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Recent Advances in High Pressure Processing of Milk and Milk Products - A review

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ABSTRACT

As the global consumers' demand towards minimally processed fresh-like foods has been continuously increasing, efforts to develop novel food processing technologies have been intensified. Among non-thermal food processing technologies, high pressure processing (HPP) seems to be more advantageous due to its environmentally friendly nature, cost efficiency, suitability for processing foods in any form and its positive impacts on foods' shelf-life as well as providing efficient microbial safety. Microbiological inactivation efficiency of HPP has been well documented but the role of this technology in digestion efficiency of milk compounds is yet to be elucidated in detail. Also, the potential safety hazards and challenges of HPP in foods require more intense studies. This review deals with the recent developments in HPP treatment to milk and milk products.

Keywords: High hydrostatic pressure, Shelf-life, Quality, Dairy, Nutrition

1. Introduction and Technological Background of High Hydrostatic Pressure

Food consumption paradigms have changed dramatically during the last two decades. Today, consumers are keener on consuming safer, healthier and minimally processed fresh-like foods and this demand has become more pronounced during coronavirus disease-2019 pandemic. The efficiency of thermal applications to foods including thermization, pasteurization, ultra-high pasteurization and ultra-high temperature treatments on providing food safety has been known for many decades. Although thermal technologies are widely employed in food processing worldwide, possible adverse effects of heat treatment on some food components have motivated the food community to develop alternative technologies to heating. Efforts have been intensified to adapt some non-thermal food processing technologies from lab/pilot scale to industrial scale, and some non-thermal food processing technologies are now enjoying market success and consumer acceptance. High hydrostatic pressure (HPP), pulsed electric field (PEF), ultrasound, cold plasma, ozonization, irradiation and ultraviolet light are some examples of non-thermal food processing technologies. These technologies are employed in shorter processing time and at lower temperatures which leads to improvement of the nutritional quality of foods as well as sensory quality. Also, the shelf-lives of non-thermally processed foods are improved without impairing the food safety parameters (Mahalik & Nambiar 2010; Alexandre et al. 2012).

HPP has gained popularity in food processing faster than the other non-thermal technologies. About 75% of the scientific papers and patents on non-thermal food processing technologies are directly related with HPP. The jam was the first commercial HPP-processed food introduced into the markets in 1990 followed by retort rice products, cooked hams, and sausages (Yamamoto 2017). HPP is based on the Le Chatelier principle:

"If a dynamic equilibrium is disturbed by changing the conditions, the position of equilibrium moves to counteract the change"

In HPP treatment, isostatic pressure is transmitted to the product through water. Since the water used in HPP is recyclable, it brings about an advantage of reduced energy consumption and environmental protection (Toepfl et al. 2006). The pressure is transmitted uniformly and instantaneously throughout the product, therefore achieving an effect equivalent to pasteurization. A HPP equipment is made up of a high-pressure vessel and its closure, a pressure generator and a material handling mechanism equipped with a temperature control system (Datta & Deeth 1999). The pressure range applied to foods may vary between 300 MPa and 900 MPa depending on the structure and initial microbial load of the product. In practice, the pressure range of 400-600 MPa is sufficient enough to provide microbial safety in many foods (Trujillo 2002). HPP has minimal effect on sensory, nutritional and textural characteristics of the foods (Rendueles et al. 2011; Grundy et al. 2016). The pressure, holding time and temperature applied are the major parameters determining the effectiveness of microbial inactivation.

HPP allows drastically reducing or eliminating the use of preservatives or additives in food. Also, HPP prevents food waste on the retail shelf and in the consumer's refrigerator since it has an extended shelf life (Trujillo et al. 2002). Depending on the processing conditions and product characteristics, the shelf-life of the HPP-treated foods may be extended up to three-fold. HPP may be applied to pre-packed liquid or solid foods. Some high-pressure equipment are suitable for continuous production as well. In the latter case, an aseptic filling may be required depending on the shelf-life expectations and/or safety requirements of the end product. Although HPP has many advantages over traditional heating systems regarding microbial inactivation, it is not effective in the elimination of spores (Pinto et al. 2020). Also, HPP may cause some textural and colour changes in foods under question. Finally, HPP technology is not recommended for the processing of dry products and to ensure microbiological safety, a minimum water activity of 0.8 in foods is required.

Non-thermal food processing technologies are accepted as novel technologies in many countries. Some countries mandate risk analysis of the products manufactured using one or more of the non-thermal food processing technologies. European Union (EU) accepts HPP technology as a 'novel' food processing technology (EC 258/97). As in the standard applications of the EU, the regulations are in the form of 'roof regulation' and the applicability of novel technologies including the implementation details are determined by country regulations. At this point, a position paper published by French authorities in 2010 on HPP technology includes the statement that applications performed at room temperature and around 5 minutes up to 600 MPa pressure are harmless (Jung & Tonello-Samson 2018). This means no risk analysis is required for such foods as long as the conditions stated above are strictly followed. According to the EU framework agreement (European Community Treaty), products produced in any community member country have the right of free circulation within the EU. Today, according to "Regulation of the European Parliament and of the Council on novel foods, amending Regulation (EU) No 1169/2011 of the European Parliament and of the Council and Repealing Regulation (EC) No 258/97 of the European Parliament and of the Council and Commission Regulation (EC) No 1852/2001", application of HPP in food processing is allowed. By now, no chemical and/or microbiological risk that is directly associated with HPP application has been reported. While the number of industrial-scale HPP equipment was 2 in 1994, this figure has reached just over 600 today. In the dairy industry, HPP is being currently used to process cheese milk (Pastoret brand Spanish cheese) or packaged cheese after production (Mu brand Cheddar sticks and Duetto Hot Pepperoni + cheese bars, United Kingdom). In Mexico and Lebanon, two companies are using HPP at the industrial scale to extend the shelf-life of vacuum-packed cheeses. In Australia, cold-pressed milk equivalent to heat-pasteurized milk is being produced commercially (Made by Cow®, Australia). In New Zealand, a bovine colostrum product branded as Col+® is being manufactured by directly processing colostrum with HPP. The last two examples are devoid of any form of heat treatment and approved as safe by country authorities.

2. HHP Treatment of Milk

2.1. Effects of HPP on microbial inactivation in milk

Milk and dairy products are suitable mediums for the growth and survivability of a range of contaminant microorganisms. The degree of contamination and the processing temperature in different milk processing stages significantly determine the diversity of the microbial community in milk (Dash et al. 2022).

Some spoilage and pathogenic microorganisms including Shiga toxin-producing *Escherichia coli, Salmonella* spp., *Listeria monocytogenes, Campylobacter* spp., and *Yersinia* spp. which are known to cause food-borne infections, intoxications and toxicoinfections in humans, are frequently associated with milk and milk products (Dhanashekar et al. 2012; EFSA 2016; Melini et al. 2017; Lee et al. 2019). Therefore, it is important to take every possible measure to avoid such microbiological safety hazards in industrial food processing. The application of HPP generally damages microbial cell walls and membranes, inactivates enzymes,

degrades chromosome DNA, and unfolds and/or dissociates proteins, causing gelation, especially, at higher pressures (i.e., >600 MPa) (Chiozzi et al. 2022; Yang et al. 2012).

It is known that bacterial cells, yeasts and molds are more sensitive to pressure than spores. While pressure treatment at 400-600 MPa at the ambient temperatures inactivate the former microbial groups, much higher pressures and/or longer treatment time are required for the elimination of spores. Specifically, pressure conditions higher than 1200 MPa are considered capable of inactivating spores with relative success; but this very high pressure is not preferred by the food industry since gelation or textural weaknesses in the products are likely to occur. In general, Gram-positive bacteria (*i.e.*, *Listeria monocytogenes*, *Staphylococcus aureus*) are more resistant to high pressure than Gram-negative bacteria (*i.e., Pseudomonas, Salmonella* spp., *Yersinia enterocolitica*, *Vibrio parahaemolyticus*). This is due to the presence of teichoic acid in the peptidoglycan structure of gram-positive cell wall, giving cell wall a more rigid structure (Katsaros et al. 2016).

The degree of resistance of milk associated pathogenic and/or spoilage microorganisms against high pressure is affected by multiple factors such as species and strains, bacterial shape, inoculum level, physiological state of bacteria, milk composition, processing pressure, holding time, temperature, and growth phase (Zagorska et al. 2021; Serna-Hernandez et al. 2021).

HPP treatment of donkey milk at 400 MPa for 180 s resulted in <10 colony forming unit/mL of *Pseudomonas* spp., *Enterobacteriaceae*, and *Bacillus cereus* after 30-day storage at 4 °C (Giacometti et al. 2016). HPP application to goat's milk at 600 MPa for 7 minutes at 15 °C did not cause an increase in coliforms, *B. cereus*, yeast and molds during 22 days of storage at 8 °C (Tan et al. 2020). HPP treatment of milk at 345 MPa for 5 min at 50 °C was reported to result in 8 log reduction of *E. coli* and *L. monocytogenes*; however, the reduction in *S. aureus* counts was rather limited (5.33 log) under the same conditions (Alpas et al. 2000).

A summary of the recent studies on the pressure resistance of microorganisms commonly found in different milk categories is given in Table 1. On the other hand, potential impacts of HPP treatment on microorganisms that are less frequently associated with milk such as *Staphylococcus aureus*, *Coxiella burnetii*, and *Mycobacterium*, have been subjected to limited number of scientific evaluations. In a recent project run by Özer et al. (2022), the effectiveness of HPP treatment at 200, 400 or 600 MPa for 5 or 10 min on inactivation of *Mycobacterium tuberculosis*, *Eschericia coli* O157:H7 and spore-forming *Bacillus* spp. in bovine colostrum microfiltrate was investigated. Results demonstrated that except for the treatment at 200 MPa for 5 or 10 min, all other pressure conditions totally inactivated the target pathogens. On the other hand, HPP at 200 MPa for 5 or 10 min failed to inactivate *E. coli* O157:H7 and *M. tuberculosis*. Recently, Yang et al. (2020) showed that multi-cycle HPP treatment (2 x 2.5 min at 600 MPa) resulted in higher level of microbial eradication with satisfactory level of preservation of milk quality than single-cycle treatment (1 x 5 min at 600 MPa). Extra care must be taken in the eradication of pathogenic microorganisms by HPP as their toxins may withstand HPP conditions applied. For example, the number of *Aeromonas hydrophila* AH191 may well be reduced by at least 9 orders of magnitude after HPP treatment at 250 MPa for 30 min at 25 °C, but their toxins may remain unaltered, as concluded by Durães-Carvalho et al. (2012).

2.2. Effect of HPP on milk compounds

Due to the high water activity (i.e., >0.9 a_w), most dairy products including raw or processed milk and fermented dairy products are classified as highly vulnerable foods with a short shelf-life. The HPP successfully extend the shelf-life of milk and milk products without impairing the physical and/or organoleptical characteristics of end product to a large extent (Wang et al. 2016). However, milk components, including fat, casein, whey proteins, enzymes, and minerals can be affected by HHP treatments (Ravash et al. 2022). The method and conditions of processing or the type of milk affect the changes in milk fat. Huppertz et al. (2011) reported that high pressures treatments of cow's milk at 100-600 MPa at 20 °C for 60 min do not influence fat globules. Kiełczewska et al. (2020) demonstrated that HPP treatment of caprine milk at 200-500 MPa for 10 min at 20 °C did not affect the particle size during storage, the colour values, and the overall fatty acids (FAs) profile, but the ratio of branched chain FAs increased. A decrease in FAs upon high pressure treatment to ewe's milk was reported by Gervilla et al. (2001). Milk fat globule membrane size in the range of 1-2 μm tended to increase but those in the range of 2-10 μm decreased after being pressurized at 100-500 MPa at 25 or 50 °C.

HHP may cause modifications in milk proteins. Depending on the HHP conditions, casein micelles may be disintegrated into smaller sub-micelles which are further re-associated (Anema et al. 2005a; Huppertz et al. 2004ab; Orlien 2021; Cadesky et al. 2017). While pressures between 100-200 MPa have limited (if any) modifications in casein micelles, pressures around 250 MPa led to the aggregation of casein micelles and increased the average size of micelles (approximately 25%). Pressures above 400 MPa resulted in breakdown in hydrophobic interactions and decreased the average size of micelles (up to 50%) (Bravo et al. 2015). Whey proteins, especially β-lactoglobulin, may undergo a pressure-driven denaturation (Anema et al. 2005b; Lopez-Fandiño et al. 1996). Whey proteins

Table 1. Recent studies of applications of high-pressure processing in different milk categories

Dairy products	*Pressure*	*Exposure time*	*Temperature*	*Effect*	*Reference*
Raw milk	600 MPa	5 min	18 °C	5 log reductions for *E. coli*, *Salmonella* and *L. monocytogenes*	Stratakos et al. (2019)
Cow and goat milks	450 MPa	7 min	15 °C	Increased shelf life (up to 22 days to 8 ◦C), with no increase in *Bacillus cereus*, mesophilic aerobic spores, coliform, yeast and mold	Tan et al. (2020)
Raw milk	300 MPa	30 min	25 °C	Inactivation in *Salmonella* spp., *E. coli*, *Shigella*, and *Staphylococcus aureus*	Yang et al. (2012)
Raw whole milk	600 MPa	5 min	40 °C	3 log reductions for total bacterial count and *E. coli*	Liu et al. (2020)
UHT whole milk	500 MPa	10 min	25 °C	6.20 log reductions for *L. monocytogenes*	Misiou et al. (2018)
Reconstituted milk powder	500-600 MPa	3-5 min	25 °C	Inactivation of *Escherichia coli*, *Pseudomonas fluorescens* and *Enterobacter aerogenes* Increases in the levels of microorganism-derived lipopolysaccharides	Machado et al. (2019)
Human milk	593.96 MPa	233 s	25 °C	6-7 log reductions for *Bacillus cereus, Bacillus aureus*	Rocha-Pimienta et al. (2020)

UHT: Ultra-high temperature treatments

may undergo pressure-driven denaturation of which level is affected by time, temperature and pH of the milk and milk products being pressurized (Huppertz et al. 2004ab; Hinrichs & Rademacher 2004; Arias et al. 2000). Under relatively harsh the processing conditions (i.e., at >300 MPa for >30 min), β-LG irreversibly unfolds which leads to increase in hydrophobicity and hence protein aggregation (Pittia et al. 1996). As the hydrophobic groups buried inside the globular structure of whey proteins become unmasked, the hydrophobicity of the whey proteins increases (Lim et al. 2008). In general, secondary structure of whey proteins is relatively more stable against HPP than the tertiary and quaternary structures (Velez-Ruiz et al. 1998). It has been shown that changes in solubility of whey protein isolate (WPI) is dependent on HPP conditions (Kanno et al. 1998). While no change was observed in solubility of WPI at 400MPa for 10 min, a clear decrease in solubility after HPP at 690 MPa for 5 to 30 min was evident (Lee et al. 2006). Any change in protein leads to a variety of functional characteristics which in turn contribute to the improvement of the organoleptic properties of HPP-treated dairy products (Ravash et al. 2022).

Compared to thermal pasteurization, HPP treatment produces little or no damage on various classes of immunoglobulins (Ig) present in dairy products, which contribute positively to human health (Huang et al. 2020). The HPP treatment of human colostrum at 200 MPa for 2.5, 15 and 30 minutes at 8 °C resulted in insignificant changes in IgA, IgM and IgG (Sousa et al. 2014). It was recently reported that immunoglobulin concentration in human milk remained unchanged after HPP processing at 400 MPa for 5 min and at 593.96 MPa for 233 s (Rocha-Pimienta et al. 2020).

Effects of HPP on milk enzymes show a dependency on HPP conditions. This is an important feature because HPP can be used to control enzymatic activities in dairy products such as mature cheeses, i.e., activation or deactivation of proteolytic and lipolytic enzymes. For example, high pressure application at 400MPa in processing of bovine milk (Munir et al. 2020) and at 200-300 MPa in ewe milk (Alonso et al. 2012) stimulated the proteolysis during cheese maturation (Munir et al. 2020; Alonso et al. 2012).

3. HPP-mediated Chemical and Physicochemical Modifications in Milk and Milk Products

3.1. Effects on milk pH

As a result of increase in the concentration of ionized calcium in milk serum caused by HHP-driven solubilization of micellar calcium phosphate, changes the pH of milk (Huppertz et al. 2004a, 2002; Liepa et al. 2016). The composition of milk-more specifically the buffering capacity of casein micelles- also influences the changes in the pH levels of milk subjected to HPP (Iturmendi et al. 2020). Fat

may have a protective role on HPP-mediated casein micelles' dissociation, thereby diminishing the variation in the milk's pH (Yang et al. 2020; Iturmendi et al. 2020).

3.1.1. Effects on emulsion stability

Emulsion stability is related with the changes in physical parameters of macromolecules including size distribution, flocculation and droplet arrangement over time. Emulsion destabilization is often linked with the changes in physical appearance of a food product, development of undesirable flavors and rapid degradation of nutrients. These changes are due to the exposure of the fat fraction to oxidation and other chemical reactions triggered by emulsion destabilization. HPP affects native milk proteins including ones located on the milk fat globule membrane; hence these modifications affect the emulsion stability of milk. Milder pressure applications can improve emulsion stability *via* exposure to hydrophobic groups *via* the mechanisms explained above. Exposure of hydrophobic groups yields reductions in droplet size as well as modifications in molecular flexibility of milk proteins. This eventually leads to more evenly distribution of polar and non-polar amino acid residues and improves the emulsifying properties. In addition, protein denaturation generates the formation of low-molecular-weight components, and these seem to promote emulsion stability. However, higher pressure treatments, i.e., >400 MPa, seem to adversely affect the emulsification capacity of native milk proteins which leads to decrease in solubility of whey proteins (Gharibzahedi et al. 2019).

3.1.2. Effects on viscosity

While unconcentrated whole and skimmed milk show generally a Newtonian fluid characteristic, concentrated milk display a pseudoplasticity with a shear-thinning flow behavior. Milk viscosity is affected by HPP at varying levels depending on the HPP pressure applied and the time of exposure (Janahar et al. 2021; Serna-Hernandez et al. 2021). Another key factor is the increase in casein micelle hydration caused by HPP-triggered partial disintegration of casein micelles (Zhang et al. 2020).

4. Effect of HPP on Digestion Profiles of Milk Compounds

Although the digestion efficiency of milk components such as proteins and lipid have been well documented (Kopf-Bolanz et al. 2014; Lorieau et al. 2018; Mat et al. 2016; Mulet-Cabero et al. 2019; He et al. 2015), the impact of HPP on digestion profiles of milk components has been subjected to the limited number of scientific studies so far. The effect of HPP on *in vitro* digestion efficiency of β-lactoglobulin (Maynard et al. 1998; Chicón et al. 2008a), α-casein (Hu et al. 2017), whole milk (Liu et al. 2020) and whey protein isolate (Chicón et al. 2008a) were investigated. The level of tryptic hydrolysis of β-lactoglobulin B increased with increasing pressure and the highest tryptic hydrolysis was obtained at 300 MPa treatment (Stapelfeldt et al. 1996). The peptide profiles of β-lactoglobulin and whey protein isolate treated with HPP in the range of 100-800 MPa and at 400 MPa, respectively, were not affected by the pressure treatment (Maynard et al. 1998; Chicón et al. 2008a). HPP treatment at 200 MPa for 5 min resulted in the highest pepsin digestibility of α-casein (Hu et al. 2017) and gastric digestion profiles of whole milk remained unchanged after 600 MPa for 5 min (Liu et al. 2020). As the duration of HPP extends, the digestion efficiency of α-casein decreases (Hu et al. 2017). More recently, Aalaei et al. (2021) investigated the *in vitro* static gastric digestion profiles of milk proteins subjected to HPP at 400 MPa for 15 min, 600 MPa for 5 or 15 min. The authors used two different static digestion models simulating adult and elderly people's gastric system. Overall, digestion of proteins subjected to HHP at 600 MPa for 5 or 15 min was slower in the elderly model than the adult model evidenced by the high concentration of long chain peptides in the former. Interestingly, HPP at 400 MPa for 15 min improved the protein hydrolysis in the elderly model and yielded more or less similar peptide profiles to the adult model. Increasing pressurizing time at 600 MPa did not cause any further increase in the digestion efficiency of proteins. The majority of the peptides yielded upon *in vitro* simulated gastric digestion of whey proteins had a length of 16-20 amino acids, indicating high digestion efficiency in whey proteins. In general, caseins, α-lactalbumin and bovine serum albumin are resistant to HHP at 400-600 MPa (Liu et al. 2020; Yang et al. 2020; Lopez-Fandiño et al. 1996). Above 600 MPa, an interaction between caseins and β-lactoglobulin occurs *via* thiol-disulphide bonds (Bogahawaththa et al. 2018). This eventually slows down the digestion of milk proteins. Regarding milk protein digestion efficiency, 400 MPa seems to be suitable at which microbiological safety is also ensured (Özer et al. 2022). Digestion efficiency of α-casein subjected to HHP at 200 MPa for 5 min decreased by 36-43 % when pressure conditions were set to 600 MPa for 15 min due to casein aggregation (Hu et al. 2017). Similarly, digestion of κ-casein was reduced by half after being processed at 600 MPa (17%) compared with 400 MPa treatment (36%), due possibly to its aggregation with β-lactoglobulin at 600 MPa (Bogahawaththa et al. 2018). In general, although major whey proteins are denatured by HPP, their digestibility does not change at a significant level. Vilela et al. (2006) found that digestibility of single-cycle or triple-cycle pressure-treated WPI with pepsin was higher than untreated WPI after 30 min of digestion (51% and 68% vs 30.9% reduction in WPI, respectively). These findings were further supported by Iskandar et al. (2015) who showed that after 30

min of pancreatic digestion, the degree of pressure-treated WPI hydrolysis reached 95% but the degree of hydrolysis of untreated native WPI was 83%. Most recently, Zhang et al. (2022) demonstrated that the digestion profile and level of retention of nutrients of donor human milk were similar to raw milk but not the samples treated with holder pasteurization at 62.5 °C for 30 min. Protection of lactoferrin in donor human milk by HPP was also higher than those thermally treated (Pitino et al. 2019; Sergius-Ronot et al. 2022). In contrast, a decrease in lactoferrin at very high-pressure applications (550 to 800 MPa) was evident in bovine milk (Bravo et al. 2015). Upon HPP treatment to human donor milk at 350 MPa at 38 °C, the levels of metabolic hormones including insulin, nesfatin-1, cortisol and leptin remained unchanged, glucagon-like peptide 1 level increased and apelin and adiponectin levels decreased (Marousez et al. 2022). However, classical holder pasteurization (62 °C for 30 min) caused dramatic decreases in those metabolic hormones except for adiponectin which remained unchanged.

HPP also effectively reduces the allergenicity of milk proteins depending on the pressurizing conditions (Beran et al. 2009; Huang et al. 2014). In an extensive study, Kleber et al. (2007) demonstrated that the allergenicity of β-lactoglobulin- one of the major allergenic proteins in milk- increased as the pressure and treatment time extended from 200 MPa to 600 MPa and from 0 min to 30 min at <25 °C, respectively. However, when the treatment temperature was increased above 25 °C, the allergenicity of β-lactoglobulin decreased but still higher than the untreated samples. This may be due to the pressure-driven unfolding of whey proteins resulting in the generation of new epitopes which are buried in three-dimensional (3D) structure of folded native proteins (Mills & Mackie 2008). On the other hand, there is no clear correlation between the degree of protein denaturation and the allergenicity, indicating the complexity of food ingredients in food matrices. In most cases, the combination of HPP and enzymatic treatment (i.e., trypsin or chymotrypsin) is applied to reduce allergenicity of proteins (Chicón et al. 2008b).

5. HPP Treatment of Dairy Products

5.1. Impact of HPP on physical stability of whey-based beverage formulations

Fermented whey-based beverages, a promising way to valorize by-products of dairy manufacturing, are often associated with limited shelf-life due to the post-acidification occurring during storage. Time-dependent sedimentation as a result of heat-induced denaturation of whey proteins is another major challenge for whey-based fermented and non-fermented beverages. Severe thermal treatments applied above 70 °C result in protein denaturation, accompanied by loss of aqueous solubility and foaming properties (Kester & Richardson 1984; Pittia et al. 1996). Pega et al. (2018) investigated the effects of HPP at 200 MPa for 10 min or 400 MPa for 1 min on the properties of a fermented beverage manufactured from sweet whey using the starter lactic acid bacteria *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. The authors concluded that the flavor and texture of beverages treated by HPP were maintained up to 45 days post-processing with no changes in chromatic parameters. Sampedro et al. (2009) studied the effects of heat treatment, PEF and HPP processing on pectin methyl esterase (PME) activity and levels of volatile compounds in an orange juice - milk mix beverage. The conditions for inactivation of PME at >90% were as follows: thermal treatment at 85 °C for 1 min, PEF treatment at 25 kV/cm at 65 °C or HPP treatment at 650 MPa at 50 °C. After HPP treatment, the average losses of volatile compounds were between 14.2% and 7.5% at 30 °C, 22.9%, and 42.3% at 50 °C.

Barba et al. (2012) developed an orange juice-milk beverage using HPP at 100-400 MPa for 2 to 9 min. The authors demonstrated that the loss of ascorbic acid in the beverages was <10%, soon after HPP treatment and high-pressure treatment time had no significant effect on ascorbic acid losses. Similar results were reported by Bull et al. (2004) who showed that the ascorbic acid concentration of fruit and vegetable juices was not influenced significantly by HPP at mild temperatures. On the contrary, the colour changes in the HPP-treated samples were more remarkable as the pressure and treatment time increased (Barba et al. 2012).

HPP was demonstrated to better protect the antioxidant capacity of whey-based sweet lime beverages compared with thermal treatment (45.8% vs 76.7%). Sensory parameters of the same beverage remained unchanged during storage as well (Bansal et al. 2019).

5.2. Cheese

HPP has been demonstrated to affect the enzymatic coagulation time, acceleration of ripening, increasing of yield, and modifications to physicochemical and sensory properties of cheese (Chawla et al. 2011; San Martín-González et al. 2006; Naik et al. 2013; López-Pedemonte et al. 2007; Huppertz et al. 2002; Lopez-Fandiño et al. 1996; Nuñez et al. 2020; Chopde et al. 2014; Martínez-Rodríguez et al. 2012; Costabel et al. 2016). Early studies showed that HPP treatment to milk resulted in increased yield in Cheddar (Drake et al. 1997) and semi-hard goat cheese (Trujillo et al. 1999) without impairing the cheese flavor compared to those made from pasteurized milk. There is no clear consensus on the effects of HPP on cheese quality and processing time. For example, Delgado

et al. (2011) demonstrated that the original cheese flavor was not maintained when HPP was applied to cheese made from raw milk at the early stages of maturation. However, this does not mean the development of inferior quality in the end product. Escobedo-Avellaneda et al. (2021) failed to detect any impact of HPP treatment on the coagulation time of milk in Oaxaca cheese production (a pasta-filata type variety). As discussed above, HPP triggers interactions between β-lactoglobulin and κ-casein, leading to retarding caseinomacropeptide release by chymosin. Milder pressure treatments, i.e., ≤200 MPa cause a reduction in rennet coagulation time of milk. This situation is possibly associated with the increase in the surface area of the casein micelles due to the decrease in the size of the casein micelle with the effect of high pressure, and thus the expansion of the area for the action of chymosin (San Martín-González et al. 2006; Naik et al. 2013). Accelerating cheese ripening without impairing the quality characteristics of cheese is highly desirable by cheesemakers. HPP has been proved to have positive effects on accelerating cheese ripening without altering the quality and sensorial attributes (Chopde et al. 2014; San Martín-González et al. 2006). Cheese-ripening involves a series of complex biochemical reactions mainly regulated by milk enzymes, and proteolysis is the most important biochemical event largely determining the flavor and texture changes in cheese (San Martín-González et al. 2006). The high pressure alters the bacterial cell wall as discussed earlier and modifies the casein matrix, making it more susceptible to proteolytic enzymes activities. Also, HPP-triggered bacterial lysis results in release of bacterial enzymes at higher rates. In addition, the shifts in pH levels and modification of water distribution provide better conditions for enzymatic activity (Chopde et al. 2014; Martínez-Rodríguez et al. 2012). Acceleration of goat's milk cheese ripening by HPP at 400 MPa was also reported by Saldo et al (2000), but the HPP-treated cheeses had crumbly body and bitter flavor defects compared to the untreated cheeses.

The HPP-treated cheeses when compared to traditionally processed cheeses have smaller and uneven compartments in their matrices; however, during the aging process, the differences are almost non-existent, and the final products are relatively similar (Nuñez et al. 2020). Additionally, the interior color of the cheeses is modified by HPP treatment. HPP treatment causes a loss of brightness and an increase in yellowness in the cheeses. Readers are recommended to refer to Nuñez et al. (2020) for an extensive review on the effects of HPP on cheese characteristics.

5.3. Yogurt & fermented milks

The effect of HPP treatment on acid-type dairy gels has been subjected in many scientific reports up to now (Ferragut et al. 2000; Harte et al. 2002; Lanciotti et al. 2004; Vieira et al. 2019; Walsh-O'Grady et al. 2001). In general, within the pressure range of 300-700 MPa, the rheological properties of yogurt-type gels improve which eliminates the necessity for stabilizers and contributes to clean label productions (Loveday et al. 2013). Formation of yogurt matrix relies mainly on the interactions between denatured whey proteins and caseins *via* thiol-disulfide bonds. During the fermentation, the pH is reduced by the action of yogurt starter bacteria to the isoelectric point of caseins and a 3D gel matrix consisting of interacted milk proteins is formed (Soukoulis et al. 2007; Harte et al. 2003). The viscosity of yogurt milk increases immediately after HPP treatment and remains almost stable for a period of 60 days under cold storage conditions. Yogurt samples made from milk treated with high pressure treatment at 676 MPa for 30 min had similar rheological and water holding properties to gels made from heat treated milk at 85 °C for 35 min, with very different microstructural characteristics (Harte et al. 2002). This feature stemmed from the reaggregation of disrupted micellar fragments by high pressure during fermentation process. On the other hand, when skim milk was supplemented with whey protein hydrolysate or concentrate (whey protein concentrate-80) prior to HPP treatment, the resulting yogurt had inferior gel properties (Sakkas et al. 2019).

HPP reduces syneresis, a common defect in yogurt and causes thicker and smoother body in yogurt. In addition, in yogurt treated with HPP, the color characteristics -specifically to the L* and b* values- are altered, and the yield stress and the water holding capacity of yogurt matrix are increased. Regarding lipolysis and proteolysis, no clear differences between HPP-treated and thermally treated yogurt samples were reported (Walker et al. 2006; Harte et al. 2003).

Kefir made from HPP-treated whole milk had lower elastic and viscous characteristics, and lightness and color intensity than untreated control and HPP-treated kefir made from skim milk (Renes et al. 2020).

6. Conclusion

Although the advantageous of HPP of foods to food processors, consumers and environment are beyond doubt, more scientific evidence is required to be confident about its chemical safety. Majority of the studies, so far, have focused on the microbiological safety of the food processed by HPP. However, chemical interactions triggered by HPP are yet to be evaluated more deeply. In many countries, food regulations directly related with non-thermal food processing technologies are lacking. This eventually limits the level

of industrialization of HPP and similar non-thermal food processing technologies. Also, the effects of HPP on digestion efficiency of milk compounds deserve more attention.

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Evaluation of the Possible Effects of the European Green Deal Process on Agricultural Policies in Türkiye

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ABSTRACT

The European Union (EU) not only carries out some studies to ensure economic and social development but also frames policies to find solutions to emerging problems both within its borders and concerning the countries. The most important and recent of these policies is the European Green Deal, which is also intended for the agricultural sector and many others to tackle global warming and climate change better. This concept is on the agenda as the EU has made the Deal mandatory due to adverse circumstances such as an unprecedented pandemic, climate change, and droughts. One of the most important topics within the scope of the Deal is "From Farm to Fork": for a fair, healthy, and environmentally-friendly food system. Farm to Fork aims to make food systems fair, healthy, and environmentally friendly. Considering its relations with the EU, Türkiye has started to create several arrangements in its policies in line with this title. However, since academic studies on the effects of the Deal on agriculture are limited, this study has been mainly conducted to address this shortcoming. Therefore, this review aims to evaluate the possible impacts of the Deal process developments within the framework of Türkiye - EU economic relations, especially in the agricultural sector. In conclusion, the Deal will not only offer solutions to climate change but also secure a lot of support for the development and growth of economies. Türkiye should make good use of these assets within the framework of a win-win approach. Türkiye should see the Deal as an opportunity for transition into a low-carbon economy in the context of agriculture and climate change.

Keywords: European Green Deal, Farm to Fork Strategy, agricultural policies, cohesion, Türkiye, European Union

1. Introduction

The European Commission has expressed its commitment to tackling these environmental challenges in the context of global warming, climate change, the risk of species extinction, pollution, and the destruction of forests and oceans. At this point, European Green Deal (EGD) has been introduced to respond to these challenges. The EGD is a new growth strategy aiming to transform the European Union (EU) into a just and prosperous society with a modern, resource-efficient, and competitive economy without net greenhouse gas emissions and where economic growth is decoupled from resource use by 2050 (Anonymous 2019a).

The EGD, put forward by the European Commission on 11.12.2019, aims to decarbonize Europe by 2050 as the EU's long-awaited final climate action plan. For this purpose, it envisages a radical transformation in the economy and achieving climate neutrality (harmlessness) in the European continent (Ecer et al. 2021). Following Europe's footsteps; China and Japan announced carbon-neutral deadlines in September 2020. In addition, Joe Biden, who is the current president of the United States of America (USA), made the USA a party to the Paris Agreement first thing when he came to power in January 2021 and announced an incentive package afterward whose content revealed the size of the business globally (Aşıcı 2021a).

The EU intends to be a climate-neutral continent by 2050 with the EGD to zero its net greenhouse gas emissions, protect employment and industrial production, and become an influential player in reducing global greenhouse gases (Uçak & Villi 2021). In this targeted

direction, it has also expressed that it plans to reduce net greenhouse gas emissions by at least 55% by 2030 compared to 1990 levels. The EGD is the economic transformation model to be used as a roadmap to achieve this goal (Diriöz 2021).

As far as innovation and competitiveness for the development of climate-compatible technologies, a healthy and fair food system with the Farm to Fork Strategy, and the Fair Transformation Mechanism designed to "leave no one behind" are concerned, it is seen that the EGD includes policy areas such as economy, employment, health, food, social justice as well as ecology (Aşıcı 2021b).

The EGD offers an action plan on issues such as investing in eco-friendly technologies, supporting industry for innovation, and decarbonizing the energy sector to increase the efficient use of resources by switching to a clean, circular economy, restoring biodiversity, and reducing pollution (Kandemir 2021). The EGD has emerged intending to meet the EU's vision of a standard "green growth" strategy; the definition of "reaching climate neutrality on a continental scale" is used for this Deal. The EGD is based on political, legal, and economic processes that center on international cooperation (Çayırağası & Sakıcı 2021).

In addition to the Paris Climate Agreement, which Türkiye has signed, Türkiye's Medium-Term Program (2022-2024) also includes the issue of green economy adaptation and transformations within the scope of the EGD (Anonymous 2022a). Therefore, it can be said that the beginning of an application that would mean a policy change in Türkiye's transformation into a green economy has been made.

In addition to these, the fact that one of the most important partners of Türkiye in terms of foreign trade is the EU also reveals how significant and at the forefront this issue is. Therefore, there is a potential to create a substantial and new additional financial burden on exports to EU countries, especially if the necessary harmonization measures are not taken due to the border carbon regulation within the EGD.

The EGD is designed not only for improving people's health, quality of life, and taking care of nature but also for tackling climate change, a roadmap, and a Strategy for achieving economic growth by doing all this. One of the EGD's key topics is the "From Farm to Fork" strategy: for a fair, healthy, and environmentally-friendly food system." Having mapped out 20.5.2020, it is directly related to the agricultural sector. Nowadays, food systems that account for approximately one-third of global greenhouse gas emissions consume large amounts of natural resources and cause biodiversity loss and adverse health effects. Therefore, they need to be redesigned. Farm to Fork Strategy is described as the EGD, which aims to make food systems fair, healthy, and environmentally friendly (Anonymous 2022b).

With this Strategy, it is clearly stated that the sustainable regulation of food systems will also bring inspiring opportunities for businesses in the food value chain. Moreover, it is evident that new technologies and scientific developments plan to benefit all stakeholders and increase public awareness and demand for sustainable food. Thanks to this Strategy, the EU also plans to support the global transition to sustainable agri-food systems through trade policies and instruments of international cooperation.

Considering Türkiye's entry into the EU and the harmonization process between them, it will be inevitable for Türkiye to make arrangements in the relevant policies within the framework of the EGD rules. There are basically eight titles in the EGD. One of these titles, which is directly related to the agricultural sector, is "From Farm to Fork": for a fair, healthy, and environmentally-friendly food system. In addition to this title, "increasing the EU's climate target for 2030 and 2050" and "protecting and improving ecosystems and biodiversity" are other ones that can be considered in connection with the agricultural sector.

Considering that EGD policies have just begun, it can be said academic studies on this subject have just started. When the studies on the EGD are discussed in detail, it is seen most publications debate the political aspect and general scope of the process. Although the studies aim to draw a framework for climate change, circular economy, and the energy sector, academic publications and evaluations on agriculture on a sectoral basis seem to be limited. This lack of research has been the main focus in the initiation of the study.

This article is prepared to evaluate the process of EGD in terms of Türkiye, focusing on the strategies in the agricultural sector in the EGD. Besides these, when Türkiye-EU relations are considered, it becomes essential for Türkiye, which is in a strategic market, to define the implications and effects of the EGD concerning the whole world and to evaluate within the framework of these effects. This study will be able to contribute to filling this gap (lack of relevant studies evaluating the literature) with the strategies drawn up for the agricultural sector and their implementation.

This study has been prepared based on the literature. The obtained works of literature were evaluated, interpreted, and synthesized in terms of Türkiye-EU agricultural policies within the framework of the EGD. Based on these determinations, the study aims to assess the possible effects of the developments in the EGD process within the framework of Türkiye - EU economic relations, especially in the agricultural sector, and to offer suggestions on what measures are to be taken in the sector in this context.

2. European Green Deal

The crippling effects of environmental problems such as global warming, climate change, increasing air pollution, the gradual decrease in biodiversity, and the gradual depletion of natural resources have been deeply felt, especially since the 1990s. The adverse effects of economic growth and development on the environment and the relationship between the environment and energy have been the subject of intense debate in parallel with those issues on international platforms. However, in recent years, with the effect of environmental pollution and global warming, the search for severe alternatives to understanding economic growth and development has accelerated, even if not to capitalism and the free market system. The new green paradigm should be seen as a result of these alternative searches (Yalçın & Gök 2021).

There are renewable energy sources such as natural gas, which emits less carbon, and green energy, which does not emit any carbon at all, in place of coal and oil, which were used as energy sources in the period that can be called the old world. In this context, putting itself at the focal point of the world, the EU has even taken the world's leaders on the EGD, which has been put into effect on the issues of combating global warming and climate change. Therefore, it's fair to say that the EGD has emerged as one of the most significant agreements adopted by the EU.

With the EGD, the EU has announced its ambitious roadmap regarding climate change for the next 30 years. Moreover, the EGD should not be seen as just a climate policy. It also outlines reshaping the economic transformation on the axis of climate change. Policies and actions within the scope of the EGD set new targets and practices in many areas, from energy to transportation, industry to agriculture, and financing mechanisms to social life. The EGD is seen as both a new growth strategy and an economic model within the framework of green growth. This EGD is about improving people's well-being while setting out the objectives of making Europe climate neutral and protecting its natural habitats as part of its new growth strategy. In this context, it follows an approach that plans to make Europe climate neutral by 2050, reduce pollution, protect human life, animals and plants, help companies become world leaders in clean products and technologies, and help ensure a just and inclusive transition (Anonymous 2019b).

The EU has revealed its projects to reduce carbon emissions by 50% by 2030 and to reduce it to zero (the first climate-neutral zone in the world) by 2050 with the EGD and with the climate law enacted on 29.7.2021 and these targets have become binding for the Member States and the EU (Gönen & Uzun 2021).

In general terms, these policy changes are listed in Figure 1 (Anonymous 2019a). Figure 1 fully illustrates the different elements of the EGD.

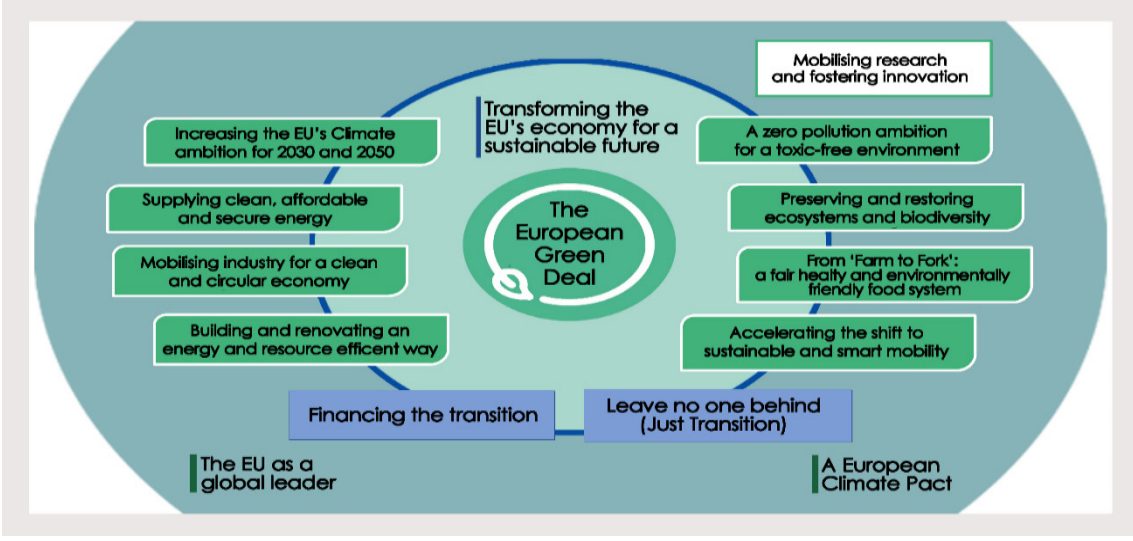

Figure 1. General Framework of the EGD (Anonymous 2019a)

All regions and sectors within the EU will need funding resources to fulfill the rules of this EGD. Although the EGD Investment Plan will respond to this need, the Just Transition mechanism will also allocate at least 100 billion Euros in the 2021-2027 period in the regions most affected by this EGD (Anonymous 2020a). Moreover, The EGD has also been expressed as an essential point of recovery from the coronavirus disease-2019 (COVID-19) pandemic, and it has been stated that one-third of the 1.8 trillion Euro investment within the scope of the Next Generation EU Recovery Plan and the seven-year budget of the EU will be financed by the

EGD (Anonymous 2022c).

The EU has implemented many measures, especially climate law and carbon border tax, in conjunction with the EGD. In addition to these measures, various transition funds have simultaneously been created for companies to switch to green energy and move away from fossil and carbon-based energy and fuel. The size of the funds allocated by Europe for environmental projects over the next ten years is over one trillion dollars. This amount of funding constitutes the enormous amount put forward for regulation on climate change in the history of the world and the EU (Uçak & Villi 2021).

Thirty percent of the long-term budget and Next Generation EU will be spent on fighting climate change-the highest share ever, from the most extensive EU budget ever. These funds are part of an effective investment plan the EU will implement to green the economy (Anonymous 2021a). The Next Generation EU is an €806.9 billion temporary rescue measure to help repair the immediate economic and social damage caused by the coronavirus pandemic. As a result, it is stated post-COVID-19, Europe can be greener, more digital, more resilient, and better suited to current and future challenges (Anonymous 2022d).

These funds are part of a significant investment plan the EU will implement to green the economy. This long-term budget will combine EU and national public funds and public and private investment to support the EU on its path to climate neutrality by 2050. Besides these, an additional €15 billion will be earmarked for the European Rural Development Fund in conjunction with the European Investment Bank and Common Agricultural Policy (CAP) to support rural areas in making the necessary structural changes in line with the EGD and achieving targets in line with the new biodiversity and Farm to Fork Strategies (Anonymous 2021a).

Considering the economic potential of the EU throughout the world, the decisions and policies taken in conjunction with the EGD may become challenging for all countries with commercial, financial, and political relations with the EU. To successfully Deal with the issues included in this EGD, which the EU has put into practice, it is paramount to include other countries. The EU establishes relations with many countries and regions worldwide in this context. As far as the EGD is concerned, it is stated that "as long as other countries do not share the same passion with the EU on a global scale, there will be a risk of an increase in carbon emission levels" and added that success would come as a result of joint efforts (Anonymous 2019a).

When the trade volume and economic and political intense relations between Türkiye and the EU are evaluated, Türkiye, which has close relations with Europe, should seriously consider the issues concerning the agricultural sector. Considering these aspects, Türkiye - EU relations may also be affected by these changes.

3. Evaluation of the Agricultural Policies in the Scope of the EGD

After the EGD was announced on 11.12.2019, which concerns agriculture and all other sectors, the EU took it to the next level by integrating environmental and climate change elements into other policy areas. Furthermore, following the announcement of the EGD, the EU has published a series of sub-strategies that establish links between it and other sectors. One of them was the "From Farm to Fork Strategy", published on 20.5.2020, and found the connection between the agricultural sector and the EGD (Akyüz 2021).

The Farm to Fork Strategy is one of the most critical issues of the EGD within the framework of a new growth target shaped by improving the living standards of individuals, being more sensitive to the environment, and having an integrative understanding. The Farm to Fork Strategy, as a new and comprehensive approach to the value Europeans place on food sustainability, comprehensively addresses the challenges of sustainable food systems and recognizes the essential link between healthy people, healthy societies, and a healthy planet. Creating a suitable food environment that facilitates choosing a healthy and sustainable diet will benefit consumers' health and quality of life while reducing health-related costs for society (Anonymous 2020b).

As the EU moves toward a healthier and more sustainable food system with the Farm to Fork Strategy, which is meant to be the cornerstone of the EGD, the following goals are pursued (Anonymous 2020c);

- Making sure Europeans get healthy, affordable, and sustainable food,

- Tackling climate change,

- Protecting the environment and preserving biodiversity,

- Ensuring fair economic return in the food chain,

- Boost in organic agriculture.

The Farm to Fork Strategy will enable the transition to a sustainable EU food system that maintains food security and ensures access to healthy food sourced from a healthy planet. The EU plans to reduce the food system's environmental and climatic footprint and strengthen its resilience, protecting citizens' health and ensuring farmers' livelihoods. It also proposes many measures for EU citizens, including improved labeling to meet better consumers' needs for information about healthy and sustainable food (Anonymous 2020d).

The targets of the strategy until 2030 have become more definite. These are (Anonymous 2021b):

- Reducing the overall use and risk of chemical pesticides by 50% and the use of more hazardous pesticides by 50%,

- Achieving at least 25% of the EU's agricultural land under organic farming and a significant increase in organic aquaculture,

- Reducing sales of anti-microbials for farm animals and in aquaculture by 50% by 2030,

- Reducing nutrient losses by at least 50% while ensuring no deterioration in soil fertility, which will cut down the use of fertilizers by at least 20%,

- Bringing back at least 10% of agricultural area under high diversity landscape features.

When the details of the specific targets given above under the main headings are examined, it is seen separate targets are determined for each title. For example, pesticides and fertilizers are particularly important in the Farm to Fork Strategy. The Commission will take some precautions to protect farmers' incomes and create alternatives such as reviewing the sustainable use of pesticides directive, the carbon farming initiative, and the certification of carbon removals that should create new business models and reward those farmers and foresters that adopt more climate-friendly practices.

Together with the Member States, the Commission will promote the recycling of food as fertilizer from organic waste, which will develop an integrated food management action plan to reduce and prevent pollution from the overuse of fertilizers. This could contribute to delivering the "zero pollution ambition" of the EGD. In addition, the new regulations on veterinary medicinal products and medicated feed to be implemented in 2022 will provide a wide range of measures to limit the use of anti-microbials in animals further and encourage their cautious and responsible use (Anonymous 2020e).

To accomplish the goals of organic agriculture, it is vital to ensure not only the sustainable economic development of the sector but also to encourage the demand. Therefore, the Commission will put forward an action plan on organic farming in addition to CAP measures such as eco-plans, investments, and advisory services. This will be an opportunity to revive both the supply and demand for organic products. At the same time, this could contribute to gaining consumer trust through promotional campaigns and green public procurement. Furthermore, aiming to achieve the UN Sustainable Development Goal, the Commission will propose legally binding targets to diminish food waste across the EU by 2023. Moreover, the Commission will consider additional opportunities as part of relevant EU policies to integrate food loss and waste prevention and take action to strengthen the evidence base for food waste prevention interventions (Anonymous 2020e).

Farm to Fork Strategy will be able to put forward actions to help consumers choose healthy and sustainable diets. In particular, the Commission will explore new ways to give consumers better information on the nutritional value of foods (Anonymous 2020f). In addition, to enhance the availability and price of sustainable food and to promote healthy and sustainable diets, including organic products, in schools and public institutions, the Commission will determine the best modalities to adopt minimum mandatory criteria for sustainable food procurement.

The EU is committed to leading by example on the transition to sustainable food systems within its borders and outside. Through international cooperation, bilaterally and multilaterally, the EU will promote more sustainable farming and fishery practices, decrease deforestation, enhance biodiversity, and boost food security and nutrition outcomes. The Commission will incorporate these Farm to Fork priorities in the programming guidance for cooperation with third countries in the period 2021-2027. The EU's bilateral trade agreements also offer a means to foster EU environmental standards in third countries in addition to food safety standards (Anonymous 2020e).

In addition to the objectives mentioned above of the Farm to Fork Strategy, it has been stated that the transition to this process can be achieved by making conscious consumer choices. In this process, it is emphasized that studies can be carried out under sub-headings such as creating a healthy food environment that makes healthy and sustainable choices easy, labeling foods to encourage consumers to pick healthy and sustainable options, and accelerating the fight against food waste, supporting research and innovation and promoting

global transition.

It has been affirmed that the EGD may have some benefits for farmers, which are (Anonymous 2020g):

- Sustainable business models, labeling plans, and marketing standards will link production methods to premium consumer demand, resulting in higher returns for farmers and food producers.

- Tools that will contribute to improving the position of farmers in the food supply chain.

- Changing consumer demand and sustainable new systems by bringing new business opportunities such as the plant protein sector or the bioeconomy.

- Advances in innovation, technology, and digital solutions (precision farming) resulting in higher productivity and lower costs through lower inputs.

- Meeting the increasing demand for sustainable food means making a stronger connection with consumers.

- Labeling and marketing initiatives to heighten awareness of EU high standards, opening up additional export opportunities.

On 15.1.2020, the European Parliament announced its resolution on the EGD, which includes a specific request to the Commission to analyze the contribution of the CAP reform proposal to the EU's environmental, climate, and biodiversity protection commitments to fully align it to the goals set in the EGD (Anonymous 2020h). In line with the CAP and this EGD, for example, Member States will have to use a new tool to encourage farmers to adopt or maintain practices that benefit the environment and climate. To exemplify, one of the priorities of the Farm to Fork Strategy is to help consumers choose healthy and sustainable diets and reduce food waste, which means that Member States should examine their actions and propose how to use the different CAP tools to address any difficulties that may arise.

The Farm to Fork Strategy of the EGD and the EU CAP are closely related to each other. On 2.12.2021, the Deal on reform of the CAP was formally adopted. The new legislation is due to begin in 2023. This new Deal brings a fairer, greener, more animal-friendly, and more flexible CAP. It will seek to ensure a sustainable future for European farmers, provide more targeted support to smaller farms, and allow greater flexibility for EU countries to adopt measures to local conditions. Agriculture and rural areas are central to the EGD, and the new CAP will be a crucial tool in reaching the ambitions of the Farm to Fork strategies (Anonymous 2022e).

It should be stated that the targets set under the new CAP are entirely in line with the EGD, and the national CAP strategic plans will contribute to the EGD targets. Meanwhile, it should be noted that the Farm to Fork Strategy is financially supported through the CAP and the budget for the European Agricultural Fund For Rural Development includes €8 billion from Next Generation EU to help rural areas make the structural changes necessary to achieve the goals of the EGD and the digital transition.

The Commission services are working intensively with the Member States and stakeholders to ensure substantial environmental and climate ambition is achieved through the CAP Strategic Plans. For example, a range of workshops has been co-organized to maximize the potential of the proposed new tools. However, in light of the EGD challenge, the role of the Commission in this preparatory phase needs to be further reinforced (Anonymous 2022e).

It should also be noted that some problems may arise during the implementation of the EGD by the Member States. Some of these vulnerable areas include the problem of food safety and security around the world and its reflections on the EU, the COVID-19 pandemic process, high inflation rates in the EU, the economic problems caused by the Russia-Ukraine war, and the energy crisis (high dependency on fossil fuels) and changes in consumer preferences due to these. Finally, it is stated that there can be some particular issues, such as the need for a large amount of infrastructure in the transition to a Farm to Fork Strategy, the possibility of a decrease in productivity in the first years of the adaptation of the farmers to the new strategy, and the need for new farm equipment.

The implementation of the EGD will require some sort of regional cross-border approach. A climate-neutral continent will require, by definition, cooperation between the EU, the Energy Community, and neighboring countries such as Türkiye. Climate neutrality over time will also raise the issue of (regional) carbon leakage. A regional approach is essential to ensure that the transition co-occurs throughout the region to avoid, for example, the risk that more ambitious countries replace domestic higher carbon electricity production with other carbon-intensive imports from their neighbors. Also, the potential EU climate-neutrality target for 2050 is unprecedentedly ambitious, especially for this region; all Member States will face challenges in delivering the required transformational changes under the EGD (Cătuţi et al. 2020).

4. Evaluation of the Türkiye's Agricultural Policies in the Scope of the EGD

Considering Türkiye - EU relations, the EU is one of the most important partners of Türkiye in terms of foreign trade. According to the data provided by "Trade Map" in 2021, The EU is the most important commercial partner of Türkiye, with which 48% of its total exports, 33,6% of its total imports, 29,7% of its total agricultural exports, 19,9% of its total agricultural imports are made. In addition, Türkiye is the 2nd most popular Foreign Direct Investment (FDI) destination after Poland. Despite the severe effects of COVID-19, Türkiye has secured %16 shares of the EU's FDI, and its largest and most significant investment source is the EU. "Türkiye is the EU's 6th largest export partner and a key export market" also shows how close Türkiye - EU relations are (Anonymous 2022f).

It can be inevitable for Türkiye to innovate in its agricultural policies by carefully examining the developments in the EU within the framework of EGD. Otherwise, there could be a high risk of disruption in the commercial agreements and the harmonization process between Türkiye and the EU in the following years due to the issues that directly concern the EU, such as agriculture, climate change, and biodiversity, especially greenhouse gas emissions. Furthermore, it is required to adapt to the new regulations the EGD brought to protect, strengthen and ensure the sustainability of Türkiye's trade relations with the EU, both in agricultural products trade and in other sectors related to agriculture. When it comes to agricultural and food products, which constitute a very considerable part of annual exports, inevitably, the targets set for the agricultural sector with the "From Farm to Fork Strategy" introduced within the scope of the EGD will affect Türkiye's agricultural sector.

The changes envisaged in the policies implemented by the EU with the EGD, the transformation in international trade and the economy, the protection and development of competitiveness in exports in line with Türkiye's development goals, and the relations established within the scope of the EU Customs Union are of great importance in terms of strengthening the contributions to the global economy (Official Journal 2021). From this point of view, inevitably, Türkiye - EU relations may also be affected by these changes. Furthermore, Türkiye needs to make many adjustments in the agricultural policies, which will have to comply with the EGD ones in the foreseeable future. Therefore, compliance with the agricultural sector requirements of the EGD also has an important place in front of Türkiye.

The Green Deal Action Plan was published by the Ministry of Commerce in line with the Presidential Circular No. 2021/15 in the Official Journal numbered 31543 on 16.7.2021. Then, The Green Deal Work Group consisted of the representatives of the Ministries of Labour and Social Security, Environment, Urbanization, and Climate Change, Foreign Affairs, Energy and Natural Resources, Treasury and Finance, National Education, Industry and Technology, Agriculture and Forestry, Transport and Infrastructure was established (Official Journal 2021).

This action plan establishes a framework that sets out the period from 2021 to 2027, aiming to support Türkiye's green transformation in all relevant policy areas (Anonymous 2022g). After the Circular, the Green Deal action plan 2021 document was published by the Ministry of Commerce. The plan emphasizes the need for a holistic approach to the effects of policy changes- especially the EU-on industry, agriculture, energy, and transportation policies in connection with Türkiye's foreign trade and draws a road map that will ensure harmonization by taking Türkiye's Customs Union relationship into account (Ministry of Commerce 2021).

The action plan aims to comply with the regulations and principles adopted under the EGD to contribute to Türkiye's transition to a more sustainable, resource-efficient, and green economy in a way that preserves and advances its current integration (Anonymous 2022g). Türkiye's action plan outlines the actions planned to be carried out under nine headings, including 32 targets and 81 actions. These headings are border carbon regulations, a green and circular economy, green financing, clean, economical, and secure energy supply, sustainable agriculture, sustainable smart transportation, battling climate change, diplomacy, EGD information, and awareness activities (Ministry of Commerce 2021).

Specialized work groups have been established under the coordination of relevant public institutions on various issues to monitor the functioning of the Green Deal Action Plan published by the Ministry of Commerce and to facilitate the coordination of the appropriate parties. One of these work groups is the Sustainable Agriculture Specialization Work Group.

Within the scope of the Green Deal Action Plan, the title closely related to the agricultural sector has been included under the name "sustainable agriculture." Given the fact that Türkiye is among the countries that will be most affected by climate change due to its geographical location and the steps to be taken by its biggest trade partner within the scope of being the first climate-neutral continent, it will contribute to Türkiye's efforts to combat climate change and its consequences, as well as to the EU's. Therefore, it is pivotal for Türkiye to take actions for sustainable agriculture so that the measures to be taken by the EGD do not hinder the sustainability of Türkiye's agricultural trade with the EU (Ministry of Commerce 2021).

The actions determined under the title of "Sustainable Agriculture" of Türkiye's action plan in line with the objectives set for agriculture within the scope of the EGD can be summarized as follows EU (Ministry of Commerce 2021):

- The research will be carried out to reduce the use of chemical fertilizers, pesticides, and anti-microbials.

- Within the framework of efforts to reduce pesticides, it is aimed to expand the use of biological and biotechnical control methods.

- Studies will be conducted by considering the target and policy changes in reducing the EU's use of chemical fertilizers.

- Imposing initiatives with the EU aims to develop organic agricultural production, complete the harmonization of legislation and ensure mutual recognition.

- It is envisaged that land consolidation activities will be carried out.

- Geothermal resources will be used in Organized Industrial Zones based on Agriculture (geothermal greenhouse). Additionally, greenhouses and production facilities using renewable energy will be supported.

- Research and development (R&D) will be carried out on reusing waste and residues in agricultural production.

- It is aimed to do awareness-raising and consumer awareness activities for the recycling of food waste and residues and to carry out R&D on the reuse of waste and residues in agricultural production.

- Awareness-raising activities that will contribute to developing sustainable agriculture between Türkiye and the EU will be carried out through information sessions on the Farm to Fork Strategy and Biodiversity Strategies included in the EGD.

In addition to these, it is seen that there are objectives in the action plan on climate change that are very closely related to the agricultural sector. Among these goals are training on sustainable agricultural techniques, conducting R&D projects on this subject, disseminating good practices, and increasing the nature-based approach in land applications.

When other policy documents are examined for Türkiye, it is pointed out that some projects and practices, namely the New Economic Program covering the years 2021-2023 and the Presidential Annual Program for 2021, will be implemented by referring to climate change. For example, in the New Economic Program, it is discussed that "to ensure compliance with the EGD in Türkiye's exports to the EU in the context of the EU Customs Union, vital research will be conducted, and required preparations will be made in coordination with not only the public and private sector but also non-governmental organizations and universities" (Ministry of Treasury and Finance 2020).

Another policy document is the Medium-Term Program covering the years 2022-2024. In this Program, it is emphasized that the significance of climate change has increased in global economic policies under the leadership of developed countries with approaches such as the EGD, and the necessity of green transformation has been openly expressed within the framework of policies to be implemented by other countries, especially the EU, which is Türkiye's primary export market. It is also added in this Program that investments to be made in Türkiye will be shaped by considering the objectives and actions included in the Green Deal Action Plan.

To achieve the goals in the action plan, institutions (Ministry of Agriculture and Forestry and Ministry of Environment, Urbanization and Climate Change) in charge are required to follow a specific scheduled process. Türkiye's roadmap for EGD within the scope of the action plan and what has been done so far are given in detail in Table 1.

Going through an economic transformation, which is the main target within the framework of the EGD, will not only mean compliance with EU criteria for the agricultural sector but also for others. What is more, it will pave the way for the goal of increasing Türkiye's competitiveness in the international market.

The circular economy desired by the EU will also be able to contribute to Türkiye's level of economic development. Furthermore, considering the export potential of Türkiye - EU agricultural products, this transformation in the agricultural sector may also yield desired results such as increasing production and productivity. Therefore, it is probable that the projects to be created in the agricultural sector in light of the EGD will contribute more to the export of agricultural products. However, for these possibilities to be realized, government and private sector policies and practices must be reconsidered concerning the circular economy.

Policies to be implemented in this direction will not only prevent the trade of agricultural products between Türkiye and the EU from being adversely affected but also ensure the growth in business between these two partners. Otherwise, Türkiye - EU economic

Table 1. The process* of the goals related to the agriculture in the action plan

*Goals and deadline**	*Progress made*
A green and circular economy	
Improving the sustainable use of water in production and consumption and the reuse of wastewater (2021 IV. Quarter-2024 IV. Quarter)	Legislative works continue. Project studies under the coordination of General Directorate of Agricultural Research and Policies (TAGEM) continue. Negotiations, evaluations, and preliminary studies with municipalities are continuing in terms of the reusability of the effluent of wastewater treatment plants.
Calculation of sectoral water footprint on a basin basis (2023 IV. Quarter)	Projects have been prepared within the scope of the basin scale; blue, green, and grey water footprints will be calculated. A general methodology has been developed in this regard.
Calculation of sectoral water footprint for water uses (2023 IV. Quarter)	Projects have been prepared within the scope of sectoral water use; blue, green, and grey water footprints will be calculated. A general methodology has been developed in this regard.
Researching the use, benefits, and improvement aspects of remote sensing, sensors, and informatics applications in the management of water resources (2022 IV. Quarter)	The "National Project for the Development of the Digital Irrigation Management System" carried out under the coordination of TAGEM continues in 6 different regions (Kırklareli, Samsun, Mersin, Ankara, İzmir, and Şanlıurfa, Adana) in 2022. Data transfer studies by DSI continue. Work on installing limnigraphs for groundwater level measurements continues.
Reducing endocrine-disrupting chemicals (2021 IV. Quarter-2023 IV. Quarter)	The project to monitor endocrine-disrupting chemicals is at the tender stage. A revision of the regulation is planned.
Sustainable agriculture	
Reducing the use of pesticides and anti-microbials (2021 III. Quarter-2023 IV. Quarter)	Within the scope of the pesticide control program, 24.525 production places were inspected in 2021. Training and extension studies are carried out. "National Veterinary Antibiotic Resistance Monitoring Project" has implemented antimicrobial resistance. An electronic Prescription System and Pharmaceutical Tracking System were implemented for antimicrobials. The vaccine Tracking System started to be implemented.
Development of organic agriculture (2023 IV. Quarter)	An increase of 25-40% was made in support payments in 2021 for biotechnical and biological control. The organic Agriculture Sector Meeting was held in 2021. It is aimed to expand organic agriculture in 81 provinces. "Project Based Approach" is adopted. Legislative harmonization studies continue. The studies of the working groups continue.
Reducing the use of chemical fertilizers (2021 III. Quarter-2023 II. Quarter)	Four projects are carried out on olive plants in İzmir, peanut plants in Osmaniye, grape plants in Manisa, and cotton plants in Mersin. Three projects are carried out in Kırklareli, Ankara, and Malatya provinces to increase the efficiency of the use of fertilizers.
Land consolidation activities (2023 IV. Quarter)	In total, 6.02 million hectares of land were registered. In the first quarter of 2022, registration procedures were completed on 50.442 hectares.
Increasing the use of renewable energy in agriculture (2021 III. Quarter-2023 IV. Quarter)	Studies on the use of existing geothermal resources, which were started in Aydın, Denizli, İzmir, and Ağrı Provinces, in Specialized Organized Industrial Zones based on Agriculture (geothermal greenhouse), are continuing.
Improving waste and residue management in agricultural production (2024 II. Quarter)	Various projects are being carried out for the completion of R&D studies.
Reducing food loss and waste (2021 IV. As of Quarter)	Awareness and information activities and legislation studies continue.
Raising awareness of the EU Farm to Fork Strategy and Biodiversity Strategies (2021 IV. Quarter)	Training, information activities, and presentations were held.

Fighting climate change	
	One national project is ongoing, and maps have been produced for figs, apricots, and cherries.
	A Strategic Plan for Adaptation to Climate Change in forestry has been prepared.
	Two projects are planned to start in the 4th quarter of 2023.
Evaluation of the effects of climate change on terrestrial and marine areas and specific water resources through ecosystem-based approaches and practices (2022 IV. Quarter-2023 IV. Quarter)	Carbon stocks continue to be determined in protected areas. Therefore, one project proposal was presented on this subject.
	A total of 6 wetland management plans were approved in 2021.
	A total of 6 wetland management plans have been prepared in 2022 and are at the approval stage.
	Studies on land degradation leveling have continued.
	R&D studies continue.
	Awareness, education, and implementations continue.
	R&D studies for the measurement and monitoring of greenhouse gases continue.
Compliance with EU Chemicals Legislation (2021 III. Quarter-2024 I. Quarter)	Legislative harmonization studies continue.
Fighting climate change (2021 IV. Quarter)	Fighting Climate Change Report will be prepared (it has not yet been declared).
	Türkiye's National Climate Change Strategy and National Climate.
	Work on updating the Change Action Plan continues.

Source: Ministry of Commerce 2021; Ministry of Agriculture and Forestry 2022

relations may be negatively impacted by this process.

One of the most critical constraints in Türkiye's transition to a green economy within the scope of the circular economy envisaged in the EGD is related to the cost of the process. Türkiye has initially earmarked 271 million Euros for green transformation within the scope of the EGD. It has been announced that until 2030, 260 million Euros will be invested each year, and the source will be largely carbon tax at the border (Yayman & Demir 2021).

Regarding compliance with the EGD, institutions in charge and organizations have started to take the preliminary steps for a transition to a circular economy by making arrangements in their fields within the framework of the environmentally friendly and green economy. It is imperative to take initiatives in this direction without wasting time so that cost of the implementations in the transition to a green economy can partially be covered by the incentives to be given by the EU.

The National Pre-accession Economic Reform Programs (2021-2023 and 2022-2024) is another policy document on the EGD. To support the green transformation and transition to a circular economy during the program period, investments in environmentally friendly production that is both energy and resource efficient will be promoted, and the targets and actions in the Green Deal Action Plan will be taken into account (Anonymous 2022g).

For these goals and actions to be successful, supporting R&D is essential. For this purpose, additional points were provided to the projects submitted in the 1st term of 2021 under the ARDEB (Research Support Programs Presidency) 1001 Program, one of The Scientific and Technological Research Council of Türkiye's Research Support Programs directly related to the priority R&D and innovation issues within the scope of compliance with the EGD; for example decreasing of pesticide fertilizer, developing of organic farming, recycling of waste and waste in agricultural production and food sector.

Another point is that Türkiye has declared a net zero greenhouse gas emission target by 2053. A long-term climate change strategy and National Climate Change Action Plan will be prepared with the support of the United Nations Development Program project. Türkiye's Intended National Contribution (Intended Nationally Determined Contributions-INDC) will also be updated within the project's scope (Anonymous 2022g). The following five years will be an intense period in which significant decisions are taken, and legal arrangements are made and turned into actions for the EU to reach the 2050 targets.

5. Conclusion and Recommendations

With the EGD, the EU intends not only to zero its net greenhouse gas emissions by 2050 but also to curtail global greenhouse gases while protecting industrial production and employment. To achieve these goals, the EGD has been announced to transition to a "carbon-

free economy" model in many primary and sub-sectors, from agriculture to industry, from transportation to energy. The EGD is seen as the EU's economic growth and development strategy in the 21st century.

Given worldwide concerns about food security and accessibility, food accessibility and affordability are among the EU's main challenges. Thus, from Farm to Fork Strategy is currently driving consumer choices. Furthermore, the expected difficulties in meeting the need for imported gas and fertilizers from Ukraine and Russia in producing inputs required for agricultural production have also raised the issue of self-sufficiency within the EU. These can be outlined as the adverse effects from Farm to Fork Strategy might face. However, it should be noted that the stated purpose of this strategy is to ensure the future competitiveness and resilience of European agriculture.

To implement planned studies on from Farm to Fork Strategy successfully, the following policy recommendations should be taken into account: i) Society should be informed and trained about from Farm to Fork and what impacts it will have on their lives; ii) Cattle breeders and farmers who will be harmed by policy changes should be provided with the necessary information about how new production methods will be and what kind of life they will lead; iii) Research on the strategy's economic, social, or environmental impacts should be conducted. More concrete steps to compensate for the welfare and income losses that farmers may experience should be discussed. Another step that needs to be taken is the new CAP (2023-2027). Under the new CAP, Member States must develop national CAP strategic plans that detail how the new CAP will be implemented in their country.

With the EGD, the EU is committed to being a global leader and providing financial support for the necessary actions to be taken to work out solutions to the factors leading to climate change and environmental problems. In this context, considering that Türkiye is a significant stakeholder in agricultural trade with the EU, it is clear that the targets determined by the EGD can directly impact Türkiye's agricultural sector. In this respect, to succeed in the transition and adaptation process to the EGD, a road map should be drawn for the agricultural sector, which is the subject of this article.

Third countries with ties with the EU will have to take action to adapt to the next transformation with the EGD since the economic structure, which is expected to change with the green transformation, will turn into a global initiative rather than EU policies in the following years. Therefore, for Türkiye to achieve sustainability by minimizing economic losses which might be suffered during the transition to "green production" within the scope of the EGD, the EGD needs to assess its sectoral impacts and plan and support the transition to harmonization. In this context, the action plan prepared under the leadership of the Ministry of Commerce is expected to strengthen Türkiye's competitiveness, increase green investments and promote green transformation.

The policies Türkiye will put into effect for harmonization with the EGD must be efficient in many areas. Since the EGD is meant to protect and increase the Member States' competitiveness upon their adaptation to the changing world, Türkiye also needs to keep up legislation and practice innovations to ensure more favorable conditions for competition exist. Some studies have started to be carried out by public authorities in Türkiye within the framework of harmonization with the policies of the EU, which is Türkiye's largest export market. For this reason, the need to prepare sectoral impact analyses, determining how the sectors can be affected, and the government support mechanisms and processes related to legislative change has emerged. As mentioned in the related section of the text, the Ministry of Agriculture and Forestry and other relevant institutions have put into practice their actions on the issues they are responsible for at certain date intervals. At this point, it should be stated there may be problems in adapting to EGD in terms of some issues. For example, some targets such as organic farming area determined as 25%, dissemination of the use of biological and biotechnical control methods, land management planning, and practices seem difficult for Türkiye.

Concerning the economic transformation planned within the framework of the EGD, the crucial role falls to the governments. However, it should be noted that the support of all social stakeholders such as producers, consumers, and NGOs should be included in the legal regulations and projects to be implemented in this regard to rendering all these efforts to be rewarding. As EGD is meant to bring about significant change and active community participation, building trust is essential in this transition period for the planned practices and policies to work smoothly and be widely accepted.

It should be explained that the Deal will not only offer solutions to climate change but also secure a lot of support for the development and growth of economies. In this context, it is considered necessary for Türkiye to assess these assets well within the framework of a win-win approach. If Türkiye cannot make the requirements within the scope of this rapid transformation, it may inevitably be negatively affected by this incapability.

Türkiye should see the EGD as an opportunity for transition into a low-carbon economy in the context of agriculture and climate change, rather than just aligning with European rules. In this context, green agricultural development policies which will enable Türkiye to achieve its goals in the field of agriculture and climate change should be urgently put into effect.

Any delay experienced in the process of harmonization with the EGD may cause Türkiye to sustain severe losses in its exports to the EU, and at the same time, the domestic labor market can be adversely affected. Therefore, while the trends in the EU and the world are moving toward a greener economy, it has become a necessity for Türkiye to switch to a production model which respects nature and people and reduces carbon emissions to maintain and develop its current status in the areas where it is the leader in the world.

On a final note, considering the effects of the energy crisis created first by the COVID-19 pandemic and then the Russia-Ukraine war on EU countries, it can be concluded the climate change and agricultural sector of the EGD will feel this crisis much more closely. EU countries have started to put nuclear power plants on the agenda again, and coal-fired power plants will be used for a while to overcome the energy crisis. Europe has attained the goals of the EGD between 2020-2022; nevertheless, these latest developments on the way to the commitments in 2030 have emerged as an obstacle to the Deal.

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Identification of Bacteria Obtained from *Dactylorhiza urvilleana* Rhizoid Region, Metal Tolerances, Bioremidant Characteristics and Effects on Maize Germination in Copper Presence

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ABSTRACT
Rapidly increasing industrialization and technological developments cause hazardous wastes to spread to the environment at a high rate. In our study, three bacterial (5O1, 5O8, 112O1) strains isolated from the rhizoid region of the orchid plant (*Dactylorhiza urvilleana*) were characterized by conventional and molecular methods (nuclear 16S rDNA). In order to characterize the isolates, primarily macroscopic, microscopic, some biochemical and physical properties of the strains were investigated. The usability of the strains screened for their general properties as bioremediation strains, in the prevention of high copper accumulation in agricultural soils was investigated. With traditional and molecular studies, two of the strains were defined as species level (*Bacillus mycoides, B. popilliae*) and one at genus level. They were determined that strains were tolerant to the tested all metal salts (Fe, Zn, Cu, Pb and Ag salts in the 1-10 mM range) except for the 5O1 strain to Ag salt, and 112O1 strain to Zn salt. The highest copper tolerance was observed in 5O1, 112O1 and 5O8 strains, respectively. The strains were determined that the copper minimum inhibition concentration values were 12.5-25 mM and the minimum bactericidal concentration value was 50 mM. The examined in terms of properties such as Indole-3-acetic acid (IAA), 1-aminocyclopropane-1-carboxylic acid (ACC) Deaminase activities, and phosphate solubility, it was determined that they promoted plant germination and growth. When the germination success of maize seeds in the presence of copper was examined, it was concluded that positive results were obtained, there was no significant difference between strains and therefore strains could be used in copper bioremediation.

Keywords: *Bacillus*, Metal tolerance, Plant growth-promoting bacteria, *Zea mays*

1. Introduction

The use of biostimulant microorganisms, which can improve physiological processes in plants, increase nutrient uptake, and promote tolerance to stresses (abiotic and biotic), is important for meeting the demands in agriculture, increasing the use of fertilizers, and more sustainable agricultural activities. When heavy metal accumulation in water and soil reaches undesirable levels, it causes universal health problems to occur and threaten societies. For this reason, there is a need for alternative biological control methods and biological resources to prevent heavy metal pollution, especially in soils used for agriculture. Rapidly increasing industrialization and contamination of industrial wastewater with heavy metals are among the most increasing environmental problems (Cetinkaya Dönmez et al. 1999; Sahan et al. 2010). This is characterized by a series of problems that progress by causing accumulations in food chains and occur with many different health problems. Researchers who want to develop effective strategies in combating pollution caused by heavy metals have turned to microorganisms. Copper metal which used in the study is one of the basic trace nutrients. However, metal levels in all environments, including air, water and soil, rise to toxic levels with the addition of a wide variety of industrial and domestic sources (Tanzadeh & Shareghi 2017). Copper in particular is found in serious amounts in various industrial wastes (Ho et al. 2002; Özer et al. 2004). It has been reported that the presence of high copper in the soil, passing to water resources and indirectly to food sources can cause serious problems in terms of human health (Saha et al. 2010). Since the massive accumulation of environmental pollutants adversely affects life, potential methods are being investigated to control the release of pollutants and to break down existing pollutants or transform them into harmless forms with appropriate recovery techniques. Bioremediation refers to the biological processes applied to decontaminate ecological resources such as soil and especially groundwater (Gillespie & Philp 2013). Many microorganisms, as an important component of the environment, develop complex mechanisms against the toxic effects of metals and make significant

contributions to the cleaning of contaminated areas (Nies 1999). Various microorganisms have a high affinity for heavy metals. Even if toxic metals remain in the soil, once they are attached to microorganisms, their uptake by plants or animals living in the soil is less. In this way, the presence of bioaugmentation and biostimulation involves the removal or reduction of heavy metals from the soil with bioremediation strategies (Das & Chandran 2011).

It is thought that obtaining new biostimulant strains that promote plant growth and increase resistance to biotic and abiotic stress will contribute significantly to the agricultural economy. In this research, we purposed to describe of physical, biochemical and plant growth promoting (PGPR) characteristics of 112O1, 5O8, 5O1 strains. Thus, 112O1, 5O8, 5O1 rhizoid bacteries isolated from *Dactyloriza urvilleana* on Ovit plateau, Rize, Turkey was identified with traditional (cultural, Gram staining and biochemical characteristics) and molecular methods (16S rRNA analysis). Heavy metal (Fe, Zn, Cu, Pb and Ag) tolerance and the ability to improve in different pH range in the presence of copper were investigated. The influence of bioremediation properties on the germination of maize plant was determined in the presence of copper.

2. Material and Methods

2.1. Conventional and molecular characterization of the isolates

For the isolation and conventional identification of bacteria, a set of morphological, physical and chemical test methods from Bergey's Systematic and Determinative Bacteriology Guide were used (Kandler & Weiss 1986; Holt et al. 1994). Mueller–Hinton (MH) agar and broth were used for the culture of the strain. Culture conditions were carried out under aerobic incubation conditions at 36 ºC for 24-48 hours. Total genomic DNA was performed according to the standard phenol and chloroform protocol (Sambrook et al. 1989). For the phylogenetic analysis and molecular characterization of bacterial isolates, specific primers 27F (5 'AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') were used for nuclear 16S rDNA intragenic gene regions. The specific gene region was amplified by Polymerase Chain Reaction (PCR) and sequence analysis was performed. The DNA sequences obtained were identified by comparing their similarities with the BioEdit program and other DNA sequences in *National Center for Biotechnology Information* GenBank (Baker et al. 2003; Rivas et al. 2004).

2.2. Determination of pH and NaCl tolerance

To determine the pH and salt tolerance of the isolates, Mueller Hinton broth medium prepared at different pH (4.5, 5.0, 6.8 and 8.0) ranges and salt (10%, 15%) concentrations were used. McFarland 0.5 turbidity bacterial suspensions were prepared from overnight cultures of isolates in MHB medium and used. Chemical tests were carried out by adding 20 µL of culture to 200 µL MHB medium in ELISA plates and incubating for 24-48 hours at 36 °C. Growth was evaluated as positive in wells with turbidity. The number of repetitions for all samples was determined as three.

2.3. Determination of metal tolerance

Metal salts ($AgNO_3$, $CuSO_4.5H_2O$, $FeCl_3.6H_2O$, $ZnCl_2$ and $Pb(NO_3)_2$) at different concentrations (1, 2.5, 5 and 10 mM) were used to determine metal tolerance (Velásquez & Dussan 2009). First of all, MacFarland 0.5 turbidity 10 µL of concentration was added to MH agar media containing metal salts prepared at different concentrations from overnight cultures of 112O1, 5O1 and 5O8 isolates and incubated for 5 days at 36 °C. Minimum inhibition concentration (MIC) and minimal bactericidal concentration (MBC) tests were performed to determine the dose of copper salt inhibiting bacterial growth by microdilution methods (NCCLS 1999).

2.4. Growth curve in copper presence

After determining the optimum copper tolerance value of the strains, their ability to grow at this concentration (3 mM $CuSO_4$) and in liquid (MHB) medium at different pH (5.0, 5.5, 6.0, 6.5, 7 and 7.5) ranges were investigated. It was incubated in a Bioscreen C (Labsystems, Helsinki, Finland) incubator set to shake and take measurements (at 420-580 nm) every 30 minutes for 24 hours at 36 °C, the optimum growth temperature as culture conditions. Growth curves were calculated by transferring the measurement data to the excel file. Positive and negative controls were used for each pH concentration and experiments were performed in triplicate.

2.5. Plant growth-promoting features

Phosphate dissolution activity was carried out according to the Fürnkranz et al. (2009) and Aydoğan et al. (2013) methods. ACC (1-Aminocyclopropane-1-Carboxylate) deaminase activity was measured using the basic method of Dworken & Foster (1958). Chrome azurol S agar assay (Cas agar) was used to test the production of bacterial siderophore proteins (Dworken & Foster 1958; Alexander & Zuberer 1991). Indole Acetic Acid (IAA) test was performed to determine whether the strains produce indole acetic acid hormone, which is an important factor for plant growth (Fürnkranz et al. 2009). The experiments were done in 3 replicates and the mean values were used. Ammonium liquid medium (ALM) was used to measure bacterial ammonium

production by colorimetric method. Color change from light yellow to red was evaluated as positive in the presence of ammonium (Aydoğan et al. 2013).

2.6. Germination test

The effect of Cu and bacterium on the success of germination of maize (May RX9292) was investigated. Maize seeds with equal weight were determined, disinfected using 70% ethanol and 3% sodium hypochlorite after then dried in the cabinet for 20 min. The bacterial suspensions (McFarland; 2) were prepared from overnight culture and 5 mL was added on maize seeds. A 10% gum arabic acid solution were added to the seed surface to allow the bacterium to adhere. The same amount of sterile water was added to the control group (Karunakaran et al. 2013). Maize seeds were shaken at 30 °C, 150 rpm for 2 hours. Ten maize seeds were placed in sterile petri dishes with filter paper. The experimental groups are listed below.

Control group

1. Maize (without Cu and bacteria).
2. Maize and 1.5 mM Cu (without bacteria).
3. Maize and 5O8, 5O1, 112O1 bacteria (without Cu).

Experimental group

Maize and Bacteria (each separately) and 1.5 mM Cu; 10 maize seeds separately for each bacterium were placed in three petri dishes and a solution of 1.5 mM Cu in 10 mL of water was added. All petri dishes were allowed to germinate at 26 °C for 16 hours of daylight and 8 hours of night in a climate cabinet with 70% humidity for a week.

3. Results and Discussion

Biochemical properties of the isolates (5O1, 5O8 and 112O1) used in the study are given in Table 1. It has been observed that the isolates are Gram positive, sporulated, Aerobic or facultative anaerobic and immobile. When physical and biochemical properties of isolates are examined; It has been observed that they can reproduce in a wide range of pH. Each has different biochemical properties, the 5O1 and 5O8 strains have a wide growth temperature range (10-45 °C), the 5O1 strain strong lecithinase activity and the ability to reduce 112O1 strain nitrate to nitrite. Microorganisms can be classified as psychrophiles, mesophiles, and thermophiles, according to the temperature range in which they can grow best. The growth temperature of microorganisms may vary according to environmental factors, but the most suitable temperature is known as the optimum growth temperature.

The metabolism of microorganisms is controlled by a wide variety of environmental factors, including pH, temperature, salinity, nutrient availability, and geographic locations (Lennon & Jones 2011; Amend et al. 2013). Among these environmental factors, pH plays an important role especially in the chemical activity of protons, redox reactions, mineral dissolution, and other geochemical reactions (Zhalnina et al. 2015). Environmental pH has the potential capability to shape interactions among microbial groups by promoting or inhibiting microbial redox reactions. The ability of our bacterial isolates to grow in a wide pH range shows that they have good adaptability to changing environmental conditions. In addition, it is thought that it can be used as growth-promoting microorganisms in plants that have the ability to grow in different pH environments.

The minimum and maximum growth temperatures of *Bacillus mycodies* 5O1 and *Bacillus popilliae* 5O8 were determined to be between 10 to 45 °C. It has been reported that heat stress tolerance can be increased in plants through growth-promoting thermotolerant endophytic bacteria and can be used as an alternative environmentally friendly way (Khan et al. 2020).

Table 1- Macroscopic, microscopic, some biochemical and plant growth promoting properties of bacterial isolates

Tests	*5O1*	*5O8*	*112O1*	*Tests*	*5O1*	*5O8*	*112O1*
Colony type	R	R	S	%10 NaCl	+	+	±
O_2 utilization	A	FA	FA	%15 NaCl	±	++	-
Oxidase	+	-	-	10 °C	+	++	-
Catalase	+	+	+	35 °C	+++	+++	+++
Motility	-	-	-	45 °C	+	+	-
Gram	+	+	+	pH 4.5	+	±	++
Spore	Cent.	Subter.	Cent.	pH 5.5	++	+++	++
Indole	-	-	-	pH 6.5	+++	+++	+++
Methyl red	+	-	+	pH 7.5	++	++	++
Citrate	-	-	-	pH 8.5	++	+	++
KIA	A/A	Al/Al	Al/Al	Glucose	ND	ND	+
H_2S	-	-	-	Lactose	ND	ND	+
Gas	-	-	-	Sucrose	ND	ND	+
Nitrate	-	-	+	Trehalose	ND	ND	+
Amylase	-	-	-	Maltose	ND	ND	-
Urease	-	-	-	Species	*Bacillus mycoides*	*Bacillus popilliae*	*Bacillus spp.*
Lecithinase	+++	-	-				

-: Negative, ±: weak positive, +: Positive, 2-3 +++: Strong Positive, A: Anaerobic, FA: Facultative anaerobic, KIA: Kligler Iron Agar, A: Acid, Al: Alkali, Cent: Central, Subter: Subterminal

The spore-forming property of *Bacillus* species provides an important advantage in the field of biological control in terms of their ability to withstand extreme environmental conditions. Therefore, developing cost-effective and stable sports-based products is an important consideration (Shafi et al. 2017). Among the many classes of biosurfactants, lipopeptides, usually produced by *Bacillus* strains (Ghojavand et al. 2008), are specifically interesting because of their remarkable surface properties (Afsharmanesh et al. 2013). Sinha & Mukherjee (2009) showed that especially Gram-positive spore-forming bacteria (*Bacillus* spp.) cell wall components with phosphate residues i.e. polysaccharides, teichoic and teichuronic acids, or phospholipid layers of the membranes can bind more heavy metals. *Bacillus* species contribute to the production of antibiotics and the emergence of plant systemic resistance against various plant pathogen diseases. It can also produce enzymes such as chitinase and β-1,3-glucanase, which have strong lytic activity. These lytic enzymes synthesized by *Bacillus* species have proven to be effective in breaking down the fungal cell wall (Leelasuphakul et al. 2006). Chitinases and glucanases are fungal cell wall hydrolytic enzymes produced by some *Bacillus* species. Many *B. subtilis* strains have been reported due to their chitinolytic activity (Das et al. 2010). Defense-related activities of enzymes have been proven against a variety of plant species and a variety of plant pathogens (Jayaraj et al. 2004). Most soils contain sufficient amounts of plant nutrients, but they are in an insoluble form, so plants cannot take up this undissolved nutrient from the soil (Francis et al. 2010). Beta-1,3-glucanases and chitinases play an active role in plant defense against various plant pathogens (Vidhyasekaran et al. 2001). Pathogenic and non-pathogenic microorganisms compete for both space and nutrients in the space around host plant roots. Nitrogen is the most essential nutrient for the development and productivity of crops. Bacteria located in the rhizosphere act as PGPR; They have the ability to improve atmospheric N fixation, hormone production, specific enzymatic activity, and plant and insect protection by producing antibiotics and other pathogen suppressants (Kamnev & Lelie 2000).

3.1. Plant growth promoting properties

Bacillus species, in addition to having a wide biocontrol ability, can multiply rapidly and are resistant to adverse environmental conditions. The most important bioactive molecules of the *Bacillus* genera are lipopeptides, polyketide compounds, bacteriocins, siderophores, and peptides not synthesized as ribosomes. Mostly, they have a broad spectrum of antagonistic activity against plant pathogenic bacteria, fungi, and viruses. *Bacillus* strains exhibit their biocontrol capacity through the induction of systemic resistance in plants and competition for ecological niches with plant pathogens, as well as inhibitory activity on the growth of plant pathogens. Apart from its antagonistic mechanism, *Bacillus* species have an important role in promoting plant growth by increasing the biosynthesis of plant hormones such as gibberellic acid (GA3) and indole 3 acetic acid (AA), which are closely related to plant nutrient availability (Chen et al. 2007). Plant growth-promoting microorganisms colonizing the host plant roots has direct and indirect strategies that affect plant growth and function by providing the plant with microbial compounds (ammonium production) and facilitating the uptake of nutrients. Many scientists have argued that *Bacillus subtilis* has the ability to increase plant growth and product yield. It also increases the production of plant hormones and nutrient uptake by reducing ethylene production in plant roots of *B. subtilis* (Chen et al. 2007; Idris et al. 2007). It was determined that our strains have strong siderophore and ammonium production ability and that 5O8 from our strains had strong IAA activity. In study results showed that the 112O1 isolate has the properties that support plant growth-promoting (such as IAA Activity, Ammonium and siderophore production, Phosphate solubility) (Table 2).

Table 2- Properties of bacterial isolates that support plant growth

Strain	*R/Z (mm)*		*ACC Deaminase*	*Ammonium Production*	*IAA Activity*
	Siderophore	*Phosphate*			
5O1	13/30	-	3/3	2+	-4.44±0.43
5O8	4/13	3/-	3/-	3+	28.68±2.33
112O1	20/43	4/8	3/3	+	13.53±0.31

R: Reproductive, Z: Zone, -: Negative, +: Positive, 2-3 +: Strong Positive

IAA has an important role in the formation and emergence of plant adventitious roots. It also increases shoot development by affecting cell expression, division and differentiation (Gardner 2009). These plant growth-promoting hormones increase the nutrient uptake ability of plants and help protect the plant against various biotic and abiotic stresses (Vessey 2003; Ghanashyam & Jain 2009).

3.2. Copper tolerance and determination of bacteria absorption ability

The surfaces of microbial cells contain various anionic structures and can be charged with a negative charge so that they can bind metal cations. This means that microorganisms have high affinity for metals. This feature, which also means heavy metal tolerance, is one of the basic conditions for a microorganism to be used in waste treatment processes (Jjemba 2004). Environmental heavy metal pollution has reached serious levels in many regions especially with industrialization. Heavy metals such as copper, lead, zinc, arsenic, chromium, mercury, manganese and cadmium, industrial, domestic and agricultural residues are contaminated into soils and waters in various ways (Jjemba 2004). The increase in environmental concentrations of metals such as the essential element Cu can cause them to become potentially dangerous. Since they cannot be metabolically degraded, they can accumulate in the food chain and reach dense amounts. When it is present in high amounts in food consumed by humans, it can lead to toxicity, cellular dysfunctions, long-term loss of workforce, disability and eventual death (Naja et al. 2010). In our study, the strains' tolerance to copper, lead, silver, iron and zinc salts were investigated in order to determine their bioremidant potential. It was observed that the strains were good growth in 10 mM iron concentrations, 5O1 and 112O1 strains showed very good reproductive potential at 10 mM Fe concentration, and the iron salt was not toxic at the concentrations tested (Table 3). In addition, the high siderophore activity of 5O1 and 112O1 strains parallels this result.

Table 3- The growth ability of bacteria in the presence of heavy metals (1, 2.5, 5 and 10 mM) in solid agar medium and determination of copper tolerance of isolates by agar-well dilution, MIC and MBC methods

Strains	*$AgNO_3$*	*$FeCl_3$*	*$CuSO_4$*	*$Pb(NO_3)_2$*	*$ZnCl_2$*
5O1	-	≤10/ + +	≤5/ ++ +	≤2.5/ +	≤2.5/ +
5O8	≤10/ +	≤10/+	≤1/ +	≤10/ +	≤2.5/ +
112O1	≤1 / +	≤10/ ++	≤2.5 / +	≤10/ +	-

	Inhibition of Copper (mM) by Agar Well Method (mm)						**Inhibition value (mM)**	
	100	**50**	**25**	**12.5**	**6.25**	**3.12**	**MIC**	**MBC**
5O1	20	10	6	-	-	-	12.5±0	50
5O8	16	12	8	6	6	-	12.5±12	50
112O1	13	11	8	6	-	-	25±0	50
B.subtilis W168*	16	14	14	10	6	-	6.3±0	50

-: No Reproductive, +: Reproductive, ++: Good Reproductive, +++: Very Good Reproductive, *: Used as a control.

It was determined that 5O1 did not grow in the presence of silver nitrate, 112O1 has a tolerance of up to 1 mM, but 5O8 was resistant to silver at 10 mM. It has been observed that 5O8 and 112O1 strains show high tolerance in the presence of lead nitrate, while 5O1 strain can reproductive at 2.5 mM. While the 112O1 strain did not reproductive in the presence of zinc chloride, it was observed that the 5O1 and 5O8 strains were only tolerant to 2.5 mM concentration. In general, it can be said that the strains have high metal tolerance. Iron is mostly in ferric form in oxidized and gaseous soils, insoluble in water at pH 7.4, and its concentration can be as low as 10^{-8} mol L^{-1}. This concentration is insufficient to support the growth and development of microorganisms. Microorganisms develop different ways to survive in these conditions, which enables their iron needs from the microenvironment (Shafi et al. 2017). Villegas et al. (2018) reported that eight of the 90 bacterial strains they isolated from the

mined soil in Mogpog formed biofilm and tolerated Cu, Pb, Zn and Cd, and soil-based isolates had great potential in the treatment of wastewater contaminated with Cu. *Bacillus* species are known to be tolerant of high metal concentrations and are used to remove cation contaminants from wastewater (Cheung & Gu 2005). It has been observed that the tolerance of bacteria against copper sulfate is very good at 5O1 at 5 mM, whereas other strains are less. It was aimed to determine the Cu tolerance MIC and MBC values and it was observed that it was effective in the range of 25-100 mM when examined with the agar well inhibition test (Table 3). The fact that the 112O1 strain has 25 mM and the other strains have 12 mM MIC values and these values are higher than the control strain shows that these strains have strong copper bioremidantion potential. The fact that the growth-inhibiting Cu concentration value (MBC) was 50 mM in all strains, including the control strain, confirms these results.

Reproduction of microorganisms in the presence of metal; changes depending on factors such as time, metal concentration, and pH. It was aimed to determine the copper absorption / tolerance properties of strains at different pH values (pH range of 5.0-7.5) in liquid media to be used in metal studies. For this purpose, the growth ability of bacteria in liquid medium (Brain heart infusion broth) pH 5.0-7.5 in the presence (3 mM) and absence of copper was spectrophotometrically measured (Figure 1).

It was observed that *B. mycoides* 5O1 was generally able to grow between pH 5.0-7.5 in the presence and absence of copper. It was affected by low pH in the presence of copper and also the best growth in both environments was determined in pH 7.0-7.5. *B. popilliae* 5O8 strain showed good growth in the range of pH 5.0-7.5 in a copper-free environment. It was affected by low pH in the presence of copper, and the best growth was in the range of pH 6.5-7.5 (Figure 1). Compared according to Cu absorption properties at various pH ranges *Bacillus* sp. It was observed that 112O1 strain completed the logarithmic phase in 8 hours at all pHs in the absence of copper, while this period extended up to 18 hours in the presence of 3 mM Cu. The results also show that *Bacillus* sp 112O1 continued to grow in the pH range of 6.0-7.5 in the presence of metal after 24 h while transitioning to the stationary phase between pH 5.0-5.5. In a study by Esertaş et al. (2020), it was observed that the growth rate and density were affected at different pH values in the presence of metal, reaching the logarithmic phase in 8 hours in the presence of copper, while this period extends at all pHs (12 hours and above times) in the presence of copper. The results observed in *B. popilliae* 5O8 strain were found to be consistent with this study.

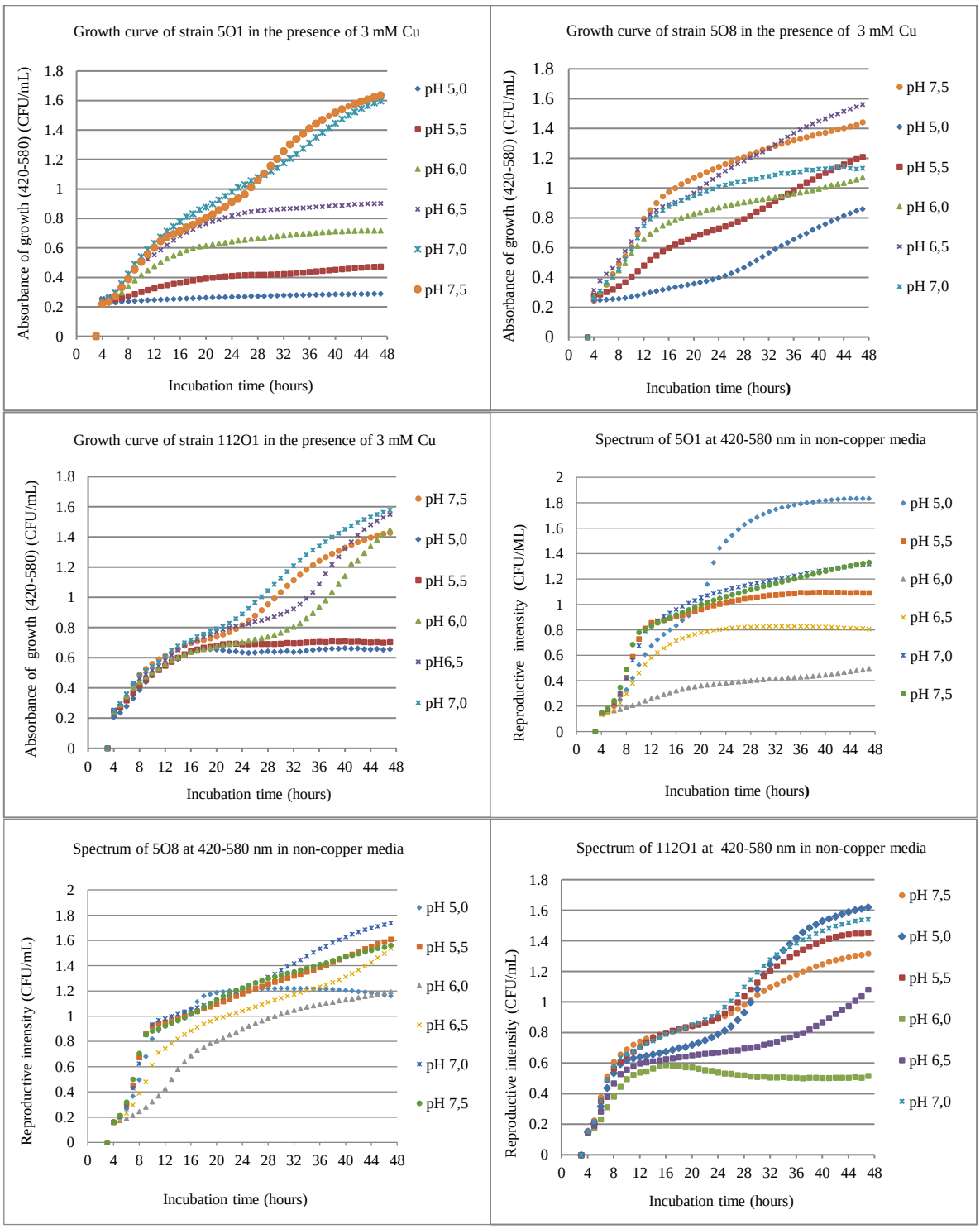

Figure 1- Growth graph of 5O1, 5O8, and 112O1 strains in BHIB medium with different pH, with and without Cu

3.3. The germination success of maize Seed in the presence of copper and bacteria

Some plants, such as *Secale cereale*, *Helianthus annuus*, *Brassica juncea*, and *Zea mays* which can absorb high concentrations of metal are called hyper accumulator plants (Visoottiviseth et al. 2002). In a study by Huang & Cunningham (1996), when the effect of the combination of maize and *Bacillus* spp. strains on Cu removal was tested, it was reported that maize accumulated Cu in its shoots and roots while reaching high biomass and growing well. In our study, the effect of metal-tolerant bacterial strains (in the presence of 1.5 mM Cu) with plant growth-promoting properties on the germination of maize, which is a hyperaccumulator plant, was investigated. For this purpose, germination success, stem, and root development parameters were measured by germinating a total of 30 (10 x 3) maize seeds for a week, 3 replicates for each group. The best germination success was determined for the 5O1 strain and the lowest for the 112O1 strain (Table 4, Figure 2). The germination success of 112O1, 5O1, and 5O8 strains, which have high plant growth-promoting properties, was determined in the range of 90-96.7% in the

presence of bacteria on the seventh day. It was observed that not affected in the presence of Cu, and it was between 93.3% and 100% in the presence of Cu and bacteria. When the effectiveness of 3 different bacterial strains tested in the presence of copper on maize germination (stem and lateral root) was examined statistically (ANOVA), it was observed that it was significant ($P<0.01$) in terms of parameters other than stem weight ($p= 0.117$).

Table 4- Determination of bacteria and copper (1.5 mM) activity on maize germination

Groups	*Germination Percentage by Day*			*Coleoptile (Body)*		*Radicle (root)*		*Lateral Root*
	3.	*5.*	*7.*	*Weight(g)*	*Length(cm)*	*Weight(g)*	*Length(cm)*	*%*
Ma	87	100	100	0.199	3.63	0.199	10.04	3.6
501-Ma	70	83.3	90	0.146	4.64	0.250	8.68	8.0
5O8-Ma	80	86.7	90	0.148	4.95	0.323	10.42	8.9
112O1-Ma	76.7	83.3	96.7	0.161	4.48	0.195	7.30	5.2
Ma-Cu	96.7	100	100	0.120	2.60	0.085	1.69	8.3
501-Cu-Ma	83.3	96.7	100	0.113	2.92	0.407	1.10	7.9
5O8-Cu-Ma	80	96.7	96.7	0.132	3.10	0.041	1.18	6.8
112O1-Cu-Ma	80	90	93.3	0.108	2.63	0.062	1.94	5.7

Ma: Maize

Figure 2- Seventh day views of maize (*Zea mays* L.) germination test groups in the presence of Cu

It was observed that the length of the coleoptile in the presence of bacteria was better than all groups, and it was higher in the presence of Cu with bacteria compared to the Cu control. It was observed that the length and weight of the radicle in the presence of bacteria was better in the 5O8 strain compared to the negative control, while the other 2 strains were good in the copper-free environment, but were affected in the presence of copper (Figure 3).

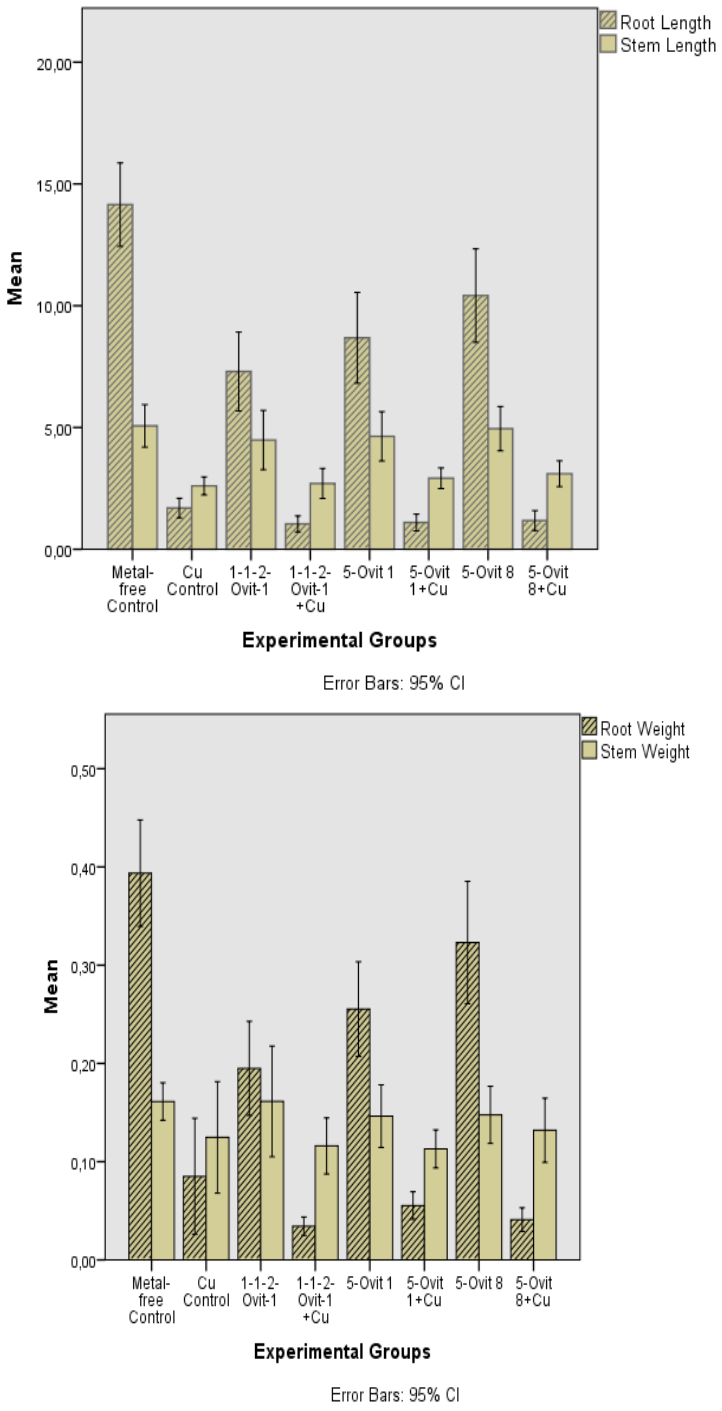

Figure 3- The effects of bacteria on root-stem length and root-stem weight in the presence and absence of Cu

It was determined that there is a significant difference ($P<0.01$) in the combination of both copper and bacteria with copper in the number of fringes among all parameters examined compared to the control group. It has been observed that the bacteria alone increase the fringing and there is a low change in the presence of copper (Figure 4). These results show that the bacteria are of rhizoid origin, stimulate plant growth and can perform metal bioremediation.

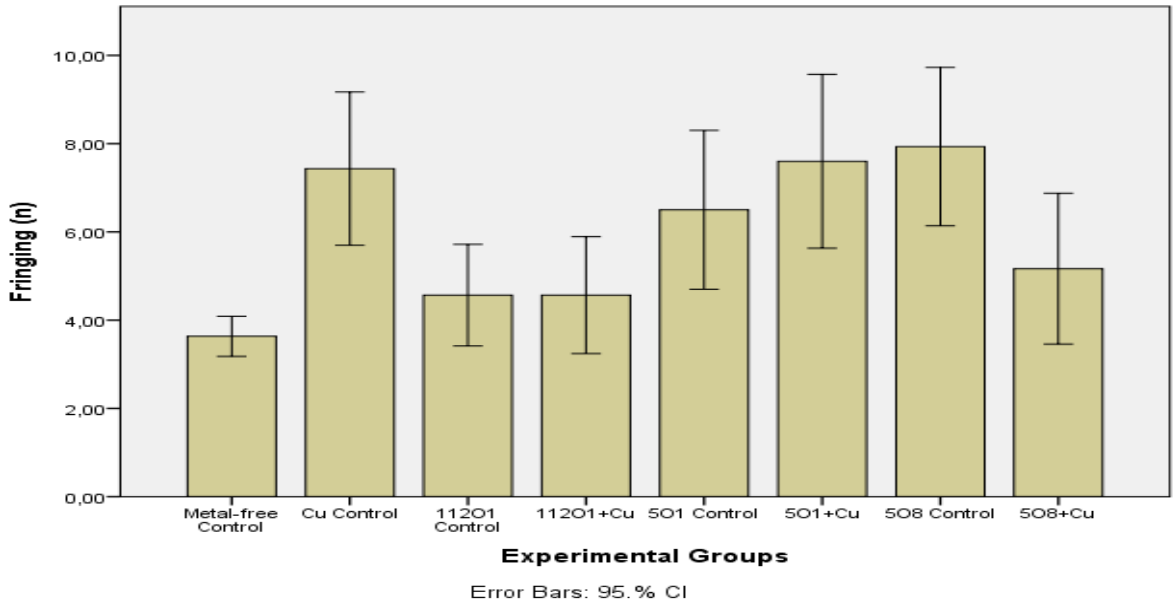

Figure 4- Effects of bacteria on fringing in the presence and absence of Cu

All seeds germinated at the end of 5th day in maize control groups with and without copper metal. Although germination was not affected in the presence of copper metal compared to the copper-free control medium, it was observed that copper metal decreased the root and shoot growth of maize, but increased fringe root formation. In general, when the germination success is examined, it is seen that germination decreases in the presence of bacteria compared to control, germination is better in the presence of copper only compared to control, and the germination success increases in copper and bacteria associations. According to these results, it was observed that the presence of 1.5 mM copper increased the germination rate, decreased the germination rate of bacteria alone, and the presence of bacteria+copper increased the germination rate of the seed.

Bacillus bacteria produce various compounds that can be used to combat many plant pests. Growth and development of biological agents in field conditions is a problem due to adverse environmental conditions. With the excellent stabilizer that can optimize its activity in field conditions, the formulation of the active product is an alternative and more effective strategy for the management of plant pests rather than using live bacteria directly. Bacterial formulated products can be harmful to live bacteria when used in conjunction with other synthetic chemical pesticides. It is important in the formulation that can extend the shelf life of the bacterial product during storage, transportation as well as field application. For a stable and effective formulation, the bacterial active compound must be recognized correctly (Shafi et al. 2017). Systemically derived resistance and plant growth promoting properties of *Bacillus* strains play a fundamental role in biological plant protection. A large number of *Bacillus* strains have been developed to effectively combat plant pests and diseases. In our study, it was concluded that the strains isolated and conventional / molecularly diagnosed are potential biocontrol agents with high metal and salinity tolerances and capable of reproducing in a wide range of temperature and pH.

4. Conclusions

Since the intensive accumulation of environmental pollutants, especially in agricultural areas and water resources, negatively affects life, the subject of researching potential new bioremidant microbial resources is always up to date. It is considered that the importance of increasing or improving productivity in agriculture will increase in the future. Based on this, the effect of strains on germination in the presence and absence of copper was tested in our study. While germination in the presence of 1.5 mM Cu did not change much due to the trace element properties of copper, the bacteria slowed the growth by taking the copper in this range. It suggests that high concentrations of metal that will affect plant growth will be absorbed by bacteria and contribute to the continuity of development. It is obvious that the evaluation of plant-bacteria relationships and selection of bacteria with plant growth promoting properties, will significantly contribute to development of new microbial preparations for agricultural purposes, bioremediation of the soil, obtaining healthier and higher quality agricultural products. In the present study, the bioremediation potential of the three strains was evaluated, and it is thought that different metal groups can be tested in future studies. In addition, it is anticipated that germination experiments can be tried on different plant species and that suitable plants will be directed to greenhouse and field studies.

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Macro and Micro Element Composition of Some Peanut (*Arachis hypogaea* L.) Varieties in Turkey

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ABSTRACT
This study was conducted to determine the macro and micro element contents of fourteen peanuts in Turkey. Virginia (NC-7, Halisbey, Arıoğlu-2003, Sultan Flower-22, Osmaniye-2005, Brantley, Wilson, Batem-5025, Batem-Cihangir, NC V 11 and Polen) Runner (Georgia Green) and Spanish (Florispan) market types have been evaluated. The research was conducted for two years (2015 to 2016) under main crop conditions in the trial areas of belonging to the Oil Seed Research Institute. The highest nitrogen content is from the Florispan (4.56%) variety, the highest phosphorus and sodium content is from the Halisbey (0.10%) variety, the highest potassium content is from the Sultan (0.46%) variety, the highest calcium content is from the Flower-22 (0.07%) variety and the highest the magnesium content was taken from Arıoğlu-2003 (0.26%) variety. The highest iron content is in Batem-Cihangir (27.34 mg kg^{-1}) variety, the highest copper content is in Flower-22 (7.08 mg kg^{-1}), the highest zinc content is in Sultan (29.35 mg kg^{-1}), the highest manganese content NC-7 (20.61 mg kg^{-1}) variety, the highest boron content was found in Florispan (26.99 mg kg^{-1}) variety. According to the results of this study, varieties with different chemical compositions can be used in food and breeding studies.

Keywords: Macro Nutrients, Micro Nutrients, Market Type, Osmaniye, Peanut Pods

1. Introduction

Peanut is oil-seed legume that is used in many ways and its seeds are also important economically and medicinally (Akram et al. 2018). India, China, America, Senegal, Indonesia, Nigeria, Brazil and Argentina are important countries producing peanuts (FAO, 2020). Peanut has become one of the major global oil-seed crops cultivated on approximately 26 million ha in about 120 countries yielding about 35 to 40 million tons of peanut pods annually (Patel et al. 2016; Sarkar et al. 2016; FAO 2020). In Turkey, peanuts are grown mostly in Adana, Osmaniye, Hatay, Mersin and Gaziantep regions.

The major constituents of peanut seeds are carbohydrates (15% to 20%), oil (36% to 54%) and protein (16% to 36%) (Davis et al. 2016). They also contain other beneficial minerals such as phosphorus, potassium, iron, manganese, calcium, sodium, selenium, copper and zinc are also present in peanuts (Ayoola et al. 2012). Moreover, multivitamins including tocopherols, thiamine and folic acid are present in peanut seeds (Isanga & Zhang 2007). The presence of flavonoids, vitamin E, resveratrol and different hydroxycinnamic acids including chlorogenic, caffeic, ferulic acids and coumaric give peanut its high antioxidant capacity (Hasan et al. 2013; Jonnala et al. 2006; Sales & Resurreccion 2010; Zhang et al. 2017).

Peanuts are cultivated in almost all soil types around the world and require macro and micronutrients for growth and development compared to many other crops (Cox et al. 1970, 1982; Dwivedi 1988; Hartzog & Adams 1988; Singh 1999, 2004).

For an adequate fertilization management, the designation of nutrient uptake and congregation during the plant development are important. So that, it is possible to identify the times at which elements are needed want during crop development and the dispensation of the elements in different parts of the plant. In order to determine the nutritional needs of a plant, both the chemical composition and accumulation of nutrients in fruits and leaves should be considered. This information may facilitate the estimation of the amount of nutrients to supply during fertilization.

Its cultivation improves soil fertility through atmospheric nitrogen fixation (Lal 2008). There are numerous peanut varieties that are usually preferred on the basis of high-blanch abilities, high fat content, high yield and low-shelling (Deshpande et al.

2008, Mulando & Resurreccion 2006). Morover, the quality and composition of peanut oil varies among genotypes and developmental stages and in response to environmental factors (Andersen & Gorbet 2002; Krishna et al. 2015).

In the study, the nutrient content of fourteen different peanut varieties were compared. The results obtained will reveal which variety will need which nutrient element is more sensitive or more in peanut cultivation.

2. Material and Methods

2.1. Material

Polen, Halisbey, Sultan, Arıoğlu-2003, Flower-22, Osmaniye-2005, Brantley, Wilson, Georgiya Green, Florispan, Batem-5025, Batem-Cihangir, NC V 11 and NC-7 peanut varieties used as materials in the study were provided from Çukurova University Faculty of Agriculture Field Crops Department. After the harvest, the pods of peanut varieties were broken and the seeds were removed. And then plant nutrient analysis of the seeds was made. In the province of Osmaniye where the study was conducted, the average climate data for the long years of April-September for the trial years of 2015-2016 are given in Table 1.

Table 1- Monthly temperature, total precipitation, relative humidity in 2015-2016 growing seasons and long-term (1994-2016) averages in Osmaniye Province

Months	*Average Temperature (^0C)*			*Total Precipitation (mm)*			*Relative Humidity (%)*			*Soil Temperature at 10 cm (^0C)*	
	2015	*2016*	*Average (1994-2016)*	*2015*	*2016*	*Average (1994-2016)*	*2015*	*2016*	*Average (1994-2016)*	*2015*	2016
April	16.1	19.6	16.08	76.6	11.7	85.60	60.0	51.3	61.77	18.2	21.2
May	21.9	20.6	20.41	42.0	87.1	67.84	58.3	67.1	60.18	22.6	24.3
June	24.4	26.3	23.23	31.8	9.3	36.08	64.1	62.2	55.63	28.6	31.4
July	27.9	28.7	28.00	-	0.8	12.01	62.3	65.1	65.75	34.9	36.5
August	29.0	29.2	27.32	1.9	10.0	7.48	56.2	66.8	61.82	35.9	36.2
September	27.5	24.8	24.11	2.6	79.1	35.33	54.5	60.8	58.66	33.3	31.1

When the months of the experiment are examined, it is seen that the temperature values of 2016 are generally higher. The highest temperature in both years occurred in August. However, when the average of the long term is examined in terms of temperature, it is seen that the highest temperature value is in July. The province of Osmaniye has a typical Mediterranean climate with warm and rainy winters and dry and hot summers. When the total amount of precipitation for the years of the experiment is examined, it is seen that the most important difference occurred in April 2015. This difference in precipitation that occurred in April 2015 caused the planting to take place on April 9. According to the long-term average, the total precipitation values varied between 7.48 mm and 85.60 mm. It is seen that rainfall values vary according to years, months and years.

Soil samples were taken from 0-30 cm depth of the fields where peanuts are grown. Soil analyzes were carried out by Bursa Uludağ University Faculty of Agriculture Soil Science and Plant Nutrition Department in the first year and by Osmaniye Korkutata University Soil Laboratory in the second year (Kacar 1994). Soil analysis results are given in Table 2.

Table 2- Some physical and chemical characteristics of the soils of the experimental sites

Properties	*Year 2015 (Toprakkale)*	*Year 2016 (Alahanlı)*
pH	7.30	7.80
Lime, $CaCO_3$, (%)	6.93	0.82
Organic matter (%)	1.161	1.692
Total N (%)	0.061	0.071
NH_4-N, mg kg^{-1}	21.6	36.3
NO_3-N, mg kg^{-1}	4.4	8.4
$NaHCO_3$-P, mg kg^{-1}	11.3	4.1
Exc. K, mg kg^{-1}	55	72
Exc. Ca, mg kg^{-1}	2714	2602
DTPA-Cu, mg kg^{-1}	0.5	0.8
DTPA-Zn, mg kg^{-1}	1.0	0.3
DTPA-Mn, mg kg^{-1}	2.4	4.7
DTPA-Fe, mg kg^{-1}	5.8	4.0

As a result of the soil analysis, the pH value of Toprakkale (7.30) and Alahanlı (7.80) soils were evaluated as slightly alkaline and salt-free in both soils according to the EC values. In terms of lime content, Toprakkale soil (6.93%) is calcareous and Alahanlı soil (0.82%) is in the lime-free class. The organic matter content of both soils (1.161% - 1.692%) was evaluated as a low (Tüzüner 1990). According to the plant nutrient analysis results; It was determined that the nitrogen content of both soil samples (0.061% - 0.071%) was low (Silanpaa 1990). In terms of P (phosphorus), Toprakkale soil (11.3 mg kg^{-1}) was sufficient, while Alahanlı soil (4.1 mg kg^{-1}) was determined to be low (Silanpaa 1990). K (potassium) was found to be low in both soils (55-72 mg kg^{-1}), Fe (iron) was found to be very high in Toprakkale (5.8 mg kg^{-1}), and high in Alahanlı (4.0 mg kg^{-1}) (Lindsay & Norwell 1978). Toprakkale soil is evaluated as high in terms of Cu (copper), Zn (zinc) and low Mn (manganese) content. Alahanlı soil is evaluated as high in Cu and low in Mn and Zn content.

2.2. Method

In the research, field trials were carried out for 2 years (in 2015-2016) in the irrigable test areas of Oil Seed Research Institute in Osmaniye province in Toprakkale and Alahanlı during the main crop growing periods. The experiment was set up with 4 replications according to the randomized blocks trial pattern. The parcels are 5 m long and consist of 4 rows (5.0 m x 2.8 m = 14.0 m^2). The planting density is arranged as 70 x 15 cm. Before planting, the seeds were disinfected with Korconil 75W (Chlorothalonil) and Korban 25W (25% Chlorpyrifos-Ethyl) as 1.0 kg drug per 100 kg of seeds. Before planting, soil preparation operations were carried out. 200 kg 20-20-0 compound fertilizer per hectare was used according to the analysis results. A total of 40 kg da^{-1} of CAN 26% (calcium ammonium nitrate) fertilizer was applied, with the first top fertilizer being 20 kg da^{-1} at the beginning of flowering and the second top fertilizer being 20 kg da^{-1} at the beginning of pod formation. It has been used in fertilizers containing microelements during cultivation. Sowing was done manually on April 8 and April 9, in 2015 and 2016, on a date when soil and weather conditions were appropriate. Sprinkler irrigation system has been used for sufficient plant germination after planting. During the growing period, the necessary activities (hoeing, irrigation and spraying) were carried out on time in accordance with the technique. The harvest of varieties in the Virginia group is between 140-160 days, and Flower-22 from this group can be harvested in 125-130 days because it is early, Georgia Green in the Runner group in 135-140 days, and the Florispan variety in the Spanish group in 120-130 days (August in the middle of the second week of September). After the harvest, 20 plants were removed randomly from each plot and their fruits were ground, and their macro and micro element contents were determined.

2.3. Analysis methods of macro and micro elements of peanut pods

The seed samples of the plant were dried until they reached a constant weight in 65 ^oC air-dried oven. Total nitrogen in plant samples was determined with a Buchi K-437/K-350 Digestion/Distillation Unit according to the Kjeldahl method (Bremner 1960). Seed samples were digested at 180 degrees with 4 ml HNO_3 and 3 ml H_2O_2 (Berghof MWS 2 DAP 60 K microwave oven). Macro and micro nutrients were analyzed from extracts using the ICP OES (Perkin Elmer OPTIMA 2100 DV) (Kacar & İnal 2014).

The research data were analyzed through use of JUMP 5.0.1 statistics package program according to the randomized block trial design. The differences between the average values were compared to significance level by using LSD Multiple Comparison Test.

3. Results and Discussion

N, P, K, Fe, Cu, B, Ca, Zn, Mn, Mg elements are considered essential for plants (Gascho & Davis 1994). Nutrients must be in sufficient quantities in the soil in order to get enough yields. Year x varieties interactions were found to be significant ($P<0.05$) in N, K, Zn and B (Table 3 and 4). On the other hand, P, Ca, Mg, Na, Fe, Cu and Mn were found to be insignificant in the years x cultivars interaction of the average of years. The two-year averages of the peanut product were evaluated in the study.

Nutrient element determined as the highest nitrogen and the lowest copper in the plant as a 4.38 % and 3.08 mg kg^{-1}. In varieties, nitrogen content range from 3.67% to 4.38%, P content of the cultivars varied between 0.460% and 0.558%, K ranged from 0.367% to 0.458%, Ca content of the cultivars is between 0.052% and 0.072%, the Mg content of the varieties varied between 0.237 and 0.262%. Na element content of the varieties varied between 0.080 and 0.099%. The highest N content was obtained from the Florispan variety with 4.38%, while the lowest N content was obtained from the Georgia Green variety with 3.67% (Table 3). Of all of the necessary mineral nutrients, plants require N in the greatest amounts. Regulation of N uptake in plant is complex, with carefully controlled integration of NH_4^+ and NO_3 uptake, besides occurs at both genetic and physiological levels. In leguminous plants, where most of the required N is obtained from close associations with N2-fixing bacteria in root nodules, the regulatory system is even more complicated and is likely to involve proteins and signals specific for the transfer of $NH4^+$ across the peribacteroid membrane (Tyerman et al. 1995; Marini et al. 2000). Steer & Hocking (1984) reported that the N content of the seeds, besides the Napplied to the plant, the variety characteristics of the plants and the environmental conditions were effective.

The average P content of the cultivars varied between 0.460% and 0.558%. Jonnala et al. (2005) determined P content between 0.356 and 0.427% in different pistachio lines; Shokunbi et al. (2012) determined the P content between 0.372% and 0.403% in their study on Boro Light, Boro Red, Mokwa, Campala and Ela peanut cultivars. While the highest average P content was obtained from Halisbey (0.56%) cultivar, the lowest P content (0.46%) was obtained from Batem-5025 variety (Table 3). The results found by the researchers (0.25-0.66%) are consistent with the results we found. Although it is extracted in smaller quantities compared to other macronutrients, P is considered the main productivity factor of the peanut crop (Bolonhezı et al. 2005). The accumulation of more than 70% of the P taken by the peanut plant in the fruits shows how important this element is in the formation and development of the fruits (Feitosa et al. 1993). Regulation of the uptake of P appears to be via the internal nutrient status of the plant. Gene expression and kinetics studies both suggest that the high-affinity P transporters are more responsive to nutrient status than low-affinity transporters.

The average K content varied from 0.367% to 0.458%. The highest K content was observed in Sultan (0.458%) cultivars, while the lowest average K content was observed in Florispan (0.367%) (Table 3). Jonnala et al. (2005) found the K content between 0.564% and 0.615, Shokunbi et al. (2012) found the K content between 0.575% and 0.611%. The amount of K element can vary. K is very important to plants and is the second-most absorbed element, overcome only by N. K has the physiological function of an enzymatic activator and, once absorbed, can be transferred from the older parts of the aerial portions to the newer parts (Tasso et al. 2004). K plays an important role in the formation of fruits, acting in the transport of photoassimilates in the phloem (Taiz & Zeiger 2013). The deposition of biomass in fruit is necessarily accompanied by the accumulation of K. In addition, K is a required nutrient in the activation of several enzymes essential to the synthesis of organic compounds, among them starch (Laviola & Dias 2008).

Regulation of K uptake is still poorly understood. It has been shown that plants respond to low concentrations of K by increasing the capacity for high-affinity K transport (White 1997), which might be due either to an increase in the number of transporters or to a higher activity of existing transporters.

The average Ca content of the cultivars is between 0.052% and 0.072% (Table 3). The highest Ca content was obtained from Flower-22 variety, which is in the Virginia group, while the lowest average Ca content was obtained from Halisbey and Arıoğlu-2003 cultivars (Table 3). Cobb & Johnson (1973) found that the Ca content in the seed was in the range of 0.01-0.08%. The results are consistent with the results found by the researchers. Shokunbi et al. (2012) also found that the Ca content Boro Light, Boro Red, Mokwa, Campala and Ela in the peanut varieties was in the range of 0.044-0.063%. Shibli et al. (2019) reported that the Ca content BARD-9, BARD-479 and Local 334 in the peanut seed was in the range of 0.039-0.048%.

Ca has chemical attributes that give it a special role in controlling many aspects of plant growth and development. Ca is rather unusual among plant nutrients in that most Ca is localized outside of the cell where it has an important role in stabilizing cell walls by cross-linking acidic side groups on adjacent pectic polysaccharide molecules. In plants without pectin in cell walls, essentiality for Ca is difficult to demonstrate (O'Kelley 1968).

The highest Mg element content was obtained from Arıoğlu-2003 (0.262%) variety, while the lowest Mg content was obtained from Polen, Osmaniye-2005, Batem-5025, Florispan and Batem-Cihangir varieties with 0.237%. Gaines and Hammons (1981) found the Mg content between 0.20-0.25%. They conducted with Early Bunch, Florigiant, Florunner and Tifrun peanut varieties. Jonnala et al. (2005) found Okrun peanut and 188, 540, 654 in the peanut lines Mg content between 0.189 and 0.219%, Shokunbi et al. (2012) also found that the Mg content Boro Light, Boro Red, Mokwa, Campala and Ela in the peanut seed was in the range of 0.098-0.112%, Shibli et al. (2019) reported that Mg content between 0.068 and 0.083%. These results are consistent with our study. Mg is present in plant cells in high concentrations and has a range of important functions: it is a cofactor in reactions involving ATP, stabilizes DNA and RNA molecules and cellular membranes, and is a component of chlorophyll. Despite this, our understanding of Mg uptake is very poor, with almost nothing reported in the literature about the mechanism of Mg entry into plant cells. The Na element content of the varieties varied between 0.08-0.099%. The highest average Na content was obtained from Halisbey variety, the lowest average Na content was obtained from NC-7, Wilson, Batem-5025 and Georgia Green varieties.

Peanut plant also contains different amounts of micro nutrients. Micronutrients are essential elements that are used by plants in small quantities. In spite of this low requirement, critical plant functions are limited if micronutrients are unavailable, resulting in plant abnormalities, reduced growth and lower yield. In such cases, expensive, high requirement crop inputs such as nitrogen and water may be wasted. Because of higher yields, higher commodity prices and higher costs of crop inputs, growers are reviewing all potential barriers to crop production, including micronutrient deficiencies. Micro element analysis results are given in Table 4.

Table 3- Macro Element Content of Peanut Varieties

Macro Elements	*Years*	*Varieties*													
		Polen	*Halisbey*	*NC-7*	*Sultan*	*Flower-22*	*Osmaniye-2005*	*Brantley*	*Wilson*	*Batem-5025*	*Arıoğlu-2003*	*Georgia Green*	*Florispan*	*NC V 11*	*Batem-Cihangir*
N (%)	1. Years	3.72 bc	3.54 e-g	3.44 g	3.58 ef	3.50 fg	3.77 b	3.70 b-d	3.53 e-g	3.62 c-e	3.61 d-f	3.25 h	3.94 a	3.44 g	3.56 ef
	2. Years	4.35 b-e	4.25 c-e	4.33 b-e	4.46 b-d	4.20 c-e	4.15 d-e	4.41 b-e	4.34 b-e	4.53 a-c	4.42 b-e	4.09 e	4.83 a	4.61 ab	4.37 b-e
	Means	4.04 bc	3.89 b-d	3.88 cd	4.02 b-d	3.85 de	4.06 bc	4.05 bc	3.94 b-d	4.07 b	4.02 b-d	3.67 e	4.38 a	4.03 b-d	3.96 b-d
P (%)	1. Years	0.470 d-e	0.563 a	0.480 c-e	0.500 b-e	0.510 bc	0.503 b-d	0.520 b	0.480 c-e	0.465 e	0.493 b-e	0.505 b-d	0.505 b-d	0.480 c-e	0.510 bc
	2. Years	0.470 c-e	0.555 a	0.473 c-e	0.500 b-d	0.503 b-d	0.495 b-e	0.515 ab	0.463 de	0.455 e	0.478 b-e	0.475 b-e	0.495 b-e	0.480 b-e	0.510 bc
	Means	0.470 gh	0.558 a	0.476 e-h	0.500 b-e	0.506 b-d	0.498 b-f	0.517 b	0.471 f-h	0.460 h	0.485 c-h	0.490 b-g	0.500 b-e	0.480 d-h	0.510 bc
K (%)	1. Years	0.400 c-e	0.425 cd	0.395 de	0.465 a	0.380 e	0.430 bc	0.413 cd	0.395 de	0.375 e	0.415 cd	0.458 ab	0.405 c-e	0.420 cd	0.430 bc
	2. Years	0.370 c	0.428 ab	0.343 de	0.453 a	0.378 c	0.430 ab	0.358 cd	0.380 c	0.363 cd	0.418 b	0.438 ab	0.330 e	0.418 b	0.438 ab
	Means	0.385 de	0.426 c	0.368 de	0.458 a	0.378 de	0.430 bc	0.385 de	0.387 d	0.368 de	0.416 c	0.447 ab	0.367 e	0.418 c	0.433 bc
Ca (%)	1. Years	0.060 b-d	0.053 d	0.063 a-d	0.070 ab	0.073 a	0.060 b-d	0.070 ab	0.068 ab	0.065 a-c	0.053 d	0.060 b-d	0.065 a-c	0.055 cd	0.070 ab
	2. Years	0.050 c	0.053 bc	0.063 ab	0.060 bc	0.073 a	0.060 bc	0.058 bc	0.060 bc	0.058 bc	0.053 bc	0.058 bc	0.050 c	0.060 bc	0.053 bc
	Means	0.055 cd	0.052 d	0.062 bc	0.065 ab	0.072 a	0.060 b-d	0.064 b	0.064 b	0.061 bc	0.053 d	0.059 b-d	0.057 b-d	0.057 b-d	0.061 bc
Mg (%)	1. Years	0.238 bc	0.255 ab	0.243 a-c	0.245 a-c	0.245 a-c	0.243 a-c	0.245 a-c	0.248 a-c	0.243 a-c	0.258 a	0.245 a-c	0.238 bc	0.250 a-c	0.235 c
	2. Years	0.245 bc	0.250 bc	0.258 ab	0.255 ab	0.255 ab	0.243 bc	0.245 bc	0.250 bc	0.243 bc	0.268 a	0.255 ab	0.238 c	0.243 bc	0.250 bc
	Means	0.241 bc	0.252 ab	0.250 a-c	0.250 a-c	0.250 a-c	0.242 bc	0.245 bc	0.248 bc	0.242 bc	0.262 a	0.250 a-c	0.237 c	0.246 bc	0.242 bc
Na (%)	1. Years	0.093 bc	0.103 a	0.093 bc	0.095 a-c	0.090 b-d	0.095 a-c	0.093 bc	0.083 d	0.083 d	0.098 ab	0.088 cd	0.093 bc	0.098 ab	0.093 bc
	2. Years	0.080 c-e	0.095 a	0.075 e	0.085 b-d	0.080 c-e	0.085 b-d	0.080 c-e	0.078 de	0.080 c-e	0.090 ab	0.080 c-e	0.080 c-e	0.088 a-c	0.088 a-c
	Means	0.086 c-e	0.099 a	0.084 de	0.090 b-d	0.085 de	0.090 b-d	0.086 c-e	0.080 e	0.081 e	0.093 ab	0.084 de	0.086 c-e	0.084 a-c	0.090 b-d

CV (N) years x cultivars (P<0.05): 4.6, CV (P) years x cultivars (P<0.05): 5.6, CV (K) years x cultivars (P<0.05): 5.0, CV (Ca) years x cultivars (P<0.05): 13.16, CV (Mg) years x cultivars (P<0.05): 5.1, CV (Na) years x cultivars (P<0.05): 7.6

2015 Year: Toprakkale; 2016 Year: Alahanlı

Table 4- Micro Element Content of Peanut Varieties

Micro Elements	*Years*	*Varieties*													
		Polen	*Halisbey*	*NC-7*	*Sultan*	*Flower-22*	*Osmaniye-2005*	*Brantley*	*Wilson*	*Batem-5025*	*Arıoğlu-2003*	*Georgia Green*	*Florispan*	*NC V 11*	*Batem-Cihangir*
Fe (mg kg^{-1})	1. Years	17.09 g	24.99 a-d	22.29 e	23.85 c-e	19.55 f	23.88 b-e	24.29 b-e	19.84 f	18.06 fg	26.11 ab	25.97 a-c	22.92 de	24.21 b-e	26.76 a
	2. Years	16.96 f	24.74 b-d	21.44 e	26.89 ab	24.39 b-d	23.71 c-e	23.28 c-e	21.30 e	18.08 f	25.02 b-d	25.77 a-c	22.86 de	24.37 b-d	27.91 a
	Means	17.02 h	24.86 b-d	21.86 fg	25.37 b-d	21.97 fg	23.80 c-e	23.78 de	20.57 g	18.07 h	25.56 bc	25.87 ab	22.89 ef	24.29 b-e	27.34 a
Cu (mg kg^{-1})	1. Years	3.33 b	3.38 b	3.43 b	3.38 b	7.17 a	3.97 b	3.45 b	4.06 b	3.92 b	4.04 b	3.94 b	3.86 b	4.05 b	4.09 b
	2. Years	3.86 bc	2.78 c	2.64 c	3.52 bc	7.00 a	4.01 bc	3.27 bc	3.17 bc	3.30 bc	3.82 bc	3.96 bc	4.59 b	4.09 bc	4.16 bc
	Means	3.59 b-d	3.08 cd	3.04 d	3.45 b-d	7.08 a	3.99 b-d	3.36 b-d	3.61 b-d	3.61 b-d	3.93 b-d	3.95 b-d	4.23 b	4.07 b-d	4.13 bc
Zn (mg kg^{-1})	1. Years	22.55 gh	21.95 h	26.14 d-f	31.78 a	30.92 ab	29.76 a-c	28.41 cd	27.89 c-e	25.79 ef	26.29 d-f	29.39 bc	24.51 fg	25.92 ef	24.21 f-h
	2. Years	22.70 cd	22.21 c-e	17.59 hı	26.92 a	20.13 e-g	22.29 c-e	18.26 g-ı	18.78 g-ı	19.02 g-ı	24.33 bc	19.74 f-h	25.59 ab	17.27 ı	21.68 d-f
	Means	22.63 de	22.08 de	21.87 de	29.35 a	25.52 b	26.02 b	23.33 cd	23.33 cd	22.41 de	25.31 b	24.57 bc	25.05 b	21.60 e	22.94 de
Mn (mg kg^{-1})	1. Years	17.05 ef	18.97 a-e	20.66 a	17.10 e	19.76 a-c	17.77 c-e	17.58 de	17.86 c-e	18.37 b-e	14.98 f	19.29 a-d	18.91 a-e	19.39 a-d	19.97 ab
	2. Years	16.07 d-f	16.16 d-f	20.56 a	16.67 de	17.58 c-e	17.42 c-e	17.55 c-e	17.52 c-e	18.56 bc	14.53 f	15.84 ef	17.69 cd	18.87 a-c	19.99 ab
	Means	16.56 e	17.56 de	20.61 a	16.89 e	18.67 b-d	17.59 de	17.57 de	17.69 de	18.46 cd	14.76 f	17.57 de	18.30 cd	19.13 bc	19.98 ab
B (mg kg^{-1})	1. Years	18.22 e	24.83 bc	16.92 e	25.54 ab	18.01 e	22.17 d	17.02 e	17.82 e	17.10 e	16.84 e	22.36 d	26.65 a	23.34 cd	18.26 e
	2. Years	17.70 e	24.92 b	17.67 e	22.93 bc	24.72 b	21.00 d	17.21 e	16.83 e	15.85 e	23.93 b	21.35 cd	27.34 a	22.68 b-d	23.01 bc
	Means	17.96 g	24.87 b	17.29 gh	24.24 bc	21.36 ef	21.38 ef	17.12 gh	17.32 gh	16.48 h	20.38 f	21.86 de	26.99 a	23.01 cd	20.64 ef

CV (Fe) years x cultivars (P<0.05): 7.7, CV (Cu) years x cultivars (P<0.05): 27.8, CV (Zn) years x cultivars (P<0.05): 6.6, CV (Mn) years x cultivars (P<0.05): 7.8, CV (B) years x cultivars (P<0.05): 6.6

2015 Year: Toprakkale; 2016 Year: Alahanlı

Fe element, one of the micro elements, varied between the cultivars between 17.02 and 27.34 mg kg^{-1}. The highest average Fe content was 27.34 mg kg^{-1} from Batem-Cihangir variety, while the lowest average Fe content was obtained from Polen (17.02 mg kg^{-1}) (Table 4). In previous studies by researchers, it was determined that the Fe content of peanuts varied between 18 and 70 mg kg^{-1} (Hallock 1980; Cobb & Johnson 1973). In a study conducted by Gaines and Hammons in 1981, they found the rate of Fe element in peanut seed at the rate of 23-32 mg kg^{-1}. Shokunbi et al. (2012) found the content of Fe in the peanut seeds between 13.3-16.7 mg kg^{-1}. The Fe content that we found in our study is between 17.02-27.34 mg kg^{-1}, and it is consistent with the findings of the researchers.

The variety with the highest average Cu content among the varieties was Flower-22 (7.08 mg kg^{-1}). The variety with the lowest Cu content was NC-7 (3.04 mg kg^{-1}). It was determined that the Cu content of peanuts varied between 3 and 20 mg kg^{-1} by previous researchers (Hallock et al. 1971; Walker & Hymowitz 1972). James Yaw et al. (2008) were found the Cu content between 17 and 27 mg kg^{-1} with twenty peanut varieties in their study. The Zn content of 14 different peanut varieties grown under main crop conditions varied between 21.60-29.35 mg kg^{-1}. The highest Zn content was obtained from the Sultan variety with 29.35 mg kg^{-1} and the lowest Zn content was obtained from the NC V 11 variety with 21.60 mg kg^{-1} (Table 4). The Zn content was determined in previous studies to vary between 17 and 800 mg kg^{-1} (Hallock et al. 1971; Walker & Hymowitz 1972; Cobb & Johnson 1973). The Zn content was determined in previous studies to vary between 27 and 65 mg kg^{-1} (Jonnala et al. 2005; James Yaw et al. 2008; Shokunbi et al. 2012; Shibli et al. 2019). When the Mn content is evaluated; the highest Mn content is in the NC-7 (20.61 mg kg^{-1}) variety, the lowest Mn content is determined in the cultivar Arıoğlu-2003 (14.76 mg kg^{-1}) (Table 4). The Mn content was determined in previous studies to vary between 11.0 and 32.0 mg kg^{-1} (Hallock & Allison 1980; Hallock 1980). Boron (B) content of the varieties varied between 16.48-26.99 mg kg^{-1}. The highest B content was obtained from Florispan (26.99 mg kg^{-1}) and the lowest B content was obtained from Batem-5025 (16.48 mg kg^{-1}) (Table 4).

In our study, the peanut varieties were ranked as N>P>K>Mg>Ca>Na>Zn>Fe> B>Mn>Cu in terms of the amount of plant nutrients. Silva et al. (2016) reported macro and micro nutrient uptake in their study in descending order was: N > K > Ca > Mg > S > P and Fe > Zn > Mn > Cu > B, respectively. According to Malavolta et al. (1997) the majority of crops, in general, obey the N > K > Ca > Mg > P ≈ S order of macro- and Fe > Mn > Zn > Cu ≈ B order of micronutrients. However, in peanuts, an inversion of Mn with respect to Zn occurs.

There are several factors known to affect the plant nutrient composition of foods; genetic factors, climate, geography, geochemistry, agricultural practices such as fertilizer use, stage of maturity, and the growth period. There is a considerable variety of peanut varieties used for the study, and this variety provides the opportunity to select genotypes with desired characteristics for use in fertilizer programs. Further studies should be performed on these varieties to demonstrate the effects of agricultural operations on plant nutrient composition.

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Growth, Blood Parameters, Immune Response and Antioxidant Enzyme Activities in Rainbow Trout (*Oncorhynchus mykiss* Walbaum, 1792) Fed Diets Supplemented with Fumitory (*Fumaria officinalis*)

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ABSTRACT

In a feeding experiment for 75 days, the effects of fumitory (*Fumaria officinalis*) extract on growth, haematology, immune response and antioxidant enzyme activities in rainbow trout (*Oncorhynchus mykiss*) were evaluated. The aqueous methanolic extract of the plant was mixed with feeds at three different levels, 0.1% (FO1), 0.2% (FO2) and 0.3% (FO3), and feed with no plant extract donated as the control. All four groups (mean initial weight: 13.02 ± 0.02 g) were fed with the prepared diets twice daily *ad libitum* in a triplicate experiment. Blood and liver samples were taken from the fish on 15, 30, 45, 60 and 75 days. Also, overall growth parameters were determined based on body weight data recorded from all fish before and at the end of feeding trial. Results indicated that there were variations in nonspecific immune parameters (lysozyme, myeloperoxidase and oxidative radical production), but supplementation of the plant extract did not affect the immune response of the fish significantly. On the other hand, there was a general increase in the antioxidant enzyme activities (superoxide dismutase, catalase, glutathione peroxidase, and glucose-6-phosphate dehydrogenase) evaluated in this study. Furthermore, possibly the most remarkable finding of the study is that the growth performance in the FO3 group was roughly 30% higher than that of the control group. Considering all findings in the present study, we conclude that 0.3% supplementation of fumitory extract would be beneficial for rainbow trout farming with respect to elevated growth and antioxidant status.

Keywords: Growth promoter, Supplement, Organic, Medicinal plant, Feed additive

1. Introduction

Increasing world population brings along a huge demand of food worldwide (Salem et al. 2021). Undoubtedly, one of the most popular sources to meet up the food demand is seafood. In this context, the fact is that approximately 60% of animal protein requirement will be fulfilled by seafoods, especially after 2050s, which indicates the worth of aquatic food production systems. The limited or static production trend of fish products from capture fisheries stresses that production must be enhanced from aquaculture. In recent years, with the advancement in technology and application of technologies, there has been enhancement in seafood production through aquaculture (Bilen et al. 2015). However, in intensive aquaculture systems, there are emergence of several subsequent factors, such as impaired animal welfare and physiological functions, stress on species under culture as well as environmental degradation. Today, fish producers use vaccines, synthetic drugs and chemicals in order to prevent such losses and make production system efficient (Lalumera et al. 2004; Ji et al. 2007; Larragoite et al. 2016; Karga et al. 2020). However, there exist several adverse effects of these synthetic and chemical products on farmed animals (Serrano 2005; Cabello 2006; Defoirdt et al. 2011). In addition, similar negative impacts have been indicated on aquatic organisms (Defoirdt et al. 2007; Baquero et al. 2008). To address these issues, in recent years, a great number of studies have intensively focused on application of organic-based feed additives that have stress relieving, anti-inflammatory, immunostimulant, antifungal, and antimicrobial properties (Asadi et al. 2012; Balamurugan et al. 2016; Hernandez et al. 2016; Kirubakaran et al. 2016; Mohamed et al. 2018; Taştan & Salem 2021; Sönmez et al. 2022). Among various organic-origin products, microorganisms, plants, plant extracts and essential oils have been tested in several recent studies as alternatives to drugs, chemicals and antibiotics (Bilen et al. 2019, 2020; Arslan et al. 2018; Sönmez et al. 2015a, 2021; Elbesthi et al. 2020; Yilmaz et al. 2019, 2020; Amoush et al. 2021; Lakwani et al. 2021).

Fumitory (*Fumaria officinalis*) is one of the 55 plant species in Fuminaria family (Mitich, 1997). These plants are distributed in West and Middle Europe with 17 species found in Turkey (Rehman et al. 2013). Fumitory, known as a medicinal plant, is used as a digestive stimulant and diuretic (Sajjad et al. 2015), with antimicrobial properties (Bisset & Wichtl 2001). Fumitory contains isoquinolin as the dominant alkaloid (Sajjad et al. 2015), covering a total phenolic content of 10.5 mgGAE g^{-1} (dry weight) with 78.9% antioxidant activity (Şengül et al. 2009). It is speculated that with all these properties, this medicinal plant may have immune stimulating and antioxidant effects.

In this context, effects of aqueous methanolic extract of fumitory (*F. officinalis*) on growth performance, haematology, immune responses and antioxidant activities on rainbow trout (*Oncorhynchus mykiss*) have been evaluated in this study. The main purpose of this study therefore was to assess a novel immune stimulating and antioxidant product that is easy to procure and prepare, and has potential application in aquaculture industry.

2. Material and Methods

2.1. Experimental design

The study was conducted at Kastamonu University Inland and Marine Fish Production, Application and Research Centre using 12 net cages (1.5×1.5×1.5 m) designated as 4 treatments with 3 replicates. A total of 480 rainbow trout juveniles (mean initial weight: 13.02±0.02 g) were stocked into cages as 40 fish per cage. Aqueous methanolic extract of *Fumaria officinalis* was added to feeds by spraying at three levels, 0.1% (FO1), 0.2% (FO2) and 0.3% (FO3). The fish were fed *ad libitum* twice a day for 75 days. Liver tissue and blood (using heparinized syringes) were sampled from 3 fish per cage every 15 days. Immunological and haematological changes were evaluated in blood samples, whereas antioxidant activities were determined using liver samples.

2.2. Extraction method

Leaves of the plant were powdered subsequent to drying under shade. Powdered leaves (100 g) were mixed with 1 L of 40% methanol. Then the mixture was stored in a dark place for 72 days and mixed by inverting twice a day during this period. The mixture was filtered to remove particulates and the solvent was evaporated completely using an evaporator at 75 °C. To determine the exact amount of extract, 50 mL of distilled water was heated at 50 °C, added to the extract present in the evaporator flask, and the total amount of extract was determined by weighing the sample. Finally, extract was prepared in phosphate buffered saline (PBS) as per the dose quantity for this study, prepared solutions were kept at -20 °C until use and at +4 °C during the study period (Bilen et al. 2016). Analysis of composition of *F. officinalis* extract was carried out according to Özkan et al. (2017) using GC-MS QP2010 Ultra (Shimadzu) and results are presented in Table 1.

2.3. Calculation of growth parameters

The fish were weighed prior to the feeding trial and at the end of the study. Growth performances of the fish were computed as per the formulas given below:

$$Weight\ gain\ (WG) = 100\ x\ \frac{Wf-Wi}{W_i} \quad (1)$$

$$Specific\ growth\ rate\ (SGR) = 100\ \times\ \frac{\ln Wf - \ln Wi}{days} \quad (2)$$

$$Food\ conversion\ rate\ (FCR) = \frac{Feed\ intake}{Weight\ gain} \quad (3)$$

Where; Wf: Final weight, Wi: Initial weight.

2.4. Determination of haematological indices

Total erythrocyte count, haematocrit levels and haemoglobin amount were determined according to Blaxhall & Daisley (1973). Mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration values were computed by the formulas described by Lewis et al. (2006).

Table 1- Composition of the plant (*F. officinalis*) extract used in this study

Peak#	*Area%*	*Name*
1	1.15	Imidazole, 2-amino-5-[(2-carboxy)vinyl]-
2	0.92	Ethyl n-propyl ketone
3	4.18	Hydroperoxide, 1-ethylbutyl (CAS)
4	4.74	Ethanol, 2-(hexyloxy)-
5	0.54	3-HEXEN-2-ONE
6	0.18	Hexanoic acid, 2-ethyl- (CAS)
7	0.76	2,4-Decadienal, (E,Z)- (CAS)
8	1.22	Deca-2(E),4(E)-dienal
9	1.94	Tetradecane
10	0.20	Heptadecane, 2,6,10,14-tetramethyl-
11	0.29	Cyclohexanone, 2-(hydroxymethyl)-
12	0.55	Dodecanoic acid (CAS)
13	0.60	1-Tetradecanol
14	1.08	Hexadecane
15	1.22	Dodecanoic acid, 1-methylethyl ester
16	0.25	E-14-Hexadecenal
17	0.33	Heptadecane
18	2.16	Tetradecanoic acid
19	0.34	Octadecane (CAS)
20	0.99	Pentadecanoic acid
21	0.83	8-(2-Acetyloxiran-2-yl)-6,6-dimethylocta-3,4-dien-2-one
22	35.99	Oleic Acid
23	15.21	Palmitic acid
24	9.09	Octadecanoic acid (CAS)
25	0.50	11,14-Eicosadienoic acid, methyl ester
26	0.34	10-Octadecenoic acid, methyl ester
27	8.60	9,12-Octadecadienoic acid (Z,Z)-
28	4.50	Propiolic acid
29	0.71	Hexadecanoic acid, butyl ester
30	0.59	Bicyclo[4.3.0]nonan-1-ol, 7,9-bis(methylene)-2,2,6-trimethyl-
TOTAL	100	

2.5. Determination of non-specific immune parameters

Oxidative radical production was analyzed as described by Siwicki & Anderson (1993). Briefly, 0.1 mL of blood was mixed with 0.1 mL solution containing 0.2% nitroblue tetrazolium (NBT) and incubated for 30 min at 25 °C. Fifty microliter suspension was taken from the mixture and 1 mL of N, N-dimethyl formamide was added on top of it. The mixture was centrifuged at 3000 g for 5 min. Finally, the mixture was transferred to a clean tube and absorbance was recorded against N, N-dimethyl formamide blank at 540 nm. Results were calculated by multiplying with 4.

Lysozyme activity was determined by slightly modifying the method of Ellis (1990). Accordingly, 100 µl *Micrococcus lysodeikticus* suspension (prepared by dissolving 0.02 g *M. lysodeikticus* bacterial cells, Sigma-Aldrich in 100 mL phosphate buffered saline solution) was mixed with 10 µl of fish plasma in 2 replicates. Changes in absorbance values at 530 nm were recorded at 0 and 4 min.

The method described by Sahoo et al. (2005) was modified in order to determine myeloperoxidase level. Briefly, 10 µl of serum was taken from the samples and transferred to the wells with 2 replicates. Ca^{+2} and Mg^{+2} free Hank's Balanced Salt Solution (HBSS) at 125 µl was added to wells. Then 35 µl of TMB substrate solution (Sigma-Aldrich) and 35 µl of 5 mM fresh hydrogen peroxide (Sigma-Aldrich) were added. Finally, changes in absorbance values were recorded at 450 nm at 0 and 4.5 min.

2.6. Analysis of antioxidant enzyme activity

Antioxidant enzyme activities of fish were determined in liver tissues on 15, 30, 45, 60 and 75th day using commercial kits according to instructions of the manufacturers: Superoxide dismutase (SIGMA SOD Assay Kit, Item no: 19160-1KT-F); Catalase (CAYMAN Catalase Assay Kit, Item no: 707002); Glutathione peroxidase (CAYMAN Glutathione Peroxidase Assay Kit, Item no: 703102); Glucose-6-phosphate dehydrogenase (SPI-BIO G6PDH Assay Kit, Item no:0112); Lipid peroxidation (CAYMAN TBARS Assay Kit, Item no: 10009055).

2.7. Statistical analysis

Variances in obtained data for different parameters were determined using one-way ANOVA. In order to find out any difference present between groups, Duncan's multiple range test was employed. The level of significance was set at 95%. All analyses were conducted using SPSS for Windows (Version 22, IBM).

3. Results

The changes in growth performance of fish are summarized in Table 2. From the growth performance data obtained by the end of the feeding trial (day-75), the highest weight increase was observed in rainbow trout fed with 0.3% *F. officinalis* extract.

Table 2- Growth performances of rainbow trout (*Oncorhynchus mykiss*) fed with *Fumaria officinalis* supplemented feed in different doses

Groups	*Initial Weight (g)*	*Final Weight (g)*	*Weight Gain (%)*	*SGR*	*FCR*
Control	12.45±0.2	69.14±0.3^{b}	454.94±7.86^{b}	2.28±0.01^{b}	1.4±0.007
FO1	12.34±0.16	65.10±0.5^{b}	427.99±8.96^{b}	2.22±0.02^{b}	1.4±0.01
FO2	12.10±0.04	68.78±0.32^{b}	468.89±38.42^{b}	2.31±0.09^{b}	1.43±0.03
FO3	12.23±0.07	87.88±0.2^{a}	618.18±44.62^{a}	2.62±0.08^{a}	1.35±0.3

SGR: Specific growth rate; FCR: Food conversion ratio. Different superscript letters in the same column show significant differences between groups (P<0.05). Data are expressed as mean±SE.

As evident from Table 2, the highest final weight was determined in the FO3, followed by FO2, control and FO1 groups, respectively (P<0.05). Although, there was no significant difference between control, FO1 and FO2 groups, the final weight of fish in the FO3 group was significantly higher than other groups (P<0.05). Similarly, weight gain (%) and SGR of the FO3 group were significantly higher compared to other groups (P<0.05). Regarding FCR, it was observed that although the FO3 group had lower FCR in comparison with control and other treatment groups, this difference was statistically insignificant (P>0.05).

Changes in the oxidative radical production (ORP) were determined from blood samples collected on 15, 30, 45, 60 and 75th days. Results obtained are provided in Figure 1. On the 15, 30 and 45th day of the study, although there were variations in the ORP of rainbow trout fed with FO extract, these differences were not statistically significant (P>0.05). Results of day 60 revealed that control group had the highest ORP level (1.03±0.22 mg/mL), whereas, ORP levels in treatment groups did not differ. At the last sampling day of the study, the highest value was recorded in the control group (4.63±0.23 mg/mL), followed by the FO1 (4.31±0.29 mg/mL), FO2 (3.7±0.3 mg/mL) and FO3 (3.63±0.24 mg/mL) groups, respectively. In regards to the statistical analyses, FO1 was found to be similar to the control group (P>0.05), while FO2 and FO3 treatment groups exhibited lower ORP (P>0.05).

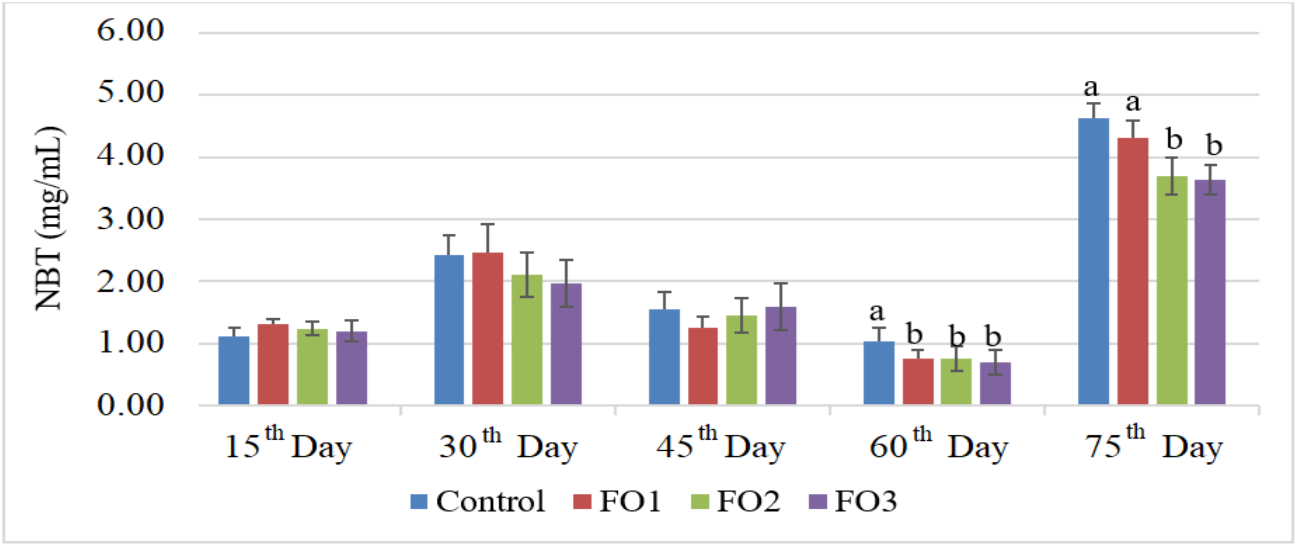

Figure 1 - Changes in oxidative radical production of rainbow trout (*Oncorhynchus mykiss*) fed with *Fumaria officinalis* supplemented feed in different doses. Different lowercase letters on the bars show significant differences between groups at a particular sampling time (n=3).

Changes in the lysozyme activity of rainbow trout are presented in Figure 2. It was observed that although lysozyme activity varied between groups, these differences were not significant (P>0.05) except for the FO1 group. On the 60th day, lysozyme activity in FO1 group increased significantly (P<0.05) compared to the control and other experimental groups.

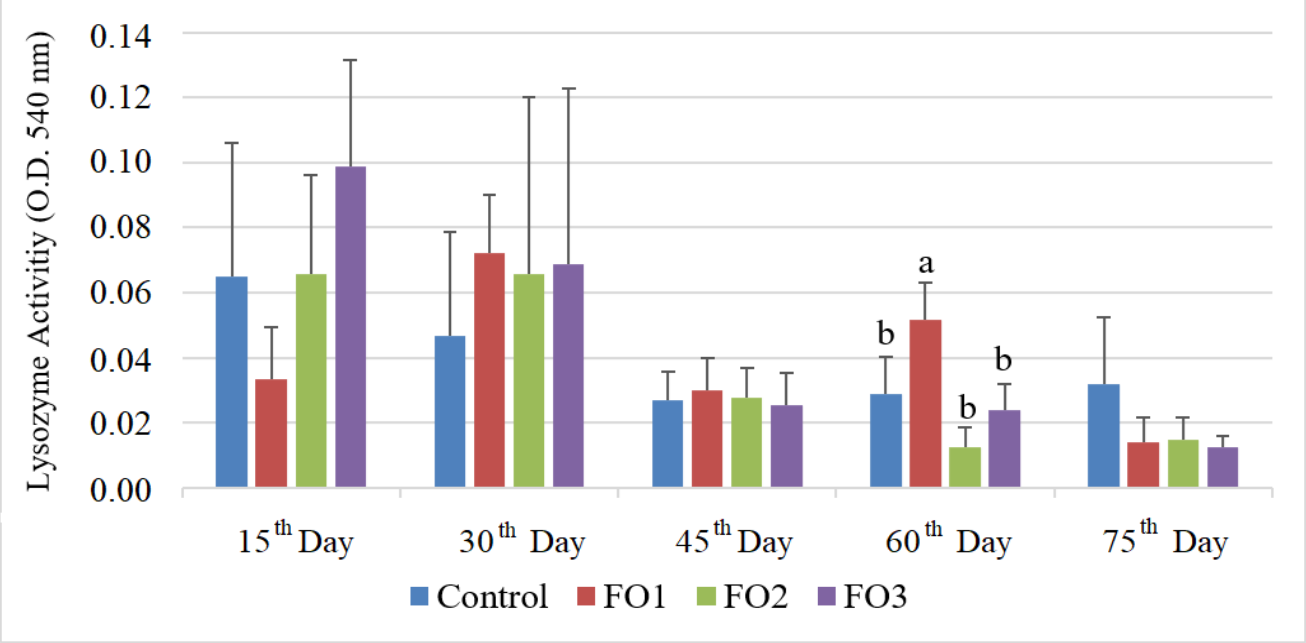

Figure 2- Changes in lysozyme activity of rainbow trout (*Oncorhynchus mykiss*) fed with *Fumaria officinalis* supplemented feed in different doses. Different lowercase letters on the bars show significant differences between groups at a particular sampling time (n=3)

Myeloperoxidase (MPO) activity was determined from the serum of fish every 15 days throughout the study and the results are presented in Figure 3. On the 15th day of the study, MPO was found as 22.53±6.77 in FO3, 18.09±10.71 in FO1, 10.63±4.06 in FO2 and 10.56±1.83 in the control group. The highest MPO activity was recorded in FO3 group followed by the FO1 group (P<0.05). In addition, although the lowest MPO activity was detected in the control group, no difference was found between control and FO2 groups (P>0.05). On the 30th day, MPO activities of the experimental groups were 50.47±23.01, 45.04±13.66, 36.80±12.27 and 19.45±13 in the FO1, control, FO2 and FO3 groups, respectively. Although, the highest myeloperoxidase value was recorded in FO1 group, it was not significantly different from that of the control group (P>0.05). Moreover, MPO values did not differ among the experimental groups on 45, 60 and 75th days of the study.

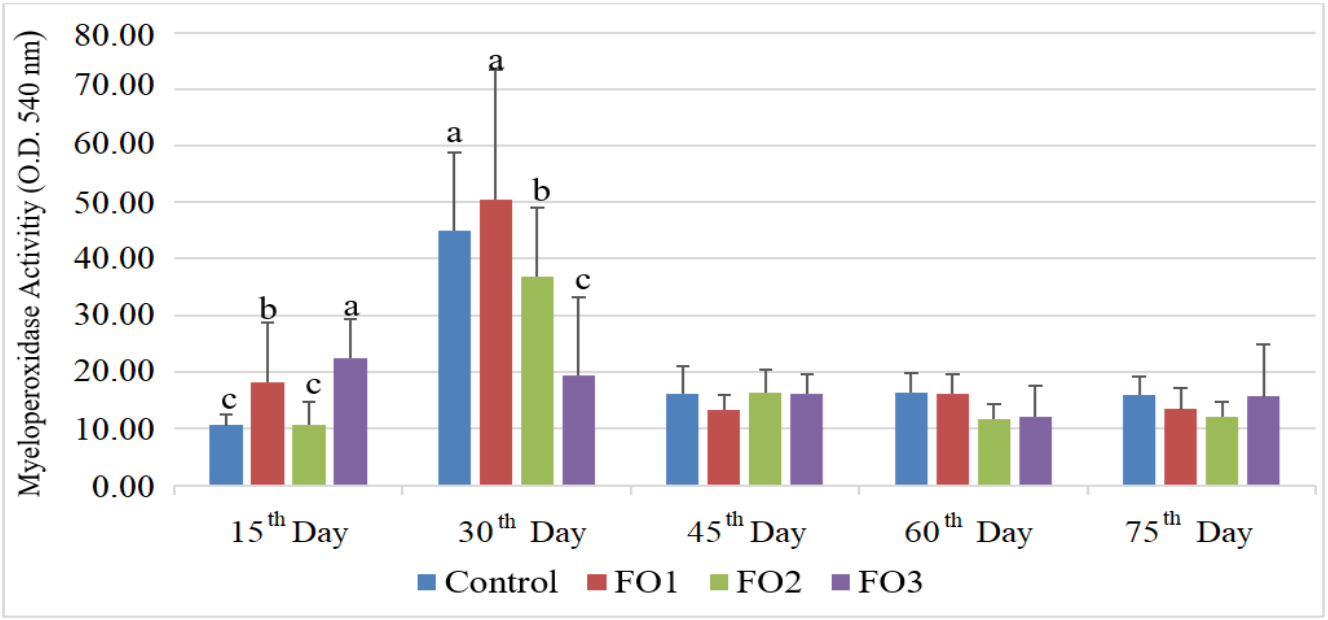

Figure 3- Changes in myeloperoxidase activity of rainbow trout (*Oncorhynchus mykiss*) fed with *Fumaria officinalis* supplemented feed in different doses. Different lowercase letters on the bars show significant differences between groups at a particular sampling time (n=3)

During the study, changes in haematological parameters were also determined from blood samples collected from fish at 15-day intervals (Table 3). Mean corpuscular haemoglobin concentration (MCHC) was the only parameter that did not differ between groups at any sampling time. On 15th day of the study, red blood cell (RBC) value of FO1 was similar to control ($P>0.05$), whereas, FO2 and FO3 groups presented decreased RBC counts ($P<0.05$). While, no change was observed in the haemoglobin (HGB) content between groups, haematocrit (HCT) levels of FO2 and FO3 groups decreased. None of the other parameters were affected on day 15.

Table 3- Blood parameters of rainbow trout (*Oncorhynchus mykiss*) fed with *Fumaria officinalis* supplemented feed in different doses

Day	*Groups*	*RBC*	*HGB*	*HCT*	*MCV*	*MCH*	*MCHC*
15	**Control**	1.19±0.03^{a}	7.63±0.45	26±0.81^{a}	217.82±3.29	67.93±1.50	67.93±0.36
	FO1	1.23±0.03^{a}	7.93±0.29	26.12±0.46^{a}	215.32±2.05	68.48±1.74	68.48±0.90
	FO2	1.01±0.04^{b}	7.70±0.37	22.50±0.83^{b}	222.26±1.72	73.01±1.28	73.01±0.37
	FO3	0.94±0.03^{b}	7.20±0.28	21.22±0.84^{b}	226.49±1.86	79.17±1.39	79.17±0.56
30	**Control**	0.37±0.04^{b}	7.27±0.02^{a}	16.75±3.08^{b}	229.22±1.92^{b}	230.19±1.03^{a}	31.88±0.00
	FO1	1.03±0.04^{a}	7.92±0.22^{a}	23.12±0.86^{a}	241.97±0.74^{a}	77.43±1.34^{b}	34.34±0.48
	FO2	0.96±0.04^{a}	5.67±0.36^{b}	20.47±1.23^{a}	248.95±4.36^{a}	62.26±0.00^{b}	29.46±0.00
	FO3	1.04±0.04^{a}	7.72±0.37^{a}	23.52±0.81^{a}	245.38±1.57^{a}	73.92±1.73^{b}	32.78±0.83
45	**Control**	1.23±0.03^{a}	8.10±0.45	28.15±0.81^{a}	229.22±1.92^{a}	65.87±2.82^{c}	28.71±1.06
	FO1	1.01±0.03^{b}	7.52±0.23	24.44±0.81^{b}	241.97±0.74^{b}	74.72±1.14^{b}	30.88±0.46
	FO2	0.96±0.04^{b}	7.58±0.47	25.20±1.63^{b}	248.95±4.36^{b}	74.65±2.31^{b}	29.56±1.01
	FO3	0.88±0.04^{c}	7.53±0.14	22.47±0.59^{b}	245.38±1.57^{b}	86.45±4.40^{a}	32.64±0.94
60	**Control**	1.11±0.08^{b}	8.10±0.15^{a}	26.1±1.81^{a}	225.19±6.28^{b}	53.15±15.70^{b}	31.57±1.98
	FO1	0.90±0.02^{b}	6.36±0.36^{b}	22.1±1.81^{b}	225.97±7.52^{b}	67.09±4.01^{a}	29.12±1.06
	FO2	0.96±0.06^{b}	5.27±0.64^{c}	23.13±1.57^{b}	240.06±2.06^{a}	63.75±1.15^{a}	26.54±0.27
	FO3	1.28±0.03^{a}	7.80±0.35^{a}	29±1.12^{a}	226.09±4.28^{b}	60.81±1.76^{a}	26.87±0.38
75	**Control**	1.45±0.10	8.95±0.15^{a}	34.83±2.06^{a}	240.88±2.60	62.69±3.49	25.98±1.18
	FO1	1.31±0.04	7.66±0.23^{b}	28.97±2.07^{b}	237.71±3.37	58.76±1.26	24.73±0.49
	FO2	1.28±0.06	6.85±0.20^{b}	30.57±1.34^{b}	238.83±1.71	54.02±2.39	22.58±0.85
	FO3	1.24±0.05	6.82±0.11^{b}	29.28±1.20^{b}	235.68±1.18	55.33±2.05	23.47±0.84

RBC: Red blood cell, HGB: Haemoglobin, HCT: Haematocrit, MCV: Mean corpuscular volume, MCH: Mean corpuscular haemoglobin, MCHC: Mean corpuscular haemoglobin concentration. Different superscript letters in the same column show significant differences between groups at a particular sampling time. Data are expressed as mean±SE.

On day 30, RBC count in all treatment groups was higher than the control ($P<0.05$). HGB value of FO2 was lower than that of control, while HGB values of FO1 and FO3 groups were not different. In contrast, all treatment groups exhibited an increase in HCT compared to the control group. Similarly, MCV values of fish in all treatment groups were higher than that of the control. In addition, all experimental groups presented decreased MCH values.

RBC, HCT and MCV values of all the treated groups were lower than that of control on 45th day of the study ($P<0.05$). HGB values did not differ among groups ($P>0.05$). In contrast, all treated groups exhibited increased MCH values.

When the haematological results of the 60th day were examined, it was noticed that FO3 group had the highest RBC count ($P<0.05$), while other treatment groups had similar counts compared to the control ($P>0.05$). HGB contents of FO1 and FO2 groups were lower than that of the control, whereas HGB of FO3 group was not different. Similarly, FO1 and FO2 groups presented decreased HCT values. In case of MCV, an increase was found only in the FO2 group, compared to the control. MCH values of all treatment groups were higher than that of the control group ($P<0.05$).

On the 75th day, the highest HGB value was recorded in the control group ($P<0.05$). While similar results were observed for HCT levels, no differences were found in other parameters examined in the study ($P>0.05$).

Changes in superoxide dismutase (SOD) activity determined at the end of the study are presented in Figure 4. It was observed that SOD activity did not differ among the groups at any sampling time ($P>0.05$).

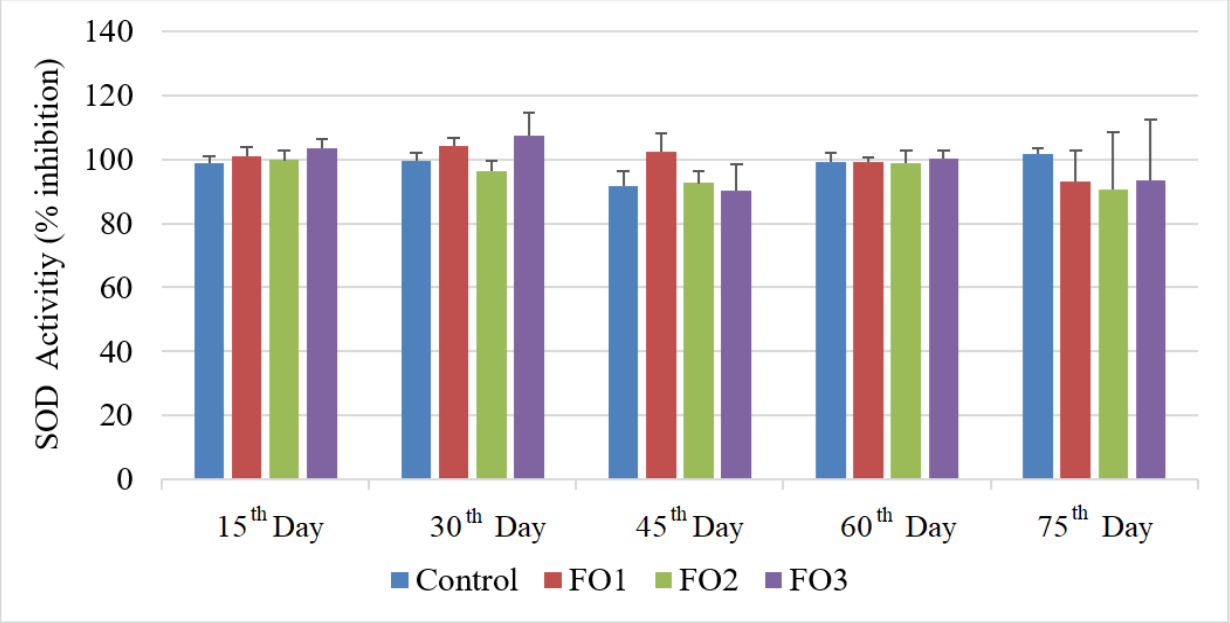

Figure 4- Changes in liver superoxide dismutase activity of rainbow trout (*Oncorhynchus mykiss*) fed with *Fumaria officinalis* supplemented feed in different doses (n=3)

Results of catalase (CAT) activity are presented in Figure 5. On 15th day of the study, no difference in CAT was observed among treated and control groups (P>0.05). On 30th day, the highest CAT value was observed in FO2 group (P<0.05). CAT activity of FO3 group did not differ in comparison to control, and the lowest CAT activity was found in the FO1 group. Results of day 45 exhibited that all treated groups had lower CAT activity than that of the control. On the 60th day, highest CAT activity was observed in FO2 group, followed by FO1, control and FO3 groups, respectively. Finally, at the last sampling time, all treated groups had decreased CAT activities in comparison to control group.

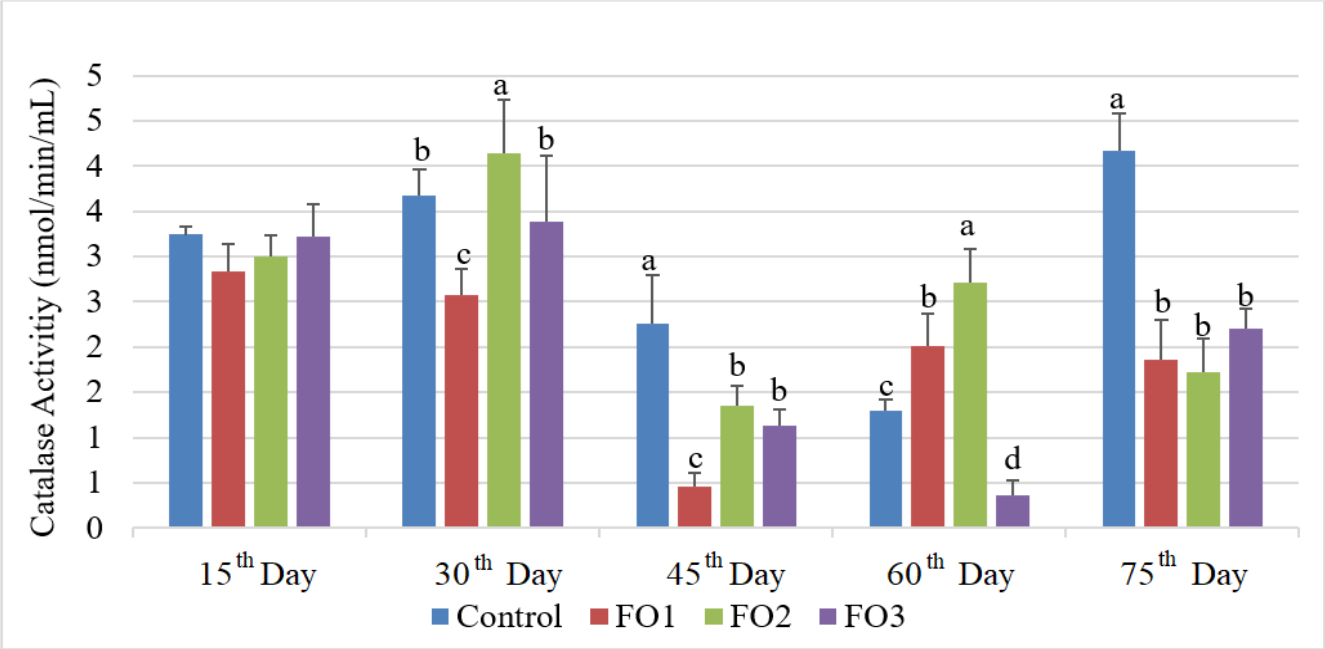

Figure 5- Changes in liver catalase activity of rainbow trout (*Oncorhynchus mykiss*) fed with *Fumaria officinalis* supplemented feed in different doses. Different lowercase letters on the bars show significant differences between groups at a particular sampling time (n=3)

Results of glutathione peroxidase (GPx) activity obtained from liver samples from fish on day 15, 30, 45, 60 and 75 are provided in Figure 6. There were no differences in GPx activities among groups on the 15, 30 and 45th days of the study (P> 0.05). On day 60, the highest GPx activity was observed in the control group followed by FO1, FO2 and FO3 groups, respectively (P<0.05). Furthermore, at the last sampling time, all treated groups had increased GPx activities compared to the control, where the highest value was in FO3 group.

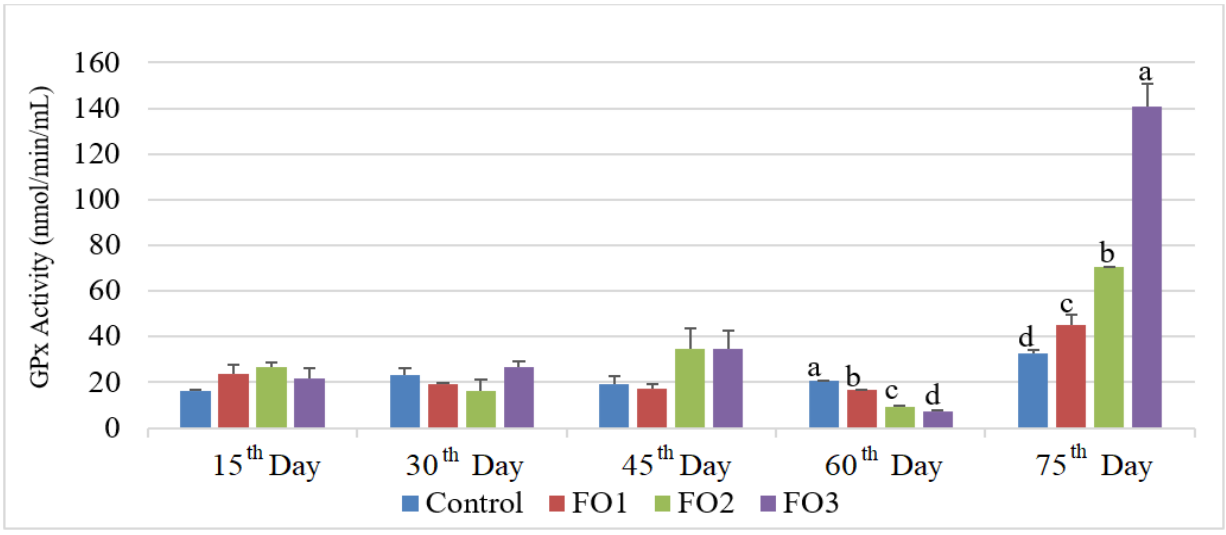

Figure 6- Changes in liver glutathione peroxidase activity of rainbow trout (*Oncorhynchus mykiss*) fed with *Fumaria officinalis* supplemented feed in different doses. Different lowercase letters on the bars show significant differences between groups at a particular sampling time (n=3)

Results of glucose-6-phosphate dehydrogenase (G6PDH) activities determined from liver samples collected from fish on day 15, 30, 45, 60 and 75 are presented in Figure 7. Regarding G6PDH activity, there was no difference observed between groups on 15, 30 and 60th days (P>0.05). On the contrary, control group showed the highest G6PDH activity on day 45 in comparison to all treated groups (P<0.05). In contrast, all the treated groups had increased G6PDH activities on the 75th day with significantly higher levels found in FO2 and FO3 groups compared to the control.

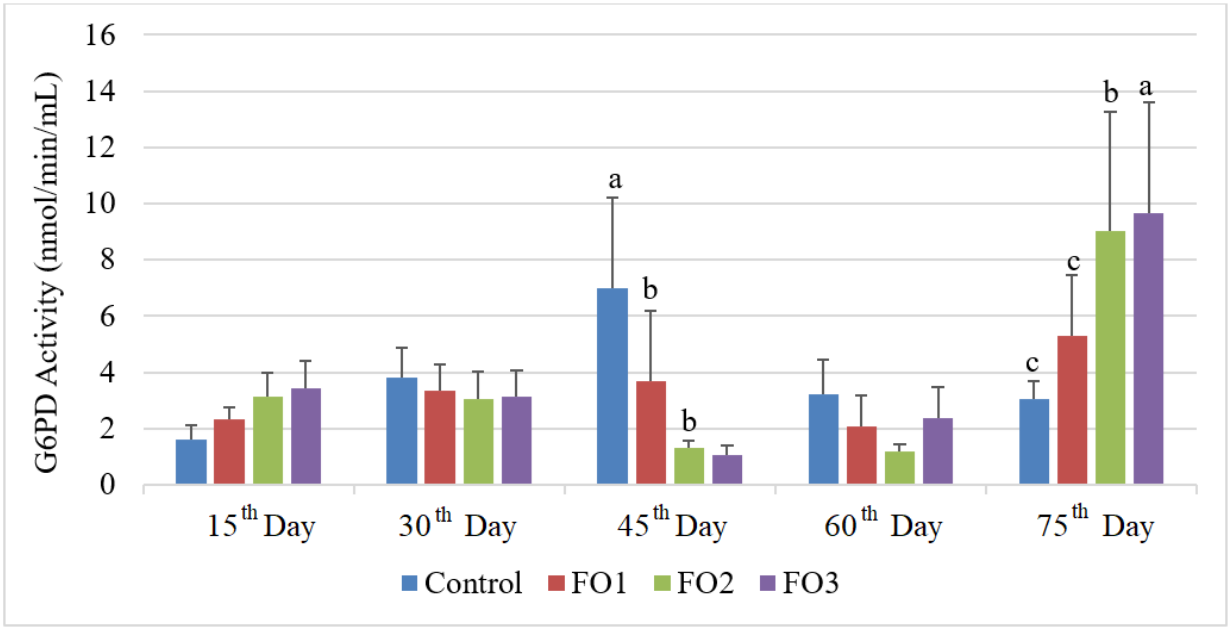

Figure 7- Changes in liver glucose-6-phosphate dehydrogenase activity of rainbow trout (*Oncorhynchus mykiss*) fed with *Fumaria officinalis* supplemented feed in different doses. Different lowercase letters on the bars show significant differences between groups at a particular sampling time (n=3)

Changes in lipid peroxidation (LPO) determined using liver samples collected from fish at 5 sampling times are presented in Figure 8. Although, there were slight variations in LPO values among groups, no difference was observed at any sampling time (P>0.05).

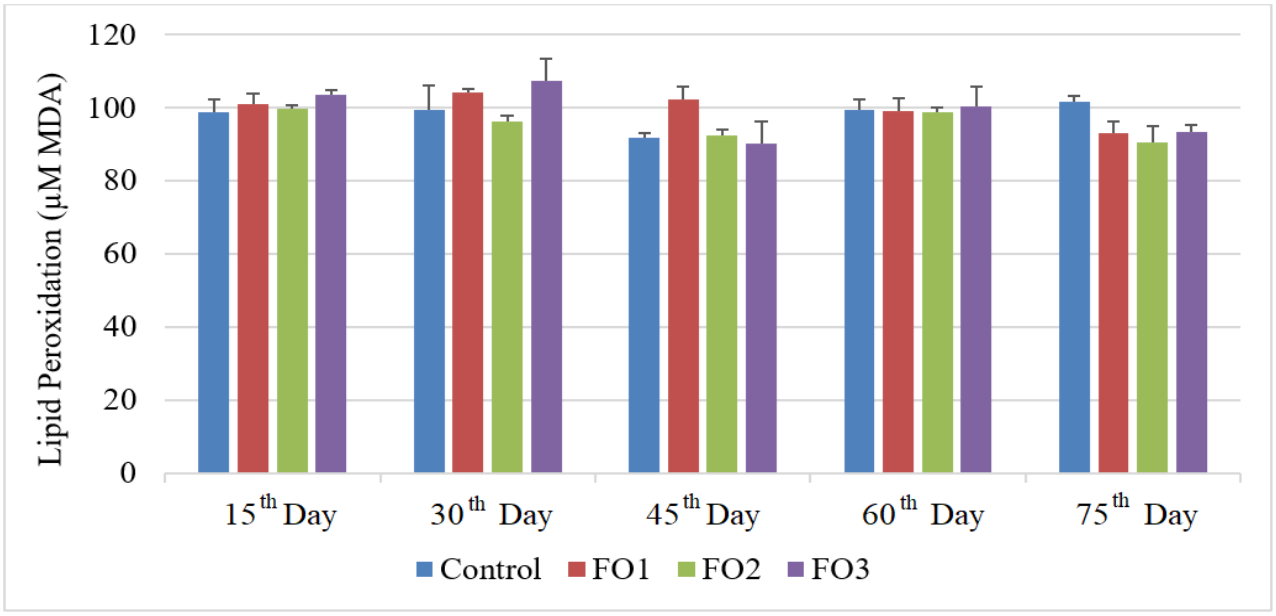

Figure 8- Changes in liver lipid peroxidation of rainbow trout (*Oncorhynchus mykiss*) fed with *Fumaria officinalis* supplemented feed in different doses (n=3)

4. Discussion

In the present work, it was observed that humoral immune responses did not change at early stages but decreased in long term, whereas antioxidant parameters were elevated at the later part of the experiment. It was noticed that control group exhibited more effective results on blood parameters. Furthermore, a significantly increased growth performance was recorded in the FO3 group. Therefore, it was inferred that the use of *F. officinalis* at 0.3% could substantially increase growth of rainbow trout.

Growth performance results of the study indicated that the best performance was obtained by the FO3 group, presenting a mean body weight of 87.88±0.2 g with an elevated growth performance around 30%. Another important point here is that the FCR decreased but not significantly ($P>0.05$), with the increase of fish growth. Several studies reported that the use of plant extracts affects growth of fish (Awad & Awad 2017). Moreover, this effect may depend on dose, feeding duration, species and physiological conditions (Harikrishnan et al. 2011, Gannam & Schrock, 1999). Elevated digestive enzyme activities can also significantly enhance the growth performance of fish (Awad et al. 2012). In this context, it can be deciphered that the phenolic compounds or other functional ingredients contained in the aqueous methanolic extract of *F. officinalis* are effective on growth hormones that promotes a faster growth in fish. It can also be stated that components of the plant might have influenced utilization of feed by activating digestive enzymes. Similar to these results, Mahdavi et al. (2013) found that *Aloe vera* extract increased growth, weight gain and SGR of carp when administered at 0.1, 0.5 and 2.5% rates. In another study, Awad et al. (2012) reported that lupine, mango and nettle supplemented into feed increased weight gain, SGR and digestive enzyme activities in rainbow trout fed for two months. In contrast, kefir administration had no effects on growth performance of Çoruh trout, *Salmo coruhensis* (Can et al. 2012).

Reactive oxygen species (ROS) such as superoxide radicals emerge as a result of immune response and they play a vital role in elimination of pathogens. Removal of ROS is extremely important for the continuation of crucial physiological and metabolic activities. SOD enzyme performs the task of removing these anions and plays a significant role in the destruction of superoxide free radicals. Increase in SOD activity in the present study may indicate elevated superoxide radical production within the cells. In this mechanism, ORP activity gets involved. Moreover, increase in ORP indicates enhanced superoxide radical production by the cells, whereas, increase in SOD indicates enhanced catabolism of superoxide radicals and the cell is induced accordingly for this. Therefore, SOD and ORP should not be evaluated independently. Theoretically, when ORP increases, an increase in SOD activity is also expected.

ORP is one of the most important cellular activities that prevent growth and proliferation of pathogens in the body (Divyagnaneswari et al. 2007). In our study, ORP activity did not change or decrease at the end of the study. This could be attributed to the increased antioxidant responses. Previously, tetra, oyster mushroom and common nettle supplementation increased ORP in rainbow trout (Bilen et al. 2011; Bilen et al. 2016). Likewise, Nya & Austin (2009) demonstrated that ginger administration increased ORP in rainbow trout. Contrary to these results, Bilen & Bulut (2010) reported that rainbow trout fed with laurel leaf extract did not exhibit any change in intracellular ORP.

The innate immune system contains antiprotease, protease, lysozyme, antibodies, complement and lytic factors that are present in the serum to prevent growth of microorganisms and prohibit their adhesion to tissues (Alexander & Ingram 1992). From our lysozyme activity results, an increase was observed initially, but it decreased at the end of the study. In this context, it can be opined that *F. officinalis* exhibited lytic feature. It is to mention that lysozyme activity in generally enhanced in fish fed with medicinal plants (Abarike et al. 2019, Almabrok et al. 2018). This fact was demonstrated earlier in different fish species using various plants, such as *Astragalus membranaceus, Angelica sinensis* and *Crataegus hupehensis* in Nile tilapia (Abarike et al. 2019); *Achyranthes aspera* in rohu (Rao et al. 2006); *Solanum trilobatum* in Mozambique tilapia (Divyagnaneswari et al. 2007); *Astragalus radix* & *Ganoderma lucidum* in carp (Yin et al. 2009).

Myeleperoxidase (MPO) activity increased on day 15 and varied thereafter. This situation may be attributed to the antioxidant property of the *F. officinalis*. MPO has a significant role in killing microorganisms. Thus, increase in MPO indicates that the immune response is mounted. Similar to this study, Christybapita et al. (2007) did not obtain any change in myeloperoxidase activity in the Mozambique tilapia fed with *Eclipta alba* extract.

SOD activity did not vary during sampling times of the study. This might have caused due to ORP effects. In a previous study, carbamazepine administration significantly reduced SOD activity in rainbow trout (Li et al. 2010). It has been demonstrated that treatment with extract obtained from *Suaeda maritime* caused decreased SOD activity (Thirunavukkarasu et al. 2010). Moreover, Sönmez et al. (2015b) observed a reduction in SOD activity in rainbow trout fed with peppermint oil. In contrast, some studies described an increased SOD activity in fish fed with different plant extract supplementations (Thirunavukkarasu et al. 2010; Keleştemur & Özdemir 2013; Sönmez et al. 2015b).

CAT is an antioxidant enzyme that performs the task of catalysing the dismutation of superoxide radicals eliminated by SOD to H_2O_2 and it is found in intra-cellular peroxisomes. CAT activity is expected to elevate when SOD activity increases. In our study, CAT activity varied independently to SOD activity and increased in treated groups at different sampling times. Gülçin et al. (2009) observed an elevation in CAT activity of rainbow trout fed with melatonin. Similarly, kefir supplementation caused an increase in CAT activity of Çoruh trout (Can et al. 2012). Contrarily, Sönmez et al. (2015b) described that mint administration did not affect CAT activity, or it decreased depending on the time.

GPx catalyses the formation of NADPH and GSSG, which are essential for glutathione reductase enzyme to function. An increase in GPx activity of rainbow trout was observed at the end of the study. Based on this, it is inferred that GPx activity is positively influenced by the long-term use of *F. officinalis* extract. Similar to our results, Zhang et al. (2015) also demonstrated an increased GPx activity of Japanese seabass (*Lateolabrax japonicus*) fed with magnesium and vitamin E supplemented feeds. Moreover, thyme and sage supplementation caused an elevation in GPx activity in rainbow trout (Sönmez et al. 2015b). Contrary to these results, Li et al. (2010) reported a decreased GPX activity in rainbow trout due to carbamazepine administration.

G6PDH catalyses the pentose phosphate pathway by producing NADPH. Produced NADPH is essential for the glutathione reductase and CAT enzymes. Therefore, G6PDH enzyme is important for decomposition of H_2O_2. G6PDH activity generally increased only at the end of the study in all treated groups. These data support the elevation in GPx activity at the end of the study and agree with the work conducted by Sönmez et al. (2015b).

LPO is determined by estimating the amount of malondialdehyde (MDA) formed by the release of free oxygen radicals and is a highly important factor in determining oxidative stress. Basically, an increase in MDA level indicates elevated oxidative stress and LPO (Yagi 1984). In this study, *F. officinalis* administration did not affect MDA level in rainbow trout. Contrary to our results, MDA levels were found to be elevated in tilapia fed with selenium and alpha-tocopherol (Keleştemur & Özdemir (2013). Şahan et al. (2017) demonstrated a decrease in MDA level in rainbow trout fed with rosehip. Similarly, significantly decreased levels of MDA were detected in Mozambique tilapia (Amer 2016) and rainbow trout (Gülçin et al. 2009) fed with spirulina and melatonin supplemented diets, respectively.

5. Conclusions

Based on the findings of the present study, it is suggested that it is pertinent to feed rainbow trout with *Fumaria officinalis* in order to strengthen its antioxidant system. Dietary supplementation of *F. officinalis* at 0.3% rate for 90 days elevated growth performance by around 30%, which is the most notable outcome obtained in this study. In addition, use of this plant extract did not have any negative effect on fish survival rate or other immune and antioxidant responses. However, this work was limited to three different doses of the plant (0.1, 0.2, and 0.3%) and further studies should investigate higher doses.

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Ethics Approval Statement

Study protocol was approved in advance by Kastamonu University Local Ethics Committee of Animal Trials with the approval number of 2017.01.

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Determination of Separation Efficiency of Hydrocyclone Used Pre-Filter in Micro Irrigation at Different Inlet Velocities and Sand Diameters

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ABSTRACT
Hydrocyclones are used as pre-filter to reduce suspended particles in irrigation water on the subsequent filters. The aim of the study was to determine separation efficiency (SE) of hydrocyclones, called H1, H2 and H3 according to inlet/outlet diameters, at water velocities of 1.0 (V10), 1.5 (V15) and 2.0 (V20) ms^{-1}, and sands diameter of 0.5 (D05), 1.0 (D10), 1.5 (D15), 2.0 (D20) and 2.5 (D25) mm. Therefore, a hydrocyclone laboratory test system was constituted using a water tank, motor pump, inverter, flowmeter, valve, hydrocyclone, disk filter, and polythene pipe. Separation efficiencies were calculated by dividing amounts of sand collected in collection box by feeding amount of sand. The lowest average separation efficiency was determined as 37% at H1V10D05 treatment and the highest ones as 97% at both H2V20D10 and H3V20D20, and the other ones changed between two values. Average separation efficiencies resulted as 69%, 88% and 88% for H1, H2 and H3 hydrocyclones, and 71%, 84% and 90% for V10, V15 and V20 water velocities, and 78%, 82%, 82%, 83% and 84% for D05, D10, D15, D20 and D25 sand diameters, respectively. Besides these, average separation efficiency for three parameters was 82%. Since the inlet size of H2 is smaller than that of H3 and its SE was higher than that of H1 and equal to that of H3, the most suitable hydrocyclone was determined as H2 to be used in the micro irrigation. The highest average separation efficiency was 90% at a water velocity of 2.0 ms^{-1}. According to separation efficiency of the hydrocyclone, the optimum water velocity in the inlet of the hydrocyclone was determined as 2.0 ms^{-1}. The separation efficiency of hydrocyclone showed that the efficiencies increased with increasing water velocity from 0.5 ms^{-1} to 2.0 ms^{-1} and sand diameters from 0.5 to 2.5 mm. In separation efficiency for micro irrigation, water velocities and suspended materials play crucial roles as well as hydrocyclone mechanical properties.

Keywords: Micro irrigation, Hydrocyclone filter, Sand diameter, Water velocity, Efficiency

1. Introduction

The shortage of water resources has always been one of the main obstacles to the development of many sectors such as hydropower, agriculture, textile, mining, urban domestic water and so on (Feng et al. 2020). Due to the climate change and drought, the allocated irrigation water to the agriculture is getting decrease year by year and accordingly, to increase the use of micro irrigation systems that have higher irrigation efficiency (Goyal & Panigrahi 2015) and require higher initial capital investment have been supported by the government over the last few decades in Turkey (Ministry of Agriculture and Forestry 2021). On the other hand, water supplied from lakes, ponds, rivers, streams and canals may contain large amounts of organic and inorganic matter. Emitters with small orifices are used to deliver the required low irrigation flow rates and these small orifices can easily be clogged by particulate matter, biological growths, chemical precipitates or combination of these present in irrigation water (Keller & Bliesner 1990; Mailapalli et al. 2007). And also plant roots, sand, rust, microorganisms and dirt in the irrigation water could be the major sources of clogging for emitters (Adin & Sacks 1991) and solenoid valves in irrigation automation system (Feng et al. 2020). This shortens the economic life of the micro irrigation system. A clogged solenoid valve cannot function and accordingly irrigation automation cannot work. Besides, the partially or complete clogging of emitters can reduce or stop a system functioning, and thus, the clogging of the emitters may cause non-uniform water and nutrient (Nakayama & Bucks 1991), which can cause problems such as reduced plant growth, development, yield and variable product quality (Yurdem et al. 2010). The sustainability of the micro irrigation depends on the separation to prevent emitter clogging. Therefore, separation is necessary in the micro irrigation to prevent emitters and solenoid valve from clogging.

Since the later part of the 19th century, hydrocyclones, called cyclone separators have been utilized as a solid-liquid separator to remove sand from well water (Bernardo et al. 2006). They were also used solid-solid (Klima & Kim 1998), liquid-liquid (Smithy & Thew 1996) and gas-solid (Fıçıcı & Arı 2008). A typical hydrocyclone consists of a cylindrical section, a conical section, an underflow cylinder section and a sand collection box. The separation is based on density difference between the

liquid/solid/gas and the material to be separated. The principle of centrifugal separation is used to remove or classify solid particles from a fluid, based on particle size, shape and density. Hydrocyclones are used as a primary filter for separation of coarse materials in irrigation water before it enters the screen or sand filters. So, these primary filters decrease the coarse suspended materials in irrigation water that passes the secondary filters such as media (gravel) and disc (screen) (Desai & Praveen 2011).

Hydrocyclone filters remove 98% of the sand particles (Keller & Bliesner 1990). It depends upon the centrifugal force to remove or eject high density particles from the water. Hydrocyclone filters cannot remove organic materials in irrigation water (Chauhdary et al. 2017).

Hydrocyclones have been extensively utilized as separation device in many areas such as the water treatment, air pollution control, agriculture, chemical, cement, power, milling and mining (Avcı & Erel 2003; Martinez et al. 2008; Yurdem et al. 2010; Desai & Praveen 2011; Parihar et al. 2012; Sakura & Leung 2015). There are many papers on the performance of hydrocyclone filters used in the industries areas (Dirgo & Leith 1985; Asomah & Napier-Munn 1997; Youngmin et al. 2000; Shukla et al. 2011; Cernecky & Plandorova 2013; Sakura & Leung 2015; Tan et al. 2015; Liu et al. 2016; Kenny et al. 2017). However, studies on hydrocyclones used as water filtration devices in the micro irrigation are limited (Yurdem et al. 2010; Feng et al. 2020).

At present, many domestic and foreign manufacturers have produced many kinds of filter products, and many experts and scholars have done a lot of research on the hydraulic performance and filtration ability of the filter, but there is no comprehensive report on the filter features and its performances (Feng et al. 2020). The most important parameters in cyclone operation are pressure drop (energy lost) and collection efficiency (Erence et al. 1994; Chauhan 1998; Fassani & Goldstein 2000; Erbaş et al. 2013). Chauhan (1998) tried to evaluate the performance of the hydrocyclone filter with clean water and with known concentration of impurities. The performance of the filter was studied by varying discharge, head loss, influent and effluent concentrations, and filtration efficiency with time of operation of the filter. Soccol & Botrel (2004) tested hydrocyclones in different sizes, using circular feeding (I) and rectangular feeding tubes (II, III and IV) for pre-filtering irrigation water under different pressure differential. In another study, Soccol et al. (2007) evaluated sand separation efficiency of hydrocyclone under varies pressure and concentration for micro irrigation system. Bulancak et al. (2006) studied to determine the efficiency of nine different filters (discs, screens, hydrocyclone, sand separator and media filter) used in drip irrigation systems. In a study of hydrocyclone filter by Mailapalli et al. (2007) studied, its performance for micro irrigation was evaluated by the variation of discharge, pressure drop, influent concentration. Firstly, the filter was tested with clean water to determine clean pressure drop and later it was tested with four concentrations of solid suspension. Results of this study showed that filtration efficiency increased with the increase of concentration. Srivastava et al. (1998) studied the hydraulic performance of commercially available drip irrigation hydrocyclone filter for various sets of discharge and pressure drop for two concentration. They found that the initial removal efficiency of higher concentration was more than lower concentration but final removal efficiency was not very much affected by the concentration of solid suspension. Desai & Praveen (2011) were designed and fabricated the six hydrocyclone models (M1 ... M6), and then tested for its performance evaluation for micro irrigation. They found that the hydrocyclone model M3 with 26° cone angle and 0.065 m underflow cylinder diameter was the best model with the removal efficiency of 95.68% among all the six models.

Since hydrocyclones have been designed in different size and shape, and produced for micro irrigation to prevent emitter clogging in many countries, further separation efficiency knowledge is needed in different hydraulic properties of hydrocyclones and size of the suspended material. For this purpose, a hydrocyclone test system was constituted in laboratory conditions.

So, the objective of the study was to determine separation efficiency for H1, H2 and H3 hydrocyclones at 3 different water velocities of 1.0, 1.5 and 2.0 ms^{-1} and sands diameter of 0.5, 1.0, 1.5, 2.0 and 2.5 mm.

2. Material and Methods

Since many kinds of hydrocyclones in different size and shape are produced for micro irrigation by many domestic manufacturers, further SE knowledge is needed in different hydraulic properties of hydrocyclones and size of the suspended material. In order to determine SE of these hydrocyclones, three widely used hydrocyclones in micro irrigation by the farmers, were chosen in the experiment. The SE of these three hydrocyclones was determined at 3 water speeds (1.0, 1.5 and 2.0 ms^{-1}, called as V10, V15 and V20, respectively) and 5 sand diameters (0.5, 1.0, 1.5, 2.0 and 2.5 mm called as D05, D10, D15, D20 and D25, respectively). Therefore, a workbench was assembled in the laboratory and it consists of reservoir (1), motor pump unit (2), inverter (3), flow meter (4), hydrocyclone (5), disc filter (6), fittings (7) and funnel (8) (Figure 1). The experiment was performed in the workbench.

1) Reservoir: Metal sheet of 2 mm, 1×1×1 m size, 1m^3 capacity and painted to prevent rust and algae growth.

2) Motor pump: Centrifugal pump with discharge flow of 44 m^3h^{-1}, pumping hydraulic head of 24 m and electrical motor with 5.5 kW (3 phases 380 VAC, 11.3 A, 50 Hz) and 2870 rpm.

3) Inverter: Used to control the speed of the motor pump to drive variable flow rate in pipeline (6 kW).

4) Flowmeter: Electromagnetic flowmeter with nominal flow of 119.4555 $m^3 h^{-1}$.

5) Hydrocyclone: Three hydrocyclones having technical properties same, except inlet/outlet pipes, were utilized in the experiment.

6) Disc filter: Capacity of 38-50 m^3h^{-1}, filtration sensitivity of 130 µm. It was assembled to pipeline after hydrocyclones to prevent the return of sand.

7) Fittings: Ø75 polyethylene (PE) pipes, resisting 405 kPa, were used to connect each unit to each other in the workbench. Fittings consist of valves (2½"), couplings (2½") and reductions (from 3" to 2½").

8) Funnel: A 130 cm long polyethylene (PE) funnel (diameter of one end ∅6 cm and other end ∅3 cm) was used to discharge sand samples into inlet of the suction pipe of the motor pump.

Hydrocyclones were referred as H1, H2 and H3 according to the inlet (Di)/outlet (Do) diameters. While nominal inlet (Di)/outlet (Do) diameters of hydrocyclones were 2", 2½" and 3", their inside diameters were 50.8, 63.5 and 76.2 in mm, respectively. The properties of hydrocyclone were given in Table 1. As seen from Table 1 and as above, only diameter of Di/Do changed but other parameters were unchanged to determine effect of diameter on SE. Diagram of the experimental hydrocyclones was given in Figure 2. In these three hydrocyclones, length of the vortex finder pipe (Lv) was lesser than cylindrical section length (Lc) too.

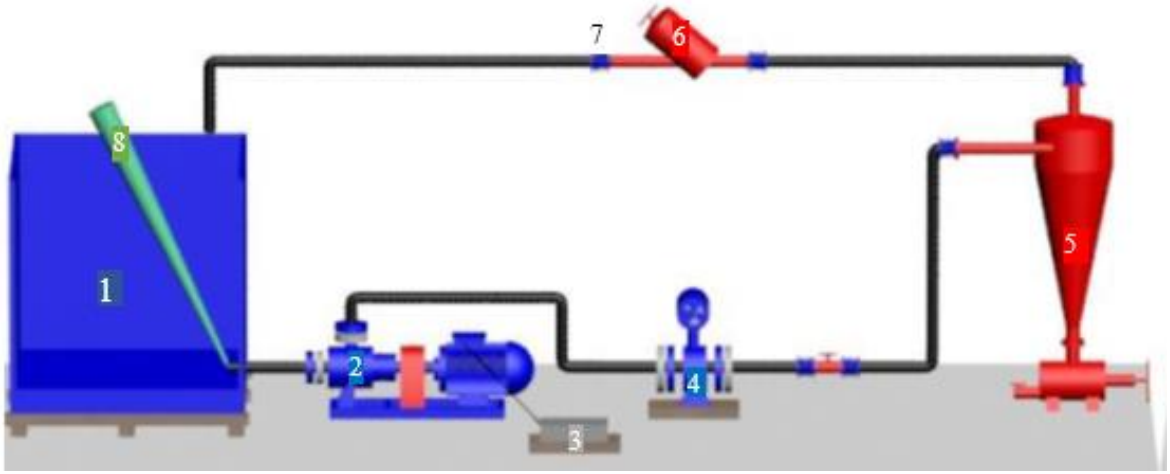

Figure 1- Diagram of the experimental workbench and components

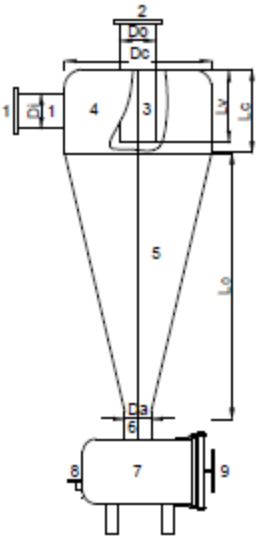

Figure 2- Diagram of the experimental hydrocyclone 1) inlet pipe, 2) outlet pipe, 3) vortex finder, 4) cylindrical section, 5) conical section, 6) apex, 7) collection box, 8) ball valve, 9) chamber cover

Table 1- Technical properties of hydrocyclone filters used in experiments

Elements of hydrocyclone	*Hydrocyclone types*		
	H1	*H2*	*H3*
Inside diameter of inlet/outlet pipe (Di/Do), mm	50.8	63.5	76.2
Cylindrical section diameter (Dc), mm	160	160	160
Cylindrical section length (Lc), mm	190	190	190
Conical section length (Lo), mm	490	490	490
Length of the vortex finder pipe (Lv), mm	150	150	150
Cone angle (ɵ°)	7.3	7.3	7.3
Apex diameter (Da), mm	35	35	35

The tests were created using 1.0, 1.5 and 2.0 ms^{-1} water velocities and 0.5, 1.0, 1.5, 2.0 and 2.5 mm sand diameters at the 2", 2.5" and 3" inlet diameters of the hydrocyclones. Each test was repeated 3 times and a total of 3*3*5*3=135 tests were conducted under 101.3 kPa therefore, a manometer of 400 kPa was mounted on pipeline. And also, the water temperature was measured as 20-22 ºC during the tests.

Although the water velocity in the main pipe in irrigation systems shows small changes according to the researchers, it was determined that the water velocity in the main pipe varies between 0.5 and 3 ms^{-1} (İşcanet al. 2001; Yıldırım 2003; Addink et al.1983). Therefore, in the hydrocyclone SE test, water velocities at inlet pipe of the hydrocyclone were chosen as 1.0, 1.5 and 2.0 ms^{-1}. The motor pump did not suck any sand at 0.5 ms^{-1} the inlet water velocity of hydrocyclone. Since flow meter available in laboratory displayed only flow rate (m^3h^{-1}), the flow rates at the inlet pipe of the hydrocyclone were calculated from Continuity Equation (Equation 1) using the water velocities (ms^{-1}) and cross section areas of inlet pipes (Table 2). The hydrocyclone inlet areas (m^2) were computed from pipe inside diameters of hydrocyclones. In order to adjust the water velocities in the inlet of the hydrocyclone, the motor pump, inverter, flowmeter and the flow rates were used. The motor pump was driven by an inverter. In this context, the velocity of the motor pump is adjusted by changing the inverter output frequency by the pot on the inverter. As the motor pump velocity was increased by inverter, so also in the system. For example, the output frequency of the inverter is gradually increased with the pot until the flow rate value on the flow meter display is equal to 14.59 m^3h^{-1}. Accordingly, this calculation the inlet water velocity of the H1 (0.0508) hydrocyclone will be equal to 2.0 ms^{-1}. The other velocities in Table 2 were set same way.

$$V = \frac{Q}{A*3600} \quad (1)$$

Where; V: Water velocity in the inlet pipe (ms^{-1}), Q: Flow rate (m^3h^{-1}), A: Pipe cross-sectional area (m^2).

Table 2- Inlet (Di)/outlet (Do) diameters and hydraulic properties of hydrocyclones

Hydrocyclones and their inlet diameters (m)	*Inlet water velocities (ms^{-1})*	*Inlet cross section area (m^2)*	*Flow rate (Q) m^3h^{-1}*
H1 (0.0508)	2.0	0.002026	14.59
	1.5		10.94
	1.0		7.29
H2 (0.0635)	2.0	0.003165	22.79
	1.5		17.09
	1.0		11.40
H3 (0.0762)	2.0	0.004558	32.82
	1.5		24.61
	1.0		16.41

Sand used in the test was obtained from a stream. Before determining the sand diameters, since the sand contained course and light materials, those course materials were removed from the sand and the sand was washed 4-5 times with tap water until light material such as silt, clay and other materials was cleared. The washed sand was dried at 105 ºC in the oven until being stable weight. In general, sand diameters vary between 0.074-2.0 mm (Kumsabar & Kip 1986). So, the dried sand was sieved on the kit of five sieves (0.5, 1.0, 1.5, 2.0 and 2.5 mm opening) to determine sand diameter of 0.5, 1.0, 1.5, 2.0 and 2.5 mm. The sand passing through sieve of 2.5 mm opening and collecting on sieve of 2.0 mm opening was referred as 2.5 mm sand and other sand diameters of 0.5, 1.0, 1.5 and 2.0 were determined using same method. Sands with different diameters were weighed at 500 g and 15 sand masses with 3 replications were formed and similarly, Tan et al. (2015) used 500 g dolomite in their study well.

There was no sand and smaller solid particles in the water and it was in the C_2S_1 class (USSL 1954). The water reservoir in the system was first filled with the water until 5-10 cm from the mouth level. Then, the valve placed on the water tank outlet pipe was opened and the motor pump was started. After the motor pump is started, the desired water velocity in the inlet of hydrocyclone is adjusted with the help of the pot on inverter. The pressure of the system was kept at 101.3 kPa in all tests. The sieved sand of different diameters was delivered through the funnel to the outlet pipe of the water reservoir (to the suction pipe of the motor pump). After the system was operated at the desired speed for 5 minutes, 500-grams-sands with the five different diameters were given to the system within 10 minutes of operation with the help of the funnel. After the sand transfer was finished, the system was operated at the same velocity for another 5 minutes. After the system was stopped, the sand in the collection box (underflow chamber) of the hydrocyclone was filtered and taken into the sample container. The collected wet sand was dried in an oven at 105º C until it reached a constant weight.

The SEs of the hydrocyclone at different water inlet velocities were calculated from Equation 2 (Soccol & Botrel 2004; Sakura & Leung 2015).

$$SE = \frac{CS}{FS} * 100 \quad (2)$$

Where; SE: Separation efficiency (%), FS: Feeding dry sand of 500 (g), CS: The collected dry sand in the hydrocyclone's collection box (g).

Hydrocyclone SE was evaluated using ANOVA and Duncan test in SPSS statistical software.

3. Results and Discussion

The laboratory tests were conducted with 3 replication using 500 g sand stacks to determine SE of H1, H2 and H3 hydrocyclones at water velocities of 1.0, 1.5 and 2.0 ms^{-1}, and sand diameter of 0.5, 1.0, 1.5, 2.0 and 2.5 mm for 10 minutes. The SEs of hydrocyclone were calculated as a percentage by dividing the dry weight of sand gathering in the collection box by the dry weight of sand supplied to the system. ANOVA test was performed using hydrocyclone efficiency values and effects of hydrocyclones, water inlet velocities and sand diameters on SE were found to be statistically significant ($P<0.05$). In addition, groups of SE of parameters were determined by Duncan test (Table 3).

SEs were determined taking into account three parameters that these were three hydrocyclones (H1, H2, and H3), three water velocities (V10, V15 and V20) and five sand diameters (D05, D10, D15, D20 and D25). The lowest average SE was resulted from 37% at H1V10D05 treatment and the highest ones from 97% at H2V20D10 and H3V20D20 and the other's changed between two values (Table 4). When averaged SE in H1, H2, and H3 treatments, their SE were 69%, 88% and 88%, respectively (Table 3) and the average SE of these three was 82%. When increased water velocity from 1.0 ms^{-1} to 2.0 ms^{-1}, SEs in H1, H2, and H3 treatments increased from 54 to 80%, 79% to 95%, and 79% from 95%, respectively. When raised sand diameters from 0.5 mm to 2.5 mm, SEs in H1, H2 and H3 changed between 59-76%, 87-89% and 86-90%, respectively (Table 4). The lowest SE was found out in H1 and the highest ones in both H2 and H3. There was significant difference statistically among SEs of the three hydrocyclones ($P<0.05$) and Duncan test divided hydrocyclones into 2 groups (Table 3). As can be seen from the Table 3, the SE of H2 and H3 constituting the first group is high and the SE of H1 constituting the second group is low. Since the size of H2 is smaller than H3 and its SE is higher than H1 and H3, the most suitable hydrocyclone was determined as H2 to be used in the micro irrigation.

In the V10, V15 and V20 treatments, the average SEs were calculated as 71%, 84% and 90%, respectively (Table 3) and the SE increases between V10 and V15, and V15 and V20, and V10 and V20 were 13%, 6% and 19%, respectively. When raised sand diameter from 0.5 mm to 2.5 mm, SE in V10, V15 and V20 changed between 65-74%, 80-86% and 87-92%, respectively (Table 4). The SE increased with both increasing water velocity (Sakura & Leung 2015) and sand diameter. The water inlet velocity had a statistically significant effect on the hydrocyclone SE ($P<0.05$). Duncan test divided SE of water speed into 3 groups (Table 3). It formed the 1st group of 2 ms^{-1}, the 2nd group of 1.5 ms^{-1} and the 3rd group of 1 ms^{-1}, respectively. The highest average SE was 90% at a water speed of 2 ms^{-1}. According to these SE, water velocity of 2.0 ms^{-1} was the optimum for the hydrocyclone.

Average hydrocyclone SEs at D05, D10, D15, D20 and D25 treatments were found to be 78%, 82%, 82%, 83% and 84%, respectively (Table 3 and Table 4). The increase in SE from D05 to D10 was of 4%, and from D10 to D20 not changed and there was an increase of 2% from D20 to D25. As sand diameter or weight increased from D05 to D25, SE increase was 6% some extent. The effect of sand diameters on SE was found to be statistically significant at $P<0.05$ level (Table 3). Duncan test divided sand diameters into 3 groups. The first group consisted of 2.5 mm sand, the second group consisted of 2.0, 1.5 and 1.0 mm, and the third group 0.5 mm sand. Hydrocyclone SE was found to be low in small sand diameters and high in large sand diameters.

The SEs in the treatments were given in Figure 3. As seen in Figure 3, SEs of H2 and H3 was close to each other and that's of H1 was lower than that's of H2 and H3. SE of H1 changed between 60% and 80% and that' of H2 and H3 changed between 80% and 97% and at five point such as 0.5, 2.0; 1.0, 2.0; 1.5, 2.0; 2.0, 2.0 and 2.5, 2.0 (sand diameter (mm), inlet velocity (ms^{-}

[1])) SE varied between 93-97% that their SEs were determined at 2.0 ms^{-1} inlet velocities. When three points (for example sand diameters and velocities= 0.5, 1.0; 0.5, 1.5; 0.5, 2.0) from the vertical axis are examined on clockwise, the SE increased as the inlet velocities and sand diameter (weight) increased. As stated by Silva (1989), when the suspension is entered the hydrocyclone, a fraction of the higher velocity of the irrigation water and heavier sands were discharged through apex. The remaining irrigation water and the lower velocity (lighter) sands were discharged throughout the vortex finder. Even though the hydrocyclone may not be separating by centrifugation all sands, a certain amount of sands was removed with 82% (all averages of the three parameters). The highest SE found out at 2.0 ms^{-1} at H2 and H3 that it was very important for choosing in water velocities for the hydrocyclones. In this subject, Addink et al. (1983) stated that the optimum flow velocity in main lines should be ranged 1–3 ms^{-1} from the point of construction and energy costs. Since our results and Addink et al. (1983)'s findings are parallel, so selection of 2 ms^{-1} is suitable for hydrocyclones of micro irrigation.

Table 3- Classification of SE in hydrocyclone, water inlet velocity and sand diameters by Duncan test

Hydrocyclones	*Mean Efficiency*
H1	69^{b}±1.95
H2	88^{a}±1.06
H3	88^{a}±1.05

Water inlet speeds (ms^{-1})	*Mean Efficiency*
V10	71^{c}±1.98
V15	84^{b}±1.32
V20	90^{a}±1.18

Sand diameters (mm)	*Mean Efficiency*
D05	78^{c}±3.30
D10	82^{b}±2.53
D15	82^{b}±2.46
D20	82^{b}±2.07
D25	84^{a}±1.98

Table 4- Average SEs for treatments

Sand diameters (mm)	*Water velocities (ms^{-1})*	*H1*	*H2*	*H3*
	V10	37	81	77
D05	V15	66	87	88
	V20	74	94	93
	V10	56	80	83
D10	V15	70	90	92
	V20	76	97	95
	V10	56	78	77
D15	V15	74	93	92
	V20	78	95	95
	V10	60	77	79
D20	V15	76	87	87
	V20	84	95	97
	V10	61	80	81
D25	V15	77	93	88
	V20	89	94	94

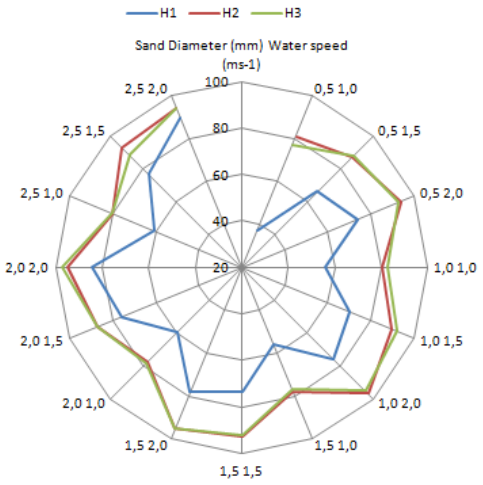

Figure 3- Effect of hydrocyclones, water velocities and sand diameters on SEs

The most upper limit of average SE with 90% was observed at water velocity treatments, and followed successively by that's SE of hydrocyclone (88%) and sand diameter (84%) treatments (Table 4). Since the highest SE was found out in water velocity treatment, it could be told that water velocity is most effective parameter in SE. Yurdem et al. (2010) reports that when the inflow

rate increase, the tangential velocity of flow in the hydrocyclone increases. Thus, the centrifugal force on the water increases and giving a high radial velocity. This creates more turbulent kinetic energy in the cyclone and finally SEs increase.

SE given in the studies conducted so far for micro irrigation changed between 9% and 97%. Examples of the studies are as follows. In the study by Desai & Praveen (2011), the SE was 95.68% in the hydrocyclone model M3 with 26º cone angle and 0.065m underflow cylinder diameter. Keller & Bliesner (1990) reported that hydrocyclones could remove up to 98% of sand particles. Soccol & Botrel (2004) resulted that in relation to the sand suspension tests the highest SE of 82.02% was obtained for the hydrocyclone I. In another study, Soccol et al. (2007) concluded the highest SE were obtained 95.48 and 97.09% at 10.8 and 22.3 kPa pressure differential in hydrocyclone, respectively. Bulancak et al. (2006) found to be 37% and 36% efficiencies for the hydrocyclone and sand separator, respectively, in drip irrigation systems. In a study of micro irrigation hydrocyclone filter with 20 cm by Mailapalli et al. (2007), the minimum and maximum efficiencies of solid suspension were 9.91-30.3, 9.93-32.96, 9.62-43.89 and 9.9-52.5%. When SE results were compared, the results of this study correlate with the SE ranges reported in the existing studies. Since hydrocyclones are produced in different sizes and operated under different management conditions such as pressure, concentration, inlet water velocity and solid suspension, the lower and upper limits of the efficiency could vary in a wide range.

4. Conclusions

SEs were determined taking into account H1, H2, and H3 hydrocyclones, V10, V15 and V20 water velocities and D05, D10, D15, D20 and D25 sand diameters.

Average SEs resulted as 69%, 88% and 88% for H1, H2 and H3 hydrocyclones, and 71%, 84% and 90% for V10, V15 and V20 water velocities, and 78%, 82%, 82%, 83% and 84% for D05, D10, D15, D20 and D25 sand diameters, respectively and also all average of SE for three parameters was 82%. These three parameters were significantly affected the results of SE. Under the tests condition, SE increased with the increase of inlet diameter, water velocities and sand diameters. The most suitable inlet diameter and water velocity of hydrocyclones were determined as H2 (0.0635m) and 2.0 ms^{-1}, respectively.

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Evaluation of Insecticide Toxicity and Enzymatic Detoxification in Neonate Larvae of European Grapevine Moth, *Lobesia botrana* Denis & Schiff. (Lepidoptera: Tortricidae)

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ABSTRACT

Lobesia botrana Denis & Schiff. (Lepidoptera: Tortricidae), also known as the European grapevine moth is a detrimental pest in grape production in Palearctic Region. Insecticides are used to control *L. botrana* infestation in grape-produced areas; however, repeated and intensive use of insecticide causes reduced efficacy in *L. botrana* management. In the present study, the efficacy of five commonly used insecticides (chlorpyrifos-ethyl, emamectin benzoate, indoxacarb, lambda-cyhalothrin, and spinosad) was evaluated in two field populations (AL and SAR) from Manisa, Turkey. In addition, detoxification enzyme activities including esterase (EST) and glutathion-S-transferase (GST) were measured via in vitro assays. LC_{50} values were found lower for chlorpyrifos-ethyl, emamectin benzoate, and spinosad and higher for indoxacarb and lambda-cyhalotrin in AL populations compared to SAR population. EST levels were slightly higher in AL population compared to SAR. GST levels were found higher in SAR population. However, no statistical difference was found in both detoxification enzyme activities and EST enzyme levels were higher than GST enzyme levels in both populations. Findings of the current study would help growers as well as the applicator to strategize their insecticide use in integrated pest management programs for *L. botrana* and possibly mitigate any insecticide resistance development.

Keywords: Detoxification enzyme, Dose-response, Grape, Insecticide toxicity, Tortricidae

1. Introduction

The European grapevine moth *Lobesia botrana* Denis & Schiff. (Lepidoptera: Tortricidae) is a major pest in grapevine production regions in Asia, Europe, North & South America (Ioriatti et al. 2011). *Lobesia botrana* can feed on approximately 40 different plant species; however, feeding on grapevine makes *L. botrana* an economically important pest in grape production. The first generations mainly feed on flower buds, which can be negligible for growers. However, the second and third generations feed on flesh and result in increased yield loss and unmarketable fruit (Pasquini et al. 2018). It is reported that in some regions 4th generation could occur due to environmental conditions (Roditakis & Karandinos 2001; Harari et al. 2007). In addition, dripping fruit juice during the feeding causes secondary damage e.g., gray mold fungal growth (*Botrytis cinerea*) (Moosavi et al. 2020). For this reason, egg and larvae stages are the primary targets to control the pest for potential crop loss.

Several control methods including mating disruption, biological control, and cultural practices are included in integrated pest management programs (IPM); however, insecticides are ubiquitously in use due to their fast and immediate effects (Ioriatti et al. 2011; Navarro-Roldan et al. 2017). Currently, commonly used insecticides include chlorantraniliprole, chlorpyrifos-ethyl, emamectin benzoate, indoxacarb, lambda-cyhalothrin, methoxyfenozide, tebufenozide, spinosad, and *B. thuringiensis*. Frequent use of insecticides causes resistance against the individual insecticide or chemical classes. In addition, factors such as canopy deposition, application rate, poor sprayer calibration, insufficient spray coverage, inappropriate timing of spray application could cause failure in insecticide application thus possibly insecticide resistance may occur (Pasquini et al. 2018).

The toxicity of insecticides has been evaluated both in the field and laboratory on *L. botrana* stages (egg, pupae, and adult) (Irigaray et al. 2005; Ioriatti et al. 2011; Civolani et al. 2014; Hatipoglu et al. 2015). Some of the insecticides were found more effective than others (e.g., emamection benzoate was highly effective compared to indoxacarb) (Civolani et al. 2014). Out of all insecticides tested, indoxacarb is the only insecticide that the resistance is observed (Civolani et al. 2014). Thus, monitoring insecticide efficacy via bioassays is necessary to strategize further insecticide used to mitigate resistance development. In addition to insecticide bioassays, enzymatic activity levels (EST; carboxylesterases and GST, glutathione S-transferases) in

neonate larvae provide detailed and consistent results on the resistance development in the field (Rodriguez et al. 2011a). Detoxification enzymes play a major role in insecticide resistance (Scott 1998; Enayati et al. 2005; Montella et al. 2012). Of the other mechanism resistance, detoxification enzymes have the ability to amplify genes through overexpression and changes in coding sequence to alter the detoxification abilities thus causing resistance (Li et al. 2007; Navarro-Roldan et al. 2017).

Insecticide detoxification in insects is undergone in two-phase metabolisms; Phase I is attaching a polar group to toxic compounds e.g., insecticides or their cleavage in the body (Navarro-Roldan et al. 2020). Phase II is adding sugar, amino acid, sulfate, or phosphate group on Phase I product to increase the polarity (hydrophilicity) to excrete from the insect body (Bernard & Philogene 1993). The main important detoxification enzyme groups are poly substrate monooxygenases (PMSO also known as cytochrome P450), EST, and GST (Despres et al. 2007). PSMO and EST belong to Phase I group enzymes while GST is in Phase II enzymes (Navarro-Roldan et al. 2020). Furthermore, detoxification enzyme levels can be measured via in vitro methods (Brown & Brogdon 1987; Navarro-Roldan et al. 2020) to monitor insecticide resistance levels. Reports about *L. botrana* enzymatic activity levels are limited (Hatipoglu et al. 2015; Navarro-Roldan et al. 2020).

The current study aims to evaluate the insecticide efficacy as well as the detoxification enzyme activity of two *L. botrana* population from Manisa, Turkey. Insecticides including chlorpyrifos, emamectin benzoate, indoxacarb, lambda-cyhalothrin, and spinosad were tested on the neonate larval stage to determine dose-response. In addition, EST and GST detoxification enzyme activities were measured via in vitro assays.

2. Material and Methods

2.1. Insect rearing

The stages of *L. botrana* (larvae, pupae, and eggs) were collected from the fields in Alaşehir (AL) and Sarıgöl (SAR), Manisa, Turkey, by visually examining the grape clusters. The area is primarily well-known with its grape (seedless and table) production. In addition, the distance between the two locations is approximately 25 km; however, types of insecticide used and frequency of the applications for *L. botrana* slightly differs due to microclimatic and geographic conditions in two locations. The insects were reared under controlled climate chamber conditions (25 °C, 60±10 RH) on a semiartificial diet (220 mL water, 4 g agar, 15 g cornmeal,15.6 g wheat germ, 15 g yeast, 1.28 g ascorbic acid, 0.4 g benzoic acid, 0.4 ml corn oil, 0.4 g nipagine, and 0.2 g benomyl) in plastic containers (Delbac et al. 2010). The photoperiod was set at 1000 lux luminosity for the first 15 h, 25 lux (twilight period) for the last hour to induce oviposition, and 8h dark period. The adults were fed with pollen: sugar: water mixture (1:1:1) to prevent starving and kept in a plastic cylinder container covered with plastic bags for egg-laying. Following laying, eggs were transferred into a container with an artificial diet for further larval feeding and adult emergence.

2.2. Chemicals

Chlorpyrifos-ethyl, lambda-cyhalothrin, indoxacarb, emamectin benzoate, and spinosad were used for mortality bioassay (Table 1). Insecticides were chosen from a different mode of action mechanism according to IRAC classification (http://www.irac-online.org/) as well as growers` preference. Insecticides were graciously donated from the corresponding companies upon request (Table 1).

Table 1- Detailed information of insecticides tested on *L. botrana* neonate larvae

Insecticides	***Active ingredient amount***	***Tested concentration range (mg AI L^{-1})***	***Formulation name***	***Chemical group****	***Manufacturer***
Chlorpyrifos-ethyl	480 g/L	60, 120, 240, 480, 960, 1920, 3840	Dursban®	Organophosphate (1B)	Dow AgroSciences, Indianapolis, IN, USA
Emamectin benzoate	5%	0.4 ,0.7, 1.5, 3.1, 6.25, 12.5, 25	Proclaim®	Avermectins (6)	Syngenta, Wilmington, DE, USA
Indoxacarb	150 g/L	2.34, 4.6, 9.3, 18.7, 37.5, 75, 150	Avaunt®	Oxadiazines (22A)	Dupont, Wilmington, DE, USA
Lambda-cyhalothrin	50 g/L	0.625, 1.25, 2.5, 5, 10, 20, 40	Karate Zeon®	Pyrethroids (3A)	Syngenta, Wilmington, DE, USA),
Spinosad	480 g/L	3, 6, 12, 24, 48, 96, 192	Laser®	Spinosyns (5)	Dow AgroSciences, Indianapolis, IN, USA

*: IRAC (Insecticide Resistance Action Committee, www.irac-online.org, 2020)

2.3. Insecticide efficacy

The neonate larvae (<24 h old) were used for bioassays. Seven or eight different concentrations of insecticides were prepared in water and mixed with a semisynthetic diet accordingly (2 µl cm^{-3}) to determine the dose-response (Bosch et al. 2007; Rodriguez et al. 2011a). A minimum of 16 larvae was used and for each concentration, four replicates were performed (Table 2). The control treatments were mixed with purified water. First, wells (24-Well Plate Costar®, Corning Inc., Corning, NY, USA) were filled

with semisynthetic diet, and then larvae were transferred to the wells individually to prevent cannibalism or any interferences between the individuals. Mortality of the larvae was determined after 24 h by touching with a fine brush to examine dead, moribund, and alive larvae (Bosch et al. 2007). Missing larvae were subtracted from the total treated larvae.

2.4. Enzymatic activity

The EST and GST enzyme activities were measured according to Rodriguez et al. (2011a). The larvae extracts were prepared in 100 µl of phosphate buffer (50 mM, pH 7.2) with a 0.4 mM final concentration of phenylmethylsulfonylfluoride. Ten neonate larvae were homogenized for each enzyme (EST and GST) with a total replicate of ten were prepared for each population. All homogenates were prepared on ice and centrifuged at 4 °C for 15 min at 15000g and supernatants were used as an enzyme source to measure EST and GST levels (Bouvier et al. 2002; Rodriguez et al. 2011a). The protein concentrations for both EST and GST were calculated by using bovine serum albumin as standard (Bradford 1976).

The total EST activity was measured in 96 well microplates and β-naphthyl acetate was used as substrate. Ninety microliters of larvae extracts were added to 90 µl of sodium phosphate buffer (50 mM, pH 6.5) containing a final concentration of 0.1 mM β-naphthyl acetate. After 15 minutes of incubation, the reaction was stopped by adding 20 µl of a staining reagent containing 3 g/L Fast Garnet and 35 g/L sodium dodecyl sulfate (Bouvier et al. 2002; Rodriguez et al. 2011a). The absorbance of the Naphthol-Fast Garnet complex was measured at 492 nm, after 15 minutes of incubation at room temperature. In addition, 10 wells were supplied with sodium phosphate buffer instead of enzyme extracts and were used as controls. The units were expressed in β-naphthol mg of protein^{-1} min^{-1} (Bouvier et al. 2002; Rodriguez et al. 2011a).

GST enzyme activity was measured in 96 well microplates (COSTAR) with UV transparent bottom and 1-chloro-2,4-dinitrobenzene (CDNB) was used as substrate. Microplate wells were filled with 4 µl of enzyme extract, 184 µl of 50 mM sodium phosphate buffer (pH 7.2), 2 µl of reduced glutathione (100 mM), and 10 µl of CDNB (30 mM). Ten wells were supplied with enzyme extract were used as control. The optical density was measured at 340 nm for both at time zero (t0) and after 1 min (t1) and the absorbance was measured at 30 °C. Optical reading results were expressed as mM glutathione conjugated mg of protein^{-1} min^{-1} (Bouvier et al. 2002; Rodriguez et al. 2011a).

2.5. Data analysis

The probit analysis (Polo Plus Program, LeOra Software LLC, Petaluma, CA, USA) was used to compare LC_{50} and LC_{90} values of the two populations (Robertson et al. 2003). Abbott formula was used to correct the mortality values (Abbott, 1925). The statistical difference of enzyme assays was evaluated via ANOVA test and means were tested with Tukey HSD test at $P < 0.05$ using R studio (R Development Core Team 2004).

3. Results and Discussion

The present study evaluates the insecticides efficacy of chlorpyrifos-ethyl, emamectin benzoate, indoxacarb, lambda-cyhalothrin, spinosad in *L. botrana* management in two grapevine production locations in Turkey. In addition to bioassays, EST and GST enzyme levels were measured for AL and SAR populations. Several reports are available on insecticide efficacy of *L. botrana* developmental stages and detoxification enzymatic levels (Irigaray et al. 2005; Vassiliou 2011; Civolani et al. 2014; Pavan et al. 2014; Hatipoglu et al. 2015; Navarro-Roldan et al. 2017; Pasquini et al. 2018; Navarro-Roldan et al. 2020). Several reports are available regarding insecticide toxicity on *L. botrana* (Irigaray et al. 2005; Vassiliou 2011; Pavan et al. 2014; Hatipoglu et al. 2015; Pasquini et al. 2018) however, up until now, there is only one insecticide resistance report available for *L. botrana* (Civolani et al. 2014). For that reason, it is important to monitor the insecticide efficacy to provide optimal *L. botrana* control and mitigate the potential resistance development.

3.1. Insecticide efficacy

The mortality bioassay results indicated that LC_{50} and LC_{90} values were found different in AL and SAR populations for the insecticides tested in the experiment (Table 2). Chlorpyrifos-ethyl LC_{50} value for AL (132.4 mg AI L^{-1}) was lower than SAR (259.3 mg AI L^{-1}). However, LC_{90} values were found slightly different for AL (900.2 mg AI L^{-1}) and SAR (919.2 mg AI L^{-1}) populations. LC_{50} values for emamectin benzoate for AL was 12.8 mg AI L^{-1} while SAR is 31.4 mg AI L^{-1}. Emamectin benzoate LC_{90} values for AL and SAR populations were 123.4 mg AI L^{-1} and 338.1 mg AI L^{-1} respectively. Indoxacarb LC_{50} values of AL (14.3 mg AI L^{-1}) were almost two times higher than SAR (8.7 mg AI L^{-1}). LC_{90} values for indoxacarb for AL (39.8 mg AI L^{-1}) and SAR (37.0 mg AI L^{-1}) were found close to each other. Lambda-cyhalothrin LC_{50} value was 100.0 mg AI L^{-1} for AL while SAR was found as 42.1 mg AI L^{-1}. LC_{90} values were followed the same trend as LC_{50} values for AL 920.4 mg AI L^{-1}and SAR 361.4 mg AI L^{-1}. LC_{50} value of spinosad for AL (0.6 mg AI L^{-1}) was almost 2.5 times lower than SAR (1.6 mg AI L^{-1}) while LC_{90} values were 18.0 mg AI L^{-1} and 7.7 mg AI L^{-1} respectively.

Table 2- Toxicity of insecticides on *L. botrana* neonate larvae for two field populations (SAR and AL)

Insecticides	*Population*	*N*[a]	*Slope*	*LC_{50} (mg AI L^{-1}; 95% CI)*	*LC_{90} (mg AI L^{-1}; 95% CI)*	*HF*[b]
Chlorpyrifos-ethyl	*AL*	227	1.540±0.025	132.4 (14.43-288.58)	900.2 (396.94-20565.41)	0.7
	SAR	128	2.332±0.352	259.3 (133.69-553.59)	919.2 (458.5-6584.12)	0.42
Emamectin benzoate	*AL*	128	1.304±0.249	12.8 (2.78-32.74)	123.4 (43.72-10125.6)	0.39
	SAR	128	1.241±0.236	31.4 (19.12-53.50)	338.1 (152.89-1679.85)	0.06
Indoxacarb	*AL*	128	2.877±0.458	14.3 (6.73-19.93)	39.8 (28.22-92.61)	0.37
	SAR	128	2.047±0.329	8.7 (3.31-21.38)	37.0 (16.67-715.37)	0.51
Lambda-cyhalothrin	*AL*	253	1.330±0.177	100.0 (57.39-220.42)	920.4 (355.19-8903.78)	0.34
	SAR	128	1.373±0.302	42.1 (7.40-105.84)	361.4 (135.48-12399.89)	0.27
Spinosad	*AL*	128	0.885±0.229	0.6 (0.04-1.55)	18.0 (5.67-275.06)	0.20
	SAR	128	1.889±0.297	1.6 (1.10-2.31)	7.7 (4.90-16.42)	0.18

[a]**:** number of individuals tested; [b]**:** heterogeneity factor divided by *dF*

Chlorpyrifos-ethyl is one of the common insecticides used for *L. botrana* control. Although chlorpyrifos-ethyl has low residual activity (<30% efficacy) in the field (Pavan et al. 2014), *L. botrana* IPM programs still include this insecticide to rotate with other insecticides. The results indicate that approximately two-fold difference in LC_{50} values were observed when comparing AL (132.4 mg AI L^{-1}) and SAR (259.3 mg AI L^{-1}) populations. The difference in LC_{50} values suggests that AL has more susceptibility to chlorpyrifos-ethyl than SAR population. Previously, a similar trend was observed among the field populations, which suggests that *L. botrana* susceptibility varies in different grapevine production regions (Hatipoglu et al. 2015). This might indicate that production areas in SAR or surrounding areas could potentially face a resistant population against chlorpyrifos-ethyl in the near future.

Emamectin benzoate is an alternative insecticide used in grape production against *L. botrana*. Results showed that AL population is reasonably susceptible to emamectin benzoate compared to SAR population, which could be speculated that SAR population could possibly be exposed to emamection benzoate more prominently. Emamectin benzoate is pronounced as more environment safely to replace other neurotoxic insecticides e.g., organophosphate insecticides. In addition, emamectin benzoate was found highly effective in controlling *L. botrana* in the field (Civolani et al. 2014).

Indoxacarb susceptibility was higher in SAR population compared to AL population. A similar trend was also observed in field populations (Hatipoglu et al. 2015). In a previous report, indoxacarb provided moderate residual activity (~75%) in the field (Pavan et al. 2014). Also, indoxacarb is the most widely used insecticide in Emilia-Romagna region in Italy and due to frequent use of indoxacarb, resistant *L. botrana* population was previously reported (Civolani et al. 2014). Results from the study indicated that indoxacarb resistance was 72-fold higher in the field population compared to control and resistance was stable even after rearing 10 generations (Civolani et al. 2014). Low or moderate (<10-fold) resistant ratios for indoxacarb were detected for multi-resistant strains of *Choristoneura rosaceana* and *Cydia pomonella* (Ahmad et al. 2002; Dunley et al. 2006; Mota-Sanchez et al. 2008).

Lambda-cyhalothrin results indicated that SAR population is more susceptible to lambda-cyhalothrin than AL population. Up until today, limited research has been done with lambda-cyhalothrin against *L. botrana* control (Navarro-Roldan et al. 2017). It has been demonstrated that adult males are more susceptible to lambda-cyhalothrin than females (Navarro-Roldan et al. 2017). In addition, AL population showed two-fold reduced susceptibility in spinosad than SAR population. The previous report indicated that spinosad also caused similar susceptibility in the field (Hatipoglu et al. 2015). In addition, the application of insecticides in combination was also evaluated and treatment combinations of lufenuron, spinosad, and indoxacarb and a combination of chlorpyrifos-ethyl, spinosad, and indoxacarb were found effective against 1st and 2nd generations in the field (Vassiliou 2011).

Overall, different mortality values were observed for the insecticides tested. Here, the present study primarily focused on the insect species and mode of action of the insecticides which are directly related to mortality. However, other factors such as insect stage (i.e., egg, immature, adult) and development, sex, mode of application, and time of exposure could also affect the mortality (Kanga et al. 1997; Lame et al. 2001; Shearer & Usmani 2001; Irigaray et al. 2005; Rodriguez et al. 2011a).

3.2. Enzymatic activity

Enzymatic activity results showed no statistical difference in EST and GST enzyme activities for AL and SAR populations (Figure 1). EST activities were measured as 9.4 nmol β-naphthol mg protein^{-1} min^{-1} and 6.3 nmol β -naphthol mg protein^{-1} min^{-1} in AL and SAR, respectively. However, there was no statistical difference in enzymatic activities in the two populations ($F_{1,24} = 1.85$, P=0.18). GST enzymatic activity of AL was found 0.032 mM glutathione conjugate mg protein^{-1} min^{-1} and it was 0.038 mM glutathione conjugate mg protein^{-1} min^{-1} in SAR. Again, there was no significant difference in GST activities when comparing two populations ($F_{1,22} = 0.42$, P= 0.52). Although there was no statistical difference between the two locations for detoxification enzymes, the amount of EST found in the populations was found more than GST amounts.

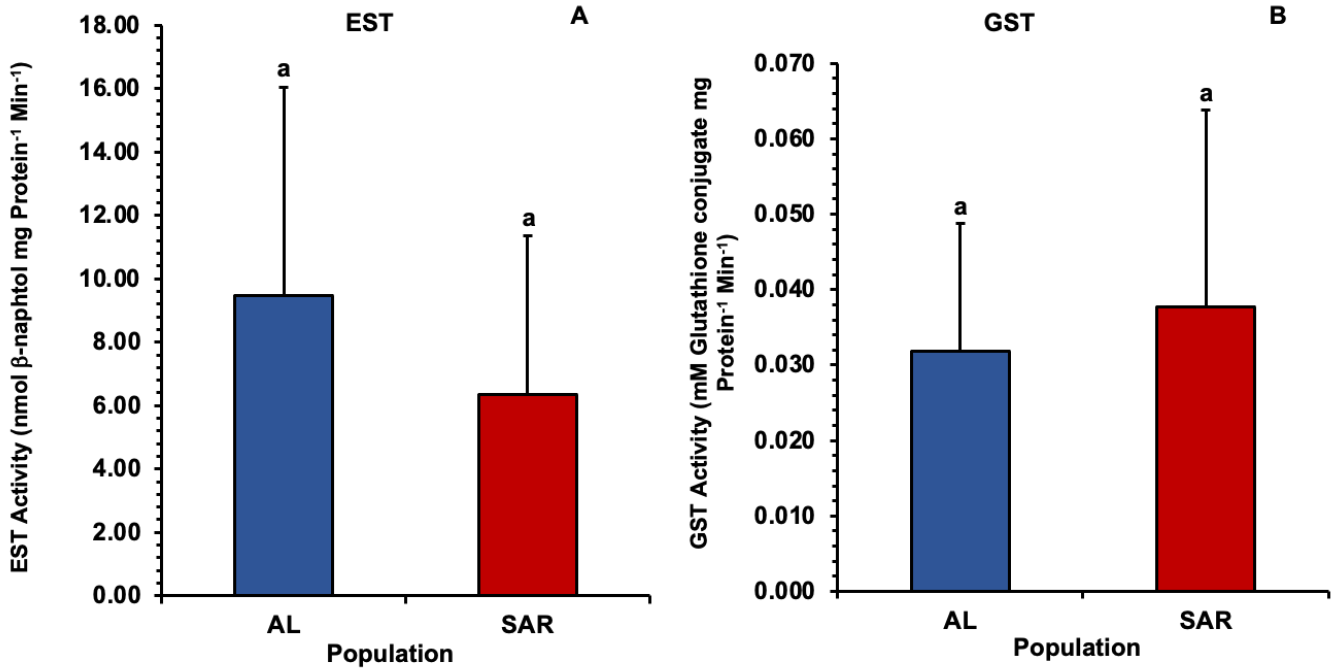

Figure 1- EST (A) and GST (B) enzymatic activities of *L. botrana* neonate larvae (n=200) from AL and SAR populations. Different letters indicate there is a statistical difference (P<0.05)

In the present study, two detoxification enzymes did not show any statistical difference between AL and SAR populations. However, EST enzyme levels were found drastically higher than GST levels for both populations. Enzymatic activity is directly related to insecticide pressure and thus it can vary in locations due to the insecticide application regime (Navarro-Roldan et al. 2020). A study reviewed 92 cases of lepidopteran detoxification mechanism and found that EST was affected only at 63% of the cases while GST was observed at 36% of the cases (Navarro-Roldan et al. 2020). Enzymatic activity of EST increased in *C. pomonella* treated with chlorpyrifos (Parra Morales et al. 2017). Several insecticides are detoxified via EST in species such as *Myzus persicae*, which could potentially induce resistance against broad-spectrum insecticides and cause cross-resistance (Devonshire & Moores 1982; Navarro-Roldan et al. 2020). On the other hand, the activity of GST was found higher when *Spodoptera littoralis* larvae treated with spinetoram (Ismail 2020). In addition, GST plays significant roles in environmental adaptation and detoxification for *Drosophila melanogaster* and *Anopheles gambiae* species (Ranson et al. 1998; Enayati et al. 2005). Previous findings suggest that EST and GST play primary roles in insecticide detoxification in lepidopteran species. Phase I enzyme families i.e., EST were actively responsible for detoxification in *C. pomonella*, *G. molesta*, *L. botrana* while Phase II enzyme i.e., GST was less changed for detoxification except for *G. molesta* (Navarro-Roldan et al. 2020). *Lobesia botrana* is the only species that the EST enzymatic activity was higher than *C. pomonella* and *G. molesta* (Navarro-Roldan et al. 2020), and different activity levels were found between sexes i.e., higher GST activity in females (Navarro-Roldan et al. 2020). As indicated in the literature, EST has the ability to detoxify a broad spectrum of insecticides in *L. botrana* compared to GST, which supports the finding from AL and SAR populations in the present study.

A positive correlation was reported between PMSO activation and chlorpyrifos-ethyl in neonate larvae from resistant *C. pomonella* population; however, there was no correlation in neonate larvae between EST and GST enzymes and chlorpyrifos-ethyl resistant population (Reyes & Sauphanor 2008). EST and GST were not involved in resistance mechanisms in neonate larvae from the field population in Spain (Rodriguez et al. 2011b). However, a significant correlation was found between EST and adult and fifth instar larvae to organophosphate resistance in *C. pomonella* (Reuveny & Cohen 2007; Reyes et al. 2007; Voudouris et al. 2011; Reyes et al. 2015). The chlorpyrifos-ethyl activity was reduced via PMSO detoxification (Feyereisen 1999) and high-level PMSO activity was detected in the field population (Dunley et al. 2006; Bosch et al. 2018). Increased mortality by chlorpyrifos at 48 h could be due to the oxidative desulfurization of the P=S group to its metabolites chlorpyrifos-

oxon (P=O) by PMSO, which can result in increased toxicity of chlorpyrifos over time (Yu 2008). Also, it is reported that the higher tolerance of *L. botrana* males to chlorpyrifos and difference in susceptibility between sexes is associated with the differences in enzymatic activities and quantities (Navarro-Roldan et al. 2017). The previous reports suggest that PMSO could be the major detoxification enzyme for chlorpyrifos-ethyl; however, whether EST and GST enzymes play an active role is still unclear for *L. botrana* larvae.

On the contrary of other lepidopterans, no relationship was found between PMSO and emamectin benzoate detoxification in *L. botrana* (Tabashnik 1991; Liang et al. 2003; Bosch et al. 2018). Reduction of emamectin benzoate efficacy detected in *C. pomonella* in Europe and found to be related to increased EST activity while *B. tabaci* resistance to emamectin benzoate is related to PMSO and GST activity (Kang et al. 2006; Reyes et al. 2007; Yu 2008). Reduced efficacy of emamectin benzoate is associated with increased EST activity in *C. pomonella*, which could possibly be similar in *L. botrana* as well. It is suggested that all three major detoxification enzymes (PMSO, EST, and GST) play important roles in indoxacarb detoxification for *C. rosaceana* larvae (Hafez et al. 2020). However, in other insects (e.g., *Spodoptera litura*, *Plutella xylostella*, *Phenacoccus solenopsis*) the primary enzymes that detoxify indoxacarb were PMSO and EST (Sayyed & Wright 2006; Sayyed et al. 2008; Afzal & Shad 2015; Hafez et al. 2020). All three major enzymes could likely be associated with indoxacarb detoxification in *L. botrana*. Reports indicated that oxidative metabolism might also be involved in the detoxification of indoxacarb for other insects including *C. rosaceana* and *C. pomonella* (Ahmad & Hollingworth 2004; Rodriguez et al. 2011a). It is highly possible that a similar detoxification pathway for indoxacarb could play role in *L. botrana*.

Previous reports indicated that two major enzymes, PMSO and EST play key roles in pyrethroid detoxification in *C. pomonella* larvae (Yu 2008). Reduced susceptibility to deltamethrin in *C. pomonella* larvae is associated with EST detoxification (Voudouris et al. 2011). Forty-one genes were demonstrated to show gene amplification by EST in *Aedes aegypti* against deltamethrin resistance (Faucon et al. 2015). This could be an indication that PMSO and EST might be responsible for lambda-cyhalothrin detoxification in *L. botrana* neonate larvae (Bosch et al. 2018). It was stated that enzymatic detoxification of spinosad was related to insect species (Wang et al. 2006) and several detoxification mechanisms were presented in the previous reports (Scott 1998; Shono & Scott 2003; Wang et al. 2006; Reyes et al. 2007). One study indicated that PMSO is associated (GST has no role) with spinosad resistance in *Musca domestica* (Scott 1998; Reyes et al. 2007). However, resistance to spinosad was reported to be related to an altered target site and not to PMSO or GST in *M. domestica* (Shono & Scott 2003) in another study. The previous report suggested that the increased activity of PMSO is related to spinosad resistance in *Bemisia tabaci* (Kang et al. 2006; Reyes et al. 2007) and *Spodoptera exigua* (Wang et al. 2006). According to the previous literature, one assumes that PMSO could be the main detoxification enzyme for spinosad in *L. botrana* neonate larval stage.

Different detoxification mechanisms were detected for *C. pomonella*. For example, increased EST activity was observed against azinphos-methyl in Argentina while increased GST (Reyes et al. 2007) activity in Chile (Fuentes-Contreras et al. 2007) and Europe (Reyes & Sauphanor 2008). Such differences in detoxification mechanisms could be related to the genetic background of insects in different locations and countries (Pashley 1983). It is obvious that genetic studies should also be incorporated in such studies to better understand the detoxification mechanisms of the target insects. Variations in previous reports may be related to different substrate affinity-binding capacities. Reduced EST activity could also be related to a reduced affinity for the non-specific naphthyl acetate (β) substrate and increased affinity for the insecticide substrate (Bush et al. 1993; Reyes et al. 2007). Binding affinity could also be different based on the substrate used during the experiment (Sole et al. 2018). Such issues can be an important factor in determining EST detoxification and potentially be minimized using another substrate e.g., p-nitrophenyl acetate. This could be a possible reason why our detoxification enzyme results are different than previous reports (Hatipoglu et al. 2015; Navarro-Roldan et al. 2020). Limited research has been conducted on *L. botrana* detoxification enzyme levels and only neonate larvae and adult stages were used in the previous reports (Hatipoglu et al. 2015; Navarro-Roldan et al. 2020). No correlation was found in EST and GST activity levels among developmental stages (neonate larvae, diapausing larvae, and adult) in *C. pomonella* (Reyes & Sauphanor 2008). However, one study indicated that using neonate larvae provided more consistent results when the field population was considered (Rodriguez et al. 2011a). This implies that mortality bioassay and enzyme activity measurements need optimization and standardization in future research. A high number of individuals (neonate larvae) were used during the bioassay and enzymatic activity (EST and GST) experiments; thus, we were unable to rear enough individuals to further evaluate PMSO activity levels in our study. Previous reports indicated that such issues could potentially happen and could be minimized by reducing the number of neonate larvae used for the experiment to obtain more detailed data on detoxification enzyme levels (Rodriguez et al. 2011a; Bosch et al. 2018).

4. Conclusions

Monitoring the efficacy of insecticide from the field population has a great impact on future insecticide use for achieving efficient pest control. Metabolic resistance is an irreversible process that could potentially limit insecticide use once it occurs. Applicators and growers should train properly to create better insecticide application programs that focus on prioritization and understanding of the potential risks of insecticide resistance. Using insecticide in proper order and efficiently could help growers to provide insecticide use for a longer-term. This could also give researchers the necessary time to discover and register new insecticides for efficient pest control. In addition, there is a need for standardization in bioassays and enzymatic analyses for *L. botrana* in order to provide comparable results between different experiments from various locations. Lastly, more research is needed to

monitor insecticide toxicity and measuring detoxification enzyme levels for effective *L. botrana* control in grape production areas.

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Abbreviations

AL	Alasehir population
CDNB	1-chloro-2,4-dinitrobenzene
EST	Carboxylesterase
GST	Glutathione S-transferases
IPM	Integrated pest management
IRAC	Insecticide resistance action committee
LC_{50}	Lethal concentration that kills 50% of the population
PMSO	Poly substrate monooxygenases
SAR	Sarigol population

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Combining Ability and Gene Action Controlling Chocolate Spot Resistance and Yield Traits in Faba Bean (*Vicia faba* L.)

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ABSTRACT
Chocolate spot disease devastatingly impacts faba bean growth and productivity. Thenceforth, genetic study of chocolate spot resistance and yield traits is crucial to conceive appropriate strategies for breeding and sustaining faba bean production particularly under abrupt climate change and a fast-growing global population. The current study was performed to identify promising resistant and high-yielding progenies, to study the mode of inheritance for chocolate spot resistance and yield traits using half-diallel mating design, and to investigate the association between seed yield and its attributes traits under conditions of chocolate spot disease. Two resistant (Nubaria-1 and Sakha-1) and two susceptible (Tribe-White and Camolina) parents previously characterized were used to generate six F_1 hybrids which were selfed to produce F_2 progenies. The parents and their F_1 and F_2 were evaluated at hot-spot location for chocolate spot disease. Significant variation was detected for chocolate spot resistance and yield traits among the evaluated parents and their cross combinations in both generations. The general (GCA) and specific (SCA) combining ability effects were highly significant for chocolate spot severity and yield traits in both generations. From the results, it is noteworthy that the cross combinations of P_1(Nubaria-1)×P_2(Sakha-1), P_1×P_4(Camolina) and P_2×P_3(Tribe-white) displayed the highest seed yield per plant in the F_1 generation (155.66, 199.96, and 147.96 g respectively) as well as the F_2 generation (172.36, 123.06, and 119.80 g respectively) simultaneously with high resistance to chocolate spot. Consequently, these crosses could be promising combinations for increasing seed yield, and resistance to chocolate spot in breeding programs of faba bean. The additive gene effect was predominant for chocolate spot resistance, plant height, days to flowering, number of branches per plant and 100-seed weight in both generations. Accordingly, selection could be effective to improve these traits in early generations. By contrast, the non-additive gene effects were preponderant for seed yield per plant, number of seeds per plant and number of pods per plant. This suggests the importance of transgressive segregation for improving these traits through breeding programs. While selection for improving these traits could be less effective in the segregated generations which should be postponed to advanced generations. A strong positive association was identified between seed yield per plant and each of number of branches per plant, 100-seed weight, plant height, days to flowering, number of pods per plant and number of seeds per plant. This signifies their significance as vital attributes for indirect selection, especially in the early generations due to their ease of evaluation in comparison with seed yield. On the contrary, linear regression analysis revealed a steeply inverse relationship between seed yield and chocolate spot disease in both generations.

Keywords: *Vicia faba*, Chocolate spot, Heterosis, Heritability, Cluster analysis, Principal components, Linear regression

1. Introduction

Faba bean (*Vicia faba* L.) is a globally essential pulse crop (Karkanis et al. 2018; Desoky et al. 2021). It is an important source of protein, dietary fibers, carbohydrates, and micronutrients for humans as well as livestock. Besides, it improves soil properties and contributes to sustainable agriculture through atmospheric nitrogen fixation (van Berkum et al. 1995; Duc 1997; Youseif et al. 2017). The gap between consumption and faba bean production is increasing due to population growth, particularly in developing countries. Accordingly, productivity and the cultivated area should be increased to limit this gap (Zohry & Ouda 2017; Mansour et al. 2021a).

Chocolate spot disease that caused by *Botrytis fabae* threatens faba bean production worldwide, through damaging its foliage and constraining photosynthetic activity (Duc 1997; Beyene et al. 2016). Severe epidemics can destructively reduce faba bean yield by over 60% (Deneke 2018). Furthermore, infected plants produce seeds with reddish-brown discoloration which reduces their market value (Kaur et al. 2018). Subsequently, developing resistant and high-yielding genotypes is a crucial goal for improving faba bean productivity alongside agricultural practices particularly under the current fast-growing global population.

The success of plant breeding programs depends principally on a better understanding genetic basis of the important economic traits (Gracia et al. 2012; Abaza et al. 2020; Kamara et al. 2021). Therefore, it is required to provide sufficient information on the inheritance nature and heritability of the important characters. Diallel mating system is a powerful biometric approach to assess the effects of general combining ability (GCA) and specific combining ability (SCA) as well as assess the gene action involved in different traits in faba bean. It also aids in the knowledge of heterotic patterns of progeny at the earlier stage of crossing programs (Şimşek & Ceyhan 2017; Salem et al. 2020; Kamara et al. 2021).

Seed yield is the most important trait, but it is a complex trait for genetic improvement (Shi et al. 2009; Ceyhan & Şimşek 2021). In this context, several yield attributes as number of seeds, number of pods, and seed weight could be exploited as indirect indices for improving seed yield (Desoky et al. 2020; Mansour et al. 2021). Consequently, it is essential to determine the interrelationship among seed yield and its attributes under chocolate spot disease conditions.

The present study aimed at identifying promising hybrids that could be exploited for improving chocolate spot resistance and productivity in faba bean, determining the type of gene action controlling chocolate spot resistance and yield traits in faba bean, and investigating the association between seed yield and its attributes traits under conditions of chocolate spot disease.

2. Material and Methods

2.1. Parental genotypes and crossing

Four diverse faba bean genotypes were chosen based on their resistance to chocolate spot, genetic diversity, growth habit, and yielding ability from preliminary trials. The selected parents included two cultivars resistant to chocolate spot (Nubaria-1and Sakha-1) and two susceptible genotypes (Tribe-White and Camolina) obtained from the national gene bank and genetic resources of Egypt. The description of used parents is presented in Table 1. During the growing season of 2016-2017, the selected parents were grown at Giza Agricultural Research Station, Agricultural Research Center (30° 01' N, 31° 12' E). Half-diallel mating design excluding reciprocal (4×4) was applied to generate 6 F1 hybrids. During the next growing season of 2017-2018, the F1 hybrids were selfed to produce F2 seeds and simultaneously the parents were crossed again to secure adequate F1 seeds.

Table 1- Description of the used parental faba bean genotypes

Genotype	*Origin*	*Pedigree*	*Botanical group*	*Reaction to Botrytis fabae*
Nubaria-1	Egypt	Single plant selection form Giza Blanka	Large	Resistant
Sakha-1	Egypt	716/724/83 × 620/ 283/8	Medium	Resistant
Tribe-White	Sudan	Individual selection	Medium	Susceptible
Camolina	Spain	Imported from Spain	Small	Susceptible

2.2. Experimental sites and agronomic practices

The parental genotypes, F1 and their corresponding F2 progenies were evaluated during the growing season of 2018-2019 at Nubaria Agricultural Research Station, Agricultural Research Center, Egypt (30° 49' N, 30° 01' E). The experimental site soil is classified as sandy loam soil throughout the profile (72.11% sand, 8.52% silt 19.37% clay) with pH 8.31 and electrical conductivity 1.95 dS/m. Nubaria is considered a hot-spot for chocolate spot disease in Egypt. Randomized Complete Block Design in three replications was applied for each generation. Each plot comprised of six rows with 3-m long and 0.60-m distance between rows and 0.2-m among plants with two seeds in each hill. The sowing date was carried out in the optimum time for faba bean growing in the region which is the end of October. Potassium and phosphorus were added before sowing at a rate of 95 kg K/ha as potassium sulfate (48% K_2SO_4) and 32 kg P/ha as superphosphate (15.5% P_2O_5). Nitrogen fertilizer was added once at sowing as a basal dose with a rate of 45 kg N/ha as ammonium sulfates (21% N). Surface irrigation was applied as the commonly used system in the region.

2.3. Measured traits

The scale outlined by Bernier et al. (1993) was used to score the degrees of chocolate spot disease (Table 2). Days to flowering was determined as number of days from planting to 50 percent of the plants in each plot have open flowers. Plant height (cm) was recorded from the soil surface to the top of the branch at physiological maturity in each plot from readings of ten plants. Likewise, number of pods number per plant was estimated in each plot and 100-seed weight was recorded from the weight of two sets of 100 seeds.

Table 2- Used scale for chocolate spot disease (Botrytis fabae)

Scale	*Description*	*Grade*
1	No disease symptom or very small specks	Highly resistance
3	Few small discrete lesions	Resistance
5	Some coalesced lesions with some defoliation	Moderately resistance
7	Large coalesced sporulating lesions, some dead plants, 50% defoliation	Susceptible
9	Extensive lesions leaves, severe defoliation, stems and pods, stem girding, heavy sporulating, blackening and death of more than 80% of plant	Highly susceptible

2.4. Statistical analysis

The analysis of variance (ANOVA) was applied for all recorded data using R statistical software version 3.6.1. Combining ability analysis was performed according to Griffing's method 2 model 1 (Griffing, 1956). The genetic components and related genetic parameters were estimated as suggested by Hayman (1954). Heritability values in the narrow and broad sense were determined according to Mather & Jinks (1971). The hierarchical cluster analysis was employed to group the assessed genotypes and the principal component analysis (PCA) was utilized to assess their relationships among the evaluated traits utilizing R statistical software version 3.6.1.

3. Results and Discussion

3.1. Analysis of variance

The analysis of variance disclosed highly significant differences among evaluated genotypes in both generations for all assessed traits (Table 3). Besides, the GCA and SCA effects were highly significant for all studied traits except number of pods per plant in F2. These findings indicate wide genetic variability among used parents and their progenies for all evaluated variables. In the light of that, similarly high genetic variability for yield traits and resistance to chocolate spot was disclosed in faba bean by Rhaïem et al. (2002); Abo-Hegazy et al. (2012); Beyene et al. (2016); Tekalign et al. (2017); Beyene et al. (2018) and Lee et al. (2020). They depicted genetic variations are valuable for developing new genotypes with high-yielding and resistance to chocolate spot.

Table 3-Mean squares of ANOVA and combining ability effects for evaluated traits for F1 and F2 populations

Source of Variance	*df*	*Chocolate spot*	*Days to flowering*	*Plant height*	*No. of branches/ plant*	*No. of pods/ plant*	*No. of seeds/ plant*	*100-seed weight*	*Seed yield/ plant*
F1 generation									
Genotype	9	6.71**	143.2**	369.1**	12.67**	2152**	14374**	2971**	6865**
GCA	3	16.41**	285.6**	908.4**	28.77**	1497**	8709**	8223**	10248**
SCA	6	1.86*	72.00**	99.51	4.62**	2479**	17205**	345.0**	5172**
Parent (P)	3	15.66**	351.4**	537.9**	34.21**	33.62	1228	4493**	11338**
Cross (C)	5	1.48*	40.2*	269.3**	1.97**	2460**	15914**	2653**	2292*
P vs C	1	5.98**	33.37	361.8*	1.55	6966**	46109**	0.12	16310**
Replication	2	0.18	20.25	9.32	0.65	107.1	639.7	65.78	77.69
Error	18	0.47	9.64	51.84	0.36	139.7	580.8	45.82	609.5
Total	29	2.39	51.82	147.4	4.20	761.9	4865	955.1	2514
F2 generation									
Genotype	9	7.52**	136.6**	565.0**	13.82**	512.0*	3022**	2851**	6070**
GCA	3	21.75**	370.5**	1321**	32.79**	509.4	1869	7761**	13548**
SCA	6	0. 93	19.59	187.1*	4.35**	513.3	3597**	395.4**	2331**
Parent (P)	3	15.66**	351.4**	537.9**	34.21**	33.62	1228	4493**	11338**
Cross (C)	5	4.13**	31.04*	643.6**	3.93**	762.9*	4016**	2398**	4122**
P vs C	1	0.07	19.93	253.5	2.11	693.1	3435*	191.2	4.61
Replication	2	0.95	9.47	44.32	0.23	26.14	642.2	52.16	238.6
Error	18	0.35	8.83	58.62	0.70	249.0	748.2	62.09	302.1
Total	29	2.62	48.53	214.8	4.75	315.3	1446	926. 9	2088

* and **: indicate significant at P≤0.05 and P≤0.01, respectively

3.2. Performance of used parents and their F1 and F2 progenies

The evaluated parents and their F1 and F2 progenies displayed a wide significant variation for chocolate spot resistance and agronomic traits (Table 4). The parental genotypes Nubaria-1 and Sakha-1 recorded the lowest severity of chocolate spot compared with Camolina and Tribe-white (Table 4). In addition, Nubaria-1 exhibited the latest flowering and highest values of

plant height, number of seeds per plant, number of branches per plant, 100-seed weight, and seed yield per plant. While Camolina displayed earliest flowering and produced the highest number of pods per plant. On the contrary, Tribe-white recorded the lowest values of seed yield per plant and all attributed traits. In the F1 generation, the hybrid P1×P2 followed by "P1×P4" and "P2×P3" displayed the lowest severity of chocolate spot. Besides, the hybrids "P1×P2" and "P3×P4" recorded the earliest flowering followed by "P2×P4" and "P1×P4". Furthermore, the hybrids "P1×P4" and "P2×P4" followed by "P1×P2" and "P2×P3" possessed the highest seed yield per plant and its components. On the contrary, "P3×P4" and "P2×P4" recorded the highest severity of chocolate spot. Besides, "P1×P3" and "P3×P4" recorded the lowest seed yield per plant and its attributed traits. Concerning F2 progenies, the cross "P1×P2" followed by "P2×P3" and "P1×P4" recorded the lowest severity of chocolate spot and displayed superiority for seed yield per plant as well as its attributes. On the contrary, "P3×P4", "P2×P4" and "P1×P3" displayed the highest susceptible values for chocolate spot and lowest seed yield per plant and its attributes.

Table 4- Mean performance for chocolate spot resistance and yield traits of the evaluated parents, F1 and F2 progenies

Genotypes	*Chocolate spot*	*Days to flowering*	*Plant height (cm)*	*No. of branches/ plant*	*No. of pods/ plant*	*No. of seeds/ plant*	*100-seed weight (g)*	*Seed yield/ plant*
F1 generation								
Nubaria-1 (P1)	1.30	63.66	130.80	10.80	44.50	152.33	129.30	187.16
Sakha-1 (P2)	1.87	42.93	112.20	4.50	42.33	131.73	100.36	132.70
Tribe-white (P3)	5.63	42.56	102.63	3.10	38.73	104.03	55.20	56.73
Camolina (P4)	5.40	40.80	102.17	5.10	46.56	137.80	47.30	64.93
P1×P2	1.86	41.90	125.80	6.53	40.73	119.00	137.20	155.66
P1×P3	2.73	51.83	127.47	5.63	44.33	132.13	93.26	129.00
P1×P4	2.20	44.96	126.10	7.56	82.46	250.86	78.10	199.96
P2×P3	2.30	46.63	119.13	5.90	67.46	205.20	71.83	147.96
P2×P4	2.86	44.63	111.90	6.96	98.50	295.83	62.53	181.20
P3×P4	3.86	42.06	103.83	5.53	111.36	265.96	54.53	134.06
LSD 0.05	1.18	3.33	5.35	1.03	9.27	35.34	7.61	30.35
F2 generation								
P1×P2	1.73	50.20	131.66	6.90	47.40	147.30	132.13	172.36
P1×P3	3.53	47.00	122.20	4.20	25.46	83.13	76.40	62.60
P1×P4	3.53	48.63	132.43	6.56	53.86	175.73	76.40	123.06
P2×P3	3.40	42.93	116.63	4.46	55.86	173.13	70.46	119.80
P2×P4	4.50	42.86	111.06	4.60	73.06	158.90	60.03	95.76
P3×P4	5.20	43.33	93.30	5.36	61.40	181.70	51.90	93.50
LSD 0.05	1.01	4.10	6.13	1.44	8.07	32.92	6.52	29.82

The severity of chocolate spot and yield traits were utilized to classify the evaluated genotypes and their cross combinations according to their performance. Using hierarchical clustering the parental genotypes and their crosses were classified into three groups (Figures 1A and 1B). The genotypes included in group (a) displayed the highest severity of chocolate spot; thereupon, they could be considered susceptible genotypes. While the genotypes in group (c) had the lowest values; hence, they could be considered resistant genotypes. In respect of yield traits, the genotypes in group (a) exhibited the highest yield traits; consequently, they could be considered high-yielding genotypes compared to the genotypes in groups b and c. Likewise, Mansour et al. (2020); Gharib et al. (2021); Moustafa et al. (2021a); Mansour et al. (2021b); employed hierarchical clustering to classify the genotypes based on their performance.

From the results of F1 and F2 generations, it is noteworthy that the cross combinations of "P1×P2", "P1×P4" and "P2×P3" displayed constant performance through both generations with high-yielding and resistance to chocolate spot. Consequently, these crosses could be promising combinations for increasing seed yield, and resistance to chocolate spot in faba bean breeding programs. Correspondingly, Beyene et al. (2016) and Tekalign et al. (2017) identified promising cross combinations in faba bean for enhancing resistance to chocolate spot and seed yield attributes.

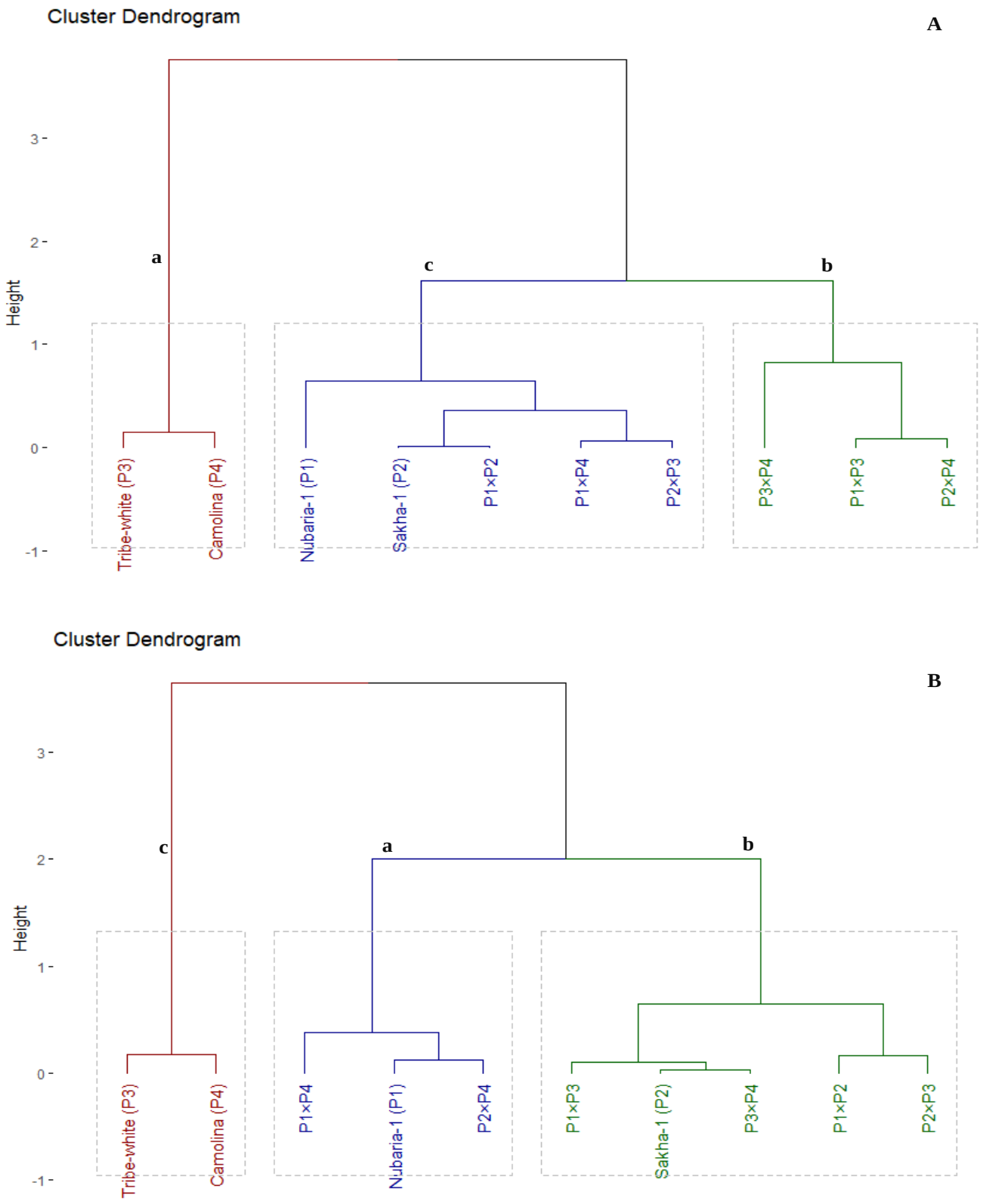

Figure 1- Dendrogram of the phenotypic distances among four faba bean parental genotypes and their six cross combinations based on their performance in chocolate spot disease (A) and seed yield traits (B).

3.3. General (GCA) and specific (GCA) combining ability effects

The GCA effects of parental genotypes revealed that Nubaria-1 and Sakha-1 expressed highly significant negative GCA for chocolate spot severity which indicates that they could be considered as good combiners for resistance of this disease (Table 5). Likewise, Sakha-1 and Camolina displayed highly significant negative GCA effects (desirable) for days to flowering which indicates that they could be considered as good combiners for earliness. Moreover, Nubaria-1 is a good combiner for plant height,

100-seed weight, number of branches per plant, and seed yield per plant. Similarly, Sakha-1 showed a positive and significant effect for 100-seed weight and seed yield per plant. Furthermore, Camolina had positive GCA for number of pods per plant and number of seeds per plant. Generally, the parental genotypes Nubaria-1 and Sakha-1 had highly significant desirable GCA effects for most assessed traits while Camolina for certain yield attributes. Subsequently, the valuable alleles of these genotypes could be considered good sources for improving yield traits and resistance to chocolate spot in faba bean breeding programs.

Table 5- General combining ability effects of the used parents for all evaluated traits

Parent	*Chocolate spot*	*Days to Flowering*	*Plant height*	*No. of branches/ plant*	*No. of pods/ plant*	*No. of Seeds/ plant*	*100-seed /plant*	*Seed yield/ plant*
Nubaria-1 (P1)	-0.94**	5.84**	9.99**	1.75**	-8.66**	-15.13**	25.39**	27.38**
Sakha-1 (P2)	-0.71**	-1.99**	0.04	-0.41**	-2.85	-2.33	9.58**	9.26*
Tribe-white (P3)	0.86**	-0.89	-4.22*	-1.25**	-1.31	-14.35**	-14.13**	-28.37**
Camolina (P4)	0.79**	-2.96**	-5.81**	-0.09	12.83**	31.80**	-20.84**	-8.27
LSD 0.05	0.29	1.33	3.08	0.25	5.06	6.33	2.90	5.58
LSD 0.01	0.403	1.82	4.23	0.35	6.94	9.16	3.97	7.50

* and **: indicate significant at P≤0.05 and P≤0.01, respectively

The estimates of SCA effects in F1 generation displayed significant negative effects for chocolate spot disease (desirable for resistance) by "P1×P2", "P1×P4" and "P2×P3" (Table 6). Additionally, the cross combinations "P1×P2", "P1×P4", "P2×P4" and "P3×P4" displayed significant negative SCA effects for days to flowering (desirable for earliness). Moreover, positive and significant SCA effects were assigned for "P1×P2", "P1×P4", "P2×P3" and "P2×P4" for seed yield per plant and certain of its attributes. In F2 generation, significant negative effects for chocolate spot disease were displayed by "P1×P2" and "P2×P3". Furthermore, positive, and significant SCA effects were recorded by "P1×P2", and "P2×P3" for seed yield per plant and certain of its components. These cross combinations were derived from parental genotypes with good×good or good×poor general combiners. It could be concluded that the three cross combinations of "P1×P2" and "P2×P3" possessed constant behavior in both generations, accordingly, they are promising for resistance to chocolate spot as well as seed yield and most of its attributes in breeding programs of faba bean. These findings are in consonance with previous studies that elucidated significant GCA and SCA effects for resistance to chocolate spot as well as seed yield and its components in faba bean as Alghamdi (2009); Ghareeb & Helal (2014) and Beyene et al. (2016) and El-Hosary (2020) and in bean as reported by Ceyhan et al. (2014), Ceyhan & Şimşek (2021) and Kepildek & Ceyhan (2021).

Table 6- Specific combining ability effects of F1 and F2 progenies for studied traits

Cross	*Chocolate Spot*	*Days to flowering*	*Plant height*	*No. of branches/plant*	*No. of pods/plant*	*No. of seeds/plant*	*100-seed weight*	*Seed yield/plant*
F1 generation								
P1×P2	-0.51*	-8.40**	0.43	-0.98**	-9.45*	-43.03**	19.26**	19.91*
P1×P3	-0.19	0.68	5.49*	-1.03**	-7.42	-17.88	0.96	-8.95
P1×P4	-0.65*	-4.12**	5.71*	0.26	16.60**	54.70**	-9.41**	41.92**
P2×P3	-0.85**	3.32**	7.11*	1.39**	9.94*	42.38**	-6.58*	28.14**
P2×P4	0.21	-3.38**	-1.47	1.29**	26.83**	86.87**	-9.17**	41.27**
P3×P4	0.78**	-4.29**	-2.34	-0.70**	38.15**	69.02**	-6.54*	-31.76**
F2 generation								
P1×P2	-0.31*	1.31	3.40	0.09	-0.56	-1.41	17.62**	15.33*
P1×P3	0.14	3.93**	1.66	-2.02**	-14.58*	-46.62**	12.42**	-50.45**
P1×P4	0.03	1.97	11.27**	0.73*	3.61	21.66*	8.04*	1.21
P2×P3	-0.32*	-0.55	6.78*	-0.63	8.28	38.05**	-4.99	19.93**
P2×P4	0.67**	-0.81	-0.59	-0.32	15.27*	0.50	-11.05**	-12.91
P3×P4	0.30	-1.86	-9.45**	1.23**	10.40	38.43**	-6.52*	-18.81**

* and **: indicate significant at P≤0.05 and P≤0.01, respectively

3.4. Components of genetic variation and heritability

The additive component (D) was significant for chocolate spot disease, plant height, days to flowering, number of branches per plant, 100-seed weight and seed yield per plant in F1 and F2 generations (Table 7). These results disclosed that the additive effect was involved in the inheritance of these characters in both generations. Moreover, the dominance components H1 and H2 were significant for all traits in F1 except for chocolate spot disease and 100-seed weight while for chocolate spot disease in F2. The obtained results revealed the importance of both additive and dominant components in the expression of the evaluated traits. While the magnitude of additive component (D) was higher than the dominance component (H1) for all evaluated traits except number of seeds per plant, number of pods per plant, and seed yield per plant in F1 which was confirmed by the average degree of dominance (H1/D)0.5 which was higher than unity for these traits. This indicates that the non-additive gene effects were

preponderant for the aforementioned traits while the additive gene effect is more important in controlling chocolate spot resistance, plant height, days to flowering, number of branches per plant and 100-seed weight. This suggests the importance of transgressive segregation for improving seed yield per plant, number of seeds per plant, and number of pods per plant through the breeding program. On the other hand, selection for these traits could be less efficient in the segregated generations which should be postponed to advanced generations. While, selection could be more effective for chocolate spot resistance, plant height, days to flowering, 100-seed weight and number of branches per plant in segregated generations. This was also confirmed by the heritability values in the narrow and broad sense. These findings concur with Ceyhan et al. (2008); Farag & Afiah (2012); Obiadalla-Ali et al. (2013); Ceyhan et al. (2014); Ghareeb & Helal (2014) and El-Hosary (2020) who disclosed that the non-additive gene action was crucial in the expression and regulating seed yield, number of pods per plant and number of seeds per plant.

Table 7- Components of genetic variance for evaluated traits

Genetic component	*Chocolate spot*	*Days to flowering*	*Plant height*	*No. of branches/plant*	*No. of pods/plant*	*No. of seeds/plant*	*100-seed weight*	*Seed yield/plant*
F1 generation								
D	5.07*	113.6**	163.4**	11.27**	-34.26	213.84	1482*	3594**
H1	1.72	92.07*	68.25**	6.38**	2829**	20090**	435.1	5410**
H2	1.47	70.56*	64.09**	4.39*	2198**	15748**	398.6	4494**
F	2.43	92.49*	-46.94	8.75**	-92.40	475.3	-481.1	2740**
E	0.15	3.57	15.86**	0.13	45.47	195. 6	15.94	185.5*
(H1/D)0.5	0.58	0.90	0.63	0.75	5.34	9.69	0.54	1.23
h 2 (ns)	0.74	0.50	0.76	0.65	0.37	0.33	0.90	0.40
H2 (bs)	0.92	0.92	0.88	0.96	0.95	0.97	0.99	0.92
F2 generation								
D	5.08**	114.2**	160.2**	11.18**	-64.36	163.5	1477**	3681**
H1	0.21	19.49*	192.3*	5.46*	500.9**	4133*	468.6*	2982*
H2	0.19	13.68*	166.9*	4.35*	349.4**	3550*	453.6*	2667*
F	0.50	54.08**	-167.2	6.77**	-102.4	215.9	-344.6	1297
E	0.14	2.96*	19.06	0.22	75.57*	245.9	20.37	98.58
(H1/D)0.5	0.20	0.41	1.10	0.70	2.78	5.03	0.56	0.90
h 2 (ns)	0.92	0.84	0.74	0.68	0.37	0.19	0.87	0.64
H2 (bs)	0.94	0.92	0.92	0.95	0.71	0.82	0.98	0.95

* and ** indicate significant at $P \leq 0.05$ and $P \leq 0.01$, respectively

3.5. Interrelationship among traits

Adequate understanding of the association between seed yield and its attributes is essential for identifying selection criteria that could be employed to improve faba bean productivity through breeding programs. Principal components (PCs) analysis is an efficient tool to assess the relationship among traits (Moustafa et al. 2021b; El-Sanatawy et al. 2021). The first two PCs showed most of the variability, about 86.31% (59.97% and 26.34% by PC1 and PC2, in the same order). Consequently, the two PCs were used to construct the PC-biplot (Figure 2). In the PC-biplot, the characters are represented by close vectors to each other signify robust positive relationships while those that were situated almost inverse (at 180°) demonstrate an extremely negative relationship. Consequently, a strong positive association was proven between seed yield per plant and each of 100-seed weight, number of branches per plant, days to flowering, plant height, number of seeds per plant and number of pods per plant. On the contrary, seed yield and all its attributes had a strong negative association with chocolate spot severity. The detected strong association between seed yield and these traits suggests their significance as vital attributes for indirect selection, especially in the early generations due to their ease of evaluation in comparison with seed yield. Similarly, a strong positive relationship between seed yield and its attributes was proved by Alan and Geren (2007); Abo-Hegazy et al. (2012); Abo-Mostafa et al. (2014); Sharifi (2014); Tekalign et al. (2017) and Elshafei et al. (2019).

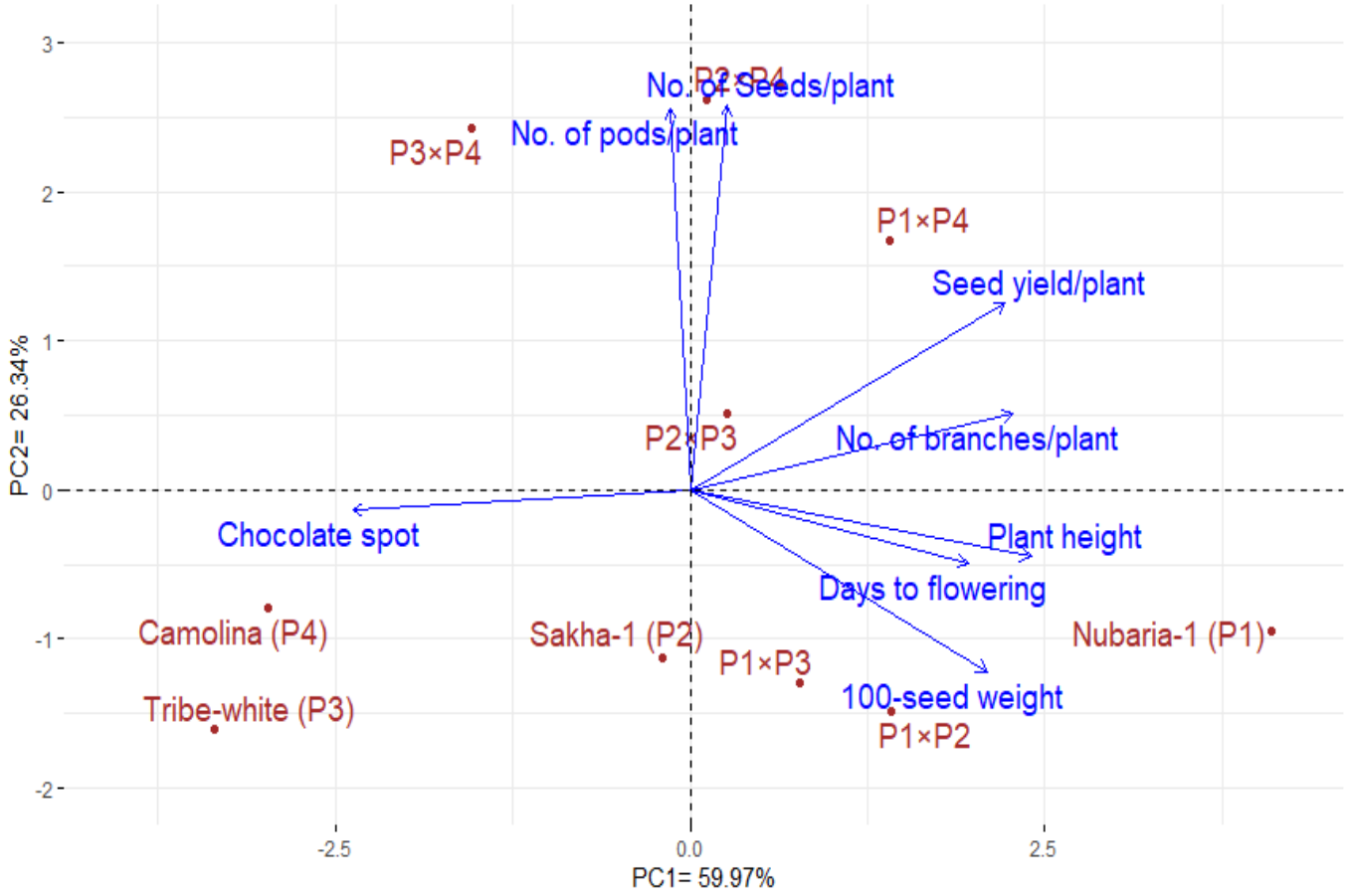

Figure 2- Biplot of PCA for the evaluated parental genotypes and their cross combinations to explore the association among the studied traits

3.6. Seed yield response to chocolate spot severity

The response of seed yield of faba bean to chocolate spot severity was determined in the early generations F1 and F2 (Fig. 3). The seed yield per plant as a dependent variable was regressed upon the incidence of chocolate spot through linear regression analysis. It was found a strong linear inverse relationship between seed yield and chocolate spot severity with a high negative slope in both generations. Hence, seed yield in both generations decreased steeply with increasing chocolate spot severity. The regression equations in both generations could be useful to predict yield loss and response to chocolate spot severity. There was yield loss of 25.56 and 21.65 g per plant in response to increasing each unit of chocolate spot disease in both generations F1 and F2, respectively. In this context, Sahar et al. (2011); Tolessa et al. (2015); Haile et al. (2016); Kora et al. (2017) demonstrated highly negative regression between seed yield and chocolate spot severity in faba bean.

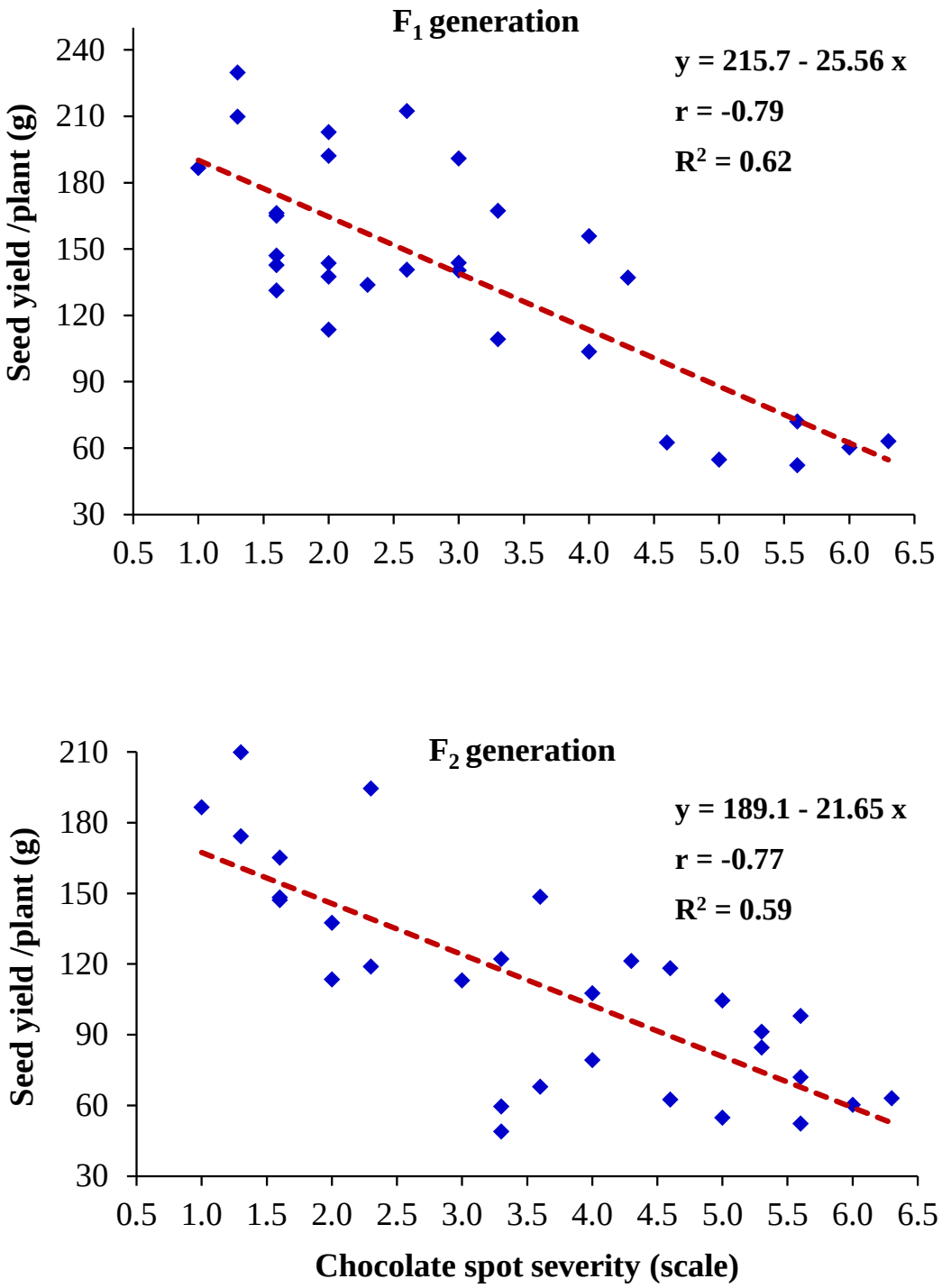

Figure 3- Response of seed yield of faba bean to chocolate spot disease in the F1 and F2 generations

4. Conclusions

Considerably genetic variations were identified among parental genotypes and their F1 and F2 progenies for chocolate spot resistance and assessed yield traits. The cross combinations of "P1(Nubaria-1)×P2(Sakha-1)", "P1(Nubaria-1)×P4(Camolina)" and "P2(Sakha-1)×P3(Tribe-white)" possessed constant performance through both generations with high-yielding and resistance to chocolate spot. Accordingly, these crosses are proposed to be exploited in faba bean breeding as valuable sources of high-yielding and resistance to chocolate spot disease. The additive gene effect was predominant for chocolate spot resistance, plant height, days to flowering, number of branches per plant and 100-seed weight in both generations. The predominance of additive effect for these traits indicates that selection could be efficient for improving them in early generations. On the other hand, the non-additive gene effects were preponderant for seed yield per plant, number of pods per plant and number of seeds per plant. This implies the significance of transgressive segregation and developing hybrids in enhancing these traits through breeding programs. Strong positive association was detected between seed yield per plant and each of number of branches per plant, 100-seed weight, plant height, days to flowering, number of pods per plant and number of seeds per plant. This reflects their importance for indirect selection, especially in the early generations due to their ease of evaluation in comparison with seed yield.

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Determination of Pipe Diameters for Pressurized Irrigation Systems Using Linear Programming and Artificial Neural Networks

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ABSTRACT
Pressurized irrigation systems are widespread among other alternatives in Mediterranean countries. Since the initial investment costs of pressurized irrigation systems are quite high, it is crucial to determine design parameters such as pipe diameter. Most of the current optimization techniques for pipe diameter selection are based on linear, non-linear, and dynamic programming models. The ultimate aim of these techniques is to produce solutions to problems with less cost and computation time. In this study, a novel approach for determining pipe diameter was proposed using Artificial Neural Networks (ANN) as an alternative to existing models. For this purpose, three pressurized irrigation systems were investigated. Different ANN architectures were created and tested using hydrant level parameters of the irrigation systems, such as irrigated area per hydrant, hydrant discharge, pipe length, and hydrant elevation. Different training algorithms, transfer functions, and hidden neuron numbers were tried to determine the best ANN model for each irrigation system. Using multilayer feed-forward ANN architecture, the highest coefficients of determination were found to be 0.97, 0.93, and 0.83 for irrigation systems investigated. It was concluded that pipe diameters could be determined by using artificial neural networks in the planning of pressurized irrigation systems.

Keywords: Machine learning, Optimization techniques, Irrigation water management, Network performance analysis, Hydraulic parameters

1. Introduction

Limited water resources impose people for modernizing irrigation systems to provide equal rights to all users and to save water. One of the main reasons for not using water effectively in irrigation networks is that water losses are very high in the systems. In light of this fact, initially, water-saving measures should be taken thoroughly in agriculture. Firstly, it is imperative to install water transmission and distribution systems that will minimize water losses. Therefore, pressurized irrigation systems instead of open channel systems must be established in new irrigation projects.

The design of pressurized irrigation systems is a complicated, and time-consuming process. For this reason, many hydraulic parameters and factors must be considered. This process consists of mainly five stages: (1) optimization of network layout to reduce total cost of network, (2) hydrant discharge calculation considering plot sizes (3) determination of design flow each pipeline, (4) calculation of the optimum pipe size diameters to minimize the investment and energy costs, (5) network performance analysis for different operating conditions to specify the potential supply failure situations of the network or of the pumping plant (Alandi et al. 2007).

The initial investment costs of pressurized irrigation systems are relatively high. A reason for this might be that the majority of the expenses are vastly spent on pipe costs. Since the size of the pipes is directly related to the pipe prices, the selection of the optimum pipe diameter is significant in terms of both project cost and system performance when designing the network.

With advances in computer technology, optimization techniques have been used by many researchers to solve complex equations and the design of hydraulic parameters efficiently. The classical optimization techniques such as linear programming (LP), non-linear programming, and dynamic programming have been commonly used for many years. Especially in large and complicated network systems, classical optimization techniques can be time-consuming to find an optimal solution. Therefore, metaheuristic optimization techniques have been proposed, such as Genetic algorithms, simulated annealing, tabu search, ant-colony optimization, and harmony search (Schaake & Lai 1969; Alperovits & Shamir 1977; Lansey & Mays 1989; Simpson et al. 1994; Cunha & Sousa 1999; Geem et al. 2002; Maier et al. 2003; Cunha & Ribiero 2004).

Artificial Neural Network (ANN) is a machine learning method gaining popularity for solving complex problems in recent years. Unlike many machine learning methods, the core idea behind ANN is to reveal the hidden interactions between features (variables) that consist of the data. An ANN learns by using numerical values that can be observed or experienced previously. Accordingly, they can predict the data that the ANN model has not seen before with its hidden network structure. This approach allows the ANN to simulate the biological nervous system. An input layer of the ANN contains neurons corresponding to the number of features in training data. Target output values are represented by one neuron in an output layer. Hidden layers are located between the input and output layers. Neurons collect information that reaches them via transfer functions and transmit it to the subsequent neurons they are connected to (Omid et al. 2009). ANNs can reveal potential and hidden correlations between the variables that make up the data. Contrary to conventional models, an ANN model can produce satisfactory results even if there are several miscalculated neuron weights. In addition to these advantages, ANN models also can learn using the outputs of multiple traditional models looking for a solution to the same problem, thereby producing better predictions by combining the solving power of different models. ANN-based approaches were reported for possible solutions to various issues in non-agricultural water networks. One of the problems that were tried to be solved with ANN was to detect bursts and leaks in urban water networks (Mounce & Machell 2006; Arsene et al. 2012). Besides, some efforts were reported for non-agricultural purpose water networks such as assessment of the water flow rate and pressure losses (Czapczuk & Dawidowicz 2018; Dawidowicz et al. 2018), pipe failure detection (Shirzad & Safari 2019), and infrastructure aging risk assessment (Cantos & Juran 2019). As of preparing this paper, there was only one study accomplished by Dawidowicz (2018) investigating to determine pipe diameters based on ANN for urban water networks to the best of the authors' knowledge. However, the literature was in lack of optimization techniques in determining pipe diameters for agricultural irrigation networks using ANN.

The aim of present study was to investigate the possibilities of determining the pipe diameter, which is an important system design parameter in pressure irrigation systems using ANN as an alternative to the currently used models. For this purpose, three different pressurized irrigation systems operated with the on-demand method were selected, and different ANN architectures were created and tested with the hydrant level parameters of the system such as irrigated area, hydrant discharge, pipe length, and hydrant elevation above sea level.

2. Material and Methods

2.1. Study areas

In this study, three different sized pressurized irrigation system data were used to re-estimate pipe diameters with ANN: (1) Gulluce-Dolluk; (2) Devecikonagi; and (3) Yolcati. The Gulluce-Dolluk and Devecikonagi pressurized irrigation systems are located within the Mustafakemalpasa district's borders in the Marmara Region, 90 km from Bursa city center. These systems supply irrigation water from Devecikonagi Dam (Figures 1 and 2). The Devecikonagi pressurized irrigation system's irrigation water is taken from the Devecikonagi Dam via the transmission line and pumped to a water collection pool at the highest point (103.6 m) of the irrigation area by using a pump. A pressurized pipe irrigation system then delivers water to the hydrants from the water collection pool. The Yolcati (Gobelye) pressurized irrigation network is located in the Marmara Region, 20 km from Bursa city center, within the boundaries of Bursa Uludag University Campus in Turkey (Figure 3). The Yolcati Pond is used for irrigation purposes of Bursa Uludag University, within the Nilufer District of the central Bursa. Irrigation water is conveyed to hydrants with a piped irrigation system by pumping from the sluice gate to the reservoir located on the highest point of irrigation area by two electro pumps. Then it is distributed to the irrigated area from the reservoir with the help of gravity. The properties of three different pressurized irrigation systems used in this study are shown in Table 1.

Table1-The properties of pressurized irrigation systems used in this study.

Study areas / ***System parameters***	***Gulluce-Dolluk***	***Devecikonagi***	***Yolcati***
Coordinate	40° 10' N, 28° 23' E	39° 54' N, 28° 34' E	40°02′ N, 28°23′ E
Irrigated area (ha)	5820	360	125
Discharge (l s^{-1})	5035	528	217
Upstream elevation (m)	77	103.6	140
Total hydrant number	741	63	54
Pipe material	HDPE (High-density polyethylene)	HDPE	HDPE

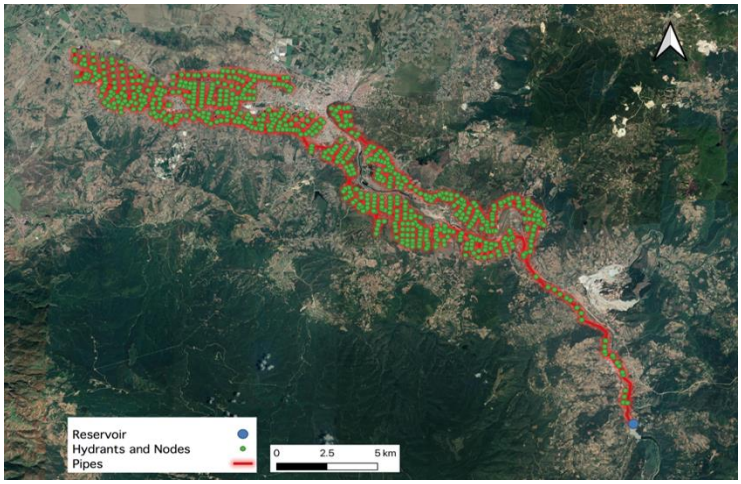

Figure 1- The layout of Gulluce-Dolluk pressurized irrigation network

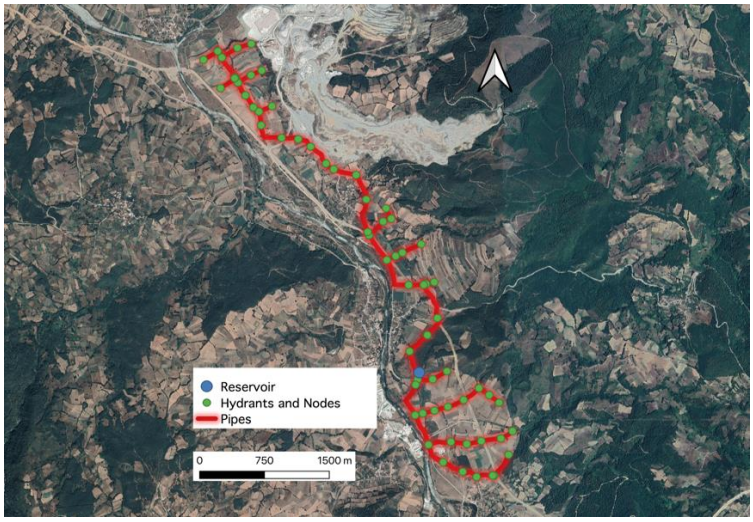

Figure 2- The layout of Devecikonagi pressurized irrigation network

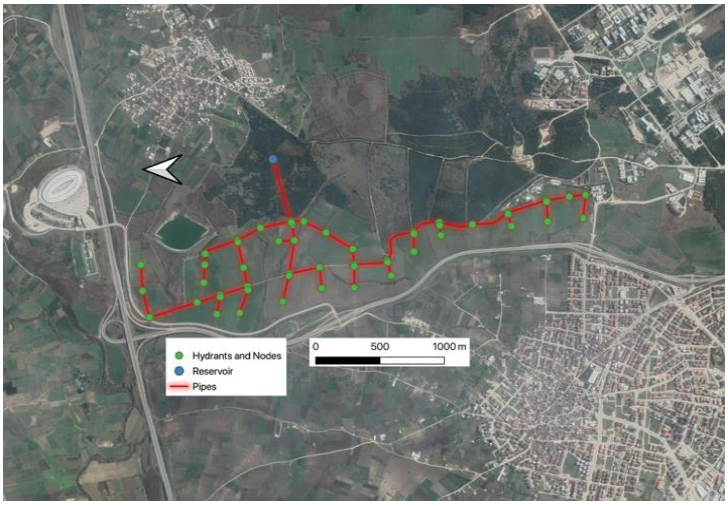

Figure 3- The layout of Yolcati pressurized irrigation network

2.2. Software

The use of ANN requires the preparation of data with an appropriate number of training examples. For this purpose, hydraulic data was collected from three different pressurized irrigation systems, and hydraulic calculations were made to create the training data for ANN. For this reason, COPAM (Combined Optimization and Performance Analysis Model) software, by

Lamaddalena & Sagardoy (2000), was applied. The ANN models were implemented and tested with Matlab (2020a, The MathWorks, Inc., Natick, Massachusetts) software.

2.3. Hydraulic calculations

Three different configurations are available with COPAM; calculation of discharge, calculation of pipe diameter, and analysis. There are two modules (Clément and random) in the structure of discharge calculation, a module (optimization) under the structure of pipe diameter calculation, and two modules (configurations and hydrants) under the analysis structure. In COPAM, pipe diameters are determined using an optimization process called Labye's iterative discontinuous method (ELIDM), which is an extension for several flow regimes models (SFR) (Labye 1981; Ait-Kadi et al. 1990). Further details about COPAM software can be found in Lamaddalena (1997), Lamaddalena & Sagardoy (2000) and Calejo et al. (2008).

COPAM has a software module for calculating an irrigation network's optimum pipe diameter under several different flow configurations and single flow regime conditions. ELIDM, used by COPAM, implements linear programming methods to cover several different flow regimes.

The ELIDM model uses the Darcy equation to calculate the pipes' friction coefficient (Eq. 1).

$$Y = 0.000857\ (1 + 2\gamma D^{-0.5})^2\ Q^2 D^{-5}\ L = u\ Q^2\ L \quad (1)$$

Where; γ, roughness parameter of Bazin (expressed by m0.5); Q, pipe discharge ($m^3\ s^{-1}$); u, dimensional coefficient of resistance ($m^{-1}\ s^2$); L, the length of pipe (m). Bazin's roughness coefficient was taken as 0.05 for HDPE pipes used (Lamaddalena & Sagardoy 2000). AKLA model was used for each hydrant's reliability analysis according to a minimum pressure head Hmin of 25 m.

2.4. Artificial neural network (ANN)

In this study, multi-layered feed-forward artificial neural network structures were employed to determine the pipe diameters of pressurized irrigation systems. While designing the prediction models, one hidden layer was used along with an input and an output layer since a hidden layer is enough to solve many complex problems. The use of more than one hidden layer is required in rare cases. However, this situation would cause the network to learn excessively and negatively affect the ability to generalize (Wang & Paliwal 2006; Nazghelichi et al. 2011). Different training algorithms can be used to update neuron weights in ANNs. The training algorithm used may affect the performance of ANN (Beale et al. 2014). Therefore, in this study, four different training algorithms were employed to determine the best ANN architecture that provides the highest prediction success in irrigation networks tested. The training algorithms used in the experiments are shown in Table 2 (Garg & Bansal 2015; Pakalapati et al. 2019).

Table 2- The training algorithms used in the experiments

Training algorithm	***Abbreviation***
Bayesian regularization backpropagation	Trainbr
Levenberg-Marquardt	Trainlm
Resilient backpropagation	Trainrp
Scaled conjugate gradient	Trainscg

The number of neurons in the hidden layer plays a crucial role in the creation of ANN models. There is no generally accepted rule for determining the number for ANNs. However, in a few studies, empirical methods were established to determine the number of neurons (Heaton 2015; Priddy & Keller 2005). In this study, while creating ANN architectures, the numbers of neurons up to 40, with five intervals starting from five, were tried. Their performances in predicting the diameter of network pipes were investigated. Another factor that affects ANN performances is the transfer function. While the linear transfer function was used in the output layer of the ANNs created, the tangent-sigmoid and logarithmic-sigmoid transfer functions were employed separately in the hidden layer. Table 3 shows the ANN architectures employed in the present study. The equations of these transfer functions are given in Eq. 2, 3, and 4 (Lertworasirikul & Tipsuwan 2008).

$$\text{logsig}(x) = \frac{1}{(1+e^{-x})} \quad (2)$$

$$\text{tansig}(x) = \frac{2}{(1+e^{-2x})} - 1 \quad (3)$$

$$\text{purelin}(x) = x \quad (4)$$

Table 3- The ANN architectures employed in the present study

	Training algorithm																															
	Trainbr								*Trainlm*								*Trainrp*								*Trainscg*							
	Number of neurons																															
	5	10	15	20	25	30	35	40	5	10	15	20	25	30	35	40	5	10	15	20	25	30	35	40	5	10	15	20	25	30	35	40
Transfer function	Tangent-sigmoid																															
	Logarithmic-sigmoid																															

The hydraulic data of Gulluce-Dolluk, Devecikonagi and Yolcati irrigation networks were used in the training and testing of the ANNs. The variables used in the training of the ANNs were start point, end point, irrigated area (ha), hydrant discharge (l s^{-1}), pipe length (m), and hydrant elevation (m). Assuming that each variable used affects determining a pipe diameter, the ANN models were expected to use hidden correlations between variables in deciding a proper diameter of a pipeline. Figure 4 shows a representative model of the ANN scheme concept in the study. There were 741 hydrants and nodes data in the Gulluce-Dolluk irrigation system, 63 in the Devecikonagi irrigation system, and 54 in the Yolcati irrigation system. For each irrigation system, there were 3 data sets, namely, training set (50% of total data), validation set (25%), and test set (25%) (Sigtia & Dixon 2014; Ucar et al. 2020). Those data sets were constructed randomly before the experiments. During the networks' training, the training and validation sets were used to update the neuron weights. The prediction performances of the trained ANN were evaluated using the test data set that the network had never seen during the training phase. Since the variable value ranges were quite different for each variable, before training, all the variables were normalized in the range of 0 to 1 to increase the prediction success. Matlab initializes the neurons' weight values randomly at the beginning of the training of any network. This causes each training to yield a different prediction model, even if the number of hidden layers and other network parameters remain unchanged. To cope with this variation, a constant random state was used to give all ANN models experimented equal chance.

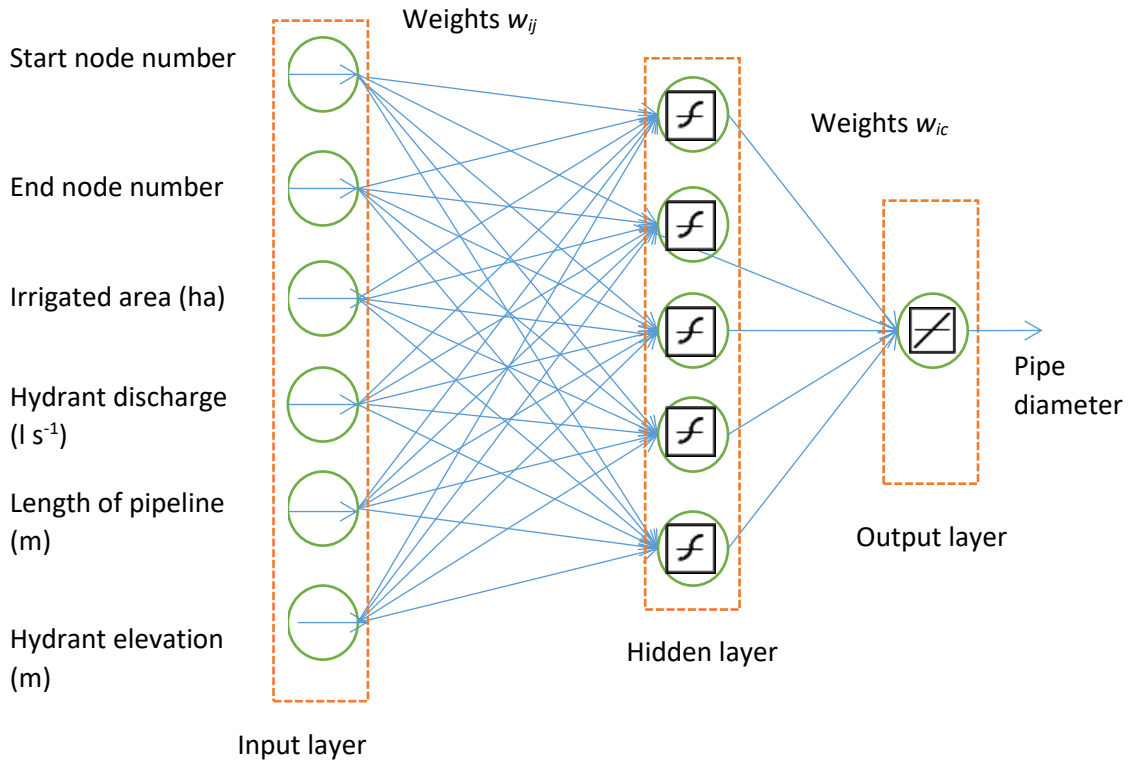

Figure 4- A hidden layered feed-forward ANN model

In the ANN models' training, mean square error (MSE) was used as a performance function. The error goal and the maximum number of epochs were set to 0.001 and 1000, respectively. In the experiments, to prevent overfitting, a training process stopped when the error goal was reached, the course of the validation error remained constant, or the validation error did not decrease over five iterations. The best ANN models were determined in predicting pipe diameters based on the highest determination coefficient (R^2), the lowest root mean square error (RMSE), and the mean absolute percentage error (MAPE) values for the test data set.

3. Results and Discussion

In the experiments, three real-world irrigation networks were investigated. A total of 64 different ANN architectures were employed for each irrigation network using eight different neuron numbers, four training algorithms, and two activation functions. Thus, the most successful ANN models were determined for each irrigation network.

3.1. The gulluce-dolluk pressurized irrigation system

A total of 741 available network sections were used for training and testing the ANN models based on the Gulluce-Dolluk irrigation network. Table 4 shows prediction successes on 185 test samples of ANN models tested with different model parameters. According to the results, the best prediction performances were observed using the ANN models with the trainbr algorithm and 15 neurons. Both transfer functions yielded very similar performance metrics. The R^2 value was obtained as 0.97 for both transfer functions providing very close error metrics. The lowest RMSE and MAPE values were also obtained for those highest R^2 scores. The MAPE values were found between 20% and 50% for these models. According to Moreno et al. (2013), these scores are interpreted as a reasonable prediction. Figure 5a shows the best performed ANN model's training record in predicting pipe diameters of the Gulluce-Dolluk irrigation network. A smooth decrease in training and testing error was obtained during the training of this ANN model. This model yielded the best training performance at the training iteration 216. The error histogram of this ANN model was shown in Figure 5b. As a useful performance indicator, it was observed that most of the error distributed close to zero error line in the error space. For this ANN model, Figure 5c shows a plot representing the linear regression of target pipe diameters relative to predicted ones on the test data set. The regression plot also supported that this ANN model was very accurate. For the experiments related to Gulluce-Dolluk, the second-best performance was obtained from the trainlm algorithm with an R^2 value of 0.96. It was also inferred from the experiments that high prediction performances were provided by the ANN models having 15 neurons in their hidden layers. The learning algorithm trainrp was an exception in this regard since the highest R^2 (0.92) was observed using 30 neurons in the hidden layer.

Table 4- Performance results of the ANN models in predicting the pipe diameters of Gulluce-Dolluk

Training algorithm	*N. of neurons in hidden layer*	*R*	*R^2*	*RMSE*	*MAPE*	*R*	*R^2*	*RMSE*	*MAPE*
		Transfer function Tangent-sigmoid				Transfer function Logarithmic-sigmoid			
Trainbr	5	0.97	0.94	113.29	0.30	0.96	0.92	126.24	0.33
	10	0.95	0.91	149.56	0.36	0.97	0.93	125.66	0.31
	15	**0.98**	**0.97**	**91.04**	**0.28**	**0.98**	**0.97**	**90.98**	**0.27**
	20	0.95	0.90	160.87	0.29	0.96	0.92	145.00	0.32
	25	0.79	0.61	483.21	0.35	0.83	0.69	398.53	0.30
	30	0.86	0.75	350.53	0.29	0.96	0.92	167.45	0.29
	35	0.97	0.94	127.80	0.32	0.94	0.89	169.64	0.35
	40	0.97	0.94	128.96	0.31	0.94	0.88	174.68	0.40
Trainlm	5	0.97	0.93	117.05	0.33	0.96	0.93	118.67	0.32
	10	0.93	0.87	211.84	0.34	0.94	0.89	173.27	0.33
	15	**0.98**	**0.96**	**93.74**	**0.29**	**0.98**	**0.96**	**100.25**	**0.30**
	20	0.96	0.92	144.44	0.37	0.96	0.92	144.32	0.36
	25	0.95	0.90	159.82	0.36	0.91	0.83	217.70	0.36
	30	0.96	0.92	151.46	0.37	0.97	0.94	137.59	0.30
	35	0.96	0.93	133.41	0.33	0.97	0.94	124.25	0.30
	40	0.96	0.92	141.57	0.36	0.96	0.93	135.77	0.38
Trainrp	5	0.90	0.81	196.61	0.60	0.89	0.80	203.41	0.58
	10	0.91	0.83	209.71	0.45	0.93	0.87	172.94	0.45
	15	0.93	0.86	181.27	0.47	0.93	0.87	174.90	0.47
	20	0.92	0.85	193.05	0.53	0.87	0.76	257.72	0.53
	25	0.89	0.80	230.00	0.49	0.94	0.89	170.74	0.47
	30	0.95	0.89	181.05	0.49	0.96	0.92	153.96	0.43
	35	0.94	0.88	176.18	0.43	0.95	0.90	159.74	0.37
	40	0.93	0.86	177.02	0.53	0.93	0.85	185.25	0.51
Trainscg	5	0.82	0.67	258.66	0.70	0.82	0.68	255.63	0.69
	10	0.86	0.74	286.98	0.51	0.88	0.75	267.07	0.50
	15	0.95	0.90	152.24	0.41	0.74	0.54	334.43	0.91
	20	0.89	0.79	234.14	0.63	0.84	0.70	291.20	0.66
	25	0.90	0.80	232.73	0.48	0.92	0.85	201.13	0.52
	30	0.87	0.76	268.12	0.52	0.93	0.87	198.64	0.53
	35	0.77	0.60	318.31	0.68	0.84	0.71	269.05	0.58
	40	0.89	0.79	220.76	0.60	0.81	0.66	280.07	0.70

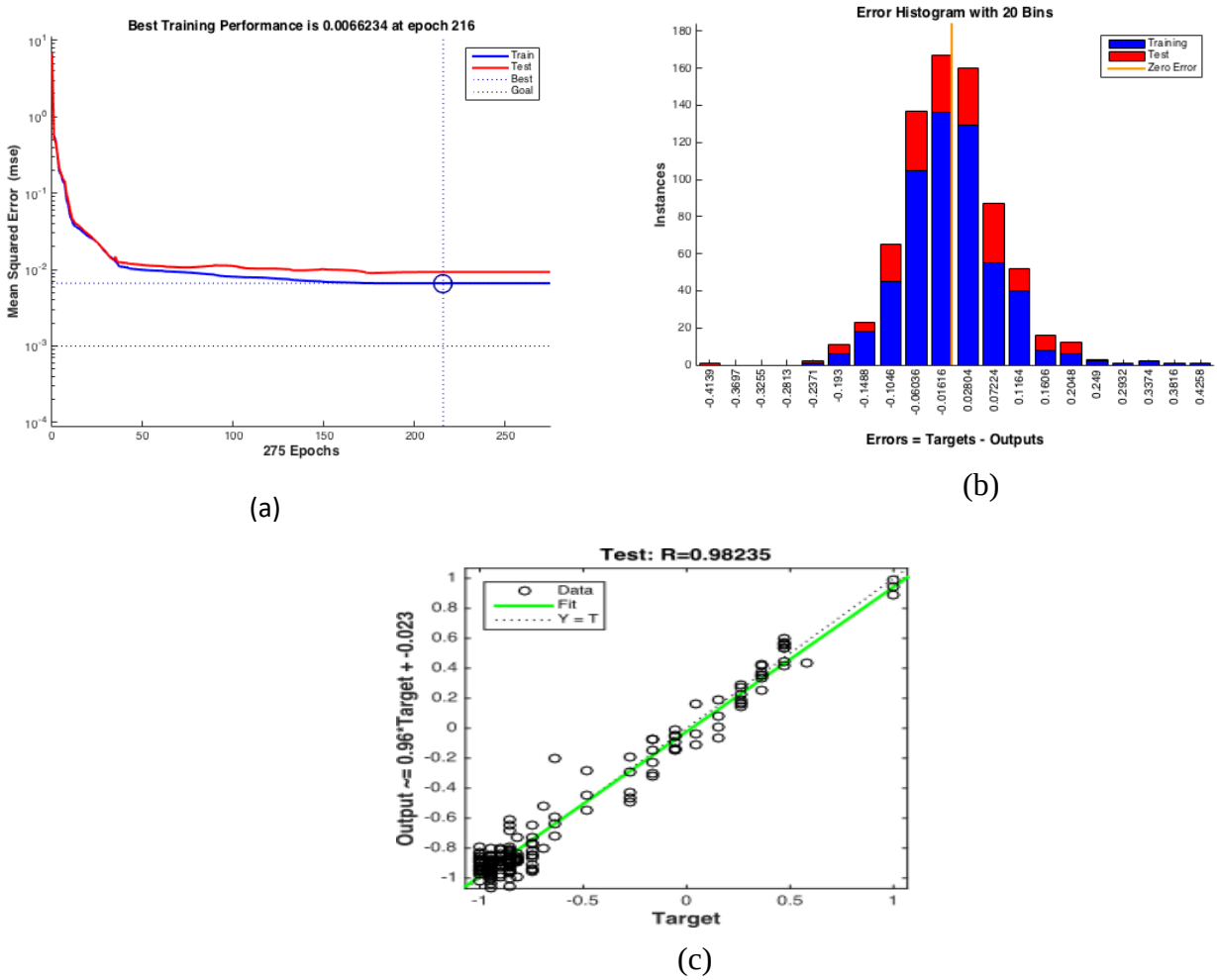

Figure 5- Training record (a), error histogram (b), and regression plot (c) related to the best ANN model performed for Gulluce-Dolluk irrigation network

3.2. The devecikonagi pressurized irrigation system

The Devecikonagi was another irrigation network studied in this study. Using 63 available sections, different ANN models were trained and tested. For this irrigation network, only 16 test sections were available. In the real-world, there are many irrigation networks with such a small number of sections. Furthermore, it was an important task to reveal the performance of neural network models predicting pipe diameters with fewer sections or pipelines. The performance results of the experiments related to this irrigation network were given in Table 5. The highest R^2 value (0.93) was obtained using the trainscg algorithm, 20 neurons, and the tangent-sigmoid transfer function. The second-highest performance metric (R^2= 0.89) for this experiment group was obtained with the trainbr algorithm using the same number of neurons. The MAPE values, obtained between 0.1-0.2, showed that these models performed good predictions (Moreno et al. 2013). Figure 6a shows that the best training performance was reached at epoch 20. This experiment's error histogram showed that the error is mainly distributed between -0.9528 and 0.5842 (Figure 6b). In Figure 6c, the regression plot related to the best-performed model also shows that model fits well with a slight shift from the perfect fit.

Table 5- Performance results of the ANN models in predicting the pipe diameters of Devecikonagi

Training algorithm	*N. of neurons in hidden layer*	*R*	*R^2*	*RMSE*	*MAPE*	*R*	*R^2*	*RMSE*	*MAPE*
		Transfer function Tangent-sigmoid				Transfer function Logarithmic-sigmoid			
Trainbr	5	0.56	0.32	103.13	0.35	0.56	0.32	96.23	0.33
	10	0.88	0.78	74.01	0.25	0.88	0.77	74.27	0.25
	15	0.78	0.61	104.88	0.37	0.79	0.62	98.31	0.35
	20	**0.94**	**0.89**	**53.59**	**0.18**	0.94	0.89	54.15	0.18
	25	0.49	0.24	143.09	0.28	0.62	0.38	121.13	0.25
	30	0.73	0.53	107.74	0.27	0.75	0.57	103.82	0.29
	35	0.83	0.68	70.27	0.20	0.87	0.76	70.67	0.22
	40	0.69	0.48	104.91	0.26	0.62	0.38	118.26	0.26
Trainlm	5	0.67	0.44	63.27	0.23	0.82	0.67	50.05	0.19
	10	0.80	0.64	112.10	0.43	0.93	0.86	71.00	0.18
	15	0.36	0.13	164.84	0.59	0.68	0.46	124.63	0.48
	20	0.76	0.58	107.57	0.32	0.91	0.82	66.68	0.23
	25	0.53	0.28	144.30	0.31	0.68	0.47	115.25	0.33
	30	0.57	0.32	162.36	0.56	0.50	0.25	142.86	0.54
	35	0.75	0.56	123.30	0.34	0.78	0.62	92.28	0.22
	40	0.56	0.32	124.62	0.30	0.63	0.40	113.22	0.28
Trainrp	5	0.34	0.12	108.55	0.40	0.55	0.31	86.98	0.36
	10	0.83	0.68	89.65	0.29	0.88	0.77	76.58	0.29
	15	0.74	0.54	105.40	0.42	0.79	0.63	127.83	0.53
	20	0.93	0.87	60.77	0.20	0.90	0.81	75.31	0.24
	25	0.73	0.53	114.89	0.27	0.65	0.42	119.72	0.25
	30	0.63	0.40	141.71	0.42	0.80	0.64	122.78	0.47
	35	0.67	0.45	97.68	0.25	0.40	0.16	152.49	0.42
	40	0.29	0.08	174.50	0.49	0.70	0.49	102.45	0.31
Trainscg	5	0.65	0.42	64.28	0.24	0.63	0.39	67.06	0.24
	10	0.74	0.54	109.67	0.42	0.86	0.75	88.13	0.32
	15	0.80	0.64	110.49	0.44	0.89	0.79	115.20	0.52
	20	**0.96**	**0.93**	**48.29**	**0.17**	0.85	0.73	90.95	0.33
	25	0.69	0.48	129.06	0.27	0.74	0.55	116.60	0.25
	30	0.55	0.30	135.84	0.42	0.60	0.37	123.31	0.45
	35	0.71	0.51	131.53	0.36	0.36	0.13	124.20	0.32
	40	0.71	0.51	104.70	0.22	0.70	0.50	103.66	0.31

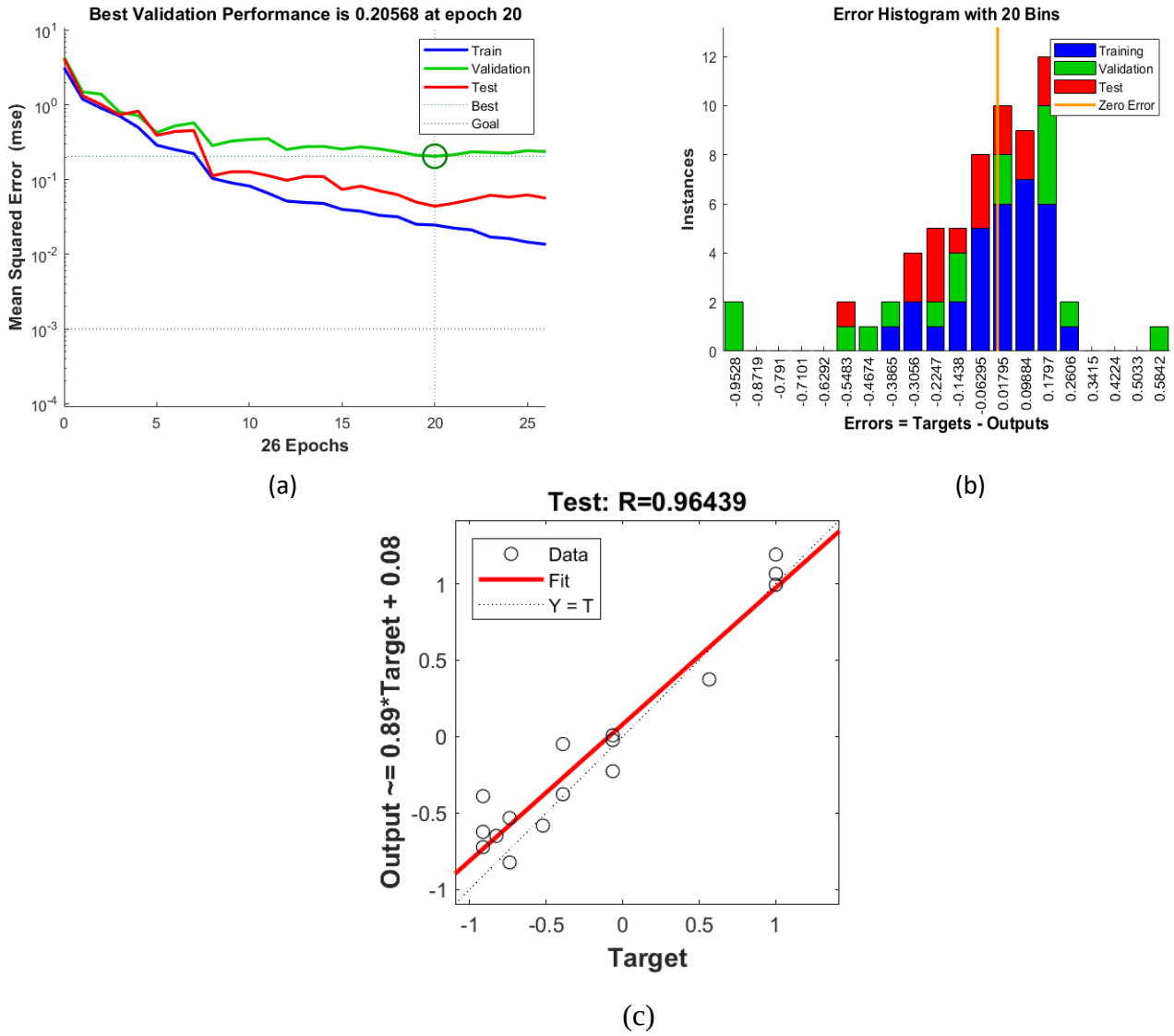

Figure 6- Training record (a), error histogram (b), and regression plot (c) related to the best ANN model performed for Devecikonagi

3.3. The yolcati pressurized irrigation system

This network has a few numbers (54) of pipeline sections. According to the experiments, the best prediction result (R^2= 0.83) was obtained using the training algorithm of trainbr (Table 6). The trainbr algorithm was responsible for the second-best performance metric (0.73). Apart from these, the rest of the training algorithms yielded R^2 values below 0.6 with this irrigation network. For this experiment group, the most successful predictions were observed with 10 neurons and the tangent-sigmoid transfer function. The best training performance was reached at epoch 20 for the model with the highest R^2 (Figure 7a). This experiment's error histogram showed that the prediction errors were distributed in a wide range (Figure 7b), which was an unwanted and a relatively low-performance result. The regression plot related to the best-performing model also shows that the model fits not as good as the other networks investigated in this research (Figure 7c).

Table 6- Performance results of the ANN models in predicting the pipe diameters of Yolcati

Training algorithm	*N. of neurons in hidden layer*	*R*	*R^2*	*RMSE*	*MAPE*	*R*	*R^2*	*RMSE*	*MAPE*
		Transfer function Tangent-sigmoid				Transfer function Logarithmic-sigmoid			
Trainbr	5	0.73	0.53	59.03	0.24	0.73	0.53	58.99	0.24
	10	**0.85**	**0.73**	**70.90**	**0.24**	0.85	0.72	72.48	0.25
	15	0.51	0.26	84.48	0.27	0.51	0.26	84.40	0.27
	20	0.80	0.64	64.20	0.25	0.80	0.64	64.17	0.25
	25	0.47	0.22	77.34	0.30	0.50	0.25	75.14	0.30
	30	0.45	0.20	67.76	0.30	0.45	0.20	67.82	0.30
	35	0.63	0.40	65.06	0.32	0.27	0.08	87.54	0.41
	40	0.72	0.52	57.14	0.23	0.72	0.52	57.17	0.23
Trainlm	5	0.66	0.44	72.45	0.31	0.67	0.45	59.79	0.30
	10	0.68	0.47	171.92	0.79	0.66	0.44	115.85	0.39
	15	0.44	0.20	95.23	0.26	0.28	0.08	123.69	0.35
	20	-0.59	0.35	158.97	0.74	0.44	0.19	94.09	0.39
	25	0.67	0.45	65.69	0.26	0.61	0.37	69.59	0.29
	30	0.14	0.02	142.67	0.73	0.29	0.08	104.87	0.46
	35	0.05	0.00	139.25	0.67	0.19	0.04	109.00	0.56
	40	0.48	0.23	111.29	0.49	0.53	0.28	89.46	0.44
Trainrp	5	0.75	0.56	62.26	0.24	0.76	0.58	55.17	0.22
	10	**0.91**	**0.83**	**75.43**	**0.26**	0.84	0.71	86.12	0.27
	15	0.38	0.14	100.81	0.26	0.25	0.06	115.44	0.34
	20	0.14	0.02	131.55	0.61	0.58	0.33	87.19	0.40
	25	0.35	0.12	108.10	0.35	0.64	0.41	68.07	0.33
	30	0.04	0.00	106.35	0.50	0.31	0.10	77.35	0.41
	35	0.21	0.04	154.05	0.61	0.35	0.12	87.20	0.37
	40	0.51	0.26	147.12	0.58	0.67	0.44	98.99	0.48
Trainscg	5	0.72	0.52	56.66	0.26	0.72	0.52	57.50	0.26
	10	0.73	0.54	96.99	0.36	0.67	0.44	98.58	0.34
	15	0.31	0.09	104.12	0.33	0.29	0.09	110.23	0.37
	20	0.55	0.31	94.52	0.49	0.63	0.39	85.72	0.37
	25	0.53	0.28	83.55	0.30	0.47	0.22	86.67	0.35
	30	-0.11	0.01	138.88	0.74	0.04	0.00	90.13	0.38
	35	0.43	0.18	127.75	0.61	0.50	0.25	90.42	0.48
	40	0.56	0.31	99.76	0.47	0.57	0.33	72.11	0.36

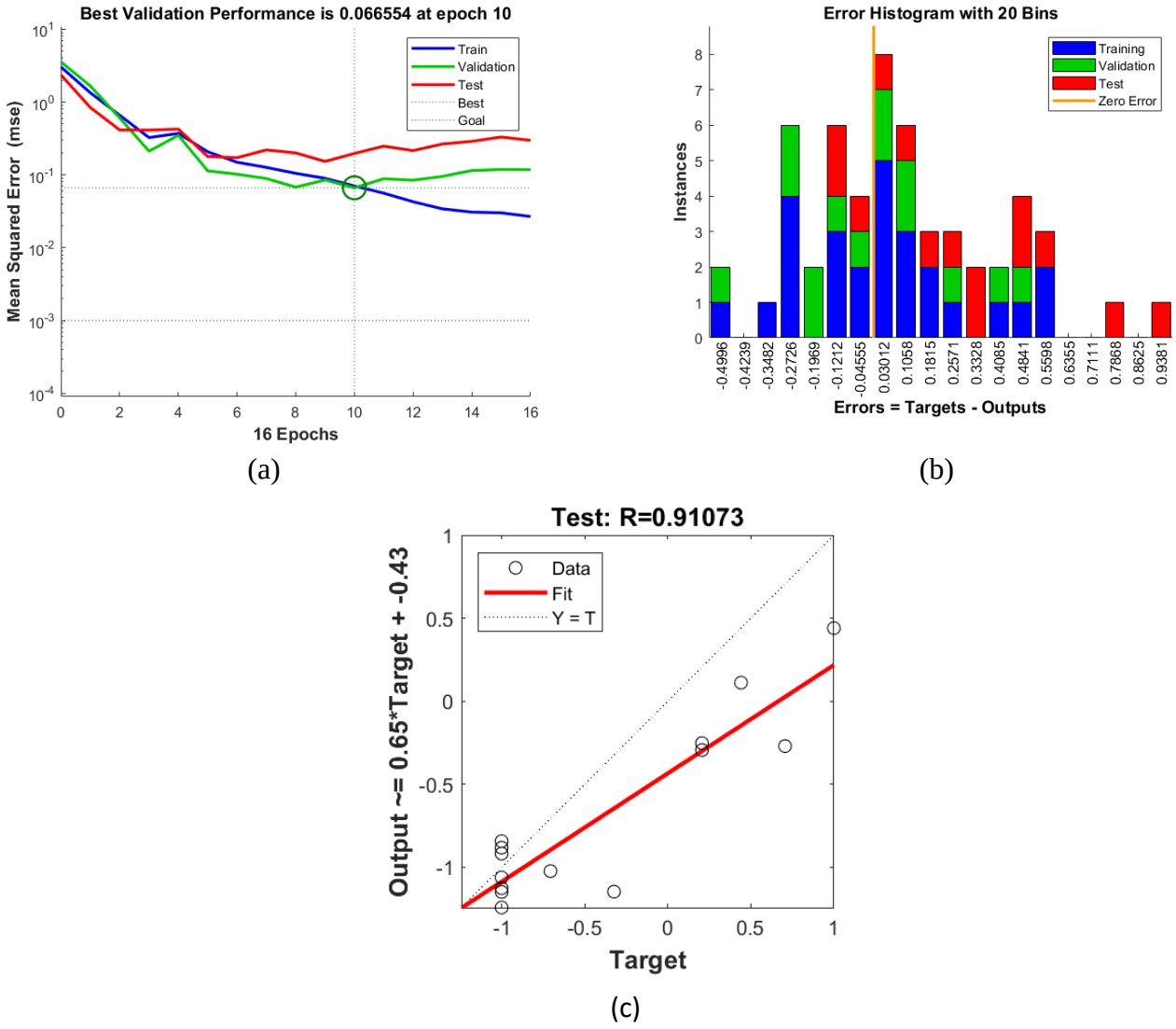

Figure 7- Training record (a), error histogram (b), and regression plot (c) related to the best ANN model performed for Yolcati

The ANN models yielded decimal numbers in the experiments, as seen in Table 7, illustrating the target and predicted pipe diameters. The pipe sections in the table were chosen randomly from the test data sets. The predicted pipe diameters were standardized as commercially available integer values, pointing out the proposed method's potential usage. The last row of Table 6 shows the pipe diameters obtained from the Network Optimization Program (NOP) used by The Republic of Turkey, the General Directorate of State Hydraulic Works (SHW). The NOP is an out-of-date program operated on MS-DOS, and it has some drawbacks, such as it is not possible to add all the pipes in production (Wang & Dal, 2017). When comparing the ANN results and the NOP results, an R^2 value of 0.95 was obtained (RMSE = 143.85, MAPE = 0.16) with a correlation coefficient (R) of 0.98. Although the results of the NOP should not be considered as a reference (ground-truth) for model verification, these findings were remarkable because statistically similar values were obtained using the ANN model and a classical method.

Table 7- Target and predicted values for pipe diameters

		Pipe Diameter (mm)			
Study Area	*Pipe section number*	*Hydraulic data*	*Predicted (ANN)*	*Predicted (standardized)*	*NOP*
Gulluce-Dolluk	50	1400	1407.95	1400	1900
	244	160	156.88	160	200
	575	1000	998.54	1000	1000
	621	450	450.40	450	500
	709	160	216.18	200	225
Devecikonagi	4	600	598.30	600	600
	15	355	371.83	355	350
	27	250	235.85	225	250
	53	355	364.51	355	355
	56	280	283.16	280	280
Yolcati	2	315	237.21	250	450
	24	110	84.83	90	160
	28	160	106.16	110	160
	35	355	299.01	315	400
	54	110	88.72	90	110

In this study, pressurized irrigation systems of different sizes were investigated to predict pipe diameters using ANN. In the Yolcati irrigation, using the least numbers of hydrants, pipe diameter prediction success was lowest with a R^2 value of 0.83. In the Gulluce-Dolluk irrigation having 741 hydrants, the pipe diameter prediction was done with the highest R^2 value of 0.97. Thus, it can be concluded that as the number of data increases in the training data set, pipe diameter estimation success also increases. Although it may not be fair to make a one-to-one comparison with the related papers employing ANN to optimize or analyze water networks, some points are worth discussing. While the approach developed in this study was to take the problem as a regression task, Dawidowicz (2018) addressed the problem as a classification task to determine the pipe diameters for urban water networks. Sadly, a prediction score was not reported in the researcher's study. However, a confusion matrix was available to compute the overall accuracy of the prediction model. Using a relatively large database of 36 urban water networks, the prediction model's overall accuracy could be computed as 0.998 based on the confusion matrix reported. In this paper, relatively high prediction scores were also obtained. Unfortunately, pressurized irrigation networks usually are not abundant, and they do not have as many nodes as urban water distribution networks. These may make it challenging to model agricultural irrigation networks with ANN compared to urban water networks. Nevertheless, the results obtained in this study were promising in terms of modeling agricultural irrigation networks using ANN.

4. Conclusions

The initial investment costs of pressurized irrigation systems are quite high, in addition, the calculation of pipe diameter in system design is very important for the performance of the irrigation system. Different ANN models were trained and tested to predict pipe diameters of three irrigation systems. Tested networks had different sizes. It was a promising result of a model fit success of 0.83 for an irrigation network with relatively low hydraulic data. The highest model success was obtained for the Gulluce-Dolluk irrigation system with an R^2 value of 0.97. Considering these findings, it was concluded that ANN could be a versatile tool to determine the pipe diameters of pressurized irrigation networks if a decent number of training samples is available. Although it is difficult to integrate a new system parameter into traditional models, new system parameters can be easily integrated into the ANN-based model implementations. Since the core idea of tubular networks is the same as the pressurized irrigation networks, the findings of the present work should also guide future studies related to the drinking water networks. Future studies should include the usage of a large hydraulic database covering a vast number of conditions to train the ANN models that can make more accurate predictions for design parameters of irrigation networks.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Essential oils of *Origanum* Species from Turkey: Repellent Activity Against Stored Product Insect Pests

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ABSTRACT
Studies on the production of plant-based pest control strategies have been growing in recent years. *Origanum* (Lamiaceae) species are important medicinal aromatic plants and many studies have been conducted on their biological activities. This study was conducted to determine the repellent effects of plant essential oils extracted from four different *Origanum* species: *O. onites*, *O. vulgare* var. *hirtum*, *O. vulgare* var. *verticium* and *O. onites* × *O. vulgare*, against four different stored product pests: *Rhyzopertha dominica* (F., 1792) (Coleoptera: Bostrichidae), *Tribolium confusum* Jacquelin Du Val, 1863 (Coleoptera: Tenebrionidae), *Sitophilus granarius* (Linnaeus, 1875) and *Sitophilus oryzae* (Linnaeus, 1763) (Coleoptera: Curculionidae), under laboratory conditions. The neo-clavenger apparatus was used to obtain essential oils. As a result of the experiment, *O. onites* essential oil showed the highest activity at a dose of 0.25 µl cm^{-2} with 68% on *T. confusum* after 2 hours. This oil showed the highest activity on *S. oryzae* with 90% at the lowest application dose of 0.025 µl cm^{-2}. When the results are evaluated generally, the highest activity was found in *O. onites* essential oil. Other essential oils have varying degrees of activity depending on time and dose. Results of the experiment show that *Origanum* oils have a significant potential to controlling this pest.

Keywords: Bioactivity, Essential oils, Lamiaceae, Repellent activity, Stored product pest

1. Introduction

Rapidly increasing global population and consequently increased food demand stands as a major problem in front of agricultural systems with limited resources. Considering that the existing agricultural areas have reached to their highest reachable limit, the current global agricultural production be protected against biotic and abiotic factors from harvest to the table. Qualitative and quantitative losses occur in the stored products due to stored product pests. The reduction of damages caused by stored product pests is based on various cultural, physicomechanical and chemical control methods. The most popular and commonly used approach for controlling these pests globally is chemical control. Methyl bromide and aluminum phosphide are the most common synthetic chemicals used to control these pests (Evans 1987; Taylor 1994; Villers et al. 2010; Mutungi et al. 2014). The use of these chemicals is being prohibited in the scope of Montreal protocol due to their toxicity against warm-blooded organisms and damage to ozone layer. For this purpose, studies have concentrated on alternative management methods against stored product pests, including diatomaceous earth, entomapathogens and plant essential oils, etc. (Tülek et al. 2015; Alkan 2020; Ertürk et al. 2020; Uçar et al. 2020; Atay et al. 2021).

Plants use different defence mechanisms to defend themselves from enemies. Among these processes are several secondary metabolites that are synthesized inside plant cells. These compounds having insecticidal and behavioral activities against various pests (Güncan & Durmuşoğlu 2004) can be classified as alkaloids, glycosides, phenols, terpenoids, tannins and saponins (Shanker & Solanki 2000). The essential oils of plants contain volatile hydrocarbons and their derivatives, terpenic or non-terpenic compounds (Baser 2009). *Origanum* (Lamiaceae) species are essential aromatic medicinal plants and many biological studies have been carried out. Different activities of *Origanum* species such as antioxidant, cytotoxic, antimicrobial, anti-acetylcholinesterase, antibacterial, repellent, antifungal, allelopathic, phytotoxic, insecticidal have been determined in a number of earlier studies (Coccimiglio et al. 2016; Lesjak et al. 2016; Govindarajan et al. 2016; Boukaew et al. 2017; La Pergola et al. 2017; da Cunha et al. 2018; Giatropoulos et al. 2018; López et al. 2018; Ibáñez & Blázquez 2018; Szczepanik et al. 2018; Wijesundara & Rupasinghe 2018; Vinciguerra et al. 2018; Benelli et al. 2019; Dutra et al. 2019; Grul'ová et al. 2019; Reyes-Jurado et al. 2019). The studies conducted to the determine the essential oil composition of *Origanum* species have reported that the main are carvacrol, thymol, γ –terpinene, terpinen-4-ol, linalool, p-cymene, sabinene, α-terpinene, trans sabinene, cis

sabinene hydrate, terpinene, α-pineneand 4-terpineol (Aligiannis et al. 2001; Martucci et al. 2015; Lesjak et al. 2016; Mechergui et al. 2016).

The repellent activities of essential oils extracted from four different *Origanum* species (i.e., *O. onites*, *O. vulgare* var *hirtum*, *O. vulgare* var *verticium*, and *O. vulgare* × *O. onites*) were determined against four important stored product pests (i.e., *Rhyzopertha dominica*, *Tribolium confusum*, *Sitophilus granarius* and *S. oryzae*).

2. Material and Methods

2.1. Pant material

The plants, i.e., *Origanum onites*, *Origanum. vulgare* var *hirtum*, *Origanum vulgare* var *verticium*, and *Origanum vulgare* × *Origanum onites* were collected from the production area of Field Crops Central Research Institute, Ankara, Turkey during 2018. Reyhan Bahtiyarca Bağdat identified the species. The herbariums of these species were prepared and deposited to Directorate of Plant Protection Central Research Institute, Ankara, Turkey.

2.2. Extraction of essential oils

The aerial parts (100g each) of the air-dried plant samples of all the species were separately subjected to hydro distillations for 4 h using a clavenger apparatus. Oils yield was 2.2, 4.56, 2.76 and 3.1% for *Origanum onites*, *O. vulgare* var *hirtum*, *O. vulgare* var *verticium*, and *O. vulgare* × *O. onites*, respectively. The extracted oils were preserved under -20 °C until analyzed. In my previous work, the main components of *O. onites* essential oil were thymol (22.9%), γ-terpinene (13.0%), p-cymene (12.9%) andcarvacrol (7.2%). Similarly, the essential oils of *O. vulgare* var. *hirtum* were composed of carvacrol (32.5%), thymol (16.1%), p-cymene (12.2%) and γ-terpinene (7.9%). Likewise, the essential oil of *O. vulgare* var. *verticium* had carvacrol (35.0%), p-cymene (11.6%), γ-terpinene (10.3%) and thymol (9.1%). Nonetheless, *O. vulgare* x *O. onites* essential oil had carvacrol (15.2%), cis-sabinene hydrate (14.6%), terpinen-4-ol (14.6%) and γ-terpinene (8.7%) (Alkan 2020).

2.3. Insect rearing

Adult insects from a stock culture of *Sitophilus oryzae*, *Sitophilus granarius*, *Tribolium confusum* and *Rhyzopertha dominica* (Plant Protection Central Research Institute, Ankara, Turkey) without exposure to synthetic insecticide for at least 10 years were used. Nutrient mixture of crushed soft bread wheat and dry yeast (*Saccaromyces cerevisiae*) was used to rear *Tribolium confusum* and *Rhyzopertha dominica*. The wheat was crushed to coarse size in feed crushing machine and kept in freezer at -18 °C, 72 hours to eliminate the risk of harmful contamination. Dry yeast was ground in a grinding mill, sieved through 100 mesh sieves and added to wheat at 5% ratio. Whole wheat grains were used for rearing *Sitophilus granarius* and *S. oryzae*. In order to obtain sufficient number of adults in the trials, 700-1000 mixed sex and two weeks old adult individuals were transferred to jars containing 250 g of whole wheat and left for a 48 h oviposition. The lid of the glass jar was punctured with a hole 1 cm in diameter and covered with a 120 mesh screen wire to provide ventilation as well as moisture exchange and prevent insect escape. After oviposition adult insects discarded and the emergence of adult insects were monitoring daily, in the trials 7-28 days old, mixed-sex adult individuals were used. Insects used in the experiment were kept in climate cabins (Nüve ID 501, Turkey) under 25±1 °C temperature, 65±5% relative humidity and continuous dark conditions.

2.4. Repellent activity assay

The method of McDonald et al. (1970) was followed in order to determine the repellent activity of plant essential oils. For this purpose, 9 cm discs were cut from Whatman No. 1 filter paper. Acetone was applied to half of the filter paper as solvent and regarded as control. Different concentrations of essential oils (0.025, 0.125, 0.06 and 0.25 µl cm^{-2}) were applied to the other half of the filter paper with pipette. The filter papers were fixed at the bottom of Petri dishes, which were kept under fume hood for 5 minutes to allow the acetone to evaporate. After that, 7-28 days old insect were released into the middle of the filter paper. Repellent effect trials were set up in a completely randomized design with five replications. 20 adult insects were used in each replication, and a total of 100 individuals were used for each dose. In the control group only acetone was used. The top of the Petri dishes were covered with muslin cloth to avoid any fumigant activity and the area where the insects were present was recorded after 2, 4, 6, 8, 10, 12, and 24 hours (Table 1).

Table 1- Treatment used repellent activity biossays

Essential oil	*Dose (µl cm^{-2})*	*Time (hour)*
Origanum vulgare	0.25	2, 4, 6, 8, 10,12, 24
Origanum onites	0.125	
Origanum vulgare var. *hirtum*	0.06	
Origanum vulgare var. *verticium*	0.025	
Control	Pure acetone	

2.5. Statistical analysis

The following formula was used to compute the percent repellent activity: Repellent activity (%)= ((Nc − Nt)) / ((Nc + Nt)) × 100, where Nc is number of insects in control, Nt is number of insects in respective essential oil treatment. After the calculation of percentage repellent activity, it was classified according to 0-V scale devised by Juliana & Su (1983). According to this scale, 0.1% repellent activity belongs to Class 0, 0.1-20% to Class I, 20.1-40% to Class II, 40.1-60% to Class III, 60.1-80% to Class IV and 80.1-100% to Class V. Statistical differences between application times were evaluated according to Tukey test using software *Minitab 16* (Ryan et al. 2012). Principle Component Analysis was performed with the help of software *GenStat* (Payne 2009).

3. Results and Discussion

The differential repellent activities of essential oils were noted against four important stored product pests depending on the application timing and dose (Table 2). The essential oil of *O. onites* showed the highest repellent activity against *S. oryzae* at the lowest application dose. i.e. 0.025 µl cm^{-2} showed an average repellent activity of 63.1% and placed in repellency class IV. At the same dose. *O. vulgare* var. *hirtum* essential oil exhibited the highest repellent activity against *S. oryzae* with an average repellent effect of 42.9%. which was placed in repellency class III. *O. vulgare* var. *verticium* essential oil displayed an average repellent effect of 38.9% against *S. oryzae* and placed in repellency class II. The essential oil of *O. vulgare* × *O. onites* showed the highest activity against *T. confusum* with 72.0% repellent effect and placed in repellency class IV.

At the highest application dose (0.25 µl cm^{-2}), essential oils had varying repellent effects depending on application timing and pest species. At this application dose, the highest activity was recorded for *O. onites* essential oil against *T. confusum* with 58.0% repellent activity and fell in repellency class III. The repellent activity of *O. onites* was followed by the essential oil of *O. vulgare* × *O. onites* with 47.7% repellent effect and placed in repellency class III. Essential oils of *Origanum* species displayed significant repellent activity against *R. dominica*. *T. confusum*. *S. oryzae* and *S. granarius* depending on the application dose and time. Kłyś et al. (2017) have studied the repellent activity of >300 plant extracts essential oil and powder against *S. oryzae*. *S. granarius*. *T. castaneum*. *R. dominica* and *Oryzaephilus surinamensis*. Kim et al. (2010) revealed that *O. vulgare* essential oil has a repulsive effect against *Tribolium castaneum*. Taban et al. (2017) investigated the insecticidal and repellent activity of essential oils obtained from *Satureja* species (1% v/v application dose) against *T. castaneum* and 98-100% repellent activity was observed. It was reported that the main components of this plant essential oil were thymol and cavracrol (Taban et al. 2017).

Thymol and cavracrol were the main components of almost all plant essential oils used in this experiment and showing repellent activity. Plant essential oils have shown varying degrees of repellent activity against the storage pest insects taken to the experiment. This may be due to the reactions of insects to the substance or substances in the chemical composition of the plants, as well as to the physiology of the insect. There are many studies conducted before in which plant essential oils were tested against stored product pests (Wang et al. 2015; Koutsaviti et al. 2018; Liang et al. 2018; Zhang et al. 2019). Considering the pest groups in which the repellent effects of plant essential oils are studied, it is seen that they are mostly tested against insects that harm humans or cause disease vectors.

It is seen that the trials against warehouse pests are less than these pests. This situation can be explained in several ways; first of all, in the application against storage membranes, the high permanence of the components in the chemical composition of some plant essential oils was a big problem. As a result of the repellent activity trials, it has been stated that plant essential oils have significant repellent effects on both *T. castaneum* and *S. granarius*. Previous studies show that different herbal essential oils have repellent activities against these pests. Wang et al. (2006) tested the essential oils they obtained from *Artemisia vulgaris* against *T. castaneum* in their study and reported that this plant essential oil showed significant repellent activity at a concentration of 0.6 µl mL^{-1}. In another study (Liu & Ho 1999), the repellent activity of the essential oils obtained from *Evodia rutaecarpa* was tested against *T. castaneum* and *Sitophilus zeamais*, and it was concluded that this plant essential oil showed repellent activity in both insects. However, they reported that *T. castaneum* is more sensitive to plant essential oil in terms of repellency than *S. zeamais*. In another study, they tested the repellent activity of essential oils obtained from *Tagetes terniflora*, *Cymbogon citatus* and *Elyonurus muticus* plants against *T. castaneum* and *S. oryzae* under laboratory conditions and reported that all plant essential oils had repellent effects against larvae and adults of *T. castaneum* and *S. oryzae* larvae (Stefanazzi et al. 2011).

Table 2- The repellent effect of different doses of essential oils against some coleopteran stored product pests

EO	*Repellency (%) ± StDev*																
	0.25 µl cm^{-2}					*0.125 µl cm^{-2}*				*0.06 µl cm^{-2}*				*0.025 µl cm^{-2}*			
	HAT	RD	TC	SO	SG	RD	TC	SO	SG	RD	TC	SO	SG	RD	TC	SO	SG
A	2	34.0±9.3a	68.0±5.8a	10.0±7.7b	28.0±9.2a	40.0±15.	40.0±9.5b	74.0±6.0a	58.0±9.7a	32.0±6.6a	56.0±13.6	74.0±11.7	44.0±18.3	40.0±10.0	72.0±6.6a	78.0±10.2	38.0±11.1
	4	22.0±9.7a	60.0±6.3a	12.0±2.0b	12.0±5.4b	32.0±14.	64.0±12.1	64.0±5.1a	38.0±13.9	28.0±4.9a	48.0±8.6a	64.0±7.5a	20.0±17.6	44.0±12.9	48.0±5.8b	90.0±3.2a	54.0±8.1a
	6	28.0±12.0	52.0±10.2	14.0±7.9b	30.0±11.0	24.0±17.	48.0±2.0b	66.0±6.0a	42.0±13.6	34.0±8.1a	20.0±10.6	42.0±10.2	18.0±20.3	46.0±11.2	42.0±13.2	64.0±9.3b	12.0±14.6
	8	30.0±15.5	56.0±12.9	14.0±5.1b	16.0±21.4	46.0±13.	50.0±3.2b	60.0±6.3a	30.0±9.5b	38.0±8.6a	28.0±8.4a	62.0±12.0	20.0±10.5	36.0±9.3a	54.0±9.3b	76.0±9.3b	13.0±8.3c
	10	16.0±11.2	58.0±9.2a	12.0±6.4b	12.0±16.2	46.0±14.	46.0±5.1b	78.0±5.8a	40.0±15.2	30.0±7.1a	32.0±11.5	46.0±13.6	14.0±6.7b	56.0±6.8a	46.0±11.2	54.0±9.3b	14.0±11.2
	12	12.0±8.0a	58.0±5.8a	6.0±2.1b	22.0±28.4	46.0±13.	60.0±4.5a	70.0±8.9a	28.0±13.9	26.0±8.1a	30.0±17.0	56.0±15.4	12.0±8.0b	34.0±6.8a	32.0±13.6	34.0±18.1	24.0±15.7
	24	2.0±1.2b	54.0±9.3a	64.0±7.5a	14.0±9.2a	44.0±14.	50.0±7.1b	74.0±9.3a	28.0±9.2b	22.0±5.8a	26.0±14.4	50.0±15.2	14.0±6.7b	44.0±9.3a	44.0±10.8	46.0±14.4	20.0±12.3
	A(RC	20.6 (II)	58.0 (III)	18.9 (I)	19.1 (I)	39.7 (II)	51.1 (III)	69.4 (IV)	37.7 (II)	30.0 (II)	34.3 (II)	56.3 (III)	20.3 (II)	42.9 (III)	48.3 (III)	63.1 (IV)	25 (II)
B	2	10.0±6.c	74.0±8.7a	40.0±7.7a	16.0±11.7	36.0±13.	46.0±11.7	60.0±5.5a	20.0±10.5	22.0±12.9	20.0±16.4	70.0±13.4	18.0±8.6a	22.0±7.2a	54.0±7.5a	48.0±3.7a	20.0±10.6
	4	60.0±5.1a	22.0±29.6	6.0±1.2c	12.0±12.8	36.0±13.	36.0±13.6	62.0±8.0a	4.0±0.8a	12.0±6.7a	48.0±8.6a	56.0±6.0a	34.0±8.7a	14.0±9.3a	36.0±13.6	58.0±11.6	24.0±9.8a
	6	30.0±8.5b	18.0±1.3a	18.0±1.5b	28.0±15.2	30.0±15.	20.0±9.5a	46.0±9.3a	2.0±1.2a	42.0±10.2	44.0±8.7a	36.0±9.3a	16.0±9.3a	16.0±5.8a	32.0±17.7	42.0±14.6	14.0±0.0a
	8	18.0±1.3b	42.0±3.7a	12.0±4.8b	32.0±13.6	46.0±14.	20.0±13.4	26.0±16.0	16.0±5.7a	54.0±6.8a	36.0±15.0	34.0±8.1a	16.0±6.3a	22.0±9.9a	42.0±12.7	42.0±7.3a	16.0±11.7
	10	15.0±4.8c	32.0±9.7a	12.0±7.1b	56.0±6.8a	40.0±12.	28.0±11.1	38.0±10.7	32.0±6.3a	56.0±6.8a	34.0±11.2	20.0±5.5b	6.0±15.0a	12.0±4.3a	16.0±11.7	36.0±6.0a	20.0±13.4
	12	12.0±	58.0±9.6a	6.0±2.2c	20.0±5.5b	42.0±16.	38.0±7.3a	26.0±13.6	14.0±5.3a	54.0±6.8a	36.0±8.1a	20.0±5.5b	28.0±11.7	8.0±3.3a	32.0±15.0	32.0±10.7	12.0±8.7a
	24	6.0±1.2c	48.0±15.0	16.0±7.5b	12.0±4.6c	54.0±10.	30.0±8.4a	24.0±12.9	22.0±6.3a	58.0±7.3a	38.0±12.4	28.0±5.8b	14.0±6.3a	14.0±8.2a	22.0±13.0	42.0±11.1	18.0±10.7
	A(RC	21.6 (II)	42.0 (III)	15.7 (I)	25.1 (II)	40.6 (II)	31.1 (II)	40.3 (III)	15.7 (I)	42.6 (III)	36.6 (II)	37.7 (II)	18.9 (I)	15.4 (I)	33.4 (II)	42.9 (III)	17.7. (ı)
C	2	36.0±12.7	28.0±8.6b	56.0±7.5a	38.0±11.6	24.0±12.	6.0±15.7a	58.0±13.2	36.0±8.1a	12.0±10.4	28.0±9.2b	52.0±7.3a	40.0±10.5	8.0±4.9a	70.0±9.5a	48.0±8.0a	40.0±13.8
	4	30.0±8.0a	24.0±12.5	20.0±4.5b	32.0±18.3	32.0±13.	20.0±8.9a	30.0±21.1	24.0±7.5b	34.0±18.9	50.0±8.9a	44.0±12.9	32.0±9.7a	14.0±8.7a	38.0±8.0b	26.0±9.8b	20.0±8.4a
	6	24.0±6.0a	22.0±9.7b	4.0±2.5c	28.0±11.1	38.0±13.	16.0±10.0	26.0±13.3	16.0±15.7	32.0±16.6	28.0±15.3	56.0±6.8a	36.0±4.0a	24.0±8.1a	22.0±9.7b	32.0±11.6	28.0±12.4
	8	30.0±14.0	12.0±8.6b	16.0±4.1b	26.0±6.0a	30.0±20.	14.0±8.7a	28.0±8.0a	12.0±5.8b	34.0±22.9	30.0±8.4b	32.0±13.9	24.0±5.1a	26.0±10.3	12.0±8.6b	32.0±10.2	28.0±13.2
	10	26.0±9.7a	46.0±6.0a	14.0±2.8b	26.0±13.6	32.0±14.	16.0±14.4	40.0±8.4a	14.0±8.1b	38.0±22.7	38.0±8.6b	42.0±9.7a	22.0±3.7a	14.6±12.0	46.0±6.0b	52.0±13.6	18.0±8.6a
	12	20.0±6.9a	20.0±10.5	16.0±4.2b	22.0±16.6	34.0±15.	20.0±8.9a	34.0±5.1a	34.0±14.0	30.0±21.5	44.0±9.3a	34.0±11.7	26.0±6.8a	22.0±15.3	32.0±8.0b	44.0±10.8	24.0±13.6
	24	24.0±8.1a	24.0±11.2	12.0±6.7b	24.0±19.1	46.0±13.	16.0±14.4	34.0±6.0a	28.0±16.6	40.0±18.7	42.0±8.6a	34.0±11.7	22.0±5.8a	42.0±8.6a	38.0±9.7b	38.0±8.0a	16.0±12.1
	A(RC	27.1 (II)	25.1 (II)	19.7 (I)	28.0 (II)	33.7 (II)	15.4 (I)	35.7 (II)	23.4 (II)	31.4 (II)	37.1 (II)	42.0 (III)	28.9 (II)	21.4 (II)	36.9 (II)	38.9 (II)	24.9 (II)
D	2	30.0±16.4	70.0±12.6	38.0±10.2	40.0±12.6	48.0±9.7a	90.0±3.2a	58.0±9.7a	48.0±6.6a	28.0±12.0	68.0±5.8a	66.0±5.1a	36.0±12.1	14.0±6.0a	88.0±2.0a	68.0±8.6a	10.0±7.1a
	4	44.0±12.1	72.0±9.2a	46.0±7.5a	44.0±7.5a	52.0±10.	88.0±7.3a	46.0±4.0a	42.0±6.6a	30.0±8.4a	62.0±15.0	44.0±10.3	42.0±13.9	12.0±8.0a	70.0±5.5b	68.0±2.0a	24.0±12.5
	6	64.0±15.7	38.0±14.3	52.0±12.4	54.0±19.6	42.0±12.	82.0±5.8a	34.0±5.1b	32.0±9.2a	28.0±11.6	54.0±17.5	28.0±5.8b	44.0±14.7	12.0±3.7a	74.0±4.0b	44.0±16.6	26.0±2.5a
	8	56.0±9.3a	30.0±8.9b	20.0±14.1	48.0±9.7a	42.0±12.	56.0±10.3	32.0±16.6	50.0±12.2	20.0±15.8	48.0±7.3a	30.0±13.0	40.0±17.0	10.0±5.2a	60.0±10.5	46.0±9.8a	36.0±14.7
	10	50.0±13.8	44.0±10.3	18.0±10.7	40.0±11.0	32.0±15.	60.0±7.1b	34.0±10.8	36.0±6.8a	22.0±13.6	62.0±10.7	36.0±16.9	34.0±19.4	10.7±4.8a	68.0±6.6b	52.0±13.6	12.0±6.0a
	12	46.0±17.5	42.0±8.6b	22.0±8.6b	44.0±14.4	44.0±11.	64.0±5.1b	28.0±10.2	34.0±6.8a	24.0±12.9	46.0±6.8a	32.0±10.2	34.0±12.1	10.0±6.3a	74.0±7.5b	58.0±10.7	28.0±16.9
	24	14.0±16.3	38.0±11.6	24.0±11.7	30.0±11.4	34.0±14.	62.0±3.7b	30.0±10.5	26.0±12.1	28.0±9.7a	54.0±10.3	44.0±15.4	24.0±13.6	18.0±8.0a	70.0±5.5b	56.0±9.8a	16.0±8.1a
	A(RC	43.4 (III)	47.7 (III)	31.4 (II)	42.9 (III)	42.0 (III)	71.7 (IV)	37.4 (II)	38.3 (II)	25.7 (II)	56.3 (III)	40.0 (II)	36.3 (II)	12.2 (I)	72.0 (IV)	56.0 (III)	21.7 (II)

[1]Different letters in the same row indicate statistically different from each other (Anova P<0,05, Tukey test). **A:** *Origanum onites*; **B:** *Origanum vulgare* var. *hirtum*; **C:** *Origanum vulgare* var. *verticium*; **D:** *Origanum vulgare* × *Origanum onites;* **HAT;** Hour after treatment; **RD**: *Rhyzopertha dominica*; **TC:** *Tribolium confusum*; **SO:** *Sitophilus oryzae*; **SG:** *Sitophilus granarius;* **A(RC):** Average (Repellency Class)

As a result of the bi-pilot analysis, it was concluded that the activity of essential oils for *Rhyzopertha dominica* gave similar results at 8, 10 and 12 hours, and the activity at the end of 2, 4 and 6 hours (Figure 1). Also, *Origanum onites* and *O. vulgare* × *O. onites* essential oils have similar activity while *O. vulgare* var. *hirtum*: *O. vulgare* var. *verticium* essential oils were included in the similar group as a result of bi plot analysis. Similar results were obtained, although there were minor changes in other insects included in the experiment.

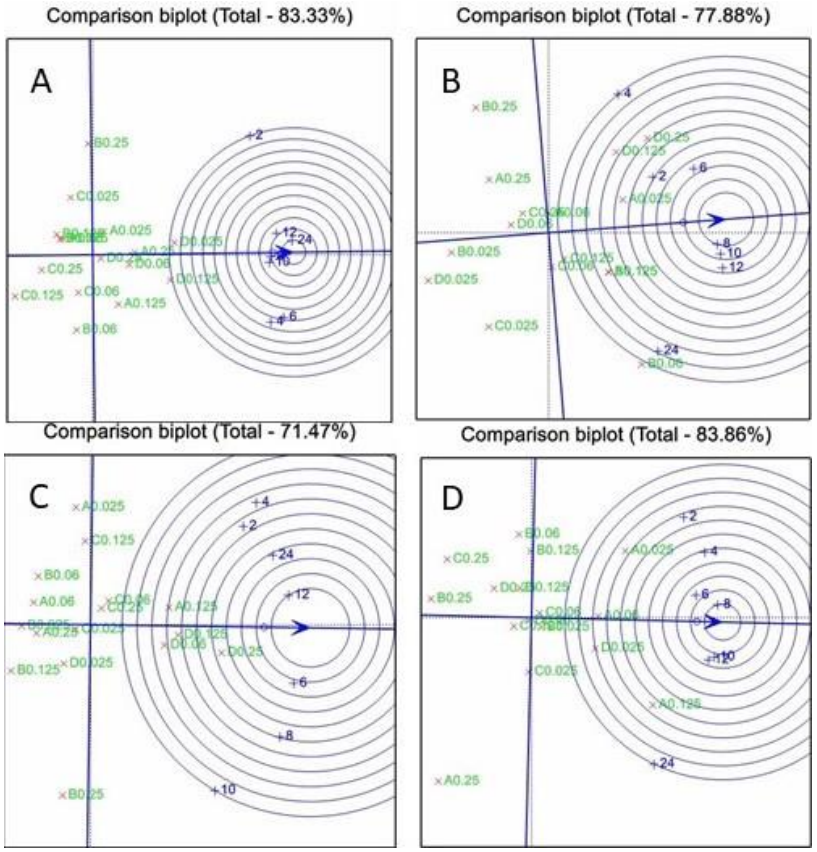

Figure 1- Principal component analysis of essential oil on the stored product pest: *Rhyzopertha dominica* (A), *Sitophilus granarius* (B), *Sitophilus oryzae* (C) and *Tribolium confusum* (D)

Parameters obtained the ANOVA (Insect*EOs*Time*Dose) determined that interaction between the factors tested for essential oils. Analysis results revealed that EOs * Dose interaction is not important but insect × EOs × dose interaction is important. In addition, as a result of the analysis, it was concluded that the quadruple interaction (insect × EOs × time × dose) is important (Table 3).

Table 3- ANOVA parameters for different applications of *Origanum* essential oils against *Sitophilus oryzae, Sitophilus granarius, Tribolium confusum* and, *Rhyzopertha dominica*

Source	*df*	*F-value*	*P-value*
Insect	3	79.93	0.000
EOs	3	63.67	0.000
Time	6	9.53	0.000
Dose	3	34.82	0.000
Insect × EOs	9	14.04	0.000
Insect × Time	18	2.40	0.001
Insect × Dose	9	10.94	0.000
EOs × Time	18	0.57	0.922
EOs × Dose	9	8.23	0.000
Time × Dose	18	0.45	0.976
Insect × EOs × Time	54	0.98	0.516
Insect × EOs × Dose	27	6.42	0.000
Insect × Time × Dose	54	0.72	0.936
EOs × Time × Dose	54	0.88	0.728
Insect × EOs × Time × Dose	162	0.73	0.996
Error	1792		
Total	2239		

In this study repellent effects of essential oils obtained from *Origanum* species against four important stored product pests which cause significant damages in warehouse were tested under laboratory conditions.

Studies on the use of essential oils in controlling agricultural pests have recently gained momentum. In parallel with the toxicity of pesticides used in pest control to hot blood and increasing consumer awareness, interest in the use of agents derived from natural or natural products is increasing. In this context, it becomes important to use plant-based control methods in warehouse pests. Essential oils are tested against storage pests as well as many other pests. In this study, the behavioural effects of essential oils obtained from four *Origanum* species that can be produced conventionally were tested against four important storage pests under laboratory conditions. Results of the experiment show that these oils have an important potential in pest control. However, in order for the studies to be put into practice, application method and formulation studies should be done.

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Effect of Yellow and Stop Drosophila Normal Anti-insect Photoselective Nets on Vegetative, Generative and Bioactive Traits of Peach (cv. Suncrest)

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ABSTRACT
The effect of anti-insect photoselective yellow (mesh size of 2.4x4.8 mm) and Stop Drosophila Normal (mesh size of 0.90x1 mm) nets on the generative and vegetative traits of peach (cv. Suncrest) was studied at an orchard near the city of Čakovec, Croatia. Netting significantly affected some vegetative parameters (leaf surface, leaf length and leaf shape index) but there was no significant effect on productivity parameters (yield, yield efficiency, fruit mass and share of decayed fruit). Regarding fruit colouration application of nets significantly affected b* and C* background and L*, b*, C* and h° additional colour parameters. Majority of inner fruit quality parameters (fruit firmness, titratable acidity and total soluble solids / titratable acidity ratio) as well as of bioactive compounds (total polyphenolic content, antioxidant activity, anthocyanin content and share of alkali-soluble pectin) was also under significant effect of netting. Since yellow net only slightly reduced peach fruit quality (compared to control) it can be recommended for application as an anti-insect net. However, Stop Drosophila Normal net more notably reduced quality parameters (especially additional fruit colour) and hence should be used only when other control strategies show to be ineffective.

Keywords: Netting, Light modification, Fruit quality, Bioactive compounds, Anti-insect nets, Sustainability

1. Introduction

Nowadays, modern agriculture is unimaginable without netting. Nets are applied due to numerous reasons, mainly for the protection against various hazards (hail and wind, excessive sun radiation, birds) (Bosco et al. 2015; Giaccone et al. 2012; Middleton & McWaters 2002) and subsequently against insects (Pajač Živković et al. 2018; Tasin et al. 2008). Anti-insect nets are similar to anti-hail nets but they differ from anti-hail nets in mesh size and application method (Pajač Živković et al. 2018). Anti-insect nets overlay the fruit tree canopy and edges of the orchard creating a barrier that disrupts pest propagation by preventing their flight (Tasin et al. 2008). Pajač Živković et al. (2018) successfully applied anti-insect nets with mesh size of 2.4x4.8 mm against oriental fruit moth (*Grapholita molesta* (Busck 1916)) and peach twig borer (*Anarsia lineatella* (Zeller 1839)), two economic pests of stone fruits in the Republic of Croatia. However, relatively recently *Drosophila suzukii* (Matsumura), a highly polyphagous invasive pest to whom peach is one of the host plant species, invaded western countries and became a challenge in the fruit production process (Cini et al. 2012).

Due to the pest's small size, dense nets with smaller mesh sizes are required for successful protection. Smaller mesh in anti-insect nets creates more shade than traditional anti-hail nets of the same colour, which can have a potentially negative effect on vegetative and generative traits of plants cultivated underneath them. However, over a decade ago a relatively new technology emerged – photoselective netting as a tool for light quality and quantity manipulation under practical field conditions (Rajapakse & Shahak 2007). Traditional black nets can influence only light quantity because of their opacity, while translucent nets additionally have the light scattering ability, but neither one can influence the quality properties of light (Ilić & Fallik 2017; Oren-Shamir et al. 2001; Shahak 2008; Shahak et al. 2004b). According to Basile et al. (2012), photoselective nets are made of partially translucent threads that selectively screen-out certain light spectra, in UV and/or visible light spectra, which pass through them and at the same time transform direct light to diffuse. Coloured nets can increase the content of scattered light for two or more times (Oren-Shamir et al. 2001). Scattered light has a better penetration possibility in dense canopies (or in the inner part of canopies), thus increasing the efficiency of light-dependent processes (Lakso & Musselman 1976; Shahak 2014; Shahak et al.

2004a). Moreover, due to light filtration ability (light quality modification) which is defined by pigments incorporated in plastic material (Shahak et al. 2016), they also promote specific plant physiological responses (Shahak 2014). Up to date, the available literature is notably insufficient regarding the effect of photoselective nets on vegetative and generative traits of peaches. Therefore, it is hard to appoint which type of photoselective net should be applied for pest protection so that multiple benefits can be achieved. Moreover, there are no studies that deal with the effect of photoselective nets on the content of bioactive compounds in peach fruit. Hence, this study aims to test the effect of two different photoselective anti-insect nets on peaches 'Suncrest' with emphasis on their effect on the synthesis of the bioactive compounds in fruits.

2. Material and Methods

2.1. Plant material and treatments

The experiment was established at a peach orchard in Vratišinec, near the city of Čakovec, Croatia in April 2015 on the 12 years old peach (*Prunus persica* (L.) Batsch.) cv. 'Suncrest' grafted on vineyard peach (peach seedling). The peaches were trained as an open vase with a spacing of 4x3 m. The experiment consisted of three treatments: uncovered trees that served as control (C), the trees that were covered with yellow photoselective nets (Tenax, Italy) after petal fall (Y) and the trees that were covered with white Stop Drosophila Normal net (Artes Politecnica, Italy) after petal fall (D). Y net had a mesh size of 2.4x4.8 mm while D nets had a mesh size of 0.90x1 mm. Due to smaller mesh sizes both nets have anti-insect properties. In this study yellow net was used because of its promising results obtained in Israel (Shahak et al. 2016). According to the manufacturer D net has protection properties against *D. suzukii* and was therefore selected. The experiment was set up according to a random block schedule in three repetitions for each net and control. Each repetition was physically separated from each other (different net cage) and included three peach trees. Fruits from both treatments and control were harvested on August 6, 2015.

2.2. Vegetative parameters

Vegetative parameters were measured after the end of the vegetative period. Trunk cross-sectional area (TCSA) was measured at a height of 25 cm above soil level and expressed in cm^2. Length of the one-year shoot (cm), internode length (cm), and thickness of one-year shoot (mm) was measured on 10 randomly selected shoots from the middle – the outer part of the tree canopy, per repetition. Thickness (mm) was measured 5 cm from the shoot base by the digital caliper Prowin HMTY0006. Internode length (cm) was measured on three internodes placed at the middle part of the shoot, at 10 randomly selected shoots per repetition. The density of internodes (number of internodes cm^{-1}) was calculated according to the formula:

$$density\ of\ internodes = \frac{number\ of\ internodes\ per\ shoot}{length\ of\ the\ shoot} \quad (1)$$

From each repetition, 10 leaves (30 leaves per treatment) were randomly sampled from the middle part of the shoots located at the middle – the outer part of the canopy. Leaf length (cm) was measured by the digital caliper Prowin HMTY0006 from the top of the leaf to the petiole insertion and leaf width (cm) on the widest part of the leaf. Leaf shape index was calculated as a ratio between leaf length and width. Petiole length (cm) of each leaf was also measured by the caliper Prowin HMTY0006. Leaf surface (cm^2) was calculated by the ImageJ software program (Image Processing and Analysis in Java), frequently used software in scientific community (Schneider et al. 2012), according to a modified method described by Padrón et al. (2016). After setting the length scale in pixels by the determination of the known length in the figure, brightness threshold was modified to highlight the leaf blade and then the leaf area was measured using a Region of Interest manager tool.

2.3. Productivity parameters

Yield, yield efficiency and share of decayed fruit were measured on three fruit trees for each repetition (9 trees per treatment). Yield per tree (kg) was determined in the orchard immediately after harvest by weighing the total yield of fruits for each tree. Yield efficiency (kg cm^{-2}) was calculated according to the formula:

$$yield\ efficiency = \frac{yield\ per\ tree\ (kg)}{TCSA\ (cm^2)} \quad (2)$$

Share of decayed fruit was determined relative to the total yield per tree. Fruit mass was measured on 30 fruit per repetition (90 fruits per treatment) using a digital analytical balance (OHAUS Adventurer AX2202, Ohaus Corporation Parsippany, NJ, USA) with an accuracy of 0.01 g.

2.4. Physico-chemical properties of fruits

Analyses of physico-chemical properties were conducted at the Department of Pomology and Department of Chemistry, Faculty of Agriculture, University of Zagreb, Croatia.

2.4.1. Fruit skin colour parameters

The fruit skin colour parameters were measured according to the CIE L*a*b* and CIE L*C*h° systems, using a colorimeter (ColorTec PCM; ColorTec Associates Inc., USA). In a three-dimensional uniform space L* value is defined as a vertical coordinate which defines lightness, and a* and b* values as a horizontal coordinate which, if negative, indicates intensity of green and blue colour (respectively), or if positive, intensity of red and yellow colour (respectively) (AN 1005.00 2012). According to the most widely accepted international criterion when h° is 0° it assigns to the semiaxis +a* (redness), when is 90° to the semiaxis +b* (yellowness), when is 180° to the semiaxis -a* (greenness) and when is 270° to the semiaxis -b* (blueness) (Carreño et al. 1995). Fruit skin colour were taken separately for the background (yellow) and additional (orange – red) fruit colour on 10 randomly selected fruits from each repetition (30 fruit per treatment).

2.4.2. Fruit firmness, total soluble solids (SSC), titratable acidity (TA) and SSC / TA ratio

All measurements were conducted on 10 randomly selected fruits from each repetition (30 fruits per treatment). The firmness was measured using PCE PTR-200 (PCE Instruments, Jupiter/Palm Beach, USA) fitted with an 8 mm diameter plunger and expressed in kg cm^{-2}. Measurements were taken at four opposite equatorial positions on each fruit at 90° after fruit skin was removed. The SSC was measured with a hand digital refractometer (Atago, PAL-1, Tokyo, Japan) and expressed as °Brix. TA was determined by the titration method with 0.1 mol dm^{-3} NaOH and expressed as % of malic acid. The SSC / TA ratio was calculated from the corresponding values of SSC and TA for each fruit.

2.4.3. Pectin

Pectin fractions (water-soluble pectin, ammonium oxalate-soluble pectin, alkali-soluble pectin) were determined by the method of Robertson (1979) and as well as Fruk (2014). Pectin fractions were isolated from the alcohol-insoluble precipitate of peaches by the distilled water, ammonium oxalate and sodium hydroxide. All absorbance readings were conducted on spectrophotometer Shimadzu UV 1700 (Shimadzu Corporation, Kyoto, Japan). Pectin content of each fraction was calculated according to the standard calibration curve of galacturonic acid. Afterwards obtained pectin content was expressed as mg kg^{-1} of galacturonic acid (mg kg^{-1} GA) and then converted into relative share of fractions according to the formula:

$$UD = \left(\frac{FP}{P}\right) \cdot 100 \quad (3)$$

Where: UD – share of appropriate pectin fraction expressed in %; FP – recorded content of appropriate pectin fraction expressed as mg kg^{-1}; P – total measured amount of pectin in the sample expressed as mg kg^{-1}

2.4.4. Total polyphenolic content and antioxidant potential

2.4.4.1. Preparation of extracts

The peach extraction was carried using a modified method by Komes et al. (2016). Randomly sampled 10 peaches from each repetition were mashed and homogenized with a laboratory mixer (FOSS homogenizer 2094 (Hillerød, Denmark)) until a homogenized fraction was obtained. Then, 10 g of the homogenized fraction was poured with 50 mL of boiled (100 °C) distilled water. Extraction was performed with constant mixing at room temperature (20 °C) for 30 minutes. The suspension was filtered through a metal strainer, cooled, and centrifuged at 900 x g for 5 min. Supernatants were filtered using Whatman No. 4 filter paper in a 50 mL volumetric flask and supplemented with distilled water. Extracts were prepared in duplicates for each sample (and for each respective repetition). The final concentration of obtained extracts was 200 g L^{-1}.

2.4.4.2. Total polyphenolic content

The modified Folin Ciocalteu's method of Singleton et al. (1999) was used for the determination of total polyphenolic content. A mixture of 0.1 mL of peach extract juice with 7.9 mL distilled water and 0.5 mL Folin Ciocalteu's reagent (diluted with distilled water in 1:2 ratio) and 1.5 mL of 20% sodium carbonate was vortexed and left for 2 h to complete the reaction. The absorbance was measured at 765 nm (Ough & Amerine 1988) on spectrophotometer Shimadzu UV 1700 (Shimadzu Corporation, Kyoto, Japan). Total polyphenolic content was determined according to standard calibration curve of galic acid. The data were expressed as gallic acid equivalents, mg GAE 100 g^{-1} of fresh fruit weight (fw).

2.4.4.3. Antioxidant activity

The antioxidant activity was determined using well-known methods with 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), according to the procedures of Brand-Williams et al. (1995) and Re et al. (1999), respectively. The obtained data was expressed as μmol Trolox equivalents (μmol TE 100 g^{-1} fw).

2.4.5. Pigments

2.4.5.1. β-carotene determination

The β-carotene content was measured according to the procedure reported by Barros et al. (2008). In a dark test tube, 0.1 g of homogenized sample of randomly selected 10 peach fruit from each repetition was mixed with 10 mL of acetone-hexane (4:6 mL) mixture, vortexed for 1 minute, and filtered through Whatman No. 4 filter paper. The final volume was set to 10 mL. Per each repetition two samples were used and per each sample measurements were conducted in parallel. The absorbance was measured at 453 nm, 505 nm, and 663 nm on spectrophotometer Shimadzu UV 1700. The data was expressed as μg β-carotene g^{-1} fw (μg g^{-1} fw). The following equation was used for the calculation of the β-carotene content:

$$\beta - carotene = 0.216 \cdot A_{663} - 0.304 \cdot A_{505} + 0{,}452 \cdot A_{453} \quad (4)$$

2.4.5.2. Total anthocyanins

The total anthocyanins content were measured in three samples per each repetition, according to the modified method described by Proctor (1974). Per each sample three discs of fresh peaches (fruit exocarp) were obtained (1 mm thick and the surface of one disc was 1.13 cm^2). The disc area was calculated based on the ellipse surface formula:

$$Disc\ surface = \pi \cdot a \cdot b \quad (5)$$

Where: a – value of the first radius; b – value of the second radius

In order to involve 10 fruits per each repetition in this measurement, discs of last two peaches were halved. The discs were then inserted into a test tube and immersed in 3 ml of acidified methanol solution (1% conc. HCl (v/v)) and vortexed. The tubes were left for three hours at room temperature (20 °C) in the dark chamber. The absorbance was measured at 532 and 653 nm on spectrophotometer Shimadzu UV 1700, and the obtained values of the optical density of anthocyanins were calculated according to the formula described by Wells (1995):

$$Total\ anthocyanins = A_{532} - (0.25 \cdot (A_{653})) \quad (6)$$

Where: A532 – measured absorbance values at 532 nm; A653 – measured absorbance values at 653 nm

The optical density values were then divided by the molecular extinction coefficient of cyanidin ($2.45x10^4$) and by the area of the disks (area of three disks - 3.39 cm^2) to convert the values into the concentration of micromoles of anthocyanins per cm^2 (μmol cm^{-2}) (Siegelman & Hendricks 1957).

2.5. Statistical analysis

Data were statistically analyzed using SAS statistical software ver. 9.4 (SAS Institute, NC) by ANOVA and Tukey's HSD test ($P \leq 0.05$).

3. Results and Discussion

3.1. Vegetative parameters

According to ANOVA, treatments did not achieve a significant effect on vegetative parameters of peach shoots (Table 1). However, according to ANOVA, treatments revealed a significant effect on the leaf surface ($P<0.01$), leaf length ($P<0.001$) and leaf shape index ($P<0.05$) (Table 2). Peaches grown under the Y net had significantly higher leaf surface and leaf length values than those grown under the D net or C. Although according to ANOVA leaf shape index was significantly affected by treatments ($P = 0.049$), according to Tukey's HSD test no significant difference occurred between peaches grown under the Y and D net and from C (Table 2). Hence, a strong non-significant trend is evident where leaves of peaches grown under the Y net tend to have higher leaf shape ratio (more elongated leaves) than leaves of peaches grown under the D net and from C (Table 2).

Table 1- Shoot length, shoot diameter, length of internodes, the density of internodes and trunk cross-sectional area (TCSA) of peaches cultivated under anti-insect photoselective nets

Treatment	*Shoot length (cm)*	*Shoot diameter (mm)*	*Length of internodes (cm)*	*Density of internodes (internodes cm^{-1})*	*TCSA (cm^2)*
C	58.53±11.61	6.26 ±1.04	1.92±0.23	0.52±0.07	110.62±27.34
Y	57.56 ±12.27	6.13±1.13	2.02±0.33	0.51±0.09	114.28±25.41
D	53.9 ±12.41	6.06±0.90	2.08±0.25	0.49±0.07	120.33±24.56
	ANOVA				
Treatment	0.34$^{n.s.}$	0.74$^{n.s.}$	0.08$^{n.s.}$	0.19$^{n.s.}$	0.74$^{n.s.}$

[1] Results are expressed as mean ± SD; [2] $^{n.s.}$, nonsignificant

Table 2- Leaf petiole length, leaf surface, leaf length, leaf width and leaf shape index of peaches cultivated under anti-insect photoselective nets

Treatment	*Petiole length (cm)*	*Leaf surface (cm^2)*	*Leaf length (cm)*	*Leaf width (cm)*	*Leaf shape index*
C	0.91±0.11	40.21±5.07 b	15.51±1.05 b	3.59±0.33	4.34±0.33 a
Y	0.97±0.21	45.00±6.79 a	17.28±1.79 a	3.81±0.43	4.54±0.31 a
D	0.89±0.12	40.63±5.81 b	16.15±1.46 b	3.70±0.43	4.38±0.33 a
	ANOVA				
Treatment	0.15$^{n.s.}$	0.008**	0.0001***	0.14$^{n.s.}$	0.049*

[1] Results are expressed as mean ± SD; [2] Means followed by different letters within columns and are significantly different (Tukey's HSD test; P ≤0.05); [3] $^{n.s.}$, *, **, ***, nonsignificant, or significant at P ≤0.05, P≤0.01, P≤0.001, respectively

In majority of other studies netting, or application of nets with higher shading properties (when there was no control), enhanced or (less frequently) did not significantly affected leaf vegetative parameters (Amarante et al. 2011; Basile et al. 2014; Brar et al. 2020; Giaccone et al. 2012; Retamales et al. 2008; Vuković et al. 2016). Results obtained in this research are following the majority of the above cited literature. Generally, application of Y net enhanced some leaf vegetative parameters and according to non-significant trend application of Y and D net enhanced internode length. The main reason for such findings is probably due to the shade avoidance mechanism, as proposed in other studies (Basile et al. 2014; Bastias 2011). Reduced red to far-red ratio, which occurs under the white and yellow net (Shahak et al. 2004a, 2004b), is according to Casal (2012) one of the main signals responsible for the shade avoidance mechanism. Shade avoidance mechanism symptoms include higher internode length and leaf elongation (Smith & Whitelam 1997), which was in this study recorded on peaches grown under the Y net. According to Oren-Shamir et al. (2001), the shade avoidance mechanism is not responsible for changes in vegetative growth under all nets. Baraldi et al. (1994) reported that at the bottom part of the peach canopy, where red to far-red ratio and fitocrome equilibrium was reduced (which are signals for triggering shade avoidance mechanism), internode length was enhanced, but leaf surface was reduced in comparison to middle and top part of the peach canopy. All this presents probable explanations why leaves of peaches grown under the D nets did not have enhanced growth as those under the Y nets.

3.2. Productivity parameters

According to ANOVA, treatments did not achieve a significant effect on any productivity trait (Table 3).

Table 3- Yield, yield efficiency, fruit mass and share of decayed fruit of peaches cultivated under anti-insect photoselective nets

Treatment	*Yield (kg / tree)*	*Yield efficiency (kg cm^{-2})*	*Fruit mass (g)*	*Share of decayed fruit (%)*
C	25.97±9.58	0.24±0.09	147.10±39,41	0.83±1.68
Y	31.50±13.69	0.27±0.08	149.50±22.94	1.16±1.60
D	30.76±8.19	0.27±0.10	146.36±26.89	1.24±2.05
	ANOVA			
Treatment	0.45$^{n.s.}$	0.59$^{n.s.}$	0.79$^{n.s.}$	0.87$^{n.s.}$

[1] Results are expressed as mean ± SD; [2] $^{n.s.}$, nonsignificant

Similarly in some other studies netting, or application of nets with higher shading properties (when there was no control), did not achieve significant effect on peach yield or yield efficiency (Giaccone et al. 2012; Vuković et al. 2016). However, the nonsignificant trend is noticeable where trees grown under nets have higher yield and yield efficiency than those from control, although high standard deviation should be also taken into account. If recalculated it presents yield increase of around 4 t per hectare. It must be highlighted that the application of nets did not cause a significant increase in the share of decayed fruit, meaning that possible increase of relative air humidity and plant wetness duration under nets, which was reported by other authors (De Paula et al. 2012; Shahak et al. 2004b), did not have a significant positive effect on fruit fungi infection under nets.

3.3. Fruit skin colour parameters

According to ANOVA, treatments had a significant effect on b* ($P<0.05$) and C* ($P<0.01$) fruit background colour parameters, while on other background colour parameters no significant difference was obtained (Table 4). Peaches grown under the D net had significantly smaller b* and C* background colour values than those grown under the Y net and from C (Table 4).

Table 4- Background colour parameters of peach fruit cultivated under anti-insect photoselective nets

Treatment	*L**	*a**	*b**	*C**	*h°*
C	63.39±2.77	6.27±3.43	40.91±2.80 a	41.53±2.74 a	81.28±4.84
Y	61.82±3.93	6.52±3.61	40.67±4.38 a	41.35±4.33 a	80.87±5.20
D	61.15±3.75	5.28±4.02	37.96±3.93 b	38.57±3.41 b	81.68±6.79
	ANOVA				
Treatment	$0.044^{n.s.}$	$0.34^{n.s.}$	0.016^{*}	0.008^{**}	$0.82^{n.s.}$

[1] Results are expressed as mean ± SD; [2] Means followed by different letters within columns and are significantly different (Tukey's HSD test; $P \leq 0.05$); [3] $^{n.s.}$, *, **, nonsignificant, or significant at $P \leq 0.05$, $P \leq 0.01$, respectively

Background colour is a primary criterion maturity used for commercial peach harvest (Lewallen & Marini. 2003), because it changes along with other important parameters such as soluble solids, flesh firmness and violate compounds (Ramina et al. 2008). In most peach cultivars assessment of fruit maturity by skin background colour changes include transformation from green to yellow colour (Crisosto & Valero 2008). Therefore, peaches grown under the D net probably delayed fruit ripening which is indicated by smallest intensity of yellow colour.

According to ANOVA, treatments had a significant effect on all additional fruit colour parameters with exception of a* colour parameter (Table 5). Peaches from C had significantly smaller values of b* and C* additional colour parameters than those grown under the Y and D net. Peaches grown under the D net had significantly higher L* and h° values than those from C, while in comparison to peaches grown under the Y net no significant difference was recorded (Table 5).

Table 5- Additional colour parameters of peach fruit cultivated under anti-insect photoselective nets

Treatment	*L**	*a**	*b**	*C**	*h°*
C	31.22±4.55 b	10.61±2.74	10.74±4.30 b	15.23±4.66 b	44.19±7.86 b
Y	33.01±5.38 ab	13.07±3.61	14.58±5.64 a	19.70±6.32 a	47.29±6.55 ab
D	35.97±5.61 a	11.85±3.27	15.47±5.64 a	19.63±6.07 a	51.62±7.00 a
	ANOVA				
Treatment	0.008^{**}	$0.09^{n.s.}$	0.015^{*}	0.04^{*}	0.0009^{***}

[1] Results are expressed as mean ± SD; [2] Means followed by different letters within columns and are significantly different (Tukey's HSD test; $P \leq 0.05$); [3] $^{n.s.}$, *, **, ***: nonsignificant, or significant at $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$, respectively

Similarly in some other studies netting, or application of nets with higher shading properties (when there was no control), generally harmed additional fruit colouration (Amarante et al. 2011; Giaccone et al. 2012; Solomakhin & Blanke 2010). Lack of significant difference of some fruit additional colour parameters between peaches grown under the Y net and from C indicates a milder negative effect of the Y net on peach additional fruit coloration. However, application of D net caused notable reduction of peach additional colour. Since red colour for retailers historically presents one of the main fruit quality components (Crisosto & Costa 2008), this is huge drawback for application of D net. Such fruit colour changes are caused by nets light environment modification since according to Hamadziripi (2012) light intensity that reaches fruit skin has crucial effect on colour development. According to Westwood (1993), exposure of the peach fruit to direct light is necessary for the development of red colour, therefore enhancement of scattered light by photoselective nets (Oren-Shamir et al. 2001; Shahak et al. 2004a, 2004b) cannot alleviate negative effects of shading on additional peach colour development. Hence, fruit additional colour parameters and indexes were highly reduced under the D net due to its high shading factor (indicated by smallest mesh size).

3.4. Fruit firmness, total soluble solids, titratable acidity and SSC / TA ratio

According to ANOVA, treatments revealed a significant effect on fruit firmness, TA and SSC/TA ratio ($P<0.001$) (Table 6). Peaches grown under the Y and D net had significantly higher fruit firmness than those from C (Table 6). Peach fruit firmness (with skin background colour) is one of the main maturity indices used to determine and supervise harvesting operations (Crisosto & Valero 2008). In available literature there is inconsistency regarding effect of netting on this fruit trait (Brkljača et al. 2016; Giaccone et al. 2012; Shahak et al. 2004a; Vuković et al. 2016), which may be contributed to the different agro-ecological conditions and hereditary factors.

Although no significant difference was recorded in SSC of peach fruits, a notable non-significant trend is evident where fruits of peaches from C (10.43±1.08%) tend to have higher SSC than those grown under the Y and D net (9.93±0.79 and 9.79±0.91%, respectively) (Table 6). According to Iglesias & Echeverría (2009; after Clareton 2000) soluble solids below 10% are generally

unacceptable to consumers. Therefore, only peaches from C scored satisfactory SSC levels, while peaches grown under the D net will probably cause negative consumer response. In most studies netting, or application of nets with higher shading properties (when there was no control), reduced peach fruit SSC levels (Shahak et al. 2004a ; Amarante et al. 2011; Giaccone et al. 2012; Brkljača et al. 2016) or did not achieve significant effect (Amarante et al. 2011; Corollaro et al. 2015; Vuković et al. 2016). Since SSC is under high influence of light availability (Corelli Grappadelli & Marini 2008) it is clear that the highest average reduction of SSC occurred under the D net due to the highest shading factor (indicated by smallest mesh size).

Peaches from C (0.54±0.09%) had significantly smaller TA than those grown under the Y and D net (0.63±0.06 and 0.65±0.08%, respectively) (Table 6). According to a study conducted by Bassi & Selli (1990) variety 'Suncrest' has high levels of acids and they may contribute to its unsatisfactory taste. In available literature there is inconsistency regarding effect of netting on this fruit trait (Shahak et al. 2004a; Giaccone et al. 2012; Lobos et al. 2013; Vuković et al. 2016, 2020).

Peaches from C had a significantly higher SSC / TA ratio than those grown under the Y and D net (Table 6). SSC / TA is important factor in consumer acceptance (Crisosto & Kader 2000).

Table 6- Firmness, total soluble solids (SSC), titratable acidity (TA) and SSC/TA ratio of peach fruits cultivated under different anti-insect photoselctive nets

Treatment	*Firmness (kg cm^{-2})*	*SSC (°Brix)*	*TA (% as malic)*	*SSC / TA*
C	4.29±0.62 b	10.43±1.08	0.54±0.09 b	19.96±4.19 a
Y	4.87±0.46 a	9.93±0.79	0.63±0.06 a	15.83±2.45 b
D	5.20±0.63 a	9.79±0.91	0.65±0.08 a	15.34±2.05 b
	ANOVA			
Treatment	<0.0001***	0.15$^{n.s.}$	<.0001***	<.0001***

[1] Results are expressed as mean ± SD; [2] Means followed by different letters within columns and are significantly different (Tukey's HSD test; P ≤0.05); [3] $^{n.s.}$, ***, nonsignificant, or significant at P≤0.001, respectively

3.5. Pectin

No significant difference was recorded in a share of water-soluble and ammonium oxalate-soluble pectin in peach fruits (Table 7). However, peaches grown under the Y net had a significantly higher share of alkali-soluble pectin than those grown from C, while between those grown under the D net in comparison to those grown under the Y net and from C no significant difference was recorded. However, a strong non-significant trend must be noted where peaches grown under the D net tend to have higher share of alkali-soluble pectin than those from C (Table 7).

Table 7- The share of water-soluble pectin (SWP), the share of ammonium oxalate-soluble pectin (SAOP), the share of alkali-soluble pectin (SAP) of peach fruits cultivated under different anti-insect photoselective nets

Treatment	*SWP (%)*	*SAOP (%)*	*SAP (%)*
C	41.26 ± 6.77	51.15 ± 9.12	7.60 ± 5.10 b
Y	34.31 ± 6.01	34.69 ± 5.57	31.00 ± 9.71 a
D	41.07 ± 5.65	42.93 ± 9.17	15.99 ± 7.67 ab
	ANOVA		
Treatment	0.3$^{n.s.}$	0.1$^{n.s.}$	0.017*

[1] Results are expressed as mean ± SD; [2] Means followed by different letters within columns and are significantly different (Tukey's HSD test; P≤0.05); [3] $^{n.s.}$, *, nonsignificant, or significant at P≤0.05, respectively

Around 15 to 17 days after the full bloom of peaches, protopectins insoluble to water are hydrolysed into pectin fractions that are soluble in water (Selli & Sansavini 1995). Softening of peach fruits is accompanied by the transformation of pectin insoluble in water to water-soluble pectin that gives the fruit the characteristic texture of ripe fruit (Jia et al. 2006). Since peaches grown under the D and Y net also had significantly higher fruit firmness and TA than those from C as well as reduced b* (significantly) and a* (notable non-significant trend) value of background colour for peaches cultivated under the D net, together with pectin fractions results, it may indicate delayed fruit ripening of peaches grown under the D and Y net in contrast to those from C. Similarly, Ordóñez et al. (2016) reported that at commercial maturity apples 'Golden Delicious' grown under the white and black nets (6-7 and 16% of shading, respectively) significantly differ in fruit firmness, SSC and TA. However, according to the same author if data was compared at the beginning of climacteric rise in fruits then no significant difference was recorded. Possibly, by later harvest date, some of negative effects of nets on inner peach fruit quality parameters can be lessen. Additional peach coloration (which is important parameter for peaches) under D net almost certainly cannot be considerably improved by delayed harvest date because direct light exposure of the peach fruit is necessary for the development of red colour (Westwood 1993) and D net has smallest mesh size (hence highest shading factor). Rapid ripening of peaches should also be taken into account.

3.6. Total polyphenolic content, antioxidant potential

According to the ANOVA, treatments achieved a significant effect on the total polyphenol content, ABTS and DPPH antioxidant activity ($P<0.001$) and total anthocyanin content (Table 8). Total polyphenol content and ABTS antioxidant activity were significantly higher in peaches from the C than in those grown under the Y and D net. DPPH antioxidant activity was significantly smaller in fruits grown under the Y net than in those under the D net and from C (Table 8).

In south Italy, Basile et al. (2012) reported significantly smaller content of total polyphenols and antioxidant activity in the flesh of kiwi 'Hayward' grown under the white net than from C. The scarcity of studies related to these biochemical parameters emphasizes the importance of the results obtained in this study. Light intensity and quality can affect the biosynthesis of antioxidants and phenols (Bakhshi & Arakawa 2006; Jurić et al. 2020), and the concentration of certain polyphenols is being increased when fruits are exposed to UV light because flavonoids can absorb UV radiation and therefore prevent tissue damage (Arakawa et al. 1985). Since it was reported that white and yellow nets absorb UV radiation (Shahak 2008; Shahak et al. 2004b), it can be assumed that the Y and D nets by reduction of UV light transmittance caused reduction in total polyphenol content and ABTS antioxidant activity of peach fruits. Interestingly, fruits of peaches grown under the D net did not significantly differ in DPPH antioxidant activity with peaches from C. Moreover, it must be highlighted that peaches grown under the D net had a significantly smaller amount of total polyphenols and ABTS antioxidant activity than those from C. The main reason for this occurrence is probably because ABTS radical can react with both hydrophilic and lipophilic antioxidants (Prior et al. 2005), while DPPH only with lipophilic antioxidants (Jatoi et al. 2017). Since peach fruit contains a significant amount of carotenoids (Oliveira et al. 2016) (which are lipophilic antioxidants) it is possible that such results were obtained due to a higher amount of carotenoids of peaches grown under the D net (indicated by a non-significant trend).

3.7. Pigments

Regarding the β-carotene levels, in peach fruits, no significant difference was recorded between treatments (Table 8). However, according to a non-significant trend, peaches grown under the D net tend to have a somewhat higher amount of β-carotene. Peaches grown under the D net had significantly smaller total anthocyanin content than those from C, while between peaches grown under the Y net and other treatments no significant difference was recorded (Table 8).

Table 8- Total polyphenol content (TPC), ABTS antioxidant potential (AOP – ABTS), DPPH antioxidant potential (AOP – DPPH), β Carotene content and total anthocyanin content (TAC) of peach fruits cultivated under different anti-insect photoselective nets

Treatment	*TPC (mg GAE 100 g^{-1} fw)*	*AOP –ABTS (µmol TE 100 g^{-1} fw)*	*AOP - DPPH (µmol TE 100 g^{-1} fw)*	*β Carotene (µg g^{-1} fw)*	*TAC (µmol cm^{-2})*
C	21.45±3.06 a	67.38±4.56 a	40.68±3.16 a	5.02±1.30	1.75±1.01 a
Y	15.64±2.26 b	45.08±6.76 b	35.00±4.22 b	5.65±1.81	1.21±0.60 ab
D	18.14±2.18 b	44.90±6.84 b	40.02±1.75 a	7.35±2.44	0.71±0.32 b
	ANOVA				
Treatment	0.0018**	0.0011**	0.007**	0.1$^{n.s.}$	0.047*

[1] Results are expressed as mean ± SD; [2] Means followed by different letters within columns and are significantly different (Tukey's HSD test; $P\leq0.05$); [3] $^{n.s.}$, *, **, nonsignificant, or significant at $P\leq0.05$, $P\leq0.01$, respectively

According to Iglesias et al. (1999) anthocyanin content is directly related to a / b fruit colour ratio and inverse with h and L colour values. It was also the case in this study (Tables 5 and 8) meaning that reduction of anthocyanin content in peaches grown under D net was the main reason for poor additional colour development. Similar results were obtained in Germany by Solomakhin & Blanke (2010) on sun-exposed part of the apple 'Pinova' protected by different types of photoselective nets. In peaches, exposure of fruit to direct light is necessary for the development of red colour (Westwood 1993) and hence for the synthesis of anthocyanins. Therefore, it is evident that D net, due to its high shading factor (indicated by smallest mesh size), achieved the highest average reduction of total anthocyanin content in peach fruits. Changes in light quality can also influence fruit anthocyanin content. Shorter wavelengths, in a range from blue to UV light, show the most prominent influence on the accumulation of flavonoids (anthocyanins) in fruit (Zoratti et al. 2014). White and yellow nets absorb UV light (Shahak 2008; Shahak et al. 2004b) and it may also contribute to the reduction of total anthocyanin content in fruit skin. Since D net has relatively small mesh size, it might had caused the highest reduction of UV light transmittance.

4. Conclusions

Application of nets significantly affected a notable part of studied vegetative and generative peach traits. Application of Y net significantly enhanced some leaf vegetative parameters which were not the case for the D net, while shoot vegetative parameters were not significantly affected by the net application. A significant effect on productivity parameters was not achieved by the application of nets. Application of Y net generally achieved a smaller reduction of peach fruit quality traits than D net, and in some cases did not significantly differ with results obtained by peaches from C. Bioactive compounds and antioxidant activity of fruits were generally reduced as a consequence of net application. It can be concluded that application of Y net only slightly

reduced peach fruit quality compared to C (trees grown without net), and therefore can be recommended for application as an anti-insect net. Moreover, due to its additional properties (anti-hail, anti-insect, etc.), its application will overall have a positive effect. However, D net more notably reduced peach fruit quality parameters (and especially additional fruit colour which is important parameter for peaches). Therefore, it should be used only when other control strategies show to be ineffective.

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Abbreviations

C	Control
Y	Yellow photoselective net
D	Stop Drosophila Normal net
SSC	Total soluble solids
TA	Titratable acidity

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Energy and Exergy Analysis of Palm Tree Pruning Residues Gasification

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ABSTRACT
Gasification is the process of obtaining syngas containing combustible gases such as H_2, CO and small amounts of CH_4 from biomass by performing partial combustion with a limited oxygen supply or with the help of suitable oxidants such as CO_2 and water vapor. The efficiency of the gasification process is the most important parameter that determines the success of the system. In this study, the performance of the system in the gasification of palm pruning residues was evaluated by energy and exergy analysis methods. The gasification process was carried out at 7.6 Nm^3/h and 10.2 Nm^3/h air flow rates in the laboratory type fixed bed downdraft gasification unit manufactured in the Biosystems Engineering Department. The lower heating value of the syngas obtained as a result of gasification was found to be 4.09 MJ/Nm^3 at 7.6 Nm^3/h air flow rate and 3.76 MJ/Nm^3 at 10.2 Nm^3/h air flow rate. It has been observed that the lower heating value of the syngas is lower at high air flow rate. Energy efficiencies of the gasification system at 7.6 and 10.2 Nm^3/h air flow rate were calculated as 47.6% and 52.8%, and exergy efficiencies were calculated as 43.7% and 48.1%, respectively. Exergy efficiencies were found to be lower than energy efficiencies in both air flow rates. However, as the air flow rate increased, the energy and exergy efficiencies also increased. The results obtained are similar to the results of previous studies on gasification of biomass.

Keywords: Biomass, Syngas, Heating value, Irreversibility, Partial combustion, LHV

1. Introduction

Biofuels which are obtained from biomass have considerable potential as a clean and renewable energy source, which can be converted into secondary energies such as, generate electricity, heat and power. However, the most important challenge in biomass-based energy conversion systems is to develop efficient conversion technologies (Ptasinski et al. 2007). Energy conversion technologies of biomass are gathered under basic topics such as thermochemical, biochemical and extraction. Main thermochemical conversion methods are direct combustion, gasification and pyrolysis. The gasification process is also one of the thermochemical conversion methods of biomass, and the syngas which contains such as H_2, CO, CH_4, and CO_2 gases obtained as a result of the process can be used to obtain electrical energy, heat energy, and enriched hydrogen to fuel cells (Hao et al. 2003). In gasification process, air is generally used as gasification agent due to its low cost. When the air is used in the gasification process of the biomass, the low heating value (LHV) of obtained syngas is about 4-7 MJ Nm^{-3} depending on the raw material. 12-28 MJ Nm^{-3} of LHV can be obtained with using pure O_2, but the cost of syngas production increases due to cost of O_2 production (Laurence & Ashenafi 2012; Manatura et al. 2017).

Energy and exergy analysis are conducted to estimate the efficiency of the system in all energy conversion systems. Efficiency estimation, known as energy efficiency based on the first law of thermodynamics, is the standard measure of an energy producing system. Exergy is the maximum amount of work a system can do reversibly until it becomes dead state under ideal conditions (Thermodynamic equilibrium). Efficiency evaluation with exergy analysis is more meaningful than energy analysis since it is a measure of the approach to ideal conditions (Zhang et al. 2013). This thermodynamic analysis technique estimates the efficiency of the process and determines its energy quality and availability. Exergy analysis, on the other hand, is used to discover new ways to increase energy efficiency. This estimate is made in terms of the second law of thermodynamics and irreversibility. In recent years, exergy analysis has been used to evaluate the performance analysis of different systems and to improve their efficiency (Cohce et al. 2011; Saidur et al. 2012). Many exergy and energy researches have been conducted on biomass gasification.

Exergy and energy analyzes have been made in the gasification of municipal wastes in fluidized bed gasifier. It is stated that the maximum energy and exergy efficiency is achieved at 0.4 of ER and 650 °C temperature (Tang et al. 2016). Pre-drying the biomass in the gasification process increases energy and exergy efficiency (Karamarkovic & Karamarkovic 2010). In the

gasification process performed with high temperature steam, energy and exergy analyzes were carried out at different steam/biomass (S/B) ratios. It was stated that as the S/B ratio increases, exergy and chemical energy efficiency are negatively affected, while high preheating process positively affects the efficiency (Wu et al. 2014). Coconut shell, coir pith, bamboo and eucalyptus are gasified with steam. The highest exergy efficiency was achieved in the gasification process of coir pith with 79.2%. The exergy efficiencies of coconut shell, bamboo and eucalyptus were 77.5%, 74.4% and 68.3%, respectively (Sreejith et al. 2013). In the research conducted to compare exergy analysis in the gasification process of different biofuels, materials of different vegetable origin, vegetable oils and fertilizer were used. It is stated that exergy efficiency of solid biomass is lower than coal in the gasification process, and exergy efficiency increases when using obtained syngas in the drying process of the feedstock (Ptasinski et al. 2007). The type of biomass, the moisture content and the temperature of the gasification agent significantly affect exergy efficiency, the composition and heating value of the syngas. The performance of the gasification process improves as the moisture content of the biomass decreases and the temperature of the gasification agent increases. Exergy efficiency and working temperature are also significantly affected by the moisture content of the biomass. These values decrease as the humidity increases. Therefore, before the gasification process, the biomass containing excess moisture must be dried and brought to an acceptable humidity level (Wang et al. 2013). Because of its rich hydrogen production, the gasification process with steam generally has higher exergy efficiency than partial oxidation (Zhang et al. 2012). As the gasification temperature increases, the exergy efficiency increases, while the increase in the particle size of the material decreases the exergy efficiency. The steam/biomass ratio and flow rate initially increase exergy efficiency but later decrease it (Zhang et al. 2019). Chern et al. (1989) gasified wood chips at different air temperatures (25 °C, 293 °C and 348 °C) in their study. Researchers stated that as the air temperature increases, the exergy efficiency increases. Exergy efficiency of wood chips gasification at the highest air temperature was 65%. Moisture is an important problem in the gasification of sugar beet bagasse. In order to realize the gasification process, the moisture content in the bagasse must be below 30%. In addition, the preheating process increases exergy efficiency (Pellegrini & de Oliveira 2007). Hosseini et al. (2012) also stated that exergy efficiency in the gasification process of sawdust is significantly affected by the moisture content of the biomass.

It is stated that there are approximately 700 000 palm trees in cities located in the Mediterranean region of Turkey (Hazir & Buyukozturk 2013). In a study conducted to determine the physical and thermal properties of palm pruning residues in the province of Antalya, located in the Mediterranean region, it was stated that an average of 50 kg of pruning residue per year was obtained from a palm tree (Yilmaz et al. 2021). As it can be understood from these studies, 35 000 tons of palm pruning residue is generated every year in the Mediterranean region. The energy value of these residues is around 582 400 GJ/year. As can be seen, although palm pruning residues have a significant energy potential in the region, there is not enough research on their conversion to energy. In this research, it is aimed to determine the possibilities of evaluating this energy potential by gasification method. For this purpose, the pellets obtained from palm pruning residues were gasified in a micro-scale gasification unit at two different air feeding rates, and energy and exergy analyzes were carried out.

2. Material and Methods

2.1. The gasifier system

Experimental set up of the gasifier system includes gasifier reactor, cyclone, gas cooling unit, and vacuum pump and flare unit (Figure 1). Flare is a syngas burning unit. In addition, there are measurement and control components, gas chromatography device and its components in the system. The gasifier reactor is fixed-bed and downdraft type and has 170 mm in diameter, 750 mm in height and is made of high temperature resistant 5 mm thick stainless steel. Temperature sensors are installed in the reactor inlet and outlet. In addition, there are K-type thermocouples at 5 different points in the reactor. Air and syngas flow rates were measured by orifice flow meters. Experimental data was collected and monitored by PLC control system. The vacuum pump is located in the gas outlet line and also provides the suction of syngas. The pump operates with three-phase current and has a rated power of 0.37 KW and a maximum flow rate of 30 m^3/h. During the gasification process, the temperature of core region in the gasifier was kept about at 800 °C.

Figure 1- The gasification system units

2.2. Feedstock

In this research, pruning residues of palm trees were used in the gasification process. The pruning residue pellets were supplied from a company that produces pellets in Antalya.

Some physical properties and ultimate analysis results of pellets have been given in Table 1.

Table 1- Some physical properties and ultimate analysis results of palm residue pellets

Physical properties	Values
Moisture (% db.)	5.95
Average pellet diameter (mm)	6
Average pellet length (mm)	35
pellet density (kg/m^3)	1000
Ultimate analysis results	
Ash Content (%)	6.30
Volatile substance content on the original basis (%)	69.33
Volatile matter content on a dry basis (%)	73.12
Fixed carbon content (%)	20.58
Carbon (%)	44.15
Hydrogen (%)	5.63
Nitrogen (%)	1,02
Oxygen (%)	42.09

The lower heating value of biomass was calculated by the following equation (Rupesh et al. 2020);

$$LHV_{Biomass} = 0.0041868(1 + 0.15[O]\left(7837.667[C] + 33888.889[H] - \frac{[O]}{8}\right) \quad (1)$$

Where; O, H, and C are the weight percentage of oxygen, hydrogen, and carbon elements in the biomass obtained from ultimate analysis.

2.3. Syngas and Air flow rate measurements

The electric motor operating the vacuum pump during gasification was operated at two different frequencies (25 Hz and 35 Hz). Syngas flow measurement with orifice type flow meter placed in the gas line before the gas sample outlet point was carried out in accordance with EN ISO 5167-2 standard.

After the syngas flow rate measurement, the air flow rate has been determined by the equation below (Dalmis et al. 2018);

$$AFR = \frac{GFR * N_{syngas}}{N_{air}} \quad (2)$$

2.3. Syngas analysis

The syngas sample with the help of a pipe from the main gas output line were taken and analyzed with Agilant 7890A GC model gas chromatography device. The device gives percentages by volume of gas components (H_2, CO, CH_4, CO_2 and N_2) contained in the syngas.

2.4. Energy and exergy analysis

The mass and energy flows used in the energy and exergy calculations in the gasification process are shown schematically in Figure 2.

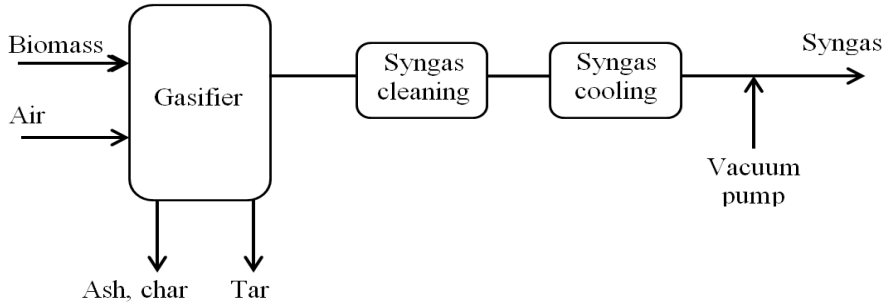

Figure 2- Schematic view of mass and energy flows in the gasification process

2.4.1. Energy analysis

Kinetic $(\dot{Q}_{ki})$ and potential $(\dot{Q}_{po})$ energy flows of syngas can be neglected. Thus, total energy flow can be shown as below (Manatura et al. 2017);

$$\dot{Q}_{syngas} = \dot{Q}_{ph} + \dot{Q}_{ch} \quad (3)$$

The physical (sensitive) energy of syngas is calculated as below;

$$\dot{Q}_{ph} = \dot{m}_{syngas}\, \Delta h = \dot{m}_{syngas} \int_{T_0}^{T} C_p dT \quad (4)$$

The chemical energy flow of syngas is calculated as below;

$$\dot{Q}_{ch,syngas} = \dot{m}_{syngas}\, LHV_{syngas} \quad (5)$$

The chemical energy flow of biomass, tar and syngas can be calculated by equations given below;

$$\dot{Q}_{ch,biomass} = \dot{m}_{biomass}\, LHV_{biomass} \quad (6)$$

$$\dot{Q}_{ch,tar} = \dot{m}_{tar}\, LHV_{tar} \quad (7)$$

The LHV of syngas is calculated by equations given below (Wang et al. 2013);

$$LHV_{syngas} = 12.622 x_{CO} + 35.814 x_{CH_4} + 10.788 x_{H_2} \quad (8)$$

The energy efficiency of the gasification can be calculated by the following equation;

$$\eta_{En} = \frac{\dot{Q}_{syngas}}{\dot{Q}_{biomass} + \dot{Q}_{electricity}} \quad (9)$$

2.4.2. Exergy analysis

Exergy analyzes of the gasification of palm pruning pellets were performed by the method applied by Tang et al. (2016), Wang et al. (2013) and Zhang et al. (2010).

Only chemical exergy was considered for biomass. The exergy of biomass can be defined as;

$$\dot{E}x_{biomass} = \beta \dot{m}_{biomass}\, LHV_{biomass}$$

Chemical exergy $(\dot{E}x_{ch})$ and physical exergy $(\dot{E}x_{ph})$ are the sum of exergy $(\dot{E}x)$ of syngas;

$$\dot{E}x_{syngas} = \dot{E}x_{ch} + \dot{E}x_{ph} \quad (11)$$

The chemical exergy of syngas can be determined by the composition analysis of syngas and the flow rate. Its value is obtained from the following equation;

$$\dot{E}x_{ch} = \dot{m}_{syngas}(\sum_i (y\ ex_{ch})_i + RT_0 \sum_i (y\ \ln y)_i) \quad (12)$$

The standard chemical exergy of gases (ex_{ch}) can be obtained from any thermodynamics book. $\dot{m}_{syngas}$ is the syngas output rate from the gasifier.

The physical exergy of syngas is determined as:

$$\dot{E}x_{ph} = \dot{m}_{syngas}\ \sum_i \left(y\ ex_{ph}\right)_i \quad (13)$$

For each gas components, the specific physical exergy in kJ kmol^{-1} is defined as:

$$ex_{ph} = (h - h_0) - T_o(s - s_0) \quad (14)$$

The exergy efficiency of the gasification system can be calculated by the following equation;

$$\eta_{Ex} = \frac{\dot{E}x_{syngas}}{\dot{E}x_{biomass} + \dot{E}x_{electricity}} \quad (15)$$

The irreversibility of the gasification system is calculated as below;

$$I_r = \dot{E}x_{input} - \dot{E}x_{output} \quad (16)$$

3. Results and Discussion

The lower heating value (LHV) of pellets was calculated as 16640 kJ/kg by Equation 1. The air mass flow rate (AFR), syngas flow rate (GFR) and fuel consumption rate (FCR) obtained as a result of the gasification process performed by operating the electric motor connected to the vacuum pump at 25 Hz and 35 Hz frequencies are given in Table 2. These values express the performance of the gasification process. The specific gas production rates (SGPR) which are also given in Table 2 were calculated by using GFR and FCR values. Gunarathne et al. (2013) gasified the wood particles of rubber trees in a downstream gasifier and found the specific gas production rate between 2.84 Nm^3/kg-biomass and 2.91 Nm^3/kg-biomass. The specific gas production rate in the gasification of the grass pellet was found between 1.57 Nm^3/kg-biomass and 1.96 Nm^3/kg-biomass (Diken & Kayişoğlu, 2020). Specific gas production rates provided in the gasification of palm pruning wastes in this study were seen to be closer to the values obtained in the gasification of rubber tree wood particles. In the gasification process at both stages, the temperature of the core region varied between 700 °C and 800 °C.

Table 2- Performance parameters of palm pruning residues gasification

AFR (Nm^3/h)	*GFR (Nm^3/h)*	*FCR (kg/h)*	*SGPR (Nm^3/kg biomass)*
7.6	9.57	3.89	2.46
10.2	12.08	4.27	2.83

Gas components of syngas and heat values are given in Table 3. Therefore, the lower heating value of syngas at 7.6 Nm^3/h air flow rate were higher than 10.2 Nm^3/h air flow rate. The higher CH_4 rate in the syngas at this air flow rate was the most important factor that increased the lower heating value. Samadi et al. (2020) carried out a research in order to estimate the energy to be obtained in the gasification of biomass. Researchers stated that the specific heat values are high at small air flow rates, and the specific heat values decreases as the air flow rate increases.

Table 3-Rate of gas components and heat values of syngas

AFR(Nm^3/h)	*Gas components in syngas(%)*					*Heating value(MJ/Nm^3)*	
	H_2	*CO*	*CH_4*	*CO_2*	*N_2*	*LHV*	*HHV*
7.6	13.79	13.21	2.62	15.49	54.88	4.09	4.47
10.2	12.77	13.29	1.97	12.95	59.03	3.76	4.08

Dalmis et al. (2018) found the lower heat value of the syngas between 3.61 MJ/Nm^3 and 4.59 MJ/Nm^3 in the study where they gasified rice straw pellets. (Diken & Kayişoğlu, 2020) stated that the lower heat value of syngas obtained from gasification of grass pellets changes between 3.83 MJ/Nm^3 and 3.92 MJ/Nm^3. The lower heat value in the gasification of wood sawdust pellet was around 5.7 MJ/Nm^3 (Simone et al. 2012). Pellegrini and Oliveira (2007) found the lower heat value between 4.7 MJ/Nm^3 and 5.1 MJ/Nm^3 in the gasification of sugar cane with steam + air. Rao et al. (2004) determined the upper thermal values of the synthesis gases obtained by gasification of different biomass. Researchers found that these values are 5.6 MJ/Nm^3 in urban waste, 5.0 MJ/Nm^3 in wood sawdust, 4.82 MJ/Nm^3 in soybean pellets, 4.95 MJ/Nm^3 in corn cobs, 4.76 MJ/Nm^3 in pea stems and 4.80 MJ/Nm^3 in peanut shells. In this study, where the palm pruning wastes were gasified, heat values of obtained syngas were seen to be close to the values obtained in the gasification processes previously made using different biomass.

Energy and exergy balances at 7.6 Nm^3/h and 10.2 Nm^3/h air flow rate are given in Table 4 and 5. Energy and exergy efficiencies at 7.6 Nm^3/h were 50.1% and 43.2%, respectively. These values were 52.6% and 45.1% at 10.2 Nm^3/h. Energy efficiencies were higher than exergy efficiencies at both air flow rates. In addition, irreversibility value was higher than lost energy at both stages. In many previous studies, it is seen that the energy efficiency in the gasification of biomass is higher than the exergy efficiency (Cohce et al. 2011; Wu et al. 2014).

Table 4- Energy and exergy balances at 7.6 Nm^3/h air flow rate

Energy input	***(kW)***	***Exergy input***	***(kW)***
Biomass	17.98	Biomass	20.46
Electricity	0.37	Electricity	0.37
TOTAL	**18.35**	**TOTAL**	**20.83**
Energy output		**Exergy output**	
Chemical energy	8.56	Chemical exergy	8.49
Physical energy	0.12	Physical exergy	0.0021
Tar	0.51	Tar	0.51
Lost energy	9.16	Irreversibility	11.83
Energy efficiency (%)	**50.1**	**Exergy efficiency (%)**	**43.2**

Table 5- Energy and exergy balances at 10.2 Nm^3/h air flow rate

Energy input	***(kW)***	***Exergy input***	***(kW)***
Biomass	19.73	Biomass	22.46
Electricity	0.37	Electricity	0.37
TOTAL	**20.10**	**TOTAL**	**22.83**
Energy output		**Exergy output**	
Chemical energy	9.92	Chemical exergy	9.78
Physical energy	0.15	Physical exergy	0.0026
Tar	0.51	Tar	0.51
Lost energy	9.52	Irreversibility	12.53
Energy efficiency (%)	**52.6**	**Exergy efficiency (%)**	**45.1**

Beno et al. (2020) stated that the maximum thermal efficiency and exergy at the gasification of paddy husks are obtained at ER = 0.20 and decreases rapidly above this value. Researchers reported that thermal efficiency is 78% and exergy value is around 70%. In a study where poplar sawdust was gasified, it was stated that the average exergy efficiency was 48% and this value was close to coal gasification (Iribarren et al. 2014). Coconut shell and charcoal were gasified by mixing in certain proportions. When the coconut shell was gasified alone, the exergy efficiency was around 36%. Exergy efficiency in the mixture of 70% coconut shell and 30% charcoal exceeded 50% (Monir et al. 2018). Gu et al. (2019) used air, oxygen-enriched air and pure oxygen as gasifying agents to gasify the paddy stalk. The researchers found exergy yields in gasification as 55%, 58% and 63% respectively. Zhang et al. (2018) conducted exergy analyzes in conventional and partial gasification of biomass and found it to be 65.6% and 68.0%, respectively. The highest thermal energy and exergy efficiencies in the gasification processes performed with preheated air and steam at different temperatures were found as 81.5% and 76.2%, respectively, at the 1.83 steam/biomass ratio (Wu et al.2014). The maximum gasification exergy efficiency of pine with 10% moisture content is 73.73%, which is 1.45% and 2.67% higher than that of pine with 20% and 30% moisture content. The maximum gasification exergy efficiency of sawdust with 10% moisture content is 65.12% which is 1.14% and 1.96% higher than that of sawdust with 20% and 30% moisture (Wang et al. 2013). As can be seen, the energy and exergy efficiency in the gasification of biomass can be in a very different range depending on the composition of the biomass, its moisture content and the properties of the gasification agent. It is possible to say that the energy and exergy efficiency values obtained in this study are within acceptable limits.

4. Conclusions

In this study, palm pruning residues were gasified at two different air flow rates and the energy and exergy efficiencies of the gasification process were investigated. Energy efficiency was higher than exergy efficiency at both air flow rates. It was observed that the gas has a higher thermal value at a lower air flow rate. Despite the differences in the structure of the biomass, the thermal value, energy and exergy efficiency values obtained in this research have remained within the limits of the results obtained in the previous studies. Palm is grown as an oil plant in Far East Asian countries. In Turkey there are quite a lot, especially the palm populations in parks and gardens in the Mediterranean region. With the pruning of palm trees, thousands of tons of waste are generated each year. The evaluation of these wastes as an energy source through gasification will make important contributions to the economy and the environment.

This article is derived from the master thesis which prepared by Demirtas (2019), "Gasification of Palm Tree Pruning Waste (in Turkish) ".

Abbreviations and Symbols

AFR	Air flow rate (Nm^3/h)
C_p	The specific heat at constant pressure of the syngas (kJ kmol/K)
$\dot{E}x_{biomass}$	The exergy flow of the biomass (kW)
$\dot{E}x_{syngas}$	The exergy flow of the syngas (kW)
$\dot{E}x_{ch}$	The chemical exergy flow of the syngas (kW)
$\dot{E}x_{ph}$	The physical exergy flow of the syngas (kW)
ex_{ch}	The standard chemical exergy of gas components (kJ/kmol)
ex_{ph}	The specific physical exergy of gas components (kJ/kmol)
GFR	Syngas flow rate (Nm^3/h)
h, h_0	The specific enthalpy at the current and dead state of syngas components (kJ/kmol)
I_r	The irreversibility of the gasification system (kW)
$LHV_{Biomass}$	The low heating value of the biomass (kJ/kg)
LHV_{tar}	The low heating value of the tar (kJ/kg)
LHV_{syngas}	The low heating value of the syngas (kJ/Nm^3)
$\dot{m}_{Biomass}$	The mass flow rate of the biomass (kg/s)
$\dot{m}_{tar}$	The mass flow rate of the tar (kg/s)
$\dot{m}_{syngas}$	The mass flow rate of the syngas (kmol/s in Equation 3 and Equation 11, Nm^3/s in Equation 4)
N_{syngas}	Volumetric ratio of nitrogen in the syngas (%)
N_{air}	Volumetric ratio of nitrogen in the air (%)
$\dot{Q}_{syngas}$	The total energy flow of the syngas (kW)
$\dot{Q}_{ph}$	The physical energy flow of the syngas (kW)
$\dot{Q}_{ch,syngas}$	The chemical energy flow of the syngas (kW)
$\dot{Q}_{ch,biomass}$	The chemical energy flow of the biomass (kW)
$\dot{Q}_{ch,tar}$	The chemical energy flow of the tar (kW)
$\dot{Q}_{ch,syngas}$	The chemical energy flow of the syngas (kW)
$\dot{Q}_{electricity}$	The electrical input to gasification system (kW)
s, s_0	The specific entropy at the current and dead state of syngas components (kJ/kmol K)
T_0	The temperature at dead state (298.15 K)
T	The syngas outlet temperature (K)
y_i	The molar fraction of each gas composition (i) in the syngas.
x_{CO},x_{CH_4}, x_{H_2}	Molar fractions of gas components in syngas, respectively
β	The quality coefficient of the biomass
Δh	The specific enthalpy change of the syngas (kJ/kmol)
η_{En}	The energy efficiency of the gasification system
η_{Ex}	The exergy efficiency of the gasification system

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Antioxidant Activity and Essential Amino acid Content of Bread Wheat (*Triticum aestivum* L.) Varieties

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ABSTRACT
In recent years, consumption of healthy and nutritious products has come to the forefront with the increased consumers' awareness because of sensitivity to human health. Bread wheat provides the important proportion of protein, fiber, mineral, and antioxidant compounds that detected wheat and wheat-based products in daily consumption. The study aimed to provide new insights and results on the antioxidant properties and essential amino acid profile of 45 bread wheat varieties collected from different ecological regions of Turkey. Antioxidant activity was measured using DPPH radical and total phenolic content was determined by using the Folin-Ciocalteu method used gallic acid as standard. Essential amino acid content was evaluated by oxidation and hydrolysis procedure by HPLC analysis. As a result of the study, significant differences and correlations were found between the varieties and all evaluated parameters. Total phenolic content and antioxidant activity values respectively ranged between 102.4 and 211.8 µg GAE/g (gallic acid equivalent/g), 11.8 and 26.3% inhibition in whole wheat flour. Amino acid amount and profile were significantly affected by variety and the most limiting amino acid lysine showed a wide range (0.39-1.47 g/100 g flour) and the concentration of leucine and phenylalanine were found to be higher in wheat compared to the other essential amino acids. Correlation analysis between protein and essential amino acids demonstrated a positive correlation with high values. Clustering analysis highlighted an important genetic diversity, suggesting that winter growth habit varieties have valuable nourishment properties with high protein and amino acid composition. These data suggest that health and nourishment properties influenced differently by genotype potential of wheat grain.

Keywords: Whole wheat, Antioxidants, Protein quality, Lysine, DPPH, HPLC, Cluster

1. Introduction

Wheat is a major crop of cereals grown across the world because widely adapted to most climate and soil conditions. Bread wheat is an important food for daily consumption to provide energy and essential nutrients due to its high content of carbohydrate and protein content. In addition to basic nutrients, wheat is also a good source of phytochemicals which can provide additional health benefits (Narwal et al. 2012). Whole grain products are recommended for healthy diets as a source of dietary fiber and antioxidant substances. Biotic and abiotic stress factors lead to the formation of free radicals in plants and our bodies that damage body cells, cell walls or even DNA. Some chemicals (phenolic acids and vitamins) which are taken from the nutrients show antioxidant activity in the human body, prevent these radicals from causing damage. It is known that cereals (especially whole grain products) also have antioxidant activity potential compared to fruits and vegetables and effective in preventing many chronic diseases (Miller et al. 2000; Adom & Liu 2002; Ryan et al. 2011). Consumption of whole-grain products shown to several health benefits such as the reduced risk of cancer, chronic diseases, and cholesterol-lowering effect (Liyana-Pathirana & Shahidi 2005; Gasztonyi et al. 2011; Ma et al. 2014). Dietary fiber is associated with natural antioxidants and reported that it could be metabolized in the colon and utilized by the microbial microbiota for providing health benefits for humans (Xu et al. 2021). Genotype, environment or the interaction and individual environment parameters (temperature stress, precipitation, and drought level) can significantly affect antioxidant properties of wheat grain (Moore et al. 2006; Lv et al. 2013). As well as environmental and climate conditions grain structure and chemical properties of wheat may vary widely by agronomic treatments such as N fertilizer, organic/conventional farming system, pesticide application, and variety growth habit (long/short straw, winter/spring type). As the researchers mentioned that use of nitrogen fertilization, herbicides, and modern short straw varieties can all have a negative effect on the antioxidant content of wheat (Wang et al. 2020). Phenolic content has been found higher in organic growing condition than conventional farming system. Growing conditions and variety-specific properties have a certain effect on the biosynthesis and accumulation of phenolic acids. Total phenolic content of spring wheat varieties grown in organic conditions had higher than that of the wheat grown in conventional conditions (Vaher et al. 2010). Some studies have focused on the relationship between phenolic content or antioxidant activity with grain color and size. Ma et al. (2014a) reported that highly significant genotypic differences were observed in phenolic content and antioxidant activity among wheat varieties which are classified as black

wheat with the highest antioxidant activity followed by red then white grain color wheat. However, antioxidant capacity and phenolic compounds in bran and flour highly depend on milling processes and products. Wheat grain parts such as the aleurone layer has the highest antioxidant activity wheat fraction, followed by the bran (Žilić 2016). Antioxidant activity of wheat flour extracts varies with cultivar and environmental conditions. Therefore, wheat variety might be the primary consideration for the enhanced antioxidant activity of wheat products. (Iqbal et al. 2007; Okarter et al. 2010; Tian & Li 2018). Different environmental conditions affect the phenol and antioxidant activity of wheat, and each antioxidant may respond to the environmental changes differently (Zhou & Yu 2004).

Besides contribution to health, amino acids of wheat, as a part of daily intake of amino acids constitute the importance of daily nutrition. Both in humans and animals nutrient quality is determined by protein content and the balance of amino acids in wheat grain. However, essential amino acids can not be synthesized by animals and in our bodies and hence must be provided in the diet (Shewry 2009; Zhang et al. 2017). The amino acid distribution of wheat is quite unbalanced and protein quality is associated with lacking essential amino acids like lysine, threonine, and methionine (Siddiqi et al. 2020). Lysine is considered the most important and limiting essential amino acid in cereal grains (Ufaz & Galili 2008; Konvalina et al. 2011). The nutritional quality can be improved by increasing protein content and limiting amino acids especially lysine. Due to the inverse relationship between lysine and protein content, a big challenge occurs so lysine can be used as a measure of protein quality (Anjum et al. 2005). Among the few studies that have been done about antioxidant and amino acid content of wheat grain, it is the first result of varieties grown in Turkey. In this study, we investigated the nutrient quality (protein and essential amino acid) and antioxidant properties of wheat varieties commonly grown in Turkey to facilitate the selection of varieties to produce high-quality wheat in breeding and agronomic studies and to give new impulses to future wheat quality efforts.

2. Material and Methods

2.1. Wheat samples

Bread wheat varieties widely grown all-around of Turkey were obtained from Trakya Agricultural Research Institute (Thrace region), Bahri Dağdaş International Agricultural Research Institute (Central Anatolia Region), Field Crops Central Research Institute (Central Anatolia Region), Transitional Zone Agricultural Research Institute (Central Anatolia Region), Maize Research Institute (Marmara Region) and Eastern Mediterranean Agricultural Research Institute (Southeastern Anatolia Region). Forty-five bread wheat varieties are collected after harvesting and stored +4 °C for chemical analysis (Table 1). Wheat varieties were classified due to grain color, spike color and type, growth habit, kernel weight, and adapted climate classification are given in Table 2. Climate zones and growth habits of the varieties were determined based on Köppen-Geiger classification (Öztürk et al. 2017; Yılmaz & Çiçek 2018). According to the adapted growth climate conditions of varieties seven subclimate types (Bsk: arid, steppe and cold summer, Csa: temperate, dry and hot summer, Cfa: temperate, without dry season but hot summer, Dsa: cold, dry and hot summer, Dsb: cold, dry and warm summer, Dfa: cold, without dry season and hot summer, Dfb: cold, without dry season and warm summer) were detected for different regions of Turkey. The Central Anatolia region is described as; the largest subclimate type of B zone (cold, semi-arid climate with cold winters and dry summer). Type C climate is dominant in coastal regions (Thrace, Marmara, Aegean, and Southeastern Anatolia) described as; dry and hot summer without dry season but hot summer. The four subclimate types (Dsb, Dsa, Dfa, Dfb) belong to type D is identified as cold winter, dry and warm summer season observe in Central Anatolia, Black Sea and Eastern Anatolia.

Table 1- Origin and description of the wheat varieties

Variety	***Label***	***Breeder Institute/Company***	***Variety***	***Label***	***Breeder Institute/Company***
Adana 99	adn	Eastern Mediterranean ARI	Karatoprak	krtprk	Bahri Dağdaş International ARI
Ahmetağa	ahmt	Bahri Dağdaş International ARI	Kate A 1	katea	Eastern Mediterranean ARI
Ak 702	ak702	Transitional Zone ARI	Kıraç 66	kirac	Transitional Zone ARI
Aldane	aldne	Trakya ARI	Konya 2002	konya	Bahri Dağdaş International ARI
Alpu 01	alpu	Transitional Zone ARI	Kutluk 94	kutluk	Transitional Zone ARI
Altay 2000	altay	Transitional Zone ARI	Lütfübey	ltfby	Field Crops Central ARI
Altınbaşak	abasak	Eastern Mediterranean ARI	Mesut	mesut	Transitional Zone ARI
Bayraktar 2000	byrktr	Field Crops Central ARI	Momtchill	mmtchl	Maize Research Institute
Beşköprü	bskpru	Maize Research Institute	Müfitbey	mftby	Transitional Zone ARI
Bezostaja-1	bztja	Maize Research Institute	Nacibey	ncby	Transitional Zone ARI
Ceyhan 99	cyhn	Eastern Mediterranean ARI	Osmaniyem	osmny	Eastern Mediterranean ARI
Çukurova-86	ckrova	Eastern Mediterranean ARI	Pamukova 97	pmkov	Maize Research Institute
Dağdaş 94	dagdas	Bahri Dağdaş International ARI	Pandas	pandas	Eastern Mediterranean ARI
Demir 2000	demir	Field Crops Central ARI	Sagittario	sgttario	Tasaco Tarım Company
Doğankent-1	dgnknt	Eastern Mediterranean ARI	Selimiye	slmy	Trakya ARI
Ekiz	ekiz	Bahri Dağdaş International ARI	Seri 2013	seri13	Eastern Mediterranean ARI
Gerek 79	gerek	Transitional Zone ARI	Seri 82	seri82	Eastern Mediterranean ARI
Gökkan	gokkan	Eastern Mediterranean ARI	Seyhan 96	syhn	Eastern Mediterranean ARI
Hanlı	hanlı	Maize Research Institute	Sönmez 01	snmz	Transitional Zone ARI
Harmankaya	hrmnkya	Transitional Zone ARI	Tahirova	throva	Maize Research Institute
İkizce	ikizce	Field Crops Central ARI	Tosunbey	tsnby	Field Crops Central ARI
İzgi 01	izgi	Transitional Zone ARI	Yüreğir 89	yrgir	Eastern Mediterranean ARI
Karahan 99	krhn	Bahri Dağdaş International ARI			

ARI: Agriculture Research Institute

2.2. Extraction of wheat samples

Wheat samples were milled in UDY Corporation, USA (Cyclone sample mill) miller and whole wheat flour was used for the determination of total phenolic content, free radical scavenging activity, and fiber content. Methanol extracts were prepared adding 50 mL methanol to 5 g each sample and shaked for 30 min. After shaking the methanolic extract was centrifuged at 5000 rpm for 20 min. The supernatants were collected and stored +4 °C until analysis (Ragaee et al. 2006).

2.3. Total phenolic contents

The total phenolic content of each whole wheat sample was determined by using the colorimetric method used by Kaluza et al. (1980) and Ragaee et al. (2006) and modified for the study. Briefly, extracts were reacted with Folin-Ciocalteu reagent and then neutralized with sodium carbonate. After 30 min. the phenolic mixture was centrifuged at 2000 rpm for 10 min. and was measured at 725 nm at the spectrophotometry. Gallic acid was used as the standard and phenolic content was expressed as µg gallic acid equivalents/g sample.

2.4. Total antioxidant activity

The total antioxidant activity was measured by DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method described by Brand-Williams et al. (1995). 3900 µl DPPH solution was added to 100 µl of methanolic wheat extract and the samples were put in 36 °C in water bath for 30 min. The absorbance of the mixture at 517 nm was measured and methanol was used as a blank, antioxidant activity was calculated as percent discoloration described below:

$$AAC\ (Inhibition\ \%) = (Absorbance_{control} - Absorbance_{sample}) \times 100/Absorbance_{control}$$

2.5. Crude fiber ratio

Whole milled wheat samples were scanned for crude fiber ratio (%) by using Near Infrared Reflected Spectroscopy (NIRS) method with Bruker MPA device (Oliveira & Franca 2011).

2.6. Crude protein content

Total protein content of flour samples was measured by CN elemental analyzer (Elementar-group, Hanau, Germany) using the high-temperature combustion method.

2.7. Essential amino acid analysis

Seven essential amino acids were identified by an amino acid analyzer (Biochrom 20 Plus, Biochrom Ltd. Cambridge, UK) in Berlin Freie Universität, Institute of Animal Nutrition and procedure chosen depends on the oxidation (for methionine and cysteine) and hydrolysis of samples (EU Commission Regulation 2009). In both oxidation and hydrolysis procedures, grain samples were ground to pass through a 0.8 mm (Perten Lab. Mill) sieve and flour sample (0.5 g) was added to a 100 mL bottle fitted with a screw cap. The samples placed in an ice-water bath and added 5 mL of oxidation mixture the left for 16 hours in refrigerator at 0 °C. After 16 hours samples removed from the refrigerator and decomposed the excess oxidation reagent by the addition of 0.84 g sodium disulfide. 25 mL of hydrolysis mixture (6 mol HCl/l containing 1 g phenol/l) was added to the oxidized samples and placed the bottle containing the mixture in an oven at 110 °C for 24 hours. On completion of hydrolysis, the mixture samples were removed and placed in an ice-water bath. After oxidation and hydrolysis procedure sample pH level adjusted to 2.20 at room temperature using sodium hydroxide (7.5 mol/l) solution and pH adjusted hydrolysate with sodium buffer to a 100 mL flask. Each sample was filtered through a 0.45 µm cellulose acetate membrane filter (Millipore) and injection volume was 200 µl.

Table 2- General characteristics and climate classification of the varieties

Variety	*Grain color*	*Spike type*	*Growth habit*	*Spike color*	*TKW (g)*	*Climate classification* (Köppen Geiger)*
Adana 99	White	Awned	Spring	White	28-39	Csa
Ahmetağa	Red	Awned	Winter	White	33.4	Bsk, Dsb, Dsa, Dfa
Ak 702	-	-	-	-	-	-
Aldane	Red	Awnedness	Spring	White	42.5	Cfa, Csa, Dsb
Alpu 01	White	Awned	Winter	White	40-44	Bsk, Dsb, Dsa, Dfa
Altay 2000	White	Awned	Winter	Brown	36-40	Bsk, Dsb, Dsa, Dfa
Altınbaşak	White	Awned	Spring	White	29.7-37.8	Csa
Bayraktar 2000	White	Awned	Winter	White	32-34	Bsk, Dsb, Dsa, Dfa
Beşköprü	Red	Awnedness	Winter	Red	37.2	Cfa, Dsb
Bezostaja-1	Red	Awnedness	Winter	White	38-44	Cfa, Bsk, Dsb, Dsa, Dfb
Ceyhan 99	Red	Awned	Spring	White	28-38	Csa, Cfa
Çukurova-86	-	-	-	-	-	-
Dağdaş 94	White	Awned	Winter	White	36-42	Bsk, Dsb, Dsa, Dfa
Demir 2000	Red	Awned	Spring	White	35.5	Bsk, Dsb, Dsa, Dfa
Doğankent-1	-	-	-	-	-	-
Ekiz	Red	Awned	Spring	White	36.4	Bsk, Csa, Cfa, Dsb, Dsa, Dfa,
Gerek 79	White	Awned	Winter	Brown	32-38	Bsk, Csa, Cfa, Dsa, Dsb, Dfa
Gökkan	White	Awned	Spring	White	35.7-40	Csa
Hanlı	Red	Awned	Spring	White	30-40	Csa
Harmankaya	Red	Awned	Winter	White	38-44	Bsk, Dsd, Dsa, Dfa
İkizce	Red	Awned	Winter	White	28-32	Bsk, Dsd, Dsa, Dfa
İzgi 01	White	Awned	Winter	White	36-42	Bsk, Dsd, Dsa, Dfa
Karahan 99	White	Awned	Winter	White	32-38	Bsk, Dsd, Dsa, Dfa
Karatoprak	White	Awned	Spring	White	32-40	Csa
Kate A 1	Red	Awnedness	Spring	White	35.9	Csa, Cfa
Kıraç 66	White	Awned	Winter	White	40.3	Bsk, Dsd, Dsa, Dfa
Konya 2002	Red	Awned	Winter	White	40-49	Bsk, Dsd, Dsa, Dfa
Kutluk 94	White	Awned	Winter	White	34-36	Bsk, Dsd, Dsa, Dfa
Lütfübey	Red	Awned	Winter	Brown	38	Bsk, Dsd, Dsa, Dfa
Mesut	Red	Awnedness	Winter	White	37-41	Bsk, Dsd, Dsa, Dfa
Momtchill	Red	Awnedness	Winter	White	42-45	Csa, Cfa
Müfitbey	White	Awned	Winter	White	38-42	Bsk, Dsd, Dsa, Dfa
Nacibey	Red	Awned	Winter	White	36-38	Bsk, Dsd, Dsa, Dfa
Osmaniyem	Red	Awned	Spring	White	35-42	Csa
Pamukova 97	Red	Awned	Spring	White	30-40	Csa
Pandas	Red	Awned	Spring	White	25.2-38.7	Csa
Sagittario	Red	Awned	Spring	White	40-45	Csa
Selimiye	Red	Awnedness	Winter	Red	38.5	Csa, Cfa
Seri 2013	White	Awned	Spring	White	36.2-40.9	Csa
Seri 82	-	-	-	-	-	-
Seyhan 96	White	Awned	Spring	White	40-42	Csa
Sönmez 01	White	Awnedness	Winter	White	38-44	Bsk, Dsd, Dsa, Dfa
Tahirova	White	Awned	Spring	White	34-46	Csa, Cfa
Tosunbey	White	Awned	Spring	White	30-35	Bsk, Dsb, Dsa, Dfa
Yüreğir 89	White	Awned	Spring	White	28.8-43.0	Csa

General characteristic information base on breeder institute/company. (-: lack of information)
Bsk: arid, steppe and cold summer, Csa: temperate, dry and hot summer, Cfa: temperate, without dry season but hot summer, Dsa: cold, dry and hot summer, Dsb: cold, dry and warm summer, Dfa: cold, without dry season and hot summer, Dfb: cold, without dry season and warm summer.

2.8. Statistical analysis

Statistical analysis was performed using SPSS 19 statistical software (SPSS Inc. Chicago, USA). Data for each of the samples were evaluated by a one-way ANOVA procedure with 3 replications using the Duncan's multiple range test and linear correlation analysis were carried out to examine the relationships between the factors. Wheat varieties were differentiated using PC-ORD software through hierarchical clustering analysis (Mc Cune & Mefford 2006). Cluster analysis was performed using a tree diagram based on Euclidean distances was developed by Ward's method (Ward 1963; Arslan et al. 2019).

3. Results and Discussion

3.1. Total phenolic contents

Total phenolic content results indicate that statistically significant differences were obtained between varieties ($P<0.01$). Total phenolic content of the varieties ranged from 102.4 to 211.8 µg GAE/g (>2-fold) and the highest amount of phenolic content was measured in İzgi variety and the lowest in Sönmez variety. Values of varieties changed in a wide range and intensely between 140-200 µg GAE/g. In all evaluated varieties İzgi (211.8 µg GAE/g), Tahirova (209.0 µg GAE/g) and Demir 2000 (207.8 µg GAE/g) had the highest phenolic content and the lowest values obtained from Gökkan (135.0 µg GAE/g), Pamukova (130.4 µg GAE/g) and Sönmez (102.4 µg GAE/g), respectively (Table 3). A comparison of the total phenolic contents of varieties showed that İzgi, Demir 2000, Konya 2002, Osmaniyem, Ahmet ağa, Ceyhan 99, and Mesut varieties can be classified as highest total phenolic content whose grain color is red (Table 2). As mentioned by researchers grain color and bran fraction are related to phenolic content and antioxidant activity in wheat (Ma et al. 2014a; Žilić 2016). The results obtained by the previous studies are partially similar and have higher values compared to phenolic content results (Ragaee et al. 2006; Li et al. 2008; Revanappa & Salimath 2011) and also the widely ranging phenolic content may be due to different extracting methods (Engert & Honermeier 2011). It has been reported that phenolic content in wheat grain has positive contributions to human health and the antioxidant potential of wheat bran, a product in wheat milling industry emphasized by many studies. However, there are other categories of wheat products such as germ and shorts (Liyana-Pathirana & Shahidi 2006; Vaher et al. 2010).

3.2. Total antioxidant activity

Total antioxidant activity analysis of wheat grain based on DPPH radical scavenging activity and explained as % inhibition rate. As statistical results free radical scavenging activity of wheat grain changed by variety ($P<0.01$). The mean value of antioxidant activity was 17.19% and it varied from minimum 11.89% (Doğankent) to maximum 26.33% (Tosunbey) in all participated varieties. Besides Tosunbey variety, Momtchill (25.90%), Selimiye (23.78%), Ahmetağa (23.21%), Kutluk (23.13%), Nacibey (22.85%), Seri 82 (22.87%), Ceyhan 99 (21.99%) and Kıraç (21.60%) varieties have more than 20% inhibition value so these varieties more important in terms of antioxidant activity among other varieties (Table 3). The other 37 bread wheat varieties got 11-19% inhibition values. According to cluster analysis results (Figure 2) varieties with high antioxidant activity (ranged between 15.22% and 26.3%) are mainly located in Group C and has winter growth habit as described in Table 2. From these results, it can be concluded that growth habit of variety and growing conditions have a certain effect on the accumulation and content of the phenolic compounds (Vaher et al. 2010). Total antioxidant activity values are higher than results from several previous studies (Mpofu et al. 2006; Ryan et al. 2011). These findings could explain differences in grain phenol and antioxidant activity mainly change by genotype. Although environmental factors play an important role in antioxidant activity of cereals, genetic component is a key element with high heritability values to obtain higher antioxidant values (Lachman et al. 2012).

3.4. Crude fiber content

The primary components of dietary fiber of wheat are non-starch polysaccharides including insoluble dietary fiber and soluble dietary fiber. Health benefits attributed to the antioxidant capacity of phenolic and flavonoid compounds in cereals detected in crude fiber fractions (Sumczynski et al. 2015). Most bread products are made with refined white flours which are lack of important nutrients such as dietary fiber, vitamins, minerals and antioxidants due to removal of wheat bran and germ fractions (Xu et al. 2021). Wheat bran is a part of grain and milling product that has phenolic compounds mostly concentrated and may contribute to the total antioxidant activities of wheat. Most of the antioxidants in grains located in the bran and germ fractions (Miller et al. 2000). As obtained results, crude fiber mean value is 2.76% and highest fiber content obtained from Nacibey (3.30%) while minimum value noted in Mesut variety (2.25%). The fiber content of wheat grain remained limited (2-3% crude fiber content) and received not variable values but statistically differences observed between varieties ($P<0.01$). Apart from Nacibey in terms of crude fiber Gökkan (3.18%), Sönmez (3.25%), Karahan (3.03%), Ak 702 (3.18), Kıraç (3.17%) and Tosunbey (2.93%) varieties have come to the fore with high values (Table 3).

Table 3- Total phenolic, antioxidant activity, fiber, and protein content of varieties

Variety Name	*Phenolic content (µg GAE/g)*	*Antioxidant activity (DPPH %)*	*Fiber content (%)*	*Protein content (%)*
Adana 99	156.6^{ai}	15.68^{d-j}	2.72^{e-j}	13.00^{H*}
Ahmet Ağa	192.8^{af}	23.21^{a-d}	2.44^{g-k}	16.60^{j}
Ak 702	160.4^{ah}	14.36^{g-j}	3.18^{abc}	14.23^{z}
Aldane	164.8^{ah}	15.30^{e-j}	2.66^{e-k}	16.26^{o}
Alpu	173.9^{ah}	18.82^{b-j}	2.45^{g-k}	16.46^{l}
Altay	193.4^{af}	15.91^{d-j}	2.64^{e-k}	17.34^{h}
Altınbaşak	138.9^{ei}	16.40^{c-j}	2.75^{d-j}	13.24^{F}
Bayraktar	141.0^{di}	14.38^{g-j}	2.86^{b-h}	17.37^{g}
Beşköprü	176.9^{ah}	13.82^{hij}	2.63^{e-k}	12.99^{I}
Bezostaja 1	178.2^{ah}	16.54^{c-j}	2.44^{h-k}	17.34^{h}
Ceyhan 99	191.4^{ag}	21.99^{a-g}	2.50^{f-k}	13.43^{D}
Çukurova	153.7^{bi}	19.87^{a-i}	2.35^{jk}	12.83^{K}
Dağdaş 94	187.3^{ah}	16.77^{c-j}	2.86^{b-i}	15.10^{v}
Demir2000	207.8^{abc}	16.48^{c-j}	3.03^{a-e}	18.23^{d}
Doğankent	160.8^{ah}	11.89^{j}	2.69^{e-j}	12.76^{M}
Ekiz	153.6^{bi}	16.06^{c-j}	2.51^{f-k}	16.17^{q}
Gerek 79	187.5^{ah}	15.07^{f-j}	2.82^{c-i}	16.29^{n}
Gökkan	135.0^{ghi}	13.22^{ij}	3.18^{abc}	13.75^{B}
Hanlı	150.3^{ci}	14.97^{f-j}	2.80^{c-i}	13.01^{G}
Harmankaya	143.2^{di}	15.64^{d-j}	2.71^{e-j}	15.67^{r}
İkizce	166.4^{ah}	13.54^{ij}	2.57^{f-k}	14.50^{y}
İzgi	211.8^{a}	15.22^{f-j}	2.72^{e-j}	18.52^{c}
Karahan	137.9^{fi}	16.08^{c-j}	3.03^{a-e}	18.08^{e}
Karatoprak	183.2^{ah}	14.75^{g-j}	2.54^{f-k}	14.01^{A}
Kate A1	173.8^{ah}	16.24^{c-j}	2.90^{a-f}	16.43^{m}
Kıraç	178.3^{ah}	21.60^{a-h}	3.17^{a-d}	18.71^{b}
Konya	197.1^{ad}	18.02^{c-j}	2.84^{b-i}	16.66^{i}
Kutluk	195.9^{ae}	23.13^{a-e}	2.75^{d-j}	19.78^{a}
Lütfübey	189.0^{ag}	19.29^{a-j}	2.82^{c-i}	12.79^{L}
Mesut	194.4^{af}	17.96^{c-j}	2.25^{k}	16.51^{k}
Momtchill	189.1^{ag}	25.90^{ab}	2.75^{d-j}	11.99^{Q}
Müfitbey	155.5^{ai}	16.02^{c-j}	2.43^{ijk}	17.41^{f}
Nacibey	150.7^{ci}	22.85^{a-f}	3.30^{a}	16.25^{p}
Osmaniyem	195.0^{af}	15.42^{d-j}	2.92^{a-f}	14.96^{w}
Pamukova	130.4^{hi}	13.93^{hij}	2.87^{b-g}	12.35^{O}
Panda's	162.8^{ah}	13.35^{ij}	2.90^{a-f}	14.80^{x}
Sagittario	144.3^{di}	17.07^{c-j}	2.74^{d-j}	13.55^{C}
Selimiye	186.2^{ah}	23.78^{abc}	2.56^{f-k}	15.61^{s}
Seri 2013	175.5^{ah}	14.75^{g-j}	2.87^{b-h}	15.15^{u}
Seri 82	182.2^{ah}	22.78^{a-f}	2.63^{e-k}	12.39^{N}
Seyhan 95	190.3^{ag}	15.48^{d-j}	2.65^{e-k}	12.14^{P}
Sönmez	102.4^{i}	16.80^{c-j}	3.25^{ab}	15.34^{t}
Tahirova	209.0^{ab}	12.79^{ij}	2.89^{b-f}	12.39^{J}
Tosunbey	187.9^{ag}	26.33^{a}	2.93^{a-f}	16.60^{j}
Yüreğir	159.6^{ah}	13.93^{hij}	2.76^{c-j}	13.25^{E}
ANOVA	**	**	**	**
Std. Deviation	30.88	4.56	0.34	13.03
Maximum	211.8	26.33	3.30	19.78
Minimum	102.4	11.89	2.25	11.99
Average	171.4	17.19	2.76	15.16

*: the letters shown with the big font are the continuation of the alphabet, **: significance at P<0.01

3.5. Crude protein content

Most studies showed approximately 3-fold variation in protein content (from 7-22%) with about one-third of this being under genetic control (Shewry 2009). Grain quality is a complex of physical and chemical characteristics and affected by mainly genotype, environmental conditions and their interactions (Erekul et al. 2012). Crude protein values ranged between 11.99-19.78% that can be expressed as high protein content. The highest protein value was obtained from Kutluk variety which belongs to Central Anatolian Region and the lowest protein ratio was obtained from Momtchill variety. Karahan (18.08%), İzgi (18.52%) and Kıraç (18.71%) varieties also come to the forefront with high protein level (18%) between all evaluated varieties (Table 3). In breeding studies, there is a big challenge for breeders that there is found to be dilution effect between protein content and grain yield (Erekul & Köhn 2006). This inversely effect may cause to result high protein ratio that obtained from low yield potential varieties. Wheat grain yield value is low (approx. 2744 kg/ha) in Turkey compared to the European countries due to the variability in climate conditions of regions (Faostat 2018). Heat stress during grain filling period can cause a decrease of thousand-grain weight which means lower yield values that could be an explanation of high protein content. Therefore, regional weather conditions have a great impact on protein content due to the wheat yield. Turkish Grain Board (TMO) determines quality standards and purchases prices of farmers so 14% standard set in protein content for bread wheat grain. In our study, 28 varieties were found above this standard value and described as high protein content grains and differentiated as Group C in cluster dendrogram (Figure 2.) which are mostly adapted to winter growth habit in Central Anatolia Region (Bsk, Dsd, Dsa, Dfa sub climate type) described as arid and cold winter with dry and hot summer climate in Table 2.

3.6. Essential amino acid content

Wheat varieties showed a uniform essential amino acid profile that was characterized by lower concentrations of threonine, lysine, and methionine, and leucine obtained the highest average value between essential amino acids. While protein is major quality emphasis in wheat, it also enhances nutritional value of grain. Kutluk variety has higher threonine (0.491 g/100 g), valine (0.761 g/100 g), isoleucine (0.633 g/100 g), leucine (1.167 g/100 g), phenylalanine (0.896 g/100 g), lysine (0.464 g/100 g) and methionine (0.344 g/100 g) values than other varieties. Selimiye had remarkably high values from threonine (0.546 g/100 g), valine (0.802 g/100 g), isoleucine (0.876 g/100 g), phenylalanine (1.021 g/100 g), and lysine (1.475 g/100 g) amino acids. Statistically significant differences were observed ($P<0.01$) according to varieties and obtained essential amino acid values widely changed by genotypic variation (Table 4). Especially in terms of Selimiye lysine content, maximum value is (approx.) 5 times higher than minimum value but this situation was not seen for threonine and methionine amino acids. Higher leucine content made difference with highest average value compared to other essential amino acids. Therefore, varieties with high leucine content (İzgi, Karahan, Kıraç, Kutluk and Müfitbey) come to the forefront with high total essential amino acid content (Figure 1). These results extend our knowledge of high protein content caused an increase in the amount of amino acids. Concentrations of protein and essential amino acids were found significantly higher in winter growth habit compared to spring growth habit varieties and tree diagram of cluster analysis indicate that varieties in Group C have more nutritional value than others (Figure 2). Therefore, there is evidence that amino acid content of wheat grain basically depending on the protein content of variety also affected by growing conditions, climate and environment (Anjum et al. 2005; Konvalina et al. 2011).

Table 4- Essential amino acid content of varieties (g/100 g flour)

Variety name	*Threonine*	*Valine*	*Isoleucine*	*Leucine*	*Phenylalanine*	*Lysine*	*Methionine*
Adana 99	0.353^{t}	0.432^{J}	0.295^{F}	0.769^{C}	0.536^{B}	0.345^{xy}	0.228^{D}
Ahmet Ağa	0.389^{n}	0.496^{A}	0.366^{w}	0.893^{r}	0.660^{p}	0.370^{p}	0.281^{n}
Ak 702	0.382^{o}	0.508^{vw}	0.384^{s}	0.847^{v}	0.605^{x}	0.362st	0.260^{uv}
Aldane	0.443^{g}	0.523^{p}	0.380^{t}	0.983^{g}	0.733^{g}	0.399^{k}	0.235^{C}
Alpu	0.402^{l}	0.499^{z}	0.390^{q}	0.888^{t}	0.620^{v}	0.372^{o}	0.261^{tu}
Altay	0.405^{l}	0.504^{x}	0.365^{wx}	0.952^{k}	0.689^{m}	0.370^{p}	0.290^{l}
Altınbaşak	0.332^{y}	0.429^{K}	0.308^{C}	0.728^{IJ}	0.508^{l}	0.316^{E}	0.240^{B}
Bayraktar	0.413^{k}	0.553^{n}	0.420^{n}	0.951^{kl}	0.706^{k}	0.377^{n}	0.325^{e}
Beşköprü	0.346^{w}	0.413^{N}	0.275^{I}	0.729^{l}	0.513^{G}	0.344^{y}	0.264^{r}
Bezostaja 1	0.399^{m}	0.510^{tu}	0.404^{o}	0.928^{m}	0.664^{o}	0.370^{p}	0.293^{k}
Ceyhan 99	0.332^{y}	0.406^{O}	0.281^{G}	0.715^{L}	0.511^{H}	0.328^{B}	0.259^{v}
Çukurova	0.341^{x}	0.451^{G}	0.309^{C}	0.766^{D}	0.526^{D}	0.330^{A}	0.220^{F}
Dağdaş 94	0.348^{uv}	0.464^{E}	0.336^{A}	0.827^{y}	0.598^{y}	0.346^{xy}	0.264^{r}
Demir2000	0.434^{h}	0.571^{k}	0.448^{j}	1.003^{f}	0.710^{j}	0.401^{j}	0.313^{f}
Doğankent	0.350^{tu}	0.436^{l}	0.299^{E}	0.752^{E}	0.530^{C}	0.361^{t}	0.246^{A}
Ekiz	0.389^{n}	0.490^{B}	0.357^{z}	0.890^{s}	0.639^{t}	0.354^{w}	0.310^{gh}
Gerek 79	0.390^{n}	0.561^{m}	0.442^{k}	0.905^{n}	0.642^{s}	0.376^{n}	0.311^{g}
Gökkan	0.357^{s}	0.514^{rs}	0.384^{s}	0.802^{A}	0.553^{A}	0.361^{t}	0.267^{q}
Hanlı	0.331^{y}	0.456^{F}	0.337^{A}	0.748^{F}	0.485^{K}	0.337^{z}	0.247^{zA}
Harmankaya	0.366^{r}	0.507^{w}	0.387^{r}	0.837^{w}	0.585^{z}	0.365^{r}	0.309^{h}
İkizce	0.364^{r}	0.548^{o}	0.425^{m}	0.852^{u}	0.585^{z}	0.387^{l}	0.239^{B}
İzgi	0.464^{d}	0.692^{c}	0.553^{c}	1.085^{b}	0.808^{d}	0.464^{b}	0.374^{a}
Karahan	0.453^{f}	0.610^{f}	0.484^{h}	1.018^{e}	0.725^{h}	0.432^{d}	0.333^{c}
Karatoprak	0.367^{r}	0.488^{C}	0.377^{u}	0.812^{z}	0.553^{A}	0.326^{C}	0.283^{m}
Kate A1	0.443^{g}	0.549^{o}	0.405^{o}	0.981^{h}	0.739^{f}	0.407^{i}	0.327^{d}
Kıraç	0.459^{e}	0.645^{d}	0.518^{e}	1.020^{d}	0.757^{e}	0.441^{c}	0.301^{i}
Konya	0.424^{j}	0.567^{l}	0.438^{l}	0.967^{j}	0.698^{l}	0.373^{o}	0.296^{j}
Kutluk	0.491^{b}	0.761^{b}	0.633^{b}	1.167^{a}	0.896^{b}	0.464^{b}	0.344^{b}
Lütfübey	0.334^{y}	0.502^{y}	0.373^{v}	0.742^{G}	0.492^{J}	0.367^{q}	0.262st
Mesut	0.406^{l}	0.602^{h}	0.487^{g}	0.950^{l}	0.651^{q}	0.410^{h}	0.278^{o}
Momtchill	0.346^{w}	0.512^{s}	0.385^{s}	0.772^{B}	0.529^{C}	0.385^{m}	0.252^{x}
Müfitbey	0.473^{c}	0.598^{i}	0.449^{ij}	1.068^{c}	0.816^{c}	0.441^{c}	0.311^{g}
Nacibey	0.446^{g}	0.644^{d}	0.522^{d}	0.978^{i}	0.709^{j}	0.417^{f}	0.293^{k}
Osmaniyem	0.429^{i}	0.606^{g}	0.490^{f}	0.903^{o}	0.644^{r}	0.413^{g}	0.277^{o}
Pamukova	0.350^{tu}	0.509^{tu}	0.389^{q}	0.767^{D}	0.520^{E}	0.363^{s}	0.254^{w}
Panda's	0.391^{n}	0.582^{j}	0.450^{i}	0.888^{t}	0.626^{u}	0.433^{d}	0.267^{q}
Sagittario	0.331^{y}	0.445^{H}	0.336^{A}	0.740^{H}	0.515^{F}	0.300^{G}	0.253^{wx}
Selimiye	0.546^{a}	0.802^{a}	0.876^{a}	0.899^{p}	1.021^{a}	1.475^{a}	0.263^{rs}
Seri 2013	0.397^{m}	0.522^{p}	0.364^{x}	0.895^{q}	0.673^{n}	0.408^{i}	0.271^{p}
Seri 82	0.317^{z}	0.417^{M}	0.306^{D}	0.663^{N}	0.467^{L}	0.307^{F}	0.217^{G}
Seyhan 95	0.308^{A*}	0.424^{L}	0.313^{B}	0.669^{M}	0.456^{M}	0.338^{z}	0.225^{E}
Sönmez	0.373^{p}	0.513^{rs}	0.396^{p}	0.835^{x}	0.616^{w}	0.357^{v}	0.268^{q}
Tahirova	0.342^{x}	0.475^{D}	0.361^{y}	0.727^{K}	0.509^{l}	0.359^{u}	0.249^{y}
Tosunbey	0.443^{g}	0.621^{e}	0.490^{f}	0.978^{i}	0.720^{i}	0.420^{e}	0.248^{yz}
Yüreğir	0.349^{uv}	0.621^{e}	0.257^{H}	0.716^{L}	0.515^{F}	0.321^{D}	0.220^{F}
ANOVA	**	**	**	**	**	**	**
Std. Deviation	0.052	0.087	0.105	0.118	0.118	0.167	0.035
Maximum	0.546	0.802	0.876	1.167	1.021	1.475	0.374
Minimum	0.306	0.406	0.275	0.663	0.456	0.390	0.217
Average	0.390	0.532	0.405	0.866	0.627	0.399	0.273

*: the letters shown with the big font are the continuation of the alphabet, **: significance at P<0.01

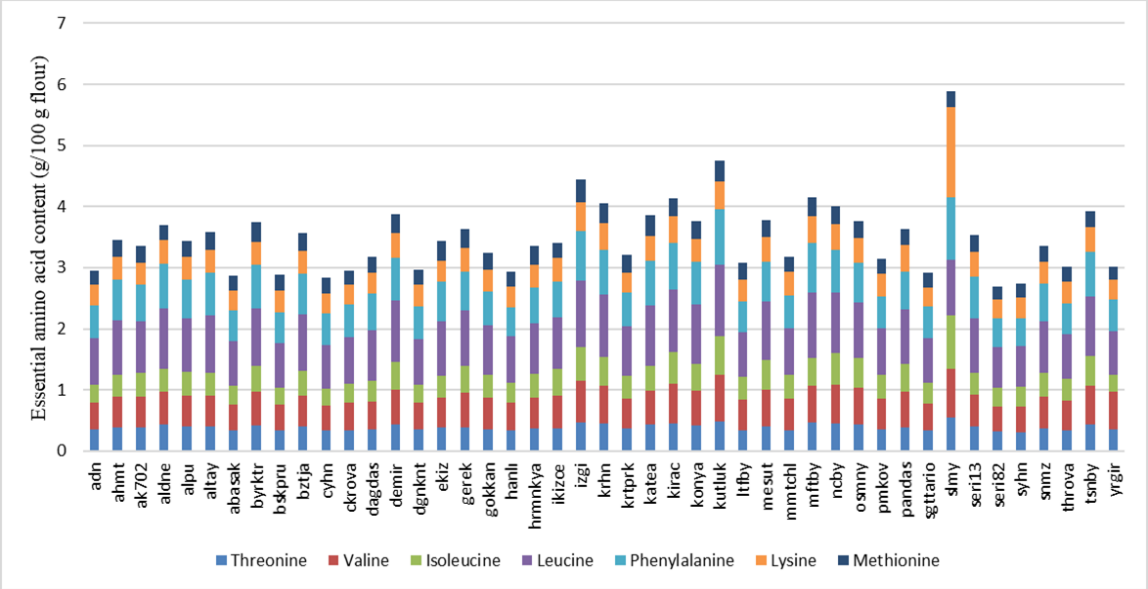

Figure 1- Essential amino acid distribution of varieties (g/100 g flour)

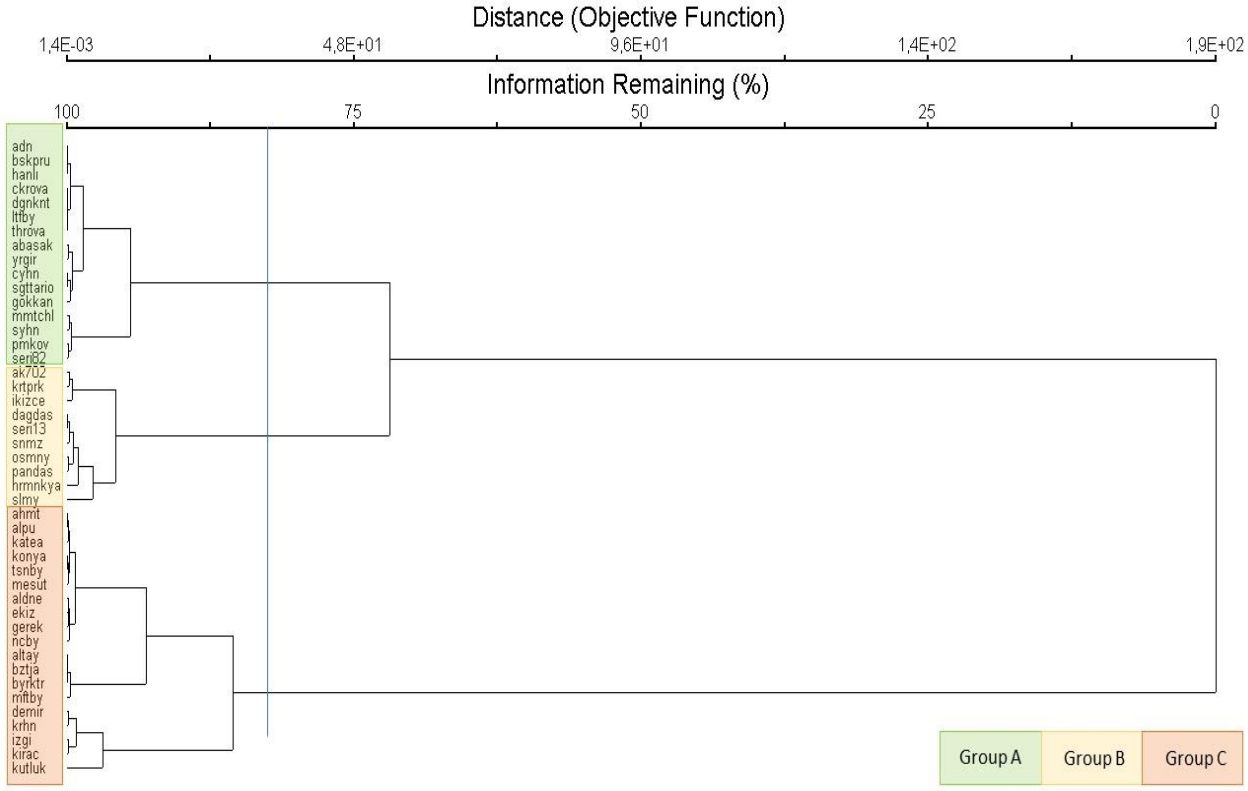

Figure 2- Dendrogram of cluster analysis for 45 varieties based on antioxidant and essential amino acid content

3.7. Correlation and cluster analysis of parameters

Phenolics are known as the most significant bioactive compounds with high antioxidant activity value in wheat grain (Žilić 2016). In this respect, it was found that total antioxidant activity was influenced by the phenolic content of grain in this study. The analysis showed a positive significant correlation (r= 0.196**) between total antioxidant activity and total phenol content of wheat grain. On the other side crude fiber content also had a significant correlation (r= 0.171**) with total phenolic content of grain. This result is also supported by few studies that bran fraction has many health-beneficial compounds such as vitamins, fiber, and whole-grain cereals. Bioactive compounds are mainly concentrated outer layer of grain and potentially health-beneficial compounds such as phenolics are mostly found in the aleurone layer (Mazzoncini et al. 2015; Serea & Barna 2011; Žilić et al. 2012). However, amino acid content of wheat grain is positively related to protein content that showed a strong correlation between crude protein and essential amino acids (Table 5). Grain protein concentration depends on the combination

of many factors as well as amino acid concentration affected by protein exchange. In our study, we also found a positive correlation between total antioxidant activity and essential amino acids (except leucine and methionine) this could be important from a nutritional physiology perspective. Dendrogram (Figure 2) showed that the 45 wheat varieties were gathered into three main clusters (Group A, Group B and Group C) taking into existing similarities for antioxidant and amino acid properties. The first cluster (Group A) comprised mainly spring wheat varieties (Adana 99, Hanlı, Tahirova, Altınbaşak, Yüreğir, Ceyhan 99, Sagittario, Gökkan, Seyhan 96, Pamukova) and three winter wheat varieties (Beşköprü, Lütfübey and Momtchill). The second cluster (Group B) comprised both spring wheat varieties (Karatoprak, Seri 2013, Osmaniyem, Pandas) and winter wheat varieties (İkizce, Dağdaş 94, Sönmez 01, Harmankaya, Selimiye). The third cluster (Group C) comprised mainly 14 winter wheat varieties (Ahmet ağa, Alpu 01, Konya 2002, Mesut, Gerek 79, Nacibey, Altay 2000, Bezostaja-1, Bayraktar 2000, Müfitbey, Karahan 99, İzgi 01, Kıraç 66, Kutluk) and 5 spring wheat varieties (Kate A1, Tosunbey, Aldane, Ekiz, Demir 2000). Group A is differentiated by spring wheat varieties mainly adapted to dominant subtropical semihumid and humid coastal climate type of Turkey recognized as tempered, dry and hot summer (Csa climate classification in Table 2) while third cluster group comprised mainly winter wheat varieties mainly adapted to arid and cold winter with dry and hot summer climate type seen in Central Anatolia Black Sea and Eastern Anatolia regions (Table 2). According to these hierarchical associations, it could be confirmed that antioxidant and amino acid properties are related to mainly growth habit (spring vs winter) and genetic variability.

Table 5- Linear correlation (r) of the parameters studied

	Aac	*Fiber*	*Pro*	*Thr*	*Val*	*IsoLeu*	*Leu*	*PheAl*	*Lys*	*Meth*
Phe	0.196**	0.171*	0.150*	0.151*	0.152*	0.164*	0.148*	0.154*	0.111	0.118
Aac		0.060	0.136	0.215**	0.226**	0.286**	0.137	0.227**	0.233**	-0.008
Fiber			0.010	-0.025	-0.014	-0.014	0.002	-0.013	-0.023	0.003
Pro				0.817**	0.662**	0.584**	0.943**	0.824**	0.206**	0.805**
Thr					0.868**	0.856**	0.887**	0.977**	0.625**	0.633**
Val						0.905**	0.745**	0.848**	0.629**	0.534**
IsoLeu							0.657**	0.857**	0.810**	0.483**
Leu								0.873**	0.247**	0.783**
PheAl									0.663**	0.642**
Lys										0.111

Phe: Total phenolic content; Aac: Total antioxidant activity; Pro: Protein; Thr: Threonine; Val: Valine; IsoLeu: Isoleucine; Leu: Leucine; PheAl: Phenylalanine; Lys: Lysine; Meth: Methionine. Significance at P<0.05 (*) and P<0.01 (**)

4. Conclusions

To our knowledge, there are only a few studies published on the phenol, antioxidant, and amino acid potential of wheat. Thus, the first data of wheat grain about health and nourishment value obtained and aimed to be used in terms of further food and agriculture studies by scanning widely grown Turkish varieties. The results of the present study indicate that antioxidant activity and amino acid content of wheat grain mainly change by genotype. In evaluated varieties Ceyhan 99, Ahmetağa, Kutluk, Momtchill, Selimiye and Seri 82 had higher values in terms of phenolic content and antioxidant activity. Kutluk and Selimiye varieties exhibited high essential amino acid profile and in terms of protein quality to any other varieties. Central Anatolia stands out in terms of varieties with high protein and essential amino acid content across all regions. Clustering analysis also underlined varieties with winter growth habit as higher nutritional value compared to spring growth habit varieties. In correlation results the most attractive result is increasing fiber content promotes higher phenolic content and almost all essential amino acids increased as the protein of the wheat grain increased. Genotypical variation was found to be an important factor to achieve healthy and nutritionally superior wheat and its products can be evaluated in breeding studies.

By the research, it is tried to be revealed a new approach with interdisciplinary study about essential amino acid, phenol and antioxidant properties of bread wheat and thus obtained new results revealed the strength of relationships between parameters.

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Determination of the Seed Yield and Quality Characteristics of Some Advanced-Generation Field Pea (*Pisum sativum* L.) Lines

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ABSTRACT
This study was conducted to determine the seed yield, yield components, and seed quality values of some field pea genotypes grown under Bursa ecological conditions. The trials were carried out at Bursa Uludag University, Faculty of Agriculture, Agricultural Research and Application Center in the years 2013-2014 and 2015-2016. The present study was conducted with three replications according to the randomized complete block design. The 7 pea lines (PS1, PS2, PS3, PS4, PS5, PS6, PS7) and Golyazi cultivar were used as the plant material. These lines were obtained as a result of the hybridization studies that were started in 2007. The Golyazi cultivar, which was developed by Bursa Uludag University, Faculty of Agriculture, Department of Field Crops, was used as the control cultivar. In this study, plant height, the number of pods per plant, the number of seeds per pod, the number of seeds per plant, 1000-seed weight, seed yield, crude protein ratio, and crude protein yield were determined. PS1 line gave the highest values in terms of 1000-seed weight (288.87 g), seed yield (3336.3 kg/ha), and crude protein yield (619.1 kg/ha) based on two-year results. It was also determined that the PS1 line is a good cultivar candidate especially, in terms of seed yield.

Keywords: Genotypes, Plant height, 1000-seed weight, Crude protein ratio, Crude protein yield

1. Introduction

Field pea (*Pisum sativum* L.) is an annual plant of the legume family. The adaptation ability of field pea, which is a cool-season plant, is high. Field pea is a good intermediate crop, and it is utilised as a green fertilizer plant, is suitable for mixed cultivation. This plant is commonly grown for forage and seeds (Krga et al. 2019; Lakić et al. 2019; Halil & Uzun 2020). Field pea is resistant to cold, its seedlings can survive at -20 °C, and can be planted in many areas in winter (Shereena & Salim 2006). Pea seeds can be used as a valuable protein and energy source in feed rations instead of soybean which has the risk of GMO (Genetically Modified Organism). Cultivation of pea is easier compared to soybean; the oil content of pea seeds is lower than soybean seeds (Marohnić 2006; Rapčan et al. 2010; Krizmanić et al. 2020).

Field pea was included in the data of TUIK (Turkish Statistical Institute) for the first time in 2014 in Turkey. The cultivation areas of the field pea are increasing more and more. The cultivation areas of field pea are 24319 ha in 2020. As can be seen here, the cultivation area of the field pea increased approximately 6.5-fold in seven years. Despite this increase, the share of field pea in total forage crop cultivation areas is only 0.80% (Anonymous 2020). Field pea cultivars have been registered in Turkey since 2007; and according to official data, 30 cultivars have been registered until today (Anonymous 2021).

To increase animal production, it is necessary to increase the cultivation areas and production of forage crops. This can be accomplished largely by breeding productive and high-quality varieties. In this respect, new cultivars must be developed in field peas, and the field pea cultivation areas must be increased. It is necessary to develop productive and quality genotypes or to improve the insufficient sides of the existing cultivars with breeding studies (Ceyhan 2003).

Characteristics such as plant height, the number of seeds per plant, the number of pods per plant, the number of seeds per pod, and 1000-seed weight provide high seed yield in field pea as in the other plants. Kumar et al. (2013) submitted that pea grain yield might be increased by selecting genotypes that have a large number of pods per plant and a large number of seeds per pod. Researchers conducted studies on different plants and reported that the germination and seedling establishment of large seeds is better than small seeds; plants obtained from large seeds have high competition ability for plant-growth re-sources as soil water, soil nutrients, and light, field performance is better; seed yield could increase by 15%-20% with the use of quality seeds (Gan et al. 2006; Ambika et al. 2014). Tawaha & Turk (2004) reported that heavy seeds provided 12% more 100-seed

weight than light seeds. Smithcher & Weeden (2018) reported that the seed yield in large-seeded peas would be higher than small-seeded peas due to the higher harvest index however, they reported that the cost of seeds would also increase in such a case.

In this research, the main aim was to investigate the seed yield and quality properties of some pea lines obtained by hybridization method and to reveal a new variety candidate with superior characteristics.

2. Material and Methods

Field trials were performed in 2013-2014 and 2015-2016 at Bursa Uludag University, Faculty of Agriculture, Agricultural Research and Application Center in two years. The climatic data of the trial years are given in Table 1. As shown in Table 1, the total precipitation was 496.20 mm in the first year and 459.30 mm in the second year in the November-June period. Also, it is seen in Table 1 that the long term period total annual precipitation (558.30 mm) is more than in the period of these experiments. The average temperature in the plant development period was 12.50 °C in the first year, 13.13 °C in the second year, and 11.50 °C in the long term. In both years, it was determined that the average heats were more than the long term. The relative humidity values were found to be 71.17% and 72.64%, respectively. This value was reported as 71.00% as the average of long term (Anonymous 2016).

Table 1- Climate data of Bursa province in the years of research and the long term

	Precipitation (mm)			*Temperature (°C)*			*Relative Humidity (%)*		
Months	*Long Term*	*2013-14*	*2015-16*	*Long Term*	*2013-14*	*2015-16*	*Long Term*	*2013-14*	*2015-16*
November	74.40	60.80	26.40	12.2	11.80	12.74	74.30	75.40	78.05
December	101.80	38.60	3.00	7.60	4.90	5.60	73.60	66.80	76.60
January	92.50	30.80	122.20	5.30	8.98	5.20	75.00	70.42	80.70
February	78.40	20.40	80.70	6.30	8.55	11.10	73.10	73.66	76.00
March	70.30	42.40	75.60	8.30	10.65	11.20	72.20	69.57	71.00
April	59.20	112.00	22.80	12.8	14.51	16.40	69.50	71.09	65.30
May	50.40	96.80	67.30	17.50	18.32	18.30	68.80	71.73	71.20
June	31.30	94.40	36.40	22.20	22.29	24.50	61.30	70.66	62.30
Total/ Average	558.30	496.20	459.30	11.50	12.50	13.13	71.00	71.17	72.64

The soils of the trial area were generally clayey, had low organic matter content, and had no salinity, alkalinity problems.

The field pea genotypes were provided from different sources were crossed according to the semi-diallel hybridization method in 2007. The genotypes with large seeds, long pods, the high number of seeds in pod, and high seed yield per plant were selected in the later years. Seven advanced-generation field pea lines (PS1, PS2, PS3, PS4, PS5, PS6, PS7) were used in this study. The Golyazi cultivar was used as the control material in this study. Golyazi was a cultivar registered by Bursa Uludag University Faculty of Agriculture, Department of Field Crops, in 2007. This study was conducted to investigate the seed yield and quality characteristics of some field pea genotypes and to determine superior lines. The experiments were performed in a randomized block design with 3 replications.The study started in 2013-14, and the second year of the study was established in 2014-15. However, the experiment in the 2014-15 year was canceled because of winter damage. The trial was re-sown for winter in 2015-16, and the results of two years were used in the study. The seeds were sown manually at 100 seeds per square meter on 11.11.2013 and 06.11.2015.

The plot size was 6 m^2. The row spacing was 20 cm, and there were 6 rows in the plots. Fertilizer (30 kg/ha N) was applied after sowing. Weed control was done manually twice. Plants were sprayed twice with 10 days intervals against *Bruchus pisorum* at the flowering stage. The plant height, the number of pods per plant, the number of seeds per pod, and the number of seeds per plant was determined in 10 plants that were selected from each plot before the harvest. The harvest was performed using Hege 125 C Plot Combine (Hans-Ulrich Hege Maschinenbau, Hohebuch, Germany) on 10.06.2014 and 17.06.2016. After the harvest, the seeds were fumigated against *Bruchus pisorum* with Phostoxin tablets. After this process, the seeds were cleaned, and the seed yield and 1000-grain weights were determined. The nitrogen contents of the seeds were determined by the Micro Kjeldahl Method. By this method, the crude protein contents (Nx6.25) and the crude protein yields were calculated. The variance analysis was conducted using the JUMP 7.0 (SAS 1989-2002) package program. Comparisons between means were made using least significant differences (LSD) at 0.05 probability level.

3. Results and Discussion

The plant height, the number of pods per plant, the number of seeds per pod, the number of seeds per plant, 1000-seed weight, seed yield, crude protein ratio, and crude protein yield characteristics were examined in both years of this study.

3.1. Plant height

The results for the plant heights of the field pea genotypes are shown in Table 2. As seen in the table, the differences between plant heights were statistically significant at 1% probability level in genotype, year, and genotype x year interaction. As seen in the genotype averages, the highest plant height was obtained at the Golyazi cultivar with 145.12 cm and the lowest plant height value was at the PS7 with 78.95 cm (Table 2). Uzun et al. (2005) determined that the highest plant height was obtained at Golyazi cultivar with 158.6 cm in a study they conducted with different pea genotypes. The plants were taller in the first year (average 115.94 cm) than in the second year (average 78.77 cm) (Table 2). Since the total precipitation in the first year (496.20 mm) was higher than in the second year (459.30 mm) and the average temperature especially in the spring months in the first year was lower than in the second year plant height was higher (Table 1). In genotype x year interaction, the highest plant height was detected in the first year of the Golyazi cultivar (158.03 cm), and the lowest plant height was detected in the second year of the PS4 (64.20 cm), PS7 (64.00 cm), PS5 (60.93 cm), and PS2 (60.80 cm) lines (Table 2). In many studies conducted on peas, plant height values were reported to vary between 39.5 and 137.7 cm (Ibrahim et al. 2019; Krga et al. 2019; Prasad et al. 2019; Kadıoğlu et al. 2020; Krizmanić et al. 2020). Krizmanić et al. (2020) reported that the plant height was important as well as the number of seeds per pod, the number of pods per plant, and the 1000-seed weight in developing a productive and quality cultivar in field pea.

Table 2- Plant height and the number of pods per plant values of the pea genotypes

	Plant height (cm)			***The number of pods per plant***		
Genotypes	***2013-2014***	***2015-2016***	***Genotype average***	***2013-2014***	***2015-2016***	***Genotype average***
PS1	107.90 de	73.93 h	90.92 C	19.73 bc	11.60 fg	15.67 B
PS2	111.43 d	60.80 ı	86.12 D	21.67 ab	15.93 e	18.80 A
PS3	103.50 e	82.27 g	92.88 C	17.47 de	11.60 fg	14.53 BC
PS4	105.90 de	64.20 ı	85.05 D	20.23 bc	10.53 g	15.38 BC
PS5	126.60 b	60.93 ı	93.77 C	19.43 cd	10.40 g	14.92 BC
PS6	120.27 c	91.80 f	106.03 B	16.33 e	13.60 f	14.97 BC
PS7	93.90 f	64.00 ı	78.95 E	23.00 a	15.93 e	19.47 A
Golyazi	158.03 a	132.20 b	145.12 A	17.33 de	10.70 g	14.02 C
Year average	115.94 A	78.77 B		19.40 A	12.54 B	
LSD (%5)	G: 4.09	Y: 3.19	GxY: 5.79	G: 1.52	Y: 1.17	GxY: 2.15
P<0.05	G: **	Y: **	GxY: **	G: **	Y: **	GxY: **
CV (%)	3.56			8.08		

The difference between averages with the same letter is not significant (P<0.05) (LSD); * and **: significant at P= 0.05 and 0.01 (F-test) respectively; ns: not significant (F-test)

3.2. *Pods/Plant*

As seen in Table 2, the genotype, year, and genotype x year interaction were found to be statistically significant on the number of pods per plant at 1% probability level. According to two years average, the highest number of pods per plant was obtained at PS7 line with 19.47 and PS2 line with 18.80. The least number of pods per plant was determined at the Golyazi cultivar (14.02). According to the the differences between years, the highest number of pods per plant were taken in the first year with 19.40. In the trial, the number of pods per plant detected in the first year was higher than in the second year (Table 2). Especially the fact that the total precipitation in the flowering period in the first year (208.8 mm) was more than in the second year (90.1 mm) increased the number (Table 1). Harvey (1980) stated that peas are very sensitive to water during flowering and pod filling periods. As seen in genotype x year interaction in Table 2, the highest number of pods per plant was obtained from the PS7 line (23.00) in the first year of the study. The PS2 line (21.67) was also in the same statistical group with it. The number of pods per plant increased in the first year due to high precipitation especially in April and May (Table 1). Golyazi (10.70), PS4 (10.53), and PS5 (10.40) genotypes had the lowest number of pods per plant in the second year (Table 2). The number of pods per plant was varied between 2.6 and 30.4 in previous studies made with different pea genotypes in different years and locations (Singh et al. 2018; Ton et al. 2018; Jiang et al. 2020; Jiang et al. 2020; Kadıoglu et al. 2020). Lakić et al. (2019) reported that Kosev (2014) had explained that the number of pods per plant was important at increasing the yield. Georgieva et al. (2015) indicated that the effect of the number of pods per plant on the seed yield in peas was positive and significant. Furthermore, these researchers reported that field pea breeders should pay attention to the number of pods per plant in developing high-yield genotypes.

3.3. Seeds/Pod

The effects of genotypes and years were significant at 1% probability level for the number of seeds per pod (Table 3). According to the results of the study, the highest number of seeds per pod was obtained in the PS4 (4.97), PS7 (4.80), and PS1 (4.78) lines,

followed by PS3 (4.67) and PS6 (4.58) lines, which were in the same group. The lowest value was obtained in the Golyazi cultivar with 3.87 value. The number of seeds per pod obtained in the first year (4.71) was higher than in the second year (4.25) (Table 3). In the second year of the trial, the high-temperature values at the flowering time decreased the number of seeds per pod (Table 1). Although the number of seeds per pod is hereditary, it is affected by stress conditions (Egli & Bruening 2002). The number of seeds per pod decreases as stress continues (Jiang et al. 2020). The number of seeds per pod was varied between 3.73 and 5.40 in the genotype x year interaction (Table 3). Uzun et al. (2005) reported that the number of seeds per pod in pea ranged between 4.0 and 4.7 in a study conducted under Bursa ecological conditions. On the other hand, Prasad et al. (2019) found the number of seeds per pod between 2.1 and 5.2. These values are similar to the values in our study. Some researchers reported that the number of seeds per pod ranged between 5.27 and 7.5 (Ibrahim et al. 2019; Lakić et al. 2019; Kadıoglu et al.2020). Krizmanić et al. (2020) reported that the number of seeds per pod in field peas was one of the characteristics affecting the seed yield. Kumar et al. (2013) reported that when the number of seeds per pod was higher, it would increase the seed yield in peas.

Table 3- The number of seeds per pod and the number of seeds per plant values of the pea genotypes

	The number of seeds per pod			***The number of seeds per plant***		
Genotypes	***2013-2014***	***2015-2016***	***Genotype average***	***2013-2014***	***2015-2016***	***Genotype average***
PS1	4.87	4.70	4.78 A	68.40 a	52.87 cde	60.63 A
PS2	4.60	3.53	4.07 BC	54.67 bcd	36.87 ı	45.77 CD
PS3	4.63	4.70	4.67 AB	52.33 cde	38.60 hı	45.47 DE
PS4	5.40	4.53	4.97 A	59.27 b	38.20 ı	48.73 BCD
PS5	4.50	3.73	4.12 BC	56.10 bc	43.47 gh	49.78 B
PS6	4.67	4.50	4.58 AB	49.67 ef	34.80 ı	42.23 E
PS7	5.00	4.60	4.80 A	59.33 b	38.60 hı	48.97 BC
Golyazi	4.00	3.73	3.87 C	50.33 de	44.87 fg	47.60 BCD
Year average	4.71 A	4.25 B		56.26 A	41.03 B	
LSD (%5)	G: 0.62	Y: 0.25	GxY: 0.88	G: 3.47	Y: 2.86	GxY: 4.91
P<0.05	G: **	Y: **	GxY: ns	G: **	Y: **	GxY: **
CV (%)	11.83			6.04		

The difference between averages with the same letter is not significant (P<0.05) (LSD); * and **: significant at P= 0.05 and 0.01 (F-test) respectively; ns: not significant (F-test)

3.4. Seeds /Plant

The number of seeds per plant values are given in Table 3. As shown in Table 3, there were significant differences at 1% probability level between genotypes, years, and genotype x year interaction. In genotypes, the highest number of seeds per plant was in the PS1 line (60.63) due to high the number of seeds per pod and the number of pods per plant. The lowest number of seeds per plant was measured in the PS6 line (42.23). In the first year, the number of seeds per plant (56.25) was higher than in the second year (41.03). As seen in the genotype x year interaction, the highest number of seeds per plant was obtained in the first year of PS1 line and the lowest number of seeds per plant was obtained in the second year of PS4, PS2, and PS6 lines (Table 3). The number of seeds per plant varied from 24.5 to 102.8 at previous works (Uzun et al. 2005; Ton et al. 2018). Georgieva et al. (2015) explained that the number of seeds per plant was important in developing high-yield pea genotypes.

3.5. 1000-Seed weight

There were significant differences at 1% probability level between genotypes, and 5% probability level between years for the 1000-seed weight (Table 4). The highest 1000-seed weight was obtained from PS1 (288.87 g) and PS4 (282.51 g) lines. The lowest 1000-seed weight was determined at PS6 (180.82 g), PS7 (180.10 g) lines, and Golyazi (176.72 g) cultivar. It was found that the average 1000-seed weight of the genotypes in the first year (239.83 g) was higher than in the second year (226.86 g) (Table 4). 1000-seed weight decreased in the second year due to very low precipitation and slightly higher temperature during April and May (Table 1). In Bursa conditions, field peas usually begin to bloom and fill seeds in April-May. Smaller seeds are obtained under unfavorable environmental conditions (Singh et al. 2009). In a trial conducted by Gantner et al. (2008), it was reported that there was a negative relationship between pea yield with temperature in May and June. It was reported in previous studies conducted in peas that there is a positive relation between seed yield and 1000-seed weight, and 1000-seed weight should be considered as an important characteristic in achieving a high-yield cultivar (Kosev & Mikic 2012; Goa & Ashamo 2014; Georgieva et al. 2015). It was observed that 1000-seed weights varied between 175.25 and 296.33 g in the genotype x year interaction (Table 4). In previous studies conducted in peas, the 1000-seed weights of genotypes varied between 67.3 and 285.0 g (Tan et al. 2012; Ton et al. 2018; Lakić et al. 2019; Prasad et al. 2019; Krizmanić et al. 2020). The reason for this variation were different pea genotypes, sowing seasons, and climatic factors. Rapčan et al. (2010) reported that the location affected the 1000-seed weight at a significant level.

Table 4- 1000-seed weight and seed yield values of the pea genotypes

	1000-seed weight (g)			*Seed yield (kg/ha)*		
Genotypes	***2013-2014***	***2015-2016***	***Genotypes average***	***2013-2014***	***2015-2016***	***Genotypes average***
PS1	296.33	281.40	288.87 A	3529.3	3143.3	3336.3 A
PS2	286.57	262.42	274.49 B	2424.0	2194.3	2309.2 E
PS3	248.67	228.85	238.76 C	2525.0	2386.0	2455.5 D
PS4	290.43	274.58	282.51 A	3250.3	3053.3	3151.8 B
PS5	252.70	236.24	244.47 C	2587.0	2389.0	2488.0 CD
PS6	182.13	179.50	180.82 D	2166.7	2118.0	2142.3 F
PS7	183.57	176.62	180.10 D	2655.0	2562.7	2608.8 C
Golyazi	178.20	175.25	176.72 D	2066.3	2032.0	2049.2 F
Year average	239.83 A	226.86 B		2650.5 A	2484.8 B	
LSD (%5)	G: 7.47	Y: 8.13	GxY: 10.57	G:1.28	Y: 0.53	GxY: 1.82
P<0.05	G: **	Y: **	GxY: ns	G: **	Y: **	GxY: ns
CV (%)	4.24			2.72		

The difference between averages with the same letter is not significant (P<0.05) (LSD); * and **: significant at P= 0.05 and 0.01 (F-test) respectively; ns: not significant (F-test)

3.6. Seed yield

As shown in Table 4, the effect of only genotypes and years on the differences between seed yield was found to be statistically significant at 1% probability level. As seen in the genotype mean the highest seed yield was obtained in the PS1 line with 3336.3 kg/ha (Table 4). The seed yield of this line was higher than the other genotypes due to the high number of seeds per plant and 1000-seed weight. Some researchers reported that there is a positive relation between seed yield with the number of seeds per plant, and 1000-seed weight in field pea (Kosev & Mikic 2012; Anwar et al. 2014; Goa & Ashamo 2014). In this study, the lowest seed yield was found at the Golyazi cultivar (2049.2 kg/ha) and the PS6 line (2142.3 kg/ha) (Table 4). As understood from the table, the seed yield of the PS1 line was found to be 63% higher than the Golyazi cultivar, which was used as the control. The mean seed yield of the first year (2650.5 kg/ha) was 7% higher than the second year (2484.8 kg/ha) (Table 4). The values of all characteristics that were examined, including seed yield, were found to be higher in the first year. The high rainfall in April and May (112.0 mm and 96.80 mm, respectively), which are the flowering and seed-filling times, and the low temperatures in April (14.51 °C) increased the seed yield in the first year (Table 1). Rapčan et al. (2010) reported that a sufficient amount of rainfall and air temperature particularly during flowering is important in obtaining the high seed yield in pea. According to several studies, it has been observed that pea are sensitive to high temperature and low precipitation, especially in the generative period. (Uzun et al. 2005; Gullap et al. 2017). The average daily air temperature is 15-18 °C for the flowering of peas. Temperatures above 26 °C immediately after flowering decrease seed yield in pea because of the flower falling (Popović et al. 2002; Rapčan et al. 2010). Plants are vulnerable to heat stress in the pre-pollination and post-pollination stage. Heat stress causes yield loss by limiting seed filling time or seed filling rate (Uzun et al. 2005; Hedhly 2011; Kaushal et al. 2016). In our study, according to the State Meteorology Department, in the second year, the maximum temperatures recorded in February, March, April, and May were 25.4, 25.7, 30.5, and 32.4 °C, respectively. Whereas, in the first year, the maximum temperatures were 14.39, 16.05, 20.6, and 23.93 °C, respectively. As is seen, the maximum temperatures of the second year were higher than the maximum temperatures of the first year (Anonymous 2016). Therefore, results of research were shown that the seed yield in the first year was more greater compared to the second year. Also, a pea is sensitive to water. Water sensitivity increases especially during flowering and pod settings. Inadequate water in the flowering period affects negatively fertilization causing flower losses (Harvey 1980). In our study, although the total precipitation in the flowering and pod setting periods was 208.80 mm in the first year, the second year was only 90.10 mm (Table 1). Uzun et al. (2005) reported that drought in the flowering and pod-filling period reduced seed yield of field pea. The mean seed yields were varied between 2032.0 and 3529.3 kg/ha in the present study. Previous studies in different years and locations with different pea genotypes have shown that seed yield varied from 1040.0-5130.0 kg/ha (Gullap et al. 2017; Ton et al. 2018; Ibrahim et al. 2019; Lakić et al. 2019; Kadıoglu et al. 2020; Krizmanić et al. 2020).

3.7. Crude protein ratio

In terms of crude protein ratio, only the differences between genotypes were significant at the 1% probability level (Table 5). The highest crude protein ratio was obtained from the PS6 line with 21.12%, and Golyazi (20.15%), PS7 (20.12%), PS3 (19.43%), and PS5 (19.42%) genotypes were also in the same statistical group (Table 5). Crude protein ratios of these genotypes were high, due to low 1000-seed weight. The crude protein ratio of Golyazi cultivar varied from 11.6 to 22.8% in previous experiments conducted in the same (Uzun et al. 2012) and different (Cacan et al. 2018) locations. The lowest crude protein ratio (17.82%) was observed in the PS4 line. In our study, the crude protein ratio varied between %18.47-21.13% (Table 5). In different

studies conducted on peas, this value was reported to vary between %16.4-27.3% (Uzun et al. 2012; Gullap et al. 2017; Księżak et al. 2018; Singh et al. 2018; Ada et al. 2019; Goswami & Shukla 2019).

Table 5- Crude protein ratio and crude protein yield values of the pea genotypes

	Crude protein ratio (%)			*Crude protein yield (kg/ha)*		
Genotypes	***2013-2014***	***2015-2016***	***Genotype average***	***2013-2014***	***2015-2016***	***Genotype average***
PS1	18.47	18.67	18.57 BC	651.6	586.6	619.1 A
PS2	18.87	19.23	19.05 BC	458.0	422.1	440.0 DE
PS3	19.20	19.67	19.43 ABC	485.4	469.6	477.5 CD
PS4	19.30	16.33	17.82 C	627.4	497.4	562.4 AB
PS5	19.87	18.97	19.42 ABC	514.0	453.1	483.6 CD
PS6	21.10	21.13	21.12 A	457.1	447.6	452.4 DE
PS7	20.57	19.68	20.12 AB	545.4	504.3	524.8 BC
Golyazi	20.10	20.20	20.15 AB	415.4	410.4	412.9 E
Year average	19.68	19.24		519.3 A	473.9 B	
LSD (%5)	G: 1.75	Y: 1.00	GxY: 2.48	G: 0.57	Y: 0.31	GxY: 0.81
P<0.05	G: *	Y: ns	GxY: ns	G: **	Y: *	GxY: ns
CV (%)	7.61			9.81		

The difference between averages with the same letter is not significant (P<0.05) (LSD); * and **: significant at P= 0.05 and 0.01 (F-test) respectively; ns: not significant (F-test)

3.8. Crude protein yield

The crude protein yield differences were found to be significant at 1% probability level in genotypes, and at 5% probability level in years; and were found to be insignificant in genotype x year interaction. The highest average crude protein yield was obtained from the PS1 line with 619.1 kg/ha. The PS4 line (562.4 kg/ha) was also in the same group with the PS1 line. The Golyazi cultivar had the lowest crude protein yield compared to the other genotypes. Previous studies have shown that the crude protein yield of the Golyazi cultivar varied from 370.0 kg/ha to 853.0 kg/ha (Uzun et al. 2005; Cacan et al. 2018). As seen in Table 5, the average crude protein yield in the first year (519.3 kg/ha) was higher than the average crude protein yield in the second year (473.9 kg/ha) due to higher seed yield and crude protein ratio in the first year. It was reported in various studies conducted on peas that crude protein yield values ranged between 119.0-1049.0 kg/ha (Rapčan et al. 2010; Uzun et al. 2012; Cacan et al. 2018).

4. Conclusions

This study aimed to determine the seed yield and quality characteristics of some field pea genotypes at Bursa ecological conditions and to propose a new field pea cultivar candidate. According to the data obtained in this study, it was determined that the PS1 line surpassed the control cultivar and other lines in many characteristics. Based on seed yield and quality values at two years of Bursa ecological conditions, PS1 line can be considered for a new cultivar candidate.

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Effects of Goat Manure, Biochar, and NPK Applications on Growth and Nutrient Concentrations of Lettuce

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ABSTRACT

During the last decades, biochar (BC) gained increasing importance amongst scholars. Though, only a few studies explored the effects of the combined application of BC with manure on plant growth and nutrient concentrations. This study investigates the effects of separate and joint applications of goat manure (GM) and its derived BC on the growth and mineral element concentrations in lettuce. A completely random design was used in the field experiment. Two factors consisted of GM, BC at 5 Mg ha^{-1} and 10 Mg ha^{-1} combined with inorganic fertilizers [nitrogen, phosphorus, and potassium (NPK)] at 100%, 50%, and 0% were applied to the soil before planting the lettuce and, ANOVA analysis and Duncan test (at α=5%) were conducted. The results showed that a joint application of inorganic and organic fertilizers affects significantly the yield and yield parameters of the lettuce whilst the separate application of organic fertilizer affects significantly the lettuce content in N, P, K, Ca and Mg. Compared to GM, the application of GM-derived BC increases significantly the lettuce content in Zn and decreases its content in Cu. In addition, the highest total yield of lettuce was obtained with a joint combination of GM, BC, and 100% NPK (100 kg N ha^{-1}, 100 kg P_2O_5 ha^{-1} and 100 kg K_2O ha^{-1}). Consequently, this combination of organic and inorganic fertilizers is favorable fertilization in producing lettuce.

Keywords: Biochar, Goat manure, Lettuce, Inorganic fertilizer, Nutrients

1. Introduction

For long decades, inorganic fertilizers were used as one of the most essential inputs in agricultural systems. Though the resources consisting of the raw materials of some fertilizers such as phosphorus have declined (Gunes et al. 2006) and other inorganic fertilizers composed of heavy metals such as Hg, Cd, As and Pb accumulated in the different parts of the plants while they are applied at a high dose to the agricultural soil (Savci 2012). Besides, the pollution of soil and groundwater resources due to NO_3 constitutes serious issues (Pawar & Shaikh 1995) so that the increase of fertilizer efficient use becomes crucial. Accordingly, Palm et al. (1997) indicated that the combination of organic materials and inorganic fertilizers is a reliable method.

On the other hand, processed or unprocessed farmyard manures could be used in agricultural systems. Most studies focused on biochar (BC) obtained from wood materials (Lentz & Ippolito 2012; Borchard et al. 2012; Schomberg et al. 2012; Hansen et al. 2016). Besides, Sarmento et al. (2019) investigated the effects of goat manure (GM) application on the development and yield of lettuce plants in Brazil.

BC is a porous substance rich in carbon produced from organic materials such as; animal droppings, wood, green waste, sludge by a means of pyrolysis process under limited oxygen supply (Lehmann & Joseph 2009). Sohi et al. (2010) mentioned that the International Biochar Initiative (IBI) indicated that BC differs from charcoal due to it is especially used as a soil amendment. In addition, Lehmann et

al. (2006) noted that BC is a material obtained from black fragments, light, porous, dry, and easy to transport. Zhao and Nartey (2014) defined BC as a porous, high specific surface area, negatively charged, containing functional groups (hydroxyl, carboxylic, phenolic) material.

Recently, BC has gained much importance from scholars due to it is considered a means to combat global climate change and to improve soil fertility. Besides Lehmann (2007) stated that BC is an inorganic and biological product that could remain active in the soil for more than 1000 years. Therefore, the application of BC in the soil offers opportunities for atmospheric carbon sequestration and increases the soil capacity in nutrient retention. This is due to that a high specific surface area coupled with the presence of numerous surface functional groups increase the cation exchange capacity (CEC) of the soil (Sohi et al. 2010; Verheijen et al. 2009). Krull et al. (2009) mentioned that the BC physico-chemical properties depend on the pyrolysis conditions and type of used raw material and Wang et al. (2012) noted that the BC effects on the availability of plant nutrients in soil depend on the type of raw material as well as the content of BC nutrients. Most studies focused on BC application, soil physico-chemical properties, and plant yield (Sohi et al. 2010; Jeffery et al. 2011) whilst only a few scholars explore the effects of BC and manure application jointly (Sarkhot et al. 2012; Lentz et al. 2014).

On the other hand, the application of BC could improve soil water retention, reduce the toxicity of certain pollutants and stimulate soil biodiversity (macro and micro-fauna) (Rees et al. 2014). Biederman and Harpole (2013) mentioned that the presence of BC in the soil increases crop productivity is usually observed in the presence of BC whilst Jeffery et al. (2011) stated that BC has potential negative effects such as; increasing soil salinity and fortifying soil contaminants. The use of BC in agriculture and the environment gained increasing importance due to it improving plant growth, sequestering carbon, and capturing the soil contaminants (Verheijen et al. 2009). Allaire and Lange (2013), highlight that this interest in BC comes from its ability to improve soil fertility, water content, and soil microbiology and therefore BC contributes to increasing plant productivity both in agricultural soils, artificial soils, or degraded soils.

In addition, the importance of BC depends on its properties, type of soil, plant species, and climate (Mounirou et al. 2020). Accordingly, the BC properties depend on the used raw material and the type of pyrolysis method used for its production. The advantages of the pyrolysis technique include easy liberation of nutrients to the soil and the BC can be lonely used or combined with organic or mineral fertilizers to improve soil fertility (Mounirou et al. 2020). In addition, BC can be applied to soils polluted by heavy metals to restore them. Besides, BC contains a high proportion of P, K, Ca, Mg, Fe, Zn, Cu, and Mn twice than manure, and can sequester carbon for millennia due to its high carbon content. BC improves soil pH, CEC, soil water retention capacity, biological activity, and structure so that it could significantly improve the physico-chemical and biological soil parameters (Kaya et al. 2019).

Many techniques are used in producing BC but they can be ranged into modern or artisanal techniques. Modern techniques, relatively affordable are developed in India, Canada, Japan, China, Turkey, France and Rwanda, which are countries are where there is increasing interest in BC. However, previously it was shown that BC can increase crop yields by 100-300% according to its raw materials, the temperature of pyrolysis techniques and the type of soil and the plant. This study aims to evaluate the efficiency of BC, manure, and inorganic fertilizers and their combination on lettuce cultivation.

2. Materials and Methods

2.1. Materials

2.1.1. Manure and biochar

The GM used in this study was procured at Ankara University hovel and it was sieved at 2 mm and then it was weighted before application. In addition, this sieved GM was used for obtaining BC. For this, the GM was pyrolyzed in a stainless-steel skip under limited O_2 conditions. First, the GM was filled into the skip and tilted horizontally on 2 bricks. Wood was fired perpetually under skip for 3 hours and rotated every 30 minutes and then after 3 hours, it was waited to cool room temperature and weighted for application. The BC yield was determined as described by Sadaka et al. (2014) and the physicochemical properties of the GM and BC were shown in Table 1.

Table 1- Physicochemical property of GM and BC

Characteristics	*GM*	*BC*
pH, (1:10 w/v)	9.22	12.2
EC, mS cm^{-1} (1:10 w/v)	16.3	26.0
Organic matter, %	65.9	41.3
Ash, %	34.1	58.7
C, g kg^{-1} (elemental analyzer)	273	281
N, g kg^{-1} (Kjeldahl)	26.8	17.8
P, g kg^{-1} (wet digestion)	8.46	15.9
K, g kg^{-1} (wet digestion)	34.5	69.4
Ca, g kg^{-1} (wet digestion)	41.7	64.7
Mg, g kg^{-1} (wet digestion)	9.25	12.6
S, g kg^{-1} (elemental analyzer)	6.60	7.85
Fe, g kg^{-1} (wet digestion)	5103	7860
Zn, g kg^{-1} (wet digestion)	220	410
Cu, g kg^{-1} (wet digestion)	50.0	92.0
Mn,g kg^{-1} (wet digestion)	341	609

GM: goat manure, BC: biochar

2.1.2. Site of study

This study was conducted at the Research Farm of Ankara University. The soil of the site of the experiment consists of clay loam and contains about 4.4 g kg^{-1} organic matter (wet digestion), 0.83 g kg^{-1} total N (Kjeldahl), 5.90 mg kg^{-1} available P ($NaHCO_3$ extraction), 743 mg kg^{-1} K, 5653 mg kg^{-1} Ca, 2387 mg kg^{-1} Mg (NH_4OAc extraction). Moreover, the soil of the site of the experiment had a pH value of 8.22, contained lime at 153.2 g kg^{-1} and its EC was 358 µS cm^{-1}. The available Fe, Zn, Cu, and Mn (DTPA extraction) were 3.22, 0.55, 1.43, and 11.6 mg kg^{-1}, respectively. Furthermore, the average temperatures were 18.8 °C maximum and 11.8 °C minimum whilst the average of rainfalls recorded in September was 26.8 mm and 19.5 in October.

2.1.3. Experimental design

This study was conducted in September and October 2017 at the Ayaş Horticultural Research and Application Station of Ankara University. A completely random experiment design consisting of 2 factors (organic and inorganic fertilizers) was used. GM and BC were applied separately and together at 5 Mg ha^{-1} and 10 Mg ha^{-1}, respectively, 2 days before planting the lettuce. In addition, inorganic fertilizers 100% [nitrogen, phosphorus, and potassium (NPK)] composed of 100 kg N ha^{-1}, 100 kg P_2O_5 ha^{-1} and 100 kg K_2O ha^{-1} was applied at the same date with inorganic fertilizer. Other NPK doses 50% NPK and 0% NPK (control) were used. Accordingly, soil and fertilizers were mixed with grabber and 30-day-old lettuce (*Lactuca sativa* L. var. Crispa) seedlings were planted spaced 30x30 cm between the rows and each plot was distant 1.5x1.5 m width and separated by a 0.5 m buffer. Then, the experiment area was watered with a drip irrigation system.

2.2. Methods

2.2.1. Measurements

Sixty days after planting, the plants were harvested, the total yield, the weight and diameter of the head, the weight of fresh and dry leaves and the total concentrations of the nutrient (N, P, K, Ca, Mg, Fe, Zn, Cu, Mn) were determined. A total of 10 mature plants randomly chosen from experiment plot, the weight of the plant, (g $plant^{-1}$) was determined by weighing on the balance. In addition, the head diameter was determined by selecting randomly 10 plants from each experiment plots as suggested by Sarmento et al. (2019). Accordingly, this head diameter was the average value obtained by measuring with a ruler by turning the heads upside down. Three leaves of lettuce were pulled off and 4^{th} and 5^{th} healthy leaves to determine the leafs fresh and dry weight. For this, the leaf samples were dried at 65 °C until to have a constant weight whilst the concentration of the leaves N and P were determined according to Kjeldahl

procedure and spectrophotometrically, respectively and other parameters determined by AAS (Analytik Jena Vario 6) in a solution prepared form wet digested (4:1-HNO_3:$HClO_4$) plant subsamples (Isaac and Kerber 1971).

2.2.2. Statistical analysis

MINITAB 17 was used for data analysis, and the ANOVA test and Duncan test (at α=5%) were used to compare the study variables.

3. Results

3.1. Effects of NPK and organics manures applications on head weight and diameter of the lettuce

The combination between organic manures and NPK effects significantly the head weight and diameter of the plants of lettuce ($p<0.01$). The highest head weight was observed with the combination of 100% NPK + GM + BC (Table 2). Compared to 50% NPK + organic manure, 100% NPK didn't affect the head weight, but there were some similarities between head weight and diameter of lettuce. All the combinations increased head diameter as well as 100% NPK, and 50% NPK + organic manure combination.

Table 2- Effects of NPK and organic manure applications on head weight and diameter of the lettuce

Head weight (g plant^{-1})			
Applications	100% NPK	50% NPK	0% NPK
Control	96.8±1.64^{bc}	73.5±0.44^{d}	32.7±4.05^{e}
BC	114.3±1.49^{b}	96.2±7.21^{bc}	78.2±3.95^{d}
GM	103.4±11.2^{bc}	109.7±11.1^{b}	86.2±4.65^{cd}
BC + GM	133.6±2.33^{a}	109.5±3.83^{b}	74.0±5.57^{d}
F value	NPK	57.85***	
	OM	24.45***	
	NPK x OM	3.76**	
Head diameter (cm)			
Applications	*100% NPK*	*50% NPK*	*0% NPK*
Control	17.9±0.43^{bcde}	16.2±0.18^{e}	12.3±1.14^{f}
BC	19.7±0.35^{abc}	18.5±0.59^{bcd}	17.8±0.49^{cde}
GM	18.8±0.45^{bcd}	19.2±0.87^{abcd}	18.0±0.09^{bcde}
BC + GM	20.9±0.25^{a}	19.8±0.61^{ab}	17.5±0.67^{de}
F value	NPK	26.65***	
	OM	27.19***	
	NPK x OM	3.51**	

GM: goat manure, BC: biochar, NPK: inorganic fertilizer, OM: organic manures
Values are the average of 4 replicates
**p<0.01, ***p<0.001
The difference between the means indicated with the same letter in the same column is not significant ANOVA test and Duncan test (at α=5%)

3.2. Effect of NPK and organics manures applications on wet weight and dry weight of lettuce

Table 3 showed the effect of NPK and organic manure applications on wet weight and dry weight of lettuce. Accordingly, the application of both NPK, organic manure applications, and NPK x organic manures increased significantly the leaf fresh and dry weights and the interaction was significant at 5% ($p<0.05$).

Table 3- Effect of NPK and organic manure applications on wet weight and dry weight of lettuce

Wet weight (g leaf^{-1})			
Applications	*100% NPK*	*50% NPK*	*0% NPK*
Control	7.95±0.15^{cde}	6.35±0.15^{f}	3.25±0.39^{g}
BC	9.50±0.24^{ab}	8.10±0.31^{cd}	6.75±0.39^{ef}
GM	8.90±0.82^{bc}	9.15±0.79^{abc}	7.20±0.18^{def}
BC + GM	10.30±0.37^{a}	9.05±0.33^{abc}	6.55±0.34^{f}
	NPK		59.53***
F value	OM		27.14***
	NPK x OM		2.68*
Dry weight (g leaf^{-1})			
Applications	*100% NPK*	*50% NPK*	*0% NPK*
Control	0.43±0.01^{bcd}	0.36±0.01^{e}	0.21±0.02^{f}
BC	0.49±0.01^{ab}	0.44±0.01^{bc}	0.37±0.01^{de}
GM	0.48±0.04^{ab}	0.49±0.03^{ab}	0.41±0.01^{cde}
BC+GM	0.54±0.02^{a}	0.48±0.01^{ab}	0.37±0.02^{e}
	NPK		59.66***
F value	OM		29.47***
	NPK x OM		3.27*

GM: goat manure, BC: biochar, NPK: inorganic fertilizer, OM: organic manures
Values are the average of 4 replicates
*p<0.05, ***p<0.001
The difference between the means indicated with the same letter in the same column is not significant ANOVA test and Duncan test (at α=5%)

3.3. Effect of NPK and organics manures applications on the total yield of lettuce

The application of organic manures and NPK dosages affected significantly the total yield (p<0.01). The application of the organic manures and inorganic fertilizers affected the yield and the highest yield was obtained with the combination of 100% NPK + GM + BC (Figure 1). Furthermore, compared to the combination of 50% NPK + organic manure, the combination of 100% NPK did not affect the total yield.

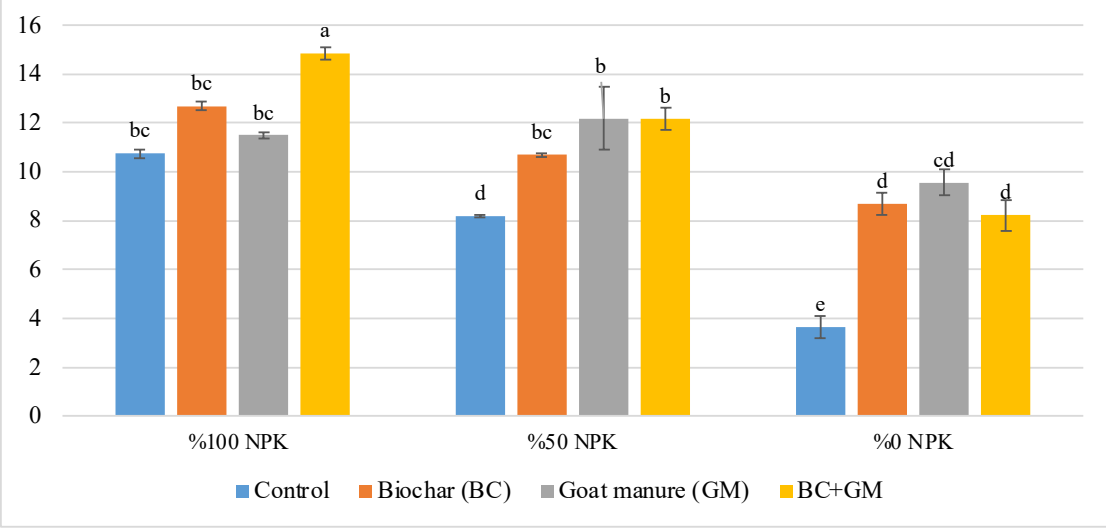

Figure 1- Effect of NPK and organics manures applications on total lettuce yield
Column showed mean of three repeats and bars showed standard error (p<0.05)

3.4. Effect of NPK and organics manures applications on total NPK of lettuce

Table 4 showed that the applications of inorganic and organic fertilizer on the total N content of the plant lettuce were statistically significant. With regards to the control treatment, the application of inorganic and organic fertilizers increased significantly the plant total N content. Likewise, the application of organic fertilizers increased the total N content compared to the control, the applications of 100% and 50% inorganic fertilizer did not show the effect on the plant total N.

On the other hand, the applications of inorganic and organic fertilizer affected significantly the total P content. Compared to the control treatment, the application of both inorganic, and organic fertilizers increased the total P content of the plant of lettuce. Besides, the application of organic fertilizers has increased significantly the lettuce content in P whilst the applications of inorganic and organic fertilizer and their interactions have affected significantly on the total K content in the trial area. Compared to the control, the total K content of lettuce plants increased significantly in all the treatments. For the combinations; 100% and 50% inorganic fertilizer, the total K content was statistically the same due to the applications of organic fertilizer Compared to the control, the total K content of lettuce plants receiving the organic fertilizers increased significantly.

Table 4- Effect of NPK and organics manures applications on total NPK of lettuce

Total N (g kg^{-1})			
Applications	*100% NPK*	*50% NPK*	*0% NPK*
Control	36.0±0.54^{abc}	34.9±1.00^{abc}	29.6±0.93^{d}
BC	35.6±0.41^{abc}	35.5±0.43^{abc}	34.0±0.94^{c}
GM	36.9±0.50^{a}	36.4±0.74^{ab}	34.6±0.43^{bc}
BC + GM	36.1±0.55^{abc}	36.4±0.71^{ab}	35.1±0.47^{abc}
F value	NPK	20.52***	
	OM	8.59***	
	NPK x OM	3.64*	
Total P (g kg^{-1})			
Applications	*100% NPK*	*50% NPK*	*0% NPK*
Control	3.50±0.32^{b}	2.81±0.16^{c}	2.03±0.06^{d}
BC	4.89±0.16^{a}	4.82±0.18^{a}	4.43±0.23^{a}
GM	4.40±0.16^{a}	3.82±0.12^{b}	3.27±0.12^{bc}
BC + GM	5.02±0.07^{a}	4.75±0.33^{a}	4.84±0.15^{a}
F value	NPK	18.22***	
	OM	77.55***	
	NPK x OM	2.64*	
Total K (g kg^{-1})			
Applications	*100% NPK*	*50% NPK*	*0% NPK*
Control	87.8±1.45^{a}	85.5±1.93^{ab}	66.7±1.80^{c}
BC	86.8±0.77^{ab}	88.3±1.92^{a}	84.5±1.37^{ab}
GM	83.3±0.94^{ab}	88.2±4.34^{a}	83.6±3.13^{ab}
BC + GM	86.2±2.11^{ab}	89.2±3.92^{a}	79.5±2.46^{b}
F value	NPK	16.48***	
	OM	4.16*	
	NPK x OM	4.21**	

GM: goat manure, BC: biochar, NPK: inorganic fertilizer, OM: organic manures

Values are the average of 4 replicates

$^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$

The difference between the means indicated with the same letter in the same column is not significant ANOVA test and Duncan test (at α=5%)

3.5. Effect of NPK and organics manures applications on total Ca and Mg of lettuce

The interaction effect between inorganic and organic fertilizer applications on lettuce total Ca content in the trial area was statistically significant. In all applications, the lettuce plant total Ca content increased significantly compared to the control. Application of 50% inorganic fertilizer only in plants treated with BC increased plant Ca content compared to 100% inorganic fertilization. In plants that do not apply inorganic fertilizers, all organic fertilizers applied significantly increased the total Ca content compared to control (Table 5). It showed that the effect of inorganic and organic fertilizer interaction on the total Mg content of the lettuce plant was statistically significant. In 100% to 50% inorganic fertilizer applications, organic fertilizer applications significantly reduced total Mg compared to their Controls. In plants without inorganic fertilizer application, total Mg content increased significantly with the application of BC and goat fertilizer.

Table 5- Effect of NPK and organic manure applications on total Ca and Mg of lettuce

Total Ca (g kg^{-1})			
Applications	*100% NPK*	*50% NPK*	*0% NPK*
Control	32.4±1.78^{bc}	34.7±0.08^{abc}	24.9±2.20^{d}
BC	29.9±0.53^{c}	38.6±1.86^{a}	33.4±1.83^{bc}
GM	32.3±2.10^{bc}	35.3±1.46^{ab}	36.4±0.61^{ab}
BC + GM	31.6±1.83^{bc}	35.1±0.65^{abc}	31.3±2.11^{bc}
F value	NPK	10.2***	
	OM	3.70*	
	NPK x OM	3.74**	
Total Mg (g kg^{-1})			
Applications	*100% NPK*	*50% NPK*	*0% NPK*
Control	8.52±0.22^{b}	9.47±0.09^{a}	6.33±0.20^{ef}
BC	7.18±0.13^{cd}	7.50±0.33^{c}	6.56±0.15^{ef}
GM	5.51±0.13^{h}	6.17±0.14^{fg}	6.74±0.07^{de}
BC + GM	5.73±0.08^{gh}	6.12±0.06^{fg}	8.64±0.14^{b}
F value	NPK	12.9***	
	OM	77.7***	
	NPK x OM	68.5***	

GM: goat manure, BC: biochar, NPK: inorganic fertilizer, OM: organic manures
Values are the average of 4 replicates
$^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$
The difference between the means indicated with the same letter in the same column is not significant ANOVA test and Duncan test (at α=5%)

3.6. Effect of NPK and organic manure applications on total Fe and Zn of lettuce

The effect of inorganic fertilizer and organic fertilizer applications on the total iron content of the lettuce plant in the trial area was statistically significant, but the effect of the interaction was insignificant. Total iron increased significantly with 50% inorganic fertilizer application. With regards to the application of organic fertilizer, there was not any statistically significant difference between the different treatments. However, the content in Fe was low in the plant receiving the goat fertilizer compared to plants with BC (Table 6). It showed that the effect of inorganic and organic fertilizer applications interaction on the total zinc content of the lettuce plant was significant. Through, the application of 50% inorganic fertilizer, only the BC plant increased the Zn content.

3.7 Effect of NPK and organic manure applications on total Cu and Mn of lettuce

The interaction of inorganic fertilizer applications to the total Cu content of the lettuce plant in the trial area was insignificant, but the effect of organic fertilizer applications was statistically significant. With the application of BC alone, the total Cu content of the lettuce plant decreased compared to the control (Table 7). Additionally, Table 7 showed that the application of only inorganic fertilizer on the total Mn content of the lettuce plant in the trial area was statistically significant and all inorganic fertilizer applications performed increased compared to the control.

Table 6- Effect of NPK and organic manure applications on total Fe and Zn of lettuce

Total Fe (mg kg^{-1})				
Applications	*100% NPK*	*50% NPK*	*0% NPK*	*Mean*
Control	122.6±10.5	126.6±3.52	138.6±18.7	129.3±6.85^{ab}
BC	134.1±15.1	153.1±4.12	127.4±9.55	138.2±6.44^{a}
GM	101.4±3.84	125.6±15.0	107.6±5.12	111.5±5.81^{b}
BC + GM	118.6±12.2	141.4±7.88	107.4±8.83	122.4±6.67^{ab}
Mean	119.2±5.85^{b}	136.7±4.94^{a}	120.2±6.26^{b}	
	NPK		3.39*	
F value	OM		3.39*	
	NPK x OM		0.94^{NS}	
Total Zn (mg kg^{-1})				
Applications	*100% NPK*	*50% NPK*	*0% NPK*	
Control	16.85±0.57^{bcd}	16.33±0.70^{cd}	19.65±0.35^{bc}	
BC	14.18±0.24^{d}	20.35±1.23^{b}	17.48±0.75^{bcd}	
GM	16.60±0.56^{cd}	15.08±1.15^{d}	24.37±3.24^{a}	
BC + GM	16.15±0.59^{cd}	15.43±0.25^{d}	16.78±0.12^{bcd}	
	NPK		10.99***	
F value	OM		2.54^{NS}	
	NPK x OM		6.04***	

GM: goat manure, BC: biochar, NPK: inorganic fertilizer, OM: organic manures

Values are the average of 4 replicates

^{NS}Non-significant, *p<0.05, ***p<0.001

The difference between the means indicated with the same letter in the same column is not significant ANOVA test and Duncan test (at α=5%)

Table 7- Effect of NPK and organic manure applications on total Cu and Mn of lettuce

Total Cu (mg kg^{-1})				
Applications	*100% NPK*	*50% NPK*	*0% NPK*	*Mean*
Control	18.15±0.67	17.93±0.66	18.33±0.80	18.14±0.38^{a}
BC	17.23±0.25	17.83±0.76	15.50±0.65	16.85±0.43^{b}
GM	20.20±0.40	18.08±0.73	18.90±0.53	19.06±0.40^{a}
BC + GM	19.88±0.41	18.65±0.75	19.00±1.02	19.18±0.43^{a}
	NPK			
F value	OM	7.81***		
	NPK x OM	1.55^{NS}	2.18^{NS}	
Total Mn (mg kg^{-1})				
Applications	*100% NPK*	*50% NPK*	*0% NPK*	
Control	53.83±0.97	54.35±0.57	48.15±2.81	
BC	54.10±1.62	53.35±1.41	51.18±1.84	
GM	56.40±1.65	57.15±1.41	53.78±2.20	
BC + GM	56.88±2.78	57.75±0.66	51.13±2.93	
Mean	55.30±1.22^{a}	55.65±0.68^{a}	51.06±0.91^{b}	
	NPK			
F value	OM	2.64^{NS}		
	NPK x OM	0.37^{NS}	7.23**	

GM: goat manure, BC: biochar, NPK: inorganic fertilizer, OM: organic manures

Values are the average of 4 replicates

^{NS}Non-significant, **p<0.01, ***p<0.001

The difference between the means indicated with the same letter in the same column is not significant ANOVA test and Duncan test (at α=5%)

4. Discussion

The application of organic manure applications increases the efficient use of inorganic fertilizer. In addition, the application of organic amendments has improved the nutrient supplements, the capacity of soil water retention, bulk density and CEC. These results are consistent with Yadav et al. (2019) who indicated that the application of BC with inorganic fertilizers increased the biomass, whilst the application of farmyard manure combined with inorganic fertilizers reduced this biomass.

The application of alkaline BC and GM in calcareous soil increased significantly the lettuce total yield Similarly Gunes et al. (2014), Inal et al. (2015), Sahin et al. (2017) and Najafi-Ghiri et al. (2019) indicated that the application of BC increased barley yield. Besides, Kammann et al. (2011) found that the application of the BC on sandy soil at 0, 100 and 200 t ha^{-1} increase the plant development and the leaves content in N content under greenhouse conditions.

Table 4 showed that the applications of inorganic and organic fertilizer lettuce increase significantly the total N and P content of the plant and the applications of inorganic and organic fertilizer and their interactions have affected significantly on the total K content. Previously, Spokas et al. (2012) mentioned that BC acts as an N-trap in the soil so that it increased the rate of nitrogen utilization of plants. Clearly, Jones et al (2012) emphasized that the application of BC increased significantly the N content of the meadow and Deluca et al. (2009) explained that the conversion of organic matter to BC presents high soluble P content that increased significantly the plant phosphorus concentration. Furthermore, Wang et al. (2018) note that the application of bamboo BC prepared at 450 °C at 0.5, 10, and 25 g kg^{-1} increases the plant concentration in potassium.

Gunes et al (2014 and 2015) state that the application of BC and phosphorus improves the concentrations of N, P, K in the plant of lettuce plant and the application of chicken manure BC obtained under different temperatures increases P and K concentrations and total yield of lettuce and corn.

Compared to the control, the application of the organic fertilizers increases the total Ca content with the 100% to 50% inorganic fertilizer applications reduce significantly reduced total Mg in the plant. Major et al. (2010) found that the application of BC (0.8 and 20 tons ha^{-1}) increases the calcium intake of the plant and Lehmann et al. (2006) mentioned that the use of BC in tropical areas increases the P, K Ca uptake by the plants.

On the other hand, Rees et al. (2014) and Zhou et al. (2017), and Gunes et al. (2014) indicated that the intake of microelements decreased with the application of BC in the soils slightly alkaline and Mielki et al. (2016) stated that the application of the BC increases soil pH by decreasing the beneficial Fe concentration which decreases the iron concentration in the plant. Gunes et al. (2015) found that application BC caused a decrease in iron concentration in lettuce plants and Salmani et al. (2014) noted that the application of BC reduces the copper uptake of the plant because it adsorbs the amount of useful copper in the soil. Karami et al. (2011) found the application of BC reduces the copper concentration on the plant of meadow grass. Likewise, Park et al. (2011) reported that application of BC on the plant cabbage reduces copper intake and Guneş et al. (2015) mentioned that the development of chicken manure BC obtained at different temperatures on the plant of lettuce plant increases the concentration of the plant in Zn with the BC obtained at 300 °C and 350 °C. Previously, Mandal et al. (1988) stated that the application of BC increases soil organic matter.

Lentz and Ippolito (2012) highlight that the application of organic fertilizer at 42.4 ha^{-1} and BC obtained from hardwood pyrolysis at 22.4 t ha^{-1} increased the content of the soil in Mn 1.5 times, while the application of organic fertilizer increases 1.2-1.7 times other plant nutrients except Fe. Peng et al. (2011) and Dong et al. (2011) noted that the application of BC decreases the usefulness of some microelements by increasing soil pH according to the material used to produce this BC and Chirenje and Ma (2002) indicate that the increase in soil pH after BC application is thought to be related to the ash content and pH of the BC.

5. Conclusions

This study investigates the effects of separate and joint application of GM and BC on the growth, mineral concentrations and efficient use of inorganic fertilizer on the lettuce. The findings showed that the application of whether GM or BC alone improved significantly the parameters of the growth of the plants and the content of the nutrient of the plants in N, P, K and Ca. Though the application of inorganic fertilizers reduced significantly the content of plants in Mg, Fe, Zn, Cu, and Mn according to the applied doses of and partially decreased with the applications of BC. This study revealed that a joint application of 100% of inorganic fertilizer, BC and GM had the highest yield. Also, a joint application of 50% inorganic fertilizer combined whether with BC or the GM affected similarly the plant yield than 100% of inorganic fertilizer, BC and GM. Additionally, these doses affected the growth of the plants and the nutrients content of leaves. An application of BC or GM combined with a low dose of inorganic fertilizers, especially in the soil with low organic matter

improves the physical, chemical, and biological of the soil. Therefore, it increases the productivity of the plants as well the content in nutrient of the plants.

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Determining Alternative Crops with Multi Criteria Decision Making Methods within the Framework of Land Risk Criteria

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ABSTRACT
Natural, societal, and economic hazards have a negative impact on agricultural production. In the field of agriculture, productivity studies are common, but election studies are rare. The goal of the study was to figure out which product to plant based on the region's characteristics by anticipating risk factors in advance. The most appropriate crop kind to grow based on the risk variables faced in agricultural production was explored in this study. The nine risk factors in the Çukurova region, as well as three alternative crops, were determined for this study. Input costs, changes in climatic conditions, changes in yield loss due to pests, agricultural tools and machinery failure, theft, fire, crop damage due to excessive water, crop loss due to drought and lack of technical information were chosen as criteria. Citrus, cereal, and legume were chosen as alternative crops. First, using the Analytical Hierarchy Process method, the weights of the score were determined. As a consequence of the weighing, the input costs criterion had the greatest weight value of 0.29. The criterion with the lowest score was a lack of technical information (0.01). Then, using the steps of the Elimination and Choice Translating Reality English method, which is one of the Multi Criteria Decision Making methods, the best relevant alternative ranking was determined. The comparison was also done using the Technique for Order Preference by Similarity to Ideal Solutions method. The cereal alternative was the best in both methodologies as a result of their application. In the first method, legumes and citrus were chosen, however in the second method, the opposite outcome was obtained.

Keywords: Risk criteria, Alternative crops, AHP, ELECTRE, TOPSIS

1. Introduction

Economic, social, political, technological, and human hazards all have an impact on the agriculture industry, which has a very sensitive and unique structure. Some variables may be taken into account for efficient production (Rani et al. 2020). From this perspective, agriculture's effective performance in terms of human nutrition is inextricably linked to the management of hazards that endanger agricultural productivity (Tümer et al. 2010).

Controlling and examining agricultural risks can be beneficial (Harvey et al. 2014, Duong et al. 2019). Some measures must be done beforehand in order to limit the risk, alleviate its impact, or maintain the sector's survival amid unfavourable conditions. It's impossible to imagine a world without risk, and being risk averse isn't an option. However, with the right policies in place, the risk may be managed and its impact minimised (Akçaöz & Özkan 2002).

Decision making is challenging in today's continuously changing and increasingly difficult life conditions. Because there are so many factors in decision making, Multi Criteria Decision Making (MCDM) procedures are more accurate in elections. The MCDM is a set of strategies for attempting to find the best or most appropriate solution to a decision problem that fits many criteria. The MCDM strategies can be applied to a variety of situations. Analytical Hierarchy Process (AHP), Technique for Order Preference by Similarity to Ideal Solutions (TOPSIS), and Elimination and Choice Translating Reality English (ELECTRE) are among the most popular (Sindhu et al. 2017; Çiçek et al. 2020). For example, Karaca (2013) recommended employing AHP, ELECTRE, and TOPSIS methodologies to choose from six dealer applicants for an automotive company in Turkey. The study by Pourkhabbaz et al. (2014) has two objectives: The first goal was to apply the Analytic Network Process (ANP) and Simple Additive Weighting (SAW) methodologies to determine the ecological capabilities of agricultural land utilization. Second, the integrated VIseKriterijumska Optimizacija I Kompromisno Resenje (VIKOR) and AHP models were used to rank the most acceptable agricultural solutions in this territory. According to the research, the north areas of the study region (Takestan-Qazvin Plain) are unfavorable for agricultural development. Tunca et al. (2015) investigated for the appropriate method for choosing the best accounting package programme by considering three main sets of criteria and fifteen

sub criteria. Ömürbek et el. (2016) used the AHP, ELECTRE, and SAW methodologies from MCDM methods to pick a building audit business in the construction sector in Isparta. Agricultural decision-making strategies use MCDM methods. Sánchez-Lozano et al. (2016) identified the Murcia coast in southern Spain as a potential location for solar photovoltaic fields. TOPSIS and ELECTRE, two MCDM methodologies, were used to explore for appropriate sites. Using MCDM methods, a very valuable database was developed for tackling complicated locations such as the evaluation and selection of viable places. Widiatmaka et al. (2016), the goal of this study was to examine at the agricultural land that was available in Bogor, Jakarta. Two steps of analysis are used in the methodology: Land suitability and land availability analysis utilizing AHP. In agriculture, Papathanasiou et al. (2016) demonstrated the deployment of a web-based decision support system that includes TOPSIS and VIKOR and allows decision makers to compare the outcomes of both methodologies. Alper & Başdar (2017) used the ELECTRE and TOPSIS methodologies to assess the financial effectiveness of factoring companies listed on the Istanbul Stock Exchange. The criterion weights in the study were obtained using the AHP approach by Yalçıner & Karaatlı (2018), while deposit bank selection was done using the TOPSIS and ELECTRE methods. Seyedmohammadi et al. (2018) used MCDM methods to assess locations appropriate for maize, rapeseed, and soybean crop growing planning in Iran. The incorporation of created framework as an effective instrument could help in executing better control over soil, land and environment losses. Deepa & Ganesan (2019) designed a decision making mechanism for determining the best crop to grow on agricultural land. The study by Tork et al. (2021) aims to use the AHP method to estimate the effectiveness and rate the possibilities for upgrading the surface water distribution system.

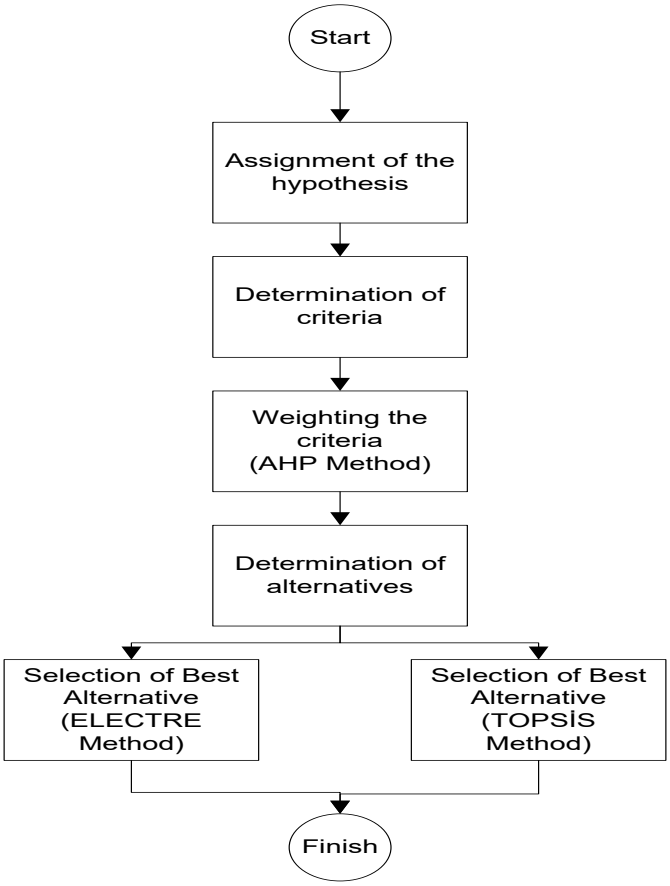

Figure 1- The flowchart of hybrid MCDM

The study's hypothesis is that risk factors in agricultural output are increasing as a result of global warming (fires, floods). Figure 1 depicts the study's hypothesis utilizing hybrid approaches. In this perspective, cereal is the most sustainable plant species with risk factors due to its high level of environmental adaptation. Cereal should be planted first among all crops, according to the study's research question. Input costs, changes in climatic conditions, changes in yield loss due to pests, agricultural tools and machinery failure, theft, fire, crop damage due to excessive water, crop loss due to drought, lack of technical information were set for criteria in this study (Akçaöz & Özkan 2002; Tümer et al. 2010). In the Çukurova region, alternative citrus, cereal, and legume crops were chosen. Weighting was accomplished using the AHP approach by scoring experts after the criteria had been determined. Then, using the ELECTRE and TOPSIS methods, the rank of obtaining optimal solution was discovered.

2. Material and Methods

The importance of MCDM in terms of guiding the decision maker cannot be overstated. In order to compute MCDM scores, one should seek expert advice. The experts' opinions are taken into account during scoring. People who have worked in agriculture in the Çukurova constituency have been interrogated.

One of the MCDM, AHP, was first suggested by Myers and Alpert in 1968 and developed as a model for solving decision-making challenges by Saaty in 1977 (Myers & Alpert 1968, Saaty 1977). The following are the steps that can only be followed to address a decision-making problem using AHP:

Step 1: The decision making problem is defined.

Step 2: The matrix of inter-factor comparison is constructed. Comparisons are made for values above the diagonal of the comparison matrix. The matrix elements on the diagonal are 1. It is used to Equation 1 for the components below the diagonal. *a* values are matrix elements which is scored in Table 1.

$$a_{ji} = \frac{1}{a_{ij}} \tag{1}$$

Table 1 shows a comparison of factors based on their respective relevance levels.

Table 1- Importance scale (Saaty 1977)

Importance	*Value definitions*
1	Equal Importance
3	Weak Importance of one over another
5	Essential or strong importance
7	Demonstrated Importance
9	Absolute Importance
2, 4, 6, 8	Intermediate value

Step 3: The significance distributions of elements are calculated as a percentage. The column vectors that generated the comparison matrix are utilised to estimate the weights and percentage significant distributions of the components, and the matrix *b* is produced in Equation 2.

$$b_{ij} = \frac{a_{ij}}{\sum_{i=1}^{n} a_{ij}} \tag{2}$$

In Equation 3, the column vector, referred to as the priority matrix *w*, is obtained by taking the arithmetic mean of the row constituents of the resulting matrix *c*.

$$w_i = \frac{\sum_{j=1}^{n} c_{ij}}{n} \tag{3}$$

Step 4: Consistency in factor benchmarks is measured. After calculating the total mean (λ), the Consistency Index (*CI*) can be calculated using in Equation 4. *n* is number of sampling.

$$CI = \frac{\lambda - n}{n-1} \tag{4}$$

Step 5: For each factor, percentage significance of distributions at the decision point is obtained.

Step 6: The distribution of the results at the decision points is determined.

The ELECTRE method was first used in 1966 introduced by Benayoun et al. (1966). Below are the steps of the ELECTRE method (Triantaphyllou 2000).

Step 1: The decision matrix is composed.

Step 2: The standard decision matrix is calculated using the Equation (5). To calculate, for example, the y_{11} element of matrix Y, the b_{11} element of matrix B is obtained by dividing by the square root of the sum of squares of the one column elements of the matrix.

$$y_{ij} = \frac{b_{ij}}{\sqrt{\sum_{k=1}^{r} b_{kj}^2}} \tag{5}$$

Step 3: The weighted standard decision matrix is generated by multiplying the elements in each column of the standard decision matrix corresponding weight values. The AHP methodology mentioned above is the most popular for these weights.

Step 4: Concordance and discordance determination of sets are encountered. The decision points for the evaluation factor are compared with another one.

Step 5: Concordance and discordance matrix are calculated. The concordance index (m_{kl}) is the sum of the weights associated with the criteria contained in the concordance set. Discordance matrix index (n_{kl}) is defined the Equation (6).

$$n_{kl} = \frac{\max_{j \in N_{kl}} \left| z_{kj} - z_{lj} \right|}{\max_{j} \left| z_{kj} - z_{lj} \right|} \tag{6}$$

Step 6: Composing concordance superiority and discordance superiority matrices size are r*c. The concordance threshold value ($\underline{m} = \frac{1}{r(r-1)} \sum_{k=1}^{r} \sum_{l=1}^{r} m_{kl}$) and the discordance threshold value ($\underline{n} = \frac{1}{r(r-1)} \sum_{k=1}^{r} \sum_{l=1}^{r} n_{kl}$) are obtained.

Step 7: The overall dominance matrix has values of 1 or 0 depending on whether it is more or less than the concordance and discordance matrices threshold value.

Step 8: The order of importance of decision points is determined.

Hwang and Yoon devised TOPSIS in 1981, and it contained the same core methods as the ELECTRE method (Hwang & Yoon 1981). The steps of the TOPSIS method as follows;

Step 1: The decision matrix is composed.

Step 2: The standard decision matrix is calculated the Equation (5).

Step 3: The weighted standard decision matrix (x_{ij}) is created by multiplying the elements in each column of the standard decision matrix corresponding weight values.

Step 4: In order to compose an ideal set (x_j^*), the largest of the evaluation factors in the weighted standard decision matrix, are selected. The negative ideal (x_j^-) solution is composed by selecting the smallest of the evaluation factors in the weighted standard decision matrix. If our goal is minimization, the values obtained will be the exact opposite.

Step 5: Calculation of separation measures obtained from are called the ideal distinction ($T_i^* = \sqrt{\sum_{j=1}^{c} (x_{ij} - x_j^*)^2}$) and the negative ideal distinction ($T_i^- = \sqrt{\sum_{j=1}^{c} (x_{ij} - x_j^-)^2}$) measure.

Step 6: Calculation of relative proximity ($U_i^* = \frac{T_i^-}{T_i^- + T_i^*}$) of each decision point to an ideal solution, ideal and negative ideal discrimination measures are used.

3. Results and Discussion

Weighting calculations were encountered in the Çukurova region as a result of expert evaluations within the scope of risk criteria selected from the literature using the AHP technique in this study (Akçaöz & Özkan 2002; Tümer et al. 2010). The decision matrix is composed by using weights with Equations (1-3).

The challenge is complicated by the definition of criteria, and the solution may comprise inconsistencies. The definition of criteria complicates the problem and there may be inconsistencies in the solution. In furthermore, the findings may be prejudiced as expert opinion is sought. Calculating the consistency index minimizes these complications, and Equation (4) can be used to properly apply the strategy (Table 2). Table 2 indicates the Random Index (RI) numbers corresponding to the number of *n* sampling. The AHP consistency index is 0.09, which is less than the desired 0.10 value.

Table 2- Consistency values

n	*3*	*4*	*5*	*6*	*7*	*8*	*9*
RI	0.58	0.90	1,12	1.24	1.32	1.41	1.45

AHP scores were computed by a brainstorming technique while AHP matrices are generated in the study. Points are determined by a total of 3 people, two experts with 3-5 years of work experience and one expert with 5-10 years of experience working in the field of agriculture. When making the scores, the experts included other disciplines in the brainstorming when necessary. Each criterion and its weighting values are given in Table 3 (Bold values). These criteria are determined by considering citrus, legume and cereal alternatives.

Table 3- Weighting by using AHP method

Criteria	*Input costs*	*Changes in climatic conditions*	*Changes in yield loss due to pests*	*Agricultural tools and machinery failure*	*Theft*	*Fire*	*Crop damage due to excessive water*	*Crop loss due to drought*	*Lack of technical information*	*wi*	***Wi***	*V1*	*V2*
Input costs	1	1	5	5	4	3	9	7	9	3.81	**0.29**	2.95	10.30
Changes in climatic conditions	1	1	2	3	5	1	7	7	9	2.87	**0.22**	2.12	9.85
Changes in yield loss due to pests	0.20	0.50	1	2	5	2	7	3	9	1.93	**0.15**	1.51	10.42
Agricultural tools and machinery failure	0.20	0.33	0.50	1	1	2	5	7	9	1.40	**0.11**	1.05	9.93
Theft	0.25	0.20	0.20	1	1	1	3	5	9	1.03	**0.08**	0.77	9.91
Fire	0.33	1.00	0.50	0.50	1.00	1	3	5	8	1.29	**0.10**	0.95	9.74
Crop damage due to excessive water	0.11	0.14	0.14	0.20	0.33	0.33	1	1	7	0.41	**0.03**	0.30	9.81
Crop loss due to drought	0.14	0.14	0.33	0.14	0.20	0.20	1.00	1	6	0.39	**0.03**	0.30	10.13
Lack of technical information	0.11	0.11	0.11	0.11	0.11	0.13	0.14	0.17	1	0.15	**0.01**	0.13	10.80
												λmax=	10.10

The yellow background indicates the scores given by the experts.

Table 1 is principally in the first stage, in light of the scales in the Çukurova region, according to the ELECTRE method; after that, the decision matrix is in Table 4. It is proved by rating the criteria specified for the selection of each alternative crop. The importance scale in Table 1 is used to rate the decision matrix.

Table 4- Composed a decision matrix according to the ELECTRE method

Criterion/Alternatives	*Citrus*	*Cereal*	*Legume*	*Weights*
Input costs	7	3	8	0.29
Changes in climatic conditions	9	8	7	0.22
Changes in yield loss due to pests	8	7	6	0.15
Agricultural tools and machinery failure	5	9	7	0.11
Theft	9	5	7	0.08
Fire	3	9	7	0.10
Crop damage due to excessive water	7	5	9	0.03
Crop loss due to drought	5	7	9	0.03
Lack of technical information	6	3	9	0.01

Each cell in Table 5, the standard decision matrix, has been calculated and rearranged according to Equation (5) in Step 2 of the ELECTRE method.

Table 5- Standard decision matrix by ELECTRE method

Criterion/Alternatives	*Citrus*	*Cereal*	*Legume*	*Weights*
Input costs	0.63	0.27	0.72	0.29
Changes in climatic conditions	0.65	0.57	0.50	0.22
Changes in yield loss due to pests	0.66	0.57	0.49	0.15
Agricultural tools and machinery failure	0.40	0.72	0.56	0.11
Theft	0.72	0.40	0.56	0.08
Fire	0.25	0.76	0.59	0.10
Crop damage due to excessive water	0.56	0.40	0.72	0.03
Crop loss due to drought	0.40	0.56	0.72	0.03
Lack of technical information	0.53	0.27	0.80	0.01

A weighted standard decision matrix is computed by multiplying each cell by the findings of the standard decision matrix discovered using the AHP methodology (Table 6).

Table 6- Weighted standard decision matrix by ELECTRE method

Criterion/Alternatives	*Citrus*	*Cereal*	*Legume*
Input costs	0.18	0.08	0.21
Changes in climatic conditions	0.14	0.13	0.11
Changes in yield loss due to pests	0.10	0.09	0.07
Agricultural tools and machinery failure	0.04	0.08	0.06
Theft	0.06	0.03	0.04
Fire	0.03	0.08	0.06
Crop damage due to excessive water	0.017	0.012	0.022
Crop loss due to drought	0.012	0.017	0.022
Lack of technical information	0.005	0.003	0.008

The following concordance sets (m) were discovered by comparing each row to each other. For items that are not in the concordance set, discordance sets (n) were established. The concordance and discordance sets are shown in Table 7.

Table 7- Concordance and discordance sets according to ELECTRE method

Concordance Sets		**Discordance Sets**	
m_{12}	1,2,3,5,7,9	n_{12}	4,6,8
m_{13}	2,3,5	n_{13}	1,4,6,7,8,9
m_{21}	4,6,8	n_{21}	1,2,3,5,7,9
m_{23}	2,3,4,6	n_{23}	1,5,7,8,9
m_{31}	1,4,6,7,8,9	n_{31}	2,3,5
m_{32}	1,5,7,8,9	n_{32}	2,3,4,6

Table 8 shows the concordance and discordance matrix after establishing their sets.

Table 8- Calculation of concordance and discordance matrix according to ELECTRE method

	Concordance matrix				*Discordance matrix*		
	Citrus	***Cereal***	***Legume***		***Citrus***	***Cereal***	***Legume***
Citrus	-	0.78	0.45	**Citrus**	-	0.5	1
Cereal	0.24	-	0.58	**Cereal**	1	-	1
Legume	0.57	0.44	-	**Legume**	1	0.15	-

The values of concordance and discordance threshold are calculated. The concordance threshold value is 0.51 and discordance threshold value is 0.78. In Table 9, cells that are greater than the concordance and discordance superiority matrix value are given 1 otherwise 0.

Table 9- Concordance and discordance superiority matrix according to ELECTRE method

	Concordance superiority matrix				*Discordance superiority matrix*		
	Citrus	***Cereal***	***Legume***		***Citrus***	***Cereal***	***Legume***
Citrus	-	1	0	**Citrus**	-	0	1
Cereal	0	-	1	**Cereal**	1	-	1
Legume	1	0	-	**Legume**	1	0	-

By multiplying the corresponding cells of the concordance superiority matrix and the discordance superiority matrix, the total dominance matrix in Table 10 is formed.

Table 10- Total dominance matrix by ELECTRE method

	Citrus	**Cereal**	**Legume**
Citrus	-	0	0
Cereal	0	-	1
Legume	1	0	-

As a result of this finding, legumes were preferred over citrus, and the preferred cereal is likewise preferred over legumes. In this instance, cereal is the best option, followed by legume and citrus.

TOPSIS, the MCDM method, uses the same weighted standard decision matrix as the ELECTRE method's first three steps. Table 11 displays the ideal and negative ideal values. Because the risks are not requested to be of significant value, the higher number in the row is the negative ideal result.

Table 11- The ideal and negative ideal values from the weighted standard decision matrix according to the TOPSIS method

Criterion/Alternatives	***Citrus***	***Cereal***	***Legume***	***Ideal***	***Negative Ideal***
Input costs	0.18	0.08	0.21	0.08	0.21
Changes in climatic conditions	0.14	0.13	0.11	0.11	0.14
Changes in yield loss due to pests	0.10	0.09	0.07	0.07	0.10
Agricultural tools and machinery failure	0.04	0.08	0.06	0.04	0.08
Theft	0.06	0.03	0.04	0.03	0.06
Fire	0.03	0.08	0.06	0.03	0.08
Crop damage due to excessive water	0.017	0.012	0.022	0.012	0.022
Crop loss due to drought	0.012	0.017	0.022	0.012	0.022
Lack of technical information	0.005	0.003	0.008	0.003	0.008

After calculations according to TOPSIS method, ideal distinction (T^*), negative ideal distinction (T^-) and relative proximity ($T^-/(T^*+T^-)$) are shown the ranking from the largest value to the smallest value is found in Table 12.

Table 12- Ranking alternatives according to TOPSIS method

Alternatives	T^*	T^-	$T^-/(T^*+T^-)$	*Ranking*
Citrus	0.12	0.07	0.38	2
Cereal	0.07	0.14	0.68	1
Legume	0.14	0.05	0.27	3

Cereal is chosen first in the TOPSIS approach, followed by citrus and legume. This disparity is predicted because both strategies were based on subjective judgements. Depending on the approach and locality, the decision maker can pick which alternative to choose. In the Çukurova region, the subject of which product should be preferred first, given the risk concerns, is a point of contention. The resolution of this debate can be clarified with the help of MCDM methods. The answer to this case is that cereal is a better option with the help of MCDM methods.

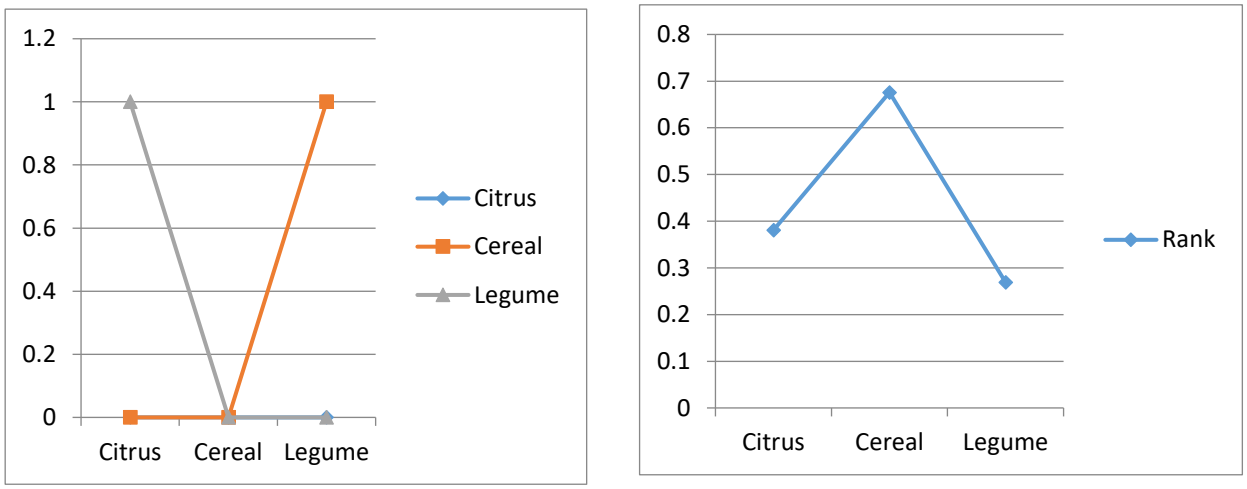

Figure 2- Benchmarking with ELECTRE and TOPSIS

Although the ELECTRE and TOPSIS approaches have identical initial phases, the final steps differ. For both techniques, cereal is the preferable crop (Figure 2). In this study, while cereal priority was observed, Gulluoglu et al. (2017), Bakal et al. (2017) and Arıoglu et al. (2018) focused on legumes (peanut, soybean) in the Mediterranean region. In terms of hazards, our results are preferable to the sort of crop they employed in their experiments. There are also studies on wheat and eucalyptus using MCDM method (Reubens et al. 2011; Sarkar et al. 2014). According to Sarkar et al. (2014), the watershed region was relatively appropriate for wheat crop development, with factors such as inadequate soil depth and inefficient drainage. The input cost criterion, on the other hand, has a high weight value in our analysis. Despite the fact that the criteria were given various weights in these researches, a MCDM method was utilized for crop selection.

4. Conclusions

In terms of environmental preservation and resource sustainability, efficient agricultural land management is critical. Taking into account the hazards in agricultural production might lead to more efficient production. MCDM methods can be used to identify risk strategies in agriculture, which is challenging and complex. The most acceptable crop type for production based on risk parameters encountered in agricultural production was explored in this study. In the Çukurova region, this research was examined at the criteria and alternatives. Input costs, changes in climatic conditions, changes in yield loss due to pests, agricultural tools and machinery failure, theft, fire, crop damage due to excessive water, crop loss due to drought, and lack of technical information were all used to determine AHP weights. The criterion weights were 0.29, 0.22, 0.15, 0.11, 0.08, 0.10, 0.03, 0.03 and 0.01, respectively. The input cost criterion was given the highest weight. The lack of technical information criterion, on the other hand, earned the lowest score. Citrus, cereal, and legume crops were chosen for the study based on geographical conditions. The ELECTRE method's most suited alternate sequencing was determined. By taking the value of cell 1 where legumes cross citrus, it was proposed that legumes would be chosen based on the entire matrix result. Similarly, by assigning a value of 1 to the cell where cereal and legume overlap, it was determined that the cell where cereal and legume intersect would be preferred over the legume in the cereal. It has also been proposed TOPSIS approach, which is another effective decision-making methodology. The respective rankings were 0.68, 0.38 and 0.27. Cereal was the first option, followed by legume. Following the use of the TOPSIS and ELECTRE methodologies, the cereal alternative was shown to be the best in both ways. The ELECTRE approach yielded legumes and citrus, whereas the TOPSIS method yielded the opposite outcome. Cereal can be determined to be the optimal choice, as indicated in our hypothesis. TOPSIS has produced more acceptable outcomes based on expert observations for the region. Another MCDM method can be used to determine the different regions for future crops to be planted. It can be integrated not only in the Çukurova region, but also in locations like the Black Sea, Marmara, and Aegean, based on risk factors. Risk variables (flood, landslide, etc.) and their degrees, for

example, can be updated based on precipitation in the Black Sea region, and alternatives for cultivating tea, hazelnut, and chestnut can be calculated.

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The Combined Effects of Salinity and Drought on Young Almond Trees and Physiological Parameters

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ABSTRACT
In drought years, almond growers have to restrict fresh water application, either stressing the trees with inadequate water or adding saline water and reducing water stress but causing salt stress. Tree response to combined water and salt stress are critical consideration on management decisions but there is no appropriate information currently. That's why, it was investigated the water and salt stress and combined water-salt stress on two almond varieties in a two year (2015 and 2016) outdoor experiment with young trees. Trees were 1 year old at the beginning of the experiment. The experiment was conducted USDA (United States Department of Agriculture) Salinity Laboratory, Riverside, California, USA. Drought treatments consisted of 100%, 80% and 60% of tree evapotranspiration (ET) and salt treatments of Electrical Conductivity (EC= 0.55, 1.20, 2.40 and 3.0 dS m^{-1}), for a total of 120 trees in twelve treatments with two varieties and five replicates. We examined water use, trunk diameter and physiological parameters (leaf net photosynthetic rate, stomatal conductance and leaf water potential). Photosynthetic rate values (Pn) ranged between 3.53 and 11.08 µmol CO_2 m^{-2} s^{-1} for Nonpareil and 4.58 and 9.48 µmol CO_2 m^{-2} s^{-1} for Aldridge. Stomatal conductance values ranged between 0.076 and 0.283 mol H_2O m^{-2} s^{-1} for Nonpareil and 0.097 and 0.302 for Aldridge. All parameters showed significant decline starting at 80% water application and EC 1.2 dS m^{-1}. In terms of growth rather than survival, almond was sensitive to water as well as salt stress. We evaluated combined stress using three stress response models: additive stress, dominant stress model and a multiplicative stress model where the predicted growth loss is obtained by multiplying the relative growth response for the individual stresses. Equation (2) for reduction in trunk growth were developed for treatments with either salinity only or water only stress. Both varieties grafted to Nemaguard rootstock were very sensitive to salinity with growth loss starting at EC 1.2 dS m^{-1}. The results indicate that the Nonpareil is more sensitive to drought and salt stress than Aldridge. Aldridge almond variety can be recommended for areas where water supplies are scarce and salinized.

Keywords: Almond, Combined stress, Photosynthesis, Stomatal conductance, Trunk growth

1. Introduction

Salinity is one of the most important problems threatening both arid and semi-arid agricultural lands. Throughout the world, more than twenty percent of the agricultural lands are irrigated and approximately 20% are under direct threat of salinity (Worldbank 2021). Increased salinity levels significantly limit crop quality and yields and when associated with increased sodicity, also deteriorate soil structure. In addition, salinity-induced stress influences plant growth through various physiological, biochemical and molecular changes exerted in plant internal mechanisms (Ashraf & Foolad 2007). Impacts of salinity on plant and soil mechanisms should clearly be identified in order to grow a crop with saline water or saline soil (Düzdemir et al. 2009). Salinity management in irrigation requires knowledge of the irrigation water amounts and salinity levels which avoid or minimize decrease in yield. Species and varieties of a genus of crops (plants) have wide variability in their resistance to salinity enabling growers to utilize this information when making crop or varietal crop selections.

Drought is another factor threatening agricultural production. The major part (approximately 70%) of water consumption in the world is used for agricultural production. However, as the ratio of water use increases due to rapidly increasing population and developing industry, water amounts used in agriculture decreases (Önder et al. 2005). Thus, there is a high likelihood that plant species cultivated in irrigated agricultural areas will face water deficit in coming years. In this case, researchers need to determine the resistance of different plant species to both drought and salt stress.

Both salinity and drought stress cause plants to limit water uptake and result in reduction of the growth rate associated with metabolic changes. When irrigating with saline water, reduced water application reduces salt leaching and increases salt

accumulation in the soil. Thus, not only water and salt stress needed to be examined but also plant response to combined salt and water stress.

Almond is considered sensitive to salt stress and its productivity rapidly reduces at salt concentrations above 1.5 dS m^{-1}, with a 50% yield reduction at a soil salinity concentration of 4 dS m^{-1} (where data are reported as EC_e, the salinity of a soil saturation extract), (Maas & Hoffman 1977; Ottman & Byrne 1988; Grieve et al. 2012). However almond response to salt stress has been shown to vary considerably as related to rootstock (El-Motaium et al. 1994; Zrig et al. 2016; Sandhu et al. 2020), as well as genotype of scion (Momenpour et al. 2018).

Contrary to the salt sensitivity of almonds, it is regarded as tolerant to water deficit conditions (Fereres & Goldhamer 1990; Torrecillas et al. 1996; Yıldırım et al. 2021). Almond has always been traditionally considered a drought tolerant crop, grown in areas with limited water supply (Gispert et al. 2011). However, this consideration is based primarily on survival as related to seasonal drought. Evaluation and identification of the tolerant cultivars of fruit trees are very important for drought stress and their ability to grow under these conditions. Drought stress generally has significant effects on plant physiology of almonds. Plant physiological characteristics such as photosynthesis and transpiration rate are dependent on the severity and duration of drought stress (Ranjbar et al. 2019). It has also been noted that response to water stress is dependent on rootstock (Isaakidis et al. 2004). The spectacular vegetative and productive response of this crop to irrigation (Leon et al. 1985) justifies the interest in the knowledge of the plant water relations in almond trees under drip irrigation conditions.

According to production data of year 2018, USA with 1,872,500 tons of annual production is first in world almond production (58.8% of total world production) (FAO 2020), with almost all the production in California. The almond growing regions in California face periodic restrictions on fresh water supply (surface water) and in drought years will need to evaluate if it is best to just reduce water application causing drought stress or to supplement with saline ground water causing reduced drought stress but adding salt stress. Our objectives were thus to 1) examine the salt, drought and combined salt and drought response of two different almond scions grafted to a commonly used rootstock and 2) evaluate predictive models for the response to the combined stresses to enable decision making regarding optimal quantities of applied saline water when fresh water is inadequate to meet tree ET demand.

2. Material and Methods

This project was conducted consecutively for two years (2015 and 2016) at an experimental area in USDA Salinity Laboratory, Riverside, California, USA. Two very commonly used varieties of almond, Nonpareil and Aldridge were grafted on a widely used rootstock, Nemaguard. Recent information indicates that Nemaguard is a relatively salt sensitive rootstock (D. Sandhu, personal communication).

Almond trees were planted in March 2015 into pots having 100 L volume. Soil mixture was including soil from the westside of the San Joaquin Valley and peat moss (1:1 ratio), with soil analysis provided in Table 1. Each tree was irrigated with control water (Riverside Gage Canal water, EC_w = 0.55 dS m^{-1}) until soil water level reached field capacity up to the beginning of June for each year. This simulates typical winter-spring conditions in Central California and Mediterranean climate where rain is sufficient during this period to avoid need for irrigation.

Table 1- Properties of soil mixture in pots

Texture	*Clay loam*
Saturation (%)	45.4
Salinity (dS m^{-1})	2.13
pH	7.94
Na ($mmol_c$ l^{-1})	6.629
K ($mmol_c$ l^{-1})	0.565
Ca ($mmol_c$ l^{-1})	13.180
Mg ($mmol_c$ l^{-1})	5.050

There were 12 different drought and salinity treatments in this experiment: Three different drought treatments for each irrigation salinity (D_0; full irrigation, 100% evapotranspiration of almond trees, no stress, D_1; 80% evapotranspiration of almond trees, 20% deficit irrigation, moderate stress, D_2; 60% evapotranspiration of almond trees, 40% deficit irrigation, severe stress). We had four different salinity treatments (S_0; EC= 0.55 dS m^{-1}, S_1; 1.20 dS m^{-1}, S_2; 2.40 dS m^{-1}, S_3; 3.0 dS m^{-1}) for each of the drought treatments. There were five replications for each treatment and each replication had one trees. 60 trees were used for each variety, totally 120 trees were used in the study. Maximum air temperature of vegetation period in 2015 and 2016 are shown in Figure 1a and 1b, respectively.

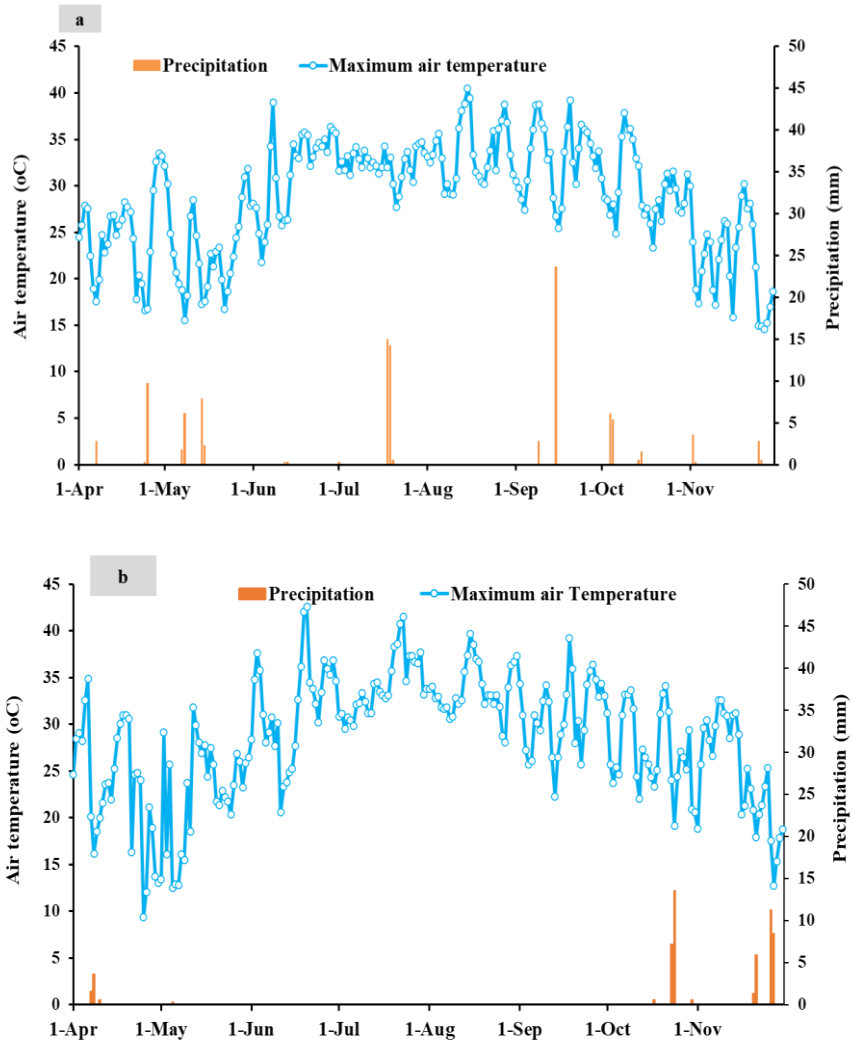

Figure 1- Maximum daily air temperature and precipitation values of experimental area in a) year 2015 and b) year 2016

2.1. Salt composition

In order to obtain the saline irrigation waters, KNO_3, KCl, $MgCl_2$, $CaCl_2$, NaCl, and $NaSO_4$ salts were added to the canal water during study years (2015 and 2016). During the preparation of saline waters, sodium adsorption ratio (SAR) values of each treatment were maintained less than 5.0 in order to avoid the adverse effect of increasing SAR on soil structure and water gas movement. There were four tanks for different salt treatments, each tank had a1400 L volume. All the salts were mixed and added to tanks located near the experimental area (Table 2). The canal water (EC=0.55 dS m^{-1}) was used as control treatment (S_0). All trees were fertilized with NPK and micronutrients per agronomic recommendations.

Table 2- Salt amounts used for preparing of saline water

Treatments	*Salts (g L^{-1})*					
	KNO_3	*KCl*	*$MgCl_2$*	*$CaCl_2$*	*NaCl*	*Na_2SO_4*
S_1 (1.20 dS m^{-1})	0.051	0.037	0.212	0.154	0.112	0.217
S_2 (2.40 dS m^{-1})	0.051	0.037	0.470	0.344	0.262	0.471
S_3 (3.0 dS m^{-1})	0.051	0.037	0.600	0.438	0.339	0.595

2.2. Measurements

2.2.1 Soil water measurement

Soil water content was measured using soil moisture sensors (Decagon 5TE Soil Moisture Corp.). One sensor was used for each replication. The soil field capacity and wilting point were 25.36 g g^{-1} and 17.20 g g^{-1}, respectively. Irrigation interval was 3-4 days (two irrigations in a week). Soil water was measured before each irrigation and amounts of irrigation water was calculated as liter per pot. We started to apply drought and salinity treatments as of July in 2015 and June in 2016.

Evapotranspiration volume (ET) between two consecutive irrigations was calculated by using the water balance Equation (1) as follows:

$$ET = [(W_n - W_{n+1}) / P_w] + (I - R) \quad \text{(Eq. 1)}$$

are the pot weights before the n^{th} and n + 1^{th} irrigation (kg), Pw is water bulk density (1 kg dm^3 or 1 kg l^{-1}), I and R are amounts of applied and drainage water (litres).

2.2.2. Leaf net photosynthetic rate and stomatal conductance

The Leaf Net Photosynthetic rate and leaf stomatal conductance (Li-Cor 6400 instrument) were measured before irrigations at 11^{00}-14^{00} PM. The measurements were made four times on July 29, August 12, August 29 and September 17 in 2015 and three times in 2016, June 16, July 14 and August 11 (there were no leaves on some treatments in September 2016).

2.2.3. Leaf water potential

Leaf water potential (LWP) was made by pressure chamber (Soil Moisture Company) before irrigations at pre-dawn (05^{00}-06^{30} AM) during the years of study. LWP was measured four times in 2015 and two times in 2016 because there were no leaves on some treatments in 2016.

2.2.4. Trunk diameter

Trunk diameter were measured two times each year using digital caliper. Trunk diameter was measured on east-west and north-south orientation at 10 cm above the graft point and the average of the two values was calculated and taken as trunk diameter. Covariance analysis was made for trunk diameter.

2.2.5. Experimental design and statistical analysis

The experiment was designed as a split plot. Main plots were drought treatments (D) and sub plots were saline water (S). There were five replications for each treatment and each replication had one trees. All the trees were pruned in February 2016.

3. Results and Discussion

3.1. Plant water consumption (Evapotranspiration, ET)

ET of Nonpareil variety varied from 99.7 L to 218.3 L in 2015 and 70.6 and 248.5 L in 2016 (Table 3). ET decreased as drought and salinity stress levels increased for both years. High salt content of irrigation water increases osmotic potential around root zone. Due to high osmotic potential, roots cannot use water efficiently (Suarez 2012). Excessive amounts of soluble salts in the soil are known to reduce plant water use (Yang et al. 2002). Many researchers have established that plant water consumption was affected by water salinity and water consumption decreased with increasing water salinity (Germana et al. 2000; Murkute et al. 2005). In addition to salinity stress, drought stress also affected plant water consumption. ET decreased with decreased amounts of irrigation water.

In the absence of salinity, the measured ET progressively decreased with water deficit (20% and 40%), as shown in Table 3. Drought stress thus affected ET of young almond trees even at 20% deficit. Salinity decreased tree ET starting at the lowest salinity level EC=1.2 dS m^{-1}. These data confirm the high sensitivity of almond to salt stress. The ET increased for second year of the study in S_0 and S_1 treatments (for D_0 and D_1 drought levels), not unexpected as the trees developed in second year of the experiment. Despite increasing salinity, almond trees continued growth but the S_2 and S_3 salinity treatments caused decrease in ET of almond trees compared to the first year of the experiment (Table 3).

Amounts of daily ET in all treatments were close to each other up to beginning of August in 2015 (Figure 2), suggesting that trees endured one month of reduced water before adverse effects commenced. After that date, daily ET changed depending on drought and salinity levels. Water and salinity stress affected after beginning of August for the first year. But when considered

second year, daily ET of all treatments were very different (Figure 3). ET was affected more in 2016 than in 2015 due to cumulative stress effects.

Table 3- Total plant water consumption values in 2015 and 2016

Treatments		*Plant water consumption (Liters per tree)*	
Drought levels	*Salinity levels*	*2015*	*2016*
D_0	S_0	218.3	248.5
	S_1	193.1	205.8
	S_2	174.4	155.7
	S_3	159.8	92.4
D_1	S_0	172.3	212.3
	S_1	154.3	164.6
	S_2	142.6	119.8
	S_3	130.2	75.4
D_2	S_0	132.2	156.7
	S_1	122.3	120.1
	S_2	111.6	93.7
	S_3	99.7	70.6

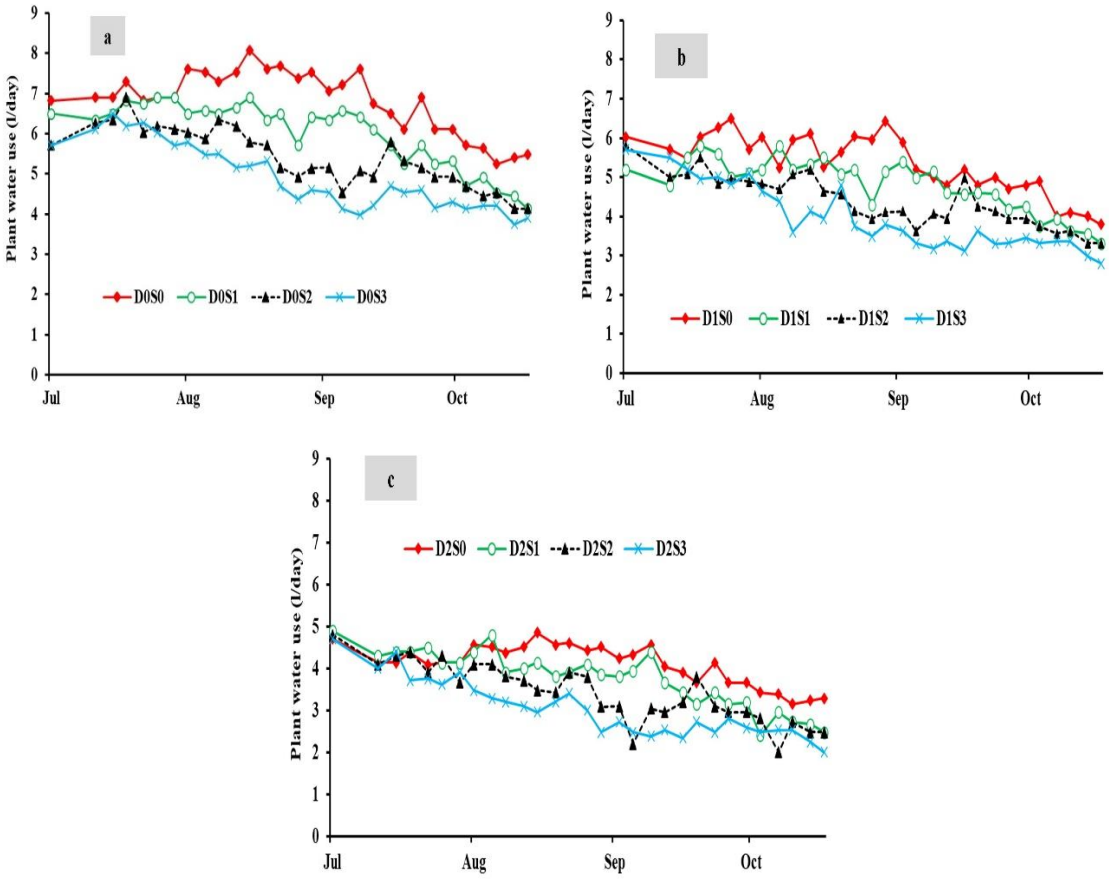

Figure 2- Daily ET of treatments after applying drought and salinity treatments in 2015 (D0; full irrigation, D1; moderate stress, D2; severe stress, S0; EC= 0.55 dS m^{-1}, S1; 1.20 dS m^{-1}, S2; 2.40 dS m^{-1}, S3; 3.0 dS m^{-1})

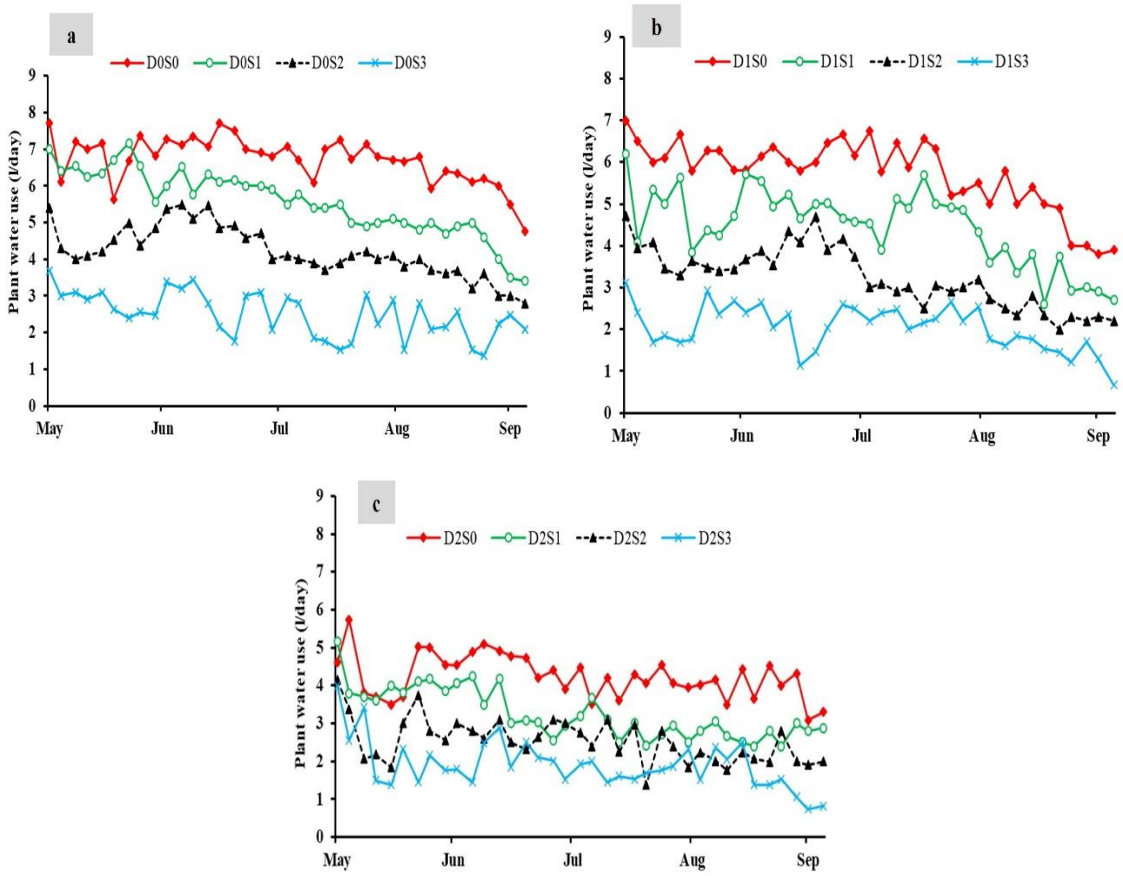

Figure 3- Daily ET of D_2 treatments after applying drought and salinity treatments in 2016

3.2. Photosynthetic rate (Pn)

Drought, salinity and drought salinity interaction had significantly adverse effects on photosynthetic rates (Pn) of Nonpareil and Aldridge almond in 2015 ($P<0.01$). The effects of drought and salinity on Pn were separate in 2016 ($P<0.01$) (Table 4 and Table 5).

Pn results were generally similar for both varieties in 2015 but they were different in 2016. This reason may be that cumulative effect of drought and salinity stress was more pronounced in second year. The values of Pn decreased continuously after the first measurement for each year. The response to drought and salinity stress became more severe with time.

Salt stress causes decreasing photosynthetic effectiveness (Sayed 2003). Increasing salt stress has earlier been reported a higher salinity level to decrease Pn values in almond variety and rootstocks (Zrig et al. 2015; Zrig et al. 2016). They found no significant effect until EC= 9.95 dS m^{-1} treatment. We attribute our reported sensitivity at much lower salinity to our longer-term application of salt (2 year versus 4 weeks in Zrig et al. 2016).

When 80% of ET was applied as irrigation water, drought stress also negatively affects Pn (Anjum et al. 2011). Romero et al. (2004) reported that long-term water stress led to a progressive decline in a with significant reductions after 21 days in the RDI (regulated deficit irrigation) treatment.

On the other hand, Pn was slightly higher in 2016 than that in 2015 in D_0S_0 treatments (no stress) for both varieties (Figure A1 and Figure A2). The reason may be that almond trees growth at second year of the study. Considered the last measurements of D_2S_3 treatments, Pn decreased in 2016. Drought and salinity stress had a significant impact in 2016 as compared to 2015 due to cumulative effects. For example, there were no leaves on the trees in D_2S_2 and D_2S_3 treatments of Nonpareil variety at the last measurement (August 11) in 2016, so Pn could not be measured in those treatments.

Figure 4a and 4b show the rates of Pn results according to D_0S_0 treatments (100%) in 2015 and 2016 respectively. The rates of Pn decreased as drought and water salinity level increased. The rates of Pn were close in similar treatments for both varieties in the first year. They were very different from each other in the second year. the results of Pn measurements of Aldridge were higher than Pn measurements of Nonpareil. Nonpareil did not resist to drought and salinity stress (D_2S_2 and D_2S_3 stress levels) conditions after beginning of August so no leaves on the trees were seen in that treatments. The results indicate that the Nonpareil is more sensitive to drought and salt stress than Aldridge.

Table 4- Photosynthetic rate of Nonpareil at last measurement (µmol CO_2 m^{-2} s^{-1})

Drought levels	*Salinity levels*			
	S_0	*S_1*	*S_2*	*S_3*
	2015			
D_0	9.85 a**	7.82 b	6.81 c	6.20 def
D_1	7.71 b	6.35 cde	5.78 f	5.28 g
D_2	6.56 cd	5.92 ef	5.07 gh	4.60 h
	2016			
Drought levels		Salinity levels		
D_0	10.93 a**	S_0	11.08 a**	
D_1	7.85 b	S_1	8.69 b	
D_2	3.53 c	S_2	5.96 c	
		S_3	4.02 d	

**: P<0.01 Values with common letters do not differ significantly

Table 5- Photosynthetic rate (Pn) of Aldridge at last measurement (µmol CO_2 m^{-2} s^{-1})

Drought levels	Salinity levels			
	S_0	S_1	S_2	S_3
	2015			
D_0	9.90 a**	7.82 b	6.78 c	6.18 de
D_1	7.83 b	6.23 cde	5.61 fg	5.30 gh
D_2	6.65 c	5.90 fg	4.95 hı	4.58 ı
	2016			
Drought levels	Pn	Salinity levels	Pn	
D_0	9.33 a**	S_0	9.48 a**	
D_1	8.00 a	S_1	8.59 ab	
D_2	5.64 b	S_2	6.88 bc	
		S_3	5.68 c	

**: P<0.01 Values with common letters do not differ significantly

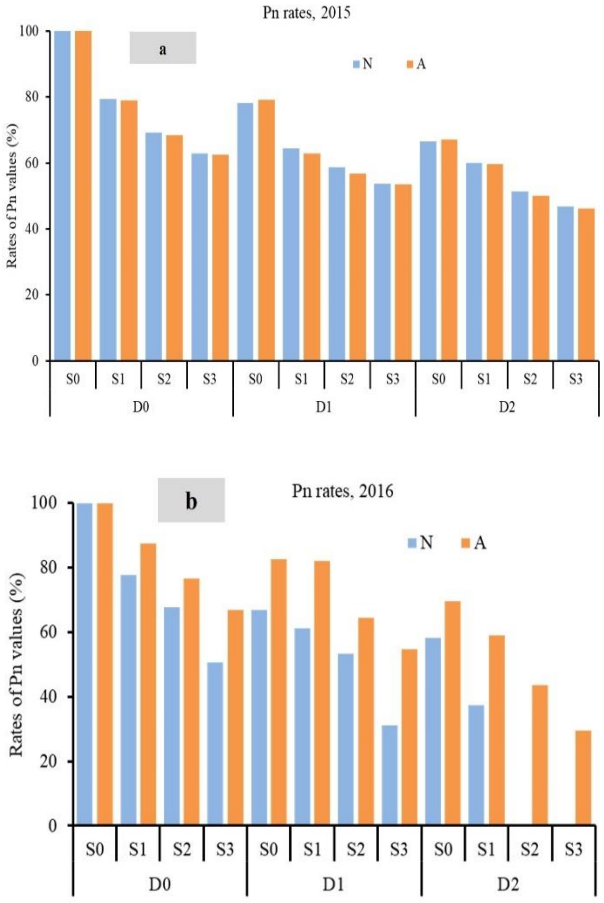

Figure 4- The Pn rates relative to control treatment for Nonpareil (N) and Aldridge (A) at the last measurement

3.3. Stomatal conductance (gsw)

The stomatal conductance (gsw) results varied between 0.135 and 0.350 mol H_20 m^{-2} s^{-1} for Nonpareil and between 0.120 and 0.330 mol H_20 m^{-2} s^{-1} for Aldridge in 2015 (Figure A3). There was no interaction effects of salinity and drought on gsw, according to variance analysis of Nonpareil for both years ($P<0.01$). There are 3 different groups for drought treatments (Table 6). The lowest value was obtained from D_2 treatments. Salinity treatments were divided into three different groups. S_2 and S_3 treatments had the lowest gsw results. Drought and salinity treatments had separate effects for the first year of Aldridge ($P<0.01$). There were 3 different statistical groups for both drought and salinity stress treatments. In the second year drought and salinity stress and interaction was statistically significant ($P<0.01$).

The results of different treatments for both varieties were similar in the first year of the study. The gsw was higher in second year than first year in no-stress treatment. But gsw were less in second year than first year for other treatments. The decrease in gsw was significantly different from the control even at the lowest salinity level and at the 20% reduction in water for Nonpareil (Table 6) as well as Aldridge (Table 7) in 2015 and 2016. The effects of stress applications on gsw were more evident in second year. The gsw values decreased continuously after the first measurement for each year. As with the photosynthesis data discussed earlier, the impact of drought and salinity stress became more severe in year two but was significant in both years and varieties at the lowest salt stress applied.

As the treatment drought and salinity stress level increased, gsw decreased. Nonpareil and Aldridge varieties had similar results for gsw. As salinity level increases, decreasing of photosynthesis is in relationship with closure of stomata (Sibole et al. 1998). Zrig et al. (2015) stated that gsw was affected negatively by increasing salt stress in almonds. When plants are exposed to salinity stress, they close their stomata firstly, in prevent water loss. Stomatal closure gives rise to decreased gsw (Ashraf 2004; Munns & Tester 2008).

The gsw was not measured at the last measurement in 2016 due to lack of leaves on Nonpareil almond trees in S_2 and S_3 salinity levels of D_2 treatments but it was measured in Aldridge (Figure A4).

Table 6- Stomatal conductance (gsw) of Nonpareil at the last measurement (mol H_2O m^{-2} s^{-1})

Drought treatments	gsw	Salinity treatments	gsw
		2015	
D_0	0.243 a**	S_0	0.249 a**
D_1	0.203 b	S_1	0.213 b
D_2	0.163 c	S_2	0.183 bc
		S_3	0.168 c
		2016	
D_0	0.283 a**	S_0	0.279 a**
D_1	0.182 b	S_1	0.202 b
D_2	0.076 c	S_2	0.144 c
		S_3	0.096 c

**: P<0.01 Values with common letters do not differ significantly

Table 7- Stomatal conductance (gsw) of Aldridge at the last measurement (mol H_2O m^{-2} s^{-1})

Level	gsw	Level	gsw
		2015	
D_0	0.237 a**	S_0	0.248 a**
D_1	0.204 b	S_1	0.210 b
D_2	0.163 c	S_2	0.180 bc
		S_3	0.170 c
		2016	
	S_0	S_1	S_2	S_3
D_0	0.302 a**	0.226 b	0.157 cd	0.132 cdef
D_1	0.217 b	0.269 c	0.140 cde	0.134 cdef
D_2	0.159 cd	0.128 def	0.116 ef	0.097 f

**: P<0.01 Values with common letters do not differ significantly

Figure 5 and Figure 6 show the gsw results final measurements in 2015 and 2016, respectively, relative to the control (D_0S_0 treatments =100%). The gsw rates were also similar to each other at the end of the first year (Figure 5). The gsw rates which had the highest drought and salinity stress were very close. The rates were 47.7% and 48.4% for Nonpareil and Aldridge almond varieties, respectively. The rates obtained in 2016 were significantly different for the two varieties (Figure 6). Aldridge was affected less than Nonpareil by drought and salinity stress according to gsw results, consistent with Pn results discussed above.

If water applications through either irrigation or rainfall are not adequate to meet water requirements, stomatal closure will be initiated, reducing gas exchange and rate of photosynthesis (Doll & Shackel 2015). Gsw decreased in non-watered almonds as compared to watered almonds (Gomes-Laranjo et al. 2006). Due to drought and salinity stress in our experiment, gsw and Pn results were negatively affected for both almond varieties.

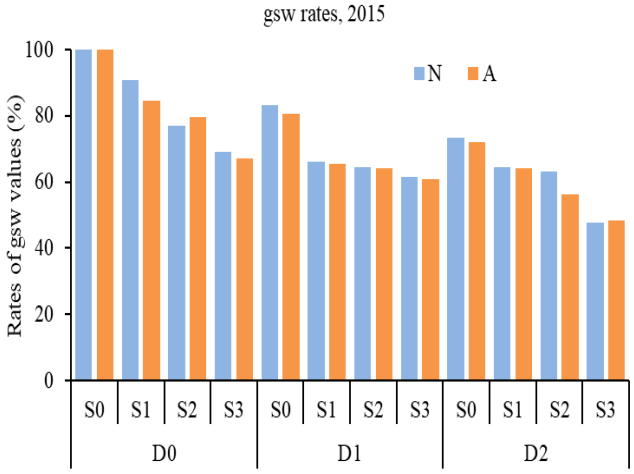

Figure 5- The gsw rates relative to control treatment rates for Nonpareil (N) and Aldridge (A) varieties at the last measurement in 2015

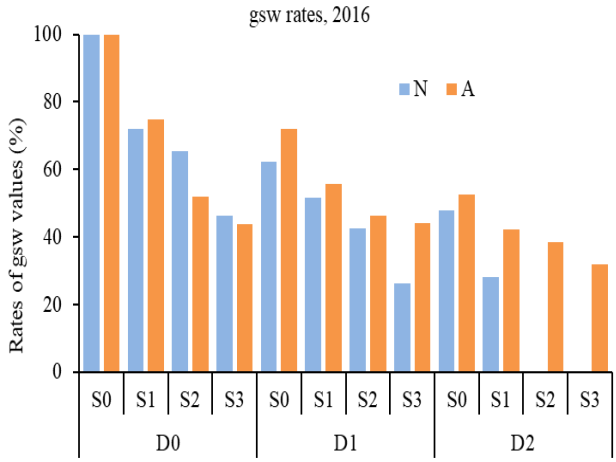

Figure 6- The gsw rates relative to control treatment rates for Nonpareil (N) and Aldridge (A) varieties at the last measurement in 2016

3.4. Leaf water potential (LWP)

There were three different statistical groups for LWP in salinity treatments of Nonpareil and Aldridge varieties in 2015. The 1.2 dS m^{-1} and 2.4 dS m^{-1} salinity levels did not have significantly different LWP but the LWP was greater with increased salinity in the order $S_3 > S_2 > S_1 > S_0$. The 2.4 dS m^{-1} and 3.0 dS m^{-1} salinity levels had almost similar LWP results in Aldridge in 2016. Cumulative salt effects may explain the shift of the 2.4 dS m^{-1} treatment in the second year of experiment.

Drought and salinity stress had significant effects on almond trees ($P<0.01$). There were no significant interactions between drought and salinity stress interaction on Nonpareil and Aldridge varieties. According to results of data for drought stress, there were three different groups for drought treatments in 2015 (Table 8 and Table 9). Each water deficit level had a significantly different increasing adverse effect on LWP of Nonpareil and Aldridge trees. In 2016, 20% (D_1) and 40% (D_2) water deficit levels were in the same group for Nonpareil, no-stress (D_0) and 20% (D_1) water deficit levels were in the same group for Aldridge. In 2016 a 20% water deficit did not affect LWP on Aldridge trees, likely due to trees adjusting growth to decreased water for two years.

Table 8- LWP of Nonpareil at the last measurement

Level	*Least Sq Mean*	*Level*	*Least Sq Mean*
	2015		
D_0	1.13 c**	S_0	1.23 c**
D_1	1.36 b	S_1	1.40 b
D_2	1.73 a	S_2	1.42 b
		S_3	1.57 a
	2016		
D_0	0.96 b**	S_0	1.05 c**
D_1	1.21 a	S_1	1.12 bc
D_2	1.25 a	S_2	1.16 ab
		S_3	1.23 a

**: P<0.01 Values with common letters do not differ significantly

Table 9- LWP of Aldridge at the last measurement

Level	*Least Sq Mean*	*Level*	*Least Sq Mean*
	2015		
D_0	1.16 c**	S_0	1.23 c**
D_1	1.31 b	S_1	1.38 b
D_2	1.68 a	S_2	1.40 b
		S_3	1.52 a
	2016		
D_0	1.23 b**	S_0	1.21 b**
D_1	1.27 b	S_1	1.29 ab
D_2	1.41 a	S_2	1.35 a
		S_3	1.38 a

**: P<0.01 Values with common letters do not differ significantly

LWP values varied from -0.75 to -1.85 MPa and from -0.75 to -1.75 MPa for Nonpareil and Aldridge varieties in 2015, respectively (Figure 7). LWP measurements in 2016 varied from -0.9 to -1.3 MPa and from -0.7 to -1.4 MPa for Nonpareil and Aldridge varieties, respectively (Figure 8). Because there were no leaves on some trees after early August 2016, only two measurements were taken that year.

LWP values decreased towards the end of the season for all treatments. LWP values were affected negatively by increasing drought and irrigation water salinity. Decrease in LWP for almond with water stress (Karimi et al. 2015,) and salt stress (Shibli et al. 2003) has been reported earlier, however their decreases in LWP were relatively small in contract to our large negative values. LWP decreased in none-watered almond trees (Gomes-Laranjo et al. 2006). Our study included combined drought and salinity stress at levels that eventually killed some trees, thus the treatments affected almond trees more negatively than earlier studies.

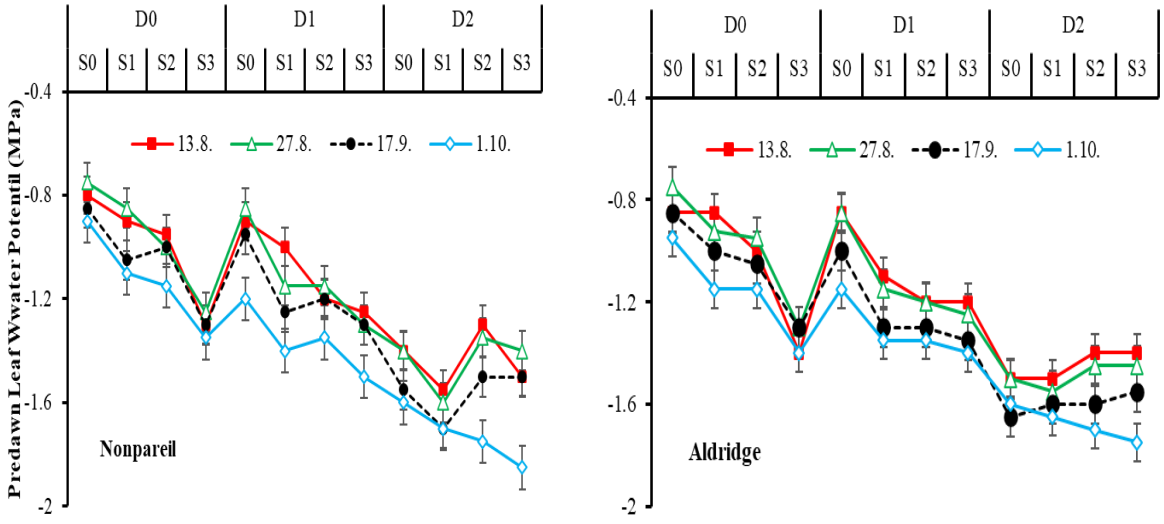

Figure 7- LWP of almond trees during growing period in 2015. (*Error bars indicate standard errors of the means)*

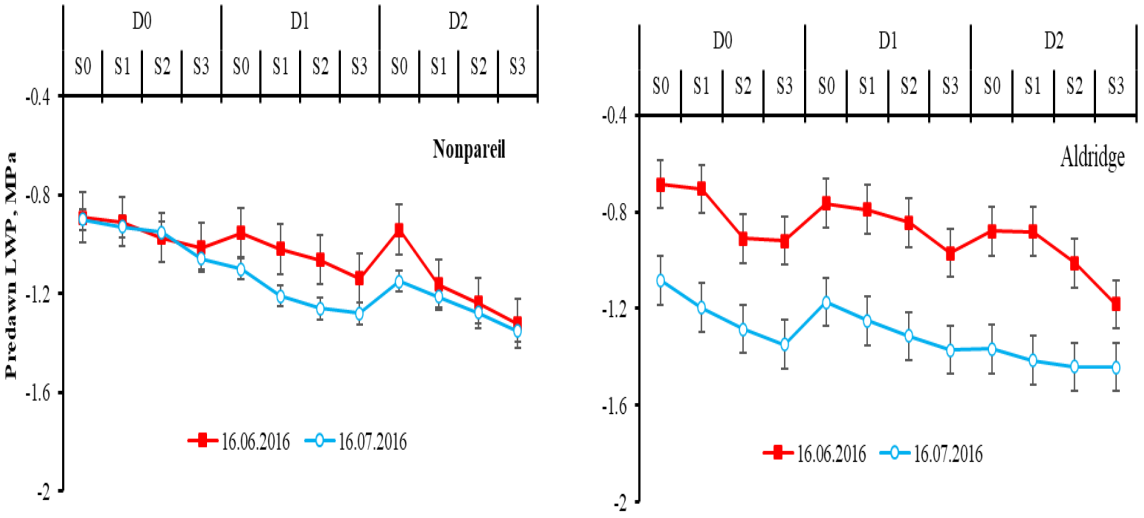

Figure 8- LWP of almond trees during growing period in 2016. Error bars indicate standard errors of the means

3.5. Trunk diameter

Drought and salinity stress interaction had no significant effects on Nonpareil and Aldridge varieties in first year of the study (Table 10 and Table 11). Only drought stress affected Nonpareil variety in 2015 ($P<0.01$). The D_0 levels (no water deficit) had the highest trunk diameter with 20-.35 mm. Drought and salinity interaction had a significant effect on trunk diameter in 2016 ($P<0.01$). Combining the highest water deficit (D_2, 40%) and salinity levels (S_2, 2.4 dS m^{-1} and S_3, 3.0 dS m^{-1}) resulted in the lowest trunk diameters in 2016 (Table 10).

Drought and salinity stress had adverse effects on trunk diameter of Aldridge separately in the first year of the study ($P<0.01$ and $P<0.05$, respectively). There were two different statistical groups (Table 11). The D_1 (20% water deficit) and D_2 (40% water deficit) were in the same statistical group. The S_0 (0.50 dS m^{-1}) and S_1 (1.2 dS m^{-1}) treatments had the same effects on trunk diameter for the Aldridge.

Drought and salinity interaction was significant ($P<0.01$) in 2016. Combining the highest water deficit (D_2, 40%) and salinity levels (S_2, 2.4 dS m^{-1} and S_3, 3.0 dS m^{-1}) had the lowest trunk diameters in 2016 (Table 11). Trunk of almond trees continued to grow even under drought and salinity stress conditions for the first year of the study (Figure 9). Drought and salinity stress decreased growth of trunk diameter for both varieties. The lowest increasing rates (%) were obtained in D_2 and S_3 treatments for all almond trees. Water deficit and salinity affected almond trees after planting date (Figure 10). Considering increasing rates of trunk diameter, trunk shrinkage can be ~~seen~~ considered in drought and salinity stress treatments at the second year. The highest trunk shrinkage was determined in S_3 treatments for all drought treatments.

Prediction of the impact of multiple stresses on biomass yield or crop yield has been made with various response models (Jin et al. 2020; Shahhosseini et al. 2021). The most commonly utilized are the dominant stress models, where the response is considered to be impacted only by the most dominant stress, the additive response model where the stresses are taken to be the sum of the individual stresses and the multiplicative model where the combined stress is taken as the product of the response of the individual stresses.

Earlier Dudley & Shani (2003) found that corn yield in the presence of salinity and water stress could be satisfactorily predicted using the UNSATCHEM (Suarez & Simunek, 1996) model, which considers the biomass response to osmotic and matric stress to be multiplicative. Similarly, Örs & Suarez (2017) found the multiplicative model to have better predictive capability than the additive or dominant stress models for spinach response to water and salt stress. While there is no theoretical basis for the model, it reflects the observation that response to one stress is generally less severe in the presence of another stress, for example the influence of salinity on boron toxicity of broccoli (Smith et al. 2010), elevated pH effect on response to EC (Huang et al. 2017).

Evaluation of the ability to predict the effect of combined stress based on the individual stress response functions was made by using the values of the changes in trunk diameter over the total time of the experiment (from the initial to final measurements) for each individual tree. This approach provided a more sensitive response to the stresses as it considered a longer time frame (than analysis of individual years) and was not impacted by initial variations in trunk diameter among plants.

Shown in Figure 11a is the response of trunk diameter to salinity, expressed as increase in diameter relative to the non- saline control, in the absence of water stress. These data indicate that trunk diameter changes were linear with salinity and that

substantial decrease in growth occurred even at the lowest salinity treatment. In a similar manner the response to water stress in the absence of salinity stress showed a decrease in growth with reduced water application, which was represented by the linear relationship (Figure 11b). A 25% decrease in water application resulted in a 37% reduction in growth (Figure 11b). These data indicate that the combinations of rootstock and scion were very sensitive to both salinity and water stress.

Table 10- Trunk diameter (mm) of Nonpareil

Drought treatments	*Trunk diameter*	*Salinity treatments*	
		2015	
D_0	20.35 a**	S_0	20.21 *ns*
D_1	19.66 ab	S_1	19.70
D_2	19.05 b	S_2	19.64
		S_3	19.19
		2016	
	S_0	S_1	S_2	S_3
D_0	23.62 a**	19.97 d	20.10 d	22.63 bc
D_1	21.92 c	20.00 d	23.34 ab	18.52 e
D_2	20.12 d	18.43 e	17.7 e	15.30 f

**: P<0.01 Values with common letters do not differ significantly; Ns: no-significant

Table 11- Trunk diameter (mm) of Aldridge

Drought treatments	Trunk diameter	Salinity treatments	
		2015	
D_0	22.54 a**	S_0	22.17 a*
D_1	21.40 b	S_1	21.95 a
D_2	21.04 b	S_2	21.5 ab
		S_3	21.02 b
		2016	
	S_0	S_1	S_2	S_3
D_0	26.90 a**	24.87 b	21.82 c	19.37 efg
D_1	19.97 def	20.07 cde	21.67 cd	17.27 h
D_2	19.93 def	21.49 cd	18.00 gh	18.22 fgh

**: P<0.01; *: P<0.05 Values with common letters do not differ significantly

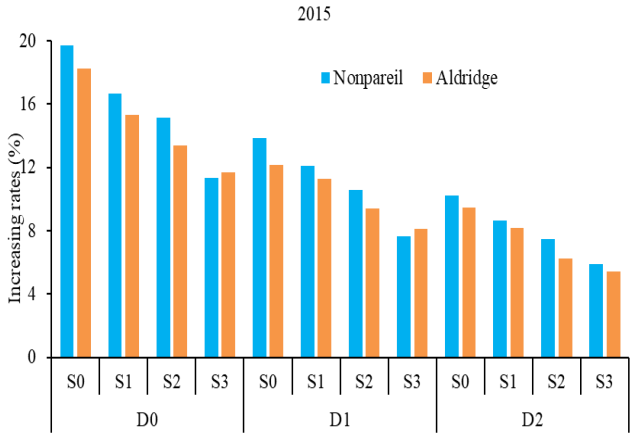

Figure 9- Increasing rates of trunk diameter measurements in 2015

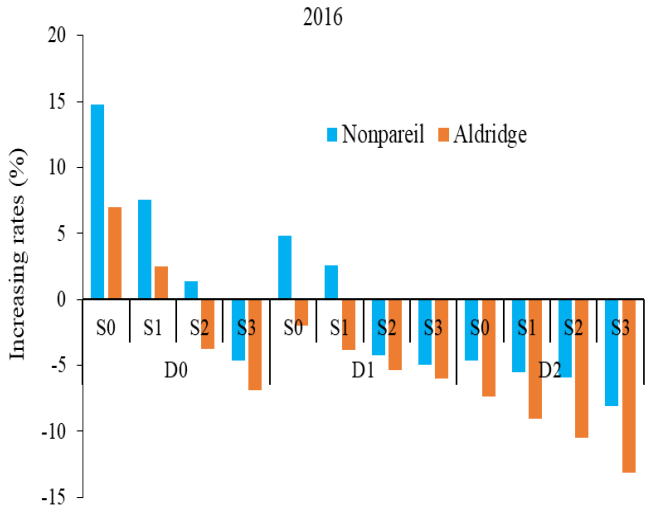

Figure 10- Increasing rates of trunk diameter measurements in 2016

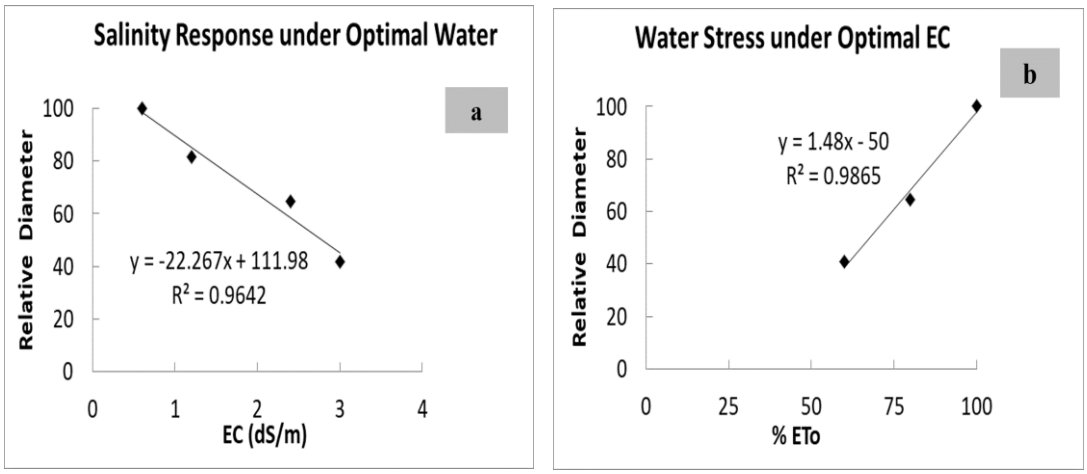

Figure 11- Relative trunk diameter as related to a) salt and b) water stress

Using the results of Figure 11 (a and b) we compared the predictions of the three stress response models to measurements in the change in trunk diameter for the treatments with combined stress. In each instance we used only data from the combined stress treatments, so no experimental data used to develop the predictive equations for water and salt stress (shown in Figure 11a, b) were used in the evaluation of the combined stress models. For the additive stress model, we added the predicted decreases from the separate stresses to predict the response to combined stress. For the major stress model, we evaluate the decrease in growth expected from the water and salt stress and selected the response from the stress with the greater response. For the multiplicative model we calculated the response expected from water and salt stress and multiplied them.

As shown in Figure 12a, the additive model predicted lower growth than observed, consistently overpredicting loss of growth, indicating that the presence of one stress reduces the impact of another stress. Predictions from the major stress model overestimated growth (Figure 12b) as might be expected since presence of a major stress did not eliminate the growth reduction due to other stresses. The multiplicative model predictions shown in Figure 12c also overestimated growth (underestimated the impact of combined stress) but appeared to be the most satisfactory model. As shown in Table 12 we evaluated the model fit to experimental data using a number of statistical tests. In all instances the multiplicative model provided the best fit. Perhaps the statistic of most interest to those interested in predicting growth is the value for mean absolute deviation. The multiplicative model had a low value of 5.4 versus 10.1 for the additive model and 21.4 for the major stress model. The overall predictive equation is

$$RG= 32.96\ EC\ (V_a/V_c) +165.7(V_a/V_c) – 1113.4EC -5599 \quad \text{(Eq. 2)}$$

Where: RG is relative growth; V_a is volume of water applied (mm): V_c; is volume needed to meet crop demand under no stress condition (mm): EC; is irrigation water electrical conductivity (dS m^{-1}).

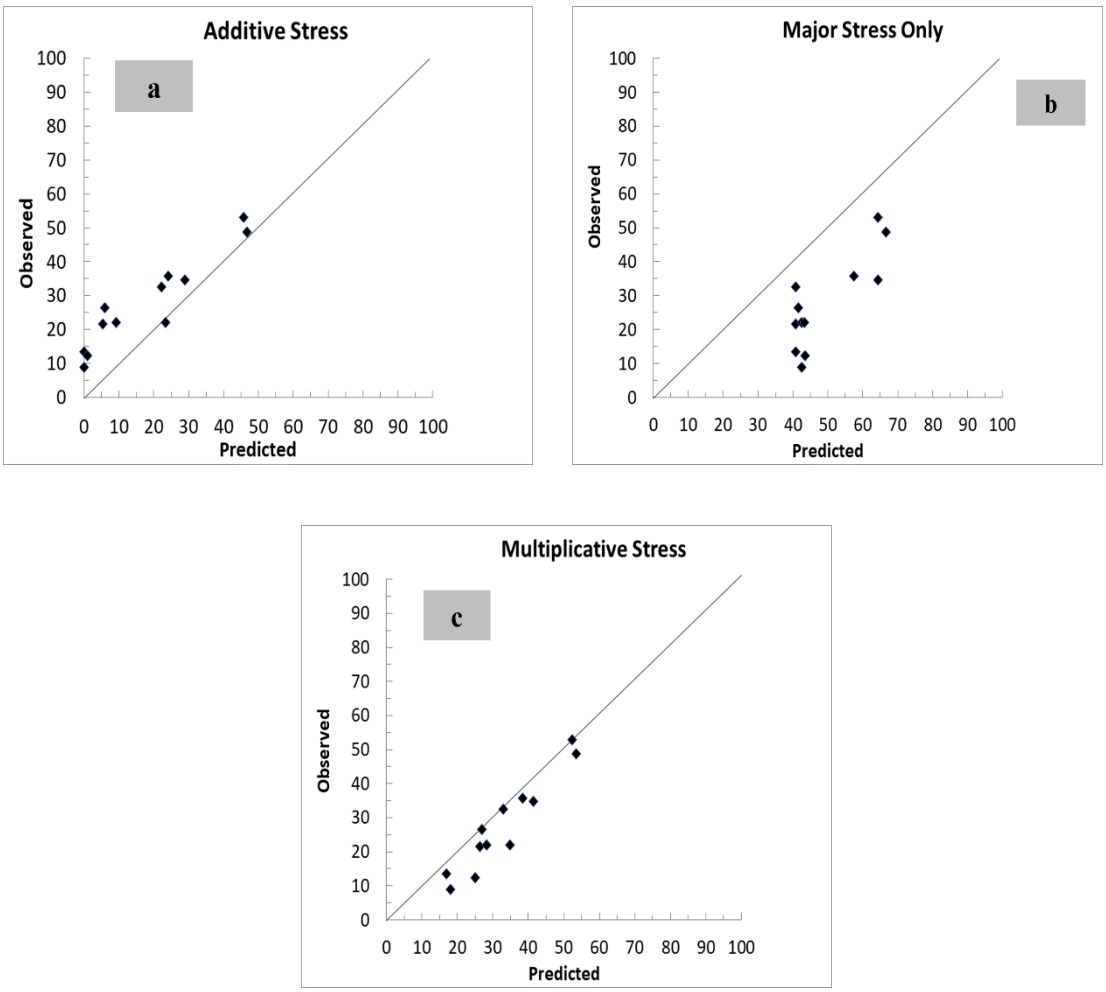

Figure 12- Measured relative trunk growth versus predicted values using a) additive stress model, b) major stress only model, and c) multiplicative stress model.

Table 12- Statistical evaluation of model predictions

Statistical parameter	*Additive stress*	*Major stress*	*Multiplicative stress*
Mean absolute deviation	10.1	21.4	5.4
Mean square error deviation	131	515	46.5
RMSD	11.4	22.7	6.82
Mean error	-9.9	21.4	5.3
Correlation	0.94	0.83	0.95

4. Conclusions

Drought and salinity stress levels affected plant water consumption, growth, and physiological parameters of Nonpareil and Aldridge almond varieties. The results suggest that almond may not be as drought tolerant as currently considered. Trunk growth significantly decreased with a 20% decrease in applied water. The data indicated that even for very young trees the adverse effects increased in year two as compared to the first year of stress, suggesting that one year studies are not sufficient to characterize tree response to water or salt stress. The growth data was consistent with results from the physiological measurements. The data for both varieties were similar in first year but it was established that scion varieties not just rootstock are relevant to improved stress tolerance. Nonpareil was more sensitive than Aldridge in the second year of stress. For example Nonpareil almond trees had mortality in August of year two at and above EC 2.4 dS m^{-1} in 40% water deficit treatment. Both varieties grafted to Nemaguard rootstock were very sensitive to salinity with growth loss starting at EC 1.2 dS m^{-1}.

All parameters showed significant decline starting at 80% water application and EC 1.2 dS m^{-1}. In terms of growth rather than survival, almond was sensitive to water as well as salt stress. Trunk growth under combined water and salt stress treatments was well predicted only when using a multiplicative stress response function. Equation (2) for reduction in trunk growth were developed for treatments with either salinity only or water only stress. The results indicate that the Nonpareil is more sensitive to drought and salt stress than Aldridge. Aldridge almond variety can be recommended for areas where water supplies are scarce and salinized.

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Appendix

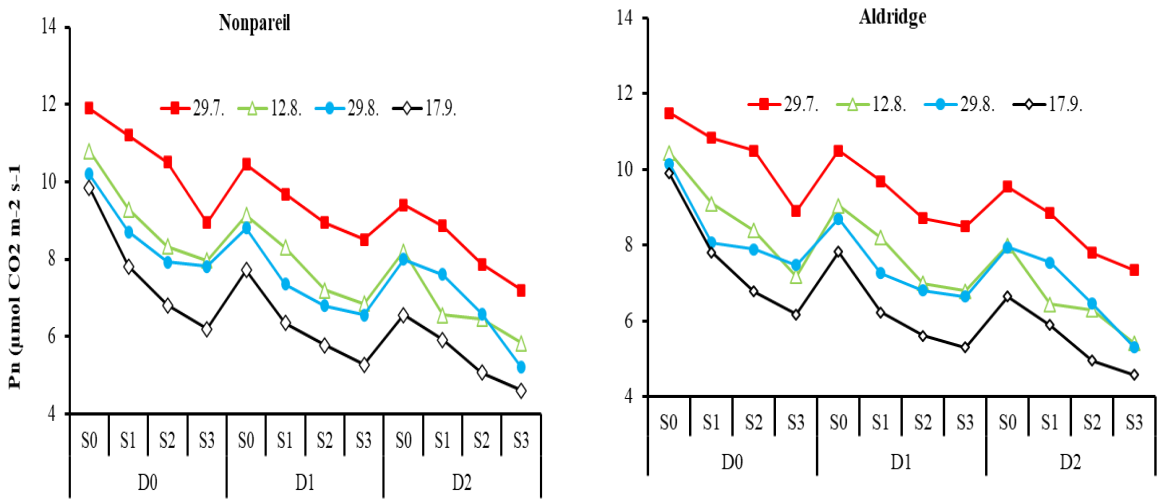

Figure A1. Seasonal fluctuation of Pn for Nonpareil and Aldridge in 2015

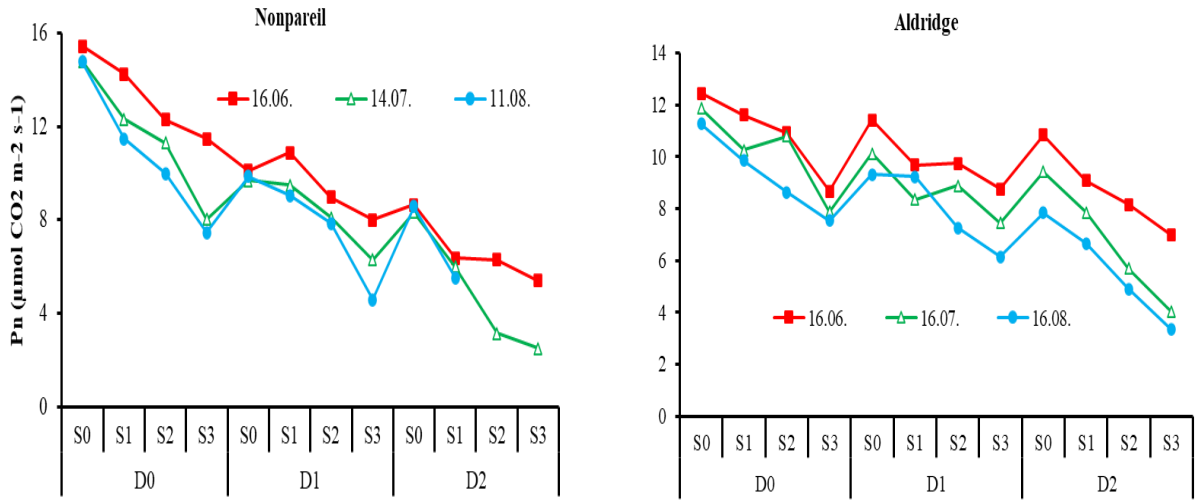

Figure A2. Seasonal fluctuation of Pn for Nonpareil and Aldridge in 2016

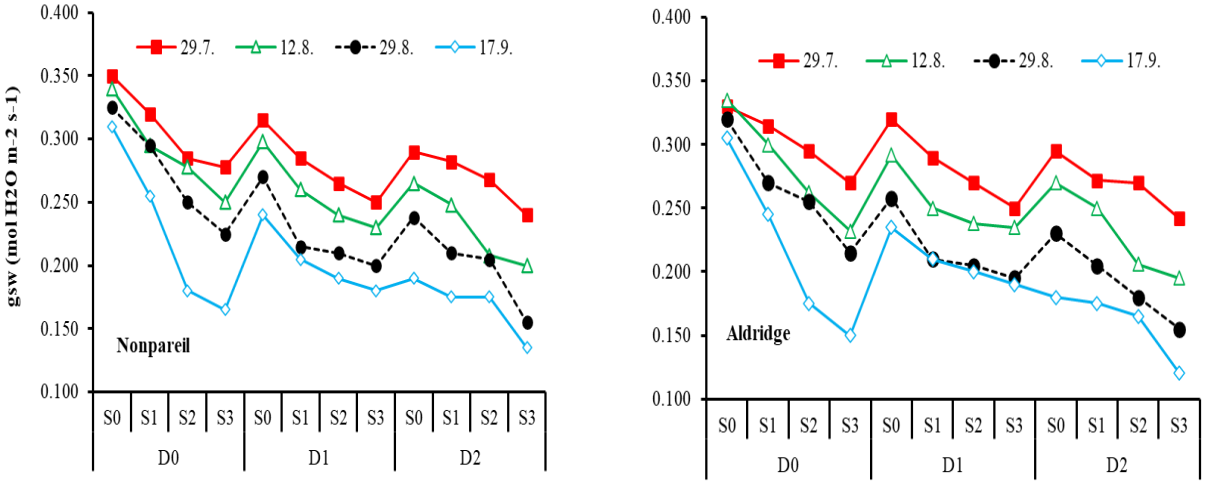

Figure A3- Seasonal fluctuation of gsw for Nonpareil (a) and Aldridge (b) in 2015

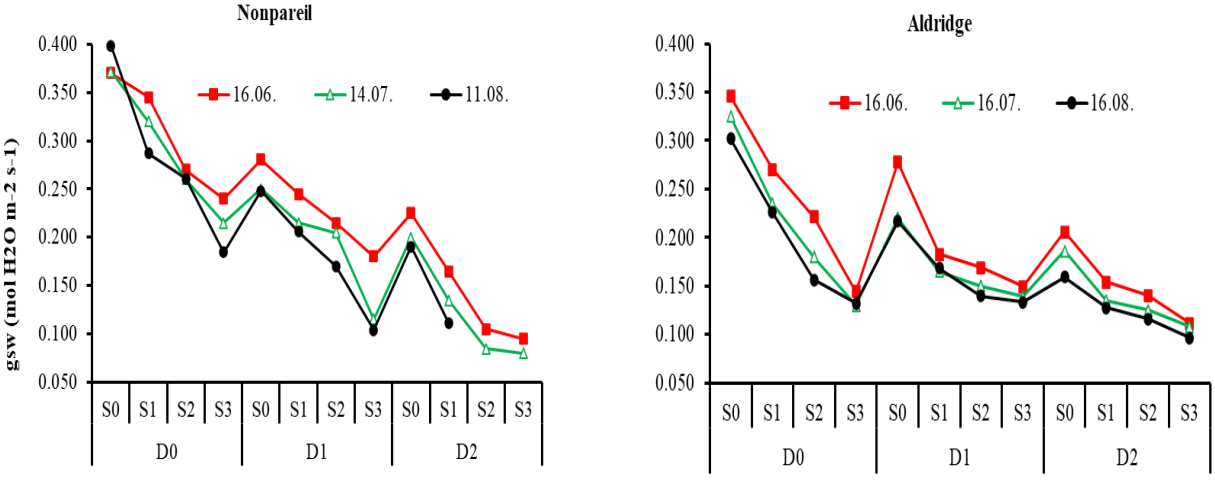

Figure A4- Seasonally fluctuation of gsw for Nonpareil (a) and Aldridge (b) in 2016

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Modelling Yield Response and Water Use to Salinity and Water Relations of Six Pepper Varieties

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ABSTRACT
Better understanding of crop yield response under salinity and water deficit conditions is essential to meet food need under the circumstance of population growth and climate extremities. It has been well known that plant species response differently under stress conditions. Recent studies show that these different responses occur not only among species but also in different varieties within the same species. The aims of the study are to examine and to compare yield, yield response factors (k_y), salinity thresholds, biomasses, and water productivity responses of six varieties of pepper plant (Sürmeli-Hot, Yalova, BT016-Hot, BT 016, BT Ünsal, BT Demok) under salinity conditions. In another experiment under the same conditions (location, time, growth media etc.), water deficit was applied to two of these six varieties (BT Ünsal and BT Demok) separately, and their responses to salinity and water deficit conditions were compared. The experiment was carried out in containers. The amount of irrigation water was determined manually by weighing each container. Water deficit treatments were consisted of meeting 120, 100, 70 and 50% of soil water depleted from field capacity. Water salinity levels were 0.25 (control), 2, 4 and 6 dS m^{-1}. There was no difference in yield under non-stress and excessive stress conditions, but the yield difference was as high as 38.9% under moderate stress conditions. Varietal differences were also observed for water productivity. Salinity threshold values vary between 0.89 and 1.83 dS m^{-1}. Yield response factor (k_y) were high for all varieties under salinity. Comparing the k_y values obtained under water deficit and salinity experiments, sensitivity to salinity induced water stress was found higher than that of applied water deficit itself. Using salinity (Model 1) and water deficit (Model 2) data set of two varieties, two models were created plotting relative yield and water potentials (osmotic potential + matric potential) and compered their predications statistically. Statistically better predictions were obtained from Model 2.

Keywords: Irrigation, Stress response modelling, Relative yield, Yield response factor, Salinity tolerance, Water deficit

1. Introduction

Beginning in the 1960s and 70s, the “Green Revolution” lead dramatic increase in total global food production. In this period, the world has witnessed extraordinary productivity increase. While agricultural land has increased by only 30%, grain production has tripled during this period (Wik et al. 2008). This productivity increase is generally attributed to the development of productive crop varieties and management practices (Allen et al. 1998), but a significant part of this increase is related to increasing in irrigated areas. Irrigated lands have higher productivity and economic returns per unit area compared to non-irrigated lands. Globally, irrigated lands are estimated to account for 15% of the cultivated area yet produce between 30% and 40% of the world's food needs (Ghassemi et al. 1995; Postel 1999). While the annual increase in irrigated areas was 1.5% by the time “Green Revolution”, it is reported that this rate is 0.6% in 1998-2030 (UNESCO 2009). In arid regions, the effect of irrigation is generally much greater. On the other hand, arid and semi-arid regions are the most effected lands by water scarcity and salinization. Scientists have been drawing attention on food security questions raised by climate extremities, population growth, water, and land degradations etc. (Kiremit & Arslan 2016). Fifty years ago, this problem was overcome to some extent, but the next fifty years will be more difficult. Due to the limited conditions in all aspects, careful use of lower quality and quantity of water has vital importance for upcoming years. The issues underlined above show that it is more important than ever to carry out studies on salt and drought sensitive plants that cannot be grown without irrigation in arid and semi-arid regions. Many scientists have repeatedly stated that water stress and salinity are the most important abiotic stresses limiting plant growth and yield (Wang et al. 2003; Saleem et al. 2020; Alam et al. 2021; Heydari et al. 2021; Atiya et al. 2022). Therefore, understanding, selecting, and developing crops with high osmotic tolerance may provide a solution to the problem (El-Beltagy & Madkour 2012).

Economically and nutritionally important solanaceae vegetables such as pepper, eggplant, and tomato etc., occupied 39% and 66% of horticultural production globally and in Europe, respectively. Solanaceae family members are also utilized as model

crops for several traits (Sharma et al. 2017). Pepper (*Capsicum annuum*) production recently increased from 17 to 36 million tons with 35% increase in cultivated area (Tripodi & Kumar 2019; López-Serrano et al. 2021). Pepper plants were considered very sensitive to drought and sensitive to salinity stress (Ayers & Westcot 1989; De Pascale et al. 2003) but some other studies reported response variations to salinity and drought (Aktaş et al. 2006; Kurunç et al. 2011; Semiz et al. 2014; Ünlükara et al. 2015). Plant genetic resources help breeders developing varieties adapting to those different climatic conditions, but more knowledge about unexplored resources is required. Pre-breeding research, to first identify and then characterize those resources for all crops, will need to be at the center of the next research and innovation projects (Economidis et al. 2010).

To counterbalance the predicted increase in the world population to up to nine billion people by 2050 and the related expected impact of climate change, science must produce the knowledge on more productive water use and understand plant response to drought and salinity via plant water use models. Forecasts based on the integration of crop yield models for water stress associated with climate change will be more important than ever for sustainable production and food security. Crop yield models would lead us to make better decision to increase yields and productivity in a sustainable way and adapting crops to match the effects of changes in the environment. Reports of salt tolerance for crops were mostly based on relatively few studies, sometimes just one. However, overall varietal differences were not reported in these databases (Suarez et al. 2021). Suarez et al. (2021) also pointed out the difficulties in comparisons salinity-yield response data in consequence of nonstandard experimentation procedure.

Yield potential of a crop means the maximum yield obtained under non stress conditions in a particular location (equal solar energy, air temperature, longitude etc.). Where the environmental conditions are equal, the yield difference among varieties is a result of their genetical factors (Watson 1952; Sinclair et al. 2005; Prado et al. 2018). Capacity building in terms of standard methods, modelling, and comparable data features on measuring yield potentials under increasing stress conditions, need to be discussed thoroughly. Biotechnology and genetic tools/methods for breeding have been gained tremendous acceleration but salinity and drought resistant crop breeding are relatively slow. The reason for this is generally expressed as the complex response of plants under stress conditions (Kumar et al. 2020; Godoy et. al. 2021). This statement may be true but is it sufficient? The partial results of our study surprisingly revealed the importance of the magnitude of the stress. The method of the applied stress is also important to be comparable real word condition. The method applied water deficit studies on plant (sometimes called drought) need to be comparable to field condition. For example, reporting the results should be referred depleted water from field capacity (FC), not continuous drought or keeping soil water at some % of FC which are not practical. In terms of salinity, utilized salts to prepare saline water should be scrutinized as well. For example, using only NaCl salt would lead infiltration problems then resulting in water stress in the root zone and this situation could be underestimated. Or specific ion toxicity occurs, which may be incorrectly thought of as the osmotic effect of salinity (Grattan & Grieve 1992; Munns & Tester 2008; Semiz & Suarez 2019). Navarro et al. (2002) reported that the use of salt in irrigation water was more harmful than NaCl salts in irrigation water on pepper plants than Na_2SO_4 at the same electrical conductivity (EC) levels. These issues, which have not been emphasized much, need to be discussed in the scientific arena. More comparative studies are needed on these issues. The methodical problems listed above are for modelling and pre-breeding studies. Practical approaches used in plant physiology studies are not criticized.

In arid and semi-arid areas, irrigation has become even more dependent on poorer quality and deficit quantity water. Moreover, differences in resistance to drought and salinity between varieties, which were not covered extensively before, have become more important than before. Demonstrating the tolerance differences among varieties with the help of models will provide technical support to the primary users in economic planning and irrigation programming. In addition, the method followed here is especially recommended for plant breeding as it gives comparable results in selecting drought- and salinity-resistant varieties.

The aim of the study are to (1) determine and compare biomass and fruit yield of six pepper plant varieties under increasing salinities (2) determine and compare the response of increasing water deficit levels of two pepper plant varieties (3) modelling plant-water-soil salinity relations under salinity and water deficit conditions (4) compare the model outputs for water salinity and water deficit (5) suggest and discuss the importance of methodology on pre-breeding and stress tolerance modelling studies. Thus, our research outputs will contribute to the agricultural sciences by developing models that make adaptive yield estimation for primary users and planners in their own fields, as well as discussing and making recommendations for methods used for breeding salinity and drought tolerant plants.

2. Material and Methods

Pepper (*Capsicum annuum*) seedlings (standard and registered) were purchased Atatürk Horticultural Central Research Institute and Bursa Seed Company, Turkey. The list of varieties and registered names are shown in Table 1. Pepper seeds were sown in seedling viols in a greenhouse in April 2018, in Ankara. The greenhouse mean temperatures were 10-15 °C at night and 20-25 °C daytime. When the pepper seedling reached three-true leaf stage, the seedlings were transferred to the experimental containers in May 2018. The containers had been well-watered with tap water (0.25 dS m^{-1}) for two weeks to provide good establishment. Then, the experiment was initiated in accordance with the treatments.

Table 1- Registered and standard varieties used in the experiment

Plant abbreviation	*Variety*	*Maintainer*
YS	Sürmeli- Hot	Atatürk Horticultural Central Research Institute, Turkey
YT	Yalova	Atatürk Horticultural Central Research Institute, Turkey
KC	BT 016- Hot	Bursa Seed, Turkey
KT	BT 016	Bursa Seed, Turkey
HT	BT Ünsal	Bursa Seed, Turkey
GT	BT Demok	Bursa Seed, Turkey

Salinity-water use-yield relations of 6 pepper varieties and deficit water applications-water use-yield relations of HT and GT varieties were determined. For this purpose, fresh and biomass yield, evapotranspiration (Semiz et al. 2014), soil osmotic (Allison et al.1954) and matric potentials (Saxton & Rawls 2006), water productivity (Allen et al. 1998), salinity threshold and slope values (Maas & Hoffman 1977), yield response functions (Stewart & Hagan 1973) were examined.

The experimental setup was completely randomized factorial design with three replications, totally 96 containers. The experiment was conducted 350 mm in diameter and 300 mm in depth containers, in Ankara University Faculty of Agriculture, Department of Farm Structures and Irrigation experimental area, Ankara, Turkey. The soil was top layer of an agricultural land (30 cm of surface soil). Air dried soil were sieved (4 mm). The soil texture was sandy clay loam (Bouyoucos 1951). Experimental soil media was consisted of 1:0.5:0.5, soil: peat (Klasmann TS 1): perlite. Initial soil extract salinity, EC_e and pH_e were 0.255 dS m^{-1} and 7.49, respectively. To provide free drainage conditions, 5 cm thick gravel was filled at the bottom of the containers. Each container was filled with 11 kg of air-dried soil media. Randomly selected 6 containers used to evaluate the weight at field capacity. These 6 containers initially saturated with tap water and covered to prevent evaporation. The containers were weighed again immediately after drainage had stopped and the average weight was taken as the weight at field capacity (Ünlükara et al. 2010; Semiz et al. 2012). At the beginning of the study, the weight of each lysimeter at field capacity was known, Evapotranspiration (ET) could be calculated based on the weight lost between consecutive irrigations (as detailed by Semiz et al. 2014). The amount of irrigation water to be applied was calculated as follows (Eq. 1):

$$AW = \frac{\frac{(W_{fc}-W)}{\rho_w}}{1-LF} \quad \text{(Eq. 1)}$$

Where; AW is applied water (L): W_{fc} and W; are the weight of each lysimeter at field capacity and the weight of each lysimeter just before irrigation: (g); respectively: ρ_w ; unit weight of water (1000 g L^{-1}) and LF is the leaching fraction, which is not applicable for water deficit treatments.

Irrigation waters were applied in accordance with salinity levels and the percentage of total water needs, manually. Each container was weighed at 3–4-day intervals. ET was determined by weighting each container thus each plant received a different quantity of water based on their actual water consumption during the previous time interval. Irrigation water amounts were selected 120, 100, 70 and 50% of depleted water from field capacity. Thus, only 70% or 50% of the evapotranspiration being met, the water deficit was created. Irrigation water salinity levels were 0.25 (tap water), 2, 4 and 6 dS m^{-1}. Saline irrigation water was prepared using Na_2SO_4, NaCl, $CaCl_2$, $MgSO_4$ salts to mimic natural saline conditions and not to lead additive specific ion toxicity rather than the salinity (Grattan & Grieve 1992). The SAR value was kept below 5 because in addition to salinity, infiltration problems would not arise and the negative effect of Na and/or Cl as specific ions would not interfere with the salinity effect (Ayers & Westcott 1994). SAR (sodium adsorption ratio, defined as $Na^+/((Ca^{2+}+Mg^{2+})/2)^{0.5}$ where concentrations are expressed in m mol L^{-1}). The EXTRACTCHEM model (Suarez & Taber 2012) was used to calculate the amount of salt to be dissolved in tap water.

At the end of the experiment, soil samples were collected from each container, air dried, sieved 4 mm, saturated for 24 h, extracted and then salinities were measured (Allison et al.1954). The salt tolerance model (Eq. 2) proposed by Maas and Hoffman (1977) was applied to determine threshold and slope values (relative yield decrease per unit increase in salinity for each variety).

$$Y_r = 100 - b(EC_e - EC_{e\,treshold}) \quad \text{(Eq. 2)}$$

Where: Y_r; relative yield: % EC_e; soil salinity beyond the threshold value dS m^{-1}: $EC_{e\ threshold}$; threshold soil salinity dS m^{-1}: b; slope value which is the % yield loss per unit increase in electrical conductivity of the saturated soil extract beyond the threshold value.

The following equations (Eq. 3, 4, 5 and 6) proposed by Saxton & Rawls (2006) were used to determine the matrix (MP) and osmotic potential (OP).

$$\Psi_{(1500-33)} = \Psi_\Theta = A(\Theta)^{-B} \quad \text{(Eq. 3)}$$

$$A = \exp(\ln 33 + B\ln\Theta_{33}) \quad \text{(Eq. 4)}$$

$$B = \left(\frac{\ln(1500)-\ln(33)}{\ln(\Theta 33)-\ln(\Theta 1500)}\right) \quad \text{(Eq. 5)}$$

$$\Psi_o = 40EC_e \quad \text{(Eq. 6)}$$

Where, tension at moisture Ψ_Θ (matric potential) is in kPa; 33 kPa moisture is Θ_{33}; A and B are coefficients of moisture-tension: %v; 1500 kPa moisture is Θ_{1500}: %v; Ψ_o is the osmotic potential in kPa; and EC_e is the electrical conductance of a saturated soil extract at 25° C in dS m^{-1}.

The total matric and osmotic potential values for each treatment was plotted against their relative yield values. Then, regression equations were created namely Model 1 and Model 2. The data used for Model 1 and Model 2 were salinity and water deficit experimental results of HT and GT varieties, respectively. Next, using Model 1 and Model 2 outputs and measured relative yields were plotted against predicted versus observed relative yields for the rest of 6 varieties.

Two-way ANOVA and Bonferroni (1936) multiple range test were performed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA).

3. Results and Discussion

3.1. Fruit and biomass yield

The interaction of genotypes and water amount was found statistically significant at P<0.005 significance level (Table 2). GT and HT had similar fruit yield, where water requirement was fully met (100% and 120%). The fruit yield difference was only occurred at moderately water deficit condition (70%) with 12.1% higher yield of GT. At 50% of water requirements are met, both genotypes had similar fruit yield. It is worth mentioning here that in this study, free drainage conditions are provided. Thus, there were similar amount of water in the root zone for both 100% and 120% treatments. This result shows that both genotypes give similar fruit yields under optimum conditions. At the moderately water deficit condition (where 70% water requirement met), genotypic difference may have arisen thus more fruit yield decline occurred in HT genotype. At the excessive water deficit condition (where 50% water requirement met), the fruit yield declines were similar in both genotypes, 60% and 59% for GT and HT, respectively. These findings suggested that it is also important to select the magnitude of the water deficit to find out genotypic response to water deficit. Kurunç et al. (2011) reported 33.7 and 40.4% yield reduction for bell pepper, where 70 and 50% water requirement met, respectively. They also reported no yield difference between the treatments of 145% and 100% water requirement met.

Table 2- Yield, biomass, ET, and WP statistical results under water deficit conditions

Applied Water		*120%*	*100%*	*70%*	*50%*	
Yield g plant^{-1}	GT	116.3 A a	115.2 A a	86.2 A b	46.2 A c	90.97 A
	HT	112.4 A a	111.8 A a	75.8 B b	46.7 A c	86.68 B
	Mean	114.34 a	113.50 a	80.99 b	46.45 c	
ET, mm	GT	350.0 B a	304.5 B b	259.0 A c	164.50 A d	269.50 B
	HT	381.1 A a	322.6 A b	273.6 A c	178.6 A d	288.98 A
	Mean	365.54 a	313.56 b	266.29 c	171.50 d	
Biomass, g plant^{-1}	GT	8.20 A a	8.32 A a	5.33 A b	3.20 A c	6.288 A
	HT	7.36 B a	7.58 B a	4.88 B b	2.98 A c	5.625 B
	Mean	7.780 a	7.950 a	5.005 b	3.090 c	
WP, kg m^{-3}	GT	4.70 A b	5.35 A a	4.71 A b	3.98 A c	4.684 A
	HT	4.18 B b	4.90 B a	3.92 B b	3.72 B	4.180 B
	Mean	4.441 b	5.126 a	4.314 b	3.848 c	
A ↓ Vertical comparisons of means; a→ Horizontal comparisons of means **Water amount P< 0.005, genotype P>0.005, genotype x WD P<0.005**						

Interaction of water amount and genotypes were found significant on biomass production. Biomass productions of both genotypes showed similar trend as continuous reductions in biomass yield under moderate and excessive water deficit conditions and did not differ under full irrigations. Comparing full irrigations, GT's biomass reductions were 32.4% and 61.1% at 70 and 50% treatments and HT's biomass reductions were 34.6% and 60.1% at 70 and 50% treatments, respectively.

Comparing genotypes, genotypic variations have arisen at full irrigation and moderate water deficit treatments. Biomass yields of GT were 10.2, 9.0 and 8.0% higher than HT under 120, 100 and 70% treatments respectively and statistically similar under 50% treatment. Unlike the fruit yield, genotypic differences occurred in biomass yield under no and moderate water stress.

Salinity experiment (increasing salinity with 20% regular leaching) revealed some genotypic variations (Table 3) as well. Genotype and salinity levels interaction was found statistically significant on biomass and fruit yield (P<0.005). In irrigation with control water (non-saline), genotypes had statistically similar fruit yields. Increasing salinity levels caused to decrease in economical yield for all genotypes, in different rate. At 2 dS m^{-1} salinity level, YT (103.0 g) and KC (105.5 g) genotypes had the similar (statistically) highest fruit yields. The fruit yield reductions by genotypes were 0.08%, 21.4%, 18.7% and 32.5% for KT, YS, HT, and GT, respectively. At 4 dS m^{-1} salinity levels KC (87.4 g) had the highest fruit yield. The fruit yield reductions by genotypes were 0.07%, 17.0%, 21.1%, 34.1% and 38.9% for YT, KT, YS, HT, and GT, respectively. At 6 dS m^{-1} salinity levels YT (55.5 g) had the highest fruit yield. The fruit yield reductions by genotypes were 0.19%, 20.9%, 21.1%, 22.7% and 31.7% for KC, KT, YS, HT, and GT, respectively. De Pascale et al. (2003) utilized sea salts diluting in irrigation water on the Laser variety (pepper) and reported a yield reduction of 46% at a salinity level of 4.4 dS m^{-1}. The authors also reported that 8.5 dS m^{-1} salinity level and non-irrigated (drought) treatments showed similar plant-water relations. Salinity in the root zone causes excessive ion accumulation and high osmotic and matric stress in plants. Thus, a decrease in growth and development occurs. This results in lower soil matrix potential and leads to drought conditions, i.e., conditions that cause physiological limitation for plant growth (Munns & Tester 2008; Abrar et al. 2020).

Table 3-Yield, biomass, ET, and WP statistical results under salinity conditions.

Irrigation Water Salinity (dS m^{-1})		*0.25*	*2*	*4*	*6*	*Mean*
Yield g $plant^{-1}$	YS	110.5 A a	82.1 C b	69.0 C c	43.9 B d	76.37 C
	YT	114.6 A a	103.0 AB b	79.5 B c	55.5 A d	88.17 A
	KC	116.6 A a	105.5 A b	87.4 A c	45.1 B d	88.64 A
	KT	114.0 A a	96.1 B b	72.5 BC c	42.9 B d	81.38 B
	HT	112.4 A a	84.9 C b	57. 6 D c	37.9 BC d	73.18 C
	GT	116.3 A a	70.5 D b	53.4 D c	35.2 C d	68.84 D
	Mean	114.04 a	90.34 b	69.91 c	43.42 d	
Biomass g $plant^{-1}$	YS	8.14 B a	8.22 B a	7.03 AB a	4.30 B b	6.92 B
	YT	12.21 A a	12.23 A a	7.07 AB b	5.84 A b	9.34 A
	KC	11.00 A a	10.34 A ab	8.31 A b	7.06 A b	9.18 A
	KT	11.08 A a	10.11 A a	7.11 AB b	6.98 A b	8.82 A
	HT	7.36 C a	3.87 D b	3.51 C b	3.33 B b	4.52 C
	GT	8.20 B a	6.54 C ab	5.16 B b	3.51 B c	5.85 B
	Mean	9.67 a	8.52 b	6.37 c	5.17 d	
ET, mm	YS	403 A a	372 A b	341 A c	246 B d	340 AB
	YT	407 A a	373 A b	336 AB c	289 A d	351 A
	KC	389 A a	346 AB b	309 B c	277 A d	330 B
	KT	374 AB a	360 AB b	309 B c	238 B d	320 B
	HT	381 A a	341 B b	284 BC c	203 C d	302 C
	GT	350 B a	289 C b	255 C c	206 C d	275 D
	Mean	383 a	347 b	306 c	243 d	
WP, kg m^{-3}	YS	3.88 B a	3.12 C bc	2.87 C cd	2.54 A d	3.212 C
	YT	4.00 B a	3.91 AB a	3.35 B b	2.72 A c	3.602 AB
	KC	4.24 AB a	4.31 A a	4.00 A a	2.30 A b	3.821 A
	KT	4.32 AB a	3.77 B b	3.32 BC c	2.56 A d	3.601 A
	HT	4.18 B a	3.53 BC b	2.87 C c	2.64 A c	3.413 B
	GT	4.70 A a	3.45 BC b	2.97 C c	2.421 A d	3.493 B
	Mean	4.221 a	3.681 b	3.280 c	2.530 d	
A ↓ Vertical comparisons of means; a→ Horizontal comparisons of means **Salinity P< 0.005, genotype P<0.005, genotype x Salinity P<0.005**						

Biomass yield for six cultivars under increasing salinity levels showed also different responses (Table 3). At control salinity level, highest-mean biomass yield (statistically similar) was 11.43 g $plant^{-1}$ for YT, KC and KT cultivars. Biomass yield strongly affected both salinity levels and genotypes. YS and GT genotypes had 8.17 g $plant^{-1}$ mean biomass yield (statistically similar) but increasing salinity levels affected GT more strongly than YS. Comparing to control treatment, biomass yield reductions were 20, 37, and 57.2% for GT at 2, 4, and 6 dS m^{-1}. There was no statistical difference in biomass productions of YS at control, 2 and 4 dS m^{-1} salinity levels but there was 47.2% yield reduction at 6 dS m^{-1} salinity level. The lowest biomass yield at control treatment was observed in HT genotype with 7.36 g $plant^{-1}$ and a sharp biomass yield decrease occurred at 2 dS m^{-1} with 47.4%. Interestingly, there was no furthered biomass yield decrease under increasing salinity levels for HT. Baath et al. (2017) reported that biomass productions of 5 pepper cultivars decreased under increasing salinity conditions up to 5 dS m^{-1} salinity level. At the control treatment (120% water application rate and 0.25 dS m^{-1} salinity level), the six genotypes gave similar fruit yield responses, indicating that genotypic variations on crop yield potentials under optimal conditions were not significant. Rameshwaran et al. (2016) stated that under water or salinity stress conditions, biomass/yield differences among varieties were higher. Ficiciyan et al. (2021) also reported similar fruit yield responses under full irrigation and variable responses under drought treatments of 15 sweet pepper varieties. These results indicated genotypic variations in both biomass and fruit yields occurred at moderate salinity

level. These results strengthen our hypothesis that genotypes of peppers may response variable to moderate stress but similarly response to excessive and non-stress condition. Other outcome of the current study is that it is important to carefully assign the salinity and drought stress levels to reveal genotypic differences when selecting candidate lines for breeding.

3.2. Water use and productivity

Irrigation water was applied in accordance with individual plant water requirements, meaning that each plant received water according to own stress response. Evapotranspiration rate decreased with decreasing water amount applied, in accordance with the nature of the treatments. The treatment of 100% and 120%, where water requirement fully met, water consumption was higher HT than GT (Table 2). The treatment of 70% and 50%, where water requirement partially met, both genotypes were in the same statistical group. Water productivity (WP) was higher in GT than HT under excessive, moderate, and non-stress conditions. The highest WP (5.35 kg m^{-3}) was observed in GT at 100% of water requirement met and, the lowest (3.72 kg m^{-3}) in HT at 50% of water requirement met.

ET reduced with increasing salinity levels for six pepper cultivars. ET reductions is directly proportional to salinity induced water use stress (Table 3). Under control treatment (0.25 dS m^{-1}), the lowest ET (350 mm) was observed in GT genotype, and rest of the genotypes were at the same statistical group. Under control treatment (non-saline), although KC, KT and GT at the same statistical group, the highest WP was observed in GT (4.70 kg m^{-3}) but decreased sharply under moderate and excessive salinity treatments. Except KC, WPs of each genotype continually decreased with increasing salinity level, statistically. WP of KC decreased only at excessive saline condition. Rameshwaran et al. (2016) investigated two pepper cultivars under different water regimes and salinity levels. They reported that increasing salinity level and water deficit caused reductions in WP, but they did not report varietal differences. Varietal differences in WPs were reported for different water regimes such as for wheat van den Boogard et al. (1997); for peanut Bhatnagar-Mathur et al. (2007), Ratnakumar et al. (2009) and Devi et al. (2009); for pepper Erwin et al. (2019). Erwin et al. (2019) stated that differences in water use properties among pepper varieties are higher than previously reported.

3.3. Crop stress modelling

Under optimal conditions, plant ET is maximum (ET_m), and the relative yield (RY_m) is 100% (Vaux & Pruitt 1983). Under abiotic stress conditions, ET and RY decrease by different rates related the magnitude of the environmental stresses and the plant type or variety (Suarez et al. 2021). It has been suggested different approaches to predicting or modelling RY under stress conditions. First approach is estimating the relationship between ET and RY reductions (Doorenbos & Kassam 1979; Vaux & Pruitt 1983). Second approach is establishing a relation which directly estimates RY under quantitative measures of the stress, in our study matric and/or osmotic potentials. Both approaches were adopted, and their predictions were analysed.

First approach includes soil extract salinity-relative yield and water deficit-relative yield estimations. Ayers & Westcot (1989) salinity threshold and slope modelling are used to determining and forecasting yield reduction under saline conditions. Ayers & Westcot (1989) reported 1.5 dS m^{-1} pepper salinity threshold. The method proposed by Ayers & Westcot (1989) was adopted, and similar but variable results were found. Graphical models and outputs are shown in supplementary files-S2 and Table 4, respectively. The most sensitive genotype was found to be KT with a slope value of 13.5% and a threshold of 0.89 dS m^{-1}. The most resistant genotype was GT with 1.83 dS m^{-1} threshold with 13.9% slope value. These variable responses to salinity are suggesting that it is important to select not only crop species but also its variety. Reporting crop response to salinity suggested and standardized by Ayers & Westcot (1989) are of great important since outputs of this approach are comparable and applicable to directly economical and water management decisions. No publications were found addressing salt tolerance modelling of different pepper varieties under this standard approach. Most pepper salinity tolerance studies solely NaCl was used as salinity agent such as Aktaş et al. (2005), Chartzoulakis & Klapaki (2000) Penella et al. (2015) and Giorio et al. (2020) and so on.

Table 4- Ayers and Westcot (1989) and Stewart and Hagan (1973) model outputs

Plant variety	*Threshold (dS m^{-1}) and slope (%)*	*Yield Response Function (k_y)*
YS	1.51 and 13.5	1.70
YT	1.43 and 12.8	1.73
KC	1.55 and 16.2	1.75
KT	0.89 and 13.5	1.78
HT	1.12 and 13.8	1.56
GT	1.83 and 13.9	1.83
HT-WD	-	1.39
GT-WD	-	1.35

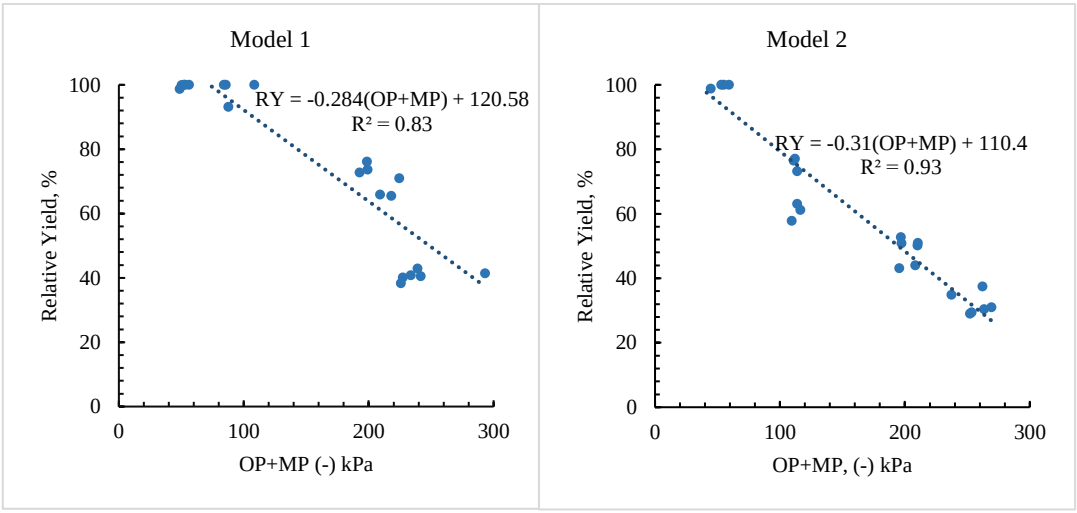
Stewart & Hagan (1973) proposed yield response function model, to predicting relative decrease of crop yield under water deficit conditions. Yield response functions of crops are beneficial modelling since they give important sense of water use and yield predictions under non-standard conditions. The model procedure was first proposed to determine yield response to water deficit but later it has been also implemented on water use modelling under saline conditions (Khataar et al. (2018) for bean and

wheat; Kurunç et al. (2011) for bell pepper; Düzdemir et al. (2009) for cowpea). Yield response functions (ky) were determined for all genotypes under salinity and for HT and GT genotypes under water deficiency conditions. Graphical models and outputs are shown S1 and Table 4, respectively. All varieties were found very sensitive to water deficit caused by salinity. The most sensitive and the most resistant varieties were GT with 1.83 and HT with 1.56 k_y values, respectively. Under water deficit conditions, on the contrary to salinity, GT and HT gave similar responses, 1.35 and 1.39 k_y values, respectively. Those results indicated that both varietal responses occur under salinity and, GT and HT are more sensitive to water deficit caused by salinity. In other words, salinity induced ET reduction was found more pronounced (or severe) than water deficit itself. No studies reporting and comparing salinity and water deficiency conditions for different varieties were found in the same experiment. Kurunç et al. (2011) examined and compered k_y values under salinity and water deficit for a bell pepper variety. Although their findings are not compatible to our study, they reported that salinity and drought effected bell pepper yield response similarly. Qiu et al. (2017) reported that under increasing salinity level k_y value of hot pepper was 1.65.

Githu & Goodwin (2020) stated that studies on physiological feature needed to carry along with yield-water response functions to provide a better knowledge of crop response. They also pointed out the difficulty in put into practice the results from different studies. Apparently, more modelling and standardized studies need to be carried out.

To establish the second approach (quantitative relationship), soil osmotic and matric potentials were calculated, as detailed in the Allison et al. (1954) and Saxton & Rawls (2006), respectively. Since only the HT and GT varieties were exposed to water deficit, the datasets were used under salinity and water deficit (separately) to construct the models (Figure 1).The total water potential (OP+MP) was considered as a quantitative stress measurement. Two different models were created, the results of which were combined for HT and GT. Model 1 and Model 2 represent salinity and water deficit experimental results, respectively. Then, in order to examine whether any model would (statistically) predict relative yield better, both models were applied to the entire dataset for 6 varieties. Some comparative statistical analyses are shown in Table 5. In addition, a graphical presentation was prepared to demonstrate the accuracy of the estimates. The lines of x=y represent perfect predictions and dots represents prediction via models.

Figure 1 clearly shows that Model 2 produces more reliable predictions than Model 1. Comparative statistical analyses also showed that Model 2 has better predictions than Model 1. The reason of the better predictions of Model 2 might be pepper yield's being relatively more sensitive to MP under these ranges of MP and OP. Since salinity experimental results were used in Model 1, MP's relative impact on OP+MP value was lower. Similarly, relative impact of MP on MP+OP was more since water deficit experimental results were used.



(a) (b)

Figure 1- (a) Saline treatment data set (b) Water deficit treatments data set used

Table 5- Comparative statistical analyses for predicted and observed relative yield

Statistical Analyse	*Salinity-Model 1*	*Water Deficit-Model 2*
Mean Absolute Deviation	11.88	5.57
Mean Squared Error-Deviation	183.39	48.41
Root Mean Square Error	13.54	6.96
Mean Absolute Percentage Error	16.26	9.63
Mean Error	11.82	-2.22
Mean Percentage Error	16.08	-5.28
Correlation	0.95	0.95

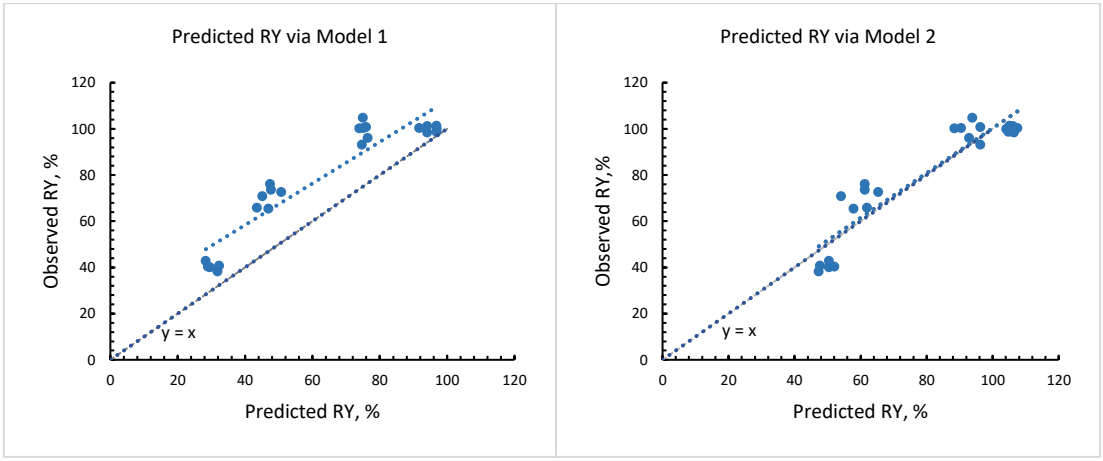


Figure 2- Predicted versus observed relative yields for six varieties

4. Conclusions

The fruit yields of GT and HT varieties showed similar responses under non-water stress and excessive water stress conditions, but GT had higher yield under moderately water stress condition. Under control treatment, no yield differences were observed between the varieties, but increasing salinity levels caused a larger difference in yield responses. The yield difference observed as high as 38.9% and as low as 0.08% at 4 dS m^{-1} salinity level. These results clearly demonstrated the yield response under salinity were varietal. GT had higher water productivity than HT variety under both non-water stress and water deficit conditions. Varietal differences have also arisen for WP under salinity condition, except the highest salinity level. The crop stress model was examined for each variety. Salinity threshold values ranged 1.83 and 0.89 dS m^{-1}. This large difference suggests the importance of variety selection under low water quality conditions. Yield response functions were determined under both salinity and water deficit. Higher sensitivity was found in the water use-yield relationships under salinity stress than under water stress conditions. This result may indicate that pepper is more sensitive to salinity induced water stress, or that the water stress caused by salinity is higher than the applied water deficit itself. Two models were created for HT and GT variants using the water deficiency treatment and salinity treatment dataset. Total soil potentials (osmotic + matric potentials) were determined and plotted against the relative yield of HT and GT varieties. Both models were applied to predict RY for the rest of the cultivars. Then, predicted and observed RY were cross-checked and their results evaluated statistically. Interestingly, statistically better estimates were found generated through Model 1 using data based on water deficit. This might be the result of higher water stress sensitivity under saline conditions.

As a conclusion, it is aimed to reveal varietal differences by proposing standardized studies on salinity and water deficit in the same experiment and to benefit the practical use of the models.

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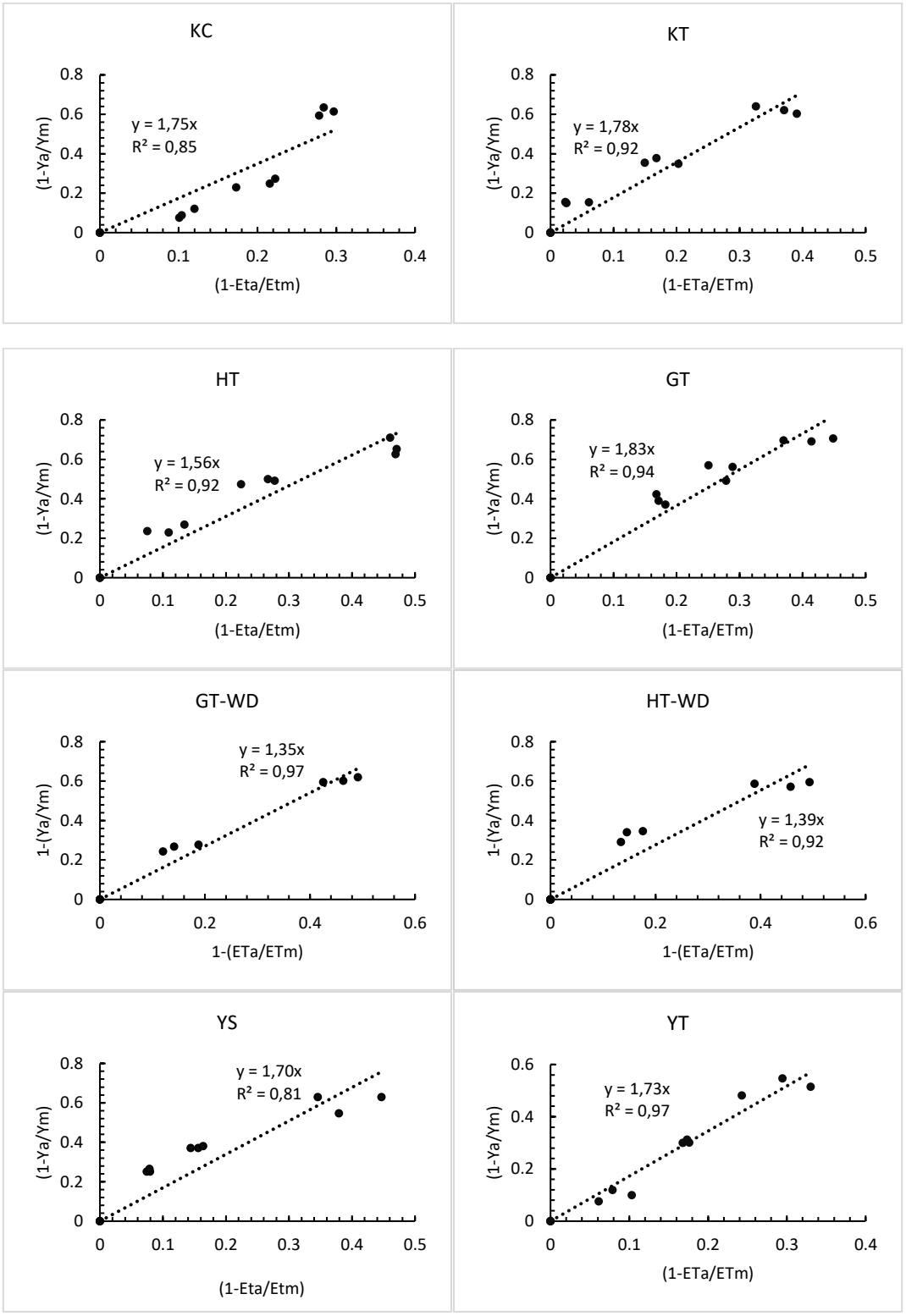
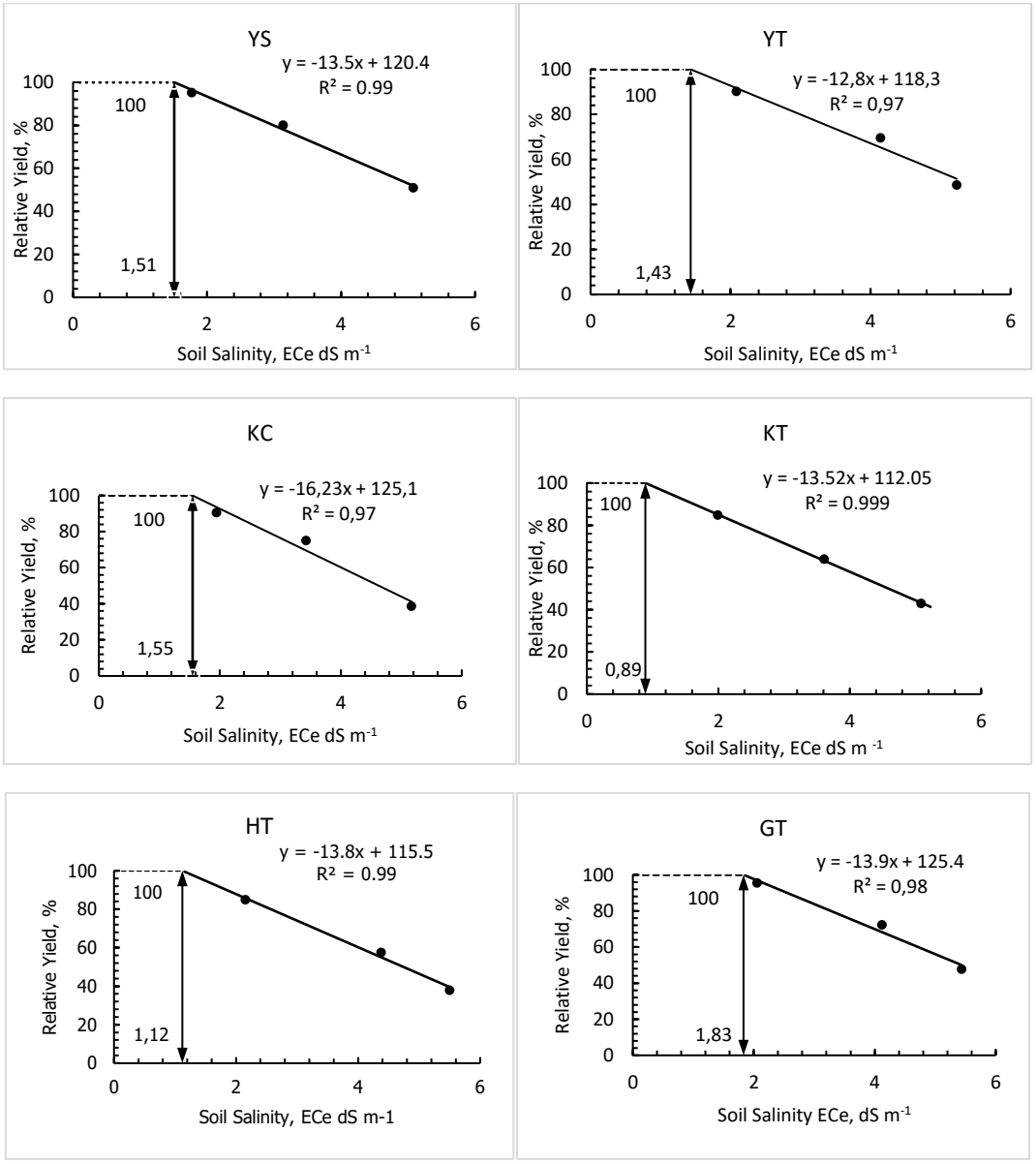
SUPPLEMENTARY



S 1- Yield response functions model for each variety



S 2- Salt tolerance models for each variety

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Functional and Physicochemical Properties of Milled and Microfluidized Bulgur and Chickpea Brans

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ABSTRACT
Dietary fiber plays a crucial role in human diet due to their health-promoting effects. Cereal brans are widely used for fiber enrichment of bakery products; however, their high phytic acid content, mostly localized in the aleurone layer, lowers the nutritional value of the end-product. Therefore, the functional and physicochemical properties of two aleurone-free brans, bulgur and chickpea brans, were investigated as alternative fiber sources. Furthermore, effect of particle size reduction by means of milling and the microfluidization process on these properties were determined. The microfluidization reduced the particle sizes of bulgur and chickpea brans to 13.12 and 14.25 µm, respectively. The results indicated that the microfluidization significantly increased the soluble dietary fiber content of brans. Thus, the insoluble/ soluble dietary fiber ratios of bulgur and chickpea brans decreased to 8.42 and 6.13 from 19.20 and 15.33, respectively. The phytic acid contents ranged from 230.8 to 247.9 mg/100g for bulgur bran, and 112.1 to 113.1 mg/100g for chickpea bran. After the microfluidization, these contents decreased to 107.1 and 47.9 mg/100g for bulgur and chickpea brans, respectively. The milled samples did not show any differences in terms of phenolic contents and antioxidant activity, but the microfluidization increased the phenolic content of bulgur and chickpea brans as 73.80% and 59.62%, respectively. In addition, the antioxidant activity values increased 73.08% for bulgur bran, and 76.70% for chickpea bran with this process. Chickpea bran had higher swelling and water holding capacity than that of bulgur bran, but the oil holding capacities of both types of brans were close to each other. Conventional milling had no significant effect on these properties, whereas the microfluidization improved them. Therefore, it can be said that the applied microfluidization process enhanced physicochemical properties along with their functional properties, and it is possible to degrade phytic acid with microfluidization process.

Keywords: Bulgur bran, Leblebi, Antioxidant activity, Dietary fiber, Phytic acid

1. Introduction

Over the last decades, numerous studies have reported that consumption of an adequate amount of dietary fiber can help regulate blood glucose level, lower blood cholesterol, and reduce the risk of chronic diseases such as coronary heart disease, obesity, and colon cancer. Due to these striking health-promoting effects, once considered to be just a by-product of milling industry, cereal brans, now are regarded as significant food additives owing to their high dietary fiber content. These health benefits have also been attributed to their high phytochemical contents such as phenolic compounds that exhibit antioxidant activity including gallic, vanillic and ferulic acids (Adom & Liu 2002). However, these cereal brans are far from being ideal fiber sources. They all contain high levels of phytic acid, an antinutrient which has a strong affinity to form insoluble complexes with amino acids, and mineral cations including iron, magnesium, zinc, and calcium, thereby impairing the bioavailability of these micronutrients. Phytic acid is mostly localized in the aleurone layer of cereal grains, thus remain in the bran after the milling process (Stevenson et al. 2012). Therefore, these brans reduce the nutritional value significantly when they are incorporated into food products. In order to overcome these drawbacks regarding the use of cereal brans as functional ingredients, it is essential to find new alternative fiber sources without any limitations caused by the aleurone layer.

This paper presents two aleurone-free fiber sources; bulgur and chickpea brans. Bulgur is a traditional whole grain semi ready-to-eat product, preferably produced from durum wheat by cleaning, cooking in water, followed by drying, partially dehulling and grounding. Bulgur bran is the part disposed during partial dehulling stage in bulgur process. Roasted chickpea or simply leblebi, is a traditional snack, has been widely consumed in Turkey and the Middle East Region. The leblebi process includes cleaning and grading of chickpea, soaking, tempering, boiling, resting, roasting, and dehulling (Deshpande & Damodaran 1990). During this dehulling stage, chickpea bran is removed and disposed as the same manner as bulgur bran. These undervalued by-products may have great potential use as a functional ingredient since they do not contain the aleurone layer and consequently, should have considerably low phytic acid content compared to other cereal brans.

Particle size distribution of bran also plays a vital role regarding end-products properties. Novel micronization techniques such as microfluidization alters the properties of bran, enhance some micronutrients and impair others. Brewer et al. (2014) showed that the phenolic acid content of whole wheat bran increased as the particle size decreased, while Rosa et al. (2013) reported that the increase in antioxidant capacity of wheat bran is well correlated with the decrease of the particle size. Ultrafine grinding slightly decreased phytic acid content of wheat bran from 7060 mg/100g to 6750 mg/100g (Hemery et al. 2011). Several studies showed that microfluidization improves antioxidant activity by loosening the microstructure of fiber-matrix (Chen et al. 2013; Wang et al. 2013; Wang et al. 2014) and alters the ratio between insoluble and soluble fiber by increasing the soluble fiber content (Chen et al. 2013). Furthermore, the microfluidization process can enhance the hydration properties of fiber as well as their oil holding capacity (Wang et al. 2012; Wang et al. 2013). These physicochemical properties are of importance due to their ability to modify viscosity, texture and health-promoting effects of the end-products.

Hence, the main objectives of this study are as follows: (a) to investigate the functional and physicochemical properties of two possible fiber sources with low phytic acid content, bulgur bran and chickpea bran and to reveal their potentials, (b) to determine if the particle size distribution obtained by conventional milling and microfluidization process affects these properties. To best to our knowledge, there has been no study regarding the functional and physicochemical properties of bulgur and chickpea brans. Moreover, the effects of milling and microfluidization on these properties are expected to be different considering the composition of these brans is quite different from wheat and cereal brans since they do not contain aleurone layer which is rich in some micronutrients including vitamins, minerals, and enzymes.

2. Material and Methods

2.1. Materials

Bulgur bran (11.3% moisture, 11.58% protein, 2.12% ash) and chickpea bran (9.80 % moisture, 6.55% protein, 4.78% ash) were purchased from local suppliers. Bran samples were ground by a laboratory mill (Laboratory Mill 120, Perten, Germany), and subsequently separated into 3 different particle sizes (100 µm; 200 µm; 350 µm) using a Retsch AS 200 tap sieve shaker (Retsch GmbH, Haan, Germany) and stored at 4 °C until used. All reagents were analytical grade and were purchased from Merck (Dermasdat, Germany).

2.2. Microfluidization of bran

Microfluidization process was performed with a Microfluidizer M-110P (Microfluidics, Newton, MA, USA). Bulgur and chickpea brans with a particle size of 100 µm were chosen for the microfluidization process to prevent the obstruction of the interaction chambers. Brans with a particle size of 100 µm were prepared as mentioned above, dispersed in distilled water in a ratio of 1:20 (w/w) separately. The bran suspensions subsequently processed through the Microfluidizer with a Z type interaction chamber with a diameter of 100 µm (IC 100) for five passes at 30000 psi as pre-trials showed the further passes did not decrease the particle size of the brans. The interaction chamber was cooled down with icy water to keep the temperature of processed material at about 30 °C. Microfluidized bran suspensions were collected after designated passes and were freeze-dried to the moisture content of a maximum of 4%. The dried samples were stored at 4 °C until used.

2.3. Particle size distribution

The particle size distribution of microfluidized samples was measured by laser scattering method using Microtrac Bluewave particle size analyzer (Microtrac, Montgomeryville, PA, USA) (Wang et al. 2012). D90, D50, and D10 were the selected D-values which respectively represent the diameter of the particle that 90%, 50% and 10% of the sample's volume is smaller than the indicated diameter. All measurements were conducted in triplicate.

2.4. Chemical analysis

The moisture, protein and ash contents of bran fractions were determined by AACCI approved method 44-01.01, 08-01.01and 46-12.01, respectively (AACCI 2010). Phytic acid and phytate phosphorus contents were measured according to the colorimetric method described by Haug & Lantzsch (1983). The insoluble (IDF), soluble (SDF) and total dietary fiber (TDF) contents were determined by AOAC method 991.43 (AOAC 2012). The extraction of phenolic compounds of samples was performed according to Adom and Liu (2002) with slight modifications as described in our previous report (Özkaya et al. 2017a). After the extraction, phenolic content was determined by the Folin-Ciocalteu spectrophotometric method whereas antioxidant activity was measured using 2.2-di-phenyl-2-picryl-hydrazyl (DPPH) according to Yu et al. (2002).

2.5. Physicochemical analysis

The physicochemical properties of brans including swelling capacity (SC), water holding capacity (WHC) as well as oil holding (OHC) capacity was measured according to Wang et al. (2012). SC is defined as the settled bed volume occupied by a sample immersed in excess water. Accurately weighted dry samples (0.5+0.001 g) immersed in water (20 mL) and left

undisturbed overnight in a volumetric cylinder for complete hydration. The volume attained by bran was recorded and the swelling capacity was expressed in mL/ g dry sample.

WHC and OHC is the quantity of water and oil retained by a known amount of sample under the conditions used. WHC and OHC are measured by mixing the accurately weighted dry samples (0.5+0.001 g) with 20 mL water for 24 h and with 20 mL vegetable oil 30 min, respectively. After the centrifugation at 2000 g for 10 min, the supernatant was carefully removed. The WHC and OHC were expressed as g water/ g dry sample and g oil/ g dry sample, respectively.

2.6. Statistical analysis

All analyses were conducted in triplicate and data presented as the mean of measurements. The data were analyzed with SPSS software (V.22.0 for Windows, SPSS Inc., Chicago, IL) using one-way analysis of variance (ANOVA), followed by Duncan's post-hoc test to verify any significant differences among means. Significance of differences was defined as $P<0.05$.

3. Results and Discussion

3.1. The particle size distribution of microfluidized bulgur and chickpea brans

Table 1 shows the particle size distribution of 100 µm bran samples before and after the microfluidization treatment. After the microfluidization, the particle size of bulgur and chickpea bran decreased to 13 µm and 14 µm from 94 µm, respectively. It shows that the microfluidization process dramatically reduced D50 values (<2 µm) and narrowed the particle size distribution of brans.

Table 1- The particle size distribution of 100 µm and microfluidized bulgur and chickpea brans

Bran samples	*Particle size (µm)*	*Particle size distribution (µm)*		
		D90	D50	D10
Bulgur bran	100	93.45±2.15	5.40±0.08	2.81±0.05
	MF(<15)	13.12±0.13	1.89±0.04	0.97±0.03
Chickpea bran	100	94.36±2.83	6.22±0.07	3.12±0.04
	MF(<15)	14.25±0.08	1.92±0.03	0.98±0.01

MF: Microfluidized; D90: 90% of the volume that is smaller than the size indicated; D50: 50% of the volume that is smaller than the size indicated; D10: 10% of the volume that is smaller than the size indicated; Values are means ± standard deviations

3.2. The dietary fiber content of bulgur and chickpea brans

An adequate amount of dietary fiber intake provides many health benefits, however soluble and insoluble fibers play different roles in human health and soluble fiber provides more essential health benefits than insoluble ones in many aspects (Galisteo et al. 2008). Cereal brans typically have low soluble fiber (2-4%) and high insoluble fiber (25-48%) content and it is possible to redistribute these fiber fractions using novel milling techniques and microfluidization process. Table 2 indicates that both bulgur and chickpea brans are rich in dietary fiber, mainly in IDF similar to the other cereal brans. The IDF, SDF, and TDF contents of 350 µm bulgur bran were 65.22%, 3.39% and 68.61%, respectively. Saka et al. (2020) also reported that TDF contents of 200 µm, 400 µm and 850 µm bulgur bran quite high as 72.67%, 77.00% and 83.04%, respectively. Bulgur bran has considerably high TDF compared to wheat bran (36.5%-54.2% TDF) probably due to being aleurone-free. Aleurone is the very outside layer of endosperm, which contains low dietary fiber content compared to the other bran layers (Esposito et al. 2005).

IDF, SDF, and TDF contents of 350 µm chickpea bran were 64.34%, 4.97% and 69.32%, respectively. For the sake of comparison values of the dietary fiber content of chickpea bran are not available in the literature. However, some studies reported that the total fiber contents of whole grain were found between 16.1% and 21.6% (Rincón et al. 1998). It would be reasonable to assume that dietary fiber is also mainly localized outer layer of the grain; therefore, chickpea bran has higher fiber content than the whole grain.

Fiber contents of bulgur and chickpea brans decreased with decreasing particle size from 350 µm to 100 µm; however; these decreases were found to be significant for only SDF content of chickpea bran ($P<0.05$). It is probable that the part which has lower fiber content was more easily broken down to smaller particles. Saka et al. (2020) reported similar results that TDF content of 850 µm bulgur bran decreased to 72.67% from 83.04% when it was milled to 200 µm.

Table 2- Insoluble, soluble, and total dietary fiber contents of bulgur and chickpea brans with different particle sizes

Bran samples	*Particle size (μm)*	*IDF* (%)*	*SDF* (%)*	*TDF* (%)*	*IDF/SDF*
Bulgur bran	350	65.22± 2.40[a]	3.39±0.16 [a]	68.61±2.56 [a]	19.24
	200	63.42±1.56 [a]	3.29±0.24 [a]	66.71±1.80 [a]	19.28
	100	61.43± 1.27[a]	3.20±0.17 [a]	64.63±1.10 [a]	19.20
	<15**	60.94±1.98[a]	7.24± 0.21[b]	68.18± 2.19 [a]	8.42
Chickpea bran	350	64.35±2.55 [a]	4.97±0.13 [b]	69.32± 2.42 [a]	12.95
	200	62.73±2.12 [a]	4.45±0.23 [ab]	67.18±2.35 [a]	14.10
	100	62.38±1.13 [a]	4.07± 0.18[a]	66.45± 0.95[a]	15.33
	<15**	60.29± 1.84[a]	9.83± 0.28 [c]	70.12±1.56 [a]	6.13

IDF: Insoluble dietary fiber; SDF: Soluble dietary fiber; TDF: Total dietary fiber; * On a dry basis; ** Microfluidized; Values are means ± standard deviations; The lower-case letters 'a-c' in the same column indicate differences between the averages of the same bran samples with the different particle sizes are statistically significant (P<0.05)

Microfluidization process significantly increased SDF contents of bulgur and chickpea brans up to 7.24% and 9.83%, respectively while decreased IDF mildly and consequently increased TDF. However, these changes in IDF and TDF contents were not found to be statistically significant (P<0.05). It is reasonable to assume that this process changed the soluble and insoluble fiber ratio by causing a redistribution of fiber fractions. This result is parallel to the finding of Zhu et al. (2010) who stated that ultrafine grinding decreased insoluble fiber of wheat bran while it increased SDF content significantly. Chau et al. (2007) also reported a similar trend of IDF/ SDF ratio change for carrot dietary fiber with high-pressure micro-size grinding at 11600 psi. This ratio change is probably due to degrading of hemicellulose, cellulose and lignin to smaller particles (Zhu et al. 2010).

3.3. The phytic acid content of bulgur and chickpea brans

The high amount of phytic acid content of cereal bran is regarded as one of the attributes limiting their use as a fiber source for enrichment of the food products. Therefore, the aim was to investigate novel fiber sources with low phytic acid content as an alternative for cereal brans. Table 3 shows that milled bulgur brans have extremely low phytic acid content ranged from 230.8 to 247.9 mg/100g considering phytic acid content of wheat bran changing between 2500 to 6000 mg/100g (Özkaya et al. 2017a). The findings are similar to those of Saka et al. (2020) who reported phytic acid content of 200 μm, 450 μm and 800 μm bulgur brans are 228.6 mg/100g, 215.4 mg/100g and 215.7 mg/100g, respectively. The phytic acid contents of bulgur brans are considerably low probably due to removing phytic acid-rich aleurone layer from bran during bulgur process. In addition, phytic acid may be degraded during the bulgur process due to the exposure to the high temperature in the cooking stage. Although phytic acid is heat-stable, it may be easily degraded while heating in aqueous media (Cheryan & Rackis 1980).

Table 3- Phytic acid, phytate phosphorus and total phosphorus contents of bulgur and chickpea brans with different particle sizes

Bran samples	*Particle size (μm)*	*Phytic acid* (mg/100g)*	*Phytate phosphorus* (mg/100g)*	*Total phosphorus* (mg/100g)*
Bulgur bran	350	230.8±5.5 [b]	65.1±1.6 [b]	119.0±1.0 [a]
	200	231.6±7.0 [b]	65.3±2.0 [b]	118.9±1.8 [a]
	100	247.9±8.1 [b]	69.9±2.3 [b]	121.2±1.7 [a]
	<15**	107.1±4.0 [a]	30.2±1.1 [a]	122.6±1.1 [a]
Chickpea bran	350	112.1±4.0 [b]	31.6±1.1 [b]	242.6±2.0 [a]
	200	112.6±5.5 [b]	31.8±1.6 [b]	243.4± 2.1[a]
	100	113.1±3.5 [b]	31.9±1.0 [b]	242.7±2.5 [a]
	<15**	47.9 ±6.5 [a]	13.5±1.8 [a]	243.2±3.0 [a]

*: On a dry basis; **: Microfluidized; Values are means ± standard deviations; The lower-case letters 'a-b' in the same column indicate differences between the averages of the same bran samples with the different particle sizes are statistically significant (P<0.05)

Chickpea bran, which had even less phytic acid than bulgur bran, has its content ranged from 112.1 to 113.1 mg/100g. Similar to bulgur bran, phytic acid of chickpea bran is probably degraded during the leblebi process; boiling stage and additionally resting stage considering phytic acid content of whole grain chickpea is between 121 and 403 mg/100 g (Hussain et al. 1989; Zia-Ul-Haq et al. 2007). These results are consistent with Hussain et al. (1989) who reported that phytic acid and phytate phosphorus contents of unprocessed whole chickpea decreased of 40% and 38%, respectively, after the autoclave process in an aqueous media.

No significant differences were noted between phytic acid, phytate phosphorus and total phosphorus contents of 350 μm, 200 μm and 100 μm (P>0.05). However, even though total phosphorus content remained constant, a striking decrease occurred

in phytic acid contents of both bran samples with the microfluidization process. This dramatic decrease probably occurred due to degrading of phytic acid with the high pressure in the aqueous media during microfluidization process. Our previous work has shown that thermal degradation of phytic acid occurs under low pH and high-pressure conditions (Özkaya et al. 2017a). In this present work, pH was not lowered; however; the pressure used in the microfluidization process was quite high up to 30000 psi and probably resulted in degradation of phytic acid under low-temperature conditions.

3.4. Phenolic compounds content of bulgur and chickpea brans

Cereal brans have attracted much attention over the last decades not only because of their high dietary fiber content but also because of their high phenolic compounds content. Phenolic compounds are secondary metabolism products of plants and have plenty of reported health-promoting effects. As seen in Table 4, the total phenolics content of milled bulgur bran fractions ranged between 3675.68 and 3720.55 µg GAE/g. These results are approximately 30% lower than those reported for wheat bran (Özkaya et al. 2017a), indicating that the bulgur process might have decreased the phenolics content due to the high temperature during the cooking stage of bulgur process. The lower phenolic compound could also be attributed to the differences between wheat cultivars and the fact that bulgur bran does not contain micronutrients-rich aleurone layer. However, bulgur bran has still a high amount of phenolics ranged between the phenolic compound contents of rice bran (4209.8 µg GAE/g) and oat bran (2892.8 µg GAE/g) (Özkaya et al. 2017a; 2017b). As seen from Table 4, chickpea bran is also a good source of phenolic compounds with the total phenolic contents of 1727.48-1756.11 µg GAE/g. It is likely that phenolics are located mostly in the outer layer of chickpeas similar to the cereal grains considering total phenolic compounds of raw whole grain chickpea is ranged between 980 and 1800 µg GAE/g (Xu & Chang 2007, 2008). Xu & Chang (2008) stated that approximately 40–50% of phenolics decreased in chickpea by leaching into soaking and cooking water. Therefore, it would be reasonable to assume that phenolic compounds content of chickpea bran decreased during the process (leblebi) in the same manner as those of bulgur bran.

Table 4- Free, bound and total phenolic compounds contents and antioxidant activity of bulgur and chickpea brans with different particle sizes

Bran samples	*Particle Size (µm)*	*Phenolic compounds* (µg GAE/g)*			*Antioxidant activity* (µmol TE/100 g)*		
		Free	*Bound*	*Total*	*Free*	*Bound*	*Total*
Bulgur bran	350	725.0±8.8^{ab}	2950.7±22.1^{a}	3675.7±30.9^{a}	145.0±6.6^{a}	274.3±7.4^{a}	419.2±0.8^{a}
	200	705.2±11.6^{a}	2974.9±26.0^{a}	3680.2±37.6^{a}	148.3±6.0^{a}	280.1±5.9^{a}	428.0±11.9^{a}
	100	755.7±10.1^{b}	2964.9±32.3^{a}	3720.6±22.2^{a}	149.6±4.5^{a}	284.5±6.4^{a}	433.1±10.9^{a}
	<15**	1723.8±21.8^{c}	4743.8±27.4^{b}	6467.5±49.2^{b}	323.2±8.7^{b}	426.1±9.7^{b}	749.6±1.0^{b}
Chickpea bran	350	353.2±5.9^{a}	1374.3±16.2^{a}	1727.5±22.2^{a}	175.2±4.9^{a}	91.2±3.5^{a}	266.3±1.4^{a}
	200	383.1±7.4^{b}	1360.2±19.2^{a}	1743.3±11.8^{a}	183.8±5.6^{a}	89.7±3.9^{a}	272± 9.5^{a}
	100	399.4±7.3^{b}	1356.7±20.1^{a}	1756.1±27.4^{a}	189.4±5.4^{a}	86.1±4.2^{a}	275.1± 9.6^{a}
	<15**	918.6±14.0^{c}	1884.4±17.6^{b}	2803.0±31.6^{b}	348.1±8.4^{b}	138.2±4.5^{b}	486.1±12.9^{b}

GAE: Gallic acid equivalent; TE: Trolox equivalent; *: On a dry basis; **: Microfluidized; Values are means ± standard deviations; The lower-case letters 'a-c' in the same column indicate differences between the averages of the same bran samples with the different particle sizes are statistically significant (P<0.05)

No significant differences were noted between the phenolics contents of milled bran fractions apart from the free phenolics (P>0.05). However, a clear trend was not observed among free phenolics of bulgur bran fractions while a slight increase occurred for those of chickpea bran with decreasing particle size from 350 µm to 100 µm.

Microfluidization process dramatically enhanced phenolic compound contents of bulgur and chickpea bran, especially free phenolics (up to 56% and 57%) and consequently total phenolics (up to 42% and 37%). Several studies have also mentioned this enhanced phenolics content phenomenon with the microfluidization application (Wang et al. 2013; Wang et al. 2014) and stated that this process increases the extractability of phenolic compounds due to loosening structure of the dietary fiber. It is worth noting that advance dry milling technique like jet-milling might result in collapsing of dietary matrix, and hence decrease the extractability of phenolic compounds (Zhu et al. 2010), while microfluidization process causes a loosen matrix structure due to the rapid pressure release at the end (Wang et al. 2013).

3.5. Antioxidant activity of bulgur and chickpea brans

Free, bound and total antioxidant activity of bulgur bran ranged from 145-149 µmol TE/ 100 g, 274-284 µmol TE/ 100 g to 419-431 µmol TE/ 100 g, respectively (Table 4). The total antioxidant activity of bulgur bran is 54% lower than those of wheat bran (Özkaya et al. 2017a). Chickpea bran has a lower total antioxidant activity compared to wheat bran as expected due to their relatively low phenolic compound contents; however, despite their lower free phenolic compounds content, their free antioxidant activity was found slightly higher than those of bulgur bran as ranged from 175 to 189 µmol TE/ 100 g. There were not any significant differences noted among the different fractions of milled brans; however, microfluidization process again

strikingly enhanced the antioxidant activity of brans parallel to their phenolic compound contents. The applied microfluidization process increased their total antioxidant activity, approximately two-fold, indicating again the loosen structure of the dietary fiber. Due to this loosen structure, antioxidant functional groups might be more exposed to the surrounding environment (Wang et al. 2013).

3.6. Physicochemical properties of bulgur and chickpea brans

Physicochemical properties of fibers including SC, WHC, and OHC are important characteristics due to their ability to influence the end product quality and their health-promoting effects. SC and WHC of 350 µm bulgur bran were found as 6.96 and 6.62 g water/ g bran, respectively (Figure 1). Hydration properties of bulgur bran are higher than the findings of Wang et al. (2012) who reported that untreated wheat bran has SC of 5.96 mL water/ g bran and WHC of 4.02 g water/ g bran. These higher hydration properties probably originate from the higher dietary fiber content of bulgur bran. Chickpea bran has slightly higher SC and WHC as 7.21 mL water/ g bran and WHC of 7.69 g water/ g bran, respectively. Milling process did not affect the hydration properties of brans; however, microfluidization process significantly improves hydration properties, especially of chickpea bran (P<0.05). SC and WHC are strongly related to the chemical composition, porosity and particle size of fibers. As mentioned before, the microfluidization process may loosen the structure of the fiber matrix, even might cause cavities and pores inside the matrix. These structural changes may result in a more exposing surface to outside media and binding of more water, and consequently enhance hydration properties (Chau et al. 2006).

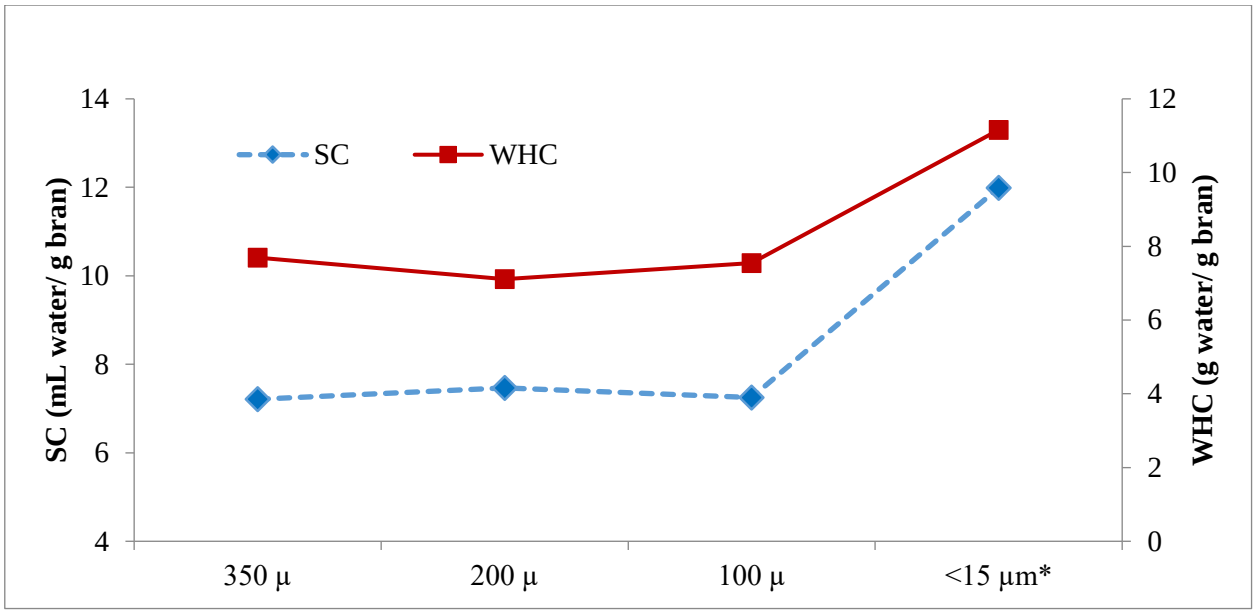

Figure 1- Swelling capacity (SC) and water holding capacity (WHC) of bulgur (A) and chickpea (B) brans with different particle sizes * *Microfluidized*

OHC of 350 µm bulgur and chickpea brans were found as 2.35 g oil/ g bran and 2.31 g oil/ g bran, respectively (Figure 2). As in hydration properties, grinding did not affect OHC of bulgur bran; however, it enhanced OHC of chickpea bran with no clear trend. Similar to hydration properties, microfluidization process again dramatically increased OHC probably due to the increased porosity, surface area and capillary attraction of fiber and lead to an increase of physical entrapment of oil (Chau & Huang 2003). These results suggest that these fiber sources might be a useful functional ingredient to modify viscosity and texture, avoid syneresis, stabilize of fat-food products and emulsions.

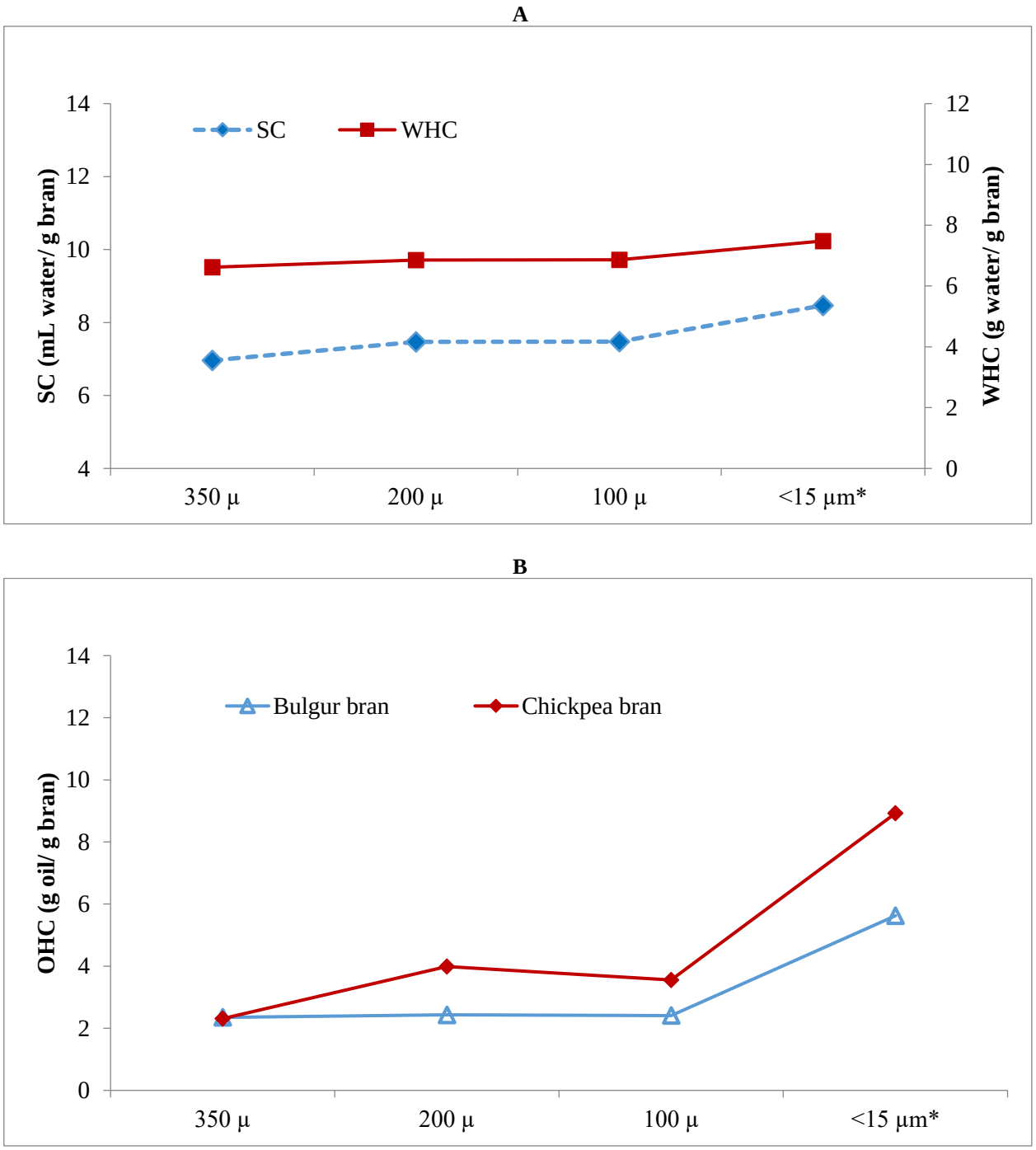

Figure 2- Oil holding capacity (OHC) of bulgur and chickpea brans with different particle sizes * *Microfluidized*

4. Conclusions

Cereal brans have been widely used to produce fiber enriched food products. Numerous studies conducted through the years reported their health-promoting effects as well as their adverse impacts on end-products. However, their severe effects on end-product are not the only limitation regarding their use in the food industry. Their dramatically high phytic acid content is an important issue considering phytic acid is an antinutrient and may cause nutritional problems when a large amount of fiber enriched products is consumed. Therefore, it is necessary to find new alternative dietary fiber sources with low phytic acid. Consequently, this study investigates the functional and physicochemical properties of bulgur and leblebi processes by-products, bulgur and chickpea brans as new dietary fiber sources. The results have shown that both brans contain a considerable amount of fiber as well as phenolic compounds along with a low amount of phytic acid. The milled bran fractions (350 µm; 200 µm; 100 µm) exhibited similar functional properties; however; further particle size reduction of brans with microfluidization process dramatically improved these functional properties. The microfludization process did not only increase phenolic compounds content and their antioxidant activity but also altered their insoluble/soluble dietary fiber ratio by increasing soluble fiber content and most importantly degraded phytic acid. In addition, the applied microfluidization process

enhanced the hydration properties along with the OHC. This study indicates that these brans, both milled and microfluidized, could be valuable functional ingredients for health-oriented food products considering their high dietary fiber content, physicochemical properties and low phytic acid contents and clearly should not be considered as disposable by-products. Furthermore, this process can be used successfully to alter functional and physicochemical properties of other fiber sources. Further studies should focus on the effects of bulgur and chickpea brans on end-products and investigate if the particle size has an impact on the quality, texture and functional properties of the products.

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Abbreviations

IDF	Insoluble dietary fiber
SDF	Soluble dietary fiber
TDF	Total dietary fiber
SC	Swelling capacity
WHC	Water holding capacity
OHC	Oil holding capacity
GAE	Gallic acid equivalent
TE	Trolox equivalent
DPPH	2.2-di-phenyl-2-picryl-hydrazyl

Conflict of interest

The authors have declared no conflict of interest.

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Development of AI Based Larvae Transfer Machine for Royal Jelly Production

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ABSTRACT
Honeybees produce many different products beneficial to humans. One of these of is royal jelly which is the bee product with highest nutritional value but is most difficult to produce. The most time-consuming procedure in royal jelly production involves removing larvae with ideal size from the honeycomb cells and transferring them to queen cups. In order to increase the speed of the larva transfer process and perform it without labor power, a machine autonomically performing larva transfer was developed in three stages. Firstly, a CNC platform that can move on three axes above the honeycomb was created. In the second stage, a camera device was developed to image the larvae and mounted on the platform. Later larvae were photographed with this device and labelled. Tagged photos have been quadrupled by data augmentation methods. A Mobiledet+SSDLite deep learning model was trained with these photographs and this model identified larvae with ideal size with 96% success. Additionally, the central points of the honeycomb cells were identified with the Hough circles method. In the third and final stage, a device which can transfer the identified larvae from the honeycomb cells to the queen cups was developed and mounted on the platform. Later general software controlling the platform and devices was developed. At the end of this study, for the first time in the literature, an artificial intelligence-supported machine was developed for automatic transfer of ideal larvae from natural honeycombs for royal jelly production.

Keywords: Bee larvae transfer, Image processing, Mobiledet SSD Lite, Royal jelly

1. Introduction

As a result of apiculture activities, led by honey, pollen, beeswax, propolis, honeybee venom and royal jelly are produced (Alvarez-Suarez 2017). Royal jelly is a produced by the hypopharynx of bees as a result of transfer via blood of the nutrition from honey and pollen consumed by 4-12-day-old young worker bees after digestion in the digestive organs (Pirk 2018; Pereiraet al. 2019). It is one of the most important products among bee products (Yeung & Argüelles 2019). At the same time, it is the bee product with most difficult production and highest price (Bruneau 2017).

Royal jelly is used in health products, for healthy nutrition and in the cosmetic industry (Ramadan & Al-Ghamdi 2012). Clinical tests and studies researching the effects of royal jelly on a variety of diseases obtained positive results for treatment of many diseases like cancer, diabetes, ulcers, infertility, hypertension, tumors, etc. (Bincolettoet al. 2005; Silici et al. 2009; Shirzad et al. 2013; Miyata & Sakai 2018; Ahmad et al. 2020; Sofiabadi & Samiee-Rad 2020). Royal jelly is used for different purposes in the food, health and cosmetic industries in many countries. Studies investigating the effects on health stated it has positive effects like antibacterial, antimicrobial, anti-inflammatory, antioxidant, antihypertensive, antiseptic and antitumor properties (Okamoto et al. 2003; Fratini et al. 2016; Park et al. 2019). Additionally, royal jelly was revealed to strengthen the immune system and have age-preventive effects in different studies (Vucevic et al. 2007; Kunugi & Mohammed Ali 2019).

Royal jelly is a yellowish-white and acidic secretion from the hypopharyngeal and mandibular glands of nurse bees used to feed young worker larvae during the first three days and the queen bee during her entire life (Ahmad et al. 2020). For royal jelly production, firstly artificial queen cells called queen cups are created. Later, larvae hatched 1-2 days before found in normal honeycomb cells are transferred to the queen cups (Gençer & Fıratlı 1999). The larvae are examined for size 1-2 days after hatching. The reason for use of larvae hatched 1-2 days before is that the production amounts for royal jelly are highest for larvae of this size (Gemeda et al. 2020).

For royal jelly production, production increases in proportion to the number of larvae that can be transferred and the number of hives used. However, the process to transfer the larvae by hand from the honeycomb to the queen cup lasts 5-15 seconds (Grafting 2016). The duration and quality of the procedure are affected by factors such as manual skills, location of the larvae in

the honeycomb and fatigue. In the apiary where the study was performed, nearly 2000 larvae can be transferred in one day. Considering that one person may transfer one larva in mean 10 seconds, this job requires 5-6 working hours without breaks.

In order to transfer larvae for royal jelly production, firstly it is necessary to identify the honeycomb cells and larvae with image processing methods. There are multiple studies about image processing in the agricultural field for classification and detection of marks on fruit like apple, pear, etc., determination of the placement of fruit on branches, and detection of the dimensions and quality of agricultural products (Dubey & Jalal 2016; Capizzi et al. 2016; Yang et al. 2017; Zhang et al. 2017; Ponce Aquino & Andújar 2019; Songaet al. 2020). In the apiculture field, there are image processing studies related to counting bees, determining the gender of bees and observing bee movements (Dembski & Szymański 2019; Ngoa et al. 2019). Additionally, there are studies related to classification of honeycomb cells, detection of size and detection of only large larvae present in the cells in the literature (Sparavigna 2016; Alves et al. 2020; Giraud 2020). However, there is no study related to identification of larvae with sizes for use in royal jelly production. This study has the feature of being the first in the literature from this aspect.

In this study, a machine is proposed to autonomously complete larva transfer to accelerate and increase royal jelly production and lower costs. This machine is a platform that can move on 3 axes with a camera mounted on the platform, a larva transfer tool and an integrated system providing control. The system detects larvae with ideal size with computer imaging and the larva transfer tool is controlled on the platform which can move over the honeycomb to remove larvae from the honeycomb and transfer them to queen cups.

2. Material and Methods

2.1. Larvae transfer system setup

The larva transfer system comprises 3 sections. These are a mechanism that can move in 3 axes, a camera setup linked to this mechanism which can image the larvae and a tool to transfer the larva. The schematic for the larva transfer system is presented in Figure 1.

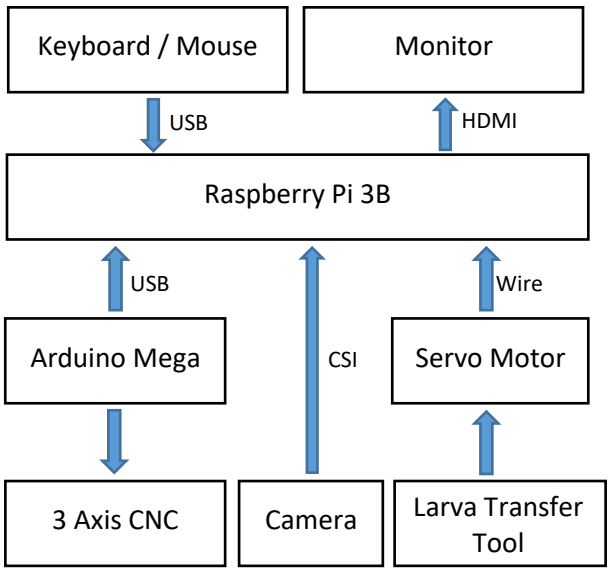


Figure 1- Schematic for larva transfer system

2.2. Triaxial mechanism

A system which can move in 3 axes above the honeycomb was developed to identify and transfer the larvae in the honeycomb. Design of this system noted the standard honeycomb frame size and dimensions of the rod where the queen cups are located. Images of the natural honeycomb dimensions and the placement of the mechanism above the honeycomb and cup rod are given in Figure 2.

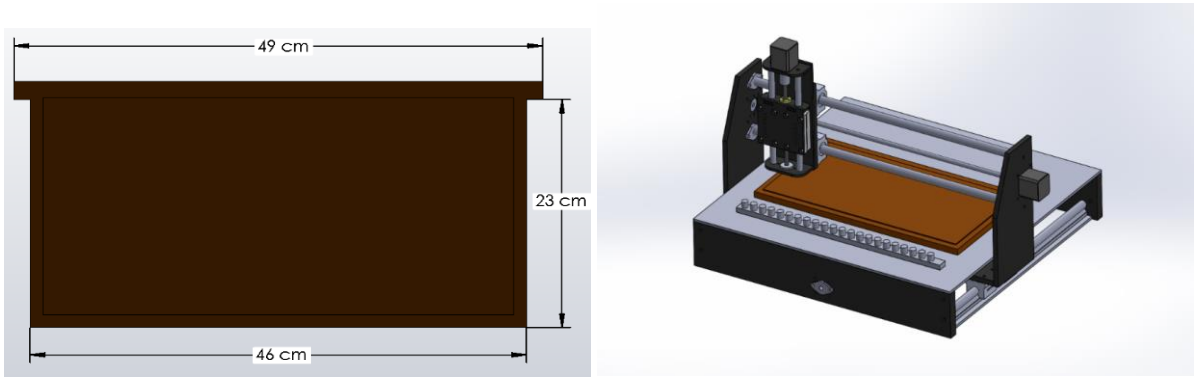


Figure 2- Honeycomb frame dimension and system design

The system used a classic cartesian movement mechanism. Movement on the X, Y and Z axes was provided by a belt and pulley mechanism powered by Nema 23 step motors for the X and Y axes and a Nema 17 series step motor for the Z axis. To drive the motors, TB6560 series step motor driver cards were used with control by an Arduino Mega microcontroller (Arduino Mega 2560 Rev3). The combined form of the system is given in Figure 3.

Figure 3- The combined form of the system

The Arduino Mega embedded system, which drives the motors for the movement of the platform, was run on the GRBL software. GRBL is an open-source coded, embedded, high performance g-code-parser and CNC milling controller written in optimized C that will run on a straight Arduino (grbl). When the system is to be moved, G codes are sent to the Arduino Mega through serial connections and the system moves to the required point.

2.3. Imaging area

A camera was mounted on the mobile head of the system which can move in 3 axes for use to image the larvae. Many alternatives were attempted for the camera (Go Pro Hero 5 Black, A4Tech Pk-910P 720P WEB CAM, Microscope Cam 500X); however, due to the small dimensions of the larvae (~2 mm) and the deep and dark honeycomb cells, cameras with automatic focus could not clearly photograph of larvae. For this, a 5 MP resolution camera operating via the camera serial interface with manually adjustable zoom features was used (Raspberry Pi Camera). The first photographs taken with this camera could not view the larvae in the dark-colored cells. The reason for this was the deep and dark honeycomb cells. For this reason, a lighting LED was added to the system. The mounted view of the camera system is presented in Figure 4.

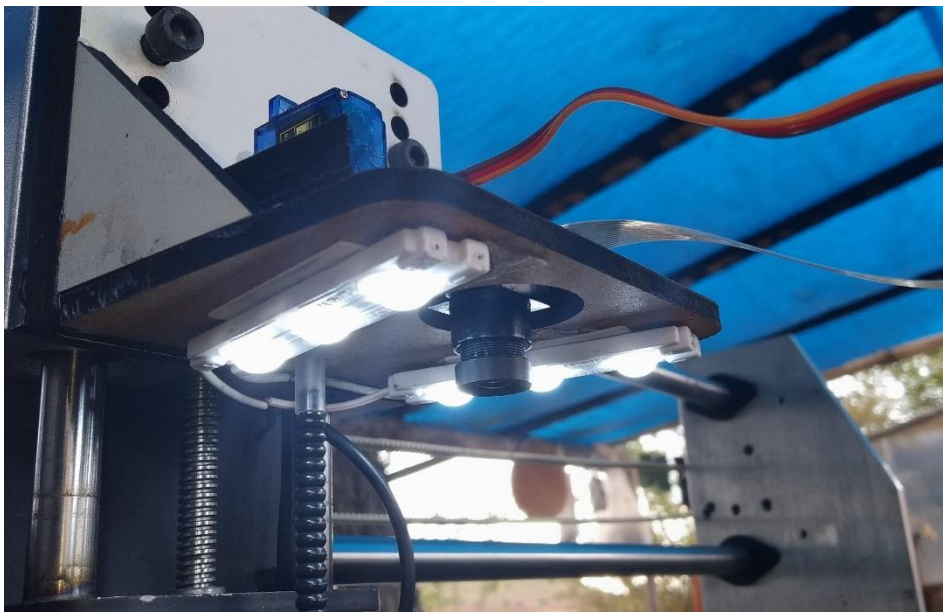


Figure 4- Camera and lighting LED

In this stage to operate the software providing imaging with the camera, platform movement and all controls, a Raspberry Pi 3B was added to the system (Raspberry Pi 3 Model B).

2.4. Larva transfer tool

Larvae are transferred by hand with a specialized tool similar to a ballpoint pen shown in Figure 5. This tool is first placed in the honeycomb cell and slowly moved to scoop the larva onto the tip of the tool. Due to the adhesion of the royal jelly surrounding the larva, they stick to the tip of the tool. Then, the larva is brought to the queen cup and the button on the other end of the tool is pressed to push the tongue forward and drop the larvae into the queen cup. Thus, the transfer process is completed (Grafting 2016).

Figure 5- Larva transfer tool

The larva transfer tool was mounted with the camera on the mobile platform in the study. Operation of the larva transfer tool was completed with an arm linked to the shaft of an RC servo motor. This mechanism can press the button on the tool and drop the larvae. The appearance of the larva transfer tool on the mechanism is shown in Figure 6.

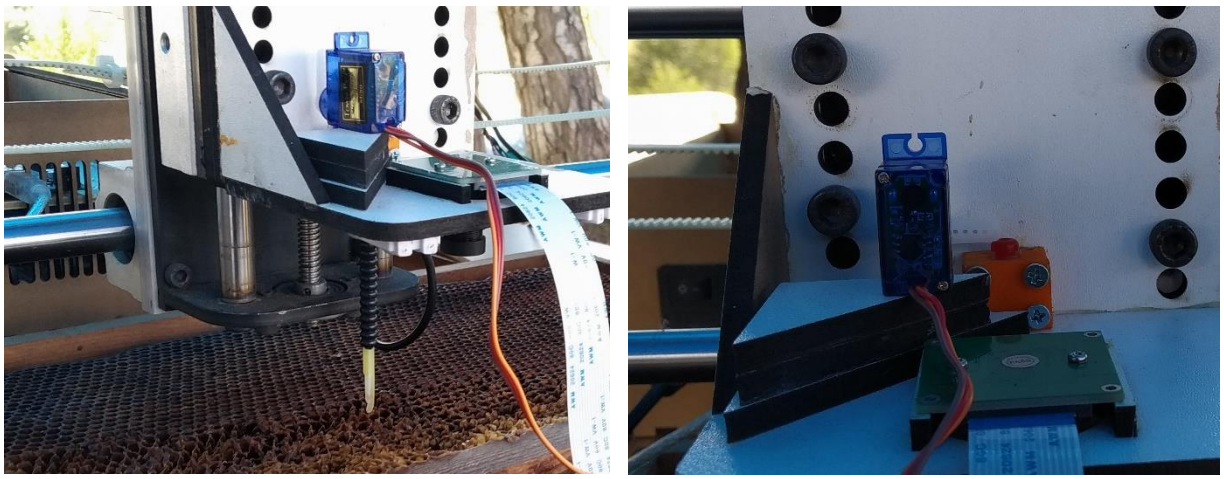


Figure 6- Appearance of larva transfer tool on the mechanism

3. Larva detection and transfer

In order to perform the transfer procedure, firstly it is necessary to identify the larva within the honeycomb cells. Then it is necessary to place the larva transfer device at the point where the larva is identified, take the larva from the cells and finally leave them in the queen cups.

3.1 Photographing and detecting larvae

For detection of larva, photographs were taken of both sides of 25 different honeycombs. A total of 1356 photographs were taken from different regions in each honeycomb. The camera was placed at 10 cm distance from the honeycomb in order for the system to clearly image 65 honeycomb cells. The best focusing and rate of honeycomb cells in focus was obtained from this distance in the photography process. For the photography process, honeycombs that were both old and new were chosen and that contained dense larvae. The new honeycombs were yellow in color, while they darkened over the years to reach a color close to black. Figure 7 shows photographs of old and new honeycombs.

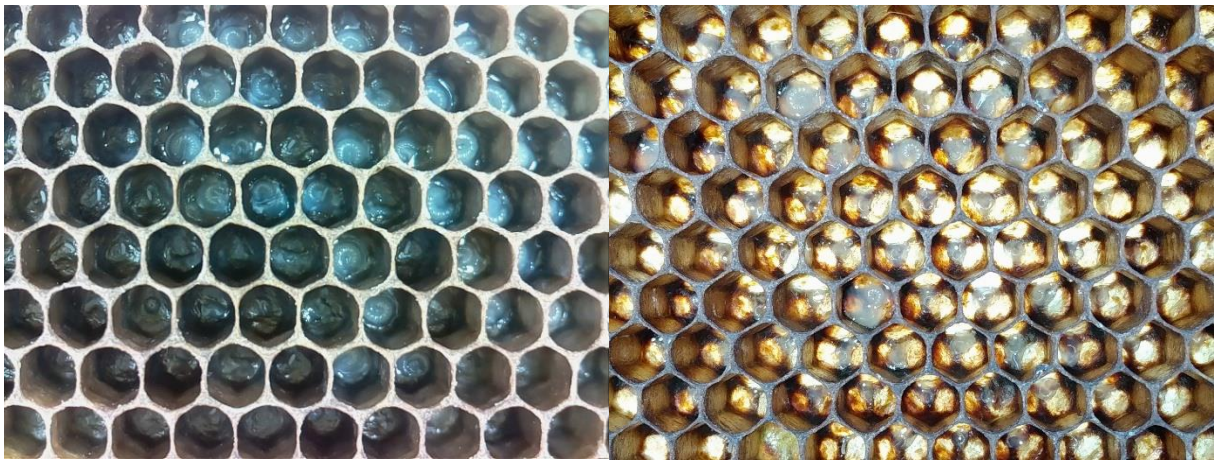


Figure 7- Larvae in honeycomb cells

When honeycombs are first created by bees, they have a yellow color and have pronounced rates of transparency. In this situation, it is difficult to distinguish the white-colored and small larvae from the background color. Additionally, as the honeycombs darken, the light rate in the cells containing larvae reduces and makes photography difficult. Honeycombs with different colors were photographed for the system to operate in all these different conditions.

The resolution of the photographs was 640*480 and 96 dpi. The resolution was low in order to use a computer with low system resources, like Raspberry Pi, for the larva detection process in the system.

The larva detection process is actually an object finding process. Currently one of the most commonly chosen object finding methods is deep learning-based object identification. The general feature of these methods is that they find the desired objects

themselves after being trained in the distinguishing features with many samples. Generally, these deep learning methods use convolutional artificial neural networks (O'Shea & Nash 2015; Albawi et al. 2017; Agarwal et al. 2019).

CNNs (Convolutional Neural Networks) are generally designed to consist of convolution, sampling and full link layers and work by performing operations in these layers respectively (Amidi & Amidi 2019). In the convolution layer, some filters are applied to the image to extract low- and high-level features from the image. In the pooling layer, methods such as max and avg are applied to the data, reducing the size of the data and reducing the number of transactions that need to be made on the network. In the full connection layer, there is a neural network in which every data coming out of the pooling layer is given as input to all neurons and is in principle the same as a traditional multilayer perceptual neural network (MLP). This neural network performs the image classification process.

There are many available deep learning-based object finding models and studies in this area continue intensely. However, as the imaging process in this study used Raspberry Pi 3B, it was necessary for the model to be used for the platform to be operable in systems with low processing power.

Currently, there are many object finding CNN models that can be used with Raspberry Pi. Examples of these include SSD (Single-shot Detector), Faster R-CNN and YOLO (You Look Only Once) (Zhang et al. 2018; Chandan et al. 2018; Huang et al. 2018; Foley & R, 2018). Studies comparing the operating performance of these models observed successful object detection and accurate location with many algorithms according to the dimensions of the photographs, dimensions of the objects and training durations (Desai et al. 2020; Bouguettaya et al. 2019). When performance with Raspberry Pi is considered, the MobileNet+SSDLite-based models were identified to produce better results (Gunnarsson 2019; Brokate 2019; Huang et al. 2017). Within the scope of the study, one of the most up-to-date and successful versions of these models of Mobiledet+SSDLite was used (Xiong et al 2021).

MobileDet is one of the most advanced image classifier model architectures for mobile devices that emerged with the work of University of Wisconsin-Madison and Google researchers (Xiong et al. 2021). MobileDet uses three main blocks in the design of its backbone: inverted bottleneck (IBN), fused convolution, and Tucker/CP decomposition. In this study, a pre-trained deep learning model SSDLite MobileDet-CPU (ssdlite_mobiledet_cpu_320x320_coco) was used. The structure of this model is presented in Figure 8.

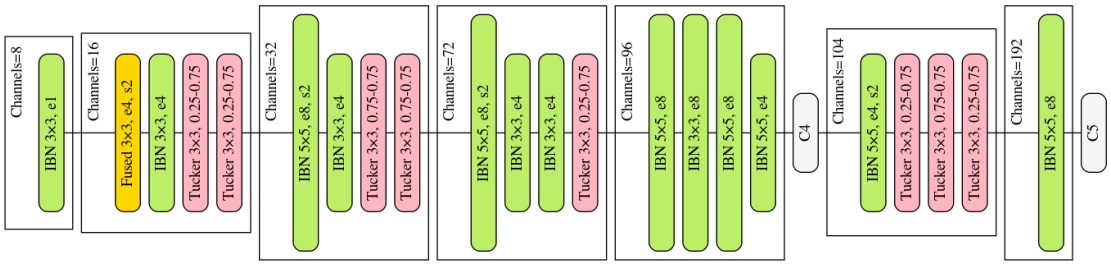


Figure 8- Mobiledet CPU Architecture

In order for Mobiledet+SSDLite to be able to detect larva, it is necessary to train the model with images of larva from the honeycomb photographs. For this, a total of 6373 larvae with ideal size for royal jelly production were labeled on 640*480 photographs taken of the honeycombs with the Labellmg software (LabelImg). The labelling process is presented in Figure 9.

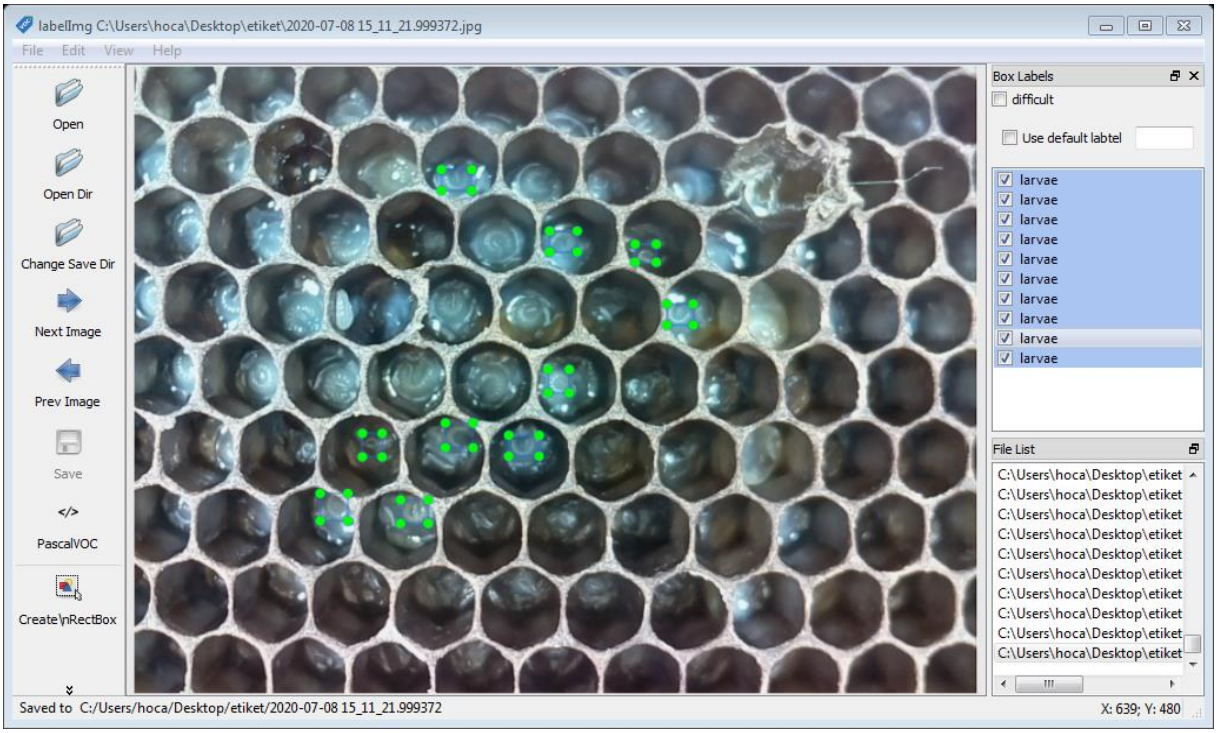


Figure 9- Labelling of larvae with ideal dimensions

In order to increase the success rate of the model to be trained after the labeling process, the data augmentation process was applied to the data set in our study. Using data augmentation techniques, a relatively smaller dataset is transformed into a larger database and deep learning algorithms are trained with these datasets. The basic principle in the data duplication is generate additional training data by applying some deformation, rotation, translation etc. to the existing data (Dieleman et al. 2015; Salamon & Bello 2016). There are many data augmentation techniques, such as rotating the image at different angles, *horizontally vertically*, adding noise and color manipulation to the image. In this study, in order to increase the data, all the marked photos were rotated 90, 180 and 270 degrees, also considering the shapes of the larvae on the honeycomb, and the tag files were automatically changed with the written python script. With this process, the number of photos increased to 5424 and the number of larvae to 25492.

70% of tagged photos are reserved for training, 20% for verification and 10% for testing. Augmented data was used to in the separation process. Images were randomly selected from each honeycomb in the proportions specified for each data set. The training process was carried out using TensorFlow framework and Google Colab platform. The graphics processor used is Tesla P100 GPU. The model is trained using stochastic gradient descent with an initial learning rate of 0.001, 0.9 momentum, 0.0005 weight decay, and batch size 32. The training was carried out in fifteen thousand steps. As a result of the training, the success rate on the validation data was calculated as 96%.

The trained model, after the training process was tested to measure the performance of the model with all the photos reserved for the test. In this test process, 2439 larvae in 542 photographs were tried to be detected and 2347 of them were successfully tested. In addition, 45 false larvae were detected. Accuracy, f1-score, precision, recall values are also used to measure the performance of CNN-based models (Sokolova & Lapalme 2009). To calculate these values, True Positive (TP), True Negative (TN), False Positive (FP) and False Negative (FN) values were determined and the formulas in Table 1 were used.

Table 1- Model results

Metric	***Equation***	***Values***	***Results***
Accuracy	$\frac{TP+TN}{TP+FN+TN+FP}$	$\frac{2347+0}{2347+93+0+45}$	0.962
Precision	$\frac{TP}{TP+FP}$	$\frac{2347}{2347+45}$	0.981
Recall	$\frac{TP}{TP+FN}$	$\frac{2347}{2347+93}$	0.961
F1-score	$\frac{2*precision*recall}{precision+recall}$	$\frac{2*0.981*0.961}{0.981+0.961}$	0.971

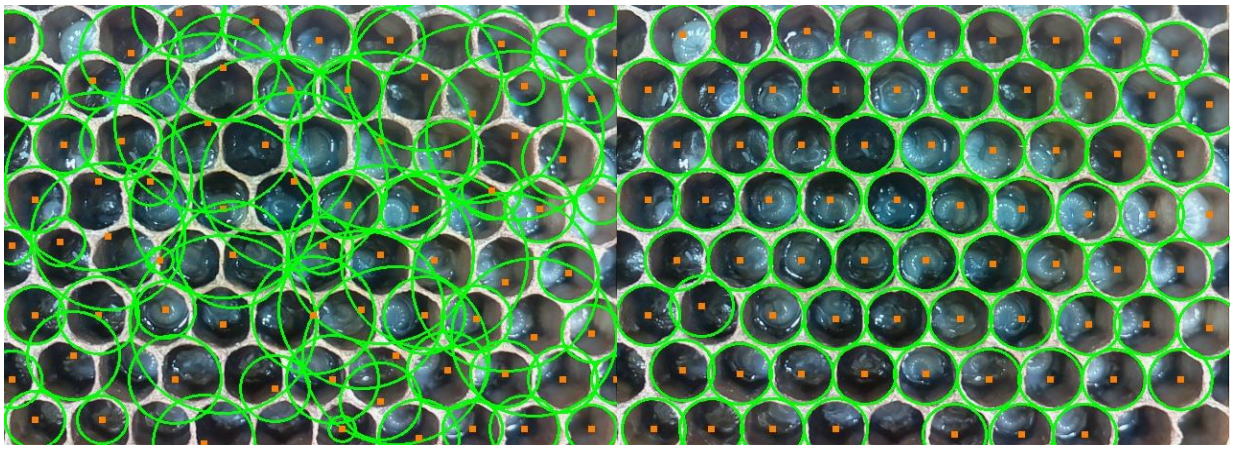
The Mobiledet+SSDLite model used in the study was more successful than the Faster R-CNN model developed by Gungormus (2020) to identify developing larva in terms of both performance (96.2%, 80.4%) and speed (Gungormus 2020).

One of the most important performance criteria for a model that will work in real time is the detection time of the larvae in a photograph taken. In the test process, the average detection time of the larvae in all photographs was determined as 2.75 sec.

3.2. Detection of honeycomb cells

Larva are located within the honeycomb cells at the base. However, larvae with ideal size are located at different points of the honeycomb based on the point where the queen bee laid the eggs (Figure 4). In order to transfer larvae with the larva transfer tool, it is necessary to place the mechanism at the correct point above the honeycomb and to lower the larva transfer tool to the correct point of the cell. Lowering the larva transfer tool at the wrong location may cause damage to the honeycomb or larva, in addition the damaging the device. For this, it is necessary to ensure detection of the central points in the honeycomb cells. Honeycomb cells have hexagonal shapes. However, when examined carefully, the shape is very close to a circle. Based on this, round shapes were placed on the cells using the Hough Circles technique and the center points of the circles and hence the cells could be identified (Söylemez 2012).

For detection of honeycomb cells, the Open CV image processing library was used. Firstly, photographs were converted to greyscale. Later 'Hough circles' were placed on the greyscale photographs. As a result of this process, the center points and radius values for many circular areas placed on the photographs according to the parameters were identified. In accordance with these values, circles were drawn in green color on the photograph with OpenCV and the image observed in Figure 10a was created. However, there were too many circles as can be seen from the figure. At this point, based on the information that the dimensions of the honeycomb cells were the same size, circles with radius above or below a certain value were deleted. As a result, the central points of all cells located in the photograph were identified and labelled and are presented in Figure 10b. The same process was used for detection of the center points of the queen cups and was successful.



a) Detection with circles on honeycomb image b) Circles at honeycomb cell dimensions

Figure 10- Cell detection

3.3 Mechanism and movement algorithm for larva transfer tool

Operating system software was developed to monitor and control the whole system. The pseudo algorithm explaining operation of the operating system is as follows:

1:	$N_{qc} = 22 \leftarrow Total\ number\ of\ Queen\ Cup$
2:	Place larva transfer tool at central point of cell
3:	Take photograph of cell
4:	Identify larva with suitable size
5:	Identify cells
6:	Number larva with suitable size within cells
7:	$larvae = 1$
8:	**repeat**
9:	move to cell containing larva
10:	Take larva
11:	Move to queen cup
12:	Drop larva
13:	$larvae \leftarrow larvae + 1$

14:	**until** $larvae > N_{qc}$
15:	Finish

When the software is first operated, the system is moved to the center point of the honeycomb because queen bees generally lay in spiral form beginning from the center point of the honeycomb. Then the photograph of the honeycomb is taken and larva with ideal dimensions and the center of the cells are determined. Larva with ideal dimensions located in the honeycomb cells are numbered. The identified and numbered larvae are presented in Figure 11.

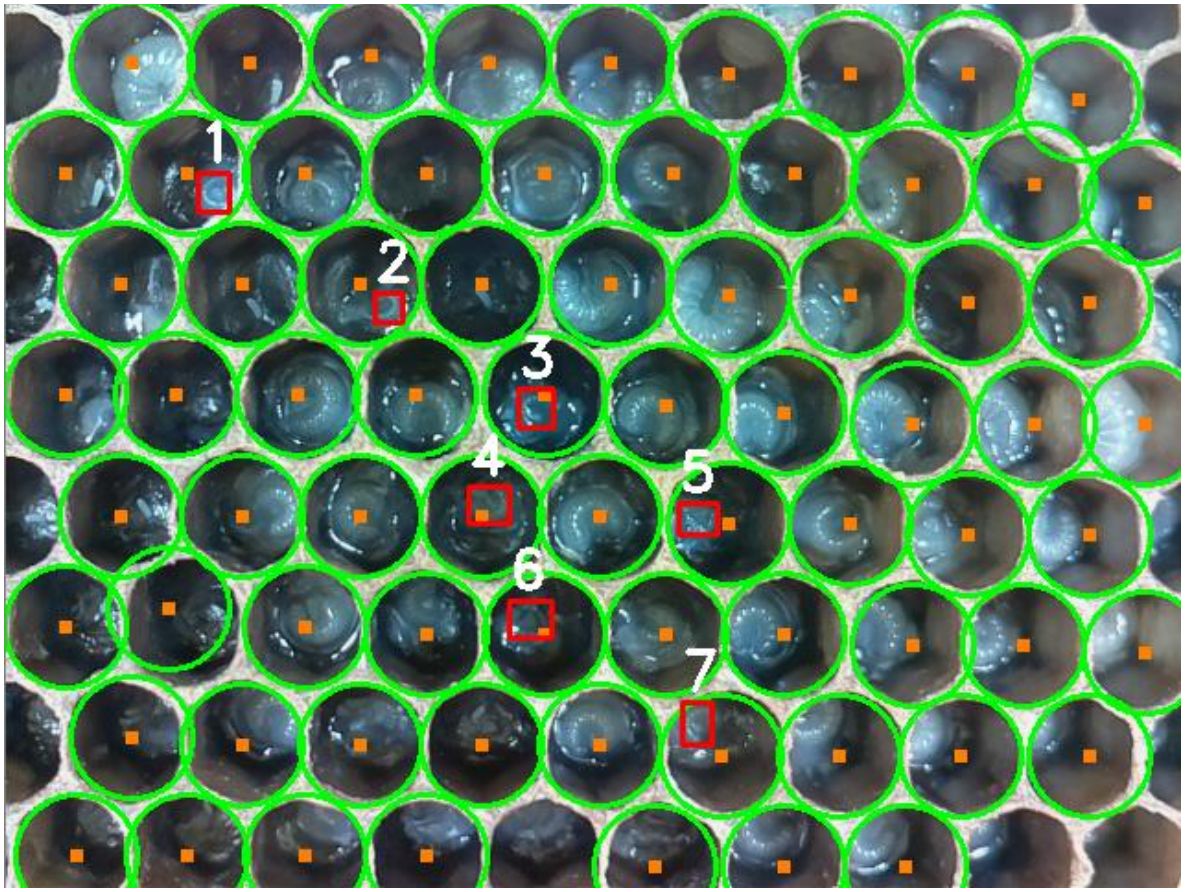
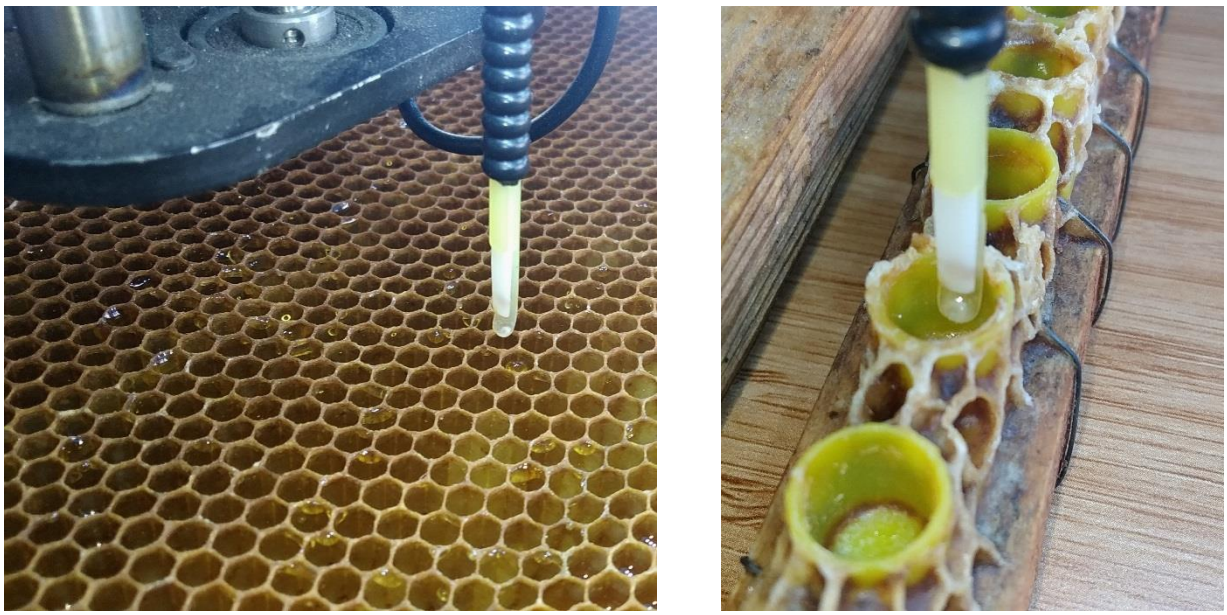


Figure 11- Detection and numbering of larvae

After numbering the larvae, the system begins the transfer process for all larva in order. For this, firstly the larva transfer tool moves to a point between the edge of the cell and the larva. Then the larva transfer tool is lowered 4 cm downward and moved toward the larva to ensure the larva is placed on the transfer tool tip. After taking the larva, the larva transfer tool is moved upwards by 4 cm and then moved to the point where the queen cups are located. The rod containing the queen cups and the cups have standard size and are continuously at the same point, so the system moves to the next cup in order. Then the tool is lowered 3 cm, the button on the transfer tool is pressed and the larva is dropped. The image taken after removing the larva is shown in Figure 12a, and the image for the larva being dropped is presented in Figure 12b.



a) Retrieving larva from honeycomb cells b) Leaving larva in queen cup

Figure 12- Larva transfer process

After the end of the larva transfer process, the same procedure is repeated until the number of queen cups is reached. After filling all cups, the operator simply needs to place a new rod and restart the system.

4. Results

The developed larva transfer tool was prepared to complete the larva transfer process automatically. Then it was tested with 5 dark and 5 light honeycombs. The tested honeycombs contained mean 400 larva with ideal size, with 200 on each side of the honeycomb (these values vary according to season, work by the queen, duration of use of honeycombs). After photographing the ideal size larvae, detection with the MobileDet+SSDLite model took mean 2.75 s. The same process lasted nearly 7 s with Faster R-CNN.

The larva transfer system successfully identified 95.56% of larvae in the 10 honeycombs. Attempts were made to transfer 440 of the identified larvae and 217 (50%) were successfully transferred. At this point, no difference was revealed in performance between dark- and light-colored honeycombs. Images of a successful transfer are presented in Figure 11. Additionally, it was determined that the mean duration for successful transfer processes was 20 s from when larvae were identified. A normal person has mean larva transfer rate of 7 s (this was the mean rate for workers in the apiary where the study was performed).

5. Conclusions

There was no study in the literature about the detection and autonomic transfer of bee larvae with appropriate size for royal jelly production. This study used a trained convolutional artificial neural network using Mobiledet+SSDLite to achieve larva detection with high performance. The larva transfer mechanism developed transferred the identified larva with nearly 50% performance. The basic cause of unsuccessful transfer attempts was the variability in the depths of the honeycomb cells. Some larva did not adhere to the larva transfer tool and were pushed to the edge of the cell and could not be retrieved. Additionally, some honeycomb cells were morphologically deformed which caused errors.

Future studies to increase the performance of the system:

- An end effector (perhaps with vacuum) design may be used to pay attention to variations in depths of the honeycomb cells and to retrieve the larvae from the cells without damage.
- Faster embedded system (FPGA-supported systems etc.) and mechanical equipment may be used to increase the transfer rate.
- The rods of queen cups used in the apiary studied contain 22 cups, but there are designs containing 30 or 60 cups and with different shapes. An algorithm may be developed which calculates the number of cups and operates accordingly.

Acknowledgment

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Machine Learning Approaches for One-Day Ahead Soil Temperature Forecasting

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ABSTRACT

Present study investigates the capabilities of six distinct machine learning techniques such as ANFIS network with fuzzy c-means (ANFIS-FCM), grid partition (ANFIS-GP), subtractive clustering (ANFIS-SC), feed-forward neural network (FNN), Elman neural network (ENN), and long short-term memory (LSTM) neural network in one-day ahead soil temperature (ST) forecasting. For this aim, daily ST data gathered at three different depths of 5 cm, 50 cm, and 100 cm from the Sivas meteorological observation station in the Central Anatolia Region of Turkey was used as training and testing datasets. Forecasting values of the machine learning models were compared with actual data by assessing with respect to four statistic metrics such as the mean absolute error, root mean square error (RMSE), Nash−Sutcliffe efficiency coefficient, and correlation coefficient (R). The results showed that the ANFIS-FCM, ANFIS-GP, ANFIS-SC, ENN, FNN and LSTM models presented satisfactory performance in modeling daily ST at all depths, with RMSE values ranging 0.0637-1.3276, 0.0634-1.3809, 0.0643-1.3280, 0.0635-1.3186, 0.0635-1.3281, and 0.0983-1.3256 °C, and R values ranging 0.9910-0.9999, 0.9903-0.9999, 0.9910-0.9999, 0.9911-0.9999, 0.9910-0.9999 and 0.9910-0.9998 °C, respectively.

Keywords: ANFIS, Daily soil temperature, LSTM, Elman neural network (ENN), Feed-forward neural network

1. Introduction

Annual, monthly, daily, and hourly meteorological data are among the most critical atmospheric parameters for many engineering systems and agricultural activities. As one of these meteorological parameters, soil temperature (ST) has crucial importance in distinct disciplines, including soil science, meteorology, agronomy, environmental studies, atmospheric, hydrological, and agricultural numerical models, ecological applications, and agricultural management (Mehdizadeh et al. 2017). Also, ST is a significant meteorological factor for agricultural activity, solar energy technologies, geothermal energy systems, ground source heat pumps, etc. The chemical structure of the soil and organic components are highly affected by ST. The soil heats inwards from the surface and cools by losing heat from inside to outside (Feng et al. 2019). Therefore, daily and seasonal temperature changes are high, although not as high as air. These changes decrease towards the depths, and the temperature remains constant after a certain level. Although the effects of the surface, in general, affect up to 10 m depth, temperature changes are negligible at depths more than 1.5~2 m. For these reasons, many studies have focused on ST forecasting and modeling (Araghi et al. 2017; Shahabi et al. 2021).

Meteorological parameters are measured at meteorological stations located at specific points in many parts of the world. ST measurements are generally made with soil thermometers and soil thermographs at depths of 5, 10, 20, 50, and 100 cm. Many meteorological and atmospheric variables are more easily measured than ST, and therefore more widely accessible. Measuring the ST of a specific location when needed is not as easy as measuring the air temperature of that point. Therefore, estimating ST based on various meteorological parameters, which can be measured much more quickly, has facilitated many engineering problems (Xing et al. 2018).

The thermal changes and energy balances between the soil and ground surface at a certain depth are highly affected by the ST (Araghi et al. 2019). Accurate ST forecasting is recognized as crucial information and foresight for this reason (Zeynoddin et al. 2019). Various studies have recently been conducted on short and mid-term ST forecasting (Penghui et al. 2020). In the first category, statistical approaches such as numerical weather prediction (NWP) methods are used, assuming that future

variations in the statistical characteristics of the ST data set will be similar to those in the past. However, these approaches often require much data that may not be available for long-term forecasts. In the second category, artificial intelligence (AI) and machine learning models such as an artificial neural network (ANN) (Citakoglu 2017; Singhal et al. 2021; Zhou et al. 2020), support vector machine (Xing et al. 2018), gene expression programming (GEP) (Mehdizadeh et al. 2017), genetic programming (Gill & Singh 2015; Stajkowski et al. 2020), adaptive neuro-fuzzy inference system (ANFIS) (Mehdizadeh et al. 2020a) and hybrid models (Sattari et al. 2020; Shamshirband et al. 2020) are used. Various studies have modeled ST as a non-linear physical aspect (Li et al. 2020; Xu et al. 2020; Zeynoddin et al. 2020; Hao et al. 2021).

There are many approaches to predict ST using numerical, analytical, and data-driven models based on the literature. In the early 18th century, Fourier suggested a one-harmonic analytical method that accepts the STs as a function of depths and year's date (Xing et al. 2018). This model is derived according to one-dimensional heat conduction equations, considering the ST on the surface as the boundary conditions. Analytical models can be applied for any desired location, but many parameters such as the soil's heat conductivity, density, and thermal capacity must be known. A particular study is needed to obtain this information correctly. Therefore, analytical models cannot be easily adapted wherever desired. Numerical methods are also utilized to predict the ST. While only conduction heat transfer is considered in analytical methods, convection heat transfer and mass transfer can be included in the mathematical model and conduction heat transfer in numerical methods. However, developing the mathematical model in numerical methods is complex, and at the same time, the model calculation time is comparatively long-time. Naranjo-Mendoza et al. (2018) investigated analytical and numerical methods for forecasting ST, and they presented that the sinusoidal approach was not appropriate for estimating the short-term temperature variations. However, the finite difference method was the most appropriate approach for long- and short-term temperature forecasting. Kayaci & Demir (2018) have presented a numerical model of transient ST distribution for a horizontal ground source heat pump. They obtained steady periodic ST and investigated the impacts of distinct parameters on the ST profile.

Different types of ST prediction models based on the correlation between the ST and meteorological parameters using the data-driven statistical methods can be listed as linear regression (LR), non-linear regression (NLR), ANFIS, ANN, wavelet neural network (WNN), and deep learning. George (2001) presented a study to estimate ST using ANN algorithms. Wind speed, relative humidity and atmospheric temperature data were utilized to predict ST. Gang et al. (2014) estimated the efficiency of a hybrid ground source heat pump unit with the ANN predictive control method. Yan et al. (2016) used data monitoring and mining techniques for predicting the long-term performance of a ground heat pump system using short-term data. Chen et al. (2018) developed an ANN model to estimate the vertical ground heat exchangers. A data-driven model was used by Xing et al. (2018) for daily ST estimations. They estimated the daily or monthly ST of a single site with great accuracy considering solar radiant, air temperature, and time as inputs. Samadianfard et al. (2018) applied the GEP, and WNN approaches to estimate short-term ST at distinct depths. Zeynoddin et al. (2020) used the linear-based stochastic model for ST estimation.

As seen recently, machine-learning approaches have been efficiently used to estimate ST. Considering previous studies, most of the studies modeling or forecasting STs use many environmental and atmospheric variables as inputs. However, the availability of these models may be limited in countries where atmospheric and environmental data are scarce. The STs estimation for any future short or long-term without the need for any other meteorological or geographical data by establishing a relationship between previous STs and future ST can have a significant advantage. Therefore, the main scope of this study is to predict the next day's ST based solely on the previously measured ST data. For this purpose, a univariate procedure was used. Creating such a univariate time series information is very important for areas where meteorological data are limited and can contribute to many previously mentioned applications. In this respect, in this work, six distinct machine learning techniques such as ANFIS-SC, ANFIS-GP, ANFIS-FCM, ENN, FNN, and LSTM were used in one-day ahead ST forecasting.

2. Material and Methods

This section describes the methods used for soil temperature prediction, and provides information about the study area and materials.

2.1. Study area and data

In this study, daily STs at 5, 50, and 100 cm depths were obtained from the Turkish Meteorological Service's station, located in Sivas province, Turkey. The climate of the study area, illustrated in Figure 1, is a continental climate with warm and dry summers and cold and snowy winters. The average air temperatures in wintertime and summertime are recorded as -1.7 °C and 18.7 °C, respectively (www.sivas.climatemps.com). Total annual precipitation is 427.4 mm. The ST data used in this study cover 10-year daily records from 2010 to 2020. In the machine learning applications detailed above, the first 80% of the dataset was used for training and 20% of the dataset was utilized for testing.

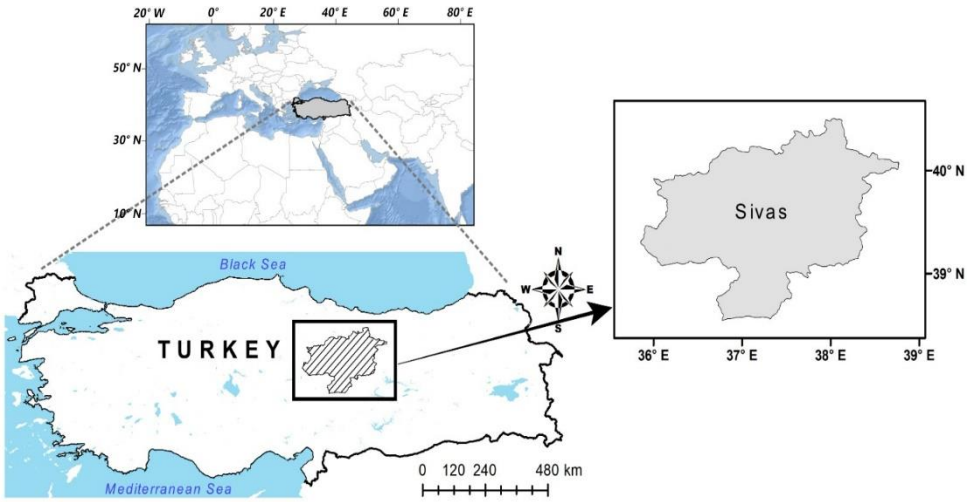

Figure 1- Location map of the study area, showing the place of the ST measurement station.

Table 1 presents descriptive statistics for the daily ST data used in training and testing phases. In Table 1, the skewness is a measure of the symmetry condition of distribution, and zero skewness stands for a completely normal (Gaussian) distribution. In this study, the ST data also indicate an almost normal distribution since the skewness values of both training and testing data sets are close to zero. Concerning the maximum and minimum ST values at different depths, Table 1 reveals that maximum values are higher and minimum values are lower in the training phase than in the testing phase. The standard deviation values show that the ST variation decreases as the depth decreases. Figure 2 illustrates the variation in 10-year ST data sets used in this study at 5, 50, and 100 cm depths. The figure also reveals the training and testing data sets, covering 80% and 20% of all data, respectively. Besides, it is clear from Figure 2 that the ST variation at different depths shows the almost normal distribution for each year, and the more the depth increases, the lower the ST variation is observed.

Table 1- The descriptive statistics for the daily ST data used in training and testing phases.

Data	*Depth*	*Unit*	*Min.*	*Max.*	*Avg.*	*Std Dev.*	*Skewness*
Training Data	5 cm	°C	-7.2	34.8	14.0	10.6	0.13
	50 cm		1.2	27.5	14.0	7.9	0.09
	100 cm		3.9	24.3	13.9	6.1	0.11
Testing Data	5 cm	°C	-0.6	33.5	15.0	9.9	0.14
	50 cm		3.7	26.7	15.2	7.5	0.08
	100 cm		6.3	24.0	14.9	5.8	0.08

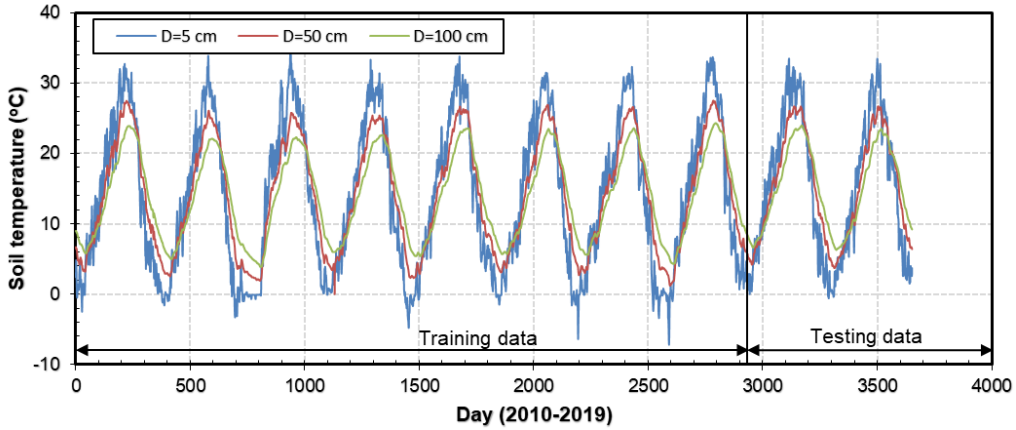

Figure 2- 10 year ST data sets at 5, 50 and 100 cm depths used in the study. Training and testing data cover 80% and 20% of all data, respectively.

2.2. Artificial neural networks (ANNs)

ANNs are strongly applied in many scientific disciplines by using the dispersed storage features and large-scale parallel local processing techniques available in the human brain. Recently, they have become more effective tools, particularly for non-linear modeling processes, which are difficult to express with statistically or physically defined equations (Inyurt & Sekertekin 2019).

Figure 3 presents the structure of a primary neuron with *R* inputs. Each input *p* is identified by a weight value *W*. The sum of bias and weighted inputs constitutes the transfer function *f*. The different transfer functions f can generate neuron outputs (Mathworks 2020a).

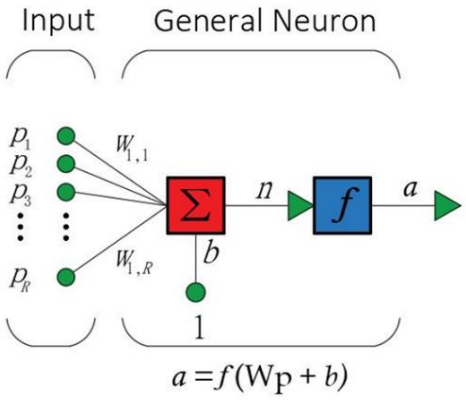

Figure 3- Structure of a basic neuron with *R* inputs (Mathworks 2020a)

Different ANNs with different algorithms and structures can be formed in simulating and modeling linear or non-linear parameters. Usually, ANNs can be classified into two categories: (a) feed-forward neural networks (FNNs) and (b) recurrent networks. The FNNs do not have feedback or delay elements. In recurrent networks, the network's current and previous inputs or outputs affect the output. The recurrent networks, unlike the FNNs, can use internal memory to process handling random input data series and exhibit transient behavior. This capability makes them applicable to time series forecasting with satisfactory predictive results. As a particular recurrent neural network, Elman neural network (ENN) has been widely and successfully used in time series estimation and forecasting (Mehdizadeh et al. 2017**)**.

2.2.1. Feed-Forward Neural Network (FNN)

The simplest and first of artificial neural networks is FNN. In the structure of the FNN, information flows forward, moving in one direction from input nodes to output nodes. Feed-forward backpropagation ANN covers supervised learning, but there are no feedback loops or connections in the network. In the multi-layer FNNs, several neuron layers are connected in a forward direction. As shown in Figure 4, each node unit in one layer connects directly to neurons in the next layer (Mathworks 2020a).

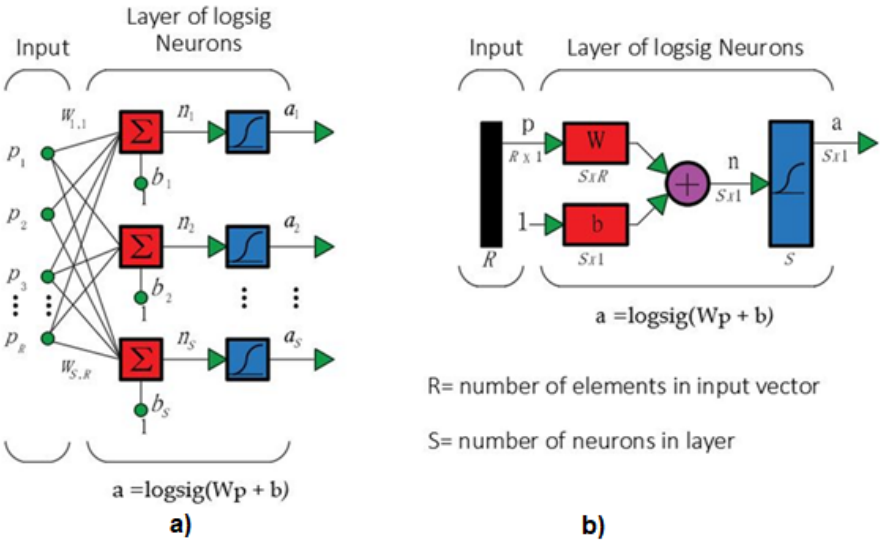

Figure 4- A single-layer network of *S* logsig neurons with R inputs (Mathworks 2020a)

FNNs usually consist of hidden layers followed by an output layer. Multiple neuron layers provide the non-linear relationships between the input and output vectors of the network with non-linear transfer functions. The linear output layer is usually applied for non-linear regression or function fitting cases. If it is desired to limit the outputs of a network between 0 and 1, the sigmoid transfer function should be preferred in the output layer. With this situation, a decision is made by the network, or the network's pattern recognition problem is solved. The two-layer logsig/purelin network is presented in Figure 5 (Mathworks 2020a).

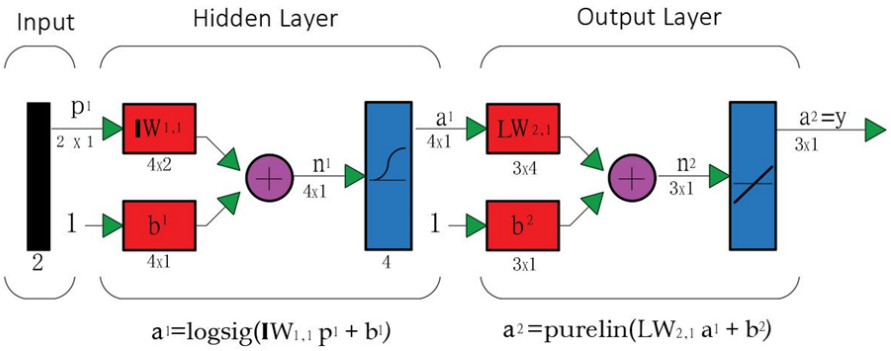

Figure 5- The two-layer logsig/purelin network (Mathworks 2020a)

2.2.2. Elman neural networks (ENN)

ENN consists of several layers and the additional set of connection units in an elementary FNN. A fixed weight value connects the hidden layer units and these context units. Both context units and input nodes activate the nodes in the hidden layer. As shown in Figure 6, content units are enabled to be activated with the feedback of hidden units. At time step, *t+1*, context units record a copy of the hidden unit values obtained at step *t*. The behavior of the context layer achieves the improvement of the dynamic information processing capacity of the network as local feedback (Mathworks 2020b).

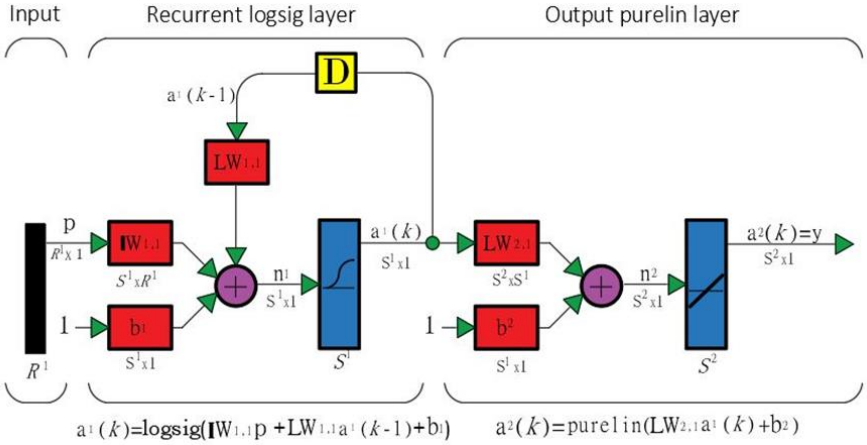

Figure 6- Structure of a two-layer Elman neural networks (Mathworks, 2020b)

Feedback is usually provided from the first layer output to the first layer input in a two-layer ENN. Thanks to this recurrent connection, the Elman network both detects and produces patterns that change over time. Logsig neurons and purelin neurons can be utilized in the recurrent or hidden and output layers of the ENN, respectively. This approach is special because two-layer networks with these transfer functions can estimate any function with random accuracy. However, the number of hidden layers must have sufficient neurons. The greater the complexity of the function available, the more hidden neurons are required. ENN differs from traditional two-layer networks in that the first layer has a recurrent connection. The delay in this connection reserves the values from the previous time step available in the current time step. Therefore, even if the same inputs are supplied to two ENN with the same biases and weights at a given time step, their outputs may differ due to different feedback situations. The network can learn spatial patterns and temporal patterns to store knowledge for future reference (Mathworks 2020b).

2.3. Adaptive neuro fuzzy inference system (ANFIS)

An ANFIS model combines two statistical systems: The Fuzzy Inference System (FIS) and the ANN. Figure 7 shows Type-3 fuzzy reasoning and corresponding equivalent ANFIS architecture (type-3 ANFIS), respectively. A circle describes a fixed node in the structure, while a square denotes an adaptive node. As a simple structure, *x* and *y* inputs and *f* output can be considered. The Sugeno model type is the most extensively applied fuzzy model found in the related works (Jang 1993; Karakuş et al. 2017; Tabari et al. 2012).

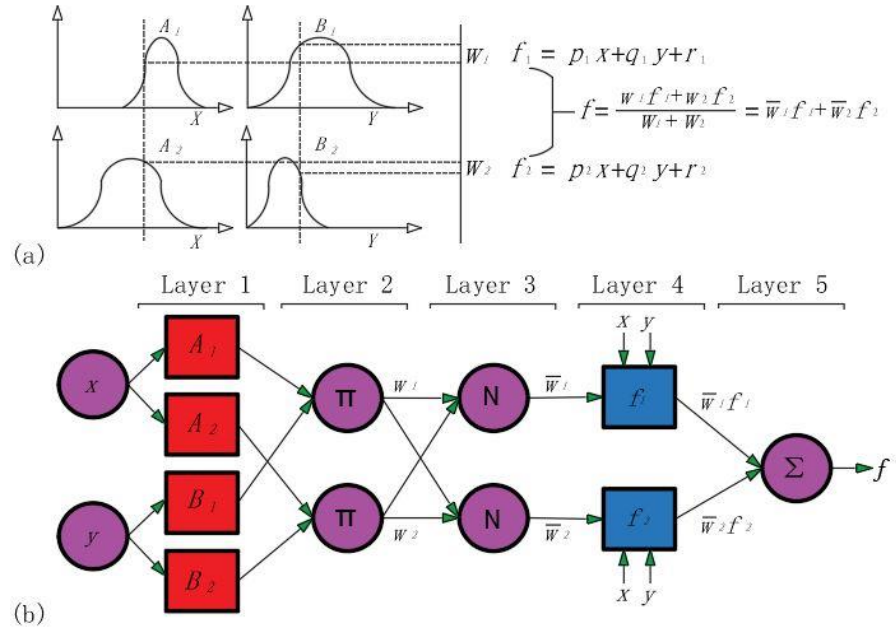

Figure 7- (a) First-order Takagi-Sugeno fuzzy model (b) equivalent ANFIS (Type-3 ANFIS) (Jang 1993)

In general, an ANFIS model contains two phases: **c**onstruction and training. Membership functions' types and numbers are determined during the construction phase, and the input and output data are divided into rule patches. Therefore, clustering methods are employed for understanding and classifying inputs facilitating the training phase with the help of the ANFIS model. Three clustering approaches comprising the methods of subtractive clustering (SC), fuzzy c-means (FCM), and grid partitioning (GP) are used for this purpose. Fuzzy c-means (FCM) is a clustering method allowing each data point to have multiple clusters and belong to different degrees of membership. The Subtractive Clustering (SC) algorithm considers each data point a candidate cluster center, and the potential of each data point is calculated by measuring the density of the data point surrounding the cluster center. The algorithm uses an iterative process, assuming each point is potentially a cluster center considering their location for other data points (Benmouiza & Cheknane 2019). Grid partitioning (GP) algorithm divides the input data space into a rectangular subspace with the help of an axis-paralleled partition. Each input is split into identically shaped membership functions. The grid is created without using any physical meaning or data density repartition. According to system input-output training data, fuzzy rules are generated using each grid part, thus achieving rapid learning and optimized calculation time. However, the size of the inputs and grid considerably affects the method's performance. A finer grid usually yields higher performance**,** as expected. The size and location of the fuzzy grid regions can be optimized using adaptive grid partitioning (Benmouiza & Cheknane 2019). Further information regarding ANFIS may be obtained from the work of Jang (1993).

2.4. Long short-term memory (LSTM) neural network

LSTM neural network is a type of Recurrent Neural Network that addresses problems by adding memory cells with persistent errors. This way, errors can be regenerated without disappearing gradients. Three different gates are present in the LSTM neural network. An input gate learns to preserve the persistent error flow in the memory cell from irrelevant inputs. An output gate learns to protect other units from unrelated memory content saved in the memory cell. A forget gate teaches how long the value is in the memory cell (Hochreiter & Schmidhuber 1997; Piotrowski et al. 2015; Salman et al. 2018; Zahroh et al. 2019; Cai et al. 2020; Cho et al. 2020).

Figure 8 shows the LSTM layer architecture, indicating the flow of an *X* time series with *S*-length *C* properties (channels) across an LSTM layer. In this architecture diagram, h_t is also known as the hidden element and is the output. c_t is the cell state at time step *t*. (Mathworks 2020c).

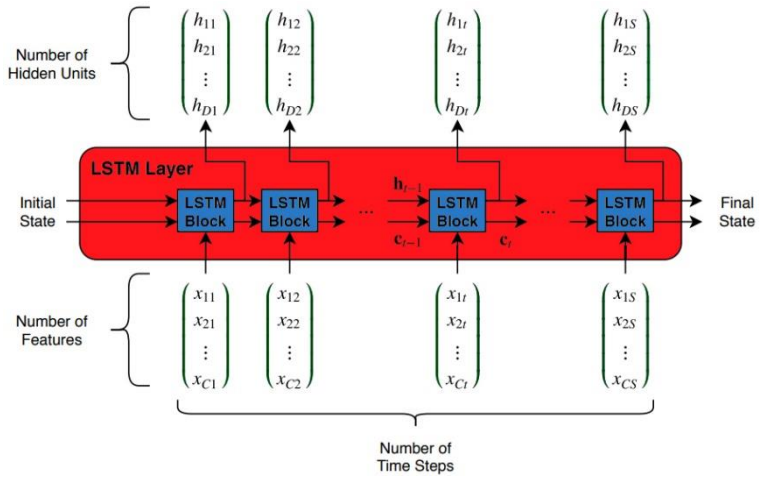

Figure 8- LSTM layer architecture (Mathworks 2020c)

Figure 9 presents the flow of data at time step *t*. This figure indicates how the gates forget, update, and output the cell and the hidden states. Some components in the LSTM layer architecture are used to control the cell state and the hidden state of the layer. For example, input gate (*i*) and output gate (*o*) control the cell state update and level of cell state added to the hidden state, respectively. Besides, the forget gate (*f*) checks the level of cell state reset (forget). On the other hand, cell candidate (*g*) adds the information to the cell state. Further information regarding LSTM can be obtained from Mathworks (2020c) study.

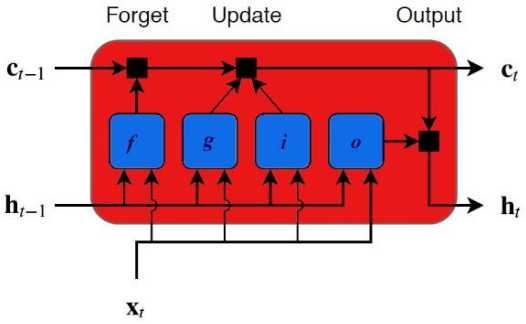

Figure 9- The flow of data at time step *t* (Mathworks 2020c)

2.5. Statistical parameters

In this study, four statistical error parameters such as mean absolute error (MAE), root mean square error (RMSE), Nash−Sutcliffe efficiency coefficient (NSE), and correlation coefficient (R) are used for the assessment of the accuracy of the models in forecasting the observed output variable. MAE, RMSE, NSE, and R parameters, respectively, are expressed as follows (Başakın et al. 2021; Citakoglu 2021):

$$MAE = \frac{1}{N}\sum_{i=1}^{N}|p(i) - o(i)| \quad (1)$$

$$RMSE = \sqrt{\frac{1}{N}\sum_{i=1}^{N}[p(i) - o(i)]^2} \quad (2)$$

$$NSE = 1 - \frac{\sum_{i=1}^{N}[o(i) - p(i)]^2}{\sum_{i=1}^{N}[o(i) - \bar{o}]^2} \quad (3)$$

$$R = \left(\sum_{i=1}^{N}[p(i) - \bar{p}][o(i) - \bar{o}]\right) / \left(\sqrt{\sum_{i=1}^{N}[p(i) - \bar{p}]^2}\sqrt{\sum_{i=1}^{N}[o(i) - \bar{o}]^2}\right) \quad (4)$$

Where: *p(i)* and *o(i)* are the predicted value and observed value at the time *i*, respectively; $\bar{p}$ and $\bar{o}$ are the means of the predicted values and observed values, respectively, and the total number of data is represented by N.

3. Results and Discussion

In this study, a time-series analysis was applied to predict one-day ahead ST. This is a technique for using time series data values to predict future values based on our historical data points. The proposed time series method is the univariate modeling based on time series data for the modeled variable. The most important advantage of univariate modeling is that there is no need to obtain independent variables. It is known that time-series tools may capture the stochastic component of the time series data, besides machine learning tools may forecast the determinative part of the time series data. In this respect, the used ANFIS-FCM, ANFIS-GP, ANFIS-SC, ENN, FNN, and LSTM techniques were developed based on past observations of the ST parameter as an input to train the model and to predict future values.

The performances of six machine learning techniques, namely, FNN, ENN, LSTM, ANFIS-SC, ANFIS-FCM, and ANFIS-GP, were investigated based on one-day ahead ST forecasting at three different depths of 5 cm, 50 cm, and 100 cm. The trial and error method determined the optimal parameters in each method. The results of the error criteria for determining the optimal parameters in each method by trial and error method are given in Table 2. In order to deal with over-training, a known disadvantage in machine learning models, the data sets were categorized into training and testing. All models were calibrated based on the training data, and then their performances were evaluated using the testing data, which did not take part in model training. Figure 10 represents measured and forecasted ST variations by six machine learning methods at the testing phase. It is clear from Figure 10 that daily ST variations at 5 cm depth show abrupt changes. The general overview of the model results in the central figure of Figure 10 reveals that almost all models overlap with the measured data; however, the zoomed images explain the differences more clearly. The zoomed images show that ANFIS-GP (blue dashed line) explicitly differs from the other methods.

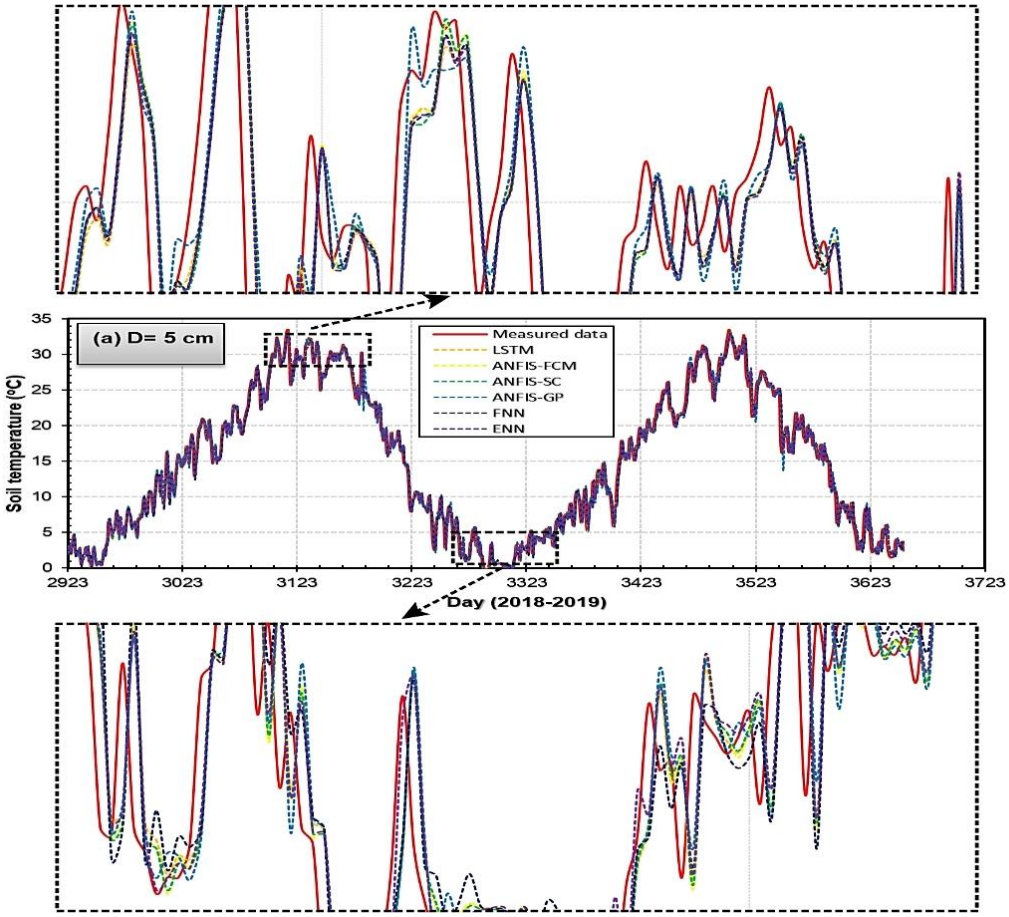

Figure 10- Illustration of the measured and forecasted ST by machine learning models at 5 cm depth

Table 2- The results of the error criteria for determining the optimal parameters in each method by trial and error method

Method	*Depth (cm)*	*HLN**	*MAE (ºC)*	*RMSE (ºC)*	*R*	*Method*	*Depth (cm)*	*HLN**	*MAE (ºC)*	*RMSE (ºC)*	*R*
LSTM	5	5	1.0145	1.3391	0.9908	FNN	5	6	1.0088	1.3308	0.9909
		10	1.0012	1.3292	0.9910			7	1.0078	1.3308	0.9910
		15	**0.9978**	**1.3256**	**0.9910**			**8**	**1.0065**	**1.3281**	**0.9910**
		20	1.0039	1.3292	0.9910			9	1.0136	1.3340	0.9909
		25	1.0039	1.3310	0.9910			10	1.0151	1.3366	0.9909
	50	**5**	**0.1783**	**0.2251**	**0.9995**		50	6	0.1087	0.1395	0.9998
		10	0.1838	0.2331	0.9995			7	0.1090	0.1396	0.9998
		15	0.1900	0.2400	0.9995			**8**	**0.1081**	**0.1389**	**0.9998**
		20	0.2065	0.2645	0.9994			9	0.1085	0.1391	0.9998
		25	0.2009	0.2532	0.9994			10	0.1105	0.1418	0.9998
	100	5	0.0809	0.1016	0.9998		100	6	0.0523	0.0633	0.9999
		10	0.0838	0.1060	0.9998			7	0.0521	0.0635	0.9999
		15	**0.0773**	**0.0983**	**0.9998**			**8**	**0.0519**	**0.0635**	**0.9999**
		20	0.0885	0.1123	0.9998			9	0.0523	0.0635	0.9999
		25	0.0887	0.1103	0.9998			10	0.0520	0.0638	0.9999
ANFIS-FCM	5	**2**	**1.0036**	**1.3276**	**0.9910**	ENN	5	6	1.0087	1.3300	0.9909
		4	1.0111	1.3334	0.9909			7	1.0074	1.3241	0.9910
		6	1.0161	1.3377	0.9909			**8**	**0.9967**	**1.3186**	**0.9911**
		8	1.0097	1.3365	0.9909			9	1.0368	1.3481	0.9907
		10	1.0132	1.3476	0.9907			10	1.0105	1.3305	0.9909
	50	**5**	**0.1087**	**0.1388**	**0.9998**		50	6	0.1167	0.1471	0.9998
		10	0.1097	0.1403	0.9998			7	0.1101	0.1406	0.9998
		15	0.1108	0.1415	0.9998			**8**	**0.1092**	**0.1409**	**0.9998**
		20	0.1106	0.1416	0.9998			9	0.1097	0.1405	0.9998
		25	0.1113	0.1431	0.9998			10	0.1099	0.1402	0.9998
	100	**5**	**0.0525**	**0.0637**	**0.9999**		100	6	0.0526	0.0636	0.9999
		10	0.0528	0.0642	0.9999			7	0.0525	0.0635	0.9999
		15	0.0530	0.0647	0.9999			**8**	**0.0521**	**0.0635**	**0.9999**
		20	0.0531	0.0649	0.9999			9	0.0523	0.0636	0.9999
		25	0.0529	0.0649	0.9999			10	0.0529	0.0641	0.9999
ANFIS-SC	5	0.1	1.0124	1.3331	0.9909	ANFIS-GP	5	**2**	**1.0407**	**1.3809**	**0.9903**
		0.3	1.0132	1.3354	0.9909			3	1.1600	1.6971	0.9853
		0.5	1.0101	1.3327	0.9909		50	**2**	**0.1106**	**0.1408**	**0.9998**
		0.7	1.0045	1.3294	0.9910			3	0.1120	0.1438	0.9998
		0.9	**1.0036**	**1.3280**	**0.9910**		100	**2**	**0.0517**	**0.0634**	**0.9999**
	50	0.1	0.1135	0.1470	0.9998			3	0.0520	0.0638	0.9999
		0.3	0.1109	0.1417	0.9998						
		0.5	0.1096	0.1398	0.9998						
		0.7	**0.1083**	**0.1387**	**0.9998**						
		0.9	0.1087	0.1389	0.9998						
	100	0.1	0.0539	0.0661	0.9999						
		0.3	0.0528	0.0648	0.9999						
		0.5	0.0528	0.0644	0.9999						
		0.7	0.0529	0.0645	0.9999						
		0.9	**0.0528**	**0.0643**	**0.9999**						

** HLN: Hidden layer number / Number of MFs / Influence radius / Number of neurons in the hidden layer*

Concerning the visual interpretation of the daily ST forecasting at 50 cm depth (Figure 11), the temporal variations in ST at 50 cm depth are not as dynamic as the ST at 5 cm depth. As observed in Figure 10, the general overview of the model results in the central figure of Figure 11, showing that almost all models are in good agreement with the measured data; however, the zoomed images reveal the differences between the models. The zoomed images show that LSTM (orange dashed line) explicitly differs from the other methods. On the other hand, all other methods present the same results with minor differences at breaking points. As in the 50 cm ST results, similar trends were observed in ST at 100 cm depth (Figure 12), presenting that the LSTM results were different from all other methods, while the other methods acted almost in the same manner. These results also indicate that the temporal variations in ST decrease with the increasing depth, enabling the methods to provide higher accuracy with better modeling capacities.

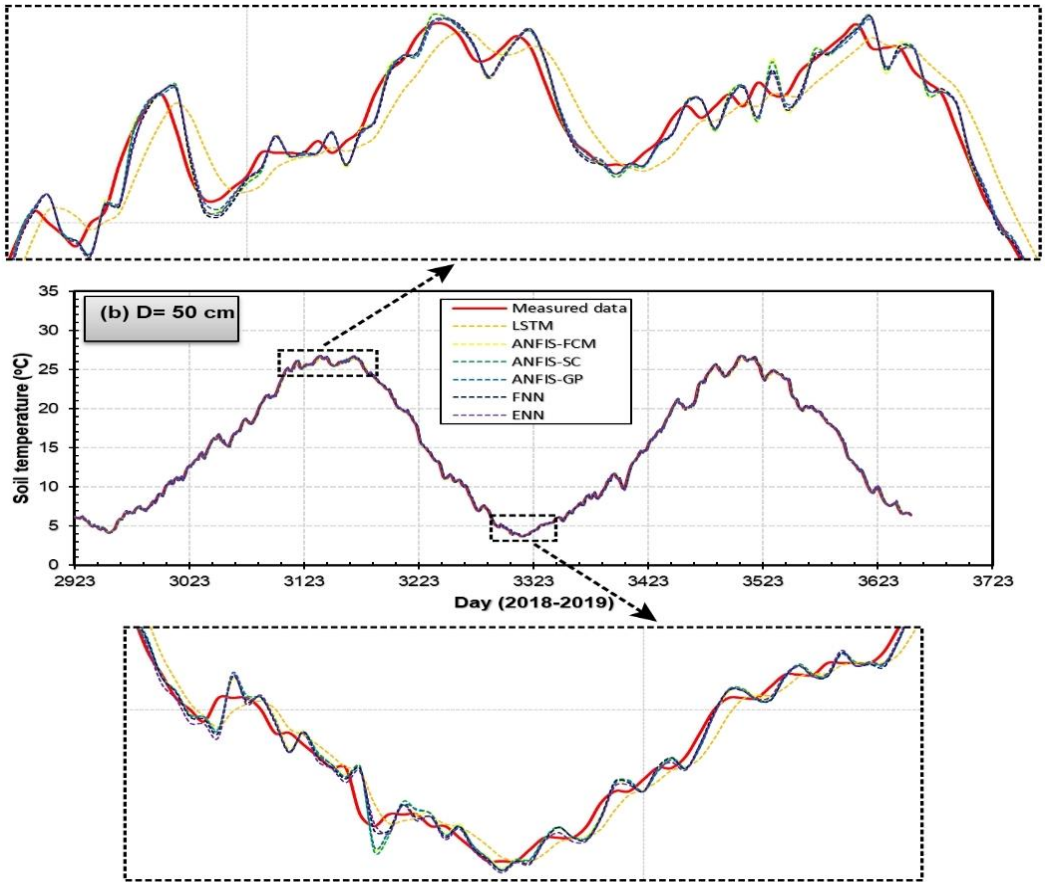

Figure 11- Illustration of the measured and forecasted ST by machine learning models at 50 cm depth.

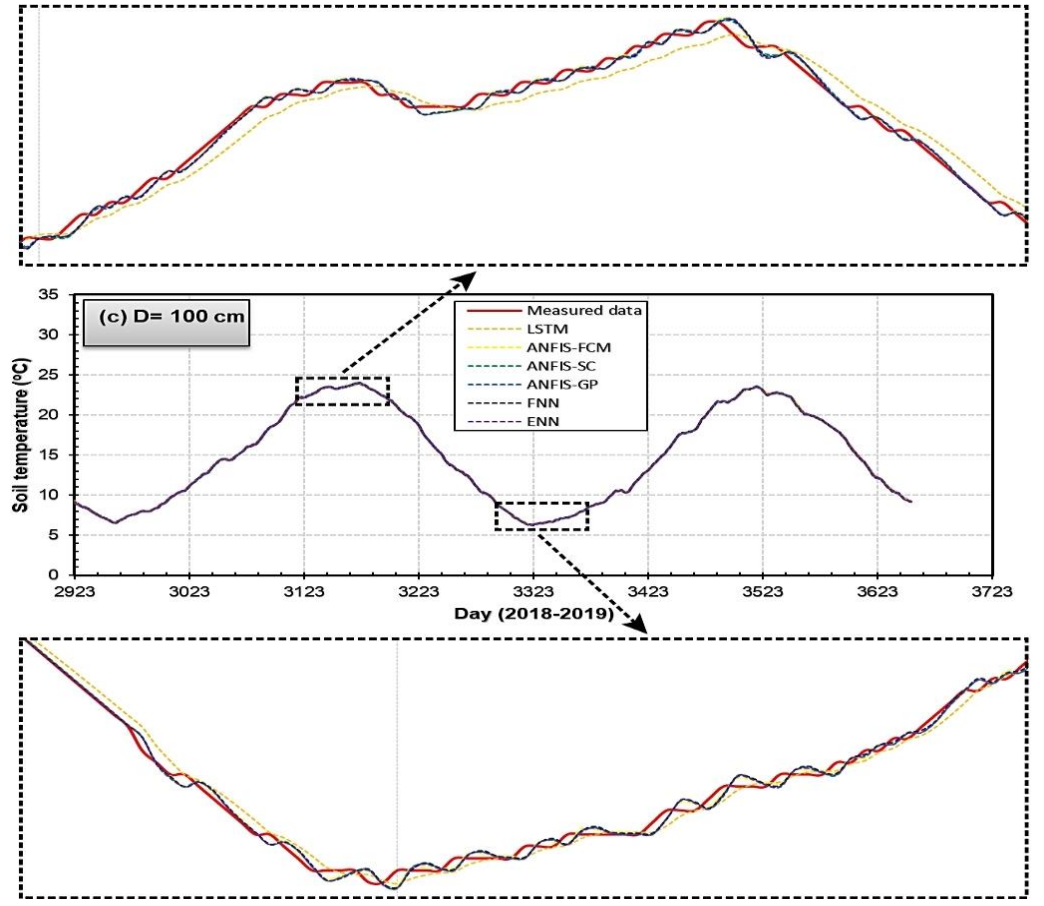

Figure 12- Illustration of the measured and forecasted ST by machine learning models at 100 cm depth.

In addition to the visual results above for forecasting the ST, Figures13-15 represent the regression analyses of the measured and forecasted ST data at 5 cm, 50 cm, and 100 cm, respectively. In the figures, X-axis presents measured ST values, while Y-axis corresponds to forecasted ST in °C. These figures provide the distribution of the measured and forecasted values. Concerning R for the ST forecasting at 5 cm depth (Figure 13 and Table 3), LSTM, ANFIS-FCM, ANFIS-SC, ANFIS-GP, FNN, and ENN presented 0.9910, 0.9910, 0.9910, 0.9903, 0.9910, and 0.9911. Although all results are satisfactory, ENN provided slightly better results than the other 5 cm ST forecasting methods. It is clear from Figures 14 and 15 that the regression plots show that all methods forecasted the ST at 50 cm and 100 cm depth at a higher level than the ST at 5 cm depth due to high temporal variations in ST. The highest R values for the ST forecasting at 50 cm and 100 cm depth were provided by the FNN and ANFIS-GP with the values of 0.9998 and 0.9999, respectively (Figures 14-15 and Table 3).

Table 3- Statistical accuracy results of the ST forecasting with six machine learning methods at various depths.

Depth (cm)	*Method*	*MAE (ºC)*	*RMSE (ºC)*	*R*	*NSE*
5	LSTM	0.9978	1.3256	0.9910	0.9822
	ANFIS-SC	1.0036	1.3280	0.9910	0.9821
	ANFIS-FCM	1.0036	1.3276	0.9910	0.9821
	ANFIS-GP	1.0407	1.3809	0.9903	0.9807
	FNN	1.0065	1.3281	0.9910	0.9821
	ENN	**0.9967**	**1.3186**	**0.9911**	**0.9824**
50	LSTM	0.1783	0.2251	0.9995	0.9991
	ANFIS-SC	0.1083	0.1387	0.9998	0.9997
	ANFIS-FCM	**0.1087**	**0.1388**	**0.9998**	**0.9996**
	ANFIS-GP	0.1106	0.1408	0.9998	0.9996
	FNN	0.1081	0.1389	0.9998	0.9996
	ENN	0.1092	0.1409	0.9998	0.9996
100	LSTM	0.0773	0.0981	0.9998	0.9997
	ANFIS-SC	0.0528	0.0643	0.9999	0.9999
	ANFIS-FCM	0.0525	0.0637	0.9999	0.9999
	ANFIS-GP	**0.0517**	**0.0634**	**0.9999**	**0.9999**
	FNN	0.0519	0.0635	0.9999	0.9999
	ENN	0.0521	0.0635	0.9999	0.9999

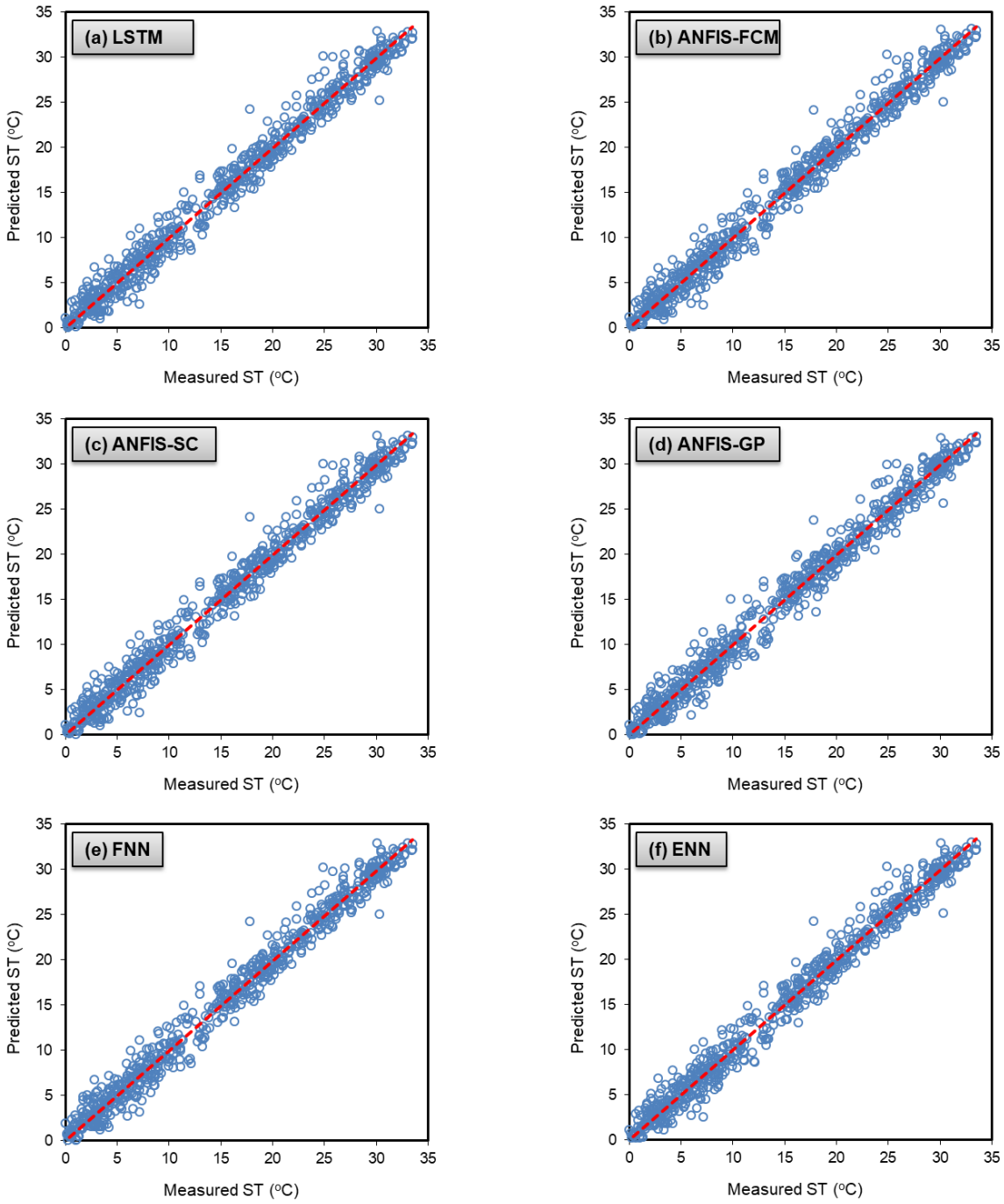

Figure 13- Regression analyses of the measured and predicted data of ST at 5 cm depth with various methods: a) LSTM, b) ANFIS-FCM, c) ANFIS-SC, d) ANFIS-GP, e) FNN, f) ENN methods.

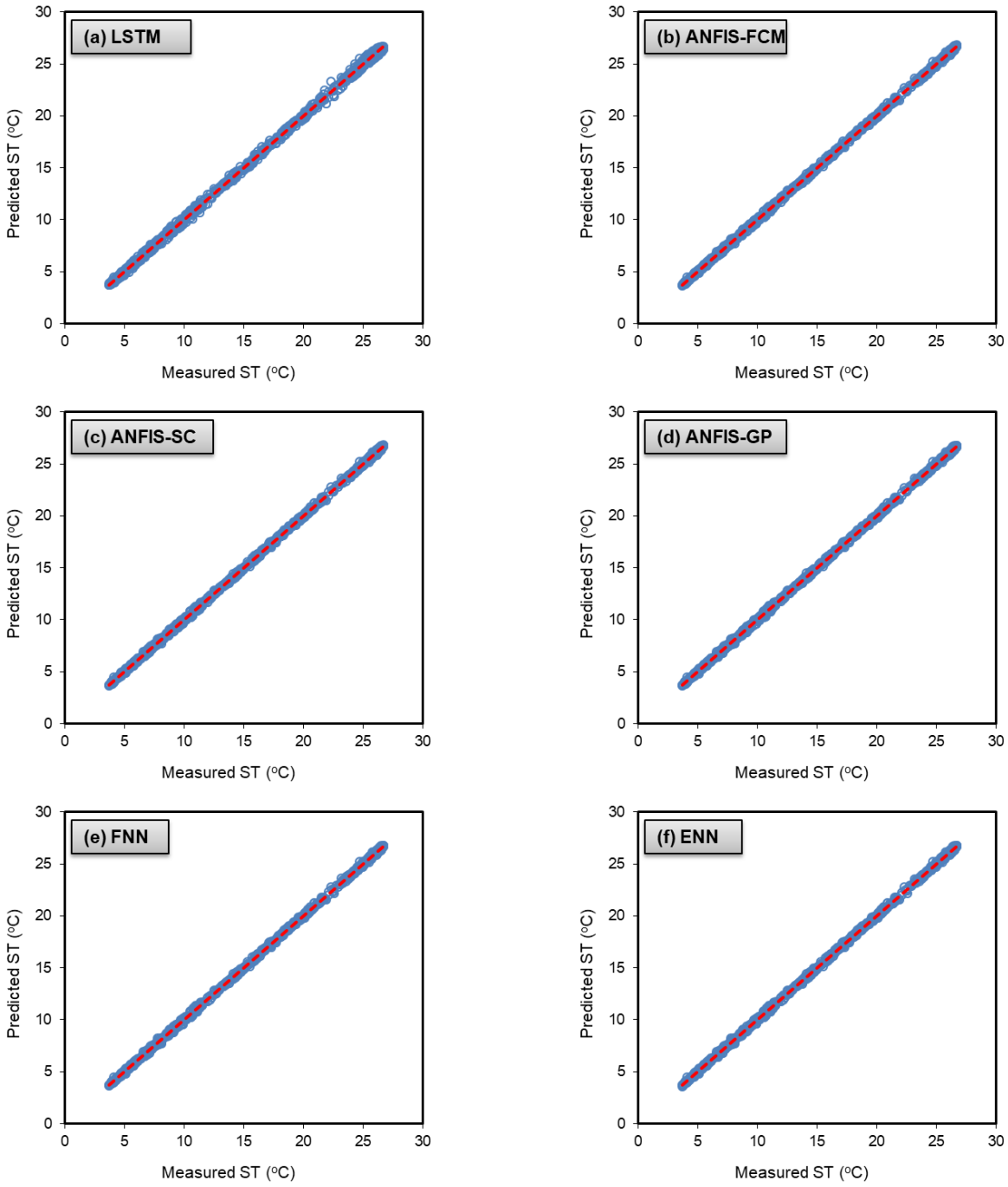

Figure 14- Regression analyses of the measured and predicted data of ST at 50 cm depth with various methods: a) LSTM, b) ANFIS-FCM, c) ANFIS-SC, d) ANFIS-GP, e) FNN, f) ENN methods.

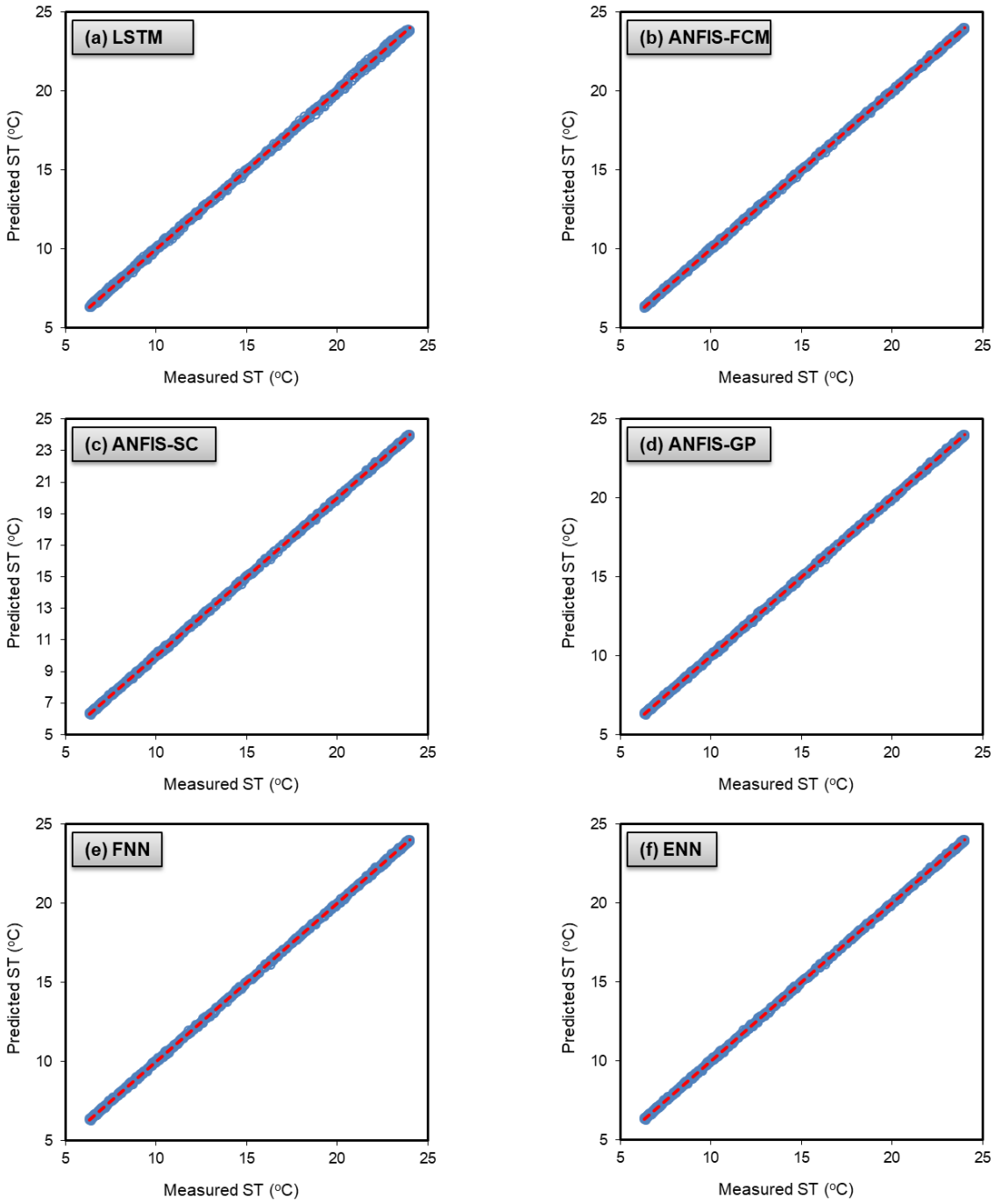

Figure 15- Regression analyses of the measured and predicted data of ST at 100 cm depth with various methods: a) LSTM, b) ANFIS-FCM, c) ANFIS-SC, d) ANFIS-GP, e) FNN, f) ENN methods.

In summary, Table 3 highlights the statistical accuracy results of the ST forecasting with six machine learning methods at various depths. It is clear from the table that every method provided satisfactory results at each depth compared to each other**.** However, we would like to demonstrate which method will give the best results, even with small differences. Considering the ST forecasting at 5 cm depth, ENN provided the best results with the statistical metrics of 0.9967 ºC MAE, 1.3186 °C RMSE and 0.9824 NSE. On the other hand, the second-best method was the LSTM with 0.9978 ºC MAE, 1.3256 ºC RMSE, and 0.9822 NSE, and also the ANFIS-GP offered the worst results with 1.0407 °C MAE, 1.3809 ºC RMSE, and 0.9807 NSE. For ST forecasting at 50 cm depth, the performance of the methods from the best to the worst can be listed as FNN, ANFIS-SC, ANFIS-FCM, ENN, ANFIS-GP, and LSTM, respectively. While the FNN presented the best results with 0.1081 °C MAE, 0.1389 °C RMSE, and 0.9996 NSE, the LSTM was the worst method with 0.1783 °C MAE, 0.2251 °C RMSE, and 0.9991 NSE in forecasting the ST at 50 cm depth. Concerning the ST forecasting at 100 cm depth, the effectiveness of the methods from the best to the worst, respectively, were ANFIS-GP, FNN, ENN, ANFIS-FCM, ANFIS-SC, and LSTM. The ANFIS-GP provided the best accuracy results as 0.0517 °C MAE, 0.0634 °C RMSE, and 0.9999 NSE, whereas the worst results obtained from the LSTM were 0.0773 ºC MAE, 0.0983 °C RMSE, and 0.9997 NSE. Although the LSTM was effective in forecasting the ST at 5 cm depth, this performance was not the same as in forecasting the ST at 50 cm and 100 cm depths, which reveals it works better

in complex environments. Another important point of the performance results is that the optimal parameters of each method were determined by trial and error, which may affect the accuracy results, but the study was already organized under this assumption. Even though we listed the best and the worst methods based on the statistical accuracy metrics for every depth, in general, all methods provided identical and satisfactory results with slight differences. The RMSE results at 5 cm, 50 cm, and 100 cm depths varied from 1.3186 °C to 1.3809 °C, from 0.1388 °C to 0.2251 °C, and from 0.0634 °C to 0.0983 °C, respectively.

Taylor diagram based on statistical analysis was utilized while comparing six models in order to assess the consistency of the predicted data from the measured data. Thus, further comparisons of all models were provided using the Taylor diagram. Figure 16 shows the Taylor diagrams for the ST variable of LSTM, ANFIS-FCM, ANFIS-SC, ANFIS-GP, FNN, and ENN methods. As can be seen from the figure, it is understood that the 6 models used in the modeling of ST data at 5, 50, and 100 cm depths are similar to each other, the data lines overlap and it is difficult to separate from each other. However, it is clear that the closest results to the measurement values are obtained at a depth of 100 cm.

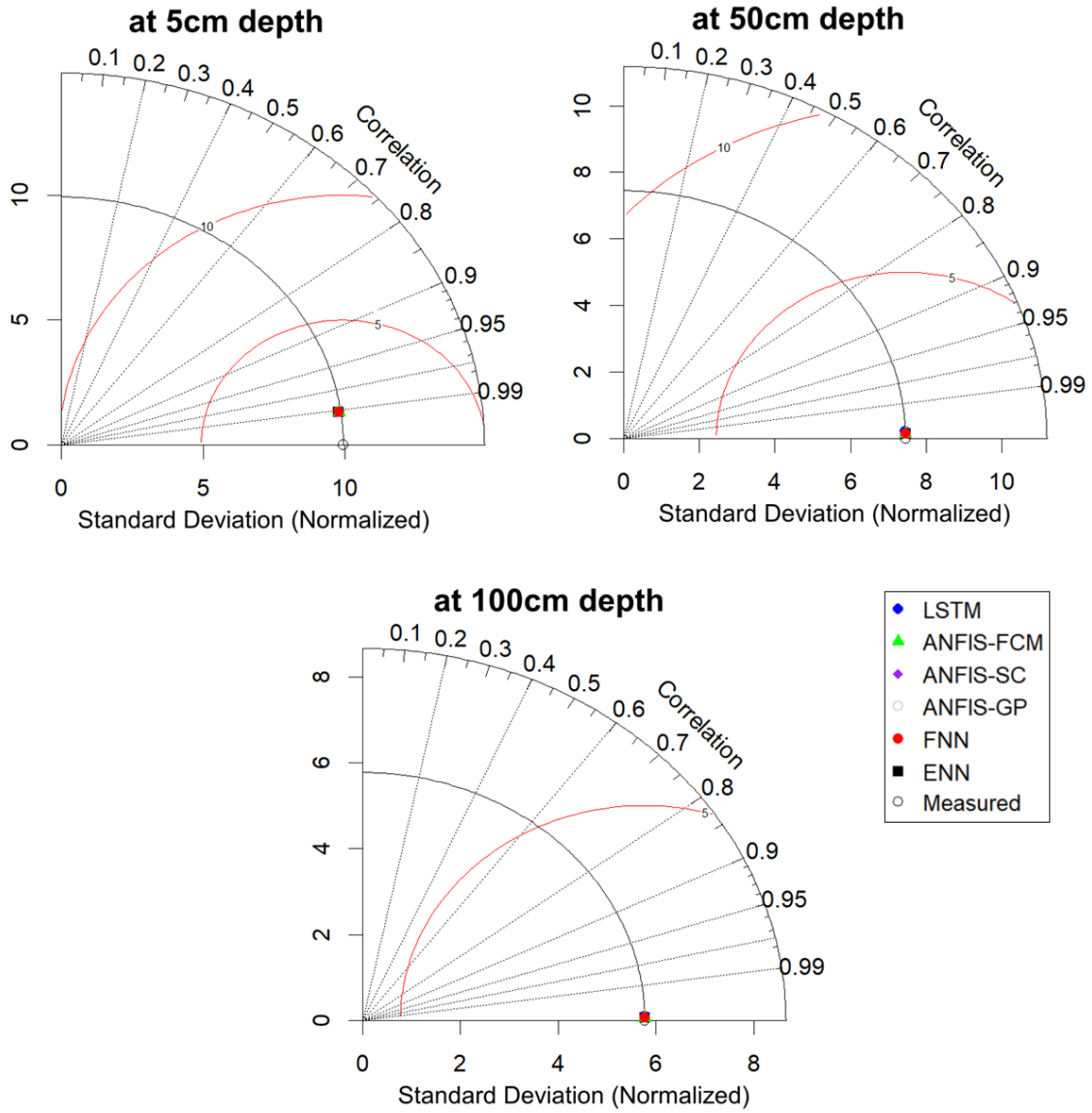

Figure 16- Taylor diagrams for ST variable of LSTM, ANFIS-FCM, ANFIS-SC, ANFIS-GP, FNN, and ENN methods.

Additionally, a comparison with previous studies was carried out to verify the accuracy of our results and models. The literature review showed that previously published studies mainly applied machine learning models using some meteorological parameters to predict ST. However, few studies have been reported in the literature for ST prediction using time series-based models. According to this, the prediction results of the proposed models are compared with the results from some published studies based on daily ST estimates in Table 4. The results showed that the models proposed in this study predicts daily ST with close accuracy to the models in other studies, and even more accurately than most. In summary, the results in this table show that the proposed time series forecasting models have been successfully applied for one-day ahead forecasting of ST.

Table 4- Summary of typical studies on daily ST forecasting

Reference	Country	Data	Method	Depth (cm)	Error criteria	
					R	RMSE (ºC)
Singhal et al. (2021)	India	Daily (2016 - 2017)	ANN	10	0.9560	0.5520
				20	0.9840	0.2950
				45	0.9740	0.3980
Hao et al. (2021)	Switzerland	Daily (2004 - 2014)	EEMD-CNN	5	0.9970	0.4660
				10	0.9980	0.3750
				25	0.9990	0.2760
Zeynoddin et al. (2019)	Iran	Daily (2013 - 2015)	Linear stochastic method	5	0.9953	1.3300
				10	0.9971	0.9800
				20	0.9983	0.7000
Yu et al. (2021)	China	Daily (2012 - 2020)	EEMD-Conv3d	0-7	0.9946	1.3096
Mehdizadeh et al. (2020b)	Iran	Daily (1998 - 2017)	GEP-FARIMA	5	-	0.2400
				10	-	0.1300
				50	-	0.0500
				100	-	0.1600
This study	Turkey	Daily (2010 - 2019)	ENN	5	0.9900	1.3186
			ANFIS-FCM	50	0.9988	0.1388
			ANFIS-GP	100	0.9999	0.0634

4. Conclusions

In this study, the performances of the six machine learning methods, namely, LSTM, ANFIS-SC, ANFIS-FCM, ANFIS-GP, FNN, and ENN, were evaluated based on one-day ahead ST forecasting at three different depths (5 cm, 50 cm, and 100 cm). The ST data at all depths cover the 10 years, and for all methods, the training and testing data sets were split into 80% and 20%, respectively, based on the whole data set. Concerning the data structure at different depths, it was observed that the daily temporal variation in ST increases with the decreasing depth. In other words, the daily ST at 5 cm depth shows more changes compared to the ST at 50 cm depth, and the variations in daily ST at 50 cm depth are higher than the ST at 100 cm depth. Considering the responses of the machine learning methods to these variations at different depths, it was proved that the methods provided high accuracies when the depth of the daily ST increased**,** such as from 5 cm to 100 cm. The visual and statistical results revealed that all methods presented satisfactory forecasting results at each depth**,** with only slight differences. The best performances in forecasting the daily ST at 5 cm, 50 cm, and 100 cm depths were obtained from ENN, FNN, and ANFIS-GP. In addition, the worst accuracies for the same depths were provided by the ANFIS-GP, LSTM, and LSTM, respectively. Even though LSTM was the second-best method in forecasting the ST at 5 cm depth, it did not respond well at 50 cm and 100 cm depths, which shows that it is good at forecasting the ST in complex environments. Overall, the obtained results indicate that all methods used in this study can be performed for the daily ST forecasting at different depths.

Acknowledgments

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Genetic Diversity of Cherry Laurel (*Laurocerasus officinalis* Roemer) BY SSR Markers

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ABSTRACT

Cherry laurel (*Laurocerasus officinalis*) belongs to the Rosacea family. The main distribution area for edible cherry laurels is the Blacksea shores in Turkey. In the study, it was aimed to reveal the differences among the various cherry laurel genotypes by using the SSR molecular marker technique. Cherry laurel genotypes were selected from the Black Sea Region of Turkey. A total of 15 SSR primer pairs were developed and used for *Prunus* species, and the phylogenetic relationship and polymorphism rates were also demonstrated. As a result, 13 SSR primers resulted in scorable DNA band profiles. UDAp-401 SSR primer was detected with a minimum of 3 alleles and BBCT001 primer with a maximum of 17 alleles. The average number of alleles was observed at 9 per locus. Whereas, the average number of polymorphic bands per SSR marker was calculated as 8.38. Additionally, 109 polymorphic DNA profiles were obtained from a total of 117, and the polymorphism rate was calculated as 93.5%. The band patterns resulting from SSR analysis showed multiple alleles, suggesting polyploidy in cherry laurel. In conclusion, we determined that the SSR molecular markers could be used to identify the different cherry laurel genotypes. Furthermore, these results depicted that among the different genotypes sampled there is significant genetic variability that can be useful for future research and breeding programs.

Keywords: *Prunus laurocerasus* L, DNA, Polymorphism, primer

1. Introduction

The Rosaceae family's cherry laurel (*Laurocerasus officinalis* L.) is a small tree or also known as an evergreen shrub. Different cherry laurel species or genotypes are grown naturally and used as a fruit and ornamental plants. It grows as a single tree in many places in the Blacksea Region. Cherry laurel cultivated tree's height can range from 5 to 10 m. These plants may differ from each other in terms of leaf size and shape, fruit colour, size and taste (İslam & Vardal 2006).

Cherry laurel might differ in terms of leaves, flower, fruit color and taste. The leaves of the cultivated types are larger and some types are narrow and long. White flowers collect in an upright cluster on an axis of 5 - 15 cm. Each flower has 5 sepals, 5 petals, 1 female and 15-20 male organs (İslam et al. 2020; Turna & Güney 2006). The people consumed it as table fruit, because of its taste & healthy properties of this unique fruit (İslam 2002; Halilova & Ercisli 2010; İslam et al. 2010; Tarakci et al. 2013; Temiz et al. 2014; Eser et al. 2014). The fruits are consumed as fresh after ripening. In addition, its fruits are made into jam, molasses and pickles. It is also used in the pharmaceutical industry (Guven & Geçgil 1961; Islam 2008; Eser et al. 2014). Since, it is an evergreen tree, it isused as an ornamental plant in parks and gardens in floriculture, and as a windbreaker for orchards and houses (İslam & Deligöz 2012).

In recent years, some morphological, biochemical, and molecular studies have been carried on cherry laurel. Different types of molecular markers like RAPD (Aka Kaçar 2001; Sandallı 2002; Aksu et al. 2012; Yılmaz et al. 2012; Pınar et al. 2018), and SSR (Cipriani et al. 1999; Downey & Lezzoni 2000; Aka Kaçar et al. 2005; Kaçar et al. 2006; Wünsch 2009; Türkoğlu at al. 2010; Ercişli et al. 2011; Stanys et al. 2012; Hajyzadeh et al. 2013; Köse 2013) have been used to characterize the genetic diversity in cherry laurel.

Microsatellite markers, known as Simple Sequence Repeats (SSRs), are co-dominant and stable markers, demonstrates high polymorphism, repeatable and suitable for automation. SSRs have a cross-species feature and are an informative marker system.

(Weber & May 1989; Yamamoto et al. 2001; Wünsch & Hormaza 2002). The present study aimed to investigate the genetic diversity of cherry laurel genotypes growing naturally in the regions of the Black Sea using SSR markers.

2. Material and Methods

2.1. Material

A total of 43 cherry laurel genotypes were used as plant materials (Table 1). The plant materials were selected from the Blacksea Region in Turkey considering the morphological, phenological and fruit characteristics with the support of the TUBITAK project (No:107O252) in 2007-2010 and planted at Ordu University, Faculty of Agriculture, Research Field (Figure 1).

Figure 1- Cherry laurel orchard in Ordu University Faculty of Agriculture, Research Field

Table 1- The accession list of cherry laurel used in the present study

Code Numbers	*Genotypes*	*Sampling site*	*Code Numbers*	*Genotypes*	*Sampling site*
1	R126	Rize	23	R137	Rize
2	R135	Rize	24	R19	Rize
3	T214	Trabzon	25	G40	Giresun
4	T203	Trabzon	26	R25	Rize
5	R27	Rize	27	R149	Rize
6	A19	Artvin	28	S24	Samsun
7	R20	Rize	29	S37	Samsun
8	O44	Ordu	30	T193	Trabzon
9	R24	Rize	31	T219	Trabzon
10	A4	Artvin	32	T217	Trabzon
11	T87	Trabzon	33	S16	Samsun
12	A14	Artvin	34	T94	Trabzon
13	O20	Ordu	35	R142	Rize
14	R27x	Rize	36	S51	Samsun
15	R5	Rize	37	R24X	Rize
16	O26	Ordu	38	T159	Trabzon
17	O29	Ordu	39	O27	Ordu
18	T303	Trabzon	40	T216	Trabzon
19	Keller	Trabzon	41	S3	Samsun
20	Sarı	Trabzon	42	A23	Artvin
21	S25	Samsun	43	R14	Rize
22	S21	Samsun			

2.2. Method

The research was carried out in Plant Biotechnology Laboratory, Department of Horticulture, Çukurova University, Adana, Turkey

2.2.1. DNA isolation

Young leaves were collected from each accession, immediately frozen in liquid nitrogen (-196 ºC), and stored at –80 °C. Genomic DNA was extracted from the leaf samples following the protocol for minipreps using CTAB (Simsek et al., 2008). The DNA quality and quantity were measured using a NanoDrop ND 100 spectrophotometer (NanoDrop Technologies) and agarose gel electrophoresis.

2.2.2. PCR amplification and gel electrophoresis

A total of 15 SSR primers (PMS49, PceG25, PMS40, PMS67, PceGA34, UDP98-21, PMS2, PMS3, PceGA59, UDP96-005, UCD-CH17, UDAp-401, BBCT001, BBCT002 and BBCT005) were tested. Information about SSR primers were shown in Table 2. Amplification was conducted at a total volume of 20 µl (25 ng of genomic DNA, 1X PCR buffer, 0.02 mM of each dNTP, 2.5 µmol primer (forward + reverse) and 0.8 units of DNA Taq polymerase, 5 µl dd H_2O). The amplifications were as follows: 94 ºC /5 min, 35 cycles/94 ºC /1 min, 55 ºC /30 sec, 72 ºC /1 min, and a final extension at 72 ºC / 5 min. Blue stop solution (95% formamide, 25 mM EDTA, and 2% bromophenol blue) was added to each PCR reaction as well. The PCR products were denatured at 95 ºC for 3 min and resolved in denaturing gel containing 6.5% polyacrylamide (40:2 acrylamide/bisacrylamide), 8.4 gr urea, 10 mL ddH_2O, and 2 mL 10X TBE buffer. PCR products were run in a 4.300 DNA Analyzer (LI-COR). Parameters for each run were 1.5 h, 1.500 V, 40 W, 40 mA, and 45 ºC respectively.

Table 2- Information about SSR primers used in the present study

No	*SSR Marker*	*Primers Sequence*	*Tm (°C)*	*Type name*	*Bp*	*References*
1	PMS49	F: TCA CGA GCA AAA GTG TCT CTG	50	Cherry laurel	79-185	Hajyzadeh et al. 2013
		R: CAC TAA CAT CTC TCC CCT CCC		Cherry		Cantini et al. 2001
2	PceGA25	F: GCA ATT CGA GCT GTA TTT CAG ATG	49	Cherry laurel	141-198	Hajyzadeh et al. 2013
		R: CAG TTG GCG GCT ATC ATG TCT TAC		Cherry		Cantini et al. 2001
3	PMS40	F: TCA CTT TCG TCC ATT TTC CC	50	Cherry laurel	181-226	Hajyzadeh et al., 2013
		R: TCA TTT TGG TCT TTG AGC TCG		Cherry		Cantini et al. 2001
4	PMS67	F: AGT CTC TCA CAG TCA GTT TCT	48	Cherry laurel	144-191	Hajyzadeh et al. 2013
		R: TTA ACT TAA CCC CTC TCC CTC C		Cherry		Cantini et al. 2001
					149-161	Struss et al. 2003
5	PceGA34	F: GAA CAT GTG GTG TGC TGG TT	45	Cherry laurel	140-174	Hajyzadeh et al. 2013
		R: TCC ACT AGG AGG TGC AAA TG		Sour cherry		Downey & Lezzoni 2000
6	UDP98-021	F: AAG CAG CAA TTG GCA GAA TC	54	Cherry laurel	145	Hajyzadeh et al. 2013
		R: GAA TAT GAG ACG GTC CAG AAG C		Peach		Testolin et al. 2000
7	PMS2	F: CAC TGT CTC CCA GGT TAA ACT	-	Cherry laurel	132-152	Hajyzadeh et al. 2013
		R: CCT GAG CTT TTG ACA CAT GC		Cherry		Cantini et al. 2001
					127-151	Struss et al. 2003
8	PMS3	F: TGG ACT TCA CTC ATT TCA GAG A	-	Cherry laurel	152-200	Hajyzadeh et al. 2013
		R: ACT GCA GAG AAT TTC ACA ACC A		Cherry		Cantini et al. 2001
					153-203	Struss et al. 2003
9	PceGA59	F: AGA ACC AAA AGA ACG CTA AAT C	-	Cherry laurel	181-256	Hajyzadeh et al., 2013
		R: CCT AAA ATG AAC CCC TCT ACA AAT		Cherry		Cantini et al. 2001
10	UDP96-005	F: GTA ACG CTC GCT ACC ACA AA	55	Cherry laurel	93-101	Türkoğlu et al. 2010
		R: CCT GCA TAT CAC CAC CCA G		Cherry	115-135	
				Sour cherry	99-113	
				Mahaleb	117-119	
				Silverberry	156-186	
				Peach	155	Cipriani et al. 1999 Testoline et al. 2000
11	UCD-CH17	F:TGG ACT TCA CTC ATT TCA GAG A	58	Cherry	180-200	Türkoğlu et al. 2010
		R: ACT GCA GAG AAT TTC CAC AAC CA		Sour cherry	178-202	
				Mahaleb	164	
				Silverberry	14-160	
				Cherry	186-190	
12	UDAp-401	F:AAA CCC TAG CCG CCA TAA CT	60	Cherry laurel	106-116	
		R: GCT AAA GGC CTT CCG ATA CC		Cherry	260-270	
				Sour cherry	262-272	
				Mahaleb	138-146	
				Silverberry	146-162	
				Apricot	201	Messine et al. 2004

2.2.3. Data analysis

The SSR primer pairs which produced clear PCR fragments were scored and indicated as present (1) or absent (0). Genetic similarity was calculated using Jaccard's coefficient to obtain a pairwise similarity matrix (Jaccard 1908). The Principle Coordinate (PCoA) and cluster analysis was constructed using the unweighted pair-group method with arithmetic mean (UPGMA) using the PAST program (Hammer et al., 2001). The bootstrap values for the clusters were calculated by 1000 replicates using the PAST program. The representativeness of the dendrogram was evaluated by estimating cophenetic correlation for the dendrogram and comparing it with the similarity matrix, using Mantel's matrix correspondence test (Mantel, 1967). PIC values for each locus were measured by using the following equation: $PIC=1 -\Sigma Pi^2$, where Pi is the frequency of the i^{th} allele) (Perrier & Jacquemond-Collet 2006).

3. Results and Discussion

Allele sizes of 15 SSR markers are presented in Table 3.

Table 3- Allele sizes (bp) of the SSR markers used in the study

SSR Marker	*Amplicon size found in this study*	*Amplicon size reported in the literature*		*Reference*
	Cherry laurel	**Cherry laurel**	**Cherry**	
PMS67	154-180	-	144-191 149-161	Cantini et al. 2001 Struss et al. 2003
PMS49	180-186	-	79-185	Cantini et al. 2001
UDAp-401	150-154	106-116	260-270	Messiani et al. 2010
PMS2	146-162		132-152 127-151	Cantini et al. 2001 Struss et al. 2003
UDP98-021	108-124	-	-	Hajzadeh et al. 2013 Testolin et al. 2000
UDP96-005	112-146	93-101	115-135	Türkoğlu et al. 2010
PceGA59	220-236	-	181-256	Cantini et al. 2001
PMS3	135-160	-	152-200 153-203	Hajzadeh et al. 2013 Struss et al. 2003
UCD-CH17	150-162	-	180-200	Türkoğlu et al. 2010
PceGA34	142-155	-	-	Hajzadeh et al. 2013 Downey & Lezoni 2000
BBCT001	315-350	-	-	-
BBCT002	194-222	-	-	-
BBCT005	220-246	-	-	-
PceGA25	-	-	141-198	Cantini et al. 2001
PMS40	-	-	181-226	Cantini et al. 2001

15 SSR primer pairs were used to determine the genetic relationship among the cherry laurel genomes. Among these 15 primers, 13 SSR primer pairs produced a scorable amplicon. The number of scorable bands from 13 SSR primer pairs ranged from 3 (UDAp-401) to 17 (BBCT001), as presented in Table 4. The total number of bands obtained was 117. The average number of bands per primer was 9. The average number of polymorphic bands per SSR marker was 8.38. The highest polymorphic band number was 17 and was obtained from BBCT001 primer. 109 polymorphic fragments were obtained from a total of 117 bands, and the polymorphism rate was calculated as 93.5%.

Table 4- Information obtained with SSR markers

SSR Primer	*Total number of bands*	*Number of polymorphic bands*	*Polymorphism rate*	*PIC*
PMS67	11	9	81.81	0.93
PMS49	4	4	100	0.92
UDAp-401	3	3	100	0.85
PMS2	9	9	100	0.76
UDP98-021	9	8	88.88	0.68
UDP96-005	14	13	91.85	0.92
PceGA59	7	7	100	0.78
PMS3	10	8	80	0.86
UCD-CH17	6	5	83.33	0.74
PceGA34	7	7	100	0.82
BBCT001	17	17	100	0.85
BBCT002	10	10	100	0.81
BBCT005	10	9	90	0.83
Average	**9**	**8.38**	**93.52**	
TOTAL	**117**	**109**		

3.1. Dendrogram and evaluation of SSR analysis

The similarity index was calculated according to the Jaccard dissimilarities (Figure 3). Dendrograms were constructed based on the UPGMA method using Past software (Figure 4).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42
2	0.46																																									
3	0.50	0.51																																								
4	0.50	0.52	0.06																																						
5	0.61	0.44	0.47	0.48																																					
6	0.62	0.59	0.42	0.43	0.49																																				
7	0.53	0.47	0.45	0.40	0.48	0.55																																			
8	0.62	0.59	0.61	0.62	0.63	0.64	0.60																																		
9	0.60	0.60	0.47	0.48	0.54	0.62	0.49	0.60																																	
10	0.53	0.39	0.46	0.44	0.40	0.57	0.41	0.55	0.48																																
11	0.59	0.63	0.39	0.37	0.56	0.57	0.56	0.53	0.54	0.50																															
12	0.50	0.55	0.08	0.13	0.46	0.42	0.49	0.62	0.46	0.44	0.37																														
13	0.59	0.61	0.47	0.48	0.56	0.53	0.62	0.61	0.61	0.57	0.45	0.45																													
14	0.68	0.71	0.58	0.59	0.64	0.61	0.66	0.64	0.67	0.62	0.57	0.57	0.65																												
15	0.68	0.71	0.45	0.49	0.58	0.5	0.66	0.60	0.66	0.66	0.52	0.43	0.60	0.57																											
16	0.65	0.56	0.37	0.38	0.55	0.48	0.43	0.66	0.51	0.54	0.39	0.35	0.50	0.64	0.53																										
17	0.59	0.61	0.44	0.47	0.53	0.53	0.65	0.59	0.65	0.56	0.46	0.42	0.19	0.60	0.52	0.52																									
18	0.56	0.56	0.17	0.18	0.53	0.48	0.52	0.65	0.49	0.47	0.38	0.17	0.51	0.60	0.52	0.33	0.50																								
19	0.56	0.60	0.32	0.37	0.53	0.56	0.53	0.55	0.54	0.54	0.27	0.33	0.47	0.61	0.55	0.41	0.42	0.41																							
20	0.58	0.51	0.22	0.24	0.40	0.42	0.51	0.64	0.55	0.51	0.48	0.23	0.50	0.62	0.46	0.41	0.42	0.30	0.42																						
21	0.54	0.43	0.49	0.52	0.43	0.54	0.44	0.67	0.60	0.33	0.62	0.50	0.63	0.62	0.64	0.55	0.55	0.50	0.59	0.50																					
22	0.62	0.58	0.37	0.38	0.44	0.52	0.54	0.70	0.59	0.55	0.46	0.40	0.64	0.63	0.52	0.50	0.59	0.41	0.47	0.34	0.50																				
23	0.65	0.50	0.48	0.49	0.28	0.61	0.53	0.66	0.61	0.48	0.55	0.49	0.60	0.59	0.57	0.54	0.55	0.52	0.55	0.49	0.46	0.48																			
24	0.51	0.55	0.43	0.48	0.53	0.54	0.59	0.59	0.58	0.50	0.51	0.42	0.46	0.54	0.47	0.60	0.42	0.54	0.48	0.46	0.55	0.57	0.45																		
25	0.57	0.61	0.15	0.20	0.52	0.46	0.55	0.67	0.50	0.48	0.42	0.12	0.52	0.58	0.45	0.39	0.46	0.20	0.42	0.25	0.51	0.37	0.53	0.46																	
26	0.53	0.62	0.16	0.18	0.50	0.43	0.51	0.68	0.51	0.51	0.43	0.13	0.48	0.61	0.46	0.40	0.47	0.25	0.40	0.27	0.57	0.41	0.56	0.50	0.09																
27	0.50	0.57	0.33	0.35	0.52	0.49	0.50	0.68	0.56	0.43	0.50	0.34	0.50	0.61	0.56	0.50	0.49	0.38	0.48	0.37	0.49	0.55	0.60	0.53	0.39	0.32															
28	0.57	0.53	0.39	0.40	0.51	0.57	0.50	0.57	0.42	0.50	0.46	0.40	0.54	0.65	0.60	0.52	0.56	0.46	0.53	0.45	0.55	0.50	0.52	0.55	0.44	0.45	0.47													
29	0.55	0.57	0.21	0.22	0.5	0.44	0.58	0.68	0.58	0.53	0.42	0.18	0.52	0.58	0.42	0.43	0.46	0.26	0.45	0.14	0.54	0.36	0.53	0.43	0.14	0.19	0.39	0.44											
30	0.53	0.60	0.21	0.23	0.53	0.47	0.57	0.67	0.56	0.56	0.42	0.18	0.50	0.60	0.43	0.44	0.44	0.30	0.43	0.18	0.60	0.41	0.54	0.40	0.18	0.196	0.37	0.45	0.08										
31	0.50	0.57	0.16	0.18	0.48	0.43	0.52	0.64	0.51	0.49	0.40	0.13	0.50	0.61	0.43	0.38	0.47	0.25	0.40	0.24	0.57	0.39	0.51	0.44	0.16	0.15	0.37	0.45	0.15	0.12									
32	0.44	0.48	0.37	0.41	0.55	0.52	0.47	0.62	0.54	0.37	0.57	0.39	0.54	0.63	0.62	0.52	0.56	0.45	0.55	0.46	0.41	0.54	0.59	0.53	0.46	0.44	0.33	0.50	0.49	0.50	0.44								
33	0.50	0.53	0.48	0.51	0.52	0.56	0.61	0.66	0.53	0.53	0.63	0.46	0.59	0.68	0.61	0.61	0.61	0.54	0.57	0.46	0.59	0.58	0.63	0.49	0.50	0.51	0.53	0.55	0.48	0.51	0.51	0.48							
34	0.53	0.61	0.33	0.37	0.55	0.59	0.58	0.64	0.54	0.56	0.53	0.30	0.58	0.63	0.52	0.55	0.52	0.41	0.50	0.34	0.60	0.40	0.54	0.43	0.30	0.34	0.48	0.53	0.32	0.26	0.31	0.50	0.45						
35	0.55	0.46	0.39	0.40	0.49	0.50	0.58	0.63	0.62	0.42	0.57	0.40	0.53	0.61	0.55	0.55	0.50	0.41	0.55	0.35	0.47	0.54	0.51	0.47	0.40	0.40	0.37	0.47	0.34	0.34	0.40	0.40	0.50	0.46					
36	0.57	0.60	0.41	0.42	0.50	0.57	0.54	0.57	0.38	0.48	0.43	0.39	0.48	0.64	0.55	0.49	0.54	0.45	0.53	0.46	0.57	0.49	0.53	0.54	0.39	0.40	0.50	0.26	0.43	0.44	0.42	0.50	0.51	0.51	0.50				
37	0.58	0.55	0.49	0.50	0.55	0.56	0.59	0.64	0.32	0.60	0.58	0.50	0.55	0.74	0.66	0.55	0.59	0.55	0.60	0.42	0.64	0.58	0.67	0.58	0.51	0.47	0.53	0.44	0.46	0.50	0.50	0.54	0.50	0.59	0.57	0.38			
38	0.56	0.58	0.29	0.31	0.58	0.59	0.60	0.68	0.55	0.53	0.52	0.28	0.57	0.64	0.54	0.51	0.54	0.29	0.55	0.35	0.60	0.46	0.56	0.50	0.21	0.27	0.46	0.50	0.26	0.25	0.29	0.47	0.51	0.25	0.34	0.44	0.56		
39	0.56	0.57	0.32	0.33	0.47	0.47	0.53	0.63	0.45	0.52	0.50	0.30	0.47	0.66	0.48	0.37	0.46	0.42	0.51	0.33	0.53	0.47	0.55	0.52	0.30	0.31	0.45	0.42	0.32	0.32	0.31	0.44	0.48	0.48	0.45	0.37	0.34	0.40	
40	0.50	0.56	0.41	0.40	0.52	0.52	0.51	0.66	0.51	0.47	0.52	0.40	0.52	0.68	0.66	0.50	0.54	0.47	0.57	0.44	0.56	0.59	0.60	0.56	0.41	0.37	0.48	0.43	0.43	0.44	0.40	0.45	0.47	0.51	0.50	0.40	0.41	0.45	0.40
41	0.53	0.58	0.25	0.23	0.53	0.52	0.55	0.70	0.51	0.50	0.44	0.22	0.55	0.62	0.55	0.40	0.53	0.26	0.48	0.34	0.57	0.44	0.52	0.51	0.21	0.23	0.41	0.46	0.27	0.27	0.23	0.48	0.52	0.44	0.47	0.43	0.52	0.23	0.34	0.43	
42	0.54	0.53	0.44	0.42	0.44	0.51	0.49	0.67	0.50	0.38	0.52	0.44	0.50	0.69	0.63	0.56	0.54	0.47	0.57	0.44	0.52	0.55	0.53	0.53	0.46	0.42	0.41	0.46	0.48	0.49	0.47	0.42	0.53	0.55	0.48	0.36	0.45	0.50	0.42	0.39	0.47	
43	0.52	0.56	0.20	0.21	0.47	0.44	0.50	0.67	0.49	0.48	0.42	0.16	0.52	0.60	0.50	0.4	0.49	0.25	0.43	0.26	0.51	0.40	0.50	0.50	0.16	0.18	0.37	0.42	0.22	0.22	0.18	0.44	0.50	0.40	0.42	0.39	0.49	0.28	0.27	0.39	0.12	0.42

Figure 3- Similarity (Jaccard) index of cherry laurel genotypes

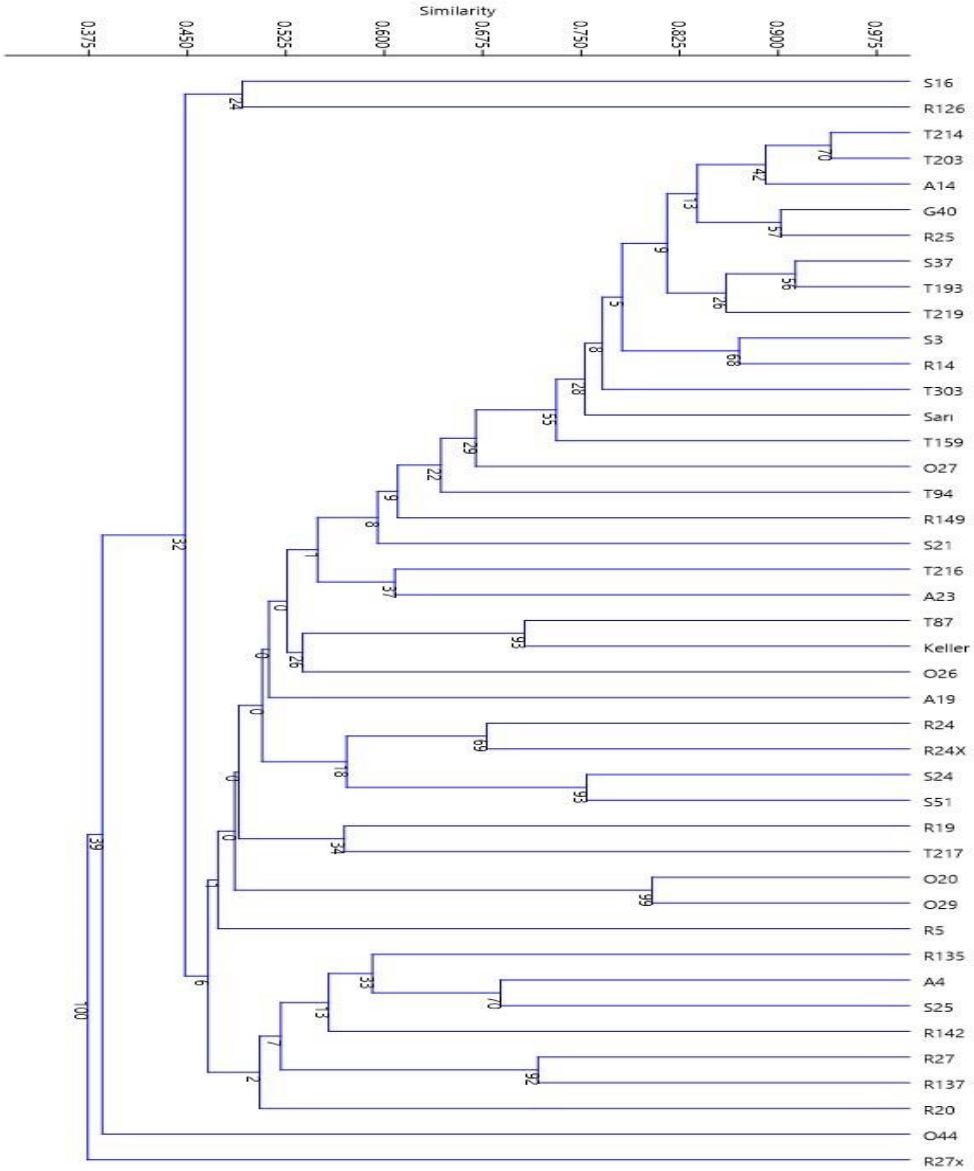

Figure 4- SSR dendrogram of cherry laurel

The dendrogram separated 43 accessions of cherry laurel collected from 6 different sites in the Black Sea Region of Turkey. UPGMA cluster analysis showed that all cultivars are clustered in two major groups. The similarity rate was found to be between 0.94 and 0.26. In the first major group, the R27x genotype selected from Rize was separated from the other 42 genotypes. Its fruits ripen too late. In the second major group, the O44 genotype selected from Ordu separated all other genotypes. This genotype has pointed and dark black fruit and produces large trees. The validity of the dendrogram in reflecting the genetic relationships among the cherry laurel genotypes is indicated by a high cophenetic correlation coefficient (r) of 0.98. Principle coordinate analysis (PCoA) was also performed using the similarity matrix, and the two-dimensional dendrogram corroborated UPGMA analyses (Figure 5).

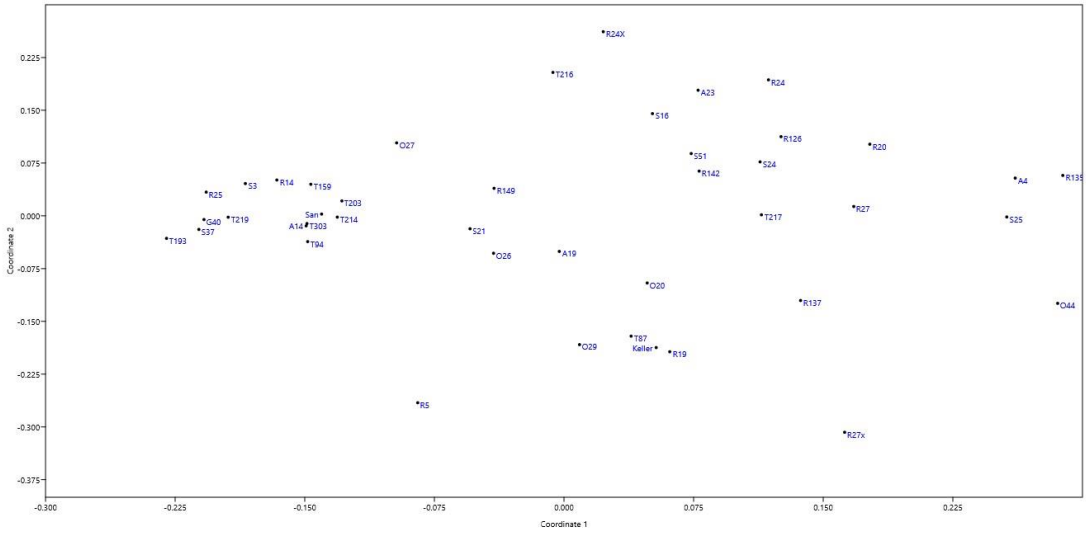

Figure 5- Principle coordinate analysis (PCoA) of 43 cherry laurel genotypes generated by the data from SSR analyses

When all genotypes were evaluated, the most distant were R27x and R24x genotypes with a ratio of 0.26. In our study, when genotypes were evaluated in terms of the province where they were collected, the genotypes selected from Rize had the lowest similarity by 0.49, while the genotypes selected from Trabzon had the highest similarity with a mean ratio of 0.61. The genotypes selected from Rize showed the most distant distribution. When the genotypes selected from all the provinces are evaluated as a whole, it can be said that there is a gene flow in the cherry laurel genotypes among the provinces. The UCDCH-17, PMS67, PMS49, PMS2, PMS3 loci, which were developed from cherry (Struss et al. 2003), the UDAp-401 locus developed from apricot (Messina et al. 2004), the UDP 98-021, UDP 96-005 loci developed from peach (Cipriani et al. 1999), and PceGA34 and PceGA59 loci developed from sour cherry were used in the definition.

DNA markers are frequently used in the genetic identification of *Prunus* species. Of the 15 SSR primers used in the study of Mnejja et al. (2004), 13 SSR primers yielded a scorable band pattern. This study showed that SSR markers can be used among the *Prunus* species (Mnejja et al. 2004; Wünsch 2009). Also, Celikkol (2011), and Hayaski et al. (2009) stated that crossing between species could be possible. The primers used by Hajyzadeh et al. (2013) included PMS 49, PMS 67, Pce GA34, UDP98-021, PMS2, PMS3, PceGA59, and the number of alleles supported our work. The scorable band number of the SSR primers was found to be a total of 117. They ranged from 3 alleles (UDAp-401) to 17 (BBCT001) alleles. The average number of bands was recorded as 9 per primer. The average number of polymorphic bands per primer was 8.38. The highest polymorphic band number, obtained from the BBCT001 primer, was 17. 109 bands were polymorphic, and the polymorphism rate was calculated as 93.52% (Table 4). The polymorphism rates of the SSR markers used in this study were close to those of Testolin et al. (2000) and Cantini et al. (2001).

Cherry, sour cherry and cherry laurel belong to the family Rosaceae. The haploid chromosome number of the cherry progenitor was eight (n= 8) and cultivated cherry varieties were obtained by selection from this progenitor (Acunalp 2012). Peach, apricot and plum species are diploid, with chromosome number 24, 16 and 16 respectively (Çelikkol 2011). However, when the gel images of SSR analysis were examined it was determined that more than 2 DNA band profiles were formed in a single genotype suggesting polyploidy in *Prunus laurocerasus* L. Furthermore, SSR analyzes were carried out on a diploid plant species, up to two DNA bands are obtained in the case that the amplified region is heterozygous. When SSR-PCR results were examined, it was determined that more than 2 DNA band profiles were formed in a single genotype. Meurman (1929) stated that the number of chromosomes increased up to 2n= 22x in cherry laurel species. Likewise, Zahra et al. (2010) stated that the basic chromosome sequence was 8x in *P. laurocerasus*. Additionally, Hajyzadeh et al. (2013) observed that more than 2 DNA band profiles were detected in a single cherry laurel genotype.

Based on the dendrogram, the genotype of O44 sampled in Ordu and the genotype R27x sampled in Rize were found to be quite different from the other genotypes (Figure 4). Except for these two genotypes, all the other genotypes (selected from Trabzon, Artvin, Rize, Giresun, Samsun and Ordu) were clustered close to each other. Moreover, Türkoğlu et al. (2010) exhibit a similarity ratio of 0.95 among five *P. laurocerasus* and 20 cherry rootstock genotypes in the Black Sea and Northeast regions of Turkey. When the similarity index was examined, the highest ratio was recorded between T203 and T214, with a ratio of 0.94. It was followed by a ratio of 0.92 between T193 and S37 genotypes, and a ratio of 0.91 between the R25 and G40 genotypes. These values are the closest of all the genotypes. The most distant individuals are R27x-R24x genotypes with a ratio of 0.26, followed by R27x-R135 and R27x- R5 with a ratio of 0.29. Additionally, Ercişli et al., (2011) determined the genetic variation

of 18 wild cherry genotypes with 10 SSR primers. They found that 9 genotypes had a high similarity ratio, and 2 genotypes differed in terms of tree and fruit characteristics.

4. Conclusions

In conclusion, we determined that by SSR analysis it is possible to differentiate the 43 different cherry laurel genotypes sampled in the Black Sea region. Furthermore, these results depicted that among the different genotypes sampled there is significant genetic variability that can be useful for future research and breeding programs. Since studies related to cherry laurel are limited around the world these results will provide the pathway for future studies.

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Do Agricultural Supports Affect Production? A Panel ARDL Analysis of Turkey

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ABSTRACT
One of the controversial issues in the trade negotiations carried out with the World Trade Organization deals with agricultural supports related to production, which is claimed to disrupt the market system by creating an imbalance in supply and demand in the domestic market and to cause world trade contraction. In this context, the aim of this study is to examine the production effect of the deficiency payments and land-based direct supports widely used in Turkey. The study conducts a panel autoregressive distributed lag analysis for 11 selected agricultural products (wheat, corn, sunflower, seed cotton, paddy, soybean, canola, safflower, tea, dried beans, and olives) for the 2002-2019 period. The findings from the study are as follows: i) Increases in deficiency payments and land-based direct supports increase short and long run production. However, land-based direct supports have less of an effect on production. ii) While increases in input prices have a negative short run effect on production, the long run effect is the opposite. iii) Agricultural product price is not an important indicator for producers. This finding can be explained by the fact that farmers accept agricultural supports as a complement to the price variable in their production decisions. iv) Increases in the minimum wage added to the model based on Turkey's structural characteristics have a negative long run impact on production. v) No statistically significant relationship exists between the number of tractors used to represent agricultural mechanization and the amount of production.

Keywords: Production effects of the agricultural supports, Decoupled payments, Deficiency payments, Land-based direct supports, Minimum wage

1. Introduction

Agricultural supports are one of the policy tools that guide production and producers as well as an important matter of contention. The distorting effect agricultural supports have on production in domestic and foreign trade in particular have caused them to be questioned globally. A certain discipline was established through the World Trade Organization (WTO) Uruguay Round, in particular with regard to agricultural supports' distorting effect on trade, thus aiming to increase transparency and predictability in international trade policy (OECD 2001).

The Agreement on Agriculture (AoA) signed at the end of the Uruguay Round significantly limited coupled payments, which directly affect production and trade and are also known as the Amber Box. Starting in 1995, developed countries agreed to cut decoupled payments in this context by 20% within 6 years, and developing countries including Turkey by 13% within 10 years. Underdeveloped countries were exempted from making any cuts. Meanwhile, WTO member countries were allowed freedom in implementing decoupled payments, supports that have little or no effect on production and trade and also known as the Green Box (WTO 2016).

Whether or not an agricultural support is allocated is directly linked to its impact on production and trade. Agricultural supports are decoupled if they have no or minimal impact on production and trade. According to the AoA, decoupled payments must compensate the following criteria.

- Support must be provided through a publicly funded government program that does not include transfers from consumers,
- The support does not have the effect of providing price support to the producers,
- Support must comply with certain criteria and measures set out in the AoA.

For example, direct payments to producers, decoupled income supports and rural development supports are decoupled payments (WTO 1994).

After the AoA, developed countries in particular started taking some serious steps with regard to coupled agricultural supports. The United States of America (USA) under the 1996 Farm Act began to implement Production Flexibility Contract (PFC) and Market Loss Assistance (MLA) supports in this context independent of current output level. The 2002 Farm Act contributed to the institutionalization of decoupled payments by introducing counter-cyclical payments (CCP) in the USA (Westcott & Young, 2004). Meanwhile, the European Union (EU) decreased market price supports under the 1992 McSharry Reforms of the Common Agricultural Policy (CAP) while increasing direct supports targeting producers' income. The Agenda 2000 CAP Reforms drew attention to environmental and market-oriented support policies (Castellano-Alvarez et al. 2021).

The transition process in agricultural supports began in the early 2000s in Turkey. Budget deficits and imbalances in the domestic market caused by the market price supports that had been implemented until that time are seen as the reason behind the economic crises Turkey has been experiencing. This is why agricultural support reforms in Turkey, unlike in other countries, are based on economic crises. In this context, Turkey has turned to land-based direct payments targeting income from production-based price supports to deficiency payments (Demirdogen & Olhan 2017). The reasons for such a policy change are to reduce the impact supports have on production and to ensure the dominance of market signals while deciding on production (Egri 2014).

Currently, the supports granted by developed countries in particular are independent of price variations due to their distorting effect on domestic and foreign trade. Variables such as income level, planted area, and constant production levels are targeted instead. However, this has been accompanied by debates on whether the supports granted by developed countries are actually decoupled. Studies have shown even supports that are claimed to be decoupled to be able to affect production. In fact, agricultural supports are one of the most controversial issues in the Doha Round, which is still taking place within the WTO (Ritcher 2015).

Turkey's agricultural support does not directly affect the price system. On the other hand, land-based direct payments are based on planted area, while deficiency payments are based on agricultural output. Theoretically, it is expected that the production effect of land-based direct payments is less than output-based supports. This is an important discussion topic for Turkey, because both support policy have an important role in Turkey's agricultural support policy. Thus, this study indicates the production effect of land-based direct payments and deficiency payments applied in Turkey.

This study uses the panel autoregressive distributed lag (ARDL) analysis as the econometric method and discusses the effect the land-based direct supports and deficiency payments implemented in Turkey between 2002 and 2019 have had on 11 products. The study is expected to contribute to the relevant literature in the following aspects: i) Comparatively analyzing the degree to which deficiency payments and land-based direct supports are decoupled is an important innovation, because the level to which both supports are decoupled has great importance for production policies and WTO negotiations. ii) Econometrically analyzing the production effect of deficiency payments and land-based direct supports to cover a wide product group for Turkey in general is an important distinction in the study. iii) This first use of the panel ARDL method, which provides consistent and robust short and long run results even in with a limited number of the observations is an important innovation for the literature. iv) The effects minimum wage and agricultural mechanization have on agricultural production has also been shown by taking into account Turkey's unique situation.

2. Material and Methods

2.1. Agricultural supports in Turkey and literature review

Minimum price policies have been the main theme of agricultural support policies implemented up to the 2000's. Minimum price policies were first started with wheat in 1932, with the number of supported products increasing to 26 by 1992. However, the excess supply and financing problem created by minimum price policies led to the need for transformations in agricultural supports (Cakmak et al. 1999). As a matter of fact, at the beginning of 2000, land-based direct supports and deficiency payments began widely being used (Yuceer et al. 2020).

Direct supports are income payment made to producers without establishing a direct relationship between production amount and payment. Because the state does not intervene directly in prices, the market mechanism determines prices (Yukseler 1999). Direct supports are one of the least costly economic support tools because they have a minimal impact on production (Winters 1988).

Deficiency payments are a support policy tool used to bring prices to a reasonable level when the cost of production exceeds the market price. In deficiency payments, the prices consumers pay are not interfered with. Therefore, cost problems such as storage and product disposal are eliminated through the subsidy's market-distorting effect (Cakmak et al. 1998). In addition, while deficiency payments are given directly according to the production amount of a particular product, the land-based direct payments are paid according to planted area. Theoretically, payments based on output affect production more than supports based on planted area (OECD 2005).

One of the objectives of the agricultural supports is to ensure continuity in the agricultural production. As a matter of fact, concerns about food safety that came to the fore with the COVID-19 pandemic, the increase in food prices and the breaking of

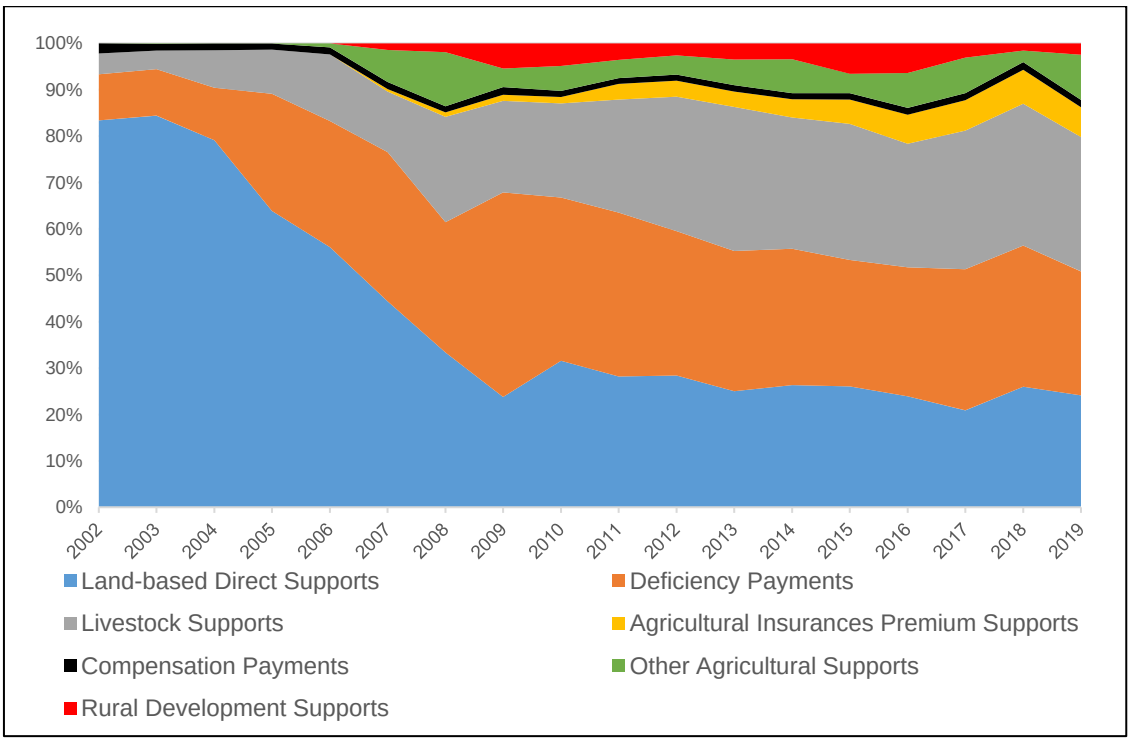
the link between agricultural inputs and outputs brought sustainability in agricultural production back to the world agenda (Arumugam et al. 2021). Thus, the agricultural supports are still an important policy tool. In this context, Table 1 shows the quality and quantity of agricultural supports provided in Turkey between 2002 and 2019.

Table 1- Agricultural Supports Provided in Turkey (in millions of TL)

Years	*Agricultural Support Type*							
	Land-based Direct Supports	*Deficiency Payments*	*Livestock Supports*	*Agricultural Insurances Premium Supports*	*Compensation Payments*	*Other Agricultural Supports*	*Rural Development Supports*	*Total*
2002	1 558	186	83	0	41	0	0	1 868
2003	2 253	268	106	0	39	3	0	2 669
2004	2 443	350	249	0	44	1	0	3 087
2005	2 352	928	352	0	47	2	0	3 681
2006	2 661	1 290	678	2	72	40	0	4 743
2007	2 461	1 782	722	31	84	382	79	5 541
2008	1 953	1 646	1 330	55	80	683	109	5 856
2009	1 078	2 002	895	59	76	181	246	4 537
2010	1 858	2 071	1 192	80	76	320	284	5 881
2011	1 996	2 503	1 727	239	90	280	249	7 084
2012	2 166	2 378	2 216	263	98	319	195	7 635
2013	2 189	2 642	2 721	290	120	479	307	8 748
2014	2 406	2 690	2 589	357	122	666	312	9 142
2015	2 605	2 726	2 935	528	139	415	655	10 003
2016	2 694	3 128	3 002	704	168	845	718	11 259
2017	2 696	3 927	3 847	853	197	986	393	12 899
2018	3 747	4 391	4 403	1 060	239	362	219	14 421
2019	4 157	4 602	4 993	1 104	271	1 671	424	17 222

Source: Republic of Turkey, Ministry of Agriculture and Forestry

As can be seen in Table 1, the total amount of agricultural supports in 2019 was 17.2 billion TL, of which 4.1 billion TL was for land-based direct supports, 4.6 billion TL for deficiency payments, and 4.9 billion TL for livestock supports. Figure 1 shows the proportional distribution and change in agricultural supports provided in Turkey between 2002 and 2019. The percentage of land-based direct payments, deficiency payments and livestock supports have of the total support in 2019 was 24.1%, 26.7%, and 30.1%, respectively.



Source: Republic of Turkey Ministry of Agriculture and Forestry

Figure 1- Agricultural Supports Provided in Turkey (%)

Turkey, especially in the recent years, increased agricultural supports to a great extent. According to OECD data, Turkey is the country granting the most agricultural support compared to GDP among the OECD countries. This ratio was approximately 1.5% for the 2019. This situation brings the production effect of the agricultural supports to the agenda given by Turkey (Bayraktar & Bulut 2016).

Early studies on the effect of agricultural supports and the limitation of this effect go all the way back to the end of the Second World War, when there was surplus supply. For instance, in order to limit the effect agricultural supports have on production, Nicholls & Gale (1946) proposed that agricultural supports be made according to a fixed production level. Swerling (1959) stated that supports can be given with respect to producer income. Nash (1961) proposed an unconditional income-based payment. Meanwhile, Van Donkersgoed (1988) stated that providing supports for rural development purposes would limit the production effect.

Although Organization for Economic Co-operation and Development (OECD) Committee of Ministers officially attempted first steps for distorting effect of agricultural supports on international trade in 1987, WTO regulated agricultural supports with the AoA in 1994 (OECD 1998). In the AoA, agricultural supports with the lowest production impacts were considered independent of production, and member states could freely provide these supports. However, this situation was accompanied by research on the effect these newly applied supports had on production.

Hennessy (1998) claimed that decoupled payments cannot be fully decoupled even if they are not related to production. According to Hennessy, decoupled payments can raise production through taking more risk by farmers with the increase in income (wealth effect) and reducing uncertainty in the income (insurance effect). If decoupled payments change the slope of the supply curve, demand shocks can increase output. This definition is called effectively decoupled (Cahill 1997) and also accepted by the OECD (2000).

In the following period, developed countries took crucial steps regarding decoupled payments. For example, Adams et al. (2001) investigated the production effect PFC and MLA supports had for 8 products in the USA. According to the analysis, a 1 unit increase in PFC and MLA supports increases planted areas by 0.026 acre. The effect of product price and agricultural credits on the planted area was statistically insignificant. Frandsen et al. (2003) made a projection for the EU covering 1997-2013 and found wheat production would decrease by 6.9%, grain production by 5.6% and oilseed production by 8.9% if internal support was eliminated. Anton & Mouel (2004) investigated the production effect of CCP and loan deficiency payments for the USA and pointed out that both payments increased production. Beckman & Wailes (2005) examined the effect direct supports and CCP have on rice production in the USA. Their results showed direct supports to be decoupled. Goodwin & Mishra (2005) surveyed 4 125 farmers to investigate the effect of direct payments and CCP on corn, wheat and soybeans. While 21% of the farmers stated that direct payments affect their production decision, 12.3% of farmers pointed out that CCP affects their production decision. Goodwin & Mishra (2006) investigated the effect Agricultural Market Transition Act (AMTA) and MLA

supports provided between 1998-2001 in the USA. The results from that study showed a one-dollar increase in AMTA supports to increase the planted area of corn by 0.92 acres, of soybean by 0.61 acres, and of wheat by 0.36 acres; only the results for wheat were statistically insignificant. Each dollar per acre increase in MLA payments was statistically significant for corn, increasing its planted area by 5.7 acres; these increases were insignificant for soybeans and wheat. Katranidis & Kotakou (2008) investigated the impact the reforms in the EU, CAP had on Greece's cotton production for 1994-2002. Their study's findings showed the production effect of decoupled payments to be low. Key & Roberts (2008) calculated that for each 1% increase in decoupled supports, the planted area of eight agricultural product increased between 0.014% and 0.027 %. According to the findings from O'Donoghue & Whitaker (2010), who analyzed 11 agricultural products in the USA, direct supports increase planted area by 9%-16%. Weber & Key (2012) calculated the effect direct supports given to 10 agricultural products in the USA have on the amount of production and planted area for 1997, 2002, and 2007. Accordingly, 1% increase in direct supports increases country-wide production by 0.20% and planted area by 0.19% acre. When the same analysis was made for the Heartland Region, 1% increase in direct support was found to increase production by 0.29% and planted area by 0.27% acre. Becker & Judge (2014) examined rice production in Arkansas, Mississippi, and the Gulf Coast in the USA. Their findings show that for each 1% increase in direct supports, rice planted areas had increased by 0.19%, 0.08% and 0.092% for each respective region, as well as a 1% increase in conjuncture supports to increase production by 0.046%, 0.040% and 0.028%, also respectively. Haß (2021) simulated the effects of the coupled and decoupled payments given to sugar beet in the EU. Accordingly, the simulated increase in sugar production of EU countries providing coupled support raised totals 258 000 tonnes of sugar (5.7%), while sugar production in EU countries without coupled payments declined by only 21 000 tonnes (0.2%).

However, similar studies have been conducted in Turkey on the production effect of agricultural supports. For instance, Aktas et al. (2015) calculated the effect of market price supports and input supports for 12 countries using OECD data for 1995-2010. Their study's findings showed a 1% increase in both support types to increase this ratio by 0.005%; only the result for Turkey was negative. Demirdogen et al. (2016) investigated the effect deficiency payments and input supports had on corn and cotton production in Turkey's Ceyhan and Yuregir regions of Adana Province for 2008-2012. Their findings show input support to be more effective on production compared to deficiency payments. Yildiz (2017) examined the effect agricultural supports made from the Turkey central government budget had on agricultural production for 2006-2016 using quarterly data. As a result of the causality analysis, a long run correlation was found between agricultural supports and the level of agricultural production. Dogan et al. (2018) examined the effect deficiency payments had on the planted areas of wheat, barley, and corn. The causality analysis using data from 1994-2016 showed deficiency payments to affect the planted areas of barley and maize but not wheat. Canbay (2021), who investigated production effect of agricultural supports between 1995 and 2018 in Turkey, found a statistically significant and positive relationship between agricultural supports and production in the short and long run. On the other hand, Erdal et al. (2021) and Erdal et al. (2020) showed that agricultural supports have effect not only on crop production but also on livestock existence.

The literature shows that, despite the qualitative transformations, agricultural supports are an important variable affecting production. This study, unlike previous ones, aims to fill an important gap in the literature by concentrating on the decoupled degree of deficiency payments and land-based direct supports for the first time. In addition, the use of Panel ARDL, which provides consistent and robust results in the short and long run even in situations where the number of observations is limited due to frequent policy changes, will bring a different methodological perspective to the literature. Finally, the relationship between minimum wage, agricultural mechanization and agricultural production is explained by taking into account the unique situation of Turkey for the first time.

3. Data, Model and Methodology

3.1 Data and model

This study examines the production effect of deficiency payment supports and land-based agricultural supports. The latter has the highest percentage of total supports at 65%; 33% of the remaining supports are livestock, and approximately 3% are rural development supports. The study examines supports over 11 agricultural products: wheat, corn, sunflower, seed cotton, paddy, soybean, canola, safflower, tea, dried beans, and olives. The period under review is 2002-2019, when land-based direct supports and deficiency payments had mainly been used. The study uses annual and nominal data. The reason for using nominal data is the expectation that farmers will act by looking at nominal data.

The data for the variables of number of tractors (representing production amount), product price, diesel price, and agricultural mechanization have been obtained from the Turkish Statistical Institute (TurkStat), support amount data from the Ministry of Agriculture and Forestry, fertilizer price data from the Agricultural Credit Cooperatives of Turkey and minimum wage data from the Ministry of Labour and Social Security.

Two different models have been created to measure the production effect of agricultural supports. The first model is shown in Equation (1):

$$PR_{it1} = \beta_0 + \beta_1 DefPay_{it} + \beta_2 DirSup_{it} - \beta_3 Fuel_{P_{it}} - \beta_4 Fer_{P_{it}} + \beta_5 P_{it} + \varepsilon_{it} \quad (1)$$

Where; *PR* is the dependent variable of the model showing the annual production amount. The independent variables are *DefPay* (deficiency payment supports), *DirSup* (land-based direct supports), *Fuel* (fuel price), *Fer* (fertilizer price), and *P* (price of the relevant product). In determining the variables, studies of Weber & Key (2012), Goodwin & Mishra (2006), Goodwin & Mishra (2005) and Adams et al. (2001) were benefited.

Considering Turkey's unique situation, the variables of *MinSal* (minimum wage) and *Mech* (agricultural mechanization), which are considered to be effective on agricultural production, were then added to the first model to create the second model shown in Equation (2):

$$PR_{it,2} = \beta_0 + \beta_1 DefPay_{it} + \beta_2 DirSup_{it} - \beta_3 Fuel_{P_{it}} - \beta_4 Fer_{P_{it}} + \beta_5 P_{it} - \beta_6 Min_{Sal_{it}} + \beta_7 Mech_{it} + \varepsilon_{it} \quad (2)$$

Where: ε_{it} ; in both models shows the independently distributed error term for all time periods.

While deficiency payments are given on the production level of a specific product, direct payments are paid according to land where any product is planted. Deficiency payments and direct payments are expected to affect the production positively. Therefore, the β_1 and β_2 coefficient signs are shown as positive. However, deficiency payments are expected to affect production more than direct supports because deficiency payments aim directly at the output while direct payments aim at the planted area. β_3, β_4 and β_6 coefficient signs are expressed as negative, since increase in input prices, which causes an increase in production costs, is expected to adversely affect production. β_5 coefficient sign is shown as positive, since theoretically, increases in product prices are expected to encourage production. β_7 coefficient sign is positive since the agricultural mechanization variable is expected to positively affect production through increase in agricultural productivity.

3.2. Methodology

In agricultural production, previous year prices are an important factor for producers. Therefore, Panel ARDL analysis which is a dynamic model including lagged values of the variables is preferred. Panel ARDL analysis also allows to the separation short and long run effect of independent variables on the dependent variable (Pesaran et al. 1999). In other words, Panel ARDL analysis offers important opportunities to display short and long run results of the variables that are effective in agricultural production. Panel ARDL analysis also presents robust results even when the number of observations is limited. Another reason for choosing Panel ARDL analysis is the limited number of observations in the study. In addition, in the Panel ARDL analysis, it is not necessary for the variables to be stationary at the same level, provided that they do not exceed second degree differentiations (Pesaran et al. 1999). All these reasons demonstrate that Panel ARDL Analysis is a suitable method for this study.

Firstly, horizontal cross-section dependency was tested. In case the shocks affect all cross-section units, parameter estimations that don't take into consideration the presence of inter-unit correlations become biased and inconsistent (Pesaran 2004). The Breusch & Pagan Lagrange multiplier test (LM test), which is used to determine whether a cross-sectional dependence exists between units, tests the basic hypothesis of no correlation between units (Baltagi et al. 2012). The Pesaran cross-section dependence (CD) test is recommended as an alternative to the LM test to determine the presence of inter-unit dependence in cases where the cross-section size is smaller than the number of observations ($T < N$). The Pesaran CD test is calculated as shown in Equation (3) (Pesaran 2004).

$$CD = \sqrt{\frac{2T}{N(N-1)}} \left(\sum_{i=1}^{N-1} \sum_{j=i+1}^{N} \hat{\rho}_{ij} \right) \quad (3)$$

In case of cross-section dependency, second generation unit root tests are applied. In this context, a different method was developed by Pesaran (2007) to eliminate the correlation between units. In this method, the cross-sectional mean of the lagged value and first difference of the series for each unit is added to the Dickey Fuller (DF) or augmented Dickey Fuller (ADF) regression equation. The cross-sectional augmented Dickey Fuller (CADF) regression equation can be represented as follows:

$$\Delta Y_{it} = \alpha_i + \rho_i \bar{Y}_{i,t-1} + d_0 \bar{Y}_{t-1} + d_1 \Delta \bar{Y}_t + \mu_{it} \quad (4)$$

In the CADF test, the unit root test statistics (CIPS) that are valid for the entire panel are calculated after calculating the individual unit root parameters for each cross-section. The calculation method of CIPS is shown in Equation (5):

$$CIPS\ (N,T) = \underline{\tau} = 1/N \sum_{i=1}^{N} \bar{\tau}\ (N,T) \quad (5)$$

In addition, the *P* statistic as calculated by Maddala & Wu (1999) and the *T* statistical value for the entire panel can be calculated as shown in Equation (6):

$$P(N,T) = -2 \sum_{i=1}^{N} ln\ (\rho_{iT})$$

Making stationarity by addressing a difference causes the long run relations among series to weaken. Cointegration analysis assumes a stationary linear combination of non-stationary series to be possible even if the series are not stationary (Wooldridge 2012).

The Pedroni cointegration test allows diversification and heterogeneity of the cointegration vector for the explanatory variables. In addition, Pedroni presented seven cointegration tests in order to show the effects within and among groups in the panel data (Pedroni 1999).

While no cointegration exists for any cross sections in the null hypothesis of the Pedroni cointegration test, a cointegration relationship is present in the alternative hypothesis. In the Kao cointegration test handles the DF and ADF tests have been handled in two ways. DF type tests can be calculated from their estimated residues as shown in Equation (7) (Baltagi & Kao 2001).

$$y_{it} = \alpha_i + \beta x_{it} + e_{it} \quad (7)$$
$$y_{it} = y_{it-1} + u_{it}$$
$$x_{it} = x_{it-1} + \varepsilon_{it}$$

In tests used to analyze the long run relationship among series, all series should be stationary at the same level. Therefore, Pesaran & Shin (1999) and Pesaran et al. (2001) developed the panel ARDL approach, which allows the cointegration test to be applied to series at varying degrees of integration.

Pesaran et al. (2001) proposed a boundary test with two stages. The first stage investigates whether long run relationships exist among the variables in the model. In the second stage, the error correction model provides the short run equation among variables and is estimated using Pesaran & Shin's (1999) panel ARDL method with the error terms obtained from the long run equation of the model.

When the series are stationary at different levels, the panel ARDL (p, q_1, q_2,…,q_n) model to be estimated is shown in Equation (8) under the assumption that the number of units is I = 1, 2, …, N with time period t =1, 2, …, T (Pesaran et al. 1999):

$$y_{it} = \sum_{J=1}^{p} \lambda_{ij}\, y_{i,t-j} + \sum_{J=0}^{q} \delta'_{ij}\, x_{i,t-j} + \mu_i + \varepsilon_{it} \quad (8)$$

Equation (9) is reached when the time series observations for each group are aggregated.

$$\Delta y_i = \phi_i y_{i,t-1} + X_i\beta_i + \sum_{J=1}^{p-1} \lambda^*_{ij}\, \Delta y_{i,t-j} + \sum_{J=0}^{q-1} \Delta X_{i,-j}\delta^{*\prime}_{ij} + \mu_i \iota + \varepsilon_{it} \quad (9)$$
$$y_i = (y_{i1}, y_{i2}, \dots, y_{iT})'$$

Where: i = 1, 2, …, N, i. is the Tx1 vector of the group's observations on the dependent variable and $X_i = (x_{i1}, x_{i2}, \dots, x_{iT})'$ is the bit Txk matrix of the observations varying between units and time periods.

Panel ARDL analyses use the mean group (MG) estimation method developed by Pesaran & Smith (1995), the pooled mean group (PMG) estimation method developed by Pesaran et al. (1999), and the dynamic fixed effects (DFE) estimator to estimate the long run coefficients of the model. Which methods will be used is decided by the Hausman test. The Hausman test is based on the consistency, efficiency, and accuracy of the results when selecting among the estimators (Samargandi et al. 2015).

4. Empirical Findings

Firstly, regarding the presence of correlations between the units that make up the panel, the cross-section dependency test was performed first, and the results are shown in Table 2.

Table 2- Results of the Cross-Section Dependency Tests

Variables	***Cross-Section Dependency Tests***[4]							
	Breusch-Pegan LM		***Pesaran Scaled LM***		***Bias-Corrected Scaled LM***		***Pesaran CD***	
	Stat.	***Prob.***	***Stat.***	***Prob.***	***Stat.***	***Prob.***	***Stat.***	***Prob.***
PR	293.8436	0.0000*	22.77284	0.0000*	22.42909	0.0000*	13.41372	0.0000*
Def_Pay	549.8977	0.0000*	47.18664	0.0000*	46.84289	0.0000*	22.82688	0.0000*
P	816.6761	0.0000*	72.62297	0.0000*	72.27922	0.0000*	28.54858	0.0000*

[4]Significance levels are denoted as follows: *; 1%, **; 5%, ***; 10%

According to the findings, the null hypothesis stating that the variables do not include cross-sectional dependence has been rejected at the 1% significance level. For this reason, second-generation unit root tests should be applied to the variables containing cross-sectional dependence.

Table 3 shows the results from the second-generation unit root test. According to the findings, while PR_{it} is stationary at the level, the first-order differences for $DirSup_{it}$ and P_{it} are stationary. In the next step, both models were analyzed with the Pedroni and Kao cointegration tests to examine whether the variables are cointegrated**.** Table 4 presents the results from the cointegration tests for both models.

Table 3- Results of the Second-Generation Unit Root Tests

Variables	***Lag***	***Maddala and Wu***[↓]				***Peseran***[↓] ***(CIPS)***			
		Trend excluded		***Trend included***		***Trend excluded***		***Trend included***	
		χ^2	***Prob.***	χ^2	***Prob.***	***Zt-bar***	***Prob.***	***Zt-bar***	***Prob.***
PR	0	34.944	0.039**	65.725	0.000*	-2.140	0.016	-1.439	0.075***
	1	34.645	0.042**	41.363	0.007*	-2.958	0.002*	-2.918	0.002*
Def_Pay	0	13.787	0.909	28.871	0.149	-1.554	0.060***	0.541	0.706
	1	23.949	0.350	14.487	0.883	0.867	0.807	3.843	1.000
P	0	0.455	1.000	9.433	0.991	-0.477	0.317	1.039	0.851
	1	0.268	1.000	15.669	0.832	-0.641	0.261	0.751	0.774
Primary Differences									
PR	0	260.516	0.000*	212.946	0.000*	-6.155	0.000*	-5.231	0.000*
	1	63.129	0.000*	36.376	0.028**	-4.102	0.000*	-2.386	0.009*
Def_Pay	0	254.070	0.000*	284.375	0.000*	-8.954	0.000*	-9.206	0.000*
	1	62.391	0.000*	54.581	0.000*	-0.715	0.237	-0.859	0.195
P	0	96.699	0.000*	83.387	0.000*	-5.162	0.000*	-3.608	0.000*
	1	60.433	0.000*	53.241	0.000*	-1.031	0.151	0.779	0.782

[↓]The significance levels are denoted as follows: *; 1%, **; 5%, ***; 10%

Table 4- Results of the Cointegration Tests

Model 1[↓]	***Stat.***	***Prob.***	***Weighted Stat.***	***Prob.***
Panel-v Statistic	-1.457243	0.9275	-2.317253	0.9898
Panel-rho Statistic	2.129174	0.9834	3.322190	0.9996
Panel-PP Statistic	-5.034373	0.0000*	-2.829790	0.0023*
Panel-ADF Statistic	-4.310517	0.0000*	-1.999547	0.0228
Between-dimensions				
Group -rho Statistic	4.159058	1.0000	-	-
Group-PP Statistic	-5.290071	0.0000*	-	-
Group-ADF Statistic	-3.016186	0.0013*	-	-
Model 2[↓]	**Stat.**	**Prob.**	**Weighted Stat.**	**Prob.**
Panel-v Statistic	-2.769146	0.9972	-3.308102	0.9995
Panel-rho Statistic	2.922129	0.9983	3.641252	0.9999
Panel-PP Statistic	-4.106087	0.0000*	-3.861696	0.0001*
Panel-ADF Statistic	-4.377019	0.0000*	-3.648529	0.0001*
Between-dimensions				
Group -rho Statistic	4.613099	1.0000	-	-
Group-PP Statistic	-4.200117	0.0000*	-	-
Group-ADF Statistic	-3.419192	0.0003*	-	-

The significance levels are denoted as follows: *1%, **5%, ***10%.

According to the Pedroni cointegration results from Table 4, the null hypothesis H_0, which states no cointegration to exist among the series, is rejected. When examining the group statistics, those apart from the group-rho statistic are seen to be significant at the 1% level. After Pedroni Cointegration test, Kao cointegration test was used and results are presented in Table 5.

Table 5- Results of the Kao Cointegration Test

		t-Stat.	**Prob.**
Model 1[↓]	ADF	-2.843520	0.0022*
	Residual Variance	0.603933	
	HAC Variance	0.422462	
Model 2[↓]	ADF	t-Stat.	Prob.
		-3.102577	0.0010*
	Residual Variance	0.603174	
	HAC Variance	0.410215	

[↓]The significance levels are denoted as follows: *1%, **5%, ***10%.

The Pedroni and Kao cointegration tests in Tables 4 and 5 show the presence of a cointegration relationship among the series at the 1% significance level for both models.

Table 6 shows the results from the Hausman test performed for deciding which of the PMG, MG, and DFE estimators estimated by the panel ARDL are the most effective.

Table 6- Results of the Hausman Test

Variables	*Coefficients*		*(b-B)*	*St. Error*
	(b)	*(B)*		
	MG	*PMG*	*Difference*	
Model 1				
Def_Pay	-0.0439904	0.1091276	-0.153118	0.509349
Dir_Sup	0.381128	0.1779722	0.2031558	1.08979
Fuel_P	-0.5833855	0.2106551	-0.7940406	2.280452
Fer_P	0.7148702	0.4243131	0.2905571	1.101366
P	0.1605679	0.1131583	0.0474096	1.504058
Model 2				
Def_Pay	-5.504829	0.16895	-5.673779	1.71e+20
Dir_Sup	3.377491	0.221532	-3.599023	1.38e+20
Fuel_P	11.75163	0.4125583	11.33907	3.60e+20
Fer_P	-2.938338	0.6282212	-3.566559	1.19e+20
P	-18.12046	0.1286967	-18.24915	5.12e+20
Mech	5.570274	-0.1823304	5.752604	2.12e+20
Min_Sal	11.04657	-0.3466583	11.39322	2.81e+20

The obtained results indicate that the PMG estimator has the most efficient estimators. In this context, the Panel ARDL results of both models are given in Table 7.

Table 7- PMG Panel ARDL Regression Results

Variables	*First Model*[↓]				*Second Model*[↓]			
	Long run estimation				*Long run estimation*			
	Coef.	*St. Error*	*t-Stat.*	*Prob.*	*Coef.*	*St. Error*	*t-Stat.*	*Prob.*
Def_Pay	0.177972	0.070267	2.532.811	0.0128**	0.221532	0.076092	2.911.371	0.0046*
Dir_Sup	0.109128	0.049123	2.221.499	0.0285**	0.168950	0.056509	2.989.802	0.0037*
Fuel_P	0.210655	0.101463	2.076.186	0.0403**	0.412558	0.128950	3.199.375	0.0020*
Fer_P	0.424313	0.133224	3.184.953	0.0019*	0.628221	0.166196	3.779.993	0.0003*
P	0.113158	0.102582	1.103.104	0.2725	0.128697	0.108987	1.180.841	0.2411
Mech	-	-	-	-	-0.182330	0.205195	-0.888571	0.3769
Min_Sal	-	-	-	-	-0.346658	0.180746	-1.917.936	0.058***
Variables	**Short run estimation**				**Short run estimation**			
Cointeq01	-0.720429	0.135497	-5316944	0.0000*	-0.732724	0.152099	-4817411	0.0000*
D(Def_Pay)	0.288277	0.174398	1.652.988	0.1013	0.281516	0.170280	1653256	0.1021
D(Dir_Sup)	0.178551	0.068923	2.590.600	0.0109**	0.209458	0.069593	3009736	0.0035*
D(Fuel_P)	-0.276946	0.142532	-1.943.051	0.0547***	-0.238423	0.144505	-1649924	0.1028
D(Fer_P)	-0.044743	0.193255	-0.231521	0.8174	-0.261590	0.161843	-1616314	0.1099
D(P)	-0.537364	0.436143	-1.232.081	0.2207	-0.259699	0.376227	-0.690271	0.4920
C	0.090427	0.077410	1.168.152	0.2454	-	-	-	-
D(Mech)	-	-	-	-	-0.469318	0.318651	-1.472.830	0.1447
D(Min_Sal)	-	-	-	-	-0.327104	0.227534	-1.437.606	0.1544
C	-	-	-	-	0.204048	0.102370	1.993.247	0.0496**

Note: The maximum length of lag is 1; [↓]The significance levels are denoted as follows: *1%, **5%, ***10%.

The error correction coefficients in the equations of Models 1 and 2 are respectively -0.72 and -0.73 and are significant with an error margin of 1%. This result means that 72% of the short run imbalances in the first model and 73% in the second model are eliminated over the long run.

According to the results, the findings from both models are similar. This is important in terms of showing that the findings from both models are robust and consistent. Production effect of the both kinds of supports is positive in the short and long run in line with expectations. According to Model 1, a 1% increase in deficiency payments increases production by 0.28% in the short run and by 0.17% in the long run. A 1% increase in land-based direct supports increases production by 0.17% in the short run and 0.10% in the long run. According to the results from Model 2, while a 1% increase in deficiency payments increases

production by 0.28% in the short run, it increases by 0.22% in the long run. A 1% increase in land-based direct supports increases production by 0.20% in the short run and 0.16% in the long run. In the short run, the results are statistically significant, considering that the probability values of deficiency payments in both models are slightly above 10%. The results confirm the hypothesis that deficiency payments and land-based direct supports affect the production.

The results for fuel and fertilizer, which are used as input items, are similar for both models. Accordingly, while the coefficients are negative in the short run, they become positive in the long run. However, the short run results for the variable of fertilizer in Model 1 are statistically insignificant. The findings show that the increase in input prices negatively affects production in the short run, but this trend is reversed over the long run. The findings show that the expectations regarding increase in the production costs are not valid especially in the long run.

In addition, the variable of price, which has negative coefficients in the short run and positive in the long run, is statistically insignificant in both models. In other words, the hypothesis that increases in product prices raise production is rejected.

While the effect of minimum wage on agricultural production in Model 2 is statistically insignificant in the short run, it is negative and significant with a 10% probability in the long run. Accordingly, a 1% increase in minimum wage reduces agricultural production by 0.34% in the long run. The results reflect the expectation that increases in the minimum wage affect production negatively, especially in the long run.

Representing agricultural mechanization, the effect of the number of tractors on production as used in Model 2 is found to be statistically insignificant for both the short and long run. According to the results, the hypothesis that agricultural mechanization affect production positively is rejected.

5. Conclusions and Policy Advice

One of the ways to limit the distorting effect of agricultural supports in domestic and foreign markets is to separate production and supports. As a matter of fact, as a result of the commitments made to the WTO, developed countries in particular have given weight to decoupled payments. However, Turkey has switched from price supports to direct and deficiency payment supports in the early 2000's. This study has analyzed the effect of deficiency payments and land-based direct supports on 11 agricultural products using the Panel ARDL method with annual data from 2002-2019. The study's findings and policy recommendations based on these findings are as follows:

i) Land-based direct supports and deficiency payments implemented in Turkey affect the production. As a matter of fact, even if agricultural supports are decoupled, a great majority of the studies on the production effect of the agricultural supports confirm these findings. To give an example, O'Donoghue & Whitaker (2010), Weber & Key (2012), Becker & Judge (2014), Yildiz (2017), Canbay (2021), Erdal et al. (2021) and Erdal et al. (2020) suggested that the agricultural supports affect production positively. Especially, Katranidis & Kotakou (2008) and Haß (2021) concluded that production effects of coupled payments are more than decoupled payments. Demirdogen et al. (2016) and Aktas et al. (2015) found a very small effect between agricultural production and supports. However, Beckman & Wailes (2005), Goodwin & Mishra (2006) for soybeans and wheat and Dogan et al. (2018) for wheat did not find any significant correlations between the supports and the agricultural production. In fact, the vast majority of studies are regarding direct payments that do not depend on the current output level. However, the results indicates that direct payments affect production. These findings indicate that direct payments are not decoupled. Findings for Turkey indicate that deficiency payments and land-based direct payments increase agricultural production. This is an expected result for Turkey, because deficiency payments depend on output while land-based direct payments depend on production. Therefore, both supports can be used as a policy tool to direct production. However, the positive production effect of supports may pose a disadvantage for Turkey and especially other developed countries in its negotiations carried out with the WTO.

ii) Short and long run effects of the deficiency payments have a greater effect on production than land-based direct supports. In other words, land-based direct supports are more decoupled than deficiency payments because land-based direct supports are paid per acre regardless of what is produced. Indeed, Beckman & Wailes (2005) and OECD (2005) pointed out that land-based direct supports had less production effect compared with output based payments. Also, Haß (2021) found that decoupled payments affect production less. On the other hand, Demirdogen et al. (2016) indicate that the production effects of the deficiency payments are less than input supports while Aktas et al. (2015) concluded that there are negative relationship between market price supports, input supports and agricultural production.

In contrast with these studies, Dogan et al. (2018) did not find any relationship between deficiency payments and wheat production. There can be several reasons why the production effect of deficiency payments are low or insignificant. Methodological differences, such as focusing on one region or agricultural product, limit overall effects of the deficiency payments. In particular, the substitution of products for which the difference payment is paid, limits the results of product-based studies. As a matter of fact, when the deficiency payments are evaluated as a whole, deficiency payments increase production in the short and long run. This being the case, the most effective policy tool in terms of production policy is the deficiency payments. However, output-based supports such as the deficiency payment support is also an important matter of contention for the WTO.

iii) While the increase in input prices affects production negatively in the short run, it has a positive impact over the long run in Turkey. Suh & Moss (2021), Komarek et al. (2017) and Srivastava (2017) concluded that input prices affect agricultural production negatively. On the other hand, Taylor & Koo (2008) emphasized that producers respond to the high input and commodity prices by increasing supply for compensating decrease in farm income. In Turkey, these findings can be explained by the fact that farmers, whose production and income decreased in the short run, increased their production in the long run to compensate for the decrease in their incomes. Various policy measures from tax benefits to supports can be implemented to avoid the negative impact these increases have on input prices.

iv) The relationship between product prices and production is statistically insignificant. Xie & Wang (2017) and Borawska et al. (2021) conducted that agricultural product prices are an important indicator for production, whereas Goodwin & Mishra (2006) and Adams et al. (2001) could not reach a statistically significant relationship between agricultural product prices and production. As a consequence of researches, it can be said that relationship between product price and production level differs from each other according to structural characteristics of the countries. For example, farmers in Turkey can prefer produce more in order to compensate for the decrease in their income. Since agricultural enterprises in Turkey are mostly small-scale family businesses, their income sources are largely dependent on agricultural production. Under such a circumstance, farmers avoid risky attitude by acting according to agricultural supports instead of taking risks by looking at price level in production decisions. Also, disruptions of relative prices of the agricultural products, producers' future expectations regarding product prices and farmers' production habits may also cause distorting effects in the production decisions. These results indicate that agricultural supports, as a variable, play more significant role than product prices in production decision. However, the findings on the production effect of price are not consistent with theory and the results are different in the literature, so more studies on the production effect of product price are needed.

v) Considering Turkey's unique situation, minimum wage variable was added to the model. The long run results from the variable of minimum wage are negative and statistically significant. This situation can be explained by the fact that producers turn to sectors other than agricultural when the minimum wage rises. Again, this situation can be seen as one of the reasons explaining the decreasing trend of agricultural employment in Turkey over the years. Therefore, the minimum wage policy is important not only for workers and employers but also for the agricultural sector and agricultural policy.

vi) Considering Turkey's unique situation, the agricultural mechanization variable was added to the model. The relationship between the number of tractors, which has been used to represent agricultural mechanization and agricultural production is statistically insignificant. The results show that Turkey has reached the saturation point in terms of number of tractors and thus the law of diminishing returns is valid. In fact, Tokgoz (2018) pointed out a similar situation in Turkey in the 1950-1960 periods. During this decade, Turkey started to implement liberal economic policies and number of imported tractor increased significantly. The increasing number of tractors had a positive impact on agricultural production until the mid-1950s. However, in the late of 1950s, current account deficit and spare parts supply problems caused an adverse change. Using the number of tractors to represent agricultural mechanization in the model may also mean that the production effect of the number of tractors is sufficiently utilized as a result of the investments and incentives made in this field. For this reason, incentives should be determined by looking at the positive production effects obtained from agricultural machinery of varying quality and technologies in order to increase agricultural production and productivity.

As a result, the production effect of deficiency payments applied in Turkey is higher than land-based direct supports. At this point, the issue that the uncertainty in the agricultural incentive policy can affect the production decisions by guiding producers toward risky behaviours stands out as a subject worth investigating in terms of guiding policymakers.

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Classification of Some Barley Cultivars with Deep Convolutional Neural Networks

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ABSTRACT
The homogeneity of the seeds is an important factor in terms of processing, transportation, storage, and product quality of agricultural products. It is possible to classify the grain polymorphism of barley cultivars, which are economically important among cereal crops, in a short time with computer vision methods with high accuracy rate and almost zero cost. In this research, a novel image database consisting of 2800 images were created to classify 14 barley cultivars. Six different deep convolutional neural network models were designed based on a transfer learning method with pretrained DenseNet-121, DenseNet-169, DenseNet-201, InceptionResNetV2, MobileNetV2 and Xception networks. The models were trained and evaluated with test-time augmentation method, the best performance was obtained from DenseNet-169 model with average 96.07% recall, 96.29% precision, 96.07% F1-score, and 96.07% accuracy on a test set independent of the training set. The results showed that the transfer learning method performed using additional layers such as dropout and data augmentation with sufficient data samples in these images with high similarities prevented overfitting by increasing the model performance. As a result, it can be suggested that the provided web tool based on the transfer model has an encouraging performance in identifying seeds with a high number of cultivars such as barley.

Keywords: Computer vision, Automatic seed recognition, Transfer learning, Barley classification

1. Introduction

Barley is the genus *Hordeum* from the Triticeae tribe of the *Poaceae* family (El Rabey et al. 2014). There are 30 species in the genus *Hordeum*, about 3/4 of them are perennial. The cultivated barley cultivars are located under the *Hordeum vulgare* L. taxon. Although the majority of Hordeum species are diploid (2n = 14), there are also tetraploid (2n= 28) and hexaploid (2n= 42) species (Bothmer 1992). One of the first cultivated plants, barley is primarily used in animal feed and malt industry (Kün 1988).

Artificial intelligence development efforts which first started with the mathematical modelling of the human nerve cell in 1943 (McCulloch & Pitts 1943) pave the way for very successful results in many disciplines today. Convolutional Neural Network (CNN) can also be regarded as the milestone of these efforts with their successful results in recent years. Although it was developed by LeCun et al. (1998), as the first convolutional neural network as it is today, it has not been able to achieve sufficient success for many years because of hardware deficiencies due to the excessive computational load required and the lack of high-resolution digital image sources.

In recent years, developments in graphic processing unit hardware and increasing digital image sources have enabled the development of deeper network structures with high performances by developing layer types, layer numbers, and connection types in the convolutional neural networks. LeNet, the introduction of the CNN concept, has 7 layers (LeCun et al. 1998), AlexNet with parallel computing on 2 GPUs has 8 layers (Krizhevsky et al. 2012), VGG model with multi-layer structure has 19 layers (Simonyan & Zisserman 2014), GoogleNet with inception module has 22 layers (Szegedy et al. 2015), ResNet with skip connection has 50 layers (He et al. 2016), and DenseNet with connected all layers has 169 layers (Huang et al. 2017).

Studies on computer-based image recognition of cultivars of cereal plants are showed difference in parallel with the improvement in methods and hardware in this field. The cultivars belonging to 5 different wheat classes cultivated in Canada are classified in scores ranging from 15 to 96% with the help of plan-form spatial shape features and elliptic Fourier descriptors of kernel perimeters (Neuman et al. 1987). A discriminant analysis-based algorithm has been developed using colour, texture, and morphological properties to classify Canadian western summer wheat, Canadian western amber durum

wheat, barley, oat, and rye seeds. In the classification according to performance ratios, success rates for 5 cultivars/species were determined as 98.9, 93.7, 96.8, 99.9, and 81.6% (Majumdar & Jayas 2000a) using 23 morphological features, 94.1, 92.3, 93.1, 95.2, and 92.5% using 18 colour features (Majumdar & Jayas 2000b), 85.2, 98.2, 100, 100, and 76.3% using 15 most prominent tissues (Majumdar & Jayas 2000c), and 99.7% mean accuracy using 20 most significant morphological, colour, and texture features, respectively (Majumdar & Jayas 2000d).

Paliwal et al. (2001) developed 9 different neural network architectures in order to classify 5 different cereal cultivars: hard red summer wheat, Canadian western amber durum wheat, barley, oats, and rye. They carried out the training and testing of the neural network with 8 different morphological features obtained from a dataset consisting of 7500 grains from 1500 grains of each cultivar. According to their test results, they were able to distinguish barley and rye by 88%, wheat and oats by 97%. Choudhary et al. (2008) classified accuracy from 89.4% to 99.3% using linear and quadratic statistical classifiers, with 135 wavelets, 93 colours, 56 textures, and 51 morphological properties of wheat, barley, oat, and rye grains with a total of 335 properties. Mebatsion et al. (2013) used the least squares method to classify grains of wheat, barley, oat, and rye varieties, and they used geometric and colour attributes obtained with elliptic Fourier identifiers. According to the results of the analysis, the researchers differentiated the grain varieties from 98.5% to 100%. Martinez et al. (2018) classified 5 different olive cultivars (Picudo, Lucio, Cortijuelo, Manzanillo de Montefrio, and Negrillo de Estepa) with an average accuracy rate of 89% using a dataset consisting of 250 images with various texture features and least squares discriminant analysis method. Demir et al. (2019) compared the longitudinal, surface and gravitational properties of 7 almond cultivars (Bertina, Ferragnes, Ferradual, Ferrostar, Glorieta, Lauranne, and Marta) with the elliptic Fourier descriptors of their shapes. They observed that the greatest difference feature is in horizontal orientation and thickness dimension in suture orientation of almond shape. Three different deep learning architectures (AlexNet, GoogleNet, and ResNet) were investigated on a dataset consisting of 4800 grains for the classification of 4 different sunflower (Reina, Sirena, Armada, and Palanci) seeds (Kurtulmuş 2021). Accordingly, the GoogleNet model gave the best result with a 95% accuracy rate. A computer image analysis technique was applied to identify the seeds (100 grains for each genotype) of three maize (*Zea mays* L.) genotypes (Beyaz & Gerdan 2021). They reported that the success of prediction accuracy was found as 99% for Random Forest and Gradient Boost Decision Tree, 97.66% for Multilayer Perceptron, 96.66% for Decision Tree, and 97.40% for Majority Voting algorithms by using the Knime Analytics Platform.

With the advances in image recognition, while the studies for image recognition of different grain types were carried out in the past, the studies for the recognition of different varieties belonging to the same grain have increased in recent years. For the classification of barley varieties, Zapotoczny et al. (2008) used linear and nonlinear discriminant analysis and principal component analysis methods with the help of 74 morphological features obtained from grains of 5 different summer barley varieties cultivated in Poland. According to the test results, the researchers obtained the least classification error rate of 5.06% using the linear discriminant analysis method. Szczypiński et al. (2015) classified the cultivars belonging to 11 different barley classes cultivated in Poland with the help of 590 shape, colour, and texture features using linear classifiers and Artificial Neural Network (ANN) with an accuracy ranging from 67% to 86%.

This study aims to develop an effective computer vision system to classify some barley cultivars using Deep Convolutional Neural Network and help reduce the errors of traditional methods by avoiding human intervention. Few studies have been conducted on the classification of barley cultivars using artificial neural networks (Szczypińskia et al. 2015; Hailu & Meshesha 2016; Dolata & Reiner 2018; Kozlowski et al. 2019; Shi et al. 2021; Singh et al. 2021). For this aim, a novel image dataset of the seeds were created in order to classify the 14 barley cultivars. The images obtained were cropped in small sizes and made suitable for neural network training, and then data augmentation methods were applied to the training datasets. Six different deep convolutional neural networks were trained and evaluated using the Fine-Tuning and test-time augmentation method. A web-based barley classification tool was developed from the most successful network.

2. Material and Methods

2.1. Obtaining images of barley cultivars

In this study, the seeds of 14 barley (*Hordeum vulgare* L.) cultivars (Akar, Başgül, Burakbey, Bülbül 89, Çetin 2000, Durusu, Efes 98, Erciyes, Özen, Tarm 92, Tosunpaşa, Yalın, Yıldız and Zeynelağa) were evaluated which were obtained from Ankara Field Crops Research Institute in 2017. The barley seeds were photographed at 24 Bit 3456 × 5184 resolution using Canon EOS 600D camera to diagnose cultivars due to differences in surface morphology. A total of 56 high-resolution images (each include ~3000 barley seeds) of 14 barley cultivars were cropped, then the cropped images that did not contain barley seeds were deleted and a dataset of 2800 images was obtained. Figure 1 shows sample images of the seeds of 14 barley cultivars that differ morphologically.

Figure 1- Morphological surface images of 14 barley cultivars

2.2. Image preprocessing and dataset preparation

Image sizes used to train the convolutional neural network are crucial for training time and system performance. For this purpose, the images, each occupying 6 ~ 7 MB in memory, were cropped into 432 × 648 small images before being given to the neural network. Finally, images were resized to 256 × 256 dimensions and given to neural network input. Figure 2 shows the preprocessing of an image in the dataset.

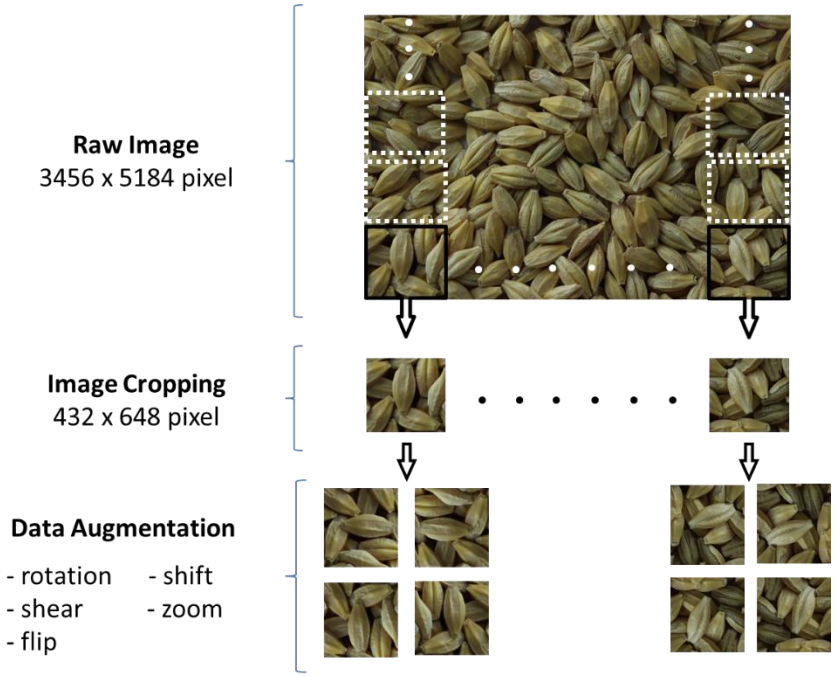

Figure 2- Preprocessing steps of raw image in a barley cultivar

All dataset was divided into 3 parts: training (60%), validation (20%), and testing (20%). Then, the images in the training dataset were subjected to 5 different data augmentation processes: rotation (90º), shear (25%), flip (horizontal, vertical), shift (width, height), and zoom (25%) (Table 1).

Table 1- Dataset for image classification of barley cultivars

	Number of images in the dataset			
Barley cultivars	***Train***	***Validation***	***Test***	***Total***
Akar	120	40	40	200
Başgül	120	40	40	200
Burakbey	120	40	40	200
Bülbül 89	120	40	40	200
Çetin 2000	120	40	40	200
Durusu	120	40	40	200
Efes 98	120	40	40	200
Erciyes	120	40	40	200
Özen	120	40	40	200
Tarm 92	120	40	40	200
Tosunpaşa	120	40	40	200
Yalın	120	40	40	200
Yıldız	120	40	40	200
Zeynelağa	120	40	40	200
Total number of augmented images	11760		**Total number of original images**	2800

2.3. Convolutional neural network architecture and transfer learning

The difference of convolutional neural networks from classical neural networks is that it determines which feature is more important and which feature is less important, with the millions of parameters it has obtained using different filters and methods. During these operations applied in layers: The initial and middle layers are revealed the edge features of the image and the shape and texture features of the image, respectively. The entire object is acquired towards in the final layers.

CNN now achieve high success in many common computer vision problems such as object detection, tracking, recognition, segmentation, classification, and so on compared to other traditional methods. In this success, the deepening of the network plays an important role by improving the layer structures and connections. In this study, six different deep neural network models consisting of DenseNet-121, DenseNet-169, DenseNet-201, InceptionResnetV2, MobileNetV2, and Xception were developed. These models have a functional network structure. In this structure, the data flow can branched and layer outputs can concatenated. Among these models, in addition to the traditional neural network connection of DenseNet networks, all layers had connections with all other layers, providing high accuracy and generalization potential for this network (Figure 3).

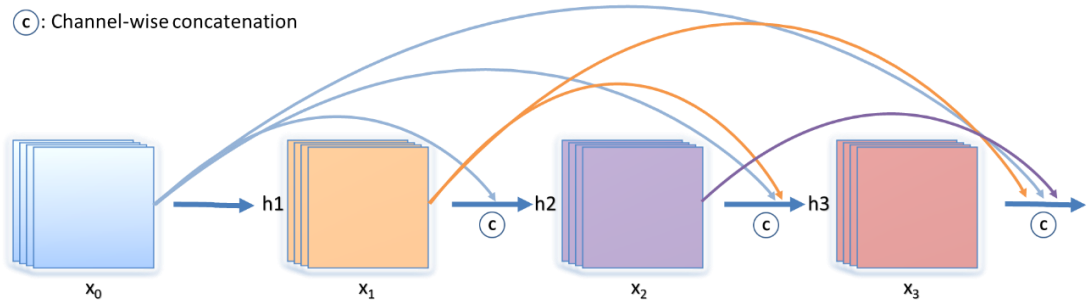

Figure 3- DenseNet neural network architecture (modified from Huang et al. 2017)

Unlike the shortcut links in the ResNet network, these links are not just $x_n = h_n(x_{n-1}) + x_{n-1}$ the sum of the values in the previous layer, but a combination of their values in all their previous layers $x_n = h_n([x_0, x_1, \ldots, x_{n-1}])$. With these links, each layer uses as input feature-maps of all previous layers and sends its own feature-maps as input to all subsequent layers (Huang et al. 2017).

To create a modern deep CNN from scratch, determining many parameters such as filter dimensions, weights, layers, connections, and then training and testing the entire model requires high speed hardware and is a very time-consuming process. Instead, using a pre-trained network with thousands of categories and millions of images provides faster and more successful results. In our study, 6 different pre-trained models were used in the ImageNet dataset. Except for the top layers of Deep Convolution Neural Network (DCNN) architectures, the rest is called the 'base model'. To build our models, we replaced the original top layers with the following layers in sequence: The last layer of each base model is connected to the dropout layer

with a probability of 0.5 and global average pooling layer, respectively, thus reducing the risk of overfitting. The feature map obtained from the previous layer was then fed into the first dense (fully connected) layer, which consists of 1024 neurons and has the ReLU activation function. Then it connects to the last dense prediction layer which has SoftMax activation function. The number of neurons in this layer is 14 according to the number of barley cultivars (Figure 4).

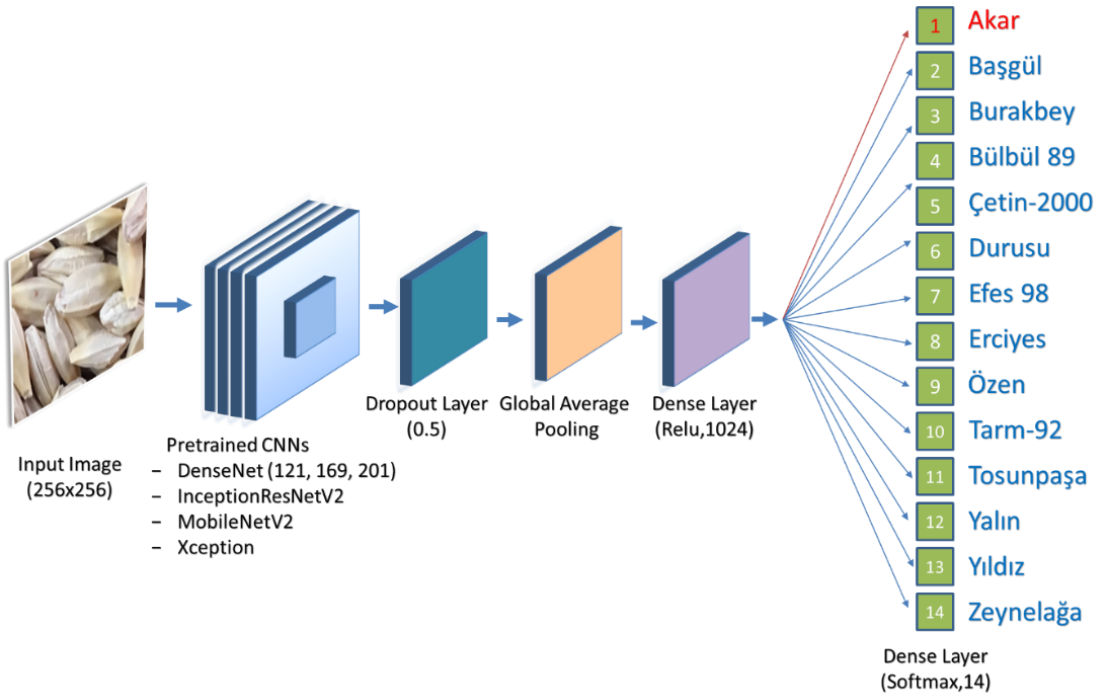

Figure 4- Identification of a barley cultivar with the transfer learning architecture of six different CNN models

A confusion matrix is used for evaluating the performances of classification model. This matrix, with as many rows and columns as the number of classes, compares the actual target values with those predicted by the model. With the help of this created matrix, the number of true positives (TP) (that is, the sum of the samples correctly predicted by the model in the calculated class), the number of true negatives (TN) (that is, the sum of the samples correctly predicted by the model in the except calculated class - the values shown in bold in the matrix), the number of false positives (FP) (that is, the sum of samples that the model predicted in this class even though it is not in the calculated class - values below and above the gray values in the matrix), and the number of false negatives (FN) (that is, in the calculated class, but the model predicted in a different class - values to the right and left of the gray values) were calculated for each class. With the help of these values, the precision (1), recall (2), F1 Score (3), and accuracy (4) rates of each barley cultivars of the CNN model were calculated (Table 4).

$$Precision = \frac{TP}{TP + FP} \quad (1)$$

$$Recall = \frac{TP}{TP + FN} \quad (2)$$

$$F1\ Score = \frac{2 \times Precision \times Recall}{Precision + Recall} \quad (3)$$

$$Accuracy = \frac{TP + TN}{TP + TN + FP + FN} \quad (4)$$

3. Results and Discussion

The code in this study was written by Python and run using the Colab environment. Tensorflow and Keras deep learning frameworks were used to define the CNN models. Six different ImageNet pre-trained CNN models such as DenseNet-121, DenseNet-169, DenseNet-201, InceptionResnetV2, MobileNetV2, and Xception have been fine-tuned. To train the models, the maximum epoch value among 30, 60 and 90 was determined as 60. Since the model could not be trained any more, the program stopped automatically and the maximum epoch value of 30 was reached. In the models, stochastic gradient descent

was used as a learning algorithm and categorical cross entropy as loss function. The learning rate starts with 0.01. If the validation loss does not decrease for a total of 5 epochs the learning speed automatically reduce by 0.75. If it does not decrease for a total of 10 epochs, training is automatically stopped. In this way, the model with the lowest loss value was calculated in every 5 epochs and the weight matrix was saved without overfitting. The epochs, accuracy and loss values of the models converging to the lowest validation loss value were obtained during the training performances (Table 2).

Table 2- Training performances of the CNN in current study

Model	*Epochs to Converge*	*Training Loss*	*Training Accuracy (%)*	*Validation Loss*	*Validation Accuracy (%)*
DenseNet-121	22	0.1940	94.01	0.1972	93.96
DenseNet-169	26	0.1504	94.82	0.1902	94.29
DenseNet-201	28	0.1988	93.75	0.2352	92.71
InceptionResNetV2	21	0.2125	93.12	0.2428	91.45
MobileNetV2	30	0.2407	91.46	0.2543	91.23
Xception	18	0.2824	90.76	0.2975	90.18

According to the training performance results of the models (Table 2), it is understood that DenseNet models were obtained lower errors and higher performance results compared to other models. Among all models, the DenseNet-169 model was the best performing model with 94.29% validation accuracy. In order to determine the best epoch value, the DenseNet-169 model was trained with 3 different epoch values (30, 60, and 90) and early stopping method, and the highest accuracy rate was obtained in 60 epochs (Figure 5a). In addition, the model was trained with and without the dropout layer to observe the effectiveness of the dropout layer in the DenseNet-169 model (Figure 5b). Without the dropout layer, it was observed that the model was tendency to overfitting so that although the training loss continued to decrease after a while in the loss graph of the model, the validation loss remained constant and the gap between the training loss and the validation loss increased (Figure 5b).

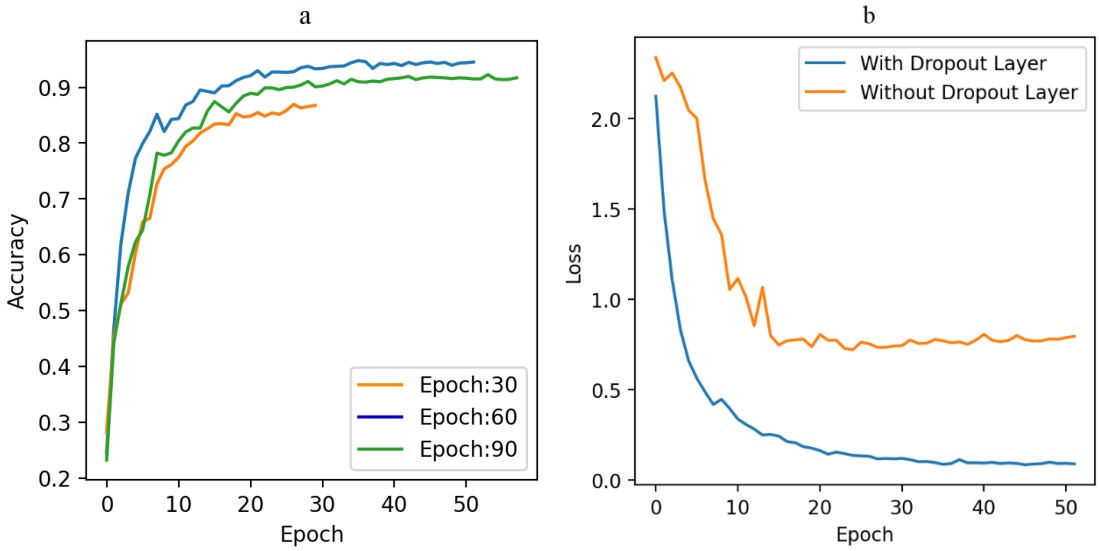

Figure 5- The graph of training accuracy with respect to different epoch values (a) and loss graph with and without the dropout layer in the DenseNet-169 model (b) using the data set of 14 barley cultivars

Recently, various methods have been developed to improve the performance of deep learning models, the test-time augmentation method is one of them (Howard 2013). During the testing of the trained model in this method, unlike the traditional network test method, in addition to a single prediction output for the original image, more predictions output is obtained as the number of augmented images. These predictions are then aggregated and the class with the highest prediction percentage becomes the classification final result (Figure 6).

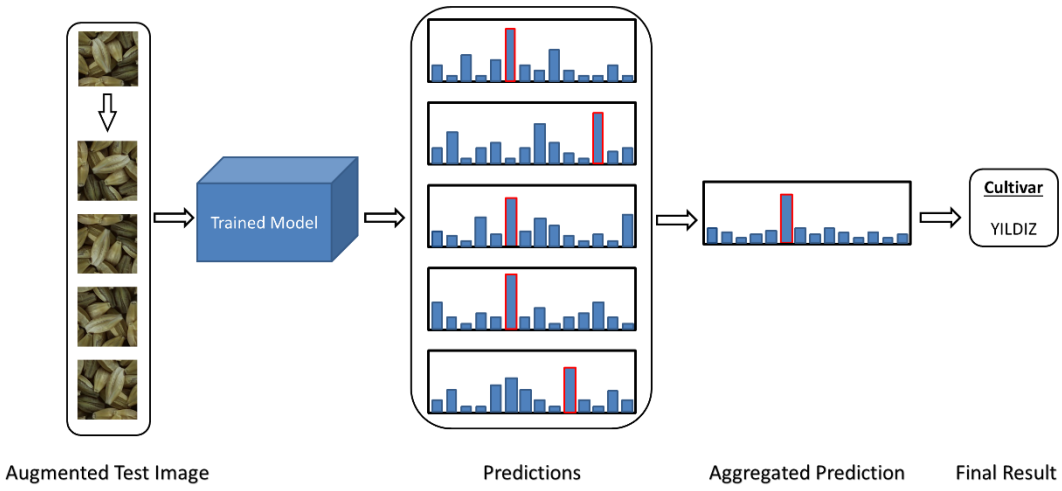

Figure 6- Flowchart designed to identify a barley cultivar at test time by image augmentation

As a result of the experiment performed with the 4 different image augmentations (rotation, vertical flip, horizontal flip, shear) used in the test-time augmentation method on the test data set, the classification accuracy increased by 1.78% and reached 96.07%. In our current study, confusion matrix was created from the classification results in order to perform detailed performance analyses of the DenseNet-169 model (Table 3).

Table 3- Confusion matrix of the tested CNN model for the classification of barley cultivars

Barley cultivars	*Akar*	*Başgül*	*Burakbey*	*Bülbül 89*	*Çetin 2000*	*Durusu*	*Efes 98*	*Erciyes*	*Özen*	*Tarm 92*	*Tosunpaşa*	*Yalın*	*Yıldız*	*Zeynelağa*
Akar	**34**	0	0	0	0	2	0	0	0	0	4	0	0	0
Başgül	0	**36**	4	0	0	0	0	0	0	0	0	0	0	0
Burakbey	0	0	**40**	0	0	0	0	0	0	0	0	0	0	0
Bülbül 89	0	0	0	**38**	0	0	2	0	0	0	0	0	0	0
Çetin 2000	0	0	0	0	**40**	0	0	0	0	0	0	0	0	0
Durusu	0	0	0	0	0	**40**	0	0	0	0	0	0	0	0
Efes 98	0	0	0	0	0	0	**40**	0	0	0	0	0	0	0
Erciyes	0	0	0	2	0	0	0	**38**	0	0	0	0	0	0
Özen	0	0	0	0	0	0	0	0	**40**	0	0	0	0	0
Tarm 92	0	0	0	0	0	0	0	0	0	**40**	0	0	0	0
Tosunpaşa	2	0	0	0	0	0	0	0	0	0	**38**	0	0	0
Yalın	0	0	0	0	0	0	0	0	0	0	0	**40**	0	0
Yıldız	0	0	0	0	0	4	0	0	0	0	0	0	**36**	0
Zeynelağa	2	0	0	0	0	0	0	0	0	0	0	0	0	**38**

When model classification performance rates (Table 4) are examined, it is seen that Akar and Tosunpaşa cultivars have the lowest F1 scores. It can be argued that this result is due to the fact that these two cultivars are very similar to each other polymorphically.

Table 4- Classification performance rates of the tested CNN model for the recognition of barley cultivars (%)

Barley Cultivars	*Precision*	*Recall*	*F1 Score*
		%	
Akar	89.47	85.00	87.18
Başgül	100.0	90.00	94.74
Burakbey	90.91	100.0	95.24
Bülbül 89	95.00	95.00	95.00
Çetin 2000	100.0	100.0	100.0
Durusu	86.96	100.0	93.02
Efes 98	95.24	100.0	97.56
Erciyes	100.0	95.00	97.44
Özen	100.0	100.0	100.0
Tarm 92	100.0	100.0	100.0
Tosunpaşa	90.48	95.00	92.68
Yalın	100.0	100.0	100.0
Yıldız	100.0	90.00	94.74
Zeynelağa	100.0	95.00	97.44
		Accuracy	**96.07**

3.1. Web-based model deployment

A web application has been developed to eliminate the different needs of the trained model, such as complex background applications, architectural connections, and communication protocols and to enable to be used the platform-independent by the end user. TensorflowJS application was used to convert the model to API format. Based on the developed web application, the classification process of the image uploaded by the user was performed without requiring any additional installation, such as any driver, library, and so on. In this context, the web-based classifier is available at https://fatih-tr.github.io/barley.

The performances of the convolutional neural networks highly depend on the characteristics of the training data. Our methods such as using images containing more seeds in a single image, data augmentation in the training data set, and adding a dropout layer to the network structure (because CNN tends to overfitting it causes generalization to decrease) provide the CNN model to perform better than similar studies. Demir et al. (2019) used some size and shape features of almond cultivars and comparison of the almond shapes with elliptic Fourier descriptors. Kurtulmuş (2021)has determined the best DCNN models to identify sunflower seeds and showed that the identification of sunflower seeds was feasible using deep learning technology. This researcher suggested that the main limitation of this work is that it requires manual adjustment of sunflower seeds before they are captured on camera. In Table 5, the results obtained in this study were compared with the studies on the classification of barley cultivars in recent years (Hailu & Meshesha 2016; Dolata & Reiner 2018; Kozlowski et al. 2019; Shi et al. 2021; Singh et al. 2021). Dolata & Reiner (2018) reported an accuracy rate of 97.24%. If they calculated according to the confusion matrix as in our study (Equation 4), the accuracy rate would have been calculated as 87.68%. According to our research with 14 barley cultivars, the fine-tuned DenseNet-169 model was performed better than other models and the state-of-the-art literature. On the other hand, Singh et al. (2021) achieved the best accuracy rate of 98.38% with CNN because of near-infrared hyperspectral imaging.

Table 5- Comparison of accuracy rates of barley cultivars classified using ANN and CNN

References	*Number of barley cultivars*	*Method*	*Accuracy (%)*
Szczypińskia et al. (2015)	11	ANN	86.90
Hailu & Meshesha (2016)	4	ANN	95.10
Dolata & Reiner (2018)	8	CNN	97.24
Kozłowski et al. (2019)	6	CNN	93.21
Shi et al. (2021)	9	CNN	95.70
Singh et al. (2021)	35	CNN	98.38
Current Study	**14**	**CNN**	**96.07**

4. Conclusions

This study consisted of literature review, obtaining of seeds, taking images, preprocessing images, defining, training, and testing of CNN models, and deploy of web-based application. At the first stage, the seeds brought together were preserved in appropriate conditions in the laboratory environment, the photos were taken, then the images were cropped to small sizes and the training images were augmented. With this image data set, six different networks were trained and evaluated. According to

the test results performed, the DenseNet-169 CNN model showed the best performance by classifying 14 different barley seed cultivars with 96.07% test accuracy. At the last stage, a web-based application was developed, enabling the classification process from all web tools to be done independently from the platform.

The successful result obtained from the classifier will be the basis for the classification of other grain cultivars such as wheat, rice, and corn. In later studies, the success rate can be increased with the different deep learning models and classification techniques. The performance rate could be improved by increasing the number and quality of the images using the different types of microscopes. The classification of other cereal cultivars and the detection of foreign objects in their contents were targeted as the next studies.

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The Effects of Different Irrigation Levels and Nitrogen Doses on Growth, Quality and Physiological Parameters of Warm-season Turfgrasses

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ABSTRACT

This research was conducted to determine to effects of different irrigation levels and nitrogen doses (ND) on the various warm-season turfgrasses at the Agricultural Training and Research Centre of the Bursa Uludag University Faculty of Agriculture for two years in a row. The experimental design was the randomized blocks in a split-split plot design with three replications. The main plot was irrigation levels (I_1=25%, I_2=50%, I_3=75%, and I_4=100% of pan evaporation), subplots were turfgrass species [hybrid Bermudagrass (*Cynodon transvaalensis x Cynodon dactylon*) cv. Tifdwarf, seashore paspalum *(Paspalum vaginatum* Sw.) cv. Seaspray, zoysiagrass (*Zoysia japonica* Steud.) cv. Zenit], and sub subplots were ND's (monthly 0.0, 1.25, 2.5, and 5.0 g N m^{-2}). Visual turfgrass color and quality, clipping yield, leaf relative water content (RWC), loss of turgidity (LT), chlorophyll content (CC), and electrolyte leakage were measured. According to the results, significant differences were determined among irrigation levels, turfgrass species, and ND's for color, quality, clipping yield and physiological parameters. Turfgrass visual color, quality and clipping yield were shown to decrease significantly with decreases in irrigation water and N fertilizer. The study findings demonstrated that under a non-limiting water supply, irrigation could be decreased by adjusting N fertilizer rates with I_3N_3 treatments can maintain acceptable turfgrass visual color and quality under Mediterranean climatic conditions. In addition, at 25% (I_1) deficit irrigation level, leaf RWC, CC decreased significantly, while an increase was determined in LT. This research indicated that under 75% (I_3) deficit irrigation and N_3 ND, acceptable quality can be maintained with 'seaspray' seashore paspalum under Mediterranean climate performed.

Keywords: Deficit irrigation, Nitrogen fertilization, Turf color, Turf physiology

1. Introduction

Many turfgrass species have been used in Turkey's arid and semi-arid regions, where rainfall is restricted to only four or five months during winter and fall of the year. This situation keeps the issue of efficient use of water lawns on the agenda. Contrary to popular belief, Turkey is neither a country rich in freshwater resources nor the wealthiest country in the region. Turkey lies in a semi-arid zone, with just about one-fifth of the water available per capita in water-rich places like North America and Western Europe. Countries with 10.000 cubic meters of water per capita per year are considered to be water rich. This is far more than Turkey's per capita consumption of 1350 cubic meters. With a population of 100 million people predicted by 2030, this amount will drop to 1000 m^3 per capita/year (Turkey's Policy on Water Issues, 2021).

In order to provide the required functional and aesthetic characteristics, irrigation in turfgrass-covered leisure sites or sports grounds is important (Kneebone et al. 1992). However, water requirements are high, and maintaining a decent grade of turfgrass quality throughout the summer season is costly. Irrigation which was done carefully, substantially impacts the management costs and environmental

impact of turfgrass. Turfgrass managers and scientists wish to devise ways to maintain a given level of turf quality and reduce irrigation supplies considerably (Ervin & Koski 1998; Cereti et al. 2009). Previous studies with deficit irrigation have shown that satisfactory turf quality can be maintained substantially by reducing water rather than providing full evapotranspiration (ET) (Fu et al. 2004; DaCosta & Huang 2006; Fu et al. 2007a; Fu et al. 2007b; Colmer & Barton 2017). Wilt-based irrigation has been demonstrated to cut irrigation water use in half, minimize thatch mat layers, and improve lawn quality when compared to non-limiting soil moisture-based irrigation (Fu & Dernoeden 2009).

Due to scarce water resources in semi-arid climates, turfgrasses tolerant of lower irrigation regimes have gained popularity. Heat and drought stress can cause grasses to lose their leaf water potential, close their stomata, raise their canopy temperature, and produce reactive oxygen species (White et al. 1992; Leung & Giraudat 1998; Wang & Huang 2004).

Warm-season turfgrasses, which are the main subject of our research, are more resistant to drought and their water use efficiency is higher than cool-season turfgrasses (Huang 2008; Zhou et al. 2012). However, they can show the expected performance with less amount of fertilization. Therefore, it has an important position in terms of preventing some environmental problems such as water pollution and especially nitrate pollution. Excess nitrogen (N) fertilization pollutes groundwater and rivers because of N leaching (Snyder et al. 1984; Pathan et al. 2007).

Turfgrass cultivation requires the use of N fertilizer to provide fast and uniform growth, good color, high shoot density, and sufficient strength for harvesting (Beard 1973). N management is another essential aspect of water conservation that is sometimes disregarded (Brown et al. 2004). When turf lacks N, it loses quality faster than turf that is properly fertilized when exposed to moisture stress (Feldhake 1981).

In the Mediterranean climate zone, where the experiment is conducted, warm-season turfgrasses are less common than cool-season turfgrass because of low temperatures in the fall and winter seasons, their availability on the seed market, and limited availability of vegetative production sources (Kir et al. 2010; Severmutlu et al. 2011; Aydinsakir et al. 2016). Previous studies in this region focused only on cool-season turfgrass (Bilgili et al. 2011; Uzun & Bilgili 2011; Bilgili & Yonter 2016; Zere & Bilgili 2016).

The objectives of this research were to determine the effects of different irrigation levels and nitrogen doses (ND) on growth, quality and physiological characteristics of three warm-season turfgrass species including seashore paspalum, zoysiagrass and hybrid Bermudagrass under Mediterranean-type environments in Turkey.

2. Materials and Methods

This study was conducted at the Agricultural Training and Research Centre of the Bursa Uludag University Faculty of Agriculture experimental turfgrass plots in 2014 and 2015. The research site was situated in Bursa/Turkey (40° 13' 36" N latitude, 28° 51' 35" E longitude, 112 m elevation), with a Mediterranean climate.

The climate data was slightly different between the growing seasons with average temperatures of 16.0 °C and 15.4 °C in 2014 and 2015, respectively (Table 1). The long-term (1950-2015) average temperature, average relative humidity, and annual precipitation were 14.6 °C, 64.7%, and 694.1 mm, respectively.

In the first growing season, relative humidity was slightly higher (9%) and the average temperature was 1 °C above the long-term average (Table 1).

Regarding soil analysis reports, the upper 20 cm of the soil was considered loamy and rich in K. Receivable p level was 30.9 mg kg^{-1} and soil pH was 8.5 (Table 2). Undisturbed and disturbed soil samples were used to determine field capacity and wilting point according to Cassel & Nielsen (1986) and Tuzuner (1990). Total available moisture and average bulk density for a 0-30 cm soil profile were 45.0 mm and 1.35 g cm^{-3}, respectively.

The experiment consisted of three warm-season turfgrass species including, seashore paspalum (cv. Seaspray), zoysiagrass (cv. Zenith) and hybrid Bermudagrass (cv. Tifdwarf). The trials were organized in a split-split plot design with three replications in randomized blocks. The main plots were laid out in accordance with the irrigation levels, and the subplots were the turfgrass species. Finally, the ND's (0.0, 1.25, 2.5 and 5.0 g N m^{-2} $month^{-1}$) were placed into sub-sub plots, which possessed dimensions of 1 x 2=2 m^2 (Table 3).

Four irrigation levels [25% (I_1), 50% (I_2), 75% (I_3), and 100% (I_4) of pan evaporation] were applied, during the experiment period each year from May to September as described by Emekli et al. (2007). The US Weather Bureau Class A pan evaporation method (Epan) was

Table 1- The climate data of the experimental area

Months	*Temperature (°C)*			*Precipitation (mm)*			*Relative humidity (%)*		
	2014	*2015*	*LT*[*]	*2014*	*2015*	*LT*	*2014*	*2015*	*LT*
January	9.0	5.4	5.5	30.8	112.0	82.9	70.4	79.0	70.0
February	8.6	7.3	6.1	20.4	74.2	70.7	73.7	76.5	68.7
March	10.7	9.1	8.6	42.4	78.2	66.1	69.6	79.1	67.7
April	14.5	11.5	13.0	112.0	95.6	66.0	71.1	70.1	66.1
May	18.3	19.3	17.4	96.8	36.0	43.4	71.7	64.2	62.0
June	22.3	21.7	22.5	94.4	37.8	36.5	70.7	72.0	57.8
July	25.6	25.5	24.8	4.6	0.0	17.7	64.5	60.7	56.2
August	25.7	26.4	24.5	45.4	5.6	13.8	67.9	61.5	57.3
September	20.6	23.6	20.2	115.6	98.1	40.8	76.6	73.2	63.8
October	16.4	16.4	15.0	68.6	93.2	75.5	80.2	83.7	68.7
November	11.3	12.7	10.5	72.4	26.4	79.9	83.0	78.1	69.3
December	9.3	5.6	7.2	143.2	3.0	100.8	87.3	76.6	68.7
Total	-	-	-	846.6	660.1	694.1	-	-	-
Average	16.0	15.4	14.6	-	-	-	73.9	72.9	64.7

[*]LT: long term mean (1950-2015)

Table 2- Soil properties of the experimental field

%Sand	46.25	EC, µS cm^{-1}	468
%Silt	30.99	%Organic matter	2.091
%Clay	22.76	%N	0.106
Texture class	Loam	P, mg kg^{-1}	30.95
pH	8.48	Total K	5180

used to calculate the irrigation levels. The experimental area was irrigated at 3-day intervals utilizing a pop-up sprinkler (PSU-04-15A, Hunter, USA) irrigation system. To create a 90° wetting pattern, four corners of each 4 x 6-meter plot were placed on sprinkler heads, and sprinkling was done at a rate of 7.5 mm h^{-1}, which was lower than the soil infiltration rate (8.0 mm h^{-1}). N (Ammonium nitrate, 26%) fertilization was applied monthly from May to September, which was broadcasted by hand in the middle of each month.

Before irrigation, in the center of each plot at depths of 0.15 m and 0.45 m neutron probe (503 DR Hydroprobe, CPN International, Inc., Martinez, CA, USA) was used to assess the soil water content (SWC). Between two consecutive soil water measurement dates, the ET of turfgrass was calculated as the residual of the soil water balance. Each irrigation treatment's ET was figured out separately using the formula:

$$ET = I + P - RO - DP + CR \pm \Delta SF \pm \Delta SW$$

Where *I* represent the depth of irrigation water (mm), *P* represents precipitation (mm), *SF* represents water transferred in or out of the root zone horizontally by subsurface flow, *SW* represents the change in *SWC* (mm), *RO* represents the depth of runoff (mm), *DP* represents deep percolation below the root zone (mm), and *CR* represents capillary rise. Water meters were used to measure irrigation water, and *P* was calculated using meteorological station data. Because the spreading rate was under the infiltration rate, no runoff occurred. Given the difficulties in determining *SF, DP*, and *CR* from a water table over short periods (Allen et al. 2007), *SF* and *CR* were set to zero. Using moisture measurements for the 0.6 m soil profile to account for percolation, the soil water balance was calculated. Doty et al. (1990) observed on cool-season turfgrasses that a significant portion of grass water uptake occurs in the 0-25 cm soil profile; thus, effective rooting depth was assumed to be 0.3 m. Emekli et al. (2007), Wherley et al. (2015) and Amgain et al. (2018) also used in their studies with similar effective rooting depth.

Turf color, turf quality and clipping yield were all determined in this study. Prior to mowing, turfgrass color was assessed visually on a scale of 1-9 (1= completely yellow, 6= light green; 9= dark green) on each clipping date. Turfgrass quality ratings were evaluated

Table 3- Experimental design of the study

	I			*II*			*III*		
	TS-1	*TS-2*	*TS-3*	*TS-2*	*TS-1*	*TS-3*	*TS-1*	*TS-3*	*TS-2*
25% (I_1)	1.25	2.50	1.25	1.25	5.00	0.00	0.00	2.50	0.00
	2.50	0.00	5.00	5.00	1.25	5.00	2.50	0.00	1.25
	0.00	1.25	0.00	0.00	2.50	2.50	5.00	1.25	2.50
	5.00	5.00	2.50	2.50	0.00	1.25	1.25	5.00	5.00
	TS-2	TS-1	TS-3	TS-1	TS-2	TS-3	TS-3	TS-1	TS-2
50% (I_2)	1.25	1.25	1.25	0.00	2.50	0.00	2.50	2.50	0.00
	2.50	0.00	5.00	5.00	0.00	5.00	0.00	0.00	5.00
	5.00	5.00	0.00	2.50	1.25	2.50	1.25	1.25	1.25
	0.00	2.50	2.50	1.25	5.00	1.25	5.00	5.00	2.50
	TS-3	TS-2	TS-1	TS-2	TS-1	TS-3	TS-1	TS-3	TS-2
75% (I_3)	5.00	1.25	0.00	0.00	2.50	0.00	5.00	2.50	2.50
	1.25	5.00	2.50	2.50	5.00	2.50	0.00	5.00	0.00
	0.00	0.00	5.00	1.25	1.25	1.25	2.50	0.00	5.00
	2.50	2.50	1.25	5.00	0.00	5.00	1.25	1.25	1.25
	TS-1	TS-3	TS-2	TS-2	TS-1	TS-3	TS-1	TS-2	TS-3
100% (I_4)	5.00	0.00	5.00	0.00	1.25	2.50	2.50	0.00	5.00
	1.25	1.25	1.25	2.50	0.00	5.00	5.00	5.00	0.00
	0.00	2.50	2.50	1.25	5.00	1.25	0.00	2.50	2.50
	2.50	5.00	0.00	5.00	2.50	0.00	1.25	1.25	1.25

Irrigation Levels: 1. %25 (I_1), 2. %50 (I_2), 3. %75 (I_3), and 4. %100 (I_4) of pan evaporation, Turfgrass Species: TS-1. Hybrid Bermudagrass, TS-2. Seashore paspalum TS-3. Zoysiagrass, Nitrogen Doses: 1. 0.0, 2. 1.25, 3. 2.5 and 4. 5.0 g N m^{-2} $month^{-1}$

according to National Turfgrass Evaluation Program (NTEP) guidelines using a 1–9 visual rating scale, where 1= poorest, 6= acceptable, and 9= best (NTEP, 2010) based primarily on texture, uniformity, density, and turf color. Drought-induced leaf wilting, rolling, and browning were also considered when rating turfgrass quality, with a score of 6.0 or higher considered acceptable. For each year, clipping yields were evaluated monthly. After reach to 6-8 cm height (18 June, 24 July, 29 August and 09 October in the first year and 05 June, 15 July, 24 August and 05 October in the second year), a 0.5 m × 1.0 m strip of turf at the center of each sub subplot was clipped to 4 cm height. The clipped turf samples were taken to dry. Samples were dried at 70 °C for 24 h and then weighed (Bilgili et al. 2017).

Leaf relative water content (RWC), loss of turgidity (LT), chlorophyll content (CC) and electrolyte leakage (EL) were analyzed to determine irrigation and fertilizer application effects on warm-season turfgrasses. Physiological analyses were done in July 2015, which was mostly high temperature and almost entirely drought month regarding the region.

Leaf RWC and LT were measured by a previously described method by Salisbury & Ross (1992) and Gulen & Eris (2003). Leaf tissues 1.0 cm long were cut from each of the three fully expanded and uniform leaves (replicates) per treatment. Initially, the fresh weight was enrolled, and then samples were settled in a petri dish of distilled water for four hours. After gently blotting the leaf surface with paper, turgid weights were recorded. At the end of this stage, leaf examples have put a drying at 70 °C for 24 h to determine the dry weight. Leaf RWC and LT were measured as follow;

RWC (%) = [(fresh weight - dry weight) / (turgid weight - dry weight)] × 100

LT (%) = [(turgid weight - fresh weight) / turgid weight] × 100

The relative CC was measured on the ten whole leaves with the portable chlorophyll meter (Minolta, SPAD-502) for each plot. First, leaves were measured individually at three points: on the upper, middle, and lower parts, and then, average SPAD readings were calculated by Gulen & Eris (2003); Moran & Porath (1980).

Primarily, turfgrass samples were cut 1 cm long three replications per fertilization treatment to determine EL. Leaf tissues were washed kindly in pure water, blotted gently with paper, and put in test tubes. Purified water (20 mL) was filled in test tubes. Then, the samples were leached by vacuum infiltration to let uniform diffusion of electrolytes and shaken on a gyratory shaker (at 250 rpm) overnight at room temperature. After incubation, solutions' electrical conductivity was analysed using a conductivity meter (WTW TetraCon 325; InoLab, Weilheim, Germany). Afterward, leaf tissues were killed by autoclaving solution likewise, and the total conductivity was gauged at room temperature. Finally, percentage of injury was calculated for all temperatures from the EL data using that notation (Arora et al. 1992):

% Injury = [(% L (t) - % L (c)) / (100 - % L (c)] 100 where % L (t) and %L (c) are the percentage EL data for the treated and control examples, respectively.

All data were subjected to analysis of variance (ANOVA). Irrigation level, turfgrass species, N doses and their interactions were separated statistically different groups by using the least significant difference (LSD) test at the 0.05 or 0.01 probability levels by using JMP Pro 13.

Results and Discussion

3.1. Applied Irrigation Water and Evapotranspiration

The total amounts of irrigation water and rainfall (mm) for the first and the second year are shown in Table 4. Total irrigation water amounts ranged from 144.3-577.2 mm in the first year to 188.5-753.8 mm in the second year, while total rainfall amounts were 226.2 mm and 88.3 mm, respectively. Table 5 shows the seasonal ET rates for each treatment group.

Maximum seasonal ET values were obtained in the second season for the I_4 x N_4 treatment in Bermudagrass, seashore paspalum, and zoysiagrass being 860 mm, 856, and 838, respectively. Based on the applied irrigation water amounts and precipitation data, the seasonal ETc values were determined to be ranged from 356 mm to 860 mm in the first season and from 282 mm to 856 mm in the second season in terms of all turfgrasses (Tablo 5).

Table 4- The total amount of irrigation water and rainfall in 2014 and 2015

Irrigation	*Irrigation water applied (mm)*	*Rainfall (mm)*
	2014	
I_1	144.3	226.2
I_2	288.6	
I_3	432.9	
I_4	577.2	
	2015	
I_1	188.5	88.3
I_2	376.9	
I_3	565.4	
I_4	753.8	

In a study conducted with Bermudagrass under a Mediterranean climate, it was reported that the total irrigation water and seasonal water use in 100 percent of Class A pan was 1168.2 mm and 1186 mm, respectively (Emekli et al. 2007). In another study conducted in Mediterranean climate conditions, it was observed that the total amount of water applied to the golf course turf and the total ET were 780.3 mm and 896 mm, respectively (Bastug & Buyuktas 2003). The applied irrigation water and ETc values in our study were found lower than research results of Bastug & Buyuktas (2003) and Emekli et al. (2007).

The relationships between N doses and seasonal ETc values obtained for each irrigation treatments and species are given in Figure 1. Linear relationships with a high correlation coefficient in the increasing direction were determined between N dose and ETc (except for seashore paspalum under irrigation treatment I_1 for 2015). However, the linear relationships obtained for both years in Bermudagrass and seashore paspalum species under irrigation treatment I_4 (100% of pan evaporation) were statistically significant. Turfgrass uses

Table 5. Seasonal evapotranspiration (mm) was determined in irrigation levels, turfgrass species, and nitrogen doses for the 2014 and 2015 experimental years

Irrigation	*Nitrogen*	*Bermudagrass*		*Seashore paspalum*		*Zoysiagrass*	
		2014	*2015*	*2014*	*2015*	*2014*	*2015*
I_1	N_1	356	313	349	295	344	299
	N_2	360	312	356	282	333	298
	N_3	380	315	356	304	343	308
	N_4	377	326	367	308	368	313
I_2	N_1	499	474	472	467	463	461
	N_2	506	476	467	460	485	458
	N_3	505	498	471	483	499	476
	N_4	523	507	494	487	500	477
I_3	N_1	632	673	603	646	638	637
	N_2	635	694	612	649	640	644
	N_3	641	692	649	651	646	655
	N_4	664	696	653	679	648	674
I_4	N_1	764	821	757	810	743	802
	N_2	782	837	769	833	777	826
	N_3	787	844	782	832	788	825
	N_4	814	860	800	856	794	838

more water when the N fertilization dose increases due to the higher growth encouraged by the fertilizer (Carrow & Duncan 2003). Some researchers have observed that reducing the N fertilizer dose can reduce turfgrass ET in the presence of an unlimited water supply; however, these studies have not consistently provided quantitative data on how the N dose affects turfgrass quality (Shearman & Beard 1973; Feldhake et al. 1983). As a result, it's not always obvious how much water may be saved by changing N fertilizer doses. The including turfgrass quality indicators is critical for determining the extent of N fertilizer doses may be utilized to reduce turfgrass water usage.

3.2. Turfgrass Color, Quality and Clipping Yield

During both experimental seasons, irrigation levels, turfgrass species, and N doses substantially impacted visual turfgrass color, quality, and clipping yield. IL x TS and IL x ND two-way interactions were important in some observation for color and quality. Therefore, TS x ND interaction was not important in all date. In addition, most of all two-way, and all three-way interactions were significant for clipping yield (Table 6). Regardless of the effects of the year, the data were analyzed individually for each year because the length of the observation dates varied from year to year (Kopp & Guilard 2002). Irrigation levels, turfgrass species and N doses affect the observed parameters (Table 7 and 8).

The lowest irrigation level (I_1) produced the lowest color, quality, and clipping yield were taken from throughout the trial I_4 irrigation treatment produced the highest turf color and quality ratings, and clipping yield in general. In a similar study that used color as key quality criteria, a reduction in the ET rate of Kentucky bluegrass was related to a decline in quality (Feldhake et al. 1984). Acceptable color and quality were maintained at I_3 and I_4 treatments, whereas I_1 did not produce desirable color quality each experimental season. The turf color and quality values of all turfgrasses included in the study were either not statistically significant or very close to each other according to the month in both experimental years (Table 7 and 8).

The N doses had a substantial impact on turf color, quality, and clipping yield (Table 7 and 8). Overall, the 5 g N m^{-2} treatment outperformed the other N treatments in turf color, quality, and clipping yields. In this study, the 2.5 g N m^{-2} dose produced acceptable turf color and quality while yielding lower clipping yields than the high N dose both years.

Control (0 g N m^{-2}) N doses, on the other hand, resulted in the lowest evaluations for turf color, quality, and clipping yields. According to Bilgili & Acikgoz (2011), increasing doses of N application consistently improved color, quality ratings, and clipping yields on a turf mixture (50% perennial ryegrass, 30% Kentucky bluegrass, 10% chewing red fescue, and 10% creeping red fescue). Salman and

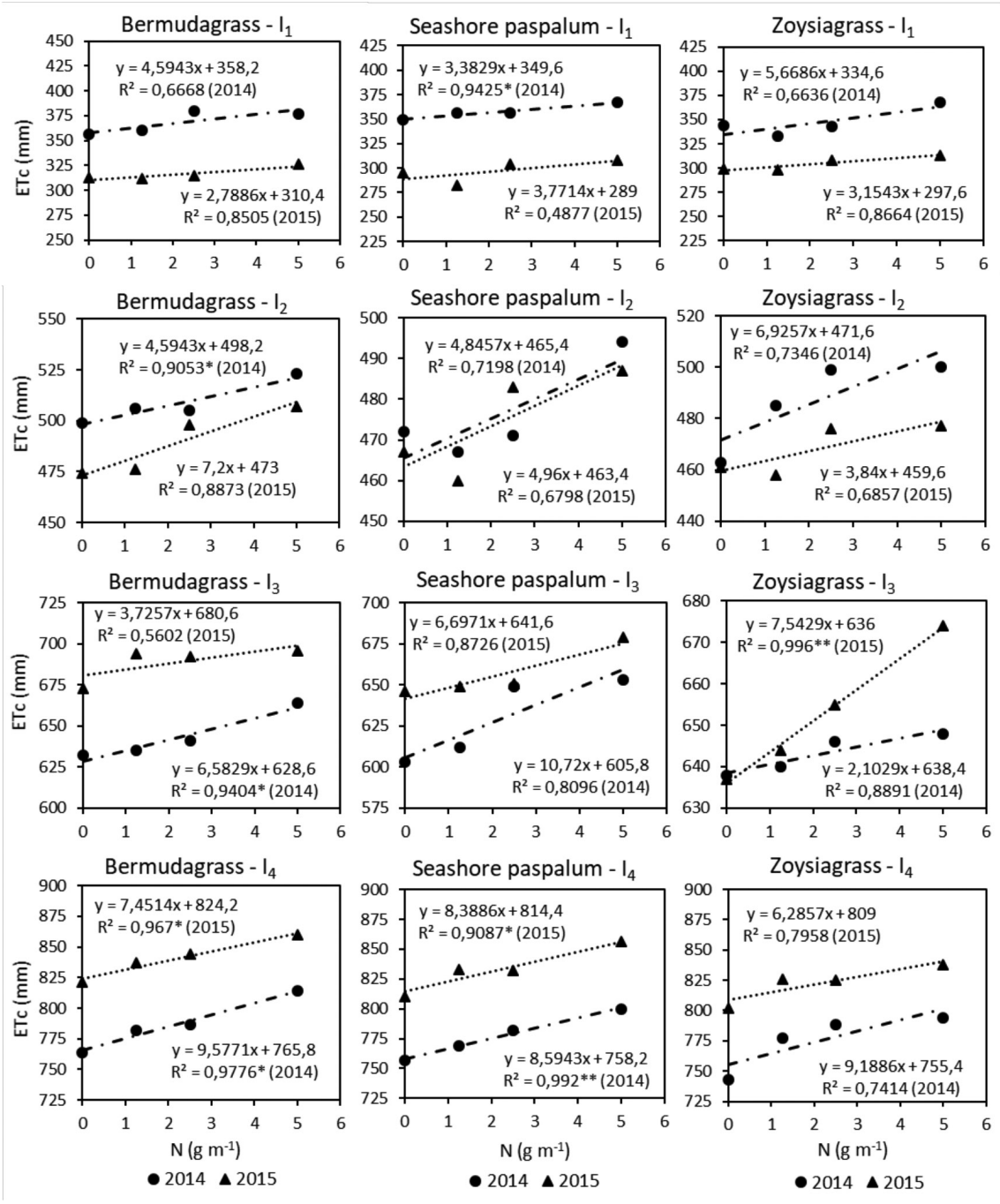

Figure 1- The relationships between N rates and seasonal ETc values obtained for each irrigation treatments and species

Avcioglu (2010) determined that ND of 10 g N m^{-2} was the best dose for turf color and quality on tall fescue and perennial ryegrass in a Mediterranean climate on loamy soil. The I_3 irrigation level and 2.5 g N m^{-2} dose treatment maintained acceptable turfgrass color and quality throughout each experimental season.

The best clipping yield values were obtained from I_4 irrigation treatment in both years, but the lowest values were obtained I_1 treatment. Generally, high values were taken regarding clipping yield from zoysiagrass in both years. Highest clipping yield values were taken from 5 g N m^{-2} dose like I_4 irrigation treatment in both years.

IL x TS interaction color ratings in the first year was found significant only in the July observation. The highest color ratings were obtained from seashore paspalum and zoysiagrass at I_4 irrigation level, and Bermudagrass at I_3 irrigation level. In the second year, IL x

Table 6- Results of variance analysis of some warm-season turfgrass species (TS) color, quality, and clipping yields under different irrigation levels (IL), and nitrogen doses (ND) in the 2014-2015 experimental years

Sources of variation	*2014*				*2015*			
	Color							
	18 June	*24 July*	*29 August*	*09 October*	*05 June*	*15 July*	*24 August*	*05 October*
IL	**	**	**	*	**	**	**	**
TS	ns	ns	ns	ns	**	**	**	**
ND	**	**	**	**	**	**	**	**
IL x TS	ns	*	ns	ns	**	*	ns	*
IL x ND	*	ns	*	ns	ns	ns	ns	*
TS x ND	ns	ns	ns	ns	ns	ns	ns	ns
ILx TS x ND	ns	ns	ns	ns	ns	ns	ns	ns
	Quality							
IL	**	**	**	**	**	**	**	*
TS	*	**	ns	**	**	*	**	**
ND	**	**	**	**	**	**	**	**
IL x TS	ns	**	*	*	**	ns	ns	ns
IL x ND	*	**	**	ns	**	**	*	ns
TS x ND	ns	ns	ns	ns	ns	ns	ns	ns
ILx TS x ND	ns	ns	ns	ns	ns	ns	ns	ns
	Clipping yield							
IL	**	**	**	**	**	**	**	**
TS	**	**	**	**	**	**	**	**
ND	**	**	**	**	**	**	**	**
IL x TS	**	**	**	**	**	**	**	**
IL x ND	ns	**	**	**	**	**	**	**
TS x ND	**	**	ns	**	**	**	**	**
ILx TS x ND	**	**	**	**	**	**	**	**

*,**F-test significant at p≤0.05 and p≤0.01, respectively. ns: not significant

TS interaction color ratings were found significant in the June, July, and October observations. Generally, the highest color ratings were found at I_4 irrigation level in all turfgrass species. However, I_1 irrigation level and zoysiagrass interaction gave the lowest color ratings.

IL x ND interaction color ratings in the first year were found significant only in the June and August observations. I_3 irrigation level x N_4 ND and I_4 irrigation level x N_4 ND gave the highest color ratings both observation dates. In the second year, IL x ND interaction color ratings were found significant only in the October observation and I_4 irrigation level x N_4 ND interaction gave the highest turfgrass color ratings at the same date. IL x ND interaction quality ratings were found significant in the June, July and August observation dates, and in general, I_4 irrigation level x N_4 ND interaction gave the highest turfgrass quality ratings at the same dates in both years. IL x ND interactions clipping yield values were found important in July, August, and October observation dates in the first year but in the second year all dates. Generally, I_4 irrigation level x N_3 ND, I_4 irrigation level x N_4 ND and I_3 irrigation level x N_4 ND interactions gave the highest clipping yield in both years.

IL x TS interactions turfgrass quality ratings were found significant in the July, August and October observation dates in the first year. I_4 irrigation level x all turfgrass species interactions gave the highest quality ratings by taking 6.5 and above ratings. In the second year, IL x TS interaction quality ratings were found significant only in the June observation, and the highest quality ratings were obtained from I_4 x seashore paspalum and I_4 x zoysiagrass interactions at the same date. IL x TS interactions clipping yield values were found significant in all observation dates in both years. Generally, zoysiagrass x I_4 irrigation levels interactions gave highest clipping yields both years.

Table 7- Turf color of some warm-season turfgrass species (TS) under different irrigation levels (IL), and nitrogen doses (ND) in the 2014-2015 experimental years

IL	*Color*							
	2014				*2015*			
	18 June	*24 July*	*29 August*	*09 October*	*05 June*	*15 July*	*24 August*	*05 October*
I_1*	6.1 c	6.0 c	5.8 c	5.7 b	5.5 c	5.8 c	5.8 d	5.3 c
I_2	6.8 b	6.6 b	6.5 b	5.9 ab	6.1 b	6.4 b	6.2 c	6.1 b
I_3	6.9 b	7.0 a	6.8 ab	6.0 a	6.5 a	6.5 b	6.4 b	6.3 ab
I_4	7.2 a	7.0 a	7.0 a	6.1 a	6.8 a	7.0 a	6.7 a	6.6 a
LSD (0.05)	0.26	0.18	0.38	0.25	0.34	0.17	0.18	0.34
TS								
1**	6.7	6.6	6.5	5.9	6.1 b	6.5 a	6.2 b	5.9 b
2	6.8	6.7	6.6	6.0	6.7 a	6.6 a	6.5 a	6.3 a
3	6.8	6.6	6.5	6.0	5.9 b	6.2 b	6.1 b	5.9 b
LSD (0.05)	Ns	Ns	Ns	Ns	0.17	0.20	0.21	0.19
ND								
0.00***	5.1 d	5.1 d	5.0 d	4.8 d	4.7 d	4.9 d	4.7 d	4.5 d
1.25	6.7 c	6.5 c	6.2 c	5.7 c	5.9 c	6.2 c	5.8 c	5.7 c
2.50	7.3 b	7.1 b	6.9 b	6.3 b	6.7 b	7.0 b	6.9 b	6.6 b
5.00	8.0 a	7.9 a	8.0 a	7.0 a	7.6 a	7.7 a	7.7 a	7.4 a
LSD (0.05)	0.20	0.23	0.19	0.22	0.19	0.19	0.20	0.18

*I_1=25%, I_2=50%, I_3=75%, I_4=100%, **1: Hybrid Bermudagrass (Cynodon transvaalensis x Cynodon dactylon) Tifdwarf, 2: Seashore paspalum (Paspalum vaginatum Sw) Seaspray, 3: Zoysiagrass (Zoysia japonica Steud.) Zenith, ***g m^{-2}

Table 8- Turf quality of some warm-season turfgrass species (TS) under different irrigation levels (IL), and nitrogen doses (ND) in the 2014-2015 experimental years

IL	*Quality*							
	2014				*2015*			
	18 June	*24 July*	*29 August*	*09 October*	*05 June*	*15 July*	*24 August*	*05 October*
I_1	6.1 c	5.8 c	5.5 d	5.3 c	4.8 d	5.2 d	5.1 d	5.2 b
I_2	6.2 bc	6.3 b	5.9 c	5.4 c	5.5 c	5.7 c	5.7 c	5.6 ab
I_3	6.5 b	6.5 b	6.6 b	5.8 b	6.1 b	6.1 b	6.2 b	6.0 a
I_4	7.0 a	6.9 a	6.8 a	6.3 a	6.8 a	6.7 a	6.5 a	6.0 a
LSD (0.05)	0.35	0.25	0.16	0.24	0.25	0.30	0.15	0.49
TS								
1	6.3 b	6.3 b	6.2	5.5 b	5.8 b	5.8 b	5.8 b	5.6 b
2	6.7 a	6.5 a	6.2	5.7 a	6.1 a	6.1 a	6.1 a	5.9 a
3	6.3 b	6.3 b	6.2	5.8 a	5.6 c	5.8 b	5.7 b	5.5 b
LSD (0.05)	0.32	0.14	ns	0.16	0.19	0.22	0.22	0.21
ND								
0.00	4.9 d	4.9 d	4.9 d	4.8 d	4.6 d	4.7 d	4.5 d	4.4 d
1.25	6.3 c	6.1 c	5.9 c	5.5 c	5.5 c	5.6 c	5.4 c	5.3 c
2.50	7.0 b	6.9 b	6.5 b	6.0 b	6.2 b	6.3 b	6.3 b	6.1 b
5.00	7.6 a	7.6 a	7.5 a	6.5 a	7.0 a	7.1 a	7.3 a	6.9 a
LSD (0.05)	0.26	0.19	0.19	0.20	0.19	0.20	0.20	0.21

In the first year except for August observation, the rest of all observation dates TS x ND interactions clipping yield values were found important. Both years generally gave the highest clipping yield values were obtained from seashore paspalum x N_4 and N_3 irrigation level, zoysiagrass x N_4 and N_3 irrigation level interactions.

3.3. Physiological Parameters

An ANOVA indicated that statistically significant ($p<0.01$) differences for all physiological parameters. All physiological parameters were affected from irrigation levels, turfgrass species and ND's treatments and two-way interactions and three-way interaction (Table 9).

The results showed that irrigation levels, turfgrass species, nitrogen doses, and their interactions significantly affected RWC (Table 10).

According to IL x TS, the seashore paspalum gave the highest RWC at all irrigation levels. The lowest RWC was generally obtained from I_1 deficit irrigation in all warm-season turfgrasses, while the well-watered irrigation level (I_4) gave the highest RWC. In terms of IL x TS interaction, the lowest RWC in IL x TS interaction was obtained from the lowest irrigation level of zoysiagrass. The lowest RWC value in IL x ND interaction was obtained from the I_1 deficit irrigation level. The well-watered irrigation level (I_4) gave the highest RWC at all N doses. The highest RWC was obtained from the highest dose (5 g m^{-2}) in all irrigation levels. The lowest RWC value in TS x ND interaction was generally obtained from the control fertilization (0 g m^{-2}). In comparison, high N fertilization (5 g m^{-2}) was given the highest RWC in all turfgrass species. The highest leaf RWC values were taken in seashore paspalum among the turfgrasses at all N doses.

Table 9- Results of variance analysis of leaf relative water content, loss of turgor, relative CC, and electrolyte leakage for irrigation levels, turfgrass species, and ND's in the 2015 year

Sources of variation	*2015*			
	RWC	*LT*	*CC*	*EL*
Irrigation levels (IL)	**	**	**	**
Turfgrass species (TS)	**	**	**	**
Nitrogen doses (ND)	**	**	**	**
IL x TS	**	**	**	**
IL x ND	**	**	*	**
TS x ND	**	*	*	**
ILx TS x ND	**	**	**	**

Table 10- Leaf relative water content, loss of turgor, CC and electrolyte leakage regarding irrigation levels (IL), turfgrass species (TS) and nitrogen doses (ND) mean values in the 2015 year

IL	*RWC*	*LT*	*CC*	*EL*
I_1	48.2 d	39.1 a	22.8 b	21.7 a
I_2	55.9 c	32.5 b	23.3 b	15.5 b
I_3	66.8 b	27.5 c	26.6 a	14.1 c
I_4	74.3 a	16.3 d	26.1 a	11.7 d
LSD (0.05)	4.93	3.20	1.44	0.75
TS				
1	51.3 c	36.7 a	20.2 c	23.8 a
2	79.0 a	15.9 c	30.6 a	9.0 c
3	53.7 b	33.8 b	23.4 b	14.5 b
LSD (0.05)	2.26	2.29	0.98	0.80
ND				
0.00	58.1 b	31.9 a	20.9 d	18.5 a
1.25	60.2 b	30.2 a	23.6 c	15.7 b
2.50	57.7 b	30.0 a	25.7 b	15.2 b
5.00	69.3 a	23.2 b	28.6 a	13.7 c
LSD (0.05)	2.95	3.18	1.18	0.67

Leaf RWC is commonly used to measure the water status (Pu et al. 2003). In the last decade, many studies directly estimate leaf parameters such as leaf RWC (Penuelas et al. 1993; Pu et al. 2003). Plants respond to decreased soil and cellular water content with changes in their morphological, physiological, and biochemical properties. Where water is scarce, these characteristics determine the survival of plants (Nilsen & Orcutt 1996). Many other articles described the relationship between drought tolerance and leaf water (Huang et al. 1998; White et al. 2001). Variations in drought tolerance were found among hybrid Bermudagrass, seashore paspalum, and zoysiagrass cultivars. The highest leaf RWC values were determined from seashore paspalum in I_4 irrigation level.

In our study, RWC decreased during drought stress, while grass species treated with high N compared to low N treatment showed higher leaf RWC under all irrigation conditions. The highest LT was observed in all ND's under deficit irrigation, while the lowest LT was kept in full irrigation conditions. Turfgrasses treated with high N showed higher leaf RWC during the drought than turfgrasses treated with low N (Chang et al. 2016). Fertilization is a practice that could increase water use efficiency or reduce water stress effects in arid areas (Carrow 2006; Ahmad et al. 2014). However, there was no substantial relation between lowering fertilization rates and water savings (Ebdon et al. 1999).

LT of turfgrass species used in this study had significant differences. The results showed that irrigation levels, turfgrass species, ND's, and interactions significantly affected LT (Table 9).

The present study results clearly showed that LT increased in all turfgrass species under water deficit compared to the well-watered conditions. While the highest value of LT was obtained from the I_1 deficit irrigation level, the lowest LT was obtained at a well-watered level (I_4). The result showed that LT of hybrid Bermudagrass was highest among the three species, followed by zoysiagrass and seashore paspalum. Five g m^{-2} N dose gave the lowest LT value among N doses. However, other N doses were included in the same statistical group. Our results showed that without N fertilizer and low N fertilizer doses increased LT (Table 10).

According to IL x TS interaction, zoysiagrass gave the highest LT at I_1 deficit irrigation level. The lowest LT was obtained from the well-watered level (I_4) in all warm-season turfgrasses, while the I_1 deficit irrigation level gave the highest LT. The highest LT in IL x ND interaction was obtained from the I_1 deficit irrigation level and low N fertilization levels. The well-watered irrigation level (I_4) gave the lowest LT at all N doses. The lowest LT was obtained from the highest N dose (5 g m^{-2}) in all irrigation levels. The lowest LT value in TS x ND interaction was obtained from the high N fertilization (5 g m^{-2}) in all warm-season turfgrass species. Especially zoysiagrass had the least loss of turgor in all N doses. However, hybrid Bermudagrass generally suffered the most LT.

In our study, the highest LT was observed in hybrid Bermudagrass under I_1 deficit irrigation. Especially seashore paspalum has been the species with the lowest LT under I_2, I_3 and I_4 irrigation levels. On the other hand, the least LT was observed under all irrigation levels in seashore paspalum among the all warm-season turfgrasses. Although the most tolerant turfgrass was a seashore paspalum and the least susceptible was a zoysiagrass, hybrid Bermudagrass and zoysiagrass had the same irrigation levels same drought tolerance levels, suggesting that both species have a range of drought tolerance. Similar to our results, paspalum genotypes were found to have better drought performance than hybrid Bermudagrass (Huang et al. 1997). However, Sever Mutlu et al. (2011) stated that Bermudagrass and buffalograss species/cultivars may perform better than buffalograss, bahiagrass, seashore paspalum, zoysiagrass, centipedegrass warm-season species. Changes in environmental conditions such as soil type or weather can create these differences. They could be due to genetic differences between cultivars with different levels of natural drought tolerance (Jespersen et al. 2019). Drought tolerance and recovery of various warm-season turfgrasses during drought were associated with osmotic adjustment (Qian et al. 1997). More specifically, drought tolerance involves maintaining positive turgor pressure at low tissue water potential and includes osmotic adjustment and dehydration tolerance achieved through protoplasm resistance (Gibeault et al. 1989; Carrow 1996; Jones et al. 2004). Carrow (1996) found that further drought tolerance before stress was associated with low basal osmotic potential, osmotic adaptation, maintenance of positive turgor pressure, and delayed leaf rolling during stress.

Comparative water use efficiencies in turfgrass species differ markedly from their relative drought tolerance because each is a more specifically different physiological phenomenon. Turfgrass species and cultivars have been found to respond differently to drought stress through drought resistance (Levitt 1980; Minner & Butler 1985). Under limited water conditions, plants may survive or even continue to grow by avoiding drought stress, showing tolerance to cellular water shortage, and both together (DaCosta & Huang 2006). Beard (1981) states that turfgrass characteristics and physiological hardiness associated with drought resistance can be much different than those plant characteristics that contribute to reduced water use rates and stresses. It should be recognized that a reduced water use rate will delay the onset of drought, as will a turfgrass species whose root system extends through a more significant portion of the soil profile. However, once the available soil moisture is exhausted, the ability of perennial grasses to survive a subsequent drought is the ultimate concern. Therefore, turfgrasses with severe drought resistance may not necessarily be those possessing the lowest water use rates.

The leaf CC measurements showed a significant difference among irrigation levels, turfgrass species, ND's and interaction effects (Table 9). CC significantly decreased under deficit irrigation conditions compared to the well-watered treatment (I_4). Water stress negatively influenced the CC in some species. The lowest LT was observed at a high N dose (5 g m^{-2}) in all turfgrass species.

In our study, seashore paspalum under all irrigation levels gave the highest CC values in IL x TS interaction. However, hybrid Bermudagrass in all irrigation levels gave the lowest CC. The well-watered irrigation level (I_4) in the seashore paspalum gave the highest CC, while the I_1 deficit irrigation level gave the lowest CC in hybrid Bermudagrass. The highest CC was generally taken from the 5 g m^{-2} N dose and I_3 and I_4 irrigation levels. On the other hand, control plots without N application and I_1 and I_2 deficit irrigation levels gave the lowest CC. The highest CC in TS x ND interaction was obtained seashore paspalum in all N doses. However, hybrid Bermudagrass gave the lowest CC in all N doses. The 5 g m^{-2} N dose gave the highest CC in the seashore paspalum, while the control N dose gave the lowest CC in hybrid Bermudagrass.

In the present study, the CC decreased according to the water stress in all turfgrass species. The CC was statistically in the same group at I_3 and I_4 irrigation levels. However, CC was generally lower in deficit irrigation levels such as I_1 and I_2. Especially under water stress conditions accompanied by extreme heat and radiation, chlorophyll is degraded, so the total content of this pigment in the leaves can determine the stress in the plant (Chylinski et al. 2007). It has been widely reported that decreased CC under limited water supply conditions leads to significant inhibition of leaf photosynthetic rates (Farooq et al. 2009).

The lowest CC was observed in both deficit irrigation and low N doses. The highest CC was obtained from 5 g m^{-2} N dose and I_3 and I_4 irrigation levels. CC in plants is a good indicator for leaf N concentration and is strongly correlated with leaf N and chlorophyll concentration (Blackmer & Schepers 1995; Saud et al. 2017). CC and its function is a direct measure of plant health (Brock, 2005). Chlorophyll levels increase linearly as applied N levels increase; thus, chlorophyll concentration correlates with N concentration in leaves, plant nutrition, and production (Benett et al. 2008).

The results showed that irrigation level, turfgrass species, ND's, and interaction significantly affected EL (Table 9).

The highest EL in IL x TS interaction was obtained from hybrid Bermudagrass in I_1 deficit irrigation level. Seashore paspalum generally shared the lowest EL. All warm-season turfgrasses gave the lowest EL in the well-watered (I_4), while the I_1 deficit irrigation level showed the highest EL. The lowest EL was obtained from the highest N dose (5 g m^{-2}) in all irrigation levels. The lowest EL was obtained with the highest ND (5 g m^{-2}) in all irrigation levels. However, the highest EL was obtained with the control N dose under I_1 deficit irrigation. The well-watered irrigation level (I_4) gave the lowest EL at all ND's. The seashore paspalum gave the lowest EL in TS x ND interaction in all N doses among warm-season turfgrass species. However, the highest EL was obtained from hybrid Bermudagrass in the control N dose.

Similar to our findings many researchers reported that increased drought stress increases EL (Guo et al. 2006; Liu et al. 2008). An increase in EL occurs when the rise in cell permeability (Blum & Ebercon, 1981). Lee et al. (2009) carried out their study deficit irrigation level by 100%, 80%, 60%, and 40%. There were significant differences among turfgrass varieties at all treatment of 100% irrigation level except 80%. The EL results of our research were similar to this research. Jiang & Huang (2002) showed that EL of tall fescue increased during drought stress conditions.

In summary, this research indicated that turfgrass requiring the least water to maintain quality in periods of hot summer months was seashore paspalum. Furthermore, the higher tolerance of seashore paspalum was associated with more effective osmotic regulation, reflected by the minor decrease in leaf RWC and cell membrane stability.

4. Conclusions

A two-year study was carried out to evaluate the visual turfgrass color and quality, as well as clipping yield and except for physiological parameters responses of some warm-season turfgrasses to various irrigation levels and ND's under Mediterranean climatic conditions. From the findings reported the following conclusions could be drawn.

1. The results of this study showed that the I_3 and I_4 irrigation levels under the 2.5 and 5 g N m^{-2} doses produced significantly higher ratings of color, quality and, clipping yields during both years.

2. The 100% (I_4) irrigation and 5 g N m^{-2} doses treatment resulted in a sufficiently dark turf color and quality; to achieve better seasonal turf quality under Mediterranean climatic conditions, this schedule can be adapted by evaluating the level of irrigation and N rate.

3. Based on the results of this study, it is concluded that under deficit irrigation conditions an acceptable turf quality can be sustained at 75% of Class A pan evaporation, and 2.5 g N m-2 doses treatment.

4. This research indicated that turfgrasses requiring the least water to maintain acceptable quality in periods of hot summer months were seashore paspalum cv. Seaspray.

5. Consequently, it was concluded that statistically significant differences were determined physiological effects of different irrigation levels and ND's on various warm-season turfgrasses.

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Identification of Self Incompatibility (*S*) Alleles in Turkish Apple Gene Sources using Allele-specific PCR

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ABSTRACT
Self-incompatibility (SI) is a genetic mechanism in many flowering plants by which generative reproduction is prevented. The self-incompatibility caused by the genetic functions of the cell is controlled by genes called S genes or self-incompatibility genes. Self-incompatibility results in decreased pollination and ultimately yield loss. In apple (*Malus domestica* L.), self-incompatibility is controlled by multi-allelic S-locus. Approaches in the S-glycoprotein profiles and allele-specific PCR methods using the gene profiles and S-glycoprotein profiles for determination of the incompatibility levels are of great importance. In current study, the self-incompatibility status of 192 apple genotypes (such as, Amasya, Hüryemez, Şah elması, Tokat, Demir elması etc.) obtained from the National Collection of Atatürk Horticultural Central Research Institute, Yalova, Turkey, has been determined. For this purpose, genotype-specific allele status and compatibility levels were screened via PCR (Polymerase Chain Reaction) using 4 different S-alleles (Sd, Sf, S26 and S9). 181 genotypes containing at least 1 S-allele were identified as 'Partially Incompatible' and 12 genotypes involving 4 S-alleles were assigned 'Totally Incompatible'. No S-alleles were observed in 2 genotypes (Pancarlık and Hüryemez) which exhibited 'Compatibility' status.

Keywords: *Malus domestica* L., Self-incompatibility, Anatolian gen resourses, *S*-alleles

1. Introduction

Apple (*Malus domestica* Borkh), belonging to the family *Rosaceae*, originates from the temperate countries of the Western Asia, between Black Sea and Caspian Sea. Apple trees are medium-sized, defoliating trees (Nour et al. 2010; Shaheen et al. 2017) and have economic significance worldwide (Shulaev et al. 2008). In addition to the commercial varieties production in the world, identification of individual species superior to natural gene sources is important for the production and improvement of the new variety candidates. Turkey possesses very rich apple gene sources. A National Collection including about 200 apple genotypes has been established within the Atatürk Horticultural Central Research Institute (Yalova, Turkey). No studies have been conducted yet about the self-incompatibility status of the genotypes/varieties in this collection.

Self-incompatibility is one of the cellular functions that protect intraspecific genetic diversity by preventing/decreasing self-pollination in flowering plants (Silva & Goring 2001). In most plant species, self-incompatibility is typically regulated by a gene locus containing several alleles (*S*-locus), on which at least two genes, pistil S and pollen S, are located and pollen tube inhibition occurs when the specificity of the same "*S*-allele" is expressed by both pollen and pistil. Gametophytic Self-Incompatibility (GSI) is the most common type of self-incompatibility (Franklin-Tongand & Franklin 2003; Abdallah et al. 2019) detected in large numbers of flowering plant species (Ma et al. 2018) so that, whenever the *S*–haplotype of pollen is homogeneous with one of the pistil *S*-haplotypes, pollen tube fails to grow in the style. In the apple GSI, the *S-RNase* gene and an *F-box* gene called *SFBB* (*S*-locus F-box brothers) act as pistil and pollen factors, respectively (Broothaert et al. 1995; De Franceschi et al. 2012). At the same chromosomal locus, a series of *SLF* (*S*-locus F-box protein) genes are aligned with *S-RNase*, which have been presumably obtained by gene exchange and duplication. The product of the pistil is *S-RNase*, which is considered an extracellular and polymorphic ribonuclease encoded by *S* gene (de Nettancourt 2001), whereas, the pollen *S* gene encodes a protein including F-box motif, called S haplotype-specific F-box protein (SFB). Later investigations have shown that *Rosaceae* SI system consists

of two distinct mechanisms, for example, in *Prunus* from *Amygdaleae* tribe, the SFB recognizes self *S-RNase*, through a self-recognition manner whereas, *Pyrus* and *Malus* from tribe *Pyreae* exhibit a non-self-recognition system in which a subset of non-self *S-RNases* are recognized specifically by various SFBB proteins of the SI system. Additional biological and biochemical description of the *S*-locus genes, along with the other SI-related genes located elsewhere than *S* locus, could elucidate the evolution, origin and molecular mechanisms of *Rosaceae* SI system (Sassa 2016).

In pollination of distantly related species, the SI ratio is considered to be one of the most important determinants of the diversity in evolutionary development of flowering plants (Whitehouse 1951). Pollinators are required for commercial production of many self-incompatible species and use of inappropriate pollinators results in economic loss.

Many fruit species (including *Malus* and *Pyrus*) in the family *Rosaceae* exhibit typical GSI (Shulaev et al. 2008). In terms of molecular mechanism of self-incompatibility in apple, *S*-allele genes have been isolated and characterized (Broothaerts et al. 1995). In many studies, apple specific self-incompatibility alleles were identified and *S*-genotypes were determined by using allele-specific PCR applications which are fast and useful methods (Sakurai et al. 1997, 2000; Verdoodt et al. 1998; Matsumoto & Kitahara 2000; Broothaerts &Van Nerum 2003; Broothaerts et al. 2004).

There are also a number of researches on proteins of self-incompatibility alleles. S-glycoproteins have been studied in Japanese pear (*Pyrus pyrifolia*) and it has been revealed that *S-RNase* (S_1, S_3, S_5, S_6, and S_7) regions that break the pollen tube growth were similar in *Pyrus* and *Malus* (Sassa et al. 1994; Janssens et al. 1995; Ishimizu et al. 1998; Van Nerum et al. 2001). Recent studies have shown that *S-RNases* interact with a conserved protein (MdROP) in the pistil of apple (Meng et al. 2014). Also, it is specified that, in both types of SI, programmed cell death (PCD) which is known as an active and genetically mechanism for the controlled elimination of targeted cells, plays a key role in the rejection of self-incompatible pollen (Serrano et al. 2015). In this regard, nitric oxide (NO) and reactive oxygen species (ROS), have been found as important regulators that are required for PCD in plants (Sadhu et al. 2019). In this sense, the H_2O_2 to NO ratio, determines the activation time of cell death (Delledonne et al. 2001) and ROS, which is produced from the degradation of O_3 in the apoplast is involved in both the initiation and progression of cell death (Overmyer et al. 2003; Sharma et al. 2012; Serrano et al. 2015).

In order to investigate the possible involvement of polyamines (PAs) and transglutaminase (TGase) in the reproduction of *Pyrus communis* L. plants, Mandrone et al. (2019) studied the content of free, soluble-conjugated and insoluble-bound PAs as well as the activity, abundance and immunolocalization of TGase. Results clearly indicate that during the SI response, TGase activity is increased, resulting in the accumulation of PAs conjugated to hydroxycinnamic acids and other small molecules. Li et al. (2018) also reported that treating with self *S-RNases*, leads to a marked growth inhibition in apple pollen tubes, as well as a decrease in endogenous soluble pyrophosphatase activity (MdPPa) and elevated levels of inorganic pyrophosphate (PPi). *S-RNase* binding to two variable regions of MdPPa leads to silencing of MdPPa expression and results in a reduction in pollen tube growth.

In the current study, compatible/incompatible allele profiles of 192 apple genotypes obtained from the National Collection of Atatürk Horticultural Central Research Institute, Yalova, Turkey, have been determined using 4 different *S*-alleles. Due to the successful amplification results and widely usage in *S*-allele screenings of the different geographic apple populations; S_d (Matsumoto & Kitahara 2000; Sakurai et al. 2000), S_f (Matsumoto & Kitahara 2000; Sakurai et al. 2000), S_{26} (Janssens et al. 1995; Halász et al. 2011; Brancher et al. 2020) and S_9 (Janssens et al. 1995; Halász et al. 2011; Brancher et al. 2020) *S*-allels were employed in this study to screen Turkish apple gene sources. The genotypes were classified according to compatibility levels and the possible correlations between compatibility and genetic similarities of the genotypes were revealed.

2. Material and Methods

2.1. Plant material

In this study, 192 apple genotypes obtained from Atatürk Horticultural Central Research Institute, Yalova, Turkey, were used as plant material. Genomic DNA was extracted from apple leaves using Lefort et al. (1998) method. DNA quantification was performed with Nanodrop ND-100 spectrometer and the DNA was visualized on 1% agarose gel.

2.2. PCR reactions

Allele-specific primers (S_9, S_{26}, S_f, S_d) were used to identify single alleles. Primers and their nucleotide sequences were used as described by Sakurai et al. (2000) for S_{26}, S_f and S_d alleles and Janssens et al. (1995) for S_9 allele (Table 1). The optimized PCR reactions for the mentioned primers were performed using 5 pmol primers, 25 mM $MgCl_2$, 100 µM dNTP mix, 5X PCR Buffer, 5U Taq polymerase and 50-250 ng genomic DNA in a total volume of 15 µL. Negative control was used to monitor contamination in each PCR reaction. TouchDown PCR program was applied in BioRad T100TM brand thermocycler as: 3-min pre-denaturation at 94 °C followed by 1-min denaturation at 94 °C, 1-min 45-sec annealing at annealing temperature of each primer pairs and 2-min extension at 72 °C (10-min final extension at 72 °C).

2.3. Evaluation of S-Alleles by band profiles

Amplified PCR products were run using 2% agarose gel electrophoresis with 100 bpDNA Marker (Solis Byodyne) at 100V for 1 hour and then visualized using agarose gel imaging system (Gene Genius Bio Imaging System).

Table 1- Oligonucleotide sequences for allele-specific PCR

Primer name	*Primer sequences*	*Expected length (bp)*
MalusS_{26}*	GAAGATGCCATACGCAATGG ATGAATTCTTAATACCGAATATTGGCC	193bp
MalusS_9**	CAGCCGGCTGTCTGCCACTT CGGTTCGATCGAGTACGTTG	343bp
MalusS_d*	ATCGAACTGATCATGTAGGC TATCGTGAACCTTGTGGTGG	355bp
MalusS_f*	CAATCGAAACGATCATGAAG TCCGTGTATAGGCCATCGAC	493bp

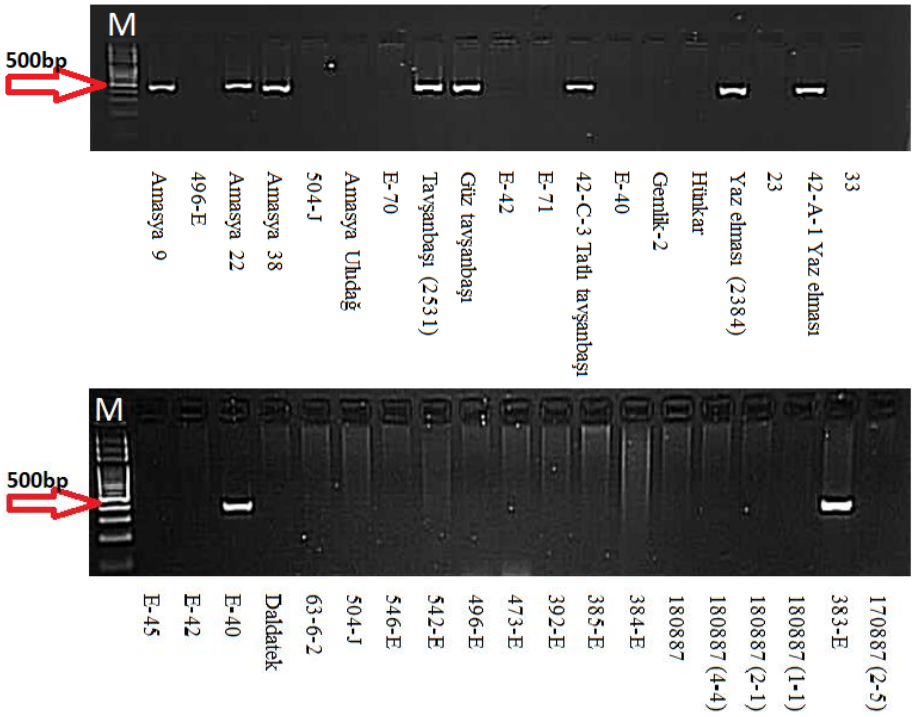
*: Sakurai et al. (2000), **: Janssens et al. (1995)

After agarose gel imaging, genotypes containing *Malus* S_{26} allele with193 bp, genotypes containing *Malus* S_9 allele with 343 bp, genotypes containing *Malus* S_d allele with 355 bp, and genotypes containing *Malus* S_f allele with 493 bp were found to be self-incompatible with the corresponding *S*-alleles. The results were evaluated based on the allele sharing criteria described by Broothaerts et al. (1996) and Ishimizu et al. (1999). Genotypes containing at least 1, 2, and 3 out of 4 *S*-alleles studied were partially incompatible, the genotypes in which these regions could not be amplified were compatible, and those in which all regions could be amplified by PCR were totally incompatible.

3. Results

3.1. Identification of S-Alleles

Four different *S*-alleles (S_9, S_{26}, S_f, S_d) were successfully amplified by PCR in 192 apple genotypes and after agarose gel electrophoresis, visualized using agarose gel imaging system (Figure 1). 181 out of 192 genotypes containing at least 1 *S*-allele were 'Partially Incompatible' and of the remaining, 12 genotypes which contained 4 *S*-alleles were 'Totally Incompatible'. No *S*-alleles were observed in 2 genotypes (Pancarlık and Hüryemez) which exhibited 'Total Compatibility' contrary to the rest of the genotypes (Table 2).



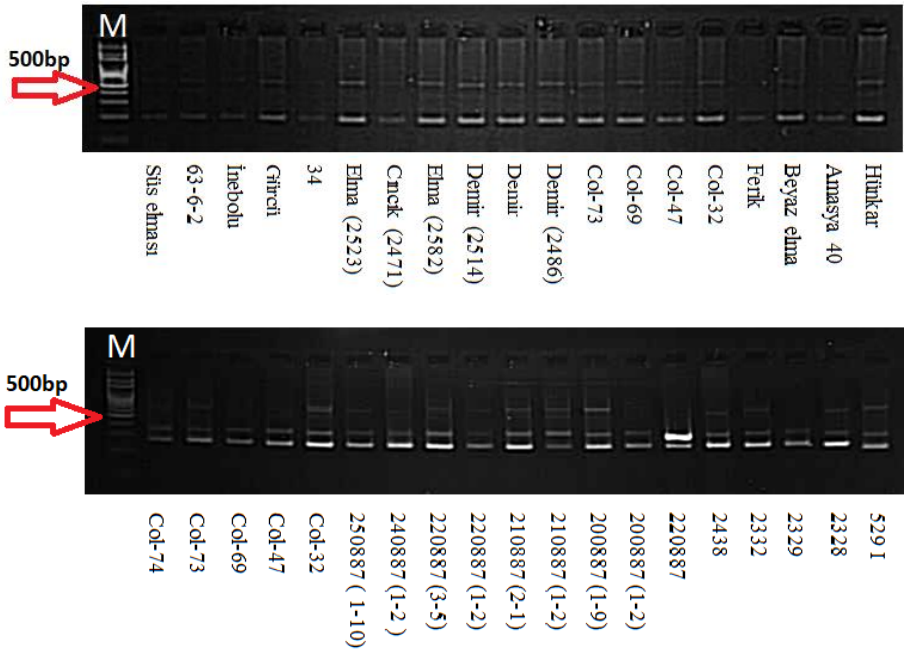


Figure 1- *S*-allele-specific PCR analysis (S_f, S_d, S_{26} and S_9, respectively, M: 100 bp DNA Marker)

Table 2- *S*-allele compositions of apple genotypes

NO	*Genotype*	*S-allele*	*Compatibility status*	*Region*
1	Amasya 9	S_fS_{26}	Semi compatible	Central B.S
2	Amasya 21	S_fS_{26}	Semi compatible	Central B.S
3	Amasya 22	S_fS_{26}	Semi compatible	Central B.S
4	Amasya 38	$S_9S_fS_{26}$	Semi compatible	Central B.S
5	Amasya 50	S_fS_{26}	Semi compatible	Central B.S
6	Amasya Uludağ	$S_{26}S_{26}$	Semi compatible	Central B.S
7	Şah elması	$S_9S_fS_{26}$	Semi compatible	Eastern B.S
8	Tavşanbaşı (2531)	$S_9S_fS_{26}S_d$	Incompatible	Unknown
9	Güz tavşanbaşı	$S_9S_fS_{26}$	Semi compatible	Unknown
10	Yaz tavşanbaşı	$S_9S_fS_{26}$	Semi compatible	Unknown
11	42-KP-1 Mayhoş tavşanbaşı	$S_9S_fS_{26}$	Semi compatible	Unknown
12	42-C-3 Tatlı tavşanbaşı	S_fS_{26}	Semi compatible	Unknown
13	Tokat-1	S_fS_{26}	Semi compatible	Unknown
14	Tokat-2	$S_9S_fS_{26}$	Semi compatible	Unknown
15	Tokat-4	$S_9S_fS_{26}$	Semi compatible	Unknown
16	Yaz elması (2384)	S_fS_{26}	Semi compatible	Unknown
17	Yaz elması (2563)	S_fS_{26}	Semi compatible	Unknown
18	42-A-1 Yaz elması	$S_{26}S_{26}$	Semi compatible	Unknown
19	Kaba elma (42-E-6)	$S_9S_fS_{26}$	Semi compatible	Central A.
20	130887	$S_9S_fS_{26}$	Semi compatible	Aegean
21	130887 (2-3)	$S_9S_fS_{26}$	Semi compatible	Aegean
22	130887 (3-4)	$S_9S_fS_{26}S_d$	Incompatible	Aegean
23	170887 (2-5)	$S_9S_fS_{26}$	Semi compatible	Aegean
24	180887	$S_9S_fS_{26}$	Semi compatible	Aegean
25	180887 (1-1)	$S_9S_fS_{26}$	Semi compatible	Aegean
26	180887 (2-1)	$S_9S_fS_{26}$	Semi compatible	Aegean
27	180887 (4-4)	$S_9S_fS_{26}$	Semi compatible	Aegean
28	180887 (5-4)	$S_9S_fS_{26}S_d$	Incompatible	Aegean
29	372-E	S_fS_{26}	Semi compatible	Unknown
30	383-E	$S_9S_fS_{26}S_d$	Incompatible	Unknown
31	384-E	$S_9S_fS_{26}$	Semi compatible	Unknown
32	385-E	$S_9S_fS_{26}$	Semi compatible	Unknown
33	392-E	$S_9S_fS_{26}$	Semi compatible	Unknown
34	473-E	$S_9S_fS_{26}$	Semi compatible	Unknown
35	496-E	S_9S_{26}	Semi compatible	Unknown
36	542-E	$S_9S_fS_{26}$	Semi compatible	Unknown
37	546-E	$S_9S_fS_{26}$	Semi compatible	Unknown
38	504-J	$S_{26}S_{26}$	Semi compatible	Unknown
39	63-6-2	S_9S_f	Semi compatible	Marmara
40	Daldatek	$S_{26}S_{26}$	Semi compatible	Unknown

Table 2 (Continue)- *S*-allele compositions of apple genotypes

NO	*Genotype*	*S-allele*	*Compatibility status*	*Region*
41	E-70	$S_{26}S_{26}$	Semi compatible	Unknown
42	E-42	$S_{26}S_{26}$	Semi compatible	Unknown
43	E-71	$S_{26}S_{d}$	Semi compatible	Unknown
44	E-40	$S_{9}S_{26}S_{d}$	Semi compatible	Unknown
45	E-45	$S_{9}S_{26}$	Semi compatible	Unknown
46	55	$S_{9}S_{26}$	Semi compatible	Marmara
47	52	$S_{9}S_{26}$	Semi compatible	Marmara
48	51	$S_{9}S_{26}$	Semi compatible	Marmara
49	60	$S_{9}S_{26}$	Semi compatible	Marmara
50	12	$S_{9}S_{26}$	Semi compatible	Marmara
51	49	$S_{9}S_{26}$	Semi compatible	Marmara
52	61	$S_{9}S_{26}$	Semi compatible	Marmara
53	82	$S_{9}S_{26}$	Semi compatible	Marmara
54	57	$S_{9}S_{26}$	Semi compatible	Marmara
55	62-1	$S_{26}S_{26}$	Semi compatible	Marmara
56	78	$S_{9}S_{26}$	Semi compatible	Marmara
57	66	$S_{9}S_{26}$	Semi compatible	Marmara
58	47	$S_{9}S_{26}$	Semi compatible	Marmara
59	62-2	$S_{9}S_{26}$	Semi compatible	Marmara
60	56	$S_{9}S_{26}$	Semi compatible	Marmara
61	37	$S_{9}S_{26}S_{d}$	Semi compatible	Marmara
62	63	$S_{9}S_{f}S_{26}$	Semi compatible	Marmara
63	41	$S_{9}S_{26}$	Semi compatible	Marmara
64	81	$S_{9}S_{26}S_{d}$	Semi compatible	Marmara
65	9	$S_{9}S_{26}$	Semi compatible	Marmara
66	76	$S_{9}S_{26}$	Semi compatible	Marmara
67	48	$S_{9}S_{26}$	Semi compatible	Marmara
68	67	$S_{9}S_{26}$	Semi compatible	Marmara
69	73	$S_{26}S_{26}$	Semi compatible	Marmara
70	17	$S_{9}S_{26}$	Semi compatible	Marmara
71	72	$S_{9}S_{26}S_{d}$	Semi compatible	Marmara
72	65	$S_{9}S_{26}$	Semi compatible	Marmara
73	29	$S_{9}S_{26}S_{d}$	Semi compatible	Marmara
74	21	$S_{9}S_{f}S_{26}$	Semi compatible	Marmara
75	24	$S_{9}S_{f}S_{26}$	Semi compatible	Marmara
76	14	$S_{9}S_{f}S_{26}S_{d}$	Incompatible	Marmara
77	20	$S_{9}S_{26}$	Semi compatible	Marmara
78	13	$S_{9}S_{f}S_{26}$	Semi compatible	Marmara
79	Candır	$S_{9}S_{f}S_{26}S_{d}$	Incompatible	Unknown
80	19	$S_{9}S_{26}$	Semi compatible	Marmara
81	25	$S_{9}S_{26}$	Semi compatible	Marmara
82	11	$S_{9}S_{26}$	Semi compatible	Marmara
83	32	$S_{9}S_{f}S_{26}$	Semi compatible	Marmara
84	458 S (Ciğit)	$S_{9}S_{f}S_{26}$	Semi compatible	Unknown
85	23	$S_{9}S_{26}$	Semi compatible	Marmara
86	33	$S_{9}S_{26}$	Semi compatible	Marmara
87	15	$S_{9}S_{26}S_{d}$	Semi compatible	Marmara
88	7	$S_{9}S_{f}S_{26}$	Semi compatible	Marmara
89	2	$S_{9}S_{26}S_{d}$	Semi compatible	Marmara
90	1	$S_{9}S_{f}S_{26}$	Semi compatible	Marmara
91	6	$S_{9}S_{26}S_{d}$	Semi compatible	Marmara
92	34	$S_{9}S_{9}$	Semi compatible	Marmara
93	4	$S_{9}S_{f}S_{26}$	Semi compatible	Marmara
94	18	$S_{9}S_{f}S_{26}$	Semi compatible	Marmara
95	31	$S_{9}S_{26}$	Semi compatible	Marmara
96	Gemlik-2	$S_{9}S_{26}$	Semi compatible	Marmara
97	Gemlik-3	$S_{9}S_{f}S_{26}$	Semi compatible	Marmara
98	Almıla (42-BS-9)	$S_{9}S_{26}$	Semi compatible	Central A.
99	Hanım teni (42-E-3)	$S_{9}S_{26}$	Semi compatible	Central A.
100	Karapınar elması (42KP-3)	$S_{9}S_{26}$	Semi compatible	Central A.
101	Hünkar	$S_{9}S_{26}$	Semi compatible	Unknown
102	Amasya 40	$S_{9}S_{26}$	Semi compatible	Central B.S
103	Beyaz elma	$S_{9}S_{26}$	Semi compatible	Eastern B.S
104	Ferik	$S_{9}S_{26}$	Semi compatible	Marmara
105	Bey elması (2477)	$S_{9}S_{26}$	Semi compatible	Eastern B.S
106	Gelin elması (2475)	$S_{9}S_{26}S_{d}$	Semi compatible	Eastern B.S

Table 2 (Continue)- *S*-allele compositions of apple genotypes

NO	*Genotype*	*S-allele*	*Compatibility status*	*Region*
107	Göbek (2475)	$S_{26}S_{26}$	Semi compatible	Eastern B.S
108	Altınok elması (2490)	S_9S_{26}	Semi compatible	Aegean
109	Demir (2486)	S_9S_{26}	Semi compatible	Unknown
110	Demir	S_9S_{26}	Semi compatible	Unknown
111	Demir (2514)	S_9S_{26}	Semi compatible	Unknown
112	Elma (2582)	S_9S_{26}	Semi compatible	Eastern B.S
113	Cıncık (2471)	$S_9S_fS_{26}$	Semi compatible	Eastern B.S
114	Elma (2523)	S_9S_{26}	Semi compatible	Eastern B.S
115	Haşhaş elması (2596)	$S_9S_fS_{26}$	Semi compatible	Eastern B.S
116	Gürcü	$S_{26}S_{26}$	Semi compatible	Eastern B.S
117	5	S_9S_9	Semi compatible	Marmara
118	38	S_9S_9	Semi compatible	Marmara
119	529 I	S_9S_{26}	Semi compatible	Unknown
120	2328	S_9S_{26}	Semi compatible	Unknown
121	2329	S_9S_{26}	Semi compatible	Unknown
122	2331	$S_{26}S_{26}$	Semi compatible	Unknown
123	2332	$S_9S_fS_{26}$	Semi compatible	Unknown
124	2438	S_9S_{26}	Semi compatible	Unknown
125	220887	S_9S_{26}	Semi compatible	Aegean
126	200887 (1-2)	$S_9S_fS_{26}$	Semi compatible	Aegean
127	200887 (1-9)	S_9S_{26}	Semi compatible	Aegean
128	210887 (1-2)	S_9S_{26}	Semi compatible	Aegean
129	210887 (2-1)	$S_9S_fS_{26}$	Semi compatible	Aegean
130	220887 (1-2)	S_9S_{26}	Semi compatible	Aegean
131	220887 (3-5)	S_9S_{26}	Semi compatible	Aegean
132	240887 (1-2)	$S_9S_fS_{26}$	Semi compatible	Aegean
133	250887 (1-10)	$S_9S_fS_{26}$	Semi compatible	Aegean
134	42-E-2 (Ankara güzeli)	$S_9S_fS_{26}$	Semi compatible	Central A.
135	Arpa elması (2482)	$S_9S_fS_{26}$	Semi compatible	Central B.S
136	Col-32	$S_9S_fS_{26}$	Semi compatible	Unknown
137	Col-47	$S_9S_fS_{26}$	Semi compatible	Unknown
138	Col-69	$S_9S_fS_{26}$	Semi compatible	Unknown
139	Col-73	$S_9S_fS_{26}$	Semi compatible	Unknown
140	Col-74	S_9S_{26}	Semi compatible	Unknown
141	Cidagut	$S_9S_fS_{26}S_d$	Incompatible	Unknown
142	El-23035 (Amasya)	$S_{26}S_{26}$	Semi compatible	Central B.S
143	Hüryemez	-	Compatible	Eastern B.S
144	J/5/4/59 Bel.	$S_{26}S_{26}$	Semi compatible	Unknown
145	Kadir-Hatice	$S_fS_{26}S_d$	Semi compatible	Unknown
146	Kalkandelen	S_fS_f	Semi compatible	Unknown
147	Karpuz	S_9S_f	Semi compatible	Unknown
148	Kavun (425 E)	S_fS_f	Semi compatible	Eastern B.S
149	Kış elması (2590)	$S_9S_fS_{26}S_d$	Incompatible	Eastern B.S
150	Laz elması (2570)	$S_9S_fS_{26}$	Semi compatible	Eastern B.S
151	Mahsusa elması	$S_9S_fS_{26}$	Semi compatible	Eastern B.S
152	Mektep elması (2565)	S_9S_f	Semi compatible	Eastern B.S
153	Niğde İngiliz	$S_9S_fS_{26}$	Semi compatible	Unknown
154	Oltu elması (2594)	S_9S_{26}	Semi compatible	Eastern B.S
155	Paşa elması	S_fS_{26}	Semi compatible	Eastern B.S
156	Petek (2577)	$S_{26}S_d$	Semi compatible	Eastern B.S
157	Petevrek elması (2566)	$S_fS_{26}S_d$	Semi compatible	Eastern B.S
158	Piraziz	S_fS_{26}	Semi compatible	Eastern B.S
159	Portakal	S_fS_{26}	Semi compatible	Unknown
160	Reçel elması (2506)	S_fS_{26}	Semi compatible	Eastern B.S
161	Rize demir	S_fS_{26}	Semi compatible	Eastern B.S
162	Sandık	$S_9S_fS_{26}$	Semi compatible	Eastern B.S
163	Sarı elma	S_fS_{26}	Semi compatible	Unknown
164	Sinop	$S_9S_fS_{26}$	Semi compatible	Central B.S
165	Susuz elma	$S_9S_fS_{26}$	Semi compatible	Eastern B.S
166	Şeker	S_fS_{26}	Semi compatible	Unknown
167	Tatlı elma (2492)	$S_9S_fS_{26}$	Semi compatible	Eastern B.S
168	Tatlı elma (2511)	$S_9S_fS_{26}$	Semi compatible	Eastern B.S
169	Uzun yorma	$S_9S_fS_{26}$	Semi compatible	Unknown
170	Yenişehir	$S_9S_fS_{26}$	Semi compatible	Unknown
171	42-E-7 Yıldızkıran	$S_9S_fS_{26}S_d$	Incompatible	Central A.
172	42-E-4 Mayhoş yıldızkıran	$S_9S_fS_{26}S_d$	Incompatible	Central A.

Table 2 (Continue)- *S*-allele compositions of apple genotypes

NO	*Genotype*	*S-allele*	*Compatibility status*	*Region*
173	Adsız	$S_9S_fS_{26}$	Semi compatible	Unknown
174	Orak	$S_9S_fS_{26}$	Semi compatible	Unknown
175	Yenice	$S_{26}S_{26}$	Semi compatible	Unknown
176	Süs elması	S_9S_f	Semi compatible	Eastern B.S
177	Amasya 37	$S_9S_fS_{26}$	Semi compatible	Central B.S
178	10	$S_9S_fS_{26}$	Semi compatible	Aegean
179	Söğüt elma	$S_9S_fS_{26}$	Semi compatible	Eastern B.S
180	Samsun	$S_9S_fS_{26}$	Semi compatible	Central B.S
181	528 J	$S_9S_fS_{26}$	Semi compatible	Unknown
182	YB-2	$S_9S_fS_{26}S_d$	Incompatible	Unknown
183	Yaz elması (2482)	$S_{26}S_{26}$	Semi compatible	Eastern B.S
184	Gelendost	S_9S_{26}	Semi compatible	Unknown
185	Pozmer 20	$S_9S_fS_{26}S_d$	Incompatible	Unknown
186	32-E-1	$S_9S_fS_{26}$	Semi compatible	Unknown
187	42-C-5	$S_{26}S_{26}$	Semi compatible	Unknown
188	Daldabir	S_fS_{26}	Semi compatible	Unknown
189	Pancarlık	-	Compatible	Unknown
190	359 (11)	$S_{26}S_d$	Semi compatible	Unknown
191	Cidagut	$S_9S_fS_{26}$	Semi compatible	Unknown
192	İnebolu	S_dS_d	Semi compatible	Unknown

-: Genotype does not carry any of the *S*-alleles. Central B.S: Central Black Sea; Eastern B.S: Eastern Black sea; Central A.: Central Anatolian.

S_{26} allele was the most frequent *S*-allele (49%) followed by S_9 allele (31%). S_d was found to be the least frequent *S*-allele (5%). In terms of *S*-allele combinations, the most common 2-allele combination was detected as "S_9-S_{26}" *S*-genotype (29%) out of total *S*-genotypes. The most common 3-allele combinations, was observed in "S_9-S_f-S_{26}" alleles (39%). 4-allele combination was only reported in 12 genotypes (6%).

In studied apple genotypes, there were 2 genotypes (Pancarlık and Hüryemez) (1%) containing none of the 4 alleles (S_9, S_{26}, S_f, S_d), 22 genotypes containing 1 type of *S*-alleles (11%), 81 genotypes containing 2 types of *S*-alleles (42%) and 75 genotypes containing 3 types of *S*-alleles examined (39%) and 12 genotypes (6%) contained a total of 4 *S*-alleles.

3.2. Relationship with Type-Clonal level similarity

Homonymous 8 Amasya apple genotypes (Amasya 9, Amasya 21, Amasya 22, Amasya 37, Amasya 38, Amasya 40, Amasya 50, Amasya Uludağ) contained mostly S_f and S_{26} *S*-alleles in 4 loci, homonymous 3 Demir apple genotypes (Demir, Demir (2486) and Demir (2514)) contained mostly S_9 and S_{26} alleles in 4 loci, homonymous 3 Tokat apple genotypes (Tokat-1, Tokat-2 and Tokat-4) contained mostly S_f and S_{26} alleles, homonymous 5 Tavşanbaşı apple genotypes (Tavşanbaşı (2531), Güz Tavşanbaşı, Yaz Tavşanbaşı, 42-KP-1 Mayhoş Tavşanbaşı and 42-C-3 Tatlı Tavşanbaşı) contained mostly S_9, S_f and S_{26} alleles, homonymous 4 Yaz apple genotypes (Yaz (2384), Yaz (2563), Yaz (2482) and 42-A-1 Yaz) contained mostly S_{26} allele and all of these genotypes were found to be 'Partially Incompatible' (Tavşanbaşı (2531)-Totally Incompatible).

3.3. Relationship with SSR based similarity

SSR based similarity rates of 58 binary comparisons have been formerly calculated (Burak et al. 2014). From which, 12 (20.6%) cases were totally similar (S_9S_{26}), 34 (20%) were similar in terms of 2 *S*-alleles (S_9S_{26} and S_fS_{26}), 1 (1.7%) was similar in terms of 3 *S*-alleles ($S_9S_fS_{26}$) and 22 (37.9%) were similar in terms of single *S*-allele (S_{26}) (Table 3).

Table 3- Genetic similarity rates based on SSR analysis

No	*Genotypes with similar genetic origin*	*Similarity rates %*
1	1 – 52	90.6
2	42-E-6 (Kaba elma) – 180887	
3	42-E-6 (Kaba elma) - 180887 (5-1)	
4	392-E – 496-E	
5	458 S – 76	
6	65 – 61	
7	65 – 62-1	
8	65 – 62-2	
9	65 – 11	
10	65 – Amasya 38	
11	65 – Amasya 40	
12	65 – 2329	
13	Amasya 50 – Amasya Uludağ	
14	Demir (2486) – Demir	
15	4 – 31	93.8
16	1 – 130887 (2-3)	
17	25 – Demir (2486)	
18	37 – 76	
19	55 – Göbek (2475)	
20	Amasya 50 – 180887 (5-4)	
21	Amasya 50 – Amasya 9	
22	Amasya 50 – Amasya 21	
23	Amasya 50 – Amasya 22	
24	Amasya 50 – Amasya 37	
25	Daldatek – 180887	96.9
26	180887 (5-4) – 62-1	
27	180887 (5-4) – 62-2	
28	180887 (5-4) – 11	
29	180887 (5-4) – Amasya 38	
30	180887 (5-4) – Amasya 40	
31	180887 (5-4) – 2329	
32	Amasya 9 – 62-1	
33	Amasya 9 – 62-2	
34	Amasya 9 – 11	
35	Amasya 9 – Amasya 38	
36	Amasya 9 – Amasya 40	
37	Amasya 9 – 2329	
38	Amasya 21 – 62-1	
39	Amasya 21 – 62-2	
40	Amasya 21 – 11	
41	Amasya 21 – Amasya 38	
42	Amasya 21 – Amasya 40	
43	Amasya 21 – 2329	
44	Amasya 22 – 62-1	
45	Amasya 22 – 62-2	
46	Amasya 22 – 11	
47	Amasya 22 – Amasya 22	
48	Amasya 22 – Amasya 38	
49	Amasya 37 – 62-1	
50	Amasya 37 – 62-2	
51	Amasya 37 – 11	
52	Amasya 37 – Amasya 38	
53	Amasya 37 – Amasya 40	
54	Amasya 37 – 2329	
55	Amasya 50 – Amasya 38	
56	Amasya 50 – Amasya 40	

3.4. Relationship with triploidy

Based on the genetic analysis of the same apple population, Burak et al. (2014) reported that 12 genotypes were potentially triploid. Of the triploid genotypes, except 1 genotype (Cidagut), remaining 11 genotypes contained at least 2 different incompatibility alleles and were identified as 'Partially Incompatible'. High diversity of incompatibility alleles can be attributed to triploid genotypes (Table 4).

Table 4- Incompatibility states of triploid genotypes identified through SSR analysis

Triploid genotypes identified through SSR analysis	*Incompatibility alleles*	*Incompatibility status*
25	S_9S_{26}	Semi compatible
37	$S_9S_{26}S_d$	Semi compatible
76	S_9S_{26}	Semi compatible
52	S_9S_{26}	Semi compatible
Beyaz Elma	S_9S_{26}	Semi compatible
1	$S_9S_fS_{26}$	Semi compatible
Mektep Elması (2565)	S_9S_f	Semi compatible
Susuz Elma	$S_9S_fS_{26}$	Semi compatible
20	S_9S_{26}	Semi compatible
240887(1-2)	$S_9S_fS_{26}$	Semi compatible
Col-32	$S_9S_fS_{26}$	Semi compatible
Cidagut	$S_9S_fS_{26}S_d$	Incompatible

4. Discussion

The apple reproductive mechanism is regulated genetically by the *S*-locus through the *S-RNase* based gametophytic self-incompatibility system (De Franceschi 2018). Accurate recognition of the *S*-genotypes through *S*-genotyping by *S-RNase* alleles is important for the economic and permanent apple production because in order to apple fertilization, at least two genotypes without or only with one common *S*-haplotype are required. Moreover, *S*-alleles are used for supporting new genotypes, and also help recognizing the parental ones (Kasajima et al. 2017; Matsumoto et al. 2018).

There are two different labelling of *S*-alleles: European labelling uses figures like S_1 and S_2, and Japanese labelling uses characters like S_a and S_b. S_a, S_b, S_c, S_d, S_f, S_g, S_i, S_h, and S_z are identical with S_2, S_3, S_9, S_7, S_1, S_{20}, S_{24}, S_{10}, and S_{25}, respectively, but the identity of S_e is not clear (Hegedűs 2006).

S-genotypes of 192 diploid, triploid or tetraploid Turkish genotypes were discriminated using four different *S*-alleles. In current study, most of the analysed apple genotypes (53%) having two *S*-alleles were diploid and 40% of which including three or four *S*-alleles were determined as triploid and tetraploid genotypes. The frequency of occurrence of the examined *S*-alleles displayed a wide variation in the apple germplasm. Two *S*-alleles (S_{26} and S_9) were very common among the evaluated genotypes, presumably as a result of the prevalent use of the same breeding parents, and two (S_d and S_f) alleles were very rare so that, there was almost a 10-fold difference in frequency between the most prevalent (S_{26}) and rare (S_d) alleles. In this regard, it could be said that rare alleles (S_d and S_f) may belong to non-commercial or old species.

Recently, in a study conducted by Broothaerts et al. (2004) over *S*-genotyping of 150 European, American and Japanese apple cultivars, S_3 allele was reported as the most prevalent allele, followed by S_2 and S_9 while the S_{26} allele was found as rare allele. The authors concluded that the high frequency of the S_3 allele is due to the large-scale utilization of 'Golden Delicious' (S_2S_3) and its descendants in many present and past breeding programs (Broothaerts et al. 2004). Hegedűs (2006) also reported that the S_2, S_3, S_5, S_7, S_9, and S_{10} alleles were the most common among the commercial apple cultivars, and the high abundance of these alleles is attributed mainly to the prevalent application of the 'Golden Delicious', 'Delicious', 'Jonathan', 'McIntosh', and 'Cox's Orange Pippin' genotypes in apple breeding programs worldwide. Whereas, Matsumoto et al. (2007) found S_1, S_7 and S_9 to be the most common alleles, Dreesen et al. (2010) identified S_2, S_3, S_5, and S_9 as the most frequent *S*-alleles among European apple cultivars. Larsen et al. (2016) reported a high frequency of S_3 allele (28%) among 432 *Malus* genotypes. Brancher et al. (2020) also found the most frequent allele to be S_3, followed by S_5, since the most genotypes studied were indirect or direct derivatives of the 'Golden Delicious' (S_2S_3), 'Gala' (S_2S_5), and 'Imperatriz' (S_3S_5) cultivars. In our study, the high frequency of S_9 allele may be derived from the 'Delicious', 'Cox's Orange' and/or 'Fuji' cultivars all having S_9 which are also ancestors to many American, European and Japanese cultivars.

As represented in Table 2, eight diploid incompatibility groups were identified while, theoretically it is possible to form 10 incompatibility groups out of 4 alleles. Besides, 190 out of 192 studied genotypes (98%) were fully genotyped with mentioned 4 *S*-alleles. Although a large number of *S*-alleles are available in apple, artificial selection or repeated use of the same genotypes as parents appears to significantly restrict the number of compatibility groups associated with commercial clones. Overall, it could be noted that the actual number of cross-incompatible groups is not already large enough therefore, many incompatibility problems are expected in natural environments.

In the current study, it is required to new markers for identifying the two genotypes with unknown *S*-genotypes (Hüryemez and Pancarlık). The *S*-allele used for this purpose should be selected from the alleles that have a lower frequency among the apple genotypes.

According to studies by Halász et al. (2011) and De Franceschi et al. (2016), alleles S_2, S_3 and S_5 were found to be associated with apple scab (*Venturia inaequalis*) resistance. Since, the S_{26} allele has been identified as a rare one among the European apple cultivars in numerous studies, this allele is not yet fully characterized in literature. A comprehensive study should be performed to identify possible association of the S_{26} allele with important traits in Turkish apple genotypes. For example, this allele may be related to the taste of the fruit, as in the case of "Jonica" cultivar (Mir et al. 2016), or it may be related to woolly apple aphid resistance or other important traits.

5. Conclusions

The assignment of *S*-genotypes of apple cultivars needs important consideration in selecting proper pollen donors in breeding practices and orchard management (Li et al. 2011). For most genotypes, evidences for the correct *S*-allele discrimination seems to be strong and is often supported by studies at the DNA and protein levels, as well as by functional assessments through pollinations. Furthermore, the selection of suitable pollinizers may now more accurately include the correct compatibility relationships in addition to other factors that need to be considered, such as the overlapping of the blooming periods.

The present study has provided the first comprehensive discrimination of *S*-allele genotyping of common Turkish apple genotypes and presented optimized methodologies for genetic studies which will be very helpful for future researches on apple incompatibility. Additional data was acquired for identification of Turkish apple gene sources and incompatible genotypes were determined. It is suggested that obtained data can be utilized in cultivation, breeding and pollinator selection activities regarding the studied apple genotypes.

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IsVoNet8: A Proposed Deep Learning Model for Classification of Some Fish Species

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ABSTRACT

In the classification of fish, both knowledge and great effort are required to determine the characteristics of fish. Traditionally, however, manual classification of extrinsic characteristics of different fish species has been a difficult and time-consuming process due to their close resemblance to each other. Recently, deep learning methods used in the light of developments in the field of computer vision have facilitated the training of fish image classification models and the recognition of various fish species. In this study, a new convolutional neural network model classifying 8 different belonging to 6 families (Mullidae, Sparidae, Carangidae, Serranidae, Clupeidae, Salmonidae) fish species using deep learning methods was proposed. The species include *Clupeonella cultriventris* N., *Sparus aurata* L., *Trachurus trachurus* L., *Mullus barbatus* L., *Pagrus major* T & S., *Dicentrarchus labrax* L., *Mullus surmuletus* L. and *Oncorhynchus mykiss* W. The proposed model (IsVoNet8) is compared with the ResNet50, ResNet101 and VGG16 models. The success accuracies obtained as a result of the comparison are respectively; 98.62% in the IsVoNet8, 91.37% in the ResNet50 model, 86.12% in the ResNet101 model and 97.75% in the VGG16 model. However, it was obtained that the loss rates of ResNet50 0.3646, ResNet101 0.5811, VGG16 0.0696 and with the IsVoNet 0.0568. As a result, it has been observed that the IsVoNet classifies marine fish, which is widely consumed in Türkiye.

Keywords: Artificial intelligence, Deep learning, Convolutional neural network, CNN, Fish taxonomy

1. Introduction

Fish are very diverse animals of all the vertebrate groups of animals with more than 33000 species in the world. There are different types of fish in the four major geographical regions of Turkey. Classification of different fish species is very important for aquaculture, stock management of water bodies, monitoring of aquatic organisms and conservation of marine biology. The fish species numbers of Türkiye's four major geographic regions is as follows: Aegean Sea coasts 389 species, Mediterranean Sea coasts 388 species, Sea of Marmara coasts 249 species and Black Sea coasts 151 species (Bilecenoglu et al. 2002). The classification of different fish species is very important for the preservation and protection of aquaculture and marine biology. Global warming and climate change have a negative impact on the amount of fish species, their habitats and stock distribution. Traditionally, manual classification of different species fish is quite difficult and time-consuming. Species identification in morphometric studies of fish species creates a database for biologists, scientists and aquaculture. However, these studies took a long time, which led to the emergence of new techniques. Today, with the use of automatic identification systems, systems that analyse and classify fish species are developed without any human intervention. In the light of the developments in computer technologies, computerized fish classifiers have been widely used for the classification of fish. Advanced technology equipped with artificial intelligence using deep learning methods facilitates the recognition of a diverse range of fish species. Deep learning is a branch of machine learning which uses multiple layered neural network topology to represent high-level abstractions of data (Sarigül & Avci 2017). The deep learning structure is based on the learning of more than one feature level of data. It is based on learning from the representation of the main data (Lecun et al. 2015). The representation of an image can be considered to comprise a vector of density values

per pixel or features such as clusters of edges and custom shapes, with some representing the data better (Song & Lee 2013). Convolutional neural network (CNN) is one of the most popular deep learning methods used currently (Hridayami et al. 2019). Among the most accepted and used CNN models in the literature are Resnet50, Resnet101, VGG16 models.

CNN used as basic deep learning tools have obtained significant success in classifying fish species. In the literature, there are many studies based on CNN on the classification of fish species. Montalbo & Hernandez (2019) proposed a methodology to recognize fish species using VGG16 Deep CNN (DCNN). Hridayami et al. (2019) applied VGG16 DCNN, which is pre-trained on ImageNet via transfer learning method to recognize fish images. In this approach, 50 species of fishes are recognized with different accuracies on four different datasets. Zhang et al. (2019) studied the classification of fish and realized the rapid and accurate identification of common freshwater fish species by machine vision technology. Rauf et al. (2019) have carried out the experimental comparison with other deep learning frameworks involving VGG16 for transfer learning, one block VGG, two block VGG, three block VGG, LeNet-5, AlexNet, GoogleNet, and ResNet50 on the Fish-Pak data set. Simonyan & Zisserman (2014) VGGNet is one of the top CNN models used for classification as it integrates better learning capabilities compared with AlexNet. Shah et al. (2019) a dataset containing six different fish types (grass carp, common carp, mori, rohu, silver carp, thala) is collected in Pakistan. Each class in the dataset contains three dominant features of fish types (body, scale and head) with different number of images. CNNs are utilized to classify fish from their body images. There are several examples of this model being used in other fields of research. Ranzato et al. (2007) combined hierarchical tree with Gaussian mixture model to recognize 15 species of fish in underwater videos. Marini et al. (2018) estimated the abundance of the fish using an autonomous imaging device and genetic-programming-based classifier. Vabø et al. (2021) applied CNNs with transfer learning on a novel dataset of 9056 images of Atlantic salmon scales for four different prediction tasks.

In this study, a new model has been proposed by using deep learning methods in order to avoid the difficulty of species identification and loss of time in classifying fish species with traditional methods. The use of 8 fish species is limited, the use of more fish specimens might increase the applicability of the model to field studies. One of the disadvantages of this model is that the success of model classification may decrease if the number of fish species is hundreds. Model success may vary in real-time applications. In summary, some studies have been carried out in the literature using machine learning and image processing techniques to classify fish (İşçimen et al. 2014; Kutlu et al. 2017). However, there are few studies on the use of deep learning methods in the classification of fish species in Türkiye increases the importance of the study (Sarigül & Avci 2017; Kayaalp & Metlek 2021). For this reason, the proposed model provides superiority in classifying fish species due to the lowest error rate and high success accuracy. In addition, the proposed model to determine the success accuracy of this model; compared with Resnet50, Resnet101 and VGG16 models. Firstly, the aim of this study is to determine the CNN model performance that can be achieved for fish species classification. Therefore, this model will offer a new approach to the identification of marine fish that are widely consumed.

2. Material and Methods

2.1. Acquisition of image

In this study, a Large-Scale Fish Dataset was used (except for shrimp). This dataset consists 8 species belonging to 6 families (Mullidae, Sparidae, Carangidae, Serranidae, Clupeidae, Salmonidae). There are; black sea sprat (*Clupeonella cultriventris* N.), gilt head bream (*Sparus aurata* L.), horse mackerel (*Trachurus trachurus* L.), red mullet (*Mullus barbatus* L.), red sea bream (*Pagrus major* T & S.), sea bass (*Dicentrarchus labrax* L.), striped red mullet (*Mullus surmuletus* L.) and trout (*Oncorhynchus mykiss* W.) (Ulucan et al. 2020).

2.2. Image data preprocessing

This dataset contains 9 different seafood types collected from a supermarket in Izmir, Turkey for a university-industry collaboration project at Izmir University of Economics (Ulucan et al. 2020). In this study, there are 1000 fish images of 590x445 pixels from each class belonging to 8 fish species and a total of 8000 fish images in RGB format. The fish images in the original dataset were converted to 224x224 pixel gray color format by pre-processing and labelled under the same class. The sample images of fish images obtained as a result of pre-processing are given in Figure 1.

2.3. Convolutional neural networks model

In this study, along with the classification models Resnet50, Resnet101, VGG16 an alternative model (IsVoNet8) has also been proposed. ResNet50, classical neural network model and frequently used in the training of deep learning networks, and shows superior

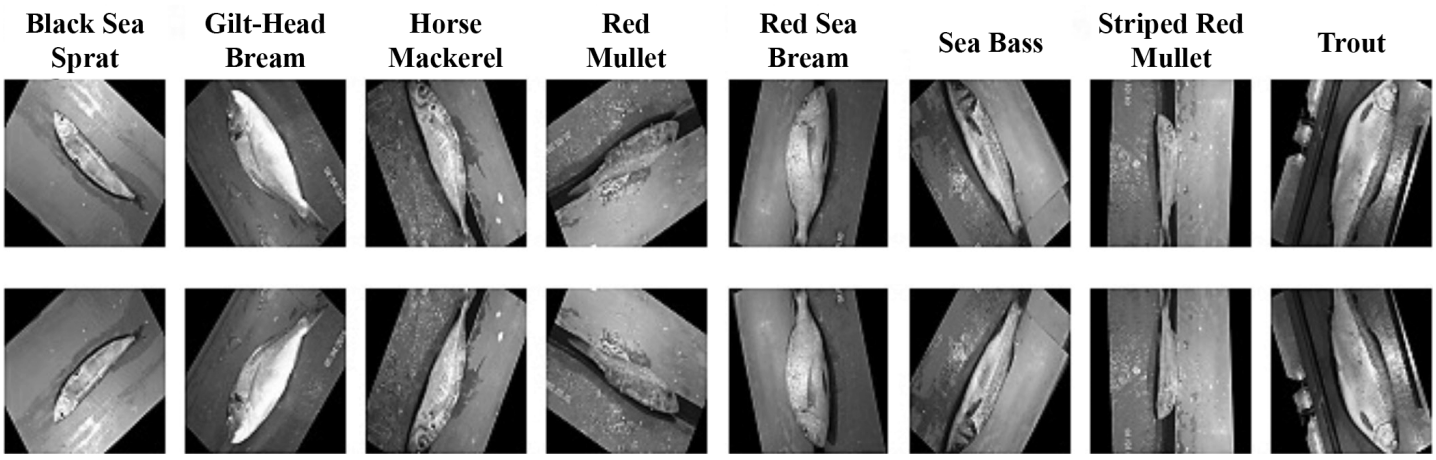

Figure 1- Image examples from the dataset

success among ResNet (He et al. 2016) architectures, consisting of 50 layers and 23M parameters, and ResNet101 models consisting of 101 layers and 42M parameters were preferred. However, the VGG16 model, one of the VGGNet architectures that forms the basis of object recognition models, consisting of 41 layers and 134M parameters, was preferred.

The proposed model in Figure 2 consists of 18 layers (convolution, maximum pooling, dropout, ReLU, flattening, fully connected and classification layers) and a total of 6.813.064 parameters.

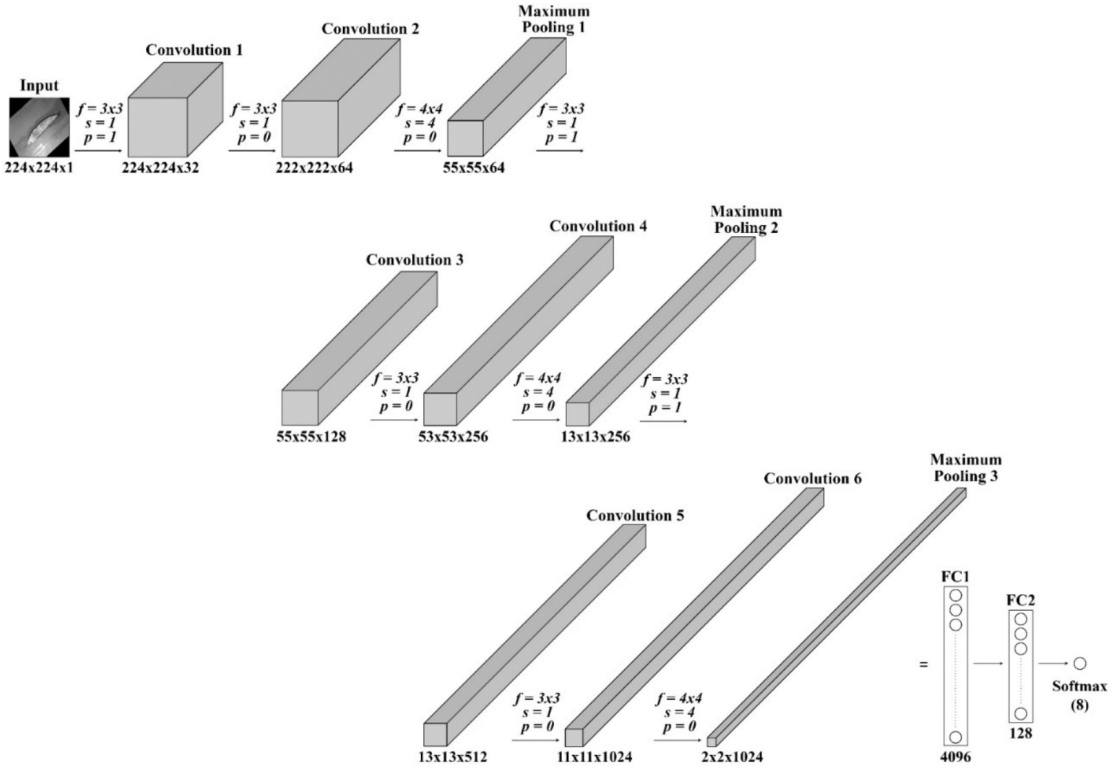

Figure 2- Proposed CNN model (IsVoNet8) (f: filter size, s: stride, p: padding, FC: fully connected)

In Figure 2, an image of a fish in a gray color format with a size of 224x224 pixels is given to the input of the proposed model. Initially, the first convolution of the input image (convolution) filter size 3x3 convolution layer 32, the second convolution layer convolution

filter size 3x3, 64 applying the filter size 4x4 with a maximum dry docking has been obtained by applying 55x55x64 feature map pixels. Then, the image obtained by applying 128 and 256 two convolution layers, respectively, in 3x3 filter size and maximum pooling in 4x4 filter size, with the size of 13x13x256 pixels, the feature map was given as input to next block. As a result of this process, the image obtained by applying 512 and 1024 two convolution layers, respectively, in 3x3 filter size and maximum pooling in 4x4 filter size, with the size of 2x2x1024 pixels, the feature map was given as input to the full connection layer (FC1), and 4096 neurons were obtained. Then, 128 neurons were obtained using the full connection layer (FC2) and 8 output fish classes were obtained by applying the Softmax activation function to the neurons. In addition, the ReLu activation function, which regulates non-zero input values to zero in each convolution operation, and a 15% dropout layer are used to prevent the network from over-memorizing after each maximum pooling and FC2.

The low number of layers used in the proposed model reduces the computational complexity of the neural network. In this way, the proposed model takes less time in classifying fish species, making its use in real-time applications superior.

2.4. Training and testing

The models used in the study were coded using the Python programming language and the proposed model codes were published as open access on a website for other researchers to use (GitHub 2022). All models used in the study were run in Google Colaboratory (Colab 2021) environment with a high-performance NVIDIA Tesla K80 graphics processor. In addition, a dataset of 8 different fish species; divided into three groups as training, testing and validation. The number of fish images belonging to each group is given in detail in Table 1. The dataset consisting of fish images was randomly divided into three as 80% training, 10% testing and 10% validation data group. As a result of this process, a total of 8000 fish images were used, of which 6400 in the training data group, 800 in the test data group and 800 in the validation data group.

Table 1- Dataset of 8 different fish species

	Training (80%)	*Test (10%)*	*Validation (10%)*	*Total (100%)*
Dataset	6400	800	800	8000

Epoch (20), Mini Batch Size (32) and Optimization Algorithm (Adamax) training parameters were used for each model training in the study. However, in the proposed model, dropout (15%) and ReLU were used as activation functions.

2.5. Performance metrics

As a result of the model training process, the mathematical expressions of the precision, recall, f1-score and accuracy performance criteria were given between Equations 1 and 4. In the formulas given below, TP represents the true positive, TN represents the true negative, FP represents the false positive and FN represents the false negative.

$$Precision = \frac{TP}{TP+FP} \quad (1)$$

$$Recall = \frac{TP}{TP+FN} \quad (2)$$

$$f1-score = \frac{2*Precision*Recall}{Precision+Recall} \quad (3)$$

$$Accuracy = \frac{TP+TN}{TP+FP+FN+TN} \quad (4)$$

3. Results and Discussion

The traditionally defined methods are those that require professional skill and take a long time. Therefore, feature extraction methods based on image processing technologies are used in order to eliminate human-induced errors in fish images efficiently, in a short time. In this study, a new evolutionary neural network model recognizing and classifying 8 different fish species using deep learning methods was proposed. The species include *Clupeonella cultriventris* N., *Sparus aurata* L., *Trachurus trachurus* L., *Mullus barbatus* L., *Pagrus major*

T & S., *Dicentrarchus labrax* L., *Mullus surmuletus* L. and *Oncorhynchus mykiss* W. from Mullidae, Sparidae, Carangidae, Serranidae, Clupeidae, Salmonidae family.

According to the experimental results obtained using the training and test data groups, the precision, recall, f1-score and accuracy performances criteria of the models as a result of the training process are given in Table 2. In addition, the accuracy and loss graphs of each model are shown in Figure 3.

Table 2- Performance criteria for each model using training and test data groups

Model	*Class*	*Precision*	*Recall*	*F1-Score*	*Model*	*Class*	*Precision*	*Recall*	*F1-Score*
ResNet50	Black Sea Sprat	0.84	1.00	0.91	ResNet101	Black Sea Sprat	0.78	1.00	0.88
	Gilt-Head Bream	0.98	0.80	0.88		Gilt-Head Bream	0.89	0.85	0.87
	Horse Mackerel	0.97	0.92	0.94		Horse Mackerel	0.98	0.94	0.96
	Red Mullet	0.94	1.00	0.97		Red Mullet	0.77	0.96	0.85
	Red Sea Bream	0.74	0.99	0.85		Red Sea Bream	0.99	0.87	0.93
	Sea Bass	1.00	0.75	0.86		Sea Bass	0.74	0.99	0.85
	Striped Red Mullet	0.99	0.86	0.92		Striped Red Mullet	1.00	0.40	0.57
	Trout	0.97	0.99	0.98		Trout	0.94	0.88	0.91
	Accuracy			0.91		Accuracy			0.86
	Macro avg	0.93	0.91	0.91		Macro avg	0.89	0.86	0.85
	Weighted avg	0.93	0.91	0.91		Weighted avg	0.89	0.86	0.85
Model	*Class*	*Precision*	*Recall*	*F1-Score*	*Model*	*Class*	*Precision*	*Recall*	*F1-Score*
VGG16	Black Sea Sprat	0.97	0.98	0.98	IsVoNet8	Black Sea Sprat	0.98	0.99	0.99
	Gilt-Head Bream	0.99	0.97	0.98		Gilt-Head Bream	0.99	1.00	1.00
	Horse Mackerel	0.97	0.98	0.98		Horse Mackerel	1.00	0.98	0.99
	Red Mullet	1.00	0.99	0.99		Red Mullet	1.00	0.97	0.98
	Red Sea Bream	0.95	0.97	0.96		Red Sea Bream	0.98	0.99	0.99
	Sea Bass	0.98	0.97	0.97		Sea Bass	0.97	1.00	0.99
	Striped Red Mullet	0.97	0.96	0.96		Striped Red Mullet	0.99	0.97	0.98
	Trout	0.99	1.00	1.00		Trout	0.98	0.99	0.99
	Accuracy			0.98		Accuracy			0.99
	Macro avg	0.98	0.98	0.98		Macro avg	0.99	0.99	0.99
	Weighted avg	0.98	0.98	0.98		Weighted avg	0.99	0.99	0.99

When the graphs in Figure 3 are examined, the training process of the CNN in ResNet50 and ResNet101 models is realized with higher learning, however, as a result of the last epoch, the training rate in the ResNet50 model is 99.34% and the test rate is 92.37%; In the ResNet101 model, the training rate reached 98.50% and the test rate reached 88.13%. Since the training and testing rates are inconsistent in these two models, it seems that the testing process is not successful enough. However, the same situation is observed in the loss rate of these two models. In the VGG16 model, it seems that the training and testing process takes place well and the network learns properly. As a result of the last epoch of the VGG16 model, it was seen that the reached training rate 1.00% and the test rate 97.37%. Thus, it is seen that the training and testing ratio in the VGG16 model is more consistent than in the ResNet50 and ResNet101 models. This consistent situation is also seen in the loss graph of the VGG16 model (Figure 3c). In the proposed IsVoNet8 model, it is seen that the training rate is 98.89% and the test rate is 98.37% as a result of the last epoch. In the proposed model, it has achieved superior results due to the lower number of layers and parameters used and the cost lower compared to other models. Successful results were obtained in the loss graph of the proposed model compared to other models (Figure 3d).

After the training and testing process of each of the four models discussed in the study, the accuracy of the models was tested using a validation data group consisting of a total of 800 fish images never seen by the model nets (Table 3). In addition, a confusion matrix for 8 different fish classes obtained for each model using the same validation data group is given in Figure 4.

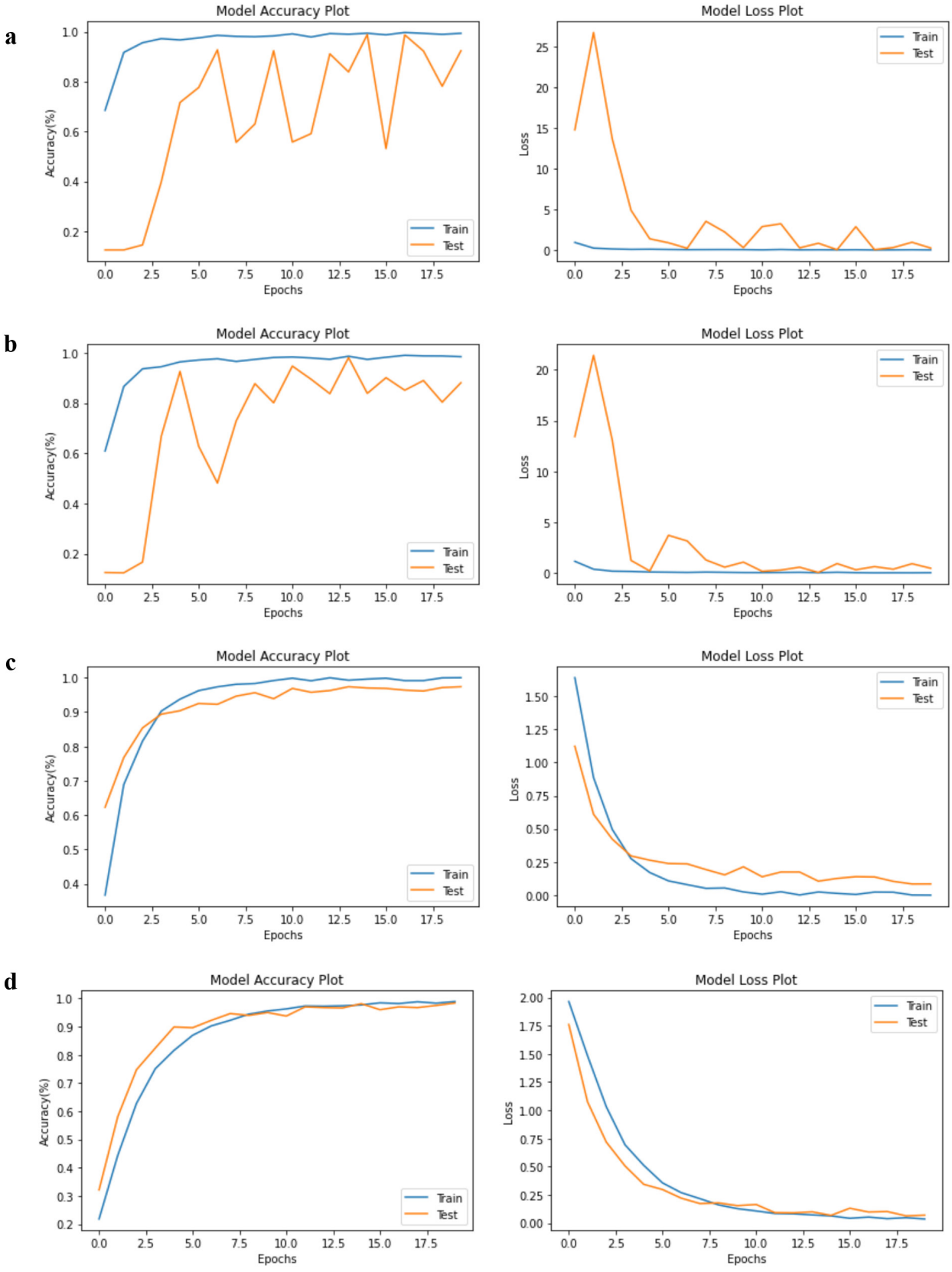

Figure 3- Accuracy and loss graphs for each model using training and test data groups: (a) ResNet50, (b) ResNet101, (c) VGG16, (d) IsVoNet8

Table 3- Accuracy and loss values for each model using the validation data group

Model	*Accuracy*	*Loss*
ResNet50	91.37%	0.3646
ResNet101	86.12%	0.5811
VGG16	97.75%	0.0696
IsVoNet8	98.62%	0.0568

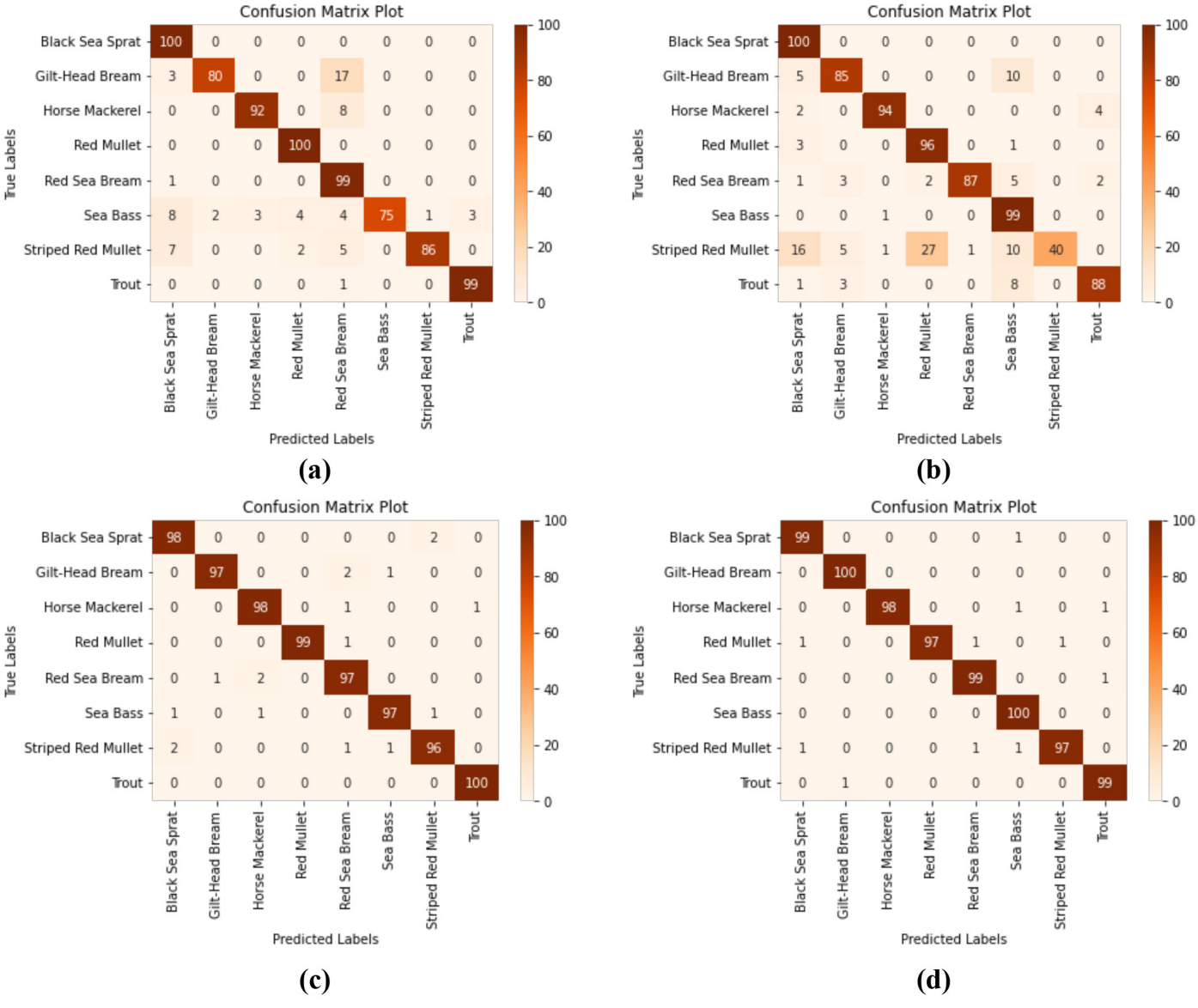

Figure 4- Confusion matrix for each model using the validation data group: (a) ResNet50, (b) ResNet101, (c) VGG16, (d) IsVoNet8

When the accuracy values given in Table 3 were examined, it was seen that the highest success accuracy rate was obtained from the IsVoNet8 model proposed with 98.62%. Rathi et al. (2017) developed a novel method based on CNN to classify 21 species of fishes and achieving an accuracy of 96.29 percent. In another study Khalifa et al. (2018) used a simplified AlexNet to identify multiple species of fishes. The data used was composed of eight species, with 191 sub-species trained. The results achieved 85.59 percent accuracy and 85.41 percent using AlexNet.

In addition, 91.37%, 86.12% and 97.75% success accuracy rates were obtained in ResNet50, ResNet101 and VGG16 models, respectively. The authors obtained the highest accuracy of 98.81% to recognize the Bangladesh freshwater fish for InceptionResnetV2 and Xception On the other hand, ResNet152V2 achieved 90.24% accuracy which is the lowest among all the working approaches (Majumder et al. 2021). When the loss rates of each model were examined, it was found that the ResNet50 model had 0.3646, the ResNet101 model was 0.5811, the VGG16 model was 0.0696, and the loss ratio of the proposed IsVoNet8 model was 0.0568. Kratzert & Mader (2018) proposed that an enhanced version of VGG16 model for the automated classification of the underwater fish species. The authors proposed a technique based on the monitoring system of FishCam for the observation of underwater objects. They classified 10 fish species and obtained 93.3% accuracy. Santos & Gonçalves (2019) compared results of VGG16 and VGG19 in classifying fish images and had VGG19 with 83%, which is 2% higher than VGG16 of 81%. Montalbo & Hernandez (2019) proposed a methodology to recognize fish species using VGG16 Deep Convolutional Neural Network (DCNN). Though this approach gets 98.67% accuracy, they have used synthetic augmented data for training and testing the proposed model for three different fish species. It has been seen that the model we proposed provides a better performance than the studies in the other literature due to both the low learning time of the neural network and the high accuracy of success.

In addition, in the confusion matrixes given in Figure 4, the results of estimating the fish class according to the validation data group of each model are seen. It seems that the best classification is in the proposed IsVoNet8 model. Therefore, according to the results given in Table 3 and Figure 4, it is seen that the IsVoNet8 model classifies the fish classes considered in the study with higher success accuracy. According to the results obtained; Selecting the layer and hyperparameter values used in the proposed model in accordance with the model network provided a superior performance compared to other studies used in the classification of fish species (Rathi et al. 2017; Khalifa et al. 2018; Kratzert & Mader 2018; Montalbo & Hernandez 2019; Santos & Gonçalves 2019; Majumder et al. 2021).

4. Conclusions

In this study, the common species of fish, which have an important role in the nutrition of human beings, have been predicted through the images of deep learning algorithms, which is one of today's popular machine learning methods. In this context, 8 different fish species were classified using deep learning architectures. A new model was developed in the classification process, and the proposed model was compared with the ResNet50, ResNet101 and VGG16 models accepted in the literature to prove the success accuracy of the model. While comparing, 8000 fish images in the data set; analyses were made by using 6400 of them in the model training, 800 in the model testing part and 800 in the validation data group.

As a result of the model training, according to the validation data group, 91.37% success accuracy in the ResNet50 model, 86.12% in the ResNet101 model, 97.75% in the VGG16 model and 98.62% in the proposed IsVoNet8 model were obtained. It has been observed that the proposed IsVoNet8 model has achieved high success accuracy compared to other models, despite the fact that both the number of parameters is little and the cost is low. The higher success of the proposed model compared to the other models discussed in the study highlights the superiority of the model. When the study was compared with other similar studies (Sarıgül & Avcı 2017; Iqbal et al. 2021; Ju & Xue 2020; Mathur & Goe 2021; Guo et al. 2020 and Kayaalp K & Metlek S 2021), although the number of classes was small, the high number of images in the data set led to a better success rate.

As a result, it was seen that the IsVoNet8 model proposed in the study classified fish images with a superior success rate compared to other models. The fact that the IsVoNet8 model is also applicable to classify different fish species increases the original value of the study. In further studies, the IsVoNet8 model is possible to be studied and subsequently evaluated with images of other fish species. Furthermore, the model is also possible to be developed to estimate fish biodiversity, species richness and size, weight, and age essential for stock assessment and management. Moreover, the study results could be used to develop mobile determination and classification applications depending upon deep learning in order to identify fish species.

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Isolation and Characterization of Rhizospheric Bacteria from *Vuralia turcica* Rhizospheric Soil

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ABSTRACT

Plant growth-promoting rhizobacteria are highly active in soil ecosystems for legumes due to their biotic activities. *Vuralia turcica* (Kit Tan, Vural & Kucukoduk) Uysal & Ertugrul is a Turkish endemic legume plant with potential value as ornamental and food crops. However, reports of plant growth-promoting rhizobacteria in *V. turcica* rhizosphere are lacking in the literature. The purpose of this study was the isolation and characterization of endophytic bacteria from *V. turcica* rhizospheric soil. Ten bacterial strains were isolated and identified by comparing the 16S rRNA and 16S-23S rRNA ITS region. 4 isolates belonged to *Bacillus megaterium*, 3 strains belonged to *Stenotrophomonas rhizophila*, 1 strain belonged to *Rhodococcus erythropolis*, 1 strain belonged to *Xanthomonas albilineans*. The remaining 1 strain belonged to *Lysobacter enzymogenes*, respectively.

Keywords: Growth-promoting, 16S rRNA, 16S-23S rRNA ITS region, Molecular characterization

1. Introduction

Plant growth-promoting rhizobacteria (PGPRs) can stimulate plant growth, colonize the rhizosphere, and thrive and compete with other soil microorganisms accounting for their plant growth-enhancing properties (Kloepper 1994). They can be subdivided according to their function into plant growth-friendly phytostimulators, plant-enhancing biofertilizers, organic waste degraders in soil, and biopesticides controlling fungal and microbial pathogens (Antoun & Prevost 2005). In addition, they can be divided into two groups in terms of their position to anchor and colonize: the first one, extracellular PGPR, colonizes the rhizosphere or unoccupied spaces between root cortex cells (Bhattacharyya & Jha 2012), and the second one, intracellular PGPR, where rhizobia occur in root cell nodular structures (Figueiredo et al. 2010; Bhattacharyya & Jha 2012).

Different genera of PGPR, such as *Bacillus*, *Enterobacter*, *Azospirullum*, *Pseudomonas*, and *Azotobacter*, improve the growth of chickpea (Roopa et al. 2012), tobacco (Zhang & Kong 2014), squash (Yildirim et al. 2006), lettuce (Barassi et al. 2006), and common bean (Barros et al. 2018), respectively. PGPRs are highly effective on legumes in soil ecosystems due to their biotic activities. In terms of application, they account for the increase in the yield of legumes by enhancing the concentration of mineral elements such as N, P, and K available in the soil.

In recent years, sudden climate changes have started to bring irreversible damages such as forest fires that are more common and affecting more expansive areas, the emergence of epidemics that threaten plant and animal species, the shift of biodiversity to the north to adapt to increasing temperatures, and the increase in drought and water scarcity due to decreased precipitation. However, it has been determined that there has been a decrease in agricultural lands per capita worldwide in recent years. It is stated that this decrease rate is 14.3% in developed countries, but it increases to 40% in developing countries (Aydinalp & Cresse 2008; Farooq et al. 2009). Therefore, the importance of PGPRs has been increasing in recent years. Some researchers have suggested that PGPRs have positive effects on plant development in many plant species by a series of mechanisms such as early germination, plant height, weight, development of shoot tissues, root growth, leaf area, early flowering, increase in chlorophyll amount, delay in the formation of the abscission layer in the leaf, and balancing the nutrient content (Sharafzadeh 2012; Singh et al. 2014; Fan et al. 2017). Plant growth-promoting rhizobacteria are becoming more widely used in agriculture, and they offer

an enticing alternative to chemical fertilizers, herbicides, and minerals. Also, excessive amounts of expensive fertilizers are used in intensive agricultural practices to produce optimum crop yields. Biological nitrogen fixation by symbiotic and nonsymbiotic bacteria, on the other hand, can help increase soil fertility and crop output while minimizing the use of chemical fertilizers. Legumes play a critical role in agricultural production because of their ability to fix nitrogen in conjunction with rhizobia.

Vuralia turcica (Tan et al. 1983) Uysal et al. (2014) is a Turkish endemic plant of legume that is potentially valuable to increase yield. However, reports of PGPRs in *V. turcica* rhizosphere are lacking in the literature. Therefore, this study aims to isolate and characterize PGPRs in rhizospheric soil where *V. turcica* grows.

2. Material and Methods

2.1. Soil and rhizome sampling

Four rhizospheric soil samples of approximately 100 g each and four rhizomes were collected from four *V. turcica* fields in April 2017 during the plant's blooming period. The fields were located close to the Eber and Akşehir lakes in Konya Province in Turkey. The coordinates of each sample collected are: L1 (Gölçayır, 38° 28′ 10.5′′ N/31° 21′ 04.4′′ E), L2 (Akşehir, 38° 28′ 17.328′′ N/31° 20′ 52.468′′ E), L3 (Dereçine, 38° 30′ 36.702′′ N/31° 17′ 56.702′′ E), L4 (Sultandağı, 38° 32′ 43.2168′′ N/31° 16′ 54.4728′′ E). A Global Positioning System (GPS; Magellan eXplorist 310) was used to determine the coordinates of the samples collected. Soil samples were obtained from the depth of rhizomes (~30 cm) and taken in sterilized ziplock containers, stored in an ice chest, and transported to the laboratory to use within 12h from the collection. Simultaneously, rhizome samples from the depth (~30 cm) were collected for mineral element analysis. The sample collection was implemented by the workers of Nezahat Gökyiğit Botanical Garden in Istanbul, Turkey.

2.2. Physicochemical properties of the rhizospheric soil samples

The collected soil samples were aseptically separated from rhizomes to determine the physicochemical properties of the soils. 5 g of air-dried soil was passed through a 2 mm mesh used for pH and electrical conductivity (EC) determination. Soil pH was measured in a 2.5:1 water/soil suspension using a pH meter (Hanna instruments-HI 2211) described by Jackson (1959). EC of each soil sample was determined using the method described by Richards (1954) using electrical conductivity (EC) meter (WTW series-inoLab-Cond-720) in a 5:1 distilled water: soil dilution.

2.3. Nutrient analysis of the rhizospheric soil samples and the collected rhizomes

The mineral element content of the collected soils and rhizomes was determined using the methods described by Tekdal et al. (2018). Rhizome samples were finely ground after drying at approximately 65°C for 48h. Soil samples were air-dried for two days and then separated through a 2 mm mesh for analysis. The ground rhizome and air-dried soil samples were used to determine mineral element content according to the methods described by Lindsay & Norvell (1978) and Olsen et al. (1954). Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) (Vista-Pro Axial, Varian Pty Ltd, Mulgrave, Australia) was used to determine the mineral concentrations of the samples. Certified standard reference materials obtained from the National Institute of Standards and Technology (NIST; Gaithersburg, MD, USA) were used to calibrate the measurements.

2.4. Endophytic bacteria isolation

Endophytic bacteria were isolated from the collected soil samples. Modified solid Yeast Extract Mannitol (YEM) medium described by Vincent (1970) was used to screen PGPRs in the soil. 10 grams of the soil sample from the rhizosphere was diluted to 10−5–10−6 suspension using 100 mL sterilized water. The soil suspension of 100 µL was then spread over a petri dish (100 × 15 mm) containing a culture medium for PGPRs. The Petri dishes were left in the incubator for 48h at 28°C. Screened strains were transferred to a fresh YEM agar medium for bacteria, which were then stored at 4°C for future use. For the pre-selection of PGPRs, YEM agar medium containing either Bromothymol blue or Congo red was used. Gram staining of the isolates was also conducted.

2.5. Microbial identification system (MIS) analysis of isolated bacteria

Bacterial fatty acid methyl ester (FAME) analysis was carried out utilizing gas chromatography (6890N GC, Agilent Technologies INC., USA) and MIS tools (Sherlock 6.0 MIDI, Inc., Newark, DE, 2005).

2.6. DNA isolation and PCR analysis

DNA was extracted from approximately 200 mg of the sample using the commercially-available kit (QIAamp DNA Minikit (Cat#51304, Qiagen, Valencia, CA) following the manufactures' instructions. DNA quality was checked on 1.5% agarose gel and was quantified spectrophotometrically. It was then stored in TE buffer (10 mM Tris, 1 mM ethylenediaminetetraacetic acid, pH 8.0) at –20°C for use in PCR analysis. 16S rRNA and 16S-23S rRNA Internal Transcribed Spacer (ITS) primers of bacteria

were used for PCR amplification. 16S rRNA genes were amplified using the sense strand primer 5' AGAGTTTGATCCTGGCTCAG -3' (D1F) and the anti-sense strand primer 5' AAGGAGGTGATCCAGCC -3' (D1R). 16S-23S rDNA ITS region was identified using the sense strand primer 5' TGCGGCTGGATCCCCTCCTT -3' (FGPS1490-72) and the anti-sense strand primer 5' CCGGGTTTCCCCATTCGG -3' (FGPL132-38). The PCR mixture (25 µL) consisted of 5 ng of DNA, 0.125 U/µL Taq DNA polymerase (Fermentas), 0.2 mM dNTP mixture, 0.8 µM of forward and reverse primer each, the polymerase reaction buffer (1X). The PCR was performed as follows: 35 cycles of 94 °C for 7min, 94 °C for the 30s, 55 °C for 30s, 72 °C for 1min, and 72 °C for 5min. PCR products were sequenced by BM Laboratory Systems, Ankara, Turkey (https://www.bmlabosis.com).

The 16S sequences of the isolates L1S2, L1S3, L1S4, L2S1, L2S2, L2S3, L3S1, L4S1, L4S2, L4S3, and L4S4 were submitted to GenBank under accession numbers MT477132, MT477133, MT477134, MT477135, MT477136, MT477137, MT477138, MT477139, MT477140, MT477141, respectively whereas the GenBank accession numbers of 16S-23S rDNA ITS sequences of the isolates L1S2, L1S3, L1S4, L2S1, L2S2, L2S3, L3S1, L4S1, L4S2, L4S3, and L4S4 are MT460379, MT460380, MT460381, MT460382, MT460383, MT460385, MT460386, MT460387, MT460388, and MT460389, respectively.

2.7. Phylogenetic analysis

The sequencing results were analyzed using the NCBI database for homologous sequences of 16S rRNA and 16S-23S rRNA ITS region in GenBank at the National Center for Biotechnology Information (NCBI), Bethesda, USA, using the BLAST search program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al. 1990). The Tamura 3-parameter and the Kimura 2-parameter methods were used to calculate the differences in nucleotides (Kimura 1980; Tamura 1992). The neighbor-joining method (Saitou & Nei 1987) was used to build a phylogenetic tree by the Molecular Evolutionary Genetics Analysis (MEGA) program version 7.0 (https://www.megasoftware.net/). Aligned 16S rDNA and 16S-23S rDNA ITS sequences were evaluated with bootstrap analysis (1000 replicates) (Felsenstein 1985).

3. Results

3.1. pH, soil nutrient, and salt content determination

The first step of this work was soil sampling. In addition to the bacterial isolation, the soil samples were used as soon as possible to analyze the pH, salt composition, and nutrient components. The pH and salt composition of the samples are reported in Table 1. The results showed that all soil samples collected from four different points were alkaline, as the pH range of analyzed samples was from 7.50 to 8.20. The soil sample taken from location 2 (L2) had a high EC, 1654 µs/cm (Table 1).

Table 1- pH and salt levels of the soil samples

Depth (cm)	*Sample*	*Region*	*pH*	*Salt (µs/cm)*
0-30	L1	Gölçayır	7.50	3.18
0-30	L2	Akşehir	7.98	1654
0-30	L3	Dereçine	8.05	512
0-30	L4	Sultandağı	8.20	396

3.2. Determination of rhizome and soil nutrient components

The mineral element content of the rhizomes and soils collected was obtained by ICP-OES analysis, and the results are reported in Table 2. B, Mo, Cu, Fe, Mn, Ni, and Zn levels are much higher in rhizomes than in soil samples. In contrast, macronutrients are present at much lower levels in rhizomes as compared to the soil, while the reverse is true for N and C levels.

Table 2- The mineral nutrient content of the rhizome and soil samples of *V. turcica* collected from different natural habitats

*Samples**	*B*	*Mo*	*Cd*	*Cu*	*Fe*	*Mn*	*Ni*	*Zn*	*Ca*	*K*	*Mg*	*N*	*C*
	mg kg^{-1}								*%*				
	Rhizomes												
L1	28	1.11	0.04	11	404	16	0.52	30	0.33	0.72	0.37	1.29	47.39
L2	16	0.03	0.05	11	604	22	0.74	15	0.52	0.24	0.34	1.29	47.08
L3	27	0.49	0.04	15	380	52	0.67	33	0.38	0.53	0.18	1.72	46.72
L4	36	0.46	0.03	9	379	23	0.65	32	0.59	1.06	0.56	2.08	47.30
	Soils												
L1	4.22	0.05	0.01	1.43	8.71	0.05	0.05	0.05	616	24	92	0.37	7.99
L2	2.36	0.03	0.01	2.46	9.03	0.03	0.03	0.03	697	35	135	0.16	4.27
L3	0.54	0.01	0.02	3.99	7.79	0.01	0.01	0.01	253	12	53	0.06	4.07
L4	0.64	0.04	0.01	1.89	12.12	0.04	0.04	0.04	7038	10	1517	0.42	7.68

***:** The coordinates of each sample; L1: (Gölçayır, 38° 28′ 10.5′′ N/31° 21′ 04.4′′ E), L2: (Akşehir, 38° 28′ 17.328′′ N/31° 20′ 52.468′′ E), L3: (Dereçine, 38° 30′ 36.702′′ N/31° 17′ 56.702′′ E), L4: (Sultandağı, 38° 32′ 43.2168′′ N/31° 16′ 54.4728′′ E)

3.3. Pre-selection of isolated bacteria from samples of the soil from different environments of V. turcica

There were a total of 10 isolates. Among these isolates, 3 isolates were obtained from sample 1, 2 isolates were obtained from sample 2, 1 strain was obtained from sample 3, and 4 isolates were obtained from sample 4. Table 3 indicates the responses to red, bromothymol blue, and gram-staining. As a result of MIS analysis, various colonized bacteria are given in Table 4 on the rhizospheric soils of *V. turcica* were determined.

Table 3- Reactions of isolates to Congo red, Bromothymol blue and Gram-staining

Location	*Strain Code*	*Gram-staining*	*Congo red*	*Bromothymol blue*
1	L1S2	Negative-Coccus	Red	Orange-Basic
	L1S3	Negative-Coccus	Black	Fungus-Acidic
	L1S4	Positive-Coccus	Orange	Yellow-Orange-Basic
2	L2S1	Negative-Coccus	Red	Yellow-Orange-Basic
	L2S2	Negative- Coccus	no growth	Yellow-Basic
3	L3S1	Positive- Coccus	Red	Yellow-Green-Basic
4	L4S1	Negative- Coccus	Red	Yellow-Orange-Basic
	L4S2	Negative- Coccus	Red-Black	Yellow-Orange-Acidic
	L4S3	Negative-Bacillus	Red	Yellow-Orange-Acidic
	L4S4	Negative-Bacillus	Red	Yellow-Orange-Acidic

Table 4- MIS results of the bacteria isolated from soil samples

Location	*Strain Code*	*MIS result*
1	L1S2	*Kocuria kristinae*-GC subgroup A (0.692)
	L1S3	*Bacillus megaterium*-GC subgroup A (0.805)
	L1S4	*Virgibacillus pantothenticus* (0.675)
2	L2S1	*Bacillus megaterium*-GC subgroup A (0.792)
	L2S2	*Pseudomonas syringae*-syringae (0.380)
3	L3S1	*Stenotrophomonas maltophilia* (0.305)
4	L4S1	*Kocuria erythromyxa* (Deinococcus) (0.374)
	L4S2	*Bacillus viscosus* (0.496)
	L4S3	*Pseudomonas huttiensis* (0.605)
	L4S4	*Pseudomonas huttiensis* (0.552)

3.4. Phylogenetic analysis of soil-isolated bacteria

The 16S rRNA and 16S-23S ITS sequences of soil-isolated bacteria were compared to the 16S rRNA and 16S-23S ITS sequencing identified in the GenBank database and BLAST. The results of the 16S rRNA analysis showed that the 10 isolates could be attributed to five groups. Among them, 6 isolates were *Pseudomonas* sp., 1 isolate was *Arthrobacter* sp., and the other 3 were *Bacillus* sp., *Aurantimonas* sp., and *Sphingomonas* sp., respectively. One isolate was closely phylogenetically related to *Agrobacterium tumefaciens*, showing 100% similarity in their 16S rRNA sequencing. One strain was found to be closely phylogenetically related to *Bacillus megaterium* (Figure 1).

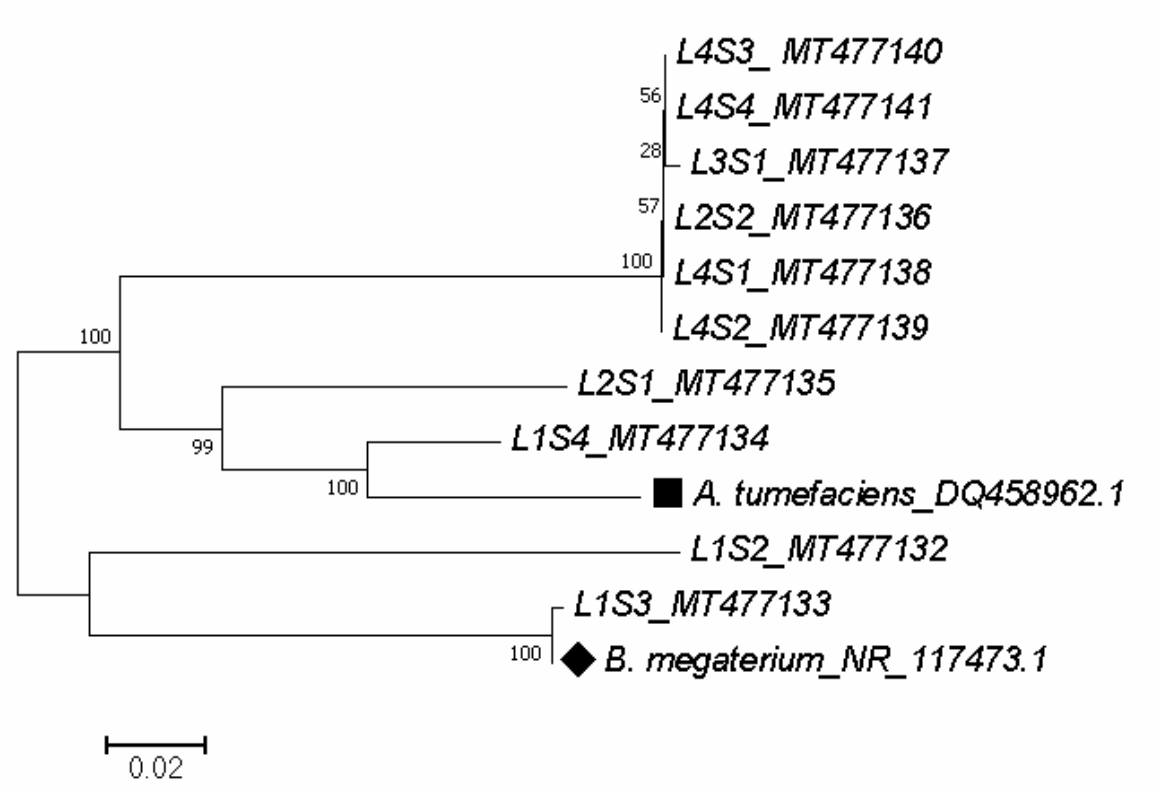

Figure 1- Neighbor-joining tree based on partial sequences of 16S rDNA region of the isolate for phylogenetic inference. As the outer group, DQ458962.1 and NR_117473.1 were chosen to depend on their 16S rRNA sequence similarities

On the other hand, the 16S-23S ITS analysis showed that the dominant genus was *Bacillus* in 6 isolates and *B. Megaterium* in the remaining 4 isolates. The strain L1S3 was also closely phylogenetically related to *B. megaterium* due to 16S-23S ITS analysis (Figure 2).

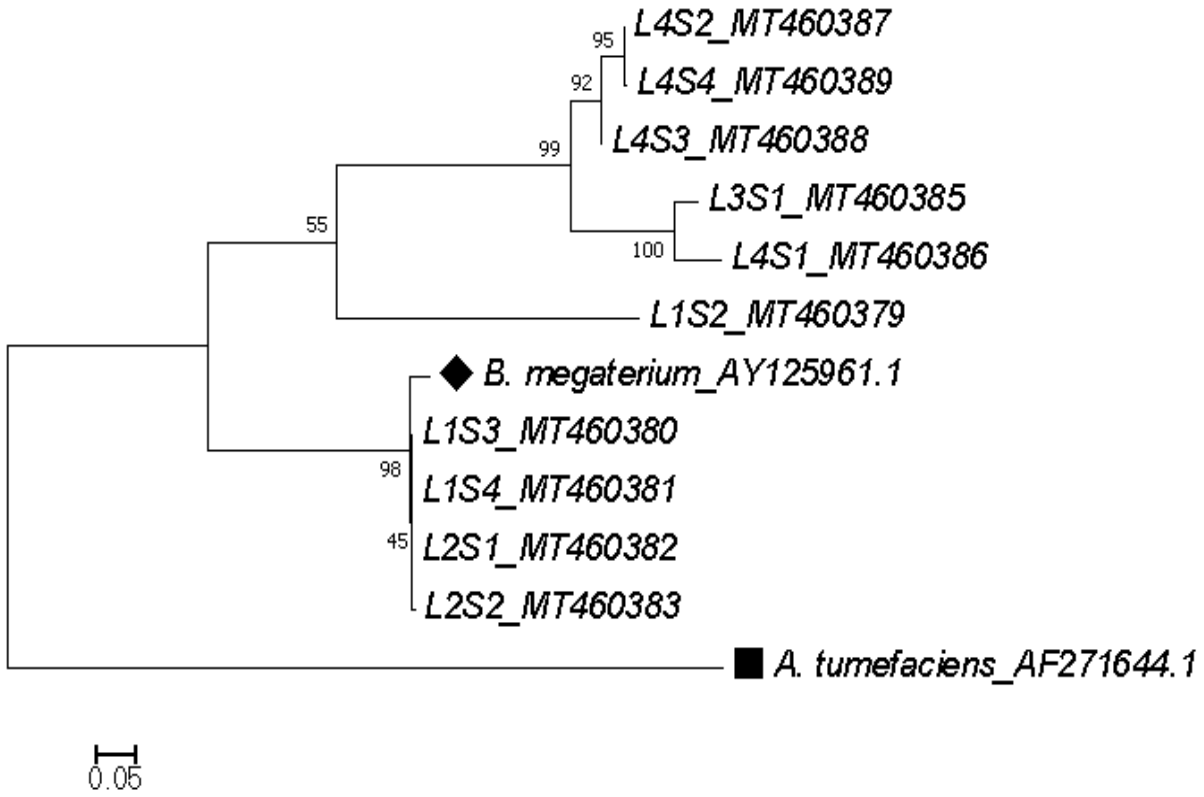

Figure 2- Neighbor-joining tree based on partial sequences of the ITS region of the isolate for phylogenetic inference. As the outer group, AF271644.1 and AY125961.1 were chosen to depend on their ITS sequence similarities

4. Discussion

The results of the pH and EC of collected soil samples, summarized in Table 1, indicate that all soil samples were alkaline.

Significant variations were found in the mineral analysis of rhizomes and soil samples. The quantities of minerals boron (B), copper (Cu), iron (Fe), zinc (Zn), and carbon (C) were elevated in rhizomes but in reduced concentrations in soil samples (Table 2). This discrepancy is most possibly due to the growth-promoting ability of bacteria present in *V. turcica* rhizospheres. In the phytoremediation of heavy metal-contaminated soils, *B. megaterium* is beneficial as this bacterium boosts the B desorption from the soil by enhancing its deposition in plants (Esringu et al. 2014). Also, *B. megaterium* can metabolize the Cu in the environment (Hohapatra et al. 1993). Therefore, high levels of Cu and B found in the rhizome samples are likely to be associated with *B. megaterium* activity.

Production and secretion of siderophores are an activity of *B. megaterium*, like most other PGPRs (Cornelis & Simon 2010). Increased secretion of siderophores by *B. megaterium* is reported in soils with iron deficiency (Arceneaux & Byers 1980). Siderophores can chelate ferric ions present in the environment into a siderophore-iron complex from which plants can acquire the Fe ion. The siderophore is then released into the rhizosphere and recycled (Santos et al. 2014). The amount of available iron found in *V. turcica* rhizomes while there is the facilitation of iron uptake can explain an iron scarcity in the soil through released siderophores of *B. megaterium*. Bacterial siderophores probably chelate the iron that is unavailable to *V. turcica* into plant-available forms.

In the literature, it is also mentioned that *B. megaterium*-related siderophores can ease Zn uptake for plants by solubilizing it (Kucey et al. 1989). Zn chelating behavior of *B. megaterium* is interpreted as the main reason for the high amount of Zn found in rhizomes in Zn deficient soil. Indirect growth-promotion pathways of *B. megaterium* include the activation of induced systemic resistance, the synthesis of antibiotics, the synthesis of siderophores, and the secretion of lytic enzymes (Ngoma et al. 2012). Besides providing iron ions to the host plants, *Bacillus spp*. produced siderophores also decrease the availability of the metal ions for competing species or pathogens present around the rhizosphere (Mathiyazhagan et al. 2004). Iron ions can easily be deprived of the rhizosphere because of the high affinity of siderophores with metal ions. This causes iron deficiency for surrounding pathogens and suppresses them (Kloepper et al. 1980). While protecting the host plant, this suppression enables *Bacillus spp*. to compete better for nutrients and gets more room to colonize (Labuschagne et al. 2010). The Fe ions were found in different compounds in the rhizomes than in the soil. With siderophores, *B. megaterium* competes better for Fe than pathogens and indirectly promotes plant growth through pathogen suppression.

The chlorophyll content of a plant represents the N-fixation activity and the plant's total N content (Kumawat et al. 2000). The photosynthetic capacity of a plant could be enhanced through *Bacillus sp.* N-fixing ability increases chlorophyll content. Thus, more photosynthates would be produced in the plant body to be further exuded from the roots (Elkoca et al. 2007). Rhizobacteria metabolize root exudates mostly for their carbon content (Vacheron et al. 2013). Regarding energy production, *B. megaterium* can metabolize more than 62 different carbon sources, including mono and disaccharides. This versatile carbon consumption-ability increases this species' survivability by facilitating colonization in different environments (Stülke & Hillen 2000; Vary et al. 2007).

Phytohormones induce and regulate plant growth, and PGPR are able to produce them. *Bacillus spp.* can directly influence root growth and shoot development by secreting auxins and cytokinins, which represent the main classes of plant growth-promoting compounds (Persello-Cartieaux et al. 2001; Arkhipova et al. 2005). In the literature, many reports show the ability of *B. megaterium* to produce plant hormones like auxins, gibberellins, cytokinins, and abscisic acid (Karadeniz et al. 2006). *B. megaterium* can induce shoot growth and ensure robust root development in plants through cytokine signaling (Ortiz-Castro et al. 2008). This bacterial species accounts for an increase in the root surface area of its host plant by supporting a robust root development, lateral root growth, root hair elongation, and root growth with an auxin/ethylene independent mechanism (López-Bucio et al. 2007). In this context, it is reasonable to assume that the growth of *V. turcica* is promoted by hormone-signaling pathways related to this bacterium as the soil is deficient in nutrients. Confirming this assumption requires a determination of the hormone levels and molecular interactions, including signaling pathways between the bacterium and the plant.

B. megaterium is a well-known PGPR species that promotes plant growth by N-fixation, P-solubilization, increased mineral uptake, and antimicrobial activity (López-Bucio et al. 2007). Many bacterial species were isolated from the soil samples taken around *V. turcica*'s natural habitat, but the most intriguing species were *B. megaterium*, *Rhodococcus erythropolis*, *Xanthomonas albilineans*, *Lysobacter enzymogenes*, and *Stenotrophomonas rhizophila* (Figure 1). *R. Erythropolis* is known to promote its host plant's growth in cold climate conditions (Trivedi et al. 2007). *X. albilineans* were reported to have pathogenic effects on sugarcane plants (Zhang et al. 2017). It is unclear whether this bacterium is pathogenic or not for *V. turcica*. However, even if it is, the antimicrobial activity of *B. megaterium* was probably useful in suppressing this bacterium, as healthy plant growth was naturally achieved. *B. megaterium* is an important bacterium frequently used in the industrial processing of fungicides, viral inhibitors, vitamins, and enzymes (Vary et al. 2007). It carries a high potential as a biofertilizer because of its plant growth-inducing abilities (Patel et al. 2016). It would be agriculturally and industrially interesting to collect more information on the molecular relations and secondary metabolite interactions between *B. megaterium* and the plant *V. turcica*, a resident of an

environmentally unfavorable place. Nutrient uptake for plants could be relieved through the secondary metabolites that PGPR secrete into the environment. *L. enzymogenes* exerts antifungal activity through lytic enzyme secretion (Jochum et al. 2006). This activity could account for the survival of *V. turcica* in its natural habitat by suppressing its fungal pathogens. *S. rhizophila was reported* to help plants to overcome salt stress enabling their growth in salty soils (Egamberdieva et al. 2016). Since the soil of *V. turcica*'s natural habitat is salty (Table 1), it could be supposed that *S. rhizophila* is helping *V. turcica* in relieving salt stress in its natural environment.

MIS is based on quantity as a percentage of fatty acids in microorganisms' cells (Şahin et al. 1999). Evaluation of MIS analysis is shown in Table 4. Moreover, the variety of isolated bacteria was different in soil samples at MIS analysis results. This could be due to the reduction in hydrocarbon sources in the soils. The number of FAME groups was high when the soils were collected, and then it started to decrease based on time. This study relies more on sequence analysis because of the narrow fatty acid library that is used in MIS analysis (1000 elements).

The role of isolated bacteria is unknown, so some speculations on the possible physiological roles of isolated bacteria were considered. Remarks on the estimation of the bacterial role should be proved experimentally.

5. Conclusions

With antibiotics potentially secreted, *B. megaterium* may restrict growth and increase the colonization rate of other microorganisms on the rhizome. Further analysis is needed to make certain conclusions on this subject, which reveals the antibiotics produced in the natural ecosystem of *V. turcica*. *B. megaterium* may be essential to *V. turcica*'s survival. As this plant's natural habitat is limited to an area, there is a lack of *B. megaterium* may be the cause of why *V. turcica* is not present elsewhere. The soil in which *V. turcica* naturally exists is also where the bacterium is found. By affecting the previous literature findings on this bacterial species, we strongly propose that *B. megaterium* plays a critical role in *V. turcica* plant growth and production in its natural environment. This is the first report of the presence of plant growth-promoting rhizobacteria in natural habitats and endemic plant *V. turcica* rhizospheric soils.

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Comprehensive Stability Analysis of Wheat Genotypes through Multi-Environmental Trials

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ABSTRACT
In rainfed areas, due to variable environmental factors, improving the yield stability of the introduced cultivars along with increasing yield should be considered. The main aim of this study is to obtain high-yield wheat genotypes that are stable and adaptable to cold climatic conditions in Iran. For this purpose, 25 wheat genotypes were evaluated in a randomized complete blocks design with three replications during three cropping seasons (2013-2016) under supplementary irrigation and rainfed conditions. PBSTAT-GE software was used for genotype × environment interaction (GEI) analysis and comprehensive sustainability analysis. The results showed that G5, G14, G16 and G18 genotypes had good stability and general adaptation based on parametric and non-parametric stability statistics. Combined analysis of variance based on the Additive Main Effect and Multiplicative Interaction (AMMI) model showed that GEI is significant in the term of grain yield. Also, the ratios between the sum of squares G, GE and IPC1 showed that the AMMI is suitable for data analysis. GGE biplot analysis identified five mega-environments (MEs), in which ME I including E1, E2, E3, E4, E5, E6, and G7, G5, G14, G13, G16, G18, G20 being the superior ME I genotypes. According to AMMI and GGE biplote stability methods, lines G20, G18, G13, G16, G14 and Saein cultivar (G5) can be considered as desirable genetic resources in wheat production programs under variable environments in Iran, due to having the appropriate combination of yield and stability.

Keywords: Adaptability and stability, AMMI, ANOVA, GE interaction, GGE biplot, Wheat

1. Introduction

To analyze genotype × environment interaction (GEI) and determine genotypes adapted to different climatic conditions, the evaluation of genotypes obtained from breeding programs at the national level has an important role in identifying suitable genotypes for target environments (Ayed et al. 2016). Breeders usually evaluate advanced genotypes using their similar responses under different environments to classify environments into similar groups and to determine the best genotype for each environmental group and introduce stable high yield genotype for different environments (Yan et al. 2000). GEI is one of the biggest challenges for researchers, which reduces the efficiency of selection and prediction of genotype yield in target environments (Yan & Fregeau-Reid 2018). One of the methods to reduce GEI and also increase yield is to select and introduce high-yield and stable genotypes in different regions (Kang 1993). Many researchers have used the combined analysis of variance method to estimate GEI, however, combined ANOVA and interaction test lacks the ability to determine the stable genotype (Reynolds et al. 2016). Therefore, various methods have been used to identify stable genotypes, including univariate and multivariate parametric and non-parametric stability methods (Elias et al. 2016). Breeders use parametric stability analysis to reach a series of multivariate experiments. Calculation of stability based on non-parametric rankings does not require statistical assumptions, such as normal distribution of model residuals as well as interaction effects. In addition to being easy to use and interpret, these methods have the least noticeable error in calculations compared to parametric calculations (Huehn 1996). Although parametric and non-parametric univariate statistics are easy to calculate and use, however, these methods cannot well interpret the complex and multidimensional nature of the interaction. Therefore, the use of complementary multivariate methods has been suggested to solve this problem (Naroui Rad et al. 2013). AMMI model as a multivariate parametric method is one of the most widely used and popular methods (Malosetti et al. 2013). The AMMI method distinguishes the main effects (genotype and environment) and interactions and is well used to determine GEI (Lai 2012;

Sadiyah & Hadi 2016). The graphical tools of this method have a special role in the simultaneous evaluation of yield and stability, as well as the selection of MEs and specific adaptability (Ajay et al. 2020). The AMMI model by puncturing genotypes and environments on the biplot, identifies the position of the genotypes relative to each other and the studied environments (Elakhdar et al. 2017). Mohammadi et al. (2015), Ajay et al. (2020), Khan et al. (2020), Lozada & Carter (2020), Mekonnen et al. (2020), Mohammadi et al. (2020) and Verma & Singh (2021) by examining the stability of wheat genotypes reported that the AMMI method with high fitting power, is more suitable than other methods for determining genotypes with general and specific adaptability for different locations. Ahakpaz et al. (2021) by examining 18 rainfed barley genotypes for three cropping years in cold rainfed regions of Iran, reported that the effects of G, E and GEI on grain yield were significant. Also, according to AMMI analysis, there was a significant difference between the first two main components.

Another multivariate method is the Genotype plus GE interaction (GGE) biplot (Yan et al. 2000), which is a powerful graphical model for identifying the best-yield cultivars among different environments. GGE biplot is an analytical method of GEI that simultaneously evaluates the main effect of genotype and GEI. In this method, genotypes are evaluated based on yield in different environments, the combination of stability and yield, the ability of environments to distinguish genotypes, and their representativeness (Yan & Kang 2003). The difference between these two multivariate methods is that GGE biplot is based on environment-centered principal component analysis, while AMMI is based on double-centered PCA (Yan et al. 2007). Ashraful et al. (2017), Bornhofen et al. (2017), Bavandipour et al. (2018) and Singh et al. (2019) reported that the combined application of AMMI and GGE biplot methods provide a powerful tool for identifying high-yielding and adaptable wheat genotypes as well as analyze and interpret multi-environment trials (MET) data in breeding programs.

Phenotypic stability can be divided into two principal types, that is, stability in the biological and agronomic sense. Stability in the biological (static) sense refers to the ability of genotypes to maintain constant production in different environments, with low variation between them, that is, genotypes exhibit "homeostasis". Stability in the agronomic (dynamic) sense indicates that the genotype positively responds to improvements in edaphoclimatic conditions of the environment and can perform above the mean in different locations (Sabaghnia et al. 2015). This behavior is of interest to plant breeders and farmers. It is important for the plant breeder to adopt methods in which genotype stability is associated with high grain yield average. The combination of these two concepts is known as the "ideal genotype" (Yan et al. 2007; Yan 2016).

Today, several statistical packages are used by plant breeders to perform stability analysis, such as CropStat, PBTools, GEA-R and SAS. PBSTAT-GE (Bayuardi-Suwarno et al. 2008) is a web-based software package for comprehensive stability analysis available at http://www.pbstat.com. PBSTAT-GE includes program code development from R packages, including Agricolae and GGE biplot. About twelve univariate parametric and non-parametric stability measures with AMMI and GGE biplot analysis can be performed using this software. PBSTAT-GE also estimates heritability, the correlation between stability parameters, principal components with genotypes biplot, stability statistics and genotypes biplot, G × E heatmap, genotypes and stability parameters heatmap. The simplicity of PBSTAT-GE operation significantly saves researchers time and effort in performing stability analysis and select top genotypes in the breeding programs. The objectives of this study were (i) to evaluate GEI on grain yield of wheat genotypes in the rainfed areas of Iran using univariate and multivariate stability methods (ii) to identify genotypes that have high mean yield and stable performance over test environments, and (iii) to investigate the relationships between the most commonly used stability statistics.

2. Material and Methods

Field trials were conducted in the Miandoab Agricultural Research Station, West Azerbaijan Province, Iran, located at 36°58´ N, 46°06´ E and 1 314 m above sea level. The soil texture of this site was loamy silt with pH 7.9 and the soil field capacity (FC) at a depth of 30 cm was 28.7. Climatic parameters are shown in Figure 1 and Supplementary Table S1. A total of twenty-three wheat genotypes (Table 1) containing 5 cultivars and 18 promising lines along with check varieties Sardari and Azar 2, were included in the stability study. These lines were developed by several breeders at various research stations/institutes of Iran and the International Maize and Wheat Improvement Center (CIMMYT).

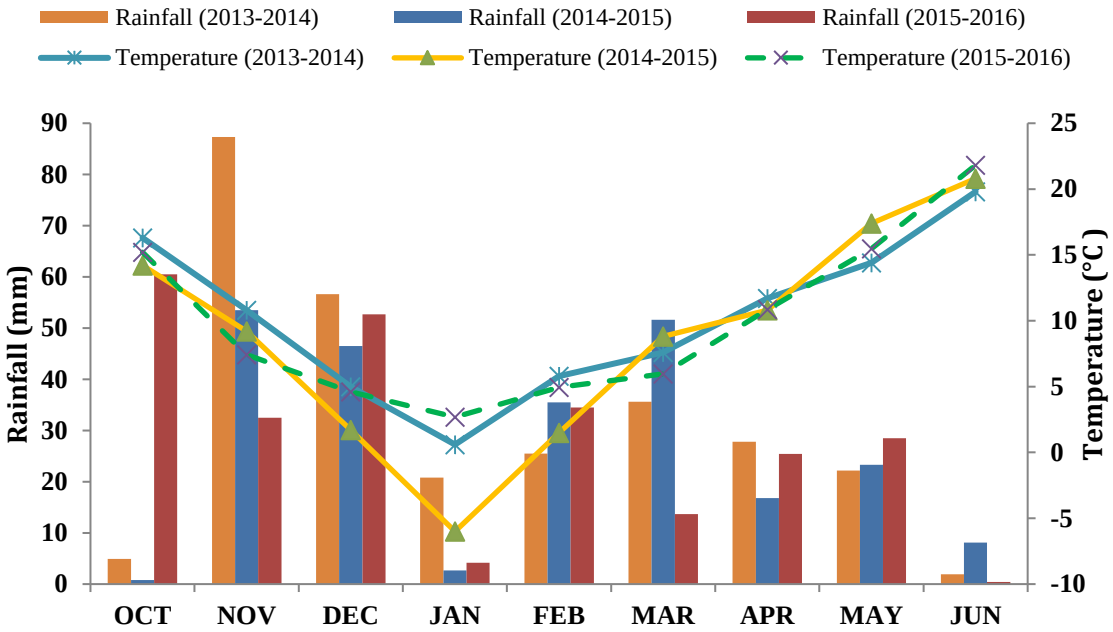

Figure 1- Monthly patterns of temperature of air and rainfall recorded during the course of the experiment

Test materials were phenotyped comprising two trials under drought-stressed (rainfed, RF) and supplementary irrigated (SI) conditions. Each field experiment was arranged based on RCBD with three replications and conducted over three consecutive cropping seasons (2013-2016). A total of 6 environments (combinations of environmental conditions and years) were examined including E1, E3 and E5, representing SI conditions in 2013/2014, 2014/2015 and 2015/2016 seasons, respectively, and E2, E4 and E6 were RF conditions in 2013/2014, 2014/2015 and 2015/2016 seasons, respectively. Under SI conditions, the genotypes were irrigated when the mean soil water content fell to 80% of FC. Fertilizer rates were 90 kg ha^{-1} $CO(NH_2)_2$, 90 kg ha^{-1} $(NH_4)_2HPO4$ and 60 kg ha^{-1} K_2SO_4 used before planting. Each plot consisted of six rows, 4 meter long and 20 cm row spacing (plot size = 4.8 m^2). Grain yield (Y_i, kg ha^{-1}) data were obtained from two middle rows of each plot for each genotype in each test environment.

Table 1- Name, pedigree and origin of tested wheat cultivars and promising lines

Code	*Pedigree / Name*	*Type*	*Origin*
G1	Sardari	Cultivar	IRAN
G2	Azar 2	Cultivar	IRAN
G3	Rasad	Cultivar	IRAN
G4	Ohadi	Cultivar	IRAN
G5	Saein	Cultivar	IRAN
G6	Azar2/87Zhong291-149	Promising line	IRAN
G7	Varan	Cultivar	IRAN
G8	Homa	Cultivar	IRAN
G9	F10S-1//ATAY/GALVEZ87	Promising line	IWWIP
G10	Seafalah/3/Sbn//Trm/K253	Promising line	IRAN
G11	Sardari-101	Promising line	IRAN
G12	Unknown 11	Promising line	IRAN
G13	Sabalan/4/Vrz/3/Or F1.148/Tdl//Blo	Promising line	IRAN
G14	Sabalan//Cno79/Prl"S"/3/Pf82200/4/Ebvd99-1	Promising line	IRAN
G15	SARDARI-HD84//UNKN/HATUSHA	Promising line	IRAN
G16	F130-L-1-12/LAGOS	Promising line	IWWIP
G17	Sara-PBWYT-85-86-22-5	Promising line	IWWIP
G18	PYN/BAU//BONITO	Promising line	IWWIP
G19	Sabalan/84.40023//Seafallah	Promising line	IRAN
G20	SUBEN-7	Promising line	IWWIP
G21	Azar2/78Zhong291-99	Promising line	IRAN
G22	Sardari//Ska/Aurifen	Promising line	IRAN
G23	TIRCHMIR1/LCO//SABALAN	Promising line	IWWIP
G24	TAST/TORIM/3/MLC/4/CWW339.5/SPN/5	Promising line	IWWIP
G25	BJN C 79/4/KVZ/CUT75/3/YMH//61.15	Promising line	IWWIP

IWWIP: International Winter Wheat Improvement Program

After checking the assumption of data normality using SPSS software, comprehensive stability analysis was done for yield data in all environments using PBSTAT-GE software. In this study, the parametric stability measures include CV_i (Coefficient of variability) (Francis & Kannenberg 1978), b_i (Linear regression coefficients) (Finlay & Wilkinson 1963), S^2d_i (Residual MS value) (Eberhart & Russel 1966), W^2_i (Wricke's ecovalence) (Wricke 1962), D_i (Hanson genotypic stability) (Hanson 1970) and σ^2_i (Shukla stability variance) (Shukla 1972) were calculated. Non-parametric stability methods based on yield ranks of genotypes in each environment were Kang yield-stability statistics (YS_i) (Kang 1993), $S_i^{(1)}$, $S_i^{(2)}$, $S_i^{(3)}$ and $S_i^{(6)}$ methods introduced by Nassar & Huehn (1987), $NP_i^{(1)}$, $NP_i^{(2)}$, $NP_i^{(3)}$ and $NP_i^{(4)}$ introduced by Thennarasu (1995) and the TOP ranking statistic method (Fox et al. 1990). In addition, PBSTAT-GE performed the ANOVA of the AMMI model for grain yield along with AMMI biplots including $AMMI_1$ biplot (IPC1 vs. yield) and $AMMI_2$ biplot (IPC1 vs. IPC2). GGE biplots were also drawn based on the first two main components obtained from stability analysis by GGE biplot.

3. Results and Discussion

Unpredictable environmental changes produce unpredictable results, therefore, it is necessary to select different types of genotypes that are fully adaptable and able to respond to environmental changes optimally. Climate changes over the years leads to environmental variations and, consequently, the occurrence of significant GEI. The GEI that affects yield is shown in Figure 2. Differences in grain yield between rainfed and supplementary irrigation conditions indicate that there is an interaction between genotypes and environments. Mean yield of genotypes in various environments showed that E1, E3 and E5 had high yield mean than the average of all genotypes (2877.63 kg ha^{-1}) in which maximum grain yield (3593.24 kg ha^{-1}) was related to E1 and the minimum of it (2197.82 kg ha^{-1}) was belonged to E4 environment.

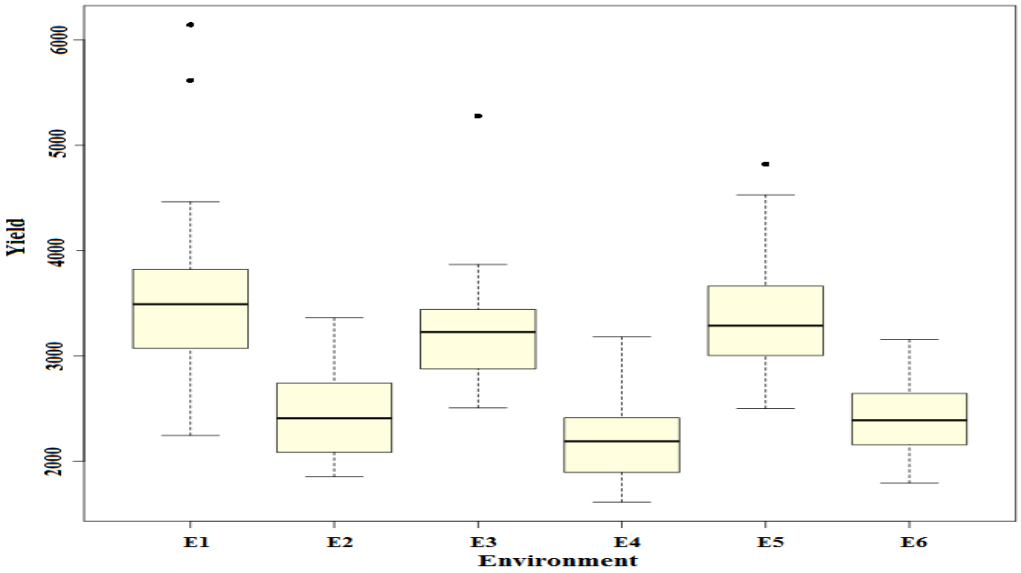

Figure 2- Average grain yield of wheat genotypes across six environments

The grain yield values of the wheat genotypes under supplementary irrigation and rainfed conditions over 3 years are listed in Table 2. In this study, the stress intensity (SI) was calculated according to Fischer's method (Fischer & Maurer 1978) that it was equal to 0.309. Genotype G10 followed by G7 and G5 showed the highest mean yield in both supplementary irrigation and rainfed conditions, while genotypes G12 and G17 had the lowest yield in both experimental conditions. Despite the high mean yield in both environmental conditions, genotypes G10 and G7 showed relatively low stability. Breeders can select genotypes with high mean yield but low stability that have the best response to certain environments (Mohammadi et al. 2012). Saein (G5) was better than the control cultivars Sardari and Azar 2 due to its higher mean yield and general adaptation to the studied environments (Table 2). In this study, genotypes G2, G13, G14 and G16, with high average yield in both supplementary irrigation and rainfed conditions, showed the lowest decrease of relative yield changes during 3 years due to drought stress (Table 2). In a study conducted by Roostaei (2015) on the genotypes of wheat studied in the present study, it was reported that these genotypes performed better under rainfed conditions and traits such as number of kernels per spike and to some extent thousand kernel weight and peduncle length, had a significant role in the yield stability of G2, G13, G14 and G16 genotypes.

According to G × E heatmap (Figure 3), the studied genotypes were divided into two main groups that first group consisting of G10, G6 and G7 genotypes. The second group was divided into 2 subgroups that G23, G25, G22, G17, G4, G15 and G12 genotypes were assigned to the first subgroup and the rest of the genotypes to the second subgroup. The genotypes with equal yield in all environments can be considered as stable genotypes. Accordingly, G22, G23, G25 and G17 genotypes can be considered as stable genotypes. The performance of genotypes in each environment indicates the diversity within that

environment, so that the difference in colors related to the genotypes in an environment indicates a variety between genotypes in that environment. Accordingly, under E1, E3 and E5 (SI conditions), genotypic diversity was high (Figure 3).

Visual considering of the distribution of genotypes in the studied environments showed the difference of various genotypes in terms of grain yield in one environment and also the difference between their means from one environment to another, which shows the selection of genotypes based on yield in one place, does not have high validity, thereby, in order to get accurate result, genotypes must be evaluated over years and places to estimate their stability (Eberhart & Russell 1966).

Table 2- The values of grain yield in wheat genotypes under supplementary irrigation and rainfed conditions over three years (2013-16)

	2013/14			*2014/15*			*2015/16*		
Gen.	Y_p	Y_s	*RYC*	Y_p	Y_s	*RYC*	Y_p	Y_s	*RYC*
G1	3034.3	2052.6	0.32	3820.4	1895.2	0.50	3161.4	2158.3	0.32
G2	3491.7	2923	0.16	3227.8	2311.9	0.28	3737.8	2944.3	0.21
G3	3556.5	2469.3	0.31	2954.6	1612.8	0.45	3020.3	2227.4	0.26
G4	2763	2409.1	0.13	2598.1	1960	0.25	2863.1	2265.7	0.21
G5	4297.2	2742.4	0.36	3164.8	2628.5	0.17	3378.3	2518.7	0.25
G6	4463.9	2772.5	0.38	3297.2	1872	0.43	4529.5	3155.7	0.30
G7	5616.7	2793.3	0.50	3868.5	2413.7	0.38	4259.5	2891.2	0.32
G8	3144.4	2659.1	0.15	2672.2	1964.6	0.26	3288.2	2452.8	0.25
G9	3459.3	2015.6	0.42	3473.1	2423	0.30	3835.1	2644.6	0.31
G10	6144.4	3362.8	0.45	5278.7	2520.2	0.52	4821.9	2690.2	0.44
G11	3059.3	1950.7	0.36	3627.8	3182.2	0.12	3134.2	2540.1	0.19
G12	2246.3	1853.5	0.17	2507.4	1807.2	0.28	2721.7	1792.9	0.34
G13	3623.7	2869.1	0.21	3225	2535.1	0.21	3533.1	2356.5	0.33
G14	3760.2	2659.6	0.29	3346.6	2340.5	0.30	3664.1	2869.3	0.22
G15	2871	2370.8	0.17	2555.2	2110	0.17	2501.6	2027.7	0.19
G16	3685.5	2402.2	0.35	3280.1	2375.8	0.28	3542.5	2717.8	0.23
G17	3005.7	2183.4	0.27	2675.1	1965	0.27	3061.4	2022.2	0.34
G18	3868.4	2505.4	0.35	3442.9	2220.5	0.36	3399.9	2390	0.30
G19	3822.7	2218.5	0.42	3405	2190.8	0.36	3812.4	2584	0.32
G20	3899.1	2580.2	0.34	3470.2	2322.2	0.33	3658.1	2379.9	0.35
G21	3083.9	2852.1	0.08	2744.7	2688.3	0.02	2755.2	2286.8	0.17
G22	3184.7	2086	0.34	2987.8	1848.8	0.38	3004.9	2148.7	0.28
G23	3104.1	2036.5	0.34	2933.2	1789.2	0.39	2934.7	1890.3	0.36
G24	3572.4	2339.9	0.35	3311.9	2082.6	0.37	3195.2	2496.5	0.22
G25	3073.6	1901.9	0.38	2877.5	1885.4	0.34	2939	1906.7	0.35
Mean	3593.3	2440.4	0.30	3229.8	2197.8	0.31	3390.1	2414.3	0.28
$LSD_{5\%}$	611.3	536.1	0.12	586.4	470.4	0.18	593.9	463.6	0.15

Y_p: Grain yield under supplementary irrigation conditions (Kg ha^{-1}); Y_s: Grain yield under rainfed conditions (Kg ha^{-1}); RYC: decrease of relative yield changes

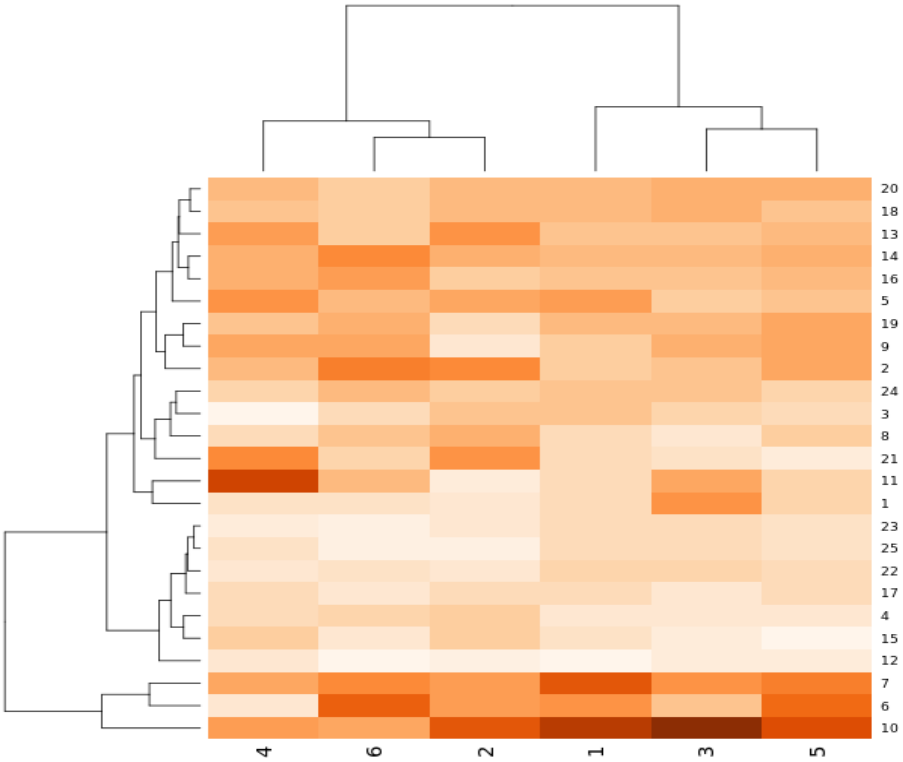

Figure 3- GEI in wheat genotypes under six environments and heatmap for indication of PCs contribution in G+GE

3.1. Univariate indices of parametric stability

According to Francis & Kannenberg (1978), genotype whose yield is higher than the average of all genotypes and its coefficient of variation is lower than the mean of genotypes is introduced as a stable genotype. Therefore, based on the results of Table 3, the genotypes G2, G14, G13, G16 and G5 were considered as stable genotypes compared to other genotypes. Stability of a genotype indicates the genetic potential of a plant that is adapted to growing in the environment (Shahzad et al. 2019). According to the Eberhart & Russel stability method (1966), by assessing the genotypes for general adaptation parameters (dynamic stability), genotypes G5, G24, G23, G22, G25 and G16 had b values close to 1. However, in this method, genotypes with low variance of deviation from regression (S^2d_i), high mean performance and regression coefficient (b_i) close to 1 show better general adaptability across environments (Finlay & Wilkinson 1963; Eberhart & Russel 1966). Accordingly, G5, G14 and G16 with above-average grain yield, were found to be more stable than other genotypes and had higher reliability. Four genotypes G4, G8, G15 and G12, not only were found to be among the lowest yielders but also showed poor adaptation to the test environments. Based on W_i, D_i and σ^2_i parameters, which measure the sums of squares contributed by each genotype to the interaction effect, some high-yielding genotypes, G16 and G18, and low-yielding genotypes, G22, G23, G25, G17 and G24, had the lowest values of these indices and were found to be the most stable across environments (Table 2 and Table 3). This finding offers that selection for genotypic stability based on W_i, D_i and σ^2_i statistics (also called type I stability or static stability) favors below-average-yielding over high yielding wheat genotypes. Similarly, these parameters distinguished stable wheat genotypes in another studies (Farshadfar et al. 2012; Bornhofen et al. 2017; Mohammadi et al. 2020).

The W_i, D_i, CV_i and σ^2_i indices appear to be conservative in introducing stable high-yielding cultivars across environments (Table 2 and Table 3). According to the previous studies, both high yield and type I stability rarely occur in multi-location variety experiments (Karimizadeh et al. 2012; Temesgen et al. 2015). However, these methods can be used to test the reliability of genotypes against yield fluctuations under various environments (Altay 2012). Static stability may be more effective than dynamic stability in a wide range of environments especially in developing countries (Simmonds 1991).

Table 3- Parametric measures of wheat genotypes across six environments

Gen.	*CV$_i$*	*b$_i$*	*P_b$_i$*	*S^{2}d$_i$*	*P_S^{2}d$_i$*	*W$_i$*	*D$_i$*	*σ^2_i*
G1	28.53	1.10	0.357	104051	0.077	803074	1597.06	506084
G2	16.12	0.76	0.034*	-31751	0.624	344889	1416.83	207268
G3	25.9	1.09	0.428	-32089	0.627	253512	1416.35	147674
G4	13.54	0.52	0.000**	-72244	0.931	480397	1358.47	295642
G5	21.25	1.00	0.994	15663	0.324	430231	1482.25	262925
G6	30.43	1.55	0.000**	150897	0.034*	1496592	1654.69	958378
G7	32.79	1.90	0.000**	88632	0.100	2164666	1577.64	1394079
G8	17.77	0.70	0.010**	-23237	0.561	430460	1428.8	263075
G9	24.04	1.08	0.473	30766	0.257	502356	1502.49	309964
G10	36.04	2.43	0.000**	71907	0.132	4280565	1556.29	2774013
G11	20.11	0.50	0.000**	228127	0.008**	1724950	1745.54	1107308
G12	18.52	0.57	0.000**	-38765	0.679	531684	1406.89	329091
G13	17.3	0.83	0.140	-55797	0.814	94170	1382.47	108973
G14	18.43	0.94	0.608	-74110	0.942	77096	1355.72	32620
G15	12.87	0.47	0.000**	-68583	0.907	593983	1363.85	369720
G16	19.26	0.95	0.678	-75699	0.951	68683	1353.37	27133
G17	19.83	0.81	0.099	-79418	0.969	112304	1347.86	55582
G18	22.97	1.14	0.223	-81844	0.979	73998	1344.26	30599
G19	25.51	1.26	0.021*	-62178	0.862	241450	1373.21	139808
G20	23.02	1.18	0.122	-86169	0.993	77695	1337.81	33010
G21	9.52	0.26	0.000**	-36007	0.657	1202544	1410.81	766608
G22	22.72	0.97	0.765	-86913	0.995	21945	1336.7	-3347
G23	24.63	1.01	0.963	-83928	0.987	31908	1341.15	3150
G24	21.34	1.00	0.993	-76046	0.952	63390	1352.86	23681
G25	24.15	0.97	0.825	-81068	0.976	44409	1345.41	11303

CV_i: Coefficient of variability; b_i Linear regression coefficient; S^2d_i: Residual MS value; W_i: Wricke's ecovalence; D_i: Hanson genotypic stability; σ^2_i: Shukla's stability variance; * and **: Significant at 5% and 1% probability level, respectively

3.2. Univariate indices of non-parametric stability

Several non-parametric stability methods have been proposed based on the genotype ranking ratio in each environment. Genotypes with the same rank in all environments are considered as stable genotypes (Thennarasu 1995; Huehn 1996). Simultaneously selection based on yield and stability (YS_i) is the combination of mean yields and Shukla stability parameter in a statistical test (Kang 1993). The genotype with the highest yield and the lowest Shukla stability parameter is assigned the rank of one. Genotypes with high YS_i values above the mean of all genotypes were selected as the most stable genotypes, which included G5, G14, G2, G10, G20, G13, G7, G19, G16, G6, G9 and G18 genotypes, respectively (Table 4). In fact, this method of stability analysis introduces high yielding genotypes as stable cultivars. Nassar & Huehn (1987) proposed the values of $S_i^{(1)}$ and $S_i^{(2)}$ to test the stability of genotypes based on the genotype ranking in the environment. Genotypes with minor changes in ranking are the most stable (Kaya & Sahin 2015). For each genotype, $Z_i^{(1)}$ and $Z_i^{(2)}$ measures were computed according with the rank of adjusted data and summed on the genotypes to gain Z-scores. Based on Table 4 results, both of these indices were more than the critical value of $\chi^2_{(0.01,\ 25)} = 44.31$, which showed that the stability rank was significantly different between the studied genotypes. Based on $Z_i^{(1)}$, $Z_i^{(2)}$, G6, G10 and G11 genotypes were the most unstable genotypes in terms of grain yield. Vaezi et al. (2018) pointed out that $S_i^{(1)}$ and $S_i^{(2)}$ display static concept of stability. So, $S_i^{(1)}$ and $S_i^{(2)}$ could be used as an agreement method that select genotypes with moderate yield and yield stability. The other two non-parametric indices $S_i^{(3)}$ and $S_i^{(6)}$, combine stability and yield based on yield ranks of genotypes in each environment. The lowest value for each of these measures indicates the maximum stability for a particular genotype (Huehn 1996; Kaya & Sahin 2015). In this experiment, based on $S_i^{(1)}$, $S_i^{(2)}$, $S_i^{(3)}$ and $S_i^{(6)}$ indices, G18, G14 and G16 were the most stable genotypes with yields higher than total mean (Table 4). Fox et al. (1990) proposed a non-parametric superiority statistics for evaluating general adaptability. Accordingly, the ranking of genotypes was done separately in each environment and the number of environments in which each genotype was in the top (TOP), middle (MID) and low (BOT) third of the ranks was calculated. A genotype with a higher rank in the upper third is considered a genotype with wide adaptability. In this study, G7, G10, G6, G2 and G5 genotypes had high adaptability and in contrast, G8, G19 and G9 genotypes showed the lowest adaptability. In fact, stable genotypes which are selected by TOP method have high mean yield (Mohammadi et al. 2009). Sabaghnia et al. (2015) and Mohammadi et al. (2020) reported that the TOP and YS_i indices were associated with mean yield and the dynamic concept of stability, so these procedures could be used to recommend cultivars adapted to desirable conditions. Genotypes with lowest values of Thennarasu's (1995) non-parametric stability measures, which were calculated from the ranks of adjusted yield means, are considered more stable. According to the $NP_i^{(1)}$, G22 followed by G14, G16, G25 and G23 were identified as stable in comparison to other genotypes. Based on $NP_i^{(2)}$, $NP_i^{(3)}$ and $NP_i^{(4)}$, genotype G22 followed by G25, G23, G17 and G12 had the lowest values and were the most stable. The more unstable genotypes based on $NP_i^{(1)}$, $NP_i^{(2)}$, $NP_i^{(3)}$ and $NP_i^{(4)}$ were G10 followed by G7, G6, G11 and G2 (Table 4). The coincidence of the $NP_i^{(2)}$, $NP_i^{(3)}$ and $NP_i^{(4)}$ indices was also seen by Mohammadi et al. (2009) and Golkar et al. (2020) in wheat and safflower, respectively. The results showed that Thennarasu and Nassar & Huehn measures are part of the concept of static stability and the selected stable genotypes by these indices may

not have high yield (Ahmadi et al. 2015; Vaezi et al. 2018). In present study, genotypes G14, G16 and G18, with the lowest Thennarasu's statistics and high mean yields, were introduced as genotypes with general adaptability (Table 4). According to Farshadfar et al. (2012), Thennarasu's (1995) non-parametric stability parameters do not add important information to statistics obtained by Nassar & Huehn (1987). Therefore, the use of Huehn non-parametric stability measures could be a method of selection as there is a statistical procedure available to examine the significance of $S_i^{(1)}$ and $S_i^{(2)}$. However, Thennarasu's stability indices would be effective alternatives to parametric procedures.

Table 4- Non-parametric measures of wheat genotypes across six environments

Gen.	*YS$_i$*	*S$_i^{(1)}$*	*Z$_i^{(1)}$*	*S$_i^{(2)}$*	*Z$_i^{(2)}$*	*S$_i^{(3)}$*	*S$_i^{(6)}$*	*TOP*	*NP$_i^{(1)}$*	*NP$_i^{(2)}$*	*NP$_i^{(3)}$*	*NP$_i^{(4)}$*
G1	8	7.53	0.15	52.17	0.00	14.15	1.77	1	4.17	0.22	0.41	0.47
G2	21	10.20	0.85	77.37	1.19	17.21	3.45	2	7.83	0.92	1.03	1.30
G3	7	8.47	0.01	48.57	0.02	6.82	1.18	0	5.17	0.30	0.37	0.50
G4	3	10.87	1.56	83.37	1.83	4.59	1.08	0	8.17	0.41	0.43	0.56
G5	23	10.27	0.91	75.60	1.03	12.88	2.71	1	7.67	0.90	0.93	1.21
G6	17	13.33	6.04	126.67	10.34	39.45	4.73	3	10.00	2.22	1.40	1.82
G7	19	11.20	1.99	91.47	2.89	5.27	2.55	4	7.67	2.56	2.38	3.05
G8	9	10.27	0.91	69.47	0.57	6.93	1.42	0	7.00	0.48	0.51	0.69
G9	16	10.13	0.79	70.27	0.62	25.38	3.45	0	6.33	0.97	0.79	1.05
G10	20	13.93	7.57	144.17	15.76	11.00	4.80	4	10.83	10.83	4.38	5.57
G11	6	13.07	5.41	121.47	8.95	32.45	3.70	1	9.67	0.77	0.83	1.07
G12	-2	7.87	0.05	51.87	0.00	0.14	0.16	0	4.33	0.17	0.27	0.32
G13	19	8.20	0.00	45.77	0.07	13.21	2.50	1	5.17	0.52	0.66	0.88
G14	22	5.47	1.96	22.00	1.67	2.33	1.07	0	3.00	0.38	0.57	0.73
G15	0	11.00	1.73	81.37	1.60	5.48	1.15	0	7.50	0.34	0.41	0.54
G16	17	5.20	2.34	18.80	2.05	5.21	1.47	0	3.00	0.33	0.42	0.55
G17	4	5.20	2.34	19.87	1.92	1.83	0.72	0	4.00	0.21	0.21	0.27
G18	15	7.07	0.38	34.67	0.56	4.00	1.40	0	4.67	0.42	0.54	0.71
G19	18	9.13	0.16	54.97	0.02	10.28	2.21	0	5.83	0.73	0.70	0.94
G20	20	7.20	0.30	37.07	0.41	5.98	1.70	0	4.67	0.52	0.63	0.82
G21	6	12.60	4.40	112.70	6.84	27.53	3.13	1	8.50	0.50	0.70	0.91
G22	5	3.07	6.63	6.80	3.79	1.81	0.65	0	1.67	0.09	0.13	0.17
G23	2	5.53	1.87	21.77	1.70	2.06	0.64	0	3.17	0.15	0.20	0.27
G24	11	7.07	0.38	34.27	0.58	2.40	1.01	0	5.00	0.40	0.42	0.55
G25	1	4.67	3.21	14.80	2.57	1.10	0.51	0	3.00	0.15	0.17	0.22
Test measures												
$Z^{(1)}$-sum = 51.92		E ($S^{(1)}$) = 8.32		E ($S^{(2)}$) = 52		$\chi^2_{(0.01, 25)}$ = 44.31						
$Z^{(2)}$-sum = 66.96		Var ($S^{(1)}$) = 4.16		Var ($S^{(2)}$) = 539.07		$\chi^2_{(0.01, 1)}$ = 6.64						

YSi: Rank-sum stability index; $S^{(i)}$, $NP^{(i)}$: Nassar & Huehn's and Thennarasu's non-parametric stability statistics, respectively; Z_1 and Z_2: the standard values of $S^{(1)}$ and $S^{(2)}$, respectively

3.3. Rank correlation between yield, parametric and non-parametric stability indices

Spearman's rank correlation between stability parameters and mean yield are given in Table 5. There was a very significant positive correlation between yield with YS_i and TOP ($P < 0.01$) which showed that these measures allow simultaneous identification of stable and high yielding genotypes. Farshadfar et al. (2012) and Khalili & Pour-Aboughadareh (2016) also reported a strong positive correlation between yield and TOP, indicating that TOP is a suitable statistics for identifying high-yield genotypes. Yield improvement is possible by changing the yield stability by increasing the TOP measure that can be directly related to the development of specific genotypes by improving the growing environmental conditions. However, the $S_i^{(3)}$, $S_i^{(6)}$, $NP_i^{(2)}$, $NP_i^{(3)}$ and $NP_i^{(4)}$ showed a positive significant correlation with each other and a strong negative correlation with yield. These results are in agreement with the work of Temesgen et al. (2015). Therefore, selection based on these stability statistics will be less effective when yield improvement is the main target of selection. $S_i^{(1)}$ had a significant positive correlation with Thennarasu and Nassar & Huehn statistics which can be considered as an alternative and useful parameter for selection of stable genotypes. Mohammadi et al. (2009) reported that the $S_i^{(1)}$ and $S_i^{(2)}$ are related to the static concept (biological) of stability. However, association among $S_i^{(1)}$ and $S_i^{(2)}$ with yield was not significant (Table 5), which indicating that these measures could be used as a mediating method for the selection of highly stable genotypes (Vaezi et al. 2018).

Table 5- Spearman rank correlation among mean yield and stability parametric and non-parametric measures in wheat genotypes across six environments

	Yield	*CV_i*	*b_i*	*S^2d_i*	*W_i*	*D_i*	*σ^2_i*	*YS_i*	*$Si^{(1)}$*	*$Si^{(2)}$*	*$Si^{(3)}$*	*$Si^{(6)}$*	*TOP*	*$NP_i^{(1)}$*	*$NP_i^{(2)}$*	*$NP_i^{(3)}$*
CV_i	-0.30															
b_i	-0.17	-0.07														
S^2d_i	-0.41*	0.20	0.50*													
W_i	-0.27	0.08	0.80**	0.87**												
D_i	-0.41*	0.20	0.50*	1.00**	0.87**											
σ^2_i	-0.27	0.08	0.80**	0.87**	1.00**	0.87**										
YS_i	0.94**	-0.19	0.04	-0.27	-0.08	-0.27	-0.08									
$Si^{(1)}$	-0.37	0.00	0.76**	0.78**	0.89**	0.78**	0.89**	-0.19								
$Si^{(2)}$	-0.38	0.01	0.77**	0.80**	0.92**	0.80**	0.92**	-0.20	0.99**							
$Si^{(3)}$	-0.52**	0.05	0.39	0.75**	0.63**	0.75**	0.63**	-0.43*	0.72**	0.72**						
$Si^{(6)}$	-0.74**	0.19	0.46*	0.74**	0.66**	0.74**	0.66**	-0.61**	0.74**	0.75**	0.92**					
TOP	0.62**	-0.22	-0.48*	-0.72**	-0.67**	-0.72**	-0.67**	0.45*	-0.65**	-0.68**	-0.65**	-0.76**				
$NP_i^{(1)}$	-0.41*	-0.04	0.70**	0.71**	0.80**	0.71**	0.80**	-0.24	0.97**	0.96**	0.73**	0.76**	-0.65**			
$NP_i^{(2)}$	-0.82**	0.17	0.50*	0.63**	0.61**	0.63**	0.61**	-0.69**	0.76**	0.76**	0.73**	0.89**	-0.66**	0.80**		
$NP_i^{(3)}$	-0.86**	0.13	0.49*	0.66**	0.62**	0.66**	0.62**	-0.73**	0.74**	0.76**	0.74**	0.91**	-0.74**	0.77**	0.97**	
$NP_i^{(4)}$	-0.86**	0.14	0.49*	0.65**	0.61**	0.65**	0.61**	-0.73**	0.75**	0.76**	0.72**	0.89**	-0.71**	0.78**	0.98**	1.00**

* and **: Significant at the 5% and 1% probability level, respectively

The strong positive correlation of S^2d_i with W_i and σ^2_i showed that S^2d_i can be used not only to evaluate the predictability of the estimated response obtained from linear regression, but also to assess the relative contribution of genotype to GEI and indirectly its biological stability (Mohammadi et al. 2012). The positive and highly significant correlation of S^2d_i, W_i, σ^2_i and D_i with Thennarasu and Nassar & Huehn non-parametric indices (Table 5) showed that these parameters have a similar role in ranking the stability of genotypes as previously confirmed by Roostaei et al. (2014); Dia et al. (2016), Bornhofen et al. (2017), Vaezi et al. (2018) and Golkar et al. (2020). According to Temesgen et al. (2015), various stability parameters justify genotypic efficiency differently, regardless of yield performance. Therefore, GEI evaluation and yield stability should be based on a combination of sustainability measures.

In order to better understand the relationships between stability statistics, Heatmap of genotypes and stability parameters were drawn (Figure 4). In this figure, genotypes were ranked based on stability statistics. In this way, the parameters were categorized in two main clusters. The first cluster was assigned to mean yield (Y_i), TOP and YS_i. The second cluster consisted of three sub-clusters with CV_i and b_i parameters in the first sub-cluster, S^2d_i, D_i, W_i and Stabvar parameters in the second sub-cluster and $S_i^{(3)}$, $S_i^{(6)}$, $NP_i^{(2)}$, $NP_i^{(3)}$, $NP_i^{(1)}$, $NP_i^{(4)}$, $S_i^{(1)}$ and $S_i^{(2)}$ were placed in the third sub-cluster. According to previous reports, $S_i^{(3)}$, $S_i^{(6)}$, $NP_i^{(2)}$, $NP_i^{(3)}$ and $NP_i^{(4)}$ criteria have shown similar stability ranking patterns in various crop species (Mohammadi et al. 2009; Farshadfar et al. 2012; Khalili & Pour-Aboughadareh 2016; Vaezi et al. 2018).

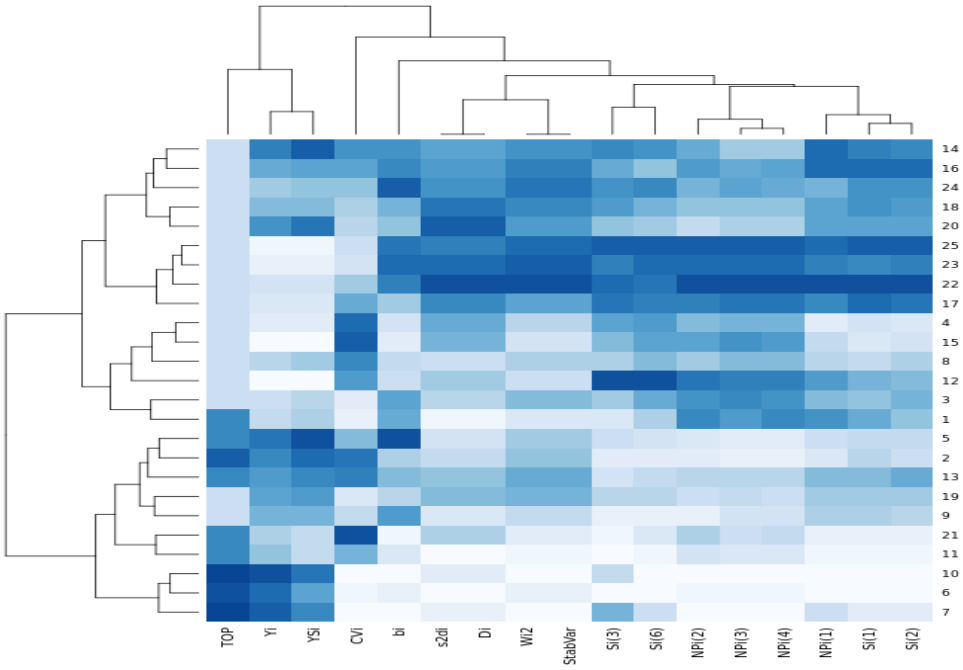

Figure 4- Heatmap of wheat genotypes distribution and stability parameters

3.4. AMMI analysis

The main effects of genotype, environment and G × E interaction were very significant ($P < 0.001$) (Table 6). The magnitude of the SSGE was 3.59 times more than that for genotype effect, demonstrating there were considerable deferential response among the genotypes to change of environment and the differential discriminating ability of the test environment (Shukla et al. 2015; Heidari et al. 2017; Bavandpori et al. 2018; Tekdal & Kendal 2018; Mekonnen et al. 2020). The value of the GEI is mostly due to variations in environmental conditions from supplementary irrigated to rainfed and from year to year. Furthermore, part of the GEI is due to genetic diversities among the studied genotypes. Analysis of GEI showed that only the first interaction principal component (IPC1) was highly significant ($P < 0.01$) and explained 58.6% of variability of GE. In the current study, the first two principal components (IPC1 and IPC2) explained about 77.8% of SSGE (Table 6). Thus, investigation of the distribution of environments and genotypes based on these two IPCs can prepare useful information for visualizing the response patterns of environments and genotypes. Hagos & Abay (2013) and Khan et al. (2020) affirmed that the first two IPCs were enough to justify the GEI. The G6, G7 and G10 genotypes showed the highest GEI while, the lowest interaction were obtained for G12, G15 and G25 genotypes.

Table 6- Combined analysis of variance for grain yield using AMMI model in wheat genotypes across six environments

Source	*Df*	*SS*	*MS*	*Prob.*	*GE expl. (%)*	*Cum. (%)*
Environment (E)	5	832512628	166502526	0.000		
Genotype (G)	24	28449331	3268722	0.000		
G × E	120	102238124	406174	0.000		
IPC1	28	59911540.7	2139697.9	0.007	58.6	58.6
IPC2	26	19629719.8	754989.2	0.148	19.2	77.8
IPC3	24	13393194.2	558049.8	0.506	13.1	90.9
IPC4	22	7054430.6	320655.9	0.922	6.9	97.8
IPC5	20	2249238.7	112461.9	0.957	2.2	100
Residuals	288	29196262	101375.9	0.989		

$AMMI_1$ model biplot shows the distribution of environments and genotypes based on IPC1 values and mean yields (Figure 5). A genotype that has a higher value of yield (horizontal axis) and a smaller amount (near zero) of IPC1 (vertical axis), will be a more desirable genotype, because this genotype has both high yield and stability. The G14, G5, G13, G16 and G20 genotypes appear to have high performance stability. The highest yield differences between genotypes were G10 and G12 and among environments were E1 (highest mean yield) and E4 (lowest mean yield). The correlation between the mean of genotypes and their IPC1 values was positive and highly significant ($r = 0.76$, $P < 0.01$), which revealed that medium-yield genotypes are stable, while high-yield and low-yield genotypes are relatively unstable. These results make it possible to use both aspects of adaptability, namely general and specific adaptability of genotypes, which is the main approach of plant breeders in selecting genotypes during MET (Gauch 2013).

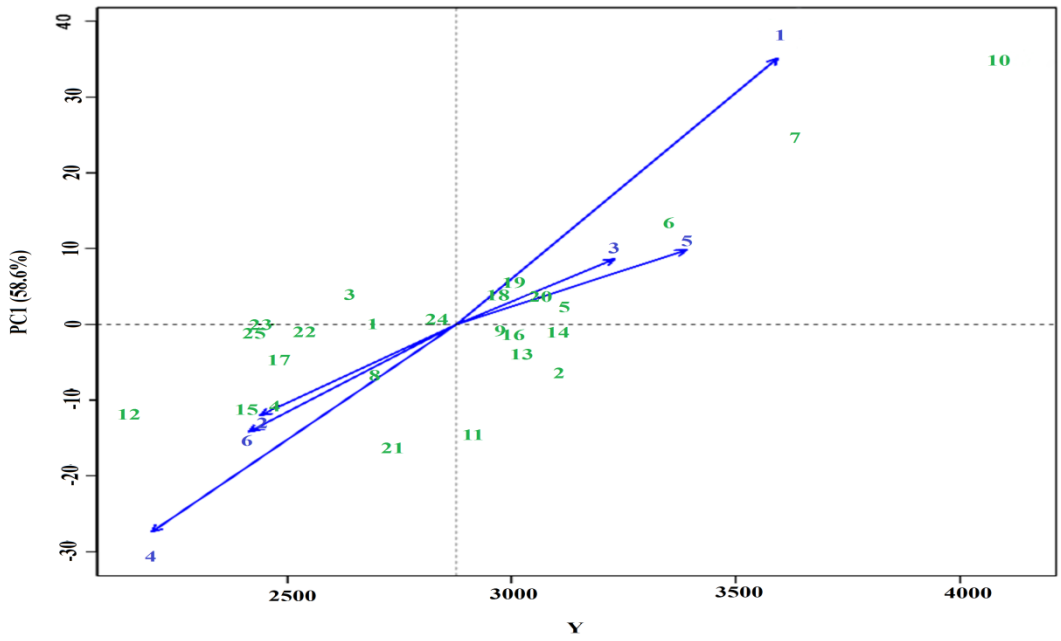

Figure 5- Biplot of yield and first principle component for wheat genotypes and environments ($AMMI_1$ model) [Genotype numbers are green and environment numbers are blue]

The $AMMI_2$ model biplot shows the position of genotypes and environments in terms of the first two IPCs, which accounted for about 77.8% of GEI variations (Figure 6). Genotypes and environments close to the origin have less impact and those far from the origin have a greater role in GEI (Lai et al. 2012). Thus, genotypes G6, G10, G11, G7 and almost G8 and G21, far from the origin, have the most fluctuation in environmental changes, while genotypes G13, G14, G16, G17, G18, G19, G20, G22, G23, G24, G25 and G5 within the orient and close to the biplot origin had a smaller share of the GEI and were generally adaptable with all environments. In addition to stability, high yielding criteria must also be considered for the final choice. Accordingly, G14, G13, G16, G19, G18, G20 and G5 genotypes can be introduced as genotypes with higher yield and general stability. The discriminating ability of the environments can be clarified by the magnitude of IPC1 and IPC2. Among the environments, E1 with the highest IPC1 and the lowest IPC2, was the most discriminatory and had the greatest effect on the GEI (Figure 6). The high distribution of environments compared to the genotypes indicated a high environmental diversity compared to the genotypes (Oliveira et al. 2014). The $AMMI_2$ biplot has better fit and accuracy for studying the complex GEI pattern. Also, the $AMMI_2$ with two main components justifies the highest rate of GEI changes (Khan et al. 2020).

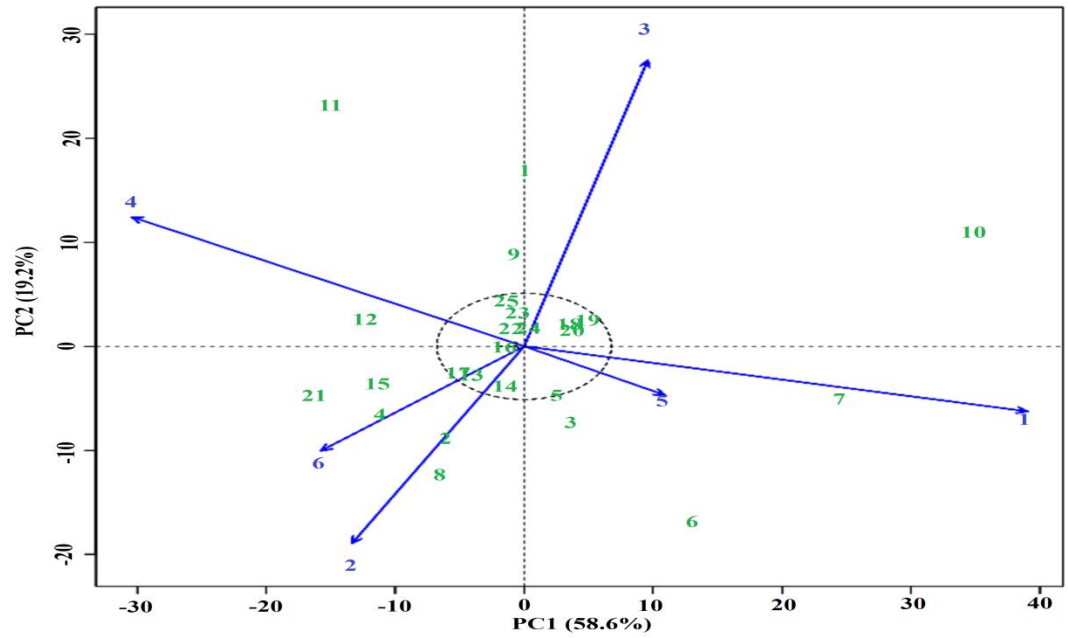

Figure 6- Biplot of the first two principal components of wheat genotypes and environments ($AMMI_2$ model) [Genotype numbers are green and environment numbers are blue]

Some researchers have reported $AMMI_2$ as a better model than $AMMI_1$ for the GEI investigation (Rodrigues et al. 2016; Kumar et al. 2018). A genotype is suitable for a specific environment when it is able to adapt well to and close to that environment. Thus, G7 genotype had specific adaptation to E1 and E5 environments, G8 and G2 genotypes to E2 environment, G12 genotype to E4 environment, G1 genotype to E3 environment and G4, G21 and G15 genotypes to E6 environment (Figure 6). In general, AMMI clarifies GEI and provides a summary of patterns and relationships between genotypes and environments.

3.5. GGE biplot analysis

The GGE biplot method is able to analyze Which-Won-Where Pattern of genotypes in which GEI, differentiation of MEs and recommendation of specific genotypes for each MEs are presented graphically (Rakshit et al. 2014; Oral et al. 2018). GGE biplot polygon view of yield for the studied genotypes in six environments is shown in Figure 7, which is formed by connecting the farthest genotypes from the biplot origin with straight lines and the rest of the genotypes within the polygon. The results show that GGE biplot justifies 85.1% of the total GEI changes by the first two IPCs. According to Yan & Kang (2003), it can be used to extract MEs if this biplot justifies at least 60% of GEI changes. By investigation the polygon diagram, the G8, G6, G10, G11, G11, and G12 genotypes at the vertex of the polygon were identified as superior or weak genotypes in some or all environments. By examining Figure 7, five MEs were identified that ME I including E1, E2, E3, E4, E5 and E6 environments, of which G7, G5, G14, G13, G16, G18 and G20 were the top ME I genotypes. Therefore, these genotypes were suitable for both SI and RF conditions. A ME refers to a group of environments in which one or more genotypes have the best performance (environmental response) (Yan et al. 2000). The G11, G12 and G8 genotypes at the top of the polygon were not included in any of the MEs, indicating low performance of these genotypes under all environments. The rest of the studied genotypes in the other four MEs did not show any specific adaptability with any of the environments (Figure 7). GGE biplot enables simultaneous visualization of the performance and stability of genotypes as well as the discriminating power and representativeness of environments. On the other hand, this procedure allows the ranking of cultivars based on yield in a specific environment and the comparison of them with the ideal cultivar (Yan et al. 2007; Aktas 2016). Due to the simplicity of graphical interpretation of GGE biplot results, this method is widely used in GEI analysis. In Ethiopia, to evaluate the stability of 22 bread wheat genotypes in six environments, using polygonal diagram, two MEs and five superior genotypes were identified (Temesgen et al. 2015). In one ME, genotypes must be evaluated for mean yield and stability over environments. Yield and stability of genotypes are assessed by Average Tester Coordinate (ATC) method in a biplot (Yan et al. 2007; Mehari et al. 2015; Singh et al. 2019). The line that passes through the origin of the biplot and the mean of the environments (mean scores of PC1 and PC2 environments) is called the ATC axis (Figure 8). The vertical dimension of ATC, which passes through the origin of the biplot and is perpendicular to the horizontal axis of the ATC, estimate the GEI and is an indicator of the instability of the genotypes. Genotypes located on the right side of the ATC vertical dimension have higher mean yield (Yan & Tinker 2006). According to this biplot view, it is possible to study the effect of G and GEI simultaneously. Figure 8 shows the visualization of each genotype on ATC and is an approximation of the yield rank of the genotypes. In this ranking, G10, G7, G6, G5, G20, G14, G19, G18, G2, G16, G13 and G9 genotypes had higher yield than total mean, respectively. The genotypes have a greater distance from ATC horizontal axis indicates a greater role in the GEI and they are less stable. Accordingly, the genotypes G11 followed by G6, G1 and G8 were more unstable than other genotypes. According to Yan & Tinker (2006), an ideal genotype should have both high mean yield and high stability in a ME. In fact, an ideal genotype should have the highest PC1 score (high yielding performance) and lowest PC2 score (high stability) (Yan & Tinker 2006; Oral et al. 2018). According to Figure 8, genotypes G10, G6 and G7 had high yield and low stability, and genotypes G1, G8, G11 and G3 had low yield and stability and genotypes G24, G21, G22, G23 and G15 had low yield and high stability. Breeders can select genotypes with high mean yield but low stability that have the best response to certain environments. For example, genotypes G7 and G10 had the best response to environments E1 and E5, while genotype G6 showed a weak reaction to environment E2. In addition to high stability under different environments, G20, G18, G13, G16, G14, G19 and G5 genotypes had mean yield higher than the total mean (Figure 8). Previously, GGE biplot has been used and emphasized to investigate adaptability and simultaneous combination of yield and stability in bread wheat by Naroui Rad et al. (2013), Mehari et al. (2015), Bornhofen et al. (2017), Bavandpori et al. (2018) and Singh et al. (2019). Evaluation of test environments for discriminating power vs. representativeness presented in Supplementary Figure S1. The small arrow shown on the ATC line is where there is ideal environment. This point is considered as an ideal virtual environment so, desired environment has short distance vector from the ATC axis is consider as an ideal environment (Yan et al. 2000). According to Figure 8, environments E5, E1, E3 and E6 can be considered as desired environments for wheat genotypes. The favorable conditions of these environments can be clearly seen in Figure S1. Environments close to the arrow represent ideal environments in the experiment. The small length of the environment vector, shows the less power of the environment to discriminate and diversify between genotypes. Accordingly, the E6 environment had the least ability to discriminating and representativeness genotypes. Whereas, E3 and E1 environments had the highest discriminating power and diversify between genotypes, indicating their sufficiency as test environments for multi-environmental trials. On the other hand, for selecting genotypes with general adaptability, E1 environment was the most suitable in terms of representativeness of environments, while E6 was the most suitable based on representativeness for yield (Figure S1). Generally, both the AMMI and GGE biplot methods performed the same in identifying stable and high-yielding genotypes (G20, G18, G13, G16, G14, G19 and G5). This result was in agreement with the findings of Aktas (2016), Bornhofen et al. (2017) and Singh et al. (2019). Moreover, GGE biplot has the advantage of better discriminating power and representativeness than AMMI biplot (Yan et al. 2007; Singh et al. 2019).

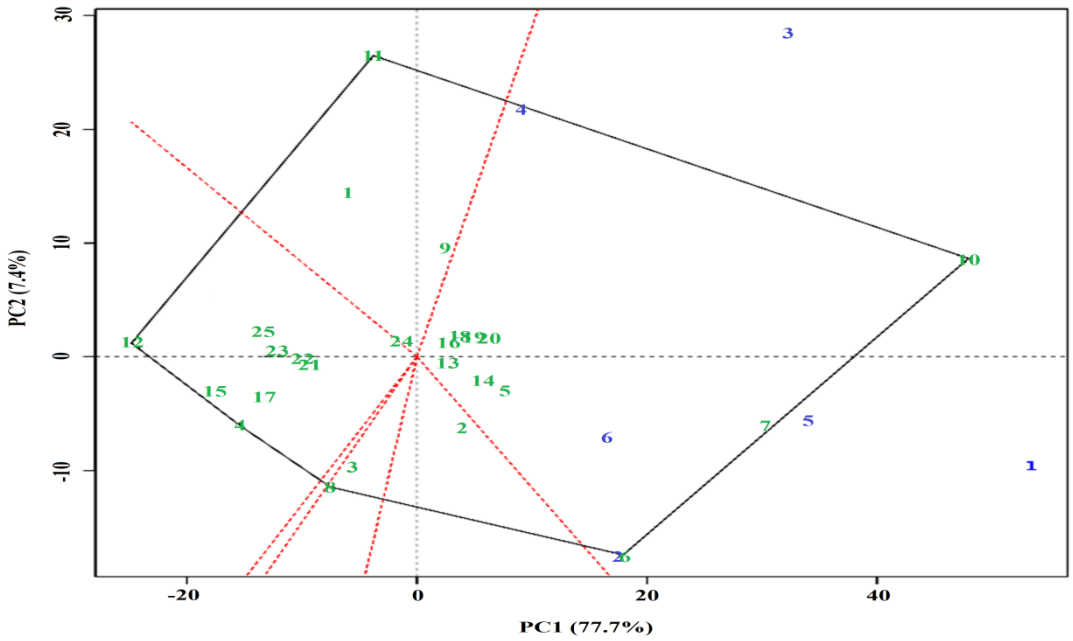

Figure 7- Graphical display for coincidence of wheat genotypes with environments and grouping test environments [Genotype numbers are green and environment number are blue]

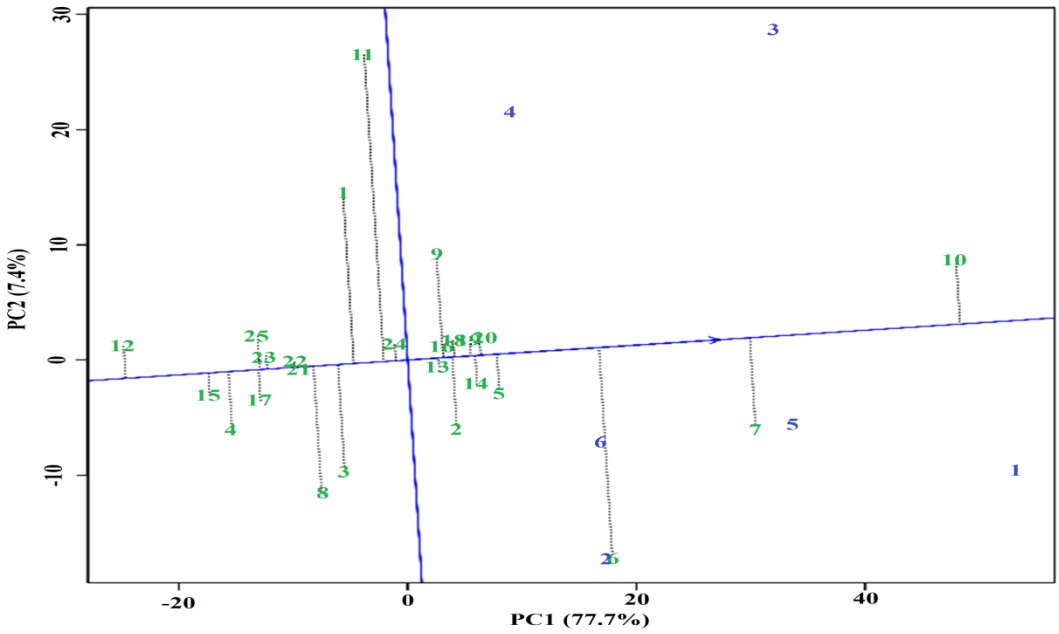

Figure 8- Average tester coordinate (ATC) view of the GGE biplot based on yield of wheat genotypes in 6 environments [Genotype number are green and environment number are blue]

4. Conclusions

Several of the univariate parametric and nonparametric stability statistics employed in the present investigation quantified stability of wheat genotypes with respect to yield, stability or both. However, both mean yield and stability should be considered simultaneously to exploit the useful effects of GEI. According to Eberhart & Russel method, G5, G14 and G16 with above-average yield, were found to be more stable than other genotypes. The results showed selection for genotypic stability based on Wi, Di and σ2i measures favors below-average-yielding over high yielding wheat genotypes. Genotypes G14, G16 and G18, with the lowest Thennarasu and Huehn non-parametric stability measures and high mean yields, were introduced as genotypes with general adaptability. The results of graphical analysis of the GEI in present study showed that the studied environments explain a large part of changes in the matrix of GEI. Therefore, the AMMI and GGE biplot methods were suitable tools for grouping diverse environments and determining stable and adaptable genotypes to different environmental conditions. Accordingly, lines G20, G18, G13, G16, G14, G19 and Saein cultivar (G5) can be considered as desirable genetic resources in wheat production programs under variable environments in Iran, due to having the appropriate

combination of yield and stability. Also, G10, G7, G2, G8, G4, and G1 genotypes with specific adaptation, are only recommended for use in certain environments.

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Supplementary data:

Table S1- Some of climatic parameters from October to June at the Agricultural Research Station of Miandoab

	2013/2014				2014/2015				2015/2016			
	Average temperature			Freezing	Average temperature			Freezing	Average temperature			Freezing
	Min	Max	Mean	dayes[a]	Min	Max	Mean	dayes	Min	Max	Mean	dayes
OCT	7.1	25.6	16.3	0	5.5	23	14.2	2	8.4	22.1	15.2	0
NOV	4.9	16.8	10.8	3	2.7	15.7	9.2	9	0.9	13.9	7.4	12
DEC	0.7	9.3	5	13	-2.4	5.7	1.7	19	1.2	8	4.6	10
JAN	-4.4	5.7	0.6	23	-10.6	-1.5	-6	30	-2.7	8.1	2.7	24
FEB	0.4	11.2	5.8	14	-4.2	7.2	1.5	22	-1.3	11.1	4.9	23
MAR	1.3	13.8	7.6	13	2.6	15	8.8	8	-0.8	12.8	6	18
APR	3.9	19.5	11.7	2	3.7	17.8	10.8	7	4.1	21.4	10.9	3
MAY	7.3	21.4	14.4	1	9.5	25.4	17.4	0	7.4	29.6	15.5	3
JUN	11	28.6	19.8	0	12.3	29.3	20.8	0	12.8	33.4	21.8	0

[a] Days below 0°

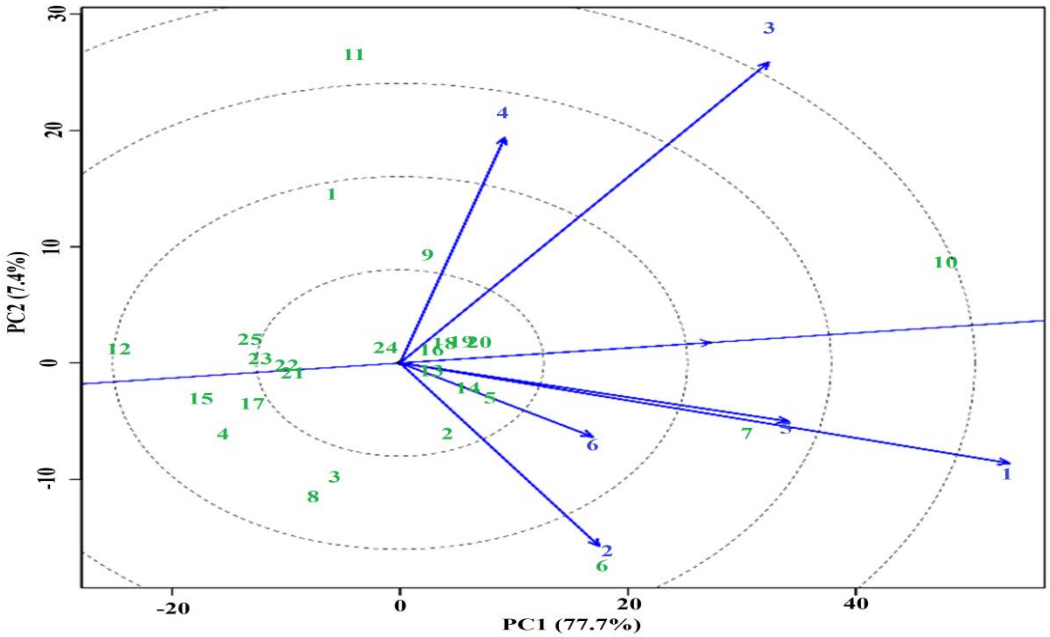


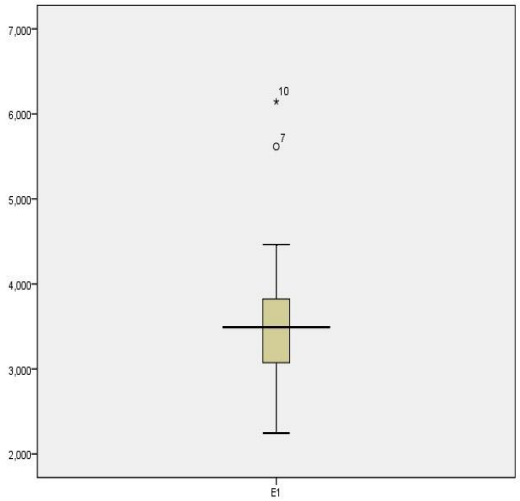
Figure S1- Evaluation of test environments in terms of their "discriminating power vs. representativeness" [Genotype number are green and environment number are blue]

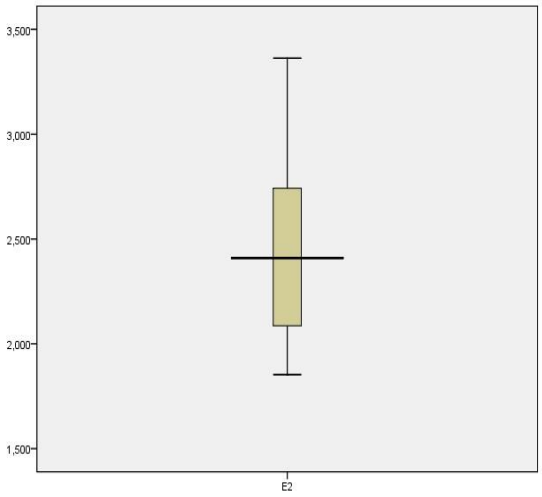
Tests of Normality

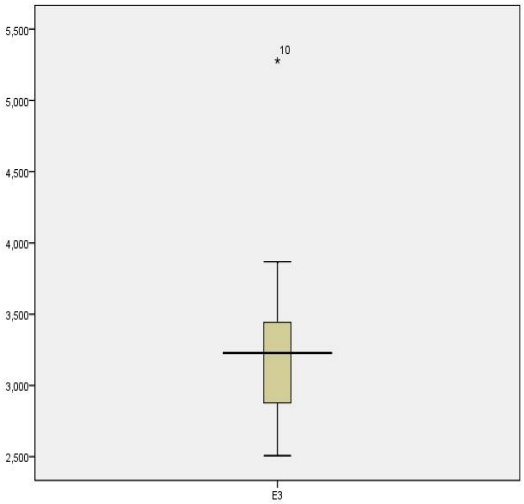
	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
E1	.199	25	.012	.853	25	.002
E2	.103	25	.200*	.964	25	.490
E3	.176	25	.045	.846	25	.001
E4	.143	25	.200*	.951	25	.270
E5	.115	25	.200*	.943	25	.175
E6	.063	25	.200*	.986	25	.971

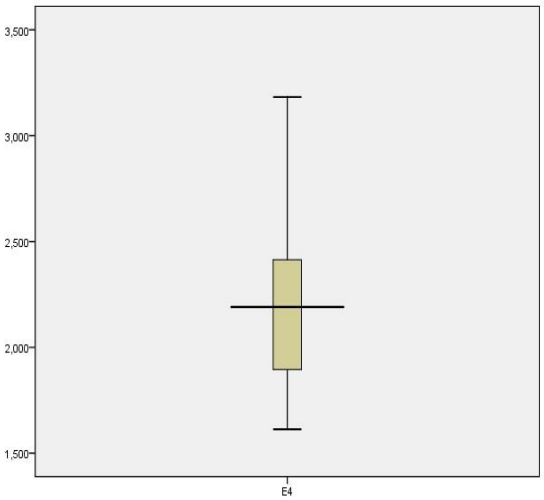
a. Lilliefors Significance Correction

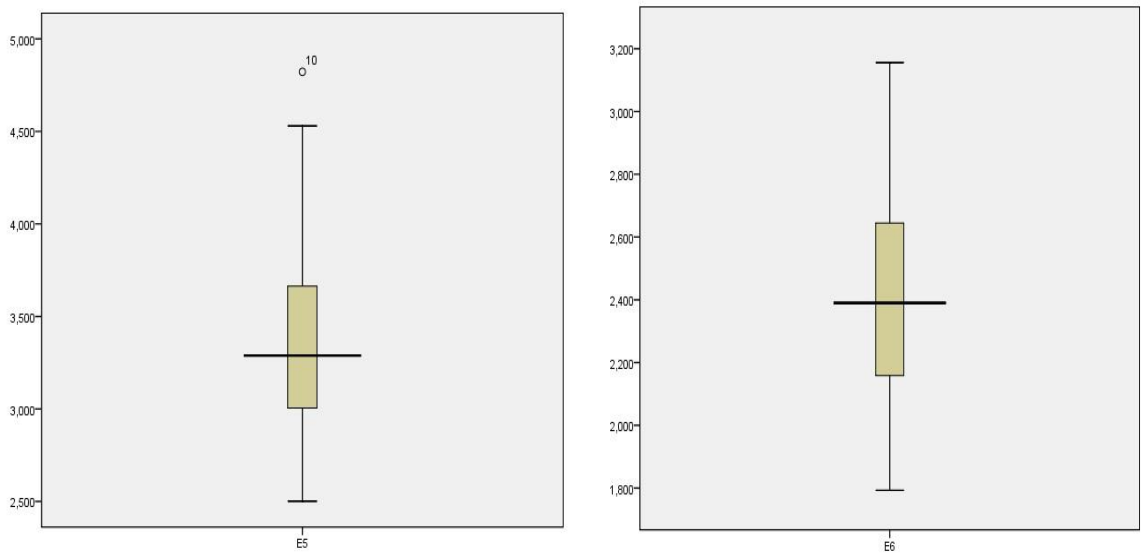
*. This is a lower bound of the true significance.











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Determination and Comparison of Bioactive Compounds in Different Parts of *Glycyrrhiza* Species

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ABSTRACT
Glycyrrhiza spp., one of the most widely used herbal medicine for centuries in the world, contain a large number of bioactive such as triterpene saponins and flavonoids which are the main constituents and show broad biological activity. The present study aimed to evaluate the phytochemical profile of extracts from different parts (roots, stems and leaves) of all wild *Glycyrrhiza* spp. grown in Turkey and to reveal that other parts besides the roots are a rich source of bioactive compounds with potential use in the pharmaceutical and food industries. For this purpose, extracted bioactive compounds from different parts of five *Glycyrrhiza* spp. collected in different provinces of Turkey were determined and compared. The microwave-assisted extraction method, which is mentioned as a green technique and requires low solvent and extraction time, was applied. Determination of bioactives was performed using liquid chromatography- electrospray ionisation tandem mass spectrometry (LC-ESI-MS/MS). Among the collected *Glycyrrhiza* spp.; in leaf parts, the highest glycyrrhizin (GL) (2.05±0.07 mg g^{-1}) and glycyrrhetinic acid (GA) (0.107±0.005 mg g^{-1}) contents were found in *Glycyrrhiza flavescens* ssp. *flavescens*; the highest carbenoxolone (CBX) (0.133±0.006 mg g^{-1}) and liquiritin (LQ) (1.644±0.014mg g^{-1}) contents were in *Glycyrrhiza glabra* grown in Polatlı. In stem parts, the highest GL (2.735±0.04 mg g^{-1}), CBX (0.069±0.004 mg g^{-1}) and LQ (0.602±0.010 mg g^{-1}) contents were determined in *G. glabra* plant growing in Ankara. In root parts, the highest GL (14.68±0.09 mg g^{-1}) and LQ (9.735±0.046 mg g^{-1}) contents were detected in *G. glabra* plant growing in Gaziantep while the highest GA (0.136±0.005 mg g^{-1}) and CBX (0.188±0.067 mg g^{-1}) contents in *Glycyrrhiza flavescens* ssp. *antalyensis*. Thus, it was determined which location in Turkey and which parts of *Glycyrrhiza* spp. that grow wild in Turkey can be used as a priority for the food and pharmaceutical industry with this study.

Keywords: *Glycyrrhiza, microwave-assisted extraction, bioactive compounds, LC-ESI-MS/MS*

1. Introduction

The *Glycyrrhiza* genus consists of about 30 species, which are distributed worldwide and are mainly native to the Mediterranean countries, central to southern Russia, and certain regions of Asia (Messier et al. 2012), is a species of perennial plant belonging to the *Leguminosae* family (Russo et al. 2014). Licorice has been used commercially as a depigmentation agent in cosmetics and as a flavoring and sweetening agent in food products which has been classified as "generally recognized as safe" by the Food and Drug Administration (Jiang et al. 2016).

Licorice is one of the oldest and most popular herbal medicines in the world. More than 20 triterpenoids and 300 flavonoids, which possess several pharmacological properties including anti-inflammatory (Cheel et al. 2010), antispasmodic (Cho et al., 2010), expectorant (Kim et al. 2006), antiallergic (Liao et al. 2012), antidepressive (Zhang & Ye 2009), antiviral, antioxidative, antimicrobial, antidiabetic, antiasthma, and anticancer activities as well as immunomodulatory, gastroprotective, hepatoprotective, neuroprotective, and cardioprotective effects (Hosseinzadeh & Nassiri-Asl 2015), have been isolated from *Glycyrrhiza* spp. (Yang et al. 2015). In the flora of Turkey, the genus *Glycyrrhiza* is represented by six species (*Glycyrrhiza glabra*, *G. echinata*, *G. aspera*, *G. iconica*, *G. flavescens*, *G. asymmetrica*) three of which are endemic (*G. iconica*, *G. flavescens* ssp. *antalyensis*, *G. asymmetrica)* (Duran et al. 2012). But, *G. aspera* is no longer available as the area where the plant grows is permanently under water. The distribution of these species in Turkey is presented in Figure 1.

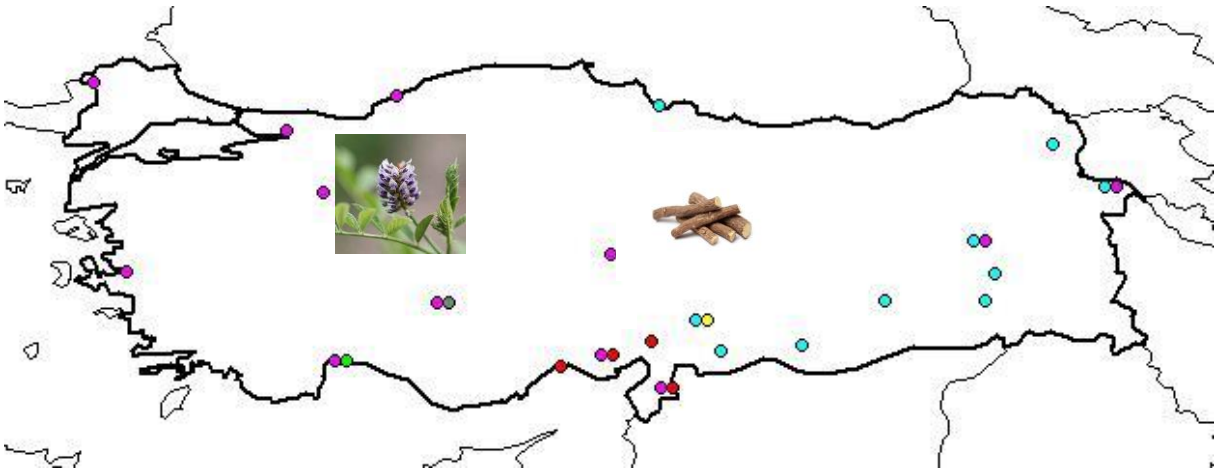

Figure 1- The distribution of *Glycyrrhiza* species in Turkey

Glycyrrhizin (GL), the most important constituent of licorice, is the saponin of the pentacyclic triterpene derivative of the oleanane type (Maatooq et al. 2010). It exhibits potent hydrocortisone-like anti-inflammatory, antiulcer, antiviral and antihepatotoxic activities (Cinatl et al. 2003; Sasaki et al. 2002). Glycyrrhetinic acid (GA), aglycone metabolite of GL, is a potent antibiotic against ulcer-causing *Helicobacter pylori* (Krausse et al. 2004). Also, GA and its derivatives have cytotoxic, anti-inflammatory, immuno-modulating, antitumor and apoptosis-inducing effects (Maatooq et al. 2010; Csuk et al. 2010). In addition, liquiritin (LQ), which is among the most important phenolic compounds, is mostly responsible for antioxidant and antitumor activities of licorice (Wang & Nixon 2001). Furthermore, carbenoxolone (CBX), a semi-synthetic compound derived from GL, has a wide scope of pharmacological activities. Although it possesses anti-inflammatory, antimicrobial and antiulcer properties at low doses, it induces adverse effects including cytotoxicity (Bharathala et al. 2021).

The present study aimed at the evaluation of the phytochemical profile of bioactive compounds in extracts from different parts (roots, stems and leaves) of five *Glycyrrhiza* spp. Thus, it was determined which locations and parts of all *Glycyrrhiza* spp. that grow wild in Turkey can be used as a priority for the food and pharmaceutical industry with this study.

To date, several methods have been developed for the quantification of bioactive in licorice, including high-performance thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) coupled with other techniques, capillary electrophoresis (CE) and gas chromatography (GC) in tandem with other methods. But it is the first time, the detection of the GL, GA, CBX and LQ contents in different parts of all species and varieties of *Glycyrrhiza* growing wild in Turkey via liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) was carried out at the same time.

2. Material and Methods

2.1. Plant materials and chemicals

Glycyrrhiza spp. were collected from different provinces of Turkey. Only *G. aspera* could not be collected because the area where the plant grows was permanently under water during the field studies. Identification and collection of the plant material were performed by botanist Dr. Ozlem CETIN. Voucher specimens were deposited at Gazi University Herbarium (GAZI), Ankara. The species, codes, collection dates, collected parts, locations and coordinates of licorice plants grown in Turkey were shown in Table 1. *Glycyrrhiza* spp. were air-dried before being analysed.

GL, GA, CBX and LQ were obtained from Sigma Aldrich (St. Louis, MO, United States). Methanol (for HPLC, ≥ 99.9%) was purchased from Merck (Darmstadt, Germany).

Table 1- The species, codes, collection dates, locations, coordinates and collected parts of licorice plants grown in Turkey

Plant	*Codes*	*Collection date*	*Location*	*Coordinates*	*Part*
Glycyrrhiza x iconica (endemic)	GXI	01/06/2010	B4 Konya: Sarayonu, between Sarayonu-Gozlu Farm, 992 m, field edge	38º 26' 51' 'N 32º 27' 58' 'E	Leaf, Stem, Root
G. echinata ssp. *echinata*	GEE	10/06/2011	C3 Konya: Seydisehir, Golyuzu village, 1100 m	37º 19' 57' 'N 31º 56' 19' 'E	Leaf, Stem, Root
G. echinata ssp. *macedonica*	GEM	01/06/2010	C2 Mugla: Dalyan, Iztuzu beach road	36º 49' 27.82' 'N 28º 38' 48.43' 'E	Leaf, Stem
G. flavescens ssp. *antalyensis* (endemic)	GFA	07/05/2011	C3 Antalya: Kemer, Between Tekirova-Cirali, Ancient road, 4 m	36º 28' 07' 'N 30º 30' 17' 'E	Leaf, Stem, Root
G. flavescens ssp. *flavescens*	GFF	16/04/2011	C6 Osmaniye: Osmaniye-Yarpuz road, 10th km, 835 m	37º 016' 20' 'N 36º 27' 433' 'E	Leaf, Stem, Root
G. asymmetrica (endemic)	GA	23/05/2010	C3 Antalya: Kemer	36º 37' 47.35' 'N 30º 33' 14.32' 'E	Root
	GAE	22/05/2011	C3 Antalya-Aksu road, 1 km before Aksu, roadside	36º 56' 45.34' 'N 30º 50' 30.98' 'E	Leaf, Root
G. glabra	GGC6	15/06/2010	C6 Gaziantep: Nizip, 7 km before Nizip	37º 02' 21.69' 'N 37º 54' 21.66' 'E	Root
	GGC2	24/06/2010	C2 Denizli: Pamukkale-Denizli road, roadside	37º 53' 52.09' 'N 29º 07' 32.69' 'E	Root
	GG1036	22/05/2011	C6 Kahramanmaras: Between Kahramanmaras-Turkoglu, Before reaching the Aksu bridge, 448 m, roadside	37º 29' 38' 'N 36º 53' 42' 'E	Root
	GG1047	22/05/2011	C6 Kahramanmaras: Between Kahramanmaras-Turkoglu, After the Aksu bridge	37º 29' 38' 'N 36º 53' 42' 'E	Root
	GGC7	01/02/2012	C7 Adıyaman: Isikli village, field edge	37º 26' 13.87' 'N 38º 13' 45.21' 'E	Root
	GG1055	01/06/2012	C7 Sanliurfa: Between Sanliurfa-Suruc, 12 km before Suruc	37º 06' 603' 'N 38º 49' 934' 'E	Leaf, Stem
	GGB4	23/06/2012	B4 Ankara: Polatlı-Eskişehir road, Sakarya riverside, 680 m	39º 35' 00' 'N 31º 56' 58' 'E	Leaf, Stem

2.2. Preparation of licorice extracts

According to the data obtained as a result of the study conducted by Özcan et al. (2020), the extraction process was performed by microwave-assisted extraction method, which is the extraction method with the highest yield compared to other extraction methods. Finely ground 1 g of *Glycyrrhiza* samples was added to 10 mL of 50% MeOH (MeOH: H_2O; 1: 1, v/v). The samples were extracted at room temperature (25±1 ºC) in a microwave oven (Milestone Start D Microwave Digestion System, USA) for 7 minutes with 100-watt energy. The extracts obtained were diluted 10000 times and passed through a 0.2 µm membrane filter. The same process was repeated without diluting the extracts to determine bioactive compounds which exist at low concentration. Filtered extracts were taken into vials and given to LC-ESI-MS/MS.

2.3. Preparation of calibration standard solutions

Stock solutions (mg mL^{-1}) of the four bioactive standards were prepared by dissolving in methanol. Stock solutions were mixed and then diluted with methanol to give solutions containing 1, 25, 100, 500 and 1000 ng mL^{-1} of mixed standard solution. Calibration curves were constructed by injecting each standard solution at each concentration level in triplicate and plotting peak areas versus concentrations. A representative chromatogram is given in Figure 2.

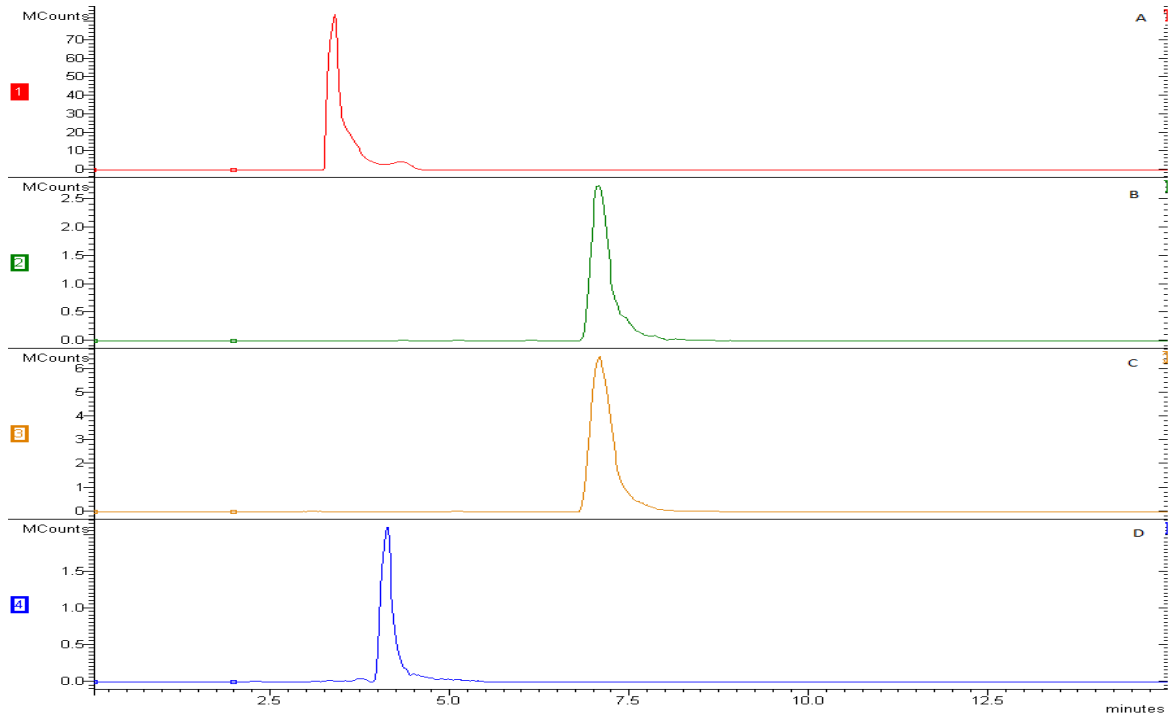

Figure 2- Representative chromatogram of 25 µg/L standard solutions. A: Liquiritin, B:Glycyrrhetinic acid, C: Carbenoxolone, D: Glycyrrhizin

2.4. Chromatographic conditions and mass spectrometric determination

Quantitative analyses of four bioactive compounds were performed on Zivak Tandem Gold (Istanbul, Turkey) system equipped with a Waters Symmetry C18 column (2.1 mm × 150 mm I.D., particle size 5 µm; Waters, Milford, MA, USA). The mobile phase consisted of deionised water (A) and methanol (B) and a gradient elution program was programmed as the following steps for chromatographic separation; 0:00 – 2:30 min, 10% B; 2:30 – 4:00 min, 50% B; 4:00 –10:00 min, 90% B; 10:00 – 12:00 min, 90% B; 12:00 – 15:00 min, 50% B; 15:00 – 20:00 min, 10% B with a constant flow 300 µL min^{-1}.

The instrument was operated in negative ion mode with an ion spray voltage of 5000 V, nebuliser gas (nitrogen) of 35 psi, source temperature of 50 ºC, drying gas temperature of 350 ºC. The column effluent was injected directly into the ESI source and spotted with multiple reaction monitoring (MRM). Mass parameters were optimised by using standard solutions of GL, GA, CBX and LQ in Özcan et al. (2020). All the transition ions and parameters, which were determined after optimisation by using standard solutions of each compound, were listed in Table 2.

Table 2- Precursor-product ions and parameters for MRM of compounds used in the present study

Analytes	*Precursor ion (m / z)*	*Screening ion (m / z)*	*Confirmation ion (m / z)*	*Capillary voltage (V)*	*Collision energy (eV)*
Glycyrrhizin	822.1	351	193	80	50
Glycyrrhetinic acid	470	425	355	60	40
Liquiritin	417.5	254.9	135.1	60	20
Carbenoxolone	570.1	469.7	100.2	80	30

2.5. Statistical analysis

Each sample was analyzed in three replicates and all results were presented as mean values ± standard deviations and analyzed by SPSS Statistics software 23 (IBM, Armonk, NY, USA). One-way analysis of variance (ANOVA) was performed using the Tukey test for any significant differences between the means. Differences between the means at 1% ($P<0.01$) level were evaluated significantly.

3. Results and Discussion

The content of GL, GA, CBX and LQ in different plant parts of *Glycyrrhiza* spp. was calculated and listed in Table 3 which showed significant variations among the contents ($P< 0.01$).

Table 3- Quantitative LC-MS/MS analysis of bioactive compounds from different parts of *Glycyrrhiza* spp. grown in Turkey

Codes	*Glycyrrhizin (GL)*			*Glycyrrhetinic acid (GA)*			*Carbenoxolone (CBX)*			*Liquiritin (LQ)*		
	Leaf	***Stem***	***Root***	***Leaf***	***Stem***	***Root***	***Leaf***	***Stem***	***Root***	***Leaf***	***Stem***	***Root***
GXI	0.67±0.07 ^{C}	0.75±0.04 ^{C}	2.60± 0.09 ^{G}	0.074±0.01 ^{A,B}	0.063±0.002 ^{A}	0.062± 0.009 ^{C}	0.070±0.005 ^{C}	0.019±0.030 ^{B}	0.013± 0.003 ^{D}	0.32±0.001 ^{D}	0.323±0.005 ^{B}	0.266± 0.006 ^{G}
GEE	0.33±0.03 ^{D}	0.65±0.06 ^{C}	7.65±0.05 ^{E}	0.067± 0.022 ^{B}	0.064±0.02 ^{A}	0.088±0.010 ^{B,C}	0.023± 0.006 ^{D}	0.015±0.010 ^{B}	0.026±0.013 ^{D}	0.292± 0.012 ^{E}	0.230±0.009 ^{D}	0.260±0.004 ^{G}
GEM	0.91±0.05 ^{B}	1.01±0.09 ^{B}		0.066±0.008 ^{B}	0.070±0.004 ^{A}		0.010±0.003 ^{D}	0.012±0.003 $^{B. C}$		0.392±0.003 ^{B}	0.262±0.008 ^{C}	
GFA	< LOD ^{E}	< LOD ^{E}	1.59±0.02 ^{H}	0.070± 0.003 ^{B}	0.065± 0.004 ^{A}	**0.136±0.005** ^{A}	0.020± 0.004 ^{D}	0.015± 0.008 ^{B}	**0.188±0.067** ^{A}	< LOD ^{F}	< LOD ^{E}	0.378±0.003 ^{F}
GFF	**2.05±0.07** ^{A}	0.441±0.02 ^{D}	1.22±0.07 ^{I}	**0.107±0.005** ^{A}	0.065±0.011 ^{A}	0.068±0.003 ^{B,C}	0.098±0.004 ^{B}	0.013±0.006 $^{B. C}$	0.086±0.065 ^{C}	0.397±0.008 ^{B}	0.266±0.004 ^{C}	0.261±0.005 ^{G}
GA			0.87±0.03 ^{J}			0.070±0.003 ^{B,C}			0.014±0.005 ^{D}			0.259±0.002 ^{G}
GAE	< LOD ^{E}		1.08±0.07 ^{I,J}	0.070±0.01 ^{B}		0.065±0.012 ^{B,C}	0.014±0.006 ^{D}		0.128±0.126 ^{B}	< LOD ^{F}		0.264±0.002 ^{G}
GGC6			**14.68±0.09** ^{A}			0.081±0.008 ^{B,C}			0.012±0.017 ^{D}			**9.735±0.046** ^{A}
GGC2			11.10±0.07 ^{D}			0.090±0.009 ^{B}			0.012±0.005 ^{D}			3.380±0.011 ^{D}
GG1036			12.02±0.06 ^{C}			**0.133±0.006** ^{A}			0.012±0.001 ^{D}			4.917±0.014 ^{C}
GG1047			7.28±0.09 ^{F}			0.076±0.007 ^{B,C}			0.015±0.001 ^{D}			3.212±0.018 ^{E}
GGC7			12.34±0.12 ^{B}			0.072±0.007 ^{B,C}			0.012±0.001 ^{D}			8.424±0.053 ^{B}
GG1055	< LOD ^{E}	< LOD ^{E}		0.060±0.009 ^{B}	0.058±0.007 ^{A}		0.015±0.003 ^{D}	< LOD ^{C}		0.352±0.005 ^{C}	0.248±0.005 $^{C, D}$	
GGB4	0.718±0.1 ^{C}	**2.735±0.04** ^{A}		0.061±0.004 ^{B}	0.075±0.01 ^{A}		**0.133±0.006** ^{A}	**0.069±0.004** ^{A}		**1.644±0.014** ^{A}	**0.602±0.010** ^{A}	

mg g^{-1}, LOD<1, *Values are mean ± SE. Different letters mean the statistical difference between groups

The quantitative analysis results indicated GGC6 (14.68±0.09 mg g^{-1}) had the highest GL content in roots of *Glycyrrhiza* spp., followed by GGC7 (12.34±0.12 mg g^{-1}) and GG1036 (12.02±0.06 mg g^{-1}). As shown in Table 3, GL was quantified maximum in stems of GGB4 (2.735±0.04 mg g^{-1}) and then GEM (1.01±0.09 mg g^{-1}), respectively. Likewise, GL was detected highest in the leaves of GFF (2.05±0.07 mg g^{-1}). The GL was identified as the most prominent compound among quantified bioactive compounds of *Glycyrrhiza* spp. In the comparison among different parts of each *Glycyrrhiza* spp., GL was found maximum in roots of GGC6 (14.68±0.09 mg g^{-1}).

As stated in Table 3, GA was determined maximum in roots of GFA (0.136±0.005 mg g^{-1}) and GG1036 (0.133±0.006 mg g^{-1}). On the other hand, according to the comparative analysis results on stem parts, no significant difference was observed in GA contents among *Glycyrrhiza* spp. ($P<0.01$). In addition, the highest GA content in leaf parts was obtained in GFF (0.107±0.005 mg g^{-1}) and GXI (0.074±0.01 mg g^{-1}).

Among the roots of all *Glycyrrhiza* spp., the highest CBX content was found in GFA (0.188±0.067 mg g^{-1}), followed by GAE (0.128±0.126 mg g^{-1}) and GFF (0.086±0.065 mg g^{-1}), respectively. Also, CBX was quantified maximum in stems of GGB4 (0.069±0.004 mg g^{-1}). No significant difference was observed in CBX contents among other *Glycyrrhiza* spp. ($P<0.01$). Comparing the leaf parts of all species, the highest CBX content was found in GGB4 (0.133±0.006 mg g^{-1}), followed by GFF (0.098±0.004 mg g^{-1}) and then GXI (0.070±0.005 mg g^{-1}).

LQ content was detected maximum in roots of GGC6 (9.735±0.046 mg g^{-1}), followed by GGC7 (8.424±0.053 mg g^{-1}) and GG1036 (4.917±0.014 mg g^{-1}). In the comparison of the leaf and stem parts of all species, it was observed that the highest LQ contents were in GGB4 (1.644±0.014 mg g^{-1} and 0.602±0.010 mg g^{-1}, respectively). For the stem parts, GXI (0.323±0.005 mg g^{-1}) was the second species with the maximum LQ after this species. On the other hand, for leaf parts, GFF (0.397±0.008 mg g^{-1}) was the second with the highest LQ content, following GGB4.

Esmaeili et al. (2020) collected 28 licorice (*Glycyrrhiza glabra* L.) populations from various wild areas across of Iran and quantified the bioactive compounds by high-performance liquid chromatography–photodiode array detection (HPLC–PDA). According to the findings of their study, minimum (11.7 mg g^{-1}) and maximum (74.0 mg g^{-1}) amount of GL were determined in Haji Abad and Eghlid populations, while minimum (0.14 mg g^{-1}) and maximum (2.01 mg g^{-1}) amount of LQ were quantified in Saqez and Eghlid populations. Furthermore; Souri et al. (2016) reported that average content of GL varied between 1.38% and 3.40% in their study with different populations from Iran. In another study, Hayashi et al. (2003) the quantity of GL in their study was higher than reported in Italy (1.6–3%), Spain (0.7–4.4%) and Uzbekistan (4.76%–6.13%).

On the other hand, Alsaadi et al. (2020), extracted *G. glabra* collected from Hatay province of Turkey by sonication with 50% ethanol and then quantified three bioactive compounds (GL, LQ and glabridin) via HPLC. According to their results, GL content varied from 0.54% to 2.40%, while LQ content varied from 0.18% to 1.85%.

In addition, Quintana et al. (2020) also extracted phytochemicals from *G. glabra* L. by supercritical antisolvent (SAS) technique. The LQ content ranged from 0.71 mg g^{-1} to 9.61 mg g^{-1} while GL content ranged from 0.18 mg g^{-1} to 0.56 mg g^{-1} in their work.

Li et al. (2016) determined the bioactive substances by UHPLC-MS/MS in six different *G. glabra*, five different *G. uralensis* and *G. inflata*. In their study, the GL content of the *G. glabra* samples ranged from 16.28 mg g^{-1} to 31.40 mg g^{-1} and LQ content varied from 1.27 to 8.14 mg g^{-1}. On the other hand, GA content obtained a maximum of 0.10 mg g^{-1}, the other five samples were detected below the LOD value (Li et al., 2016).

Montoro et al. (2011) determined the bioactive compounds in *G. glabra* collected from Turkey by LC- ESI-MS/MS and found GL content as 32.52 mg g^{-1} and LQ content as 2.38 mg g^{-1}.

In the light of all these results, large variations in these bioactive compounds have been mainly thought to be related to differences in used extraction methods and parameters as well as genetics (Esmaeili et al., 2020), harvesting time and also environmental and/or soil factors (Hou et al. 2018; Hosseini et al. 2014). In addition to all this, Jt et al. (2011) noted that the physicochemical characterization of soil is the foremost factor concerning the accumulation of bioactive ingredients in this plant.

4. Conclusions

In this study, for the first time leaf, stem and root parts of different species of *Glycyrrhiza* grown wild in Turkey were compared with each other in terms of the highest GL, GA, CBX and LQ contents. As a result of the comparison of the leaf parts of the plant, it was found that *Glycyrrhiza flavescens* ssp. *flavescens* contained the highest GL and GA, while *Glycyrrhiza glabra* included the maximum CBX and LQ. Among the *Glycyrrhiza glabra* spp. collected from different provinces of Turkey, the species grown especially in Ankara was determined as the species with the highest bioactive substance content. In a

comparison of stem parts of the plant; *Glycyrrhiza glabra*, grown in Ankara, had the highest content of bioactive substances and there was no statistically significant difference between species in terms of GA content.

Comparing the root parts of the plant, *Glycyrrhiza glabra* was the species with the highest bioactive substances except for CBX. *G. glabra*, which had the highest GL and LQ contents in the root parts of the plant, were collected from Nizip district of Gaziantep, and *G. glabra* with the highest GA was collected from Kahramanmaras. On the other hand, the species with the highest CBX in the root part was *Glycyrrhiza flavescens* ssp. *antalyensis*.

Glycyrrhiza glabra spp., grown in Gaziantep and Kahramanmaras provinces, was ahead of other species due to the fact that the leaf parts were less than the other parts of the plant and it had the highest efficiency in terms of these four bioactive substances content.

This is the first study to evaluate and compare the extracts obtained from the leaves, roots and stems of all different wild *Glycyrrhiza* spp. grown in Turkey in terms of bioactive substances.

Furthermore, we have demonstrated that different parts of *Glycyrrhiza* spp., which are a rich source of bioactive compounds with potential usage in pharmaceuticals and the food industry. Briefly, in this work, the populations with high-amount of each bioactive ingredient in root, stem and leave parts of *Glycyrrhiza* spp. were identified, which can be exploited depending on the purpose of usage.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Comparison of Dry and Wet De-Feathering Methods on the Quality Characteristics and Shelf Life of Broiler Carcasses

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ABSTRACT
In poultry slaughterhouses, carcasses can be contaminated with microorganisms at various points during the slaughtering processes, affecting some quality characteristics and shelf life of chicken meat. The present study was conducted to investigate the effects of different de-feathering methods on the meat quality characteristics and shelf life of broiler chickens. Forty male broilers 42 days of age (Ross 308) were used in the experiment. After slaughtering, they divided into 2 groups and first group was de-feathered by simple dry plucking method (DPM) and the other was a classic wet method (CWM). 5 fillets and 5 drumsticks from each treatment group were analyzed 0, 3, 5, and 7th days of storage time. The water holding capacity (WHC), color, pH and Warner-Bratzler shear force value (W-BSFV) were analyzed for meat quality characteristics of the raw meat samples. Total aerobic mesophilic bacteria, total psychrophilic bacteria, numbers of microorganisms assessed critically for food safety such as coliform bacteria, *E. coli*, *Enterococcus* spp. *Campylobacter* spp. also, the presence of *Salmonella* spp. in carcasses was determined. The results indicated that the skin colors of the fillets and drumsticks were yellower and the meat color of the drumsticks was darker in the DPM group than CWM. On the other hand, no significant effects of the plucking method were detected on the WHC and W-BSFV of the samples. The pH value of the fillets was higher in CWM group ($P<0.01$), but there were no differences between the pH values of drumsticks of two groups. The microorganism levels, however, were influenced significantly ($P<0.01$) by the plucking methods and the storage time. The shelf life of the carcasses was shortened, due to the high microbial load in DPM group. It is concluded that simple DPM may be used by low capacity farms which produce the broilers for consumers who prefer yellow-skinned chicken meat at the expense of reduction in the shelf life due to increased microbial load.

Keywords: Dry feather plucking, Meat quality, Microbial properties, Texture, Water holding capacity

1. Introduction

Poultry meat is the most important source of low-cost animal protein, rich in nutrients, minerals and vitamins. This protein source may contain a high level of pathogenic and spoilage bacteria, and may have risk for human health. Therefore, the level of microorganisms in chicken meat is important in terms of food safety.

In general, chicken feathers, skin, feet and digestive tracts are contaminated with microorganisms due to the farm conditions and production methods. Therefore, it is important to be careful during harvesting, transporting and also slaughtering processes after bleeding. In classic wet method which is widely used, there is a risk of contamination of the broiler carcasses at various points during the slaughtering process, mainly as scalding (immersion in water), de-feathering and evisceration. At the stage of evisceration, especially when the internal organs are mechanically separated, the intestines are mostly damaged by the equipment, thus causing fecal contamination of the carcasses. In addition, scalding and plucking processes may remove the epidermis layer of the skin. This may cause bacteria contamination, quick growth of bacteria and increased colonization on the carcass surface during the intestines removal and water cooling stages, which affects the shelf life of the meat (Erginkaya & Yurdakul 2010; Var et al. 2011). At the same time, there is also cross-contamination risk of other carcasses via contaminated equipments in slaughterhouses (Rivera-Perez et al. 2014; Hernandez et al. 2018; Perez-Arnedo & Gonzalez-Fandos 2019).

These bacterial contaminants have been known to affect some quality characteristics and the shelf life of chicken meat. Furthermore, it threatens human health. Studies showed that chicken meat can be contaminated with different pathogenic microorganisms especially *Salmonella* spp. and *Campylobacter jejuni*, which causes food infections in humans (Erol 2007).

Dry plucking is another method used for de-feathering of poultry. In this commercially applied method, a hot water boiler is not used to soften the feathers of the chickens. Instead of being immersed in the hot water, the chickens are passed through steam tunnels to loosen their follicles. Softened feathers are cleaned with the help of automatic plucking machines (Anonymous 2021). Another dry plucking method we used in our research is the simple dry plucking method. In this method, a dry plucking machine is used, consisting of a system on which gears are used to catch and pluck the feathers. The stems, whose cutting process has been completed, are taken one by one without wetting the feathers, and their feathers are plucked in the dry plucking machine. After the carcass is thoroughly washed, it is rested at +4 ºC until it cools down. Since feather plucking can be done hot or cold in the animals applied DPM, microbiological problems do not occur in the carcass due to burning and moisture, so the shelf life of the carcass is long.

In some studies, it has been reported that there is an increase in the level of microorganisms in carcasses after the application of the classical wet plucking (Anıl et al. 1989; Pacholewicz et al. 2015; Althaus et al. 2017; Ae Kim et al. 2017; Corry et al. 2017; Mota-Gutierrez et al. 2022). On the contrary, the DPM is a more hygienic method since immersion in hot water is not carried out after cutting and therefore the risk of contamination by pathogenic microorganisms is limited (Riggs et al. 2011).

The present study is important and original in the lack of scientific research on the comparison of the effect of dry and wet plucking methods on the quality and shelf life of the broiler carcasses. In addition, it is also important to reveal which one is a more hygienic method and for producing healthy meat. The DPM can be preferred as an alternative method for them. Therefore, the present study was conducted to determine the effects of different feather plucking methods on the meat quality characteristics and shelf life of the broiler carcasses.

2. Material and Methods

2.1. Breeding and De-feathering of animals

Forty male broilers (Ross 308) at 42 days of age obtained from a private company were used in the experiment. All the animals were raised up in the same flock using the same feeding regimes, in which four types of feeds were used such as, broiler starter feed (0-11 day, 3 000 Kcal/kg ME; 23% CP), grower feed 1 (11-21 day, 3 100 Kcal/kg ME; 22% CP), grower feed 2 (22-37 day, 3 250 Kcal/kg ME; 20% CP) and finisher feed (38-42 day, 3 250 Kcal/kg ME; 20% CP) during the fattening period. The selection process ensured the same breeding and feeding period, feed and feeding conditions, age, sex and approximately similar weight. All vaccinations of the animals have been made and care has been taken to ensure that they are healthy. The birds were divided into two groups and each group was included twenty birds. After the birds were cut from the neck, they were left for 1-2 minutes for bleeding. In the classical wet method, the birds were scalded by placing the carcasses in a 50-60 °C hot water tank for 60-90 seconds to ensure that the feathers are easily plucked. The wetted bodies were subjected to the first cooling process in cold water after they were plucked in the plucking machine. Later, they were eviscerated, washed, rested in cold water and drained respectively (Türkoğlu & Sarıca 2014).

In the simple dry plucking method, a dry plucking machine consisting of a system with gears to catch and pluck the feathers was used. The birds were de-feathered one by one in the dry plucking machine without scalding step. Afterward, they were eviscerated, washed and stored at + 4 °C up to analysis was done.

All the carcass of two groups were placed in sterile bags individually and transferred to Cukurova University Animal Nutrition Laboratory, and stored at +4 °C up to analysis was done. Each group was stored in a different refrigerator so that there is no contamination from one group to another. A total of 10 carcasses at week 0, five from each group, were used to perform the meat quality study after cutting. The rest of 30 carcasses, 15 from each group, were stored at +4 °C for 7 days (as the current legislation could let chicken meat to consume maximum 7 days from the slaughter) (GSO 2013) to carry out the shelf life studies.

Five breast and five drumsticks samples from each treatment group were analyzed for pH, WHC and W-BSFV during storage at day 0, 3, 5, and 7 of the shelf life. Only colors of breast and drumstick were analyzed for their muscle and skin separately.

The experimental procedure was approved by the Ethics Committee of Cukurova University (Approval No: 2022-5)

2.2. pH value

To determine pH values of the raw meat obtained from the drumstick and breast meat taken from each carcass was separately minced. One hundred mL of pure water was added to 10 g of meat samples taken from the minced meat, and homogenizers were used to homogenize the samples for 1 min, after which the pH values were read by means of a pH meter (HANNA HI99163).

2.3. Color

The basic color parameters *(L*, a*, b*)* of the breast and drumstick skins as well as meat samples (minced) from the left side muscle were measured by using a spectrocolorimeter (HunterLab, ColorFlex EZ) (Hunt et al. 1991).

2.4. Warner-Bratzler shear force value

Muscle samples were removed from the left side of the *pectoralis major*. Raw meat samples were cut into 1 cm × 2 cm × 3 cm (height × width × length) pieces with the length following the fiber direction. The texture analyzer which has an HDP/WBV Warner Bratzler blade set with V slot table was used for determining of the shear force value (TA/XT Analyzer Plus of Stable Micro Systems, Vienna Court, UK). Samples were sheared at a test speed of 5 mm/s, and perpendicular to the longitudinal orientation of the muscle fibers. The probe's pre-test speed was 10 mm/s, test speed 5 mm/s and cutting distance 5 cm. The mean of recorded peak shear force (kg) of samples was used for statistical analysis (Barbanti & Pasquini 2005; Carvalho et al. 2013; Schwarz et al. 2021).

2.5. Water holding capacity

WHC was estimated by centrifuging 1 g of the sample placed on tissue paper in a tube for 4 minutes at 1500 rpm. The water remaining after centrifugation was measured by drying the samples overnight at 70 °C (Castellini et al. 1998).

WHC has been calculated as follows (Eq 1).

$$WHC\ (\%) = (M1 - M2) / m \times 100 \quad (1)$$

M1: Filter paper + sample weight
M2: Filter paper + dry weight
m: Initial sample weight

2.6. Microbiological analysis

In the experiment, the microbiological quality of carcasses was determined by using the method of Ransom et al. (2002). Samples were collected from the muscle and skin parts of the 5 randomly selected carcasses in each group at days 0, 3, 5, and 7th of the shelf life and analyzed to determine the microorganism levels. Ten grams of muscle and skin pieces were taken from the drumstick and breast areas per carcass for microbiological analysis. Samples were added to 90 mL of 0.1% sterile peptone water and homogenized using a stomacher for 2 minutes. Then serial dilutions were made up to 10^{-8} in the tubes containing 9 mL of sterile peptone water.

Total mesophilic and psychrophilic bacteria counts were calculated using the petri dish spread method. The prepared dilutions spread to the Petri dishes they were incubated for 2 days at 30 °C and 10 days at 10 °C for mesophilic and psychrophilic bacteria growth (respectively). Violet Red Bile Agar VRBA, Oxoid, CM 0107) was used to state the total coliform bacteria count using the double pouring plate method. Petri dishes were incubated for 24 h at 37 °C. Total *Enterococcus* spp. counts were determined by pouring method with Kanamycin Aesculin Azide Agar Base (OXOID) (Oxoid CM 485) and were incubated for 18 hours at 37 °C, BD 151 *Campylobacter* Agar was used to determine *Campylobacter* spp counts 37 °C, in a jar and microaerobic atmosphere 42-48 hours. *E. coli* isolation was made in Tryptone Bile X–Glucuronide Medium (TBX) (Oxoid CM 945), for 24 hours at 30 °C. The presence of *Salmonella* spp. was determined by the ISO (2007) 6579 procedure (ISO 6579:2002/amd 1). The first step of the study; 25 g (mL) carcass samples were incubated in 225 mL peptone water (Peptone Water; Buffered (TPS) * 1.07228.0500) pre-enrichment (18±2 hours at 37 ºC), from which 0.1 mL is taken and 10 mL *Salmonella* Enrichment Broth acc. To Rappaport- Vassiliadis ((RVS Broth) * 1.07700.0500) is inoculated into the liquid medium and selective pre-enrichment is performed (24±3 hours at 41.5 ºC). Seeding was done on XLD agar and the empty colonies were confirmed on TSI agar. Seeding was done on Xylose Lysine Deoxycholate (BD, XLD agar) Agar and the empty colonies were confirmed on BD Triple Sugar Iron Agar (TSI Agar).

2.7. Statistical analysis

Data were processed using a general linear model of the ANOVA that included: plucking methods (group), the shelf life of the carcass (day), and its interaction as fixed effects. The data were analyzed statistically using the GLM (General Linear Model) procedure of SAS (2004). Statistical significance was declared at $P \leq 0.05$. All data are reported as least squares means with pooled standard errors (SEM).

3. Results and Discussions

3.1. Meat quality results

The color values of the meat and skin samples of the drumstick and breast parts were determined and analysis results were summarized in Table 1 and Table 2. According to the research results, the plucking method had significant effects on the drumstick meat and skin color *L**, *a** and *b** values (P<0.01). Also, an interaction was observed between the plucking method and the shelf life with respect to drumstick meat redness (P<0.01) and yellowness (P<0.05).

Table 1-The effect of plucking methods on the drumstick skin and meat color in shelf life studies

Drumstick Skin Color	*Shelf Life (day)*	*Group (Plucking Method)*		*SEM*	*P*		
		Dry Plucking Method	*Wet Classic Method*		*Group*	*Day*	*Group x Day*
L*	**0**	64.3	67.4	0.81	< 0.01	0.014	0.317
	3	64.6	67.8				
	5	63.6	64.1				
	7	63.1	65.8				
a*	**0**	2.1	2.3	0.56	< 0.01	< 0.01	0.082
	3	2.5	-0.2				
	5	1.9	0.6				
	7	0.4	-1.3				
b*	**0**	23.8	11.6	1.33	< 0.01	0.013	0.430
	3	22.3	6.4				
	5	21.9	12.3				
	7	20.5	6.6				
Drumstick Meat Color							
L*	**0**	59.0	67.4	0.87	< 0.01	0.053	0.245
	3	58.8	67.8				
	5	58.5	64.1				
	7	57.7	65.8				
a*	**0**	4.6	2.3	0.53	< 0.01	0.098	< 0.01
	3	4.6	-0.2				
	5	5.9	0.6				
	7	7.7	-1.3				
b*	**0**	21.3	11.6	1.13	< 0.01	0.074	0.028
	3	20.5	9.4				
	5	21.8	12.3				
	7	22.2	6.6				

***L**:** Lightness, ***a**:** Redness, ***b**:** Yellowness

While the effect of the plucking method on the breast skin color was found to be significant in terms of *a** and *b** values (P<0.01), the effect of the shelf life stage was determined to be significant only in terms of *a** value (P<0.01). On the other hand, the effect of the plucking method on the breast meat color was significant in terms of the *L** value (P<0.01), in the meantime, the effect of the shelf life stage was significant with respect to *a** (P<0.01) and *b** (P<0.05) values. Our finding shows that the drumstick and breast skin colors of the CWM group were brighter than those of the DPM group. In light of these results, it can be said that water treatments (scalding and cooling) cause the skin to shine. It has been reported that high water temperatures at scalding affect the appearance and color of the skin (Heath & Tomas 1973). If the scalding temperature is 60-66 °C and the exposure time is 45-90 s, the epidermis or cuticle of the carcass is removed. So, the yellow skin color turns into pale white (Heath & Tomas 1973; Perez-Vendrell 2001). In this study, the epidermis layer was intact and the skin was yellow, because scalding treatment was not applied during slaughtering in the DPM group. Also, the DPM group's skin colors were found to be redder and yellower. These increases in *a** and *b** values may be a result of bloody tissue formation, depending on the pressure applied to the skin during plucking.

Table 2-The effect of plucking methods on the breast skin and meat color in shelf life studies

Breast Skin Color	*Shelf Life (day)*	*Group (Plucking Method)*		*SEM*	*P*		
		Dry Plucking Method	*Wet Classic Method*		*Group*	*Day*	*Group x Day*
L*	**0**	63.4	66.4	0.71	0.141	0.073	0.054
	3	63.9	63.8				
	5	63.4	62.7				
	7	63.1	63.9				
a*	**0**	3.0	3.0	0.60	< 0.01	< 0.01	0.071
	3	3.0	-0.2				
	5	2.6	1.2				
	7	0.6	-0.6				
b*	**0**	24.7	13.3	0.92	< 0.01	0.159	0.863
	3	23.4	11.0				
	5	23.6	13.5				
	7	22.0	10.6				
Breast Meat Color							
L*	**0**	62.1	58.0	0.92	< 0.01	0.056	0.112
	3	62.9	60.9				
	5	59.6	60.1				
	7	60.4	58.7				
a*	**0**	4.9	2.8	0.43	0.768	< 0.01	< 0.01
	3	4.0	6.1				
	5	6.6	6.6				
	7	6.1	6.4				
b*	**0**	23.5	20.6	0.36	0.324	0.018	< 0.01
	3	21.5	23.4				
	5	22.9	23.5				
	7	23.1	22.5				

***L**:* Lightness, ***a**:* Redness, ***b**:* Yellowness

Drumstick meat color was brighter and light colored in the CWM group, while it was redder and yellower in the DPM group. It is reported that lighter color of meat was associated with low WHC just as dark color is related to high WHC (Qiao et al. 2001; Mudalal et al. 2014; Tijare et al. 2016; Cai et al. 2018). This may be related to the light color of the drumstick meat in our study. Unlike the drumstick, the breast meat color was brighter and lighter in the DPM group. It is reported that there is a significant negative correlation between the light color and pH values of breast meat (Barbut 1993; Allen et al. 1997; Qiao et al. 2001). Just like our results, low pH causes the spread of proteins in the muscle and the light is reflected differently from the surface and causes light color (Mir et al. 2017).

The effects of different feather plucking methods and shelf life stage on pH, W-BSFV and WHC are presented in Table 3. According to the results obtained from the present experiment showed that the plucking methods and shelf life stage have no significant ($P>0.05$) effects on W-BSFV and WHC. On the other hand, the W-BSFV was higher in the breast meat of DPM group on the first day and no differences were observed between the means of all groups for the shelf life duration. In both DPM and CWM pH value of the drumstick has no significant differences, although higher pH value tendency was observed in the breast part of the DPM group ($P<0.01$). The shelf life studies showed that, a significant decrease tendency was observed pH value of the drumstick (DPM: 6.15; CWM: 6.18) and the breast meat of the DPM application group.

Table 3-The effects of different feather plucking methods on pH, W-B shear value (kg) and water holding capacity (%) in shelf life studies

Parameters	*Shelf Life (day)*	*Group (Plucking Method)*		*SEM*	*P*		
		Dry Plucking Method	*Wet Classic Method*		*Group*	*Day*	*Group x Day*
Drumstick pH	**0**	6.1	5.9	0.10	0.613	0.103	0.145
	3	6.0	6.2				
	5	6.3	6.2				
	7	6.2	6.4				
Breast pH	**0**	5.8	5.9	0.05	< 0.01	0.256	0.166
	3	5.7	5.9				
	5	5.8	5.9				
	7	5.8	5.8				
Breast W-B Shear Value	**0**	35.0	34.0	1.79	0.533	0.221	0.997
	3	34.1	33.1				
	5	33.4	32.6				
	7	31.1	30.7				
Drumstick WHC	**0**	76.0	76.4	0.68	0.504	0.844	0.308
	3	76.3	76.2				
	5	76.3	75.5				
	7	74.8	76.6				
Breast WHC	**0**	75.1	75.2	0.57	0.962	0.287	0.247
	3	75.3	76.4				
	5	75.1	75.0				
	7	76.5	75.3				

W-B: Warner-Bratzler**, WHC:** Water Holding Capacity

It has been reported that there is a negative correlation between the pH and *L** value of breast meat. Although the pH value of light colored meat shows a marked tendency towards a decrease, there is a reverse tendency in dark colored meat (Allen et al. 1997; Barbut 1997; Fletcher 2002; Petracci 2004; Kralik et al. 2014). Additionally, there are some reports stating that if the pH value of the muscle after slaughtering is high, it causes the meat to be dark, hard and dry as well as shortens its shelf life. On the other hand, low pH values at 24 hours after slaughtering causes the WHC and color intensity of the meat to be lower but leads to a longer shelf life of the meat (Yang 1993; Allen et al. 1997). Similarly, in our study the pH value of drumstick meat was higher in the DPM group on the slaughtering day, and so the meats became harder (DPM: 74.0; CWM: 72.9) and darker (DPM 59.0 - CWM 67.4), and the deterioration occurred earlier than the other group. On the other hand, the low pH value of drumstick meat in the CWM group caused high WHC and *L** values.

However, our findings suggesting low pH value and WHC of breast meat caused high *L** values in DPM group do not support the previous findings (Yang 1993; Allen et al. 1997).

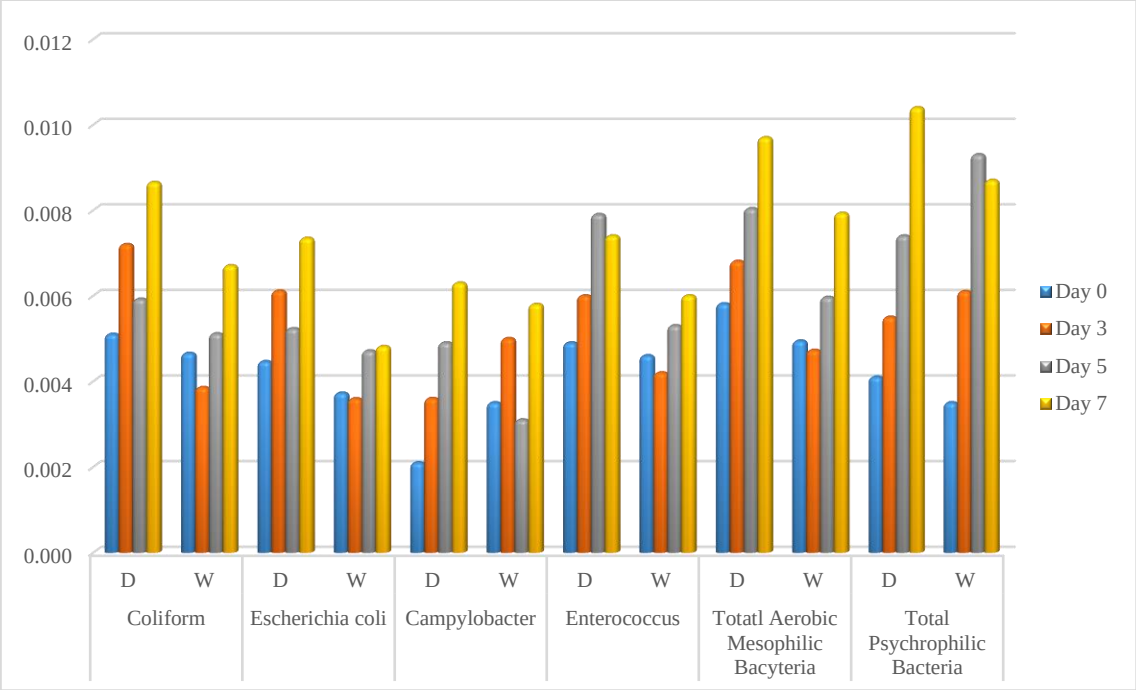
In our study, in the DPM group, the W-BSFV of the breast meat increased after slaughter, and the meat was hardened when compared with the CWM group. Anton et al. (2019) report that, after death, muscle cells are producing lactic acid and release proteolytic enzymes. This causes the disruption of connective tissue and results in the softening of the meat. This degradation occurs only when the meat is warm. In our research, the CWM group was scalded in hot water. So the cooling rate of the carcass has been slower than the DPM group. This resulted in the muscles being softer than the DPM group.

According to the results of shelf life studies, the plucking methods did not have a significant effect on the WHC. The average WHC of the drumstick was higher in the CWM group (DPM: 75.85; CWM: 76.20), but in the breast meat, the WHC was higher in the DPM group (DPM: 75.50; CWM: 75.48). The pH value of meat becomes low due to changes in myofibrillar structure in the muscle, which also causes low WHC (Petracci, 2004). The reason for the lower WHC of the drumstick in the DPM group (DPM: 75.85; CWM: 76.20) is because the mean pH value was lower (DPM: 6.15; CWM: 6.18). Also, the pH value of the breast meat was lower in the DPM group (DPM: 5.78; CWM: 5.88), while the WHC is lower in CWM group (DPM: 75.50; CWM: 75.48). It is reported that a low pH value of poultry meat is related to low water-holding capacity (WHC), so cook-loss, drip loss, shelf life is increased and tenderness is decreased (Barbut, 1993). The broiler breast meat is so light colored when the muscle holds much water (Petracci 2004). According to the research findings, it was determined that the WHC of breast meat in the CWM group after slaughtering was higher (DPM: 75.10; CWM: 75.20) and the color was darker (*L** value) (DPM: 62.10; CWM: 58.00), contrary to report.

3.2. Microbiological results

In the present study, samples were collected from the meat and skin parts of the carcasses at day 0, 3, 5 and 7th of the shelf life

and analyzed to determine the microorganism levels. Our results indicate that, the effects of plucking methods and shelf life stage on total mesophilic aerobic bacteria, total psychrophilic bacteria, coliform bacteria, *E. coli*, *Campylobacter* and *Enterococcus* spp. levels of the carcass after slaughtering were very significant (P<0.01). At the same time, the level of microorganisms was higher in the DPM group, compared with the CWM group, both after slaughtering and at the end of the shelf life studies (Figure 1). *Salmonella* was, also, found in the DPM and the CWM groups at day 0, 3, 5 and 7th of the shelf life of studies.



D: Dry Plucking Method, **W:** Classic Wet Method

Figure 1- The effect of different feather plucking methods on carcass microbial load (Log10 CFU / g)

The microbial contamination level of chicken meat is determined its shelf life (Morshedy & Sallam 2009). Some studies reported that the prevalence of *Campylobacter* decreases after scalding but increases after plucking and evisceration (Guerin et al. 2010). In the scalding stage of slaughterhouses, the high temperatures of the water expand the hair follicles and relax the skin. In the next processes, bacteria transfer to the enlarged follicles, and cooling of the carcasses may cause the bacteria to be trapped. This increases the bacterial load of chicken meat (Demirok et al. 2013). On the contrary, it has been reported that the process of poultry slaughterhouses, such as the scalding process in hot water reduces the level of *Salmonella* and microbial load in the carcass. It is recommended for the control of microbial growth that the scalding water temperature is above 47 °C. High scalding water temperatures greatly decrease the levels of the microorganisms in the carcass compared with low water temperatures (Dickens et al. 1999; Rivera-Perez et al. 2014; Rouger et al. 2017; Maharjan et al. 2019). Total aerobic mesophilic bacteria, total psychrophilic bacteria, coliform bacteria, *E. coli*, *Enterococcus* spp. and *Campylobacter* levels have decreased significantly. It is also worth noting that; the microbial load of the DPM group was significantly high. It is because; microorganisms on the feathers may be infected to the carcass surface during the dry feather plucking since the carcass had been turned with a hand during plucking. Similarly, the gut microorganisms may be infected inside the carcass during the evisceration. So, the shelf life of the carcasses in the DPM group was shorter than CWM group. Anıl et al. (1989) noted that the DPM is a more hygienic method than the CWM, and this application may lead to microbial contamination. Our findings were opposite the observations of Anıl et al. (1989). The reason for the incompatibility of the results may be that the DPM application in our study was different from commercial slaughterhouses.

The wet plucking method applied in the conventional broiler industry is not preferred today by some people with the suspicion of being halal-haram. This is because the scalding process is considered unhealthy for hygiene and carries a microbial risk. The main problem is that scalding water is thought to be contaminated with blood and feces, which causes an increase in microbial load and contamination of carcasses.

Many studies have shown that the increase of microorganism levels during the storage time can affect the pH value of meat (Nychas et al. 2008). Similarly, some studies have shown that there is a positive correlation between the pH value and the shelf life of the meat. When the pH value of the breast meat is high, the shelf life is prolonged (Yang 1993). Moreover, there is also a significant relationship between the number of microorganisms and meat color. While the pH value of the dark breast meat is high, that of the light color is low. High pH meat is dark, hard and dry, whereas low pH meat is pale, softer and has PSE (Pale

Soft Exudative) (Yang 1993; Allen et al. 1997; Fletcher 2002; Karaoglu et al. 2006; Garcia et al. 2010). Elliott & Heiniger (1965) reported that the minimum temperature that limits *Salmonella* proliferation is 46.2 °C. According to this for the prevention of increasing *Salmonella* load, wetting water temperature, higher than 47 °C, should be sufficient (Buhr 2014). In our study, hot water scalding did not prevent the growth of *Salmonella*. The appearance of *Salmonella* in both dry and CWM groups after slaughter and during shelf life studies suggests that *Salmonella* is present in the flock.

4. Conclusions

The results indicated that the feather plucking methods have significant effects on the skin and meat *L**, *a**, *b** values. When DPM is applied, the color of the drumstick and breast skin is more yellow. Likewise, the CWM increased the brightness of the drumstick and breast skin colors, and water applications caused the shine of the skin. It was observed that the DPM had no significant effect on the water-holding capacity of the drumstick and the breast meat. On the other hand, decreased pH value caused the meat to harden a little.

Our results suggest that scalding in hot water reduced the microbial load. The high level of microorganisms associated with the DPM caused the shelf life of the carcass to become shorter when stored under the conditions of +4 °C refrigerator. It is concluded that simple DPM may be used by low capacity farms which produce the broilers for consumers who prefer yellow-skinned chicken meat at the expense of a reduction in the shelf life due to increased microbial load.

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Evaluation of the Antioxidant Capacity, Antimicrobial Effect, and *In Vitro* Digestion Process of Bioactive Compounds of Cherry Laurel Leaves Extracts

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ABSTRACT
Cherry laurel is a less known fruit species with an astringent taste and is mostly consumed as fresh fruit only in the Black Sea and Marmara regions of Turkey. Cherry laurel (*Laurocerasus officinalis* Roemer) leaves can be prepared in different forms such as infusion by steeping the dried leaf in boiled water and as an extract for its further use as a food supplement or ingredient. In this study, aqueous and ethanol extracts of cherry laurel leaves were prepared and examined in terms of total phenolic compound (TPC), total flavonoid compound (TFC), antioxidant capacities using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and the copper reducing antioxidant capacity (CUPRAC) assays after submitting to *in vitro* digestion. Additionally, the antimicrobial potential of the leaves extract was evaluated. The TPC of ethanol and aqueous extracts were found at 17.62 and 0.83 mg gallic acid equivalent GAE.g^{-1} leaves and the TFC of ethanol and aqueous extracts were determined as 11.61 and 0.47 mg catechin equivalent CE.g^{-1} leaves, respectively. In terms of antioxidant activity results, ethanol and aqueous extracts had 41.11 and 0.77 mg Trolox equivalent TE.g^{-1} leaves for the DPPH assay, and 67.05 and 1.63 mg TE.g^{-1} leaves for the CUPRAC assay. After gastric digestion post-gastric (PG), compared to the initial values significantly lower recovery of the TPC (11.2 and 41%) and TFC (5.8 and 14.9 %) was observed for ethanol and aqueous extracts. The recovery of TPC, TFC values after the intestinal fraction was lower compared to the PG fraction for ethanol extracts, whereas for aqueous extracts they were higher compared to the PG fraction. The highest inhibition zone was observed against *Listeria monocytogenes* and *Aspergillus niger* when 10% extract concentration was applied. The experimental data verified that these extracts displayed remarkable antioxidant and antimicrobial activities, and the extraction method was important in terms of the bioaccessibility of bioactive compounds.

Keywords: *Laurocerasus officinalis* Roemer, Gastrointestinal, Phenolics, Flavonoid, Bacteria, Molds

1. Introduction

Plants have been used for medicinal purposes for over a thousand years. In traditional medicine around the world, 28.187 plant species have been identified and documented (Başer 1998; Dalar et al. 2018; Antonelli et al. 2019).

Cherry laurel (*Laurocerasus officinalis* Roemer), locally called as *taflan* or *karayemis*, has an astringent taste and is primarily consumed as fresh fruit only in local markets; it can also be dried, pickled, and sometimes processed into different products such as pekmez, jam, and marmalade (Capanoglu et al. 2011). The cherry laurel tree is also an appealing ornamental plant with dark, evergreen foliage and clusters of white flowers in the spring (Macit & Demirsoy 2012). In Turkish folk medicine, the fruit, seeds, and leaves have been used for different purposes, such as decoctions of leaves have been used for the treatment of food poisoning, stomach pain, prevention of excessive oil secretion in hair; leaf infusions have been applied for stomach ache, sore throat, and hemorrhoids and the seeds have been used for the treatment of descensus ventriculi (gastric descent) (Basri 1864; Hami 1864; Yesilada et al. 1999; Uslu et al. 2018).

Only few reports have been published on the bioactive contents of cherry laurel genotypes sampled in the West Black Sea Region (Beyhan et al. 2018; Islam et al. 2020). The effects of preharvest calcium chloride ($CaCl_2$) treatment on several quality features and bioactive substances of sweet cherry fruit were examined in a study by Erbaş & Koyuncu (2022), and it was discovered that all treated kinds had lower antioxidant activity than control fruit. Compared to the fruits of cherry laurel, it was reported that the leaves

also contain a higher level of phenolic compounds (Alasalvar et al. 2005; Orhan-Erdogan & Akkol-Kupeli 2011; Karabegovic et al. 2014). A major phenolic compound identified in cherry laurel leaves was chlorogenic acid, ranging from 25.44 to 36.49 mg/g of dry extract. Other phenolic compounds determined in cherry laurel leaves were o-coumaric acid, quercetin 3-glucoside, luteolin 7-glucoside, apigenin 7-glucoside, kaempferol 3-glucoside, and naringenin (Karabegović et al. 2014). Akkol et al. (2012) also identified three phenolic compounds, hydroxyphenyl acetic acid, kaempferol, and catechin derivatives, in the cherry laurel leaf extracts. Many studies have also shown the potential use of plant extracts as antimicrobial ingredients. The use of such compounds as antimicrobial additives in mildly processed food products has also been popular (Cui et al. 2010). *Laurocerasus officinalis* Roemer has been found to have anti-inflammatory, antifungal, and anti-nociceptive effects when prepared with ethanol and water extracts (Uslu et al. 2018).

Traditional consumption of medicinal plants was preparing the infused liquid by adding the plants to the boiled water, leaving them too steep, and filtering the plants (Suna et al. 2019). However, due to advancements in the functional food and nutraceutical industries, medicinal plant extracts have been created and used in a variety of forms, including pills, tablets, liquid extracts, powders, and the development of various value-added food products in recent years. When the aqueous infusion of medicinal plants and their phenolic extracts were consumed, they would be subjected to gastrointestinal digestion. *In vitro* digestion assays have been developed as an alternative approach to *in vivo* studies since they are simple, cheap, and reproducible tools to assess the stability of different food constituents against digestion fluids. Therefore, it's crucial to figure out how digestion impacts the stability of phenolic compounds and their antioxidant properties. As a result, their bioaccessibility depends on whether the medicinal plant's aqueous infusion or ethanolic extract is taken first. There have been some studies in the literature about the determination of antioxidant potential and bioactive compounds in cherry laurel leaf extracts (Akkol-Kupeli et al. 2012; Celep et al. 2013; Karabegović et al. 2013; Karabegović et al. 2014; Uslu et al. 2018). However, to the best of our knowledge, there has been no study focusing on the antioxidant potential of leaves extracts through *in vitro* gastrointestinal digestion process and their antimicrobial potential. As a result, cherry laurel leaves were made as an ethanol extract and an infusion in this investigation (aqueous extract; dried leaves steeped in boiled water). Then subjected to *in vitro* digestion, and their antioxidant properties, total phenolic, and flavonoid contents were assessed. Additionally, the antimicrobial activity of cherry laurel leaf extract was determined.

2. Materials and Methods

2.1. Chemicals

Folin-Ciocalteu's phenol reagent, sodium carbonate, sodium nitrite, aluminum trichloride, copper (II) chloride, ammonium acetate, sodium bicarbonate, sodium hydroxide, ethanol, hydrochloric acid (37%), nutrient broth, nutrient agar, potato dextrose agar (PDA) obtained from Merck (Darmstadt, Germany). Gallic acid, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), (±)-6- Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), catechin hydrate standard, neocuproine, pepsin (P6887), pancreatin (P7545), and all other reagents used to prepare simulated gastric fluid and simulated intestinal fluid were obtained from Sigma-Aldrich (Steinheim, Germany).

2.2. Plant material and extraction

Cherry laurel (*Laurocerasus officinalis* Roemer) leaves were obtained from Istanbul, Turkey was verified (Gul Herbarium Identification #46/4/1-1) by Prof. Dr. Hasan Ozcelik, senior botanist, Department of Biology, Suleyman Demirel University, Isparta, Turkey.

The fresh leaves were washed up with tap water and dried in the shadow at room temperature (25±2 °C) for three days and, the final moisture content of the leaves was 8.50±1.24%. The extraction conditions were based on preliminary experiments. The extracts from dried leaves were extracted by two different procedures. In the first procedure, 10 g of milled dried leaves were extracted with 100 mL ethanol mixture (80% ethanol + 20% water) for 2 h on a magnetic stirrer at room temperature. For the second procedure (aqueous extracts), the traditional method to prepare the herbal infusion was simulated by steeping 10 g of unmilled dried leaves with 100 mL of freshly boiled water for 5 min. Both ethanol and aqueous (infusion) extracts were filtered (Whatman no 1), and the clear phase was used for further analysis.

2.3. Simulated in vitro gastrointestinal (GI) digestion assay

The *in vitro* gastrointestinal digestion model of McDougall et al. (2005) and Kamiloglu et al. (2014) was applied for cherry laurel leaves extracts. The change in the antioxidant activity and release of polyphenolics of the ethanolic and aqueous extracts were examined at a

gastric stage and an intestinal stage of digestion, and the *in vitro* gastrointestinal digestion procedure is shown in Figure 1. Briefly, 2.5 mL extracts were combined with 20 mL distilled water and 5 M HCl was used to alter the pH to 1.7. 1.5 mL of pepsin solution (315 units/mL) was added and incubated for 2 hours at 37 °C in a hot water bath with 100 rpm shaking. After 2 h, 2 mL aliquots of the post-gastric (PG) digestion were collected. 4.5 mL of 4 mg/mL pancreatin solution (2 units/mL) and 25 mg/mL bile salt mixtures were added to the remainder in the 250 mL glass beaker. Segments of dialysis bags (MWCO 12,000 Da) were cut and filled with sufficient sodium $NaHCO_3$ (0.5 M) to neutralize the sample's titratable acidity (pH 7). Samples were incubated in a shaking water bath (100 rpm) at 37 °C for another 2 h to complete the intestinal phase of the *in vitro* digestion process.

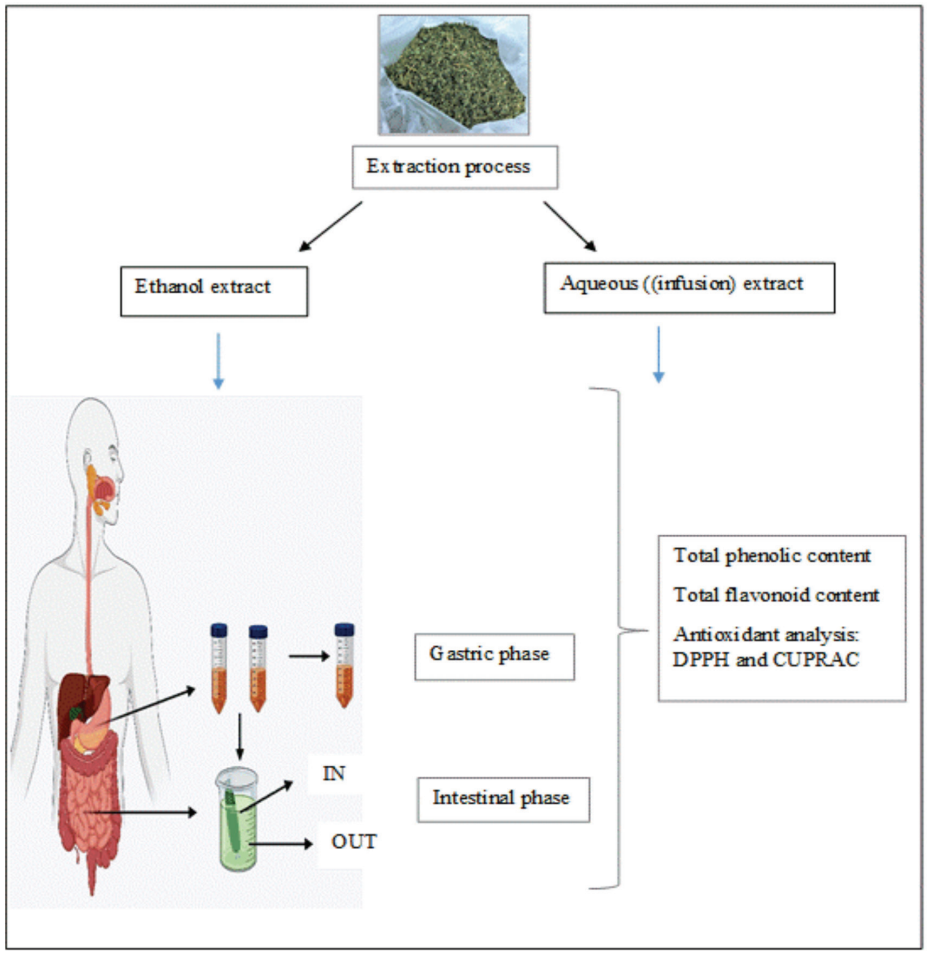

Figure 1- Flow chart outlining the steps involved in the *in vitro* gastrointestinal digestion procedure

After the intestinal phase, the content of the dialysis membrane was taken as the IN fraction, while the medium outside the membrane was referred to as the OUT fraction. The blank was also prepared with identical chemicals but without samples and underwent the same conditions. Then, the sample taken at each digestion step was centrifuged at 2268 g for 20 min and stored at -20 ºC for the analysis. Total phenolic compound (TPC), total flavonoid compound (TFC), and antioxidant activities were determined for ethanol and aqueous (infusion) extracts, as well as for each of the PG, IN and OUT fractions, using the methods described below.

2.4. Determination of total phenolic and total flavonoid content

The TPC and TFC were determined according to the method Singleton et al. (1999), Zhishen et al. (1999), respectively. The absorbances at 760 nm for TPC, 510 nm for TFC were measured using a Shimadzu 150 UV-1800 spectrophotometer (Kyoto, Japan). For TPC, the results were given as mg GAE per g dried leaves. The linear range of standard curve was from 0.01 to 0.1 $mg.mL^{-1}$ (r^2=0.993). For TFC, the results were given as mg CE per g dried leaves. The linear range of standard curve was from 0.01 to 0.35 $mg.mL^{-1}$ (r^2=0.996).

2.5. Antioxidant capacity assays

The DPPH radical scavenging capacity of cherry laurel leaves extracts was evaluated according to the method of Sanchez-Moreno (2002) and Singh et al. (2002). 0.1 mL of extract was mixed with 4.9 mL of DPPH solution (6×10^{-5} M) and incubated at room temperature for 20 min in the dark. The absorbance was measured at 517 nm, and the results were given as mg TE per g dried leaves. The linear range of standard curve was from 0.05 to 0.5 mg.mL^{-1} (r^2=0.996).

The copper reducing antioxidant capacity (CUPRAC) assay was carried out according to the method of Apak et al. (2004). One mL of each $CuCI_2$ solution (0.01 M), neocuproine (7.5 mM), and 1 M of ammonium acetate buffer (pH 7.0) solutions were added to a test tube. After the addition of 0.1 mL of extract, 1 mL of distilled water was added. All samples were incubated at room temperature for 1 h in the dark. The absorbance was measured at 450 nm, and the results were given as mg TE per g dried leaves. The linear range of the standard curve was from 0.05 to 1 mg.mL^{-1} (r^2=0.991).

2.6. Antimicrobial activity assays

Antimicrobial activity of the extracts was determined by using the agar diffusion method against five bacteria (*Bacillus cereus* FMC19, *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* ATCC 19118, *Salmonella* Typhimurium ATCC 14028, *Escherichia coli* O157: H7 ATCC 33150) and three molds (*Penicillium carneum, Aspergillus flavus, Aspergillus niger*. Bacteria were inoculated in nutrient broth and incubated at 37 °*C for 24 h, while the* molds were activated in PDA solid medium at 27 °C for 3 days. Then 1% of bacteria were added in nutrient broth and re-incubated for 18 h. Final cell concentrations were measured as 10^6-10^7 *colony-forming unit/*mL. One hundred *µL of microbial cultures* were pipetted, sowed by the spread plate method, and left for 20 min. Then, 4 equidistant wells were bored by sterile cork borers (*Ø*=5 mm) (Sagdic et al. 2013). According to our preliminary study, extract with 80% ethanol of leaves was found most effective against bacteria and mold whereas the aqueous (infusion) extract of leaves had no or limited inhibiting effect on any of the tested bacteria and mold. This might be owing to the excessive heat generated by the high boiling temperature during water extraction, as well as the chemical changes that resulted. That's why ethanol extract of cherry laurel leaf only was used. Dried leaves (6 g) were extracted successively with 60 mL of ethanol mixture (80% ethanol 20% water) by using a Soxhlet extractor (Buchi Extraction Unit E-816) for 4.5 h at 60 °C temperature, and then concentrated under vacuum at 40 °C. The dried plant extracts were dissolved in ethanol as a final concentration of 1%, 5%, 10% (w:v), and 20 µL of extract and negative control was prepared using ethanol. The petri dishes were incubated at 27 ºC for 3-4 days for the molds, and at 37 ºC for 18-24 h for the bacteria. After the incubations, the zones were measured as mm and the analyzes performed in triplicate.

2.7. Statistical analysis

The statistical analysis was carried out by means of SPSS Statistics (IBM SPSS 17.0, USA). All data were presented as a mean of at least three measurements, i.e. ± standard deviation for each extract. The differences of bioactive compounds or antioxidant capacity between cherry laurel leaves obtained by ethanol and aqueous (infusion) extracts were evaluated by t-test, the differences among value of bioactive compounds or antioxidant activity that obtained in different steps of the *in vitro* digestion assay and the differences among concentration of antimicrobial effects were analyzed by one-way ANOVA combined with the Duncan comparison test at $p<0.05$ significance level.

3. Results and Discussion

3.1. Total phenolic, total flavonoid contents, and antioxidant capacity of cherry laurel leaves extracts

The TPC, TFC, DPPH radical scavenging capacity, and CUPRAC of ethanol and aqueous extracts (infusion) of cherry laurel leaves were given in Table 1. The TPC and TFC of ethanol and aqueous extracts were determined as 17.62 and 0.83 mg GAE g^{-1} of leaves, and 11.61 and 0.47 mg CE g^{-1} of leaves, respectively. In terms of antioxidant capacity results, ethanol and aqueous extracts had 41.11 and 0.77 mg TE g^{-1} leaves for DPPH assay, and 67.05 and 1.63 mg TE g^{-1} leaves for CUPRAC assay (Table 1). In a study by Karabegović et al. (2014), the effects of different extraction techniques on the composition and antioxidant capacity of cherry laurel fruits and leaves were studied. TPC and TFC content of dried cherry laurel leaves were found as 36.65±0.6 mg of GAE.g^{-1} leaf and 26.31±0.72 mg rutin/g leaf when the soxhlet extraction technique was applied with methanol for 2 hours. Orhan-Erdogan & Akkol-Kupeli (2011), found the TPC content of dried cherry laurel leaves as 10.04±0.06 and 14.93±0.98 mg of GAE.g^{-1} leaves when methanol and distilled water (not boiling water) were used as the extraction solvents. The TPC result of ethanol extract (17.62±0.14

mg GAE.g^{-1} leaves) determined in our study was higher than those determined by Orhan-Erdogan & Akkol-Kupeli (2011), but lower than Karabegović et al. (2014). The differences in the results can be explained with differences in the extraction conditions, type of extraction solvent, as well as with environmental factors, plant varieties, and age of the trees, post-harvesting conditions, or storage (Kolayli et al., 2003). In our study, the TPC and TFC of the infusion (aqueous extract) were lower than the ethanol extract as expected due to the procedure applied for its preparation. The decrease in phenolic acids and flavonoids found in water extraction (infusion) could be due to oxidation of this chemical in the presence of water at high temperatures. Flavonoids are known to breakdown in the water at temperatures of 100 °C and above. The thermal stability of flavonoids is affected by the quantity and type of substituents as well as the position of the hydroxyl group, with compounds with fewer substituents being less stable at high temperatures (Kasmi et al. 2021). As a result, the higher bioactive compounds in the ethanol extract than in the infusion extract can be explained by the fact that the leaves are not ground and boiled water is used in the extraction process. Similarly, Martins et al. (2014) discovered that the hydroalcoholic extract of *Origanum vulgare* has higher antioxidant activity than the aqueus extract (infusion). In the same way, Ozkan (2009) found that ethanol extracts of *Sideritis* species had higher amount of phenolics as compared to aqueous infusions. In theory, the bigger the particle size of leaves the lower the yield of extraction will be due to the lower contact surface area of the plant with the water. Also, 100 °C treatments produce lower extraction yield and bioactive compounds (Vuong et al. 2011; Geoffroy et al. 2017).

Table 1- The TPC, TFC, and antioxidant capacities of ethanol and aqueous (infusion) extract of cherry laurel leaves

	Unit	***Ethanol extract***	***Aqueous extract (infusion)***
TPC	mg GAE.g^{-1}	17.62±0.14^{a}	0.83±0.10^{b}
TFC	mg CE.g^{-1}	11.61±0.30^{a}	0.47±0.16^{b}
DPPH	mg TE.g^{-1}	41.11±0.57^{a}	0.77±0.70^{b}
CUPRAC	mg TE.g^{-1}	67.05±1.30^{a}	1.63±0.02^{b}

The results are given as mean ± standard deviation of triplicate measurements. Means with different letters in the same row are significantly different ($p<0.05$). The results are expressed for per g dried leaves, *TPC* total phenolic content, *TFC* total flavonoid content

In terms of antioxidant capacity, Orhan-Erdogan & Akkol-Kupeli (2011) found that at 2000 g.mL^{-1} of extract, methanol and aqueous (not infusion) extracts of cherry laurel leaves had 64% and 31% of DPPH radical scavenging capacity, respectively. The aqueous extract likewise had a lesser antioxidant capacity than the ethanol extract in our study. The ethanol is frequently used to extract tannins, polyphenols, and flavonols, and ethanolic extracts have high antioxidant activity. With only a tiny amount of flavonols leached into the polar solvent during water infusion, such preparations have the inferior antiradical-scavenging capability (Kobus et al. 2009). The higher antioxidant capacity of leaves compared to the fruits of cherry laurel had been determined in previous studies, Karabegović et al. (2014) determined EC_{50} values of the extracts of cherry laurel fruits and leaves found that leaf extracts showed higher antioxidant capacity than those of the fruit extracts. Antioxidant capacity values of any compound would differ depending on the measurement method. In our study, the CUPRAC values of both extracts were higher than the values obtained by the DPPH method. Cu^{2+} ion takes part in the formation of free radicals; the reduction of cupric ion indicates another mechanism than that of the DPPH method reflecting the antioxidant potential. This could be because the CUPRAC assay can detect both the hydrophilic and lipophilic antioxidant capacity of extracts because the reagent is soluble in both aqueous and organic solvents. DPPH assay with hydrophobic systems because it uses a radical that is only dissolved in the organic solvent (Capanoglu et al. 2018).

3.2. The TPC, TFC, DPPH and CUPRAC of cherry laurel leaves extracts obtained after simulation in vitro gastrointestinal digestion

In Figure 2, the recovery (%) of the TPC, TFC, and antioxidant activities of the extracts were given at each step of simulated gastrointestinal digestion, namely PG (after stomach digestion), IN (the material entered the serum), and OUT (the material remained in the GI tract) after intestinal digestion.

The TPC, TFC, and antioxidant capacity recovery (%) was calculated by dividing the values obtained for the PG, IN, and OUT fractions by the initial values of undigested extracts. For a better evaluation of the change, the initial values of ethanol and aqueous (infusion) extract were considered as 100%. After gastric digestion (PG), compared to the initial values a significantly lower ($p<0.05$) amount of the TPC (11.2 and 41%) and the TFC (5.8 and 14.9%) were recovered from ethanol and aqueous extract, respectively. When DPPH radical scavenging capacity and CUPRAC values of initial values and those obtained after gastric digestion (PG) were compared, significantly lower recovery values were observed for ethanol extract (71% and 30%, respectively). The trend was similar for CUPRAC

values of aqueous extract (32% recovery), whereas it was different for the aqueous extract in the DPPH values. The initial and PG values for DPPH recovery were not significantly different for aqueous extract.

After the intestinal digestion, for the IN fractions of both ethanol and aqueous extracts, the recovery (%) values for the TPC (3.9 and 15.7%) and the TFC (0.4 and 6.4%), respectively, significantly lower than those obtained at the initial values. Except for the recovery (%) of the antioxidant capacity value of aqueous extract attained by the CUPRAC assay, the antioxidant capacity values of the IN fractions were reduced (6.5-19.5%) compared to the values obtained after the initial values.

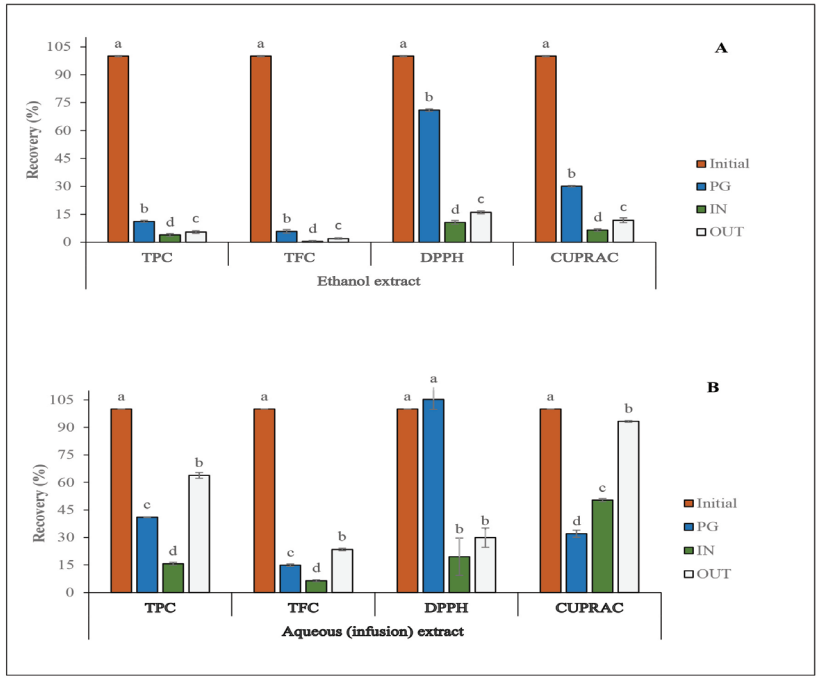

Figure 2- Changes in the TPC, TFC, and antioxidant capacity (determined by DPPH and CUPRAC assays) of cherry laurel leaves ethanol extract (A) and aqueous (infusion) extract (B) during *in vitro* gastrointestinal digestion, expressed as recovery percentage. The terms represent; initial, undigested sample (100%); PG (post-gastric), recovered after gastric digestion; IN, dialyzed fraction; OUT, non-dialyzed fraction recovered after intestinal digestion. Data represent the average values±standard deviation of four independent samples. Different letters above the bars represent the statistically significant differences ($p<0.05$)

The recoveries of the TPC and TFC of OUT fractions were significantly higher than those of the IN fractions. Non-dialyzed phenolic fraction (OUT) for ethanol extract accounted for 5.4 and 2% of the initial TPC and TFC, respectively. For aqueous extracts, the OUT fraction was 63.9 and 23.5% of the initial TPC and TFC. Similar to the outcomes of TPC and TFC, for all samples, the recovery values for antioxidant capacity obtained for OUT samples were higher than the IN values.

In our study, after gastric digestion (PG) 88.2 and 94.2% of the TPC and TFC in ethanol extract was lost, and correspondingly the antioxidant capacity values were decreased by 30 and 70% obtained by the DPPH and CUPRAC assays compare to the initial values. Whereas for aqueous extract, although the initial TPC and TFC were lower compared to the ethanol extract, the loss of TPC and TFC (59 and 85.11%) was lower at the PG stage. Compared to the initial values, the antioxidant capacity value was reduced to 32% when determined by the CUPRAC assay, whereas it wasn't statistically significant difference when determined by the DPPH assay for the aqueous extract. This difference could be related to the difference in the mechanisms related to the methods of determination, which means employing a method depending on one mechanism may not reflect the true antioxidant capacity. Furthermore, the accessibility and metabolism of dietary antioxidants influence their effect in biological systems. As a result, the differences in the conditions of antioxidant activity/capacity models applied to biological systems, as well as potential interactions between food-derived and endogenous antioxidants, must be considered (Capanoglu et al. 2018).

The loss of TPC and TFC in the digested samples were in agreement with the results of other authors. Ortega-Vidal et al. (2019) studied the effects of *in vitro* digestion on the phenolic profile and antioxidant capacity of *Jasonia glutinosa* herbal tea infusion and found that

the TPC content was reduced approximately 86% after *in vitro* digestion and a similar trend was observed for antioxidant capacity values obtained by the DPPH and ABTS, approximately 57 and 86% reduction compared to initial values obtained before digestion.

Donlao & Ogawa (2018) showed that *in vitro* digestion reduced antioxidant activity in all tea infusion samples. They also revealed that whereas polyphenols in tea infusions were reasonably steady during digestion, the DPPH values were reduced by 16.0 to 25.7 percent after digestion.

The sum of IN and OUT fractions was representing the total amount of TPC and TFC at the intestinal stage, and IN (dialyzed fraction) was taken as serum available, and OUT (non-dialyzed fraction) was referred to as colon available. When TPC and TFC content was analysed after the intestinal stage (IN + OUT), there was a decrease in TPC compared to the PG stage (from 1.97±0.01 to 1.64±0.01 mg GAE. g^{-1} leaves) and TFC values (from 0.67±0.03 to 0.27±0.01 mg CE.g^{-1} leaves) for ethanol extracts, interestingly whereas for aqueous extracts there was an increase in both TPC (from 0.34±0.02 to 0.66±0.01 mg GAE.g^{-1} leaves) and TFC values (from 0.07±0.01 to 0.14±0.04 mg CE.g^{-1} leaves).

For both ethanol and aqueous extracts, the TPC and TFC of OUT fractions were significantly higher than of the IN fractions representing those larger amounts of compounds could be metabolized by the microflora in the colon. The recovery (%) of TPC and TFC in the OUT fraction in the aqueous extract (64 and 23%) was significantly higher than the values obtained by ethanol extract (5.44 and 2%). Similar to the outcomes of TPC and TFC, the recovery values for antioxidant capacity obtained for the OUT samples were higher than of the IN fractions.

The recovery (%) of TPC, TFC, and antioxidant capacity at the intestinal stage (IN + OUT fraction) were lower compared to the PG fraction for ethanol extracts. Whereas for aqueous extracts, at the intestinal stage, the recovery (%) of TPC, TFC, and CUPRAC were higher compared to the PG fraction. Except for the recovery value of DPPH, it is thought that the transition from an acidic to an alkaline environment improves the ability of phenolics to donate protons present on their aromatic rings, enhancing their antioxidant properties (Ucar & Karadag, 2019). This was only observed for aqueous extracts. The differences in the recovery of antioxidant capacity at the intestinal stage might be related to possibly different individual phenolics obtained in ethanol and aqueous extract. Karabegović et al. (2014) investigated the phenolic profile of methanolic from cherry laurel leaves and fruit Regardless of plant materials or extraction techniques, chlorogenic acid was the most common component in cherry laurel leaf and fruit extracts, ranging from 25.44 to 36.49 mg.g^{-1} dry extract, although quercetin 3-glucoside, o-coumaric acid, luteolin 7-glucoside, kaempferol 3-glucoside, apigenin 7-glucoside, and naringenin were present in the leaf extracts, while rutin, caffeic acid, and vanillic acid were detected in fruit extracts.

Phenolics are highly sensitive to pH changes and, thus differences in the TPC and TFC, and antioxidant capacity after digestion could be due to the stability of each type of phenolic compound present in the sample matrix. In our study, the extraction solvent and preparation methods were different, the individual phenolics would be different in ethanol and aqueous extract of cherry laurel leaves, therefore this difference obtained *in vitro* digestion study could be related to the effects of food matrix (such as particle size, food components) and the presence of individual compounds in the extract. A similar trend was reported by Siracusa et al. (2011) who evaluated the antioxidant capacities and phenolic composition of *Capparis spinosa* L. and *Crithmum maritimum* L. aqueous extracts before and after the submission to an *in vitro* digestion process. They found that the amount of total phenol in digested infusions significantly decreased (<1%) in both samples, suggesting that the dominant phenolic components detected in original samples are not stable under the gastrointestinal tract. Total loss of chlorogenic acid after intestinal digestion was only 33% in the caper extract whereas it was 81.7 and 95.7% for the standard mixture and sea fennel extract. Although the initial chlorogenic acid amount was the lowest in the caper extract, after digestion its recovery was the highest in the caper extract, indicating that the extract composition, the presence of other constituents may strongly influence the behavior of a single compound. Similarly, when rutin was submitted to gastric digestion as a part of the standard mixture, at PG the loss was 88%, whereas it was negligible (1.7%) when it was in the composition of the caper extract. Even for the same particular phenolic component, the extract composition had an effect on its stability after digestion, according to their study. When the standard combination, aqueous extract of caper, and sea funnel were subjected to gastric digestion (PG), the loss of chlorogenic acid was 58, 5.8, and 66%, respectively.

3.3. Antimicrobial activities of cherry laurel leaf extract

The antimicrobial activity of ethanol extract of cherry laurel leaves at different concentrations was given in Table 2. Generally, upon increasing the ethanol extract concentration, the inhibition zone was also increased, and the highest inhibition zone was observed at 10% extract concentration applied against *Listeria monocytogenes.* Between 5 and 10% of extract concentration, the differences

in the inhibition against *Bacillus cereus* and *Escherichia coli* O157: H7 were not significant ($p>0.05$). The leaves extract showed good antimicrobial activity and can be used to explore novel antimicrobial activities. In terms of antifungal properties, the inhibition zone was the highest against *Aspergillus niger*, and it was increased by increasing the extract concentration from 1 to 10%. Against *Penicillium carneum*, at 1% extract concentration no inhibition was observed, whereas the inhibition was increased with the higher concentrations applied. The ethanol extract of cherry laurel leaves was not effective against *Aspergillus flavus* at any concentration applied in this study.

Table 2- Antimicrobial activity of the cherry laurel leaves extract (inhibition zone, mm)

		Extract concentration (%)		
Bacteria	***Negative control (Ethanol)***	***1***	***5***	***10***
B. cereus	nz	8.33±1.15^{b}	13.00±1.00^{a}	13.33±0.58^{a}
S. aureus		10.33±1.15^{b}	10.67±0.58^{b}	12.67±0.58^{a}
L. monocytogenes		9.00±1.00^{b}	8.33±1.15^{b}	18.33±3.06^{a}
S. Typhimurium		7.67±1.15^{b}	8.00±1.00^{b}	11.67±1.15^{a}
E. coli O157:H7		8.33±1.15^{b}	10.00±1.00^{a}	10.83±0.76^{a}
Molds				
P. carneum	nz	nz	11.00±1.00^{b}	17.33±2.52^{a}
A. flavus		nz	nz	nz
A. niger		6.33 ± 0.58^{c}	12.00±1.00^{b}	15.33±1.53^{a}

The results are given as mean ± standard deviation of triplicate measurements. Means with different letters in the same row are significantly different ($p<0.05$). nz: no inhibition zone

In the study of Ayla et al. (2019), the antimicrobial activity of methanol extracts of the fruit part of *L. officinalis* against *E. coli*, *S. aureus*, *B. cereus* was determined by the agar well diffusion technique. While the strongest antimicrobial activity was found for *S. aureus* (34 mm), the weakest antimicrobial activity was found for *E. coli* (23 mm) when the extract concentration was 25%, in our study, when the leaves the part of *L. officinalis* was used at 10% of extract concentration, inhibition zones were obtained, 12.67±0.58 and 10.33±0.58 against *S. aureus* and *E. coli*. In the study of Sahan (2011), among all the extracts (methanol, ethanol, acetone, chloroform), the ethanol extract of *Prunus laurocerasus* L. leaves was found most effective against *Fusarium oxysporum, Penicillium solitum,* and *Rhizopus oligosporus*. The antibacterial activity of different concentrations of *Laurus nobilis* leaves extract (1, 2.5, 5, 7.5, 10, and 20%) against *S. aureus* was investigated in another study utilizing a Vernier Calliper to measure the zone of growth inhibition. *S. aureus* growth was inhibited at doses of *L. nobilis* leaves extract ranging from 2.5 to 20 (v/v) (Hamdan & Masoud 2020).

4. Conclusions

The innovation of this study is the evaluation of change in total phenol, total flavonoid content, and antioxidant capacities of cherry laurel leaves subjected to a simulated *in vitro* digestion model. Although the initial levels of TPC, TFC, and antioxidant capacities were higher for ethanol extracts, the highest recovery (%) for PG, IN, and OUT fractions was obtained by the aqueous extract, after digestion. As a result, when medical plants and their extracts were consumed, the fate of desirable phenolic components after digestion would be critical in determining which medicinal plant consumption method, such as infusion, to use. Additionally, the growth inhibition of the tested molds and bacteria by the cherry laurel leaf extract indicated its potential as a novel antimicrobial food ingredient. Further studies should include the determination of individual phenolic compounds in the cherry laurel leaf extracts depending on the method of preparation either by aqueous infusion or organic solvent extraction, and how the composition of the extract would affect their stability during *in vitro* digestion.

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The effects of Fennel (*Foeniculum vulgare*) Essential Oils on Growth Performance and Digestive Physiological Traits in Black Sea Salmon (*Salmo labrax* PALLAS 1814) Juveniles

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ABSTRACT
The present study was carried out in the freshwater recirculating aquaculture system to determine the effects of the fennel (*Foeniculum vulgare*) essential oil on Black Sea salmon (*Salmo labrax*). Fish were distributed randomly to 50 L experimental tanks, and 45 fish were in each tank. The experiment was triplicate in each group, and the results were averaged. Five diets of equal isonitrogenous and isocaloric content with 50, 100, 200, and 400 mg kg^{-1} of fennel essential oil were prepared. Fish were fed with diets at the rate of 3% of live body weight four times daily by hand for 90 days. The results revealed that dietary supplementation with fennel essential oil did not have any significant effect on the growth performance. Supplementation with 200 mg fennel kg^{-1} increased lipase activity in contrast with control group. Supplementation with 400 mg kg^{-1} fennel showed similar results with the control group in terms of pepsin activity. The intestinal villi length of fish fed 200 mg kg^{-1} fennel was higher than the control group. The thickness of muscularis in group fed with 50, 100 and 200 mg kg^{-1} fennel was similar to each other and higher than the control group. Lactic acid bacteria were reduced by fennel essential oil supplementation. Results showed that fennel essential oil can be used in diets of Black Sea salmon without the growth performance.

Keywords: *Salmo labrax*, Feed additive, Essential oil, Nutrition

1. Introduction

Black Sea salmon aquaculture started in 2007, and today has been reached the level of 2311 tons/year in Turkey (TÜİK 2021). Currently, Black Sea salmon widely cultivated in the Southeastern Black Sea, has quite suitable cultural characteristics for global aquaculture (Çakmak et al. 2013). The importance of aquaculture is quite high in meeting animal protein needs which tends to increase constantly in human nutrition. Increasing demand and consumer preferences to seafood has prompted researchers to carry out new studies to increase species diversity along with the amount of production in aquaculture. As well as the rainbow trout and the Atlantic salmon, the Black Sea salmon have been expected to play an important role in the future in meeting the global demands which are in an ever increasing trend.

Numerous feed additives used to improve growth performance in fish, are chemical substances such as hormones and antibiotics that have undesirable side effects (Khalafalla 2009). However, using natural supplements in aquaculture have begun to gained importance due to the adverse effects of antibiotics and synthetic antioxidants on human health (Bilal et al. 2008). The natural and harmless compounds have the potential to be used in aquaculture as an alternative to antibiotics (Navarrete et al. 2010). World Health Organization encourages medicinal herbs to substitute or minimize the use of chemicals (Tonsy et al. 2011). The increasing concern about the use of antibiotics in animal production is the search for aromatic herbal and essential oil alternatives, which have a stimulating effect on both microbial activity and animal digestion (Kim et al. 2011). It has been stated that the plant extracts could be used as alternative additives as a result of both in vivo and in vitro studies (Bilal et al. 2008). Essential oils and their extracts derived from medicinal and aromatic plants increase the absorption of nutrition and the benefit of fishmeal by having a stimulant effect on the enzyme activities of pancreas and bile. They also increase intracellular absorption, accelerate the renewing of epithelium cells in the small intestine, regulate the intestinal microflora and prevent the habitation of

disease-causing bacteria into the digestive system. Therefore, these oils provide both improvements in growth performance and strengthening the immune system (Khalafalla 2009; Tonsy et al. 2011). Essential oils are recognized as safe according to the Food and Drug Administration (Snuossi et al. 2016). The essential oil derived from herbs and species are an alternative feed additive, and have antimicrobial, antioxidant and antifungal properties (Çabuk et al. 2014). Fennel (*Foeniculum vulgare*) essential oil is a biennial medicinal and aromatic plant belongs to Apiaceae (Umbelliferae) family (Hassaan & Soltan, 2016), and also has antioxidant, anticancer, antibacterial, antifungal properties, antidiabetic (Sotoudeh & Yeganeh 2016), hepatoprotective effects and antioxidant activities (Hassaan & Soltan 2016).

The goal in the aquaculture sector is to achieve maximum profit with minimum expense under optimum conditions. The use of products involving natural compounds which has short term effects are preferable in aquaculture by both producers and consumers. In order to commercialize the use of such products in aquaculture, it is important to investigate these medicinal-aromatic plants in all aspects. However, studies carried out on essential oils in fish nutrition are limited. In this study, it was aimed to investigate the effect of fennel essential oil on the growth performance and digestive physiology of Black Sea salmon, which is an endemic for Turkey and an increasing demand for farming.

2. Material and Methods

2.1. Fish, maintenance and feeding experiment

The study was performed at the freshwater recirculating aquaculture system at the Central Fisheries Research Institute. Fifth filial generation (F5) of Black Sea salmon (*Salmo labrax*) with average initial weights of 3.52±0.01g were used in the study. Every experiment was carried out in triplicate. Treatments were performed in 15 experimental tanks. The 675 fish (135 individuals per group) were randomly distributed into 50 L tanks at a density of 45 fish per experimental tank. Fish were fed by hand 3% of body weight four times daily. Experiments were carried out in square tanks with refreshing used water 22 times daily. Water temperature (15.10±0.98 °C), oxygen (8.78±0.21 mg/L), pH (7.43±0.18) and mortality were recorded daily. Ammonia (0.05±0.05 mg/L) was measured weekly.

2.2. Diet formulation

Fennel oil was supplied from Talya Herbal Products which are extracted from cultured *Foeniculum vulgare* provided from in the Mediterranean region of Turkey. The analyses were carried out by Anadolu University with the Agilent GC/MS system (7890B-5977B model) having an HP-Innowax column (60 m x 0.25 mm x 0.25 µm). In the analysis, carrier gas was selected as helium (0.7 mL/min), injection temperature was set as 250 ºC, ion source temperature was set as 230 ºC, and 70 eV electron was used for ionization. Ultimately, obtained results were evaluated with Wiley 9-Nist 11 Mass Spectral Database in Anadolu University. The volatile components of fennel essential oil were shown in Table 1. Experiment diets were prepared to contain fennel essential oils at levels of 50, 100, 200 and 400 mg kg^{-1}. All raw materials except fish oil and fennel essential oil were homogeneously blended with grinder. The mixture was extruded at 70 °C. Then the fennel oils were first included into the fish oil and then were penetrated into the extruded baits by vacuum coating. Similarly control diet without fennel oil was prepared by being penetrate into fish oil extruded post. Extruded feeds were cut 2.0 mm pellet size. Five diets were formulated, involving control. The control diet did not contain fennel oil. Fish meal was a mixture of European sprat (*Sprattus sprattus)* and Atlantic herring (*Clupea harengus*) meals containing 65.37% of crude protein and 10.7% of crude lipid, whereas fish oil was derived from European anchovy (*Engraulis encrasicolus*) the most used oil source for feed ingredients. Ingredients and nutrient compositions of diets were shown in Table 2.

Table 1- Volatile components of fennel essential oil*

Compound	*%*
(E)-Anethole	72.6
Limonene	6.3
Anisaldehyde	4.4
Methyl chavicol	3.7
(E,E)-2,4-Decadienal	3.2
(E)-2-Heptenal	2.3
(E,Z)-2,4-Decadienal	2.2
Carvone	1.4
α-Fenchone	1.3
(E)-2-Decenal	1.0
Anisketone	0.9
α-Pinene	0.7

*: The most abundant chemical compounds of essential oils were listed according to amounts that were found higher than 0.5%.

2.3. Fish performance

Before weight measurements, fish were starved for 1 day, then lightly anesthetized with 50 ppm benzocaine. The growth performance was calculated with equations shown below.

$$\text{Specific growth rate (SGR)}\ \% = 100 \times [(\ln \text{Final weight} - \ln \text{Initial weight})/\text{days}]$$
$$\text{Weight gain (WG)}\ g = (\text{Final weight - Initial weight})$$
$$\text{Feed conversion ratio (FCR)} = (\text{Feed intake}/\text{Weight gain})$$
$$\text{Survival rate (SR)}\ \% = 100 \times (\text{Final number of fish}\ /\text{Initial number of fish})$$ (Hoseinifar et al. 2014).

Table 2- Formulation and chemical parameters of the basal diet (%)

Ingredients	*%*
Fish meal	31
Soybean meal	20
Wheat gluten	6
Pea protein concentrate	12
Sunflower seed meal	7
Wheat flour	12.5
Fish oil	11
Vitamin mix[1]	0.22
Mineral mix[2]	0.16
Vit C	0.12
Chemical parameters	
Crude protein	46.20
Crude lipid	14.97
Crude Ash	9.38
Moisture	6.14

[1]: Supplied the following: inositol 300 mg, biotin (Vit B7) 200 mg, tocopherol (Vit E) 200 mg, calcium pantothenate (Vit B5) 50 mg, riboflavin (Vit B2) 30 mg, pyridoxine (Vit B6) 20 mg, thiamine (Vit B1) 20 mg, menadione (Vit K3) 12 mg, niacin (Vit B3) 6 mg, retinol (Vit A) 0.6 mg, folic acid (Vit B9) 0.5 mg, cholecalciferol (Vit D3) 0.05 mg, cobalamin (Vit B12) 0.05 mg. **[2]:**Supplied the following: ferric sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) 50 mg, manganese (II) oxide (MnO) 50 mg, zinc oxide (ZnO) 50 mg, copper sulfate pentahydrate ($CuO_4S \cdot 5H_2O$) 10 mg, calcium iodate (Ca_2IO_6) 0.8 mg, cobalt carbonate hexahydrate ($CoCO_3 \cdot 6H_2O$) 0.15 mg, sodium selenite (Na_2SeO_3) 0.15 mg.

2.4. Digestive enzyme assays

Midgut samples were taken in 45th minutes after feeding and were kept at -80 °C until analyses. Tissue samples brought to Çanakkale Onsekiz Mart University, Faculty of Arts and Science, Biology Department, Water Ecology Laboratory in the cold chain. It was necessary to prepare homogenate from the digestive tract to be used and to obtain cytosolic fractions to analyse the digestive enzymes. The tissues were weighed and homogenized in a 1:5 ratio with homogenization buffer (0.05 phosphate buffer pH 7.4). The specific activity of each enzyme evaluated in the study was measured spectrophotometrically. Obtained values were proportioned to the protein value in homogenate and interpreted in terms of mU / mg.protein^{-1}. For this, Bradford (1976) method was used to calculate the amount of protein.

For the measurement of trypsin enzyme activity, Tseng et al. (1982), the analysis method used in their study and Na-Benzoyl-DL-arginine-p-nitroanilide (BAPNA) was used as substrate. Enzyme activities of the samples were measured in a spectrophotometer at 253 nm wavelength for 5 minutes. Measurement of pepsin enzyme activity was performed using a revised version of the analysis method used by Worthington (1982) by Infante & Cahu (1994). Besides, bovine hemoglobin was used as a substrate. Samples were measured at a wavelength of 280 nm for 5 minutes. Monitoring the α-amylase enzyme activity depended on the study conducted by Bieth & Metais (1968) which they used soluble starch as a substrate. Samples were measured at 540 nm wavelength for 5 minutes. To measure lipase enzyme activity, α -naphthyl caprylate was used as the analysis method and substrate used in the study conducted by Versaw et al. (1989). Spectrophotometric measurement at 490 nm wavelength for 10 minutes.

2.5. Histomorphological studies

Intestinal sampling was sampled from the beginning part of the middle intestine, which is final point of section attached to the intestine of pyloric caeca. Tissue samples were taken from 6 fish with each group cut into 1.0 cm pieces and placed into 10% formalin for further processing. Then, tissue samples were carried to Kırsehir Ahi Evran University, Faculty of Agriculture, Zootechni Department to tissue processing. Tissues were placed into tissue cassettes for dehydration process and were embedded in paraffin blocks, then subsequently cut 5-µ thickness and placed on a slide. A tissue sample of each intestine was prepared and stained with hematoxylin and eosin solution by using the standard paraffin-embedding procedure. After the embedding process,

the muscularis layer, villi length and villi width were photographed with ZEISS Primostar HD Light microscope and evaluated by using an image processing and analysis system.

2.6. Enumeration of intestinal microbiota

A total of 25 fish (5 fish from each diet group and control) were used for bacterial examination. The intestinal tract of fish was aseptically removed in the Fish Health laboratory of Trabzon Central Fisheries Research Institute. A gram of digestive content was homogenized with 9 ml of 0.1% peptone-water containing 0.9% NaCl using the stomacher apparatus (BagMixer CC, Interscience). Five-fold serial dilutions of content were prepared and streaked on de Man, Rogosa and Sharpe (MRS, Merck) for the count of the lactic acid bacteria (LAB). LAB was allowed to incubate at 30 °C for 48h in anaerobic jars (Merck), (Harrigan and McCance 1976). The dilution (100µl) was also streaked on Coliform Agar (CES, Merck) and incubated for 24 h at 35 °C for the count of the total aerobic mesophilic bacteria (TAMB) and *Escherichia coli* (Ture et al. 2018). At the end of incubation, the total number of LAB, *E. coli* and TAMB were calculated by counting the colony-forming units.

2.7. Statistical analyses

Data are presented as means with standard errors. The data were statistically analysed by one-way ANOVA. Duncan's multiple range test was performed for the significance of differences of means among groups. The intestine microbiota data were log10 transformed, then analysed. The result was considered significant at $p<0.05$. Data analysis performed using SPSS 21.0.

3. Results

3.1. Growth and feed utilization

The results obtained from measurements at the end of the experiment are shown in Table 3. No differences were observed in growth performance between the control diet and fennel oil levels ($p>0.05$). Fish exposed to the 100 mg kg^{-1} fennel oil group reached a final weight of 31.26 g, but not higher statistically from the other group. Besides insignificance results among the groups, all trials had high survival rates at the end of the experiment.

Table 3- Growth parameters of Black Sea salmon fed with fennel oil supplemented diets

Performance	*Levels of fennel (Foeniculum vulgare) oil (mg kg^{-1})*					*P values*
	0	*50*	*100*	*200*	*400*	
FW	29.83±1.11	30.46±0.69	31.26±1.06	29.72±0.25	30.62±0.45	0.646
FI	23.30±0.72	23.71±0.55	24.18±0.44	23.43±0.45	23.91±0.22	0.730
WG	26.31±1.11	26.95±0.69	27.74±1.05	26.20±0.25	27.10±0.45	0.644
FCR	1.07±0.04	1.00±0.05	0.97±0.04	1.08±0.04	1.00±0.04	0.486
SGR	2.30±0.04	2.32±0.03	2.35±0.04	2.29±0.01	2.33±0.01	0.600
SR	91.85±2.67	97.04±1.48	91.11±2.22	96.30±0.74	94.82±0.74	0.133

No difference between means ($P>0.05$), values are given as means with standard errors. FW (g): Final weight, FI (g): Feed intake, FCR: Feed conversion ratio, WG (g): Weight gain, SGR (%): Specific growth rate, SR (%): Survival rate.

3.2. Enzyme status

Table 4 illustrates the results obtained from the digestion enzyme activities. Supplementation with fennel essential oil affected on pepsin and lipase enzyme activities. α- amylase activity was similar among trial groups, which was higher in fish fed 50 mg kg^{-1} fennel oil versus the control. The same observation was done for the trypsin activity, which was higher in fish fed 200 mg kg^{-1} fennel oil versus the control although there was no statistical difference among groups. Pepsin enzyme activity decreased lightly in Black Sea salmon fed with 400 mg kg^{-1} fennel oil, but it was in those fed with 50 and 200 mg kg^{-1} fennel oil that the lowest rate was determined.

Table 4- The activity of digestion enzymes of Black Sea salmon fed with fennel oil supplemented diets, U mg^{-1}

Enzymes	*Levels of fennel (Foeniculum vulgare) oil (mg kg^{-1})*					*P values*
	0	*50*	*100*	*200*	*400*	
Pepsin	$69.95±7.29^{a}$	$15.83±2.91^{b}$	$27.24±8.90^{b}$	$16.40±2.65^{b}$	$58.13±2.67^{a}$	0.000
Trypsin	34.52±5.93	30.99±0.61	35.48±3.15	44.17±5.51	22.39±3.81	0.052
Amylase	1.46±0.46	7.08±2.90	5.21±1.45	6.69±2.50	2.05±0.63	0.177
Lipase	$0.02±0.00^{bc}$	$0.04±0.00^{ab}$	$0.02±0.01^{bc}$	$0.06±0.02^{a}$	$0.01±0.00^{c}$	0.013

Means with different superscript letters in a row are significantly different at $P<0.05$, values are given as means with standard errors.

3.3. Intestine histomorphology

The results are summarized in Table 5 and shown in Figure 1. The essential oil groups except for 400 mg kg^{-1} fennel oil decreased intestinal villi length. In addition to, the intestinal villi length of the 200 mg kg^{-1} fennel oil was higher than that of the control one ($P<0.05$). Although there was no statistical difference among them in terms of villi length, it was higher in fish fed 50 and 100 mg kg^{-1} fennel oil compared to control. Moreover, adding fennel essential oil to the diet increased the muscularis layer. Muscularis layer was found to be higher for fish fed all other fennel oils except for 400 mg kg^{-1} fennel oil compared to fish fed control diet. However, supplementation with fennel essential oil did not effect on villi width.

Table 5- Intestine histomorphology of Black Sea salmon fed with fennel oil supplemented diets, µm

Histomorphology	*Levels of fennel (Foeniculum vulgare) oil (mg kg^{-1})*					*P values*
	0	*50*	*100*	*200*	*400*	
VL	223.80±5.35^{bc}	252.58±16.30^{ab}	255.57±16.36^{ab}	281.43±10.01^{a}	191.17±7.66^{c}	0.000
VW	65.15±2.88	69.67±4.65	78.72±7.26	75.78±5.65	82.14±4.32	0.145
Muscularis	44.53±1.82^{b}	57.48±4.55^{a}	56.61±2.77^{a}	57.71±2.72^{a}	51.87±1.70^{ab}	0.008
VL/VW	3.58±0.25^{a}	3.85±0.33^{a}	3.87±0.56^{a}	4.03±0.34^{a}	2.46±0.21^{b}	0.020

Means with different superscript letters in a row are significantly different at $p<0.05$, values are given as means with standard errors. VL: Villi length, VW: Villi width

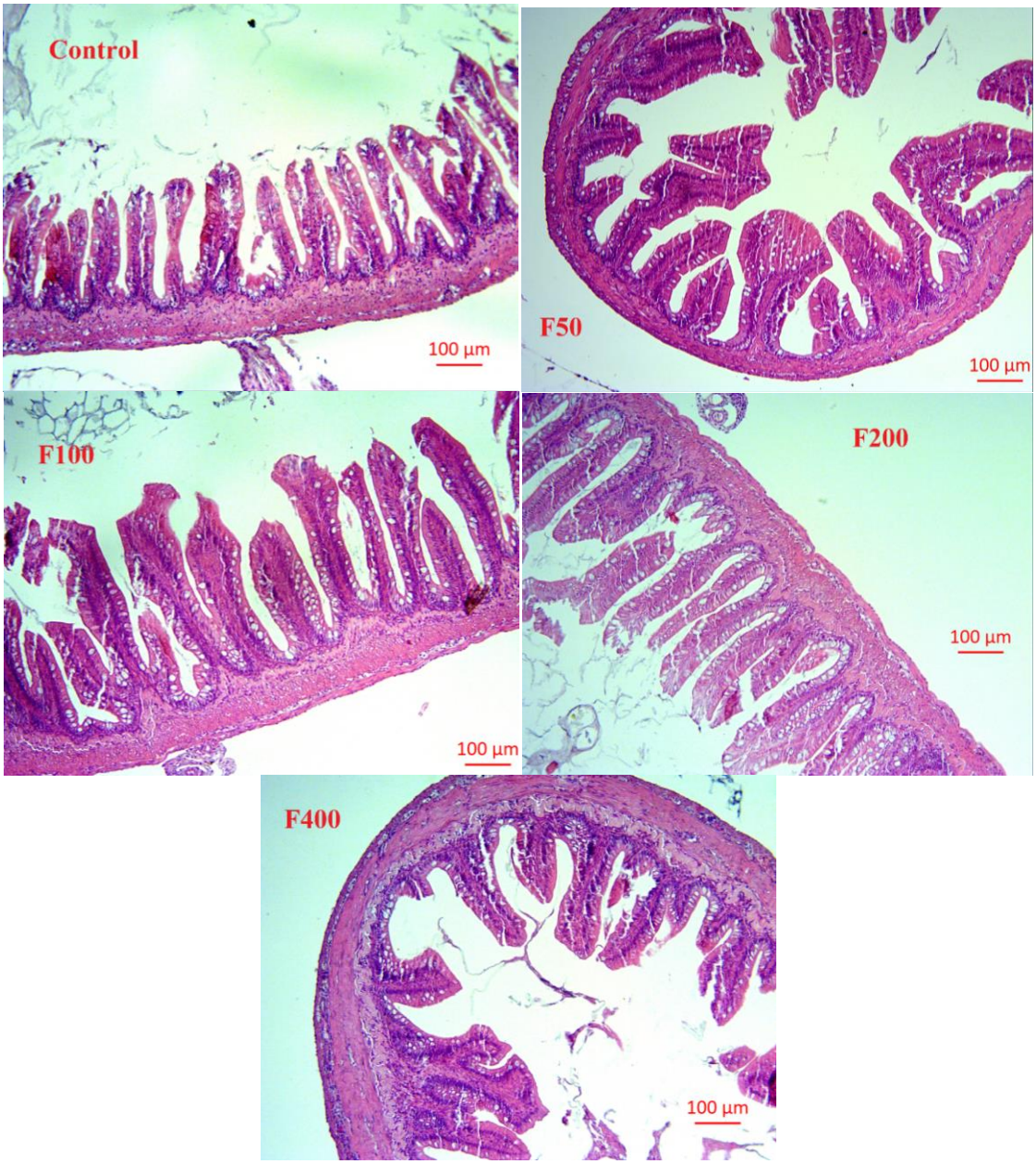

Figure 1- Intestine histology of Black Sea trout fed with fennel oil supplemented diets (4x, H&E)

3.4. Intestinal microbiota

Administration of fennel essential oil to the diet led to significant differences among the groups in terms of intestine microbiota. Feeding with a diet containing different levels of fennel essential oil decreased significantly the amount of lactic acid bacteria. The control group showed higher lactic acid bacteria than all other fennel oils. Conversely, the 200 and 400 mg kg^{-1} fennel oils showed a higher *E. coli* and coliform than the other groups. The lowest *E.coli* and coliform counts were found in fish fed 100 mg kg^{-1} fennel oil among groups. However, the lowest lactic acid bacteria were found in fish fed 400 mg kg^{-1} fennel oil (Table 6).

Table 6- Intestine microbiota of Black Sea salmon fed with fennel oil supplemented diets, CFU log/g

Microbiota	*Levels of fennel (Foeniculum vulgare) oil (mg kg^{-1})*					*P*
	0	*50*	*100*	*200*	*400*	*values*
E.Coli	5.46±0.16^{b}	4.90±0.35^{bc}	4.38±0.52^{c}	6.95±0.22^{a}	6.97±0.18^{a}	0.000
Coliform	12.47±0.15^{b}	12.07±0.30^{b}	11.36±0.55^{b}	13.86±0.39^{a}	13.96±0.45^{a}	0.003
LAB	11.05±0.05^{a}	5.29±0.10^{c}	5.98±0.09^{b}	5.28±0.15^{c}	4.58±0.18^{d}	0.000

Means with different superscript letters in a row are significantly different at P<0.05, values are given as means with standard errors.

4. Discussions

In our study, all the experimental diets were willingly consumed by fish during the trial. This has shown that fennel oil was attractive for Black Sea salmon. Abo-State et al. (2017) stated that phytogenic feed additives increase the flavour and palatability of feed. Additionally, Seden et al. (2009) stated that increased feed intake is due to high demand for nutrients or increased appetite. Phytogenic feed additives have been recognized in aquaculture as natural eco-friendly growth promoters in recent years (Abd El-Naby et al. 2019). In the present study, no effect of dietary fennel essential oil at levels 50, 100, 200 and 400 mg kg^{-1} was observed on the fish performance (P>0.05). The findings of the present study in terms of final weight, weight gain and specific growth rate are in accordance with those reported by Cruz Villeda (2013). Hassan & Soltan (2016) found that dietary supplementation of fennel oil at the level of 1 ml kg^{-1} increased the growth performance of Nile tilapia fry. Besides, Sotoudeh & Yeganeh (2016) found that feeding with dietary fennel oils (75, 100, 125 and 150 mg kg^{-1}) of convict cichlid (*Cichlasoma nigrofasciatum*) did not make a difference on growth performance (final weight, weight gain and specific growth rate) except FCR. However, our results with fennel oils were similar to the results of Mahdavi et al. (2014) found that feeding with fennel essential oil at levels of 100, 200, 400 and 600 mg kg^{-1} did not find significant differences in the growth performance of the Caspian kutum (*Rutilus frisii kutum*) fry and those of Abo-State et al. (2017) found that feeding with phytogenic feed additive containing oregano oil at levels of 1 g kg^{-1} did not find significant differences in the growth performance of the Nile tilapia (*Oreochromis niloticus*) fingerling. Being obtained different results on growth performance in the studies conducted on essential oils may change depending on essential oils or their composition and levels.

The activity of the digestive enzymes of fish is an important indicator in understanding the digestive physiology and revealing the digestion and absorption capacity of the nutrients taken (Wei et al. 2010). Digestion of nutrients begins with the activities of digestive enzymes in the stomach and continues in the intestine by trypsin, chymotrypsin, amylase and lipase enzymes secreted by the pancreas (Mohamed et al. 2018). The profiles or activity levels of digestive enzymes can be affected by size, age, origin, temperature, season and food (Hani et al. 2017). Medicinal herbs or their derivatives increase digestive enzyme secretions (Amhamed et al. 2018). In a previous study, Mohamed et al. (2018) found that trypsin, amylase and lipase activities in common carp (*Cyprinus carpio*) fed diets with 0.1%, 0.5% or 1% *Apium graveolens* extract at a level of 0.1%, 0.5% or 1% increased significantly. An additional study, De Souza et al. (2019) reported that, amylase enzyme activity in Nile tilapia increased at the level of 1 ml kg^{-1} *Ocimum basilicum* essential oil, but did not change at levels of 0.25, 5 or 2 mL kg^{-1}. The findings obtained from our study demonstrated that feeding with fennel essential oil affected activity of pepsin and lipase enzymes, but not trypsin, amylase. The highest lipase enzyme activity was determined in 200 mg kg^{-1} fennel essential oil. Moreover, our results with the amylase enzyme activity of Black sea salmon fed with fennel essential oil is in accordance with those reported by Magouz et al. (2021). Also, our results obtained on trypsin and amylase activities are similar to those reported by Heidarieh et al. (2012). The pepsin activities in the carnivorous is higher as compared to herbivorous fish. Unlike, the amylase enzyme in carnivorous fish is moderate level due to low or lack of carbohydrate intake in natural environment, and lowest than herbivorous (Natalia et al, 2004). According to Mohamed et al. (2018), trypsin activity in common carp (*Cyprinus carpio*) fed with garlic (*Allium sativum*) powder was not higher than amylase and lipase. Our study showed that trypsin enzyme activity was higher than amylase and lipase in all the trial groups. A similar result was seen in those obtained with the administration of *Apium graveolens* extract in Japanese seabass, *Lateolabrax japonicus* (Xu et al. 2019).

The small intestine is the primary site for absorption of nutrients, and thus playing a vital role in fish growth (Abdel-Latif et al. 2020). Intestinal villi play an important role in the digestion and absorption of nutrients exposed to digestive enzymes (Munglue 2016). In fish, the healthy intestinal tract can improve growth performance and gut health. In general, high intestinal villi play an important role in the feed efficiency and absorption of nutrients (Alagawany et al. 2020). The findings of our study about the intestinal morphology demonstrated that the addition of fennel essential oil to the diet increased the thickness of the

muscularis, but had no effect on intestinal villi width. Besides, intestinal villi length was enhanced in levels in except for 400 mg kg^{-1} fennel essential oil. Valladao et al. (2019) found that, in Nile tilapia, supplementation with thyme essential oil did not alter the intestinal morphology including villi height and width. According to Munglue et al. (2019), intestinal villi length and width in catfish (*Clarias macrocephalus* × *Clarias gariepinus*) fed with the diet containing rice paddy herb (*Limnophila aromatica*) extract were significantly higher than those of the control. Similarly, Addam et al. (2018) found that villi length in the anterior intestine significantly enhanced in Nile tilapia fed *Lippia origanoides* essential oil diet. In fish, intestinal villi height is related to the digestive and absorptive functions of the intestines (Munglue & Dasri 2015). In our study, the increased villi length may cause improve absorption of nutrients as stated by Alagawany et al. (2020), the increase of the intestinal villi height and width may increase the surface area for absorption. Also, in terms of intestinal villi width, our results are harmonious with those of reported by Addam et al. (2018). In Black sea salmon, muscularis layer decreases gradually from the beginning of the anterior intestine to the end of the posterior intestine (Özel et al. 2019). One of the functions of this structure contribute to the mixing of feed with digestive enzymes (Mumford et al. 2007). In the posterior intestine, also, enhanced muscularis thickness assists defecation and moisture reabsorption (Munglue 2016). Our results with fennel essential oil were similar to those reported by Munglue (2016), dietary supplementation of lotus (*Nelumbo nucifera*) stamen extract enhanced muscularis thickness in anterior and posterior intestines in the Catfish (*Clarias gariepinus*).

In fish, the intestinal microbiota is influenced by genetic, nutritional, microbiological and environmental factors. Among these factors, the diet one of the main factors responsible for changes in bacterial diversity of the digestive tract (Sutili et al. 2017). Diet ingredients are important for the composition and activity of gut microbiota in fish (Yusuf et al. 2017). According to Giannenas et al. (2012), the inclusion of phytogenic substances to the diet can affect the intestine populations of trout. However, little is known about the antimicrobial effect of essential oils in fish (Cruz Villeda 2013). Findings of our study showed that feeding with dietary supplemented fennel essential oil had a significant effect on intestinal microbial activity including *E.coli*, coliform and lactic acid bacteria. According to Yusuf et al. (2017), the inclusion of 4 g kg^{-1} fumaric to the diet in juvenile tilapia (*Oreochromis niloticus*) increased the lactobacillus count and decreased the fecal coliform. In our study, suppressive effect on lactic acid bacteria of diets with fennel essential oil can be explained according to Alagawany et al. (2020), the antimicrobial effect of essential oils is depended to the effective and phenolic substrates obtained from some herbs. Lactic acid bacteria are not among the dominant bacterial communities due to found in low abundance in fish intestine, but can have positive effects on fish health and disease resistance due to can inhibit the growth of pathogenic bacteria (Hoseinifar et al. 2019). Findings of our study shown that fennel essential oil had a decreasing effect on intestinal lactic acid bacteria in the Black Sea salmon. Besides, diets supplemented with 200 and 400 mg kg^{-1} fennel oil increased *E.coli* and coliform counts. This result may be due to antimicrobial, antifungal and antibacterial activities of fennel essential oil as stated by Sotoudeh & Yeganeh (2016).

5. Conclusions

Results of our study showed that feeding the juvenile Black Sea salmon with fennel essential oil had not an effect on growth performance including feed intake, final weight, weight gain, feed conversion ratio and specific growth rate. Dietary supplementation of fennel oil affected the enzymes except for trypsin and amylase. Feeding with 200 mg kg^{-1} fennel oil increased lipase activity. Moreover, feeding with 50, 100 and 200 mg kg^{-1} fennel essential oil may have the potential to increase the surface area required for digestion by increasing intestinal villi length. Dietary supplemented fennel essential oil showed antimicrobial properties by decreasing lactic acid bacteria count. Similarly, 50 and 100 mg kg^{-1} fennel oil had the same effect on *E.coli* and coliform counts. In aquaculture, the number of studies on the possible effects of essential oils on the digestive physiology of fish is quite limited. Detailed studies are required to better understand of the mechanism of action of these oils in fish.

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Data Availability Statement

Research data are not shared. All the related data has been given with the article.

Animal Welfare Statement

The authors confirm that the ethical policies of the journal have been adhered to and the appropriate ethical review committee approval has been received from the Animal Ethics Committee of Central Fisheries Research Institute, Turkey (application number ETIK-2017/1). The authors confirm that they have followed EU standards for the protection of animals used for scientific purposes and feed legislation.

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