". The field experience is based on the fall of two consecutive years (2018 and 2019), using the method of fractional plots. Phenological observations were made: beginning of stem formation, beginning of flowering, duration of flowering, full maturity; vegetation period.

Biometric measurements include: the height of the first bean, the number of beans per plant, the number of seeds per bean and the weight of seeds per plant. The measurements of each indicator are made on 10 plants from each repetition, and for the year the average of them is taken for the respective indicator. The yield of green mass is reported in the phase of mass flowering - the beginning of fruiting.

## RESULTS AND DISCUSSION

In the autumn of the two years (2018 and 2019) no differences were reported between organically and conventionally grown peas when registering the full germination phase. Visual observations revealed that, in general, the plants of the studied cultivation systems are characterized by only slight damage to the leaves and drying of some of the branches. During the three years of study, their vital functions were not impaired and their overwintering was successful. The results of the phenological observations on average for the period are presented in Table 1.

Table 1. Phenological observations in winter peas

| Phenophase                            | Conventional  |               |         | Organic       |               |         |
|---------------------------------------|---------------|---------------|---------|---------------|---------------|---------|
|                                       | 2018/<br>2019 | 2019/<br>2020 | average | 2018/<br>2019 | 2019/<br>2020 | average |
| Sowing                                | 14.11         | 08.11         |         | 14.11         | 08.11         |         |
| Germination                           | 02.12         | 28.11         |         | 02.12         | 28.11         |         |
| Beginning of stem formation           | 15.03         | 10.03         |         | 15.03         | 10.03         |         |
| Early flowering, date                 | 14.05         | 07.05         |         | 12.05         | 05.05         |         |
| Duration of flowering, average - days | 32            | 35            | 33,5    | 32            | 35            | 33,5    |
| Beginning of fruiting                 | 27.05         | 29.05         |         | 27.05         | 29.05         |         |
| Full maturity, date                   | 30.06         | 01.07         |         | 30.06         | 01.07         |         |
| Vegetation period, average – days     | 228           | 234           | 231     | 228           | 234           | 231     |

The data show that there are no differences and variations between organically and conventionally grown peas in terms of the occurrence of individual phenophases. Beginning of stem formation occurs in the first half of March, flowering begins in late April to early May. Fruiting begins after May 20. The phase of full maturity was registered at the end of June. In the conditions of the town of Chirpan, the Mir variety is characterized by a vegetation period of an average of 231 days for the two years of study. According to the description of the Mir variety (Milyanchev et al., 1986), it belongs to the group of early-maturing varieties. Similar results for the length of the growing season have been reported by other authors (Kertikova and Kertikov, 2016). The results of the characteristics of forage peas by main elements of productivity are presented in Table 2.

The analysis of the data regarding the height of the first bean bet shows that conventionally grown peas bet higher than the first bean than organically grown peas. The values of the number of fertile nodes vary from 7.2 to 7.8 for conventional peas, while for organic peas they are from 6.1 to 6.7. The number of beans per plant also varies from 12.7 for 2019 in organic farming to 14.5 for 2020 in conventional. Data on the number of seeds in one bean show that there are no significant differences between organically and conventionally grown peas. Most likely this is due to the fact that this indicator is related to the genetic characteristics of the variety "Mir" and does not depend on the conditions in different cultivation systems.

More significant are the differences in the weight of seeds per plant. The Mir variety grown conventionally has a higher yield (8.36 g / plant) than that of organic production (6.56 g / plant).

Table 2. Structural analysis of winter peas

| Indicator                    | Conventional  |               |         | Organic       |               |         |
|------------------------------|---------------|---------------|---------|---------------|---------------|---------|
|                              | 2018/<br>2019 | 2019/<br>2020 | average | 2018/<br>2019 | 2019/<br>2020 | average |
| Height of the first pod (cm) | 99.9          | 101.5         | 100.7   | 92.5          | 98.3          | 95.4    |
| Number of fertility nodes    | 7.2           | 7.8           | 7.5     | 6.1           | 6.7           | 6.4     |
| Number of pods per plant     | 13.9          | 14.5          | 14.2    | 12.7          | 13.5          | 13.1    |
| Number of seeds per pod      | 5.1           | 5.7           | 5.4     | 4.9           | 5.3           | 5.1     |
| Seed weight per plant (g)    | 7.85          | 8.87          | 8.36    | 6.1           | 7.02          | 6.56    |

The analysis of biometric measurements shows that in organically grown peas almost all indicators are lower than in conventional cultivation. An exception is the indicator number of seeds in beans where the average for the two years of the study the number is very small.

According to similar studies (Kertikova, 2012; Kertikova and Kertikov, 2013a; 2016; Barbieru, 2020) a key factor in terms of productivity is the duration of the flowering phase. However, the present study did not show a difference in the duration of this phase in organically and conventionally grown peas. The higher productivity of conventional peas is mainly due to the fruit elements - buttons, flowers and beans. The yield of green mass is reported in the phase of mass flowering - the beginning of fruiting. The data are presented in Table 3.

Table 3. Yield from green mass, t/da

| Agriculture system | 2018/2019 | 2019/2020 | average |
|--------------------|-----------|-----------|---------|
| Conventional       | 4,62      | 4,95      | 4,785   |
| Organic            | 3.45      | 3.90      | 3.675   |

The analysis of the obtained results shows that conventional peas form a larger green mass than organic peas. On average for the two years of research, the yield of green mass in winter forage peas of the Mir variety, grown conventionally, averaged 4,785 t / da, while in the case of the biological cultivation system it was 3,675 t / da.

## CONCLUSIONS

During the two years of testing of organic and conventional forage peas, no difference was observed in the occurrence and duration of the individual phenological phases. The different systems of pea cultivation do not affect the occurrence of the individual phenophases and the duration of the vegetation period of the crop.

In terms of the main elements of productivity, conventionally grown peas have higher rates than organically grown peas. An exception is the number of seeds in beans, which is almost the same for peas in both farming systems.

Differences were also reported in the production of green mass. During the years of research, the yield of green mass in winter forage peas variety Mir, grown conventionally, averaged 4,785 t / da, while in the biological system of cultivation was 3,675 t / da.

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## HARMFUL ENTOMOFAUNA BY CHICKPEAS-CICER ARIETINUM L. (FABALES, FABACEAE)

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### ABSTRACT

Chickpeas are attacked by determined circle pests, which have permanent character in individual years are multiply en masse and are able to compromise the harvest. In this regard clarifying the species composition of the most common pests, as well as its successful on time control of the struggle with them have important meaning in its cultivation. The research was carried out in the area of the town of Rouen. For reporting density of insect pest use standard entomological methods. In fields with chickpeas settled down 21 pest, belonging to 10 families and 5 orders. From them of economic importance are: the cotton bollworm (*Helicoverpa armigera* Hub.) and the chickpea leafminer (*Liriomyza cicerina* Rond.). Because higher temperatures in 2020 extend chickpea vegetation resulting in the species have multiplied en masse. In 2020 14.66% of damaged beans were reported from cotton bollworm and 24% damaged leaves from the chickpea leafminer. In 2021, respectively, 15% damage was registered on beans and 7.33% on leaves.

**Key word:** chickpeas, standard entomological methods, cotton bollworm (*Helicoverpa armigera* Hub.), chickpea leafminer (*Liriomyza cicerina* Rond.)

### INTRODUCTION

The harmful entomofauna of chickpea is very specific and rich in species. About 60 insects are known in the world that are enemies of chickpeas both under field conditions and during storage in warehouses: black cutworm (*Agrotis ipsilon* Hfn., *Agrotis segetum* Schiff., *Spodoptera exigua* Hub., *Spilarctia oblique* Walker, *Helicoverpa armigera* Hub. and *Helicoverpa punctigera* Wallengren), chickpea leafminer (*Liriomyza cicerina* Rond.), aphids (*Aphis craccivora* Koch.) and the bruchids of the genus *Callosobruchus* spp. (Reed et al., 1987; Clement et al., 2000; Sharma et al., 2007).

Sharma et al., (2007) indicated the following economically important chickpea species: cutworms of the genus *Gonocephalum* spp.; nightshades – *Agrotis ipsilon* Hfn., *Agrotis segetum* Schiff., *Trichoplusia ni* Hub., *Spodoptera exigua* Hub., *Spodoptera oblique* Walker, nightshades: *Helicoverpa armigera* Hub., *Helicoverpa punctigera* Wallengren and *Helicoverpa zea* Boddie.; *Microtermes obesi* Holm. and *Odontotermes* sp.; *Autographa nigrisignia* Walker; leafmining flies: *Liriomyza cicerina* Rondani and *Liriomyza congesta* Becker; aphids: *Aphis craccivora* Koch and *Acyrtosiphon pisum* Harris; *Metopina ciceri* Disney; bruchids: *Callosobruchus chinensis* L., *Acanthoscelides obtectus* Say., *Callosobruchus analis* F. and *Callosobruchus phaseoli* Gylh.

In Haryana, India the following have been found on chickpeas pests: *Aphis craccivora*, *Odontotermes obesus*, *Agrotis ipsilon*, *A. flammata* (*Ochropleura flammata*), *Plusia*

*nigrisigna* (*Autographa nigrisigna*), *Spodoptera exigua* and *Heliothis armigera* (*Helicoverpa armigera*), *Lrom agiomyampisicer Ligromis*. Of these, the most economically important are: *Aphis craccivora*, *O. obesus* and *H. armigera* (Neresh and Malik 1986).

In Asia, Africa and Australia economic significant pest by chickpeas are: the cotton boll weevil (*Helicoverpa armigera* Hubner), *Aphis craccivora* Koch (*Hemiptera: Aphididae*) and grain eaters of the genus *Callosobruchus* (*Callosobruchus chinensis* L., *Callosobruchus maculatus* F., *Callosobruchus analis* F.) (Van Emden et al., 1988).

Dubey et al., (1993), Tripathy et al., (1999) studied the preferences on the cotton bollworm to different food hosts and establish that chickpeas is the most preferred crop.

In Australia, Syria, the Mediterranean region of Europe, Transcaucasia and Central Asia, North Africa, Bulgaria, the key pests of chickpeas are: the chickpea leafminer *Liriomyza cicerina* and species of genus *Helicoverpa spp.* (Miroshnichenko and Pavlova, 1953; Al-Soud et al. 1990; Pimbert, 1990; Weigand and Tahhan, 1990; Saxena, 1993; Weigand and Pimbert, 1993; Weigand et al., 1994; El Bouhssini et al., 1998; Matthews, 1999; Knights and Siddique, 2002; Dimitrov, 2008, Tebkew et al., 2006; Fite et al., 2021; Fite et al., 2022).

In chickpea, worldwide losses caused by cotton bollworm have been estimated at US\$ 330 million per year (ICRISAT, 1992).

Of the leafmining flies the most widely common species is *Liriomyza cicerina* Rondani (Hariri, 1979; Reed et al., 1987; Pimbert, 1990; Weigand and Tahhan, 1990; Garrido et al., 1992; Tamer et al., 1998). In parallel they are with her two more have been established species: *Chromatomyia horticola* Goureau and *Agromyza spp.* It is economic the most important enemy of chickpea in the Mediterranean region, Eastern Europe and in northern India ((Neresh and Malik, 1986, Weigand and Tahhan, 1990), Garrido et al., 1992; Weigand et al., 1994; Tamer et al., 1998).

Listomining flies attack in a different degree the individual varieties chickpeas, like the difference in the yield reduction is negligible. Is not established correlation between density of the larvae and yield losses (Cikman and Civilek, 2007).

From aphids (family *Aphididae*), with the greatest importance being *Aphis craccivora* Koch. (Reed et al., 1987; Kaiser et al., 1990; Weigand and Tahhan, 1990). The species causes both direct as well as indirect damages. The direct damages are connected with nutrition, and the indirect ones as a virus carrier.

In many areas of the world in the warehouses cereals inflict considerable losses (Labeyrie, 1981). Of these, the most common chickpeas in the warehouses are: *Callosobruchus chinensis* L. and *Callosobruchus maculatus* F. (Reed et al., 1987; Weigand and Tahhan, 1990; Weigand and Pimbert, 1993, Fite et al., 2022).

In India from cereals are found 13% damage (Mookherjee et al., 1970; Dias and Yadav, 1988).

## **MATERIAL AND METHODS**

The studies are conducted during the period 2020-2021 in chickpea crops in the area of the city of Rouen. To establish the species composition and density of pests, standard entomological methods were used: visual observations, test plots, mowing with an entomological bag, etc., and were applied throughout the growing season from sowing to harvest.

## RESULTS AND DISCUSSION

Determinant for the development and the multiplication of the enemies the chickpeas are the weather conditions. After sowing until stem formation and growth, chickpea pests are representatives of the family *Elateridae* and *Noctuidae*. They were found in mixed populations. (Table 1).

Table 1. Species composition and density of pest of chickpeas in the area of the city of Rouen (numbers/m<sup>2</sup>, bale) for the period 2020-2021.

| Order, Family                                  | Species                               | 2020   | 2021   |
|--|---------------------------------------|--------|--------|
| <i>Coleoptera, Curculionidae</i>               | <i>Sitona lineatus</i> L.             | 2      | 2      |
|  | <i>Sitona macularius</i> Marsh.       | 2      | 1      |
|  | <i>Tychius quinguepunctatus</i> L.    | 1      | 1      |
| <i>Elateridae</i>                              | <i>Agriotes lineatus</i> L.           | 0,5    | 0,5    |
|  | <i>Agriotes ustulatus</i> Schall.     | 1      | 1      |
|  | <i>Agriotes obscurus</i> L.           | 1      | 0,5    |
| <i>Diptera, Agromyzidae</i>                    | <i>Liriomyza cicerina</i> Rond.       | 5      | 2      |
| <i>Hemiptera, Heteroptera<br/>Pentatomidae</i> | <i>Lygus pratensis</i> L.             | 1      | 1      |
|  | <i>Lygus rugulipenis</i> Popp.        | 1      | 1      |
|  | <i>Dolycoris baccarum</i> L.          | 2      | 3      |
| <i>Hemiptera, Sternorrhyncha<br/>Aphididae</i> | <i>Aphis fabae</i> Scop.              | Bale 1 | Bale 1 |
|  | <i>Aphis craccivora</i> Koch.         | Bale 1 | Bale 1 |
| <i>Cixiidae</i>                                | <i>Hyalesthes obsoletus</i> Sig.      | 3      | 1      |
| <i>Lepidoptera, Noctuidae</i>                  | <i>Helicoverpa armigera</i> Hübner    | 4      | 2      |
|  | <i>Agrotis segetum</i> Schiff.        | 1      | 1      |
|  | <i>Agrotys ypsilon</i> L.             | 1      | 1      |
| <i>Crambidae</i>                               | <i>Loxostege sticticalis</i> L.       | 1      | -      |
| <i>Orthoptera, Acrididae</i>                   | <i>Calliptamus italicus</i> L.        | 4      | 1      |
|  | <i>Dociostaurus maroccanus</i> Thung. | 3      | 1      |
| <i>Gryllidae</i>                               | <i>Gryllus campestris</i> L.          | 2      | 0      |
|  | <i>Melanogryllus desertus</i> Pall.   | 2      | 1      |

The species of the genus *Agriotes*: *Agriotes lineatus* L., *Agriotes ustulatus* Schall. and *Agriotes obscurus* L. were found in a low density of 0.5-1 pcs./m<sup>2</sup> during the two years of study. Significant

damage wireworms cause in wet spring years. The reporting period characterized by drought, therefore their density is low. At the same time, from the family *Noctuidae*, established caterpillars of *Agrotis segetum* Schiff. and *Agrotys ypsilon* L. They were recorded in a low density - 1 pc./m<sup>2</sup>.

After formation of the true leaves until budding, chickpeas were damaged by grasshoppers and crickets. The following crops were found: *Calliptamus italicus* L., *Dociostaurus maroccanus* Thung, *Gryllus campestris* L. and *Melanogryllus desertus* Pall. They have a wide food specialization and attack a number of field crops (Chorbadjiev, 1936; Dirimanov, 1962). The omnivorous pests were found in higher density in 2020: *C.italicus*- 4 pcs./m<sup>2</sup>, *D. maroccanus* - 3 pcs./m<sup>2</sup>, *G. campestris* and *M.desertus* - 2 pcs./m<sup>2</sup>. We associate this with both the weed associations and the ecological conditions and the structure of the cultivated areas in the area.



Their presence in the chickpea crop can be explained by the adjacent sunflower crops, which are known to be a preferred host for species of the order *Orthoptera*.

In the phenophases stem formation to formation of chickpea beans occurred species of the genus *Sitona*: *Sitona lineatus* L., *Sitona macularius* Marsh and *Tychius quinguepunctatus* L. The reported one density is: *S.lineatus*, *S.macularius* - 2 pcs./m<sup>2</sup> each and *T.quinguepunctatus* - 1 pc./m<sup>2</sup>. The species were established in higher numbers in 2020. We associate this with the weed vegetation in and around the chickpea crops, as well as with the presence of favorable conditions for the development of the crops located adjacent to the chickpeas.

Parallel with them they found bed bugs, aphids and leafhoppers. Among the bedbugs, the following were found: *Lygus pratensis* L., *Lygus rugulipenis* Popp. and *Dolycoris baccarum* L.. They are permanent residents of chickpea fields. Of these, only the *D.baccarum* was found in a high density of 2-3 pcs./m<sup>2</sup>. The species of the genus *Lygus* were observed throughout the growing season, but in insignificant numbers, 1 pc./m<sup>2</sup>, which can be connected to the adjacent alfalfa crops, in which they have a constant presence. According to Glogoza et al., (2004), species of the genus *Lygus* are potential enemies of chickpea.

Among the cicadas *Hyalesthes obsoletus* Sig. was found. In the first year, the density was 3 pieces/m<sup>2</sup>, and in the second - 1 piece/m<sup>2</sup>. The presence and in a chickpea crop we explain it with the wide I food specialization. It is characteristic of cicadas that they are enemies of a number of cultivated plants and carry phytoplasmas.

Damage from the chickpea leafminer fly (*Liriomyza cicerina* Rond.) was detected on chickpeas as the leaves formed. Their larvae feed on the parenchyma of the leaves and cause the formation of mines. The density during the survey period ranged from 5 pcs/m<sup>2</sup> in 2020 to 2 pcs/m<sup>2</sup> in 2021.

Aphids were found during the budding - flowering phases: *Aphis fabae* Scop. and alfalfa aphid *Aphis craccivora* Koch. They were found in mixed populations. *A.craccivora* emerged as the dominant species. Aphids formed colonies on the leaves and vegetative tops of the plants. Affected parts faded, deformed and stunted.

In the phenophase of the beginning of milk maturity, damage by the caterpillars of the cotton bollworm (*Helicoverpa armigera* Hübner) was observed. They burrow into the beans and fill them with limbs and silk threads. Comparing order Lepidoptera insects with the studies of other authors Lateef (1985), Singh and Yadav (1999) it is evident that regardless of the area where chickpea is grown, it is a particularly preferred host of the cotton bollworm. It refers to the economically important pest of the generative organs of the chickpea. This species showed relatively constant density in both years of the study. The number of units moved as follows: 4 units/m<sup>2</sup> in 2020. and 2 pcs./m<sup>2</sup> in 2021.

During the observations during the growing season, single caterpillars of (*Loxostege sticticalis* L.) were found. We associate the presence of the species in the chickpea field with its wide food specialization.

As a polyvolt type with perennial type population dynamics, the *H.armigera* has significant fluctuations in numbers and multiplication. They are highly dependent on environmental factors and food hosts. The first cotton bollworm eggs laid in 2020 were found on June 29, and the first caterpillar damage was found on July 5 (Table 2). The average day and night temperatures for the period reached 22°C, and the maximum 38°C. As a result of the created conditions, the enemy multiplied massively and was found in high density.

Table 2. Indicators for the cotton bollworm in 2020-2021

| Indicators        | year    |        |
|-------------------|---------|--------|
|                   | 2020    | 2021   |
| First eggs        | 29 June | 1 July |
| First damage      | 5 July  | 9 July |
| Damaged beans (%) | 14,66   | 7,33   |

In 2021, environmental conditions, namely frequent rainfall and lower mean day-night temperatures, inhibited cotton nightshade development. The first eggs in 2021 were found on July 1, 2 days later than the previous year. Average day-night temperatures in June reached 19°C, and maximum 32°C. The first bites caused by the caterpillars of the second generation were found in early July (July 9).

The differences in the development and numbers of the cotton nightshade during the two years of study are due to environmental factors, mainly temperature. It is higher in 2020, therefore the cotton nightshade has multiplied massively, it was found in high density and hence the damage is greater.

Table 3 shows that in 2020 the percentage of damaged beans was higher by 14.66% compared to 2021, respectively – 7.33%. We explain this by the higher density of the enemy and the extended spawning period. Due to the high temperatures, the chickpea growing season was extended, which in turn favored the development of the caterpillars.

Table 3. Chickpea beans damaged by cotton bollworm caterpillars in 2020-2021

| Parcels  | Total number of beans | 2020          |               | 2021          |               |
|----------|-----------------------|---------------|---------------|---------------|---------------|
|          |                       | healthy beans | damaged beans | healthy beans | damaged beans |
| I        | 100                   | 87            | 13            | 90            | 10            |
| II       | 100                   | 88            | 12            | 95            | 5             |
| III      | 100                   | 81            | 19            | 93            | 7             |
| average% | 100                   | 85,33         | 14,66         | 92,66         | 7,33          |

The first flies in chickpeas in 2020 were detected at the beginning of May - May 4. During this period, the mid-night air temperatures at the end of March and the beginning of April are higher, which is why the start of the flight of the fly began earlier (Fig. 1).

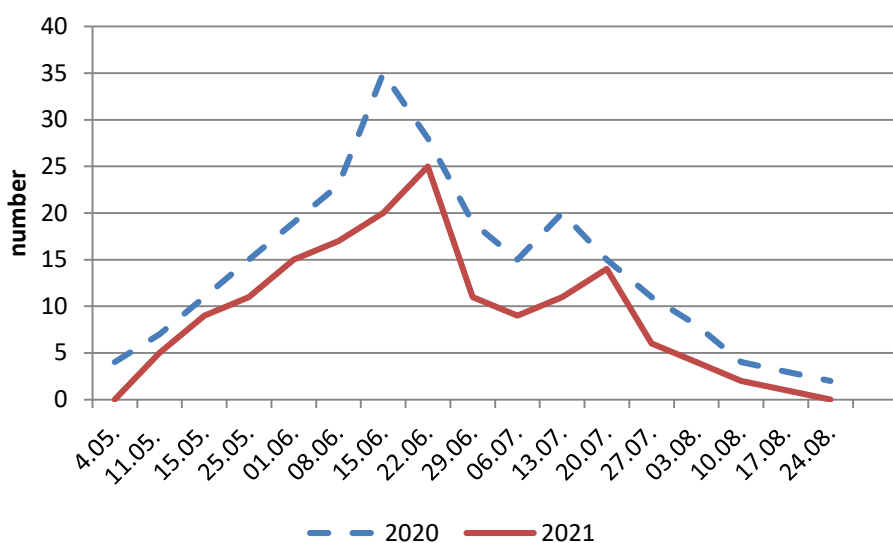


Fig. 1 Population dynamics of the chickpea leafminer fly in 2020-2021 in the area of the city of Rouen

Favorable environmental conditions favored the multiplication of the species. A peak was recorded in the second ten days of June, after which the number of flies began to decrease. In 2021, an enemy appeared in the chickpea fields later than the previous year - May 11. Air temperatures at the end of March and in April of 2021 are lower than in 2020, and therefore flies start later. As a result of the environmental conditions, the species multiplied massively and peaked on the third ten days of June. After this period, the density decreased. In the second ten days of July, in both years, the density of the fly increased again, and at the end of August, single species were recorded.

Table 4. Chickpea leaves damaged by *Liriomyza cicerina* larvae in 2020-2021

| Parcels  | Total number leaves | 2020           |    | 2021           |    |
|----------|---------------------|----------------|----|----------------|----|
|          |                     | Damaged leaves |    | Damaged leaves |    |
|          |                     | number         | %  | number         | %  |
| I        | 100                 | 25             | 25 | 20             | 20 |
| II       | 100                 | 20             | 20 | 15             | 15 |
| III      | 100                 | 27             | 27 | 10             | 10 |
| verage % | 100                 | 24             | 24 | 15             | 15 |

At the beginning of chickpea ripening, before the leaves start to turn yellow, the percentage of the attacked leaves was recorded. Table 4 shows that in 2020, 24% of damaged leaves were recorded, and in 2021, respectively, 15%. The conditions in 2020 had a favorable effect on the development of the species, as a result of which the density increased and the percentage of damaged plants increased. Economically the most significant are the damages caused by the larvae of the first and second generation flies.

## CONCLUSIONS

As a result of the conducted research, the following conclusions can be drawn:

- 21 species belonging to 10 families and 5 orders were found in chickpea crops. Among them for the region of the city of Rouen, of economic importance are: *Liriomyza cicerina* Rond. and *Helicoverpa armigera* Hub.

- In 2020, the appearance of cotton nightshade in chickpea fields is earlier than in 2021. Due to the higher temperatures in 2020, the growing season of chickpea was extended, resulting in massive multiplication of the species and damage to the beans reached 14.66%, and in 2021 respectively 7.33%.

- The first leaf-mining flies in the area of Rouen were found at the beginning of May. In 2020, 24% damaged leaves were reported, and in 2021 – 15%. The most significant damage is caused by the larvae of the first and second generation flies.

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## PRODUCTION OF BIODIESEL FROM WASTE VEGETABLE OILS

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### ABSTRACT

In Turkey, 1,500,000 tons of vegetable oil is used annually for food purposes. Approximately 350,000 tons of waste oil is produced from these oils. Since these wastes are recycled and used in the production of vegetable oil and/or mixed with animal feed, they pose a danger to the environment and public health and adversely affect the food chain. Biodiesel production for the recovery of used vegetable oil is very important for this waste assessment and is more environmentally friendly than petroleum diesel, which is an alternative to the collected product. Today, only 9% of our country's oil needs are met from domestic sources. This situation forces our country to seek alternative energy sources. For this reason, biodiesel is becoming more and more important for the present and future of our country, which was dependent on oil in the past, oil and natural gas today, and natural gas in the future. In addition, air pollution in some big cities has made the use of fuels with biodiesel additives mandatory.

**Keywords:** biodiesel, waste vegetable oil, energy source, natural gas

### INTRODUCTION

Used vegetable and animal oils are an important raw material. Used vegetable and animal oils are usually black in color, have a strong odor and are free of excessive solids. It is known that one liter of used vegetable oil pollutes 1 million liters of water. Vegetable and animal oils have very high caloric value and chemical oxygen demand (COD). When these waste oils are thrown into the water or sewer, they damage the aquatic ecosystems by covering the surface of the water, prevent the passage of oxygen from the air to the water, decompose in the water over time and accelerate the depletion of oxygen in the water. Waste water sticks to the pipelines, causing frequent narrowing and clogging of the pipe section. Waste vegetable and animal oils constitute 25% of wastewater pollution. Vegetable oils used in seas, streams and lakes harm birds, fish and other living species (Ozturk, 2004). The amounts of waste vegetable and animal oil produced in some countries are shown in Table 1.

Vegetable and animal oils (including waste oils) can be converted into biodiesel by simple reactions. First, the solids in vegetable and animal oils must be filtered out. It is then transferred to a storage tank, left to stand for 24 hours, the colloids precipitate, then any water that may be present in the oil is removed with gentle stirring for 12 hours. The mixing process is carried out at 50°C and 1 atm pressure. Then, sodium hydroxide is added to the oil, which has been dehydrated and solid, and glycerine and fatty acid are obtained from the oil. The resulting fatty

acid is reacted with methyl alcohol to form the methyl ester of the fatty acid. As a result of the reaction, biodiesel is obtained (Ozdemir and Mutlubas, 2016)

Table 1. Amount of waste vegetable and animal oil produced in some countries

| Country       | Amount (million tone/year) |
|---------------|----------------------------|
| China         | 4.5                        |
| Europa        | 0.7-1.0                    |
| United States | 10.0                       |
| Japan         | 0.45-0.57                  |
| Malesia       | 0.5                        |
| Canada        | 0.12                       |
| Taiwan        | 0.1                        |
| Turkey        | 0.3                        |

### Structure of Fats

Fats (triglycerides) are esters of fatty acids with the trivalent alcohol glycerin ( $C_3H_5(OH)_3$ , 1,2,3 trihydroxypropane) (Tahir 1982). If the triglyceride consists of fatty acids of the same type, it is called simple triglyceride, and if it consists of different types of fatty acids, it is called mixed triglyceride. Although oils are mostly composed of triglycerides, they contain a small amount of monoglyceride and diglyceride (Artukoglu 2006).

The physical and chemical structure of oils is largely determined by the type and amount of fatty acids they contain. Fatty acids are divided into three according to their bond shape:

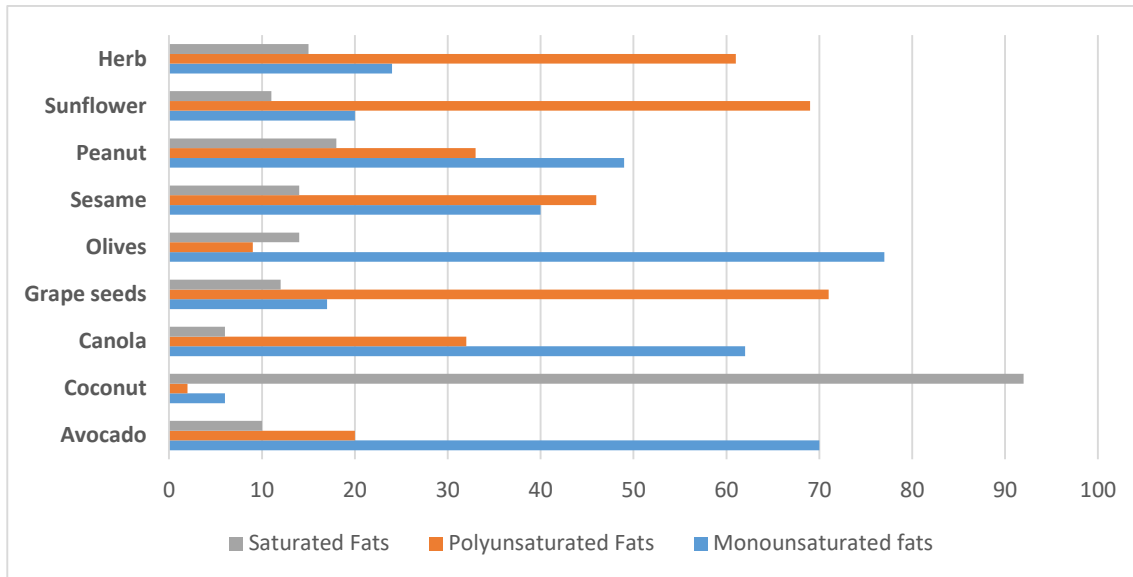
- Saturated fatty acids
- Monounsaturated fatty acids
- Polyunsaturated fatty acids

The structural formulas of saturated fatty acids have no double bonds, only single bonds, their general formula is  $R-COOH$ , and R is a hydrocarbon chain, the melting point and boiling point of saturated fatty acids increase molecularly. It is also known that saturated fatty acid is less sensitive to chemical reactions.

Unsaturated fatty acids are fatty acids that contain one or more double bonds in their structure. When fatty acids contain only one double bond, they are called monounsaturated fatty acids, and when they contain more than one double bond, they are called polyunsaturated fatty acids (Artukoglu, 2006).

Figure 1 shows the distribution of monounsaturated fats, polyunsaturated fats and saturated fats in the products.





**Figure 1.** Distribution of monounsaturated fats, polyunsaturated fats and saturated fats in products

Vegetable waste oils; Infrastructure systems are faced with serious problems if it turns into waste after being used and is thrown into the sewer, and the consequences can go up to the clogging of the pipelines in the future. It is a proven result that these wastes entering the treatment plant through the channels have a negative effect on the treatment efficiency of the treatment plant (Picture 1)

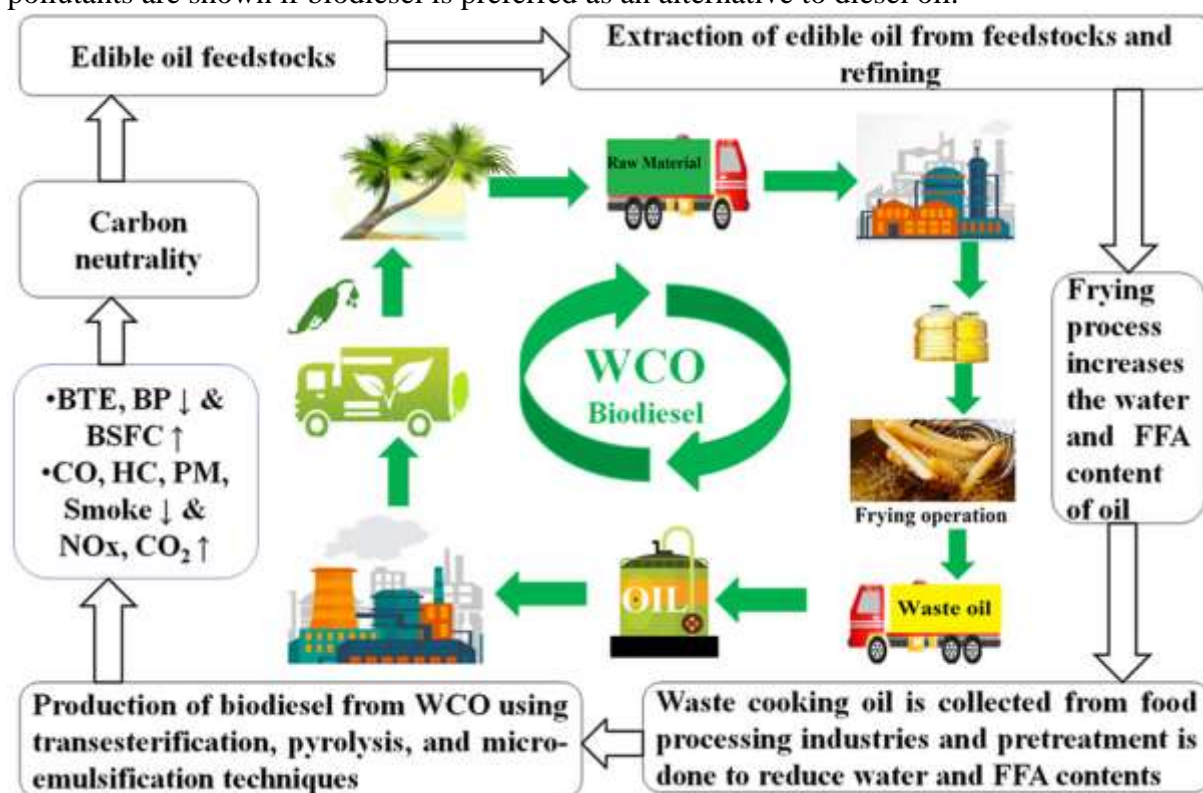


**Picture 1.** Destruction of oils in pipes

The evaluation of waste vegetable oils has many main goals and these goals contribute to the Turkish economy. These; by collecting used vegetable oils and turning them into a high value added product, providing great benefits to human health and the environment, opening a new chain with high added value for agricultural products, contributing to the improvement of the socio-economic situation of rural areas and reducing migration, reducing Turkey's dependence on foreign oil. has been determined.

### Conversion of Waste Vegetable Oils to Fuel

Biodiesel is an alternative energy source used as an alternative to diesel. Biodiesel is a less toxic biofuel than diesel. Biodiesel can be used directly in vehicles with diesel engines without modification. The higher the quality of vegetable and animal oils, the better the quality of the obtained biodiesel. Biodiesel; It is a renewable, locally produced fuel used in diesel engines and obtained from vegetable oils such as soybean oil, canola oil, and waste animal and vegetable oils. Biodiesel, a biodegradable biofuel, lubricates the engine and helps keep the fuel system clean. It is safer than fossil diesel fuel due to its high flash point. If a certain amount of biodiesel is used instead of diesel fuel, a significant reduction in pollutants such as HC, CO and PM will be achieved. There is a slight increase in the amount of NOx. In Figure 2, changes in some pollutants are shown if biodiesel is preferred as an alternative to diesel oil.



**Figure 2.** biodiesel production from waste cooking oil and its use as fuel in compression ignition engines: 3rd generation cleaner feedstock (Singh et al., 2021).

Due to the direct use of vegetable oils in diesel engines as fuel, some engine problems such as injector clogging, deposits and carbon soot coating are observed as a result of long-term use. The main causes of these engine problems are; vegetable oils have high viscosity, poor cold flow properties and low volatility.

The main factor that negatively affects the direct use of vegetable oils as diesel fuel is their high viscosity. This value is about 10 times that of diesel fuel. The injection system of modern diesel engines is very sensitive to changes in viscosity. High viscosity disrupts the fuel molecular process in the combustion chamber and prevents complete combustion with increased droplet size. Incomplete combustion causes accumulation in the combustion chamber, coking and clogging of the injectors, and contamination of the lubricating oil, and thickening and thickening of the lubricating oil are observed (Balo et al., 2006).

Another problem with using vegetable oils as fuel is that they contain unsaturated bonds. The mixing of unsaturated structures into the lubricating oil and polymerization in this environment causes an increase in viscosity, which can destroy the engine. In addition, the tendency of vegetable oils to solidify at low temperatures causes problems in their use as fuel. In order for vegetable oils to be used as an alternative fuel in diesel engines, their high viscosity and volatility must inevitably be improved. For this, four chemical methods can be applied: pyrolysis (thermal cracking), microemulsion formation, dilution and ester exchange (metabolism) (Knothe et al. 2005).

Pyrolysis is the process of breaking chemical bonds to form smaller molecules. Pyrolysis can be applied in two ways. One is the thermal cracking of the vegetable oil in a closed container, and the other is the application of the thermal cracking effect with the standard ASTM distillation method. (Sanli 2005).

Another method of reducing the high viscosity of vegetable oils is to form microemulsions with short-chain alcohol solvents such as methanol and ethanol. With this method, it is possible to produce diesel fuel as a completely independent alternative to petroleum. Microemulsions reduce injector clogging because they contain alcohol, but they cool the combustion chamber due to the high latent heat of alcohol evaporation (Ma and Hana 1999, Srivastava and Prasad 1999).

The dilution and extraction process is the process of diluting vegetable oils and mixing them in certain proportions with substances such as diesel fuel or solvents or ethanol. As a result of diluting the sunflower oil with diesel fuel at a ratio of 1:3 by volume, the viscosity of the mixture was found to be 4.88 mm<sup>2</sup>/s at 40°C. However, it was concluded that this mixture is not suitable for use in severe injector clogging and direct injection diesel engines. (Sanli 2005).

### **Environmental effects of Biodiesel**

One of the most important environmental advantages of biodiesel is that it is obtained from renewable raw materials and has sustainable energy potential. Since it can be biodegraded quickly and easily in nature, it does not accumulate and does not cause toxic effects. Studies have shown that almost all of biodiesel and 40% of diesel can be degraded in water in about a month. Since it is produced from vegetable and animal oils, the amount of waste produced can be reduced, therefore, biodiesel production can be considered as an environmentally friendly process in which energy is recovered from waste. Biodiesel emissions are chemically different from diesel in typically lower CO, CO<sub>2</sub>, SO<sub>x</sub>, polyaromatics and particulate emissions. According to the results of the research report published by the EU, while 1 liter of diesel consumption generates 3.2 kg of CO<sub>2</sub> emission, this amount decreases to 0.7 kg/L when biodiesel is consumed.

CO<sub>2</sub>, which constitutes a large part of greenhouse gases, causes global warming, which is one of the most important environmental problems in the world. CO, SO<sub>x</sub>, NO<sub>x</sub> emission values formed as a result of combustion with CO<sub>2</sub> increase the greenhouse effect and harm human health. Since biodiesel is produced from agricultural products, it accelerates the carbon cycle by converting CO<sub>2</sub> with photosynthesis in the biological carbon cycle and thus does not increase the greenhouse effect. It has also been shown that less CO, SO<sub>x</sub>, particulate and unburned hydrocarbon (HC) emissions are emitted.

NO<sub>x</sub> emissions from biodiesel are higher than diesel fuel. Emissions vary depending on the engine's compatibility with biodiesel fuel. It has been tested that NO<sub>x</sub> emissions will increase up to 13%. However, biodiesel does not contain sulfur. Therefore, NO<sub>x</sub> control technologies can be applied to systems using biodiesel fuel. Conventional diesel fuel contains sulfur and is

not suitable for NO<sub>x</sub> control technologies. The negative effects on the ozone layer in the use of biodiesel are less than half that of diesel fuel. The sulfur compounds that cause acid rain are almost absent in biodiesel fuels. The level of CO (toxic gas) formed as a result of the combustion of biodiesel fuel is 50% less than the level of CO formed due to the combustion of diesel fuel.

### **Examples around the World and Turkey**

There is no legal requirement regarding the use of biodiesel in Turkey for biodiesel blended diesel fuel applications. In applications in Turkey, the aim is to blend biodiesel at a maximum rate of 7% in accordance with the TSE EN 589 standard. As an alternative, biodiesel is produced from waste crude and vegetable oils in accordance with TS EN 14 214 A+1 in the production facility where the biodiesel investment will be made. National marker biodiesel is transported to the main warehouse of the fuel distribution company by land or sea. In the production process of biodiesel, 99.8% pure glycerine, which is used in the pharmaceutical and cosmetic industry, is also produced in addition to biodiesel.

Many countries around the world, especially developed countries, are trying to increase the share of renewable energy resources due to the energy policies they implement. For this reason, incentive and support programs are defined by law. Biodiesel is legally tax-exempt in Austria, France, Germany, Italy, Ireland, Norway, Sweden, Poland, Slovakia and the Czech Republic.

### **RESULTS**

In our country, vegetable and animal oils are disposed of indiscriminately, polluting both domestic and underground water resources. Waste oils spilled into the sewer increase the treatment cost of the wastewater treatment plant and damage the wastewater treatment plant. Used oil is a valuable substance. It can be used for various purposes after being collected. Therefore, it is possible to minimize the damage to the environment. In big cities such as Istanbul, primarily used vegetable and animal oils should be collected separately from hotels, motels, restaurants, restaurants and ready-made food centers. These wastes should be stored in separate clean containers. This waste should be entrusted to approved biodiesel production companies. They must donate the waste oil they collect to companies producing biodiesel. Approximately 150,000,000 kg. 150.000.000 kg per year with the recovery of used oils formed around the biodiesel and 15,000,000 kg. It contributes to the economy by producing glycerin. With the recovery of vegetable and animal oils, domestic wastewater is polluted 25% less.

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## CURRENT APPROACHES TO BIOREMEDIATION OF LANDFILL LEACHATE

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### ABSTRACT

The main source of leachate is water formed as a result of organic degradation. Dissolved organic carbons, which can be formed wherever anaerobic decomposition takes place, also consist of organic wastes in landfills. Since leachate affects the aquatic ecosystem and human health, it must be kept in the sanitary landfill or treated before it is released into the aquatic ecosystem. The methods developed for the treatment of leachate are physical, chemical, biological and advanced treatment methods, and it is difficult to obtain high treatment efficiency and wastewater quality by using any of these methods alone. Chemical methods used in the treatment of leachate are coagulation-flocculation, chemical precipitation and chemical-electrochemical oxidation. Biological methods are a combination of aerobic, anaerobic and anoxic processes. Physico-chemical methods are also often used in conjunction with biological methods to remove non-biodegradable substances from leachate. The aim of this review is to present the current methods used in leachate remediation

**Keywords :** Leachate, organic degradation, landfill, treatment efficiency

### INTRODUCTION

The leachate, which is formed by the storage of solid wastes in heaps and the leakage of rain water falling on these areas through the waste piles, is waste water with extremely diverse pollution load, both quantitatively and qualitatively. Since it can contain organic, inorganic substances and heavy metal species in high concentrations, it has a more concentrated pollution load than many domestic and industrial wastewater. Since the leachate formed contains pollutants and nitrates arising from the waste composition, it reaches underground and surface waters through the soil and carries a high amount of pollution load to the receiving environment it reaches. For this reason, the amount of solid waste leachate in a region may adversely affect the quality of underground and surface waters in that area. The transport of these dissolved substances in the leachate is an important hazard for groundwater and surface waters (ShuJun et al., 2008).

Since garbage leachate affects the aquatic ecosystem, environment and public health, it must be treated before being discharged into receiving environments. Various methods have been developed for the treatment of leachate. The methods to realize this treatment are physical, chemical, biological and advanced treatment methods. Due to the complex structure of leachate,

these methods, which have been developed, do not allow an effective treatment efficiency to be achieved by using them alone. Therefore, a combination of biological, physical-chemical and chemical processes is generally recommended for the treatment of leachate (Cossu et al., 2001).

### **Leachate**

Many of the organic matter in solid waste can be biodegraded and broken down into simpler compounds by microorganisms (Tokmakkaya, 1998). Solid waste piles, which are exposed to water above their water holding capacity by the effect of rain water, cannot accommodate this excess water. This excess water released from the waste is defined as leachate. Leachate waters are high-strength wastewater that shows extreme variability both quantitatively and qualitatively (Gülşen, 2009). For this reason, leachate is a potential source of pollutants for surface and groundwater, and is also very harmful to human health. It has negative effects such as kidney diseases, digestive diseases, carcinogenic effects (Nordin, 2006).

### **Leachate Formation**

In case of deterioration of biologically degradable organics in solid waste content as a result of microbial activities, the waters it creates contribute to the waste moisture for leachate formation. The water formation in question consists of both aerobic and anaerobic reactions (Hui, 2005). These reactions take place in the body of the landfill in solid waste landfills. The end products of these complex reactions are carried along with landfill gas and leachate. Leachate containing many and high concentrations of organic compounds is also highly variable in terms of quality. Organic pollutants measured as heavy metals, ammonia, COD and BOD indicate that leachate is toxic (Wichitsathian, 2004).

### **Biological Processes in Leachate Formation**

Biological processes in leachate formation generally occur in two main stages as aerobic and anaerobic.

Stage I - Aerobic: This first stage is the process that covers the first few days during the filling of the waste brought to the landfill to the storage cell. During this time, the large molecular and complex fragments are broken down into their essential components; During these processes, while the temperature increases, the pH value decreases (Arıkan et al., 2003).

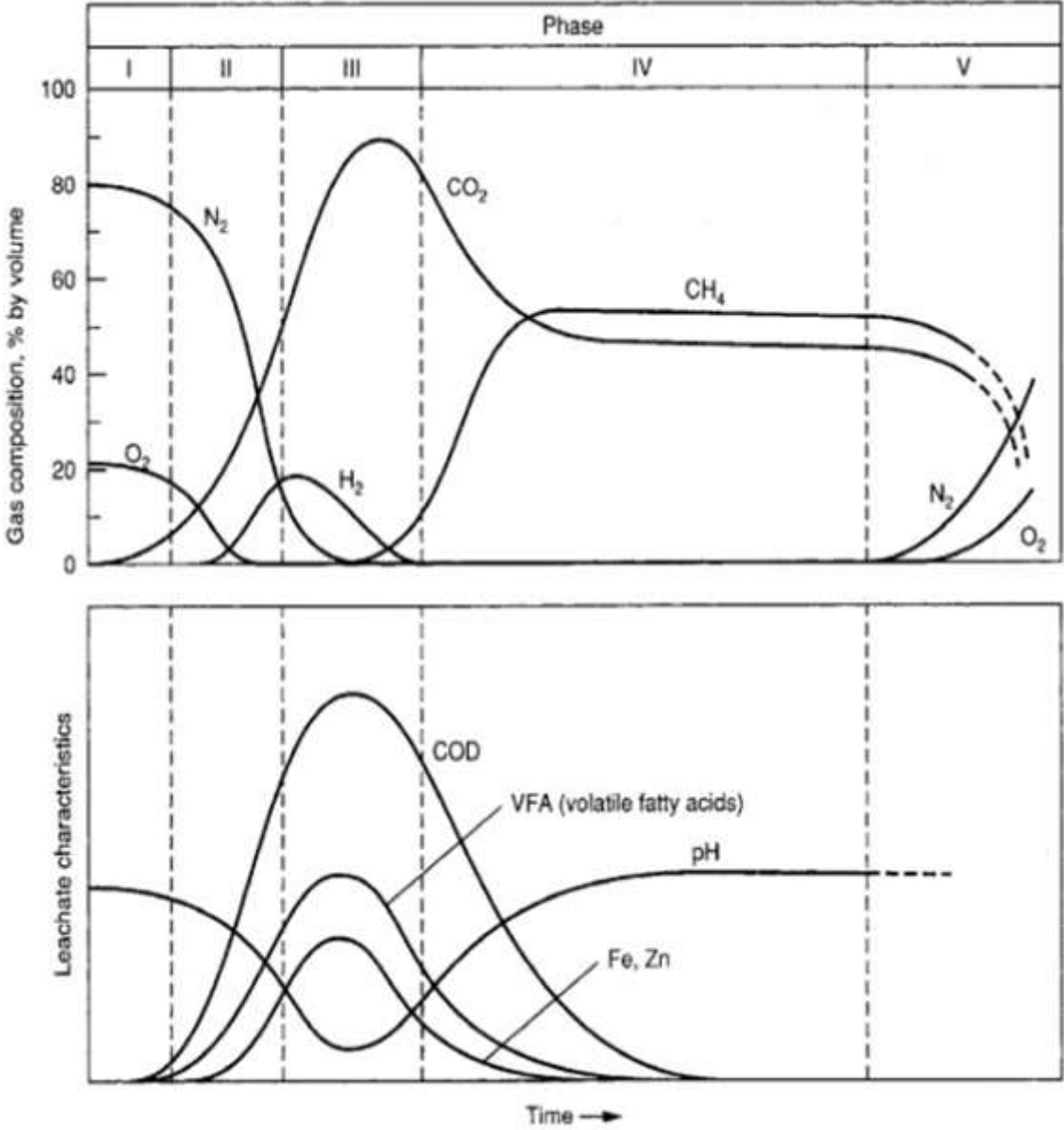
II. Stage - Anaerobic/Acid Absorption Cycle: In this stage, volatile organic acids decrease as a result of anaerobic microorganism activities. In addition to the decrease in pH and redox potential, inorganic ion concentrations also decrease at this stage. It precipitates heavy metals (Fe, Mn etc.) formed as a result of fermentation of sulfites and acids. In this phase, which is longer than the first phase, the aerobic phase, an increase in BOD, BOD/COD ratio and ammonia concentration is observed (Christensen and Kjeldsen, 1989).

III. Stage - Anaerobic / Intermediate anaerobiosis (Initial Methane Production Phase): This step, which lasts relatively long compared to the other stages that take place, is known for the much slower growth of methanogenic bacteria. Slow growth of bacteria increases the production of methane gas, causes a decrease in the volatile acid density and raises the pH value. The heavy metal concentration begins to decrease slowly. Although the growth of methanogen bacteria slows down, ammonia production continues to increase (Arıkan et al., 2003)

IV. Stage-Anaerobic/Methanogen: In this stage where methanogen bacteria are dominant, methane continues to be produced despite the completion of the biological decomposition of the main organic materials. The pH value reaches values close to neutral. Low heavy metal concentration and BOD/COD ratio are observed (Arıkan et al., 2003).

Stage V – Aerobic/ Maturity Period: Maturity stage is observed in aged landfills. This stage is seen in old landfills. Due to the decrease in the rate of methane formation, the chemical components of the air increase under the influence of the new substances mixed. Mobility of metal ions is of particular importance for the reactions at this ongoing stage (Martenson et al., 1999). After the gas diffusion, it is observed that the organic matter remaining as a result of the degradation is converted into CO<sub>2</sub> and the pH value decreases. This event, which causes an increase in the oxidation potential, triggers the mixing of metals and toxic substances into the leachate. On the other hand, in laboratory-scale studies, it has been determined that leachate in the old storage areas where aeration is performed has a low pH value and has a heavy metal concentration of approximately twice its former value (Arıkan et al., 2003). In Figure 1, the process in question is given below on the graph.





**Figure 1.** Sequence of Leachate Stabilization4 fazı (Tchobanoglous et al., 1993)

## Leachate Treatment Methods

Leachate containing high organic matter (heavy metal, sulphate, chloride, etc.) and toxic substances contaminate surface and groundwater by being carried from solid wastes (Paredes, 2003). Leachate treatment depends on factors such as leachate input to the process to be used for treatment, system requirements and discharge limits, leachate quality and quantity. Leachate water can be treated by biological, physical and chemical methods or by combinations thereof (Table 1). In terms of leachate characteristics, it is not sufficient to treat it using a single method. Therefore, it is of great importance to perform an effective treatment and to determine the cost-effective method (Saetang, 2009).

It is possible to treat the leachate at a certain rate by returning it to the landfill. The operating principle of this application is that the leachate returned to the landfill uses the landfill as a closed reactor. In regions where sewage systems are available, leachate can be discharged into sewer systems, provided that the leachate discharge values specified in the Water Pollution Control Regulation are met (Dölgen, 1998).

**Table 1.** Physical processes used in leachate treatment (Tchobanoglous et al., 1993)

| Treatment process | Purpose   |                        |
|-------------------|---|------------------------|
| Biological        | Active sludge   | Organic carbon removal |
|                   | Sequential batch reactors                                   |                        |
|                   | Aeration lagoon/stabilization pool                          |                        |
|                   | Biofilm systems (trickle filter, rotating biological discs) |                        |
|                   | Airless lagoon and contact tanks                            |                        |
|                   | Airless (upstream sludge bed, filter or hybrid) reactors    |                        |
|                   | Nitrification/denitrification                               |                        |

## Biological Treatment

In the most general sense, biological treatment is the process of converting dissolved and undissolved compounds in the wastewater environment into final products as a result of the reactions occurring during microorganism activities under oxygen (aerobic) or oxygen-free (anaerobic) conditions. In this treatment process, aerobic and anaerobic lagoons, activated sludge process, anaerobic sludge digester, biodisks and anaerobic filters are used (Cossu et al., 1992).

While biological processes are effective for young leachate containing volatile fatty acids, they provide less removal for old leachate (stabilized).

## Activated Sludge Process

In the activated sludge process, aerobic lagoon system operation is also available. It is another process where microorganisms transform organic materials into harmless end products under aerobic conditions. The difference from ventilated lagoons is the sludge return of the system in the activated sludge process. Therefore, it can be designed by keeping the hydraulic retention time shorter. System efficiency varies according to temperature and organic matter loading amount. BOD<sub>5</sub>/COD > 0.4 in laboratory, pilot and full scale activated sludge applications; BOD<sub>5</sub> removal was 99% at F/M < 0.05 kgBOI<sub>5</sub>/kgMLSS.day conditions (Andreottola et al., 1989)

## **Anaerobic Lagoon, Digester and Filters**

Anaerobic treatment is the decomposition of organic polluting compounds in wastewater into short-chain organic acids (butyric acid, propionic, acetic acid, etc.) which are intermediates under anaerobic conditions by acid-reducing bacteria, and then into final products such as H<sub>2</sub>S, CH<sub>4</sub>, CO<sub>2</sub> and NH<sub>3</sub> by methane bacteria. Anaerobic treatment has several advantages. These;

- They require less energy
- Less sludge (biomass) formation is observed
- Less space requirement
- It is the ability to decompose resistant organic compounds that are difficult to decompose.

Anaerobic treatment systems commonly used in leachate treatment; anaerobic lagoons, digesters and anaerobic filters.

Anaerobic lagoons are mostly among the first units of the treatment plant. It is aimed to reduce the peaks of leachate quality to lower levels and to obtain a more homogeneous leachate content. In this way, it is aimed to reduce the peaks of leachate quality and to obtain a more homogeneous leachate characteristic.

Anaerobic digesters have the same working principle as lagoons. The difference between them; digesters are constantly mixed and closed systems. This mixing process is provided mechanically or by returning the formed biogas to the system (Dölgen, 1996).

## **RESULTS AND DISCUSSION**

Leachate waters formed in solid waste storage areas contain many pollutants as content. For this reason, various difficulties are still encountered in the treatment of these wastewaters today. In order to eliminate and reduce these difficulties in leachate characterization and treatment, it is necessary to control and well control the leachate formation. One of the most effective methods for this situation is to control solid waste, and to reduce the amount of waste by pre-treatment of this type of waste at its source. It is the establishment of leachate treatment plants that should be put into practice quickly in our country. Appropriate planning should be carried out considering the receiving environment discharge standards, the maximum volume of the receiving environment, the geological and topographic characteristics of the region where the treatment plant will be established, as well as the economic situation. If one of these parameters is ignored, the leachate that will be mixed with the surface and underground waters as a result of direct infiltration will make it difficult to access healthy and consumable water and cause us to face a situation that threatens the life of living things. The methods applied for leachate treatment are physical, chemical, biological and advanced treatment methods. It is not possible to apply any of these developed methods alone to obtain a successful treatment efficiency and effluent quality. For this reason, it is necessary to use physical, chemical and biological treatment methods together or in combination in leachate treatment.

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## **THE FACTORS THAT EFFECTS MICROORGANISMS IN THE RECOVERY OF RARE EARTH ELEMENTS**

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### **ABSTRACT**

Rare earth elements (REEs) are found in nature as compounds, especially as oxides. REEs are also referred to as rare earth metals “REM” or “rare earth oxides, REO.” Wastes that serve as potential sources of REEs can be classified into three main categories: industrial waste, mining waste, and electronic waste. Common industrial wastes used include mineral processing wastes (phosphogypsum, red mud) and coal processing wastes (fly ash). In the biological recovery of REEs, microorganisms are affected by species as well as by physical and chemical factors. Physical factors include aeration, temperature and pulp density, chemical factors include pH, redox potential and metal toxicity. All these factors can work separately or simultaneously to affect the entire REE biological recovery process. In this study, some basic physical and chemical factors affecting the microorganisms involved in the recovery of REEs.

**Keywords:** rare earth elements, NTE extraction, redox potential, metal toxicity, pulp density

### **INTRODUCTION**

Rare earth elements (REE) is a metal group consisting of the lanthanide series and its use in high-tech industries around the world is becoming more and more important day by day. When there is a need to expand the production of such elements, since the production chain of rare earth elements is associated with many environmental factors and the problems it brings, processes that do not harm the receiving environment are investigated to obtain them.

Many of the REEs' features are similar to each other and they are mostly located together geologically. REE metals are generally marketed as "oxide". REE reserves in the world are concentrated in 8 countries and its total reserves are around 140x10<sup>6</sup> tons. In terms of REE reserves, China is the leading country in the world with 55x10<sup>6</sup> tons. When the data on REE in the literature are researched, changes in the figures are striking. The main reason for this is that countries do not strategically share their real figures with other countries. Countries only share as much as they want. This leads researchers to think that the real REE reserve in the world is much higher than this.

### Usage areas of REEs

Because REEs are similar to each other, they are used in metal, alloy or machined forms (Figure 1).



Figure 1. Some REE and sources

REEs are offered to the market in the form of oxides, metals and various chemical compounds, and they are also preferred in the production of high quality metal alloys due to their sensitivity to high temperature ranges (Figure 2).

### Rare Earth – Key Applications



Figure 2. REE usage areas (Awais, 2016)

Materials containing REEs, if used as additives, are stable and resistant to high temperature and corrosion, and are lightweight materials. These features make REEs computers, hybrid vehicles, rechargeable batteries, mobile phones, flat television screens, laptops, wind turbines, medical imaging equipment, radar systems, catalytic converters, more corrosion resistant metal alloys, aircraft engines, medicine, ceramics, glass (Figure 3).

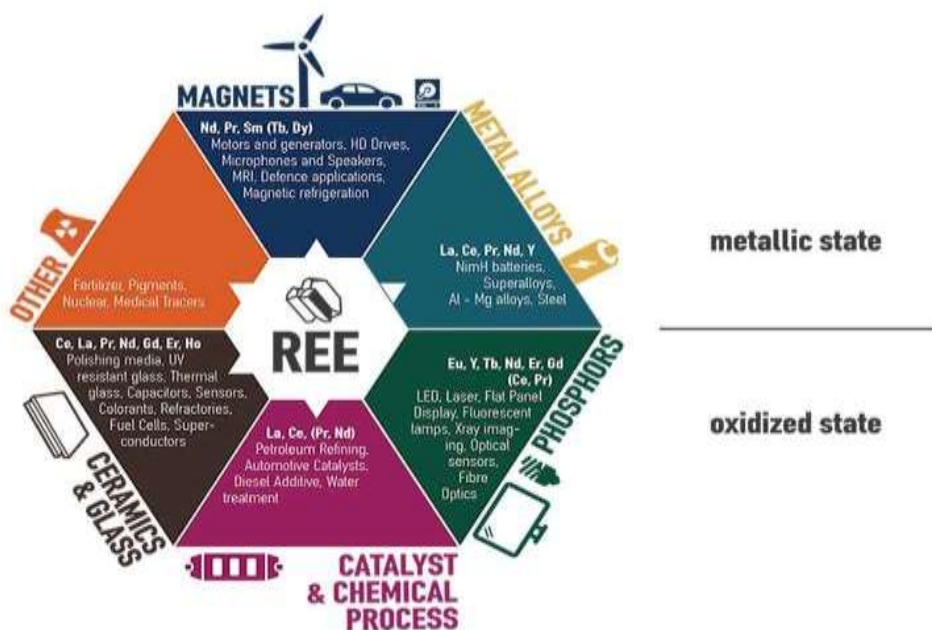


Figure 3. REE composition by end use (Coint and Dahlgren, 2019)

### Presence of REEs in waste

The reason why REEs are considered as strategic elements is that they are used in very different sectors in the production of advanced technological materials that are resistant to high temperature, abrasion, and corrosion. The 15 lanthanide groups also have similar chemical properties, and if we include scandium and yttrium in this group, REEs consist of 17 elements (Figure 4) (Jha et al., 2016). Most of the REE production comes from minerals such as bastnasite, monazite, and xenotime, and it is present in different concentrations in many minerals including oxidized, carbonate, silicate and phosphate compounds (Jordens et al., 2013). Flotation, magnetic and gravity enrichment methods are used for the recovery of REE from minerals, as well as hydrometallurgical methods are preferred (Krishnamurthy and Gupta, 2015). The elements cerium and lanthanum are mostly used in catalytic converters. Rare earth elements are also widely used in metallurgy due to their corrosion resistance at high temperatures and their anti-oxidation properties (Yildiz, 2016).

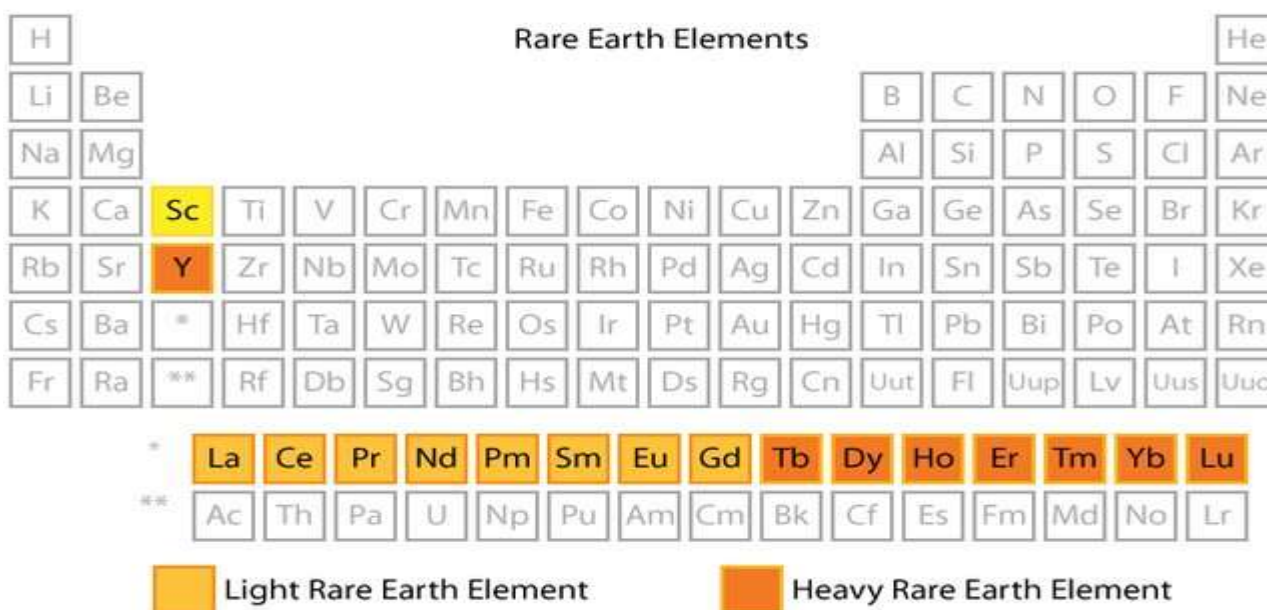


Figure 4. Rare earth elements in periodic table (Coint and Dahlgren, 2019)

## **Factors affecting microorganisms in the recovery of REEs**

### **Aeration**

O<sub>2</sub> works as organisms that are involved in the biological recovery of REEs and also receive terminal electrons for acidophilic and chemolithotrophic bacteria to maintain their metabolic activities. The aeration or oxygenation process is an important parameter for the growth and proliferation of microorganisms active in REE biorecovery (Rawlings, 2005). CO<sub>2</sub> and O<sub>2</sub>, which act as carbon source and electron acceptor for the autotrophic microorganism *A. ferrooxidans* and *A. thiooxidans*, respectively, should be provided with aeration during the bioleaching process until they reach saturation (Gu et al., 2017). The internal diffusion of O<sub>2</sub> results in thickening of the oxide layer (Zhang et al., 2019), and the literature indicates that active elements and their alloys can efficiently reduce the internal diffusion rate of O<sub>2</sub> (Fu et al., 2019). If we give an example of this; Appropriate addition of rare earth elements to the system can decompose oxygen atoms at metal-oxide interfaces and oxide scale grain boundaries, and as a result, diffusion is prevented by reacting with oxygen (Majumbar et al., 2013). It also improves the adhesion of lime, as the oxide scale can also optimize the microstructure (Naumenko et al., 2007). All these factors are influenced by the successful and effective operation of a bioleaching process and the selection of optimized aeration depending on the process configuration and the specific microorganism used in the process.

### **Pulp density**

Sludge density is an important parameter that affects the growth and proliferation of microorganisms, which determines the fate of the biological recovery of metals. High sludge density, high shear force affects biological growth as a parameter that limits O<sub>2</sub> and CO<sub>2</sub> flux and also increases toxic metal load (Wang et al., 2014). Dough density also affects pH as it has a high buffering capacity (Arshadi and Mousavi, 2015). It was previously determined in Jowkar et al., (2018) that the adaptation of *A. thiooxidans* with increasing pulp density from 2% to 4% (w/v) of LCD dust increases microbial tolerance to toxic metals. The inhibitory effect of high pulp density can be mitigated by adding it to pre-grown active microbial culture. The optimum pulp density for maximum REE biorecovery varies depending on the source of the minerals and microbial diversity.

### **Temperature**

The effect of REEs on the biorecovery process is also important because of the direct effect of temperature on microbial growth, kinetics, and microbial metabolic activity. It is known that microorganisms classified as psychrophilic, mesophilic or thermophilic depending on the temperature requirement exhibit optimum growth temperatures around -40°C, 20°C, 25-47°C or 41-68°C, respectively (Zhao et al., 2018). Mineral oxidative properties of various microbial groups also vary depending on optimum growth temperatures (Muravyov et al., 2015). The optimum growth temperature of *A. ferrooxidans*, which is widely used especially for biological leaching of REEs, is between 28–30 °C (Zhan et al., 2019). Other variants of the *Acidithiobacillus* genera, such as *Acidithiobacillus ferrivorans*, *Acidithiobacillus caldus*, have optimum growth temperatures of 5°C and 45°C, respectively (Ccorahua-Santo et al., 2017). The optimum growth temperature also increases the adhesion of the enhanced microbial cells to the mineral surface. The binding of *A. ferrooxidans* to the surface of pyrite minerals increases fourfold when the temperature is increased from 17 °C to 28 °C (Bellenberg et al., 2015). When *L. ferrooxidans* is developed at 25°C, it can biorecycle 100% of Dy and Pr from end-of-life magnets (Auerbach et al., 2019).

### **pH**

The mobility of trace elements in soil is driven by various mechanisms, including precipitation-dissolution, sorption-desorption, and reduction-oxidation (chemical or microbial) processes. However, both pH and redox potential have long been recognized as critical parameters



controlling the fate of pollutants in the environment. Meanwhile, some experimental devices have been developed to control the redox and pH of the soil suspension (Patrick et al., 1990; Chuan et al., 1996). Most previous research efforts have focused on heavy metals that make up a redox chemistry. The effect of redox potential and pH on As, Cr and Se speciation and mobility in contaminated soil has been well reported (Patrick et al., 1990). It was pointed out that the solubility of these metals was directly correlated with the changes in pH and redox potential, providing the dominant species change. It is stated that pH and redox potential directly affect the release of Pb, Cd and Zn, which is caused by the reduction and dissolution of Fe-Mn oxyhydroxides under low pH.

### **Redox potential**

The redoxolysis process is defined as the process mediated by the dissolution of REE from a mineral phase by a microorganism, and this process is related to the redox potential of the aqueous medium. Bacteria such as *A. ferrooxidans*, *A. thiooxidans* or *L. ferriphilum*, which are iron oxidizing bacteria, can oxidize Fe<sup>2</sup> to Fe<sup>3</sup> at a very high redox potential (Huynh et al., 2019). It has been reported that the bioleaching experiment using waste LED lamps as a mineral source in the oxidation of Fe<sup>2</sup> to Fe<sup>3</sup> by *A. ferrooxidans* occurred at >600 mV (Pourhossein and Mousavi, 2019). Similarly, *A. caldus* bacteria obtained 52% Sc, 52.6% Y and 59.5% La extractions from coal slag at 845-855 mV redox potential. A low redox potential was required for REE to dissolve through the acidolysis process mediated by sulfur-oxidizing bacteria (Masaki et al., 2018). Yahya and Johnson (2002) also found that sulfur oxidation of *Sulfobacillus* sp occurred efficiently at a redox potential of 100-150 mV. For the purpose of selecting a microbial strain based on mineral properties, it is necessary to have the desired redox potential for enhanced REE biological protection.

### **Metal toxicity**

According to Gwenzi et al. (2018), many previous studies have evaluated the toxicity of REEs from a single exposure, and therefore very few publications deal with combinations of REEs. In some studies, He et al. (2020) were able to predict the toxic effects of REE mixtures testing combinations of Y, La, Ce, Pr, Nd, Gd and/or Lu. Literature information on REE toxicity in fungi is not sufficient (Kang et al., 2019). Baschien and Hyde (2018) found that fungi are cosmopolitan and can be used to monitor water pollution as they biotransform REEs throughout the year. Fungi are concentrated in all ecosystems, including primarily aquatic environments (Grossart et al., 2019).

## **RESULTS**

The demand for REEs is increasing in industrialized countries. REEs are found in limited numbers and in natural ore bodies. REE needs to be biologically recovered from different wastes such as phosphogypsum, red mud, fly ash, mine residues, acid mine drainage and e-waste, and for this, bioprocess approaches using microorganisms need to be developed. The interaction of microorganisms between minerals and REEs in many chemical or biological processes such as redoxolysis, acidolysis, complexolysis, biosorption, bioaccumulation and bioprecipitation has been noted as important mechanisms for REE extraction from source minerals. The main types of microorganisms that are frequently used in the biorecovery of REEs are mentioned above. It has been determined that different physico-chemical operational parameters such as aeration, sludge density, temperature, pH, redox potential and metal toxicity significantly affect REE biorecovery. These methods, such as the optimization of a number of physical, chemical or biological parameters, the development of genetically modified microorganisms, their adaptation to highly toxic environments, and the use of suitable substrates, may shed light on future studies to significantly improve the sustainable biorecovery of REE from waste materials or ores.

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## DETECTION AND GENETIC INVESTIGATION OF POTATO LEAFROLL VIRUS IN KAZAKHSTAN

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### ABSTRACT

*Potato leafroll virus* (PLRV) causing leafroll disease is one of the most dangerous viral pathogens of potato. Therefore, the investigation of viral diseases plays important role in maintaining the quality and quantity of the yield. Previously, *potato virus X*, *potato virus M*, *potato virus Y* and *potato virus S* were detected in different regions of Kazakhstan. In the present work, the identification and genetic study of PLRV were performed for the first time in the South and North of Kazakhstan. Between 2021 and 2022, a total of 117 samples were collected from three potato growing areas followed by RT-PCR testing for PLRV presence. Sixty-one viral isolates were identified and analyzed. For detection and investigation of PLRV, sets of specific primers were developed for capsid protein (CP) gene of PLRV. The primers for virus detection were located in the gene regions highly conserved across the different strains and isolates of PLRV. Also, the genetic analysis of complete CP of PLRV was performed by sequencing coding region of gene. The primers for CP sequencing also included the sites for endonucleases *NdeI* and *XhoI* for further cloning and investigation of biological activities of capsid proteins. Phylogenetic analysis revealed that isolates from South Kazakhstan were closely related to the isolates from South Asia (especially Pakistan and India), whereas isolates from northern Kazakhstan formed the clade with isolates from Pakistan and Serbia.

### INTRODUCTION

Potato is one of the most important agricultural crops grown worldwide. In Kazakhstan, potato production in 2020 amounted to more than 4 million tons with a growing area of 193,806 hectares. (FAOSTAT, 2020) The main growing areas are located in the north and south of Kazakhstan. Export and import of potatoes in 2020 amounted to 347.1 thousand tons and 40.9 thousand tons, respectively. In 2021, the main volume of potatoes was imported to Kazakhstan from Pakistan and Iran, whereas the main countries for export included Uzbekistan, Turkmenistan, and Tajikistan. Seed potatoes are mainly imported to Kazakhstan from Europe. Potato is affected by more than 30 viruses. *Potato virus X*, *potato virus M*, *potato virus Y* and *potato virus S* are dangerous pathogens that could reduce yield by 30-80% (Khurana et al., 1988). *Potato leafroll virus* belongs to the family *Luteoviridae*, genus *Polerovirus* (D'Arcy et al., 2000) and leads to yield reductions of 90%. PLRV is transmitted by several species of aphids, including the green peach aphids *Myzus persicae* (Gildow et al., 2000). Plant infection usually occurs from infected tubers and symptoms appear first on the lower leaves in the form of upward rolling of the leaf margins. The infected plant is stunted and in most cases the color of plant leaves is slightly pale with purpling or reddening.

The genome of PLRV consists of positive sense single stranded RNA with 10 open reading frames (ORF) coding for proteins P0, P1, Rap1, P1-P2, P3a, P3, P4, P3-P5, P6, and P7. ORF0 encodes a 28 kDa suppressor of RNA interference, ORF3 – 23 kDa capsid protein, and ORF4 – 17 kDa movement protein. ORFs encoding capsid proteins or proteins important for life cycle of viruses are frequently used for detection and evolution analysis of pathogens. In this study, we have

developed specific primers for ORF3 to detect PLRV and evaluated the genetic diversity of local isolates in broad context.

## MATERIAL AND METHODS

Between 2021 and 2022, a total of 117 leaf samples were collected from potato growing areas in south (Almaty region) and north (Nur-Sultan, Pavlodar) Kazakhstan. The plant material was placed for storage at  $-80^{\circ}\text{C}$  until RNA isolation.

Total RNAs were extracted from the potato leaves using cetyltrimethylammonium bromide (CTAB) buffer and tissue homogenization in liquid nitrogen (Rahmani et al., 2020). The quality of total RNA samples was analyzed by agarose gel electrophoresis (2% w/v).

cDNA was synthesized in two steps. In the first step, 200 ng RNA, 0.5  $\mu\text{M}$  Oligo-dT, and 0.5  $\mu\text{M}$  random hexamer primers were mixed in a final volume of 15  $\mu\text{l}$  for subsequent denaturation at  $72^{\circ}\text{C}$  for 10 min followed by cooling on ice. In the second step, cDNA was synthesized using 5x RT reaction buffer, 0.5 mM dNTPs, and 200 U reverse transcriptase at  $45^{\circ}\text{C}$  for 1 h.

For detection and genetic investigation of PLRV, set of specific primers were developed for capsid protein (CP) gene of PLRV: forward – AAACATATGAGTACGGTCGTGGTT; reverse - TTTCTCGAGCTATTTGGGGTTTTGC. The PCR mix contained 0,2 mM of each primer, 12,5  $\mu\text{l}$  Platinum™ SuperFi II Green PCR Master Mix, and 2  $\mu\text{l}$  of cDNA as a template for amplification in a 25  $\mu\text{L}$  reaction volume. The PCR conditions were set to  $96^{\circ}\text{C}$  for 3 min as initial denaturation temperature followed by 30 cycles of  $96^{\circ}\text{C}$  for 20 s,  $50^{\circ}\text{C}$  for 20 s, and  $72^{\circ}\text{C}$  for 1 min, respectively, and a final elongation step at  $72^{\circ}\text{C}$  for 5 min. PCR products were analyzed by electrophoresis in 1.5% (w/v) agarose gel. Sequencing reaction was performed in 10  $\mu\text{l}$  of mix containing 2  $\mu\text{l}$  of the PCR product, 3.2 mM of either the forward or reverse primer for each isolate, 1  $\mu\text{l}$  BigDye™ Terminator Reaction Mix, and 1.5  $\mu\text{l}$  BigDye™ Terminator sequencing buffer. Cycling conditions were  $96^{\circ}\text{C}$  for 1 min followed by 25 cycles of 10 s at  $96^{\circ}\text{C}$ , 5 s at  $50^{\circ}\text{C}$ , and 4 min at  $60^{\circ}\text{C}$ . Capillary sequencing was carried out on the 3500 Genetic Analyzer (Applied Biosystems, California, USA) using the StdSeq50\_POP7 sequencing run module.

The obtained sequences were verified by the nucleotide BLAST search program (Altschul et al., 1990). Sequences were aligned using MAFFT software (Katoh et al., 2013), and neighbor-joining trees were constructed using MEGAXI software under the default settings.

## RESULTS AND DISCUSSION

In most cases, PLRV infection occurs directly through tubers. Therefore, it is important to prevent the planting of infected seed material in order to reduce the spread of the virus from other countries. The development of detection test-systems will help in epidemiological studies, post entry quarantine, disease monitoring, seed potato certification, and advanced virus resistant breeding programs.

In the result of PLRV detection by developed specific primers, 37% and 5% of the samples collected from South and North of Kazakhstan, respectively, were positive for virus (Fig.1). Previously, *potato virus X*, *potato virus M*, *potato virus Y*, and *potato virus S* were detected in different regions of Kazakhstan.

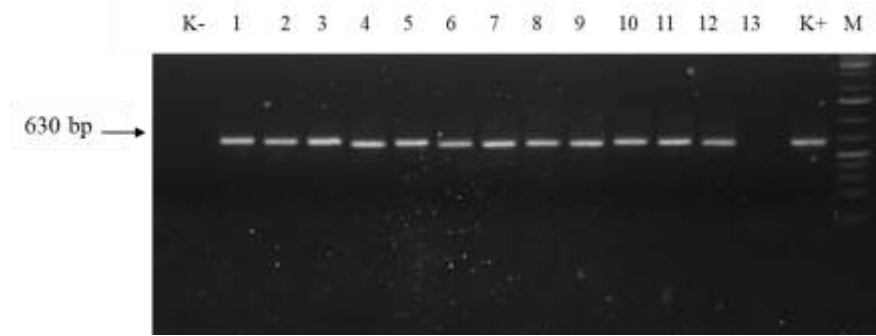


Fig. 1 Results of PLRV detection: lanes 1-6 are samples from South Kazakhstan; lanes 6-12 are samples from North Kazakhstan. K – negative control, K+ positive control, M – marker DNA (GeneRuler™ 1kb plus)

Kazakhstan isolates and isolates obtained from Genbank (NCBI) were subjected to phylogenetic analysis. Phylogenetic analysis demonstrated that most of the local PLRV isolates formed a group with the isolates of Asian origin (mainly Pakistan, China and India). One of North Kazakhstan isolates (KZ3-N) belongs to the group with isolates from South Kazakhstan (KZ44, KZ19, KZ33, KZ42), KZ4-N isolate formed the clade with isolates from Pakistan, Serbia and India (Fig.2).

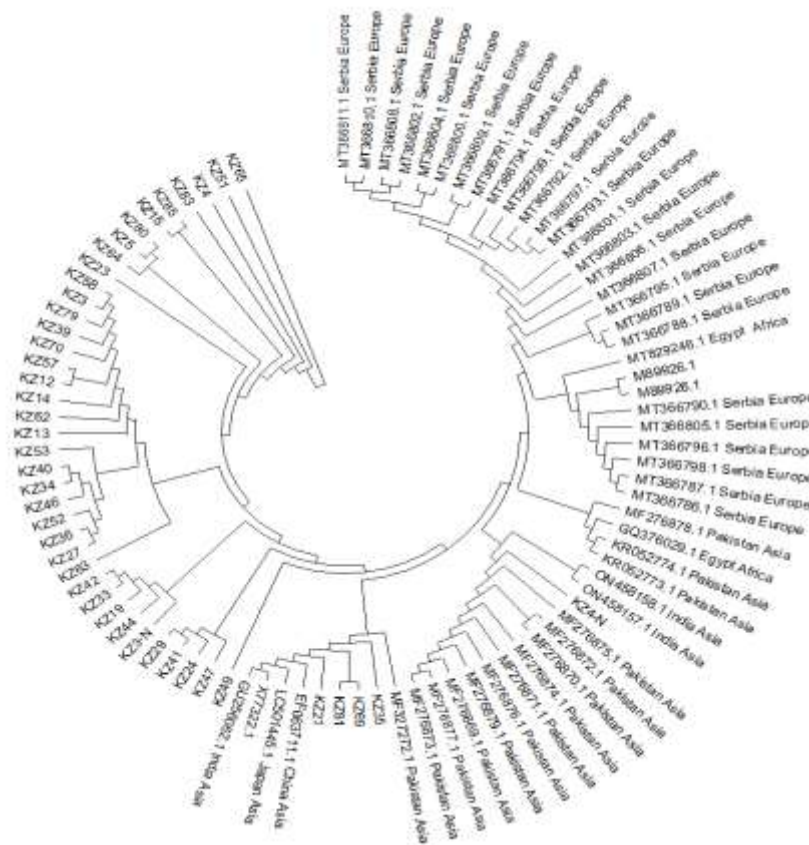


Fig. 2 Neighbor joining tree based on coat protein-coding sequences of PLRV of local isolates (Kazakhstan) and Genbank (NCBI) isolates.

As the results show, most of the South Kazakhstan isolates are grouped with the Asian isolates, differed by only 6 nucleotides from the Pakistani isolates. It can be assumed that potato tubers imported from Pakistan could have been used by local farms as an alternative to seed planting material. Isolates from Kazakhstan KZ35, KZ69, KZ81, KZ21 were grouped into one

cluster with isolates from China, Japan, and India and have a maximum overlap of 3 nucleotides. At the same time, the rest of the isolates from northern Kazakhstan are also in the group with Asian and European isolates (Serbia) differed by only 4 nucleotides. This may be due to the planting of seed potatoes obtained from abroad because local farmers are not ready to produce high quality seeds. According to the study Hossain et al., 2013 Pakistani isolates had high homology with European and Canadian isolates, the same data are presented for isolates from India (Jeevalatha et al. 2013). From this it can be assumed that the main focus of virus spread could be European seeds, which could lead to the global spread of the virus.

The investigation and detection of viral pathogens play important role in maintaining the quality and quantity of the yield. In the present work, the identification and genetic study of PLRV were performed for the first time in the South and North of Kazakhstan.

## CONCLUSION

PLRV is one of the dangerous pathogens affecting potatoes, it spreads at a high rate and could reduce yield by 90%. Therefore, special attention should be paid to the timely detection of PLRV in seed material. The identification and genetic study of PLRV were performed for the first time in the South and North of Kazakhstan. In conclusion, the highly specific primers and optimized PCR conditions described in this study could be used for research and commercial propose to certify seed potato. The results of phylogenetic analysis allow to clarify the picture of PLRV evolution and distribution in the world. It could be assumed, that local isolates have the origin from different infected sources since PLRV from Kazakhstan formed clades with Asian and Europe isolates.

## ACKNOWLEDGMENT

This work was funded by the Ministry of Agriculture of the Republic of Kazakhstan as a part of the targeted funding program BR10765038: “Development of methodology and implementation of scientifically based system of certification and inspection of seed potatoes and planting material of fruit crops in the Republic of Kazakhstan”.

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## EVALUATION OF RASPBERRY VIRUSES' SPREADING IN KAZAKHSTAN

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### ABSTRACT

Raspberry is a berry crop widely cultivated in many countries of the world. *Raspberry ringspot virus* (RpRSV), *Raspberry leaf mottle virus* (RLMV), *Raspberry leaf blotch virus* (RLBV) and *Raspberry bushy dwarf virus* (RBDV) are globally widespread viruses and could lead to crop yield losses by 20-50%. In Kazakhstan raspberry planting material is mainly imported from Russia and European countries, thus there is an urgent need in detection of viral pathogens to prevent spreading of new species, strains and isolates. In the present work, the sets of primers for multiplex RT-PCR detection of RpRSV, RLMV, RLBV and RBDV were developed. The plant material of cultivated raspberry including 187 samples was collected in Pomological Garden (Almaty) and in private farms. Additionally, 35 wild raspberry samples were obtained in mountains of the Trans-Ile Alatau. The specific primers for RpRSV, RLMV, RLBV and RBDV were targeted to the coat or nucleocapsid protein genes. In the result, seven samples of the cultivated raspberry were positive for RLMV and 58 samples for RBDV. Among the wild raspberry samples, 15 samples were infected by RBDV, and two samples were positive for RLMV and RLBV. RpRSV is a quarantine virus in Kazakhstan and has not been identified in the analyzed samples. In the present work it was revealed for the first time that raspberry viruses have been widely spread both in the fields and in wild forests in the country. The spreading of viral pathogens in raspberry fields probably occurs with an infected planting material or common plant hosts including weeds. Thus, the monitoring of the plant material and plants in the fields using fast and sensitive molecular methods is important task in managing the spreading of viral infections.

**Keywords:** raspberry viruses, RNA, RT-PCR

### INTRODUCTION

Raspberry is a berry crop widely cultivated in many countries of the world. *Raspberry ringspot virus* (RpRSV), *Raspberry leaf mottle virus* (RLMV), *Raspberry leaf blotch virus* (RLBV) and *Raspberry bushy dwarf virus* (RBDV) are globally widespread viruses and can lead to crop yield losses by 20-50%.

*Raspberry ringspot virus* belongs to the nepovirus group and is transmitted by plant nematodes. The virus induces abnormal shapes and colors of plant leaves, dwarfing of whole plant. In Kazakhstan RpRSV is known as quarantine virus. *Raspberry leaf mottle virus* is a member of *Closteroviridae* family causing raspberry mosaic disease and crumbly fruit disease in raspberry plants (Cieslinska, 2020). *Raspberry bushy dwarf virus* is known as a pollen-borne idaeovirus infecting raspberry and blackberry plants. It may cause leaf curling and necrosis. *Raspberry leaf blotch virus* causes raspberry leaf blotch disorder (RLBD) and induces the yellow rings and blotches on plant leaves. This virus can be transmitted by raspberry leaf and bud mites (*Phyllocoptes gracilis*) (Dong et al., 2016). RLBD symptoms have observed in many countries including Germany, France, Norway, Netherlands, Scotland, England and others (McGavin et al., 2012; Paunović and Jevremović, 2017; Bi et al., 2012; Mavrič et al., 2014).



In Kazakhstan, the information related to the spreading of raspberry viruses was absent until this work. The importance of detection of viral pathogens consists in the prevention of spreading of new species, strains and isolates since the raspberry planting material is mainly imported from Europe.

## MATERIALS AND METHODS

The plant material including 187 samples of cultivated raspberry was collected in Pomological Garden (Almaty) and in private farms. Additionally, 35 wild raspberry samples were obtained in mountains of the Trans-Ile Alatau. RNA from leaf tissue was isolated by CTAB method (Rahmani and Amraee, 2020). The quality of isolated RNA was confirmed by agarose gel electrophoresis (2% w/v). Reverse transcription was conducted by using the RevertAid Reverse Transcriptase (Thermo Scientific, USA). The mix of 200 ng RNA, 0.5  $\mu$ M Oligo-dT, and 0.5  $\mu$ M random hexamer primers in a final volume of 15  $\mu$ l was incubated for 10 min at 72 °C and then cooled on ice. Then 5x RT reaction buffer, 0.5 mM dNTPs, and 200 U reverse transcriptase were added followed by incubation for 1 h at 45 °C.

Four sets of specific primers for detection of raspberry viruses were developed (Table 1). The known nucleotide sequences of each virus were retrieved from NCBI. Then alignment has been carried out by using Muscle, ClustlW and MAFFT methods to reveal conservative sites for primer design (Edgar, 2015). Specificity of all promising primers was tested by using NCBI-Blast and Primer-BLAST, and the most suitable primer sets were selected for detection. The amplicon sizes for each virus is differ from each other by about 30 nucleotides for the visual detection on gel.

Table 1. Nucleotide sequences of specific primers for raspberry viruses

|      | Forward primer             | Reverse primer                |
|------|----------------------------|-------------------------------|
| RpRV | 5'-CAGAGTATGGGTGATTTCT-3'  | 5'-GAAACAGCGCACTCTT-3'        |
| RLMV | 5'-TAGCGTACTTGTACTGTTC-3'  | 5'-ACGTCATGAAGGGAGAA-3'       |
| RLBV | 5'-TACACTTGTAGCATGTTTGG-3' | 5'-CCAACCCTTGTCAATTTTGAT-3'   |
| RBDV | 5'- AGATCCATGACGGATGTGG-3' | 5'- AACTAAGTTAGAACTATTGTGG-3' |

PCR reaction mix in volume of 25  $\mu$ l contained 0,2 mM of specific forward and reverse primers for each virus, 2  $\mu$ l of cDNA as a template. The PCR cycling conditions were 95 °C for 3 min followed by 30 cycles of 30s at 95 °C, 20 s at 52°C, and 40 s at 72 °C. After a final extension of 5 min at 72 °C, 10  $\mu$ l of each PCR reaction was separated on a 2.0% (w/v) agarose gel to confirm the presence of viruses.

## RESULTS AND DISCUSSION

The increase of the production of berry crops and the domination of particular genotypes, lead to the emerge of new viruses, strains and isolates overcoming immune system of plants. The development of new rapid methods of virus detection makes it possible to detect on time the viral pathogens and prevent the spreading of infections (Martin and Tzanetakis, 2015; Martin and Tzanetakis, 2006; Martin et al., 2013).

In this work, we analyzed 222 samples of raspberry plants collected in Almaty region including samples of wild raspberry. RNA from leaf tissues for every sample was isolated by CTAB method (Rahmani and Amraee, 2020) (Figure 1).

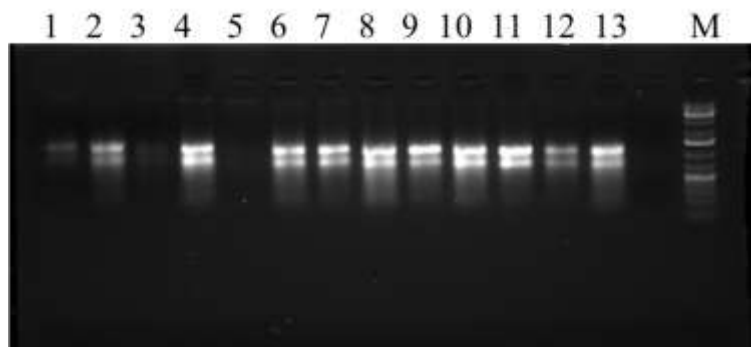


Figure 1. RNA isolated from raspberry leaves by CTAB method  
1-10 - raspberry samples from Pomological Garden; 11-13- samples of wild raspberry; M- GeneRuler 1kb Plus DNA Ladder

The specific primers for RpRSV, RLMV, RLBV and RBDV were targeted to the coat or nucleocapsid protein genes. In the result, seven samples of the cultivated raspberry were positive for RLMV and 58 samples for RBDV. Among the wild raspberry samples, 15 samples were infected by RBDV, and two single samples were positive for RLMV and RLBV. RpRSV is a quarantine virus in Kazakhstan and has not been identified in the analyzed samples (Figure 2).

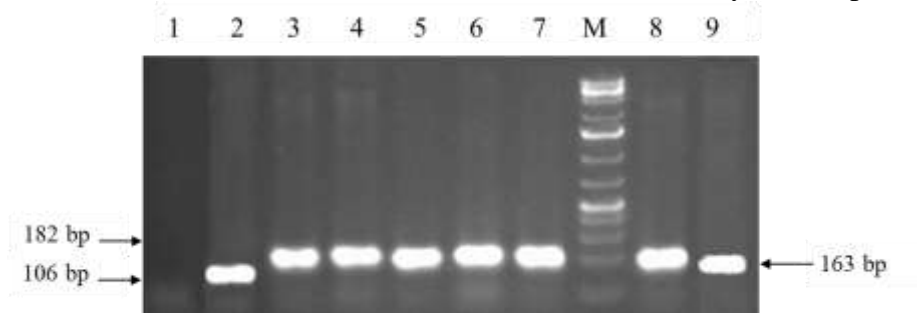


Figure 2. Detection of RLMV (163bp), RBDV (182bp) and RLBV (106bp) in raspberry samples.

1- Negative control; 2- sample of wild raspberry; 3-9- raspberry samples from Pomological Garden; M- GeneRuler 1kb Plus DNA Ladder

To date, several ways of transmission of plant viruses are described, the most complex of them is occurred through the pollen and seeds. The effective way to manage spreading of viruses is to detect pathogens on time in the planting material before cultivation. In Kazakhstan, the planting material of raspberry is mainly imported from European countries and Russia in most cases without certification for absence of dangerous viruses.

Viral infection of raspberry can be asymptomatic and thus undetectable by visual inspection in nurseries, but when moved to production fields and infected with additional pathogens, berry crops can quickly die. Therefore, it is necessary to detect these viruses on time by molecular methods, such as RT-PCR and sequencing to prevent their spread.

## CONCLUSION

In the present work, it was revealed for the first time that raspberry viruses have been widely spread both in the fields and in wild forests in the country. RLMV and RBDV were identified in cultivated raspberry plants while RLMV, RBDV and RLBV were detected in wild raspberries. Detection of viruses was performed by primers developed in this study for every virus. The

confirmation of PCR product specificity was conducted via sequencing followed by searching for similarity between biological sequences in NCBI-Blast.

The spreading of viral pathogens in raspberry fields probably occurs with an infected planting material or common plant hosts including weeds. Thus, the monitoring of the plant material and plants in the fields using fast and sensitive molecular methods is important task in managing the spreading of viral infections.

### ACKNOWLEDGMENT

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## **DETERMINATION OF LAND USE AND LAND COVER CHANGES USING REMOTE SENSING: GEBZE-IZMIR HIGHWAY KARACABEY ROUTE**

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### **ABSTRACT**

National wide development projects known to have apparent impacts on land use land cover (LULC) status within a certain area. It is important to foreseen the probable effects on natural and agricultural lands within the planning step of such projects for sustainable agricultural activities. In Turkey, these effects can be seen partially around the highway lines which provide transportation networks between metropolitan cities. In present study, it was aimed to determine the LULC changes occurred due to Karacabey route of Gebze-Izmir highway project, a hotspot that presents an important location for remote sensing researches, for further synthesis using remote sensing. The construction process of the route has started in 2015 and has completed in 2018 by transferring the responsibility for operation and maintenance. Landsat ETM+ (1999) and Landsat OLI (2013 and 2021) imageries with 30 m spatial resolution were pan-sharpened to 15 m prior to the analysis, and then clipped using 2 km width buffer zone along the route to obtain the study area. Subsequently, study area was classified into seven classes including built-up area, bare area, water surface, olive orchard, agricultural field, highway, and forest area using maximum likelihood algorithm. Accuracies of each classification were determined via 200 stratified randomized control points using Google Earth and CORINE maps considering the closest date, and overall accuracies were over 80%. The changes between classes were determined and interpreted for the study years. Findings revealed that most obvious changes were the decreases in agricultural fields in both years whereby there were considerable increases in transportation network and built-up class areas. The study presents preliminary work of ongoing research.

**Keywords:** Change detection, Highway, Karacabey, LULC, Remote sensing.

### **INTRODUCTION**

Land use and land cover (LULC) changes are known to occur as results of natural causes and anthropogenic activities. Regional actions related to transportation (Meneses et al., 2017; Feng et al., 2021) and urban development (Hailu et al., 2020) projects have a trigger impact on LULC status, which usually led to uncontrolled and unplanned changes, particularly in developing countries. The construction process of the highways, which aim to make the transportation between mega-cities, has caused the conversion from vegetative cover into roads and settlements. The rapid and relatively economic determination of such changes and

environmental consequences occurred due to the mentioned investments provides essential determinant in the local and regional planning studies, whereas remote sensing technologies provides valuable tools for these purposes. Determination of historical LULC changes are enabled with NASA-Landsat 1-3 (60 m) and Landsat 4-9 (30 m) imageriessince they collect land surface data for fifty years with different resolutions. It is possible to determine the changes in LULC caused by the construction of highways over time, with the help of Landsat images especially with 30 m medium resolution from these satellites, which are presented to users as open source.

The LULC changes against urban related investments are detailed in different studies (Justice et al., 2015; Kantakumar et al., 2016; Riad et al., 2020; Alharthi & El-Damaty, 2022; Mansour et al., 2022). Environmental problems may ocured especially construction of highways or road networks around city centers (Feng et al., 2021), sourced by the needs of growing population and urban expansion process requirements. For instance, it was denoted that there is a strong relationship between construction of highways and the carbon footprint (Zhao et al., 2016). Furthermore, there can be losses in forest areas dependent to highway construction, as it is reported by Klarenberg et.al. (2018). In addition to large area losses in highway constructions, LULC conversions between different land classes are a common result. In Turkey after national wide highway constructions news take place on press, LULC change has immediately starts to occurred around constructed regions, as a general result.

The main objective of the study was quantitative determination of impact rate of Gebze-Izmir highway construction process on LULC changes in the specified route of Karacabey.

## MATERIAL AND METHOD

The construction of the Gebze-Izmir Highway was started in 2015, and it is started to service on 04 August, 2019 by transferring the responsibility for operation and maintenance. Figure 1 represents the location and the length of specified part of the highway. The study was conducted in a buffer zone, which is obtained by composing 1 km double-sided zone (2 km total) to the 167 km highway center line, and the LULC changes occurred only within this buffer zone were investigated. It was foreseen that the study will be effective in determining the change in the months when agricultural production is intense, depending on the previous studies conducted around the specified area (Inalpulat et. al., 2018; Inalpulat and Genc, 2021).

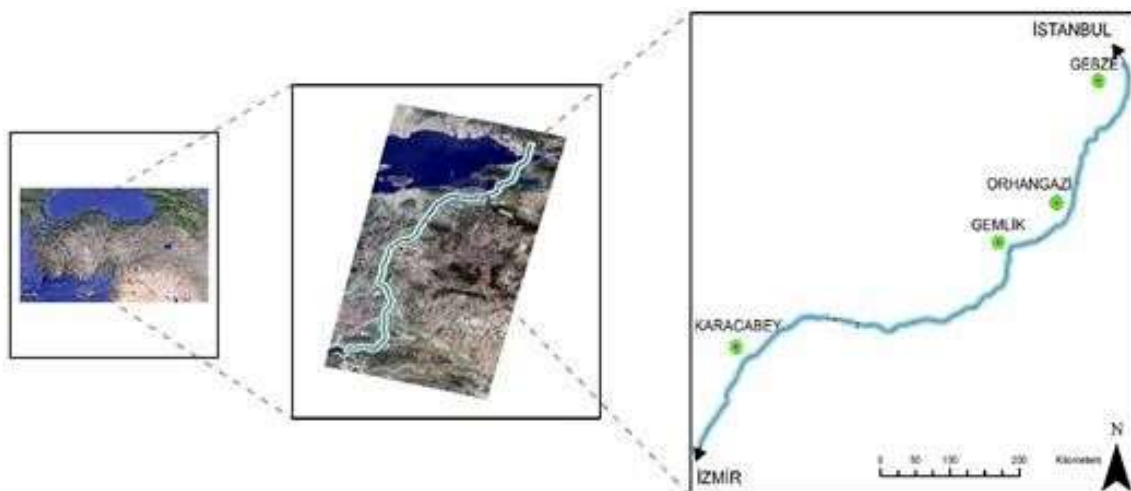


Figure 1. Location of Karacabey Route of Gebze-Izmir Highway

The satellite images used in the study were downloaded as open source from the USGS (United States Geological Survey) Earth Explorer website. The images were taken on 04 October, 1999 before the Gebze-Bursa West junction construction starting date, 30 July, 2013 after the

Gebze-Bursa West Junction end, and 11 August, 2021 after the Gebze-İzmir highway completion date. The steps of the study are given in Figure 2.

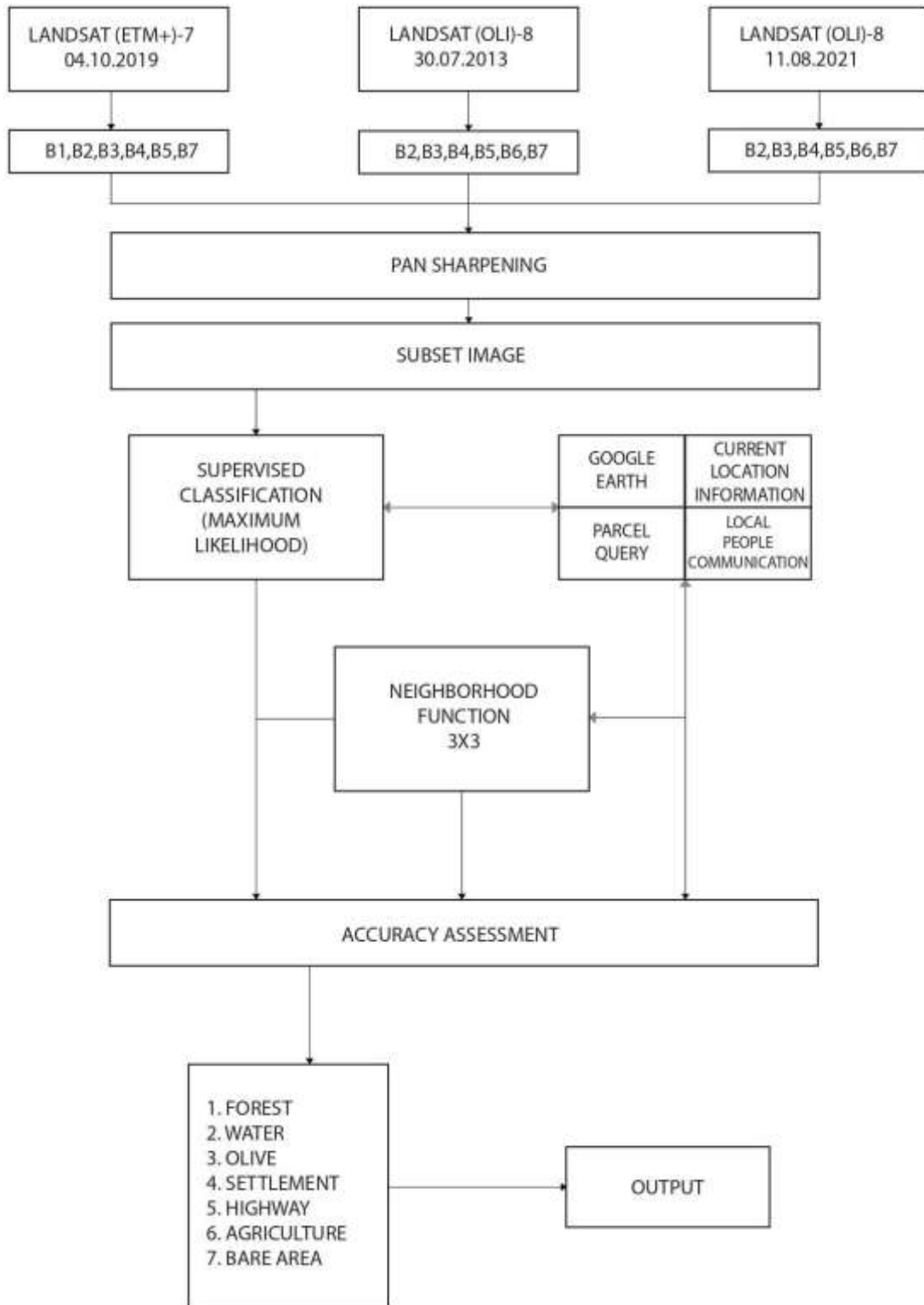


Figure 2. The flowchart of the steps followed in the study

Used images were obtained for all three dates by utilizing 1<sup>st</sup>-5<sup>th</sup>, and 7<sup>th</sup> bands for Landsat ETM+, and 2<sup>nd</sup>-7<sup>th</sup> bands for Landsat OLI from the original Landsat images. Since the studied

area is on a narrow line, the terrestrial resolution of satellite images with a terrestrial resolution of 30 meters was reduced to 15 meters by using pan-sharpening by applying pan sharpening process. Supervised classification procedures were applied via maximum likelihood algorithm (MLA) with the help of the local data, parcel query, Google Earth and the opinions of the people living in the region. In MLA method, the probabilities of being belonged to each class for a candidate pixel are calculated separately, and the pixel is assigned to the class with the highest probability. The main LULC classes used in the study were selected by considering the dominant vegetation and land use in the region. These are namely, forest, water, olive, settlement, highway, agriculture, and bare area. Subsequent to the image classification, neighborhood analysis with 3x3 pixels was applied to organize pixel values defined as noise in classified images. Each processed cell is scanned when the raster is visited and a new value of the scanned cell is calculated relative to its neighbors in consequence, and LULC maps were obtained.

The accuracies of LULC maps were assessed by procedures defined in the Congalton and Green (2009). The most common way to express the accuracy of classified images is to check and compare remotely sensed data with onsite observations using random reference points belonging to different classless of the maps. Stratified randomized control points were used in accuracy assessment step in the study.

## RESULTS AND DISCUSSION

The results of accuracy assessments are given in Table 1. Accordingly, the accuracies of 1999 and 2013 LULC maps were increased with implementation of 3x3 pixel neighborhood function while accuracy of 2021 has reduced in an acceptable rate. The situation is expected to be sourced from relatively enlarged width of 2021 highway would affected adversely from the noise reduction since the salt and pepper pixels were currently low in comparison with more narrow structure of the road in the past years. Consequently, all the classifications were good since the kappa values were over the threshold of 0.75 as denoted in the literature (Lillesand and Kiefer, 2000).

Table 1. Accuracies of original band derived LULC maps.

| LULC Map              | Overall Accuracy | Overall Kappa |
|-----------------------|------------------|---------------|
| 1999                  | 83.00%           | 0.7808        |
| 2013                  | 85.00 %          | 0.8034        |
| 2021                  | 87.00 %          | 0.8341        |
| 1999 3x3 Neighborhood | 86.50 %          | 0.8252        |
| 2013 3x3 Neighborhood | 88.00 %          | 0.8243        |
| 2021 3x3 Neighborhood | 85.50 %          | 0.8104        |

The LULC maps of 1999, 2013, and 2021 before and after neighborhood analysis has applied are given in Figure 3a-c and Figure 4a-c, respectively. In addition, Figure 5 shows the LULC class areas in graphical version. The changes in LULC statuses are investigated in two steps. The first step included the overall changes in the specified buffer zone, and the second step covers the changes occurred only within the latest coverage of Highway class area. A standard post-classification change detection method was applied to identify the two-step changes, and the results of the change analysis are given in Table 2 to Table 5, individually. There were conversions between all classes in both periods. However, there is confusion especially between highway and

other classes in the earlier dates. For instance, over 28% of highway coverage designated to convert into olive class, which was an unexpected situation. This result was thought to be sourced from relatively narrow structure of the old highway status, and it was not easy to discriminate between H and other class pixels using Landsat imageries with moderate resolution. On the other hand, considering only the changes under H class areas gave more satisfactory results for analyzing the effects of highway construction on other LULC classes. In this context, the most observable changes were transitions from bare area, agriculture and forest areas with 11.78%, 9.79% and 8.29%, respectively from 1999 to 2013, and agriculture, bare area and olives with 49.27%, 46.64% and 43.41%, whereby the percentages represents the gains of new highway pixels from to prior LULC class pixels.

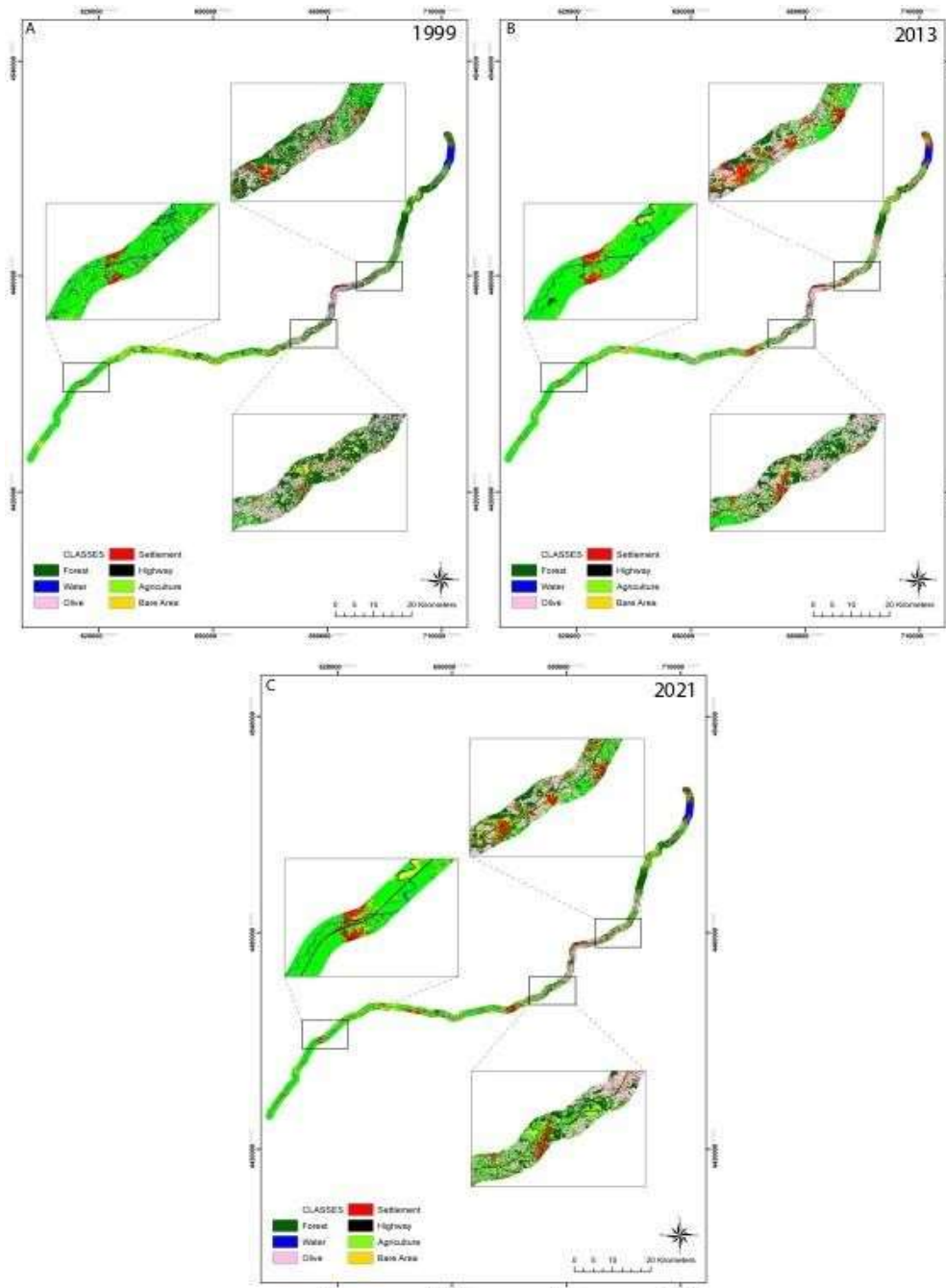


Figure 3. LULC maps derived from A) 04 October,1999 B) 30 July, 2013, C) 11 August, 2021



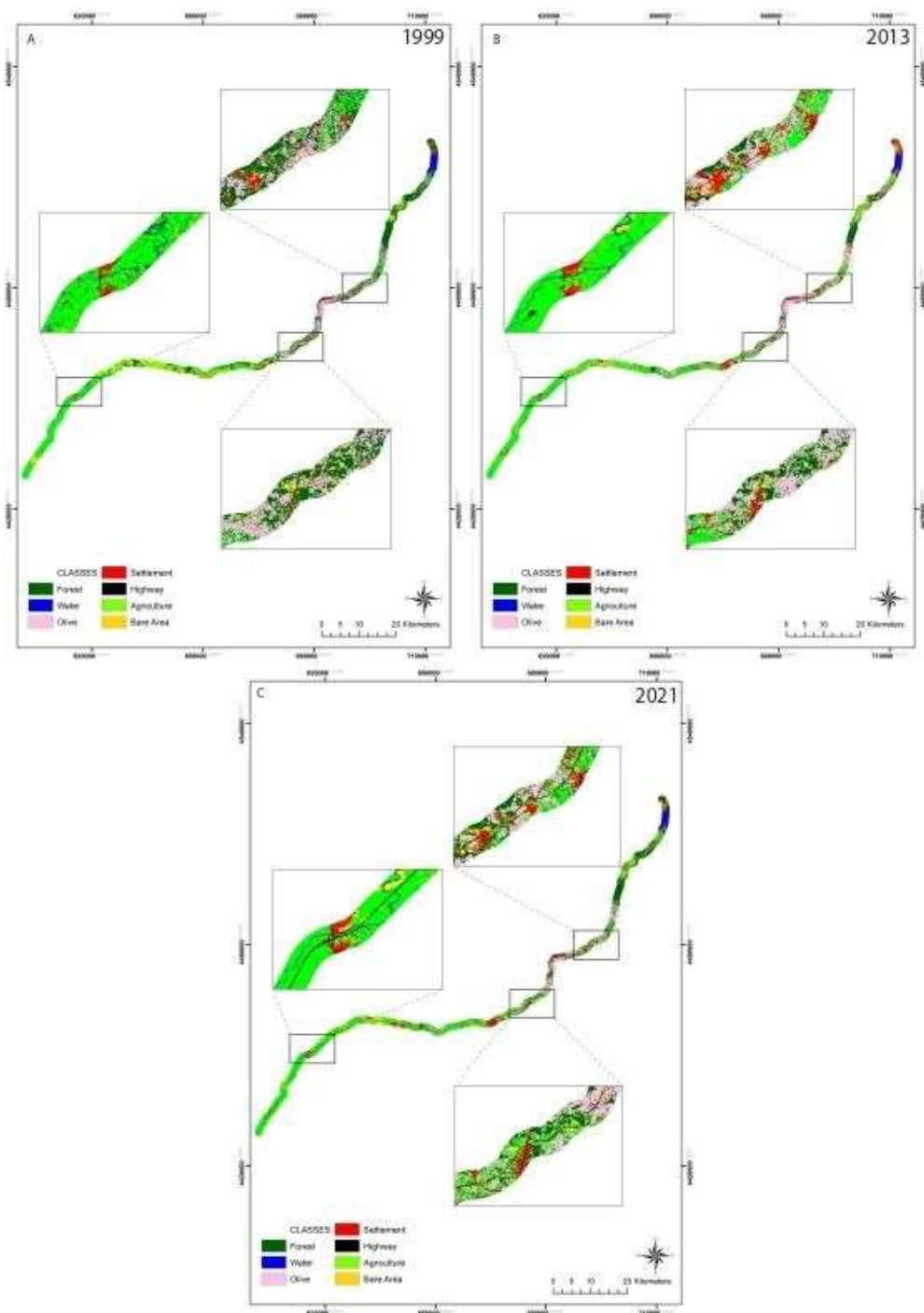


Figure 4. 3x3 Neighborhood applied LULC maps derived from LULC maps derived from A) 04 October,1999 B) 30 July, 2013, C) 11 August, 2021

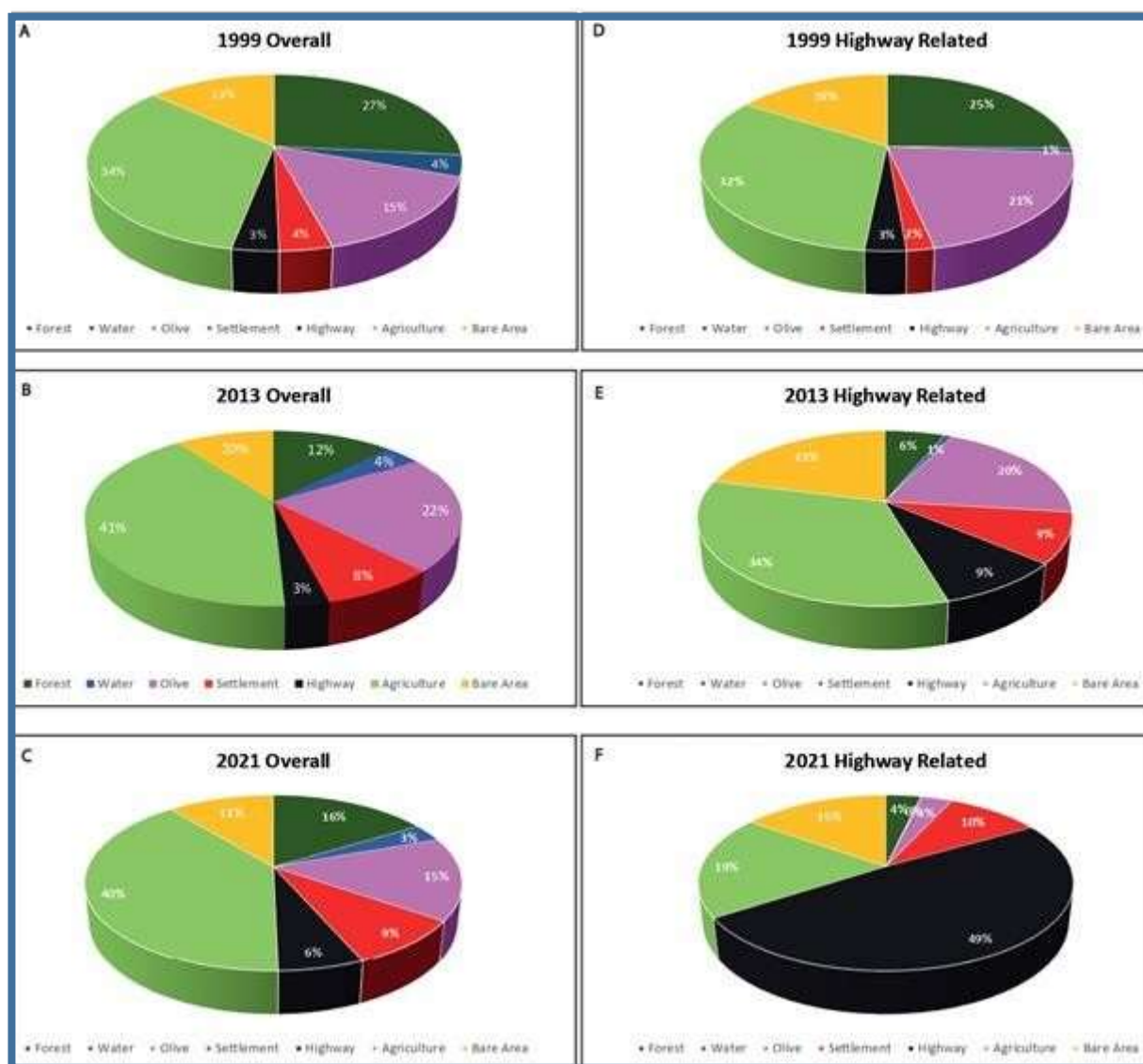


Figure 5. LULC class areas of A) LULC 1999 B) LULC 2013, C) LULC 2021, D) LULC Highway 1999, E) LULC Highway 2013, F) LULC Highway 2021.

Table 2. Overall LULC changes between 1999 and 2013 (%) from 3x3 neighborhood maps

| Class | F            | W            | O            | S            | H            | A            | B            |
|-------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|
| F     | <b>41.24</b> | 0.95         | 20.98        | 4.60         | 2.36         | 18.88        | 10.98        |
| W     | 2.15         | <b>77.54</b> | 4.46         | 8.15         | 2.15         | 4.15         | 1.38         |
| O     | 4.22         | 1.11         | <b>59.36</b> | 6.14         | 3.55         | 21.46        | 4.16         |
| S     | 0.54         | 0.85         | 7.45         | <b>61.57</b> | 6.68         | 16.77        | 6.13         |
| H     | 0.88         | 0.62         | 28.79        | 25.26        | <b>15.32</b> | 23.33        | 5.81         |
| A     | 1.73         | 0.54         | 10.28        | 6.24         | 2.37         | <b>68.44</b> | 10.40        |
| B     | 0.75         | 0.20         | 14.64        | 4.10         | 2.67         | 58.03        | <b>19.62</b> |

Table 3. Overall LULC changes between 2013 and 2021 (%) from 3x3 neighborhood maps

| Class | F            | W            | O            | S            | H            | A            | B            |
|-------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|
| F     | <b>81.10</b> | 0.10         | 6.29         | 1.73         | 1.22         | 5.50         | 4.06         |
| W     | 4.20         | <b>80.37</b> | 2.50         | 2.10         | 2.91         | 6.87         | 1.05         |
| O     | 13.23        | 0.08         | <b>50.56</b> | 3.76         | 4.09         | 20.08        | 8.20         |
| S     | 1.79         | 0.86         | 3.54         | <b>50.21</b> | 18.04        | 16.46        | 9.11         |
| H     | 7.31         | 0.84         | 9.84         | 13.78        | <b>34.58</b> | 27.09        | 6.56         |
| A     | 4.96         | 0.24         | 6.01         | 5.32         | 3.86         | <b>70.66</b> | 8.95         |
| B     | 7.77         | 0.12         | 3.59         | 9.15         | 8.59         | 31.51        | <b>39.28</b> |

Table 4. Highway-dependent LULC changes between 1999 and 2013 (%) from 3x3 neighborhood maps

| Class | F            | W            | O            | S            | H            | A            | B            |
|-------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|
| F     | <b>21.00</b> | 0.00         | 11.75        | 14.07        | 8.29         | 14.07        | 30.83        |
| W     | 0.00         | <b>50.00</b> | 11.11        | 22.22        | 5.56         | 5.56         | 5.56         |
| O     | 2.38         | 0.71         | <b>55.24</b> | 8.10         | 6.43         | 11.43        | 15.71        |
| S     | 0.00         | 0.00         | 12.20        | <b>36.59</b> | 7.32         | 24.39        | 19.51        |
| H     | 0.00         | 0.00         | 20.00        | 20.00        | <b>25.00</b> | 18.33        | 16.67        |
| A     | 1.20         | 0.30         | 7.38         | 5.27         | 9.79         | <b>58.58</b> | 17.47        |
| B     | 0.30         | 0.00         | 14.94        | 4.57         | 11.28        | 47.56        | <b>21.34</b> |

Table 5. Highway-dependent LULC changes between 2013 and 2021 (%) from 3x3 neighborhood maps

| Class | F            | W           | O           | S            | H            | A            | B            |
|-------|--------------|-------------|-------------|--------------|--------------|--------------|--------------|
| F     | <b>42.19</b> | 0.00        | 2.34        | 5.47         | 26.56        | 10.94        | 12.50        |
| W     | 0.00         | <b>7.14</b> | 0.00        | 7.14         | 7.14         | 71.43        | 7.14         |
| O     | 2.20         | 0.00        | <b>6.83</b> | 7.56         | 43.41        | 18.54        | 21.46        |
| S     | 1.06         | 0.00        | 3.19        | <b>11.17</b> | 57.45        | 18.09        | 9.04         |
| H     | 0.52         | 0.00        | 2.62        | 4.71         | <b>73.30</b> | 12.04        | 6.81         |
| A     | 0.58         | 0.00        | 1.02        | 12.35        | 49.27        | <b>23.26</b> | 13.52        |
| B     | 0.93         | 0.00        | 3.48        | 10.44        | 46.64        | 19.72        | <b>18.79</b> |

## CONCLUSIONS

In this study, the LULC changes against highway construction process within a buffer zone of Karacabey route, Gebze-Izmir highway was investigated in two periods between 1999 and 2013, and 2013 and 2021 using pan-sharpened Landsat imageries. The results demonstrated that there were considerable changes in all classes within both time periods. On the other hand, use of the highway class area for change detection analysis reduced the misinterpretations. In conclusion, it is recommended to use imageries with higher resolutions for improvement of the findings by

eliminating spectral mixtures between relatively narrow samples of highway route and its surrounding pixels. Ongoing study is focused on short-term changes in the whole of Gebze-Izmir Highway using Sentinel-2 imageries.

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## **MONITORING OF URBAN AREA AND AGRICULTURAL LAND CHANGES AFTER GOLCUK EARTHQUAKE (17 AUGUST, 1999)**

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### **ABSTRACT**

Earthquakes cause irreversible consequences, especially damaging the residential areas. During the reconstruction process, agricultural lands around the existing residential areas are dramatically under pressure of change. Present study focused on investigation of reconstruction process impacts on agricultural lands after earthquake eventuated in 17 August, 1999, Golcuk, Turkey, using remote sensing techniques. Pre- (1999) and post-disaster (1999, 2000, 2005, 2010, 2015, and 2020) land use land cover (LULC) statuses of the area was evaluated through Landsat TM and OLI imageries with 30 m spatial resolution, which were sharpened with a 15 m resolution pan- image. The LULC maps were generated using Landsat TM and Landsat OLI pan sharpened images with total of 6 bands of visible (3 bands), near infrared (1) and mid infrared bands (2). In addition, Normalized Vegetation Difference Index (NDVI) was calculated from these images for each date. Then NDVI bands were added to the 6-band images, and 7-band images were obtained. The study area clipped from both 6- and 7- band images and classified into six main LULC classes, namely, built-up area, road, greenhouse area, open agricultural field, forest, and water surface via supervised classification algorithm. Accuracy assessments showed that addition of NDVI band improved overall accuracies except the most recent post-disaster imagery date (September, 1999). The results revealed that there were great changes in all classes except water surface between the dates before and after the 1999 disaster, whereas the built-up class decreased due to changes in spectral reflectance of collapsed buildings (September, 1999). As a conclusion, while there were gradual increases in residential area throughout the five-year periods between 2000 and 2020, it was observed that agricultural lands decreased due to the development in the residential area.

**Keywords:** Agricultural land, Earthquake, Golcuk, Landsat, Urban area.

### **INTRODUCTION**

Earthquakes are known to be one of the most destructive disasters. Numerous earthquakes caused death of millions of people all around the world within the ends of last century, whereas economical costs were also devastating due to damaged natural terrain together with infrastructures and collapsed buildings, which are also led to drastic changes on earth surface properties (Turker and Sumer, 2008).

In the earthquake of Golcuk, Izmit almost 19 thousand people died, approximately 300 thousand houses and 43 thousand establishments were damaged according to latest reports (Anonymous, 2022). Moreover, according to the study conducted by Aydoner and Akinciturk (2013), over 90% of total Golcuk district area is found to be under settlement suitability rate of 20%, as it is cited in Bolat and Dogan (2022).

One of the most important actions after earthquakes, as well as other disasters, is the determination of the damage amount. In this context remotely sensed imageries or aerial photos presents valuable data source to acquire highly accurate and rapid results (Korkmaz and Abualkibahs, 2018), and thus used in various areas of the world. In addition, monitoring of the renewal process after the event is another important part of management strategies. In present study, it was aimed to determine the rapid and periodic changes occurred due to Golcuk earthquake between 1999 and 2020 using Landsat series.

## MATERIAL AND METHOD

The study was conducted around the main residential site of Golcuk, Turkey, where the impacts of the earthquake (17 August, 1999) was highly disastrous. Figure 1 represents the location and coverage of the study area. Landsat 5 (TM), Landsat 7 (ETM+), and Landsat 8 (OLI) imageries comprised main data source in the study. The acquisition dates of imageries were 25 July, 1999; 27 September, 1999; 27 July, 2000; 19 September, 2005; 16 August,

2010; 14 August, 2015; 27 August, 2020. Since the earthquake eventuated in 17 August, 1999, the first and second image presented pre-disaster and immediate post-disaster land use land cover (LULC) status of the area, respectively. Furthermore, the LULC changes were monitored within every five year period between 2000 and 2020 years. The visible, near infrared, and short wave infrared bands of each image were stacked, pan-sharpened to 15 m, and then study area was clipped prior to the supervised classification process. Six main LULC categories were defined as forest (F), agriculture (A), greenhouse (G), road (R), urban (U), and water surface (W). Maximum likelihood algorithm was used as classifier to obtain LULC maps. In addition to the original image classification, Normalized difference vegetation index (NDVI) of each date were calculated and added to original bands, then classified to evaluate the NDVI effect on classification accuracy in the area of interest.

$$NDVI = (NIR - RED)/(NIR + RED)$$

Accuracy assessments were conducted to determine the reliability of each classification via stratified randomized control points using Google Earth application depending on Congalton and Green (2009). Overall accuracy and kappa statistics values were used to assess the accuracies.

## RESULTS AND DISCUSSION

The LULC maps from original band of different dates are given in Figure 2 a-g, LULC<sub>NDVI</sub> maps are given in Figure 3 a-g, and the classification accuracies of original and NDVI-added imageries can be seen on Table 1 and Table 2, respectively. Addition of NDVI band seemed to change classification accuracy. Therefore, results of more accurate classifications were considered in the further analyses. Findings of the study revealed that there were considerable changes in all LULC classes except W class between the pre- and immediate post-disaster statuses due to the unstable conditions in the area since wreckages of collapsed buildings were transferred to proper places temporarily, which led to changes in spectral signatures of other LULC types such as agriculture. According to the results, particularly, areas of U and R classes, which are related to impermeable and concrete structures, decreased immediately after earthquake, while the

areas of the other classes increased slightly. However, the drastic decrease in F covers seemed to source from misclassification since there were spectral similarities between dense and green agricultural plants and natural vegetation cover, as the overall accuracy and kappa of the LULC map were quite lower (Lillesand and Kiefer, 2000).

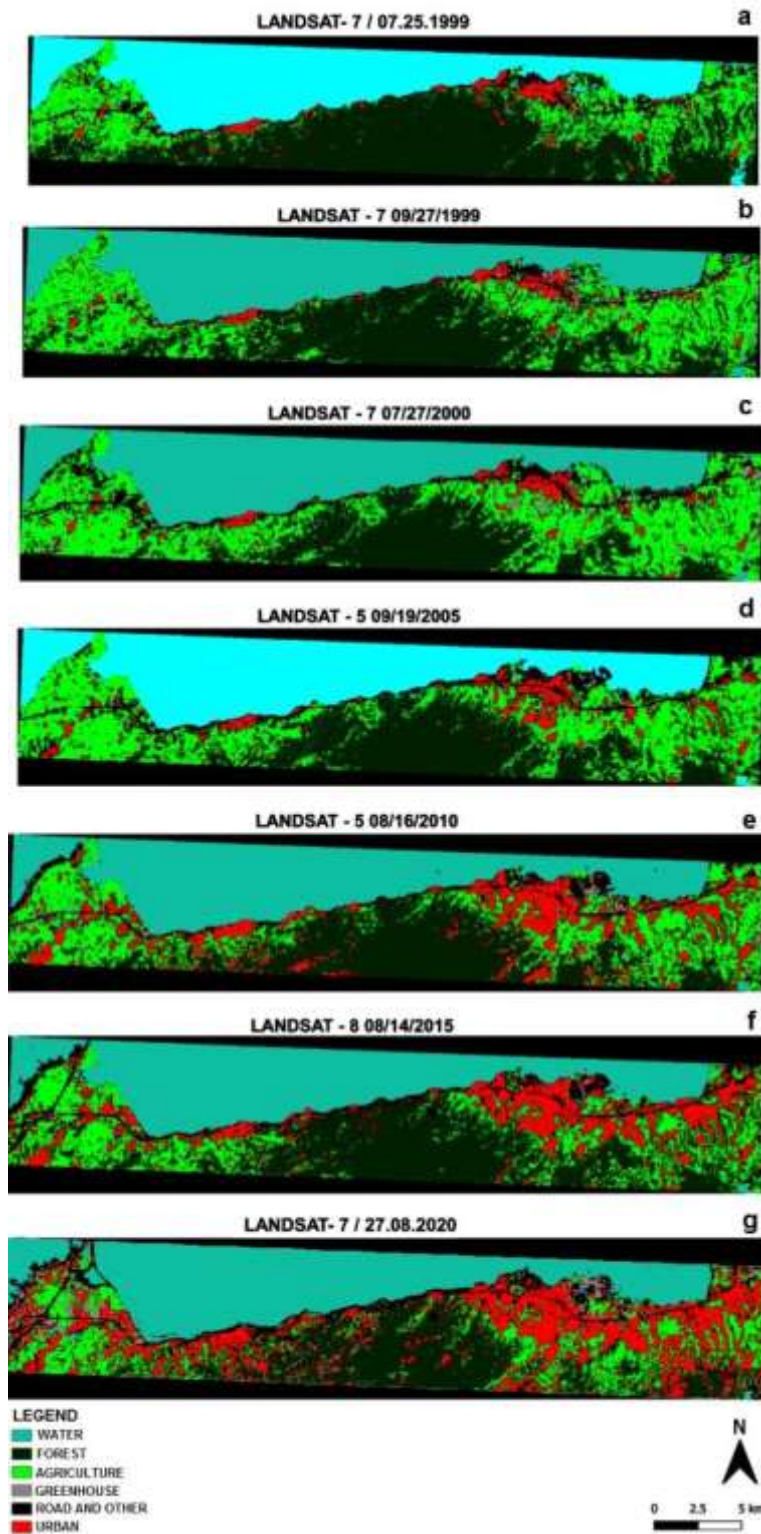


Figure 2. LULC maps derived from original bands a. pre-disaster in 1999, b. post-disaster in 2019, c. 2000 year, d. 2005 year, e. 2010, f. 2015, and g. 2020 years

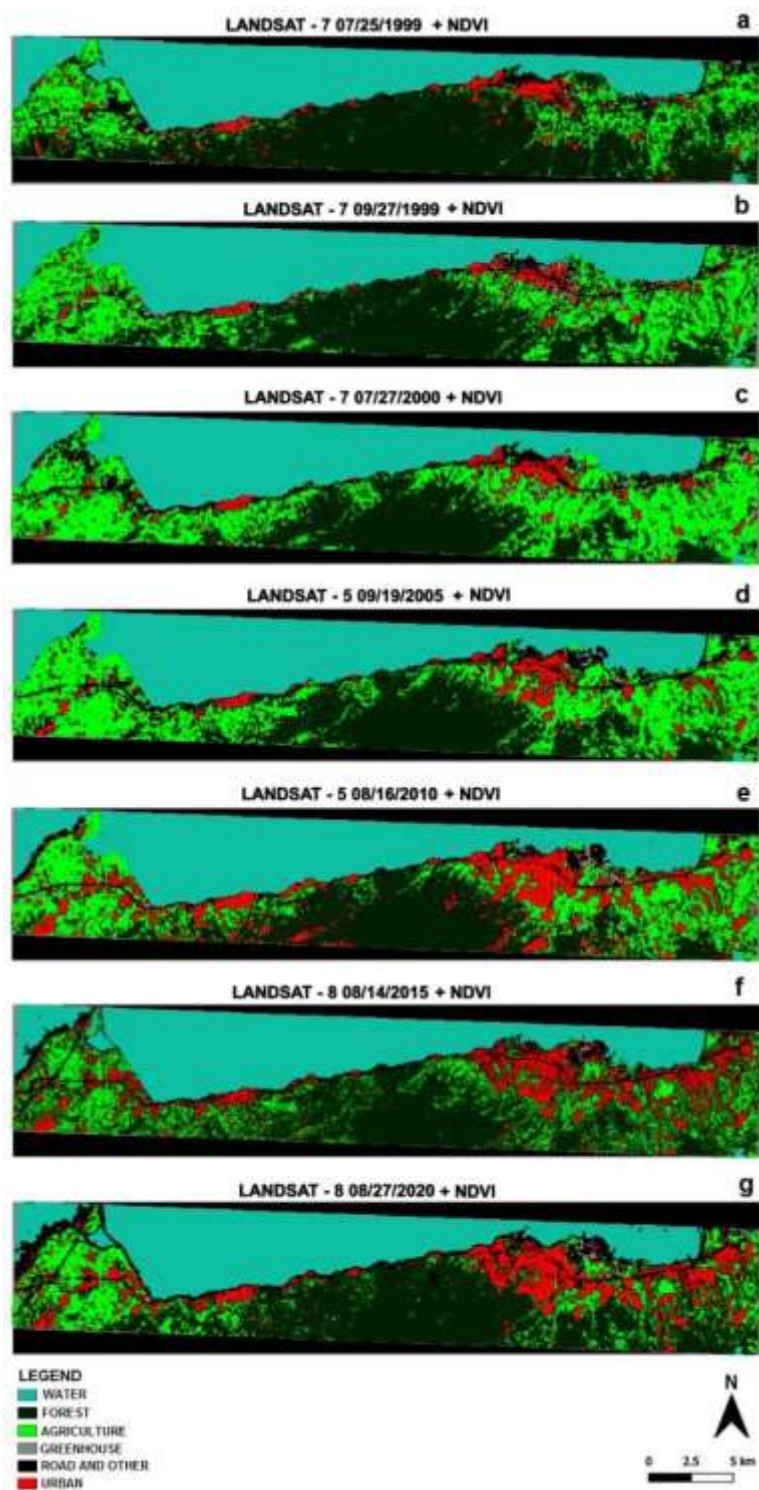


Figure 3. LULC maps derived from NDVI-added bands a. pre-disaster in 1999, b. post-disaster in 2019, c. 2000 year, d. 2005 year, e. 2010, f. 2015, and g. 2020 years



Table 1. Accuracies of original band derived LULC maps.

| LULC Maps (Original)              | Overall Accuracy (%) | Overall Kappa |
|-----------------------------------|----------------------|---------------|
| LULC <sub>Pre-disaster1999</sub>  | 82,00                | 0,7497        |
| LULC <sub>Post-disaster1999</sub> | 88,00                | 0,8461        |
| LULC <sub>2000</sub>              | 83,00                | 0,8000        |
| LULC <sub>2005</sub>              | 85,29                | 0,8235        |
| LULC <sub>2010</sub>              | 84,31                | 0,8118        |
| LULC <sub>2015</sub>              | 83,33                | 0,8000        |
| LULC <sub>2020</sub>              | 81,00                | 0,7641        |

Table 2. Accuracies of NDVI-added band derived LULC maps.

| LULC Maps (NDVI-Added)                 | Overall Accuracy (%) | Overall Kappa |
|--|----------------------|---------------|
| LULC <sub>NDVI_Pre-disaster1999</sub>  | 86,27                | 0,8353        |
| LULC <sub>NDVI_Post-disaster1999</sub> | 85,29                | 0,8235        |
| LULC <sub>NDVI_2000</sub>              | 86,27                | 0,8353        |
| LULC <sub>NDVI_2005</sub>              | 85,29                | 0,8235        |
| LULC <sub>NDVI_2010</sub>              | 88,24                | 0,8588        |
| LULC <sub>NDVI_2015</sub>              | 86,27                | 0,8353        |
| LULC <sub>NDVI_2020</sub>              | 84,31                | 0,8118        |

Monitoring of 5-year changes between 2000 and 2020 years has revealed that the most drastic changes are occurred in A, R, and U classes, while F class area observably decreased consequent to the first year of the event and changes in G areas mostly sourced from misclassifications. Considerably, the areas of R and U classes seemed increased gradually due to construction activities in the area after the earthquake (Figure 4). On the other hand, there was a considerable fluctuation in the area of A class. The situation is probable to be sourced from uncertain and continuous recovering activities after in the area after earthquake event, which are observable on the LULC<sub>Post-disaster</sub> and LULC<sub>2000</sub> maps. However, there was a decreasing tendency during the studied years in A class area with the increasing of especially U class, since new settled areas constructed on these areas. Moreover, the progressive expansion of urban areas can be seen on Figure 5. There were great changes in both amounts and directions of new urban areas between 2000 and 2020. Similarly, Urban related areas noted to be increased whereas green areas reported to decrease in different studies conducted within different parts of the area (Aslan and Ince, 2019; Bolat and Dogan, 2022; ) The process expected to be continued in the near future, and agricultural lands around the settled areas would be transform into U class.

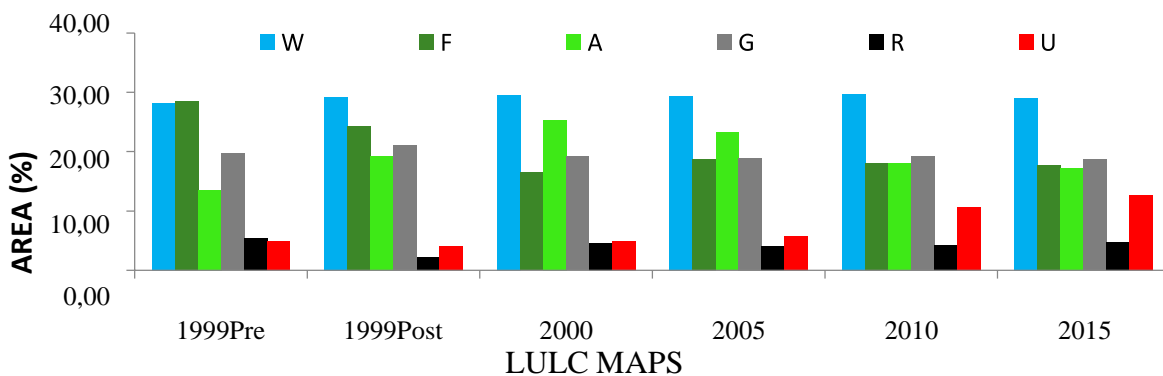


Figure 4. Temporal changes in class areas

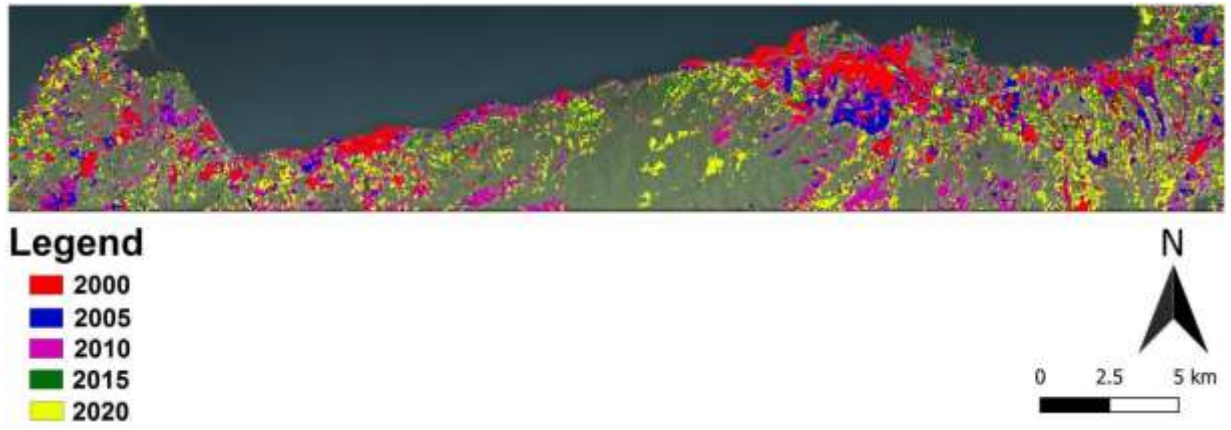


Figure 5. Urban expansion between 2000 and 2020 years

## CONCLUSIONS

Depending on the findings of the study, it was concluded that there were drastic decreases in settlement, road and other concrete structures due to unexpected earthquake event in 1999. Subsequent to the disaster, restructuring activities had great impact on LULC status in the area immediate after the event. It was seen that vegetative cover, especially agricultural lands faced with rapid expansion of urban areas, and the ongoing process is estimated to be continued in the future. Therefore, well-planned environmental friendly approaches and management strategies are recommended to be required for sustainable development in the area. A study is planned for simulation of future LULC status of the area depending on the historical status and ancillary data of population, terrain properties, socioeconomic approaches, and structural proximities, depending on different scenarios.

## ACKNOWLEDGEMENT

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## **VISUAL BASIC STUDY BASED ON AUTOMATION PROGRAM FOR THE MAPPING OF THE SPATIAL DISTRIBUTION OF SOIL PARAMETERS USING GIS AND KRIGING INTERPOLATION METHOD OF THE SURFER SOFTWARE**

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### **ABSTRACT**

This work has focused on creating a computerized bank of pedologic data of the plain of Chemora-Boulhilet, in which we selected 101 pedologic profiles involving 281 horizons. This operation may require several years of effort makes sense only if the information that it conveys is complete and reliable. In this context, some attempts of consistency will be used. The chosen methodological approach focused on the creation of the database dedicated initially to characterize the size, structure and distribution of analytical pedologic descriptors soil's rates. Second, it is statistically process the information collected using two different statistical approaches: descriptive statistics and elementary statistical design based on a correlation matrix mainly devoted to research possible links between some soil parameters. Representations of thematic maps were obtained using an interpolation method known as kriging of the SURFER software (version 11) to allow obtaining a vision of the spatial distribution of a pedologic element in the plain of Chemora- Boulhile.

**Keywords:** Plain of Chemora-Boulhilet, data-base, GIS, Kriging.

### **INTRODUCTION**

Soil is no longer considered an inert medium; it evolves in space and time. This evolution gives it variability in its properties (morphological, physical, chemical and biological, etc.). At the same time; the ground is above all a thin and fragile asset. It is today, by society, considered as a consumable resource, although essential and very slowly renewable. It is therefore essential to be able to have information on the properties of soils and on their spatial organization. This serves as a support for the production of a soil reference system based on the constitution of a geographical database controlled by a Geographic Information System.

The use of the geographic information system (GIS) integrating geostatistical analysis models constitutes a new approach for the spatial analysis of information and the updating of cartographic works (Drapeau, 2000; Ferrandis et al. 2000; Le Corre, 2000).

Geostatistics is a relatively recent interpolation method for studying spatial variability developed by Krige (1951), Matheron (1965), Journel Huijbregts (1978), and Cressie (1993). It

is a branch of applied statistics that measures the spatial dependence and spatial structure of a measured property (Mulla, 2000).

## MATERIAL AND METHODS

### *Presentation of the study area*

The plain of Chemora-Boulhilet is located in the heart of the Hautes Plaines Constantinoises; Halfway between the heights of Tell Constantine and those of the Aurès. This plain straddles the regions of Batna (communes of Chemora and Boulhilet), and Oum El Bouaghi. The study area (Chemora-Boulhilet) is located between parallels 35°35'N and 35°50'N and meridians 6°30'E and 6°55'E (Figure 1).

The climate of the Chemora Plain is characterized by semi-arid continentality with cool winters, frosts, snowfalls and dry, hot summers with a very dry and drying southerly wind (siroco) which corresponds to the major part of the high plateaus and high plains of the interior of the country.

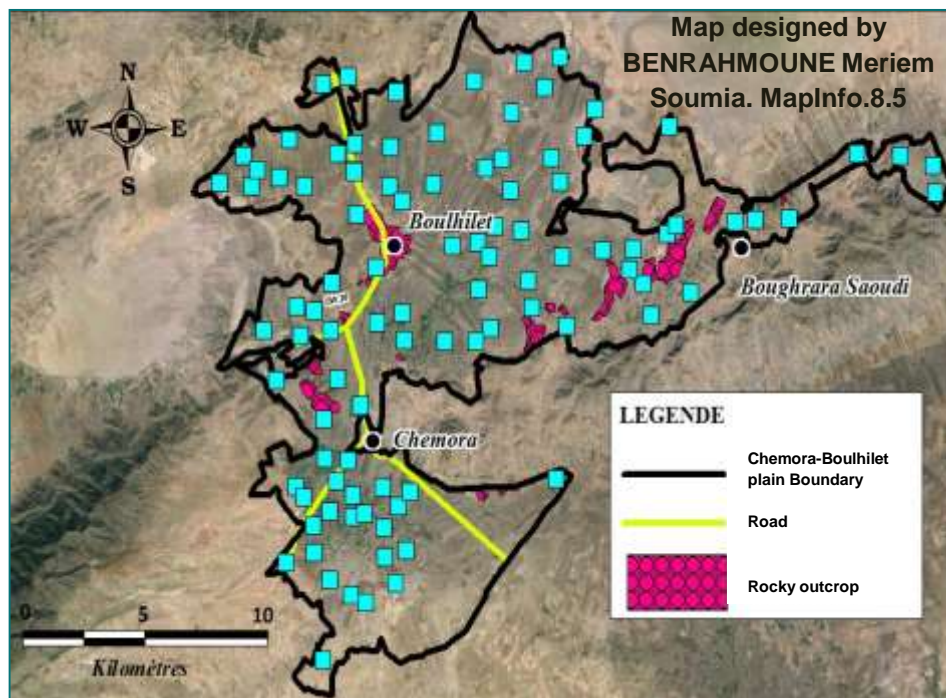


Figure 1. Implementation of the profiles of the Chemora-Boulhilet plain

### *Data-processing*

Two types of processing were carried out: Descriptive statistical processing and correlations between the soil parameters studied (clay, CEC and sand). The descriptive statistics used are: the mean, the variance and the standard deviation. From the standard deviation and the mean, the coefficient of variation can be calculated using the following formula:

$$CV (\%) = (\text{standard deviation/average}) \times 100$$

The correlations between the various parameters consist in determining the degree of association or the connection which exists between the variables of the bank carried out on the variables taken two by two. This relationship is assessed using a coefficient defined as the correlation coefficient 'r' which is the square root of  $R^2$ .

$$r = \sqrt{R^2}$$

According to Dagnelie (1975), the correlation coefficient can take values between -1 and 1. The closer the correlation coefficient «r» approaches to +1 or -1, the linear relationship is strong. Conversely, the closer the «r» is to 0, the linear relationship is weak. It is a question of looking for the relations which can exist between the pedological parameters without consideration of horizons, the data are taken as a whole:

### ***SURFER (11)***

Two major steps are summarized: Development of the Variogram and the production of a Krigean map of the data. The Variogram is created, adjusted by a basic linear model, which will have to be improved (Figure 2).

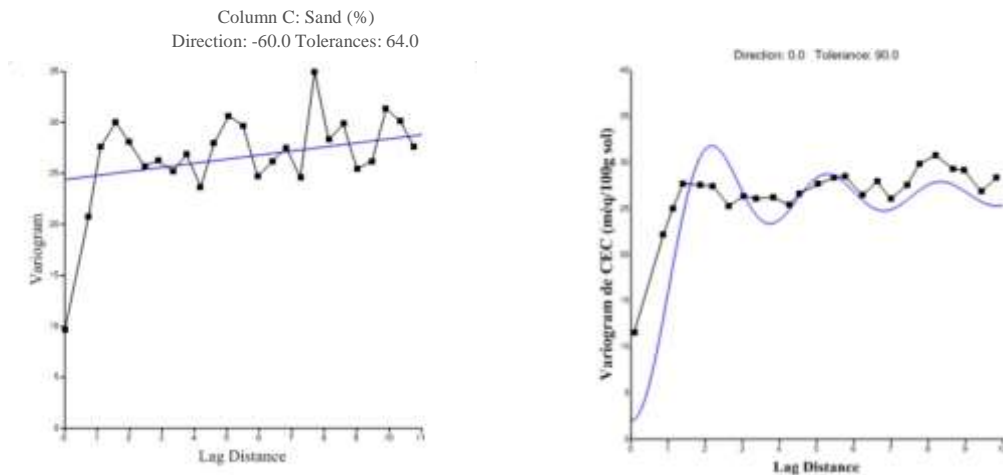


Figure 2. Variogram of CEC values created before and after adjustment.

Once the variogram has been established, and its model adjusted, we can make a map of the values by Kriging, ie by interpolation of the values between the measurement points (a regular mesh of the interpolated values). Kriged data is stored in a file with the “.grid” extension. The Surfer software uses the “.grid” file to establish the cartography of the studied parameter. For this, it is necessary to retrieve the parameters of the variogram, then to create the interpolated data. Then we can display the data in the form of a map, called a Krigea map.

## **RESULTS AND DISCUSSION**

We present in alternation the results acquired for the parameters studied (clays, sands and CEC). The thematic analysis of each descriptor will be exhibited in the preamble at the level of the surface horizons in cartographic form (Figure 3).

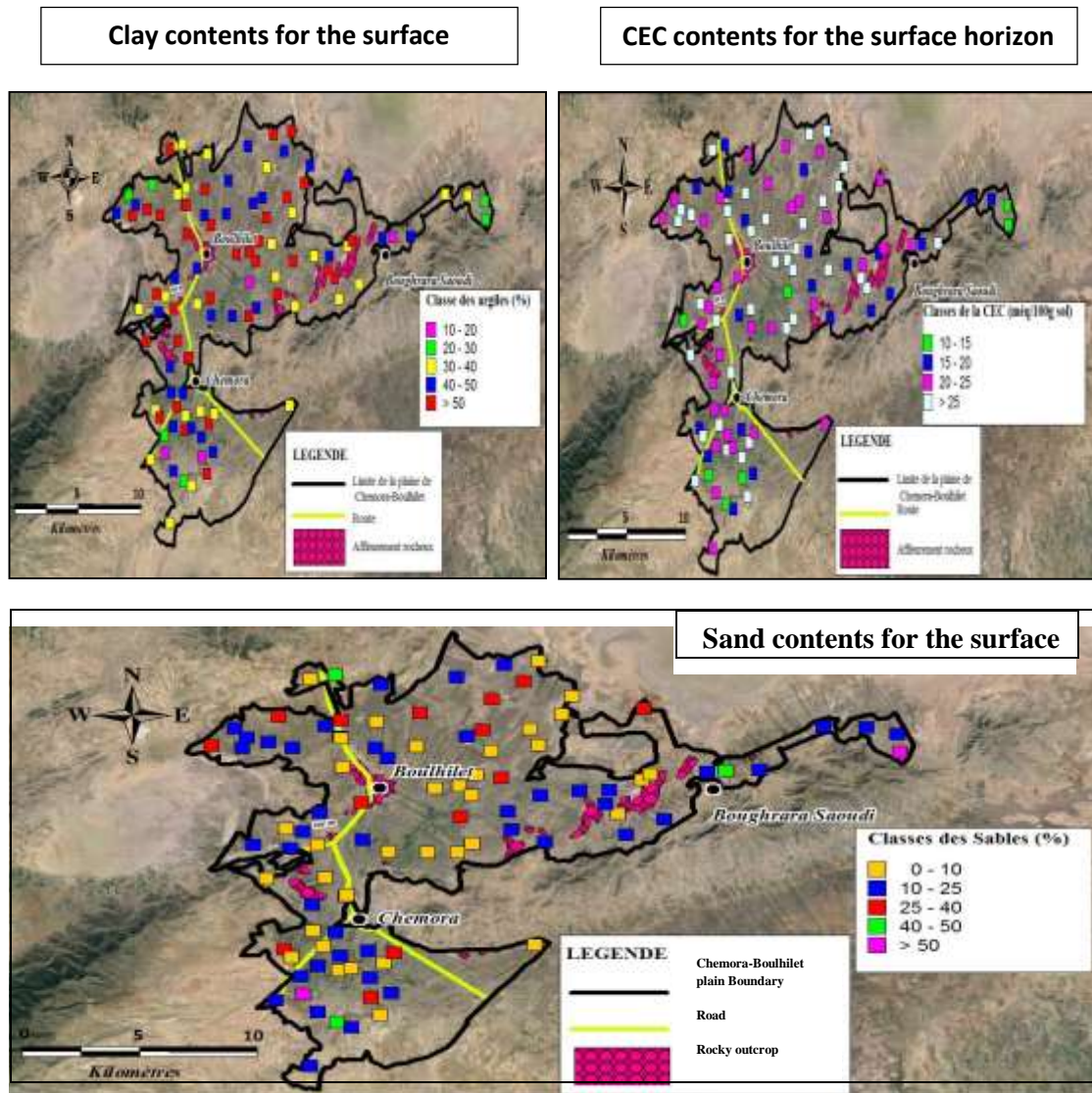
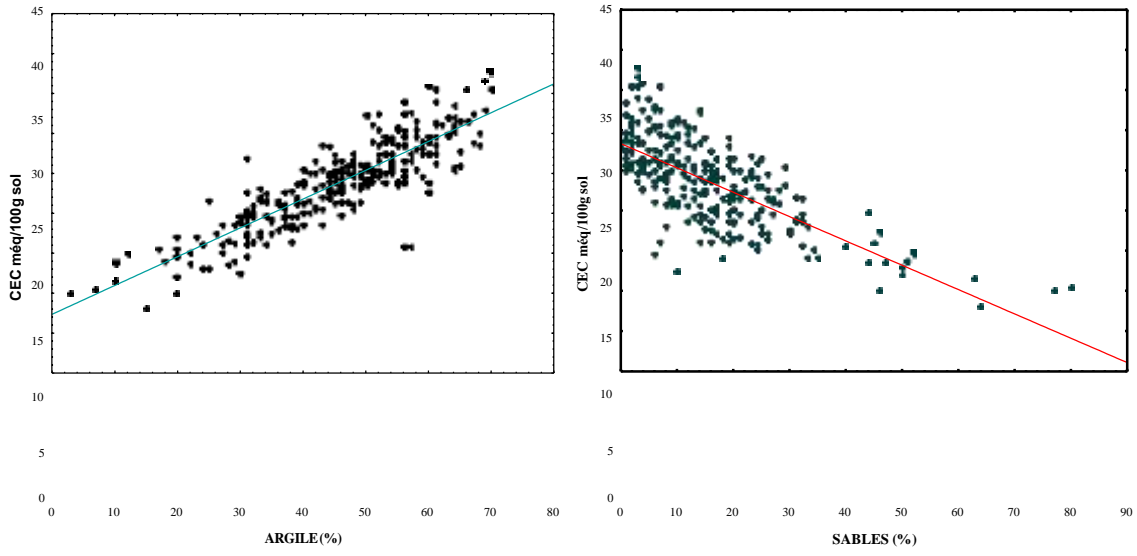


Figure 3. Classes of sands, clays and CEC for the surface horizon.

In the soils of the study area, there are colossally clayey classes around the Bouhilet area and south of Chemora. These grades become less frequent towards Bouhrara Saoudi. The spatial fugacity of the cation exchange capacity shows that the horizons of the size of the study area have high quotients in this element. In addition, in space, the contents of the sands of the surface horizons are practically minimal throughout the area (rate between 0 and 25%). To know if there is a relationship between two variables, it is necessary to identify the form of a possible relationship by establishing a correlation diagram. Our analysis is essentially based on correlation coefficients and simple linear regressions with a number of pairs of 281. We will attempt to discuss the relationships of certain parameters through their correlation coefficients and their regression lines (Figure 4).





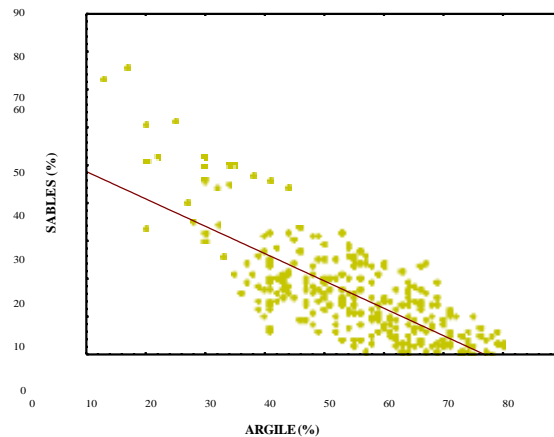


Figure 4. Correlations between the different soil parameters studied.

The scatter plot lines up along the regression line. There is a tendency for CEC to increase when clay levels increase with a positive, highly significant and proportional relationship ( $r = 0.87$ ). The regression equation is of the type:

$$\text{Clay} = 7,3526 + 0,3604 \times \text{CEC}$$

The cation exchange capacity (or CEC) measures the number of electronegative sites on the clay-humus complex. There are permanent exchanges of cations ( $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{K}^+$ ,  $\text{Na}^+$ ) between these electronegative sites and the soil solution. The cation saturation rate corresponds to the percentage of electronegative sites (CEC) that they occupy. These cations are therefore retained by the clay-humus complex (Masson, 2012 in Bouneau and Souchier, 1979).

The relationship between clay content and sands is negative, inversely proportional and very highly significant. The coefficient of this correlation equals ( $-0.75$ ), and the linear regression equation is expressed as follows:

$$\text{Clays} = 48,2713 - 0,7221 \times \text{Sands}$$

Clays and sands are characterized by diametrically opposed properties (sands have low retention capacity unlike clays, sands are highly permeable whereas clays are not, sands do not fix cations against clays, etc.). This phenomenon indicates that the cloud of points stretches along the straight line and shows that high clay content corresponds to low sand content. Thus, when the threshold of 30% sand is exceeded, the point cloud is rather scattered and this relationship is less readable.

There is a negative and significant correlation between the sands and the cation exchange capacity with a torque of 281 ( $r = -0.71$ ). The regression equation generates:

$$\text{Sands} = 28,3593 - 0,303 \times \text{CEC}$$

Some soils (particularly light sandy soils) have a low cation exchange capacity, because sands are rigid particles with no negative charges, which does not allow them to fix cations (Bockman et al., 1990). This confirms the presence of a strong inversely proportional relationship of the CEC with the sands as the regression line indicates. In order to allow us to obtain a vision of the spatial distribution of the cation exchange capacity of the Chemora- Boulhilet plain, the SURFER software makes it possible to produce thematic maps revealing the spatial distribution by producing the variogram relating to the data extracted. of the selected parameter (CEC) using the kriging interpolation method with smoothing of the iso- lines in 2D, then a cartographic

representation of the surface in 3D. All profiles are staked accordingly (comparing them with those staked by MapInfo.8.5 previously) (Figure 5).

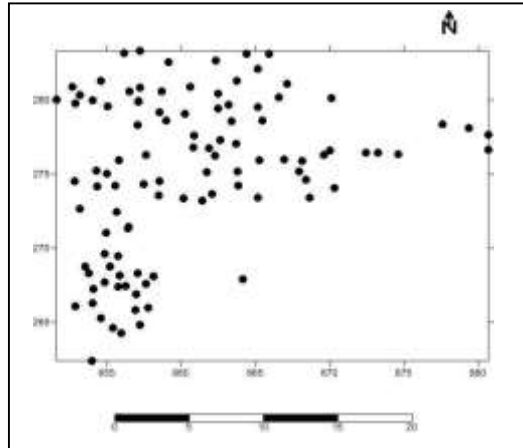


Figure 5. Spatial distribution of profiles by SURFER. 11.

The experimental clay variogram is fitted to a 'Wave Hole Effect' model representing a step (scale) of 24.5 and range (length) equal to 1 km with an anisotropy of ratio 4.4 and an angle  $-25^\circ$  and a nugget effect equal to 1, indicating a micro-variance microregionality) equal to 1 (Figure 6). According to this variogram, the spatial structure shows a regional autocorrelation between the different pairs of CECs up to a distance of 1 km, the latter was taken as a limit to fix the dimensions of the mesh (mesh) used as a step. interpolation by the Kriging method. The plateau indicates the total variance of this parameter and its residual dispersion.

The modeling of this structure by experimental variogram was made with the following spatial characteristics:

- The maximum class distance (Max lag Distance) = 10
- Number of classes (Number of lags) = 25
- Class width (Lag width) = 1
- The vertical value (Vertical scale) =  $40^2$

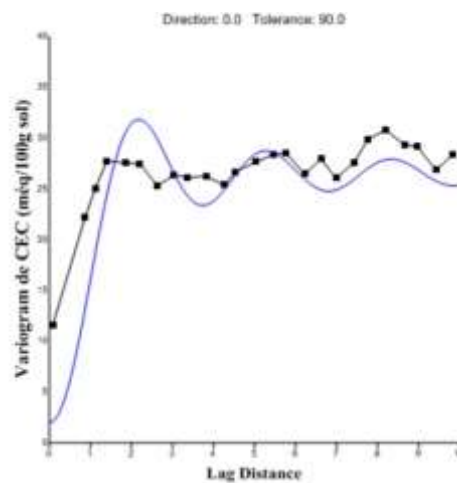


Figure 6. Variogram of sand values created before and after adjustment.

Figure 7 characterizes the areas of high to very high grades illustrated by the dark parts. The majority of the profiles analyzed are characterized by the presence of very high values according to the standards established by Chamayou and Legros (1989), reflecting high levels of exchangeable elements between the clay-humic complex and the soil solution. With the exception of a few which are distributed irregularly in the extremities of the plain (South, South-West of Chemora, and towards Boughrara Saoudi). Zones with high to very high grades are characterized on the map by the dark parts ( $> 20$  meq/100g soil). However, the medium to low contents are characterized by the sparse parts (contents between 10 and 20 meq/100g soil). The values vary from 8 to 39 meq/100g soil.

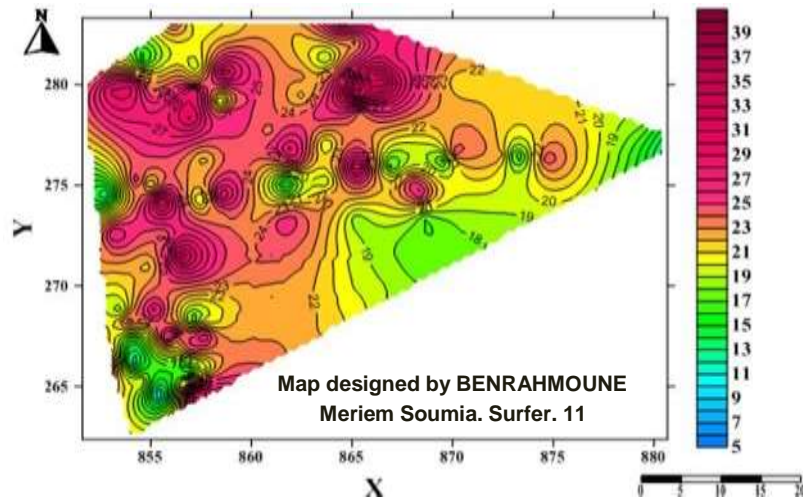


Figure 7. Distribution of CEC contents in iso-lines (2D model).

The SURFER.11 software also allows, from the Krigea map, to visualize the contents of the cation exchange capacity on the space delimited by the available data, and to bring out the model in three (3) dimensions (Figure 8). The observation of the two models shows that the cation exchange capacity has colossal levels throughout the study area. It is mainly controlled by the nature and the clay content (Madani, 2007). In other words, the CEC is therefore influenced in a very highly significant way by the richness of the soils studied in clays. Indeed, our results confirm those found previously in the thematic analysis of the CEC carried out by the MapInfo8.5 software, and in the descriptive statistical analyzes «correlations between the CEC and the clays.

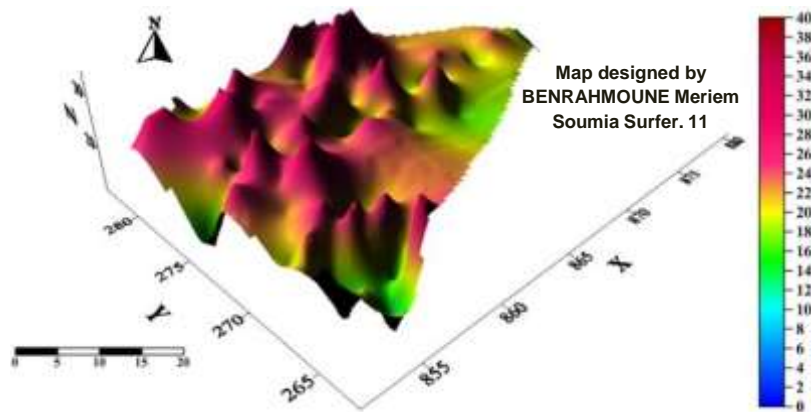


Figure 8. 3D model of CEC grade distribution.

## CONCLUSION

The thematic study of three soil parameters (clays, sands and CEC) in three categories of horizon (H1, H2, and Hp), showed that the soils of the study area have high to very high CEC contents. and clay whereas they are strongly very weakly to weakly sandy. Finally, the calculations showed that each of these descriptors varies more or less strongly in space and in the profile. The relationships between the different parameters show that clays are positively correlated with CEC and total potassium, but not correlated with sands. However, CEC is negatively correlated with sands and statistically highly significant. The maps established the interpolation method by kriging of the SURFER software (version 11) by two types of modeling, one in iso-lines grid (2D) and the other in 3D surface, made it possible to give a vision on the spatial distribution of the Cation Exchange Capacity of the Chemora-Boulhilet plain. In conclusion, SURFER becomes an indispensable tool for researchers in different fields of science by offering maps of good visual quality.

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## **CONTRIBUTION TO THE DIACHRONIC STUDY OF THE SPATIOTEMPORAL EVOLUTION OF THE VEGETATION COVER OF BELEZMA NATIONAL PARK (BATNA, ALGERIA)**

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### **ABSTRACT**

Monitoring the intensity of photosynthetic activity in the Belezma National Park (BNP) is necessary to preserve a better knowledge of the distribution and spatio-temporal dynamics of vegetation. This allows, through the interpretation of remote sensing data and the use of GIS, to detect changes in the state of the cover. For monitoring on a regional scale, spatial remote sensing is required. In this work, we analysed the diachronic evolution of the vegetation cover from the Normalized Difference Vegetation Index (NDVI) data for a period of thirty-four years from 1987 to 2021. However, there is still no consensus on the most effective method for such monitoring. The method implemented is based on twenty Landsat satellite images (TM and OLI/TIRS), forming five study scenes "1987-2021", these are essentially visual analysis of images, after eliminating those containing a high rate of cloud cover. This diachronic analysis revealed that the forest ecosystems of the Belezma National Park have undergone changes that provide information on the different mutations that this region has experienced.

**Keywords:** BNP, Remote sensing, GIS, NDVI, diachronic analysis.

### **INTRODUCTION**

Created in 1984 but only became operational in 1987, north of Batna, the Belezma National Park (PNB) aims to safeguard the 15,000 hectares of cedar threatened with extinction, to maintain the rise of animal species (Bessah, 2005). The vegetation is abundant and varied (the Aleppo pine, the cedar with its floristic procession represented by the holly. The particularity of the park is the presence of the only stand of Etruscan honeysuckle species in danger and the presence of various Orchids. The avifauna is represented by the pink-winged bullfinch, a fairly rare bird in Algeria. The PNB brings benefits that go beyond their borders, its mission, like any protected area in the world, is the protection of wildlife and the preservation and conservation of maintenance of environmental services.

The study of the dynamics of forest vegetation in the Belezma National Park is a subject that generates very relevant questions that deserve to be explored, among which: does the phenomenon evolve in time and space? ? How can it be quantified and by what method?

The management of this floristic richness requires in-depth knowledge of the current state of the vegetation. This knowledge requires the choice of adequate methods. Among these current methods, we cite digital mapping. Of all the vegetation indices proposed since the origins of spatial remote sensing, the normalized vegetation index (Rouse et al., 1974) has become the standard tool for describing the spectral behavior of vegetation cover. Its

usefulness for describing the plant cover is based on the fact that, on the one hand, the latter preferentially (but not exclusively) absorbs light energy in the red wavelengths for photosynthesis, and on the other hand strongly reflects this energy in the near infrared, depending on the inter-cellular structure of the photosynthesizing plant material (Gausman, 1985). Moreover, this index minimizes the variation of illumination according to the relief (Holben and Justice, 1981), but remains sensitive to the turbidity of the atmosphere (Jackson et al., 1983; Crippen, 1988). Various alternatives have been proposed to replace the NDVI, but the latter remains probably the most widely used (Huete, 1988). The normalized difference index has been related to various traits descriptive of canopy physiology, the three most important being leaf area index, percentage of solar energy intercepted by the canopy for photosynthesis, and percentage canopy vegetal. Abnormal variations in NDVI may be linked to an environmental disturbance that has resulted in leaf loss or foliage desiccation. This index, whose robustness has been demonstrated, is strongly correlated with the biophysical parameters of the vegetation. It can be considered as a good indicator of chlorophyll activity and therefore of the vigor of the vegetation. Sensitive to spatial and temporal variations, it is suitable for monitoring the dynamics of vegetation (Huete et al., 2002). Applications have shown its interest in the evaluation of forest dieback, the detection of damage due to drought or the mapping of tree defoliation, in the event that these phenomena lead to a sudden and significant change in photosynthetic activity (Sellers, 1985; Deshayes et al., 2006; Solberg et al., 2007).

The application of computer processing techniques of geographical data from aerospace remote sensing, on the forest ecosystem of the Belezma National Park, the subject of this work, is envisaged in order to provide the various actors (decision-makers, managers, etc.) basic tools that can contribute to the sustainable management of the heritage in question, by providing elements of response to the questions that arise and which constitute the problem of the study. For this purpose, a diachronic study of the images acquired over a period of thirty-four years (1987-2021) was carried out to analyze the changes in the vegetation cover and quantify the evolution of its regression and/or progression. This approach is part of the conservation and integrated management of this ecosystem.

## **MATERIAL AND METHODS**

### *1. Presentation of the study area*

The Belezma National Park is located in the mountainous massif of Belezma which is at the western end of Mont Aurès on the eastern part of northern Algeria; it occupies an area estimated at 26250 ha of the national forest of the massif of which 16091.9 ha constituting the peripheral zone (Beloula, 2010). It is located between longitude ( $X_1$ : 5° 55' 10",  $X_2$ : 6° 10' 45") and latitude ( $Y_1$ : 35° 32' 40",  $Y_2$ : 35° 37' 46"): The national park Belezma is characterized by very rugged relief and slopes greater than 25% (Figure 1). These are rocky bars, cliffs on both sides, culminating peaks up to 2136 m altitude (djebel Tichaou) and narrow valleys. Formed by two 60 km long ridge lines, these mountain ranges are in decreasing altitude going from south to north at any point of the territory of the park (Falek, 2017).

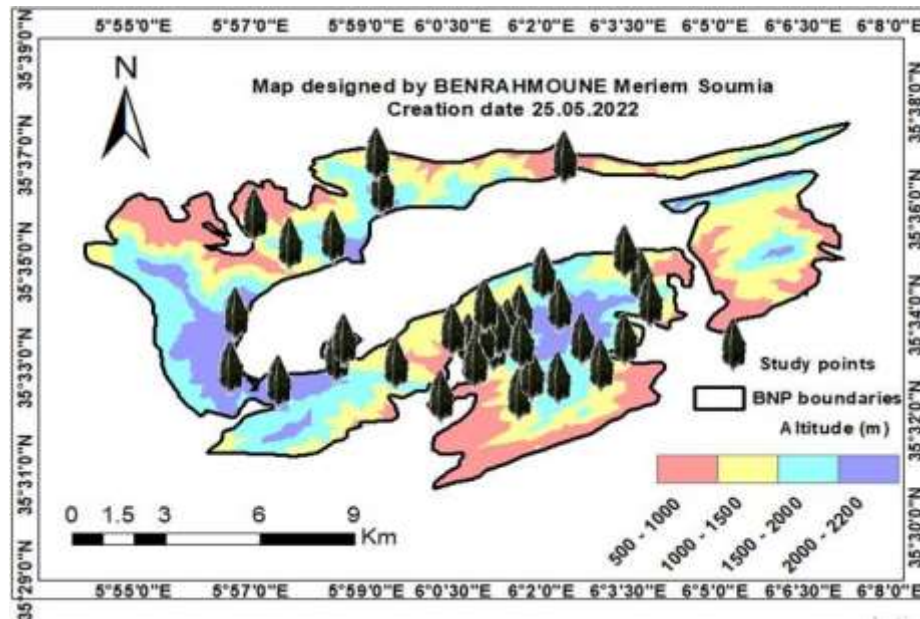


Figure 1. Elevation Map of Belezma National Park (Benrahmoune, 2022).

### 2. Acquisition of satellite images

The Landsat satellite program, managed by the USGS (United States Geological Survey) and NASA (National Aeronautics and Space Administration), allowed us to collect satellite images of Belezma National Park.

### 3. Selection of image dates

For any diachronic study of the vegetation, it is very important to choose the shooting date carefully, because the field conditions vary greatly during the seasons of the year. The remote sensing of the vegetation being based on the photosynthetic activity, we opted for the dry season (from May to August), because the majority of the formations such as the cedar and the holm oak during this season present a very high quantity of green biomass. In addition, during the dry season, the rate of cloud cover (cloudiness) is the lowest, thus making cloud-free image acquisition possible.

### 4. Georeferencing

The downloaded satellite images were imported by changing the projected coordinates by the geographical coordinates of the international system WGS 1984. For each month of the period studied, we imported a scene which presents the monthly values of the vegetation index studied. Thus, by averaging these monthly values for each pixel and for each year of the period studied (1987-2021) to finally obtain a time series of 20 images. The values of this series for a given plant pixel then make it possible to create the interannual profile of the NDVI over this period.

### 5. Image processing

Changes are required for each image; the first corresponds to the extraction of the working area on each image of the dates selected in order to be able to superimpose them, knowing that the influence of each of the scenes covers a surface of a quadrilateral with a side of 185 km. Thus, it is important to reduce this influence to that which includes the entire Belezma park (by applying a mask) in order to lighten future treatments using the same mask (Figure 2).



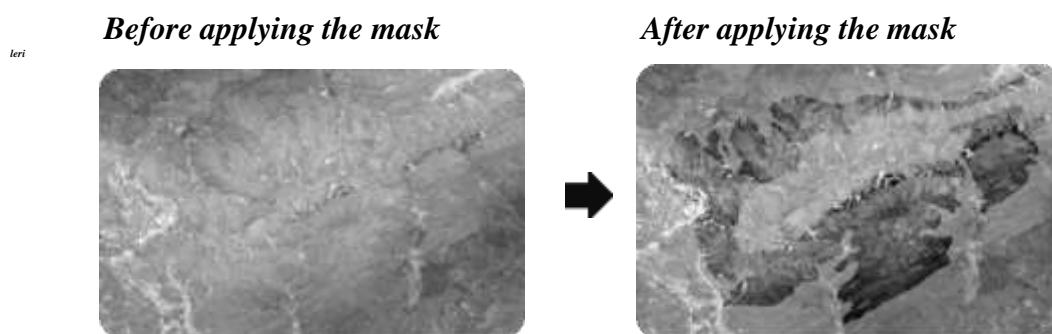


Figure 2. Landsat 8 satellite image (Benrahmoune, 05/19/2022).

#### 6. Calculation of the NDVI vegetation index

In our approach, the main step is to characterize the state of the vegetation by an index that combines, at any point of the image, the reflectance values of different wavelengths. There are a large number of indices constructed for various applications: most combine reflectance values measured in the near infrared (NIR: near infrared) and in the red (R) and the most widely used is the NDVI (Normalized Difference Vegetation Index or Normalized Vegetation Index):

$$NDVI = (PIR - R)/(PIR + R)$$

The result of an NDVI takes the form of a new image, the value of each pixel being between -1 and +1. Negative values correspond to surfaces other than plant cover, such as water, buildings and clouds for which the reflectance in the red is greater than that of the near infrared. For bare soils, the reflectances being roughly of the same order of magnitude in the red and the near infrared, the NDVI presents values close to 0. As for the plant formations, they have positive values, generally between 0, 2 and 0.8. The highest values correspond to the densest cover (Sellers, 1985; Ronald, 2006). It is the analysis of the palette of nuances extending between these extreme values (very infrequent) that informs the observer about the density of the plant cover and the quantity of green biomass.

The images obtained illustrate, spatially and quantitatively, the significant changes in vegetation that have occurred over time in the study region. These changes can be a progression, a regression or a stability of the vegetation cover.

## RESULTS AND DISCUSSION

In this section, we consider that the NDVI is time invariant. Figure 3 shows the intra-annual variability of this index for holm oak, Atlas cedar and the association of the two BNP forest species. The highest values of the NDVI index are always displayed for the association (Atlas cedar + holm oak), while the lowest are recorded for holm oak for the entire study period. In addition, the month of the year 2022 is characterized by a maximum vegetative activity of the forest species of the BNP; the maximum NDVI rate is around 0.236 for the combination of holm oak and Atlas cedar. There is a significant reduction in this activity which results in a significant drop in the values of the NDVI index for the same month of the year 1987. This reduction varies according to the forest stand analyzed, it is significant in the cedar association and holm oak (0.0750).

We mapped the seasonal NDVI index in order to spatially determine the production of forest biomass between the spring season, presented by the month of May, and the summer season presented by the dry period (June, July and August). The pixels that represent the forest surfaces have been segmented into 4 NDVI classes. Negative values correspond to surfaces other than plant cover, such as water, buildings and clouds for which the reflectance in the red is greater than that of the near infrared.

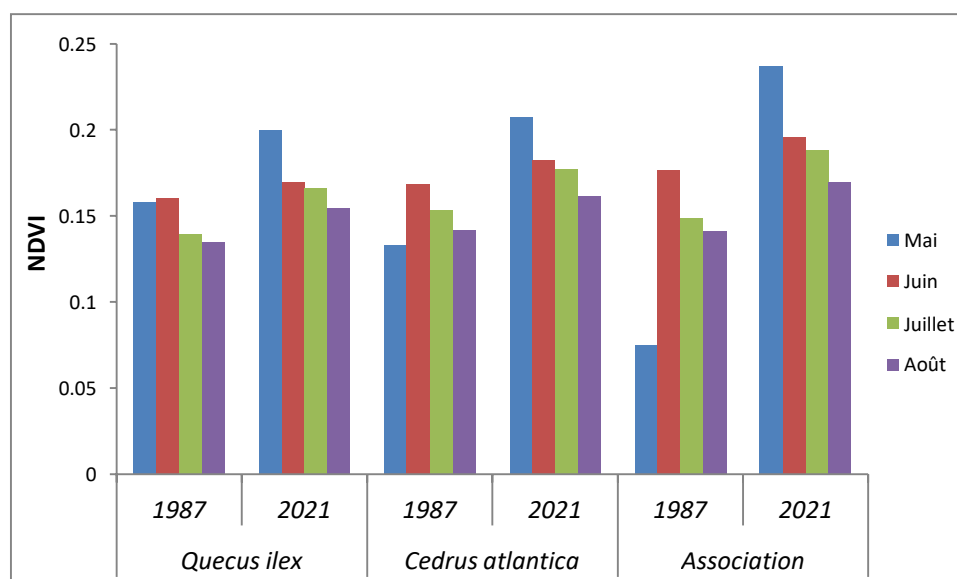
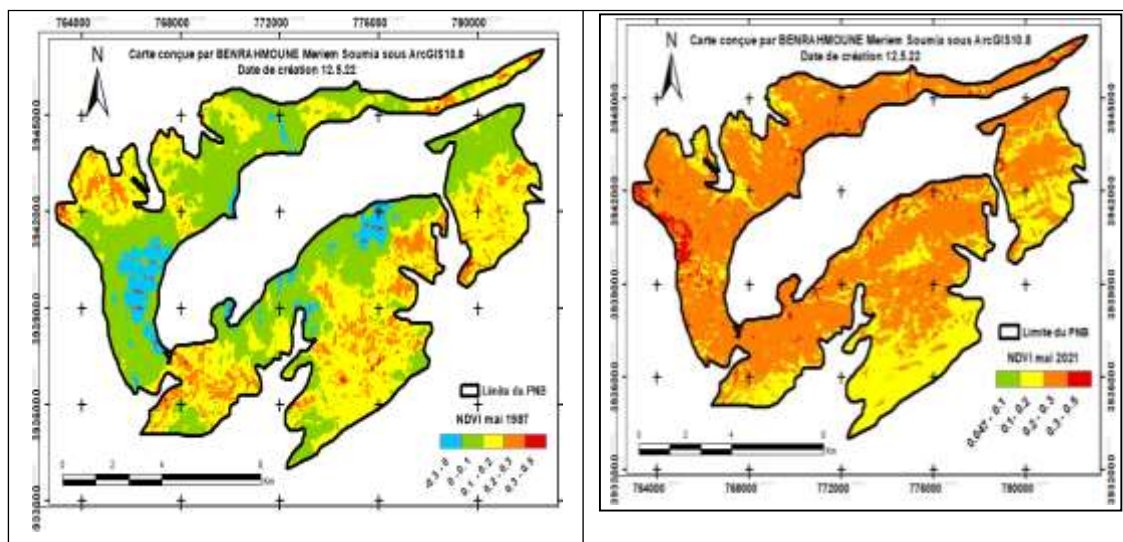


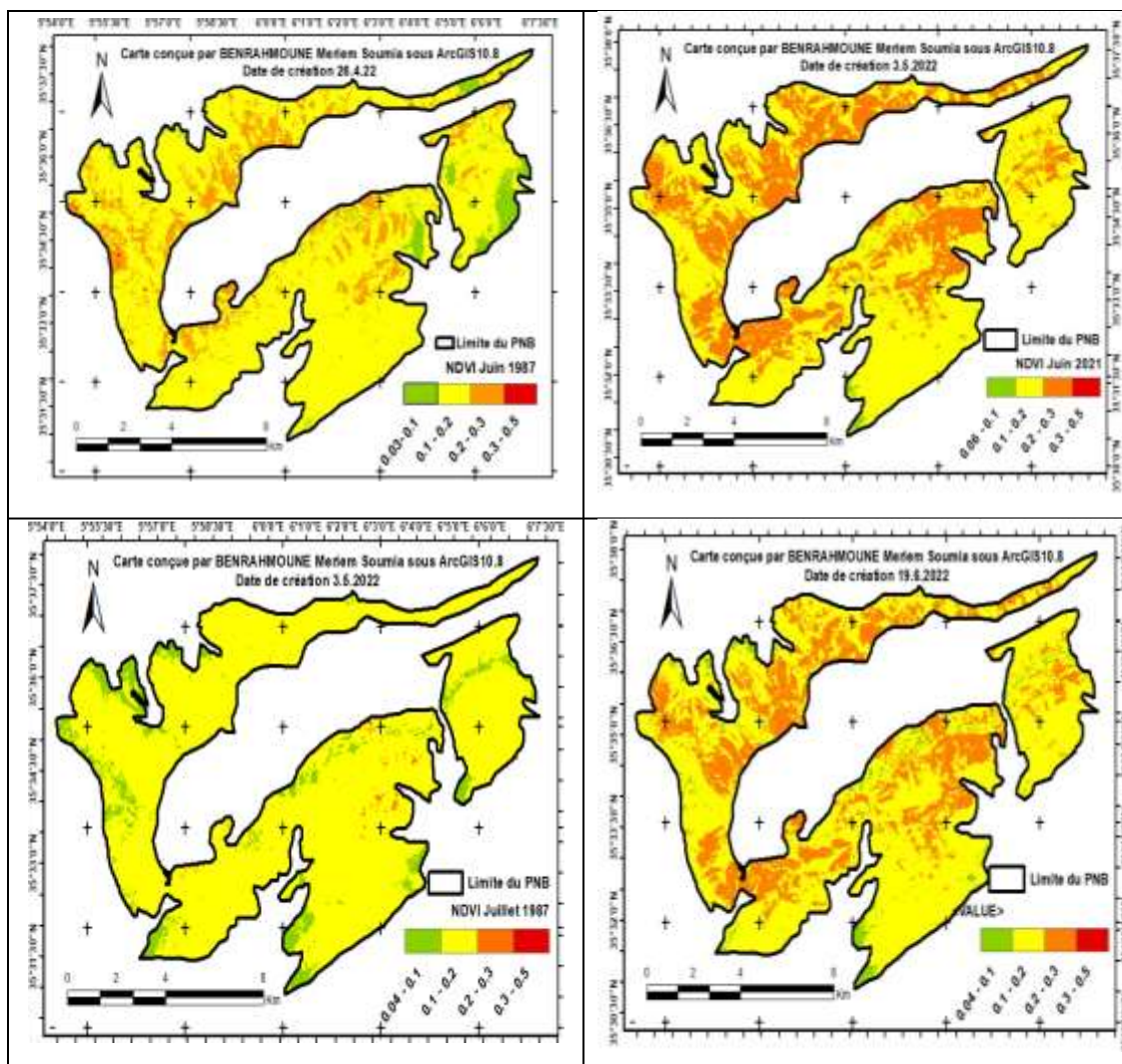
Figure 3. Average intra-annual variation of the NDVI index of different forest ecosystems between 1987 and 2021 in BNP.

We tried to characterize the temporal variability of the NDVI index of forest ecosystems through graphic curves during the spring season and to identify spatially via thematic maps the stressed forest plots where the chlorophyll activity of the leaves presents fluctuations and abnormalities. Part of the forest vegetation of Belezma National Park records an interannual progression of photosynthetic activity which increases between 1987 and 2021 when the NDVI values become more important for this period. According to Pettorelli et al. (2005), NDVI is directly linked to vegetation productivity, biomass and the dynamics of ecosystems, particularly forest environments (Carvalho et al., 2004).

It is widely used as a proxy for estimating plant cover state variables and biophysical processes such as photosynthesis (Tucker and Sellers, 1986). In terms of forest species, the inter-monthly variation of NDVI, presented in Figure 4, was calculated over a period of 34 years. The results obtained show that the three forest stands of the BNP are characterized by a fluctuation of the values of the vegetation index NDVI during the dry period. The results obtained indicate that the maximum value of NDVI is recorded at the level of the month of June for the three forest stands for the two years 1987 and 2022 (Figure 5), while the lowest values are observed at the level of the month of August for holm oak (0.134).



Map 4. Interannual evolution of the NDVI index for the spring season (1987-2021).



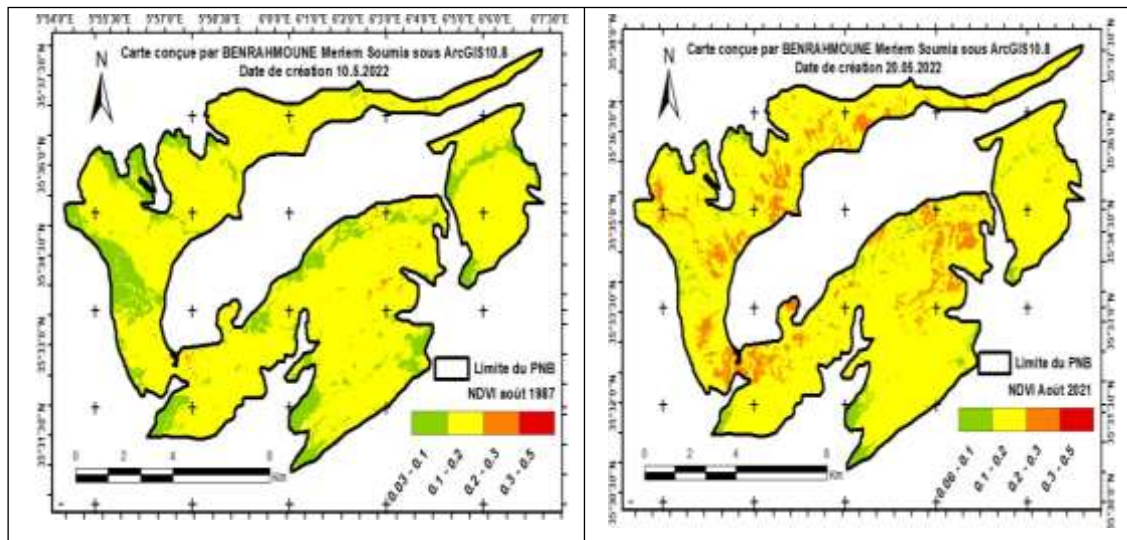


Figure 5. Interannual evolution of the NDVI index for the summer season (1987-2021) (Benrahmoune, 2022).

In terms of the inter-seasonal variability of the forest vegetation, we can see significant NDVI values of part of the forest vegetation of the BNP during the months of June and the month of July of the summer season, reflecting the variability in seasonal vegetative activity and indicate the irregularity and seasonal heterogeneity of NDVI. However, the forest ecosystem displays a strong inter-seasonal irregularity in vegetative activity. Forest areas where vegetation is stressed are easily identifiable.

## CONCLUSION

Geographic Information System (GIS) and remote sensing techniques for processing LANDSAT ETM+ satellite images of Belezma National Park taken from 1987 and 2021, have made it possible to say that it is possible to derive relevant information on the dynamism of forest vegetation. The maps produced give an idea of the spatial distribution of green biomass and the intensity of vegetative activity in the forest stands of Belezma National Park. The NDVI calculated over a period of 34 years (1987-2021), made it possible, on the one hand, to understand the spatio-temporal variability at the interannual and seasonal scale of the forest cover of the National Park of Belezma and on the other hand, to spatially geolocate the forest areas that are experiencing stress peaks and disturbances of photosynthetic activity. In conclusion, precise data, adequate software, remote sensing and geographic information system are important tools to deal comprehensively with vegetation dynamics. In addition, the analysis of the spatio-temporal dynamics of forest vegetation from satellite remote sensing has proven to be an excellent means of investigation to assess the intensity of the natural and anthropogenic factors that contribute to the regression of the Algerian forest.

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## RECOVERY OF RARE EARTH ELEMENTS FROM INDUSTRIAL WASTE

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### ABSTRACT

The use of rare earth elements in industrial products has increased worldwide. Advanced industrial wastes have led researchers to focus on REE recovery. Recent advances in bioprocess technology have created viable avenues for REE recovery from waste materials. In this study, the recovery of REE resources from industrial wastes was investigated. As a result of the analyzes it is suitable to obtain REE biological recovery from waste materials. Available literature includes REE contaminated soils near REE mines, coal mines, heavy traffic roads and agricultural soils (due to REE incorporation with phosphate fertilizers). First, the traditional separation methods used in the mining industry and their main methods of removing/precipitating REE are described. Then, soil improvement techniques used to improve REE are highlighted. Soil remediation techniques that enable REE extraction are one step closer to resource recovery, which contributes to the cyclicity of REE. Techniques such as phytoremediation, soil washing, and electrokinetic treatment show promising extraction results.

**Keywords:** rare earth elements, bioprocess, biological recovery, remediation techniques

### INTRODUCTION

Rare earth elements (REE) are found in “green technology” (Dodson et al., 2012). From a sustainable and clean production perspective, it is imperative that all types of waste are harvested and used throughout their life cycle (Markard et al., 2012). REE consists of 17 elements. REEs are soft, machinable and good electrical conductors. This makes them suitable for applications in a variety of key technologies such as automotive, nuclear, petroleum, electronics, renewable energy, phosphorus, Ceramic, fertilizers and even biological materials (Ramos et al., 2016). REE production has increased from 124,000 tons in 2010 to 167,000 tons in 2018, a 35% increase over the past 8 years (USGS, 2000). Worldwide reserves are around 99 million tons (UNEP, 2012). Because REE recycling is so weak, pressure on mining and intact resources intensifies as demand for REEs increases. In 2018, Jowitt et al. (2018) suggest that only 2.8% (10,683 out of 375,000 tonnes) of all disposed REE is recycled.

Today, global production of REEs is led by China (85%), followed by Australia (10%), Russia (2%), India (1%), Brazil (1%), Malaysia and Vietnam (Zhou et al., 2017). The global production, reserves and imports of REEs to the USA are presented in Figure 1. In order to

eliminate such supply shortages, intensive efforts are also made to remove REEs from industrial wastes (Önal and Binnemans, 2019). The specific REE requirements for some common industrial products are presented in Table 1; and the production of REEs from major wastes are also presented in Table 2.

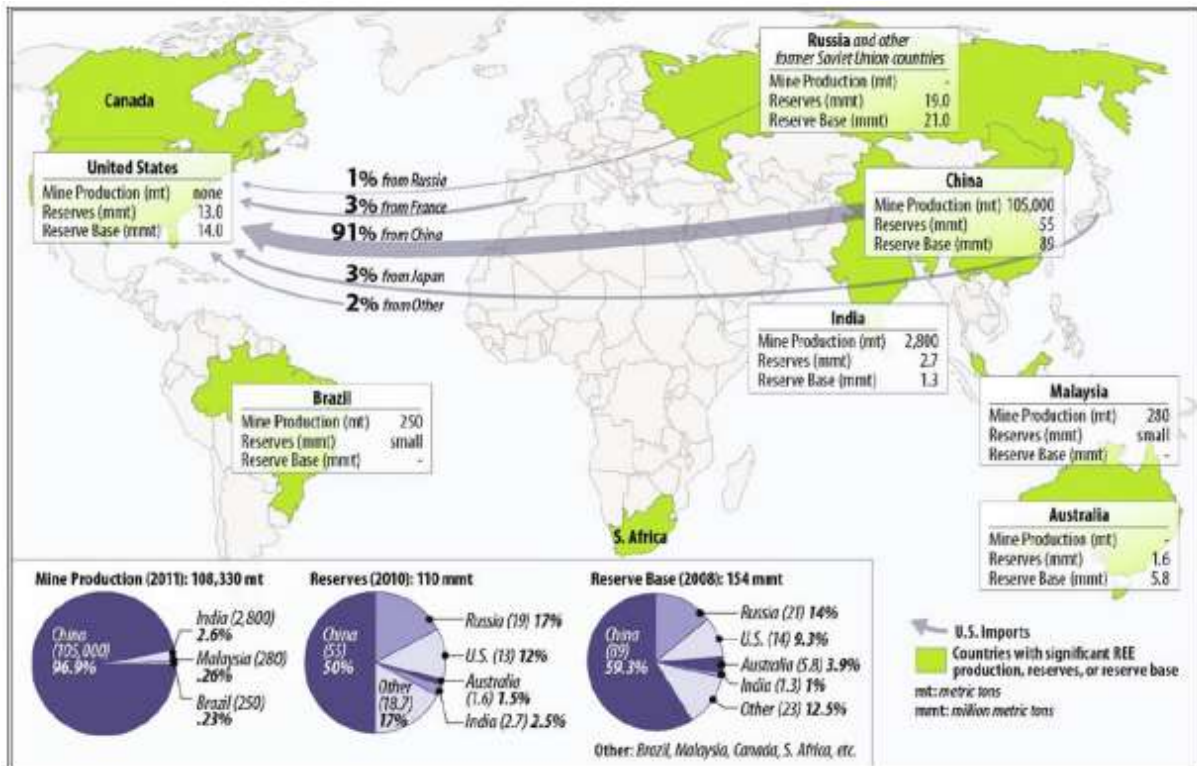


Figure 1. Global production and storage of REEs and their import into the United States.

Note: The reserve base represents the total amount of economic, sub-economic and marginal economic resources, while reserves represent the portion of the resource base that can be economically mined and produced on the market (adapted from Humphries, 2013).

In this article study, the general characteristics of REEs, the presence of REEs from industrial wastes, the recovery of REE from primary and secondary sources, the biological recovery of REEs and the factors affecting it, the issues that will be used in the future for the improvement of the areas exposed to REE lock are stated.

**Table 1.** Percentage of REE used in the production of some common industrial products (Binnemans et al., 2013)

| Production                  | NTE%* |    |    |    |    |    |    |    |    |   |        |  |
|-----------------------------|-------|----|----|----|----|----|----|----|----|---|--------|--|
|                             | La    | Ce | Pr | Nd | Sm | Eu | Gd | Tb | Dy | Y | Others |  |
| Magnet                      |       |    | 23 | 69 |    |    | 2  |    | 5  |   |        |  |
| Batteries                   | 50    | 33 | 3  | 10 | 3  |    |    |    |    |   |        |  |
| Metalurgy                   | 26    | 52 | 5  | 16 |    |    |    |    |    |   |        |  |
| Auto catalizor              | 5     | 90 | 2  | 3  |    |    |    |    |    |   |        |  |
| Liquid catalysis extraction | 90    | 10 |    |    |    |    |    |    |    |   |        |  |
| Polishing dust              | 31    | 65 | 3  |    |    |    |    |    |    |   |        |  |
| Glass additives             | 24    | 66 | 1  | 3  |    |    |    |    |    | 2 | 4      |  |
| Phosphorus                  | 8     | 11 |    |    |    | 5  | 2  | 5  |    |   | 69     |  |
| Ceramic                     | 17    | 12 | 6  | 12 |    |    |    |    |    |   | 53     |  |
| Others                      | 19    | 39 | 4  | 15 | 2  |    | 1  |    |    |   | 19     |  |

**Table 2.** Concentration of REEs in different waste materials/waste streams (Dev et al., 2020).

| Source             | PPM       |           |            |          |            |            |           |            |            |            |             |            |              |       |  |
|--------------------|-----------|-----------|------------|----------|------------|------------|-----------|------------|------------|------------|-------------|------------|--------------|-------|--|
|                    | La        | Ce        | Pr         | Nd       | Sm         | En         | Gd        | Tb         | Ho         | Er         | Tm          | Yb         | Ln           | Y     |  |
| phosphogypsum      | 1450      | 2310      | 235        | 899      | 163        | 34.9       | 98.7      | 7.4        | 7.4        | 15.7       | 1.4         | 5.6        | 0.6          | 180   |  |
| Red bulk           | 114       | 368       | 28         | 98.6     | 21.3       | 5          | 22        | 3.5        | 4.3        | 13.5       | 1.9         | 14         | 2.4          | 75.7  |  |
| Fly dust           | 91.4      | 195.6     | 23.5       | 88.6     | 18.2       | 3.4        | 16.4      | 25         | 2.8        | 6.9        | 1.2         | 6.5        | 1            | 62.1  |  |
| Mine waste         | 903       | 2047      | 239        | 906      | 148        | 19.1       | 138       | 16.2       | 17.3       | 54.1       | 5.3         | 38.3       | 4            | 664   |  |
| Acid mine drainage | 0.01-0.09 | 0.07-0.09 | 0.006-0.03 | 0.02-0.1 | 0.006-0.06 | 0.001-0.02 | 0.008-0.1 | 0.001-0.02 | 0.001-0.01 | 0.002-0.03 | 0.0000-2.00 | 0.001-0.01 | 0.0002-0.002 | NA    |  |
| NdFEB magnet       | NA        | NA        | 3.4        | 259.5    | NA         | NE         | NA        | NA         | NA         | NA         | NA          | NA         | NA           | NA    |  |
| NiMH batteries     | 237       | 67        | NA         | 36       | NA         | NA         | NA        | NA         | NA         | NA         | NA          | NA         | NA           | NA    |  |
| Phosphorus         | 3.8       | 4.9       | NE         | NA       | NA         | 4.4        | 25        | 27         | NA         | NA         | NA          | NA         | NA           | 112.2 |  |

Na: Not available

## METHODS OF SEPARATION OF NTE FROM ORE

### Presence of REEs in Industrial Wastes

Industrial wastes can be considered as potential sources of REE. Common industrial wastes used for REE extraction include mineral processing wastes (phosphogypsum, red mud) and coal processing wastes (fly ash). Mine tailings and acid mine drainage sources are the main sources of mining waste for REEs.

It is essential to develop new technologies with high added value in order to ensure technological development and increase international competitiveness. In order to achieve this goal, critical raw materials such as REE should be researched, produced and used in advanced technological products in order to meet the raw material needs in priority areas.

In addition to flotation, magnetic and gravity enrichment methods, hydrometallurgical methods are also used in the recovery of REE from minerals (Krishnamurthy and Gupta, 2015). In catalytic converters, mostly cerium and lanthanum elements are preferred. Europium is used in



compact fluorescent light bulbs, televisions and mobile phone displays. Rare earth element alloys such as ScAl, Sc-Mg, Y-Al, Y-Mg and Nd-Mn are also widely used in metallurgy due to their resistance to corrosion at high temperatures and their anti-oxidation properties (Yıldız, 2016; MTA, 2017).

## **RECOVERY OF RARE SOIL ELEMENTS FROM PRIMARY AND NECONDARY SOURCES**

Although many minerals contain REE, the most common minerals from which REE is produced are monazite, bastnasite, and xenotime. In addition to these, an important REE source is ion adsorbing clays (Haque et al., 2014). Loparit is used in REE production in Russia, but its share in world total production is less than 3% (Kosynkin et al., 1993). In addition to these minerals, secondary sources containing important REE are waste magnets, fluorescent lamps, catalysts and rechargeables. In addition, coal and fly ash stand out as potential REE sources (Kurşun and Terzi, 2018).

### **Recovery of rare earth elements from primary sources**

Flotation, magnetic, electrostatic and gravity separation (shaking table, spirals) methods or combinations of these methods are highly preferred in REE recovery from ores. Ores containing REE are found together with barite, fluorite, calcite, silicate and iron minerals, which makes mineral enrichment processes difficult.

### **Physical/physicochemical methods**

The world's largest REE producing ore deposits are located in Mountain Pass (USA, California) and Bayan Obo (China). In addition to REEs from ores, magnetite, fluorite, hematite and niobium oxides are also recovered as by-products. Bayan Obo ore containing bastnasite and monazite, which was started to be mined as iron ore in 1927, is accepted as the largest iron deposit in China (1.5 billion tons of reserves). The Bayan Obo plant has three different process streams. After grinding the ore to  $-74 \mu\text{m}$  (d90), flotation is applied. In the coarse flotation process,  $\text{Na}_2\text{CO}_3$  is used as a pH regulator,  $\text{Na}_2\text{SiO}_3$  is used to suppress iron and silicates, and sodium salt (paraffin soap) is used as a collector. It is possible to achieve 80% REE gain after rough flotation and gravity enrichment. Finally, a monazite concentrate containing 47% REE and bastnasite concentrate containing 68% REE are produced (Jiake and Xiangyong, 1984).

### **Hydrometallurgical methods**

Another REE recovery method is suitable hydrometallurgical methods from ores or concentrates (60-70% REE) produced by physical separation methods. Acidic ( $\text{H}_2\text{SO}_4$ ,  $\text{HNO}_3$ ,  $\text{HCl}$ ) or alkaline ( $\text{NaOH}$ ) reactive systems are preferred in the leaching of REEs, and purification and recovery methods including REE precipitation/solvent extraction/ion exchange methods are preferred for the recovery of REE from charged leaching (Akkurt et al., 1993; Kumar et al., 2014).

### **Recovery of rare earth elements from secondary sources**

The most common magnets containing REE are those containing Nd-Fe-B. The increase in the prices of strategically important elements such as cobalt has led to the production of cobalt-free permanent magnets. Depending on these developments, Nd-Fe-B based permanent magnets were obtained by choosing traditional powder metallurgy and melting methods in 1983 (İçin, 2016). The sintering method is the most common powder metallurgy method among production methods.  $\text{Nd}_2\text{Fe}_4\text{B}$  sintered magnets have high magnetic properties. The amount of energy

produced by this phase per unit volume is approximately 512 kJ/m<sup>3</sup>. The most important reason why NdFeB magnets have high permanent magnetism comes from a strong ferromagnetic interaction between REE (Nd) and the transition metal (Fe) that forms the composition of the magnet (Icin, 2016).

## BIOLOGICAL RECOVERY OF RARE SOIL ELEMENTS

### Bioleach

Bioleaching is carried out by both autotrophic and heterotrophic microorganisms; The type of mineral is associated with the selection of microorganisms. Autotrophic microorganisms are employed for the extraction of Sc from ore minerals containing polymetallic sulfide, while heterotrophic microorganisms are mostly preferred for carbonates and phosphate-rich minerals. Heterotrophs have been preferred in most studies for REE extraction by bioleaching and require different supplements to provide electron donors and carbon sources (Zhuang et al., 2015). For the heterotrophic microorganism-mediated extraction process, the production of organic acids and metal-binding molecules is required. Organic acids include oxalic, gluconic, acetic, citric, formic and malic acids (Fathollahzadeh et al., 2019) that lower the pH and help REEs leach, while metal-binding molecules are also used as chelating agents to separate target molecules from solution (Hopfe et al., 2018). *Pseudomonas*, *Enterobacter*, *Serratia* and *Bacillus* bacteria have been found to exhibit a wide variety of REE recovery capacity from monazite ores (Shin et al., 2015; Brisson et al., 2016). The syntrophic relationship of native microorganisms in ore bodies may describe a higher bioleaching capacity than pure culture alone (Corbett et al., 2018) (Figure 2).

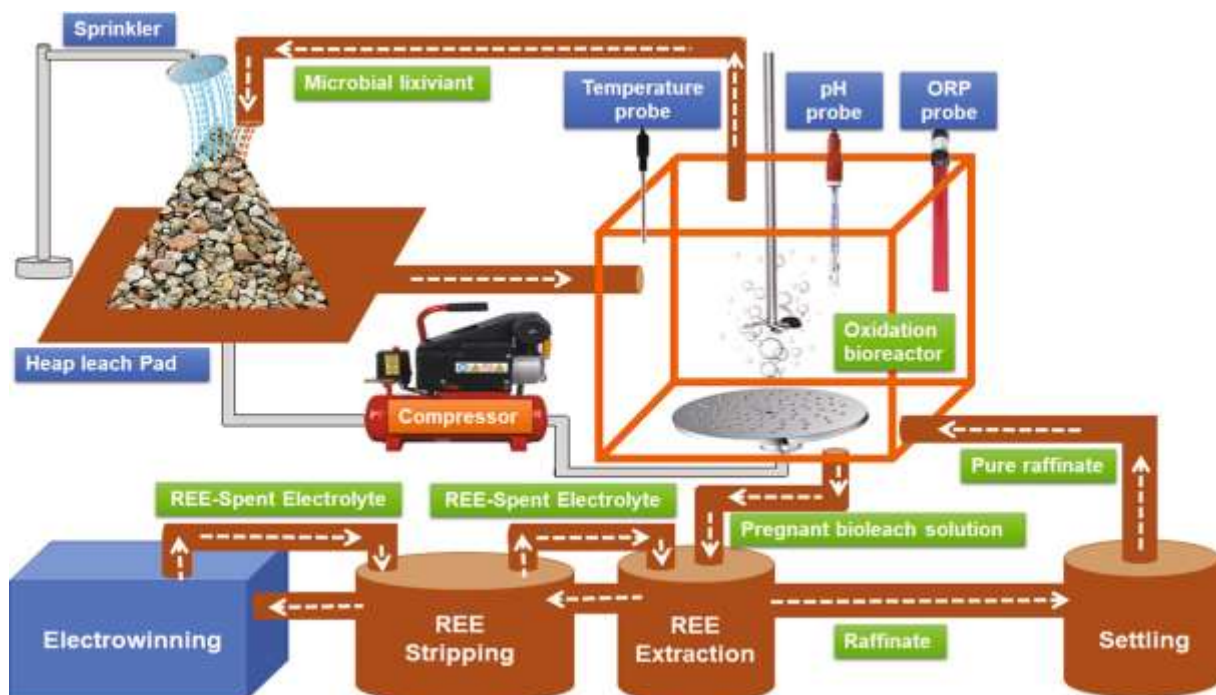


Figure 2. Schematic diagram of the field unit operation of heap-bioleaching system (Dev et al., 2020).

## **Biosorption**

Biosorption is highly preferred in the stages of recovery of REEs from aqueous solutions due to its various advantages such as recovery efficiency, minimum sludge production and simple processing (Das and Das, 2013). Adsorption of REEs on the surface of a microbial cell includes electrostatic interactions, ion exchange, surface complex formation and precipitation (Andres and Gerente, 2011).

## **Bioaccumulation**

Bioaccumulation is a metabolically active process involving the intracellular uptake of REEs adsorbed on the cell surface. REEs are first adsorbed on the cell surface, and then the importer complex in the membrane lipid bilayer transfers the REEs into the intracellular space (Maleke et al., 2019). Subsequently, REEs are extracted by proteins and peptide ligands located in the intracellular space (Merroun et al., 2003). It has been stated in previous studies that a wide variety of microorganisms bioaccumulate REEs from environmental sources. Bioaccumulation of Ce and Nd of *Bacillus cereus* bacteria isolated from rare earth enriched soil Emmanuel et al. (2008) and it was also found that exposure to Ce increased the expression of the COOH group on the bacterial cell surface. However, the low solubility of REEs in lipids is also considered a limiting factor for REE bioaccumulation (Ochsner et al., 2019).

## **RESULTS**

This study includes technologies that rehabilitate and remove REE polluted receptors. REE and metals are pollutants in the soil environment and are in high demand in industrial and technological applications. Remediation methods should aim to recover and recycle REE.

REE contaminated sites include REE mines, coal mines, farmland, landfills and roads. Concentrations detected in contaminated sites are lower than in ores from which REE is mined, and the soil binding mechanisms of these concentrations are also different. In addition, soils can be a good source of REE, albeit dilute, although the concentrations detected are lower than the ores from which REE is extracted. As a result, there is a need for new technologies besides the traditional REE separation methods used in the mining industry. Therefore, the center of future research should be on REE extraction from contaminated receiving environments mainly targeting critical REE (Y, Nd, Eu, Tb, Dy and Er). There are very few studies on extracting REEs from polluted soils. Research so far has focused on subtraction or immobilization. Higher level studies include bioremediation and soil remediation with REE adsorbing chemicals or particulate materials, but these methods also run counter to the goal of recovery. Phytoremediation, soil washing and electrokinetic treatment methods have demonstrated the ability to simultaneously activate, remove and improve soils REE, so the future development of combined methods for REE recovery from polluted sites is at the top of the list.

Bioprocess technologies have generally been applied to extract REEs from key sources such as monazite ores. When it is necessary to work with low-grade REE in industrial wastes, the content of future research topics comes from the application of microbial bioleaching technology for high-efficiency REE recovery. Integrating lab-scale research at pilot and field scale will result in the successful implementation of a less costly bioprocess technology for industrial-grade REE biorecovery. The development of genetically engineered and metal-resistant bacteria or the gradual adaptation of bacteria to high concentrations of heavy metals can also increase heavy metal toxicity to a reasonable level during REE bioleaching. At the same time, using pure substrates such as sucrose, tryptone and glucose to support microbial growth during the bioleaching process will increase the operating cost. Given all these reasons, managing a cost-effective biorecovery process of REE on an industrial scale will require a

diverse cross-discipline research effort, including genetic engineering, microbiology, chemistry, and biohydrometallurgy. When a cost assessment is made among all these methods; REE provides a significant income from biological leaching compared to traditional chemical leaching. Methods such as the adaptation of microorganisms to high metal concentrations and the use of cost-effective substrates will shed light on future studies to achieve REE biorecovery and improve sustainable biorecovery of REE from waste materials.

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## FOOD WASTAGE IN A TUNISIAN UNIVERSITY CANTEEN: IMPACT OF AN EDUCATION AND AWARENESS CAMPAIGN ON STUDENTS' ATTITUDES AND BEHAVIORS

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### ABSTRACT

**Background:** Food wastage has tremendous negative consequences on food security and nutrition, as well as on environment and economy. Because of the changes in food consumption patterns, collective caterings such as University canteens and restaurants are becoming a significant source of losses and waste. Thus, implementing intervention strategies become urgent. Such strategy could be reached through a combination of educational, technical and administrative measures.

**Objective:** This study aimed at evaluating the impact of an *online* and *onsite* education and awareness campaign about food wastage on students' attitudes and behaviors.

**Methodology:** The study was undertaken in a University canteen "Les Jardins" (University of Carthage, National Institute of Agronomy of Tunisia, Tunisia). An awareness and education raising campaign named "*Allech Hakka*" ("*Why like this*") was designed, launched online on social media (Facebook/Meta© and Instagram©) and onsite, through a Day of Awareness of Food Loss and Waste. Posters were posted online weekly for two months. The Day consisted on three conferences and a discussion on food wastage and 3R (reduce, reuse, recycle) strategies. After interventions, face to face interviews based on a structured questionnaire, were conducted on 143 student respondents (mean age 23 years old, 60.4% women, 70.3% regular canteen users) during two weeks.

**Results:** Survey respondents admitted to always (6.3%) or regularly (42.3%) discard food products. They estimated their level of wastage to be less (40.5%) or similar (52.2%) to those of other students. However, they affirmed to be shocked (72.1%) by the canteen food wastage level, presented in one post. About the online campaign, 65.8% of respondents have followed it, on social media. Most of respondents (73.8%) have appreciated it (mean score 3.94/5), in particular its information clarity (82%, mean score 4.08/5) and its graphic design (76.5%, mean score 4.08/5). About 73.9% have "liked", and 26.1% have "shared" posts. However, a small minority have attended to the awareness raising day, indicating a higher adherence of youngsters of actual communication channels, namely the Internet and possibilities offered by smartphones. Interestingly, most of respondents affirmed that the campaign "*Allech Hakka*" would lead to change surely (28.8%) or probably (67.6%) their behaviors toward food wastage. A majority affirmed they would reduce (64%) or even stop (26.1%) their food waste. Main reasons were ethical (77.5%), economical (71.2%), religious (63.1%), and environmental (54.1%).

**Conclusion:** Our study has shown that launching a simple and inexpensive information and education campaign about reducing food waste has increased students' awareness and improved their attitudes. It also pointed out the importance of the target-oriented choice of communication channels. This campaign should be duplicated across other Tunisian universities. The outcome of these studies would help organizations and policymakers design better targeted actions and measures for food waste reduction.

**Keywords:** Food waste, awareness campaign, students, *online* and *onsite* education.

## INTRODUCTION

Food loss and waste is a challenging issue. Recent estimates of the Food and Agriculture Organization of the United Nations suggest that globally around one third of food never reaches a human stomach (FAO, 2011), and global food waste is associated with large amounts of greenhouse gas emissions (FAO, 2013). Accordingly, consequences of food waste reach the social, economic and environmental scale. Reducing food waste may contribute in reducing hunger that rose to 874 million in 2022 (FAO, 2022). Sustainable development goal set food waste reduction as a target. The 12.3 goal aims by 2030, to halve per capita global food waste at the retail and consumer levels and reduce food losses along production and supply chains, including post-harvest losses (Reynolds et al., 2019). Such fact, highlight that food waste is a universal problem.

This waste concerns not only production, harvesting, transport and storage but it also involves mainly processing, distribution and consumption (FAO, 2013). In Europe, the main food wastage is generated by households (53%), food industries (19%), collective catering (12%) and by distribution (5%) (European Parliament, 2017). In Near East and North Africa region, food waste has been estimated to 250 Kg per year per individual and, at the consumption stage, to be 34% (FAO, 2015). Thus, it is urgent for researchers, practitioners and policy makers to understand how to prevent food waste across supply chain, particularly consumption stage (Reynolds et al., 2019). Several strategies could be suggested such as improving planning and purchasing, repurposing kitchen leftovers, offering customers smaller portion sizes and takeaway bags, launching awareness campaigns (Vizzoto et al., 2021).

As mentioned, food waste occurs also at collective catering. Particularly, in the context of university, food waste could be higher weather at preparation stage because of the high quantities to prepare or also at consumption level. In fact, the low meal fees may encourage students to waste food (Ellison et al., 2019). To overcome, waste problem both actors are concerned (canteen staff and consumers: students). At staff level, efforts could be conducted on like: reducing overcooking, reusing leftover, adjusting portion size, serving style adjustment (Vizzoto et al., 2021). Regarding consumer, improving his behavior is the key action. To do so, different strategies could be adapted. For example, offering financial incentives could encourage consumers to improve their behavior (Neff et al., 2015). Or, contrarily, applying penalties would also have a positive impact on consumers' behavior. Awareness campaigns represent another way of affecting consumer behavior as it targets his beliefs (Kuo & Shih, 2016). Moreover, such approach at university scale could be considered a low cost solution contributing in sustainability at university campus (Ellison et al., 2019). A review by Reynolds et al. (2019) reported that information campaigns could reduce up to 28% of food waste. On the same context, a student-focused education campaign conducted by Martins et al. (2016) lead to a 33% waste reduction in main dishes. Accordingly, this research aimed at evaluating the impact of education and awareness campaign about food wastage on students' attitudes and

behaviors. Considering students' specificity, campaigns were diffused in two ways: *online* and *onsite*.

### MEHODOLOGY:

The study was undertaken in a University canteen "Les Jardins" (University of Carthage, National Institute of Agronomy of Tunisia, Tunisia). An awareness and education raising campaign called "*Allech Hakka*" ("*Why like this*") was designed, launched online on social media (Facebook/Meta© and Instagram©) and onsite by sticking posters at different sites on the campus: entry, canteen, classrooms, administration, and departments. Posters were posted online weekly for two months from May to July 2020. Then, a Day of Awareness of Food Loss and Waste was organized: three conferences and a discussion on food wastage and 3R (reduce, reuse, recycle) strategies. Both online and onsite campaign were evaluated through a survey. Answers were collected online and also through face to face interviews with 143 students: mean age 23 years old, 60.4% women, 70.3% regular canteen users.

### RESULTS:

#### *Students' attitude toward food waste:*

As shown on Table 1, only 7.2% declared to never waste food while 92.8% do weather it is always, often or rarely. Although, discarding food is a negative behavior. Admitting it may represent a promoting result. In fact, to solve a problem it is crucial to admit its existence. More than half interviewed students reported that their food waste level is the same as other students and persons on their age. Such finding could explain why we can observe such phenomenon among students: I behave like other, and then it does not represent a problem. Discarding food would be common. Interestingly, 72.7% were choked by statistics communicated through published posters. Such fact might be considered as an indicator of campaign success and efficiency as students recognized the importance of this issue. Consequently, their attitude may change as they could see clearly the impact of a behavior they used to consider common and to which they did not use to attribute a high importance.

**Table1:** Student perception toward food waste (n=143)

|   | <b>% Respondents</b> |
|---|----------------------|
| Food waste frequency  | 6.3 Always           |
|   | 42.2 Often           |
|   | 44.1 Rarely          |
|   | 7.2 Never            |
| Esteemed food level compared to other students and persons on the same age        | 7.2 Higher           |
|   | 52.3 The same        |
|   | 40.5 Less            |
| Regarding food waste all over the world and at university do you find statistics? | 72.7 chocking        |
|   | 25.2 Common          |
|   | 2.1 No opinion       |

*Evaluation of rising awareness campaign:*

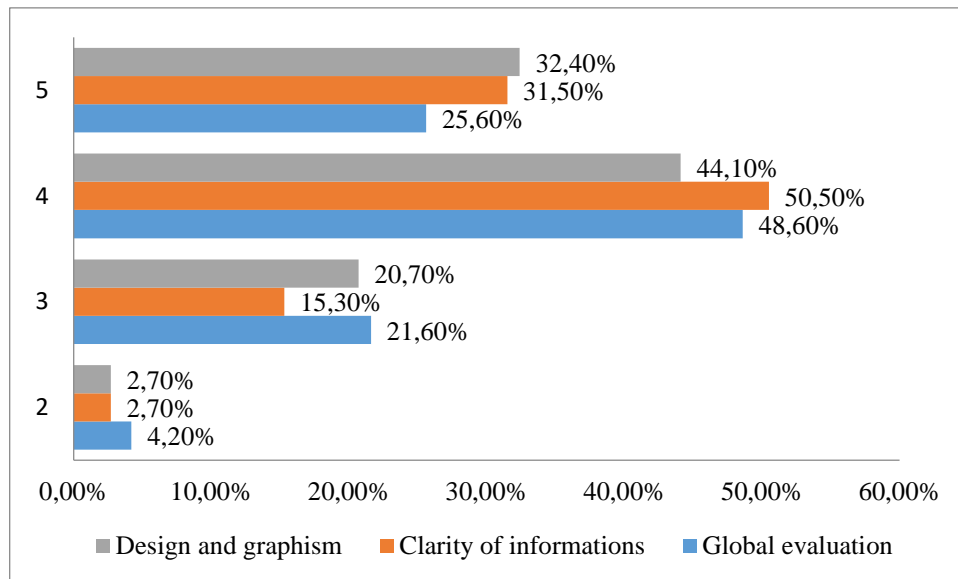
To assess the efficiency of the conducted measures a post-survey was conducted. Almost 60% of respondents noticed the onsite measures and 65.8% followed the campaign on social media. Thus, we can consider that interventions were noticed by students. On the same context, social media seem the best channel of communication with students. When asked about the onsite event, 81.2% reported that they heard about it thanks to social media. This finding might be explained by the high use of social media in Tunisia. According to Meta-insight platform: 72.75% of Tunisians actively use social media where 46.5% are women and 53.5% are men. Moreover, 24.3% of users are aged between 18-24 and 32.4% between 25-34 year (Digital-discovery, 2022).

**Table 2:** Penetration of awareness campaign (n=143)

|   | <b>% of respondents</b>        |
|---|--------------------------------|
| Did you notice the presence of posters on the campus?                               | 59.8 Yes                       |
|   | 40.2 No                        |
| Did you follow the awareness campaign on social media?                              | 65.8 Yes                       |
|   | 34.2 No                        |
| How did you know about the organization of Day of Awareness of Food Loss and Waste? | 81.2 Social media              |
|   | 13 Colleagues                  |
|   | 5.8 Institute official website |
| Did you attend this in person event?  | 26.9 Yes                       |
|   | 73.1 No                        |

Students were asked to evaluate the quality of the posters used by attributing a mark from 1 to 5 for several criteria like: clarity of information, the design and their global evaluation. As shown on Fig.2, regardless the criteria all marks are above the average particularly for information clarity where half respondents (50.5%) attributed 4/5 and 31.5% gave 5/5. These results highlight that content of the intervention was appreciated by students and basically observed through social media. However, this was not enough to get students involved in the onsite intervention. Students knew that Day of Awareness of Food Loss and Waste would be held at university through conferences on related topics meanwhile only 26.9% attended this event. This could be considered as a limitation for conducted interventions. The low participation on scientific organized conferences reveals that it would not be the appropriate action to use with students. Implication should be in a different approach considering age. Similar finding were previously reported by Soma, Li & Maclaren (2020) about implication on workshops in the context of intervention for food waste reduction.





**Fig.1** Evaluation of awareness campaign

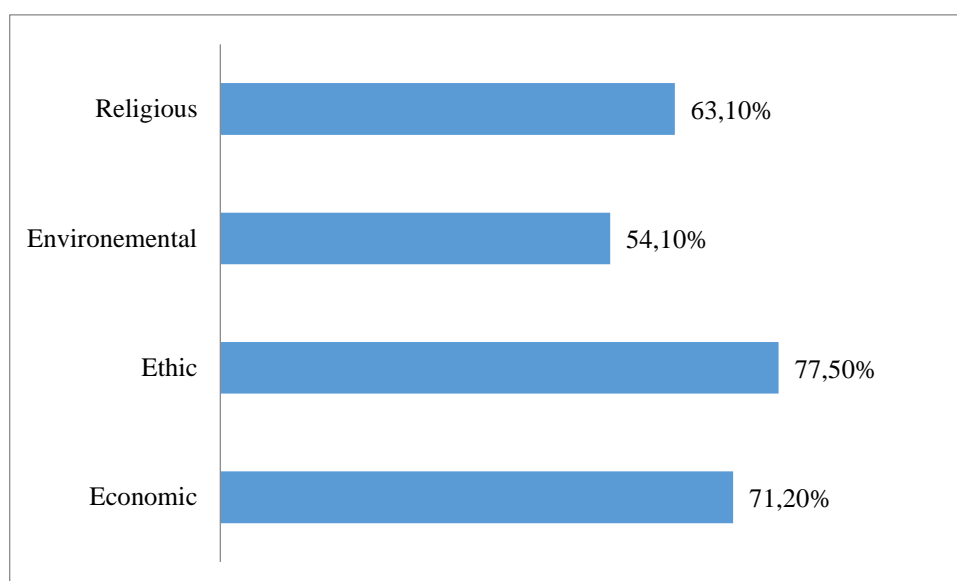
*Impact of the rising awareness campaign on students' behavior in the future:*

Food waste is partly attributable to consumers (FAO, 2015) on the post-survey an interest was accorded to potential changes in the future after conducted interventions. Interestingly, a positive impact was observed. In fact, only 3.6% of respondents declared that they will not change their behavior while 67.6% would probably do. Moreover, 64% of students reported that they will reduce food waste. Ethical and economical reasons seem to be the main drivers to do so (Fig.2).

**Table 3:** Impact of the awareness campaign

|   | <b>% of respondents</b>   |
|---|---|
| After this campaign would you change your behavior toward food waste? | 28.8 Absolutely<br>67.6 Probably<br>3.6 No  |
| Which attitude would you adapt?                                       | 27% I will immediately stop discarding food<br>64% I will reduce food waste<br>9% I will keep my current habits |

These results are confirming previous ones. In fact, thanks to informations communicated through posters students recognized the importance of food issues and its consequences. Probably, this is why 72.7% were shocked. Accordingly, students would be willing to adjust their behavior because they understand clearer its impact at different scales and levels.



**Fig.2** Main reasons to change behavior toward food waste

## CONCLUSION:

This study evaluated the impact of a food waste online and onsite education campaign on students' attitude toward food waste. Results showed that the use of social media and digital tools would be recommended to raise students' awareness as students reported a potential positive change was reported by students. Considering the costs of online campaign such results are promising. In perspective, this research needs to be completed by some quantitative measurements to completely assess efficiency of such approach.

## ACKNOWLEDGMENTS:

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## MICROALGAE CULTURE FOR FOOD SUPPLEMENTATION: AN INSIGHT INTO LIPID FRACTION

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### ABSTRACT

**Background:** Climate change and natural resources scarcity are challenging food security. Accordingly, attention must be addressed to food production. Microalgae cultivation does not compete with land and resources required for traditional crops and has a superior yield compared to terrestrial crops. Moreover, microalgae metabolites may represent high interest to food industry. **Objective:** This research aimed to assess microalgae lipid composition for potential food supplementation with polyunsaturated fatty acids (omega-3 and omega-6). **Methodology:** Samples were firstly collected from water. Then, samples were grown on BG11 media. Identification of microalgae was made through microscopic observation and determination of some growth-kinetic parameters like pH and chlorophyll content. Samples then were cultivated in controlled conditions, harvested and lipid fraction composition was determined through Gas chromatography-mass spectrometry (GC-MS). **Results:** Microalgae growth-kinetic analysis showed significant evolutions in pH and chlorophyll levels during 15 days of culture. Such evolution was probably related to photosynthesis reactions. Regarding microalgae lipid composition, three main components were identified: Methyl palmitate (70.24%), Palmitoleic acid (16.84%) and Stearic acid (12.41%). Unfortunately, no *polyunsaturated fatty acid* was detected. **Conclusion:** Dominance of methyl palmitate could suggest the use of *microalgae* lipid fraction as a natural additive in food industry as an emulsifier, stabilizer, wetting agent. Further investigations on microalgae culture conditions and its impact on fatty acid composition would be recommended.

**Keywords:** Microalgae, Gas chromatography-mass spectrometry (GC-MS), fatty acid, sustainable production.

### INTRODUCTION

Hunger is the distress associated with lack of food. Unfortunately, the number of people affected by hunger globally rose to as many as **828 million** in 2021, an increase of about **46 million** since 2020 (FAO et al., 2022). Such increase may result from climate change, crises and conflicts and also COVID-19 pandemic. In fact, the number of undernourished people grew by 150 million from 2019 to 2022 (FAO et al., 2022). Such facts are challenging for the 2030 agenda for sustainable development where eradicating hunger represent the second goal: Zero hunger. Accordingly, there is a huge effort to make to overcome this situation and reach food security.

Meanwhile, planetary boundaries for sustainable food supply has been already exceed (Campbell et al., 2017). Furthermore, according to the Food and Agriculture Organization (FAO): “expanding food production and economic growth have often come at a heavy cost to

the natural environment” (FAO, 2017). Thus, food production is more problematic and challenged. On one hand, the environmental factor is crucial to take into consideration on a global sustainable approach. On another hand, World population grew continuously to reach 7.7 billion in 2019 and it is expected to reach 10 billion people by 2050 (Vandermeer et al., 2018).

Algae, a multicellular or unicellular form of living organisms, can be divided into either macro or micro algae based on its size (Sathasivam et al., 2019). Microalgae are extremely diverse group with estimated number of species ranging from 200,000 to 800,000 (Koyand et al., 2019). These micro-algae grow in strongly humid environments and are characterized by their ability to grow on soils not arable and according to different trophic regimes. Interestingly, they can transform light energy, carbon dioxide into algal biomass through photosynthesis (Remize et al., 2021). Microalgae have various applications in different fields: agriculture, pharmaceutical, food, cosmetic, water treatment and energy production (Nasoloniaina, 2017). From a nutritional point of view, microalgae are rich in many bioactive molecules with high added value, including: lipid (omega 3 fatty acids), antioxidants, protein, carbohydrates, nucleic acid, vitamins, chlorophylls and carotenoids (Stirk & van Staden, 2020). Thus, using microalgae for food supplementation may represent a promising opportunity in a context of sustainable production.

Particularly, regarding lipid fraction hite lamp connected to a timer in order to ensure day/night cycles of 16/18h. The process was repeated until a pure microalgae colony was obtained. These colonies were observed under microscope for morphologic identification.

### **Monitoring of chlorophyll a content**

The monitoring of the chlorophyll a content is done by taking from the samples in culture every two days for 15 days a volume of 5mL, putting them in conical tubes after centrifugation at 4500 rpm for 15min the recovered pellet is separated and re suspended with a quantity of 5ml 90% methanol.

Thereafter the mixture was incubated at 60°C for 10 min. The extract was analyzed using a UV spectrophotometer (UV-2401PC) at 665 nm for chlorophyll at, 652 nm

Therefore, the pigment concentration was calculated as follows (Daneshvara, 2019):

$$\text{Chlorophyll a ( } \frac{\text{mg}}{\text{L}} \text{)} = 16,82 \times A_{665} - 9,28 \times A_{652}$$

### **pH determination:**

The pH measurement was done every two days using a pH meter (OHAUS Starter 2100). The equipment was calibrated with certified solutions before each determination.

### **Analysis of fatty acids composition by GC-MS:**

The fatty acids profile of the different samples of this strain is determined by an Agilent Technologies model GC-MS (MSD5975-GC7890). After this analysis, a chromatogram is obtained for each sample. Each chromatogram indicates the different methylated organic compounds and their retention times. This identification is carried out by the database listed in the measuring device.

## RESULTS AND DISCUSSION

### Sample preparation and identification:

The samples taken are maintained in an enriching medium which is known for its performance in increasing the growth of micro-algae which is BG11 (Blue-Green medium).



**Fig.1** Collected samples on BG 11 Medium

A strain of micro-algae (EK) was isolated from the freshwater of a valley, the isolation is done by successive transplanting on solid medium BG11. The purification of the isolated strain consists in taking apart all the colonies green which appear on the surface of the Petri dishes using a handle, then transplant them again in streaks on the same medium which was used for its isolation.



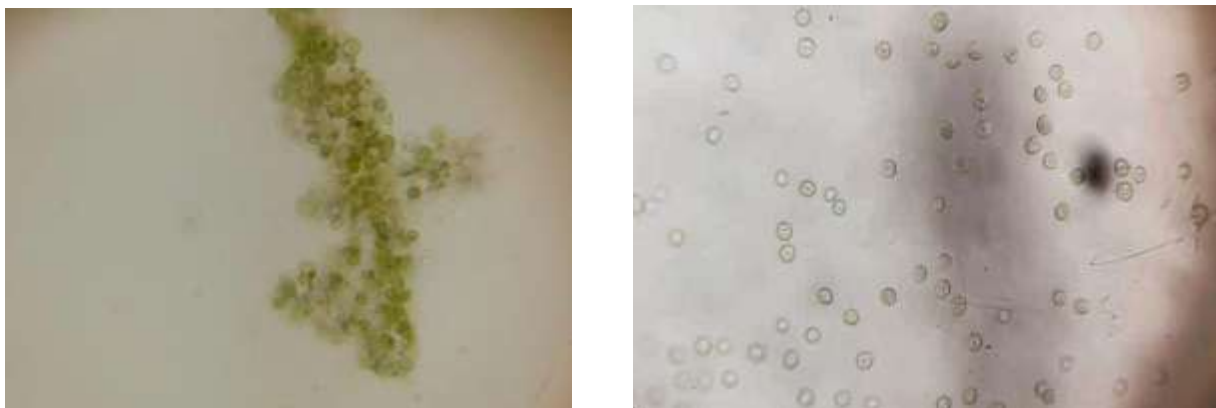
**Fig.2:** Example of isolated sample

The characterization of the strain (EK) is made according to the morphological characters, biochemical characters (pigment, reserve molecules). The characteristics of this strain are presented in Table 1:

**Table 1:** Characteristics of the first isolated sample

| Parameter  | Observation |
|------------|-------------|
| Color      | Green       |
| Cells form | Spherical   |
| Mobility   | Yes         |
| Core       | Yes         |

The observed samples are spherical cells, some more or less elongated, with a flagellum facilitating their mobility. Each cell consists of a thin wall which is surrounded by a generally parietal cup-shaped plastid, made up of chlorophyll pigment.



**Fig.3:** Microscopic observation of the strain (Gr×40)

According to the microscopic observation and the morphological characterization of the cells, the isolated strains could belong to the class of chlorophyceae, order chlorococcale family chlorellacee. So at this level it may be Chlorella, for confirmation it is necessary to carry out molecular analyzes.

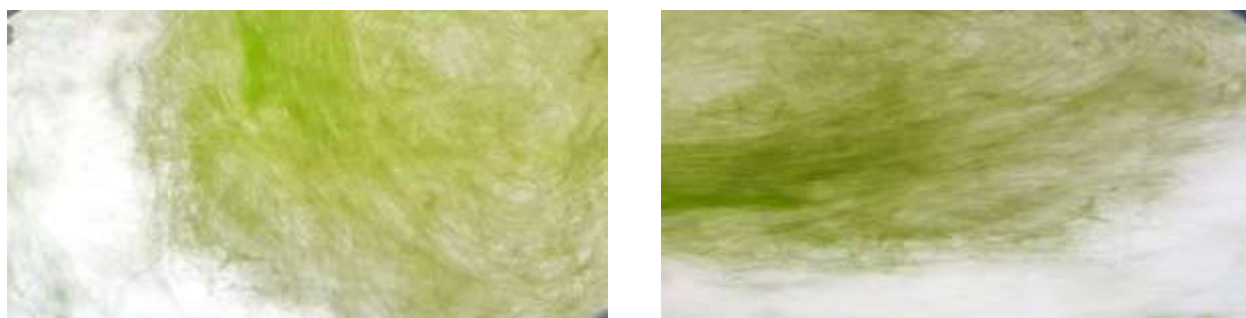
Chlorella strain is a unicellular genus, formed from a spherical or oval cell, containing a single cup-shaped chloroplast with pyrenoids. Their mode of reproduction is asexual. It is characterized by its high photosynthetic potential as well as its rapid growth rate (Jo et al., 2020).

The characterization of the second strain (EO) is also made according to the morphological characters, biochemical characters (pigment, reserve molecules). The characteristics of this strain are presented in Table 2.

**Table 2:** Characteristics of the second isolated sample

| Parameter  | Observation |
|------------|-------------|
| Color      | Green       |
| Cells form | Filamentous |
| Mobility   | No          |
| Core       | No          |

According to the microscopic observation and the morphological characterization of the cells, the isolated strains could belong to the class of Prokaryotes, family of Cyanobacterium.

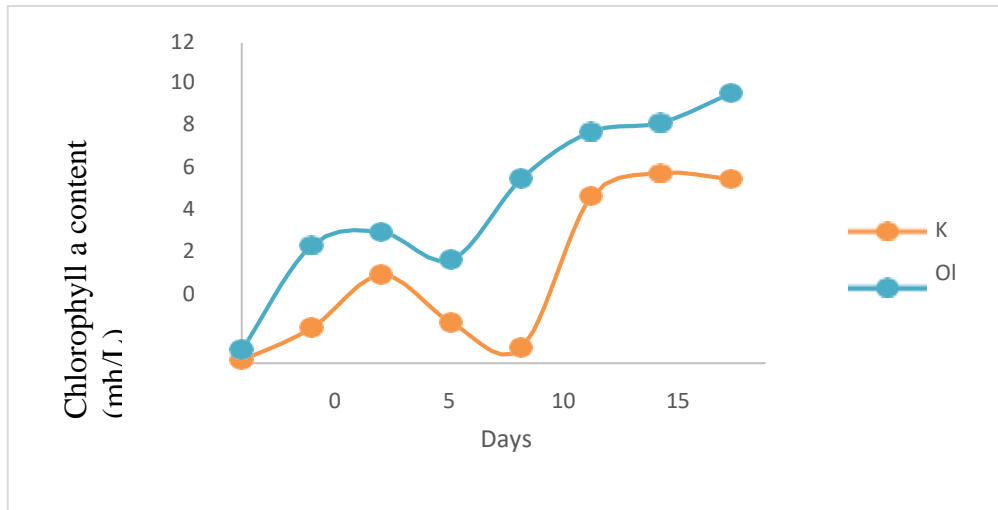


**Fig.4:** Microscopic observation of the second strain (Gr×40)

**Monitoring of chlorophyll a content:**

Microalgae are characterized by the presence of chloroplasts, chlorophyll "a" is known as the most powerful pigment to intercept the light energy taken by microalgae to do their photosynthesis. (Sialve and Steyer, 2015).

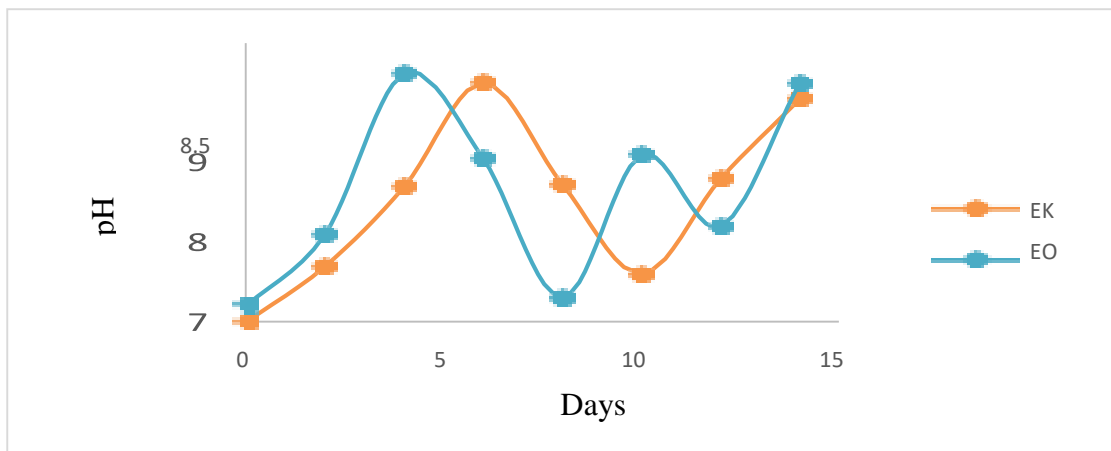
The level of pigment (chlorophyll a) in the OL strain increases from Day 1 to Day 15 until it reaches an approximate value of the order of 11mg/L whereas for the K strain in the same period it was able to reach a quantity of about 8mg/L. So this reflects the good progress of photosynthesis in these two strains as well the increase in biomass (Venckus et al., 2021).



**Fig. 5:** Evolution of Chlorophyll a during 15 days

**pH monitoring:**

Figure 6 shows the variation in pH over 15 days. From these graphs it can be seen that the pH fluctuation was also observed during the cultivation of microalgae, the pH value varies between 7 and 9. This pH fluctuation is a consequence of the photosynthesis process. The increase in pH proved to be due to the photosynthetic uptake of inorganic carbon, such as CO<sub>2</sub> by the microalgae. The drop in pH observed at the end of the culture could be explained by a decrease in activity (Costache et al., 2013).



**Fig.6:** Evolution of pH during 15 days



**Fatty acids composition:**

Lipid droplet in microalgae could represent a potential feedstock for several products. Fatty acids composition is presented in Table 3. As shown, methyl palmitate was the dominant component, followed by Palmitoleic acid (monounsaturated fatty acid) and Stearic acid. Unfortunately, no polyunsaturated fatty acids were obtained. This could be attributed to condition culture as lipid production depends on growth conditions (Ferreira et al., 2019). Though, obtained component may represent high interest. Results of Shen et al. (2021) showed that methyl palmitate contributes in protecting heart against ischemia/reperfusion-induced injury. Similarly, El Demerdash (2011) reported that methyl palmitate has an anti-inflammatory and antifibrotic effect. Thus, incorporation of microalgae lipid might be recommended for health benefits rather than technological ones (emulsifier, stabilizer, wetting agent). Moreover, a systematic review of Hunter et al. (2010) highlighted that compared to other saturated fatty acids stearic acid lowered LDL cholesterol, was neutral with respect to HDL cholesterol and consequently lowered the ratio of total to HDL cholesterol. Palmitoleic acid is the only unsaturated fatty acid detected (monounsaturated). This fatty acid also beneficial health effect mainly on some metabolic diseases like diabete and also it ameliorated and prevents from insulin resistance (Frigolet et al., 2017). Although, polyunsaturated fatty acids were not detected, somme factors may modify obtained fatty acids profie. In fact, trophic mode may affect lipid production. In fact, heterotrophy or mixotrophy may lead to microalgae rapid growth, high production of biomass, and low harvesting costs, due to the higher cell densities achieved (Nicodemou et al., 2022). Accordingly, further investigation in this context would recommend t evaluate its impact on lipid production of tested strain.

**Table 3:** Fatty acid composition

| Fatty acid       | Molecular formula | Content (%) |
|------------------|-------------------|-------------|
| Methyl palmitate | $C_{17}H_{34}O_2$ | 70.24       |
| Stearic acid     | $C_{18}H_{36}O_2$ | 12.41       |
| Palmitoleic acid | $C_{16}H_{30}O_2$ | 16.84       |

**CONCLUSION**

Microalgae are a promising feedstock for many valuable bioactive compounds. Results provided by this research showed the presence of promoting health effect component obtained from cultivation of a freshwater strain sample. To optimize production, it is crucial to take into account several factors such as: strain selection (fatty acid profile, photosynthetic efficiency), microalgae cultivation conditions and system performance (cost efficient, capacity, biomass, extraction method...) and final product quality (nutritional quality, physico-chemical properties, stability...). Accordingly, in perspective, it would be recommended to optimize culture conditions and investigate other trophic mode and their impact on fatty acid profile.

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## INVESTIGATION OF FIN DESIGNS FOR ENHANCING THERMAL EFFICIENCY OF PV

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### ABSTRACT

One of the most important obstacles to increasing the performance of PV (Photovoltaic) panels is the high panel temperature. Most of the study on PV is about solving the problem of decrease in thermal efficiency of the overheated panel. In order to eliminate this obstacle, the aim of this study is to perform a cooling design to increase the thermal efficiency of a monocrystalline solar panel. First of all, the drawing of a reference PV panel was made in Solidworks program. Then, validation of thermal analysis of PV panel based on the STC (standard test conditions) values was performed by using the ANSYS Steady State Thermal program. After the validation, two PV models with different cooling parts were designed. The cooling parts were made of aluminum and consist of holed and non-holed fins. Analysis of new design PV models was made with STC values again. When the simulations were examined, an efficiency increase of 0.91% was obtained on the panel as a result of the cooling system integration. The holed fin has not been significantly affect the efficiency results compared to the non-holed design. It was determined that adding a cooling parts to the PV panel causes a significant decrease in the panel temperature.

**Keywords:** PV module, solar power, cooling system, thermal efficiency

### INTRODUCTION

Today, due to increasing energy needs, changing climatic conditions and occasional energy crises, attention has been turned to alternative energy sources in many countries of the world (Michaelides, E. E. S., 2012). While alternative sources are expected to be cleaner and more sustainable due to changing climate conditions, it is aimed at increasing energy production in a sustainable way by reducing the dependence on foreign countries on energy for energy export countries. Considering the desired conditions, the systems benefiting from solar energy become one of the most prominent alternative energy sources (Tiwari, G. N., & Tiwari, A., 2016).

Photovoltaic panels (solar panels) absorb sunlight and turn electricity through photovoltaic cells that they have on them. Although PV panels absorb most of the rays from the sun, only a certain part of the energy can be converted into electrical energy, while the non-electrically convertible part dissipates as heat. Heat and increased ambient temperatures on the panel cause increased cell temperatures on the panel resulting in reduced electrical efficiency of the panel (Hernandez-Perez, J. G., et al., 2020). The PV panel is lost in power output and energy efficiency conversion for every 1°C degree increase above 25°C (Karakaya, H., & Şen, İ. E., 2019). It is important to improve the thermal efficiency of panels by working on panel cooling to minimize these losses, to make panels suitable in different climate conditions and to promote the use of solar panels (Pearsall, N., 2016).

In this study, 2 fin designs (with-hole and no-hole) were designed to be mounted on the solar panel to reduce adverse effects, and then the effect of the designed fin models on the

modeled panel was examined. The change in panel efficiency was calculated with the help of numerical analysis. Numerical analysis was done in Ansys Steady State Thermal program.

## MATERIAL AND METHOD

The study was started by modeling the PV panel through the SOLIDWORKS program, based on the JKM260P-60 panel (Table 1). Tempered glass, pv cells, eva, tedlar and frame parts are modeled through the program and then a panel model is completed by performing the assembly process. The fin designs (with-hole and no-hole) were also carried out through the same program. The diameter of the hole for fin was determined as 8mm. The designed fin models are prepared to be installed on the panel we create for cooling purposes.

Table 1. Technical specifications of solar PV module (JKM260P-60).

| Features              | Value          |
|-----------------------|----------------|
| Maximum power voltage | 31.1 V         |
| Maximum power current | 8.37 A         |
| Short circuit current | 8.98 A         |
| Open circuit voltage  | 38.1 V         |
| Open circuit voltage  | (-10C) 42.7V   |
| Polycrystalline cell  | 156x156 mm     |
| Number of cells       | 60 (6x10)      |
| Dimensions            | 1650x992x40 mm |

Firstly, the experimental study of Akal and Türk was used for validation (Akal, D., & Türk, S., 2022). The energy efficiency solution of the PV model was made using the meteorological data of Edirne province on 31 July. Boundary conditions are radiation rate, wind velocity, outdoor temperature etc. The data obtained from ANSYS is in good agreement with the experimental study of Akal and Türk (Figure1).

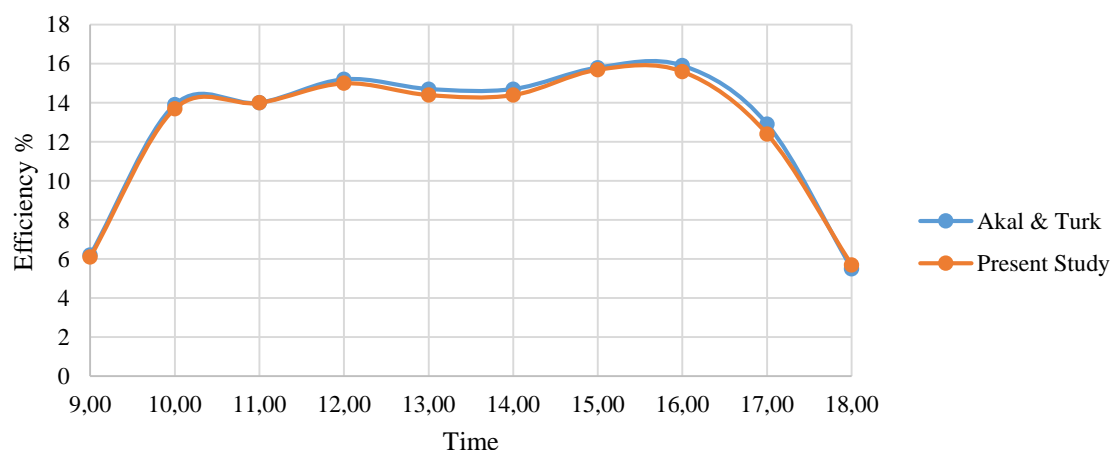


Figure 1. Changing of Energy Efficiency of PV according to meteorological data of 31 July

After the validation work, changes were made in the panel geometry to provide cooling in order to increase the panel efficiency. The newly designed model is based on fin insertion.

Properties of PV component was showed in Table 2. According to Table 2, the designed Fin models are mounted on Tedlar with 13 pieces. When the Fins are mounted under the Tedlar, the length of the edges for the triple fin groups assembly is 23 mm to 36 mm respectively, and the distance between the fins is 239 mm. For Dual fin groups, their distance to the edges is 220.3 mm to 391.23 mm respectively, and the distance between the fins is 239.54 mm (Figure 2).

Then, for the boundary conditions of the panel we modeled (Figure 3), we performed the thermal analysis using the ANSYS steady-thermal program based on the STC (standard test conditions) values of the panels (Montes-Romero, J. et al., 2016). Analysis were made with three different heat transfer coefficient. These values are 25, 15 and 5 W/m<sup>2</sup>K. After the completed analysis, we collected the data obtained from the panel.

After examining the obtained data, we carried out the cooling design in order to increase the thermal efficiency. After performing the assembly of the 2 different cooling parts we designed, they were subjected to thermal analysis according to the STC (Standard test conditions) values and the results of the data were collected for comparison.

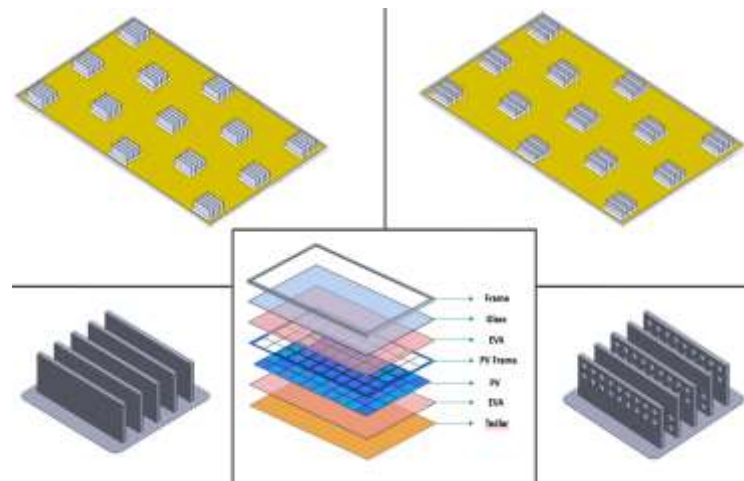


Figure 2. Panel model and fin assembly on panel

Table 2. Properties of PV component

| Component  | Height [mm] | Width [mm] | Depth [mm] | k [W/mK] |
|------------|-------------|------------|------------|----------|
| Glass      | 1650        | 992        | 3.20       | 1        |
| Eva        | 1650        | 992        | 0.80       | 0.26     |
| Pv Cell    | 156         | 156        | 0.30       | 148      |
| Pv Frame   | 1650        | 992        | 0.30       | 190      |
| Tedlar     | 1650        | 992        | 0.50       | 0.26     |
| Fin (base) | 156         | 156        | 0.50       | 190      |
| Fins       | 136         | 50         | 6          | 190      |

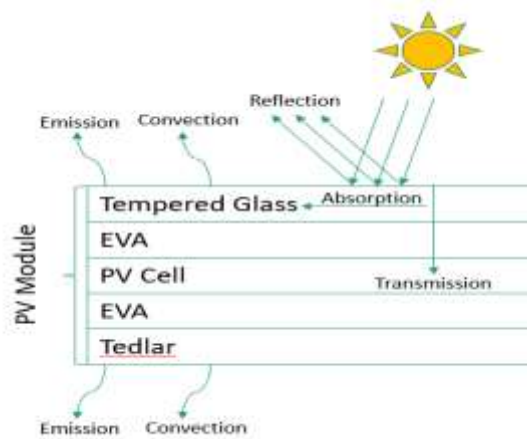


Figure 3. Analyze module for the PV panel

## RESULTS AND DISCUSSION

With the data collected during the study process, graphs such as temperature distribution depending on the heat convection coefficient and temperature distribution depending on the cooling type were created.

When the temperature distributions were examined by looking at the graphic (Figure 4), it was observed that the panel temperature increased significantly with the decreasing wind and the component with the maximum temperature on the panel was the cells. Depending on the increasing temperature, it is seen that the efficiency of the panel decreases considerably in windless and hot climate conditions. In order to improve this and make it a more efficient panel in adverse weather conditions, it has been determined that panel cooling is a necessity for widespread and effective use factors.

Looking at the graphic (Figure 5) after cooling, it was observed that the temperature distribution of the panels operating in a windless environment, the temperature on the cells and the panel module in general decreased and accordingly the efficiency of the panel cells increased.

Again, when the graphics after cooling are examined, it is deduced that the panel efficiency can approach the level in standard test conditions by decreasing to much lower temperatures for the locations where there is wind effect.

When the temperature distribution graph is examined (Figure 6) from corner to corner, it is observed that the maximum cooling has occurred on the no-hole Fin design. As the with-hole fin design causes a decrease in the surface area caused by the holes, the amount of air in contact with the surface has also decreased, accordingly, it has been observed that the no-hole design cools better than the with-hole design.

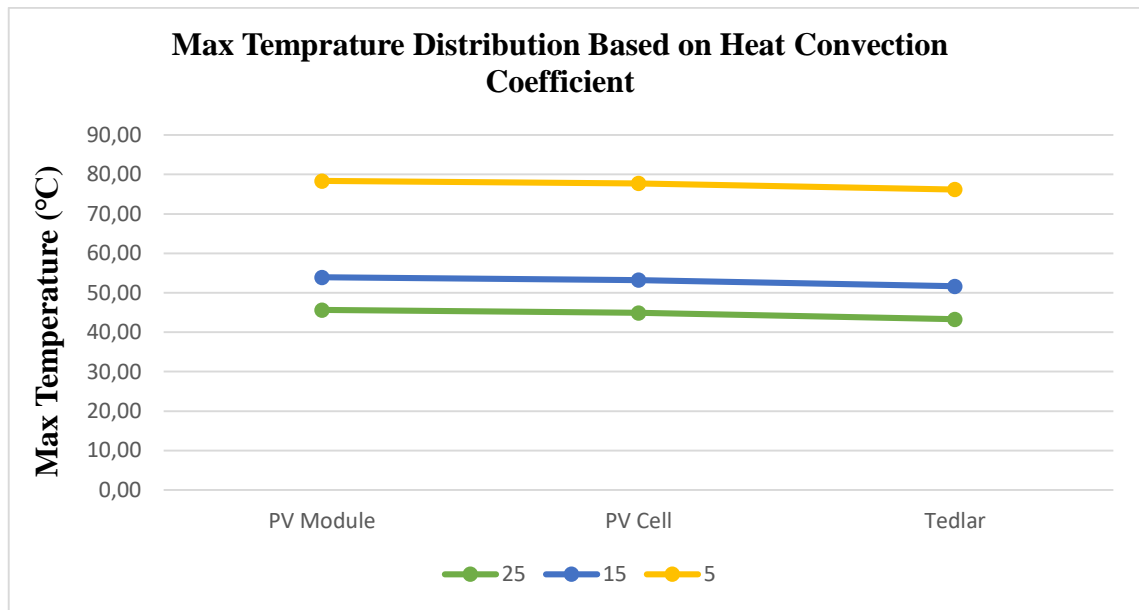


Figure 4. Max temperature distribution based on heat convection coefficient

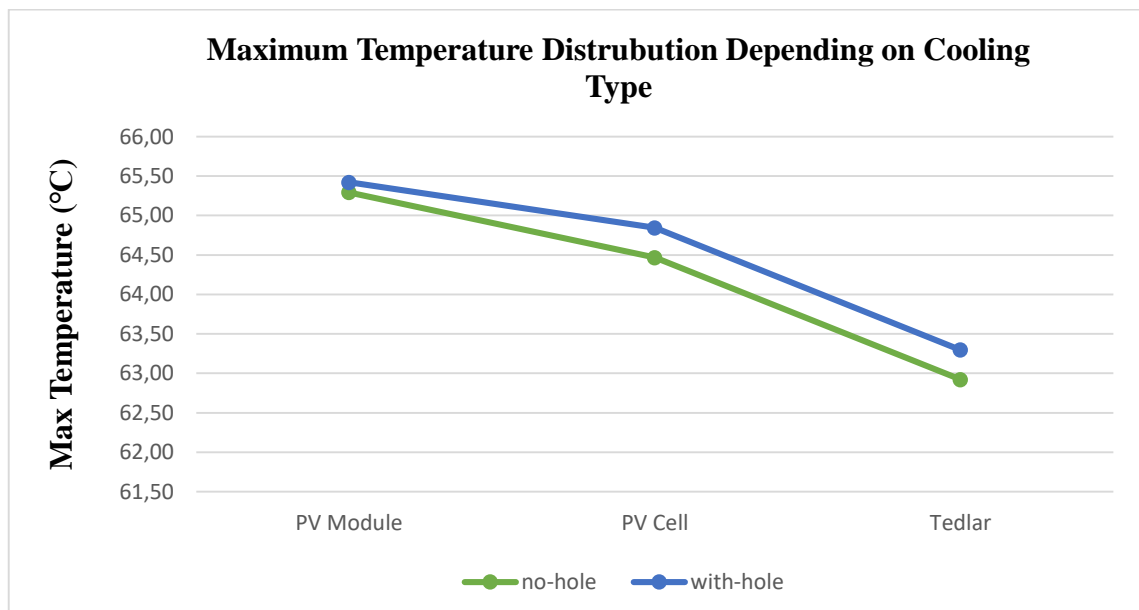


Figure 5. Maximum temperature distribution depending on cooling type



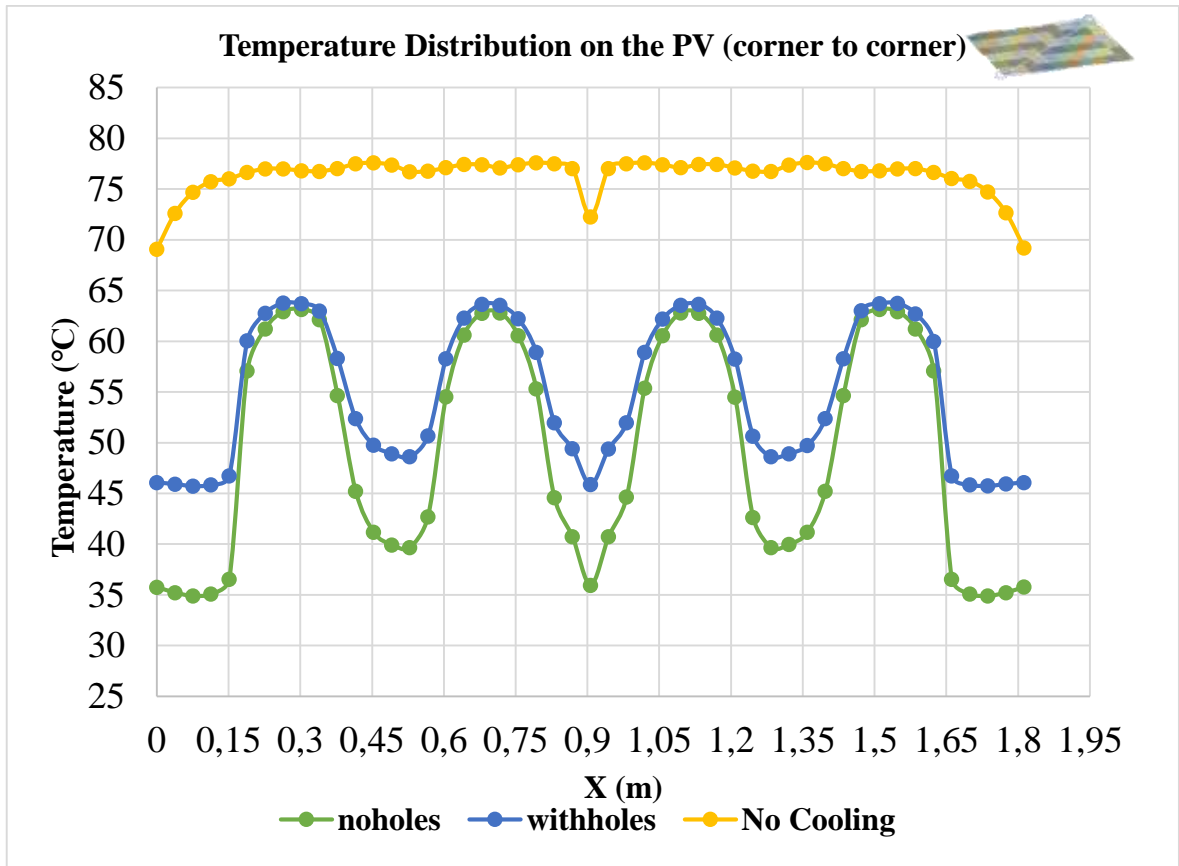


Figure 6. Temperature distribution on the PV (corner to corner)

## CONCLUSIONS

It has been observed that the maximum temperature on PV Panel, PV Cell, Tedlar belongs to the heat transfer coefficient of  $5 \text{ W/m}^2\text{°C}$ . These temperatures were measured as 78.326, 77.751, 76.196 °C respectively. The panel efficiency was calculated using the measured maximum cell temperature.

$$\eta_c = 0.15[1 - 0.0045(77.751 - 25)] = 0.1143 = \%11.43$$

After the cooling design addition, the maximum cell temperature dropped to 64,467 °C and 13,284 °C cooling was provided on the cells. When the panel efficiency is calculated over the determined new cell temperature;

$$\eta_c = 0.15[1 - 0.0045(64.467 - 25)] = 0.123359775 = \%12.34$$

When the results are examined, an efficiency increase of 0.91% was obtained on the panel as a result of the cooling system integration. Studies are carried out on different cooling systems in order to increase the experimental efficiency on the PV Panel above 2%-3%.

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## THE EFFECT OF FRONT WING MODELS ON AERODYNAMICS OF F1 CARS

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### ABSTRACT

One of the main topics of the automotive industry has been the improvement of vehicle design aerodynamics to reduce the drag coefficient. In order to reduce lap times in F1 (Formula 1) races, it is of great importance to improve car aerodynamics. In this study, the effect of front wing design on aerodynamics in F1 cars was investigated. Three different front wing models were designed in Solidworks program. Then three-dimensional airflow simulation was applied on the Petronas F1 car model. Simulations were made by using Ansys Fluent software. The free flow velocity was calculated as 75 m/s and the solutions were carried out in the k- $\epsilon$  turbulence model. According to simulations, drag force and drag coefficient were calculated and streamlines on different wing models were demonstrated. As a result of analysis it was concluded how the changes in the front wing model affect the aerodynamics of the F1 car and the best wing model for enhancing aerodynamics efficiency were determined.

**Keywords:** Front wing, Aerodynamics, Formula 1, Drag coefficient, Drag force.

### INTRODUCTION

Formula 1 cars are some of the fastest road cars in the world. Although these open wheeled vehicles are only 30-40 km faster than the fastest sports cars, they can turn even the fastest corners 5-6 times faster than these cars due to their aerodynamic structure (Aniruddha P. et al., 2014).

One of the key factors in determining a success of an open wheel race car such as Formula 1, is its aerodynamic efficiency (Mokhtar & Lane, 2008). The aerodynamics of race cars can be improved using computational fluid dynamics (CFD) tools. These tools provide highly accurate results on very short timescales. CFD can be used many engineering areas such as fluid flow, heat transfer and chemical reactions etc. by means of computer based simulation. The technique is very powerful and spans a wide range of industrial and non-industrial application areas. Thanks to CFD, designers working on aerodynamics can instantly see how the different geometric structures of the parts affect the vehicle (Arrondeau, B., et al., 2020).

The front wing, which is the first part of the Formula 1 car, also manages the aerodynamics of the F1 car. The front wing is one of the most valuable aero parts of the car and improves handling (Versteeg H K, Malalasekera W., 2007). The working principle of the front wing is quite simple. Air molecules passing over the inclined pillars pass more slowly than air molecules passing below. In this way, the air molecules exert a net force on the pillars, called downforce (Petkar, R. Et al., 2014). Another factor affecting the F1 car is air resistance. Air passing over the surface of the F1 car passes by friction and creates a friction force there. If the air is scattered over the surface, the drag effect increases, causing the F1 car to slow down (Kieffer, W. et al., 2004). Simply, the aerodynamic goal in Formula 1 is; to increase the downforce as much as possible, but also to reduce the drag effect as much as possible (Syazrul,

M. 2010). In this study, three different front wings modeled and analysed with CFD. According to analyses results, determined which model has the more suitable drag force.

## **MATERIAL AND METHOD**

### **Drawings Processes**

Computer-aided design program Solidworks was used to draw the front wing of the F1 vehicle. Solidworks is one of the leading CAD programs to date. It is a solid modeling program which has good interconnectivity with many grid generation systems. It has an easy to use system of part design, followed by integrating parts together into an assembly. In order to design 3 different wing models, firstly, 2D drawings were made in Solidworks program and dimensioned. These dimensions were entered with reference to the dimensions of the official Formula 1 vehicle. Material assignments were made in the drawings and the masses and surface areas of the models were calculated (Arrondeau, B. et al., 2020). Render images of the Formula 1 model, whose drawing was completed, were taken from Solidworks Visualize and KeyShot programs (Figure 1 and 2).



Figure 1. Rendered isometric view of Formula 1 car



Figure 2. Rendered front view of Formula 1 car

The differences in these designed wing models were the number of wings and the spacing between the wings. In the first model, the gaps between the wings and the number of wings were modeled as few (Figure 3). In the second model, the gaps between the wings were increased and the number of wings was modeled the same number as in the first model (Figure 4). In the third model, the number of wings was increased and the gaps between the wings were designed to be larger than in the first model (Figure 5).



Figure 3. First wing model



Figure 4. Second wing model

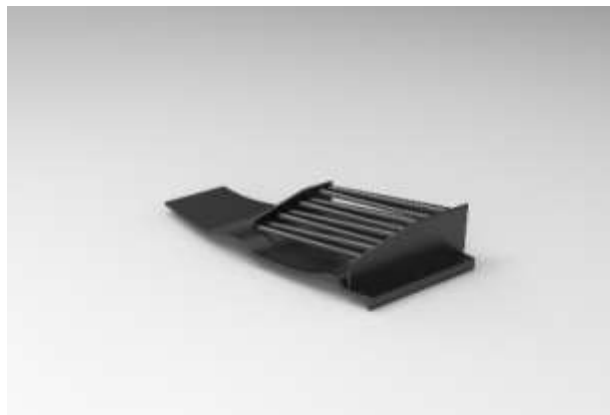


Figure 5. Third wing model

## Analyses Processes

As a first step, the wing models drawn from the Solidworks program were transferred to the Ansys program. Then we created a wind tunnel for the transferred model. The purpose of creating a wind tunnel is to create the area for certain air flow for the wing model.

The next step was to assign a quality mesh to the model. Mesh generation is a very important part of the CFD process. The good quality of mesh determines the accuracy of solution as well as rate of convergence. The finer of the grid, the more accurate result, however the computational time increases significantly (Figure 6).

After the mesh process was completed, the step of determining the turbulence model was started. The  $k-\epsilon$  turbulence model was choosed fort the analyses beacuse it gives more accurate results and it is easier and more affordable.

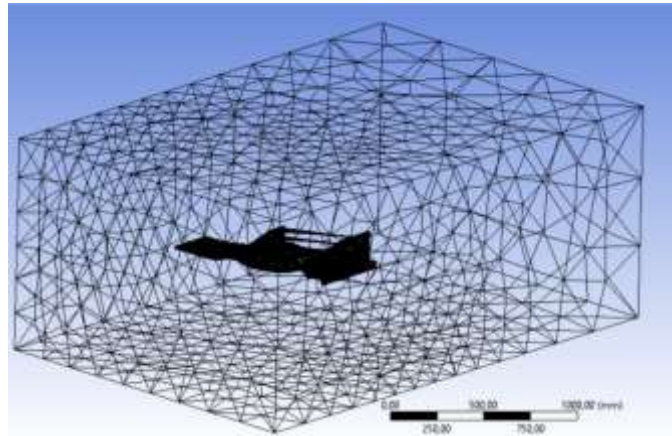


Figure 6. Mesh of the model

Only the speed at the entrance of the wind tunnel and only the pressure values at the exit are entered into the program. In the wind tunnel, velocity boundary condition is given to the inlet and it is assumed that its value is 75 m/s. Zero pressure outlet boundary condition is given to the outlet of the tunel. Surface of the wings is defined stationary wall. In the next step, in order to define the Reference Values, after assigning the material of the part drawn from Solidworks, the surface areas of the wing models were calculated from the mass properties section through the program. Coupled was chosen as the solution method. Turbulence kinetic energy and momentum were chosen as the first order for to be more quick solutions.

In order to find the drag force and drag coefficients, the existence of these coefficients must be entered into the program. As the last step, hybrid initialization was chosen as the initialization method and the analysis were started.

## RESULTS AND DISCUSSION

The two main forces affecting the efficiency of aerodynamics are drag coefficient and drag force. In fluid dynamics, the Drag coefficient is a number that aerodynamicists use to model all the complex dependencies of shape, gradient and flow conditions on the drag of Formula 1 vehicles. As a result of the CFD analysis, drag coefficient values of three different front wing models are showed in Figure 7.

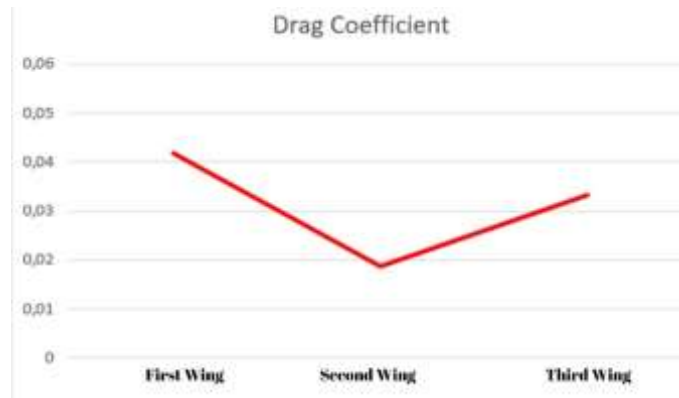


Figure 7. Drag Coefficient Values

When comparing Drag Coefficient values, the wing model with the lowest Drag Coefficient value is the most efficient wing model. For this reason, the second wing model is the most efficient wing model.

The Drag Force is the resultant force applied to the front surface of the structure moving in the amount of particulate matter. In sports cars, on the other hand, since high speeds are aimed, drag is tried to be reduced as much as possible in order to prevent the vehicle from rolling and to reach higher speeds easily. As a result of the CFD analysis, drag force values of three different front wing models are showed in Figure 8.

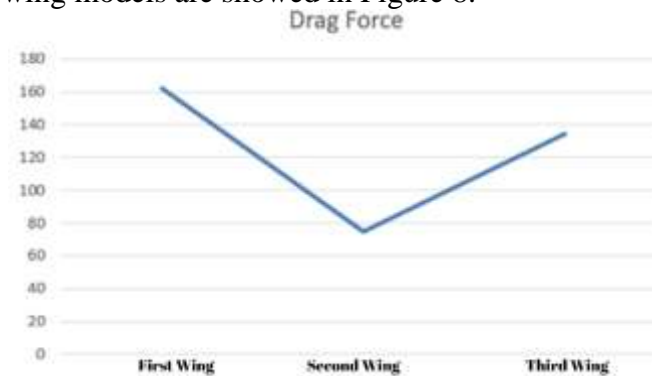


Figure 8. Drag Force Values

In order to minimize the drag effect that will affect the deceleration of the vehicle, the wing with the least drag force should be used. For this reason, the most efficient value is the value at which Drag Force is low. If the graph is examined, the wing model with the lowest Drag Force value is the 2nd Wing.

Since providing a uniform air flow will allow the racing car to be more stable and faster, it is necessary to look at the "velocity streamlines" on the wing (Szudarek & Piechna, 2021). Therefore, velocity streamlines are shown on all wings at  $z=0.2$  m plane (Figure 9). When Figure 9 is examined, the model with the most uniform air flow is seen in the second front wing model, as in drag force.

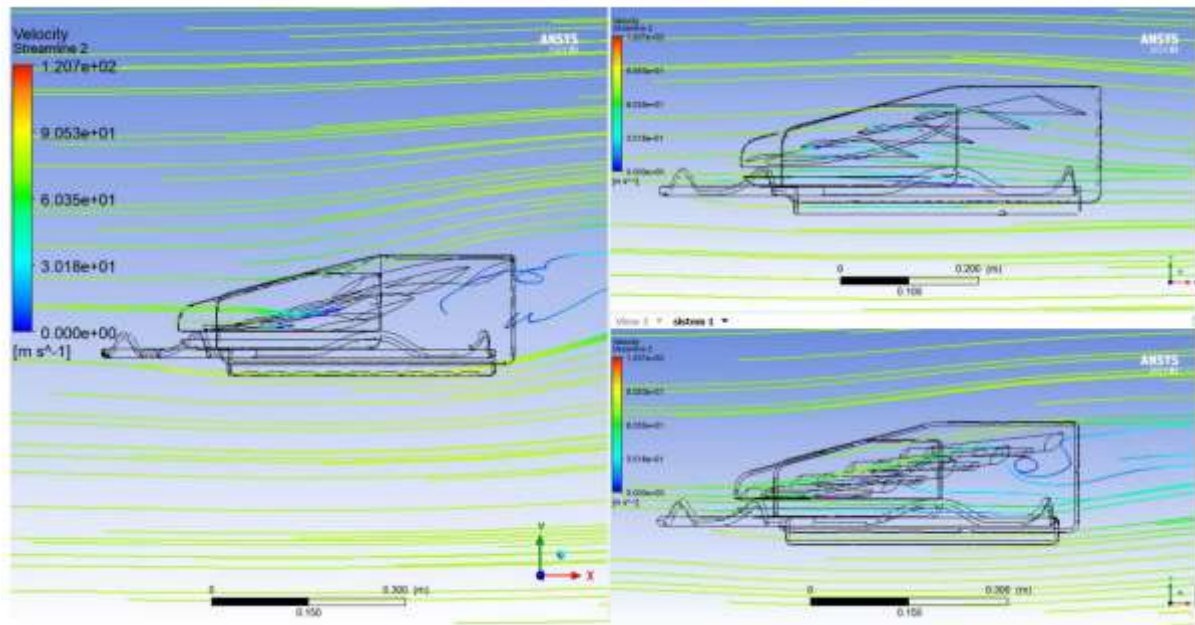


Figure 9. Velocity streamlines on  $z=0,2\text{m}$  plane

## CONCLUSIONS

In this study, while the front wing models of the Formula 1 vehicle were designed, the number of wings, the distances between them and their thickness were changed. Flow analyzes of these three front wing models were made using the Ansys program. As a result of the analysis, Drag Coefficient and Drag Force values were calculated in the Formula 1 front wing model.

In these analysis results, Drag Coefficient and Drag Force parameters play an important role in the Formula 1 front wing model. It is preferred that the numerical value of these parameters be small, and the most efficient wing model for these two values was the wing with the gap between the wings and also the wing (Second Wing Model) with the smallest Drag Values. The model with the most uniform air flow on the wing was also in the second front wing model.

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## **EFFECT OF TOBACCO WASTE ON YIELD AND MINERAL NUTRITION OF BARLEY**

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### **ABSTRACT**

The aim of this study was to investigate the effect of tobacco waste (TW) application on plant growth and mineral nutrition of barley (*Hordeum vulgare*). Four doses of TW (0, 2, 4 and 6%) were applied into the 1 kg pots in a randomized plots experimental design with three replications. After the germination of seeds, soil moisture level in the pots was kept at the field capacity with irrigating distilled water after weighing the pots daily. The experimental study was ended at the stem elongation stage when the flag leaf just visible after 6 weeks of sowing. The dry weight of barley increased from 0.77 g/pot in control treatment to 2.57 g/pot in 4% doses of TW treatment, significantly ( $P<0.01$ ). Macro and micro nutrient contents in plant samples generally increased with the farmyard manure application over the control. It was determined that the highest nutrient contents in plant samples were N (4.66%), P (0.21%), Fe (326,64 ppm), Zn (56.31 ppm) and Cu (40.60 ppm) in 4% doses, Ca (0.39%), Mn (108.40 ppm) in 2% doses, Mg (0.15%) in 6% doses and K (0.45%) in the control application. On the other hand all macro and micro nutrient uptake values were generally higher in 4% TW application ( $P<0.01$ ). It can be concluded that tobacco waste can be used as a soil conditioner to improve plant nutrition and growth in organic farming systems.

**Keywords:** Tobacco waste, barley, macro-micro nutrients, yield.

### **INTRODUCTION**

Improvements in soil chemical properties have been reported for re-use of organic by-products and include; increased plant available potassium, calcium and magnesium (Jakobsen, 1996; Wen et al., 1999; Miyasaka et al., 2001; Erhart and Hartl, 2003). Compost applications increased organic status of the soil and nutrient contents, and produced strongest correlations between soil nutrient levels and barley yield (Courtney and Mullen, 2007). In another study, 50 Mg/ha organic amendment in soil increased barley yield (Zhang et al., 2000). Okur et al. (2008) reported that the application of tobacco waste and farmyard manure significantly increased lettuce yield when compared to the control due to improving soil chemical and biological parameters. Chaturvedi et al. (2008) found that dried tobacco waste application into soil significantly increased the yield of tomato fruit and critical nutritional parameters; the highest yield of tomato was obtained with 3% tobacco waste application.

In Turkey, 6000-6500 Mg of tobacco waste per year is generated at various stages of post-harvest processing of tobacco and during the manufacture of tobacco products (Okur et al., 2008). The objective of this study was to determine the effects of tobacco waste application on plant growth, nutrient contents and uptakes in barley (*Hordeum vulgare*) under a greenhouse condition.

## MATERIAL AND METHOD

The surface soil sample (0-20 cm) used in this study was taken from the Agricultural Experimental Field of Ondokuz Mayıs University, Samsun-Turkey. Some properties of the soil sample are given in Table 1.

Table 1. Some physical and chemical properties of the soil

|                             |       |   |       |
|-----------------------------|-------|---|-------|
| Sand, %                     | 27.43 | Org. Matter, %                          | 2.33  |
| Silt, %                     | 24.57 | Exc. K, cmol kg <sup>-1</sup>           | 0.77  |
| Clay, %                     | 48.00 | Exc. Ca, cmol kg <sup>-1</sup>          | 28.10 |
| pH (1:1)                    | 6.60  | Exc. Mg, cmol kg <sup>-1</sup>          | 6.69  |
| EC 25°C, dS m <sup>-1</sup> | 0.13  | Cation Exc. Cap., cmol kg <sup>-1</sup> | 52.80 |

After drying in the air condition and sieving from 2 mm screen, 1 kg of soil sample as oven dry weight basis was filled into each pot. Four doses of tobacco waste including a control treatment (0, 2, 4 and 6%) were applied into soil in a randomized plots design with three replications. Eight barley seeds were sown after the germination 5 plants were left in each pot. Soil moisture level in the pots was kept around the field capacity with irrigating distilled water after weighing pots daily. The experimental study was ended at the stem elongation stage when the flag leaf just visible after 6 weeks of sowing. After weighing the harvested plant samples, they were washed, dried and crushed for macro and micro nutrient element analysis. Total N by Kjeldahl method, P by spectrophotometric method and the other macro and micro element contents in plants were analyzed after wet digestion of samples using atomic absorption spectrophotometer (Kacar and Inal, 2008).

Variance analyses of the data were run using SPSS program and pairs of mean values compared using Duncan test.

## RESULTS AND DISCUSSION

Effect of tobacco waste (TW) application on barley yield is given in Figure 1. The dry weight of barley increased from 0.77 g/pot in control treatment to the highest 2.57 g/pot in 4% doses of tobacco waste treatment, significantly ( $P < 0.01$ ). The yield of barley decreased to 2.29 g/pot in the highest doses (6%) of tobacco waste application.

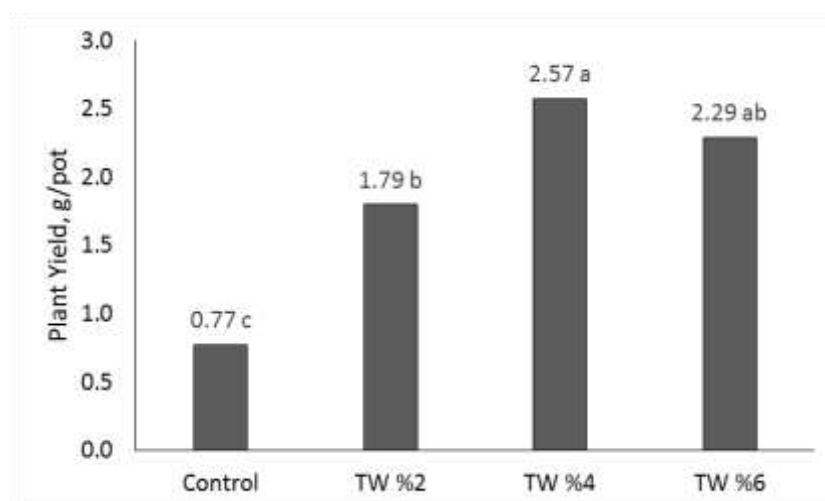


Figure 1. Effect of tobacco waste (TW) application on barley yield.

Effects of TW application on macro nutrient contents of barley are given in Table 2. Macro nutrients, except K, in plant samples generally increased with the tobacco waste application over the control statistically at 0.01 level. It was determined that the highest macro nutrient contents in plant samples were Mg (0.15%) in 6% doses, N (4.66%) and P (0.21%) in 4% doses, Ca (0.39%) in 2% doses of TW application, and K (0.45%) in the control application.

Effects of TW application on micro nutrient contents of barley are given in Table 3. Micro nutrients, except Cu, in plant samples generally increased with the tobacco waste application over the control statistically. It was determined that the highest micro nutrient contents in plant samples were Fe (326.64 ppm), Zn (56.31 ppm), Cu (40.60 ppm) in 4% doses, and Mn (108.40 ppm) in 2% doses of TW application.

Table 2. Effects of TW application on macro nutrient contents of barley

| App. Doses | N, %** | P, %** | K, %**  | Ca, %** | Mg, %** |
|------------|--------|--------|---------|---------|---------|
| Control    | 1.75 b | 0.14 b | 0.45 a  | 0.24 b  | 0.06 c  |
| TW %2      | 3.99 a | 0.16 b | 0.30 bc | 0.39 a  | 0.08 bc |
| TW %4      | 4.66 a | 0.21 a | 0.22 c  | 0.37 a  | 0.11 b  |
| TW %6      | 3.98 a | 0.15 b | 0.31 b  | 0.32 ab | 0.15 a  |

\*\*statistically significant at 0.01 level.

Table 3. Effects of TW application on micro nutrient contents of barley

| App. Doses | Fe, ppm* | Zn, ppm* | Mn, ppm** | Cu, ppm <sup>ns</sup> |
|------------|----------|----------|-----------|-----------------------|
| Control    | 291.14 b | 46.86 b  | 14.00 c   | 28.70                 |
| TW %2      | 267.70 b | 49.29 b  | 108.40 a  | 37.80                 |
| TW %4      | 326.64 a | 56.31 a  | 85.20 ab  | 40.60                 |
| TW %6      | 274.67 b | 48.39 b  | 60.40 b   | 36.40                 |

\*statistically significant at 0.05 level; \*\*statistically significant at 0.01 level; ns: non significant

The effects of TW application doses on macro and micro nutrient uptakes by barley plant are given in Figures 2, 3, 4 and 5. While the lowest nutrient uptakes by plant were determined in the control application, all macro and micro nutrient uptakes by the plants significantly increased with tobacco waste application ( $P < 0.01$ ). The highest N (118.55 mg/pot), P (5.48 mg/pot) and Ca (9.50 mg/pot) uptakes by the plants were determined in 4% TW application. The highest K (7.22 mg/pot) and Mg (3.40 mg/pot) uptakes by the plants were found in 6% TW application. Similarly, the highest Fe (840.29  $\mu$ g/pot), Zn (143.43  $\mu$ g/pot), Mn (223.25  $\mu$ g/pot) and Cu (102.35  $\mu$ g/pot) uptakes by the plants were determined in 4% TW application (Figures 4 and 5).

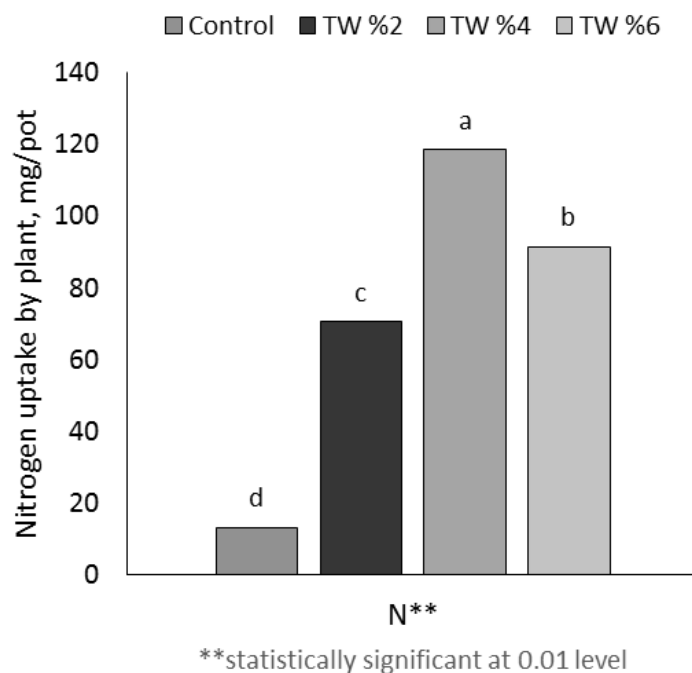


Figure 2. Effect of tobacco waste (TW) application on N uptake by plant

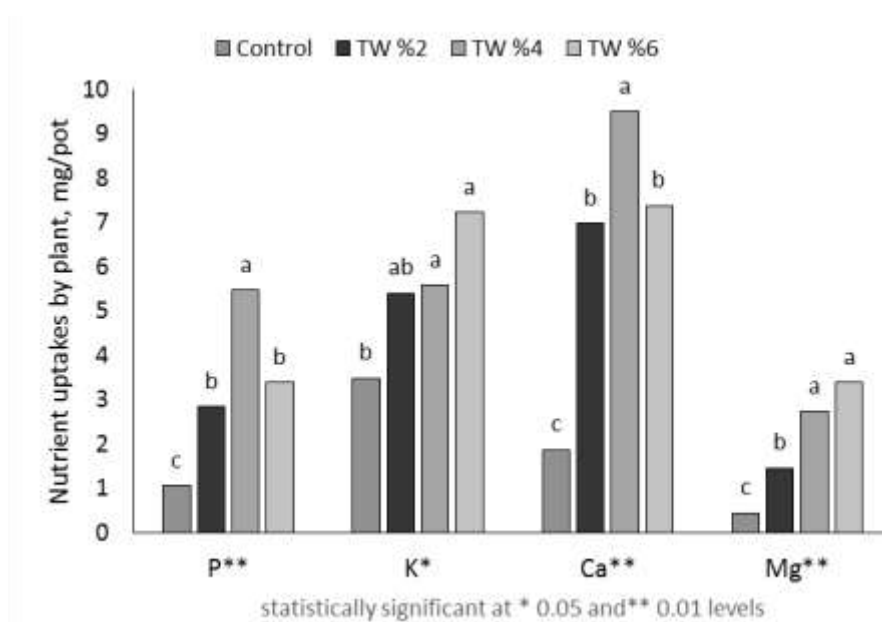


Figure 3. Effect of tobacco waste (TW) application on macro nutrient uptake by plant

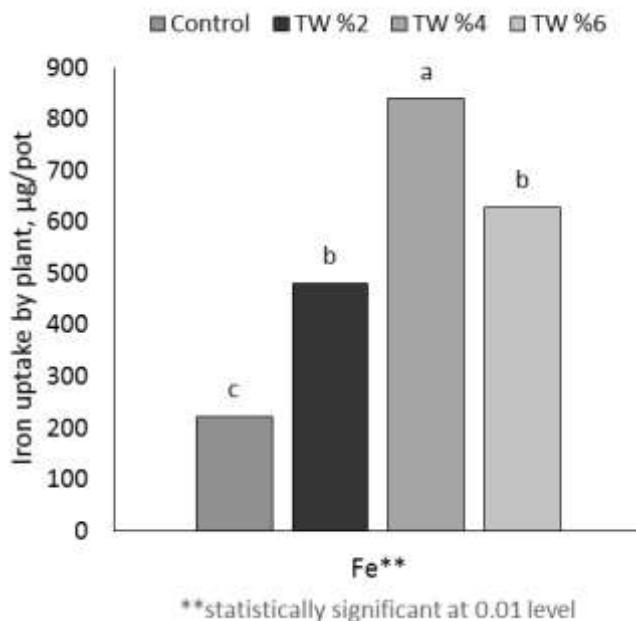


Figure 4. Effect of tobacco waste (TW) application on Fe uptake by plant

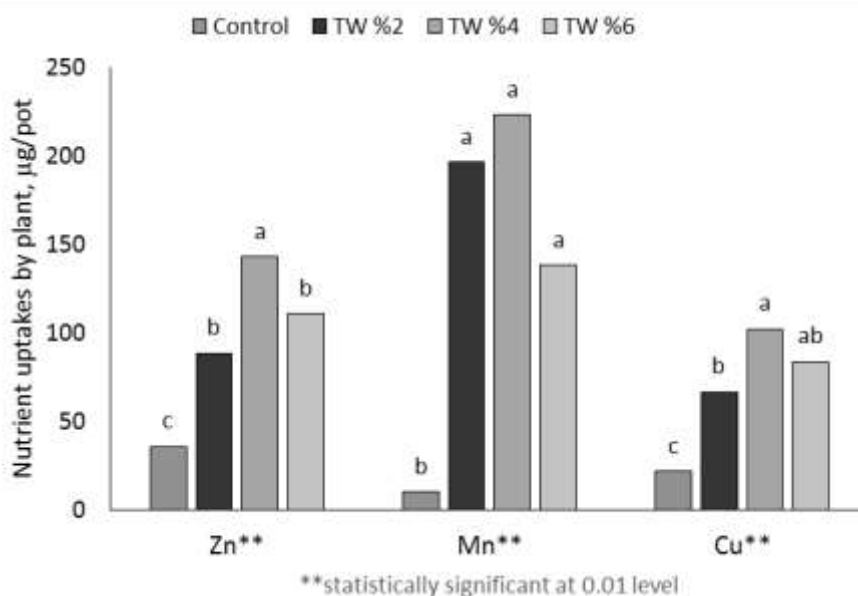


Figure 5. Effect of tobacco waste (TW) application on micro nutrient uptake by plant

Increasing the yield generally reduced K content in barley plant samples. However, all macro and micro nutrient uptakes by the plants increased with increasing the yield. Generally, 3% of TW application had the highest yield and macro and micro nutrient uptakes. Results of this study is similar to results of other studies completed on the effects of tobacco waste application on yield and nutrient contents of different crops (Okur et al. 2008; Chaturvedi et al. 2008).

## CONCLUSIONS

In this study, tobacco waste application into a clay textured soil increased barley yield over the control. Macro and micro nutrient contents generally increased with increasing TW application doses. It seems that the optimum doses of TW application are 3% for clay textural soils to have

the highest yield and nutrient uptakes by barley plant. It can be concluded that tobacco waste can be used in organic farming to help plant nutrition and increase yield in fine textural soils.

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## **EFFECT OF CULTIVATION ON SPATIAL VARIABILITY OF SOIL ORGANIC CARBON CONTENT IN A SMALL SCALE FIELD**

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### **ABSTRACT**

Soil cultivation is generally done to form a homogeneous media to supply optimum growth conditions for seeds and plants. In this study, spatial variability of soil organic carbon content (SOC) in a cultivated field was determined by geostatistical method. Conventional tillage was used with a mouldboard plough at a depth of 15 cm in a field. A total number of 49 soil samples were taken from a square grid at 5 m spacing of a 30 x 30 m<sup>2</sup> plot selected in the center of field. The SOC contents of soil samples varied between 1.18% and 1.73%. Clay content (31.48 to 43.97%), bulk density (BD) (1.12 to 1.41 g/cm<sup>3</sup>), total porosity (F) (46.79 to 57.73%), volumetric water content ( $\theta$ ) (19.64 to 43.86%), soil pH (6.47 to 7.40) and electrical conductivity (EC) (0.31 to 0.80 dS/m) values also showed variations among the sampling points. In kriging interpolation for the spatial variability of SOM, the biggest  $r^2$  (0.766) and the smallest RSS (0.0013) values were determined with Gaussian model. Spatial dependences of the SOC was strong in the field with 6.4 of nugget/sill ratio. The semivariogram of SOC showed spatial dependence with a range of 157.61 m. The SOC had significant positive correlations with clay (0.365\*\*), F (0.287\*) and significant negative correlations with soil BD (-0.286\*),  $\theta$  (-0.362\*) and silt (-0.429\*\*) content. This study showed that spatial variability of SOC in arable fields can be predicted with monitoring soil organic carbon in cultivated fields for global warming researches.

**Keywords:** Soil organic carbon, tillage, soil properties, spatial variability.

### **INTRODUCTION**

Benefits from soil tillage are known as i) improvement of soil-air-water relations in seedbeds, ii) control of undesired vegetation, and iii) reduction of the mechanical impedance to root growth (Gardner et al. 1999). Soil tillage practices causes changes to soil structure and hydraulic properties dynamically in space and time (Mueller et al., 2003; Strudley et al. 2008). The ordinary kriging is one of the most common methods in spatial interpolation of soil properties after estimating semivariogram parameters of soil properties using geostatistical tools (Goovaerts, 1998; Utset and Greco, 2001; Castrignano et al., 2003; Zhao et al., 2009). There are numerous studies about tillage effects on soil hydraulic properties in space and time. Authors reported that there is a little work on small-scale spatial variability in soil hydraulic properties resulting from tillage practices (Strudley et al. 2008). The objective of this study was to determine changes in spatial variability of soil organic matter content in a cultivated field by geostatistical method.



## MATERIAL AND METHOD

This study was carried out on Vertic Haplustoll in the Experimental Field having a 4% slope north to south (41°21' N, 36°10' E) direction in Samsun-Turkey. Conventional tillage in 4 ha field was used with a mouldboard plough at a depth of 15 cm. Soil properties were measured in a randomly selected small-scale plot near the center of the field 20 days after soil tillage. The measurements in 49 different soil sampling points were made in a square grid at 5 m spacing in the 30 x 30 m<sup>2</sup> plot. After determining the bulk density (BD) by undisturbed soil core method (Demiralay, 1993), total porosity (F) was calculated using the equation;  $F=1-(BD/2.65)$ . Gravimetric (W) and volumetric water ( $\theta$ ) contents, soil pH (1:1) and Electrical Conductivity (EC) values were determined (Tüzüner, 1990). Particle size distribution of the surface soil samples (0-15 cm depth) was determined by hydrometer method (Demiralay, 1993). Organic matter contents of the soil samples were analyzed by Walkley-Black method (Kacar, 1994).

The geostatistical analyses were performed with the GS+ version 9, and the correlations among the soil properties were calculated using SPSS program. The semivariance ( ) describing degree of spatial dependence of random variable  $Z(x_i)$  over a certain distance was estimated from (Trangmar et al., 1985):

$$\gamma(h) = \frac{1}{2N(h)} \sum_{i=1}^n [Z(x_i) - Z(x_i+h)]^2$$

Where  $\gamma(h)$  is the semivariance for the interval distance class  $h$ ,  $N(h)$  is the number of pairs,  $Z(x_i)$  and  $Z(x_i + h)$  are the measured sample values at position  $i$  and  $(i + h)$ , respectively.

## RESULTS AND DISCUSSION

According to the results of soil analyses, while soil organic carbon (SOC) content values varied between 1,18 and 1,73%, bulk density (1.12 to 1.41 g cm<sup>-3</sup>), clay (31.48 to 43.97%), silt (14.49 to 36.38%), sand (30.11 to 47.57%), volumetric water content (15.19 to 32.56%), pH (6.47 to 7.40) and EC (0.31 to 0.80 dS m<sup>-1</sup>) values showed variations among the sampling points at the cultivated field (Table 1). Ogunkunle (1993) reported that soil properties having a coefficient of variation (CV) between 0 and 15 % are considered least variable, 15 and 35%, moderately variable, and bigger than 35% highly variable. The CV values of the soil properties indicated that the most soil properties are least variable while EC values having 22.41% of CV was more variable than the other soil properties at the field.

Skewness and kurtosis values and frequency distributions for clay, BD, SOC,  $\theta$ , pH and EC indicated that the soil properties usually showed normal distribution (Table 1, Figure 1). Therefore, the original values of soil properties were not transformed. Warrick and Nielsen (1980) reported that the spatial variability of the static soil physical properties is commonly fitted to normal probability distributions; whereas the dynamic properties, related to water or solute movement, are usually lognormally distributed. Veronese-Junior et al. (2006) reported that moisture content values for Brazilian Ferralsol showed normal distribution. Utset and Greco (2001) found that BD in 30 x 30 m<sup>2</sup> plot of Rhodic Ferralsol is normally disturbed.

Table 1. Descriptive statistics for the soil properties.

|                        | Min.  | Max.  | Mean  | Std. Dev. | CV, % | Skewness | Kurtosis |
|------------------------|-------|-------|-------|-----------|-------|----------|----------|
| Clay, %                | 31.48 | 43.97 | 38.31 | 2.92      | 7.62  | 0.030    | -0.785   |
| Silt, %                | 14.49 | 36.38 | 22.54 | 3.42      | 15.17 | 1.266    | 4.907    |
| Sand, %                | 30.11 | 47.57 | 39.15 | 3.74      | 9.55  | 0.209    | -0.463   |
| BD, g cm <sup>-3</sup> | 1.12  | 1.41  | 1.27  | 0.067     | 5.28  | 0.016    | -0.109   |
| W, %                   | 15.19 | 32.56 | 24.32 | 3.24      | 13.32 | -0.069   | 0.681    |
| Θ, %                   | 19.64 | 43.86 | 30.87 | 4.70      | 15.22 | 0.223    | 0.625    |
| SOC, %                 | 1.18  | 1.73  | 1.46  | 0.13      | 9.13  | -0.254   | -0.419   |
| pH(1:1)                | 6.47  | 7.40  | 6.84  | 0.184     | 2.69  | 0.446    | 0.851    |
| EC, dS m <sup>-1</sup> | 0.31  | 0.80  | 0.531 | 0.119     | 22.41 | 0.151    | -0.476   |

SOC: soil organic carbon, BD: bulk density, W: gravimetric water, Θ: volumetric water.

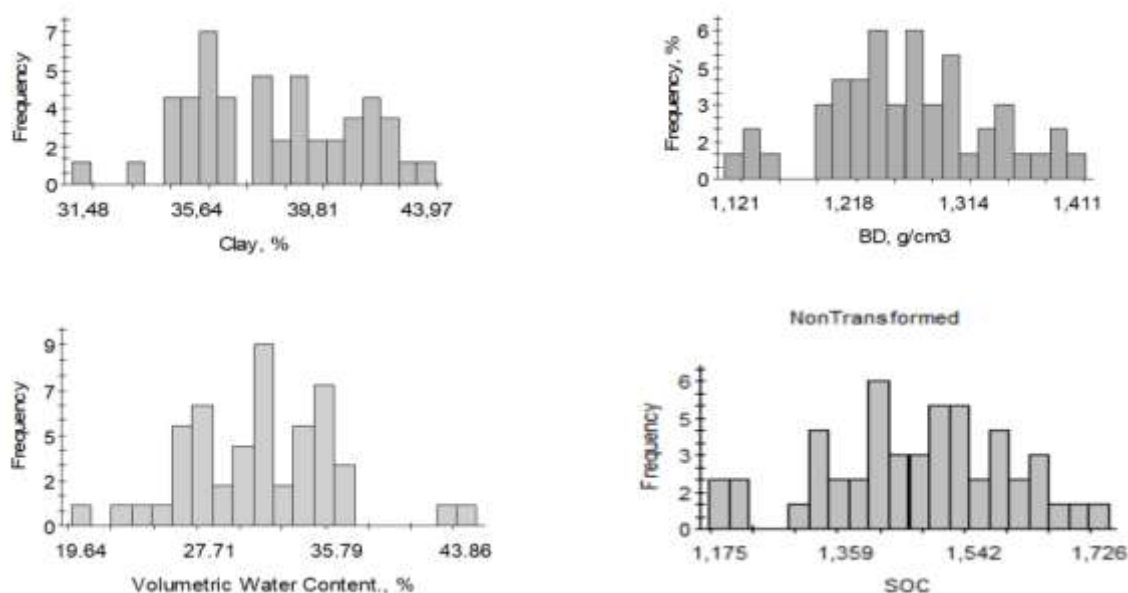


Figure 1. Frequency distribution of the soil properties.

To evaluate the spatial variability of the soil properties, the exponential model for clay content and BD and the Gaussian model for SOC and  $\theta$  were selected with their biggest  $r^2$  values and the smallest residual sum of squares (RSS) values using the GS+9 package program (Table 2). The semivariograms of the soil properties indicated that the range in spatial correlation varied among soil properties. The range indicates the distance in a field where measured properties are no longer spatially correlated. Measured properties of the samples at a distance less than the range become more alike with decreasing distances between them (Tabi and Ogunkunle, 2007). The shortest range (19.67 m) was observed for BD and the longest range (157.61 m) was observed for SOC content.

The nugget effect, which represents random variation caused mainly by the undetectable experimental error and field variation within the minimum sampling space (Cerri et al., 2004; Aşkın and Kızılkaya, 2006), was higher in  $\theta$  content than in the other soil properties. Generally, the nugget values close to zero for the physical properties revealed that all variances of the soil

properties were reasonably well explained at the sampling distance used in this study by the lag. A variable has strong spatial dependency if the ratio of nugget/sill is equal or less than 25%, moderate spatial dependency if the ratio is between 25 and 75%, and weak spatial dependency if the ratio is greater than 75% (Cambardella et al., 1994; Bo et al., 2003). Generally, strong spatial dependency of soil properties is related to structural intrinsic factors such as texture, parent material and mineralogy, and weak spatial dependency is related to random extrinsic factors such as plowing, fertilization and other soil management practices (Zheng et al. 2009). The ratios of nugget/sill in the soil physical properties, except BD, were less than 25% in Table 2. Therefore, spatial dependence values for SOC and the other soil properties were strong. Spatial dependence of BD was moderate due to having 44.91% nugget/sill ratio. This indicated that soil plowing as an extrinsic factor weakened spatial dependency of BD in the field. Cressie and Horton (1987) found that there was a strong spatial dependence (12 m lag distance) in infiltration rates for a silty clay loam undergoing moldboard plowing.

Table 2. Models and parameters for the soil properties

|      | Model       | Nugget,<br>(C <sub>0</sub> ) | Sill,<br>(C <sub>0</sub> +C) | C <sub>0</sub> /(C <sub>0</sub> +C) | a     | r <sup>2</sup> | Cross Val.<br>r <sup>2</sup> |
|------|-------------|------------------------------|------------------------------|-------------------------------------|-------|----------------|------------------------------|
| Clay | Exponential | 3.750                        | 28.490                       | 13.16                               | 80.19 | 0.723          | 0.541                        |
| BD   | Exponential | 0.00269                      | 0.00599                      | 44.91                               | 19.67 | 0.786          | 0.122                        |
| SOC  | Gaussian    | 0.00055                      | 0.015                        | 3.66                                | 8.61  | 0.214          | 0.309                        |
| θ    | Gaussian    | 12.90                        | 76.80                        | 16.80                               | 79.07 | 0.750          | 0.040                        |

(SOC: soil organic carbon, BD: bulk density, volumetric water content)

Block-kriged maps of the soil properties were created by GS+9 program (Gamma Design, 2010), using 0.32 x 0.32 m<sup>2</sup> grid system with 8836 points (Figure 2). While the SOM content values increased from the east to the west part of field, the lowest SOM content values were obtained at the northeast part of field. Similarly, clay content in soil generally increased in the east to west direction of the plot. On the contrary, high BD is found in the eastern part of the plot. It is known that the variation in bulk densities is the result of differences in soil texture, organic matter contents and management practices (Wolf and Snyder, 2003).

Soil OC content had significant positive correlations with clay (0.365\*\*) and total porosity (0.288\*), while it gave negative correlations with BD (-0.286\*), silt (-0.429\*\*), W (-0.288\*) and θ (-0.362\*) contents (Table 3). Organic matter increases the soil's capacity to hold water by direct absorption of water and by enhancing the formation and stabilization of aggregates containing abundance of pores that hold water under moderate tensions (Weil and Magdoff, 2004). Volumetric water content had a significant positive correlation with BD (0.480\*\*).

Table 3. Correlation matrix among the soil properties

|          | Clay    | Silt     | Sand     | BD      | F        | $\theta$ | pH     | EC       |
|----------|---------|----------|----------|---------|----------|----------|--------|----------|
| SOC      | 0.365** | -0.429** | 0.157    | -0.286* | 0.288*   | -0.362*  | -0.034 | 0.178    |
| Clay     |         | -0.495** | -0.313*  | -0.358* | 0.362*   | -0.431** | 0.176  | 0.007    |
| Silt     |         |          | -0.671** | 0.139   | -0.127   | 0.232    | -0.139 | 0.007    |
| Sand     |         |          |          | 0.153   | -0.170   | 0.114    | 0.002  | -0.013   |
| BD       |         |          |          |         | -0.995** | 0.480**  | -0.138 | 0.018    |
| F        |         |          |          |         |          | -0.466** | 0.134  | -0.015   |
| $\theta$ |         |          |          |         |          |          | 0.019  | 0.082    |
| pH       |         |          |          |         |          |          |        | -0.602** |

\*\*correlation is significant at 0.01 level, \*correlation is significant at 0.05 level. (SOM: soil organic matter, BD: bulk density,  $\theta$ : volumetric water content, )

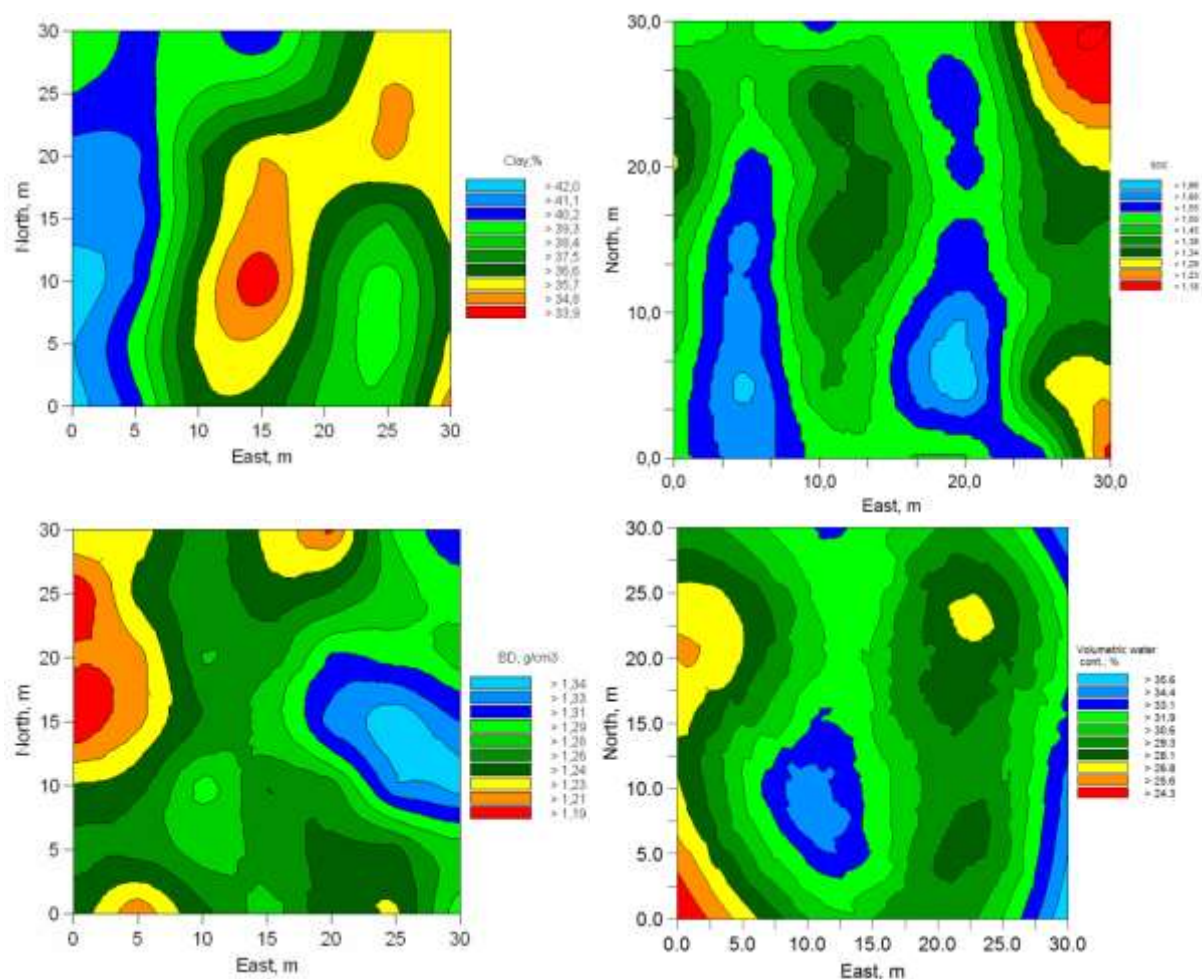


Figure 2. Block kriged maps for clay content, bulk density (BD), soil organic carbon content (SOC) and volumetric water content.

## CONCLUSIONS

According to the CV values, SOC and the other soil properties, except clay content, showed less variation in the field. Generally, the range or the distance of spatial dependence for the soil physical parameters, except BD, varied between 79 m and 157 m. These are the distance between two sample-collecting points for soil properties in the field. While the BD had moderate spatial dependence, the other soil physical properties had strong spatial dependence with having lower nugget/sill ratio less than 25%. Strong spatial dependency of the soil properties may be attributed to clay content, and moderate spatial dependency of BD can be attributed to effect of soil tillage. There were strong relationships among the soil properties. Kriged maps illustrated positional similarity between the SOC content and related with other soil properties along the small scale plot of cultivated field. As a result, SOC showed high spatial variability even if in the small-scale plot cultivated for preparing suitable seed bed and plant growth soil conditions. Therefore, in precision agricultural, heterogeneity and variation of soil properties such as, SOC, BD and water content in a field due to soil plowing should be taken into consideration for a successful site specific management. The spatial variability of SOM in cultivated fields can also be predicted for monitoring organic carbon in global warming researches.

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## THE IMPORTANCE OF MOLECULAR METHODS USED IN DETERMINING HERITAGE DISEASES IN CATTLE

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### ABSTRACT

Hereditary diseases in cattle are a very important group of diseases that cause significant yield and economic losses and hereditary diseases are mostly caused by autosomal recessively inherited alleles. Disorders (physiological and morphological) in the genetic material transferred to the offspring by heredity, diseases that negatively affect the health and yield of the animal or that reduce fertility by causing embryonic death are defined as hereditary diseases. Hereditary diseases cause the cessation of protein synthesis by changing the genetic codes as a result of mutations in the transferred genes spontaneously or due to environmental effects. Understanding the molecular mechanisms of hereditary diseases and diagnosing them with molecular techniques at the gene level is a cheap, practical, fast and precise method. It is very important in reducing economic losses, removing diseases from the herd and detecting hereditary disease carriers in the herd in the breeding programs to be applied. Determination of carrier individuals in terms of hereditary diseases is a very important step for the genetic improvement of production in cattle breeding in Turkey. In particular, it should be checked whether the breeds are free from important hereditary diseases. It should be considered that mutant alleles causing hereditary diseases known to be breed-specific, such as Bovine Leukocyte Binding Deficiency (BLAD), Factor XI deficiency (FXID), Spinal muscular atrofi (SMA), Limber legs, *Brachyspina* syndrome (BS), Uridine monophosphate synthetase deficiency (DUMBS), Mule foot (Syndactyly), Bovine citrullinemia (BC) and Complex vertebral malformation (CVM), can also be spread to other cattle breeds by crossbreeding.

**Key words:** Bovine, hereditary disease, molecular method, mutation.

### INTRODUCTION

Hereditary diseases in cattle are a very important group of diseases that cause significant yield and economic losses and hereditary diseases are mostly caused by autosomal recessively inherited alleles (Hacıhasanoğlu Çakmak and Yardibi, 2019; Aksel et al., 2021). Holsteins are one of the most preferred cattle breeds around the world, also in Turkey. Autosomal genetic diseases are an important issue for dairy cattle owners (Patel et al., 2006). Disorders - physiological and morphological- in the genetic material transferred to the offspring by heredity diseases that negatively affect the health and yield of the animal or that reduce fertility by causing embryonic death are defined as hereditary diseases (Arthur et al., 2001). Hereditary diseases cause the cessation of protein synthesis by changing the genetic codes as a result of mutations in the transferred genes spontaneously or due to environmental effects (Başaran 1996). Understanding the molecular mechanisms of hereditary diseases and diagnosing them with molecular techniques at the gene level is a cheap, practical, fast and precise method. It is very important in reducing economic losses, removing diseases from the herd and detecting

hereditary disease carriers in the herd in the breeding programs to be applied (Akyüz and Arslan, 2009; Adamov et al., 2014; Hacıhasanoğlu Çakmak and Yardibi, 2019).

### SOME HEREDITARY DISEASES IN CATTLE

Determination of carrier individuals in terms of hereditary diseases is a very important step for the genetic improvement of production in cattle breeding in Turkey. In particular, it should be checked whether the breeds are free from important hereditary diseases. It should be considered that mutant alleles causing hereditary diseases known to be breed-specific, such as Bovine Leukocyte Binding Deficiency (BLAD), Factor XI deficiency (FXID), Spinal muscular atrophy (SMA), Limber legs, *Brachyspina* syndrome (BS), Uridine monophosphate synthetase deficiency (DUMBS), Mule foot (Syndactyly), Bovine citrullinemia (BC) and Complex vertebral malformation (CVM), can also be spread to other cattle breeds by crossbreeding. The genotypes for BLAD, FXID, SMA, Limber legs, BS, DUMPS, MF (Syndactyly), BC, and CVM were identified by using microsatellite, sequencing, and PCR-RFLP method. The primers and restriction enzymes used in this study for each genetic disorder were shown in Table 1.

Table 1. Primers and restriction enzymes (RE) used for identification of BLAD, DUMPS, CVM, FXID, MF, SMA, BC and BS genotypes

| Hereditary diseases | Primers  | Rest Enzyme                   | References                                     |
|---------------------|--|-------------------------------|--|
|                     | F; forward, R; reverse   |                               |  |
| BLAD                | F: 5' GAATAGGCATCCTGCATCATATCCACCA 3'<br>R: 5' CTTGGGGTTTCAGGGGAAGATGGAGTAG 3'                     | TaqI                          | Meydan et al., (2010)                          |
|                     | F: 5' CCTGCATCATATCCACCAG 3'<br>R: 5' GTTTCAGGGGAAGATGGAG 3'                                       | <i>TaqI</i> ,<br><i>HaeII</i> | Adamov et al., (2014)                          |
| DUMPS               | F: 5' GCAAATGGCTGAAGAACATTCTG 3'<br>R: 5' GCTTCTAACTGAACTCCTCGAGT 3'                               | <i>AvaI</i>                   | Schwenger et al., (1994)                       |
| CVM                 | F: 5' CACAATTTGTAGGTCTCAATGCA 3'<br>R: 5' CGATGAAAAGGAACCAAAAGGG 3'                                | <i>EcoT22I</i>                | Kanae et al. (2005)                            |
|                     | <i>CVM-PIRA-F</i> : CACAATTTGTAGGTCTCACTGCA 3'<br><i>CVM-PIRA-R</i> :<br>CGATGAAAAGGAACCAAAAGGG 3' | <i>PstI</i>                   | Adamov et al., (2014)                          |
| FXID                | F:5' CCCACTGGCTAGGAATCGTT 3'<br>R:5' CAAGGCAATGTCATATCCAC 3'                                       | Sequencing                    | Marron et al., (2004)                          |
| MF                  | F: 5' AGCGTGTGGACAAGTACTCAG 3'<br>R: 5' ACCTCAAGCTCAAAGCTCCTA 3'                                   | Sequencing                    | Duchesne et al., (2006)                        |
| SMA                 | F: 5' CCCTGGGCTGAAAGGAATCA 3'<br>R: 5' TGGGGCAGGGTACCTGAG 3'                                       | <i>MvaI</i>                   | Krebs et al., (2007)                           |
| BC                  | F: 5' GGCCAGGGACCGTGTTCATTGAGGACATC 3'<br>R: 5' TTCCTGGGACCCCGTGAGACACATACTTG 3'                   | <i>AvaII</i>                  | Grupe et al. (1996)                            |
| BS                  | F: 5' GCTCAAGTAGTTAGTTGCTCCACTG 3'<br>R: 5' ATAAATAAATAAAGCAGGATGCTGAAA 3'                         | pBR322 /<br><i>MspI</i>       | Charlier et al., (2012)<br>Fang et al., (2013) |



### **Bovine Leukocyte Binding Deficiency (BLAD)**

It is a lethal autosomal recessive inherited disease in Holstein cattle (Ackermann et al., 1993; Citek et al., 2006; Czarnik et al., 2007). Bovine leukocyte binding deficiency disease is a point mutation that causes the conversion of aspartic acid, the 128th amino acid of this gene, into glycine as a result of the replacement of adenine nucleotide with guanine nucleotide at position 383 of the gene. The allele that causes BLAD is frequency of c.383A>G (Kehril et al., 1992; Nagahata et al., 1994). It encodes the  $\beta$ 2 integrin (CD18) glycoprotein, the subunit of the CD11/CD18 complex, which provides endothelial-leukocyte binding. This amino acid change causes disruption of the synthesis of the  $\beta$ 2 integrin molecule, which binds to the receptors in the vascular endothelial cells of the leukocytes during infection and allows the leukocytes to pass out of the vessel (Kehril et al., 1992; Nagahata et al., 2004; Schuster et al., 1992)

Clinical signs of BLAD in sick calves: Ulcers in the mouth, gingivitis, persistent diarrhea, pneumonia, bronchitis are detected in sick calves immediately after birth (Akyüz and Ertuğrul 2006; Czarnik et al., 2007). In patients, there is a sustained increase in leukocytes. There are 8000/1mm<sup>3</sup> leukocytes in the blood of a healthy cattle, but there are more than 100,000/1mm<sup>3</sup> leukocytes in sick cattle. Sick animals die at an early age. However, under good care conditions, they can live up to two to three years. (Kehril et al., 1992; Akyüz and Ertuğrul 2006; Czarnik et al., 2007).

BLAD first was reported in 2006 in Turkey by Akyüz and Ertuğrul. The prevalence of BLAD carriers among Holstein bulls and bull candidates was found 0.84%. The researchers determined that the mutant allele for the frequency of BLAD disease was 24% (Schütz et al., 2008). Later, Akyüz et al. 2010 researched BLAD in Holstein cattle in Turkey. The prevalence of BLAD carriers among Holstein cows was found to be 2.2%. Prevalence of BLAD carrier among Holstein bulls in Turkish Holstein population is much lower than the prevalence of BLAD carrier among Holstein cows in Turkey. The prevalence of BLAD carriers for bulls and cows in Japan (Patel et al., 2007a) and some other European countries (Citek et al., 2006; Czarnik et al., 2007) much higher than the values reported in Turkey. The frequency of mutant BLAD allele in Holstein cattle was estimated as 0.035 by Meydan et al., (2006) and 0.02 by Meydan et al., (2010). BLAD alleles were not found in Turkish native cattle breeds (Akyüz and Ertuğrul 2006). Similarly, BLAD carriers were not found in Gir cattle in Brazil (Riberio et al., 2000) and in Brown Swiss bulls in Iran (Norouzy et al., 2005). The presence of mutant allele causing BLAD has been shown for the first time except of pure Holstein breeds (Aksel et al., 2021). The prevalence of BLAD carriers was found to be 5% (Patel et al., 2011) and 1.69% (Roy et al., 2012) in India, 1.37% in China (Zhang et al., 2012), 6.6% in Iran (Hemati et al., 2015). It has been reported to be as 0.0025% in Lithuanian dairy cattle (Morkūnienė et al., 2019), 0.82% in Czech cattle (Citek et al., 2006), 0.69% in China Holsteins (Sun et al., 2011), 3.23% Indian Holstein in Fresian cattle (Patel et al., 2007a), 3.3% in Iranian Holsteins (Norouzy et al., 2005), 14.1% in American Holstein (Shuster and et al., 1992), in Polish 7.9% Holstein Fresian cattle; (Czarnik et al., 2007), 13.4% in Japan (Nagahata et al., 1997). In Turkey, Şahin et al. (2013) and Korkmaz Ağaoğlu et al. (2015) reported the prevalence of BLAD carriers in Holsteins to be 2.18%, 2% respectively. the study of Çakmak and Yardibi (2019) the incidence of BLAD carriers among the Holstein cattle was calculated at 1.33%. According to the results they have obtained, their incidence is found to be lower than those reported in studies from China, India, Iran and Turkey.

### **Uridine Monophosphate Synthase Deficiency (DUMPS)**

It is an autosomal recessive inherited disease in Holstein cattle. Embryonic death in homozygotes about day 40 of pregnancy (Schwenger et al., 1993; Citek et al., 2006). It is especially important in dairy cattle breeding as it causes embryonic death (Shanks et al., 1992). This inherited disease causes more insemination per calving and longer calving intervals



(Akyüz and Ertuğrul, 2008). Orotic acid, which emerges in the last step of pyrimidine nucleotide synthesis, is converted to uridine monophosphate by the uridine monophosphate synthetase enzyme (UMPS). A point mutation (C → T) at codon 405 within exon 5 causes an early stop codon in the UMPS gene in cattle, a functionally impaired enzyme is synthesized (Kaminski et al., 2005; Ghanem et al., 2006; Patel et al., 2006; Citek et al., 2006). The UMP synthase gene was mapped to the bovine chromosome 1 (Schwenger et al. 1994; Citek et al. 2006). The amount of orotic acid in animals can be detected in milk, urine and blood (Robinson and Shanks, 1990).

The mutant allele that causes this hereditary disease has also been found in Holsteins breed in India, USA, Canada, Germany, Hungary (Schwenger et al., 1993; Kaminski et al., 2005; Citek et al., 2006; Ghanem et al., 2006). Akyüz and Ertuğrul researched DUMPS in Holstein and native cattle in Turkey in 2008. There were no positive results indicating the presence of DUMPS allele in any sample from the breeds and samples studied. It was determined that 1.79% of the bulls and 0.96% of the cows examined in Argentina in 1996, and two of the 1468 Holsteins examined in Taiwan in 2001 carried the DUMPS allele (Patel et al., 2006). On the other hand, no DUMPS carrier was found in a study conducted in Poland in 2005 using 2209 animals (Kaminski et al., 2005). In a study conducted in India, it was reported that none of the samples examined were carriers of DUMPS (Patel et al., 2006). It was determined that none of the 509 cattle examined were carriers of DUMPS genetic defects in Turkey (Meydan et al., 2013). DUMPS carriers were not found in Brazilian cattle (Riberio et al., 2000), in Polish dairy cattle (Kaminski et al., 2005), in Iran Brown Swiss bulls (Norouzy et al., 2005), in Czech Republic cattle (Citek et al., 2006) and in Indian cattle (Patel et al., 2006). These results supported that these genetic disorders are specific to only Holstein cattle worldwide. DUMPS allele was not found in Holstein cattle (Akyüz and Ertuğrul 2008; Meydan et al., 2010; Öner et al., 2010) as well as Turkish native cattle breeds.

### **Complex Vertebral Malformation (CVM)**

CVM is a lethal disease characterized by autosomal recessive inheritance in Holstein cattle (Agerholm et al., 2004a; Ghanem et al., 2008; Nagahata et al., 2009). The disease results in fetal death and abortion (usually before day 260 of pregnancy) with spinal cord anomaly (Agerholm et al. 2001). It is a point mutation (G → T) that causes the substitution of the guanine nucleotide with the thymine nucleotide at position 559 of the SLC35A3 gene located bovine chromosome 3 that encodes the uridine diphosphate-N-acetylglucosamine transporter (Agerholm et al., 2001; Steffen 2001; Thomsen et al., 2006).

The most prominent pathologies seen in calves affected by CVM; Twisted rigid ankles, crippled legs, a shortened neck, and a curvature of the spine. Joint curvatures, spinal cord curvature, malformations in the vertebral column (Agerholm et al., 2001, 2004b; Nielsen et al. 2003; Kanae et al., 2005).

The presence of the disease was first reported in Denmark (Agerholm et al., 2001) and later, the presence of this hereditary disease was detected in countries such as United Kingdom (Revell, 2001), USA (Duncan et al., 2001), Japan (Nagahata et al., 2002), Sweden (Berglund et al., 2004), Czech Republic (Citek et al., 2006), Poland (Rusc and Kaminski, 2007), Iran (Rezaee et al., 2008). Adamov et al. (2014) studied in Holstein-Friesian cattle population in Republic of Macedonia. This study demonstrates that carriers of CVM is present in Macedonia, although at low frequency. The frequency of mutant CVM allele in 350 Holstein cow was calculated as 0.017 (Meydan et al., 2010) but in another study the results of Meydan et al.'s (2013) demonstrated that there was no carrier for CVM in 509 Turkish native cattle examined. The prevalence of the results of Meydan et al. (2010) is very low compared to Denmark (31.0%) (Thomsen et al., 2006), Poland (24.8%) (Rusc and Kaminski, 2007), Japan (32.5%) (Nagahata et al., 2002), Sweden (23.0%) (Berglund et al., 2004), and Germany (13.2%) (Konersmann et

al., 2003). CVM carriers were identified frequency of 21.6% (Saber et al., 2014) and 34% (Hemati et al., 2015) respectively in Iran. It has been reported that the frequency of the carrier animals are 10.48% (Zhang et al., 2012), 2.92 % (Wang et al., 2011) and 15.58% (Wang et al., 2012) in Holstein cattle in China. Kulaklı and Akyüz (2011) did not find carriers of CVM in Turkey. Hacıhasanoğlu Çakmak and Yardibi (2019) also didn't find CVM carriers.

#### **Factor XI Deficiency (FXID)**

It is an autosomal recessive bleeding disorder caused by the addition of an adenine-rich 76 base pair long fragment to the 12th exon of the Factor XI gene located on chromosome 27 in cattle (Liptrap et al., 1995; Mukhopadhyaya et al., 2006). Factor XI (FXI) is a protein that plays a key role in plasma coagulation. Factor XI, one of the proteins involved in blood coagulation, is also called serine protease (Marron et al., 2004; Ghanem et al., 2005). Coagulation is a balanced system which has two important roles; It limits blood loss by forming fibrin after injuries and enables blood coagulation in the veins, thus protecting against deep vein thrombosis or myocardial infarction (Brush et al., 1987). The sick animals have a high susceptibility to mastitis, metritis, pneumonia, excessive bleeding, and reproductive problems, calves show a lower birth weight and survival rate than the norm. The prolonged bleeding time after injection, bloody milk intake, and anemia are observed (Marron et al., 2004; Citek et al., 2006; Korkmaz Ağaoğlu et al., 2015).

Meydan et al., (2009) studied 225 Holstein cows in Turkey and they determined carriers of the FXID. As a first attempt, this study found that the mutant FXI allele frequency in Holstein cows in Turkey is 0.9% and the prevalence of carrier cattle is 1.8%. Later The frequency of mutant FXID allele in 350 Holstein cow was detected as 0.006 and the FXI carrier prevalence was 1.2 % (Meydan et al., 2010). In another study, Öner et al., (2010) studied 170 Holstein cow in Turkey and the frequency of the FXI mutant allele and the FXI carrier prevalence were 0.06 and 1.17 % respectively The carrier prevalence and FXID mutant allele frequency were as 0,4 % and 0.002 of 504 Holstein cows (Karlı et al., 2011) respectively, 1,8% and 0.009 of 500 Holstein cattle (Korkmaz Ağaoğlu et al., 2015), respectively. These results are quite similar to each other. On the contrary Avanus and Altinel (2016) researched 287 Holstein cow and FXID carrier in Thrace region of Turkey and they didn't find carrier cattle. Aksel et al., (2021) also investigated FXID in 48 Holstein cross in Turkey but No FXID carriers were found. FXID carriers were not found in 509 Turkish native cattle (Meydan et al., 2013). Hacıhasanoğlu Çakmak and Yardibi (2019) also didn't find FXID carriers. Yaşar investigated FXID in 150 Holstein cows in 2011 and found the prevalence of FXID to be approximately 0.7%. It was determined that FXID prevalence was 1.7% in the investigated Holstein bulls by Akyüz in 2013. The allele frequency data of FXID were studied in Holstein cattle of various countries and reported as; 1,2% of 419 cattle in USA (Marron et al., 2004), 0.36% of 279 cattle in Czech Republic (Citek et al., 2008), 0,61% of 330 bulls in India (Patel et al., 2007b) and 1% of 500 cows in Japan (Ghanem and Nishibori, 2009). The prevalences in this studies are also similar but in India, any of 307 Holstein cattle was not identified as FXI deficient or a carrier (Mukhopadhyaya et al., 2006). Two carriers and a FXID animal of 576 Holstein cows were reported in China (Zhang et al., 2010).

#### **Mule Foot (Syndactyly)**

Mulefoot disease (MFD) in cattle is an autosomal recessive disorder of phenotypically variable in different cattle breeds that causes syndactyly (Johnson et al., 2006; Drögmiller et al., 2006). Syndactyly in cattle is an autosomal recessive abnormality characterized by the fusion of the functional digits. In cattle, syndactyly, also called "mulefoot," refers to the fusion or nondivision of the two functional digits of the bovine foot and consists mainly in synostotic phalanges. There are single quotes instead of two and sick calves cannot stand or walk (Hart-

Elcock et al., 1987). Syndactylies and polysyndactylies were malformations of the distal limbs that result in fusion with or without the presence of supernumerary digits (Hamosh et al., 2005). This disorder has been previously mapped to the telomeric part of bovine chromosome 15 (Charlier et al., 1996). Syndactyly has been reported in different cattle breeds such as Holstein, Simmental, Aberdeen–Angus, Japanese Native, Brown Swiss, Chianina, and Czech Black Pied (Millar et al., 2000). LRP4 mutations in the pathogenesis of congenital syndactyly in different cattle was studied. Duchesne et al., (2006) presented solid evidence that a 2-bp substitution in exon 33 of the LRP4 gene is a strong candidate causal mutation for syndactyly in Holstein cattle. Megf7/Lrp4 encodes a member of the multifunctional low-density lipoprotein (LDL) receptor gene family (Herz et al., 2002; Nykjaer et al., 2002). Johnson et al.'s (2006) showed that a G → A transition at the first nucleotide in the splice donor site of intron 37 completely disables this splice site. Their findings provide precise evidence that mutations in Megf7/Lrp4 cause MFD in cattle. Drögemüller et al. (2006) studied genetic analysis of syndactyly in German Holstein cattle using Microsatellite genotyping markers RM004, BM848 and BMS820. Congenital syndactyly with a variable number of affected feet was observed in eight black and white German Holstein calves. Two mutations in the bovine LRP4 gene have been reported as the primary cause of syndactyly in the Holstein and Angus cattle breeds and four novel mutations in the bovine exon 33 of the LRP4 gene were detected by Drögemüller et al. (2007).

### **Spinal Muscular Atrophy (SMA)**

SMA is a neurodegenerative disease of Brown Swiss cattle that is inherited as a simple autosomal recessive trait and is characterized by progressive degeneration and death of alpha motor neurons. Axial muscles are also affected (El-Hamidi et al., 1989). The first consistent description of SMA in cattle was reported in 19 Brown Swiss calves in 1989 by El-Hamidi et al. Later, Spinal muscular atrophy (SMA) is a heritable condition in Brown Swiss cattle characterized by profound muscular atrophy affecting appendicular muscles, particularly of the rear limb (Troyer et al., 1993). Agerholm et al. (1994) described SMA in Red Danish calves. SMA is a motor neuron disease (MND) in humans and diverse animal species (He et al., 2005). Clinically, MNDs are characterized by neurogenic muscle atrophy, paresis, and hyporeflexia (Garg et al., 2017). Degeneration and loss of motor neurons cause progressive weakness and neurogenic muscular atrophy. It has been associated with a genetic mutation of the FVT1 gene on chromosome 24 (BTA24), also known as 3- keto dihydrosphingosine reductase (KDSR). A transition from G to A at the first nucleotide of codon 36 of exon 6 which determines the substitution of Ala175 with Thr175 is thought to be the causative mutation of SMA (Krebs et al., 2007). FVT1 is part of the glycosphingolipid metabolism and catalyzes the second step in the biosynthesis of the central precursors of this pathway, sphingosine and ceramide. Bovine SMA has been reported in Brown Swiss, Holstein, Friesian, and Red Danish breed. Cagnotti et al., (2020) studied genetic analysis of five Blond d'Aquitaine calves diagnosed with SMA and to determine whether the mutation was associated with the disease. The findings confirmed the diagnosis of SMA but No mutation of the FVT1 gene was found on genetic analysis

### **Limber Legs**

Limber leg is a hereditary condition of Jersey cattle, apparently controlled by a simple lethal autosomal recessive gene (Lamb et al., 1976). Some affected calves are born dead. Alive calves appear normal at birth but are unable to stand because of incompletely formed muscles, ligaments, tendons, and joints. its front legs are entwined or crossed, and its hind legs are open to the side (Lamb et al., 1971). Cattle have abnormal position of legs. Joints appear larger than normal due to muscle atrophy. In non-hereditary abnormalities there are caused by multiple gene factors. Abnormalities have occurred naturally as a result of environmental factors such as ingestion of toxic substance, faulty nutrition or radiation. this condition termed linger legs

because of the lack of control of legs, is characterized clinically by abnormal flexure and extension of the joints, especially the shoulders, elbow, knee, hip, stifle and hock. Generally all four legs affected (Warkany, 1960; Shupe et al., 1967 ). In 1969, nine abnormal female calves were born in a registered Jersey herd in Utah. These were characterized by abnormal flexure and extension of the shoulder, elbow and hip joints. Calves were normal in outward appearance except they had little or no control over the movement of their limbs. This lack of control and abnormality of the limbs led to calling the condition "limber legs" (Lamb et al., 1971).

### **Brachyspina Syndrome (BS)**

BS is a rare monogenic autosomal recessive hereditary disorder identified in the Holstein breed (OMIA, 2020). BS was first described in 2006 by Danish researchers in Denmark (Agerholm et al., 2006). Later, the cases were reported in the Netherlands, Italy, Germany, and Canada (Agerholm and Peperkamp, 2007; Agerholm et al., 2010; Buck et al., 2010; Testoni et al., 2008). Brachyspina is a single gene recessive trait, which can cause embryonic death, stillbirth, or other physical deformities. It is characterized by a shortened spinal cord, long legs, and abnormal organs in calves (Agerholm et al., 2006; Agerholm and Peperkamp, 2007; Testoni et al., 2008). There are clinical signs such as stillborn calves, Obvious shortening of the spine, long and slender limbs, Inferior brachygnathism, and internal organ malformation, such as renal and gonadal dysplasia. High proportion of abortions, Long calving interval (Agerholm et al., 2006; 2010; Charlier et al., 2012). Some of the consequences of BS are the reduction of the fertility rate and milk production (Federici Rodriguez et al., 2021).

Brachyspina syndrome is caused by a mutation in the FANCI gene (Fanconi Anemia complementation group I) that is located on chromosome 21. FANCI encodes for a monoubiquitin protein with a fundamental role in DNA repair processes (Fang et al., 2013). This deletion of 3.3Kb in the FANCI gene which leads to a frame-shift and premature stop codon (Agerholm et al., 2006; 2010; Testoni et al., 2008). BS carriers for the allele of the disease were determined with Many other studies (Vanraden et al., 2011; Fang et al., 2013; Sahana et al., 2013; Li et al., 2016; Artigas et al., 2020). Fang et al., (2013) studied in China 206 Holstein bulls and 136 Holstein cows for BS, identified 10 BS carrier bulls and 3 carrier cows. The frequency of BS carriers in the present study (4.9% in bulls and 2.2% in cows) is lower than that reported in the United States (6%) and the Netherlands (7.4%) (VanRaden et al., 2011; Charlier et al., 2012). The haplotypes identified in Nordic Holsteins are linked to Brachyspina syndrome and the haplotype 21–276 carries the disease gene (Sahana et al., 2013). They observed the carrier frequency of 4% in the Nordic Holstein population. The lowest frequency of BS was reported in the Nordic Holstein population. Federici Rodriguez et al., (2021) also detected Brachyspina mutation in 72 Uruguayan Holstein cows.

### **Bovine Citrullinaemia (BC)**

BC is a rare and Holstein-Friesian specific metabolic genetic disorder of cattle (Windsor, 2009). The disease occurs as a result of the deficiency of the argininosuccinate synthetase (ASS) enzyme, which enables the conversion of citrulline to arginosuccinate in the urea cycle. In the urea cycle due to the absence of the arginosuccinate synthetase enzyme, It causes the accumulation of citrulline which is a more toxic product than ammonia in the body during the conversion of ammonia to urea (Padeeri et al., 1999). ASS is a urea cycle enzyme which is expressed at high levels in the liver. The enzyme catalyzes the conversion of citrulline, aspartate, and ATP to yield argininosuccinate, the immediate precursor of arginine (Husson, 2003). It has been determined that this hereditary disease develops as a result of a C86T transition mutation in the 5th exon of the gene encoding the ASS enzyme. The mutation responsible for this disorder has been characterized as a single-base substitution (C → T), converting the CGA codon that codes for arginine-86 to TGA, a translation- termination codon.

(Dennis et al., 1989). The BC gene coding for ASS was mapped to chromosome BTA11 in the bovine genome (Grupe et al., 1996). This genetic disorder is autosomal recessive and that is lethal in the early postnatal period (Robinson, 1993). Affected calves display severe neurological dysfunction and death within 1 week. BC animals show impaired urea cycle leading to ammonia accumulation in blood and tissues. There are symptoms such as unsteady gait, aimless wandering, visible blindness, head pressing, and convulsions (Dennis et al., 1989; Ilie, 2011).

BC, first was described in the Australian Holstein cattle by Dennis et al., in 1989. Later, BC studies continued ve Robinson et al., (1993) 367 US Holstein bulls evaluated, and one carrier was identified (bull C). This result corresponds to an incidence of 3%. Patel et al. in 2006 researched BC in Indian Holstein cattle and none of the animals were carriers of citrullinaemia. 350 Holstein cows examined for BC in Turkey and carriers of BC was not detected (Meydan et al., 2010). Öner et al., (2010) investigated 170 Holstein cows and they didn't find BC carrier. Meydan et al., (2013) studied on BC in Turkish native cattle breeds and their results demonstrated that the mutant allele of BC is absent in Turkish native cattle. Similarly, BC carriers were not found in Gir cattle in Brazil (Riberio et al., 2000).

## CONCLUSION

It is necessary to investigate the genetic structures of cattle in Turkey in terms of hereditary disorders. Determination of carrier individuals in terms of hereditary diseases is a very important step for the genetic improvement of production in cattle breeding in Turkey. It should be considered that mutant alleles causing hereditary diseases known to be breed-specific, such as BLAD, FXI, BC, SMA, Limber legs, BS, DUMPS, Mule foot (Syndactyly) and CVM, can also be spread to other cattle breeds by crossbreeding. In particular, it should be checked whether the breeding male and female breeds are free from these hereditary diseases.

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## COMPARISON OF TWO APPS BUILT WITH UNITY AND VUFORIA

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### ABSTRACT

Augmented reality is to show computer-generated virtual objects simultaneously in the real world by using the camera feature of our smart devices. This research aims to provide information and example usage of augmented reality in order to reduce paper waste by using technology. Since the studies in this field are mostly in the gaming and entertainment sector, it is a striking deficiency that the advantages of this technology are not explained. An electronic device will be selected in the research and its user manual will be taken as reference, then this user manual will be turned into an application using augmented reality technology. At the same time, a new manual will be prepared for the user manual that has been turned into an application, and this manual will only contain markers and the manual will definitely be less than half the number of pages of the original manual.

**Keywords:** Augmented reality, user manual, mobile application, Vuforia, Unity

### INTRODUCTION

With technological advancement, visuality has become necessary in our age. Specific applications in our technological devices such as computers, smartphones and tablets interact with users through an interface. The interface can be thought of as a bridge that meets the visual need between the user and the application. However, in line with the increasing needs, interface designs are insufficient in some application designs.

Applications usually have a 2D design. This offers the user a virtual environment completely disconnected from the real world. Although 2D virtual environments do not trigger people's visual perception much, their memorability is lower than in 3D environments. With the development of technology in recent years, it has begun to move from 2-dimensional environment designs to 3-dimensional environment designs that are more memorable and appealing to the senses.

3D designs can be examined under the name of "Augmented Reality". Augmented reality is rapidly rising among the technologies that are frequently used by well-established companies such as Google and Apple in recent years, on which development has been made and people want to use more.

User manuals produced for electronic devices are booklets consisting of pages of text, and today most people try to start using the device directly without reading it. These booklets are generally just a waste of paper. In this article, the solution to the problem has been determined as getting rid of the paper waste of these user manuals and turning them into useful ones. In order to solve this problem, augmented reality technology, whose use has increased frequently today, has been determined as a resource.

The purpose for solving the identified problems;

It has been determined to provide information about the use of augmented reality technology, to provide ease of understanding by embodying soft information using this technology, and to prevent paper waste by using this technology as the ultimate goal.

Many scientists have put forward different definitions of augmented reality. For example, according to Azuma, augmented reality provides information that cannot normally be perceived by human senses in order to develop people (Somyürek, 2014).

According to Behringer, Mizell, and Klinker, this technology increases situational awareness by placing virtual objects or information in the real world (Somyürek, 2014).

Dias defined augmented reality as a multidisciplinary field that includes computer-human interaction areas where computer-generated data blends with the real world (Demirel, 2017).

Based on these definitions, we can say that augmented reality, in its simplest definition, is the real-time display of virtual objects created in the virtual environment in the real world.

## USAGE AREAS OF AUGMENTED REALITY TECHNOLOGY

With the increase in the frequency of use of augmented reality technology in recent years, its usage areas have increased at the same rate.

In the field of education, Augmented Reality is used to embody intangible information and to increase learning by providing unique visual interaction.

Table 1. Usage Areas of Augmented Reality Technology

| Author                                  | Date | Field  |
|---|------|--|
| Kerawalla, Luckin, Seljeflot, & Woolard | 2006 | Comparison of augmented reality and traditional classroom applications                       |
| Abdüsselam & Karal                      | 2012 | The effect of using augmented reality in physics teaching on academic achievement            |
| Cuendet, Bonnard, Do-Lenh & Dillenbourg | 2013 | A study showing that augmented reality applications can be made in the classroom environment |
| Çetinkaya & Akçay                       | 2013 | Augmented reality use in educational environments  |
| Serio, Ibáñez, & Kloos                  | 2013 | The effect of augmented reality on students' motivation                                      |

In the field of marketing, companies use this technology to market their products more memorably and engagingly with the user.

Augmented reality in artistic events, as an exhibition method, offers a unique experience by attracting more attention from the audience.

In the game industry, games have started to use augmented reality technology to increase the interest of the players.

## AUGMENTED REALITY APPLICATION TYPES

An augmented reality application can be developed in 4 different ways according to the working principle of the application. These species are;

- Marker based AR
- Marker-less AR
- Projection based AR
- Superimposition based AR

**Marker Based AR:** Marker based augmented reality applications are the most commonly used augmented reality applications. Simply put, the camera will scan the marker and show the 3D object that needs to be displayed on the screen (Deniz, 2020). It can also be called image recognition (Erençin, 2020). The biggest advantage of applications designed in this way is that they adjust the position and orientation of the content according to the marker. With these applications, pictures in a magazine are modelled in 3D, allowing the user to have a unique experience.

**Marker-less AR:** Marker-less augmented reality apps are augmented reality apps that work without the need for a marker. They are also known as ground-based or location-based augmented reality (Erençin, 2020). It uses data from the user's location, which can be GPS data or ground identification data. This data used actually acts as a marker, the rest of its work is not different from cursor-based augmented reality.

**Projection Based AR:** This kind of augmented reality application, which does not have much usage area, is a newly developed one, which is formed by interacting with the light reflected on concrete surfaces without the need for a device.

**Superimposition Based AR:** It is a type of augmented reality close to virtual reality. It partially or completely changes the original image (Erençin, 2020).

## **TECHNOLOGIES THAT CAN BE USED FOR DEVELOPING AUGMENTED REALITY APPLICATION**

There are many frameworks that can be used for developing augmented reality applications. These frameworks are differentiated from each other according to the convenience they provide, the frequency of use, the platforms they support, and on what basis they are used.

**UNITY :** UNITY, which is the most used software to develop augmented reality applications, is actually a game engine that is often heard by players, publishers and developers. Unity is free software up to a certain profit and allows to development of a wide variety of applications for a wide variety of platforms. Unity, which has succeeded in being the software preferred by developers in this respect, is also a software that provides rich content for all kinds of applications thanks to its wide variety of packages. Unity seems to have a diverse structure with the ability to develop applications for PC, Mac & Linux Standalone, Android, Universal Windows Platform, tvOS, PS4, iOS, Xbox One, WebGL, and Facebook platforms.

Unity is a preferred platform for augmented reality applications as it allows application development with C# language and can use C# Scripts in augmented reality applications.

Unity also offers an easy-to-use interface. The interface can be adjusted according to the screen size of the platform to be developed. Thus, problems of resolution are prevented in the applications to be made.

Each project consists of at least 1 scene. Everything in the scene is called an object, and there is at least one camera object in each scene. The camera is the window to the user's simulation world.

Components can be added or removed from each object in multiple ways. Objects can gain many features thanks to these components and can be made closer to reality.

**Components in Unity:** Transform Component, Three and two-dimensional graphic components (Mesh Renderer and Mesh Filter, Sprite Renderer), Physics manager and physics components (Rigidbody, Collider, Wheel Collider, Physics Material, Joints), UI Components (Canvas, Image, Button, Text, Script Component) (Doğan, 2019).

**Advantages and Disadvantages of the UNITY Platform :** Unity is one of the most frequently used platforms by developers. Advantages to getting here:

- Support for high level and frequently used languages such as C# and JavaScript
- Visual Studio support

- Easy addition of Android SDK and JDKs
- Ability to manufacture for multiple platforms
- Being free software up to a certain profit
- Hosting packages for augmented reality technology
- Having a very large store such as Asset Store

Disadvantages:

- In terms of performance, it is slightly worse than other software.
- Since it receives frequent updates, it is necessary to learn something new every time we open the application.

VUFORIA: Vuforia is a library that uses computer vision to detect 3D objects. The library has been developed for many languages and can be easily added to the unity project. Planar targets are stored in the device target database in image format. Thanks to the library, it is ensured that the device displays the 3D object when it sees this picture. When the target image is out of the camera angle, it is excluded from being displayed in the 3D object (Baykara et al., 2017).

Vuforia has been developed for mobile environments. It is designed to recognize multiple target images and 3D objects in real-time. It also has advanced technologies that enable the scanning of physical or complex solid objects.

Vuforia is a development kit that offers a lot of features for augmented reality applications. Just like Unity, it is free software until the earnings from the product reach a certain point. Thanks to Vuforia, we can use a picture we want as a marker. Because of this feature, it would not be wrong to say that it is software that allows us to produce markers for Vuforia. An augmented reality application is made by adding virtual objects to these produced markers.

If the images are black and white with high resolution and do not contain much detail, more successful markers are created because it is easier for a camera to detect and identify images with these characteristics than other images. The generated markers can be downloaded as in the last step and used directly in the Unity program.

ARCORE : It was released by GOOGLE in August 2017 as designed for android devices. As soon as ARCore starts to work, it scans all surfaces detected by the phone camera and collects information about this environment (Güngör, 2017). A flat surface is needed to place the 3D object in the augmented reality application in the real world, and ARCore determines the flat floor thanks to the information it has acquired and places the 3D object on the floor with the user's click on the flat surface.

ARCore uses 3 basic features to integrate with the real world (ARCORE, 2020):

- Motion Tracking: allows the phone to understand and track its position relative to the world.
- Environmental Understanding: allows the phone to detect the size and position of any surface: horizontal, vertical and angled surfaces such as floors, coffee tables or walls.
- Light Estimation: this allows the phone to predict the current lighting conditions of the environment.

ARCore supports development on 4 different platforms. These platforms are Android, Unity, Unreal, and iOS (ARCORE, 2020).

While ARCore was supported on very few devices until recently, it is now supported on almost all devices on the market, with the release of Android devices very often. The complete list of devices supported by ARCore technology is available at <https://developers.google.com/ar/discover/supported-devices>.

ARKit : Having almost the same features as ARCore, the feature that distinguishes this platform from ARCore is that; it is iOS-based, not Android. Thanks to the Lidar Scanner technology that Apple devices have in recent years, it provides depth detection and offers users an enhanced augmented reality experience. At the same time, thanks to its high-resolution maps, 3D objects



can be added to the maps and the user will have a better experience thanks to the high-resolution values.

**AR Foundation:** AR Foundation allows you to work with augmented reality platforms in a multi-platform way within Unity. This package presents an interface for Unity developers to use, but doesn't implement any AR features itself. To use AR Foundation on a target device, you also need separate packages for the target platforms officially supported by Unity:

- ARCore XR Plug-in on Android
- ARKit XR Plug-in on iOS
- Magic Leap XR Plug-in on Magic Leap
- Windows XR Plug-in on HoloLens

Packages need to be added to Unity along with AR Foundation (UNITY, 2020).

We can see the parts supported by the AR Foundation package for each package in Table 2 below (UNITY, 2020).

Table 2. The parts supported by the AR Foundation package for each package

|                                  | ARCore | ARKit | Magic Leap | HoloLens |
|----------------------------------|--------|-------|------------|----------|
| <b>Device tracking</b>           | ✓      | ✓     | ✓          | ✓        |
| <b>Plane tracking</b>            | ✓      | ✓     | ✓          |          |
| <b>Point clouds</b>              | ✓      | ✓     |            |          |
| <b>Light estimation</b>          | ✓      | ✓     |            |          |
| <b>Environment probes</b>        | ✓      | ✓     |            |          |
| <b>Face tracking</b>             | ✓      | ✓     |            |          |
| <b>2D Image tracking</b>         | ✓      | ✓     | ✓          |          |
| <b>3D Object tracking</b>        |        | ✓     |            |          |
| <b>Meshing</b>                   |        | ✓     | ✓          | ✓        |
| <b>2D &amp; 3D body tracking</b> |        | ✓     |            |          |
| <b>Raycast</b>                   | ✓      | ✓     | ✓          |          |
| <b>Pass-through video</b>        | ✓      | ✓     |            |          |

## DEVELOPMENT OF APPLICATIONS

**Augmented Reality Application Using Image Target:** The technologies to be used for the first application using Image Target were determined as Unity 2019.2.0f1, Vuforia, and 3ds Max, and it was decided to buy the 3D model from the Sketchfab site or Asset Store in Unity. Working steps with the general plan are determined as follows:

1. An electronic device will be selected, and a user manual will be taken.
2. Markers will be created for the user manual by using Vuforia.

3. The 3D model of the selected electronic device will be downloaded.
4. If necessary, the 3D model will be run using 3ds Max to add animation and animation will be added.
5. The ready-to-use 3D model will be transferred to the Unity workspace.
6. The user manual will be made into a complete application.

For the application to be made, it is necessary to choose a technological tool that has a user manual. While making this selection, attention was paid to ensure that the user manual is simple, understandable and modelable.

It has been determined that the user manual planned for the application needs 5 markers. The cover page and the first page of the user manual are not designed as markers. For this, a license key must first be created. To get a license key, we need to open the "Develop" tab from the Vuforia website and then say "Get Development Key". When we approve the necessary conditions, a license key is created where we can use the Vuforia Engine. For the markers, black and white images with clear outlines and images with a rating of 3 stars or more on Vuforia were selected. Selected markers have been uploaded to the system through the Vuforia official site and added to the database. Then, the database for the Unity editor was downloaded by clicking the Download Database (All) button.

Since the product chosen for the user manual is a hair dryer, a 3D model was easily found and downloaded from the Asset Store.

The non-visual written parts of the user manual were visualized with the 3ds Max program and turned into 3D models.

Since the model downloaded from the Asset Store and the 3D models we prepared from the 3ds Max program were saved in fbx format, they were transferred to the Unity environment by dragging and dropping.

For the application, a scene called "Homepage" was created in the Unity program. The "Contents" scene was prepared in the same way as the home scene. Unlike the homepage and contents scene, scenes where the created 3D models will be displayed, are prepared. If we consider the "Important safety and environmental instructions" section, which is the first of the pages where our 3D models will be used, 2 buttons have been added to the canvas to go back to the scene first and move forward. Then, the main camera was deleted from the hierarchy menu and replaced with ARCamera, which will show our AR application. For ARCamera to work, the "Open Vuforia Engine Configuration" button in the inspector panel was clicked and our License Key was entered in this field.

After these operations, it's time to add our marker. Image Target from Vuforia Engine has been added to the Hierarchy menu to add our marker. By following the Assets, Import Package, and Custom Package paths, the database that we prepared and downloaded on the Vuforia site was added to the study.

In the Image Target Inspector panel, under Image Target Behavior, Type = Predefined, Database = ThesisUserGuideDB, Image Target = Security and Environment Instruction is set, and our sign is ready for use. The last thing we need to do after these operations is to set the 3-dimensional object that our marker will call. Our object that we prepared in 3Ds Max has been set as "child" by dragging and dropping under Image Target and its dimensions have been adjusted.

All the necessary adjustments for the AR application are completed with this process. A small script has been written to set the transitions for the user interface. Visual Studio 2017 is used for this script. The script has been added to work in the click event separately for all buttons in the application interface.

All of these processes were applied separately for all prepared objects, scenes and the entire user manual and the work was completed.

In the setting for the working user, the resolution value is set to 1080 x 2340 px in the vertical position (Portrait), which is the screen resolution value of the Xiaomi Redmi Note 8 Pro. Afterwards, the final settings of the work done before the build were made in the player settings option of build settings. Company Name has been determined as "CSU" and Product Name has been determined as ThesisUserGuide. Under Allowed Orientations for Auto Rotation, only the Portrait option is selected to allow the application to be used only in the portrait position of the phone. Under Other settings, Graphics APIs have been set by removing Vulkan because Vuforia Vulkan is not compatible with image graphics. Since Android 7.0 'Nougat' (API LEVEL 24) and higher is recommended for AR applications, this setting was selected and the application was built and turned into an apk.

### **AUGMENTED REALITY APPLICATION USING MODEL TARGET**

It has been decided to make an application that has the same purpose but will also include machine learning and to compare these two applications. As a result of our research, it was decided that the most suitable program was the Vuforia Model Target Generator and the application steps were determined in the following order:

1. The model will be added to be used in the Vuforia Model Target Generator.
2. The second model will be ready for training by completing the necessary steps.
3. It will be trained with the cloud training provided by Vuforia, which is ready for training.
4. The resulting Unity Package will be added to the Unity environment.
5. The user manual will be made into a complete application.

Model Target Generator is an application that converts a 3D model into Model Target. A Model given to the training can be used on Target Unity.

The operations were carried out on the 3D model used in the previous application and it was used for training.

Steps:

1. The 3D model is loaded into the Model Target Generator program.
2. The axes of the model are set, and the Up vector y-axis is selected.
3. The model's real-world dimensions are selected from the Model Units tab.
4. From the Motion Hint tab, the appropriate option is selected, depending on whether our object is in motion or not. The default is left for this project.
5. From the Guide View tab, if our object will not be trained and will be recognized with the help of guidelines, a Guide View is created. If our object is to be trained and guidelines are not used, an Advanced View is created. An advanced view has been created for this project. Recognition range values are set after the advanced view is created.
6. The created model is given to the training by creating a database on the target application.
7. If we get the "Completed" printed at the end of the training, it means that our training has been completed successfully.

As a result of the processes above, our model was trained on Vuforia Cloud Training with the training of around 45 minutes and made ready for use.

Our trained model was exported as Unity Package via the Vuforia Model Target Generator program. As in the first application, the Vuforia license key was obtained and the Vuforia Engine was installed in the Unity project.

The model target was selected from the Vuforia engine from the Hierarchy panel and added to the project. The database and model that we trained were selected from the Inspector panel.

Following these steps, the necessary processes for the object recognition part on Unity have been completed. For the remaining parts of the user manual that cannot be visualized, the Book-

Page Curl asset has been downloaded from the assets store. User guide pages have been added to this asset.

## CONCLUSION

Although augmented reality technology dates back to the 1950s, this technology has just begun to enter our lives. There is much more work and research to be done in this area. Since the use of technology is directly connected to our smartphones, it is an area that is progressing in parallel with the development of smartphones.

This study gives information about the usage areas, limitations, advantages and how it is put into practice of augmented reality technology and shows the results about how it can be used against paper waste, which is one of the many wastes in life.

The electronic product chosen for use in the study is an electric hairdryer. The user manual booklet prepared for this electronic device, whose working mechanism is very simple, is 14 pages. When we put this booklet into practice using augmented reality technology, this waste can be avoided with 5 markers. 5 markers can be converted to a single marker by overloading if desired, but a section is assigned to each marker to make it more understandable. If desired, a pdf-format file can be prepared for markers without wasting any paper and displayed on the product's website, or a booklet with a minimum of 1 and a maximum of 5 pages can be arranged according to the user's request. Thanks to the second application, there is no need to use paper at all, so it has been shown that paper waste can be completely avoided by using machine learning algorithms.

For the hair dryer user guide, 2 applications were made. One is the image target, and the other one is the model target. Among these applications, the one containing the image target works as a result of defining the markers on the image, while the one containing the model target works as a result of the recognition of the object using machine learning.

The application that works with Image target takes up less space and does not affect the performance of the mobile phone much. The application working with the model target takes up more space than the application working with the image target and affects the performance of the mobile phone more.

While no training is required for the application working with the image target, training is required for the application working with the model target.

Even though the paper usage is reduced, it still continues as the marker is required for the application to work with the image target. For the application working with the Model Target, since no marker is required, the object itself is sufficient for the user, and there is no need to use paper.

While it is easier for software developers to make an application that works with Image Target, it is necessary to have object recognition knowledge from machine learning branches for the application that works with Model Target.

While it is sufficient to upload an image from the Vuforia website for the application working with the image target, it is necessary to use the Vuforia Model Target Generator application and to have a 3D model for the application working with the Model Target.

Since the object is sufficient for the application working with the model target and it recognizes all the objects in the form of the trained model, it works for almost all hair dryers. The application working with Image target, on the other hand, can only work in those environments where markers exist, as it consists of certain markers.

Similar studies can be diversified in more complex electronic devices, in more diverse sector groups and usage areas, as explained in different areas of use.

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## **STUDY ON METHANE COMBUSTION UNDER OXYGEN DILUTED CASES IN A TUBE BURNER**

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### **ABSTRACT**

The methane combustion under oxygen diluted cases in a tube burner is numerically investigated in this paper. For the inlet condition of the tube burner, the combustion air was assumed to be diluted with CO<sub>2</sub> for 20% on molar base and O<sub>2</sub> fraction was assumed to be 10% on molar base. The simulations were run for the different preheating values of the oxidizer mixture. The results show that the preheating process of the diluted oxidizer mixture extends the flame zone as the preheating degree is increased. This proves that using a preheated and diluted oxidizer mixture for methane combustion has the advantage to increase the heat transfer rate due to the extended flame zone and to reduce the NO<sub>x</sub> emissions by lowering the maximum flame temperature value. In addition, it is determined from the results that the diluted oxidizer mixture has a limited preheating value in order to maintain combustion, which is 1000K for the cases investigated in this paper.

**Keywords:** Methane, combustion, oxygen dilution

### **INTRODUCTION**

Combustion is reaction process of a fuel and an oxidizing gas under proper physical and chemical conditions and producing heat energy in the combustion environment. Oxidizing gas may in practice be pure oxygen or air. Necessary conditions for the combustion of a gaseous fuel with air are the proper proportions of fuel and air in the combustion environment, a turbulent mixture, sufficient interaction time and threshold energy. The main purpose of combustion systems in many industrial applications that require heat treatment is to provide high combustion and thermal efficiency. In addition, minimizing the emissions and pollutants formed in the combustion environment is one of the criteria that has come to the fore in the last few decades. The researches to reduce the emission of NO<sub>x</sub> and CO<sub>2</sub> emissions with appropriate combustion efficiency is generally on modifying combustion systems geometrically. On the other hand, oxygen-diluted or flameless combustion forms are among the methods of increasing thermal efficiency and reducing emissions, which are less studied. The necessary condition for flameless combustion is to reduce the oxygen level in the oxidizing air with the addition of gases such as CO<sub>2</sub> or N<sub>2</sub>. In order to combust fuel with an oxidizing gas mixture with diluted oxygen level, it is necessary to preheat the oxidizing mixture. In this way, combustion takes place in a larger volume that increases the thermal efficiency in the combustion environment, while on the other hand, thanks to the oxygen dilution, the combustion temperature can be lower than in conventional combustion systems. In this way, it is possible to reduce thermal NO<sub>x</sub> emissions. Some of the studies related to the subject are reviewed briefly in this section. Cavaliere and de Joannon (2004) examined the oxygen-diluted combustion cases in their study

from chemical, thermodynamic and physical point of view and evaluated the potential of the applicability of this combustion form. Fordoei et.al (2018) numerically investigated the properties of oxygen-diluted combustion, such as heat transfer and flame structure. According to the results, the decrease in maximum flame temperature in diluted oxygen combustion cases was greater than the decrease in methane-air combustion. This was explained by changing the physical and chemical properties in the oxidizer mixture by adding CO<sub>2</sub> instead of N<sub>2</sub>. Wünnig and Wünnig (1997) studied the properties of flameless combustion and its potential in application areas. Cheong et.al (2019) experimentally studied MILD non-premixed combustion without preheating and dilution. The effects of the distance between the fuel and air ports and the excess air coefficient on the combustion regimes were investigated. Tu et.al (2019) studied MILD methane combustion with numerical and kinetic computation. More favourable MILD conditions were formed thanks to the different physical and chemical properties obtained as a result of replacing N<sub>2</sub> gas with CO<sub>2</sub> in the oxidizing mixture. In addition, the flame length in the combustion environment increased with the addition of CO<sub>2</sub>.

In this study, the combustion process of methane fuel with air and a gas mixture obtained by diluting the air with CO<sub>2</sub> for 20% on molar basis in an experimental combustion tube was investigated numerically. The molar ratio of O<sub>2</sub> gas was assumed to be 10% by dilution of the air. The main purpose of the study was to examine the effect of the inlet temperature of the diluted oxidizing gas mixture on the combustion performance and flame structure.

## NUMERICAL STUDIES

In this study, the Fluent code was used as a numerical analysis tool. The combustion tube, in which the study was carried out, was modelled as two-dimensional and axially symmetrical. The combustion tube modelled in two dimensions is shown in Fig.1.



**Figure 1** 2D Combustion Tube

The combustion tube measures 2 m in length and 0.155m in diameter. The inlet diameter of the fuel port is 0.004m. The dimension values used are the same as the model in the study of Brookes and Moss (1999). In the study, the combustion cases of methane fuel with atmospheric air and diluted air with different inlet temperatures were simulated. Four different analyses were conducted in total. In the simulations, it was assumed that the combustion air was diluted with CO<sub>2</sub> for 20% on molar base and the resulting oxidizer mixture had an O<sub>2</sub> ratio of 10% on molar base. In addition, the inlet conditions for diluted air were 800K, 900K and 1000K, respectively. The atmospheric air inlet temperature was 290K for the case without dilution. In order to determine the temperature distribution in the combustion environment, the radiation heat transfer mechanism was taken in account in the calculations and the wsggm-domain based approach was used for the absorption coefficient. The standard k- $\epsilon$  model was used for turbulence modelling. While the convergence criterion was 10<sup>-6</sup> for the energy and radiation

equations in the calculations, it was  $10^{-3}$  for the other equations. The mass, momentum and energy conservation equations are given in Eq. (1), Eq. (2) and Eq. (3), respectively.

$$\frac{\partial \rho}{\partial t} + \nabla \cdot (\rho \mathbf{v}) = 0 \quad (1)$$

$$\frac{\partial (\rho \mathbf{v})}{\partial t} + \nabla \cdot (\rho \mathbf{v} \mathbf{v}) = -\nabla \cdot \mathbf{P} + \rho \sum_{i=1}^N Y_i \mathbf{f}_i \quad (2)$$

$$\frac{\partial (\rho h)}{\partial t} + \nabla \cdot (\rho \mathbf{v} h) = \nabla \cdot \left[ \frac{k_t}{c_p} \nabla h \right] + S_h \quad (3)$$

In these equations,  $\rho$  is density,  $\mathbf{v}$  is velocity vector,  $\mathbf{P}$  is stress tensor,  $Y_i$  is mass fraction of  $i$  species,  $\mathbf{f}_i$  is force acting on unit mass of  $i$  species,  $h$  is enthalpy,  $t$  is time,  $k_t$  is heat conduction coefficient,  $c_p$  is specific heat and  $S_h$  is the radiation source term. The radiation intensity equation used is given in Eq. (4) (ANSYS Fluent 12 Theory Guide 2009).

$$\frac{dI(\vec{r}, \vec{s})}{ds} + (\alpha + \sigma_s) I(\vec{r}, \vec{s}) = \alpha n^2 \frac{\sigma T^4}{\pi} + \frac{\sigma_s}{4\pi} \int_0^{4\pi} I(\vec{r}, \vec{s}') \Phi(\vec{s}, \vec{s}') d\Omega' \quad (4)$$

In Eq.(4),  $\vec{r}$ ,  $\vec{s}$ ,  $\vec{s}'$ ,  $s$ ,  $\alpha$ ,  $n$ ,  $\sigma_s$ ,  $\sigma$ ,  $I$ ,  $T$ ,  $\Phi$  and  $\Omega'$  are position vector, direction vector, scattering direction vector, path length, absorption coefficient, refractive index, scattering coefficient, Stefan-Boltzmann constant ( $5.672 \times 10^{-8} \text{ W/m}^2\text{K}^4$ ), total radiation intensity, local temperature (K), phase function and solid angle, respectively.

In addition to the conservation equations, the non-premixed combustion model was used as a chemistry model. In this model, the average mixture fraction parameter was solved as follows; (ANSYS Fluent 12 Theory Guide 2009);

$$\frac{\partial (\rho \bar{f})}{\partial t} + \nabla \cdot (\rho \mathbf{v} \bar{f}) = \nabla \cdot \left( \frac{\mu_t}{\sigma_t} \nabla \bar{f} \right) + S_m + S_{\text{user}} \quad (5)$$

The values of average scalar quantities such as temperature and density were calculated as follows; (ANSYS Fluent 12 Theory Guide 2009);

$$\bar{\phi} = \int \phi(f, \bar{H}) P(f) df \quad (6)$$

In this equation,  $P(f)$  is the probability density function of mixture fraction. The laminar flamelet approach was used in order to obtain  $\phi(f, \bar{H})$  function. GRI-Mech 3.0 mechanism (Smith et.al.) was used for the calculations of flamalets. In addition, the average mixture fraction variance was solved in the Fluent code for the solution of the mean value of scalar  $\bar{\phi}$  in Eq.(6)

For the solution algorithm, "coupled" was used for velocity-pressure coupling, "least-square cell based" was used as the gradient method and "second-order upwind" was used for equation discretization.

## RESULTS

The numerical analyses were performed for four different cases. These cases are given in Table 1. In the analysis, the fuel consumption rate was taken as 0.000172 kg/s and the excess coefficient of oxidizing gas was taken as 1.4 for all the cases. In addition, before starting the

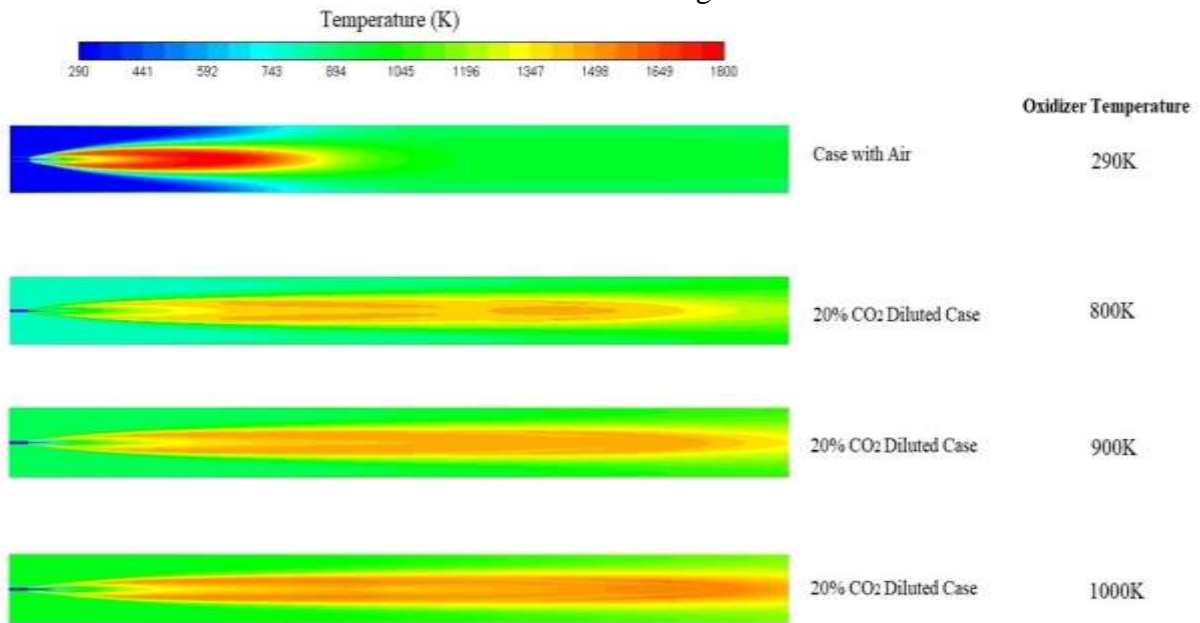


analysis, the mesh element number independence study was carried out using the methane-air case given in Table 1. The parameter considered in this study was the temperature change in the central axis. The number of cells used in the mesh was 639,000.

**Table 1** Studied Cases

| Fuel    | Oxidizer<br>(Molar Base)                                      | Oxidizer Inlet Temperature |
|---------|---|----------------------------|
| Methane | Air   | 290K                       |
| Methane | 10% O <sub>2</sub> - 20% CO <sub>2</sub> - 70% N <sub>2</sub> | 800K                       |
| Methane | 10% O <sub>2</sub> - 20% CO <sub>2</sub> - 70% N <sub>2</sub> | 900K                       |
| Methane | 10% O <sub>2</sub> - 20% CO <sub>2</sub> - 70% N <sub>2</sub> | 1000K                      |

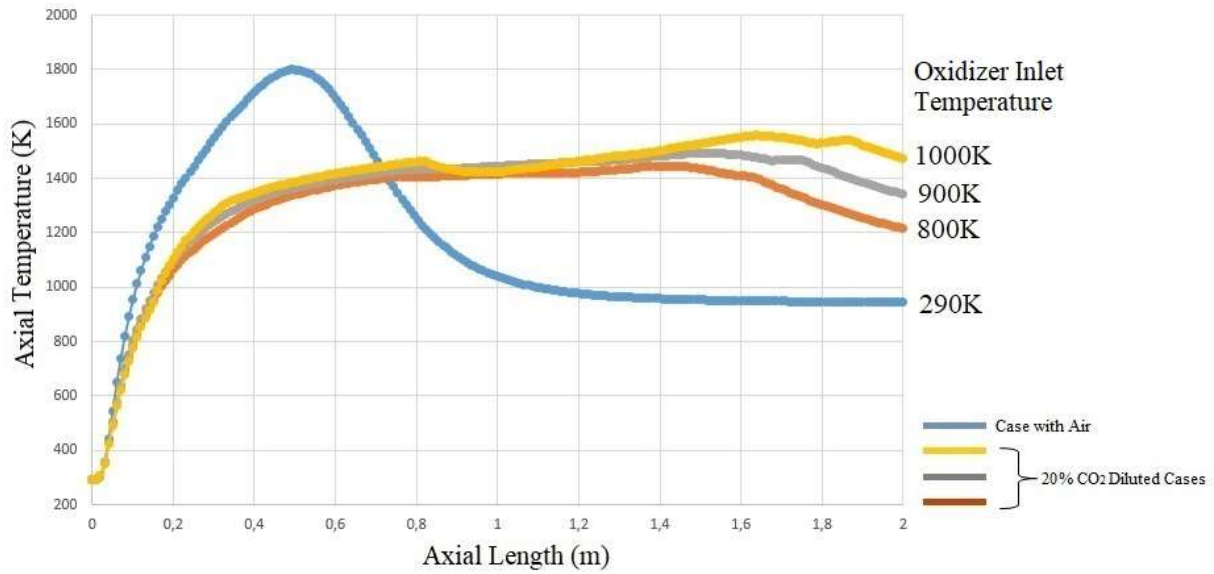
The obtained results from the simulations are presented in Fig.2. The combustion process with atmospheric air gives a conventional flame form as expected. This flame form is short and intense in the centre. On the other hand, the flame forms give a different appearance when the atmospheric combustion air is diluted with CO<sub>2</sub> for 20% on molar base and the O<sub>2</sub> content is accepted as 10% on molar base. As can be seen from Fig.2, the flame lengths are longer than the conventional flame and the flame temperature intensity is reduced. This is due to the decrease in the reaction rate and incomplete reaction of methane fuel, since there is less O<sub>2</sub> gas in the oxidizing mixture than in normal air. Thus, unreacted methane fuel, as well as intermediate products and radicals take a longer path in the flame tube. In this way, the combustion reactions occur in a larger volume in the flame tube. Thus, it is possible to decrease the formation rate of thermal NO<sub>x</sub> emissions with decreasing flame temperature. In addition, an increase in the heat transfer rate can be expected, in the absence of adiabatic conditions, from the flame to the combustion tube walls with the increasing flame volume.



**Figure 2** Obtained Results

The results given in Fig. 2 show that the higher the inlet temperature of the oxidizing gas, the wider the flame form and the higher the outlet temperature, as expected. However, when the inlet temperature of the oxidizing gas is 1000K, it is seen that the temperature value on the central axis decreases rapidly after a point and rises again. This is essentially an indicator that

shows the rate of reaction is very slow in the respective regions. This is due to the high axial flow velocity of the oxidizing gas, and its inability to pass through enough to sustain the reaction in the radial direction. This result shows that, in a real application, when the inlet temperature of the oxidizing gas reaches a certain value, the reactions will stop completely and the flame formation will disappear. For the cases given in this study, this temperature value is 1000K. The axial temperature values for the different cases are given in Fig.3. For 1000K inlet temperature, the temperature change in the central axis shows a tendency that the flame form cannot be sustained in the combustion tube.



**Figure 3** Calculated Axial Temperature Values

## CONCLUSIONS

The results obtained from this study are as follows;

- Adding an inert gas to the combustion air in a way that dilutes the oxygen in it increases the flame length and decreases the temperature of the flame. This is one of the factors that can reduce thermal NO<sub>x</sub> emission formation.
- In the case of using a preheated oxygen-diluted air, the volume of the combustion flame is larger than in normal air, which can have an effect that will increase the rate of heat transfer from the flame to the combustion environment wall.
- There is a certain limit temperature value at which the oxygen-diluted oxidizing gas can be preheated for the combustion process of methane fuel. It is expected that there will be no combustion beyond this limit value. The limit preheating degree determined in this study is 1000K.

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## **PRODUCTION OF STARCH-BASED BIOPLASTIC AND ITS USE IN 3D PRINTERS**

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### **ABSTRACT**

It is an obvious fact that synthetic plastics have a wide range of uses, but this global use is quite damaging to the environment and human health. In order to prevent this situation, scientific researchers have succeeded in producing bioplastics that can degrade in nature and do not harm health by doing different studies. This study examines the mechanical and physical behaviors of bioplastics such as tensile strength, electrical conductivity, modulus of elasticity and morphological structure, obtained by adding graphite and some fiber materials, as reinforcement material to bioplastics whose matrix material is starch, as well as the use of produced bioplastics in three-dimensional printers. After the preparation of the starch matrix and graphite reinforced plastic samples, they were examined under a microscope to examine the morphological and internal structure of the samples.

**Keywords:** corn starch, biodegradable materials, bioplastic, carbon fiber, 3D Printer

### **INTRODUCTION**

Bioplastics (Biodegradable plastics) are polymers produced from renewable biological resources such as oil, starch, plant structures and food waste. It participates in the cycle in nature in a much shorter time compared to its derivatives. Combinations of bioplastics with various reinforcing materials and agents have been produced in order to meet the desired mechanical properties. In this way, a wide variety of properties can be added to bioplastics (Özdenur and Erkmen, 2013; Karakuş and Ayhan, 2019; Ashothaman et al. 2021).

Today, with the increase in the use of 3D printer technology, which can manufacture using plastic raw materials, the importance of choosing alternative raw materials to be used in production has increased at the same rate. The selection of the material to be produced depends on many factors such as the desired performance values of the final product, conditions of use, cost, suitability of the material to the production process of the printer. Today, studies are carried out continuously in order to improve the types of materials currently used and to add desired properties (Yedrissov et al., 2022).

According to Garcia et al (2021) world plastic consumption is currently estimated to exceed 700 million tons per year and will reach one billion by 2021. The massive use of petrochemical plastics has become a real problem for health and the environment. Packaging materials are an essential part of product processing and because of this the number of investigations on the development and use of new alternatives has increased. Bioplastics (Biodegradable plastics) are polymers produced from renewable biological resources such as oil, starch, plant structures and food waste. In recent years, research on biodegradable plastic has progressed considerably (Zhang et al.2021; Jiang et al.2020). Much attention is given today

to developing green of green and sustainable materials to demonstrate the potential of biocompatible extraction-separation techniques in both laboratories and industrial sections. Biodegradable packaging is one that is capable of decomposing into carbon dioxide, methane, water, inorganic compounds, or biomass, the dominant mechanism of decomposition being the enzymatic action of microorganisms and that the resulting products can be obtained and measured in a period of a certain time (Brozio and Masek, 2020).

Al-qahtani et al (2021) aimed to develop and investigate the physical, mechanical, barrier, morphological, and antioxidant properties of the biodegradable films made from corn starch. Abbasi and Haeri (2021) introduced the source and properties of biodegradable materials and discussed their analytical applications in liquid phase microextraction and sorbent-based microextraction techniques. In his MSc. thesis Kuz (2017) investigated some characteristic properties of biopolymer and composite materials obtained by adding starch-based biopolymer carbon fiber and TiO<sub>2</sub>. García et al (2021) added copolymer pluronic F127 in certain proportions to the mixtures of corn starch and Chitosan (CTS) polymers obtained from crab shells separately and together.

The use of starch-based bioplastics in the food industry, which is one of the production methods, and the effect of additives such as glycerol, chitosan, citric acid, which are used to increase the functionality of these plastics, on bioplastics were mentioned by Cheng, et al. (2021). Cheng et al. (2021) carried out a deeper insight to predict the precision and textural quality of printed products through the molecular structure of starch, and presented useful information for designing personalized starch-based food products by 3D printing. Cheng et al. also investigated the relationship between rheological properties and printability of three types of starch-based staple food (potato, rice and corn starch) for hot-extrusion 3D printing (HE-3DP). In their study, Dimonie et al. (2019) found that Poly Vinyl Alcohol (PVOH) component can be produced by a method called melt modification, which provides a similar melting of corn starch. It was found that very good quality 3D printing filaments could be obtained from these thermoplastics.

Since there is a polymer binder such as glycerine in the structure of starch-based simple bioplastics obtained from corn starch, they are flexible and unstable. It is very important to increase the strength of such bioplastics and to be able to produce them continuously. In this study, the changes in the mechanical properties and electrical conductivity of the polymer formed by adding graphite powder to starch matrix biopolymers and removing glycerol from the mixture were analyzed. This polymer mixture was produced in a three-dimensional printer with a "core -xy" type and screwed chamber extruder. Characterization analyzes of the produced samples were carried out with the help of tensile test, microscopy and electrical conductivity conduction tests.

#### **MATERIAL AND METHOD**

The A screw-chamber 3D printer was designed for the fabrication of bioplastic samples. "AutoCAD Fusion360" Solid Modeling Program was used for the skeleton design of the printer (Figure 1). During the manufacturing, St 37 black steel 20x20 Al sigma profile was preferred, the sh

ets were cut on CNC benches. MGN12 linear guide and carriage are used for bedding. GT2 belt, T8 Ball Screw and Nema 17 Stepper motors were used for the drive, Arduino control card with Atmega 2560 microprocessor was used for control. A chambered extruder was manufactured.

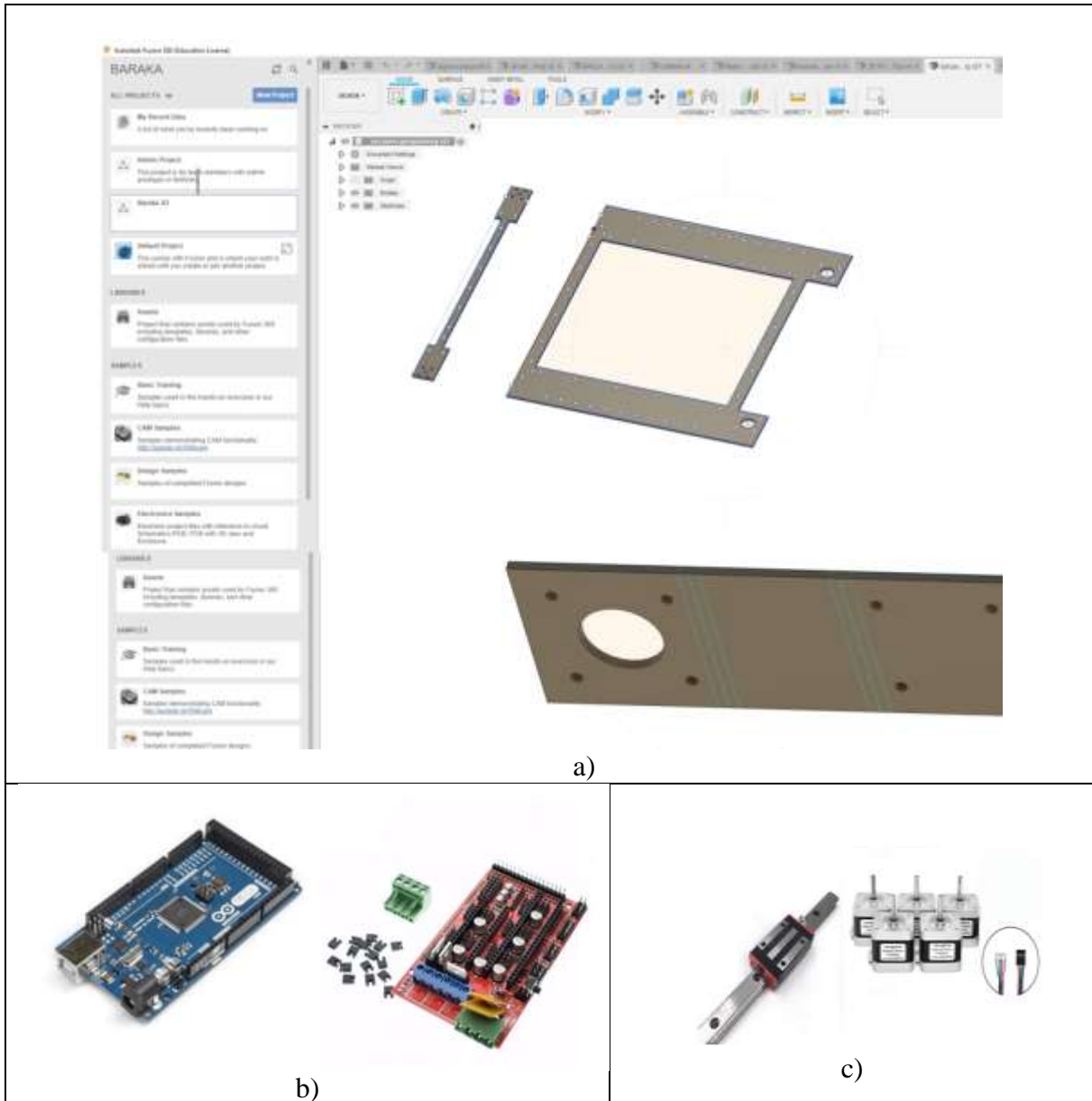


Figure 1. Production of 3D printer a) Designs of motor bearings in the solid modeling program b) Arduino mega control card c) MGN12 linear guideway, trolley and Nema 17 stepper motors.

After the assembly and electrical connection, the necessary sensor controls were made. The operating type of the printer is a Core XY type system, and the Marlin library was used for the necessary software. Figure 2 depicts the assembled 3D printer parts.



Figure 2. 3D printer assembled.

## RESULTS AND DISCUSSION

Reinforcement materials caused visible changes in the change of mechanical properties of biopolymers. The samples were subjected to different tests in order to learn exactly how much and how the reinforcement materials worked.

There was no problem in terms of viscosity and extrusion process in the use of the obtained bioplastics in FDM technology in three-dimensional printers. In addition, some of the bioplastics we produce have gained electrical conductivity. This feature obtained is not suitable for the smooth and stable operation of all the plastics we produce. Figure 3 shows the starch based composite's visual structure after print process.



Figure 3. a) Three-dimensional printer output of 11.7% graphite powder reinforced bioplastic  
b) Printout of 10% fine sand and 15% graphite powder reinforced bioplastic.

If we look at the structure of the bioplastic viewed under the microscope, graphite is in a crystalline structure like diamond, since graphite is an allotrope of the element carbon. The plate structure formed by intertwining of graphite and starch molecules can be seen in the image as can be seen in the images under (c) 200 X and (d) 500X focusing, the lattice structures formed by the graphite molecules shine by creating reflection under the light (Figure 4). In Figures 4 the micro-structures of the starch-based 11.7% Graphite powder-reinforced bioplastic sample are seen under the light microscope, 50x, 100x, 200x, 500x, respectively, in the Nikon NIS Elements program.

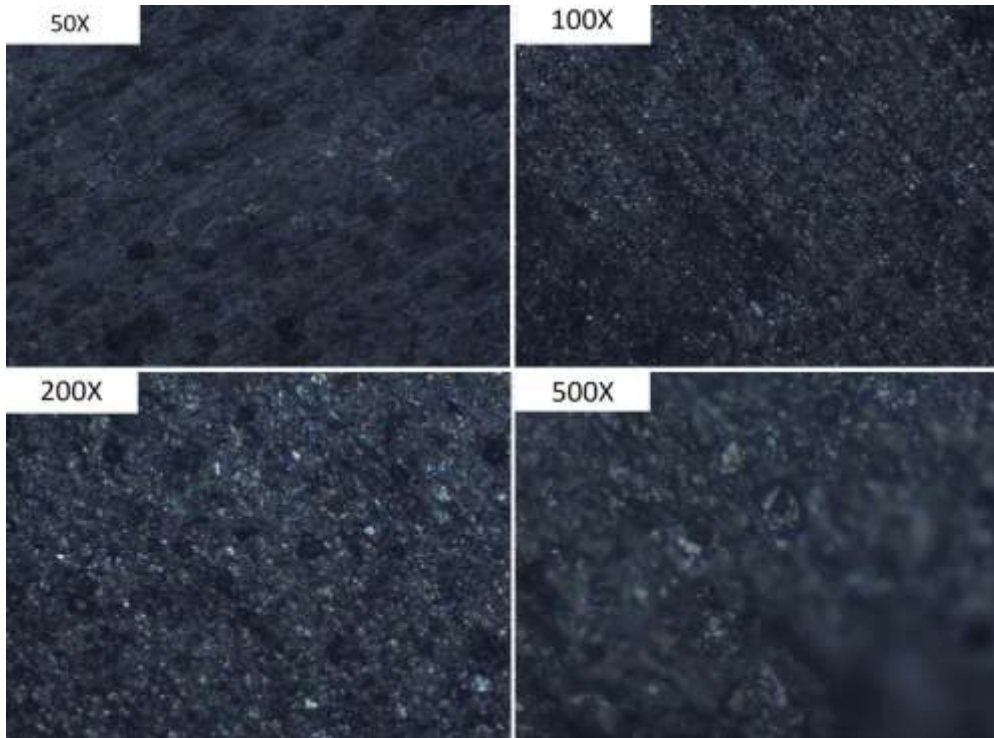


Figure 4. (a) 50X, (b) 100X, (c) 200X and (d) 500X zoom view of Graphite-reinforced Bioplastic under Microscope, respectively.

Improvements in electrical conduction properties were depicted in Figure 5 and 6 via graphs. It was observed that 11.7% Graphite-reinforced biodegradable plastic can be operated at all voltages of 24V and below without any problems, compared to the fact that the internal tension of 11.7% Graphite reinforced bioplastic is lower than 10% fine sand and graphite added bioplastic and 25% Graphite reinforced plastics.

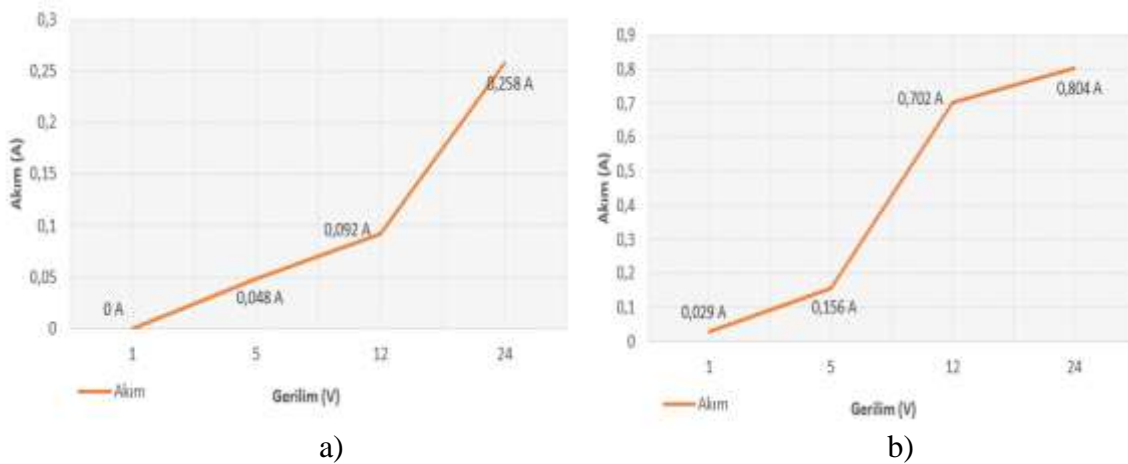


Figure 5. a) Electrical measurements of 11.7% g graphite Reinforced bioplastic b) Electrical conduction graph of 10% Fine Sand and 15% Graphite Reinforced bioplastic



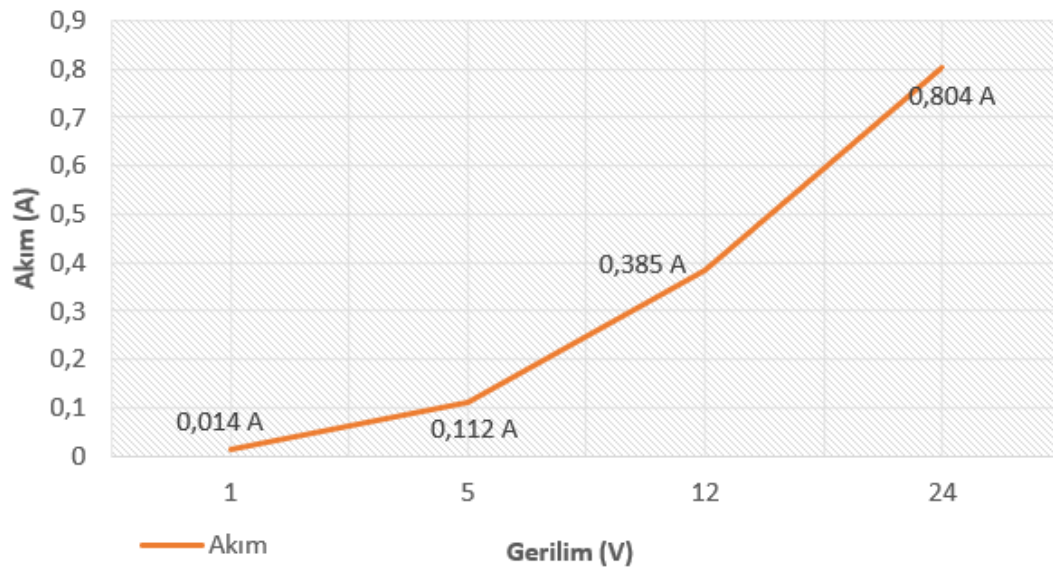


Figure 6. Electrical conduction graph of 25% Graphite Reinforced Bioplastic under voltages.

Figure 7 depicts the tensile strength of unreinforced starch bioplastic sample. In order to test the tensile strength values of the samples, it was examined that how the carbon addition affected the obtained values. The test results showed that Graphite reinforcement had a positive effect on the tensile strength of bioplastics. Figure 8 shows the tensile test result of the graphite reinforced bioplastic sample. As a result of the tests, graphite reinforced starch composite exhibited higher strength value compared to non-reinforced starch-based bioplastics.

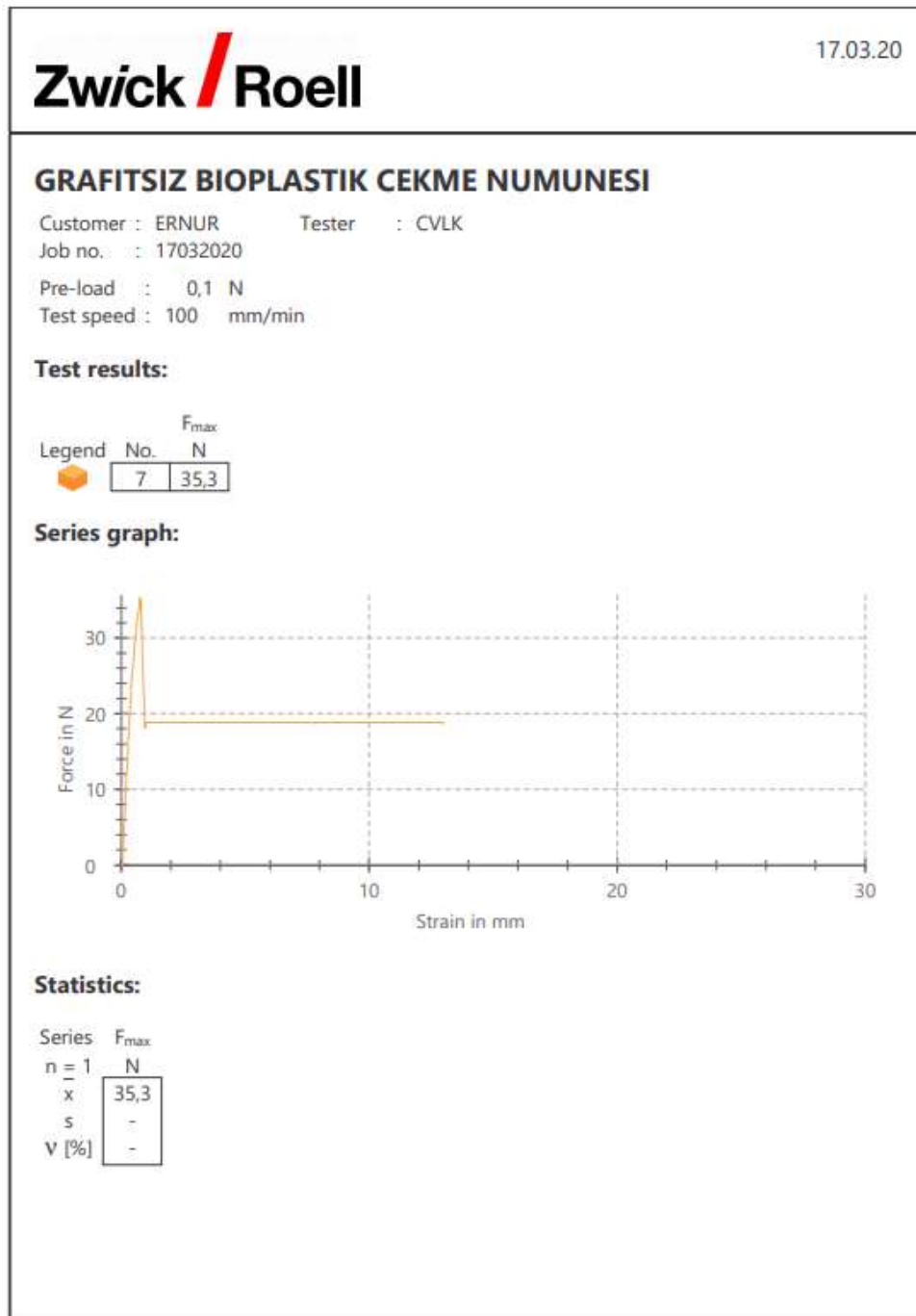


Figure 7. Tensile strength test results of graphite-free plastic

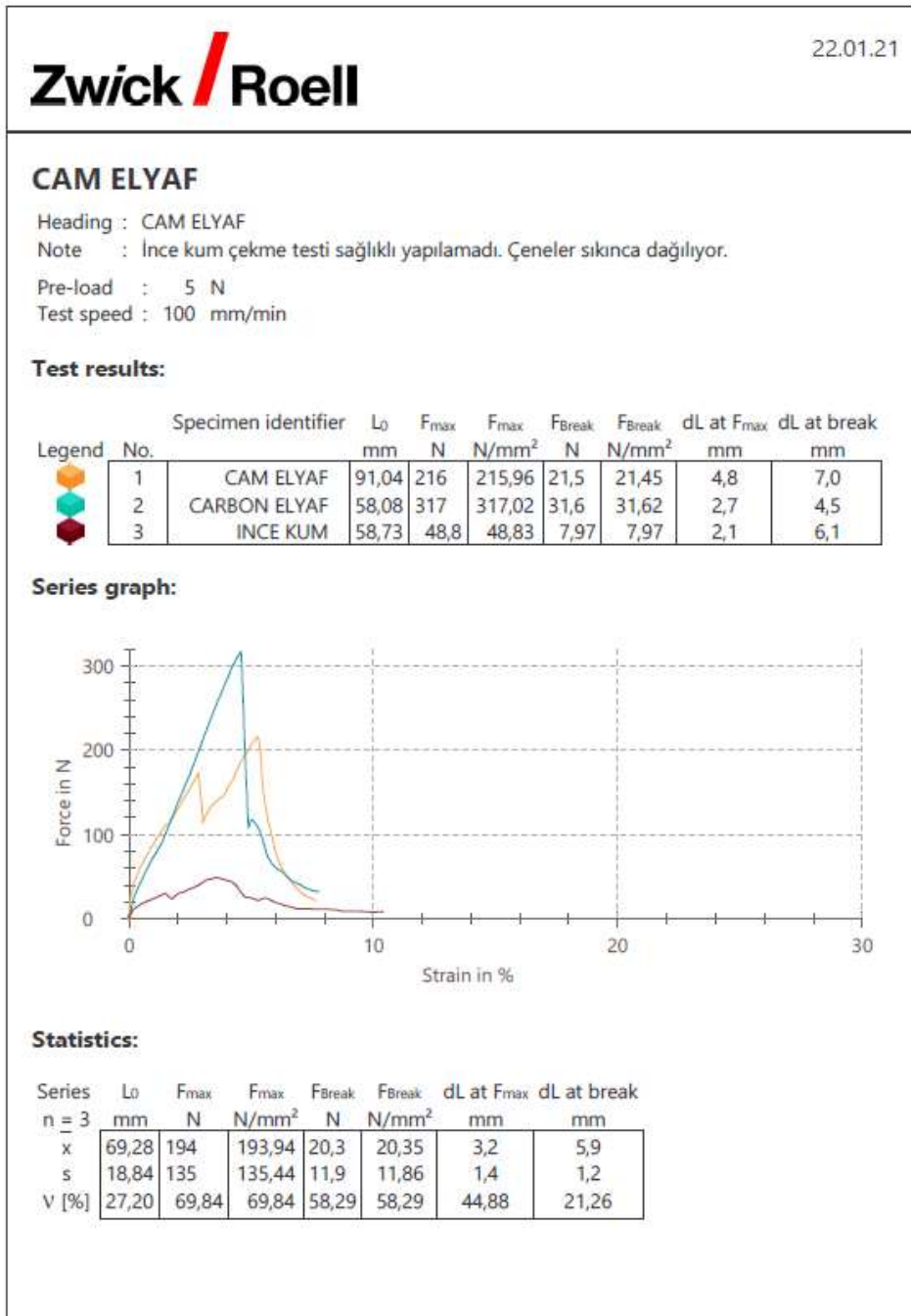


Figure 8. Tensile strength test results of graphite reinforced samples

## CONCLUSIONS

The graphite powder has electrical conductivity and high hardness strength, in this study, bioplastics were prepared with these materials in order to investigate the effect of other materials such as carbon fiber, glass fiber clippings, and fine sand on bioplastics, in addition to graphite powder.

Although bioplastics reinforced with 10% Fine Sand and 15% Graphite Powder and bioplastics reinforced with 25% Graphite powder are superior to other plastics in terms of electrical conductivity, they have shown that they cannot work healthily.

It was concluded that the 11.7% Graphite Powder reinforced bioplastic sample could work with a desired performance in the tests. The current required to light the LED bulb in an LED lamp circuit is approximately 20mA or 0.02 Amperes. The electrical conduction results of our plastic with 11.7% graphite ratio showed that it could transmit 0.092 Ampere electric current under 12V voltage in a healthy way without any heating or deformation problems.

Only 11.7% graphite powder-reinforced bioplastic material was found to be suitable in tests for its applicability in three-dimensional printer manufacturing. Improvements should be made for the printing process of 10% glass fiber clipped and 15% graphite powder-reinforced bioplastic material in three-dimensional printers. Other bioplastic blends have had problems with the discharge stage of the printer's nozzle and are not suitable for printing. It can be interpreted that in future technologies, it is possible to use customized bioplastics in designs where electrical conduction is required or for heating needs. As a result of the improvements, the bioplastic materials we work on can be designed in specific geometries and produced as intermediate or finished products in three-dimensional printers with FDM technology.

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## **EFFECT OF WELDING CHAMBER GEOMETRY ON THE PRODUCT QUALITY FOR HOLLOW ALUMINUM PROFILES**

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### **ABSTRACT**

Hollow profiles are used in many engineering applications. Porthole dies are widely used in the manufacturing of the hollow and complex shaped profiles by extrusion. One of the most important parameters in die design that affects the quality of the profile is the amount of residual aluminum, which is called the dead zone, which will occur during billet transitions in the welding chamber. In this study, a new design was made in the welding chamber of the current die in order to prevent material accumulation in the billet transitions, and the results of the new design were examined. 3D finite element model for modelling porthole die extrusion was carried out based on Inspire Extrude Metal 2021.2 software using ALE algorithm. AA6063 aluminium alloy was chosen as material. The temperature of billet was selected as 460°C and the ram speed of 5 mm/sec was set up. Finally, the FEM results were analyzed and the new die design was evaluated primarily in terms of profile quality.

**Keywords:** Porthole die, extrusion, welding chamber

### **INTRODUCTION**

Extrusion of aluminum alloys with porthole dies is widely applied to produce profiles with complex shapes and hollows due to its high efficiency and flexibility (Chen et al., 2015). Hollow profiles play a key role in many important fields such as high-speed trains, buildings, boats and ships, engineering structures. In the method, the hot billet is split into several metal streams at the die portholes. These metal streams then recombine in the weld chamber (the weld zone), and excessive plastic deformation and strong hydrostatic pressure at the contact surface lead to a highly complex extrusion welding process. Finally, profiles of the desired shape and size flow out of the lower die. As a result of the process, one or more weld seams will be formed along the extrusion direction (Fan et al., 2017). The weld quality of the weld seams determines the performance of the profiles, and the quality of the weld seams depends on the degree of adhesion of the weld interfaces, the microstructure of the weld zone and the extrusion die geometry (Den Bakker et al., 2014; Kim et al., 2002). Extrusion pressure and temperature increase with increasing ram speed, which has a positive effect on weld quality during porthole die extrusion (Zhang et al., 2012a; Bingöl and Keskin, 2007).

Die design is very important in porthole dies. In traditional trial and error design, extrusion die design is mainly based on the practice experience and expertise of die designers. Usually, after a porthole die is produced, it is subjected to various modifications and tests until an acceptable profile is achieved, resulting in additional cost and time loss (Liu et al., 2012). With the rapid development of digital technology, many researchers have carried out some simulation studies on the extrusion of aluminum alloys with porthole dies for die design. Xue

et al. (2018) proposed optimization strategies to develop a multi-output porthole die extrusion process for a thin-walled hollow profile with industrial application. Zhang et al. (2012b) investigated the extrusion process of a hollow tube geometry from aluminum material with a three-hole porthole die using a FEM simulation. In his studies, he revealed that both the number of portholes and the die hole significantly affect the material flow, extrusion force and weld pressure. Sun et al. (2015) investigated the numerical simulation of the extrusion process for a complex hollow door frame using HyperXtrude software. Bastani et al. (2010) carried out a simulation of the aluminum extrusion process based on the ALE algorithm to examine the process parameters affecting the flow balance and exit temperature. Chen et al. (2011) performed FE analysis for the extrusion process of a wide-hole profile. Yi et al. (2018) simulated a porthole die extrusion process based on HyperXtrude software using the ALE algorithm to solve the concave defect in the lower part of the extrusion of a complex hollow aluminum profile. Yi et al., who quantitatively analyzed the velocity distribution at the die exit and the pressure distribution in the welding chamber, added partition plates to the design, changed the die and prevented the defect.

In the last few decades, many studies have also been conducted to investigate the weld quality of extruded profiles using porthole dies. Very shallow weld chambers will cause defects in extruded profiles (Yu et al., 2016). For a porthole die extrusion process, the depth of the extrusion die's weld chamber is an important factor in the presence or absence of micro gas defects. A weld chamber depth that is too small will result in a gas cavity behind the die. This leads to the formation of the bonding interface with nanoscale defects and amorphous layers, which have a negative impact on the weld quality. Increasing the welding chamber depth and extrusion speed prevents the presence of micro defects. It is also mentioned in the literature that increasing the volume of welding chambers also contributes to the improvement of welding quality (Donati and Domesani, 2005).

Al-Mg-Si alloy hollow profiles also play an important role in the automotive industry and civil construction fields due to their low density and high performance (Li et al., 2019). Yu and Zhao (2018) obtained different types of welding seams by changing the extrusion die geometry and process parameters in the extrusion of different types of Al-Mg-Si alloys with porthole dies. The interface structures of the weld seams were systematically investigated using optical microscope and transmission electron microscope (TEM). At the end of the study, the effects of extrusion die geometry and process parameters on the formation processes of weld seams, solid state bonding conditions and interface structures were revealed. Fan et al. (2017) tried to develop a porthole die extrusion-based method for joining dissimilar Al alloys. In the mentioned study, 1060/6063 Al alloys were extruded with porthole dies and a good weld quality was obtained. The study presented a different perspective on joining different materials.

In the presented study, a design change was made in a porthole die, in which billet extrusions are performed successively, in order to prevent material residue in the welding chamber during the billet transitions, and the results of the change were examined.

## **DEFINITION of the PROBLEM and NEWLY DESIGN**

As mentioned before, porthole dies play a key role especially in the production of hollow aluminum profiles. The quality of the porthole die directly affects both the product quality and the service life of the die. If the metal flow rate at the die exit is not uniform, the performance and quality of the extruded profiles will be seriously affected. In the traditional trial-and-error method, extrusion die design primarily relies on the designers' practical experience and expertise. In such an application, a porthole die is often subjected to various modifications and tests after it is produced until an acceptable profile is achieved. This naturally causes additional cost and time loss. The part chosen for this study, which was carried out to prevent material

residual in the die, is a part that is generally used with rubber in the automotive sector and whose surface quality and visuality must be at a high level since its entire surface can be seen. Another reason for choosing this profile is that the amount of waste obtained in the current design is 3 times more than the desired amount. One of the main purposes of the study is to minimize this amount of waste. This amount of waste also gives us the length of the region where the welding problem is experienced. In Figure 1, the length of this waste amount in the process using the existing die is seen.

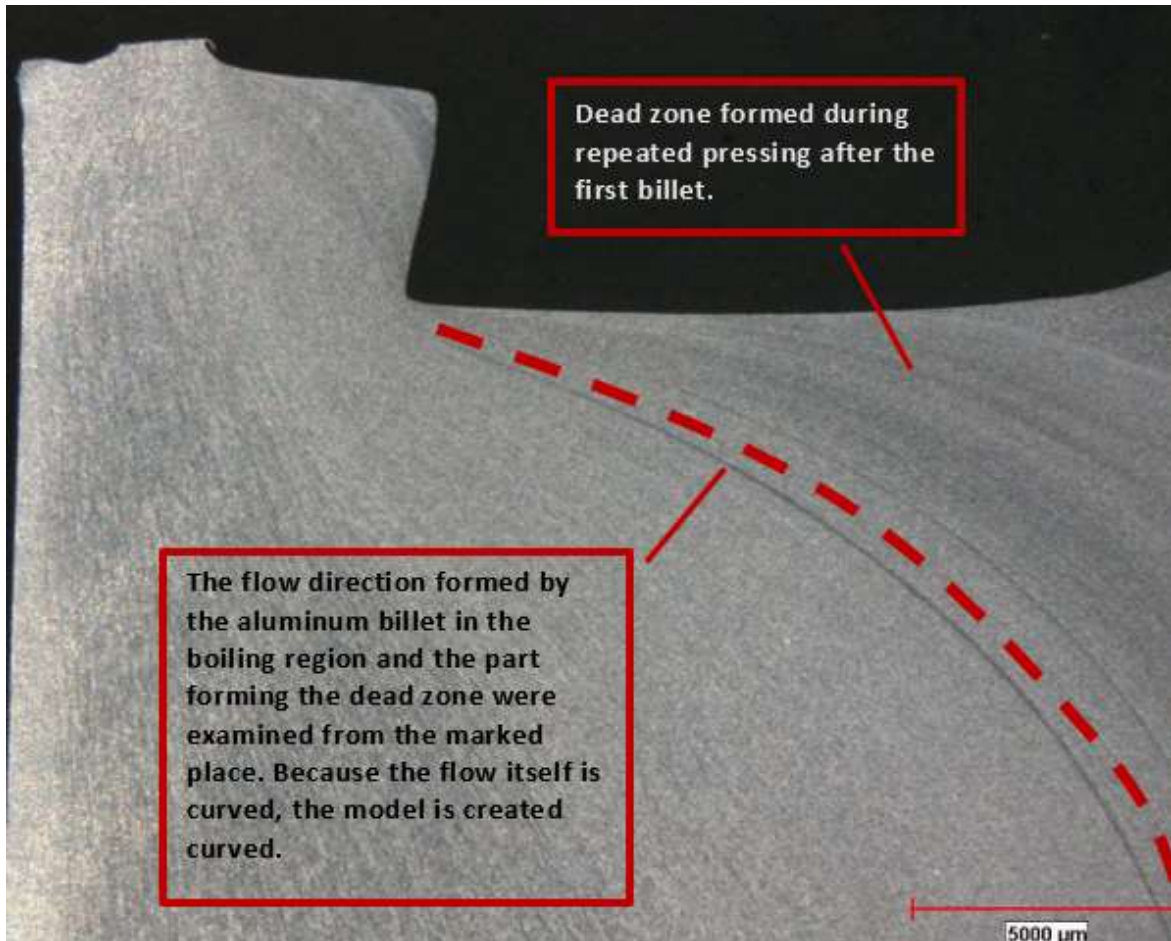


Figure 1. Waste zone due to welding chamber

The main purpose of this study is to carry out an optimization study in the die in order to minimize the residual aluminum that will occur during billet transitions on the welding chamber, which is one of the most important parts in die design that affects the quality of the profile, and is called the dead zone seen in Figure 1. The porthole die model was simulated in Inspire Extrude Metal 2021.2.

In order to eliminate the problems described above, it is designed to modify the geometry of the welding chamber in the current die. In this way, it is aimed to minimize the amount of Al waste in the welding chamber during the transitions between the billet. The newly designed welding chamber and the current design are presented in Figure 2.a and Figure 2.b. The primary goal in the design is to avoid sharp corners. In this respect, the 12 mm high welding chamber with sharp corners and a flat surface, shown in Figure 2.a, is designed with curvilinear geometry in the form of a whole die. The new form is presented in the circled area in Figure 2.b as a technical drawing image.



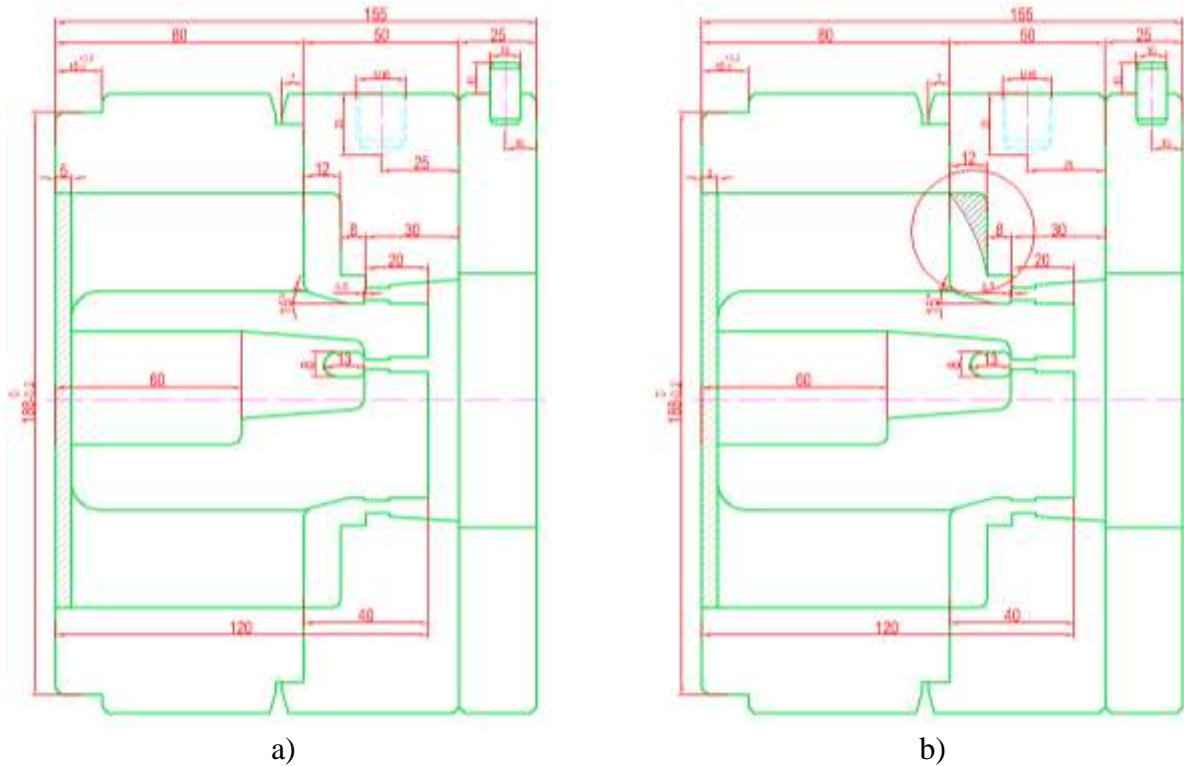


Figure 2. Geometrical dimensioning of the porthole die  
 a) Current die b) Newly design

The extrusion die and product profile used in the presented study are shown in Figure 3.a and Figure 3.b, as the current state and the new welding chamber design, respectively. There are 6 portholes in the current die and sufficient volume is created for the aluminum billet to be divided and recombined in the welding chamber. In the welding chamber profile shown in Figure 3.a; when the die corners are sharp, the aluminum billet is added to the next billet continuously after the first time. While the first billet cools towards the end of the process, the mixture of the two billet causes cracks as the new billet enters hot. This causes distortions on the profile surface and unwanted traces that may occur on the surface. Moreover, metal deposits remaining in the welding chamber necessitate frequent cleaning of the molds in the caustic pool. For this, solid model analysis of the welding chamber with curvilinear surface was made in comparison with the current mold. Flow analysis was performed with the Inspire Extrude Metal program for both die designs. Parameters such as the flow rate of the profile coming out of the die, the stresses formed in the profile, the ratio of remain aluminum in the welding zone were examined comparatively. For the solid model analysis, AA6063 was chosen as the billet material as it actually is.

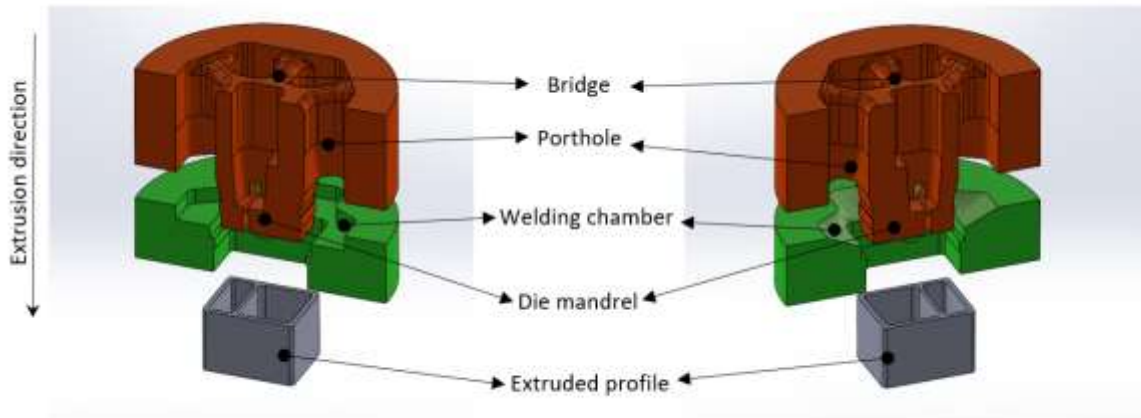


Figure 3. Three dimensional representation of the porthole extrusion die and product profile

Figure 3 has three dimensional representation of the porthole extrusion die and product profile for two different die profile

a) Current die b) Newly design

The other extrusion simulation parameters are as follows:

Billet diameter: 184 mm

Billet length: 800 mm

Profile transition length: 15mm

Billet temperature: 460°C

Die and container temperature: 420°C

Ram speed: 5mm/sn.

Extrusion ratio: 24

Mechanical properties of the H13 hot work tool steel was defined to the programme as die material.

### SIMULATION RESULTS

In Figure 4, the simulation during the extrusion of the product coming out of the sharp die used in the company is presented for 3 separate stages. In the simulation, the blue color represents the first extruded billet and the red color represents the second billet.

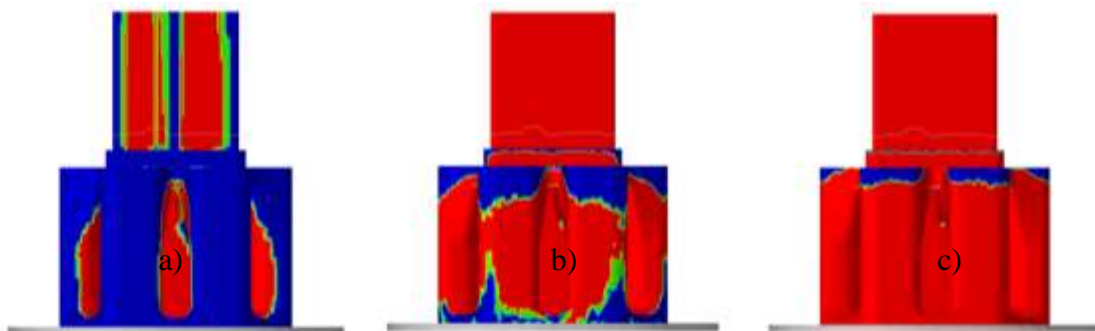


Figure 4. The amount of residual aluminum in the welding chamber as a result of the simulation for the current die

Figure has the amount of residual aluminum in the welding chamber as a result of the simulation for the current die

a) The stage the second billet enters the die b) The intermediate stage image c) The stage the second billet completely extruded

Figure 4.a simulates the stage just after the second billet enters the die, and Figure 4.b simulates the middle stage of the joining process. Particularly in Figure 4.c, at the stage where the second billet completely fills the die, the pieces of the first billet remain in the sharp corners at the point close to the product exit (blue areas). Thus, the dies designed and manufactured with sharp corners and flat surfaces, the aluminum billet in the previous stage is mixed with the next billet piece by piece in each pressing, causing a heterogeneous structure in the profile.

In order to eliminate these problems and reduce the remaining aluminum ratio, the welding chamber profile was designed to have a curved geometry as shown in Figure 2 and the simulation was repeated under the same conditions. While taking images for Figure 5.a, 5.b and 5c, attention was paid to ensure that the number of iterations and process steps were exactly the same as in Figures 4.a, 4.b and 4.c. Although there are not great differences in the intermediate stages, the most important difference is seen in the last stage. As can be seen from Figure 5.c, the blue colored areas, which are the indicators of the remaining parts of the first billet, have decreased considerably. When the extrusion process was completed under the same conditions for both models, the difference between Figure 4.c and Figure 5.c was determined by measuring the remaining amount of the old billet. Accordingly, in the current design, 0.55 kg of material remains from the first billet at the stage when the second billet completely fills the die (Figure 4.c), while the amount of residual material remaining under the same conditions in the new design with curvilinear welding chamber is 0.23 kg. (Figure 5.c). Thus, an inclined welding chamber has reduced the amount of residual material by 58% and it is clear that products with smoother profiles will result.

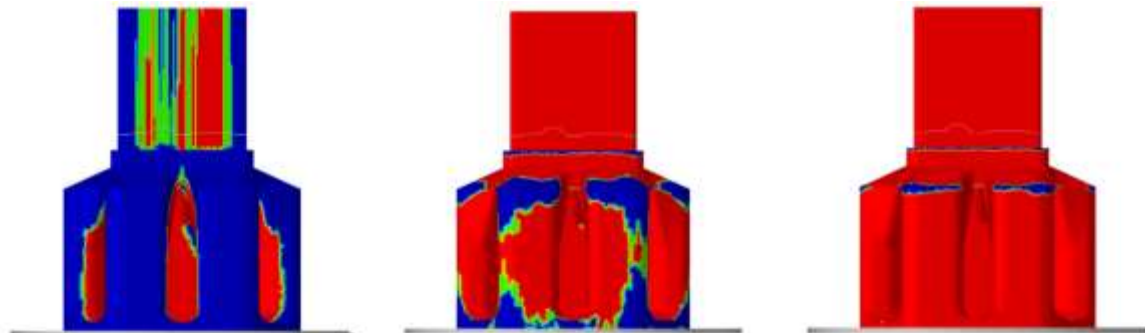


Figure 5. The amount of residual aluminum in the welding chamber as a result of the simulation for the newly designed die

a) The stage the second billet enters the die b) The intermediate stage image c) The stage the second billet completely extruded

In order to evaluate the stress effect of two different welding chamber geometries on the porthole, welding chamber and product profile, the total stress values for both cases were investigated. When Figures 6.a and 6.b are examined, there is no significant difference between the stress values and there is an equivalent stress of approximately 45 MPa at the die exit. Although the stress value in the curvilinear welding chamber is slightly higher than in the flat surface design with sharp corners, it is clearly seen that there is no difference that will affect the operation of the process or the product quality.

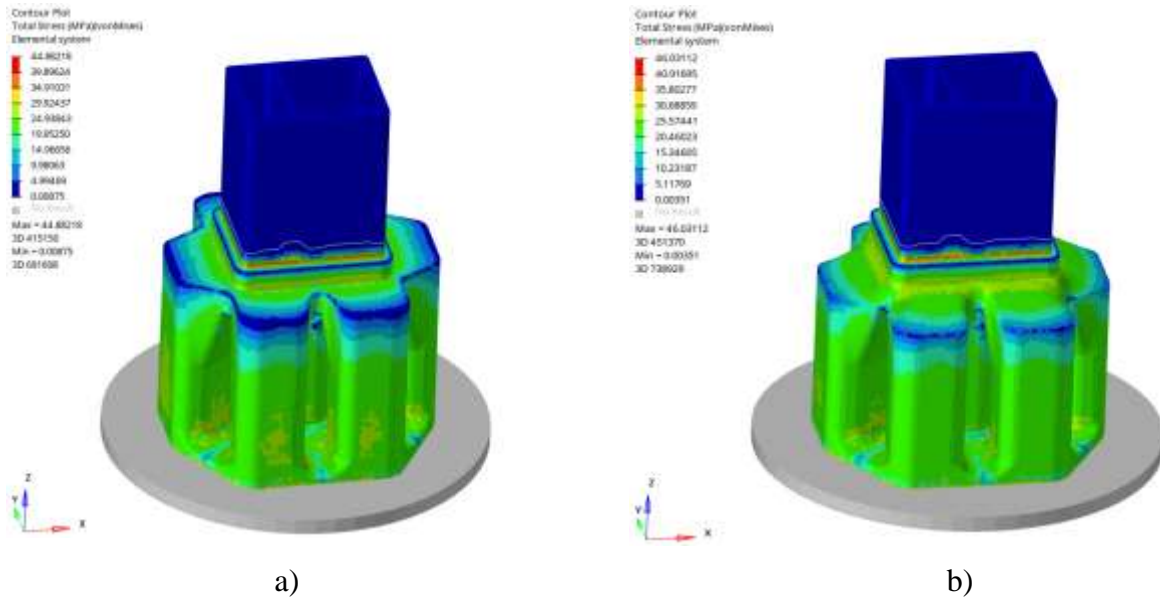


Figure 6. Maximum Von-Mises stress on the part  
 a) Current die b) Newly design

The extrusion force was investigated as the final comparison parameter for two different welding chamber designs. It can be seen in Figure 7 that the extrusion force for both designs is almost the same throughout the entire stroke. However, in the flat weld chamber design, during the transition from the first billet to the second billet, it is predicted that the force will be 13% higher, since more aluminum remains due to the geometry of the welding chamber.

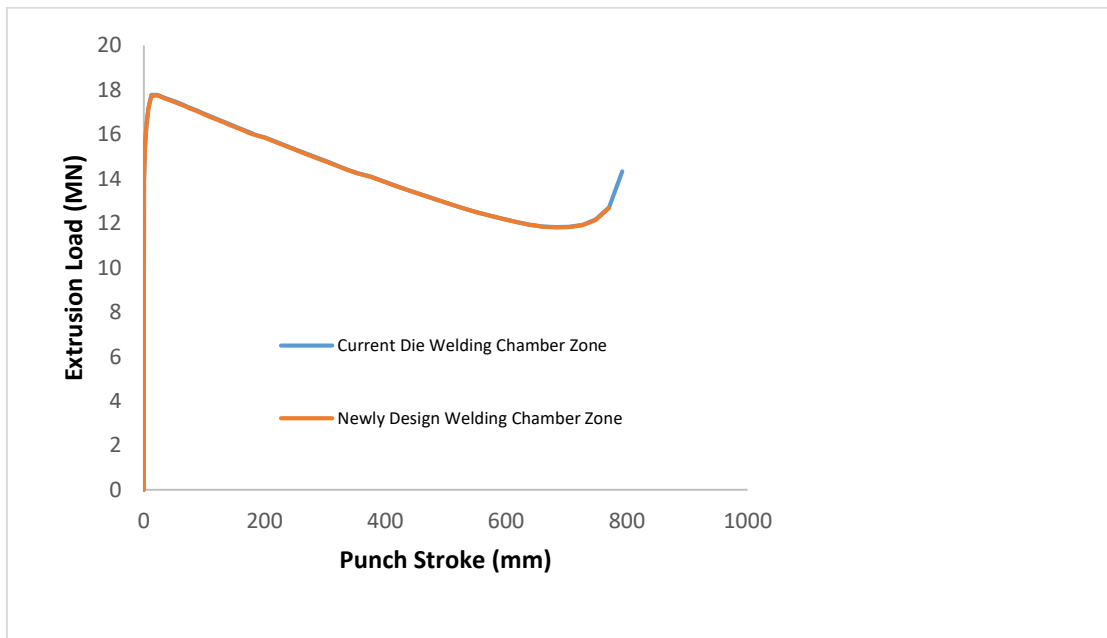


Figure 7. Theoretical extrusion loads as a function of punch stroke for two different welding chamber geometry

## CONCLUSION

- 1) In this study, the flow characteristic of the metal was investigated by performing a 3D FE modelling to simulate the porthole die extrusion of hollow aluminum profiles with the Inspire Extrude Metal 2021.2 program based on the HyperXtrude software using the ALE algorithm.
- 2) Two different welding chamber designs are compared in the light of various parameters. It is aimed to solve the problem of residual material, especially in the transition between billets.
- 3) Based on the surface quality of the product, it has been shown that extruded products with higher surface quality can be obtained with the newly designed welding chamber profile with curvilinear geometry.
- 4) However, since sharp corners still remain in the die, the residual material problem is not completely resolved and different design models related to this are left to the next studies.

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## **MANUFACTURING STRATEGIES ON FOOD SUPPLEMENT EFFERVESCENTS AND BIOACTIVE COMPONENT RETENTION**

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### **ABSTRACT**

Plant based food effervescence as dietary supplements, and/or fortified foods, food byproduct based food powders may be great value-added products for getting healthy bioactive constituents. Activated phenolic bioactives and probiotics which have been naturally extracted to be highly potent and easily absorbed by using food tablets. The unit dose, temper evident, solid preparations of active ingredient and ingredient mix, powder quality for safety manufacturing; should be monitored. Through the powder forming, the flexible design of dosage forms as technical manufacturing parameters has been considered. Owing to the supplement manufacturing, consumer demand, routes of drug delivery, oral utilization capacity should be examined. In this context, the bulk density, the tapped density as pre-compression parameters have been confirmed while thickness, hardness, % weight variation, % friability, % invitro drug release as post-compression parameters have been carried out as physiochemical properties. Besides chemical profiles, functional properties, and detailed clinical nutrition data of expressed effervescent. In this presentation, agrifood and plant based supplement manufacturing strategies and research data on mulberry-based, citrus-based, tomato-based, honey-based effervescent chemistry and nutritional quality have been carried out.

**Keywords:** Plant foods, effervescent, strategy, quality, property, supplement

### **INTRODUCTION**

Food tablets as dietary supplements, and/or fortified foods, food by-product based food powders may be great value-added products for getting healthy bioactive components. The functional constituents of the foods, some preferable functional foods or some functional plant/fruits/ vegetables/spice foods has been standardized as the nutraceutical product and generate under good manufacturing practices (GMPs). Meanwhile the mentioned plants have been used in effervescent food supplements such as effervescent food tablets (Tokusoglu 2018; Tokusoglu and Swanson,2015; Tokusoglu and Hall,2011).

Nutraceutical food tablets has been prepared by direct compression method through selected tablet machines and has been manufactured according to established prescription methods. The functional constituents of the foods, some preferable functional foods or some functional plant/fruits/ vegetables/spice foods has been standardized as the nutraceutical product and generate under good manufacturing practices (GMPs). Primarily, a nutraceutical or selected food must be detected for "non-toxic food constituent strategy" by advanced toxicity analyses,

then it must be detected and analyzed in terms of health benefits including disease treatment and/or prevention (Tokusoglu 2018; Pham,2015; Prabhakar and Krishna,2011; Stahl,2003)

Activated phenolic antioxidants are derived from the healthiest of plants, fruit, vegetable and/or spices. A wide range of free radicals are neutralized by absorption of antioxidant phenolics through effervescent; hence body cells are protected from damage and inflammation.

Uniquely containing activated phenolic antioxidants that have been naturally extracted to be highly potent and easily absorbed by your body in food tablets. Activated phenolic antioxidants are derived from the healthiest of plants, fruit, vegetable and /or spices. A wide range of free radicals are neutralised by absorption of antioxidant phenolics through effervescent; hence body cells are protected from damage and inflammation. Effervescent tablet has been proved its utility as an oral delivery system in the pharmaceutical and dietary industries for decades

### **Food Effervescent Tablet Properties**

In effervescent nutraceutical technology; a balanced ratio of acids and carbonates are used for forming a buffer and it has optimal compatibility with the stomach. Gas bubbles occur from the liquid after chemical reaction by adding water; alkali metal bicarbonates and acids (majorly citric or tartaric acids) are utilized to produce effervescence. In effervescent system, when organic acid and bicarbonate get together in the water, CO<sub>2</sub> is released; the solving process is performed in 17–20°C water. The foam of them helps to kill the local bacteria.

Food tablet is described as unit dose, temper evident, solid preparations including one or more active ingredients or whole food powder. Patient and/or consumer demand, routes of drug delivery, oral utilization capacity, the flexible design of dosage forms as technical manufacturing parameters has been considered; also the bulk density (g/ml), the tapped density (g/ml) as pre-compression parameters have been confirmed while thickness (mm), hardness

(kg/cm<sup>2</sup>), % weight variation, % friability, % in- vitro drug release as post-compression parameters have been carried out as physiochemical properties. Among the alkali sources, sodium carbonate is preferred due to its low cost, high solubility and intensity of reaction than potassium carbonate and bicarbonate.

Polyvinylpyrrolidone (PVP) is used as binder in effervescent; its form is as dry powder or wet forms of aqueous or hydroalcoholic solutions Water-soluble lubricants, colors, flavorings and sweeteners are also added as other ingredients. Mannitol and PEG 6000 are other utilized effective binders. At production stage in tablet machine, relative humidity should be low (25%) and ambient temperature should be at room temperature (24±1°C). Tablet forming quality parameters (weight, hardness, pH, solution time and friability) are inspected.

Dyes or lake color pigments have been added to manufacture colored solutions or products. In effervescent tablets, color stability is also significant. These should be chosen as anhydrous materials. Generally dried flower bud, herbs, chamomile extract may be utilized for this purpose, their percentage may be lower than 1–2%. The remain part of the 0.1–2% in effervescent should be consisted of vitamin E, squalene, almond oil and cosmetic esters. Besides, PEG-30 castor oil, laureth 4, polysorbate 80 or 85 can be used as emulsifiers.



### **Probiotic Using in Effervescent Manufacturing**

Probiotics are living microorganisms that confer benefits on the recipient health when administered in appropriate amounts. Probiotics are mainly utilised in nutritional supplements owing to their positive impacts on health (Do Espirito et.al,2011) An adequate selection among different probiotic strains has to be performed to allow manufacturing probiotic-supplemented food and plant consuming products. The aim in administering probiotics is to induce a balanced enteric microbiota, that will have a favorable effect on consumer health.

The strain selection process guarantees the survival of the probiotic microorganisms in the demanded products during its manufacturing and storage, in addition to provide that the product will confer sufficient technological properties (Bansal and Garg,2008).

The food effervescent matrix selected for incorporating probiotic strains should be carefully fortified so that an adequate selection of the probiotic strain-food pair is attained. The compatibility and adaptability among the selected strains and matrixes is principal. Varied lactic probiotic products, chiefly the fermented as well as some non-lactic products, have been improved as fruit effervescent containing probiotics and have been achieving interest in the global marketplace. To define the composition of effervescent probiotics in tablet form, an assessment of the component`s effects on the viability of the microorganisms are performed.

### **Prebiotic Using in Effervescent Manufacturing**

Prebiotics are food ingredients that induce the growth or activity of beneficial microorganisms (e.g., bacteria and fungi). Approximately 47% of chicory root fiber contain the prebiotic fiber inulin; inulin nourishes the gut bacteria, improves digestion and helps relieve constipation. Tablets including prebiotics have metabolic properties and improves the intestinal ecosystem and colon cells, stimulating the peristalsis, improves lipids and reducing the cholesterol and triglyceride serum levels and also facilitates the mineral salt absorption (Brunser and Gotteland,2010).

The powder blend has been thoroughly mixed with talc and magnesium stearate and compressed into 300-400 mg tablet using single rotatory punching machine based on tablet processing strategy. Among the trial /serial tablet formulations; "mesir effervescent tablet" could be more efficacious owing to majorly cinnamaldehyde (as v/v) whereas "black mulberry effervescent tablet" could be more beneficial due to the presence of morusin and apigenin phenolic anticarcinogenics and also "mandarin peel effervescent tablet" could be salutary because of its naringenin and hesperidin flavonone phenolic bioactives, also "tomato peel effervescent tablet" could be important due to its lycopene and anthocyanidin phenolic antioxidants.

### **CONCLUSION**

In this context, the innovative and conventional food tablet processing strategies has been focused to chemical characterization, functional properties, their unique bioactive features, antioxidative, anticarcinogenic reports of above-mentioned developed tablets. General usage, bioactive materials from plants and probiotic /prebiotic fortification are so important than ordinary supplement materials. Specific applications are more efficient and are required to meticulous manufacturing.

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## **IMPACT OF COVID-19 PANDEMIC ON MENSTRUAL CYCLE IN YOUNG WOMEN IN EDIRNE PROVINCE, TURKEY**

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### **ABSTRACT**

The COVID-19 pandemic has profoundly affected the lifestyle of the global population, albeit to varying degrees in every society. During the pandemic period, the stress and anxiety faced by women, who are the important part of society. It was inevitable that it would have an impact on reproductive health. It is known that stressful periods and psychological distress can change women's menstrual periods. Therefore, an observational study was conducted on the menstrual cycles of young women throughout the pandemic. 482 volunteer young women aged between 18 and 32 living in Edirne participated in the study online and were asked to complete the survey questions. 431 participants were able to answer all questions completely. The mean age was  $22.7 \pm 1.6$  years. 39% of respondents reported recording their menstrual cycles using a mobile app or diary. 23% were using hormonal contraception. 49% reported changes in their menstrual cycle since the start of the pandemic. 23% reported worsening of premenstrual symptoms. Compared to the pre-pandemic period, 28% reported new onset menorrhagia and 32% reported new dysmenorrhea. 29% of respondents reported experiencing irregular menstrual delays. Cycle length ranging from 28 days to 31 days and bleeding days from 5 days to 9 days, greater variability ( $p = 0.01$ ) was detected. There was a median increase of 2 days ( $p < 0.0001$ ) at the minimum. The COVID-19 epidemic, especially menstrual irregularities in women; significantly affected reproductive health. The long-term effects of this on women's health have not yet been adequately determined. Future studies should address this in detail in the female population of all ages.

**Keywords:** COVID-19 pandemic, menorrhagia, menstrual abnormalities, Young Adult

### **INTRODUCTION**

The COVID-19 pandemic has profoundly affected the lifestyle of the global population, albeit to varying degrees in every society. During the pandemic period, the stress and anxiety faced by women, who are the most important part of society; It is inevitable that it will have an impact on reproductive health. Stressful periods and psychological distress; It is known that women can change their menstrual patterns and amounts. Therefore, an observational study was conducted on the menstrual cycles of young women throughout the pandemic in Edirne.

## **MATERIAL AND METHOD**

482 volunteer young women aged between 18 and 32 living in Edirne participated in the study online and were asked to complete the survey questions. Our work; It was held between October 2021 and March 2022. 431 participants were able to answer all questions completely.

### *Data Analyze*

SPSS 20.0 Package Program was used in evaluating the collected data and in all statistical analyzes. The level of significance was determined as 5% in all statistical analyzes. Data were studied with appropriate descriptive statistics. Numerical variables were analyzed with mean and standard deviation. Categorical variables were evaluated with frequency and percentage. The normal distribution control of the data was evaluated with the Shapiro-Wilk test. Student's t test was used for pairwise comparisons of the groups. Chi-square test was used for the relationships between categorical variables. Descriptive statistics for numerical variables were given as mean and standard deviation. Descriptive statistics for categorical variables were given as percentage and frequency.

## **RESULTS**

Our work was based on volunteerism. The mean age of the participants was  $22.7 \pm 1.6$  years. 39% of respondents reported recording their menstrual cycles using a mobile app or diary. 23% were using hormonal contraception. 49% reported changes in their menstrual cycle since the start of the pandemic. 23% reported that their premenstrual symptoms got worse during the pandemic. Compared to the pre-pandemic period, 28% reported new onset menorrhagia and 32% reported new dysmenorrhea.

29% of respondents reported experiencing irregular menstrual delays. Of those who could not menstruate "occasionally" before the pandemic, 9% could not menstruate "often" during the pandemic. A wider variation ( $p = 0.01$ ) was found in cycle length, ranging from 28 days to 31 days and bleeding days from 5 days to 9 days. There was a median increase of 2 days ( $p < 0.001$ ) at the minimum.

Women reported a 15-minute decrease in median daily exercise. 22% reported working more and 16% working less. Participating women reported a significant increase in low mood ( $p < 0.001$ ). Increased appetite ( $p < 0.001$ ), poor concentration ( $p < 0.001$ ), anxiety disorder ( $p < 0.001$ ), sleep disturbance ( $p < 0.001$ ), and feeling of loneliness ( $p < 0.001$ ) were increased.

The specific stressors reported were as follows. School or work stress (52%), anxiety in accessing health centers (37%), change in finances (201/19%), difficulties in homeschooling (36%), family or partner conflict (18%), familial illness or age (19%). Participating women reported a significant increase in low mood ( $p < 0.001$ ). Increased appetite ( $p < 0.001$ ), poor concentration ( $p < 0.001$ ), anxiety disorder ( $p < 0.001$ ), sleep disorder ( $p < 0.001$ ), sense of loneliness ( $p < 0.001$ ) increased.

Participating women reported a 15-minute reduction in average daily exercise. 22% reported working more, 16% less. 9% of those who could not menstruate "occasionally" before the pandemic, could not menstruate "frequently" during the pandemic.

## **DISCUSSION**

The COVID-19 epidemic, especially menstrual irregularities in women; significantly affected reproductive health. The long-term effects of this on women's health have not yet been

adequately determined. Future studies should address this in detail in the female population of all ages.

Psychosocial stress and other women's health disorders have been associated with changes in duration corresponding to the number of days of menstruation and the amount of menstruation reflected in heavier or lighter bleeding patterns. Women who perceived themselves in high-stress jobs reported both longer and shorter menstrual periods, while women suffering from depression reported longer menstrual periods (1-3).

In this study, stressors related to COVID-19 could be a contributing factor to menstrual cycle changes, as more than half of all women with high stress scores reported a change in menstrual duration. Additionally, participants recorded significant changes in menstrual bleeding during the COVID-19 pandemic. Previous studies have linked abnormal bleeding patterns and other menstrual irregularities to stress in adolescent and young adult populations, and stress is known to contribute to changes in menstrual bleeding patterns among adult populations as well (4). Our work; that states that the COVID-19 pandemic may have directly contributed to menstrual cycle irregularities in women experiencing both moderate and high stress.

These data corroborate the experiences of women reporting COVID-19-related menstrual cycle irregularities to healthcare professionals, online forums or through other media outlets, and can foster a discussion between healthcare professionals and their patients about reproductive health during the COVID-19 outbreak. The relationship between high perceived stress and menstrual cycle irregularities is important because it supports the findings that stress has downstream effects on reproductive function (5, 6)

The stress from the pandemic could potentially put women at higher risk for endocrine dysregulation. It is important for healthcare providers to be aware of this association, as low estrogen levels can adversely affect metabolic and bone health (7). More research is needed to better understand the underlying impact of the COVID-19 pandemic on the menstrual cycle and, more broadly, on women's reproductive health. Our study identifies and highlights a critical need to assess the long-term reproductive effects of the COVID-19 pandemic.

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*Financial support:*

No support was received from any person, institution or organization for the study.

*Limitations:*

Our study is an example of Trakya University Health School. Further studies can also be carried out with broader participation, health undergraduate students from all over the country.

*Conflict of Interest:*

There is no conflict of interest in the study.

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## **GENDER INEQUALITY DURING THE COVID-19 PANDEMIC; FROM THE WINDOW OF UNIVERSITY STUDENTS**

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### **ABSTRACT**

The COVID-19 pandemic has caused unprecedented disruptions in the global sociocultural space. Especially in developing countries, access to health institutions have been delayed, resulting in socioeconomic income loss and high unemployment rates. However, evidence for gender differences in economic outcomes, if any, in economic outcomes such as income, spending, savings, and job loss in a multi-country pandemic was limited. We conducted a study involving university students living in different geographical regions and from families of various income levels. Our study with Trakya University Health Science School students; made with online interviews. 179 female and 216 male students participated in the study. In our study, first of all, sociodemographic characteristics were recorded. The data obtained from the survey we prepared on gender were used. The effects of COVID-19 on gender inequality in these results were evaluated. During the pandemic, women were in greater need of health services ( $p < 0.001$ ). At the bivariate level, in the pandemic process; women had lower self-determination control to go out (87.2% versus 70.0%). Women had less consistent access to secure, private internet (57.1% vs. 69.3%). Women also expected their labor incomes to fall 30 percent more than men. In young adults, the COVID-19 pandemic has been found to increase underlying inequalities. Young women should have equal access to resources, including economic, health and technology-based support. Gender-sensitive remediation efforts are needed. The unique and unavoidable academic, economic and social needs of young people must be addressed.

**Keywords:** COVID-19 pandemic, Gender Inequality, Young People, COVID-19, gender; Gender Analysis, Women

### **INTRODUCTION**

In response to the epidemic, Turkey, like many other countries in the world, asked its citizens to stay at home. The dizzying spread of the pandemic was being tried to be prevented in this way. As a result of the curfew, many students had to do their school duties at home as well as housework. The students were located in various regions with different backgrounds and cultures. For this reason, it has become even more important that information seeking behavior is not exposed to negative gender discrimination during the pandemic period. Students who had reached the university level needed a large amount of information in a new era (Cui, Ding, & Zhu, 2022).

However, access to quality and sufficient information could be limited during the Covid-19 pandemic, due to the limitations of countries and the world (Cui et al., 2022; Prowse et al., 2021). In the past, libraries were important sources of information for university students. But in the university environment, the continuous development and change of science shows its

effects. The information needs of university students are also changing. Unfortunately; the researches that examines this in depth is limited.

However, with the advancement in technology, the tendency to seek information in the academic field, especially in the field of gender, has changed.

The aim was to examine the information seeking behaviors of university students in the field of gender in terms of their information needs and to emphasize the role of the Covid-19 pandemic in this area.

## **MATERIAL AND METHODS**

We conducted a study involving university students from different geographical regions and from different income families. Our study with Trakya University School of Health Sciences students; Made with online interviews over Microsoft Teams. 179 female and 216 male students participated in the research between December 2021 and May 2022. In our study, first of all, sociodemographic characteristics were recorded.

The data obtained from the questionnaire we prepared on gender were used. These results evaluated the effects of COVID-19 on gender inequality.

Within the scope of the research, "Personal Information Form" was used to evaluate the demographic information and lifestyles of the students. In the personal information form, besides sociodemographic characteristics, questions about sleep times, smoking and alcohol use, eating habits, and physical activities were asked to the participants.

Ethical approval was obtained from the scientific research ethics committee of Trakya University and it was recorded that they volunteered with the informed consent form obtained from all participants.

All statistical analyzes evaluating the collected data were performed using the SPSS 20.0 Package Program. Data were summarized with appropriate descriptive statistics. Paired group comparisons were made with Student's t test. The level of significance was determined as 5% in all statistical analyzes.

## **RESULTS**

COVID-19 risk perception was high, with a gender gap to the detriment of young women (63.6% versus 72.2%;  $p < 0.001$ ). Participants' concerns about the pandemic and challenges to prevention were examined. During pandemic, restricted access to contraception was detected among women using hormonal contraceptives (34.6%). Gender asymmetry was observed in depressive symptoms (21.8% male; 34.3% female) in the participants. Gender inequalities disproportionately made young women unable to meet their basic economic needs ( $p < 0.005$ ).

Gender-specific concerns for women included difficulties in accessing menstrual hygiene (49.8%). Qualitative results identified the augmenting effect of economic disruption and isolation on premenstrual syndrome in women.

It was observed that privacy restrictions increased among young women. Our findings showed that, due to the COVID-19 pandemic, women were 19 percent more likely than men to worry about not being able to find a job or permanently losing their job. The study was conducted with university students aged 18-25.

COVID-19 risk perception was high, with a gender gap to the detriment of young women (63.6% versus 72.2%;  $p < 0.001$ ). Participants' concerns about the pandemic and challenges to prevention were examined. During COVID-19, restricted access to contraception was detected among women using hormonal contraceptives (34.6%).



Gender asymmetry was observed in depressive symptoms (21.8% male; 34.3% female) in the participants. Women had less consistent access to secure, private internet (57.1% vs. 69.3%).

Gender-specific concerns for women included difficulties in accessing menstrual hygiene (49.8%). Increasing privacy restrictions were observed among young women. Qualitative results identified the augmenting effect of economic disruption and isolation on premenstrual syndrome in women. It was observed that privacy restrictions increased among young women.

The students living in different cities from their families during their university education were examined. Looking at the change with staying in dormitories or houses; It was observed that students staying at home had higher sleep quality and positive mood than students living in dormitories. As the income level of the families of the students increases; It was observed that sleep quality increased, while concerns about the pandemic were decreasing.

Gender inequalities disproportionately made young women unable to meet their basic economic needs ( $p < 0.05$ ). During the pandemic, women were in greater need of health services ( $p < 0.001$ ). At the bivariate level, in the pandemic process; women had lower self-determination control to go out (87.2% versus 70.0%). Our findings showed that women were 19 percent more likely than men to worry about not being able to find a job or permanently losing their job due to the COVID-19 pandemic. Women also expected their labor incomes to fall 30 percent more than men. Perhaps because of these concerns, women tend to reduce their current consumption and increase their savings. In the pandemic process, the curfew applied in cities to maintain social distance; It also changed the way students conduct their academic and social activities. Many countries have closed their universities. Therefore, both faculty and students had to do research and teach from home.

A student's productivity and academic success; The efficiency of research and education is determined jointly by available time. First, given the unequal distribution of domestic tasks, the pandemic is more likely to engage young women with more domestic tasks and to devote less time to education. Second, scientific education and teaching often require a quality and uninterrupted environment.

As a result of the pandemic, women are more likely to multitask between college education and household duties. For this reason, it was seen that they had lower efficiency in conducting their academic life.

All these factors indicate that female students' productivity is more likely to be affected disproportionately than males.

## **DISCUSSION**

Factors such as the different rates of participation in business sectors for men and women and the widening of this gap during the pandemic period may play an important role in explaining the gender gap (Prowse et al., 2021). This points to a growing pandemic in gender disparities, possibly due to changing infection rates and women's share of socioeconomic and academic power.

In young adults, the COVID-19 pandemic has been found to increase underlying inequalities. They also face the gendered impact of the pandemic, which reflects the negative economic, social and academic impacts. Young women should have equal access to resources, including economic, health and technology-based support.

Gender-sensitive remediation efforts are needed (Cui et al., 2022; Feng & Savani, 2020). The unique and unavoidable academic, economic and social needs of young people must be

addressed (Cui et al., 2022). Also, problems in a new pandemic can cause mental problems and psychological distress, especially for female students.

Our findings show that if the epidemic and quarantine continues for too long, young and female academics in top-ranked universities may become significantly disadvantaged. This could become a fairness issue that could expose women to a higher unemployment or career risk. We hope that our findings will increase awareness of the subject among the academics of the society. Measures can be taken to balance domestic responsibilities between male and female young adults. Our study showed a slower increase in academic development among students, with a 14 percent increase in the gender gap. Therefore, our findings raise a concern for gender inequality, not for overall productivity.

We also hope that our work will inspire researchers to explore other forms of inequality resulting from the COVID-19 pandemic. In this way, universities; plays an active role in the information search of both male and female students (Gillis & Krull, 2020). However, more work needs to be done. Therefore, there is a gap in the literature regarding the knowledge seeking behavior of students in the field of gender (Browning et al., 2021). First, in the pandemic, there were studies on information-seeking behavior. However, it was observed that there was a lack of research in the field of gender equality in order to solve the problems of young people.

At the same time, they are faced with the gender-based effects of the pandemic, which reflect the negative economic, social and academic impacts. It was seen that the use of social media was common among young people during the pandemic process. It was concluded that if online communication and education opportunities are increased, it will both contribute to their social development and increase the interest of students in gender equality in education.

Factors such as the different rates of participation in the business sectors for men and women and the widening of this gap in the pandemic can play an important role in explaining the gender gaps (Feng & Savani, 2020). This indicates an increased pandemic in gender differences, probably due to varying rates of infection and women's share of socioeconomic and academic power. Perhaps because of these concerns, women tend to reduce their current consumption and increase their savings.

Therefore, in order to solve the problems of female students in Turkey, it is necessary to conduct such studies and to obtain information about their knowledge seeking behaviors in university vocational education. It was observed that female students should be supported with various programs in areas needed as a tool to facilitate the educational environment, which was much more difficult in the pandemic, and to learn the basics of functionality.

As a result, universities may consider providing additional support, such as housing and financial advantages, to women whose productivity is disproportionately affected. Universities and faculty should keep this inequality in mind when evaluating exams.

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## VAGINISMUS - IS IT POSSIBLE TO SCREEN YOUNG WOMEN?

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### ABSTRACT

Despite its universal prevalence, it is observed that vaginismus has not been adequately studied in young women in the premarital period. Vaginismus is considered to be a spasmic response that underlies most of the symptom - focused behavioral and cognitive therapy approaches that have dominated the sex therapy field to date. However, there is a prevailing opinion that it would be more accurate to think of vaginismus as multifactorial. This descriptive cross-sectional study was conducted with the students of Trakya University in Edirne in 2022. Volunteer participants who were sexually active (with or without penetration) were included as a condition of participation in our study. 218 students completed all the questions. In this study, a preliminary diagnosis of vaginismus was made by applying a questionnaire by the specialist. Demographic and gynecological information forms were used among the data collection tools. The "Vaginal Penetration Cognition Questionnaire", which is a valid and reliable assessment tool, was included. The scale is used to examine the perspectives and feelings of women with vaginismus and dyspareunia. The other scale used is; It was "Sexual quality of life-female". Getting a high score on the scale; means higher quality of sexual life. According to the results of this study, the mean age of women was  $21.52 \pm 1.49$ . The results of this study showed that fear of sexuality, positive cognition and negative self-perception, sexual intimacy, sexual quality of life, and education variables were the final predictors of vaginismus diagnosis score. Therefore, this disorder is considered multidimensional. A multidisciplinary approach to vaginismus treatment is better suited to effectively respond and resolve the complex nature of this distressing sexual challenge for men and women in adult intimate relationships.

**Keywords:** Vaginismus, Sexual Dysfunction, Sexual Behavior, Disorders of Sexual Development

### INTRODUCTION

The symptom-only approach has been criticized as reductionist. Various physical, psychological, social and cultural factors contribute to vaginismus (Mühlrad, Haraldson, Harlow, Anell Olofsson, & Bohm-Starke, 2021). Therefore, considering that this disorder is multidimensional and that more attention should be paid to all biological, psychological and social dimensions in its treatment, this study was conducted to evaluate the bio-psychological factors contributing to vaginismus in our country.

Despite its universal prevalence, it is observed that vaginismus has not been adequately studied in young women in the premarital period. Vaginismus is considered to be a spasmic response that underlies most of the symptom-focused behavioral and cognitive therapy approaches that have dominated the sex therapy field to date. However, there is a prevailing opinion that it would be more accurate to think of vaginismus as multifactorial.

The symptom-only approach has been criticized as reductionist. Vaginismus, as a sexual dysfunction, prevents sexual penetration through involuntary and frequent spasms of the muscles in the outer third of the vagina. Negative beliefs about gender, cultural factors, fear of pain, injury, bleeding, etc. factors play an important role in the prediction of vaginismus (Vicente-Neira et al., 2022). For this reason, it is necessary to consider all biological, psychological and social dimensions in the multidimensionality and treatment of vaginismus. Considering the wide range of etiology; bio-psychosocial model can be designed (Mühlrad et al., 2021). Thus, since there is a strong framework of factors that contribute to sexual problems in women, recognition at an early age will lead to positive developments. Our study was conducted to investigate the bio-psychological factors associated with vaginismus.

In this study, fear of sex, sexual intimacy, sexual quality of life, and experience from the bio-psychosocial model were the final predictors of vaginismus diagnostic score. Therefore, this model can be used to design interventions for the treatment of vaginismus, particularly in the psychological and interpersonal domains. Patients with vaginismus often experience pain with some form of avoidance phobia, fear, and involuntary muscle contraction. Indeed, this phobia precedes the onset of vaginismus and may be accompanied by negatively associated cognitions and expectations. Various physical, psychological, social and cultural factors contribute to vaginismus. The study was conducted to evaluate the bio-psychological factors contributing to vaginismus in Edirne province.

## **MATERIAL AND METHODS**

First of all, 275 female students participated in the study, but 218 students completed all the questions. Those who were married and had a known psychiatric illness were not included in the study. This descriptive cross-sectional study was conducted with the students of Trakya University in Edirne between February 2022 and June 2022.

Volunteer participants who were sexually active (with or without penetration) were included as a condition of participation in our study. Demographic and gynecological information forms were used among the data collection tools. Those who were married and had a known psychiatric illness were not included in the study. In this study, a preliminary diagnosis of vaginismus was made by applying a questionnaire by a gynecologist.

Two scales were used in the study. The "Vaginal Penetration Cognition Questionnaire", which is a valid and reliable assessment tool, has been included (Klaassen & Ter Kuile, 2009). The other scale used is; It was "Sexual quality of life-female" (Symonds, Boolell, & Quirk, 2005).

### *"Vaginal Penetration Cognition Questionnaire"*

The first version of the "Vaginal Penetration Cognition Questionnaire" had 40 questions. The scale was reduced to 22 questions after initial validity and reliability by Klaassen and Ter Kuile. This scale evaluates factors related to the perception of vaginal penetration (Klaassen & Ter Kuile, 2009). It is used to examine the perspectives and feelings of women with vaginismus and dyspareunia.

### *"Sexual quality of life-female"*

The other scale used is; It was "Sexual quality of life-female". This scale was developed by Symonds et al. Developed by (Symonds et al., 2005). The other scale used is; It was "Sexual quality of life-female". This scale was developed by Symonds et al. The scale is a 6-point Likert type scale ranging from strongly disagree to strongly agree. It consists of 18 items. Getting a high score on the scale; means a higher quality of sexual life ((Symonds et al., 2005).

## **RESULTS**

In our study, the mean age of women was  $21.52 \pm 1.49$  years. The results of our study showed that fear of sexuality, positive cognition and negative self-perception, sexual intimacy, sexual quality of life and education variables were the final predictors of vaginismus diagnosis score. Therefore, this disorder is considered multidimensional. First, univariate linear regression was used, in which the dependent variable was the total score for the diagnosis of vaginismus, and the independent variables were entered into the model one by one.

Education, depression, stress, anxiety, fear of sex, sexual self-efficacy, sexual quality of life, sexual function, sexual violence, sexual intimacy, sexual knowledge and attitude, destructive cognition and control, positive cognition and mental image cognition variables "Vaginismus" ' was significantly associated with the diagnosis variable.

Vaginismus diagnosis score increased by 0.141, 0.651 and 0.115, respectively, with all variables remaining constant for each increase in fear of the opposite sex, self-image, and quality of life scores. For each increase in the education level of the person, the vaginismus diagnosis score increased by an average of 3.038 points, with all variables remaining constant.

Finally, the vaginismus diagnostic score decreased by 0.183 for each increase in the vaginal penetration positive cognition score, provided all variables remained constant. Bio-psychological factors associated with vaginismus were evaluated in our study. The contribution of various physical, psychological, social and cultural factors to the development of vaginismus was examined.

## **DISCUSSION**

Vaginismus is considered as a multidimensional disorder. Women with vaginismus often experience severe anxiety and stress at any penetration, leading to severe avoidance behaviors such as pushing, squeezing their legs, and screaming. Interestingly, only a relatively small number of women report a history of sexual or other trauma. Therefore, pre-existing sexual trauma does not definitively predict these conditions (White, 2018).

The role of interpersonal relationships in vaginismus is the least researched in the literature (Kovacs & Briggs, 2015). Vaginismus can be an indicator of relationship difficulties. For this reason, unresolved relationship problems should be addressed before vaginismus treatment in couple therapy.

However, the difficulties experienced by women with vaginismus; There is evidence that it may predate the partner relationship and may have its origins in complex attachment relationships in the family. In particular, the mother-daughter relationship has been emphasized

as a vital component of the diagnosis and treatment of vaginismus (White, 2018). Therefore, it may be necessary to explore the role of early attachment relationships to understand vaginismus dynamics within the couple relationship.

It is suggested that women who can be assertive and form an identity independently of their mothers during late adolescence are less afraid of sexual and non-sexual intimacy with their romantic partners (McEvoy, 2021; White, 2018). Considering the role of the mother-daughter relationship in the development of vaginismus, therapists may be aware of the inherent difficulty of girls being psychologically separated from their mothers when the mother is overly intrusive and has no boundaries (McEvoy, 2021).

In order to fully understand vaginismus, it is important to investigate it as complicated. Interpersonal and cultural levels continue to be explored. Many scientists support a more holistic approach to both understanding and treating vaginismus (SadatiKiadehi, Pazira, & Alavijeh, 2020). It may be helpful to incorporate a biopsychosocial and experiential approach to vaginismus perspectives.

A multidisciplinary approach to vaginismus treatment is better suited to effectively respond and resolve the complex nature of this distressing sexual challenge for men and women in adult intimate relationships.

In addition, this study showed that education variables in the interpersonal domain of sexual life quality and bio-psychosocial model were predictors of vaginismus diagnosis score. It reveals that intimacy with spouse, sexual satisfaction and marital satisfaction are significantly associated with genito-pelvic pain/penetration disorder. It has been observed that sexual problems can be the cause or result of dysfunctional or unfriendly relationships. According to recent studies, one of the causes of vaginismus in women is the fear of intimacy with the husband, which prevents close relationships and sexual intercourse (McEvoy, 2021; SadatiKiadehi et al., 2020). As mentioned earlier, sexual self-expression and talking about sexual issues, desires, and sexual preferences are among the interpersonal factors considered important in establishing a desirable sexual relationship. In contrast, couples will get less pleasure when they don't talk about sexual matters, which will lead to more sexual problems. This is because if a problem is not addressed by the couple, it cannot be resolved and can lead to sexual dissatisfaction (SadatiKiadehi et al., 2020). On the other hand, people who express more positive emotions, feel higher levels of sexual satisfaction and intimacy with their spouses, experience less stress, and are less likely to divorce because they can reconcile marital conflicts more effectively (8). A "one size fits all" approach to vaginismus treatment is insufficient to effectively respond to and resolve the complex nature of this troubling sexual challenge for men and women in adult partner relationships. It is the duty of medical professionals to provide a seamless, multidisciplinary response to women and their partners seeking help in overcoming the physical and psychological pain associated with vaginismus.

Many scientists support a more holistic approach to both understanding and treating vaginismus (Mühlrad et al., 2021). It may be helpful to incorporate a biopsychosocial and experiential approach to vaginismus perspectives. The most important building block in the success of vaginismus treatment is that the problem is not only attributed to physical but also to psychological causes.

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the success of vaginismus treatment is that the problem is not only attributed to physical but also to psychological causes.

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## COMPARATIVE RESISTANCE OF STORED CEREALS AND PULSE TO *Sitophilus zeamais* MOTSCHULSKY (COLEOPTERA: CURCULIONIDAE)

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### ABSTRACT

We hypothesized the degree of resistance of stored grains and pulse to *Sitophilus zeamais*, a cosmopolitan insect pest of stored foods in the tropics. Three varieties each of maize (TZPB SR, DMR 9943, DMR 9928), sorghum (NGBO1354, NGBO1469, NGBO1582) and wheat (NGBO1123, NGBO1124, NGBO1224) and a commonly grown cowpea variety (Ife brown) were used for the experiment. In a no-choice experiment, 20 g of each food variety was weighed into each of ten 1 L Kilner jar and five pairs of *S. zeamais* were introduced and covered with muslin cloth. Similarly, a free choice experiment was conducted on a white circular cardboard divided into ten equal sectors with each containing the food sample. All experiments were laid out in a CRD, r = 4. Data were collected on F<sub>1</sub> emergence, percentage survival, days to emergence, seed weight loss and susceptibility index and analyzed using ANOVA and means were separated with the NDMRT (p<0.05). The highest mortality (90%) of *S. zeamais* was observed on Ife-Brown and wheat variety, NGBO 1123 in all the days of the trials. Significantly (p<0.05) higher numbers (10.67, 9.86) of adult *S. zeamais* emerged from NGBO 1582 (sorghum) and NGBO 1124 (wheat) respectively. Susceptibility indices ranged from 0 to 5.8 in both no-choice and free-choice experiments. Cowpea variety (Ife Brown) and the wheat variety (NGBO 1123) were the least suitable host to *Sitophilus zeamais*. Desirable characteristics from these resistant grains could be useful in breeding programs to develop varieties that are resistant to the insect pests.

**Keywords:** Free choice experiment, Developmental time, Susceptibility index, Breeding programs

### INTRODUCTION

Cereals are a good source of rich-dietary fibre, vital nutrients like vitamin E, omega 3 fatty acid, phosphorous, magnesium and zinc (Macauley, 2015), constituting the largest source of food for human beings, as well as for animals, especially in Africa. Each cereal, such as maize, rice and sorghum, has its important nutrients that help to boost the body health (Klopčič *et al.*, 2020; Baniwal *et al.*, 2022). Cowpea is one of the most versatile food legumes in the tropics and subtropical regions of the world; the most important seed legume in Africa (Dakora and Belane, 2019), with a particularly high demand in Nigeria. The incessant rise in human population necessitates growing need for human food and animal feed, and consequently, there is a high demand for the maintenance of quality and quantity grain food products (Garcia-Correia, 2002).

Insect pests are among the main biotic agents that disrupt food substances in storage. They do these either by eating grains, contaminate commodities with their faeces, webbing, as well as their body parts (Hodges *et al.*, 2011; Berhe *et al.*, 2022). The maize weevil (*Sitophilus zeamais*) is an important cosmopolitan pest of grains, including, maize, wheat, rice and sorghum, in the tropics. Its infestation begins in the field when the grain moisture content is between 50–55%, allowing the weevils to already complete one generation, and lay eggs for the second generation (Adedire, 2001). Earlier reports have also shown that it attacks other plants like *Carya illinoensis* and *Prunus persica* (Bloem *et al.*, 2002).

During harvest of crops, many smallholder farmers in Africa, especially Nigeria allocate sections of their local storage facilities for their produce and do not usually construct separate structures for each produce, leading to cross infestation of the produce by insect pests. Although cereal grains are the main host crops, infestation and damage caused by *Sitophilus zeamais* of cereal crop varieties are not well documented. As well, members of the genus *Sitophilus*, have been reportedly found in some other classes of crops. For example, in Nigeria, a strain of *S. zeamais* was found in some cowpea cultivars and laboratory observation revealed that the strain utilized the cowpea cultivars as its food, evidenced by the leftover powdery materials in the jars containing the cowpea seeds (Babarinde *et al.*, 2008). Gupta *et al.* (1985) reported that *S. rugicollis*, infested the seeds of sal (*Shorea robusta*) and caused damage on the oil crop and Coombs *et al.* (1977) reported that some strains of *S. oryzae* were found to develop on grain legumes such as peas, lentils and black grams. These developments necessitated the conduct of this current study in other to determine and compare the level of resistance of food host crops to *Sitophilus zeamais*.

## **MATERIALS AND METHODS**

### **Study location**

This study was carried out in the Entomology Research Laboratory of Department of Crop Protection and Environmental Biology, University of Ibadan, Ibadan under ambient temperature of  $27 \pm 5^\circ\text{C}$ , relative humidity of  $72 \pm 6\%$  and 12 hours photoperiod.

### **Sources of Crop varieties**

Three maize varieties (TZPB SR, DMR 9928, DMR 9943) and one cowpea variety (Ife Brown) were obtained from the Institute of Agriculture Research and Training (IAR&T), MOOR plantation, Ibadan, while three sorghum varieties (NGBO1354, NGBO1469, NGBO1582) and three wheat varieties (NGBO1123, NGBO1124, NGBO 1224) were obtained from the National Centre for Genetic Resources and Biotechnology (NACGRAB) Moor Plantation, Ibadan, Nigeria. The food hosts were cleaned and kept in a refrigerator for 7 days to kill existing storage insect pest.

### **Insect colony**

Colony of *Sitophilus zeamais* was established in the laboratory with initial stock obtained from Nigerian Stored product Research Institute (NSPRI), Onireke, Ibadan. Fifty (1 male: 1 female) weevils were introduced into 150 g maize grains in each of three 1- Litre Kilner jars with mesh lids. Old weevils were removed after 10 days of mating and oviposition. Teneral adults were used for the experiments.

### **Survival of *S. zeamais* on food hosts**

Twenty (20) grams of each food varieties (Maize, sorghum, wheat and cowpea), were weighed and placed into each of ten 1 L Kilner jars and five pairs (1:1) of one week old *S. zeamais* were

introduced into each jar and covered with lids having muslin cloth. Each jar was replicated four times and the experiment was laid out in a Completely Randomized Design (CRD). The set up was left undisturbed in the laboratory for ten days and data on mortality were collected at 6, 8 and 10 days after infestation.

Percentage mortality was calculated as,

$$\frac{\text{Number of dead weevils}}{\text{Total number of weevils}} \times \frac{100}{1}$$

### No - choice experiments

Food varieties (20 g each), were weighed and placed in forty jars and ten one-week old *S. zeamais* were introduced and the jars were covered with muslin cloth. All insects, both dead and living, were removed after 12 days. The set-up was left undisturbed until the emergence of F<sub>1</sub> progeny. Daily count of the emerged adults was done (until emergence ceased) and every insect was removed to prevent further egg laying on food samples. Adult *S. zeamais* that emerged from each jar were summed up and compared among food hosts. Median developmental time (MDT) was calculated as the time (days) from the middle of the oviposition period to the emergence of 50% of the F<sub>1</sub> progeny (Akinbuluma and Ewete, 2019). Susceptibility index (SI) for each treatment was then calculated using the formula:

$$SI = \frac{\log_{10} F \times 100}{MDT}$$

The grains were later sieved to remove the dust produced from adult feeding and reweighed using a Digital Pocket Weighing balance and percentage weight loss was determined as follows:

$$W (\%) = \frac{WI - WF}{WI} \times \frac{100}{1}$$

Where, W (%) = weight loss (%), WF = Final weight, WI = Initial weight

### Free choice experiments

The same set up as the no-choice experiment was repeated with some modifications. Briefly, a white cardboard was cut to give a circular shape fitting into the bottom of a bowl and ten equal sectors were traced on the circular cardboard. Twenty (20) g of each grain was randomly placed in each sector. Adult insects (100) were placed at the centre of each bowl and covered with muslin cloth. The set up was replicated 4 times. The bowls were left undisturbed for 7 days for insect to oviposit, after which the grains were carefully transferred into Kilner jars and covered with muslin cloths. As described above, data were collected on emergence of F<sub>1</sub> adults (until completion of emergence), MDT and SI and weight loss and compared among food hosts.

## RESULTS

### Mortality of *S. zeamais*

Table 1 shows the percentage mortality of *S. zeamais* infested on the different varieties of food maize, sorghum, wheat, and cowpea (Ife brown) varieties. There was no mortality of *S. zeamais* on the infested DMR 9943 (maize) and NGBO 1224 (wheat) up till the 10<sup>th</sup> day of trial. Mortality was significantly higher (p < 0.05) in NGBO 1123 (wheat) and Ife brown (cowpea) than in other food host varieties on all days of trials.

**F<sub>1</sub> Emergence in *S. zeamais* and weight loss in food hosts**

The number of F<sub>1</sub> emergence of adult *S. zeamais* as well as weight loss in maize, sorghum, wheat and cowpea in a no choice experiment and free choice experiments are presented in Tables 2a and 2b. The highest significant ( $p < 0.05$ ) number of adult *S. zeamais* (10.67, 9.86) emerged on NGBO 1582 (sorghum) and NGBO1124 (wheat), respectively, while the least emergence was observed on NGBO1123 (wheat) and Ife brown (cowpea). Maize variety, TZPB SR-W and sorghum variety, NGBO 1582 had the longest developmental period of 38.63 and 35.75 days, respectively. The highest weight losses in food varieties were recorded on DMR 9943 (maize), NGBO 1582 (sorghum) and NGBO 1124 (wheat), while the least weight losses were found in NGBO 1123 (wheat) and Ife brown (cowpea) (Table 2a). As shown in Table 2b, the highest significant number of adult *S. zeamais* in a free choice test, emerged from maize varieties, DMR 9928 and DMR 9943. The sorghum variety, NGBO 1354 had the longest median developmental time (34.50), even though not significantly different from developmental time in other food hosts apart from NGBO 1224 (wheat) and DMR 9928 (maize). Significantly higher weight losses were recorded on maize varieties, DMR 9943 and TZPB SR, while Ife brown (cowpea) and NGBO1123 (wheat) recorded the least percentage weight loss (Table 2b).

Fig. 1 shows the susceptibility indices of the food host varieties used in this study. As we had similar susceptibility indices for the no-choice and free choice experiments, we decided to use a representative index for our reports. Ife Brown (cowpea) and NGBO 1123 (wheat) had the lowest (0) susceptibility index, while NGBO 1469 (sorghum) had the highest (5.8) susceptibility value (Fig. 1).

Table 1: Survival of *Sitophilus zeamais* infested on varieties of food hosts in the laboratory

| Food host | Varieties | Mortality ( $\pm$ S.E) at days after infestation |                   |                   |
|-----------|-----------|--|-------------------|-------------------|
|           |           | 6  | 8                 | 10                |
| Maize     | DMR 9928  | 0.00 $\pm$ 0.00a                                 | 0.00 $\pm$ 0.00a  | 5.00 $\pm$ 0.30a  |
|           | DMR 9943  | 0.00 $\pm$ 0.00a                                 | 0.00 $\pm$ 0.00a  | 0.00 $\pm$ 0.00a  |
|           | TZBP SR W | 4.61 $\pm$ 4.61a                                 | 9.22 $\pm$ 5.32a  | 9.22 $\pm$ 5.32a  |
| Sorghum   | NGBO 1354 | 32.53 $\pm$ 4.91b                                | 41.99 $\pm$ 5.07b | 49.87 $\pm$ 5.07b |
|           | NGBO 1469 | 4.61 $\pm$ 4.61a                                 | 9.22 $\pm$ 5.32a  | 16.22 $\pm$ 5.32a |
|           | NGBO 1582 | 0.00 $\pm$ 0.00a                                 | 0.00 $\pm$ 0.00a  | 7.00 $\pm$ 3.40a  |
| Wheat     | NGBO 1123 | 78.75 $\pm$ 6.70c                                | 90.00 $\pm$ 0.00c | 90.00 $\pm$ 0.00c |
|           | NGBO 1124 | 9.22 $\pm$ 5.32a                                 | 9.22 $\pm$ 5.32a  | 9.22 $\pm$ 5.32a  |
|           | NGBO 1224 | 0.00 $\pm$ 0.00a                                 | 0.00 $\pm$ 0.00a  | 0.00 $\pm$ 0.00a  |
| Cowpea    | IFE-BROWN | 80.78 $\pm$ 5.32c                                | 90.00 $\pm$ 0.00c | 90.00 $\pm$ 0.00c |

Means within a column with the same letter(s) are not significantly different at  $p < 0.05$  using New Duncan's Multiple Range Test

Table 2a: Adult Emergence, Median Developmental Time of *Sitophilus zeamais* and weight loss in food hosts in a no-choice test

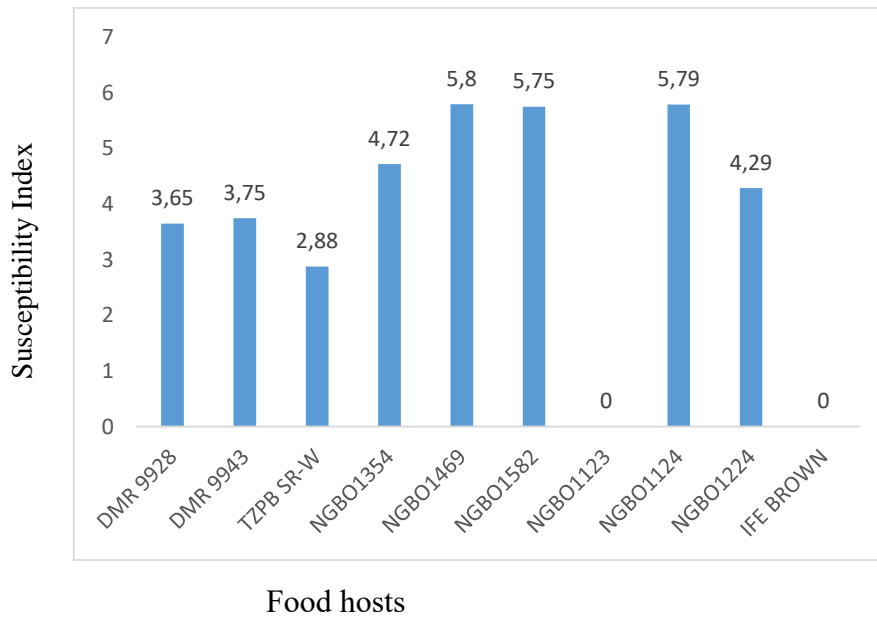
| Food hosts | Varieties | F <sub>1</sub><br>Emergence | Median<br>Development Time | Loss<br>in weight (%) |
|------------|-----------|-----------------------------|----------------------------|-----------------------|
| Maize      | DMR 9928  | 4.38±0.12bc                 | 34.5±0.83cd                | 10.57±0.66bc          |
|            | DMR 9943  | 4.41± 0.15bc                | 33.75± 0.41bc              | 15.44±1.12d           |
|            | TZBP SR   | 3.67±0.42b                  | 38.63±1.69e                | 8.51±0.97b            |
| Sorghum    | NGBO 1354 | 5.77±0.55d                  | 32.25±1.11bc               | 7.37±1.03b            |
|            | NGBO 1469 | 7.95±0.44e                  | 31.00±0.5b                 | 8.06±0.97b            |
|            | NGBO 1582 | 10.67±0.62f                 | 35.75±1.38de               | 13.89±1.57d           |
| Wheat      | NGBO 1123 | 1.00±0.00a                  | 0.00± 0.00a                | 1.11±0.21a            |
|            | NGBO 1124 | 9.86±0.18f                  | 34.25±0.95cd               | 13.39±1.36cd          |
|            | NGBO 1224 | 5.21± 0.45cd                | 33.25±0.25bcd              | 7.50±1.03b            |
| Cowpea     | IFE-BROWN | 1.00±0.00a                  | 0.00±0.00a                 | 0.88±0.09a            |

Means within a column with the same letter(s) are not significantly different at  $p < 0.05$  using New Duncan's Multiple Range Test

Table 2b: Adult Emergence, Median Developmental Time of *Sitophilus zeamais* and weight loss in food hosts in a free-choice test

| Food hosts | Varieties | F <sub>1</sub><br>Emergence | Median<br>Development Time | Loss<br>in weight (%) |
|------------|-----------|-----------------------------|----------------------------|-----------------------|
| Maize      | DMR 9928  | 5.07±0.13de                 | 31.5±0.86bc                | 36.12±1.75de          |
|            | DMR 9943  | 5.58± 0.31e                 | 32.5± 0.86bcd              | 41.97±2.48ef          |
|            | TZPB SR   | 4.31±0.22c                  | 32.0±0.91bcd               | 49.28±1.29f           |
| Sorghum    | NGBO1354  | 4.56±0.42cd                 | 34.5±0.65d                 | 17.73±1.79b           |
|            | NGBO1469  | 4.52±0.16cd                 | 31.75±1.1bcd               | 28.25±1.34b           |
|            | NGBO1582  | 4.87±0.21cd                 | 31.75±0.94bcd              | 24.67±7.49cd          |
| Wheat      | NGBO1123  | 1.00±0.00a                  | 0.00± 0.00a                | 4.18±0.89a            |
|            | NGBO1124  | 3.67±0.09b                  | 33.75±0.95cd               | 17.82±2.60bc          |
|            | NGBO1224  | 3.55± 0.19b                 | 30.75±0.75b                | 20.57±1.88b           |
| Cowpea     | IFE-BROWN | 1.00±0.00a                  | 0.00±0.00a                 | 3.13±1.09a            |

Means within a column with the same letter(s) are not significantly different at  $p < 0.05$  using New Duncan's Multiple Range Test



Scales: 0–4.0 = resistant, 4.1–6.0 = moderately resistant, 6.1–8.0 = moderately susceptible, 8.1–10.0 = susceptible and  $\geq 10.1$  = highly susceptible (Dobie, 1974)

Fig. 1.: Susceptibility index of food host varieties in a no choice test

## DISCUSSION

Significantly high mortality of *S. zeamais* was recorded on Ife-Brown and on wheat variety, NGB0 1123, suggesting that *S. zeamais* must have been starved to death since the food host varieties could not supply the appropriate nutrients to the insect. Conversely, little or no mortality was recorded on all the maize host varieties and other wheat and sorghum hosts, agreeing with earlier reports that *Sitophilus zeamais* preferred some food varieties (especially maize) than others.

There was considerable variation in the F<sub>1</sub> progeny of *S. zeamais* across the food hosts. Significantly higher number of adults emerged from the three sorghum varieties than from the other food varieties ( $p < 0.05$ ) could be as a result of the small sizes of sorghum grains, which provided an increase in the number of grains available for oviposition. This finding is in accordance with earlier reports that oviposition and emergence of *S. zeamais* was density dependent (Richards, 1947; Pederson, 1979; Mathias *et al.*, 2015). In this study, *S. zeamais* completed its development on the food hosts between 33 - 39 days, on the choice and no-choice experiments. Similar observations have also been made by Ojo and Omoloye, (2016) where they reported that the comparative developmental cycle of *S. zeamais* from egg to adult was 34.7 days (on maize). Akinbuluma and Ewete (2019) also reported a median developmental time of 33.0 days of *S. zeamais* on DMR-ESY maize variety.

Losses in maize hosts were consistently higher than those in other food hosts and this might be due to the high survival rate of *S. zeamais* on the maize grains. Infestation of *S. zeamais* can cause weight loss of above 30% in stored maize (Paneru *et al.*, 1996; Sharma *et al.*, 2016). Similarly, a range of between 7 – 29% was observed to be lost to *S. zeamais* feeding on sorghum, agreeing with the reports of Patrick and McClure (2009) that stored sorghum incur losses of up to 20%. Suleiman *et al.* (2013) reported that *S. zeamais* is a serious insect pest of sorghum causing grain damage of up to 65.5% after four months of exposure to *S. zeamais* under laboratory conditions, while Goftishu and Belete (2014) also recorded grain damage of

about 30.0% after 2 months of *S. zeamais* infestation. The least percentage weight loss in Ife brown (cowpea) and NGBO 1123 (wheat) varieties might be due to the low survival of the insect pest on these food hosts. The range of susceptibility indices suggests that all the food host varieties used in this study were moderately resistant to *S. zeamais* apart from NGBO1123 (wheat) and Ife brown (cowpea) which were highly resistant (Dobie, 1974; Akinbuluma *et al.*, 2019). From this study, maize, sorghum and two wheat varieties NGBO 1124 and NGBO 1224 can serve as suitable hosts, while cowpea and wheat variety (NGBO1123) are the least suitable host of *S. zeamais* in relation to feeding and development.

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## WEED MANAGEMENT PRACTICES OF THE BAYINDIR DISTRICT OUTDOOR ORNAMENTAL PLANTS' PRODUCERS

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### ABSTRACT

Approximately 500 growers from the Bayındır District of Izmir Province, Türkiye share one quarter of outdoor ornamental plant production in Türkiye. Weeds at pots can significantly affect growth of ornamental plants and results with income loss. A face-to-face grower survey was carried out with 46 growers from Bayındır to find out weed problems and management practices of pot production of outdoor ornamentals. Furthermore, two nurseries were observed for their management practices in 2020 and 2021. Growers are mostly 30-50 years old range and 65% of enterprises are family farms approximately one hectare premises. More than half of the growers has over 10 years of experience in outdoor plants production. The highest produced species are *Cupressus macrocarpa*, *Cupressocyparis leylandii*, and *Viburnum tinus* out of ~30 species. Weed management and irrigation were considered as the fourth foremost problem following to production costs, selling prices, and marketing. Sprinkler irrigation is followed by drip irrigation and flood irrigation. One third of growers stated that weed control is a must while half of the growers it should be done if needed. Herbicide applications can be delivered two to 12 times in a season, but mostly five to seven times. Due to loss of herbicide efficiency, hand weeding and hoeing are followed by herbicide applications. Tarping on soil is done by ~20% of producers which helps the weed control. Tarping is considered mostly as an expensive method by farmers who do not apply. Glyphosate is the most common herbicide followed by pendimethalin. Experienced farmers thought weed control is done in all stages of production and showed tendency of using overdoses of herbicides comparing to less experienced ones. On the contrary, more less experienced farmers thought herbicides has lost their efficiency. Different weed species were recorded from observed enterprises and observations supported survey results. It is concluded that continues irrigation increases weeds that lead herbicide use often which ends up herbicide efficiency lost or herbicide resistance in weeds (that is not verified in this study) that causes use of overdoses and environmental pollution, increases costs and labors.

**Keywords:** Herbicide inefficiency, Glyphosate, Outdoor ornamentals, Survey, Tarping, Weed management

### INTRODUCTION

Changing life styles in aggregated and larger cities have caused increase in demand on ornamentals and pets (Uludag and Ertürk, 2012). The biggest share in ornamental plant acreages covered by outdoor ornamental production (Gülçür, 2015). Türkiye has been a growing market and export hub for ornamental plants, which triggers ornamental production as well. Outdoor ornamental production covers 3770 ha in Türkiye, which is  $\frac{3}{4}$  of total ornamental production area (TÜİK, 2020). Bayındır district of İzmir Province meets 22% of outdoor ornamental plants

demands of Türkiye on 300 ha open field and 80 ha covered production area by 500 producers (TÜİK, 2020; ITO, 2016; Çakar et al., 2019).

Outdoor ornamental market for nursery plants, particularly container-grown production highly demand weed-free plants (Case et al., 2005). The limited sources of the container environment and weed pressure are capable to inhibit the growth up to 60% (Fretz, 1972; Sidhu et al., 2020). Nevertheless, weed management is a tremendous problem not only because of weed competition but also the aesthetic concerns of the market (Derr, 1994; Steward et al., 2017). Therefore, weed control thresholds are literally zero for container-grown productions that are set by the end-user preferences (Creager, 1982; Steward et al., 2017). Weed and water management can be very challenging and costly for container ornamental production due to the limited supplies such as air, nutrients, water, and soil (Shen and Zheng, 2017). In a survey of a palm nursery in Bursa, Türkiye, *Cyperus rotundus* and *Portulaca oleraceae* during summer-autumn, and *Urtica dioica* and *U. urens* during winter-spring were identified as the most dense and difficult to control weeds (Inci and Uludag, 2017). Beyond the lack of postemergence herbicides registered for container-grown ornamental plants, any potential non-selective herbicide drift can cause an injury and reduce the prices due to the high-demands of ornamental market (Case et al., 2005; Khamare et al., 2022; Inci and Uludag, 2017). Similarly, the preemergence herbicides can significantly increase the growth length by months even with minor injuries (Khamare et al., 2022).

This study aimed to address current weed management methods, challenges, and trends with the growers' perspective using as a case outdoor ornamental growers views and implementations from Bayındır district.

## MATERIALS AND METHODS

This research was conducted at two outdoor ornamental nurseries (Table 1) at Bayındır District of Izmir, Türkiye during the 2022-2021 seasons. All production practices including weed control were recorded during the length of the research. Additionally, a face to face grower survey was carried out with 46 local ornamental producers.

Table 1. Outdoor ornamental plant nurseries' specifications at Bayındır, Izmir.

|           | Size (ha) | Time (years) | Plants   |
|-----------|-----------|--------------|--|
| Nursery 1 | 10        | 13           | <i>Phottinia fraseri</i><br><i>Viburnum tinus</i><br><i>Berberis thunbergia</i><br><i>Jasminum sambac</i><br><i>Ligustrum japonicum texanum</i><br><i>Cupressus macrocarpa</i><br><i>Cupressocyparis leylandii</i><br><i>Euonymus japonica</i> |
| Nursery 2 | 8         | 8            | <i>Buxus sempervirens</i><br><i>Cupressocyparis leylandii</i><br><i>Cupressus macrocarpa</i><br><i>Washingtonia filifera</i><br><i>Viburnum tinus</i>  |

Observations were carried out from September 15, 2020, to April 29, 2021, for two seasons. No pest control advisership was given to growers for consistency purposes during the

field visits. Soil maintenance, irrigation, fertilization, pest management including weed control practices were noted as well as interviews with growers if needed. Growing pots were hand-weeded at 15-20 days intervals due to high demands to weed-free pots for marketing. This phenomenon is almost a must for all pot-sale ornamentals. Weeds were observed separately with tarp covered and no tarp areas. Recorded weeds were identified based on the Flora of Turkey (Davis, 1965-1988) and accepted plant names were taken from the World Flora Online (WFO, 2022).. Plant saplings were irrigated every three days during the early vegetative growth stages. Yet, they are nearly irrigated every 20 days either with drip- or sprinkler-irrigation once reach to mature growth stages. Weeds were pulled ~15-20 days intervals from the growing pots.

Furthermore, 46 growers were interviewed with a survey to correspondence ~10% of the local growers. Grower survey included 20 questions about the demography, production expenses, challenges, and pest problems. Grower responses were subjected to statistical analysis with SPSS software and results were interpreted.

## RESULTS AND DISCUSSION

### Nursery observations

Weeds from 18 families recorded from both nurseries between November 2, 2020, to November 6, 2020, and March 12, 2021, to April 29, 2021.

Table 2. Observed weeds from outdoor ornamental plant nurseries in 2020.

|           | <b>Plants</b>  |
|-----------|--|
| Nursery 1 | <i>Althernanthera caracasana</i><br><i>Amaranthus viridis</i><br><i>Cynodon dactylon</i><br><i>Cyperus fuscus</i><br><i>Cyperus rotundus</i><br><i>Digitaria sanguinalis</i><br><i>Eleusine indica</i><br><i>Erigeron sumatrensis</i><br><i>Heliotropium europaeum</i><br><i>Plantago major</i><br><i>Setaria verticillate</i>                   |
| Nursery 2 | <i>Amaranthus retroflexus</i><br><i>Amaranthus viridis</i><br><i>Cyperus rotundus</i><br><i>Echinochola crus</i><br><i>Eclipta sp.</i><br><i>Eleusine indica</i><br><i>Euphorbia nutans</i><br><i>Parietaria officinalis</i><br><i>Plantago major</i><br><i>Portulaca oleraceae</i><br><i>Sonchus oleraceus</i><br><i>Tripodion tetraphyllum</i> |

Table 3. Observed weeds from outdoor ornamental plant nurseries in 2021.

|           | <b>Plants</b>  |
|-----------|--|
| Nursery 1 | <i>Amaranthus viridis</i><br><i>Avena fatua</i><br><i>Chenopodium album</i><br><i>Convolvulus arvensis</i><br><i>Cyperus rotundus</i><br><i>Diploaxis erucoides</i><br><i>Equisetum variegatum</i><br><i>Euphorbia lathyris</i><br><i>Lysimachia arvensis</i><br><i>Malva sylvestris</i><br><i>Matricaria chamomilla</i><br><i>Melilotus officinalis</i><br><i>Papaver rhoeas</i><br><i>Phalaris minor</i><br><i>Poa annua</i><br><i>Polypogon monspeliensis</i><br><i>Ranunculus repens</i><br><i>Rumex crispus</i><br><i>Vebaricum thapsus</i> |
| Nursery 2 | <i>Bromus tectorum</i><br><i>Chenopodium album</i><br><i>Cynodon dactylon</i><br><i>Cyperus rotundus</i><br><i>Equisetum variegatum</i><br><i>Malva sylvestris</i><br><i>Melilotus officinalis</i><br><i>Poa annua</i><br><i>Polypogon monspeliensis</i>   |

In two fields 31 weed species were identified, which many similar plants with Sakarya Province ornamental plant production areas that have 92 weed species (Küçük et al., 2020).

### **Grower surveys**

More than half of the farmers between 30 and 50 years old (Figure 1). Education level is mostly limited with compulsory education (70%) which was elementary before and now middle school (Figure 2). In another study in Bayındır, average age was 43.2 and education duration was 8.1 years, which is in agreement with our study (Çakar et al., 2019). Two third of growers described themselves farmers, the remaining mentioned other professions as their main jobs.

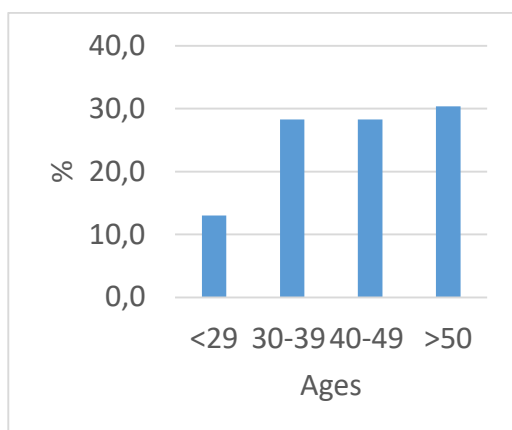


Figure 1. Ages of Producers

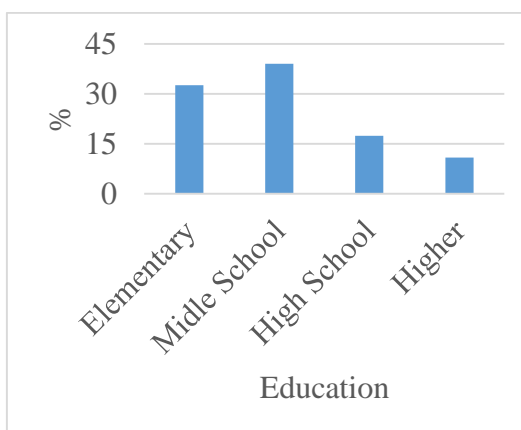


Figure 2. Education of Producers

Growers' experience on agriculture is mainly over 10 years including over 20 years but on ornamental plants is mainly less than 20 years with huge amount of less than nine years (Figure 3).

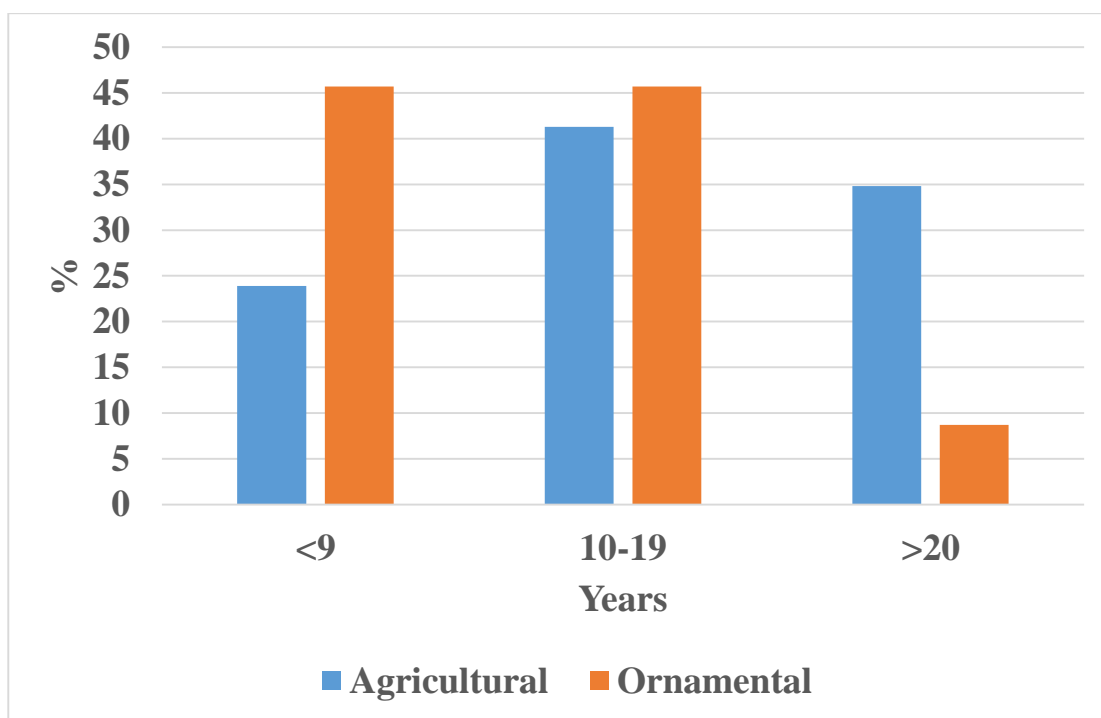


Figure 3. Growers' experiences on agriculture and ornamental plant producing

Outdoor ornamental production nurseries are mostly around 0.7-1.2 ha (52.2%) and half of the remaining owned less than 0.7 ha and the other half has over 1.2 ha. Farms were mostly family farms without any labor from outside. The applications were mostly were not depended on demographic information such as age, education, or size of field if it is mentioned separately in the text.

The production share of the most produced species was recorded as *Cupressus macrocarpa* with 10.37%; *Cupressocyparis leylandii* with 9.76%; *Viburnum tinus* with 6.1%; *Berberis thunbergii*, *Washingtonia filifera*, *Neirum oleander*, and *Pittosporum tobirana nana* with 4.8%; *Buxus sempervirens*, *Laurus nobilis*, and *Cupressus arizonica* with 4.27%;

*Euonymus japonicus* with 3.6%; *Phottinia fraseri Redrobin*, *Jasminum sambac*, and *Bougainvillea spectabilis* with 3.05%; *Euonymus japonica aurea*, *Thuja plicata*, and *Hydrangea macrophylla* with 2.44%.

Challenges in the first place were stated as cost with 28.3%, sales prices with 19.6%, marketing with 17.4%, irrigation with 13%, weed management with 13%, and labor with 8.7% (Table 4). Most growers mentioned more than one problem and stated them most important to less important (upto 4<sup>th</sup> rank). Growing costs are main challenge for all farmers in varying importance (rank). Economical problems took the first three place followed by two agronomic problems: irrigation and weeds.

Table 4. Problems according to importance level

| Problems           | Score (%) according to rank |      |      |     |
|--------------------|-----------------------------|------|------|-----|
|                    | 1st                         | 2nd  | 3rd  | 4th |
| Costs              | 28.3                        | 32.6 | 17.4 | 4.3 |
| Marketing          | 17.4                        | 28.3 | 15.2 |     |
| Price              | 19,6                        | 6.5  | 2.2  |     |
| Irrigation         | 13.0                        | 8.7  | 6.5  | 6.5 |
| Weeds              | 13.0                        | 6.5  | 4.3  |     |
| Labor              | 8.7                         | 2.2  | 2.2  |     |
| Management         |                             | 2.2  |      |     |
| Support            |                             | 2.2  | 6.5  |     |
| Prunning           |                             |      | 2.2  |     |
| Pests and Diseases |                             |      | 2.2  |     |

Necessity of weed management was depended on growers' experience (Table 5). Two third of less experienced growers responded as "when needed". The most experienced farmers divided to two groups between "always necessary" and when needed". The growers with 10-19 years of experience showed high fluctuation which could be arose from their self confidence that lets them experience new personal ideas.

Table 5. Necessity of weed management

| Experience with ornamentals<br>(Years) | Weed Management     |                |                  |            | Total |
|--|---------------------|----------------|------------------|------------|-------|
|  | Always<br>Necessary | When<br>Needed | Not<br>Necessary | No<br>Idea |       |
| <9                                     | 19.0                | 66.7           | 4.8              | 9.5        | 100   |
| 10-19                                  | 38.1                | 28.6           | 19.0             | 14.3       | 100   |
| 20>                                    | 50.0                | 50.0           | 0,00             | 0.0        | 100   |
| Total                                  | 30.4                | 47.8           | 10.9             | 10.9       | 100   |

Weed control methods are important because of cost, timing, and environment. More than half of the growers heavily rely on herbicides in their intensive weed management programs (Table 6) with around five to seven applications during the season. Likewise, 30.4% of the growers said two to four applications whereas 15.2% of them apply herbicides eight to twelve times. Application times were related to following herbicide labels. Recommended rate and lesser rate applying growers tend to repeat herbicide application less. Furthermore, 41.3% carefully follow the label, 32.6 use higher rates than the label, and 21.7% of them declared to use very higher rates whereas only 4.3% of them stated to use less rates than the herbicide labels. The growing season herbicide applications are 54.3% in March, 37% in April, and 8.7% in June. Glyphosate is the most common herbicide followed by pendimethalin. Pendimethalin and oxyfluorfen also have recommended in some ornamental plant experiments (Kaçan et al., 2018).

Table 6. Herbicides used

| Active Ingredients     | Usage (%) | Producers (%) |
|------------------------|-----------|---------------|
| Glyphosate             | 69,3      | 108.8         |
| Pendimethalin          | 15,3      | 23.9          |
| Oxyfluorfen            | 7.0       | 10.9          |
| Clethodim              | 4,2       | 6.5           |
| Metolachlor +Benoxacor | 1,4       | 2,2           |
| Fluazifop-p-butyl      | 1,4       | 2,2           |
| Quizalofop-p-ethyl     | 1,4       | 2.2           |

Growers also declared that 32.6% prefer the same commercial herbicide, 30.4% prefer the cheapest option, 23.9% follow the pesticide dealers' recommendations, and 6.5% rely on either a pest control adviser or a colleague (Table 7). But it was related to education as well. Primary and secondary school graduates mentioned "my brand" mainly but high school and higher educated growers preferred inexpensive once.

Table 7. Herbicide choice methods

| Education   | Herbicide Choice |          |                         |                  |                          |
|-------------|------------------|----------|-------------------------|------------------|--------------------------|
|             | Inexpensive      | My Brand | Recommended by a Fellow | Pesticide Dealer | Agriculture Organization |
| Primary     | 26,70            | 46,70    | 0,00                    | 20,00            | 6,70                     |
| Secondary   | 22,20            | 33,30    | 5,60                    | 33,30            | 5,60                     |
| High School | 50,00            | 25,00    | 12,50                   | 12,50            | 0,00                     |
| Higher      | 40,00            | 0,00     | 20,00                   | 20,00            | 20,00                    |
| Total       | 30,40            | 32,60    | 6,50                    | 23,90            | 6,50                     |

Producers used more than one method for weed control in addition to herbicides (Table 8). Uprooting by hand was the most popular non-chemical method which was applied 84.8% of producers. Mulching, tillage and solarization were not popular methods. In earlier studies in ornamental plant production, solarization was not effective enough although tree saplings was benefited (Kaçan et al., 2018; Kavgacı ve ark, 2019).

Tarping the soil surface was around 20% among the growers, ~65% of the others stated that they do not prefer traps due to high costs. Additionally, 11.8% of them do not believe tarping can be a solution, 11.7% of them stated tarping is not suitable for ornamentals, and 8.8% of them told tarping requires more labor.

Table 8. Non-chemical weed management methods applied

| Methods      | The Number of application | Usege (%) | Producers (%) |
|--------------|---------------------------|-----------|---------------|
| Uprooting    | 39                        | 40,2      | 84,8          |
| Hoeing       | 27                        | 27,8      | 58,7          |
| Cutting      | 17                        | 17,5      | 37,0          |
| Mulching     | 8                         | 8,2       | 17,4          |
| Tillage      | 5                         | 5,2       | 10,9          |
| Solarization | 1                         | 1,0       | 2,2           |
| Total        | 97                        | 100       |               |



Vast amount of growers (78.3%) mentioned efficiency decrease of herbicides. Their main methods for controlling weeds under efficiency loss were hoeing, uprooting, fertilizer adding to herbicide and increasing herbicide rates. The method applied was related to experience (Table 9).

Table 9. Methods employed to increase herbicide efficiency

| Experience (year) | Measures to increase control |                 |                   |           |       |
|-------------------|------------------------------|-----------------|-------------------|-----------|-------|
|                   | Hoeing                       | Increasing dose | Mixing fertilizer | Uprooting | Other |
| <9                | 22,20                        | 33,30           | 0,00              | 22,20     | 22,20 |
| 10-19             | 33,30                        | 26,70           | 6,70              | 26,70     | 6,70  |
| 20-29             | 11,10                        | 11,10           | 44,40             | 33,30     | 0,00  |
| 30>               | 33,3                         | 33,3            | 0                 | 33,3      | 0     |
| Total             | 25,00                        | 25,00           | 13,90             | 27,80     | 8,30  |

## CONCLUSIONS

Weeds are more problematic and difficult to control in container-grown ornamentals than woody ornamentals (Fretz, 1972). Weed suppression of container-grown ornamentals vary among weeds, ornamentals, and environmental conditions (Gallitano and Skroch, 1993). The competition among those plants can enormously decrease the growth of ornamental plants or even inhibits the growth (Berchielli-Robertson et al., 1990). Due to the high possibility of off-target negative impacts of herbicides, non-chemical weed management is the most realistic solution in container-grown ornamentals. Even the granular herbicides could cause severe injury via runoff water following applications (Keese et al., 1994). Container-grown ornamental growers' practices are supporting this argument in our survey results. Nearly half of the growers stated that the weed management is essential in nurseries with container production.

It is apparent that weed control is getting more difficult due to getting less herbicide choices and difficulties in labor market. In addition, green solution has become important part of recent policies worldwide, which studies related to alternative techniques required for weed control.

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## **SIMULATION STUDY OF LECITHIN BASED NANOLIPOSOME ENCAPSULATING COENZYME Q10**

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### **ABSTRACT**

The liposomal materials has been under study for a long time and has many advantages. The superior properties are their non-toxicity and its success in transporting sensitive drugs. In this study, we created a system with three liposome concentrations to observe the best encapsulation properties for transporting drugs. Coenzyme Q10 (CoQ10) is encapsulated within the liposome, and their interactions with water have been studied by performing Dispersive Particle Dynamics (DPD) technique, which is a coarse-grained (CG) simulation method. Moreover, various analyzes such as Voronoi analysis, radial distribution function, density profile and encapsulation efficiency were performed. With these analyses, the encapsulation quality was computed and it was observed that the critical micelle density could not be reached between certain values.

### **INTRODUCTION**

Nanotechnology studies cover particles of 10-100 nm in size [1]. With the contribution of nanotechnology, drug-delivery systems such as nanoliposomes have been developed. Nanoliposomes are preferred because they successfully deliver low drug concentrations to the targeted sites. Mathematical models of these systems provide the advantage of studying physicochemical and biophysical processes [2]. In this study, the liposome was created from a compound called lecithin. Lecithin is also known as phosphatidicolin. Phosphatidylcholines belong to the phospholipid class. It has a hydrophilic head (polar) group and a hydrophobic tail (apolar) group. If we look at the formation of liposomes from phospholipid molecules, adding water to the molecule increases the hydration of the outer layer and expands the polar groups. With this expansion, water begins to fill in, and this situation creates multilayer [3]. The generated bi-structural liposome was also migrated to CoQ10. CoQ10, a fat-soluble vitamin-like substance, has recently been used as a dietary supplement [4]. In this study, liposome was formed at three different concentrations. CoQ10 was kept constant. At one concentration, the micelle formation threshold was below, and a complete liposome was not seen. The average volume of CoQ10 that it could encapsulate was calculated by calculating the average volumes of the formed liposomes.

### **MATERIALS**

The chemical structure and coarse-graining procedure are schematically shown in Fig.1.

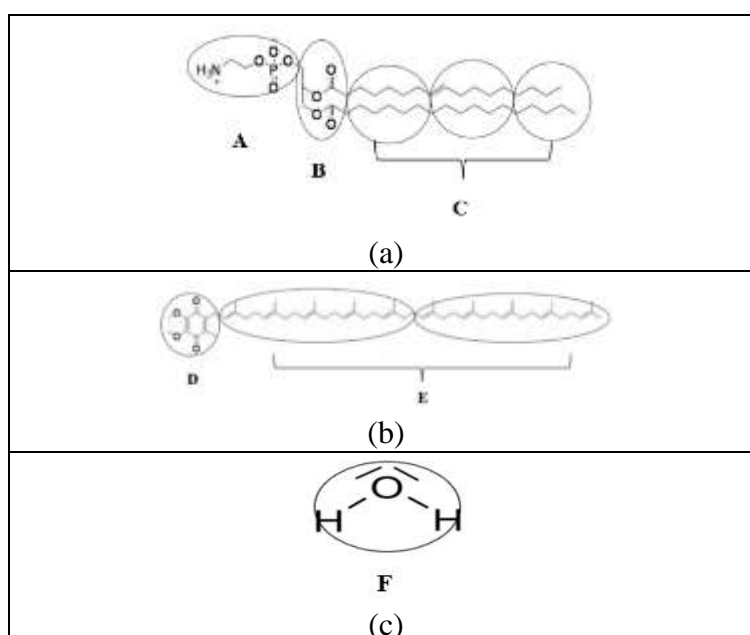


Figure 10: Schematic representation of the coarse-graining and the beads of (a) CoQ10, (b) Lecithin, (c) solvent (water).

The coarse-grained structures were selected as similar in volume. The lipid in the form of lecithin consists of five coarse-grained beads, CoQ10 includes three coarse-grained beads, and the water molecule consists of one coarse-grained bead.

Table 1: Interactions of DPD parameters used in the system [5].

| $a_{ij}$ | A     | B     | C      | D     | E      | W      |
|----------|-------|-------|--------|-------|--------|--------|
| A        | 78.33 | 80.25 | 95.21  | 78.34 | 85.72  | 89.25  |
| B        | 80.25 | 78.33 | 85.85  | 79.51 | 81.48  | 92.79  |
| C        | 95.21 | 85.85 | 78.33  | 89.85 | 78.34  | 101.21 |
| D        | 78.34 | 79.51 | 89.85  | 78.33 | 85.35  | 89.49  |
| E        | 85.72 | 81.48 | 78.34  | 85.35 | 78.33  | 100.83 |
| W        | 89.25 | 92.79 | 101.21 | 89.49 | 100.83 | 78.33  |

## METHODS

Coarse-grained DPD simulations method were used in the study. The initial structure was created with MAPS 4.0, and simulations were performed with the coarse-graining method in a computer environment. The simulation was performed in the NVE conditions.

## RESULTS

As a result of coarse-grained simulations, liposome formation was observed in all three concentration levels. As expected, water enters into the polar part of the formed liposome. CoQ10 was seen in the apolar part of the liposome, and 100% efficiency in encapsulation was observed in two concentration levels (%9, %13). In all three concentration levels, the Voronoi analysis results were somewhat similar apart from a lower profile for the 13% system (see Figure 2). The coordination numbers also follow a similar trend apart from the 9% system.

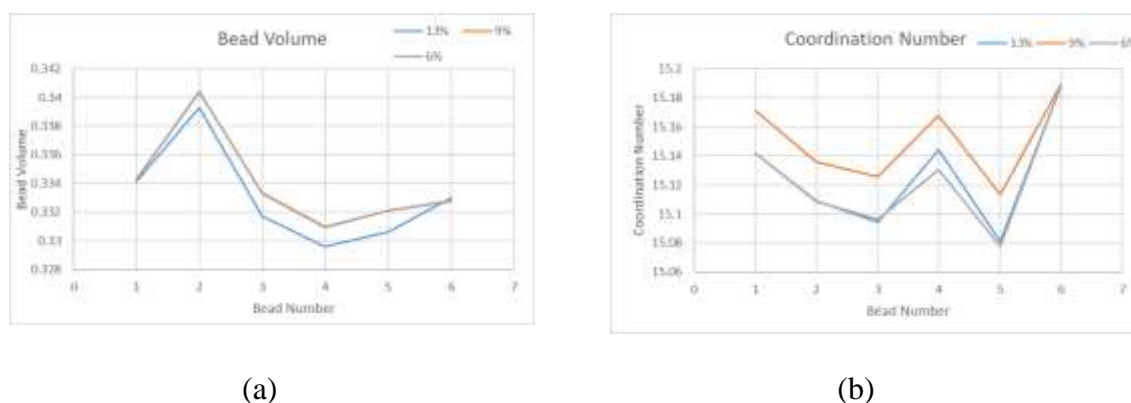


Figure 2: (a) The average coordination number of beads in the system, (b) The volume of each bead is shown in the sum of the system for all systems.

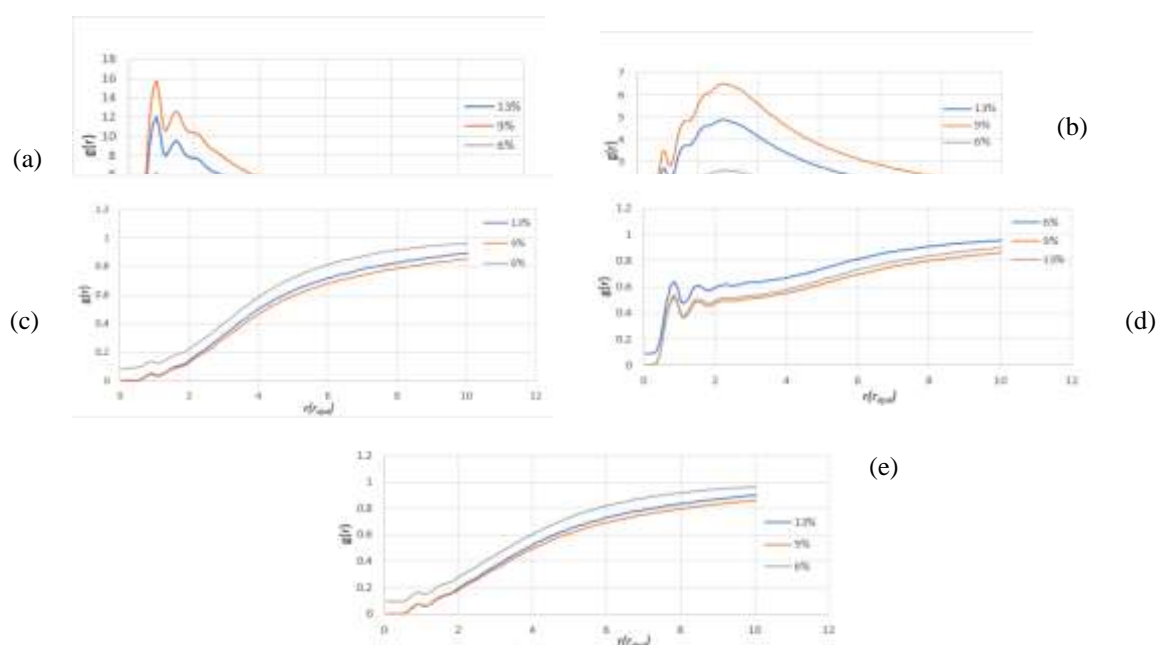


Figure 3: Interactions between hydrophilic, hydrophobic and CoQ10 are shown by RDF plots. (a) RDF of hydrophobic-CoQ10 interactions, (b) RDF plot of hydrophilic-CoQ10 interactions, (c) RDF plot of hydrophobic-Water interactions, (d) RDF plot of hydrophilic-water interactions, (e) RDF plot of CoQ10-Water interactions.

Radial Distribution Function RDF plots of hydrophobic bead-CoQ10 interactions, hydrophilic-CoQ10 interactions, CoQ10-water interactions, hydrophilic-water interactions, and hydrophobic-water interactions for different concentrations of liposomes are shown in Figure 2. CoQ10, which is a hydrophobic molecule, was located in the hydrophobic part of lecithin liposomes. In Figure 3, we depict the simulation snapshots of the liposomes. Proper liposomal structures are formed as a result of the DPD simulations. For the 6% system, we also notice a bilayer structure apart from the spherical liposomes. As can be seen from the simulation snapshots, all CoQ10 molecules are encapsulated in the hydrophobic (yellow colored) part of the liposomes. Therefore, we estimate a 100% encapsulation efficiency for all the systems.

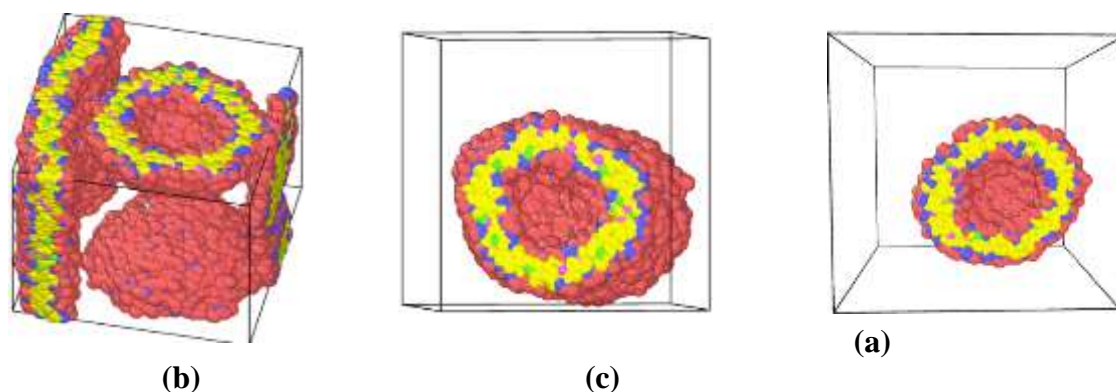


Figure 3: Simulation snapshots of (a) 6% mass fraction, (b) 9% mass fraction, (c) 13% mass fraction.

## CONCLUSIONS

In this work, the encapsulation efficiencies are computed for CoQ10 encapsulated lecithin-based liposomes at different concentrations. In all systems, spherical liposomes are obtained with all CoQ10 molecules are encapsulated within the hydrophobic core. Interactions such as hydrophilic-hydrophobic, hydrophilic-CoQ10, hydrophobic-CoQ10, and water-CoQ10 were investigated by means of RDF plots of attractive interactions between hydrophilic/hydrophobic beads and CoQ10/water. Since CoQ10 is hydrophobic, RDF plots regarding the CoQ10 and hydrophobic pairs show a dominant attraction. On the other hand, CoQ10 shows limited affinity to water due to the existence of repulsive forces. Our work can be seen as an attempt to model a particular liposomal drug-carrier encapsulating hydrophobic CoQ10 molecules. The results are expected to highlight the molecular structure and interactions for this specific system.

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## DISSIPATIVE PARTICLE DYNAMICS SIMULATIONS OF MRNA DELIVERY WITH IONIZABLE CATIONIC LIPID DOTAP

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### ABSTRACT

mRNA is a single-stranded molecule. It is synthesized by transcription and carried to the ribosome for translation. mRNA, which is necessary for protein synthesis, is used for the treatment of many diseases. The transportation of this sensitive molecule into the cell is a problem. Lipid nanoparticles can be used as a solution for this. Since lipids are also present in the cell membrane, they are biosafe as well as increase transfection. Recent studies have shown that ionizable lipids can adapt to changing ambient pH. Because of these properties, they stabilize and transfection of nucleic acids better than other lipids. It aims to simulate a nanostructure formed with ionizable cationic lipids, with the coarse-grained DPD method to examine the intermolecular and intramolecular interactions in detail.

### INTRODUCTION

Prokaryotic and eukaryotic cells all synthesize proteins. The synthesized protein participates in the structure of many molecules, especially enzymes, hormones, cell membranes, and signal molecules. Protein synthesis begins in the nucleus. Pre-mRNA synthesized from DNA by transcription contains exon and intron regions. A cap is added to the 5' end of the pre-mRNA. The poly-A tail is added to the 3' end of the mRNA. By splicing, intron regions that do not encode are discarded. Mature mRNA is obtained. It passes into the cytoplasm for the translation process. The ribosome located in the cytoplasm reads the mature mRNA. As a result of translation, a polypeptide chain is synthesized. The polypeptide chain becomes functional through post-translational processes. The mRNA carries the codes for the synthesis of the protein that the cell needs. It obtains these codes by synthesizing them from the genes in DNA. In some cases, the DNA does not have the genes to code for the protein needed. In cases where DNA does not carry the necessary synthesis genes, there are two solutions. One of the two solutions is adding the necessary genes to DNA. The other way is to insert the mRNA, which carries the codes that will enable the synthesis of the necessary protein, into the cell. Adding genes to DNA is riskier in terms of possible mutations compared to the other way. mRNA transport into the cell is safer. The mRNA cannot pass into the nucleus, so the transported information is degraded in the cytoplasm after reading it in the ribosome. The transport of sensitive mRNA into the cell by crossing biological barriers is a problem. Today, many technologies are used to increase transfection. Lipid nanostructures are an option for the transport of sensitive mRNA [1].

Lipids are found in the cell membrane as well. Due to these features, they ensure the safe transport of the molecule they carry, especially nucleic acids, to target organs and tissues. Both hydrophilic and hydrophobic active substances can be encapsulated with high efficiency with lipid nanoparticles. It can increase bioavailability because the liposome can be coated with inert and biocompatible polymers that prolong the circulatory half-life in the body. They can

be functionalized with specific ligands so that they can target specific cells, tissues and organs [2]. Lipid-based carriers protect mRNA from RNase. They are nanostructures consisting of lipid-based surfactants and have an average diameter of 10 to 100 nm. Lipids are amphiphilic molecules. It has head and tail parts. Head parts show polar (hydrophilic) properties. The tail parts show apolar (hydrophobic) properties. In water, lipids tend to clump together so that a liposome is formed. Because water is polar, it interacts repulsively with nonpolar tails. It interacts attractively with the fleece head. Hydrophobic interactions between lipid and water molecules and van der Waals interactions between lipid molecules lead to the formation of a double-layered liposome structure.

Especially with pandemic vaccines, lipids that can be ionized draw attention [3]. Ionizable lipids are positively charged at low pH. Thus, it stabilizes negatively charged nucleic acids better than other lipids. The pH of the blood is normally between 7.35 and 7.45. The ionizable lipids become neutral when they are introduced into the blood. With these properties, they do not stimulate the immune system and increase transfection. With ionized lipids, both the stability of the genetic material is ensured and the entry into the target cell is facilitated.

In this work, we are interested in DOTAP (Dioleoyl-3-trimethylammonium propane) cationic lipids that are ionizable as mRNA carriers. We choose DOTAP because it is used in the formulation of drugs approved for gene therapy. We use the dispersive particle dynamics (DPD) method in the computer environment as a coarse-grained (CG) simulation [4]. It is a useful method for simulating polymers, copolymer systems, polymer blends, and large systems on a mesoscopic scale. In this method, the particles are coarse-grained in the liquid elements. These are called DPD particles or beads. Beads are defined according to their chemical properties.

The schematization of our Coarse-graining model is in Fig. 1.

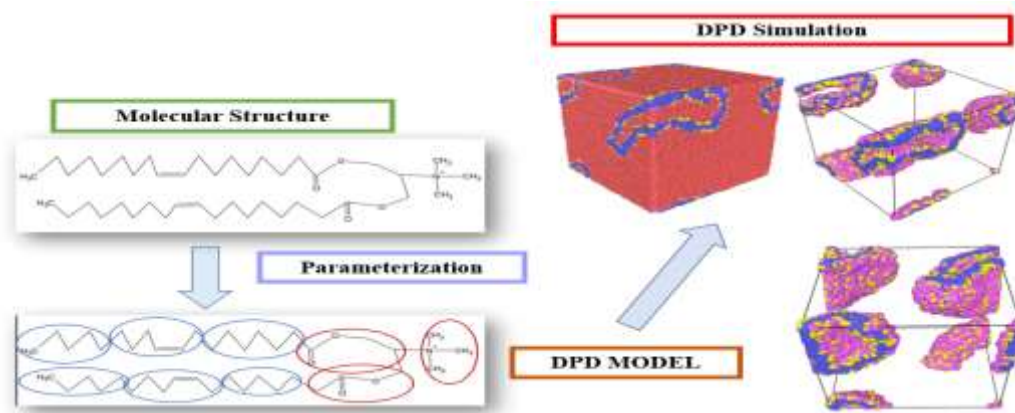


Figure 1: We schematize the DPD method, starting with the coarse-graining of the lipid.

This work aims to simulate DOTAP and mRNA interaction with the DPD method. We obtained liposomes by gradually reducing the interaction parameters between water and hydrophilic groups. Our study will provide us with more information about the mechanisms of liposome formation of ionizable lipids.

## MATERIALS

DOTAP (1,2-dioleoyl-3-trimethyl ammonium-propane) is one of the most widely used lipids for nucleic acid transport. That's why we chose it as our material.

Coarse-grained structure of DOTAP (Dioleoyl-3trimethylammonium propane) in Fig. 2.



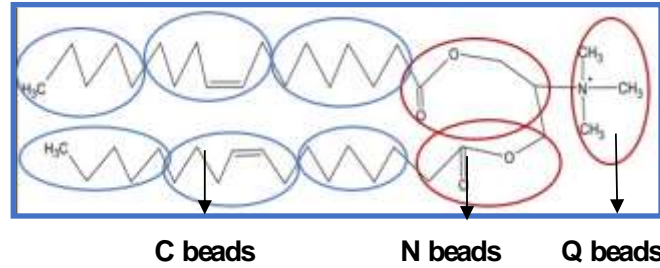


Figure 2: We show the coarse-graining of our lipid DOTAP. The Q bead is polar and represents the head portion of the lipid. The N beads act as hinges between the tails and the head of the lipid. The N beads are polar. C beads represent groups of atoms in the tail. C bead is nonpolar.

We divided DOTAP into 3 different bead types, in total 9 beads. Red beads are polar (hydrophilic). Our Q and N beads are polar. Blue beads are apolar (hydrophobic). We accepted the two tails, which are made up entirely of C atoms, as a single bead type and divided them into a total of 6 beads.

## METHODS

The simulation method we employed in our study is coarse-grained DPD. Dissipative Particle Dynamics (DPD) is a simulation technique for simulating polymers, lipids, biopolymers, and many other complex systems. In DPD, molecules are separated into beads. The idea of coarse-graining is dividing the molecules into molecular sub-units, which are called coarse-grained beads [5]. Every DPD bead has a specific character and every bead have a like feature. Coarse-grained (CG) is a method for reaching long-time scales in simulations of biomolecular simulation systems.

In the DPD system, the force acting on a bead is calculated as the sum of the protective ( $F_{ij}^C$ ), dispersive ( $F_{ij}^D$ ) and random ( $F_{ij}^R$ ) forces. The conservative force is responsible for the end structure, and the dispersive and random forces are responsible for controlling the temperature of the system as they act as a thermostat. The interaction parameters of the beads are adjusted by adapting the Flory-Huggins parameters  $\chi_{ij}$  to the DPD interactions. The obtained interaction parameters allow us to define the attractive and repulsive interactions of the determined beads with each other. The repulsion parameter for cross-interactions is calculated as in the equation using the Flory-Huggins interaction parameter  $a_{ij}$ .

$$\frac{dr_i}{dt} = v_{i,m} \frac{dv_i}{dt} = f_i$$

$$f_i = \sum_{j \neq i} (F_{ij}^C + F_{ij}^D + F_{ij}^R)$$

$$F_{ij}^C = \begin{cases} a_{ij} (1 - r_{ij}) \hat{r}_{ij} & (r_{ij} < 1) \\ 0 & (r_{ij} \geq 1) \end{cases}$$

We determined it based on reference examining the interaction of water and hydrophilic groups at the mesoscopic scale [6,7]. We re-determined the interaction parameters according to our ionizable lipid.

*Table 1: The table shows the interaction parameters we used for each bead type.*

| $a_{ij}$ | W  | C   | N    | Q    |
|----------|----|-----|------|------|
| W        | 78 | 104 | 79.3 | 79.3 |
| C        |    | 78  | 86.7 | 104  |
| N        |    |     | 78   | 79.3 |
| Q        |    |     |      | 78   |

W bead stands for water. The Q bead represents the group containing the nitrogen atom in the head of DOTAP. The N bead is our bead that contains both oxygen atoms. The N, W and Q beads are polar. Interaction parameters are smaller because they show attractive interaction. All of our C beads are beads containing the carbon atom in the tail. Due to their nonpolar (hydrophobic) properties, they need to apply repulsive forces to each other with polar beads. For this reason, higher interaction parameter values were given.

## RESULTS

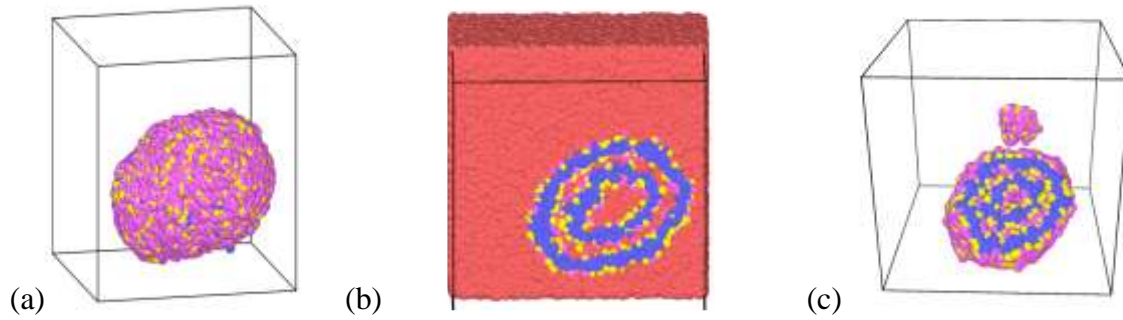
We tried different angles and interaction parameters to understand the liposome formation rules of ionized lipids. We reduced the interaction parameters (W-N, W-Q) between the water and polar groups. Thus, the water and polar groups were less repulsive to each other. We also examined the effect of the angle between the tails on the formation of liposomes by the bi-tailed lipid. We have obtained liposome structures with many different combinations.

We performed our simulations, where we took the box size as  $50 \times 50 \times 50 \text{ r}_{\text{DPD}}^3$  at 10% (mol.%) concentration. We performed many simulations by changing the interaction parameter between water and hydrophilic groups. Red colours represent water in the simulation. The yellow colour represents our N beads and the pink colour represents our Q beads. Purple represents our C beads.

When we take the interaction parameter between water-hydrophilic groups as  $74.3 k_B T$  and the angle between the tails (formed between N-C groups) as 30 degrees, we obtained the liposome structure.

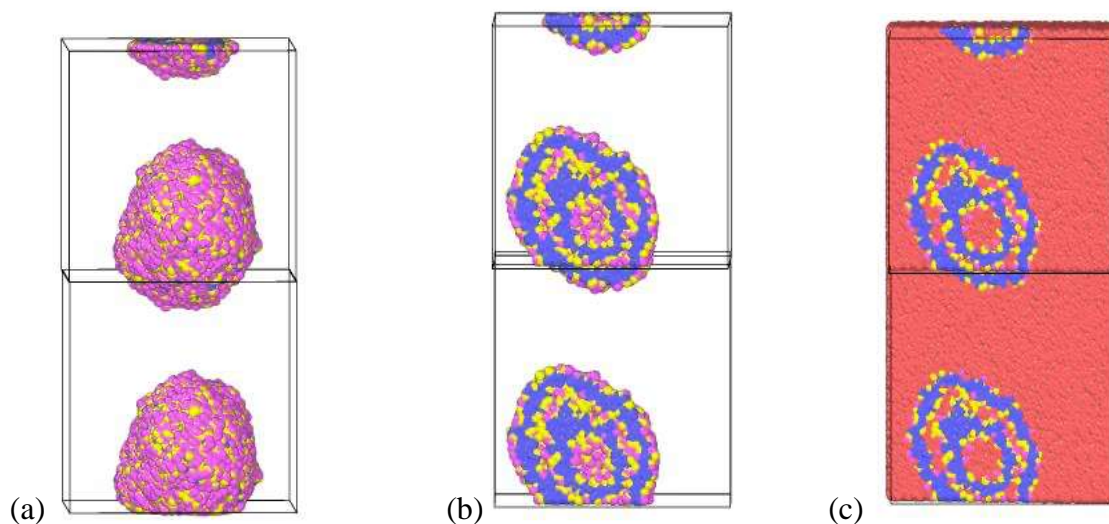
*Table 2: We show the interaction parameters from which we obtained the liposome. We reduced the interaction parameters between the W-N, and W-Q groups we used at the beginning. Thus, we have reduced the repulsive force between these groups.*

| $a_{ij}$ | W  | C   | N    | Q    |
|----------|----|-----|------|------|
| W        | 78 | 104 | 74.3 | 74.3 |
| C        |    | 78  | 86.7 | 104  |
| N        |    |     | 78   | 79.3 |
| Q        |    |     |      | 78   |



*Figure 3: We showed 3 different snapshots in 14 million DPD steps. (a) It shows the liposome in the middle of the simulation box. (b) A view from the middle relative to the y-axis of the liposome in water. It is seen that the double-layered liposome can trap water in each layer. (c) A view from the middle of the liposome. The liposome is multi-layered. The water beads have been removed for the clarity of the image.*

We kept the interaction parameters between the water and polar groups constant at  $74.3 k_B T$  and increased the angle between the N-C beads to 60 degrees.



*Figure 4: 3 different snapshots in 3 million DPD steps. We combined the same simulation box with the same box angle from the top and bottom so that the resulting structure can be seen exactly. (a) The structure of the formed liposome is seen. We've removed the water so that its structure can be viewed. (b) It is an image taken right in the middle of the resulting liposome. In the image, it is seen that the formed liposome is multi-layer. (c) A view from the middle of the liposome with water in the medium. It appears to be able to trap water in each layer of the liposome.*

We wanted to understand the liposome structure as the interaction of lipids between water and polar groups diminished. Therefore, we have taken the interaction parameter between polar groups of water and lipid as  $73.3 k_B T$ . In this interaction parameter, the best liposome structure was obtained in our simulation where we set the angle between N-C beads to be 60 degrees.

Table 3: The interaction parameters of the beads are given in the table. The table shows the 6 kT reduction of the interaction parameters of the polar groups of water and lipid from the values used at the beginning. With this reduction, less repulsive interactions occur between the water and polar groups.

| $a_{ij}$ | W  | C   | N    | Q    |
|----------|----|-----|------|------|
| W        | 78 | 104 | 73.3 | 73.3 |
| C        |    | 78  | 86.7 | 104  |
| N        |    |     | 78   | 79.3 |
| Q        |    |     |      | 78   |

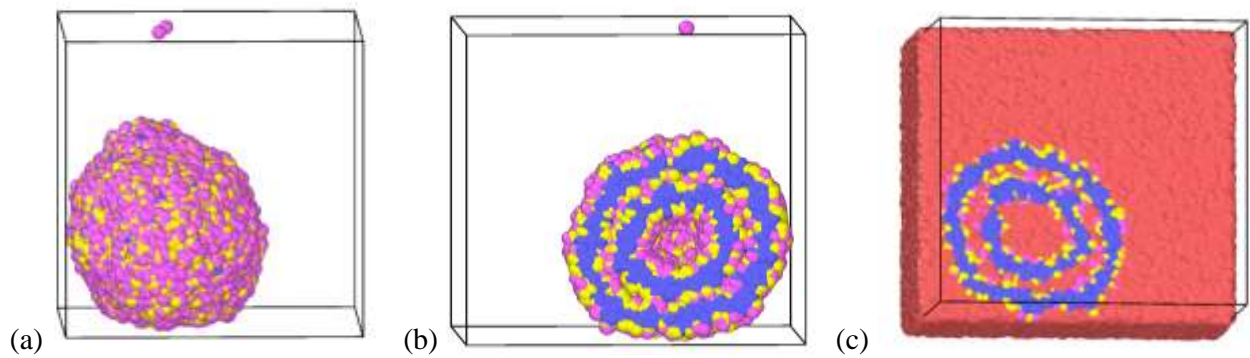
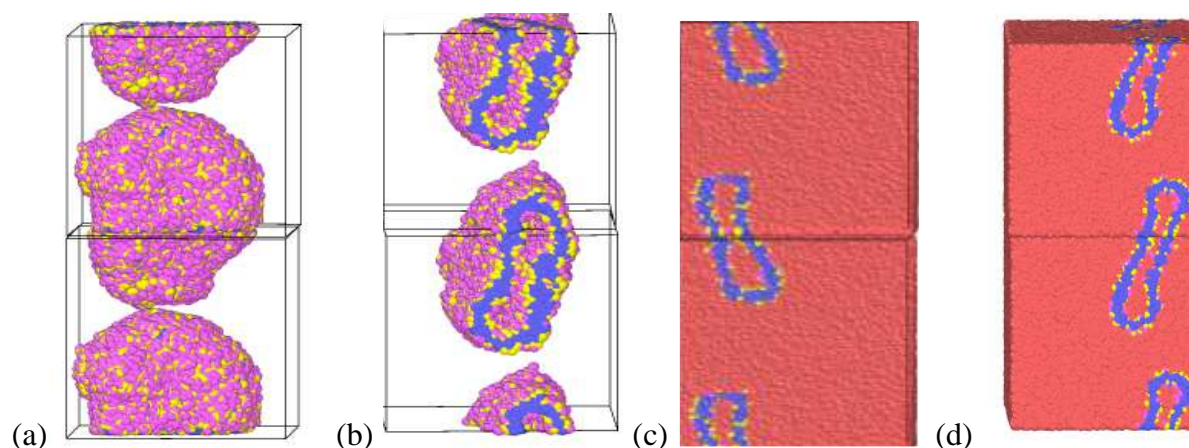


Figure 5: 3 different snapshots in 3 million DPD steps. (a) The interaction parameters are given in the table and the image of the liposome was obtained with an angle of 60 degrees. The water beads in the medium were removed to see the liposome. (b) The middle of the liposome is seen. The cavities formed in the interior can be easily seen. The water molecules in the environment have been removed. (c) In this image, which presents a section through the middle of the liposome, the water beads have not been erased. Thus, water between the layers of the liposome was visualized.

From the simulation results, we also obtained the single-layer liposome structure. We have taken the interaction parameter between the polar groups of water and lipid as 72.3 k<sub>B</sub>T. We have taken the angle between the tail beads and the N beads of the lipid 30 degrees.

Table 4: The interaction parameters between lipid and water beads are given in the table.

| $a_{ij}$ | W  | C   | N    | Q    |
|----------|----|-----|------|------|
| W        | 78 | 104 | 72.3 | 72.3 |
| C        |    | 78  | 86.7 | 104  |
| N        |    |     | 78   | 79.3 |
| Q        |    |     |      | 78   |



*Figure 6: 5 different perspectives in 22 million DPD steps In this simulation, we have obtained a single-layer liposome. (a) We combined the same simulation box with the same box angle from the top and bottom so that the resulting structure can be seen exactly. (b) The middle of the formed structure. (c) The image of the resulting structure in water (d) The interior of the structure in the water is shown. It can be seen that it traps water inside and is single-layered.*

## CONCLUSIONS

In this study, we investigated the liposome formation rules of the ionizable cationic lipid DOTAP in water using the DPD method. We have defined the interaction parameters and tail-head angle by which the DOTAP ionizable cationic lipid will obtain the multilayered liposome structure. Multilayer liposomes were obtained when the interaction parameters between the polar groups of the lipid and water were  $74.3 k_B T$  and  $73.3 k_B T$  and the angle between the tails was 60 degrees. In addition, with our study, we also defined the interaction parameter and angle at which the ionizable cationic lipid DOTAP creates a monolayer liposome structure. When we take the interaction parameter between the water and polar groups as  $72.3 k_B T$  and the angle between the tails 30 degrees, we obtained a monolayer liposome.

With our study, we showed that ionizable cationic lipids should have less repulsive interaction parameters with water than phospholipids. We show that the angle between the tail and head of bi-tailed ionizable cationic lipids affects the structure of the liposome.

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## **SEXUAL AND REPRODUCTIVE HEALTH EDUCATIONAL NEEDS AND RELATED FACTORS AMONG UNIVERSITY STUDENTS IN EDİRNE, EASTERN THRACE**

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### **ABSTRACT**

The sexual and reproductive health education and training needs of university students were evaluated. To understand the necessity of reproductive health education and training for young people by exploring the attitudes and perceptions of university students in Edirne towards reproductive health. Determining the needs of young people about sexual health; was to raise awareness. From May 2022 to July 2022, 642 Trakya University students in Edirne participated in our study. Of the 567 students who provided valid answers, 57.03% were female and 42.97% male. To the participants; Some of their socio-cultural characteristics, their demands for reproductive health services, their attitudes and experiences towards sex, and whether they were exposed to audio-visual materials were asked. Participants' knowledge and awareness about sexual health and sexually transmitted infections (STI; AIDS, Syphilis, etc.), HPV (Human Papilloma Virus) and cervical cancer were evaluated. 14.86% of them had graduated from health high schools. 85.14% had a background other than health high schools. A total of 21.2% of the respondents had previously received reproductive health training. Although 92.2% knew how HIV was transmitted, only 64.7% knew when to use a condom and 70.3% knew that condom use could reduce the risk of HIV infection. Reproductive health education lags behind the current attitudes and demands of university students. Suggested sexual and reproductive health education programs, which include various interactive activities, should be used effectively in university-based practices under the leadership of health professionals and academics who are experts in their fields.

**Keywords:** Reproductive Health, Reproductive Health Education, Sexuality, University Students

### **INTRODUCTION**

Many young people around the world often lack the strong and stable relationships needed to openly discuss reproductive health issues with their parents or other adults. Therefore, many young people do not have access to reliable information about their reproductive health needs.

In most cultures, parents and family members are an effective source of knowledge, beliefs, attitudes, and values for children and young people (Wakasa, Oljira, Demena, Regassa, & Daga, 2021). Parents often have the power to guide their children's development in sexual health issues, encouraging them to engage in sensible sexual behavior and to develop good personal decision-making skills (Bastien, Kajula, & Muhwezi, 2011). Communication within the family seems to be particularly important during the adolescence, especially on reproductive health issues. Family communication influences adolescent identity formation and role-taking ability (Habte, Melku, & Alemayehu, 2019) .

Most people become sexually active during puberty. However, hormonal contraceptive and condom use is low among teens. Unprotected sex makes the second largest contributor to health risk in young people in terms of disease burden. As a result, at least 100 million cases of sexually transmitted infections among young people are recorded each year, as well as more than 2.5 million cases of unsafe abortions for adolescents (Organization, 2009; Wudineh, Chekole, & Tesfu, 2021).

Young mothers are more likely to experience adverse pregnancy outcomes and have less ability to pursue educational opportunities than young women who delay childbearing.

As far as we know; There is no study on this subject in our region. For this reason, it is important to know the prevalence of parent-adolescent discussions on sexual and reproductive health in countries.

### ***Aim***

The aim of our study; By examining the example of Edirne province; The aim was to explore the attitudes and perceptions of university students towards reproductive health.

In the study, the sexual and reproductive health education and training needs of university students were evaluated. This study also; This study was conducted to evaluate parent-adolescent communication on sexual and reproductive health issues and related factors.

## **METHODS**

In the study; The participants were asked about some socio-cultural characteristics, their demands for reproductive health services, their attitudes and experiences towards gender, and whether they were exposed to audio-visual materials. Participants' knowledge and awareness of sexual health and sexually transmitted infections (STI; AIDS, Syphilis, etc.), HPV (Human Papilloma Virus) and cervical cancer were evaluated. 642 Trakya University students in Edirne participated in our study between May 2022 and July 2022. Of the 567 students who gave adequate and valid answers to all questions, 57.03% were girls and 42.97% were boys.

### ***Ethical approval***

The study was approved by Trakya University Faculty of Medicine Scientific Research Ethics Committee. Written informed consent was obtained from all participants.

### ***Data analysis***

Data were analyzed using the statistical software package. Results were tested with an analysis of variance. Results were calculated as mean, standard deviation. Descriptive statistics for numerical variables were given as mean and standard deviation. Student's t-test was used. Descriptive statistics for categorical variables were given as percentage and frequency. The normal distribution of the data was checked with the Shapiro-Wilk test, it was seen that they were not normally distributed. Mann-Whitney U test was used if two groups were compared. Relationships between quantitative variables were analyzed with Spearman's rho correlation coefficient. The level of significance was determined as 5% in all statistical analyzes.

## **RESULTS**

14.86% of them graduated from health high schools. 85.14% of them have a background other than health high school. A total of 21.2% of the participants had previously received reproductive health training. Majority of the students support school-based reproductive health education and learned about sexual health mostly from books, school friends and internet, especially social media. Regarding who the student discussed sexual and reproductive health issues, most of the study participants had the discussion with their peers, then with their siblings.

#### *Communication about STIs/HIV/AIDS*

19.2% of the study participants opposed premarital sexual behavior and 29.1% were able to identify the types of sexually transmitted infections listed in the questionnaire. Although 92.2% knew how HIV was transmitted, only 64.7% knew when to use a condom. 70.3% knew that the use of condoms could reduce the risk of HIV infection. About one-fifth of the students (19.1%) discussed two or more sexual and reproductive issues with their faculty members. The main reasons stated by students were embarrassment, lack of communication skills of lecturers and lack of knowledge about adult reproductive health as the reasons for students not discussing sexual and reproductive health issues. The majority of the students supported school-based reproductive health education and also learned about sexual health mainly from books, schoolmates and the internet, especially on social media. 19.2% of the survey participants opposed premarital sexual behavior and 29.1% were able to identify the types of sexually transmitted infections listed in the survey.

#### *Unwanted Pregnancy Communication*

10.2% of the students discussed the issue of unwanted pregnancy with the instructor. On the other hand, 89.8% of them did not discuss unwanted pregnancy. However, these participants argued with their peers (29.8%) and their siblings (16.9%).

#### *Premarital Sex Communication*

18.2% of the students discussed premarital sexual intercourse. Majority, 58.1% argued with their peers and 30.2% with their siblings. On the other hand, 81.8% did not discuss premarital sexuality with their parents as embarrassing, and the lack of communication skills of the parents was expressed as 63.5%. In this study, with whom the student lived; It was found to be significantly associated with adult-youth communication on sexual and reproductive issues. Participants whose living arrangements were with friends or students living alone were less likely to discuss sexual and reproductive issues than students living with both parents. This supported that they preferred to receive information from their families.

Condom was one of the most discussed sexual and reproductive health issues.

The majority of the students exchanging ideas on sexual and reproductive health issues were their peers and then their siblings.

#### *Menstrual Cycle Communication*

39.2% of the students talked to the faculty members about the menstrual cycle. The majority, 77.4%, spoke to their mother. 23.6% did not enter into an argument with his family. 24.8% expressed the lack of communication skills of the parents. 39.9% of these participants argued with their peers and 63.9% with their siblings. However, most of the participants did not discuss the issue of menstruation as a result of the discomfort and embarrassment of the discussion with the faculty members.

#### *Communication on reproductive issues*

Lack of communication was among the main reasons why young people did not discuss sexual and reproductive issues with their families. Because about three-quarters of young people consider it embarrassing; It was seen that they did not talk to their parents about sexual intercourse. According to the opinion of 76.0%; their parents had a lack of communication skills.

Bivariate logistic regression analysis was performed to evaluate the relationship between each independent variable and outcome variable. The analysis results showed that grade level, student's gender, mother's occupation, mother's education level, father's education level, life style and family size; showed that it was significantly related to the instructor-student communication.

Family education level showed a strong statistical relationship with communication about sexual and reproductive health. Adolescents whose parents have a university degree or



higher are 2.05 times more likely to communicate with faculty members about sexual health than students whose parents are illiterate.

The gender of the participants in the study was found to be statistically related to adult-youth communication on sexual and reproductive health issues. Female participants were nearly 2 times more likely to discuss sexual and reproductive health issues with faculty members, compared with male students.

## **DISCUSSION**

It was important to present the evidence for the necessity of reproductive health education and training for young people. Identifying the sexual health needs of young people; raise awareness; will improve the well-being of young people (Binu, Marama, Gerbaba, & Sinaga, 2018).

Although students' attitudes towards sexual issues were liberal, their education on reproductive health and STI / AIDS, HPV, Cervical cancer was still limited. For this reason, it is necessary to provide effective and quality reproductive health education and training to young people, especially in universities.

In general, embarrassment and parents' lack of knowledge about sexual and reproductive health are among the most important reasons why students do not discuss sexual and reproductive health issues with their families. The reason why reproductive issues are not discussed is due to many socio-cultural norms and expectations. In our culture, discussing sexual issues is not as frequent as it should be. The research revealed that adult-youth communication on sexual and reproductive health issues is low (Bikila, Dida, Bulto, Debelo, & Temesgen, 2021).

The most important reasons why students do not discuss sexual and reproductive health issues with their parents and adults are embarrassment, lack of communication skills, and lack of knowledge about sexual and reproductive health.

It was determined that gender, students' education level, students' life style and father's education level were significantly related to parent-adolescent communication on sexual and reproductive health issues. For this reason, it is recommended to provide information, training and communication to increase the awareness and practices of young people and teaching staff on sexual and reproductive health issues.

Reproductive health education lags behind the current attitudes and demands of university students. Although students' attitudes towards sexual issues were liberal, their education on reproductive health and STI/AIDS, HPV, cervical cancer was still limited.

For this reason, effective and quality reproductive health education and training should be given to young people, especially in universities.

## **CONCLUSION**

This study tried to draw attention to the need to increase the level of faculty-student communication and related factors on sexual and reproductive health issues.

In universities, lecturers play an important role in improving the lives of young people by communicating various issues, especially sexual and reproductive health issues. They have a unique role in influencing the decisions and behavior of young people on sexual and reproductive health issues based on their willingness to communicate on these issues.

Suggested sexual and reproductive health education programs, which include various interactive activities, should be used effectively in university-based practices under the leadership of expert health professionals and academics.

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## **RELATIONSHIP BETWEEN STRESS LEVEL AND VAGINITIS SYMPTOMS; A STUDY WITH TRAKYA UNIVERSITY HEALTH SCIENCES UNDERGRADUATE STUDENTS**

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### **ABSTRACT**

The aim of our study was to determine the relationship between stress levels and vaginitis symptoms in 2021 university students at Trakya University Health School. In our study, we used a cross-sectional approach. Data were collected through face-to-face interviews with 583 female students at Trakya University Health School between February 2022 and June 2022. The sociodemographic characteristics of the participants were recorded. Those with chronic gynecological disease, psychiatric disease, married, and those who applied to the hospital due to vaginitis were not included in the study. The study was carried out on a voluntary basis. Stress levels of the participants Depression Anxiety Stress Scale (DASS 42) was used. Vaginitis symptoms were evaluated with the "Genital Hygiene Behavior Scale". Approximately one-third (n= 192) (32.93%) of the 583 participants were experiencing stress. Among the participants evaluated with DASS 42; With 115 female students (67.04%), moderate stress level was the highest percentage. Participants; 40 (20.83%) were scored mild, 37 (19.27%) scored high. Most of the participants experiencing moderate and high levels of stress (84.04%); had experienced vaginitis symptoms in the last 1 year. The statistical test results obtained were significant (p= 0.019). What is remarkable for our results; in younger women, vaginitis symptoms were increased during periods of stress. Being able to manage stress well will play a preventive role in the formation of vaginal discharge in young women. More awareness should be created on stress and vaginitis in young women. Early recognition of vaginitis symptoms and prompt initiation of treatment; It is important to prevent possible complications.

**Keywords:** Genital Hygiene, Genital Hygiene Behavior Scale, Stress, Vaginitis, Young Female, Depression Anxiety Stress Scale (DASS 42)

### **INTRODUCTION**

Vaginitis is an inflammatory condition that occurs in the vagina where gynecological pathologies such as vaginosis, bacterial, candidiasis vulvovaginal and trichomoniasis are the most common vaginitis cases (Jackowich, Poirier, & Pukall, 2020). About 5 – 10 million women in the world are sick for vaginitis every year. and goes to the examination with the obstetrician (Bitew, Abebaw, Bekele, & Mihret, 2017). It is stated that 75% of women experience vaginitis at least once in their life, and 40-45% of them experience recurrent episodes with some risk factors

The most obvious symptom of female external genital tract infections is itching with a thick secretion. An itchy, irritating, painful pathology is observed on the skin. The discharge may be coagulated and odorous, whitish, grayish or greenish in places.

Women with high stress levels are more likely to experience vaginitis than those with low stress levels. Stress is an emotional feeling that is interrupted by the biological, psychological and social changes of the person (Cauci, 2004).

### **Aim**

The aim of our study was to determine the relationship between stress levels and vaginitis symptoms in university students at Trakya University Undergraduate Health School.

### **METHODS**

In our study, we used a cross-sectional approach. Data were collected through face-to-face interviews with 583 female students at Trakya University School of Health between October 2021 and February 2022. The sociodemographic characteristics of the participants were recorded. Those with chronic gynecological disease, psychiatric disease, married, and those who applied to the hospital due to vaginitis were not included in the study. The study was carried out on a voluntary basis. Stress levels of the participants Depression Anxiety Stress Scale (DASS 42) was used (Lovibond & Lovibond, 1995). Vaginitis symptoms were evaluated with the "Genital Hygiene Behavior Scale".

#### *1. Genital hygiene behaviors scale*

Validity and reliability in 2017 Karahan (Karahan, 2017). It is a five-point Likert-type scale filled by the women themselves. The scale consists of 23 items in total. Genital hygiene behaviors scale. The scale has 3 sub-dimensions; "General Hygiene Habits (first 12 items)", "Menstrual Hygiene (Articles 13-20)", and It is "Awareness of Abnormal Findings (Articles 21-23)". Scale items are scored from 5 to 1 from "strongly agree" to "strongly disagree". The 7th, 14th, 19th, 20th, and 23rd items of the scale are reverse scored (Karahan, 2017). The lowest 23 and the highest 115 points are obtained from the scale, and high scores indicate that genital hygiene behavior is positive.

#### *2. Depression Anxiety Stress Scale (DASS)*

The scale consists of 42 items. It was developed by Lovibond and Lovibond (Lovibond & Lovibond, 1995). It provides self-report of anxiety, depression and stress in participants. Turkish validity and reliability was done by Bilgel and Bayram (Bilgel & Bayram, 2010). The scale is a four-step Likert-type scale and items are evaluated between 0 and 3. The total scores of the scale range from 0 to 42 for each sub-dimension. In the scale, depression, anxiety and stress are expressed in 5 categories as normal, mild, moderate, severe and very severe. has been done.

### **RESULTS**

Approximately one-third (n= 192) (32.93%) of the 583 participants were experiencing stress. Among the participants evaluated with DASS 42; With 115 female students (67.04%), moderate stress level was the highest percentage.

The DASS consists of 14 items for depression, 14 items for anxiety, and a total of 42 items, 14 of which are stress. Stress items, on the other hand, measure the level of symptoms such as difficulty in relaxation, nervous stimulation, easy upset and boredom, discomfort, overreaction and intolerance. Anxiety items assess an individual's level of autonomic arousal, situational anxiety, subjective anxiety, and muscle response. Depression items measure discontent, helplessness, worthlessness, loss of interest and low energy level.

The participants; 40 (20.83%) were scored mild, 37 (19.27%) scored high. Most of the participants (84.04%) who experienced moderate and high levels of stress; had experienced

vaginitis symptoms in the last 1 year. The statistical test results obtained were significant ( $p=0.019$ ).

That is remarkable for our results; younger women were also found to have increased vaginitis symptoms during periods of increased stress. However, these symptoms caused the women not to be admitted to the hospital, either in terms of their age or because the symptoms did not exceed three days.

## DISCUSSION

Women who experience stress without symptoms of vaginitis probably have a fairly strong body immune system to fight vaginal bacteria in their bodies (Jackowich et al., 2020).

However, these symptoms caused no hospital admissions, both in terms of age and because the symptoms did not exceed three days (Calabrò et al., 2019). If abnormal vaginal discharge is ignored, serious consequences can occur.

Abnormal vaginal discharge can cause serious complications such as sepsis, infertility, and chronic pelvic pain. Stress seems to be one of the triggering factors.

Other risk factors that may cause the symptom of vaginitis; Inadequate hand washing can increase bacterial contamination of the vulva and cause irritation. Tight clothing, the use of underwear fabrics that do not absorb sweat may cause irritation (Loh, Hendricks, Hsiao, & Shi, 2021).

If abnormal vaginal discharge is ignored, serious consequences can occur. Abnormal vaginal discharge can cause serious complications such as sepsis, infertility, and chronic pelvic pain.

Stress seems to be one of the triggering factors. Being able to manage stress well will play a preventive role in the formation of vaginal discharge in young women.

More awareness should be created on stress and vaginitis in young women. Early recognition of vaginitis symptoms and prompt initiation of treatment; It is important to prevent possible complications.

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## EVALUATION OF SUBTERRANEAN CLOVER FROM A POINT OF VIEW POSSIBILITY TO USE IN BULGARIAN ENVIRONMENTAL CONDITIONS

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### ABSTRACT

Subterranean clover (*Trifolium subterraneum* L.) is an annual drought resistant legume with winter-spring type of development and ability for self-sowing. It is a widespread component in the pastures of the temperate areas of Central and Northern Europe and America. Subterranean clover was assessed as pure crop and component of perennial mixtures with traditional forage crops in the environmental conditions of Bulgaria. It was found the dry mass yield from pure grown subclover ranging between 3600 and 4300 kg/ha. In addition, subclover forage biomass was well balanced by principal chemical composition with optimal Ca:P ratio. Mixed crops are more effective than pure grown in using environmental resources, better withstand adverse conditions and are more productive. The suitability of subclover as a component of both mixtures with perennial grasses and with perennial legumes was studied. The results showed it is suitable component of mixtures contributing to as follows: better botanical composition, weed reducing, higher dry mass yield, longevity. In addition, its effect on the quality characteristics of forage biomass from mixtures was assessed. Leaf to stem ratio as important characteristics for the intake of the forage by animals was calculated. As a legume it contributes to increase of leaf to stem ratio, both in legume-subclover mixtures and in grass-subclover mixtures. For birdsfoot trefoil it was found from 13.0 to 20.1% higher for mixtures with subclover compared to the leaf to stem ratio for pure grown birdsfoot trefoil. The same tendency was found for sainfoin-subclover mixtures, where the leaf to stem ratio was from 9.5% to 12.8% higher, and also for alfalfa-subclover mixtures. The content of cyanoglycosides as antinutritional compounds causing poisoning in animals was determined. It was found high level in birdsfoot trefoil grown alone. However, in mixtures of birdsfoot trefoil with subclover the cyanoglycosides content was more than 50% reduced and the forage mass obtained was not toxic. The palatability of the crops tested was assessed in *in vivo* experiments with sheep. The subterranean clover was grazed at 100%, as from legumes, sheep prefer this crop the most, followed by birdsfoot trefoil and sainfoin, and cocksfoot from grasses. Subterranean clover is an appetizing and preferred species for grazing by sheep due to its low crude fiber content and higher crude protein compared to the same for other crops. Due to its self-seeding capacity, it may be used for undersowing of degraded seed production swards. The direct undersowing of degraded seed production swards of perennial legumes and grasses, apart from giving the opportunity to improve the botanical composition of the grass swards, their use for forage obtaining, but also led to significantly increase the amount of nitrogen accumulated in the soil. When degraded seed production perennial ryegrass swards were undersowed with subterranean clover, the amount of nitrogen accumulated in the soil increased to 10.6%, and in degraded seed production alfalfa swards to 18.7%, respectively. The above showed the possibility subterranean clover to be used as a component of sown pasture swards in Bulgarian environmental conditions. It is adaptable to the changing climatic conditions and responsible to

the challenges of agriculture nowadays for bigger resource use efficiency.

**Keywords:** Subterranean clover, *Trifolium subterraneum*

## INTRODUCTION

Agriculture is facing significant challenges in our century, largely due to the need to increase global food supply under the declining availability of soil and water resources and increasing threats from climate change, which places it at the center of many of society's most important debates including those highlighted by Barsby (2012) as: global food security (enhanced productivity, increased yield and sustainable production); water availability (drought-tolerant crops); global warming (CO<sub>2</sub> footprint and fertilizer use).

The permanent changes having occurred in the last years in the climate (increase of average yearly temperatures, long droughts in the spring and summer, increase of CO<sub>2</sub> concentration in the atmosphere) present a serious risk to the agricultural crops (Aranjuelo et al., 2014; FAO, 2015). This requires to study new herbaceous forage species having pronounced resistance to unfavorable abiotic factors and good adaptive capacity towards the new conditions. Only a significantly enhanced biological adaptation potential of crops, and consequently improved plasticity of agroecosystems, can help to improve the current agricultural production levels and meet future food needs (Folke et al., 2012; Banga and Kang, 2014).

The environment, its conservation, the efficient use and the improvement of the quality of natural resources are gaining in importance. At present the main interest is directed towards more drought resistant and drought tolerant components. Plant breeders have to put continuous efforts to accelerate the development of new crop varieties. Legumes species that can provide self-sowing and persist continuously in the sward become of practical importance.

Subterranean clover (*Trifolium subterraneum* L.) is a widespread component in the pastures and other grasslands of the temperate areas of Central and Northern Europe and America (Frame et al., 1998; Pecetti and Piano, 1998, 2002; Kyriazopoulos et al., 2008). The species is found in Bulgaria in open, dry grasslands in the plains and lowlands. It is known from the Black Sea coast, Danube Plain (areas of Byala Slatina and Svishtov), Northeastern Bulgaria, Sofia Region, Western Border Mountains, Struma Valley, Mesta River Valley, Thracian Lowland, Tundzha Plain, Eastern Rhodopes, Rila, Strandzha to 500 m above the sea level (Figure 1) (Assyov et al., 2012). The area of the species also includes Western and Southern Europe (in the north to Transylvania, in the east to the Crimea), the Mediterranean countries, Southwestern Asia, Northern Africa, Macaronesia (Coombe, 1968).

The subterranean clover is an annual drought resistant ephemeral legume with winter-spring type of development and ability for self-sowing (Yakimova and Yancheva, 1986; Piano et al., 1996; Frame et al., 1998). Its reproductive organs are formed in early May, and the seeds ripen before the end of the spring in hedgehog-shaped heads that remain on the soil surface. The precipitations during the late summer contribute to emergence of new self-sown plants.

An important element of the biology of this species is that a big part of the seeds germinated during the autumn. Other part germinated during the spring of the next year but because of the winter-spring type of the development the plants took part in the sward during most of the vegetation season, some of them wintering and on the next spring formed seeds (Vasilev, 2006). Substantial part of the formed seeds is solid and germinates after two-three years. This biological specificity turns the superficial soil layer into an original seed bank which makes the species even more flexible one (Pecetti and Piano, 1994).

Subterranean clover is distinguished for very good winter resistance, and in the spring it grows up quickly after the forced winter dormancy forming a dense sward (Porqueddu et al.,



2003). The effective utilization of autumn-winter soil moisture, successful seed formation and self-sowing at the end of spring allow to the subterranean clover to avoid summer droughts (Piano et al., 1996; Porqueddu et al., 2003). As a legume species, the subterranean clover is nitrogen fixing (Ferreira and Castro, 2005). It develops symbiotic relations with bacteria from the species *Rhizobium leguminosarum biovar trifolii*. The amount of fixed nitrogen per year varied from 50 to 188 kg/ha (Sanford et al., 1994; Bolger et al., 1995).

In the paper some important both biological and ecological characteristics of subclover were reviewed as well the assessment of crop in point of view possibility to use in Bulgarian environmental conditions as pure growing and as a component in mixtures with traditional perennial grass and legume forage crops was done.

## **MATERIAL AND METHOD**

The studies were performed in the Institute of Forage Crops, Pleven (43° 23'N, 24° 34'E, 230 m altitude), Bulgaria during the last 15 years. The field experiments were carried out on haplustoll and podzolized soil subtype.

## **RESULTS AND DISCUSSION**

### **Biological characteristics**

Subterranean clover is an annual plant, with several to numerous stems. Stems are 5.00-30.0 (45.0) cm long, branched, creeping, semi-appressed hairy. Stipules are ovate (egg-shaped), short sharp-pointed, often with auricles, with whole edges or slightly indented, membranous, with clear veins, 1/3-1/2 of their length accrete, much shorter than the leaf petioles, glabrous. Leaf petioles are 5.0-25.0 mm long, a little longer to much longer than leaflets, often filiform (thread-like), more or less hairy. Leaflets are 1.0-1.3 mm long and 6.00-9.00 mm wide, cordate (heart-shaped), broadly obovate (inversely egg-shaped), more rarely broadly triangular cuneate (wedge-shaped), with 8-12 lateral, protuberant veins, on the edge in the upper third part short indented, on the two sides or mostly on the underside semi-appressed rust hairy, on 0.5 mm long petioles, equal between them. Inflorescence peduncles are curved, shorter, more rarely longer than the bracts (leaves associated with inflorescences), axillary, sparsely long rust white or light rust hairy. Heads are 10.0-15.0 mm spherical, loose, appressed on the soil, self-burying. Flowers are 8.0-14.0 mm long, 2-5 (7) fertile, outer and numerous inner sterile, developing after fall of the fertile ones, almost sessile or on pedicles shorter than 0.5 mm without bracts (leaves associated with flowers). Calyx is 5.0-6.0 mm long, glabrous; tube is pale, often red purple, membranous, at its upper end often violet, cylindrical bell-shaped, with 5-10 unclear veins, teeth are slightly unequal between them, filiform (thread-like), longer than the tube, outspread hairy. Corolla is 2 times longer than the calyx, pale pink, after fall yellow brown. Standard is fused to the wings and keel in a long tube and is a little longer than them, blade is narrowly obovate (inversely egg-shaped), smooth. Wings are with an obovate (inversely egg-shaped), shorter than the filiform (thread-like) claw, slightly veined blade, ligule is unclear. Keel is blunted with an elliptically cordate (heart-shaped) blade and a claw longer than it, with a poorly developed ligule. Barren flowers are of calyxes only, with branched teeth, during fruiting of the fertile calyx teeth they accrete, spread out radially and surround the pod forming a 10.0-15.0 mm head that at the end of the flowering is completely buried in the soil. Pod is elliptical, one-

seeded, skin-like, protruding above the calyx. It flowers in March-April, and fruits in April-May (Kozhuharov, 1996).

### **Ecological characteristics**

Two associations have been described by the dominant method where subterranean clover is a co-dominant in the literature in Bulgaria. The association of *Cynodon dactylon-Lolium perenne-Trifolium subterraneum* is from the valley of Studena River near village of Batak (Pavlikeni area) (Ganchev and Kochev, 1962). It is a slightly sloping (4-5°) ground with southwesterly exposure. Soils are chernozemic, trampled. The ground is a pasture. Characteristic species are *Trifolium repens*, *Trifolium campestre*, *Plantago lanceolata*, *Erodium cicutarium*. The characteristic for the associations is that they have moderate ruderalization mainly due to the effect of overgrazing by domestic animals. This shows that the species is influenced rather favourably by the effects of overgrazing and trampling.

Association of *Poa bulbosa-Trifolium subterraneum* - it was described by Ganchev (1958). It is a pasture on some alluvial-meadow soils. By the method of Braun-Blanquet (Braun-Blanquet, 1964) it is mentioned among the species composition of sparse forests of pubescent oak in Southwestern Bulgaria (association of *Cisto incani-Quercuetum pubescentis* and *Teucrium polium-Quercus pubescens*) (Gogushev, 2009). This is an indicator for the high ecological flexibility of the species and its presence in habitats in sparse oak forests, but also in pastures differing in slope, soil cover, degree of grazing and nitrification.

Subterranean clover consists of three subspecies: ssp. *subterraneum* (adapted to well-drained, acid soils); ssp. *yanninicum* (adapted to waterlogged, acid soils) and ssp. *brachycalycinum* (adapted to neutral-alkaline cracking or stony soils). The two keys to its widespread use are its tolerance of heavy grazing and a suite of cultivars differing in flowering time, enabling it to be grown in environments with a wide range of growing season lengths.

Subterranean clover was assessed as pure crop and component of perennial mixtures with traditional forage crops in the environmental conditions of Bulgaria. It was found the dry mass yield from pure grown subclover ranging between 3600 and 4300 kg/ha (Figure 1).

In addition, subclover forage biomass was well balanced by principal chemical composition with optimal Ca:P ratio and the lowest degree of lignifications. The analysis of enzyme *in vitro* digestibility of forage biomass showed the lowest degree of lignifications (11.67 coef.) and the highest forage quality belong to *Trifolium subterraneum ssp. yaninicum*, followed by *Trifolium subterraneum ssp. brachycalycinum* (14.96 coef.) and *Trifolium subterraneum ssp. subterraneum* (15.08 coef.) (Figure 2). The forage biomass of *Trifolium subterraneum ssp. brachycalycinum* is distinguished the highest energy feeding value (FUM, feed units for milk, 0.712 g/kg dry matter) (Naydenova and Vasileva, 2015).

Mixed crops have an essential role in building a system of sustainable and ecologically friendly farming (Luscher et al., 2014; Kusvuran et al., 2014). They are more effective than pure grown in using environmental resources, better withstand adverse conditions and are more productive and long-term (Shiferaw et al., 2004; Elesesser, 2004; Mahapatra, 2011). In addition, mixed cropping are more effective over pure with regard to the nitrogen transfer from legume to grasses (Frame, 2005; Frame and Laidlaw, 2005; Vasilev et al., 2005; Graham, 2008; Nyfeler et al., 2005, 2006; Peeters et al., 2006; Pozdissek et al., 2011) (for grass-legume mixtures).

The suitability of subclover as a component of both mixtures with perennial grasses and with perennial legumes was studied. The results showed it is suitable component of mixtures contributing to as follows: better botanical composition, weed reducing, higher dry mass yield,

longevity.

Botanical composition of cocksfoot and tall fescue, pure and in mixtures with subterranean clover were investigated in field trial. It was found that inclusion the subterranean clover as a component of mixtures with cocksfoot and tall fescue had positive effect on the botanical composition and decreased the weed infestation in the swards from 28.0% to 50.0% (Figure 3 and Figure 4).

Subterranean clover covered the soil surface and due to the prostrate habit retains the moisture which influences the accompanying component in the mixture. In the mixtures with *Trifolium subterraneum ssp. brachycalicinum* the reduction of weeds was by 28.1% and almost 50.0% in mixtures with cocksfoot. Probably a weaker development of subterranean clover in mixtures with tall fescue was due to the greater competitiveness of tall fescue because of its allelopathic potential.

When the effect of subclover on the quality characteristics of forage biomass from mixtures was assessed we found the next: for cocksfoot and with tall fescue - the addition of subterranean clover in the composition of mixtures, forage biomass showed more balanced basic chemical composition (Table 1): higher crude protein content (with 0.32% units) and lower crude fiber content (with 0.63% units), lower degree of lignifications; for birdsfoot trefoil - the forage biomass obtained from the mixtures compared to pure grown birdsfoot trefoil had lower crude fiber content (with 1.38%), higher *in vitro* digestibility of dry matter (with 2.21%); for alfalfa and sainfoin - *Trifolium subterraneum ssp. brachycalicinum* and *Trifolium subterraneum ssp. yaninicum* contributed to reducing the degree of lignifications. The forage from alfalfa - *Trifolium subterraneum ssp. yaninicum* mixture was found to be with the highest protein content (18.97%) and the lowest crude fiber one (25.96%), the highest digestibility (65.94%).

Leaf to stem ratio as an important characteristic for the intake of the forage by animals. Subterranean clover as a legume component contributed to increasing the leaf to stem ratio of cocksfoot and tall fescue mixtures (Figure 5). This ratio is an important factor affecting quality and forage intake and it was calculated on the base of fresh weight. More leaf biomass formation from grasses in mixtures with legume component was associated to better nitrogen assimilation (Ledgard and Steel, 1992). As a legume it contributes to increase of leaf to stem ratio, both in legume-subclover mixtures and in grass-subclover mixtures. For birdsfoot trefoil it was found from 13.0 to 20.1% higher for mixtures with subclover compared to the leaf to stem ratio for pure grown birdsfoot trefoil. The same tendency was found for sainfoin-subclover mixtures, where the leaf to stem ratio was from 9.5% to 12.8% higher, and also for alfalfa-subclover mixtures. The components included in the mixtures tested have different type of nitrogen metabolism. Nitrogen in grasses is the result solely of nitrate nitrogen assimilation through the roots due to the activity of nitrate reductase, while in subclovers nitrogen fixation process was included. Upon successful competition among the components for available nitrogen, the efficiency of use of nitrogen is higher.

The cyanogenic glycosides are harmless under normal conditions, but during enzymatic digestion they release the strongest poison - hydrocyanic acid that causes disorder or poisoning. The specific enzyme is contained in plant cells, but acts as a rupture of the tissues or when digests the green mass in the rumen of the animals. The amount of free hydrocyanic acid directly depends on the amount of cyanogenic glycosides contained in the plants (Vough and Cassel, 2002). The content of cyanoglycosides as antinutritional compounds causing poisoning in animals was determined in birdsfoot trefoil grown alone. Birdsfoot trefoil, besides having a high content of crude protein and good digestibility of the dry matter (Chourkova, 2012) contains also cyanogenic glycosides. Cyanogenic glycosides content in stems of birdsfoot trefoil was found 38.40 mg HCN/100 g DM, in leaves 90.39 mg HCN/100 g DM and in flowers

173.60 mg HCN/100 g DM, respectively. It is known that grazing animals avoid the birdsfoot trefoil when it is in the flowering stage.

The content of cyanogenic glycosides in plants of three subclover subspecies was found very low, i.e. *Trifolium subterraneum ssp. subterraneum* (2.84 mg HCN/100 g DM), *Trifolium subterraneum ssp. brachycalycinum* (3.18 mg HCN/100 g DM), *Trifolium subterraneum ssp. yananicum* (7.87 mg HCN/100 g DM). However, in mixtures of birdsfoot trefoil with subclover the cyanoglycosides content was more than 50% reduced and the forage mass obtained was not toxic. According to the scale of toxicity, The Merck Veterinary Manual (2018) (Table 2) the leaves in flowering stage are highly toxic to animals, the flowers are very toxic and can cause death.

Due to the prostrate growth habit subterranean clover is adapted to intensive grazing systems and has high tolerance to grazing (Evers and Newman, 2008; Ovalle et al., 2008). It can flower and set seed under close grazing. In *in vivo* trials the palatability or the preference of sheep to the subterranean clover and to other commonly used perennial forage crops was studied (Kirilov and Vasileva, 2016). The palatability of the crops tested was assessed in *in vivo* experiments with sheep. Results obtained showed that subterranean clover was grazed at 100% and from the legumes sheep prefer most this crop followed by birdsfoot trefoil and sainfoin, and from the grasses - cocksfoot (Table 3). Subterranean clover consist higher crude protein and lower crude fiber content, thus is a palatable and preferred for grazing by sheep forage crop compared to sainfoin, cocksfoot and tall fescue.

Because of the self-seeding capacity of subclover it may be used for undersowing of degraded seed production swards. The direct undersowing of degraded seed production swards of perennial legumes and grasses, apart from giving the opportunity to improve the botanical composition of the grass swards, their use for forage obtaining, but also led to significantly increase the amount of nitrogen accumulated in the soil. Direct undersowing is using for the recovering and/or prolonging the persistence of the degraded pasture and meadows. It is applying in degraded stands, where the legume components are dropped due to the short duration, less adaptability to over use, adverse soil and climatic conditions or other factors. The inclusion of legumes crops through this method expect that it prolongs the durability of the stands, enhance the productivity and quality of the forage (Springer, 1997).

The possibility for undersowing with subterranean clover of degraded alfalfa, birdsfoot trefoil and white clover seed production stands was studied in a 7-years field trial. Three subterranean clover subspecies mentioned above were used. The undersowing was performed during the autumn of the fourth year of using of seed production stands with 400 germinated seeds/m<sup>2</sup> and interred spacing of 12 cm. The stands were used for forage for three years after undersowing and every year two cuts were harvested (from alfalfa four cuts were obtained during the first and second year after undersowing and three cuts during the last). It was found that subterranean clover did not negatively effect the main crop development, increased dry mass productivity with app. 10% (9.12 to 11.7%) (Table 4) and reduced the weed infestation (for alfalfa two- and three fold).

Moderate during establishment but prostrate growth habit and spreading nature of stems in established swards enables subclover to compete aggressively with weeds. Subterranean clover effectively used the autumn-winter soil moisture, formed a sufficient number of seeds for self-seeding and germinated plants occupied the sites of the dropped plants. *Trifolium subterraneum ssp. brachycalycinum* showed the best self-seeding ability. It is concluded that the undersowing of degraded seed production stands with subterranean clover is possible agrotechnical measurement and the stands thereafter could be used for forage.

In addition, when degraded seed production perennial ryegrass swards were undersowed with subterranean clover, the amount of nitrogen accumulated in the soil increased to 10.6%,

and in degraded seed production alfalfa swards to 18.7%, respectively.

Subclover can improve forage productivity, regenerate the soil through nitrogen fixation and increase farm profitability (Grigg et al., 2008). The amount of N fixed by the *Rhizobium*-legume symbiosis can be as high as 140 kg N/ha/year (Ledgard and Steele, 1992) or approximately 30 kg of N per tonne of clover biomass produced (Høgh-Jensen et al., 2004; Lucas et al., 2010). To date it is a recommended practice to introduce the subclover specific strain group to ensure successful N fixation, especially for new sown areas (Department Primary Industry NSW, 2004). The optimization of biological nitrogen fixation depends on the host plant, the bacterium and a favourable environment for both. Drew and Ballard, 2010 reported the interaction between legume varieties and *Rhizobium* strains and confirmed that nodulation differs among plant lines. (depends from the cultivar). The main reason for the higher sub clover N was its greater growth due to its ability to exploit moisture available early in spring (Lucas et al., 2010).

The above showed the possibility subterranean clover to be used as a component of sown pasture swards in Bulgarian environmental conditions. It is adaptable to the changing climatic conditions and responsible to the challenges of agriculture nowadays for bigger resource use efficiency.

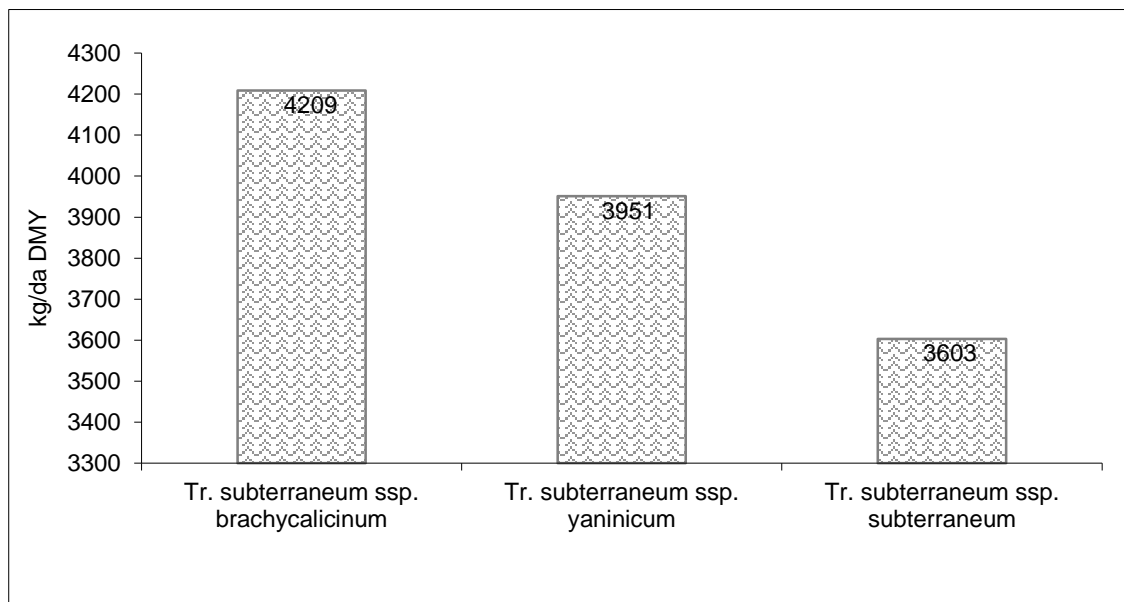


Figure 1. Annual dry mass yield from subclover

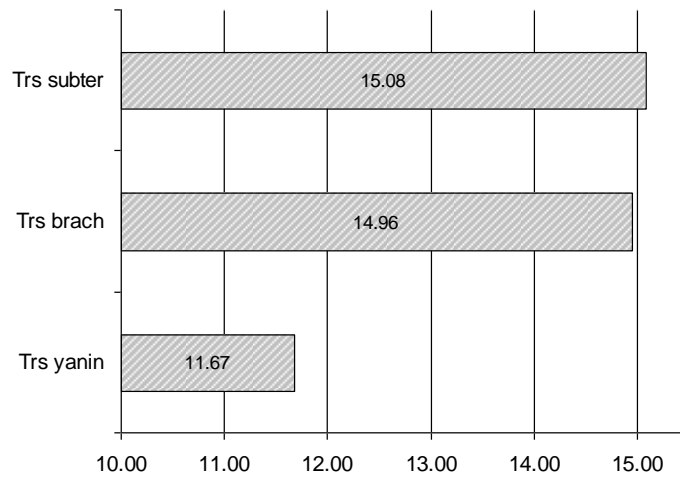


Figure 2. Degree of lignifications of dry matter of forage of three subspecies of subterranean clover  
 (*Trs yanin* - *Trifolium subterraneum* ssp. *yaninicum*; *Trs brach* - *Trifolium subterraneum* ssp. *brachycalicinum*; *Trs subter* - *Trifolium subterraneum* ssp. *subterraneum*)

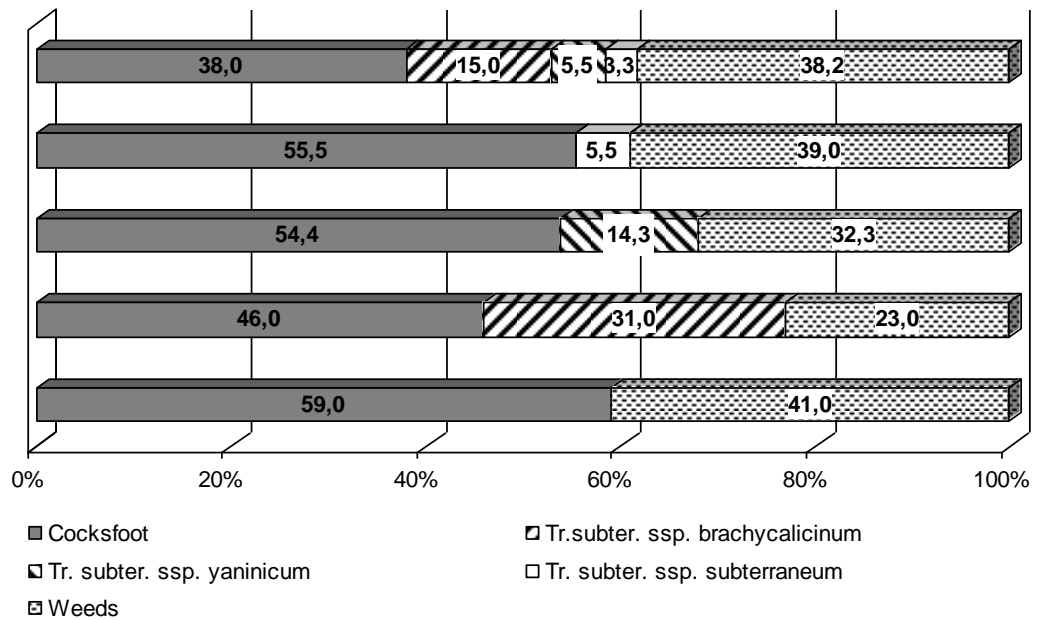


Figure 3. Botanical composition of cocksfoot pure and in mixture with subterranean clover  
 (one year after sowing)

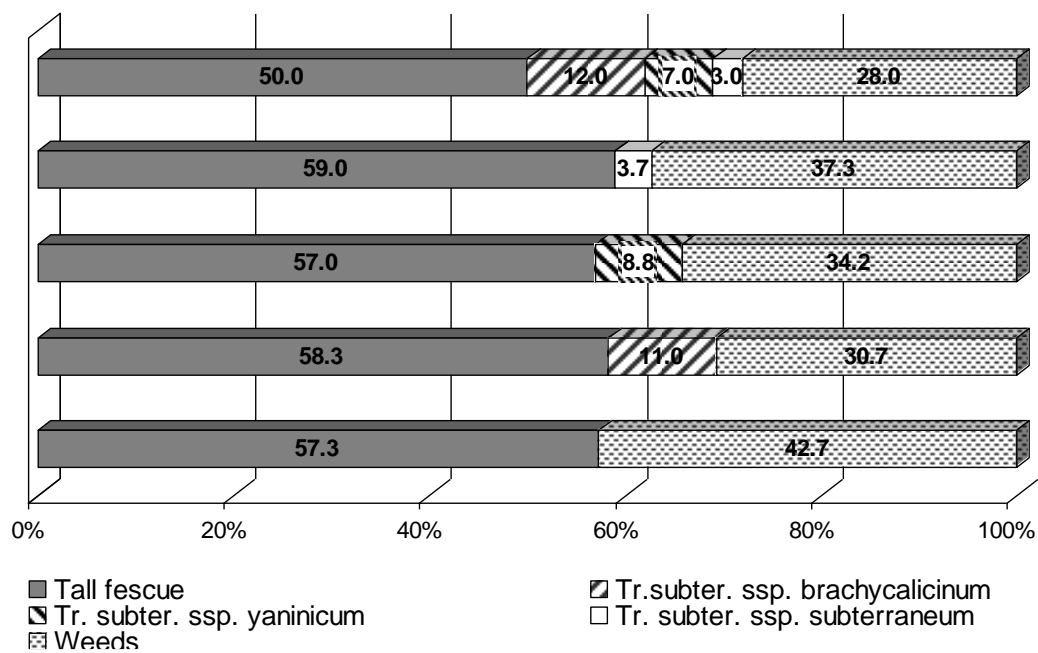


Figure 4. Botanical composition of tall fescue pure and in mixture with subterranean clover (one year after sowing)

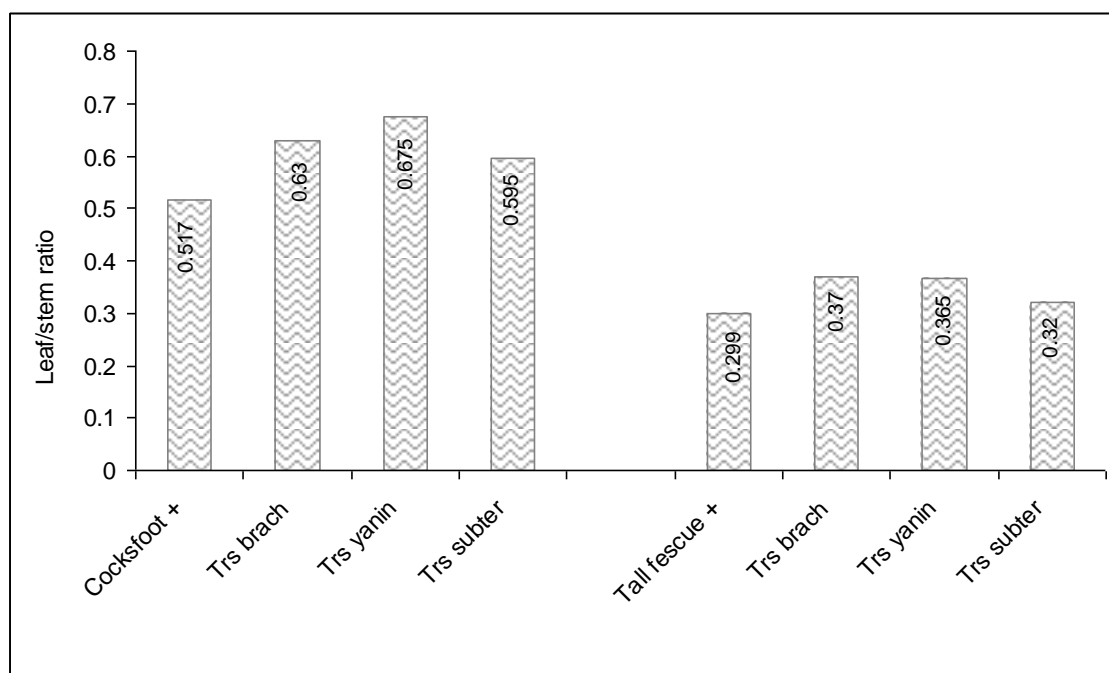


Figure 5. Leaf to stem ratio of cocksfoot and tall fescue pure and in mixture with subterranean clover

Table 1. *In vitro* digestibility (IVD) of dry matter of forage biomass (%)

|                                  | IVD   | + Subclover | Subspecies        |
|----------------------------------|-------|-------------|-------------------|
| Alfalfa                          | 61.41 | 65.94       | <i>Trs yanin</i>  |
| Sainfoin                         | 59.28 | 61.64       | <i>Trs subter</i> |
| Birdsfoot trefoil                | 60.64 | 63.33       | <i>Trs subter</i> |
| Alfalfa+cocksfoot                | 46.33 | 48.89       | <i>Trs subter</i> |
| Alfalfa+tall fescue              | 47.60 | 49.14       | <i>Trs brach</i>  |
| Sainfoin +cocksfoot              | 61.74 | 62.81       | <i>Trs brach</i>  |
| Sainfoin + tall fescue           | 55.23 | 56.74       | <i>Trs yanin</i>  |
| Birdsfoot trefoil +<br>cocksfoot | 62.29 | 65.96       | <i>Trs brach</i>  |

Table 2. Scale of toxicity (The Merck Veterinary Manual, 2018)

| Cyanoglycoside content (mg HCN/100 g DM) | Degree of toxicity   |
|--|--|
| Less than 50                             | Low, generally considered safe   |
| From 50 to 75                            | Average, potential for toxicity exists, thus should not be the sole source of feed |
| From 75 to 100                           | High   |
| Greater than 100                         | Very high, dangerous and will cause death  |

Table 3. Comparative data for the palatability during the grazing of some forage crops by sheep

| Crops       | Dry matter     | Dry matter    | Before grazing | Grazed grass | Grazed grass |     |
|-------------|----------------|---------------|----------------|--------------|--------------|-----|
|             | before grazing | after grazing |                | kg/da        | %            |     |
|             | %              |               |                |              |              |     |
| Subclover   | 21.63          | -             | 55.4           | 55.4         | 100.00       | (1) |
| B. trefoil  | 30.70          | 40.20         | 265.6          | 209.6        | 78.92        | (3) |
| Sainfoin    | 29.46          | 41.18         | 196.8          | 136.7        | 69.46        | (4) |
| Cocksfoot   | 29.02          | 38.78         | 193.9          | 165.4        | 85.30        | (2) |
| Tall fescue | 31.42          | 35.85         | 154.9          | 103.4        | 66.75        | (5) |
| SE (P=0.05) | 1.7            | 1.16          | 34.4           | 26.2         | 5.98         |     |

Table 4. Dry mass yield from undersowed with subclover seed production stands

| Stands            | DMY, kg/da         |                   | Increase |
|-------------------|--------------------|-------------------|----------|
|                   | before undersowing | after undersowing | %        |
| Alfalfa           | 962.2              | 1061.7            | 10.3     |
| Birdsfoot trefoil | 149.6              | 163.2             | 9.12     |
| White clover      | 126.9              | 141.8             | 11.7     |

## CONCLUSIONS

Subterranean clover could be grown both, alone and in mixtures. It was found as a suitable component for mixtures with traditional perennial grass and legume forage crops contributing to weed infestation decreasing, higher productivity, better quality of forage biomass obtained. An annual leguminous species, but because of the biological ability for self-seeding with the



presence in the sward at the beginning and end of vegetation, subterranean clover increases the persistence of the pasture swards. As a legume it contributes to increase of leaf to stem ratio, both in legume-subclover mixtures and in grass-subclover mixtures. In mixtures of birdsfoot trefoil with subclover the cyanoglycosides content was more than 50% reduced and the forage mass obtained was not toxic. It is considered as highly tolerant to grazing due to the prostrate growth habit and has high voluntary intake and nutritive value characteristics. After the addition of subterranean clover in the composition of mixtures, forage biomass showed more balanced basic chemical composition: higher crude protein and lower crude fiber contents, higher digestibility, lower degree of lignifications and higher energy feeding value. Subterranean clover is an appetizing and preferred species for grazing by sheep. Subterranean clover may be used for undersowing of degraded seed production swards of perennial legumes and grasses giving the opportunity to improve the botanical composition. It is a tool to increase the persistence of the pasture systems and to the prolongation of stands to allow for forage provision. Some units of the technology of artificial and natural perennial swards as weed control and nitrogen fertilization could be replaced by alternative, ecologically friendly ones including subterranean clover as a component. Subterranean clover has practical applicability under the climatic conditions of the country. It is adaptable to the changing climatic conditions and responsible to the challenges of agriculture nowadays for bigger resource use efficiency.

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## EXPERIMENTAL INVESTIGATION OF OUTPUT POWER USING PHASE CHANGE MATERIAL (RT-44HC) IN POLYCRYSTALLINE SOLAR PANELS

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### ABSTRACT

The demand for renewable energy sources is increasing day by day due to the gradual decrease of fossil-based energy sources, increasing costs and environmental problems. Recently, electricity generation from solar and wind energy has become more preferred in many countries. Photovoltaic panels are used in the conversion of solar energy directly into electricity. During energy conversion in photovoltaic panels, the module cell temperature also increases with the effect of ambient temperature. This reduces the output power obtained from the photovoltaic panel. It is important to obtain maximum energy from photovoltaic panels due to the variable solar radiation during the day and the losses due to operating conditions. Especially in summer, increasing temperature significantly reduces the output power of photovoltaic panels. In this study, it is aimed to reduce the temperature increase during energy conversion in polycrystalline photovoltaic panels with the application of phase change materials (PCM) Rubitherm RT44HC and to increase the PV Panel power. Energy production values were recorded for one month from the photovoltaic panels installed in Edirne climatic conditions. 9.85% more electrical energy was obtained from a single PV panel with PCM compared to the standard panel.

**Keywords:** Energy, Photovoltaic, Phase change material, Temperature, Efficiency

### INTRODUCTION

Although fossil-based energy sources such as coal, oil and natural gas are still used in many parts of the world, the demand for renewable energy is increasing day by day. Nuclear and hydroelectric systems, which are other energy sources, have many negative aspects. From this point of view, the sun is a clean and endless source of energy. However, the amount of energy to be obtained from solar energy varies depending on the radiation and sunshine duration. For this reason, attention should be paid to the correct location, seasonal characteristics and operating conditions in the installation of solar power plants. Photovoltaic panels are used in the direct conversion of solar energy to electrical energy. Photovoltaic panel efficiency is around 20% under today's conditions (Gokeri, 2019). If suitable operating conditions are not provided, this efficiency decreases further. There are many factors affecting the efficiency. One of the most important of these is the temperature increase on the photovoltaic surfaces. At high temperatures, the low circuit voltage ( $V_{oc}$ ) decreases significantly (Gokeri, 2019) One of the methods used to prevent this temperature increase in photovoltaic cells is phase changing

material (PCM) applications. When the studies on PCM applications in photovoltaic panels are examined;

In a study with non-PCM applied to the back surface of the photovoltaic panel, 31.94% lower temperature was obtained compared to the standard pv panel. As an hourly efficiency, 1.2% more energy was obtained compared to the standard panel. In total, it was observed that the daily efficiency of the PV panel applied Nano-PCM increased by 8.51% (Stalin et al.,2022).

In a study conducted in India to improve the performance of PV panel output power, an aluminum container was designed to hold the PCM on the back surface of the photovoltaic panel. After adding copper and silicon carbide powders to the PCM material to increase heat transfer, an average of 2.8% more energy was obtained compared to the Standard PV panel, and an average of 4.3% more energy was obtained when combined PCM was used (Kumar, et al.,2021). In order to increase the thermal and electrical efficiency of the photovoltaic panel, thermoconductor filled phase change material (PCM) was used in a study. Different proportions of beeswax and coconut oil have been added to the PCM to increase conductivity. It has been observed that when 12% terephthalic acid powder is added to these oils added to PCM, it provides higher electrical and thermal efficiency (Azimi et al.,2022). In a study using RT-30 as a phase change material on the back surface of the modules to reduce the cell temperature in photovoltaic/thermal panels (PV/T), different systems were used. These are respectively; Paraffin wax RT-30 was compared as phase change material in water-based PV/T-PCM. It has been observed that higher efficiency is obtained from the PV/T-PCM system compared to conventional PV/T (Preet et al., 2017). In order to reduce the loss of efficiency in PV modules, an experimental study was carried out on the back surface of which a heat pipe and calcium chloride hexahydrate phase change material was applied. While the temperature of 30-80°C was measured on the surface of the standard module during the day, a temperature of 30-40°C occurred in the modules using phase change material. While the efficiency loss due to the surface temperature reached 20% in standard modules, 1% efficiency loss was achieved in the modules using phase change material with heat pipes (Kayabasi and Kaya,2020).

In addition to all these, in the case of integrating photovoltaic panels on the building facades, since ventilation cannot be provided behind the PV surface, the module surface temperature increases and causes a decrease in efficiency. Photovoltaic modules to examine this situation; Pure PCM and PCM /Al<sub>2</sub> O<sub>3</sub> nanoparticles were added to the building wall. By using pure PCM and PCM/ Al<sub>2</sub> O<sub>3</sub> nanoparticles in the integration of the modules into the building wall, an increase in PV panel efficiency of 5.7% - 13.2% was achieved (Nada et al.,2018).

In the literature, no study has been found on the use of phase change materials (PCM) in order to reduce the surface temperature of the photovoltaic panel and increase the output power in real climatic conditions of Edirne province. Our aim in this study; The aim is to reduce the module temperature and increase energy production by applying (PCM) Rubitherm RT44HC to the back surface of a polycrystalline photovoltaic panel in real climate conditions.

## METHODOLOGY

### *Photovoltaic panel efficiency and Convective heat transfer*

Technically, there is no difference between the temperature changes in a conventional photovoltaic cell and a combined photovoltaic and thermal collector cell except for the heat sink due to the passing fluid absorbing the dissipated heat from the cell back surface. Hottel–Whillier equation is used to describe the thermal efficiency (Ventura et al., 2021).

$$\eta_{th} = F_R \cdot (\tau \cdot \alpha) - F_R \cdot U_L \left( \frac{T_{min} - T_{amb}}{G} \right) \quad (1)$$

where  $F_R$  =collector heat removal factor;

$U_L$  =collector overall heat loss coefficient factor (W/m<sup>2</sup>K);  
 $(\tau \cdot \alpha)$  =transmittance-absorptance product without electrical output  $T_{amb}$  =ambient air temperature (K);  
 $T_{min}$  =inlet temperature (K);  $G$  =irradiance (W/m<sup>2</sup>).  
 $\dot{E}_{solar} = I_s \cdot A$  (2)

Here,  $I_s$  is the global solar radiation and  $A$  is the PV panel area. On the other hand, the output energy is the electric energy obtained from PV panel ( $\dot{E}_{out} = \dot{E}_{elect}$ ).

Energy efficiency can be defined as the ratio of the input energy of the system to the output energy of the system (Sahin et al., 2007; Gao and Meng, 2020).

$$\eta = \frac{\dot{E}_{out}}{\dot{E}_{in}} = \frac{\dot{E}_{elect}}{\dot{E}_{solar}} = \frac{FF \cdot V_{OC} \cdot I_{SC}}{I_s \cdot A} \quad (3)$$

where,  $FF$  is fill factor,  $V_{OC}$  is open-circuit voltage and  $I_{SC}$  is short circuit current.

Figure 1 shows the working theory of the heat transfer mechanism with a Phase Change Material (PCM).

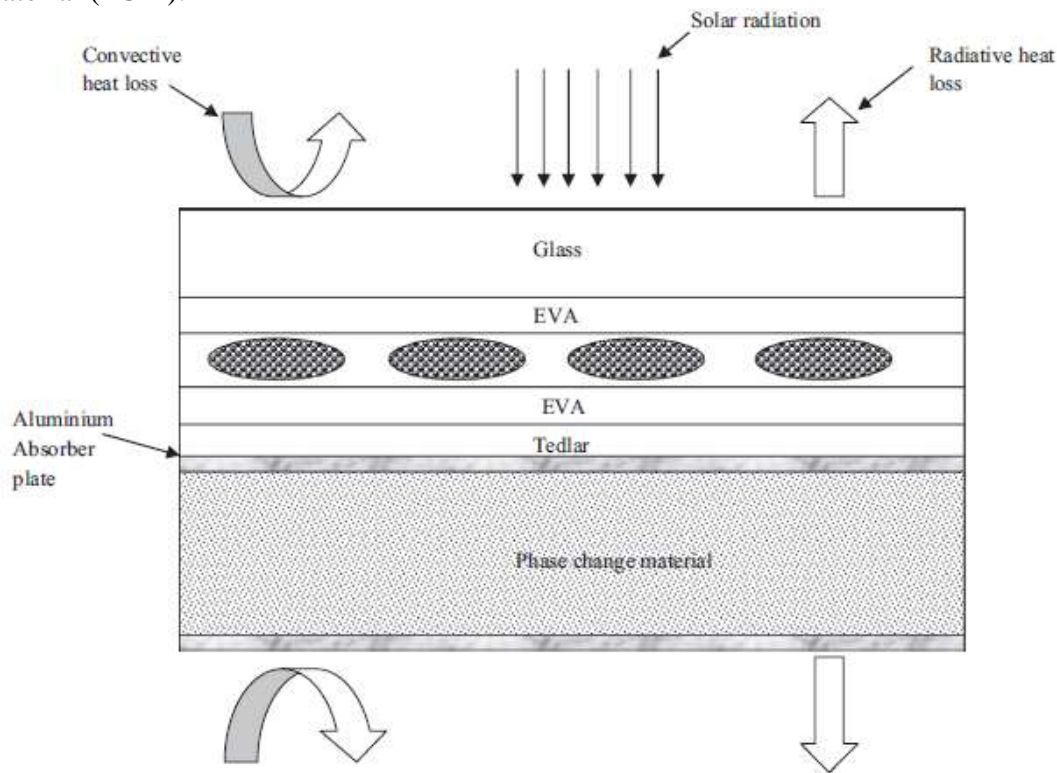


Figure 1 Heat transfer path to and from the photovoltaic with PCM (Preet et al.,2017)

Convective heat transfer are also considered for Photovoltaic front and back sides which takes place by two methods, natural and forced convection. Total heat loss by convection at the surface of photovoltaic panels is expressed as (Preet et al.,2017);

$$q_{conv} = q_n + q_f = (h_{free} + h_{forced}) A (T_{pv} - T_{amb}) \quad (4)$$

$q_n$  is heat losses due to natural convection ,

$$q_n = h_{free} A (T_{pv} - T_{amb}) \quad (5)$$

and forced convection heat transfer,

Heat losses by natural convection is given by Newton's law of cooling ;

$$q_f = h_{\text{forced}} A(T_{\text{pv}} - T_{\text{amb}}) \quad (6)$$

### Experimental Setup

The experimental design in Figure 1 was set up to compare the power generation from photovoltaic panels. Since the experiments were carried out in Edirne province Trakya University Engineering Faculty Campus, the carrier was fixed at an inclination angle of 30°. The system consists of a datalogger system that measures the current and voltage values of the electricity produced simultaneously in the solar panels and records them on the memory card, sensors, batteries, 3 Jinko brand polycrystalline panels with 260 Watt power, and connecting wires (Figure 2). In order to reduce the temperature of the first photovoltaic panel (PV Panel A) in the system, phase change material Rubitherm RT44HC was applied to the back surface. The second photovoltaic panel (PV Panel B) has the power to operate the electronic components of the system. The third photovoltaic panel (PV Panel C) is in standard form to compare the amount of electrical energy produced. In addition, the third photovoltaic panel charges the battery for uninterrupted operation of the system on days without solar radiation. Data were collected regularly over a month at 10-minute intervals. Monthly daily electricity production amounts were calculated for both panels. According to the data obtained, daily/monthly electricity production amounts for both photovoltaic panels are shown in graphics.



Figure 2. Photovoltaic Panel Installation



Table 1 Shows the technical features of the solar panel installed.

**Table 1.** Technical specifications of solar PV module

| <b>Solar Module Type: JKM260P-60</b>             |                  |
|--|------------------|
| <b>Maximum Power (P<sub>max</sub>)</b>           | 260 W            |
| <b>Power Tolerance</b>                           | 0~+3%            |
| <b>Maximum Power Voltage (V<sub>mp</sub>)</b>    | 31.1 V           |
| <b>Maximum Power Current (I<sub>mp</sub>)</b>    | 8.37 A           |
| <b>Open Circuit Voltage (V<sub>oc</sub>)</b>     | 38.1 V           |
| <b>Short Circuit Current (I<sub>sc</sub>)</b>    | 8.98 A           |
| <b>Nominal Operating Cell Temperature (NOCT)</b> | 45±2°C           |
| <b>Maximum System Voltage</b>                    | 1000 VDC         |
| <b>Maximum Series Fuse Rating</b>                | 15 A             |
| <b>Operating Temperature</b>                     | -40°C~+8°C       |
| <b>Application Class</b>                         | A                |
| <b>Weight</b>                                    | 18.5 kg          |
| <b>Dimension</b>                                 | 1650×992×40 (mm) |

Compact storage module (plates) made of aluminum is used as a capsule on the bottom surface of the photovoltaic panel. The dimensions of these plates are 450x300 mm, the thickness is 14.65 mm, and the weight is 0.35 kg. These plates were filled with 1 kg of RT44HC in the liquid phase and sealed. The plates control the changes in the volume of the paraffins, the storage material, as the phase change occurs. Figure 3 shows 6 compact storage modules containing phase change materials fixed to the photovoltaic panel substrate.



Figure 3. Modules Using Phase Change Material

The technical specifications of the RT-44HC phase change material (PCM) belonging to the Rubitherm brand are shown in Table 2.

**Table 2.** Technical specifications of the RT-44HC phase change material ([https://www.rubitherm.eu/media/products/datasheets/Techdata\\_RT44HC\\_EN\\_09102020.PDF](https://www.rubitherm.eu/media/products/datasheets/Techdata_RT44HC_EN_09102020.PDF)).

| <b>The most important data (Typical Values)</b> |                  |
|---|------------------|
| <b>Melting area</b>                             | 41-44(°C)        |
| <b>Congealing area</b>                          | 44-40(°C)        |
| <b>Heat storage capacity ±7.5 %</b>             | 250(kj/kg)       |
| <b>Specific heat capacity</b>                   | 2(kj/kg K)       |
| <b>Density solid (at 25°C)</b>                  | 0.8(kg/l)        |
| <b>Density liquid (at 60°C)</b>                 | 0.7(kg/l)        |
| <b>Heat conductivity (both phases)</b>          | 0.2<br>[W/(m.K)] |
| <b>Volume expansion</b>                         | 12.5 %           |
| <b>Flash point</b>                              | >180 (°C)        |
| <b>Max.operation temperature</b>                | 70 (°C)          |

## RESULT AND DISCUSSION

The daily energy totals obtained from photovoltaic panels are shown in Figure 4. More daily energy was obtained from panel A, in which phase-change material was used, compared to the standard panel (PV Panel C).

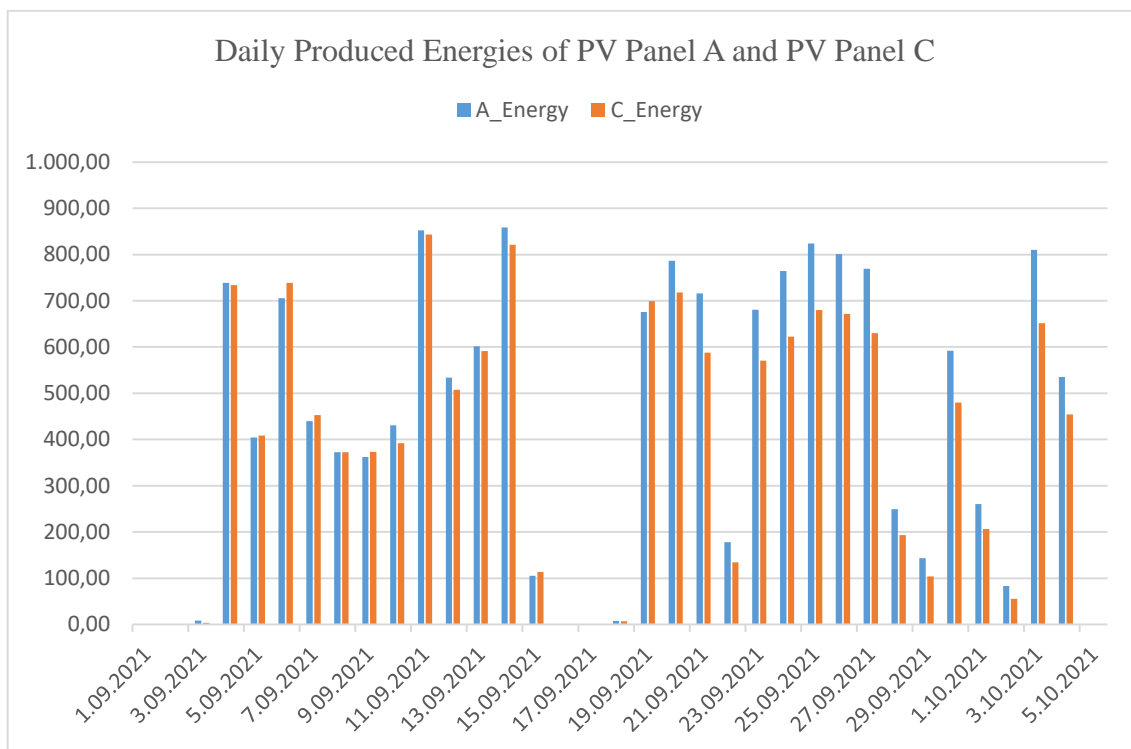


Figure 4. Daily produced energies of PV Panels

When the daily energy amounts obtained from photovoltaic panels are examined, the highest amount of energy was obtained from PV Panel A on 14.09.2021. The hours at which the maximum energy is produced are shown in Figure 5.

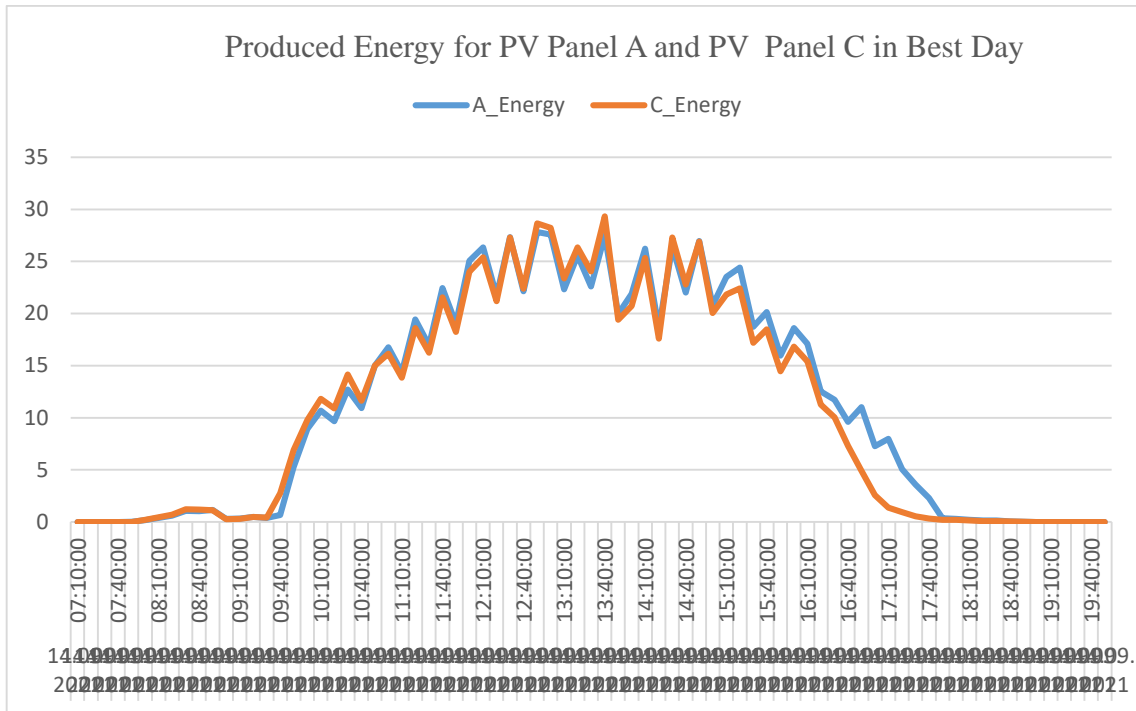


Figure 5. Produced energy for PV Panels in best day

When the daily energies obtained from photovoltaic panels are examined, the lowest production was obtained on 02.10.2021. As seen in Figure 6, more energy was obtained from PV panel C even on the day with the least energy production.

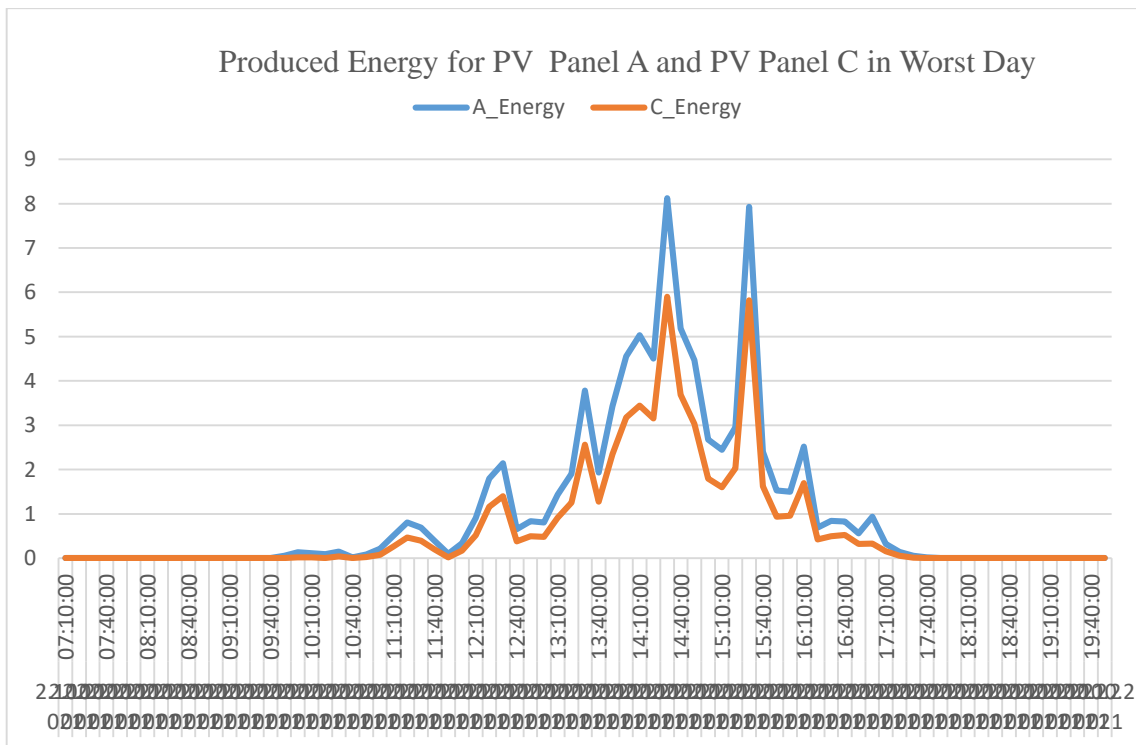


Figure 6. Produced energy for PV Panels worst day

The date 23.9.2021 was chosen randomly to examine the daily amount of energy obtained from photovoltaic panels. As seen in Figure 7, more energy was obtained from PV Panel A in a randomly selected day.

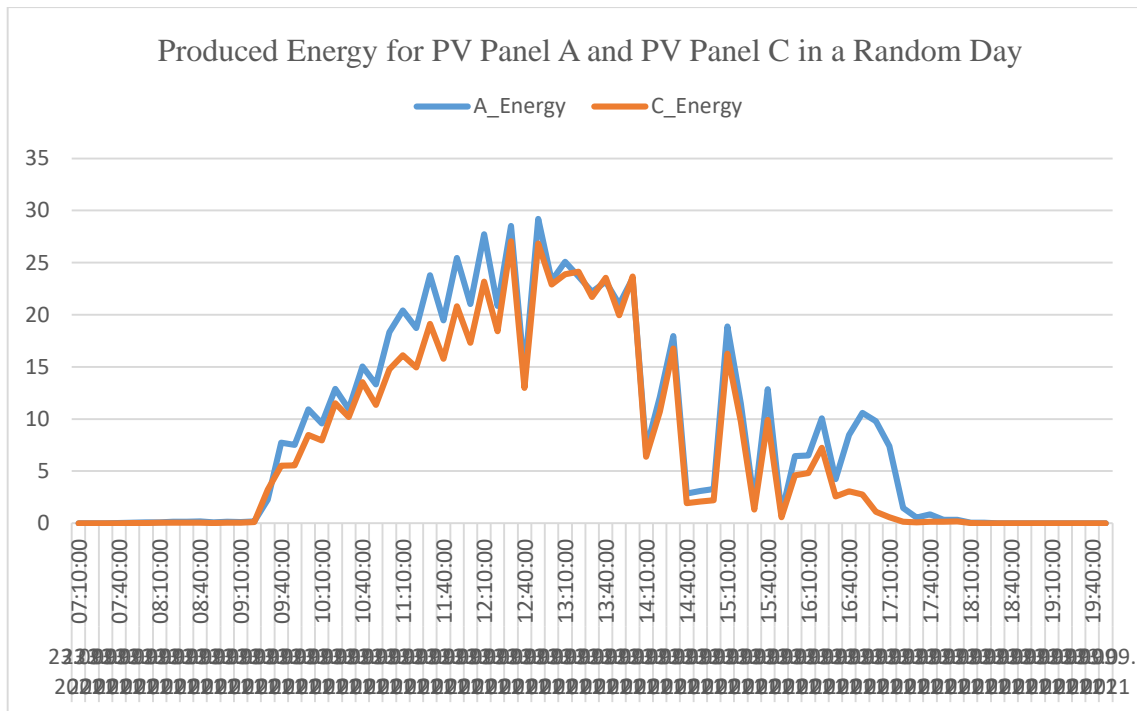


Figure 7. Produced energy for PV Panels ia a random day

Total energies obtained from photovoltaic panels are shown in Table 3. When Table 3 is examined, it is seen that 9.8% more energy is obtained from PCM-applied Panel A compared to standard PV Panel C.

**Table 3.** Total amount of energy obtained from Pv panels

|  | <b>Panel A</b> | <b>Panel C</b> |
|--|----------------|----------------|
| <b>Overall Average Power (W) (only from 7:00 to 20:00 daily)</b> | 48,33          | 43,68          |
| <b>Maximum produced peak power (W)</b>                           | 184,95         | 205,37         |
| <b>Overall Produced Energy (Wh)</b>                              | 15.290,75      | 13.919,02      |

## CONCLUSION

In this study carried out in real climatic conditions, the effect of temperature, which reduces the PV panel output power, is reduced by using phase change material (PCM). With RT44HC (PCM) applied to the back surface of the PV panel, 9.85% more electrical energy was obtained in one month compared to the standard PV panel. Maximum, minimum and random energy production dates were selected for daily comparisons of PV panels. It is seen that more energy is obtained from the phase-changing PV panel on these selected reference days.

In order to reduce the surface temperature of photovoltaic panels and to obtain more energy, it will be useful to evaluate the results by using phase change materials with different properties in future studies.

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## PHENOLOGICAL DEVELOPMENT AND GRAIN YIELD PRODUCTIVITY OF WINTER PEA GENOTYPES IN TWO LOCATIONS

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### ABSTRACT

Forage peas hybrid lines №PL, №6, №11, №12A, №13, №14, Taskent, Tore and Mir variety (standard) were studied in point of view phenological development, viz. the beginning of flowering stage after sowing, technical maturity, vegetation period duration. The study was performed in two locations both situated in the Central part of the Danube hilly plain region of Bulgaria (2017-2019). Location A (43° 23'N, 24° 34'E, 230 m altitude), podzolized soil subtype; location B (43.41° N, 24.61° E), haplustoll soil subtype. The lines and cultivars studied differ in the phenological development. For the location A, the earliest lines at the beginning of flowering stage were found №14, followed by №PL, №12, Mir and №6, Tore, №13, №11. The difference in days was 13. For the location B the earliest lines at the beginning of flowering stage were found №14, №PL, №12A, Mir, №6, Tore and №11. The difference in days was 16. Vegetation period of the genotypes studied was found shorter for the location B with 17 days despite the late sowing data. For both locations the earliest lines/cultivars were found №14 (coef. 1.00, followed by №PL (coef. 1.42), №12A (coef. 1.46), Mir (coef. 1.50) and №6 (coef. 1.60). From the tested samples pea line №14 and №PL were highly productive in grain. Lines №6, №12A and the Taskent variety were responsive with average grain yield. Therefore, the line №14 and line №PL are of interest for both earliness and grain productivity. The data obtained can be a contribution to the selection of winter forage pea to create new genotypes with pronounced both early maturity and grain productivity.

**Keywords:** Pea, Variety, Selection, Phenological development

### INTRODUCTION

Peas are a crop that has long been grown and cultivated, and is certainly of growing importance for global agricultural production. The need for protein-rich products for food for both humans and farm animals has led to greater interest in this crop. It is resistant to adverse climatic conditions and is one of the main crops suitable for growing in different soil and climatic conditions. It uses abiotic factors very effectively, such as soil and atmospheric temperature, and soil and air humidity. Pea has a relatively short vegetation period and provides the possibilities for additional economic use of the agricultural land (Zotikov and Borovlev, 2008). This legume crop plays an important role as a source of vegetable protein in solving the protein problem of livestock production.

In addition to its nutritional and fodder value, peas are used in crop rotation as they contribute to increasing soil fertility and are a factor in the biological intensification of crop production. As a legume, under favorable conditions it can fix up to 150 kg N/ha and accumulate in the soil 45-70 kg N/ha (Unkovich and Pate, 2000; Voisin et al., 2003; Clayton et al., 2004). Its use allows to reduce the use of synthetic mineral fertilizers due to the nitrogen fixing potential (Kosev and Vasileva, 2014, Vasileva and Kosev, 2015). Its advantage is not

only for obtaining plant production with a high protein content, but also more stable yields of both grain and green mass (Brezhneva and Brezhnev, 2014).

The biological characteristics of pea enable it to be successfully grown as a winter crop. Winter pea play an important role in the southern regions. The intensive growth and development of winter pea varieties occur during the period May-June, when rainfall is sufficient to ensure an intensive growth without irrigation. The winter forms are suitable in regard to the climate change occurred and the interest in them justifiably increases (Christensen et al., 2013; Elbehri et al, 2015).

The purpose of the work was to study the phenological development and grain yield productivity of winter pea genotypes in two locations.

## **MATERIAL AND METHOD**

Forage peas hybrid lines №PL, №6, №11, №12A, №13, №14, Taskent, Tore and Mir variety (standard) were studied for their phenological development, viz. the beginning of flowering stage after sowing, technical maturity, vegetation period duration. The study was conducted on the experimental field of the Institute of Forage Crops – Pleven (2017-2019), situated in the Central part of the Danube hilly plain region of Bulgaria. Two locations were used, viz. location A (43° 23'N, 24° 34'E, 230 m altitude), podzolized soil subtype; location B (43.41° N, 24.61° E), haplustoll soil subtype.

The seeds were sown in plots in six repetitions with a plot size of 10 m<sup>2</sup> for location A and in plots in two repetitions with a plot size of 2 m<sup>2</sup> for location B. The sowing was done during October. During the vegetation all observations were performed for phenological dates periods of sowing-beginning of flowering and sowing-technical maturity, vegetation period duration (days). The degree of earliness was calculated by Dimova and Marinkov (1999). Criteria for assessing the degree of earliness was adopted the date of the beginning of flowering, and for the quantitative assessment the coefficient of earliness was used. Grain yield (kg/da) was recorded. Experimental data are presented by year and average for the study period. They were statistically processed using STATGRAPHICS Plus for Windows Version 2.1.

## **RESULTS AND DISCUSSION**

It is common knowledge that the level of plant productivity depends on the interaction of the genotype with environmental factors. Essential for the formation of productivity in pea, is sufficient moisture during the growing season and the required amount of active temperature. The sowing of the winter forage pea was carried out at the end of the second decade of the month of October. The whole month of October is characterized by precipitation (108.9 mm/m<sup>2</sup>) (Table 1), which exceeds by two times the mean value for the 20-year period (52.3 mm/m<sup>2</sup>) (1996-2016) (Table 3), as well as normal daily average temperatures. In February and March the amount of rainfall is above the norm for the month. The months of April and May are extremely dry. In May the temperatures are high, with a rainfall of economic significance (18 l/mm<sup>2</sup>). Stages of beginning of flowering to full bottom pods running in these conditions. The many rainfalls fallen in June have not influenced the yield, as it has already been formed. In the first location the quantity of rainfall fell is greater, which determined the longer vegetation period, as well as the higher plant height and higher green mass yield of the late cultivars/lines.

The winter months of 2019 (Table 2) were characterized by below-normal rainfall compared to the 20-year period and higher average daily monthly temperatures. Small amounts of precipitation were reported in mid-March. This favored the initial growth of the crop. In April,



a higher average temperature and precipitation was more than twice as high as for a twenty-year period.

Throughout May, precipitation was evenly distributed with amounts slightly above the average for the month and a favorable average daily temperature. This affected the growth and development of pea. The month of June was characterized by precipitation during all three days and the amount of precipitation for the month was slightly above the value for a twenty-year period.

Table 1. Agro meteorological conditions for the period of September 2017 to June 2018 (Pleven, Bulgaria)

| Months | Rainfall | toC  |
|--------|----------|------|
| 2017   | mm/m2    |      |
| IX     | 37.4     | 19.5 |
| X      | 108.9    | 12.6 |
| XI     | 55.4     | 7.2  |
| XII    | 50.6     | 5.0  |
| 2018   |          |      |
| I      | 30.9     | 2.2  |
| II     | 72.0     | 2.2  |
| III    | 98.1     | 5.3  |
| IV     | 20.2     | 16.9 |
| V      | 47.7     | 19.6 |
| VI     | 155.2    | 21.8 |

Table 2. Agro meteorological conditions for the period of September 2018 to June 2019 (Pleven, Bulgaria)

| Months | Rainfall | toC  |
|--------|----------|------|
| 2018   | mm/m2    |      |
| IX     | 15.4     | 18.9 |
| X      | 16.1     | 13.6 |
| XI     | 61.9     | 5.4  |
| XII    | 30.6     | 1.2  |
| 2019   |          |      |
| I      | 17.7     | 0.3  |
| II     | 22.6     | 4.4  |
| III    | 19.0     | 10.1 |
| IV     | 116.8    | 12.0 |
| V      | 82.8     | 17.0 |
| VI     | 89.6     | 22.4 |

Table 3. Agro meteorological conditions for 20-year period (1996-2016) (Pleven, Bulgaria)

| Months | Rainfall<br>mm/m <sup>2</sup> | t <sub>0</sub> C |
|--------|-------------------------------|------------------|
| I      | 35.7                          | 0.1              |
| II     | 39.1                          | 2.6              |
| III    | 50.9                          | 7.5              |
| IV     | 55.5                          | 13.0             |
| V      | 61.8                          | 18.4             |
| VI     | 56.3                          | 22.1             |
| VII    | 65.0                          | 24.3             |
| VIII   | 48.8                          | 23.8             |
| IX     | 70.2                          | 18.3             |
| X      | 52.3                          | 12.7             |
| XI     | 29.5                          | 7.2              |
| XII    | 41.5                          | 1.5              |

Table 4 and Table 5 show data on the beginning dates of the onset of phenological stages, beginning of flowering and technological maturity of peas for the two locations. The lines and cultivars studied differ in the phenological development. The beginning of flowering for location A continues from 21.04. to 04.05. and the vegetation period (Figure 1) varied between 184 and 197 days, or averaged 191 days. The earliest lines/cultivars were found №14, followed by №PL, №12A, Mir and №6, Tore and №13, №11 and the latest was Taskent (Figure 2). The difference in days for the vegetation period was 13.

Table 4. Phenological development of the winter forage pea cultivars/lines (Location A)

| Cultivar/Line | Data of sowing | Beginning of flowering | Technical maturity |
|---------------|----------------|------------------------|--------------------|
| Mir           | 19.10.2017     | 27.04.2018             | 18.06.2018         |
| №PL           | 19.10.2017     | 25.04.2018             | 22.06.2018         |
| №6            | 19.10.2017     | 27.04.2018             | 20.06.2018         |
| №11           | 19.10.2017     | 02.05.2018             | 25.06.2018         |
| №12A          | 19.10.2017     | 26.04.2018             | 18.06.2018         |
| №13           | 19.10.2017     | 30.04.2018             | 26.06.2018         |
| №14           | 19.10.2017     | 21.04.2018             | 11.06.2018         |
| Taskent       | 19.10.2017     | 04.05.2018             | 29.06.2018         |
| Tore          | 19.10.2017     | 30.04.2018             | 26.06.2018         |

The technical maturity stage occurring from 11.06. to 29.06. and earliest lines/cultivars were found №14, followed by №12A and Mir, №6, №PL, №11, Tore and №13. The latest was found Taskent. The difference for vegetation period in days was 18.

Table 5. Phenological development of the winter forage pea cultivars/lines (Location B)

| Cultivar/Line | Data of sowing | Beginning of flowering | Technical maturity |
|---------------|----------------|------------------------|--------------------|
| Mir           | 02.11.2017     | 27.04.2018             | 15.06.2018         |
| №6PL          | 02.11.2017     | 27.04.2018             | 18.06.2018         |
| №6            | 02.11.2017     | 30.04.2018             | 16.06.2018         |
| №11           | 02.11.2017     | 30.04.2018             | 21.06.2018         |
| №12A          | 02.11.2017     | 27.04.2018             | 14.06.2018         |
| №13           | 02.11.2017     | 04.05.2018             | 22.06.2018         |
| №14           | 02.11.2017     | 18.04.2018             | 08.06.2018         |
| Taskent       | 02.11.2017     | 02.05.2018             | 25.06.2018         |
| Tore          | 02.11.2017     | 30.04.2018             | 22.06.2018         |

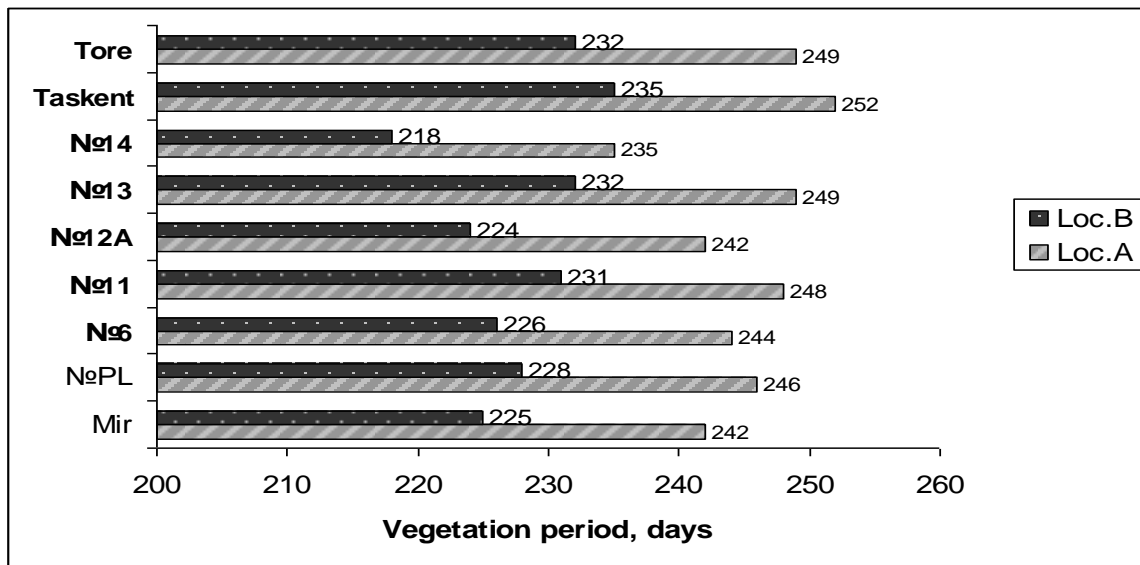


Figure 1. Vegetation period of winter forage pea cultivars/lines for two locations

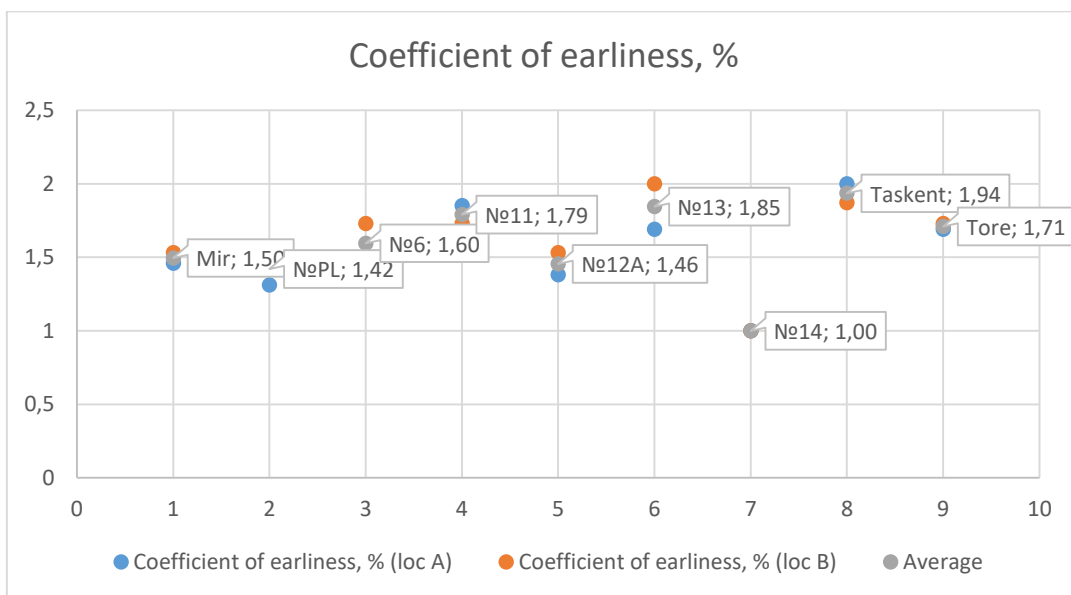


Figure 2. Coefficient of earliness of winter forage pea cultivars/lines for two locations

The duration of vegetation period affects not only grain yield and timely crop harvest, but also the production of high quality seed material. Its duration, as well as the individual phenological stages, depends to a large extent on environmental conditions. Knowing the duration of the vegetation period is necessary first and foremost to gain an idea of the earliness of the variety and to establish the possibility of growing it in one or another soil climatic zone (Ashiev, 2014). Beginning of flowering for location B varied from 18.04. to 04.05. The earliest lines/cultivars was №14, followed by №PL, №12A and Mir, and №6, Tore and №11, Taskent. The latest was line №13. The difference in vegetation period days was 16. The technical maturity stage occurring varied from from 08.06. to 25.06. and the earliest was line №14, followed by №12A, Mir, №6, №PL, №11 Tore and №13 and the latest was Taskent. The difference in days for the vegetation period was 17.

As a whole for both locations the earliest were found lines №PL, №12A, №6, and Mir cultivar. Vegetation period of assessments was found shorter for the location B with 13 days despite the late sowing data. In addition, there were more precipitations in October when the sowing for location A was done.

The observed phenological differences between the lines, regardless of the fact that they are genetically determined, are primarily due to the changes in climatic conditions during the year. The differences in the occurrence and duration of the individual phenological stages, especially the later ones, are preserved until the end of the growing season, following the individual biological characteristics of the plants.

Early maturity is a varietal characteristic, but it is also very dependent on weather conditions. A variety with a long growing season can be early-ripening and form a high grain yield, under favorable weather conditions, where it avoids drought in critical stages.

In the perspective lines of winter forage pea one of the goals is early maturity. More of cultivars were improved on this basis. Those with improved early maturity are also characterized by faster growth, development and a short growing season. The early maturity and after the possibility of earlier ripening is very important direction of selection of winter forage pea. However, the number of winter varieties is limited and it is difficult to create a varietal structure, according to the big climatic diversity of the country. The goal of the breeders in terms of winter forage pea is to increase winter hardiness also. Due to frequent droughts in the fall, it germinates later and is subject to adverse conditions after germination.

The results of Tiurin (2014) in vetch show that the more the plant forms a leaf-stem mass, so the period of germination to flowering is longer. This shows that the selection of earliness due to the shortening of the interphase period of germination - the beginning of flowering without compromising the productivity of the green mass will be associated with a number of difficulties.

Figure 3 and Figure 4 showed the obtained grain yields of the winter pea varieties and lines tested in the two locations. Yields were found directly dependent on rainfall during the growing season. Comparing the grain yields from the two locations, it is noticeable that for location A the excess compared to the Mir variety (control) was from 12.1 to 40.2%. Up to 533 kg/ha more grain was obtained than the control. For location B, the excess was from 12.3 to 43.2%, obtaining up to 432 kg/ha more grain than the control.

Proven highest yield compared to the Mir variety (control) at both locations were found line №14 and №PL, followed by line №12A. The excess for line №14 reaches 40% compared to the standard. Of the Turkish varieties, only Taskent in terms of grain yield approaches the sample average.

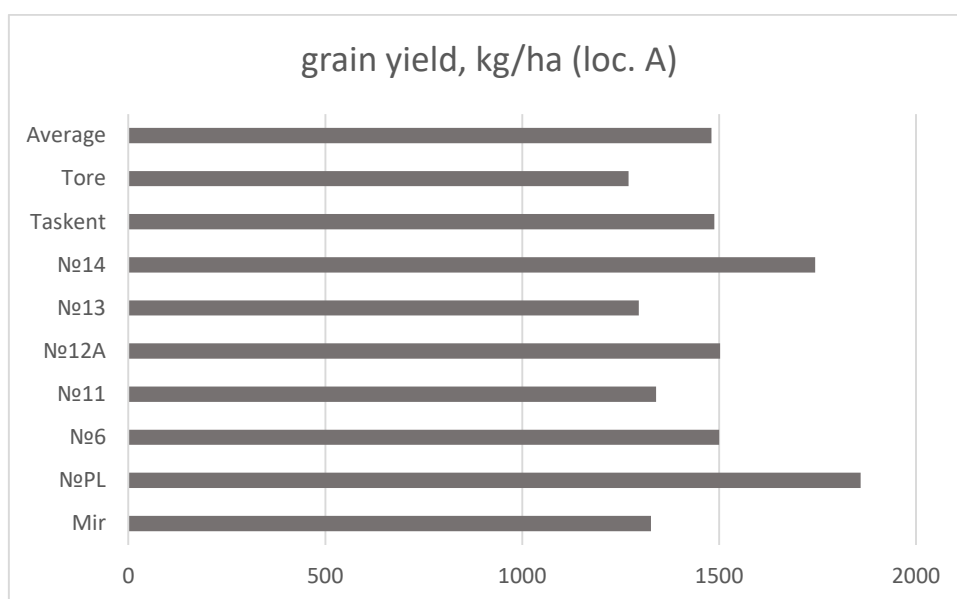


Figure 3. Grain yield of winter forage pea cultivars/lines for location A

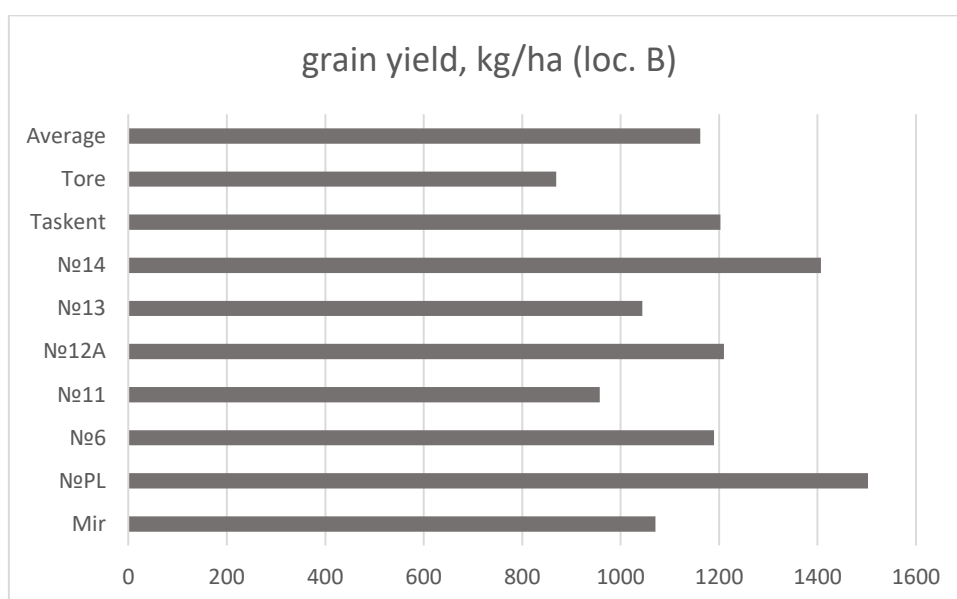


Figure 4. Grain yield of winter forage pea cultivars/lines for location B

From the tested samples, pea line №14 and №PL were highly productive. Lines №6, №12A and the Taskent variety are responsive with average grain yield. Line №13, varieties Mir and Tore are the least productive.

In another study, we found a close relationship between the amount of precipitation during flowering and grain yield ( $r = 0.73-0.85$ ) (Vasileva et al., 2020). Pea productivity depends more on the amount of rainfall that falls during flowering and seed filling. A similar dependence was observed between the yield level of the studied varieties and the temperature values. The seed productivity of a given genotype consists of a harmonious combination of all the elements of its components. Analyzing the morphological-productive elements and the influence of climatic factors, Vozian et al. (2007) found a weak relationship between temperature values and the number of pods per plant.

## CONCLUSIONS

For the location A, the earliest lines at the beginning of flowering stage were found №14, followed by №PL, №12, Mir and №6, Tore and №13, №11. The difference in days was 13. For the location B the earliest lines at the beginning of flowering stage were found №14, №PL, №12A and Mir, №6, Tore and №11, Taskent. The difference in days was 16. Vegetation period of the genotypes studied was found shorter for the location B with 17 days despite the late sowing data. For both locations the earliest lines/cultivars were found №14 (coef. 1.00), followed by №PL (coef. 1.42), №12A (coef. 1.46), Mir (coef. 1.50) and №6 (coef. 1.60). From the tested samples pea line №14 and №PL were highly productive in grain. Lines №6, №12A and the Taskent variety are responsive with average grain yield. Therefore, the line №14 and line №PL were found of interest for both earliness and grain productivity. The data can be used for the further breeding process in pea.

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## AQUATIC FLORA OF VJOSA RIVER

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### ABSTRACT

The paper provides an overview of the Vjosa river besides major gaps in knowledge Vjosa is one of the richest river in Albania, sheltering a high diversity of habitats and species, most of them of international significance. A variety of protected areas is an important ecological corridor. The aquatic flora has attracted the interest of botanists for many years, however, the botanical studies regarding aquatic plants have been limited. The study area is partly included in the European network of protected areas ‘Natura 2000’ and is of great scientific interest due to Vjosa’s biodiversity. In the floristic inventory of the studied areas two taxa, *Potamogeton sp.* and *Chara sp.* are reported more abundant. Furthermore, the bryophytes taxa. Several floristic parameters related to life forms and geographical distributions of plant species (chorology) were examined and some ruderal, and invasive species were described. Additionally, human activities in river basins in recent years have changed the structure and composition of plant communities with the introduction of ruderal and alien species. Under the motto “save Vjosa as the last wild river in Europe” we should inventory, study, and save Vjosa for the better future.

**Key words:** Vjosa, ecological corridor, European network, chorology.

### INTRODUCTION

Vjosa River Valley is not only magnificent ecosystem but such these kind of habitats services for human well-being. It is one of Europe’s last living wild rivers. Along its entire course of over 270 kilometers it is untamed and free flowing and characterized by beautiful canyons, braided river sections, islands, oxbows and meandering stretches. In some areas the riverbed expands over more than 2 km in width. What makes this river really outstanding internationally is the fact, that almost all its tributaries are free flowing and intact as well, creating a living rivers network in Europa. However, very little is known about its biodiversity flora and vegetation. There is a lot to do for river management under the EU regulations, e.g. the Water Framework Directive, in the main part is focused on the biological parameters highlighting the main flora species and coordinate the chemical-physical-biological parameters in the same language in order to present an ecological statues regarding the EU standards. From the riverbank to the forest are some habitats with specific statues that should be evaluated and treated like treasure ecosystems.

### MATERIAL AND METHODS

The taxonomic survey was done during the period 2021-2022. Investigations included data collected during field trips regarding the project, “Assessment of the ecology of the waters of the Vjosa River catchment based on microalgae and Physico-chemical parameters” PKKZH.



Habitat parameters (bank structure, land use type and riverine areas) were assessed in each survey unit. Number, size, distribution pattern of the stations and transects depend on the size and heterogeneity or diversity of habitats situated in the Vjosa valley, as well as on the bio-ecological characteristics of the species or group of species.



**Fig. 1.** The station sites are selected in the significant points of habitats: *S-01-Tre Urat*, *S-02 Sajmole*, *S-03 Memaliaj*, *S-04 Pocem*, *S-05 Ura e Mifolit*.

Data on vegetation were obtained through 50 relevés carried out in June–July and September 2021–2022. At each site the relieves were selected in relation to the homogeneity of physical features and vegetation structure. Plot sizes ranged from 10 m<sup>2</sup> to 1m<sup>2</sup> depending on the plant community. Vascular plants were identified using the “Flora e Shqipërisë” [1], [2], [3], [4], [5]. Plant communities of Vjosa valley were identified only according to expert knowledge and scientific literature. The survey methods used were entirely comparable [7], [8], [9], [10], into the approach developed by [10]. Field surveys were generally shorter (around 10 m) with longer reaches with similar sampling areas. All macrophytes (mostly vascular plants) growing in the water or rooting below the water surface were recorded along relatively short river stretches. Abundance-Dominance of taxon are calculating through Natura 2000 code.

We also included plant’s statue based on the IUCN Codes [6].

- Extinct (EX),
- Extinct in the wild (EW)
- Critically endangered, (CR)
- Extremely high risk of extinction in the wild, endangered (EN)
- High risk of extinction in the wild, Vulnerable (VU)
- High risk of endangerment in the wild, near threatened (NT)
- Likely to become endangered in the near future, least concern (LC)
- Lowest risk (does not qualify for a more at-risk category; widespread and abundant taxa are included in this category), Data deficient (DD)
- Not enough data to make an assessment of its risk of extinction,
- Has not yet been evaluated against the criteria, Not evaluated (NE)

The species extinction risk assessment is based on the Guidelines for IUCN Categories and Criteria Application version 4.0 (IUCN 2012a, 2012b).

## RESULTS

The main macrophytes identified are listed below. The Table 1 showed the abundance-dominance and the main sites where the vegetation is presented.

Table 1. The main macrophytes identified

|     | <b>Macrophytes</b>                 | <b>A-D</b> | <b>Status</b> | <b>Location</b>    |
|-----|------------------------------------|------------|---------------|--------------------|
| 1.  | <i>Alisma plantago-aquatica</i> L. | +          | EN (A1b)      | S-05 Ura e Mifolit |
| 2.  | <i>Chara</i> sp                    | ++         | VU (A1b)      | S-01-Tre Urat      |
| 3.  | <i>Ceratophyllum demersum</i> L    | +++        | VU (A1b)      | S-05 Ura e Mifoli  |
| 4.  | <i>Mentha aquatica</i> L           | +++        | EN (A1b)      | S-02 Sajmole       |
| 5.  | <i>Myriophyllum spicatum</i> L.    | ++         | EN (A1b)      | S-03 Memaliaj      |
| 6.  | <i>Phragmites australis</i> L.     | ++         | VU (A1b)      | S-05 Ura e Mifolit |
| 7.  | <i>Plantago lanceolata</i> L.      | +          | EN (A1b)      | S-02 Sajmole       |
| 8.  | <i>Potamogeton crispus</i> L       | ++         | EN (A1b)      | S-03 Memaliaj      |
| 9.  | <i>Potamogeton gramineus</i> L.    | +++        | EN (A1b)      | S-03 Memaliaj      |
| 10. | <i>Potamogeton lucens</i> L.       | ++         | EN (A1b)      | S-05 Ura e Mifolit |
| 11. | <i>Potamogeton pectinatus</i> L.   | +          | EN (A1b)      | S-04 Pocem         |
| 12. | <i>Potamogeton perfoliatus</i> L.  | +          | LR (cd)       | S-02 Sajmole       |
| 13. | <i>Sagittaria sagitifolia</i> L.   | +          | LR (cd)       | S-04 Pocem         |
| 14. | <i>Salvinia natans</i> (L.) All.   | +++        | LR (cd)       | S-04 Pocem         |
| 15. | <i>Trapa natans</i> L.             | +          | VU (A1b)      | S-02 Sajmole       |
| 16. | <i>Typha minima</i> L.             | +          | LR (cd)       | S-05 Ura e Mifolit |
| 18. | <i>Vallisneria spiralis</i> L.     | +          | LR (cd)       | S-01-Tre Urat      |
| 19. | <i>Zannichellia palustris</i> L.   | +          | LR (cd)       | S-01-Tre Urat      |

- + Rare, ++ Abundant, +++ Frequent
- The code corresponds to the NATURA 2000 code.

In the Vjosa River valley the species richness of the aquatic vegetation is at a medium level as compared to other reaches in the river. Yet, little plant mass is developed in the near-bank littoral. Some species are ubiquitous, but their plant mass is also minimal. Regarding these conditions, it is a surprise to find the high number of 16 aquatic species. Although the plant stands were small there are enough niches in the littoral near the banks of the main river, as well as in the oxbow, in which non-rooted plants can survive. *Salvinia natans* occurred in two survey units in the main channel and even *Ceratophyllum demersum*, a species of much larger size, was present in most of the stretches. All other species occurred to a much smaller extent. However, there is no development of larger plant mass and further investigations may reveal the possible reasons for this unexpected situation.

Plants association in the Vjosa river can group on 5 typical riverine habitats:

1. The first group of non-aquatic plant associations or the bottoms of rivers or (Aquatic Bed) mainly dominated by different types of Algae (*Chara* sp.)

2. The group of submerged plant associations dominated by species of the genera *Potamogeton*, *Myriophyllum*, *Najas*, *Vallisneria*, etc. known as (Rooted Vascular)
3. The third group includes associations of helophytic plants, semi-submerged plants that have their roots and part of their stems in water, but most of their bodies are above water. Among the main plants of this group are reeds (*Phragmites australis*), (*Typha*) etc. (Persistent emergents).
4. The fourth group includes types with floating leaves, (*Trapa natans*) known like (Floating-leaved). In general, these plant associations are more widespread or find the most optimal conditions in the waters of the lower flow of rivers, with calmer and slower waters, generally in bays protected from winds and waves or "pockets", and in places not rocky.
5. The fifth group refers to scrub or woody formations or environments that are seasonally flooded (Temporarily emergents) on the banks of rivers (Scrub-Shrub and Forested) dominated by the species *Salix sp.*, *Alnus glutinosa*, *Platanus orientalis* etc.

The ecological potential of river ecosystems has been damaged and continues to be damaged by:

- Inappropriate use of natural resources,
- Uncontrolled development,
- Deviated hydrology,
- Irrigation practices that ignore ecological issues,
- Massive deforestation in river valleys,
- Overgrazing,
- Erosion,
- Water and land pollution
- Consequences in Biodiversity
- Habitat change/degradation,
- Increased deposition of alluvium,
- Loss of water surfaces,
- Change in terrestrial and aquatic vegetation,

## CONCLUSION

the highly undisturbed river dynamics and the river-floodplain ecosystems along the Vjosa are in an excellent conservation status. All riverine habitats typical for the Vjosa are listed in the Annex 1 of European Union Habitats Directive, underpinning their importance for conservation at a European scale. They harbor viable community of species that have largely or completely disappeared from other European rivers systems.

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