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Glutathione and Proline Attenuates Injury Induced by Boron Toxicity in Wheat

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ABSTRACT

Given the increasing importance of boron (B) toxicity, the present study investigates the roles of glutathione (20 mM, GSH) and proline (20 mM) in the improvement of wheat (*Triticum aestivum* cv. Altındane) resistance to B toxicity (10 mM B). The plants were raised in hydroponic culture with control, B toxicity, B+glutathione, B+proline, glutathione and proline. B+glutathione and B+proline resisted the detrimental influences of B toxicity on the root and shoot lengths, the total chlorophyll, and phenolic contents. B toxicity increased superoxide radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), lipid peroxidation (MDA), and proline contents while B+glutathione and B+proline applications diminished the mentioned parameters with the exception of the proline content. Individual B toxicity and combined B+glutathione and B+proline applications increased generally total ascorbic acid and glutathione levels in the wheat while the B+proline application decreased GSH content. The B toxicity decreased superoxide dismutase, catalase and guaiacol peroxidase activities in compared with control with the exception of the ascorbate peroxidase activity. Exogenous glutathione and proline augmented all enzyme activities in the wheat exposed to B toxicity. As a result, it can be suggested that glutathione and proline mitigates B toxicity; by preventing oxidative damage in the membrane, by increasing enzymatic and non-enzymatic antioxidant and by decreasing $O_2^{\cdot-}$, H_2O_2, and MDA contents. Glutathione is generally more effective than proline in mitigating the above detrimental effects of B toxicity. The datum submitted in the current work are significant and the first to indicate that effects of exogenous glutathione and proline in improving a culture plant strength to B toxicity.

Keywords: Antioxidants, B toxicity, Glutathione, Proline, Wheat

1. Introduction

Boron (B) toxicity is a noticeable agricultural problem that limits crop productivity in different regions of the world. It can occur in B-rich soils or in soils exposed to B-rich irrigation waters, fertilizers, sewage sludge, or fly ash (Cervilla et al. 2012; Çapar et al. 2016; Nable et al. 1997). B is a unique essential micronutrient (Marschner 1995) that has a narrow concentration between its deficiency and toxicity. B toxicity causes phytotoxicity in plants and considerably decreases crop productivity and quality worldwide; Peru, Chile, Iraq, California, India, Israel, South Australia, West Asia, Morocco, Egypt, Malaysia Jordon, North Africa, Libya, Syria and Turkey (Yau & Ryan 2008).

A thiol tripeptide glutathione (with the formula γ- L-glu- L-cys-gly; a crucial multifunctional metabolite in plants) has been localized and measured in mitochondria, chloroplasts, peroxisomes, the apoplast, and vacuoles of different plant species (Zechmann 2014). Glutathione is a precursor of phytochelatins and is involved in several physiological processes; the arrangement of growth, development and cell cycle regulation, enzymatic regulation and pathogen resistance, abiotic stress tolerance, detoxification of xeneobiotics and heavy metals, protection of thiol groups, regulating the expression of stress defence genes and signaling for sulfur metabolism, regulation of sulfate transport, signal transduction and conjugation of metabolites (Jozefczak et al. 2012; Hasanuzzaman et al. 2019).

Under stress conditions, proline as an excellent osmolyte accumulates in the cytosol. Proline has acts as an antioxidative defense molecule, a metal chelator and a signaling molecule (Xiong & Zhu 2002).

There are some applications, such as phytoremediation and the effective management of water and vegetation that can prevent the harmful effects of B toxicity (Balal et al. 2017; El-Shazoly et al. 2019). As an alternative approach, the application of stimulants such as glutathione and proline enhances oxidative defenses and increase B toxicity-tolerance in plants. For this purpose, this research sought to understand the effects of exogenous glutathione and proline applications on the growth (root-shoot), proline, total chlorophyll and phenolic contents, and oxidant ($O_2^{\cdot -}$, H_2O_2, MDA) accumulation, non-enzymatic [ascorbic acid (AsA) and GSH] and enzymatic antioxidative system [superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX) and ascorbate peroxidase (APX)] in the wheat grown under the oxidative stress related with B toxicity. Additionally, this study provides a strategy to maintain sustainable and friendly production and to provide tolerance to B toxicity-exposed plants. As far as we know, this is the first study demonstrating the effects of exogenous glutathione and proline in the improvement of wheat resistance to B toxicity. Silva et al. (2016) (Proline but not glutathione actively participates in the tolerance mechanism of young *Schizolobium parahyba* var. amazonicum plants exposed to B toxicity) don't investigates effects of exogenous glutathione and proline in their study.

2. Material and Methods

Before germination, the wheat seeds (*Triticum aestivum* cv. Altındane) were surface sterilized in ethyl alcohol (95%) for 2 min and then transferred to sodium hypochlorite activated with 1% Cl for 10 min and washed with sterile dH_2O. The seeds were grown in control conditions for 11 days in a hydroponic culture system in which each container was filled with 2.8 L of Hoagland and Arnon nutrient solution. The hydroponic systems were continuously aerated with an air pump. The pH of hydroponic growth mediums was adjusted to 6.0. On the 11th day, the entire foliar region of the plants was foliar sprayed with glutathione (20 mM) and proline (20 mM) (an approximate volume of 2 mL) or simply with distilled water as a control (repeated three times at 2-h intervals). All the experiments were kept in a growth room at 22±2 ^{o}C under fluorescent white light (100 µmol m^{-2} s^{-1} at leaf level) with a 14-h light/10-h dark photoperiod. Afterward the plants were exposed to B toxicity stress [10 mM, boric acid (H_3BO_3)] for 3 days (preliminary studies showed that 20 mM glutathione and proline solutions were optimum to increase B tolerance in the wheat seeds). On the 14th day after the treatment began, all the plants were harvested (the tissues were rinsed three times in distilled water after harvesting) and analysed.

The total chlorophyll content was assayed according to Witham et al. (1971). The total soluble phenolic contents were determined according to Dewanto et al. (2002). Proline was determined according to the method described by Bates et al. (1973). Each experiment was repeated at least three times.

The superoxide level was measured according to Elstner & Heupel (1976). Sodium nitrite was used as a standard solution to calculate the production rate of superoxide anion. The hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) contents were measured according to Loreto & Velikova (2001). The MDA level was calculated using an extinction coefficient of (ε=155 mM^{-1} cm^{-1}) and was expressed as µmol g^{-1} fresh mass.

For the enzyme assays (SOD, CAT, GPX and APX), the leaf tissues (0.5 g) were homogenized in liquid nitrogen, and 5 mL 10 mmol L^{-1} K–P buffer (pH 7.0) containing 4% (w/v) polyvinylpyrrolidone and 1 mmol L^{-1} disodium ethylenediaminetetraacetic acid was added. The homogenates were centrifuged at 12.000×g and 4 ^{o}C for 15 min, and the supernatant was used to determine enzymes activities. The activity of SOD was assayed by monitoring the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) according to the methods of Agarwal & Pandey (2004). One unit of SOD (unit mg^{-1} protein min^{-1}) was defined as the amount of enzyme activity that inhibited the photoreduction of NBT to blue formazan by 50%. CAT activity was measured at A_{240} for H_2O_2 decomposition rate using the extinction coefficient of (ε=40 mM^{-1} cm^{-1}) according to the method of Gong et al. (2001). One unit of CAT activity (µmol H_2O_2 mg^{-1} protein min^{-1}) was assumed to be the amount of enzyme that decomposed 1 nmol of H_2O_2 per mg of soluble protein per minute. GPX activity was determined in the homogenates by measuring the increase in absorption, and colour development at 470 nm due to the guaiacol (hydrogen donor) oxidation was recorded for 5 min, as described by Yee et al. (2002). The GPX activity (µmol g'col mg^{-1} protein min^{-1}) was estimated by the increase in absorbance of oxiguaiacol at 470 nm (extinction coefficient of ε = 26.6 mM^{-1} cm^{-1}) and was expressed as nmol of guaiacol consumed per mg of soluble protein per minute. APX activity was determined by monitoring the decrease in absorbance at 290 nm as reduced AsA was oxidized (extinction coefficient of ε = 2.8 mM^{-1} cm^{-1}) according to the method described by Nakano & Asada (1981). The APX activity (µmol AsA mg^{-1} protein min^{-1}) was calculated as the amount of enzyme that oxidizes 1 nmol of ascorbate consumed per mg of soluble protein per minute (ε=2.8 mM^{-1} cm^{-1}).

Tissue samples (0.2 g) were powdered in liquid nitrogen and then 2 mL of 5% (w/v) trichloroacetic acid was added and homogenized. After centrifugation at 12.000×g for 10 min at 4 ^{o}C, the supernatant was collected to determine the total AsA and GSH contents. The total AsA content (AsA+DHA) was estimated as described by Mukherjee & Choudhuri (1983). The reduced total GSH (µmol g^{-1})

content was determined according to Griffith (1980). The levels of GSH were estimated as the difference between total GSH and oxidised glutathione (GSSG).

The experiment was organized as a completely random design with three replications. All data obtained were subjected to a two-way analysis of variance (ANOVA) and the significant differences between treatment means were determined by the Duncan multiple range test using the SPSS 20.0 to separate the means. Data are shown as means with three replicates and the significance was determined at the 95% confidence (α=0.05) limits.

3. Results

As shown in Figure 1 and Table 1, B toxicity significantly inhibited the root and shoot lengths of the wheat seedlings by approximately 13.00% when compared to the control. Glutathione and proline applications reverted these B toxicity-based inhibitions to a degree of 8.36% in glutathione and 7.86% in proline. Individual glutathione and proline showed an important increase in the growth parameters compared to their control by 9.05% in glutathione and 2.73% in proline.

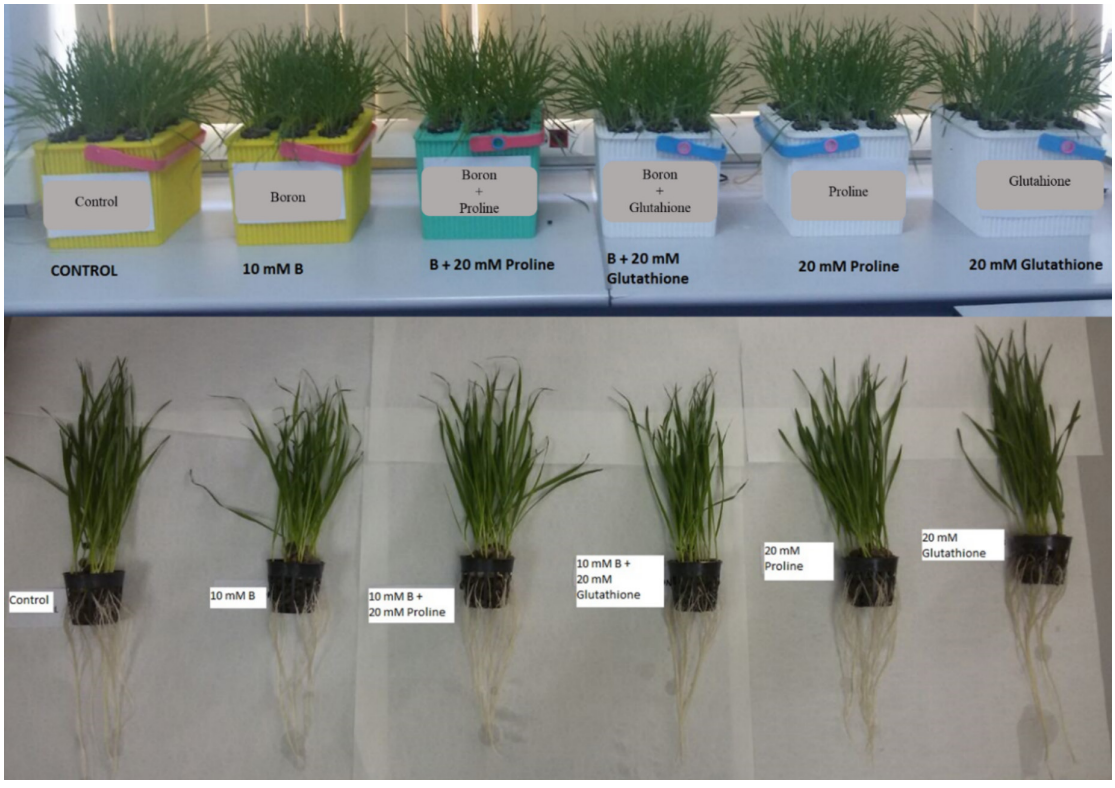

Figure 1- Effects of glutathione and proline on root-shoot lengths on the 14th day the wheat had been exposed to B toxicity in hydroponic culture

The B toxicity markedly reduced the total chlorophyll content by 26.65% compared to the control (Table 1). Glutathione and proline applications remarkably inversed the B toxicity-based inhibition in total chlorophyll content by 22.69% in glutathione and 14.15% in proline. Similarly, individual glutathione and proline applications resulted in significant a rise in total chlorophyll content by 6.88% in glutathione and 2.11% in proline when compared with the control.

The B toxicity markedly reduced total the phenolic substance by 5.61% compared to the control (Table 1). Glutathione and proline applications remarkably inversed the B toxicity-based inhibition in total phenolic substance by 19.80% in glutathione and 26.73% in proline. Similarly, individual glutathione and proline applications resulted in a noticeable elevation in total phenolic substance by 2.80% in glutathione and 11.2% in proline when compared to the control.

The B toxicity remarkably boosted proline content by 40.79% compared to the control (Table 1). B+glutathione and B+proline applications significantly more increased the proline content by 19.63% in glutathione and 36.45% in proline. Similarly, individual

glutathione and proline applications caused an increase in proline content by 3.95% in glutathione and 28.95% in proline when compared to the control.

Table 1- Effects of glutathione and proline on root-shoot lengths, total chlorophyll, total phenolics and proline contents on the 14th day of the wheat exposed to B toxicity in hydroponic culture

Treatments	*Root lengths (cm)*	*Shoot lengths (cm)*	*Total chlorophyll (mg.g^{-1} FW)*	*Total phenolics (µg.g^{-1} FW)*	*Proline (µg.g^{-1})*
Control	20.87±0.55^{c}	22.11±0.34^{c}	7.48±0.25^{c}	10.7±0.34^{e}	76±70.0^{f}
B toxicity	18.06±0.32^{e}	19.24±0.55^{f}	5.48±0.40^{e}	10.1±0.43^{f}	107±5.4^{c}
B+Proline	19.48±0.18^{d}	20.56±0.23^{e}	6.26±0.15^{d}	12.8±0.28^{a}	146±6.6^{d}
B+Glutathione	19.57±0.34^{d}	21.44 ±0.30^{d}	6.73±0.26^{d}	12.1±0.14^{b}	128±5.8^{b}
Proline	21.44±0.47^{b}	23.06±0.36^{b}	7.63±0.40^{b}	11.9±0.10^{c}	98±3.9^{a}
Glutathione	22.76±0.66^{a}	24.46±0.66^{a}	7.99±0.33^{a}	11.0±0.18^{d}	79±4.6^{e}

Data are the means ± standard deviation of three independent replicates. The different small letters indicate significant differences at $p<0.05$ according to Duncan's multiple range test at $p<0.05$

As determined in Table 2, $O_2^{\cdot-}$ production and H_2O_2 level were significantly increased by B toxicity respectively 35.50% and 63.76% compared to control in wheat while glutathione and proline applications significantly reduced B toxicity-induced increases in these parameters (12.75% and 28.63% in glutathione and 8.42% and 21.40% in proline, respectively). Similarly, individual glutathione and proline applications caused decreases in $O_2^{\cdot-}$ production and H_2O_2 levels by 1.27% and 12.27% in glutathione and 5.55% and 5.32% in proline, respectively when compared to the control.

When compared with the control, the MDA content aggressively increased up to 27.44% under the B toxicity. However, glutathione and proline applications reduced MDA content by 8.08% and 5.92% ratio, respectively when compared to B toxicity (Table 2). Similarly, individual glutathione and proline applications caused decreases in MDA content by 7.55% in glutathione and 5.49% in proline when compared to the control.

As indicated in Table 2, B toxicity significantly augmented the total AsA and GSH amounts by 2.21-fold, and 46.07%, respectively. B+glutathione and B+proline applications significantly more increased total AsA and GSH contents by 26.42% and 17.10% in glutathione and 14.40% and 7.88% in proline, respectively when compared to the control. Similarly, individual glutathione and proline applications significantly increased the total AsA and GSH contents by 52.45% and 22.73% in glutathione, and 83.04% and 9.37% in proline, respectively.

Table 2- Effects of glutathione and proline on superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), lipid peroxidation (MDA), total AsA and GSH contents on the 14th day the wheat exposed to B toxicity in hydroponic culture

Treatments	*$O_2^{\cdot-}$ (nmol.min g^{-1})*	*H_2O_2 (µmol g^{-1})*	*MDA (nmol ml^{-1})*	*Total AsA (nmol g^{-1})*	*Total GSH (nmol g^{-1})*
Control	6.31±0.24^{d}	23.48±1.00^{d}	5.83±0.28^{c}	572±46^{f}	1782±89^{f}
B toxicity	8.55±0.28^{a}	38.45±1.20^{a}	7.43±0.22^{a}	1264±68^{c}	2603±111^{b}
B+Proline	7.83±0.21^{b}	30.22±0.80^{b}	6.99±0.15^{b}	1446±54^{b}	2398±73^{c}
B+Glutathione	7.46±0.24^{c}	27.44±0.60^{c}	6.83±0.19^{b}	1598±77^{a}	3048±146^{a}
Proline	5.96±0.44^{e}	22.25±1.20^{e}	5.51±0.36^{d}	1047±39^{d}	1949±51^{e}
Glutathione	6.23±0.56^{d}	20.60±0.94^{f}	5.39±0.24^{e}	872±74^{e}	2187±99^{d}

GSH: Glutathione, AsA: Ascorbic acid. Data are the means ± standard deviation of three independent replicates. The different small letters indicate significant differences at $p<0.05$ according to Duncan's multiple range test at $p<0.05$

As shown in Table 3, B toxicity remarkably reduced SOD, CAT and GPX activities by 29.4%, 18.2% and 36.2% respectively while increasing APX activity by 52.2%. B+glutathione and B+proline applications remarkably augmented SOD, CAT, GPX and APX activities by 18.1%, 5.6%, 27.4% and 41.3% in glutathione, and 26.2%, 8.8%, 14.1% and 23.0% in proline, respectively when compared

to the control. Similarly, individual glutathione application increased SOD and APX activities by 8.3% and 24.8% and decreased CAT and GPX activities by 8.2% and 21.1%, respectively. Individual proline applications increased SOD and APX activities by 5.6% and 10.1% and decreased CAT and GPX activities by 3.4% and 14.7%, respectively.

Table 3- Effects of glutathione and proline on the enzymatic antioxidative system (SOD, CAT, GPX and APX enzyme activities) on the 14th day the wheat exposed to B toxicity in hydroponic culture

Treatments	*SOD (unit mg^{-1} protein min^{-1})*	*CAT (µmol H_2O_2 mg^{-1} protein min^{-1})*	*GPX (µmol g'col mg^{-1} protein min^{-1})*	*APX (µmol AsA mg^{-1} protein min^{-1})*
Control	27.46±1.00^{c}	39.46±1.20^{a}	1452±79^{a}	14.09±2.30^{f}
B toxicity	19.38±0.90^{f}	32.28±0.78^{f}	926±42^{f}	21.44±1.40^{e}
B+Proline	24.46±1.60^{d}	35.11±1.28^{d}	1057±54^{e}	26.37±1.10^{b}
B+Glutathione	22.89±1.40^{e}	34.07±1.14^{e}	1180±66^{c}	30.30±3.50^{a}
Proline	28.99±2.10^{b}	38.12±2.00^{b}	1239±75^{b}	15.52±1.90^{e}
Glutathione	29.75±1.30^{a}	36.22±1.60^{c}	1145±38^{d}	17.58±1.00^{d}

SOD: Superoxide dismutase, CAT: Catalase, GPX: Guaiacol peroxidase, APX: Ascorbate peroxidase. Data are the means ± standard deviation of three independent replicates. The different small letters indicate significant differences at $p<0.05$ according to Duncan's multiple range test at $p<0.05$

4. Discussion

In this study, the effect of B toxicity on physiological and biochemical parameters in wheat was investigated in the presence and absence of glutathione and proline supplementation. The B toxicity significantly reduced the root and shoot growth of wheat compared to the control (Figure 1 and Table 1). The reduction in the growth of root and shoot in many plants is the typical syndrome of plants exposed to B toxicity (Landi et al. 2013; Seth & Aery 2017; El-Shazoly et al. 2019). The results are coherent with previous studies demonstrating that B toxicity reversely affects plant fruitfulness by disrupting membrane stability, photosynthetic pathways, photosynthetic pigments and the generation of reactive oxygen species (ROS) (Seth & Aery 2017; El-Shazoly et al. 2019). B toxicity can delay elongation and cell division by binding to ATP and NADPH thereby disturbing their proper working in plant metabolism (Cervilla et al. 2012). B can cause metabolic disruption, also an inhibition and/or dysfunction of the enzyme, and disruption of cell division, and elongation. Exogenous glutathione and proline notably decreased B toxicity-induced inhibitions in the root-stem lengths. The positive effects of glutathione and proline applications on wheat growth occurred not only in the existence of B toxicity but also in non-stressed wheat. The highest valuations in root-shoot lengths were registered at individual glutathione and proline applications (Figure 1 and Table 1). Exogenous GSH application improved the germination and growth of Arabidopsis, tobacco, and pepper under mercury (Hg) stress. Exogenous GSH also conferred Cd, Cu and Zn stress tolerance (Hasanuzzaman et al. 2019). Anjum et al. (2015) reported that plants tailor to excessive situations of abiotic stresses either by synthesizing S-rich complexes, such as reduced glutathione or by osmotic arrangement owing to proline accumulation (proline acts as a nitrogen welding in the course of the plant growth).

B toxicity decreased the total chlorophyll content in the wheat leaves (Table 1). Other studies have also recorded that B toxicity reduces carotenoid, chlorophyll, biomass contents, and the internal carbon dioxide (CO_2) concentration in some plants (El-Shazoly et al. 2019; Silva et al. 2016). B toxicity can cause oxidative stress by producing excess ROS or diminishing pigment biosynthesis through different mechanisms, including altering the enzyme activities and limiting the uptake of elements, thereby decreasing the total chlorophyll content (Catav et al. 2022). The above-mentioned effects are not only related to a specific target of B toxicity at the cellular level but also are the monitored replies of the capability of B to compose complexes to molecules that are involved in different cellular processes. Glutathione and proline applications reversed considerably the B toxicity-based decreases in total chlorophyll content in the presence and absence of B toxicity. Glutathione and proline can contribute to increased stability of the thylakoid membranes and plastid biogenesis. Glutathione and proline can preserve membranes from the destructive effect of B toxicity by increasing the enzyme activities or through overexpression of some responsible genes in photosynthesis, or by maintaining the photosynthetic device (Xia et al. 2009).

Phenolics have multiple biological effects such as antioxidant activity and the markers of stress in plants. B toxicity decreased the total phenolic content, but exogenous glutathione and proline applications increased the total phenolic content in all applications (Table 1). Phenolics can behave as a direct antioxidant, an absorption strainer for radiation, and can restrict the stimulation of chlorophyll under

stress terms for the photosynthetic device (Cervilla et al. 2012). GPX activity and phenolic content decreased in the B toxicity although glutathione and proline applications increased both of them (Tables 1, 3). GPX activity is one of the most "noticeable signs" of the actuation of phenolic metabolism under B toxicity. Exogenous glutathione and proline can augment the pentose phosphate pathway, which protects a high percentage of reduced antioxidants like glutathione for the scavenging of ROS and supply erythrose-4-phosphate for the biosynthesis of phenolic compounds (Lu et al. 2014; Mishra & Heckathorn 2016).

Proline maintains cellular homeostasis and rehabilitates plant toleration under abiotic stresses by scavenging ROS and stabilizing protein structure (Seth & Aery 2017; Catav et al. 2022;). To analyze the relationship between exogenous glutathione and proline applications and B toxicity tolerance, we examined the stress-stimulated proline backlog and the impacts of exogenous glutathione and proline. In this study, B toxicity enhanced the quantity of proline in the wheat (Table 1), This increase may be due to augmented proline biosynthesis and protein degradation and/or the decreased degradation of proline, or by inducing osmotic stress (Seth & Aery 2017; Catav et al. 2022;). Exogenously proline application has been used as a joint way to provide more proline welds and enhancement proline accumulation. That proline increase under B toxicity is significant since a decline in proline can lead to greater lipid peroxidation (MDA) (hence, membrane damage) (Molassiotis et al. 2006). Proline is constantly associated as a ROS antagonist that decreases oxidative stress. Proline also inhibits apoptosis-like cell death. Proline protects the protein structure against denaturation and strengthens the cell membranes during interaction with phospholipids (Silva et al. 2016). Similar results have also been noted in how toxic B concentrations enhanced proline content in peppers and tomatoes (Eraslan et al. 2007). Our study suggests that proline and photosynthetic pigments are both synthesized from a similar substrate. In this way, the decrease in chlorophyll content under the B toxicity could be due to an increase in proline accumulation (Balal et al. 2017). Exogenous glutathione and proline applications also further increased the proline content in all applications (Table 1). In our study, the improved toleration to B toxicity was correlated with an increase in the activities of antioxidant enzymes and in the proline accumulation in wheat like other abiotic stresses, particularly drought and salt. Previous studies also reported that proline accumulation is related to stress toleration under abiotic stresses (Balal et al. 2017; Khan et al. 2015). Exogenous glutathione and proline applications can increase abiotic stress tolerance by mitigating the adverse effects of ROS, preventing MDA in the membranes, and by reducing MDA content in wheat. The effects of glutathione and proline on the reduction of B stress were also partially due to the stimulatory impact on the proline accumulation. Proline can be used by plants as a source of endogenous nitrogen to strengthen the structure of the protein, enzymes, and photosynthetic apparatus and maintain cellular homeostasis under conditions of abiotic stress (Seth & Aery 2017). Thus, glutathione and proline applications may be a possible cause of increased proline accumulation in response to glutathione and proline in B toxicity. It was concluded that the role of proline as a free radical scavenger is more important than simply as an osmolyte in stress reduction (Hong et al. 2000).

To monitor the oxidative damage in wheat under B toxicity, MDA content as used as an indicator of oxidative stress in the different abiotic stresses was calculated (Aghaleh et al. 2011). Our study has shown that MDA content was increased significantly by B toxicity (Table 2). With respect to the existing outcomes, some researchers declared that B toxicity increased MDA content (Balal et al. 2017; El-Shazoly et al. 2019). An increase in MDA content in exposed and non-exposed to B toxicity was alleviated by exogenous glutathione and proline applications decreasing the production of extremely disruptive free radicals (that is by decreasing the $O_2.^-$ and H_2O_2 contents).

The phytotoxicity induced by B toxicity causes the generation of ROS that is required for different biological processes in plants, including cellular proliferation, stress acclimation, and signal transduction (Catav et al. 2022). B toxicity significantly increased $O_2.^-$ production together with enhanced H_2O_2 content in wheat (Table 2). Some researchers also found identified increases in the MDA and H_2O_2 contents and electrolyte leakage in reply to B toxicity (Balal et al. 2017; El-Shazoly et al. 2019). The increased $O_2.^-$, H_2O_2 and MDA levels by B toxicity showed antioxidative systems could not bring concentrations of ROS within normal ranges (at a steady and secure grade for plant oxidative stress). For this reason, glutathione and proline applications may be appropriate for the plants to scavenge extreme ROS and to hinder MDA. Glutathione and proline applications in exposed and non-exposed to stress B toxicity have reduced the $O_2.^-$, H_2O_2, and MDA quantities while augmenting the activities of antioxidant enzymes, suggesting that these two compounds may play a role in the ROS quenching or stopper of ROS production. Our results support those of previous studies (Hasanuzzaman et al. 2019).

The total AsA and GSH amounts as non-enzymatic antioxidants found in chloroplast and cellular compartments significantly increased in B toxicity when compared to the control (Table 2). Cervilla et al. (2007) recorded AsA and GSH intensified with an increment in the B concentration in the culture ambiance, and Mittler (2002) recorded that its concentrations differ in the many abiotic stress conditions. Our results support previous studies that also indicated an increment in total GSH level in pear leaves under B-toxicity (Wang et al. 2011). Some researchers declared that the upregulation or overexpression of AsA-GSH pathway enzymes

and the enhancement of the AsA and GSH levels conferred plants better tolerance to abiotic stresses by reducing the ROS ($O_2^{\cdot-}$ and $OH^{\cdot}$) (Hasanuzzaman et al. 2019). Although defensive measures against stress occasionally occur regardless of glutathione, an increase in the GSH content is interrelated with the capability of plants to stand against B stress-induced oxidative stress (Foyer & Noctor 2011). B+glutathione and B+proline applications significantly increased the total AsA and GSH contents with respect to B toxicity alone (Table 2). The exogenous application of proline upregulates the enzyme activities in the AsA-GSH cycle (such as APX in this study). A multifunctional metabolite GSH is a crucial transport and repository form of non-protein reduced sulphur and has a protective role against the protein degradation sourced by the oxidation of protein thiol groups. GSH also has a key role in intracellular antioxidative defense and protection mechanisms by regenerating ascorbic acid via the AsA-GSH cycle during stress in plants (Hasanuzzaman et al. 2019). In addition, some researchers reported that increasing GSH biosynthesis augmented cadmium and nickel stress toleration in the various plants, and the Arabidopsis mutant that produces less GSH was hypersensitive in both cadmium and copper stresses (Yadav 2010). Increased antioxidant activity resulting from GSH accumulation may preserve many photosynthetic enzyme activities in B toxicity. In this study, less oxidative damage was reported in glutathione and proline applicated plants by increasing non-enzymatic compounds (total AsA and GSH). Finally, the stimulation of GSH synthesis like AsA as the strongest ROS scavenger could prevent B toxicity. These results are partially in accord with Ruiz et al. (2003) that B stress decreased glutathione accumulation in the sunflower leaves but an external application of GSH diminished the harmful effects stimulated by B toxicity.

B toxicity decreased SOD, CAT and GPX activities but increased APX activity in wheat plants (Table 3). The response of enzymatic antioxidants against to B toxicity significantly relies on the species and stresses. A decrease in SOD activity in the B toxicity shows that detoxification of $O_2^{\cdot-}$ radicals by SOD under B toxicity is not enough. In addition, the decreases in CAT and GPX activities suggest that these enzymes are unable to completely detoxify H_2O_2 generated by B toxicity. Our results don't support the previous results that found increases in SOD, CAT and GPX activities in the different plants (Molassiotis et al. 2006; Balal et al. 2017; Sent & Aery 2017). Similarly, an increment of APX activity was observed in citrus while a decline in CAT activity was registered after B toxicity in diverse plants (Oluk et al. 2012). In this study, elevated APX activity (has a higher affinity for H_2O_2) likely aided in maintaining the H_2O_2 amount at normal levels. However, exogenous glutathione and proline applications and individual glutathione and proline treatments enhanced SOD, CAT, GPX and APX activities (Table 3). Exogenous glutathione and proline provide protection by boosting the SOD, CAT and GPX activities against to B toxicity. The increased SOD activity with B+glutathione and B+proline applications may enhance the ability to scavenge $O_2^{\cdot-}$ reducing membrane damage in the wheat. Exogenous glutathione and proline applications suppress H_2O_2 accumulation accompanied by a rise in the CAT and GPX activities. An increase in GPX activity demonstrates its role as a defensive measure to counteract B toxicity-induced oxidative damage in wheat. Thus, we observed that CAT, GPX and APX enzymes in the wheat with B+glutathione and B+proline were successfully detoxified H_2O_2 induced by B toxicity. Other studies have also found that exogenous proline applications significantly enhanced these enzyme activities in some stress conditions (salinity and Cd stresses) (Hayat et al. 2012). Proline is known to act as an enzyme protector under abiotic stress conditions. The roles of glutathione and proline in preventing the deleterious effects of B toxicity can be the result of the activation of key antioxidant enzymes mediated owing to the arrangement at the levels of transcriptional, translational and/or enzyme activities. The results of this and previous studies demonstrate that plants are needed an efficient antioxidative system to protect from oxidative damage and to increase resistance to environmental stresses (Cervilla et al. 2012; Hasanuzzaman et al. 2019).

5. Conclusions

This study suggests that glutathione and proline confer toleration to B toxicity in the wheat by boosting the SOD, CAT, GPX and APX activities and decreasing the $O_2^{\cdot-}$, H_2O_2, and MDA amounts. Therefore, this work provides an efficient eco-friendly route for farmers to minimize the B-toxicity worldwide.

Data availability: Data are available on request due to privacy or other restrictions.

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Analysis of the Relationships Between Agricultural Producer Protection and Macroeconomic Variables in Fragile Five Countries by Bootstrap Panel Causality Test

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ABSTRACT

Agriculture is a leading sector that provides capital accumulation to sustain the economic development processes of developing countries' economies. The low supply and demand elasticity of agricultural products cause fluctuations in agricultural product prices and producer income. Therefore, the first negative reflection of price instability that may arise from fluctuations is generally observed in farmers. Hence, policymakers intervene in the agricultural sector to reduce the instability in agricultural product prices and protect agricultural producers against these effects, as well as the capital accumulation needed for growth and development. Based on this background, this study analyzes the causality relationship between agricultural producer protection (PP) and the macroeconomic variables of Brazil, Indonesia, India, Türkiye, and South Africa, which are referred to as the Fragile Five countries, using the panel bootstrap panel causality test developed by Kónya (2006) with the data between 2000 and 2020. The study findings differ among the countries in the sample. It was determined that there are causality relationships between agricultural PP and economic growth, economic development and inflation variables in all countries involved in this study. Although it is difficult to generalize the main findings of the study to all countries in the sample, it can be concluded that economic growth, economic development and inflation, and agricultural PP variables interact with one another. The study also concludes that the protective and supportive measures for agriculture, a significant sector for the macroeconomic performance indicators of the country's economies, are too important to be neglected.

Keywords: Agricultural economics, Support policy, Subsidies, Food shortages, Economic growth, Inflation

1. Introduction

The agricultural sector, which includes soil cultivation, animal husbandry, fisheries, and forestry, is a critical economic growth dynamic that cannot be ignored by the economies of industrialized and emerging countries (Awokuse & Xie 2015). Furthermore, it makes significant contributions to the economy, particularly at the start of economic growth and development, by feeding the populace and transferring resources to other sectors (Schultz 1964; Kuznets 1972). The fact that agricultural products (food) are both a commercial commodity and a vital resource for human well-being maintains and deepens the sector's role in the economy (Page 2013). The agriculture sector's capital accumulation, in particular, helps the initial stage of the economic growth process. Agricultural products find consumers in international marketplaces, bringing in foreign exchange while improving the country's gross domestic product (GDP). In this regard, the agricultural sector aids in the development of other industries (Johnston & Mellor 1961; Schultz 1964; Schneider & Gugerty 2011; Taylor & Lybbert 2020).

Agriculture growth is a significant tool for regional development and poverty reduction (Johnston & Mellor 1961; Kumar et al. 2011; Oyakhilomen & Zibah 2014; Dogan et al. 2015; Wickramasinghe 2018; and Bekun & Akadiri 2019). However, as globalization spreads the increase in income and welfare, population, and life expectancy around the world has led to an increase in the rate of food consumption. This was particularly apparent in the in the 2000s when food consumption outpaced production (Abbott et al. 2008), resulting in inflationary pressure.

In recent years, the primary battle for policymakers in national economies has been tackling inflation. Inflation in food prices has risen far faster than the consumer price index (CPI) in all regions of the world due to supply interruptions caused by coronavirus disease-2019 (COVID-19) (Valentina 2020; Gruère & Brooks 2021). Many variables, including increases in input costs (Algan et al. 2021), global climatic changes (Pelling et al. 2002; Singh-Peterson & Lawrence 2015), and losses in agricultural output, have caused agricultural output prices to rise globally (Edwards et al. 2011). The increase in food prices, which makes up a significant share in the household's disposable income, has led to a decrease in welfare levels. According to the Food and Agriculture Organization (FAO 2022) report, in February 2022, the food price index increased by an all-time high of 3.9% compared to January 2022 and was 20.7% higher than 2021.

Excessive fluctuations in agricultural product supply or prices have made the agricultural sector more fragile than before and destabilized regional-global markets (Bekkers et al. 2017). This instability exacerbates the CPI increase problem for national economies on a global scale, and if CPI increases caused by food price increases are not addressed, this effect can produce a domino effect and lead to an increase in agricultural product prices (Güloğlu & Nazlıoğlu, 2013). Based on this background analysis, the price increases in agricultural outputs in recent years have seriously affected low-income consumers. These price increases have managed to become a current issue once again, especially in developing countries, as they negatively affect the welfare of low-income consumers (Wodon et al. 2008; Albers & Peeters 2011; Jomo & Chowdhury 2020).

Agriculture is crucial in many developing nations for decreasing poverty and hunger (OECD 2005). Food price increases may spark social discontent by lowering the per capita income (GDPC) of the inhabitants in this group of countries living in countries that spend a large amount of their income on food. As a result, the agricultural sector indisputably affects the macroeconomic factors such as economic growth (Gollin 2010). It is a fundamental determinant of CPI and welfare, particularly economic growth. For these reasons, factors arising from the unique structure of the agricultural sector (such as the uncertainty of climatic conditions, the amount of agricultural output, fluctuations in supply-demand and prices, inadaptability for competition, and being an important source of income for the government) necessitate the active intervention of the state in the agricultural sector. Although the economic activities in today's world operate in the form of a free market, the fragile structure of the agricultural sector does not provide much opportunity for this. Especially with the 2008 crisis, many researchers began to discuss seriously the idea of returning to Keynes to solve the crisis. This understanding, which is conceptualized as "Keynes Resurrected", has led to the emergence of fiscal policy as an important and effective policy tool and the re-emergence of debates concerning state interventionism (Alesina 2012). Therefore, almost every developed and developing country adopts the intervention (support) in agriculture as an active policy tool for economic and social reasons (Stiglitz 1987; OECD 2005; Lundberg 2005; Birner & Resnick 2010; Albers & Peeters 2011; Searchinger et al. 2020). Policymakers support the agricultural sector with various market regulations such as direct support for agricultural production, tax advantages, technology and knowledge transfers, measures to protect markets, non-tariff barriers, quantity restrictions, and price supports (Andreosso-O'Collaghan 2003; Lundberg 2005; Clapp 2017; Searchinger et al. 2020). The major goal of such tools is to promote agricultural development and expand overseas agricultural commerce (Vozarova & Kotulic 2016). Thus, the housing need is fulfilled first, and excess supply is traded internationally. This, in turn, helps to solve the problem of insufficient capital accumulation, which is the most serious impediment to economic growth and development in emerging countries. These initiatives may help to lessen the recent food-induced CPI rises that have hit national economies.

When the prices of agricultural products are more unstable than the prices of products produced by other sectors outside of agriculture, the first negative reflection of price instability, which may arise from fluctuations, is usually on the farmers. Therefore, one of the reasons for governments to intervene in the agricultural sector is to reduce the instability in agricultural product prices and protect agricultural producers against these effects (Özdemir 1989).

Economic development is one of the ultimate goals of the economies of developed and developing countries. It can, however, only be achieved with stable economic growth momentum. There is a consensus that the capital accumulation that can be obtained in the agricultural sector, which is indicated for the first stage of economic development, leads to economic growth, and economic growth leads to economic development (Schultz 1964; Kuznets 1972; Taylor & Lybbert 2020). Additionally, it is among the issues that have been discussed recently that the agricultural sector is a fundamental sector for increasing welfare, reducing poverty and hunger, as well as the means by which the increase in output prices of this sector negatively affects household welfare (Wodon et al. 2008; Bekun & Akdiri 2019; Jomo & Chowdhury 2020). These effects are experienced far more in underdeveloped and developing countries. Based on these discussions, the extent to which the protective measures applied to the agricultural sector affect the economies has led some researchers to turn their attention to this area.

Studies on the effects of agricultural support on macroeconomic performance are limited in the literature. Among these studies, Balisacan & Roumasset (1987) examine the relationship between the calculated nominal protection rates of major grains and economic development with the 1979-1981 period data of 68 countries. The researchers concluded a strong relationship between economic development and agricultural protection. The study conducted by Köse & Meral (2021) for Türkiye determined no relationship between agricultural support and economic growth. On the other hand, Albers & Peeters (2011) reported that agricultural supports have a reducing effect on food inflation in their study of the period of 2002-2010 in 9 countries in the Mediterranean region. Caracciolo et al. (2014) investigated the links between the world and national prices of some important staple crops such as maize, rice, and wheat in 4 developing countries. They found no direct correlation between wheat and rice subsidies and the world prices of these products. However, subsidies for maize seeds have an impact on global prices (Caracciolo et al. 2014). Furthermore, the benefits of agricultural support in the agricultural sector both enhance food supply and lower food prices and this is projected to aid in poverty reduction (Oyakhilomen & Zibah 2014).

Gollin (2010) emphasizes that the increase in agricultural productivity is the first and most important source of economic growth in many developing countries. To this end, studies on this topic have been carried out in the form of the contribution of various agricultural supports to agricultural productivity. Having investigated the effects of agricultural supports on agricultural productivity and agricultural growth in the literature, Hennessy (1998), Skuras et al. (2006), McCloud & Kumbhakar (2008), Henningsen et al. (2009), Terin et al. (2013), Gu (2014), Gautam (2015), Ela et al. (2016), Vozarova & Kotilic (2016), Erdal et al. (2021), Canbay (2021) determined that the government's agricultural support policies positively impact agricultural productivity. On the other hand, Bezlepkina & Oude (2006), Nastis et al. (2012), and Uslu & Apaydın (2021) suggested that supports for the agricultural sector have negative effects on agricultural productivity. In addition, Rad-Tüzün & Aslan (2018) reported that agricultural supports have positive effects on some products and negative effects on others. Kalabak & Aslan (2021) found that this effect is limited. Besides, Yıldız (2017) reached the findings of bidirectional causality between the variables in their study, while Roe et al. (2002), and Uslu & Apaydın (2021) could not find a statistically significant relationship between the variables. As can be deduced from the literature, the findings of the studies on the subject, which are few in number, reveal different results. The reasons for the different results mainly stem from the rate differences between the countries/groups of countries discussed in terms of agricultural support from GDP, the fragile structure depending on climatic conditions, and technological infrastructure deficiencies for the sector. In addition to these reasons, marketing strategy mistakes, input price increases due to cyclical developments, the lack of physical and human capital, and the inability to consistently maintain the implemented support plans lead to differentiation in results.

To that end, this paper investigates the causality relationships between economic growth, economic development, and inflation, which are macroeconomic performances of protective policies toward producers in the agricultural sector. The main hypothesis of the study is that the practices aimed at protecting the producers in the agricultural sector lead to an increase in the amount of output, which eventually suppresses the rate at which inflation increases as well as contributes to economic growth and development. For this purpose, the panel bootstrap causality test developed by Kónya (2006) is used with data from 2000 to 2020 for the Fragile Five (Brazil, Indonesia, India, Türkiye, and South Africa). Considering the overall economic performance of the Fragile Five countries in 2020, the Indian economy shrank by 7.25%, South Africa by 6.43%, Brazil by 4.05%, and Indonesia by 2.06% compared to the previous year. Among these countries, only the Turkish economy grew by 1.79% (World Bank 2022a). With the exception of Türkiye, the GDPC (constant 2015 US$) of the Fragile Five tends to decrease again in 2020 when compared with the previous year. In addition, the GDPC data of these countries for 2020/2021 is far below the OECD average. Among these countries, the GDPC from highest to lowest is ordered as Türkiye, Brazil, South Africa, Indonesia, and India (World Bank 2022b). The annual inflation rates indicate that while Brazil's annual inflation rate was 3.21% in 2020, this rate increased to 8.30% in 2021. Annual inflation for 2020 and 2021 rose from 1.92% to 1.56% in Indonesia, from 5.56% to 4.89% in India, from 12.27% to 19.59% in Türkiye, and from 3.21% to 4.61% in South Africa (OECD 2022a). Figures regarding the share of the agricultural sector in GDP in 2020 reflect that it is 5.9% in Brazil, 13.70% in Indonesia, 18.31% in India, 6.68% in Türkiye, and 2.52% in South Africa (World Bank 2022c). In addition to being among the developing countries, major economic problems such as high inflation, unstable growth, and lack of capital are the common features of the Fragile Five (Kırca & Canbay 2020). The fundamental reason for selecting these countries as the study's sample group is that the agricultural sector is critical to their economies, and their economic performance is still volatile. This is a unique study since there are very few studies in the literature that focus on the links between agricultural producer protection (PP) and macroeconomic variables. It also differs from the others in that the subject is tested for the first time in the context of the Fragile Five, the time span under consideration, and the method employed.

2. Material and Methods

2.1. Material

For this study, the 2000 and 2020 period of the Fragile Five (Brazil, Indonesia, India, Türkiye, and South Africa) is discussed in the model. This period is used in the model due to the restriction of the time series of the agricultural PP variable of some countries in the sample of the research. In the model, PP, GDP, GDPC, and CPI variables are used. GDP represents economic growth, and economic development is represented by the GDPC variables used in the model. Although there are many indicators of economic development, GDPC is among the most commonly used (Şaşmaz & Yayla 2018). In addition, one of the main reasons for choosing GDPC as an indicator of economic development as a variable in the model is the use of this variable to represent economic development in the studies of Kuznets (1955) and Grossman and Krueger (1991).

Among the variables, PP was obtained from the Organization for Economic Co-operation and Development (OECD 2022b) database, and GDP (World Bank 2022d), GDPC (World Bank 2022b), and CPI (World Bank 2022e) were accessed on the World Bank database. PP is defined as the ratio between the average price received by producers (measured at the farm gate), including net payments per unit of current output, and the border price (measured at the farm gate). For instance, a coefficient of 1.10 suggests that farmers, overall, paid prices that were 10% above international market levels. This indicator reflects the level of price distortions and is measured by the Producer Nominal Protection Coefficient expressed as the ratio of farm price to the border reference price. GDP data are in constant 2015 prices, expressed in US dollars. GDPC is based on purchasing power parity. Data are constant with 2017 international dollars. Logarithms of both variables were taken and included in the analysis. The data of CPI (2010=100) are period averages.

Descriptive statistics of the variables of the countries are given in Table 1.

Table 1- Descriptive statistics

Variables	***PP_Brazil***	***PP_Indonesia***	***PP_India***	***PP_Türkiye***	***PP_ South Africa***
Mean	1.03	1.17	0.83	1.27	1.03
Median	1.03	1.18	0.83	1.28	1.02
Max.	1.06	1.34	0.93	1.41	1.11
Min.	1.00	0.87	0.72	1.06	1.00
Std. Dev.	0.02	0.12	0.06	0.09	0.02
Jarque-Bera	2.26	1.24	1.26	1.43	6.76
J-B Prob.	0.32	0.53	0.53	0.48	0.03
Variables	***GDP_ Brazil***	***GDP_ Indonesia***	***GDP_ India***	***GDP_ Türkiye***	***GDP_South Africa***
Mean	28.08	27.20	28.03	27.20	26.42
Median	28.16	27.21	28.06	27.14	26.46
Max.	28.25	27.67	28.62	27.64	26.60
Min.	27.80	26.70	27.40	26.68	26.12
Std. Dev.	0.15	0.32	0.39	0.31	0.15
Jarque-Bera	2.48	1.55	1.41	1.40	2.29
J-B Prob.	0.28	0.46	0.49	0.49	0.31
Variables	***GDPC_ Brazil***	***GDPC_ Indonesia***	***GDPC_ India***	***GDPC_ Türkiye***	***GDPC_ S.Africa***
Mean	9.82	17.15	11.10	9.67	11.21
Median	9.84	17.16	11.11	9.62	11.25
Max.	9.95	17.51	11.57	9.97	11.29
Min.	9.64	16.78	10.62	9.28	11.04
Std. Dev.	0.10	0.24	0.31	0.22	0.08
Jarque-Bera	1.97	1.60	1.42	1.41	3.46
J-B Prob.	0.37	0.44	0.49	0.49	0.17

Table 1- Continued

Variables	*CPI_ Brazil*	*CPI_ Indonesia*	*CPI_ India*	*CPI_ Türkiye*	*CPI_ S.Africa*
Mean	107.59	100.19	107.72	113.00	105.02
Median	100.00	100.00	100.00	100.00	100.00
Max.	172.77	154.08	184.32	263.22	164.05
Min.	52.53	44.01	54.33	20.59	59.89
Std. Dev.	38.14	35.85	44.44	65.21	33.50
Jarque-Bera	1.47	1.46	2.06	2.03	1.68
J-B Prob.	0.47	0.48	0.35	0.36	0.42

2.2. Method

In this study, in which the relationships between the variables in the model are examined, the relationships between the variables are tested with the panel bootstrap causality test developed by Kónya (2006). In this test, the existence of a cointegration relationship between the variables is not mandatory, and at the same time, there is no need to focus on the stationarity levels of the variables. This test, however, does have two important prerequisites. The first is the existence of cross-section dependence in the models, while the other prerequisite is that the coefficients of the models are heterogeneous.

Among the cross-sectional dependence test, the BP_{LM} test developed by Breusch & Pagan (1980), the CD_{LM} test developed by Pesaran (2004), the LM_{adj} test developed by Pesaran et al. (2008), and finally determined by LM_{BC} tests developed by Baltagi (2012) are frequently used tests. In order to determine the homogeneity/heterogeneity of the coefficients, the $\tilde{\Delta}$ and $\tilde{\Delta}_{adj}$ test statistics suggested by Pesaran & Yamagata (2008) are generally used.

Holding the view that the seemingly unrelated regression (SUR) estimator is more effective than ordinary least squares estimator, Kónya (2006) developed a causality test based on this SUR estimator developed by Zellner (1962). In fact, each equation in the SUR system is based on Sims's (1980) Vector Autoregressive (VAR) approach. The relationships between the variables used in the study are modeled using the SUR system as follows (Kónya 2006):

$$\left.\begin{aligned} PP_{1,t} &= \varphi_{1,1} + \sum_{l=1}^{ml\_PP_1} \alpha_{1,1,l} PP_{1,t-1} + \sum_{l=1}^{ml\_GDP_1} \beta_{1,1,l} GDP_{1,t-1} + \xi_{1,1,t} \\ PP_{2,t} &= \varphi_{1,2} + \sum_{l=1}^{ml\_PP_1} \alpha_{1,2,l} PP_{2,t-1} + \sum_{l=1}^{ml\_GDP_1} \beta_{1,2,l} GDP_{2,t-1} + \xi_{1,2,t} \\ &\vdots \\ PP_{N,t} &= \varphi_{1,N} + \sum_{l=1}^{ml\_PP_1} \alpha_{1,N,l} PP_{N,t-1} + \sum_{l=1}^{ml\_GDP_1} \beta_{1,N,l} GDP_{N,t-1} + \xi_{1,N,t} \end{aligned}\right\} \quad 1$$

$$\left.\begin{aligned} GDP_{1,t} &= \varphi_{2,1} + \sum_{l=1}^{ml\_GDP_2} \beta_{2,1,l} GDP_{1,t-1} + \sum_{l=1}^{ml\_PP_2} \alpha_{2,1,l} PP_{1,t-1} + \xi_{2,1,t} \\ GDP_{2,t} &= \varphi_{2,2} + \sum_{l=1}^{ml\_GDP_2} \beta_{2,2,l} GDP_{2,t-1} + \sum_{l=1}^{ml\_PP_2} \alpha_{2,2,l} PP_{2,t-1} + \xi_{2,2,t} \\ &\vdots \\ GDP_{N,t} &= \varphi_{2,N} + \sum_{l=1}^{ml\_GDP_2} \beta_{2,N,l} GDP_{N,t-1} + \sum_{l=1}^{ml\_PP_2} \alpha_{2,N,l} PP_{N,t-1} + \xi_{2,N,t} \end{aligned}\right\} \quad 2$$

$$\left.\begin{aligned} PP_{1,t} &= \varphi_{3,1} + \sum_{l=1}^{ml\_PP_1} \alpha_{3,1,l} PP_{1,t-1} + \sum_{l=1}^{ml\_GDPC_1} \beta_{3,1,l} GDPC_{1,t-1} + \xi_{3,1,t} \\ PP_{2,t} &= \varphi_{3,2} + \sum_{l=1}^{ml\_PP_1} \alpha_{3,2,l} PP_{2,t-1} + \sum_{l=1}^{ml\_GDPC_1} \beta_{3,2,l} GDPC_{2,t-1} + \xi_{3,2,t} \\ &\vdots \\ PP_{N,t} &= \varphi_{3,N} + \sum_{l=1}^{ml\_PP_1} \alpha_{3,N,l} PP_{N,t-1} + \sum_{l=1}^{ml\_GDPC_1} \beta_{3,N,l} GDPC_{N,t-1} + \xi_{3,N,t} \end{aligned}\right\} \quad 3$$

$$\left.\begin{aligned} GDPC_{1,t} &= \varphi_{4,1} + \sum_{l=1}^{ml\_GDPC_2} \beta_{4,1,l} GDPC_{1,t-1} + \sum_{l=1}^{ml\_PP_2} \alpha_{4,1,l} PP_{1,t-1} + \xi_{4,1,t} \\ GDPC_{2,t} &= \varphi_{4,2} + \sum_{l=1}^{ml\_GDPC_2} \beta_{4,2,l} GDPC_{2,t-1} + \sum_{l=1}^{ml\_PP_2} \alpha_{4,2,l} PP_{2,t-1} + \xi_{4,2,t} \\ &\vdots \\ GDPC_{N,t} &= \varphi_{4,N} + \sum_{l=1}^{ml\_GDPC_2} \beta_{4,N,l} GDPC_{N,t-1} + \sum_{l=1}^{ml\_PP_2} \alpha_{4,N,l} PP_{N,t-1} + \xi_{4,N,t} \end{aligned}\right\} \quad 4$$

$$\left.\begin{aligned} PP_{1,t} &= \varphi_{5,1} + \sum_{l=1}^{ml\_PP_1} \alpha_{5,1,l} PP_{1,t-1} + \sum_{l=1}^{ml\_CPI_1} \beta_{5,1,l} CPI_{1,t-1} + \xi_{5,1,t} \\ PP_{2,t} &= \varphi_{5,2} + \sum_{l=1}^{ml\_PP_1} \alpha_{5,2,l} PP_{2,t-1} + \sum_{l=1}^{ml\_CPI_1} \beta_{5,2,l} CPI_{2,t-1} + \xi_{5,2,t} \\ &\vdots \\ PP_{N,t} &= \varphi_{5,N} + \sum_{l=1}^{ml\_PP_1} \alpha_{5,N,l} PP_{N,t-1} + \sum_{l=1}^{ml\_CPI_1} \beta_{5,N,l} CPI_{N,t-1} + \xi_{5,N,t} \end{aligned}\right\} \quad 5$$

$$\left.\begin{aligned} CPI_{1,t} &= \varphi_{6,1} + \sum_{l=1}^{ml\_CPI_2} \beta_{6,1,l} CPI_{1,t-1} + \sum_{l=1}^{ml\_PP_2} \alpha_{6,1,l} PP_{1,t-1} + \xi_{6,1,t} \\ CPI_{2,t} &= \varphi_{6,2} + \sum_{l=1}^{ml\_CPI_2} \beta_{6,2,l} CPI_{2,t-1} + \sum_{l=1}^{ml\_PP_2} \alpha_{6,2,l} PP_{2,t-1} + \xi_{6,2,t} \\ &\vdots \\ CPI_{N,t} &= \varphi_{6,N} + \sum_{l=1}^{ml\_CPI_2} \beta_{6,N,l} CPI_{N,t-1} + \sum_{l=1}^{ml\_PP_2} \alpha_{6,N,l} PP_{N,t-1} + \xi_{6,N,t} \end{aligned}\right\} \quad 6$$

Model 1 is used to test the causality relationship from GDP to PP, from PP to GDP in Model 2, from GDPC to PP in Model 3, from PP to GDPC in Model 4, from CPI to PP in Model 5, and from PP to CPI in Model 6.

N represents the number of countries (i=1, 2, 3, ..., 5) expressed in the equations, and t represents the time interval (t=2000, 2001, 2002, 2003, ..., 2020). In addition, "ml" represents the lag length and $\xi_{1,1,t}$, $\xi_{1,2,t}$,..., $\xi_{1,N,t}$, $\xi_{2,1,t}$, $\xi_{2,2,t}$,..., $\xi_{2,N,t}$, $\xi_{3,1,t}$, $\xi_{3,2,t}$,..., $\xi_{3,N,t}$... are the error terms which are supposed to be white noises.

In Kónya's (2006) panel bootstrap causality test, Wald test statistics are calculated using the VAR equations estimated for each country in the SUR system above. However, critical values for each country are derived by the bootstrap method. The following hypotheses are tested by comparing the calculated Wald test statistics with the critical values calculated by the bootstrap method. By applying constraints to the coefficients as shown below, causality relationships between the variables can be determined.

If not all $\beta_{1,N,l}s$ are zero, but all $\alpha_{2,N,l}s$ are zero; there is unidirectional Granger causality from GDP to PP. If not all $\alpha_{2,N,l}s$ are zero, but all $\beta_{1,N,l}s$ are zero; there is unidirectional Granger causality from PP to GDP. If all $\beta_{1,N,l}s$ and $\alpha_{2,N,l}s$ are zero; there is no causality relationship between GDP and PP. If neither $\beta_{1,N,l}s$ nor $\alpha_{2,N,l}s$ are zero; there is bidirectional Granger causality. Similarly, if not all $\beta_{1,N,l}s$ are zero, but all $\alpha_{2,N,l}s$ are zero; there is unidirectional Granger causality from GDPC to PP. If not all *s* are zero but all s are zero; there is unidirectional Granger causality from CPI to PP.

3. Results and Discussion

Table 2 shows the results of the cross-sectional dependence and homogeneity test, which is the prerequisite of the Kónya (2006) panel bootstrap causality test.

Table 2- Cross-section dependence (CSD) test and slope homogeneity (SH) tests results

Tests	*CSD*				*SH*	
Models	BP_{LM}	CD_{LM}	LM_{BC}	LM_{adj}	$\tilde{\Delta}$	$\tilde{\Delta}_{adj}$
Model 1	23.99* (0.007)	3.12* (0.001)	3.00* (0.002)	-0.36 (0.713)	6.54* (0.001)	7.13* (0.001)
Model 2	158.21* (0.001)	33.14* (0.001)	33.01* (0.001)	12.54* (0.001)	5.84* (0.001)	6.37* (0.001)
Model 3	23.78* (0.008)	3.08* (0.002)	2.95* (0.003)	0.99 (0.318)	6.14* (0.001)	6.69* (0.001)
Model 4	42.17* (0.001)	7.19* (0.001)	7.07* (0.001)	4.75* (0.001)	5.71* (0.001)	6.22* (0.001)
Model 5	21.91* (0.001)	2.66* (0.001)	2.53* (0.001)	-0.19* (0.841)	3.83* (0.001)	4.17* (0.001)
Model 6	201.99* (0.001)	42.93* (0.001)	42.80* (0.001)	14.21* (0.001)	5.59* (0.001)	6.10* (0.001)

*Indicates cross-sectional dependence and heterogeneity at 1% and 5% statistical significance level. Figures in (parentheses) are probability values

Test results in Table 2 show that there is a cross-sectional dependence in all the models. In Model 1, Model 3, and Model 5, except for the LM_{adj} test developed by Pesaran et al. (2008), findings on three cross-sectional dependencies were obtained, while in the other four models results on four cross-sectional dependencies were acquired. The BP_{LM} test can be used when the time dimension is larger than the cross-sectional dimension (t>N), while the CD_{LM} test can be used when both the time dimension is larger than the cross-sectional dimension and the cross-sectional dimension is larger than the time dimension (t>N, N>t). Since it is (t>N) in all study models, the BP_{LM} test can be taken as a reference. However, in CD_{LM} and LM_{BC} test results cross-sectional dependence was detected in all models. According to these results, shocks that can occur in any of the five countries included in the model may affect other countries in the future. Moreover, as can be seen in Table 1, the homogeneity test results of the slope coefficients of Pesaran & Yamagata (2008) in all models are significant at the 1% level. Therefore, the alternative hypothesis, the slope coefficients, is heterogeneous. As a result of the findings obtained from the tests in Table 2, there is no obstacle to performing the Kónya (2006) panel bootstrap causality test.

The causality results between PP and GDP are presented in Table 3.

Table 3- Causality between PP and GDP

H_0: GDP is not the Granger causality of PP (Model 1)					
Countries	***Coefficients***	***Test statistics***	***Critical values***		
	GDP	***Wald***	***10%***	***5%***	***1%***
Brazil	-0.078	8.057***	6.838	10.29	17.365
Indonesia	0.266	8.346**	5.876	8.492	14.779
India	0.03	1.221	8.817	12.605	22.472
Türkiye	-0.134	9.040***	6.459	9.566	16.841
South Africa	-0.186	27.197*	5.166	7.905	16.51
H_0: PP is not the Granger causality of GDP (Model 2)					
Countries		***Test Statistics***	***Critical Values***		
	PP	***Wald***	***10%***	***5%***	***1%***
Brazil	0.56	6.289***	6.169	9.421	17.602
Indonesia	0.03	0.987	6.956	11.474	27.316
India	0.09	2.116	6.135	9.474	19.847
Türkiye	0.27	9.434***	6.963	10.389	21.934
South Africa	0.35	13.097**	7.483	11.496	25.582

*,**,***Indicate rejection of the null hypothesis at the 1, 5, and 10 percent levels of significance, respectively

According to the test results in Table 3, there is a bidirectional causality relationship between PP and GDP variables in Brazil, Türkiye, and South Africa. However, there is a positive causality relationship to PP in GDP in Indonesia. In Model 1, there is a negative causality from GDP to PP in Brazil, Türkiye, and South Africa, and in Model 2, positive causality from PP to GDP in Brazil, Türkiye, and South Africa.

While the agricultural sector has an important share in the economies of countries that are in development efforts at the beginning, this weighted share is replaced by the manufacturing and service sectors as the countries develop. In the development literature, this change is referred to as a natural and structural transformation (Polat 2011). The main reason for this structural transformation is the low-income demand elasticity of agricultural products compared to industrial and high-tech products (Fisher 1939). Additionally, as countries' income levels increase, the total demand shifts relative to industrial and high-tech products, and foreign trade rates follow a course against agricultural products (Singer 1950; Prebish 1962). The primary reason why economic growth negatively affects PP in Brazil, Türkiye, and South Africa may be that as the economies of the countries grow, more importance is given to sectors with high added value, especially manufacturing and service industry, rather than the agricultural sector. Particularly, under the same conditions, the share of the agricultural sector in national income or total production decreases (Uslu & Apaydın 2021). This may also be a possible result of reducing the share of agricultural support in GDP proportionally. PP increases economic growth in Brazil, Türkiye, South Africa, and Indonesia. The protection policies applied to the producers in the agricultural sector contribute to the country's GDP and the increase in productivity.

The causality results between PP and GDP per capita are given in Table 4.

Table 4- Causality between PP and GDP per capita

H_0: GDPC is not the Granger causality of PP (Model 3)					
Countries	***Coefficients***	***Test statistics***	***Critical values***		
	GDPC	***Wald***	***10%***	***5%***	***1%***
Brazil	-0.100	4.417	7.971	11.276	21.540
Indonesia	0.409	8.873***	6.316	9.124	17.778
India	0.056	2.160	9.660	13.358	22.256
Türkiye	-0.248	11.382**	7.409	10.779	18.307
South Africa	-0.299	12.807**	6.829	10.116	27.448
H_0: PP is not the Granger causality of GDPC (Model 4)					
Countries		***Test statistics***	***Critical values***		
	PP	***Wald***	***10%***	***5%***	***1%***
Brazil	0.59	11.194**	5.760	8.285	16.298
Indonesia	0.02	0.541	6.087	9.853	24.434
India	0.06	1.484	5.697	8.611	15.936
Türkiye	0.36	8.920**	5.816	8.744	17.375
South Africa	0.39	9.020***	6.366	9.517	19.437

,*Indicate rejection of the null hypothesis at the 5 and 10 percent levels of significance, respectively

According to the current findings in Table 4, a bidirectional causality relationship was observed between PP and GDPC variables in Türkiye and South Africa. The direction of these relationships is negative from GDPC to PP and positive from PP to GDPC. The existence of a positive and unidirectional causality relationship from GDPC to PP in Indonesia and from PP to GDPC in Brazil was found.

According to Ernst Engel's (1857) law, as consumer income rises, the share of the budget devoted to basic items drops, while the share assigned to luxury goods rises. As a result, increasing welfare and income levels with economic development raise household luxury expenditure. According to Engel's Law, it is possible that the share of consumption for food, which is the basic need, in income decreases. As a result of all these developments, the support for the agricultural sector may decrease or cause it to remain weaker than before. In particular, the study findings for Türkiye and South Africa can be based on Engel's law. In Indonesia, economic development leads to increased protective measures for farmers. Indonesia's basic agricultural policy is primarily aimed at meeting domestic demand,

and the government follows a protective policy, especially in the agricultural sector, against producers (Poernomo 2017). Policies to protect producers in Brazil, Türkiye, and South Africa both positively affect economic growth and lead to economic development.

Finally, the causality results between PP and CPI are presented in Table 5.

Table 5- Causality between PP and CPI

H_0: CPI is not the Granger causality of PP (Model 5)					
Countries	***Coefficients***	***Test statistics***	***Critical values***		
	CPI	***Wald***	***10%***	***5%***	***1%***
Brazil	-0.01	14.783**	6.187	9.168	16.212
Indonesia	0.01	9.069**	5.732	8.133	14.846
India	0.01	2.519	8.483	12.141	20.944
Türkiye	-0.01	11.891**	6.480	9.124	16.122
South Africa	-0.01	6.355***	5.270	7.967	14.396
H_0: PP is not the Granger causality of CPI (Model 6)					
Countries		***Test statistics***	***Critical values***		
	PP	***Wald***	***10%***	***5%***	***1%***
Brazil	-78.73	15.379*	5.177	7.706	14.061
Indonesia	2.40	0.415	4.984	7.514	14.770
India	-34.947	36.731*	5.137	7.472	14.308
Türkiye	-28.00	6.096***	5.201	7.716	15.064
South Africa	-15.10	0.854	5.677	8.445	16.238

*,**,***Indicate rejection of the null hypothesis at the 1, 5, and 10 percent levels of significance, respectively

In Table 5, a negative and bidirectional causality relationship was found between PP and CPI variables in Brazil and Türkiye. A negative and unidirectional causality relationship from PP to CPI was also observed in India. Therefore, the measures to protect the farmers in these three countries have a reducing effect on inflation. As Özdemir (1989) states, policymakers apply policies to reduce production costs to prevent agricultural producers from increasing income and food price fluctuations. According to our study findings, the practices aimed at protecting farmers decrease during periods of increased inflation in both countries. In addition, negative causality from CPI to PP in South Africa and positive and unidirectional causality in Indonesia were determined. Furthermore, in South Africa's inflationary climate, farmer protection protections are being reduced. This result can be seen as the possibility that inflationary pressure will impair the effectiveness of preventive measures. In Indonesia, rising inflation exacerbates protective measures.

4. Conclusions

In this study, the causality relationships between agricultural PP and GDP, GDPC, and CPI variables in the agricultural sector were investigated using the panel bootstrap causality test developed by Kónya (2006) with the data of the Fragile Five from between 2000 and 2020. The study findings determined a bidirectional causality relationship between agricultural PP and GDP in Brazil, Türkiye, and South Africa. This relationship is negative from economic growth to agricultural PP and positive from PP to economic growth. A positive causality relationship was found from GDP to PP in Indonesia. When the relationships between PP and economic development variables are examined, there is a bidirectional causality relationship in Türkiye and South Africa. The direction of these relations is negative from economic development to PP and positive from PP to economic development. There is a positive and unidirectional causality relationship from economic development to PP in Indonesia and from PP to economic development in Brazil. Finally, a negative and bidirectional causality relationship was found between PP and CPI variables in Brazil and Türkiye. A negative and unidirectional causality relationship from PP to CPI was also observed in India. Besides, a negative and unidirectional causality relationship from CPI to PP was found in South Africa and positive in Indonesia.

There was no causal relationship found in the analytical findings between the variables other than the CPI variable of the PP variable in India. As a result, India's farmer-protection policies have little effect on inflation. Agriculture, on the other hand, is a major economic activity for India, one of the world's most densely populated countries, for a variety of reasons, including considerable employment

opportunities. Despite this, India's abundance of food production legislation has a negative impact on the agriculture sector. Additionally, the State Agricultural Produce Markets Regulation prevents successful farming ventures. While agricultural price policy and related instruments have motivated farmers to adopt new technologies to increase the physical and economic access to food, they have also restricted private sector initiatives and produced a few other economic challenges (Malaisamy 2021).

Among the countries included in the model, South Africa is the one that is most susceptible to drought. This puts small-scale farmers in a difficult position, especially in the agricultural sector, which already has a weak infrastructure. For instance, in 2015, agricultural production in South Africa decreased by 8.4% due to drought and by 15% in livestock herd stock. This, of course, adversely affect farmers' agricultural outputs and incomes (Matlou et al. 2021). For this reason, farmers need assistance to alleviate the detrimental consequences of these shocks. The South African government is an important supporter of agriculture, which is seen as critical to all of these difficulties and economic development. Although governmental investments in agriculture have expanded since 2004-2005, the rate of growth has recently slowed and infrastructure spending has been prioritized (Mncina & Agholor 2021). In a study conducted in South Africa, the government's activities to support the agricultural sector, including drought period, led to an improvement in the welfare of small-scale farmers. However, it is thought that introducing these support measures only during periods of drought will not be enough for the sector's future (Matlou et al. 2021).

In the last ten years, Türkiye has continued to make necessary internal and external structural reforms to increase income level and productivity by increasing the use of sustainable natural resources with comprehensive government support and policy interventions in agriculture. Due to the fragile nature of agriculture, policies directing the producers in line with the demand of market conditions have been adopted instead of policies involving government intervention in the prices of products that adversely affect the market price formation. Although agricultural support in Türkiye has always increased based on the current prices, the increase in support has not been sustainable and has stayed below the targeted figures. The fundamental issue in Türkiye's agricultural sector is the lack of agricultural structural frameworks and the frequent changes in support policies (Demirtas 2021). Additionally, despite many beneficial improvements in the agricultural sector and subsidies, the number of farmers and agricultural lands supported by support instruments account for a small portion of the total data. At the same time, the quantity of support supplied to producers is a very small proportion of total agricultural support, raising concerns about the sustainability of agriculture in Türkiye (Yeni & Teoman 2022).

In Indonesia, policymakers follow a protective policy against producers, particularly in the agricultural sector, and the government control rice price policies and thus effectively controls domestic rice prices. These protective measures can drive the price of agricultural output much higher than the social price (shadow). Therefore, farmers get more profit. This causes agricultural production output to be cheaper overall. Indonesia's basic agricultural policy is primarily aimed at meeting domestic demand. The way in which agricultural products can be exported is through a series of regulations. In particular, there is a need to reorganize the agricultural system, strengthen agricultural technology and support an adequate institutional system as a whole. However, in this case, output prices may approach world market prices (Poernomo 2017).

As a crucial agricultural producer and exporter, Brazil provides a low level of support and protection to agriculture. Support to producers in Brazil was 2.6% of gross farm income in 2016-18. This level is well below the Organization for Economic Co-operation and Development (OECD) average. This support is 5.7% in the 2000-2002 period. Total support for agriculture, including producer support and general services, fell to 0.4% of GDP (OECD 2019).

Underdeveloped countries must import new techniques and technology from developed ones in order to achieve economic growth and development. The quantity of foreign exchange obtained from exports determines an impoverished country's ability to meet its purchases. The ability of these countries to increase their foreign exchange income depends on their competitiveness in international markets. However, each state aims to produce its food needs domestically to reduce its dependence on foreign countries. Additionally, the negative effects of the instability in the prices of agricultural products may prevent the enterprises from turning into modern agricultural enterprises, as well as hinder the development of the agricultural sector. As a result of this, a country's growth/development rate decreases or does not increase. Eliminating these negative effects on agricultural product prices forces governments to interfere in agricultural product pricing first to accelerate agricultural growth and thereafter to offer general development (Özdemir 1989).

Most farmers in developing countries are low-income, small-scale businesses. The farmers in these countries face unfair competition against agricultural products that receive high support from developed countries. This is one of the biggest obstacles to agricultural development in developing countries (Sharma & Das 2018). For this reason, developing countries need to show sufficient interest in the agricultural sector, supporting the acceleration of industrialization, economic growth, and development (Schultz 1964; Kuznets 1972).

In this context, it is necessary to increase the supportive, protective, regulatory, and stable measures for agriculture. Only in this way can the increase in capital accumulation of small-scale farmers, who are predominantly active in this group of countries, bring output efficiency, economic growth, development, and price stability.

In the databases of state institutions or international organizations, the data on agricultural support are not detailed in a way that can support academic research. Existing data, on the other hand, include cuts in both country and time dimensions. All these constraints do not allow the analysis of the effects of agricultural support on macroeconomic performance indicators for a longer period of time and for wider country groups. However, despite this limitation, this study is significant as it is an additional study to the few existing studies on the effects of agricultural support policies on macroeconomic indicators. In addition, this study sets an example for other researchers, and the potential results that could be obtained by them could encourage new studies in the field and guide policymakers. Moreover, in the analysis period of the study, a single year from 2019 to 2020 corresponds to the COVID-19 pandemic. Considering the data in the OECD database during this 1-year period, a slight decline is observed in the support applied to producers in all countries except Brazil. It is considered that this period did not have much effect on the results of the analysis part of the study. Hence, conclusions on the effects of the COVID-19 pandemic can only be made if micro-scale data are available.

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A Meta-analysis of Bentonite Efficacy on Performance, Carcass Yield, Giblet, and Blood Constituents of Broiler Fed Contaminated Aflatoxin

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ABSTRACT

Aflatoxins can easily grow and develop in many feed ingredients and influence the risk of several animal chronic diseases. The present study aimed to evaluate the effect of bentonite inclusion on performances, carcass yield, giblet, and blood constituents of broiler-fed contaminated aflatoxin through a meta-analytical approach. A database was developed based on scientific publications that were searched using several search tools such as Science Direct, PubMed, Scopus, and Google Scholar with "bentonite", "clay", "montmorillonite", "aflatoxin" and "broiler" as keywords. A total of 31 studies were retrieved and included in the analysis. Data analysis was based on the mixed model in which dietary bentonite inclusion was treated as the fixed effect and different studies were considered as random effects. Bentonite used were sodium bentonite, calcium bentonite, and montmorillonite form with levels ranging from 0 to 5%. Meanwhile, aflatoxin B1 levels in the diet ranged from 0 to 5 mg/kg. In this study, body weight gain, feed intake, and feed efficiency showed a linear increase ($p<0.001$) in all phases. The mortality rate linearly decreased ($p<0.05$) in the cumulative. Bentonite inclusion linearly increased ($p<0.001$) the carcass, breast, leg, bursa of Fabricius, and gizzard weight proportion. The percentage of kidney, liver, heart, pancreas, uric acid, and gamma-glutamyltransferase linearly decreased ($p<0.001$) by increasing bentonite levels. Dietary bentonite linearly increased ($p<0.001$) total protein, albumin, aspartate aminotransferase, calcium, glycogen, globulin, and hemoglobin ($p<0.05$). It was concluded that bentonites have the ability to eliminate the detrimental effects of aflatoxin, which may increase the broiler performance, carcass yield and prevent the change in abnormal giblet weight. For effectiveness and animal safety, the EFSA recommends using a maximum of 2% bentonite in the feed.

Keywords: Aflatoxin, Bentonite, Body weight gain, Feed efficiency, Mixed model, Montmorillonite

1. Introduction

Aflatoxins are secondary metabolites produced by *Aspergillus* species that can easily grow and develop in many feed ingredients and influence the risk of several animal chronic diseases. Aflatoxin B1 is the most toxic among the aflatoxins types such as aflatoxin B2, aflatoxin G1, and aflatoxin G2 (Manafi 2012). Acute aflatoxicosis in broilers occurs in excessive and long-term consumption of aflatoxin, and it has effects such as fatigue, anorexia, anemia, bleeding, respiratory distress, pubescence, bloody diarrhea, and high mortality in poultry (Mohamadi & Alizadeh 2010). In low levels and long-term consumption, aflatoxin causes poor performance in poultry and a decrease in egg production, feed consumption, and egg weight (Nasaruddin et al. 2021). In the poultry industry, aflatoxins cause serious economic losses due to these negative effects (Bryden 2012). Therefore, effective methods are urgently needed for the detoxification of feeds containing aflatoxin.

For this purpose, some adsorbents are used in the poultry diet to reduce their absorption from the digestive system by binding aflatoxin. Some adsorbents have been tested *in vitro* as well as *in vivo*, including bentonites. The main advantages of using bentonite as an adsorbent in the feed are its affordability, safety, and convenience. Bentonite belongs to the smectite family, which is mostly composed of montmorillonite clay (Pouraboulghasem et al. 2016). Bentonite primarily contains sodium montmorillonite or calcium montmorillonite,

which sodium montmorillonite can absorb huge amounts of water and has greater swelling potential than calcium montmorillonite (Park et al. 2016). It has been reported that bentonite has been found effective in counteracting mycotoxins (Jaynes & Zartman 2011), and improves broiler weight gain (Ani et al. 2014) and egg production (Darmawan & Ozturk 2022). Dietary bentonite also increased feed efficiency but did not affect blood constituents and carcass yield (Khanedar et al. 2013). However, a meta-analysis study that can describe quantitatively summarize the effectiveness of dietary bentonite on broiler has not been provided yet. Therefore, our study aimed to evaluate the bentonite influence on performances, carcass yield, percentage of giblet, and blood constituents of broiler-fed aflatoxin-contaminated feed using data from published articles in a meta-analysis study. It was hypothesized that bentonite would eliminate the negative effects of aflatoxin by binding the mycotoxin in the digestive tract and it would improve broiler performance.

2. Material and Methods

2.1. Development of the database

The journal's search engines (Web of Science, Science Direct, Scopus, and Pub Med) were used to find articles that discussed the different dosages used of bentonite in broiler diets (Table 1). "Bentonite", "clay", "montmorillonite", "aflatoxin", and "broiler" were used as keywords. The criteria article used to be included into the database were: (a) conducting *in vivo* trials on broiler chicken, (b) adding bentonite to basal diets, (c) containing aflatoxin as positive control and/or without aflatoxin as negative control treatments (d) reporting broiler performances, carcass yield, percentage of giblet, blood parameter, (e) the data were not presented in the graph, and (f) articles in English. A total of 100 studies were found from the electronic database. Thus, the studies were screened based on their title and abstract, with 32 deleted related to unsuitable articles such as duplicate articles, review articles, data in graph form, and articles not written in English. Finally, 31 studies were fully reviewed and included in the analysis.

Table 1- Description of the studies included in the database

Authors	*Birds (n)*	*Bentonite Form*	*Dosage (%)*	*Aflatoxin Level (mg/kg)*	*Period (day)*
Tauqir et al. (2001)	240	Sodium bentonite	0-4.5	0	1-42
Rosa et al. (2001)	100	Sodium bentonite	0-0.3	0-5	30-52
Xia et al. (2004)	240	Montmorillonite	0-0.15	0	1-49
Miazzo et al. (2005)	160	Sodium bentonite	0-0.3	0-2.5	23-50
Thalij (2005)	100	Sodium bentonite	0-0.5	0-2.5	1-21
Bailey et al. (2006)	900	Montmorillonite	0-0.5	0-4	1-42
Shi et al. (2006)	160	Montmorillonite	0-0.3	0-0.1	1-42
Pasha et al. (2007)	300	Sodium bentonite	0-0.1	0-0.1	1-42
Pasha et al. (2008)	300	Sodium bentonite	0-1	0	1-42
Kermanshahi et al. (2009)	288	Sodium bentonite	0-1	0-0.5	1-42
Ghahri et al. (2010)	500	Sodium bentonite	0-0.5	0-0.5	1-35
Katouli et al. (2010)	448	Bentonite	0-3	0	1-42
Damiri et al. (2010)	288	Sodium bentonite	0-3	0	1-42
Manafi (2012)	336	Sodium bentonite	0-0.75	0-0.5	1-35
Damiri et al. (2012)	288	Sodium bentonite	0-3.75	0	1-42
Safaeikatouli et al. (2012)	448	Sodium bentonite	0-3	0	1-42
Indresh et al. (2013)	360	High-grade bentonite	0-0.75	0-0.5	1-35
Khanedar et al. (2013)	260	Sodium bentonite	0-1.5	0	1-42
Eckhardt et al. (2014)	1056	Ca Montmorillonite	0-0.5	0-3	1-42
Pappas et al. (2014)	300	Bentonite	0-1	0.02	1-42
Ani et al. (2014)	360	Bentonite	0-5	0	1-28
Dos Anjos et al. (2015)	250	Bentonite	0-0.75	0-2	1-21
Azizpour & Moghadam (2010)	300	Sodium bentonite	0-3	0-0.2	1-42
Malekinejad et al. (2015)	224	Bentonite	0-0.5	0-0.5	1-24

Table 1- Continued

Authors	*Birds (n)*	*Bentonite Form*	*Dosage (%)*	*Aflatoxin Level (mg/kg)*	*Period (day)*
Shannon et al. (2017)	180	Natural bentonite, Concentrated bentonite	0-0.5	0-2	1-21
Attar et al. (2017)	540	Sodium bentonite	0-1.5	0	25-42
Rafiu et al. (2019)	180	Sodium bentonite	0-0.15	Unknown	1-56
Basseboua et al. (2018)	420	Sodium bentonite	0-5	0	1-50
Abadi et al. (2019)	256	Sodium bentonite	0-3	0	1-42
Attar et al. (2019)	540	Sodium bentonite	0-1.5	0	11-42
Saleemi et al. (2020)	125	Bentonite	0-0.3	0-0.3	1-42
Mgbeahuruike et al. (2018)	80	Sodium bentonite	0-0.2	0-0.12	1-56

2.2. Data extraction and description

The variables used in the database were average daily gain (ADG), feed conversion ratio (FCR), daily feed intake (DFI), mortality, carcass yield, breast weight, leg weight, giblet weight (liver, heart, kidney, gizzard, pancreas, bursa of Fabricius), and blood constituents (albumin, total protein, uric acid, calcium, gamma-glutamyltransferase (GGT), aspartate aminotransferase (AST) glycogen, globulin, hemoglobin, packed cell value (PCV). Microbial population and intestinal shape were excluded in the database due to the small number of studies that provided the related parameter. All measured data were converted into the same units for statistical analysis and the dosage of bentonite was expressed in percentage (%) of diets. The data on broiler performances were divided into the starter phase, the final phase, and the cumulative. The database used in meta-analysis and descriptive statistics are presented in Table 1 and Table 2. The database was published between 2001 and 2020 with a total of 10 527 broiler chickens. Bentonite used were sodium bentonite, calcium bentonite, and montmorillonite form with levels ranging from 0 (control) to 5 %. Meanwhile, aflatoxin B1 levels in the diet ranged from 0 to 5 mg/kg. The minimum period of the evaluated study lasted 21 days, while the maximum period lasted 50 days. The broiler strains used were Cobb 500, Ross 308, Arbor Acres, Faobro, and Vencobb (Table 1).

Table 2- Regression equations for the effect of bentonite dose on broiler performances

			Parameter estimates					*Model statistics*		
Parameter	*Unit*	*n*	*Model*	*Intercept*	*SE intercept*	*Slope*	*SE slope*	*p-value*	*RMSE*	*AIC*
Starter phase										
ADG	g/bird/day	56	L	30.16	1.66	0.7115	0.554	<0.0001	9.642	351.0
DFI	g/bird/day	56	L	47.13	2.57	1.1235	0.703	<0.0001	12.114	380.5
FCR		56	L	1.57	0.06	0.001	0.009	<0.0001	0.156	-74.5
Mortality	%	10	L	3.83	2.66	-1.0158	4.021	0.3864	8.828	51.2
Finisher phase										
ADG	g/bird/day	70	L	59.6014	5.1638	1.2407	0.763	<0.0001	15.221	522.0
DFI	g/bird/day	68	L	111.67	10.2945	2.4961	1.265	<0.0001	25.243	596.0
FCR		70	L	2.0423	0.1008	-0.01552	0.014	<0.0001	0.280	-19.6
Cumulative										
ADG	g/bird/day	86	L	44.0454	1.9219	1.3514	0.666	<0.0001	13.859	601.2
DFI	g/bird/day	86	L	90.2034	4.4378	1.6860	1.145	<0.0001	23.489	700.9
FCR		86	L	2.1092	0.1104	-0.02356	0.0117	<0.0001	0.236	-40.2
Mortality	%	30	L	11.8190	3.4268	-1.1562	1.605	0.025	20.410	222.7

n: Number of treatments, RMSE: Root mean square error, SE: Standard error, AIC: Akaike information criterion, L: Linear

2.3. Statistical analysis

The data in the database were analyzed statistically in a meta-analysis using a mixed model methodology (St-Pierre 2001). The level of bentonites was fixed effects while the studies were random effects. The procedure for the mixed model was as follows:

$$Yij= B0+ B1Xij+ si+ eij$$

where Yij was the dependent variable, B0 was overall intercepted across all experiments (fixed effect), B1 was the linear regression coefficient of Y on X, Xij was the value of the continuous predictor variable (bentonite level), si was a random effect of experiment i, and eij was the unexplained residual error. The statistical analysis was conducted using SAS software version 9.1 and the p-value 0.05 criteria to assess the significant effect of each variable.

3. Results and Discussion

3.1. Performances

The regression equations between the level of bentonite and broiler performances are presented in Table 2. In general, our meta-analysis study shows that dietary bentonite supplementation indicates positive effects on broiler performances. ADG and DFI showed a linear increase ($p<0.001$) and FCR represented a linear decrease ($p<0.001$) in all phases. A higher bentonite level was correlated with an increase in ADG (Figure 1) with linear equation Y= 44.05+1.351X and a decrease in FCR with linear equation Y= 2.11–0.024X (Figure 2). The mortality rate linearly decreased ($p<0.05$) in cumulative by increasing bentonite level.

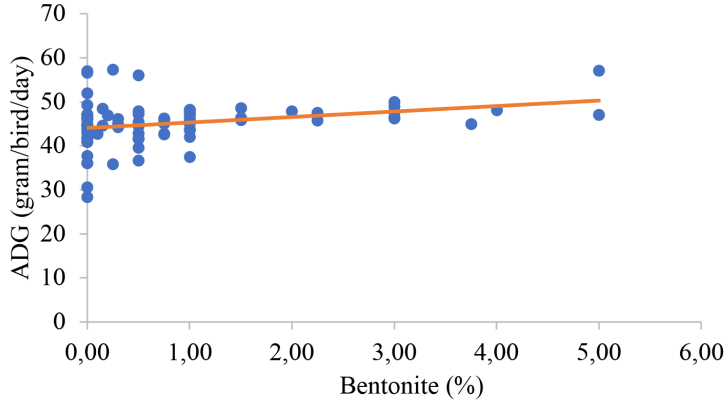

Figure 1- Relationship between bentonite levels and ADG cumulative based on the results of the meta-analysis. Equation: ADG cumulative (gram/bird/day) = 44.05+1.351×bentonite (%) (n=86; p<0.0001; RMSE=13.86)

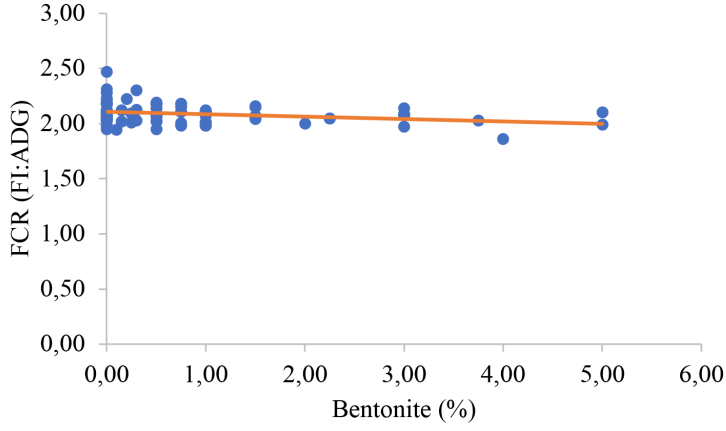

Figure 2- Relationship between bentonite levels and FCR cumulative (FI: ADG) based on the results of the meta-analysis. Equation: FCR cumulative =2.11–0.024 × bentonite (%) (n=86; p<0.0001; RMSE=0.24)

The improvement in ADG, DFI, and feed efficiency indicates that adding bentonite to the diet improves nutrient utilization and also suggests that bentonite has a beneficial effect on chickens fed an aflatoxin-contaminated diet. This result was supported by previous studies that used calcium bentonite and sodium bentonite (Khanedar et al. 2013; Besseboua et al. 2018). The positive impact of dietary bentonite could be attributed to the effect of bentonite in improving the digesta retention time, water absorption, and digestive enzymes in the intestine (Damiri et al. 2012; Khalifeh et al. 2012), nutrient digestibility (Pasha et al. 2008) and lowering in total counts of pathogenic bacteria (Shehata & Abd El-Shafi 2011) hence improving broiler performance. Moreover, bentonite improved intestine health and nutrient absorption by increasing villus height and surface area of broilers and enhancing regeneration of the intestinal epithelium (Wu et al. 2013; Besseboua et al. 2018).

Additionally, the efficacy of bentonite is related to its high mineral content including macro minerals (calcium, sodium, magnesium) and micro minerals (iodine, selenium, iron, zinc) which are needed to improve the enzymes activity and hormones to support growth (Smith et al. 2018). The presence of silicate minerals activity, which enhanced the transit time of feed in digestive tract and nutrient metabolism, might be responsible for performance improvement (Safaeikatouli et al. 2012). Suzanne et al. (2017) stated that clay groups have anionic microstructures in which most of the alkali metal ions and trace elements can be used as sources of animal minerals.

Another mechanism of bentonite in increasing the performance of broilers was through its ability to absorb aflatoxins B1 (Eckhardt et al. 2014; Mgbeahuruike et al. 2018). The previous study revealed that high doses of aflatoxin lead to acute clinical symptoms, increase liver damage, and decrease immune system. In broilers, aflatoxin was able to eliminate several enzymes activity involved in protein, starch, and lipids digestion, resulting in lower body weight increase and feed efficiency (Dos Anjos et al. 2015). The mechanisms of mycotoxins binding by bentonite had been widely investigated including ion interactions, electron donors, selective chemisorption, hydrogen and furan rings bonds as well as the interaction between carbonyl groups and exchange cations (Wang et al. 2018; Deng et al. 2010).

3.2. Percentage of carcass yield and giblet weight

The regression equations between the level of bentonite, carcass yield, and giblet percentage are presented in Table 3. The positive effects of bentonite addition in diet with a linear pattern ($p<0.001$) resulted in increasing carcass weight percentage, breast weight percentage, leg, bursa of Fabricius, and gizzard. Meanwhile, the percentage of kidney, liver, heart, and pancreas linearly decreased ($p<0.001$) by increasing bentonite level. The increase in the carcass yield revealed the efficacy of bentonite in improving digestive enzymes and nutrient digestibility in the intestine as well as protecting the giblet against aflatoxins, as result, it was able to work normally during digestion, absorption, and metabolism. The efficacy of bentonite may also be recognized in the reduction of liver and kidney size that is susceptible to aflatoxins. This argument is confirmed by Dos Anjos et al. (2015) who claimed that the inclusion of bentonite to aflatoxin B1 diet reduced the prevalence of histological lesions, the proportion of kidney and liver weights of chicks. Aflatoxins are recognized as irritating to the digestive tract and causing intestinal dysfunction, increasing the relative weights of giblets, including liver, kidneys, heart, bursa fabricius, and pancreas (Valchev et al. 2013). Additionally, histological results revealed hepatocellular degeneration lesions in the livers of aflatoxin-exposed birds, as well as vacuolar degeneration and significant fat accumulation caused by decreased lipid transport rather than enhanced lipid biosynthesis (Cruz et al. 2019). In birds, the kidney is involved in the clearance of hazardous metabolic waste products from the blood as well as the preservation of biochemical homeostasis. Because of these activities, the kidney is the primary organ in poultry that accumulates Aflatoxin B1. Several studies have found that Aflatoxin B1 induces renal dysfunction, which might explain the lower 1, 25-dihydroxycalciferol level, calcium, and phosphorus (Gholami-Ahangaran et al. 2016; Fouad et al. 2019). This statement could also explain why the calcium content of the blood increased with bentonite inclusion in this study.

Table 3- Regression equations for the effect of bentonite dose on giblet and carcass yield

			Parameter estimates					*Model statistics*		
Parameter	***Unit***	***n***	***Model***	***Intercept***	***SE intercept***	***Slope***	***SE slope***	***p-value***	***RMSE***	***AIC***
Carcass	%	24	L	60.94	4.24	0.594	0.747	<0.0001	3.376	115.1
Breast	%	22	L	21.81	2.91	0.036	0.294	<0.0001	2.162	84.7
Leg	%	22	L	23.81	3.35	0.116	0.269	<0.0001	1.972	82.4
Bursa of Fabricius	%	34	L	0.15	0.04	0.008	0.009	<0.0001	0.051	-104.9
Gizzard	%	45	L	2.29	0.26	0.028	0.039	<0.0001	0.329	25.2
Heart	%	24	L	0.59	0.03	-0.009	0.026	0.0354	0.143	-34.2
Kidney	%	52	L	0.75	0.08	-0.098	0.098	<0.0001	0.446	17.2
Liver	%	80	L	3.36	0.35	-0.172	0.197	<0.0001	2.353	287.0
Pancreas	%	26	L	0.37	0.049	-0.014	0.013	<0.0001	0.063	-68.1

n: Number of treatments, RMSE: Root mean square error, SE: Standard error, AIC: Akaike information criterion, L: Linear

3.3. Blood constituents

The regression equations between the level of bentonite and metabolites parameters of broiler chickens are presented in Table 4. The increase of dietary bentonite increased total protein, albumin, hemoglobin, aspartate aminotransferase, calcium, glycogen, and globulin (linear pattern; $p<0.001$). There was a linear decrease ($p<0.001$) in uric acid and gamma-glutamyltransferase. Our study shows that the indication of bentonite inclusion in the broiler diet increases the absorption of aflatoxin in the digestive system, which inhibits the decrease of blood serum albumin, globulin, hemoglobin, PCV, and total protein when using a contaminated diet. In previous studies, the effect of bentonite inclusion in the broiler chicken diet increased albumin, total protein, and globulin in the serum (Aghashahi 2015; Shannon et al. 2017). The efficacy of bentonite may be attributed to its composition, which is mostly in the form of adsorption montmorillonite (Pouraboulghasem et al. 2016). Montmorillonite has the ability to inhibit uric acid absorption in the gut by increasing uric acid diffusion from the bloodstream to the intestine (Ma et al. 2009). The addition of bentonite to the aflatoxin diet was also beneficial in avoiding alterations in GGT enzyme concentrations that were highly correlated with the damage of the liver as the main target organ. As a result, the decrease in GGT suggested that bentonite alleviated the negative effects of aflatoxin (Shannon et al. 2017). The decrease in hemoglobin and PCV were markedly reduced in the broiler chicks fed containing aflatoxin reported by Rathod et al. (2017). The low proportion of PCV might be due to aflatoxin-induced hemolysis caused by plasma membrane lipid peroxidation (Umar et al. 2012). Meanwhile, the decrease in hemoglobin concentration during aflatoxicosis might be attributed to a combination of decreased iron absorption and hematopoiesis suppression (Abeena et al. 2015).

Table 4- Regression equations on the influence of bentonite dosage blood parameter

			Parameter estimates					*Model statistics*		
Parameter	***Unit***	***n***	***Model***	***Intercept***	***SE intercept***	***Slope***	***SE slope***	***p-value***	***RMSE***	***AIC***
Albumin	g/dL	51	L	1.32	0.17	0.119	0.106	<0.0001	0.570	49.2
Total Protein	g/dL	51	L	2.74	0.29	0.116	0.185	<0.0001	1.001	103.2
Calcium	mg/dL	15	L	9.92	0.80	0.13	0.09	<0.0001	0.54	19.0
Uric acid	mg/dL	25	L	5.77	0.66	-0.414	0.414	0.0001	2.218	85.1
AST	U/L	15	L	39.96	6.57	1.210	1.281	<0.0001	6.104	85.4
GGT	U/L	21	L	11.49	2.42	-1.891	2.288	0.0009	5.819	109.5
Glycogen	mg/dL	14	L	224.16	17.95	24.846	20.904	<0.0001	56.695	119.2
Globulin	g/dL	22	L	1.69	0.41	0.069	0.144	<0.0001	0.449	18.6
Hemoglobin	g/dL	12	L	9.60	1.34	1.132	2.428	0.0141	2.694	48.0
PCV	%	12	L	34.36	3.74	11.300	13.019	0.2879	14.559	77.5

n: Number of treatments, RMSE: Root mean square error, SE: Standard error; AIC: Akaike information criterion, L: Linear

Generally, by meta-analysis, the use of bentonite benefited broiler performance in an aflatoxin-contaminated diet. Nonetheless, it should be recognized that bentonite is also able to absorb the nutritional content in animal diets including micronutrients as well as veterinary drugs (Alam & Deng 2017). Because bentonite has no specific effects on mycotoxin binders, The European Food Safety Authority (EFSA) has advised that oral veterinary drugs be evaluated in the diet containing a mycotoxin binder to ensure their safety in terms of binding to the drug components. Therefore, EFSA recommended using no more than 20 g/kg of bentonite in the diet with the composition of smectite (>70%), opal and feldspar (<10%), and calcite and quartz (4%) for effectiveness in binding aflatoxin B1 and safe for all animals (EFSA 2012).

4. Conclusion

The bentonite addition has the ability to eliminate the detrimental effects of aflatoxin which may increase the broiler performance (ADG, DFI, FCR, and mortality rate). Bentonite is also effective in improving carcass yield and preventing changes in abnormal giblet weights caused by aflatoxin. However, for effectiveness and animal safety, the EFSA recommends using a maximum of 2% bentonite in the feed.

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Impact of Roasting on Quality and Compositional Characteristics of Fig Seed Oil

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ABSTRACT

This study investigates the effect of roasting time and temperature on the chemical parameters and bioactive compound content of fig seed oil. For this purpose, fig seeds were ground and roasted in an electrical oven at 100, 150 and 200 °C for 10 and 20 minutes. After roasting, the seeds were processed into oil using a laboratory scale mill. The obtained fig seed oil samples were evaluated for their peroxide value, K232 and K270 spectrophotometric indices; fatty acid, triacylglycerol, sterol and tocopherol profiles. That results show that peroxide values, spectrophotometric indices, α- and γ-tocopherol levels were negatively affected by the roasting process. The major tocopherol was γ-tocopherol and ranged in 3914-4016 mg kg-1. The main fatty acid was linolenic acid constituting 40% of the fatty acids; followed by linoleic (31.76-31.97%), oleic (17.06-17.30%) and palmitic (7.00-7.11%) acids. The major triglycerides were LnLO (12.27-12.46%), LnLnL (12.18-12.40%), LnLnLn (9.32-9.52%) and LnLL (9.34-9.50%) confirming the fatty acid profile. β-sitosterol was the predominant phytosterol ranging in 2985.07-3623.24 mg/kg followed by Δ-5-avenasterol (19.52-20.60%) and Δ-7-avenasterol (4.16-4.81%). The principal component analysis results enabled a clear discrimination between unroasted, mildly-roasted and intense-roasted oil samples.

Keywords: γ-tocopherol, Fig seed oil, Roasting, sterol, Triacylglycerol

1. Introduction

Fig (*Ficus carica* L.) is a world-famous fruit tree from the Moraceae family and has been largely cultivated for its fruits (Solomon et al. 2006) because figs have a higher nutritional value than most other fruits (Varhan et al. 2019). The fruit has been consumed both in fresh and dry forms as a dietary food product since at least the earliest civilizations. The various parts of *Ficus carica* L. can be used for a number of different purposes (Palmeira et al. 2019). Fig syrup has been used for therapeutic purposes (Khare 2007), the plant latex is used as curdling agent in the production of various dairy products, the leaves are traditionally used as animal feed and the wood is used for ornamental purposes (Badgujar et al. 2014).

The fruit from fig trees provide high concentrations of biochemicals and antioxidants (Hssaini et al. 2020). Phytochemical studies have shown that the fruit contains phenolic compounds, fatty acids, amino acids, anthocyanins, aliphatic alcohols, organic acids, volatile components, phytosterols, hydrocarbons and a number of secondary metabolites (Badgujar et al. 2014). The fruit is a rich source of phenolics that affect the antioxidant capacity, sensory features and health-promoting properties (Caro and Piga, 2008). Rutin has been reported to be the major phenolic of the figs, followed by (+)-catechin, (−)-epicatechin, chlorogenic acid, syringic and gallic acid (Veberic et al. 2008). The fruit has also been reported to contain iron, calcium, potassium and vitamin K (Joseph & Raj 2011). Due to its prosperous composition, the health attributes of the fig have been the subject of numerous studies and various studies have noted the antispasmodic, antiplatelet (Gilani et al. 2008), anticonstipationon (Kim et al. 2010) and hypoglycemic effects (El-Shobaki et al. 2010) of the fruit. In addition, the anthocyanin content of figs have been shown to prevent obesity, cardiovascular disease and certain cancers in addition to diabetes (Wojdyło et al. 2016). For this reason, consumer demand has increased in recent years for figs and fig-based products (Teruel-Andreu et al. 2021).

Figs contain a broad number of tiny seeds that give the characteristic nutty taste of the fruit. The number of the seeds may be up to 1600 and vary depending on the size and the maturity of the fruit. Fig seeds have been shown to be good sources of carbohydrate (52.62-53.66%), protein (14.74-15.07%), ash (2.99%) and oil (23.06-23.67%). Moreover, the seeds are important reserves of minerals including Mg, Mn, Zn, Fe, Ca, Cu, Na, K and P (Nakilcioğlu-Taş 2018). A reasonable amount of waste is produced during pureeing and juice production from the figs. The obtained by-product is appreciably rich in terms of fig seeds and typically used for obtaining fig seed oil, which is characterized by its high linolenic acid content as well as by its oleic, linoleic, stearic and palmitic acid levels (İçyer et al. 2017). Dietary fat composition is known to play fundamental roles in biological activities and the replacement of saturated fatty acids with polyunsaturated acids has shown to have the potential to eliminate the risk of type-2 diabetes and cardiovascular disorders (Lenighan et al. 2019). Fig seed oil is a viable ingredient for common dietary items as a good source of plant based polyunsaturated fatty acids. In addition to its fatty acid composition, fig seed oil has been designated as a very rich source of γ-tocopherol (Güven et al. 2019). γ-tocopherol has recently gained considerable interest due to its possible complementary effects to those of α-tocopherol with respect to health issues (Wagner et al. 2004).

Oilseeds from diverse sources have gained increased interest with increasing demand in vegetable oils for use as a food ingredient or for cooking purposes. Fats & oils are staple foods in many countries as lipids are critical nutrients that affect the taste & structure of the foods (Issaoui & Delgado 2019). The oils are extracted from the seeds via processing techniques that include cleaning, drying, size reduction, roasting, solvent extraction or pressing steps. Roasting is a key step for assisting the release of oil from the seeds and affects the oxidative stability, quality, nutritional characteristics and shelf life of the oil. Various studies have analyzed the influence of pre-roasting prior to the extraction of oil from sunflower (Goszkiewicz et al. 2020), safflower (Taha & Matthäus 2018), rapeseed (Jing et al. 2020), sesame (Ji et al. 2019), pumpkin (Potočnik et al. 2018), black cumin (Suri et al. 2019a), chia (Ghafoor et al. 2018) and other seeds. However, there is no study showing the effects of the roasting process before the extraction on the stability, quality and chemical composition of fig seed oil. For this reason, the aim of this study is to monitor the changes in composition (fatty acid and triacylglycerol profiles) and bioactive compound content (sterols and tocopherols) of fig seed oil by roasting process before mechanical extraction. Since the heat intensity affects the composition of the oil, different roasting temperatures (100, 150, 200 °C) and periods (10, 20 min) were considered.

2. Material and Methods

2.1. Materials

Fig seeds were obtained from a local supplier (Egesia Natural Products, Aydın/Turkey). Sodium chloride, potassium hydroxide, sodium thiosulphate, acetic acid, chloroform, ethyl alcohol, acetone, acetonitrile, pyridine, chloroform and phenolphthalein were from Merck (Darmstadt, Germany). n-hexane, isooctane, diethyl ether, hydrochloric acid, methanol, sodium hydroxide, 2,2-diphenyl-1-picrylhydrazyl, α-,β-, γ- and δ-tocopherol standards, 5α-cholestan-3β-ol and β-sitosterol were purchased from Sigma-Aldrich (St-Louis, USA). N,O-Bis(trimethylsilyl)trifluoroacetamide-BSTFA with trimethylchlorosilane and 37 fatty acid methyl ester mix were from Supelco (Bellefonte, USA). The reagents used for high-performance liquid chromatography (HPLC) analyses were of HPLC grade.

2.2. Methods

2.2.1. Roasting of fig seeds and oil extraction

The fig seeds were first milled in an electrical coffee miller (Sinbo, Turkey) and then roasted at 100, 150 and 200 °C for 10 and 20 minutes in an electrical oven (Nüve, Turkey). After the roasting process, the milled seeds were cooled to room temperature. The oil samples were obtained by pressing with a laboratory scale (12 kg seed h-1 capacity, single head, 1.5 kW power, 2 hp) screw press (Koçmaksan KMS 10; Izmir, Turkey). After the extraction, the oils were centrifuged and then stored in dark glass bottles at 4 ºC in nitrogen atmosphere until analyses.

2.2.2. Peroxide value

The peroxide values of the samples were determined according to AOCS Official Method Cd 8-53. Briefly, 1 g of oil was weighed in a 250 mL erlenmayer flask with glass stopper and dissolved with 30 mL of acetic acid-chloroform solution (3:2 v/v). Saturated potassium iodide solution (0.5 mL) was poured into that solution and agitated gently for 1 min followed by the addition of 30 mL of distilled

water. The mixture was then titrated with 0.01 N $Na_2S_2O_3$ until the yellow color faded. Starch indicator was added and the titration was continued until the blue color disappeared. The peroxide value was calculated using the following equation:

PV (meq O_2/kg oil)=(S-B)xNx100/mass of sample (g)

B: volume of titrant, mL of blank

S: volume of titrant, mL of sample

N: normality of sodium thiosulfate solution

2.2.3. UV spectrophotometric indices (K232 and K270)

The K232 and K270 values were obtained according to American Oil Chemists' Society (AOCS) Official Methods Ch 5-91 (AOCS 2003). Each oil sample was weighed in a 10 mL volumetric flask and diluted in isooctane. The resulting solution was filled in quartz cuvette (optical light path of 1 cm) and the extinction values were determined using a ultraviolet-visible (UV-VIS) spectrophotometer (UV-1800, Shimadzu, Japan).

2.2.4. Antioxidant activity

The antioxidant activity of fig seed oils was determined utilizing the free radical diphenylpicrylhydrazyl (DPPH) method of Atoui et al. (2005). For this purpose, 2.5 g of fig seed oil was diluted with 2.5 mL of methyl alcohol. Fifty microliters of the extract was mixed with 1.950 mL of DPPH solution (6x10-5 M). The mixture was kept in dark cabinet at 25 °C for 60 minutes. After the incubation period, the absorbance was measured at 515 nm with a UV-VIS spectrophotometer (UV-1800, Shimadzu, Japan), using methyl alcohol as blank. The antioxidant activity was determined using the equation given below and expressed as the percentage inhibition of the DPPH radical:

AA (%)=[(Abscontrol – Abssample) x 100]/(Abscontrol)

2.2.5. Tocopherol composition

The oil sample of 1.0 g was dissolved in 10 mL of n-hexane. The tocopherol profile was determined with an HPLC instrument (Shimadzu, Kyoto, Japan) equipped with an InertSustain NH2 column (250 mm x 4.6 mm, 5 µm particle size, GL Sciences, Tokio, Japan). The mobile phase was was n-hexane:isopropyl alcohol (99.5:0.5) with a flow rate of 1.2 mL min-1. The peaks were detected with a UV detector and the detection wavelength was 290 nm. The injection volume was 20 µl. Tocopherol homologoues were identified and quantified using external standards. The results were given as mg kg-1.

2.2.6. Fatty acid composition

The methyl esters of fatty acids were prepared using the method of the International Union of Pure and Applied Chemistry (IUPAC) (IUPAC 1987). The oil sample of 0.4 g was dissolved in 4 mL isooctane and later mixed with 0.2 mL of 2 N methanolic KOH. The mixture was agitated and kept in the dark for 6 min. 0.45 mL of 1 N HCl solution was added to the mixture with a few drops of methyl orange. The mixture was left for 30 min and the upper layer was collected for chromatographic analyses. The esters were then analysed with a gas chromatograph (GC 2010, Shimadzu/Japan). DB-23 fused silica capillary column (60 m x 0.25 mm i.d. and 0.25 µm film thickness) (J&W Scientific) was used for the elution of individual fatty acids. A FID was used to detect the peaks. The carrier gas was nitrogen (99.99% purity) with a flow rate of 1 mL min-1 and the split ratio was 80:1. The detector, column and injector temperatures were 240 °C, 195 °C and 230 °C, respectively. The fatty acid peaks were identified by comparing the retention times with the ones obtained for the respective standards. The results were expressed as a percent of the total methylated fatty acids.

2.2.7. Triacylglycerol composition

The triacylglycerol composition of the fig seed oils was determined according to the AOCS Official Method Ce 5b-89 (AOCS 2003). 0.5 g of oil was dissolved in 10 mL acetone. A chromatographic analysis of the oil solution was carried out using a HPLC instrument (Shimadzu, Kyoto, Japan) equipped with a differential refractometer detector (RID). An ACE 5 C18 column (250×4.6×5µm particle size, ACE, Aberdeen, Scotland) was employed for the elution of the peaks. The mobile phase was acetone:acetonitrile (100:100)

with a flow rate of 1.5 mL min-1 and the injection volume was 10 µl. The oven temperature was 30 °C. Triacylglycerol peaks were identified through comparison with literature data (Holčapek et al. 2005). The results were expressed as area % of the total triacylglycerols.

2.2.8. Sterol composition

The sterol composition of the oils was determined according to AOCS Official method Ch 6-91 (AOCS 2003). The sterol fraction was obtained through saponification with ethanolic potassium hydroxide solution, then extracted three times with diethyl ether and separated by thin layer chromatography. Afterwards, trimethyl silyl ether derivatives of the sterols were analyzed using a gas chromatograph (GC 2010, Shimadzu, Japan) equipped with a flame ionisation detector (FID). Then, the sterol fractions were separated with a HP-5 fused silica capillary column (30 m, 0.25 mm i.d. and 0.25 micrometer film thickness, Chrom Tech., Apple Walley, M, USA). Injector, column and detector temperatures were 280 °C, 260 °C and 290 °C, respectively. Nitrogen was the carrier gas with a flow rate of 0.8 mL min-1. The split ratio was adjusted as 50:1. 5α-cholestan-3β-ol was used as the internal standard for quantification.

2.2.9. Statistical analysis

The statistical analysis was carried out using SPSS 15 packaged software (SPSS Inc., Chicago, USA). Data were evaluated by one-way analysis of variance (ANOVA) using Duncan's multiple range test to check if there were any significant differences among the analysed parameters. A p value of less than 0.05 was considered as significant. Data were also processed by principal component analysis (PCA) using XLSTAT 2021 version (Addinsoft, New York, NY, USA).

3. Results and Discussion

The changes in peroxide values, spectrophotometric indices, tocopherol contents and antioxidant activities of the oils obtained from seeds roasted at different temperatures and times were given Table 1. The peroxide value, which is a measure of the hydroperoxides (primary products of the lipid oxidation) in the oil, increased with the ascending roasting times and temperatures. A significant difference was observed in the peroxide value when the roasting time was extended from 10 to 20 minutes at 200 °C ($p<0.05$). The K232 value, an indicator of the formation of the conjugated dienes, statistically remained unchanged at 100 and 150 °C of roasting temperatures; however, a significant increase was determined with increasing process time at 200 °C ($p<0.05$). K270, a marker of secondary oxidation products namely aldehydes and ketones, had a similar trend with K232 and increased statistically at the most intense roasting conditions ($p<0.05$).

Table 1- Peroxide value, UV spectrophotometric indices and tocopherol contents of oils obtained from fig seeds roasted at different temperatures and times (%)

Roasting temperature (°C)	*Roasting time (min)*	*Peroxide value (meq O2 kg^{-1} oil)*	*K232*	*K270*	*α-tocopherol (mg kg^{-1})*	*γ-tocopherol (mg kg^{-1})*	*Antioxidant activity (%)*
Unroasted		6.23±2.33A	2.81±0.09A	0.83±0.06A	114.07±0.89A	3952.39±7.64A	92.55±1.85A
100	10	10.71±1.27BC	2.86±0.17A	0.87±0.13A	108.47±2.91B	3889.77±10.23A	90.57±1.77A
	20	10.75±1.23BC	2.98±0.16A	0.73±0.09A	108.99±3.82B	3960.04±91.43A	90.82±1.66A
150	10	9.27±0.47B	3.12±0.13A	0.88±0.05A	107.14±1.96B	3954.73±48.29A	90.13±1.27A
	20	10.75±0.54BC	3.26±0.13A	0.81±0.27A	108.09±1.61B	3914.25±61.17A	90.57±1.10A
200	10	12.18±0.51C	3.12±0.05A	0.95±0.03A	108.86±1.51B	4016.40±133.08A	89.53±5.72A
	20	16.26±2.38D	4.86±0.85B	1.95±0.40B	101.63±2.29C	3629.66±123.86B	77.32±3.40B

Data in the same column followed by different letters are significantly different at $p<0.05$

Tocopherols are efficient antioxidants responsible for the stability of edible oils. α-, β-, γ-, δ- tocopherols are known to be different forms of vitamin E, and α-tocopherol is the preferred one due to its higher vitamin E activity (Delgado et al. 2020). The fig seed oil samples contained 101.63-114.07 mg kg-1 of α-tocopherol, slightly higher than the findings of İçyer et al. (2017) and Baygeldi (2018) who reported 46 mg kg-1 and 4.6 mg 100 g-1 of α- tocopherol for fig seed oils, respectively. The major tocopherol was γ-tocopherol ranging in 3914-4016 mg kg-1 in accordance with former works (Şentürk and Karaca 2021; Tarlacı 2021). The α-tocopherol contents of fig seed oils decreased by 10.90 % and γ-tocopherol quantities decreased by 8.17% through the roasting process indicating a possible

destruction in tocopherol homologues which may be attributed to thermal induced oxidation (Ji et al. 2019). The highest loss was determined at the most intense roasting process with the highest temperature (200 °C) and longer process time (20 min). Time was more effective on the reduction of both α- and γ-tocopherol contents when the temperature was increased.

The antioxidant activity of the unroasted fig seeds was 92.55% and decreased by the raise in roasting temperatures. The lowest antioxidant activity was determined at sample roasted at 200 °C for 20 minutes. Güven et al. (2019) reported lower antioxidant capacity (52.54%) for fig seed oil. The decrease in antioxidant activity is likely due to the loss of tocopherols by the extended roasting process. Antioxidant activity was found to be high and positively correlated with α- (r=0.87) and γ- (r=0.92) tocopherol contents.

The changes in fatty acid composition by roasting at different temperatures and times, were presented in Table 2. The major fatty acid was linolenic acid ranging among 40.68-40.95% in accordance with previously published studies (İçyer et al. 2017; Duman & Yazıcı 2018; Duman et al. 2018). The variation in roasting conditions did not cause any significant change on the linolenic acid ratio of the oil samples ($p>0.05$). Linoleic acid was the second dominating fatty acid covering 31.76-31.97% of the fatty acids and was found to be the lowest in unheated oil sample. Oleic acid was the main monounsaturated fatty acid with a range of 17.06-17.30%, and, similar with the findings of Nakilcioğlu-Taş (2018), found to be the highest at the most intense roasting conditions. Modest changes were determined for palmitoleic, heptadecanoic and arachidic acids; wheras myristic, palmitic, heptadecenoic, stearic and gadoleic acids remained unchanged. Previous studies have revealed either no marked differences between unroasted and roasted seeds (Ji et al. 2019; Zhang et al. 2020), or slight modifications in the fatty acid profiles of various oils through a roasting process (Hama 2017; Suri et al. 2019b).

Table 2- Fatty acid composition of oils obtained from fig seeds roasted at different temperatures and times (%)

Fatty acids	*Unroasted*	*100 °C*		*150 °C*		*200 °C*	
		10 min	*20 min*	*10 min*	*20 min*	*10 min*	*20 min*
C 14:0	0.02±0.01A	0.01±0.00A	0.01±0.00A	0.01±0.00A	0.01±0.00A	0.01±0.00A	0.01±0.00A
C 16:0	7.02±0.08A	7.06±0.04A	7.08±0.06A	7.00±0.03A	7.04±0.09A	7.00±0.07A	7.11±0.03A
C 16:1	0.12±0.02A	0.14±0.03AB	0.18±0.01B	0.15±0.00AB	0.13±0.04AB	0.15±0.04AB	0.18±0.02AB
C 17:0	0.03±0.01A	0.04±0.01AB	0.04±0.00AB	0.04±0.00AB	0.04±0.00A	0.03±0.00B	0.04±0.00AB
C 17:1	0.02±0.01A	0.02±0.00A	0.03±0.01A	0.02±0.01A	0.03±0.01A	0.02±0.00A	0.02±0.01A
C 18:0	2.53±0.04A	2.50±0.05A	2.45±0.03A	2.52±0.06A	2.50±0.06A	2.49±0.05A	2.50±0.03A
C 18:1	17.23±0.27AB	17.13±0.10AB	17.06±0.10AB	17.21±0.05AB	17.10±0.04AB	17.17±0.08AB	17.30±0.06B
C 18:2	31.76±0.09A	31.94±0.10B	31.97±0.09B	31.90±0.08AB	31.92±0.03AB	31.90±0.12AB	31.89±0.06AB
C 18:3	40.74±0.29A	40.86±0.09A	40.95±0.08A	40.83±0.11A	40.95±0.19A	40.93±0.12A	40.68±0.09A
C 20:0	0.10±0.02AB	0.11±0.01B	0.08±0.01A	0.11±0.01B	0.10±0.02AB	0.11±0.01B	0.10±0.01AB
C 20:1	0.18±0.03A	0.19±0.01A	0.16±0.01A	0.13±0.01A	0.18±0.02A	0.19±0.02A	0.18±0.02A

Data in the same line followed by different letters are significantly different at $p<0.05$

Triglycerides are the major components of edible oils and represent 95-99% of the structure. The changes in the triacylglycerol composition of fig seed oils during roasting were given in Table 3. The major triglycerides of fig seed oils were determined to be LnLO (oleolinoleolinolenin), LnLnLn (trilinolenin), LnLLn (linoleodilinolenin) and LnLL (dilinoleolinolenin). Additionally, LnLnO (oleodilinolenin), LnLP (palmitolinoleolinolenin), LLO (oleodilinolein), SLLn (stearolinoleolinolenin), LnLnP (palmitodilinolenin), LLP (palmitodilinolein), LOP (palmitooleolinolein), LOO (dioleolinolein), LLL (trilinolein), LnOO (dioleolinolenin), LnLnS (stearodilinolenin), SLO (stearooleolinolein), SOLn (stearooleolinolenin) and OOO (triolein) were detected in smaller ratios. The major triglyceride, LnLO ranged in 12.27-12.46% and slightly increased by the roasting process. The other three main triglycerides, namely LnLnL, LnLnLn and LnLL, varied in 12.18-12.40%, 9.32-9.52%, 9.34-9.50% respectively and decreased by roasting at 200 °C for 20 minutes. LnLnP, LnLnS, LLO, LOP, SOLn, OOO, SLO were determined to remain unchanged, wheras slight changes were detected for the remaining triacylglycerols.

Table 3- Triacylglycerol profile of oils obtained from fig seeds roasted at different temperatures and times (%)

Triacylglycerols	*Unroasted*	*100 °C*		*150 °C*		*200 °C*	
		10 min	*20 min*	*10 min*	*20 min*	*10 min*	*20 min*
LnLnLn	9.52±0.07A	9.45±0.03A	9.50±0.04A	9.48±0.02A	9.43±0.07AB	9.47±0.14A	9.32±0.09B
LnLnL	12.40±0.13A	12.38±0.04A	12.36±0.05A	12.38±0.06A	12.29±0.07AB	12.38±0.11A	12.18±0.12B
LnLL	9.47±0.07AB	9.50±0.07A	9.45±0.11AB	9.37±0.04AB	9.34±0.12B	9.48±0.13AB	9.39±0.06AB
LnLnO	8.73±0.13AB	8.73±0.09AB	8.81±0.05AB	8.76±0.07AB	8.86±0.12A	8.75±0.13AB	8.68±0.08B
LnLnP	4.92±0.05A	4.93±0.01A	4.92±0.06A	4.87±0.04A	4.90±0.05A	4.86±0.08A	4.90±0.07A
LLL	3.86±0.10A	3.83±0.05AB	3.74±0.06ABC	3.78±0.09ABC	3.59±0.12D	3.68±0.07CD	3.72±0.08BCD
LnLO	12.27±0.13A	12.32±0.05AB	12.39±0.12AB	12.38±0.11AB	12.46±0.09B	12.39±0.05AB	12.37±0.08AB
LnLP	7.13±0.10A	7.18±0.04A	7.15±0.12A	7.16±0.09A	7.12±0.16A	6.94±0.11B	7.03±0.14AB
LnLnS	2.24±0.04A	2.16±0.05A	2.01±0.19A	2.09±0.22A	2.05±0.15A	2.03±0.19A	2.24±0.19A
LLO	6.79±0.06A	6.76±0.08A	6.86±0.15A	6.78±0.05A	6.87±0.08A	6.89±0.05A	6.88±0.07A
LnOO	3.02±0.20A	2.85±0.09AB	2.84±0.03AB	2.80±0.04B	2.76±0.16A	2.81±0.12A	2.86±0.09AB
LLP	4.05±0.07A	4.30±0.09B	4.33±0.09B	4.27±0.10B	4.29±0.09B	4.36±0.10B	4.33±0.07B
SLLn	5.41±0.14A	5.44±0.05A	5.64±0.17B	5.64±0.05B	5.70±0.09B	5.77±0.03B	5.73±0.09B
LOO	3.15±0.12AB	3.12±0.09AB	3.05±0.11A	3.26±0.21AB	3.33±0.15B	3.28±0.21AB	3.31±0.18AB
LOP	4.08±0.04A	4.05±0.05A	4.01±0.03A	4.06±0.07A	4.08±0.10A	4.04±0.07A	4.10±0.06A
SOLn	0.74±0.05A	0.77±0.04A	0.73±0.06A	0.72±0.08A	0.71±0.06A	0.69±0.04A	0.74±0.02A
OOO	0.66±0.01A	0.63±0.03A	0.66±0.01A	0.64±0.04A	0.65±0.01A	0.66±0.05A	0.65±0.03A
SLO	1.58±0.02A	1.60±0.03A	1.55±0.01A	1.55±0.10A	1.59±0.04A	1.52±0.03A	1.58±0.0A

Data in the same line followed by different letters are significantly different at $p<0.05$

The sterol profiles of fig seed oils obtained by oven-roasting are given in Table 4. The main sterols of fig seed oils were β-sitosterol, Δ-5-avenasterol and Δ-7-avenasterol. In addition, campesterol, sitostanol, clerosterol, stigmasterol, 24-methylene-cholesterol, Δ-7-stigmastenol, campestanol, Δ-5-24-stigmastadienol and Δ-7-campesterol were detected in lower amounts. The total sterol contents varied in 4582.46 and 5499.98 mg kg-1 and the extension in roasting time increased the total sterol contents of fig seed oils at 100 and 200 °C of processing ($p<0.05$). The results may be attributed to the damage of the cell membrane that increases the release of sterols and enrich their contents in the extracted oil, as was also previously shown by Azadmard-Damirchi et al. (2010). Güven et al. (2019) determined 6516.20 mg kg-1 of sterols in fig seed oil samples. Roasting process has been reported to cause either increments (Rękas et al. 2015) or decreases (Amaral et al. 2006) in the total sterol contents of resulting oils. β-sitosterol accounted for 64.66-65.85% of the sterols and had a similar tendency with the total sterol content. The prolongation of process time, increased β-sitosterol content at 100 and 200 °C of roasting ($p<0.05$). Δ-5-avenasterol was the second dominating sterol and covered 19.52-20.60% of the sterols. Different process temperatures and times were found to be uninfluential on Δ-5-avenasterol content. Δ-7-avenasterol was the third noticeable sterol ranging in 4.16-4.81% and was not affected by the roasting process. Campesterol and stigmasterol were the other two sterols varying in 2.92-3.15% and 2.20-2.45%, respectively. Sitostanol was not significantly affected by the roasting process, whereas modest changes were determined for the remaining 24-methylene cholesterol, campestanol, Δ-7-campesterol, Δ-5,24 stigmastadienol clerosterol and Δ-7-stigmastenol.

Table 4- Sterol content of oils obtained from fig seeds roasted at different temperatures and times (mg kg^{-1})

Sterols	*Unroasted*	*100 °C*		*150 °C*		*200 °C*	
		10 min	*20 min*	*10 min*	*20 min*	*10 min*	*20 min*
24-methylene cholesterol	15.54±3.04AB	12.33±4.58A	18.69±3.01B	15.26±2.68AB	16.81±1.61AB	16.35±2.43AB	17.11±4.00AB
Campesterol	150.69±8.62ABC	134.85±2.99A	172.01±25.06C	154.42±7.75ABC	146.31±5.24AB	142.75±9.51AB	165.90±20.53BC
Campestanol	1.13±0.63A	1.06±0.64A	2.14±0.53AB	1.48±0.36A	1.31±0.58A	1.57±0.32A	2.69±1.26B
Stigmasterol	115.92±13.30AB	105.63±2.10A	119.93±10.59AB	116.24±6.12AB	118.11±5.93AB	109.59±6.80AB	121.38±10.59B
Δ-7-campesterol	5.41±0.60AB	6.10±1.33AB	6.87±1.03AB	5.21±1.30A	7.10±1.26B	6.05±0.81AB	6.14±0.45AB

Table 4- Continued

Sterols	*Unroasted*	*100 °C*		*150 °C*		*200 °C*	
		10 min	*20 min*	*10 min*	*20 min*	*10 min*	*20 min*
Clerosterol	29.26±3.66AB	25.53±0.65A	33.15±3.36B	25.33±4.33A	28.71±2.38AB	26.48±3.51A	30.52±1.79AB
β-sitosterol	3314.47±585.85AB	2985.07±96.37A	3623.24±319.16B	3448.72±227.99AB	3173.30±293.05AB	2990.86±205.45A	3616.35±513.77B
Sitostanol	15.13±4.33A	15.51±9.79A	15.99±7.07A	11.85±1.70A	16.30±3.61A	18.97±6.58A	14.10±7.25A
Δ-5-avenasterol	990.86±129.97A	950.82±11.73A	1048.55±100.65A	1055.15±45.62A	934.97±105.67A	914.35±70.30A	1072.70±144.76A
Δ-5,24 stigmastadienol	95.04±4.22A	85.88±2.16A	95.21±9.23A	90.00±4.41A	88.83±8.54A	83.47±7.63A	123.59±39.40B
Δ-7-stigmastenol	55.24±8.26A	50.79±3.36A	56.51±5.58AB	50.27±3.62A	53.26±5.76A	48.49±3.94A	68.42±17.86B
Δ-7-avenasterol	227.86±31.07A	242.25±18.69A	263.63±14.98A	242.05±32.21A	244.02±29.05A	223.52±19.13A	261.09±22.82A
Total sterols	5018.69±751.63AB	4615.83±85.75A	5455.92±434.04B	5215.98±316.79AB	4829.04±427.41AB	4582.46±207.49A	5499.98±778.20B

Data in the same line followed by different letters are significantly different at $p<0.05$

The PCAs is a multivariate analysis method that reduces the variables to a smaller number of factors with maximum variation. In this study PCA was carried out to provide an overview of the roasting pretreatment, oil quality and compositional parameters. To perform the analysis, analytical data were arranged in a matrix. The variables which had the Kaiser-Meyer-Olkin measure of sampling adequacy index lower than 0.5 were removed and the remaining adequate variables were the peroxide value, K232, K270, α- and γ-tocopherols, antioxidant activity, C14:0, C16:0, C16:1, C17:0, C17:1, C18:0, C18:1, C18:2, C18:3, C20:0, LnLnLn, LnLnL, LnLnO, LLL, LnLO, LnLP, LnLnS, LLO, LnOO, LLP, SLLn, LOO, SOLn, 24-methylene cholesterol, campesterol, stigmasterol, campestanol, Δ-7-campesterol, β-sitosterol, clerosterol, sitostanol, Δ-5-avenasterol, Δ-5,24 stigmastadienol, Δ-7-avenasterol, Δ-7-stigmastenol and total sterols. The factor score plot is given in Figure 1. The first two factors explained 62.83% of the total variance (Factor 1: 37.70%, Factor 2: 25.13%). F1 showed high and positive correlations with peroxide value, K232, C16:1, campestanol, Δ-5,24-stigmastadienol, Δ-7-stigmastenol and Δ-7-avenasterol. The factor score plot showed that unroasted and intense-roasted (200 °C, 20 min) oil samples were clearly discriminated from the others. Mildly roasted samples for a short period (100 °C and 150 °C for 10 minutes) were clustered together, whereas a similar group was detected for samples roasted for longer time (100 °C and 150 °C for 20 minutes).

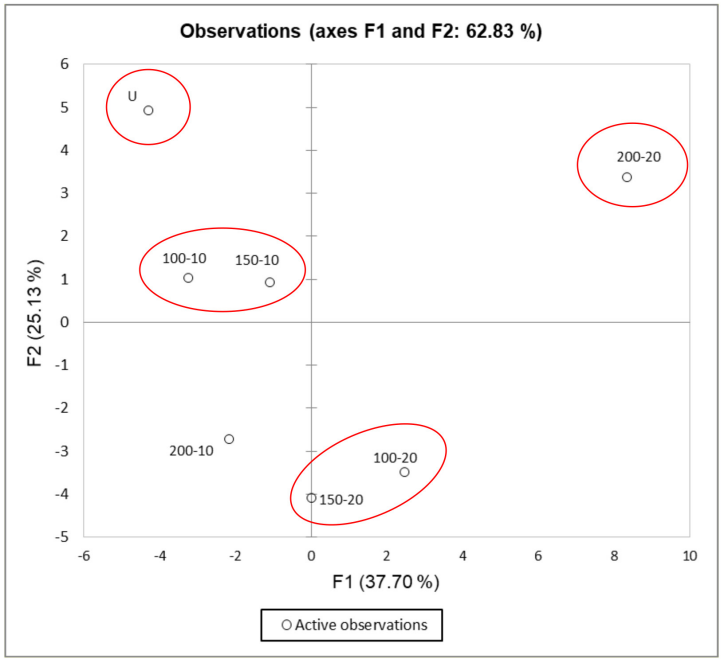

Figure 1- PCA score plot presenting the relations between unroasted and roasted (100, 150 and 200 °C for 5, 10and 15 minutes) fig seed oils

4. Conclusions

The current study reports the changes in quality parameters, antioxidant activity, chemical composition and bioactive contents of the fig seed oil by roasting process. Significant losses were detected in quality parameters, antioxidant activities and tocopherol contents of oils by roasting process. Although modest changes were observed for fatty acid, triacylglycerol and sterol profiles of oils; no clear pattern was determined by increasing time and temperature. Principle component analysis enabled an obvious separation between unroasted, mildly heated and intense heated samples. A great number of studies have been published about the effect of roasting on the composition of different vegetable oils. Yet, this is the first report investigating the effect of roasting process and parameters on fig seed oil. Further studies should be conducted to investigate the unenlighted compositional parameters of fig seed oil.

Data availability: Data are available on request due to privacy or other restrictions.

Authorship Contributions: Concept: A.Y., Design: D.D.Ş., A.Y., Data Collection or Processing: A.Y.V., D.D.Ş., A.Y., Analysis or Interpretation: A.Y.V., D.D.Ş., A.Y., Literature Search: A.Y.V., A.Y., Writing: A.Y.

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Genome-wide Analysis and Characterization of *Eucalyptus grandis* TCP Transcription Factors

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ABSTRACT

Teosinte branched/Cycloidea/Proliferating cell factors (TCPs), a small transcription gene family, serve in developmental processes such as branching, flowering, and growth of plants. In this study, the TCP transcription gene family of eucalyptus, which is considered important for its medicinal and industrial uses, was bioinformatically investigated. A total of 16 *Eucalyptus grandis* TCP (Egra-TCP) genes were found to be distributed on chromosomes 1, 2, 4, 6, 7, 9, 10 and 11. Several segmentally-duplicated gene couples including Egra-TCP-7/Egra-TCP-11, -13 and -16, Egra-TCP-6/Egra-TCP-12 and -15, Egra-TCP-12/Egra-TCP-15 and Egra-TCP-11/Egra-TCP-13 were discovered. Egra-TCPs were divided into three main clades based on phylogenetic analysis, motif, and gene structure. While Egra-TCP-10 has the highest molecular weight with 47.19 kDa, the lowest was Egra-TCP-1 with 21.68 kDa. Twelve Egra-TCP genes were found to have no introns, while the Egra-TCP-7, -15, and -16 genes had a single intron. The orthologous relationships among *E. grandis*/*Arabidopsis thaliana* and *E. grandis*/*Vitis vinifera* were identified through a synteny analysis. Digital gene expression profiles of Egra-TCP genes in tissues such as xylem, phloem, shoot tips, young and mature leaf revealed a high expression pattern. The findings of this study contributes to existing knowledge in the biotechnology field by providing contributing to our understanding of the molecular basis of the TCP gene family in the eucalyptus plant.

Keywords: bHLH domain, CYC/TB1 clade, Digital gene expression, Phylogenetic analysis, TCP transcription factors

1. Introduction

Teosinte branched/Cycloidea/Proliferating cell factor (TCP) is a plant-specific transcription factor (TF). TCPs consists of teosinte branched 1 from corn, Cycleoidea from *Antirrhinum majus*, and PCF (proliferating cell nuclear antigen factor) from *Oryza sativa*. The members of TCP contain a DNA binding motif called the TCP domain, and a basic helix loop helix conserved at the N-terminal end (Ilhan et al. 2018; Zheng et al. 2018). TCP TF plays a role in embryonic development, leaf development, branching, flowering, circadian rhythm, hormone, and stress response mechanisms in plants (Liu et al. 2018; He et al. 2020). The gene family of TCP is divided into two subgroups, class I includes PCF or TCP-P, while class II includes CYC/TB1 and CIN (CINCINNATA) or TCP-C (Ilhan et al. 2018; Ding et al. 2019; Leng et al. 2019; Lin et al. 2019). Members of the class I group differ from class II in the 4 amino acid sequences. Some class II members, however, contain an arginine-rich R domain, which is thought to be involved in protein-protein interaction (Wang et al. 2018; Jiu et al. 2019).

Class I TPCs such as TCP20 in *Arabidopsis* and PCF1/PCF2 in rice (*Oryza sativa*) are involved in increasing plant growth and cell proliferation (Xu et al. 2014). When plants with mutant TCP genes were compared with wild-type plants, no phenotypical difference was detected. It is also known that TCP20 is involved in leaf senescence and jasmonic acid synthesis pathway, while TCP16 is involved in other stages of floral development (Li et al. 2005; Danisman et al. 2012; Wang et al. 2018). In *Arabidopsis*, some TCPs, such as TCP7, 8, 22, and 23, have near expression levels in young leaves (Aguilar-Martinez & Sinha 2013).

Class II TCPs are examined in 2 subgroups, the CYC/TB1 and CIN (Martin-Trillo & Cubas 2010; Feng et al. 2019; Jiu et al. 2019). Unlike class I, class II TCPs have been found to inhibit mutant cell growth and proliferation (Jiu et al. 2019). *Arabidopsis* and tomato CIN clade mutants showed that leaf blade cells divide for a longer period of time and show larger leaves and shape changes when compared to wild types (Jiu et al. 2019; Leng et al. 2019). The TB1 gene inhibits lateral branching and growth in maize and increases lateral branching in mutants (Doebley et al. 1997). Class II TCP gene family members have roles in the response mechanism to abiotic stress conditions (Ilhan et al. 2018). For this reason, some of the TCP TF is targeted by miR319 (Palatnik et al. 2003; Nag et al. 2009; Xu et al. 2014).

Eucalyptus, the most dominant genus in the Australian flora, belongs to the *Myrtaceae* family. The genus contains more than 800 species and dominates the much of Australia (Macphail & Thornhill 2016). With its rapid growth and superior tree characteristics, eucalyptus is used for tree planting in more than 100 countries on six continents. *Eucalyptus grandis* and *E. globulus* are preferred in breeding programs worldwide (İlhan 2018). Eucalyptus offers renewable resources for the paper industry, biomaterials, and bioenergy production; its high concentrations of mono- and sesquiterpenes also provide ecological functions as well as medical and industrial uses (Myburg et al. 2014).

So far, TCP TF members have been detected in *Arabidopsis* (Li 2015), *O. sativa* (Yao et al. 2007), *Solanum licopersicum* L. (Parapunova et al. 2014), cotton (Zheng et al. 2018), apple (Xu et al. 2014), sorghum (Francis et al. 2016), common bean (Ilhan et al. 2018), soybean (Feng et al. 2018), wheat (Zhao et al. 2018), grape (Jiu et al. 2019; Leng et al. 2019; Min et al. 2018), alfalfa (Wang et al. 2018), carrot (Feng et al. 2019). While the importance of TCP genes on growth and development is widely-known, a detailed analysis has yet to be performed on *Eucalyptus grandis*. In this study, a comparative bioinformatics and in silico gene expression analysis in different tissues of the TCP gene family members in *Eucalyptus grandis* was performed,and putative Egra-TCPs were identified by genome-wide scans. Additionally, phylogenetic relationships, chromosomal distribution, gene structures, conserved motif, and cis-acting element analyzes were performed. The findings from this study will allow for a better understanding of the potential functions and classification of Egra-TCPs. In addition, it aid future functional studies of the eucalyptus plant.

2. Material and Methods

2.1. Identification of TCP proteins in the eucalyptus genome

The Pfam Accession Number for TCP TF (PF03634; https://www.ebi.ac.uk/interpro/entry/pfam/PF03634/) was obtained from the Pfam database. Protein sequences of the TCP gene family in the genome of Eucalyptus (Myburg et al. 2014), *Arabidopsis thaliana* (Lamesch et al. 2012), and *Vitis vinifera* (Jaillon et al. 2007) were retrieved from Phytozome Database v13 (https://phytozome-next.jgi.doe.gov/) using the Pfam Accession Number PF03634. Both blastp in the Phytozome Database v13 and a hidden Markov model (HMM) (http://www.ebi.ac.uk) were used to scan the *E. grandis* genome with default parameters to find all probable TCP proteins in the eucalyptus genome. TCP protein sequences belonging to maize (*Zea mays*), common snapdragon (AmCYC, AmCIN) and rice (*Oryza sativa*) (OsPCF1: LOC_Os04g11830, OsPCF2: LOC_Os08g43160) were obtained form Ilhan et al. (2018). The molecular weight and theoretical isoelectric point (pI) of the obtained Egra-TCP proteins were determined using the "ProtParam tool" (https://web.expasy.org/protparam/) according to Kasapoğlu et al. (2020).

Structure, physical locations, gene duplications, identification of conserved motifs, and phylogenetic analyzes of Egra-TCP genes

The Gene Structure Display Server v2.0 (http://gsds.gao-lab.org/) was utilized to define on the exon and intron regions of the Egra-TCP proteins (Hu et al. 2015). Genomic and coding DNA sequences have been used to predict the position information of Egra-TCP genes. Using the Phytozome Database v13, the chromosomal locations and sizes of the Egra-TCP genes were determined. All TCPs were mapped onto the Eucalyptus chromosomes by Circos (Krzywinski et al. 2009; a syntonic map was subsequently displayed by TBtools) (Chen et al. 2020).

The "Multiple EM for Motif Elimination (MEME) Tool" was used to identify additional conserved motifs of the Egra-TCP proteins (Bailey et al. 2006). The parameters of the MEME tool were set as previously described (Ilhan et al. 2018). Identified motifs were scanned using the default settings of the InterPro database (Quevillon et al. 2005). In addition, for conserved region sequence analysis, sequence logo analyzes of the bHLH domains were drawn using the WEBLOGO online web tool (Crooks et al. 2004).

Phylogenetic analyzes were performed according to the neighbor-joining (NJ) method with 1000 replicated bootstrap values. A protein sequence alignment of the Egra-TCP was performed using ClustalW (Thompson et al. 1997). In addition, a phylogenetic tree was

obtained using the MEGA v7 program (Kumar et al. 2016). The tree was shaped using the Interactive Tree of Life (iTOL) interface (Letunic & Bork 2011).

2.2. Subcellular localization and promoter analyses of eucalyptus TCP gene family

PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used to perform cis-acting element analysis in 5' upstream regions (Supplementary File 1) encompassing roughly 2 kilobases DNA segments of each sequence of the Eucalyptus TCP gene members (Lescot et al. 2002), after which TBTools software was used to draw the phenogram (Chen et al. 2020). Subcellular localizations were estimated via WoLFPSORT (Horton et al. 2007).

2.3. The Orthologous Relationships

Using MCScanX (Wang et al. 2012) with default parameters, gene duplication events between *E. grandis*, *A. thaliana,* and *V. vinifera* were determined. The substitution rates of Ka (non-synonymous substitution rate), Ks (synonymous substitution rate), and Ka/Ks between duplicate pairs of Egra-TCP genes were calculated and synteny maps were drawn using TBTools.

2.4. In silico gene expression analysis

Illimuna RNAseq datasets were obtained using the Phytozome Database v13. Expression profiles of the Egra-TCP genes were analyzed in special tissue libraries obtained from six different plant tissues (Floem, immature xylem, xylem, mature leaf, shoot tips and young leaf). In silico expression profiles were calculated with Cufflinks in units of FPKM (Expected number of fragments per kilobase of sequenced transcript per million base pairs) (Trapnell et al. 2013). The values of FPKM were converted to logarithm base 2, and a heatmap was created using the CIMMiner (https://discover.nci.nih.gov/cimminer/).

3. Results and Discussion

3.1. Identification of E. grandis TCP Genes

The sequences of TCP gene family members were obtained from *Eucalyptus grandis*, *Arabidopsis thaliana,* and *Vitis vinifera* genomes using the Phytozome Database v13 with the TCP gene family accession number acquired from the Pfam server. The TCP domains in the obtained sequences were confirmed through an HMM analysis. Through these analyzes, 16 TCP genes were identified in eucalyptus. The amino acid numbers, theoretical pIs, and instability indices of these genes are provided on Table 1.

The lengths of the eucalyptus TCP proteins range from 194 to 464. The longest amino acid sequence was Egra-TCP-10 with 464, while the shortest sequence was Egra-TCP-11 with 194 amino acids. Similarly, the highest molecular weights of Egra-TCP proteins were obtained in Egra-TCP-10 with 47.19 kDa, while the lowest was in Egra-TCP-1 with 21.68 kDa. While the theoretical pIs vary between 6.44and 10.07, the lowest value was determined in Egra-TCP-7 and the highest value was determined in Egra-TCP-4. The instability indices were altered from 30.63 (Egra-TCP-11) to 72.42 (Egra-TCP-4). According to these results, the instability index of Egra-TCP-11 was lower than 40, indicating that this protein is relatively stable.

Table 1- The information about TCP gene family members found in Eucalyptus genome

Gene ID	*Phytozome ID*	*Chromosome location*	*aa lenght*	*MW (Da)*	*pI*	*Instability index*	*Classifies*	*Subcellular localization (WolfPSORT)*	*NCBI accession no*
Egra-TCP-1	Eucgr. A01143.1	Chr01:25174892..25175768 (+)	206	21680.53	7.70	62.41	unstable	nucl: 5, mito: 5, cyto: 2, chlo: 1, plas: 1	XP_010046620.1
Egra-TCP-2	Eucgr. A02843.1	Chr01:43511787..43513420 (+)	343	35580.41	9.05	63.56	unstable	nucl: 14	XP_010025203.1
Egra-TCP-3	Eucgr. B00471.1	Chr02:4499527..4501083 (-)	427	44907.43	6.70	67.88	unstable	nucl: 14	XP_010029552.1
Egra-TCP-4	Eucgr. B03427.1	Chr02:52680182..52680991 (+)	270	28668.77	10.07	72.42	unstable	nucl: 11, mito: 2, cyto: 1	XP_010046658.1
Egra-TCP-5	Eucgr. B03529.1	Chr02:55205056..55206564 (+)	286	30207.42	6.45	52.72	unstable	nucl: 14	XP_010044850.1

Table 1- Continued

Gene ID	*Phytozome ID*	*Chromosome location*	*aa lenght*	*MW (Da)*	*pI*	*Instability index*	*Classifies*	*Subcellular localization (WolfPSORT)*	*NCBI accession no*
Egra-TCP-6	Eucgr. B00608.1	Chr02:6164848..6167321 (+)	405	44419.28	6.60	60.86	unstable	nucl: 11, chlo: 2, extr: 1	XP_010045604.1
Egra-TCP-7	Eucgr. B00699.1	Chr02:7267363..7269590 (-)	383	42610.25	6.44	48.51	unstable	nucl: 13, cyto: 1	XP_010031709.1
Egra-TCP-8	Eucgr. D02422.1	Chr04:37891043..37892367 (+)	300	32028.58	7.26	57.79	unstable	nucl: 14	XP_010053872.1
Egra-TCP-9	Eucgr. F01204.1	Chr06:16221067..16223636 (-)	414	44803.27	6.66	63.06	unstable	nucl: 13, cyto: 1	XP_010060659.1
Egra-TCP-10	Eucgr. F02587.1	Chr06:37967375..37969007 (-)	464	47191.71	8.09	60.85	unstable	nucl: 14	XP_010064787.1
Egra-TCP-11	Eucgr. G02354.1	Chr07:44218919..44219500 (+)	194	21807.44	9.62	36.63	stable	nucl: 13, cyto: 1	XP_010068762.1
Egra-TCP-12	Eucgr. I02038.1	Chr09:29927883..29930010 (+)	338	37109.33	6.85	61.83	unstable	nucl: 10, chlo: 1, cyto: 1, extr: 1, vacu: 1	XP_010029390.1
Egra-TCP-13	Eucgr. J01466.1	Chr10:18021225..18022148 (-)	308	35211.63	9.27	59.34	unstable	nucl: 12.5, cyto_nucl: 7, chlo: 1	XP_010034424.1
Egra-TCP-14	Eucgr. K01089.1	Chr11:13919096..13921146 (-)	358	39055.27	7.23	59.46	unstable	nucl: 14	XP_010035834.1
Egra-TCP-15	Eucgr. K02535.1	Chr11:33244993..33246287 (+)	342	38224.07	8.44	49.06	unstable	cyto: 8, nucl: 4, plas: 1.5, golg_plas: 1.5	XP_010037244.1
Egra-TCP-16	Eucgr. K02654.1	Chr11:33796218..33797790 (-)	367	42209.24	8.38	52.99	unstable	nucl: 14	XP_010037346.1

3.2. Physical locations and structure of Egra-TCP genes, gene duplications, identification of conserved motifs, and phylogenetic analyzes

The positions of the Egra-TCP genes in the genome were obtained from the Phytozome Database v13. All Egra-TCP genes were mapped to eucalyptus chromosomes using Circos (Figure 1). The Egra-TCPs are located on chromosomes 1, 2, 4, 6, 7, 9, 10, and 11, with most of their 5 genes on chromosome 2 and at least 1 gene on each of chromosomes 4, 7, 9, and 10.

The exon and intron numbers were determined through a structural analysis performed on the Egra-TCPs using the Gene Structure Display Server v2.0 (Figure 2). According to the gene structure analysis, 2 introns were detected in Egra-TCP-8 and 1 intron in Egra-TCP-7, -15, and -16 (TCP-C member genes). In addition, Egra-TCP-7 and -16 genes are in a similar group based on the phylogenetic tree. Again, Egra-TCP-7, -8, -15, and -16 genes were found to contain 2, 3, 2, and 2 exons, respectively. Twelve of these 16 genes are entirely intronless. Studies have shown that intronless genes are characteristic of a prokaryotic genome. Additionally, the existence of intronless genes in eukaryotic genomes has been known for past 20 years (Makeyev et al. 1999; Sugiyama et al. 1999). Many plant species have genes with no intron, including model organisms such as *A. thaliana*, *O. sativa*, and *Populus* (Yang et al. 2009). It has been reported that retrogenes are also intronless, and many retrogenes are found in eukaryotic genomes (Zhang et al. 2005). These intronless genes in the eukaryotic genomes are known to be important in comparative genomic and evolutionary studies (Zou et al. 2011; Ilhan et al. 2018). The intronless genes have been found in studies with TCP genes in different plant genomes. Approximately 77.8% of *Phaseolus vulgaris* TCP genes are intronless (Ilhan et al. 2018). Similarly, thirteen genes have been discovered in *Prunus meme* (Zhou et al. 2016), thirty-two in apple (Xu et al. 2014), forty in tobacco (Chen et al. 2016), thirty-two in *Gossypium raimondii* (Min et al. 2018), twelve in *V. vinifera* (Jiu et al. 2019), sixty-eight in *Gossypium barbadense* (Zheng et al. 2018), and fifty-one in *Brassica juncea* var. *tumida* (He et al. 2020).

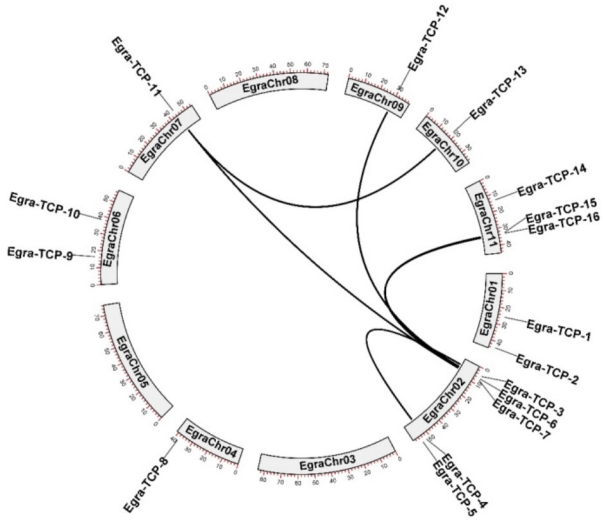

Figure 1- Chromosomal distribution and gene duplication events of Egra-TCP genes
The black curves indicate segmentally-duplicated genes. The lengths of eucalyptus chromosomes can be predicted according to the scale given

One of the most crucial evolutionary mechanisms that produce genetic diversity and functional novelty is gene duplication (Huo et al. 2018). Tandem and segmental duplications have an important role in the proliferation of plant gene families and the acquisition of new gene functions in the evolutionary process (Kondrashov et al. 2002; Cao et al. 2021). Gene duplication events have been a sunject of study in order to better determine the evolutionary relationships of members of the *E. grandis* TCP gene family. A total of 6 duplicate gene pairs were identified between Egra-TCPs using the MCScanX (Table 2, Figure 1). All of the duplicating gene pairs identified were found to be whole genome duplication or segmental duplication genes. Previous studies on the TCP gene family reported that there are both segmental and WGD duplications in apples (Xu et al. 2014), and tandem duplications in addition to these duplication events in *Arabidopsis* and rice (Yao et al. 2007). Moreover, it has been suggested that these duplications occurred during the evolution of the angiosperm (Yao et al. 2007). New gene functions obtained during these widespread plant duplication events lead to significant evolutionary changes (Navaud et al. 2007).

TBTools software was used to compute the Ka, Ks and Ka/Ks values, as a good indicator of the selection pressure at the protein level. A Ka/Ks value less than 1 means purifying selection, while a value greater than 1 means positive selection. If Ka/Ks are equal to 1, it indicates neutral selection in the evolutionary process (Juretic et al. 2005; İlhan 2018; Kizilkaya et al. 2020). The fact that Ka/Ks is less than 1 among WGD or segmental duplication gene pairs found among Egra-TCP genes in this study suggests that they are under purifying selection in the evolutionary process (Table 2). As seen in Table 2, the Ka/Ks value could not be calculated between the Egra-TCP-7/Egra-TCP-11 gene pair.

The "MEME tool" was performed to discover more Egra-TCP conserved motifs (Bailey et al. 2006; Aygören et al. 2022). In the preserved motif analysis, 15 preserved motifs were detected. The length of the determined motifs varied between 2 and 50. The most motifs were detected in Egra-TCP-10 (10 motifs), while the least motifs were detected in Egra-TCP-5 and Egra-TCP-14 with 2 motifs. The sequence obtained from Motif-1 in InterProScan searches included the TCP domain. Motif-5 shows the CYC/TB1 and R domains (Figure 3, Supplementary File 1). Motif -1 is found in all Egra-TCP genes, while Motif-5 containing the R domain, is found in Egra-TCP-4, -7, -11, -13, -15, and -16 genes.

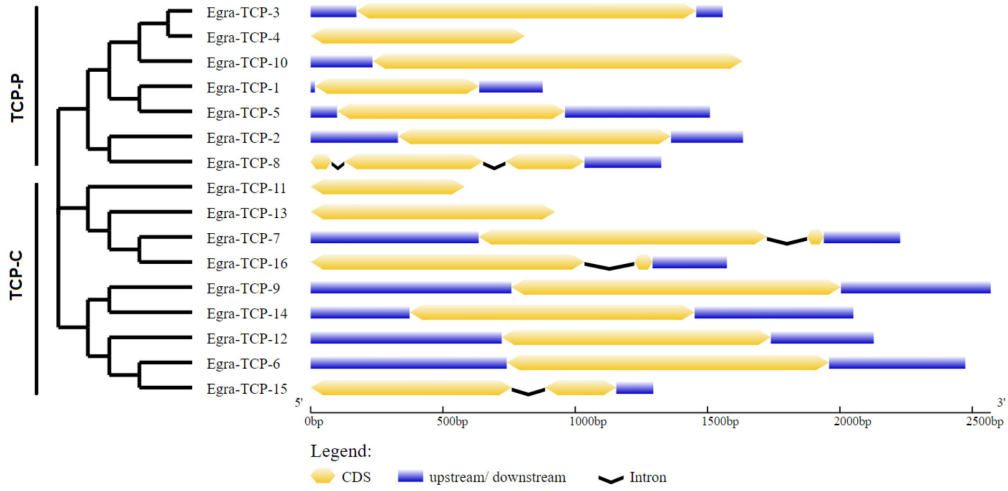

Figure 2- Exon and intron structures of Egra-TCP genes. The dark-blue and yellow boxes indicate UTRs and exon regions, respectively, and the black lines represent introns

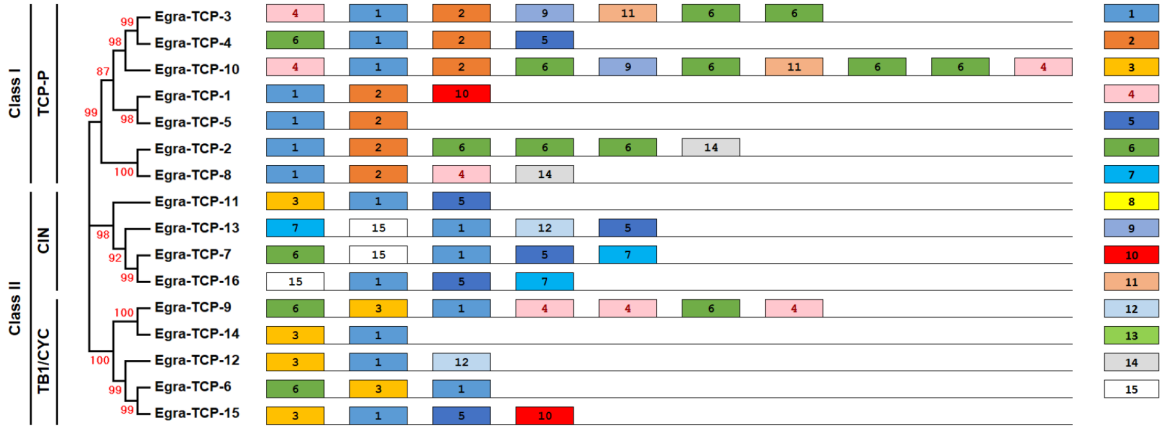

Figure 3- Additional motif analysis of Egra-TCPs. Fifteen motifs are displayed in different colors

Table 2- Ka, Ks, Ka/Ks values, Selection pressure, and duplication type of Egra-TCPs

Gene 1	*Gene 2*	*Ka*	*Ks*	*Ka/Ks*	*Selection pressure*	*Duplication type*
Egra-TCP-3	Egra-TCP-4	0.464	1.094	0.424	Purifying	WGD or Segmental
Egra-TCP-6	Egra-TCP-12	0.533	2.618	0.204	Purifying	WGD or Segmental
Egra-TCP-6	Egra-TCP-15	0.582	1.582	0.368	Purifying	WGD or Segmental
Egra-TCP-7	Egra-TCP-16	0.502	2.248	0.223	Purifying	WGD or Segmental
Egra-TCP-11	Egra-TCP-13	0.571	3.596	0.159	Purifying	WGD or Segmental

Ka : Non-synonymous substitution rate, Ks: Snonymous substitution rate

A sequence alignment analysis showed that all TCPs contain conserved basic helix loop helix. In addition, according to this analysis, the members of the PFC group have a deletion of four amino acids compared to the members of the TCP-P group in terms of the basic helix loop helix domain (Figure 4). This result is compatible with the phylogenetic analysis results.

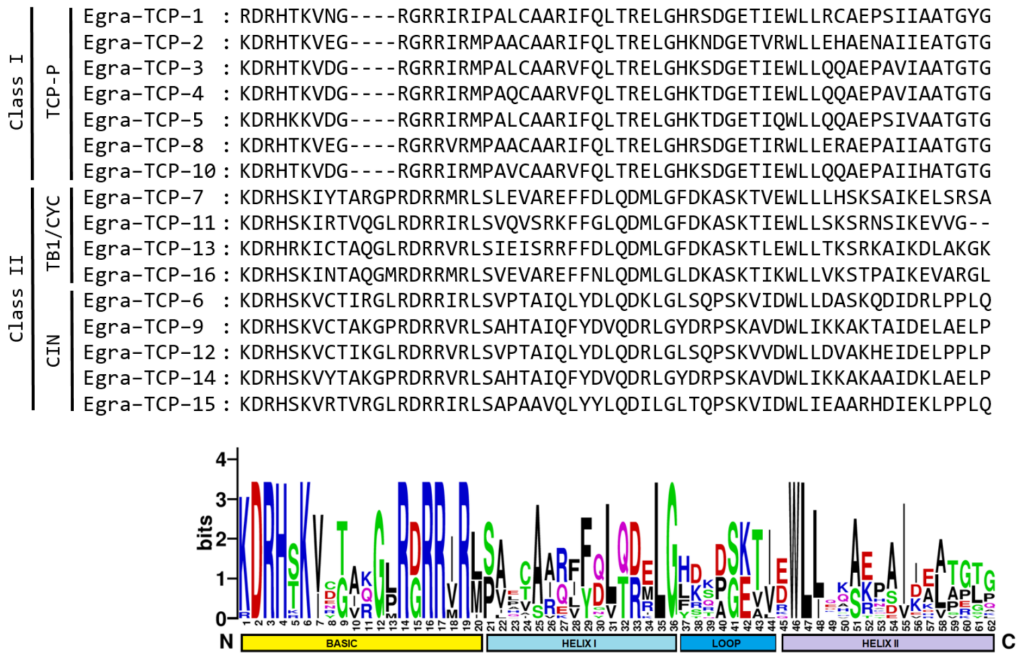

Figure 4- Sequence alignment of Egra-TCPs containing bHLH domain

To further examine the function of Egra-TCPs, the phylogenetic relationships were analyzed. TCP proteins from *Arabidopsis thaliana*, *Vitis vinifera*, maize (TB1), the common snapdragon (AmCYC, AmCIN) and rice (OsPCF) plants, and *E. grandis* were aligned based on multiple sequence alignment in MEGA v7 program with bootstrap values using the Neighbor-Joining method. A supported rootless tree is drawn. The tree was organized with iTOL (Figure 5).

Egra-TCP proteins are divided into 2 different major groups, Class I and Class II. Class I clan is known as TCP-C clan, while Class II clan is known as TCP-P. Class I group TB1/CYC clades, including TB1 and AmCYC, contain Egra-TCP-7, -11, -13, and 16 proteins. The CIN clade of the Class II group contain AmCIN contains Egra-TCP-6, -9, -12, -14, and -15 proteins, while the TCP-C clade contains OsPFC-1, OsPFC-2, Egra-TCP-1, -2, -3, -4, -5, -8 and -10 proteins.

3.3. Subcellular Localization and promotor analysis of Egra-TCP gene family

The subcellular localization of the Egra-TCPs was estimated using the WoLFPSORT program (Table 1). The Egra-TCP-2, -3, -5, -8, -10, -14, and -16 proteins are estimated to be located in the nucleus, while some Egra-TCP proteins have been estimated to be located in organelles such as the nucleus, mitochondria, vacuoles, golgi apparatus, and chloroplasts. Previous studies predicted that TCP proteins are predominantly located in the nucleus (Leng et al. 2019; Lin et al. 2019).

To further examine the function and regulatory mechanism of the Egra-TCP genes, a promoter region analysis was performed. The approximately 2000 bp genomic sequence of the Egra-TCP genes has been uploaded to the PlantCARE database. Using the data obtained as a result of the analysis, a phenogram was drawn with the help of TBTools (Figure 6).

The cis-acting elements in the promoter sequence of the Egra-TCPs were collected in six groups:environmetal stress, development, promoter-related, hormone-related, light responsiveness, and site binding-related. Whereas each Egra-TCP had an average of 130 cis-acting elements, Egra-TCP-13 had the most, with 165; the lowest cis-acting number was Egra-TCP-4 with 106. The GCN4_motif, which is one of the elements involved in endosperm expression, was determined in Egra-TCP-2, 3, -7, -9, and -14 genes. The element associated with meristem expression, CAT-box, were found in Egra-TCP-1, -2, -9, 10, -11, -13, -14 and -16 genes. Hormone-related cis-acting elements such as ABRE, TCA-element, CGTCA-motif, TGACG-motif, GARE-motif, P-box, TATC box, ERE, AuxRR-core, and TGA-element have been detected in some of the Egra-TCP genes' promoter regions. The fact that many hormonal-related elements have been found in the promoter regions of the Egra-TCP genes suggests that these genes have important roles in growth and development. Elements such as ARE, MBS, MBS, and LTR were determined as elements related to environmental stress (Table S2 and S3). This supports our findings in previous studies (Ilhan et al. 2018).

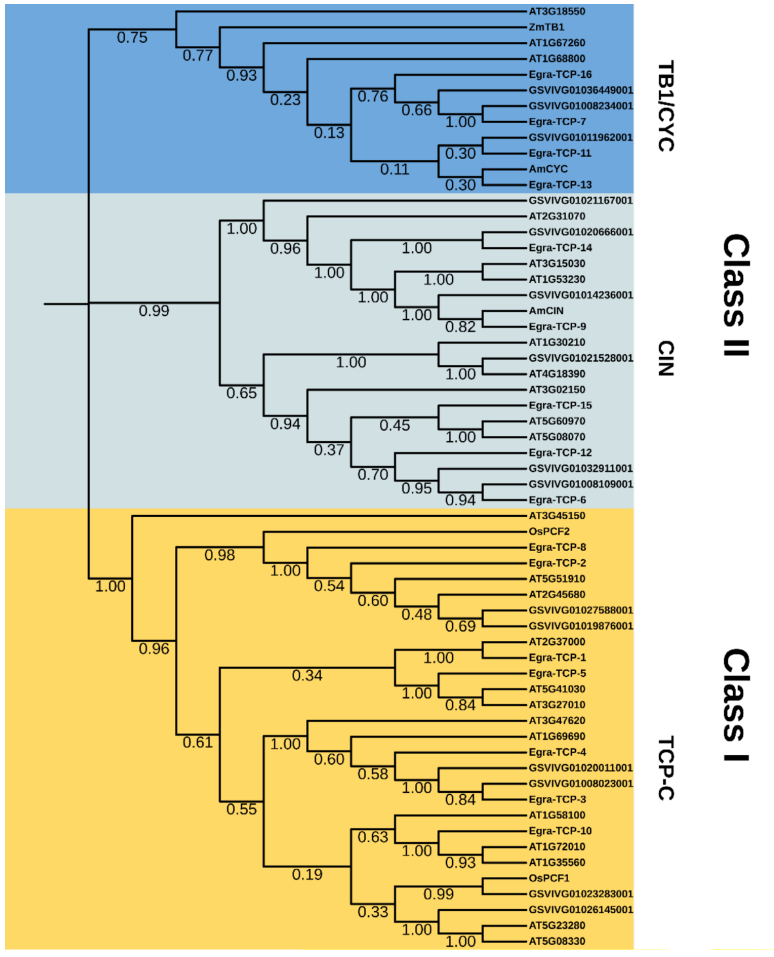

Figure 5- A phyologenetic tree of *Eucalyptus grandis*, *Arabidopsis thaliana*, and *Vitis vinifera* TCP proteins, maize (TB1), common snapdragon (AmCYC, AmCIN), and *O. sativa* (OsPCF1 and OsPCF2) proteins. The tree was drawn using the MEGA v7 and Neighbor-joining (NJ) algorithm with 1000 replicated-bootstrap values

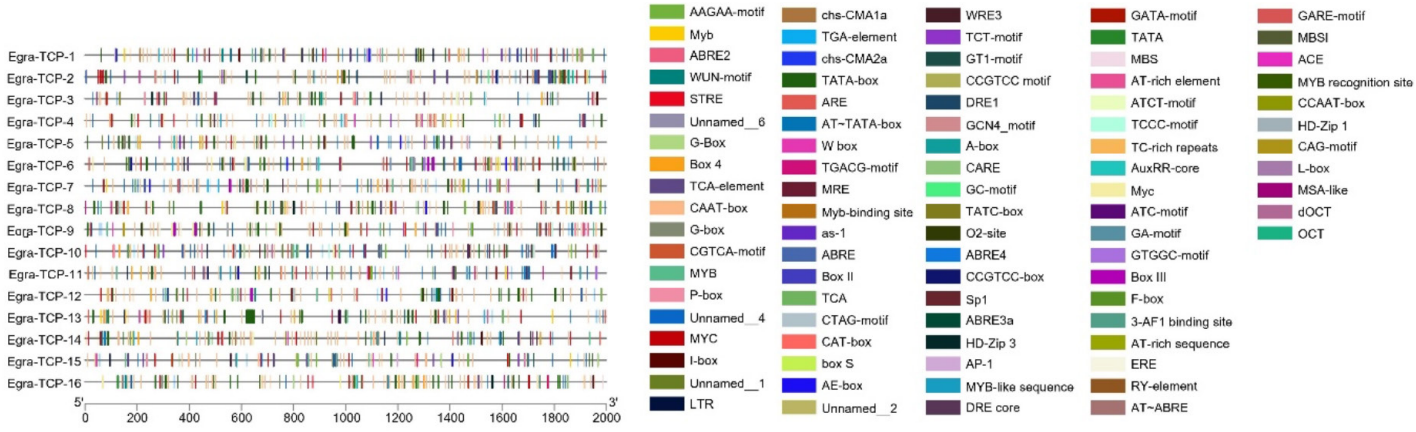

Figure 6- Cis-acting element analysis of Egra-TCP genes according to information obtained from the PlantCARE database

3.4. The Orthologous Relationships

TCP proteins are a conserved gene family among plant genomes. The orthologous association using the TCP genes *Eucalyptus grandis*, *Vitis vinifera,* and *Arabidopsis thaliana* was determined using MCScanX with default parameters. Our results showed orthologous pairs between the TCP genes of these three genomes (Table 3). In order to show the selection pressure in the evolutionary process, the Ka/Ks values of each orthologous gene pair were calculated. However, this value could not be calculated for some gene pairs. The calculated orthologous gene pair are under strong purifying selection pressure.

To predict the TCP's evolutionary pathways, a syntenic analysis of the TCP genes was performed in *Eucalyptus grandis*, *Vitis vinifera,* and *Arabidopsis thaliana* plants (Figure 7). The homology between *E. grandis* and Arabidopsis was higher than that between *E. grandis* and *V. vinifera*. In addition, when compared with the phylogenetic tree, these orthologous genes were determined to be in similar groups. Previous studies have revealed that TCP genes in similar groups show similar functions (Mondragon-Palomino and Trontin 2011; Citerne et al. 2013; Feng et al. 2018). For this reason, it is thought that these genes may have similar functions.

Table 3- Ka, Ks, Ka/Ks values, and Selection pressure among *E. grandis*, *V. vinifera*, and *A. thaliana* orthologous genes

Gene 1	***Gene 2***	***Ka***	***Ks***	***Ka/Ks***	***Selection Pressure***
E. grandis-V. vinifera					
Egra-TCP-2	GSVIVT01027588001	0.472	2.796	0.169	Purifying
Egra-TCP-2	GSVIVT01019876001	0.426	3.008	0.142	Purifying
Egra-TCP-4	GSVIVT01008023001	0.432	1.859	0.232	Purifying
Egra-TCP-4	GSVIVT01020011001	0.412	1.770	0.233	Purifying
Egra-TCP-7	GSVIVT01008234001	0.309	1.521	0.203	Purifying
Egra-TCP-8	GSVIVT01027588001	0.509	1.408	0.361	Purifying
Egra-TCP-9	GSVIVT01014236001	0.315	1.567	0.201	Purifying
Egra-TCP-12	GSVIVT01008109001	0.371	1.993	0.186	Purifying
Egra-TCP-12	GSVIVT01032911001	0.431	2.222	0.194	Purifying
Egra-TCP-13	GSVIVT01036449001	0.575	3.086	0.186	Purifying
Egra-TCP-14	GSVIVT01020666001	0.467	3.824	0.122	Purifying
Egra-TCP-14	GSVIVT01021167001	0.606	2.738	0.221	Purifying
Egra-TCP-15	GSVIVT01008109001	0.359	2.528	0.142	Purifying
Egra-TCP-16	GSVIVT01008234001	0.483	2.093	0.231	Purifying
E. grandis-A. thaliana					
Egra-TCP-7	AT1G68800.1	0.655	3.981	0.164	Purifying
Egra-TCP-7	AT3G18550.1	0.790	3.100	0.255	Purifying
Egra-TCP-13	AT3G18550.1	0.751	2.834	0.265	Purifying
Egra-TCP-15	AT5G60970.1	0.747	2.884	0.259	Purifying

Ka : Non-synonymous substitution rate, Ks: Snonymous substitution rate

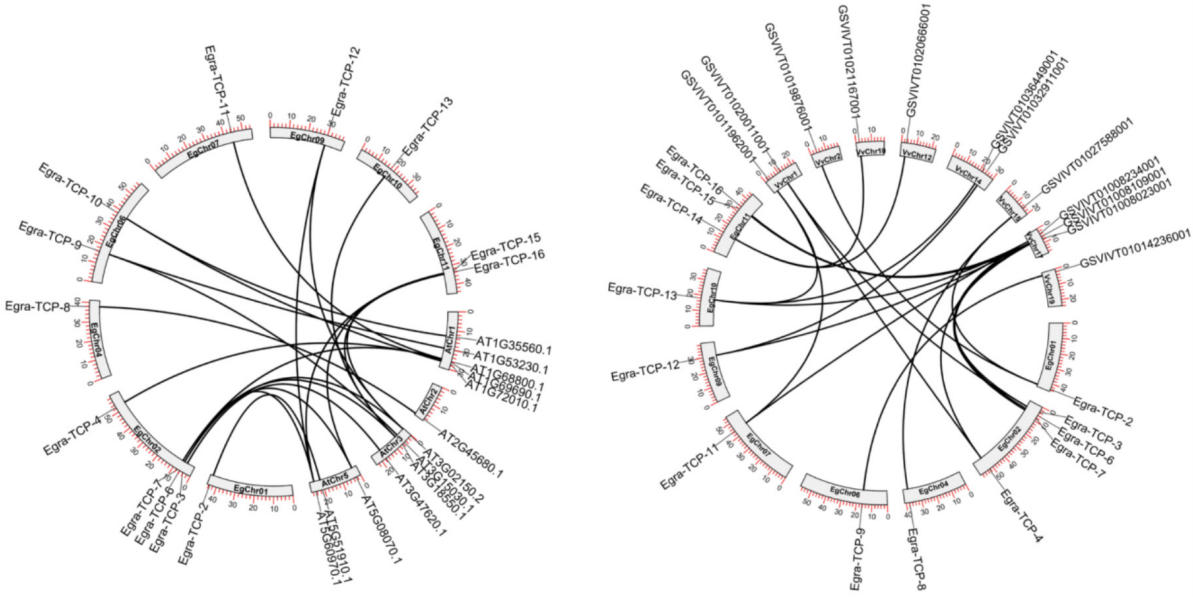

Figure 7- The orthologous relationships among *E. grandis*/*A. thaliana* (left) and *E. grandis*/*V. vinifera* (right). Black linker lines represented the syntenic relationships between *E. grandis*, *V. vinifera*, and *A. thaliana* TCP genes

3.5. In silico gene expression analyzes of TCP genes across various tissues

Different members of gene families are involved in different physiological processes among different tissues. In order to understand the expression differences and functions of TCP genes in plant growth and development, the expression values of tissue-specific TCP genes were analyzed with the data obtained from the Phytozome Database v13 (Figure 8).

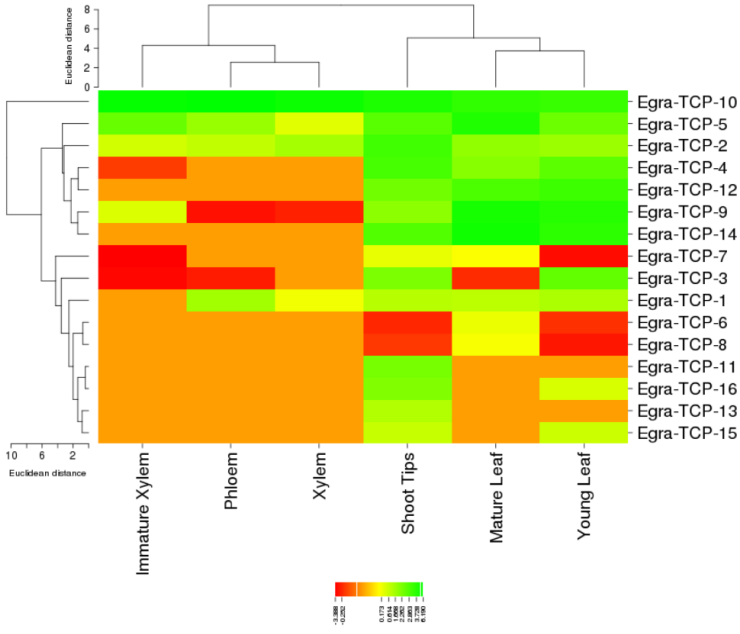

Figure 8- The expression levels of Egra-TCP genes in different tissues. The heatmap below indicates where the 16 Egra-TCPs are grouped in the six tissues. On the right are the names of the genes. Scale bars at the top display each gene's log 2 transformed FPKM values

It is seen that Egra-TCP-2, -5, and -10 have high expression levels in immature xylem, phloem, xylem, shoot tips, mature leaf, and young leaf tissues. While the remaining Egra-TCPs were not expressed in tissues such as immature xylem, phloem, and xylem, it was observed that they had little expression in shoot tips, mature leaf, and young leaf tissues. This result reveals that TCP genes are expressed tissue-specifically. Similar results have been recorded in other studies (Feng et al. 2018; Leng et al. 2019; Ling et al. 2020). A number of studies, however, have shown that TCP genes have different expression levels in different developmental stages of the plant (Pestana-Calsa et al. 2012; Xu et al. 2014; Min et al. 2018; Zheng et al. 2018; He et al. 2020). In addition, when the expression levels of duplicate Egra-TCP paralogs were compared, differences in gene expressions were detected, for example, the WGD or segmentally duplicated Egra-TCP-6/Egra-TCP-12 gene pair. While Egra-TCP-12 has a high expression level in shoot tips, mature leaf, and young leaf tissues, the expression level of Egra-TCP-6 is almost absent in shoot tips and young leaf tissues, and has been detected very little in mature leaf tissue. Tissue-specific expression differences are considered to be an indicator of functional transformations between genes (Makova and Li 2003; Li et al. 2005; Nag et al. 2009). The duplicated genes and the member of gene family and can acquire new biological functions during the evolution of the plant (Nag et al. 2009). Due to, Egra-TCP paralog gene pairs can acquire different functions in different tissues.

4. Conclusions

In this study, using in silico approaches in the eucalyptus genome, 16 TCP gene family members were identified. These genes are distributed in 7 different eucalyptus chromosomes. According to the gene expression analyses performed in immature xylem, phloem, xylem, shoot tips, mature leaf, and young leaf tissues, it was determined that Egra-TCPs have different expression levels in different tissues. In additiom, members of this gene family can take on different physiological functions in the growth and development processes of the plant. This contributes to our understanding of the functions and classification of TCP genes in the eucalyptus plant.

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Evaluation of Image Processing Technique on Quality Properties of Chickpea Seeds (*Cicer arietinum L.*) Using Machine Learning Algorithms

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ABSTRACT
Chickpea is an important edible legume consumed worldwide because of rich nutrient composition. The physical parameters of chickpea are crucial attributes for design of processing and classification systems. In this study, effects of seven different irrigation treatments (I_1-rainfed, I_2-pre-flowering single irrigation, I_3-beginning of flowering single irrigation, I_4-50% pod set single irrigation, I_5-irrigation at 50% flowering and 50% pod fill, I_6-irrigation before flowering and at 50% pod set, I7-full irrigation) on size, shape, mass, and color properties of chickpea seeds were investigated, and machine learning algorithms were used to estimate mass and color attributes of chickpea seeds. In terms of physical attributes, the best results were obtained in I_1 and I_5 irrigation treatments. According to the findings, among the irrigation treatments, I_5 had the greatest mass, volume, geometric mean diameter, projected area with the values of 0.50 g, 394.86 cm^3, 9.10 mm and 65.03 mm^2, respectively. In addition, I_1 had the highest shape index and elongation as 1.33 and 1.34, respectively. The results showed that multilayer perceptron (MLP) had the greatest correlation coefficients for mass (0.9997), chroma (0.9998) hue angle (0.9998) and color index (0.9992). The MLP yielded better outcomes than random forest for both mass and color estimation. Additionally, single or couple irrigation treatment at different physiological stages instead of full irrigation treatment might be sufficient to improve the physical attributes of chickpea.

Keywords: Chickpea, Mass, Color, Irrigation treatments, Machine learning

1. Introduction

Chickpea (*Cicer arietinum* L.) is an important grain legume for human nutrition and animal feeding (Gaur et al. 2015; Kirnak et al. 2017). It has a great place in daily diets of low-income countries and is largely grown in the Mediterranean countries, Asia, Africa, and Europe (Sastry et al. 2019). Healthy and balanced nutrition is among the most significant problems of developing countries (Hawkes 2006). Daily protein intake per capita is around 70.9 g worldwide and a balanced and healthy nutrition can be mentioned when 60% of such intake come from plant-originated and 40% come from animal-originated foodstuffs (Onder et al. 2014). Chickpea seeds contain 29% protein, 59% carbohydrate, 5% oil, 4% ash and 3% fiber (Iqbal et al. 2006). Seeds are used in imitation milk, infant formulas, bakery products and ready-to-eat products (Ashokkumar et al. 2015). Chickpea has also various health benefits and prevents various diseases such as obesity, colon cancer, diabetes, and cardiovascular diseases (Yildirim and Oner 2015; de Camargo et al. 2019). Among the edible legumes, chickpea had the third place (14,776,827 tons) worldwide. However, in Turkey, chickpea has the first place (470,000 tons) among the edible legume grains (FAOSTAT 2019).

The main chickpea types include Indian-originated desi type with small seeds, colored seed coat and angular shape; Mediterranean and Middle East-originated kabuli type with large seeds, beige color, owl's head shape; intermediate type with medium-to-small seeds, cream color (Sastry et al. 2019). Just because of larger seeds, kabuli type is generally preferred by consumers (Masoumi & Tabil 2003).

Agbola et al. (2002), indicated the seed quality characteristics of Indian chickpea varieties as color, size and "dhal (half a kernel)" recovery rate.

Chickpea is generally grown under rainfed conditions. However, supplementary irrigations especially in dry seasons may improve yield levels (Varol et al. 2020). Irrigation also improves the availability of nutrients within the rootzone (Ronnenberg & Wesche, 2011). There are significant relationships between soil moisture and available plant nutrients (Kaplan et al. 2019). Limited water resources and current water deficits exert serious stress on cultivated crops. Chickpea has a relatively shorter growing season, thus consume less water than many other broadleaf crops (Benjamin & Nielsen 2006). In any case, drought is the most important abiotic stressor also in chickpea farming (Mehta et al. 2015).

Shape, size and color parameters of chickpea seeds are used in design of transportation, classification, drying, storage and separation systems. Such parameters also play a great role in breeding studies, consumer demands and culinary preferences (Mirzaee et al. 2009; Cetin et al. 2020). The seeds with greater weight and thickness generally have greater mechanical resistance (Sastry et al. 2019). Computer vision techniques has great potential for the agricultural industry. This technology has been applied in numerous applications because of the low cost, quick inspection rate, the ability to provide reliable and consistent information (Beyaz et al. 2010; Beyaz & Ozturk 2016; Martinez et al. 2018). In addition, image processing system, which is a practical technique for automatic evaluations, was used to determine the physical properties of the seeds (Kara et al. 2013). This method has broadly contributed to relevant agricultural morphological analyzes in different products (Kupe et al. 2021).

General appearance, especially colors, greatly influence overall impression of consumers. Thus, color is considered as an important criterion in selection of foodstuffs (Costa et al. 2011). International Commission on Illumination (CIE) color space is largely used to measure color parameters (L^*, a^*, b^*) of foodstuffs. Quality classifications are successfully performed in food and agricultural industry based on color, shape, and size parameters (Omid et al. 2010).

Machine learning (ML) approaches are effective tools used in the design of accurate and reliable predictors. Such applications include various algorithms such as artificial neural network (ANN), DT, genetic algorithm, fuzzy logic, and regressions. Furthermore, there are verified models for training several ML algorithms and for adapting difficult input-output mapping strategies as well as selecting and removing useful features. These algorithms are mostly utilized for the correct selection of descriptive features in the quality assessment of agricultural products (Omid et al. 2010; Mollazade et al. 2012). ANN are consisted of interconnected processing elements like biological neurons and weighted connections corresponding to brain snaps (Karray & Silva 2004). Multilayer perceptron (MLP) is a feed-forward neural network (FFNN). Data flow through input layers toward to output layers in a single direction in FFNN (Omid et al. 2010). ANN and random forest (RF) most popular ML algorithms used in estimation of food properties (Marini et al. 2004; Mollazade et al. 2012). RF algorithm generates more than one DT with the use of bootstrap samples from the original training data to train each tree and is a good separator (Breiman 2001).

Several researchers previously investigated physical parameters (shape, size, and color) of chickpea seeds (Masoumi & Tabil 2003; Nikoobin et al. 2009; Kibar et al. 2014; Queiroz et al. 2015; Eissa et al. 2010; Jogihalli et al. 2017; Sastry et al. 2019; Soares et al. 2013; Rad et al. 2017; Gurbuz et al. 2018; Kus et al. 2017; Demir 2018; Cetin et al. 2021). Also, the image processing method applied in the present study was used in studies such as walnut (Ercisli et al. 2012; Demir et al. 2018), bean (Kara et al. 2013), orange (Sayinci et al. 2012), cherry laurel (Sayıncı et al. 2015a), hazelnut (Sayıncı et al. 2015b; Cetin et al. 2020), almond (Demir et al. 2019), grape (Küpe et al. 2021), corn (Beyaz & Gerdan 2021), soybean (Çetin 2022) and rice (Cinar & Koklu 2022). These parameters were used to estimate some other critical aspects of chickpea seeds. However, there are any studies in literature about color and mass estimation of chickpea seeds grown under different irrigation regimes. Image processing which is common technique for the identification of some physical attributes of the agricultural products. Therefore, objectives of the present study were set as to:

- Determine the effects of different irrigations performed in different physiological stages on physical quality traits which is determined computer vision techniques of chickpea seed,

- Estimate seed mass from the physical attributes with the use of different machine learning algorithms (MLP and RF),

- Estimate color parameters [color index (CI), chroma (C^*) and hue angle (h^o)] from CIE color values (L^*, a^* and b^*) with the use of machine learning algorithms.

2. Material and Methods

2.1. Field experiments and samples

Present research was implemented at the Agricultural Research and Implementation Center of Erciyes University in Kayseri, Turkey in growing season of 2017 (Figure 1). Chickpea cultivar of Aksu, commonly used by local farmers, was used in present experiments. Aksu cultivar has owl's head seeds with about 8.1 mm diameter. Each pod general has 1-2 seeds. It is a mid-early cultivar with about 109 days of vegetation period. It is highly tolerant to drought and cold temperatures. Plant growth is semi-erect, branching is intense and leaf type is normal. The cultivar is resistant to wilt disease and anthracnose. Experimental soils were clay-loam in texture with an EC_e of 0.220, 0.173 and 0.258, pH of 8.13, 8.17 and 8.14, bulk density of 1.27, 1.24 and 1.22 g cm^{-3} and organic matter of 1.25, 1.05 and 0.69 % for soil depth of 0-30, 30-60 and 60-90 cm, respectively, field capacity (FC) of 30.3%, permanent wilting point of 10.5% and infiltration rate of 23.3 mm h^{-1}. The average temperature and relative humidity at growing season were 21.5 °C and 51.9%, respectively. Total precipitation through the growing season (April - August) was 137.0 mm (Table 1).

Figure 1- Some photos of the experimental area

Table 1- Weather conditions during the study period

Climatic Data	*April*	*May*	*Jun*	*July*	*August*
T_{mean} (°C)	24.2	14.9	19.6	23.7	25.3
T_{max} (°C)	20.2	21.9	27.9	33.0	34.3
T_{min} (°C)	4.4	7.8	11.3	14.4	16.2
Wind Speed (m sn^{-1})	1.6	1.6	1.4	2.0	1.6
Precipitation (mm)	25.9	57.2	50.6	0	3.3
RH_{max} (%)	81.9	87.3	87.8	68.5	73.1
RH_{min} (%)	25.7	30.8	25.5	16.5	22.2

Experimental design was randomized blocks with 3 replicates. Sowing was performed manually on 13th of April 2016. Experimental plots (5x1.75 m) had 6 rows spaced 35 cm apart and plant spacing was 5 cm. Fertilization was practiced at sowing as to have 15 kg ha^{-1} diammonium phosphate (18-46-0). Harvests were performed manually from the inner 4 rows and two side rows were committed as to consider side effects. The photographs of the experiment are given in Figure 2.

Figure 2- The map of the experimental location

Drip irrigation system with 16 mm dripper lines and 2l h^{-1} inline emitters with 25 cm spacing was used in irrigations. Seven different irrigation treatments were practiced (I_1-rainfed, I_2-pre-flowering single irrigation, I_3-beginning of flowering single irrigation, I_4-50% pod set single irrigation, I_5-irrigation at 50% flowering and 50% pod fill, I_6-irrigation before flowering and at 50% pod set, I_7-full irrigation) from sowing to full grain fill period as to bring the deficit moisture to FC when 40% (±5) of available moisture at 60 cm soil profile was depleted.

A time domain reflectometer (TDR) (Minitrase TDR, Soilmoisture Equipment Corp. USA) device was used to monitor soil moisture continuously. Soil moisture measurements were performed at 10 cm by the plant rows with a 60 cm uncoated TDR probe. Measurements were carried out manually, once a week. TDR calibrations were performed under field conditions in accordance with Akpinar (2016) and calibration equation of P_{vp}=1.922, K-0.2186 was used. Following equation was used to calculate irrigation water quantity of each irrigation:

$$d = \frac{(P_{vfc} - P_{VP})}{10} \times D \times P$$

where; d is irrigation water quantity to be applied, mm; P_{vfc} is moisture at FC, %; P_{vp} is moisture before irrigation, %; D is soil depth to be irrigated, cm; P is cover ratio.

2.2. Image acquisition and processing for dimensional attributes

In order to determine the dimensional attributes of the images of chickpea, an acquisition method described in the present study. In this method, there is a digital camera (Nikon D300, Japan) and illumination system. The chickpea images were captured without a shadow on the background in a dark room. The chickpeas were placed on a white fiberglass at horizontal and vertical orientations. The digital camera was vertically positioned at a constant height of approximately 45 cm (Kara et al. 2013; Sayıncı et al. 2015a; Cetin et al. 2020). For the image processing analysis, 100 chickpea seeds were sampled from each irrigation treatment (Cetin et al. 2020). The length (L, mm), width (W, mm), thickness (T, mm), projected area (PA, mm^2), aspect ratio (AR) and roundness (R) at both orientation of each chickpea were identification by image processing. The equations used for calculation of the volume (mm^3), geometric mean diameter (D_g, mm), surface area (S, mm^2), shape index (SI), sphericity (φ, %) and elongation (E) were presented in Table 2. Seed mass was measured with a precision electronic scale (±0.001 g).

Table 2- Equations used for size, shape and color attributes

Variables	*Equations**	*Literature*
Shape Index (SI)	$SI = (2 \cdot L) / (W + T)$	Ozkan & Koyuncu (2005)
Volume (V, mm^3)	$V = (\pi / 6) \cdot D_g^3$	Volume of ellipse
Surface area (S, mm^2)	$S = \pi D_g^2$	Sayıncı et al. (2015)
Sphericity (φ)	$\varphi = (D_g \ / \ L) \cdot 100$	Mohsenin (1986)
Geometric mean diameter (D_g, mm)	$D_g = (L \cdot W \cdot T)^{(1/3)}$	Mohsenin (1986)
Elongation (E)	$E = L / W$	Fıratlıgil-Durmuş et al. (2010)
Chroma (C*)	$C^* = \sqrt{(a^*)^2 + (b^*)^2}$	McGuire (1992)
Hue angle (hº)	$h^\circ = \tan^{-1}(b^* / a^*),$ $(if \ \ a^* > 0 \ \ and \ \ b^* \geq 0)$	McGuire (1992)
Color index (CI)	$CI = \frac{1000 \cdot a^*}{L^* b^*}$	Jimenez-Cuesta et al. (1982)

*L: Length (mm), W: Width (mm), T: Thickness (mm), D_g: Geometric mean diameter (mm), A_p: Projected area (mm^2), A_c: The biggest circular area (mm^2)

2.3. Chromatic parameters

Chromatic parameters (L^*, a^* and b^*) were measured with the use of a chromameter (Konica Minolta CR-400, Japan). Measurements were made in CIE color space. L^* (lightness; 0 dark, 100 light), a^* (+ values are redness, - values are greenness) and b^* (+ values are yellowness, - values are blueness) values were measured. CI, hº and C^* were calculated from provided in Table 2 (Jimenez-Cuesta et al. 1982; McGuire 1992).

2.4. Statistical analysis

Experimental data were subjected to one-factor analysis and significant means were compared with the use of Tukey's multiple comparison test at 95% significance level. Differences between the treatments were assessed with linear discriminant analysis (LDA). Group centroids of treatments obtained from LDA were used to generate a scatter plot. The principal components were evaluated for multivariate tests (MANOVA). Similarities or dissimilarities of irrigation treatments were tested with the use of Hotelling's pair-wise comparisons with squared Mahalanobis distances and Bonferroni correction. Statistical analyses were performed by using SPSS v20.0 (IBM SPSS® 2010) and PAST v3.20 software (Hammer et al. 2001).

2.5. Validation methodology

In the study, to validate the generated estimation models, the k-fold cross-validation technique was applied. The k value is usually preferred as 5 or 10 in the ML estimation (Ataş et al. 2012) which is 10 was chosen in the present study. Cross-validation evaluates the generalization ability of each model by comparing its performance in a dataset not used during training to fit the parameters of different ML algorithms. This technique is applied effectively in estimation (Stegmayer et al. 2013). In this technique, dataset was divided into

10 subsets by 10-fold cross-validation technique and every subset had an equal proportion of each class. Training and testing were performed with 10 iterations. In each iteration, 1 subset was used for testing and the rest of the subsets which is 9 subsets were used for training and with each of the k subsamples used exactly once as the testing respectively (Figure 3).

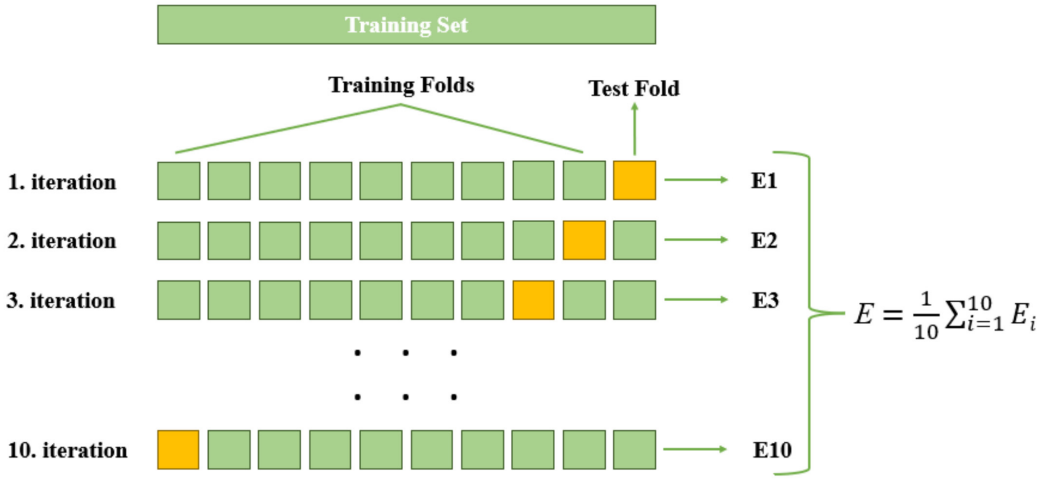

Figure 3. 10-fold cross validation methodology

2.6. Machine learning algorithms

In the current study, an estimation technique of ML algorithms was utilized by the Weka® v3.8 software (Hall et al. 2009). Two ML estimators were performed as ANNs and random forest. The estimation of seed mass belong to different irrigation treatments by ML was based on the main physical attributes. Size (V, L, W, T, D_g, SA and PA) and shape (R, φ, E, SI and AR) were used as criteria for estimation. In addition, the estimation of C*, h° and CI were based on the L*, a* and b* color properties. In this study, 100 samples were measured for each feature. A total of 9100 values were used for mass estimation, and a total of 2100 values were used for C*, h° and CI estimation for each irrigation treatments.

2.6.1 ANNs

In the present study, a MLP was used as feedforward ANN. The neural network parameters of the MLP structure were chosen as momentum 0.2, learning rate 0.3, and the number of periods 500. In addition, 12-6-1 MLP structure consisting of neurons in 12 input, 6 hidden and 1 output layers for mass estimation and 3-6-1 MLP structure consisting of neurons in 3 inputs, 6 hidden and 1 output layers for mass estimation for color estimation were considered. Applied MLP model structure is presented in Figure 4.

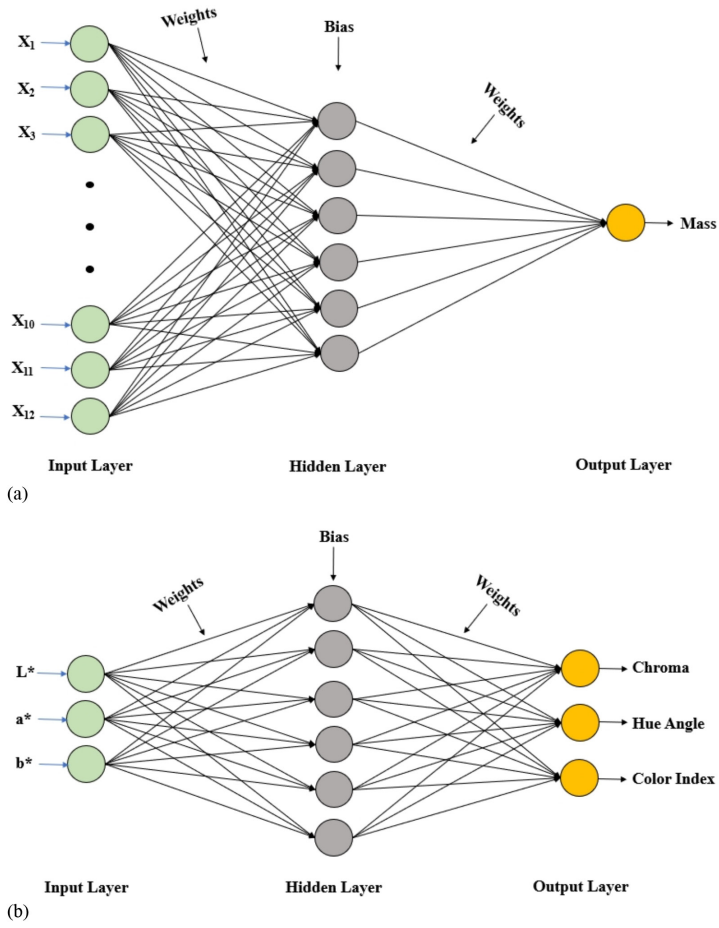

Figure 4- Model structures of the MLP for estimation of the mass (a) and color (b) attributes

In this study, RF algorithms was also utilized for estimation. RF contrary to decision tree (DT), a decision is performed with the majority of ensemble of trees built by RF in data sets assigned class (Berhane et al. 2018). Afterwards, bootstrap and ensemble scheme could overcome overfitting problem inherited from DT, there is no pruning step in RF. In addition, RF has a high estimative correlation coefficient and is robust against noise (Breiman 2001; Rodriguez-Galiano et al. 2012).

2.7. Model Performance Evaluation

Performance of MLP and RF models were assessed based on the following statistical indices: correlation coefficient (r), root mean square error (RMSE), mean absolute error (MAE), and mean absolute percentage error (MAPE):

$$r = \frac{1}{n-1}\sum_{i=1}^{n}\frac{(M_i - \dot{M})\,(E_i - \dot{E})}{S_M\ S_E}$$

$$MAE = \sum_{i=1}^{n}\frac{|\,E_i - M_i\,|}{n}$$

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n}(E_i - M_i)^2}{n}}$$

$$MAPE = \frac{1}{n}\sum_{i=1}^{n}\left|\frac{E_i - M_i}{E_i}\right| \times 100$$

Where is the n; data instances number, M_i; measured target value, E_i; predicted target value, M; measured target values mean, Ė; predicted target values mean, SM; measured target values sum, and SE; predicted target values sum. The correlation coefficient (r) was analyzed for assessing the goodness of the prediction according to Colton (1974). The correlation coefficient of between 0-0.25 indicate "little - no relationship", 0.25-0.50 indicate "fair relationship", 0.50-0.75 indicate "moderate - good relationship" and 0.75-1.0 indicate "very good - excellent relationship".

3. Results and Discussion

3.1. Shape, size, mass and chromatic parameters

This study was carried out to determine the effects of irrigations at different physiological stages on chickpea physical quality parameters. Gravitational, dimensional and area attributes are given in Table 3. Effects of irrigation treatments on shape, size, mass and color attributes were found to be significant at a 1% level. The greatest mass values were obtained from I_5 (0.50 g) while the lowest values were obtained from I_4 (0.42 g) treatments. The greatest length, width and thickness values were determined from I_5 as 11.01, 8.27 and 8.27 mm, respectively. Also, I_5 had greatest geometric mean diameter (9.10), PA (65.03 mm^2) and surface area (260.11 cm^2). PAs of chickpea seeds varied between 61.80 (rain-fed -I_1) and 57.49 mm^2 (50% pod set single irrigation-I_4). Similar findings were also reported by Masoumi & Tabil (2003) for PA between 60.87 mm^2 and 72.84 mm^2. Authors indicated that average length values were changed between 9.76 mm and 10.47 mm. George et al. (2007) stated that drying rate increased with increasing surface areas of the seed. Kibar et al. (2014) indicated that water deficits reduced average surface area and volume values from 236.99 mm^2 to 223.18 mm^2 and form 213.97 mm^3 and 200.62 mm^3 and significantly influenced thousand-seed weights of dry bean. Present findings comply with the results of Nikoobin et al. (2009) reporting decreasing geometric mean diameter and mass values with increasing from to 4.32 mm to 8.59 mm and 0.28 g to 0.42 g under different seed moisture contents.

Table 3- Mass, Dimension and Area attributes

Variables	*Mass (M, g)*	*Volume (V, cm^3)*	*Length (L, mm)*	*Width (W, mm)*	*Thickness (T, mm)*	*Geometric mean diam. (D_g, mm)*	*Projected area (PA, mm^2)*	*Surface area (SA, cm^2)*
I_1	0.47±0.04^{b}	365.99±34.97^{b}	10.70±0.47^{b}	8.18±0.38^{ab}	7.97±0.33^{b}	8.87±0.29^{b}	61.80±3.96^{b}	247.18±15.82^{b}
I_2	0.45±0.03^{bc}	350.80±29.27^{bc}	10.39±0.39^{cd}	8.12±0.39^{abc}	7.93±0.32^{bc}	8.74±0.24^{bc}	60.09±3.33^{bc}	240.36±13.33^{bc}
I_3	0.44±0.05^{c}	346.15±39.26^{c}	10.5±03.45^{bc}	7.95±0.38^{c}	7.88±0.41^{bc}	8.70±0.33^{c}	59.52±4.50^{c}	238.07±18.01^{c}
I_4	0.42±0.04^{d}	328.59±37.36^{d}	10.00±0.47^{f}	8.05±0.46^{bc}	7.78±0.39^{c}	8.55±0.32^{d}	57.49±4.36^{d}	229.95±17.44^{d}
I_5	0.50±0.03^{a}	394.86±30.80^{a}	11.01±0.43^{a}	8.27±0.32^{a}	8.27±0.31^{a}	9.10±0.24^{a}	65.03±3.38^{a}	260.11±13.50^{a}
I_6	0.44±0.05^{cd}	345.15±42.06^{c}	10.22±0.50^{de}	8.11±0.42^{abc}	7.93±0.40^{bc}	8.69±0.36^{c}	59.39±4.85^{c}	237.56±19.40^{c}
I_7	0.44±0.06^{cd}	338.83±48.07^{cd}	10.05±0.60^{ef}	8.07±0.45^{bc}	7.94±0.48^{bc}	8.63±0.42^{cd}	58.63±5.62^{cd}	234.50±22.47^{cd}
Mean	0.45±0.05	352.91±42.75	10.41±0.58	8.11±0.41	7.96±0.40	8.75±0.36	60.28±4.90	241.11±19.61
Min-max	0.24-0.63	178.25-488.26	7.85-12.12	6.20-9.95	6.37-9.57	6.98-9.77	38.29-74.97	153.17-299.86
F values	34.499**	32.884**	58.496**	6.460**	15.771*	31.788**	32.3880**	32.388**

Means indicated with different letters in the same column are significantly different (p<0.05). Min: Minimum, Max: Maximum
*Significant at p<0.05
**Significant at p<0.01

The shape in all treatments was described as oval because their average SI values were greater than 1.25. The greatest SI was obtained from I_1, I_3 and I_5 treatments while the greatest r was obtained from I_4 and I_7 treatments. Contrary to present findings, Kibar et al. (2014) reported increasing volumes with increasing irrigation water quantities. Comply with the present study, Sastry et al. (2019) reported average sphericity of desi, kabuli and intermediate type chickpea respectively seeds as 79.5, 85.7 and 84.5%. Surface area is closely correlation with the evaporation from the seed surfaces. r values close to unity indicate an almost circular shape. Contrary to present findings, Eissa et al. (2010) reported decreasing r values with decreasing moisture contents. AR, R, and sphericity values decreased, but SI and E values increased with increasing water deficit (Table 4).

Table 4- Shape attributes

Variables	*Sphericity (%)*	*Shape Index*	*Roundness*	*Aspect ratio*	*Elongation*
I_1	82.96±2.29^{c}	1.33±0.06^{a}	0.69±0.04^{c}	0.75±0.04^{d}	1.34±0.07^{a}
I_2	84.20±2.95^{b}	1.30±0.07^{b}	0.71±0.05^{b}	0.76±0.04^{bc}	1.31±0.07^{bc}
I_3	82.69±2.31^{c}	1.33±0.06^{a}	0.68±0.04^{c}	0.75±0.04^{cd}	1.34±0.07^{ab}
I_4	85.57±2.72^{a}	1.26±0.06^{c}	0.73±0.05^{a}	0.78±0.04^{ab}	1.29±0.07^{cd}
I_5	82.67±2.90^{c}	1.33±0.06^{a}	0.68±0.05^{c}	0.75±0.04^{cd}	1.33±0.07^{ab}
I_6	85.12±2.54^{ab}	1.27±0.06^{bc}	0.73±0.04^{ab}	0.78±0.03^{ab}	1.29±0.06^{cd}
I_7	85.99±3.09^{a}	1.26±0.07^{c}	0.74±0.05^{a}	0.79±0.05^{a}	1.27±0.08^{d}
Mean	84.17±3.02	1.30±0.07	0.71±0.05	0.77±0.04	1.31±0.07
Min-max	72.33-100.68	0.99-1.62	0.52-1.01	0.62-1.01	0.99-1.62
F values	27.111**	27.321**	26.894**	18.085**	17.718**

Means indicated with different letters in the same column are significantly different ($p<0.05$). Min: Minimum, Max: Maximum
**Significant at $p<0.01$

Color attributes are provided in Table 5. The greatest CI (8.46) and a^* (8.68) values were obtained from I_4 treatment (50% pod-set). However, the greatest C^* value was obtained from I_7 treatment (full irrigation) with values of 23.87. Queiroz et al. (2015) reported increasing L^* values under drying conditions. But in present study, water deficits reduced L^* values and the lowest value was obtained from the 50% pod-set (I_4) treatment as 55.26. Nevertheless, irrigation generally had a positive effect on color properties. Similarly, Jogihalli et al. (2017) in a study investigating the effects of roasting at different time and temperature conditions, reported b^* values (22.43-26.07) of close to the present values. The results showed that the change of physical attributes of chickpea seed grown in different supplementary irrigation treatments with the novelty of this study was revealed.

Table 5- Color attributes

Variables	*L^**	*a^**	*b^**	*Chroma*	*Hue angle*	*Color index*
I_1	60.25±7.50^{a}	7.43±1.05^{d}	21.32±2.94^{b}	22.60±2.92^{b}	70.64±2.80^{a}	6.01±1.57^{d}
I_2	58.92±4.92^{a}	7.66±.76^{cd}	20.21±2.11^{c}	21.64±2.06^{bc}	69.12±2.43^{b}	6.55±1.11^{cd}
I_3	60.14±5.83^{a}	8.09±0.99^{bc}	20.19±2.62^{c}	21.80±2.43^{bc}	67.92±3.65^{bc}	6.91±1.73^{bc}
I_4	55.26±5.47^{b}	8.68±1.20^{a}	19.01±2.19^{d}	20.95±2.05^{c}	65.31±3.90^{d}	8.46±1.80^{a}
I_5	56.00±11.00^{b}	7.57±1.14^{d}	19.98±3.00^{cd}	21.41±2.87^{c}	68.99±3.75^{b}	7.48±3.47^{b}
I_6	57.95±6.44^{ab}	8.11±0.98^{b}	19.41±2.05^{cd}	21.07±1.92^{c}	67.19±3.25^{c}	7.48±2.07^{b}
I_7	58.95±5.28^{a}	7.80±1.12^{bcd}	22.52±2.86^{a}	23.87±2.69^{a}	70.65±3.46^{a}	6.06±1.49^{d}
Mean	58.21±7.11	7.91±1.11	20.38±2.78	21.91±2.62	68.55±3.78	6.99±2.17
Min-max	21.86-75.81	1.60-12.18	13.44-34.96	14.40-35.52	54.51-86.30	1.18-24.31
F values	7.869**	16.594**	21.442**	17.496**	32.600**	19.192**

Means indicated with different letters in the same column are significantly different ($p<0.05$). Min: Minimum, Max: Maximum
**Significant at $p<0.01$

3.2. Discrimination of the irrigation treatments

The results of discriminant functions are presented in Table 6. The highest the eigen values, higher function gives dependent variable. Square of the correlation explains the effect size of functions. The first two functions explained 79.5% of total variation (respectively as 52.8 and 26.7%). Wilks' lambda explains best estimations. Wilks' lambda is significant for each estimator variables that is ideal, in this case, it was significant for 5 results. In the Wilks's lambda statistics, unexplained part of the differences between the groups was 34.6%.

Table 6- Discriminant analysis results

Eigenvalue statistics of discriminant functions	***Function 1***	***Function 2***	***Function 3***	***Function 4***	***Function 5***	***Function 6***
Eigenvalues	0.677	0.342	0.158	0.056	0.038	0.012
% of variance	52.8	26.7	12.3	4.3	3.0	0.9
% of cumulative variance	52.8	79.5	91.8	96.1	99.1	100.0
Canonical correlation	0.635	0.505	0.369	0.230	0.191	0.108
Significance test of canonical functions	1-5	2-5	3-5	4-5	5-6	6
Wilks' Lambda	0.346	0.580	0.779	0.902	0.952	0.988
Chi-square	730.934	374.777	171.902	71.107	33.739	8.013
df	78	60	44	30	18	8
p (sigma)	0.000**	0.000**	0.000**	0.000**	0.014**	0.432
Standardized canonical discriminant function coefficients	***Function 1***	***Function 2***	***Function 3***	***Function 4***	***Function 5***	***Function 6***
Mass	0.221	0.323	-0.137	-0.703	-0.899	0.695
Volume	1.287	1.730	6.769	4.057	3.568	-3.280
Length	3.529	-0.405	2.844	-3.589	-6.478	-5.662
Width	-2.527	-2.931	-6.976	-3.042	2.297	3.301
Thickness	-2.276	0.372	-4.692	1.941	0.875	6.167
Sphericity	2.784	1.716	4.484	1.691	-1.145	1.064
Shape index	-0.643	-2.282	-3.523	0.134	6.432	5.981
Elongation	-0.065	3.501	2.035	4.641	-2.650	2.479
L^*	0.548	-0.515	-0.213	0.034	0.718	-0.875
a^*	0.620	0.362	-1.039	-0.284	1.114	0.637
b^*	8.453	6.300	-6.132	-4.412	11.232	1.398
C^*	-8.431	-5.491	6.503	4.791	-11.362	-1.726
CI	0.953	-0.212	0.509	-0.118	0.891	-0.759

**Highly significant ($p<0.01$)

The discriminant function coefficients give relative importance of 13 estimators. According to the loadings, the function 1 and 2 had the highest loading for the b^* and C^*. For function 3, width and volume had greatest function coefficients. Figure 5 shows the centroids of 7 different irrigation treatments based on their canonical discriminant functions. Differences between components, color and size properties was considered as a significant distinguishing trait. The traits of sphericity and r for I_4, I_6 and I_7 treatments confirmed the location on the left of the canonical function 1 axis. In addition, b^* and C^* for I_1 and I_7 treatments were located on the bottom of canonical function 2 axis. Canonical function 3 had greatest load for width and volume with the negative and positive correlation, respectively. According to these attributes, I_1 and I_5 treatments and I_2 and I_3 treatments together constituted a separate group.

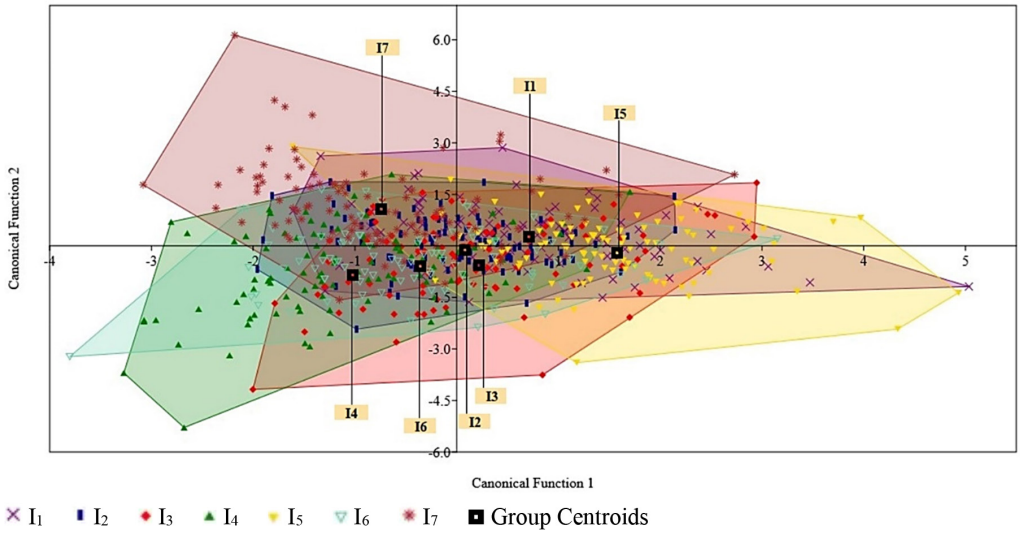

Figure 5- Scatter plots of the irrigation treatments in terms of group centroids of canonical function 1 and 2

3.3. MANOVA and pair-wise comparison

All treatments were found to be significant in terms of the shape, size and color as indicated by Wilks' Lambda, Pillai Trace and Hotelling Trace statistics ($p<0.01$). The results of the MANOVA test, Bonferroni corrected and Mahalanobis distances are provided in Table 7. Wilks' Lambda statistics expressed the percentage of variance in dependent variables and explained them with differences in independent variables. The smaller "Wilks' Lambda" statistic show that differences between groups to be analyzed increase and varies between 0 and 1 (Sayıncı et al. 2015a). Pillai Trace statistics, considered to be the most reliable among multivariate evaluations, takes into account the sum of the variance that explains the greatest discrimination of independent variables in dependent variables. Generally, the treatments with Mahalanobis distance of lower than 3 indicate significantly similar physical attributes ($p>0.05$). It concluded that the I_1 and I_2 treatments with the lowest Mahalanobis distances had the similar attributes. In the reference of Mahalanobis distances among the irrigation treatments, the closest distances were observed between I_1 and I_2 treatments, and between I_2 and I_3 treatments, while distance among the I_4 and I_5 treatments had highest value. Pair-wise comparisons revealed that there were not any similarities among the irrigation treatments.

Table 7- Differences among the irrigation treatments based on chickpea outlines

The results of MANOVA							
Effect	***Statistics***	***Value***	***Hypothesis df***	***Error df***	***F***	***p (sigma)***
	Pillai's trace	0.960	108	4086	7.204	0.000**
Variables	Wilks' Lambda	0.322	108	3881	7.867	0.000**
	Hotelling Trace	1.368	108	4046	8.542	0.000**
Hotelling's pairwise comparisons. Bonferroni corrected p values in upper triangle, Mahalanobis distances in lower triangle						
Variables	***I_1***	***I_2***	***I_3***	***I_4***	***I_5***	***I_6***	***I_7***
I_1		1.33E-03	3.10E-04	7.80E-21	2.45E-08	1.38E-09	1.60E-15
I_2	0.88		7.59E-02	5.26E-12	1.36E-16	1.05E-02	5.12E-12
I_3	1.38	0.93		3.85E-11	7.65E-15	6.13E-03	2.41E-18
I_4	4.78	2.85	2.67		2.52E-30	7.17E-03	5.63E-17
I_5	2.14	3.81	3.43	7.37		1.75E-19	5.39E-30
I_6	2.37	1.10	1.14	1.13	4.46		2.57E-13
I_7	3.57	2.85	4.20	3.89	7.27	3.11	

**Highly significant ($p<0.01$)

3.4. Estimation results of machine learning algorithms

Machine learning models were separately built based on data from 7 irrigation treatments. The results were evaluated based on the correlation coefficient of the estimation of mass and color properties. A 10-fold cross-validation was followed to test estimation correlation coefficient. The dataset of 700 measurements was divided randomly into 10 equally-sized subsets. Mass estimation results by MLP and RF were tabulated in Table 8. The best estimation criteria were higher correlation coefficient (r) and lower RMSE, MAE. Overall, each base learner performed all evaluation parameters very well with all achieving an r value of >0.98 for MLP. In the MLP algorithm, the greatest correlation coefficient for mass estimation was 0.997 and 0.996 in I_2 and I_7 treatments, respectively. The lowest RMSE and MAPE values obtained from I_2 treatment as 0.0010 and 0.0980, respectively. In addition, the lowest MAE was determined as 0.0008 for I_2 and I_6 treatments. In the RF algorithm, all r values obtained higher than 0.87. The highest r values was 0.9850 and 0.9755 for I_2 and I_7 treatments, respectively. The lowest RMSE, MAE and MAPE was found as 0.0079, 0.0053 and 0.6876 in I_2 treatment.

Table 8- Neural network parameters of the MLP structure and performance results of machine learning algorithms*

NN Type	*η*	*A*	*NoE*	n_i	n_h	n_o	*g (.)*	*Inputs*	*Outputs*	*Total records*
MLP	0.3	0.2	500	12	6	1	sigmoid	L, W, T, V, Dg, SA, PA, S, SI, R, AR, E	Mass	9100
MLP	0.3	0.2	500	3	6	1	sigmoid	L^*, a^*, b^*	Chroma Hue Angle Color Index	2100

	Mass				***Chroma***				***Hue angle***				***Color index***			
MLP	***R***	***MAE***	***RMSE***	***MAPE (%)***	***R***	***MAE***	***RMSE***	***MAPE (%)***	***R***	***MAE***	***RMSE***	***MAPE (%)***	***R***	***MAE***	***RMSE***	***MAPE (%)***
I_1	0.9992	0.0009	0.0015	0.1173	0.9998	0.0019	0.0028	0.0003	0.9996	0.0007	0.0011	0.0001	0.9988	0.0092	0.0141	0.0012
I_2	0.9997	0.0008	0.0010	0.0980	0.9996	0.0020	0.0029	0.0002	0.9998	0.0006	0.0008	0.0001	0.9992	0.0053	0.0076	0.0006
I_3	0.9989	0.0010	0.0018	0.1273	0.9998	0.0019	0.0025	0.0003	0.9997	0.0010	0.0014	0.0003	0.9981	0.0082	0.0128	0.0009
I_4	0.9989	0.0012	0.0023	0.1463	0.9972	0.0035	0.0095	0.0004	0.9985	0.0015	0.0031	0.0002	0.9963	0.0103	0.0295	0.0011
I_5	0.9848	0.0017	0.0082	0.1976	0.9996	0.0021	0.0037	0.0002	0.9978	0.0015	0.0035	0.0001	0.9982	0.0224	0.0785	0.0025
I_6	0.9994	0.0008	0.0012	0.0990	0.9998	0.0014	0.0019	0.0002	0.9997	0.0009	0.0013	0.0002	0.9945	0.0078	0.0164	0.0010
I_7	0.9996	0.0010	0.0016	0.1141	0.9984	0.0025	0.0050	0.0003	0.9994	0.0010	0.0018	0.0001	0.9980	0.0111	0.0192	0.0012
RF	***R***	***MAE***	***RMSE***	***MAPE (%)***	***R***	***MAE***	***RMSE***	***MAPE (%)***	***R***	***MAE***	***RMSE***	***MAPE (%)***	***R***	***MAE***	***RMSE***	***MAPE (%)***
I_1	0.9531	0.0064	0.0115	0.8470	0.9826	0.0152	0.0269	0.0015	0.9430	0.0103	0.0153	0.0010	0.9484	0.0607	0.0868	0.0061
I_2	0.9850	0.0053	0.0079	0.6876	0.9775	0.0128	0.0201	0.0013	0.9619	0.0081	0.0124	0.0008	0.9612	0.0411	0.0589	0.0041
I_3	0.9563	0.0062	0.0114	0.8319	0.9789	0.0158	0.0232	0.0016	0.9618	0.0116	0.0164	0.0012	0.9449	0.0484	0.0717	0.0049
I_4	0.9381	0.0089	0.0160	1.1296	0.9697	0.0179	0.0314	0.0018	0.9234	0.0129	0.0243	0.0013	0.8672	0.0655	0.1391	0.0065
I_5	0.8745	0.0066	0.0212	0.8007	0.9889	0.0143	0.0229	0.0014	0.8971	0.0112	0.0224	0.0011	0.9613	0.0472	0.0591	0.0097
I_6	0.9591	0.0063	0.0100	0.8005	0.9742	0.0135	0.0204	0.0013	0.9523	0.0114	0.0168	0.0011	0.9253	0.0528	0.0820	0.0053
I_7	0.9755	0.0082	0.0121	1.0385	0.9399	0.0156	0.0320	0.0016	0.9676	0.0091	0.0145	0.0009	0.9477	0.0657	0.1635	0.0066

*η: Learning rate, α: Momentum, NoE: Number of epochs, n_i: Number of input layers, n_h: Number of hidden layers; n_o: Number of output layers, g (.): Activation function, MLP: Multilayer perceptron; RF: Random forest, RMSE: Root mean square error, MAE: Mean absolute error, MAPE: Mean absolute percentage error

Similar to the present study, Soares et al. (2013) reported that in the mass estimation of banana, the R^2 were found between 0.63 and 0.91 in ANN algorithm. Authors also indicated the lowest mean prediction-error (MPE, %) values were obtained as 0.41. Rad et al. (2017) presented the best R^2 and MPE (%) values for ANN estimation of eggplant mass were 0.93 and 2.01, respectively. Gurbuz et al. (2018) used Find Laws algorithm for the mass estimation of almonds. The authors reported the greatest R^2 of 0.9561. Demir et al. (2020) to estimate the mass of the walnut was applied ANN algorithm and RMSE of MNN structure ranged from 0.60 to 0.89, while RMSE of Radial Basis Neural Network structure was found to be very low (0.0002) in all walnut varieties. Saglam & Cetin (2021) were applied MLP, k-nearest neighbor (kNN), RF, Gaussian processes (GP) to estimate mass (nut and kernel) of six different pistachio cultivars. Shape and size attributes were used as the input parameters and GP had the greatest correlation coefficients 0.976 for nut and 0.948 for kernel and the lowest RMSE values 0.038 for nut and 0.029 for kernel. This result conforms to the present study.

In the mass estimation, MLP yielded the best results in all treatments. ANN topology is an important factor in designing MLPs because it has an important effect on estimation. The number of hidden layers, neurons and epochs is also important. Additionally, it is preferable that the number of neurons in the hidden layer was low since it leads to an increase in the network learning speed and a decrease in the network size. In this case, ANN estimation of the individual mass in advance could make it possible for growers to prefer economical support with full assurance of a timely refund (Soares et al. 2013).

The highest r value in C^* estimation was determined in I_3 (0.9996) and I_5 (0.9997) treatments for MLP. However, the lowest RMSE, MAE and MAPE were found as 0.0718 (I_3), 0.0487 (I_6) and 0.0002 (I_2, I_5 and I_6) for MLP. In the RF algorithm, while the highest r values were obtained in I_1 (0.9826) and I_5 (0.9889) treatments the lowest was obtained in I_7 (0.9399) treatment. The lowest prediction error values were found as 0.4349 (RMSE) in I_5, 0.2792 (MAE) in I_2 and 0.0013 (MAPE) in I_2 and I_6 treatments.

The greatest r value for h° estimation determined from I_7 treatment as 0.9993 and 0.9676 for MLP and RF, respectively. In the MLP algorithm, the lowest RMSE and MAE values obtained in I_7 treatment as 0.1404 and 0.0693, respectively. I_1 treatment had the lowest RMSE for RF algorithm as 1.0587. Additionally, MAE and MAPE values were found in I_2 treatment as 0.5544 and 0.0008, respectively.

In the CI estimation, I_2 treatment had the highest r (0.9989) and the lowest RMSE (0.0528), MAE (0.0387) and MAPE (0.0006) values for MLP. The lowest RMSE, MAE and MAPE values for CI estimation were observed in I_2 as 0.3761, 0.2673 and 0.0041, respectively. However, the greatest r value was seen in I_5 treatment as 0.9613.

Comply with the present study, Kus et al. (2017) estimated C^*, hº and CI from L^*, a^* and b^* in 6 different apple varieties and reported the lowest RMSE findings as 0.5463 and 0.0001 for ANN and adaptive neuro-fuzzy interface system (ANFIS) algorithms, respectively. Demir (2018) estimated CI, C^* and hº parameters of 10 different walnut cultivars with the use of ANFIS. The author indicated that RMSE values ranged between 0.01 and 0.02, and the highest R^2 values 0.999 for h°. Germšek et al. (2017) was estimated fruit skin color (especial a color parameter a^*), for three apple varieties with the use of six different algorithms. The authors reported that the highest estimation accuracy values as 96.65% in logistic model tree algorithm. van Roy et al. (2017) estimated the color of tomatoes using hyperspectral imaging. The authors reported that partial least square (PLS) method was found to achieve the best R^2 results as 0.86, 0.93, 0.42, 0.95 and 0.51 for L^*, a^*, b^*, h° and C^*, respectively. Huang et al. (2014) used PLS regression algorithm to estimate of soybean color during drying from hyperspectral imaging and the better color estimation results obtained from mean reflectance as 0.862 (R) and 1.04 (RMSE). Present findings revealed that all ML methods had sufficient success in mass and color estimation of irrigation treatments. In the present study, physical and color properties of chickpea seeds grown under different conditions were estimated. It is thought that these data will facilitate the classification and discrimination of the seeds.

3.5. Limitations of the proposed study and the future research directions

Limitations of this study were the laboriousness of the data acquisition process. In addition, processing the obtained data and handling the applications separately caused the processes to take longer in estimations. Also, the choice of structure for ANN was also a limitation of the study. In future studies, it is recommended to preprocess the data before estimation or to reduce the data. Besides, different ML algorithms such as support vector machine, GP and kNN could be tried for similar studies. In fact, the usage of deep learning methods with image recognition and classification instead present methods of could contribute to the rapid of the process.

4. Conclusion

In this study, discrimination and estimation was performed for mass and color estimation of chickpea seeds at 7 different irrigation treatments using LDA and ML. The MLP yielded better outcomes as compared to the RF in both mass and color estimation. MLP with a 12-6-1 topology for mass estimation and 3-6-1 topology for color estimation also yielded quite a well discrimination for chickpea seeds. Present findings showed single or couple irrigations at different physiological stages could be sufficient to have desired yields and quality traits. The best results were achieved in I_5 for size and mass, I_4 and I_7 for shape and I_7 for color attributes. Present findings should also be considered in irrigation treatments and food processing technologies for chickpea.

Data availability: Data are available on request due to privacy or other restrictions.

Authorship Contributions: Concept: İ.S.V., N.Ç., H.K., Design: N.Ç., Data Collection or Processing: İ.S.V., N.Ç., Analysis or Interpretation: N.Ç., H.K., Literature Search: İ.S.V., Writing: İ.S.V., N.Ç., H.K.,

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Effects of Shade Nets on the Microclimate and Growth of the Tomato

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ABSTRACT

This study evaluates the effects of shading nets with photoselective features, with an open-field control. A green-shade net with a shading intensity of 40% intensity and blue, pearl, and yellow-shade nets with shading intensity of 75% were used as netting materials and the total radiation and photosynthetically active radiation (PAR) transmittance of the shade nets were analysed. The environmental conditions such as air temperature, relative humidity, and canopy temperature were measured with the aid of appropriate sensors. Tomatoes were grown to determine the effects of shade nets on crops cultivated under the four shade nets, and an additional treatment (control) was used in which tomatoes were grown under full sun conditions. The vegetative growth parameters, fruit quality parameters and yields were assessed and the findings showed that shading resulted in a significant increase in the total yield and pearl-shade nets are the best choice for producing a high quality tomato crop based on PAR transmittance (between 44.8% and 52.8%).

Keywords: Coloured shade nets, Radiation transmittance, Climate change, Soil water content, Crop growing

1. Introduction

Shade cloths are often used in agricultural production to protect crops from excessive solar radiation, environmental hazards (e.g. hail, strong winds, sand storms), or flying pests (bird, fruit-bats, insects) (Shahak et al. 2004; Castellano et al. 2008a; Castellano et al. 2008b). The preferred shading material for ornamental crops and nurseries is a black-shade net that diminishes light intensity by 40-80%. Anti-hail and insect-proof nets are typically made of clear or white threads or a combination of both, with initial shading factor ranges between 8% and 25% (Shahak et al. 2004). The shade nets typically used in common agricultural practices are transparent, black, or green coloured. Transparent nets are usually shading nets that allow sufficient light to enter, while black or green-shade nets are used over or under the greenhouse cladding materials, predominantly in the Mediterranean countries, to reduce high air temperatures inside the greenhouses during the spring and summer seasons of the year. Shade nets are also used as covering material of special structures, referred to as “shade houses”, and protect plants from intense solar radiation. The mesh size of these nets varies typically between 0.6 mm and 4 mm with a light transmittance ranging from 20% to 70% (Briassoulis et al. 2007b).

The photo-selective (coloured) shade nets increase the relative proportion of scattered light, and also absorb vari-ous spectral bands (visible, far-red light and beyond), thereby modifying the light environment (Shahak 2008; Nemera et al. 2015). Shade net decreases incoming solar radiation, air, leaf and root zone temperature while conserving soil water and thus reducing crop water requirements and increasing water use efficiency (WUE) (Möller & Assouline 2007; Diaz-Perez 2013).

Shade nets may contribute to an increase of crop yield as well as the quality of the harvest by modestly influencing the microclimate under the cover material. Principally shade nets are used to improve the productivity, quality and homogeneity of the plants and fruits by acting as a greenhouse and windbreak, thus providing a comfortable microclimate for the plants and fruits (Briassoulis et al.

2007a). Shahak et al. (2004) reported that blue-shade nets had a wide peak of transmittance (400-540 nm) in the blue-green region of the visible spectrum while yellow nets and red-shade nets transmitted light from 500 nm and above and from 590 nm and above, respectively. Schettini (2011) assessed the radiometric properties of coloured shade nets used to protect peach cultivation; their results showed that grey and pearl-shade nets were less transparent to photosynthetically active radiation (PAR) radiation with a PAR total transmissivity of 46.1% and 50.8% respectively. They found that the red, pearl, yellow and grey-shade nets increased the growth of the peach trees. Statuto & Picuno (2017) studied the effects of two different commercial plastic nets with shading factor of 36% and 60% respectively, on the internal microclimate of a greenhouse. They suggested that the selection of the most appropriate shading factor could significantly impact upon the protection of heat-sensitive crops from both high temperature and sunburn. Löpez et al. (2007) evaluated the correlation between dissimilar spectral compositions of red and pearl-shade nets and the lycopene content of "Bodar", "Cherry" and "Cocktail" tomato cultivars. They found that light quality and the colour of the net used as a covering material noticeably altered the lycopene content of tomatoes in addition to the variety of tomatoes grown.

This study investigates the effects of different coloured shade nets on the total radiation, PAR transmittance, environmental microclimate conditions, and development of the tomato plant.

2. Material and Methods

2.1. Study area

This study was carried out under open field conditions in 1500 m^2 area of land at the research and application farm of the Faculty of Agriculture, Akdeniz University, Antalya, Turkey (latitude 36° 54' N, longitude 30° 38' E, with an average altitude of 54 m). The research area has a typical Mediterranean climate; hot, dry summers and mild, relatively rainy winters. The annual average temperature is 18.1 °C. The annual average relative humidity and total precipitation are 60.6% and 881.7 mm, respectively. Some physical and chemical properties of the soil are given in Table 1.

Table 1- Physical and chemical properties of the soil

Depth (cm)	*Texture class*	*FC[a] (%)*	*PWP[b] (%)*	*Bulk Density (gr/cm³)*	*EC (mmhos/cm)*	*pH*	*$CaCO^3$ (%)*	*Organic Matter (%)*
0-15	CL	24.3	16.0	1.45	1.22	7.4	21.0	2.0
15-25	CL	20.0	18.0	1.29	0.284	8.1	28.2	1.6
25-35	CL	24.6	18.0	1.33	0.163	8.0	30.8	1.7

[a]FC: field capacity, [b]PWP: permanently wilting point, EC: electrical conductivity, $CaCO_3$: calcium carbonate, CL: Clay loam

Four different coloured shade nets (a green-shade net with 40% shading of the natural sunlight and pearl, yellow and blue-shade nets with 75%) were used in this study. Field tests for evaluating the effects of these nets were performed in an area around 720 m^2. The total land area covered by each coloured shade net was 180 m^2 (three repetitions of 60 m^2 for each one). For control treatment, 180 m^2 of the 720 m^2 remaining area was used. A 54 m long, 2.7 m high and 14 m wide flat-roofed iron skeleton was constructed to lay the nets in the study area. The shading nets were mounted above and around the iron frame (Figure 1).

Figure 1- The coloured shade nets (yellow, green, pearl and blue) used in the study

2.2. Measurements

The following climatic data were measured at the central part of each 60 m^2 of land designated for each coloured shade net and 780 m^2 of land assigned as the open field treatment group:

- Global solar radiation (GSR W/m^2), by means of 5 pyranometers (model CMP 3, Kipp and Zonen, Delft, The Netherlands), at a wavelength range of 300-2800 nm, placed 2.0 m above the ground and in the center of each treatment (Kittas et al. 2006);

- PAR ($mmol/m^2/s$), using 5 quantum sensors (model PQS 1, Kipp and Zonen, Delft, The Netherlands), at a wavelength range of 400-700 nm, placed 2.0 m above the ground and in the centre of each treatment (Kittas et al. 2006);

- Air temperature (T, ^oC) and humidity (RH, %), using 5 capacitive, negative temperature coefficient thermistor sensors and relative humidity sensors (model 175-H2, Testo Electronics, Istanbul, Turkey), placed at 1.5 m above the ground and in the centre of each treatment (Barroso et al. 1999) (Figure 1).

- Canopy temperature (T, ^oC), was monitored using an infrared thermometer (model Omega OS530HRE, USA).

Canopy temperature measurements were taken with a field of view estimated to be 15 degrees. The infrared thermometry was held 1 m above the ground with a horizontal angle of 45 degrees during measurements, viewing an elliptical surface having approximately a minor axis of 6 cm, a major axis of 9.5 cm and an area of 40.05 cm^2 (O'Toole & Real 1984). The measurements were taken at 12 PM and 2 PM, in four directions (east, west, north and south) in each treatment.

Total radiation and PAR transmittance were calculated using the following formula (Kittas et al. 1999):

$$\tau_T = \frac{T_i}{T_o} \times 100 \qquad \text{(Eq. 1)}$$

Where; τ_T = total radiation permeability of the shade net (%): Ti = radiation reached the surface of land under the shade net (W/m^2): To = radiation reached the surface of the land in the control group (area with full sun) (W/m^2).

$$\tau_P = \frac{P_i}{P_o} \times 100 \qquad \text{(Eq. 2)}$$

Where; τ_P = PAR permeability of the shade net expressed as a percentage: Pi = PAR reached the surface of land under the shade net ($mmol/m^2/s$): Po = PAR reached the surface of land in the control group (area with full sun) ($mmol/m^2/s$).

All GSR and PAR measurements were performed on days with clear skies. Time periods from 6 AM to 6 PM on five days under clear sky conditions were considered for the determination of monthly GSR and PAR transmittance of the shade nets (Geoola et al. 1998; Geoola et al. 2004; Kittas et al. 2006). The temperature and relative humidity data were analyzed for 24 h during selected days with clear skies in conjunction with the GSR and PAR transmittance measurements. All measurements were collected at intervals of 10 min using a data logger (model DL2e Delta-T Devices, Cambridge, UK) during the study period. The outside sensors for PAR, total radiation, temperature and relative humidity are located on the meteorological station platform which was positioned next to the shade nets.

The tomato crops [industrial tomato (cv. Ancon)] were planted with a distance of 140 cm between the rows and 40 cm between the plants. The transplanting and the last harvest date were April 27 and August 17, 2018 respectively. Nine plants from each plot were randomly selected and tagged for recording plant height (cm), the total number of leaves, plant and stem diameter (mm); these parameters were measured on the plants first at 27 days after transplanting and then at intervals of 15 days until the termination of the study. Five harvests were conducted from the 6th of June to the 17th of August on full grown fruits of at least 50% red colouration. The total yield (t/ha), fruit quality parameters [average fruit weight (gr) and size, EC and pH of the fruit juice and water soluble dry matter (%) (WSDM)] were measured seasonally. To determine the physical properties of the fruit, ten tomatoes were selected randomly from each treatment. The average fruit weight was calculated as the weight of all fruits for each tomato plant divided by the total number of fruits and recorded in grams by a sensitive digital weighing balance. The fruit diameter was measured using callipers. The biochemical parameters of the fruit (such as WSDM, EC, pH and fruit skin colour) were analysed to determine the fruit quality and the quality parameters (such as WSDM, EC and pH) were measured in the juice extracted from 10 fruits from each replication. The WSDM was estimated using a digital refractometer (ATAGO, RX 5000, Tokyo, Japan). The EC and pH values were measured in homogenized juice obtained from the sample tomato fruit via EC and pH probe of a portable multimeter (HACH LANGE HQ40D.99). Each fruit

had a specific skin colour indicating its maturity. The fruit's skin colour was measured to determine its ripeness using a "Minolta 200" chroma meter.

Water and fertilisers were supplied via a drip irrigation system. The dripper flow rate was 2 L/h at 1 atm pressure. Intermittent irrigation was carried out at intervals of every 2 days from the beginning of the experiment until the last harvest. The method for determining the irrigation water level was based on evaporation data (Epan, mm) obtained from a Class A pan located next to the control plot. The pan was mounted on a wooden platform at a height of 15 cm above the soil surface and readings were recorded daily. The irrigation level was chosen as 100% of Class-A Pan evaporation for all plots. In this way, by applying the water equally for the parcels, unwanted fluctuations in the irrigation were prevented. 100% of the two-day evaporation values taken from Class A evaporation pan were measured in mm. The irrigation level required for the plants was calculated in mm and then converted into litres (Kırda et al. 2004).

$$I = kp \times kc \times Ep \times A \qquad \text{(Eq. 3)}$$

Where; I = irrigation water (liter/plant): kp = Class A Evaporation Pan coefficient (taken as 1.0 in this study) (Öner et al. 2002): kc = plant coefficient which takes values from 0.45 to 1.25 depending on the plant's growth stage (seedling period 0.45, vegetative period 0.75, flowering 1.15, fruity 0.85, ripening 0.6) (Doorenbos & Kassam 1979): Ep = total evaporation from the A-Class Pan corresponding to the irrigation range and presented in mm; A = area of a parcel in square meters.

The soil water status was monitored gravimetrically from depths of 0-15 cm, 15-25 cm and 25-35 cm at 15 days intervals to determine the effects of the shade nets on soil moisture. First, the weight-based soil water content was computed for each depth (i.e 0-15, 15-25, 25-35 cm). Next, the weight based soil water content was multiplied by the bulk density in each depth to calculate the volumetric water content. Finally, the volumetric water content was multiplied by the depth to measure the soil water content in mm for the entire depth (35 cm).

A suitable fertilization program was prepared based on the soil analysis in the experimental area (228, 152, 290, 56, and 14 kg/ha of N, P_2O_5, K_2O, CaO, and MgO respectively). The fertilizers containing microelements were applied to the soil using the drip irrigation system. Fertilization was carried out the day before planting using 15-15-15 compound fertilizer with 50 kg NPK (15% of N, 15% of P_2O_5 and 15% of K_2O) (half of the requirement).

Data were analyzed using SPSS 16.0. According to the results of variance analysis, the classification of possible differences between the averages of subjects was made using the Duncan test at a significance level $p \leq 0.05$ (5%).

3. Results and Discussion

3.1. Solar radiation and PAR transmittance under the nets

Shading nets were placed over the tomato plants 52 days after planting (Kittas et al. 2009) and the selected physical properties of the shade nets, such as total radiation and PAR transmittance, were evaluated after the shade nets had been placed over the tomatoes. The yellow-shade nets showed the highest PAR transmittance while the green-shade net showed the lowest. The total radiation of the yellow-shade nets ranged between 57.5% and 65.6%, whereas the total radiation of green-shade nets ranged between 42.3% and 48.9%, respectively. The blue and pearl-shade nets showed a moderate transmittance (Figure 2). In their 2007 study, Briassoulis et al. (2007b) reported that the transmittance of shade nets to be between 20-70%. Kotilainen et al. (2018) noted that the spectral composition (light quality) transmitted by climate screens and shade nets used in horticultural applications is affected by shade nets material properties. They reported that shade nets reduce solar radiation transmittance and that green-shade nets can be used for plants where solar radiation is accepted as a limiting factor for them.

In this study, Figure 3 shows the daily evolution of solar radiation both in an open field and under the shade nets throughout the daylight hours of a summer day. The transmittances of the shade nets changed depending on the incidence angles of the sun's rays varying during the day.

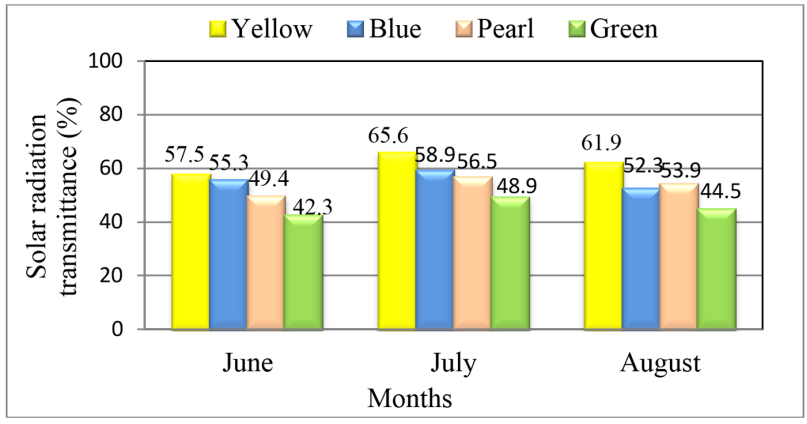

Figure 2- Solar radiation transmittance of different coloured nets (%)

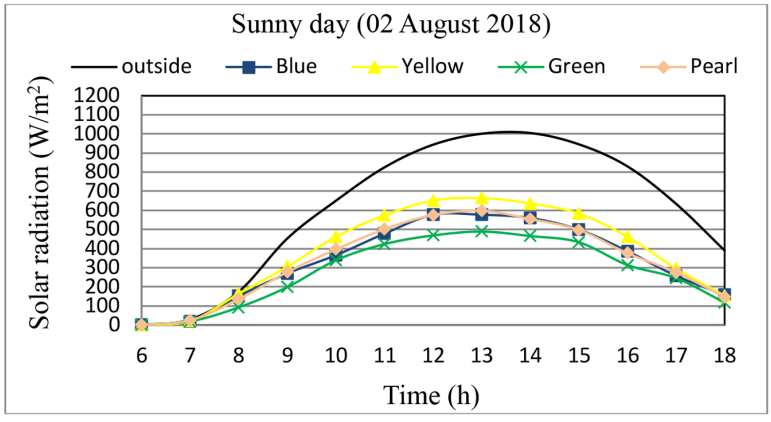

Figure 3- Solar radiation outside and under the shaded nets; on a typical summer day

As shown in Figure 4, the yellow-shade nets showed the highest PAR transmittance (56.5%) in June, while the pearl-shade nets showed the highest transmittances in July and August (48.7 and 44.8%, respectively). The pearl-shade net appears to be the most suitable net in terms of PAR region because it showed the highest transmittance in both July and August. At the same time, the total yield under the pearl-shade net was the highest of all the shade nets. The blue-shade nets showed the lowest PAR transmittance throughout the study. The PAR transmittance values of the blue-shade net varied between 28.8% and 41.1%. Figure 5 shows the daily evolution of PAR radiation in the open field and under different coloured shade nets on a typical summer day.

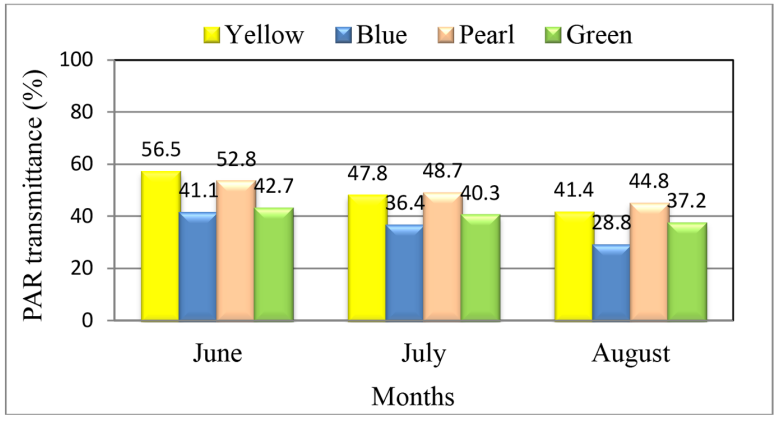

Figure 4- PAR transmittances of different coloured shade nets (%)

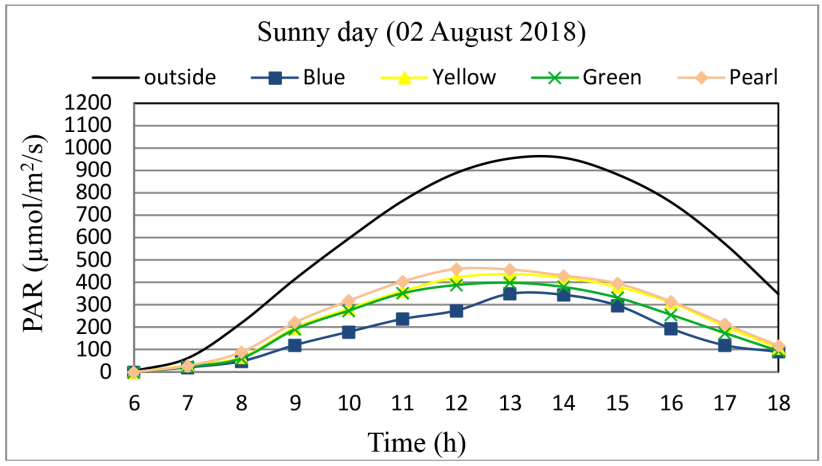

Figure 5- PAR radiation outside and under the shade nets; on a typical summer day

These findings are in line with those of Kittas et al. (2009) who investigated the spectral properties of four different-coloured shade nets with different shading intensities (two black nets with a shading intensity of 40% and 49%, a green net of 34% and mixed green and black net of 40%) in a study conducted along the coastal areas of Eastern Greece using a pyranometer and spectra radiometer to calculate measurements. Their study found no significant differences the transmittance of the nets in different wavebands (PAR: 400-700 nm, near infrared radiation: 700-1100 nm, total radiation: 400-1100 nm). Schettini (2011) examined the influence of the radiometric properties of shade nets (blue, red, pearl, gray, yellow and transparent) on peach tree morphogenesis, by studying the modification of the spectral distribution of transmitted radiation. Their results found that red, blue, yellow and pearl-shade shade nets showed a non-uniform transmittance in the PAR region. Ilic et al. (2017) studied the effects of four different coloured shade nets (red, pearl, blue and black) with a shading intensity of 40% on environmental conditions and plant development in the "net houses" and "plastic greenhouse + shade net" conditions in southern Serbia. They noted that the pearl and blue-shade nets showed 59.8% and 53.6% transmittance in the PAR region, respectively. Based on the findings obtained from our study, all shade nets significantly reduced total radiation between 34.4% and 57.7%. The reduction in PAR transmittance was found to be between 43.5% and 71.2% when compared to the uncovered area; in addition, the transmittance of shade nets in the PAR region was not constant over the entire 3 months period. Furthermore, PAR transmittance of the pearl-shade nets was found to be higher than the other nets in July and August.

3.2. Microclimate under the nets

The effects of shade nets on the microclimate environment were assessed by the recorded values of the relative humidity, and the environment and leaf temperatures. The monthly variations of the temperature and relative humidity under different coloured shade nets from June to August 2018 are shown in Table 2 and Table 3. As seen in Table 2, the average daily minimum temperature was similar among the different coloured shade nets but lower to that of the open field. The average daily temperature was also similar among the different coloured shade nets but higher to the open field group. The results suggest that average daily maximum temperature values correlate strongly with the microclimate environment. With the exception of the green-shade net for July, the average daily maximum air temperatures under the nets were higher when compared to the open field (Table 2). The highest air temperatures were recorded under blue-shade nets in June (4.5 ^{o}C higher than outside) and under yellow-shade nets in July and August (1.7 ^{o}C and 2.6 ^{o}C higher than outside, respectively). The green and pearl-shade nets were found to have similar temperature values. The lowest air temperatures among the nets in June, July and August were recorded under yellow, blue and pearl-shade nets, respectively.

The average daily relative humidity was found to be higher under the nets when compared with the control treatment without shade nets (Table 3). This may be due to the limited air circulation within the shade nets. The average daily relative humidity values among the nets varied between 54.4% and 84.2%, whereas the average daily relative humidity values recorded outside fluctuated between 49.3% and 71.9% (Table 3).

Table 2- Temperature recorded inside and outside the net houses

Months	Temperature inside shade nets (°C)												Outside (oC)		
	Yellow			Blue			Pearl			Green					
	Min	Max	Avg	Min	Max	Avg	Min	Max	Avg	Min	Max	Avg	Min	Max	Avg
June	17.9*	33.0	26.0	18.0	36.2	27.1	17.7	34.3	26.6	18.0	34.1	26.4	19.1	31.7	25.8
July	18.5	43.7	30.2	18.9	41.6	30.5	18.7	42.9	30.3	18.9	42.0	30.1	20.0	42.0	30.3
August	19.4	43.1	31.1	19.4	41.9	31.1	19.2	41.1	30.6	19.2	42.0	30.8	21.0	40.4	30.6

*Values in the table represent the average temperatures of five clear sky days. Max: Maximum, Min: Minimum, Avg: Average

Table 3- Relative humidity recorded outside and inside shade nets

Months	Humidity inside shade nets (%)												Outside (%)		
	Yellow			Blue			Pearl			Green					
	Min	Max	Avg	Min	Max	Avg	Min	Max	Avg	Min	Max	Avg	Min	Max	Avg
June	43.2*	99.9	84.2	38.0	99.9	74.7	48.1	99.9	82.6	43.0	99.9	75.3	45.8	90.2	71.9
July	15.5	99.9	58.2	20.5	99.9	54.4	21.7	99.9	60.2	19.7	97.8	55.2	15.8	84.7	49.3
August	18.9	99.9	63.1	18.2	99.9	56.7	27.5	99.9	61.3	24.0	99.9	65.4	18.3	90.0	51.6

*Values in the table represent the average relative humidity of five clear sky days. Max: Maximum, Min: Minimum, Avg: Average

The relative humidity values under the shade nets predominantly ranged in levels that maintain optimal conditions for crop growth during the study period. Ozturk (2008) suggested that the optimal relative humidity for plant growth typically varies between 50% and 80%. Figure 6 shows the daily evolution of temperature and relative humidity outside and under the shade nets on a typical summer day.

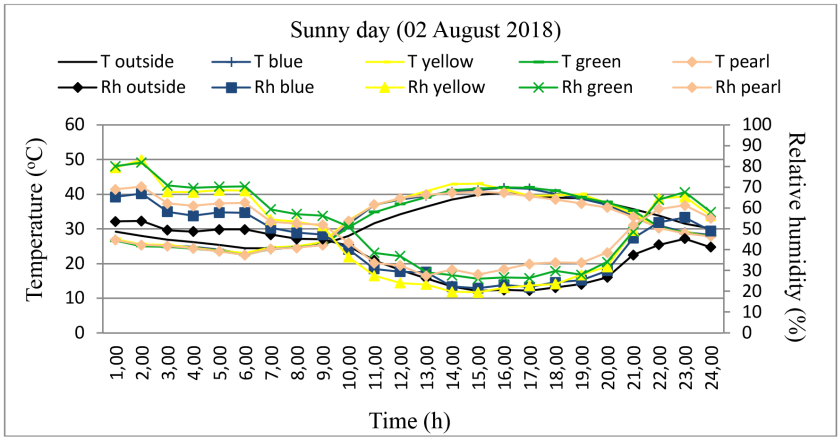

Figure 6- Temperature and relative humidity outside and under the shade nets on a typical summer day
T: temperature; Rh: relative humidity

Arthurs et al. (2013) studied environmental factors including (air temperature and humidity) both in the net houses (red, blue, black, pearl) and under open field conditions. Their study evaluated the average daily maximum temperature values and found the values in the net houses to be higher than those in the external environment. In their 2009 study, Kittas et al. (2009) noted that the average air temperature values recorded under the nets (29.5 °C) and outside (29.6 °C) were remarkably close. Their study reported that this was because the shading nets were only mounted above and not around the structures. Abdrabbo et al. (2010) found that the use of shade nets exerted a limited influence on mean air temperature and noted that air temperatures were typically lower under the nets (2-6 °C). In their 2014 study, Gaurav (2014) found that the temperature and light intensity decreased with the increase in shading density, while relative humidity increased. They determined that temperature reduction was highest in 90% shade followed by 75%, 50% and 35%. Meena et al. (2014a) investigated the change in the microenvironment under different colour shade nets and noted that light intensity, incoming radiation, canopy temperature, air temperature, and soil temperature were found to be lower under different coloured shade nets compared to those without shade netting. Ilic et al. (2017) found that the microclimates under shade nets (red, blue, pearl and black)

were noticeably similar and temperature and relative humidity values were slightly lower than those of the outside environment. They also reported that shading nets were useful in controlling the rise in temperature as the nets prevented the plants from damage from excessive sunlight; this protection from excessive sunlight also significant affected productivity. In another study, Shahak et al. (2004) reported that shading in several locations in Israel caused decreases from 1 °C to 5 °C in maximum daily temperature and an increase in the daily maximum relative air humidity by 3% to 10%. Nangare et al. (2015) investigated the effects of three green shade nets with a shading intensity of 35%, 50% and 75% on the growth of tomato crops growing in Pakistan. They found that the average monthly maximum temperatures varied substantially among these nets between 15 °C and 33.4 °C from November to April, whereas the relative humidity was between 30.4% and 61.2%. Their results showed that these values were not significant when compared to those under open field conditions.

The plant leaf temperature measurement values obtained under the shade net treatments are given in Table 4.

Table 4- Monthly mean canopy temperature values of the treatments

Months	*Shade nets (oC)*				*Outside (oC)*	*P > F*
	Yellow	*Blue*	*Pearl*	*Green*		
June	30.3[†c£]	32.1[a]	31.4[b]	30.9[bc]	32.6[a]	*
July	33.0[b]	32.0[c]	33.1[b]	32.8[b]	34.2[a]	*
August	35.7[b]	34.3[c]	36.1[b]	35.6[b]	37.9[a]	*

[†]Data are mean of 3 replicates, [£]Data in rows followed with different letters are significantly different based on the Duncan test ($p<0.05$). Values in the rows with different letters are significantly different ($p<0.05$) based on one-way ANOVA post-hoc Duncan's Multiple Range tests. *Significantly important at $\alpha=0.05$ probability level

The canopy temperature was 37.9 °C in the open field in August, the highest among all treatments ($p<0.05$). In addition, the differences between the groups were relatively significant for July and August. In the months of July and August, while the blue-shade shade net showed the lowest canopy temperature value, no significant difference was found in the canopy temperature values between yellow, pearl and green-shade net. Comparable results were verified in a study by Kittas et al. (2009) who noted that the canopy temperature was significantly lower in coloured shade net groups when compared to the open field treatment. Diaz-Perez & John (2019) found that the leaf temperature was highest in the unshaded treatment (34.2 °C) and lowest under the red net (33.2 °C).

As shown in Figure 7, the soil water contents were higher for shading applications when compared with those under the open field conditions. The difference in soil water content may be the result of reduced evapotranspiration caused by the effects of the shade nets. Throughout the growing season, the total soil water content of the pearl-shade net was found to be higher than those of the blue-shade net. The lowest value of soil water content among all the treatments was observed in the control group (Figure 7).

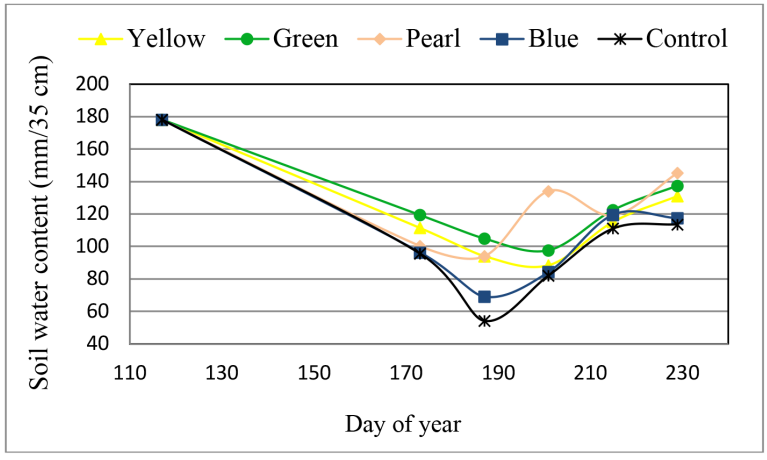

Figure 7- The changes in the soil water content during the study

Jifon & Syvertsen (2003) found that crops grown in open fields in a semi-arid climate are subjected to direct sunlight, high temperatures, and wind, resulting in high crop evapotranspiration (ETc). For this reason crops grown in these conditions require larger amounts of water. In contrast, shade-houses promoted the growth of plants by providing them a more pleasant environment with less stress and

protection from direct sunlight and high temperatures. The impact of strong winds was reduced, ETc values were lower and humidity was relatively higher. The irrigation water requirement of 23% to 31% pan evaporation was used for plants grown under 70% light reduction. In addition, water-use efficiency increases under shady conditions. Meena & Vashisth (2014b) reported that WUE was higher under nets (black, white, red and green) than the control during the summer season. Tezcan (2018) studied the influence of four shade factors (95%, 75%, 55% and 40%) of green shade netting on soil water content and plant root distributions. The highest soil moisture was obtained from 95% green shade nets and the lowest soil moisture was obtained from 40% green shade nets in covered plots. Soil moisture of 95%, 75%, and 55% green shade nets was found to be similar during the study. In addition, Tezcan (2018) noted that soil water content for the open field condition was the lowest among all treatments and showed a fluctuating trend.

3.3. Effect on the plant growth

Table 5 shows some vegetative growth parameters, yield and fruit quality parameters of the tomato plant for the four coloured shade nets and outside conditions. Statistical analysis revealed that significant differences were found between the crops grown under the shade nets and outside.

Table 5- Plant growth, yield and fruit quality parameters for all the treatments

Parameters		***Shade nets***				***Outside***	***P > F***
Yellow		***Green***	***Pearl***	***Blue***			
Plant height (cm)		69.0†	66.7	77.8	74.5	70.5	ns
Stem diameter (mm)		25.1†	25.6	23.0	25.0	25.8	ns
Number of leaves per plant		72†	83	110	101	70	ns
Total yield (t/ha)		26.3$^{c£}$	29.3^{b}	30.0^{a}	20.9^{d}	20.8^{d}	*
Average fruit weight (g)		89.3$^{b£}$	91.2^{a}	88.4^{c}	75.6^{d}	67.5^{e}	*
Fruit diameter (mm)		51.5$^{c£}$	52.3^{b}	53.4^{a}	49.1^{d}	46.7^{e}	*
WSDM (%)		4.6$^{b£}$	4.0^{d}	4.2^{c}	4.0^{d}	4.7^{a}	*
EC (dS/m)		4.6$^{a£}$	4.2^{b}	4.2^{b}	4.2^{b}	4.1^{c}	*
pH		4.4^{b}	4.4^{b}	4.4^{b}	4.4^{b}	4.5^{a}	*
Fruit skin color	L	43.4	44.8	44.2	43.5	44.4	ns
	a	33.9	34.0	33.5	33.1	35.1	ns
	b	31.0	33.8	32.5	32.4	34.1	ns

†Data represent the mean of 3 replicates, *Significantly important at α=0.05 probability level, £Data in rows followed with different letters are significantly different based on the Duncan test ($p<0.05$), ns: Non-significant. WSDM: water soluble dry matter, EC: electrical conductivity, CIE L a*b*: CIELAB color space defined by the International Commission on Illumination (CIE)

As shown in Table 5, the effects of shade nets on vegetative growing parameters such as plant height, stem diameter and the number of leaves were not statistically significant. The differences between the total yield values were considered statistically significant at a value of less than 0.05. Shading with yellow, green and pearl-shade nets increased the total yield by about 26%, 41% and 44%, respectively when compared to non-shade conditions. Shade nets also eliminated sun scalds appearing on tomatoes during the hot summer season more effectively than the open field conditions. The effects of shade nets on fruit quality parameters, such as average fruit weight, fruit diameter, WSDM and EC-pH in the fruit juice, were found to be statistically significant. The greatest fruit weight (91.2 g) was obtained from the green-shade net group whereas the lowest fruit weight (67.5 g) was recorded in the control group. The highest fruit diameter (53.4 mm) was measured in the pearl-shade nets group while the lowest fruit diameter (46.7 mm) was observed in the control group. The highest WSDM (4.7%) and pH values (4.5) were found in the control group, while the highest EC (4.6 dS/m) value occurred in the yellow-shade net group.

De Castro Ferreira et al. (2010) found that the best results in terms of growth rate, productivity, and energy efficiency for cucumbers were obtained under T3 conditions (30% red filter net in the visible and 40% transmission in the far red spectrum). Milenkovic et al. (2012) reported that total and marketable yield increased with a 40% shading level and then decreased (with 50% shade). The relative difference between the coloured and the black shade nets with regards to export-quality fruit yield was even more prominent. Milenkovic et al. (2012) noted that total fruit yields (t/ha) under the coloured shade nets were higher by 113% to 131%, relative to the equivalent black shade net. Ombodi et al. (2016) reported that shade net coloured significantly affected the vitamin C and total polyphenol contents of pepper fruits due to the effect of shade nets on the light level and light quality. Tafoya et al. (2018) reported that

the cucumber yield obtained with blue, aluminized, red or pearl net increased from 46% to 71% compared to conventional black net. Zhang et al. (2022) found that a red shade was effective in improving green tea quality by increasing the content of L-theanine and free amino acids in tea leaves collected in spring and fall when compared to the unshaded control.

This study has demonstrated that the use of shade nets for open-field tomato production increased the total crop yield, with pearl-shade nets being the most suitable type of net for tomato production when taking into account PAR transmittance and total yield values. During the study period, working under the shade nets also provided a far more comfortable work-environment for the growers and researchers participating in the study.

4. Conclusions

Our results showed that the lowest light transmittance (ranging from 42.3% to 48.9%) was obtained from 40% green-shade net and the highest light transmittance (ranging from 57.5 to 65.6%) was obtained from 75% yellow-shade net in covered plots. The light transmittance of the blue and pearl-shade nets was determined as moderate. The pearl-shade net should be the optimal choice for agricultural applications in terms of PAR transmittance since it demonstrated the best performance regarding crop yield. The shade application of colour nets affected environmental variables and provided a more favorable microclimate for tomato plants compared to non-shading conditions during the spring and summer seasons in Antalya. Shade nets were found to have a positive impact on both fruit quality parameters and yield. This study also found a statistically significant decline between 0.5 ^{o}C and 3.6 ^{o}C in the leaf temperature under the shade nets compared to the open field conditions. Soil moisture was significantly higher under the shade nets than soil moisture measured in the open fields. For this reason, the application of shade nets may allow farmers to use less irrigation water on their crops and contribute to the wider global efforts towards water conservation. In addition, other related studies reported that shade nets are also used to protect the plants from wind, hail, birds and viral diseases transmitted by insects. Due to the noticeable increase in plant viral diseases, future studies may wish to examine the effects of the shade nets on the transmission of insect mediated plant viral diseases. According to the data obtained in this study, green-shade nets are better used for plants that thrive in shade, while the agricultural applications of yellow-shade nets are more suited for plants whose growth is promoted by low solar radiation. Finally, concerning the effects of shading on the crop, this study found that shading applications with coloured nets clearly led to an increase in total yield.

Data availability: Data are available on request due to privacy or other restrictions.

Authorship Contributions: Concept: N.Y.T., Design: N.Y.T., Data Collection or Processing: N.Y.T., H.T., C.K., Analysis or Interpretation: N.Y.T., H.T., C.K., Literature Search: N.Y.T., H.T., Writing: N.Y.T.

Conflict of Interest: No conflict of interest was declared by the authors.

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The Massive Impact of Ram's Sperm Starvation on the Fertilization and Blastocyst Rates in Terms of Sperm Quality and Capacitation

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ABSTRACT

During sperm incubation *in vitro*, the sperm capacitation medium must play a key role in establishing conditions that perfect the required physiological requirements for sperm metabolic activities to obtain a high *in vitro* fertilization rate ensured by the ideal development of the embryos. Therefore, sperm collected from the caudal epididymis of rams should acquire progressive motility and fertilization. This process occurs through physiological and biochemical changes known as capacitation, a prerequisite for fertilization. In this work, we have studied a new way to incubate sperm, applied for the first time in ram, using four different treatments in terms of energy substrates and different incubation methods. In this sperm energy restriction and recovery treatment, sperm was let starving for 50 min until it lost its capacity for motility, and then was provided with glucose and incubated for 60 min. In the other three treatments, the sperm was not exposed to starvation and was incubated by the standard methods of maturation and capacitation by evaluating different energy substrates. According to the obtained results, the sperm was hyperactive and showed a significant increase in the ability to fertilize oocytes in the treatment that involved starvation and then re-activation of the sperm by adding glucose, compared to other treatments. In conclusion, the effects of this treatment persisted until after fertilization, which led to the production of a high percentage of blastocysts.

Keywords: Sheep, Sperm Starvation, Blastocyst, IVF Capacitation, Rescue Protocol

1. Introduction

The use of assisted reproductive technologies in small ruminants, such as sheep, allows for rapid and sustained growth in animals of genetic quality, increasing sheep production efficiency (Zhu et al. 2018). However, the in vitro embryo production (IVP) system in sheep lags behind its *in vivo* counterpart and *in vitro* systems produced in other mammalian species in terms of efficiency and quality, limiting the advancement of this promising technology (Ledda & Gonzalez 2018; Ledda et al. 2019). Many essential aspects limit sheep IVP, including the environment supplied by sperm capacitation, in vitro fertilization (IVF), and poor embryo culture conditions, resulting in low numbers of viable blastocysts for transfer (Amiridis & Cseh 2012; de Souza et al. 2014; Souza et al. 2021). IVF in sheep occurs through several steps. First, sperm for IVF are obtained from the epididymis of slaughtered rams, as slaughterhouses are one of the cheapest sources of biological material collection (Hajihassani et al. 2019; Merati & Farshad 2020); secondly, sperm should undergo a process known as capacitation, characterized by a sequence of physiological and biochemical processes (sperm membrane destabilization, motility changes, and acrosome reaction). Although in vitro capacitation can be accomplished by incubating sperm in a simple medium containing a capacitation-supporting component at a specific temperature and pH level, however, the media currently utilized for sperm capacitation is not entirely appropriate for IVF (García-Álvarez et al. 2015; Umehara et al. 2018; Roldan 2019). Cross-reactivity between metabolic and signaling pathways is required for sperm capacitation (Goodson et al. 2012). Consequently, in a sperm capacitation medium (CAP), energy substrates and chemicals that increase sperm motility, capacitation are required; these typically include heparin (García-Álvarez et al. 2015), glucose (Leahy et al. 2016), inositol (Vazquez-Levin et al. 2020), caffeine plus heparin (El-Shahat et al. 2016).

An appropriate supply of adenosine triphosphate (ATP) for sperm is a prerequisite for many processes required for successful fertilization (Visconti et al. 2011). Sperm motility consumes over 70% of total ATP (Bohnensack & Halangk 1986), making sperm more energetically demanding than other cell types (Garrett et al. 2008). Several investigations in several species have found strong links between sperm ATP concentration and sperm motility and swimming velocity (Tourmente et al. 2015), and reductions in specific sperm characteristics are linked to decreases in internal ATP levels (Tourmente et al. 2019). Thus, ATP consumption rate is essential to sperm performance (Sansegundo et al. 2022). Studies in primates (humans and chimpanzees) (Anderson et al. 2007), felids (Terrell et al. 2011), and strains of laboratory mice (Odet et al. 2013) found the main pathway used for ATP production in sperm differs between species. Different methods for improving mice sperm functionality have recently been reported. The first method required transient exposure to the Ca2+ ionophore A23187 (Sánchez et al. 2021), while the second required sperm incubation without energy nutrients (starvation) (Navarrete et al. 2019). Both methods were linked to a loss of motility followed by a rescue step involving the removal of ionophores or the addition of energy nutrients.

IVF requires crucial capacitation changes, which by utilizing intracytoplasmic sperm injection can be overcome, but there are some extremely obstacles in applying this technique in sheep-like it being economically expensive. This study aimed to design new strategies of capacitation by starving ram's sperm *in vitro* and estimate the effects of its quality. The absence of previous studies of ram sperm in this area boosts our study goals by using a CAP devoid of energy nutrients and glucose reintroduction after the sperm has ceased to move to determine how these starvation and rescue protocols help them to become more fertile and enhance embryonic development.

2. Material and Methods

The study was conducted in the Reproductive Biology and Animal Physiology Laboratory at Ankara University, Faculty of Agriculture, Department of Animal Science. From February 1, 2021, to January 1, 2021. The experiment was repeated seven times, in which 225 ovine ovaries were collected randomly from 120 slaughtered ewes and 27 fresh testes rams in the slaughterhouse. Although the sheep were of different ages, their reproductive status was unknown before slaughtering.

2.1. Sample collection

2.1.1. Testes collection

Fresh testes-epididymides were recovered in the scrotal sacs of mature rams slaughtered at an abattoir in Ankara, Türkiye. Individually packed testes were placed on ice and brought to the laboratory in an ice chest 1 to 2 h post-mortem. The epidermis tail was rinsed with phosphate-buffered saline (PBS) (P-5493, Sigma) to eliminate blood clots and debris upon arrival at the laboratory. The epididymis samples were then kept refrigerated at 4 °C until processing. Then ram epididymides were manipulated 18 h after the post-mortem.

2.1.2. Collection of ovaries

Ovaries were also collected from an abattoir in Ankara, Türkiye. They were placed in a cooling box containing 0.9% saline solution at 4° C and transferred to the laboratory within 1-2 h. First, the ovaries were separated from all the surrounding ligament tissues. Then, all were washed for the first time in a PBS (P5493, Sigma) to remove blood clots and remaining dirt, wash twice with distilled water, and finally put in a sterile glass container containing the oocyte collection medium (OCM).

2.2. Sperm Collection and preparation

After 18 h of transport to the laboratory, the epididymis was placed in a sterile Petri dish. HEPES-TL was injected into the epididymal tail by using a G-18 needle. The surface of the epididymis tail was removed via a sharp scalpel, and epididymis contents were withdrawn by using a sterile syringe of 5 mL (Lone et al. 2011). For sperm maturity, cauda epididymides contents were collected and disseminated in four different treatments (Table 1). The first treatment was 100 µL of sperm was diluted with 2 mL of the non-CAP containing glucose (G7021, Sigma); placed for 60 minutes in the incubator at 37 °C, 5% CO_2, then heparin (P4562, Sigma) was added to it and placed in the incubator for 50 minutes at 37 °C, 5% CO_2. The second treatment was performed by adding 100 µL of sperm to the CAP, which contains bovine serum (A3311, Sigma) and glucose, and the sperm was incubated as in the first treatment. In the third treatment 100 µL the contents of the epididymis were added to media devoid of all nutrients (which do not contain glucose). Once sperm became motionless, 2 mL of the sperm energy recovery medium (RSE) (glucose and heparin) were added to it and placed in the incubator for 50 minutes at 37 °C, 5% CO_2. Finally, in the fourth treatment, the contents of the epididymis were added to the minimum essential medium (MEM) culture medium, and the sperm has incubated as in the first treatment. The quality of the sperm was assessed by individual and

collective sperm movement. Sperm with an individual motion of less than 60% were rejected. The presence of the protoplasm droplet at the end of the sperm tail was estimated as evidence of sperm maturity and capacitation.

Table 1- Composition of sperm maturity and capacitation media

Component (g)	*Non-CAP*	*CAP*	*RSE*	*MEM*
NaCl	6.976	6.976	6.976	10%
KCl	0.356588	0.356588	0.356588	-
$CaCl_2 \cdot 2H_2O$	0.2513871	0.2513871	0.2513871	-
KH_2PO_4	0.161959	0.161959	0.161959	-
$MgSO_4 \cdot 7H_2O$	0.2933052	0.2933052	0.2933052	-
$NaHCO_3$	2.1061	2.1061	2.1061	-
Na pyruvate	0.11	0.11	-	-
*Supplements				
Glucose	1.0017	1.0017	-	-
HEPES	4.766	4.766	4.766	-
BSA	-	0.005	-	-

*On the day of use, supplements were added capacitation media

2.3. Oocytes collection

The ovaries were collected by long and sterile forceps and placed in an OCM. The surface of each ovary was sliced with a sharp blade. After that, the ovaries were transferred to a sterile glass container containing OCM. Oocytes were assessed under the microscope (LEICA DM IL LED; Wetzlar, Germany) to select oocytes to conduct our research. A total of 800 oocytes were cultured in this research; 200 oocytes were distributed per treatment.

2.4. Oocytes evaluation

Based on the number of cumulus cells and cytoplasmic homogeneity, the quality of the collected oocytes was graded as good (class A), fair (class B), and poor, as described by Wani et al. (2000).

2.5. Oocytes maturation

After collection, assessment and classification, all oocytes from class A and B were selected. Oocytes were washed twice in OCM before being incubated for 24 h in TCM199 media supplemented with 10 µL/mL Na Pyruvate, 4 µL/mL Gentamycin, 100 µM Glutamax, 1 IU/mL follicle-stimulating hormone, 1 IU/mL luteinizing hormone, 10 mg/mL Estradiol, and 10% fetal calf serum covered with mineral oil at 38.5 °C, 5% CO_2, and 90% humidity. At the end of the maturation period, plates were examined via an inverted microscope (LEICA DM IL LED; Wetzlar, Germany), then the appearance of the first polar body was estimated as an indication of oocyte maturation.

2.6. In Vitro fertilization

After capacitation, the sperm were centrifuged for 6 min at (500 g), the supernatant was removed, and the sperm were resuspended in 1 mL of sperm CAP. Then IVF drops containing mature oocytes (20-45 oocytes) were subjected to IVF by four treatments for the sperm capacitation media, each treatment separately, with sperm concentrations of $10x10^6$/mL. In an incubator with 5% of CO_2, fertilization dishes were kept at 38.5 °C for 18-22 hours after fertilization, after that fertility rates were obtained by dividing total number of fertilized oocytes by total number and multiplying by one hundred.

2.7. In Vitro culture

Zygotes were transferred into SOF media. The media containing zygotes were incubated at 38.5 °C, 5% CO_2, and 90% humidity. The embryonic development was monitored every 24 hours (Figure 1) with the replacement of 50% of the media with a new sterile medium every 24-hour. During the monitoring time, undeveloped zygotes were removed in order to maintain only developing embryos in the media.

2.8. Assessment of sperm (live/dead)

The integrity of the sperm plasma membrane was assessed using a combination of Hoechst 33,342 and propidium iodide (PI) staining (Sutradhar et al. 2010). 5µL/mL of Hoechst 33,342 was added to each tube containing sperm suspension in the medium and was incubated at 37 °C for 15 minutes. After that, the suspension was given 50 µL of PI and incubated for 5 minutes at 37 °C. Then, a 20 µL sperm suspension was put in a 1:1 glycerol/PBS solution on a glass slide and covered with a cover slip (Eskandari & Momeni 2016). The slides were then examined under a microscope (LEICA DM IL LED; Wetzlar, Germany) with the proper excitation and emission filters. PI red staining on the head of sperm indicates a damaged plasma membrane, whereas Hoechst blue staining indicates a healthy membrane (Figure 2).

2.9. Statistical analysis

Experiment was replicated seven times. Data were analyzed by one-way ANOVA using SPSS version 23.0 statistical software and results are presented as the mean (± SEM). Duncan's multiple range test was carried out for comparing means, and p-value <0.01 was considered significant.

3. Results and discussion

The study results showed in Table 2 a significant increase in the percentage of fertilization ($p<0.01$) in RSE compared to the other treatments (79.95%). There were no significant differences between CAP and non-CAP in the fertilization percentage of oocytes with 59% and 53%, respectively. These were followed by MEM with a 34% fertilization rate.

Table 2- The impacts of sperm capacitation treatment on fertilization rate (%)

Treatment	*Number of oocytes*	*Number of fertilized oocytes*	*Fertilization rate (%)*
non-CAP	200	106	53.00±3.63^{b}
CAP	200	118	59.00±3.84^{b}
RSE	200	159	79.95±7.25^{a}
MEM	200	68	34.00±6.69^{c}

^{a,b}Different superscripts indicate significant differences between sperm capacitation treatments ($p<0.01$)

As shown on Table 3, a significant superiority was observed ($p<0.01$) for RSE regarding cleavage rate with 76.72%. The other three treatments (CAP, non-CAP and MEM) have shown 50.84%, 45.28%, and 19.11% of cleavage rate, respectively. The treatment MEM exhibited the lowest cleavage percentage (19.11%) compared to the others.

Table 3- The impacts of sperm capacitation treatment on embryonic development rate (%)

Treatment	*Number of fertilized oocytes*	*Cleavage rate (%)*	*Morula rate (%)*	*Blastocysts rate (%)*
non-CAP	106	45.28±2.30^{b}	27.35±1.78^{bc}	7.43±2.07^{b}
CAP	118	50.84±3.082^{b}	38.13±2.00^{b}	11.11±3.34^{b}
RSE	159	76.72±8.79^{a}	59.74±7.64^{a}	27.70±4.00^{a}
MEM	68	19.11±1.14^{c}	2.94±0.54^{c}	0

^{a,b}Different superscripts indicate significant differences between sperm capacitation treatments ($p<0.01$)

Morulae rate was a higher ($p<0.01$) in RSE treatment (59.74%) compared to the other treatments. CAP has shown similar result with non-CAP treatment. However, the rate for non-CAP was not different from MEM treatment, which was found as 27.35 %, and 2.94%, respectively. However, MEM showed a significantly lower Morulae rate (2.94%) in comparison with RSE ($p<0.01$).

A significant superiority ($p<0.01$) was observed in RSE treatment regarding the rate of blastocysts conformation (27.70%). Moreover, results indicate that the rate of the blastocyst formation was similar in CAP and non-CAP, which were found as 11.11% and 7.43%, respectively.

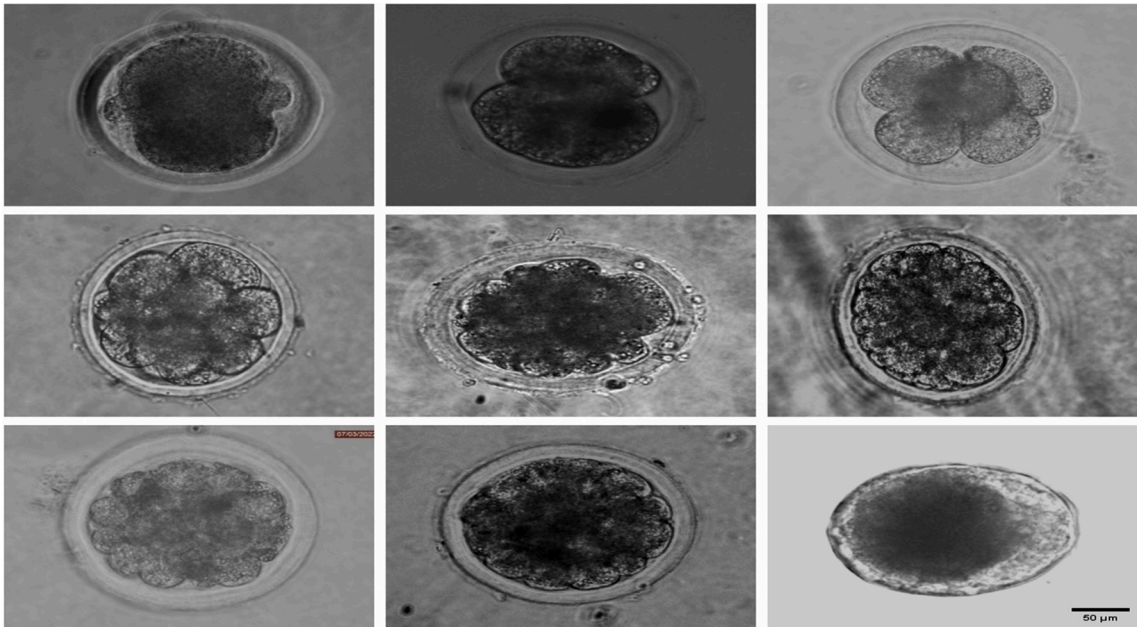

Figure 1- Representative images of IVF-derived embryos development

For well-known substances, such as glucose, the role and the mechanism on capacitation are still poorly understood (Jin & Yang 2017). Although some publications report that a lack of glucose affects negatively capacitation (Hidalgo et al. 2020; Qiu et al. 2016), others claims that glucose per se has no effect on capacitation *in vitro* (Zhu et al. 2019). Glucose is carried into the sperm cell by glucose transporter and helps generate ATP through glycolysis. ATP is used for sperm hyperactivation motility and permeability transition pore (Bucci et al. 2011; Jin & Yang 2017). During the capacitation, different conditions for sperm incubation have an impact on the development of an embryo (Küçük et al. 2020; Ferré et al. 2017). Energy substrates, ions, and a cholesterol-binding source in a particular medium can be used to achieve capacitation *in vitro* (Sajeevadathan et al. 2019; Visconti et al. 2011). A TYH medium containing only pyruvate and glucose as energy substrates can achieve capacitation and IVF in mice (Toyoda & Yokoyama 2016).

In this study, it was observed that ram sperm becomes immotile when cultured without some exogenous nutrients such as glucose and pyruvate for less than 50 min and motility can be restored by adding these energy sources.

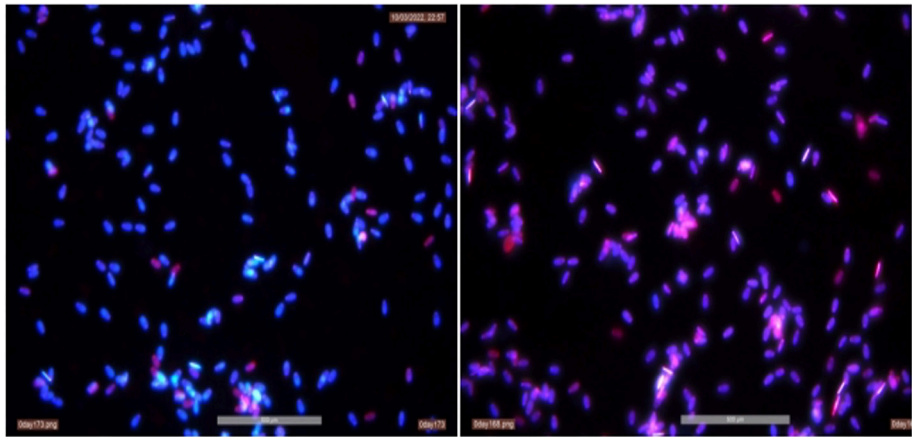

Figure 2- Ram sperm stained fluorescently. On a merged image, sperm cells with red-colored heads are dead sperm

Once re-activated, starved sperm performed higher IVF rates and hyper activation after being recovered. This increase in sperm functional properties has an unclear molecular-based mechanism. Two possible explanations may be suggested: the molecules that impair capacitation efficiency are consumed during the starving phase owing to a shortage of energy sources, or molecules that improve capacitation are increased after rescue. Another hypothesis is that the starvation followed by the salvage procedure produces metabolite changes, increasing specific metabolites that may encourage the formation of hyperactive sperm motility and fertilization (Otasevic et al. 2020). Surprisingly, oocytes fertilized with the RSE treatment produced a higher proportion of two-celled embryos that reached the

blastocyst stage. Moreover, blastocysts obtained with the RSE treatment showed an excellent growth rate and a higher number of total cells at 192 h after fertilization, both of which are indicators of optimal implantation potential (Binder et al. 2015). It has been suggested that sperm incubation conditions for IVF can alter embryonic development, which aligns with our findings (Li et al. 2006; Gu et al. 2013; Zhao et al. 2017; Zheng et al. 2018). The molecular basis for embryo post-fertilization effects, on the other hand, is unknown. The post-fertilization effects observed after RSE treatment may be attributed to epigenetic modifications in the male gamete (Figure 3). Epigenetic markers such as histone modifications and DNA methylation, essential in embryo development, are distributed differently in male and female gametes, indicating that environmental factors can alter epigenetic processes, affecting gene expression and having a significant impact on development (Zeng & Chen 2019; Canovas et al. 2017). Small non-coding RNAs in sperm may play a function in non-genomic paternal trait transfer (Gross et al. 2019).

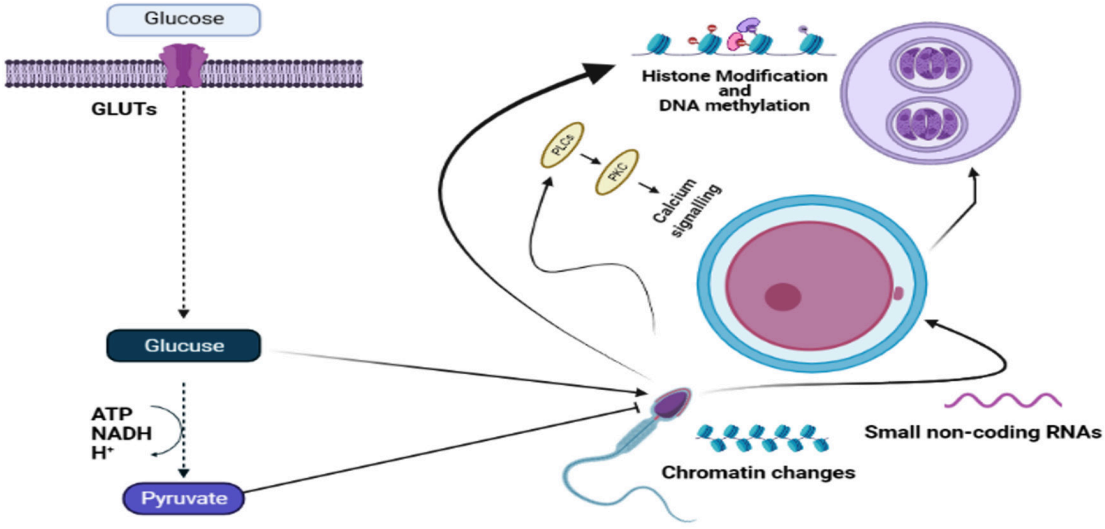

Figure 3- Possible molecular basis for the effect of glucose on capacitation and post-fertilization

One theory is that RSE treatment influences post-fertilization development by altering RNA levels, specifically the pool of non-coding RNAs, and another theory is that RSE treatment causes chromatin changes in sperm before fertilization, which has a direct impact on embryonic development (Gannon et al. 2014). These results imply that as ram sperm moves through the layers of cumulus cells and ZP, they undergo modification that assists or promotes nuclear reorganization, allowing phospholipase C zeta (PLCζ) to be exposed and activated (Saleh et al. 2020; Kashir et al. 2013). Our data has shown that treatment of ram sperm by RSE improved their capacity to activate oocytes after fertilization. We suppose that RSE circumstances cause modification of the head in ram sperm, promoting nuclear decondensation and PLCζ release, probably similar to what happens during natural sperm entrance and fertilization. In all assisted reproductive technologies, viable embryos which are capable for implantation are the most crucial step for a successful pregnancy. Implantation failure is more familiar with poor-quality embryos. These findings show that sperm quality before fertilization affects embryo growth. Similarly, sperm treated with RSE should provide improved embryo development in early stage; however, the treatment of RSE should improve overall fertilizing sperm quality boosting the likelihood for producing embryos of higher quality, rather than optimum sperm selection. Generally, sperm energy restriction and recovery RSE is a new technique that improves sperm fertilization and embryo growth after IVF.

4. Conclusions

Our findings highlight several crucial yet unanswered problems in reproductive biology and embryo development disciplines that require additional investigation. It was also demonstrated that sperm capacitation conditions persist after fertilization, opening new avenues for research into the association between sperm capacitation and embryo development in the early stages. Overall, sperm function become improved due to treatment with RSE before and following fertilization, which could be applied in other mammals, including humans, with substantial implications for ART operations.

Data availability: Data are available on request due to privacy or other restrictions.

Authorship Contributions: Concept: S.A-H., F.C., Design: S.A-H., F.C., Data Collection or Processing: S.A-H., F.C., Analysis or Interpretation: S.A-H., F.C., Literature Search: S.A-H., F.C., Writing: S.A-H., F.C.,

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Evaluation of Irrigation Experiments with GGE Biplot Method and Economic Analysis of Drip Irrigation System: A Case Study of Peanut Production

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ABSTRACT

Besides irrigation water (IW) quantity and quality, there is a decrease also in soil quality and fertility. Agricultural production lands are decreasing both in quantity and quality. There is a search for different operational alternatives in sandy soils with low production potentials. Drip irrigation is primarily practiced attain get greater yields per unit area. This study was conducted under sand soil conditions, commonly preferred for peanut farming, in the 2017-2018 growing seasons. A drip irrigation system was used in production. Halisbey, NC-7, and Sultan cultivars were used as plant materials. Two different irrigation intervals (2 and 4 days) and four different irrigation levels (I_{50}, I_{75}, I_{100} and I_{125}, calculated based on cumulative evaporation from the class-A pan) were applied. The data of five vegetative traits, including grain yield, were evaluated by regression and GGE biplot analysis. In addition, applied IW quantity, evapotranspiration and water use efficiency (WUE) were assessed by econometric analysis. Evapotranspiration values varied between 402-832 mm, applied IW quantities between 313-783 mm, yields between 5,269-8,269 kg ha^{-1}, WUE values between 0.63-1.55 kg ha^{-1} m^{-3}, economic water productivity over gross revenues between 1.29-3.81 \$ m^{-3} and benefit and cost ratios varied between 4.73-10.95. The GGE biplot statistical method is a useful tool in the evaluation of irrigation research where the number of applications and materials is high. As a results of the study, a 2-day irrigation interval and 75% of pan evaporation could be used in the irrigation of peanut plants grown under sandy soil conditions.

Keywords: Evapotranspiration, WUE, EWP, Benefit-cost ratio

1. Introduction

Peanuts, or “groundnuts”, as they are known in some parts of the world, are the edible seeds with high oil content from the legume plant family (Leguminosae, Fabaceae). In addition to its high oil content, peanuts are also a very rich nutrient in terms of protein and fiber (Suchoszek-Lukaniuk et al. 2011). They are widely used around the world in a variety of forms such as the production of oil, snacks (peanut butter, roasted peanuts etc.), and as fillers for meat products, soups, and desserts. In addition to oil, peanuts are widely used in the production of peanut butter, sweets, roasted peanuts, snacks, and fillers in recipes for meat products, soups and desserts. Peanuts are eaten around the world in a variety of forms, most of which are traditional cuisine. Moreover peanuts are also used as a complete dietary source for people on expeditions to various areas such as Antarctica, outer space and trekking. This is, in particular, the source of the elimination of malnutrition among the population in many African countries in recent years (Guimon & Guimon 2012; Arya et al. 2015). In addition, postharvest vegetative parts constitute an important source of animal feed.

Irrigation water (IW) is the basic input of agricultural production and it is getting worse both in terms of quantity and quality. It is also getting harder and harder to supply proper quality and sufficient quantity of water for production. The correct management of existing water resources is a vital issue and the highest income and benefit per unit of water has become a critical issue. According to 2020 Food and Agriculture Organization data, the world annual peanut production is about 47 million tons. China, with an annual production of 17.5 million tons, is the leading peanut producer in the world. It is respectively followed by India with approximately 6.7 million tons of production and Myanmar with 1.6 million tons. In Turkey, annually 215,928.53 tons of peanuts are produced from 54,775 ha land area and the average yield is 3,942.1 tons ha^{-1} (FAOSTAT, 2022).

The existing literature reviews have revealed that through the use of drip irrigation significant savings can be obtained especially in water, fertilizer, pesticides and energy (Soni et al. 2019; Halim et al. 2016). In addition to these savings, drip irrigation offers increases in product yield and quality. In a study conducted by Narayanamoorthy and their team in 2020, the efficiency and viability of the drip irrigation method in peanut cultivation were investigated. The results show that compared to traditional border irrigation, drip irrigation provided about 34% savings in production costs and 36% savings in IW and electrical energy, and approximately 79% increase in yield levels. An additional income of 862 $ ha-1 has also been obtained (Narayanamoorthy et al. 2020). A similar case is valid also in peanut production. For instance, Narayanamoorthy et al. (2020) conducted a study on peanuts and reported that as compared to traditional border irrigation, drip irrigation provided about 34% savings in production costs, and 36% savings in IW and electrical energy and approximately 79% increase in yield levels. In addition, with the use of drip irrigation, an additional income of 862 $ ha^{-1} was achieved.

Rathod and Trivedi (2011) used the drip irrigation method at 6 different IW/class a pan evaporation (CEP) ratio (0.6, 0.7, 0.8, 0.9, 1.0 and 1.2) in peanut production in the Junagadh region of India for 3 years. The lowest yield was measured as 1,917 kg ha^{-1} at an IW/CEP ratio of 0.6 and the highest as 2,927 kg ha^{-1} at an IW/CEP ratio of 0.9. The amount of IW applied to relevant treatments was measured as 502 and 757 mm, respectively. The highest water use efficiency (WUE) was obtained as 4.148 kg ha^{-1} mm^{-1} at an IW/CPE ratio of 0.8. This treatment (IW/CPE ratio of 0.8) was determined as the economic water application level. It was determined that drip irrigation was not a profitable application under excessive (IW/CPE=1.2) and insufficient water (IW/CPE=0.6) conditions. Similarly, Sri Ranjitha et al. (2018) used drip and furrow irrigation methods at 5 different IW/CEP ratios (0.4, 0.6, 0.8, 1.0 and 1.2) in peanut production. The lowest yield (1,223 kg ha^{-1}) was obtained from the IW/CEP ratio of 0.4 and the greatest yield (4,005 kg ha^{-1}) obtained from the IW/CEP ratio of 1.0. In terms of optimum water and economical water use, an IW/CEP ratio of 0.8 (3,765 kg ha^{-1}) was identified as the most appropriate treatment. The highest WUE (21.0 kg ha^{-1} mm^{-1}) was observed in the IW/CEP ratio of 0.8.

Regression analysis is used in agricultural research to determine the most appropriate dose in different agronomic applications (Sharma et al., 2022; Akçura, 2019). GGE biplot analysis is a method used to test hypotheses such as which genotype is better adapted to which environment and which environment is more effective in genotype selection, by using the quantitative features examined in trials established in many different environments (Akçura et al. 2019). In this study, the GGE biplot method was used to determine the interaction of the ideal irrigation level and irrigation dose with the changes of the investigated characteristics according to the combination of irrigation level and irrigation interval.

In this study, peanuts were produced by the drip irrigation method in sandy soil conditions. Two different irrigation intervals (2 and 4 days) and four different irrigation levels based on cumulative evaporations from a Class-A evaporation pan (I_{50}, I_{75}, I_{100} and I_{125}) were used. Effects of these experimental treatments on plant morphological traits and yield levels were investigated. An economic analysis of production with drip irrigation was also performed.

2. Material and Methods

2.1 Study area and climate

Experiments were conducted in the research fields of the Farmer's Training Branch of the Rural Affairs Department of Balkesir Greater City Municipality in the years 2017 and 2018. The research fields are located at 39°.52'N latitude and 27°.01'E longitude and have an average altitude of 12.0 m. Soil samples were taken from 0-120 cm soil profile in 30 cm intervals and samples were analyzed for soil physical properties (Table 1). Groundwater was used in irrigation and the IW quality class was identified as (C_3S_1) (USSL, 1954) (Table 2).

Table 1- Soil physical characteristics

Soil depth	*Sand (%)*	*Silt (%)*	*Clay (%)*	*Texture*	*FC_{Pw} (%)*	*PWP_{Pw} (%)*	*BD (g/cm^3)*	*Available water (mm)*
0-30	60.7	24.0	15.3	SL	16.87	7.98	1.54	41
30-60	95.0	2.4	2.6	S	10.87	4.83	1.59	29
60-90	58.7	30.1	11.2	SL	17.78	8.21	1.56	45
90-120	57.8	20.8	21.4	SCL	16.75	8.67	1.36	33

FC: Field capacity, PWP: Permanent wilting point, BD: Bulk density

Table 2- Irrigation water quality parameters

Cations	*Results (me/L)*	*Anions*	*Result (me/L)*
Na	2.96	CO_3	-
K	0.17	HCO_3	4.12
Ca	3.94	Cl_2	1.55
Mg	4.02	SO_4	5.41
Total	11.09	Top	11.08
pH	6.86	RSC	<1.24
EC (dS/m)	1241	SAR	1.49

Long-term (1938–2017) climate data of the research site are provided in Table 3. Meteorological data for the experimental years are provided in Table 4.

Table 3- Long-term climate data (1938-2017)

Months	*Temperature (°C)*			*Relative humidity RH (%)*	*Wind speed R (m/s)*	*Precipitation P (mm)*
	T_{aver}	T_{min}	T_{max}			
January	7.1	-12.1	22.9	70.1	2.5	82.1
February	7.5	-8.8	25.5	67.3	2.7	78.5
March	10.1	-5.6	28.5	64.3	2.6	58.2
April	14.3	-2.5	32.1	62.1	2.4	49.8
Mat	19.3	2.1	36.1	57.1	2.4	36.2
June	24.1	4.7	40.2	51.1	2.4	18.7
July	26.5	10.1	43.1	47.1	2.8	8.6
August	26.3	10.1	41.8	49.2	3.1	9.2
September	22.3	0.1	39.1	54.1	2.4	24.8
October	17.2	1.3	35.2	62.6	2.5	49.8
November	12.2	-3.8	28.2	69.4	2.6	103.1
December	8.5	-6.4	24.3	71.1	2.5	110.4
Average	16.2	-12.1	43.1	60.3	2.6	629.1

Table 4- Climate data for the experimental years (2017-2018)

Months	*T (°C)*			*RH (%)*	*R (m/s)*	*P (mm)*	*T (°C)*			*RH (%)*	*R (m/s)*	*P (mm)*
	T_{aver}	T_{min}	T_{max}				T_{aver}	T_{min}	T_{max}			
	2017						*2018*					
January	2.2	-6.1	14.2	70.1	2.4	172.3	5.5	-3.4	16.2	70.1	2.1	37.4
February	6.8	-5.6	19.3	67.3	2.3	52.3	8.9	-2.4	19.1	67.3	2.3	70.2
March	10.2	-1.5	25.2	64.3	2.3	58.8	12.3	-0.2	23.9	64.3	2.4	100.8
April	13.1	1.8	31.1	62.1	2.1	34.7	16.1	2	30.6	62.1	2.1	17.6
Mat	18.1	7.1	34.5	57.1	2.5	40.5	20.2	6.6	34.3	57.1	2.7	32.6
June	23.7	13.8	41.5	51.1	2.5	13.1	23.6	11.3	37.5	51.1	2.7	64.7
July	25.8	16.1	41.1	47.1	3.8	7.3	26.5	17	38.4	47.1	2.7	38.9
August	25.5	14.5	36.9	49.2	4.2	11.3	26.7	16.8	34.9	49.2	4.3	0.7
September	22.8	8.8	38.9	54.1	2.5	3.4	22.2	12.8	35.5	54.1	3.2	19.4
October	15.1	2.5	29.5	62.6	2.2	37.2	17.2	0.1	27.8	62.6	2.8	26.3
November	10.3	-1.5	22.5	69.4	1.4	50.1	12.5	5.7	26.7	69.4	3.1	84.9
December	9.3	-1.8	20.9	71.1	2.5	85.1	5.5	-3.4	16.2	70.1	2.1	114.3
Average	15.2	-6.3	41.5	60.5	2.6	566.1	16.4	-2.4	19.1	60.4	2.7	607.8

2.2 Irrigation and irrigation system

A drip irrigation system with inline emitters (2 Lh^{-1}, spaced 20 cm apart, operated at 2 atm) was designed for peanut irrigation. Gravimetric soil moisture measurements were performed through 0-120 cm soil profiles in 30 cm intervals. Experiments were conducted in a split-split-plot experimental design with three replications. The main plots included cultivars, sub-plots included irrigation intervals, and sub-sub-plots included irrigation. Irrigation intervals (2 and 4 days) were placed into main plots and irrigation levels (Kcp1=0.50, Kcp2=0.75, Kcp3=1.00 and Kcp=1.25) were placed into subplots. A Class-A Pan was placed within the meteorological station next to the experimental plots. Evaporations from the pan were measured daily. The amount of IW to be applied was calculated by using the following equation (Sezen et al. 2005);

$$I = A\ x\ Epan\ x\ Kcp$$

Where;

I = Amount of IW to be applied (L),

A = Plot area (m^2),

E pan = Cumulative evaporation from the pan during the irrigation interval (mm),

Kcp = Plant-pan coefficient.

About 2 m spacing was provided between the plots and 4 m between the blocks. Experimental plots were 5 m long and each plot had 4 rows. Sowing was performed at 70x20 cm (row spacing x on-row plant spacing) spacing.

2.3. Water use efficiency and irrigation water use efficiency

With the use of IW and yield data, WUE and IW use efficiency (IWUE) values were calculated through the use of the following equations (Maximov 1929; Viets 1962; Howell et al. 1990).

$$IWUE = \frac{Y}{I}$$

$$WUE = \frac{Y}{ETa}$$

Where;

IWUE: Irrigation water use efficiency (kg ha mm^{-1}),

Y = Yield (kg ha^{-1}),

I = Amount of irrigation water applied (mm),

WUE = Water use efficiency (kg ha mm^{-1}),

ETa: Actual evapotranspiration (mm).

2.4. Crop water productivity

Crop water productivity (CWP) is defined differently by various researchers (French & Schultz 1984, Bessembinder et al. 2005; Passioura 2006). CWP could be defined as the production quantity or value per unit of consumed or diverted water. It is calculated as the ratio of actual yield to the volume of water utilized:

$$CWP = \frac{Y}{ETa}$$

CWP = Crop water productivity (kg m^{-3})

Y = Yield (kg ha^{-1})

ETa = Actual evapotranspiration (m^3 ha^{-1})

2.5. Economic water productivity

Economic water productivity (EWP) was calculated with the equation given in Mengiste (2015) and Tewelde (2019):

$$EWP = \frac{GI}{IW}$$
$$GI = (PTG * YLDg) + (PTS * YLDg)$$

Where;

EWP = Economic water productivity ($ m^{-3})

GI: Gross income ($ ha^{-1})

IW: Irrigation water (m^3 ha^{-1})

PTG: Peanut sale price ($ ton^{-1}),

YLDg: Yield (ton ha^{-1}),

PTS: Herbage price ($ kg^{-1})

2.6. Agronomic practices

Halisbey, NC-7, and Sultan cultivars with Virginia-type peanut seeds commonly grown in Turkey were used as the plant material of the study. Pod yield (t ha^{-1}), seed yield (t ha^{-1}), branch length (cm), number of branches per plant, and number of pods per plant were investigated.

Sowing was performed on the 20th of April in 2017 and the 23rd of April in 2018 to have two seeds in each seedbed. Following the emergence, the number of plants was thinned to one in each seedbed. Before sowing, 250 kg ha^{-1} DAP (45 kg ha^{-1} N, 115 kg ha^{-1} P_2O_5) fertilizer was applied to experimental plots. Then, 50 kg ha^{-1} ammonium nitrate was applied through 3rd and 4th irrigations. For weed control, Befuraline active-ingredient herbicide (60% w/w) was applied, and hoeing was practiced after emergence. Harvest was practiced manually on the 15th of October in 2017 and the 20th of October in 2018.

2.7. Statistical analysis

Experimental data were subjected to analysis of variance with the use of JMP software (SAS Institute 2014). Regression analyses were conducted for morphological traits Biplot analyses were generated through the use of of GGE biplot software to see the effects of different irrigation interval x irrigation level combinations on morphological traits of different cultivars (Yan 2001).

3. Results

3.1. Irrigation water use efficiency, economic analysis, and evaluation

The amount of IW applied in experimental treatments of I_{50}, I_{75}, $I_{100,}$ and I_{125} was respectively measured as 319, 478, 637, and 796 mm in 2017 and as 308, 462, 616, and 770 mm in 2018 (Table 1). However, in Table 5, evapotranspiration values varied between 412-827 mm at 2-day irrigation intervals and between 431-849 mm at 4-day irrigation intervals in 2017; values varied between 393-789 mm at 2-day irrigation intervals and between 417-815 mm at 4-day irrigation intervals in 2018. The change in soil moisture increased with decreasing IW at both irrigation intervals of both years. The greatest water uptake from the root zone has been observed at 4-day irrigation intervals in both years.

Table 5- Irrigation water quantity, evapotranspiration and moisture change in root zone

Irrigation interval (day)	*Irrigation water level (%)*	*Seasonal irrigation (mm)*		*Soil water depletion (mm)*		*Evapotranspiration (mm)*	
		2017	*2018*	*2017*	*2018*	*2017*	*2018*
2	I_{50}	319	308	93	85	412	393
2	I_{75}	478	462	76	69	554	531
2	I_{100}	637	616	49	41	686	657
2	I_{125}	796	770	31	19	827	789
4	I_{50}	319	308	112	109	431	417
4	I_{75}	478	462	96	91	574	553
4	I_{100}	637	616	79	70	716	686
4	I_{125}	796	770	53	45	849	815

Since there were no significant differences in the yields of both growing seasons, an average of two years was used in the economic analysis (Table 6). The irrigation labor cost was calculated over the number of irrigations and hourly labor costs. For IW costs, traditional sprinkler irrigation was taken into consideration and 4 irrigations were practiced throughout the growing season. Total IW was adapted to drip irrigation over the volume of water (m^3). Peanut production costs (sowing, maintenance, harvest, etc.) and irrigation system costs were calculated over a 1 ha land area. As Enciso et al. (2005), recommended irrigation system cost was taken as 2,100 \$ ha^{-1} and system economical service-life was taken as 7 years. The annual cost was then calculated as 2,100/7=300 \$ $year^{-1}$. In addition to seed yield in peanut cultivation, post-harvest grass yield was also considered as an important source of income.

The lowest IWUE and WUE values (0.67 and 0.63 kg ha^{-1} m^{-3}) were obtained from I_{125} irrigation treatments of the Sultan cultivar at 4-day irrigation intervals and the greatest values (1.99 and 1.55 kg ha^{-1} m^{-3}) were obtained from I_{50} irrigation treatments of the Sultan cultivar at 2-day irrigation intervals. Similarly, the lowest EWP value (1.29 \$ m^{-3}) was obtained from I_{125} irrigation treatments of the Sultan cultivar at 4-day irrigation intervals and the greatest value (3.81 \$ m^{-3}) was obtained from I_{50} irrigation treatments of the Sultan cultivar at 2-day irrigation intervals. This was also observed in the CWP parameter and calculations were made on net profit. As it was in gross incomes, the lowest value (1.02 kg m^{-3}) and the greatest value (3.46 kg m^{-3}) were obtained from the same treatments of the same cultivar. The lowest gross income (10,133 \$) was obtained from I_{125} irrigation treatments of Sultan cultivar at 4-day irrigation intervals and the greatest value (15,773 \$) was obtained from I_{75} irrigation treatments of Sultan cultivar at 2-day irrigation intervals. Similarly, the lowest net income (7,991 \$) was obtained from the I_{125} irrigation treatments of the Sultan cultivar at 4-day irrigation intervals and the highest value (14,332 \$) was obtained from the I_{75} irrigation treatments of Sultan cultivar at 2-day irrigation intervals. Benefit-cost ratios (B/C) varied between 4.73-10.95.

Table 6- Applied irrigation water quantity, evapotranspiration, water use efficiency and economic analysis results

Treatments	*Amount of irrigation water (mm)*	*Irrigation water (m^3 ha^{-1})*	*Irrigation duration for the irrigation season (h)*	*Labor cost for irrigation (\$ h^{-1})*	*Total cost for irrigation labor (\$) (4x5)*	*Water price (\$ m^{-3})*	*Water cost (\$ ha^{-1}) (3x7)*	*Crop production costs (\$ ha^{-1})*
1	2	3	4	5	6	7	8	9
Two-HalisBey-I_{50}	313	3,130	44	1.70	75	0.20	626	89
Two-HalisBey-I_{75}	470	4,700	66	1.70	112	0.20	940	89
Two-HalisBey-I_{100}	627	6,270	88	1.70	150	0.20	1,254	89
Two-HalisBey-I_{125}	783	7,830	110	1.70	187	0.20	1,566	89
Two-NC7-I_{50}	313	3,130	44	1.70	75	0.20	626	89
Two-NC7-I_{75}	470	4,700	66	1.70	112	0.20	940	89
Two-NC7-I_{100}	627	6,270	88	1.70	150	0.20	1,254	89

Table 6- Continued

Treatments	Amount of irrigation water (mm)	Irrigation water (m^3 ha^{-1})	Irrigation duration for the irrigation season (h)	Labor cost for irrigation ($ h^{-1})	Total cost for irrigation labor ($) (4x5)	Water price ($ m^{-3})	Water cost ($ ha^{-1}) (3x7)	Crop production costs ($ ha^{-1})
Two-NC7-I_{125}	783	7,830	110	1.70	187	0.20	1,566	89
Two-Sultan-I_{50}	313	3,130	44	1.70	75	0.20	626	89
Two-Sultan-I_{75}	470	4,700	66	1.70	112	0.20	940	89
Two-Sultan-I_{100}	627	6,270	88	1.70	150	0.20	1,254	89
Two-Sultan-I_{125}	783	7,830	110	1.70	187	0.20	1,566	89
Four-HalisBey-I_{50}	313	3,130	44	1.70	75	0.20	626	89
Four-HalisBey-I_{75}	470	4,700	66	1.70	112	0.20	940	89
Four-HalisBey-I_{100}	627	6,270	88	1.70	150	0.20	1,254	89
Four-HalisBey-I_{125}	783	7,830	110	1.70	187	0.20	1,566	89
Four-NC7-I_{50}	313	3,130	44	1.70	75	0.20	626	89
Four-NC7-I_{75}	470	4,700	66	1.70	112	0.20	940	89
Four-NC7-I_{100}	627	6,270	88	1.70	150	0.20	1,254	89
Four-NC7-I_{125}	783	7,830	110	1.70	187	0.20	1,566	89
Four-Sultan-I_{50}	313	3,130	44	1.70	75	0.20	626	89
Four-Sultan-I_{75}	470	4,700	66	1.70	112	0.20	940	89
Four-Sultan-I_{100}	627	6,270	88	1.70	150	0.20	1,254	89
Four-Sultan-I_{125}	783	7,830	110	1.70	187	0.20	1,566	89

Treatments	Irrigation system cost for 1 ha ($ ha^{-1})	Yearly cost of the irrigation system ($ ha-1) (10/7 years)	Total cost for 1 year ($ ha^{-1}) (6+8+9+11)	Yield (kg ha^{-1})	Product sale price ($ kg^{-1})	Gross peanut kernel income per ha ($ ha^{-1} $year^{-1}$) (13x14)	Net income ($ ha^{-1} $year^{-1}$) (15-12)	Straw yield (kg/ ha^{-1})
1	10	11	12	13	14	15	16	17
Two-HalisBey-I_{50}	2,100	300	1,090	5,915	1.88	11,120	10,030	3,250
Two-HalisBey-I_{75}	2,100	300	1,441	8,269	1.88	15,546	14,105	3,250
Two-HalisBey-I_{100}	2,100	300	1,793	6,501	1.88	12,222	10,429	3,250
Two-HalisBey-I_{125}	2,100	300	2,142	6,421	1.88	12,071	9,929	3,250
Two-NC7-I_{50}	2,100	300	1,090	5,537	1.88	10,410	9,320	3,250
Two-NC7-I_{75}	2,100	300	1,441	6,053	1.88	11,380	9,938	3,250
Two-NC7-I_{100}	2,100	300	1,793	6,082	1.88	11,434	9,642	3,250
Two-NC7-I_{125}	2,100	300	2,142	5,452	1.88	10,250	8,108	3,250
Two-Sultan-I_{50}	2,100	300	1,090	6,224	1.88	11,701	10,611	3,250
Two-Sultan-I_{75}	2,100	300	1,441	7,213	1.88	13,560	12,119	3,250
Two-Sultan-I_{100}	2,100	300	1,793	6,790	1.88	12,765	10,973	3,250
Two-Sultan-I_{125}	2,100	300	2,142	6,218	1.88	11,690	9,548	3,250
Four-HalisBey-I_{50}	2,100	300	1,090	5,937	1.88	11,162	10,072	3,250
Four-HalisBey-I_{75}	2,100	300	1,441	6,934	1.88	13,036	11,595	3,250
Four-HalisBey-I_{100}	2,100	300	1,793	6,571	1.88	12,353	10,561	3,250

Treatments	*Irrigation system cost for 1 ha ($ ha^{-1})*	*Yearly cost of the irrigation system ($ ha-1) (10/7 years)*	*Total cost for 1 year ($ ha^{-1}) (6+8+9+11)*	*Yield (kg ha^{-1})*	*Product sale price ($ kg^{-1})*	*Gross peanut kernel income per ha ($ ha^{-1} year^{-1}) (13x14)*	*Net income ($ ha^{-1} year^{-1}) (15-12)*	*Straw Yield (kg/ ha^{-1})*
1	10	11	12	13	14	15	16	17
Four-HalisBey-I_{125}	2,100	300	2,142	6,140	1.88	11,543	9,401	3,250
Four-NC7-I_{50}	2,100	300	1,090	5,358	1.88	10,073	8,983	3,250
Four-NC7-I_{75}	2,100	300	1,441	5,818	1.88	10,938	9,497	3,250
Four-NC7-I_{100}	2,100	300	1,793	5,684	1.88	10,686	8,893	3,250
Four-NC7-I_{125}	2,100	300	2,142	5,446	1.88	10,238	8,096	3,250
Four-Sultan-I_{50}	2,100	300	1,090	6,192	1.88	11,641	10,551	3,250
Four-Sultan-I_{75}	2,100	300	1,441	6,621	1.88	12,447	11,006	3,250
Four-Sultan-I_{100}	2,100	300	1,793	5,724	1.88	10,761	8,969	3,250
Four-Sultan-I_{125}	2,100	300	2,142	5,269	1.88	9,906	7,764	3,250

Treatments	*Straw sale price ($ kg^{-1})*	*Straw income per ha ($ ha^{-1} year^{-1}) (17x18)*	*Gross total income per ha ($ ha^{-1} year^{-1}) (15+19)*	*Net income ($ ha^{-1} year^{-1}) (20-12)*	*ET (mm)*	*Irrigation water use efficiency (kg ha^{-1} m^{-3})*	*Water use efficiency (kg ha^{-1} m^{-3})*	*Economic water productivity over gross income ($ m^{-3})*	*Crop water productivity (kg m^{-3})*	*The benefit-to-cost (B/C) ratio*
1	18	19	20	21	22	23	24	25	26	27
Two-HalisBey-I_{50}	0.07	227.5	11,348	10,258	402	1.89	1.47	3.63	3.28	10.4
Two-HalisBey-I_{75}	0.07	227.5	15,773	14,332	542	1.76	1.53	3.36	3.05	10.9
Two-HalisBey-I_{100}	0.07	227.5	12,449	10,657	672	1.04	0.97	1.99	1.70	6.9
Two-HalisBey-I_{125}	0.07	227.5	12,299	10,157	808	0.82	0.79	1.57	1.30	5.7
Two-NC7-I_{50}	0.07	227.5	10,637	9,547	402	1.77	1.38	3.40	3.05	9.8
Two-NC7-I_{75}	0.07	227.5	11,607	10,166	542	1.29	1.12	2.47	2.16	8.1
Two-NC7-I_{100}	0.07	227.5	11,662	9,869	672	0.97	0.91	1.86	1.57	6.5
Two-NC7-I_{125}	0.07	227.5	10,477	8,335	808	0.70	0.67	1.34	1.06	4.9
Two-Sultan-I_{50}	0.07	227.5	11,929	10,839	402	1.99	1.55	3.81	3.46	10.9
Two-Sultan-I_{75}	0.07	227.5	13,788	12,347	542	1.53	1.33	2.93	2.63	9.6
Two-Sultan-I_{100}	0.07	227.5	12,993	11,200	672	1.08	1.01	2.07	1.79	7.2
Two-Sultan-I_{125}	0.07	227.5	11,917	9,775	808	0.79	0.77	1.52	1.25	5.6
Four-HalisBey-I_{50}	0.07	227.5	11,389	10,299	424	1.90	1.40	3.64	3.29	10.5
Four-HalisBey-I_{75}	0.07	227.5	13,263	11,822	563	1.48	1.23	2.82	2.52	9.2
Four-HalisBey-I_{100}	0.07	227.5	12,581	10,788	701	1.05	0.94	2.01	1.72	7.0
Four-HalisBey-I_{125}	0.07	227.5	11,771	9,629	832	0.78	0.74	1.50	1.23	5.5
Four-NC7-I_{50}	0.07	227.5	10,301	9,211	424	1.71	1.26	3.29	2.94	9.5
Four-NC7-I_{75}	0.07	227.5	11,165	9,724	563	1.24	1.03	2.38	2.07	7.7
Four-NC7-I_{100}	0.07	227.5	10,913	9,121	701	0.91	0.81	1.74	1.45	6.1
Four-NC7-I_{125}	0.07	227.5	10,466	8,324	832	0.70	0.65	1.34	1.06	4.9
Four-Sultan-I_{50}	0.07	227.5	11,868	10,779	424	1.98	1.46	3.79	3.44	10.9
Four-Sultan-I_{75}	0.07	227.5	12,675	11,234	563	1.41	1.18	2.70	2.39	8.8
Four-Sultan-I_{100}	0.07	227.5	10,989	9,196	701	0.91	0.82	1.75	1.47	6.1
Four-Sultan-I_{125}	0.07	227.5	10,133	7,991	832	0.67	0.63	1.29	1.02	4.7

3.2. The effect of the treatments on marketable yield and morphological traits

The irrigation intervals and IW levels had significant effects on seed yield, pod yield, and the number of pods per plant of the peanut cultivars ($p<0.01$) (Table 7). The values of all three parameters were higher at 2-day irrigation interval than at 4-day irrigation interval. The highest values in terms of morphological traits of the cultivars, were obtained from the Halisbey cultivar, followed by the Sultan cultivar. In irrigation treatments, the lowest values were obtained from I_{50} and I_{125} treatments at both irrigation intervals and the greatest values were obtained from I_{75} treatments, followed by I_{100} treatments.

Table 7- Morphological traits of the peanut cultivars under different irrigation intervals and levels

Irrigation interval (day)	*Cultivar*	*Irrigation levels*	*Pod yield (t ha^{-1})*	*Seed yield (t ha^{-1})*	*Branch length (cm)*	*Number of branches per plant*	*Number of Pods per plant*
Two	HalisBey	I_{50}	5.92±0.18	3.77±0.12	46.67±1.45	27.67±2.10	53.17±5.90
Two	HalisBey	I_{75}	8.27±0.12	5.67±0.11	41.50±1.14	43.67±2.98	66.67±3.16
Two	HalisBey	I_{100}	6.50±0.14	4.31±0.10	43.33±1.66	25.67±2.11	65.00±3.55
Two	HalisBey	I_{125}	6.42±0.11	4.39±0.15	42.17±2.05	29.17±2.45	55.17±2.05
Two	NC7	I_{50}	5.54±0.14	4.26±0.11	51.50±1.78	24.00±1.51	55.67±2.70
Two	NC7	I_{75}	6.05±0.09	4.56±0.06	44.33±3.38	39.83±1.79	64.17±2.25
Two	NC7	I_{100}	6.08±0.09	4.49±0.05	45.33±2.54	24.17±1.90	65.83±3.01
Two	NC7	I_{125}	5.45±0.13	4.16±0.10	42.50±2.21	28.00±2.23	51.83±2.27
Two	Sultan	I_{50}	6.23±0.25	4.17±0.17	48.67±1.15	19.67±1.44	44.50±2.14
Two	Sultan	I_{75}	7.21±0.19	4.89±0.16	41.17±2.57	35.17±2.37	71.67±4.73
Two	Sultan	I_{100}	6.79±0.13	4.67±0.10	40.00±2.28	24.00±1.31	55.33±2.44
Two	Sultan	I_{125}	6.22±0.16	4.14±0.10	41.50±1.65	25.67±0.68	52.50±2.86
Four	HalisBey	I_{50}	5.94±0.05	3.84±0.05	34.00±1.50	22.83±0.88	48.50±2.78
Four	HalisBey	I_{75}	6.93±0.10	4.71±0.07	32.67±1.81	23.83±1.23	65.50±2.33
Four	HalisBey	I_{100}	6.57±0.09	4.24±0.06	36.33±0.39	21.67±1.60	55.50±1.63
Four	HalisBey	I_{125}	6.14±0.19	4.10±0.13	37.67±1.44	22.67±1.29	49.17±2.25
Four	NC7	I_{50}	5.36±0.14	3.78±0.10	34.83±1.39	25.67±1.48	48.00±3.09
Four	NC7	I_{75}	5.82±0.15	4.44±0.12	35.33±1.04	32.00±3.25	65.50±5.06
Four	NC7	I_{100}	5.68±0.09	4.06±0.08	43.67±1.59	17.33±1.36	48.00±2.44
Four	NC7	I_{125}	5.44±0.14	3.92±0.11	36.67±2.23	18.00±1.75	39.33±2.98
Four	Sultan	I_{50}	6.19±0.07	4.13±0.06	33.83±0.88	20.00±0.58	47.83±1.72
Four	Sultan	I_{75}	6.62±0.10	4.56±0.07	32.33±1.35	18.33±1.45	67.33±1.88
Four	Sultan	I_{100}	5.73±0.09	3.89±0.07	33.67±0.60	21.00±1.62	51.83±1.77
Four	Sultan	I_{125}	5.27±0.05	3.49±0.06	33.50±1.40	15.17±1.53	40.00±2.30

There R^2 values for the regressions between seed yield and irrigation levels were quite high at both irrigation intervals of the Sultan cultivar (0.955 and 0.842, respectively). In the NC7 cultivar, a high R^2 value (0.993) observed at the 2-day irrigation interval, but a low R^2 value (0.659) observed at the 4-day irrigation interval (Figure 1).

The regression analysis results for changes in the number of branches with irrigation levels are presented in Figure 2a. The cultivars exhibited trends for changes in the number of branches with irrigation treatments at 2-day irrigation intervals. In general, the lowest number of branches per plant was obtained from I_{50} treatments and the greatest from I_{75} treatments. However, at a 4-day irrigation interval, cultivars had different trends for the changes in the number of branches per plant with irrigation levels. In the NC-7 cultivar, the greatest value was obtained from the I_{75} treatment and the lowest from the I_{125} treatment. A distinctive change in the number of branches per plant was not encountered in Halisbey and Sultan cultivars (Figure 2a).

Figure 2b shows branch lengths of the cultivars under different irrigation intervals and levels A distinctive change has been observed in branch lengths of the Halisbey cultivar with irrigation intervals. The highest value in the 2-day irrigation interval was obtained from I_{50} treatment, and no significant change was found in branch lengths at the other three irrigation levels. There was an increase from I_{50} to I_{125} in the 4-day irrigation interval. In the NC-7 cultivar, significant changes were observed in branch lengths with irrigation levels. At the 2-day irrigation interval, there was a decrease from I_{50} to I_{125}. At the 4-day irrigation interval, there was an initial increase from I_{50} to I_{125}, peaked at I_{100} and then decreased. In Sultan cultivar, branch lengths at 2-day irrigation interval first decreased then, increased from I_{50} to I_{125}. At the 4-day irrigation interval of this cultivar, there was no significant change in branch lengths with irrigation levels (Figure 2b).

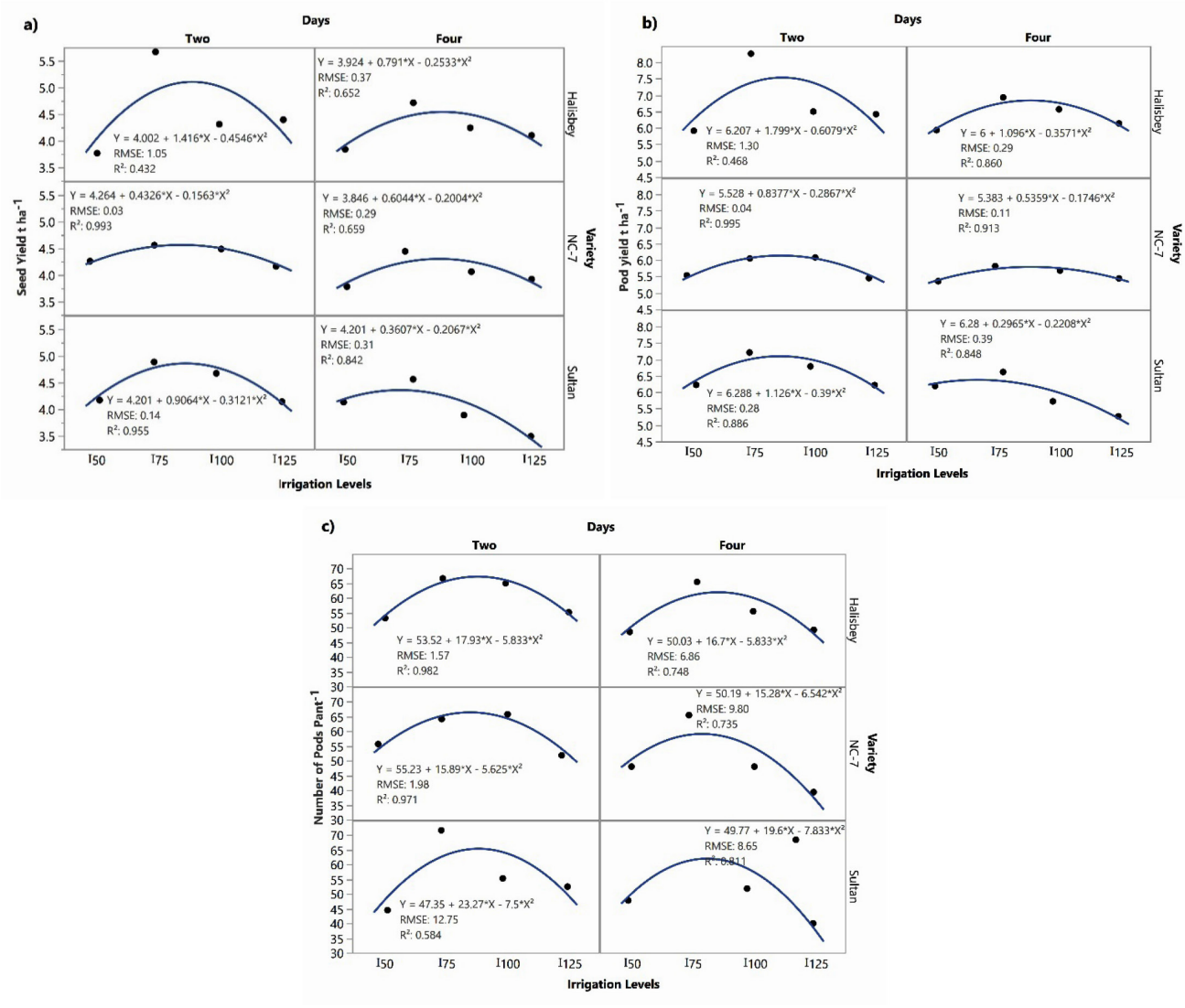

Figure 1. Regression graphs for changes in a) seed yield, b) pod yield and c) number of pods per plant with the cultivars, irrigation intervals and levels

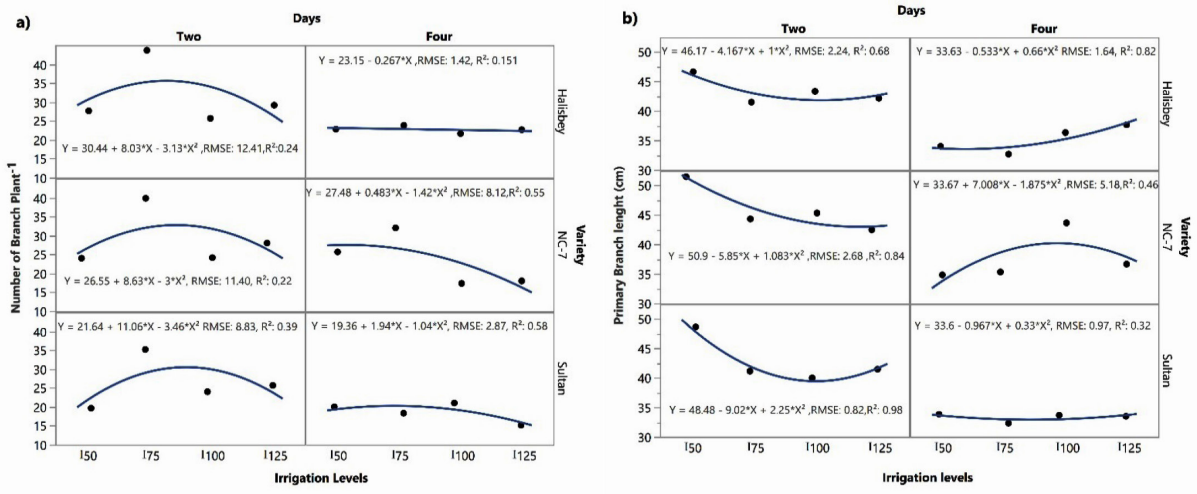

Figure 2. Regression graphs for changes in a) number of branches per plant and b) branch lengths with the cultivars, irrigation intervals and levels

The irrigation interval x trait biplot explained 83.2% of the total variation (Figure 3). Two basic groups were created on the biplot. While there was a main branch length in one of these groups, the I_{50} IW level was located diagonally at a 2-day irrigation interval. This shows that in three peanut cultivars, the highest branch length was obtained from the I_{50} IW level of a 2-day irrigation interval.

All the other traits examined formed a group and a 2-day irrigation interval and I_{75} IW level were located as diagonal. In this case, it was determined that there was a positive and significant correlation between seed yield and pod yield, number of branches per plant and number of pods per plant. All these traits had high values in the I_{75} irrigation level of the 2-day irrigation interval (Figure 3).

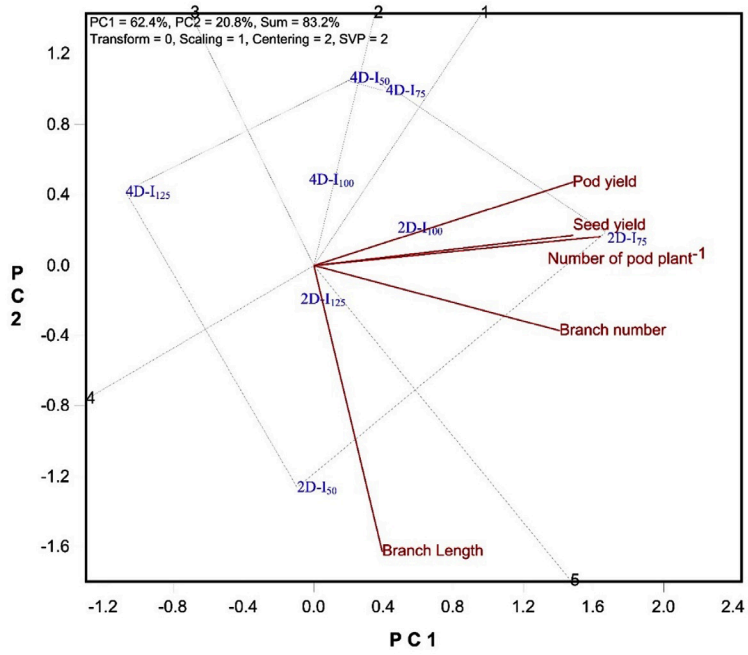

Figure 3. Visual evaluation of morphological traits of peanut cultivar under different irrigation intervals and levels
D: Irrigation interval (day), I: Irrigation level

For the interaction of interval x IW level (Figure 4) obtained over the years in three peanut cultivars, it was determined that the 2-day irrigation interval and I_{75} irrigation level of the Halisbey cultivar (H-2DI_{75}) were placed in the ideal interaction zone. In addition, it was determined that the 2-day irrigation interval and I_{75} irrigation levels of Sultan and NC-7 cultivars were placed close to the ideal interaction zone.

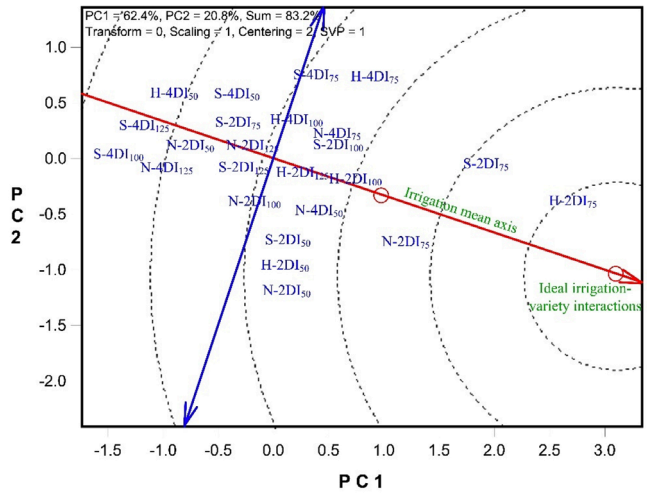

Figure 4. Biplot created for ideal irrigation interval x irrigation level x cultivar interaction
H: Halisbey, N: NC-7, S: Sultan, D: Irrigation interval (day), I: Irrigation level

3. Results and Discussion

Drip irrigation offers significant water savings in irrigations. Soni et al. (2019) used to drip, micro-sprinkler and surface irrigation methods in India and reported that as compared to surface irrigation, 40.08-55.0% water savings were achieved in drip irrigation and 25.10-43.83% in micro-sprinkler. Halim et al. (2016) conducted a study in Egypt and reported that 6-11% water saving was achieved in drip irrigation as compared to sprinkler irrigation. With the use of drip irrigation, significant cost reductions are achieved in fertilizers, pesticides, and labour.

Rathod and Trivedi (2011) conducted a 3-year study on peanuts in the Junagadh region of India and reported applied IW quantities as between 502-1,011 mm, seed yields as between 1,917-2,586 kg ha^{-1}, pod yields as between 3,710-6,640 kg ha^{-1} and IWUE values as between 0.26-0.41. Sorensen and Butts (2014) used a sub-surface drip irrigation system with different lateral spacings and IW levels (50, 75 and 100% of ET_0 values calculated by the Jensen-Haise method) for 10 years and indicated that there were no significant differences in seed yields of 75 and 100% irrigation treatments. Seed yields were reported as between 2,711-4,272 kg ha^{-1}. While the average rainfall in the ten-year production season was 477 mm, the average amount of IW applied was 295 mm in 100%, 213 mm in 75% and 154 mm in 50% of irrigation treatments. Sri Ranjitha et al. (2018) reported peanut seed yields as between 1,234-4,005 kg ha^{-1} and WUE values as between 0.80-2.1 kg ha m^{-3}. Yield levels decreased to 3805 kg ha^{-1} when 1.2 times of cumulative Epan value was applied. On the other hand, WUE values were determined to vary. Choudhary et al. (2020) reported WUE values as between 0.47-0.56 kg ha m^{-3} in the Rajasthan region of India, Kh (2017) used sprinkler irrigation under Egyptian conditions and reported WUE values as between 0.53-0.58 kg m^{-3}. Shoman and Bughdady (2018) reported WUE values as between 0.64-0.81 kg m^{-3}. Manzano Jr (2020) used the drip irrigation method and reported applied IW quantities as between 375 - 600 mm, yields as between 2,220-6,130 kg ha^{-1} and WUE values as between 0.47-0.66 kg m^{-3}. El-Metwally et al. (2020) conducted a similar study in Egypt under sandy soil conditions and reported applied IW quantities as between 154-386 mm, yields as between 3287-5391 kg ha^{-1} and WUE values as between 1.32-2.14 kg m^{-3}. El- Borai et al. (2009) reported evapotranspiration as 983 mm, yield as 4382 kg ha^{-1} and WUE values as between 0.17-0.53 kg m^{-3}. Sezen et al. (2019) reported evapotranspiration values as between 516-1067 mm, applied IW quantities as between 406-1,059 mm, yields as between 1,960-5,300 kg ha^{-1}, IWUE values as between 0.40-1.05 and WP values as between 0.32-0.74 kg m^{-3}. Bandyopadhyay et al. (2005) reported that WUE values of peanuts grown for two seasons in India varied between 0.48-0.60 kg m^{-3}. Kheira (2009) conducted a deficit irrigation study in Egypt and reported WUE values as between 0.45-0.61 kg m^{-3}. Aydınşakir et al. (2016) conducted a study in the South of Turkey and reported evapotranspiration values as between 193-809 mm, applied IW quantities as between 95-892 mm and WUE values as between 4.7-7.5 kg ha^{-1} mm^{-1}. Soni et al. (2019) used surface, micro-sprinkler and drip irrigation methods and reported applied IW quantities as between 165.12-367.44 mm and WUE values as between 5.18-19.28 kg ha^{-1} mm^{-1}. For morphological traits, Aydınşakir et al. (2016) reported branch lengths as between 33-76.7 cm, number of branches per plant as between 6.5-12.4 and number of pods per plant as between 17.3-51.8; Shoman and Bughdady (2018) reported number of pods per plant as between 33.16-44.47; Sri Ranjitha et al. (2018) reported number of pods per plant as between 11.78-24.78; El-Metwally et al. (2020) reported number of branches per plant as between 17.80-24.17 and number of pods per plant as between 23.16-37.80; Canavar and Kaynak (2013) (2013) reported branch lengths as between 41.33-55.23 cm, number of branches per plant as between 10.33-11.33 and number of pods per plant as between 28-71.77; Wang et al. (2016) reported branch lengths as between 24.8-33.9 cm, number of

branches per plant as between 7.1-14.2, number of pods per plant as between 20.3-35; Jin et al. (2021) reported branch lengths as between 35.82-47.30 cm, number of branches per plant as between 5.18-7.57 and number of pods per plant as between 11.14-16.59.

In terms of economic analysis, the present findings comply with the results of earlier studies. Manzano (2020) reports that as compared to furrow irrigation, drip irrigation increased the pod yields by 31.45%. It was determined that drip irrigation increased the pod yields up to 70.21% in dry season as compared to furrow irrigation. These increases in pod yields were mainly attributed to more homogeneous water application and less water stress. However, fixed costs were 58.75% and operating costs were 75.32% higher in drip irrigation. In the long run, the benefits of drip irrigation include increased efficiency as well as water savings. Choudhary et al. (2020) reported benefit-cost ratios (B/C) as between 1.50-1.95; Mishra et al. (2008) as between 2.46-3.10 and Soni et al. (2019) as between 1.66-2.41. Sorensen and Butts (2014) reported gross revenue as between 1,804-1,899 \$ ha^{-1} in subsurface drip irrigation and as 1,478 \$ ha^{-1} in non-irrigated treatments. The current findings were higher than some of aforementioned researchers' results and lower than some others. These differences were attributed to differences in cultivars, climate, ecological conditions, and cultural practices.

4. Conclusion

It has been found that 2-day irrigation intervals and I_{75} irrigation levels had the greatest yield levels in all three cultivars of peanut cultivation with drip irrigation in sandy soil conditions. Thus, a 2-day irrigation interval and 75% of pan evaporation could be used in the irrigation of peanut plants grown under sandy soil conditions. The GGE biplot statistical method can be used safely in the evaluation of irrigation experiments. It has been determined that it is a very useful method in determining the most appropriate doses/levels, especially in research where the number of applications and materials is high. Since the method reveals the effect and applications visually, compared to classical statistics it offers a more easily understandable explanation. The method reveals the effect and differences of the applications visually. Compared to classical statistics, the GGE biplot method offers more easily understandable explanations.

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The Effect of Cultivar and Stage of Growth on the Fermentation, Aerobic Stability and Nutritive Value of Ensiled Quinoa

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ABSTRACT

Quinoa has the potential to be an important alternative source of silage as a forage crop. However, there is limited information on the ensiling of quinoa in the literature. This study investigates the silage fermentation quality, nutritive value and aerobic stability of quinoa cultivars harvested at different plant growing stages. The experiment was carried out in the experimental area of the Hatay Mustafa Kemal University, Faculty of Agriculture in the 2019 and 2020 growing seasons. The experiment was laid out in a split plot with a randomized block design with three replications, the three main plots were based on harvesting times (flowering, milky and dough stages) and the five sub-plots were based on cultivars (Mint Vanilla, Cherry Vanilla, French Vanilla, Red Head and Titicaca). Traits such as pH, ammonia nitrogen, lactic acid bacteria (LAB), LA, acetic acid (AA), butyric acid (BA), propionic acid, ethanol (EtOH), dry matter (DM), neutral detergent fiber, acid detergent fiber, acid detergent lignin, crude protein, ash, ether extract, water soluble carbohydrate (WSC) and relative feed value were analyzed in order to determine silage fermentation quality and nutritive value. In addition, all silages were evaluated in terms of aerobic stability. In reference to the interaction effects, pH, ammonia nitrogen, LAB, AA, BA and EtOH, the silage fermentation quality parameters were between 3.83-4.16, 5.57-14.83%, 4.69-5.80 $\log_{10}$cfu/g DM, 1.37-2.10%, 0.32-0.51% and 0.79-1.63, respectively. On the other hand, DM, ADF, ash and WSC changed between 21.95-33.36%, 22.39-28.36%, 15.41-17.70% and 2.35-9.50%, respectively, as silage nutritive composition features. The carbon dioxide production values of silages exposed to air were between 5.49 g/kg and 10.26 g/kg according to interactions. Among the evaluated quinoa cultivars, cv. Titicaca and cv. French Vanilla provided superior results in terms of fermentation quality compared to other cultivars. It was also determined that it would be more appropriate to harvest these superior quinoa cultivars during the dough stage for quality silage. Among the silages, the cv. Titicaca had the best aerobic stability. As a result of this study, it was concluded that cv. French Vanilla and cv. Titicaca should be harvested during the dough stage in order to obtain better silage quality. According to the results of this study, it was deduced that the quinoa plant could be an alternative ensiling crop.

Keywords: Aerobic stability, Alternative forage crops, Fermentation quality, Harvesting time, Quinoa cultivars, Silage

1. Introduction

Ensiling is one of the most widely used roughage storage techniques in many parts of the world (Silva et al. 2020). The loss of nutrients is relatively lower in silages obtained in accordance with silage making rules, when compared to the hay-making method. In many countries, silage production tends to be increasing in comparison to hay-making (Wilkinson & Taivonen 2003). Ensiling is a method applied to reduce nutrient loss, improve feed intake and to provide long-term preservation through the use of lactic acid (LA) fermentation under anaerobic conditions (Ertekin et al. 2022). An effective fermentation in the silo can be achieved thanks to sufficient dry matter (DM) (30-35%), water-soluble carbohydrates and epiphytic lactic acid bacteria (LAB) on chopped crop material (Kızılsimsek et al. 2016). With the proliferation of LAB in the silo, the water soluble carbohydrates (WSC) in the ensiled material are converted into various organic acids, mainly LA (Khota et al. 2016). Thus, there is a rapid pH drop in the silo and the growth of undesirable microorganisms is prevented. Finally, conditions for the conservation of the roughage are stabilized (Bao et al. 2016). The presence of sufficient fermentable carbohydrates among plant material is crucial in producing LA which is necessary in order to reduce pH and increase the silage quality during the fermentation process (Bai et al. 2011).

The quality of the silage depends plant genotypic characteristics, chopping length, DM content, crop digestibility and silage additives. The stage of maturity at harvest is another immensely significant factor in terms of the nutritional value of silage. The maturing process is highly complicated and contains numerous changes in the distribution and structure of plant organs which affect the fermentation process (Atis et al. 2013). The DM content of the crops at the time of harvest is also an important factor in obtaining better quality silage. The DM content of the plants is directly related to harvest time (Carmi et al. 2006).

The increasing importance of silage has revealed the need to investigate the ensiling properties of alternative forage plant sources. Quinoa (*Chenopodium quinoa*) is a crop basically produced for its seed. In recent years, it has been suggested that quinoa has also a high nutrient content, and its potential to be used as a whole-plant forage crop has been evaluated worldwide (Galwey 1992; Peiretti et al. 2013). On the other hand, it has been reported that using the quinoa plant a as a silage material in dairy farms would provide more protein and produce a better milk yield (Darwinkel 1997). Many studies have evaluated quinoa plants being an alternative feed source in animal nutrition in recent years and its superiority of feed efficiency and quality has been emphasized in various research articles (Fuentes & Bhargava 2011; Peiretti et al. 2013; Liu et al. 2017; Asher et al. 2020; Kaya & Kizil-Aydemir 2020; Tan & Temel 2020; Temel & Yolcu 2020; Shah et al. 2020; Liu et al. 2021). However, there only a few examples of research on quinoa silage (Erdogan & Koca 2020). There is no comprehensive study however on the preservation of the quinoa plant by ensiling. This suggests that the determination of the appropriate harvesting time is a very important factor for successful quinoa ensiling.

Quinoa is an important plant with the potential to be used as an alternative forage crop yet there is no comprehensive study on the preservation of quinoa by ensiling in the current literature. Therefore, the aim of the present study is to evaluate the effect of cultivar and stage of growth on the fermentation, aerobic stability and nutritive value of ensiled quinoa.

2. Material and Methods

2.1. Experimental area and its climatic conditions

This study was carried out in the Experimental Area of Hatay Mustafa Kemal University Faculty of Agriculture located in Reyhanlı, Hatay (36°15'13.56"N 36°30'7.96"E, altitude 96 m above sea level) in the 2019 and 2020 growth seasons. The experimental soil was clay loam with a pH of 7.12 (slightly alkaline), and an organic matter reading of 1.93% (low), P 7.41 mg/kg soil (moderate), lime 6.45% (moderate) and total salt 0.0078% (low). When climatic data (Figure 1) are investigated, the means of the temperature values of the growing seasons of 2019 and 2020 were recorded as 21.9 °C and 22.2 °C, and the total precipitation amounts were recorded as 162.4 mm and 101.8 mm, respectively.

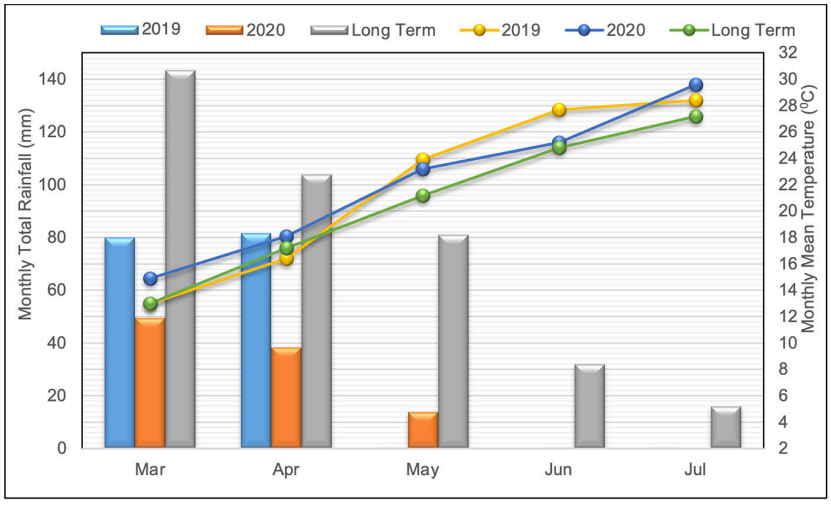

Figure 1- Monthly means of air temperature and total rainfall during the period from March to July of 2019 and 2020, as well as the long-term averages (1940-2019) in Hatay, Turkey. In the figure, the bars and lines show the monthly total precipitation and the monthly average temperature, respectively

2.2. The experimental design, cultivation and harvesting times

The experiment was laid out on a split plot in a randomized block design with three replicates, three main plots treatments aligned with harvesting times (flowering, milky and dough stages) and five sub-plots treatments were made up of the cultivars (mint vanilla, cherry

vanilla, french vanilla, red head and titicaca). Each sub-plot had 7 rows with 20 cm row spacing and 4 m row length. The sowing was performed by hand on 25 March 2019 and 27 March 2020. The seed rate was 10 kg/ha. The seeds were sown at a depth of 1-2 cm. When the seeds were sown, the soil was fertilized with 60 kg/ha N, P and K and then when the plants reached 50 cm in height, they were fertilized with 60 kg/da N as urea (Tan & Temel 2020). The plants were irrigated three times on 15 May and 30 May and 15 June in both years at field capacity with a drip irrigation system. The plants were harvested with a hand harvester at the flowering, milky and dough stages of quinoa plants.

2.3. The silage making, storage conditions and opening procedures

The quinoa plants were harvested according to the harvesting times, were chopped into 2-3 cm size for ensilaging via a chopping machine (CAN SP255, CANTEK MAKINA, Sinop, Turkey) and the chopped materials were ensilaged into 25 cm x 35 cm polyvinyl bags via an industrial vacuum packaging machine (CromPack VM 42 D. Istanbul, Turkey) with five replications for each treatment containing 400 g of fresh material. All silages were stored at 25 °C in a conditioning chamber for 90 days. The mini silos (bags) were opened after 90 days of fermentation.

2.4. Microorganism counting

The quinoa silages from all treatments were homogenized in 180 mL of Ringer's solution. Serial dilutions (from 10^{-1} to 10^{-10}) were prepared from this extract and inoculated into disposable sterile Petri dishes (90x15 mm) containing De Man Ragosa and Sharpe (MRS) agar for LAB, Malt Extract Agar (MEA) for yeast and Violed Red Bile Agar Glucose (VRBA-G) for *Enterobacteria* (Santos et al. 2014). These were incubated at 37 °C for 48 h for MRS and MEA and at 33 °C for 18 h for VRBA-G. The petri dishes presenting colonies' proliferation between 10 and 300 CFU (colony forming unit) were counted and recorded. The numbers of colonies of LAB, *Enterobacteria* and yeast were given as $\log_{10}$cfu/g DM.

2.5. Silage pH and fermentation end products

To determine pH changes of quinoa silages, a homogenized Ringer's solution obtained from quinoa silages was tested via a table-type pH meter (Inolab 8F93, Weilheim, Germany). Ammonia nitrogen (NH_3-N) was detected using distillation (Behrotest S2 KAS20, Dusseldorf, Germany) and titration methods based on the Kjeldahl procedure. The organic acids and alcohol such as lactic acid (LA), acetic acid (AA), butyric acid (BA), propionic acid (PA) and ethanol (EtOH) were detected using the methodology described by Siegfried et al. (1984) in a Shimadzu high-performance liquid chromatography system (Shimadzu KC-811, Kyoto, Japan) at a 42 °C, 0.6 mL/min flow rate via refractive index detector following a cleaning of the samples.

2.6. The aerobic stability

After a 90-day ensiling period, the laboratory silages and the bottle system described by Asbell and Stenson (1982) were opened, which is one of the most commonly used methods (Filya 2003; Filya 2004; Koç et al. 2021). This bottle system is based on trapping CO_2 gas in the KOH solution, and was used to determine the aerobic stability of quinoa silages. With this method, the silages were exposed to air for 5 days, and the aerobic stability of the silages was evaluated in terms of CO_2 production, pH level, number of *Enterobacteria* and yeast and mold.

2.7. The dry matter of the silages

Samples dried at 65 °C for 48 hours in an oven-drying cabinet were milled to pass a 1 mm sieve for the preparation for a chemical analysis. The DM contents of samples from quinoa silages were determined by a drying-oven cabinet at 105 °C for 24 hours (AOAC 2019).

2.8. The nutrient content

The crude protein (CP), ether extract (EE), ash, neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL) and WSC properties of quinoa silages were investigated in order to determine their nutrient content. The CP and EEs were determined according to the Kjeldahl method and an extraction method using diethyl ether solvent, respectively (AOAC 2019). The ash content was detected by burning the samples in an ash furnace at 550 °C for 4 hours. The cell-wall components such as NDF, ADF and ADL were analyzed with an ANKOM Fiber Analyzer (ANKOM Technology Corp., Fairport, NY, USA) according to the method described by Van Soest et al. (1991). WSC were analyzed according to the phenol-sulfuric acid method described by Dubois et al. (1956).

2.9. The nutrient compositions of fresh quinoa

The nutrient content data of the fresh quinoa were presented in our previously published article (Yilmaz et al. 2021). This data can be reviewed from this article if necessary.

2.10. Statistical analysis

The statistical analysis of the data obtained from the current study was performed using the statistical program of JMP 13 software. The general linear model was used to determine the differences among the means of cultivar, harvesting time and their interactions according to the split plot in the randomized complete blocks model. The Tukey pairwise ($p<0.05$) test was used to group factor levels.

3. Results

3.1. The silage pH, lactic acid bacteria and fermentation end-products

The effects of cultivar, harvesting time and interaction on pH, ammonia nitrogen and LAB were determined to be statistically significant (Table 1). The pH levels of the interactions are given in Figure 2a. The pH level among the interactions varied between 3.83 and 4.16. The lowest pH was obtained from cv. Titicaca, harvested at the flowering stage. However, the cv. Titicaca harvested at the flowering stage was statistically in the same group as the cv. Red Head harvested also at the flowering stage. The highest pH level was found in cv. Mint Vanilla harvested at also flowering stage. The ammonia nitrogen content among the interactions ranged from 5.57% to 14.83% (Figure 2b). The highest ammonia nitrogen content was obtained from cv. Red Head harvested at the dough stage, whereas the lowest value was found in the cv. French Vanilla harvested at flowering stage. The LAB numbers among the interactions ranged from 4.69 $\log_{10}$cfu/g DM to 5.80 $\log_{10}$cfu/g^{-1} DM (Figure 2c). While the highest LAB number was obtained from cv. Mint Vanilla harvested at milky stage, the lowest value was recorded in cv. French Vanilla harvested at dough stage.

Table 1- Anova test results of all the characteristics examined in this study

Items	*Characteristics*	*Cultivar*	*Harvesting time*	*Interaction*
Fermentation quality	**pH**	<0.0001	0.0001	<0.0001
	Ammonia nitrogen	0.0001	<0.0001	<0.0001
	Lactic acid bacteria	<0.0001	0.0405	0.0001
	Lactic acid	0.0035	0.0002	ns
	Acetic acid	<0.0001	0.0034	0.0125
	Butyric acid	ns	<0.0001	0.0325
	Propionic acid	ns	0.0010	ns
	Ethanol	<0.0001	0.0002	0.0007
Aerobic stability	**Carbon dioxide**	<0.0001	ns	<0.0001
	pH	<0.0001	<0.0001	<0.0001
	Enterobacteria	<0.0001	<0.0001	<0.0001
	Yeast and Mold	<0.0001	<0.0001	<0.0001
Nutritive value	**Dry matter**	<0.0001	<0.0001	<0.0001
	NDF	<0.0001	0.0247	ns
	ADF	<0.0001	0.0036	0.0002
	ADL	ns	0.0029	ns
	Crude protein	ns	0.0008	ns
	Ash	<0.0001	0.0017	<0.0001
	Ether extract	0.0008	0.0021	ns
	WSC	<0.0001	0.0020	<0.0001
	Relative feed value	<0.0001	0.0460	ns

pH: Power of hydrogen, NDF: Neutral detergent fiber, ADF: Acid detergent fiber, ADL: Acid detergent lignin, WSC: Water soluble carbohydrate

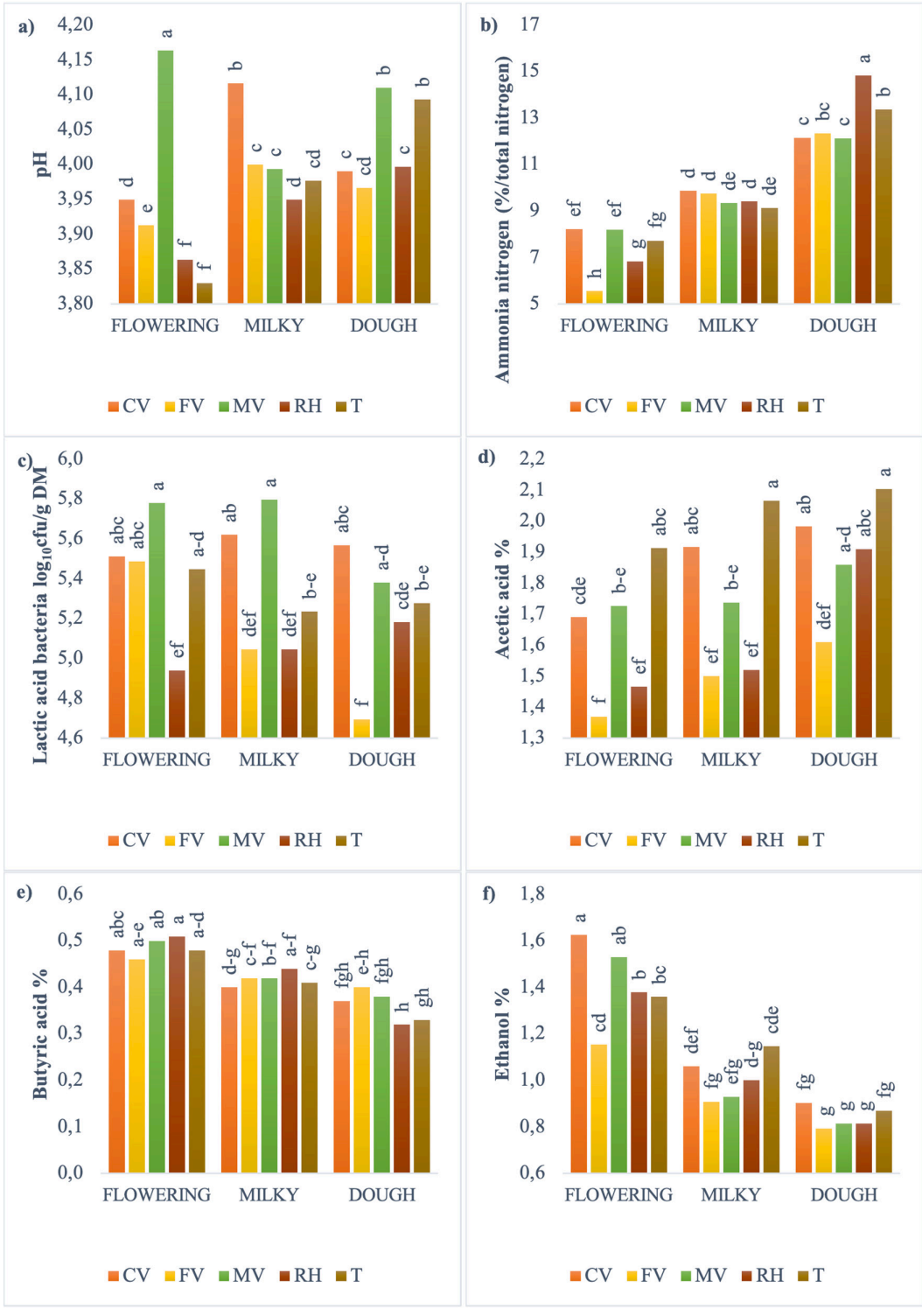

Figure 2- pH, ammonia nitrogen, lactic acid bacteria, acetic acid, butyric acid and ethanol characteristics of the evaluated silages according to the interactions

The cultivar and harvesting time changed the LA content of the silages while their interactions did not (Table 1). The LA ratio among the cultivars varied between 4.28% and 4.85%. The highest LA content was obtained from cv. French Vanilla (Table 2). Moreover, except for cv. Cherry Vanilla, other cultivars were statistically similar with cv. French Vanilla. The LA content of cultivars which had varying harvesting times increased as the harvesting time was delayed. The dough stage gave a very high LA content (5.48%). The effects of the harvesting time on PA were significant yet the cultivar and interaction were insignificant (Table 1). The PA values varied

between 0.67% and 0.86% among the harvesting times (Table 2). The highest PA was obtained from the flowering stage whereas the lowest was at the dough stage. The effects of cultivar, harvesting time and their interactions on AA content were significant (Table 1). The AA contents in the interactions were determined to be between 1.37% and 2.10% (Figure 2d). The highest AA content was obtained from cv. Titicaca, harvested at the dough stage, whereas the lowest value was found in cv. French Vanilla, harvested at the milky stage. The harvesting time and interaction changed the BA content of silages while cultivar did not (Table 1). As the harvesting times were delayed, the BA contents of all the cultivars decreased (Figure 2e). The highest BA content in the interaction was recorded in cv. Red Head harvested at the flowering stage, while the lowest was in the same cultivar harvested at the dough stage. There was a significant effect of cultivar, harvesting time and their interaction on the EtOH content of quinoa silages (Table 1). It was found that the EtOH contents in interactions were between 0.79% and 1.63% (Figure 2f). The highest EtOH content was obtained from cv. Cherry Vanilla harvested at flowering stage while the lowest EtOH content was recorded in cv. French Vanilla, harvested at the dough stage.

3.2. The aerobic stability of the silages

The carbon dioxide (CO_2), pH, *Enterobacteria* and yeast and mold properties were used as silage deterioration indicators in order to evaluate the aerobic stability of the silages, and these were significantly affected by genotype, harvesting times and interaction (Table 1). The CO_2 production among the interactions varied between 5.29 and 10.26 g/kg (Figure 3a). The highest CO_2 production was obtained from cv. Mint Vanilla, harvested at the flowering stage, while the least CO_2 production was in cv. French Vanilla harvested also at the flowering stage. The pH level of the silages exposed to air ranged from 4.15 to 6.24 (Figure 3b). The highest pH level was determined in

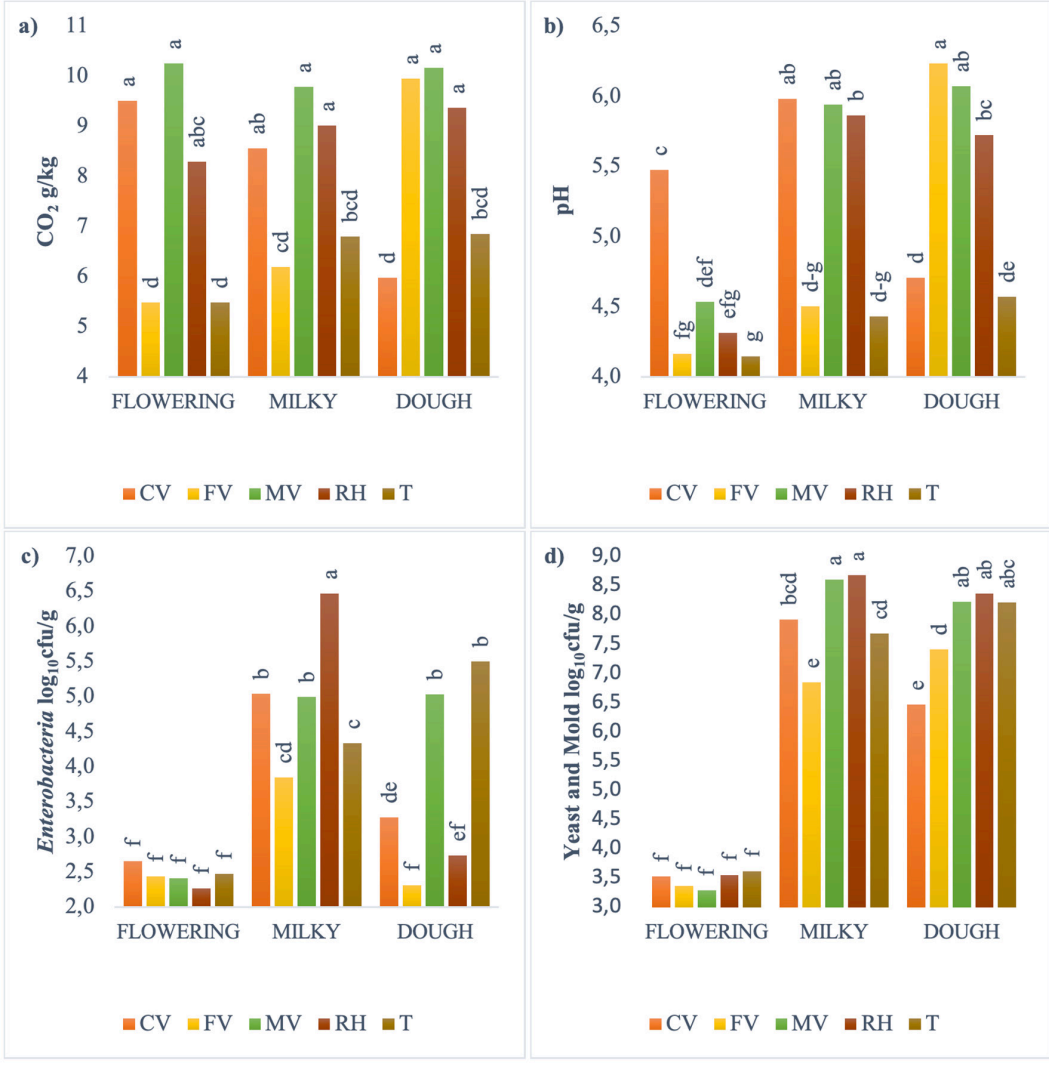

Figure 3- CO_2, pH, *Enterobacteria* and yeast and mold characteristics of the evaluated silages assessed for aerobic stability according to the interactions

cv. French Vanilla harvested at the dough stage, whereas the lowest pH level was found in cv. Titicaca harvested at the flowering stage. The *Enterobacteria* numbers of the silages under the interaction effects varied between 2.27 and 6.47 $\log_{10}$cfu/g (Figure 3c). The least and the highest *Enterobacteria* numbers were obtained from the cv. Red Head genotype, yet the highest and the lowest were found in the plants harvested at the milky stage and at the flowering stage, respectively. The yeast and mold numbers of the interactions ranged from 3.29 to 8.68 $\log_{10}$cfu g^{-1} (Figure 3d). The highest yeast and mold numbers were detected in cv. Red Head harvested at milky stage. The lowest yeast and mold numbers were obtained from cv. Mint Vanilla harvested at the flowering stage, in addition, the yeast and mold numbers of the other cultivars harvested at the flowering stage were statistically similar to those of cv. Mint Vanilla.

3.3. The nutrient contents of silages

The effects of cultivar, harvesting time and interaction on the DM of the silages were significant (Table 1). The DM contents of the interactions are given in Figure 4a. Among the interactions, the DM contents varied between 21.95% and 33.36%. The highest DM was obtained from Red Head harvested at the dough stage while the lowest DM was recorded in cv. Cherry Vanilla harvested at the flowering stage. The NDF content was affected by the cultivars and the harvesting times significantly (Table 1). The effects of the interaction on NDF content were not significant (Table 1). The NDF contents of the cultivars varied between 38.62% and 44.22% (Table 2). The lowest NDF content was obtained from cv. Titicaca. The NDF concentrations of cv. Cherry Vanilla, cv. Cherry Vanilla, cv. Mint Vanilla and cv. Red Head were statistically in the same group. The lowest NDF content among the harvesting times was recorded at the flowering stage. The NDF contents at the milky and dough stages were similar to each other.

The cultivar, harvesting time and their interaction on the ADF content of silages all had an effect on the results (Table 1). The ADF concentrations of interactions varied between 22.39% and 28.36% (Figure 4b). The lowest ADF content was obtained from cv. Titicaca harvested at the dough stage whereas the highest value was determined in cv. Mint Vanilla harvested at the milky stage. The ADL content was affected by harvesting time, yet not affected by cultivar and interaction (Table 1). The ADL contents of harvesting times were recorded between 4.07% and 4.79% (Table 2). It was observed that the ADL content of the silages decreased as the harvesting time was delayed.

Neither cultivar nor interaction had a significant effect on the CP contents of the silages (Table 1). However, the harvesting time significantly influenced the contents of the CP of the silages evaluated (Table 1). The highest CP (10.98%) among the harvesting times was recorded at the flowering stage (Table 2). As the harvesting time was delayed, the CP content of the silages decreased.

Table 2- Averages and mean comparison test results of lactic acid, propionic acid, NDF, ADL, crude protein and ether extract properties of silages depending on the main effects

Cultivars	***Lactic acid (DM%)***	***Propionic acid (DM%)***	***NDF (DM%)***	***ADL (DM%)***	***Crude Protein (DM%)***	***Ether Extract (DM%)***
Cherry Vanilla	4.28±0.25^{B}	0.75±0.03	44.21±0.50^{A}	4.53±0.11	9.70±0.32	2.43±0.06^{A}
French Vanilla	4.85±0.27^{A}	0.74±0.03	42.38±0.82^{A}	4.36±0.17	10.16±0.35	2.39±0.05^{A}
Mint Vanilla	4.61±0.28^{AB}	0.77±0.04	44.22±0.84^{A}	4.52±0.13	9.78±0.32	2.45±0.06^{A}
Red Head	4.64±0.29^{AB}	0.75±0.03	41.91±0.47^{A}	4.56±0.19	9.68±0.37	2.32±0.05^{B}
Titicaca	4.41±0.29^{AB}	0.77±0.04	38.62±0.45^{B}	4.32±0.11	10.01±0.28	2.30±0.04^{B}
Harvesting times						
Flowering	3.69±0.09^{C}	0.86±0.02^{A}	40.70±0.45^{B}	4.07±0.06^{B}	10.98±0.13^{A}	2.20±0.02^{C}
Milky	4.50±0.10^{B}	0.74±0.02^{B}	43.44±0.57^{A}	4.52±0.09^{A}	9.61±0.13^{B}	2.42±0.02^{B}
Dough	5.48±0.08^{A}	0.67±0.01^{C}	42.67±0.89^{A}	4.79±0.09^{A}	9.01±0.12^{C}	2.52±0.03^{A}

^{A,B,C}Means in the same column with different superscripts are significantly ($p<0.05$) different from each other. NDF: Neutral detergent fiber, ADL: Acid detergent lignin, DM: Dry matter

The effects of the cultivar, harvesting time and interaction on the ash content of the silages were significant (Table 1). The ash contents of the interactions were recorded between 15.41% and 17.70% (Figure 4c). The highest ash concentration was obtained from cv. Mint Vanilla harvested at the flowering stage while the lowest value was found in cv. French Vanilla harvested at the milky stage.

The cultivar and harvesting time had an effect on the EE contents of the silages, while the EE content was not influenced by interactions (Table 1). The EE values among the cultivars ranged from 2.30% to 2.45% (Table 2). The highest EE content was obtained from cv.

Mint Vanilla whereas the lowest value was recorded in cv. Titicaca. There was an increase in the EE content of the silages from the flowering stage to the dough stage (Table 2). The highest EE content was determined to be 2.52%.

The effects of the cultivar, harvesting time and their interactions on the WSC content of the silages were significant (Table 1). The WSC concentrations among the interactions ranged from 2.35% to 9.50% (Figure 4d). While the highest WSC content was found in cv. Titicaca harvested at the dough stage, the lowest WSC amount was determined to be in cv. Cherry Vanilla harvested at the flowering stage.

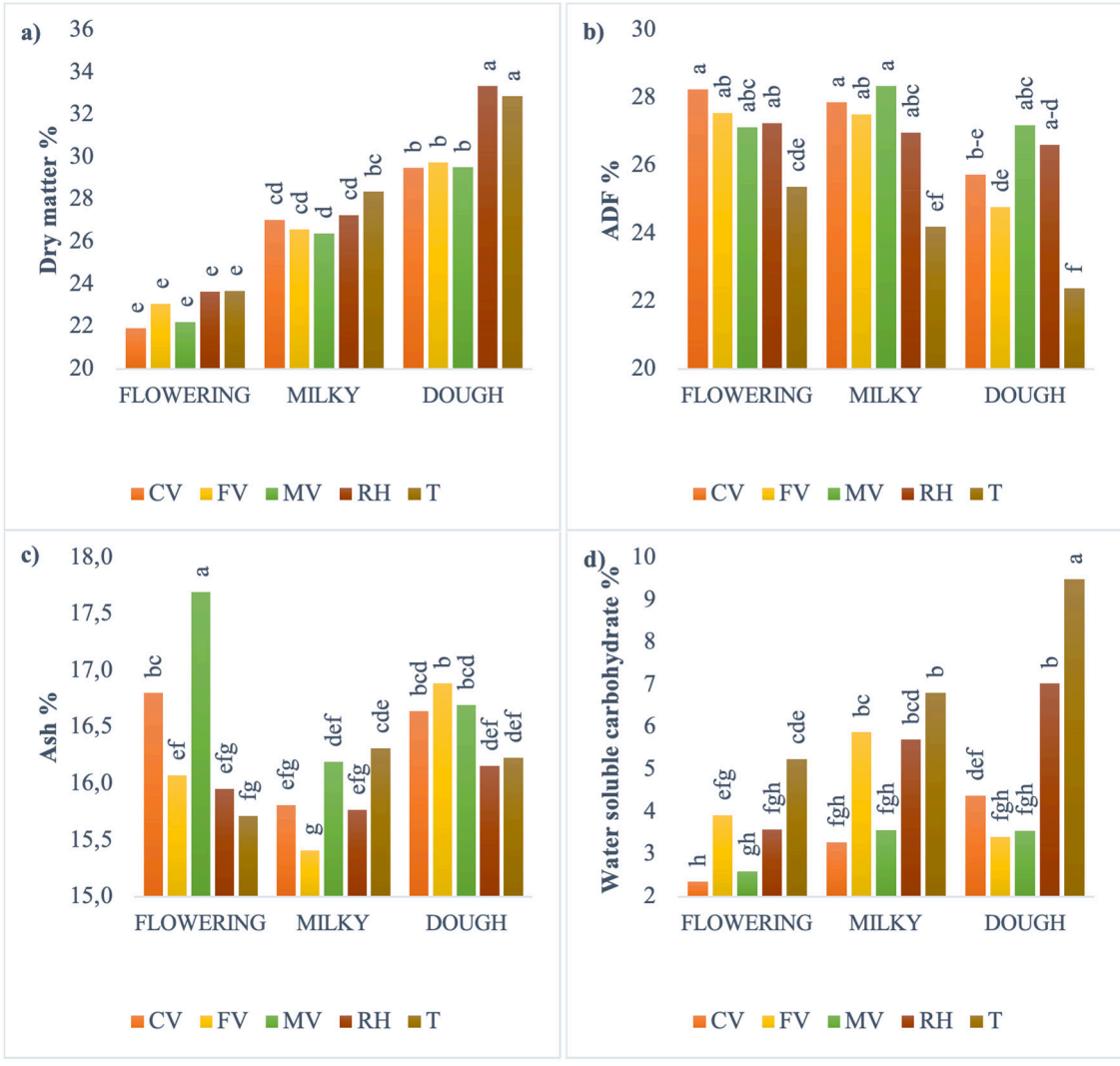

Figure 4- Dry matter, ADF, ash and water soluble carbohydrate characteristics of the evaluated silages according to the interactions

4. Discussion

4.1. The silage pH, lactic acid bacteria and fermentation end products

The difference in the response of the cultivars in terms of pH during the harvest period caused the interaction to be significant. A similar situation was reported for Kenaf by Ryu et al. (2016). Generally, the pH level of silages made from quinoa cultivars interestingly increased as the harvesting time was delayed. This result was probably due to a low DM content at the early stage and the high ash content (Table 1) of the fresh plants during the dough stage (Kung & Shaver 2001; Kung et al. 2018). In previous studies, it was determined that the silage pH levels changed according to the genotypic characteristics of different plant species (Ryu et al. 2016; Tolentino et al. 2016). Also, it was stated that the pH level of the two wheat cultivars changed with a slight fluctuation from early to late harvesting time (Filya 2003). Filya (2004) and Demirel et al. (2006) reported that the pH levels of the various silages increased as the harvesting time was delayed, similar to the pH results obtained from the present research.

The pH level of the silages is the most basic feature in explaining the fermentation quality. Generally, most plants have a pH level of 5.5-6.0 when chopped for silage. The final pH of the silages is affected by many factors, but the most important factors are the LA concentration of silages and the buffering capacity of the forage crops (Kung et al. 2018).

The amounts of LAB in quinoa silages at early harvesting times were higher than those of later harvesting times. The amounts of LAB in quinoa cultivars were affected by cultivar variation. The numbers of LAB obtained from the current study decreased as the harvesting time was delayed. The number of LAB in the plant epiphytic flora and the number of LABdetected in the silo are inversely related (Table 1). Probably the environmental conditions in the silo changed the proliferation of LAB. As a matter of fact, Whiter and Kung (2001) observed that the amount of LAB may be low in silages with high DM. During the fermentation process, LA is produced by LAB, especially homo-LAB, which is the major acid indicator for silage quality (Kung et al. 2018). Generally, the LA contents of silages vary between 2% and 4% (Kung & Shaver 2001). The LA contents of the quinoa cultivars changed according to both the cultivars and harvesting times in this study (Table 1). As the water-soluble carbohydrate content of fresh plants increased, the LA contents of the silages increased as expected. Podkówka et al. (2018) reported that the LA content of quinoa silages was between 1.83% and 1.92%. In addition, Salama et al. (2021) reported that the LA content of their quinoa silage was 3.02%. The LA contents obtained from the present study were higher than those of the previous literature reports (Table 2). Although the DM of the silages was lower (<30%), the LA contents obtained from the silages were higher (>4%) than the reported limit values. Liu et al. (2021) reported that the LA content was 5.69% in silage made from barley crops containing approximately 30% DM. LAB produce some organic acids, mainly LA, using the water-soluble carbohydrates in the plant (Kung & Shaver 2001). Depending on the epiphytic flora content of forage crops, these bacterial species may vary and the fermentation end products occurring in silage may differ (Kung et al. 2018).

The AA content varied between 1.37% and 2.1% among all treatments. These values were within the values reported for silages (Demirel et al. 2006). In general, the AA content tends to increase with the advancement of plant maturity. AA is the acid found in the second higher fermentation end product in the silages, and AA concentrations of the silages change from 1% to 3%. The moderate amount of AA in the silages delays their deterioration when they are exposed to air (Kung et al. 2018). Higher than normal concentrations (3-4%) of AA are usually found in silages dominated by *L. buchneri* bacteria (Kung 2010). Silages with high buffering capacity (high ash and protein content) may contain higher AA (Kung et al. 2000).

The BA contents of the cultivars decreased as the harvesting times were delayed (Figure 2e) and the BA contents of the quinoa silages were higher than 1.0 g/kg DM, as reported by Pinho et al. (2016). The BA contents found in the silages in this study may be considered to be high (Figure 3b). This may be due to the high moisture (Table 1) content of the silage materials of the quinoa cultivars, and may indicate that there was a *clostridial* activity in the silo during the fermentation process. The presence of BA in the well-fermented silages is indicative of the metabolic activity of undesirable *clostridial* microorganisms, which causes a large loss of DM and low energy recovery (Pahlow et al. 2003). Especially in the wetter silages, the activity of *clostridial* microorganisms is likely and they can convert the LA in the silage into BA (Kung et al. 2018).

The silage PA contents obtained from this study were higher than the values reported within optimistic limits (Table 2). The decrease in the PA content due to the delay in harvesting indicates that a late harvest may be more suitable for the purposes of this study due to increased silage quality. This can be explained by the low DM content. A high PA (0.3-0.5% DM) was found in wetter silages associated with poor fermentations (Kung & Shaver 2001). This may be due to the PA bacteria activity in the silo, as the crops used as silage material in the study were wetter (>70%). PA is generally absent or very little (<0.01%) in good silages and this acid may be undetectable in the silages with higher concentrations of DM (35-45% DM) (Kung 2010). The PA in the silages is formed by the conversion of LA and this acid is created by PA bacteria (Kung et al. 2018). Although the PA content varied depending on the cultivars, the EtOH content decreased with the delayed harvesting time (Figure 2f). As expected, the EtOH content of the silages decreased as the DM of the silage materials (fresh crops) increased. The highest EtOH content value detected in the current study was 1.63% and this value is within the acceptable limits for silage. Normal amounts of EtOH in the silages range from 1% to 2% DM (Kung 2010). A small amount of EtOH like this (<2% DM) is converted to AA in the rumen by the animal and this acid can be absorbed in the rumen wall of the animal (Bruning & Yokoyama 1988). AA can be converted into milk fat or used for body metabolism or growth (Kung et al. 2018). EtOH is the most common alcohol in silages and a high EtOH content in silages is usually associated with the activity of hetero-LAB, *Enterobacteria* and yeasts in the silo or the water soluble content of the silage material (Kung & Shaver 2001).

4.2. The aerobic stability of the silages

After 120 h of exposure to the air, the CO_2 emissions and the pH of the quinoa silages were harvested at different harvesting times, and this amount increased as the harvesting time was delayed (Figure 3a and Figure 3b) Also, as the harvesting time was delayed, the

number of *enterobacteria* (Figure 3c) and yeast and mold (Figure 3d) increased in the silages. It was determined that silages were more prone to aerobic deterioration as the harvest time was delayed. This may be due to the higher yeast count and water-soluble carbohydrate content in the fresh plants harvested later (Table 1). The aerobic stability of the cv. Titicaca genotype was found to be better at the flowering stage. The presence of oxygen during the storage and opening of the silage supports the growth of aerobic microorganisms in the silage and the growth of these microorganisms on the silage surface reduces the quality of the end product, and causes high nutrient loss (Pozza et al. 2011). Yeasts and molds grow on substrates such as sugars, as well as LA, both of which are important for silage. In the most cases, yeasts and molds are the first community of microorganisms to develop when silage comes into contact with oxygen (Muck 2010). AA has a powerful antifungal activity against aerobic deterioration (Kung & Ranjit 2001) and this acid was found to be higher in cv. Titicaca than that of the other cultivars (see Figure 2d). Therefore, the aerobic stability in cv. Titicaca was better than others.

4.3. The nutrient contents of silages

The DM of the silages is very important in obtaining a high silage nutrient quality (Borreani et al. 2018). As the harvesting times were delayed, the DM contents of the silages increased (Figure 4a). The DM contents of the silages varied significantly among the cultivars. Moreover, the DM contents of the silages were similar to those of the fresh plants (Table 1). Tolentino et al. (2016) determined that the DM contents of the silages obtained from different sorghum cultivars changed according to the cultivars. It has been reported that the silage DM content increases with the delay in the harvest time of the plants for silages made with different plants (Filya 2004; Demirel et al. 2006; Atis et al. 2013). As a matter of fact, the DM contents obtained from the present study were similar to that which was reported in the previous literature. The NDF and ADF contents of forages were fairly significant since cellulose and hemicellulose are digested to a certain extent by ruminant animals (Canpolat & Karaman 2009). The NDF content of the silages fluctuated as the harvesting times of the cultivars were delayed (Table 2). The NDF and ADF contents of the silages were slightly lower than those of the fresh plants (Table 1). This result can probably be explained by the presence and diversity of some enzymes that can exist in silages (McDonald et al. 1991). Also, the loss in the NDF and ADF content during fermentation process is minimal (McDonald et al. 1991), and this loss generally does not constitute a disadvantage in animal nutrition for most forage crops (Khota et al. 2016). Shah et al. (2020) reported that the NDF contents of different quinoa cultivars varied depending on the harvesting time. The ADF content decreased as the harvesting time was delayed in the different quinoa cultivars (Figure 4b). Shah et al. (2020) reported that the ADF content in the anthesis and grain filling stages of different quinoa genotypes ranged from 17.5% to 26.8% and 21.8% to 30.6%, respectively. Peiretti et al. (2013) determined that the ADF content of quinoa in six different harvesting times varied significantly. The ADF values in this study were within the limits reported in the above mentioned literature. As the harvesting time was delayed, the ADL content of the different quinoa cultivars decreased (Table 2). It was reported that the cell wall components in the feeds are directly affected by the cutting time of the harvested plants (Yavuz 2005; Tekce & Gül 2014). The CP content of quinoa genotypes decreased as the harvesting time was delayed (Table 2). The CP values of ensilaged quinoa cultivars were not significant. Similar results were reported for different forage plants by Butler and Muir (2003) and Nabi et al. (2006), and for different quinoa cultivars by Uke et al. (2017) and Liu et al. (2021). The results of the research indicated that quinoa had a higher protein content compared to forage maize and sorghum (Atis et al. 2013; Uke et al. 2017). The ash content of the silages fluctuated as the harvesting time was delayed in the different quinoa genotypes (Figure 4c). Liu et al. (2021) reported that the crude ash content of quinoa forage varied according to the phonological stages. In the present study, the ash content of the quinoa silages was higher than silages of many forage crops. The higher ash content in the feeds reduces the metabolizable energy content (Kung et al. 2018). The EE content of the silages increased as the harvesting time was delayed (Table 2). On the contrary, Uke et al. (2017) found that the EE contents of quinoa decreased with the delayed harvesting time. As the harvest time was delayed, the seed formation rate in the plants increased and, accordingly, the EE contents of silages (Table 2) increased. As a matter of fact, Liu et al. (2021) reported that the EE contents of quinoa plants increased as the harvest time was delayed. In addition, the high ether EE increases the total digestible nutrients and, as a result, the metabolizable energy content of the forage is increased (Khota et al. 2016). During the ensiling process, the WSC plays a critical role in silage fermentation, and it is used as a fermentable substrate in the silo in fermentation's the early stages (Silva et al. 2020). Feeds such as silages rich in water-soluble carbohydrates are an ideal energy source during early lactation, as they provide both the energy for milk production and the structural fiber needed to support chewing and rumen buffering (Klevenhusen et al. 2019). Therefore, it is very important to know the water-soluble carbohydrate content of the silages. The WSC content varied according to the cultivar (Table 1), and increased as the harvesting time was delayed in the different quinoa cultivars (Figure 4d).

5. Conclusions

This study was intended to address the silage fermentation quality, aerobic stability and nutritive value of different quinoa cultivars (harvested at varying times), which may have the potential to help address deficits in roughage production, especially in developing countries. Among the evaluated quinoa cultivars, cv. Titicaca and cv. French Vanilla gave superior results in terms of fermentation quality than the other cultivars. Among the quinoa cultivars, cv. Titicaca was the cultivar with the highest silage nutritive value. It was determined that it would be more appropriate to harvest these superior quinoa cultivars (cv. French Vanilla and cv. Titicaca) during the dough stage for a better silage quality (for fermentation quality, as well as the nutritive value and aerobic stability). Among the silages' forms, the cv. Titicaca that were harvested at the flowering stage had the best aerobic stability. As a result of this study, it was determined that cv. French Vanilla and cv. Titicaca should be harvested during the dough stage to obtain better silage quality.

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Effect of Priming on Germination Traits and Antioxidant Enzymes of Pumpkin (*Cucurbita pepo L.*) Seeds with Different Vigor under Drought Stress

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ABSTRACT

Drought stress is a critical environmental stress that limits the productivity and sustainability of agriculture in arid and semi-arid regions by reducing the germination rate and delaying the start of germination and seedling establishment. There are various methods to increase the yield of agricultural plants, each of which somehow improves the yield of seeds in terms of germination indicators and seedling establishment. One of the most common methods to increase poor seed germination is priming before planting. To investigate the effects of priming on aspects of germination of aged pumpkin seeds under drought stress, a factorial experiment was conducted based on a completely randomized design with four replications in the laboratory. Treatments that include seed aging (control, 85% and 75% of control germination), drought stress (0, -0.75 and -1.5 MPa), and priming (control, hydro, gibberellin, GR24, benzyl aminopurine and spermidine). The results of a mean comparison between drought stress, seed ageing and priming showed that the lowest germination percentage (GP) of pumpkin (43.6%), protein (0.82 mg.g^{-1}Fw), radical length (RL, 9.24 mm), plumule length (PL, 1.19 mm), relative water content (RWC, 35.5%), carotenoid (1.253 mg.g^{-1}Fw), catalase (CAT, 7.03 µmol.gmin^{-1}.mgprotein^{-1}), peroxidase (POX, 47.72 µmol.gmin^{-1}.mgprotein^{-1}) and superoxide dismutase activity (SOD, 53.48 mmol.gmin^{-1}.mgprotein^{-1}) were associated with drought treatment at -1.5 MPa and with 75% aging in the absence of priming. In addition, the lowest content of malone dialdehyde (MDA, 3.75 mmol.g^{-1}Fw) and proline (1.573 µg.g^{-1}Fw) were obtained from the absence of drought and aging with priming using spermidine. The highest GP (98.03%) and protein (9.54 mg.g^{-1}Fw) were obtained from gibberellin treatment, RL (15.73 mm) and PL (4.26 mm) in priming using GR24 hormone, RWC (83.64%), carotenoid (6.277 mg.g^{-1}Fw), CAT (40.44 µmol.gmin^{-1}.mg protein^{-1}), POX (95.71 µmol. gmin^{-1}.mgprotein^{-1}) and SOD activity (131.68 µmol.gmin^{-1}.mg protein^{-1}) in priming with spermidine with non-drought stress and aging conditions. The highest MDA (32.42 mmol.g^{-1}Fw) and proline (0.55 µg.g^{-1}Fw) were observed in drought conditions with an intensity of -1.5 MPa and 75% aging in the absence of priming.

Keywords: Abiotic stress, Catalase, Deterioration, Seed vigor, Water potential, Strigolactone

1. Introduction

Seed germination as the first stage of plant growth is one of the most sensitive stages of crop life. So that successfully passing this period will play a very important role in the subsequent optimal growth of the plant and the damage at this stage cannot be rectified in any way. In many fields, seeds are exposed to environmental stresses during germination. Drought is one of the most important abiotic stresses, limiting plant growth and crop yields (Khan et al. 2010).

Another important factor influencing the yield of crops is the agronomic quality of seeds or seed bulks. Factors such as temperature, relative humidity, and seed moisture during storage lead to seed deterioration, which ultimately causes oxidative stress (Sharma et al. 2007). Catalase is an antioxidant enzyme that detoxifies free radicals. In fact, catalase uses hydrogen peroxide as a substrate. In undeteriorated seeds, these radicals are at steady state levels due to the action of superoxide dismutase (SOD), catalase and peroxidase, which use these electron donors, and the activity of these enzymes increases during germination. For this reason, seed aging may depend on the effectiveness of the seed to maintain a sufficient level of the enzyme system to protect against oxidative stress (Zhan et al. 2014).

There are a number of methods employed to increase crop yield, each of which in some wayt improves the seed yield in terms of germination characteristics and seedling establishment. One of the most common methods of improving poor germination of seeds pre-sowing treatment (priming) before planting. The purpose of priming is to increase the germination percentage (GP) and reduce the average germination time and improve the growth and germination power of seeds in both favorable and unfavorable environmental conditions (Sedghi et al. 2010). The treatment of seeds with GR24 (a synthetic strigolactone) can strengthen a plant's tolerance to drought and salinity stress (Kapulnik & Koltai 2014). The exogenous use of spermidine reduces the adverse effects of drought stress (Hu et al. 2012). In addition to eliminating free radicals and stabilizing membranes, spermidine can increase the activity of antioxidant enzymes (catalase, peroxidase, superoxide and dismutase) as well as proline (Ndayiragije & Lutts 2006). Hydropriming treatment increases in seedling germination index, can be due to the effects of increasing in seed imbibition time. Gibberellin is a known hormone in the germination process (Graeber et al. 2012; Nee et al. 2017; Shu et al. 2016). Gibberellins can minimize water loss during drought stress by maintaining membrane stability and reducing permeability. Thereby, the activity of lipase and protease enzymes increases, releasing storage substances and converting them into transportable substances of sucrose and glucose, improving vigor and germination index. As the level of drought stress increased, the germination index decreased, but the germination index of the treated seeds decreased only slightly.

Pumpkin (*Cucurbita pepo L.*) is a new variety of that Cucurbitinae first appeared in the mid-nineteenth century in the Austrian state of Ashtria due to a natural mutation. This plant belongs to the family Cucurbitaceae, subfamily Cucurbitinae, genus *Cucurbiteae* and subfamily Cucurbitinae (Jellin et al. 2000). One of the most noticeable characteristics of this plant is its skinless seeds. Mutations in this plant led to thinning of the seed coat, which facilitated the extraction of green oil (Fruhwirth & Hermetter 2008). The oil obtained contains unsaturated fatty acids, vitamin A, vitamin E, minerals, phytosterols, carotenoids and protochlorophyll. Linoleic, oleic and palmitic acids account for 90% of the seed oil content. The content of linoleic acid (50%) is higher than other fatty acids. (Fruhwirth & Hermetter 2008). Geravandi et al. (2010) noted that there is a significant correlation between the GP, germination rate and seed vigor in wheat seeds. Ghiyasi & Tajbakhsh (2013) showed that the application of priming reduced the negative effect of drought stress on the germination and growth of soybean seedlings and improved the yield under stressful conditions in Urmia of Iran. Study of Ansari and Sharifzadeh (2012) has been performed on the effects of seed aging on germination. Basra et al. (2003a) showed that the GP of cotton seeds decreases with the acceleration of aging. Antioxidant enzymes reduce the rate of membrane lipid peroxidation during germination, thereby increasing in the germination rate. For this reason, it is expected that seed priming can be an effective way to improve the germination traits of pumpkin seeds in adverse environmental conditions.

One problem in developing countries is soil heterogeneity and the lack of suitable soil conditions, which causes problems such as reduced germination rate and lack of uniform crop growth, uneven growth of germinated plants and their Unequal competition in the use of sources such as light, food and water leads to differing performance among species of plants. To address these problems, seed priming is a potential solution. We expect that the use of these mechanisms in Iran will have a significant impact on performance and production and will improve the country's share of the global trade of this profitable plant. This study evaluates the seedlings obtained from the deteriorated seeds of pumpkins under drought stress are evaluated and different pretreatment materials are analyzed to reduce their adverse effects or improve their properties under stress conditions.

2. Material and Methods

The effect of priming on the germination of aged pumpkin seeds was studied under low osmotic potential conditions at the University of Mohaghegh Ardabili, Ardabil, Iran, in 2018-2019. A factorial experiment conducted on a completely randomized design with four replications and treatments included: 1- different levels of aging (control, 85% and 75% of control germination), 2- water potential (0, -0.75 and -1.5 MPa) and 3- priming (control, hydro, gibberellin, GR24 (a strigolactone), benzyl aminopurine and spermidine). In order to apply priming, the seeds were soaked for 24 hours at 25 °C and at a concentration of 100 µM for each hormone. Control group was without priming and distilled water was used for hydropriming. The seed bulks reached different germination degrees of 85% and 75% by being placed in an oven at 40 °C and 100% humidity for 5 and 10 days using a rapid aging procedure. For control treatment, seeds were prepared with 95% viability.

In order to apply priming, a hydropriming method with distilled water and hormone priming including gibberellin, GR24, benzyl aminopurine and spermidine were used. The seeds were then soaked in hormones for 16 hours in a germinator at 20 °C and after the priming period, the seeds were washed with distilled water and dried. Polyethylene glycol 6000 was used to prepare the osmotic solutions. The Michel and Kaufmann relationship (Michel & Kaufmann 1973) was used to create the desired osmotic potential for drought stress as below:

$\Psi s = -(1.18 \times 10^{-2})\ C - (1.18 \times 10^{-4})\ C^2 + (2.67 \times 10^{-4})\ CT + (8.39 \times 10^{-7})\ C^{2T}$

Which, Ψs is osmotic pressure (Bar), C is the concentration of polyethylene glycol 6000 (g kg^{-1} H_2O) and also T is the temperature (°C). By substituting the optimum germination temperature (25 °C) and the desired drought concentrations (7.5 and 15 bar), the amount of polyethylene glycol obtained was 127 g and 185 g, respectively (1 MPa= 10 bar).

To perform a standard germination test, 25 seeds from each treatment were planted between paper (BP) in disposable containers. The dishes were then transferred to an IKH.RI model germinator with a temperature of 25 °C and in dark. The germinated seeds were counted daily for 7 days. The criterion for the germination of a seed was radicle (RL) growth and its emergence at a rate of 2 mm from the seed coat.

2.1. Germination percentage

The GP was obtained from the following equation (ISTA 2002):

$GP = 100 \times (ni_{ti}/s)$

Which, GP is the GP, ni germinated seeds at considered time ti and S is the total number of seeds.

2.2. Radicle and plumule length

At the end of the seventh day of planting and after the complete opening of the cotyledon leaves, the normal seedlings from each treatment were selected and the radical length (RL) and plumule length (PL) were measured in millimeters.

2.3. Relative water content of leaves

To measure the relative water content (RWC), 0.5 g of isolated leaf samples were immediately weighed to determine the fresh weight using an accurate scale (0.001 g). The leaf samples were then immersed in closed test tubes containing 100 mL of distilled water and kept in a dry and light-free environment for 6 hours. After this period, the leaves were removed from the test tubes and quickly dried with a paper towel and re-weighed. Then, the leaf samples were transferred into an oven for 72 hours at 70 °C, after which the dry weight (DW) of the leaves was determined. The relative moisture content of the leaves was calculated using the formula of Qasim et al. (2003) as below:

RWC (%)= (FW-DW/TW-DW) ×100

Which, FW is leaf fresh weight, DW is the dry weight and TW is the leaf weight in the full turgor state.

2.4. Extraction and measurement of catalase activity

Catalase activity (CAT) was measured using the Aebi method (Aebi 1984). A 3 mL of the reaction complex consisted of 0.5 mL of 7.5 mM hydrogen peroxide, 1.5 mL of 100 mM potassium phosphate buffer (pH=7) and 50 µl of enzyme extract. The reaction was started by adding hydrogen peroxide. The specific activity of the enzyme was measured at a wavelength of 240 nm by spectrophotometer and expressed in micromoles of hydrogen peroxide decomposed per minute per milligram of protein.

2.5. Peroxidase enzyme assay

Peroxidase enzyme activity (POX) was measured according to the Kato and Shimizu method (Kato & Shimizu 1985). The reaction mixture contained 100 mM sodium phosphate buffer (pH=5.8) and 7.2 mM guaiacol, 11.8 mM H_2O_2 and 0.1 mL of enzyme extract. The reaction was started by adding hydrogen peroxide and changing the optical density. Changes in absorption were recorded using a spectrophotometer at 470 nm. The buffers required to measure the peroxidase were: 45 mM guaiacol, and 225 mM oxygenated water (H_2O_2). Enzymatic activity was calculated using Lambert-Beer's law and the extinction coefficient of the product of guaiacol peroxidase reaction (13.3 mM^{-1} cm^{-1}) as below:

$$(\text{Unit mg}^{-1}) = \frac{\text{POX/min}}{13.3}$$

2.6. Assessment of superoxide dismutase enzyme activity

The activity of SOD was measured using the Giannopolitis and Ries method (Giannopolitis & Ries 1977). The buffers used in the SOD enzyme assay were: (1) 50 mM K-phosphate buffer containing 0.1 mM EDTA, 13 mM methionine and 75 µM nitroblutetrazolium at pH=7 and (2) 0.12 mM Riboflavin solution. The control sample was a combination of 885 µL of buffer 1+15 µL of buffer 2+100 µL of phosphate buffer and the blank sample was a combination of 885 µL of buffer 1+15 µL of buffer 2+100 µ of phosphate buffer. In addition, an enzyme sample was obtained from a combination of 885 µL of first buffer + 15 µL of second buffer and 100 µL of enzyme extract. The absorbance was read at 560 nm with a spectrophotometer and SOD activity was calculated as below:

2.7. Measurement of malondialdehyde content

The amount of malondialdehyde (MDA) was measured according to McCue and Shetty (McCue & Shetty 2002). In the test tubes, 200 mL of homogeneous tissue was mixed with 800 mL of distilled water. 500 mL of 20% trichloroacetic acid was mixed with 1 mL of 10 mM thiobarbituric acid. The test tubes were incubated at 100 °C for 30 min. Then, they were centrifuged at 13000 g for 10 min. The resulting supernatant was used to measure the amount of MDA. The amount of light absorption was read at 532 nm and the concentration of MDA was expressed in terms of mM g^{-1}.

2.8. Measuring the amount of protein

Seed protein was measured using the Kjeldahl method (Kjeldahl 1883). In this method, first the organic nitrogen of the seeds was calculated by adding concentrated sulfuric acid and catalyst to 0.5 g of the sample and then multiplied by 6.25 to obtain the protein content of the sample in mg g^{-1}.

2.9. Measurement of proline content

Proline as an osmotic regulator and stress reagent was measured using the method of Bates et al. (1973). First, 0.02 g of fresh tissue was homogenized in 100 mL of 3% sulfosalicylic acid solution. Then, 2 mL of the filtered solution was combined with 2 mL of ninhydrin reagent and 2 mL of pure acetic acid and then 64 mL of toluene was added. The solution was kept constant until two layers formed. Then, a certain amount of supernatant was obtained and its adsorption was determined at 520 nm in the spectrophotometer and reported as µM per gram of fresh weight.

2.10. Carotenoid measurement

To measure the amount of carotenoid pigments, 1 g of the leaf was separated and homogenized with 15 mL of acetone. The light absorption of carotenoids was read at 480, 645 and 663 nm and was expressed as mg g^{-1} fresh weight according to Bruisma (1963) using the formula below:

Carotenoid content=A_{480}+ [(0.114) (A_{663}) - (0.638) (A_{645})]

2.11. Statistical analysis

Statistical calculations of data and their analysis were performed using SAS 9.4 software. Before performing the statistical calculations, the normality of the experimental error variance was evaluated using SAS software for each trait. Analyses of variance performed based on factorial experiment in which three treatments (priming, aging and water potential) and their interactions were included. To compare the means of the treatments, the least significant difference test was used at the probability level of p=0.05 and the least squares means and pdiff were used for interactions.

3. Results and Discussion

3.1. Germination percentage

The effect of different levels of drought, aging and priming as well as their interactions on GP was significant) $p \leq 0.05$) (Table 1). The results of the mean comparison table (Table 2) indicated that the lowest GP (43.6%) was related to drought treatment with an intensity of -1.5 MPa and with 75% aging. This was while seed priming significantly compensated for the damage caused by stresses and led to an increase in GP compared to the control, so that the highest GP (98.03%) were obtained from gibberellin treatment with non-drought

stress and aging conditions. The results showed that with aging seed in a drought-stress situation, the GP decreased that the effect of drought stress on reducing the GP was greater than the effect of aging. Different priming compensated for this decrease, making the role of gibberellins more affected.

Table 1- Analysis of variance for the effect of water potential and seed deterioration on some physiological and morphological traits of primed pumpkin seeds

SOV	*Df*	*MS*							*F Value*							*Pr > F*
		GP	*RL*	*PL*	*RWC*	*Pro*	*Cart*	*PR*	*GP*	*RL*	*PL*	*RWC*	*Pro*	*Cart*	*PR*	
D	2	8124.8**	133.4**	6.3**	7146.7**	52.67**	190.43**	2.71**	64562.7	54070	11144	21195	3348	29564	2134	<0.0001
P	5	202.06**	0.47**	0. 1**	312.1**	4.92**	0.37**	0.51**	1605.72	192	174	928	313	58	405	<0.0001
A	2	6483.3**	116.5**	111.1**	3132.8**	257.4**	31.43**	1.2**	51519.3	47240	196553	9291	16360	4881	949	<0.0001
D*P	10	6.43**	0.03^{ns}	0.012**	4.43**	0.82**	0.05**	0.008**	51.14	12	21	13	52	8	6	<0.0001
D*A	4	84.9**	6.33**	2.7**	255.1**	13.02**	1.53**	0.0006^{ns}	674.61	2569	4730	756	827	237	0.5	<0.0001
P*A	10	3.31**	0.01**	0.005**	1.28**	0.68**	0.03**	0.0004^{ns}	26.38	4	10	3	43	4	0.3	<0.0001
D*P*A	20	2.24**	0.01**	0.006**	2.11**	0.7**	0.03**	0.002*	17.86	4	12	6	44	5	1.8	<0.0001
Error	108	0.12	0.26	0.0005	0.58	0.01	0.006	0.001								
%CV		0.47	1.3	0.8	0.93	3.8	2.05	3.35								

^{*,**,ns}Statistically significant at p≤0.05, p≤0.01, respectively and non-significant.
SOV: Source of variation, Df: Degrees of freedom, MS: Mean square, D: water potential, P: Priming, A: Ageing, GP: Germination Percentage, RL: Radical Length, PL: Plumule Length, RWC: Relative Water Content, Pro: Protein, Cart: Carotenoid, PR: Proline

Low water potential reduces the GP and average germination rate as the reduced water uptake leads to slow metabolism in embryonic tissues resulting in reduced seedling emergence and vigor (Larcher 2001). Moreover, Ghaderi-Far et al. (2020) in their a study of pumpkin seeds found that higher temperatures and humidity reduced the germination persentage.

Numerous biochemical and metabolic reasons have been suggested for the reduced germination capacity of aged seeds, including lipid peroxidation, damage to cell membranes, damage to the RNA synthesis process, DNA degradation, as well as the deposition and

Table 2- Means of physiological traits of primed Pumpkin seeds affected by water potential and aging

Drought	*Priming*	*Ageing*	*GP (%)*	*RL (mm)*	*PL (mm)*	*RWC (%)*	*Pro (mg. g-1Fw)*	*Cart (mg. g-1Fw)*	*PR (µg. g-1Fw)*
Non-D	**Non-P**	0	92.3 f	15.11 f	4.15 c	72.48 ef	5.24 f	6.27 a	0.55 i
		85%	83.4 l	14.03 k	3.76 e	68.2 h	2.66 j	5.87 g	0.72 ef
		75%	71.53 r	13.54 q	1.33 h	65.9 i	1.76 m	4.93 m	0.84 d
	Hydro-P	0	94.46 e	15.35 d	4.21 b	75.5 d	6.21 e	6.27 a	0.59 hi
		85%	84.4 k	14.15 i	3.81 d	71.91 fg	2.76 j	5.98 e	0.753 e
		75%	75.03 q	13.72 o	1.38 fg	66.3 i	1.96 l	5.06 k	0.87 cd
	GA3	0	98.03 a	15.22 e	4.19 bc	77.64 c	9.55 a	6.27 a	0.63 gh
		85%	89.5 g	14.08 j	3.77 e	73.4 e	3.58 g	6.04 d	0.76 e
		75%	78.33 m	13.64 p	1.36 f-h	68.02 h	2.67 j	5.12 j	0.907 c
	GR24	0	95.93 c	15.73 a	4.26 a	79.32 b	8.45 c	6.27 a	0.68 fg
		85%	87.6 h	14.12 ij	3.83 d	75.32 d	2.97 hi	5.92 f	0.83 d
		75%	77.26 n	13.78 n	1.39 f	69.2 h	2.23 k	5.01 l	0.97 b
	BAP	0	95.03 d	15.52 c	4.18 bc	83.01 a	7.26 d	6.27 a	0.75 e
		85%	85.13 j	14.34 g	3.77 e	78.04 c	2.83 ij	6.09 c	0.89 cd
		75%	75.76 p	13.87 l	1.35 gh	70.91 g	1.94 l	5.18 i	1.12 a
	SP	0	97.3 b	15.62 b	4.22 b	83.64 a	9.22 b	6.27 a	0.82 d
		85%	86.5 i	14.27 h	3.84 d	80.47 b	3.08 h	6.15 b	0.97 b
		75%	76.43 o	13.8 m	1.4 f	72.85 ef	2.28 k	5.26 h	1.16 a

Table 2. Continued

Drought	*Priming*	*Ageing*	*GP (%)*	*RL (mm)*	*PL (mm)*	*RWC (%)*	*Pro (mg. g-1Fw)*	*Cart (mg. g-1Fw)*	*PR (µg. g-1Fw)*
-0.75 MP	**Non-P**	0	80.63 e	14.46 c	4.02 de	65.08 e	5.56 e	4.61 b	0.76 k
		85%	74.36 j	12.97 f	3.84 f	57.01 l	2.42 j	3.81 e	0.87 ij
		75%	60.56 p	11.34 h	1.3 i	51.93 p	1.22 n	3.04 f	0.98 g
	Hydro-P	0	82.36 d	14.5 b	4.17 b	65.3 e	5.62 de	4.73 ab	0.83 k
		85%	75.36 i	13.08 ef	3.98 e	58.4 k	2.53 ij	3.9 c-e	0.95 gh
		75%	61.23 o	11.4 h	1.36 gh	53.32 o	1.26 n	2.85 gf	1.09 ef
	GA3	0	86.13 a	14.67 b	4.15 bc	67.47 d	6.23 a	4.81 ab	0.91 hi
		85%	79.9 f	13.17 e	3.97 e	59.46 j	3.17 f	4.01 c-e	1.04 f
		75%	71.3 k	11.47 h	1.34 hi	55.12 n	1.84 k	2.94 gf	1.2 ef
	GR24	0	85.13 b	14.85 a	4.23 a	69.55 c	5.91 bc	4.67 ab	0.98 g
		85%	78.26 g	13.41 d	4.06 d	60.58 h	2.76 h	3.87 de	1.13 e
		75%	64.9 m	11.6 g	1.41 g	56.37 m	1.44 m	2.77 g	1.31 c
	BAP	0	83.53 c	14.8 a	4.12 c	70.48 b	5.77 cd	4.86 a	1.06 f
		85%	76.9 h	13.41 d	3.87 f	62.18 g	2.68 hi	4.06 cd	1.21 d
		75%	63.13 n	11.71 g	1.31 i	58.2 k	1.35 mn	3.01 gf	1.38 b
	SP	0	85.5 ab	14.82 a	4.18 b	71.27 a	6.06 b	4.91 a	1.13 e
		85%	79.56 f	13.3 d	4.04 d	63.14 f	2.96 g	4.15 c	1.29 c
		75%	67.1 l	11.68 g	1.38 gh	60.07 i	1.61 l	3.06 f	1.45 a
-1.5 MP	**Non-P**	0	70.8 e	13.2 d	3.8 d	58.88 f	3.44 d	2.29 e	0.91 i
		85%	58.6 j	11.18 h	2.43 j	44.54 l	1.65 g	1.54 kl	1.05 g
		75%	43.6 n	9.24 k	1.19 m	35.5 p	0.82 j	1.25 n	1.24 e
	Hydro-P	0	71.5 e	13.26 c	3.96 a	60.78 e	3.55 cd	3.07 c	0.98 h
		85%	60 i	11.28 g	2.55 h	45.95 k	1.77 fg	1.67 i	1.15 f
		75%	44.07 n	9.34 j	1.26 l	36.57 o	0.93 ij	1.38 m	1.32 d
	GA3	0	78.1 a	13.2 d	3.89 c	62.81 d	4.16 a	3.14 b	1.06 g
		85%	68.43 f	11.2 h	2.53 h	48.02 j	2.34 e	1.75 h	1.23 e
		75%	53.9 k	9.28 k	1.22 m	38.72 n	1.35 h	1.43 m	1.39 c
	GR24	0	74.4 c	13.33 b	3.92 bc	64.78 c	3.78 bc	2.9 d	1.15 f
		85%	62.8 h	11.31 g	2.61 g	49.89 i	1.96 f	1.61 j	1.31 d
		75%	47.13 m	9.34 j	1.31 k	40.44 m	1.14 hi	1.3 n	1.46 b
	BAP	0	72.5 d	13.4 a	3.54 e	66.53 b	3.64 b-d	3.23 a	1.25 e
		85%	60.4 i	11.48 e	2.46 i	53.51 h	1.83 fg	1.82 g	1.4 c
		75%	46.7 m	9.44 i	1.21 m	43.67 l	1.07 h-j	1.5 l	1.5 b
	SP	0	76.6 b	13.3 ab	3.95 ab	67.53 a	3.87 b	3.2 a	1.3 cd
		85%	64.7 g	11.4 f	2.65 f	54.9 g	2.57 e	1.9 f	1.5 c
		75%	49.5 l	9.4 i	1.34 k	45.8 k	1.27 h	1.5 jk	1.57 a
LSD			0.5	0.07	0.2	0.78	0.13	0.13	0.3

Non-P: non-Priming, Hydro-P: Hydro Priming, GA3: Gibberellin, BAP: Benzyl Amino Purine, SP: Spermidine, GP: Germination Percentage, RL: Radical Length, PL: Plumule Length, RWC: Relative Water Content, Pro: Protein, Cart: Carotenoid, PR: Proline, LSD: Least Significant Difference. In each column the comparison of means have been performed with SAS software using SLICE procedure so, letters in every level of water potential have begun from a

inactivation of enzymes (Basra et al. 2003b). Gibberellin is a known hormone in the germination process (Nee et al. 2017; Shu et al. 2016). Gibberellin may minimize water loss during drought stress by maintaining membrane integrity and reducing its permeability, thereby increasing in the activity of lipase and protease enzymes, leading to the release of stored materials and their conversion

into transferable materials such as sucrose and glucose and improvement of vigor and germination index (Mohamed et al. 2010). TavakolAfshari et al. (2009) reported that the decrease in seed vigor is due to decreased synthesis and degradation of cellular proteins and increased the electrical conductivity and cell permeability during aging, which priming repairs seeds and increases its vigor. The effect of different seed pretreatments on increasing enzyme activiy has been reported by other researchers studying other plants and it has been shown that increasing antioxidant enzymes is associated with increasing in germination indices under stress and the reason for improvement in the germination index in treated seeds has been attributed to the increase in consumption of seed storage materials and the activity of antioxidant enzymes in treated seeds (Ansari et al. 2013).

3.2. Radical and plumule length

The effect of different levels of drought, aging and priming as well as their interactions was significant on RL, but the results obtained from the combined effect of drought and priming on RL were not significantly different (Table 1). The lowest RL (9.24 mm) was observed in drought with an intensity of -1.5 MPa, 75% aging and control priming (Table 2). The highest RL (15.73 mm) was observed in the absence of drought and aging as well as priming with the GR24 hormone (Table 2). The results of this experiment showed that aging and drought stress had a negative effect on RL. The reduction in RL due to aging was more significtant than drought stress. In addition, each priming increased in the RL compared to the control and stress conditions, which had the least effect on hydropriming and the greatest effect on GR24 hormone. The effect of different levels of drought, aging and priming as well as their interactions was significant on PL (Table 1). The results of the mean comparison table (Table 2) showed that the lowest PL (1.19 mm) was observed in drought with an intensity of -1.5 MPa and 75% aging in the absence of priming. The results of the data showed that the effect of drought stress on PL was more than the effect of aging. The highest PL (4.26 mm) was obtained from the absence of drought and aging as well as priming with the GR24 hormone. It seems that the application of water potential and consequent drought stress (-1.5 MPa) reduces water uptake by seeds, which disrupts the process of hormone secretion and the activity of enzymes that affect seed germination and growth and RL eventually decreases. Usually, RL decreases significantly with in the increase in PEG concentration (Almaghrabi 2012). Mortazavi et al. (2008) showed that RL and PL decreased in aging seeds. One way to reduce the adverse effects of oxidative stress is through seed priming (Krishna et al. 2021). The use of certain polyamines and hormonal priming may be associated with increased ability to reduce oxidation. GR24 as a type of strigolactones plays an important role in plant development through various processes (Dun et al. 2009). Strigolactones act as positive regulators in plant response to abiotic stresses such as drought (Ha et al. 2014). One of the effects of Gr24 is to increase soluble sugars, including glucose, as well as to increase the expression of protein kinase regulation (Modi et al. 2017). Protein kinase (Price et al. 2012) and soluble sugars (Hao et al. 2021) have a major effect on the tolerance of different stresses. Therefore, it is possible that Gr24 improved drought stress tolerance in pumpkin seeds by increasing soluble sugars and protein kinase expression.The results of the present study were in line with the findings of Ahmadi et al. (2016) in investigating the effect of seed priming on salinity and drought stress on the growth characteristics of *Dracocephalum* Spp. seedlings. Their results showed that seed ageing and drought stress had a detrimental effect on RL and PL. The reduction in seedling length due to ageing was greater than that of drought stress. The reason for the reduced RL and PL of aged seeds is the decreased quality of storage materials during the period of aging (Mortazavi et al. 2008).

PL is one of the traits that indicates seed vigor. Seeds with low vigor may germinate, but due to reduced PL they cannot emerge, thus reducing the percentage of green establishment in the field. However, short stems have less emergence power due to lower DW compared to long stems (Matthews & KhajeHosseini 2006). The reduction of water uptake by seeds under stress conditions reduces the secretion of hormones and the activity of enzymes and thus impairs seedling growth, including RL and plumule (Masoumi et al. 2010). Zamani et al. (2010) in a study of lipid peroxidation and enzyme activity in aged safflower seeds noted that the relationship between lipid peroxidation and an increase in the leakage of electrolyte solutions due to degradation of cytoplasmic membrane structure reduces RL and PL. Masoumi et al. (2010) found that priming increases RL and PL by increasing the rate of use of seed storage materials. An increase in the PL of aged canola seeds has been reported with priming (Najafi et al. 2016). Therefore, it seems that the increase of such enzymes may be due to the increase in RL and PL of primed seeds.

3.3. Relative water content

The effect of different levels of drought, aging and priming as well as their interactions was significant on RWC (Table 1). The mean values showed that the lowest RWC (35.5%) was observed in drought with an intensity of -1.5 MPa and 75% aging in the absence of priming. The highest RWC (83.64%) was in the absence of drought and aging and in priming with spermidine (Table 2). The reduction of RWC under drought stress has also been reported in wheat (Liheng et al. 2011) and barley (Bandurska et al. 2012). Plants under drought

stress minimize the intercellular space and the amount of water in their organs by increasing the osmotic content within the tissues so that ambient water enters them with greater force. This reduces the RWC under drought stress (Bayoumi et al. 2008). Chadordooz et al. (2015) stated that the reason for the decrease in RWC was the delay in seedling emergence and the reduction of rooting in aged lentil seeds that had been subjected to drought stress. It appears that due to the delay in seedling emergence and reduced root growth in aged seeds, the amount of RWC decreases. Considering that drought indirectly leads to an increase in plant water transpiration therefore, the intensity of its effect on RWC was greater than aging. The RWC in Seedlings of barley (*Hurdeum vulgare*) that were exposed to drought stress, decreased compared to control plants, but exogenously applied spermidine increased in RWC (Kubis, 2003). In rice, the use of spermidine also compensated for the decrease in RWC due to drought stress (Farooq et al. 2009). The results show that priming can help reduce water loss in dry conditions, which may be largely due to the reduction of transpiration and help the root grow more effectively to absorb sufficient water. Since polyamines (spermidine) are involved in the protection of plants against environmental stresses (Bouchereau et al. 1999), they can maintain the required moisture in the plant in stressful environmental conditions.

3.4. Protein content

The effect of different levels of drought, aging and priming as well as their interactions was significant on the rate of protein changes (Table 1). A comparison of the means shows that the lowest protein content (0.82 $mg.g^{-1}Fw$) was measured in drought with an intensity of -1.5 MPa and 75% aging in the absence of priming and the highest content (9.54 $mg.g^{-1}Fw$) was observed in the absence of drought and aging and in priming with gibberellin (Table 2). One of the important biochemical changes that occur as a result of the reduction of water in plants is the change in plant protein production. To break down after stress or interfere with the synthesis (Sharma et al. 2019). A reduction in protein content during aging was also reported by Murthy et al. (2002). In addition, the decrease in primary amino acids due to ROS attack is another reason for the decrease in protein levels during the ageing process (Jacoby et al. 2012). The effect of different seed pretreatments on increasing the activity of enzymes has been reported by other researchers in different seeds (Ansari et al. 2012; Bailly 2004). In other words, seed pretreatment by increasing in the activity of antioxidant enzymes in the seed, causes resistance under stress conditions and leads to an increase in germination index (Ansari et al. 2013). According to the data obtained, it was found that in the first level of drought stress, the content of protein increased and decreased with the increasing severity of drought stress. It seems that seedlings in the first stage to combat stress reduce metabolites such as proteins to reduce osmotic pressure, but as the severity of stress increases, this ability is lost. During seed ageing, due to the high affinity of reactive oxygen species and other aldehydes produced by vital biomolecules such as proteins, they are denatured (Kapoor et al. 2010). For this reason, it seems that with the increase of ageing, the proteolysis by protease enzymes increases and leads to a decrease in its quantity. In addition, an increase in the germination index by gibberellin can be associated with an increase in the activity of enzymes and proteins.

3.5. Carotenoid content

The effect of different levels of drought, aging and priming as well as their interactions was significant on the rate of carotenoid changes (Table 1). A comparison of means shows that the lowest carotenoid content (1.253 $mg.g^{-1}Fw$) was measured in drought with an intensity of -1.5 MPa and 75% aging in the absence of priming and the highest content (6.277 $mg.g^{-1}Fw$) was observed in the absence of drought and aging and in priming with spermidine (Table 2). Drought stress led to a decrease in carotenoids. In addition, placing the seeds in an environment with both high temperature and high humidity reduced the amount of pigments. Moreover, the effect of drought stress in reducing the carotenoid content was greater than that of the aging. Chlorophylls are sensitive to oxidation and light inhibition, while carotenoids play an antioxidant and protective role for chlorophylls (Ramadan & Omran 2005). Similar results were obtained on olives (Ben Ahamed et al. 2007), sugarcane (Suriyan & Chalermpol 2009) and melons (Korkmaz et al. 2007), indicating a decrease in carotenoid content due to drought stress. Seed aging during improper storage disrupts the enzyme system that suppresses reactive oxygen species and leads to damage to the pigments (Masoumi et al. 2010). A reduction in carotenoids during aging have been reported in tomatoes (Najafi et al. 2009) and maize. Compensation for decreases and increases in chlorophyll a, b and carotenoids in aged seeds has been reported by priming in eggplants (Sardoei et al. 2014) and wheat (Shaddad et al. 2013). It has been reported that priming by plant hormones can affect carotenoid synthesis by affecting the genes encoding the biosynthesis pathway of geranyl pyrophosphate (Shaddad et al. 2013). Increasing in carotenoid content during priming can increase resistance to drought stress by maintaining essential photosynthetic activities (Abid et al. 2017). It seems that the activity of ROS following stress causes discoloration or loss of pigments such as chlorophylls and other pigment compounds. Application of polyamine pretreatment prevents damage to the dye by inhibiting lipid oxidation.

3.6. Proline content

The effect of different levels of drought, aging and priming as well as the interaction effects of priming and drought, as well as the interaction effects of drought, aging and priming were significant on proline content (Table 1). The lowest amount of proline (0.55 µmol.g^{-1} Fw) was observed in the control seeds and the highest amount (1.573 µmol.g^{-1} Fw) was observed in drought with an intensity of -1.5 MPa and 75% aging in priming with spermidine (Table 2). Proline is known as a key amino acid in osmotic regulation to increase salinity and drought resistance, the rate of increase of which varies between different varieties (Woodward & Bennet 2005). An increase and accumulation of proline under stress conditions in many plant species is associated with stress resistance and its concentration in stress-resistant plants is greater than that of sensitive plants (Zebarjadi et al. 2010). Increased proline levels under drought stress in primed safflower seeds have also been reported (Ashrafi & Razmjoo 2010). The results of our study are consistent with studies on chickpea cultivars (Alexieva et al. 2001), European borage (Zahed-Chekovary & Gasemov 2015) and wheat (Pireivatlou et al. 2010). In general, proline accumulation is believed to play an adaptive role in plant stress tolerance (Verbruggen & Hermans 2008). It was found that with increasing the rate of seed aging in wheat, proline content increases (Shaaban 2016). The intensity of proline increase resulting from drought stress was greater than seed aging stress. The findings of Ashrafi and Razmjou (2010) show that the proline content increased in safflower vegetative parts in hydro primed seeds under both stress and non-stress conditions. Polyamines can protect membranes and large molecules from oxidative stress and provide biological membrane stability under stress conditions (Farooq et al. 2009). For this reason, it can be concluded that spermidine, as a type of polyamine, facilitates the accumulation of phenolic compounds and free proline to protect against oxidative damage.

3.7. Catalase activity

The effect of different levels of drought, aging and priming as well as their interactions was significant on CAT activity (Table 3). The lowest CAT activity (7.03 µmol.g min^{-1}.mg protein^{-1}) observed in drought with an intensity of -1.5 MPa and 75% aging in the absence of priming. The highest rate (40.44 µmol.g min^{-1}.mg protein^{-1}) was achieved in drought with an intensity of -7.5 MPa in the conditions of non-aged and in priming with spermidine (Table 4). The mean comparison Table 4 indicates that the activity of CAT decreased in aging seed. However, with the application of drought, an increase in the activity of this antioxidant was observed and its activity decreased with the intensification of drought stress. An increase in CAT has been observed in olive leaves under water stress (Sofo et al. 2008). Plants increase in the amount of antioxidant enzymes during drought stress to reduce damages from reactive oxygen species (Farooq et al. 2009). Jyoti and Malik (2013) reported a decrease in CAT during the seed aging process. Researchers have also shown that priming can compensate damages through the formation of catalase subunits in the cytoplasm and complete its synthesis and reduce the severity of oxidative stress, in part by improving the activity of antioxidant enzymes (Xia et al. 2015). Catalase enzyme is one of the most important components of the antioxidant system, which increases in drought stress conditions. However, by using the seed priming technique, the quantity of this enzyme can be increased in plants under stress (Khan et al. 2020). Increased activity of the catalase enzyme with the help of polyamines under water stress has been reported by Amraiyatbar et al. (2016). Polyamines, as signaling molecules, are likely to trigger a chain of defense reactions that result in the increased activity of antioxidant enzymes (Toumi

Table 3- Analysis of variance for the effect of water potential and seed deterioration on some physiological and biochemical traits of primed pumpkin seeds

SOV	*Df*	*MS*				*F Value*				*Pr > F*
		CAT	*POX*	*SOD*	*MDA*	*CAT*	*POX*	*SOD*	*MDA*	
D	2	4883.1**	3562.9**	9719.8**	2119.9**	65906	72574	70456	47204	<.0001
P	5	123.33**	658.8**	734.8**	61.62**	1664	13419	5326	1372	<.0001
A	2	550.45**	5329.3**	21580.4**	2092.8**	7429	108555	156430	46601	<.0001
D*P	10	23**	13.48**	1.72**	1.16**	310	274	12	25	<.0001
D*A	4	9.95**	22.52**	45.22**	62.9**	134	458	327	1401	<.0001
P*A	10	0.49**	3.4**	2.15**	13.84**	6	70	15	308	<.0001
D*P*A	20	1.48**	2.7**	2.9**	1.05**	20	55	20	23	<.0001
Error	108	0.07	0.04	0.13	0.04					
%CV		1.13	0.29	0.39	1.5					

*, **, nsStatistically significant at p≤0.05, p≤0.01, respectively and non-significant.
SOV: Source of variation, Df: Degrees of freedom, MS: Mean square, D: water potential; P: Priming, A: Ageing, CAT: Catalase, POX: Peroxidase, SOD: Superoxide dismutase, MDA: Malone dialdehyde

et al. 2010). Seed antioxidant enzymes may increase in the first days of aging, but as the aging process increases, they lose their ability to defend and their quantity decreases (Amirjani 2010). Therefore, its presence as a pre-treatment agent can be a reason to increase the amount of catalase in these conditions.

3.8. Peroxidase activity

The effect of different levels of drought, aging and priming as well as their interactions were significant in POX activity (Table 3). According to the mean comparison table, the lowest POX activity (47.72 µmol.g min^{-1}.mg $protein^{-1}$) observed in drought with an intensity of -1.5 MPa and 75% aging in the absence of priming and the highest rate (95.71 µmol.g min^{-1}.mg $protein^{-1}$) was obtained from the absence of drought and aging in seed priming by spermidine (Table 4). Drought stress and aging led to the reduction of POX and the intensity of aging was greater than that of drought. Decreased peroxidase activity under drought stress has been shown in other studies (Csiszár et al. 2012). Murthy et al. (2002) showed that by increasing storage time and seed aging, the amount of MDA in the seed increases and in contrast, a decrease in the activity of catalase, glutathione reductase and ascorbate peroxidase is also observed, which has a positive correlation with seed vigor. Decreased activity of antioxidant enzymes in aged seeds has been reported due to an increase in free radicals in corn (Siadat et al. 2012) and rye (Ansari & Sharifzadeh 2012). TavakolAfshari et al. (2009) also reported in their study that the activity of peroxidase enzyme in canola seedlings is reduced due to seed aging. Decreased activity of antioxidant enzymes due to seed aging will ultimately reduce protein production for various reasons such as damage to RNA synthesis. The addition of reduced sugars to proteins, which is non-enzymatic and is known as the Maillard reaction, also inactivates antioxidant enzymes under seed aging conditions (Murthy et al. 2003). The application of polyamine in rice under drought conditions activated hydrogen peroxide purification enzymes, thereby increasing water tolerance while preventing protein degradation and membrane peroxidation (Farooq et al. 2009). In spermidine-treated barley grains, the activity of catalase and guaiacol peroxidase enzymes increased in under dehydration, and thus polyamines were able to activate H_2O_2-purifying enzymes and tolerance to water depletion (Kubis 2003). Increased in peroxidase activity during drought stress is associated with plant tolerance to dehydration (Xiong et al. 2002) and due to the sensitivity of pumpkin to drought stress, the increase of peroxidase enzyme can be justified.

Table 4- Means of antioxidant enzyme and malondialdehyde content of primed Pumpkin seeds affected by water potential and aging

Drought	*Priming*	*Ageing*	*CAT (µmol.min^{-1}.mg^{-1}protein)*	*POX (µmol.min^{-1}.mg^{-1}protein)*	*SOD (µmol.min^{-1}.mg^{-1}protein)*	*MDA (µmol.min^{-1}.mg^{-1}protein)*
Non-D	**Non-P**	0	26.6 g	85.17 h	118.67 f	4.4 m
		85%	24 k	78.9 k	103.12 j	9.31 g
		75%	21.51 m	66.13 p	76.37 p	15.71 a
	Hydro-P	0	28.46 d	89.35 d	122.4 d	4.31 m
		85%	26.64 g	83.19 i	105.2 i	8.44 i
		75%	24.7 j	71.3 n	81.8 n	13.9 c
	GA3	0	30.7 b	92.02 c	125.6 c	4.24 m
		85%	27.3 f	85.7 g	108.8 h	7.85 j
		75%	25.19 ij	73.93 m	85.62 m	12.56 d
	GR24	0	27.7 e	88.06 e	121.4 e	4.41 m
		85%	25.67 h	81.8 j	103.53 j	8.75 h
		75%	23.1 l	69.36 o	79.63 o	14.3 b
	BAP	0	29.02 c	93.2 b	128.1 b	3.94 n
		85%	25.34 hi	87.19 f	113.21 g	5.53 k
		75%	22.95 l	76.2 l	89.5 l	10.78 e
	SP	0	32.26 a	95.71 a	131.6 a	3.75 n
		85%	29.37 c	88.01 e	119.14 f	4.93 l
		75%	27.09 fg	76.34 l	92.18 k	9.95 f

Table 4. Continued

Drought	*Priming*	*Ageing*	*CAT (µmol.min^{-1}.mg^{-1}protein)*	*POX (µmol.min^{-1}.mg^{-1}protein)*	*SOD (µmol.min^{-1}.mg^{-1}protein)*	*MDA (µmol.min^{-1}.mg^{-1}protein)*
-0.75 MP	**Non-P**	0	29.3 hi	81.18 h	110.8 f	7.73 m
		85%	25.48 l	73.06 m	93.2 l	16.57 g
		75%	23.68 m	61.38 q	72.2 r	23.13 a
	Hydro-P	0	34.5 d	85.05 f	113.67 d	7.72 m
		85%	31.23 g	79.28 i	97.67 j	14.42 i
		75%	28.27 j	67.48 q	76.28 p	20.53 c
	GA3	0	35.07 d	88.95 d	115.85 c	7.59 m
		85%	33.18 f	81.26 h	100.7 i	12.03 j
		75%	31.46 g	70.23 o	79.83 o	19.25 d
	GR24	0	32.9 f	83.36 g	112.7 e	7.64 m
		85%	29.8 h	76.47 k	95.84 k	15.58 h
		75%	26.06 k	64.6 p	74.35 q	21.1 b
	BAP	0	36.73 c	92.46 b	119.03 b	7.54 m
		85%	32.65 f	86.4 e	104.24 h	10.22 k
		75%	28.93 i	74.05 k	83.74 n	18.46 e
	SP	0	40.44 a	93.95 a	121.89 a	7.55 m
		85%	37.99 b	89.96 c	107.08 g	8.64 l
		75%	33.8 e	77.57 j	87.21 m	17.44 f
-1.5 MP	**Non-P**	0	16.17 d	72.4 h	94.31 f	13.23 m
		85%	11.95 h	61.18 m	75.5 l	21.71 g
		75%	7.03 m	47.72 r	53.4 q	32.42 a
	Hydro-P	0	17.42 c	75.5 e	97.59 d	13.33 m
		85%	12.74 g	67.37 j	78.9 j	21.09 h
		75%	8.75 l	53.84 p	55.2 p	31.26 b
	GA3	0	17.56 c	76.06 d	101.06 c	13.33 m
		85%	12.83 g	71.5 i	82.5 i	18.63 j
		75%	9.24 k	55.3 o	57.7 o	28.22 d
	GR24	0	17.42 c	73.1 g	95.7 e	13.34 m
		85%	12.88 g	63.9 k	76.3 k	20.32 i
		75%	10.33 j	50.17 q	54.6 p	30.37 c
	BAP	0	17.98 b	80.01 b	104.8 b	13.32 m
		85%	13.34 f	74.66 f	85.27 h	16.32 l
		75%	10.87 i	58.02 n	61.8 n	27.22 e
	SP	0	18.36 a	83.04 a	107.7 a	13.37 m
		85%	13.7 e	78.4 c	88.62 g	17.76 k
		75%	11.12 i	63.15 l	64.6 m	26.6 f
LSD			0.34	0.25	0.49	0.23

Non-P: non-Priming, Hydro-P: Hydro priming, GA3: Gibberellin, BAP: Benzyl amino purine, SP: Spermidine, CAT: Catalase, POX: Peroxidase, SOD: Superoxide dismutase, MDA: Malone dialdehyde, LSD: Least significant difference

3.9. Superoxide dismutase activity

The effect of different levels of drought, aging and priming as well as their interactions were significant on SOD activity (Table 3). The lowest activity of SOD (53.48 µmol.g min^{-1}.mg protein^{-1}) was measured in drought with an intensity of -1.5 MPa and 75% aging in the absence of priming and the highest amount (131.68 µmol.g min^{-1}.mg protein^{-1}) was obtained from the absence of drought and aging with seed priming by spermidine (Table 4). Drought stress and seed deterioration decreased the activity of SOD enzyme. Decreased

SOD activity under drought stress has also been reported in pumpkins (Yasar et al. 2014). Cakmak et al. (2010) observed that in the long-term aging of alfalfa seeds, the amount of hydrogen peroxide and the activity of CAT and SOD enzymes decreased, citing a decrease in the germination capacity of aged alfalfa seeds. Mehravar et al. (2014) also noted the decrease in SOD activity due to seed aging. Polyamines can increase in stress resistance by stimulating the activity of various antioxidant systems (Yiu et al. 2009). The maximum activity of SOD enzyme in rice was obtained through the use of priming under drought stress (Farooq et al. 2010). According to Kubi (2005), the antioxidant system, can be changed with the help of polyamines, this system is able to modify the system of radical correction under oxidative stress. In addition, an increase in the amount of superoxidase dismutase and peroxidase due to priming in aged onion seeds has been proven (Yalamalle et al. 2019). Priming enhances antioxidant enzymes, a process that appears to occur through the optimization of defense mechanisms during germination. Seed priming reduces the activity of antioxidant enzymes. This has been proven in experiments on sesame seeds (Somasundaram et al. 2009).

3.10. Malone dialdehyde content

The effects of different levels of drought, aging and priming as well as their interactions were significant on the amount of MDA (Table 3). The highest amount of MDA (32.42 mmol. g^{-1}Fw) was observed in drought with an intensity of -1.5 MPa and 75% aging in the absence of priming and the lowest amount of MDA (3.75 mmol. g^{-1}Fw) was obtained from the absence of drought and aging in priming with spermidine (Table 4). The effect of drought on lipid peroxidation and increase in MDA, which is one of the products of lipid peroxidation in *Populus euphratica* has been reported (Bogeat-Triboulot et al. 2007). In drought-sensitive species, the MDA content is much higher than in resistant species (Bogeat-Triboulot et al. 2007), which is an internal physiological regulation in response to environmental stimuli (Salazar-Parra et al. 2012). MDA is an indicator of cell membrane peroxidation and is positively correlated with the leakage of electrolytes from the membrane. The higher the value of this index, the greater the peroxidation of the membrane and the leakage of electrolytes from the membrane, resulting in more potential damage (Jyoti & Malik 2013). MDA is the product of linoleic acid peroxidation and has the ability to damage membrane proteins through cross-linking (Varier et al. 2010). Ghahremani et al. (2017) in their study of pumpkin seeds found that with increasing temperature and aging, the number of free radicals increases and the accumulation of these harmful compounds leads to the lipid peroxidation of cell membranes and organelles, which increased the amount of MDA. Lipid peroxidation and cell membrane damage are reduced during stress by seed priming (Meng et al. 2014). The reduction of lipid peroxidation through a reduction of MDA by GR24 has also been reported in stress conditions (Ma et al. 2017). Li et al. (2015) reported that resistance to oxidants in spermidine-treated plants resulted in a lower production of O^{-}_{2}, H_2O_2, and MDA content to improve cell membrane stability. Bakheet et al. (2017) in a study on flax seeds aging concluded that priming can reduce the production of MDA by reducing the rate of peroxidation through increasing the activity of antioxidant enzymes. In general, it can be said that MDA is an indicator of the degree of damage caused by various oxidants during stress. In addition, it may be that spermidine can impact upon the ability of the activities of inhibitory systems to affect the severity of oxidative stress.

4. Conclusions

Pumpkin is classified as a plant sensitive to drought stress, however, its oily seeds are more sensitive to seed ageing conditions. In this study, germination characteristics were affected by drought stress more than seed deterioration. In the case of morphological characteristics, the decrease in burnout stress exceeded that of drought stress. While the reduction of morphological characteristics, due to seed deterioration was greater than that of drought stress. The activity of antioxidant enzymes under drought stress increased and decreased in aging seed. The carotenoid content decreased during stress and the decrease due to drought stress was more noticeable than seed ageing. Accumulated MDA and proline content make seedlings more resistant to stress, the effect of seed deterioration on these compounds was greater than that of drought stress. In general, in the case of seed pretreatment, gibberellin had the greatest effect on germination traits. Seedlings obtained from the Gr24 hormone priming had significant differences in weight and size with other treatments. In addition, seed pretreatment with spermidine reduced all the negative effects of stress, particularly that of drought stress.

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Amino Acid Content and Effect of Different Preservation Methods on Some Biochemical Properties in Black Myrtus communis L. Fruits

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ABSTRACT

In this study, fruits of black *Myrtus communis* L. were subjected to different preservation methods [frozen, sun and microwave (MW) dried] and, its biochemical properties were examined. All measurements were triplicated. It was observed that the vitamin levels decreased in sun and MW dried samples ($p<0.05$). On the other hand, total phenolic compounds, flavonoids and antioxidant capacity of fresh and frozen *M. communis* L. fruits were found to be higher in sun and MW dried fruits ($p<0.05$). The amount of oxidized glutathione and malondialdehyde (MDA) increased while the amount of ghrelin and reduced glutathione decreased in the dried fruits ($p<0.05$). Obtained results indicate that, it is rich in terms of vitamins, amino acids, and some elements. These results suggest that the black *M. communis* L. is a balanced source of amino acids in terms of the total essential amino acid/total amino acid ratio. Experimental findings show that the most suitable preservation method for *M. communis* L. fruits is freezing. In addition, MW drying seems more advantageous than sun drying in terms of vitamin loss and time.

Keywords: *Myrtus communis* L., food preservation, phenolic substance, antioxidant capacity, elements, HPLC

1. Introduction

The use of plants for human health is a well-established tradition and medicinal plants are in the center of interest around the world. *M. communis* L., commonly known as "myrtus", are grown as mainly black and white types. While white fruits of *M. communis* L. are generally consumed fresh, on the other hand the black fruits are consumed as dried. The myrtus fruits have been reported to have a higher level of vitamins, phenolic compounds, and antioxidant (Çakmak et al. 2021). The leaves and fruits of the plants are also used for curing constipation, haemorrhoids, gum infections, urinary tract infections, and chest diseases (Fadda & Mulas 2010). Generally, colored fruits have higher phenolic compounds therefore they might have higher antioxidant capacity led to health benefits (Karadeniz et al. 2005). The black myrtus fruits have no commercial values, but in recent years it drew attention because of health benefit due to the higher antioxidant capacity.

Vitamins are the nutrients that required for many biochemical functions, must be taken alongside diet. Plants are the main sources of vitamins and deficiencies of vitamins can cause various diseases (Asensi-Fabado & Munne-Bosch 2010). Lycopene is an essential carotenoid found in fruits and vegetables such as tomatoes, watermelon, and grapefruit (Yapaing et al. 2002). Elements selected in the study have functions such as antioxidants, cofactor of enzyme, stabilizers of cell membranes, structural components of metallo-enzymes and metallo-proteins, and protection against toxicity (Soetan et al. 2010). Amino acids are involved in neurotransmitter and biosynthesis processes in biological systems. Dietary supply of essential amino acids is necessary for protein synthesis, so it is important to determine the amount of amino acids in foods (Garlick 2004; Davidson 2019).

M. communis L. is a seasonal fruit, to be able to consume it all year around different preservation methods applied. Preservation techniques have an important effect on the nutritional value and medicinal benefits of the fruits. Although sun drying is widely used natural drying technique, different drying methods such as drying in ovens, drying tunnels and vacuum as well microwave (MW) drying which is a relatively new technique also be used for many foodstuffs (Ahmed et al. 2013). Temperature, time, light intensity, and humidity's are important factors on the nutritional content of fruits (Maisnam et al. 2016).

Since the black *M. communis* L. is a seasonal fruit, to consume all year around, various preservation procedures are being applied. Vitamin content of fruits depends on many factors. The importance of these factors is genetic and ecological including the manner in which the fruit is collected, the preservation methods and the shelf life. It is reported that some biochemical contents of fruits change depending on different preservation methods (Kamiloglu et al. 2015). To our knowledge, the study here is the first to investigate the biochemical content of black myrtus fruit to this extent in relation to preservation methods.

The study is based on the investigation of effect of different preservation methods (sun or MW drying and freezing) on the vitamins (A, B, C, E), carotenoids (β-carotene and lycopene), functional peptides (glutathione, ghrelin), oxidative stress markers [oxidized glutathione (GSSG) and malondialdehyde (MDA)], total phenolics and flavonoids, antioxidant capacity [α-diphenyl-β-picrylhydrazyl (DPPH), trolox equivalent antioxidant capacity (TEAC)]. In addition, some elements such as Se, Cu, Fe, Mn, Zn and amino acids also determined.

2. Material and Methods

Ripe black *M. communis* L. fruits were harvested from Osmaniye, Turkey (37.269020 N, 36.120391 E), in December 2019. The fresh fruit samples were analysed after the fruits were collected. During the study, 2.5 kg of samples were processed beforehand for each treatment. Dried samples were stored in desiccator. Frozen (-20 °C) samples were analysed in ten days after drying. 20.0 gram of each homogenized by the blender and used throughout the analysis. All measurements were triplicated.

2.1. MW and sun drying

The fresh fruit samples were dried either in MW or under the sun. For the MW drying, each portion of sample exposed to MW radiation 6 times for 5 min at full power (800 watts), while in sun-drying they were kept in a well-ventilated indoor area under sunlight for 5 days until 60% weight loss of the total weight in both cases.

2.2. Determination of vitamins A, E, β-carotene, and lycopene

1.0 g of homogenized *M. communis* L. fruit samples were introduced to a tube then, 5.0 mL C_2H_5OH was added, and the sample was sonicated for 10 minutes then vortexed and centrifuged for 6 minutes at 7,500 rpm. After that, 1.0 mL n-hexane was added to the sample and vortexed followed by the extraction of n-hexane phase and this process was repeated twice. The collected n-hexane phases were dried under vacuum, the residue was dissolved in 1.0 mL of methanol and analysed using a Supelcosil LC-18 column (25.0 cm x 4.6 mm x 5.0 µm), methanol: acetonitrile (ACN): water (63:33:4.0 v/v) as mobile phase (Mukhtar et al. 2019). Vitamin A is the sum of retinol and retinoic acid. A sample chromatogram is given in Figure 1 for the fat-soluble compounds.

2.3. Determination of B vitamins

The filtrate obtained in material section was used for the analysis of vitamins B, utilizing a Supelcosil LC-18-DB column (150 mm x 4.6 mm ID, 5 µm). 5.0 mM sodium heptanesulfonate: 0.1% TEA at 25:75 (v/v) and pH adjusted pH 2.8 by H_3PO_4 used as the mobile phase (Amidžić et al. 2005).

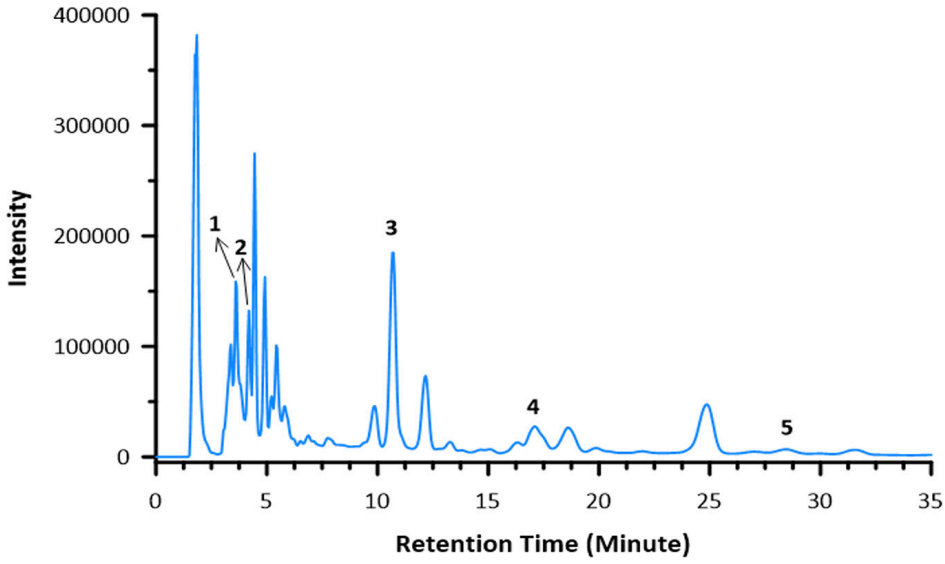

Figure 1- Sample chromatogram for fat soluble vitamins, (1: Retinol, 2: Retinoic acid, 3: Vitamin E, 4: Lycopene, 5: β-carotene)

2.4. Determination of Vitamin C, ghrelin, glutathione and MDA

Vitamin C, ghrelin, glutathione and MDA were determined, by using high performance liquid chromatography (HPLC) (Mukhtar et al. 2019; Dogan et al. 2016).

2.5. Extraction of M. communis L. fruit

The *M. communis* L. fruits samples, were subjected to different pre-treatments, were homogenized then 7.50 grams of sample extracted with 150 mL methanol in Soxhlet apparatus for 4 hours. Then methanol was removed by the rotary evaporator at 40 °C at a reduced pressure. The extract was dried, weighed dissolved in 75 mL of CH_3OH and the solution was stored in the fridge at 4 °C until analysis (Wang & Weller 2006)

2.6. Determination of total phenolic content

Total phenolic substance was determined spectrophotometrically according to Folin-Ciocalteu method modified by Dewanto et al. (2002). 0.50 mL of distilled water, 0.250 mL of sample or gallic acid and 0.125 mL of Folin-Ciocalteu reagent were mixed and shaken. After 6 minutes, 1.250 mL of 7% sodium carbonate solution was added and the total volume completed to 3.00 mL with distilled water. After 90 minutes, the absorption was measured at 760 nm by a ultraviolet-visible spectrophotometer. A working graph of gallic acid solutions prepared at different concentrations was established. The total phenolic content of the samples was determined, and the results were given as µg gallic acid per g dry weight sample (µg GAE/g dw).

2.7. Determination of total flavonoids

The total flavonoid substance was determined spectrophotometrically as described by Dewanto et al. (2002). 0.025 mL sample or quercetin, 1,250 mL distilled water and 0.075 mL 5% sodium nitrite solution, 0.150 mL 10% solution of aluminium chloride were mixed in a glass tube and allowed to stand for 5 minutes then 0.500 mL 1.0 M sodium hydroxide solution was added and total volume was completed to 2,500 mL with distilled water followed by measurement of absorbance at 510 nm. A working graph was formed with quercetin solutions prepared in different concentrations. The total phenolic content of the samples was determined using the working graph and the results were given as µg QE/g dw.

2.8. Total antioxidant capacity

Total antioxidant capacity was determined according to two different methods, DPPH and TEAC.

2.9. DPPH method

The antioxidant capacity was measured according to the method based on the scavenging activities of the stable DPPH free radical as described by Nile et al. (2013). A solution of 25 µg ml^{-1} DPPH in methyl alcohol was prepared, and the absorption of DPPH solution was measured at 510 nm. Then different amount of the sample extracts was added to DPPH solution and kept in dark for 30 minutes before measurement of absorbance at 510 nm. Results were given as IC_{50} (µg/mL), which indicates the concentration of the antioxidant substance that inhibits 50% of the DPPH radical in the medium. Low IC_{50} values indicate high antioxidant activity.

2.10. TEAC method

The ABTS free radical-scavenging activity was determined according to the method described by Re et al. (1999). The stock solutions including 7.0 mM ABTS solution and 2.4 mM potassium persulfate allowed to stand in the dark at room temperature for 12-16 h. The ABTS•+ solution was diluted with phosphate buffer (pH=7.4) to obtain an absorbance of 0.800±0.010 at 734 nm. Then 20 µL of the sample or Trolox standard was added to 2.0 mL ABTS•+ solution and allowed to stand at room temperature for 15 minutes then absorption was measured at 734 nm. Previously prepared ABTS•+ solution used as the control group. The antioxidant capacity of the sample was calculated as Trolox equivalent as µmol Trolox/g dw.

2.11. Analysis of selenium

20.0 grams of fresh *M. communis* L. fruit was homogenized and 2.5 g was transferred to a Teflon bomb then 6.0 mL of HNO_3: $HClO_4$ mixture (1: 4, v/v) was added and kept at 100 °C for 12 hours. After that, 2.0 mL of concentrated H_2O_2 was added, allowed to stand at room temperature for 24 hours. Mixture was transferred into tubes and a 4.0 N HCl concentration was achieved by adding concentrated HCl. The mixture was held at 90 °C for 15 min to reduce Se(VI) to Se(IV). To this mixture, 2 mL 2.5 M formic acid, 4 mL 0.1 M EDTA and 1.5 mL freshly prepared 3,3-diaminobenzidine solution were added and the pH was adjusted to 1.7 with the addition of 4 M NH_3. This was left to stand in the dark for 1.5 h for the formation of a metal-ligand complex. Selenium was analysed fluorimetrically according to the method of Dogan et al (2016).

2.12. Analysis of Cu, Fe, Mn and Zn

3.0 g of homogenized *M. communis* L. fruit sample was taken and 5.0 mL of $HCLO_4$ and HNO_3 mixture (1:4 v/v) was added, vortexed and sonicated for 30 minutes then left to stand for 24 hours followed by the addition of 2.0 mL H_2O_2. Final volume was completed to 25 mL with 1.0% triton-X 100 solution then the metal contents were determined by a flame AAS (Tüzen 2003).

2.13. Determination of amino acids

Hydrolysis: Approximately 2.0 grams of ground fresh fruit samples were taken into a glass tube and 5.0 mL 6.0 N HCl was added and vortexed thoroughly then, samples were kept at 110 °C for 24 hours to break peptide bonds (Elkin & Wasynczuk 1987). After that the samples cooled to room temperature, filtered and the filtrate volume was completed to 25 mL with distilled water.

Derivatization: Standard amino acid solutions were prepared using 0.10 N HCl at different concentrations between 1.0 to 5.0 µg/mL. Fifty µL standard amino acid solutions or hydrolysed fruit samples transferred to 5.0 mL glass tubes and dried under vacuum at 65 °C. Then 50 µL of "reagent 1" solution [(2: 2: 1 mixture of ethanol: water: Triethylamine (TEA) (v/v)] was added and vortexed and dried again under vacuum at 65 °C. The dried samples were vortexed by adding 50 µL of "reagent 2" solution [7: 1: 1: 1 mixture of ethanol: water: TEA: phenyl isothiocyanate (v/v)] and left at room temperature in the dark for 30 minutes for complex formation. At the end of this period, the samples were dried again under vacuum at 35 °C (Kwanyuen & Burton 2010). 1.0 mL mobile phase A and ACN mixture (8: 2 v/v) was added to each dried sample, vortexed and the samples were taken into HPLC vials for analysis.

2.14. Chromatographic procedure for amino acid analysis

Amino acid analysis was performed by HPLC by modifying Elkin and Wasynczuk (1987) with Kwanyuen and Burton (2010) methods. Nucleodur 100-5 C18 column (250x4.6 mm, 5 µm) was used. The analyses were carried out by applying the gradient program at 40 °C. The mobile phase consists of eluent A and eluent B mixture with a flow rate of 0.8 mL/min and measured at 254 nm (Table 1). Eluent A is 0.07 M $CH_3COONa \cdot 3H_2O$ (pH=6.4 with CH_3COOH) and eluent B is a mixture of ACN and water (60:40 v/v)

Table 1- Gradient program used for the separation of Phenylthiocarbamyl-amino acids

Time (minute)	*Flow rate (mL/min)*	*% Eluent A*	*% Eluent B*
0.01	0.8	90	10
12.00	0.8	70	30
16.00	0.8	65	35
16.01	0.8	50	50
25.00	0.8	100	0
30.00	0.8	50	50
30.01	0.8	10	90
35.00	0.8	10	902

2.15. Equipment and chemicals

Experiments were carried out by SHIMADZU HPLC, Prominence-I LC- 2030C 3D Model equipped with PDA detector, Perkin-Elmer, LS 55 fluorescence spectrophotometer and Perkin-Elmer flame atomic absorption spectrophotometer (AAnalyst-400), Sonicator (Wise Clean, WUC-AO3H, 170 W), Blender (Fakir Hausgrate 220 W), and Vestel brand MW (M.D-60x30, with 800-W power). Double distilled (H_2O) water was used throughout the work. All the chemical used are reagent or analytical grade and obtained from Merck or Sigma-Aldrich.

2.16. Statistical analysis

All measurements were triplicated and mean ± standard error was determined. The results were subjected to One-Way ANOVA by SPSS 10.0 for Windows. Differences between the group's means were analyzed for significance using Turkeys HSD test. The level of statistical significance was expressed as $p<0.05$. Insignificant change was indicated as $p>0.05$. Statistical difference indicated in table and figures with the different letter while the same letter indicates no statistical difference.

3. Results and Discussion

The amounts of vitamins, lyco *communis* pene ghrelin, stress biomarkers, total phenolic and flavonoids substance with total antioxidant capacity, selected elements and amino acids were measured in black *M. communis* L. fruits. The results are given in Table 2 and Figures 2-6.

Experimental results indicate that, there is no significant difference in the amount of vitamins and lycopene in fresh and frozen *M. communis* L. fruits ($p>0.05$). The lowest amounts of vitamins and lycopene lost were found in frozen fruit samples, while the highest lost found in sun-dried samples. Vitamin loss of black *M. communis* L. fruits dried in the sun and MW, ranged from 30 to 51 percent, (Table 2 and Figure 2).

The deterioration of vitamins is particularly dependent on the process condition such as temperature, presence of oxygen, light and time. Photochemical and enzymatic reactions can cause vitamins to decompose during sun drying process (Marszałek et al. 2015). Vitamin loss of sun-dried *M. communis* L. fruit samples are higher than MW-dried samples due to the longer exposure to sunlight during drying process. This can be explained by higher energy of sunlight to break down the vitamins and longer exposure time to dry (Sheraz et al. 2014). Because of shorter process time required for MW drying, lesser extend of vitamin loss is obtained and therefore it can be said that MW drying have an advantage over sun-drying process.

Table 2. The biochemical properties examined in wild black M. communis L.

Biochemical properties	*Fresh*	*Frozen*	*Sun-dried*	*Microwave-dried*	*MSE*
Vitamin A (µg/g dw)	2.15±0.06^{a}	1.92±0.07^{b}	1.10±0.05^{c}	1.18±0.05^{c}	0.015
Vitamin E (µg/g dw)	186.96±7.05^{a}	180.21±7.66^{a}	95.17±3.77^{b}	102.25±4.41^{b}	145.7
β-Carotene (µg/g dw)	7.00±0.12^{a}	6.70±0.11^{a}	4.16±0.18^{b}	4.87±0.15^{b}	0.089
Lycopene (µg/g dw)	9.34±0.31^{a}	8.95±0.26^{a}	5.92±0.19^{b}	6.42±0.25^{b}	0.292
Vitamin B_1 (µg/g dw)	78.72±3.03^{a}	73.59±2.47^{a}	48.91±1.97^{b}	54.59±1.92^{b}	25.67
Vitamin B_2 (µg/g dw)	118.20±5.52^{a}	112.00±4.39^{a}	60.14±2.38^{b}	63.48±3.26^{b}	74.22
Vitamin B_3 (µg/g dw)	344.27±14.60^{a}	336.81±10.84^{a}	176.16±8.54^{b}	188.71±7.24^{b}	499.2
Vitamin B_6 (µg/g dw)	40.80±1.36^{a}	36.42±1.45^{a}	26.82±0.98^{b}	27.00±1.23^{b}	7.231
Vitamin B_9 (µg/g dw)	5190.0±55.2^{a}	5125.0±29.2^{a}	2550.0±9.0^{b}	2568.0±7.1^{b}	4534
Vitamin B_{12} (µg/g dw)	71.39±2.05^{a}	69.61±1.65^{a}	36.21±1.27^{b}	38.69±2.14^{b}	14.8
Vitamin C (µg/g dw)	1323.0±31.9^{a}	1278.0±26.2^{a}	669.0±14.6^{b}	697.0±11.7^{b}	2310
Ghrelin (µg/g dw)	34.40±1.22^{a}	31.4±1.65^{a}	24.90±0.87^{b}	26.30±1.10^{b}	6.95
GSH (µg/g dw)	806.10±8.83^{a}	815.50±7.69^{a}	436.70±17.25^{b}	440.40±21.30^{b}	999.1
GSSG (µg/g dw)	190.10±6.27^{a}	196.70±6.59^{a}	232.70±5.26^{b}	216.20±5.90^{a,b}	163.3
MDA (µg/g dw)	5.32±0.23^{a}	6.00±0.28^{a}	6.80±0.20^{a,b}	7.00±0.14^{b}	0.210
Total phenolic substance (µg GAE/g dw)	43.01±1.97^{a}	40.94±1.73^{a}	38.63±1.36^{a}	39.94±1.71^{a}	39.17
Flavonoid (µg QE/g dw)	23.42±0.90^{a}	22.94±0.79^{a}	20.0±0.87^{a,b}	18.68±0.83^{b}	9.64
IC_{50} (µg/mL)	28.49±1.21^{a}	29.76±1.45^{a}	35.29±1.79^{a,b}	33.65±1.59^{b}	10.45
TEAC (µmol Trolox/g dw)	271.34±8.79^{a}	252.32±7.58^{b}	231.07±6.49^{c}	251.30±7.62^{b}	264.2
Selenium (µg/g dw)	0.63±0.01				
Zinc (µg/g dw)	268.71±9.13				
Iron (µg/g dw)	156.36±7.37				
Copper (µg/g dw)	46.55±1.94				
Manganese (µg/g dw)	29.76±0.95				

The letters in the table were used to compare the treatments in the rows at the 5% significance level for each characteristic examined, MSE: Mean square of error

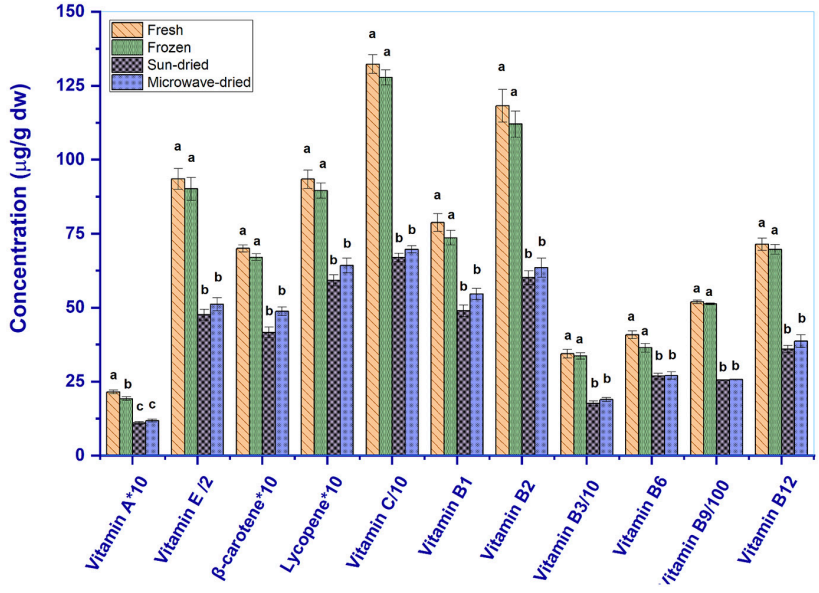

Figure 2- Fat and water soluble vitamins and lycopene content in black *M. communis* L. fruits (Vitamin A, β-carotene and lycopene values multiplied by 10, vitamin E values divided by 2, vitamin C and vitamin B_3 divided by 10, vitamin B_9 divided by 100)

It was reported that the amounts of vitamin B_1, vitamin B_2, vitamin B_3, vitamin B_6, and vitamin B_9 in some fruit and vegetables (carrot, brinjal, okra, spinach, banana, and guava) were found between 0.2-1.8, 0.16-2.0, 0.1-1.0, 0.6-2.8 and 0.16-1.9 µg/g, respectively (Ismail et al. 2013).

The results obtained showed that vitamin content in black myrtus fruits (Table 2 and Figure 2) were higher than *Opuntia ficus-indica* fruits Bakar et al. (2020), monkey apple Onivogui et al. (2014), sweet cherry and sour cherry Ferretti et al. (2010), avocado and apricot Płonka.

It has been reported that ghrelin hormone contributes to antioxidant defence in blood and brain (Omrani et al. 2015). Once it has been considered as an animal origin, the ghrelin hormone reported to be found in fruits as well Aydin et al. (2006). Experimental results show that; the least ghrelin was found in sun-dried samples, on the other hand the highest found in fresh fruit (Table 2 and Figure 3).

The amount of ghrelin in the fruits of *Crataegus laevigata* were reported to be in the range of 18.96±6.73 to 79.96±12.14 µg/g (Mukhtar et al. 2019).

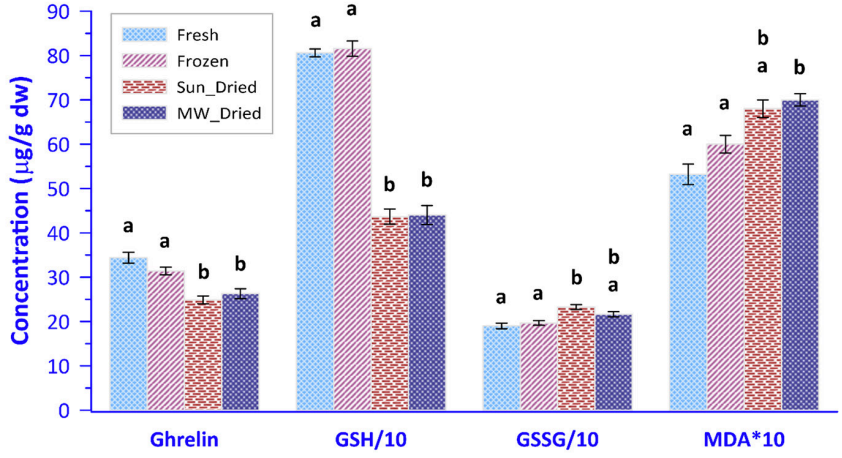

Figure 3- Ghrelin, GSH, GSSG and MDA content in black *M. communis* L fruits (GSH and GSSG values divided by 10, MDA values multiplied by 10)

Glutathione is required for the immune system of cells. Reduced glutathione is an important intracellular antioxidant molecule that is effective for transporting amino acids in metabolism and reducing sulfhydryl groups in proteins (Esterbauer et al. 1992). As seen from Table 2 and Figure 3, the amount of GSH in fresh black *M. communis* L. fruit was found to be 806.10±15.28 µg/g dw. Çakmak et al. (2021), reported that the GSH in wild white myrtus was found to be 609.90±25.80 µg/g dw. Agoreyo et al. (2017) reported that the level of glutathione in Musa *paradisiaca* L. (plantain) had 54.10±0.60 µg/g.

GSSG is an indicator of oxidative stress, the increase in the amount of GSSG inhibits protein synthesis in cells (Cnubben et al. 2001). The amount of GSSG in fresh black *M. communis* L. fruit was found to be 190.10±10.84 µg/g dw. While drying processes cause the decrease the amount of GSH, amount of GSSG had increased ($p<0.05$) (Table 2 and Figure 3).

Jones et al. (1992) reported that the amount of reduced glutathione in asparagus, avocado, apple, pear and strawberry fruits was 218, 206, 15, 33, 69, while the oxidized glutathione amount was 283, 277, 33, 50, 71 µg/g dw, respectively. GSH/GSSG ratio is used as stress biomarker, which decreases significantly as a result of drying of fruit samples ($p<0.05$) (Table 2).

Both glutathione and ghrelin are known as peptides. Preservation methods applied to foods can significantly affect the biological activity of peptides. Ultrasound, heat, and irradiation processing might affect protein structure and functions. In addition, these processes may cause Maillard reactions in food (Davis et al. 2001). As a result of the factors mentioned above, might leads to changes in the amount of peptides.

Free radicals cause lipid peroxidation by effecting the unsaturated fatty acids, resulting, MDA which is a biomarker of stress (Gaweł et al. 2004). According to the applied procedures, amount of MDA in black *M. communis* L. fruits was observed in the range between 5.32±0.40 to 7.00±0.25 µg/g dw (Table 2, Figure 3). Karatas and Kamisli (2007) reported that the drying apricot by MW and infrared increased the MDA level. The changes in the amount of MDA obtained by the drying process are consistent with the literature. Drying of *M. communis* L. fruits, cause to increase MDA level while GSH/GSSG ratio decrease which indicates the oxidative stress.

Since the moisture content of the fruits is reduced below a certain amount by drying, the shelf life of the fruits increases as they are more resistant to chemical, enzymatic and microbiological spoilage under normal atmospheric conditions. However, as a result of physical, chemical, biochemical and microbiological changes due to the effect of heat during the drying of fruits, there are losses in nutritional value.

The fact that the temperature is reduced along with the water activity in the freezing of fruits reduces the speed of chemical and biochemical reactions and microbial activities. As a result, the losses in the nutritional value of the fruits are less than in the drying process (Rickman et al. 2007).

Phenolic compounds consist of different organic molecules such as simple flavonoids, complex flavonoids, phenolic acids, and anthocyanins (Babbar et al. 2014). Total amount of phenolic substance was found to be 43.01±3.40 µg GAE/g dw in fresh black *M. communis* L. fruit. While the total phenolic substance in frozen black *M. communis* L. fruit was found to be 40.94±3.00 µg GAE/g dw, and 38.63±2.35 µg GAE/g dw in sun dried sample (Table 2 and Figure 4).

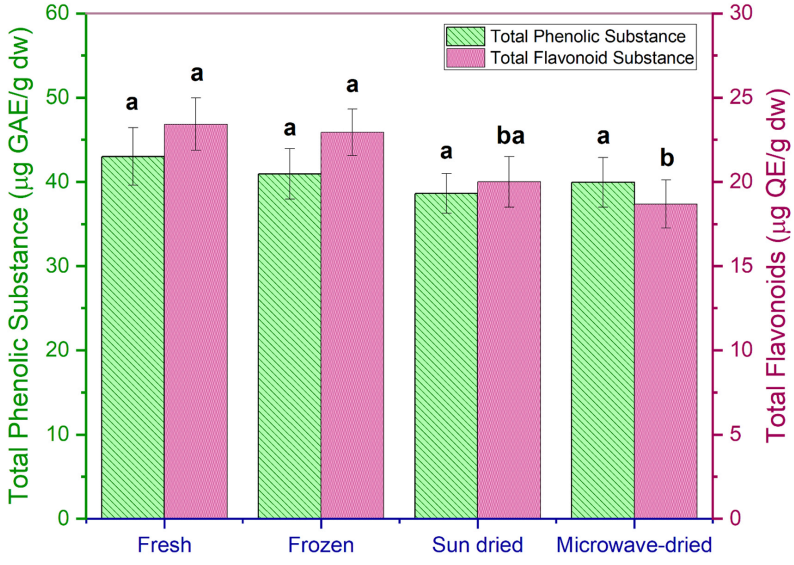

Figure 4 - Total phenolic compounds and flavonoids in black *M. communis* L fruits

The change in the amount of total phenolic compounds by different preservation methods in black *M. communis* L. fruit were found to be statistically insignificant ($p>0.05$). Zanoelo et al. (2006) reported that, after drying process the total amount of phenolic substances decrease, on the other hand, Carranza-Concha et al. (2012) found increase of phenolic compound but, Dewanto et al. (2002) reported that the total phenolic compound unchanged. As a result of the heat treatment, some phenolic compounds might be decomposed or formed a new type of phenolic compounds. Therefore, the change of the total amount of phenolic substance for different products might not be the same after drying process (Miletic et al. 2013). Flavonoids are the substances that cause the coloring of fruits and vegetables and involve in the activity of some enzymes (Panche et al. 2016). The amount of total flavonoid in fresh, frozen, sun and MW-dried black *M. communis* L. were found to be 23.42±1.55, 22.94±1.37, 20.00±1.50 and 18.68±1.43 µ*g QE/g* dw. (Table 2 and Figures 4). Hahm et al. (2015), reported that the amount of total flavonoid in *Opuntia ficus-indica* fruit as 1.91±0.29 (mg QE/g DM). The maximum loss of flavonoids was observed in MW drying process ($p<0.05$), while the least of flavonoid loss was observed in frozen fruit ($p>0.05$). This may be explained by the high temperature cause to decompose some of flavonoids during MW drying process. Drying of different vegetables and fruits under different conditions, may cause to decrease in the total flavonoid amount between 3% and 96% (Kamiloglu et al. 2015). Antioxidants are compounds that inhibit the formation of free radicals or neutralize them by transferring electrons to free radicals. Antioxidants are molecules produced from natural sources usually containing phenolic groups (Su et al. 2007).

DPPH and TEAC methods were used to determine the antioxidant capacity of the *M. communis* L. fruits. As seen from Table 2 and Figure 5, IC_{50} values in black *M. communis* L. fruits range from 28.49±2.10 to 35.29±3.10 µg/mL. Low IC_{50} values indicates high antioxidant activity. The least antioxidant capacity found in sun dried and the highest antioxidant capacity found in fresh fruit samples ($p<0.05$).

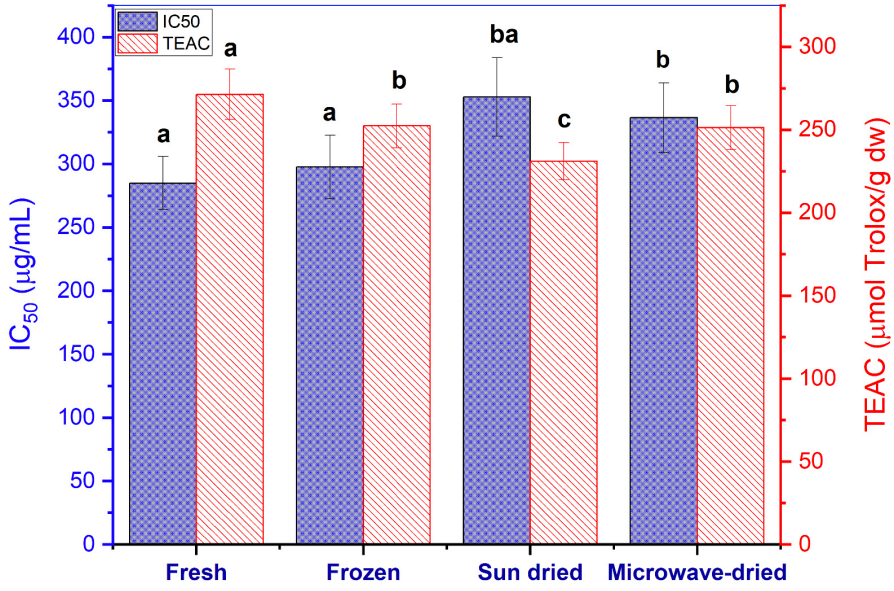

Figure 5- Total antioxidant capacity (TEAC and IC_{50} value) in black *M. communis* L fruits (IC_{50} values multiplied by 10)

IC_{50} values of mangosteen, orange, pamelo, grape and papaya fruits ranged from 11.18 to 32.80 mg/mL (Surinut et al. 2005). TEAC value of fresh black *M. communis* L. fruit was found to be 271.34±15.20 µmol troloxs/g dw. The difference of experimentally measured TEAC values of dried fruit samples in the MW was insignificant ($p>0.05$) whereas the decrease in TEAC values of sun-dried samples was significant ($p<0.05$) (Table 2 and Figure 5). Su et al. (2007) reported that the value of the antioxidant activity of rosehip fruit was 190±4.81 µmol TEAC/g. The black *M. communis* L. fruit showed higher trolox equivalents than rosehip fruit (Su et al. 2007). Major elements are found in many tissues, including the structure of bones, blood and liver, while trace elements serve as cofactors in the structure of many enzymes. Selenium is an essential trace element of several major metabolic pathways, including thyroid hormone metabolism, antioxidant defence systems, immune function. Zinc is one of the integral parts of a wide range of enzymes that is responsible catalytic, structural, and regulative role. Iron is vital for all living organisms, and it has a broad role in all metabolic activities, including oxygen transfer. Copper is one of the trace elements in the human body, and play an important role in biochemical processes. Manganese is one of the vital elements and is available in metallo proteins, such as carboxylase pyruvate and in the glial cytoplasmic enzyme, glutamine synthase (Al-Fartusie & Mohssan 2017).

The amounts of selenium, zinc, iron, copper and manganese in black *M. communis* L. were found as 0.63±0.021, 268.71±15.80, 156.36±12.75, 46.55±3.35 and 29.76±1.65 µg/g dw respectively (Table 2). Reported amount of selenium in apples, oranges, mangoes, and figs is 11, 28, 5 and 32 ng/g, respectively (Al-Ahmary 2009). Onivogui et al. (2014), reported that the amounts of zinc, iron, copper, and manganese in monkey apple (*Anisophyllea laurina R.* Br ex Sabine) were found to be 8.8, 141.4, 2.9 and 23.7 µg/g, respectively.

Essential amino acids used in protein synthesis and in metabolism must be taken with diet. Amount of amino acids determined in plants, vegetables, and fruits become important subject because one of the main source of amino acids in developing countries. In this study, the amount of essential amino acids in black myrtus fruit was found in the following order. Arginine > threonine > leucine > histidine > valine > lysine > isoleucine > methionine > phenylalanine > tryptophan. The essential amino acid content in black myrtus fruit ranges from 2.00±0.15 to 0.56±0.04 mg/g dw, while the total essential amino acid content was found to be 12.37±0.98 mg/g dw (Figure 6). Order of non-essential amino acids in the same fruit is glutamic acid > serine > aspartic acid > pyroline > glycine > alanine > cysteine > tyrosine > asparagine > glutamine. The amount of non-essential amino acids ranges from 2.69±0.20 to 0.43±0.03 mg/g dw. The total non-essential amino acid was found to be 16.04±1.38 mg/g dw.

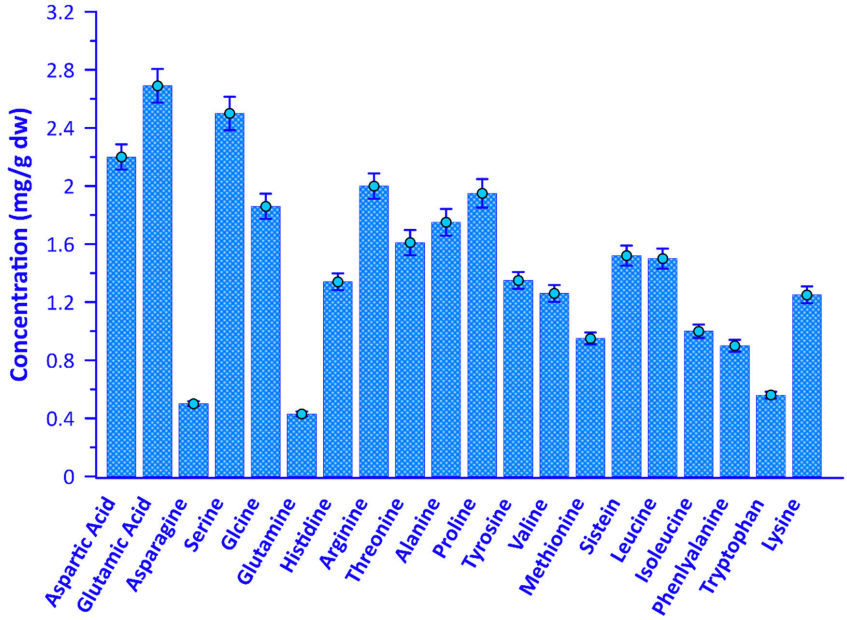

Figure 6- Amino acid composition in black *M. communis* L fruits

Glew et al (1997), reported that the amino acid content varies in between 2.64 and 0.26 mg/g, dw in *Vitex doniana* fruit and 3.94 to 0.18 mg/g dw in *Adansonia digita* (monkey bread pod). Zhou et al. (2019) studied *Nitraria tangutorum Bobr* pulp and peel grown in different regions, they reported that the total amount of essential amino acids ranged from 44.39±0.81-53.51±0.90 mg/g dw, and the total amount of non-essential amino acids ranged from 65.65±1.34-71.41±1.45 mg/g dw.

According to the Food and Agriculture Organization and the World Health Organization, the total essential amino acid/total amino acid ratio in a good protein source should be over 40%, while the total essential amino acid/total non-essential amino acid ratio should be over 60% (Zhou et al. 2019). Our results showed these ratios to be 43.5% and 77% for black myrtus fruit respectively. These results led us to say that black myrtus fruit, is a good source of essential and non-essential amino acids source.

4. Conclusions

The result obtained here in this work suggest that black *M. communis* L. fruit is a good source of nutrients in terms of fat and water-soluble vitamins, carotenes, lycopene glutathione, ghrelin, examined elements, amino acids and have a high antioxidants capacity. Content of fat and water-soluble vitamins, the total phenolic, flavonoids and antioxidant capacity in fresh and frozen black *M. communis* L. fruit samples have higher than the sun and MW-dried fruits samples.

It is also found that, while GSH/GSSG ratio is decreased, MDA level was increased in dried fruits. It can be said that drying process, induces stress to the fruits and as a result lipid peroxidation occurs. From the experimental findings, it can be said that the most suitable method for preserving *M. communis* L. fruits is freezing. In addition, MW drying seems more advantageous than drying under the sun in terms of vitamins and nutrient loss.

Data availability: Data are available on request due to privacy or other restrictions.

Authorship Contributions: Concept: D.Ö., F.K., Design: S.S., Data Collection or Processing: M.Ç., B.B., D.Ö., Analysis or Interpretation: M.Ç., B.B., D.Ö., F.K., S.S., Literature Search: M.Ç., B.B., Writing: D.Ö., F.K., S.S.

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Prevalence, Serotype Diversity and Antibiotic Resistance of *Salmonella* Among Poultry Meat and Eggs in Turkiye: A Meta-analysis

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ABSTRACT

Poultry is a well-known reservoir for Salmonella, and therefore numerous outbreaks have been reported among poultry meat products and eggs. This study is aimed at determining the pooled prevalence, serotype diversity and antibiotic resistance profiles of *Salmonella* among poultry meat and eggs sold in Turkiye. For this purpose, international (Web of Science and PubMed) and national (ULAKBIM TR Index) electronic databases were searched using based on relevant keywords in English and Turkish, and out of 1,818 articles, 41 were deemed eligible for inclusion in this meta-analysis. The random effects model was accepted when a substantial heterogeneity was obtained according to Q statistics and the I2 value, however the fixed effects model was assumed valid in the opposite case. The pooled prevalence of *Salmonella* in chicken parts, chicken carcasses, chicken giblets and eggs were 24.4% [95% confidence interval (CI)=17.8-32.6], 21.9% (95% CI=14.0-32.7), 20.1% (95% CI=10.7-34.6) and 4.8% (95% CI=1.7-13.3), respectively. *Salmonella* Enteritidis was the most common serotype among eggs, chicken parts and chicken carcasses with the rates of 22.4% (95% CI=3.6-69.3), 19.0% (95% CI=3.3-61.6) and 5.8% (95% CI=2.2-14.4), respectively. The highest pooled antibiotic resistance prevalence of *Salmonella* spp., regardless of food type, was found in tetracycline (73.9%, 95% CI=51.0-88.5) ($p<0.041$) and ampicillin (31.5%, 95% CI=20.7-44.6). The high-pooled prevalence of the organism emphasized the potential threat *Salmonella* poses to public health, and also antibiotic resistance data revealed that the use of tetracyclines, quinolones and penicillin in poultry livestock should be restricted. These results will be of great use in the future epidemiological surveillance of *Salmonella* spp. presence and antibiotic resistance among poultry meat and eggs in Turkiye.

Keywords: *Salmonella*, Enteritidis, Infantis, Chicken, Resistance, Teyracycline, Ampicillin

1. Introduction

The species *Salmonella enterica* consists of six subspecies with more than 2600 serovars, and among them *S. enterica* subsp. *enterica* is the leading cause of infections in humans and animals (Issenhuth-Jeanjean et al. 2014). *Salmonella* ranked second among the most reported food-associated infections in humans, behind *Campylobacter* in the European Union (EU), and behind norovirus in the United States of America (USA) (Ferrari et al. 2019). According to the EU One Health 2019 Zoonoses Report, 926 salmonellosis outbreaks were reported in 2019 (9,169 illnesses, 1,915 hospitalizations, and seven deaths), which corresponded to 17.9% of all foodborne outbreaks in the EU in the same year. The report also stated that *S. Enteritidis* caused 72.4% of food-borne salmonellosis cases, and the most common salmonellosis associated foods were eggs and egg products (EFSA & ECDC 2021a). Also, as poultry is a well-known reservoir for *Salmonella*, many cases of contaminated poultry meat have been reported to date (CDC 2021). Although poultry meat can be contaminated at any stage from farm to fork, transmission to humans often occurs in food preparation areas because of inadequate sanitation, insufficient cooking, improper storage conditions and/or cross-contamination (Luber 2009). Consequently, poultry meat and eggs are among the main foods involved in the spread of *Salmonella* to humans.

Drug resistance is becoming a big concern, for scientists as humanity nears the edge of the post-antibiotic era. Each year, antimicrobial resistant bacteria account for over 700,000 deaths worldwide, and this number is expected to increase to 10 million by 2050 (O'Neil

2015). In meat production, antibiotic usage is almost inevitable for therapeutic and prophylactic reasons. It is known that poultry do not show any clinical signs when infected by non-typhoidal *Salmonella,* and therefore antibiotic treatment is not required. In this case, however, the bacteria can be exposed to other antibiotics applied to the animals, meant to treat other diseases, and consequently may develop resistance (Voss-Rech et al. 2017). Additionally, although the use of antibiotics for prophylaxis and growth promoter in poultry has been banned in many countries, including Turkey, it is estimated that 60% of all antibiotics produced are used in livestock as they improve the performance effectively and general economics of the production process (Agyare et al. 2018). In this respect, it is important to monitor the antibiotic resistance patterns of *Salmonella* in foods where the pathogen is frequently found. If antibiotic resistance is not monitored well and effectively combated, the list of antibiotics kept as reserve for use in human medicine can be expanded and the number of antibiotics banned for use in veterinary medicine may increase. The prohibition of the use of many antibiotics in veterinary medicine may be on the agenda in the near future, especially colistin, macrolides, fluoroquinolones, and third and fourth generation cephalosporins, which are defined in the publication, WHO Highest Priority Critical Important Antimicrobials, (WHO 2018; European Parliament 2021).

Chicken is the best-integrated farm animal in Turkiye. Approximately 2.2 million tons of broiler meat are produced every year and consumption amounts to 20.5 kg/person (BESD-BIR 2021a; BESD-BIR 2021b). Moreover, in 2018, the number of laying hens in Turkiye reached up to 124 million (TUIK 2021), ranking 8th in the world for hen egg production with 1.2 million tones (YUM-BIR 2021). Considering this high amount of production and consumption, the prevalence of *Salmonella* spp. and their antibiotic resistance profiles among chicken meat and eggs has always been studied in Turkiye in some depth. However, the scattered nature of the data complicates the possibility of an all-encompassing interpretation, as these independent studies represent different periods of time and geographic areas. Therefore, we have sought to carry out a meta-analysis in order to determine the pooled prevalence, serotype diversity and antibiotic resistance profiles of *Salmonella* spp. among poultry meat and eggs in Turkiye between 1996 and 2020.

2. Material and Methods

2.1. Search strategy and study selection

A systematic search was conducted between May and June 2021 using the terms "*Salmonella*" and "Turkiye" or "Türkiye" or "Turkish" and "antimicrobial resistance" or "microbial resistance" or "bacterial resistance" or "resistance pattern" or "resistance" or "susceptibility" or "prevalence" and "chicken" or "poultry" or "broiler" or "turkey" or "egg" in the Web of Science, PubMed and ULAKBIM TR Index databases. Also, a further search was conducted by checking the reference list of relevant papers.

Cross-sectional studies that report the prevalence of any species of *Salmonella* in related foods were included in this systematic review and meta-analysis. Two authors (G.C. and A.N.D.) independently carried out screening in order to identify relevant abstracts and article titles. The advice of the senior author was sought (N.D.A.), as an expert, in case of any disagreement. All the articles were uploaded to Endnote X9.2 (Clarivate Analytics) and duplicates were removed.

Articles written in English or Turkish were included without any date restriction. However, only studies reporting prevalence from Turkiye were included in our study. Reviews, book chapters, letters, theses and conference abstracts were excluded. Additionally, studies with unclear/wrong results, undefined/unclear positive samples, and incomplete information about the prevalence, sample size and/or type of food were also excluded.

2.2. Data extraction

One of the authors (A.N.D.) extracted the data for *Salmonella* spp. prevalence among poultry meat and eggs. The second author (G.C.) validated the extracted data according to the systematic literature review flowchart (Figure 1). Any doubts were resolved by consulting the senior author (N.D.A.). The extracted data included author, publication year, city/region, sample source, sampling year, food type, number of samples, number of positive *Salmonella* spp. samples, identified *Salmonella* serovars, antibiotic resistance profiles of *Salmonella* spp. isolates, and the antibiotic susceptibility test method. Accordingly, six food items were tested for their pooled prevalence estimation separately: chicken carcass, chicken parts (drumstick, wing, breast, neck etc.), chicken giblets, chicken ready-to-eat (RTE) foods, turkey meat, and eggs.

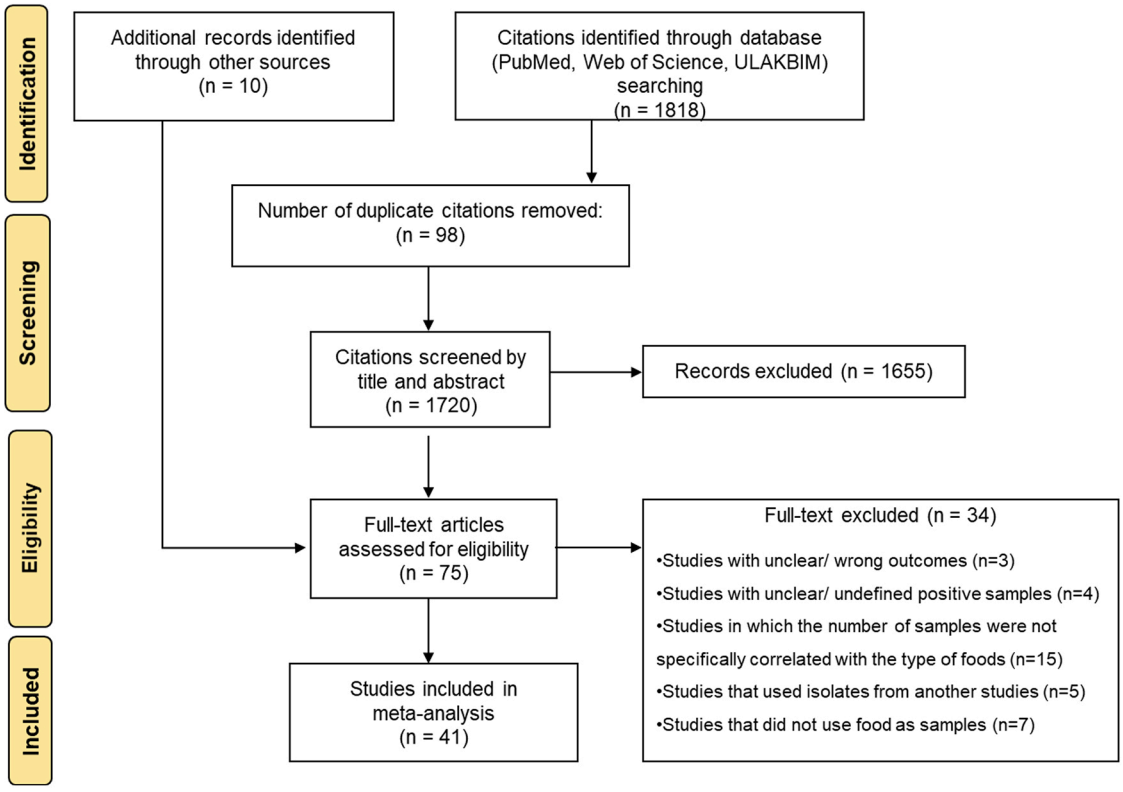

Figure 1- Flowchart of article selection process

2.3. Statistical analyses

The pooled prevalence rates of *Salmonella* spp. among several food types were determined using a fixed and random effects meta-analysis, and the results of these effects are displayed in the manuscript. The effect size measure was determined as the prevalence rate. The distribution of the individual effect and pooled effect sizes were shown with forest plots. On a forest plot, the effects of the individual studies were plotted as boxes with horizontal lines on both sides. The bigger boxes indicate the bigger weights of individual studies and the longer lines indicate the wider confidence intervals (CI). In addition, the diamond at the bottom of the graph presents the combined result. Variations among the trial-level prevalence ratios were evaluated by Q statistics following an χ^2 distribution with a (k-1) degrees of freedom. The Q statistics were calculated as follows:

$$Q = \sum_{i=1}^{k} w_i(\hat{\theta}_i - \bar{\theta})^2$$

Where; $w_i = 1/\sigma_i^2$, θ is the estimated effect measure and k is the number of studies. In determining the heterogeneity, the I^2 (Inverse variance index) value was used in addition to Q statistics (Higgins & Thompson, 2002). The I^2 value was calculated as follows:

$$I^2 = \frac{Q - (k-1)}{Q} \times 100\%$$

A value greater than 50% was considered to be high heterogeneity, between 25-50% was considered to be moderate heterogeneity and lower than 25% was considered to be low heterogeneity for the I^2 value (Patsopoulos et al. 2008). The random effects model was accepted when a substantial heterogeneity was obtained according to Q statistics and I^2 value, however the fixed effects model was assumed valid in the opposite case.

The sampling source and sampling year of the studies were taken as sources of heterogeneity. Accordingly, in order to detect the effects of sampling source and sampling year on the overall prevalence rates of *Salmonella* spp. in each food type, a series of meta-regression analyses were conducted. The sampling source variable was defined according to where the samples were obtained, and therefore into three groups: retail markets, farms and slaughterhouses. For meta-regression analysis the retail market was selected as a reference

category. The year variable was described in two groups. The first group was defined as the group for which sampling was done before the last decade, while the second group was defined as the group for which sampling was performed in the last decade. The first group was chosen as the reference category for meta-regression analysis.

Funnel plots were used to assess publication bias. Any asymmetrical scattering of the effect sizes of individual studies and their standard errors were interpreted as evidence of publication bias (Mavridis & Salanti 2014). No funnel plot was created for food groups with less than five studies. Comprehensive Meta-Analysis Version 3.3.070 was used to perform all statistical analysis and graphical representations. $P<0.05$ was considered as statistically significant.

3. Results

3.1. Description of articles

A total of 1,818 articles from three databases were identified. After removing duplications, and screening the titles/abstracts, 10 studies identified through the reference list of relevant articles were included. Subsequently, the full-texts of 75 papers were examined and 34 of them were excluded because of the reasons that given in Figure 1. Eventually, 41 articles were found eligible for inclusion in this meta-analysis. The studies were published between 1996-2020 and cover a total of 1,451 *Salmonella* spp. positive isolates out of 9,542 isolates. The characteristics of included studies are shown in Supplementary Table 1.

Table 1- Meta-analysis results of *Salmonella* prevalence

Food type	*Salmonella serovars*	*% (95% CI) - fixed*	*% (95% CI) - random*	*n*	*I^2*	*Q*	*Sig. of the model*
Chicken parts	*Salmonella* spp.	31.9 (29.8-33.9)	24.4 (17.8-32.6)	24	93.93	378.95*	<0.001[b]
	Salmonella Infantis	10.9 (8.2-14.4)	6.9 (2.2-20.1)	3	89.77	19.57*	<0.001[b]
	Salmonella enteritidis	10.7 (7.7-14.8)	19.0 (3.3-61.6)	2	91.73	12.09*	<0.001[b]
	Salmonella typhimurium	19.2 (13.0-27.5)	9.6 (0.9-56.7)	2	93.74	15.98*	<0.001[b]
	Unknown	22.9 (18.0-28.8)	2.5 (0.5-11.3)	7	93.49	92.22*	<0.001[b]
Chicken carcass	*Salmonella* spp.	20.4 (18.7-22.2)	21.9 (14.0-32.7)	13	95.34	257.70*	<0.001[b]
	Salmonella Infantis	2.5 (1.7-3.7)	2.6 (1.0-6.5)	3	74.48	7.84*	<0.001[b]
	Salmonella enteritidis	5.0 (3.5-7.0)	5.8 (2.2-14.4)	4	76.49	12.76*	<0.001[b]
	Salmonella typhimurium	5.7 (4.5-7.2)	2.8 (0.8-8.9)	5	93.20	58.86*	<0.001[b]
	Salmonella hadar	1.2 (0.7-1.9)	1.2 (0.7-1.9)	3	0.00	1.83	<0.001[a]
	Salmonella agona	0.3 (0.1-0.9)	0.3 (0.1-0.9)	2	0.00	0.48	<0.001[a]
	Salmonella virchow	0.8 (0.4-1.6)	0.6 (0.1-3.1)	2	77.75	4.45*	<0.001[b]
	Unknown	0.5 (0.2-1.5)	0.5 (0.1-2.2)	6	45.66	9.20	<0.001[a]
Chicken giblets	*Salmonella* spp.	30.0 (25.2-35.2)	20.1 (10.7-34.6)	7	83.77	36.97*	<0.001[b]
	Salmonella typhimurium	12.2 (8.2-17.7)	11.3 (5.0-23.7)	2	74.09	3.86*	<0.001[b]
	Unknown	22.9 (18.5-28.0)	22.0 (14.6-31.9)	3	70.87	6.87*	<0.001[b]
Chicken RTE food	*Salmonella* spp.	7.1 (3.3-14.5)	5.4 (1.2-20.9)	3	52.31	4.19*	<0.001[b]
Egg	*Salmonella* spp.	11.3 (9.5-13.5)	4.8 (1.7-13.3)	10	95.91	220.10*	<0.001[b]
	Salmonella enteritidis	14.0 (11.2-17.4)	22.4 (3.6-69.3)	4	98.11	158.92*	<0.001[b]
	Unknown	1.0 (0.3-2.8)	0.7 (0.1-4.0)	4	56.56	6.91*	<0.001[b]
Turkey meat	*Salmonella* spp.	22.1 (15.0-31.2)	16.7 (5.4-41.1)	3	74.66	7.89*	<0.001[b]

[a]p-value for fixed-effect model, [b]p-value for random-effects model, *Substantial heterogeneity, CI: Confidence interval, n: Number of the isolates, I: Inverse variance index, Q: Q statistics, sig.: Significance, RTE: Ready-to-eat, spp.: Species

Among the 41 studies, *Salmonella* spp. prevalence was extracted from chicken parts in 24, chicken carcasses in 13, chicken giblets in seven, chicken RTE food in three, eggs in 10 and turkey meat in three studies. Since there is only one study reporting the prevalence of *Salmonella* spp. in quail meat, a meta-analysis could not be performed for this subset.

3.2. Pooled prevalence of Salmonella spp.

The estimated pooled prevalence rates, heterogeneity findings, and number of studies included in the *Salmonella* spp. meta-analysis across all food types are shown in Supplementary Table 1 and Supplementary Figure 1. Most of the included studies reported the prevalence of *Salmonella* spp. for more than one food group.

In chicken parts, the overall prevalence of *Salmonella* spp. Among the 2,807 isolates was 24.4% (95% CI:17.8-32.6). *Salmonella* Enteritidis had a higher pooled prevalence compared to *S.* Infantis and *S.* Typhimurium. Additionally, the pooled prevalence of unknown serovars among chicken parts was 2.5% (95% CI:0.5-11.3) (Table 1). The effect of year on the pooled prevalence of *Salmonella* spp. was statistically significant according to the random effects meta-regression model (p=0.033, df=1, Q=4.56, τ^2=0.691). The pooled prevalence of the second group [33.7% (95% CI: 21.1-49.1)] was higher than the first group [19.8% (95% CI: 14.4-26.7)]. Nonetheless, the sample source had no effect on the prevalence of *Salmonella* spp. in chicken parts (p=0.313, df=1, Q=1.02, τ^2=0.911).

Among chicken carcasses, the overall prevalence of *Salmonella* spp. among 2,685 isolates was 21.9% (95% CI:14.0-32.7). While the pooled prevalence rates of the identified serovars were observed to be quite low, the highest was found in *Salmonella* Enteritidis with 5.8% (95% CI:2.2-14.4). The pooled prevalence of unknown serovars was found to be 0.5% (95% CI:0.2-1.5) (Table 1). The effect of year and the source of the sample on the pooled prevalence of *Salmonella* spp. was not found to be statistically significant according to the random effects meta-regression model as with the chicken parts (p=0.161, df=1, Q=1.96, τ^2=0.848 and p=0.935, df=1, Q=0.01, τ^2=1.034).

Among the chicken giblets, the overall prevalence of *Salmonella* spp. Among the 395 isolates was 20.1% (95% CI:10.7-34.6). Among the included studies, *Salmonella* Typhimurium was the only serovar identified in chicken giblets, with a pooled prevalence ratio of 11.3% (95% CI:5.0-23.7). In addition, the unknown serovars' pooled prevalence ratio was found to be 22.0% (95% CI:14.6-31.9) (Table 1). In the meta-regression analysis, year had no significant effect on the pooled prevalence of *Salmonella* spp. (p=0.935, df=1, Q=0.01, τ^2=1.219). The meta-regression analysis could not be performed as all samples of chicken giblets were collected from retail markets in the included studies.

Among eggs, the overall prevalence of *Salmonella* spp. among 3,258 isolates was 4.8% (95% CI:1.7-13.3). Among the included studies, *Salmonella* Enteritidis was the only serovar identified among eggs, with a pooled prevalence rate of 22.4% (95% CI:3.6-69.3). Moreover, the pooled prevalence of unknown serovars was 0.7% (95% CI:0.1-4.0) (Table 1). In the meta-regression analysis, neither the year nor the sample source had a significant effect (p=0.857, df=1, Q=0.03, τ^2=3.233 and p=0.242, df=1, Q=1.37, τ^2=2.007).

Among chicken, RTE food and turkey meat, the overall prevalence rates of *Salmonella* spp. were 5.4% (95% CI:1.2-20.9) and 16.7% (95% CI:5.4-41.1), respectively. The included studies did not have any serovar identification for these food types (Table 1). Additionally, we were unable to perform meta-regression analysis for these two food types due to the small sample sizes.

Although asymmetrical patterns were observed on the funnel plots, it was not possible to interpret it as concrete evidence of publication bias. Asymmetry in funnel plots might occur by alternative mechanisms such as heterogeneity, small study effect, selective outcome reporting or chance (Supplementary Figure 2).

3.3. The pooled prevalence of antimicrobial resistance for Salmonella spp.

The results of the meta-analysis of antimicrobial resistance in *Salmonella* spp. are shown in Table 2. The highest resistance was found to clindamycin and oxacillin [0.98 (95% CI:0.92-0.99)], while the lowest resistance was found to be to imipenem and ceftriaxone [0.9% (95% CI:0.1-6.0) and 0.9% (95% CI:0.2-4.3), respectively] among the statistically significant models. The most frequently tested antibiotics were tetracycline, nalidixic acid and ampicillin in the included studies (n=13, n=12, and n=12, respectively). On the other hand, the less-tested antibiotics were vancomycin, imipenem, meropenem, colistin, cefoxitin, sulfamethoxazole, oxacillin, clindamycin, and enrofloxacin, with two studies available for each antibiotic.

Table 2- Meta-analysis results of antimicrobial resistance in *Salmonella* species

Antibiotic	*% (95% CI) - fixed*	*% (95% CI) - random*	*n*	*I^2*	*Q*	*Sig. of the model*
Tetracycline	61.8 (57.1-66.2)	73.9 (51.0-88.5)	13	95.87	290.31*	0.041[b]
Nalidixic acid	63.2 (58.2-67.9)	73.4 (48.7-88.9)	12	95.67	253.86*	0.062[b]
Ampicillin	33.9 (30.5-37.4)	31.5 (20.7-44.6)	12	91.28	126.08*	0.007[b]
Chloramphenicol	19.9 (16.9-23.2)	14.0 (9.1-21.1)	10	78.11	41.11*	<0.001[b]
Gentamicin	8.5 (6.6-10.9)	8.8 (4.4-16.9)	10	84.58	58.38*	<0.001[b]
Streptomycin	67.0 (62.4-71.3)	60.3 (35.5-80.7)	10	95.80	214.29*	0.422[b]
Trimethoprim-sulfamethoxazole	52.8 (47.6-57.9)	39.6 (18.1-66.0)	9	95.92	195.99*	0.444[b]
Ciprofloxacin	11.1 (8.4-14.5)	0.11 (5.1-21.9)	8	81.97	38.61*	<0.001[b]
Kanamycin	35.7 (29.0-43.1)	46.6 (24.9-69.6)	5	87.61	32.28*	0.781[b]
Trimethoprim	64.3 (59.2-69.0)	73.4 (46.2-89.9)	5	95.64	91.79*	0.088[b]
Amikasin	13.7 (8.6-21.2)	14.7 (1.8-61.7)	4	93.40	45.47*	0.123[b]
Cefotaxime	30.0 (25.2-35.5)	16.5 (5.3-40.9)	4	93.82	48.51*	0.011[b]
Cephalothin	37.2 (30.9-43.9)	33.5 (18.0-53.6)	4	87.22	23.47*	0.106[b]
Cefazoline	20.4 (16.2-25.5)	15.9 (6.0-36.0)	4	91.93	37.15*	0.003[b]
Amoxicillin	13.1 (8.8-19.2)	11.5 (4.8-25.0)	4	73.60	11.37*	<0.001[b]
Neomycin	65.6 (57.8-72.6)	70.4 (55.1-82.1)	4	67.68	9.28*	<0.001[b]
Erythromycin	87.0 (80.5-91.5)	88.9 (77.9-94.8)	4	56.43	6.87*	<0.001[b]
Penicillin	68.7 (55.1-79.7)	95.0 (41.1-99.8)	3	87.08	15.48*	0.081[b]
Sulfonamide	98.2 (93.9-99.5)	98.2 (93.9-99.5)	3	0.00	0.56	<0.001[a]
Vancomycin	85.9 (77.4-91.5)	87.0 (67.3-95.6)	2	75.44	4.07*	0.002[b]
Imipenem	0.9 (0.1-6.0)	0.9 (0.1-6.0)	2	0.00	0.29	<0.001[a]
Meropenem	17.0 (11.8-23.9)	7.8 (0.7-50.3)	2	70.35	3.37*	0.051[b]
Colistin	2.6 (1.0-6.7)	2.6 (1.0-6.7)	2	0.00	0.44	<0.001[a]
Cefoxitin	9.2 (6.0-14.1)	6.6 (1.8-21.5)	2	78.42	4.64*	<0.001[b]
Sulfamethoxazole	45.5 (37.5-53.8)	91.1 (4.4-99.9)	2	93.31	14.95*	0.399[b]
Oxacillin	97.6 (92.0-99.3)	97.6 (92.0-99.3)	2	0.00	0.37	<0.001[a]
Clindamycin	97.6 (92.0-99.3)	97.6 (92.0-99.3)	2	0.00	0.37	<0.001[a]
Ceftriaxone	0.9 (0.2-4.3)	0.9 (0.2-4.3)	2	0.00	0.24	<0.001[a]
Enrofloxacin	8.3 (0.4-16.4)	8.3 (0.4-16.4)	2	0.00	0.42	<0.001 [a]

[a]p-value for fixed-effect model, [b]p-value for random-effects model, *Substantial heterogeneity, CI: Confidence interval, n: Number of the isolates, I: Inverse variance index, Q: Q statistics, sig.: Significance

4. Discussion

According to the results, the highest pooled prevalence of *Salmonella* spp. belonged to chicken parts with 24.4%. Also, chicken parts were the most studied food type (n=24) and consisted of drumsticks, wings, breasts, necks, skins, and other meat pieces (Supplementary Table 1). In order to interpret this prevalence more accurately, it should be considered together with the pooled *Salmonella* prevalence of chicken carcass, which was found to be 21.9%. These close percentages showed that chicken meat maintains its importance as a risky food for *Salmonella* contamination in Turkiye as well as throughout the world. There are some meta-analyses that report the prevalence of *Salmonella* spp. among chicken meat from another countries and regions. These are as follows: 20% for retail broiler in the USA (Golden & Mishra 2020), 3.2% for chicken meat in Europe (Gonçalves-Tenório et al. 2018), 13.2% for poultry meat/organ in Africa (Thomas et al. 2020), 14% for chicken meat in Ethiopia (Zelalem et al. 2019) and 13.5% for retail chicken in Ethiopia (Tadesse & Gebremedhin 2015). As can be seen, the prevalence in Turkiye is higher than in these countries/regions. Exceptionally, in the study of Golçalvez-Tenório et al. (2018), the overall prevalence among chicken meat was stated to be as high as 58.3% for Turkiye. However, this big difference from our finding is probably due to the very small number of studies that researchers included in their meta-analysis from Turkiye.

Further contamination can occur during slaughtering. In a survey published by EFSA, it was reported that 5% more bacteria were present in the intestines of chickens before slaughter than were found in the carcass at the end of the slaughter line (EFSA 2010). This shows that contamination from carcass to parts is highly probable, especially in integrated plants, where slaughtering and cutting processes take place together. In this context, the very close rates of chicken parts (24.4%) and chicken carcasses (21.9%) in our results can be considered as an indication of the fact that slaughtering and cutting is done in the same facility. Although chicken meat is cooked before consumption, the risk of direct contact with humans and cross-contamination with surfaces or kitchen utensils is high during the pre-cooking process.

Chicken giblets have been associated with *Salmonella* outbreaks several times in many countries (CDC 1984; CDC 2012; Lanier et al. 2018). The chicken liver is considered to be one of the more common locations for foodborne infections. The Food Safety and Inspection Service (2022) highly recommends consuming chicken liver dishes after being cooked to an internal temperature of 74 °C as pathogens can exist both on the external surface and in the internal parts of the liver (FSIS 2021). In this meta-analysis study, the *Salmonella* prevalence rate was found to be 20.1%, which is almost as high as in chicken parts and carcasses. This rate can be explained by cross-contamination during slaughtering, thus much more care needs to be taken while handling chicken.

The pooled *Salmonella* prevalence of chicken RTE food and turkey meat were found 5.4% and 16.7%, respectively. However, the low number of the studies (two for chicken RTE food and three for turkey meat) prevents the interpretation of these ratios accurately. Nevertheless, when compared with the 2019 EFSA report, these rates are quite higher than the mean incidence of *Salmonella* spp. in RTE foods (0.27%) and fresh turkey meat (5.3%) (EFSA & ECDC 2021a).

Eggs have a distinct place among other foods in terms of the way it is contaminated with *Salmonella*. Two possible routes for the *Salmonella* contamination of eggs were defined; horizontal (penetration through the eggshell) and vertical (transovarian, direct contamination of the inner egg while passing through the reproductive organs) (Cardoso et al. 2021). Therefore, some studies both investigate egg contents and eggshells. However, because of the low number of egg studies conducted in Turkiye, the presence of *Salmonella* in eggs was included as a whole in this meta-analysis. Thus, the pooled *Salmonella* prevalence for eggs was determined to be 4.8%. This ratio is quite higher than in the EFSA report (0.13%) (EFSA & ECDC, 2021a). On the other hand, Hosseininezhad et al. (2020) report an overall prevalence of *Salmonella* in eggs of 6.89% in Iran, which is almost the same as our result. Diker et al. (2020) report the prevalence of *Salmonella* spp. among table eggs to be 3.3% (24/726), when purchased from varied regions in Turkiye. Since this prevalence was obtained from a project carried out within the scope of Turkiye's national *Salmonella* control program, it is noteworthy that both of the reported figures are close to each other. However, although the prevalence for eggs is not as high as in chicken parts and carcasses, it is essential to reduce this rate, considering that Turkiye ranks 3rd in the world for egg exports (YUM-BIR 2021).

Salmonella Enteritidis was found to be the most common serotype among eggs, chicken parts and chicken carcasses with the rates of 22.4%, 19.0% and 5.8%, respectively. The results are not surprising because *S.* Enteritidis is considered to be the main serotype associated with both human salmonellosis (Ferrari et al. 2019) and infections associated with foodborne outbreaks including eggs (Cardoso et al. 2021). Moreover, in the Turkish Food Codex Regulation on Microbiological Criteria "*Salmonella* spp.," which is required to be checked for in raw poultry meat and prepared poultry meat mixtures, was changed to "*S*. Entetiridis and *S*. Typhimurium" in 2018 (Turkish Food Codex 2018). On the other hand, according to the results of the project carried out to develop a national *Salmonella* monitoring program (TUBITAK 2017), *S*. Kentucky was found as the predominant serotype detected in all of the sample matrices collected from laying hens, while *S*. Infantis was reported to be the dominant serotype in broilers, slaughterhouses, breeder flocks, turkey meat and food samples in Turkiye. It is thought that the most probable reason for this incompatibility between the results of the nationwide project and this meta-analysis is due to the scarce and scattered studies on prevalence conducted based on *Salmonella* in chicken meat and eggs. Also, the difference between the predominant serotypes is probably due to the inclusion of studies conducted in the last 24 years. Undoubtedly, the serotype distribution has started to change in recent years and *S*. Infantis is becoming the most frequently isolated serotype in poultry in Turkiye. Moreover, as it can be seen in Table 1 only a few studies report *Salmonella* serotypes in foods, and the prevalence of unknown serotypes is noteworthy. It can be observed that most of the included studies presented serotyping for *S*. Entetiridis and *S*. Typhimurium specifically, but only two of the studies further investigated *Salmonella* spp. isolates using a Kauffmann-White classification (Supplementary Table 1). Therefore, more comprehensive studies should be performed in order to determine the *Salmonella* serotype distribution in Turkiye.

The highest pooled antibiotic resistance prevalence of *Salmonella* spp., regardless of food type belonged to tetracycline and ampicillin with 73.9% and 31.5%, respectively (Table 2). Although the percentages were found to be quite higher for sulfonamide, erythromycin and vancomycin, the prevalence of these antibiotics was not taken into account due to the small number of studies on the topic. Also,

resistance to streptomycin (60.3%) and trimethoprim-sulfamethoxazole (39.6%) were found to be high but not significant ($p>0.05$). Tetracycline, ampicillin and sulfamethoxazole are widely used in veterinary medicine for the treatment of infections in food-producing animals. High resistance to tetracycline, sulfamethoxazole and ampicillin of *Salmonella* spp. were also found at an alarming level in the 2018/2019 EFSA report (EFSA & ECDC 2021b). According to data obtained from 28 EU Member States ampicillin, sulfamethoxazole and tetracycline resistance was reported to be 13.7%, 33.9% and 35.5% for broiler carcasses, respectively. Notably, a very high level of tetracycline (57.3%) in Turkish carcasses and a high level of resistance to nalidixic acid (48.8%) was reported. In the meta-analysis of Voss-Rech et al. (2017), the highest resistance of poultry-related *Salmonella* isolates was found for nalidixic acid, sulfonamide, and tetracycline with 48.2%, 43.8% and 32%, respectively.

In this study, the pooled prevalence of *Salmonella* spp. was compared for the last two decades by year. A significant increase was observed in pooled chicken parts prevalence after 2011, from 19.8% to 33.7%. Although the prevalence is expected to decrease due to stricter rules and intensified controls, with changes made to regulations in the last decade, it is thought that the reason for this dramatic increase is mainly due to the more sensitive and advanced laboratory methods used in the detection of foodborne pathogens (Cufaoglu, Ambarcioglu et al. 2021).

5. Conclusions

To the best to our knowledge, this is the first meta-analysis study on the prevalence, serotype diversity and antibiotic resistance profiles of *Salmonella* spp. isolated from poultry meat and eggs in Turkiye. The high-pooled prevalence of the organism highlighted the potential threat *Salmonella* poses for public health. Additionally, findings related to the antibiotic resistance profiles of *Salmonella* spp. show that the tetracycline, quinolones and penicillin groups of antibiotics used with poultry livestock should be restricted. Overall, the general lack of research makes it difficult to estimate the genuine prevalence of *Salmonella* among poultry meat and eggs. Moreover, the lack of evidence on *Salmonella* serotypes has impeded our attempt at a comprehensive outcome. Therefore, a more comprehensive studies on the presence and serotype distribution of the pathogen in foods is required within the framework of the Turkish national *Salmonella* control program.

Data availability: Data are available on request due to privacy or other restrictions.

Authorship Contributions: Concept: G.C., N.D.A., Design: G.C., Data Collection or Processing: G.C., P.A., A.N.D., Analysis or Interpretation: G.C., P.A., N.D.A., Literature Search: G.C., A.N.D., Writing: G.C., P.A., N.D.A.

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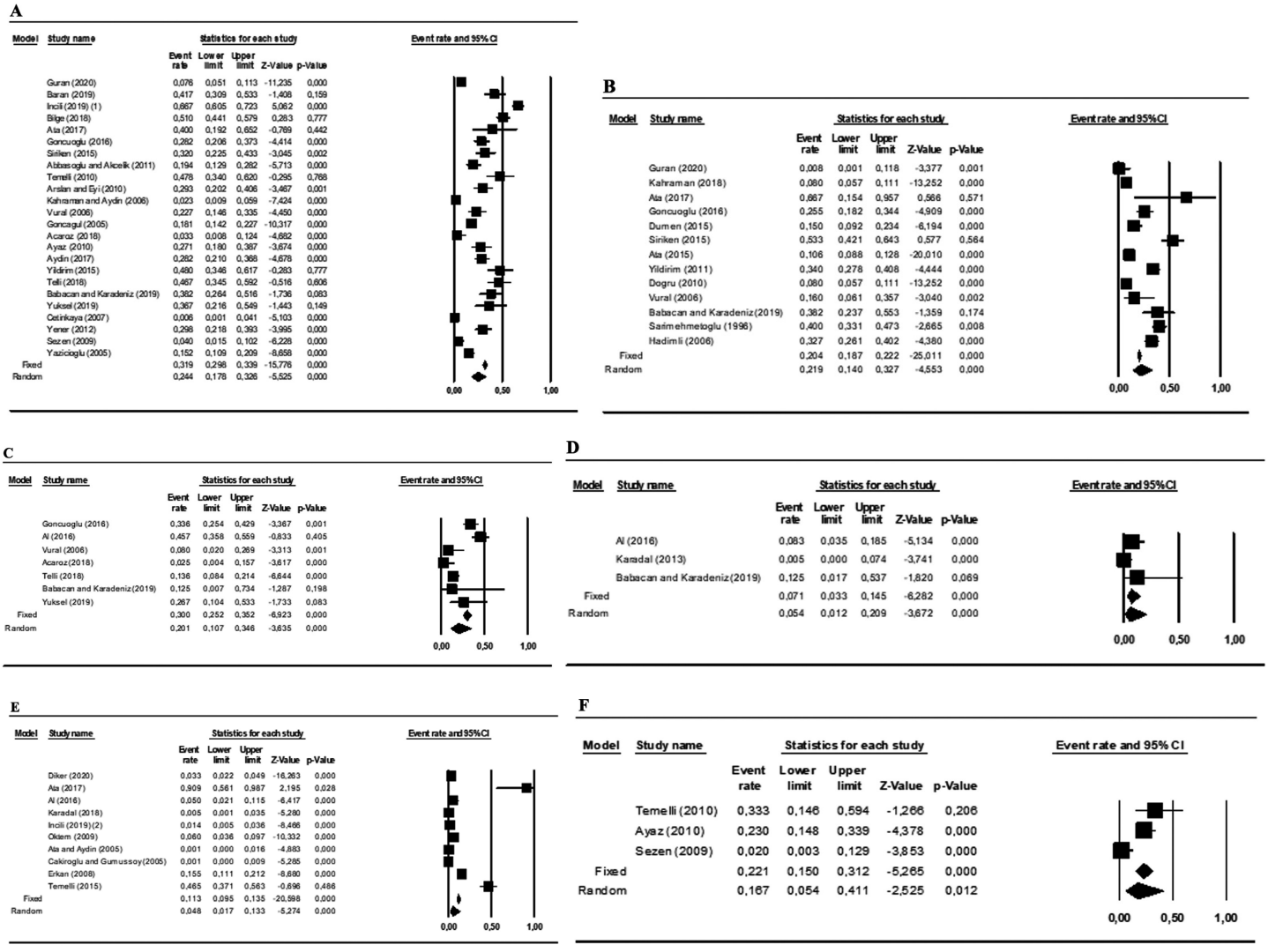
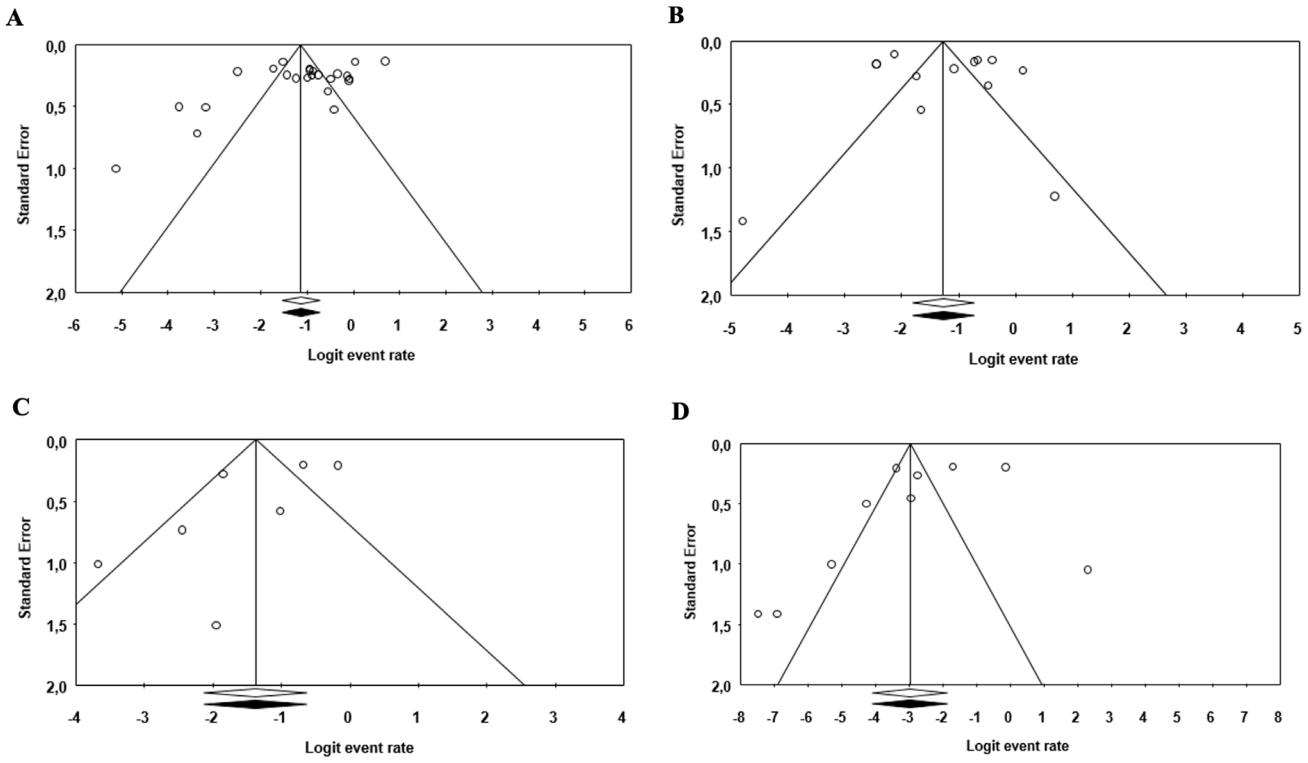


Supplementary Figure 1- Forest plots of the prevalence of *Salmonella* spp. in food types. Black boxes: effect size of each study; horizontal lines: 95% confidence interval; diamonds: overall effects for fixed and random effect models. (a) chicken parts, (b) chicken carcass, (c) chicken giblets, (d) chicken RTE food, (e) egg, (f) Turkey meat



Supplementary Figure 2- Funnel plots of the prevalence of *Salmonella* spp. in food types. Dots: individual studies; triangular regions: 95% confidence interval area; vertical lines: overall effect. (a) chicken parts, (b) Chicken carcass, (c) chicken giblets, (d) egg, chicken RTE food contains less than five studies, turkey meat contains less than five studies

Supplementary Table 1- Characteristics of included studies

Author/year	*Sample source*	*Sampling year*	*Food type*	*Number of samples*	*Salmonella spp. positive samples*	*Identified serovars*	*Antibiotic resistance profiles of Salmonella isolates*	*Antibiotic susceptibility test method*
Abbasoglu & Akcelik 2011	Retail markets	-	Chicken meat	103	20	*S.* Infantis	KAN, TET, NEO, SPEC, S, NA, TMP,	Disk diffusion
Acaröz et al. 2018	Retail markets	June-December 2017	Chicken parts Chicken giblets	100	3	-	-	-
Al et al. 2016	Retail Markets and Farms	July - August 2014	Egg Chicken RTE Chicken giblets	252	52	*S. typhimurium S. enteritidis*	AMP, TET, AMC, CFZ, ERY, GEN, NEO, NA, ENR, STX	Disk diffusion
Arslan & Eyi 2010	Retail markets	-	Poultry meat	75	22	*S. typhimurium S. bongori*	-	-
Ata & Aydin 2008	Farms	-	Egg	500	-	-	-	-
Ata et al. 2015	Slaughterhouse	January 2008 - January 2010	Chicken carcass	930	99	*S. typhimurium,* *S.* Infantis*, S. hadar, S. branderburg, S. kentucky, S. corvallis, S. agona, S. chincol, S. dabou, S. emek, S. essen, S. seftenberg, S. kingston, S. virchow*	AMP, TET, AMC, NA, C AZT, STX, GEN, CEF	Disk diffusion

Supplementary Table 1- Continued

Author/year	*Sample source*	*Sampling year*	*Food type*	*Number of samples*	*Salmonella spp. positive samples*	*Identified serovars*	*Antibiotic resistance profiles of Salmonella isolates*	*Antibiotic susceptibility test method*
Ata et al. 2017	-	2000 - 2015	Chicken parts Egg	29	18	*S. enteritidis*	-	-
Ayaz et al. 2010	Retail markets	June 2008 - May 2009	Chicken parts	214	36	*Salmonella* spp.	-	-
Aydın, 2017	Retail markets	-	Chicken parts	124	35	*Salmonella* spp.	-	-
Babacan and Karadeniz, 2019	Retail markets	-	Chicken parts Chicken RTE	100	35	*Salmonella* spp.	S, AMS, STX, NEO, OXT, TET, OFL, FLO, AMX, C, CIP, DOX, ENR	Disk diffusion
Baran et al. 2019	Retail markets	May-December 2016	Chicken parts	72	30	*Salmonella* spp.	STR, NA, CIP, TMP, C, GEN, STX, AMP, KAN, TET	Disk diffusion
Bilge et al. 2018	Retail markets	March-August 2017	Chicken parts	200	102	*Salmonella* spp.	GEN, CEF, AMP, C, CFZ, CTX, CIP, STR, STX, TET, NA, TMP	Disk diffusion
Cakıroğlu & Gümüşsoy, 2005	-	July-December 2003	Egg	882	0	-	-	-
Cetinkaya et al. 2008	Retail markets	December 2004 - June 2005	Chicken parts	168	1	*S.* Infantis	NA, STR, S, TET, TMP, STX	Disk diffusion
Diker et al. 2020	Farms	2015 - 2017	Egg	726	24	*S. Enteritidis, S. salamae*	-	-
Dümen et al. 2015	Retail markets	-	Chicken carcasses	100	15	*S. enteritidis, S. typhimurium*	-	-
Erkan et al. 2008	Retail markets	-	Egg	200	31	-	-	-
Goncagül et al. 2005	Retail markets	-	Chicken parts	315	57	*S. enteritidis*	-	-
Goncuoglu et al. 2016	Retail markets	January 2009 - March 2012	Chicken parts Chicken carcass Chicken giblets	330	96	*S. typhimurium*	AMC, FOX, IPM, AMP, C, CFZ, STX, TET, NA, GEN, KAN, EFT, CIP, SUL, TMP, CFZ, AK, CRO, STR, S	Disk diffusion
Guran et al. 2020	Retail markets	December 2016 - April 2018	Chicken parts Chicken carcass	348	22	*S.* Infantis	AK, AMC, AMP, CIP, CT, GEN, NET, TGC, STX	Phoenix NMIC-400/ID Panel
Hadimli, 2006	Retail markets	-	Chicken carcass	168	55	*Salmonella* spp.	-	-
İncili et al. 2019	Slaughterhouse	October 2013 - July 2014	Chicken parts	240	160	*Salmonella* spp.	AMP, CIP, NA, STR, GEN, SUL, TMP, C, TET, CT, CTX, CEF, FOX, CFP, MEM	Disk diffusion
İncili et al. 2019	Retail markets	January-December 2018	Egg	288	4	*Salmonella* spp.	-	-

Supplementary Table 1- Continued

Author/year	*Sample source*	*Sampling year*	*Food type*	*Number of samples*	*Salmonella spp. positive samples*	*Identified serovars*	*Antibiotic resistance profiles of Salmonella isolates*	*Antibiotic susceptibility test method*
Kahraman et al. 2018	Retail markets	July 2014 - December 2016	Chicken carcass	400	32	*Salmonella* spp.	AMP, CTX, MEM, IMP, KAN, STR, NA, CIP, TET, C, ERY, STX	Disk diffusion
Kahraman and Aydin, 2009	Retail markets	March 2007 - February 2008	Chicken parts	175	4	*Salmonella* spp.	-	-
Karadal et al. 2013	Retail markets	September-December 2012	Chicken RTE	100	0	-	-	-
Karadal et al. 2018	Retail markets	-	Egg	200	1	*Salmonella* spp.	-	-
Kasimoglu-Dogru et al. 2010	Retail markets	2003 - 2005	Chicken carcass	400	32	*S. enteritidis, S. vircho, S. typhimurium, S. hadar*	PG, NA, CEP, STR, TET	Disk diffusion
Öktem et al. 2009	Retail markets	-	Egg	250	15	*S. enteritidis*	-	-
Sarımehmetoğlu et al. 1996	Slaughterhouse	May - July 1995	Chicken carcass	180	72	-	-	-
Sezen, 2009	Retail markets	February -June 2007	Chicken parts Turkey meat Quail meat	175	6	*Salmonella* spp.	-	-
Siriken et al. 2015	Retail markets	2008 - 2009	Chicken carcass Chicken parts	150	64	*Salmonella* spp.	GEN, VAN, C, STR, CRO, TET, NA, AMP, STX	Disk diffusion
Telli et al. 2018	Retail markets	January 2015 - January 2017	Chicken parts Chicken giblets	170	43	*S. enteritidis, S. typhimurium*	AK, AMP, CEP, CEZ, CIP, CLI, C, ERY, GEN, KAN, NA, OX, P, STX, TEI, TET, VAN	Disk diffusion
Temelli et al. 2010	Retail market	-	Chicken parts	61	27	-	-	-
Temelli et al. 2015	Retail markets	-	Egg	101	47	*S. enteritidis*	-	-
Vural et al. 2006	-	-	Chicken carcasses Chicken parts Chicken giblets	125	23	*Salmonella* spp.	-	-
Yazıcıoglu et al. 2005	Slaughterhouse	-	Chicken parts	197	30	*S. virchow, S. bsilla, S. enteritidis, S. typimurium*	NA, STR	Disk diffusion
Yener et al. 2012	Retail markets	-	Chicken parts	104	31	-		
Yildirim et al. 2011	Retail markets	April 2005 - March 2006	Chicken carcass	200	68	*S.typhimurium, S.*Infantis*, S.heidelber, S.hadar, S.enteritidis, S.newport, S.thompson, S.montevideo, S.agona, S.ohio, S.rough, S.strain*	PG, OX, CLI, VAN, ERY, AMP, TET, STR, NEO, CEP, GEN, C, CTX, AK	Disk diffusion
Yildirim et al. 2015	Retail markets	-	Chicken parts	50	24	*Salmonella* spp.	-	-

Supplementary Table 1- Continued

Author/year	*Sample source*	*Sampling year*	*Food type*	*Number of samples*	*Salmonella spp. positive samples*	*Identified serovars*	*Antibiotic resistance profiles of Salmonella isolates*	*Antibiotic susceptibility test method*
Yüksel et al. 2019	Retail markets	-	Chicken parts Chicken giblets	90	15	*Salmonella* spp.	CIP, STX, C, STR, AMP, GEN, KAN, NA, TMP, TET	Disk diffusion

AK: Amikacin, AMC: Amoxicillin/clavulanic acid, AMP: Ampicillin, AMS: Ampicillin sulbactam, AZT: Aztreonam, CFZ: Cefazoline, CFP: Cefepime, CE: Ceftazidime, EFT: Ceftiofur, FOX: Cefoxitin, CTX: Cefotaxime, CEF: Cefoperazone, CRO: Ceftriaxone, CEP: Cephalothin, CEZ: Cephazolin, C: Chloramphenicol, CIP: Ciprofloxacin, CLI: Clindamycin, CT: Colistin, DOX: Doxycycline, ENR: Enrofloxacin, ERY: Eryhtromycin, FLO: Florphenicol, GEN: Gentamicin, IPM: Imipenem, KAN: Kanamycin, MEM: Meropenem, NA: Nalidixic acid, NEO: Neomycin, NET: Netilmicin, OFL: Ofloxacine, OX: Oxacillin, OXT: Oxytetracycline, PG: Penicillin G, STR: Streptomycin, SPEC: Spectinomycin, SUL: Sulfamethoxazole, S: Sulphonamide, TEI: Teicoplanin, TET: Tetracycline, TGC: Tigecycline, TMP: Trimethoprim, STX: Trimethoprim-sulfamethoxazole, VAN: Vancomycin

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Does Dry or Fresh Bee Bread Contain Clinically Significant, and Antimicrobial Agents Resistant Microorganisms?

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ABSTRACT

Bee bread is fermented and naturally preserved pollen that is enriched with digestive enzymes and organic acids from both honey and the salivary gland secretions of honeybees. As yet, there is insufficient information concerning which bacteria and yeasts are involved in the fermentation. This study seeks to determine the contents of microorganisms in fresh and dry bee bread samples and to ascertain the antimicrobial resistance of these isolated microorganisms. Fresh and dry bee bread samples were obtained from 8 different colonies that were cultivated in suitable medium to reproduction the aerobic microorganisms, anaerobic microorganisms, and fungi. The isolated strains in bee bread samples were identified by conventional and MALDI-TOF MS methods. The minimal inhibitory concentrations (MIC) of the antimicrobial agents for strains were determined according to the Clinical and Laboratory Standards Institute (CLSI). The 34 strains were isolated from fresh bee bread samples. There were no microrganisms reproduced in the dried bee bread samples. The 34 isolated strains were; *Aspergillus* spp. (12), *Rhizopus oryzae* (6), *Mucor circinelloides* (1), *Bipolaris* (2), *Trichoderma* (3), *Paecilomyces variotii* (1), *Penicillium chrysogenum* (1), *Kodamaea ohmeri* (1), *Bacillus altitudinis/pumilus* (3), *Bacillus licheniformis* (1), *B. megaterium* (1), *Micrococcus luteus* (1) and *Serratia marcescens* (1). The MIC values of itraconazole (IT), voriconazole, anidulafungin (AND), and caspofungin (CS) for *Mucor* and *Rhizopus* strains were higher (≥32 µg/mL), with the exception of amphamphotericin B posaconazole. All antifungal agents had lower MIC values compared to the *Aspergillus, Bipolaris, P. variotii*, and *K. ohmeri* strains. The *trichoderma* strains had low MIC values (≤0.50 µg/mL), with the exception of IT. The *P. chrysogenum* strains were found to have low MIC value (≤0.25 µg/mL) compared to posaconazole, AND, and CS. In this study, there were no microorganisms reproduce in the dried bee bread samples stored under suitable conditions. In addition, it was concluded that yeast, mold, and bacteria isolated in fresh bee bread samples may be resistant to antibiotics and antifungal drugs.

Keywords: Perga, Bacteria, Yeast, Molds, Antimicrobial susceptibility

Introduction

Honeybees use bee bread rather than honey and pollen for their nutrition. Because both nectar and pollen undergo some biochemical changes before being consumed by honey bees. While the pollen brought to the hive by the bees is filled into the honeycomb cells, honey, organic acids in the salivary gland secretions of the bees and digestive enzymes are added to the pollen (Deveza et al. 2015). Then, lactic acid fermentation caused by *Lactobacillus* bacteria takes place under anaerobic conditions. An important reason for fermentation is the dissolution of the outer layer (exine) of the pollen and the easy absorption of the nutrients in the pollen interior. Thus, the fermentation process serves not only to preserve the pollen content but also to form new compounds. During fermentation, bee pollen proteins are broken down into peptides and amino acids. DeGrandi - Hoffman (2013) reported that the protein concentration of pollen is higher than that of bee bread, while the amino acid concentration is lower. In another study, the lactic acid concentration in bee bread was found to be 6 times higher than in pollen (Nagai et al. 2005). It is also reported that bee bread contains vitamin K, which is not found in fresh pollen and is richer in B vitamins (Gilliam 1979a,b).

Bee bread has higher nutritional value, better digestibility, and richer chemical composition than bee-collected pollen (Habryka et al. 2016). Bee bread contains carbohydrates (24-34%), proteins (14-37%), lipids (6-13%), and other nutrients such as minerals and vitamins (Stanciu et al. 2009; Tomas et al. 2017; Kieliszek et al. 2018; Belina-Aldemita et al. 2019). In addition, it provides the essential amino acids humans cannot synthesize (Bonvehi & Escola 1997; Human & Nicolson 2006). However, the chemical composition of bee bread varies depending on the botanical origin, geographical location, climatic condition, soil type, beekeepers' activities, or storage treatments in commercial production (Pascoal et al. 2014; Ares et al. 2018). Since it contains easily digestible sugars, oil, mineral components, and higher free amino acids compared to bee pollen, it has a higher bioavailability and is easily digestible (Nagai et al. 2005). The antibacterial and antioxidant activity, immune system benefits, intestinal regulator, anti-fatigue, lipid regulator in blood and tissue, and antiaging effects of bee bread has been discussed in various studies (Villanueva et al. 2002; Nogueira et al. 2012; Kaplan et al. 2016; Urcan et al. 2018; Bakour et al. 2019).

The fact that fresh bee-collected pollen contains 21-30% water causes the development of microorganisms and the deterioration of the pollen. For this reason, it is recommended to dry it at the appropriate temperature (40 °C) and store it in a deep freezer to preserve its biological activity (Barene et al. 2015). Bee bread is more acidic than pollen and is more durable because it is a fermented product. Therefore, bee bread and pollen products should be dried, and water activity should be reduced to prevent microbial growth. In the studies carried out to date, *Candida parapsilosis, Cryptococcus neoformans, Pichia dispore, Saccharomyces heterogenicus, Torulopsis etchellsii, Torulopsis magnoliae, Torulopsis stellata, Zygosaccharomyces bailii, Bacillus subtilis, B. pumilis*, *B. licheniformis,* Penicillium spp. *Assporiformis cladosporioides,* and *Scopulariopsis brevicaulis* are among the microorganisms isolated from bee bread (Egorova 1971; Gilliam et al. 1989; Sinpoo et al. 2017). Although the fresh form of Bee bread, which is a fermented product, is the best way of consumption. It is necessary to find out the microbial content of both fresh and stored forms of bee bread.

This study seeks to determine the contents of microorganisms in fresh and dry bee bread samples, and to identify the antimicrobial resistance of these isolated microorganisms.

2. Material and Methods

2. 1. Bee bread samples

Bee bread samples were obtained from bee colonies in Erciyes University Agricultural Research Center and Erciyes Technopark (Nutral Therapy, Co), Kayseri. Fresh bee bread samples collected from 8 different colonies were brought to the laboratory under hygienic conditions. Half of each sample (one colony) was divided into two. The first half (fresh bee bread) was analyzed as soon as collected, and the second half (stored bee bread) was kept in a deep freezer for three months after being dried at 40 °C (Figure 1). Afterwards all samples were analyzed.

Figure 1- Bee bread samples

2. 2. Microbiological analysis

A microbiological analysis of each bee bread (fresh and stored) was performed according to the method described by Gilliam et al. (1989). Each sample was divided into four sub-samples of approximately 1 g and studied in 4 replicates. Each of these four replicates was homogenized in 2.5 mL of sterile 0.85% NaCl on a glass shredder, taking care of sterilization at each stage of the study. The homogenates (100 µl) were incubated in Sabouraud's dextrose agar (SD, Oxoid, UK) medium with and without antibiotics (cycloheximide and chloramphenicol) in incubators adjusted to 37 °C and 25 °C to determine the fungi contents of samples. When yeast and mold colonies appeared, their purity was checked.

The identification of yeasts were performed according to phenotypical methods with macroscopic morphology on SDA, microscopic morphology on corn meal agar, germ tube test, growth ability at 37 ºC, urea hydrolysis, and a carbohydrate assimilation test using API 20C AUX (BioMérieux, France) kits.

Mold identification was performed according to phenotypical methods with macroscopic morphology on SDA, microscopic morphology on corn flour agar and potato dextrose agar, growth ability at 37 ºC, and sensitivity to cycloheximide.

Molecular identification of all isolates at the species and genus level was performed with MALDI-TOF MS (VITEK® MS, BioMérieux, Marcy l'Etoile, Made by France) (Mutlu Sariguzel et al. 2016). The strains were tested by depositing one colony on a steel MALDI target slide by loop and a drop of formic acid was placed on the slide. The spot was dried and then overlaid with 1 µl MALDI matrix solution (VITEK MS-CHCA) and air-dried. For the mold colony, the colony was mixed with 70% alcohol. After centrifugation at 10000 rpm for 2 minutes, the upper liquid was poured out, 40 microliters of 70% formic acid and 40 microliters of 99% acetonitrile was added to the bottom sediment and 1 microliter of the top liquid was added after centrifugation at 10000 rpm for 2 minutes. The top liquid was dropped into the slide well and after a steel MALDI target slide dried, the Matrix was dropped. The prepared slide was inserted into the VITEK®MS system. Identification of yeast, mold and bacteria was analysed using the VITEK® MS database. The peaks from these spectra were compared to the characteristic pattern for a particular species, genus or family of microorganisms and this allowed for organism identification. The quality control strains were *C. albicans* ATCC 90028. *E. coli* ATCC 8739 was used to calibrate the instrument for each run (Mutlu Sariguzel et al. 2016).

To investigate aerobic and anaerobic bacteria microorganisms, 100 µl of homogenate was inoculated on 5% sheep blood agar (Oxoid, UK), chocolate agar, MacConkey agar (Oxoid, UK), Schaedler +5% sheep blood agar (BioMérieux, Marcy, France). Schaedler Neo Vanco +5% sheep blood agar (BioMérieux, Marcy, France) followed by plates were incubated under aerobic and anaerobic conditions in 37 °C and 25 °C incubators. The bacterial isolates were identified using conventional microbiological methods (Gram stain, oxidase, catalase, aerotolerance testing), automated systems with VITEK2 cards (BioMérieux, France), and MALDI-TOF MS (VITEK® MS, BioMérieux, Marcy l'Etoile, France).

2. 3. Antimicrobial susceptibility tests of strains

2. 3. 1. Antifungal susceptibility tests of strains

The gradient diffusion test (E-test strips) and broth microdilution method were used for antifungal susceptibility testing. The MIC values of itraconazole (IT), voriconazole (VO), amphotericin B (AP), fluconazole (FLU) and ketoconazole (KTZ) (Sigma Chemical Co, St. Louis, USA) were determined using the the broth microdilution method. The MIC values of posaconazole (POS), anidulafungin (AND), and caspofungin (CS) were determined via the gradient diffusion test (Etest^{R}, bioMerieux, Marcy Etoile, France). FLU and KTZ susceptibility were investigated only in yeast strains. The *in vitro* efficacy of antifungal drugs has been studied according to the recommendation of the CLSI M27-S4 for yeast and CLSI M38-A2 for molds (Clinical and Laboratory Standards Institute 2008; 2017).

1. 3. 1. 1. Broth microdilution method

RPMI 1640 medium with 34.53 g MOPS (3-N-morpholinepropanesulfonic acid) (PanReac & AppliChem, USA) containing L-glutamine, free of sodium bicarbonate and phenol red (Sigma-Aldrich, UK) was used for the broth microdilution method. Two-fold dilutions of drugs were made and dispensed into 96-well flat-bottom plates at concentrations ranging from 64-0.125 µg/mL for FLU, 16-0.03 µg/mL for AP, IT, VO, and KTZ. These plates were incubated at 35 °C for 24-48 hours. The fungal inoculum was prepared from a 24-hour culture of SDA (Oxoid, UK) incubated at 35 °C, and mold suspensions were prepared from well-spored cultures grown on potato dextrose agar and adjusted spectrophotometrically to a turbidity.

The MIC of the antifungal agents used were determined according to the CLSI recommendations.

2. 3. 1. 2. Gradient diffusion test (E-test strips)

The suspension of fungal strain (0.5 MacFarland) was spread on Mueller-Hinton agar (BD Diagnostics, France), and POS, AND, and CS MIC gradient E-test strips were applied. All media were then incubated at 37 °C. The diameters of the inhibition zones were measured after 24-48 hours of incubation.

2. 3. 2. Antibacterial susceptibility tests of strains

2. 3. 2. 1. Disk diffusion method

In vitro, antibiotic susceptibility patterns of bacterial isolates were determined by the disk diffusion method, and the results were interpreted according to CLSI (Clinical and Laboratory Standards Institute, 2015). Sterile swabs were dipped in bacterial suspensions (0.5 MacFarland) and plated on Müller Hinton agar. Antibiotic discs were placed on the plate, and all media were incubated at 37 °C. The diameters of the inhibition zones were measured after 24 hours of incubation. The antibiotics were investigated: penicillin (1 µg/mL), ampicillin (2 µg/mL), amoxicillin-clavulanic acid (20/10 µg/mL), levofloxacin (5 µg/mL), erythromycin (15 µg/mL), clindamycin (2 µg/mL), co-trimoxazole, cefoxitin (30 µg/mL), ceftriaxone (30 µg/mL), cefuroxime (15 µg/mL), gentamicin (10 µg/mL), vancomycin (30 µg/mL), meropenem (1 µg/mL). Meropenem has been studied only for gram-negative strains. All discs are Oxoid, a UK brand.

2. 4. Quality control strains

C. albicans ATCC 90028. and *E. coli* ATCC 25922 were used as quality control strains.

3. Results

All 34 microorganisms, 7 bacteria, 1 yeast, and 26 molds, were isolated from fresh bee bread samples (n=8). Anaerobic microorganisms were not detected in the samples. Bacteria, yeast, and mold did not grow in stored bee bread samples (n=8). Bacteria, yeast, and mold species isolated from fresh bee bread are shown in Table 1. Bee bread samples are shown in Figure 1. The number of strains isolated from bee bread samples collected from different colonies is shown in Figure 2a. *A. niger* colonies is shown in Figure 2b.

Molds isolated from fresh bee bread were *Aspergillus niger* (5), *Aspergillus fumigatus* (1), *Aspergillus nidulans* (2), *Aspergillus terreus* (2), *Aspergillus flavus* (2), *Rhizopus oryzae* (6), *Mucor circinelloides* (1), Bipolaris spp. (2), Trichoderma spp. (3), *Paecilomyces variotii* (1), *Penicillium chrysogenum* (1); yeast: *Kodamaea ohmeri* (1); bacteria: *Bacillus altitudinis/pumilus* (3), *Bacillus licheniformis* (1), *Bacillus megaterium* (1), *Micrococcus luteus* (1) and a yeast; *Serratia marcescens* (1). Figure 3 shows the MALDI-TOF MS protein spectra of *A. fumigatus* strain.

The yeast fungus *Kodamaea ohmeri* was isolated and obtained from only one of the eight colonies. *Rhizopus oryzae,* which is the most common mold fungus, was isolated from six different colonies. and *Aspergillus niger* was isolated from 5 different colonies. The majority of microorganisms were isolated from fresh bee bread of colony 1, including seven molds (3 *Aspergillus* spp., *Rhizopus*, *Mucor, Bipolaris* spp., *Trichoderma* spp.), a yeast (*Kodamaea ohmeri*), and one bacteria (*Serratia marcescens*). In addition, three *Aspergillus* spp., one Rhizopus, and one *Bacillus altitudinis/pumilus* strains were isolated in colony 5. In colonies 2 and 3, four different microorganisms as mold and bacteria were isolated. In the other colonies, three different microorganisms as mold and bacteria were isolated (Table 1).

Bacillus altitudinis/pumilus strains were isolated from colonies 2, 5, and 7. *Bacillus licheniformis* was isolated from colony 2, and *Bacillus megaterium* from colony 4. *Micrococcus luteus* strain was only isolated from colony 3.

All *Aspergillus* and *Bipolaris* strains had low MIC values for all tested antifungal drugs. *Trichoderma* strains isolated from colonies 1 and 2 had low MIC values, with the exception of ITR. The *Paecilomyces variotii* strain isolated from colony 3 had low MIC values for other antifungal drugs, except CS and VO (8 µg/mL).

The MIC values of antifungal drugs compared to the *Mucor* and *Rhizopus* strains were determined to be high, with the exception of AP-POS (MIC ranges 2-0.125 µg/mL). For the *Penicillium chrysogenum* strain isolated from colony 2, the MIC values of POS, AND, and CS were found to be low (MIC range 0.008-0.25 µg/mL)

Kodamaea ohmeri was sensitive to all the antifungal drugs studied, including KTZ and FLU. The FLU and KTZ MIC values for the *Kodamaea ohmer* strain were 8 µg/mL and 0.064 µg/mL, respectively. Table 2 shows the Minimal Inhibitor Concentrations (µg/mL) for six antifungal agents of fungal strains isolated from fresh bee bread samples.

Table 1- Bacteria, yeast and mold species isolated from fresh bee bread samples collected from eight different colonies

Microorganisms	*Colonies*							
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
Molds								
Aspergillus fumigatus	+							
Aspergillus niger	+		+		+	+		+
Aspergillus nidulans	+						+	
Aspergillus terreus				+	+			
Aspergillus flavus					+			+
Rhizopus oryzae	+		+		+	+	+	+
Mucor circinelloides	+							
Bipolaris spp.	+			+				
Trichoderma spp	+	+				+		
Paecilomyces variotii			+					
Penicillium chrysogenum		+						
Yeast								
Kodamaea ohmeri	+							
Bacteria								
Bacillus altitudinis/pumilus		+			+		+	
Bacillus licheniformis		+						
Bacillus megaterium				+				
Micrococcus luteus			+					
Serratia marcescens	+							

+: There was growth

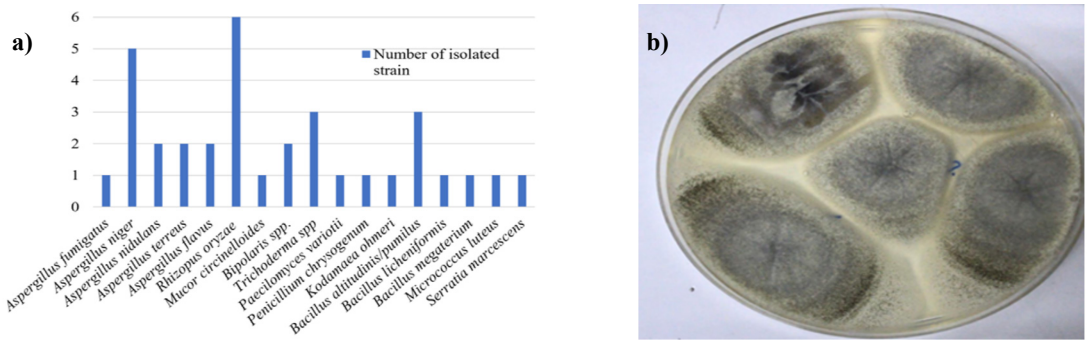

Figure 2- a)- The number of isolated strains from bee bread samples collected from different colonies, b) *A. niger* colonies

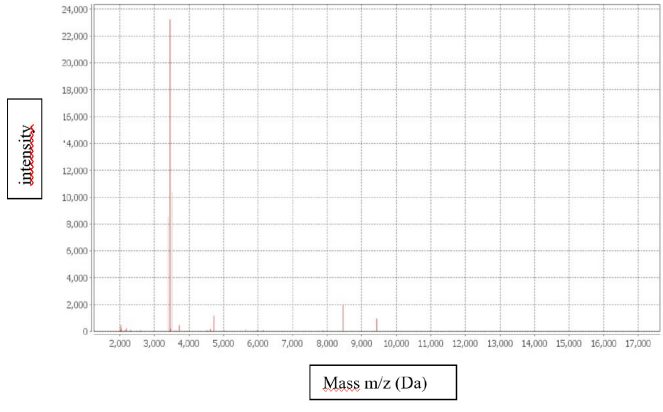

Figure 3- MALDI-TOF MS protein spectra of *A. fumigatus* strain

The epidemiological value (ECV) for six antifungal drugs (IT, VO, POS, AND, AP and CS) of the *Aspergillus* strains were found to be below the values determined according to CLSI. However, the six antifungal drugs' MIC values of the *Mucor* and *Rhizopus* strains were found to be higher than the *Aspergillus* strains. The *Mucor circinelloides* strain isolated from colony 1 was found to be 0.125 µg/mL for AP, but high MIC values were determined for other antifungal drugs. The *Rhizopus oryzae* strains were found to have high MIC values for other antifungals with the exception of AP and POS. The MICs (µg/mL) for IT, VO, POS, AND, AP, and CS of fungal strains isolated from bee bread samples are shown in Table 2.

Serratia marcescens was observed to be sensitive to ceftriaxone, meropenem, gentamicin and cotrimoxazole. The *Bacillus* spp. and *Micrococcus luteus* strains do not have susceptibility limit values determined in CLSI, only the zone diameters of antibiotics are given (Table 3).

4. Discussion

There is a heterogeneous microorganism community consisting of bacteria, yeast and molds in the ripening stage of bee bread (Haydak 1958; Di Cagno et al. 2019; Disayathanoowat et al. 2012). During intense incubation activities, bee bread is consumed in a few days, while the surplus can be stored in the honeycomb cells for several months (vanEngelsdorp et al. 2009; Podriznik & Bozic 2015). However, consumption of 21-day-old bee bread compared to 14-day-old bee bread has been reported to cause harm to the colony health and gut microbiome (Maes et al. 2016).

Table 2- Minimal inhibitor concentrations (µg/mL) for six antifungal agents of fungal strains isolated from fresh bee bread samples

Colonies	*Microorganisms*	*IT*	*VO*	*POS*	*AND*	*AP*	*CS*
		µg/mL					
1	*Aspergillus fumigatus*	0.50	0.125	0.064	0.002	0.25	0.016
	Aspergillus niger comp.	0.50	0.064	0.125	0.002	0.25	0.004
	Aspergillus nidulans	0.125	0.004	0.016	0.032	1	0.125
	Rhizopus oryzae comp.	>32	>32	2	>32	2	>32
	Mucor circinelloides	>32	>32	2	>32	0.125	>32
	Bipolaris spp.	0.50	1	0.002	0.002	0.125	0.016
	Trichoderma spp.	4	0.25	0.50	0.004	0.016	0.064
	Kodamaea ohmeri	0.25	0.064	0.032	0.25	0.008	0.50
2	*Trichoderma* spp	>32	0.25	0.50	0.008	0.25	0.032
	Penicillium chrysogenum	>32	>32	0.25	0.002	12	0.008
3	*Aspergillus niger com.*	0.5	0.50	0.25	0.002	0.25	0.006
	Rhizopus oryzae com.	>32	32	2	>32	1	>32
	Paecilomyces variotii	0.016	8	0.032	0.002	0.064	8
4	*Aspergillus terreus*	0.064	0.125	0.016	0.008	0.5	0.5
	Bipolaris spp.	0.008	0.064	0.002	0.008	0.008	0.002
5	*Aspergillus niger comp.*	0.50	0.125	0.064	0.002	0.25	0.004
	Aspergillus terreus	0.064	0.125	0.016	0.008	0.50	0.50
	Aspergillus flavus	0.125	0.125	0.064	0.002	1	0.064
	Rhizopus oryzae comp.	>32	>32	2	>32	2	>32
6	*Aspergillus niger comp.*	1	0.25	0.002	0.002	0.125	0.008
	Rhizopus oryzae comp.	>32	>32	0.50	>32	2	>32
	Trichoderma spp	32	0.25	0.50	0.002	0.125	0.064
7	*Aspergillus nidulans*	0.125	0.008	0.002	0.002	0.25	0.002
	Rhizopus oryzae comp.	>32	>32	0.50	>32	1.5	>32
8	*Aspergillus niger comp.*	1	0.025	0.008	0.002	0.25	0.002
	Aspergillus flavus	0.50	0.125	0.064	0.002	0.50	0.032
	Rhizopus oryzae comp.	>32	>32	0.50	>32	1	>32

In our study, molds were found to be the most frequently detected microorganism in bee bread samples. Molds are multicellular fungi that are common in nature on air, soil, water and organic materials and form mycelium. Molds have low water activity and optimum breeding temperatures are between 22-32 °C. Although the optimum pH requirements for molds vary between 5-6, some species of molds can grow in highly acidic environments such as pH 2.5. Molds have become industrially important due to both positive and negative changes in foods. The *Aspergillus* species are the most common molds and are used in the production of citric and gluconic acid from sucrose, as well as in the production of essential enzymes for the food industry such as amylase and pectinase. The *Aspergillus* species are used in the production of lipase and protease enzymes and in the ripening of cheeses in fermented food production. Although the pathogenic and toxic effects of molds in foods are negligible, some molds are important because they make mycotoxins and are pathogenic. The *Aspergillus flavus* and *A. parasiticus* species produce a deadly mycotoxin known as aflatoxin, which is a health risk. Mucor is a class of mold that is abundant in soil, plants, rotting fruits and vegetables. *Rhizopus oryzae* is used in the production of prebiotic and probiotic fermented soy products and lipase enzyme production (Matthews et al. 2017).

In one of the earliest studies on the microbiological content of bee bread, Burnside reported that some molds (*Cladosporium, Mucor, Penicillia* and *Aspergillus*) were found in the honeycombs and in the pollen stored in the hive (Burnside 1929). *Penicillium* was found to be the most common mold, *Aspergillus* less common and *Mucor* spp. was not isolated in their study. Yoder et al. (2013) reported that *Aspergillus, Penicillium, Rhizopus* and *Cladosporium* were the most commonly isolated strains in bee bread. Also, the researchers reported that it was found the presence of molds such as *Absidia, Alternaria, Aureobasidium, Bipolaris, Fusarium, Geotrichum, Mucor, Nigrospora, Paecilomyces, Scopulariopsis* and *Trichoderma*. In our study, while the *Aspergillus* species was the most frequently isolated in fresh bee bread, followed by *Rhizopus* spp. A study, it was reported that molds such as *Cladosporium, Aspergillus* and *Penicillium* were found in pollen but disappeared after six weeks of storage in the hive (Sinpoo et al. 2017).

In this study, only one yeast was isolated from the bee bread samples. Gilliam (1979 a) reported that yeast strains isolated from bee bread were less than pollen due to the different chemical and physical properties of bee products. Since the acidic character of bee bread creates a suitable environment for the development of yeast, yeast is crucial in transforming pollen into bee bread. The pH of the environment is important in the transformation of pollen into bee bread. Yeasts are the ones that provide this acidic environment. At the same time, yeasts play a role in the synthesis of B vitamins found in bee bread. It has been reported that bacteria such as lactic acid bacteria found in bee bread benefit from amino acids and vitamins produced by yeast strains in bee bread (Egorova 1971). It has been reported that different genus and types of yeasts were isolated in different studies investigating microorganisms in bee bread (Gilliam et al. 1977; Čadež et al. 2015). To our knowledge, *Kodamaea ohmeri* was first isolated from bee bread in our study, and this strain was found to be susceptible (a low MIC value) to all antifungals, including FLU (8 µg/mL) and KTZ (0.064 µg/mL). In a study done in 2020, a yeast isolation protocol was developed in pollen samples stored with bees from two apiaries in Belgium. Yeast isolates

Table 3- Zone diameters for various antibiotics of *Bacillus* spp. and *Micrococcus luteus* strains

	≤10 mm	***11-24 mm***	***≥25 mm***
Bacillus altitudinis/pumilus	Clindamycin	Vancomycin Cefuroksim Ceftriaxone	Penicillin Erythromycin, Co-Trimoxazole, Levofloxacin, Ampicillin, Cefoxitin, Amoxicillin, Gentamicin
Bacillus licheniformis	Penicillin Clindamycin	Vancomycin Cefuroksim Ceftriaxone Ampicillin	Erythromycin, Co-trimoxazole, Levofloxacin, Cefoxitin, Amoxicillin, Gentamicin,
Bacillus megateriumw	Clindamycin	Vancomycin Penicillin Ampicillin	Erythromycin, Co-trimoxazole, Cefoxitin, Amoxicillin, Gentamicin, Ceftriaxone,
Micrococcus luteus	-	Vancomycin	Erythromycin, Co-trimoxazole, Cefoxitin, Amoxicillin, Gentamicin, Ceftriaxone, Ampicillin, Ceftriaxone, Clindamycin

were grouped according to their macro and micromorphology and representative isolates were identified using DNA sequences. Most of the 252 isolates identified were found to belong to the genera Starmerella, Metschnikowia, and Zygosaccharomyces. According to the results of this study, Detry et al. (2020) reported that high yeast abundance in fresh bee bread decreased rapidly with storage time, Candida species were dominant in fresh bee bread, and Zygosaccharomyces members were mostly isolated from aged bee bread.

While studies on the microbiological analysis of bee bread are limited, it is clear that there is an relationship between bacteria, yeast, and molds. This study detected only 7 bacteria (5 Bacillus species) in the bee bread samples Vasquez and Olofsson reported that the lack of pathogenic microorganisms in bee bread may be due to the accumulation of various metabolites during fermentation by lactic acid bacteria. These metabolites reduce the number of microorganisms by lowering the pH of the environment (Vasquez & Olofsson 2009). The group of microorganisms remaining in the finished product are Gram-positive aerobic bacilli and some molds that can survive in adverse environmental conditions (Audisio et al. 2005). Some lactic acid bacteria produce hydrogen peroxide at a concentration that inhibits the growth of many pathogenic microorganisms (Bang et al. 2003). DeGrandi-Hoffman et al. (2013) reported that bee bread contains bactericidal compounds, as well as carbohydrates and lactic acid, that are effective in reducing the growth of microorganisms, which include mold, and spoilage bacteria. *Bifidobacterium* and *Lactobacillus* inhibitor the growth of *Staphylococcus aureus,* Gram-negative bacteria like *Listeria monocytogenes, Escherichia coli,* and *Camplylobacter jejuni*, rods like *Salmonella, Shigella, Vibrio,* and *Klebisella* species and yeast like *Candida albicans.* Lowering the pH value during fermentation is attributed to the activity of lactic acid bacteria that are introduced into the pollen from the gastrointestinal tract of the bees. Moreover, it has been reported that lactic acid bacteria isolated from the honeybee digestive tract inhibit the growth of the pathogen *Paenibacillus larvae* (Vásquez et al. 2012). The reason why no pathogenic microorganisms were detected in bee bread is thought to be a result of the accumulation of various metabolites by the fermentation process carried out by lactic acid bacteria. These metabolites reduce the number of microorganisms by lowering the pH of the environment (Vásquez & Olofsson 2009). In addition, some lactic acid bacteria are reported to produce hydrogen peroxide at concentrations that inhibit the growth of some pathogenic species (Bang et al. 2003). After the fermentation of the pollen, *Bacillus* spp. spores and fungi have been the dominant microorganism group in the pollen (Gilliam 1979 a,b). Fungal growth causes degradation of the outer wall of the pollen, potentially changing the nutritional value of the pollen (Gilliam 1979a). In addition, bacteria and fungi isolated from pollen contribute to the stabilization and transformation of pollen by producing enzymes, vitamins, antibacterial substances, organic acids, and lipids (Anderson et al. 2014). Bee pollen and bee bread inhibit the growth of antibiotic-resistant microorganisms. This effect is higher in gram positive bacteria than in Gram-negative bacteria. In studies with ethanol extract of pollen, it has been shown that it is effective against many bacteria such as *Pseudomonas aeruginosa, Staphylococcus aureus, Paenibacillus larvae* (Carpes et al. 2007; Fatrcovaa-Sramkovaa et al. 2016). Lactic acid bacteria accepted in GRAS status gain energy with the transformation of saccharides in the environment with typical fermentation metabolism, and was produced bacteriocins and metabolic products such as organic acids, diacetyl, acetoin, acetaldehyde (Forsgren et al. 2010; Wasko et al. 2012).

Bobis et al. (2010) reported that raw pollen contains 5.10^5 cfu/g of aerobic mesophilic microorganisms and about 1.10^2 cfu/g yeast and mold. A 2015 study by De-Melo et al. (2015) demonstrated mesophilic bacteria's presence at a level of <10 to 1.1 10^4 cfu/g in dried Brazilian pollen. Nogueira et al. (2012) showed that psychrophilic bacteria and bacilli, respectively, ranged from <10 to 1.1 103 cfu/g, <10 to 2.8 103 cfu/g, while the number of yeasts and molds in pollen ranged from <10 to 7.6 103 cfu/g. *Zygosaccharomyces rouxii* yeast was found in commercial samples of dried bee pollen from Portugal and Spain (Nogueira et al. 2012). Deveza et al. (2015) showed the presence of *Aspergillus* and *Cladosporium*, among the most common molds, in Brazilian bee pollen.

Pollen contamination by microorganisms is deppendent on the harvesting practices, cleaning, drying, and storage of the crop as well as its nutrient composition. As yet, there are no international microbiological limits on bee bread. In addition, according to International Honey Commission (IHC) and quality control standards, acceptable microorganism load in pollen can be <10 cfu/g for aerobic microorganism, <5 104 cfu/g for yeast and mold, and max 1 cfu/g for *Enterobacteriaceae*. Pollen should not contain *E. coli, Salmonella* spp. and *Staphylococcus aureus* (Campos et al. 2008; De-Melo et al. 2015).

In our study, mold was isolated in all bee bread samples. Detry et al. (2020) found mold and yeast in their study on bee pollen in 50% of 28 samples. Mold fungi such as *Aspergillus, Penicillium, Rhizopus,* and *Mucor* are inflammatory pathogens, especially in immunocompromised individuals, and can potentially cause severe diseases. *Aspergillus* spp. affects patients with asthma, cystic fibrosis, sinusitis, and acute invasive aspergillosis in some cancer patients and chemotherapy patients (Chen et al. 2015). *Rhizopus* and *Mucor* are found in soil and decayed plant and organic material. The routes of transmission to humans are respiratory, digestive, and cutaneous. Thrombus formed due to vascular invasion in immunocompromised individuals causes distal necrosis and infarcts. Since these strains are resistant to antifungals, they are difficult to treat, and because deep debridement is required in their treatment, patients experience tissue and organ losses such as the eyes and nose (Garcia-Hermoso et al. 2015).

It has been reported that there was a significant difference in antifungal susceptibility between the strains isolated from patients and the strains isolated from the Aspergillus medium (Sabino et al. 2016). However, in our study, the epidemiological threshold value (ECV) of *Aspergillus* strains for six antifungal drugs (IT, VO, POS, AND, AP and CS) was found below the values determined by CLSI. The MIC values of the *Mucor* and *Rhizopus* strains for six antifungal drugs were found to be higher than those of the *Aspergillus* strains. While the AP value for the *Mucor circinelloides* strain isolated from colony 1 was 0.125 µg/mL, high MIC values were detected for the other antifungal agents. We found that the *Rhizopus oryzae* complex strains have high MIC values for other antifungals compared to AP and POS. The results of our study are similar to the antifungal susceptibility of the *Mucor* and *Rhizopus* strains, which were isolated from clinical samples of immunocompromised patients and considered infectious agents (Sağıroğlu et al. 2019). The *Bipolaris* strains were found to have low MIC values for all antifungal drugs. The *Trichoderma* spp. strains were found to have low MIC values for other antifungal drugs except for IT. 8 µg/mL, VO and CS MIC values were found for *Paecilomyces variotii*, but the MIC values of other antifungal drugs were below 0.064 µg/mL. IT ve VO MIC values for *Penicillium chrysogenum* was >32 µg/mL, AP MIC value was 12 µg/mL, but the MIC values of the POS, AND, and CS drugs were below 0.25 µg/mL (Table 2).

This study isolated five *Bacillus* strains from four fresh bee bread samples. The colony-isolated *Bacillus* strains were identified as *Bacillus alttitudinis/pumilus, Bacillus licheniformis,* and *Bacillus megaterium*. The sensitivity limit values for CLSI were not determined according to CLSI criteria, only zone diameters were given and no sensitivity-resistance distinction was made. The susceptibility of the *Bacillus alttitudinis/pumilus* strains isolated from three different colonies was the same. The Food and Drug Administration states that *Bacillus subtilis, Bacillus licheniformis,* and *Bacillus pumilus* are safe to use as probiotics (Salminen et al. 1998; Schallmey et al. 2004). Gilliam et al. (1979 b) reported that *Bacillus subtilis, Bacillus licheniformis,* and *Bacillus pumilus* were isolated from bee bread stored in honeycomb cells for periods of one, three, and six weeks. Bee bread is used more and more as a food supplement due to its beneficial effects on health and probiotic properties, and in the treatment of infectious diseases due to its immune system strengthening properties.

In this study, *M. luteus* and *S. marcescens* were isolated from fresh bee bread samples, but they were not detected in stored bee bread. The sensitivity breakpoints for *M. luteus* were not determined according to CLSI criteria, only zone diameters are given (Table 3). *S. marcescens* was found susceptible to CRO, MER, CN, SXT, and resistant to AMP and CXM, according to CLSI criteria. Disayathanoowat et al. (2012) investigated bacterial and fungal communities in corbicular pollen and colony-preserved bee bread of two commercial honeybees (*Apis mellifera* and *Apis cerana*) in China. During pH reduction in bee bread stored in the hive, they observed that the bacterial population (*Enterobacteriaceae bacterium*) decreased with both traditional culture methods and next-generation sequencing. The fungal population, however, remained stable (especially *Cladosporium*) and filamentous fungi had the potential to inhibit the growth of both common/contaminant bacteria and pathogens by releasing organic acids. Our study results were consistent with Disayathanoowat's study; for fresh bee bread samples, the bacterial population in our study is very low compared to the fungi.

As a result, bee bread can be easily contaminated with mold spores, considered opportunistic pathogens, by environmental factors (climate, temperature, oxygen level) in which the hive is located. For this reason, it is necessary to prevent contamination during the production and storage of bee bread samples and to eliminate the conditions that create an environment for the growth of fungi and other microorganisms. Therefore, it is recommended to store bee bread in a deep freezer or by drying.

5. Conclusions

Most molds, a few yeasts, and bacteria were isolated from fresh bee bread samples in this study. The clinically important *Aspergillus* species were the most common mold species detected in all but one of the samples (colony 2). To increase the quality and safety of all bee products, not just bee bread, the optimization of hygienic procedures throughout the production chain should be ensured. Hygiene is essential to reduce the risk of food spoilage and accompanying disease and poisoning.

It was determined that the yeast, mold, and bacteria isolated may be resistant to antibiotics and antifungal drugs. Another striking point in our study, the bee bread sample stored under appropriate conditions such as keeping in a freezer for maturation and reducing the microorganism load was the absence of yeast, mold, and other microorganisms.

The development of new technologies in food production, processing, and storage conditions are critical elements in the global market. Systems for the control of production practices are provided by continuous monitoring for consumer protection against microbiological risks that may occur at all stages of bee bread production.

Data availability: Data are available on request due to privacy or other restrictions.

Authorship Contributions: Concept: F.M.S., S.S., N.K., Design: F.M.S., S.S., N.K., Data Collection or Processing: F.M.S., S.S., N.K., P.S., B.D., Analysis or Interpretation: F.M.S., S.S., N.K., P.S., B.D., Literature Search: F.M.S., Writing: F.M.S.

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The Effect of Different Depths of Salty Groundwater on Yield and Soil Salinity of Some Pasture Crops

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ABSTRACT
Salinity is one of the most serious environmental factors limiting the yield of plants. Because many crops experience yield losses due to the harmful effects of high salt content in soil and water. The increase in the area of land affected by salt has the potential to create problems in terms of food safety. In this context, it is necessary to develop some cultural practices to prevent or reduce the harmful effects of salinity. This study investigated the effects of salty groundwater at different depths on the yield and soil salinity of some forage crops grown in semi-arid regions for three years. The experiment was conducted using the randomized block split-plot design. The effect of water table depths on the yields of the cultivars in the first year was found to have a statistically significant ($p<0.01$) effect while it was insignificant in the second year. According to the Duncan test results, it was determined that the H1 (40 cm groundwater depth) treatment in the first year provided the highest yield in all three cultivars and formed the first group ($p<0.01$) in the Duncan test. The interaction between water table depths and plant species was statistically significant at the $p<0.05$ level. At the end of the experiment, the salt concentration of the topsoil (40 cm depth) increased significantly ($p=0.025$) at all water table depths. Moreover, although the sodium adsorption ratio of the inlet water was low, it was determined that the exchangeable sodium percentage of the soils increased significantly at all groundwater levels at the end of the trial.

Keywords: Forage crops, Pasture lands, *Festuca elatior, Lotus strictus, Puccinella distans*, Groundwater salinity

1. Introduction

Flatlands in arid and semi-arid regions and alluvial plains on the coasts have inadequate drainage due to topographic and soil structure. These lands have been salted by high groundwater levels and insufficient drainage. This type of land that covers extremely large areas of the world, is in the form of pastures or abandoned lands with low, and poor-quality grass yields. These unproductive pastures were degraded by negative factors such as overgrazing, erosion, drainage, and barrenness. These lands have reduced plant diversity due to the high-water table and excessive salt, and natural vegetation has become poor-quality pastures dominated by barren-halophyte plants of low nutritional value that are not liked by animals.

In many countries where livestock is based on meadow and pasture farming, it is aimed at producing cheap and healthy food by growing salt-resistant forage plants and their mixtures in meadows and pastures under the influence of high groundwater (Dieleman 1977). Establishing artificial pastures consisting of acceptable forage crops, currently used as pastures, may significantly increase the agricultural potential of these lands.

Another way to benefit from these lands is to rehabilitate them. However, although salt-affected soils are chemically recovered, it takes a long time to restore the physical properties of these soils. Therefore, by cultivating some salt-tolerant forage crops during this period, both the recovery of the physical properties of the soil and the farm income will be contributed.

In this context, halophytes are important because of the prevalence around the Lake Tuz and arid soils in central Anatolia (Birand, 1961). For example; *Puccinella distans* can provide a good yield of up to 26 dS m^{-1} salinity in salty soils (Öztan 1965; Akhazari et al. 2012). Bennett & Barrett-Lennard (2013) reported that samphire (*Tecticornia pergranulata*) and *Puccinellia* (*Puccinellia ciliata*) grow in areas with a salinity of 16 dS m^{-1}. The increased salinity causes only a decrease in plant height (Ashkan & Jalal 2013; Kuşvuran et al. 2014). *A. elongatum* species are less damaged by salinity conditions than other *Agropyron* species because it is more adaptable to salinity conditions in morphological terms (Koç & Acar 2017).

In addition, *Agropyron* species (*Agropyron* spp.) can naturally grow in areas with salinity issues in the Central Anatolia rangeland (Acar et al. 2016). The grass yield of 70% of the natural pastures in the Konya plain is very low, and the average dry grass yield is approximately 200 kg ha^{-1}. High-quality forage plants with high grass yields have been replaced by sour marsh plants such as *Carex* and *Juncus* (Tosun 1967).

Tall wheatgrass (*A. elongatum*) and crested wheatgrass (*A. cristatum*) from *Agropyron* spp. give a yield with a 50% loss in high salinity levels, which is not available to grow other plant species. (Akhazari et al. 2012; Ashkan & Jalal 2013). The presence of saline groundwater at 25 cm depth had a detrimental effect on the production of biomass and its components on Rhodes grass (*Chloris gayana*), whereas the effect at 125 cm and greater depths was neutral (Chiacchiera et al. 2016). *Leptochloa fusca*, *Spartina patens*, and *Sporobolus virginicus* (Smyrna) have been reported to be promising halophytic plants for feeding goats and sheep in desert areas (Ashour et al. 1997).

For the reasons mentioned above, this study was carried out to determine the effects of salty groundwater at different depths on the yield and soil salinization of some forage crops in pasture areas in semi-arid regions and to contribute to the determination of forage plant varieties suitable for semi-arid conditions.

2. Material and Method

2.1. Trial site

The trial site is the salty lands in Konya Plain with a high-water table. These lands are widely located in different parts of the Central Anatolia (Figure 1), especially around Lake Salt, in Aslım pastures, in the plains of Karapınar, Ereğli plain, and around Lake Hotamış (Meester 1970).

Figure 1- The geographical location of the research site

The trial site soils are in the hydromorphic soil group, with medium depth, it is flat land (Atalay & Secerli 1971). The upper soil is silty loam, and the lower soil is silty and organically rich. Its lime content is high, it is excessively salty and salt content decreases with depth (Table 1). The water conductivity of light sodium soils is in the middle group of the upper layers and the medium-slow group of the lower layers (Meester 1970).

Table 1- Some physical and chemical properties of trial soils

Soil depth cm	*Texture*				*pH*	*EC_e*	*NaX*	*CEC*	*ESP*	*Lime*	*OM*
	S	*Si*	*C*			*dS m^{-1}*	*cmol$^+$*	*kg^{-1}*	*%*	*%*	*%*
0-20	24.5	68.7	6.7	Si	7.8	17	3.1	13	24	22.0	4.35
20-40	20.7	70.3	9.0	Si	7.0	15	2.3	10.9	21	23.3	2.90
40-70	8.0	87.9	4.0	Si	6.5	12	1.4	11.4	13	41.7	2.56
70-110	13.3	87.0	0.0	Si	6.4	6.9	0.3	8.5	3.5	43.1	2.04

EC_e: Electrical conductivity of soil extract, NaX: Exchangeable Na, CEC: Cation exchange capacity, ESP: Exchange Na percentage, OM: Organic material, S: Sandy, Si: Silty, C: Clay

2.2. Climate

The Konya Plain has the typical characteristics of the continental climate; the summers are dry and hot, and the winters are snowy and cold. The highest annual temperature average is 11.6 ºC, the annual precipitation is 327.7 mm and the average relative humidity is 63% (Anonymous, 2018). During the trial, precipitation was 386 mm in the first year, and 277.8 mm in the second year, the evaporation was 1,171 in the first year and 1,243 mm in the second year and the average temperature was 11.1 in the first year and 10.5 ºC in the second year.

2.3. Method

Trials were carried out with 3 forage plants, A- *Lotus sitrictus*, B- *Puccinella distans*, C- *Festuca elatior* and 4 groundwater depth levels, H1=40 cm H2=60 cm H3=80 cm H4=110 cm.

2.3.1. Setting up the experiment

The experiment was carried out in 3 replicated, randomized blocks split plots trial design. The experiment was conducted in cylindrical steel barrels with a depth of 120 cm and with a diameter of 56 cm. The plot areas are 0.2462 m^2 for planting and harvesting.

In the first place, the volume weights of the soils at the trial site were determined by the layers of 20 cm. The soils were dug, stored, and allowed to dry in the shade, depending on the soil layers. Ten cm thick sand gravel was laid on the bottoms of the barrels. To measure the depth, 10 cm of soil was placed on the top of the sand-gravel layer. It was compacted until the original bulk weight was achieved with a mallet. Then the second 10 cm layer was laid, and the same operations continued until the barrel was full.

To create a water table in the barrels, holes in the specified depth are drilled and the outlet drainage pipes were located. Groundwater was pumped through inlet pipes placed under the tanks to a 4 m high water tank. The salt content of the water entered and discharged from the barrels was measured every month.

2.3.2. Properties of the plants used in the experiment

Festuca elatior, Puccinellia distans, and *Lotus strictus*, which are under the shallow water table around Konya in the natural vegetation of Aslım pasture lands, were used in the experiment (Figure 2).

Festuca elatior; high meadow ball, salt resistant, 40-60 cm tall, also called meadow ball (Tosun 1974). Its multi-sibling, bundles are 30 cm in diameter, growing vertically upwards, between 20 cm and 1 meter in length. Leaves are curled, cylindrical leaf sheathed. The underside of the leaf is brightly colored. Flowers are unique with many large and elongated spikelets together to form compound clusters**.**

Puccinellia distans is a green-colored forage crop with wheatgrass spikes resistant to salt (Werner & Senghas 1973). Five-forty cm in length with body characteristics of herbaceous plants native to Turkey. It is common in semi-marshy areas around inland salty lakes. It generally grows in sandy loam, silty loam, and loamy soils and has a strong root system and anti-erosion feature, which is important for grazing in salty areas.

Lotus strictus is a salt-resistant, yellow-flowered forage plant that reproduces with the rhizome and is 50-100 cm tall and highly branched (Kyell Vist 1971). Glabrous, with short hair only on top, perennial, erect, thick, woody below, with umbrella-like inflorescences bloom in August - September.

2.3.4. Sowing, fertilizing, and harvesting

The seeds were sown by hand, but as there was not adequate germination, the *Lotus strictus* plots were replanted. Before planting, 40 kg N, and 100 kg P_2O_5 were given per hectare and mixed into the soil. During the summer, the trial plots were irrigated 3 times with groundwater.

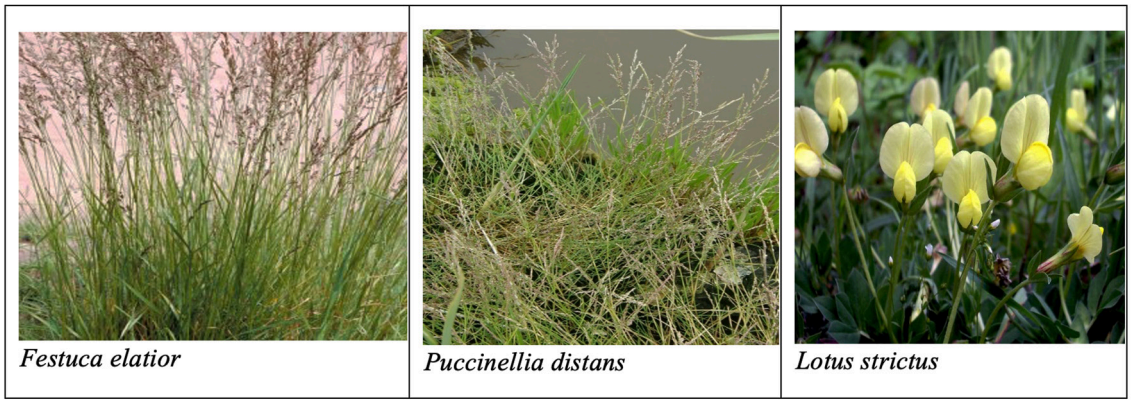

Figure 2- Pasture plants used in the experiment

When the plants reached grazing maturity, *Festuca elatior*, and *Agropyron elongatum* 20 cm, *Puccinella distans*, and *Lotus strictus* 15 cm in length, were harvested, and wet and dry grass weights were determined.

In order to determine the dry grass yield, the wet grass samples were dried in an oven at 65 ºC until they reached a constant weight, and their moisture content was determined.

2.3.5. Statistical analysis

Grass yields of the cultivars were analyzed in randomized blocks with a split-plot design. The effect of groundwater on salt accumulation was evaluated through regression analysis and pairwise comparisons (Yurtsever 1984).

3. Results and Discussion

3.1. Grass yields of crop varieties

The highest yield was obtained for 40 cm water table depth. The dry grass yields of *F. elatior, P. distans*, and *L. strictus* are 744, 642, and 615 kg da^{-1}, respectively. When the groundwater level fell to 60 cm depth, the hay yields decreased to 407, 383, and 454 ka da^{-1}, respectively. The effect of water table levels on plant yield was found to be significant, and H1=40 cm water table depth formed the first group ($F_{0.01}$=34.57** and p<0.01) in both years, and also the interaction between water table and plant species (p=0.05) was found to be significant (Table 2) and crop varieties (A, B, C).

Table 2. Dry grass yields versus different water table depths (kg da^{-1})

Groundwater depth, cm	*Crop varieties: 1st year*			*Average*	*Crop varieties: 2st year*			*Average*
	A	*B*	*C*		*A*	*B*	*C*	
40	744	642	615	667a	816	173	494	494a
60	407	383	454	415ab	543	72	378	331b
80	214	318	334	289b	792	125	209	375b
110	224	270	314	269b	509	99	206	271c

However, while *L. strictus* regenerated itself with rhizome and maintained its productivity at all water levels, the decrease in the yield of *P. distans* and *F. elatior* was significant statistically (Figure 3). Due to a decrease from 60 cm to 110 cm in the water table, the efficiency decrease continued, but the decrease was not statistically significant.

In these pasture areas, a groundwater depth of 40 cm should be considered a critical level. It should be noted that increasing the depth of the water table, in addition to damaging the existing vegetation due to water stress, may also accelerate the oxidation of organic matter in the upper soil layers.

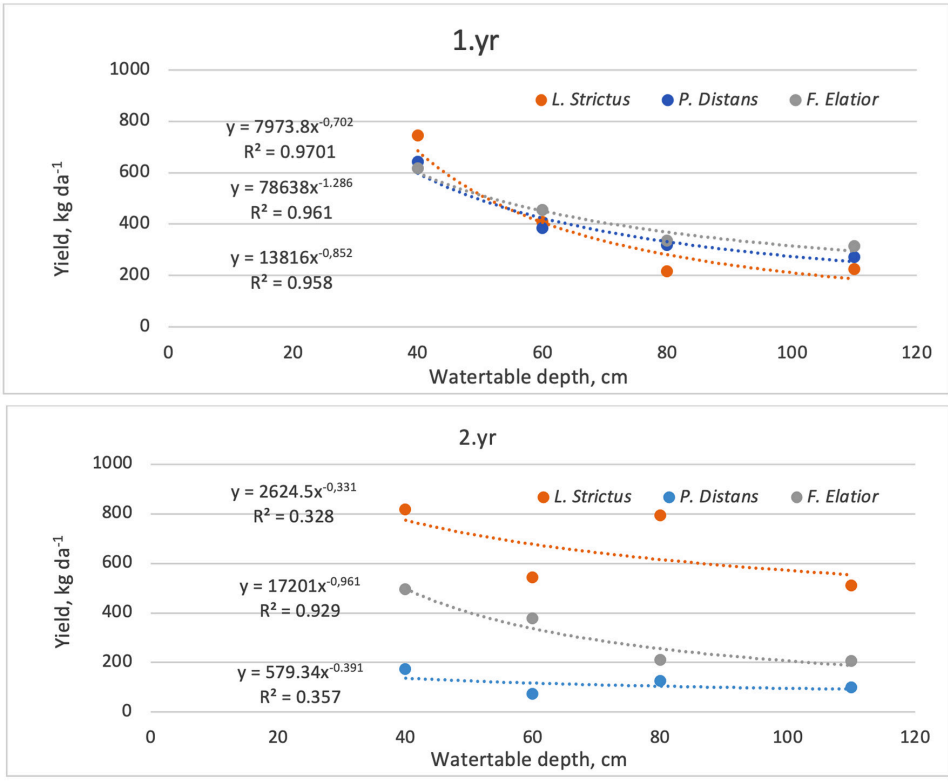

Figure 3- Dry grass yields versus water table depths in different years

The high correlation coefficients of the relationship between the decrease in the water table level and the grass yields indicated that the main reason for the decrease in the crop was water stress. Although similar results were obtained in the second year of the trial, the negative effects of the increase in soil salinity were also observed. However, although *L. strictus* regenerated by rhizome and maintained its productivity at all water levels, there were significant decreases in the yield of *P. distans* and *F. elatior* (Figure 3).

Many halophytic plants thrive optimally at 20 to 25 g l^{-1} soil salinity, and at 30 to 40 g l^{-1} show 25% to 50% growth reduction but they can sustain their lives in soils containing 60 to 90 g l^{-1} salt (Miyamoto et al. 1994). *Leptochloa fusca*, *Spartina patensand*, *Sporobolus virginicus* (Smyrna) are shown as promising halophytic plants for feeding goats and sheep in desert lands using available saline water for irrigation areas (Ashour et al. 1997). In a land with a drainage problem, 85% of the yield from tall wheatgrass was obtained from forage plants irrigated with saline drainage water, while the yield for alfalfa was 43%. (Suyama et al. 2007). Tall wheatgrass has less tolerance to salinity, and the yield is severely reduced (by 55%) under high salinity (Grattan et al. 2004, Robinson et al. 2004). To maintain high bermudagrass yields, it is recommended that soil salinities should not exceed Ec_e 12 dS m^{-1} (Kaffka et al. 2004).

Many salt-tolerant forage crop species, such as the forage crops discussed in this study, can be grown successfully in semi-arid conditions dominated by shallow groundwater. On lands with similar conditions, artificial pastures can be established before improvement. *Puccinellia distans* is a perennial cool-season salty grass, that can adapt to arid climates and extremely saline-alkaline soil conditions (Hughes 1972; Brotherson 1987; Scalia et al. 2009; Ehsani et al. 2016), and, spread in salty meadow-pasture areas. This plant produces between 4 and 10 tons of hay per hectare per year (Warren et al. 1994) and this delicious species (Shidaei & Namati 1978) is generally consumed by sheep [Peng et al. 2004; Akhazari et al. 2012, Ashkan & Jalal 2013], but, it has been reported that increasing salinity causes a decrease in plant height (Acar et al. 2016). Considering that 0.4 hectares of artificial pasture per sheep provides the most economical live weight gain in the shallow water table conditions of *A. elongatum*, *P. capillaris* and *F. elatior* in this research area (Uçar 1982), the usefulness of such an application can be easily understood that it can make a significant contribution to increasing feed production.

3.2. Change in soil salinity and sodium

The salt distribution in the soil profile was similar for all groundwater levels. The distribution of salts in the soil profile was typical, as in soils under the influence of a high water table. That is, the salt concentrations were decreased from the upper layers to the lower

layers. Statistical analysis after the experiment showed that the interaction of soil layers and water levels was significant ($F_{0.01}$=34.57** p<0.01) in the first year. Analysis by year showed that the effect of water levels on salinization was insignificant ($F_{0.01}$=124.44** p<0.01), but salt accumulation in soil layers was significantly different.

In other words, the water levels were effective in the salinization of the soil layers. In the beginning, the topsoil layer had the highest salt content and the salinity have been decreased with increasing depth (Table 3, Figure 4). Differences in soil salt contents between treatments were insignificant (p=0.202>0.05). However, there was a statistically (p<0.05) significant difference between the salt contents of the soil layers.

Table 3- Variation of salinity in soil layers versus different water table depths and crop varieties (A, B, C)

Groundwater depth, cm	*Soil depth,*	*Before trial*				*After trial*			
		ECe, dSm-1				*ECe, dSm-1*			
		A	*B*	*C*	*Mean*	*A*	*B*	*C*	*Mean*
40	0-40	15.3	14.8	15.4	15.2	18.3	21.2	24.7	21.4
	40-70	10.8	6.9	8.5	8.6	7.9	9.9	12.4	10.1
	70-110	5.9	9.0	9.4	8.1	4.9	6.7	6.4	6.0
60	0-40	14.7	16.7	14.5	15.3	20.3	28.8	23.4	24.2
	40-70	8.8	14.3	12.9	12.0	13.1	14.0	16.6	14.6
	70-110	6.7	10.0	6.0	7.6	8.1	7.8	9.2	8.4
80	0-40	16.7	16.4	16.4	16.5	13.9	17.3	12.4	14.5
	40-70	11.1	10.9	12.0	11.3	13.3	9.7	11.0	11.3
	70-110	6.3	7.0	6.3	6.5	6.4	6.9	8.9	7.4
110	0-40	16.5	15.8	16.6	16.3	19.3	16.7	17.7	17.7
	40-70	10.8	7.7	9.2	9.0	14.3	9.6	11.5	11.8
	70-110	7.1	6.7	6.7	6.8	10.4	6.7	8.1	8.4

Before and after the trial all soil layers and all treatments, p=0.0036 <0.01
Before and after the trial topsoil layer and all treatments, p=0.025 <0.05

Although the salt accumulation in the upper soil layers was slightly higher than the water table at 80 and 110 cm in the treatments where the water table was kept at a depth of 40 and 60 cm, there was no significant difference observed between them.

On the other hand, when we consider all trial treatments, the salt concentration of the top layer increased significantly at the end of the trial (p=0.025). In comparison to the baseline, salt accumulation at depths of 40-70 and 70-110 cm is insignificant (p=0.326, p=0.715).

It was determined that there was a significant difference (p=0.006-0.04) between the salt load of the incoming water and the salt load of the drained water, while there was no difference between the effects of the water table levels on the salt load of the drainage water (Table 4).

Table 4- The salinity of entering and drainage water

Water table depth, cm	*March*	*April*	*May*	*June*	*July*	*March*	*April*	*May*	*June*	*July*
	Inlet water, dS m^{-1}									
	3.3	*3.6*	*4.4*	*4.1*	*4.0*	*3.6*	*3.6*	*3.1*	*4.4*	*4.4*
	Drainage water (1st year)					*Drainage water (2st year)*				
40	5.2	4.3	5.1	4.2	4.0	5.1	5.2	6.0	8.5	5.2
60	6.3	6.1	6.5	6.7	5.5	4.7	8.1	5.4	4.8	
80	4.1	3.6	3.6	4.1	4.1	6.8	6.4	6.7	6.6	4.5
110		10.2	10.2	5.6	5.6	7.50	.	.	.	.

As shown in Table 5, before the experiment, the soil exchangeable sodium percentage (ESP) was 24% in the upper layer and decreased to 4% at 110 cm in depth. At the end of the trial, the ESP values in the topsoil increased significantly in all treatments compared to the initial values and reached 50-60%. In plots with a water table level of 80-110 cm, the ESP values are closer to each other in the entire profile. Crop varieties have not been shown affect ESP change.

Table 5- ESP status before and after trial according to groundwater depths

Groundwater depth, cm	*Soil depth*	*L. sitrictus*	*P. distans*	*F. elatior*	*Soil depth*	*Groundwater depth, cm*	*L. sitrictus*	*P. distans*	*F. elatior*
	ESP status before trial, (%)								
Before trial	0-40	24	24	24					
	40-60	21	21	21					
	60-80	13	13	13					
	80-110	4	4	4					
	ESP status after trial, (%)								
40	0-40	68	61	51	80	0-40	39	94	42
	40-60	22	20	81		40-60	61	53	35
	60-80	16	12	25		60-80	24	74	36
	80-110	13	14	16		80-110	18	29	33
60	0-40	55	50	68	110	0-40	53	60	31
	40-60	38	12	15		40-60	39	53	56
	60-80	28	9	43		60-80	28	21	50
	80-110	21	15	17		80-110	26	23	24

Since there was no difference between the ESP values according to the crops ($p=0.970^{ns}$-0.470^{ns}), the average values are taken and the changes in ESP according to the depths of the water table and soil layers are shown in Table 5 and Figure 5.

Whether there was a difference between the ESP values of the soils before the trial and the ESP values at the end of the trial was determined in the form of pairwise comparisons, taking into account the water table levels.

A statistically significant difference ($p<0.01$; (0.000-0.007^{**}) was found between the initial ESP values of all soil layers and the ESP values at the end of the experiment at different water table levels. However, the effect of different water table levels on ESP in soil layers was found to be statistically insignificant ($p>0.05$; 0.95-0.719^{ns}).

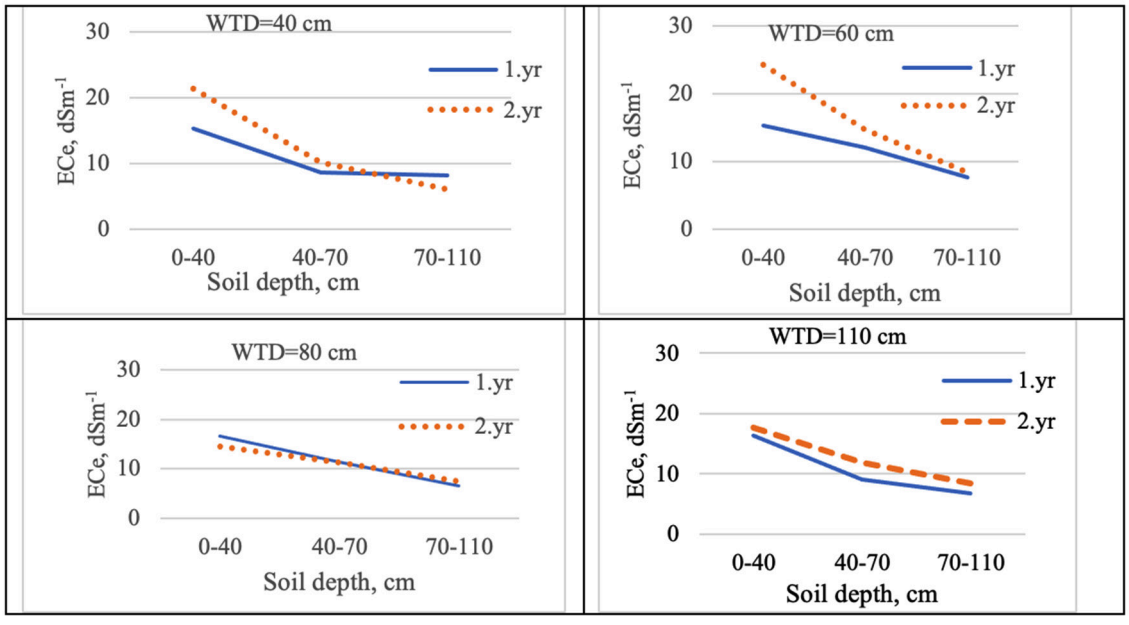

Figure 4- Change of soil salinity at different water table depths

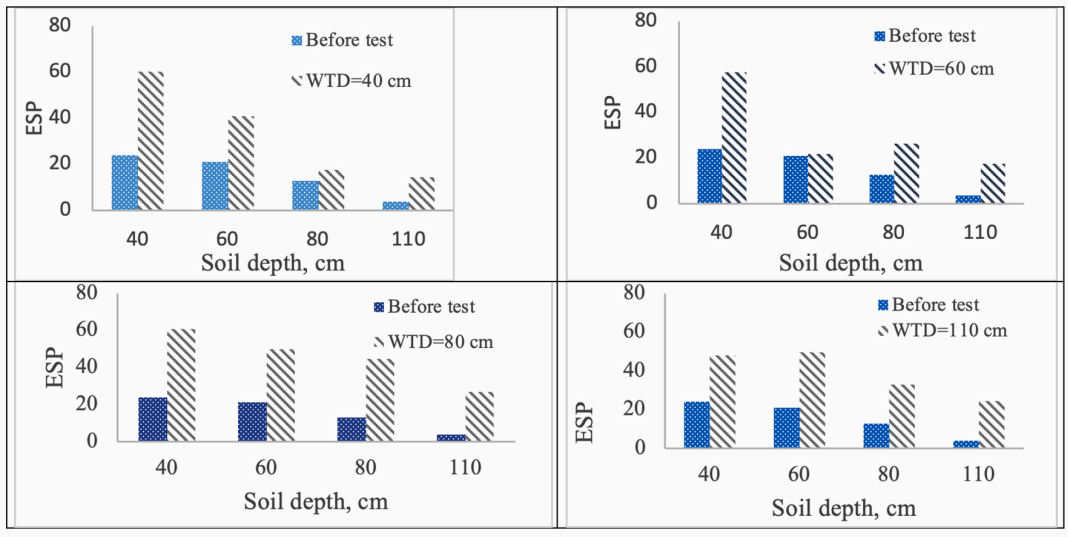

Figure 5- ESP status before and after trial according to different groundwater depths

4. Conclusion

The high groundwater level significantly increased the yield of pasture crops used in the experiment. Significant differences have been found between salt concentrations before and after the experiment in the trial soils. On the other hand, no difference was found between the effects of water table levels on salt and sodium accumulation in the soil.

Even if the high-water table increases the grass yield in the artificial pastures planned to be created, salt and sodium accumulation should be controlled for a sustainable yield. In such areas, it seems possible to create sustainable pastures if managed drainage and groundwater are provided as leaching water at the end of the season and salt-resistant forage crops are grown.

In arid and semi-arid areas under the influence of shallow groundwater for many years, uncontrolled lowering of the water table will accelerate the oxidation of organic matter as well as cause a loss of yield in these pastures. The irrigation and drainage systems in these regions should therefore be planned together, and the drainage systems to be built should be controlled drainage rather than free-flowing.

Data availability: Data are available on request due to privacy or other restrictions.

Authorship Contributions: Concept: İ.B., Design: İ.B., Data Collection or Processing: İ.B., Analysis or Interpretation: B.B., A.F.T., Literature Search: B.B., A.F.T., Writing: B.B., A.F.T.

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Effects of Different Lactic Acid Bacteria Inoculants on Alfalfa Silage Fermentation and Quality

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ABSTRACT

Alfalfa (*Medicago sativa* L.) is a crucial perennial forage plant with high protein and mineral content and may be mowed several times through the vegetation period. Along with having a large cultivation area in Türkiye, it constitutes approximately 61% of the total green forage produced. Silage is the most effective method for preserving herbage and using it as a source of roughage throughout the year. However, ensiling alfalfa, especially with low dry matter (DM) content, is difficult due to its low water-soluble carbohydrate (WSC) and buffering capacity. This study was carried out to improve the alfalfa plant's silage fermentation process by inoculating new lactic acid bacteria (LAB) strains. When the alfalfa plant reached 50% flowering, six different LAB strains were inoculated and compared with the uninoculated alfalfa silage. According to the results obtained, it was determined that LAB inoculants improved the fermentation properties of alfalfa silage in general. All inoculated strains caused a significant decrease in the pH of the resulting silage. The strain *Lactobacillus buchneri* (LS-31-1-4) was superior in terms of DM recovery (96.82%) and protein recovery (94.00%). At the same time, *Lactobacillus brevis* (LS-55-2-2) and *Leuconostoc citerum* (LS-70-6-1) were the most restrictive strains of yeast and enterobacteria growth in silage, respectively.

Keywords: LAB isolates, Microorganisms, Silage quality, Enterobacteria

1. Introduction

The livestock sector is the fastest-growing branch in developing countries' agricultural economies. However, it is known that the most important factor negatively affecting production in this sector remains the animal feeding (Kızılşimşek et al. 2016). The number of cattle units in Türkiye is around 24 million, and their dry matter (DM) needs about 110 million tons/year. The total forage production from rangelands and agricultural areas is 31 million tons, clearly indicating insufficient production, which is far from meeting the quality roughage needs of animals. The alfalfa (*Medicago sativa* L.) plant provides approximately 61% of the total forage field (TÜİK 2022). Alfalfa, with a wide cultivation area, is a perennial plant that can be harvested more than once a year, is rich in protein, and is often used as fodder for animals (Ertekin et al. 2017; Ertekin & Kızılşimşek 2020; Kızılşimşek et al. 2020).

Mowing alfalfa during its appropriate maturity period and preserving its quality is critical for livestock nutrition. Typically, nutrient losses may occur during mowing, negatively affecting quality. There is a potential risk of rain when drying, predominantly in rainy and humid regions, especially for the plant's first and last mowing times (Yakışır & Aksu 2019). For this reason, making silage is the most effective method for preserving herbage quality and enabling the use of forage in animal rations as a source of roughage in all four seasons. The advantages of ensiling are that its less labor intensive has a long supply time, and provides favorable opportunities for using additives to facilitate fermentation (Ding et al. 2020). However, ensiling the alfalfa plant is difficult due to its low DM rate and inadequate water-soluble carbohydrate (WSC) content (McDonald et al. 1991). Lactic acid bacteria (LAB) break down WSCs into acetic acid and CO_2, as well as predominantly lactic acid, accelerating fermentation and contributing to the rapid reduction of the pH in the medium. LAB also inhibits the growth of many aerobic bacteria and thus ensures feed preservation. Lee et al. (2018) noted that

inoculated LAB just before ensiling might support lactic acid fermentation and improve the feed quality of the resulting silage. LAB species associated with silage generally belong to the *Lactobacillus, Pediococcus, Leuconostoc* and *Enterococcus, Streptococcus,* and *Lactococcus* (Pahlow et al. 2003) families.

Due to its low WSC structure, DM content, and high buffering capacity, the alfalfa plant is difficult to ensilage. There is a need for practices and studies to increase the silage quality of the alfalfa plant. One of these applications, LAB inoculant application, is a method that improves the alfalfa plant's silage quality and fermentation properties. This study was carried out to investigate the effects of inoculation of different LAB strains, which were selected within the context of our study group's previous research project, on the fermentation profile and the feed quality parameters of alfalfa silage.

2. Material and Methods

2.1. Material

The Bilensoy-84 alfalfa variety, as a second cut, was used as plant material grown on agricultural fields in Kahramanmaras under irrigated conditions. At sunrise, the alfalfa plants, which are in a 50% blooming period, were harvested and removed from the weeds in them. Six LAB isolates selected among 695 isolates within the scope of the The Scientific and Technological Research Council of Türkiye (TUBITAK) project were used as microorganism material for inoculation. Table 1 shows the LAB isolates' physiological characteristics and the study's isolates numbers. The *Lactobacillus bifermentans, Lactobacillus gasseri, Pediococcus citerum*, and *Leuconostoc citerum* isolates were homofermentative, while *Lactobacillus brevis* and *Lactobacillus buchneri* were heterofermentative.

Table 1- Characteristics of lactic acid bacteria isolates

Bacteria no	***Bacteria name***	***Physiological characteristics***
LS-65-2-1	*Lactobacillus bifermentans*	Homofermentative
LS-51-2-1	*Lactobacillus gasseri*	Homofermentative
LS-55-2-2	*Lactobacillus brevis*	Heterofermentative
LS-8-1	*Pediococcus citerum*	Homofermentative
LS-31-1-4	*Lactobacillus buchneri*	Heterofermentative
LS-70-6-1	*Leuconostoc citerum*	Homofermentative

2.2. Method

The harvested plant material was first cut into 2-3 cm lengths with a chopper machine and divided into seven 2 kg treatment groups, one controlled without inoculation. After the previously determined LAB were revitalized and developed in MRS broth media, they were adjusted to a density of 10^7 cfu/g and diluted in 20 mL of distilled water. A homogeneous distribution was obtained by spraying and mixing the solution on the chopped forage. The control group was sprayed with 20 mL of distilled water without bacteria to manipulate the DM content in the inoculation treatments. Three silage packages containing 400 grams of chopped plant material from each treatment group were ensiled to be opened after 60 days (T_{60}). To determine the microorganism composition and other characteristics in each treatment group at T_0, 20 grams of sample was first blended for 1 minute at high speed with a blender in 180 mL of Ringer's solution. The samples were filtered using Whatman 54 filter paper, and the pH was measured. For the microorganism counts from the LAB strains, 1/10 dilution series were prepared and planted in MRS agar, VRBG agar, and MEA agar media for counting LAB, enterobacteria, yeast, and molds, respectively. The MRS agar media were incubated for 48 hours at 37 °C, VRBG media at 32 °C for 18 hours, and MEA agar at 32 °C for 48 hours. After incubation, microorganism counts were made.

A sample of 70 g silage was taken from the resulting silage and left to dry for 48 hours in an oven set at 78 °C, and the DM content of the samples was determined by weighing with a precision balance; then, the samples were ground in a grinding machine with a 1 mm sieve and made ready for further analysis. The nitrogen content of the feeds was determined using the Kjeldahl method, and the crude protein (CP) ratios were calculated by multiplying with the coefficient of 6.25 (AOAC 1990).

The pH, DM, microorganism counts, and CP ratio analyses mentioned above were performed for T_{60} plant samples by applying the same methods as T_0.

All statistical analysis, variance analysis, and LSD techniques for comparing averages were performed by JMP statistical analysis software.

3. Results and Discussions

The results of the pH value, DM ratios, and CP values of crop material before (T_0) and after (T_{60}) ensiling samples of alfalfa silages strained with different bacterial isolates are given in Table 2.

Table 2- Average values of pH, dry matter, and crude protein of silages belonging to different bacterial inoculants at T_0 and $T6_0$ opening times

Bacteria inoculant	*Dry matter (%)*		*pH*		*Crude protein (%)*	
	(T_0)	*(T_{60})*	*(T_0)*	*(T_{60})*	*(T_0)*	*(T_{60})*
Control	27.32^{bc}	24.01^{d}	6.27	4.87^{a}	21.70^{b}	18.56^{b}
L. bifermentans	27.01^{bc}	24.97^{cd}	6.21	4.54^{b}	21.03^{b}	19.25^{b}
L. gasseri	28.72^{a}	26.64^{a}	6.24	4.52^{b}	21.34^{b}	19.64^{ab}
L. brevis	27.43^{b}	26.19^{ab}	6.21	4.57^{b}	22.88^{a}	20.55^{a}
P. citerum	26.68^{cd}	25.38^{bc}	6.18	4.64^{b}	21.88^{ab}	18.76^{ab}
L. buchneri	26.17^{d}	25.34^{bc}	6.22	4.64^{b}	20.84^{b}	19.59^{b}
L. citerum	26.92^{bc}	25.44^{bc}	6.22	4.52^{b}	20.99^{b}	18.70^{b}
Average	27.18	25.42	6.22	4.62^{b}	21.52	19.29
LSD	0.64**	1.08**	NS	0.20*	1.11*	1.21*

a,b,c: There is a significant difference between the mean values with various symbols.
**p<0.01, *p<0.05 statistically significant
NS: Non-significant, LSD: Least significant difference

Table 2 shows that the DM contents of alfalfa before ensiling (T_0) were significantly affected by different bacterial inoculations, and the average DM content was 27.18%. In addition, it was seen that *L. gasseri* (28.72%) is superior to other inoculants in the DM content of alfalfa at T_0. The DM content of resulting silages (T_{60}) varied between 24.01-26.64%, and the highest DM content was obtained from the *L. gasseri* (26.64%) isolate, followed by *L. brevis* (26.19%) isolate (p<0.01). The DM contents of mature silages increased with bacterial isolates compared to the control. Indeed, Agarussi et al. (2019) and Blajman et al. (2020) stated that bacterial inoculants increased the DM content of silage compared to the control. In addition, Silva et al. (2020) reported that silages with low DM content had low LAB numbers and higher pH values. Moreover, it is known that silages with high DM content have relatively better DM preservation. It can be numerically calculated from Table 2 that while the loss in alfalfa silage was 3.17% in the *L. buchneri* isolate, it was 12.11% in control. In other words, 96.83% of DM was recovered by the *L. buchneri* inoculation, while only 87.89% of DM was recovered in control. Filya (2004) reported that fermentation losses, especially ensiling crop material with low DM content, might occur.

The difference between the pH values of alfalfa silage before ensiling (T_0), in which different bacterial inoculants were applied, was not statistically significant. However, when the values are examined, it is observed that the pH value decreased slightly with all LAB inoculations compared to the control (6.27). It is well known that reducing pH values in alfalfa silage is difficult. According to the data from Table 2, the pH values of the control silage were 4.87, which is a satisfying level for alfalfa silage; however, pH values for all inoculated silage were significantly lower than that of the control. All bacterial isolates are statistically in the same group. It can be stated that homofermentative bacteria isolates generally have a positive effect on the decrease in the pH of hard-to-ensilage legume plants such as alfalfa. Filya et al. (2007) reported similar observations in their experiments that homofermentative bacteria are more effective in producing lactic acid than heterofermentative, which ensures low pH values. Likewise, in a study conducted by Zielińska et al. (2015), it was shown that high pH (4.8) in alfalfa silage decreased with bacterial inoculants (4.0-4.2). Furthermore, Uher et al. (2019) demonstrated that commercial lactic acid inoculants lowered the pH compared to the control. In addition, Kuppusamy et al. (2020) reported that the *Lactobacillus plantarum* RJ1 and *Pediococcus pentosaceus* S22 LAB strains decreased the pH in alfalfa silage.

The CP value before ensiling (T_0) was statistically affected by different bacterial inoculants, and the CP value of the *L. brevis* bacterial isolates increased (p<0.05) compared to the uninoculated alfalfa (21.70%). It was observed that other bacterial isolates were statistically in the same group as the control. This was also seen in matured silages. The highest CP content was determined at 20.55% in the *L. brevis* isolate (p<0.05), followed by the *L. gasseri* at 19.64% and the *P. citerum* isolates at 18.76%. Only the *L. brevis* inoculant preserved the CP content of alfalfa silage significantly. All other inoculants and the control were comprised of the same statistic group. The CP content is lower in uninoculated (control) alfalfa compared to the *L. brevis* inoculation, indicating that less proteolysis occurred in inoculated silages. This may be associated with high pH values causing protein degradation (McDonald et al. 1991). Many recent

studies have shown that LAB isolates cause an increase in the CP content of silage compared to the control (Ergin & Gumus 2020; Li et al. 2020). Their study shows that the *L. brevis* isolate is superior to other isolates in terms of the CP content. Similarly, Laslo et al. (2019) applied six different LAB isolates. They reported that the *L. brevis* isolated on the 30th day of ensiling increased CP compared to the control and positively affected the ensiling process.

The results of LAB, yeast, enterobacteria, and mold counts before (T_0) and after 60 days (T_{60}) ensiling samples of alfalfa silages strained with different bacterial isolates are shown in Table 3.

Table 3- Presence of lactic acid bacteria, yeast, and enterobacteria at T0 and T60 opening times of silages belonging to different bacterial inoculants

Bacteria inoculants	*LAB*		*Yeast*		*Enterobacteria*		*Mold*	
	(T_0)	*(T_{60})*	*(T_0)*	*(T_{60})*	*(T_0)*	*(T_{60})*	*(T_0)*	*(T_{60})*
Control	3.70^{bc}	5.19	6.93^{bc}	5.45^{a}	5.87^{bc}	4.52^{a}	5.00	ND
L. bifermentans	4.32^{ab}	5.61	6.87^{c}	5.32^{a}	6.47^{a}	3.39^{bc}	5.10	ND
L. gasseri	4.53^{a}	5.51	7.76^{ab}	3.67^{c}	5.97^{abc}	3.69^{bc}	5.20	ND
L. brevis	3.77^{bc}	5.43	8.48^{a}	3.47^{c}	5.94^{bc}	3.20^{bc}	5.30	ND
P. citerum	4.88^{a}	5.65	7.37^{bc}	5.31^{ab}	6.37^{ab}	3.62^{bc}	5.30	ND
L. buchneri	3.40^{c}	5.52	5.31^{d}	5.11^{ab}	5.48^{c}	3.81^{b}	5.00	ND
L. citerum	3.30^{c}	5.98	5.87^{d}	4.54^{b}	5.74^{c}	3.10^{c}	5.00	ND
Average	3.98	5.56	6.94	4.70	5.98	3.62	5.13	ND
LSD	0.72**	NS	0.85**	0.77**	0.53*	0.68**	NS	ND

a,b,c: There is a significant difference between the mean values with various symbols.
**p<0.01 statistically significant
NS: Non-significant, LSD: Least significant difference, ND: Not detected

The LAB count before ensiling (T_0) varied between 3.30-4.88 ($\log_{10}$ cfu/g silage); the highest LAB count was obtained from *P. citerum* (4.88 $\log_{10}$ cfu/g) and *L.gasseri* (4.53 $\log_{10}$ cfu/g) bacterial inoculants, followed by *L. bifermentans* as (4.32 $\log_{10}$ cfu/g) bacterial inoculants ($p<0.01$). The lowest LAB count was obtained from *L. buchneri* and *L. citerum* isolates inoculations. After ensiling (T_{60}), there was no statistically significant difference between the LAB numbers of different bacterial inoculants. Table 3 shows a slight increase in LAB numbers in LAB inoculations compared to the control (5.19 $\log_{10}$ cfu/g) group. This finding may be related to LA production. Ertekin and Kızılşimşek (2020), in their study investigating the effects of different inoculants on alfalfa silage, reported that *L. citerum* and *L. bifermentans* became prominent in LA production compared to the other isolates. In addition, other studies revealed that inoculant silages increased LAB counts compared to the control (Huo et al. 2021; Silva et al. 2016). Queiroz et al. (2013) reported that the number of the LAB naturally found in the plant is low, and this amount increases with LAB isolates. However, other studies have noted that the number of undesirable microorganisms decreased, and the fermentation process improved with LAB, which became dominant in the silage (Jung et al. 2022; Muck et al. 2018).

In this study, the difference between the yeast numbers before ensiling (T_0) was statistically significant, the yeast numbers ranged between 5.31-8.48 ($\log_{10}$ cfu/g silage), and the highest yeast count was obtained from the *L. brevis* isolate ($p<0.01$). The lowest yeast count was extracted from the *L. buchneri* and *L. citerum* isolates, which were statistically in the same group. Driehuis et al. (1999) reported that the *L. buchneri* isolate inhibits yeast that enhances the silage's aerobic stability. However, this does not match the CP content we detected in the silage. The diversity of isolates can explain this difference between studies. It was observed that the yeast count decreased in silages opened after the 60th day, and the lowest yeast count was obtained at the end of the 60th day, especially in the *L. gasseri* and *L. brevis* isolates, which had the highest yeast count in fresh silage material ($p<0.01$). Therefore, the acid production potential of bacteria rather than the amount of LAB number is debatable. Even though the LAB count was low, especially in the *L. gasseri* isolate, it reduced the presence of yeast and achieved the lowest pH. At the same time, at the beginning of ensiling, the enterobacteria count in silages with the *L. bifermentans* and *P. citerum* inoculants was higher than the control, and the enterobacteria count decreased considerably on the 60th day of silage. These results indicate the inhibitory effects of the two isolates on the growth of enterobacteria. It was found that the number of enterobacteria decreased with all inoculants compared to the control. Therefore, it can be said that LAB inoculation positively affects the presence of undesirable enterobacteria in the silage. The study determined that the mold in the fresh material ranged between 5.00-5.30 ($\log_{10}$ cfu/g silage), and there was no statistical difference. In mature silages, no mold was detected in MEA plantings.

Compared to the control, the LAB isolates used in this study positively affected the fermentation properties and CP content. For quality silage fermentation, it is expected that LAB, the most critical species during silage, will have higher numerically, lower enterobacteria, yeast, and mold numbers.

4. Conclusions

This study investigated the effects of alfalfa plant silage on fermentation properties by inoculating six different LAB strains compared to untreated silage. The *L. brevis* (LS-55-2-2) and *L. citerum* (LS-70-6-1) strains were the most limiting for yeast and enterobacteria growth in silage, respectively. Distinctively, *L. gasseri* (LS-51-2-1) became prominent for recovering DM, and *L. brevis* conserved CP more significantly than the other inoculants and the control. In addition, all strains increased the number of LAB in the obtained silages and control group but did not show any statistical difference. It is concluded that LAB inoculations improved the fermentation properties of alfalfa silage in terms of pH, DM recovery, and protein content, as well as decreasing undesired microorganisms.

Data availability: Data are available on request due to privacy or other restrictions.

Authorship Contributions: Concept: T.G., F.A., M.K., Design: T.G., M.K., Data Collection or Processing: T.G., F.A., S.A., M.K., Analysis or Interpretation: T.G., F.A., S.A., M.K., Literature Search: T.G., S.A., Writing: T.G., M.K.

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Determination of Some Quality Characteristics and Rheological Properties of Yoghurts Made Using Cow Milk and Soy Drink Mixture Enriched with Pomegranate Peel Extract

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ABSTRACT

This study examined the applicability of pomegranate peel extracts (PPE) and microencapsulated PPE extracts (MPE) in yoghurts which are made with cow's milk and soy drink mixtures. For preparing PPE, pomegranate peel powders were extracted by 50% ethanol in an ultrasonic water bath. PPE was encapsulated by using a spray dryer. Phytochemical composition and antioxidant activity of PPE and MPE were determined and PPE and MPE were added to cow milk and soy drink mixture (4:1) for yoghurt production at 0.5% and 1% rates, respectively. Physicochemical, rheological, microbial and sensory properties of the yoghurt samples stored at 4 °C were determined during storage. Extract addition affected storage modulus (G') values and lost tangent (tanδ) values. The extract additive prevented the growth of yeasts and moulds and extended the shelf life of the samples. The favorable effect of the extract on taste and aroma was determined in sensory tests. The overall acceptability scores for PY1 and MY1 samples increased during the storage time and the higher scores was determined on the 28th days of storage. However, the extract contributed to the loss of textural properties such as syneresis and visible viscosity.

Keywords: Yoghurt, Ultrasound, Microencapsulation, Antioxidant, Rheology, Sensory

1. Introduction

Functional foods are defined as food or food ingredients that provide additional benefits to human physiology and metabolic functions, beyond providing basic nutritional requirements of the body, thereby preventing diseases and achieving a healthier life (Kotilainen et al. 2006). Most of the agri-food industry wastes are composed of bioactive polyphenolic phytochemicals and these waste products have the potential to become significant functional food (Amyrgialaki et al. 2014).

Pomegranate (*Punica granatum* L.), which originates from Iran, is known as one of the health-beneficial fruits with its phenolic component content (Akhtar et al. 2015; Kazemi et al. 2016). Pomegranate peel, is a valuable waste of pomegranate juice production, constitutes 26-30% of the total weight of the fruit and contains large amounts of important phenolic compounds such as flavonoids and hydrolysable tannins (Ismail et al. 2012). Due to its rich amounts of vitamins, polysaccharides, polyphenols and minerals, pomegranate peel is used in many countries such as India and Egypt in the field of ethnopharmacology especially in the treatment of diseases such as diarrhea, dysentery and dental plaque (Tripathi et al. 2014). Pomegranate peel shows higher antioxidant activity, phenolic component and therefore bioavailability compared to pomegranate fruit and it can be used as a nutritional supplement or functional input in food formulations (Surek & Nilufer-Erdil 2016). Researchers suggest that pomegranate peels should be used as low-cost nutritional supplements in the low-income countries rather than wasted in the environment (Gullon et al. 2016).

The evaluation of plant-derived extracts as food additives has been a popular subject for many studies (Caleja et al. 2016; Çam et al. 2014; Karaaslan et al. 2014). But plant extracts cannot show the desired stability in food processes and storage (Robert et al.

2010). The stabilization of extracts rich in phenolic compounds by microencapsulation and constituting the desired properties of food systems also has been the subject of many studies (Çam et al. 2014; Kaderides et al. 2015; Robert et al. 2010).

Soy drink is widely consumed especially in Far East countries and it is equivalent to cow's milk in terms of many essential nutrients. In addition to being a functional nutrient, lactose intolerance, milk protein allergy, and vegetarian diet requirements also increase the interest in this product. Although soy drinks are widely consumed in Far Eastern countries sensory problems limited the use of this products in societies that soy-based nutrition is not widespread (Trindade et al. 2001; Vij et al. 2011; Wang et al. 2003).

Soy yoghurt produced from soy drink cannot provide the expected sensory and physicochemical properties due to its chemical composition (Gu et al. 2015). To eliminate these negative properties many methods such as mixing with cow's milk (Temiz & Çakmak 2018), using aromatic herbal sources (Ye et al. 2013), stabilizers (Cho et al. 2013) and new technologies (Ferragut et al. 2009) have been tried.

In this study, we aimed to develop a new product with the use of pomegranate industry wastes that has an important functional composition. The primary aim of the study is to evaluate the pomegranate peel, which has numerous benefits to health, as a food additive and to present it to human nutrition. The pomegranate peel extract (PPE) is microencapsulated to increase the stability and functional properties of the extract during food processing and storage. By using microencapsulated PPE (MPE) and liquid PPE as a functional ingredient in yoghurt made with cow milk and soy drink mixture, it is aimed to eliminate the bad aroma based on soy and increase the functional properties of yoghurts.

2. Material and Methods

2.1. Materials

Pomegranate peels and soybeans obtained from the local market were used in this study. Without damaging the peels, the fruit part and the peel part were separated and the peels were stored at -18 °C until use. Soy drink production was carried out according to the method described by Temiz & Çakmak (2018). The dry matter of soybean drink produced is 12.42%, the fat content is 3.2%, protein content is 5.8%, and the pH is 6.72. Raw cow milk (dry matter 10.23%, fat 3.1%, protein 4.63% and pH 6.74) was obtained from the dairy enterprise operating in region. Mixed starter culture Y 410 500 I: *Streptococcus thermophilus, Lactobacillus delbrueckii subsp. bulgaricus* (Maysa Gıda San. Tic. AŞ. İstanbul, Türkiye) was used in the production of yoghurt samples. Maltodextrin having DE of 13-17 was obtained from Aldrich Company (St. Louis, MO, USA). The rest of the chemicals and standards were analytical grade and procured from Sigma or Merck (Darmstadt, Germany).

2.2. Extraction of pomegranate peels

To prepare PPE, dried peel powders were extracted by 50% ethanol at a solvent to peel powder ratio of 4:1 (v/w) in an ultrasonic water bath (Isolab Laborgerate GmbH, Germany) for 30 minutes at 4 °C. The temperature control of the ultrasonic water bath was made by the water circulation at 0 ºC. The extract was centrifuged at 10,000*xg* for 15 minutes at 4 °C (Hettich Zentrifugen Universal 320 R, Germany) and the supernatant was separated. The solvent was evaporated under 40 ºC using a rotary evaporator (Buchi Rota Vapor K-3, Buchi, Switzerland). The concentrated PPE (Brix 30) was stored at 4 ºC until analysis (Kaderides et al. 2015).

2.3. Microencapsulation of extract

PPE was microencapsulated by a spray drying technique using maltodextrin (DE 13-17) as the coating material. The coating solution that used in the microencapsulation process was prepared according to the method described by Çilek et al. (2012). After the preparation, the coating material was mixed with PPE (4:1 v/v) and homogenized for 10 minutes (IKA-Werke GmbH & Co. KG, Staufen, Germany). Drying was carried out in a laboratory-scale spray dryer (Buchi Mini Spray Dryer B290, Switzerland). The drying process was carried out according to Çam et al. (2014) method with slight modification. For the production of MPE, process conditions were determined as air inlet temperature at 160±5 ºC and outlet temperature 70±2 ºC, extract coating material ratio 1:4 (v/v) and solid feed ratio 30% (w/w).

2.4. Spectrophotometric analysis

MPE was extracted for spectrophotometric analysis. 0.2 g MPE was dissolved with 20 mL methanol: acetic acid: water (50:8:42 v/v/v). The mixture was vortexed for 1 minute and then incubated twice at 4 ºC for 20 minutes in an ultrasonic water bath (Isolab Laborgerate GmbH). The supernatant was centrifuged at 12,000*x g* for 5 minutes (Hettich Zentrifugen Universal 320 R, Germany), then filtered

and stored at 4 °C until analysis was performed (Robert et al. 2010). The total phenolic content (TPC) was determined according to the Folin-Ciocalteu colorimetric method (Fawole & Opara 2016). The calibration curve (R^2=0.9967) was determined using different gallic acid concentrations (20, 40, 60, 80, 100 ppm). TPC of PPE was expressed as gallic acid equivalent (GAE) in milligrams per mL. TPC of MPE was expressed as GAE in milligrams per g.

For surface phenolic content (SPC) of MPE, Robert et al. (2010) method was applied with little modification. 0.5 g of MPE and 25 mL of ethanol: methanol (1:1) were vortexed. The mixture was centrifuged at 3500 rpm for 3 minutes (Hettich Zentrifugen Universal 320 R, Germany) and the SPC in the clear portion was determined according to the Folin-Ciocalteu colorimetric method (Fawole & Opara 2016).

Total flavonoid concentration (TFC) was determined as described by (Fawole & Opara 2016), and the results were expressed as catechin equivalents (CE) per mL (g) sample. The results were calculated according to the catechin standard curve (R^2=0.9997). Total anthocyanin content (TAC) was measured as described by El Kar et al. (2011), and the results were expressed as mg cyaniding 3-O-glucoside per 1,000 mL (g) of the sample.

The antioxidant activity was evaluated with the scavenging method of DPPH as reported by Kazemi et al. (2016). The extract solutions were allowed to stay in the dark for 30 minutes at room temperature and then their absorbance at 517 nm was read by spectrophotometer. Two parallel studies were performed for each solution. Different concentrations of Trolox solutions were used to create linear regression equations (R^2=0.9927). Results were given in Trolox equivalents (TE) per 100 mL of extract. Antioxidant activity of the samples was also measured using the $ABTS^+$ radical cation capture activity method described by Mushtaq et al. (2015). Different concentrations of Trolox solutions were used to create linear regression equations (R^2=0.9927). Results were given in TE per 100 mL of extract. All spectrophotometric measurements were conducted in duplicate.

2.5. Yield and efficiency of microencapsulation

The yield and efficiency of the microencapsulation process were determined by the calculation method stated by Kaderides et al. (2015). The following equations are used for the yield and efficiency of the microencapsulation process.

$$\text{efficiency}=[(\text{TPC-SPC})/\text{TPC}]\times 100 \quad (1)$$

$$\text{yield}=(\text{total weight of microencapsules/total weight of input})\times 100 \quad (2)$$

2.6. Yoghurt production

Yoghurt production was carried out as stated in our previous study (Temiz & Çakmak 2018) in the Ondokuz Mayıs University Faculty of Agriculture Milk Processing Plant. As a result of the preliminary experiments and literature knowledge the mixing milk ratios to be used in the production of yoghurts were determined as 4:1 cow milk:soy drink. Yoghurt milk mixture was heat-treated at 85±2 °C for 20 minutes. After cooling the yoghurt milk to 65 °C, the mixture was added to 0% (control sample, CC0), 0.5%, 1% PPE (PY1 and PY2, respectively) and MPE (MY1 and MY2, respectively). MPE was added to the milk to make it equal to the TFC of the PPE. Yoghurt samples were stored for 28 days at 4 ºC for analysing and analyses were made on the 1st, 7th, 14th, 21st and 28th days of the storage periods. But, the 28th day analyses of the control yoghurts, it was not conducted due to the signs of mold.

2.7. Physicochemical analysis

The pH of the samples was determined by using a digital pH meter (Cyberscan PC 510, Eutech instruments, Ayer Rajah Crescent, Singapore). The lactic acid content in the samples was measured by titration using NaOH (0.1 mol/L) and expressed in lactic acid (%). For the determination of syneresis (%), 5 g of the sample was centrifuged at 2,500*x g* for 10 min at 4 °C (Sigma Model 3K30, Osterode am Harz, Germany) and the results were expressed as % syneresis based on the percentage of the amount of supernatant in the weighed sample amount.

2.8. Rheological measurements

Rheological measurements in yoghurt were performed using parallel plates (diameter: 35 mm, gap: 1 mm) in a rheometer (HAAKE Mars III; Thermo Scientific, Germany) at 4 °C. The samples were mixed in a magnetic stirrer at 100 rpm for 1 minute before rheological tests were performed on yoghurts. For all tests, a 1 mL yoghurt sample was taken and placed between the plates and allowed to equilibrate (4 °C) for 2 minutes. Two types of tests were conducted on yoghurts. For steady sweep tests, the samples were sheared

continuously at a rate ranging from 1-100/s at a constant stress of 1 Pa and shear stress values were recorded according to shear rates in this range. Apparent viscosity value (η_{50}) was expressed to be the same as the feeling of shear in the mouth, calculated at a shear rate of 50/s viscosity value was determined (Morris 1994). To determine the dynamic viscoelastic properties, frequency sweep tests were carried out over a frequency range of 0.1 to 10 Hz at 1 Pa. The rheological parameters storage modulus (G'), loss modulus (G"), and loss tangent (tanδ) which is equal to G"/G' were determined during the test. Calculation of these measurements was performed using Rheowin 4 Data Manager software (version 4.20, Haake). All the rheological parameters were the mean of two measurements of samples.

2.9. Microbiological analysis

Ten g of yoghurt sample was mixed with 90 mL 0.1% peptone (Merck Darmstadt, Germany) water in a Stomacher. Decimal dilutions were prepared with values of 10^{-2} to 10^{-9} for each sample. The viable lactobacilli count was determined according to the pour plate method using de Man, Rogosa and Sharpe agar. *Streptococci* count was determined by the pour plate method using M17 agar. Total yeast and mould count were determined by the spreading method using yeast extract glucose chloramphenicol agar (Temiz & Dagyildiz 2018). Two replicates were performed for each sample.

2.10. Sensory evaluation

For the sensory evaluation of the samples, before sensory analysis a panel of 10 people was informed about the evaluation. Each of the yoghurt samples was coded with a different number and presented to the panelists. Sensory evaluations were performed on the 1st, 7th, 14th, 21st and 28th days of storage. Panelists were asked to rate yoghurts according to their liking. Scoring categories were color and appearance, texture, taste and aroma, and overall acceptability. In the color and appearance and texture category, the samples were evaluated by 1-5 points (5= like extremely, 1= dislike extremely) scale. In the taste and aroma category, samples were asked to be evaluated in the range of 1-9 points hedonic scale (9= like extremely, 1= dislike extremely). Overall acceptability scores are presented as the average of the scores of the samples evaluated.

2.11. Statistical analysis

Samples were analyzed using SPSS Statistical Software (2000) (SPSS Inc., Chicago, IL, USA), and the results were offered as mean ± standard deviation. Significance differences ($p<0.05$) among the different types of samples and the effect of storage time were analyzed with ANOVA, followed by Duncan's multiple range tests. All measurements were conducted in duplicate.

3. Results and Discussion

3.1. Chemical composition and antioxidant activity of PPE and MPE

TPC, TAC and TFC analysis results of PPE and MPE are given in Table 1. As seen from the Table, there were significant differences between treatments ($p<0.05$). TPC content was determined higher in the PPE samples while TAC and TFC contents were determined higher in MPE samples. TPC in PPE was calculated as 108.44 GAE/mL, TFC was 9.55 mg CE/mL and TAC was 223.43 mg Cyn-3-glu/L extract. In MPE, TPC was calculated as 105.30 GAE/mL, TFC was 16.29 mg KE/mL and TAC was 410.68 mg Cyn-3-glu/L. The majority of phenolics in PPE are gallic acid, ellagic acid, punicalin and punicalagin and other condensable tannins (Ismail et al. 2012). Surek and Nilufer-Erdil (2016) reported TPC, TFC and TAC of pomegranate peel as 18029.2 mg GAE/100 g; 21,758 mg CE/100 g and 51.8 mg Cyn-3-glu/100 g, respectively. The results are different from our study and this is due to many reasons such as extraction process and pomegranate variety. The extraction of pomegranate peel phytochemicals in methanol mixtures is more efficient (Ismail et al. 2012). Fawole and Opara (2016) calculated that TPC in the PPE in ethanol: water (1:1) mixtures was 2992.93 mg GAE/100 mL. In the same study, it was stated that the amount of phenolic substance was highest in extracts prepared in alcohol-water mixtures compared to pure ethanol (2458.03 mg GAE/100 mL) and pure water (2658.00 mg GAE/100 mL). TFC in PPE was expressed as 1505.00 mg CE/100 mL. Orak et al. (2012), the average TPC of three different types of Hicaznar peel extract is expressed as 160.70 mg GAE/g. TFC in peel was reported to be between 9.44 and 19.93 mg quercetin/g.

In other studies, TPC of pomegranate peel was reported to be 101,856 mg GAE/g (Fischer et al. 2011) and 118.2-370 mg GAE/g Amyrgialaki et al. 2014). TPC in the PPE may vary for many reasons; pomegranate species, pomegranate maturity; extraction method, duration, temperature; solvent ratio, solvent type, etc.

Radical cation reduction activity is associated with the amount of phenolic compounds in many studies (Amyrgialaki et al. 2014; Surek & Nilufer-Erdil 2016; Turgut et al. 2016). Pomegranate peel has more phenolic components than all other parts of the fruit (pulp,

seed, and leaf). Fawole and Opara (2016) stated that pomegranate extract has 5-30 times more radical reduction power than fruit pulp. Therefore, it is recommended that PPE be used for the functional ingredient in food systems (Fawole & Opara 2016; Surek & Nilufer-Erdil 2016). The evaluation of antioxidant activity by a single method is very difficult because of the complexity of the antioxidant mechanism. Additionally, many factors such as temperature, the chemical structure of phenolics, and the pH of the environment affect this mechanism. Therefore, the measurement of antioxidant activity cannot be adequately assessed by a single method (Surek & Nilufer-Erdil 2016).

The results of the antioxidant activity analysis of PPE and MPE are given in Table 1. Although there was not much decrease in antioxidant activity because of the microencapsulation process, $ABTS^+$ radical cation capture activity reduction power of both PPE and MPE was found to be higher than DPPH reduction power. Surek & Nilufer-Erdil (2016) and Fischer et al. (2011) calculated the DPPH and ABTS activities in pomegranate peels as 45099.6 mg TEAC/100 g and 51100.8 mg TEAC/100 g, respectively.

Mushtaq et al. (2015) stated that ABTS reduction power in pomegranate peels extracted by using different enzymes is between 118.25-445.02 mM TE/g. In the same study, it was stated that enzyme supported extraction was more efficient than solvent extractions, especially in $ABTS^+$ reduction power. $ABTS^+$ and DPPH reduction power of PPE determined in our study did not show much difference according to the literature, but it was lower than the enzyme-assisted extraction study.

Table 1- Chemical composition and antioxidant activity of pomegranate peel extracts and microencapsulated pomegranate peel extracts

Parameter	***PPE***	***MPE***
TPC (mg GAE/mL)	108.44 ± 0.28^a	105.30 ± 1.21^b
TFC (mg CE/mL)	9.55 ± 0.02^b	16.29 ± 0.16^a
TAC (mg Cyn-3-glu/L)	223.43 ± 3.34^b	410.68 ± 2.56^a
DPPH (TE/100 mL)	71.24 ± 0.16^a	69.90 ± 0.65^a
ABTS (TE/100 g)	100.97 ± 0.21^a	93.30 ± 0.26^b

PPE: Pomegranate peel extracts, MPE: Microencapsulated pomegranate peel extracts, TPC: Total phenolic compound, TFC: Total flavonoid content, TAC: Total anthocyanin content, DPPH: Scavenging activity of DPPH; ABTS; radical cation capture activity of ABTS. Small letters show the significant difference ($p<0.05$) between treatments. Analytical results are the means ± standard deviation of three replicates

3.2. Encapsulation efficiency and encapsulation yield

As a result of microencapsulation process analysis, microencapsulation yield and efficiency were calculated as 37.6% and 78.21%, respectively. Çam et al. (2014) investigated microencapsulation optimization of PPE in spray dryer and stated that process yield was calculated as 49.8% and process efficiency as 98.8% at 160 °C inlet temperature. The efficiency and yield of microencapsulation in the spray dryer were dependent on many different parameters. Although the inlet temperature is almost the same, it can be said that the yield and efficiency of our system were low due to the different parameters such as feed rate, outlet temperature, and coating rate. In the same study, the amount of phenolic compounds in the microcapsule at 160 °C was expressed to be 94.6 mg GAE/g and this value was similar to our study. Kaderides et al. (2015) optimized the microencapsulation of PPE with different materials, the efficiency of microencapsulation was reported to be between 69.80-99.80%. Although it depends on many other factors, it was stated that using only maltodextrin as a coating material decreased the efficiency of the process, but the protein-containing coating materials (whey powder, skimmed milk powder) were used with maltodextrin increased the efficiency of the system.

The efficiency and yield of the system in the microencapsulation technique with the spray dryer depend on many factors. Studies indicated that increasing the inlet temperature reduces the system efficiency whereas decreasing the temperature prevents effective drying. However, many parameters affect the system, such as the type of coating material and coating ratio and feed flow rate (Goula & Lazarides 2015).

3.3. First fermentation time of yoghurt

pH and time factors effects on the yoghurt formation of the milk samples are given in Figure 1. As can be seen from the Figure 1, a faster pH decrease occurred in the control sample when compared to the other samples, and the time to decrease to pH 4.71 was 120 minutes. For the extract-added samples this time was 240 minutes while the time reached to 4.71 pH value for microencapsulated samples was 270 minutes. These results show that the addition of extract and microencapsulated extract had an effect on the first fermentation period of yoghurt, and microcapsules caused to prolong the growth times of bacterial cultures. Resources have shown that pomegranate peel phenolic causes lysis of cell membrane proteins (Ismail et al. 2012; Akhtar et al. 2015).

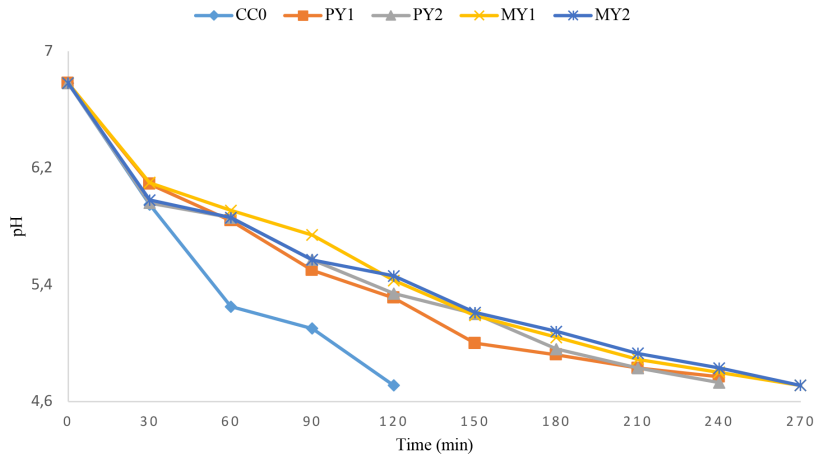

Figure 1- First fermentation time of yoghurt. CC0: control sample; PY1 and PY2: soy yogurts supplement with 0.5% and 1% pomegranate peel extract, respectively; MY1 and MY2: soy yogurts supplement with 0.5% and 1% microencapsulated pomegranate peel extract, respectively

3.4. Physicochemical characteristics of yoghurts

The pH values and titratable acidity (% lactic acid) values of samples during storage are given in Figure 2. The pH values of the samples varied between 4.02-4.65. In all samples, a decrease in pH values was observed during the storage time. The decrease in pH during storage in yoghurts is due to starter cultures producing organic acid by fermenting carbohydrates (Bedani et al. 2014a). It was a significant effect at 1st, 7th and 14th days of storage time ($p<0.05$) while the effect of PPE and MPE on pH change was not significant at the 1st, 21st and 28th days of the storage time ($p>0.05$). Osman and Razig (2010) stated that in yoghurt produced from soy-cow milk mixture, pH values decreased more as the cow milk ratio increased in the mixture. The reason for the slower decrease in pH values of yoghurt with soy drink is that the starter cultures used to metabolize lactose faster than soy oligosaccharides (Cruz et al. 2009).

The amounts of titratable acidity of yoghurt samples in terms of lactic acid are given in Figure 2. There was no significant difference in titration acidity values between samples except the first day ($p>0.05$). During storage, the titration acidity of the samples was statistically significant except for the PY2 sample ($p<0.05$). Research shows that this is due to the β-galactosidase enzyme produced by yoghurt starter cultures (Shori 2013). This naturally increases the titration acidity while lowering the pH values.

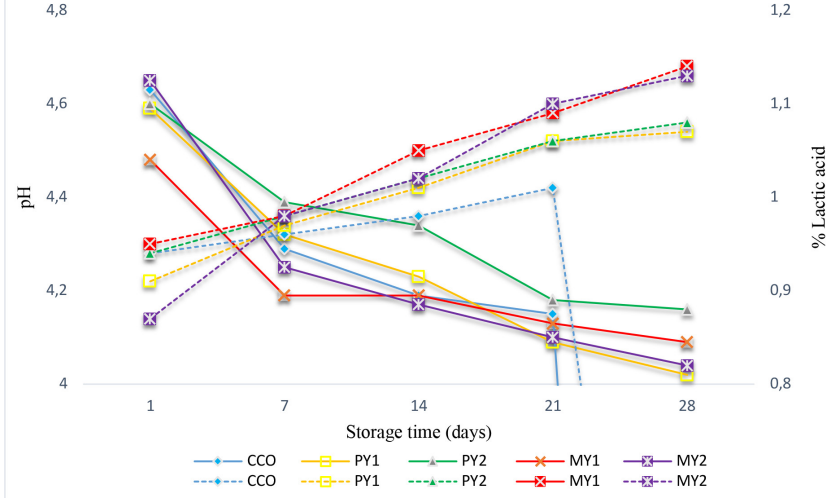

Figure 2- The pH values of the samples during storage (straight line); Changes in titratable acidity of samples (dashed line); CC0: control sample; PY1 and PY2: supplement with 0.5% and 1% pomegranate peel extract, respectively; MY1 and MY2: supplement with 0.5% and 1% microencapsulated pomegranate peel extract, respectively

The syneresis values of yoghurt samples are given in Figure 3. Syneresis values between samples and during storage were statistically significant ($p<0.05$). In yoghurt samples, syneresis increased up to the 14th day, but at the end of the 28th day, small decreases were detected. It was determined that syneresis values increased with an increasing amount of extract. Tseng and Zhao (2013) stated that

the syneresis values of yoghurt enriched with grape pulp increased during storage. However, it is stated that the amount of syneresis increases with increasing grape pulp amount. Ingredients and amounts of ingredients to be used in yoghurt are important as they reorganize the protein gel matrix. During storage, pH values were reduced more slowly in extract-added samples than the control sample. The tightening of the gel structure of yoghurt and the decrease in syneresis were related to pH values (McCann et al. 2011). This expression is consistent with the slow decrease of pH values in the extract added samples until the 14th day and consequently increase of syneresis values.

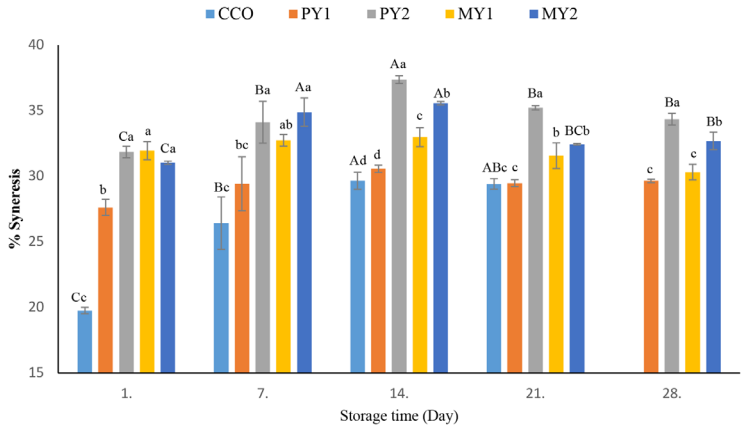

Figure 3- % syneresis values during storage of samples; CC0: control sample; PY1 and PY2: supplement with 0.5% and 1% pomegranate peel extract, respectively; MY1 and MY2: Supplement with 0.5% and 1% microencapsulated pomegranate peel extract, respectively. Capital letters show the significant difference ($p<0.05$) between storage times of same treatment at some storage time. Small letters show the significant difference ($p<0.05$) between treatments. Values are means ± standard deviation of three replicates

3.5. Rheological properties of samples

The apparent viscosity of all the samples decreased as the shear rate increased, indicating a shear-thinning fluid behaviour (Figure 4). The viscosity of the control sample decreased continuously with increasing shear rate throughout the whole shear rate range studied. However, extract addition tended to alter the shape of viscosity curves. The addition of the extract caused a decrease in the apparent viscosity values of the samples. The rheological properties reflect the mouthfeel of yoghurts and are important because they significantly affect consumer preferences. The apparent viscosity values of yoghurt samples (η_{50}) are given in Figure 5. In all samples, η_{50} values of yoghurts increased during storage. The apparent viscosity values of the control and PY1 samples during storage were found to be statistically similar. All yoghurts exhibited characteristics of a weak viscoelastic gel with G'>G" at all frequencies investigated (Figure 6). G' is associated with the energy stored before deformation during the frequency sweep test and is related to the hardness of the gel structure network (Ferragut et al. 2009). G' values of all samples were always greater than G" values. Due to this situation the yoghurt system exhibited a solid-like behaviour. G' value is associated with the number and strength of the links between casein distributions in yoghurt (Sendra et al. 2010). G' values increased on the first day with the addition of 0.5% extract and microcapsule.

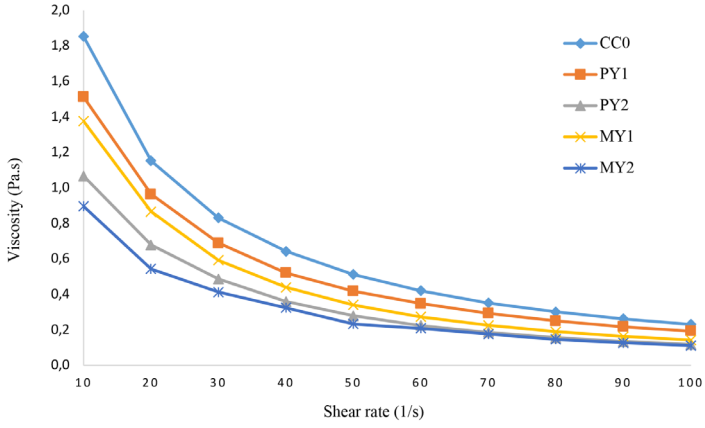

Figure 4- Apparent viscosity change due to shear rate in the 1st day samples. CC0: control sample; PY1 and PY2: yogurts supplement with 0.5% and 1% pomegranate peel extract, respectively; MY1 and MY2: Yogurts supplement with 0.5% and 1% microencapsulated pomegranate peel extract, respectively

tanδ (loss tangen) is a result of storage and loss modulus values (G"/G') and can provide more information about the viscoelastic properties of the samples (Figure 7). Low tanδ values mean stronger elastic behaviour of samples. Wang et al. (2020) stated that samples with low tanδ values can be perceived as more mouthful than higher ones. During storage, tanδ values decreased in all samples and are matched by solid-phase behaviour in this flow (Sendra et al. 2010).

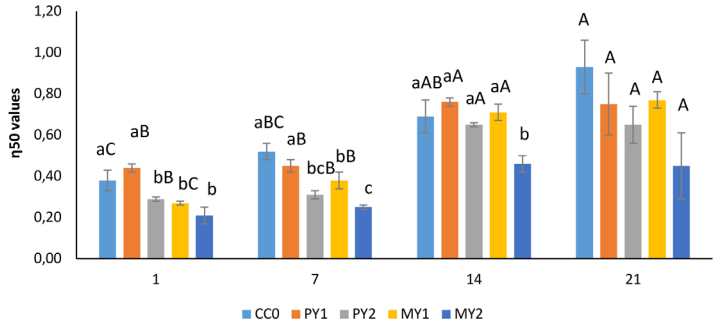

Figure 5- The apparent viscosity values of yogurt samples during storage (Pa.s). CC0: control sample; PY1 and PY2: yogurts supplement with 0.5% and 1% pomegranate peel extract, respectively; MY1 and MY2: Yogurts supplement with 0.5% and 1% microencapsulated pomegranate peel extract, respectively. Capital letters show the significant difference (p<0.05) between storage times of same treatment at some storage time. Small letters show the significant difference (p<0.05) between treatments

The addition of extract in yoghurt caused rising in G' values compared to the control. A similar result was reported by Pan et al. (2019). On the 14th day, there was an increase in G' values and a decrease in tanδ values in all samples. This shows that yoghurts exhibit solid behaviour depending on storage.

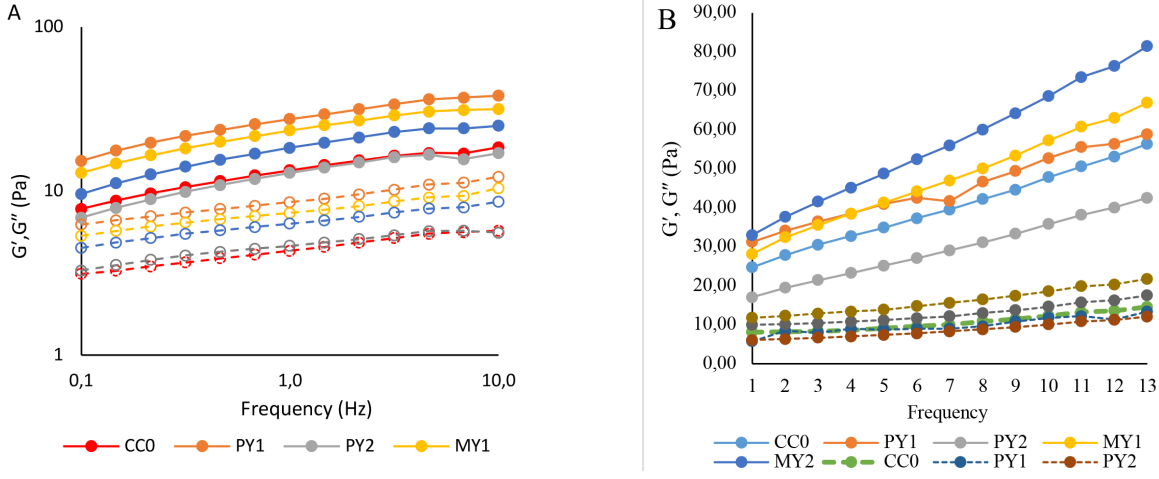

Figure 6- Frequency sweep curves of samples stored for 1 day (A), 14 days (B). CC0: control sample; PY1 and PY2: yogurts supplement with 0.5% and 1% pomegranate peel extract, respectively; MY1 and MY2: yogurts supplement with 0.5% and 1% microencapsulated pomegranate peel extract, respectively. G', storage modulus, Pa (straight line) G", loss modulus, Pa (dashed line). G' and G" were obtained from 0.1 to 10 Hz of frequency sweep at 4 °C

3.6. Microbiological analyses

The average initial microbial counts on yoghurt samples were ~10^9 cfu/g. Counts of lactobacilli and streptococci were given in Table 2. Counts of lactobacilli and streptococci were significantly higher ($p<0.05$) in the control samples. However, it was found that the durability of PPE added yoghurts during storage was higher. There were statistically significant differences in lactobacilli numbers between samples and storage time ($p<0.05$). In the yoghurt supplemented with MPE, the number of lactobacilli was lower in all analysis days. The antimicrobial properties of PPE slowed the growth of lactobacilli but did not prevent the growth of the dominant flora. Research has shown that microencapsulated phenolic compounds retain their antiradical and antimicrobial properties for a longer time (Kaderides et al. 2015). In our study, the viability of lactobacilli in MPE added yoghurts was relatively low compared to other samples. At the end of 28 days of storage, lactic streptococci and streptococci numbers were decreased in all samples.

The added extract adversely affected the viability of Streptococci. Streptococci viability was less in MPE added yoghurts than PPE added yoghurts. As previously mentioned, microcapsules retain their antibacterial properties for a longer period and consequently limit microbial growth. Similar results were obtained by Bedani et al. (2014b). Alexandre et al. (2019) stated that fruit extracts are rich in phenolic compounds which are known as antimicrobial agents, inhibiting the growth of pathogenic bacteria and fungi. However, polyphenol-rich PPE exhibited low inhibitory activity against Lactobacillus and Streptococci strains.

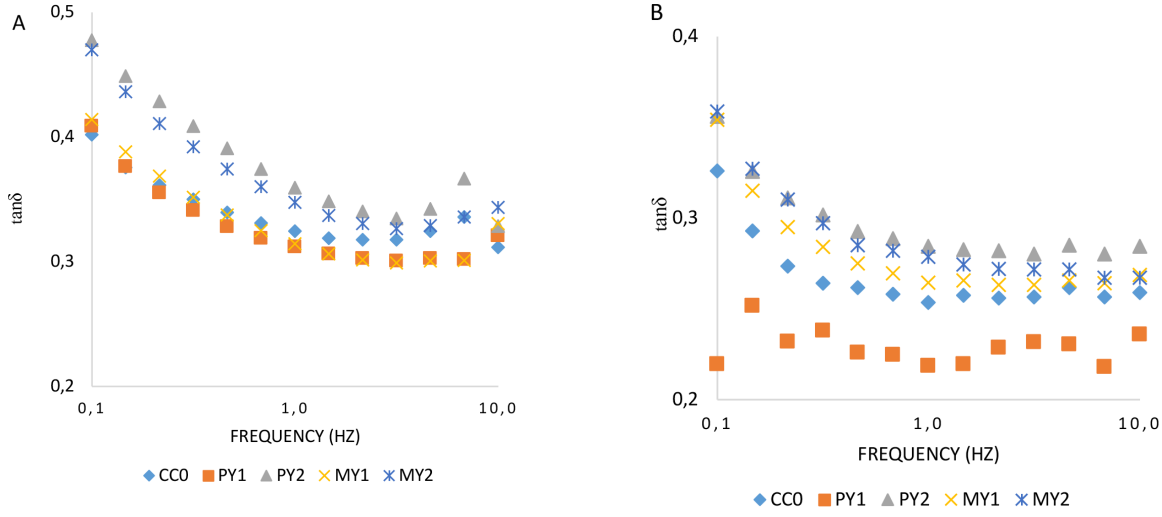

Figure 7- Loss tangent (tanδ) curves of samples stored for 1 day (A), 14 days (B). CC0: control sample; PY1 and PY2: yogurts supplement with 0.5% and 1% pomegranate peel extract, respectively; MY1 and MY2: yogurts supplement with 0.5% and 1% microencapsulated pomegranate peel extract, respectively. tanδ were obtained from 0.1 to 10 Hz of frequency sweep at 4 °C

Any yeast-mould growth was observed in any yoghurt sample during 21 days of storage. On the 28th day of the storage period, only 2.6 log CFU/g of yeast and mould was observed in the control sample. On the 28th day, yeast and mould formation did not occur in PPE and MPE added yoghurts. These results show that PPE and MPE additive prevents yeast and mould growth in yoghurts and prolongs the shelf life of yoghurts (results not shown).

Table 2- Changes in microbial counts of yoghurt samples during the storage time (log cfu/mL)

		Days				
		1	***7***	***14***	***21***	***28***
Lactobacilli	**CC0**	9.08 ± 0.16^{Aa}	9.07 ± 0.06^{Aa}	8.53 ± 0.07^{bB}	$8.16\pm0.08C^{a}$	-
	PY1	8.87 ± 0.07^{abA}	8.85 ± 0.04^{Aa}	8.68 ± 0.01^{aB}	8.29 ± 0.09^{Ca}	7.63 ± 0.02^{Da}
	PY2	8.55 ± 0.20^{Abc}	8.76 ± 0.01^{Abc}	8.55 ± 0.01^{Ab}	8.21 ± 0.03^{Ba}	7.50 ± 0.00^{Cb}
	MY1	8.33 ± 0.11^{Ac}	8.44 ± 0.21^{Ab}	8.33 ± 0.04^{Ac}	7.93 ± 0.03^{Bb}	6.92 ± 0.06^{Cc}
	MY2	8.26 ± 0.15^{Ac}	8.40 ± 0.22^{Ab}	8.00 ± 0.01^{Bd}	7.65 ± 0.03^{Cc}	6.61 ± 0.18^{Dd}
Streptococci	**CC0**	9.23 ± 0.16^{Aa}	9.19 ± 0.05^{A}	9.24 ± 0.04^{Aa}	8.07 ± 0.01^{Ba}	-
	PY1	8.99 ± 0.08^{Ab}	8.95 ± 0.12^{A}	8.94 ± 0.01^{Ab}	7.87 ± 0.01^{Bb}	7.63 ± 0.00^{Ca}
	PY2	8.81 ± 0.01^{bc}	8.17 ± 0.72	8.88 ± 0.02^{b}	7.58 ± 0.06^{c}	6.88 ± 0.15^{bc}
	MY1	8.73 ± 0.00^{Ac}	8.88 ± 0.14^{A}	8.98 ± 0.11^{Ab}	7.86 ± 0.09^{Bb}	7.32 ± 0.12^{Cab}
	MY2	8.79 ± 0.06^{Abc}	8.55 ± 0.22^{A}	8.55 ± 0.14^{Ac}	7.26 ± 0.04^{Bd}	6.59 ± 0.28^{Cc}
Yeasts/mould	**CC0**	nd*	nd	nd	nd	2.60
	PY1	nd	nd	nd	nd	nd
	PY2	nd	nd	nd	nd	nd
	MY1	nd	nd	nd	nd	nd
	MY2	nd	nd	nd	nd	nd

CC0: Control sample, nd: Not determined, PY1 and PY2: Yoghurts supplement with 0.5% and 1% pomegranate peel extract, respectively; MY1 and MY2: Yoghurts supplement with 0.5% and 1% microencapsulated pomegranate peel extract, respectively. Capital letters show the significant difference ($p<0.05$) between storage times. Small letters show the significant difference ($p<0.05$) between treatments. Values are means ± standard deviation of three replicates

3.7. Sensory evaluation

Changes in sensory properties of samples during storage were given in Table 3. The highest score was determined as 4.80 at control samples on the 7th day of storage while the lowest color and appearance score was determined as 3.00 at MY1 on the 1st day of storage. Although the color and appearance scores of the extract added yoghurts were higher than the 3 value, they were lower than the control samples. The differences between PPE and MPE added yoghurts were generally similar in terms of color and appearance scores. Similar situations were determined for texture scores of yoghurt. In the "taste and aroma" category, the sample that reached the highest score with 7.50 points was PY1 on the 28th day of storage. The sample with the lowest score was the control sample with a score of 5.58 on the 21st day of storage. The overall acceptability scores were detected between 5.57 and 7.38. For CCO sample was determined on the 21st day of storage while the lowest score for PE added yoghurts was determined at the MY2 sample on the 28th day of storage. The overall acceptability scores of the yoghurt decreased with PE adding but for PY1 and MY1 samples these scores increased during the storage time and the higher scores were determined on the 28th day of storage. Due to lack of habit to use soy products by panelists, the researchers suggested that the information given before the sensory tests of soy products would contribute to a more accurate assessment of the scores of the products (Bedani et al. 2014a; Drake & Gerard 2003).

Table 3- Changes in sensory properties of yoghurt samples during the storage time

	Days					
		1	***7***	***14***	***21***	***28***
Color and appearance	CC0	4.60 ± 0.14^{Aa}	4.80 ± 0.00^{A}	4.13 ± 0.00^{Ba}	4.50 ± 0.24^{ABa}	-
	PY1	3.70 ± 0.28^{Db}	3.90 ± 0.71^{C}	4.06 ± 0.08^{Ba}	4.09 ± 0.12^{Aab}	4.07 ± 0.50^{Ba}
	PY2	3.75 ± 0.35^{Ab}	3.38 ± 0.25^{E}	3.57 ± 0.09^{Db}	3.59 ± 0.12^{Cbc}	3.71 ± 0.01^{Bb}
	MY1	3.00 ± 0.14^{Ec}	3.82 ± 0.55^{B}	3.13 ± 0.18^{Dc}	3.89 ± 0.40^{Ab}	3.45 ± 0.05^{Cc}
	MY2	3.35 ± 0.21^{Abc}	3.35 ± 0.21^{A}	3.25 ± 0.00^{Bc}	3.25 ± 0.11^{Bc}	3.20 ± 0.10^{Cd}
Texture	CC0	4.65 ± 0.07^{a}	4.60 ± 0.00^{a}	4.44 ± 0.27^{a}	4.59 ± 0.12^{a}	-
	PY1	4.25 ± 0.07^{Ab}	3.85 ± 0.35^{Bb}	3.63 ± 0.35^{Db}	3.50 ± 0.00^{Ebc}	3.71 ± 0.20^{Cc}
	PY2	3.90 ± 0.00^{Ac}	3.25 ± 0.07^{Cc}	3.00 ± 0.00^{Db}	3.25 ± 0.35^{Cc}	3.85 ± 0.20^{Bb}
	MY1	3.55 ± 0.21^{Ed}	4.00 ± 0.14^{Bb}	3.59 ± 0.42^{Db}	3.95 ± 0.35^{Cb}	4.15 ± 0.02^{Aa}
	MY2	3.00 ± 0.14^{De}	3.79 ± 0.13^{Ab}	3.38 ± 0.00^{Cb}	2.92 ± 0.12^{Ec}	3.65 ± 0.01^{Bd}
Taste and aroma	CC0	6.45 ± 0.21^{BC}	7.40 ± 0.00^{aA}	6.98 ± 0.50^{AB}	5.58 ± 0.35^{bC}	-
	PY1	6.60 ± 0.42^{D}	6.70 ± 0.14^{Cb}	6.56 ± 0.25^{E}	7.42 ± 0.57^{Ba}	7.50 ± 0.30^{Aa}
	PY2	7.05 ± 0.50^{B}	6.84 ± 0.06^{Cb}	6.44 ± 0.08^{E}	7.33 ± 0.00^{Aa}	6.74 ± 0.10^{Dd}
	MY1	6.70 ± 0.28^{D}	7.00 ± 0.00^{Bb}	6.57 ± 0.81^{E}	7.17 ± 0.47^{Aa}	6.90 ± 0.33^{Cb}
	MY2	5.70 ± 0.28^{E}	7.45 ± 0.21^{Aa}	6.31 ± 0.03^{C}	6.84 ± 0.23^{Ba}	6.14 ± 0.20^{Dc}
Overall acceptability	CC0	7.30 ± 0.14^{Aa}	6.95 ± 0.00^{A}	7.38 ± 0.35^{Aa}	5.92 ± 0.35^{B}	-
	PY1	6.70 ± 0.57^{Cab}	6.65 ± 0.00^{D}	6.69 ± 0.08^{Cb}	6.84 ± 0.47^{B}	7.36 ± 0.01^{Aa}
	PY2	6.55 ± 0.07^{Bab}	6.40 ± 0.00^{D}	5.94 ± 0.08^{Ec}	6.58 ± 0.35^{A}	6.50 ± 0.01^{Cc}
	MY1	6.40 ± 0.28^{Db}	7.00 ± 0.42^{B}	6.07 ± 0.26^{Ec}	6.75 ± 0.35^{C}	7.31 ± 0.02^{Ab}
	MY2	5.95 ± 0.07^{Bb}	6.65 ± 0.50^{A}	5.75 ± 0.00^{Cc}	5.75 ± 0.11^{C}	5.57 ± 0.02^{Dd}

CC0: Control sample; PY1 and PY2: Yoghurts supplement with 0.5% and 1% pomegranate peel extract, respectively; MY1 and MY2: Yoghurts supplement with 0.5% and 1% microencapsulated pomegranate peel extract, respectively. Capital letters show the significant difference ($p<0.05$) between storage. Small letters show the significant difference ($p<0.05$) between treatments. Values are means ± standard deviation of three replicates

4. Conclusion

The addition of the extract also negatively affected the syneresis and apparent viscosity values. The addition of the extract increased the shelf life of the products by preventing the formation of yeast and mold in yoghurt samples. The yeast-mold formation did not occur in samples with a 1% extract addition even after 28 days. The addition of PPE and MPE was provided with reduced sensory scores due to its distinctive sour flavor when compared to the control. However, while the addition of PPE and MPE provided better preservation of sensory properties during storage, it caused weaknesses in the textural properties of yoghurts. This situation was also expressed by the panelists in their sensory analysis. In addition, it shows that they can be used as an antioxidant activity increaser in yogurt production

when the antioxidant values of PPE and MPE are examined. This is a promising conclusion that if the structure and textural properties can be improved, the use of PPE and MPE additives in yoghurts will become possible.

Data availability: Data are available on request due to privacy or other restrictions.

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Verification of QuEChERS Method for the Analysis of Pesticide Residues and Their Risk Assessment in Some Fruits Grown in Tokat, Turkey

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ABSTRACT

This study sought to determine pesticide residues in some fruits in Tokat province, Turkey and to assess the health risks associated with the determined residues. QuEChERS analytical method was verified to determine 260 pesticides by using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Pesticide solutions corresponded 10 and 50 µg kg^{-1} were applied to the pesticide-free apple matrix for the method verification. The linearities (R^2), limit of detections, limit of quantifications and mean recovery values of the pesticides ranged between 0.990-0.999, 0.83-3 µg kg^{-1}, 2.76-9.99 µg kg^{-1} and 70.3-119% (with relative standard deviation ≤20%), respectively. The results were evaluated according to the European Union maximum residue limits (EU-MRL). The residues were lower and higher than the EU-MRL with a ratio of 37.7% and 32.1% of the tested samples, respectively. The residue levels of diflubenzuron in apples, permethrin in cherries, and dimethoate in pears, apples, and peaches were higher than the EU-MRL. The results of health risk assessments indicated that omethoate and dimethoate have acute and chronic toxicity potential for consumers. Hence, the pesticide use in the study area must be reduced to avoid health risks. Furthermore, alternative management methods should be developed to lower the use of pesticides.

Keywords: LC-MS/MS, Fresh fruit, Method verification, Health risk, QuEChERS

1. Introduction

Fruits are an integral part of human nutrition for better health (Akhtar et al. 2010). The daily consumption of fruits reduces the risk of cardiovascular diseases, stroke, and cancers of the mouth, pharynx, esophagus, lungs, stomach, and colon (Aberoumand & Deokule 2010). Although fruits are very beneficial for consumers, pesticide residue problems occur in fruits from time to time and causes concerns for consumers. As in other crops, fruits are infected by numerous pests and diseases during the fruit maturation phase and post-harvest period (Sircu et al. 2019). Fruit producers intensively use pesticides in both field and warehouse against these pests. However, these chemicals cause residue problems on/in the product (Arias-Estévez et al. 2008) and they seriously threaten human health (Balkan & Yılmaz 2022b).

Since fruits are mainly consumed fresh (raw) and semi-processed, they contain higher pesticide residues in comparison to other foods of plant origin (Claeys et al. 2011). For this reason, it is important to assess the health risks associated with the intake of pesticides. Health risk assessments are a priority of food regulatory agencies to ensure consumers' food safety (Fan et al. 2019). The detection and monitoring of pesticide residues are extremely important. The monitoring of pesticide residues allows control over crop quality by identifying the potential risks of pesticides to public health. The pesticide residues data is often compared with the European Union (EU) standards which refer to maximum residue limits (MRL). Despite legal provisions concerning pesticide use in Turkey, incomplete and defective approaches persist. While studies on pesticide residues have been growing in Turkey, they remain unsatisfactory. The EU pesticides database provides technical guidance for legislation through EU database where >656 MRLs of pesticide residues can be found (EC 2023). The Federal Institute for risk assessment (BfR 2013) recommends that the accumulative risk should be evaluated

by hazard index (HI) and hazard quotients for the individual pesticides (Hamzawy 2022). The risk assessment of pesticide residues is vital for health by ensuring food quality.

The current study aimed at; 1) exploring pesticide residue levels in some fresh fruits grown in Tokat province, Turkey, by verified QuEChERS method and 2) health risk assessment based on pesticide exposure by evaluating the residue levels in fruit samples.

2. Material and Methods

2.1. Reagents and chemicals

Pesticide reference standards were supplied by Dr. Ehrenstorfer Laboratories GmbH (Bgm-Schlosser-STr. 6A, Augsburg, Germany). Acetonitrile (ACN), methanol (MeOH), magnesium sulfate anhydrous ($MgSO_4$), sodium acetate (NaOAc) and acetic acid (AcOH) were procured from Merck (Darmstadt, Germany). Primary-secondary amine (PSA) was purchased from Supelco Analytical (595 N Harrison Rd, Bellefonte, PA, USA).

2.2. Standard solution preparation

A mixture of 260 certified pesticide reference standards was used for the quantification of pesticide residues. The individual stock solution of each pesticide (1 mg mL^{-1}) was prepared in MeOH and stored at -18 °C. The mixed stock solution was prepared in extracts of blank samples (apple) at 1,000 µg L^{-1} and working standard solutions were prepared by serial dilutions with six levels of concentrations. All solutions were stored in amber vials at -18 °C.

2.3. Sample collection and storage

The samples were collected randomly from the orchards and vineyard located in Tokat, Turkey. Apple, pear, peach (at least 10 units) and cherry samples each of 1 kg, and grape samples of 2 kg (at least 5 bunches) were collected (EC 2002). The collected samples, which were placed in clean bags providing secure protection against contamination, damage and leakage, were immediately transported to the laboratory and stored in a freezer at -18 °C.

2.4. Sample extraction and clean up

The official QuEChERS AOAC Method 2007.01 was used for the extraction and clean-up procedures (Lehotay 2007). The QuEChERS steps followed are illustrated in Figure 1. Each of the samples were analyzed in triplicates with liquid chromatography-tandem mass spectrometry (LC-MS/MS).

For recovery studies, approximately 1 kg of apple sample was homogenized with a blender and 15 g of the homogenized sample were weighed in a 50-mL Falcon tube. Then, 150 µL of pesticide mixture was spiked to 15 g of sample and vortexed for 60 seconds. Fifteen minutes was waited for the pesticides to interact with the matrix. The next steps followed are illustrated in Figure 1 (Polat & Tiryaki 2019; Dülger & Tiryaki 2021).

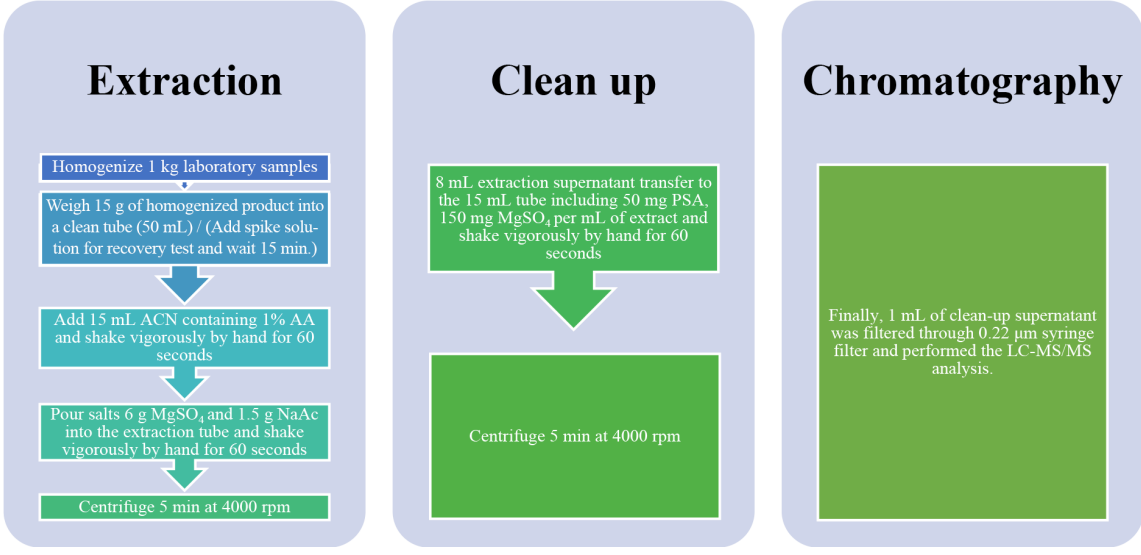

Figure 1- Analytical steps of the QuEChERS-AOAC Official Method 2007

2.5. LC-MS/MS analyses

The analyses were conducted on a LC-MS 8050 model (Shimadzu®. LC-MS/MS) equipped with UPLC: LC-30AD pump x 2, SIL-20A autosampler, DGU-20A3R degasser, CTO-20ACV column oven and triple quadrupole MS/MS detector. The LC column was an Inertsil (ODS IV) C_{18} column (2.1 mm x 150 mm, 3 µm particle size) from GL Sciences Inc (Tokyo, JAPAN). Chromatographic separation was performed using a gradient elution program with eluent A consisting of distilled H_2O + 5 mM ammonium formate, eluent B consisted of MeOH + 5 mM ammonium formate. Analyses began with 5% eluent B, which was linearly increased to 60% in 3 min, 70% in 4 min, 80% in 6 min, and 95% in 7 min (held 1.50 min), and decreased to initial stage (5% of B) at 8.51 min, holding until 15 min. The flow rate, injection volume and total run time were 0.40 mL min^{-1}, 10 µL and 15 min, respectively. The column and autosampler temperatures were maintained at 35 °C and 4 °C, respectively. For MS/MS detection, the electro spray ionization (ESI) interface used positive polarity with the following: 3 kV of capillary voltage, 3V of extractor voltage, 350 °C of heat block temperature, 250 °C of desolvation line temperature, nitrogen (N_2) as nebulizer gas of 2.9 L min^{-1} and drying gas of 10 L min^{-1}. The N_2 gas of 99% purity produced by a peak scientific nitrogen generator (Billerica, MA, USA) was used in the ESI source and the collision cell. The collision induced dissociation gas is argon (Ar, 99.99%) of 230 kPa with flow rate 0.15 mL min^{-1}. All parameters of instrument were controlled using LabSolution® software (version 4.91) (Balkan & *Yılmaz 2022*a).

2.6. Method verification

Method verification is the process of confirmation, through the provision of objective evidence, that specified requirements have been fulfilled. If a laboratory applies a standarized method or prevalidated method into its condition without any change in the procedure, the laboratory simply needs to verify that it can perform the method by meeting the method performance criteria. In that case "method verification" is more appropriate than "method validation" (Magnusson & Örnemark 2014; Dülger & Tiryaki 2022). The analytical method was in-house validated using the European SANTE/11312/2021 Guideline (SANTE 2021) by assessing linearity, mean recovery, limit of detection (LOD), limit of quantification (LOQ), and precision (repeatability and within-laboratory reproducibility) (Balkan & Yılmaz 2022a). The linearity of the method was determined using matrix-matched calibration standards at six level corresponding to 5-200 µg kg^{-1}. Linear regression coefficients (R^2) values of >0.99 were regarded as acceptable. To determine LOD and LOQ, a multi-standard working solution was spiked to the blank sample with a final concentration of 10 µg kg^{-1} and analyzed in 10 replicates. The LODs were calculated as three times the corresponding standard deviation (SD). The LOQs were calculated as ten times the SD (Magnusson & Örnemark 2014). The recovery of pesticides from the matrix and precision of the method were determined by the analyses of blank samples spiked at two concentration levels (10 and 50 µg kg^{-1}) in five replicates. The repeatability (RSD_r) was evaluated on the same day. The within-laboratory reproducibility (RSD_{wR}) was performed on five consecutive days. The precision values were expressed as the relative standard deviation (RSD).

2.7. Health risk assessment

Health risk assessments related to pesticides include estimated calculations of the extent to which the health of those who consume pesticide-containing foods will be at risk. Health risks from both acute and chronic exposure were included in the calculations. Dietary exposure assessments are based on the use of food consumption data in the relevant countries and data on the pesticide residues detected in the foods under study.

In assessing the acute and chronic risk of pesticide residues, estimated dietary exposure was compared to toxicological values known as acute reference dose (ARfD, mg kg^{-1} bw day^{-1}) and acceptable daily intake (ADI, mg kg^{-1} bw day^{-1}). The acute/short-term consumer health risk [acute hazard index (aHI)] was calculated based on the estimated short-term intake (ESTI, mg kg^{-1} day^{-1}) and the ARfD. The chronic/long-term consumer health risk (chronic hazard index, cHI) was calculated based on the estimated daily intake (EDI, mg kg^{-1} day^{-1}) and the ADI. The relevant formulas are given below (Liu et al. 2016);

ESTI=high residue level × food consumption/body weight (1)

aHI=ESTI/ARfD ×100 (2)

EDI=mean residue level × food comsumption/body weight (3)

cHI=EDI/ADI ×100 (4)

The average body weight of an adult was considered as 73.5 kg (TSI 2019; Balkan & Kara 2022). The daily consumption of apples, cherries, grapes, pears and peaches for the general population in Turkey were used as 0.08, 0.016, 0.077, 0.013 and 0.02 $kg^{-1}day^{-1}$ respectively (TSI 2021).

3. Results and Discussion

3.1. Method verification

In the verification experiments, blank samples taken from pesticide-free apple orchards were tested and checked for the absence of any of the target pesticides. The verification of the method was performed with the 260 pesticides listed in Table S1. The method performance criteria are also provided in Table S1.

The recovery (%) was calculated by dividing the measured concentration in the spiked blank sample by the true value (spiking level), multiplying by 100. The recovery for detected pesticides ranged varied between 80% and 117% (Table 1). Linearity was recorded for all pesticides, with coefficients of regression (R^2) ≥0.99. Method accuracy and precision were checked by the determination of within laboratory repeatability (RSD_r%) and reproducibility (RSD_{wR}%) of the recovery results (Table S1). Both RSD_r% and RSD_{wR}% were ≤20% in all cases, which is in accordance with the SANTE guidelines (SANTE 2021). The LOQs and LODs were lower than the corresponding default EU-MRLs for apples, cherries, grapes, pears, and peaches rendering the method acceptable for checking compliance to MRLs.

The method performance followed the analytical quality control criteria of the EU SANTE/11312/2021 guideline and therefore considered fit for the purpose (SANTE 2021). Therefore, the method was used for the monitoring of pesticide residues in apples, cherries, grapes, pears, and peaches.

Table 1- Method verification data for detected pesticides

				Spiking level (10 µg kg^{-1})			*Spiking level (50 µg kg^{-1})*		
Pesticide	***R2***	***LOD***	***LOQ***	***Recovery***	***RSDr***	***RSD_{wR}***	***Recovery***	***RSDr***	***RSD_{wR}***
		µg kg^{-1}	*µg kg^{-1}*	*(%)*	*(%)*	*(%)*	*(%)*	*(%)*	*(%)*
Acetamiprid	0.999	1.76	5.88	102	7.89	1.98	107	4.49	1.49
Azoxystrobin	0.999	1.45	4.82	117	11.4	10.4	108	8.00	5.89
Boscalid	0.993	1.85	6.16	80.0	14.6	3.81	111	14.9	3.04
Carbendazim	0.999	2.73	9.10	97.0	5.01	4.29	107	5.19	3.09
Cymoxanil	0.999	2.50	8.35	96.3	4.38	1.80	96.6	3.78	1.00
Cypermethrin	0.999	2.70	9.00	110	2.95	5.80	98.8	1.87	4.59
Cyprodinil	0.998	2.45	8.17	111	5.18	6.20	103	7.41	3.69
Deltamethrin	0.998	2.35	7.84	90.1	5.34	7.61	85.0	5.34	3.61
Difenoconazole	0.999	1.01	3.36	104	5.74	2.72	99.0	2.98	1.13
Diflubenzuron	0.994	1.87	6.22	80.9	13.0	4.71	113	3.64	3.10
Dimethoate	0.999	2.37	7.91	104	5.99	1.74	111	1.39	2.92
Etoxazole	0.999	1.71	5.71	112	4.59	4.64	115	6.15	3.17
Fenhexamid	0.999	1.91	6.38	82.0	11.2	1.72	90.2	7.33	1.83
Imidacloprid	0.999	2.16	7.20	104	3.79	1.58	97.5	1.54	1.70
Metalaxyl-M	0.991	2.49	8.30	105	3.53	3.32	98.4	5.13	2.43
Metrafenone	0.994	2.46	8.20	101	5.27	3.51	103	8.69	1.01
Novaluron	0.992	2.45	8.17	108	7.15	5.33	106	6.28	7.77
Omethoate	0.999	2.34	7.79	95.2	7.57	2.94	101	4.78	1.37
Permethrin	0.999	2.91	9.70	105	3.49	7.72	101	3.98	2.05
Pyraclostrobin	0.999	1.91	6.37	101	1.83	3.87	110	7.85	3.43
Pyridaben	0.999	1.90	6.33	101	4.28	2.50	100	4.30	3.12
Pyrimethanil	0.999	2.04	6.79	103	8.04	3.00	102	6.37	3.17
Thiacloprid	0.999	2.67	8.91	91.7	4.32	2.86	106	3.90	2.06
Thiophanate-methyl	0.992	2.93	9.76	102	2.20	2.72	103	12.9	1.81

LOD: Limit of detection, LOQ: Limit of quantification, RSD_r: Relative standard deviation repeatability, RSD_{wR}: Relative standard deviation within-laboratory reproducibility

3.2. Pesticide residue concentrations in real samples

The pesticide residue analysis results are given in Table 2.

Table 2- Pesticide residue amounts and frequencies

Food commodity	*Number of sample detectable residue and percentage, (%)*	*Number of sample > MRL and percentage, (%)*	*Pesticide*	*Frequency of detection*	*Pesticide residue (mg kg^{-1})*	*Number of sample > MRL*	*MRL* (mg kg^{-1})*
Apple	13 (76.5%)	8 (47%)	Acetamiprid	4	0.018-0.058		0.4
			Boscalid	4	0.014-0.041		2
			Cypermethrin	1	0.058		0.1
			Diflubenzuron	4	0.059-0.485	4	0.01
			Dimethoate	6	0.021-0.402	6	0.01
			Etoxazole	1	0.0103		0.07
			Imidacloprid	1	0.023		0.5
			Novaluron	3	0.011-0.069		2
			Pyridaben	3	0.012-0.052		0.9
			Thiacloprid	4	0.022-0.064		0.3
			Thiophanate-methyl	2	0.010-0.080		0.5
Cherry	5 (62.5%)	2 (25%)	Cymoxanil	1	0.011		0.01
			Cypermethrin	2	0.012-0.015		2
			Permethrin	2	0.163-0.194	2	0.05
			Tebuconazole	1	0.046		1
			Thiacloprid	4	0.011-0.155		0.5
			Thiophanate-methyl	2	0.011-0.038		0.3
Grape	5 (55.5%)	-	Azoxystrobin	2	0.035-0.044		3
			Boscalid	1	0.055		5
			Cypermethrin	1	0.118		0.5
			Cyprodinil	1	0.035		3
			Difenoconazole	3	0.016-0.084		3
			Fenhexamid	2	0.023-0.116		15
			Metalaxyl-M	2	0.042-0.085		0.7
			Metrafenone	1	0.014		7
			Pyraclostrobin	2	0.011-0.12		2
			Pyrimethanil	2	0.034-0.177		5
Pear	6 (60%)	4 (40%)	Boscalid	3	0.020-0.036		1.5
			Cypermethrin	3	0.029-0.146		1
			Dimethoate	4	0.014-0.269	4	0.01
			Thiacloprid	1	0.177		0.3
			Thiophanate-methyl	1	0.116		0.5
Peach	8 (88.9%)	3 (33.3%)	Boscalid	2	0.055-0.061		5
			Carbendazim	2	0.014-0.021		0.2
			Cypermethrin	1	0.235		2
			Deltamethrin	1	0.020		0.2
			Dimethoate	3	0.024-0.038	3	0.01
			Pyraclostrobin	1	0.014		0.3
			Pyrimethanil	1	0.063		10
			Tebuconazole	4	0.014-0.031		0.6
			Thiophanate-methyl	4	0.040-0.276		2

*EU pesticide database (European Commission, 2022), MRL: Maximum residue limits

A total 11 different pesticides were detected in 17 apple samples. No active ingredient was found in 4 samples. Diflubenzuron residues exceeded the EU-MRL by 5.9, 15.2, 24.8 and 48.5 times in 4 apple samples, and dimethoate residues exceeded the EU-MRL value by 2.1, 2.3, 3.6, 7.2, 27.1 and 40.2 times in 6 apple samples. Ay et al. (2003, 2007), Ersoy et al. (2011c), Lozowicka (2015), Mutangwe et al. (2016), El Hawari et al. (2019) and Sircu et al. (2019) reported residue concentrations over MRL values in apple. In contrast, the residue concentration recorded by Thamani et al. (2021) were lower than the MRL values.

A total 10 pear samples were evaluated, and 5 different pesticides were detected. No active ingredient was found in 4 samples. Unlicensed dimethoate in pear exceeded the EU-MRL value in 4 samples (1.4, 2.3, 24.3 and 26.9 times). Ersoy et al. (2011c), Li et al. (2015), Mutangwe et al. (2016), and Sircu et al. (2019) reported residue concentrations over MRL values in pear.

No pesticide was detected in 1 of 9 peach samples tested, and 9 different pesticides were detected in the remaining 8 samples. Dimethoate residues exceeded the EU-MRL value by 2.4 times in 2 samples and 3.8 times in 1 sample. Ersoy et al. (2011b), Mutangwe et al. (2016), and Li et al. (2019) determined residue concentrations over MRL values in peach. In contrast, the residue concentration reported by Stachniuk et al. (2017), Kaya & Tuna (2019), and Dülger & Tiryaki (2021), were lower than the MRL values.

No pesticide was found in 4 out of 9 analyzed grape samples, and 10 different pesticides were detected from the remaining 5 samples. None of these samples exceeded EU-MRL values. Ersoy et al. (2011a), Mutangwe et al. (2016), and Yakar (2018) reported residue concentrations over MRL values in grape. In contrast, Nalcı et al. (2018), Sircu et al. (2019), and Thamani et al. (2021) determined residue concentrations lower than the MRL values in grape.

No pesticide residues were found in 4 out of 8 analyzed cherry samples. In the other 4 samples, thiacloprid, tebuconazole, permethrin, cypermethrin, thiophanate-methyl active ingredients were detected. With the exception of permethrin and cymoxanil, the other active ingredients did not exceed EU-MRL values. Although the use of permethrin was prohibited, it was detected above EU-MRL values in 2 samples. Ersoy et al. (2011b) reported residue concentrations over MRL values in cherry samples in their study. Slowik-Borowiec et al. (2015), Stachniuk et al. (2017), Kaya and Tuna (2019) and Balkan and Kara (2020) determined residue concentrations lower than the MRL values in cherry samples. In addition, the samples taken from the cherry growing locations in Tokat were evaluated, and the pesticide residue levels were found to be below the MRL values.

3.3 Health risk assessment

A health risk analysis was conducted for 44 pesticides and the results are given in Table 3.

Table 3- Health risk estimation of pesticides residues in some fruits in Turkey

Food commodity	***Detected pesticide***	***ESTI (mg kg^{-1} d^{-1})***	***aHI (%)***	***EDI (mg kg^{-1} d^{-1})***	***cHI (%)***
Apple	Acetamiprid	6.37209E-05	0.2549	3.2061E-05	0.1282
	Boscalid	4.47189E-05	-	2.60687E-05	0.0652
	Cypermethrin	6.31604E-05	0.0316	6.31604E-05	0.1263
	Diflubenzuron	0.000532814	-	0.00026941	0.2694
	Omethoate	0.000104846	5.2423	4.50069E-05	15.0023
	Dimethoate	0.000232079	2.3208	0.000149092	14.9092
	Etoxazole	1.12979E-05	-	1.12979E-05	0.0282
	Imidacloprid	2.51565E-05	0.0314	2.51565E-05	0.0419
	Novaluron	7.4865E-05	-	4.61074E-05	0.4611
	Pyridaben	5.68081E-05	0.1136	3.18894E-05	0.3189
	Thiacloprid	7.01062E-05	0.2337	4.50129E-05	0.4501
	Thiophanate-methyl	8.77784E-05	0.0439	1.25782E-05	0.0157
Cherry	Cymoxanil	2.54376E-06	0.0032	2.54376E-06	0.0196
	Cypermethrin	3.35069E-06	0.0017	3.00981E-06	0.0060
	Permethrin	4.32796E-05	0.0029	3.98104E-05	0.0796
	Tebuconazole	1.02398E-05	0.0341	1.02398E-05	0.0341
	Thiacloprid	3.46603E-05	0.1155	1.14592E-05	0.1146
	Thiophanate-methyl	8.55668E-06	0.0043	1.15009E-06	0.0014

Table 3- Continued

Food commodity	*Detected pesticide*	*ESTI (mg kg^{-1} d^{-1})*	*aHI (%)*	*EDI (mg kg^{-1} d^{-1})*	*cHI (%)*
Grape	Azoxystrobin	8.55668E-06	0.0043	4.17501E-05	0.0209
	Boscalid	4.67334E-05	-	5.83718E-05	0.1459
	Cypermethrin	5.83718E-05	-	0.000125293	0.2506
	Cyprodinil	0.000125293	0.0626	3.72397E-05	0.1241
	Difenoconazole	3.72397E-05	-	3.96799E-05	0.3968
	Fenhexamid	8.88961E-05	0.0556	7.37927E-05	0.0369
	Metalaxyl-M	0.000123134	-	6.72699E-05	0.0841
	Metrafenone	8.96685E-05	0.0179	1.44422E-05	0.0058
	Pyraclostrobin	1.44422E-05	-	1.26541E-05	0.0422
	Pyrimethanil	1.32149E-05	0.0440	0.000111639	0.0657
Pear	Boscalid	6.76919E-06	-	4.98469E-06	0.0125
	Cypermethrin	2.72854E-05	0.0136	1.47043E-05	0.0294
	Omethoate	2.95747E-05	1.4787	2.02248E-06	0.6742
	Dimethoate	2.19561E-05	0.2196	6.49584E-06	0.6496
	Thiacloprid	3.29891E-06	0.0110	3.29891E-06	0.0330
	Thiophanate-methyl	2.16078E-05	0.0108	2.16078E-05	0.0270
Peach	Boscalid	1.73761E-05	-	1.73761E-05	0.0411
	Carbendazim	6.02231E-06	0.0301	6.02231E-06	0.0248
	Cypermethrin	6.64039E-05	0.0332	6.64039E-05	0.1328
	Deltamethrin	5.68254E-06	0.0227	5.68254E-06	0.0568
	Omethoate	6.65653E-06	0.3328	5.49284E-06	1.8309
	Dimethoate	4.23289E-06	0.0423	3.92427E-06	0.3924
	Pyraclostrobin	3.8365E-06	0.0128	3.8365E-06	0.0128
	Pyrimethanil	1.75686E-05	-	1.75686E-05	0.0103
	Tebuconazole	8.75457E-06	0.0292	8.75457E-06	0.0202
	Thiophanate-methyl	7.80918E-05	0.0390	7.80918E-05	0.0472

The symbol "-" represents that there was no authorized value for ARfD/ADI, and the corresponding risk index could not be computed. ESTI: Estimated short-term intake, aHI: Acute hazard index, EDI: Estimated daily intake, cHI: Chronic hazard index

The omethoate aHI value was 5.2423, cHI value was 15.0023, dimethoate aHI value was 2.3208, and cHI value was 14.9092 in apples. The omethoate aHI value was 1.4787 for pears, while the cHI value for peaches was 1.8309. Hamilton and Crossley (2004) mention a risk for consumers if the health risk index is greater than >1. Since the aHI and cHI values of omethoate and dimethoate for apples, the aHI value of omethoate for pears, and the cHI values of omethoate for peaches were >1, they were considered risky for consumers.

4. Conclusions

This study analyzed pesticide residues in some fruits produced in the Tokat province, and the health risks associated to the consumption of these fruits were quantified. The residual concentrations of 260 pesticides were determined in 54 fresh fruit samples. The fruit samples were monitored based on QuEChERS method followed by analysis using LC-MS/MS. The residue amounts were evaluated according to EU-MRL values. The pesticide residues were lower than EU-MRL values in 37.7% of the samples, and over EU-MRL values in 32.1% of the tested samples. The residues of diflubenzuron in apples, permethrin in cherries, and dimethoate in pears, apples, and peaches were over EU-MRL values. The aHI and cHI values of omethoate and dimethoate in apples, aHI value of omethoate in pears, and cHI values of omethoate in peaches were greater than the risk index of 1. The results indicated that chronic risk arising from pesticide exposure in fruits is significant for public health. Potential risks are possible due to prolonged dietary exposure. Residue levels of agrochemicals should constantly be monitored in the study region.

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Table S1- Method verfication data for 260 pesticides

Pesticide	*R2*			*Spiking level (10 µg kg^{-1})*			*Spiking level (50 µg kg^{-1})*		
		LOD	*LOQ*	*Recovery*	*RSDr*	*RSD$_{wR}$*	*Recovery*	*RSDr*	*RSD$_{wR}$*
		µg kg^{-1}	*µg kg^{-1}*	*(%)*	*(%)*	*(%)*	*(%)*	*(%)*	*(%)*
2.4-D	0.999	2.75	9.17	96.1	12.4	5.64	99.5	11.4	4.23
Abamectin	0.995	2.94	9.80	89.7	9.35	7.28	103	5.91	7.89
Acephate	0.998	1.24	4.13	99.7	2.17	2.06	104	3.41	1.80
Acequinocyl	0.998	2.45	8.17	103	4.72	8.19	107	16.1	13.9
Acetamiprid	0.999	1.76	5.88	102	7.89	1.98	107	4.49	1.49
Acetochlor	0.991	2.28	7.59	97.0	4.43	7.64	112	4.65	4.50
Acrinathrin	0.999	2.67	8.92	97.4	12.4	9.84	81.4	9.63	12.1
Alachlor	0.993	1.94	6.45	102	5.11	2.03	110	4.60	2.97
Aldicarb	0.998	2.26	7.53	111	4.63	11.40	110	11.6	11.9
Aldicarb-sulfone	0.999	1.39	4.64	95.8	1.86	1.61	102	2.09	0.70
Aldicarb-sulfoxide	0.999	1.66	5.54	106	10.4	6.76	106	7.54	5.06
Ametoctradin	0.999	2.96	9.86	87.5	5.48	1.55	99.4	2.77	2.36
Amitraz	0.999	1.81	6.03	83.4	12.3	6.99	100	10.9	3.05
Atrazine	0.996	2.74	9.14	85.7	9.01	1.90	107	6.18	1.76
Azinphos-ethyl	0.991	2.95	9.83	104	7.75	9.16	111	5.47	6.17
Azinphos-methyl	0.992	2.97	9.90	90.4	13.4	3.34	102	3.52	2.15
Azoxystrobin	0.999	1.45	4.82	117	11.4	10.35	108	8.00	5.89
Benalaxyl	0.997	2.03	6.78	113	1.68	1.84	102	4.83	2.00
Benfuracarb	0.999	2.53	8.43	89.5	9.33	5.28	93.3	19.7	3.66
Benomyl	0.995	2.60	8.68	98.9	1.35	1.68	109	4.26	1.77
Bensulfuron-methyl	0.996	2.00	6.66	87.9	4.76	3.24	109	8.86	2.82
Bentazone	0.995	2.84	9.47	115	6.60	1.90	110	7.60	2.14
Bifenazate	0.997	1.81	6.05	111	3.73	1.50	95.5	2.68	4.25
Bitertanol	0.998	1.69	5.63	81.6	18.1	3.90	102	9.40	4.83
Boscalid	0.993	1.85	6.16	80.0	14.6	3.81	111	14.9	3.04
Bromoxynil	0.991	2.87	9.55	90.9	4.10	6.05	111	2.75	0.67
Bromuconazole	0.999	2.15	7.15	98.1	11.1	4.11	105	10.5	4.15
Buprimate	0.992	2.50	8.34	107	2.50	3.78	112	2.59	1.47
Buprofezin	0.999	2.34	7.80	94.2	5.99	2.66	99.4	6.13	2.81
Butralin	0.999	2.53	8.45	92.0	2.68	4.67	92.7	4.37	6.39
Butylate	0.999	2.66	8.86	92.2	5.78	6.36	101	2.51	2.83
Cadusafos	0.998	2.29	7.62	102	5.54	4.65	110	11.3	6.65
Carbaryl	0.999	2.22	7.39	113	3.12	3.58	105	4.67	2.80
Carbendazim	0.999	2.73	9.10	97.0	5.01	4.29	107	5.19	3.09
Carbofuran	0.997	2.50	8.33	93.1	4.73	2.20	113	5.55	1.87
Carbofuran-3-hydroxy	0.999	2.50	8.35	96.6	8.31	2.46	105	9.85	2.41
Carbosulfan	0.999	2.47	8.22	90.5	3.13	1.74	91.3	13.2	1.45
Carboxin	0.992	1.70	5.67	90.2	4.46	1.82	112	4.05	2.71
Carfentrazone-ethyl	0.998	2.21	7.36	78.4	5.24	2.90	93.3	7.56	2.54
Chlorantraniliprole	0.994	2.62	8.74	106	9.33	6.11	102	11.1	3.11

Table S1- Continued

Pesticide	*R2*	*Spiking level (10 µg kg^{-1})*					*Spiking level (50 µg kg^{-1})*		
		LOD	*LOQ*	*Recovery*	*RSDr*	*RSD$_{wR}$*	*Recovery*	*RSDr*	*RSD$_{wR}$*
		µg kg^{-1}	*µg kg^{-1}*	*(%)*	*(%)*	*(%)*	*(%)*	*(%)*	*(%)*
Chlorbufam	0.997	2.42	8.06	94.1	5.63	6.28	106	13.2	10.1
Chlorfenvinphos	0.994	1.63	5.43	109	4.64	6.74	110	2.42	11.2
Chlorfluazuron	0.999	2.92	9.73	107	6.05	5.68	104	7.01	2.83
Chloridazon	0.998	2.22	7.40	96.8	5.91	1.12	105	4.57	2.68
Chlorsulfuron	0.996	2.23	7.42	107	5.31	3.73	106	6.64	2.84
Clethodim	0.998	1.97	6.57	79.7	11.3	4.89	93.5	6.54	2.76
Clodinofop-propargyl	0.998	2.28	7.59	99.9	3.54	2.44	105	4.78	3.16
Clofentezine	0.993	2.10	6.99	94.2	6.28	3.79	96.5	7.10	4.08
Clothianidine	0.998	2.20	7.34	76.0	14.7	1.91	93.0	11.4	4.11
Cyantraniliprole	0.999	1.80	5.99	97.9	8.29	2.78	100	3.50	4.35
Cyazofamid	0.969	1.95	6.50	107	5.07	5.10	110	6.25	2.96
Cycloate	0.999	2.85	9.49	104	9.95	5.52	110	6.72	1.00
Cycloxydim	0.996	2.93	9.75	108	3.84	2.94	106	5.20	2.75
Cyflufenamid	0.992	2.12	7.05	106	5.41	3.51	99.1	6.30	4.19
Cyhalothrin	0.995	2.57	8.56	112	10.2	5.39	115	13.6	5.13
Cymoxanil	0.999	2.50	8.35	96.3	4.38	1.80	96.6	3.78	1.00
Cypermethrin	0.999	2.70	9.00	110	2.95	5.80	98.8	1.87	4.59
Cyproconazole	0.999	1.22	4.07	88.8	11.9	3.53	98.3	5.09	2.88
Cyprodinil	0.998	2.45	8.17	111	5.18	6.20	103	7.41	3.69
Dazomet	0.999	2.11	7.04	102	4.95	1.66	99.1	5.40	3.72
Deltamethrin	0.998	2.35	7.84	90.1	5.34	7.61	85.0	5.34	3.61
Demeton-s-methyl	0.997	2.65	8.85	80.4	14.7	13.31	95.4	16.6	7.49
Demeton-s-methyl-sulfone	0.999	1.76	5.87	106	3.15	1.58	98.9	2.17	2.17
Desmedipham	0.998	1.38	4.60	92.2	5.98	2.71	112	8.42	2.07
Diafenthiuran	0.999	2.88	9.59	103	7.14	6.97	105	12.1	6.46
Diazinon	0.999	2.59	8.62	102	2.12	2.43	93.3	4.56	2.55
Dichlofluanid	0.994	2.80	9.34	88.0	9.04	7.05	101	13.2	5.45
Dichlorvos	0.999	2.33	7.78	110	4.37	3.82	118	7.92	4.25
Diclofop-methyl	0.996	2.22	7.40	103	5.73	7.85	102	10.5	3.21
Dicrotophos	0.999	2.44	8.14	98.1	3.65	2.50	109	2.73	2.71
Diethofencarb	0.999	1.96	6.52	95.7	5.03	1.59	109	3.33	0.97
Difenoconazole	0.999	1.01	3.36	104	5.74	2.72	98.9	2.98	1.13
Diflubenzuron	0.994	1.87	6.22	80.9	13.0	4.71	113	3.64	3.10
Dimethenamid	0.998	2.20	7.33	89.3	4.22	2.38	104	5.25	2.88
Dimethoate	0.999	2.37	7.91	104	5.99	1.74	111	1.39	2.92
Dimethomorph	0.996	2.00	6.68	93.4	7.31	6.59	95.3	4.82	5.27
Diniconazole	0.999	1.30	4.34	103	5.22	4.46	113	9.04	2.91
Dinocap	0.955	2.36	7.87	97.7	7.26	9.43	102	6.98	7.24
Dioxacarb	0.999	2.78	9.26	92.7	4.92	1.95	99.6	3.64	2.92
Diphenamid	0.999	2.08	6.93	96.3	6.87	2.83	114	5.25	3.17
Diphenylamine	0.998	2.46	8.19	86.9	9.93	8.45	99.1	15.5	11.3

Table S1- Continued

Pesticide	*R2*	*Spiking level (10 µg kg^{-1})*					*Spiking level (50 µg kg^{-1})*		
		LOD	*LOQ*	*Recovery*	*RSDr*	*RSD$_{wR}$*	*Recovery*	*RSDr*	*RSD$_{wR}$*
		µg kg^{-1}	*µg kg^{-1}*	*(%)*	*(%)*	*(%)*	*(%)*	*(%)*	*(%)*
Diuron	0.997	2.77	9.24	72.2	13.1	1.89	98.9	6.50	2.85
DMF	0.998	2.11	7.04	94.1	4.71	2.44	107	3.58	2.05
Dodine	0.999	2.71	9.02	112	2.40	2.21	110	2.45	3.89
Emamectin	0.999	1.95	6.51	77.4	8.59	2.79	99.8	8.24	3.40
Emamectin benzoat	0.999	2.25	7.49	95.1	9.16	3.64	101	11.5	4.26
EPN	0.996	2.62	8.75	88.1	7.92	6.99	105	3.02	2.85
Epoxiconazole	0.993	2.84	9.46	102	7.39	0.94	104	4.51	2.11
EPTC	0.999	2.23	7.42	102	13.8	9.42	112	9.23	7.97
Ethiofencarb	0.995	1.80	6.00	103	8.74	2.66	117	3.92	4.78
Ethion	0.999	1.97	6.57	96.6	3.24	2.66	96.9	7.76	3.36
Ethirimol	0.997	1.19	3.96	75.4	7.19	1.73	90.9	5.59	2.02
Etofenprox	0.997	1.15	3.83	102	5.65	4.18	111	3.84	4.38
Etoxazole	0.999	1.71	5.71	112	4.59	4.64	115	6.15	3.17
Famoxadone	0.998	2.52	8.40	112	8.41	6.40	112	10.6	4.91
Fenamidone	0.998	1.72	5.74	88.8	6.64	3.21	105	7.97	2.47
Fenamiphos	0.992	2.74	9.13	94.8	3.11	2.48	91.7	3.81	2.63
Fenamiphos-sulfone	0.995	2.49	8.29	84.7	16.6	3.62	108	8.25	1.71
Fenamiphos-sulfoxide	0.993	1.64	5.48	93.1	2.82	2.12	97.5	6.95	3.79
Fenarimol	0.999	2.95	9.82	99.5	13.1	2.53	111	10.1	4.63
Fenazaquin	0.999	0.99	3.30	91.8	2.49	1.03	90.6	6.11	0.57
Fenbuconazole	0.996	2.15	7.18	104	3.29	3.13	107	6.97	4.81
Fenbutatin oxide	0.999	1.67	5.57	112	4.13	7.44	111	3.54	3.97
Fenhexamid	0.999	1.91	6.38	82.0	11.2	1.72	90.2	7.33	1.83
Fenoxycarb	0.998	1.66	5.52	113	6.16	2.37	112	6.02	2.82
Fenoxyprob-ethyl	0.999	1.89	6.31	107	9.56	8.47	119	4.54	2.66
Fenpropathrin	0.999	2.37	7.92	102	8.12	9.75	110	13.2	5.90
Fenproxymate	0.999	0.83	2.76	100	1.63	2.35	88.9	3.19	3.95
Fenthion	0.999	1.32	4.38	108	3.75	4.67	115	2.74	3.31
Fenthion-sulfone	0.999	2.19	7.31	105	7.21	4.97	112	14.5	2.74
Fenthion-sulfoxide	0.999	2.98	9.93	89.9	4.75	3.18	99.2	5.36	1.95
Fipronil	0.999	1.62	5.41	89.2	6.06	4.79	88.1	7.29	2.24
Fipronil-sulfone	0.999	2.13	7.09	95.6	10.7	2.75	101	5.61	4.41
Fluazifop-p-butyl	0.998	2.19	7.29	85.7	10.2	7.95	110	6.04	4.98
Fluazinam	0.999	2.26	7.54	107	13.3	7.54	107	6.24	6.54
Flubendiamide	0.999	2.98	9.92	87.6	6.78	6.90	101	7.23	3.64
Fludioxonil	0.999	2.71	9.05	98.9	6.11	4.29	94.1	3.84	4.68
Flufenoxuron	0.998	2.89	9.64	96.2	4.09	4.07	99.0	4.37	5.18
Fluopicolide	0.995	2.96	9.87	117	2.80	2.76	110	4.10	3.82
Fluopyram	0.999	2.82	9.41	82.1	3.69	2.16	89.5	2.42	2.28
Fluquinconazole	0.997	2.42	8.07	118	4.92	4.30	97.1	6.38	5.73
Flurochloridone	0.999	2.85	9.50	95.4	14.7	6.05	108	5.79	4.60

Table S1- Continued

Pesticide	R2	Spiking level (10 µg kg^{-1})					Spiking level (50 µg kg^{-1})		
		LOD	LOQ	Recovery	RSDr	RSD_{wR}	Recovery	RSDr	RSD_{wR}
		µg kg^{-1}	µg kg^{-1}	(%)	(%)	(%)	(%)	(%)	(%)
Fluroxypyr	0.999	2.67	8.91	79.1	8.95	6.09	95.8	18.7	9.27
Flusilazole	0.995	2.66	8.87	101	8.30	5.49	101	5.17	5.25
Flutriafol	0.999	3.00	9.99	104	7.84	4.87	107	9.95	3.19
Forchlorfenuron	0.995	1.57	5.24	76.7	4.46	2.26	94.1	9.25	1.83
Formetanete hydrochloride	0.994	2.87	9.57	89.3	7.38	3.98	119	11.6	5.09
Fosthiazate	0.994	1.25	4.16	98.1	3.06	1.86	104	3.29	2.18
Furathiocarb	0.999	2.72	9.07	103	3.92	2.76	110	4.32	1.82
Haloxyfop-R-methyl	0.993	2.22	7.40	97.9	3.56	3.55	95.1	10.5	3.32
Hexaconazole	0.997	2.04	6.80	73.0	11.2	4.07	108	6.99	3.05
Hexaflumuron	0.993	2.66	8.87	93.6	10.1	10.54	104	6.59	6.33
Hexythiazox	0.999	2.38	7.93	88.7	5.31	5.65	92.9	5.32	5.21
Imazalil sulfate	0.999	1.11	3.71	102	13.8	8.12	104	7.31	4.91
Imazapyr	0.996	2.74	9.14	90.1	7.48	1.47	95.4	10.6	1.23
Imidacloprid	0.999	2.16	7.20	104	3.79	1.58	97.5	1.54	1.70
Indoxacarb	0.999	2.67	8.90	115	3.59	5.63	106	6.72	4.12
Iodosulfuron-methyl-sodium	0.999	2.40	8.01	83.7	6.39	2.25	99.7	4.46	4.40
Ioxynil	0.999	2.58	8.61	98.9	10.6	9.88	105	6.72	4.45
Isocarbofos	0.992	2.89	9.65	106	13.2	14.59	93.7	5.27	7.70
Kresoxim-methyl	0.997	2.50	8.35	112	2.79	2.75	110	5.87	1.88
Lenacil	0.991	2.19	7.31	95.8	3.71	2.64	112	3.86	2.71
Linuron	0.996	2.88	9.59	95.5	12.7	3.92	109	8.18	4.81
Lufenuron	0.999	2.74	9.13	99.0	5.61	4.57	98.3	4.43	2.26
Malaoxon	0.999	1.10	3.65	96.7	3.78	1.58	106	2.95	2.62
Malathion	0.999	2.15	7.15	81.5	5.93	1.16	108	3.62	0.99
Mandipropamid	0.999	2.28	7.61	92.0	7.12	2.64	101	8.16	2.72
MCPA	0.997	1.94	6.46	105	4.93	1.44	108	8.15	3.26
Mecarbam	0.999	2.75	9.18	99.6	2.34	2.00	100	5.87	1.85
Mepanipyrim	0.999	2.01	6.69	92.0	3.48	5.45	90.7	3.48	6.14
Mepanipyrim-hyroxypropyl	0.998	2.53	8.42	85.9	6.09	1.66	110	3.54	1.55
Metaflumizone	0.999	2.83	9.42	96.8	10.7	9.03	109	6.97	5.08
Metalaxyl-M	0.991	2.49	8.30	105	3.53	3.32	98.4	5.13	2.43
Metamitron	0.999	2.43	8.09	94.1	9.00	3.54	94.5	9.38	3.78
Methacrifos	0.999	2.15	7.18	100	7.36	4.69	98.2	7.78	1.58
Methamidophos	0.999	2.10	7.01	113	3.13	2.74	105	5.00	2.84
Methidathion	0.999	2.95	9.84	86.4	7.71	6.02	112	5.69	4.44
Methiocarb	0.992	1.79	5.97	80.2	7.55	2.48	111	6.35	2.58
Methiocarb-sulfone	0.999	1.75	5.84	99.6	7.29	2.58	99.5	5.27	2.17
Methiocarb-sulfoxide	0.999	1.68	5.61	103	3.80	2.95	101	3.51	2.00
Methomyl	0.999	2.06	6.86	100	4.30	1.70	105	3.19	1.17
Methoxyfenozide	0.994	1.46	4.86	110	8.27	10.05	111	13.6	8.44
Metolachlor-S	0.997	2.57	8.56	86.4	7.98	1.99	102	4.92	2.07

Table S1- Continued

Pesticide	R2	Spiking level (10 µg kg^{-1})					Spiking level (50 µg kg^{-1})		
		LOD	LOQ	Recovery	RSDr	RSD_{wR}	Recovery	RSDr	RSD_{wR}
		µg kg^{-1}	µg kg^{-1}	(%)	(%)	(%)	(%)	(%)	(%)
Metosulam	0.996	2.68	8.93	70.3	6.88	2.21	99.0	4.64	2.68
Metrafenone	0.994	2.46	8.20	101	5.27	3.51	103	8.69	1.01
Metribuzin	0.999	2.67	8.90	106	5.77	2.68	112	5.71	1.93
Mevinphos	0.991	2.30	7.67	106	6.20	13.45	106	3.69	3.08
Molinate	0.998	2.28	7.59	106	14.3	4.91	106	5.49	3.58
Monocrotophos	0.997	1.49	4.95	107	5.08	1.33	104	3.21	1.53
Monolinuron	0.997	1.10	3.67	92.9	4.87	4.20	92.4	4.37	4.32
Myclobutanil	0.996	1.76	5.86	87.1	5.56	1.99	109	7.23	2.13
Nicosulfuron	0.996	2.36	7.87	76.0	6.07	3.78	105	13.2	4.26
Novaluron	0.992	2.45	8.17	108	7.15	5.33	106	6.28	7.77
Nuarimol	0.999	1.53	5.09	105	8.11	2.62	116	12.7	3.38
Omethoate	0.999	2.34	7.79	95.2	7.57	2.94	101	4.78	1.37
Oxadixyl	0.997	2.68	8.95	88.8	9.22	1.68	104	2.42	3.13
Oxamyl	0.999	0.94	3.13	101	1.56	1.37	109	2.81	1.60
Oxycarboxin	0.999	2.80	9.34	111	2.95	1.73	106	2.99	2.91
Oxydemeton-methyl	0.999	1.17	3.90	96.2	6.64	3.33	100	11.1	3.95
Paclobutrazol	0.998	1.62	5.40	93.0	7.22	3.72	111	4.57	2.93
Paraoxon-ethyl	0.992	2.18	7.27	85.9	4.66	3.12	109	9.80	2.02
Paraoxon-methyl	0.999	2.45	8.16	90.2	14.7	4.78	108	9.75	4.02
Penconazole	0.991	2.80	9.33	106	5.91	4.28	110	3.68	2.78
Pencycuron	0.999	1.95	6.50	95.6	11.8	4.89	111	4.95	5.96
Pendimethalin	0.999	1.33	4.43	84.9	2.80	2.99	89.7	4.07	3.58
Permethrin	0.999	2.91	9.70	105	3.49	7.72	101	3.98	2.05
Phenmedipham	0.998	2.67	8.90	94.6	7.63	5.32	104	7.71	5.80
Phenthoate	0.998	2.38	7.95	97.1	5.63	2.27	98.0	4.22	4.26
Phorate	0.998	2.66	8.85	89.5	7.98	6.45	107	9.24	4.39
Phorate-sulfone	0.995	2.20	7.34	100	6.91	5.48	88.2	10.4	5.18
Phorate-sulfoxide	0.997	2.48	8.27	115	2.64	3.59	107	2.70	1.18
Phosalone	0.997	1.08	3.61	86.3	5.28	2.24	109	9.33	2.65
Phosmet	0.994	2.75	9.18	78.5	8.20	1.26	112	5.04	1.87
Phosphamidon	0.999	2.67	8.90	99.9	8.02	2.80	106	2.24	2.30
Pirimicarb-desmethyl	0.999	2.59	8.63	93.6	4.27	1.27	104	4.49	2.31
Primicarb	0.994	2.04	6.81	94.5	4.87	8.63	92.7	7.45	4.36
Primiphos-ethyl	0.999	2.92	9.74	108	7.56	2.84	100	6.10	1.77
Primiphos-methyl	0.999	2.21	7.36	91.4	8.23	2.31	110	5.83	2.94
Prochloraz	0.998	1.95	6.49	101	6.20	5.09	105	12.8	7.68
Profenefos	0.990	2.79	9.29	103	4.27	3.58	106	4.24	3.37
Profoxydim-lithium	0.999	2.24	7.47	85.4	3.27	3.47	96.6	5.09	4.56
Promecarb	0.998	2.49	8.29	94.1	6.49	2.22	108	2.56	1.68
Prometryn	0.999	2.06	6.87	95.6	4.39	5.68	97.7	5.16	3.79
Propaquizafob	0.998	2.35	7.85	93.7	15.3	3.28	101	6.45	7.35

Table S1- Continued

Pesticide	R2	Spiking level (10 µg kg^{-1})					Spiking level (50 µg kg^{-1})		
		LOD	LOQ	Recovery	RSDr	RSD_{wR}	Recovery	RSDr	RSD_{wR}
		µg kg^{-1}	µg kg^{-1}	(%)	(%)	(%)	(%)	(%)	(%)
Propargite	0.999	2.85	9.51	92.2	3.58	4.10	96.9	5.71	6.45
Propazine	0.998	2.08	6.95	93.0	3.03	2.39	103	3.09	1.76
Propiconazole	0.994	1.72	5.74	98.2	8.33	4.89	109	10.1	7.12
Propoxur	0.995	2.67	8.89	92.1	7.03	1.60	113	3.73	1.93
Propyzamide	0.995	1.22	4.07	89.3	7.02	2.04	111	9.75	1.47
Prothiophos	0.999	2.70	8.99	87.2	8.32	3.02	89.6	5.36	4.26
Pymetrozine	0.999	1.66	5.55	75.5	7.35	2.18	97.2	5.22	2.30
Pyraclostrobin	0.999	1.91	6.37	101	1.83	3.87	110	7.85	3.43
Pyrazophos	0.996	2.28	7.60	86.9	5.96	3.04	99.2	5.16	3.07
Pyridaben	0.999	1.90	6.33	101	4.28	2.50	100	4.30	3.12
Pyridaphenthion	0.999	1.65	5.50	119	2.88	1.58	108	1.58	1.69
Pyridate	0.999	2.66	8.85	88.8	4.18	2.85	95.2	11.7	1.83
Pyrimethanil	0.999	2.04	6.79	103	8.04	3.00	102	6.37	3.17
Pyriproxyfen	0.999	2.75	9.15	96.6	5.51	4.11	99.0	5.73	4.53
Quinalphos	0.998	2.03	6.78	96.1	11.5	3.53	111	7.06	1.77
Quizalofop-ethyl	0.997	2.25	7.50	87.6	14.5	4.08	105	8.34	4.49
Rimsulfuron	0.999	2.43	8.08	99.2	4.99	3.95	104	9.84	4.23
Sethoxydim	0.990	1.83	6.11	96.5	2.38	1.28	106	2.52	1.78
Simazine	0.999	2.59	8.64	102	7.74	4.53	105	11.0	1.98
Spinosyn A	0.999	2.35	7.82	103	3.18	4.44	105	1.57	3.80
Spinosyn D	0.999	2.84	9.47	103	4.06	4.02	113	5.20	2.82
Spirodiclofen	0.999	2.92	9.73	97.1	9.17	2.62	95.5	9.25	6.96
Spiromesifen	0.991	2.59	8.63	95.3	11.2	5.61	98.8	5.07	8.84
Spiroxamine	0.999	1.14	3.81	88.7	13.5	9.26	99.2	13.6	5.89
Sulfoxaflor	0.999	2.17	7.23	93.9	8.11	4.08	99.8	5.65	3.68
Tebuconazole	0.994	1.38	4.60	101	7.20	2.75	112	6.46	2.73
Tebufenozide	0.995	2.73	9.09	94.9	5.40	7.02	91.5	4.48	6.85
Tebufenpyrad	0.997	2.59	8.64	113	7.16	8.91	106	11.6	9.16
Teflubenzuron	0.999	2.13	7.10	109	7.60	6.74	106	8.77	12.4
Tepraloxydim	0.999	2.49	8.31	89.5	8.88	5.99	96.1	5.34	8.97
Terbutryn	0.999	2.04	6.79	85.3	5.31	1.52	102	3.77	2.28
Terbutylazine	0.998	2.38	7.95	96.1	3.22	2.34	109	4.25	11.5
Tetraconazole	0.999	1.83	6.10	98.6	4.58	1.91	106	2.77	2.94
Tetramethrin	0.996	2.71	9.04	85.0	4.74	1.95	95.9	7.30	3.72
Thiabendazole	0.997	2.33	7.76	106	2.59	1.38	113	6.22	0.94
Thiacloprid	0.999	2.67	8.91	91.7	4.32	2.86	106	3.90	2.06
Thiamethoxam	0.998	2.59	8.63	92.2	8.13	1.30	107	2.86	1.10
Thifensulfuron-methyl	0.999	1.03	3.43	104	3.79	1.58	101	2.72	1.99
Thiobencarb	0.995	2.34	7.81	116	5.52	6.03	114	5.90	13.5
Thiodicarb	0.995	2.30	7.66	95.1	4.53	4.81	94.2	4.57	3.59
Thiophanate-methyl	0.992	2.93	9.76	102	2.20	2.72	103	12.9	1.81

Table S1- Continued

Pesticide	*R2*	*LOD*	*LOQ*	*Spiking level (10 µg kg^{-1})* *Recovery*	*RSDr*	*RSD_{wR}*	*Spiking level (50 µg kg^{-1})* *Recovery*	*RSDr*	*RSD_{wR}*
		µg kg^{-1}	*µg kg^{-1}*	*(%)*	*(%)*	*(%)*	*(%)*	*(%)*	*(%)*
Tolclofos-methyl	0.998	2.44	8.14	95.7	16.5	8.44	103	10.5	6.06
Tolfenpyrad	0.999	2.55	8.50	80.1	5.61	4.57	95.9	4.25	2.58
Tolyfluanid	0.996	2.63	8.77	109	4.77	3.57	108	5.45	4.12
Tralkoxydim	0.996	1.04	3.47	115	2.38	3.03	111	1.62	1.95
Triadimefon	0.990	2.32	7.74	98.2	6.41	2.44	110	8.11	1.57
Triadimenol	0.996	2.47	8.22	99.2	6.65	4.85	83.1	5.28	6.28
Tri-allate	0.998	1.74	5.81	95.0	12.9	4.78	91.4	4.98	8.31
Triasulfuron	0.998	2.27	7.58	103	3.14	3.83	91.7	7.68	2.61
Triazophos	0.999	1.49	4.98	74.3	3.42	3.22	97.7	3.71	2.41
Tribenuron-methyl	0.999	2.02	6.74	87.4	4.73	3.08	108	3.60	2.56
Trichlorfon	0.999	1.80	5.99	104	5.94	2.15	106	4.61	1.67
Trifloxystrobin	0.998	1.52	5.07	85.5	6.83	3.39	108	6.27	2.66
Triflumizole	0.999	2.34	7.80	85.2	4.46	5.02	110	6.99	3.03
Triflumuron	0.992	2.24	7.46	111	5.07	6.36	111	11.1	3.42
Triticonazole	0.999	1.54	5.15	106	3.61	1.39	103	1.55	1.02

LOD: Limit of detection, LOQ: Limit of quantification, RSD_r: Relative standard deviation repeatability, RSD_{wR}: Relative standard deviation within-laboratory reproducibility

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The Performance of Some Walnut (*Juglans regia*) Cultivars in the Conditions of Bursa, Turkey

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ABSTRACT

The present study aimed to evaluate the pomological and phenological traits, as well as yield potential and incidence of external defects of some local ('Bilecik', 'Maraş 12', 'Maraş 18', 'Şebin', 'Şen 1') and foreign ('Chandler', 'Fernette', 'Fernor', 'Howard', 'Pedro', 'Serr') walnut cultivars grown in Bursa (north-west in Turkey) conditions between 2014 and 2020. The first leafing was observed in Maraş 12, Serr, Şebin, and Maraş 18 cultivars, while Fernette and Fernor were the last leafing cultivars. The female blooming period of the cultivars was recorded to be the longest in Maraş 18 (15 days) and Şebin (16 days) and that was the shortest in Bilecik and Serr (10 days). The male blooming period was recorded as the longest in Chandler, Fernette, Bilecik, and Şebin (16-18 days) and that was the shortest in Maraş 12 and Serr (10 days). The average yield per tree of cultivars ranged between 1.87 kg (Şebin) and 14.21 kg (Pedro). Walnut blight was observed on average in 15.85%, sunburn in 10.21%, codling moth in 5.11%, anthracnose in 3.23%, and bird damage in 2.35% of the fruits. The healthy nut percentage ranged from 40.06% (Şen 1) to 76.50% (Chandler). The nut weight of cultivars was between 9.91 g (Maraş 12) and 16.27 g (Şen 1). Kernel percentage varied from 36.31% (Şen 1) to 53.56% (Maraş 12). The principal component analysis conducted to distinguish the cultivars suggests that the most important factors could be reduced to five components. The results showed that Maraş 12, Chandler, Fernor, and Howard were suitable cultivars for commercial cultivation in Bursa conditions.

Keywords: Phenology, Yield, Fruit characteristics, External defects, Leafing, Blooming

1. Introduction

Anatolia is the center of origin and one of the oldest cultivation areas of walnut. *Juglans regia* is the most commonly grown walnut species for fruit production in Turkey (Akça 2005; Şen 2011; Akça 2012; Vahdati 2014). Turkey is the fourth walnut producer globally after China, United States of America (USA), and Iran, with 286 thousand tons of production (FA0STAT 2020). However, Turkey's current production quantity does not meet its consumption. Walnuts are imported from other countries, especially USA, Chile, and China. New walnut orchards have been recently established with standard high-yielding foreign and selected cultivars for increased production (Ercisli et al. 2012; Ertürk et al. 2014). Since there is a lack of knowledge about those cultivars' adaptation to different ecological regions (Kaşka & Sütyemez, 2001; Aktuğ Tahtacı et al. 2014; Erturk et al. 2014), the orchards established without adaptation trials have failed. It has been reported that low yield is obtained in the case of selecting walnut cultivars without considering the ecological factors (Pezikoğlu et al. 2012; Bilgin et al. 2018). Also, Cosmulescu et al. (2010) cited that ecological factors significantly affect walnut production as much as a cultivar and that the influence of the weather can be of a very local issue.

The favorable ecological conditions for walnut cultivation in Bursa, one of the important walnut cultivation areas in the Marmara region (north-west in Turkey), and the fact that walnut is prominent nutrition and economic value indicate that this region creates opportunities

for walnut cultivation in Turkey. This region, where spring frosts are sometimes seen, has a humid climate that encourages the spread of rain-related disease problems; therefore, selecting suitable cultivars is essential. There is a difference in cultivars' susceptibility to pests and diseases, which cause a significant yield loss under favorable conditions, and their susceptibility is greatly affected by environmental conditions (Woeste et al. 1992). Using late-leafing cultivars is an effective strategy to mitigate the severe damage caused by late-spring frost (Akça & Ozongun 2004; Fallah et al. 2022). In addition, late-leafing walnut cultivars are resistant to walnut blight *(Xanthomonas arboricola pv. juglandis*) (Bernard et al. 2018). Also, pollen shedding, which does not coincide with pistillate bloom, is the reason for the low yield of walnuts (Akça & Sen 1997; Cosmulescu et al. 2010). Obtaining sufficient yield from orchards depends on determining suitable cultivars that can overlap the appropriate walnut cultivars for the regions during blooming time. High yield, late leafing, tolerance to plant diseases/pests, and high nut quality are favorable traits in a good walnut cultivar (Aslantaş 2006; Bujdoso et al. 2016; Hassankhah et al. 2017; Mahmoudian et al. 2021; Sütyemez et al. 2021). The adaptation of standard foreign and local walnut cultivars has been reported in countries such as Romania (Botu et al. 2007; Botu et al. 2014), Australia (Vanhanen 2010), Serbia (Mitrovic et al. 2011), Bulgaria (Gandev & Dzhuvinov 2014; Gandev 2017), Georgia (Bobokasvili et al. 2017), Hungary (Bujdoso et al. 2020) and Iran (Mahmoodi et al. 2016*;* Hassani et al. 2020a; Toolir et al. 2021). Also, the phenological and pomological characteristics of walnut cultivars have been determined in Turkey's Southeastern Anatolia (Aktuğ Tahtacı et al. 2014), Marmara (Ertürk et al. 2014), Aegean (Bilgin et al. 2018; Yıldız & Sümer 2019), Eastern Anatolia (Karlıdağ et al. 2019), Central Black Sea (Akça et al. 2014; Akça et al. 2018), and Mediterranean regions (Sütyemez 2016; Türemiş et al. 2017; Sütyemez et al. 2021; Özcan et al. 2022). However, in Bursa, Marmara region, no long-term data consisting of all long-term yield potential, phenology, pomology, and incidence of fruit external defect observations have been published to determine the adaptation of cultivars. The present study aims to determine the performances of some local and foreign walnut cultivars in terms of yield, phenological and pomological characteristics, and incidence of fruit external defect observations based on long-term data.

2. Material and Methods

The study was carried out in in the Agricultural Application and Research Center of Bursa Uludağ University between 2014 and 2020. The orchard was established in 2008 with a planting distance of 7x7 m, and the plants were trained as a modified leader system. The experimental plantation is located at an altitude of 105 m, with geographic coordinates of 40°14' north latitude and 28°51' east longitude in Bursa. The area is characterized by a maximum temperature of 43.8 °C, a minimum of -19.2 °C, and an average annual rainfall of 700 mm. The soil is clay with a content of 38% $CaCO_3$ and a pH of 7.1-7.5.

Five local ('Bilecik', 'Maraş 12', 'Maraş 18', 'Şebin', and 'Şen 1') and six foreign ('Chandler', 'Fernor', 'Fernette', 'Howard', 'Pedro' and 'Serr') walnut cultivars grafted on *J. regia* L. were used in the present study. To determine the performance of the cultivars, the observations on various traits were recorded as follows:

2.1 Phenological traits

Phenological stages such as bud break, leafing and first, peak, and last male and female blooming were recorded according to UPOV (1999). The degree of dichogamy was calculated according to the formula suggested by Solar et al. (1997).

$$\text{Degree of dichogamy (\%)} = \frac{\text{1-No.of days when male and female blooming coincides}}{\text{Number of days of female blooming}} \text{ x100}$$

The time of defoliation was defined as when the tree lost over 50% of its leaves (UPOV 1999). All phenological observations were performed in comparison with Chandler cultivar.

The external defects as walnut blight (*Xanthomonas arboricola* pv. *juglandis*), anthracnose (*Gnomonia leptostyla*), codling moth (*Cydia pomonella*), sunburn, and bird damage were examined after harvest in four replicates of 50 fruits each. The presence or absence of damage was recorded and calculated as a percentage (Aleta et al. 2001; Hassan et al. 2017; Khasanov et al. 2019).

2.2. Pomological traits

The yield per tree (kg) was recorded in each cultivar's maturity time, and the average and total yield per decare (kg/da) were calculated. After harvest, walnut fruits were dried in natural conditions and stored until the analysis. Fruit traits were evaluated in terms of the weight (g), length (mm), thickness (mm), width of nuts (mm), kernel weight (g), shell thickness (mm), and kernel ratio (%) on 90 nut samples. Kernel rottenness and shrinkage (%) were recorded. The kernel color was classified as amber, light amber, light, and extra light (IPGRI 1994; Khadivi et al. 2019; Akça et al. 2020).

Statistical analysis

The data were statistically analyzed by ANOVA using the Statistical Package for the Social Sciences (SPSS) version 23.0. The mean values were compared with Duncan's multiple range test ($p<0.05$). Minitab (Software Version 17, Minitab) was employed to perform principal component analysis (PCA) and to create scatter plots of the first two factors.

3. Results and Discussion

Five years (2014-2018) of phenological observations are presented in Table 1. All cultivars' bud break, leafing, male and female blooming, except Fernor and Fernette, occurred before Chandler cultivar. Leafing dates of Maraş 18, Şebin, Serr, and Maraş 12 cultivars occurred 13, 16, 20, and 21 days before Chandler cultivar, respectively, while Fernette and Fernor leafed out 8 and 9 days after Chandler. The evaluated cultivars, Fernor and Fernette, were relatively late-leafing and blooming. In contrast, Serr, Maraş 12, Şebin, and Maraş 18 were early leafing compared to Chandler. Similarly, Aktuğ Tahtacı et al. (2014) reported that Maraş 12, Maraş 18, and Serr cultivars leafing was about 17 days earlier than Chandler. Gandev (2017) reported that the first beginning of bud break, male and female blooming occurred in Serr, while the latest was the Fernor cultivar. Also, in Tokat, Turkey, Fernette and Fernor leafing were 8 and 9 days later, whereas Maraş 18 and Maraş 12 cultivars were 20 and 22 days before Chandler (Akça et al. 2018). Among the walnut characteristics, late leafing is crucial in places with a risk of late frost in spring (Akça & Ozongun 2004).

Male and female blooming occurred from early April to May (Figure 1) and lasted 10 to 16 days, depending on the cultivar. Şebin, Chandler, Howard, and Fernette cultivar had the longest (10-11 days) pollen shedding period. Hassani et al. (2020a) stated that Chaldoran's leafing date was early to moderate, but it had a long male blooming period (15 days). The longest peak female blooming was seen in Bilecik, Pedro, Chandler, Fernette, and Howard cultivars, with 10 days. Therefore, Fernor and Fernette's peak of blooming (receptivity) of males and females occurred after Chandler. Besides, leaf defoliation of cultivars was observed to occur 2 to 18 days before Chandler. The results obtained in the present study were compatible when compared to results of previous studies conducted in other regions such as Argentina (Iannamico et al. 2006), Romania (Botu et al. 2007), Bulgaria (Gandev 2017), India (Bobokasvili et al. 2017), Iran (Hassani et al. 2020a) and Turkey (Sütyemez 2016; Akça et al. 2018; Yildiz & Sumer 2019; Sütyemez et al. 2019).

Botu et al. (2007) reported that Fernor, and Fernette cultivar's bud break and male blooming occurred later than Chandler. The first male blooming receptivity was observed in Serr. Akça et al. (2018) stated that the earliest and latest male and female blooming cultivars were Maraş 18, Maraş 12, and Fernor. Yildiz & Sumer (2019) found that the local walnut cultivars, except for Baikal genotype, leafed out earlier than the foreign walnut cultivars. Sütyemez et al. (2021) reported that new released walnut cultivar, Helete Güneşi, leafed out 2 and 10 days later than Chandler and Maraş 18, respectively. Hassani et al. (2020a) reported that, among the new four walnut cultivars, except for Caspian cultivar, leafed out before Chandler. Özcan et al. (2022) stated that the leafing date of the Kurtulus 100 cultivar was 5 days earlier than Chandler. Similar to the results of the present study, it has been found that compared to Chandler, leafing occurred 2 and 3 weeks before in Maraş 18 and Maraş 12, respectively (Sütyemez 2016; Sütyemez et al. 2019).

The degree of dichogamy of cultivars varied from one year to another. According to an average of five years of data, all the cultivars were recorded as protandrous (Table 1). Şen 1, Fernette, Howard, and Pedro (100%) had the highest dichogamy degree, followed by the cultivars of Chandler (88.89%), Fernor (66.67%), Maraş 18 (62.50%), Bilecik (60.00%) and Şebin (54.33%) in decreasing order. Our results, in accordance with those of Sütyemez (2016), indicated that Maraş 18 and Chandler cultivars' overlapping with staminate bloom was around 10%, and these cultivars showed protandry.

Özcan et al. (2022) reported that Chandler showed protandry; however, Kurtulus 100 cultivar was homogamous. Also, Bujdoso et al. (2020) and Hassani et al. (2020a) confirmed that Chandler's overlapping with staminate bloom was around 10%, and it was protandrous. Akça (2014) stated that male and female flowers' receptivity in the Şebin cultivar was not overlapping. The Bilecik cultivar has been recommended as a pollinator for Şebin (Akça 2014); however, the Bilecik cultivar could not fill this gap in the present study. Serr (37.50%) and Maraş 12 (33.33%) cultivars were slightly protandry. Sütyemez et al. (2019) stated that Maraş 12 showed homogamy in Kahramanmaraş, Turkey, whereas Akça et al. (2018) reported that the cultivar was protandrous in Tokat, Turkey.

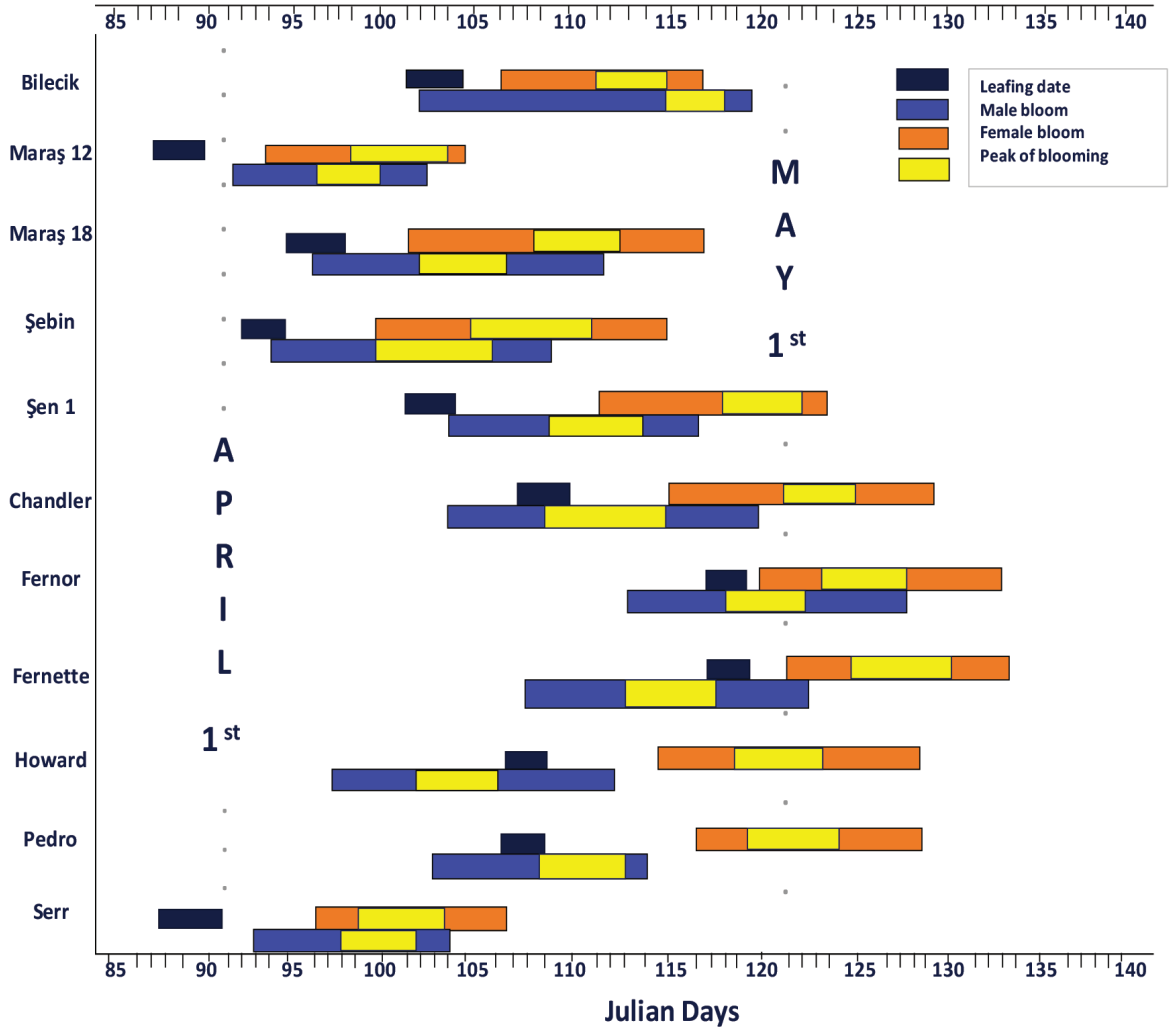

Figure 1- Leafing and blooming dates of the walnut cultivars (2014-2018)

The yield of cultivars for 2014-2020 is presented in Table 2. The average yield of cultivars varied between 1.87 kg and 14.21 kg. The highest average yield per tree was obtained from Pedro (14.21 kg) and Chandler (13.46 kg), followed by Fernor (9.30 kg), Howard (8.70 kg), and Maraş 12 (7.80 kg), while the lowest was Şebin (1.87 kg). The average yield of cultivars per decare was between 38.89 kg (Şebin) and 280.29 kg (Pedro).

The highest total yield was obtained from Pedro (2,030.14 kg) and Chandler (1,923.65 kg). The lowest total yield was obtained from Şebin (267.14 kg). There have been fluctuations in the yield in some cultivars (Figure 2).

In particular, the spring frost incident in Bursa in 2016 damaged the male flowers. This year, the lowest yield was recorded in the local cultivars except for Şebin and Şen 1. This situation may be related to damage that occurred in male flowers of Chandler and Pedro cultivars when female flowers were receptive (Figure 1). The fact that there was no fluctuation in the yield of Şen 1 may be due to its pollination with Fernette and Franquette cultivars, which were less damaged than the other foreign cultivars (Figure 1). In 2016, the highest yield was obtained from Şebin, which may have shown how sensitive this cultivar was to excessive pollen. Akça (2014) stated that Şebin's low yield problem was due to a higher female flower abortion ratio.

Akça et al. (2018) reported that the highest yield was obtained from Chandler, Pedro, and Midland cultivars. Also, Iannamico et al. (2006) stated that Tulare, Fernor, and Chandler walnut cultivars were most productive. In the conditions of the Cacak region of Serbia, high yields were obtained from Fernor (15.0 kg) followed by Fernette (13.7 kg) (Mitrovic et al. 2011). Botu et al. (2010) determined that Fernor, Fernette, and Pedro's average yields were 11.2, 11.2, and 10.0 kg, respectively. Our results, in accordance with those of Akça et al. (2014), Iannamico et al. (2006), Mitrovic et al. (2011), and Botu et al. (2010), confirmed that Chandler, Pedro, and Fernor are the most productive cultivars. Hassani et al. (2020a) reported that, in Iran, the yield of four newly released walnut cultivars was

Table 1- Phenological characteristics of the walnut cultivars (2014-2018)

Cultivar	*Bud break*		*Leafing*		*Female blooming*				*Male blooming*								*Dichogamy degree (%)*	*Nature of dichogamy*	*Defoliation*	
	B	*ND**	*B*	*ND*	*B*	*ND*	*PB*	*ND*	*EB*	*ND*	*B*	*ND*	*PB*	*ND*	*EB*	*ND*			*DD*	*ND*
Bilecik	05.04	-5	09. 04	-8	15. 04	-10	19. 04	-10	24. 04	-14	11. 04	-2	23. 04	+5	29. 04	0	60.00	Protandrous	18.11	0
Maraş 12	22.03	-18	27. 03	-21	03. 04	-22	07. 04	-22	13. 04	-26	30 .03	-13	04. 04	-13	11. 04	-18	33.33	Slightly protandry	03.11	-15
Maraş 18	30.03	-10	04. 04	-13	10. 04	-15	16. 04	-13	25. 04	-14	05. 04	-8	10. 04	-7	20. 04	-8	62.50	Protandrous	05.11	-13
Şebin	27.03	-14	01. 04	-16	08. 04	-17	14. 04	-16	24. 04	-15	03. 04	-10	08. 04	-9	19. 04	-9	54.33	Protandrous	13.11	-5
Şen 1	05.04	-5	09. 04	-8	21. 04	-4	27. 04	-3	02. 05	-7	12. 04	0	18. 04	0	25. 04	-4	100.00	Protandrous	03.11	-15
Chandler	10.04	0	17. 04	0	25. 04	0	30. 04	0	09. 05	0	12. 04	0	18. 04	0	28. 04	0	88.89	Protandrous	18.11	0
Fernor	19.04	+9	26. 04	+9	29. 04	+4	03. 05	+3	12. 05	+3	20. 04	+8	27. 04	+9	05. 05	+7	66.67	Protandrous	11.11	-7
Fernette	18.04	+8	25. 04	+8	30. 04	+5	04. 05	+4	12. 05	+4	15. 04	+3	22. 04	+4	01. 05	+3	100.00	Protandrous	10.11	-8
Howard	09.04	-1	16. 04	-1	23. 04	-1	28. 04	-2	07. 05	-2	06. 04	-7	10. 04	-7	21. 04	-8	100.00	Protandrous	14.11	-4
Pedro	09.04	-1	14. 04	-3	24. 04	0	28. 04	-1	08. 05	-1	12. 04	-1	17. 04	0	22. 04	-6	100.00	Protandrous	16.11	-2
Serr	22. 03	-18	28. 03	-20	05. 04	-20	08. 04	-21	14. 04	-24	01. 04	-11	07. 04	-11	13. 04	-16	37.50	Protandrous	13.11	-5

*ND: Number of days compared to 'Chandler' B: Beginning of blooming, PB: Peak of blooming, EB: End of blooming, DD: Defoliation date

high and very similar to that of 'Chandler'. On the other hand, Sütyemez et al. (2021) reported that in the Kahramanmaraş region of Turkey, Helete Güneşi, Chandler, and Maraş 18 cultivars' 7th, 8th, and 9th year yields were 9.36 kg, 7.03 kg, and 4.14 kg respectively. In the present study, the average yield of Maraş 18 and Chandler cultivars in these years was 5.43 kg and 13.20 kg, respectively. The reason for the differences can be the effect of ecological conditions on the cultivar. Also, in Romania (Botu et al. 2007) and Bulgaria (Gandev 2017), high yields have been obtained in Serr (12.54; 22.01 kg) except for Fernor. The differences between these studies may not be because of the lack of pollination in Serr because it has overlapping male and female blooms in the region (Figure 1, Table 1). However, this may be due to the pistillate flower abscission, observed mainly in Serr cultivar (Catlin & Olson 1990; McGranahan et al. 1994; Rovira & Aleta 1997). Pistillate flower abscission varies according to the location, and high abscission occurs yearly in some regions (Catlin et al. 1987; Gonzalez et al. 2008).

Table 2- The yield (kg/tree) and average yield (kg/tree), (kg/da) of the walnut cultivars

Cultivar	*Average yield (kg/tree)*							*Average yield (2014-2020)*	
	2014	*2015*	*2016*	*2017*	*2018*	*2019*	*2020*	*(kg/tree)*	*(kg/da)*
Bilecik	4.06 cd*	6.60 b	4.52 efg	5.82 efg	3.38 d	9.90 bcd	4.87 d	5.59 de	116.64 c
Maraş 12	5.90bc	6.49 b	4.14 efg	14.33 b	7.96 bc	11.48 bc	4.28 de	7.80 bc	171.13 b
Maraş 18	1.54ef	4.9 bc	1.96 g	9.47 de	6.51 c	8.54 d	6.69 cd	5.66 de	112.08 c
Şebin	1.48 ef	2.01 c	2.98 f	1.36 H	1.83 d	2.16 e	1.27 ef	1.87 f	38.89 d
Şen 1	2.80 de	5.21bc	6.19 de	5.67 efg	1.72 d	8.96 cd	0.71 f	4.46 e	103.31 c
Chandler	6.1 b	13.17a	12.72 ab	13.71 bc	16.24 a	17.77 a	14.51 b	13.46 a	264.51 a
Fernor	1.27 f	3.31 bc	10.42 bc	10.36 cd	13.78 a	16.60 a	9.39 c	9.30 b	189.62 b
Fernette	0.79 f	3.47 bc	5.62 def	5.01 gh	8.31 bc	12.59 b	8.73 c	6.36 cd	121.13 c
Howard	1.49 ef	11.80 a	7.96 cd	9.13 def	9.32 b	11.43 bc	9.80 c	8.70 b	173.30 b
Pedro	9.39 a	13.85 a	14.36 a	18.59 a	9.87 b	16.32 a	17.07 a	14.21 a	280.29 a
Serr	0.87 f	3.40 bc	5.30 def	5.44 Fg	2.44 d	7.85 d	4.78 d	4.29 e	85.79 c

*Different letters represent statistically different groups ($p<0.05$)

In the present study, the yields of the foreign walnut cultivars were higher than those of the local cultivars (Table 2, Figure 2). Maraş 12 and Şebin were the cultivars with the highest and lowest yield among the local cultivars, respectively. The low yield problem of the Şebin cultivar may be related to a higher female flower abortion ratio (Akça 2014). Sütyemez et al. (2019) confirmed that a cluster-bearing habit of Maraş 12 resulted in a high estimated yield. On the contrary, Akça et al. (2018) in Tokat, Turkey reported that the yield of Maraş 12, Maraş 18, and Şebin cultivars was low. However, the present study obtained higher yields from Maraş 12 and Maraş 18 among local cultivars. Also, Sütyemez (2016) and Sütyemez et al. (2019) confirmed that in Kahramanmaraş, Turkey, Maraş 18 and Maraş 12 have a intermediate and high yield, respectively. The results are consistent with those of Sütyemez (2016) and Sütyemez et al. (2019) since the climate of the Kahramanmaraş region, where the cultivars were selected, is similar to the climate in which the study was conducted, as contrasted to the climate of the Tokat region.

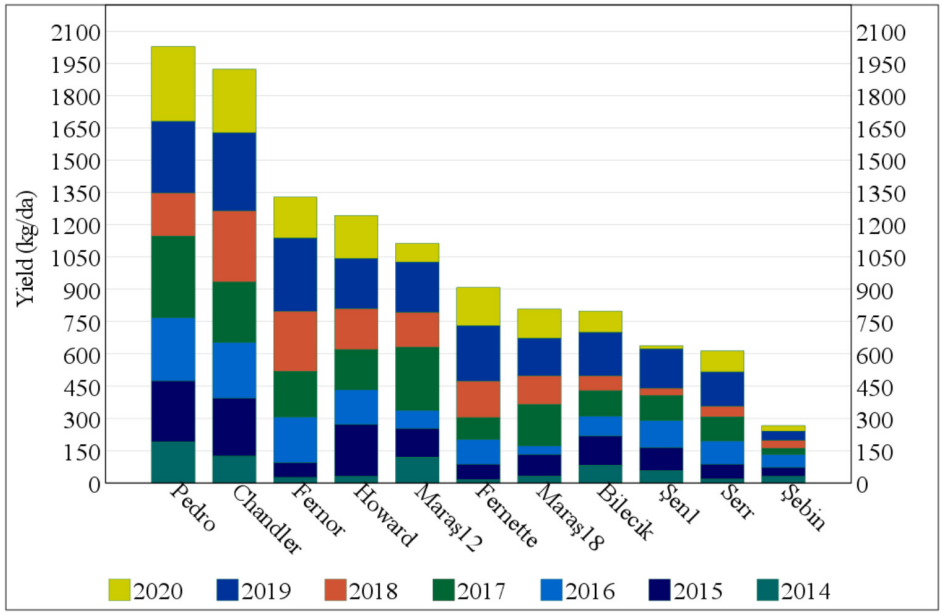

Figure 2- Total yield (kg/da) of the walnut cultivars (2014-2020)

According to the five-year data (Table 3), the highest walnut blight incidence was observed in Şen 1 (31.20%), followed by Şebin (23.81%), while the lowest was in Howard (7.83%). Anthracnose incidence was between 0.50% (Chandler) and 13.64% (Serr); codling moth ranged from 1.37% (Pedro) to 17.37% (Şen 1), and bird damage was between 0.80% (Howard) and 5.52% (Şebin). Sunburn incidence of cultivars ranged from 4.81% (Fernette) to 16.75% (Bilecik). The highest sunburn damage was seen in Bilecik (16.75%) and Howard (15.16%), whereas the lowest in Serr (6.68%), Fernor (5.62%), and Fernette (4.81%). Chandler (76.50%), Fernette (74.93%), and Howard (72.66%) cultivars were identified as having healthy nuts with a low incidence of anthracnose, bird damage codling moth, walnut blight, and sunburn. On the other hand, Şen 1 (40.06%) and Şebin (42.28%) were the cultivars with the least healthy fruit and more external defects. The present study mainly observed the walnut blight damage among the fruit's external defects.

Table 3- Incidence of external defects on walnut cultivars (2014-2018)

Cultivar	*Walnut blight damage (%)*	*Anthracnose damage (%)*	*Codling moth damage (%)*	*Sunburn damage (%)*	*Bird damage (%)*	*Healthy nuts (%)*
Bilecik	12.56 ef*	2.36 cd	1.81 cd	16.75 a	2.67 bc	65.10 d
Maraş 12	11.06 fg	3.77 c	2.54 cd	9.12 de	4.05 b	70.20 c
Maraş 18	15.00 de	3.25 c	4.12 c	10.87 cd	3.17 bc	64.75 d
Şebin	23.81 b	6.72 b	9.15 b	12.59 bc	5.52 a	42.28 f
Şen 1	31.20 a	2.00 cd	17.37 a	9.34 de	0.98 de	40.06 f
Chandler	9.43 gh	0.50 d	1.62 d	11.06 cd	1.22 de	76.50 a
Fernor	17.70 c	0.53 d	2.82 cd	5.62 f	0.43 e	73.04 abc
Fernette	15.06 de	0.75 d	3.12 cd	4.81 f	1.78 cde	74.93 ab
Howard	7.83 h	0.70 d	3.50 cd	15.16 ab	0.80 e	72.66 abc
Pedro	14.12 de	1.25 d	1.37 d	10.37 cd	2.28 cd	71.12 bc
Serr	16.54 cd	13.64 a	8.84 b	6.68 ef	2.98 bc	52.92 e

*Different letters represent statistically different groups ($p<0.05$)

Aleta et al. (2001) noted Chandler's blight incidence on fully developed nuts to be 10-14% and reported that the cultivar showed low susceptibility in field conditions. Aktuğ Tahtacı et al. (2014) stated that walnut blight was observed in all cultivars except Chandler. Bujdoso et al. (2020) reported that Chandler was moderately susceptible to walnut blight. Our results are in accordance with these reports, where Chandler has a lower walnut blight incidence (9.43%). On the contrary, Moragrega et al. (2011) and Jelev & Marinov (2016) reported that Chandler fruit was affected by walnut blight, and the blight incidence in fruit were 42.7% and 67.9%, respectively.

Some of our results agree with other studies conducted about the local cultivar's walnut blight incidence. Özaktan et al. (2007) stated that Şebin was highly susceptible, Bilecik, Şen 1, and Chandler were moderately susceptible, and Pedro was tolerant. Saracoğlu (2015) found that Chandler, Hartley, and local cultivar Şebin were highly susceptible to blight, while Pedro was classified as less susceptible. Also, in Tokat, Turkey, Chandler, Maraş 12, Şebin and Şen 1 have been found more susceptible to walnut blight than other cultivars (Akça 2018). Botu et al. (2010) indicated that Fernor, Fernette and Serr were moderately susceptible to blight. In the present study, blight incidence was lower in Bilecik, Chandler, and Maraş 12 cultivars whereas moderate in Pedro, Fernette, and Fernor.

The foreign cultivars, except for Serr, showed the lowest anthracnose incidence. Serr and Şebin cultivar had the highest anthracnose incidence among the foreign and local cultivars, respectively. In Uzbekistan, Chandler has been noted as the most resistant cultivar, with an anthracnose disease incidence of 0%. (Khasanov et al. 2019). In Bulgaria, the highest infestation index in the fruits has been observed in Serr (7.7%), while it was low in the rest of the cultivars (Fernette, Fernor, and Chandler) (Arnaudov et al. 2014). In Romania, Franquette, Fernor, and Fernette have shown very low susceptibility (Botu et al. 2010). In addition, Arnaudov and Gandev (2009) recorded that Chandler was resistant, whereas Serr was highly susceptible. These studies are consistent with the results obtained in the present study. However, Salahi & Jamshidi (2009) reported that Serr had moderate resistance, and Pedro was susceptible to the disease.

Concerning codling moth damage, the highest incidence was observed in Şen 1, Şebin, and Serr, whereas the lowest was in Chandler and Pedro. Coates (2008) reported that Serr was susceptible to codling moth, walnut blight, and sunburn. Besides, Chandler is a recommended cultivar due to the relatively low incidence of codling moth and walnut blight. Akça (2014) stated that Şebin was susceptible to codling moths. Sütyemez (2016) reported that Maraş 18 was resistant to codling moths. These studies are consistent with the obtained results.

In the present study, sunburn damage incidence was higher in Bilecik, Howard, and Şebin cultivars than the others. These results agree with Lampinen et al. (2006), who indicated sunburn occurred in Howard, followed by Chandler. Howard trees are generally smaller and, therefore, more likely to be exposed to photosynthetically active radiation conditions that can cause sunburn (Lampinen et al. 2006). Akça (2014) stated that Şebin was sensitive to sunburn and water stress, reducing kernel quality. Also, Mahmoudi et al. (2021) stated that Chandler's sunburn damage incidence was higher than the Franquette and reported that it was related to the thickness of the fruit husk of the Franquette cultivar. In the present study, the reason why less sunburn was observed on the fruits of Fernor and Fernette cultivars may be the thicker husk of these French origin cultivars such as Franquette. Early leafing cultivars experienced more external damage than late leafing cultivars (Mills et al. 2000; Bernard et al. 2018). Our results are in accordance with these reports where late leafing cultivars have lower external damage incidence. Except for Maraş 12, the ratio of healthy fruit was lower in early leafing cultivars.

The results of nut traits of the walnut cultivars are shown in Table 4. Nut weight ranged from 9.81 g (Şebin) to 16.27 g (Şen 1). Kernel weight ranged from 5.18 g (Pedro) to 7.52 g (Maraş 18), and kernel percentage varied between 36.31% (Şen 1) and 53.56% (Maraş 12). Kernel percentage was high in Şebin, Maraş 18, Maraş 12, moderate in Bilecik, low in Fernette, Serr, Howard, and Chandler, and very low in the others (Table 4). Desirable nut and kernel weight should range from 12 g to 18 g, and 6 g to 10 g, respectively, or kernel percentage should be at least 50% (McGranahan & Leslie 1990). Maraş 18 was the cultivar that had these kernel traits. Akça et al. (2014) reported that the nut weight of the evaluated 13 cultivars ranged from 12.79 g (Fernor) to 15.35 g (Midland), kernel weight from 5.80 g (Fernor) to 7.22 g (Fernette), and kernel percentage from 42.80% (Pedro) to 47.33% (Fernette). Bobokashvili et al. (2017) reported that the kernel weight of Fernette and Howard was 7.68 g and 7.80 g, and kernel percentage was 50.31% and 52.57%, respectively. In Australia, Chandler, Fernette, and Fernor's nut weights were 10.6, 11.0, and 11.4 g, respectively (Vanhanen 2010). In Iran, nut weights varied between 8.5 and 11.5 g in Serr, 11.5 and 13.5 g in Pedro, 8.5 and 11.5 g in Fernor, and 8.5 and 11.5 g in Chandler (Toolir 2021). The differences between studies in terms of nut and kernel weights can be due to the effect of environmental factors. Also, Özcan et al. (2022) reported that Kurtulus 100 obtained from a KSÜ00M5× Pedro crossing was superior to Chandler in terms of fruit quality; its nut weight, kernel weight, and kernel percentage were calculated to be 13.48 g, 7.52 g, 55.78% respectively. Similarly, Hassani et al. (2020a) stated that new cultivar Persia has a greater kernel percentage (63%) when compared to that of Chandler.

Nut width, thickness, and length significantly varied in the cultivars evaluated in the study. Nut width varied from 29.34 mm (Maraş 12) to 42.30 mm (Şen 1), nut thickness was between 30.62 mm (Maraş 12) and 42.65 mm (Şen 1), nut length ranged from 34.23 mm (Maraş 12) to 43.36 mm (Şen 1). Shell thickness, kernel rottenness, and shrinkage were also significantly affected by the cultivars. The cultivars' shell thickness was between 1.11 mm (Şebin) and 2.30 mm (Fernor). In other words, the shell strength of Şebin and Maraş 12 was comparatively weak, whereas Şen 1, Howard, and Fernor were strong. The cultivars were also assessed for kernel shrinkage and

rottenness, which are important factors that affect kernel quality. The least kernel shrinkage was observed in Maraş 12 (1.46%), Maraş 18 (1.52%), and Howard (1.84%), while the highest was observed in Pedro (15.63%). Sütyemez (2016) stated that there was no kernel shrinkage in Chandler and Maraş 18 cultivars. Kernel rottenness varied from 3.12% (Bilecik) to 21.71% (Serr).

In the present study, the nut dimension of the cultivars was similar to the results published by Akça et al. (2014) and Akça et al. (2018). Akça et al. (2014) evaluated 13 walnut cultivars grown in Tokat, Turkey, and found nut width of about 35.24 mm (Fernor) - 38.47 mm (Fernette); nut length of 40.62 mm (Fernor) - 43.50 mm (Pedro) and nut thickness of 33.73 mm (Fernor) - 36.86 mm (Fernette). Akça et al. (2018) reported that the nut width of cultivars ranged between 30.55 mm (Maraş 12) and 39.97 mm (Şen 1), and nut length varied from 32.58 mm (Şen 1) to 42.53 mm (Şen 1). In Argentina, the average nut widths of 34.10-40.80 mm, nut thickness of 33.70-40.50 mm, and nut length of 36.70-45.20 mm have been reported (Iannamico et al. 2006). Ercisli et al. (2012) in Yalova, Turkey, found nut widths of Şebin, Şen 1, Chandler, Pedro, and Serr as 33.98 mm,46.38 mm, 36.55 mm, 37.51 mm, and 37.10 mm, respectively. Türemiş et al. (2017) evaluated 10 walnut cultivars in Adana, Turkey, and nut width varied between 31.70 mm (Maraş 12) and 42.91 mm (Şen 1), nut thickness ranged from 30.19 mm (Maraş 12) to 39.59 mm (Şen 1). Karlıdağ et al. (2019) reported that the nut width of Maraş 18, Chandler, and Bilecik cultivars was 35.81, 36.24, and 38.82 mm, and nut thickness was 34.41, 34.91, and 37.19 mm, respectively. In the present study, the nut dimension of the cultivars was found larger than those determined by Ercisli et al. (2012). These differences can be due to environmental conditions on the cultivar (Vanhanen 2010). In addition to nut weight, kernel weight, kernel percentage, shell thickness, kernel color is also an important parameter in walnut cultivar, and light kernel colors are among the important objectives of walnut breeding (Vahdati et al. 2019; Hassani et al. 2020b; Sütyemez et al. 2021). In the present study the kernels of Chandler and Fernor were extra light, Şen 1 light amber and the other cultivars were light color (Figure 3).

PCA was applied to evaluate the data set and detect the most important variables for determining the data structure. The analysis showed that the eigenvalues of the first five principal components were greater than 1.0, explaining 85.20% of the total variance. The first five principal components accounted for 28.30%, 19.60%, 18.00%, 11.30%, and 8.00% of the variation.

The PC1 represented the maximum variation of the data set (Table 5). It was positively associated with nut weight, nut thickness, nut length, nut width, and shell thickness, while negatively associated with kernel percentage and bird damage values. The PC2 was negatively correlated with healthy nut ratio and yield, whereas positively connected with walnut blight damage and codling moth damage.

Yield and healthy nut ratio increased with decreasing walnut blight and codling moth damage. The negatively correlated variables with the PC3 were leafing, male and female flower blooming, on the other hand anthracnose value was determined to be positively correlated. These data reveal that the increase in anthracnose damage occurs with early leafing and flowering. The PC4 was positively correlated with kernel shrinkage and defoliation and negatively correlated with kernel weight. Besides, sunburn damage and kernel rottenness were the leading indicators for the PC5.

The plot of the PC1 versus PC2 (Figure 4) identified Şen 1 and Şebin located some distance away from the others, indicating that Şen 1's fruit weight was higher, while its yield and healthy nut ratio were lower. Also, Şebin was located on the negative side of the PC1 and the positive side of the PC2, which showed that its fruit size was smaller, while its yield and healthy nut ratio were lower than the others, similar to Şen 1. The cultivars located on the positive side of the PC1 and the negative side of the PC2, Fernor, Fernette, Pedro, Maraş 18, Chandler, and Howard, had higher yield and lower walnut blight and codling moth damage, especially Chandler and Howard.

Table 4- Some nut traits of the walnut cultivars (2014-2018)

Cultivar	*Nut weight (g)*	*Nut width (mm)*	*Nut thickness (mm)*	*Nut length (mm)*	*Kernel weight (g)*	*Kernel percent (%)*	*Shell thickness (mm)*	*Shell strength*	*Kernel color*	*Kernel rottenness (%)*	*Kernel shrinkage (%)*
Bilecik	11.12±0.07 f*	33.50±0.50 e	33.18±0.46 fg	38.92±0.62 b	5.38±0.06 hi	46.06±0.50 c	1.68±0.01 c	Intermediate	Light	3.12±0.40 i	4.15±0.49 cd
Maraş 12	9.91±0.06 g	29.34±0.19 h	30.62±0.11 h	34.23±0.16 f	5.49±0.11 ghi	53.56±0.94 a	1.40±0.02 d	Weak	Light	8.99±0.77 cef	1.46±0.24 f
Maraş 18	14.41±0.22 b	34.10±0.09 cd	35.53±0.16 b	39.15±0.12 b	7.52±0.12 a	50.89±0.31 b	1.69±0.01 c	Intermediate	Light	3.24±0.60 hi	1.52±0.37 f
Şebin	9.81±0.17 g	30.73±0.09 g	32.66±0.11 g	34.58±0.21 f	5.94±0.25 ef	50.82±2.23 b	1.11±0.02 e	Weak	Light	14.62±.48 c	2.89±0.30 de
Şen 1	16.27±0.23 a	42.30±0.12 a	42.65±0.04 a	45.36±0.18 a	6.82±0.20 b	36.31±1.16 g	2.20±0.05 a	Strong	Light amber	18.70±1.48 b	4.93±0.60 bc
Chandler	12.36±0.05 d	33.67±0.07 de	35.11±0.13 bc	39.08±0.16 b	5.73±0.02 fgh	43.92±0.14 cd	1.98±0.02 b	Intermediate	Extra light	5.84±0.51 gh	4.64±0.54 c
Fernor	13.43±0.09 c	34.00±0.13cde	35.11±0.17 bc	38.77±0.17 bc	5.84±0.04 efg	39.25±0.03 f	2.30±0.04 a	Strong	Extra light	9.34±0.66 de	2.37±0.46 ef
Fernette	13.96±0.25 b	36.16±0.04 b	35.75±0.34 b	38.02±0.08 cd	6.53±0.07 bc	40.80±0.73 ef	1.88±0.01 b	Intermediate	Light	11.50±0.55 d	4.71±0.39 c
Howard	13.17±0.29 c	34.31±0.09 c	34.04±0.27 de	36.93±0.16 e	6.14±0.16 de	43.66±0.28 cd	2.23±0.01 a	Strong	Light	6.76±0.63 efg	1.84±0.08 ef
Pedro	11.93±0.08 de	33.45±0.17 e	33.59±0.16 ef	38.08±0.25 c	5.18±0.06 i	39.60±0.40 f	1.73±002 c	Intermediate	Light	6.50±1.02 fg	15.63±0.64 a
Serr	11.66±0.13 e	32.23±0.06 f	34.60±0.09 cd	37.27±0.08 de	6.41±0.10 cd	43.14±0.47 de	1.88±0.09 b	Intermediate	Light	21.71±0.85 a	6.12±0.81 b

*Different letters represent statistically different groups ($p<0.05$)

Figure 3- Shell and kernel photos of the walnut cultivars

4. Conclusions

Late leafing was observed in Chandler, Fernor, and Fernette cultivars, while local cultivars were leafing relatively early. While the disease incidence rate was lower in foreign cultivars except for Serr, healthier fruits were obtained from Maraş 12 among local cultivars. The yields of Chandler, Pedro, Howard, Fernor, and Maraş 12 cultivars were higher, and no yield fluctuations depending on the years were observed. Yield fluctuations were detected mainly in Şebin, Serr, and partially Şen 1 cultivars. Kernel percentage was generally higher in local cultivars, and excellent result was obtained from Maraş 12, Maraş 18, and Şebin. Besides, a considerable amount of kernel shrinkage and rottenness was not seen on Maraş 18 nuts. These were followed by foreign cultivars Chandler and Howard. Pedro cultivar, which has a high yield potential, attracted attention with its high kernel shrinkage rate.

In conclusion, considering the region is affected by spring late frost, Chandler, Howard, and Fernor cultivars with late leafing, high yield potential, lower disease incidence rate, and relatively high-quality fruit may be recommended. Among the local cultivars, if the early leafing trait is ignored, Maraş 12 can be a preferable cultivar in terms of all factors. Alternatively, Maraş 18 cultivar can be recommended with lower yield potential but larger nut dimension and kernel percentage.

Table 5- Eigenvalues and cumulative variance for five factors resulted from PCA

Parameter	*1*	*2*	*3*	*4*	*5*
Nut weight	0.893	0.021	-0.246	-0.308	-0.084
Nut thickness	0.881	0.395	-0.126	-0.106	-0.080
Nut length	0.880	0.264	-0.195	0.004	0.145
Nut width	0.877	0.285	-0.297	-0.057	0.030
Shell thickness	0.773	-0.271	-0.238	0.063	-0,251
Kernel percent	-0.700	-0.048	0.354	-0.515	0.298
Bird damage	-0.677	0.351	0.341	-0.211	0.059
Healthy nut	-0.057	-0.916	-0.347	0.023	-0.051
Walnut blight damage	0.210	0.911	-0.096	-0.119	-0.149
Codling moth damage	0.348	0.811	0.235	-0.086	-0.108
Yield	0.216	-0.730	-0.207	0.224	0.057
Blooming of male flower	0.309	-0.027	-0.859	0.182	-0.040
Leafing	0.340	-0.274	-0.841	0.152	-0.167
Blooming of female flower	0.480	-0.211	-0.785	0.194	-0.084
Anthracnose damage	-0.226	0.331	0.744	0.078	-0.329
Kernel shrinkage	0,160	-0.010	0.026	0.785	-0.003
Defoliation	-0.160	-0.267	-0.208	0.720	0.106
Kernel weight	0.468	0.160	0.205	-0.663	-0.154
Sunburn damage	-0.080	-0.049	0.135	0.221	0.901
Kernel rottenness	0.141	0.463	0.392	0.199	-0.612
Eigen value	5.665	3.910	3.600	2.258	1.596
% of variance	28.300	19.600	18.000	11.300	8.000
Cumulative variance %	28.300	47.900	65.900	77.200	85.200

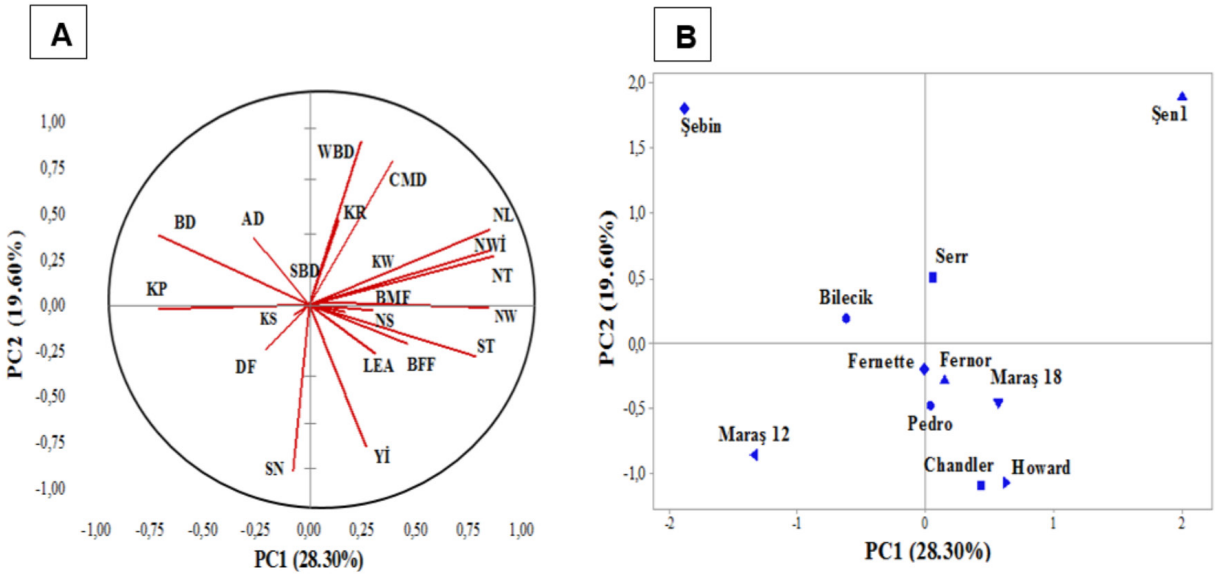

Figure 4- (A) The scatter plot of the variables of the first two principal components; (B) The scat-ter plot of cultivars YI: Yield, LEA: Leafing, BFF: Blooming of female flower, ST: Shell thickness, NW: Nut weight, BMF: Blooming of male flower, NT: Nut thickness, NWI: Nut width, NL: Nut length, KW: Ker-nel weight, CMD: Codling moth damage, KR: Kernel rottenness, WBD: Walnut blight damage, SBD: Sunburn damage, AD: Anthracnose damage, BD: Bird damage, KP: Kernel percentage, KS: Kernel shrinkage, DF: Defoliation, SN: Healthy nut ratio

Data availability: Data are available on request due to privacy or other restrictions.

Authorship Contributions: Concept: Ü.E., Design: Ü.E., Data Collection or Processing: D.A.K., M.B.K., Ö.U., C.M., Ü.E., Analysis or Interpretation: D.A.K., M.B.K., Ö.U., C.M., Ü.E., Literature Search: D.A.K., M.B.K., Writing: D.A.K.

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Volatile Compounds, Bioactive Properties and Chlorophylls Contents in Dried Spearmint (*Mentha spicata L.*) as Affected by Different Drying Methods

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ABSTRACT

This study presents a comparison of the quality characteristics of spearmint (*Mentha spicata L.*) dried by a photovoltaic thermal dryer (PVT), the shade dried spearmint (SDS), and an oven dried spearmint (ODS). The obtained samples were evaluated with respect to volatile compounds (VC), total phenolic content (TPC) and flavonoid content (TFC), antioxidant capacity (AC) and chlorophylls (Chl) contents. PDS had the highest amount of TPC, TFC and AC, while SDS and ODS did not differ significantly from each other in terms of these features. SDS exhibited the highest Chl a and Chl b contents, whereas ODS showed the lowest. The composition of VC in the dried spearmints was significantly affected by the drying methods used. The total amount of terpenoids, especially carvone, responsible for spearmint's characteristic aroma in SDS was higher than those of the others, while the concentrations of most VC were lowest in ODS. According to the results, the PVT can be recommended for drying spearmint.

Keywords: Mint drying, Photovoltaic thermal collector, Dven drying, Shade drying, Drying procedure, Quality of dried spearmints

1. Introduction

Spearmint (*Metha spicata L.*) is one of the most extensively cultivated species of the *Mentha* genus and is commonly referred to as garden mint. This aromatic and herbal spice is utilized globally in flavoring, pharmaceutical, cosmetic and fragrance applications. Additionally, they can be used in fresh or dried forms, and as essential oils (Nalawade et al. 2019). Spearmint is often used in many cuisines and the food industry mainly due to its distinct aroma. In addition, mint species are well-known for their antioxidant (Hinneburg et al. 2006) and antimicrobial (Shah & Mello 2004) characteristics. Therefore, it is estimated that the commercial significance of these plants will increase further owing to the spicing and the other diverse benefits.

Aroma is a primary factor in evaluating the quality of spices as an organoleptic characteristic. The aroma of spices primarily characterized by their contents of volatile compounds (VC) (Govindarajan & Salzer 1986). The specific aroma of spearmint is generally derived from certain terpenoid compounds (Da Porto & Decorti, 2009). The qualitative and quantitative composition of these compounds are affected by pre- and post-harvest conditions (Nalawade et al. 2019). Drying processes particularly change the profile of VC depending on temperature, humidity and time conditions (Korkmaz et al. 2020; İzli & Polat 2020; Polat et al. 2021). Nowadays, the content of bioactive substances in herbs and spices has been considered as another parameter of their quality, with a rising trend (Uribe et al. 2016). These phytochemical compounds have various beneficial effects on human physiology such as anticancer, antimicrobial, anti-diabetic, anti-inflammatory, cytotoxicity, and cytoprotective properties (Tafrihi et al. 2021). Phenolic acids and flavonoids are the primary bioactive components in mints (Lv et al. 2012). The amount of these compounds in genus *Mentha* can also differ depending on drying conditions as well as other factors including growing conditions, cultivar, harvest time (Mahendran & Rahman 2020).

Drying is one of the main processes applied to spice herbs and has critical effects on the overall acceptance of the final products. It is generally used to reduce the moisture content in the plant tissues in order to extend their shelf life. Additionally, drying treatments also facilitate the processing of spices into different forms such as powders, extracts and oils (Uribe et al. 2016). Spice herbs are dried using various methods, frequently by sun and shade drying. These traditional ways are economical, but have some disadvantages such as being time-consuming, requiring a large area, sacrificing some biochemical attributes (Mokhtarian et al. 2020), and allowing microbial (Mokhtarian et al. 2017) and other environmental (Arslan et al. 2010) contaminations. Therefore, several drying methods have been used in order to minimize the loss of quality as well as the time and energy cost of drying techniques. Herbal plants are typically dried using oven drying and hot air drying on an industrial scale (Mokhtarikhah et al. 2020). Furthermore, solar dryers are proposed as an alternative drying method for mints (Akpinar 2010; Mokhtarian et al. 2020). They are categorized as hot air convection mode (natural or forced) and energy search (direct, indirect, mixed and hybrid solar) (El-Sebaii & Shalaby 2013). The hot air from solar drying can be generated by flat plate collectors, vacuum tubes, photovoltaic (PV) and photovoltaic thermal (PVT) collectors. PVT collectors supply both thermal and electrical energy (Kovacı et al. 2020). The solar dryers designed by these collectors are recommended by several researchers to dry *Mentha* plants due to their low cost (El-Sebaii & Shalaby 2013), high efficiency (Arslan et al. 2020; Arslan & Aktaş 2020) and short drying time (Koşan et al. 2020).

The majority of published research related to dried *Mentha* genus have been primarily focused on the essential oils of these herbs (Baranauskienė et al. 2007). There are also several studies about determining some properties of dried mints using various methods. (Mokhtarian et al. 2020) found that both the total phenolic content (TPC) and chlorophylls (Chl) content of peppermint (*Mentha piperita L.*) dried using a solar collector were higher than those that were traditionally (sun and shade) dried. (Díaz-Maroto et al. 2003) demonstrated that spearmints (*Mentha spicata L.*) dried using oven (at 45 °C) and air (at ambient temperature) methods presented the best composition regarding VC compared to freeze drying. Although dried leaves of spearmint are extensively used in the food industry or in many culinary practices, the number of studies on aroma compounds and bioactive properties of this spice dried via different drying methods is limited. Moreover, to the best of our knowledge, no extensive investigations have been carried out on the VC composition and bioactive attributes of spearmint dried with a solar dryer designed with a PVT collector. Hence, the purpose of present study was to evaluate the VC composition, TPC, TFC, antioxidant capacity (AC) and Chl content of dried spearmint obtained by ODS, SDS and PDS drying methods. Additionally, the thermal and electrical efficiencies of the PVT collector were calculated.

2. Material and Methods

2.1. Plant material

Fresh spearmint (*Mentha spicata* L.) was obtained from a local market (Ankara, Turkey) and used in the experiments on the same day. A total of 300 g of fresh spearmint leaves were separated from their stems and then divided into three equal parts after being washing with water. These parts were used immediately for each drying procedure.

2.2. Standards and chemicals

Methanol, acetone, formic acid, Folin-Ciocalteau reagent, sodium carbonate, toluene, sodium nitrite, aluminum chloride, sodium hydroxide, 2,2 Diphenyl-1-picrylhydazyl (DPPH), and analytical standards of trolox, GA and catechin were purchased from Sigma Aldrich (Taufkirchen, Germany).

2.3. Photovoltaic thermal dryer (PVTD)

The PVTD used was designed as a system consisting of a PVT collector and a drying chamber (DC). The schematic view of this system is given in Figure 1.

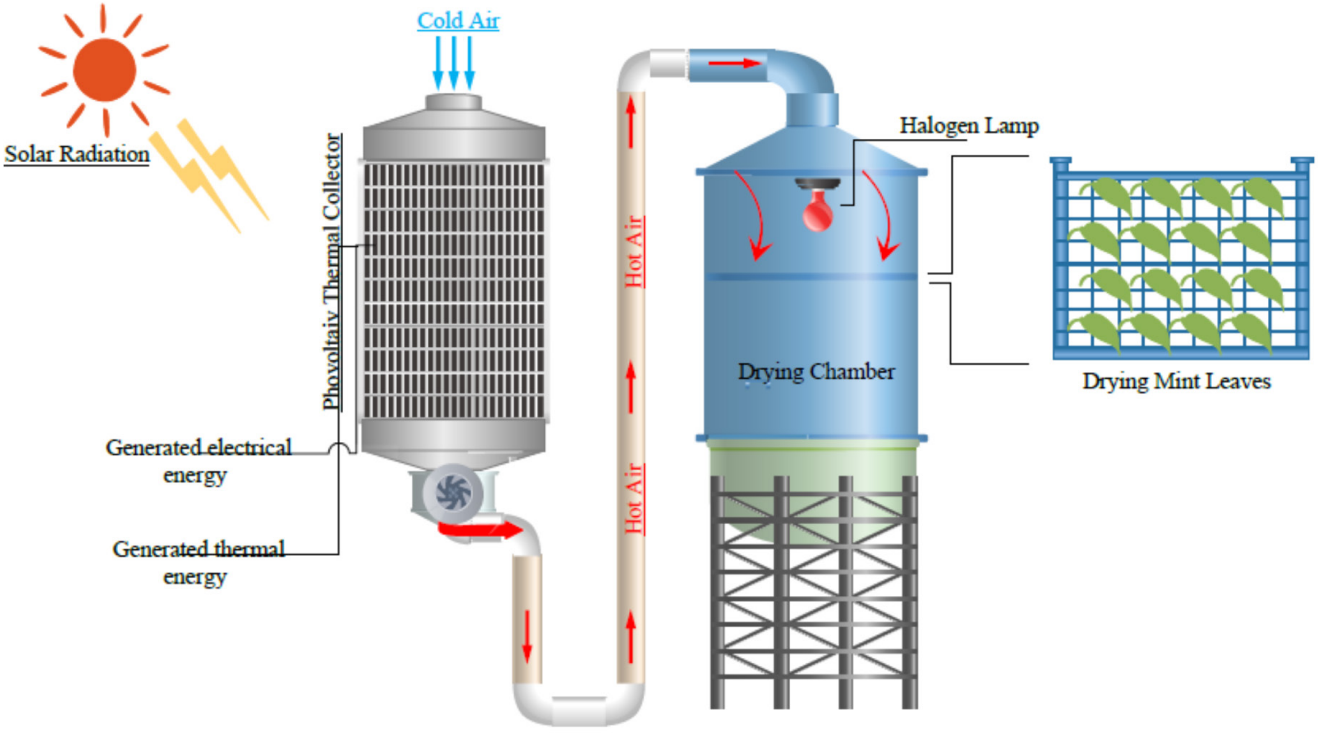

Figure 1- Schematic illustration of photovoltaic thermal collector drying system

2.3.1. Photovoltaic thermal panel

In the PVT panel design, it has been aimed to generate both heat and electricity energy from solar radiation for a sustainable solar drying system. In this way, a sustainable drying system capable of generating its own energy has been produced. The design has been created in such a way that fresh air enters from the upper part of the PVT panel and extracts the heat energy from the panel before entering the DC. Fans that pulled in fresh air were connected in series at the entrance of the PVT panel. The backside of the PV module was enclosed with an aluminum sheet, leaving 10 cm of space for the fresh air to flow homogeneously. In addition, the sides and back of the PVT panel were covered with insulation material so that no heat loss occurred. The electricity generated from the PVT panel was stored in an accumulator. It fed the system's fans and halogen lamps, which served as the auxiliary heat source in the DC, via a converter (Figure 1). Five thermocouples were located at the back of the PVT panel to observe the temperature distribution. Technical specifications of the PVT panel and other equipment used in the experimental setup are given in Table 1.

Table 1- Technical specifications of the apparatus used in the drying system

Apparatus	***Specifications***
PVT panel	275W, Vm=31.79 V, Im=8.66 A, 1640x992 mm
Charge regulator	6.9V-17.2V, 20 A, 30 Hz, -10 to+50 °C
Accumulator	Yigit battery YD12-26 12V26AH, running temperature -15 - +40 °C
Halogen lamp	50W, 12 V, AC
Fan	12 V DC - 0.32 A, -10 to +55 °C
Thermal insulation	Expanded polystyrene, 50 mm thick, 0.035 W/mK

PVT: Photovoltaic thermal, W: Watt, Vm: Maximum voltage, Im: Maximum current, V: Voltage, DC: Direct current, AC: Alternative current, mm: Millimeter, A: Amper, Hz: Hertz, mK: meter Kelvin

2.3.2. Drying chamber

The DC was constructed with 40x40x60 cm dimensions and was covered with insulation material to prevent heat loss. The heated air in the PVT panel entered from the upper part of the DC. In the DC, 50Wx4 halogen lamps, which are an auxiliary heat source, were used in order to provide sustainable drying even under conditions with no or insufficient solar radiation. Halogen lamps were

homogeneously installed on the upper part of the DC. A tray was placed 15 cm below the halogen lamps and one fan was attached to the entrance of the DC. A load cell was placed just below the tray in order to observe the amount of moisture lost by the product. Technical characteristics of the devices used in the DC are indicated in Table 2. In addition, the properties of the measurement devices used in the experimental setup are given in Table 2.

Table 2- Equipment in experimental setup and their specification's

Devices	*Brand*	*Qualification*	*Accuracy*	*Quantity (ea)*
Anemometer	Kimo, VT 200	0–20 m/s, 0.3-35 m/s, -20 to +80 °C	±0.03 m/s, ±0.1 °C	1
Data Logger	Elimko, E-680	-200 + 1200 °C	±0.5 °C	1
Load cell	Zemic, L6D, OIMLC3	Measurement capacity max. 5000 g	±0.01 g	1
Solar meter	Kimo, SL 100	0-1300 W/m^2	±5 %	1
Thermocouple	Elimko, K type NiCr-Ni	-200 + 1200 °C	±0.5 °C	9
Thermohygrometer	Kimo, HD 100	-20 to + 70 °C, 5-95 % RH	±1.8 %RH, ±0.3 °C	1

RH: Relative humidity, W: Watt, m: Meter, s: Second, g: Gram

2.4. Drying procedures

The drying experiments were performed in Ankara (39.93° N latitude and 32.86° E longitude). Using each of the three drying methods, the leaves were dried until their moisture content was below 10% (w/w) in accordance with the Turkish commercial dried spices standard. This level was achieved when the difference between two consecutive weight measurements was less than 1%. The initial and the final moisture contents of the leaves were measured using the AOAC method (1990) (method number 934.06) (Williams 1984). The moisture content in fresh leaves was 82.50% (w/w) based on wet weight. Following the drying treatments, the leaves were ground to a 200-500 µm particle size with a mill and stored in screw-capped vials at -18 °C until analysis. Each drying was performed in triplicate.

2.4.1. Oven drying

The fresh spearmint leaves were thinly spread onto a stainless-steel wire tray (38x38 cm) and dried in an oven (Memmert UN110, Schwabach, Germany) at 65 °C for 8 h.

2.4.2. Shade drying

The shade drying was carried out in the shade at room temperature. Fresh spearmint leaves were thinly spread onto a stainless-steel wire tray (38x38 cm) and left in a well-ventilated room. The leaves were turned twice to ensure a uniform drying. The drying process was complete after approximately 36 hours. The average temperatures of the room in the day and night were 22±2 and 18±2 °C, respectively, during drying. The relative humidity of the room during drying was 41±3%.

2.4.3. Photovoltaic thermal drying

Fresh spearmint leaves were evenly distributed on a stainless-steel wire tray (38x38 cm) and placed in the DC. Thereafter, all PVT experiments were initiated at 10 am and the drying process began. The experiments continued until the drying was completed (5.5 h). The DC temperature was set to 35° with the assistance of a thermostat. When the temperature dropped below 35°, halogen lamps started to operate automatically. After the drying process started, the surplus energy generated from the PVT panel continued to be stored in the accumulator. In the absence of sufficient solar radiation, the electrical energy required for the DC was provided by the accumulator. The schematic view of the experiment set is given in Figure 1. Accordingly, the ambient air enters the PVT collector from the upper side with the help of the fan and exits as hot air from the lower side. The hot air is allowed to enter the drying room, and its exhaust is delivered to the exterior as hot and humid air.

2.5. Extraction procedure of phenolics

The extraction of phenolic was carried out according to the procedure described by Capanoglu et al. (2013) with some modifications. Two hundred mg of sample was extracted with 3 mL of 75% methanol (containing 1% formic acid) using a homogenizer (Ultra-Turrax T25 Basic, IKA, Staufen, Germany) at 15000 rpm for one minute. The extract was then sonicated for 15 min at the room temperature and centrifuged (Universal 320 Hettich, Westphalia, Germany) at 1200 g for 10 min at 10 °C. The supernatant was collected and the

residue was extracted twice more. All supernatants were combined in a tube and the final volume was adjusted to 10 mL with the acidified methanol. This methanolic extract was used for TPC, TFC and AC analyses of dried spearmint samples.

2.6. Total phenolic and total flavonoid content analysis

The TPC was determined by Folin-Ciocalteau method (Singleton & Rossi 1965). Briefly, 100µL of methanolic extract was mixed with 900 µL of distilled water and 5 mL of Folin-Ciocalteu reagent (0.2 mol/L). This mixture was shaken vigorously, and left to rest for 8 min. Thereafter, 5 mL of 7.5% Na_2CO_3 solution was added, and vortexed for 20 s. This solution was left in the dark for 2 h at room temperature, and the absorbance was measured at 765 nm with a spectrophotometer (Biochrom Libra S70 Dual; Harvard Bioscience Co. Shanghai, China). TPC was calculated in terms of mg gallic acid equivalent (GAE) per g dry weight (DW) using a standard curve prepared by GA (0.0625-1 mg mL^{-1}).

TFC was determined (Uribe et al. 2016) with some modifications. 0.4 mL of the methanolic extract was added to a mixture of 4 mL distilled water and 0.3 mL of 5% $NaNO_2$. After 5 min, 0.3 mL of 10% $AlCl_3$ solution was added, and 6 min later, 2 mL of NaOH (1 mol/L) was added. The resulting mixture was stirred and the absorbance was recorded at 510 nm with a ultraviolet-visible (UV-VIS) spectrophotometer (Biochrom Libra S70 Dual; Harvard Bioscience Co., Shanghai, China). The calibration curve was plotted using quercetin (0,03125-1 mg mL^{-1}) and TFC was expressed in terms of mg quercetin equivalent per g DW.

2.7. Antioxidant capacity analysis

AC was estimated by the DPPH radical scavenging method using the protocol of Lingua et al. (2016) with minor modifications. One hundred µL of the methanolic extract was added to 3.9 mL of 60 µM DPPH solution in methanol and stirred (Lingua et al. 2016). The mixture was left in the dark for 30 min. The absorbance was then measured at 517 nm against methanol using a UV-VIS spectrophotometer (Biochrom Libra S70 Dual; Harvard Bioscience Co., Shanghai, China). The DPPH solution without the sample was used as a control. The DPPH scavenging capacity was calculated in terms of mg Trolox equivalent (TE) per g DW.

2.8. Chlorophylls content analysis

The Chl content was determined according to (Lichtenthaler 1987), with a slight modification. Briefly, 100 mg of sample was extracted via a homogenizer (Ultra-Turrax T25 Basic, IKA, Staufen, Germany) at 16500 rpm for two minutes with 10 mL of 100% acetone. The homogenate was centrifuged (Universal 320 Hettich, Westphalia, Germany) at 400 g for 5 min at room temperature. The supernatant was transferred into a tube and the extraction was repeated twice more to yield a colorless residue. Then, all supernatants were collected, filtered by Whatman filter paper, and the final volume was adjusted to 30 mL by adding 100% acetone. The absorbance of extract was measured by a UV-VIS spectrophotometer (Biochrom Libra S70 Dual; Harvard Bioscience Co. Shanghai, China) at wavelengths 645 and 663 nm. CC was calculated by the following equations [(1), (2)] and expressed in terms of mg per g DW.

$$\text{Chlorophyll a (Chl a)} = 12.25 \times A_{663} - 2.79 \times A_{645} \quad (1)$$

$$\text{Chlorophyll b (Chl b)} = 21.50 \times A_{645} - 5.10 \times A_{663} \quad (2)$$

2.9. Volatile compound analysis

VC in samples were analyzed by solid-phase microextraction (SPME) followed by a GC-MS system (Shimadzu QP2020 GC-MS; Shimadzu Corp., Kyoto, Japan), according to Korkmaz et al. (2020) with some modification. 500 mg of sample were placed in a 20 mL SPME screw cap vial (Supelco, Bellefonte, PA, USA). 10 µL toluene (84 mg L^{-1}) was added as internal standard (IS) and the vial was immediately closed with a polytetrafluoroethylene/silicon septum (Supelco). Following this stage, all SPME operations were conducted by an auto sampler (AOC 5000 Plus; AOC 5000 Plus, CTC, Switzerland) coupled with the GC-MS. The vial was kept at 40 °C for 30 min. with an agitating speed of 220 rpm, and then a 2 cm SPME fiber (Supelco, Bellefonte, PA) coated with DVB/CAR/PDMS (50/30 µm) injected into the vial. The fiber was exposed to the headspace of the vial for 30 min at the same temperature and stirring speed. Thereafter, the fiber was withdrawn and transferred to the injection port of GC-MS for desorption at 250 °C for 5 min in splitless run. Each sample was analyzed in triplicate.

VC were separated on a DB-Heavy Wax column (60 m x 0.25 mm, 0.25 µm; Agilent J&W Scientific, Folsom, CA, USA). The oven temperature program was set to 40 °C for 3 min, increased to 80 °C at 3 °C/min (held for 1 min), and then raised to 240 °C at 5 °C/min (held for 6 min). Helium was used as the carrier gas with a flow of 1.07 mL/min. Mass spectrometry was applied by a 201°C ion source temperature with a scanning range of m/z 20-450 and 70 eV electron ionization.

Identification was carried out by comparing mass spectra with the standard Wiley 9 mass spectral library and retention indices (RI) with the National Institute of Standards and Technology standard reference database. The RIs were calculated by using a series of n-alkanes (C8-C26) (Sigma-Aldrich, USA) under the same conditions in the GC-MS. VCs were semi-quantified by multiplying the concentration of IS by the ratio of the peak area of each VC to that of the IS (μg kg^{-1}).

2.10. Statistical analysis

All experiments were performed in triplicate and the data were expressed in terms of means ± standard deviation. The differences between the means were tested via One-Way analysis of variance (ANOVA) followed by Duncan's multi comparison test ($p<0.05$). In addition, the data of VC were tested by principal component analysis (PCA) to visualize the relationships between samples and VC. All statistical analyses were performed using the SPSS software package (version 16.0, IBM Inc., Chicago, IL, U.S.A.).

3. Results and Discussion

3.1. Total phenolic and total flavonoid content (TFC)

The TPC and TFC in spearmint samples are given in Figure 2. The TPC in ODS, SDS and PDS were 136.88±1.95, 139.85±1.37 and 166.79±2.05 mg GAE g^{-1}, respectively (Figure 2a). The level of TFC in ODS, SDS and PDS were 75.50±1.81, 78.24±1.54 and 101.76±2.81 mg CE g^{-1} DW (Figure 2b). As can be seen, the level of both TPC and TFC in the spearmint dried by the PVT dryer were higher than that in shade and oven dried samples ($p<0.5$). However, the difference between both TPC and TFC in ODS and SDS was not significant ($p>0.05$), most likely due to the relationship between their drying temperatures and drying times. The amount of bioactive compounds in mint leaves can decrease as a result of both longer drying time and higher drying temperature (Mokhtarian et al. 2020) The lower TPC and TFC in SDS compared to those in PDS may be caused by the longer drying time (5.55-fold) for SDS (Samoticha et al. 2016) vacuum (VD, while the lower levels of these contens in ODS could be explained by the higher drying temperature (Arslan et al. 2010). Phenolic compounds in plants may degrade because of thermal and enzymatic oxidation during drying processes. Mokhtarian et al. (2020) reported a higher (by 25%) TPC in peppermint dried by a solar dryer than that of shade dried, supporting the relationship between drying time and temperature with TPC (Mokhtarian et al. 2020).

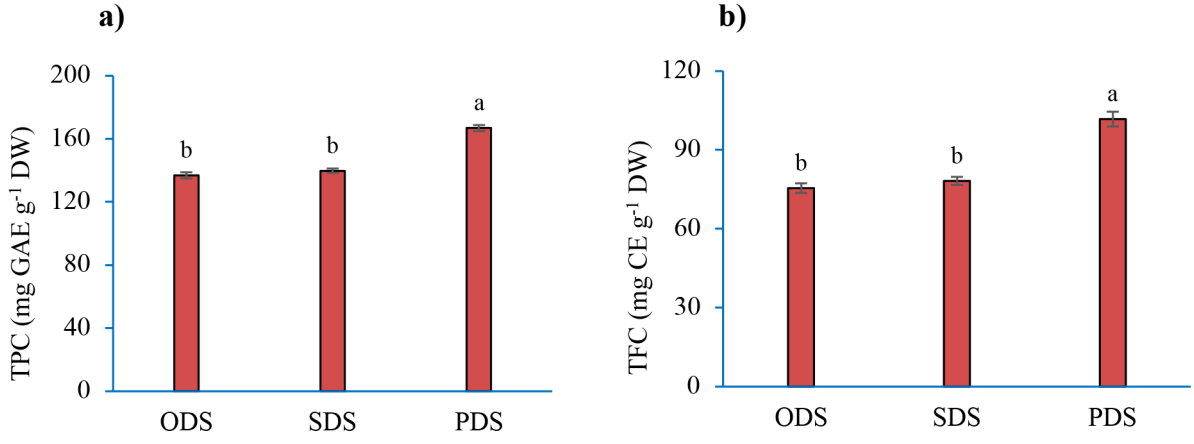

Figure 2- Total phenol (a) and total flavonoid (b) content in ODS, SDS and PDS
ODS: Oven dried spearmint, SDS: Shade dried spearmint, PDS: Photovoltaic dried spearmint, a-b Different lowercase letters were significantly different among samples ($p<0.05$)

TPC and TFC in dried *Mentha* species vary depending on breeding conditions (Lv et al. 2012), extraction methods (Koşar et al. 2005; Jeong et al. 2018; Mahendran & Rahman 2020), drying methods (Arslan et al. 2010; Uribe et al. 2016) and variety (Hinneburg et al. 2006). Uribe et al. (2016) demonstrated that TPC and TFC in a cultivar of peppermint (*Mentha piperita*) dried at different temperatures (50-90 °C) ranged between 11.56-27.12 mg GAE g^{-1} DW and 29.24-53.17 mg CE g^{-1} DW, respectively (Uribe et al. 2016). Cirlini et al. (2016) reported a TPC of 262.97 mg g^{-1} in a dry spearmint (*Mentha spicata*) (Cirlini et al. 2016). Another study reported that TPC and TFC in dried peppermint leaves accounted for 19-23% and 12% of its total weight, respectively (Jeong et al. 2018), as a similar result was observed for ODS, SDS and PDS. In a recent study (Said et al. 2022), it is reported that the TPC in leaves of a *Pelargonium graveolens* dried by shade was higher than that dried in an oven at 60 °C, similarly observed for SDS and ODS.

3.2. Antioxidant capacity

The AC values of the samples are illustrated in Figure 3. PDS exhibited the highest (133.56±2.95 mg TE g^{-1} DW) AC ($p<0.05$), while AC of ODS (93.31±2.04 mg TE g^{-1} DW) and SDS (92.72±2.80 mg TE g^{-1} DW) were not statistically different from each other ($p>0.05$). Similarly, Mokhtarian et al. (2020) found that the AC of peppermint dried in a solar dryer had 26% higher AC compared to peppermint dried by shade (Mokhtarian et al. 2020) Jeong et al. (2018) reported that the DPPH scavenging activity of dried peppermints obtained from different origins were ranged from 63.10 to 93.50 mg TE g^{-1} (Jeong et al. 2018). Lv et al. (2012) found the DPPH scavenging capacity as 233.57 and 394.17 mg TE g^{-1} for conventional and organic dry peppermint, respectively (Lv et al. 2012).

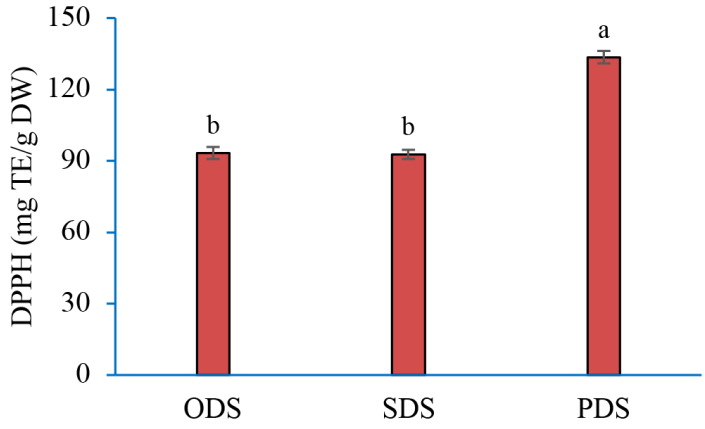

Figure 3- Antioxidant capacity of ODS, SDS and PDS
ODS: Oven dried spearmint, SDS: Shade dried spearmint, PDS: Photovoltaic dried spearmint
a,b Different lowercase letters were significantly different among samples ($p<0.05$)

It was clearly seen that the relationship between the AC values of the dried spearmints exhibited similarity with that of their TPC and TFC values. Polyphenolic compounds are one of the main components responsible for the antioxidant activities of *Mentha* plants (Riachi & De Maria 2015). In fact, there was a high correlation between the AC and both the TPC (Pearson's coefficient=990, $p<0.01$) and TFC (Pearson's coefficient=0.992, $p<0.01$). Uribe et al. (2016) also reported positive correlations between DPPH radical-scavenging capacity and the TPC (r^2= 0.97, $p<0.05$) and TFC (r^2= 0.62, $p<0.05$) of peppermint (Uribe et al. 2016). Moreover, Jeong et al. (2018) also found high correlations for both TPC (Pearson's correlation=0.785, $p<0.5$) and TFC (Pearson's correlation=0.745, $p<0.5$) in a dried peppermint genotype, although these values were lower than those observed for ODS, SDS and PDS.

3.3. Chlorophylls contents

The Chl contents in samples are depicted in Figure 4. The amount of Chl *a*, Chl *b* and the total chlorophyll (Chl *a* + Chl *b*) were significantly affected by the drying method ($p<0.05$). The PDS had the highest level of both Chl *a* (5.94±0.00 mg g^{-1} DW) and Chl *b* (3.93±0.01 mg g^{-1} DW), whereas the ODS had the lowest amount in both Chl *a* (5.01±0.01 mg g^{-1} DW) and Chl *b* (3.52±0.01 mg g^{-1} DW). The lower Chl contents in the oven dried spearmint can be explained by the higher drying temperature, causing more losses in these pigments (Uribe et al. 2016). A similar result was also observed by Yilmaz and Alibas (2022) for rosemary leaves. They found that the total Chl content in the shade-dried rosemary leaves was higher than that in the convective dried at 50 °C. In contrast, Mokhtarian et al. (2020) found that the total Chl content in peppermint dried by a solar dryer was higher than in shade dried peppermints (Mokhtarian et al. 2020) They attributed this difference to shorter drying time (2.5 h) when using solar drying compared to shade (5 h) drying.

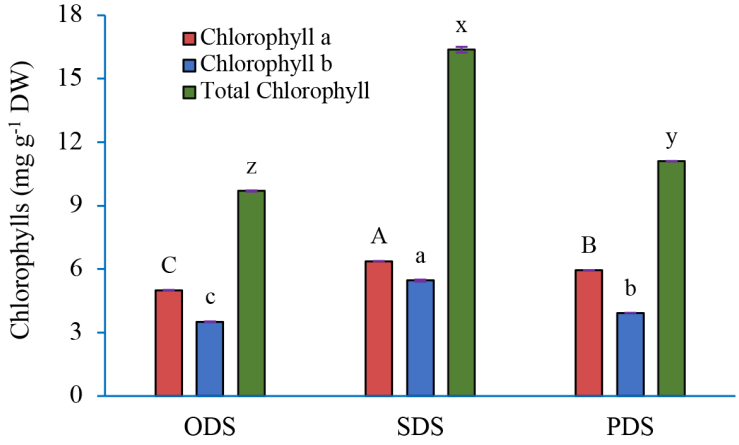

Figure 4- Chlorophylls content in ODS, SDS and PDS
ODS: Oven dried spearmint, SDS: Shade dried spearmint, PDS: Photovoltaic dried spearmint,
A-C, a-c, x-z: Different lowercase, uppercase, and Latin letters were significantly different among samples for Chl a, Chl b, and total chlorophylls contents, respectively ($p<0.05$)

The total Chl amount in dried leaves of *Mentha* types differs particularly depending on variety and the employed drying methods. For instance, Kannan et al. (2021) reported this amount in dried *Mentha arvensis* to range between 88.73-96.80 mg g^{-1} a higher range than the levels in ODS, SDS and PDS. However, Uribe et al. (2016) determined a range of 0.76-0.89 mg g^{-1} DW for dried *Mentha piperita* by vacuum drying at different temperatures (50-90 °C), a lower range than the amount in the three sample types. Another study (Nalawade et al. 2019) reported the total Chl content in a cultivar of fresh spearmint (*Mentha spicata*) leaves to be 4.09 mg g^{-1} DW and that the percentages of retention of this content in samples following different drying methods ranged between 38.97-61.12%.

3.4. Volatile compounds

The identified VC and their relative quantity in the samples are listed in Table 3. In total, 87 VC in the three spearmints were identified and grouped as monoterpenoids (Kannan et al. 2021), sesquiterpenoids (16), triterpenoid (1), aldehydes (9), alcohols (8), ketones (2), esters (9) and miscellaneous (6). The lowest total VC content was obtained from ODS (3056.48±253.45 µg kg^{-1} DW) ($p<0.05$). However, there was no statistically significant difference between the total VC content in SDS (5320.14±299.06 µg kg^{-1} DW) and in PDS (4760.46±240.67 µg kg^{-1} DW) ($p>0.05$).

Terpenoids were observed to be the most abundant group in the samples, both in terms of number (53) and quantity. The total amount of this group was found as 82.59%, 87.76% and 86.45% of the total VC concentration in the ODS, SDS and PDS, respectively. The most of the individual contents of terpenoids were the lowest in ODS ($p<0.05$). This could be due to its higher drying temperature that causes a greater decrease in monoterpenes by oxidation or evaporation (Chua et al. 2019). There were no significant differences between the contents of many compounds of terpenoids in SDS and in PDS ($p>0.05$). In general, the profile of terpenoids (oxigenated monoterpenes, monoterpenes and sesquiterpenes) in the samples of spearmints was similar to that found in previous studies on the common *Mentha* species (Silva & Câmara 2013; Verma et al. 2011).

Carvone, an oxygenated monoterpene, constituted the majority of the total amount of VC in samples, accounting for 47.68%, 45.04% and 39.24% of the total VC content in ODS, SDS and PDS, respectively. SDS contained the highest level of carvone (2.396,17±140.04 µg kg^{-1} DW) ($p<0.05$). This compound is the most responsible for the typical aroma (minty) of the cultivars of spearmint. Carvone has also been identified as the main VC in dried forms and essential oils (Díaz-Maroto et al. 2003) of spearmints (Mokhtarikhah et al. 2020; Nalawade et al. 2019). Conversely, Cirlini et al. (2016) reported that the amount of carvone in an extract of a dry spearmint was lower than that of several other VC. Additionally, Silva and Câmara (2013) have not found carvone in fresh spearmint leaves but did find it in peppermint leaves as the major VC. The other predominant compounds in samples were D-limonene (citrus) and eucalyptol (1,8-cineole) as found by Díaz-Maroto et al. (2003), Da Porto and Decorti (2009). D-Germacrene and (E)-β-bourbonene (woody) were the predominant sesquiterpenes (Da Porto & Decorti 2009; Díaz-Maroto et al. 2003). These compounds also had the highest content of SDS.

In addition to the aforementioned differences in concentrations, the samples varied based on the presence or absence of some terpenoids. For example, monoterpenes such as (D)-α-pinene (minty), menth-2-en-1-ol (herbal) and 4-terpineol (cooling-mentholic) were present only in PDS, while cis-dihydrocarvone (minty), camphol (woody-camphor) and hedycaryol were detected only in SDS.

The majority of aldehydes and alchols detected in samples were lipid derivatives such as (E)-2-hexenal, octanal, nonanal, 1-hexanol, 3-hexenol, 3-octanol, 1-octanol, 3-nonanol and 1-octanol. These compounds are produced from certain polyunsaturated fatty acides by enzymatic pathway (Silva & Câmara 2013). Overall, the content of these derivatives in both SDS and PDS were greater than in ODS ($p<0.05$), most likely due to their lower drying temepratures which were in a suitable range for enzymatic activities. The presence of fatty acides products enhanced the 'green' and 'fresh-herbal' aroma in many spices.

PDS contained a greater amount of esters than the two other samples ($p<0.05$). Carvyl acetate (minty) was detected as the major ester in all samples, but its conents in samples were close to one another ($p>0.05$). Esters of acetic acid such as dihydrocarvyl acetat (floral), 1-ethylhexyl acetate (green) and 1-pentylallyl acetate (green) were found only in PDS, while 2-methylbutyl isovalerate (fruity) was present only in SDS. Miscellaneous volatiles including anethol (licorice), dimethyl sulfide (cabbage-like), heneicosane, (E,Z)-1,3,5-undecatriene showed their highest content in SDS ($p<0.05$). As in the terpenoids, the highest VC in all others groups identified in the samples have also been reported by previous studies on different Mentha species (Chen et al. 2011; Cordero et al. 2012).

Table 3- Volatile compounds (µg kg^{-1} dry weight) in ODS, SDS and PDS

	Compound	*RI*[*]	*Samples*		
			ODS	*SDS*	*PDS*
	Monoterpenoids		2524.49±223.86^{b}	4509.72±250.84^{a}	4115.07±192.44^{a}
1	α-Pinene	1061	20.63±1.03^{b}	54.75±3.97^{a}	55.69±2.07^{a}
2	α-Thujene	1067	1.68±0.10^{b}	4.3±0.22^{a}	4.34±0.34^{a}
3	Camphene	1099	nd	1.07±0.66^{a}	nd
4	2-β-Pinene	1134	35.71±2.00^{c}	77.7±3.62^{b}	95.43±4.57^{a}
5	Sabinene	1147	28.81±1.14^{b}	70.33±4.34^{a}	76.96±8.89^{a}
6	β-Pinene	1188	48.03±7.43^{b}	117.28±5.95^{a}	122.43±3.56^{a}
7	1,8-p-Menthadiene	1191	1.8±0.23^{a}	nd	nd
8	α-Terpinene	1201	0.85±0.05^{b}	2.2±0.06^{b}	6.07±0.90^{a}
9	D-Limonene	1224	415.27±30.98^{b}	900.2±44.91^{a}	934.43±62.45^{a}
10	Eucalyptol	1230	158.48±42.6^{b}	354.44±13.43^{a}	410.63±8.69^{a}
11	1,5,8-p-menthatriene	1237	3.7±0.42^{b}	10.93±1.32^{a}	10.28±1.28^{a}
12	(E)-Ocimene	1255	45.61±4.57^{b}	102.95±9.14^{a}	nd
13	(D)-α-Pinene	1256	nd	nd	106.41±6.16^{a}
14	γ-Terpinen	1264	2.7±0.35^{b}	5.35±0.28^{a}	5.28±0.18^{a}
15	(Z)- β-Ocimene	1271	13.59±0.97^{c}	31.06±1.2^{b}	40.87±2.79^{a}
16	α-Terpinolene	1298	4.28±0.48^{c}	10.49±2.2^{b}	12.93±1.86^{a}
17	α-Pinene epoxide	1372	9.52±1.00^{b}	20.86±3.1^{a}	26.44±0.89^{a}
18	(Z)-Alloocimene	1384	1.73±0.37^{b}	4.78±0.95^{a}	5.08±1.19^{a}
19	Cosmene	1445	nd	6.78±0.69^{a}	nd
20	p-Cymenene	1450	nd	6.8±0.80^{a}	5.94±1.02^{a}
21	Limonene epoxide	1461	7.09±0.28^{a}	13.11±1.05^{a}	12.44±2.39^{a}
22	(E)-Sabinene hydrate	1473	142.06±6.56^{a}	125.11±4.88^{a}	71.1±2.15^{b}
23	β-Linalool	1552	14.62±1.00^{b}	22.57±2.15^{a}	16.22±0.91^{ab}
24	Menth-2-en-1-ol	1555	nd	nd	7.14±0.39^{a}
25	4-Terpineol	1613	nd	nd	11.83±1.04^{a}
26	cis-Dihydrocarvone	1626	nd	40.48±2.97^{a}	nd
27	trans-Dihydrocarvone	1627	18.37±3.37^{b}	nd	57.01±5.44^{a}
28	(E)-p-2,8-Menthadien-1-ol	1636	1.76±0.14^{b}	3.11±0.11^{a}	2.76±0.24^{a}

Table 3- Continued

	Compound	*RI*[*]	*Samples*		
			ODS	*SDS*	*PDS*
29	Pulegone	1666	33.01±2.98[b]	50.11±4.9[ab]	67.48±4.78[a]
30	α-Terpineol	1709	22.81±0.55[b]	33.58±2.07[a]	39.29±1.04[a]
31	Camphol	1714	nd	6.63±0.64[a]	nd
32	Isopinocarveol	1724	nd	3.95±0.47[a]	nd
33	Carvone	1767	1457.36±114.95[b]	2396.17±140.04[a]	1868.44±74.8[b]
34	6-Hydroxycarvone	1820	3.76±0.23[a]	5.43±0.42[a]	5.03±0.51[a]
35	Carveol	1873	23.5±2.2[b]	23.75±0.9[b]	30.11±1.11[a]
36	Piperitenone	1947	2.75±0.13[b]	3.55±0.41[ab]	4.14±0.14[a]
	Sesquiterpenoids		345.19±15.58[b]	519.37±31.78[a]	370.63±21.79[b]
37	α-Copaene	1507	6.33±0.34[b]	10.67±0.91[a]	10.43±0.42[a]
38	(E)-β-Bourbonene	1537	93.17±3.72[b]	131.41±10.15[a]	100.67±6.58[ab]
39	α-Gurjunene	1548	1.77±0.24[b]	3.46±0.14[a]	2.46±0.06[b]
40	β-Ylangene	1591	16.85±2.10[b]	25.68±1.58[a]	nd
41	β-Elemene	1609	2.96±0.17[b]	nd	12.48±1.51[a]
42	Caryophyllene	1619	20.22±1.62[b]	34.85±5.65[a]	15.93±0.81[b]
43	β-Gurjunene	1657	2.46±0.36[a]	nd	nd
44	Alloaromadendrene	1671	2.38±0.21[b]	3.85±0.42[a]	nd
45	Hedycaryol	1680	nd	36.97±3.98[a]	nd
46	Elemol	1681	27.1±2.91[a]	nd	39.26±3.94[a]
47	γ-Muurolene	1691	4.7±0.69[b]	9.72±0.60[a]	nd
48	Bicyclosesquiphellandrene	1699	26.5±2.07[b]	44.52±3.27[a]	28.08±3.03[b]
49	D-Germacrene	1743	127.09±5.38[b]	190.85±8.01[a]	144.28±3.86[b]
50	β-Cadinene	1777	nd	6.98±1.43[a]	nd
51	Calamenene	1855	13.67±2.37[b]	19.40±0.80[a]	14.06±1.06[b]
52	Viridiflorol	2102	nd	nd	2.986±0.55[a]
	Triterpenoids		16.17±2.32[a]	nd	5.25±0.49[b]
53	Squalene	2148	16.17±2.32[a]	nd	5.25±.49[b]
	Aldehydes		38.52±4.84[b]	77.64±4.18[a]	49.40±2.59[b]
54	Acetaldehyde	710	1.97±0.07[b]	3.134±0.02[a]	1.28±0.08[c]
55	2-Methylpropanal	898	2.24±0.10[b]	5.31±0.02[a]	1.84±0.09[b]
56	2-Methylbutanal	930	3.99±0.10[b]	7.23±0.22[a]	2.35±0.25[c]
57	3-Methylbutanal	945	4.77±1.06[b]	8.46±0.42[a]	3.21±0.20[b]
58	(E)-2-Hexenal	1241	10.61±1.48[c]	32.44±2.11[a]	18.14±1.19[b]
59	Octanal	1305	1.85±0.08[c]	2.53±0.03[b]	5.50±0.05[a]
60	Nonanal	1406	1.39±0.31[b]	nd	4.53±0.43[a]
61	Phenylmethanal	1541	3.17±0.62[b]	5.69±0.05[a]	2.13±0.05[b]
62	Perilla aldehyde	1810	8.351±1.11[b]	12.85±1.15[a]	10.44±0.45[ab]
	Alcohols		58.62±1.72[b]	91.87±5.14[ab]	109.46±13.32[a]
63	1-Penten-3-ol	1184	0.63±0.03[a]	nd	nd
64	3-methylbutanol	1226	nd	2.52±0.07[a]	nd
65	1-Hexanol	1362	0.95±0.05[b]	2.41±0.15[a]	2.26±0.16[a]
66	3-Hexenol	1392	1.91±0.03[b]	2.99±0.18[a]	nd
67	3-Octanol	1399	37.14±1.09[b]	48.25±4.65[b]	80.10±10.92[a]

Table 3- Continued

	Compound	*RI*[*]	*Samples*		
			ODS	*SDS*	*PDS*
68	3-Octenol	1457	13.55±0.41[c]	28.30±0.31[a]	19.56±1.70[b]
69	3-Nonanol	1496	0.96±0.05[c]	2.28±0.13[b]	3.46±0.33[a]
70	1-Octanol	1560	3.48±0.15[b]	5.17±0.13[a]	4.09±0.21[b]
	Ketones		3.26±0.74[b]	5.51±0.45[a]	5.62±0.47[ab]
71	3-Octanone	1273	nd	1.00±0.03[b]	1.99±0.09[a]
72	Jasmone	1962	3.26±0.74[b]	6.50±0.45[a]	3.63±0.37[b]
	Esters		40.78±2.08[b]	40.06±3.10[b]	68.34±7.28[a]
73	2-Methylbutyl 2-methylbutyrate	1295	0.89±0.10[b]	nd	2.14±0.03[a]
74	Isopentyl isovalerate	1296	nd	nd	2.74±0.46[a]
75	2-Methylbutyl isovalerate	1313	nd	1.55±0.41[a]	nd
76	1-Ethylhexyl acetate	1352	nd	nd	10.43±1.57[a]
77	3-Octanyl acetate	1352	4.31±0.08[b]	7.24±0.65[a]	nd
78	1-Pentylallyl acetate	1392	nd	nd	3.77±0.08[a]
79	(Z)-3-Hexenyl valerate	1498	3.31±0.16[b]	nd	7.89±0.75[a]
80	Dihydrocarvyl acetate	1692	nd	nd	11.11±1.45[a]
81	Carvyl acetate	1786	32.27±1.96[a]	31.27±2.05[a]	30.25±3.05[a]
	Miscellaneous		29.47±2.30[b]	74.97±3.57[a]	36.70±2.26[b]
82	Dimethyl sulfide	765	5.03±0.19[b]	10.04±1.01[a]	3.43±0.05[b]
83	Heneicosane	1260	nd	10.63±0.83[a]	5.67±0.64[b]
84	Phytane	1308	0.99±0.08[b]	1.87±0.09[a]	nd
85	(E, Z)-1,3,5-Undecatriene	1403	7.93±0.51[b]	14.46±1.06[a]	10.59±0.36[b]
86	Estragole	1685	nd	3.71±1.20[a]	nd
87	Anethol	1843	15.52±1.97[b]	34.66±3.20[a]	17.00±1.20[b]
	Total		3056.48±253.45[b]	5320.14±299.0[6]a	4760.46±240.6[7]a

[*]Calculated retention indices on DB-HeavyWax column. ODS: Oven dried spearmint, SDS: Shade dried spearmint, PDS: Photovoltaic dried spearmint, nd: not detected, [a-c]Different lowercase letters in the same row indicate significant difference among samples

3.5. Principal component analysis

The differences and similarities between samples were also evaluated by PCA regarding their VC contents (Figure 5.). 97.34% of the total variance in the data set listed in Table 1 can be explained by the two first principal components. The first component (PC1) accounted for 51.77% of the total variance, while the second component (PC2) accounted for 45.57%. As depicted in Figure 5a, ODS was characterized by 1,8-p-menthadiene, β-gurjunene, squalene and 1-penten-3-ol, while PDS was primarily characterized by (D)-α-pinene, viridiflorol, (Z)- β-ocimene, trans-dihydrocarvone, carveol, 4-terpineol, 3-octanol, 3-nonanol, octanal, 2-methylbutyl 2-methylbutyrate. On the other hand, SDS was associated with greater diversity in VC, some of which were carvone, D-germacrene, cis-dihydrocarvone, cosmene, caryophyllene, 2-methylbutanal, 3-methylbutanal 3-octenol, (E)-ocimene, α-gurjunene, phenylmethanal, dimethyl sulfide and camphol. As shown in the score plot (Figure 5b), SDS was separated from the other samples by PC1, whereas PDS was separated from ODS by PC2. PCA revealed an unequivocal discrimination between the profiles of VC in the spearmints obtained using the different drying methods.

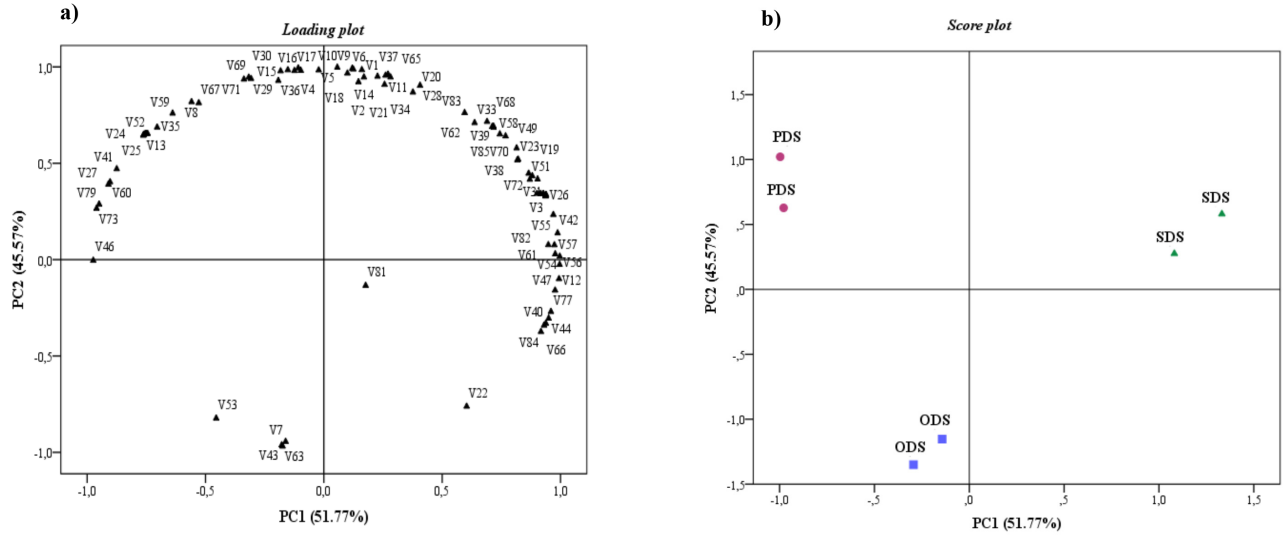

Figure 5- Loading plot (a) and score plot (b) in rotated space of the two first components of PCA for ODS, SDS and PDS samples. V: Volatile compounds codes were numbered from 1 to 87 as given in Table 3; ODS: Oven dried spearmint, SDS: Shade dried spearmint, PDS: Photovoltaic dried spearmint

4. Conclusions

In this study, a new type of dryer was developed. The mints dried in the developed dryer were compared with the products dried by the shade and oven drying methods. The products were compared in terms of consumed energy and food quality. Due to the usage of PVTD in the experiments conducted in outdoor weather conditions, the drying time was reduced by a factor of 6 compared to shade drying. The electrical (halogen lamp and fan) and thermal (heating of the drying air) energy required for the system were obtained from PVT. The sustainability of the system is ensured by storing excess energy.

The spearmint dried by PVTD had the highest TPC, TFC and AC, whereas the differences between these features of the shade-dried and oven-dried spearmints were not significant. Additionally, it was determined that the Chl content as a color agent was the highest in the shade-dried spearmint.

The VC compositions of all three samples were determined to be different from each other. The results show that the concentration of VCs representing the characteristic aroma in the shade-dried spearmint were greater compared to those in others. It was also found that the amounts of these typical VCs in the spearmint dried by shade were the lowest.

This study showed the PVTD can preserve the spice quality of spearmint.

Future studies should focus on the properties of other foods dried by PVTD in a variety of climatic conditions.

Data availability: Data are available on request due to privacy or other restrictions.

Authorship Contributions: Concept: E.A., M.K., Design: E.A., Data Collection or Processing: E.A., Analysis or Interpretation: A.K., Literature Search: A.K., M.K., Writing: A.K., E.A., M.K.

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Diagnosis of Tomato Plant Diseases Using Pre-trained Architectures and A Proposed Convolutional Neural Network Model

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ABSTRACT

Tomatoes are of the most important vegetables in the world. Presence of diseases and pests in the growing area significantly affect the choice of variety in tomato. The aim of this study is to diagnose tomato plant diseases faster and with higher degrees of accuracy. For this purpose, deep learning was used to diagnose some diseases in tomatoes, including bacterial spot, early blight, leaf mold, septoria leaf spot, target spot, mosaic virus, and yellow leaf curl virus were analyzed CNN models. A CNN model with a 2D convolutional three layers, one flatten layer approach and several Keras models, including DenseNet201, InceptionResNetV2, MobileNet, Visual Geometry Group 16 architectures were proposed. The experimental results showed that the accuracy scores were 99.82%, 92.12%, 92.75%, 91.50% and 84.12% training accuracy, respectively. The proposed CNN model provided the opportunity for rapid diagnosis for approximately 14.9 minutes. The results obtained in this study can be used in robotic spraying and harvesting operations.

Keywords: Automated diagnose, CNN modification, Deep learning, Smart farming

1. Introduction

Tomatoes are among the most widely produced and consumed foods in the world (*Solanum lycopersicum* L.). According to Turkish Statistical Institute (TSI) 2021 data, 13,095,258 tons of tomatoes were produced (TSI 2022) and they form an important agricultural product in Turkey (FAO 2019).

The cultivation of tomatoes is commonly carried out under greenhouse and in field conditions. In order to increase efficiency in production, many parameters such as climate conditions, diseases and pests should be taken into consideration. Tomato plant diseases can be divided into two groups according to their factors, physiological and pathogens. Physiological factors are typically caused by climatic conditions such as excessive irrigation and malnutrition, sunburn, cracking in fruits, and rot. Viral diseases cause damage such as black spots, brown spots, curl and deformity on the leaves. Early diagnosis of plant diseases is critical in preventing enormous economic and agricultural loss. Every disease in tomato manifests itself in different ways. Tomato early blight disease (*Alternaria solani*) occurs as spots on the leaves, stems and fruits that can be seen in every phase of the plant. Tomato mildew (*Phytophthora infestans*) disease starts as pale green spots which later turn brown and then black. Tomato leaf mold (*Cladosporium fulvum* = *Fulvia fulva*) is seen as yellow spots on the leaves, and brown mold occurs in the lower parts of these spots in the later stages (Griffiths et al. 2018). Viruses in tomato plants can cause diseases (Nitzany, 1960), one of which is the *Tomato mosaic virus* disease (ToMV) the symptoms of which manifest as light green and yellow irregular mosaic spots. The misshapen fruit causes damages such as the formation of smaller fruit than normal. Tomato yellow leaf curl virus disease, on the other hand, manifests itself in the form of shrinkage and swelling in the infected leaves, inward curving, stunting and deformity in the plant (Sade et al. 2020). According to Richard et al. (2017), the bacterial spot disease (*Xanthomonas vesicatoria*) can affect any part of the plant, including the stem, leaf, and fruit. The symptoms of this disease (*Pseudomonas syringae* pv. tomato) include the forming of stains on all above-ground organs of the plant (Abramovitch et al. 2006).

This symptom begins in the seedling period, and many brown-black spots appear on the leaves and stems of the seedlings. These spots cause drying of the entire seedling over time. Various methods are used to combat these diseases and include cultural measures if the disease is detected early, and chemical methods, in other words pesticides, are applied in the future. The early detection of diseases and determining and distinguishing exactly what factor caused the disease is critical in order to prevent devastation to large numbers of crops. Rapid diagnosis is crucial in preventing serious economic losses for tomato cultivators.

A wide variety of techniques are used for disease diagnosis and classification in tomatoes. Deep learning is one such technique and is based on Artificial Neural Networks, using multiple layers of neurons to extract attributes from raw data, and is designed to simulate the working mechanism of the human brain. Although deep learning, also known as deep neural networks or hierarchical learning, emerged in 2006 as a new field of machine learning, its foundations date back to 1940 (Deng & Yu 2014).

Diagnosing plant diseases via deep learning is popular diagnostic method for many researchers. A higher disease diagnosis rate can be obtained if this dataset is used by comparing it with images from the real environment. A further way to increase the success rate in disease diagnosis is to use hyperspectral/multispectral imaging techniques in deep learning studies. In particular, the diagnosis of diseases in their early stages is possible through the use of these technologies. Disease detection in the early stages means less pesticide application, greater economic gain, and less environmental pollution. In recent years, a number of algorithms have been used to determine plant diseases through the deep learning method (Dhakal & Shakya 2018). Ferentinos (2018) used a database consisting of 87,848 photographs taken in different conditions, both in the laboratory environment and in the field. In the database created, 58 diseases belonging to 25 plant species were examined. The trials highlighted that the VGG CNN model architecture, with a success rate of 99.53%, had the highest classification success. Another study in which the VGG19 model classified with the highest accuracy with a rate of 97.86% was carried out by Turkoglu & Hanbay (2019). Studies on real-time detection of plant diseases and the development of appropriate automation systems have made significant progress in recent years.

Some of the deep learning architectures currently in use have been successfully trained to accurately identify diseases. It is difficult to keep its generalisability because high accuracy must be addressed to the output, which may differ when implemented to real items (PC, tablet, mobile phone, various camera systems, etc.). The goal of this research is to develop a simpler convolutional neural network for diagnosing tomato plant diseases. Various deep learning models [Visual Geometry Group 16 (VGG16), MobileNet, DenseNet, Inception-ResNet] and proposed methods were investigated for this purpose. The proposed model's results were also compared to existing state-of-the-art methods.

2. Material and Methods

2.1. Dataset

In recent years, it is possible to access many data sets created by experts. In this study, the widely used PlantVillage augmented data set was used. PlantVillage augmented dataset (https://www.kaggle.com/vipoooool/new-plant-diseases-dataset) which contains 38 class of vegetable and fruit diseases (Hughes & Salathe 2015; Geetharamani & Pandian 2019). In total, 18,440 images of 7 types of diseased (bacterial spot, early blight, leaf mold, septoria leaf spot, target spot, mosaic virus, yellow leaf curl virus) and healthy leaves were selected for trials (Figure 1).

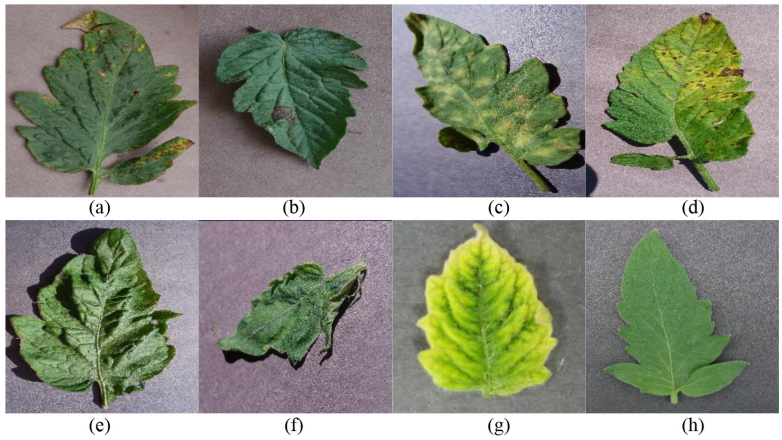

Figure 1- Bacterial spot (a), Early blight (b), Leaf mold (c), Septoria leaf spot (d), Target spot (e), Mosaic virus (f), Yellow leaf curl virus (g) and healthy (h)

This study's train and test image numbers of diseased and healthy leaves are provided in Table 1.

Table 1- Training and test dataset

Diseases	*Training images*	*Test images*
Bacterial spot	1702	425
Early blight	1920	480
Leaf mold	1882	470
Septoria leaf spot	1745	436
Target spot	1827	457
Mosaic virus	1790	448
Yellow leaf curl virus	1961	490
Healthy	1926	481

2.2. Convolutional neural network based models

Deep learning is a sub-field of machine learning and has been used in many areas in recent years. In today's engineering applications, the emphasis is on finding solutions for object recognition problems that require complex solutions. CNN is the most commonly used deep learning algorithm for object recognition applications. Generally, the basic architecture of the deep learning concept is accepted as CNN. The CNN architecture consists of convolution, pooling, fully connected, dropout and classification layers. In the convolution layer, the filtering-size reduction process is generally performed. A smaller size filter is determined for the existing object and the filtering process is performed by subjecting it to certain processes. This process continues until the system is trained. Like the convolutional layer, the pooling layer is also used for dimension reduction. In this way, the focus is on more important features. There are two different pooling techniques commonly used in CNN models, maximum and average pooling. Fully connected layers are often found towards the end of the CNN architecture and can be used to optimize goals such as classification scores. Dropout will improve learning performance by removing some connections within the network. In the fully connected layer, the visual that passes through the convolution and pooling layer and is in matrix form is transformed into a vector. After receiving the CNN input data, the training process begins and at the end of this training, it produces a final output to make a comparison with the correct result (Lawrence et al. 1997; Krizhevsky et al. 2017; LeCun et al. 2015).

In the study, pre-trained VGG16, MobileNet, DenseNet, Inception-ResNet models and a proposed CNN model were used to diagnose tomato plant diseases.

2.2.1. VGG16

VGG16, previous AlexNet (Krizhevsky et al. 2017) derivatives, focuses on smaller sizes and steps in the first convolution layer. Although VGG16 deep learning architecture provides a higher accuracy performance than AlexNet, because of the number of parameters it uses a large amount of memory. On the other hand, smaller filters were used compared to AlexNet. This architecture uses fixed 3x3 dimensional filters with variable numbers of 64, 128, and 256 filters in all convolution layers. VGG16 is a simple CNN model with GPU support that achieved 89% success in the ImageNet Large Scale Visual Recognition Challenge competition in 2014 (Simonyan & Zisserman 2014). The double or triple convolution layers are followed by the communing layers. On the input layer, the image is 224 x 224 x 3 pixels. The last layer is the classification layer. The architectural structure of VGG16 is given in Figure 2. This model consists of a total of 16 layers with 13 convolution layers, 3 fully connected layers, pooling, Relu, Dropout and Softmax layers.

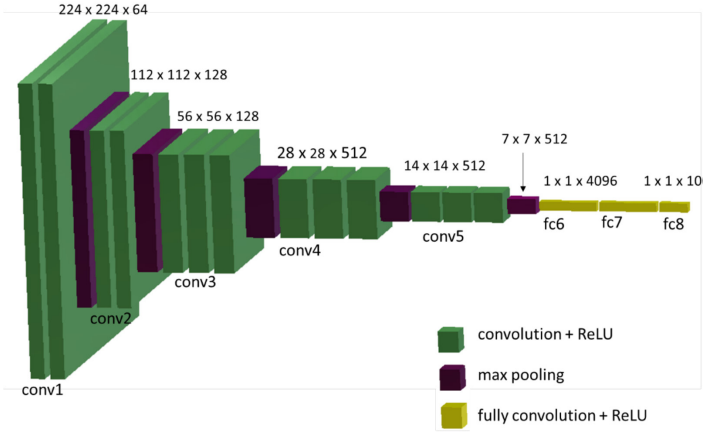

Figure 2- VGG16 architecture (Simonyan & Zisserman 2014)

2.2.2. MobileNet

MobileNet (Howard et al. 2017) has been developed to run vision applications on embedded and mobile platforms. The algorithm is based on depth-wise separable convolutional layers; these deeply separable layers are 1 x 1 convolutional layers that separate layers and are called point convolutions (Figure 3). Standard convolution filters the inputs and combines them into a new output in one step. The deeply separable convolutional layer, on the other hand, is composed of two sub-layers as in-depth convolution and point convolution. For both layers, batchnorm and ReLU non-linearities were used. Exception of the final fully connected layer, 3x3 convolution layer was followed by the batch norm and ReLU (Howard et al. 2017).

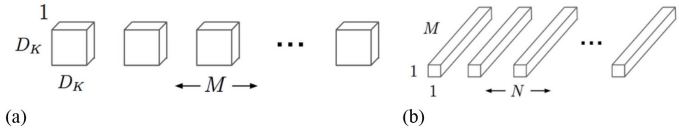

Figure 3- Depthwise convolution (a), pointwise convolution (b) (Howard et al. 2017)

2.2.3. DenseNet

Densely Connected Convolutional Networks (DenseNet) forward connects each layer to the other layers. In DenseNet architecture, each layer uses the properties of all previous layers as input and its own properties in the layer are given as input to the next layers. The advantage of DenseNet architectures is that they allow feature propagation and feature reuse, thereby reducing the number of parameters. One of the advantages of DenseNet is that the model parameters are smaller than those of ResNet. The architecture consists of dense blocks, composite function, pooling layers, bottleneck layers and compression (Figure 4) (Huang et al. 2017). This study used DenseNet201 architecture.

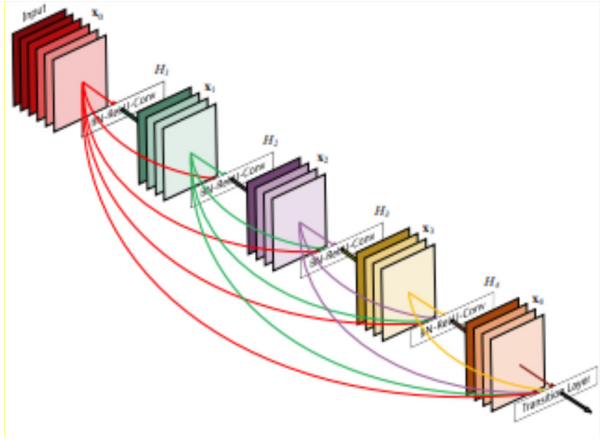

Figure 4- DenseNet architecture (Huang et al. 2017)

2.2.4. Inception-ResNet

Inception-ResNet, the structure s created by the parallel realization of 1×1, 3×3, 5×5 filters and 3×3 maximum joint operation in the convolution layers of Inception modules. Due to parallel operations, the size of the output and the number of parameters increase in complexity. To overcome this problem, 1×1 convolution layers are added before the parallel Naive Inception convolution layers to achieve size reduction. The pooling of feature is the process of centering on attributes. This is similar to the process of reducing the width and height dimensions in a normal maximum joint operation. In addition, the 1×1 convolution layers are followed by the ReLU process as the activation function. The residual layer was optimized by changing the size of the first convolution process to 1×1. ReLU is a structure based on taking a direct sum from the activation function output and transferring the previous activation value to the output even under conditions where learning stops at the convolution outputs.

2.3. Proposed CNN model

The CNN model consists of 4 convolution layers, MaxPooling, Batch Normalization, 2 Dense layers, and Dropout. While ReLU activation was used for each CNN output estimate, Softmax activation was used for the output of the final sequential estimation model sum. Softmax is usually used as the activation code at the end of the network; it is used in the process of label estimation on the data submitted to the trained network, after all stages are completed. Softmax makes it more effective and accurate in multiple classification studies (Tang 2013). The mathematical model for Softmax is presented in equations 1, 2, and 3.

$$a_i = \sum h_k W_{ki}, \quad (1)$$

$$p_i = \frac{\exp(a_i)}{\sum_j^{10} \exp(a_j)} \quad (2)$$

h: be the activation of the penultimate layer nodes

W: the weight

given by a, is

The predicted class î would be

$$\hat{\imath} = \arg\max p_i$$
$$\hat{\imath} = \arg\max a_i \quad (3)$$

The Adam model and a Keras optimizer was used with a learning rate of 0.0001. ReduceLROnPlateau and EarlyStopping have served to terminate the training when the model reach the learning epoch. In order to obtain a view of the statistics of the model, to monitor the metrics and to record them periodically at various stages of the training. The purpose of the training pause aims to minimize the loss. Certain parameters need to be adjusted to minimum loss such as monitor, patience, and verbose. When training is paused during periods of no improvement, the final epoch before the accuracy rate begins to decline is displayed. The parameters related to the model are summarized in Table 2.

Table 2- Proposed CNN model parameters

1. Conv2D layer	***2. Conv2D layer***	***3. Conv2D layer***	***Flatten layer***
Output shape	Output shape	Output shape	Output shape
62, 62, 128	29, 29, 256	12, 12, 512	18,432
MaxPooling2D	MaxPooling2D	MaxPooling2D	1. Dense and Dropout
31, 31, 128	14, 14, 256	6, 6, 512	512
Batch normalization	Batch normalization	Batch normalization	2. Dense and Dropout
31, 31, 128	14, 14, 256	6, 6, 512	256
Activation function	Activation function	Activation function	Activation function
ReLu	ReLu	ReLu	Softmax
Total	Total parameters: 3,877,000	Trainable parameters: 3,875,208	Non-trainable parameters: 1,792

In the first convolution layer, the input level is 128x128x3, in the second convolution layer is 256x256x3, and in the third convolution layer is 512x512x3. The flatten layer was composed of two dense layers and a dropout. By the end of the model summary, the total parameters were 3,877,000, with 3,875,208 trainable parameters and 1,792 non-trainable parameters.

2.4. Training-testing data and model evaluation

This study used a 80%:20% partition ratio on the models. Eighty percent of the dataset was divided up into training sets. While 20% of the dataset was partitioned as test set for model verification. For this purpose, 14,753 images were used for training and 3,687 images were used for testing.

In order to evaluate the performance of the outputs, accuracy of true positive, false positive, false negative, and true negative were used. These values evaluate the performance of the classification model including the Accuracy, Precision, Recall and F1-score. True positive correctly predicts the positive class in the model. Likewise, true negative correctly predicts the negative class. False positive incorrectly predicts the positive class in the model while false negative incorrectly predicts the negative class. Accuracy is used to measure the success of the model using ratio of true prediction in all samples. Precision measures the percentage of outcomes correctly classified. Recall measures the proportion of actual positives that are identified correctly. F1-score value shows the harmonic average of Precision and Recall values (Klinkman et al. 1998; Cinar & Koklu 2022). The formulas for the metrics used are given below.

$$\text{Accuracy} = \frac{TP}{\text{TP+TN+FP+FN}} \quad (4)$$

$$Precision = \frac{TP}{TP+FP} \quad (5)$$

$$Recall = \frac{TP}{TP+FN} \quad (6)$$

$$F1 = 2 * \frac{Precision*Recall}{Precision+Recall} \quad (7)$$

There have been chosen particular parameters for the proposed CNN model (Table 3).

Table 3- CNN training parameters

Parameter	***Value***
Batch size	32
Epoch	25
Momentum	0.9
Learning rate	0.0001
Metric	Categorical cross entropy
Patience*	2
Factor*	0.2
Verbose*	2
Optimization method*	Adam

*Specific parameters for Custom CNN model

2.5. Software

The codes were written using Python and were run on Google Colab. The computer used had a Windows 10 operating system, Intel® Core™ i7-10750H CPU 16 GB RAM with NVIDIA GeForce RTX 2060 graphics processor. The model used in the study was compiled with GPU support as TensorFlow 2.4.1 is compatible with Python 3.8.7, CUDA 11.1.0, CuDNN (deep neural networks library) 11.2.

TensorFlow as a platform was chosen because it has pre-trained models and a high-level neural network application programming interface integrated with Keras in versions 2.0 and above. Keras is an effective application programming interface for creating and training deep learning models (Gulli et al. 2019).

NumPy, SciPy, Pandas, Matplotlib, Scikit-learn, Flask, and Cython libraries were used for this study. Numpy, the open-source library of the Python programming language, provides data blocks that implement multidimensional mathematical vectors and matrices along with useful functions for mathematical operations. With Numpy, large volumes of data can be processed and advanced mathematical

operations can be performed quickly and with less code. SciPy functions are created in the Numpy library. SciPy was selected for this study as it increases data processing capabilities. Pandas library was chosen as it performs fast mathematical operations and allows the processing of different types of complex data tables through its special data structures. Matplotlib was used for data visualization. Scikit-learn, one of the best-known machine learning libraries, can be used as an independent machine learning library and can successfully create various machine learning models. SciKit (SciPy Toolkit) is an extension developed separately for SciPy. It has been used to create machine learning models as well as providing efficient tools such as data preprocessing, supervised and unsupervised learning, validation and metrics (El-Amir & Hamdy 2020).

3. Results

Table 4 shows the results of comparing the proposed CNN model to other models to confirm the results. The sequential new model was built with various hyperparameters (Momentum, Learning rate, patience, verbose etc.). It was calculated using the tomato disease dataset. The results were obtained in a short period of time and were more efficient than other CNN models in multi-classification tasks. In terms of total parameter size and time, the method has provided an advantage in terms of obtaining fast and highly accurate results.

High accuracy rates were achieved in the disease classification study performed with 4 different pre-trained models and a proposed deep CNN approach method. The models were successful, but pre-trained models achieved similar results with previous studies. The result of experimental studies consisting of 25 iterations is shown. In this experimental study, training success is calculated as 99.82% at the highest level and a rapid running time obtained as 14.88 minutes. The performance of the proposed CNN model was compared with Keras pre-trained weight architectures such as MobileNet, DenseNet201, VGG16 and InceptionResNetV2 in Table 4. The proposed model diagnosed faster than from VGG16, MobileNet, DenseNet201, InceptionResNetV2 approximately 6, 3, 23, 11 times, respectively.

Table 4- Accuracy of CNN models

Models		***Training***		***Testing***		***Time***
Architectures	Input size	Loss	Accuracy	Loss	Accuracy	Minutes
VGG16	224, 224, 3	0.2239	0.9212	0.1835	0.9388	87.06
MobileNet	224, 224, 3	0.2135	0.9275	0.1901	0.9350	42.18
DenseNet201	224, 224, 3	0.2560	0.9150	0.1729	0.9434	339.72
InceptionResNetV2	299, 299, 3	0.4629	0.8412	0.4147	0.8569	162
Proposed CNN model	256, 256, 3	0.0084	0.9982	0.0587	0.9826	14.88

Among the performance criteria, the largest training and testing loss was seen in the InceptionResNetV2 model. The lowest losses were seen in the proposed CNN model. DenseNet201 was the slowest algorithm in terms of training and testing time. Following the proposed CNN model, DenseNet201 and VGG16 algorithms have high accuracy.

The performance metrics (Precision, Recall and F1-score) of the proposed CNN model is given in Table 5.

Table 5- The performance metrics of the proposed CNN model

	Precision	***Recal***	***F1-score***	***Accuracy***
Bacterial spot	1.00	0.99	0.99	99.78
Early blight	0.95	0.98	0.97	99.1
Leaf mold	0.98	1.00	0.99	99.7
Septoria leaf spot	0.99	0.94	0.96	99.19
Target spot	0.98	0.96	0.97	99.27
Yellow leaf curl virus	1.00	1.00	1.00	99.95
Mosaic virus	1.00	1.00	1.00	99.92
Healty	0.98	1.00	0.99	99.67

When Table 4 is examined, the model with the lowest loss in the testing was the DenseNet201 architecture with a value of 0.1729 fallowing the proposed CNN model. In addition, the DenseNet201 proved to be the most successful architecture with the least loss based on the results of the training. InceptionResNetV2 was found to be the architecture with the highest loss. The plot accuracy-epochs for the models used in the experiments are given in Figure 5.

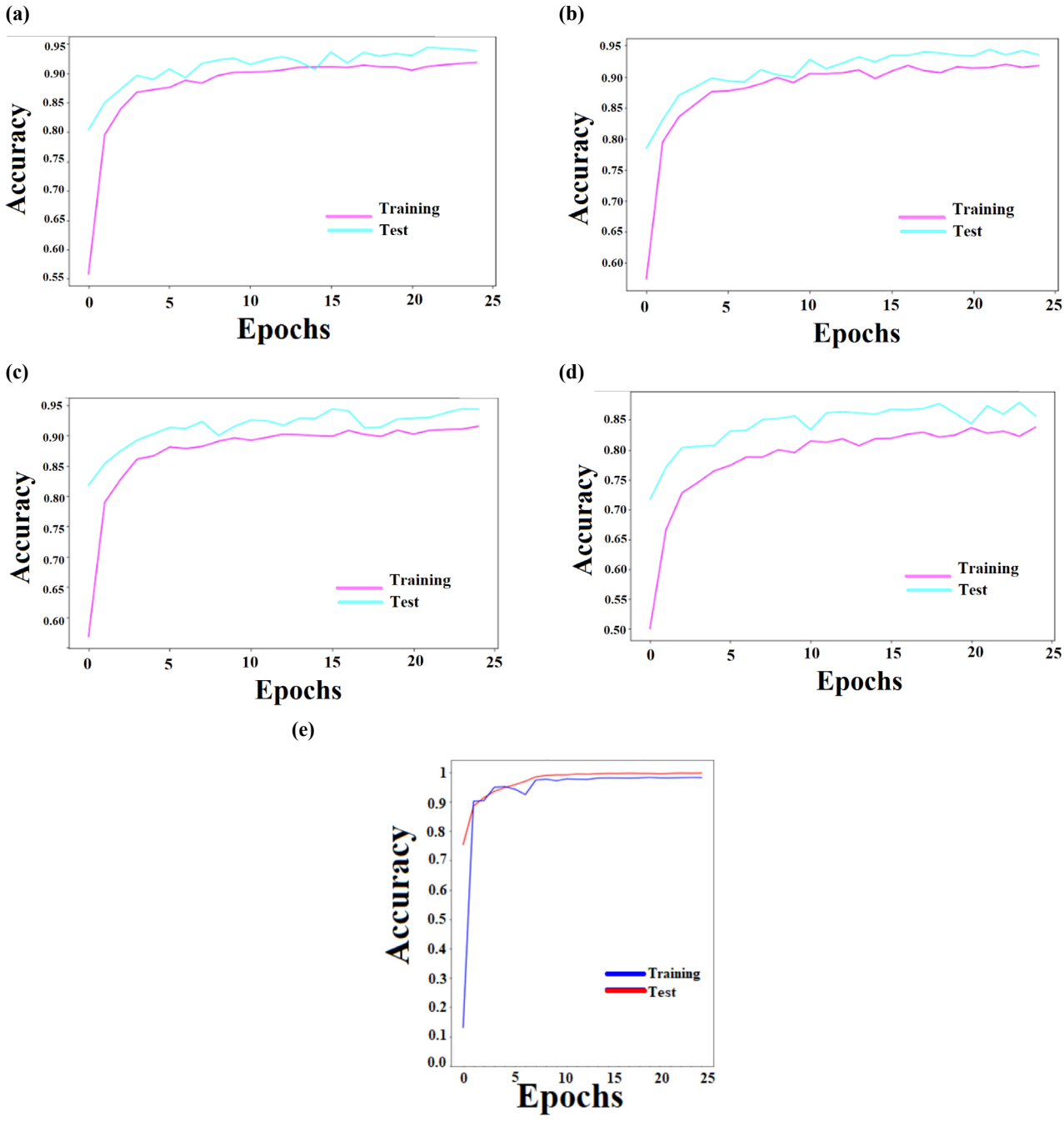

Figure 5- Plot line of train and test loss and accuracies (a) VGG16, (b) MobileNet, (c) DenseNet201, (d) InceptionResNetV2, (e) proposed CNN model

The accuracy and loss graphs of the proposed CNN model are given in Figure 5. The iteration value in the experiments was determined as 25 epochs. However, due to the callbacks, the training was completed in the 24th epoch, with an accuracy of 99.82% (Figure 5). When this result is compared with other studies in the literature, the model used has made a classification with a higher accuracy. The confusion matrix graph obtained for the CNN model is given in Figure 6. As shown in the confusion matrix, the diagnosis of the disease was found with high degrees of accuracy from the images tested. Incorrectly classified images may be the result of early symptoms of selected disease types which have a tendency to show similar symptoms (Blancard 2012).

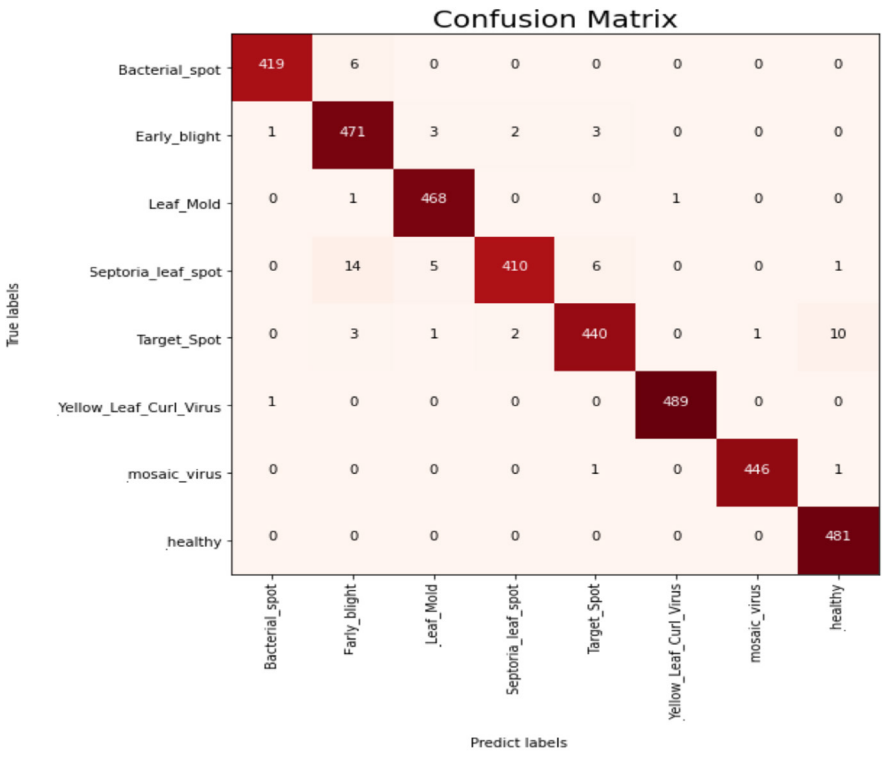

Figure 6- Confusion Matrix graph of the CNN model

Any misreading in the confusion matrix were shown in Figure 7. Similar symptoms observed in the leaf during the early stage are thought to be the cause of this. According to Figure 7, when early blight disease is predicted, it resembles bacterial spot disease about 82% of the time, while in another image of the same disease, it resembles leaf mold 58% of the time and 35% of the time. Target spot disease is thought to resemble Septoria leaf spot 88% of the time while resembling mosaic virus 17% of the time. The model discovered that the disease appeared to resemble Leaf mold in another image with the same condition 76% of the time. Early blight, target spot, leaf mold, Septoria leaf spot, and early period diseases of the mosaic virus are diseases with inaccurate predictions. This is believed to be the result of the similar symptoms that were highlighted above and displayed on the leaf in the early period.

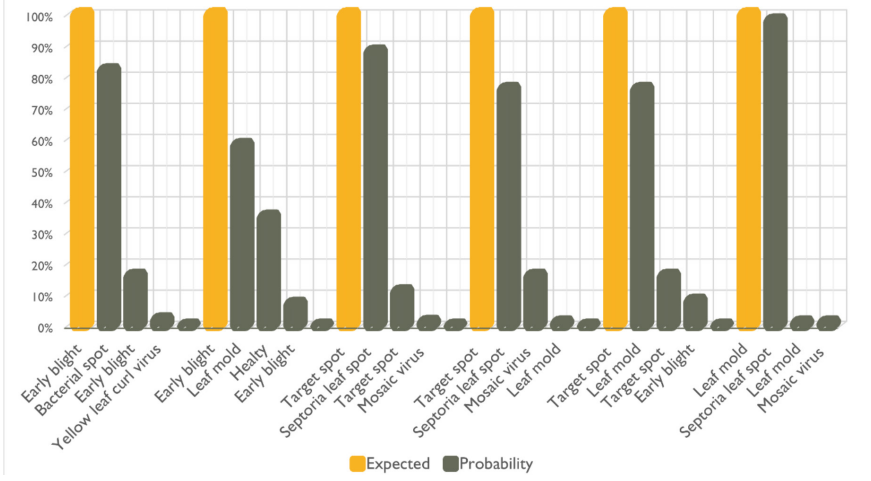

Figure 7- Wrongly predicted diseases

4. Discussion

Several studies have been conducted to improve success rates in the classification and diagnosis of fruit and vegetable diseases that affect fruits and vegetables. In Table 6, only studies related to the determination of tomato diseases are given. Table 6 also gives the number of images in the data set, the diseases that were diagnosed, the models used and their accuracy rates.

Table 6- Comparison of accuracy rates of tomato diseases using deep learning

Authour/ authors	*Model architecture*	*Images*	*Disease/diseases*	*Accuracy*
Brahimi et al. (2017)	AlexNet and GoogleNet	14.828	Yellow leaf curl virus, tomato mosaic virus, target spot, spider mites, septoria spot, leaf mold, lateblight, earlyblight, bacterial spot	98.66%, **99.18%**
Fuentes et al. (2017)	Faster R-CNN with VGG-16, ResNet-50, ResNeXt-50, R-FCN (ResNet-50 as feature extractor)	5.000	Gray mold,canker, leaf mold, plague, leaf miner, whitefly, low temperature, nutritional excess or deficiency, powdery mildew.	83%, 75.37%, 71.1%, **85.98%**
Rangarajan et al. (2018)	AlexNet and VGG16	13.262	Late blight, leaf mold, two-spotted spider mite attack, target spot, mosaic virus disease, yellow leaf curl virus disease	**97.49%**, 97.23%
Tm et al. (2018)	AlexNet, GoogleNet and LeNet	18.160	Septoria leaf spot, yellow leaf curl	A highest validation accuracy of **94.8%** (with 30 epochs) a high **99.3%** of training accuracy was obtained LeNet)
Zhang et al. (2018)	AlexNet (SGD-Adam), GoogLeNet (SGD-Adam), and ResNet (SGD-Adam)	1000	Corynespora leaf spot disease, early blight, late blight, leaf mold disease, septoria leaf spot, two-spotted spider mite, virus disease, yellow leaf curl disease	95.83%, 13.86%, 95.66%, 94.06%, **96.51%**, 94.39%
Wang et al. (2019)	Faster R-CNN (VGG-16 ResNet-50 ResNet-101) and Mask R-CNN (MobileNet ResNet-50 ResNet-101)	286	Malformed fruit, blotchy ripening, puffy fruit, dehiscent fruit, blossom-end rot, sunscald, virus disease, gray mold, ulcer disease, anthracnose)	86.09%, 88.41%, 88.53%, 88.39%, 98.52%, **99.64%**
Mkonyi et al. (2020)	VGG16, VGG19, and ResNet50	2.145	Tuta absoluta	**91.9%**
Verma et al. (2020)	AlexNet, SqueezeNet, Inception V3	2342	Tomato lLate blight (Early, middle and end stage)	90.43%, **93.40%**, 90.76%
Zhang et al. (2020)	Faster RCNN, Faster RCNN-mobile, Faster RCNN-res101 (combined k-means analysis)	4.178	Powdery mildew, blight, leaf mold fungus, and mosaic virus	97.01%, 97.31%, **98.54%**
Al-gaashani et al. (2022)	MobileNetV2 and NASNetMobile	1.152	Bacterial spot, yellow leaf curl virus, septoria leaf spot, leaf mould, healthy, late blight	97%, 97%
Tarek et al. (2022)	ResNet50, InceptionV3, AlexNet, MobileNetV1, MobileNetV2 and MobileNetV3	16,004	Target spot, septoria leaf spot, two spotted spider-mite, yellow leaf curl virus, bacterial spot, leaf mold, mosaic virus, late and early blight.	99.80%, 99.62%, 96.68%, 99.49%, 98.93%, 99.81%, 98.99%
Our proposed model	VGG16, MobileNet DenseNet201 InceptionResNetV2 Custom CNN model	18.440	Bacterial spot, early blight, leaf mold, septoria leaf spot, target spot, mosaic virus, yellow leaf curl virus	92.12%, 92.75%, 91.50%, 84.12% 99.82%

Bold numerical characteristics are the best performance of the study

When all the studies were examined, it was seen that pre-trained models gave the most successful results. The highest accuracy found in the literature is the 99.64% result achieved by Wang et al. (2019). Brahimi et al. (2017) achieved a 99.18% accuracy result in their 2017 study. In addition, among the data sets used for tomato disease detection in the literature, deep learning applications have been conducted with the highest number of samples in this study.

The proposed model achieved high accuracy rates in the classification of tomato plant diseases. The models used were successful, but pre-trained models produced comparable results in previous studies. The results of 25 iterations of experimental studies are shown. This experimental study was completed in 14.88 minutes with a success rate of 99.82%. When this result is compared to previous studies in the literature, the model used produced a more accurate classification.

5. Conclusions

The study attempted to use sequential CNN estimation to identify diseases found in tomatoes. A simpler CNN model is focused on faster and higher accuracy results, with the goal of providing better quality and efficient products with fewer errors. DenseNet201, InceptionResNetV2, MobileNet, VGG16, and Custom CNN models achieved training success rates of 92.12%, 92.75%, 91.50%, 84.12%, and 99.82%, respectively.

Artificial intelligence is being used in agriculture to identify diseases and pests, which allows for accurate and highly efficient monitoring and screening of thousands of products produced in horticulture and field crops. The findings of this study can be applied to crop growth monitoring and robotic spraying operations.

Data availability: Data are available on request due to privacy or other restrictions.

Authorship Contributions: Concept: D.G.K., C.K., M.V., Design: D.G.K., C.K., M.V., Data Collection or Processing: D.G.K., Analysis or Interpretation: D.G.K., Literature Search: D.G.K., C.K., Writing: D.G.K., C.K., M.V.

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Current antibiotic sensitivity of *Lactococcus garvieae* in rainbow trout (*Oncorhynchus mykiss*) farms from Southwestern Turkey

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ABSTRACT

The continuity of antimicrobial resistance development in bacteria changes the type and dose of effective antibiotic treatments and makes routine monitoring studies necessary for successful control of bacterial diseases. This study was aimed to determine the current antibiotic susceptibility of *Lactococcus garvieae*, which causes significant economic losses in rainbow trout (*Oncorhynchus mykiss*) farms. In the study, two consecutive visits were made to three farms operating on the banks of a stream during a disease outbreak in the fall of 2018. At each visit, 10 fish showing the signs of disease from each farm (60 fish in total) were sampled and *L. garvieae* was isolated from 16 fish. All isolates were identified using conventional and molecular methods. Then, they were examined for 5 different antibiotic resistance genes and their antibiotic susceptibility profiles were determined using the Kirby Bauer disc diffusion technique. Results of the disc diffusion test revealed that all 16 isolates had a different antibiotic susceptibility profile and the isolates with different antibiotic susceptibility profile could exist within and between farms, using the same water source. Furthermore, they revealed that highly resistant isolates that showed no susceptibility up to 82 to 100% of the 33 antibiotics at the doses evaluated exist in all farms. All isolates carried either *tetA* or *tetB* genes or both and the majority of isolates carried *tetA* gene. Together with this, 25% of the isolates which carried both or one of the resistance genes showed susceptibility to all 4 tetracycline class antibiotics at the doses evaluated. Results of the study and their comparison with previous studies in the same production area or in different regions of the country revealed the dynamic nature of antibiotic resistance development in *L. garvieae*. Additionally, it showed that monitoring studies with a limited number of isolates may not give an accurate picture of the current status of antibiotic resistance from a production area. These results of the study were also discussed in terms of the treatment strategies that trout farmers should follow when treating lactococcosis.

Keywords: Antimicrobial resistance genes, Antimicrobial susceptibility, Pathogenic bacteria, Fish farm, Fish disease

1. Introduction

Intensive culture conditions, in which a large number of individuals are kept in close contact, adversely affect fish welfare as well as weaken the immune system of fish (Sönmez et al. 2022), leading to a disease by existing pathogens in the environment. On the other hand, excessive use of antibiotics and chemotherapeutics for the treatment of diseases leads to the accumulation of antibiotics in the aquatic environment and fish, suppressing the already weakened immune system and further reducing the resistance against pathogens (Lundén et al. 2020). In addition, excessive antibiotic use may raise an important problem because it causes pathogens (bacteria) to develop resistance against antibiotics (Karayakar & Ay 2006). Once such resistance is established, it can be rapidly transferred within and between bacterial species by means of genetic elements such as plasmid and transposon (Doğancı 2001). For this reason, the ability of bacterial pathogens to develop resistance to antibiotics is a very important issue to be considered in the combat against infectious diseases (Vahaboğlu 2004). In addition, the fact that antibiotic resistance developed in aquatic environments has the potential to be easily transferred to terrestrial environments, in other words, the possible transfer of this resistance to human pathogens increases the importance of studies on the development of antimicrobial resistance in aquatic environments (Carvalho 2012).

Lactococcus garvieae is a bacterial species that can cause significant economic losses in fish farms. It was first reported from Japan in 1974 to cause an infection with high mortality rates in yellowtail (*Seriola dumerili*) (Kusuda et al. 1991; Kusuda & Salati 1991; Austin & Austin 1999). In later years, mortalities related to *L. garvieae* infections (streptococcosis or lactococcosis) were observed in many other marine and freshwater fishes from different geographic regions, and this bacterium was accepted as a common fish pathogen (Kusuda & Salati 1991). Spain, Italy, Portugal, England, South Africa, Australia, France and some Asian countries have reported that *L. garvieae* is a dangerous disease agent for rainbow trout (*Oncorhynchus mykiss*) (Ravelo et al. 2001). This pathogen was first detected in rainbow trout farms located in the western part of Turkey in 2001 and reported to cause high mortality rates (Diler et al. 2002). *L. garvieae*, which induce a systemic and rapidly spreading infection characterized by hemorrhagic septicemia, can cause significant economic losses in trout farms because it usually infects pan-sized fish (Çağırgan & Tanrıkul 1997).

Lactococcus infections generally occur in the summer months when the fish biomass is high, the culture waters get warmer and the amount of incoming fresh water decreases. The infections developing under these difficult conditions may leave trout farmers who want to protect their investments with no choice but to use prophylactic, sometimes high-dose antibiotic cures. In this context, routine monitoring studies that inform the farmers about the effective antibiotic cures against common fish pathogens in their production area carry great importance. In addition to helping farmers to protect their investments, such monitoring studies may help to reduce the unnecessary use of antimicrobials in aquaculture and its negative impacts on the environment.

Muğla province is considered as the capital of the Turkish aquaculture industry. In addition to cage farming of marine fishes in the northern part, considerable amount of pan size and larger (≥1 kg) rainbow trout are produced in the southern part of the province. According to provincial authorities, more than 40 licensed rainbow trout farms with annual production capacities ranging from 3 to 2,500 metric tons currently operate in raceway systems constructed on the banks of Eşen Stream (BSGM 2019). Although several different bacterial infections that cause important economic losses occur in these farms, *L. garvieae* is often isolated as the disease agent (Kubilay et al. 2005; Kav & Erganis 2008; Altun et al. 2013; Kurtoğlu & Korun 2018; Balta & Balta 2019).

Various studies conducted before in order to determine the effective antibiotic cure against different *L. garvieae* strains isolated from trout farms in Turkey (Akçam et al. 2004; Doğancı 2001). However, intensive use of antibiotics against bacterial pathogens leads to the development of antibiotic resistance in the aquatic environment. As this continuum of antimicrobial resistance development changes treatments in terms of the type and dose of effective antibiotics, routine monitoring studies are needed for successful control of bacterial diseases (Kum et al. 2004). The last monitoring study in Muğla province was conducted more than ten years ago (Kubilay et al. 2005). Although samples were taken from the area in a more recent study (Altun et al. 2013), only a very limited number of isolates (n=2) and antibiotics were evaluated. Therefore, this study was conducted to assess the current antibiotic susceptibility status of *L. garvieae* during a disease outbreak in the autumn of 2018, when stream flow slowed down, water temperature raised above 20 °C and fish biomass was high. In this assessment, all isolates were profiled based on their susceptibility to 33 different antibiotics and the presence of five different tetracycline resistance genes. Furthermore, the results were compared with previous studies to evaluate the dynamics of antibiotic susceptibility in *L. garvieae* over the years.

2. Material and Methods

2.1. Sample collection

During a disease outbreak, 60 rainbow trout samples, weighing an average of 165-216 g, were collected from farms operating on the banks of Eşen Stream in Muğla province, in two consecutive visits on 13 September and 15 November 2018. The samples were collected from three different farms at the beginning (farm A), middle (farm B) and end (farm C) of the stream sections where the majority of the trout farms are located. In both visits, ten fish showing disease signs (lethargic and anorexic with darkened skin, bilateral or one-sided exophthalmia or fallen eyeball, hemorrhages in the ocular, perianal area and in the fin basements) were sampled from each farm. After sampling, all fish were euthanized with high doses of MS-222 (100-200 mg/L, Priborsky & Velisek 2018 and references there) and examined for external parasites. Later, their body surfaces were disinfected with 70% ethanol and necropsy was performed. Bacterial isolation was carried out by transplanting tissue samples (anterior kidney, liver and spleen) onto tryptic soy agar (TSA) medium (Biokar-Diagnostics, France) under aseptic conditions and incubating the inoculated plates at 22 °C for 48 h. Afterwards, bacteria from tissue samples (62.5% anterior kidney, 25% spleen and 12.5% liver) that showed the most intense growth in TSA medium were transferred to tryptic soy broth (TSB) medium (Biokar-Diagnostics, France) and incubated at 25 °C for 24 h. Bacterial samples that had been grown in TSB medium were stored at -80 °C until use, after adding 20% sterile glycerin.

2.2. Morphological and biochemical identification of L. garvieae isolates

Identification of isolated bacteria by conventional methods was carried out at Isparta University of Applied Sciences, Eğirdir Fisheries Faculty, Microbiology Laboratory (IUEFF-ML). When the temperatures of the bacteria samples were taken out of the deep freezer equilibrated to room temperature, the samples were planted on TSA medium and incubated at 25 ºC for 48 h. Colony morphology, color and other characteristic features of bacteria were recorded. Afterwards, Gram staining, motility test, cytochrome oxidase, catalase, oxidation/fermentation (O/F) tests were performed (Austin & Austin 1999). For the remaining biochemical analysis, API 20 STREP (Biomerieux, France) test kit was used. API test was carried out under the protocol recommended by the company that produced the API test kit. The reference *L. garvieae* strain (ELG1) was obtained from IUEFF-ML Collection.

2.3. Molecular identification of L. garvieae isolates

Molecular identification of isolates was carried out at Akdeniz University, Faculty of Agriculture, Molecular Genetics Laboratory. For DNA isolation, 5 mL samples were prepared from the cultures incubated overnight in TSB medium. Then, the appropriate number of bacterium cells (approximately $1.5x10^9$ cells equivalent to McFarland 0.5 turbidity) specified in the total genomic DNA isolation kit (ThermoScientific, USA) protocol was taken from the culture. DNA isolation was performed using the protocol specific to Gram-positive bacteria, as suggested by the company that produced the kit. After evaluating the quality and quantity of isolated genomic DNA using a spectrophotometer (ThermoScientific, NanoDrop 1000), the DNA samples were diluted with 10 mM Tris-EDTA buffer up to 200 µl volume and stored at -20 °C until use.

B27F (5 'AGAGTTTGATCCTGGCTCAG 3') and U1492R (5 'GGTTACCTTGTTACGACTT 3') universal primers were used to amplify the targeted 16S rRNA gene sequences from total genomic DNA. The polymerase chain reaction (PCR) reaction mix was prepared by adding 2 ng of genomic DNA to 12.5 µl of 2X master PCR mix (Qiagen, Germany) as recommended in the kit protocol, 1 µl (10 nmol/µl) of each primer, and sterile distilled water to complete the total reaction volume to 25 µl. The PCR conditions applied for the multiplication of 16S rRNA genes consisted of an initial denaturation at 95 °C for 10 min, denaturation at 95 °C for 45 s, primer binding at 60 °C for 45 s, synthesis at 72 °C for 2.5 min and final elongation at 72 °C for 10 min. Denaturation, primer ligation and synthesis steps were repeated for 30 cycles. Afterwards, the PCR products were run in 2% agarose gel in order to check the reaction took place. An electric field of 8 volts/cm^2 was applied during agarose gel electrophoresis (Brody & Kern 2004) and 1 kb DNA ladder was employed for the determination of molecular size of PCR products.

2.4. Antibiotic resistance gene screening of L. garvieae isolates

Whether the *L. garvieae* that were isolated developed resistance against the commonly employed antibiotics in tetracycline class or not were confirmed by using gene-specific primers (Table 1). The PCR reaction mix was prepared as explained in section 2.3 using the same kit. The PCR conditions applied for the amplification of all tetracycline genes consisted of an initial denaturation at 94 °C for 5 min, further denaturation at 94 °C for 45 s, primer binding at 58 °C for 45 s, synthesis at 72 °C for 1 min (90 s for *tetE*) and final elongation at 72 °C for 10 min. Denaturation, primer ligation and synthesis steps were repeated for 35 cycles. Afterwards, the PCR products were run in 2% agarose gel under an electric field of 8 volts/cm^2 in order to check the presence of targeted resistance genes (Brody & Kern 2004). A 100 bp DNA ladder was employed for the determination of molecular size of PCR products. Positive-control bacteria (EAS4: *Aeromonas sobria*, EAH13: *A. hydrophila* and ELG17: *L. garvieae*) bearing targeted resistance genes were obtained from the collection of IUEFF-ML.

Table 1- Primer sequence, product size and T_m information of the screened tetracycline class antibiotic resistance genes

Targeted gene	*Primer*	*Primer nucleotid sequence (5'-3')*	*Product size (bp)*	*T_m (°C)*	*Reference*
tetA	Tet A FW	GCTACATCCTGCTTGCCTTC	210	63	Ng et al. (2001)
	Tet A RV	CATAGATCGCCGTGAAGAGG		64	
tetB	Tet B FW	TTGGTTAGGGGCAAGTTTTG	659	59	
	Tet B RV	GTAATGGGCCAATAACACCG		59	
tetC	Tet C FW	CTTGAGAGCCTTCAACCCAG	418	63	
	Tet C RV	ATGGTCGTCATCTACCTGCC		62	
tetD	Tet D FW	AAACCATTACGGCATTCTGC	787	60	
	Tet D RV	GACCGGATACACCATCCATC		60	

Table 1- Continued

Targeted gene	*Primer*	*Primer nucleotid sequence (5'-3')*	*Product size (bp)*	T_m *(°C)*	*Reference*
tetE	Tet E FW Tet E RV	GTGATGATGGCACTGGTCAT CTCTGCTGTACATCGCTCTT	1180	60 63	Schmidt et al. (2001)

T_m: Primer melting temperature

2.5. Antimicrobial susceptibility test

Kirby Bauer disc diffusion method was used to determine antibiotic resistance of the isolates. Bacterial isolates inoculated in TSB medium were incubated at 25 °C for 24 h. At the end of incubation period, turbidity of the bacterial suspensions was adjusted to 0.5 McFarland turbidity (Biomerieux, France) with physiological saline (0.9%). Then, a 0.1 mL sample taken from each suspension was distributed onto Mueller-Hinton agar (MHA) medium (Merck, Germany), containing 5% sheep blood, using a sterile swab and the plates were dried in a sterile cabinet for 5-10 min. Afterwards, antibiotic discs were placed on the plates and the plates were incubated at 25 °C for 48 h. At the end of incubation period, diameter of no growth zones formed around the antibiotic discs were measured with the aid of a millimetric ruler. Then, the isolates were classified as susceptible (S), moderately susceptible (I) or resistant (R) in accordance with the Clinical and Laboratory Standards Institute (CLSI) criteria (for *Enterococcus* spp., Enterobacterales, *Staphylococcus* spp. or *Streptococcus aureus* in the order of availability) or relevant previous studies (Table 2). Clinical breakpoints for lincomycin 2 µg (L2) and colistin 10 µg (CT10) cannot be found in the available literature. Hence the bacterial isolates were classified as "resistant" for these two antibiotics when the diameter of inhibition zones was "0". Remaining 31 different antibiotic discs were used in sensitivity detection of the isolates were ampicillin 10 µg (AM10), amoxicillin 25 µg (AX25), oxacillin 1 µg (OX1), penicillin G 10 U (P10), cephalothin 30 µg (CF30), cefoperazone 75 µg (CFP75), ceftriaxone 30 µg (CRO30), cefuroxime 30 µg (CXM30), spectinomycin 100 µg (SPT100), gentamicin 10 µg (GM10), kanamycin 30 µg (K30), streptomycin 10 µg (S10), trimethoprim 1.25 µg / sulfamethoxazole/23.75 µg (SXT25), vancomycin 30 µg (VA30), clindamycin 2 µg (CC2), apramycin 15 µg (APR15), erythromycin 15 µg (E15), pristinamycin 15 µg (PT15), tylosin 15 µg (TY15), nitrofurantoin 300 µg (FM300), chloramphenicol 30 µg (C30), florphenicol 30 µg (FFC30), ciprofloxacin 5 µg (CIP5), enrofloxacin 5 µg (ENO5), flumequine 30 µg (FLM30), norfloxacin 10 µg (NOR10), oxolinic acid 2 µg (OA2), ofloxacin 5 µg (OFX5), doxycycline 30 µg (DOX30), oxytetracycline 30 µg (T30) and tetracycline 30 µg (TE30).

Table 2- Antibiotic susceptibility test breakpoints used in the study

Antibiotics	*S*	*I*	*R*	*Reference*
β-Lactams				
Penicilins				
AM10	≥17	14-16	≤13	CLSI M100 2021
AX25	≥18	14-17	≤13	CLSI M100 2021
OX1	≥13	11-12	≤10	CLSI M31 2008
P10	≥15	-	≤14	CLSI M100 2020
Cephems				
CF30	≥18	15-17	≤14	CLSI M31 2008
CFP75	≥21	16-20	≤15	CLSI M31 2008
CRO30	≥27	25-26	≤24	CLSI M31 2008
CXM30	≥18	15-17	≤14	CLSI M31 2008
Non-β-Lactams				
Aminocyclitols				
SPT100	≥14	11-13	≤10	CLSI VET01S 2020
Aminoglycosides				
GM10	≥15	13-14	≤12	CLSI M100 2021
K30	≥18	14-17	≤13	CLSI M100 2021
S10	≥15	12-14	≤11	CLSI M100 2021
APR15	≥25	17-24	≤16	CLSI VET01S 2020
Folate pathway antagonists				
SXT25	≥16	11-15	≤10	CLSI M100 2021

Table 2- Continued

Antibiotics	*S*	*I*	*R*	*Reference*
Glycopeptides				
VA30	≥17	15-16	≤14	CLSI M100 (2020)
Lincosamides				
CC2	≥19	16-18	≤15	CLSI M31 (2008)
L2	NA	NA	NA	NA
Lipopeptides				
CT10	NA	NA	NA	NA
Macrolides				
E15	≥23	14-22	≤13	CLSI M100 (2020)
PT15	≥22	19-21	<19	Perrin-Guyomard et al. (2005)
TY15	≥26	19-25	≤18	CLSI VET01S (2020)
Nitrofurans				
FM300	≥17	15-16	≤14	CLSI M100 (2021)
Phenicols				
C30	≥18	13-17	≤12	CLSI M100 (2021)
FFC30	≥29	23-28	≤22	CLSI VET01S (2020)
Quinolones				
CIP5	≥26	22-25	≤21	CLSI M100 (2021)
ENO5	≥23	17-22	≤16	CLSI VET01S (2020)
FLM30	≥21	16-20	≤15	Korun et al. (2021)
NOR10	≥17	13-16	≤12	CLSI M100 (2021)
OA2	≥13	11-12	≤10	Balta & Balta (2019)
OFX5	≥16	13-15	≤12	CLSI M100 (2021)
Tetracylines				
DOX30	≥14	11-13	≤10	CLSI M100 (2021)
T30	≥19	15-18	≤14	Balta & Balta (2019)
TE30	≥15	12-14	≤11	CLSI M100 (2021)

S: Susceptible, I: Moderately susceptible, R: Resistant, AM10: Ampicillin, AX25: Amoxicillin, OX1: Oxacillin, P10: Penicillin, CF30: Cephalothin, CFP75: Cefoperazone, CRO30: Ceftriaxone, CXM30: Cefuroxime, SPT100: Spectinomycin, GM10: Gentamicin, K30: Kanamycin, S10: Streptomycin, SXT25: Trimethoprim/sulfamethoxazole, VA30: Vancomycin, CC2: Clindamycin, L2: Lincomycin, CT10: Colistin, APR15: Apramycin, E15: Erythromycin, PT15: Pristinamycin, TY15: Tylosin, FM300: Nitrofurantoin, C30: Chloramphenicol, FFC30: Florphenicol, CIP5: Ciprofloxacin, ENO5: Enrofloxacin, FLM30: Flumequine, OA2: Oxolinic acid, OFX5: Ofloxacin, NOR10: Norfloxacin, DOX30: Doxycycline, T30: Oxytetracycline, TE30: Tetracycline. Numbers within the abbreviations show antibiotic concentration of the discs in µg, except penicillin G, of which concentration was unit. NA: Clinical inhibition zone breakpoints are not available

2.6. Calculation of multiple antibiotic resistance index values

The multiple antibiotic resistance (MAR) index values were calculated as the ratio of the number of antibiotics to which organisms were resistant in comparison to total number of antibiotics were evaluated. Isolates with a calculated MAR value greater than 0.20 were considered to have MAR (Krumperman 1983; Çapkin et al. 2015).

3. Results

All sampled fish were euthanized with high doses of MS-222 and examined for external parasites. No external parasites were detected on the specimens.

3.1. Morphological and biochemical identification of L. garvieae isolates

Sixteen bacterial isolates (3 each from farm A and B, and 10 from farm C) were obtained from 16 out of 60 fish sampled showed morphological and biochemical characteristics compatible with *L. garvieae*. The API 20 STREP test (Table 3) confirmed that the biochemical properties of all 16 isolates were well matched with *L. garvieae*.

Table 3- API 20 STREP results of *Lactococcus garvieae* isolates

FARM ID	*ISOLATE ID*	*VP*	*HIP*	*ESC*	*PYRA*	*αGAL*	*βGUR*	*βGAL*	*PAL*	*LAP*	*ADH*	*RIB*	*ARA*	*MAN*	*SOR*	*LAC*	*TRE*	*INU*	*RAF*	*AMD*	*GLYG*
	ELG1	+	+	+	+	-	-	-	-	+	+	+	-	+	-	-	+	-	-	+	-
A	LG4, 15, 16	+	+	+	+	-	-	-	-	+	+	+	-	+	-	-	+	-	-	+	-
B	LG3, 13, 14	+	+	+	+	-	-	-	-	+	+	+	-	+	-	-	+	-	-	+	-
C	LG1, 2, 5, 6, 7, 8, 9, 10, 11, 12	+	+	+	+	-	-	-	-	+	+	+	-	+	-	-	+	-	-	+	-

ELG1: Reference *L. garvieae* strain from Isparta University of Applied Sciences, Eğirdir Fisheries Faculty, Microbiology Laboratory Collection, VP: Sodium pyruvate, HIP: Hippuric acid, ESC: Esculin ferric citrate, PYRA: Pyroglutamic acid ß-naphthylamide, αGAL: 6-bromo-2-naphthyl α-D-galactopyranoside, βGUR: Naphthol AS-BI-ß-D-glucuronic acid, βGAL: 2-naphthyl ß-D-galactopyranoside, PAL: 2-naphthyl phosphate, LAP: L-leucine-ß-naphthylamide, ADH: L-arginine, RIB: D-ribose, ARA: L-arabinose, MAN: D-mannitol , SOR: D-sorbitol, LAC: D-lactose, TRE: D-trehalose, INU: Inulin, RAF: D-raffinose, AMD: Starch, GLYG: Glycogen

3.2. Molecular identification of L. garvieae isolates

Concordant with the conventional identification methods, BLASTN algorithm yielded a perfect match (99.86-100%) with *L. garvieae*, when the 16S rDNA sequences of 16 isolates were queried with the bacterial gene sequences in the GenBank database.

3.3. Antibiotic resistance gene profiles of L. garvieae isolates

Our profiling study revealed that 15 out of 16 isolates carried *tetA* gene, 13 isolates carried *tetB* gene, and 12 isolates carried both antibiotic resistance genes (Table 4). Although we detected the presence of *tetC*, *tetD* and *tetE* genes in our reference bacteria evaluated in the same PCR reaction, these three tetracycline resistance genes were not found in any of the 16 *L. garvieae* isolates studied (Table 4).

Table 4- Antibiotic resistance gene profiles of *Lactococcus garvieae* isolates

Farm ID	*Isolate ID*	*Antibiotic resistance genes*				
		tetA	*tetB*	*tetC*	*tetD*	*tetE*
A	LG4	+	+	-	-	-
	LG15	+	+	-	-	-
	LG16	+	-	-	-	-
B	LG3	+	+	-	-	-
	LG13	+	-	-	-	-
	LG14	+	+	-	-	-
C	LG1	+	+	-	-	-
	LG2	+	+	-	-	-
	LG5	+	+	-	-	-
	LG6	-	+	-	-	-
	LG7	+	+	-	-	-
	LG8	+	+	-	-	-
	LG9	+	+	-	-	-
	LG10	+	-	-	-	-
	LG11	+	+	-	-	-
	LG12	+	+	-	-	-
Positive controls	EAS4	+	-	+	-	-
	EAH13	-	-	-	+	-
	ELG17	+	+	-	-	+

+: positive, -: negative, EAS4: *Aeromonas sobria*, EAH13: *A. hydrophila* and ELG17: *L. garvieae* strain from Isparta University of Applied Sciences, Eğirdir Fisheries Faculty, Microbiology Laboratory collection

3.4. Antimicrobial susceptibility of L. garvieae isolates

Disk diffusion tests showed that all 16 isolates had different antibiotic susceptibility profiles (Table 5). While 2 isolates (B-LG14 and C-LG11) were found to be resistant or developing resistance to all antibiotics, 11 out of the remaining 14 isolates showed resistance or have been developing resistance to more than 50% of the 33 antibiotics evaluated. Only 3 isolates (A-LG16, B-LG13 and C-LG1) showed resistance or were developing resistance to less than 50% (39, 33 and 45%, respectively) of the 33 antibiotics evaluated. It was also determined that all isolates were either resistant or developing resistance to 8 antibiotics (OA2, CC2, TY15, N0R10, APR15, FFC30, CIP15 and FLM30), and 50% or more of the isolates were resistant or developing resistance against 20 antibiotics (AM10, VA30, SXT25, E15, ENO5, GM10, K30, FM300, CT10, OFX5, T30, CFP75, CRO30, SPT100, CF30, AX25, PT15, OX1, S10 and L2). As a result, it was seen that only 5 antibiotics (TE30, C30, DOX30, CXM30 and P10) remained to which more than 50% of the isolates still showed susceptibility.

Table 5- Antibiotic susceptibility profiles of *Lactococcus garvieae* isolates according to disc diffusion method (diameter of inhibition zones in mm)

Antibiotics	***FARM A ISOLATE ID***			***FARM B ISOLATE ID***			***FARM C ISOLATE ID***										
	LG4	***LG15***	***LG16***	***LG3***	***LG13***	***LG14***	***LG1***	***LG2***	***LG5***	***LG6***	***LG7***	***LG8***	***LG9***	***LG10***	***LG11***	***LG12***	***Total I+R***
AM10*	R(13)	R(0)	S(33)	I(15)	S(35)	R(0)	R(13)	R(13)	I(14)	R(0)	S(25)	I(14)	R(0)	R(0)	R(0)	R(0)	13
AX25*	S(21)	R(0)	S(35)	I(17)	S(35)	R(0)	S(19)	S(20)	S(20)	R(0)	S(23)	S(23)	R(0)	I(17)	R(0)	R(0)	8
OX1**	R(0)	R(0)	R(0)	I(12)	R(10)	R(0)	S(16)	R(0)	R(0)	R(0)	R(0)	S(14)	R(0)	I(12)	R(0)	S(16)	13
P10**	S(20)	R(0)	S(28)	S(22)	S(30)	R(0)	S(27)	S(20)	S(22)	R(0)	S(22)	R(0)	S(15)	R(12)	R(0)	R(0)	7
CF30**	R(12)	R(0)	S(27)	R(0)	S(30)	R(0)	I(16)	I(15)	S(18)	R(0)	R(0)	S(18)	R(0)	R(0)	R(0)	R(0)	12
CFP75*	R(14)	I(19)	S(30)	R(14)	S(32)	R(0)	R(0)	I(16)	R(0)	S(22)	R(0)	R(14)	R(0)	I(17)	R(0)	R(0)	13
CRO30*	R(13)	R(0)	S(30)	R(14)	S(35)	R(0)	R(21)	R(20)	R(0)	R(23)	S(28)	R(13)	R(14)	R(11)	R(0)	R(0)	13
CXM30**	S(25)	R(0)	S(36)	S(26)	S(40)	R(0)	S(20)	S(21)	S(23)	S(28)	S(27)	R(12)	R(7)	R(8)	R(0)	S(20)	6
SPT100***	I(12)	R(0)	S(20)	S(14)	S(21)	R(0)	S(14)	R(0)	R(0)	R(0)	S(14)	I(12)	R(8)	S(14)	R(0)	S(32)	9
GM10*	S(22)	R(11)	S(17)	S(16)	S(20)	R(0)	S(16)	I(14)	I(13)	R(12)	I(13)	R(0)	S(15)	R(0)	R(0)	R(0)	10
K30*	R(12)	R(0)	S(20)	I(15)	I(15)	R(0)	I(17)	I(15)	R(13)	R(0)	R(0)	R(12)	R(13)	R(11)	R(0)	R(0)	15
S10*	R(0)	R(0)	S(17)	R(0)	S(16)	R(0)	R(0)	R(0)	R(0)	R(0)	R(7)	S(16)	R(10)	I(14)	R(0)	R(0)	13
SXT25**	I(11)	R(0)	R(0)	R(0)	S(20)	R(0)	S(16)	I(13)	I(13)	R(0)	R(0)	I(13)	S(24)	I(15)	R(0)	R(0)	13
VA30*	S(20)	I(15)	S(25)	S(18)	S(25)	R(0)	S(24)	S(20)	I(16)	R(0)	I(15)	S(22)	I(15)	I(10)	R(0)	I(16)	9
CC2**	R(0)	R(0)	R(0)	R(0)	R(0)	R(0)	R(0)	R(0)	R(0)	R(0)	R(0)	R(0)	R(12)	R(12)	R(0)	R(13)	16
L2**	R(0)	R(0)	R(0)	R(0)	-(16)	R(0)	R(0)	R(0)	R(0)	R(0)	R(0)	R(0)	-(8)	R(0)	R(0)	R(0)	14
CT10*	-(16)	R(0)	R(0)	-(18)	R(0)	R(0)	-(16)	-(17)	-(15)	R(0)	R(0)	-(10)	R(0)	-(11)	R(0)	-(16)	8
APR15*	R(0)	R(0)	R(16)	R(0)	R(14)	R(0)	R(12)	R(0)	R(10)	R(13)	R(11)	R(13)	R(0)	R(0)	R(0)	R(16)	16
E15*	S(24)	I(15)	S(30)	R(0)	S(30)	R(0)	S(28)	R(0)	S(24)	S(26)	S(26)	R(11)	R(8)	R(0)	R(0)	R(0)	9
PT15**	S(23)	R(0)	S(23)	I(20)	S(23)	R(0)	S(23)	I(19)	I(21)	I (20)	I 20	R(9)	R(13)	R(0)	R(0)	R(0)	12
TY15*	R(13)	R(0)	R(0)	R(0)	R(0)	R(0)	R(0)	R(0)	R(0)	R(0)	R(0)	R(0)	R(13)	R(11)	R(0)	R(0)	16
FM300***	S(20)	R(11)	S(22)	S(17)	S(21)	R(0)	S(23)	S(20)	S(21)	I(15)	I(15)	R(13)	R(12)	S(22)	R(0)	R(0)	8
C30**	S(22)	S(20)	S(34)	S(20)	S(30)	R(0)	S(30)	S(25)	S(23)	S(23)	R(0)	R(0)	I(14)	I(13)	R(0)	S(28)	6
FFC30**	R(17)	R(22)	R(16)	R(15)	R(0)	R(0)	R(0)	I(24)	R(22)	R(0)	I(23)	R(12)	R(0)	R(21)	I(23)	R(0)	16
CIP5*	R(13)	R(0)	I(24)	R(12)	I(22)	R(0)	R(13)	R(12)	R(14)	R(0)	R(0)	R(15)	R(13)	I(22)	R(0)	R(18)	16
ENO5*	S(23)	R(0)	S(25)	R(16)	S(25)	R(0)	S(23)	I(22)	I(21)	R(13)	I(20)	R(0)	R(12)	R(12)	R(0)	R(8)	12
FLM30*	R(7)	R(0)	R(0)	R(12)	R(12)	R(0)	R(0)	I(17)	R(0)	R(0)	R(0)	R(0)	R(7)	R(12)	R(0)	I(17)	16
OA2*	R(0)	R(0)	R(0)	R(0)	I(12)	R(0)	R(0)	R(0)	R(0)	R(0)	R(0)	R(0)	R(7)	R(9)	R(0)	R(0)	16
OFX5*	I(13)	R(0)	S(20)	R(0)	S(20)	R(0)	S(23)	S(22)	S(22)	R(0)	R(0)	I(13)	I(13)	S(22)	R(0)	R(0)	10
NOR10*	R(8)	R(0)	I(15)	R(0)	R(12)	R(0)	R(12)	R(10)	R(0)	R(0)	R(0)	R(12)	R(0)	R(10)	R(0)	R(0)	16
DOX30**	S(25)	S(16)	S(32)	S(19)	S(31)	R(0)	S(30)	S(25)	S(26)	S(25)	S(25)	I(13)	S(22)	I(12)	R(0)	R(0)	5
T30**	I(16)	R(0)	S(24)	R(0)	S(25)	R(0)	S(21)	I(17)	R(0)	R(0)	S(22)	R(0)	R(7)	I(16)	R(0)	I(16)	12
TE30**	S(22)	I(13)	S(25)	S(22)	S(23)	R(0)	S(25)	S(21)	S(20)	R(0)	R(0)	I(12)	S(19)	I(14)	R(0)	S(20)	7

Table 5- Continued

Antibiotics	*FARM A ISOLATE ID*			*FARM B ISOLATE ID*			*FARM C ISOLATE ID*										
	LG4	*LG15*	*LG16*	*LG3*	*LG13*	*LG14*	*LG1*	*LG2*	*LG5*	*LG6*	*LG7*	*LG8*	*LG9*	*LG10*	*LG11*	*LG12*	*Total I+R*
Total I+R	21	31	13	24	11	33	15	23	22	28	24	27	27	29	33	27	
Total R	16	27	10	18	8	33	13	13	16	26	18	21	24	18	32	24	
MAR	0.48	0.82	0.30	0.55	0.24	1.00	0.39	0.39	0.48	0.79	0.55	0.64	0.73	0.55	0.97	0.73	

S: Susceptible, I: Moderately susceptible, R: Resistant, -: not classified, *Critically important antimicrobials, **Highly important antimicrobials, ***Important antimicrobials, AM10: Ampicillin, AX25: Amoxicillin, OX1: Oxacillin, P10: Penicillin G, CF30: Cephalothin, CFP75: Cefoperazone, CRO30: Ceftriaxone, CXM30: Cefuroxime, SPT100: Spectinomycin, GM10: Gentamicin, K30: Kanamycin, S10: Streptomycin, SXT25: Trimethoprim/sulfamethoxazole, VA30: Vancomycin, CC2: Clindamycin, L2: Lincomycin, CT10: Colistin, APR15: Apramycin, E15: Erythromycin, PT15: Pristinamycin, TY15: Tylosin, FM300: Nitrofurantoin, C30: Chloramphenicol, FFC30: Florphenicol, CIP5: Ciprofloxacin, ENO5 Enrofloxacin, FLM30: Flumequine, OA2: Oxolinic acid, OFX5: Ofloxacin, NOR10: Norfloxacin, DOX30: Doxycycline, T30: Oxytetracycline, TE30: Tetracycline. The numbers in the abbreviated names show antibiotic concentration of the discs in µg, except penicillin G, of which concentration was unit. MAR: Multiple Antibiotic Resistance index value

3.5. Multiple antibiotic resistance index values

The MAR index values of the 16 isolates ranged from 0.24 (B-LG13) to 1.00 (B-LG14) (Table 5). Based on these high (>0.20) MAR index values, we can say that all 16 isolates evaluated in this study carried MAR.

4. Discussion

Since the first record of *L. garvieae* from rainbow trout farms located in the western part of Turkey in 2001 (Diler et al. 2002), different antibiotics have been used to control lactococcal infections (Kubilay et al. 2005; Balta & Balta 2019). Studies have been conducted since then and the results of present study clearly reveal the dynamic nature of antibiotic resistance development in *L. garvieae*. Our results showed that all 16 *L. garvieae* isolates studied had unique antibiotic susceptibility profile. In addition, it was detected that 2 of these isolates (B-LG14 and C-LG11) were either resistant or developing resistance to all 33 antibiotics at the doses evaluated. Fifty percent or more of the remaining 14 isolates also showed resistance to or were developing resistance against 28 antibiotics, indicating that only 5 antibiotics remained (penicillin G, cefuroxime, chloramphenicol, doxycycline and tetracycline) to which more than 50% of the isolates were still susceptible (Table 5). In contrast to these, previous studies reported resistance against 3 of these 5 remaining antibiotics (for penicillin G in Diler et al. 2002 and Kubilay et al. 2005, for cefuroxime in Kubilay et al. 2005, and for doxycycline in Altun et al. 2013).

Unlike previous studies that were evaluated, only a limited number of isolates from a production area (Kubilay et al. 2005; Altun et al. 2013; Kurtoğlu & Korun 2018; Balta & Balta 2019) or all 30 isolates obtained had the same antibiogram test results (Kav & Erganis 2008), our study evaluated 16 different isolates showed that *L. garvieae* with different antibiotic susceptibility profiles can be found in a farm or in different farms within the same production area. Under these circumstances, trout farmers would be unlikely to cure lactococcosis without having a prior antibiogram testing or by simply administering one type of antibiotic. Farmers who cannot treat their sick fish may contribute more to the development of resistance if they unconsciously increase the dose of antibiotics or exploit with various antibiotics. Although, it is not possible to establish a clear link without precise data on the type and intensity of antibiotic treatments applied by trout farmers over the years, our study showed that *L. garvieae* isolated from the rainbow trout raised in Muğla province either had already developed resistance or had been developing resistance to ampicillin, cephalothin, spectinomycin, vancomycin, erythromycin, pristinamycin, nitrofurantoin, chloramphenicol, enrofloxacin, ofloxacin and tetracycline (Table 5). Whereas previous studies including isolates from the region reported that *L. garvieae* isolates were susceptible to these 11 antibiotics (Table 6). Studies involving samples from other parts of the country also reported isolates of *L. garvieae* were still susceptible to cefoperazone, florphenicol, ciprofloxacin and oxolinic acid (Kav & Erganis 2008; Altun et al. 2013; Balta & Balta 2019), but 81% of the 16 isolates in this study were resistant to cefoperazone and all 16 isolates were resistant to the same doses of the last 3 antibiotics (Table 6). High MAR values ranging between 0.24-1.00 also supported these results and suggested that all 16 isolates developed MAR (Krumperman 1983). Thus, we can say that results of the present study indicate that the antibiotic resistance of *L. garvieae* has increased over the years in Muğla province. On the other hand, we should point out that the procedure used in antibiotic susceptibility tests might have contributed to outcomes of the study. Unlike previous studies, we followed the latest CLSI guidelines (CLSI M100 2021) for antibiotic susceptibility determination, thus used 5% sheep blood supplemented MHA and a 48 h incubation period. However, all previous studies presented in Table 6 used MHA only, and some used shorter (20 and 24 h) incubation periods (Kav & Erganis 2008; Balta & Balta 2019). Both procedural differences have potential to induce smaller inhibition zones, as sheep blood providing additional nutrients promotes faster and more efficient bacterial growth and a longer incubation period allow more colony formation (CLSI M100 2021).

Thus, it is possible that these procedural differences had increased our rate of classifying isolates as resistant or intermediate resistant. However, this does not mean that the latest CLSI procedure has resulted in a misclassification of the antibiotic susceptibility of bacteria. Instead, it demonstrates that the use of a standard procedure for antibiotic susceptibility testing on fish pathogens is important to obtain accurate and comparable results. Especially, considering that CLSI test breakpoints are commonly employed when classifying antibiotic susceptibility of fish pathogens.

Table 6- Antibiotic resistance profiles of *Lactococcus garvieae* in previous studies from Turkey

Antibiotics	*Location*					
	Muğla province				*Konya province*	*Eastern Blacksea region*
	Diler et al. (2002)	*Kubilay et al. (2005)*	*Altun et al. (2013)*	*Kurtoğlu & Korun (2018)*	*Kav & Erganis (2008)*	*Balta & Balta (2019)*
AM10 (81)	S	S	-	S	S	I+R
AX25 (50)	-	-	R+I	-	S	S+I+R(10 µg)
OX1 (81)	-	R	-	-	R (5µg)	-
P10 (44)	R	R	-	-	S	-
CF30 (75)	-	S+R	-	-	-	-
CFP75 (81)	-	-	-	-	S (30 µg)	-
CRO30 (81)	R	R	-	-	-	-
CXM30 (38)	-	R	-	-	-	-
SPT100 (56)	-	S*	-	-	-	-
GM10 (63)	-	-	R (120 µg)	-	R	-
K30 (94)	-	R	-	R	-	-
S10 (81)	-	R	-	R	-	R
SXT25 (81)	-	R	R	-	R	R
VA30 (56)	-	S	-	-	S (5 µg)	-
CC2 (100)	R	R	-	-	R	-
L2 (88)	-	R	R	-	R(10 µg)	-
CT10 (50)	-	R*	-	-	-	-
APR15 (100)	-	R	-	-	-	-
E15 (56)	S	S	R+I	S+I	S	S+I+R
PT15 (75)	-	S*	-	-	-	-
TY15 (100)	-	R*	-	-	-	-
FM300 (50)	-	S	-	-	-	-
C30 (38)	S	S	-	S	S	-
FFC30 (100)	-	-	R+I		S	S+I+R
CIP5 (100)	-	R	-	-	S	-
ENO5 (75)	-	S	-	-	I	S+I+R
FLM30 (100)	-	-	-	R	-	-
OA2 (100)	-	-	-	R	-	S+I+R

Table 6- Continued

Antibiotics	*Location*					
	Muğla province				*Konya province*	*Eastern Blacksea region*
	Diler et al. (2002)	*Kubilay et al. (2005)*	*Altun et al. (2013)*	*Kurtoğlu & Korun (2018)*	*Kav & Erganis (2008)*	*Balta & Balta (2019)*
OFX5 (63)	S	S+I+R	-	-	-	-
NOR10 (100)	-	R	-	-	-	-
DOX30 (31)	-	S	R+I	-	-	S+I+R
T30 (75)	-	-	R+S+I	-	S	S+I+R
TE30 (44)	S	S	-	S	-	-

S: Susceptible, I: Moderately susceptible, R: Resistant, -: not classified, AM10: Ampicillin, AX25: Amoxicillin, OX1: Oxacillin, P10: Penicillin G, CF30: Cephalothin, CFP75: Cefoperazone, CRO30: Ceftriaxone, CXM30: Cefuroxime, SPT100: Spectinomycin, GM10: Gentamicin, K30: Kanamycin, S10: Streptomycin, SXT25: Trimethoprim / sulfamethoxazole, VA30: Vancomycin, CC2: Clindamycin, L2: Lincomycin, CT10: Colistin, APR15: Apramycin, E15: Erythromycin, PT15: Pristinamycin, TY15: Tylosin, FM300: Nitrofurantoin, C30: Chloramphenicol, FFC30: Florphenicol, CIP5: Ciprofloxacin, ENO5: Enrofloxacin, FLM30: Flumequine, OA2: Oxolinic acid, OFX5: Ofloxacin, NOR10: Norfloxacin, DOX30: Doxycycline, T30: Oxytetracycline, TE30: Tetracycline. The numbers in the abbreviated names show the antibiotic concentration of the discs in µg, except penicillin G, of which concentration was unit. Numbers in parentheses after abbreviated names indicate the percentage of isolates classified as I+R in the present study. Classifications written in bold are for isolates obtained from rainbow trout raised in Muğla province. *Classified using ATB VET stript. Doses different from those used in the present study are given in parentheses

In addition to determining their susceptibility to 3 different antibiotics (doxycycline, oxytetracycline and tetracycline) from the tetracyclines class (Chopra & Roberts 2001), we investigated the presence of 5 different tetracycline resistance genes in all isolates. Among the 5 genes, *tetA* was the most common one in the studied *L. garvieae* isolates. This finding of our study is compatible with the reported results of Raissy and Shahrani (2015). Additionally, all isolates had either *tetA* or *tetB* gene and the majority of isolates (75%) carried both resistance genes. Together with this, 4 isolates (C-LG1, C-LG2, B-LG13, and A-LG16) showed susceptibility to all tetracycline class antibiotics, 4 isolates (B-LG3, A-LG4 and C-LG5 and C-LG9) showed susceptibility to doxycycline and oxytetracycline, one isolate (C-LG7) showed susceptibility to doxycycline and tetracycline, and one isolate (C-LG12) was susceptible to oxytetracycline. These results of the study suggest that the antibiotic resistance genes carried by *L. garvieae* are not sufficiently expressed to provide resistance against the antibiotic doses used in the study or antibiotic resistance genes may be silent as reported in another study (Randall et al. 2004). A similar contrast has also been reported in other studies involving *Pantoea agglomerans* (Saticioglu et al. 2018) and *L. garvieae* (Duman et al. 2020).

In addition to genes, molecules such as AmpC β-lactamases can lead to the development of MAR in bacteria (Noor ul Ain et al. 2014). This means that acquired resistance to one antibiotic can lead to the development of resistance against many other antibiotics, depending on the origin of encounter. Therefore, it is possible for existing or acquired antibiotic resistance to cause bacteria to develop resistance to many other antibiotics with the same mode of action. In the present study, we used large numbers of antibiotics, the majority of which are not used by trout farmers to treat lactococcosis or other bacterial fish diseases, to see the extent of antibiotic resistance development in *L. garvieae*. Unfortunately, we detected isolates showing resistance to all or almost all antibiotics at the doses evaluated. Determining the true causes of such a wide range of antibiotic resistance requires more extensive studies. Together with this, we should mention that there is no settlement that will cause sewage or other types of pollution before the stream section occupied by trout farms in the production area. Additionally, one of the sampled farms and some other farms in the area buy eyed eggs and fingerlings from the hatcheries located in various different regions of the country and also abroad. These two facts lead us to think fish inflows from contaminated areas could be one reason for this wide range of resistance. Nevertheless, as we stated above, further extensive research is necessary for a proper addressing of the issue.

Besides being one of the most important fish pathogens with devastating effects in aquaculture (Algöet et al. 2009), *L. garviae* is also defined as an opportunistic bacterium that can infect humans, especially the elderly (Gibello et al. 2016). Thus, it is possible that bare hand handling and raw or undercooked consumption of infected fish may have humans exposed to this zoonotic pathogen (Chan et al. 2011). Within the 33 antibiotics evaluated in this study, 18 were listed as critically and 13 were listed as highly important antimicrobials for human medicine (Table 5) by the World Health Organization (WHO 2019). And, we determined that 31-100% of the *L. garviae* strains isolated have already developed or have been developing resistant to these two groups of antibiotics. This result of the study

also suggests that antibiotic resistance developed by *L. garviae* may have reached a level that may pose significant health risks for farm workers and consumers as well.

5. Conclusions

In conclusion, no antibiotic to which all *L. garvieae* isolates are susceptible could be determined in this study. Antibiotics to which ≥50% of the strains are still susceptible (penicillin, tetracycline, pristinamycin, chloramphenicol, amoxicillin, nitrofurantoin, cefuroxime, doxycycline) should be used very carefully without forgetting that the remaining strains had already acquired or are developing resistance against them. As demonstrated in this study, several isolates of *L. garvieae* with different antibiotic susceptibility profiles can exist within and between farms using the same water source. In such a case, treatments with a single antibiotic may not be effective or the antibiotic treatment that works in one farm may not work in another. Therefore, we strongly recommend that each farm determine the effective antibiotic cure in terms of type and dose according to the antibiogram test performed specifically for each bacterial disease case. In addition, it should be kept in mind that antibiotic resistance already developed in the studied *L. garvieae* isolates may spread over time between the same or other pathogenic bacteria species in the production area. Thus, we also recommend to screen fish before all transfers between different water bodies to prevent the spreading of pathogenic bacteria. Monitoring the current antibiotic susceptibility of fish pathogens with periodic studies and preventing the spread of new or resistant fish pathogens throughout the country by establishing national monitoring programs will contribute to healthier development of the aquaculture industry. However, it should be noted that monitoring studies with a limited number of isolates may not give the full picture. Therefore, to make a more accurate assessment of current antimicrobial susceptibility in a production area, we recommend evaluating as many bacterial isolates as possible in the future studies. Finally, we would like to emphasize that handling infected fish with bare hands and consuming them raw or undercooked carry risk of exposing humans to zoonotic pathogens that are resistant to many of the antibiotics used in human medicine.

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Data availability: Data are available on request due to privacy or other restrictions.

Ethics Committee Approval: The procedures applied in this study were evaluated by the Akdeniz University Animal Experiments Local Ethics Committee and their ethical compliance was approved with the protocol number 2018.03.001 (date:26.02.2018).

Authorship Contributions: Concept: S.S.S., M.Y., A.K., Design: S.S.S., M.Y., A.K., Data Collection or Processing: S.S.S., M.Y., T.A., A.K., Analysis or Interpretation: S.S.S., M.Y., Literature Search: S.S.S., M.Y., T.A., Writing: S.S.S., M.Y., T.A., A.K.

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Evaluation of some Water, Energy and Financial Indicators: A Case Study of Esenli Water User Association in Yozgat, Türkiye

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ABSTRACT

Energy is one of the most important inputs for pressurized irrigation systems in the world's agriculture sector. As in all areas of life, energy used in irrigation has become an important research subject for researchers. In studies performed with performance indicators in Türkiye, the use of energy indicators is limited. The scarcity of energy resources makes the efficiency of energy used in irrigation schemes extremely important. This study evaluated the performance of a pressurized irrigation scheme in the Yozgat water, energy and financial indicatorsater, energy and financial. The data obtained were compared with other countries in the Mediterranean region. As a result, it was concluded that, on average, the irrigation rate was 39%, annual irrigation water supply per unit irrigated area was 8229 m3 ha−1, specific energy was 0.33 kWh m−3, energy consumed per unit irrigated was 2671 kWh ha−1, total maintenance, operation and management cost per unit irrigation water supply was 403 € ha−1 in the years (2008-2017) when the study was conducted. The finding showed that the specific energy value was about three times lower than in other countries. However, when the energy consumed per unit in the irrigated area was examined, it was found that the irrigation scheme has a high consumption of energy.

Keywords: Financial management, Energy use, Performance evaluation, Water use, Water user associations

1. Introduction

Water is essential for food, energy, poverty reduction, sustainable development and human well-being. Global projections indicate that demand for freshwater, energy and food will significantly increase over the next decades under the pressure of population growth and mobility, economic development, international trade, urbanization, diversifying diets, cultural and technological changes and climate change (Hoff 2011; Endo et al. 2017; Lu et al. 2021).

Irrigated agriculture is extremely important for providing maximum benefit with the scarce resources available. With the widespread use of pressurized irrigation systems, energy use in irrigated agriculture is increasing gradually. Total global water withdrawals for irrigation are projected to increase by 10% by 2050 (FAO 2011).

In Türkiye, rapid and extensive work has been conducted in recent years for the transition to pressurized irrigation systems instead of open channel systems. Furthermore, new irrigation projects are designed and built as pressurized irrigation systems. With these developments, agricultural energy consumption is increasing. In Türkiye, energy is used in approximately 20.5% of the total irrigated area. One of the most important problems faced by water user associations (WUA) is that the energy cost is very high, and they have payment difficulties (DSI 2019).

The data obtained as a result of irrigation performance evaluation can support the planning, implementation and management of similar projects (Bastiaanssen & Bos 1999). Performance indicators such as water, energy and financial management are to evaluate irrigation schemes and benchmark among the others or the years. The indicators help to improve water management in agricultural lands and give key clues to water decision-makers managers, policymakers and scientists. These indicators were created by Bos (1990), Molden et al. (1998), Malano & Burton (2001) and Córcoles et al. (2010) and used by many researchers.

In Türkiye, after the transfer of irrigation management (giving the management, operation and maintenance (MOM) rights of the irrigation system from the government to WUA), numerous studies have been performed to evaluate irrigation performance with these indicators and published in national and international journals.

The studies that have been done on water use, agricultural efficiency, environmental efficiency and financial efficiency indicators (Degirmenci et al. 2003; Cakmak et al. 2010; Arslan & Değirmenci 2017) have a growing body in literature (Kartal et al. 2019; Arslan et al. 2020; Çifçi & Değirmenci 2022). The studies showed that there is no energy use indicator. However, Diker (2018) calculated energy use indicators in the master's thesis on the evaluation of 18 irrigation associations in the Lower Seyhan Plain. The calculation results showed that the energy cost per unit irrigation area was between 2.79 and 123.94 $ ha^{-1}, the energy cost per unit irrigated area was between 2.93 and 132.04 $ ha^{-1} and the energy cost per unit irrigation water supply was between 0.0002 and 0.0158 $ m^{-3}, based on the data of 2011-2015. Çifçi and Değirmenci (2022) found that the highest energy cost per unit irrigation areas for five irrigation associations in the Asi Basin was 233.96 $ ha^{-1}, the energy cost per unit irrigated area was 394.94 $ ha^{-1} and the energy cost per irrigation water supply was 0-0.03494 $ m^{-3}. The literature review showed that most studies on the evaluation of the performance of irrigation associations were conducted in Spain, Türkiye, Italy and Greece.

Vanino et al. (2015) reported that in WUA in Epirus and Western Greece, Greece and Apulia, Italy, the use of surface water resources accounted for 15% of the total irrigation water consumption cost, while the use of underground water resources accounted for 60-90% of the total irrigation water consumption cost, and the energy cost should be reduced by 13%. Rodríguez-Díaz et al. (2011) used data from 10 irrigation regions in Spain, and reported that the amount of energy consumed per unit of irrigation water varied between 0.15 and 0.85 kWh m^{-3}, while Abadia et al. (2010) reported that the amount of energy supply per unit irrigated area in 22 irrigation associations varied between 92.32 and 6.229.90 kWh ha^{-1}, the energy cost per unit irrigated area was between 10.67 and 543.35 € ha^{-1} and the energy cost per unit of irrigation water varied between 0.009 and 0.264 € m^{-3}. Soto-García et al. (2013) reported that the average energy consumption per unit irrigated area in Campo de Cartagena, Miraflores & Calasparra-Cieza irrigation regions, was 1.891-2.997 kWh ha^{-1} between the years 2002 and 2011, while the energy consumption per unit water supply varied between 0.15 and 0.18 kWh m^{-3}. González et al. (2015) found the average energy consumption per unit area in the Andalusian region was 1003 kWh ha^{-1}, and the average energy consumption per unit water was 0.41 kWh m^{-3}. Alcon et al. (2017) studied 5 WUAs in the Segura River Basin District in Spain. One of these WUAs, Miraflores, which uses only groundwater, the average total MOM cost per unit irrigated area is 1014 € ha^{-1} and the average total MOM cost per irrigation water supply is 0.31 € m^{-3}, while the average values of the four other irrigation communities are 554 € ha^{-1} and 0.22 € m^{-3}, respectively. Playán et al. (2018) evaluated the success of current and future telemetry/remote control applications in the irrigation associations in Spain. They stated that these systems will become widespread in irrigation schemes in the short run.

Energy prices have been increasing dramatically in the world in recent years as well as in Türkiye. For this reason, there is a growing body of literature that recognizes the importance of using energy effectively. Energy use plays a crucial role in modern agricultural irrigation systems to use water efficiently. However, there is no considerable amount of literature that has been published on energy use in irrigation systems in Türkiye. Accordingly, researchers have shown an augmenting interest in the evaluation of irrigation schemes with energy use performance indicators.

This study focused on the analysis of the energy, water use and financial management level of Esenli Water User Association located in the Central Anatolia of Türkiye, where most sugar beet is produced and a sprinkler irrigation system has been commonly used, for over 10 years. The study results will contribute to the successful management of irrigation associations, more efficient use of energy and increased production with less water and energy consumption.

2. Material and Methods

2.1. Study area

The Esenli Water User Association of Yozgat province located in the Central Anatolian region of Türkiye was selected as the study area. A semi-arid continental climate prevails in Yozgat province. In the region, summers are hot and dry; winters are cold and rainy.

The average precipitation amount was determined as 516.7 mm (Central Anatolia Development Agency 2011). The location of Esenli Water User Association is located within the borders of Yozgat province in Türkiye (Figure 1).

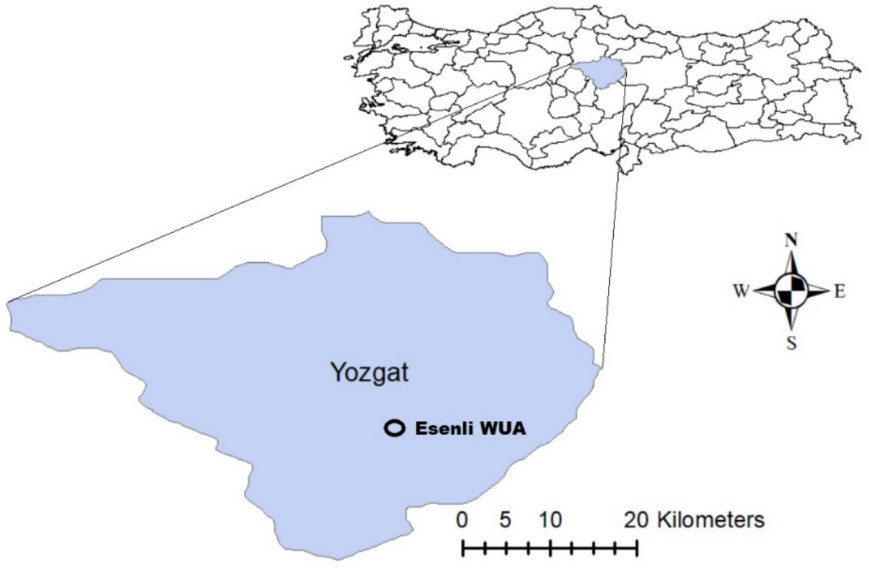

Figure 1- Esenli Water User Association location map

In the Esenli WUA, irrigation is done by pumping, and the information about the irrigation association is given in (Table 1). Sugar beet is cultivated in 98% of the irrigated area, while wheat is cultivated in dry conditions. The pumped irrigation system consists of a pipeline with a total length of 193 km including 40 km of primary pipe and 153 km of secondary pipes. Irrigation water is taken from the Gelingüllü dam, located on the Kanak stream in the Kızılırmak basin through pumping facilities. In the study, the data from 2008-2017 were taken from the irrigation association monitoring and evaluation reports (DSI 2019).

Table 1- Characteristics of Esenli Water Users Associations

Attribute	***Definition***
Command area (ha)	3296
Water distribution method	On-demand
On-farm irrigation methods	Sprinkler irrigation
Main crops	Sugar beet
Number of villages	7
Number of farmers	188
Number of parcels	879
Average parcel width (ha)	1.7
Water pricing method	ha x € (different for each crop)
Average water fee (€ ha-1)	400.67
Is water supply measured?	Yes
Is measurement done at distribution points?	No
Is there a water distribution program?	Yes

2.2. Performance indicators

Various methods have been developed and used to evaluate irrigation performance (Alcon et al. 2017; Zema et al. 2015; 2018; Abadia et al. 2010; Elshaikh et al. 2018; Carrillo-Cobo et al. 2010; Rodríguez-Díaz et al. 2011; González et al. 2015; Kukul et al. 2008). The performance indicators and calculation method used in the performance evaluation of the Esenli Water User Association are given in Table 2. In this study, the performance indicators were divided into four groups, including land use, water use, energy use and financial management. In the calculation of performance indicators, irrigation water supply (m^3), command area (ha), irrigated area (ha), energy consumed (kWh), energy cost (€), total MOM cost (the annual MOM cost of providing the irrigation service such as

salaries, communication, transportation, repairing, building, etc., €), maintenance cost (€), total revenue (€), staffing cost (€), water fee accrual (€) and water fee collection (€) data of the Esenli Water User Association over 10 years (2008-2017) were used. The changing currency of the Turkish Lira to the Euro, and the values of the year were used according to the Türkiye Central Bank.

Table 2- Description and calculation method of the performance indicators used in this study

Domain	*Indicator name*	*Abrev*	*Unit*	*Calculation*
Land use (Arslan & Değirmenci, 2018)	Irrigation ratio	ICR	%	$\frac{(Irrigated\ area)}{(Command\ area)}$
Water use (Córcoles et al. 2010; Zema et al. 2015)	Annual irrigation water supply per unit command area	VsSa	$m^3\ ha^{-1}$	$\frac{Irrigation\ water\ supply}{Command\ area}$
	Annual irrigation water supply per unit irrigated area	VsSr	$m^3\ ha^{-1}$	$\frac{Irrigation\ water\ supply}{Irrigated\ area}$
Energy use (Córcoles et al. 2010)	Specific energy	EacVs	$kWh\ m^{-3}$	$\frac{Energy\ consumed}{Irrigation\ water\ supply}$
	Energy consumed per unit irrigated area	EacSr	$kWh\ ha^{-1}$	$\frac{Energy\ consumed}{Irrigated\ area}$
	Energy cost per unit irrigated area	CENSr	$€\ ha^{-1}$	$\frac{Energy\ cost}{Irrigated\ area}$
	Energy cost per irrigation water supply	CENVs	$€\ m^{-3}$	$\frac{Energy\ cost}{Irrigation\ water\ supply}$
	Energy cost to total MOM costs ratio	EacMOMc	%	$\frac{Energy\ cost}{Total\ MOM\ cost}$
Financial management (Córcoles et al. 2010; Zema et al. 2015)	Total MOM cost per irrigation water supply	CMSVs	$€\ m^{-3}$	$\frac{Total\ MOM\ cost}{Irrigation\ water\ supply}$
	Total MOM cost per unit irrigated area	CMSSr	$€\ ha^{-1}$	$\frac{Total\ MOM\ cost}{Irrigated\ area}$
	Maintenance cost	CM	$€\ ha^{-1}$	$\frac{Maintenance\ cost}{Irrigated\ area}$
	Revenue collection performance	RCP	%	$\frac{Gross\ revenue\ collected}{Gross\ revenue\ invoiced}$

3. Results and Discussion

This study revealed that the performance indicators have varied greatly over the years. The average, range (min-max) and standard deviation values of the indicators selected in the study for the period of 2008-2017 are given in (Table 3).

3.1. Land use

The irrigation rate is an indicator used to evaluate the service area of the irrigation scheme. The irrigation rate needs to increase over the years. The average, min and max values of the irrigation rate of the study area between 2008 and 2017 are 39%, 23% and 51%, respectively. They are among the acceptable values according to the limit values given below. The irrigation rate has exceeded 50% only in 2013, over the 10 years Figure 2. Wheat is cultivated in the irrigation area in dry conditions. Farmers think that the precipitation is sufficient in these areas and do not demand irrigation water. The average ICR in the study region was 50%, 68% on average of Türkiye's WUAs according to DSI (State Hydraulic Works) reports (DSI 2019). Yercan et al. (2009) determined the acceptable limit values of ICR based on the literature and found that the irrigation rate of the irrigation associations in the Gediz river basin in Türkiye was 80%. Where, ICR is poor if in the range <30%, acceptable if 30-40%, satisfactory if between 40 and 50% and good if >50%. Zema et al. (2018) found the average ICR value to be 27.9% in 10 irrigation associations in the Calabria region of Southern Italy and considered this as poor according to the aforementioned literature.

Table 3- Average, range and standard deviation of the performance indicators

Domain	*Indicator name*	*Abrev*	*Unit*	*Average*	*Range*	*Std dev.*
Land use	Irrigation ratio	ICR	%	39	23-51	8
Water use	Annual irrigation water supply per unit command area	VsSa	m^3 ha^{-1}	3251	2355-4656	799
	Annual irrigation water supply per unit irrigated area	VsSr	m^3 ha^{-1}	8229	6458-10208	1286
Energy use	Specific energy	EacVs	kWh m^{-3}	0.33	0.12-0.48	0.09
	Energy consumed per unit irrigated area	EacSr	kWh ha^{-1}	2671	1157-3563	670
	Energy cost per unit irrigated area	CENSr	€ ha^{-1}	259	66-344	104
	Energy cost per irrigation water supply	CENVs	€ m^{-3}	0.033	0.01-0.05	0.01
	Energy cost to total MOM costs ratio	EacMOMc	%	64	15-94	26
Financial management	Total MOM cost per irrigation water supply	CMSVs	€ m^{-3}	0.05	0.03-0.07	0.01
	Total MOM cost per unit irrigated area	CMSSr	€ ha^{-1}	403	266-549	79
	Maintenance cost	CM	€ ha^{-1}	21.6	3.6-57.4	17.5
	Revenue collection performance	RCP	%	76	42-88	17

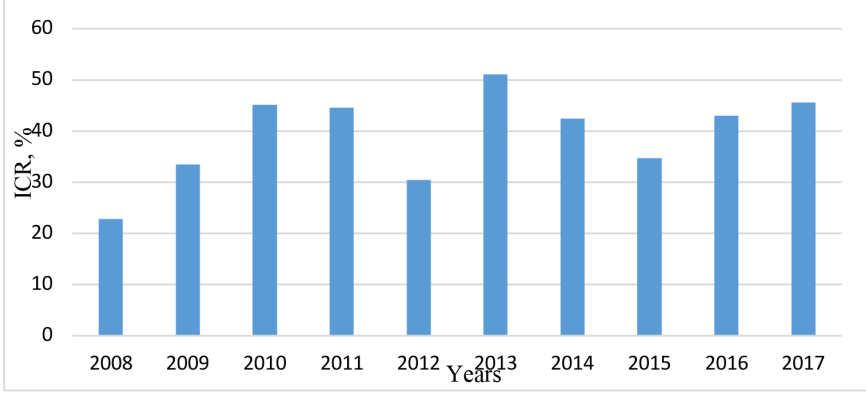

Figure 2- Irrigation ratio (ICR)

3.2 Water use

The VsSa value was 3251 m^3 ha^{-1} on average and in the range of 2355-4656 m^3 ha^{-1}. The VsSr value was 8229 m^3 ha^{-1} on average and in the range of 6458-10208 m^3 ha^{-1} Table 3. The change of VsSa and VsSr indicators over the period of 2008-2017 is given in Figure 3. As seen in Figure 3, both VsSa and VsSr values have changed considerably on a yearly basis. This shows that the irrigation association has been unable to fully apply the planned water distribution. Moreno et al. (2010) found the VsSa value in 15 irrigation associations in Spain to be in the range of 739.7 (Drip irrigation method) and 7,189.5 (sprinkler irrigation method) m^3 ha^{-1}. Alcon et al. (2017) reported that the average VsSr was 2889 m^3 ha^{-1} and the maximum amount delivered reached 4255 m^3 ha^{-1}.

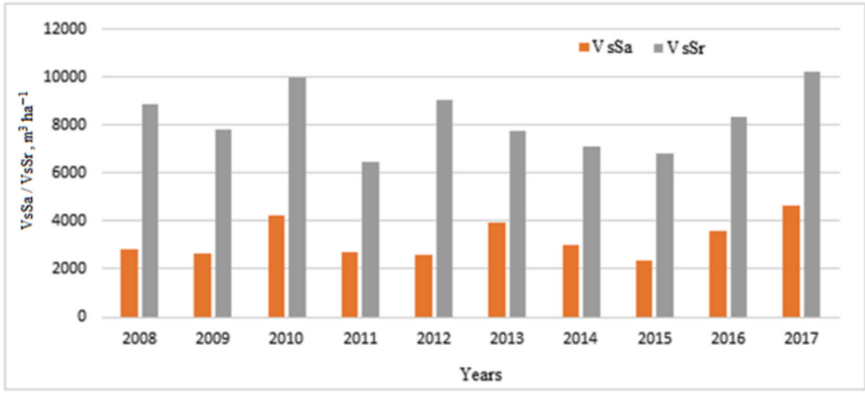

Figure 3- Annual irrigation water supply per unit command/irrigated area (VsSa/VsSr)

3.3. Energy use

The EacVs was found to be 0.33 kWh m^{-3} on average and in the range of 0.12-0.48 kWh m^{-3} and the standard deviation was found as 0.09 kWh m^{-3}. Rocamora et al. (2013) found the EacVs to be 1.71 and 1.69 kWh m^{-3} in the 2-year irrigation season (2009-2011) in Spain. The EacSr was found to be 2671 kWh ha^{-1} on average and in the range of 1157-3563 kWh ha^{-1}, and the standard deviation was found as 670. Abadia et al. (2010) defined the Esenli Water User Association as 'very large' consumer based on the scale for evaluating the energy amount consumed per irrigated area. When the change of EacVs and EacSr values between 2008 and 2017 is examined, it is seen that energy use was similarly based on area and volume, and in 2012, 2016 and 2017, EacVs decreased while the EacSr increased as seen in Figure 4. The situation resulted in lower irrigated area and higher irrigation water use can be considered as management and operation problem in the years. In Türkiye, value of average energy use is 14% (DSI 2019). The value was found that the EacVs in irrigated agriculture in Spain was between the range of 0.03 kWh m^{-3} and 0.17 kWh m^{-3} between 1950 and 2017, and the energy use increased by 2.9% between 1950 and 1979 and by 4.5% between 2014 and 2017 (Espinosa-Tasón et al. 2020). González et al. (2015) found that the EacSr in 10 irrigation associations in the Andalusian irrigation region was 1003 kWh ha^{-1} on average and in the range of 455-1901 kWh ha^{-1}, and the standard deviation was 418.1 kWh ha^{-1}.

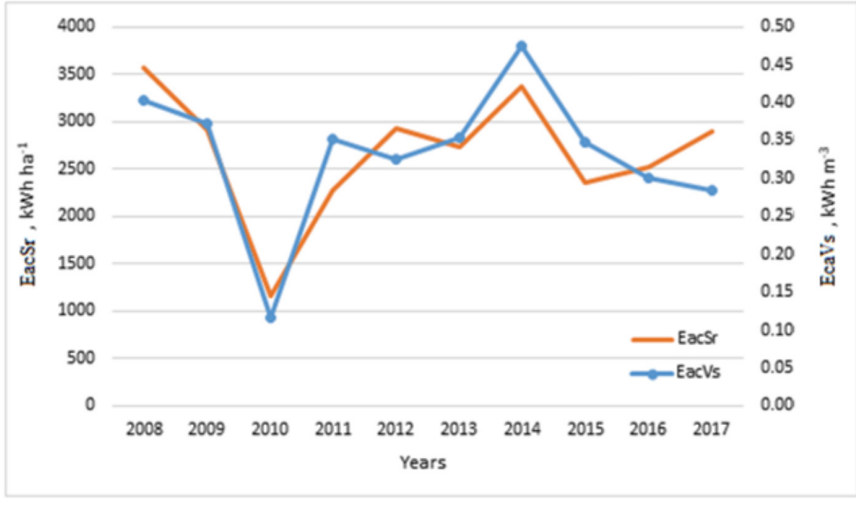

Figure 4- Specific energy (EacVs) and energy consumed per unit irrigated area (EcaSr)

The CENSr was 259 € ha^{-1} on average and in the range of 66-344 € ha^{-1}, and the standard deviation was 104. The CENVs was 0.03 on average and in the range of 0.01-0.05 € m^{-3}, and the standard deviation was 0.015 Table 3. The CENSr and CENVs rapidly increased between 2010 and 2011 as seen in Figure 5. In Asi River basin, the indicator value was found 0.007 $ m^{-3} on average which is very low comparing with the study area due to the technological backwardness used in the irrigation area (Çifçi & Değirmenci 2022). Studies on WUAs installed by only pressurized irrigation system are more suitable for benchmarking such as Spain and Italia' experience. García et al. (2014) found that the CENSr in five irrigation schemes in the Andalusian irrigation region of Southern Spain was in the range of 48.9-147.6 € ha^{-1} after the modernization of the irrigation schemes. The energy cost increased by 149% compared to before the

irrigation modernization. Carrillo-Cobo et al. (2010) determined the average CENVs as 0.05 € m^{-3} in their study with monthly data in the Fuente Palmera irrigation district in Spain in 2007. They determined that there was a significant change in energy cost in the year.

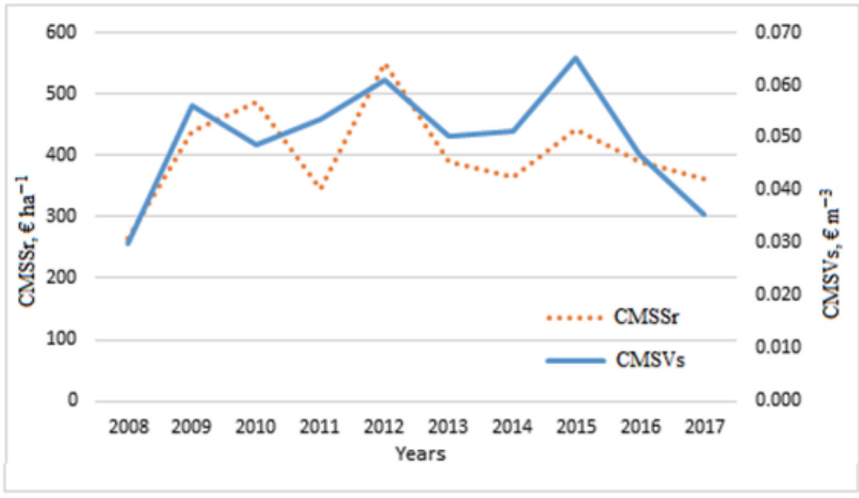

Figure 5- Energy cost per irrigation water supply (CENVs) and the energy cost per unit irrigation area CENSr

The indicators' values sharply increased due to sharp increase in energy prices after the year 2009. The EacMOMc was 64% on average and in the range of 15-94% as seen in Table 3. The energy cost has had a very high share in the total MOM cost since 2010 as depicted in Figure 6. González et al. 2015 found the EacMOMc in 10 irrigation associations in Southern Spain to be 36.4% on average. The EacMOMc is 23% on average in Türkiye according to DSI reports (DSI 2019). The higher EacMOMc may show modernization works have been continued in the irrigation area and more investment is needed to give better service to farmers. Carrillo-Cobo et al. (2010) stated that total energy cost accounts for 28% of MOM costs. Rodríguez-Díaz et al. (2011) found that the energy cost in 10 irrigation regions in the Andalusian region of Spain increased approximately ten times compared to pre-modernization, and that the post-modernization energy cost accounted for 30% of the total MOM cost. They found that the EacMOMc was 36.40% on average and in the range of 16.1-65.3% and the standard deviation value was 15.1%. Córcoles et al. (2010) found out in their study in seven irrigation associations in the Castilla-La Mancha region of Spain based on the data of 2006-2008 that the energy cost accounted for ~45% of the total MOM cost in the case of drip irrigation, while it accounted for 70% in case of sprinkler irrigation. It is seen that the 10-year average EacMOMc of the Esenli Irrigation Association, where the sprinkler irrigation method is applied, is very high.

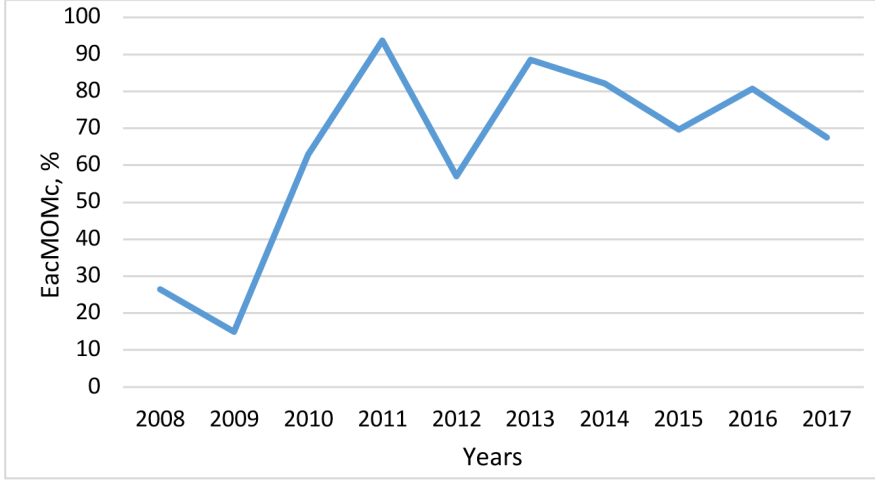

Figure 6- Energy cost to total MOM cost ratio (EacMOMc)

3.4. Financial management

Financial indicators measure how efficiently an irrigation association uses economic resources (Córcoles et al. 2010). It is essential to measure financial management performance for a sustainable irrigation association.

The CMSVs was 0.05 € m^{-3} on average and in the range of 0.03-0 between 2008 and 2017. The CMSSr was 403 € ha^{-1} on average and in the range of 266-549 € ha^{-1} (Table 3). The change of CMSVs and CMSSr over the period of 2008-2017 is given in Figure 7. González et al. (2015) found that the CMSVs in 10 irrigation Associations in Southern Spain was 0.10 € m^{-3} on average and in the range of 0.18-0.04 € m^{-3}. In sprinkler irrigation systems, energy costs account for 60-78% of total MOM costs, and in drip irrigation systems, they account for approximately 45% of total costs. Zema et al. (2018) found the CMSSr to be 1445 € ha^{-1} on average in 10 irrigation associations and stated that this variability can occur, considering that the MOM costs vary depending on different factors. The MOM cost is affected by changes in energy prices, maintenance and repair requirements, annual climate factors, water fee collection rate, the characteristics of the pumping facility, and management performance.

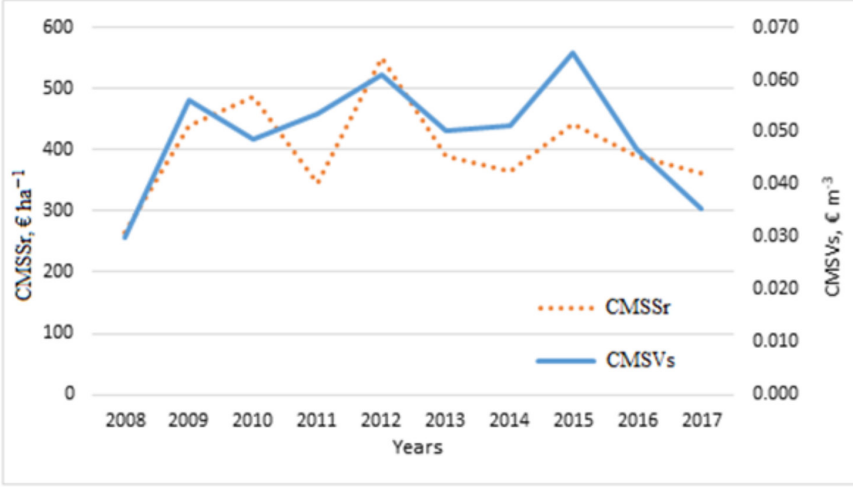

Figure 7- Total MOM cost per unit irrigated area (CMSSr) and total MOM cost per irrigation water supply

Maintenance cost is an indicator that measures the maintenance costs spent per unit area. The CM was found to be 21.6 € ha^{-1} on average and in the range of 3.6-57.4 € ha^{-1}. It is seen that the maintenance costs spent per unit area decreased from 2008 to 2013 (Figure 8). García et al. (2014) found that in five irrigation schemes in the Andalusian region of Spain, the CM was between 42.9 and 80.1 € ha^{-1} before the modernization and 76.5 and 106.4 € ha^{-1} after the modernization. It was found that the water distribution capacity was insufficient in 1/3 of irrigation schemes and deteriorated due to poor maintenance (García-Bolanos et al. 2011).

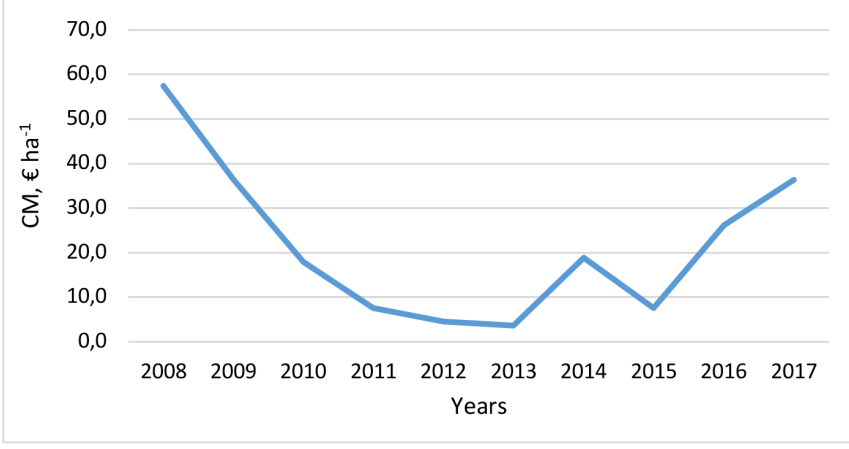

Figure 8- Maintenance cost (CM)

In the study, the RCP varied between 42% and 88% and was 76% on average. Yercan et al. (2009), based on some literature, determined the acceptable RCP value as follows; poor if <40%, acceptable if in the range 40-60%, satisfactory if between 60% and 75% and good if >75%. Based on these limit values, Esenli WUA can be considered as satisfactory. Since 2010, the RCP can be considered as very good (Figure 9). Svendsen & Murray-Rust (2001) reported that after the irrigation management transfer of irrigation schemes in Türkiye, the RCP has increased.

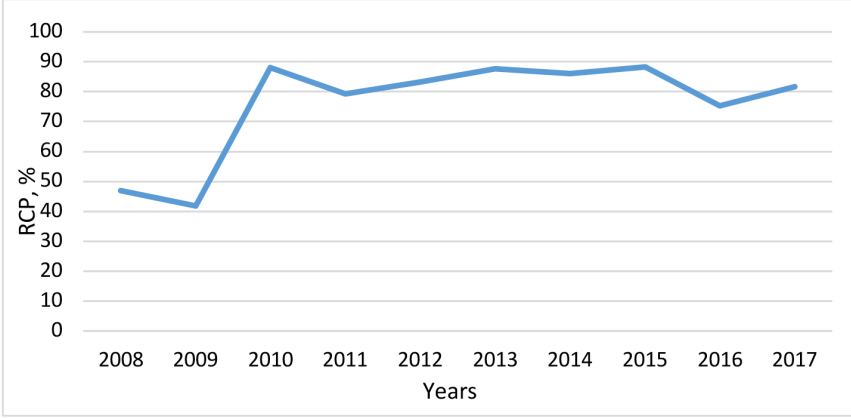

Figure 9- Revenue collection performance (RCP)

The correlation between CENSr and ICR is high and positive ($p<0.05$; $r=0.668$). In this case, it is seen that as the energy consumption per unit irrigated area increases, the irrigated area also increases (Table 4). A very high positive correlation ($p<0.01$; $r=0.862$) was found between CENSr and CENVs. Similarly, also very high positive correlation was found between CENSr and EacMOMc ($p<0.01$; $r=0.915$). In this context, as the energy consumption per unit irrigated area increases, the total MOM costs also increase. A very high negative correlation was found between CENSr and CM ($p<0.01$; $r=-0.850$). The correlation between CENSr and RCP is high and positive ($p<0.01$; $r=0.943$). It is seen that as the energy cost per unit irrigated area increases, the water fee collection rate also increases.

The observed correlation between energy and irrigated area, water consumption in this study was significant ($p<0.01$). This finding supports the findings of other studies in this field linking energy with irrigated area and water consumption (Diker 2018). The findings confirm that agricultural lands can be irrigated with less energy use when compared to the results of other studies (González et al. 2015; García et al. 2014; Carrillo-Cobo et al. 2010).

Table 4. Correlation between indicators

	ICR	*VsSa*	*VsSr*	*EacVs*	*EacSr*	*CENSr*	*CENVs*	*EacMOMc*	*CMSVs*	*CMSr*	*CM*	*RCP*
ICR	1	0.67*	-0.04	-0.31	-0.46	0.66*	0.51	0.76**	0.02	0.01	-0.48	0.644*
VsSa		1	0.66*	-0.58	-0.32	0.24	-0.12	0.28	-0.45	-0.07	0.10	0.35
VsSr			1	-0.64*	-0.157	-0.17	-0.60	-0.32	-0.44	0.19	0.41	-0.03
EacVs				1	0.844**	-0.25	0.03	-0.05	-0.02	-0.46	0.12	-0.29
EacSr					1	-0.46	-0.372	-0.30	-0.32	-0.47	0.43	-0.44
CENSr						1	0.862**	0.91**	0.34	0.35	-0.85**	0.94**
CENVs							1	0.87**	0.46	0.13	-0.80**	0.75*
EacMOMc								1	0.11	-0.03	-0.69*	0.83**
CMSVs									1	0.73*	-.67*	.29
CMSr										1	-.57	.34
CM											1	-.77**
RCP												1

*The correlation is statistically significant with the value of $p<0.10$, **$p<0.05$ and (coma) represents "0." ICR: Irrigation ratio, VsSa: Annual irrigation water supply per unit command area, VsSr: Annual irrigation water supply per unit irrigated area, EacVs: Specific energy, EacSr: Energy consumed per unit irrigated area, CENSr: Energy cost per unit irrigated area, CENVs: Energy cost per irrigation water supply, EacMOMc: Energy cost to total MOM costs ratio, CMSVs: Total MOM cost per irrigation water supply, CMSSr: Total MOM cost per unit irrigated area, CM: Maintenance cost, RCP: Revenue collection performance

4. Conclusions

It is important to evaluate the annual performance of WUAs with acceptable indicators for a sustainable irrigated agriculture. The results to be obtained can be used to improve the existing and similar irrigation schemes.

The energy consumption per unit irrigated area fluctuates. An average of 2671 kWh of energy was consumed per ha area. An average of 0.33 kWh of energy was consumed per m^3 water supplied as irrigation water. According to these values, WUA was considered a 'very large' energy consumer. The unit irrigated water supply and the energy cost per unit irrigated area have increased rapidly since 2010. The most important factor affecting energy cost is the rapid increase in energy prices. A discount should be made on the applied agricultural energy prices. Furthermore, legal arrangements should be made to allow irrigation associations to meet their energy needs from renewable energy sources (solar energy, wind energy, etc.) to reduce energy costs.

In pressurized irrigation systems, energy costs have an important place among the total MOM costs. The average EacMOMc value was found as 64%. This rate is quite high. To reduce the energy cost, necessary measures, such as reducing reactive energy costs, choosing the right pump and making pump maintenance repairs in time, should be taken. The MOM costs varied against the unit irrigated area and the supplied irrigation water. An effective monitoring and evaluation system should be established to reduce MOM costs. The irrigation system performance is mostly affected by factors in the operational phase rather than those in the planning and designing phases. Dissemination of decision support systems in irrigation associations, establishment of GIS (geographic information systems) infrastructure and establishment of a traceable management infrastructure will be effective in reducing MOM costs. The maintenance cost per unit irrigated area decreased from 2008 to 2013. It is observed that the required maintenance was performed in the irrigation system during this period. The sustainability of an irrigation system depends on the fulfilment of maintenance. A lack of maintenance for one year will create more maintenance and energy needs in the following years. Collection of irrigation water fee on time and spending it effectively for MOM activities is one of the important criteria in determining the performance of the irrigation association. In the study, the irrigation water fee collection rate was found to be 'high'. Necessary care should be taken for the efficient use of collected irrigation water fees.

In conclusion, determination of the performance levels of irrigation associations is very important for drawing lessons from the previous results and for contributing to the improvement of other irrigation associations with low performance. To increase irrigation efficiency in an efficient and successful manner, emphasis should be placed on the modernization of irrigation systems. Reducing energy costs and regular maintenance of irrigation systems emerged as the most important task in the sustainability of the irrigation system.

Data availability: Data are available on request due to privacy or other restrictions.

Authorship Contributions: Concept: S.K., H.D., F.A., İ.G., Design: S.K., H.D., F.A., Data Collection or Processing: S.K., H.D., F.A., İ.G., Analysis or Interpretation: S.K., H.D., F.A., İ.G., Literature Search: S.K., H.D., F.A., Writing: S.K., H.D., F.A.

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Assessment of Growth, Metallic Ion Accumulation, and Translocation of Lavandin *(Lavandula × intermedia)* Plant in Cadmium Stress

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ABSTRACT

Excess cadmium (Cd), which is toxic to plants, severely limits crop production in agricultural areas. For this reason, this study investigated the effect of increased Cd levels on lavandin growth, some physiological parameters, and metallic ion accumulation and translocation. In greenhouse conditions, six different levels of Cd (0, 25, 50, 100, 150, and 200 µM Cd) were applied to plants grown in perlite medium together with a complete nutrient solution. Increasing Cd levels decreased biomass production in both the shoots and roots and the contents of chlorophyll (Chl) *a, b, a+b*, and carotenoid (Car). In addition, excessive Cd decreased the concentrations of some metallic cations such as iron (Fe), zinc (Zn), manganese (Mn), and calcium (Ca) in the shoots and roots. Similarly, increasing Cd decreased the bio-concentration factor (BCF) of the metallic cations (BCF of Cd, Fe, Mn, and Zn in both the shoots and roots and the BCF of copper (Cu) in the roots. Toxic Cd levels decreased the translocation factor (TF) of Zn and Cu and the net accumulation (NA) via roots in Fe and Zn. The effect of Cd on the NA via roots in K, Ca, Mn, and Cu was not found to be significant. However, increasing Cd caused an increase in shoot and root membrane permeability and the TF of Fe and Mn. It was concluded that Cd2+ ion interacts divalent cations such as Ca2+, Fe2+, Zn2+, and Mn2+ ions and could affect the concentrations of these ions in the shoots and roots, and excess Cd has a negative effect on the growth and the photosynthetic capacity of lavandin.

Keywords: Bio-concentration, Chlorophyll, Membrane permeability, Metal translocation, Nutrient imbalance, *Lavandula hybrida*

1. Introduction

Cadmium (Cd), a non-nutritive element for plants, is regarded as toxic to plants and other living organisms and has serious pollutant effect on the environment. This metal's contamination of agricultural soils is largely driven by mining activities such as the smelting of Zn-containing ores, burning of fossil fuels, incineration of wastes, use of sewage sludge as fertilizer, contaminated irrigation water, and the applications of phosphorus fertilizers (Alloway & Steinnes 1999). The fact that it is an easily soluble element in water increases its polluting capacity and toxicity effects on the environment.

In plants, Cd toxicity causes the most visible symptoms through chlorosis, leaf curls, and stunting (Benavides et al. 2005), while it also inhibits plant growth (Hediji et al. 2021), and decreases photosynthetic activity (Sandalio et al. 2001; Irshad et al. 2021). In addition, Ekmekçi et al. (2008) reported that excess Cd can cause an increase in reactive oxygen species at the cellular level and thus damages membranes, cell molecules, and organelles. In addition, it may cause a decrease in nutrient and water uptake and ion translocation, an increase in oxidative damage, and disruptions in a plant's metabolism (Haider et al. 2021). Cd toxicity may also adversely affect the uptake and transfer of K, P, Ca, Mg, and Mn (Nazar et al. 2012). Previous studies in relation to Cd toxicity have provided significant findings concerning plant growth in pea plants (Sandalio et al. 2001), in Indian mustard (Goswami & Das 2015), and beans (Hediji et al. 2021). Excess Cd also induces leaf chlorosis accompanied by a lowering of the photosynthetic rate in maize cultivars (Ekmekçi et al.

2008) and wheat (Irshad et al. 2021). Angelova (2012) reported that some medicinal and aromatic plants, such as *Valeriana officinalis* L. and *Melissa officinalis* L., uptake heavy metals from the root zone through the root system and accumulate a large part of them in their roots. In a later study Angelova et al. (2015) states that the Lavandin plant, which is more tolerant to heavy metals, can grow in polluted soils and generally accumulate heavy metals in various organs, with the exception of essential oils.

The genus *Lavandula* belongs to the *Lamiaceae* family and includes cultivars and hybrids of about 40 species. Lavandin, which is called hybrid lavender/or hardy lavender, is widely grown in the Mediterranean basin, especially on calcareous soils, and appears as a shrub form that can grow up to 70-80 cm. The interest in this species has increased in recent years due to its high yield of essential oils, its ability to grow in contaminated or barren agricultural lands, and being wide in demand for its oil in the food and cleaning industry (Platt 2009). In addition, its appealing appearance during the flowering period makes it popular for agro-tourism and urban parks (Veeck et al. 2016). This study determines the effects of Cd on plant growth and concentration, accumulation, and translocation of ions in lavandin plants. In addition, this study not only gives an idea about the behaviour of Cd in a plant belonging to the *Lamiaceae* family but also presents the potential for the plant to be grown in Cd-contaminated soils as aromatic as well as ornamental plants.

2. Material and Method

2.1. The experimental design

The greenhouse experiment was carried out with lavandin (*Lavandula × intermedia*), a hybrid of *L. angustifolia* and *L. latifolia*, in the summer season of 2017 and lasted for 30 days. The average air temperature and relative humidity were measured at 26/17 °C (day/night) and 65%, respectively. Four-month-old seedlings were obtained from a private company producing outdoor ornamental plants and transferred to 2 L capacity polyethylene pots (one plant per pot) in the hydroponic system [modified Hoagland solution (Hoagland & Arnon 1950) and perlite as an inert media] inside a greenhouse under natural light conditions. Prior to Cd application, the lavandin saplings were irrigated with different rates of modified Hoagland solution for 12 days (four days quarter-strength, four days half-strength, and four days full-strength) for acclimatization. This solution contained calcium nitrate tetrahydrate [$Ca(NO_3)_2.4H_2O$] (5 mM), potassium nitrate (KNO_3) (5 mM), magnesium sulphate heptahydrate ($MgSO_4.7H_2O$) (2 mM), potassium di-hydrogen phosphate (KH_2PO_4) (1 mM), boric acid (H_3BO_3) (45.5 µM), iron sulphate heptahydrate ($FeSO_4.7H_2O$) (44.7 µM), sodium chloride (NaCl) (30.0 µM), manganese sulphate monohydrate ($MnSO_4.H_2O$) (9.1 µM), zinc sulphate heptahydrate ($ZnSO_4.7H_2O$) (0.77 µM), copper sulphate pentahydrate ($CuSO_4.5H_2O$) (0.32 µM), ammonium molybdate tetrahydrate [$(NH_4)_2Mo_7O_{24}.4H_2O$] (0.10 µM), and disodium EDTA dihydrate ($Na_2EDTA.2H_2O$) (54.8 µM). The pH of the nutrient solution was maintained at 6.5 throughout the experiment.

The saplings were exposed to six applications; including control (0), 25, 50, 100, 150, and 200 µM Cd (as $CdCl_2$). These levels were dissolved separately in a full-strength modified Hoagland solution in different containers. During the period of the experiment, the plants were irrigated with these solutions (100 mL per day). The Cd levels were selected based on the doses used in previous studies and the toxicity of these doses to various plant species (Cikili et al. 2016; Kaya et al. 2009).

2.2. Procedures in sampling and harvesting

At the end of the 30th day, the harvested plants were separated into shoots and roots to determine the weights of biomass (the FWs and DWs). The aerial parts were weighed for shoot FW. The roots were carefully cleaned from the soil and dipped for 15 minutes in an aerated 0.5 mM calcium chloride ($CaCl_2$) solution to remove the nutrients adsorbed from the root surface and weighted for root FW. In order to remove any particles that may have adhered to plant surfaces, samples were washed three times under running tap water and rinsed with deionized water. Afterwards, all shoots and roots were kept in an oven with regulated ventilation at 70 °C in order to dry them until their weight was stable. The shoot and root dry weights of the dried plant samples were determined separately and then ground for nutritional ion analysis.

2.3. Determination of photosynthetic pigments and membrane permeability

The membrane permeability (MP) in the disk samples taken from fresh leaves was measured by electrical conductivity (EC%) (Yan et al. 1996). Briefly, 1 cm^2 pieces of washed fresh leaf were placed in a beaker containing 10 mL (30 °C) deionized water for three hours, and the conductivity of the solution was then measured (EC_1). After boiling the same samples for 20 mins, they were cooled to room temperature and their conductivity was measured again (EC_2). The MP (EC %) was calculated using the formula [$(EC_1/EC_2) \times 100$].

The photosynthetic pigment contents were measured in fully expanded young leaves immediately before harvest. For this, fresh leaf samples (0.25 g) were cut into small pieces and homogenized using a homogenizer (Heidolph DIAX 900, Kelheim, Germany) in 10 mL of acetone (90%, v/v).

The absorbance of the filtered extract was determined at 663, 645, and 470 nm using a spectrophotometer (Shimadzu UV-1201, Japan), and then the Chl *a*, Chl *b*, and carotenoid (Car) contents were calculated, respectively, according to the formula reported by Lichtenthaler (1987).

2.4. Determination of metal ions

To measure the nutrient ion concentrations, 500 mg of each of the shoot samples were dry-ashed in a muffle furnace at 500 °C for 6 h, and then the cooled ash was dissolved in 10 N nitric acid (HNO_3) solution (Miller 1998). The concentrations of Cd, Fe, Zn, Mn, and Cu were determined using ICP-OES (Perkin Elmer Optima 2100 DV; Waltham, MA). The concentration of K and Ca were analysed using a flame photometer.

2.5. Determination of bio-concentration, translocation, and accumulation

The capacity of the lavandin plant to accumulate Cd was evaluated based on the bio-concentration factor (BCF), the translocation factor (TF), the total accumulation rate (TAR), and net ion accumulation (NA) via roots. The BCF is defined as the ratio of the total metal concentration in the aerial parts to the metal concentration in the rooting media, and it is accepted as an indicator of the ability to absorb metals and transport them to the shoots (Cikili et al. 2016). The TF value is defined as the ratio of heavy metal concentration in the shoot to that in the root (Cikili et al. 2016). The TAR, which is a useful parameter for bioaccumulation studies, is a measure of plants' heavy metal uptake (Zhu et al. 1999). The NA of ions via roots is the rate of total ion quantities in the whole plant to root DW. The last two values have been calculated using equations [1] and [2], respectively (Moradi & Ehsanzadeh 2015).

$$\text{TAR of Cd (µg/g DW/day)} = (Cd_{shoot} \times DW_{shoot}) + (Cd_{root} \times DW_{root}) / \text{growth day} \times (DW_{shoot} + DW_{root}) \quad [1]$$

$$\text{NA of ions via roots (mg or µg / g DW)} = [ion]_{shoot} / DW_{root} \quad [2]$$

where $[ion]_{shoot}$ or $_{root}$ is ion concentration in shoot or root.

2.6. Statistical analysis

The experiment was designed in a completely randomized factorial design (in three replications) and the data obtained were analysed using ANOVA with the MINITAB package program (Minitab Corp., State College, PA). The differences between applications were analysed using Duncan's multiple range test at the significance level (α=0.05).

3. Results

3.1. Shoot and root biomass

A significant decrease in shoot and root FWs and DWs of lavandin were found with increasing Cd levels (Figure 1). These decreases varied from 25.9% and 39.1% in shoot FW, 31.3% and 50.7% in root FW, 26.4% and 49.6% in shoot DW, and 33.8% and 44.1% in root DW.

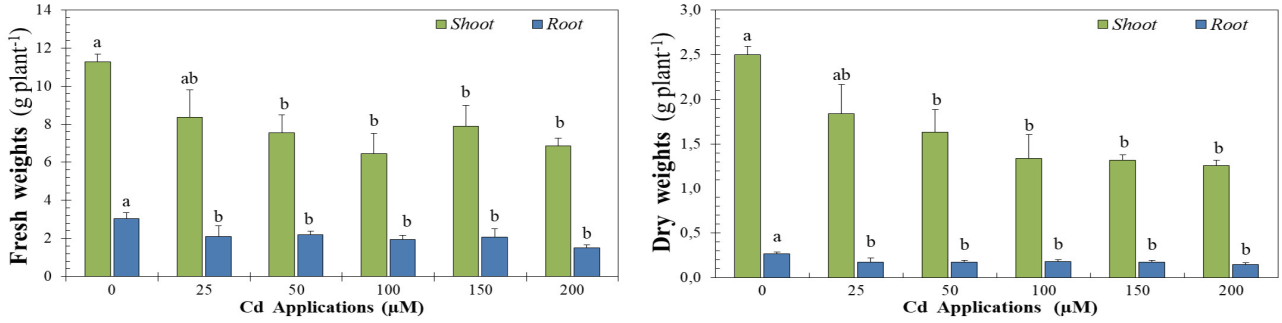

Figure 1- The effect of Cd toxicity on fresh and dry weights of leaves and roots of lavandin. (Bars indicate means of three replicates ± SE. Different letters on the bars for each parameter differ significantly according to DMRT)

3.2. Membrane permeability and photosynthetic pigment contents

The increase in Cd levels considerably affected the MP values and photosynthetic pigment contents of the lavandin leaves compared to the control (Figure 2). Particularly high Cd levels (150 and 200 µM) resulted in a significant increase in the MP values in the roots of lavandin at 29.5% and 31.6% and also in its leaves at 26.3% and 28.0%, respectively. On the other hand, increasing Cd levels (25, 50, 100, 150, and 200 µM) caused a notable decrease in Chl *a*, Chl *b*, Chl *a*+*b*, and Car content in the lavandin leaves. These decreases in Chl *a* content were found to be 13.5%, 24.6%, 43.8%, 64.8, and 57.8%, respectively. Similar decreases in Chl *b*, Chl *a*+*b*, and Car contents of lavandin were also found (Figure 2).

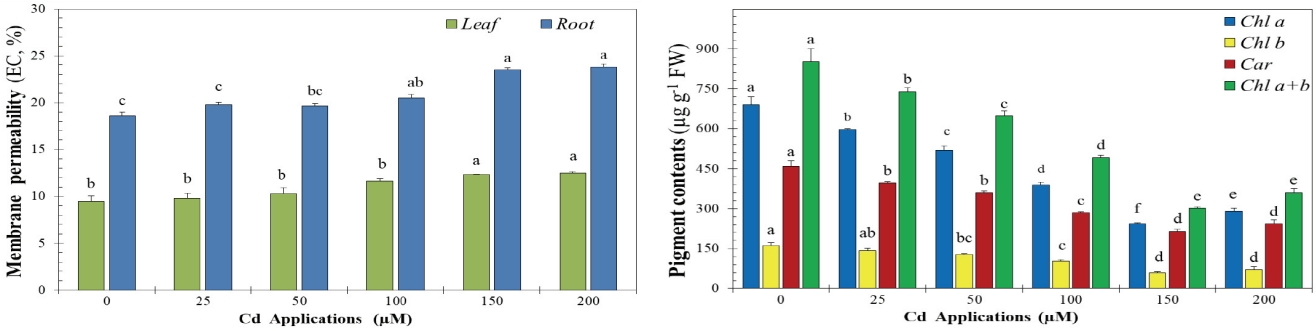

Figure 2- The effect of Cd toxicity on membrane permeability in leaves and roots, and photosynthetic pigment contents in leaves of lavandin (Bars indicate means of three replicates ± SE. Different letters on the bars for each parameter differ significantly according to DMRT)

3.3. Concentration, translocation and accumulation of metallic nutrients

Shoot and root Cd concentrations increased considerably with all Cd applications. Those increases with the lowest and highest Cd levels ranged from 7.3-fold to 27.5-fold in the shoots and from 39.8-fold to 96.0-fold in the roots (Table 1). At the same time, the shoot and root BCF of lavandin plant showed a decreasing tendency depending on increasing Cd levels (Table 1). While the root BCF value of Cd was found by 1.56 at the lowest Cd (25 µM) level, it was 0.73 at the highest Cd (200 µM) level. Similarly, these decreases in the roots ranged from 293 to 88 in the lowest and highest level of Cd. The TF of Cd significantly decreased with all Cd levels compared to the control. All Cd levels (25, 50, 100, 150, and 200 µM Cd) caused a marked increase in the TAR of Cd at 30.8-, 54.1-, 92.9-, 95.6-, and 92.4-fold, respectively.

Table 1-The effect of Cd toxicity on concentrations, bio-concentration, translocation, and accumulation of Cd in lavandin

Treated Cd (µM)	*Concentrations of Cd (µg g^{-1} DW)*		*BCF of Cd*		*TF of Cd*	*TAR of Cd (µg g^{-1} DW day^{-1})*
	Shoot	*Root*	*Shoot*	*Root*		
0	0.6±0.03^{e}	20.7±1.59^{e}	-	-	2.71±0.37^{a}	0.08±0.01^{c}
25	4.4±0.20^{d}	824.1±7.36^{d}	1.56±0.07	293±2.6	0.53±0.02^{b}	2.46±0.14^{b}
50	5.1±0.21^{d}	1278.1±40.4^{c}	0.90±0.04	227±7.2	0.40±0.02^{b}	4.33±0.29^{b}
100	10.0±0.39^{c}	1756.7±156^{b}	0.89±0.04	156±13.9	0.58±0.07^{b}	7.43±1.38^{a}
150	14.2±1.13^{b}	1847.5±51.0^{ab}	0.84±0.07	110±3.0	0.77±0.07^{b}	7.65±0.87^{a}
200	16.5±0.54^{a}	1988.0±50.6^{a}	0.73±0.02	88±2.3	0.83±0.04^{b}	7.39±0.49^{a}
F-test	***	***	-	-	***	***

All values are the average of three replicates (means ± SE, n=3). Different letters in the same column differ significantly according to the DMRT. *F*-test shows significant difference at ***, p<0.001

The effects of Cd levels on the K concentrations in the shoot and root were not found to be significant in the lavandin plant, compared to the control (Figure 3). However, Cd levels caused a notable decrease in Ca concentration in the shoots and roots. These decreases ranged from 15.8% to 28.6% in the shoots and from 24.5% to 44.8% in the roots.

Increasing Cd levels were also found to significantly affect the micronutrient (Fe, Mn, Zn, and Cu) concentrations when compared with the control (Figure 4). The shoot and root Fe concentrations were significantly reduced with the highest Cd levels. While the maximum reduction in the shoot was found with 200 µM Cd applications (25.9%), it was found with 100 µM Cd applications (42.9%) in the root

(Figure 4A). The shoot Mn concentrations showed a tendency to decrease in all Cd levels. However, all Cd levels (25, 50, 100, 150, and 200 µM) caused a dramatic decrease in root Mn concentration by 61.6%, 50.0%, 48.2%, 53.7%, and 65.3%, respectively (Figure 4B).

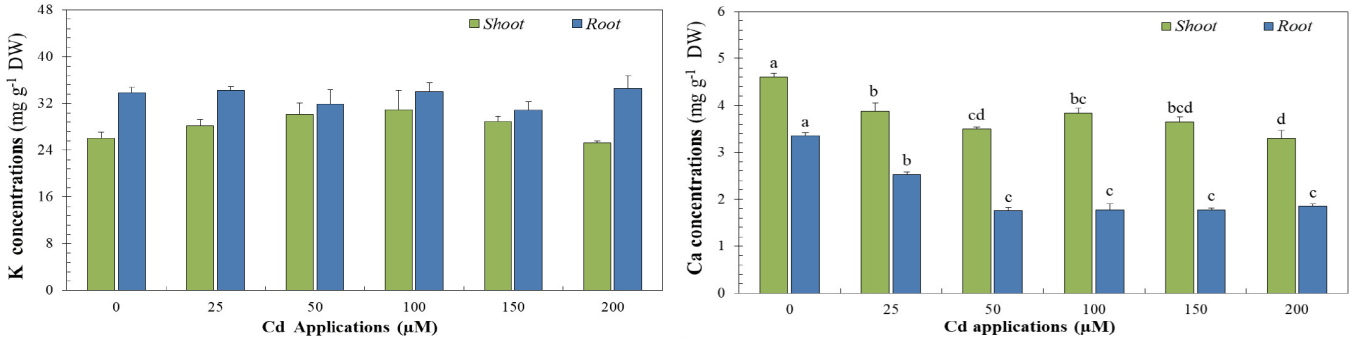

Figure 3- The effects of Cd toxicity on K and Ca concentrations in shoot and root in lavandin (Bars indicate means of three replicates ± SE. Different letters on the bars for each parameter differ significantly according to DMRT)

Likewise, increasing Cd levels caused a notable decrease in shoot and root Zn concentrations. In the shoots, these decreases at 25, 50, 100, 150, and 200 µM were 5.3%, 19.7%, 29.0%, 22.4%, and 46.6%, respectively. In the roots, similarly, the decreases were 63.3%, 67.6%, 77.5%, 79.1%, and 79.4%, respectively (Figure 4C). However, only the 100 and 200 µM Cd levels caused notable increases in the shoot Cu concentration by 38.7% and 11.1%, respectively. All Cd levels increased the root Cu concentrations and these increases were found to be over 2-fold in comparison with the control (Figure 4D).

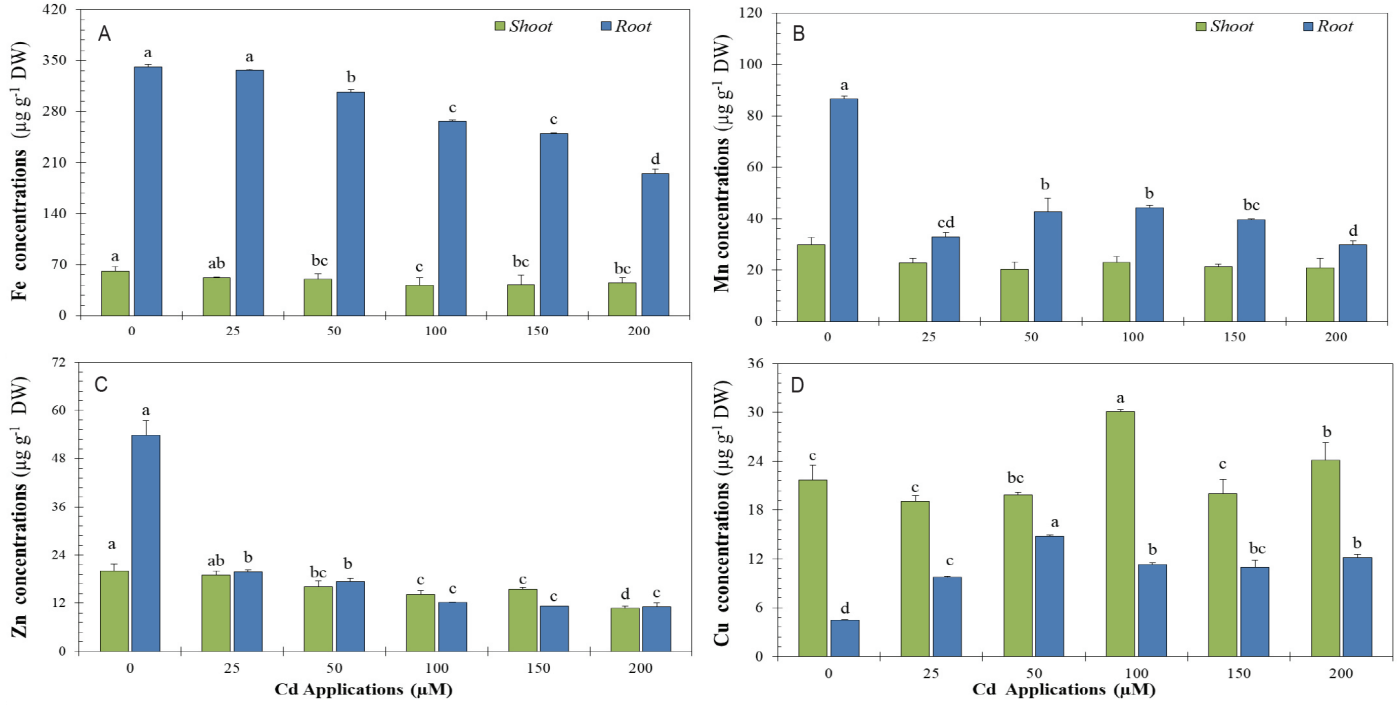

Figure 4- The effects of Cd toxicity on micronutrient concentrations in shoot and root in lavandin (Bars indicate means of three replicates ± SE. Different letters on the bars for each parameter differ significantly according to DMRT)

The BCF of Fe in lavandin (shoots and roots) reduced significantly with all Cd levels, compared to the control (Table 2). Higher Cd levels (50, 100, 150, and 200 µM) decreased the shoot BCF of Fe by 17.6%, 30.7%, 30.3%, 25.8%, respectively. These decreases were by 10.2%, 21.9%, 27.0%, and 43.1%, respectively, in the root BCF of Fe. Similar decreases in the BCF of Zn were found in both shoot and root. While 25, 50, and 150 µM Cd levels caused a decrease in shoot BCF of Cu, 100 and 200 µM Cd levels increased this parameter by 38.6% and 11.0%, respectively. All Cd levels increased the root BCF of Cu when compared to the control. The maximum

increase in root BCF of Cu was achieved with 50 µM Cd levels (Table 2). With all Cd levels (25, 50, 100, 150, and 200 µM), the BCF of Mn in roots significantly decreased at 62.0%, 50.6%, 48.8%, 54.2%, and 65.7%, respectively, compared to the control.

Table 2-The effect of Cd toxicity on bio-concentration of heavy metal ions in lavandin

Treated Cd (µM)	*BCF of Fe*		*BCF of Mn*		*BCF of Zn*		*BCF of Cu*	
	Shoot	*Root*	*Shoot*	*Root*	*Shoot*	*Root*	*Shoot*	*Root*
0	24.4±1.24^{a}	137±2.69^{a}	59.3±0.50	173.0±2.45^{a}	401±34.3^{a}	1078±71.5^{a}	1084±92.2^{bc}	226±3.21^{d}
25	20.9±0.65^{ab}	135±0.55^{a}	45.6±3.50	5.7±3.13^{cd}	380±21.4^{ab}	396±9.47^{b}	957±36.8^{c}	488±5.70^{c}
50	20.1±1.28^{bc}	123±2.87^{b}	40.2±3.61	85.4±10.1^{b}	322±28.3^{bc}	349±12.7^{b}	996±12.4^{bc}	740±4.52^{a}
100	16.9±0.89^{c}	107±4.01^{c}	45.9±2.91	88.6±1.65^{b}	285±18.5^{c}	242±3.92^{c}	1502±15.7^{a}	568±24.2^{b}
150	17.0±0.32^{bc}	100±5.12^{c}	42.9±8.31	9.2±0.87^{bc}	311±6.89^{c}	224±1.56^{c}	99±89.1^{bc}	550±41.9^{bc}
200	18.1±2.31^{bc}	78±3.03^{d}	41.8±1.71	59.4±3.48^{d}	214±11.5^{d}	222±18.8^{c}	1203±109^{b}	610±18.5^{b}
F-test	**	***	ns	***	***	***	**	***

All values are the average of three replicates (means ± SE, n= 3). Different letters in the same column differ significantly according to the DMRT. *F*-test shows significant difference at $^{***}p<0.001$; $^{**}p<0.01$; ns: not significant

Increasing the Cd levels significantly affected the TF of metallic ions in comparison to the control, except for TF of K (Table 3). The TF level of K and Ca showed an increasing tendency with all Cd levels, with the exception of TF of K at the 200 µM Cd level. A significant difference in TF of Fe with Cd levels was not found, but a 200 µM Cd level provided a 30.3% increase. All Cd levels (25, 50, 100, 150, and 200 µM) caused notable increases in TF of Mn by 2.6-, 2.5-, 3.4-, 3.5-, and 2.7-fold and in TF of Cu by 2.1-, 1.4- 1.5-, 1.6-, and 2,1-fold, respectively. However, the same Cd levels caused notable decreases in TF of Zn by 59.1%, 71.8%, 44.5%, 61.6%, and 58.9%, respectively.

Table 3- The effect of Cd toxicity on translocation (TF) of metallic ions in lavandin

Treated Cd (µM)	*TF of K*	*TF of Ca*	*TF of Fe*	*TF of Mn*	*TF of Zn*	*TF of Cu*
0	0.78±0.07	1.45±0.12^{b}	0.178±0.01^{b}	0.37±0.02^{c}	4.79±0.39^{a}	0.34±0.01^{c}
25	0.82±0.04	1.75±0.21b	0.155±0.01^{b}	0.96±0.07^{b}	1.96±0.10^{c}	0.70±0.05^{a}
50	0.95±0.03	1.93±0.14^{b}	0.164±0.01^{b}	0.92±0.07^{b}	1.35±0.02^{c}	0.48±0.05^{bc}
100	0.91±0.06	2.72±0.39^{b}	0.159±0.00^{b}	1.27±0.10^{a}	2.66±0.13^{b}	0.52±0.03^{b}
150	0.95±0.10	2.07±0.02^{ab}	0.171±0.01^{b}	1.29±0.03^{a}	1.84±0.25^{c}	0.54±0.10^{ab}
200	0.71±0.07	1.84±0.28^{b}	0.232±0.03^{a}	0.99±0.13^{b}	1.97±0.12^{c}	0.71±0.02^{a}
F-test	ns	*	*	***	***	**

All values are the average of three replicates (means ± SE, n= 3). Different letters in the same column differ significantly according to the DMRT. *F*-test shows significant difference at $^{***}p<0.001$; $^{**}p<0.01$; $^{*}p<0.05$; ns: not significant

A significant interaction was found between increasing Cd levels and net accumulation of Cd, Fe, and Zn was found as compared to the control (Table 4). All Cd levels significantly increased the NA of Cd, with the highest increase 97-fold at 150 µM Cd level. However, increasing Cd levels decreased the NA of Fe and Zn. These decreases in NA of Fe were significant only at the 150 and 200 µM Cd levels by 36.0% and 46.1%, respectively. Likewise, the NA of Zn considerably decreased with 50, 100, 150, and 200 µM by 36.6%, 54.2%, 31.7%, and 61.3%, respectively. A non-significant interaction between increasing Cd levels and NA of K and Ca was found in lavandin (Table 4).

Table 4- The effect of Cd toxicity on the net accumulation (NA) of metallic ions via roots in lavandin

Treated Cd (µM)	*NA of Cd*	*NA of Fe*	*NA of Mn*	*NA of Zn*	*NA of Cu*	*NA of K*	*NA of Ca*
	(µg g^{-1} DW)					*(mg g^{-1} DW)*	
0	25.1±1.80^{d}	841.9±23.1^{a}	332.4±22.8	217.6±7.37^{a}	181.3±3.40	249.2±18.0	41.58±4.38
25	864±10.8^{cd}	806.6±24.0^{a}	238.0±9.35	192.2±9.83^{ab}	182.6±8.79	290.8±25.4	37.26±0.77
50	1316±41.6^{bc}	679.6±36.5^{ab}	193.5±24.0	138.0±19.6^{cd}	163.4±16.5	254.0±10.3	27.10±3.73
100	1821±157^{ab}	667.9±71.0^{ab}	188.0±24.9	99.6±12.2^{de}	201.9±35.0	224.6±25.4	25. 78±4.36
150	2414±597^{a}	538.7±111^{bc}	243.5±76.3	148.6±19.0^{bc}	186.8±32.5	287.4±49.1	33.97±5.88
200	1729±411^{abc}	453.8±83.5^{c}	172.0±44.0	84.3±21.5^{e}	179.5±50.4	202.8±53.7	24.08±5.34
F-test	**	*	ns	***	ns	ns	ns

All values are the average of three replicates (means ± SE, n=3). Different letters in the same column differ significantly according to the DMRT. *F*-test shows significant difference at $^{***}p<0.001$; $^{**}p<0.01$; $^{*}p<0.05$; ns: not significant

4. Discussion

Cd, which has no known physiological function in plants, has serious toxic effects on plants (Wang et al. 2007). Depending on the species or genotype, plants may show toxicity symptoms in the presence of more than 5-10 mg kg^{-1} of Cd in the rooting media (Mengel & Kirkby 2012). Typical toxicity symptoms of Cd include chlorosis and stunted growth (Jali et al. 2016). Also, toxic Cd levels inhibit plant growth, photosynthetic activity (Ekmekçi et al. 2008), adversely affect nutrient uptake and balance, and may accumulate in plant organs (Goswami & Das 2015). In our study, increasing the Cd levels caused a significant decrease in shoot and root FW and DW (Figure 1,5) and Chl *a*, *b*, *a+b,* and Car contents (Figure 2A). These decreases may be associated with the phytotoxic effect of Cd on the synthesis of the cell wall (Parrotta et al. 2015) and enzyme activities, and photosynthetic electron transport (Hassan et al. 2005). Excess Cd also reduces Fe and Zn uptake (Figure 4A, 4C), resulting in leaf chlorosis (Haider et al. 2021). However, higher Cd levels can inhibit chlorophyll (Chl) biosynthesis and reduce the activity of enzymes involved in CO_2 fixation and thus cause a disruption in chloroplast biosynthesis (Raziuddin et al. 2011). Sandalio et al. (2001) found that Cd affected both the leaves and roots in pea plants and significantly inhibited transpiration and photosynthesis rate, as well as cause a general nutrient imbalance in the plant. In addition, Cd may lead to the impairment of photosynthetic machinery (Irshad et al. 2021) and it may displace with Mg which are central atoms of the Chl molecule (Gill 2014). On the other hand, excess Cd caused an increase in the MP value of lavandin leaves (Figure 2B). These increases could be explained by the direct effects of excess Cd on proteins and lipids, which are the main components of membranes, or by inducing lipid peroxidation (Fodor et al. 1995). Ekmekçi et al. (2008) found that Cd applications might result in membrane damage and deterioration of membrane integrity in maize leaves. Barceló & Poschenrieder (1990) stated that metal toxicity causes a reduction in water content and thus affects the plasma MP and, particularly, Cd interacts with the water balance.

Figure 5- The toxic effect of cadmium on growth of lavandin

Depending on the increasing Cd levels, shoot and root Cd concentrations also increased (Table 1). Cd concentration in roots is generally higher than in shoots (Ekmekçi et al. 2008; Ehsan et al. 2015). In this study, it was found in the roots to be over 120-fold than in the shoots. This indicated that the plants accumulated Cd in their roots and did not carry it to the aerial organs due to some barriers (Pinto et al. 2004). Di Toppi & Gabbrielli (1999) explained that the reduction in the transfer of Cd from root to shoot could be due to metal immobilization in the cell wall. Angelova (2012) reported that some medicinal and aromatic plants accumulate a large portion of heavy metals in their roots and transfer very little of these heavy metals to the upper organs, and have a high growth potential in heavy metal-contaminated areas.

The Cd uptake by plant roots occurs through the same transmembrane carriers used to uptake Ca^{2+}, Fe^{2+}, Mg^{2+}, Cu^{2+}, and Zn^{2+} (Papoyan & Kochian 2004) and Cd ion could compete with these cations to access the cell of plants through the transport systems (Barceló & Poschenrieder 1990). Thus, Cd could interfere with the uptake and transport of Ca, P, Mg, K, and Mn (Nazar et al. 2012). Decreases in Ca concentration (Figure 3) and Fe, Mn, and Zn concentrations (Figure 4) in shoots and roots could be attributed to the interactions of these nutrients with Cd. This effect could be ascribed to dysfunctions of the membrane integrity caused by the displacement of Ca in the cell wall with Cd. In line with this, Nada et al. (2007) reported that the roots and leaves of almond seedlings exposed to 100 and 150 µM Cd caused a decrease in Ca, Mg, and K.

The BCF indicates the ability of plants to absorb a heavy metal from the rooting medium, while TF indicates the ability of heavy metals to transfer from the root medium to the upper organs such as stem, leaf, flower, and fruit. Both concepts are typically used to determine the phytoremediation capacity of plants (Ghosh & Singh 2005) and tend to decrease depending on increasing concentrations of heavy metals in rooting media (Zhao et al. 2004). The results from this study reveal that metallic ion accumulation was reduced in

the shoots and roots with increasing Cd levels. The fact that the BCF of Cd in the roots was greater than the BCF of Cd in the shoot indicates that the root accumulates more Cd than in the shoot (Table 1). It is known that many plants reduce the uptake of metals to their aerial parts and preferentially accumulate and store them in their roots (Usman et al. 2019) or bind them to amino acids, proteins, and peptides (Pál et al. 2006). Likewise, the BCF of Fe, Zn, and Mn was reduced by increasing Cd levels (Table 2). The higher metallic ion concentrations in the roots compared to the shoots (Figure 4) can be explained by the inability to transport them from the roots to the shoots. Reductions in translocation from root to the shoot appear to be a common feature in plants exposed to heavy metals. The relatively high accumulation of Cd in plant roots could partially be due to the binding of the Cd ions to some specific sites in the root cell wall (Zhu et al. 1999). Chen et al. (2021) reported that heavy metal accumulation in rice and maize plants followed the order root > stem = leaf > cereal.

The net accumulation of metallic cations via roots indicates the amount of these cations deposited by a unit root in the upper organs of a plant (Moradi & Ehsanzadeh 2015). While the increase in net accumulation of Cd is likely to depend on the accumulation of Cd ions in rooting media, a decrease in net accumulation of Fe and Zn depends on excessive Cd in rooting media and its toxic effects (Table 4). It also could be related to the antagonistic effects of Cd ions on Fe and Zn ions that have been in the same redox-active group (Singh et al. 2016). Our results are in line with those reported by Moradi & Ehsanzadeh (2015) who studies Cd ions in safflower plants.

In our study increasing Cd levels caused an increase in the TAR of Cd (Table 1). This increase in the TAR of Cd could be attributed to the excess Cd^{2+} concentration present in the rooting media. Campbell (1995) stated that the metal accumulation is controlled by the free ion concentration. Likewise, Kösesakal et al. (2011) reported that higher metal presence in rooting media causes an increase in metal uptake and significantly increases TAR value.

5. Conclusion

This study concludes that lavandin accumulates increasing amounts of Cd in the tissues at increasing Cd levels in the rooting medium, with negative effects on growth and biomass production. The roots are the main metal sinks due to a low translocation from roots to the shoot, suggesting a defence or tolerance mechanism to avoid toxic levels in physiologically most active apical tissues. However, Cd could interact with metal ions, especially divalent cations (Ca, Fe, Zn, and Mn), and could prevent these ions from fulfilling their role in mineral nutrition by reducing their uptake by roots. Meanwhile, a significant correlation has been found between the net accumulation of Cd, Fe, and Zn via roots of lavandin and Cd concentrations in the rooting medium.

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Export Competitiveness of Türkiye Agri-food Products in the European Union and The Shanghai Cooperation Markets

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ABSTRACT

The purpose of this study was to investigate the competitive advantage and long-term viability of Türkiye agri-food exports to the European Union (EU) and Shanghai Cooperation Organization (SCO) markets. The Lafay index and trade ratios were utilized to investigate comparative and competitive advantage, while the survival function was employed to estimate export competition for the EU and SCO markets. The results indicate that Türkiye has a competitive edge in the export of fruits and vegetables to the EU and SCO states. However, the period of comparative advantage experienced by EU and SCO countries was not similar, nor was the benefit consistent. Despite fluctuations in Türkiye's international trade balance with SCO members, its foreign trade with EU members has increased. Hence, Türkiye should continue to trade in agri-food with the EU while simultaneously expanding business networks with SCO countries.

Keywords: Survival analysis, Comparative advantage, Agri-food products, Export-import ratio

1. Introduction

The agricultural sector is relatively important to the Turkish economy but its share has declined in comparison to the growth of other sectors. Agriculture and food production, on the other hand, remain important components of Türkiye's economy and workforce. For instance, Türkiye is the world's leading exporter of nuts, raisins, dried apricots, and dried figs (Erdem 2020). Apart from that, Türkiye exports an enormous amount of fresh fruits and vegetables to Russia and other European Union (EU) countries. However, Türkiye imports agricultural products due to inadequate supply or excess demand. Türkiye's agri-food imports are primarily raw ingredients for the food sector. Because a country's resources are insufficient to cover all of its residents' requirements, it enters into diverse economic, political, and commercial partnerships with other countries. Opportunities entering the market through mutual customs tariff rate reductions, the removal of non-tariff barriers for agri-food products, and obtaining quotas for non-traded products have positive effects on foreign trade within the scope of cross-country agreements and economic cooperation organizations.

Türkiye and the EU had signed a commercial trade agreement (the Customs Union Agreement of 1996) in which agricultural products played a significant role. However, the relationship has deteriorated since 2005, when full membership negotiations with the EU were temporarily halted. Though Türkiye and the EU have cooperated for a long time, it is no longer sustainable due to views stressing people's previous prejudices and member countries' opposition to Türkiye's full membership. As a result, the Turkish government aspired to join the Shanghai Cooperation Organization (SCO), submitting a formal application in 2011 and being accepted a year later (Pantucci & Petersen 2013). Türkiye's agricultural food exports and imports are influenced by the SCO, which represents 45 percent of the world's population (Telatar 2019; Isik 2016). The SCO has broadened its collaboration among its members to include a wide range

of topics to help them grow and develop socially and culturally. With its friendship and good neighborly connections, the SCO strives to promote mutual trust and contribute to the member nations' economic progress and social-cultural development. In this context, the SCO has broadened its collaboration among its members to include a wide range of topics (Nakiboglu et al. 2017).

The international economic relationship, including long-term competitiveness between Türkiye, the European Union (EU), and the SCO was examined in this study to determine whether the SCO could be a viable option for the EU in the case of Turkish agricultural food products. It will provide the trade competitive advantage and long-term viability of Turkish agri-food exports with EU and SCO to increase agricultural foreign trade.

2. Material and Methods

2.1. Data

In this study, data about Türkiye's export and import of agri-food goods with the EU (28 nations), the SCO (12 countries), and other countries of the world for the years 2010-2018 were analysed. The analysis took into account export and import values, changes in trade volumes, historical balances, and trends. The analysis used the dollar to represent Türkiye's overseas trade in agri-food items and transaction values. These data covering the years from 2010-2018 were obtained from the Turkish Statistical Institute (TSI). In the TSI dataset, export and import statistics are classified as "free on board" and "cost", "insurance", and "freight-delivery" with insurance and freight. The product items were analyzed according to the 2-base international harmonized system classification, and the first 24 items covering agricultural food products were considered (Table 1).

Table 1- Agricultural sector chapters in the Harmonized System Classification

Code	***HS2 classification***
HS01	Live animals
HS02	Meat and edible meat offal
HS03	Fish and crustaceans, molluscs and other aquatic invertebrates
HS04	Dairy produce, birds' eggs, natural honey, edible products of animal origin not elsewhere specified or included
HS05	Products of animal origin, not elsewhere specified or included
HS06	Live trees and other plants, bulbs, roots and the like, cut flowers and ornamental foliage
HS07	Edible vegetables and certain roots and tubers
HS08	Edible fruit and nuts, peel of citrus or melons
HS09	Coffee, tea, mate and spices
HS10	Cereals
HS11	Products of the milling industry, malt, starches, inulin, wheat gluten
HS12	Oil seeds and oleaginous fruits, miscellaneous grains, seeds and fruit, industrial or medicinal plants, straw and fodder
HS13	Lac, gums, resins and other vegetable saps and extracts
HS14	Vegetable plaiting materials, vegetable products not elsewhere specified or included
HS15	Animal or vegetable fats and oils and their cleavage products, prepared edible fats, animal or vegetable waxes
HS16	Preparations of meat, of fish or of crustaceans, molluscs or other aquatic invertebrates
HS17	Sugar and sugar confectionery
HS18	Cocoa and cocoa preparations
HS19	Preparations of cereals, flour, starch or milk, pastrycooks' products
HS20	Preparations of vegetables, fruit, nuts or other parts of plants
HS21	Miscellaneous edible preparations
HS22	Beverages, spirits and vinegar
HS23	Residues and waste from food industries, prepared animal fodder
HS24	Tobacco and manufactured tobacco substitutes

Source: TSI 2019.

2.2. Theoretical backgrounds

The Ricardian models propose that governments should better focus on developing food items with a comparative advantage when analyzing the competitiveness of agri-food sectors in the context of global or regional competition. As a result, the concept of comparative advantage would be used to determine a country's competitiveness. Lafay (1992) clarifies two key distinctions between comparative advantage and competitiveness. First, competitiveness is normally measured by comparing a product between countries, whereas comparative advantage is measured by comparing products within a country. Second, changes in macroeconomic variables have an impact on competitiveness.

2.3. Method of data analysis

The Lafay index (LFI) was adopted to identify the comparative advantages of Türkiye's agri-food exports to the EU, SCO, and other nations around the world (Lafay 1992). The LFI has an advantage in that it accounts for macroeconomic distortions (Balassa 1965; Balassa 1977; Balassa 1991). It also corrects a potential bias in other indices like the Balassa index, which can lead to erroneous conclusions about a country's trade competition when both exports and imports are considered. Furthermore, the LFI index provides an analysis of each product's position within each country's or group of nations' international trade structure.

$$LFI_j^i = 100\left(\frac{x_j^i - m_j^i}{x_j^i + m_j^i} - \frac{\sum_{j=1}^{N}\left(x_j^i - m_j^i\right)}{\sum_{j=1}^{N}\left(x_j^i + m_j^i\right)}\right)\frac{x_j^i + m_j^i}{\sum_{j=1}^{N}\left(x_j^i + m_j^i\right)} \quad (1)$$

where x_j and m_j denote exports and imports of product j from a chosen trading partner, respectively.

The number of product items examined is denoted by the letter N. The index's positive values imply that a country has a comparative advantage in a particular item. An increase in the index value implies that the product or product group exported to the country where the trade is conducted has become more specialized. Negative LFI values, on the other hand, suggest that there is no competitive advantage or specialization (Zaghini 2003). The LFI examines a country's comparative advantage (disadvantage). In this index, values ranging from $-\infty$ to $+\infty$ were used. The export-import ratio provides information on a country's export and import relationships. The higher the ratio, the greater the country's export power. This ratio, which shows the percentage of imports (M_{yi}) covered by exports (x_{yi}), can also be used to compare trade performance across countries.

$$\text{Export/import coverage ratio (\%)} = \frac{X_{y_i}}{M_{y_i}} \times 100[\%] \quad (2)$$

Duration analysis was used to address the question, "What is the length of the comparative advantage period at the product level?" (Bojnec & Fertö 2009). The starting and ending years serve as reference points for evaluating dynamics. In the agri-food product groupings, survival functions based on the LFI index have been estimated. The product boundary estimator was used to estimate the non-parametric survival function St, by Kaplan and Meier (1958). Consider the following example: (t_i; c_i), i = 1,2,..., n has n distinct observations with I = 1,2,..., n. The survival time is t_i, and the censoring indicator variable C is c_i (it takes the value 1 if a failure occurs, otherwise it is 0). We assumed, however, that the recorded failure time was correct. As a result, sequential survival time is denoted by t(1) t(2) and t(m). The n_j denotes the subject number that is in danger of failing at t(j) and d(j), respectively. The Kaplan-Meier survival function estimator can be written as follows:

$$\prod_{t(i)<t} \frac{n_j - d_j}{n_j} \quad (3)$$

If t(1) is true, then s(t)=1 (Cleves et al. 2010). Because it includes information from both censored and uncensored observations, the Kaplan-Meier estimator is robust when numerous censored observations are considered. Furthermore, the log-rank test and the Wilcoxon test, both non-parametric tests, were used to evaluate the equality of survival functions for the LFI index in the agri-food product categories.

3. Results and Discussion

3.1. The export-import ratio, the volume of foreign trade, and the foreign trade balance

For the period between 2010 and 2018, Türkiye's agri-food exports increased around the world in general and to the EU and SCO in particular (Table 2). In comparison to the previous period, the value of Türkiye's agricultural exports of food commodities to other countries has increased. This export value climbed steadily until 2014, after which it remained stable. Exports to EU countries grew at a slower pace during the same period. In terms of exports to SCO countries, the growth pace seen up until 2015 is unlikely to continue in the next few years. Although EU exports accounted for a major portion of Türkiye's agri-food product exports until 2015, the EU's share of the country's agri-food product exports began to decline after that year. Only in 2016 did the share of agri-food exports to SCO drop significantly, with similar levels of exports reported in the previous years.

Table 2- Export value of Turkish agri-food products in the period of 2010-2018 (billions of dollars)

Years	*World*	*EU*	*%*	*SCO*	*%*
2010	12.040	4.494	37.322	1.396	11.594
2011	14.427	4.792	33.213	1.690	11.714
2012	15.251	4.643	30.443	1.629	10.684
2013	16.977	4.954	29.178	1.745	10.278
2014	17.995	5.558	30.889	1.856	10.312
2015	16.789	5.525	32.909	1.696	10.102
2016	16.249	5.097	31.368	1.161	7.147
2017	16.909	5.166	30.552	1.401	8.284
2018	17.673	5.292	29.945	1.530	8.658
Average Annual Growth (%)	5.1890	2.234		2.580	

Source: Own calculations based on the TSI dataset

Türkiye's agri-food product imports have fluctuated over the same period. These changes occurred in trade with the rest of the globe, the EU, and SCO countries over the course of several years (Table 3). It should be noted that the EU and SCO are moving in opposite directions when it comes to importing shares. Despite changes over time, Türkiye's trade with the rest of the world saw a large increase in the import value of agri-food goods by the end of the period. Agri-food imports from EU countries have shown a similar average growth pattern. After 2012, however, there was no equivalent surge in agri-food imports from SCO nations. The proportion of agri-food products imported from EU nations in global food imports declined by around 2%, while the proportion imported from SCO members increased by about 8%. However, it is worth noting that, by the end of the period, the import values of the EU and SCO countries are nearly identical.

Table 3- The import value of Turkish agri-food products from 2010 to 2018 (in billions of dollars)

Import	*World*	*EU*	*%*	*SCO*	*%*
2010	7.683	2.082	27.10	1.046	13.62
2011	10.961	3.192	29.12	1.411	12.87
2012	10.734	2.805	26.13	2.780	25.90
2013	11.200	2.698	24.09	2.569	22.94
2014	12.418	2.682	21.59	3.536	28.47
2015	11.243	2.839	25.25	2.709	24.09
2016	11.038	2.786	25.24	2.322	21.04
2017	12.666	3.312	26.15	2.484	19.61
2018	12.845	3.329	25.92	2.758	21.47
Average annual growth (%)	7.5860	7.517		17.785	

Source: Own calculations based on the TSI dataset

Türkiye's international trade balance in agri-food items was summarized in Table 4. According to the results, though there was a trend toward imports in international trade with the rest of the world, the EU, and SCO countries, the tendencies differed by the year. The trade balance with the rest of the world, which had fallen to $3.47 billion in 2011, rose in 2013 due to a growth in exports, stayed at comparable levels until 2017, and then declined in the following years. The export-import balance in the agri-food trade with EU countries fluctuated, and the trade balance went below $2 billion in 2018. Except for the first few years, the shift in the trade balance of agri-food items created with SCO countries can be understood as imports are constantly at the forefront (Isik 2016). This change peaked in 2014, and by the end of nine years, the balance was in a different direction than it was at the start of the period. Despite the fact that the three categories have changed over time, there is a consistent pattern in the agri-food products trade that favors imports.

Table 4- Türkiye's foreign trade balance for agri-food products from 2010 to 2018 (in billions of dollars)

Years	*World*	*EU*	*SCO*
2010	4.358	2.411	0.350
2011	3.466	1.600	0.279
2012	4.517	1.838	-1.151
2013	5.777	2.255	-0.824
2014	5.577	2.877	-1.680
2015	5.546	2.686	-1.012
2016	5.211	2.311	-1.161
2017	4.243	1.854	-1.084
2018	4.828	1.963	-1.227

Source: Own calculations based on the TSI dataset

According to the results presented in Table 5, notwithstanding fluctuations, Türkiye's foreign trade volume of agri-food products to the globe, the EU, and SCO member nations increased. In international commerce, agri-food goods peaked in 2014, then fell by 11% for two years before beginning to rise again. Trade relations with EU countries, in particular, have remained stable since 2011. A similar trend has been seen between the SCO countries and the rest of the globe, with improved agri-food trade links. Taking into account the trade volumes of agri-food products achieved through the SCO countries, certain structural elements of Türkiye's agri-food export-import trade can be derived by looking at the export-import ratios of the world, EU member countries, and SCO members separately. The high export-to-import ratio does not imply that Türkiye's exports to certain nations or groups of countries are particularly valuable. Türkiye's export-import ratio for products such as vegetables and fruits, malt and milling products, and meat and fish products was fairly high (Table 6a).

Table 5- Türkiye's foreign trade volume for agri-food products with the rest of the world, the EU, and the SCO from 2010 to 2018 (in billions of dollars)

Trade volume	*World*	*EU*	*SCO*
2010	19.723	6.576	2.442
2011	25.389	7.984	3.101
2012	25.985	7.448	4.410
2013	28.177	7.652	4.314
2014	30.413	8.240	5.391
2015	28.032	8.364	4.405
2016	27.287	7.883	3.484
2017	29.575	8.478	3.885
2018	30.518	8.621	4.288

Source: Own calculations based on the TSI dataset

Table 6a- The export-import ratio of Türkiye in the period of 2010-2018 (world)

HS	*2010*	*2011*	*2012*	*2013*	*2014*	*2015*	*2016*	*2017*	*2018*
01	2.20	0.60	0.96	3.89	19.10	10.68	4.62	2.86	3.28
02	83.15	75.98	547.95	2432.04	10341.44	415.92	882.94	621.37	219.87
03	233.83	227.34	234.42	279.86	325.59	264.96	426.40	357.26	475.95
04	236.57	460.08	467.86	414.11	380.22	360.07	538.46	579.33	581.87
05	88.17	81.16	112.53	131.89	124.06	106.58	108.16	93.64	100.73
06	112.65	112.39	107.85	83.23	89.35	95.14	93.55	103.00	164.15
07	351.14	290.67	344.63	318.96	232.34	228.08	206.27	191.03	281.08
08	1107.68	993.35	832.51	938.35	1040.49	937.48	716.61	691.21	703.68
09	100.07	93.62	87.16	108.58	110.88	84.33	90.69	55.80	67.81
10	33.05	5.70	11.25	8.78	5.40	6.53	8.93	6.47	5.08
11	1364.50	1628.33	1280.13	1178.32	1137.40	1096.67	1014.54	889.06	1000.28
12	11.73	13.54	14.26	16.75	11.30	10.61	20.49	14.82	17.56
13	14.58	18.08	21.96	23.72	22.82	23.01	24.14	27.37	31.66
14	340.23	391.42	381.68	198.53	102.76	139.16	162.63	167.93	138.83
15	46.27	64.04	64.39	77.49	55.14	53.44	59.62	70.03	85.75
16	1307.14	2265.57	1774.32	1919.10	1040.21	590.72	687.24	1468.70	2709.75
17	738.61	747.14	518.90	592.35	525.45	336.07	205.08	282.27	350.45
18	105.94	104.17	118.27	119.59	113.97	99.87	86.36	84.50	118.43
19	475.10	522.95	647.32	695.70	748.41	706.82	726.03	743.72	824.41
20	2370.42	1829.91	2237.33	2044.10	2120.58	1913.80	2339.80	2478.74	2864.43
21	147.55	149.52	153.65	141.23	132.15	116.57	117.27	103.37	114.19
22	165.16	121.47	133.61	111.29	107.82	110.37	116.47	112.38	150.08
23	3.70	5.95	9.93	12.80	12.17	9.91	9.73	11.89	16.52
24	189.27	167.45	172.83	178.78	190.20	170.80	170.37	178.74	165.95

Source: Own calculations based on the TSI dataset

According to Yercan and Isikli (2009), Türkiye is a prominent player in the global market for several horticultural products. Fruits and vegetables account for a large percentage of Turkish agricultural exports. Furthermore, due to the country's high demand for grain, grain imports were found to be significantly higher than exports. Imports continue to outnumber exports in the coffee, tea, and spice industries. The trade balance continues to improve in favor of exports when compared to imports of sugar and sugar products.

In the trade of pastry products such as cereals, flour, starch, or milk preparations, the share of exports in trade volume has risen over time. From 2002 to 2015, according to Bashimov (2017), Türkiye was a net exporter in the HS03, HS07, HS08, HS11, HS14, HS16, HS17, HS19, HS20, HS22, and HS24 coded product groups and a net importer in the HS12, HS13, HS15, and HS23 coded product groups.

The fruit business accounts for the majority of Türkiye's agri-food exports to EU countries. Though there have been some changes in exports throughout the years, fruit and vegetable exports continue to be a significant source of revenue for Türkiye's agricultural sector (Table 6b). Moreover, despite the 2014-2015 slowdown and low imports, the export-to-import ratio was regarded as high. In the export of meat and fish, a similar structure was seen. The amount of seafood exported has increased. The opposite tendency may be seen in the export of other animal products, where imports are becoming more common.

Table 6b. Export-import ratios of Türkiye in the period of 2010-2018 (EU)

HS	*2010*	*2011*	*2012*	*2013*	*2014*	*2015*	*2016*	*2017*	*2018*
01	1.84	0.34	0.32	1.50	1.77	0.67	0.53	0.21	0.17
02	0.92	0.54	2.74	10.58	341.67	6.09	902.26	2.18	1.20
03	1370.34	1663.35	1865.60	2066.94	2290.75	2701.10	2723.16	1635.40	2067.53
04	8.20	8.94	5.26	8.73	11.67	10.43	13.39	25.01	28.25
05	413.17	399.46	346.52	456.71	481.39	354.60	281.04	193.77	131.85
06	70.16	56.42	61.67	45.83	51.67	54.79	56.58	62.57	101.42
07	1531.24	1051.57	1044.90	1565.41	1189.43	730.53	1084.52	1442.07	1756.52
08	8822.88	7376.96	7854.64	7979.10	7598.98	5278.11	6909.81	7503.07	5905.15
09	270.65	204.24	205.62	217.79	208.44	190.42	232.06	172.56	141.96
10	32.70	6.57	14.97	25.33	17.76	11.46	11.27	5.29	10.26
11	93.67	77.70	66.84	51.46	50.28	54.34	42.00	26.05	21.02
12	22.44	22.76	37.43	20.56	20.06	24.35	30.52	59.58	52.37
13	5.97	12.90	10.40	12.18	15.97	20.54	24.18	28.68	27.30
14	5540.51	11576.66	4268.54	3233.88	710.22	595.51	1370.93	1673.50	1768.17
15	121.02	64.26	94.86	231.98	21.87	26.94	47.50	120.78	139.33
16	959.71	1611.76	1472.38	2015.43	1485.29	1395.28	2105.16	1752.23	2603.62
17	471.12	406.62	430.27	414.76	490.18	344.39	141.96	211.68	203.24
18	28.47	28.47	25.21	23.14	25.84	34.82	24.70	25.56	28.57
19	75.28	67.58	70.27	69.52	77.17	71.92	82.01	82.99	89.28
20	2976.19	2107.97	2738.02	2408.88	2478.22	1935.15	2418.28	2498.24	2719.65
21	69.62	78.66	73.86	62.62	57.59	41.82	42.46	39.66	42.73
22	111.90	69.61	69.65	46.21	44.42	45.10	51.65	47.14	85.81
23	3.60	0.87	0.64	1.13	4.02	2.95	2.45	3.61	4.56
24	169.89	120.86	88.68	79.12	104.38	80.10	64.59	95.63	90.24

Source: Own calculations based on the TSI dataset

Although sugar and sugar products are exported more than they are imported, this scenario tends to shift over time. Domestic output of animal-derived items, such as dairy products, eggs, and honey, failed to fulfill domestic demand, as evidenced by imports outnumbering exports. The biggest import values were for live animals and meat products, which were also the top export values. Türkiye has been importing meat for a long time. Although grain imports are in a similar scenario, it has been observed that this commodity is insufficient to meet the need for production abroad. However, various grains, flour, starch, and milk preparations have become increasingly significant in recent years.

The export-import ratios of agricultural food items exchanged with SCO countries may differ from those of agricultural food products traded with the rest of the world or EU countries (Table 6c). For example, Türkiye's meat trade with SCO countries, has remained strong and export-oriented. Other animal-derived goods have a far lower rate. The trade balance of cocoa and cocoa products has primarily been on the export side in recent years, while the import of this product has increased. Similarly, with the recent growth in imports of dairy products, eggs, and honey products, exports have become a major part of the trade volume of agri-food items. Cereal commerce, like that of the rest of the world and the EU countries, was dominated by imports. Türkiye has also purchased coffee, tea, and spices from SCO nations. Imports of edible oil products were found to be higher than exports of milling items such as malt, starch, and wheat gluten, lacquer, chewing gum, resin, and other vegetable saps and extracts, as well as animal and vegetable fats and oils. The export-import values for fish and other aquaculture products, which were initially close to each other, have increased on the export side over time, which is why the ratio of these product items has reached such high levels. Though the export-import balance has altered several times throughout the years, this ratio did not reflect a similar tendency for sugar and sugar products.

Table 6c- Türkiye's export-import ratios from 2010 to 2018 (SCO)

HS	*2010*	*2011*	*2012*	*2013*	*2014*	*2015*	*2016*	*2017*	*2018*
01	62.22	166.40	27.57	1.00	10.59	1.86	1.15	51.49	218.56
02	*	*	*	43714.90	*	92030.53	*	*	86928.89
03	75.69	121.75	107.10	230.90	321.29	260.23	336.27	279.80	368.46
04	1849.84	12387.70	3020.09	4003.55	2989.60	4273.09	7838.50	12796.67	1652.22
05	0.03	0.17	0.10	0.05	0.59	0.59	3.33	1.59	1.39
06	259.20	323.35	212.75	275.37	494.56	435.76	1128.09	513.63	829.48
07	557.30	413.18	430.94	327.91	344.99	361.41	53.78	44.99	88.34
08	2818.83	2418.01	1342.24	1592.78	1920.80	1150.60	427.53	531.27	1304.47
09	47.76	73.71	33.85	24.26	32.90	26.80	26.01	10.85	19.27
10	0.66	0.49	0.32	0.51	0.29	0.73	1.12	0.83	0.51
11	96.05	67.60	8.38	31.15	4.39	21.20	51.39	78.69	18.13
12	101.86	74.48	31.14	75.23	38.36	31.20	39.19	8.94	57.92
13	10.85	3.93	16.16	11.46	17.32	15.62	7.96	16.14	26.97
14	317.53	226.48	381.62	177.23	93.67	194.87	160.34	119.72	104.36
15	33.40	39.82	7.34	11.88	6.50	6.50	8.56	15.37	22.80
16	942.72	3328.62	1450.62	475.29	88.48	31.86	147.35	50.21	41.65
17	294.42	305.91	121.65	153.20	74.10	105.14	69.49	98.27	110.86
18	5436.82	4407.84	1601.24	1191.26	4426.85	19205.09	7326.58	248.96	423.44
19	2812.45	2815.36	2188.37	1359.38	1536.17	1473.66	2587.07	2122.90	1716.79
20	745.74	800.72	943.32	748.80	853.86	680.57	1172.40	1583.37	1983.36
21	540.41	477.91	277.06	159.20	138.49	96.85	91.91	78.35	83.07
22	13.99	6.43	6.30	3.98	7.60	19.74	33.06	25.62	20.41
23	0.89	0.54	0.81	0.56	0.67	0.54	0.75	1.06	1.37
24	115.48	188.49	302.22	310.08	308.58	199.01	219.59	136.67	143.68

*Import was not carried out. Source: Own calculations based on the TSI dataset

3.2. Comparative advantage in relation to the EU and the SCO

In its fruit trade with both the EU and the SCO, Türkiye has a comparative advantage (Table 7). However, while Türkiye had a comparative advantage in some of the agri-food items traded with these organizations, a variety of agri-food products were traded with EU countries. There was a comparative advantage in fruit, vegetables, fish, aquaculture, sugar, and sugar products. Ekmen Ozcelik & Erlat (2014) found that Türkiye has a higher index of revealed comparative advantage in fruit and vegetable products. Moreover, when we consider products such as fruit and vegetables, sugar and honey, and raw material for sugar between 1996 and 2007, Turkey has a higher comparative advantage than the EU in the group of milk powder. When the average LFI value from 2010 to 2018 is used, comparative advantage can be seen in fruit (0.17), vegetables (0.09), fish and seafood (0.03), and sugar and sugar products (0.02). For the study period, Türkiye's exports of certain products to EU countries have been greater than its purchases. On the other hand, live animals, cereals, coffee, tea extracts, yeasts, sauces, dietary foods, food industry residues, roughages, oilseeds, chocolate, and meat products have no comparative advantage.

Table 7- Comparison of Türkiye's competitiveness against the EU and SCO in the period of 2010-2018 (the Lafay index)

HS	*2010*	*2011*	*2012*	*2013*	*2014*	*2015*	*2016*	*2017*	*2018*	*Average*
European Union										
08	0.1695	0.1917	0.1878	0.1736	0.1715	0.1767	0.1739	0.1678	0.1631	0.1751
20	0.0844	0.1007	0.1023	0.0934	0.0948	0.1083	0.0975	0.1002	0.0940	0.0973
03	0.0175	0.0258	0.0257	0.0287	0.0294	0.0322	0.0424	0.0435	0.0446	0.0322
07	0.0338	0.0288	0.0221	0.0233	0.0202	0.0166	0.0247	0.0316	0.0336	0.0261
17	0.0066	0.0087	0.0087	0.0080	0.0080	0.0048	-0.0034	0.0034	0.0027	0.0053
16	0.0019	0.0024	0.0023	0.0032	0.0025	0.0022	0.0024	0.0024	0.0025	0.0024
05	0.0013	0.0024	0.0025	0.0030	0.0028	0.0016	0.0012	0.0007	-0.0006	0.0017
14	0.0007	0.0018	0.0009	0.0007	0.0003	0.0003	0.0005	0.0008	0.0005	0.0007
09	0.0006	0.0009	0.0007	0.0007	0.0000	-0.0001	0.0012	0.0005	-0.0006	0.0004
13	-0.0037	-0.0029	-0.0035	-0.0039	-0.0039	-0.0032	-0.0034	-0.0027	-0.0032	-0.0034
15	-0.0035	-0.0040	-0.0024	0.0024	-0.0144	-0.0107	-0.0099	-0.0030	-0.0013	-0.0052
06	-0.0063	-0.0058	-0.0066	-0.0110	-0.0102	-0.0082	-0.0090	-0.0065	-0.0028	-0.0074
11	-0.0048	-0.0034	-0.0047	-0.0074	-0.0079	-0.0076	-0.0095	-0.0094	-0.0116	-0.0074
04	-0.0092	-0.0040	-0.0079	-0.0103	-0.0120	-0.0105	-0.0089	-0.0060	-0.0069	-0.0084
24	-0.0059	-0.0041	-0.0137	-0.0175	-0.0157	-0.0156	-0.0208	-0.0095	-0.0125	-0.0128
22	-0.0066	-0.0097	-0.012	-0.0200	-0.0234	-0.0222	-0.0194	-0.0192	-0.0094	-0.0158
19	-0.0211	-0.0154	-0.0172	-0.0215	-0.0204	-0.0192	-0.0171	-0.0134	-0.0121	-0.0175
02	-0.0516	-0.0767	-0.0159	-0.0039	0.0001	-0.0054	0.0002	-0.0120	-0.0263	-0.0213
18	-0.0265	-0.0181	-0.0212	-0.0241	-0.0219	-0.0173	-0.0198	-0.0217	-0.0217	-0.0214
12	-0.0552	-0.0443	-0.0316	-0.0624	-0.0612	-0.0418	-0.0380	-0.0139	-0.0213	-0.0411
23	-0.0274	-0.0392	-0.0790	-0.0468	-0.0321	-0.0365	-0.0380	-0.0337	-0.0353	-0.0409
21	-0.0374	-0.0225	-0.0292	-0.0411	-0.0487	-0.0493	-0.0500	-0.0481	-0.0477	-0.0416
10	-0.0335	-0.0465	-0.0271	-0.0426	-0.0411	-0.0579	-0.0433	-0.0626	-0.0367	-0.0435
01	-0.0235	-0.0666	-0.0810	-0.0245	-0.0167	-0.0371	-0.0537	-0.0893	-0.0911	-0.0537
Shanghai Cooperation Organization										
08	0.2087	0.1938	0.1636	0.1694	0.1578	0.1789	0.1354	0.2093	0.1962	0.1792
07	0.0813	0.0675	0.0815	0.0769	0.0804	0.0798	0.0012	-0.0044	0.0111	0.0528
24	-0.0022	0.0081	0.0309	0.0327	0.0355	0.0335	0.0549	0.0183	0.0221	0.0260
18	0.0258	0.0269	0.0255	0.0239	0.0222	0.0207	0.0284	0.0171	0.0205	0.0234
20	0.0139	0.0111	0.0135	0.0145	0.0165	0.0145	0.0179	0.0260	0.0242	0.0169
21	0.0168	0.0179	0.0185	0.0107	0.0108	0.0063	0.0097	0.0049	0.0047	0.0111
03	-0.0025	0.0001	0.0029	0.0067	0.0119	0.0094	0.0138	0.0128	0.0198	0.0083
19	0.0073	0.0074	0.0078	0.0077	0.0084	0.0096	0.0148	0.0144	0.0119	0.0099
04	0.0025	0.0172	0.0022	0.0034	0.0032	0.0044	0.0147	0.0137	0.0124	0.0082
02	0.0060	0.0097	0.0161	0.0069	0.0094	0.0048	0.0026	0.0035	0.0026	0.0068
17	0.0074	0.0090	0.0090	0.0098	0.0043	0.0048	0.0036	0.0059	0.0062	0.0067
06	0.0007	0.0009	0.0007	0.0012	0.0019	0.0015	0.0052	0.0036	0.0045	0.0022
14	0.0029	0.0018	0.0042	0.0017	0.0006	0.0021	0.0023	0.0015	0.0007	0.0020
16	0.0005	0.0019	0.0005	0.0003	0.0002	-0.0002	0.0003	0.0000	-0.0001	0.0004
01	-0.0001	0.0000	0.0000	-0.0003	0.0000	-0.0001	-0.0001	0.0000	0.0001	-0.0001
13	-0.0031	-0.0029	-0.0010	-0.0017	-0.0007	-0.0011	-0.0013	-0.0012	-0.0008	-0.0015
09	-0.0018	-0.0006	-0.0007	-0.0017	-0.0004	-0.0011	-0.001	-0.0045	-0.0017	-0.0015
11	-0.0009	-0.0023	-0.0033	-0.0027	-0.0029	-0.0037	0.0001	0.0013	-0.0016	-0.0018
05	-0.0095	-0.0110	-0.0041	-0.0047	-0.0034	-0.0037	-0.0026	-0.0033	-0.0020	-0.0049
22	-0.0195	-0.0200	-0.0080	-0.0096	-0.0057	-0.0042	-0.0016	-0.0033	-0.0043	-0.0085
12	-0.0075	-0.0157	-0.0199	0.0030	-0.0080	-0.0137	-0.0074	-0.0475	0.0012	-0.0128
23	-0.0524	-0.0619	-0.0516	-0.0566	-0.0399	-0.0505	-0.0557	-0.0576	-0.0503	-0.0529
15	-0.0367	-0.0283	-0.0972	-0.0878	-0.1000	-0.1272	-0.1175	-0.0561	-0.0268	-0.0753
10	-0.2376	-0.2307	-0.1913	-0.2037	-0.2020	-0.1648	-0.1178	-0.1544	-0.2505	-0.1948

Source: Own calculations based on the TSI dataset

In comparison to EU countries, live animals (-0.05), cereals (-0.04), coffee and tea extracts (-0.04), yeasts and sauces (-0.04), food industry residues and roughages (-0.04), oilseeds (-0.04), cocoa (-0.02), and meat (-0.02) are at a disadvantage. The majority of these traded goods are targeted for import. According to the EU, while Türkiye received positive and rather high values in vegetables and fruits, it received negative and quite low values in grains, drinks, and cigarettes, according to the LFI estimated by Cagatay & Guzel (2003). Türkiye had a comparative advantage in its agricultural food trade with SCO countries. Fruit, vegetables, cocoa, coffee extracts, tea extracts, sauces, and yeast products are all examples. Fruits, vegetables, cocoa, and coffee extracts; tea extracts; yeasts; and sauces had comparative advantage index averages of 0.17, 0.05, 0.02, and 0.01 for fruits, vegetables, cocoa, and coffee extracts; tea extracts; yeasts; and sauces, respectively.

Türkiye has mainly exported these products to these countries. On the other hand, it cannot be said that it has a comparative advantage in cereals, animal and vegetable fats and oils, edible oils, food industry residues, roughages, oilseeds, soft drinks, alcoholic beverages, and vinegar products. Their indexes were, cereals (-0.19), animal and vegetable fats and oils, edible fats (-0.07), food industry residues and forages (-0.05), oilseeds (-0, 01), alcoholic beverages, and vinegar products (-0.01), in which the import side of the trade has played a more important role over the years. As the founder of the SCO, Russia has a comparative advantage in cereals and vegetable oils compared to Türkiye (Benesova et al. 2017; Liefert and Liefert 2020).

In trading with SCO countries, Türkiye has a comparative advantage in cocoa powder, chocolate, and other culinary products containing cocoa. In commerce with EU countries, Türkiye, on the other hand, has a comparative disadvantage in certain products when trading with EU countries. In recent years, exports of this food category have increased, particularly to China. In recent years, the export of this food category to China has increased significantly. The sauce and preparations were imported from China, in addition to the export and import of protein concentrate and protein-based compounds. SCO member nations export a lot of yeast and baking powder, which means they have a competitive edge. For the same products, however, there was a comparative disadvantage compared with the EU.

3.3. Analysis of the LFI with a duration greater than zero

The stability of the relative comparative trade advantage or trade competitiveness over time was tested using duration analysis. In Figure 1, the LFI index was used to show Türkiye's 9-year survival rate in the EU and SCO markets (groups). According to the survival analysis, since 2010, the EU and SCO countries' markets (groups) have been declining. The SCO countries saw the greatest drop in survival rates for the LFI >0 indicators. In other words, the EU and SCO markets' chances of survival plummeted from 92 percent in 2010 to 54 percent in 2018 for the EU and 30 percent for the SCO. Similar conclusions on the limited continuity of Türkiye's exports to EU member countries were found by Piskin (2017) in his study. The equality of survival functions in the LIF index for the EU and SCO groups was examined using two non-parametric tests (the Wilcoxon test and the log-rank test). The results demonstrate that at a 5% significance level, the equality of survival function between the EU and SCO groups may be rejected, indicating that there is no resemblance in the comparative advantage period between the two groups (Figure 1).

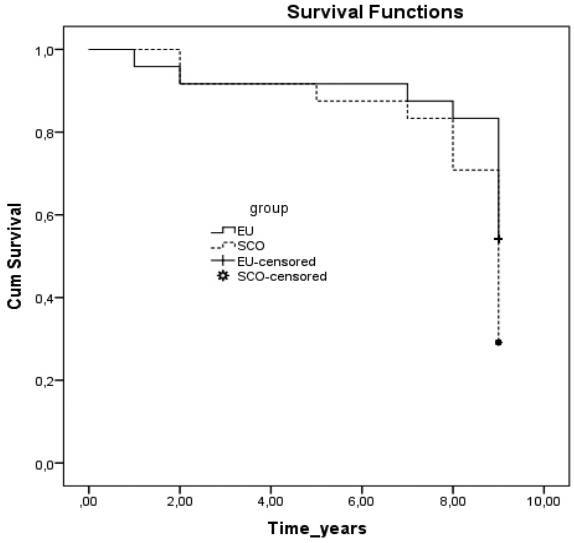

Figure 1- LFI Kaplan-Meier survival rates >0

4. Conclusions

Türkiye's overseas trade volume increased to $30 billion between 2010 and 2018. The volume of agri-food exports increased in both EU and SCO countries throughout this time. In 2010, the share of trade volume with SCO members was 37% of that with EU countries; by the end of 2018, it had risen to 52%. The reason for this was that trade volume with SCO countries increased at a faster rate than trade volume with EU countries.

Türkiye's agri-food exports increased from $12.040 billion in 2010 to $17.673 billion in 2018, while imports increased from $7.683 billion to $12.845 billion. Furthermore, the annual rates of export and import growth climbed by 5% and 7%, respectively. Türkiye's exports to EU nations grew at a slower rate than those to SCO nations. Similarly, agri-food imports from SCO countries increased at a quicker rate than imports from EU countries. In terms of import shares, the EU and SCO countries followed opposing trends. However, the import values of the SCO and EU countries were essentially the same towards the end of the study period.

Despite a foreign trade surplus in agricultural and food items with EU countries in 2018, the foreign trade balance slipped below $2 billion in 2018. Except for the first few years, the imports were higher in the adjustment of the agri-food trade balance with SCO countries. Despite these fluctuations over time, Türkiye's foreign trade balance for agri-food goods with both EU and SCO member nations continues to favor imports.

From 2010 to 2018, the vegetable and fruit trade was one of Türkiye's largest revenue generators in the agricultural food goods sector. Fruit and vegetable commerce had a higher export-import ratio than live animals and animal feed trade in Türkiye's trade with the EU and SCO countries. In other words, in the export of fruit and vegetable goods to these two nations, Türkiye possessed a comparative advantage.

Grain, food industry residues, roughages, and oilseed products, on the other hand, share a comparative disadvantage with these two groups. In addition, Türkiye had a comparative advantage and a comparative disadvantage in the SCO and EU countries, respectively, for the same cocoa products. Coffee extracts, tea extracts, yeasts, sauces, and dietary meals are all examples of this. The survivability analysis of its export competitiveness was conducted in the EU and SCO markets. Since 2010, survival rates for the LFI >0 index have been declining in the EU and SCO groupings. In other words, by 2018, the EU's and SCO countries' odds of survival, which were 92 percent in 2010, had dropped to 54 percent for the EU and 30 percent for the SCO countries. Between EU and SCO country groups, there is a variation in the comparative advantage period. During this time, it was discovered that the comparative advantage was not lasting. Policymakers should create supportive policies in this situation for sustainable exports with the EU and SCO.

Türkiye had a comparative advantage in the export of agri-food products to the EU and SCO markets for 8 and 13 food types, respectively. Exports were limited, however, due to preferential agri-food product trade adopted in the EU market for different political reasons. In contrast to the EU countries, there was a growth in foreign trade volume despite the lack of a stable foreign trade balance with the SCO countries. Given the limited supply of agricultural food items in the EU market, Türkiye could increase its export trade volume by strengthening ties with SCO nations. According to the findings, a large part of Türkiye's export of agricultural food products is to EU countries, but a significant part of its imports are from SCO countries. In this case, it seems to Türkiye's detriment that Türkiye prefers the EU to the SCO, or the SCO to the EU. It will be in Türkiye's favor if it maintains its relations with both institutions independently of each other. Finally, political structural reforms in the agri-food sector should be adopted to combat unfavorable trends in agri-food trade with EU and SCO members. In this context, having an effective institutional structure, implementing technology-oriented transformation, increasing the level of competence by creating a qualified workforce, and providing the necessary infrastructure support are the prerequisites for the increase in productivity required to compete in the international arena. Türkiye must transition from a technology-importing to a technology-producing country, it is necessary to focus on innovation activities and provide incentives to support innovation in a careful and planned manner. A sustainable competitive advantage requires the development of an innovation culture at the level of entrepreneurs, companies, industries, regions, and nations.

Data availability: Data are available on request due to privacy or other restrictions.

Authorship Contributions: Concept: S.K., B.Ö., F.Y., Design: S.K., B.Ö., F.Y., Data Collection or Processing: S.K., B.Ö., F.Y., Analysis or Interpretation: S.K., B.Ö., F.Y., Literature Search: S.K., B.Ö., F.Y., Writing: S.K., B.Ö., F.Y.

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Cropping Pattern Classification Using Artificial Neural Networks and Evapotranspiration Estimation in the Eastern Mediterranean Region of Turkey

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ABSTRACT

Determining cropping patterns is crucial for quantifying irrigation water requirements at a catchment scale. For this reason, new and innovative technologies such as remote sensing (RS) and artificial neural networks (ANNs) are robust tools for generating the spatiotemporal variation of crops. In line with this, this study aims to classify each crop type using the ANN algorithm and calculate crop evapotranspiration (ETc). This study was conducted in the Akarsu Irrigation District (9495 ha) in the Lower Seyhan Plain in southeastern Turkey in the 2021 hydrological year. Crop types were classified using the ANN algorithm in the Environment for Visualizing Images (ENVI) program based on combined data from Sentinel-2 images with a 10-m resolution and ground truth data collected during the winter and summer seasons. The image analysis results demonstrated that bare soil and citrus made up 3666 ha and 3742 ha respectively in the winter season, while first crop corn (1586 ha) and citrus (4121 ha) were preponderant in summer. The confusion matrix of the ANN algorithm showed high agreement (wheat 89.76%, onion 91.67%; citrus 97.67% in winter and 98.98% in summer; 100% for lettuce, potato, sesame-2, palm, and watermelon) and medium agreement (fruit 58.33% in winter, 42.86% in summer) with ground truth data in growing seasons. Furthermore, the agreement was more than 80% for the first and second crops (cotton, soybean, peanut, and corn) in the summer season. Annual reference evapotranspiration and ETc were around 1308 mm and 890 mm, respectively. The ETc values for wheat, citrus, first-crop corn, and second-crop soybean were found to be consistent with previous studies of direct evapotranspiration methods conducted in the Cukurova region. Overall, RS and ANNs can be used to classify crop types accurately in the growing season. This study builds upon and expands the application of RS and ANNs in large-scale irrigation schemes.

Keywords: Crop-type classification, Crop evapotranspiration, Sentinel-2, Supervised classification, Remote sensing

1. Introduction

Cropping patterns are defined as the areal coverage under different crops at different periods. It also refers to the time and spatial order or succession of crops and/or uncultivated in a specific agricultural area. As pointed out, among others, by Cetin (2020), the types of cultivated plants are subject to change spatiotemporally since market demand and climate conditions are variable in time. However, the determination of crop types in a large-scale irrigation catchment is not an easy task. Crop classification can provide farmers, irrigation water managers, irrigation authorities, engineers of water user associations, etc., with essential and precise information on the crop type by using remotely sensed data coupled with ground truth data. Zheng et al. (2015) indicated that the satellite imagery selection for crop classification is based on, among many other things, image availability, variety level in crop types, and land area extent.

Remote sensing (RS) methods based on optical and/or microwave sensors have become increasingly common in order to extract crop information that explains the vegetation conditions and biophysical crop properties (Yildirim & Asik 2018). RS technology not only provides continuous and large spatial coverage on a large scale (Aksu & Arikan 2017; Oguz 2015) but also provides precision and confidence in the final products. For this reason, RS techniques have been widely applied for drought monitoring (Aksu et al. 2022),

precipitation (P), temperature, water resource, and agricultural monitoring (Kuzay et al. 2022) in large- or small-scale areas. RS also provides availability of information (freely) on crop growth and health status to the farmers (Jayanth et al. 2021). Among other satellites, the Sentinel-2 satellite has thirteen spectral bands with 5-day temporal resolution, which makes it the most popular satellite for vegetation mapping.

A review of the literature shows that the Sentinel-2 has been widely used in precision agriculture for crop monitoring applications (Whyte et al. 2018; Sonobe et al. 2017; Belgiu & Csillik 2018). Five major crop types and non-agriculture areas in Ukraine, Mali, and South Africa and five local locations distributed over the world were classified using the Sentinel-2 and Landsat 8 data (Defourny et al. 2019). They reported that the average accuracy of classification for all areas, with one exception, was higher than 80%. Moreover, Jiang et al. (2020) obtained a mean accuracy of 94% for producing a map using Sentinel-2 satellite imagery for major crop types in three vast sites located in China (each site has between 2 and 3 crops). It must be remembered, however, that classification accuracy is subject to plant species that show changes depending on the climate change phenomenon.

In the Seyhan River basin, climate projections by Columbia University's Center for Climate System Research (CCSR) and the Metrological Research Institute (MRI) of Japan have shown increasing temperature trends (+2.7 °C and +2 °C change by CCSR and MRI, respectively) and decreasing P (159 mm by MRI and 161 mm by CCSR) for the future period up to 2080 (Selek et al. 2016). Increasing temperatures will not only accelerate evapotranspiration rates but also affects plant species. With this in mind, the areal extent of crop types and, hence, estimation of crop evapotranspiration (ETc) is of critical importance. As such, to estimate ETc precisely, crop classification is paramount. Crop data and the development stages of each crop are used to estimate ETc which is determined by multiplying reference evapotranspiration (ETo) by crop coefficient (Kc) according to the crop type and different vegetative stages (Aksu & Arikan 2017; Cetin 2020). The Penman-Monteith (PM) model is one of the best indirect methods for ETc estimation, and has been used commonly in a considerable amount of research in recent years (Santos et al. 2019). In the same context, PM is considered a standard by scientists, irrigators, and the Food and Agriculture Organization because it takes into account all the climatic variables (Santos et al. 2019; Allen et al. 1998).

This study highlights how to determine cropping patterns by using the Artificial Neural Networks (ANNs) algorithm with Sentinel-2 data and ground truth data to better understand and monitor the growth stages of crops and estimate crop biophysical parameters. This study is considered the first attempt to classify cropping patterns in the Akarsu Irrigation District (AID) in the LSP of Turkey. The overall objective of this study is to (1) classify crop types by using the ANN algorithm with the Sentinel-2 satellite images and ground truth data and (2) estimate ETc based on ETo by the PM model using the data of two meteorological stations over the study area.

2. Material and Method

The flowchart for ETc calculation, known as a "two-step" procedure, in this study is presented in Figure 1. Figure 1 summarizes the details of the data as well as the methods adopted in detail below.

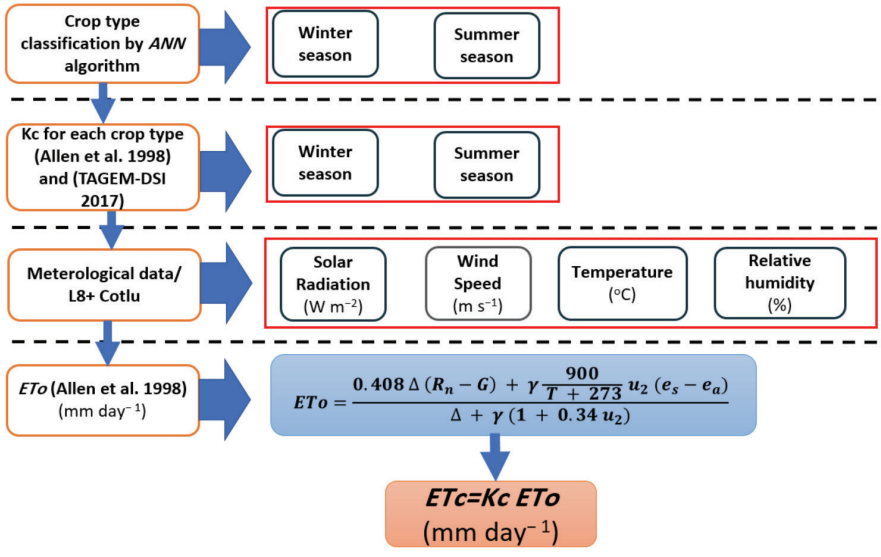

Figure 1- Methodology followed for crop evapotranspiration (ETc) calculation by a "two-step" procedure

2.1. Study area

The study area, AID, covers an area of approximately 95 km^2 (9495 ha). The AID is situated in the LSP and located between 36˚57'and 36˚51'N latitudes and 35˚40' and 35˚29'E longitudes, and is noted for its substantially flat terrain (Figure 2). The Mediterranean climate - characterized by hot and dry summers and warm and rainy winters - prevails in the study area. The annual daily average, minimum and maximum air temperatures are 18.9 °C, 9.0 ºC, and 31.0 ºC, respectively. The annual mean P of the Seyhan River basin is around 649.5 mm (Cetin et al. 2020). The hydrological year in Turkey and the AID covers the period between the 1st of October of one year and the 30th of September of the next year. Major crops grown in the AID include cereals, potatoes, onions, and lettuce in the winter season, while summer crops include corn, cotton, soybeans, groundnuts, and watermelons (Ozcan et al. 2003), regardless of whether they are a first or second crop.

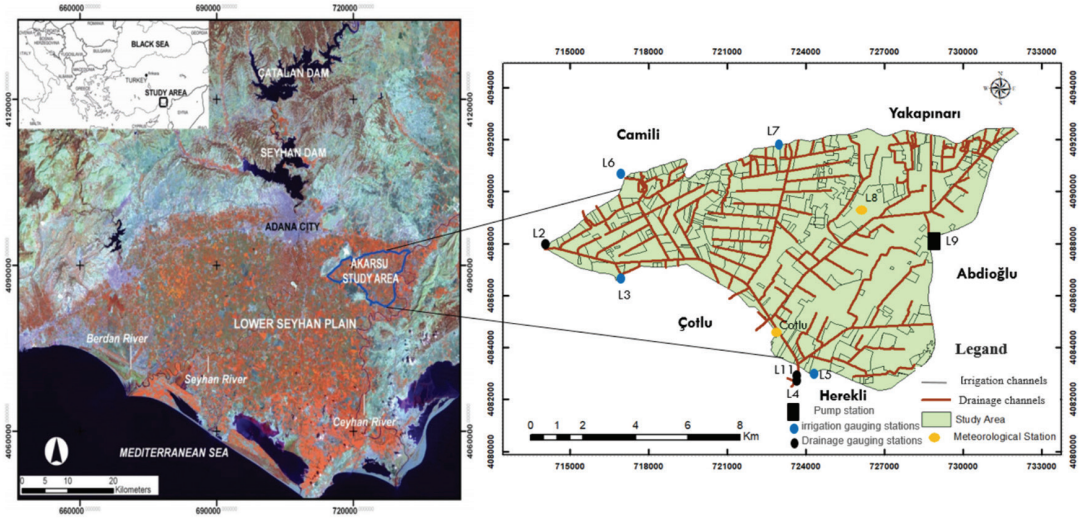

Figure 2- The Akarsu Irrigation District in the Lower Seyhan Plain, in the Eastern Mediterranean region of Turkey

2.2. Crop type processing

Crop classification results provide crucial data on crop types at the regional scale and thus on cropping patterns. The flowchart, given in Figure 3, presents the required steps to implement the ANN algorithm to classify crop types in the winter and summer seasons using remotely sensed data coupled with ground truth data on the AID. To generate the most accurate insights, we combined data from Sentinel-2 images and ground truth data obtained by surveying different croplands or fields in both the winter and summer seasons. Since splitting the data into two parts (80% for training and 20% for testing) is a generally accepted practice in data science (Mahlayeye et al. 2022) and geospatial modelling (Boken et al. 2004), the same procedure was applied in this research. Moreover, the procedure of the ANN algorithm was followed to detect field boundaries and classify different types of crops. To achieve high degrees of accuracy in the classification process, Normalized Difference Vegetation Index (NDVI), ranging from -1.0 to +1.0, was also determined and included in the study as shown by Rouse et al. (1973).

The NDVI values were calculated as the following by using the Sentinel-2 Satellite Imagery:

$$NDVI = \frac{(NIR_{band8} - Red_{band4})}{(NIR_{band8} + Red_{band4})} \quad (1)$$

where NIR_{band} and Red_{band} are the "*near-infrared*" and *"red"* reflectance, respectively.

Sentinel-2A-2B images, the details of which are given in Table 1, are obtainable at https://scihub.copernicus.eu/dhus/#/home. The crop-type classification was performed in Environment for Visualizing Images (ENVI) software using the "Neural Net Classification" module under "Supervised Classification"; the logistic activation method was used, and three hidden layers were selected. In detail, the input layers are 10 regions in the winter season and 16 regions in the summer, each region presents a crop type as a region of interest (ROI). The links between the input layers and three hidden layers take different weights and are trained depending on the required

output (classified map). To improve classification accuracy, a band is added to the calculated NDVI image as remote sensing data. Furthermore, ground truth data were converted to the ROI format and entered into the ENVI program, along with 5-band (Red, Green, Blue, NIR, and NDVI) images. The Neural Net root mean square (RMS) curve, which was drawn against the iteration value, was extracted separately for both the 2021 winter and 2021 summer classification maps, and the iteration value with the minimum RMS value, as suggested strongly by Boken et al. (2004), was used in the classification. Concordantly, following the steps given in Figure 3, pre-processing of the image data was performed before classification.

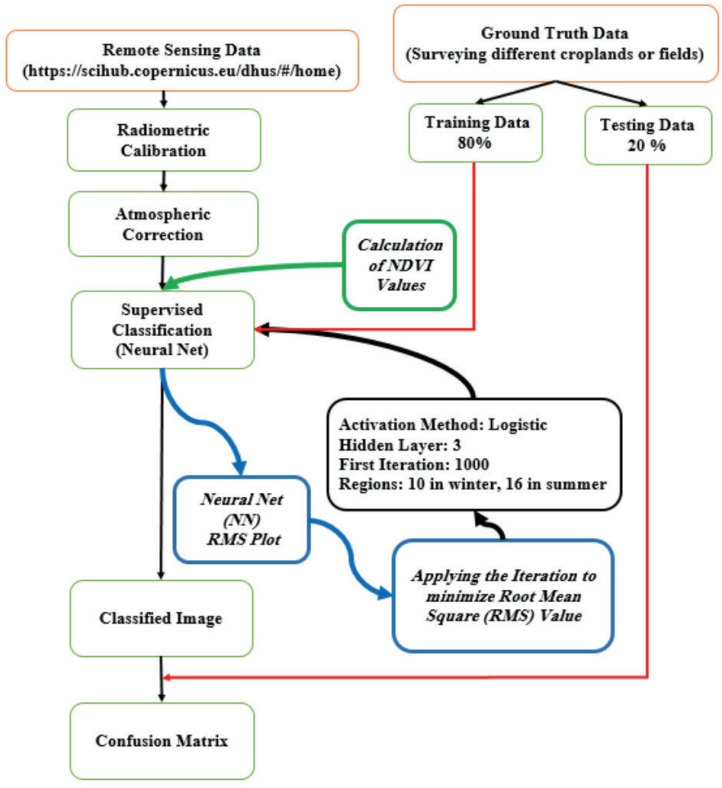

Figure 3- Methodology followed for ANN procedure by ENVI program using remotely sensed data coupled with ground truth data

Table 1- Satellite image names and dates of the images used in the acquisition of summer and winter cropping patterns in the research area

Image name	*Date*
S2B_MSIL2A_20200413T082559_N0214_R021_T36SYF_20200413T115830	13.04.2020
S2B_MSIL2A_20200702T082609_N0214_R021_T36SYF_20200702T122946	02.07.2020
S2A_MSIL1C_20200806T082611_N0209_R021_T36SYF_20200806T100018	06.08.2020
S2B_MSIL2A_20210329T082559_N0214_R021_T36SYF_20210329T111602	29.03.2021
S2B_MSIL2A_20210627T082559_N0300_R021_T36SYF_20210627T112602	27.06.2021
S2A_MSIL2A_20210811T082601_N0301_R021_T36SYF_20210811T114551	11.08.2021

2.3. Crop Evapotranspiration (ETc) Estimation

ETc, as in Equation 2, is calculated by multiplying ETo by Kc according to the crop type and different vegetative stages. This methodology is known as a "two-step" procedure (Steduto 2000).

$$ETc = Kc\ ETo \quad (2)$$

where ETc is crop evapotranspiration, ETo is reference evapotranspiration in the unit of mm day^{-1}, and Kc is crop coefficient for a single crop depending on the crop's stage of development. In this study, the standard single Kc value was chosen, as proposed by Allen et al. (1998) and the tables provided by The General Directorate of Agricultural Research And Policies-The State Hydraulic Works (TAGEM-

DSI) (2017) have been used. In addition, Kc values consider the status of the crop at the time of growth stage in different times of growth stages, i.e., initial crop development, mid-season (second and third stages), and late-season, for crops, which are common in the winter and summer seasons in the LSP of Turkey. Allen et al. (1998) modified the PM method to estimate ETo (Equation 3). Equation 3 was developed for short grass; more information, if needed, related to Equation 3 has been provided by Allen et al. (1998).

$$ETo = \frac{0.408\,\Delta\,(R_n - G) + \gamma \frac{900}{T+273} u_2\,(e_s - e_a)}{\Delta + \gamma\,(1 + 0.34\,u_2)} \quad (3)$$

where *ETo* is the reference evapotranspiration (mm day^{-1}), R_n is the net radiation at the crop surface (MJ m^{-2}day^{-1}), G is the soil heat flux density (MJ m^{-2}day^{-1}), T is the mean daily air temperature at 2 m height (°C), u_2 is the wind speed at 2 m height (m s^{-1}), e_s is the saturation vapour pressure (kPa), e_a is the actual vapour pressure (kPa), e_s-e_a is the saturation vapour pressure deficit (kPa), Δ is the slope of the vapour pressure-temperature curve (kPa°C^{-1}), and γ the psychrometric constant (kPa°C^{-1}).

In this study, climatic variables on hourly and daily time scales were obtained from two automatic meteorological stations, shown in Figure 2, and we applied quality control checks to meteorological data. For this purpose, Cotlu and L8 meteorological stations, as seen in Figure 2, were established and operated in the research area.

3. Results and Discussion

3.1 Precipitation and reference evapotranspiration (ETo)

Meteorological data observed at the meteorological stations in the study area in the 2021 water year indicated that the climatic conditions of the district and its environs are conducive to agriculture throughout the year. The study area is characterized as hot and dry in summer, and rainy and cool in the winter. Therefore, as seen in Figure 4, ETo ranged between 0.67 mm day^{-1} (minimum value on January 14, 2021) and 8.08 mm day^{-1} (maximum value on July 28, 2021). In addition, Figure 5 shows the temporal variability in monthly ETo values, ranging from 43 mm (all-time low in both December and January) to 189 mm (all-time high in July). The annual cumulative ETo was around 1308 mm for the 2021 hydrological year. As seen in Figure 4, variability in daily P and ETo is more distinctive than in monthly ones. As can be seen from the results, the annual mean ETo and its standard deviation were around 3.59 mm day^{-1} and 1.80 mm day^{-1} (S^2=3.24), respectively. A higher variance in daily reference evapotranspiration indicates greater temporal variability in the data (CV≈50%), implying more meteorological stations are needed in the District. The annual total P of the 2021 water year decreased by 183 mm to the annual mean P of the basin (Cetin et al. 2020). Based on the meteorological observations, most of the rainfalls occurred in the winter season, the cumulative P was 382 mm in the winter season of the 2021 water year, whereas rainfall events rarely took place in the summer season.

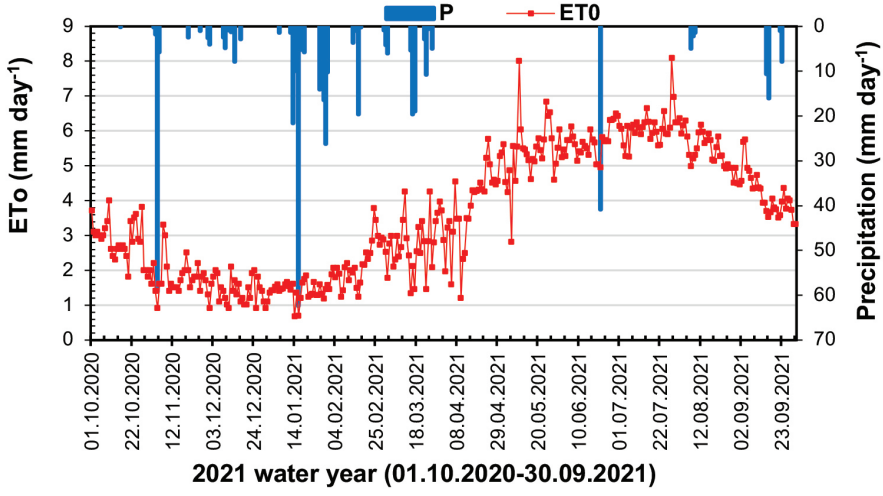

Figure 4- Temporal variations of precipitation and ETo over the study area in the 2021 water year

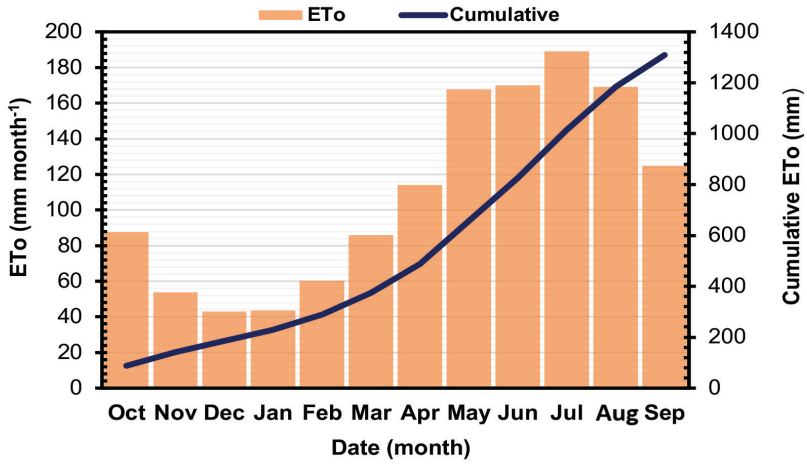

Figure 5- Monthly ETo values and cumulative reference evapotranspiration during the 2021 hydrologic year

3.2. Crop type classification

Figure 6 shows the results of the crop classification both in the winter and summer seasons. Classification of crop types by ANN algorithm coupled with the ground truth data (for each parcel) resulted in a changing cropping pattern over the study area. A total of 1,316 ground-truth data were used for crop classification in the winter and 1,469 in the summer. Eighty per cent of the dataset was used in training and twenty per cent in testing. Finally, a high classification accuracy (more than 90%), on average for most crops, was obtained over the study area, regardless of the summer or winter season. Mahlayeye et al. (2022) pointed out that cropping patterns are distinguished through their designed spatial arrangement within a field. The cropping pattern results presented in this study (Figure 6) showed parallelism with the cropping pattern examples, i.e., spatial clustering, given in Mahlayeye et al. (2022). For example, as can be seen in Figure 6, citrus-planted areas were dominant in the eastern parts of the study area throughout the year. However, a significant part of the land is empty (bare soil) in winter, and reserves are kept for the first crop in the summer season. On the other hand, corn as the first (Corn-1) and second (Corn-2) crop was the most common crop (18.6%) in the summer season. The pie charts in Figure 7 show the percentage of crop types by summer and winter seasons.

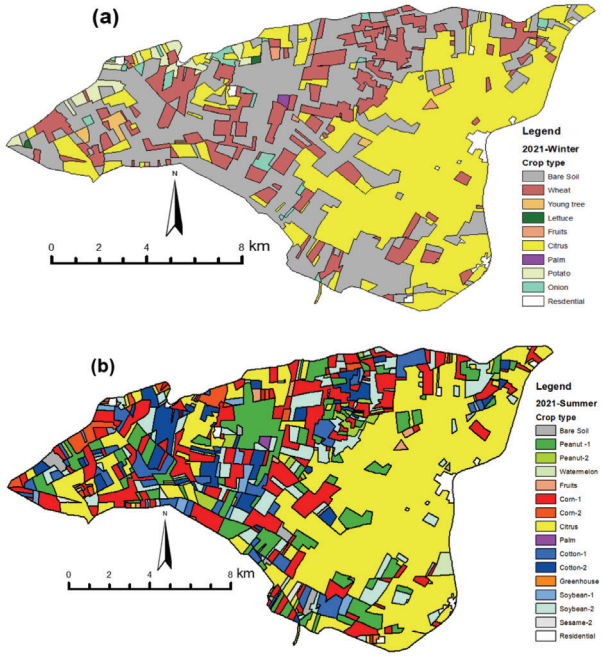

Figure 6- Crop types distribution over the study area: in winter (a) and in summer (b)

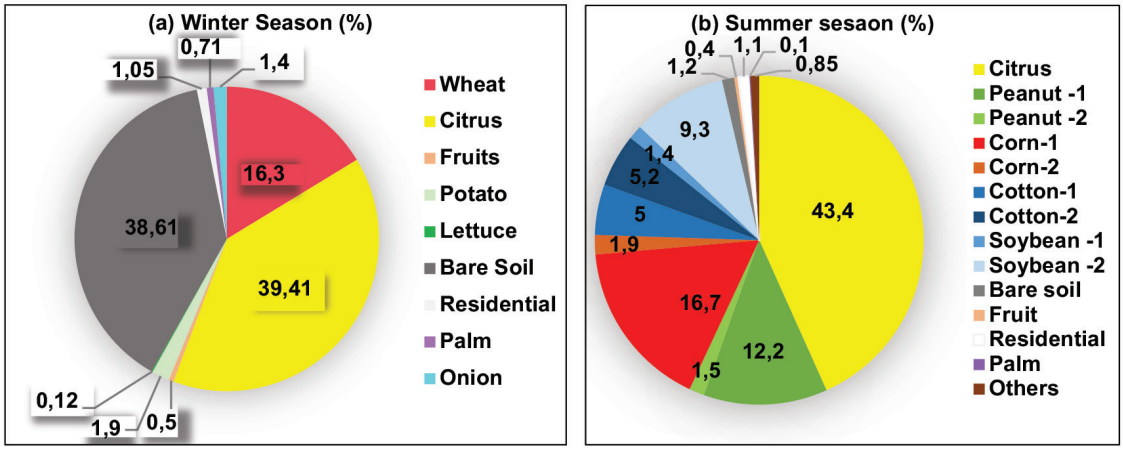

Figure 7- The percentage of area covered by plants cultivated in the research area in the winter and summer of 2021

When the ANN algorithm by the ENVI software was applied, a confusion matrix was obtained for the winter and summer seasons (Table 2,3). As seen in Table 2, wheat-grown areas were confused with citrus plantations (3.41%) and bare fields (6.83%) in the winter season while fruit plantations were confused with citrus plantations (33.33%) and bare soil (8.33%). This type of confusion can be explained by the fact that fruit plantations and small citrus trees have the same land characteristics in winter since small citrus seedlings occupy only a very small area of the land. In addition, onion-planted areas were confused with bare soil (8.33%). During the collection of the ground truth data on March 1 to 7, 2021, we observed that wheat and onion crops partially covered the land. For this reason, onion- and wheat-planted fields were faultily classified as bare soil in generated cropping pattern maps (Table 2).

Likewise, a confusion matrix was acquired for the crops grown in the summer season (Table 3). Based on results from Table 3, fruit-planted areas have the highest level of confusion with citrus plantations (42.86%) owing to either their same shape formation or coverage characteristics. Hence, the ANN algorithm in the ENVI software defectively classified fruit trees as citrus plantations. Additionally, Corn-1, Corn-2, Peanut-1, Peanut-2, Soybean-1, Soybean-2, and fruit fields were confused with citrus trees from 0.82% to 42.86% as shown in Table 3. Luckily, Soybean-1 was confused with Soybean-2 (15.38%); this confusion can be explained plainly that the first- and second-crop soybean have the same shape formation (height, width, and leaf development, etc.) at the time of ground truth data acquisition.

Table 2- Confusion matrix of crop types (%) by ANN classification in the winter season

		Truth							
	Class	*Bare soil*	*Wheat*	*Lettuce*	*Fruit*	*Citrus*	*Palm*	*Potato*	*Onion*
Predicted	*Bare Soil*	93.68	3.02	0.00	0.00	3.30	0.00	0.00	0.00
	Wheat	6.83	89.76	0.00	0.00	3.41	0.00	0.00	0.00
	Lettuce	0.00	0.00	100.00	0.00	0.00	0.00	0.00	0.00
	Fruit	8.33	0.00	0.00	58.33	33.33	0.00	0.00	0.00
	Citrus	7.33	0.00	0.00	0.00	92.67	0.00	0.00	0.00
	Palm	0.00	0.00	0.00	0.00	0.00	100.00	0.00	0.00
	Potato	0.00	0.00	0.00	0.00	0.00	0.00	100.00	0.00
	Onion	8.33	0.00	0.00	0.00	0.00	0.00	0.00	91.67

Table 3- Confusion matrix of crop types (%) by ANN classification in the summer season

		Truth												
	Class	*Citrus*	*Corn-2*	*Corn-1*	*Cotton-1*	*Peanut-1*	*Soybean-2*	*Cotton-2*	*Bare Soil*	*Sesame-2*	*Soybean-1*	*Peanut-2*	*Fruit*	*Palm*	*Watermelon*
Predicted	*Citrus*	98.98	0.00	0.51	0.00	0.51	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Corn-2	3.45	86.21	6.90	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.45	0.00	0.00
	Corn-1	5.75	0.44	85.40	1.33	2.21	3.10	0.44	0.00	0.00	0.00	0.00	1.33	0.00	0.00
	Cotton-1	0.00	0.00	2.70	89.19	0.00	2.70	5.41	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Peanut-1	6.77	0.00	3.76	0.00	85.71	2.26	0.00	0.00	0.00	0.00	0.75	0.75	0.00	0.00
	Soybean-2	0.82	0.00	4.92	0.00	0.00	90.98	1.64	0.82	0.00	0.82	0.00	0.00	0.00	0.00
	Cotton-2	4.05	0.00	2.70	1.35	0.00	0.00	91.89	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Bare Soil	7.14	0.00	10.71	0.00	0.00	0.00	0.00	85.71	0.00	0.00	0.00	0.00	0.00	0.00
	Sesame-2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	100.00	0.00	0.00	0.00	0.00	0.00
	Soybean-1	3.85	0.00	0.00	0.00	0.00	15.38	0.00	0.00	0.00	80.77	0.00	0.00	0.00	0.00
	Peanut-2	5.71	0.00	8.57	0.00	0.00	2.86	5.71	0.00	0.00	0.00	77.14	0.00	0.00	0.00
	Fruit	42.86	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	42.86	14.29	0.00
	Palm	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	100.00	0.00
	Watermelon	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	100.00

3.3. Temporal Variability in Crop Evapotranspiration

The values of ETc were estimated based on the ETo and Kc for crop types that were classified using the ANN algorithm. Table 4 shows the monthly ETc for each crop type. In addition, Figure 8 shows the variation of monthly ETc over the AID; the yearly ETc was about 890 mm for the 2021 water year. Based on the results of Table 4, it is of great importance to highlight that the highest and the lowest calculated ETc values were for Cotton-1 and onion, respectively. In this regard, the ETc on a yearly basis was highest for cotton followed by citrus, and corn. Research results conducted by Boken et al. (2004) noted that the irrigation depth for cotton was greater than that of corn, supporting our own findings. On the other hand, in the winter season, wheat and potato are the highest water-demanding crops in the AID. At the same time, Cotton-1, Corn-1 and Peanut-1 are highly water-intensive crops when compared with other crops in the summer season. For the whole year, ETc for deciduous fruit trees such as apple, pomegranate, peach, plum, etc., and evergreen trees such as citrus (lemon, mandarin, orange, grapefruit, etc.) was around 1008 mm and 889 mm, respectively. As shown in Table 4, the values of ETc for second crops like Corn-2, Cotton-2, Soybean-2, and Peanut-2 are lower than the first crop ETc. The likely reason may be that Kc values, which vary by the crop development stages, and sowing dates, hence, the length of the growing period, and harvesting periods for second crop types, are slightly smaller than those of the first crops. In this context, several studies have been done to estimate ETc in the Cukurova region in which our study area was located. Table 5 shows measured ETa by direct methods, ETa using the Soil and Water Assessment Tool (SWAT) model obtained from the existing literature, and the calculated ETc in the 2021 water year. As seen in Table 5, one of the studies estimated the actual evapotranspiration (ETa) for Soybean-2 in 2009 by the lysimeter and Bowen Ratio-Energy Balance (BREB). The seasonal cumulative ETa for Soybean-2 by the lysimeter was 354 mm in the period of the growing season (from 25.06.2009 to 06.10.2009), whereas BREB was 405 mm (Unlu et al. 2010) in the same period. As understood clearly from these two figures, the ratio of ETa by BREB to ETa by lysimeter is 1.14, indicating that lysimeter estimates are equal to 87% of BREB estimates in the same period and there exists inherited variability in ET estimation methods. In turn, the difference in ETc value for Soybean-2 (ETc=562.1 mm) in the 2021 water year in our study and ETa values in 2009 for Soybean-2 (ETa=354 mm by lysimeter and ETa=405 mm by BREB) may be explained by the inherent spatio-temporal variability in climate data and Kc selection in the ETc method. Another study by Nur (2019) was conducted to determine Corn-1 (maize) evapotranspiration in the Cukurova region, between 28.04.2012 and 02.09.2012 by using the lysimeter method and water budget method under the Cukurova conditions. The seasonal ETa for Corn-1 was determined as 618.2 mm and 488.8 mm by using the weighted lysimeter method and water budget method, respectively. In the same context, the ETc value

(841.4 mm) obtained in this study for Corn-1 in the 2021 water year was higher than the ETc values by lysimeter and water budget methods (618.2 mm and 488.8 mm, respectively) for Corn-1 in the 2012 water year. Based on the results, it may be concluded that the difference between the seasonal ETa values for first crop corn (Corn-1) obtained from the lysimeter and the water budget method is almost 26%, but a 36% difference between ETa by lysimeter and ETc by Kc in this study. It should be kept in mind that, let alone the stochastic behaviour in the ETc, the ETc values in this study are subject to climatic variables observed in the 2021 water year and regional Kc values acquired from TAGEM-DSI (2017). Therefore, a 36% difference might be quite reasonable. Another possible reason for the difference could be the values of Kc in the ETc method because Kc values are related to development growth stages. Put another way, ETc calculations do not consider the stressed conditions, but direct methods for ETa estimation. A study by Koc & Kanber (2020) estimated wheat evapotranspiration as 708 mm by using the water balance method under irrigated conditions in the Cukurova region in the 2004 water year. The Kc values for wheat may be the reason behind the difference in values of the ETc method and a possible reason to explain the difference between the ETc method (488.9 mm in this study) and the water balance method for wheat in the 2021 and 2004 water years. Akpolat (2011) estimated the ETa of wheat under rainfed conditions, by using BREB and lysimeter methods in the Cukurova region in the 2010 water year. The seasonal ETa of rainfed wheat (between 19.11.2009 and 25.05.2010) was found to be 321 mm and 376 mm for the BREB and lysimeter methods, respectively. In the 2010 hydrological year, wheat was based only on rainfalls and there was no irrigation. Therefore, the actual ETa for wheat in 2010 was less than the ETc of 488.9 mm in this study in the 2021 water year. Although ETc is crop water requirement, ETa is the water consumed actually by the crop. Hence, ETa is subject to stressed conditions and the availability of water during the growing season. For example, a study for determining the ETa of wheat was performed by Yildiz (2019) under rainfed conditions in the Cukurova region between the 2014 and 2015 growing seasons. Seasonal rainfed wheat evapotranspiration was measured as 368 mm by the lysimeter method, 312 mm by the eddy covariance (EC) method, and 335 mm by using the water budget method. The rainfed wheat evapotranspiration value (2014-2015) was ≈33% lower than the wheat ETc method (488.9 mm) in the 2021 hydrological year. In other words, seasonal wheat evapotranspiration grown under natural rainfall conditions is less than the ETc method which takes into consideration rainfalls and irrigation together. Unlu et al. (2011) estimated cotton evapotranspiration by using a water balance model for drip-irrigated full (100%) and deficit (70% and 50%) irrigation from 2005 to 2008 in the Cukurova Region. Cotton evapotranspiration varied between 477 mm and 671 mm in full irrigation and from 376 mm to 398 mm under severe water-stressed conditions. Climate data in the 2021 water year and Kc values in the ETc method could be a reason for the difference between the ETc method and the water balance model for full irrigation treatment results by Unlu et al. (2011). For citrus, Unlu et al. (2014) estimated the ETa of grapefruit trees or a 'Rio Red' grapefruit (Citrus paradisi Macfad. 'Rio Red') orchard through three different irrigation regimes in the Mediterranean environment (in Adana, Turkey) using three methods of EC, BREB, and the water balance method in 2011 and 2012. Their results showed that the annual ET of grapefruit (for full irrigation) was measured as 810.5 mm and 892.9 mm (water balance) for 2011 and 2012, respectively. Moreover, annual grapefruit ET was measured as 716.9 mm and 640.4 mm for the BREB method and EC method, respectively. As seen in Table 4, citrus evapotranspiration values (888.8 mm) are slightly in agreement with yearly grapefruit ETa values obtained by Unlu et al. (2014). On the other hand, Golpinar (2017) estimated the water budget elements, including the potential and ETa, in the AID by using the SWAT from 2009 to 2014. Based on the results, actual ETa values generated by the SWAT model at the catchment level varied from 671.4 mm to 744.7 mm (Golpinar 2017). The cumulative ETc value given in Figure 8, i.e., annual ETc at the irrigation district, in the 2021 water year was higher than the values of ETa between 2009 and 2014 for the same study area (AID). As seen in Figure 8, the monthly ETc values are less than 35 mm per month from November to February. However, it reaches its peak value as ETc=151.3 mm in July, indicating that July is the peak irrigation season in the region. These changes in the value of ETc may be to the result of inherent temporal variability in the climate data from year to year and/or spatial variation in cropping patterns over the years. For example, the difference between the monthly average temperatures observed in 2021 and the monthly average temperatures observed in previous years varied between 1.0-4.4 °C, indicating that monthly temperatures are higher in 2021 than in the previous years. On the other hand, the relative humidity increased at a rate of 6%. Furthermore, Cetin et al. (2020) pointed out that the annual total P (476.6 mm) of 2021 water year decreased by 183 mm (almost 28% less than normal) compared to the annual mean P of the basin. Despite all this, it might be possible that the SWAT model could underestimate or overestimate the ET values based on the ETo estimation method selected.

Table 4- Monthly and yearly ETc values (mm) for the crop types during the growing seasons (2020-2021)

	Oct	*Nov*	*Dec*	*Jan*	*Feb*	*Mar*	*Apr*	*May*	*Jun*	*Jul*	*Aug*	*Sep*	*Total*
Wheat	-	24.3	35.6	44.5	69.4	98.6	111.4	91.2	13.9	-	-	-	488.9
Citrus	61.4	37.7	30.1	32.7	45.2	60.0	79.8	117.5	110.6	122.9	109.9	81.1	888.8
Corn-1	-	-	-	-	-	52.0	102.9	196.0	204.1	180.4	91.6	14.5	841.4
Corn-2	66.6	-	-	-	-	-	-	-	33.4	131.3	180.4	144.2	555.9
Cotton-1	42.6	-	-	-	-	30.2	72.2	163.0	204.1	226.9	197.9	118.9	1055.9
Cotton-2	76.7	-	-	-	-	-	-	-	29.9	129.8	193.1	149.7	579.2
Watermelon	-	-	-	-	-	-	64.0	160.3	187.1	188.9	-	-	600.2
Fruit (pomegranate, apple, peach, plum, etc)	71.1	38.8	27.1	8.7	12.1	60.0	86.5	141.7	146.3	162.6	145.4	107.3	1007.5
Onion	43.9	32.5	40.3	45.8	58.5	40.6	-	-	-	-	-	-	261.5
Potato	-	-	23.6	38.9	69.4	98.6	98.1	-	-	-	-	-	328.6
Peanut-1 (groundnut-1)	-	-	-	-	-	-	42.6	129.0	188.5	217.5	148.6	28.9	755.1
Peanut-2 (groundnut-2)	60.5	-	-	-	-	-	-	-	57.3	129.4	155.5	118.5	521.3
Soybean-1	-	-	-	-	-	-	52.6	143.9	195.6	184.7	66.0	-	642.9
Soybean-2	58.9	-	-	-	-	-	-	-	45.8	133.8	186.0	137.5	562.1
Sesame-2	-	-	-	-	-	-	-	-	115.7	199.7	185.9	88.7	590.1

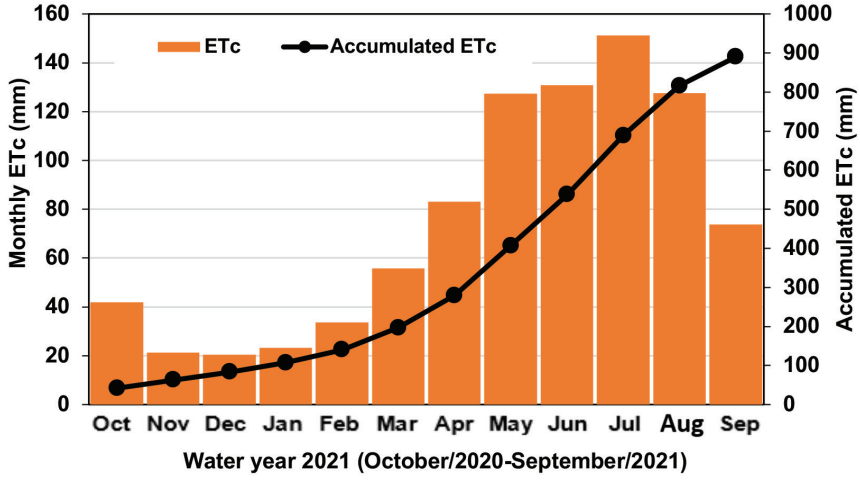

Figure 8- Monthly ETc values and cumulative ETc during the 2021 water year

Table 5- Measured ETa by direct methods, estimated ETa using the SWAT model from the existing literature, and calculated ETc in 2021 water year (mm)

Crop type and the whole study area	*Years*	*Measured ETa in the Cukurova region*				*Estimated ETa*	*Calculated ETc in 2021*
		Lysimeter	*Bowen ratio-energy balance (BREB)*	*Water budget method*	*Eddy Covariance (EC)*	*SWAT*	
Wheat	2004			708.0 Koc & Kanber (2020)			488.9
	2010	376.0 Akpolat (2011)	321.0				
	2015	368.0 Yildiz (2019)		335.0	312.0		
Citrus	2011		716.9 Unlu et al. (2014)	810.5	640.4		888.8
	2012			892.9 Unlu et al. (2014)			
Corn-1	2012	618.2 Nur (2019)		488.8			841.4
Cotton-1 for full irrigation	2005			671.0			1055.9
	2006			477.0			
	2007			587.0			
	2008			601.0			
		Unlu et al. (2011)					
Soybean-2	2009	354.0 Unlu et al. (2010)	405.0				562.1
Study area	2009	Golpinar (2017)				744.7	890.0
	2010					671.4	
	2011					713.2	
	2012					670.9	
	2013					730.0	
	2014					730.2	

4. Conclusions

This study applied up-to-date technologies for remote sensing and ANNs jointly to classify crop types by using the Sentinel-2A-2B and ground truth data obtained by field campaigns conducted in the study area in the 2021 hydrological year. In addition, daily ETc by the "two-step" procedure and ETo using the PM model were estimated. Based on crop type classification results and the confusion matrix by the ANN model, the use of Sentinel-2A-2B showed high compatibility with ground truth data. Validation results showed that the estimation accuracy was 100% for lettuce, watermelon, palm, and sesame-2, more than 93% for citrus in both winter and summer seasons, and over 80% for other crops-with the exception of fruit trees. In addition, the discrimination capability of the ANN algorithm for citrus and fruit trees did not achieve a high degree of accuracy in the winter and summer seasons when compared with other crops. The ANN approach helped us to generate sufficiently accurate classified crop distribution maps over large irrigation catchments. In turn, citrus plantations were preponderant in the winter and summer seasons of the 2021 water year with coverages of 39.4% and 43.4%, respectively. Bare soils, i.e., fallow areas, made up 38.61% of the study area in the winter, while first-crop corn (Corn-1) constituted nearly 17% of the study area in the summer. The annual total of ETc and ETo was around 890 mm and 1308 mm in the 2021 water year, respectively. ETc values (Soybean-2, Corn-1, wheat, and citrus) are (to some extent) compatible with previous studies in the literature in the same study area. Essentially, inconsistencies among ETa and ETc values obtained by different methods have been attributed to the inherited spatio-temporal variability in the meteorological data, climate change phenomena, and spatial variations in cropping

patterns over the study area, etc. Moreover, research findings led us to conclude that the use of remote sensing data in cropping pattern determination is promising for providing information frequently and freely with high spatial resolution. RS images and ANNs could be confidently used to classify crop types accurately in different growth stages throughout the growing seasons. As freshwater resources are very limited in the changing world, particularly in Mediterranean countries, disagreements often occur on how to allocate them to water-demanding sectors. For this reason, the proposed method, simple but effectively applicable, for classifying crop types and determining ETc may be a pragmatic remedy to assist water authorities in apportioning irrigation water among both irrigation schemes and, if needed, farmers optimally and equitably in a realistic manner. Furthermore, water user associations, water authorities, and others may adapt the methodology followed in this study to other large-irrigation schemes where cropping patterns are difficult to obtain.

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Lablab purpureus: Evaluation and Selection of Drought-tolerant - High-yielding Accessions in Dry Farming Systems Based on Drought Tolerance Indices and Multi-environmental Yield Trials

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ABSTRACT

Breeding for drought tolerance in crops requires responding knowledge on the moisture regimes of crops. This study was conducted to evaluate and select the drought tolerant - high yield Lablab accessions in the dry farming systems of Tanzania based on drought tolerance indices and field performance. Three sites from different agro-ecological conditions were selected for the study and each site involved trials with moisture stress-free (MSF) and early terminal moisture stress (TMS) conditions. The experimental design consisted of a randomized complete block design. The twelve accessions used in the study were the best genotypes selected from the former moisture screening experiment at the seedling stage. Data collections included days to 50% flowering and maturity, seed yield plant-1 and seed yield ha-1. The responses of the accessions to TMS conditions were quantified through ten developed indices whose correlation coefficients were computed from the mean of the seed yield plant-1 under MSF and TMS conditions. ANOVA and post-hoc tests were used to analyze the variation among the accessions and their field parameters as well as to compare their means, respectively. Based on this analysis, three indices; mean productivity, geometric mean productivity, and stress tolerance index were discovered with higher correlation coefficients (>0.5), and thus effective for selecting the TMS tolerant accessions with high seed yield plant-1 under both conditions. Through the combined rank mean method, D147, D363, HA4, D349, D352, D348, and D359 were generally selected as Lablab TMS tolerant accessions with high seed yield plant^{-1} across the evaluation sites. Although the significant difference ($p<0.05$) in field performance was noted among the accessions and across the sites, promising trends were found among the above selected TMS tolerant accessions. It is therefore suggested that, further evaluation of the above-selected accessions should involve farmers in their fields before releasing them as commercial varieties.

Keywords: *Lablab purpureus,* Field evaluation, Moisture regime, Stress tolerance indices, Terminal moisture stress

1. Introduction

Ever-increasing food demand is highly predictable over the world due to the rapid growth in the global population (UN 2019; Beltran-Pea et al. 2020). This high food demand is likewise projected in many areas due to drought stress (FAO 2017). The major effects of drought stress are likely to take place in Africa where farmers are more vulnerable to climate change (Muchuru & Nhamo 2019; Benjamin et al. 2021). Maintaining crops in such persisted conditions of climatic challenges is the most significant phase of production.

Since there are few crop species that perform well in harsh conditions, knowledge on their production in drought condition could enhance our effort to develop drought tolerant genotypes for the future sustainability of food security (Raza et al. 2019).

Lablab (*Lablab purpureus* L. Sweet) is a multi-purpose crop, useful for both human consumption and animal feeding (Naeem et al. 2020; Whitbread et al. 2011). Some essential food and nutritional materials have been found higher in Lablab compared to many other related crops. For instance, the protein content is higher in Lablab (302 g kg^{-1} in seed flour) than in other legumes (Venkatachalam & Sathe 2007). Lablab is also the best cover crop and green manure. Since it fixes more nitrogen (180-215 Kg N ha^{-1}) than other related crops (McDonald et al. 2001; Bell et al. 2017), farmers utilize it for conservation agriculture. Farmers rely on Lablab for food and income, especially during persistent drought condition when other legumes such as common beans (*Phaseolus vulgaris*), soybeans (*Glycine max*), cowpeas (*Vigna unguiculata),* and pigeon peas (*Cajanus cajan*) are no longer in the field. For this reason, Lablab is increasingly becoming an important food and cash crop (Raghu et al. 2018) particularly in rural areas in Eastern Africa (Karanja 2016; Nord et al. 2020).

Despite such potential, Lablab has been neglected in many areas in Africa, including Tanzania. This has caused fewer improved cultivars and the continual use of local landraces among farmers (Maass et al. 2010; Miller et al. 2018; D'Alessandro & Molina 2021). To enhance sustainable production of Lablab, especially in dry farming systems that dominate in many areas in the world, a need should be there to develop high-yield and drought-tolerant varieties which could accelerate its transformation to an industrial crop. Such types of varieties are highly needed in many African countries, including Tanzania, as they would serve as food and income security in large parts of their countries that produce very little food due to fewer and erratic rainfalls (FAO 2017; Mkonda & He 2017). A report by the Ministry of Agriculture and Food Security (MAFS 2015) in Tanzania has shown that about 300-600 mm of rainfall is experienced in many areas of the country to influence the production of several crops that farmers prefer to grow such as common beans, cowpeas, soybeans, maize, etc.

Since many crops suffer drought stress typically at the reproductive stages (Nadeem et al. 2019), breeding for drought-tolerant varieties in Lablab should focus on terminal moisture stress (TMS) (Susmitha & Ramesh 2020). This kind of stress is primarily due to insufficient rainfall (meteorological drought) that leads to soil moisture (SM) (agricultural drought) stress (Ding et al. 2021). MOA (2015) and Da Silva et al. (2019) reported that many crops experience drought stress when a percentage of SM drops to a range of 35-50%. This is different from Lablab which is capable of vigorous growth when provided with only little amount of SM (Miller et al. 2018). Lablab is able to grow in very low rainfalls of about 200 mm and high temperatures of around 35 $^{\circ}C$ (Maass et al. 2010). SM elicits drought stress in Lablab when it is below a range of 25-50% (Fening et al. 2009).

Since drought tolerance is organized by polygenes through a complex process (Wang et al. 2018; Missanga et al. 2021), the development of TMS tolerant cultivars has not been possible with many crops (Susmitha & Ramesh 2020). Field evaluation for TMS tolerant cultivars has mainly been performed to develop stress tolerant cultivars (Mitra 2001). The first approach involves genotypes evaluation for yield under moisture-stress free (MSF) or moisture stress (MS) environment. However, this method is proven ineffective due to low yield, significant interactions among accessions, and the MS environment, and low genetic heritability. The second approach focuses on the genetic manipulation of the crop. This method is also proven ineffective as knowledge on genetic mechanisms for crop transformation is still lacking in many crops such as Lablab. Based on such challenges, genotypes evaluation for TMS tolerant varieties at both MSF and TMS conditions is seen as the most suitable method (Susmitha & Ramesh 2020).

Ten drought tolerant indices (Table 1) generated to establish the relationship between yield under MS relative to MSF conditions are practical tools for drought tolerant studies (Talebi et al. 2009). These indices involve mean productivity (MP), geometric mean productivity (GMP), hormonic mean productivity (HMP), stress tolerance index (STI), abiotic tolerance index (ATI), stress non-stress production index (SNPI), yield index (YI), modified STI 1 and 2 (K_1STI and K_2STI), and drought susceptibility index (Susmitha & Ramesh 2020). The relationship between these indices and crop performance in the field such as days to 50% flowering, days to maturity, seed yield $plant^{-1}$ and seed yield hectare $(ha)^{-1}$ is essential for developing TMS tolerant genotypes (Bennani et al. 2017), however with limited information in Lablab. For this reason, this study aims to evaluate and select potential drought tolerant Lablab accessions in the dry farming systems based on drought tolerant indices and field parameters under MSF and early TMS conditions.

Table 1- Formerly developed indices used to quantify the accessions' responses to TMS experiments (Susmitha & Ramesh 2020; Gitore et al. 2021)

S/N	*Drought tolerance indices*	*Code reference*	*Formula*
1	Mean productivity	MP	(Yp+Ys)/2
2	Geometric mean productivity	GMP	$\sqrt{(Yp\ xYs)}$
3	Harmonic mean productivity	HMP	2* (Yp xYs) / (Yp+Ys)
4	Stress tolerance index	STI	(Yp x Ys)/ $\overline{Y}p^2$)
5	Abiotic tolerance index	ATI	[(Yp-Ys)/($\overline{Y}p$/$\overline{Y}s$) x √ (Yp x Ys)]
6	Stress non-stress production index	SNPI	[^{3}√ Yp+Ys/(Yp -Ys)] x [^{3}√Yp x Ys]
7	Yield index	YI	(Yp/$\overline{Y}s$)
8	Modified STI 1	K1STI	$Yp^2/\overline{Y}s^2$
9	Modified STI 2	K2STI	$Ys^2/\overline{Y}s^2$
10	Drought resistance index	DI	(Ys x (Yp/Ys)/$\overline{Y}s$

Yp and Ys: seed yield plant^{-1} of each accession under MSF and TMS conditions, respectively
$\overline{Y}p$ and $\overline{Y}s$: Mean seed yield plant^{-1} of all accession under MSF and TMS conditions, respectively. MSF: Moisture stress free, TMS: Terminal moisture stress

2. Material and Methods

2.1. Multi-location sites and Lablab accessions for field evaluation

This study was carried out at the three different agro-ecological production zones of Tanzania (Figure 1) in the 2021 production season. The sites were drylands and part of the Lablab production areas in the country. The village of Lengijave was the first evaluation site located (3°24'23.23" S; 36°42'1.78" E and altitude of 1794 masl) in the Arumeru district in Arusha region (in the upper side of the Northern highland zone). The second site; the village of Kavambughu (04°01'59.26" N, 37°41'44.03" E and altitude 928 masl) was located in the Same district in Kilimanjaro region (in the Northern arid zone). The Tanzania Agricultural Research Institute (TARI), Selian, in Arusha region (03°21'50.08" N and 36°38'06.29" E with an altitude of 1390 masl) was the third site (in the lower side of the Northern highland zone) (Figure 1). All three evaluation sites had rainfall predominantly taking place from March to May. A short rainfall period occurs between October and January. The field crops, including Lablab, are typically produced during the long rainfall period while few crops are produced during the short rainfall.

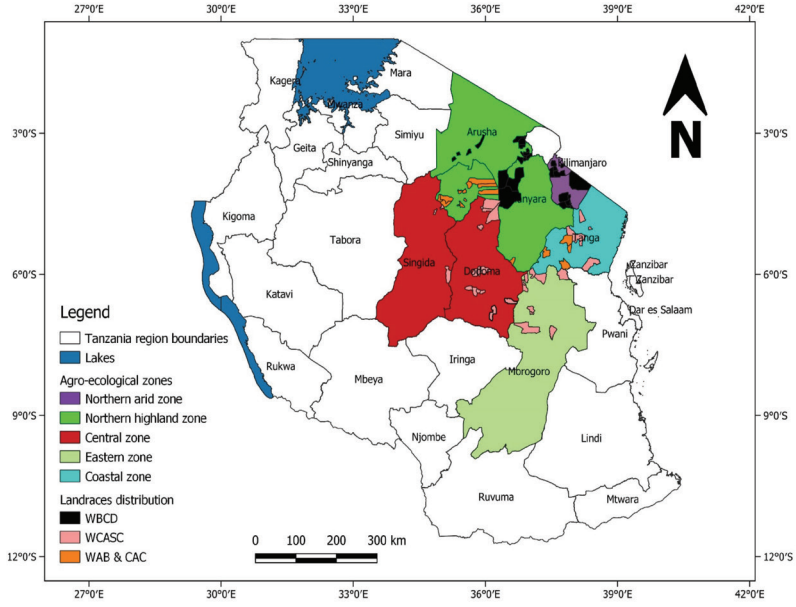

Figure 1- A map of Tanzania (mainland) showing five agro-ecological zones, wards and their villages potential in Lablab grain production along with seed color diversity in each production zone: there are wards with black seeded cultivars dominating (WBCD); wards with cultivars of all seed colors (WCASC); and wards with high availability of black cultivars and less of other cultivars (WAB & CAC)

The Lablab genotypes selected for evaluation consisted of 12 accessions (Table 2, Figure 2): 10 as drought tolerant accessions including Karamoja red and Eldoret Black-2 as recently evaluated for commercial release in Tanzania (Miller et al. 2018; Nord et al. 2020) and 2 as drought susceptible accessions. These 12 accessions were the best selections from the previous MS screening experiment in 2020 of about 300 Lablab accessions (local and exotic) performed at the seedling stage in the greenhouse (Missanga et al. 2021).

Table 2- Some characteristics of the Lablab accessions (12) selected from the moisture screening experiment at seedling stage in 2020 (Missanga et al. 2021) that have been used for high yielding and drought tolerant multi-location evaluation trials in Tanzania

S/N	*Ac- id*	*Accession name*	*Growth habit*	*Seed color*	*Flower color*	*Origin*
Drought tolerant accessions						
1	D 348	Eldoret KT Black-2*	Semi-determinate	Black	Purple	Kenya[c]
2	D 363	Karamoja Red*	Indeterminate	Red	Yellowish	Uganda (Karamoja)
3	HA4	-	Determinate	Cream	Yellowish	India[c]
4	D 352	Eldoret KT Cream	Semi-determinate	Reddish	White	Kenya[c]
5	D 349	Eldoret KT Maridadi	Semi-determinate	Black	Pink	Kenya[c]
6	D 359	ILRI. 14491[i]	Semi-determinate	Brown	White	ILRI, Kenya
7	D 311	Kondoa White	Indeterminate	White	White	Tanzania (Kondoa)
8	D 250	-	Indeterminate	Brownish-creamy	Yellow	India
9	D 55	-	Indeterminate	Brownish-creamy	White	Cambodia
10	D 147	-	Vigorous growth Indeterminate	Brown	Yellowish	Ethiopia
Drought susceptible accessions						
11	D 271	-	Indeterminate	Reddish	White	India
12	D 66	-	Indeterminate	Brownish	White	Uzbekistan

D: Dolichos lablab, Ac- id: Accession identification number, *Accessions used as checks/controls
[c]Already released as the commercial varieties; [i]Potential line at the International Livestock Research Institute

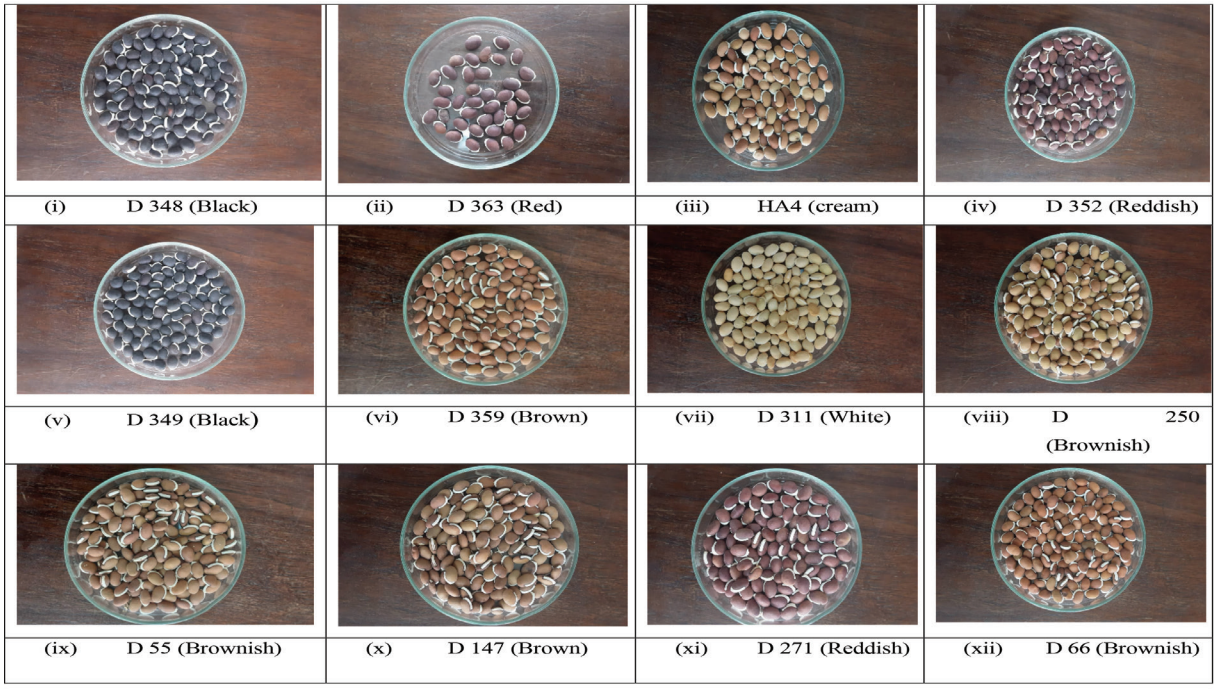

Figure 2a. Illustration of the variability of seed colors of different Lablab accessions (i-xii) that were selected from moisture screening experiment at seedling stage in 2020 and used for the high yielding and drought tolerant multi-location evalua-tion trials in dry farming systems of Tanzania (diameter of the Petri-dish: 65mm x 15mm)

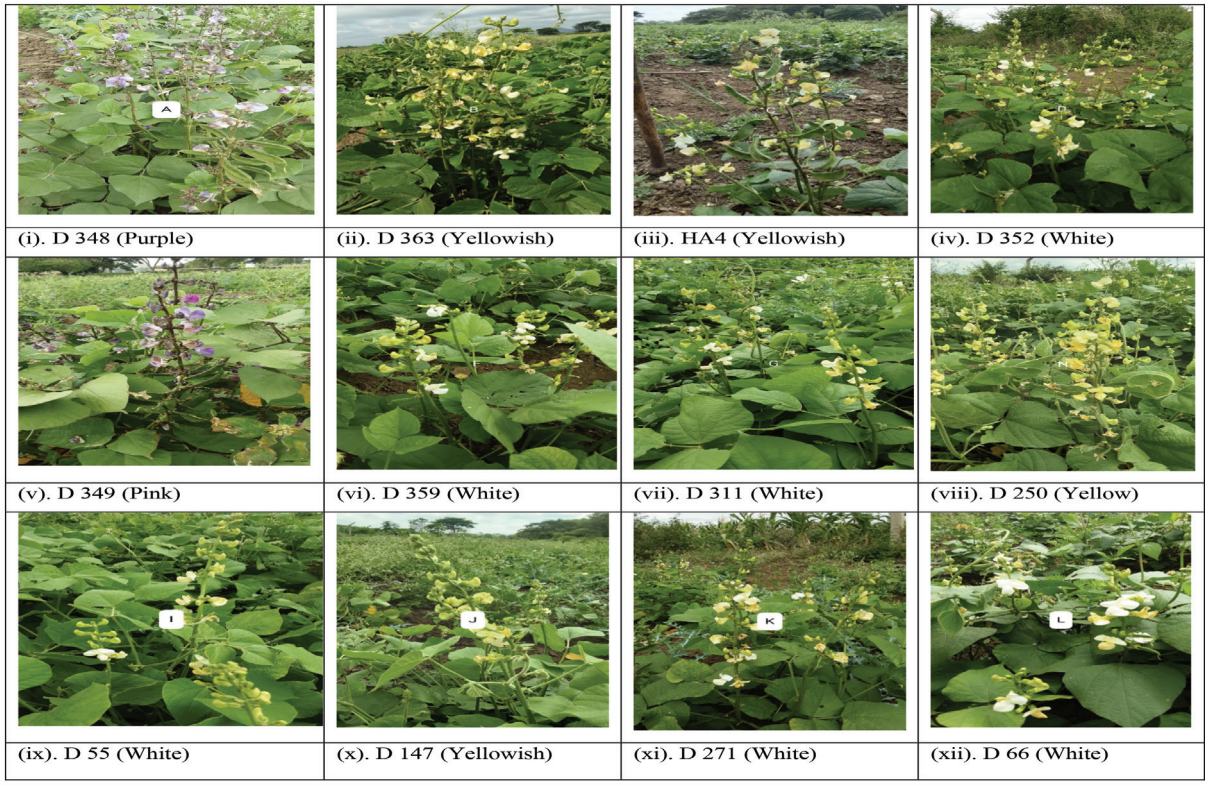

Figure 2b. Illustration of the variability of flower colors of different Lablab accessions (i-xii) that were selected from moisture screening experiment at seedling stage in 2020 and used for the high yielding and drought tolerant multi-location evaluation trials in dry farming systems of Tanzania (diameter of the Petri-dish: 65mm x 15mm)

2.2. Field experimental processes

2.2.1. Field establishment

The field experiments were initiated in early April 2021 through field cultivation using hand-hoe at the Lengijave site, and ploughing at the rest of the sites. The next was leveling and laying out of the experiments for the planting of seeds.

2.2.2. Soil physical properties, and soil nutrients

Soil from the study sites (0-30 cm) was evaluated for physical properties as well as micro and macro-nutrients (Table 3) before the experiment. The content of organic carbon (OC%) ranged from low at the Lengijave and TARI, Selian sites to medium at the Kavambughu site. Their soils were acidic (medium) and non-saline in nature. The total nitrogen (TN%) and exchangeable cations [cmol (+) kg^{-1}] for calcium (Ca), potassium (K), and magnesium (Mg) were very low at all the sites. The exchangeable cation for sodium (Na) ranged between low at the Kavambughu site and very low at the Lengijave and TARI, Selian sites. At all the sites, a high amount (mg kg^{-1}) of sulphur (S), manganese (Mn), and iron (Fe) and low level of available (mg kg^{-1}) phosphorus (P), zinc (Zn), and boron (B) were recorded. Aluminium (Al) (mg kg^{-1}) was elevated a bit at the Lengijave site compared to other sites. This classification of soil physical properties and micro and macro nutrients were established based on the soil guide by Msanya et al. (2001) and Sendhil et al. (2018).

Table 3- Soil physical properties, macro and micro-nutrients (0-30 cm) at the three evaluation sites

S/N	*Soil parameters*	*Unit*	*Evaluation sites*			*Properties*
			Lengijave	*Kavambughu*	*TARI, Selian*	
1	Organic carbon (OC)	%	1.05	1.67	1.25	Physical properties
2.	Soil pH		5.60	5.75	5.70	
3.	Electrical conductivity (EC)	dSm^{-1}	0.87	0.96	0.83	
4.	Total Nitrogen (TN)	%	0.04	0.059	0.039	Macro-nutrients
5.	Calcium (Ca)	cmol (+) kg^{-1}	0.09	0.31	0.15	
6.	Potassium (K)	cmol (+) kg^{-1}	0.02	0.021	0.013	
7.	Magnesium (Mg)	cmol (+) kg^{-1}	0.005	0.088	0.005	
8.	Sodium (Na)	cmol (+) kg^{-1}	0.048	0.292	0.046	
9.	Sulphur (S)	mg kg^{-1}	28.2	64.21	31.96	
10.	Phosphorus (P)	mg kg^{-1}	5.56	6.35	3.03	
11.	Manganese (Mn)	mg kg^{-1}	4.84	5.96	5.32	
12.	Zinc (Zn)	mg kg^{-1}	0.45	0.46	0.38	Micro-nutrients
13.	Iron (Fe)	mg kg^{-1}	41.75	38.18	37.15	
14.	Boron (B)	mg kg^{-1}	0.007	0.011	0.009	
15.	Aluminium (Al)	mg kg^{-1}	56.76	40.42	29.00	Element

2.2.3. Experimental design

The field experimental design at each site involved two separate trials that had different moisture regimes (MR) during the flowering and pod-filling stages i.e. an irrigated field that formed the moisture stress-free (MSF) experiment and a non-irrigated field that formed the early TMS experiment. Both experiments were set down under the randomized complete block design (RCBD) and each of them was laid down into three replications to make a total of 36 plots per experiment. The spacing for the experiments was 0.75 m between the rows and 0.45 m within the rows. One m was left unplanted to form a border space between the plots while 2.5 m was allowed for the space between MSF and TMS experiments.

2.2.4. Starting and management of the experiments

The seeds were sown on 16th April 2021 (Lengijave site) as well as on 19th and 22nd April, 2021 for the TARI, Selian, and Kavambughu sites, respectively. For 5 rows $plot^{-1}$ and 1 seed $hole^{-1}$, 25 seeds were sown into a single plot of 8.4 m^2 (8.4 x 10^{-4} ha). While all other agronomic processes such as weeding, insecticide application etc. were kept properly, no fertilizer was applied to the field during the experiment.

2.3. Data management

An early TMS condition was set to meet the flowering stage, and therefore weather data and the SM contents were recorded every month at all the three sites to establish the irrigation time for the MSF experiment.

2.3.1. Weather data

Rainfall was below the average and unevenly distributed during the production season (April - September) at all the evaluation sites. The levels of rainfall recorded during this period were 290.0 mm, 88.6 mm, and 503.2 mm at Lengijave (Figure 3a), Kavambughu (Figure 3b), and TARI, Selian (Figure 3c), respectively. Among the sites, the majority of rainfall occurred between April and May with little rainfall from June to September. Temperatures were also variable among the sites. The average maximum/minimum temperatures during Lablab growing period were higher at the Kavambughu site (26.7/16.9 ˚C) and lower at the Lengijave site (22.1/13.5 ˚C). The average maximum/minimum temperatures at TARI Selian were 23.3/14.8 ˚C. June and July were the coldest months, especially at Lengijave (21.1/12.2 ˚C and 20.2/12.0 ˚C), compared to TARI, Selian (22.2/14.0 ˚C and 22.0/13.2 ˚C) and Kavambughu (25.7/15.6 ˚C and 25.1/15.3 ˚C).

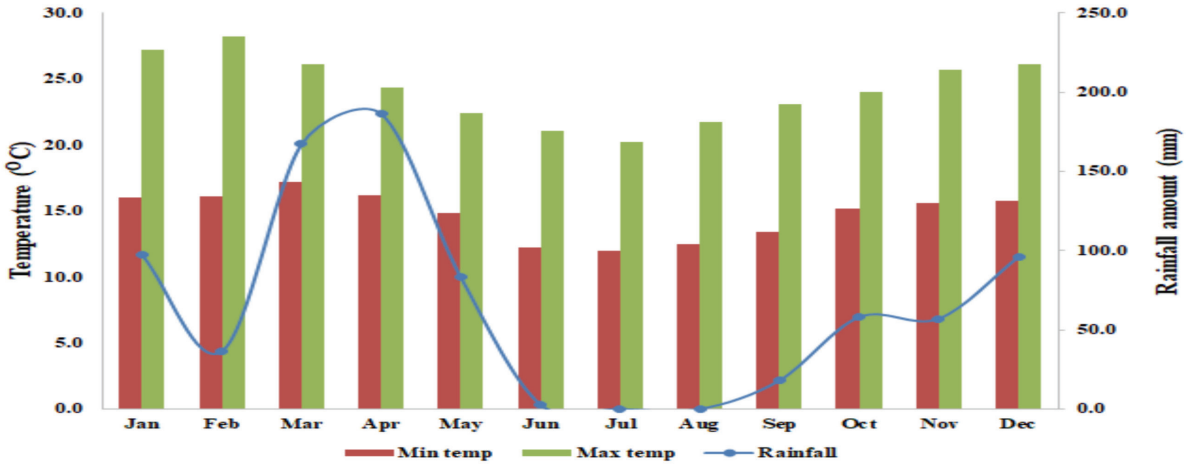

Figure 3a- Weather data (rainfall, maximum and minimum temperature) in 2021 collected from min-weather station (Korvofon seed company) closer to Lengijave evaluation site. Rainfalls dropped off from June to August to induce soil moisture stress in the field

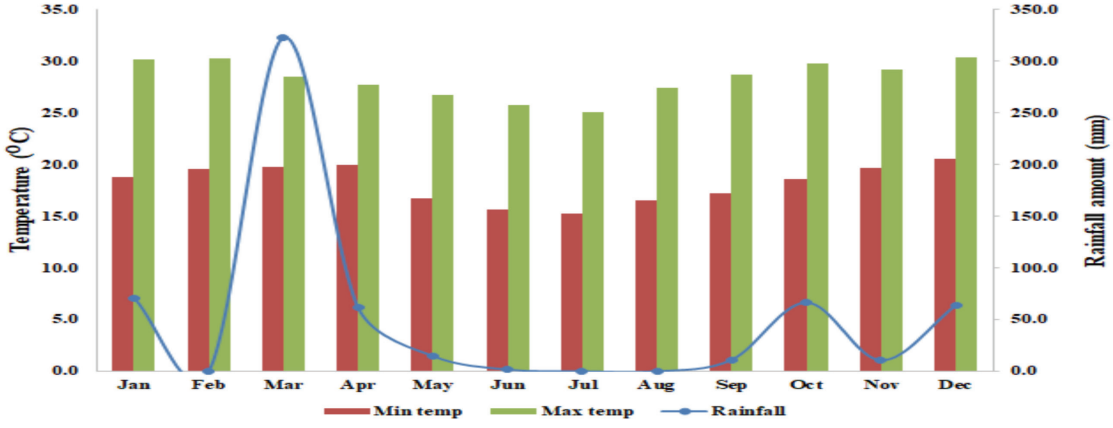

Figure 3b- Weather data (rainfall, maximum and minimum temperature) in 2021 collected from Same weather station. There was almost no rainfall from June to August that led into little moisture content in the field

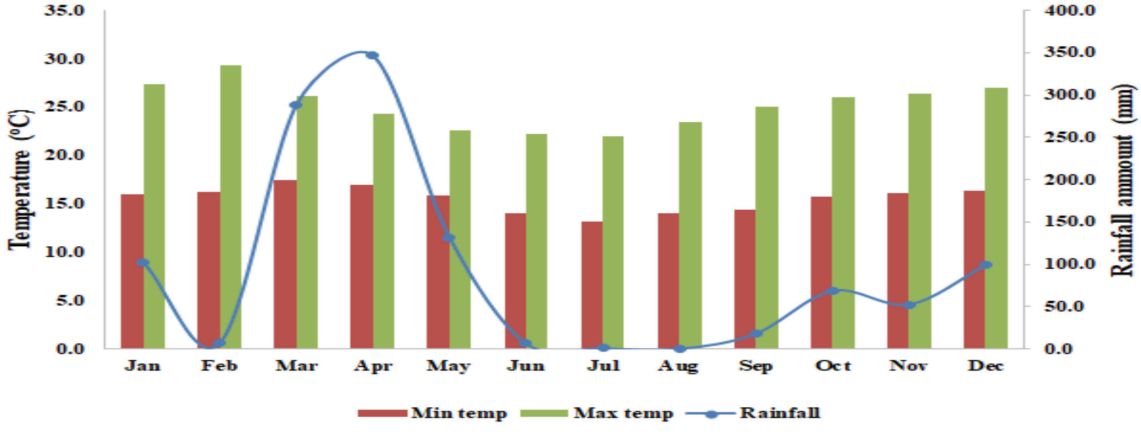

Figure 3c- Weather data (rainfall, maximum and minimum temperature) in 2021 collected from TARI, Selian mini-weather station (closer to the experiment). There was very little rainfall from June to August that caused loss of soil moisture to the experiment

2.3.2 Soil moisture content (%)

Changes in the amount of rainfall (Figure 3a-c) from June as per weather data collected from the nearby meteorological/min-weather stations altered the moisture content (MC) in the soil across all the experimental sites (Table 4). The soil MC in this month was slightly above 20% (at TARI, Selian) and below 20% for the rest of the sites. Since Lablab experiences drought stress when the soil content in below 25% (Fening et al. 2009), irrigation was initiated for the MSF experiments at the end of June (the 2nd month of the experiment).

Table 4- Monthly records of soil moisture content (%) at three evaluation sites

Month	*Lengijave*		*Kavambughu*		*TARI, Selian*		*Average*	
	MSF	*TMS*	*MSF*	*TMS*	*MSF*	*TMS*	*MSF*	*TMS*
1	35.6	34.2	30.7	32.4	37.8	38.2	34.7	34.9
2*	19.8	18.5	12.4	10.9	21.5	20.8	17.9	16.7
3	30.9	15.4	28.3	8.8	34.2	17.1	31.1	17.8
4	28.2	13.1	26.6	6.0	32.7	14.3	29.2	11.1

*Irrigation was initiated on the 2nd month of the experiment. Moisture stress is triggered in Lablab when its content is below 25%. To maintain the moisture to the MSF experiments, irrigation was also done monthly-wise. MSF: Moisture stress free, TMS: Terminal moisture stress

The amount of water [30 liters (L) plot^{-1}] to irrigate the MSF experiment at the beginning of flowering was attained through monitoring the soil absorption capacity as described in the irrigation protocols (number four) for vegetable crops at the University of Georgia, USA (Da Silva et al. 2019).

2.4 Data collection

Data collected from the experiments included different plant parameters related to production and yield including days to 50% flowering, days to maturity, seed yield (g) plant^{-1} and seed yield (kg) ha^{-1} for establishing the best high yielding and drought tolerant accessions. Seed yield plant^{-1} was used to establish drought tolerant indices for selecting the desirable accessions that would tolerate early TMS. The harvest involved hand-picking of dry pods that were thoroughly threshed and weighed for overall seed yield determination.

2.5 Statistical analysis

All the collected data were tested for normality by Bartlett's and Levene's tests and both showed $p>0.05$. Further testing for linearity by Cook's distance and collinearity ($p>0.05$) approved the normal distribution of the data. The equality of variance was tested by Shapiro-Wilk, and Kolmogorov-Smirnov tests and proved that p-values were >0.05.

2.5.1 Drought tolerance indices

Pooled (multi-way) ANOVA and post-hoc tests were performed to analyze the significance of the mean sum of the squares (MSS), F, and p-values attributable to MRs, genotypes (G), replications (R), as well as G and MR interactions (G × MR) for seed yield plant^{-1} at all the evaluation sites. The analysis was implemented using R-software version 4.1.1 (2021-08-10).

Quantification of the accessions' responses to TMS conditions: The responses of Lablab accessions to TMS conditions were quantified through the formerly 10 developed indices (Table 1) whose values were computed from the mean of the seed yield (g) plant^{-1}. The next was the correlation coefficients (Pearson) values of the indices with seed yield (g) plant^{-1} of the accessions evaluated under MSF and TMS conditions.

Desirable indices for selection of the TMS tolerant accessions: The indices that showed significant coefficient correlation (magnitude >0.5) with mean for seed yield plant^{-1} of all accessions evaluated under both MSF and TMS conditions were considered suitable for selection of TMS tolerant accessions from all the sites. Since more than one suitable index was recognized as suitable and the stress-tolerant accessions varied with the indices, the rank mean (RM) generated from the rank sum method of indices was used to identify TMS tolerant accessions at each site. The RM values on average from all the sites were combined into one index to produce the overall best TMS tolerant accessions (Farshadfar & Elyasi 2012; Susmitha & Ramesh 2020; Gitore et al. 2021).

2.5.2 Field parameters

The means and standard deviation (SD) of field parameters from all the Lablab accessions at each evaluation site were computed using Jamovi 1.2.25 for further statistical analysis. General analysis of variance and Post-hoc tests (by Gen Stat software version 12) were used to explore variations among the accessions and their selected parameters: days to 50% flowering, days to maturity, seed yield plant^{-1}, and seed yield ha^{-1} to compare their established means. The compared means and established differences were presented as "mean ± SD". Boxplots generated from statistical software (Minitab 14) were used to visualize the variation among the accessions and between the MR irrespective of the evaluation sites. In all the analyses, a significant difference was considered when $p<0.05$.

3. Results

3.1 Drought tolerance indices

3.1.1 Multi-way analysis of variance

Table 5 shows multi-way ANOVA for MR, G, R, and G x MR evaluated for seed yield plant^{-1} under MSF and TMS conditions at all three evaluation sites. Overall G at each site varied significantly for seed yield plant^{-1} as indicated from MSS, F, and p-values (significant *at p=0.05; **p=0.01; **p=0.001) attributable to accessions evaluated under two MR. Furthermore, G at all the sites performed differentially across the MSF and TMS conditions for seed yield plant^{-1} as revealed by significant MSS, F, and p-values attributable to G in interaction with MR (G x MR) and genotypes in the non-stressed experiment (G_1).

Table 5- Multi-way ANOVA for accessions evaluated under MSF and TMS conditions at the three sites for seed yield (g) plant-1

Source of effects	*Df*	*Lengijave, Arumeru*			*Kavambughu, Same*			*TARI, Selian, Arusha*		
		MSS	*F-value*	*p-value*	*MSS*	*F-value*	*p-value*	*MSS*	*F-value*	*p-value*
Moisture regime (MR)	1	0.28	2.19	0.143	0.08	0.39	0.5350	0.15	0.004	0.9495
Genotypes (G)	11	0.27**	2.69	0.0059	0.46**	2.88	0.0034	7.64***	3.595	0.0008
Replication (R)	2	0.01	0.051	0.9500	0.02	0.032	0.9680	0.63	0.082	0.9220
G x MR	23	0.14	1.57	0.1350	0.37**	3.44	0.0012	6.15**	3.181	0.0023
G_1	11	0.30***	26.69	<0.001	0.61***	281.7	<0.001	1.23**	99.39	<0.001
G_2	11	0.11	0.69	0.7430	0.21	0.99	0.4850	0.56	1.09	0.4120
R_1	2	0.004	0.04	0.9630	0.006	0.027	0.9730	0.028	0.063	0.9390
R_2	2	0.03	0.185	0.832	0.002	0.008	0.9920	0.019	0.033	0.967

Df: Degrees of freedom, MSS: Mean sum of squares, Statistically significant difference avails, MSF: Moisture stress free, TMS: Terminal moisture stress. *p=0.05, **p=0.01, ***p=0.001.
G: Overall genotypes at each station; G_1: Genotypes at non stressed experiment (MSF); G_2: Genotypes at stressed experiment (TMS)
R: Overall replications at each station; R_1: Replications at non stressed experiment (MSF); R_2: Replications at stressed experiment (TMS)

3.1.2 Desirable indices based on significant correlation of drought indices with seed yield plant-1 under MSF and MF conditions

The correlation coefficients of drought indices with seed yield plant^{-1} under MSF and TMS conditions in the three sites are displayed in Table 6. The significant indices that exhibit high (>0.5) and positive magnitude of correlation with seed yield plant^{-1} under two MR were counted as desirable for the selection of TMS tolerant accessions. From this basis, three (MP, GMP, and STI), six (MP, GMP, HMP, STI, YI, and ATI), and five (MP, GMP, HMP, STI, and ATI) indices expressing a high magnitude (>0.5) of significant correlation with seed yield plant^{-1} were considered valuable for selecting TMS tolerant accessions at Lengijave, Kavambughu, and TARI, Selian, respectively. Finally, three common indices i.e. MP, GMP, and STI were found as desirable indices for the selection of the TMS tolerant accessions from all the evaluation sites.

Table 6- Correlation coefficients of drought tolerant indices with seed yield (g) plant-1 under MSF and TMS condition in the three evaluation sites

S/N	*Drought tolerant Index*	*Lengijave*		*Kavambughu*		*TARI, Selian*	
		MSF	*TMS*	*MSF*	*TMS*	*MSF*	*TMS*
1	Mean productivity (MP)	0.55**	0.58**	0.72**	0.65**	0.66**	0.75**
2	Geometric mean productivity (GMP)	0.51**	0.61**	0.74**	0.61**	0.69**	0.68**
3	Harmonic mean productivity (HMP)	0.44	0.62**	0.75**	0.57**	0.69**	0.63**
4	Stress tolerance index (STI)	0.51**	0.60**	0.74**	0.59**	0.68**	0.65**
5	Abiotic Tolerance Index (ATI)	0.84**	-0.79**	0.83**	0.59**	0.65**	0.74**
6	Stress non-stress production index (SNPI)	0.34	-0.04	0.76**	0.17	0.35	0.13
7	Yield index (YI)	0.87**	-0.74**	0.59**	0.79**	0.48	0.76**
8	Modified STI (K1STI)	0.99**	-0.32	0.99**	0.05	0.99**	0.07
9	Modified ST2 (K2STI)	-0.37	1.00**	-0.06	0.99**	0.00	0.98**
10	Drought resistance index (DI)	1.00**	-0.36	1.00**	-0.05	1.00**	-0.01

Pearson correlation analysis at 5%, *p=0.05; **p=0.01. MSF: Moisture stress free, TMS: Terminal moisture stress

3.1.3 Selection of TMS tolerant accessions based on recognized indices

The TMS tolerant accessions were not the same under three (MP, GMP, and STI) recognized indices. Therefore, through the combined RM method i.e. an effective combination of the selective indices into a single index, HA4, D363, D147, D348, D349, D352, and D271 from Lengijave (site 1); D147, D363, D349, HA4, D311, D352, and D359 from Kavambughu (site 2); and D147, D363, D348, D352, D359, HA4, and D349 from TARI, Selian (site 3) (Table 7) were recommended as TMS tolerant accessions.

Table 7- Selection of TMS tolerant accessions based on combined RM method of desirable indices at Lengijave (Site 1), Kavambughu (Site 2) and TARI - Selian (Site 3) evaluation sites

S/N	*Indices*	*MP*			*RS*			*GMP*			*RS*			*STI*			*RS*			*Combined RM*		
	Site	*1**	*2**	*3**	*1**	*2**	*3**	*1**	*2**	*3**	*1**	*2**	*3**	*1**	*2**	*3**	*1**	*2**	*3**	*1**	*2**	*3**
1	D 348	11.32	40.96	56.74	4	9	3	10.46	37.15	56.38	4	11	3	1.09	0.51	1.879	4	11	3	4	10	3
2	D 363	12.28	74.29	68.37	2	2	2	11.94	74.16	68.37	2	2	2	1.42	2.04	2.75	1	2	2	2	2	2
3	HA4	12.50	51.15	41.22	1	4	6	11.08	45.63	31.18	1	4	6	1.22	0.77	0.57	3	4	6	1	4	6
4	D 352	9.30	44.70	43.42	6	8	4	9.29	43.36	41.72	6	6	4	0.86	0.70	1.02	6	6	4	6	6	4
5	D 349	10.05	63.25	40.84	5	3	7	9.29	62.67	30.42	5	3	8	0.86	1.47	0.56	5	3	7	5	3	7
6	D 359	7.80	44.84	42.00	9	7	5	7.76	42.93	40.96	9	7	5	0.60	0.69	0.99	9	7	5	9	7	5
7	D 311	7.15	51.12	27.60	11	5	11	6.99	44.07	24.55	11	5	11	0.48	0.72	0.35	11	5	11	11	5	11
8	D 250	7.25	26.5	12.35	10	12	12	7.18	26.36	12.32	10	12	12	0.51	0.26	0.09	10	12	12	10	12	12
9	D 55	6.84	40.49	32.22	12	10	9	6.84	38.61	29.37	12	9	8	0.46	0.55	0.51	12	9	9	12	9	9
10	D 147	12.01	77.72	70.32	3	1	1	11.52	77.38	70.10	3	1	1	1.32	2.23	2.89	2	1	1	3	1	1
11	D 271	8.87	46.00	39.04	8	6	8	8.62	40.87	30.98	7	8	7	0.74	0.62	0.56	7	8	8	7	8	8
12	D 66	8.97	40.24	31.91	7	11	10	8.45	37.88	24.73	8	10	10	0.71	0.53	0.36	8	10	10	8	11	10

MP: Mean productivity, GMP: Geometric mean productivity, STI: Stress tolerance index, RS: Rank sum, RM: Rank mean
1*, 2* and 3 imply to evaluation site number 1 (Lengijave), 2 (Kavambughu) and 3 (TARI - Selian), respectively

3.1.4 The best TMS accessions based on the combined RM method of desirable indices from all the evaluation sites

The best TMS tolerant accessions generated by the combined RM methods of desirable indices from all three evaluation sites (Table 8) involved D147, D363, HA4, D349, D352, D348, and D359.

Table 8- Overall best TMS tolerant accessions generated from combined RM method of desirable indices from for the three evaluation sites

S/N	*Ac- ID*	*RM method*			*Average positions*	*Combined RM method*
		Lengijave	*Kavambughu*	*TARI, Selian*		
1	D 348	4	10	3	5.67	6
2	D 363	2	2	2	2	2
3	HA4	1	4	6	3.67	3
4	D 352	6	6	4	5.33	5
5	D 349	5	3	7	5	4
6	D 359	9	7	5	7	7
7	D 311	11	5	11	9	9
8	D 250	10	12	12	11.33	12
9	D 55	12	9	9	10	11
10	D 147	3	1	1	1.67	1
11	D 271	7	8	8	7.67	8
12	D 66	8	11	10	9.67	10

Combined rank mean (RM) method of desirable indices to recommend the best TMS tolerant accessions

3.2 Field parameters

The Lablab accessions evaluated for high yield and drought tolerance in the present study had a wide range of genetic diversity (Table 2, Figure 2a,b). These accessions were significantly varied ($p<0.05$) when evaluated for field parameters at the three agro-ecological sub-zones under MSF and TMS conditions (Table 9a-d, Figure 4). Descriptions of the selected parameters for the specific accession at each evaluation site and each MR were as follows:

3.2.1. Days to 50% flowering

Days to 50% flowering were significantly varied among the Lablab accessions and between MSF and TMS conditions (Table 9a, Figure 4). HA4 had fewer days (56.9) of 50% flowering irrespective of the evaluation sites and MR. The next accessions with few (68-70.7) days of 50% flowering were D349, D352, D348, D147, and D359 while the rest of the accessions had longer (73.3-81.1) days of 50% flowering. Respective to the MR, almost all the accessions generally had 50% flowering earlier in TMS than in MSF conditions, except in HA4, D271, and D66 which did not show any significant variation. Moreover, concerning the evaluation sites, days to 50% flowering among all accessions were overall shorter (63 days) at the Kavambughu site in Same and longer (88 days) at the Lengijave sites in Arumeru (Table 10).

Table 9a. Variations of Lablab accessions on days to 50% flowering as compared across the evaluation sites in MSF and TMS water regimes

S/N	Ac-Id	*Lengijave*		*Kavambughu*		*TARI, Selian*		*Average*	
		MSF	*TMS*	*MSF*	*TMS*	*MSF*	*TMS*	*MSF*	*TMS*
1	D 348	84.0±2.00 r-u	81.0±1.00 rs	62.3±1.53 l-k	60.0±1.73 l-g	67.3±1.53 j-o	64.0±1.00 e-i	71.2±9.93 b-d	68.3±9.72 b
2	D 363	94.7±3.21 x-z	91.7±2.08 v-x	63.0±1.00 d-k	60.3±2.08 c-h	66.0±1.00 h-m	63.7±1.53 d-l	74.6±15.20 c-f	71.9±15.00 b-d
3	HA4	64.3±0.58 j-o	64.0±1.00 e-l	49.7±1.53 ab	49.7±1.53 ab	56.7±1.53 bc	56.7±1.53 bc	56.9±6.45 a	56.8±6.32 a
4	D 352	82.0±1.00 r-t	80.3±1.53 g-r	63.0±2.65 d-k	60.0±1.73 c-g	67.0±1.00 i-n	64.3±1.53 f-h	70.7±8.80 b-d	68.2±9.38 b
5	D 349	83.7±2.08 r-u	81.0±1.00 rs	60.3±1.15 c-h	58.0±1.00 b-d	63.3±1.53 d-k	61.3±2.08 e-l	69.1±11.10 bc	66.8±10.80 b
6	D 359	87.0±1.00 t-v	84.0±3.00 r-u	61.0±1.73 l-h	58.3±0.58 l-e	68.0±2.65 k-o	66.0±1.00 h-m	72.0±11.80 b-d	69.4±11.50 bc
7	D 311	100.0±2.65 zA	98.0±1.00 yzA	71.0±1.73 m-p	68.0±1.00 k-o	75.0±2.00 pq	72.7±0.58 n-p	82.0±13.70 h	79.6±14.00 f-h
8	D 250	97.0±1.00 y-zA	94.0±1.00 w-y	65.0±2.65 g-l	62.7±1.53 d-k	71.0±1.73 m-p	67.0±1.00 l-n	77.7±14.80 e-h	74.6±14.70 c-f
9	D 55	95.3±1.15 x-z	92.0±2.65 v-x	67.0±1.00 l-n	65.0±2.65 g-l	73.0±2.00 op	71.3±1.53 m-p	78.4±13.00 e-h	76.1±12.40 d-g
10	D 147	86.1±1.53 u-w	84.0±1.00 s-v	63.0±1.00 d-l	60.0±1.00 c-j	65.2±1.53 l-p	63.0±2.00 l-n	71.4±11.40 b-d	69.0±11.20 c-e
11	D 271	99.0±1.00 yzA	94.0±2.65 w-y	67.0±1.00 l-n	68.0±1.00 k-o	71.7±2.08 m-p	71.0±1.73 m-p	79.2±15.00 e-h	77.7±12.40 e-h
12	D 66	102.0±1.00 A	99.0±1.73 yzA	68.0±1.73 k-o	69.3±0.58 l-p	74.3±2.08 p	74.0±1.00 p	81.4±15.70 gh	80.8±13.90 gh

Table 9b. Variations of Lablab accessions on days to maturity as compared across the evaluation sites in MSF and TMS water regimes

S/N	Ac-Id	*Lengijave*		*Kavambughu*		*TARI, Selian*		*Average*	
		MSF	*TMS*	*MSF*	*TMS*	*MSF*	*TMS*	*MSF*	*TMS*
1	D 348	129.0±1.73 x-zA	127.0±0.58 t-y	114.0±0.58 h-m	111.0±0.58 e-i	119.0±2.00 op	116.0±1.00 j-o	121.0±6.63 b-e	118.0±6.84 bc
2	D 363	134.0±1.00 B-D	132.0±0.58 A-C	114.0±1.73 g-l	112.0±1.00 f-i	118.0±1.00 m-p	116.0±1.00 j-o	122.0±9.23 c-g	120.0±9.03 b-d
3	HA4	107.0±1.00 b-d	105.0±0.58 bc	99.0±1.00 a	97.3±0.58 a	107.0±1.00 b-d	104.0±1.00 b	104.0±4.09 a	102.0±3.57 a
4	D 352	128.0±1.53 w-zA	126.0±1.00 t-y	116.0±1.00 j-o	112.0±0.58 f-j	119.0±0.58 o-q	116.0±0.58 k-o	121.0±5.61 b-f	118.0±6.12 b-d
5	D 349	128.0±2.65 v-zA	126.0±1.15 t-y	111.0±1.73 e-h	109.0±1.00 d-f	115.0±1.00 i-n	114.0±0.58 g-l	118.0±7.87 bc	116.0±7.81 b
6	D 359	131.0±1.15 AB	130.0±0.58 yzA	110.0±0.58 d-g	108.0±1.00 c-e	120.0±0.58 o-r	118.0±1.00 m-p	120.0±9.14 b-e	119.0±9.42 b-d
7	D 311	139.0±1.73 E-G	135.0±0.58 C-E	119.0±1.00 op	116.0±0.58 k-o	127.0±2.31 t-y	124.0±1.00 s-u	128.0±8.87 h-j	125.0±8.30 e-i
8	D 250	142.0±1.00 GH	138.0±1.15 D-F	115.0±1.00 l-n	113.0±1.53 g-l	123.0±1.00 q-l	119.0±1.00 op	127.0±12.00 g-j	123.0±11.10 d-h
9	D 55	141.0±1.53 F-H	138.0±0.58 E-G	117.0±0.58 l-o	114.0±1.00 g-l	125.0±1.00 t-w	123.0±0.58 r-t	127.0±10.60 h-j	125.0±10.70 e-i
10	D 147	131.0±1.15 zAB	127.0±0.58 u-z	113.0±0.58 f-k	110.0±0.58 d-g	121.0±1.15 p-s	119.0±1.15 n-p	121.0±7.86 b-f	119.0±7.40 b-d
11	D 271	144.0±1.00 HI	141.0±0.58 F-H	116.0±1.00 j-o	114.0±0.58 h-m	124.0±0.58 s-v	123.0±1.15 r-t	128.0±12.50 h-j	126.0±11.60 f-j
12	D 66	147.0±1.00 I	144.0±0.58 HI	119.0±0.58 n-p	116.0±1.73 j-o	126.0±1.00 t-y	126.0±0.58 t-x	131.0±12.80 j	129.0±12.50 ij

Table 9c. Variations of Lablab accessions on seed yield (g) plant-1 as compared across the evaluation sites in MSF and TMS water regimes

S/N	Ac-Id	*Lengijave*		*Kavambughu*		*TARI, Selian*		*Average*	
		MSF	*TMS*	*MSF*	*TMS*	*MSF*	*TMS*	*MSF*	*TMS*
1	D 348	11.0±0.76 a-f	15.7±1.61 d-i	71.8±2.46 w-z	67.1±2.61 u-x	63.1±6.42 s-w	68.2±1.70 w-y	48.6±28.70 e-h	50.3±26.00 e-h
2	D 363	9.4±0.50 a-e	8.6±0.27 a-d	78.6±4.01 zA	70.4±1.65 w-z	69.4±1.80 w-z	75.9±4.11 x-zA	52.5±32.70 f-h	51.7±32.50 e-h
3	HA4	14.3±1.42 a-f	11.2±0.80 a-d	68.9±4.89 t-v	64.6±2.44 r-t	57.8±1.72 p-s	63.0±3.78 q-t	47.0±2.78 fg	40.3±2.35 f
4	D 352	8.9±0.78 a-e	7.1±1.33 a-d	33.8±0.45 no	31.9±1.64 l-o	31.4±2.32 l-o	32.7±0.76 m-o	24.7±12.00 a-d	23.9±12.70 a-d
5	D 349	12.0±0.40 a-f	10.7±0.40 a-f	58.2±3.22 r-v	55.6±1.98 r-t	52.1±2.19 qr	54.0±2.63 q-s	40.8±21.80 d-h	40.1±22.10 d-h
6	D 359	8.6±0.61 a-d	6.8±0.45 a-d	57.8±3.07 r-u	54.9±1.21 q-s	51.3±14.20 qr	50.5±6.82 qr	39.2±24.30 d-h	37.4±23.30 d-g
7	D 311	8.7±1.25 a-d	7.0±0.42 a-d	53.8±3.14 q-s	52.7±1.59 qv	40.2±2.99 op	45.5±0.82 pq	34.2±20.20 b-e	35.0±21.30 c-f
8	D 250	8.3±0.50 a-d	6.7±1.65 a-d	30.5±0.76 l-n	28.0±0.68 k-n	13.1±0.23 a-f	15.3±1.84 b-h	17.3±10.10 ab	16.7±9.38 ab
9	D 55	6.7±1.04 a-d	5.6±0.38 a	28.3±0.59 k-n	26.7±0.85 j-n	19.0±3.71 f-k	15.0±1.67 a-h	18.0±9.55 a-c	15.8±9.19 a
10	D 147	15.4±1.37 b-g	13.9±1.35 a-e	85.1±4.67 A	77.0±2.06 yzA	64.7±1.93 t-w	68.1±1.97 w-y	53.1±31.10 h	53.0±29.60 gh
11	D 271	7.0±0.86 a-d	5.9±0.15 ab	24.9±1.71 i-n	23.7±0.70 h-m	14.3±1.14 a-h	11.7±0.58 a-f	15.4±7.89 a	13.8±7.86 a
12	D 66	6.3±1.03 a-d	6.2±1.67 a-c	25.2±1.00 j-n	22.8±1.14 g-l	13.6±0.89 a-g	11.6±1.14 a-f	15.0±8.31 a	13.5±7.42 a

Table 9d. Variations of Lablab accessions on seed yield (kg) ha-1 as compared across the evaluation sites in MSF and TMS water regimes

S/N	Ac-Id	*Lengijave*		*Kavambughu*		*TARI, Selian*		*Average*	
		MSF	*TMS*	*MSF*	*TMS*	*MSF*	*TMS*	*MSF*	*TMS*
1	D 348	327.4±22.5 a-d	467.3±47.9 c-h	2137.9±73.2 t-w	1997.0±77.2 s-u	1878.0±191.0 q-t	2029.8±50.6 t-v	1447.8±854.0 f-h	1498.0±775.0 f-i
2	D 363	278.8±15.0 a-c	256.0±7.9 a-c	2340.3±119.0 wz	2096.2±49.1 t-w	2066.5±53.7 t-w	2259.9±122.0u-x	1561.8±672.0 hi	1537.4±966.0 g-i
3	HA4	425.6±42.2 a-f	333.3±23.8 a-d	2051.6±146.0 t-v	1922.6±72.7 r-t	1721.2±51.1 p-s	1874.0±112.0 q-t	1399.5±749.0 fg	1376.7±786.0 f
4	D 352	264.9±23.2 a-c	210.3±39.5 a-c	935.5±13.4 j-m	949.0±48.7 j-m	935.5±69.0 k-n	973.2±22.50 l-n	735.8±356.0 b	711.0±377.0 b
5	D 349	357.1±11.9 a-d	1006.9±12.0 lm	1732.1±95.9 p-s	1653.8±58.8 p-r	1549.6±65.3 op	1606.2±78.2 o-q	1213.0±649.0 e	1192.5±658.0 e
6	D 359	254.0±18.2 a-c	201.4±13.4 a-c	1719.2±91.5 p-s	1633.9±36.1 o-q	1526.8±423.0 op	1503.0±203.0 op	1166.7±723.0 de	1112.8±694.0 c-f
7	D 311	258.9±37.30 a-c	207.3±12.4 a-c	1567.2±93.5 op	1601.2±47.5 o-q	1196.4±88.9 mn	1354.0±24.4 no	1018.8±600.0 c	1043.0±634.0 cd
8	D 250	247.0±14.9 a-c	199.4±49.2 a-c	907.7±22.5 j-l	834.3±20.3 i-l	388.9±6.9 a-d	455.4±54.6 b-f	514.6±302.0 a	496.4±279.0 a
9	D 55	200.4±31.00 a-c	167.7±11.30 a	841.3±17.40 i-l	794.6±25.40 i-l	564.5±110.00 d-i	446.4±49.7 a-f	534.4±284.0 a	469.0±274.0 a
10	D 147	459.3±40.60 b-g	413.7±22.40.30 a-e	2531.7±139.00 x	2292.7±61.30 v-x	1925.6±57.50 r-t	2026.8±58.60 t-v	1638.9±926.00 i	1577.7±882.00 hi
11	D 271	207.3±25.70 a-c	176.6±4.55 ab	741.1±50.90 g-l	705.4±20.80 f-k	424.6±33.80 a-f	349.2±17.20 a-d	457.7±235.00 a	410.4±234.00 a
12	D 66	186.5±30.50 a-c	184.5±49.70 a-c	750.0±29.80 h-i	678.6±33.80 e-j	404.8±26.50 a-e	345.2±33.80 a-d	447.1±247.00 a	402.8±221.00 a

Ac-Id: Accession Identification number, MR: Moisture regime, Changes in alphabets refer to the significant difference at $p<0.05$

Table 10- Comparison of the values obtained from field parameters at the three evaluation sites

S/N	*Evaluation site*	*Lengijave*		*Kavambughu*		*TARI, Selian*		**Average*	
	Moisture regime (MR)	*MSF*	*TMS*	*MSF*	*TMS*	*MSF*	*TMS*	*MSF*	*TMS*
1	Days to 50% flowering	90.0	86.9	63.5	61.5	68.2	66.1	73.9	71.5
3	Days to maturity	133.5	130.9	113.6	111.1	120.4	118.1	122.5	120.1
3	Seed yield plant-1	9.7	8.8	51.4	48.0	40.8	42.7	34.0	33.1
4	Seed yield in kg ha-1	288.7	261.2	1531.0	1427.3	1215.0	1270.3	1011.6	986.3

This table compares the data values obtained from the field parameters for MSF and TMS experiments at each evaluation site. *Average data values obtained from the field parameters for all evaluation sites

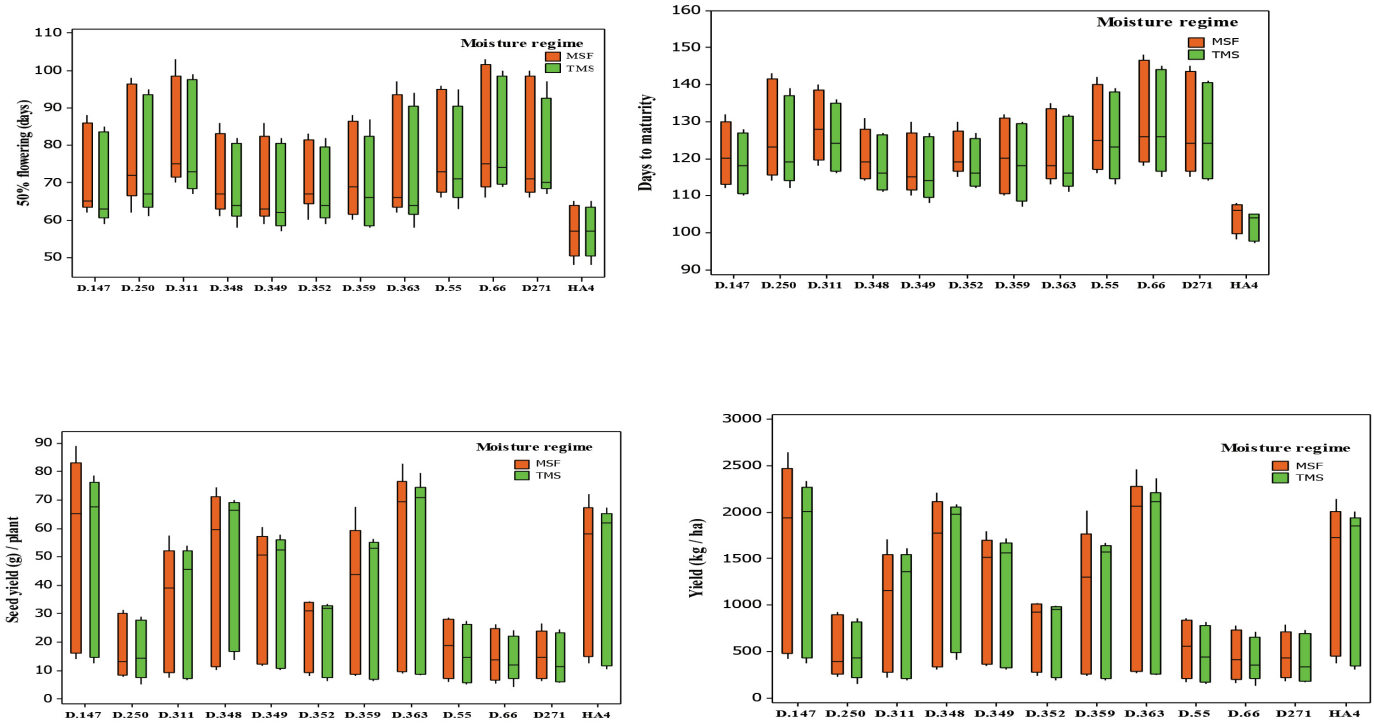

Figure 4. Sameness and variations in some field parameters; days to 50% flowering, days to maturity, seed yield (g) plant-1 and seed yield (kg) ha-1 as compared among the Lablab accessions irrespective of the evaluation sites and between MSF and TMS moisture regimes

3.2.2. Days to maturity

Table 9b and Figure 4 show days to maturity varied significantly among Lablab accessions and between MSF and TMS experiments. HA4 matured earlier (103 days) compared to the rest of the accessions irrespective of the evaluation sites. The next early (117-121 days) maturing accessions involved D349, D348, D352, D359, D147, and D363. The rest of the accessions had longer days (125-130) to maturity. Concerning the MR, most of the accessions generally matured earlier in TMS than in MSF conditions except in HA4 which showed no significant variation. Concerning the evaluation sites, the accessions generally matured earlier (112.4 days) at the Kavambughu site, in Same while taking longer (132.2 days) at the Lengijave site in Arumeru (Table 10).

3.2.3 Seed yield parameters: seed yield plant^{-1} and seed yield ha^{-1}

Seed yield plant^{-1} (Table 9c, Figure 4) and seed yield ha^{-1} (Table 9d, Figure 4) also differed significantly among Lablab accessions and between MSF and TMS conditions. Irrespective of the evaluation sites, D147, D363, D348, HA4, D349, D359, and D311 had the greatest (53.1 - 34.6 g plant^{-1} or 1608.3 – 1030.9 kg ha^{-1}) seed yield values. The low (24.3-14.3 g plant-1 or 723.4-425.0 kg ha^{-1}) seed yield values involved the rest of the accessions. Regarding the moisture conditions, a significant variation in the seed yield parameters

was observed among half of all accessions. Generally, the values were higher in the MSF experiments than in the TMS experiments for D363, HA4, D359, and D147 and the opposite of it for D348, and D311 accessions. This kind of trend where seed yield is produced higher in TMS conditions than in MSF conditions was more noticeable at TARI, Selian than at the other two sites. However, no significant variation was generally noted between MSF and TMS experiments for the other half of accessions i.e. D352, D349, D250, D55, D271, and D66. With respect to the evaluation sites, the low (9.3 g plant^{-1} or 275 kg ha-^{1}) seed yield values were noted at the Lengijave site, in Arumeru while the large (49.7 g plant^{-1} or 1479.2 kg ha-^{1}) seed yield value was obtained at the Kavambughu site in Same (Table 10).

4. Discussion

4.1. Lablab genetic resources for evaluation studies

A wide genetic diversity in crops is essential for enhanced breeding programs. Lablab is a leguminous crop with a wide range of genetic diversity (Venkatesha et al. 2007; Maass et al. 2010). The accessions selected for the present study (Table 2, Figure 2a,b) have shown morphological diversity that plays an important role in the theme of the study. The growth habits of the crop i.e. determinate and indeterminate are useful traits to determine the harvesting and long utilization of the crop in the field (Keerthi et al. 2018; Basanagouda et al. 2022). Seed characteristics in Lablab especially the color influence the consumption and marketing of the crop. Kenya has more consumers of Lablab, especially the black seeded cultivars, than Tanzania. Lablab production in Tanzania is mainly for marketing. The consumers of this crop in Tanzania are very few and they mostly prefer white or cream colored seeds. For this reason, the production of Lablab in Tanzania is focused more on Northern zone in order to seek the market in Kenya (Miller et al. 2018). Lablab flowers have not received much discussion as a production means in East Africa. However, the use of Lablab as an ornamental crop can take a further step towards utilizing their morphology and colors in future business. The results in Table 5 justify the use of the selected accessions for the planned study.

The genetic potential in Lablab has enabled its wide distribution over a range of agro-ecological zones (Vidigal et al. 2018) similarly to the landraces cultivation in five agro-ecological production zones of Tanzania (Figure 1). Farmers producing Lablab in Tanzania have been depending on their own landraces due to the unavailability of improved varieties. Rongai (black seeds), and white seeds (Kilosa, Gairo, Dodoma, Kondoa, and Karatu whites) are some of the Lablab cultivars that farmers have been growing in Tanzania. Among African countries, Kenya released its commercial varieties earlier than other countries. Through the Kirk-house Trust funded Stress Tolerant Orphan Legume project (Kirkhouse Trust 2019), and United States Agency for International Development, Canadian Food-grains Bank sponsored ECHO project (Nord et al. 2020), Lablab exotic germplasm was collected from various Lablab growing countries to enhance breeding activities in Tanzania and the rest of Africa. Such genetic resources were supplemented with local collections made by the authors of the present study in Tanzania. All these resources were screened for drought stress at the seedling stage in 2020 (Missanga et al. 2021) to obtain drought-tolerant and susceptible accessions (Table 2) for the present field evaluation study.

4.2. Drought tolerant indices: selection for desirable accessions

The ability of crops to tolerate drought stress as evaluated in different stressed conditions based on grain yield is a heritable trait controlled by the genes (Farshadfar & Elyasi 2012). The mean dry seed yield plant-1 of twelve selected Lablab accessions in the present study established different drought indices (Table 6) at the three sites under both MSF and TMS conditions. Since the selection of TMS tolerant genotypes from both MR considers the indices with high magnitude (Susmitha & Ramesh 2020), MP, GMP, and STI were selected as the overall desirable indices that established the TMS tolerant accessions in this study. These three types of indices were reported by Ferdandez (1992) and Guendouz et al. (2012) as the special drought tolerant indices that breeders use to select high-yielding cultivars under stress and non-stress conditions. The criteria and indices similar to those obtained in this study for Lablab were only reported in India (Susmitha & Ramesh 2020). However, further similar reports were found in soybean (Seyyed et al. 2014), chickpea (Uday et al. 2016), barley (Zare 2012), cowpeas (Ajayi 2020), common wheat (Moosavi et al. 2008; Farshadfar & Elyasi 2012; Bennani et al. 2017), and durum wheat (Talebi et al. 2009; Guendouz et al. 2012).

As farmers' selection for drought tolerant varieties (Table 7,8) considers high yielding cultivars in different moisture conditions (Ramesh & Byregowda 2016) i.e. accessions with the high average number of desirable indices in combination especially in MP, GMP, and STI (Golabadi et al. 2006; Guendouz et al. 2012; Susmitha & Ramesh 2020), D147, D363, HA4, D349, D352, D348, and D359 were recommended in the present study as drought tolerant and high yielding Lablab varieties.

Most of the Lablab accessions evaluated and selected in this study as overall high yielding and TMS tolerant accession (Table 8) have been widely used in production and considered in some research studies. They were already released as commercial varieties in India (HA4) (Ramesh et al. 2018), and Kenya [D349 (Eldoret KT Maridadi), D352 (Eldoret KT Cream), D348 (Eldoret KT Black-2)] (Kirkhouse Trust., 2015; KEPHIS, 2017; Cook et al. 2020). D363 (Karamoja red) and D348 (Eldoret Black-2) were recently evaluated for commercial release in Tanzania (Miller et al. 2018; Nord et al. 2020). D359 was the potential line (ILRI 14491) in the series of Lablab lines at the I*nternational Livestock Research Institute* (ILRI), Kenya (Cook et al. 2020).

4.3. Field performance evaluation in Lablab genotypes

A wide variation in Lablab growth parameters similar to this work (Table 9a-d, Figure 4) affirms the potential of this crop among smallholder farmers (Pengelly & Maass 2001; Whitbread et al. 2011). Serving as a multi-purpose crop (Naeem et al. 2020; Raghu et al. 2018), Lablab has been used for human consumption especially as tender leaves, immature pods, green seeds, and dry grains (D'Alessandro & Molina 2021) or as forage for livestock (Ewansiha & Singh 2006). The major factors that lead to the underutilization of Lablab in Tanzania and other African countries is primarily due to the unavailability of improved varieties. Many farmers' landraces in Tanzania have indeterminate growth habits taking longer to flower and mature. Harvests from these indeterminate cultivars are repetitive with low yield (Huyghe 1998; Sultana et al. 2001).

The breeders' targets similar to the goals of this study (Table 9a,b) have been to select early flowering Lablab accessions that would tolerate drought stress and provide them with early harvesting. Furthermore, other important goals have been to achieve high-yielding accessions (Table 9c,d) which can provide farmers with a good economic return. Some early flowering accessions as per this study (HA4, D349, D352, D348, D147, and D359) (Table 9a) were also early maturing cultivars (Table 9b) as well as high yielding accessions (Table 9c,d) as reflected in other studies. While no MSF and TMS experiments were conducted before to compare Lablab genotypes in East Africa including Tanzania, Miller et al. (2018) and Nord et al. (2020) from northern Tanzania only evaluated some Lablab genotypes in the field, including some accessions used in this study. Similar trends in their field performance were also discovered in this study. Ewansiha et al. (2007) from Nigeria reported 40-60 days as the early flowering period in Lablab, similar to days taken by HA4 to accomplish 50% flowering in this study (Table 9a). The rest of the accessions were classified into the second group with intermediate days of flowering. Ranges of days from 51-160 days and 90-197 days were reported by Whitbread et al. (2011) in South Africa as days to 50% flowering and days to maturity in Lablab, respectively. In Uganda, Kankwatsa and Muzira (2018) have reported 52-69 days (first year) and 56-108 days (second year) as days to 50% flowering. In her Lablab evaluation study by Kamotho et al. (2016) in Kenya, the grand mean in days to 50% flowering, and days to maturity were 98.79, and 143.45, respectively. The minimum and maximum days for maturity and seed yield (g) plant^{-1} were 117.8 and 186.4 as well as 19.7 and 126.9, respectively. Such information has a close relation to the findings obtained from this study. Although improved Lablab varieties can produce 2.5-5.0 tons (t) of green pods or 1.5-2.0 t of dry seeds per ha (Heuzé et al. 2016; Nord et al. 2020), most of their cultivars have long production cycles compared to other legumes. Early-maturing varieties in common bean and cowpeas involve an average of 60 and 90 days, respectively. Yield production in common bean is 0.88 t ha^{-1} while that of cowpeas ranges from 1.3 to 1.5 t ha^{-1}) (CIAT; World Bank 2017; Njonjo et al. 2019).

One important criteria to select for drought-resilient cultivars in crops is their similar performance in both stress and non-stress conditions. However, their selection should further consider the genotypes with desirable performance (Mehraban et al. 2019). Based on this criteria, Figure 4 displays some accessions (D147, D363, D348, HA4, D349, D352, and D359) that were generally performing well in the entire area of the study. Their minor variations between MSF and TMS condition in some accessions (Figure 4) were due to drought resilience abilities in Lablab (Robotham & Chapman 2015) and the relationship that Lablab shows between rainfall and grain production (Bakari & Pauline 2020). Zinzala et al. (2016) reported that stressful environments tend to lower plant physiological parameters in many crops. Unclear trends in Lablab performance and grain productivity (Table 10) along the three evaluation sites were triggered by rainfall variations (Figure 3a-c). Since, Lablab is a drought resilient and photoperiodic sensitive crop (Ramtekey et al. 2019), it does not need much rainfall to raise production (Nord et al. 2020). The low temperature (Figure 3a) particularly during reproduction season and soil factors (Table 3) especially a raised amount of Aluminium seem to be the limiting factors for Lablab production at the Lengijave site in the upper side of the northern highland zone. This site was not suited for Lablab grain production in the selected period of the present study. As mentioned by Forsythe (2019), some areas in Tanzania are better suited to grow Lablab from December in order to utilize early rainfall and avoid the drop in temperature from April that would influence the production.

5. Conclusion

Some potential Lablab accessions were evaluated for high yielding and drought tolerance at multi-locational field trials in the dry farming systems of Tanzania. The evaluation was completed under MSF and TMS conditions. Based on correlation criteria, three drought tolerant indices i.e. MP, GMP, and STI were discovered as the effective indices in the selection of the TMS tolerant accessions with high seed yield under the stipulated conditions. Through the combined RM method, D147, D363, HA4, D349, D352, D348, and D359 were selected as Lablab TMS tolerant accessions with high seed yield under both conditions. Moreover, their performance in multi-locational field evaluation trials showed promising trends. The field performance involved days to 50% flowering, days to maturity, and seed yield parameters i.e. seed yield plant^{-1} and seed yield ha^{-1}. Based on these findings, it was suggested that, the selected high yield - drought tolerant Lablab accessions from the present study should be further evaluated by farmers in their fields before releasing them as commercial varieties. Furthermore, the breeding programs can start to utilize these resourceful accessions to generate variability among Lablab cultivars both inside and outside of the country.

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Effects of Air Temperature and Relative Humidity on Milk Yield of Holstein Dairy Cattle Raised in Hot-Dry Southeastern Anatolia Region of Türkiye

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ABSTRACT

The aim of this study is to investigate the possibility of using meteorological data obtained from a public meteorology station in determining the effect of air temperature and relative humidity on milk yield in Holstein dairy cattle raised in Diyarbakir province of Türkiye. Records on daily milk yield obtained from a commercial farm were used in the study. Meteorological records including daily maximum and minimum temperatures and daily maximum and minimum humidity data were obtained from the nearest public weather station. A total of 185 healthy Holstein dairy cattle, with five different lactation parities, as well as details on some environmental conditions such as, year, month, lactation period, weather temperature, and the humidity that the animals are exposed to are included in the dataset relating to milk production. Five different temperature-humidity index variants, including THI_a (maximum temperature and humidity), THI_b (minimum temperature and humidity), THI_c (average temperature and humidity), THI_d (maximum temperature and minimum humidity), and THI_e (minimum temperature and maximum humidity), were considered to evaluate the effect of heat stress on milk production. The critical values at which the milk yield began to decrease due to heat stress in this study slightly deviated from the critical value of 72, which is accepted as the threshold value for the start of heat stress and determined as 77, 54, 64, 69, and 54 for THI_a, THI_b, THI_c, THI_d, and THI_e, respectively. Based on these values, the loss of milk production of one cow per year was calculated as 98.25, 157.68, 207.36, 164.30, and 190.08 kg when using THI_a, THI_b, THI_c, THI_d, and THI_e, respectively. This study confirmed that weather stations located away from farms provide useful information for research on heat stress in dairy cows. It can be concluded that THI_d, which shows the least deviation from the critical value of 72 (only 3 unit), better reflects the stress condition that animals are exposed to due to temperature and humidity. For this reason, the highest daily air temperature and lowest daily humidity appear to be the most important factors in this investigation to assess heat stress and both variables can be combined into a THI.

Keywords: Heat stress, Temperature-humidity index, Milk production loss, Meteorological data

1. Introduction

Climatic conditions are known to affect the welfare of farm animals and their productivity (Hill & Wall 2015). Climatic change, which occurs with the deterioration of the atmospheric synthesis on a global scale, is widely considered to be one of the biggest threats facing the planet. Climate models predict a temperature rise of 0.3 to 4.8 ˚C over the next century (Wankar et al. 2021) and this continual rise in temperature will have a serious impact upon food production and farming.

In many species, including cattle, the body temperature is constant. In order for the body temperature to remain constant, there is a balance that compensates for the increase in body temperature depending on the increase in ambient temperature. A disruption of this balance is referred to as heat stress (West 1994). Radiation, convection, and conduction are less effective in dissipating body heat in cattle when the ambient temperature reaches their body temperature. In order to maintain a stable body temperature, more moisture must be removed from the skin via evaporation and a higher respiratory rate (high panting score) is necessary (Kadzere et al. 2002). In addition, high relative humidity (RH) reduces the efficiency of evaporative cooling. As a result, a high ambient temperature combined

with a high humidity level reduces the cooling capacity and causes the body temperature to rise (West 1994), resulting in significant milk yield losses (Herbut & Angrecka 2012; Konyves et al. 2017; Gantner et al. 2017; Yazgan 2017). Moreover, heat stress negatively affects performance not only for dairy cattle but also for beef cattle (Yazgan et al. 2013).

The effect of heat stress on test-day milk yield can be described as a function of four variable groups or variables (Ravagnolo et al. 2000). The first of these groups is highest, average, or lowest temperature and humidity values during the 24 hour-period prior to milk yield recording. Second is heat stress measures (e.g. fan, shading, and sprinkler applications), third is duration of current heat stress and finally duration of previous heat stress. There are many methods to quantify heat stress and the simplest of these is the temperature-humidity index (THI), calculated by the combination of temperature and humidity into one value and defined by several formulas (Thom 1959; Bianca 1962; NRC 1971; Leonard 1985; Mader et al. 2006).

Ravagnolo et al. (2000) reported that meteorological data obtained from public weather stations contain useful information for studies on heat stress in dairy cattle, since daily yields are affected by weather conditions and they reflect the effect of weather temperature and humidity. This means that the impact of heat stress on animals can be determined when weather conditions, such as temperature and humidity, prior to the test days are recorded. Another important problem encountered while calculating the effect of heat stress on animals is deciding which values to consider as the maximum, minimum, or average temperature and humidity variables while calculating THI. Because the temperature and humidity values do not remain constant throughout the day, they change constantly, and it may not always be appropriate to only use average values.

A significant amount of cattle milk production is carried out in the Diyarbakir province of Türkiye. Since, however, the province is one of the hottest regions of Türkiye, milk production is adversely affected. In the summer seasons, in particular, temperatures can reach as high as 46 °C (Kallioglu et al. 2015). Accordingly, the average daily maximum air temperature is around 37 °C, which negatively affects milk production due to heat stress.

This study (1) investigates the relationship between milk production and air temperature and RH in Holstein dairy cattle raised in Diyarbakir province of Türkiye by using publicly available weather information and (2) calculates milk yield losses that occur due to heat stress.

2. Material and Methods

2.1. Data

The milk production data were obtained from a modern commercial dairy cattle farm located in Diyarbakir. The farm is located at 37°59'03" N latitude and 40°21'37" E longitude, with the altitude of 665 meters. The cattle were kept in an open-system free stall barn, fed ad libitum, had free access to water, and were milked three times a day with their yield recorded by an automatic milking system. Each cow had at least total 270 records to be part of the analysis. Records with milk production <8 kg or >50 kg, daily records of animals during the first four days of lactation and those after 350th day for extended lactations were eliminated from the data set. There were five parities in lactation records for daily milk yields and only one lactation record (non-repeated observation) for each cow. Weather data included daily maximum, minimum and average temperature, and humidity were obtained from the public weather station located in Diyarbakir that belongs to the Turkish State Meteorological Service authorised by Ministry of Environment, Urbanization and Climate Change of the Republic of Türkiye. While the distance between the weather station and the farm was 15.32 km as a straight line (crow flies), the altitude difference between the farm and the weather station was only 15 m.

The formula proposed by Mader et al. (2006) is highly correlated with the panting score. For this reason, the formula given below (Eq. 1) was used for THI calculations in this study.

$$THI = (0.8 \times T) + [(RH / 100) \times (T - 14.4)] + 46.4 \quad (1)$$

Where;

THI : Temperature humidity index;

T : Dry bulb weather temperature (°C);

RH : Relative humidity (%).

According to this formula, heat stress in dairy cattle begins when the THI value reaches 72, which corresponds to 100% humidity at 22 °C, 50% humidity at 25 °C or 20% humidity at 28 °C. Using combinations of maximum, minimum or average temperature and humidity value with this equation THI_a (maximum temperature and humidity), THI_b (minimum temperature and humidity), THI_c (average temperature and humidity), THI_d (maximum temperature and minimum humidity) and THI_e (minimum temperature and maximum humidity) were calculated daily. Figure 1 shows all calculated THI variants for each day of the year (averaged over 3 years) for the present data set. Each test-day record was assigned the daily THI_a, THI_b, THI_c, THI_d, and THI_e values of the previous days and put together with the daily milk production data. Final data comprised 46 438 various parity daily records of milk collected from 2018 through 2020 from 185 healthy Holstein dairy cattle (Tables 1, 2).

Table 1- Descriptive statistic of milk production data

			Milk yield (kg)	
OLP	***N***	***n***	***Mean***	***SD***
1	26	6,834	27.55	7.17
2	36	8,655	30.86	8.02
3	64	16,150	29.03	9.77
4	51	12,590	28.64	9.12
5	8	2,209	27.29	7.64
Total	185	46,438	28.96	8.89

Order of lactation parity (Each animal has only one lactation record), N: Number of lactations, n: number of daily milk yield records, SD: Standard deviation

Table 2- Means and standard deviations of milk yield and THI on the farm by months between 2018 and 2020

	Milk yield (kg)			*THI_a*		*THI_b*		*THI_c*		*THI_d*		*THI_e*	
Month	***n***	***Mean***	***SD***	***Mean***	***SD***	***Mean***	***SD***	***Mean***	***SD***	***Mean***	***SD***	***Mean***	***SD***
January	5,324	29.61	6.43	47.32	6.08	39.95	4.05	42.86	4.45	49.15	5.31	35.28	4.76
February	4,804	26.96	6.63	52.25	3.79	42.81	2.52	46.42	2.56	53.35	3.65	37.38	3.78
March	4,949	24.35	6.58	57.02	5.57	45.70	3.40	50.26	3.54	56.77	4.55	41.39	3.92
April	3,949	23.00	6.00	64.64	5.56	50.02	2.62	56.06	3.28	62.25	3.58	46.91	3.22
May	3,110	20.97	5.66	79.77	6.60	57.44	3.55	66.46	4.32	71.64	3.88	57.16	5.20
June	2,054	19.15	5.95	86.55	2.72	64.08	2.11	73.81	1.95	78.07	1.66	66.48	2.69
July	1,420	18.59	5.77	85.13	2.95	66.36	1.94	74.67	1.83	78.73	1.91	68.92	2.24
August	597	17.81	5.96	86.75	2.94	68.44	1.51	76.67	1.30	80.35	1.61	71.08	1.71
September	4,460	35.95	7.44	82.82	2.52	62.14	2.00	71.20	1.65	76.04	1.74	63.79	2.53
October	5,333	36.15	7.02	74.51	6.07	57.64	3.59	64.99	4.37	69.99	4.32	57.25	5.56
November	5,129	34.81	7.13	60.89	6.50	48.30	3.45	53.47	3.93	59.52	4.71	45.18	4.22
December	5,309	32.64	6.83	50.86	5.38	42.80	4.98	46.39	4.86	51.70	5.07	40.21	5.37

n: Number of observations, SD: Standard deviation

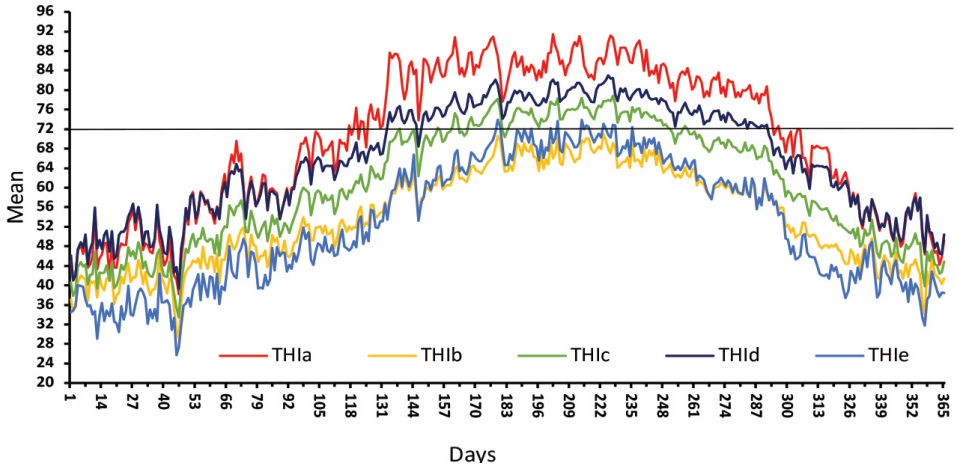

Figure 1- Three-year (2018-2020) average of THI values

2.2. Statistical analysis

The following statistical model (equation 2) was used to calculate the least square means of daily milk yield by THI variants. Since there were no repeated observations of any animals in the dataset, an element of the individual effects of animals was not added to the model.

$$Y_{ijkl} = olp_i + (ym_j) + dim_k + THI_l + e_{ijkl} \quad (2)$$

Where:

Y_{ijkl} : Daily milk yield for parity i, year-month j, days in milk class k and THI variant class l;

olp_i : Effect of order of lactation parity (Each animal has only one lactation record and i=1, 2, 3, 4 and 5);

$(ym)_j$: Year effect (2018, 2019 and 2020) nested with month (j=1 to 23);

dim_k : Days in milk class (k=1 for 5 to 35, k=2 for 36 to 65, k=3 for 66 to 95, k=4 for 96 to 125, k=5 for 126 to 155, k=6 for 156 to 185, k=7 for 186 to 215, k=8 for 216 to 245, k=9 for 246 to 275, k=10 for 276 to 305 and k=11 for >305);

THI_l : Temperature-humidity effect (l= 38 to 91 for THI_a, l=33 to 71 for THI_b, l= 35 to 78 for THI_c, l= 43 to 82 for THI_d and l= 31 to 74 for THI_e);

e_{ijk} : Random residual effect.

In order to calculate the milk yield losses, a similar approach was used as reported by Ravagnolo et al. (2000) and formulated for all THI variants as follows (equation 3);

$$MYL = [(THI_m - THI_{cr}) \times d] \times [(Y_1 - Y_2)/u] \quad (3)$$

Where:

MYL : Milk yield loss (kg) in stress zone;

THI_m : Average THI value of the interval when milk yield starts to decrease and reaches the minimum value;

THI_{cr} : Critical THI value at which milk yield starts to decrease, d is the number of days over the critical THI value (calculated from Figure 1);

Y_1 : Least squares mean of milk yield at the critical THI value;

Y_2 : Least squares mean of milk yield corresponding to THI_m;

u : Total THI unit between heat stress periods.

All analyses were conducted with the GLM procedure of SAS (2000).

2. Results and Discussion

The number of observations, mean milk yield, and standard deviation for each month of the three years are shown in Table 2. The milk yield average is the lowest (17.81±5.96 kg) in August when THI_a, THI_b, THI_c, THI_d and THI_e values reach their highest values.

The estimated values for the coefficients of determination (R^2), sums of squares (SS), and mean square errors (MSE) for the THI variants are provided in Table 3. All fixed effects in the model (equation 2) were statistically significant ($p<0.05$) for all analyses. As shown in Table 3, R^2, SS and MSE values of THI variants were determined to be very close to each other. The R^2 value of THI_e was the highest (0.4599) whereas the R^2 value of THI_a was the lowest (0.4544). Furthermore, THI_b had the lowest MSE value (43.19).

Table 3- Coefficient of determination (R^2) and sums of squares (SS) and mean square error (MSE) for THI variants

Variant	*Combination*	*R^2*	*SS*	*MSE*
THI_a	Maximum temperature and humidity	0.4544	1 666 867	43.25
THI_b	Minimum temperature and humidity	0.4567	1 671 744	43.19
THI_c	Average temperature and humidity	0.4573	1 669 838	43.22
THI_d	Maximum temperature and minimum humidity	0.4585	1 665 540	43.25
THI_e	Minimum temperature and maximum humidity	0.4599	1 671 773	43.31

3.1. Milk yield levels for THI variants

3.1.1. THI_a

Figure 2 shows the change of least square means of the milk yields by the values of THI variants. The THI_a values obtained by using the maximum temperature and maximum humidity were in the range of 38-91, and this range was the largest of all THI variants (54 units). As shown in Figure 2 and Table 4, there were fluctuations in the least square means of the milk yields, ranging from 38 to 77. When the THI_a value exceeded 77, the milk yield began to decrease, but increased slightly after 87. In this range, the milk yields decreased from 26.64±0.318 kg to 25.33±0.385 kg and the difference was 1.31 kg ($p<0.05$). However, least square means of milk yields began to decrease at the point THI 77 instead of the critical value previously stated 72.

3.1.2. THI_b

As indicated in Figure 2 and Table 4, all possible THI_b values lies in the range of 33-71 since daily minimum temperature and minimum humidity values were used in its calculation. The lowest milk yield was obtained (24.82±0.432 kg) when THI_b was equal to 34. Between 34 and 54 THI_b values, continuous fluctuations were observed in the milk yields. However, after the 54 THI_b value, there was a continuous decrease in milk yields to 67 THI_b value. When THI_b was 54, the milk yield was 26.95±0.408 kg; however, when THI_b value reached to 67 the milk yield decreased to 25.19±0.533 kg and the difference was 1.76 ($p<0.05$) due to heat stress.

3.1.3. THI_c

As indicated in Figure 2 and Table 4, THI_c values were calculated accepting that daily average temperature and humidity values ranged between 35-78. Accordingly, the threshold THI_c value at which the milk yield started to decrease continuously was determined as 64, far behind the critical value (THI=72). The milk yield tended to increase despite fluctuating values from the point where the THI_c was 35 to 64, but after this point it decreased rapidly and reached minimum at the 78 point. While the THI_c value was equal to 64, the milk yield was 27.44±0.510 kg. When the THI_c value increased to 78, decreased to 25.19±0.486 kg and the difference was 2.25 ($p<0.05$).

3.1.4. THI_d

THI_d values calculated by considering daily maximum temperature and minimum humidity values were in the range of 43-82 as observed in Figure 2 and Table 4. This range (39 units) was not found to be larger than THI_a. However, the threshold THI_d value at which the milk yield began to continuously decrease was determined as 69 and was very close to the critical value (THI=72). In other words, THI_d had the least deviation from the critical value by 3 units. Although the THI_d values fluctuated from 43 to 69, the milk yield tended to remain constant in this range, but after this point, it decreased rapidly and reached a minimum at the 82. When the THI_d value reached 69, the milk yield was 27.08±0.398 kg. When the THI_d value increased to 82, the milk yield decreased to 25.43±0.442 kg and the difference was 1.65 ($p<0.05$).

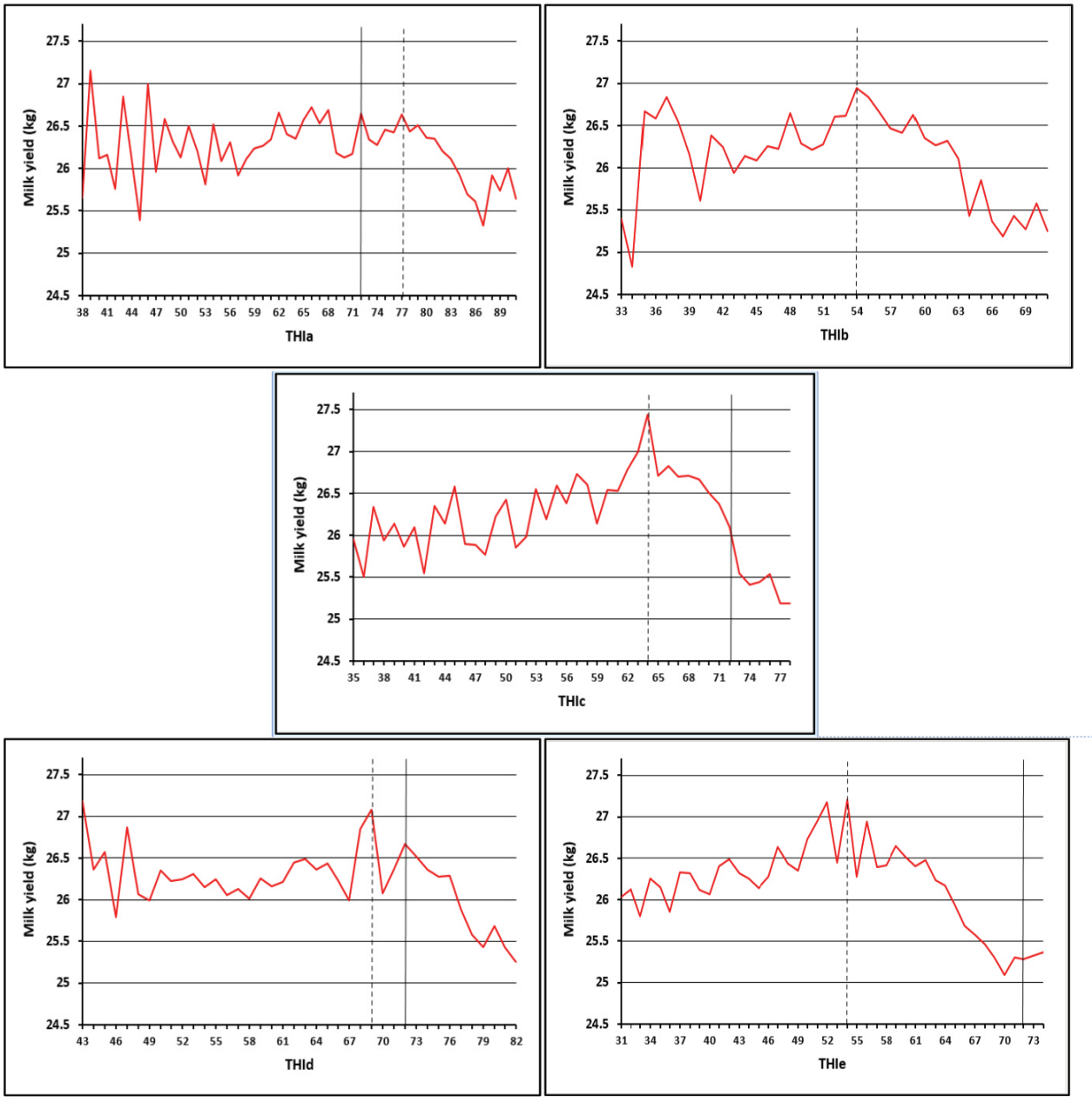

Figure 2- Least square means of milk yields by THI variants. Vertical lines show the critical THI value (72) while the dashed vertical lines show the THI value where milk yield starts to decrease continuously

Table 4- Least square means and standart errors of milk yields by THI variants*

THI	Milk yield (kg)														
	THI_a			THI_b			THI_c			THI_d			THI_e		
	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
31	-	-	-	-	-	-	-	-	-	-	-	-	517	26.03	0.375
32	-	-	-	-	-	-	-	-	-	-	-	-	850	26.13	0.327
33	-	-	-	171	25.39	0.558	-	-	-	-	-	-	1,199	25.80	0.301
34	-	-	-	339	24.82	0.432	-	-	-	-	-	-	1,192	26.25	0.303
35	-	-	-	859	26.67	0.337	341	25.96	0.468	-	-	-	1,381	26.15	0.309
36	-	-	-	515	26.58	0.372	339	25.50	0.437	-	-	-	1,163	25.85	0.298
37	-	-	-	521	26.84	0.380	177	26.34	0.561	-	-	-	1,355	26.33	0.288
38	339	25.65	0.436	523	26.54	0.389	696	25.94	0.371	-	-	-	1,500	26.32	0.285
39	177	27.16	0.669	1,009	26.16	0.307	676	26.14	0.356	-	-	-	1,331	26.12	0.289
40	512	26.12	0.385	1,191	25.61	0.295	518	25.86	0.383	-	-	-	1,487	26.07	0.281
41	528	26.16	0.398	1368	26.38	0.293	510	26.09	0.396	-	-	-	1,669	26.40	0.280
42	174	25.76	0.561	2,,657	26.24	0.261	851	25.54	0.343	-	-	-	3,753	26.49	0.247
43	513	26.84	0.392	1,874	25.94	0.275	1177	26.35	0.311	339	27.19	0.440	1,478	26.32	0.273
44	680	26.08	0.352	2,495	26.14	0.260	1184	26.14	0.308	678	26.36	0.349	1,917	26.25	0.253
45	497	25.39	0.386	2,208	26.09	0.262	1373	26.58	0.309	178	26.57	0.553	1,022	26.14	0.304
46	858	27.00	0.334	1,657	26.26	0.278	2061	25.90	0.288	527	25.79	0.368	1,747	26.28	0.269
47	1,027	25.95	0.328	2,125	26.22	0.259	2345	25.89	0.281	849	26.87	0.344	1,764	26.64	0.270
48	826	26.58	0.336	2,789	26.64	0.246	1465	25.77	0.294	1,517	26.06	0.297	1,289	26.43	0.296
49	1,839	26.32	0.286	2,133	26.29	0.256	1216	26.23	0.307	1,167	25.99	0.302	1,207	26.35	0.279
50	683	26.12	0.347	1,864	26.21	0.253	1851	26.43	0.284	1,343	26.35	0.302	745	26.73	0.328
51	1,204	26.50	0.313	1,489	26.28	0.275	2659	25.85	0.265	850	26.22	0.329	674	26.95	0.333
52	1,182	26.20	0.306	1,497	26.61	0.267	2206	25.98	0.270	1,176	26.24	0.312	170	27.17	0.554
53	1,027	25.82	0.314	613	26.61	0.338	1424	26.55	0.285	1,357	26.31	0.293	272	26.45	0.466
54	1,985	26.52	0.276	352	26.95	0.408	1393	26.19	0.297	2,022	26.15	0.276	173	27.21	0.551
55	2,212	26.09	0.280	307	26.84	0.433	1252	26.60	0.286	1,687	26.24	0.281	354	26.28	0.424
56	1,320	26.31	0.297	465	26.66	0.377	955	26.38	0.300	2,196	26.05	0.275	499	26.94	0.375
57	861	25.91	0.331	1,359	26.46	0.284	590	26.73	0.335	1,693	26.13	0.281	1,088	26.39	0.304
58	1,313	26.11	0.297	1,298	26.42	0.293	1383	26.61	0.278	1,475	26.02	0.287	1,079	26.41	0.307
59	1,938	26.23	0.272	1,411	26.63	0.282	1019	26.14	0.315	2,187	26.25	0.262	823	26.65	0.323
60	1,350	26.27	0.280	1,748	26.35	0.272	419	26.54	0.417	1,693	26.16	0.268	1,050	26.51	0.308
61	755	26.34	0.328	2,488	26.26	0.267	1297	26.53	0.284	578	26.21	0.352	1,387	26.41	0.292
62	305	26.66	0.437	1,153	26.31	0.304	389	26.78	0.405	1,523	26.45	0.270	1,200	26.48	0.301
63	602	26.40	0.351	1,384	26.11	0.315	280	26.99	0.454	1,888	26.48	0.262	1,780	26.24	0.291
64	1,180	26.35	0.282	959	25.43	0.329	219	27.44	0.510	949	26.36	0.300	982	26.17	0.327
65	1,572	26.58	0.273	1,091	25.85	0.327	518	26.71	0.375	574	26.43	0.362	938	25.93	0.346
66	345	26.72	0.431	863	25.37	0.351	391	26.83	0.402	847	26.23	0.318	792	25.68	0.346
67	471	26.53	0.376	261	25.19	0.533	992	26.70	0.311	806	25.99	0.322	903	25.57	0.343
68	446	26.69	0.383	378	25.43	0.474	1549	26.71	0.284	961	26.85	0.303	640	25.46	0.404
69	940	26.18	0.320	340	25.28	0.498	1224	26.67	0.302	396	27.08	0.398	788	25.30	0.360
70	416	26.13	0.386	247	25.57	0.550	1829	26.51	0.290	458	26.08	0.383	291	25.09	0.509
71	256	26.17	0.473	92	25.25	0.751	2228	26.38	0.294	268	26.36	0.457	338	25.30	0.487
72	454	26.64	0.380	-	-	-	1287	26.09	0.324	1,287	26.67	0.288	305	25.28	0.516
73	235	26.34	0.474	-	-	-	914	25.55	0.359	1,817	26.52	0.273	162	25.32	0.630
74	791	26.28	0.343	-	-	-	886	25.41	0.371	632	26.36	0.345	137	25.37	0.643

Table 4- continued

THI	Milk yield (kg)														
	THI_a			THI_b			THI_c			THI_d			THI_e		
	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
75	812	26.46	0.312	-	-	-	890	25.44	0.361	2,243	26.28	0.283	-	-	-
76	618	26.43	0.352	-	-	-	780	25.54	0.410	2,440	26.28	0.304	-	-	-
77	893	26.64	0.318	-	-	-	276	25.18	0.514	1,317	25.88	0.343	-	-	-
78	423	26.43	0.395	-	-	-	372	25.19	0.486	1,041	25.57	0.343	-	-	-
79	688	26.51	0.336	-	-	-	-	-	-	1,332	25.43	0.338	-	-	-
80	1,402	26.36	0.283	-	-	-	-	-	-	444	25.69	0.448	-	-	-
81	663	26.35	0.350	-	-	-	-	-	-	517	25.43	0.442	-	-	-
82	1,598	26.20	0.298	-	-	-	-	-	-	284	25.25	0.513	-	-	-
83	1,775	26.12	0.296	-	-	-	-	-	-	-	-	-	-	-	-
84	1,092	25.92	0.321	-	-	-	-	-	-	-	-	-	-	-	-
85	1,160	25.70	0.322	-	-	-	-	-	-	-	-	-	-	-	-
86	839	25.61	0.335	-	-	-	-	-	-	-	-	-	-	-	-
87	543	25.33	0.385	-	-	-	-	-	-	-	-	-	-	-	-
88	1,037	25.92	0.330	-	-	-	-	-	-	-	-	-	-	-	-
89	485	25.73	0.388	-	-	-	-	-	-	-	-	-	-	-	-
90	238	26.00	0.494	-	-	-	-	-	-	-	-	-	-	-	-
91	281	25.64	0.486	-	-	-	-	-	-	-	-	-	-	-	-

*Due to the large number of means, it is not possible to show which means in the same column are statistically different from each other. Instead, some important statistical differences were noted in the text. Furthermore, the milk yield increases from light yellow to dark yellow in all columns. n: Number of observations, SE: Standard error

3.1.5. THI_e

As shown in Figure 2 and Table 4, THI_e values calculated based on average temperature and humidity values ranged between 31 and 74. The threshold THI_e value at which the milk yield started to continuously decrease was determined as 54, and it was below the critical value (THI=72), similar to THI_c and THI_d. However, THI_e had the greatest deviation from the critical value among all variants by 18 units. Even though the THI_e had fluctuating values of 31 to 54, the milk yield tended to increase in this range. After that point, however, it rapidly decreased and reached a minimum at 70 points. While the THI_e value was equal to 54, the milk yield was 27.21±0.551 kg. When the THI_e value increased to 70, the milk yield decreased to 25.09±0.509 kg and the difference was 2.12 ($p<0.05$).

3.2. Milk yield loses for THI variants

THI_a values, obtained by using the maximum temperature and maximum humidity, were in the range of 38-91 and the stress zone in the range of 77-87 (Figure 2, Table 4). The difference between the two values is 10 units. If a THI_a of 77 was considered to cause heat stress, then the cattle would be under heat stress for more than one third of the year. On average, Diyarbakir had 125 THI_a days per year with values ranges from 77 to 87 (Figure 1), and the mean THI_a on these days was 83. The difference between the two values is 6 units. This means that a lactating cow during that entire period would be exposed to 750 units (6x125) of THI_a over the comfort zone (Equation 3). As a result, cows lose a production equal to 750 units. So, the loss of milk production of one cow per year because of heat stress would be 98.25 kg with a loss of 0.07 kg (98.25/125=0.78 and 0.78/10=0.07) per unit of THI_a greater than 77. Similarly, the losses of milk yields for one cow per year during heat stress periods were calculated as 157.68, 207.36, 164.30 and 190.08 for THI_b, THI_c, THI_d, and THI_e, respectively. In addition, the losses of milk production per unit of THI greater than the threshold were 0.08, 0.09, 0.07, and 0.08 kg for THI_b, THI_c, THI_d, and THI_e, respectively (Table 5).

Table 5- Heat stress characteristics and milk yield losses in the examined cow population

Parameters	THI_a	THI_b	THI_c	THI_d	THI_e
Heat stress period (days)	125	146	162	163	144
THI range for heat stress period	77-87	54-67	64-78	69-82	54-70
Average THI during the heat stress period	83	62	72	77	64
Losses (kg/per cow)					
Loss of milk yield during heat stress period	98.25	157.68	207.36	164.30	190.08
Loss of milk yield per unit THI increase	0.07	0.08	0.09	0.07	0.08

As mentioned earlier in Table 3, all R^2 values were around 0.45, indicating that almost half of the yield variation was explained by the model, including weather variables. However, THI_d and THI_e combinations containing extreme values together (maximum and minimum) have slightly higher coefficients of determination than the others. Ravagnolo et al. (2000) reported that while the amount of moisture in the air was constant, the lowest humidity occurred when the temperature was highest. This is consistent with the findings in this study. While the coefficients of determination values for THI variants were higher than the values reported by Ravagnolo et al. (2000), West et al. (2003), Freitas et al. (2006), they were close to the values reported by Dikmen & Hansen (2009) and Yazgan (2017).

Due to the different combinations of temperature and humidity levels (maximum, minimum, or average) being used when calculating THI variants (Figure 2, Table 4), the highest and lowest values of THI variants are different. For this reason, the highest THI_b value obtained was only 71 using the minimum temperature and humidity values. This showed that THI_b was insufficient to determine the effect of heat stress under the conditions in which this study was conducted. This indicates that it is not practical to use minimum temperature and minimum humidity variables when calculating THI values in Diyarbakir conditions.

According to the THI formula (Equation 1), heat stress in dairy cattle starts at a THI of 72 and is called the critical value; after this point the milk yield continuously decreases. In this study, deviations from critical values (THI=72) were observed for all THI variants. In comparison to 72, the point at which milk yields began to decline continuously for the THI_a, THI_b, THI_c THId and THI_e variants were 77, 54, 64, 69 and 54 respectively (Figure 2). While the deviation value of the THI_a variant was greater than the critical value of 72, all other variants (THI_c, THI_d and THI_e) had values less than the critical level. The least deviation was observed in the THI_d variant by 3 units. The reason why other the THI variants deviate more from the critical 72 value than THI_d may be due to the temperature and humidity variables used in THI_a, THI_c and THI_e calculations. In other words, combining the variables of maximum temperature and minimum humidity into one THI seems to better reflect the stress conditions to which animals are exposed in Diyarbakir conditions. Another reason for deviations from the critical value may be the distance between the farm and the weather station.

Yazgan (2017) reported the critical THI values where milk yield started to decrease as 68, 76, 80, and 70 for minimum temperature and humidity, average temperature and humidity, maximum temperature and minimum humidity, and minimum temperature and maximum humidity combinations, respectively. These results differ from the values reported in this study. However, deviations from critical values (THI=72) when combinations of mean temperature and humidity (THI_c) and maximum temperature and minimum humidity (THI_d) were used in this study were similar to the values reported by Bouraoui et al. (2002) and Bohmanova et al. (2007).

As shown in Figure 2, for all THI variants, fluctuations were observed during the comfort zone, which corresponds to the range from the starting THI values to dashed vertical lines. THI_d showed the minimum deviation from the starting point of heat stress and the minimum fluctuation during the comfort zone when compared with others. Some fluctuations may be caused by the use of fans, shading, and sprinkler equipment. When such equipment is activated, heat stress may appear to be lower at higher temperatures. This also causes the THI to appear not only linear but also of zigzag shape (Figure 2). Fluctuations in all of the THI curves could also be caused by an insufficient number of daily milk yield records with a given THI, by partial confounding with other effects in the model (Equation 2) and by ignoring herd management practices (e.g. change in feeding regimen in some animals) and other conditions (e.g. prolonged exposure of animals to direct sunlight or strong wind when animals were in the paddock).

Ravagnolo et al. (2000), reported that the maximum daily air temperature and minimum daily humidity were the most critical variables to quantify heat stress. Similar results were obtained from this study as shown in Figure 2 where THI_d seems to be less affected by the factors causing fluctuations and showed a deviation of only 3 units from the heat stress beginning point (THI=72). Moreover, THI_d

performed better than other THI variants in quantifying the heat stress in this study. Therefore, it can be said that the results obtained from the use of THI_d in calculating of milk yield losses are more reliable than the other THI variants.

Considering the THI_d, the amount of milk yield loss (0.07 kg) for each unit of THI_d increase obtained from this study was similar to that reported by Konyves et al. (2017) and Gantner et al. (2017). It was, however, slightly lower than that reported by Igono et al. (1992) and Ravagnolo et al. (2000), and much lower than that reported by Ingraham (1979), Her et al. (1988), Bouraoui et al. (2002), West et al. (2003), Bohmanova et al. (2007), Zimbelman et al. (2009), Herbut & Angrecka (2012) and Yazgan (2017). Various causes might have contributed to these discrepancies. For instance, the results may be sensitive to distances between the meteorology station and the farm, 15 km in the present study and the distances were different in all studies. In addition, while in some studies (Her et al. 1988; Igono et al. 1992), the weather conditions were measured on the farm, in this study they were obtained away from the farm. Different measures towards alleviation heat stress levels (e.g., fans, shading, and sprinkler application systems) may have been used in all studies. In addition, the use of other weather variables in some of the studies may be another reason. Apart from these, some researchers' (West et al. 2003) use of temperature and humidity values 2 or 3 days before milk yield may explain the differences in milk yield loss between this study and others. Lastly, to obtain the least squares mean of milk yields, daily yield records were used in this research. Most of the other studies mentioned above, however, used monthly data.

Temperature and humidity values vary during the day, and the characteristics of this variation are different for each region. For example, while the RH level in one region is 70% during the day for 3 hours, it may remain at this level for 14 hours in another region. This is valid for the air temperature and is a determining factor for heat stress on animals. That is, if the minimum humidity level during the day does not change for a long period of time and then suddenly drops, it would not be correct to use the maximum humidity value when calculating the THI value. This also applies to other temperature and humidity variables (maximum or minimum). Findings from this study confirm this. For this reason, this type of research should be carried out when the effect of heat stress on animal production in a region is to be determined.

4. Conclusion

This study confirmed that weather stations located away from the farms contain information useful for research on heat stress in dairy cows. Using the combination of maximum daily air temperature and minimum daily humidity in the THI formula (THI_d) performed better than other THI variants in quantifying the heat stress in this study. This combination was affected less by other environmental factors, and the results obtained from this combination appear to be more biologically meaningful. As a result, it can be used to quantify heat stress in farms with conditions similar to those in this study. However, the performance of these weather variable combinations can be different in other geographic areas. The distance between a farm and weather station is another important factor for the accurate measurement of the heat stress effect. Therefore, similar studies should be carried out on farms located in different regions.

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Data availability: Data are available on request due to privacy or other restrictions.

Authorship Contributions: Concept: O.D., Design: K.Y., Data Collection or Processing: O.D., Analysis or Interpretation: K.Y., Literature Search: O.D., K.Y., Writing: K.Y.,

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Effects of Dietary Sage, Myrtle and/or Probiotic Mixture on Growth, Intestinal Health, Antioxidant Capacity, and Diseases Resistance of *Oncorhynchus mykiss*

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ABSTRACT
It is widely known that the use of medicinal plants and probiotics as feed additives has a positive effect on growth, non-specific immune system, and resistance to diseases in aquaculture. This study examines the effects of dietary supplementation with sage (*Salvia officinalis*) and myrtle (*Myrtus communis*), alone or in combination with a probiotic mixture (PM) on growth, intestine microflora and histology, some antioxidant enzymes activities in the muscle tissues of rainbow trout (*Oncorhynchus mykiss*) and disease resistance against *Vibrio anguillarum*. For this purpose, fish were fed with a control diet of 1% sage, 1% myrtle, 1.1% probiotic mixture, 1% sage +1.1% PM and 1% myrtle+1.1% PM supplemented diets for 60 days. At the end of the trial, the fish fed the diets supplemented with myrtle and sage + PM showed a positive effect on feed conversion ratio. According to the histological assessment, the villi length, villi width and goblet cell numbers in the intestines of fish in all groups increased compared to the control. Superoxide dismutase activity in the muscles of fish in the PM group was higher than the fish in the other groups ($p<0.05$). The malondialdehyde activity was unaffected with the exception of the fish in the sage group ($p<0.05$). The lactic acid bacteria count in the intestines increased in fish fed the sage + PM ($p<0.05$). Fish fed the diets supplemented with sage + probiotic mixture, probiotic mixture, myrtle + probiotic mixture, and myrtle saw a significant reduction in mortality (0-32.5%) due to V. *anguillarum* compared to the control (63.2%) ($p<0.05$). In conclusion, the use of probiotics, sage and myrtle in combination as a feed supplement showed a positive effect on the growth performance, intestinal microflora and histology, and antioxidant enzymes activities and disease resistance in rainbow trout.

Keywords: *Salvia officinalis*, *Myrtus communis*, Probiotic, Histology, *Vibrio anguillarum*, Rainbow trout

1. Introduction

The aquaculture sector has seen a rapid expansion across the world in recent decades (Guardiola et al. 2017). In Turkey, almost all of freshwater farming is based on rainbow trout production (Okumuş 2002). The benefits of better growth rate, better feed utilization rate and enhancement against diseases on fish culture will provide high added value to the country. In addition, intensive culture has led to the stress and the outbreak of diseases (Kennedy et al. 2016) in fish. Antibiotics and chemotherapeutics agents are extensively used in the treatment of diseases in aquaculture. However, these practices have not only negatively affected the environment (Santos & Ramos 2018; Srichaiyo et al. 2020), but also formed antimicrobial resistance in bacteria. For this reason, the use of medicinal plants to manage fish pathogens is an alternative and current practice. Medicinal plants are being used in aquaculture not only as chemotherapeutics but also as feed additives, as they contain a wide variety of chemical compounds (Awad & Awaad 2017). Probiotic products may provide broad-spectrum and greater non-specific disease protection (Lara-Flores 2011). There is evidence to suggest that probiotics in aquaculture may prove effective in improving growth performance, immunostimulation and increase disease-resistance (Balcazar et al. 2006).

Myrtle (*Myrtus communis* L.) is a Mediterranean evergreen shrub that has been used since ancient times for medicinal, food and spice purposes. The dried leaves of this herb have volatile oils which contain 1.8-cineole, linalool, linalyl acetate, terpinolene, tannins and flavonoid compounds. The leaves and fruits have long been used in Turkish folk medicine for antiseptic purposes when healing wounds, and in the treatment of prostatitis, bronchitis, sinusitis, and colds (Keven-Karademir & Avunduk 2015). Previous studies have reported the growth promotion (Taee et al. 2017a), antimicrobial (Taee et al. 2017b), anaesthetic (Al-Niaeem et al. 2019), antioxidant (Safari et al. 2017) properties of myrtle in fish.

Sage (*Salvia officinalis* L.) is a plant in the Lamiaceae family (Carović-StanKo et al. 2016), it contains some components (α, β-pinene, 1,8-cineole, borneol, and α, β-thujone) which have antibacterial (Hać-Szymańczuk et al. 2014, 2015) and antioxidant (Wojdyło et al. 2007; Roby et al. 2013) properties (Bernotienė et al. 2007). A number of studies have extensively analysed the effects of sage oil on growth in different fish species (Salomón et al. 2020), immune response (Terzioğlu & Diler 2016), meat quality (Mehdizadeh et al. 2019) and blood parameters (Aydin & Harmantepe 2018).

Over the past decade, particularly in aquaculture, research has focused on the application of using aromatic plants to replace subtherapeutic antibiotics in growth promoters, disease control, immune response, and disease resistance of various fish species. In addition, studies have shown that the use of medicinal plants in a synbiotic with probiotics increases these effects even more (Abdallah et al. 2022). However, studies in aquaculture on this subject are very limited.

This study investigates the effects of dietary supplementation with sage and myrtle plants alone as well as in combination with an indigenous and exogenous probiotic mixture (PM) composed of four lactic acid bacteria, Bifidobacterium spp. and one yeast strain on rainbow trout growth, intestinal microflora and histology, antioxidant enzyme activities and disease resistance against *Vibrio anguillarum*.

2. Material and methods

2.1. Preparation of experimental diets

The PM was prepared to contain lactic acid bacteria (*Lactobacillus* spp., *Lactococcus* spp., *Lactobacillus acidophilus*), *Bifidobacterium* spp., one yeast strain (*Kluyveromyces marxianus*) was isolated from kefir (Kök Taş et al. 2012; Gümüş et al. 2017) and *Lactobacillus sakei* from rainbow trout intestines (Didinen et al. 2018). The lactic acid bacteria were grown on De Man, Rogosa and Sharpe (MRS) Broth (Merck 110661) and incubated for 24 hr at 25 °C. The cells were collected by centrifugation at 5,000 g for 15 min at 4 °C. Experimental diets were formulated based on the study of New 1987 (Table 1). Different probiotic bacteria species were added to feed at a rate of 1.1% with sunflower oil (0.05 mL kg^{-1}) to provide 1×10^8 CFU g^{-1}.

Myrtle berries and sage leaves samples were obtained from a commercial company that has official production permission in Isparta and dried at room temperature and then powdered. The powder was mixed with sunflower oil (0.05 mL kg^{-1}) and added at a rate of 1% to the feed.

Table 1- Formulation of the experimental diets (g kg^{-1})

Feed ingredients	***Groups***					
	Control	***Myrtle (1%)***	***Myrtle (1%) + PM***	***Sage (1%)***	***Sage (1%) + PM***	***PM***
Fish meal[1]	350	350	350	350	350	350
Soybean meal[2]	300	300	300	300	300	300
Wheat gluten[3]	50	50	50	50	50	50
Wheat meal[4]	124.95	114.95	103.95	114.95	103.95	113.95
Fish oil[5]	80	80	80	80	80	80
C vitamin[6]	5	5	5	5	5	5
Vitamin premix[7]	20	20	20	20	20	20
Mineral premix[8]	10	10	10	10	10	10
Pellet binders[9]	40	40	40	40	40	40
Antioxidant[10]	5	5	5	5	5	5

Table 1- continued

Feed ingredients	***Groups***					
	Control	***Myrtle (1%)***	***Myrtle (1%) + PM***	***Sage (1%)***	***Sage (1%) + PM***	***PM***
Others[11]	15	15	15	15	15	15
Additions to standard diet						
Plant powder	-	10	10	10	10	-
Lactobacillus spp.	-	-	2	-	2	2
Lactococcus spp.	-	-	2	-	2	2
Lactobacillus acidophilus	-	-	2	-	2	2
Bifidobacterium spp.	-	-	2	-	2	2
Yeast strain	-	-	2	-	2	2
Lactobacillus sakei	-	-	1	-	1	1
Sunflower oil	0.05	0.05	0.05	0.05	0.05	0.05

[1-6]Abalioglu Feed Factory, Torbalı-Izmir/Turkey
[7]Vitamin premix.; per kg, 4,000,000 IU vitamin A, 480,000 IU vitamin D3, 40,000 mg vitamin E, 2,400 mg vitamin K3, 4,000 mg vitamin B1, 6,000 mg vitamin B2, 40,000 mg niacin, 10,000 mg calcium D-pantothenate, 4,000 mg vitamin B6, 10 mg vitamin B12, 100 mg D-biotin, 1,200 mg folic acid, 40,000 mg vitamin C and 60,000 mg inositol.
[8]Mineral premix.; per kg 23,750 mg Mn, 75,000 mg Zn, 5,000 mg Zn, 2,000 mg Co, 2,750 mg I, 100 mg Se, 200,000 mg Mg.
[9]Pellet binders; lignosulfonate.
[10]Antioxidant; ethoxyquin.
[11]Others; choline chloride, methionine + cystine.
PM: Probiotic mixture

The experimental groups were be formed as sage (group I), myrtle (group II), PM (group III), sage + PM (group IV) and myrtle + PM (group V) and control (group VI).

2.2. Gas Chromatography-Mass Spectrometry (GC-MS) analysis of plant powder

A GC-MS analysis was performed to determine the phenolic content of the plants and the GC-MS analyses of the plants were carried out using a Hewlett-Packard 6890 series gas chromatograph fitted with a flame ionization detector, CPWax 52CB capillary column (50 m×0.32 mm; film thickness ¼ 0.25 Im). Pure helium gas was used as the carrier gas at a constant flow rate of 40 mL min^{-1}. The injection quantity was 1 µL, and the injector and detector temperature was maintained at 240 °C. The column oven temperature was set at 60 °C and was raised by 2 °C per min up to 220 °C. The final temperature was maintained at 220 °C for 20 min. Relative percentage amounts were calculated from chromatograms from the Turbo Crom Navigator computer program.

2.3. Fish and experimental conditions

The experiments were performed in systems with a water flow rate of 12 L min^{-1}. The water quality parameters were measured as temperature 12±2 °C, dissolved oxygen 7.4 mg L^{-1} and pH 7.3. A total of 600 (6 groups with two replicates, 50 fish per tank) average 45.44±3.11-50.65±3.26 g rainbow trout (*Oncorhynchus mykiss*, Walbaum) were randomly distributed into 600 L tanks. The experimental fish were fed daily at 2% of their biomass for 60 days. Throughout the study, the fish were weighed at 2 week intervals and their weight was determined and the amount of feed to be given was regularly updated according to their body weight. The welfare of the fish was performed according to the ethical standards of the national guidelines approved by the Isparta Applied Sciences University Animal Care and Use Committee (decision number: 001, date: 24.09.2020).

2.4. Detection of the growth performance

Growth performance was evaluated based on the following formulas (De Silva & Anderson 1995):

Weight gain (WG, g) = W2-W1;

Specific growth rate (SGR, %day) = 100 x (Ln W2-Ln W1)/T;

Feed conversion ratio (FCR) = (feed intake, g)/(weight gain, g);

Condition factor (CF) = 100 x (body weight, g)/(standard length^3, cm);

Survival rate (SR, %) = 100 x (Nf / Ni)

Feed intake (FI, g day^{-1}) = (total consumed of feed, g)/(number of fish)

W1 (Initial weight), W2 (Final weight), T (Number of days in the feeding period), Ni (Initial number of fish) and Nf (Final number of fish), respectively.

2.5. Enumeration of intestinal microbiota

To determine the effect of dietary plant powder and probiotics on the bacterial population of the intestine, 6 fish samples were taken from each experimental group at the end of 60 days. After the fish was anesthetized (clove oil, 50 mg L^{-1}), the skin was wiped with alcohol and digestive tract samples were taken by ventral incision. Samples were created by homogenizing whole intestinal samples in phosphate buffered saline (PBS) and tenfold serial dilutions of these samples were prepared and spread on Man, Rogosa and Sharpe Agar to obtain lactic acid bacteria. The plates were incubated at 37 °C for 48 hours, after which the plate count agar were incubated at 30 °C for 48 hours to acquire a total count of the aerobic bacteria. Following this, the bacteria colonies were counted and averaged for each sample (Giannenas et al. 2012).

2.6. Histological examination of intestines

At the end of the study, after anaesthesia (clove oil, 50 mg L^{-1}), a necropsy was performed on 6 fish from each experimental group. Following the aseptic dissection, the intestine in its entirety was removed. The anterior intestinal tissue was then excised and samples taken and fixed in 10% neutral formalin solution and processed by automatic tissue processing equipment (Leica ASP300S). The intestine samples were embedded in paraffin, and 5 µm longitudinal sections of the intestine were cut using a rotary microtome. Then, intestine sections were stained with hematoxylin and eosin (H&E) and examined under a light microscope.

In order to evaluate the morphometric changes of the intestines, the length and width of each villi were measured at 40x under a microscope. The morphometric evaluation was carried out using the Database Manual Cell Sens Life Science Imaging Software System. The mucous cells (goblet cells) in the anterior part of the intestine were counted for each fish and the mucous cell counts were reported as mean number ± standard deviation per 100,000 µm^2 of epithelial section area (Heidarieh et al. 2013).

2.7. Antioxidant activity in tissues

A total of 20 fish from each experimental group were decapitated under anaesthesia (clove oil, 50 mg L^{-1}) at day 60 after the feeding trial. Muscle samples were taken and washed with physiological saline. A 3 mL Tris-HCl buffer containing 0.25 mol L^{-1} sucrose was adjusted to pH 7.3 and mixed with the tissue sample and stored at -80 °C. The muscle tissues were homogenized in a motor-driven tissue homogenizer with phosphate buffer (pH 7.4). Unbroken cells, cell debris, and nuclei were sedimented by centrifugation at 2,000 g (rpm) for 10 min. The levels of malondialdehyde (MDA) and the activities of superoxide dismutase (SOD) and catalase (CAT) were defined in the supernatants. The determination of SOD activity was predicted in the supernatant according to the method described by Aebi (1974) and is referred to in kilounits per gram protein. The MDA levels in the tissues were detected from the homogenate by following the double heating method (Draper & Hadley 1990); the concentration of MDA is obtained as micromoles per gram protein in the muscle tissue. SOD, CAT activities and MDA levels were determined using a spectrophotometer (Shimadzu UV-1601).

2.8. Bacterial challenge

The pathogenicity of the *V. anguillarum* strain in rainbow trout was confirmed by intraperitoneal injecting (i.p.) and reisolating pathogens from diseased fish prior to the experiments. After 60 days of feeding, the experimental infection was performed by i.p. injection under anaesthesia (clove oil, 50 mg L^{-1}). The *V. anguillarum* pathogen was inoculated on Tryptic Soy Broth medium and incubated at 25°C for 24 hours. The cells were harvested by centrifugation (2,000f), washed with PBS and re-suspended in PBS. 40 fish from each experimental group were intraperitoneally injected with *V. anguillarum* with 0.1 ml volumes of bacterial suspension adjusted to 2.0x105^5 CFU mL^{-1} (LD_{50} dose) (Diler et al. 2017). Mortalities were recorded daily and any moribund fish were examined bacteriologically. Afterwards, the relative percent survivals (RPS) were calculated according to Amend (1981).

2.9. Statistical analysis

For the evaluation of differences in growth parameters including final weight, SGR, FCR and feed intake, the analysis of covariance (ANCOVA) was used with the incorporation of initial average weights as a covariate. The influence of treatments on survival rates, lactic acid bacteria and total bacteria counts in the intestines, antioxidant activity, mortalities, RPS values, length and width of the villi, goblet cells counts of intestines between the experimental groups were analyzed by one-way analysis of variance (ANOVA). SPSS 18.0 software was used to analyze the data. The significant variables were discriminated among the treatments using the Duncan test. In the statistical analysis, $p<0.05$ was considered as the significance level for growth parameters, survival rates, lactic acid bacteria and total bacteria counts in the intestines, antioxidant activity and mortalities and RPS values while $p<0.001$ was for the villi length, width and goblet cell numbers of the intestines.

3. Results

3.1. Chemical composition of medicinal plants

In this study, the major compounds in myrtle; hexanol 32.84%, 1.8-cineole(=eucalyptol) 31.62%, camphor 8.73%, alpha pinene 8.39% and limonene 4.63%; in sage; 1.8 cineole (56.98%) and camphor (21.15%) were determined (Table 2).

Table 2- Components of sage and myrtle

Compound	***Myrtle (%)***	***Sage (%)***
Alpha Pinene	8.39	1.93
Hexanal	32.84	-
Limonene	4.63	0.46
1.8-Cineole	31.62	56.98
1-Pentanol	2.05	-
p-Cymene	2.41	0.91
2-Heptenal	1.85	-
1-Hexanol	2.82	-
7-octen-4-ol	2.82	0.39
Camphor	8.73	21.15
Alpha-Terpineol	1.84	1.31
Camphene	-	1.74
Hexanal	-	0.43
Beta-Pinene	-	0.39
Beta-Myrcene	-	1.14
2-Hexenal	-	0.41
3-Hexen-I-ol, (Z)		0.20
Alpha-Thujone	-	1.10
Beta-thujone		0.69
Bomyl acetat	-	3.43
4-Terpineol	-	0.29
Trans-Caryophyllene	-	3.48
Aromadendrene	-	0.26
Linalyl oxide	-	0.42
Alpha-Humulene	-	0.48
Terpinyl acetate	-	0.27
Borneol	-	2.13

3.2. Growth parameters

In this study, the difference was not statistically significant between the groups in terms of final weight, weight gain, condition factor, specific growth rate, feed intake, and survival rate ($p>0.05$). The FCR values of the fish fed the diet with myrtle and sage + PM were significantly lower than the fish in the other groups and the control ($p<0.05$) (Table 3).

Table 3- Growth performance of the rainbow trout fed containing plants without or with probiotic bacteria

	Experimental groups					
	Control	***Myrtle (1%)***	***Myrtle (1%) + PM***	***Sage (1%)***	***Sage (1%) + PM***	***PM***
W1 (g)	46.26±0.91	45.44±3.11	50.50±2.97	46.62±0.41	50.65±3.26	46.80±0.16
W2 (g)*	72.85±3.79	76.69±5.65	79.95±4.90	75,24±6.33	76.12±0.05	75.20±7.46
WG (g)*	26.58±4.70	31.25±2.54	29.46±1.92	28.62±5.92	25.47±3.31	28.41±7.30
CF	1.23±0.15	1.29±0.44	1.24±0.18	1.22±0.11	1.27±0.14	1.25±0.09
FCR*	1.12 ± 0.17^{ab}	0.78 ± 0.01^{c}	1.29 ± 0.01^{a}	1.21 ± 0.02^{ab}	0.92 ± 0.13^{bc}	1.25 ± 0.07^{ab}
SGR (% day)*	0.76±0.12	0.87±0.01	0.77±0.00	0.79±0.13	0.68±0.11	0.79±0.16
FI (g day^{-1})*	0.49±0.01	0.40±0.04	0.63±0.01	0.57±0.10	0.39±0.09	0.58±0.01
SR (%)	100.00±0.00	97.50± 0.70	92.00±5.65	100.00±0.00	96.00±5.65	100.00±0.00

*Actual average values are shown but they were analyzed with ANCOVA using initial mean weights as covariate.
Data are presented as the means ± standard deviation (n-2) values within the same row having different superscipts are significantly different ($p<0.05$).
PM: Probiotic mixture, W1: Initial weight, W2: Final weight, WG: Weight gain, CF: Condition factor, FCR: Feed conversion ratio, SGR: Specific growth rate, FI: Feed intake, SR: Survival rate

3.3. Microbiological analysis

The lactic acid bacteria count in the intestine of fish in the sage + PM group was found to be higher than the fish in other groups. The total bacteria count in the intestine of fish fed with PM, sage and myrtle + PM groups were higher than the fish in other groups (Table 4).

Table 4- Lactic acid bacteria and total bacteria counts of fish in groups

Groups	***Lactic acid bacteria count (log CFU g^{-1})***	***Total bacteria count (log CFU g^{-1})***
Myrtle (1%)	2.50 ± 0.06^{b}	1.27 ± 0.43^{b}
Myrtle (1%) + PM	2.40 ± 0.37^{b}	6.05 ± 1.79^{a}
Sage (1%)	2.51 ± 0.02^{b}	7.26 ± 3.06^{a}
Sage (1%) + PM	3.13 ± 0.00^{a}	2.60 ± 1.58^{b}
PM	2.27 ± 0.24^{b}	6.25 ± 0.21^{a}
Control	2.14 ± 0.00^{b}	3.04 ± 1.14^{b}

Data are presented as the means ± standard devation (n-2) values within the same row having different superscipts are significantly different ($p<0.05$). PM: Probiotic mixture

3.4. Histology of intestine

Based on a microscopical examination of intestine samples in fish sampled from all groups, there were no pathological findings (Figure 1). In general, an increased villi length, width and numbers of goblet cell were observed in fish from all experimental groups compared to the control group ($p<0.001$) (Table 5). The best group on intestinal villi length and villi width of the fish was the myrtle + PM, while the best group in goblet cell numbers was determined to be the sage + PM group. Probiotics had an additional effect in improving the effects of the myrtle and sage groups (Figure 1).

Table 5- Villi length, villi width and goblet cell numbers of fish intestines in groups

Groups	*Villi length (µm)*	*Villi width (µm)*	*Goblet cell count*
Myrtle (1%)	588.75±15.13^{b}	92.75±2.50^{b}	101.50±1.29^{b}
Myrtle (1%) + PM	611.50±7.04^{a}	105.75±4.34^{a}	99.75±1.70^{b}
Sage (1%)	578.50±8.50^{b}	91.25±1.70^{b}	98.00±2.16^{b}
Sage (1%) + PM	595.25±4.11^{ab}	101.25±3.86^{a}	109.00±1.82^{a}
PM	591.50±8.69^{ab}	102.25±2.06^{a}	102.00±5.47^{b}
Control	481.75±35.10^{c}	85.25±5.18^{c}	92.25±3.59^{c}

Data are presented as the means ± standard deviation (n-2) values within the same row having different superscipts are significantly different ($p<0.05$). PM: Probiotic mixture

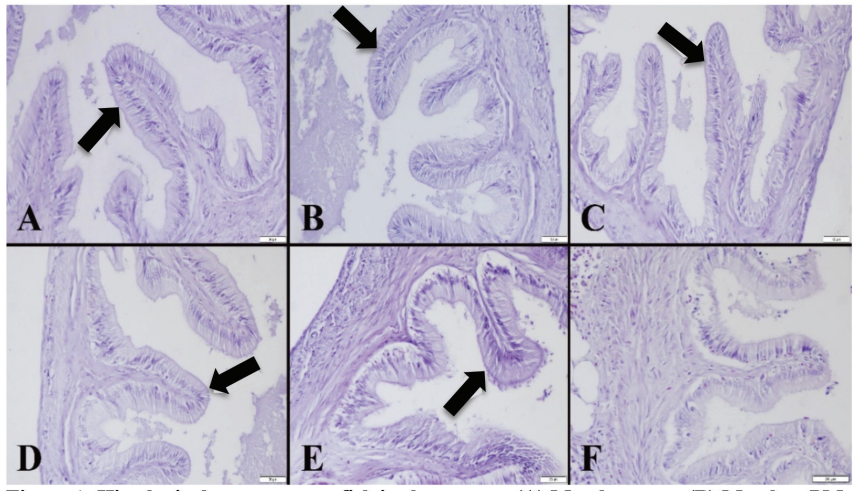

Figure 1- Histological gut structure fish in the groups; (A) Myrtle group, (B) Myrtle + PM group, (C) Sage group, (D) Sage + PM group, (E) PM group, (F) Control group, H&E, Bars=50 µm. The increase in villi length, villi width and in the number of goblet cells in the intestines of fish is indicated by the arrow

3.5. Antioxidant activity

The MDA level was unaffected with the exception of the fish in the sage group ($p<0.05$). In addition, the SOD activity of fish in the PM group was higher than those in other groups ($p<0.05$). CAT activity was not affected regardless of the treatment (Table 6).

Table 6- Antioxidant activity of fish in groups

Groups	*MDA*	*CAT*	*SOD*
Myrtle (1%)	0.48±0.37^{ab}	0.16±0.07	1.54±0.20^{bc}
Myrtle (1%) + PM	0.26±0.12^{b}	2.49±4.78	1.75±0.33^{b}
Sage (1%)	0.71±0.12^{a}	0.40±0.24	1.30±0.09^{c}
Sage (1%) + PM	0.29±0.10^{b}	0.36±0.42	1.34±0.15^{c}
PM	0.39±0.20^{b}	0.25±0.18	2.06±0.08^{a}
Control	0.29±0.10^{b}	0.36±0.32	1.74±0.14^{b}

Data are presented as the means ± standard deviation (n-2) values within the same row having different superscipts are significantly different ($p<0.05$). PM: Probiotic mixture, MDA: Malondialdehyde, CAT: Catalase, SOD: Superoxide dismutase

3.6. Diseases resistance against V. anguillarum

The statistical analysis demonstrated that fish fed with diets containing sage + PM, PM, myrtle + PM, myrtle at 21 days had significantly lower levels of mortality than those in the sage and control groups ($p<0.05$) (Figure 2). The RPS values were calculated as 100, 72.36, 60.38 and 48.62 in fish fed with diets containing sage + PM, PM, myrtle + PM, myrtle, respectively (Table 7). When resistance against *V. anguillarum* was evaluated, it was determined that the highest effect with 100% RPS value was in fish fed with diets containing sage + PM.

Table 7- Mortality and RPS values of fish in experiment groups challenged with *V. anguillarum*

	Mortality (%)	***RPS (%)***
Myrtle (1%)	32.50±3.53^{b}	48.62±4.50^{d}
Myrtle (1%) + PM	25.00±3.21^{c}	60.38±5.92^{c}
Sage (1%)	52.77±3.92^{a}	16.54±4.45^{e}
Sage (1%) + PM	0.00±0.00^{e}	100.00±0.00^{a}
PM	17.50±3.53^{d}	72.36±5.01^{b}
Control	63.20±1.33^{a}	-

Data are presented as the means ± standard deviation (n-2) values within the same row having different superscipts are significantly different ($p<0.05$). PM: Probiotic mixture, RPS: Relative percent survivals

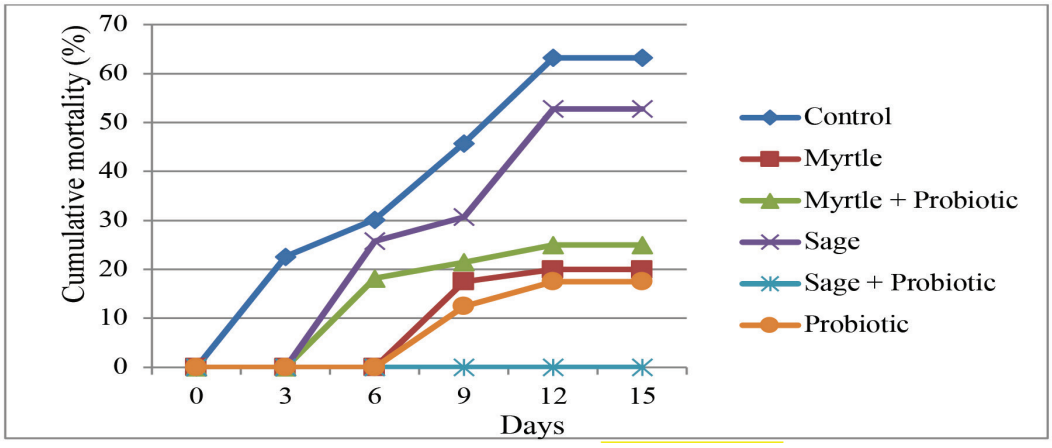

Figure 2- Fish cumulative mortality (%) against *V. anguillarum* among the groups

4. Discussion

This study found that a dietary supplementation with sage and myrtle, alone or in combination with PM increased the final fish weight but the difference was not found to be statistically significant ($p<0.05$). In a 2015 study, Sönmez et al. (2015) noted that a dietary inclusion of sage oil was effective in enhancing the growth of rainbow trout. El-Kholy (2012) also noted an increase in weight gain and feed efficiency in a tilapia hybrid (*Oreochromis niloticus* × *Oreochromis aureus*) fed with *S. officinalis* leaf powder at 150 and 300 mg kg^{-1} feed for 90 days. In contrast, Aydin & Harmantepe (2018) have reported that the administration of sage oil to tilapia (*Oreochromis niloticus*) diets (0, 0.25, 0.5 and 1%) reduced feed intake, specific growth rate, weight gain and final weight. In addition, Dadras et al. (2019) noted that the use of sage extracts (30, 60 and 120 mL kg^{-1} feed) in juvenile sturgeon (*Huso huso)* had no affect growth performance. The different results in growth performance may be related to the levels of herbal plants tested.

Taee et al. (2017a) noted a decrease in FCR in rainbow trout fed with myrtle powder. Mohamadi et al. (2016) also stated that feeding rainbow trout with 300 mg kg^{-1} of myrtle essential oils led to a decrease in FCR. In addition, Safari et al. (2017) reported that dietary myrtle at 20 g kg^{-1} improved the growth-related gene expressions in zebrafish (*Danio rerio*). Similarly, in the present study, it was determined that myrtle and sage + PM was more effective on the FCR values than other groups as well as the control.

The presence of autochthonous probiotic bacteria in the diet has changed the microbial metabolism of fish, which enhances growth performance by stimulating exogenous enzyme production (Bhatnagar et al. 2012). Similarly, Bhatnagar et al. (2012) determined that probiotic bacterium *Bacillus coagulans* was incorporated in different levels of *Mentha piperita*. Their results showed low FCR in the group fed diets with *M. piperita* and *Bacillus coagulans.*

The positive effects of lactic acid bacteria on growth were determined in different fish species such as *Lactobacillus acidophilus* in grass carp (Wang 2011); *Lactobacillus curvatus* in Persian sturgeon (*Acipencer percisus*), *Leuconostoc mesenteroides* in beluga (*Huso huso*) (Askarian et al. 2011); *Lactobacillus plantarum* in tilapia (Yu et al. 2017); *Lactobacillus casei* in *Barbus grypus* (Mohammadian et al. 2017). In present study, the final weight in rainbow trout fed with PM was found to be higher than in the control group; the difference, however, was not statistically significant ($p<0.05$).

Nutraceuticals such as probiotics, prebiotics, synbiotics, medicinal plants and immunostimulants affect the gut microbiota. Probiotics which stick with the mucosal epithelium of intestine and prevents pathogens colonization are a vitally importance modulation of intestinal microbiota (Hoseinifar et al. 2018). Giannenas et al. (2012) reported that the total counts of aerobic gut bacteria were not affected whereas the levels of *Lactobacillus* spp. were decreased by supplementation of thymol to the diet for 8 weeks. In the present study, the lactic acid bacteria counts in the intestine were unaffected by sage and myrtle groups in rainbow trout. However, the lactic acid bacteria counts of fish in the sage + PM group were found to be higher than those in other groups. Merrifield et al. (2010), concluded that the addition of different types of probiotics (*B. licheniformis*, *B. subtilis* and *E. faecium*) to the feed significantly increased the intestinal microbiota. In this study, the results were compatible with the literature, and it was determined that the fish in the group supplemented with PM increased the total number of bacteria in the intestinal microbiota. These findings suggest that a dietary inclusion of phytogenic products combined with PM may have a positive effect on the intestinal populations of rainbow trout.

The combination of probiotics and herbal products as an alternative disease control strategy is provided by improvement of hematological and biochemical parameters, disease resistance to pathogens (Ringø & Song 2016). Probiotics have been used to fight pathogens by producing inhibitory compounds such as bacteriocins, lysozymes, organic acids, proteases, hydrogen peroxide, diacetyl and other inhibitory chemicals (Karmakar et al. 2012). Van Doan et al. (2016) indicated that a dietary combination of *Lactobacillus plantarum* with Jerusalem artichoke (*Helianthus tuberosus*) in pangasius catfish (*Pangasius bocourti*) improved the serum lysozyme and respiratory burst activities, phagocytic index and disease resistance against *A. hydrophila* when compared to control fed fish. Harikrishnan et al. (2011) reported that the supplementation of *Lactobacillus sakei* and *Scutellaria baicalensis* improved the haematological, innate immune response and resistance against *Edwardsiella tarda* in barred knifejaw (*Oplegnathus fasciatus)*. Similarly, in the present study, the use of PM alone and in combination with sage in rainbow trout feeding improved disease resistance to *V. anguillarum.* Medicinal plants may have provided a protection against this pathogen due to the phenolic compounds that are known to restrict or inhibit the growth of bacteria (Dorman & Deans 2000). The antimicrobial activity of sage and myrtle was based on the 1,8-cineole component (Metin et al. 2020).

While nutritional components affect intestinal morphology, natural feed additives improve growth performance, feed efficiency and intestinal histology (Giannenas et al. 2012). Therefore, it can be supposed that the enhancement of goblet cell density could result in a higher defence mechanism against pathogens in rainbow trout. Diler & Görmez (2019) observed that the chemical components of *Artemisia absinthium* L. and *Artemisia campestris* L. are strongly associated with the efficiency of intestinal morphology in rainbow trout and the administration of *A. absinthium* L. to the diet of trout presented led to increase in higher goblet cells, villus length and width. Studies have shown that increasing the length and width of the villi increases the absorption in the intestines (Dimitroglou et al. 2010), and that the presence of goblet cells is an important indicator of intestinal health (Elsabagh et al. 2018). Similarly, the present study found increased villi length, villi width and goblet cell numbers of fish in all experimental groups compared to fish in the control group.

SOD and CAT are enzymes that play an important role in antioxidant defence mechanisms in biological systems (Livingstone 2001; Ritola et al. 2002; He et al. 2015). Safari et al. (2017) reported that dietary myrtle at 20 g kg^{-1} improved antioxidant (*SOD* and *CAT*) enzymes gene expression in zebrafish. In contrast, in the present study, the use of myrtle in rainbow trout feed did not affect SOD and CAT activities in muscles. This difference may be due to the different fish species and the doses of myrtle used in the studies. MDA, which is one of the end products of lipid peroxidation caused by free oxygen radicals, is commonly known as a marker of oxidative stress and antioxidant status. The increase in MDA levels is a crucial indicator of cell membrane injury (Yagi 1984). Our study was determined that the MDA activity in muscles of fish in the myrte+PM group was lower than fish in the other groups. The results are in line with the MDA activity of aqueous methanolic extract of pomegranate peel (*Punica granatum*) and veratrum (*Veratrum album*)

(Sönmez et al. 2022), sage and thyme oil in rainbow trout (Sönmez et al. 2015b) and mucuna (*Mucuna pruriens*) seed extract in *Botia rostrata* (Mukherjee et al. 2022). The authors argue that this may be associated with a bioactive substance in the medicinal plants and phytoconstituents exhibit radical scavenging activity.

5. Conclusion

The dietary administration of myrtle and sage + PM showed positive effects on FCR as a result of feeding. The highest value in terms of lactic acid bacteria count was obtained from the groups fed with sage, while highest value in terms of total bacteria count was obtained in groups fed with myrtle + PM, sage and PM. It was also determined that the use of both plants alone and in combination with a PM was effective on intestinal morphology. The dietary administration of myrtle, PM, myrtle + PM improved disease resistance against *V. anguillarum*, and no mortalities or diseases were observed in the fish fed with sage + PM. The combination of probiotics and plant products could be used as an alternative disease control strategy in rainbow trout culture.

Data availability: Data are available on request due to privacy or other restrictions.

Ethics Committee Approval: The welfare of the fish was performed according to the ethical standards of the national guidelines approved by the Isparta Applied Sciences University Animal Care and Use Committee (decision number: 001, date: 24.09.2020).

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Probiotic Fermentation and Organic Acid Profile in Milk Based Lactic Beverages Containing Potential Prebiotic Apple Constituents

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ABSTRACT

Probiotic milk-based matrices contain bioactive compounds required for the biochemical and physiological processes of metabolism as a result of fermentation. The present work aimed to evaluate the viability of probiotic bacteria in a lactic beverage fortified with probiotic milk/apple juice to understand the utilization of apple juice as a prebiotic source and investigate the organic acid profile. By monitoring the fermentation development and bacterial growth the results obtained indicated that the probiotic bacteria were viable over the predicted shelf life; the cell counts ranged from 7.48 to 12.00 $\log_{10}$ cfu mL^{-1}, conferring that the beneficial health effects on the host as probiotic bacteria must be at a minimum concentration of 6.0 $\log_{10}$ cfu mL^{-1} at the moment of consumption. *Lactobacillus casei* exhibited higher survival than the other lactic strains, presumably due to its higher ability to tolerate low acidity. During the fermentation and storage of milk based lactic beverages containing apple juice the formation of organic acids were determined as an indirect characteristic for growth of lactic acid bacteria. Lactic, acetic, malic, tartaric and citric acids were the primary organic acids. The quantities of propionic and butyric acids as short chain fatty acids were noted to increase during fermentation, being strain-specific. In conclusion, when probiotic milk is fortified with apple juice nutraceutical components, it can be a potential source of substrate and a synbiotic matrix for the growth of probiotic bacteria without any nutritional supplement.

Keywords: Lactic fermentation, Apple, Bioactive metabolite, Postbiotic

1. Introduction

The health-consciousness of consumers has resulted in a surge of specific foods or food components, so-called functional foods, which provide an additional physiological benefit beyond that of meeting basic nutritional needs. Functional foods and their bioactive components such as vitamins, minerals, phytonutrients, lipids, prebiotics and probiotics are being marketed to improve the quality of life and reduce the risk of disease - in both humans and animals (Cencic & Chinwaru 2010). The concern on the inclusion of probiotic strains in different food products has progressively grown over the years as many health benefits are ascribed to it, and therefore, the market share and production have increased (Das et al. 2012; Markowiak & Ślżewska 2017). Probiotics are defined as living microorganisms that exert positive influences to human health by improving the properties of intestinal microflora, when ingested in sufficient amounts (Villena & Kitazawa 2017). International standards for probiotic bacteria in food products state that a minimum dose of 6.0-7.0 log colony-forming units (cfu) of viable bacteria should be taken per g/mL product at the time of consumption to provide health benefits on the host (Ranadheera et al. 2017). Probiotics may play a beneficial role on lactose intolerance, cancer, allergies,

hepatitis, *Helicobacter pylori* infections, urinary tract infections, hyperlipidaemia, the assimilation of cholesterol, and antibiotic-associated gastrointestinal problems. Moreover, probiotics may reduce the intestinal pH, enhance the synthesis of vitamin K, folic acid, B group vitamins, short chain fatty acids and other postbiotics, and improve the absorption of certain minerals (i.e. Ca, Zn, Fe, Mn, Cu and P) (Homayouni-Rad et al. 2012). These assigned beneficial effects could be the result of the symbiotic relation between the host and gut microbiota. The intestinal microbiota contribute to the regulation of the gut health, and the enhancement of resistance against infections and differentiation of host immune system by lowering the pH through production of organic acids, such as lactate and short chain fatty acids, due to the break-down of complex carbohydrates or elaboration of antibiotic-like substances (Marco & Tachon 2013; Ranadheera et al. 2017; Narli & Ozcan 2022; Omak & Yilmaz-Ersan 2022).

Milk and dairy products are valued as the most common and traditional way for probiotic delivery. Among them dairy or milk-based drinks, fortified with probiotics, prebiotics, fibers, polyphenols, etc., were the first commercialized probiotic foods and still remain at the forefront. Dairy foods are considered as advantageous to non-dairy carrier foods in tolerating harsh gastro-intestinal conditions, due to the buffering capacity of milk and milk fat, which might protect probiotics in such stress conditions by reducing their direct exposure (Marco & Tachon 2013; Ranadheera et al. 2017; Ozdemir & Ozcan 2020).

Fruit juices have been suggested as non-dairy carrier foods for probiotic bacteria since they are rich in essential nutrients (minerals, vitamins, dietary fibers, phenolic compounds and phytochemicals) and sugars (Yoon et al. 2004; Mousavi et al. 2011). However, since for any functional probiotic food the major success and preferability criteria are to retain the viability and sensory characteristics, the survival of probiotics in fruit-based matrices to the end of shelf-life is more complex and challenging than those found in dairy products, where bacteria need more protection due to the low pH and stability (Perricone et al. 2015; Thakur & Josh 2017; Barat & Ozcan 2018).

The inclusion of prebiotics, non-digestible fibers that are resistant to digestion in small intestine and can selectively be fermented by the gut microflora, in dairy formulations was reported to stimulate the growth and activity of one or a limited number of probiotic *Lactobacillus* and *Bifidobacterium* spp., in the colon, thereby improving host health via modulating fat metabolism, obesity, and preventing constipation (Das et al. 2012; Shah et al. 2020).

Apples contain a balanced and high amount of soluble and insoluble dietary fibers. Among them, the water-soluble pectin is a polysaccharide which is not digested by enzymes in the human digestive system and provides colonization of probiotic bacteria as a prebiotic source in the large intestine (Kowalczyk et al. 2021; Zahid et al. 2021). While apples contain approximately 2-3 grams of dietary soluble fiber per 100 g, of which 50% is pectin, they are also an excellent source of phytochemicals. The fiber found in apples combined with other apple nutrients could be fermented into short-chain fatty acids (SCFAs) that help to enhance the growth of beneficiary gut bacteria (Chung et al. 2017; Kowalczyk et al. 2021).

Since the proposed uptake of probiotics is from natural sources, the hypothesis of combining the nutritional benefits of apples with the health benefits of the probiotics in a probiotic milk drink formula is a challenge. Pereira et al. (2013), Dimitrovski et al. (2015), and Zandi et al. (2016) have all investigated the viability of probiotic bacteria in fermented juices containing apples. Paredes et al. (2022), evaluated a fruit-vegetable mix including apple as a potential substrate for probiotic bacteria and stated that fermentation changed with nutraceutical properties.

Despite the large number of studies on the fermentation of fruits and vegetables as probiotic juices, few have focused on probiotic dairy products fortified with fruit juices (Barat & Ozcan 2018; Paredes et al. 2022). In this study, it is aimed to investigate the effects of apple juice pectic polysaccharides on prebiotic potential, organic acid fermentation, growth and viability of probiotic bacteria in milk matrix.

2. Material and Methods

2.1. Fruit juice preparation

Following preliminary trials, in order to create the desired sensory properties of apple pulp, a juice formulation consisting different kinds of apples was designed. The apples (Golden Delicious 60 kg, Granny Smith 20 kg and Star Crimson Delicious 20 kg apple varieties, 3:1:1, kg:kg) were hand-picked, washed (under a heavy spray application of water and rotary brush), crushed into pulp by a food processor, enzymatically mashed, and then cold-pressed. The raw juice was subjected to enzymatic clarification (Pectinex-30 mL 100 mL^{-1} at 50 $^{\circ}C$ for 2 h) which was followed by gelatine (1 g 100 mL^{-1} for 2 h) and bentonite (10 g 100 mL^{-1} for 2 h) treatments. The

clear apple juice was then filtered, glass-bottled (600 mL), pasteurized (20 min at 60 ˚C), and stored at 4±1 ˚C before use for further production.

2.2. Probiotic culture preparation

Freeze-dried cultures of *Lactobacillus casei* (Lc-11)*, Bifidobacterium animalis* subsp. *lactis* (Bl-04)*, Lactobacillus acidophilus* (La-14) and *Lactobacillus rhamnosus* (Lr-32)*,* were supplied from Danisco (Niebull, Germany), propagated as suggested by Barat and Ozcan (2018) and maintained at 4±1 ˚C until used.

2.3. Production of lactic beverage

Reconstituted skim milks 10.70% (w/w *dry matter*) were heat-treated at 90 ˚C for 10 min, cooled down to 37 ˚C and inoculated with each probiotic bacteria (*Lb. casei, B. lactis, Lb. acidophilus* and *Lb. rhamnosus*) with initial counts of 9.60, 9.15, 9.00 and 9.48 $\log_{10}$ cfu mL^{-1}, respectively. The incubation was carried out at 37 ˚C until the final pH was 4.7. The probiotic milk was stored in the refrigerator (4±1 ˚C) for 12 hours after fermentation. The pasteurized apple juice, of 12.2 ˚Brix with a titratable acidity of 0.51% as malic acid, was mixed with the milks at a ratio of 1:1 (v/v) for the production of probiotic lactic beverages. The beverages, denoted as LBC (with *Lb. casei)*, Lactic beverage with *B. lactis* (LBL), Lactic beverage with *Lb. acidophilus* (LBA) and Lactic beverage with *Lb. rhamnosus* (LBR), were stored at 4±1 ˚C for 28 days. An analysis was performed every 7th day of storage.

2.4. Enumeration of probiotic bacteria

The probiotic strains were enumerated on de Man, Rogosa and Sharpe Agar (MRS) (Merck, Darmstadt, Germany). *Lb. rhamnosus* and *Lb. casei* were counted on MRS-vancomycin agar, MRS-Bile was used for *Lb. acidophilus,* and for *B. lactis*, MRS-LP agar supplemented with lithium chloride, sodium propionate and cysteine was used. The plates were incubated for 72 h at 37 °C under anaerobic conditions (Tharmaraj & Shah 2003).

2.5. Analytical methods

The pH of the lactic beverages was recorded using a digital pH meter (Hanna HI 2211-02, RI/USA). Titratable acidity was expressed in grams of lactic acid using the method described by Oladipo et al. (2014). The color of the samples was measured using a Minolta Spectrophotometer CM-3600d (Osaka, Japan). Whey separation was expressed as the volume of drained whey (mL) per 100 mL sample (Delikanli & Ozcan 2014). The organic acids such as lactic, citric, acetic, propionic, formic and butyric acids were determined as described by Akalin et al. (2002) using a Jasco High-Performance Liquid Chromatography (Dionex ICS 3,000, LC-900 Series, Dionex Corp., Sunnyvale, CA) equipped with a model H-980-01 holder that accepts Rheodyne valves, an 7124 injector fitted with a 20 µL sample loop, a Jasco PU-980 solvent delivery system, and an ICS series UV-visible/variable wavelength detector (Dionex VWD).

2.6. Statistical analysis

The Analysis of Variance and the Tukey test ($p<0.01$) were used to determine significant differences. All of the analyses were performed using the STATISTICA Software package for Windows 8.0 (StatSoft Inc., Tulsa, OK, USA).

3. Results and Discussion

The viable counts of four probiotic strains in the lactic beverage with apple juice during 28 days of storage at 4±1 ˚C were presented in Figure 1. The changes of viable cell counts of all the strains during cold storage were significant ($p<0.01$). However, the attained viable cell number reduction was less than 1 $\log_{10}$ cfu mL^{-1}. It was observed that in the probiotic lactic beverage containing apple juice, all bacteria used were viable over the predicted shelf life, and the cell counts ranged from 7.48 to 12.00 $\log_{10}$ cfu mL^{-1}, respectively.

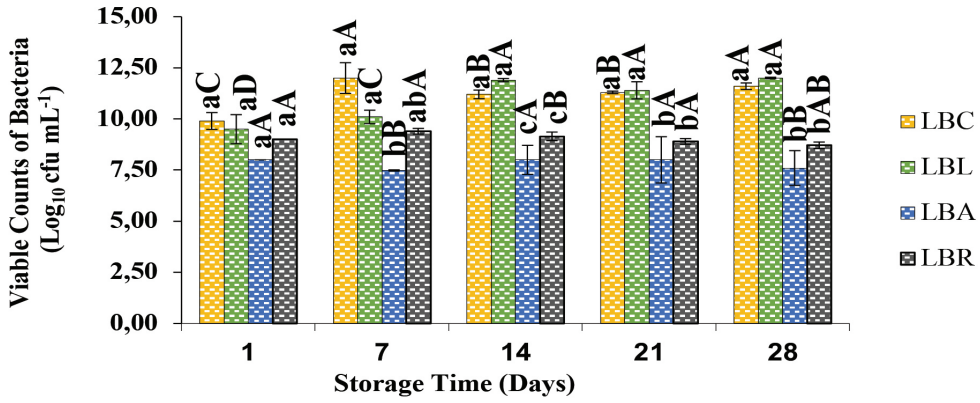

Figure 1- Viable counts of probiotic bacteria in lactic beverages
LBC: lactic beverage with *Lb. casei*, LBL: lactic beverage with *B. lactis*, LBA: lactic beverage with *Lb. acidophilus*, LBR: lactic beverage with *Lb. rhamnosus*. Different superscript (a-c) letter represent significant differences ($p<0.01$) between lactic beverage samples; Different superscript (A-C) letter represent significant differences ($p<0.01$) between different times of storage

The probiotic LAB used in this study had cell counts over 7.0 $\log_{10}$ cfu mL^{-1} at the end of the storage period which was higher than the minimum requirements by the Food and Agriculture Organization/World Health Organization to confer probiotic activity. This finding may highlight the probiotic value of the product retained and such a product could be a potential vehicle for probiotic delivery (Figure 1). Many factors may affect the viability and performance of probiotic bacteria in a complex food matrix, including the physicochemical properties of a food (carbohydrate, fat, solid non-fat and protein content, type of proteins, pH, acidity, etc.); rate and proportion of inoculation; food additives (bioactive compounds, sweeteners, stabilizers, etc.) which probiotic bacteria are exposed; the presence of organic acids, bacteriocins, SCFAs, hydrogen peroxide and other secondary metabolites produced by starter cultures; the strain used; rate and proportion of inoculation; applied temperature; fermentation and storage time; redox potential; final acidity of the product; molecular oxygen content and oxygen permeability through the packaging materials (Bazrafshan & Homayouni 2010; Kerry et al. 2018).

In the present study, *Lb. casei* and *B. lactis* respectively had greater viable cell counts than *Lb. acidophilus* and *Lb. rhamnosus*, possibly due to their higher acid tolerance and efficient utilization of essential nutrients in the apple juice such as dietary fibers, phenolics, and organic acids. These results align with those of Costa et al. (2013), Pereira et al. (2013) and Zandi et al. (2016) who studied the viability of *Lb. casei* in fermented fruit juices.

Perricone et al. (2015) suggested that the survival of probiotic species was the result of the synergistic and antagonistic action of many factors. They stated that pH and phenolic compounds exert a detrimental effect on viability, whereas protein and dietary fiber could protect cells from acidic stress.

Probiotic bacteria may utilize the carbohydrates present and produce organic acids resulting in lower pH of the product during storage. The optimum growth of *Bifidobacterium* occurs at pH 6.0-7.0; below pH 4.1 most probiotic species lose their viability within a week even at 4 ˚C, and below pH 2.5 the growth and survival of most species is limited within 3 h (Ding & Shah 2008). In general, the acid-tolerance of the *Bifidobacterium* species is strain-dependent, and hence, it can be considered that *Bifidobacteria* are highly sensitive to an acidic environment, with the exception of *Bifidobacterium animalis*, which can survive at acidic pH better than the other species (Sanchez et al. 2007). Improving the viability of probiotic bacteria in fermented dairy and non-dairy products until the time of consumption has been the concern of several studies. pH and titratable acidity are the most important factors that restrict the growth and survival of probiotics, and thus, their health benefits. Hydrogen ions may damage probiotic cells by changing the intracellular pH, disrupting mass transfer through the cell membranes and increasing the concentration of non-dissociated molecular organic acids. This results in enhanced bactericidal effect, which is pH dependent (Mortazavian & Sohrabvandi 2006).

It was observed that the pH in all beverages reduced whereas titratable acidity increased during 28 days of storage ($p<0.01$). Low pH and high acidity in the probiotic beverages containing *Lb. casei* and *Lb. rhamnosus* were associated with their ability to respond quickly to stress, high survival rate and stability (Table 1).

Ding & Shah (2008) reported that fruit juices may be an alternative vehicle for the incorporation of probiotics due to being rich in essential nutrients that have attractive organoleptic properties for consumer acceptance and preference. Fruit juices contain high amounts of carbohydrates that could encourage probiotic growth. They are often supplemented with oxygen scavenging ingredients such as ascorbic acid, thus promoting anaerobic conditions. *Lactobacilli* are generally resistant to low pH and survive in juices with pH ranging from 3.7 to 4.3; *Bifidobacteria,* however, are less acid tolerant, and a pH of about 4.6 is detrimental for their survival (Gueimonde et al. 2004; Kun et al. 2008).

Several strains of *Lb. plantarum, Lb. acidophilus* and *Lb. casei* can grow in fruit matrices due to their tolerance to acidic environments. However, the survival and storage behavior of probiotics in the fermented fruit-based matrix is more complex than in dairy products because the bacteria need more protection from the acidic conditions and other ingredients in the matrix (Ding & Shah 2008; Tamang et al. 2016). Saarela et al. (2016), reported that in apple juice the better survival and protection of *Lb. rhamnosus* were achieved in the presence of oat flour with 20% of β-glucan.

In the present study, whey separation was higher in LBC samples due to the high titratable acidity regarding storage time (Table 1). The color (L, a, b) values of the lactic beverages were found to be probiotic strain-dependent (Table 1) ($p<0.01$). LBR samples had higher L values than the other lactic beverages, since they were lighter in appearance due to the change in gelling properties. The redness (a) and yellowness intensity values (b) of lactic dairy beverages containing apple juice were higher in LBL samples at the end of storage, indicating that fermentation had a positive effect on the product color.

Various organic acids are known to be found in foods, like milk and fruit juices, including lactic, citric, orotic, benzoic, sorbic and others, which play a key role on sensorial characteristics for consumer acceptability, prevention of microbial growth, increasing the stability and quality of the product, and, especially, extending the predicted shelf life (Mato et al. 2005).

The preservation effect of lactic acid bacteria (LAB) in fermented foods is a result of the utilization of available carbohydrates and the formation of organic acids that in turn exhibit antimicrobial activity. In many studies, glucose has been introduced as the most important carbohydrate source for lactic probiotic species to enhance their growth and adoptability (Lankaputhra et al. 1996). Depending on the microorganisms involved the fermentation proceeds via the glycolysis pathway for homofermentative LAB with the almost exclusive formation of lactic acid, and via the pentose phosphate pathway for heterofermentative LAB with formation of lactic, acetic and other acids (Ozcan et al. 2021).

During the fermentation and storage of the lactic beverage containing apple juice, organic acids were formed according to the fermentation mechanisms of LAB. This biosynthetic mechanism was effective on aromatic preference. Lactic, acetic, malic, tartaric and citric acids were the major organic acids in the probiotic milk and lactic beverage samples. The amount of propionic and butyric acids, as SCFAs, had increased during fermentation in the lactic beverages depending on the activity of probiotic bacteria (Table 2, Figure 2a,b).

Lactic acid is known to be formed through the reduction of pyruvic acid, transformation of malic acid and/or lactose degradation. Lactic acid in apple juice was 1.09±0.172 mg g^{-1} and between 6.01±0.21 mg g^{-1} and in probiotic milk 11.83±0.25 mg g^{-1}, however its quantity was reduced in the lactic beverage consisting apple juice during storage with the exception of *Lb. rhamnosus*, which may account for the malolactic fermentation or favored reactions, which can potentially limit the prebiotic activity of the substrate. The differences between the lactic acid productions of the strains used may be the result of the different efficiency of the lactose-hydrolysing/galactosidase enzyme activity.

The acetic acid formation by the *Lactobacillus* strains may be the result of the biochemical pathway differentiation for carbohydrate utilisation, citrate metabolism and/or may originate from the heterofermentative pathway (Zalán et al. 2010). The acetate is an important parameter for the flavour development of many cultured milk products, and is linked to the citrate metabolism, since citric acid is stated to be metabolized into acetic acid (Torino et al. 2005).

Mousavi et al. (2011), reported that selected probiotic bacteria (namely *Lb. acidophilus*, *Lb. paracasei, Lb. plantarum* and *Lb. bulgaricus*) were capable of metabolizing citric acid when fermentation starts, while sugar consumption by all the strains was relatively low at this stage.

Table 1- Physicochemical properties of milk based lactic beverages produced with apple juice

Lactic beverage		*pH*	*Titratable acidity (g 100 g^{-1})*	*Whey separation (mL)*	*L*	*Color values a*	*b*
LBC	1st day	4.30±0.010^{bcB}	0.90±0.023^{aC}	0.00±0.000^{bD}	82.61±0.115^{cB}	0.88±0.050^{bB}	21.56±0.130^{aA}
	7th day	4.13±0.010^{dE}	1.07±0.009^{aB}	11.50±0.700^{aC}	83.27±0.075^{bA}	1.03±0.020^{bA}	20.73±0.050^{aB}
	14th day	4.22±0.010^{cC}	1.18±0.010^{aA}	20.50±0.707^{aB}	81.29±0.076^{cD}	0.64±0.010^{cC}	19.58±0.051^{bD}
	21th day	4.37±0.010^{cA}	1.09±0.009^{aB}	20.00±0.000^{abB}	81.84±0.140^{cC}	0.70±0.036^{cC}	19.86±0.115^{bC}
	28th day	4.15±0.010^{cD}	1.17±0.005^{aA}	29.00±0.000^{aA}	82.56±0.155^{aB}	0.83±0.029^{bB}	19.70±0.083^{bCD}
LBL	1st day	4.32±0.000^{bE}	0.85±0.010^{bC}	0.00±0.000^{bB}	81.67±0.352^{dB}	1.38±0.101^{aAB}	20.57±0.338^{bBC}
	7th day	4.45±0.010^{aD}	0.93±0.010^{bA}	5.75±3.889^{cB}	81.61±0.191^{cB}	1.26±0.123^{aD}	20.30±0.477^{aC}
	14th day	4.64±0.010^{aB}	0.93±0.010^{bAB}	19.00±4.242^{aA}	81.78±0.251^{bC}	1.38±0.057^{aD}	20.07±0.222^{aB}
	21th day	4.55±0.010^{bC}	0.90±0.010^{bB}	23.50±4.949^{aA}	82.76±0.075^{bA}	1.49±0.026^{aD}	21.87±0.085^{aA}
	28th day	4.66±0.010^{aA}	0.92±0.010^{cAB}	27.00±2.828^{aA}	81.64±0.272^{bB}	1.25±0.030^{aD}	20.79±0.120^{aBC}
LBA	1st day	4.56±0.020^{aA}	0.73±0.000^{cAB}	0.75±0.354^{abB}	85.17±0.026^{aA}	0.83±0.011^{bD}	19.31±0.487^{cA}
	7th day	4.39±0.010^{bC}	0.71±0.000^{cB}	6.50±4.949^{cAB}	83.64±0.103^{aB}	0.97±0.020^{bD}	19.08±0.020^{bA}
	14th day	4.46±0.030^{bB}	0.74±0.003^{cAB}	12.00±5.656^{bAB}	82.70±0.961^{bB}	0.84±0.086^{bD}	18.53±0.327^{cB}
	21th day	4.58±0.010^{aA}	0.73±0.010^{cAB}	15.50±6.363^{bA}	83.35±0.090^{bB}	1.04±0.030^{bD}	18.92±0.090^{cAB}
	28th day	4.41±0.010^{bBC}	0.75±0.010^{dA}	18.50±4.949^{bA}	82.93±0.244^{aB}	0.91±0.040^{bD}	18.87±0.157^{CAB}
LBR	1st day	4.29±0.000^{cA}	0.87±0.000^{bB}	1.50±0.707^{aE}	84.54±0.208^{bAB}	0.50±0.011^{cD}	19.09±0.037^{cB}
	7th day	4.24±0.035^{cAB}	0.92±0.010^{bB}	8.00±0.000^{bD}	83.94±0.209^{aC}	0.23±0.005^{cD}	19.12±0.056^{bB}
	14th day	4.24±0.010^{cB}	0.97±0.030^{bB}	14.50±0.500^{bC}	84.23±0.066^{aBC}	0.25±0.015^{dD}	19.40±0.073^{bB}
	21th day	4.22±0.005^{dB}	1.15±0.090^{aA}	17.00±0.000^{bB}	84.95±0.457^{aA}	0.29±0.090^{dD}	19.90±0.337^{bA}
	28th day	3.99±0.020^{dC}	1.14±0.010^{bA}	19.00±0.00^{bA}	82.73±0.198^{aD}	0.05±0.032^{cD}	19.33±0.172^{aB}

LBC: Lactic beverage with *Lb. casei*, LBL: Lactic beverage with *B. lactis*, LBA: Lactic beverage with *Lb. acidophilus*, LBR: Lactic beverage with *Lb. rhamnosus*. Different superscript (a-c) letter represent significant differences ($p<0.01$) between lactic beverage samples; different superscript (A-C) letter represent significant differences ($p<0.01$) between different times of storage

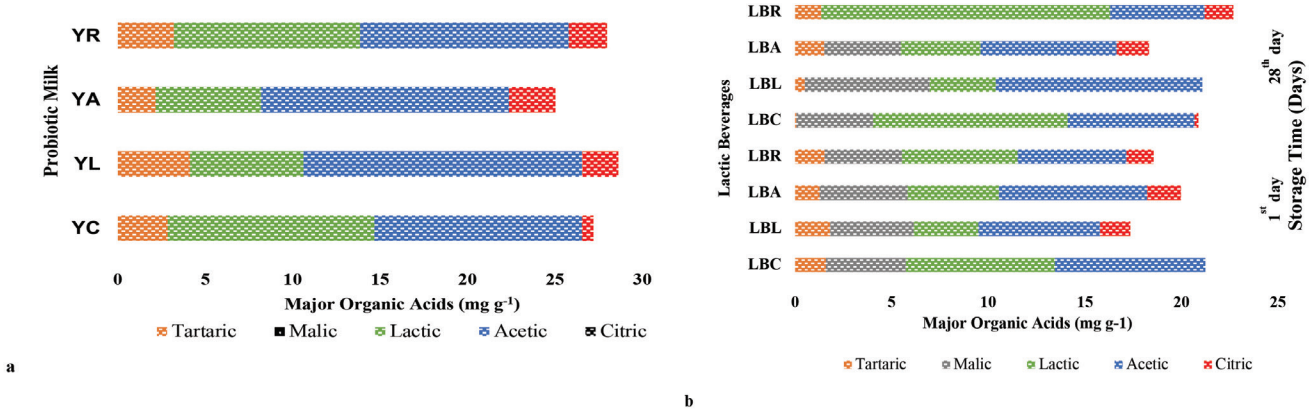

Figure 2- Major organic acids of lactic beverages (a) and (b) probiotic milk samples
LBC: Lactic beverage with *Lb. casei,* LBL: Lactic beverage with *B. lactis,* LBA: Lactic beverage with *Lb. acidophilus,* LBR: Lactic beverage with *Lb. rhamnosus.* YC: Probiotic milk with *Lb. casei,* YL: Probiotic milk with *B. lactis,* YA: Probiotic milk with *Lb. acidophilus,* YR: Yogurt with *Lb. rhamnosus*

Table 2- Organic acid profile of milk based lactic beverages produced with apple juice (mg g^{-1})

		Oxalic	*Tartaric*	*Malic*	*Lactic*	*Acetic*	*Citric*	*Fumaric*	*Propionic*	*Butyric*
Apple juice		0.029±0.010	1.367±0.174	7.076±0.242	1.091±0.172	0.310±0.093	0.114±0.015	0.001±0.000	0.329±0.075	0.048±0.009
Probiotic milk										
YC		0.057±0.003	2.847±0.024	0.000±0.000	11.827±0.247	11.863±0.763	0.672±0.037	0.001±0.000	0.047±0.014	0.016±0.004
YL		0.187±0.244	4.135±0.290	0.000±0.000	6.477±0.701	15.941±3.075	2.080±0.175	0.001±0.000	0.169±0.019	0.027±0.012
YA		0.058±0.007	2.177±0.104	0.000±0.000	6.005±0.208	14.194±0.788	2.656±0.240	0.001±0.000	0.088±0.016	0.197±0.060
YR		0.080±0.003	3.213±0.085	0.000±0.000	10.646±0.184	11.928±0.263	2.201±0.093	0.001±0.000	0.587±0.017	0.016±0.001
Lactic beverage										
LBC	1st day	0.103±0.009	1.596±0.165	4.148±0.173	7.716±0.290	7.780±0.247	0.000±0.000	0.001±0.000	0.038±0.009	0.252±0.032
	28th day	0.091±0.006	0.054±0.039	4.000±1.308	10.069±0.279	6.559±0.323	0.202±0.064	0.018±0.001	0.591±0.093	0.529±0.093
LBL	1st day	0.090±0.004	1.807±0.719	4.327±0.199	3.362±0.190	6.297±0.935	1.559±0.233	0.000±0.000	0.113±0.007	0.177±0.083
	28th day	0.092±0.004	0.500±0.106	6.473±0.294	3.411±0.241	10.697±0.384	0.000±0.000	0.005±0.001	0.173±0.014	0.042±0.002
LBA	1st day	0.101±0.012	1.280±0.097	4.551±0.210	4.721±0.087	7.680±0.310	1.749±0.244	0.000±0.000	0.118±0.008	0.120±0.017
	28th day	0.095±0.002	1.516±0.075	3.959±0.536	4.129±0.442	7.052±0.736	1.678±0.131	0.001±0.000	0.172±0.006	0.148±0.005
LBR	1st day	0.102±0.004	1.542±0.065	3.995±0.150	5.983±0.112	5.644±0.427	1.409±0.089	0.000±0.000	0.054±0.012	0.336±0.053
	28th day	0.109±0.010	1.359±0.150	0.000±0.000	14.956±0.202	4.885±0.019	1.498±0.037	0.000±0.000	0.741±0.212	0.820±0.002

LBC: Lactic beverage with *Lb. casei*, LBL: Lactic beverage with *B. lactis*, LBA: Lactic beverage with *Lb. acidophilus*, LBR: Lactic beverage with *Lb. rhamnosus*. Different superscript (a-c) letter represent significant *differences* ($p<0.01$) between lactic beverage samples; YC: probiotic milk with *Lb. casei*, YL: probiotic milk with *B. lactis*, YA: probiotic milk with *Lb. acidophilus*, YR: yogurt with *Lb. rhamnosus*. Different superscript (A-C) *letter* represent *significant differences* ($p<0.01$) between different times of storage

In the LBL sample, the acetic acid formation increased during storage, indicating that the amount and proportion of acids produced was highly dependent on the substrate metabolized (Table 2). Biedrzycka et al. (2003) and Ozcan & Eroglu (2023) reported that the generation of acetic acid by *Bifidobacteria* is much more stable than that of lactic acid, though the concentration of the latter may be higher, especially when the substrate is easily- and well-metabolized. According to the findings of the present work, the initial citrate concentrations in samples were higher than in the lactic beverage consisting apple juice at the end of the storage for each probiotic strain used; however, no relation was noted between the citrate utilization and the acetate production.

Fuleki et al. (1995), reported that malic acid, being responsible for the sour taste, was the most abundant acid in authentic apple juice. In the present study, malic acid was not detected in fermented probiotic milk samples (YC, YL, YA and YR). Its level decreased in LBC, LBA and LBR at the end of storage, whilst, for LBL beverages the malic acid content increased (Table 2). The reduction in malic acid content throughout storage for *Lactobacillus* spp. may account for the degradation of malic acid as a carbon source for bacterial growth and secondary bacterial fermentation (malolactic fermentation) (Zhang et al. 2008). Most LAB shape the decarboxylation of L-malate to L-lactate and CO_2 by a NAD^+ and Mn_2^+-dependent malolactic enzyme (MLE), although a few can convert L-malate into pyruvate by the action of a malic enzyme (ME). Biochemical evidence has shown that *Lb. casei* strains possess both ME and MLE activities. Even though the ME pathway enables *Lb. casei* to grow on L-malate, MLE does not support the proliferation (Landete et al. 2013). For *Lb. rhamnosus,* the significant decrease in malic acid occurred the more lactic acid formed, pointing to the presence of MLE. For *B. lactis* the conversion of citric acid into malic acid/acetic acid was observed.

It was found that bacterial growth in the lactic beverages containing apple juice increased due to the high sugar content of the apple juice and formed and/or metabolized organic acids, such observed in *Lb. acidophilus* and *Lb. casei* which used lactic acid as the major carbon source for growth. Moraru et al. (2007) showed that bacterial growth in vegetables juice with various concentrations led to a pH reduction and an increase in lactic acid, which was attributed to the sugar consumption and production of organic acid by the lactic acid cultures. Yoon et al. (2004) stated that *Lb. acidophilus, Lb. plantarum, Lb. casei* and *Lb. delbrueckii* subsp. *bulgaricus* were capable of rapidly utilizing tomato juice for cell synthesis and lactic acid production without nutrient supplementation and pH adjustment, even though the initial pH value was 4.1; however, the accumulation of lactic acid, diacetyl, and acetaldehyde as a result of growth and fermentation could reduce their viability.

4. Conclusion

Fermented milk has long been used as the main vehicles for probiotics containing essential nutrients and recently fruit juices have been exploited as suitable carriers for probiotics. Consequently, a milk-based lactic beverage containing apple juice could be considered as a novel probiotic beverage without the supplementation of extra nutrients.

Although many fruit juices have a low pH (pH <4.5) and may adversely affect probiotic growth, apple juice provides an ideal growth environment for probiotic microorganisms with the sugar, organic acids, phenolic compounds, dietary fiber, vitamins and other trace elements it contains. According to the results of the present work, *Lb. casei, B. lactis*, *Lb. acidophilus* and *Lb. rhamnosus* show some issues when being used as starter for production of milk fortified with apple juice, however, they showed high survival and potential prebiotic activity in probiotic milk + apple juice drink during cold storage at 4 ˚C of 4 weeks. The viable cell counts were higher than 10^7 cfu mL^{-1}, which was sufficient to confer beneficial health effects. Lactic, acetic, malic, tartaric and citric acids were highly detected, and the amount of propionic and butyric acids, especially SCFAs, increased during fermentation depending on the strain. One could say that the success of a new probiotic/lactic beverage depends particularly on the capability of the probiotic culture to provide satisfactory viable cells that beneficially modify the gut microbiota of the host. However, the challenge to meet consumer demands is related to development of milk + fruit juice fermented beverages with the introduction of probiotics through new methods, such as encapsulation, fortification with other ingredients, using non-conventional juices or non-conventional probiotics, to increase the bacterial survival. Furthermore, for designation of new generation milk beverages with potential prebiotic and postbiotic components further studies need to focus on *in vitro* and *in vivo* assays with metabolomics studies.

Data availability: Data are available on request due to privacy or other restrictions.

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