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CONTENTS **2022, 28(2)**

Research articles:

Journal of Agricultural Sciences (Tarim Bilimleri Dergisi) 2022, 28 (2) : 159 - 170 DOI: 10.15832/ankutbd.870923

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Effect of Aqueous Methanolic Extract of Pomegranate Peel (*Punica granatum*) and Veratrum (*Veratrum album*) on Oxidative Status, Immunity and Digestive Enzyme Activity in Rainbow Trout (*Oncorhynchus mykiss*)

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ABSTRACT

Aqueous methanolic extracts of pomegranate peel (*Punica granatum*) and veratrum (*Veratrum album*) were evaluated for their effects of supplementation in fish diets on oxidative status, digestive enzymes and immunity of rainbow trout fingerlings (*Oncorhynchus mykiss*). Three different concentrations of pomegranate 250 (P250), 500 (P500) and 1000 (P1000) mg kg^{-1} and veratrum 250 (V250), 500 (V500) and 1000 (V1000) mg kg^{-1} were added to the diet and the fish were fed these diets for 60 days over a control diet without any supplementation (P0 and V0, respectively). Immune responses and oxidative status of fish were evaluated every 20 days. Digestive enzyme activity and growth performances were determined at the end of study. We observed that the respiratory burst activity significantly increased ($P<0.05$) at the end of the study compared to control except in the V500 fish group. Lysozyme was increased on the 20th and 40th day of the study in all experimental groups compared to the control group ($P<0.05$). Myeloperoxidase activity was observed to increase on the 40th day in veratrum treated groups and significantly decreased ($P<0.05$) on the 60th day in all groups compared to the control. Haematological responses showed a declining trend in all the groups supplemented with veratrum extract. SOD activity increased in pomegranate groups while no differences were observed on catalase activity. G6PDH activity was observed to decline in the veratrum groups, however, GPx activity significantly increased ($P<0.05$) in V500 and V100 groups. Lipid peroxidation was determined at the lowest level on the 20th and 40th days of the study in P1000 and V250 fish groups. The study indicated a positive impact of pomegranate peel extract on the immunity of rainbow trout at moderate doses of supplementation.

Keywords: Immune response, Antioxidant activity, Digestive enzyme, Growth performance, Rainbow trout, *Punica granatum*, *Veratrum album*

1. Introduction

The aquaculture industry has progressed tremendously with the introduction of new technologies to the industry (Bilen et al. 2013). However, increasing stocking densities, other culture system intensification inevitably resulted in fish losses. Recently, several studies were conducted on fish health, including the importance of medicinal plants in increasing growth performance (Farahi et al. 2012; Heidarieh et al. 2013; Bahabadi et al. 2014; Sönmez et al. 2015a; Arslan et al. 2018; Cavdar et al. 2020), resistance to diseases (Nya & Austin 2009) and also protection against some important fish pathogens, such as *Lactococcus garvieae* (Bilen et al. 2019a) and *Aeromonas hydrophila* (Bilen & Elbeshti 2019) were evaluated after medicinal plant application. Also, increased antioxidant activity and improved health status were demonstrated (Sönmez et al. 2015b).

Veratrum album commonly known as the White Hellebore, is a characteristic plant of humid acid soils. It can form dense vegetations in a wide range of alpine habitats throughout Eurasia (Kleijn & Steinger 2002). Veratrum plants usually produce a single preformed shoot per year and they have a single rhizome (Kleijn & Steinger 2002). Veratrum contains a range of alkaloids (1.6-9%) (Rätsch 2016).

Pomegranate fruits are commonly consumed fresh and in processed forms as wine, jams, and juice. Therefore, the by-product of the pomegranate juice industry, pomegranate peel, is a cheap output. Pomegranate peel accounts for roughly 50% of the fruit weight and it is known to contain high molecular weight phenolics, appreciable quantities of microelements, flavonoids, complex polysaccharides, proanthocyanidins, and ellagitannins. These compounds were reported to display strong apoptotic, antimicrobial, antioxidant, and anti-mutagenic properties (Seeram et al. 2005; Öztürk et al. 2018). Thus, it has been often utilized as a natural antioxidant in a variety of dietary supplements.

With this background, these two plants were chosen as the materials of the study and it was aimed to examine the effects of pomegranate peel (*Punica granatum*) and veratrum (*Veratrum album*) on oxidative status, immune responses and digestive enzyme activity of rainbow trout (*Oncorhynchus mykiss*).

2. Material and Methods

2.1. The fish and medicinal plants

Experimental fish, (*Oncorhynchus mykiss* juveniles; initial weight: 6.22 g) were provided by Kastamonu University Inland and Marine Fish Production, Application and Research Centre, Turkey. Medicinal plants were procured from an herbal medicine shop at Kastamonu. Plants were shade-dried and aqueous methanolic extract was prepared following the protocol by Bilen et al. (2020). The extract was supplemented to the commercial trout feed by spraying and kept in -20 ºC until further use.

2.2. Experimental procedure

All experiments were performed in Fisheries Faculty, Kastamonu University. All treatment groups were kept in 300 L recirculated aquarium systems following a completely randomized design. During the study period, 20% of the water was exchanged every day and the parameters were maintained at 17 ºC temperature, 7.5 pH, 8.0 mg L^{-1} oxygen at ambient photoperiod. After 2 weeks of acclimation period fish were fed with plant extract supplemented diets. Fish were fed twice in a day for 60 days of study. At every 20 day of the study, blood and liver tissues were collected to determine immunological and oxidative status of the test fish.

2.3. Immune responses

Non-specific immune responses, such as respiratory burst activity (Siwicki et al. 1994), lysozyme activity (Ellis 1990), and myeloperoxidase activity (Sahoo et al. 2005) were investigated as per the mentioned methodologies.

2.4. Antioxidant activity

Superoxide dismutase, catalase, glutathione peroxidase, and glucose 6 phosphate dehydrogenase activities were determined by using commercial kits (SIGMA 19160-1KT-F SOD assay kit, Cayman 707002 Catalase assay kit, Cayman 703102 glutathione peroxidase assay kit, and SPI-BIO0112 G6PDH activity assay kit, respectively).

2.5. Digestive enzymes

Digestive enzyme activities in the stomach and intestine were assayed after termination of the feeding trial. The stomach and intestine were thoroughly homogenized in ice-cold distilled water ten times their weight and centrifuged at 15000 g for 45 min at 4 °C. The supernatant was used as a crude enzyme source. The α-amylase activity was analysed by the starch hydrolysis method of Jiang & Wang (2012) with some modifications. Lipase activity was assayed using the method described by German et al. (2004). Pepsin analysis was conducted according to the method reported by Worthington (1993). Enzyme trypsin analysis in the intestines was determined using N-a-benzoyl-DL-arginine 4-nitroanilide hydrochloride as a substrate (Faulk et al. 2007) with minor modifications. Specific activities of all digestive enzymes were calculated as milligram of protein. All enzyme activities were calculated according to the equations below:

$$Amylase = \frac{[(Sample - Blank)^2 \times 7.712] - [1.802 \times (Sample - Blank)] + 0.082}{mg\ protein}$$

$$Lipase = \frac{[(Abs\ 405\ Sample - Abs\ 405\ Blank) \times (0.2359 + 0.0153)^2]}{mg\ protein}$$

$$Pepsin = \frac{[(Abs\ 280\ Sample - Abs\ 280\ Blank) \times 1000]}{[10\ min \times mg\ protein]}$$

$$Trypsin = \frac{\left[\frac{(410\ abs)}{10\ min}\right] \times 1000 \times volume\ of\ reaction\ mixture}{8.800 \times mg\ protein}$$

2.6. Haematology

Haematological parameters were assayed using the BC3000 Plus haematology analyser. WBC count was determined using haemocytometer (Blaxhall & Daisley 1973).

2.7. Growth performance

At the end of the experiment, all fish were weighed individually on a digital scale (0.01 g) to evaluate the FCR, SGR, final weight, and weight gain as follows:
Weight gain (WG)=Wf−Wi
Food conversion ratio (FCR)=feed intake/weight gain
Specific growth rate (SGR)=100×[(lnWf−lnWi)/days]
Where, Wi is mean initial weight and Wf is mean final weight.

2.8. Statistical analyses

All data were expressed as mean ±SE (Standard error). Means were compared using one-way ANOVA. Homogeneity of variance was determined by using the Kolmogorov-Smirnov test and Levene's test. Comparisons between groups were performed by using Tukey's multiple range test with the significance level of 0.05. Statistical analyses were conducted with SPSS software version 24.0 (IBM, NY, USA).

3. Results

3.1. Immune responses

In this 60-day study, respiratory burst, lysozyme, and myeloperoxidase activities were estimated using the blood and serum. The results are summarized in Figure 1. The results showed a decreased level on the 20th day of the study in P500, P1000 and V1000 fish groups compared to control ($P<0.05$). No differences were observed in other groups' respiratory burst activity compared to the control ($P>0.05$). As the study progressed, respiratory burst activity in all experimental groups increased significantly except in P1000 and V1000 when compared to the control.

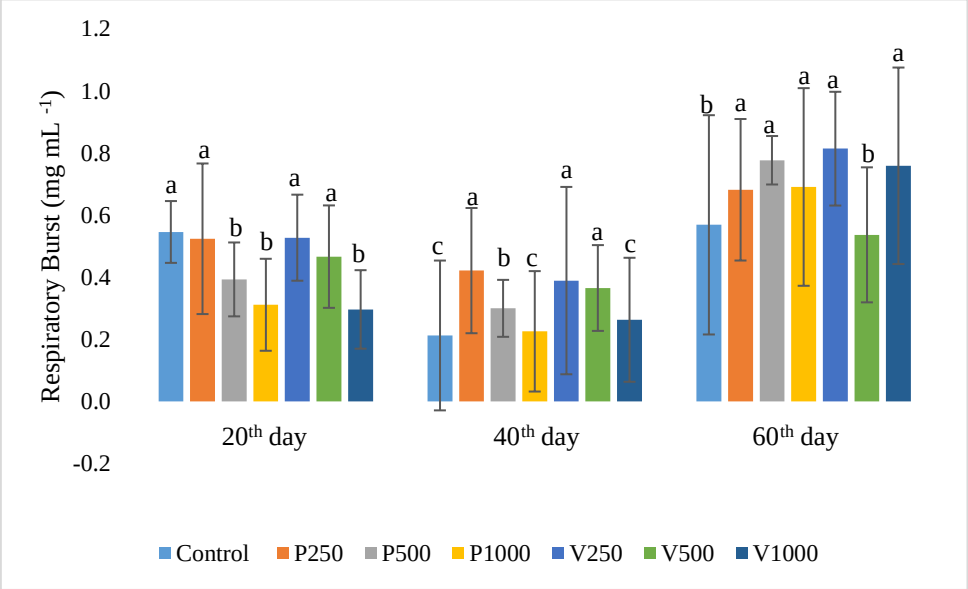

Figure 1- Respiratory burst activity in blood leucocytes of rainbow trout fed with methanolic extracts of pomegranate peel and veratrum. P250, P500, and P1000, extracts of pomegranate peel at 250, 500, and 1000 mg kg^{-1} diet, respectively; V250, V500, and V1000, extracts of veratrum at 250, 500, and 1000 mg kg^{-1} diet, respectively. Values are presented as mean ± SE. Different letters above columns express significant differences between groups ($P<0.05$)

Results on lysozyme activity are displayed in Figure 2. In all experimental groups, lysozyme activity increased significantly on the 20th and 40th days of the study compared to the control.

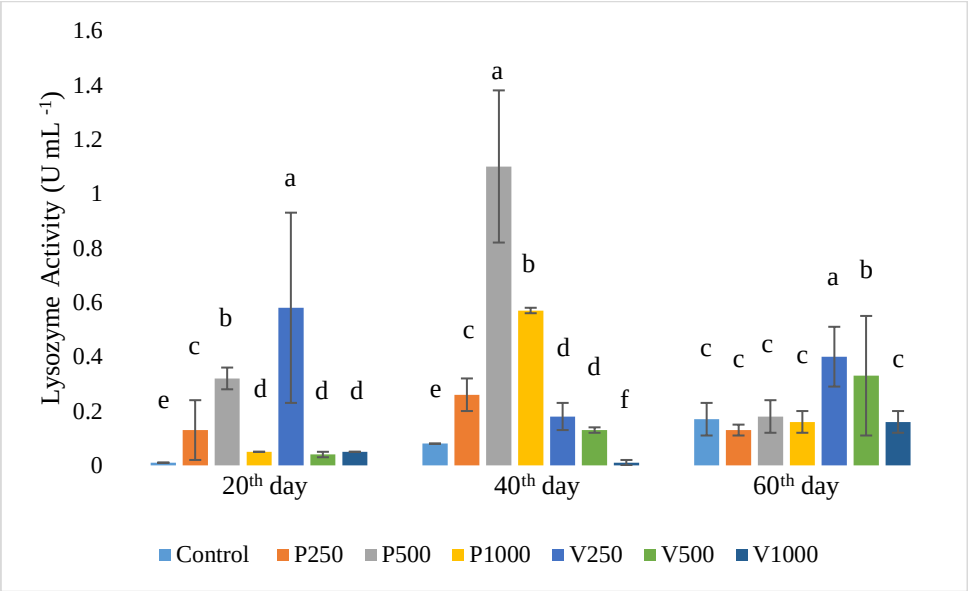

Figure 2- Lysozyme activity in blood leucocytes of rainbow trout fed with methanolic extracts of pomegranate peel and veratrum. P250, P500, and P1000, extracts of pomegranate peel at 250, 500, and 1000 mg kg^{-1} diet, respectively; V250, V500, and V1000, extracts of veratrum at 250, 500, and 1000 mg kg^{-1} diet, respectively. Values are presented as mean ± SE. Different letters above columns express significant differences between groups (P<0.05)

Myeloperoxidase activity showed varying trends in different experimental groups (Figure 3.). Although, the MPO activity in the treated fish groups increased at the beginning, it decreased by the end of the study and significantly higher MPO activity was observed in the control group.

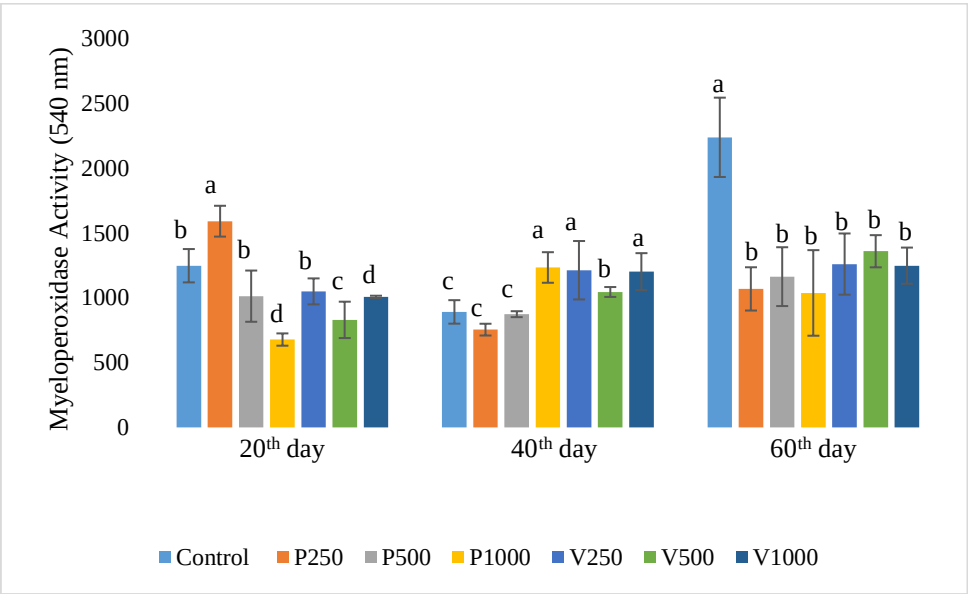

Figure 3- Myeloperoxidase activity in blood leucocytes of rainbow trout fed with methanolic extracts of pomegranate peel and veratrum. P250, P500, and P1000, extracts of pomegranate peel at 250, 500, and 1000 mg kg^{-1} diet, respectively; V250, V500, and V1000, extracts of veratrum at 250, 500, and 1000 mg kg^{-1} diet, respectively. Values are presented as mean ± SE. Different letters above columns express significant differences between groups (P<0.05)

3.2. Antioxidant activity

SOD activity was found to increase in the groups P250 and P500 compared to other treatments and the control group on the 20th day of the study (P<0.05) (Table 1). By the 40th day of the study, SOD decreased only in the P1000 fish group. In all other groups, SOD increased and significantly higher values were observed from the V1000 group compared to that of the control (P<0.05). Similar to that, V1000 group showed the highest SOD activity on the 60th day. Catalase activity had no differences at any sampling time in any treatment groups compared to the control (P>0.05) (Table 2).

Table 1- Superoxide dismutase (SOD) activities in rainbow trout fed with pomegranate peel (*Punica granatum*) and veratrum (*Veratrum album*) extracts supplemented feed

Groups	*20th Day*	*40th Day*	*60th Day*
Control	89.25±19.70[b]	65.63±17.41[b]	44.76±5.24[b]
P250	125.88±19.26[a]	102.56±7.53[a]	30.60±10.08[b]
P500	180.66±41.92[a]	95.82±18.21[a]	31.81±5.24[b]
P1000	9.59±5.79[c]	22.62±5.46[c]	41.83±13.21[b]
V250	64.94±5.29[b]	69.94±12.56[b]	92.72±0.77[a]
V500	99.38±17.59[b]	80.74±20.48[a]	21.9±8.65[b]
V1000	60±9.49[c]	128.02±10.90[a]	104.3±13.5[a]

All data are given as mean ± SE (n = 9); different letters in the same column denote statistically significant differences (P<0.05) between groups. P250, P500, and P1000, extracts of pomegranate peel at 250, 500, and 1000 mg kg^{-1} diet, respectively; V250, V500, and V1000, extracts of veratrum at 250, 500, and 1000 mg kg^{-1} diet, respectively. The SOD activity is expressed as U/mL.

Table 2- Catalase (CAT) activities in rainbow trout fed with pomegranate peel (*Punica granatum*) and veratrum (*Veratrum album*) extracts supplemented feed

Groups	*20th Day*	*40th Day*	*60th Day*
Control	0.70±0.53	0.73±0.29	0.69±0.18
P250	0.43±0.03	0.46±0.04	0.49±0.05
P500	0.46±0.08	0.50±0.00	0.48±0.03
P1000	0.49±0.01	0.66±0.17	0.58±0.09
V250	0.36±0.03	0.47±0.03	0.40±0.02
V500	0.45±0.10	0.61±0.05	0.59±0.04
V1000	0.52±0.00	0.44±0.03	0.50±0.01

All data are given as mean ± SE (n = 9); different letters in the same column denote statistically significant differences (P<0.05) between groups. P250, P500, and P1000, extracts of pomegranate peel at 250, 500, and 1000 mg kg^{-1} diet, respectively; V250, V500, and V1000, extracts of veratrum at 250, 500, and 1000 mg kg^{-1} diet, respectively. The CAT activity is expressed as nmol/min/mL.

Glucose-6-phosphatase dehydrogenase (G6PDH) activities were significantly elevated (P<0.05) in P1000, P250, and V500 fish groups compared to that in the control. In other groups, G6PDH was significantly decreased after the 20th day of the study (P<0.05). On the 40th day of the study, G6PDH activity significantly decreased in all treatment groups except P1000 compared to the control (P<0.05). On the 60th day of the study, G6PDH values of P250 and V1000 were almost similar to the control (P>0.05) (Table 3).

Table 3- Glucose-6-phosphatase dehydrogenase (G6PDH) activities in rainbow trout fed with pomegranate peel (*Punica granatum*) and veratrum (*Veratrum album*) extracts supplemented feed

Groups	*20th Day*	*40th Day*	*60th Day*
Control	0.67±0.01 [d]	0.90±0.03[a]	0.85±0.02[a]
P250	0.87±0.01 [b]	0.75±0.01[b]	0.92±0.01[a]
P500	0.57±0.01 [e]	0.72±0.01 [c]	0.64±0.01[c]
P1000	1.05±0.01 [a]	0.82±0.02 [a]	0.68±0.00[b]
V250	0.72±0 [d]	0.69±0.02 [c]	0.73±0.02[b]
V500	0.79±0.01 [c]	0.74±0 [b]	0.62±0.01[c]
V1000	0.67±0 [d]	0.67±0 [c]	0.84±0[a]

All data are given as mean ± SE (n = 9); different letters in the same column denote statistically significant differences (P<0.05) between groups. P250, P500, and P1000, extracts of pomegranate peel at 250, 500, and 1000 mg kg^{-1} diet, respectively; V250, V500, and V1000, extracts of veratrum at 250, 500, and 1000 mg kg^{-1} diet, respectively. The G6PDH activity is expressed as nmol/min/mL.

Glutathione peroxidase (GPx) activities in rainbow trout fed with different doses of experimental plants are shown in Table 4. The highest GPx activity was determined in V500 and V1000 compared to the control. The decrease in GPx activity was determined on the 40th day of study in P250 and V1000 groups compared to the control (P<0.05). No significant differences were observed in treatment groups compared to the control on the 60th day (P>0.05).

Table 4- Glutathione peroxidase (GPx) activities in rainbow trout fed with pomegranate peel (*Punica granatum*) and veratrum (*Veratrum album*) extracts supplemented feed

Groups	*20th Day*	*40th Day*	*60th Day*
Control	103.02±5.14[b]	105.73±10.52[a]	90.01±0.91
P250	57.62±7.16[c]	36.16±9.41[c]	97.02±0.41
P500	47.98±9.85[c]	118.15±19.13[a]	89.02±0.72
P1000	4.18±0.57[d]	94.97±5.26[a]	91.01±0.30
V250	51.39±13.50[c]	90.53±11.50[a]	93.02±0.56
V500	127.78±11.72[a]	104.87±7.05[a]	95.03±0.01
V1000	163.96±28.42[a]	76.34±6.17[b]	91.01±0.45

All data are given as mean ± SE (n = 9); different letters in the same column denote statistically significant differences (P<0.05) between groups. P250, P500, and P1000, extracts of pomegranate peel at 250, 500, and 1000 mg kg^{-1} diet, respectively; V250, V500, and V1000, extracts of veratrum at 250, 500, and 1000 mg kg^{-1} diet, respectively. The GPx activity is expressed as nmol/min/mL.

Lipid peroxidation on the white muscle of the treated fish groups is presented in Table 5. MDA content was significantly decreased in V250, P1000 and V1000 fish groups on the 20th day. On the 40th day of the study, P1000 had lowest value (P<0.05). Only P250 and P500 groups' MDA levels were similar to that of the control (P>0.05). In all other groups, MDA activity decreased significantly. A significant decrease in MDA levels was determined in P250 and V500 fish groups on the 60th day of study compared to that of the control (P<0.05).

Table 5- Malondialdehyde (MDA) levels in rainbow trout livers fed with pomegranate peel (*Punica granatum*) and veratrum (*Veratrum album*) extracts supplemented feed

Groups	**20th Day**	**40th Day**	**60th Day**
Control	5.45±0.13[b]	5.08±0.16[ab]	5.00±0.23[a]
P250	7.02±0.17[a]	5.89±0.18[a]	3.91±0.08[b]
P500	5.44±0.19[b]	5.11±0.10[ab]	4.35±0.15[a]
P1000	3.87±0.41[c]	2.56±0.18[c]	4.77±0.48[a]
V250	2.51±0.09[d]	2.95±0.13[c]	5.17±0.2[a]
V500	5.80±0.2[b]	4.21±0.27[b]	3.46±0.19[b]
V1000	4.01±0.10[c]	4.53±0.17[b]	4.44±0.29[a]

All data are given as mean ± SE (n = 9); different letters in the same column denote statistically significant differences (P<0.05) between groups. P250, P500, and P1000, extracts of pomegranate peel at 250, 500, and 1000 mg kg^{-1} diet, respectively; V250, V500, and V1000, extracts of veratrum at 250, 500, and 1000 mg kg^{-1} diet, respectively. MDA levels are expressed as nmol/min/mL.

3.3. Digestive enzymes

Pepsin activity was determined at the end of study (Figure 4.). The highest pepsin activity was observed in the P250 fish group (P<0.05). While no differences were observed in P500 and control groups, all other groups' pepsin activity was significantly decreased compared to the control.

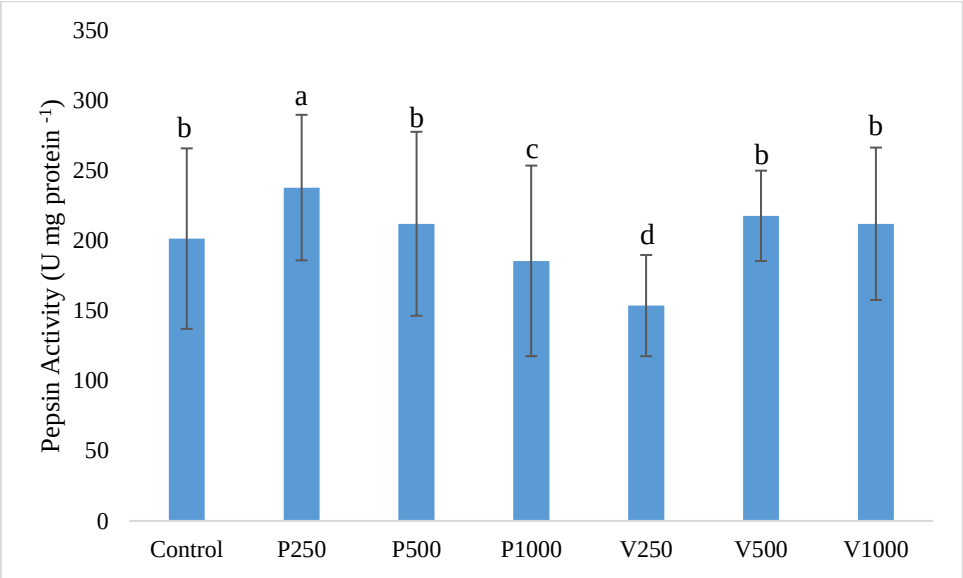

Figure 4- Pepsin activity in rainbow trout fed with methanolic extracts of pomegranate peel and veratrum. P250, P500, and P1000, extracts of pomegranate peel at 250, 500, and 1000 mg kg^{-1} diet, respectively; V250, V500, and V1000, extracts of veratrum at 250, 500, and 1000 mg kg^{-1} diet, respectively. Values are presented as mean ± SD. Different letters above columns express significant differences between groups (P<0.05)

Trypsin activity was higher than control only in the P1000 group (P<0.05) and in P500 group, no difference was observed compared to the control (Figure 5). However, in all other groups, trypsin activity significantly decreased.

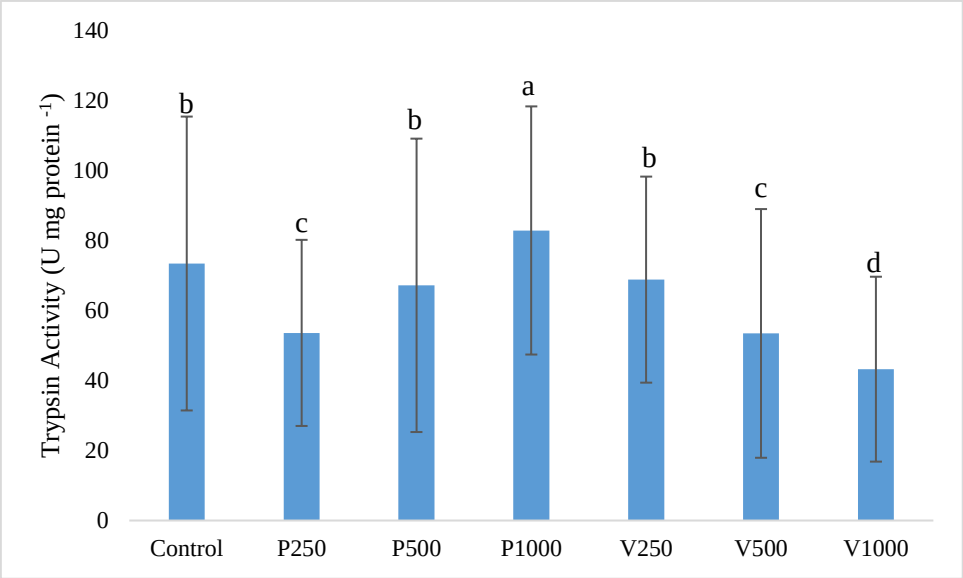

Figure 5- Trypsin activity in rainbow trout fed with methanolic extracts of pomegranate peel and veratrum. P250, P500, and P1000, extracts of pomegranate peel at 250, 500, and 1000 mg kg^{-1} diet, respectively; V250, V500, and V1000, extracts of veratrum at 250, 500, and 1000 mg kg^{-1} diet, respectively. Values are presented as mean ± SD. Different letters above columns express significant differences between groups (P<0.05)

Lipase activity showed no significant differences among the treated fish groups (Figure 6.).

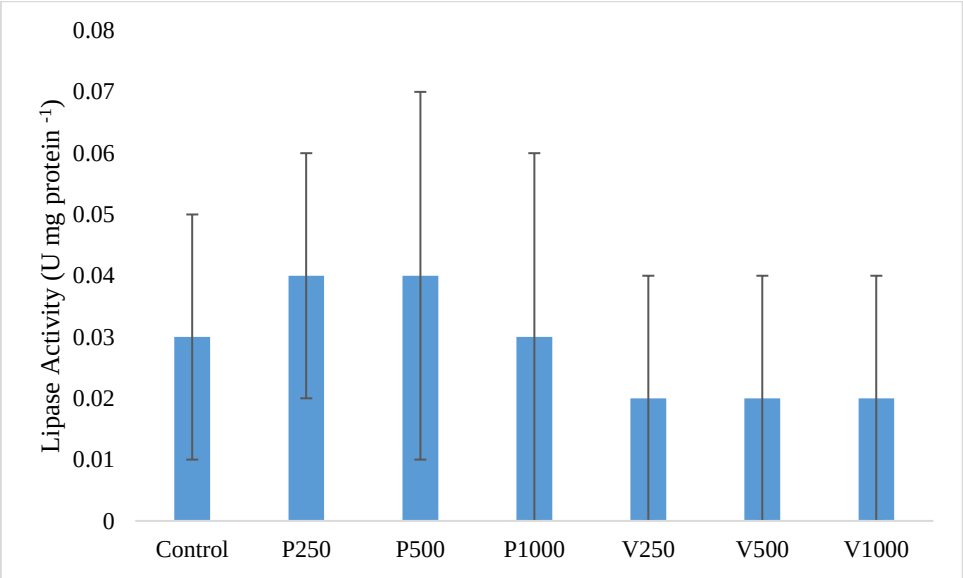

Figure 6- Lipase activity in rainbow trout fed with methanolic extracts of pomegranate peel and veratrum. P250, P500, and P1000, extracts of pomegranate peel at 250, 500, and 1000 mg kg^{-1} diet, respectively; V250, V500, and V1000, extracts of veratrum at 250, 500, and 1000 mg kg^{-1} diet, respectively. Values are presented as mean ± SD. Different letters above columns express significant differences between groups (P<0.05)

Amylase activity data are presented in Figure 7. In all treated fish groups, amylase activity decreased significantly.

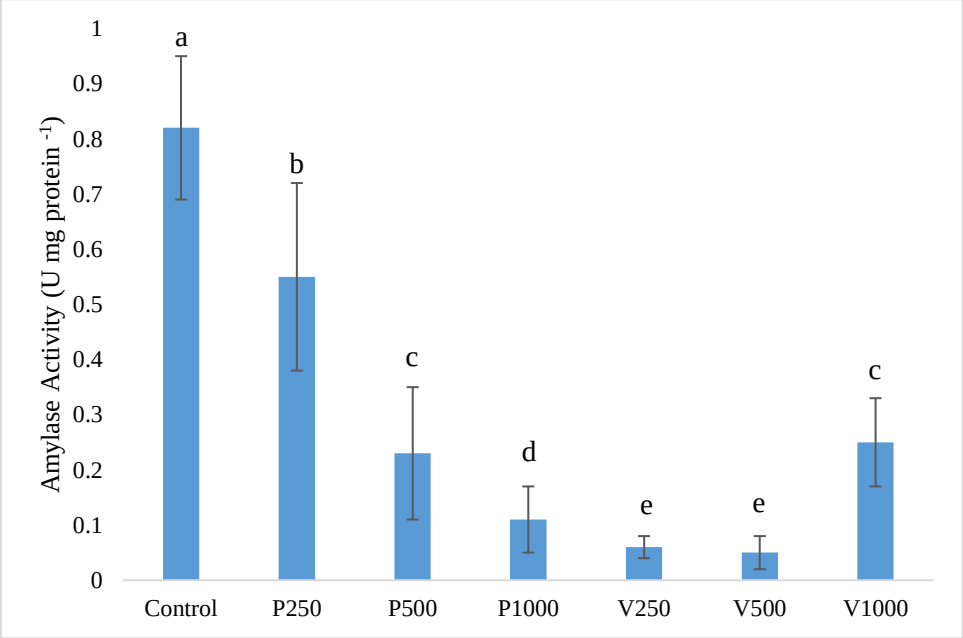

Figure 7- Amylase activity in rainbow trout fed with methanolic extracts of pomegranate peel and veratrum. P250, P500, and P1000, extracts of pomegranate peel at 250, 500, and 1000 mg kg^{-1} diet, respectively; V250, V500, and V1000, extracts of veratrum at 250, 500, and 1000 mg kg^{-1} diet, respectively. Values are presented as mean ± SD. Different letters above columns express significant differences between groups (P<0.05)

3.4. Haematology

Results on haematological responses are given in Table 6. Results showed a decreased WBC count in the P1000 fish group compared to that of the control (P<0.05). No significant differences were observed for RBC count among all the treatment groups (P>0.05). Haemoglobin content was significantly higher in the P250 group compared to the control. Other groups' haemoglobin contents did not show any differences compared to the control (P>0.05). Haematocrit values decreased significantly in P500 and V250 compared to the control (P<0.05).

Table 6- Haematological responses of rainbow trout fed with pomegranate peel (*Punica granatum*) and veratrum (*Veratrum album*) extracts supplemented feed

Groups	*WBC*	*RBC*	*HGB*	*HCT*	*MCV*	*MCH*	*MCHC*
Control	75.1±12^{a}	1.60±0.09	10.10±0.50^{b}	30.20±2.40^{a}	189.45±5.15	63.25±0.25	335.00±10.00
P250	77.2±12.5 ^{a}	1.89±0.38	13.15±0.69^{a}	36.35±10.28^{a}	213.00±9.74	65.27±7.01	306.50±23.51
P500	65.1±13.4 ^{a}	1.30±0.09	8.50±0.10^{b}	18.92±10.11^{b}	213.35±10.45	77.73±11.40	368.50±73.12
P1000	54.23±8.57 ^{b}	1.42±0.08	9.54±0.21^{b}	27.68±6.08^{a}	212.75±5.16	67.97±3.08	320.00±15.18
V250	81.21±2.14^{a}	1.43±0.12	9.30±0.54^{b}	18.80±12.83^{b}	195.10±6.97	65.60±6.96	336.25±28.71
V500	80.21±2.38^{a}	1.38±0.11	8.44±0.71^{b}	26.18±4.22^{a}	202.28±5.40	62.07±3.73	307.67±17.28
V1000	79.32±7.68^{a}	1.40±0.01	7.98±0.73^{b}	24.38±4.72^{a}	203.32±5.35	51.10±18.07	293.40±15.69

All data are given as mean ± SE (n = 9); different letters in the same column denote statistically significant differences (P<0.05) between groups. P250, P500, and P1000, extracts of pomegranate peel at 250, 500, and 1000 mg kg^{-1} diet, respectively; V250, V500, and V1000, extracts of veratrum at 250, 500, and 1000 mg kg^{-1} diet, respectively.

3.5. Growth performance

Growth performances are summarized in Table 7. The final weight of fish significantly decreased (P<0.05) in all treatment groups except P1000 compared to the control. The lowest growth was recorded in veratrum administered groups (P<0.05). Similar results were observed on weight gain and SGR values (P<0.05). However, interestingly, FCR decreased significantly in the P500 fish group compared to the control (P<0.05).

Table 7- Changes at the end of the study (60 day) in growth performance of rainbow trout fed with pomegranate peel (*Punica granatum*) and veratrum (*Veratrum album*) extracts supplemented feed

Parameter	***Control***	***P250***	***P500***	***P1000***	***V250***	***V500***	***V1000***
Initial Weight (g)	6.22 ± 0.3	6.42±0.1	6.13±0.2	6.19±0.2	6.02±0.3	6.36±0.4	6.45±0.3
Final Weight (g)	27.64±1.8^{a}	22.57±0.57^{b}	23.48±1.54^{b}	25.13±3.1^{a}	20.02±1.47^{c}	19.98±0.55^{c}	13.38±3.59 ^{d}
WG (%)	216.9±5.24^{a}	149.68±2.42^{b}	182.68±15.15^{b}	198.03±16.38^{a}	157.80±22.9^{c}	133.05±8.59^{c}	94.60±33.85^{d}
FCR	0.96±0.23^{b}	0.91±0.11^{b}	0.75±0.08^{c}	0.99±0.48^{a}	0.89±0.02^{b}	0.95±0.07^{b}	1.00±0.07 ^{a}
SGR (%)	2.48±0.11^{a}	2.15±0.04^{b}	2.21±0.11^{b}	2.31±0.21^{a}	1.94±0.12^{c}	1.94±0.05^{c}	1.21±0.46 ^{d}

All data are given as mean ± SE; different letters in the same column denote statistically significant differences (P<0.05) between groups. P250, P500, and P1000, extracts of pomegranate peel at 250, 500, and 1000 mg kg^{-1} diet, respectively; V250, V500, and V1000, extracts of veratrum at 250, 500, and 1000 mg kg^{-1} diet, respectively.

4. Discussion

In the present study, growth performances were negatively affected after the fish were fed with feeds supplemented with varying doses of pomegranate peel and veratrum aqueous methanolic extracts. On the contrary, positive effects were observed on immune response and antioxidant activity on such supplementations.

Respiratory burst is an activity that results after the production of several reactive oxygen molecules by neutrophils (Moritomo et al. 2003) and it is an important adaptive immune parameter in such studies (Bilen et al. 2019b). In the present study, the respiratory burst gradually increased in all experimental groups over time. Similar results were found in different fish species treated with other medicinal plant extracts (Amhamed et al. 2018; Mohamed et al. 2018). In line with our study, increased activity was determined in rainbow trout fed with different medicinal plants, such as *Rheum officinale*, *Panax ginseng*, *Origanum vulgare*, *Lavandula officinalis*, *Echinacea purpurea*, *Curcuma longa*, and *Aloe vera* (Bulfon et al. 2018). Lysozyme is an also important defense molecule of the fish (Bilen & Elbeshti 2019; Saurabh & Sahoo 2008). In the present study, lysozyme activity showed an increasing trend over the entire study period and this indicated that both the plant extracts contributed to such increase over 40 days of study. The medicinal plant has positive effects on lysozyme activity (Almabrok et al. 2018). Veratrum has an active ingredient known as dihydro-veratramine (Wilson et al. 2010) which is very close to cyclopamine which acts as the hedgehog signaling pathway inhibitor element (Reya et al. 2001). Cyclopamine has been reported to affect the innate immune system in rainbow trout (Sönmez et al. 2018). We believe that this content should be responsible for lysozyme activity. At the end of the study, in all treatment groups, MPO activity was found to decrease significantly. A similar trend of MPO activity was observed in rainbow trout fed with lemon balm (Bilen et al. 2019c). However, contrasting results were obtained for *Cyprinus carpio* and seabass fed with *Malva sylvestris* (Bilen et al. 2019b; Bilen et al. 2019d).

Antioxidant system plays an important role in elimination of reactive oxygen species (ROS), thereby preventing cell damage (Hoseinifar et al. 2020). In the present study, SOD activity in the P250 and P500 groups has increased, while no significant differences were observed in CAT activity. After feeding for 20 days, almost all groups showed an elevated G6PDH activity, while V500 and V1000 supplementation significantly increased GPx activity. Interestingly MDA levels decreased only in P1000 and V250 fish groups. MDA is well known as an oxidative stress indicator (Fang et al. 2002). It appears from the study that a low dose of veratrum and a high dose of pomegranate peel has antioxidative properties. The results are in line with the antioxidant activity of *Psidium guajava* leaf extracts in *Oreochromis mossambicus* (Gobi et al. 2016), and sage and thyme oil in rainbow trout (Sönmez et al. 2015b).

Digestive enzymes are important markers to determine differences in growth performance in fish. In the present study, amylase activity was lower in all of the treatment groups than in the control. Amylase increases after glycogen and starch are introduced into the digestive system and are more active in omnivore fish species, suggestive of its lower values for rainbow trout. No differences were observed in amylase activity after rainbow trout fed with stinging nettle (*Urtica dioica*), mango (*Mangifera indica*), and lupin (*Lupinus perennis*) (Awad et al. 2012).

Lipase is secreted by the pancreas and has a major role in lipid catabolism (Awad et al. 2012). No difference was observed in lipase activity in the present study as the experimental diets have similar fat contents. However, increased lipase activity was determined after lemon balm administration (Bilen et al. 2019c). In the present study, a higher level of trypsin activity was found in the P1000 fish group. However, no difference was observed after Ergosan administration in rainbow trout (Heidarieh et al. 2012).

Haematological parameters are important indicators to determine fish health. In the present study, WBC count increased in veratrum administered groups, while RBC and HBG decreased. It can be opined that an inflammatory response could have started after veratrum usage. Similar to this result, an increased WBC count was determined in rainbow trout after *Melissa officinalis* (Farahi et al. 2012) and *Echinacea purpurea* treatments (Oskoii et al. 2012).

Growth performance in the present study was affected negatively, especially in veratrum groups. However, it is interesting to note that FCR in the P500 group showed a diminishing trend. Similar to our FCR result, Heidarieh et al. (2013) found a decrease in FCR value in rainbow trout fed with different doses of *Aloe vera*. In contrast to our growth performance results, Ngugi et al. (2015), Adel et al. (2017), and Nobahar et al. (2015) found an increase in rainbow trout fed with different doses of medicinal plants. No difference was observed after the administration of ribwort plantain (Elbesthi et al. 2020).

5. Conclusions

Both *Veratrum album* and pomegranate peel extract supplementation at varying doses increased the overall immune status and antioxidant activities of rainbow trout fingerlings in a short period. Although all results were obtained under laboratory conditions, it may be suggested that short-term usage of feed supplemented especially with pomegranate peel extract could be used to improve immune responses. However, its long-term usage is not suggested. High doses of veratrum are not suggested as an additive at all. Pomegranate peel, when supplemented at 500 mg/ kg, in particular, can be beneficial as it lowers the FCR and improves the overall feed efficiency. Long-term effects are yet to be determined for different fish and feed combinations as these by-products of the food industry have huge potentials to be included in the fish feed as additives. Further studies may investigate the effects of these plant extracts on disease resistance.

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Effect of the Application of Foliar Jasmonic Acid and Drought Stress on Grain Yield and Some Physiological and Biochemical Characteristics of *Chenopodium quinoa* Cultivars

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ABSTRACT

Water shortage is a critical problem touching plant growth and yield in arid and semi-arid areas such as Iran. Plant hormones such as jasmonic acid (JA) play a crucial role in altering plant morphology in response to stress. To investigate the effect of JA and drought stress on grain yield and some physiological and biochemical characteristics of quinoa cultivars, a split-plot factorial experiment based on randomized complete block design with three replications was conducted at Kerman Agricultural and Natural Resources Research and Education Center over two crop years (2018-2019). In this experiment, drought stress as the main factor in two levels including non-stress and stress based on 60% and 90% soil available moisture depletion and JA foliar application (0, 1 and 2 mg L^{-1}) and cultivars ($Giza_1$, Titicaca, Q_{29}) respectively, as factorial were sub factor. The maximum grain yield (3775 kg ha^{-1}) was obtained in $Giza_1$ cultivar under non-stress condition and 1 mg L^{-1} JA foliar application. The greatest grain protein and total chlorophyll content were obtained in Titicaca cultivar under non-stress and 1 mg L^{-1} JA foliar application by 18.17% and 1.83 mg g^{-1} fresh leaf weight, respectively. In the opposite trend, the maximum amount of malondialdehyde was observed under drought stress and non-use of JA. In general, given the results of this study, it can be stated that JA caused an increase in grain yield in *quinoa* cultivars by reducing the harmful effects of drought stress and improving plant growth.

Keywords: Quinoa, Abiotic stress, Growth regulator, Grain yield, Protein

1. Introduction

Chenopodium quinoa is an herbaceous dicotyledonous crop belonging to the C3 group of plants. It is a member of the Amaranthaceae family and is called a pseudo-grain plant (Adolf et al. 2012). Quinoa is plant-based caviar and is gluten-free and suitable for people with gluten intolerance. Food and Agriculture Organization has compared it with powdered milk because of its high nutritional value. If all conditions are favorable, it has been reported to be 1.5-6 t ha^{-1} depending on cultivar and crop area (FAO 2011). Drought stress is one of the most important limiting factors for plant growth and production which, reduces more than 50% the average production of most products worldwide (Lata et al. 2011). The resistance of plants to drought stress is very complex due to the complicated interactions between environmental factors and the variety of physiological, biochemical, molecular phenomena affecting plant growth, so it is quintessential to recognize the effects of drought stress on plants (Hui-Ping et al. 2012). Prolongation of the duration of the water stress causes significant damage and consequently plant cell death due to the generation of excess reactive oxygen species (ROS) which causes lipid peroxidation, degradation of proteins and nucleic acids causing an alteration in plant metabolism (Hossam et al. 2020; Al-Khateeb et al. 2019). Increasing plant resistance to drought stress would be the most economical approach to improving agricultural productivity and reducing agricultural use of freshwater resources (Sofy 2015; Sperdouli & Moustakas 2012). The initial symptoms of water stress realize at stomatal level and stomas close to prevent further moisture loss through transpiration (Farooq et al. 2009). High resistance of quinoa to drought stress and salinity is caused quinoa to be adapted to different climatic conditions (Bhargava et al. 2007).

Iran is centrally located in the arid and semi-arid regions of the Earth, with more than 60% of its land area being classed as arid and semi-arid, therefore, drought is one of the most important abiotic stresses in this region, that cultivation of drought-

resistant crops such as quinoa is the best way to prevent crop failure (Vega-Galves et al. 2010). Drought-tolerant plants retain more water by absorbing water from the protoplast; accordingly, they have higher relative water content (Silva et al. 2007). Jasmonates (JA and methyl ester, methyl jasmonate) are a group of plant growth regulators that participate in many physiological processes and play a defensive role in the plant (Bari & Jones 2009). It is produced from lipid peroxide by increasing lipoxygenase activity (Maksymiec & Krupa 2002). JA can be used in limited amounts for enhancing plant growth, gene expression, osmolytes, antioxidant enzymes, and carotenoids (Sofy et al. 2020).

On the other hand, as key messengers introduce in the induction process, these compounds lead to the accumulation of secondary metabolites (Rubio et al. 2009). JA is the most important hormone for resistance to biotic and abiotic stresses. It accumulates rapidly in the wound and non-wound sites after plant injury (Bari & Jones 2009a). It has reported that JA exerts its protective role against drought stress by changes in protein, malondialdehyde content, and antioxidant activity (Yun-xia et al. 2010). In an experiment, the application of JA at a concentration of 0.5 mM on several rapeseed species *(Brassica napus)* exposed to drought stress for 10 days resulted in higher weight grain, chlorophyll content and leaf relative water content in all tested species compared to control (Mahabub Alam et al. 2014).

In this work recognizing the effects of different environmental stresses on the physiology of quinoa plant was studied for knowledge of its resistance mechanism and its survival in order to increase tolerance to stress. Due to the role of JA in increasing resistance in plants under stress, many features such as high grain quality of protein, high tolerance to drought and other stresses investigated. Therefore, the present study primary purpose was to investigate the variance effect of JA on grain yield and some physiological and biochemical parameters of quinoa cultivars under drought stress.

2. Material and Methods

This experiment has been carried out at the research farm of Joupar Station affiliated with Kerman Agricultural and Natural Resources Research and Education Center with 30° 17’ N and 57° 5’ E and 1900 m above sea level during two consecutive years (August 7th 2018 and 2019). This region's climate is cold and mild, with an average annual rainfall of 145 mm and an average annual minimum and maximum temperatures of 4 and 23 °C. Meteorological observations for two years in Joopar research station has been shown in Table 1. Before experimenting, to determine the soil's physical and chemical properties, soil samples were collected from the field for laboratory analysis and the results have been shown in Table 2. Land preparation included tillage, fertilization and furrow. Based on soil test results the amount of 46 kg ha^{-1} P_2O_5 from triple super phosphate source was given to the soil before planting also, 92 kg ha^{-1} nitrogen fertilizer from urea source of one third before planting, was given a third in the stem of longitudinal growth stage (4 to 6 leaves) and one third of the soil bud stage. The experiment was conducted as a split-plot factorial based on randomized complete block design with 3 replications. In this experiment, drought stress as the main factor in two levels including non-stress (normal irrigation) and drought stress based on 60% and 90% soil available moisture depletion and JA foliar application at three levels (0, 1 and 2 mg L^{-1}) and three varieties of quinoa (Giza$_1$, Titicaca, Q_{29}) as factorial were sub-factor. Planting operations were arranged in the middle of the stack on August 7th and the experimental designed based on number of lines 162, their distance 0.5 m, plant to plant distance 10 cm, variety to variety distance o.6 m, plant size 1 m, sample size from 30 cm to 80 cm with 16.6 plants per m^2. Plot length was 5 m and 4 rows per plot and distance between the replications was 3 m. Weeding was performed twice by the worker by hand and thinning of the plants was performed in a 10-leaf stage at a distance of 10 cm. In order to avoid interference with irrigation treatments, three rows were applied between drought stress and optimum irrigation. Drought stress was applied 20 days after of planting. At this stage the plant height was about 12 cm. Some genotypes such as Titicaca and Giza$_1$ cultivars are early genotypes, with the cultivation period ranging from 85 to 100 days, and medium-sized genotypes such as Q_{29} can be harvested over a period of 110 to 130 days. Calibrated Time Domain Reflectometry (TDR) was used for daily drought treatments in the way that in non-stress and drought stress, soil moisture is 12.2% and 8.9%, respectively. JA foliar application was done at 50% flowering stage in three times using a hand sprayer (Volume 20 L, pressure 3 bar, nozzle size 120 micrometer made by shark company of china). The duration of flowering, dough and milking periods of quinoa are the most sensitive stages to drought stress. Therefore, JA was used in these stages to combat drought stress (Geerts et al. 2008; Sofy et al. 2016a). In this experiment, German JA (SIGMA-ALDRICH) was used with purity of more than 97%. To 100 mg of the purchased solution, the amount of 5 cc of ethanol was added to make the milky solution be achieved. Then, the soluble volume of ethanol reached 30 ml and a completely transparent solution was produced. By adding sterile distilled water to solution up to 476cc volume, the solution of stoke 1 mM JA was obtained. the control solution contains 10 liters of sterile distilled water, the first treatment of the primary treated containing 48 ml stoke in 9.52 liters of sterile distilled water (equivalent 1 mg/L JA) and the second treatment containing 96 mL of stoke in 9.05 liters of sterile distilled water (equivalent to 2 mg/L JA).

Table 1- Detailed meteorological observations for two years based on the JOOPAR station

				Non-Stress (60%)	*Drought Stress (90%)*
Year	*Month*	*Temperature (C)*	*Humidity (%)*	*Precipitation (mm)*	*Precipitation (mm)*
2018	AUG.	17-33	16	0	0
	SEP.	15-30	18	2	0
	NOV.	13.5-27	24	5	0
	DEC.	11.5-22	31	16	0
2019	AUG.	18-31	19	1.5	0
	SEP.	17-29.5	20	6	0
	NOV.	14-23	25	11	0
	DEC.	10-21	33	17	0

Table 2- Some physico-chemical properties of soil (0-30 cm)

Soil Properties	*2018*	*Sandy Loam*
Soil texture	Sandy Loam	73
Sand (%)	69	12.6
Clay (%)	13	17.7
Silt (%)	18	7.9
pH	7.7	1.91
Electric Conductivity (ds m^{-1})	1.98	0.53
Organic matter (%)	0.48	18.1
Field Capacity (%)	18.8	7.3
Permanent wilting point (%)	7.6	1.35
Soil bulk density (g cm^{-3})	1.41	

2.1. Determination of chlorophyll and carotenoid

Absorbance of leaf extract at 663, 645 and 470 nm was measured using the S2100 Diod Array Spectrophotometer made in the United Kingdom using the following equation of photosynthetic pigments in mg g^{-1} leaf fresh weight (Lichtenthaler & Wellburn 1983).

$$Chlorophyll\ a = [\,12.7(A_{663}) - 2.69(A_{645})\,] \times V / 1000 \times W \quad [1]$$

$$Chlorophyll\ b = \left[\,22.9(A_{645}) - 4.68(A_{663})\right] \times V / 1000 \times W \quad [2]$$

$$Total\ Chlorophyll(a+b) = \left[\,20.2(A_{645}) + 8.02(A_{663})\right] \times V / 1000 \times W \quad [3]$$

$$Carotenoids = \left[(1000 \times A_{470}) - (2.27 \times C_a) - (81.4 \times C_b) / 226\right] \times V / 1000W \quad [4]$$

In the above equations, A is the absorbance read, V is the diluted extract volume and W is the fresh weight of the leaf sample in grams.

2.2. Malondialdehyde (MDA):

Malondialdehyde (MDA) was extracted with 5% trichloroacetic acid and determined according to (Heath & Packer 1968). MDA level was routinely used an index of lipid peroxidation and was expressed as µmol g^{-1} fresh weight using the following Equation:

$$MDA\left(\mu mol\ g^{-1} Fw\right) = \left[(A_{532} - A_{600}) / 156\right] \times 1000 \times dilution\ factor \quad [5]$$

2.3. Relative water content (RWC)

To determine the relative water content, 10 leaves of 5 random plants were used and finally the relative water content was calculated using the following equation in terms of percentage (Mata & Lamattina 2001):

$$RWC = (FW - DW) / (TW - DW) \times 100 \quad [6]$$

In the above equation, FW is leaf fresh weight, DW leaf dry weight, TW is turgidity weight, and DW is dry weight.

2.4. Seed protein

In order to measure grain protein, Kjeldahl apparatus in the laboratory first calculated the percentage of total nitrogen in the laboratory and then multiplied the percentage of nitrogen in the coefficient of 6.25 by the amount of protein in the grain (Magomya et al. 2014). Quinoa was harvested by plant yellowing and passing through the physiological ripening stage. To determine grain yield, two midline plants in each plot were harvested after removing the margin of plot. For calculation of biologic yield, 0.6 m^2 surface area was harvested and after drying and weighing, biomass dry weight and grain weight were determined. Harvest index was calculated using the following equation.

Harvest index = Grain yield / biologic yield * 100 [7]

2.5. 1000-grain weight

Four samples (250 grains) were counted for measuring 1000-grain weight using a balance with accuracy of 0.001 g. Data imported for variance analysis running SAS 9.2 software. Before performing the statistical calculations, the normality of the experimental error variance was evaluated using SAS software for each trait. Furthermore, Duncan's multiple range test (DMRT) was used to compare the mean of main effects and the least squares means (LSMEANS) and pdiff were used for interactions.

3. Results and Discussion

3.1. Grain yield

The results of the comparison of triple interaction means showed that the greatest grain yield was obtained by $Giza_1$ (3775 kg ha^{-1}) in non-stress conditions and application of 1 mg L^{-1} JA also the Titicaca cultivar by (3438 kg ha^{-1}) was the second-ranking. The lowest seed yield by $Giza_1$ (1781 kg ha^{-1}) was obtained with 53% reduction in yield under drought stress and non-use of JA (Table 4). In total, in conditions of water scarcity, the stomas are closed, as a result, the amount of carbon input to the plant reduces and photosynthesis and grain yield reduces as well. Deficit irrigation in quinoa reduced grain yield, shoot dry weight, harvest index, number of seeds and seed weight (Razzaghi et al. 2012). Application of JA improved quinoa grain yield under drought stress and non-stress conditions compared to non-application of JA in mentioned conditions (Table 4). Increasing of grain yield due to the use of JA has also been reported in a bulk of studies (Yun-xia et al. 2010a; Sofy et al. 2016a). The response of different cultivars to the foliar application of JA was affected by the plant genotype in which $Giza_1$ and Titicaca had the highest grain yield. According to the correlation table, grain yield had a positive and significant relationship with grain protein, 1000-seed weight, biologic yield, harvest index, leaf relative water content and photosynthetic pigments traits. This indicates the importance of these traits in increasing seed yield in plant so that the highest positive correlation was obtained between grain yield and harvest index. In contrast, the correlation between malondialdehyde and grain yield was negative and significant (Table 5).

3.2. 1000-seed weight

The results of the comparison of triple interaction of year, JA in cultivar for thousand seeds weight showed that the maximum 1000-seed weight in the first year of experiment was obtained from Q_{29} cultivar with 2 mg L^{-1} of JA equivalent to 3.58 g and the lowest 1000-seed weight in the second year of experiment was obtained from Q29 cultivar and non-consumption of JA equivalent to 2.54 g (Table 3). Triple interaction results also indicated that maximum thousand seeds weight under non-stress conditions and application of 2 mg L^{-1} JA in Q_{29} cultivar was 3.74 g and the lowest was obtained in Titicaca cultivar with 36% decrease under drought stress and 1 mg L^{-1}JA by 2.4 g (Table 4).

Table 3- Mean comparison of 1000-seed weight of quinoa under interaction effect of JA and cultivar

Treatments		***1000- Seed Weight (g)***	
JA (mg L^{-1})	***Cultivar***	***First year***	***Second year***
		2018	2019
0	$Giza_1$	2.82±0.013 def	2.6± 0.14 f
	Titicaca	2.81± 0.006 ef	2.82± 0.012 cde
	Q_{29}	2.81±0.008 ef	2.54± 0.106 f
1	$Giza_1$	3.001± 0.093 c	3.005± 0.091b
	Titicaca	2.79±0.176 f	2.8± 0.174e
	Q_{29}	2.98± 0.081cd	2.79±0.085 e
2	$Giza_1$	3.15± 0.023 bc	3.2± 0.002 a
	Titicaca	2.98±0.085 cde	3.28± 0.183a
	Q_{29}	3.58±0.186 a	3.13± 0.217ab

Means in each column followed by similar letters are not significantly different at the 5% probability LSD Test.

According to the results of previous research on quinoa, intensity of drought stress at seed filling stage, by reducing photosynthesis, decreases weight of thousand seeds and ultimately decreases grain yield per unit area (Gamez et al. 2019). Increasing 1000-seed weight due to the use of JA has also been reported in many studies (Swiatek et al. 2003). According to the correlation table, there was a positive and significant relationship between 1000-seed weight and biologic yield, harvest index and photosynthetic pigments traits. It should be noted that the highest correlation coefficient (r=0.63) was between 1000-seed weight and total chlorophyll content (Table 5).

3.3. Grain protein

The Comparison of triple mean interaction showed that the greatest grain protein content was obtained from Titicaca under non-stress condition and 1 mg L^{-1} JA equivalent to 18.17%. Also, the lowest seed protein content was obtained from Q29 under drought stress and 1 mg L^{-1} JA equivalent to 13.93% (Table 4). Drought stress induces the expression of genes encoding intracellular proteases and induces the breakdown of proteins and the re-mobilization of nitrogen and subsequent synthesis of soluble substances. Therefore, a decrease in protein content under drought stress is associated with a decrease in synthesis and an increase in the activity of protein-degrading enzymes (Feller 2004), which is in line with the results of this study. Application of methyl jasmonate in stress condition increased protein content and increased activity of catalase and peroxidase enzymes (Yastreb et al. 2015). According to the correlation table, there was a positive and significant relationship between grain protein and biologic yield, harvest index, relative water content and chlorophyll content traits, whereas the highest correlation coefficient (r=0.501) was between grain protein and total chlorophyll content (Table 5).

Table 4- Three-way interaction mean comparison of drought stress, JA and cultivar related to all measured traits in quinoa plants (2018-2019)

Treatments			*Seed Yield (kg ha^{-1})*	*1000-Seed Weight (g)*	*Seed Protein (%)*	*Biologic yield (kg ha^{-1})*	*Chlorophyll a (Mg g^{-1}fw)*	*Total Chlorophyll (Mg g^{-1}fw)*	*MDA (μmol g^{-1}fw)*
Drought stress	JA (mg L^{-1})	Cultivar							
Non-stress (60% evacuation humidity)	0	Giza1	2779.58 ± 114*de*	2.83 ± 0.012*efg*	17.88 ± 0.1*a*	10461.45 ± 941.47*d – g*	1.2 ± 0.089*c*	1.49 ± 0.091*ac*	1.56 ± 0.1*ij*
		Titicaca	3040.83 ± 191.7*cd*	2.82 ± 0.013*efg*	17.2 ± 0.094*abc*	11078.7 ± 482.38*cde*	1.125 ± 0.046*cde*	1.4 ± 0.039*cde*	2.25 ± 0.31*g*
		Q29	2521.24 ± 56.99*efg*	2.73 ± 0.054*ghi*	15.18 ± 0.058*ef*	9855.4 ± 6.9.6 *f – i*	0.97 ± 0.085 *fgh*	1.24 ± 0.086 *fgh*	1.31 ± 0.033 *jk*
	1	Giza1	3774.83 ± 295.65*a*	3.21 ± 0.003*c*	17.17 ± 0.021*abc*	12556.62 ± 389.36*ab*	1.4 ± 0.056*ab*	1.72 ± 0.059*a*	1.36 ± 0.057 *jk*
		Titicaca	3286.67 ± 181.3*bc*	3.19 ± 0.004*c*	18.17 ± 0.014*a*	11853.07 ± 465.72*abc*	1.48 ± 0.093*a*	1.83 ± 0.097*a*	5.26 ± 0.043*d*
		Q29	2693.83 ± 36.09*df*	2.78 ± 0.014*gh*	18.1 ± 0.149*a*	9990.41 ± 413.18*e – h*	0.97 ± 0.054 *fgh*	1.31 ± 0.053*efg*	0.49 ± 0.033*l*
	2	Giza1	2939.16 ± 203.83*cd*	3.19 ± 0.018*c*	17.4 ± 0.034*ab*	10816.63 ± 308.26*c – f*	1.15 ± 0.076*c*	1.53 ± 0.06*a*	1.09 ± 0.037*k*
		Titicaca	3437.88 ± 116.09*ab*	3.38 ± 0.141*b*	16.65 ± 0.017*bcd*	12719.75 ± 316.49*a*	1.37 ± 0.048*b*	1.74 ± 0.064*a*	1.76 ± 0.126*hi*
		Q29	2886.64 ± 172.11*de*	3.74 ± 0.138*a*	16.25 ± 0.069*b – e*	11488.05 ± 481.94*bcd*	1.09 ± 0.005*cde*	1.44 ± 0.034*bcd*	1.92 ± 0.055*h*
Drought stress (90% evacuation humidity)	0	Giza1	1780.82 ± 92.65*i*	2.59 ± 0.136*i*	16.52 ± 0.066*bcd*	8447.5 ± 494.44 *j*	0.85 ± 0.025*i*	1.07 ± 0.03 *j*	6.39 ± 0.043*a*
		Titicaca	2329.02 ± 61.7 *fgh*	2.8 ± 0.004 *fg*	15.72 ± 0.027*de*	8733.52 ± 481.97*ij*	1.04 ± 0.007*def*	1.24 ± 0.017 *fgh*	5.88 ± 0.24*b*
		Q29	2284.5 ± 48.34*gh*	2.62 ± 0.12*hi*	14.25 ± 0.026 *fg*	8403.5 ± 408.01 *j*	0.56 ± 0.045 *j*	0.71 ± 0.057*k*	4.58 ± 0.088*e*
	1	Giza1	2515.76 ± 80.78*efg*	2.79 ± 0.003*gh*	16.1 ± 0.029*cde*	10423.68 ± 298.71*d – g*	0.92 ± 0.006*ghi*	1.16 ± 0.028*hij*	5.45 ± 0.1*cd*
		Titicaca	2377.67 ± 106.79 *fgh*	2.4 ± 0.002 *j*	15.93 ± 0.027*de*	9436.7 ± 365.3*g – j*	1.11 ± 0.073*cde*	1.38 ± 0.079*cde*	5.74 ± 0.2*bc*
		Q29	2385.26 ± 102.75 *fg*	2.99 ± 0.114*de*	13.93 ± 0.059*g*	9706.04 ± 376.45 *f – i*	0.88 ± 0.052*hi*	1.07 ± 0.054*ij*	1.57 ± 0.093*ij*
	2	Giza1	2557.16 ± 126.97*efg*	3.16 ± 0.021*cd*	15.95 ± 0.033*de*	10159.3 ± 367.99*e – h*	1.02 ± 0.06*efg*	1.33 ± 0.051*def*	5.15 ± 0.058*d*
		Titicaca	2205.31 ± 135.78*gh*	2.87 ± 0.061*efg*	14.5 ± 0.05 *fg*	9499.37 ± 413.63*g – j*	1.15 ± 0.0*cd*	1.44 ± 0.012*bcd*	4.44 ± 0.11*e*
		Q29	2002.18 ± 187.09*hi*	2.96 ± 0.051*ef*	14.26 ± 0.045 *fg*	9161.4 ± 627.31*hij*	0.94 ± 0.029 *f – i*	1.19 ± 0.034*ghi*	3.51 ± 0.089 *f*

Means in each column followed by similar letters are not significantly different at the 5% probability LSD Test.

Table 5- Correlation coefficients between plant characteristics of quinoa (2018-2019)

Traits	*Seed Yield*	*Seed protein*	*1000-seed weight*	*Biologic Yield*	*Harvest Index*	*RWC*	*Chlorophyll a*	*Chlorophyllb*	*Total Chlorophyll*	*Carotenoied*	*MDA*
Seed Yield	1										
Seed protein	0.436**	1									
1,000-seed weight	0.56**	0.20^{ns}	1								
Biologic Yield	0.69**	0.35**	0.55**	1							
Harvest Index	0.72**	0.28*	0.27*	0.017^{ns}	1						
RWC	0.27*	0.47**	0.21^{ns}	0.33*	0.081^{ns}	1					
Chlorophyll a	0.55**	0.45**	0.57**	0.53**	0.29*	0.28*	1				
Chlorophyll b	0.509**	0.44**	0.56**	0.58**	0.17^{ns}	0.49**	0.47**	1			
Total Chlorophyll	0.61**	0.501**	0.63**	0.605**	0.301*	0.37**	0.97**	0.67**	1		
Carotenoied	0.31*	0.19^{ns}	0.309*	0.21^{ns}	0.23^{ns}	-0.095^{ns}	0.40**	0.32**	0.42**	1	
MDA	-0.37**	-0.206^{ns}	-0.34*	-0.37**	-0.18^{ns}	-0.43**	-0.13^{ns}	-0.39**	-0.23^{ns}	-0.13^{ns}	1

*, **, ns means significant at 5% and 1% level of probability, and non-significant, respectively

3.4. Biologic yield

The results of mean comparison of triple interaction showed the maximum biologic yield was obtained from Titicaca cultivar with 2 mg L^{-1} JA under non-stress condition by 12719.8 kg ha^{-1} and the lowest biologic yield was related to Q_{29} cultivar with 34% decrease under drought stress condition and non-consumption of JA was equivalent to 8403.5 kg ha^{-1}, which was not statistically significant with $Giza_1$ (Table 4). According to previous research, drought stress in quinoa reduced dry matter accumulation and reduced biologic yield and biomass of whole plant (Sanchez et al. 2003), which is in agreement with the results of this study. Increased biologic yield by JA growth regulators has also been reported in other studies (Harpreet & Geertika 2015; Swiatek et al. 2003a). The results of correlation table showed that there was a positive significant relationship between biologic yield, relative water content, chlorophyll a, b and total chlorophyll. It was also found that the higher the level of malondialdehyde stress index in the plant, the lower the biologic yield would be (Table 5).

3.5. Harvest index

Non-drought stress treatment with 27.32% had higher harvest index than stress treatment with 24.52%. Harvest index is a measure of the ratio of seed weight to total plant and high yielding cultivars have higher harvest index (Roshdi et al. 2009). According to the results of the researchers on quinoa plant, harvest index was significantly lower than the control conditions. Also, under drought stress, reducing grain yield, harvest index, number of seeds and grain weight in comparison with normal irrigation conditions (Razzaghi et al. 2012). These are consistent with the results of this study. According to the correlation table, there was a positive and significant relationship between harvest index and chlorophyll a and total chlorophyll, indicating that increasing chlorophyll content in plant increased harvest index, and a negative relationship between harvest index and malondialdehyde (Table 5).

3.6. Relative water content (RWC)

The results of comparison of mean triple interaction showed that the Titicaca cultivar in non-stress condition at the first year of experiment had the maximum relative water content with 91.44% whereas the lowest value was observed in Q_{29} cultivar in the second year in drought stress condition with 67.36% which showed the 26.33% reduction (Table 6). One of the most important indices of plant water balance is relative water content and plays an important role in regulating stomata conductance and consequently plant photosynthesis rate (Mitchel et al. 2001). According to the results of the researchers, increasing the amount of irrigation water in quinoa plant resulted in an increase in chlorophyll and relative water content (Sharifan et al. 2018). Correlation coefficients showed that there was a positive significant relationship between relative water content and chlorophyll a, chlorophyll b and total chlorophyll, while a negative and significant correlation was observed with malondialdehyde. As the stress index in the plant increased, the relative water content decreased significantly. The decrease in relative water content was related to the decrease in water availability under drought stress conditions and the positive and significant relationship of this trait with total chlorophyll confirms this (Table 5).

Table 6- Interaction effect of drought stress and cultivar on Relative Water Content of quinoa a_1: Non-stress, a_2: Drought stress, c_1:$Giza_1$, c_2: Titicaca, c_3:Q_{29}

Year	*Drought Stress (a)*	*Cultivar (c)*	*RWC (%)*
2018	a_1	c_1	90.61± 1.48 ab
		c_2	91.44± 0.98 a
		c_3	89.65 ± 1.58 a-c
	a_2	c_1	85.9± 1.61 b-c
		c_2	85.14± 1.38 c
		c_3	86.14± 1.28 bc
2019	a_1	c_1	79.26± 2.21 d
		c_2	76.65± 2.88 d
		c_3	86.18± 3.18 bc
	a_2	c_1	70.51± 1.70 e
		c_2	68.41± 1.38 e
		c_3	67.36± 1.48 e

3.7. Chlorophyll a, b and total

The results of mean comparison of triple interaction of drought stress, JA and cultivar showed that the highest amount of chlorophyll a with 1.48 mg g^{-1} leaf fresh weight belonged to Titicaca cultivar and application of 1 mg L^{-1} JA under non-stress conditions and the lowest chlorophyll in was observed in Q_{29} cultivar equal to 0.56 under drought stress and non-use of JA (Table 4). The results of comparison of mean triple interaction of year with drought and JA showed that the greatest amount of chlorophyll b in the second year of experiment with 2 mg L^{-1} JA from non-stress treatment equal to 0.40 mg g^{-1} fresh weight and

the lowest chlorophyll content at the first year in the stress and non-use of JA treatments were equal to 0.18 (Table 7). The results of mean comparison of triple interaction of drought stress, JA and cultivar showed that the maximum total chlorophyll content equal to 1.83 mg g^{-1} leaf fresh weight was obtained from Titicaca cultivar and 1 mg L^{-1} JA under non-stress condition which had not statistically significant difference with $Giza_1$ cultivar. The lowest total chlorophyll content was obtained with 61.2% decrease from Q_{29} cultivar with 0.71 mg g^{-1} leaf fresh weight under drought stress and non-use of JA (Table 4). The content of photosynthetic pigments, such as chlorophylls and carotenoids, which are important in converting light energy to chemical energy, varies under drought stress (Jaleel et al. 2009). These changes can somehow limit the photosynthesis that complicates the direct effect of drought on stoma closure, gas exchange, and photosynthesis. The decrease in chlorophyll and carotenoids concentrations is primarily associated with the production of reactive oxygen species (ROS) (Reddy et al. 2004), which is consistent with the results of this study. The use of JA at low concentration (1 mg L^{-1}) in non-stress conditions compared to non-application of JA and 2 mg L^{-1} resulted in the greatest chlorophyll content of whole leaf. According to the findings of researchers, the jasmonates may induce the gene expression of some key enzymes involved in chlorophyll biosynthesis through the formation of 5-aminolevulinic acid (Udea & Saniewski 2006). JA and its methyl ester (methyl jasmonate) can indirectly produce carbohydrates and other substances used in plant metabolism by increasing or preventing the plant's photosynthetic performance, in this way the plant is resistant to stress conditions (Popova et al. 2003). In the present study, due to genetic potential, Titicaca cultivar had the highest total chlorophyll content among cultivars, and under severe drought stress condition, Q_{29} cultivar had the lowest chlorophyll content compared to the other two cultivars. JA increases the chlorophyll content of the plant by increasing the activity of the enzymes involved in photosynthesis as well as decreasing the reactive oxygen species by influencing biochemical processes and antioxidant enzymes activity.

Table 7- Interaction effect of drought stress and foliar application of JA on Chlorophyll b content
a_1: Non stress, a_2: Drought stress, b_1:0 mg JA L^{-1}, b_2: 1 mg JA L^{-1}, b_3: 2 mg JA L^{-1}

Year	*Drought Stress (a)*	*JA (mg L^{-1}) (b)*	*Chlorophyll b content (mg g^{-1}fw)*
2018	a_1	b_1	0.278 ± 0.010 e
		b_2	0317 ± 0.005 cd
		b_3	0.333 ± 0.014 bc
	a_2	b_1	0.184 ± 0.012 g
		b_2	0235 ± 0.0101 f
		b_3	0294 ± 0.0106 de
2019	a_1	b_1	0.29 ± 0.0109 de
		b_2	0.356 ± 0.012 b
		b_3	0.406 ±0.031 a
	a_2	b_1	0.203 ± 0.016 fg
		b_2	0.236 ± 0.024 f
		b_3	0.275 ± 0.009 e

3.8. Carotenoids

Results of comparison of the mean interaction of drought stress and JA showed that the maximum leaf carotenoid content was observed under non-stress condition and application of 2 mg JA l^{-1} by 0.47 mg g^{-1} fresh leaf weight (Table 8). Also mean comparison of JA and cultivar showed that the highest amount of carotenoids belonged to Titicaca cultivar and application of 2 mg L^{-1} of JA equivalent to 0.51 mg g^{-1} leaf fresh weight, while the lowest amount was obtained from Q_{29} cultivar and non-use of JA (Table 9). Carotenoids are auxiliary pigments that affect the absorption and transmission of light and are chlorophyll protectors during the photo oxidation process, which reduces under drought stress, one of the most important causes of chlorophyll depletion being its degradation by reactive oxygen species. Decreasing of photosystem-2 activity lead to reduce Rubisco activity and lack of ATP synthesis; thereby reactive oxygen species in chloroplasts increases (Lawlor & Cornic 2002). Some researchers have reported that leaf chlorophyll and carotenoids content decrease under drought stress (Nayyar & Gupta 2006). In a study, the content of chlorophyll and carotenoids in cotton genotypes decreased with induction of drought stress compared to control and increased with increasing drought stress (Kumar et al. 2007).

Table 8- Interaction effect of drought stress and foliar application of JA on Carotenoieds content
a_1: Non stress, a_2: Drought stress, b_1:0 mg JA l^{-1}, b_2:1 mg JA l^{-1}, b_3:2 mg JA l^{-1}

Drought stress (a)	*JA (b)*	*Carotenoieds content (Mg g^{-1}fw)*
1	1	0.393±0.033b
1	2	0.468±0.035a
1	3	0.47±0.037a
2	1	0.323±0.025c
2	2	0.328±0.026c
2	3	0.397±0.036b

Table 9- Interaction effect of foliar application of JA and cultivar on Carotenoieds content
b_1:0 mg JA l^{-1}, b_2:1 mg JA l^{-1}, b_3:2 mg JA l^{-1}, c_1:Giza$_1$, c_2: Titicaca, c_3:Q_{29}

JA (b)	*Cultivar*	*Carotenoieds content (Mg g^{-1}fw)*
1	Giza$_1$	0.388±0.042bcd
1	Titicaca	0.363±0.031de
1	Q_{29}	0.325±0.037e
2	Giza$_1$	0.414±0.046bc
2	Titicaca	0.416±0.043b
2	Q_{29}	0.365±0.040cde
3	Giza$_1$	0.375±0.038bcd
3	Titicaca	0.51±0.047a
3	Q_{29}	0.415±0.045b

3.9. Malondialdehyde (MDA)

The results of the comparison of mean triple interaction showed that the maximum amount of malondialdehyde (MDA) in quinoa leaf under drought stress was observed in Giza$_1$ cultivar and no application of JA by 6.39 µmol g^{-1} leaf fresh weight while the lowest amount of MDA was in Q_{29} cultivar under non-stress conditions associated with 1 mg L^{-1} JA equivalent to 0.49 (Table 4). In stress conditions, excessive amounts of oxygen free radicals cause damage to the cell membrane, the most prominent of which is the peroxidation of the fatty acids present in the membrane. This change in cell membrane fatty acids result in the production of small compounds such as MDA, which is the end product of lipid peroxidation, while an increase in this compound is a sign of cell membrane damage (Jia et al. 2015). The use of JA to help the plant to cope with environmental stresses, including drought, can increase the activity of plant antioxidant systems through the expression of specific genes, thereby reducing lipid peroxidation and damage to the membrane (Shan & Liang 2010). As can be seen in the results, JA at the level of 1 mg L^{-1} resulted in a significant reduction of MDA. Increasing the amount of MDA production as a major indicator of oxidative stress and the crucial role of JA in reducing drought stress and lowering the amount of MDA in other plants have also been demonstrated (Alam et al. 2014). Correlation coefficients showed that MDA had a negative and significant relationship with grain yield, 1000-seed weight, biologic yield, relative water content and chlorophyll b, indicating that water deficit stress increased MDA in plant and decreased yield, and yield components in the plant (Table 5).

4. Conclusions

Results showed that the incidence of drought stress (90% of soil moisture discharge), decreased yield and yield components quinoa, So that cause to 53%, 36% and 34% reduction in grain yield, 1000-seed weight and biologic yield respectively, but the grain yield decrease in Giza$_1$ cultivar was due to lower chlorophyll loss and consequently higher photosynthesis than other cultivars. The application of foliar JA compared to non-spraying conditions in both favorable irrigation conditions and drought stress, had the greatest effect on performance and yield components. Application of JA at 1 mg L^{-1} increased grain yield by 16% compared to non-use. The mentioned items can be concluded that the plant quinoa has a tolerance and relatively favorable resistance to drought stress and foliar of JA on the plant under stress conditions causes the plant resistance and reduces the harmful effects of dehydration on plant growth process. Also, by applying appropriate management in farm and planting of early and yielding cultivars it is possible to guarantee the establishment of this plant in low water conditions of course, it is suggested that the experiment is also studied and investigated in greenhouse conditions.

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In Situ and *in Vitro* Nutritive Value Assessment of *Styrax Officinalis* L. as an Alternative Forage Source for Goat Feeding

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ABSTRACT
The current study aimed to determine *Styrax officinalis* L. (SO) leaf's nutritive value, collected at four phenological stages, pre-flowering (PF), flowering (FL), seed linkage (SL), and fruiting (FR) by *in situ* and *in vitro* experiments. The ruminal degradability of dry matter (DM) and crude protein (CP) and *in vitro* gas production (GP) of SO leaves were measured using three rumen fistulated mature Saanen goats. Significant differences between chemical compositions of the SO leaves collected at different phenological stages were observed (P<0.001). The DM, CP, ether extract (EE), and ash values of SO leaves ranged between 29.16 to 45.63%, 10.11 to 19.79%, 3.40 to 5.85%, and 4.71 to 6.49% during the different phenological stages (PF, FL, SL and FR, respectively). Cell wall components of SO leaves showed a cubic trend due to their capability to form new shoots after grazing. The effective DM and CP degradability of SO leaves ranged between 66.91 to 77.93% and 64.92 to 84.57%, which means an average value for animals fed at approximately maintenance level when rumen outflow rate (r) is equal to 0.02 h^{-1}. Significant differences between the SO leaves collected at different phenological stages were observed in GP at all incubation times (P<0.05 and P<0.001). After 96 h incubation, the gas produced ranged between 20.68 to 27.53 mL/200 mg DM of the substrate. The research findings clearly indicate that degradability of DM, CP, and ME content of SO leaves ranged between moderate to high and significantly affected by phenological stages, however, they could be utilized until the end of the FL stage as forage sources.

Keywords: *Styrax officinalis* L. leaves, Ruminal degradability, Digestibility, Gas production, Chemical composition

1. Introduction

Goats, well-adapted species to marginal areas, can benefit from shrubs effectively, while other species cannot utilize them. In Turkey, goat breeding is characterized by low input usage and is carried out depending on natural conditions. Thus, natural pastures have an essential role in goat feeding. Therefore, shrublands in the Mediterranean climate zone represent valuable feed sources, especially in goats (Perevolotsky et al. 1998). In general, the quality of shrubland depends on the type, distribution, and nutrient composition of the plant. Indeed, shrublands become more important in meeting these animals' nutrient demands in dry seasons (Tölü 2009).

The nutrient composition of shrubs may vary according to seasons or even months (Rogosic et al. 2006). While new shoots and leaves rich in protein content accelerate in spring, protein content decreases, and cell wall components increase in summer. Acknowledgement of the changes in the shrubs' nutrient content throughout the year makes it an important source to decide the grazing season, determine additional feed strategies, establish the pasture-animal relationship correctly, and increase the economic and biological efficiency of breeding activities (Metera et al. 2010). In Turkey, shrublands are widely used for goat feeding throughout the year in some areas. Therefore, determining the animals' grazing preferences in shrublands is critical for these areas' sustainability. While there is a limited number of studies that were performed in Turkey aimed to determine the annual feed production of the shrubs, the change of feeding potential, and the grazing capacity, there are many studies on the nutritional composition of shrublands and consumption preferences of different species in these areas and about determining the feeding potential, *in vitro* gas production, and *in sacco* degradability characteristics of some of them in the literature (Dziba et al. 2003; Boubaker et al. 2004; Kamalak et al. 2004; Ammar et al. 2005; Moujahed et al. 2005; Karabulut et al. 2006; Rogosic et al. 2006; Salem et al. 2006; Bruno-Soares et al. 2011; González-Pech et al. 2021).

Styrax officinalis L. (SO), which belongs to the *Styracaceae* family, is a deciduous plant in a dwarf tree or bush and is widely distributed in America, Mexico Mediterranean countries, e.g., Turkey, France, and Greece (Jaradat 2020). Although SO has deep roots, it has developed a root system that allows to benefit more from surface water due to their adaptation to drought (Mahall et al. 2010). The average 180-day life cycle starts with buds' blooming in early spring and ends with senescence in August-September. The main factor determining the growth and the number of leaves and flowers on the plant is the amount of rainfall in the previous year and varies according to annual rainfall (Mahall et al. 2010). The Phoenicians, Egyptians, and Romans used the resins of this plant for incense and treatment. It is widely found in the south, west, and northern parts of Turkey. Besides, the *Styracacea* family, e.g., *S. japonicum*, *S. formosanus*, *S. obassia*, *S. macranthus*, and *S. officinalis*, is known for its various biological activities in the form of insecticidal, fungicidal, antimicrobial, antidiabetic, antiproliferative, and cytotoxic effects (Łyczko et al. 2020; Yesilyurt & Cesur 2020).

Briefly, it is possible to meet some of the nutrient requirements of goats from plant leaves and fruits that grow in shrublands in dry seasons. One of them is SO, which is widely distributed in the maquis vegetation. Therefore, the current study aimed to determine the nutritive value of SO leaf, collected at four phenological stages, by *in situ* and *in vitro* experiments.

2. Material and Methods

The present study was carried out from April to August 2019 in Balikesir, Turkey. The animal care and handling procedures were reviewed and approved by the Sheep Breeding Research Institute's Ethical Committee (Approval number: 3451976).

2.1. Forage samples

The leaf samples of SO was collected by hand-clipping from at least 20-30 different individual SO shrubs at pre-flowering (PF), flowering (FL), seed linkage (SL) and fruiting (FR) stages in four different district of Balikesir, Turkey (Bandirma (B), Erdek (E), Gönen (G), Manyas (M)) via three different sampling point. The amount of dry matter (DM) in the fresh form, collected from each sampling point, was determined on the same day by drying approximately 10 g of fresh material in an oven at 102 °C for at least 16 hours until it reaches a constant weight. Fresh leaves were dried in air-conditioned rooms (24 ± 2 °C) by inverted several times a day for a week and were ground to pass through a 3 mm, 1 mm, and 0.5 mm sieve kept in the refrigerator until analysis.

2.2. Chemical analysis

The proximate analysis of SO leaves used in the *in situ* and *in vitro* studies was performed according to Weende's analysis by using AOAC (1990) methods. Briefly, dry matter (DM) of SO was determined by drying the SO leaves at 102 °C overnight, and ash content was determined by ashing the SO leaves in a muffle furnace at 550 °C for 3 h. The nitrogen (N) content of SO samples was measured by the Kjeldahl method and multiplied by 6.25 to get the crude protein (CP) ratio. To analyze the Neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) of SO, a method was used described by Van Soest et al. (1991). The condensed tannin (CT) content of SO samples were determined by Sun et al. (1998). While the samples milled through 1 mm were used for the proximate analysis with cell wall contents, milled through 0.5 mm was used to determine SO leaves' CT contents.

2.3. In situ DM and CP degradation

Three adult male Saanen goats, about 3 years old ages and 74.27 ± 1.03 kg live weight (Mean ± SE), fitted with a permanent rumen cannula were used to measure the rate and extent of digestive DM and CP loss (digestibility, effective degradability) of SO leaves by using the in situ nylon bag technique (Ørskov & McDonald 1979). Goats received a good quality alfalfa hay and concentrate (60:40) at about 1.25 times greater than maintenance level twice a day, at 09:00 and 16:30 at an equal amount. Nylon bags purchased from Ankom Technology (R510 forage bag, 5 x 10 cm size, 50 ± 10 µ porosity; ANKOM Technology, Macedon NY, USA). Approximately 3 g of SO leave, milled through 3 mm, were placed in each nylon bag and incubated in the rumen of each of the three goats for specified periods (3, 6, 12, 24, 48, and 72 h). Four bags, each containing one of the SO leaves collected from different phenological stages, were placed in each goat's rumen and then withdrawn at once after remaining in the rumen for the specified incubation time. Upon removal from the rumen, bags were immersed in a bucket of cold water, transferred into the laboratory, washed in running cold tap water for 2 minutes, and then washed in the cold rinse cycle (15 min) of a washing machine. Zero time disappearance was obtained by similarly washing un-incubated bags. The washed bags were dried in an air-forced oven (60 ± 2 °C, 48 h) and weighed to determine DM disappearance. Digestion characteristics of DM and CP of SO leaves were calculated using equations 1 and 2 (Ørskov & McDonald 1979):

$$Y = a + b(1 - e^{-ct}) \quad (1)$$

$$P = a + \frac{(b \times c)}{(c + r)} \quad (2)$$

Where: *Y*, is the disappearance rate of DM or CP of SO leaves at time t (%); *a*, an intercept representing the portion of DM or CP of solubilized SO leaves at zero time incubation (%); *b*, the portion of DM or CP of SO leaves that is readily degraded in the rumen (%); *c*, the rate constant of fraction *b* (% h^{-1}); *t*, is the time of incubation (h); *P*, is the effective degradability of DM or CP of SO leaves (%); *r*, is the rate of outflow from the rumen (% h^{-1}; an outflow rate of 0.02, 0.05, and 0.08 was applied).

2.4. In vitro gas production

In vitro incubations of SO leaves were carried out using the ANKOM RF Gas Production System (ANKOM Technology, Macedon NY, USA). Rumen fluid was obtained from three adult male Saanen goats before morning feeding. On each occasion, approximately 200 mg SO leaves sample (dry weight), milled through 1 mm, were incubated in triplicate in 250 mL glass bottles. The glass bottles were pre-warmed at 39 °C before the injection of 100 mL rumen fluid-buffer mixture (without trypticase; 20/80, v/v) by following the procedure of Goering & Van Soest (1970). All the glass bottles containing rumen fluid-buffer mixture and SO leaves samples were incubated for specified times (0, 3, 6, 12, 24, 48, 72, and 96 h). Total gas volume was corrected for blank incubation. Cumulative GP data were fitted to Ørskov & McDonald (1979) model using the NEWAY computer package program according to equation 3.

$$Y = a + b(1 - e^{-ct}) \quad (3)$$

Where: *Y*, is the gas production volume (GP) of SO leaves at time t (mL /200 mg DM); *a*, gas production volume from immediately soluble fraction of SO leaves (mL); *b*, gas production from the insoluble fraction of SO leaves (mL); *c*, gas production rate constant for the insoluble fraction of SO leaves (mL/h); *t*, the time of incubation.

The metabolizable energy (ME, MJ/kg DM) and organic matter digestibility (OMD, % DM) contents of SO leaves were calculated using equations of Menke et al. (1979) (Equations 4 and 5).

$$ME\ (MJ/kg\ DM) = 2.20 + 0.136\ GP + 0.057\ CP \quad (4)$$

$$OMD\ (\%\ DM) = 14.88 + 0.889\ GP + 0.45\ CP + 0.0651\ Ash \quad (5)$$

Where: GP, is 24 h net gas production (mL/200mg); CP, crude protein.

2.5. Statistical analysis

In situ DM and CP degradation, data of chemical components, and *in vitro* GP and kinetics were analyzed applying a randomized complete block design using SAS (2016) pocket program. The least-squares means were compared using Tukey's multiple comparison tests. The model used for the least-squares (LS) analysis was as follows:

$$Y_{ijk} = \mu + B_i + S_j + e_{ijk} \quad (6)$$

Where: Y_{ijk}, is the variable studied; B_i, is the block; (B, E, G, M), S_j, is the phenological stages (PF, FL, SL, and FR); e_{ijk} , is the residual error. Linear, quadratic, and cubic trends of the phenological stage were analyzed according to orthogonal polynomial contrasts.

3. Results and Discussion

The chemical composition of the SO leaves collected at four different phenological stages is presented in Figure 1. Significant differences were observed between chemical compositions of the SO leaves collected at different phenological stages ($P< 0.001$). DM contents of SO leaves ranged between 29.19 to 45.63% and increased with maturity, except the FL stage. In contrast to DM, SO leaves' CP content decreased with increased maturity and ranged between 10.11 to 19.79%. CP content of SO leaves collected at the PF stage was significantly higher than those gathered at the other three stages (FL, SL, and FR). While the highest CP (19.79%) and ash (6.49%) contents were observed at the PF stage; the highest DM (45.63%), EE (5.85%), ADL (10.14%), CT (22.75%) contents were observed at FR stage as expected with increased maturity. CT content of SO leaves ranged between 6.42 to 22.75%. Previous researchers have demonstrated that with the plant's increased maturity, the DM ratio increased while the CP ratio decreased (Mountousis et al. 2008; Tolunay et al. 2009; Ataşoğlu et al. 2010). The results obtained in the DM and CP ratio in the study are consistent with the literature reports on this subject.

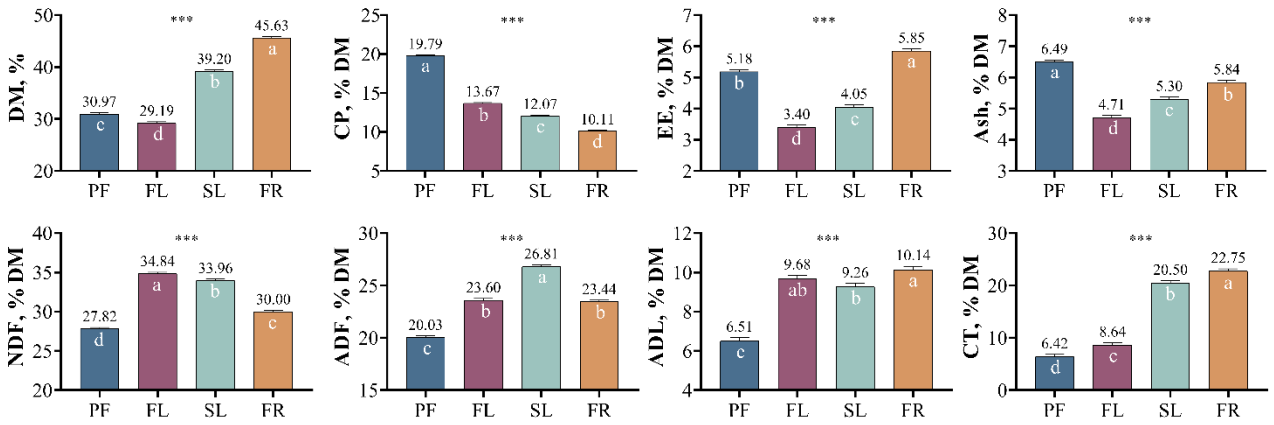

Figure 1- Chemical composition of *Styrax officinalis* L. leaves collected at different phenological stages

PF: Pre-flowering stage, FL: Flowering stage; SL: Seed linkage stage, FR: Fruiting stage; DM: Dry Matter; CP: Crude Protein; EE: Ether Extract; CA: Crude Ash; NDF: Neutral Detergent Fiber; ADF: Acid Detergent Fiber; ADL: Acid Detergent Lignin; CT: Condensed Tannin
The values with different letters (a, b, c, d) in each graph is statistically different (P<0.05);
***: P<0.001.

In contrast, the increased level of DM of SO leaves with the SL stages in parallel to weather conditions, NDF, and ADF values were found to be decreased. These results may be accepted as an indicator that SO's leaf formation continues from new shoots after grazing. It was well documented in the literature that lignification was increased in plants as the maturity was increased (Parissi et al. 2005). In the present study, the ADL content of SO leaves was increased as the maturity was increased.

In a study conducted to determine the anti-methanogenic properties of some tree leaves containing tannins by in vitro gas production test, the DM, CP, EE, Ash, NDF, and ADF content of SO leaves was found to be 34.26%, 11.64%, 9.28%, 13.33%, 28.95% and 22.94% respectively (Şimşek & Kamalak 2019). In another study conducted to determine the yield and nutrient composition of shrubs in the pastures in Anti-Taurus Mountain, the CP, ADF, and NDF content of SO leaves was determined as 10.60%, 23.9%, 40.2%, in Şavşak and 11.6%, 27.2%, 43.1% in Çandır, respectively (Kökten et al. 2010). However, it was not specified in which vegetation period or phenological stage the samples were taken in both studies. For this reason, it would not be easy to make an adequate comparison between the data obtained in our research and the literature reports. On the other hand, the results obtained in our study were found to be higher than the reported values of the SO leaves collected by Ertekin et al. (2019) at the FR stage.

The profiles for DM and CP disappearance of each phenological stage are illustrated in Figure 2, and degradability parameters are given in Tables 1 and 2. The mean rates of DM and CP disappearance were greater for the PF stage compared to the other three phenological stages (Figure 2). After a 48 h incubation period, DM disappearances between PF - FL and SL - FR were close to each other (Table 1). Except for the 12 h incubation period (P<0.01), all the incubation periods, degradability parameters, and effective degradability of SO leaves were significantly affected by the phenological stages (P<0.001). Also, significant differences between the phenological stages in terms of effective degradability of DM obtained from different rumen outflow rates were observed. While the SO leaves' highest effective degradability was observed at PF stages, the lowest effective degradability of SO leaves was marked at SL stages. Like the disappearances of DM of SO leaves, the CP degradability was significantly affected by the phenological stages (P<0.01 and P<0.001) (Table 2). There was a significant decrease in CP degradation in the rumen with increased maturity of SO. Approximately 27.94% decrease was observed between the beginning (PF) and the end of the vegetation period (FR) for the readily degradable part of CP of SO leaves.

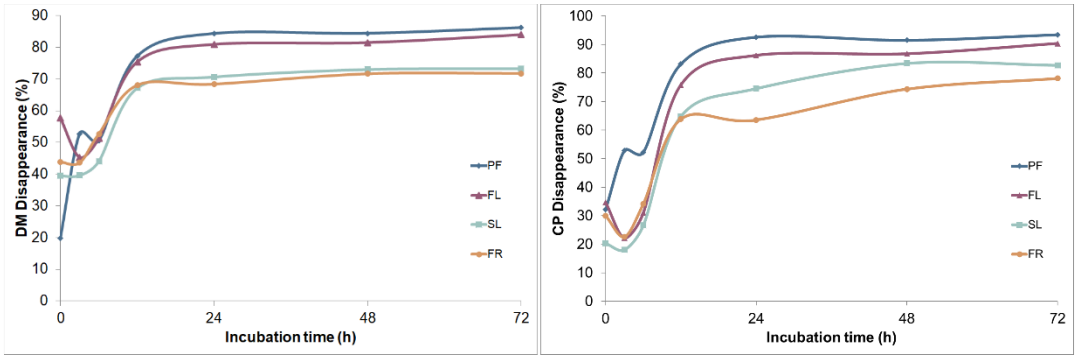

Figure 2- Degradation curve of dry matter and crude protein of *Styrax officinalis* L.

PF: Pre-flowering stage; FL: Flowering stage; SL: Seed linkage stage; FR: Fruiting stage; DM: Dry Matter; CP: Crude Protein

Table 1- Degradability coefficients and effective degradability of DM of *Styrax officinalis* L.

	PF	*FL*	*SL*	*FR*	*SEM*	*M*	*PS Effect*		
							L	*Q*	*C*
0	19.87^{z}	57.71^{x}	39.43^{y}	43.84^{w}	0.161	***	NS	NS	***
3	52.73^{x}	45.28^{w}	39.62^{y}	43.61^{wy}	0.883	***	*	***	***
6	50.51^{x}	51.19^{x}	44.03^{w}	52.68^{x}	0.699	***	NS	NS	***
12	77.15^{x}	75.34^{xw}	67.11^{y}	68.03^{wy}	1.664	**	**	**	**
24	84.29^{x}	80.89^{w}	70.65^{y}	68.38^{z}	0.233	***	***	***	***
48	84.34^{x}	81.45^{x}	73.01^{w}	71.63^{w}	0.691	***	***	***	***
72	86.13^{x}	83.93^{x}	73.23^{w}	71.74^{w}	0.826	***	***	***	***
c	0.146^{x}	0.062^{y}	0.085^{w}	0.099^{w}	0.004	***	NS	NS	NS
a	22.05^{z}	48.35^{x}	34.90^{y}	40.57^{w}	0.489	***	NS	*	NS
b	63.57^{x}	36.92^{w}	39.67^{w}	31.70^{y}	0.833	***	NS	NS	NS
ED^{1}	77.93^{x}	76.28^{x}	66.95^{w}	66.91^{w}	0.374	***	***	***	***
ED^{2}	69.36^{x}	68.81^{x}	59.79^{y}	61.58^{w}	0.303	***	**	*	***
ED^{3}	63.07^{w}	64.49^{x}	55.25^{z}	58.05^{y}	0.281	***	*	NS	***

IT: Incubation time (h); PS: Phenological stages; PF: Pre-flowering stage; FL: Flowering stage; SL: Seed linkage stage; FR: Fruiting stage; SEM: Standard error of means; M: Main effect; L: Linear effect; Q: Quadratic effect; C: Cubic effect; a: the fraction of readily degradable DM (%); b: the fraction of degradable DM at a measurable rate (%); c: degradation rate of DM (% h^{-1}); ED^{1}: effective degradability of DM (%) with a rumen outflow rate of 0.02; ED^{2}: effective degradability of DM (%) with a rumen outflow rate of 0.05; ED^{3}: effective degradability of DM (%) with a rumen outflow rate of 0.08; The values with different letters (x, w, y, z) in the same row are statistically different (P<0.05), NS: Not Significant; *: P<0.05, **: P<0.01, ***: P<0.001.

Table 2- Degradability coefficients and effective protein degradability of *Styrax officinalis* L.

IT	*PF*	*FL*	*SL*	*FR*	*SEM*	*M*	*PS Effect*		
							L	*Q*	*C*
0	32.18^{w}	34.61^{x}	20.28^{y}	29.96^{w}	0.492	***	***	NS	**
3	52.85^{x}	22.14^{w}	18.03^{w}	22.59^{w}	2.017	***	**	***	***
6	52.16^{x}	30.97^{wy}	26.57^{y}	34.13^{w}	1.646	***	*	***	***
12	83.16^{x}	75.85^{xw}	64.86^{wy}	63.86^{y}	2.501	**	***	***	**
24	92.58^{x}	86.14^{x}	74.55^{w}	63.53^{y}	1.861	***	***	***	***
48	91.48^{x}	86.69^{x}	83.39^{x}	74.36^{w}	1.816	**	***	***	***
72	93.43^{x}	90.31^{x}	82.71^{w}	78.12^{y}	0.931	***	*	*	***
c	0.110^{x}	0.072^{w}	0.072^{w}	0.063^{w}	0.005	**	NS	NS	NS
a	31.50^{x}	21.13^{w}	11.60^{y}	22.70^{w}	0.772	***	NS	***	***
b	62.72^{w}	71.45^{x}	74.15^{x}	56.00^{y}	1.339	***	NS	**	**
EPD^{1}	84.57^{x}	76.97^{w}	69.53^{y}	64.92^{z}	0.437	***	***	***	***
EPD^{2}	74.63^{x}	63.20^{w}	55.24^{y}	53.63^{y}	0.553	***	***	***	***
EPD^{3}	67.83^{x}	54.88^{w}	46.61^{y}	47.14^{y}	0.588	***	***	***	***
BP^{1}	15.43^{z}	23.03^{y}	30.47^{w}	35.08^{x}	0.437	***	***	***	***
BP^{2}	25.37^{y}	36.80^{w}	44.76^{x}	46.37^{x}	0.553	***	***	***	***
BP^{3}	32.17^{y}	45.12^{w}	53.39^{x}	52.86^{x}	0.588	***	***	***	***

IT: Incubation time (h); PS: Phenological stages; PF: Pre-flowering stage; FL: Flowering stage; SL: Seed linkage stage; FR: Fruiting stage; SEM: Standard error of means; M: Main effect; L: Linear effect; Q: Quadratic effect, C: Cubic effect; a: the fraction of readily degradable CP (%), b: the fraction of degradable CP at a measurable rate (%); c: degradation rate of CP (% h^{-1}); EPD^{1}: effective degradability of CP (%) with a rumen outflow rate of 0.02; EPD^{2}: effective degradability of CP (%) with a rumen outflow rate of 0.05; EPD^{3}: effective degradability of CP (%) with a rumen outflow rate of 0.08; BP^{1}: bypass protein (%) with a rumen outflow rate of 0.02; BP^{2}: bypass protein (%) with a rumen outflow rate of 0.05; BP^{3}: bypass protein (%) with a rumen outflow rate of 0.08. The values with different letters (x, w, y, z) in the same row are statistically different (P<0.05); NS: Not Significant;*: P<0.05; **: P< 0.01; ***: P< 0.001

The rapidly degradable (a) DM and CP fraction of SO leaves are presented in Tables 1 and 2. While the PF stage (22.05%) had a lower readily degradable DM, the FL stage (48.35%) had higher readily degradable DM. On the other hand, the lowest readily degradable CP fraction was observed at the SL stage (11.60%), while the highest one was the PF stage (31.50%). As shown in Tables 1 and 2, the effective degradability DM and CP of SO leaves, especially at heavily grazed at PF and FL stages, were moderate to high. To our knowledge, there is no information in the literature about *in situ* digestibility of SO leaves. However, compared to the *in situ* studies done with some trees and shrubs in the literature, SO leaves' digestibility, collected during the heavily grazed, for 48 h was higher than some exotic trees and shrubs *Spondias mombin* L., *Antiaris toxicaria* Lesch., *Baphia nitida* Lodd., *Grewia carpinifolia* Juss., *Griffonia simplicifolia* (DC.) Baill., *Calliandra calothyrsus* Meisn., *Acacia brevispica* Harms, *Acacia tortilis* (Forssk.) Hayne, *Acacia seyal* Delile, *Acacia nilotica* (L.) Delile, *Acacia mellifera* (M.Vahl) Benth; was similar to *Thespesia populnea* (L.) Sol. ex Corrêa, *Ficus exasperata* Vahl.; was lower than *Acacia nubica* Benth (Kaitho et al. 1993; Apori et al. 1998; Abdulrazak et al. 2000). The degradability of CP of SO leaves was higher than *S. Mombin*, *B. nitida*, *G. Carpinifolia*, *G. Simplicifolea*, *G. Oppositifolia*, *Ziziphus mauritiana* Lam.; was similar to *A. Toxicaria*, *F. Exasperata;* was lower than *T. Populnea* (Apori et al. 1998; Khan et al. 2009).

The GP, estimated parameters of GP, ME, and OMD of SO leaves collected at different phenological stages are given in Table 3. The GP of SO leaves increased with increasing time of incubation. After 96 h incubation, the gas produced ranged between 20.68 to 27.53 mL/200 mg DM of the substrate. Significant differences between the SO leaves collected at different phenological stages in terms of GP at all incubation times were observed (P<0.05 for 48, 72, and 96 h; P<0.001 for 3, 6, 12, and 24 h). In contrast to incubation time, no significant differences were observed for the estimated GP parameters (P>0.05).

Table 3- *In vitro* gas production kinetics, Metabolisable Energy and Organic Matter Digestibility of *Styrax officinalis* L.

IT	***PF***	***FL***	***SL***	***FR***	***SEM***	***M***	***PS Effect***		
							L	***Q***	***C***
3	12.24^{x}	8.53^{w}	8.52^{w}	9.20^{w}	0.31	***	***	***	***
6	16.39^{x}	12.51^{w}	12.19^{w}	12.95^{w}	0.39	***	***	***	***
12	19.36^{x}	15.78^{w}	15.39^{w}	16.65^{w}	0.48	***	**	***	***
24	21.25^{x}	18.41^{w}	17.61^{w}	19.08^{xw}	0.59	***	*	**	**
48	23.87^{x}	20.49^{xw}	18.12^{w}	20.78^{xw}	1.21	*	NS	*	NS
72	25.59^{x}	22.32^{xw}	19.52^{w}	21.45^{xw}	1.44	*	NS	NS	NS
96	27.53^{x}	23.97^{xw}	20.68^{w}	22.69^{xw}	1.67	*	NS	NS	NS
a	1.05	1.23	0.38	1.02	0.23	NS	NS	NS	NS
b	23.55	20.82	19.34	20.86	1.31	NS	NS	NS	NS
a + b	24.60	22.05	19.71	21.88	1.47	NS	NS	NS	NS
c	0.174	0.131	0.185	0.175	0.018	NS	NS	NS	NS
Gas	21.25^{x}	18.41^{y}	17.61^{y}	19.08^{xy}	0.59	***	*	**	**
ME	6.26^{x}	5.49^{y}	5.30^{y}	5.36^{y}	0.08	***	***	***	***
OMD	43.46^{x}	37.78^{y}	36.36^{y}	36.65^{y}	0.52	***	***	***	***

IT: Incubation time (h); PS: Phenological stages; PF: Pre-flowering stage; FL: Flowering stage; SL: Seed linkage stage; FR: Fruiting stage; SEM: Standard error of means; M: Main effect; L: Linear effect; Q: Quadratic effect; C: Cubic effect; a: the gas production from the immediately soluble fraction (mL); b: the gas production from the insoluble fraction (mL); c: the gas production rate constant for the insoluble fraction (b); a + b: potential gas production (mL); Gas: Gas volume after 24 h of incubation; ME: Metabolizable Energy (MJ/kg DM); OMD: Organic Matter Digestibility (% DM). The values with different letters (x, y) in the same row are statistically different (P<0.05); NS: Not Significant;*: P<0.05; **: P< 0.01; ***: P<0.001

At all incubation times, the GP of SO leaves collected at the PF stage were significantly higher than those gathered from the other three stages. The relationship between obtained GP and phenological stages from this study showed a cubic trend extending the grazing period. These results were not in agreement with such studies, which showed a decreasing trend as the plant's period increased (Sileshi et al. 1996; Lee et al. 2000; Kamalak 2006). On the other hand, the GP of SO leaves were relatively lower than other shrubs such as *Atriplex amnicola*, *Atriplex nummularia*, *Atriplex semibaccata Acacia saligna*, *Maireana brevifolia*, *Rhagodia preissii* (Norman et al. 2010). The lower GP may be related to the species of rumen fluid taken and the high propionate production levels of SO leaves during fermentation. These statements are in agreement with Van Soest (1994) and Getachew et al. (1998).

Based on the estimated parameters of GP, the readily soluble (a), insoluble (b), and gas production rate (c) were not significantly affected by the phenological stages (P>0.05). This result may be related to the large variety of chemical composition cell wall strıcture of SO leaves. Givens et al. (2000) stated that the feed's chemical composition and cell wall structure are often influenced the degradation parameters. Ndlovu & Nherera (1997) noted that approximately 25% of the gas production variation was related to changes in the feed's ADF and NDF content. The findings of this research are in agreement with the results of those researchers.

4. Conclusions

The present study was designed to better understand the importance of SO leaves for goat feeding in shrublands by using *in situ* and *in vitro* methods. Although the findings should be interpreted with caution, this study has several strengths. Firstly, the findings clearly indicate that degradability of DM, CP, and ME content of SO leaves were ranged between moderate to high and significantly affected by phenological stages. Secondly, the nutritional composition of SO leaves depicted them as good forage sources for goats until the end of the FL stage. Thirdly, SO has the ability to give new shoots in parts that are broken, cut, or grazed for any reason, allowing the grazing period to extend a little more. On the other hand, there is a limitation need to be noted regarding the grazing or feeding with SO leaves, which lies in the fact that the high CT levels. Further *in vivo* research is required to better understand voluntary consumption of SO leaves by goats and reduce the limiting effect of high CT levels on feed intake.

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Screening of Wild Strawberry Germplasm for Iron-deficiency Tolerance Under Hydroponic Conditions

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ABSTRACT

Cultivated strawberry has been developed from hybridization between *Fragaria chiloensis* and *F. virginiana*. The progenitor species exhibit significant genetic diversity. Growth attributes of progenitor species and their responses to several stress factors have been studied. However, iron-deficiency tolerance (FeDT) of different species have merely been tested under hydroponic conditions. This study evaluated FeDT of 23 genotypes belonging to super-seed collection under hydroponic conditions. Two genotypes (one Fe-deficiency tolerant and one sensitive) were selected from screening experiment and their physiological and morphological mechanisms playing role in FeDT were determined. Plant parameters associated with FeDT, i.e., pH of the growth medium, root Fe reductase activity, total and active Fe concentration of shoot were recorded. The Fe-efficiency of strawberry subspecies varied between 51% and 98%. Fe efficiency values also varied among subspecies. AukeLake and RCP37 belonging to *F. chiloensis* were highly resistant and sensitive to Fe-deficiency, respectively based on Fe efficiency values. A highly significant relationship was observed between Fe concentration and FeDT of the genotypes. Acidification of nutrient solution and root Fe reductase activity were closely related to high shoot iron concentration. Our findings indicated existence of a close relationship between root uptake and root to shoot translocation of Fe, which ultimately contribute greatly to FeDT among tested strawberry genotypes.

Keywords: Fe-deficiency, Tolerance, Fe reductase, Genetic resources, Genotype, Gensitivity, Strawberry, Physiological responses

1. Introduction

Iron (Fe) deficiency is a common nutritional problem in calcareous and alkaline soils of the Mediterranean basin, where horticultural crops are frequently cultivated (Álvarez-Fernández et al. 2006). Fe deficiency in soils and crops has been reported from various regions of Europe, including Spain (Sanz et al. 1992; Pastor et al. 2002), Greece (Tagliavini et al. 2000) and France (Ollat et al. 2003). Likewise, Turkish soils located in the Mediterranean basin are highly calcareous with 74% of the area containing >1% calcium carbonate ($CaCO_3$) (Eyupoglu 1999). Despite high total Fe content in agricultural soils, several physicochemical factors such as high pH, $CaCO_3$ and clay content, low moisture, organic matter and soil temperature reduce Fe availability for plants (Tagliavini & Rombola 2001; Marschner 2011). Low Fe availability causes significant yield and quality reduction of fruits, vegetables and grain crops (Hansen et al. 2006; Rombola & Tagliavini 2006; Álvarez-Fernández 2011).

Various plant species respond differently to Fe deficiency in soils (Vose 1982; Awad et al. 1994; Tagliavini & Rombola 2001; Chen et al. 2018). Cultivated strawberries, *Fragaria* × *ananassa* Duch., are among the most sensitive species to Fe-deficiency (Álvarez-Fernández et al. 2006; Pestana et al. 2011; Alkan Torun et al. 2013, 2014). Several studies have reported varied response of large number of strawberry cultivars to Fe deficiency in different growth mediums (Kafkas et al. 2007; Alkan Torun et al. 2013, 2014; Gama et al. 2016).

Strawberry is one of the most popular summer fruits. Strawberry fruits have unique, highly desirable taste and flavor that influence consumer preferences (Gundogdu et al. 2020). Cultivated strawberry has been originated from hybridization between *F. chiloensis* (L.) Mill. and *F. virginiana* Mill. Since that time, a few native clones have been used by the breeders; thus, cultivated strawberries have a narrow genetic base. Since parental species come from a wide geographical region and exhibit great genetic diversity, extended efforts have been made to sample (Hancock et al. 2001a) characterize (Hancock et al. 2001b; Hancock et al. 2003; Hancock et al. 2004), maintain and finally utilize (Hancock et al. 2002, Hancock et al. 2005) the native clones of *F. virginiana* and *F. chiloensis* (Hancock et al. 2001c; Hancock et al. 2010). A super core collection of native clones has been tested across several environments for horticultural attributes (Hancock et al. 2001c). Indeed, cultivated strawberry has been developed

by crossing the superior clones (Luby et al. 2008; Hancock et al. 2010). The response of super core collection to various biotic and abiotic stress factors have been thoroughly investigated (Serce & Hancock 2002; Serce et al. 2002; Serce & Hancock 2005; Lewers 2007). However, responses of the elite native clones to limiting plant nutrients merely been tested.

The objective of this study was to investigate the physiological responses of 23 strawberry genotypes of super core collection, belonging to *F. chiloensis* and *F. virginiana* subspecies to Fe deficiency under hydroponic conditions. Plants derived from the shoot-tip culture were grown in the nutrient solution with sufficient or deficit-Fe supply until the appearance of Fe chlorosis symptoms in young leaves. Changes in chlorophyll density (SPAD), shoot dry matter and concentration of total Fe in the shoot were determined to elucidate the differential responses of the genotypes to the Fe deficiency. Based on the genotypic response, the most sensitive and resistant genotypes were selected and their morphological and physiological mechanisms playing role in Fe-deficiency tolerance were investigated. The result will help breeders to improve Fe-deficiency tolerance of strawberry in the future studies.

2. Material and Methods

2.1. Plant material

Total 23 wild strawberry genotypes belonging to *F. chiloensis* and *F. virginiana* subspecies (Hancock et al. 2010) were used as plant material in the study. The subspecies and their genotypes (*n*=23) are listed in Table 1. A second experiment was conducted with one susceptible and one resistant genotypes selected from these 23 genotypes.

Table 1- List of *Fragaria chiloensis* and *Fragaria virginiana* sub-species and genotypes tested for iron-deficiency tolerance under hydroponic conditions

No.	*Subspecies/genotype*	*PI Number*	*Origin*
	F. chiloensis* ssp. *pacifica		
1	RCP 37	551445	California
2	WLH (Westport Light House-8)	551453	Washington
3	BSP 14	551459	Oregon
4	Pigeon Point (CA 1367)	551728	California
5	Auke Lake (CFRA 368)	551735	Alaska
6	CFRA 1267	612488	British Columbia
7	HM 1 (CFRA 1691)	612489	Oregon
8	Scotts Creek (CFRA 1692)	612490	California
	F. chiloensis* f. *chiloensis		
9	Darrow 72 (CFRA 24)	236579	Chile
10	CA 1541	551736	Peru
11	2 BRA 1A (CFRA 1075)	612316	Chile
12	NAH	612318	Ecuador
	F. chiloensis* f. *patagonica		
13	2 TAP 4B (CFRA 1092)	612317	Chile
	F. virginiana* ssp. *glauca		
14	BT3 (CFRA 1693; CA 1226)	612491	Utah
15	BH 2 (CFRA 1696; LH 5-1)	612494	South Dakota
16	LH 50-4	612495	Montana
17	RH 43 (CFRA 1698; N8688)	612496	Alaska
18	LH 30–4 (CFRA 1703)	612501	Montana
	F. virginiana* ssp. *virginiana		
19	NC 96-35-2	612323	Alabama
20	Eagle 14 (CFRA 1694)	612492	Ontario
21	JP 95-1 (CFRA 1435)	612570	Florida
22	RH 23	612498	Minnesota
23	NC 95-21-1	612569	Mississipi

2.2. Plant culture, treatments, tissue sampling and harvest

Shoot tips from runners of each genotype were cultured in a dedicated nutrient medium (Aka-Kacar & Cetiner 1992). When sufficient growth was achieved in the fourth subculture, tissues were transferred to main nutrient medium (Murashige & Skoog 1962). Adaptation of plant material from tissue culture to ambient conditions was performed in an inert perlite medium under greenhouse conditions for three weeks. The resulting plants were then transferred to culture pots filled with 2.7 L of nutrient solution containing 2.0 mM $Ca(NO_3)_2$, 0.7 mM K_2SO_4, 0.1 mM KH_2PO_4, 0.1 mM KCl, 0.5 mM $MgSO_4$, 1 µM H_3BO_3, 0.5 µM $MnSO_4$, 0.5 µM $ZnSO_4$, 0.2 µM $CuSO_4$ and 0.01 µM $(NH_4)_6Mo_7O_{24}$. Iron was supplied as Fe-EDTA at deficient (1 µM) or sufficient rate (100 µM) (Cakmak et al. 1987). Each pot contained four plants, and all treatments were repeated thrice. Culture solution was renewed every three days and aerated continuously. Plants were grown for six weeks and harvested as shoots and

roots when severe symptoms Fe deficiency-induced chlorosis appeared in young leaves. Fresh leaf and root tissue samples were taken for determination of chlorophyll concentration and Fe reductase activity, respectively. Fresh leaf samples were stored at 80 ºC until analysis, whereas the whole shoot samples were dried at 70 °C until a constant weight was gained.

2.3. Dry matter production, Fe efficiency and scoring of Fe deficiency symptoms

Biomass (g plant^{-1}) of the dried shoots was determined on an electronic balance. Iron efficiency was calculated by dividing shoot biomass of Fe-deficit treatment to Fe-sufficient treatment and expressed as percentage. This rate is commonly used in literature to indicate Fe-efficiency of species (Graham 1984). Visual scoring of Fe deficiency-induced chlorosis was evaluated based on the progression of chlorotic area on young leaves using a scale of 0 to 3 where 0 represented "no chlorosis" and 3 "severe chlorosis". The severity of Fe deficiency chlorosis in intact leaves was also measured using a portable chlorophyll meter (SPAD-502, Minolta, Japan).

2.4. Determination of total and active Fe concentration in shoot

Dried shoots were used for the analysis of total and active Fe concentrations after milling shoot samples to powder in an agate mill. Total Fe concentration was determined according to Ozturk et al. (2006) with slight modifications. Briefly, 125 mg (±5) ground shoot sample was digested in 2 mL of 30% H_2O_2 and 5 mL of 65% HNO_3 using a microwave reaction system (Mars Express CEM Corp., Matthews, NC) for 30 min. Following digestion, sample volume was brought to 20 mL by deionized water and filtered through quantitative filter paper. Iron concentration in extracts was analyzed with an inductively coupled plasma optical emission spectrometer (ICP-OES, Jobin-Yvon, JY138-Ultrace) and the results were checked against a standard reference material (SRM 1547 Peach Leaves, National Institute of Standards and Technology, Gaithersburg, MD, USA).

The concentration of active Fe was also analyzed by ICP-OES following extraction of 100 mg (±5) ground shoot sample in 10 mL of 1 N HCl for 2 h at 120 rpm (Takkar & Kaur 1984).

2.5. Determination of leaf chlorophyll concentration

Leaf samples of 100 mg (±5) were extracted in 10 mL of 80% acetone and centrifuged at 5000 *gravity* for 15 min. The supernatant was used to determine total chlorophyll concentration according to Lichtenthaler & Wellburn (1983) following measurement of optical densities of samples at 663 nm.

2.6. Determination of root Fe-reductase activity

The root Fe-reductase activity (the reducing capacity of roots for Fe^{3+} and Fe^{2+}) of the genotypes was measured according to Camp et al. (1987).

2.7. Experimental design and statistical analysis

The collected data was subjected to Shapiro-Wilk normality test for determining the normality, which indicated a normal distribution. Therefore, original data was used for statistical analysis. Two-way analysis of variance was carried out to determine the significance of data and least significant difference test at 5% probability was used to separate means where ANOVA indicated significant differences. Principal component analysis with Kaiser normalization was used for easier interpretation and better representation of the results. Data was analyzed using xlstat statistical software.

3. Results

Genotypes, Fe treatments and their interactions significantly affected all measured variables during screening experiment (Table 2).

Table 2- Mean square values and significance of SPAD, Shoot biomass and Fe concentration of strawberry genotypes grown with deficit (1 µM Fe) and sufficient (100 µM Fe) Fe supply

Source	*df*	*SPAD*	*Shoot biomass*	*Fe concentration*
Genotype	22	452.5*	117299*	10123*
Fe treatment	1	25808*	960815*	608250*
Genotype × Treatment	22	264.3*	18285*	5075*
Error	92	6.78	4314	435.3

df: degree of freedom; *:indicates significance at P<0.05 level

3.1. Severity of Fe deficiency symptoms

The occurrence of Fe deficiency symptoms (i.e., chlorosis in young leaves) and symptom scores were higher in genotypes sensitive to Fe-deficiency along with reduced SPAD values, shoot biomass and Fe efficiency (Table 3). Genotypes WLH and BSP-14 belonging to *F. chiloensis* ssp. *pacifica* subspecies had the highest, whereas LH 50-4 and 2BRA 1A belonging to *F. virginiana* ssp. *glauca* and *F. chiloensis* ssp. *chiloensis*, respectively observed the lowest symptom scores. Overall, *F. chiloensis* ssp. genotypes (except 2BRA 1A and 2 TAP-4B) expressed more severe symptoms of Fe deficiency compared to *F. virginiana* ssp. (Table 3).

Table 3- Iron deficiency symptom (chlorosis) scores, SPAD values, shoot biomass, Fe efficiency and total Fe concentration in shoots strawberry genotypes grown under deficit (1 µM Fe) and sufficient (100 µM Fe) Fe supply

Genotype	*Symptom Score*	*SPAD*		*Shoot biomass (mg plant^{-1})*		*Fe efficiency*	*Fe concentration (mg kg^{-1})*	
		Fe_1	*Fe_{100}*	*Fe_1*	*Fe_{100}*	*(%)*	*Fe_1*	*Fe_{100}*
				F. chiloensis* ssp. *pacifica				
RCP 37	2.5	10.5 l	48.9 cd	275 o-s	510 g-k	54	35 uv	95 l
WLH	3.0	10.2 l	53.4 a-d	169 rs	328 m-q	52	55 p-s	151 h
BSP-14	3.0	12.0 kl	53.7 a-d	116 s	186 qrs	62	30 v	154 gh
Pigeon Point	2.2	12.2 kl	52.5 bcd	339 m-q	683 b-e	50	33 uv	107 k
Auke Lake	1.5	27.3 i	51.8 cd	319 n-r	397 j-p	80	49 q-t	170 g
CFRA-1267	2.5	11.3 kl	47.7 de	320 n-q	563 e-i	57	41 tuv	121 j
HM1	2.2	13.3 kl	59.9 a	360 l-p	698 a-d	52	43 r-u	136 i
Scotts Creek	2.5	12.3 kl	52.1 cd	475 h-l	816 a	58	31 v	93 l
				F. chiloensis* f. *chiloensis				
Darrow-72	2.2	12.9 kl	48.4 d	519 f-j	601 c-h	86	44 q-u	104 kl
CA-1541	2.3	12.7 kl	49.3 cd	456 i-m	759 ab	60	40 tuv	131 ij
2 BRA 1A	0.5	50.9 cd	57.3 ab	462 i-m	637 b-g	73	59 n-q	191 f
NAH	2.2	10.3 l	47.4 de	480 h-k	723 abc	66	42 s-v	125 ij
				F. chiloensis* f. *patagonica				
2 TAP-4B	1.0	28.6 i	50 cd	318 n-r	563 d-i	56	44 q-u	205 e
				F. virginiana* ssp. *glauca				
BT 3	1.2	39.0 gh	42.0 e-h	473 h-m	683 b-f	69	56 pqr	189 f
BH 2	2.0	24.8 d	47.3 def	243 p-s	448 i-n	54	44 q-u	196 ef
LH 50-4	0.5	50.2 c	59.2 a	370 l-p	385 k-p	96	70 mno	307 a
RH 43	1.0	46.0 d-g	60.8 a	276 n-s	295 n-s	94	75 mn	315 a
LH 30-4	1.5	37.2 h	53.4 a-d	157 s	262 o-s	60	56 o-r	233 d
				F. virginiana* ssp. *virginiana				
NC 96-35-2	1.5	40.1 fgh	55.5 abc	370 l-p	378 l-p	98	61 n-q	319 a
Eagle-14	0.7	48.6 d	57.5 a	354 l-p	376 l-p	94	70 m	249 c
RH 23	2.0	23.9 ij	48.0 d	261 p-s	312 n-r	84	63 nqp	189 ef
NC 95-21-1	2.0	18.2 jk	50.8 cd	216 p-s	363 l-p	59	25 v	231 d
JP 95-1	2.2	16.2 k	50.6 cd	202 q-s	398 i-o	51	54 p-t	235 b
LSD(alpha=0.05)		4.33		106.51			33.83	

Means sharing different letters within a column statistically differ from each other.

Moreover, Fe deficiency symptom scores were in line with SPAD readings of intact leaves and chlorophyll concentration. The mean SPAD values of the *F. chiloensis* ssp. genotypes (except 2BRA 1A and 2 TAP-4B) were remarkably lower under Fe-

deficiency compared to *F. virginiana* ssp. genotypes. Lower SPAD values are considered as an indication of higher sensitivity of *F. virginiana* ssp. to Fe-deficiency. As expected, mean SPAD value significantly increased under sufficient-Fe availability, especially in sensitive genotypes to Fe-deficiency. Similar to the differences in sensitivity to Fe-deficiency between subspecies, genotypes of a given subspecies also differed in sensitivity to Fe-deficiency. The differences were highly prominent among the genotypes of *F. virginiana* ssp. *virginiana* and *F. chiloensis* ssp. *chiloensis* as indicated by broad differences in SPAD values (i.e., up to 4-5 fold). However, under sufficient-Fe treatment the differences in SPAD values among genotypes were much lower (Table 3).

3.2. Shoot biomass and iron efficiency

A great variation (116-519 mg plant^{-1}) was noted for shoot biomass among genotypes (Table 3). Variability in biomass was also similar under Fe-sufficient treatment. The subspecies with the highest and lowest biomass production under Fe-deficiency were *F. chiloensis* ssp. *chiloensis* and *F. chiloensis* ssp. *pacifica*, respectively. These results suggested that, subspecies of *F. chiloensis* were more sensitive to Fe-deficiency than *F. virginiana*. Compared to Fe-deficit conditions, biomass under Fe-sufficient treatment was 77%, 42%, 77%, 36% and 29% higher in *F. chiloensis* ssp. *pacifica*, *F. chiloensis* ssp. *chiloensis*, *F. chiloensis* ssp. *patagonica*, *F. virginiana* ssp. *glauca* and *F. virginiana* ssp. *virginiana*, respectively. Indeed, Fe-efficiency, an important variable in Fe-deficiency tolerance, was relatively higher (with few exceptions) among genotypes of *F. virginiana* ssp. *virginiana* and *F. virginiana* ssp. *glauca* subspecies with mean values of 77% and 73%, respectively (Table 3). The Fe efficiency was markedly lower in *F. chiloensis* spp., except Auke Lake and Darrow-72 genotypes, which are apparently Fe-efficient. Huge variation in biomass and Fe efficiency can be important traits in breeding programs aiming for Fe deficiency-tolerant and high yielding cultivars.

3.3. Shoot iron concentration

Shoot Fe concentration significantly varied among genotypes, especially under Fe-deficit conditions. The mean shoot Fe concentrations under Fe-deficit treatment were 60, 55, 46, 44 and 39 mg Fe kg^{-1} for *F. virginiana* ssp. *glauca*, *F. virginiana* ssp. *virginiana*, *F. chiloensis* f. *chiloensis*, *F. chiloensis* f. *patagonica* and *F. chiloensis* ssp. *pacifica*, respectively (Tables 3). Iron-deficit treatment severely reduced shoot Fe concentration in all genotypes at variable rates. For example, in NC 95-21-1 Fe concentration was reduced from 231 to 25 mg kg^{-1} as compared to Darrow-72 from 104 to 44 mg kg^{-1}, corresponding to 9.2 to 2.4 fold of difference in shoot Fe concentration, respectively (Tables 3).

The Fe-efficient subspecies and genotypes had higher shoot Fe concentrations than subspecies with lower Fe efficiency (Table 3). This finding indicated the significance of shoot Fe concentration in Fe efficiency of the genotypes under Fe-deficit conditions. Thus, a significant correlation (R^2 = 0.49, P<0.001) between the shoot Fe concentrations of genotypes under Fe-deficit treatment was observed.

3.4. Principal component analysis

Principal component analysis (PCA) yielded two principal components with eigenvalues greater than one. These two axis explained 80.55% of the total variation in the data (Table 4). All of the measured variables significantly contributed towards the variability. The genotypes were divided into 2 groups. The first group had similar SPAD values and Fe uptake, which contained 5 genotypes. The second group had genotypes with similar biomass production and contained 7 genotypes. The remaining 11 genotypes had variable values of the measured traits (Figure 1). The PCA did not group the genotypes on subspecies, indicating that genotypes belonging to different subspecies exhibit similarities in the measured traits.

Table 4- Factor loading and variability explained by first two axis of the principal component analysis.

Variables	*PC1*	*PC2*
SPADFe-	0.81	0.46
PADFe+	0.64	0.11
BiomassFe-	-0.36	0.91
BiomssFe+	-0.72	0.63
FeFe-	0.83	0.37
FeFe+	0.91	0.03
Eigenvalue	3.24	1.59
Variability (%)	53.97	26.58
Cumulative %	53.97	80.55

Fe- and Fe+ indicate Fe-deficit and Fe-sufficient treatments, respectively.

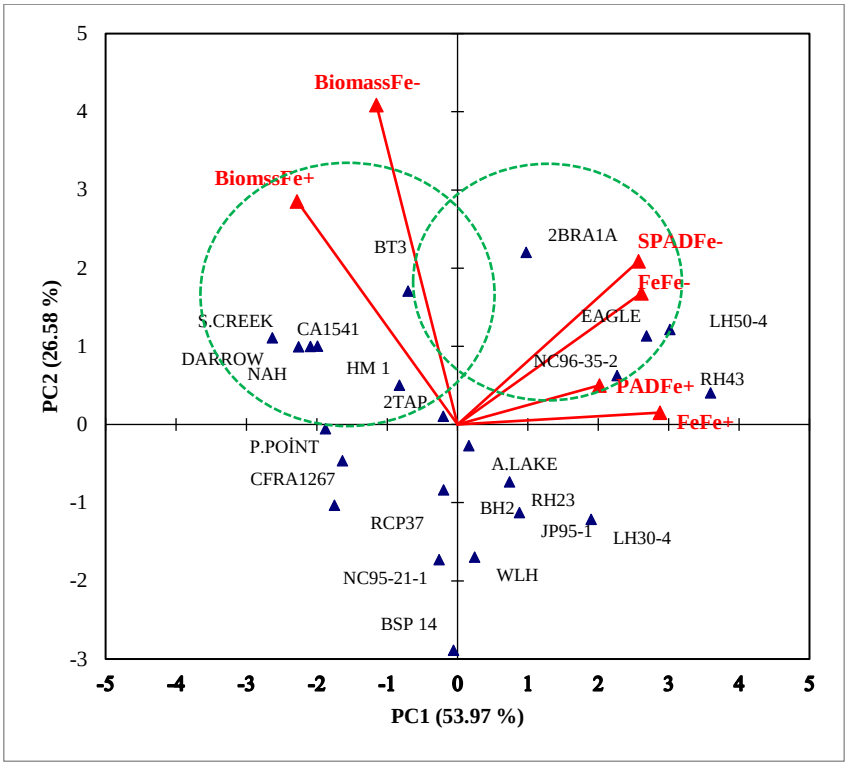

Figure 1- Biplot of first two axis of principal component analysis executed on the biomass, SPAD values and Fe accumulation of 23 strawberry genotypes

3.5. Important characteristics in iron-deficiency tolerance

The second set of experiment was conducted by using RCP37 (Fe-inefficient: 54%) and Auke Lake (Fe-efficient: 80%) genotypes of *F. chiloensis* ssp. *pacifica* subspecies to determine the important characters for Fe-deficiency tolerance. Since strawberry is a strategy-I plant, root Fe reductase activity and rhizosphere acidification are significant factors. Chlorophyll density of leaves (SPAD values), total and active Fe levels were determined. Fe-deficit (1 µM) and Fe-sufficient (100 µM) treatment significantly ($P<0.05$) affected all measured variables (Table 5). Both genotypes showed chlorosis under Fe-deficit supply; however, symptoms were more severe in Fe-inefficient RCP 37 genotype. The SPAD readings and chlorophyll values RCP 37 genotype were 13 and 0.6 mg g^{-1}, while the Fe-efficient Auke Lake had higher values for these variables (19 and 1.2 mg g^{-1}) (Table 5). The Fe-efficient genotype produced 837 mg $plant^{-1}$ dry matter under Fe-deficit supply, while Fe-sensitive genotype could only produce about half of this dry matter. However, the dry matter production of both genotypes was higher under sufficient Fe supply (1145 and 510 mg $plant^{-1}$ for Auke Lake and RCP 37, respectively) corresponding to 37% and 56% increase compared to Fe-deficit treatment (Table 5; Figure 2).

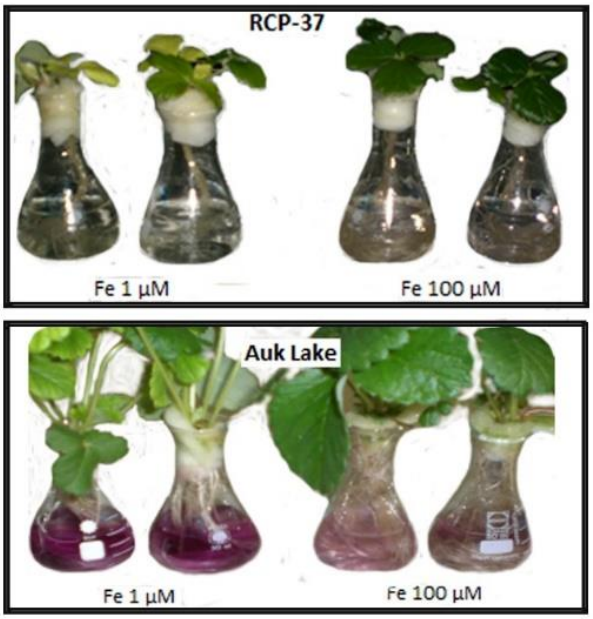

Figure 2- Changes in root Fe-reductase activity of Auke Lake and RCP-37 strawberry genotypes grown under deficit and sufficient Fe supply

Higher production of biomass under deficit and sufficient Fe supply by Auke Lake genotype can be related to its higher root Fe uptake ability (Table 5). Both genotypes maintained a similar pH level (6.3 and 6.4) in the culture solution under sufficient Fe supply. However, Auke Lake reduced the solution pH (4.8) to a higher extent compared to RCP 37 (5.3) under Fe-deficit condition (Table 4). Furthermore, Auke Lake expressed higher root Fe-reductase activity as compared to RCP 37 (8.3 and 6.6 µmol g root^{-1} 2 h^{-1}, respectively) under Fe-deficit conditions. Nonetheless, both genotypes had a similar Fe-reductase activity under Fe-sufficient condition (i.e., 1.4-1.0 µmol g root^{-1} 2 h^{-1}) (Table 5 and Figure 3).

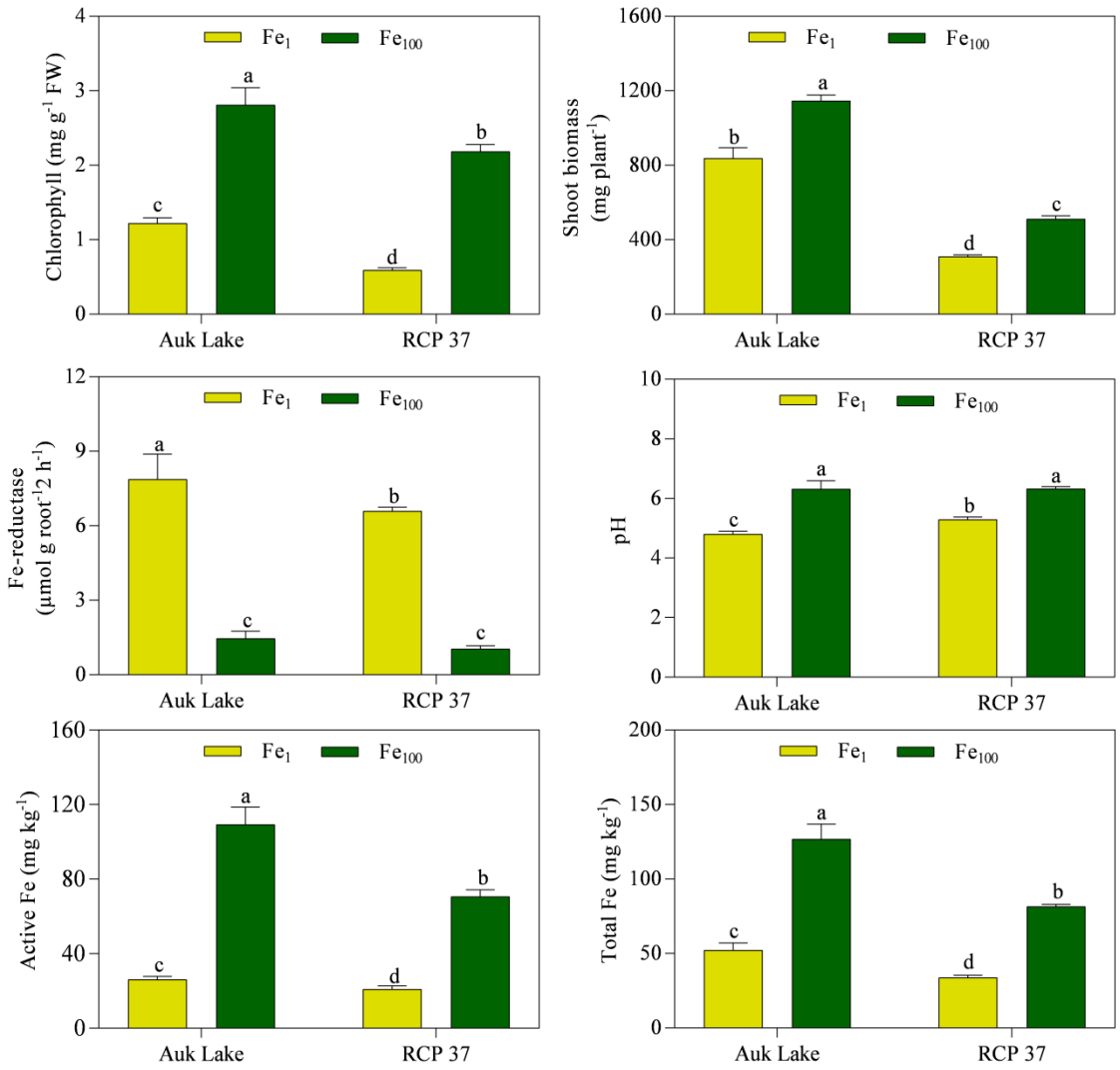

Figure 3- Changes in chlorophyll concentration shoot biomass, nutrient solution pH, root Fe-reductase activity, total and active Fe concentrations in shoots of Auke Lake and RCP 37 strawberry genotypes grown under deficit and sufficient Fe supply

Table 5- Changes in SPAD, chlorophyll concentration, shoot biomass, nutrient solution pH, root Fe-reductase, and total and active Fe concentrations in shoots of Auke Lake and RCP 37 strawberry genotypes under Fe-deficit (1 µM) and Fe-sufficient (100 µM) Fe supply

Variable	Fe_1		Fe_{100}		Mean squares and significance			
	Auke Lake	RCP 37	Auke Lake	RCP 37	Genotype (G)	Fe Treatment (T)	G × T	Error
SPAD	19 ± 1.5c	13 ± 1.2d	38 ± 0.7b	42 ± 1.7a	3.4	2275.3*	96.0*	1.80
Chlorophyll (mg g^{-1} FW)	1.2 ± 0.1	0.6 ± 0.03	2.81 ± 0.2	2.18 ± 0.1	1.17*	7.61*	0.00	0.02
Shoot biomass (mg $plant^{-1}$)	837 ± 82c	309 ± 17d	1145 ± 33a	510 ±25b	1013264*	194820*	8587*	1197
Nutrient solution pH	4.8 ± 0.4c	5.3 ± 0.1b	6.4 ± 0.2a	6.3 ± 0.1a	0.28	6.64*	0.40*	0.07
Fe-reductase (µmol g $root^{-1}$ 2 h^{-1})	8.3 ± 0.9a	6.6 ± 0.1b	1.4 ± 0.2c	1.0 ± 0.1c	3.91*	151.4*	1.41*	0.28
Total Fe (mg kg^{-1})	52 ± 4.8c	34 ± 1.5d	127 ± 10a	82 ± 1.4b	3035*	11239*	534*	32.3
Active Fe (mg kg^{-1})	26 ± 1.8c	21 ± 1.9c	109 ± 9a	71 ± 3b	1404*	13377*	870*	26.4

*indicates significance at P<0.05 level.

4. Discussion

Different genotypes significantly differed for all measured variables under sufficient and deficit Fe supply. The SPAD values indicating chlorophyll levels of the genotypes and consequently Fe-deficiency tolerance (FeDT) varied among subspecies and genotypes of the same subspecies. SPAD values were less than 20 under Fe deficiency for majority of the strawberry genotypes. Chlorophyll contents increased with Fe application and the SPAD values were generally around 50 under sufficient Fe supply. Gama et al. (2016) reported similar for SPAD values under Fe deficiency in strawberry. Pestana et al. (2012a) compared the responses of carob (*Ceratonia siliqua* L.) and three-foil lemon (*Poncirus trifoliata* (L.) Raf) tree rootstocks against Fe deficiency under hydroponic conditions with different Fe levels. Significant reductions in growth and SPAD values were reported for three-foil lemon under Fe-deficit treatment. However, growth and SPAD readings for carob were similar under all Fe treatments.

Retarded plant growth, decreased dry matter, reduced shoot, grain and fruit yields are among the major impacts of Fe chlorosis. Fe deficiency reduced biomass production of all subspecies included in the study compared to sufficient Fe supply (Table 3). In the second experiment, increased dry matter under sufficient Fe supply was 27% and 36% for Auke Lake and RCP 37 genotypes, respectively (Table 5). Results revealed that decreased biomass of tested genotypes by Fe deficiency was correlated with symptom scores under Fe deficiency. Similar decreases in yield and yield components of crops caused by Fe-deficiency chlorosis have been reported in various studies (Álvarez-Fernández et al. 2011; Gama et al. 2016). For example, fruit yield in pear trees without chlorotic leaves was 65 kg tree^{-1} compared to trees exhibiting mild chlorosis (23.7 kg tree^{-1}) (Álvarez-Fernández et al. 2011). Similarly, fruit yield in peach trees with none (SPAD values of 39-43), mild (SPAD values of 24-44) and severe (SPAD values of 18-24) Fe-deficiency chlorosis were 128, 21.8 and 33.8 kg tree^{-1}, respectively (Álvarez-Fernández et al. 2011). Consequently, overall decrease in fruit yield in trees with mild chlorosis was about 64% in pear and 83% in peach.

In the current study, Fe deficiency caused significant reduction in chlorophyll contents, biomass and leaf Fe concentrations. Average leaf Fe contents of *F. chiloensis* ssp. *pacifica* genotypes under sufficient and deficit Fe supply were 128 and 39 mg kg^{-1}, respectively, corresponding to 70% decrease (Table 3). The decrease in *F. chiloensis* ssp. *chiloensis*, *F. chiloensis* ssp. *patagonica*, *F. virginiana* ssp. *glauca* and *F. virginiana* ssp. *virginiana* subspecies were 67, 79, 76 and 77%, respectively. Similar decrease in Fe concentration has also been reported by Jelali et al. (2010) in pea and Gama et al. (2016) in strawberry. Plant tissue Fe concentration of Merveille de Kelvedon and Lincoln pea cultivars was 15.0 and 17.4 mg kg^{-1} under sufficient Fe supply compared to 9.7 and 10.4 mg kg^{-1} under deficient Fe. However, the lowest Fe concentration was observed in plants receiving lime treatments where Fe concentration was reduced by 35.3% in Merveille de Kelvedon and 40.4% in Lincon compared to the non-limed treatment (Jelali et al. 2010).

Our results indicated positive correlation between Fe efficiency values and leaf Fe concentrations under Fe deficiency. This finding indicates the existence of variation in the uptake, transport and/or utilization of Fe in strawberry subspecies and genotypes. Jelali et al. (2010) also reported variation in Fe efficiency in pea grown under sufficient and deficit Fe supply.

Growth medium acidification mediated by H^+-ATPase, Fe-reductase and release of organic acids from roots are specified as significant mechanisms for Fe uptake conferring FeDT (Han et al. 1998; Vizzotto et al. 1999; Jelali et al. 2010; Pestana et al. 2012b; Gama et al. 2016). Proton release level and ability of growth medium acidification substantially varied among strawberry genotypes tested in the current study. The initial nutrient solution pH values (6.3-6.4) decreased to 4.8 in Auke Lake and 5.3 in RCP 37 genotype under deficit Fe supply (Table 5; Figure 2). These results are in line with Pestana et al. (2012b) where similar decrease in solution pH was reported in strawberry under deficit Fe supply. Similar findings were also reported for pea cultivars under Fe deficiency (Jelali et al. 2010). Both pea cultivars acidified the growth medium under Fe-deficit conditions and the greatest decline (pH 3.35) was observed in Marveille de Kelvedon cultivar. In a study with different quince and pear genotypes grown in a calcareous soil, rhizosphere pH values of quince genotypes were higher than pear genotypes (Tagliavini et al. 1995). Fe-efficient *Malus* × *iaojinensis* (apple) genotype decreased rhizosphere pH by 2 units in calcareous soils under Fe deficiency (Han et al. 1998, 1994). Mengel & Malissovas (1982) investigated H^+ release from the roots of Huxel and Faber vine cultivars into the nutrient solution and reported severe chlorosis in Huxel grown on calcareous soils. Faber was Fe deficiency tolerant as it released 406 µmol H^+ per plant from the roots in 12 hours, whereas Huxel released only 173 µmol 12 h^{-1} plant^{-1} (Mengel & Malissovas 1982). Our results suggested that variation in proton release capacity of strawberry genotypes under Fe-limiting conditions can be exploited as a selective trait in breeding new cultivars with higher FeDT.

The Fe^{+3} reduction ability of genotypes into Fe^{+2} through Fe-reductase enzyme activity of roots is another significant character for FeDT. The Fe-reductase enzyme activity in the Fe-efficient genotype was induced to a higher extent compared to the Fe-inefficient genotype (Table 5; Figure 2). Higher Fe-reductase activities have been reported for various Fe-efficient plant species. Pear rootstock *Cydonia oblonga* is classified as Fe-sensitive and *Pyrus communis* as Fe-resistant. Fe-reductase activities of pears under Fe deficiency increased with Fe application, though a similar increase was not occurred in quince (Tagliavini et al. 1995). The effect of HCO_3^- on Fe-reductase activity varied between quince and pear and caused a higher decrease in quince compared to the pear. Variation in Fe-reductase activity was related to the higher decreases in the rhizosphere pH of Fe-resistant *P. communis* than *C. oblonga* (Tagliavini et al. 1995). In contrast, Alcantara et al. (2000) reported that Fe-reductase activity was not always related to Fe-chlorosis. These reports indicate that the mechanisms involved in FeDT could be mediated by multiple environmental factors, including but not limited to Fe availability in the growth media.

5. Conclusions

The results of the present study found substantial variation in Fe efficiency (ability to grow under limited Fe supply) among and within *Fragaria chiloensis* and *Fragaria virginiana* strawberry subspecies and genotypes. Proton (H+) release capacity and Fe-reductase enzyme activity in the roots, and total and active Fe concentration in shoots were found to be important in resistance to iron deficiency for strawberry genotypes. The results of the current study can be used to improve iron deficiency tolerance in cultivated strawberry.

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Determination of Proper Turning Frequency to Increase for Hatching Results in Hatching Eggs With Abnormal Shape Index

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ABSTRACT

The aim of this study was to determine the optimum turning frequency for increasing the hatchability results in hatching eggs with abnormal shape index. For this purpose, 4050 hatching eggs obtained from 56 weeks old ATAK-S hybrid hens were used. Shape index was divided into 3 groups as 71 and smaller, 72 – 76, 77 and higher. The research was conducted according to a completely randomized factorial experimental design. The hatching eggs were incubated with turning frequency of 30, 60 and 120 minutes. In the present study, fertility rate, hatchability, hatch of fertile, early, middle, late embryonic mortality, discard chick rate, chick quality, malformation and malposition rates were determined. The turning frequency was found to have a significant effect on hatch of fertile eggs and late embryonic mortality. It was found that turning frequency and shape index had no significant effect on the hatching results regarding discarded chicks, early embryonic mortality, malposition, malformation rate, and chick quality. Interaction between egg shape index and turning frequency did not influence hatching traits and chick quality. It was determined that there was no interaction effect between turning frequency and shape index on all the characteristics discussed. According to the results obtained from the research, it was observed that the 30 and 120 minute turning frequency of hatching eggs with different shape indexes had a negative effect on the hatchability of fertile eggs and late embryo mortality. It was concluded that even if the shape index of the hatching eggs were different, the optimum turning frequency should be every 60 minutes.

Keywords: Abnormal eggs, Hatching results, Shape index, Turning frequency

1. Introduction

Hatchery procedures is one of the important steps in poultry production, since nowadays hatching equipment of high-developed technology is available. Environmental factors such as temperature, humidity, ventilation and turning are automatically regulated, which provides more than 90% hatching efficiency (Yıldırım & Yetisir 2004; Bruggeman et al. 2007; Elibol 2009; King'ori et al. 2011).

Despite a visible effort by breeding enterprises, it has been determined that it is impossible to produce eggs at a level that will meet the incubation needs. In studies conducted up to date, it has been reported that some egg parameters could lead to embryo death (Narushin & Ramanov 2002; Durmuş 2014).

It has been reported that turning the eggs in the first 18th day of incubation is essential for the proper development of the embryonic membranes and the proper positioning of the embryo (Eycleshymer 1907; New 1957; Robertson 1961a; Robertson 1961b; Lundy 1969; Deeming 1989). While some studies have reported optimum turning frequency as 96 times daily (Elibol & Brake 2003; Wilson et al. 2003), turning 24 times a day was reported sufficient in commercial hatcheries due to similar results of 96 times a day (Freeman & Vince 1974). It was stated that the impairment of the position in the embryo may be due to insufficient turning frequency, improper turning angle (Byerly & Olsen 1931; Byerly & Olsen 1933; Robertson 1961a; Landauer 1967; Lundy 1969; Wilson et al. 2003), the absence of turning in the first week of incubation and older flock age (Elibol & Brake 2004).

Egg quality is an important factor that affects hatching results (Elibol 2009). Characteristics of the egg quality can be classified in two groups: internal and external quality. External quality characteristics are egg weight, shape index, and shell quality (Narushin & Romanov 2002; Sarıca & Erensayın 2009; Durmus 2014). Hatching eggs with a shape index of 72 - 76 have the optimum shape index value, while hatching eggs with shape index below and above these values have low hatchability and hatchability of fertile eggs, whereas late-stage embryo mortality and malposition rates are significantly increased (Ascı &

Durmus 2015). Although the low angle of turning of the eggs during incubation increases the malposition rate, it has been reported that this rate can be reduced by increasing the number of turning of the eggs (Elibol & Brake 2004).

As mentioned above, high rates of malposition have been reported to negatively affect hatching results. It has been predicted that the frequency of incubation may be the solution to this situation. In order to increase the hatching results of abnormal eggs, in this study, 30, 60 and 120 minutes of turning frequencies were applied to the eggs with low, high and normal shape indexes and the effects on hatching results have been determined.

2. Material and Methods

The research was implemented with 4050 hatching eggs obtained from 56 weeks old ATAK-S hybrid parents that were classified into shape index groups as described below with Rauch shape index measuring instrument.

1) Shape index 71 and smaller
2) Shape index 72 - 76
3) Shape index 77 and above

Hatching eggs were stored for 7 days in a room at 15 °C and 80% relative humidity. The turning frequency on the incubator was set to 30, 60 or 120 minutes. The study was carried out in random blocks in 3 * 3 factorial experimental designs with 3 replications. Incubation trays with a hatching capacity of 150 eggs were considered as replicate. Therefore 3 * 3 * 3* 150 = 4050 hatching eggs have been used. The eggs classified according to shape index were placed in trays and placed in Pas Reform brand 19.200 capacity smart type incubators, and pre-warmed in rooms with 24 °C and 75% relative humidity for 8 hours, then transferred to the incubator with a temperature of 37.8 °C and relative humidity of 50%. After incubation for 18 days and being checked for fertility, eggs were transported to hatching machines providing 36.5-37 °C temperature and 55 - 78% relative humidity. Chicks were hatched from the eggs that had been kept there for 3 days. During the research, the following characteristics have been determined and calculated with the formula below. Fertility was determined by cracking the eggs separated by lamp control on the 18th day of the incubation. Poor quality chicks that cannot be sold was described as discarded chick.

Fertility: (Number of fertile eggs / Number of eggs placed in incubator) * 100.
Hatchability: (Number of alive chicks hatched/ Number of eggs placed in incubator) * 100.
Hatch of fertile (Number of alive chicks hatched / number of fertile eggs) * 100.
Early embryonic mortality: (Number of embryos died between 0-6 days of incubation / Number of fertile eggs) * 100.
Mid embryonic mortality: (Number of embryos died between 7-18 days of incubation / Number of fertile eggs) * 100.
Late embryonic mortality: (Number of embryos died between 19-21 days of incubation / Number of fertile eggs) * 100.
Discarded chicks: (Number of discarded chicks / Number of eggs placed in incubator) * 100.
Chick quality: Pasgar score was used as chick quality determination method developed by Pas Reform (Boerjan 2006).
Malformation and Malposition: Eggs with death embryo in shell were examined and malformation and malposition rates were determined.

2.1. Statistical analysis

For all the examined parameters, normal distribution of the data was checked with the Kolmogorov-Simirnov test and the homogeneity of the group variances was assessed with the Levene test. General Linear Model (GLM) analysis was applied to evaluate the features that fulfill the assumptions, and Tukey multiple comparison tests at 5% significance level was used to detect significant differences. Minitab 16 package statistics program was used to evaluate the data.

The applied mathematical model is provided below.

$$Y_{ijk} = \mu + a_i + b_j + ab_{ij} + e_{ijk}$$

$Y_{ijk,}$The value of any of the research parameters; μ,Population average; $a_{i,}$ Effect of the turning frequency (30-60-120 minutes); $_{j,}$ Effect of the shape index (≤ 71, 72 – 76, ≥ 77); ab_{ij} = Interaction between turning frequency (i) and shape index (j); e_{ijk}, Experimental error

3. Results and Discussion

As indicated, results of the evaluation of the hatchability data are presented in Table 1. It was determined that the turning frequency, shape index and their interaction had an insignificant effect on hatchability (P>0.05).

Table 1- Effects of egg shape index and turning frequency on hatchability

Turning Frequency	*Shape Index*			*Average*
	≥ 77	*72 - 76*	*≤ 71*	
30 minutes	68.44±2.62	71.11±1.78	71.33±7.33	70.17±1.80
60 minutes	73.56±1.82	75.78±0.80	74.33±2.33	74.58±0.87
120 minutes	69.56±1.60	70.89±0.589	75.62±0.39	71.57±1.07
Average	70.52±1.29	72.59±0.99	73.76±2.15	

Hatchability of fertile eggs data is shown in Table 2. It has been determined that the effect of turning frequency on this parameter was significant (P<0.05). The egg shape index value did not have a significant effect on the hatchability of fertile eggs and turning frequency * shape index interaction was insignificant (P>0.05).

Table 2- Effects of egg shape index and turning frequency on hatchability of fertile eggs

Turning Frequency	*Shape Index*			*Average*
	≥ 77	*72 - 76*	*≤ 71*	
30 minutes	79.74±2.05	84.65±1.45	81.78±4.98	82.09±1.49 [b]
60 minutes	86.44±0.84	85.056±0.64	87.44±2.40	86.17±0.67 [a]
120 minutes	79.63±0.61	80.20±1.76	86.34±0.02	81.52±1.22 [b]
Average	81.94±1.31	83.30±1.04	85.19±1.80	

[ab] Values within a column with different superscripts differ significantly at P<0.05

The outcomes obtained as a result of the evaluation of the fertility data are presented in Table 3. It was found that the effects of turning frequency, shape index and turning frequency*shape index interaction on the fertility rate were also insignificant (P>0.05).

Table 3- Effects of egg shape index and turning frequency on fertility

Turning Frequency	*Shape Index*			*Average*
	≥ 77	*72 - 76*	*≤ 71*	
30 minutes	85.78±1.24	84.00±1.39	87.00±3.67	85.42±1.03
60 minutes	85.11±2.26	89.11±1.46	85.00±0.33	86.58±1.15
120 minutes	87.33±1.54	88.44±1.24	87.58±0.42	87.81±0.68
Average	86.07±0.93	87.19±1.05	86.53±1.08	

The findings of early embryo mortality rate are provided in Table 4. On the early embryo mortality rate, the turning frequency, shape index and turning frequency*shape index interaction were determined to be insignificant (P>0.05).

Table 4- Effects of egg shape index and turning frequency on early period embryo mortality rate

Turning Frequency	*Shape Index*			*Average*
	≥ 77	*72 - 76*	*≤ 71*	
30 minutes	12.22±1.49	6.08±1.29	9.07±6.13	9.13±1.66
60 minutes	7.01±0.74	7.48±1.31	5.49±0.76	6.81±059
120 minutes	9.91±1.23	8.75±1.66	7.91±2.61	8.97±0.89
Average	9.71±0.96	7.44±0.81	7.49±1.86	

The findings of the mid-term embryo mortality rate are illustrated in Table 5. Turning frequency, shape index and turning frequency*shape index interaction were also found to be insignificant on medium-term embryo mortality rate (P>0.05).

Table 5- Effects of egg shape index and turning frequency on mid-term period embryo mortality rate

Turning Frequency	*Shape Index*			*Average*
	≥ 77	*72 - 76*	*≤ 71*	
30 minutes	2.61±0.73	1.58±0.45	1.17±0.43	1.86±0.37
60 minutes	2.07±0.90	2.50±0.28	3.14±1.58	2.50±0.46
120 minutes	2.30±0.46	1.99±0.64	0.38±0.38	1.70±0.40
Average	2.33±0.37	2.02±0.27	1.56±0.68	

Data related to the late embryo mortality rate are presented in Table 6. It has been determined that the effect of turning frequency on this feature was significant (P<0.05). It was determined that the egg shape index value did not have a significant effect on the late embryo mortality rate and the turning frequency*shape index interaction was insignificant (P>0.05).

Table 6- Effects of egg shape index and turning frequency on late period embryo mortality rate

Turning Frequency	*Shape Index*			*Average*
	≥ 77	*72 – 76*	*≤ 71*	
30 minutes	5.43±0.72	7.43±0.82	6.81±2.01	6.53±0.62 [b]
60 minutes	3.93±0.47	4.47±0.80	3.93±1.58	4.13±0.44 [a]
120 minutes	7.65±1.19	8.56±0.58	4.99±1.83	7.32±0.77 [b]
Average	5.67±0.69	6.82±0.71	5.24±0.97	

[ab] Values within a column with different superscripts differ significantly at P<0.05

Data on discarded chicks (Table 7), malposition rate (Table 8), malformation rate (Table 9) and pasgar score chick quality values (Table 10) were not significantly affected by the turning frequency, shape index and turning frequency*shape index interaction (P>0.05).

Table 7- Effects of egg shape index and turning frequency on discarded chick rate

Turning Frequency	*Shape Index*			*Average*
	≥ 77	*72 - 76*	*≤ 71*	
30 minutes	0.00±0.00	0.22±0.22	1.00±0.33	0.33±0.18
60 minutes	0.44±0.44	0.44±0.22	0.00±0.00	0.33±0.18
120 minutes	0.44±0.22	0.44±0.22	0.33±0.33	0.42±0.12
Average	0.30±0.16	0.37±0.12	0.44±0.22	

Table 8- Effects of egg shape index and turning frequency on malposition rate

Turning Frequency	*Shape Index*			*Average*
	≥ 77	*72 - 76*	*≤ 71*	
30 minutes	2.59±0.24	3.19±0.50	3.44±0.24	3.03±0.23
60 minutes	2.60±0.65	4.72±0.86	4.32±1.19	3.83±0.55
120 minutes	5.12±1.15	6.05±0.80	3.70±160	5.11±0.65
Average	3.44±0.57	4.65±0.55	3.82±0.54	

Table 9- Effects of egg shape index and turning frequency on malformation rate

Turning Frequency	*Shape Index*			*Average*
	≥ 77	*72 - 76*	*≤ 71*	
30 minutes	0.51±0.26	0.27±0.27	0.40±0.40	0.39±0.15
60 minutes	0.53±0.27	0.00±0.00	0.00±0.00	0.20±0.13
120 minutes	0.50±0.25	0.25±0.25	0.38±0.38	0.38±0.14
Average	0.52±0.13	0.17±0.12	0.26±0.16	

Table 10- Effects of egg shape index and turning frequency on chick quality pasgar score values

	Shape Index			
Turning Frequency	*≥ 77*	*72 - 76*	*≤ 71*	*Average*
30 minutes	9.76±0.003	9.81±0.017	9.74±0.134	9.77±0.028
60 minutes	9.76±0.047	9.80±0.026	9.77±0.043	9.78±0.021
120 minutes	9.67±0.090	90.79±0.020	9.77±0.013	9.74±0.036
Average	9.73±0.032	9.80±0.011	9.76±0.037	

Late embryo mortality rate was significantly lower at 60 minutes of turning frequency than the other frequencies. The hatchability of fertile eggs was significantly higher at the turning frequency of every 60 minutes compared to the other frequencies. In the case of the eggs with different shape indexes, it was observed that the turning done at an angle of 90^0 degrees every 60 minutes gave better results. It is considered that the hatchability of fertile eggs is higher in this group than the other turning groups due to the lower rate of late embryo mortality. However, this situation does not appear to have a positive effect on hatchability. It is suggested that a positive effect will occur on hatchability by increasing the number of replicates. It has been observed that egg shape index and turning frequency have no effect on the other hatching features that are examined in the present study.

Freeman & Vince (1974) stated that it is enough to turn eggs 24 times a day in commercial hatcheries. The results obtained in our research in terms of turning frequency support this finding. Baspınar et al. (1997) reported a positive correlation (r = 0.809) between embryonic mortality and shape index in their study where the incubation characteristics of Japanese quail eggs depend on the egg weight and shape index. However, there was no significant difference in embryo mortality rates in terms of shape index. They determined that the effect was insignificant. Esen & Ozcelik (2002), Turkyılmaz et al. (2005), Yılmaz & Cağlayan (2008) and Sari et al. (2010) reported that the effect of shape index on incubation characteristics was insignificant in their studies in quails. It is observed that the results obtained for the shape index in the present study are in line with these findings. Ascı & Durmus (2015) reported that the egg shape index has a significant effect on the late embryo mortality rate, hatchability of fertile eggs, hatchability and malposition, and eggs with normal shape index have a better outcome than others. Durmus (2014), Narushin & Ramanov (2002) reported that the hatchability of fertile eggs is much lower in the round shaped eggs than the pointed ones. However, while the effect of the shape index discussed on the hatching results was found to be insignificant, it was determined that the effect of the turning frequency on the hatchability of fertile eggs and late-stage embryo mortality rate was important. Considering these findings it is concluded that the outcomes of the study differ. Further work is essential to clearly define these discrepancies.

According to the results obtained from the present study, it was determined that the 30 and 120 minute turning frequency of hatching eggs with different shape indexes had a negative effect on the hatchability of fertile eggs and late embryo mortality. It was concluded that even if the shape index of the hatching eggs were different, the optimum turning frequency should be every 60 minutes.

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Identification of *S*-Allele Based Self-incompatibility of Turkish Pear Gene Resources

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ABSTRACT
Self-incompatibility is considered to be a growth-limiting factor in fruit plants. In species with hermaphrodite flowers, *S*-locus (*S*-allele) has been accepted to control self-incompatibility, and the genetic control of this locus is provided by multiple genes (alleles). Pear (*Pyrus communis* L.) belongs to the *Pomoideae* from the *Rosaceae* family and is found to have great genetic potential in terms of ecological features in Turkey. To protect these cultivation features, national garden collections have been established across the country and *Atatürk Horticultural Central Research Institute–Yalova* collection is considered as genes bank. Identification of the different features of this collection (fruit quality, stress tolerance, self-incompatibility, grafting incompatibility, etc.) is of great importance for its utilization in pear breeding and cultivation. However, to our knowledge, this collection has not been characterized for self-incompatibility trait. In the current study, PCR (Polymerase Chain Reaction)-based amplification of the *S*-allele regions (S_1, S_6, S_7, S_8) causing the self-incompatibility in 180 pear genotypes obtained from the national pear germplasm was investigated by molecular biological methods based on the comparison of amplified products. In 180 pear genotypes, the S_6 allele was the most prevalent one with 63% frequency, while the S_8 allele was the least common allele with a rate of 4%. In allele combinations, the S_1-S_6 allele combination was the most common allele combination with a rate of 18%, and trilateral allele combinations (S_1-S_6-S_7 and S_1-S_6-S_8) were observed at a rate of 1%. Findings of the current research will enable the classification of the materials and the analysed material is likely to be used in breeding studies as well as pear cultivation.

Keywords: Pear genotypes, self -incompatibility, *S*-allel, PCR amplification

1. Introduction

Pear (*Pyrus communis* L.), is common fruit grown in almost all moderate climates especially in Europe and Asia. In Turkey, Anatolia area is considered as the homeland and/or diversity centre for many fruit trees. About 640 different pear genotypes have been found in the country. To protect these gene sources, local nominated genotypes in different eco-geographical regions have been collected and protected within this area (Akçay et al. 2014).

Self-incompatibility (SI) in flower-bearing plants is known as a failure in self-fertilization, which may be controlled genetically. Self-incompatibility is known as an unfavourable feature in successful fertilization and fruit set. This phenomenon has been found in more than 100 plant families and has been reported in nearly 40% of plant species (Igic et al. 2008) involving some major crop plants like pome, potato, canola, cocoa, olive, stone fruits, coffee, etc. and/or wild relatives of the mentioned crops. In the majority of plants, genetic control of self-incompatibility is done by a single multiallelic locus named *S*-locus however, control of the systems by two or more loci in some crops like grasses has also been identified (De Nettancourt 2001). Determinants of both female and male specificity which their products are expected to interact and operate the function of self or non-self-distinction are encoded by the *S*-locus (Iwano & Takayama 2012; Muñoz-Sanz et al. 2020).

Depending on the function time of the gene in the stamen, most SI types can be categorized as gametophytic or sporophytic. In sporophytic self-incompatibility (SSI), determination of the SI phenotype is defined by the diploid genotype of parental or pollen-donor plant (sporophyte) and in gametophytic SI (GSI), the SI phenotype is determined by the pollen gametophytic haploid genotype (gametophyte) (Hiscock & Tabah 2003). At the molecular level, three mechanisms of the SI have been identified. In *Brassicaceae*, sporophytic self-incompatibility (SSI) has been identified while two separate types of gametophytic self-incompatibility (GSI) including *S-RNase*-based SI widely elucidated in *Solanaceae* and *Rosaceae*, and the Papaver based system relating to the programmed cell death (PCD) (Muñoz-Sanz et al. 2020).

In GSI which is considered to be the most prevalent type of SI, the cross becomes completely compatible when the male parent (haploid pollen genome) and female parent (diploid pistil genome) contain entirely disparate *S*-genotypes without any common *S*-allele (for instance S_1S_2 x S_3S_4). In case one *S*-allele is shared by the parents (for instance S_1S_2 x S_1S_3), proceeding of the pollen tube containing the similar allele is inhibited by pistil and consequently, the cross is considered to be semi-compatible. Accordingly, in case the same *S*-alleles are carried by the parents at the self-incompatibility locus (for instance S_1S_2 x S_1S_2), growth of the pollen cannot be happened on stigma. In other words, in case pistil-pollen couples do not include the same alleles, include at least one similar allele, or include several alleles, full compatibility, semi-compatibility or incompatibility is encountered, respectively. In crosses with the semi-compatible behaviour, half of the existing pollens are inhibited and could affect the yield and fruit set significantly, for example in Japanese plums, European pears and apple (Schneider et al. 2005; Zisovich et al. 2005; Goldway et al. 2008; Sapir et al. 2008). Results of the incompatibility are observed in minimum seed set and consequently, higher rates of small fruit formation, and yield loss.

Though GSI system applies similar genes in different taxa to specify the pollen rejection system, the mechanism elaboration differs significantly. Moreover, in all families, at least two linked genes or often more, are involved in *S*-locus consisting of a set of pollen-expressing *SFBB* genes (*S-locus F-Box Brothers*) and a pistil-expressed *S-RNase* gene. Pistil-expressed glycoproteins which show ribonuclease activity are encoded by *S-RNase* and they function as extremely selective cytotoxins which result in inhibition of pollens germination and growth when pollen single *S*-haplotype corresponds to one of the diploid pistil *S*-haplotypes (Sanzol & Robbins 2008; Gao et al. 2015; Claessen et al. 2019; Muñoz-Sanz et al. 2020). Another is an F-box protein gene which is distinctly expressed in pollen, is named *SFB* or *SLF* according to the family (De Franceschi et al. 2012; Bagheri & Ershadi 2019; Muñoz-Sanz et al. 2020).

Ability to predict the genomic structure of the pear *S*-locus has been performed using BAC cloning and sequencing in the Japanese pear (*Pyrus pyrifolia*) (Sassa et al. 2007). This was the first study to reveal the existence of several *SFBB* genes surrounding the *S-RNase*. Comparing the genomic sequence surrounding the *S2*- and *S4-RNases*, elucidated meaningful changes in the orientation and position of the *SFBB* genes in pear *S*-haplotypes compared to the *S-RNase* gene (Okada et al. 2011; Claessen et al. 2019).

In *Rosaceae* and *Solanaceae*, *S*-haplotypes have 16 to 20 *SLF* gene sequences which collectively contribute to SI function of the pollen (Kubo et al. 2010; Kakui et al. 2011; Williams et al. 2014; Muñoz-Sanz et al. 2020). Sequence alignment of the *S-RNase* amino acid revealed five regions with the conserved characteristics (i.e. C1, C2, C3, C4, and C5) along with a highly variable region. In addition, a highly polymorphic intron located between the C2 and C3, is found in the hyper variable region (De Franceschi et al. 2012; Bagheri & Ershadi 2019).

2D-PAGE (Two-dimensional Polyacrylamide Gel Electrophoresis) technique has been used to determine mentioned *S*-genotypes earlier. Despite being a fast technique, it is not preferred by plant breeders because it is not a reliable and easy method (Ishimizu et al. 1996). In the following years, PCR technique was applied to identify protected nucleotide sequences of *S-RNase*. Zuccherelli et al. (2002) isolated 2 *S*-allele DNA fragments in Japanese pear and proved that their sequences are similar to that of databank sequences. Among them, 6 *S*-allele fragments (S_a, S_b, S_c, S_d, S_e and S_h) have been cloned and sequenced. Using these alleles, the *S*-allele genotyping was performed in 10 pear genotypes (Barlett, Cascade, Doyennedu Comice, Abbé Fétel, Beurré Hardy, Passe Crassane, Conference, Beurré Bosc, Max Red Bartlett, Eletta Morettini), and the molecular data obtained were confirmed by field crossing results. In the study, PCR based *S*-allele genotyping at molecular level has been shown to be more fast and valid method for European pears.

A similar result was reported by Sanzol & Robbins (2008), that partially *S*-genotyped European pear cultivars and semi compatible test-crosses of these cultivars resulted in the identification of 14 *S*-alleles (S_1 to S_{14}) at the phenotypic level and allele-specific PCR led to the distinction across *S-RNases* that yielded amplification products with similar size using appropriate primers. The authors concluded that these two procedures presented a system for discrimination of all fourteen *S*-alleles in European pear at the molecular level.

Although self-pollination could have occurred in pear, the commercial-scale production depends on the existence of at least two simultaneously flowering compatible cultivars to enable appropriate and effective cross-fertilization so, exploiting of self and inter-compatible cultivars are important for economic fruit set (Zisovich et al. 2010; Goldway et al. 2012). In addition, in last decades, repeated use of scant numbers of cultivars in fruit breeding programs led to increase in cross-incompatibility property as well as a narrow genetic base in new pear varieties (Sanzol & Herrero 2002; Bagheri & Ershadi 2019). In this point of view, identification of the *S*-genotypes of the given species is important for revealing the species and inter-species fertilization biology to proper utilization in breeding programs (Quinet et al. 2014).

Although the incompatibility has been investigated in many pear germplasms, no findings relating to the self-compatibility/incompatibility have been encountered in genotypes of Turkey. In this paper, we evaluated the *S*-allele profiles of the pear genotypes of Turkey national collection in terms of fertilization biology.

2. Material and Methods

2.1 Plant material

180 pear (*Pyrus communis* L.) genotypes were obtained from the Atatürk Horticultural Central Research Institute, Yalova, Turkey, as plant material (Supplementary Table 1).

Table 1- Primer sequences for allele-specific PCR

Primer	*Forward (5'-3')*	*Reverse (5'-3')*	*PCR product size*
*PyrusS$_1$***	aatgtaagactacagccctg	tccaccagtggcctgtttg	367bp
*PyrusS$_6$**	gtttgtggccttcaaacgacg	gtgatcctttaaaagaactgc	347bp
*PyrusS$_7$***	tcacccagaaaattgcactaatgc	ccagtggcctttgtattcccaa	352bp
*PyrusS$_8$***	gtcattgacggggtttgaaccc	ccaactgggctttgagtgat	218bp

*: Ishimizu et al. (1999); **: Nashima et al. (2015)

2.2 Polymerase chain reactions (PCR)

Genomic DNA was isolated from the pear leaves according to Lefort et al. (1998). Determination of the DNA quantity was performed using Nanodrop ND-100 spectrophotometer and extracted DNA was checked on 1% agarose gel.

Allele-specific primers were applied for identifying the single alleles (S_1, S_6, S_7, S_8). Nucleotide sequences of *PyrusS$_1$*, *PyrusS$_6$*, *PyrusS$_7$*, and *PyrusS$_8$* primers were obtained according to Ishimizu et al. (1999) and Nashima et al. (2015), respectively (Table 1). Optimization of PCR (BioRad T100^{TM}) was conducted for used primers as follows: 5X PCR reaction buffer, 25 mM $MgCl_2$, 100 µM dNTP, 5 pmol primer, 5U Taq polymerase and 50-250 ng of genomic DNA was performed at a total volume of 15 µL. Negative control was conducted for controlling each PCR contamination. PCR program was performed as follows; 3 minutes pre-denaturation at 94 °C, 10 cycles (94 °C 1 min; 65 °C 1 min 45s; 72 °C 2 min; reducing the annealing temperature by 1 °C each cycle), 20 cycles (94 °C 1 min; 50 °C 1 min 45s; 72 °C 2 min) and final extension was performed at 72 °C for 10 minutes.

2.3. Evaluation of S-alleles according to band profiles

Amplified PCR products were separated by 2% agarose gel electrophoresis along with DNA marker (Solis Byodyne) in 100 V for 1 hour and thereafter, DNA bands were visualized using a visualization system (Gene Genius Bio Imaging System) (Figure 1).

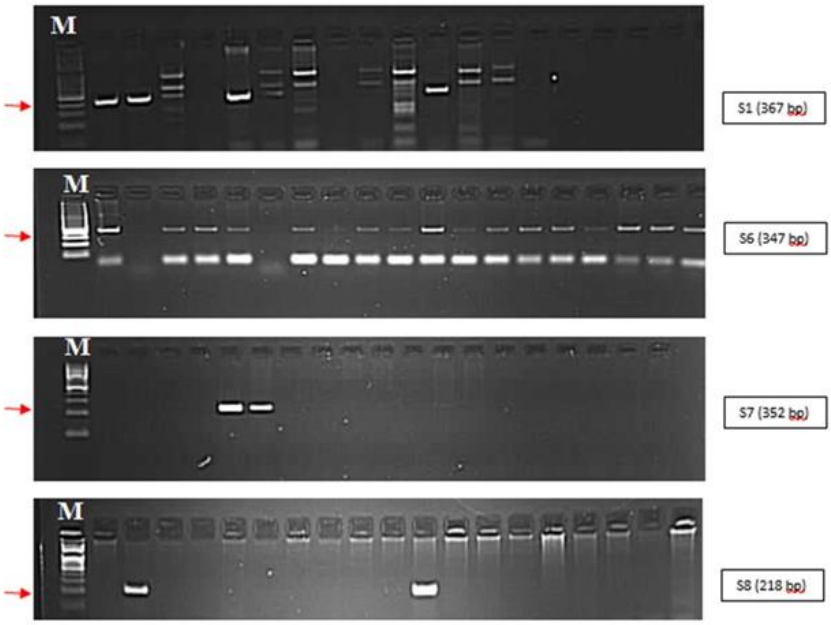

Figure 1- Examples of *S*-allele-specific PCR amplification images (S_1, S_6, S_7 and S_8 respectively). M: 100bp DNA ladder

After agarose gel visualization, genotypes with 367 bp (base pairs) in *PyrusS*$_1$ primer, 347 bp in *PyrusS*$_6$ primer, 352 bp in *PyrusS*$_7$ primer, and 218 bp in *PyrusS*$_8$ primer were considered self-incompatible for the relevant *S*-allele. Allele distribution features determined by Broothaerts et al. (1996) and Ishimizu et al. (1999) have been considered in evaluation of the results.

Among four *S*-alleles, genotypes with at least one-two of which were accepted as semi-compatible, genotypes without amplified fragments were introduced as compatible, and the genotypes with three *S*-alleles are considered to be incompatible.

3. Results and Discussion

By identifying self-incompatibility in genotypes at molecular levels using *S*-alleles, findings of the pear gene resources have been revealed in Turkey (Supplementary Table 1).

3.1. Relationship of compatibility and genetic similarity

Two homonyms of Yalova pear genotypes named "Göksulu (Malatya)" and "Göksulu" (Akçay et al. 2014), showed to possess no similar *S*-alleles and were determined to be semi-compatible.

According to Akçay et al. (2014), "150 887 (1-5)" and "240 887 (3-3)" genotypes with a similarity rate of 91.7% were observed to contain same *S* allele (S_6). Also, in "Bey Armudu" and "16" genotypes, with 91.7% similarity, the first genotype contained S_6 allele while the other one was identified to be whole compatible (Supplementary Table 1).

3.2. Relationship of compatibility and triploidy

In a trial, genetic analyses using 18 SSR loci in 11 pear genotypes revealed presence of three alleles in 4-10 loci, and these genotypes have been identified as potential triploid genotypes (Akçay et al. 2014). In the current study, high variation of SSR alleles detected earlier, was not observed in incompatibility alleles and the S_6 allele was the most observed allele in these triploid incompatible genotypes and while there was no incompatibility allele observed in other 5 genotypes (A 2411, E. Buzbağ, A 2407, 265 GFAE, 140 887 (2-2)) the rest showed at least one incompatible allele (Table 2).

Table 2- Self-incompatibility states of triploid genotypes in SSR analyses

Triploids pear genotypes in SSR analysis	***Incompatibility allele***	***PCR based- Incompatibility status***
190 887 (3-7)	S_6S_6	Semi-compatible
140 887 (2–1)	S_6S_6	Semi-compatible
A 2412	S_1S_6	Semi-compatible
A 2404	S_6S_6	Semi-compatible
A 2401	S_6S_7	Semi-compatible
A 2411	-	Compatible
E. Buzbağ	-	Compatible
A 2407	-	Compatible
265 GFAE	-	Compatible
140 887 (2-2)	-	Compatible

Table 3- *S*-Alleles, self-incompatibility cases and numbers

S allele	***Number of genotypes***	***PCR based-Incompatibility status***
S_6S_6	69	Semi-compatible
S_6S_7	4	Semi-compatible
S_6S_8	2	Semi-compatible
S_7S_7	5	Semi-compatible
S_8S_8	3	Semi-compatible
S_1S_1	14	Semi-compatible
S_1S_6	33	Semi-compatible
-	46	Compatible
$S_1S_6S_8$	2	Incompatible
$S_1S_6S_7$	2	Incompatible

3.3. Identification of alleles and compatibility

To improve the knowledge of self-incompatibility and its relations to many new pear genotypes, we elucidated *S*-genotype of 180 pear genotypes and arranged them based on their *S-RNase* alleles in 10 incompatibility groups (Table 3). Our results revealed incompatibility relations among a great number of pear genotypes in Turkey with unknown previous pollination requirements. These findings of given genotypes will be beneficial for indicating the potential of fertilization and fruit set, establishing the isolated gardens, and selecting as pollinizer.

In this research, four different *S*-alleles (S_1, S_6, S_7, S_8) whose primers have been identified in similar work (Kim et al. 2002; Nashima et al. 2015) were optimized and band size of 180 pear genotypes was determined. 130 out of 180 genotypes which contained at least one *S*-allele were identified as semi-compatible. From the rest, 4 of which containing three *S*-allele were determined as incompatible. In 46 genotypes, it was not observed any *S*-allele and being different from the others, they were identified as compatible genotypes (Table 3).

S_6 allele was observed with 63% rate followed by S_1 with 28% rate. S_8 was the less observed *S*-allele with 4% rate. The highest bilateral *S*-allele combination was observed in S_1-S_6 (18%), and the highest trilateral combinations were S_1-S_6-S_7 and S_1-S_6-S_8 (1%). Quadripartite combination was not observed in any genotype.

In terms of S_1, S_6, S_7, and S_8 alleles, 46 genotypes (25%) contained none of them, 91 genotypes (50%) contained one of them, 39 genotypes (21%) contained two of them, and 4 genotypes (2%) contained three of them. No genotype containing all of the mentioned alleles was observed (Table 3).

Similar to our work, 14 Japanese pear genotypes were investigated using 11 *S*-alleles including S_1, S_6, S_7, and S_9 alleles, and these alleles are applicable effectively for rapid identification of *S*-genotypes in similar pear breeding studies, and marker-assisted selection (MAS) performing (Nashima et al. 2015). Low sequence similarity of allele introns (average 43%) and high polymorphism in exon regions have been shown as the causes of high performance features of these *S*-alleles (Nashima et al. 2015). Nashima et al. (2015) identified existence of peculiar amplification (especially between 55-61 °C) through various PCR optimization trials of the S_1, S_6, S_7, and S_9 primers, and observed undesirable fragments on agarose gel in all primer pairs under 100 bp. In our research, these primers were similarly optimized using Touch Down PCR (*Tm* between 55-65 °C) program and some non-specific band was observed among PCR products.

Nowadays, *S*-allele genotyping trials at molecular level have been accelerated. Up until now, *S*-allele cloning and sequencing has been performed in about 26 European pear genotypes. In addition, genotyping of *S-RNase* genes in more than 150 pear genotypes has been carried out (Sanzol & Herrero 2002; Zuccherelli et al. 2002; Zisovich et al. 2004; Sanzol et al. 2006; Takasaki et al. 2006; Sanzol & Robbins 2008; Goldway et al. 2009; Sanzol 2009 a, b; Nashima et al. 2015).

In our study, since the high numbers of genotypes possess S_6 allele, so this allele was chosen as the prevalent one. The high ratio of genotypes bearing the S_6 allele (112 out of 180 genotypes) in comparison to genotypes bearing S_1 and other two alleles, could be explained by a plausible hypothesis that the S_6-locus is linked to another gene or genes encoding an important trait for pear cultivation or quality so, it could be mentioned that the S_6 allele involved a selective preference during breeding. A previous study on European pears revealed that certain *S*-alleles have considerably more frequency, and selection for commercial traits was proposed to describe this result. In this regard, Sanzol & Robbins (2008) noted that the majority of selected offspring of "Williams Bon Chrétien" cultivar with *PcS101* and *PcS102 S*-alleles comprised the *PcS101* allele rather than the *PcS102* allele. The authors suggested that the *PCS101* allele is of interest throughout selection. In addition, genetic analyses of species belonging to distant taxa are quite consistent with this feasibility (Burke et al. 2002; Gandhi et al. 2005). Nevertheless, it could be mentioned that the high use of certain genotypes as parent in breeding programs could also be one of the reasons for the abundance of certain alleles.

In our study, Williams cultivar was employed as a reference genotype and displayed an incompatible feature similar to previous studies (Sanzol & Herrero 2002). *S*-allele-specific PCR analysis (S_1, S_6, S_7 and S_8, respectively) are given in Figure 1 and the Williams cultivar representing reference genotype with known *S-RNase*s (S_1S_6), gave the expected PCR products corresponding to each allele and after electrophoresis, the higher frequency of S_6 allele was sensibly distinct among studied genotypes (Figure 1). Orcheski & Brown (2012) noted that, since specific reference cultivars are often used as parent in breeding programs of new cultivars so, their related *S*-alleles are found frequently in new developed commercial cultivars.

In the current study, three alleles were found in the four genotypes, namely 'E2480 Kızıl Bıldırcın' and 'E 2481 Kaymak' from Central Anatolian region and '21' and '52' from Marmara regions using these primers (Supplementary Table 1). According to 46 self-compatible genotypes, considering the proportion of regional samples, the highest proportion (40%) with 13 out of 32 genotypes was assigned to the Marmara region and the lowest percentage with 3 out of 24 genotypes (12% of the region samples) allocated to the Central Anatolian region so, more attention should be paid to contrivance, regarding the commercial production and orchard design in this region. The results confirmed that the self-incompatibility feature tends to

increase in species, which is contrary to the former reports indicating that most cultivars were compatible (Mehlenbacher et al. 1991; Herrera et al. 2018).

Although much progress has been gained in understanding how the SI system works, many points of ambiguity remain still unclear. It has been recently illustrated that the *Pyrus* SI response begins with the uptake of the *S-RNase* protein from the style transmitter tissue by the pollen tube in a non-allelic specific manner. The entry of both non-self and self *S-RNase*s into the pollen tube, support that the process of self-recognition takes place inside the pollen tube and subsequently results in inhibition of the non-self *S-RNase*s activity (Goldraij et al. 2006; De Franceschi et al. 2012; Meng et al. 2014a; Meng et al. 2014b, Williams et al. 2015). In *Malus* and *Pyrus* species, the simultaneous attendance of multiple *SFBB* genes on the *S*-locus as the pollen *S*-detectors (Sassa et al. 2007) indicates that each of the SFBB proteins may detect one or more non-self *S-RNase*s, targeting them for proteasome degradation (De Franceschi et al. 2012).

The recognition system has been proposed to occur *via* two models for GSI in *Pyrus* species. In the first model, the SFBB protein which identifies specifically the self *S-RNase*, relinquishing the self *S-RNase* intact to reject extension of the incompatible (self) pollen tube while mark all non-self *S-RNase*s (Williams et al. 2015). In the second model which functions in the non-self-recognition way, the multiplex *SFBB*s that each distinguish and target a subset or unique non-self *S-RNase*s, resulted in *S-RNase* decomposition so, the self *S-RNase*s which are not prohibited led to the inhibition of self-incompatible pollen growth (Kubo et al. 2010).

It should be noted that in nature, true rejection of pollen is not only controlled genetically but also is affected by various external agents, like the environmental factors and pollination grade resulting in selfed seeds in self-incompatible varieties (Visser & Marcucci 1984). Interestingly, in cases where pollination occurs by non-self pollen, the evolvement possibility of ovule or fruit is higher than in case of self-pollination, which indicates the attendance of additional post-zygotic inhibitors that inhibit the selfed seed formation (Martin & Lee 1993). It is concluded that this abortion could be caused by recessive homozygous alleles combination or accumulation of low alleles that occur as a result of self-fertilization (Pannell & Labouche 2013) or due to the lower intake strength of plant sap for energy attainment.

Similarly, crosses between two semi-compatible lines could also lead to adversities. In semi-compatible crosses, half of the pollens bearing certain *S*-genotype are inhibited, resulting in a limited number of possible *S*-genotype compositions in the progeny. This “artificial selection” significantly affects the diversity of *S*-alleles, leading to a reduction in biological and genetic diversity as well as the loss of interesting traits in pear cultivars (Claessen et al. 2019).

According to the contents, examination of the pear SI mechanism reveals that in most commercial cultivars, the fruit set is highly depend on cross-pollination and successful fertilization. For these reasons, incompatibility is a system that nature has developed to prevent the accumulation of adverse homozygous allele damages and to provide a way to create varieties with diverse characteristics while maintaining viability over the years.

4. Conclusions

S-genotyping has made it possible to classify cultivars in relevant incompatibility groups considering their compatibility relations. Self-incompatibility is displayed when the pollen genotype corresponds to one of the pistil *S*-alleles. Therefore, self-incompatible lines bearing the similar *S*-alleles by classification in the identic incompatibility category, are considered to be inter-incompatible, while lines from other categories containing at least one different *S*-allele are accepted to be inter-compatible (Muñoz-Sanz et al. 2017).

It should be noted that standardization of the *S*-alleles identification criteria is essential in various laboratories, including the full sequencing of *S* alleles and utilization of the identic primer pairs. This will lead to easy *S*-allele identification without confusion and will provide valuable information for pear breeders (Herrera et al. 2018). Obtained results in this study make it possible to organize the incompatibility relationships between pear genotypes with former unknown pollination knowledge and provide the possibility to select suitable parents in designing new crosses in pear production and breeding programs. Besides, this work could be helpful for other *Rosaceae* fruit products with the same challenges encountered by pear.

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Supplementary Table 1- *S*-allele compositions of pear cultivars

No.	*Cultivar name*	*S-Genotype*	*PCR based- Compatibility status*	*Region*
1	190 887 (3-2)	S_6S_6	Semi-compatible	Unknown
2	190 887 (3-1)	S_8S_8	Semi-compatible	Aegean
3	240 887 (3-1)	-	Compatible	Aegean
4	22	-	Compatible	Marmara
5	38	S_6S_6	Semi-compatible	Marmara
6	A-48	-	Compatible	Unknown
7	133	S_1S_6	Semi-compatible	Marmara
8	200 GFAE	S_6S_6	Semi-compatible	Black Sea
9	207 BF(G)	-	Compatible	Black Sea
10	221 GFAE (G)	S_6S_8	Semi-compatible	Black Sea
11	223 GFAE (G)	S_6S_6	Semi-compatible	Black Sea
12	A-2406	S_1S_6	Semi-compatible	Unknown
13	Azdavay	S_6S_6	Semi-compatible	Black Sea
14	Bağ (G)	-	Compatible	Black Sea
15	Bal Armut (Malatya)	S_6S_6	Semi-compatible	Unknown
16	Bey Armudu	S_6S_6	Semi-compatible	Unknown
17	Çankaya1	S_6S_6	Semi-compatible	Unknown
18	Cennet (G)	S_6S_6	Semi-compatible	Black Sea
19	E 2473 Cinci	S_8S_8	Semi-compatible	Central Anatolian
20	E 2501 Eğrişap	S_6S_6	Semi-compatible	Central Anatolian
21	E 2507 Pamukap	S_1S_1	Semi-compatible	Central Anatolian
22	E Rize	S_6S_6	Semi-compatible	Unknown
23	Fakaz (G)	S_1S_6	Semi-compatible	Black Sea
24	Gümüşhane (G)	S_1S_6	Semi-compatible	Black Sea
25	Gürpınar (G)	S_1S_6	Semi-compatible	Black Sea
26	İğnesi (Malatya)	S_7S_7	Semi-compatible	Unknown
27	İncir (Malatya)	-	Compatible	Unknown
28	Kantartopu	S_1S_1	Semi-compatible	Black Sea

Supplementary Table 1 (Continue)- *S*-allele compositions of pear cultivars

No.	*Cultivar name*	*S-Genotype*	*PCR based- Compatibility status*	*Region*
29	Karagöynük	-	Compatible	Marmara
30	Karpuz	S_6S_6	Semi-compatible	Black Sea
31	Kiraz	S_1S_1	Semi-compatible	Black Sea
32	Küpdüşen (Malatya)	S_6S_6	Semi-compatible	Unknown
33	Küre (G)	S_6S_6	Semi-compatible	Black Sea
34	Laliye	S_1S_1	Semi-compatible	Marmara
35	Malatya Limon	S_1S_6	Semi-compatible	Unknown
36	Mis (G)	S_1S_1	Semi-compatible	Black Sea
37	Turşu (G)	S_6S_6	Semi-compatible	Black Sea
38	174W (G) D.dAngulume	S_1S_1	Semi-compatible	Unknown
39	E Santa Maria	-	Compatible	Unknown
40	Williams	S_1S_6	Semi-compatible	Unknown
41	Akça	S_1S_1	Semi-compatible	Marmara
42	E. Buzbağ	-	Compatible	Marmara
43	Çiçek	-	Compatible	Marmara
44	Göksulu	S_1S_6	Semi-compatible	Marmara
45	Göksulu (Malatya)	S_1S_1	Semi-compatible	Marmara
46	Malatya (Malatya)	S_6S_6	Semi-compatible	Unknown
47	205 AF (G)	S_6S_6	Semi-compatible	Black Sea
48	188 BK (G)	S_6S_6	Semi-compatible	Black Sea
49	130 887 (1-3)	S_1S_1	Semi-compatible	Unknown
50	130 887 (3-7)	S_1S_6	Semi-compatible	Unknown
51	140 887 (1-1)	S_6S_6	Semi-compatible	Unknown
52	140 887 (2-3)	S_6S_6	Semi-compatible	Aegean
53	150 887 (1-3)	S_6S_6	Semi-compatible	Aegean
54	150 887 (1-5)	S_6S_6	Semi-compatible	Unknown
55	150 887 (2-3)	S_6S_6	Semi-compatible	Aegean
56	172 887 (2-2)	S_6S_6	Semi-compatible	Aegean
57	190 887 (2-6)	S_6S_6	Semi-compatible	Unknown
58	180 887 (4-3)a	S_6S_6	Semi-compatible	Aegean
59	180 887 (9-7)	S_6S_6	Semi-compatible	Aegean
60	190 887 (3-3)	S_1S_6	Semi-compatible	Unknown
61	190 887 (3-5)	S_1S_6	Semi-compatible	Aegean
62	190 887 (3-7)	S_6S_6	Semi-compatible	Aegean
63	190 887 (4-1)	S_6S_6	Semi-compatible	Aegean
64	190 887 (6-2)	S_1S_6	Semi-compatible	Aegean
65	200 887 (2-1)	S_6S_6	Semi-compatible	Aegean
66	200 887 (2-3)	S_8S_8	Semi-compatible	Aegean
67	200 887 (2-4)	S_1S_6	Semi-compatible	Aegean
68	200 887 (11-6)	S_6S_6	Semi-compatible	Aegean
69	210 887 (2-2)	S_1S_1	Semi-compatible	Aegean
70	210 887 (2-3)	S_1S_6	Semi-compatible	Aegean
71	210 887 (4-4)	S_6S_6	Semi-compatible	Unknown
72	210 887 (4-6)	S_6S_6	Semi-compatible	Aegean
73	220 887 (2-3)	S_1S_6	Semi-compatible	Aegean
74	220 887 (3-3)	S_1S_6	Semi-compatible	Aegean
75	230 887 (3-3)	S_6S_6	Semi-compatible	Aegean
76	240 887 (3-3)	S_6S_6	Semi-compatible	Aegean
77	A33	S_1S_6	Semi-compatible	Unknown
78	A 129	S_1S_6	Semi-compatible	Unknown
79	A 2401	S_6S_7	Semi-compatible	Unknown
80	A 2407	-	Compatible	Unknown
81	A 2409	S_1S_6	Semi-compatible	Unknown
82	A 2411	-	Compatible	Unknown
83	A 2412	S_1S_6	Semi-compatible	Aegean
84	E 2470 Kokmuş Armut	S_6S_6	Semi-compatible	Unknown
85	E 2444 Arpa	S_6S_6	Semi-compatible	Aegean
86	E 2462 Bıldırcın	S_6S_7	Semi-compatible	Central Anatolian
87	E2480 Kızıl Bıldırcın	$S_1S_6S_7$	Incompatible	Central Anatolian
88	E 2481 Kaymak	$S_1 S_6S_8$	Incompatible	Central Anatolian
89	E 2493 Kışlık Şalgam	S_1S_6	Semi-compatible	Central Anatolian
90	E 2516	S_1S_6	Semi-compatible	Central Anatolian
91	E 2539 Baymaz	S_1S_6	Semi-compatible	Central Anatolian
92	E 2540 Dalkıran	S_1S_6	Semi-compatible	Unknown
93	E 2542 Ağa	S_1S_6	Semi-compatible	Central Anatolian
94	E 2547 Armut	S_6S_6	Semi-compatible	Central Anatolian

Supplementary Table 1 (Continue)- *S*-allele compositions of pear cultivars

No.	*Cultivar name*	*S-Genotype*	*PCR based- Compatibility status*	*Region*
95	E 2556 Hocacul	-	Compatible	Unknown
96	E 2557 Mustafabey	S_6S_6	Semi-compatible	Central Anatolian
97	E Ankara	S_6S_6	Semi-compatible	Central Anatolian
98	E Giresun	S_6S_6	Semi-compatible	Central Anatolian
99	10	S_6S_6	Semi-compatible	Marmara
100	13	S_6S_6	Semi-compatible	Unknown
101	14	S_1S_6	Semi-compatible	Unknown
102	18	S_1S_1	Semi-compatible	Marmara
103	19	S_6S_6	Semi-compatible	Unknown
104	20	S_6S_6	Semi-compatible	Marmara
105	21	$S_1S_6S_8$	Incompatible	Marmara
106	30	S_6S_6	Semi-compatible	Marmara
107	37b	S_1S_1	Semi-compatible	Marmara
108	41	S_1S_6	Semi-compatible	Unknown
109	43	S_6S_6	Semi-compatible	Marmara
110	136	-	Compatible	Marmara
111	139	S_1S_1	Semi-compatible	Marmara
112	231 BC	S_1S_6	Semi-compatible	Unknown
113	P5-9	S_6S_6	Semi-compatible	Unknown
114	P5-23	-	Compatible	Unknown
115	Thompson (Malatya)	S_6S_6	Semi-compatible	Unknown
116	Andre Desportes	-	Compatible	Unknown
117	150 887 (1-1)	-	Compatible	Aegean
118	E 2545	-	Compatible	Unknown
119	140 887 (2-5)	-	Compatible	Unknown
120	140 887 (2-2)	S_6S_7	Semi-compatible	Aegean
121	E 2533 Sarı Armut	-	Compatible	Central Anatolian
122	E 509 Kış Armudu	-	Compatible	Central Anatolian
123	E 224 K. Armut	S_1S_6	Semi-compatible	Central Anatolian
124	52	$S_1S_6S_7$	Incompatible	Marmara
125	200 887 (2-1)b	-	Compatible	Aegean
126	210 887 (4-5)	S_6S_6	Semi-compatible	Unknown
127	P5 11	-	Compatible	Unknown
128	64	-	Compatible	Marmara
129	44	-	Compatible	Marmara
130	134	-	Compatible	Marmara
131	E 2537 Bulap	S_6S_6	Semi-compatible	Central Anatolian
132	220 887 (3-1)	-	Compatible	Unknown
133	213 GFAE	-	Compatible	Karadeniz
134	236 GFAE	-	Compatible	Karadeniz
135	A 2404	S_6S_6	Semi-compatible	Unknown
136	190 887 (3-6)	S_6S_6	Semi-compatible	Unknown
137	31	S_6S_6	Semi-compatible	Marmara
138	190 887 (2-3)	S_6S_6	Semi-compatible	Aegean
139	16	-	Compatible	Marmara
140	P5-2	S_6S_6	Semi-compatible	Unknown
141	14	-	Compatible	Unknown
142	A 129	S_1S_6	Semi-compatible	Unknown
143	P 522	S_6S_6	Semi-compatible	Unknown
144	A 2410	S_1S_6	Semi-compatible	Unknown
145	E 2462 Bıldırcın	-	Compatible	Central Anatolian
146	E 2480 Kızıl Bıldırcın	S_6S_6	Semi-compatible	Mediterranean
147	E 2542 Ağa	S_6S_6	Semi-compatible	Central Anatolian
148	150 887 (3-1)	-	Compatible	Aegean
149	21	-	Compatible	Marmara
150	190 887 (1-1)	-	Compatible	Aegean
151	200 887 (2-1)	S_1S_1	Semi-compatible	Aegean
152	E Ankara	S_6S_6	Semi-compatible	Central Anatolian
153	220 887 (2-3)	S_7S_7	Semi-compatible	Aegean
154	Giresun	S_7S_7	Semi-compatible	Unknown
155	150 887 (1-1)	S_6S_7	Semi-compatible	Aegean
156	240 887 (3-2)	S_7S_7	Semi-compatible	Aegean
157	200 887 (2-1)a	-	Compatible	Aegean
158	140 887 (2-1)	S_6S_6	Semi-compatible	Aegean
159	140 887 (2-2)	-	Compatible	Aegean
160	265 GFAE	-	Compatible	Karadeniz

Supplementary Table 1 (Continue)- *S*-allele compositions of pear cultivars

No.	***Cultivar name***	***S-Genotype***	***PCR based- Compatibility status***	***Region***
161	E 2533 Sarı Armut	S_6S_8	Semi-compatible	Central Anatolian
162	E 2509 K. Armut	S_1S_6	Semi-compatible	Unknown
163	190 887 (3-4)	S_6S_6	Semi-compatible	Unknown
164	200 887 (2-1)b	-	Compatible	Aegean
165	E 2525 Ekşi Armut	S_6S_6	Semi-compatible	Central Anatolian
166	44	S_6S_6	Semi-compatible	Marmara
167	134	S_6S_6	Semi-compatible	Marmara
168	E 2537 Bulap	S_6S_6	Semi-compatible	Central Anatolian
169	220 887 (3-1)	S_6S_6	Semi-compatible	Unknown
170	235 P	-	Compatible	Unknown
171	A 2404	-	Compatible	Unknown
172	31	S_1S_6	Semi-compatible	Marmara
173	M 2404	-	Compatible	Unknown
174	190 887 (2-3)	S_7S_7	Semi-compatible	Aegean
175	180 887 (4-3)b	S_6S_6	Semi-compatible	Aegean
176	220 887 (4-1)	S_6S_6	Semi-compatible	Aegean
177	45	-	Compatible	Marmara
178	32	-	Compatible	Marmara
179	138	-	Compatible	Marmara
180	A 135	-	Compatible	Unknown

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Inbreeding in Holstein Friesian Cattle Population in Turkey

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ABSTRACT
Inbreeding is generally associated with a reduction in production and profitability. Therefore, it is essential that it be monitored and kept under control. The purpose of this study was to calculate the inbreeding coefficient for Holstein Friesian cattle registered in the database of the Cattle Breeders' Associations of Turkey (CBAT). In this study, pre-herdbook and herdbook databases were combined. The database consisted of 6,935,005 individuals born between 1962 and 2012. Inbreeding coefficients were calculated using Wright's method, and ranging from zero to 43.75% with a mean of 0.0012 and standard deviation (SD) of 0.01273 for all animals, and considering the inbred animals, the mean inbreeding coefficient was 0.0106 and standard deviation was 0.03272. The average inbreeding of all animals born in the population in 2012 was found to be 0.0022. In the population, the proportion and the number of inbred individuals increased over the years, while the mean inbreeding coefficient decreased. This could be due to the fact that gene flow in the population from different countries was considerably high, and pedigree information was taken into account while importing sperm and live animals (both heifers and bulls).

Keywords: Relationship, Inbreeding, Holstein Friesian, Cattle

1. Introduction

Inbreeding is defined as the mating of individuals related to each other by ancestry (Falconer & Mackay 1997), and is a growing concern in dairy cattle breeding (Weigel & Lin 2002; Gullstrand 2015; Doekes et al. 2019). Especially the developments in the applications of artificial insemination (AI) during the past few decades have resulted in an increased use of few top sires all over the world, which has led to the spread of related offspring across different countries or even continents. For example, a few superior top Holstein bulls have sired about 250,000 milking daughters and 3,000 progeny-tested sons all over the world (Weigel 2001). In a study by Miglior (2000), it was found that the percentage of bulls born and sired by five bulls has increased from 25% to 47%, 61% and 55% in Europe, North America, and Oceania in 20 years, respectively.

Using relatively fewer bulls or increasing the number of progenies per bull in a selection program leads to an increase in genetic gain, but this may also decrease the genetic variance by inbreeding (Freyer et al. 2005; Gullstrand 2015). In other words, increased inbreeding decreases production traits (Thompson et al. 2000; Pryce et al. 2014), survivability (Sewalem et al. 2006), and reproductive performance (Adamec et al. 2006; Kaygısız & Kösetürkmen 2007; Bayram et al. 2008; Hofmannova et al. 2019), which is known as inbreeding depression.

Controlling and monitoring of inbreeding levels are important in cattle populations to minimize the inbreeding depression (Wiggans & VanRaden 1995; Weigel 2001; Freyer et al. 2005; Sorensen et al. 2005; Sewalem et al. 2006; Rokouei et al. 2010; Doekes et al. 2019). For example, the numbers of inbred cattle and their mean inbreeding coefficients are calculated every year for many breeds in the US, which are bred under the supervision of the United States Department of Agriculture (USDA), especially Holstein Friesian and Red Holstein breeds, and the results are posted on the website of the Council on Dairy Cattle Breeding (USCDCB 2021). Thus, the trend of the inbreeding coefficient of the Holstein Friesian population in the US can easily be followed.

Knowing the inbreeding coefficient is also significant for comparative studies of cattle populations from different countries. The objective of this study was to calculate the inbreeding coefficients of Holstein Friesian cattle registered by the Cattle Breeders' Associations of Turkey (CBAT). Although many studies investigated inbreeding levels of Holsteins as well as those of other breeds, no such comprehensive inbreeding analysis has been carried out in Turkey to date.

2. Material and Methods

2.1 Data

This study used pedigree information regarding Holstein Friesian cattle breed in farms which are members of the Cattle Breeders' Associations of Turkey. The database of the study is comprised of two elements: (i) pre-herdbook and (ii) herdbook, including information about, for example, ownership, breeding and some production traits. In the pedigree, certain constrains were applied to improve the quality of the data. Therefore, some data from the file were not included in the analysis, and the applied constrains are summarized in Table 1.

Table 1- Reasons for excluding certain data in the analyses although they were included in the main pedigree file

Reason for exclusion from the dataset	*Pedigree File*
Both parents were unknown	1 746 241
Dams were older than 13 years when the progeny was born	18 843
The period between the birth of sire and their progeny was shorter than two years	648
Calving interval was shorter than 235 days	1 600
Total	1 767 332

The records utilized in this study contained animal, sire and dam identification information, as well as sex, province and date of birth data. The inbreeding coefficients were calculated using pedigree records of Holsteins born between 1962 and 2012, for 6,935,005 individual animals, among which the longest ancestral path was 13. There was recorded information for at least one parent for all of these individuals. 79.93% of the animals in the pedigree file consisted of individuals of which both parents were known.

2.2 Analysis of data

In this study, the coefficient of inbreeding for each animal was calculated using the MTDFNRM module of MTDFREML (**M**ultiple **T**rait **D**erivative **F**ree **RE**stricted **M**aximum **L**ikelihood (MTDFREML) software (Boldman et al., 1995). In the MTDFNRM module, inbreeding coefficient of individuals is calculated, as done in Wright's method, by halving the numerator relationships of each parent.

The inbreeding coefficient of animal X (F_x) is calculated as follows (Wright 1922):

$$F_x = \sum_{CA=1}^{k} \left[\left(\frac{1}{2}\right)^{(n_1+n_2+1)} (1 + F_{CA}) \right] \quad (1)$$

Where; CA is a common ancestor of sire and dam of X; k is the number of common ancestors in the X's, pedigree; n_1 is the number of generations separating the common ancestor from the sire of X, n_2 is the number of generations separating the common ancestor from the dam of X, and F_{CA} , is the inbreeding coefficient of the common ancestor.

The pedigree file was rearranged so that it meets the requirements of the MTDFNRM module. All records were sorted according their birth year and recorded appropriately. Then, all animals were sorted from the oldest the youngest. Some parents had no birth dates in the main file, so new birth dates were assigned by taking their oldest offspring into consideration. Mean inbreeding was calculated per year based on the birth year of the animals.

3. Results and Discussion

The number of animals included in the analyses was 6,935,005, whereas the number of animals born after 1990 was 6,931,329. Of these, both parents were known for 5,543,259 individuals. In the pedigree, there were 278,907 full sib groups, and the average family size was 2.05. The number of animals and their mean inbreeding coefficient with its standard deviation are presented in Table 2. The mean inbreeding coefficient and standard deviation were 0.0012 and 0.01273, while, only with respect to the inbred animals, they were 0.0106 and 0.03272, respectively. The highest inbreeding coefficient in this population was 0.4375.

Table 2- Inbreeding coefficient of individuals in pedigree file

Groups	*N*	*Mean*	*Standard Deviation*	*Min*	*Max*
Whole population	6 935 005	0.0012	0.01273	0	0.4375
Inbred population	962 359	0.0106	0.03272	0.000015	0.4375

In this study, inbreeding coefficients were calculated as lower than the ones included in the previous reports, in which the inbreeding coefficient was determined to be 0.026 for Holsteins born in 1990 by Wiggans & VanRaden (1995), and the average inbreeding coefficient in elite Holstein cows and AI Holstein bulls was calculated as 0.042 and 0.044, respectively, by Weigel and Lin (2002). The difference can be attributed to the fact that Turkey has been importing both semen and heifers continuously from other countries.

In Turkey, there are some studies that investigated the inbreeding level of Holsteins in the small-scaled herds, and their results are not similar to those found in the present study. Because their data were obtained from smaller and more closed populations compared to our population, which comprises a greater number of herds from all around the country. For example, some reports of the mean inbreeding coefficients were 1.35% in 439 animals (Bayram et al. 2008), 0.31% in 293 animals (Okumuş et al. 2010), and 1.91% in 810 animals (Duru 2012).

Pedigree records used in this study started in 1962, but the records of only a small number of animals (3,676) born before 1990 were available, and only five of these animals were inbred. Therefore, mean inbreeding coefficients were presented for the period between 1990 and 2012 (see Table 3 and Figure 1). The inbreeding rate was not stable over time and consisted of three periods in which inbreeding was changing at different rates. As seen in Table 3, between 1991 and 1996, inbreeding coefficient rose steadily from 5.85% to 7.68%. However, after 1996, it gradually decreased from 6.41% until it went down to 1.45% in 2005. From 2005 to 2012, the downward trend in inbreeding coefficient continued while fluctuating between 1.45% to 0.88% (see Table 3 and Figure 1). The annual rate of change in inbreeding between 1990 and 2012 was found as -8.23%.

Table 3- Annual mean inbreeding coefficients in inbred individuals between 1990 and 2012

Birth Year	*Number of Individuals*	*Inbred Individuals, (%)*	*Mean Inbreeding Coefficient, (%)*
1990	2 196	2.91	5.85
1991	2 744	3.53	5.35
1992	4 477	4.02	6.24
1993	7 484	2.78	6.75
1994	9 217	2.94	7.03
1995	13 461	2.38	7.61
1996	19 703	3.03	7.68
1997	26 143	2.76	6.41
1998	37 556	2.42	7.00
1999	56 940	2.38	5.58
2000	80 626	2.57	5.14
2001	88 874	4.05	4.65
2002	112 612	4.62	4.03
2003	151 632	4.37	3.45
2004	225 888	4.75	2.52
2005	316 635	5.48	1.45
2006	540 579	5.49	1.10
2007	722 436	6.52	0.97
2008	744 449	10.25	0.94
2009	785 488	14.58	1.00
2010	889 773	18.80	1.00
2011	113 6811	21.08	0.93
2012	955 605	24.86	0.88

As seen in Table 3, mating of close relatives was avoided after 2003, while the number of distantly related animals with a common ancestor increased. In contrast to our findings, USCBDCB (2001) reported that the inbreeding coefficient for cows and bulls steadily increased after 1960, and the USCDCB 2021 Report showed that the inbreeding coefficient of cows for 2012 was 5.89%, while it was 8.59% for 2020. Moreover, Sorensen et al. (2005) reported the mean inbreeding coefficient for calves born in 2003 to be 3.9% in Danish Holsteins. In their study, the inbreeding trend was described as a smooth increase. Sewalem et al. (2006) reported the average levels of inbreeding for animals born in 2004, which was 3.20% for Holsteins, 3.99% for Ayrshires and 3.60% for Jerseys. In the same study, the magnitude of the inbreeding coefficient was observed to be increasing over time. In other words, inbreeding increased as pedigrees got deeper. However, in direct contrast to the studies cited above, in our study, inbreeding trends were observed to decline over time (see Figure 1). This result can be attributed to the fact that Turkey's Holstein population is not a closed one, that is, Turkey continued to import both semen and heifers every year from several other countries around the world (TUIK 2021).

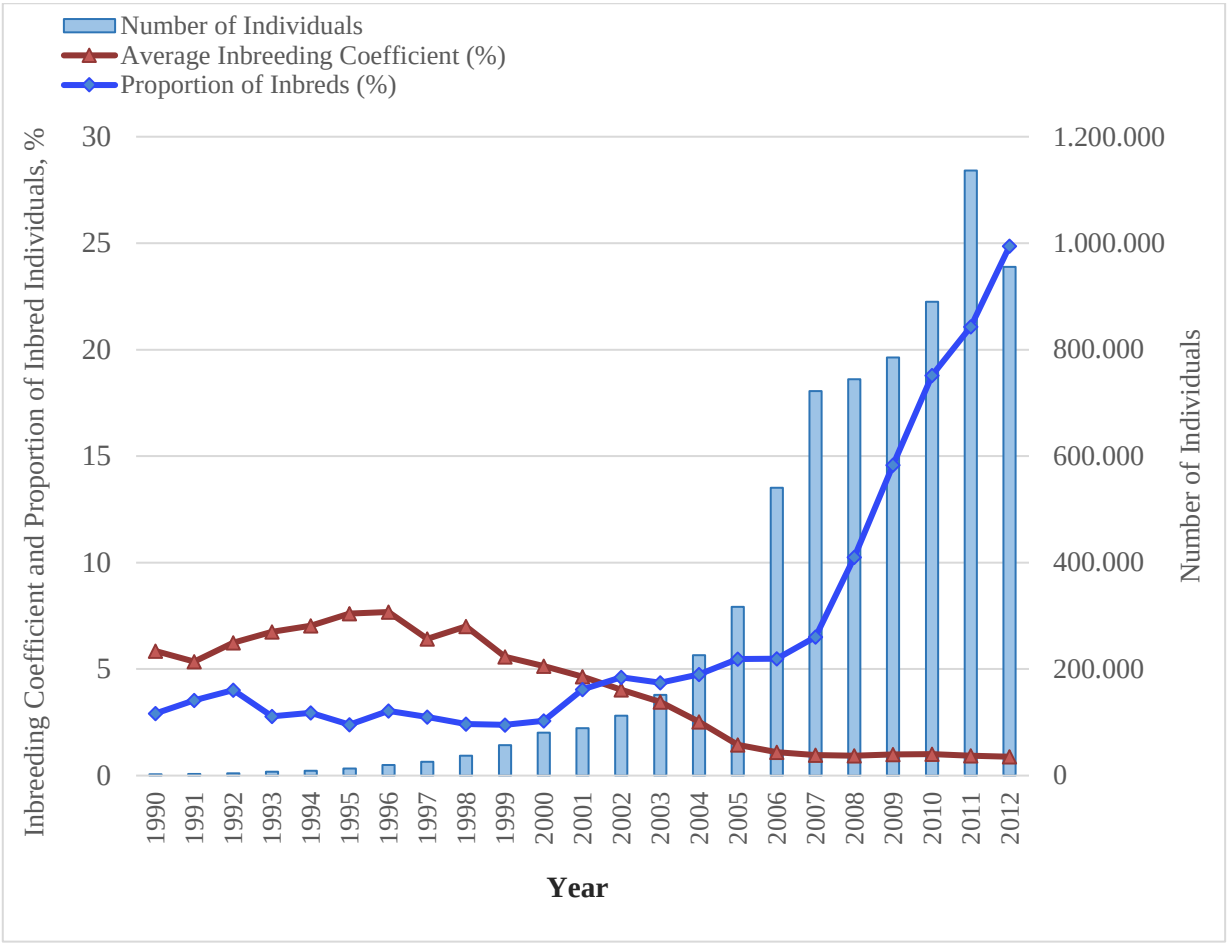

Figure 1- Number of individuals and the mean inbreeding coefficient from 1990 to 2012

The distribution of inbreeding coefficients is shown in Table 4. Most individuals (92%) had an inbreeding coefficient less than or equal to 2.50%. In this population, the inbreeding coefficient was greater than 12.50% only for 1.86% of inbred animals. In other words, an overwhelming majority of the inbred individuals had an inbreeding coefficient below 12.50%. Usually, the mating of half-sibs would result in a 12.50% expected inbreeding coefficient, but the modern practice of using the sperm from the same sires over a period of many years leads to an increase in the number of inbred animals. This results in inbreeding coefficients greater than 12.50% in the population due to accumulated relationships among animals. Mc. Parland et al. (2007) reported that 0.80% of all Holstein-Friesian cross in the population had an inbreeding coefficient greater than 12.50%. Hofmannova et al. (2019) reported that 0.39% of Czech Holsteins had an inbreeding coefficient over 10%.

Table 4- Distribution of individuals based on different inbreeding levels and birth years

Inbreeding Class[1]	*Born between 1990 and 2012*[2]	*Birth Year* 2008	2009	2010	2011	2012
F ≤ 0.025	885 442	70 356	105 969	154 274	224 359	223 450
0.025<F≤0.05	36 536	3 876	4 698	5 932	5 947	5 324
0.05<F≤0.10	22 454	1 250	2 212	4 397	5 381	4 994
0.10<F≤0.15	3 371	98	179	236	659	728
0.15<F≤0.25	11 807	485	1 137	1 920	2632	2 338
F>0.25	2 744	215	345	495	627	772
Total	962 354	76 280	114 540	167 254	239 605	237 606
Total number of individuals in the population	6 931 329	744 449	785 488	889 773	1 136 811	955 605

[1]: F=Inbreeding coefficient; [2]: Includes animals born between 1990 and 2012

The sire was known for 81.81% of the animals included in our study. The most used 10 bulls had sired 10.61% of the population, and also, 9.06% of males themselves were the offspring of these bulls. Table 5 shows the number of individuals with known sires and the progeny per bull after 2002.

Table 5- Number of individuals born between 2002 and 2012 with known sires recorded in the pedigree file

Birth Year	*Number of Individuals*		*Proportion of Individuals with Known Sires, %*	*Number of Sires*	*Average Progeny per Sire*
	Total	*Sire Known*			
2002	112 612	79 079	70.2	2 396	33.0
2003	151 632	94 045	62.0	2 982	31.5
2004	225 888	137 198	60.7	2 780	49.4
2005	316 635	201 082	63.5	2 154	93.4
2006	540 579	414 312	76.6	2 070	200.2
2007	722 436	609 863	84.4	2 376	256.7
2008	744 449	633 740	85.1	2 141	296.0
2009	785 488	667 419	84.9	2 221	300.5
2010	889 773	762 775	85.7	2 477	307.9
2011	1 136 811	954 243	83.9	2 755	346.4
2012	955 605	821 271	85.9	2 365	347.3

The proportion of individuals with known sires decreased until 2005. However, after that year, this proportion increased so much that nearly 85% of the individuals born after 2007 were the offspring of known sires. In addition, progeny per bull rapidly increased until 2012. As seen in Table 5, progeny per bull was 33 in 2002, but it went up to 347 in 2012.

Another finding of this study was that 13.88% of the registered Holstein Cattle population was inbred to some degree. After 2007, the proportion of inbred animals increased rapidly, but the inbreeding coefficient did not increase accordingly. It seems that the upward trend in the proportion of inbred animals is likely to continue, but this trend will not cause a significant increase in the inbreeding coefficient.

4. Conclusions

After calculating the inbreeding coefficients for the Holstein Friesian Cattle Population in Turkey, 13.88% of these animals were determined to be inbred in the whole population between 1962 and 2012. However, the average inbreeding coefficients of these inbred individuals was significantly low. This means that the percentage of inbred individuals in the population increased over time, while their mean inbreeding coefficient decreased. One reason for this development was that the gene flow from different countries in the population was quite high, and that the pedigree information had been considered while importing sperm, heifers and bulls. Another reason was the financial support of the government for artificial insemination and calves born from AI. Also, the AI technology has increased the use of same bulls' sperm for a long time in the population. Therefore, it has increased the number of distant relative animals. In other words, the chance of mating of distant relatives has increased. So, this has led to a large number of individuals with low inbreeding coefficients in the population. Besides, new farms were continually being added to the system, which meant that the cattle population in Turkey has gradually risen with the addition of these new animals. As a result of the study, it was determined that the level of inbreeding was not high except for some herds.

Owing to the continued import of live animals into the country, and the meticulous consideration of pedigree records in sperm imports, the inbreeding coefficient in Turkey is expected to follow the same trend without much increase.

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Screening of Antibiotic Resistance and Virulence Genes of *Enterococcus* spp. Strains Isolated from Urfa Cheese

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ABSTRACT

Enterococcus faecium, *E. durans* and *E. faecalis* species were isolated and identified from traditional Urfa cheese samples which were produced from unpasteurized raw milk. The enterococcal load of the cheese samples was between 4.4-5.6 log cfu g^{-1}. High-level tetracycline, streptomycin, erythromycin, gentamycin, and penicillin resistance was determined in the enterococcal isolates. Multiple antibiotic resistance was also determined in *E. faecalis* (20.4%) and *E. faecium* (16.3%) strains. 36.7% of the enterococcal isolates were greater than 0.2 MAR index ratio in this study. The *gel*E and *agg*2 genes were found in 40 (81.63%) of the enterococcal isolates, whereas the *van*B gene was found in 3 (6.12%) of the enterococcal isolates. The results indicate that the consumption of Urfa cheese, which is produced using raw milk, may have public health risk because of its antibiotic resistance characteristics and virulence genes of enterococcal biota.

Keywords: Urfa cheese, Traditional Turkish cheese, Enterococci, Antibiotic resistance, Virulence genes, Public health

1. Introduction

Urfa cheese, which is traditionally produced mainly from sheep, goat or bovine unpasteurized raw milk, is a semi-hard and brined cheese variety in Turkey. Starters are not added to the production of this cheese variety (Kırmacı 2016, Atasoy et al. 2021). The use of raw milk in the production of this cheese may cause serious public health risks. Conversely, it was revealed that the pasteurization process may negatively affect cheese flavor quality because of killing lactic microflora in raw milk. In a study, the lactic acid bacteria (LAB) load in Urfa cheese was reported to be between 4.78-9.68 log cfu g^{1}, while the distribution of LAB members was found to include *Enterococcus* spp. (48.95%), *Lactococcus* spp. (40.55%), *Lactobacillus* spp. (9.10%), *Streptococcus* spp. (0.69%) and *Leuconostoc* spp. (0.69%). In this study, the genus *Enterococcus* was found to be a dominant LAB member with *E. faecium* (40%), *E. durans* (32.85%), *E. faecalis* (18.57%), *E. lactis* (5.71%) and *E. hirae* (2.85%) species (Kırmacı et al. 2016).

Enterococci is a member of the gastrointestinal microbiota of humans and animals. They are also presented especially in fermented foods such as sausages and cheeses and may contribute to cheese flavor with their proteolytic and lipolytic enzyme activities. They have an ability to survive in unsuitable conditions in the food environment such as pasteurization temperature, 6.5% NaCl concentration, etc. (İspirli et al. 2017).

Enterococci may have beneficial characteristics; however, some strains are still an important issue for food industry and public health. Enterococci are also considered as a nosocomial pathogen and they have ability to develop a wide range of antibiotic resistance and to have a potential to carry some virulence determinants (Terkuran et al. 2014, Calonico et al. 2018).

Although antibiotic-resistant enterococcal species may be found in cheeses made by using raw and pasteurized milk, their presence in food chain may cause a serious public health risk of spreading antibiotic resistance from food to humans, animals and the environment (Çitak et al. 2004, Camara et al. 2020). Academic studies reinforce the argument that *Enterococcus* species isolated from European cheeses may be resistant to one or more antibiotics including chloramphenicol, tetracycline, erythromycin, penicillin, gentamycin, rifampicin, lincomycin, vancomycin and fusidic acid (Citak et al. 2004; Kürekçi et al. 2016; İspirli et al. 2017; Mrkonjic Fuka et al. 2017; Sanlibaba & Senturk 2018; Silvetti et al. 2019; Camara et al. 2020).

Virulence factors determined in enterococci, such as cytolysin (*cyl*A, *cyl*B, *cyl*M genes), aggregation substance (*agg*2 gene), and gelatinase (*gel*E gene), should be evaluated for the pathogenicity of enterococcal isolates. Cytolysin may cause the

deformation of cell membranes such as erythrocytes and other mammalian cells. An aggregation substance is a protein which is surface-localized and efficiently allows conjugal transfer in a fluid environment. Gelatinase is an enzyme that hydrolyzes bioactive compounds such as collagen, gelatin, hemoglobin (Templer & Baumgartner 2007). There were some studies about a screening of virulence genes isolated from traditional raw milk cheeses. It was reported that enterococcal strains isolated from raw milk cheese may carry at least one of the virulence genes such as *gel*E (Templer & Baumgartner 2007, Hammad et al. 2015).

The aim of this study was to determine antibiotic resistance characteristics and virulence genes of enterococcal strains isolated from traditional white-brined Urfa cheese samples.

2. Material and Methods

2.1. Isolation and identification of enterococcal strains

In the study, the cheese samples (n=20) were obtained from local producers in Urfa, Turkey. The cheese samples (10 g) were homogenized with 90 milliliters of buffered peptone water (BPW; Merck, Germany) using a laboratory stomacher for 1 min. The decimal dilutions of the samples were made in sterile BPW and spread on Kanamycin Aesculin Azide (Merck, Germany) agar and then incubated at 37 °C for 48 h. Three suspicious black colonies were taken from each cheese sample plates and purified on Trypticase Soy agar (Merck, Germany).

For the identification of the pure strains, Gram staining, catalase reaction, growth in 10 °C, 45 °C, pH 9.6, 6.5% NaCl were applied, and API 20 Strep (bioMérieux, France) biochemical test kit was used. The strains were stored at −20 °C in Brain Heart Infusion (Merck) broth with 30% glycerol (Citak et al. 2004; Jurkovic et al. 2006).

2.2. Screening of antibiotic resistant enterococcal strains

The antibiotic-resistance characteristics of the enterococcal strains were detected for some antibiotics, including erythromycin (15 µg), vancomycin (30 µg), ampicillin (10 µg), tetracycline (30 µg), chloramphenicol (30 µg), penicillin G (10 µg), and gentamycin (10 µg) on Muller Hinton agar (Merck), using a disc diffusion method as described by the Clinical and Laboratory Standards Institute (2017). The tested antibiotic discs were obtained from Oxoid (UK). The results were evaluated according to the cut-off levels in CLSI (2017) standard for the antibiotics.

The multiple antibiotic resistance (MAR) index of each isolate was also calculated (Krumperman 1983). The MAR index is the ratio of the total number of antibiotics to which the isolate was resistant to the number of antibiotics to which the isolate was exposed (Krumperman 1983). If the calculated MAR index is greater than 0.2, it means that the isolate was heavily exposed to human- or animal-sourced antibiotics; if the MAR index is equal to or smaller than 0.2, it means that the antibiotics were used very rarely or were not used at all.

2.3 *Screening of enterococcal strains for virulence and antibiotic resistance genes*

The vancomycin (*van*A and *van*B) and erythromycin (*erm*B), resistance genes, and virulence determinants (*agg*2, *gel*E, *cyl*M, *cyl*B, *cyl*A) of the enterococcal strains isolated from the Urfa cheese samples were determined by polymerase chain reaction (PCR). The genomic DNAs of the enterococcal strains were extracted by using a commercial DNA isolation kit (Qiagen). PCR primers for antibiotic resistance and virulence genes (Table 1) were selected according to Eaton & Gasson (2001), Reviriego et al. (2005); Pasquaroli et al. (2014).

PCR amplifications were performed in 25 µL reaction mixtures using 1 mM dNTP mix (Promega, Sunnyvale, CA, USA), 1 U Go Taq Flexi DNA polymerase (Promega), 1 µL of DNA and 10 pmol of each primer obtained from IDT (Integrated DNA Technologies, Coralville, IA, USA). The samples were exposed to an initial cycle of denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 53 °C for 30 s and elongation at 72 °C for 45 s (Eaton and Gasson 2001; Reviriego et al. 2005).

E. faecalis NCIMB 700584 and *Enterococcus hirae* FM 2.16 were used as positive control strains for virulence genes and *erm*B gene, respectively (Eaton & Gasson 2001; Pasquaroli et al. 2014).

Table 1- The primers for virulence and antibiotic resistance genes

Genes	*Primer sequence (5'-3')*	*Product size (bp)*
***agg*2**	F-5' GTT GTT TTA GCA ATG GGG TAT R-5' TCC TGT CAC TCC TCT TCT CAG	1210
***gel*E**	F-5' ACC CCG TAT CAT TGG TTT R-5' ACG CAT TGC TTT TCC ATC	419
***cyl*M**	F-5' TGC TTC TCC ACT GTG ACC T R-5' ATC TAG TAA ATG TTA AGA AAT ACA	742
cylB	F-5' TGG AAG CAT TAC TTC CAG CT R-5' AAC TGC AAC CTC AAG ATT GG	843
***cyl*A**	F-5' AAT CCT ATC GGT TAC TGC TTA R-5' AGC ATC ACA ACC ATC CTA AC	517
***van*A**	F-5' GTA CAA TGC GGC CGT TA R-5' GGG ACA GTT ACA ATT GC	732
***van*B**	F-5' GTG CTG CGA GAT ACC ACA GA R-5' CGA ACA CCA TGC AAC ATT TC	1145
***erm*B**	F-5' CAT TTA ACG ACG AAA CTG GC R-5' GGA ACA TCT GTG GTA TGG CG	425

3. Results and Discussion

3.1. Distribution of enterococcal strains

Urfa cheese is a traditional white cheese variety which is produced in the southeastern part of Turkey. This cheese type is produced using raw ovine or bovine milk or a mixture of these milk types. It is kept in a high-dense brine solution or boiled for 2-3 minutes to obtain the microbiological quality. However, these methods not enough for providing hygienic quality. It was mentioned that Urfa cheese was produced by raw milk heated up to 30-35 °C and that the microbial load of the cheese was, therefore, quite high (Uraz et al. 2008).

The enterococcal counts of the Urfa cheese samples were found to be between 4.4-5.6 log cfu g^{-1} in the study. A total of 54 isolates were picked from the cheese samples and 49 of them were identified as *Enterococcus* spp. The isolates were also defined as *E. faecalis* (24), *E. faecium* (22) and *E. durans* (3) using the API 20 Strep biochemical test kit (Table 2).

There were limited data about the enterococcal load and distribution of Urfa cheese samples in different studies. Uraz et al. (2008) analyzed 11 Urfa cheese samples and found out that *E. faecalis* (33%) and *E. faecium* (13%) were the dominant flora member of lactic acid bacteria in Urfa cheese samples. In another study (Kırmacı et al. 2016), the indigenous enterococcal load was determined to be between 4.78-7.04 log cfu g^{-1} in 20 Urfa cheese samples. Besides, *Enterococcus* spp. (48.95%) was a dominant part of the lactic flora of Urfa cheese with the distribution of *E. faecium* (40%), *E. durans* (32.85%), *E. faecalis* (18.57%), *E. lactis* (5.71%) and *E. hirae* (2.85%) species. Because of the resistance of enterococci to high salt content and low pH, this bacterial genus becomes a dominant member of lactic acid bacteria in Urfa cheese. Not only in Urfa cheese but also in many different traditional kinds of cheese produced from raw or pasteurized milk samples in the Mediterranean region, *Enterococcus* spp. may be found widespread (Kırmacı et al. 2016; Sanlibaba & Senturk 2018; Silvetti et al. 2019; Camara et al. 2020).

Enterococci is a part of natural gastrointestinal tract of humans and farm animals; so, these bacteria may contaminate milk directly during milking through animal faeces or indirectly through production equipment (Mrkonjic Fuka et al. 2017; Camara et al. 2020).

3.2. Antibiotic resistance characteristics and virulence genes of strains

Most of the enterococcal isolates were found to be resistant in high or intermediate level against at least one of the tested antibiotics (Table 2). Certain strains were highly resistant to tetracycline (51%), streptomycin (26.5%), erythromycin (10.2%), gentamycin (2%), and penicillin (2%), while some strains were intermediate-level resistant to erythromycin (30.6%) tetracycline (18.3%) and vancomycin (2%). The number of the antibiotic-resistant strains of E. faecalis was higher than that of the E. faecium strains. Ten (20.4%) of the E. faecalis strains and eight (16.3%) of the E. faecium strains showed multidrug resistance. In this study, it was also found that the MAR index was greater than 0.2 in 18 (36.7%) enterococcal isolates (Figure 1). Although most of the tested antibiotic resistance genes (vanA, ermB) were not detected in the strains, only the vanB gene was found in 3 (6.12%) of the enterococcal isolates (Table 2).

Table 2- Distribution, virulence genes, and antibiotic resistance characteristics of enterococci in Urfa cheese samples

No	*Isolate No*	*Cell Morphology*	*Gram staining*	*Catalase*	*Growth at*				*Species*	*Resistance* Characteristics*	*Virulence* Genes*
					15 ˚C	*45 ˚C*	*pH 9.6*	*6.5% NaCl*			
1	1.1	Coccus	+	-	+	+	+	+	*E. faecalis*	S_R, E_R, T_R	*gel*E, *agg2*
2	1.2	Coccus	+	-	+	+	+	+	*E. faecium*	T_I	*gel*E, *agg2*
3	1.3	Coccus	+	-	+	+	+	+	*E. faecium*	T_I	*gel*E, *agg2*
4	2.1	Coccus	+	-	+	+	+	+	*E. faecalis*	T_R	*gel*E, *agg2*
5	2.2	Coccus	+	-	+	+	+	+	*E. faecium*		*gel*E, *agg2*
6	2.3	Coccus	+	-	+	+	+	+	*E. faecalis*	E_R, T_R	*agg2*
7	3.1	Coccus	+	-	+	+	+	+	*E. faecalis*	P_R, T_I	*gel*E, *agg2*
8	3.2	Coccus	+	-	+	+	+	+	*E. faecium*	E_R, T_I	*gel*E, *agg2*
9	3.3	Coccus	+	-	+	+	+	+	*E. faecium*		*gel*E
10	5.2	Coccus	+	-	+	+	+	+	*E. faecalis*	T_R	*gel*E, *agg2*
11	10.1	Coccus	+	-	+	+	+	+	*E. faecalis*	S_R, T_R, E_I	*gel*E, *agg2*
12	10.2	Coccus	+	-	+	+	+	+	*E. faecalis*	T_I	*gel*E, *agg2*
13	10.3	Coccus	+	-	+	+	+	+	*E. faecalis*	T_R	*gel*E, *agg2*
14	11.1	Coccus	+	-	+	+	+	+	*E. faecalis*	T_I	*gel*E, *agg2*
15	11.2	Coccus	+	-	+	+	+	+	*E. faecalis*	T_R	*gel*E, *agg2*
16	11.3	Coccus	+	-	+	+	+	+	*E. faecium*	E_I	*gel*E, *agg2*
17	12.1	Coccus	+	-	+	+	+	+	*E. faecalis*	T_R	*agg2*
18	12.2	Coccus	+	-	+	+	+	+	*E. faecium*	S_R, T_I	
19	12.3	Coccus	+	-	+	+	+	+	*E. faecium*	T_R	*gel*E
20	13.1	Coccus	+	-	+	+	+	+	*E. faecium*	E_I	*agg2*
21	13.2	Coccus	+	-	+	+	+	+	*E. faecium*	E_I	*agg2*
22	13.3	Coccus	+	-	+	+	+	+	*E. faecalis*	T_R, *van*B	*agg2*
23	14.2	Coccus	+	-	+	+	+	+	*E. faecalis*	S_R, T_R, *van*B	*gel*E, *agg2*
24	14.3	Coccus	+	-	+	+	+	+	*E. faecalis*	T_R	*gel*E, *agg2*
25	15.1	Coccus	+	-	+	+	+	+	*E. faecium*		*gel*E, *agg2*
26	15.2	Coccus	+	-	+	+	+	+	*E. faecium*	S_R, T_R, E_I	*gel*E, *agg2*
27	15.3	Coccus	+	-	+	+	+	+	*E. faecalis*	T_R	*gel*E, *agg2*
28	16.1	Coccus	+	-	+	+	+	+	*E. durans*	G_R, *van*B	*gel*E, *agg2*
29	16.2	Coccus	+	-	+	+	+	+	*E. faecalis*	S_R, T_R, E_I	*gel*E, *agg2*
30	16.3	Coccus	+	-	+	+	+	+	*E. faecalis*	S_R, E_R, T_R	*gel*E, *agg2*
31	17.1	Coccus	+	-	+	+	+	+	*E. faecium*	T_R, E_I	

Table 2 (Continue)- Distribution, virulence genes, and antibiotic resistance characteristics of enterococci in Urfa cheese samples

No	*Isolate No*	*Cell Morphology*	*Gram staining*	*Catalase*	*Growth at*				*Species*	*Resistance* Characteristics*	*Virulence* Genes*
					15 ˚C	*45 ˚C*	*pH 9.6*	*6.5% NaCl*			
32	17.2	Coccus	+	-	+	+	+	+	*E. faecium*	S_R, T_R, E_I	
33	17.3	Coccus	+	-	+	+	+	+	*E. faecium*	V_I, E_I, T_I	*gel*E, *agg2*
34	18.1	Coccus	+	-	+	+	+	+	*E. durans*		*gel*E, *agg2*
35	18.2	Coccus	+	-	+	+	+	+	*E. faecium*	T_R	*gel*E
36	18.3	Coccus	+	-	+	+	+	+	*E. faecalis*	S_R, T_R, E_I	*gel*E
37	19.2	Coccus	+	-	+	+	+	+	*E. faecalis*	S_R, E_I	*gel*E, *agg2*
38	19.3	Coccus	+	-	+	+	+	+	*E. faecalis*	E_I	*agg2*
39	20.1	Coccus	+	-	+	+	+	+	*E. faecium*	T_R	*gel*E, *agg2*
40	20.2	Coccus	+	-	+	+	+	+	*E. faecium*	T_R, E_I	*gel*E, *agg2*
41	20.3	Coccus	+	-	+	+	+	+	*E. faecalis*	T_R	*gel*E, *agg2*
42	22.1	Coccus	+	-	+	+	+	+	*E. faecalis*	T_R	*gel*E, *agg2*
43	22.3	Coccus	+	-	+	+	+	+	*E. faecium*		*gel*E, *agg2*
44	23.1	Coccus	+	-	+	+	+	+	*E. faecium*	E_I	*gel*E, *agg2*
45	23.2	Coccus	+	-	+	+	+	+	*E. durans*		*gel*E, *agg2*
46	23.3	Coccus	+	-	+	+	+	+	*E. faecalis*	S_R, E_R, T_I	*gel*E
47	24.1	Coccus	+	-	+	+	+	+	*E. faecium*	S_R	*gel*E, *agg2*
48	24.2	Coccus	+	-	+	+	+	+	*E. faecium*		*gel*E
49	24.3	Coccus	+	-	+	+	+	+	*E. faecalis*	S_R, T_R, E_I	*gel*E, *agg2*

* S_R: Streptomycin resistance; E_R: Erythromycin resistance; E_I: Intermediate level erythromycin resistance; T_R: Tetracycline resistance; T_I: Intermediate level tetracycline resistance; P_R: Penicillin resistance; G_R: Gentamycin resistance; V_I: Intermediate level vancomycin resistance; *gel*E: gelatinase encoded gene; *agg*2: aggregation substance encoded gene; *van*B: vancomycin resistance gene

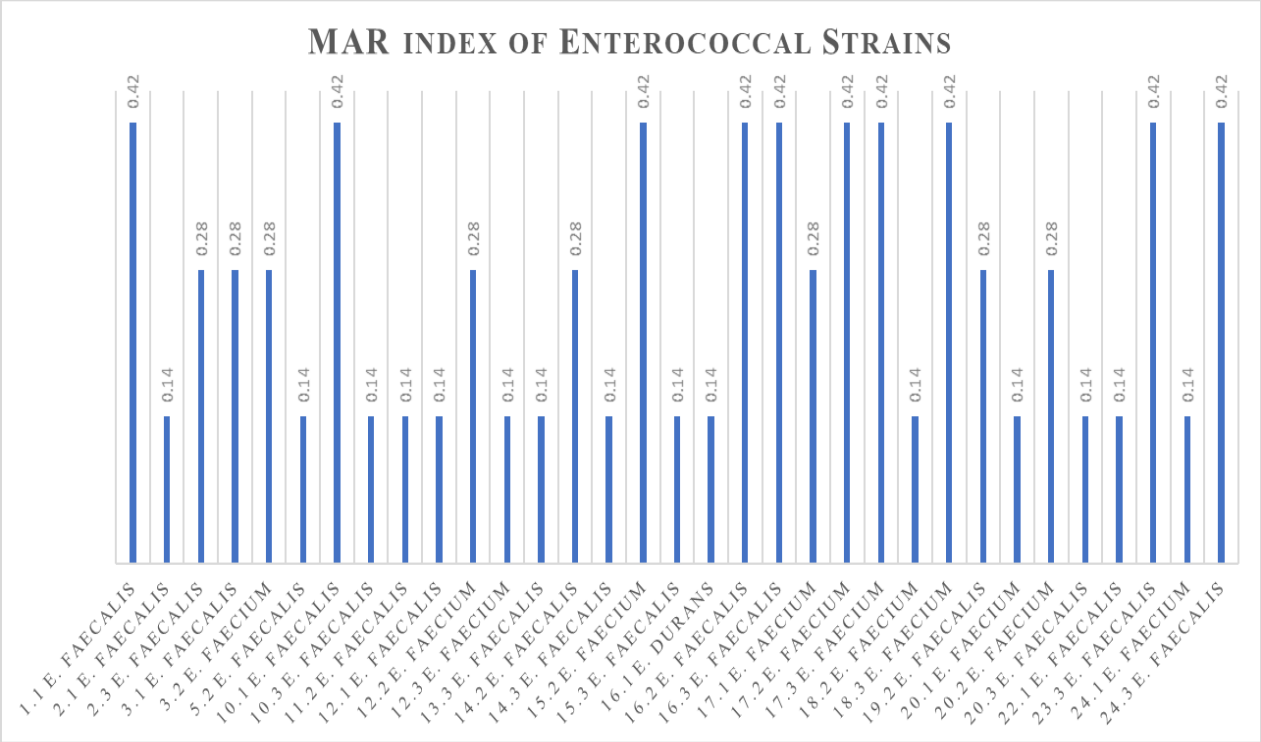

Figure 1- Multiple antibiotic resistance (MAR) index of enterococcal strains isolated from Urfa cheese

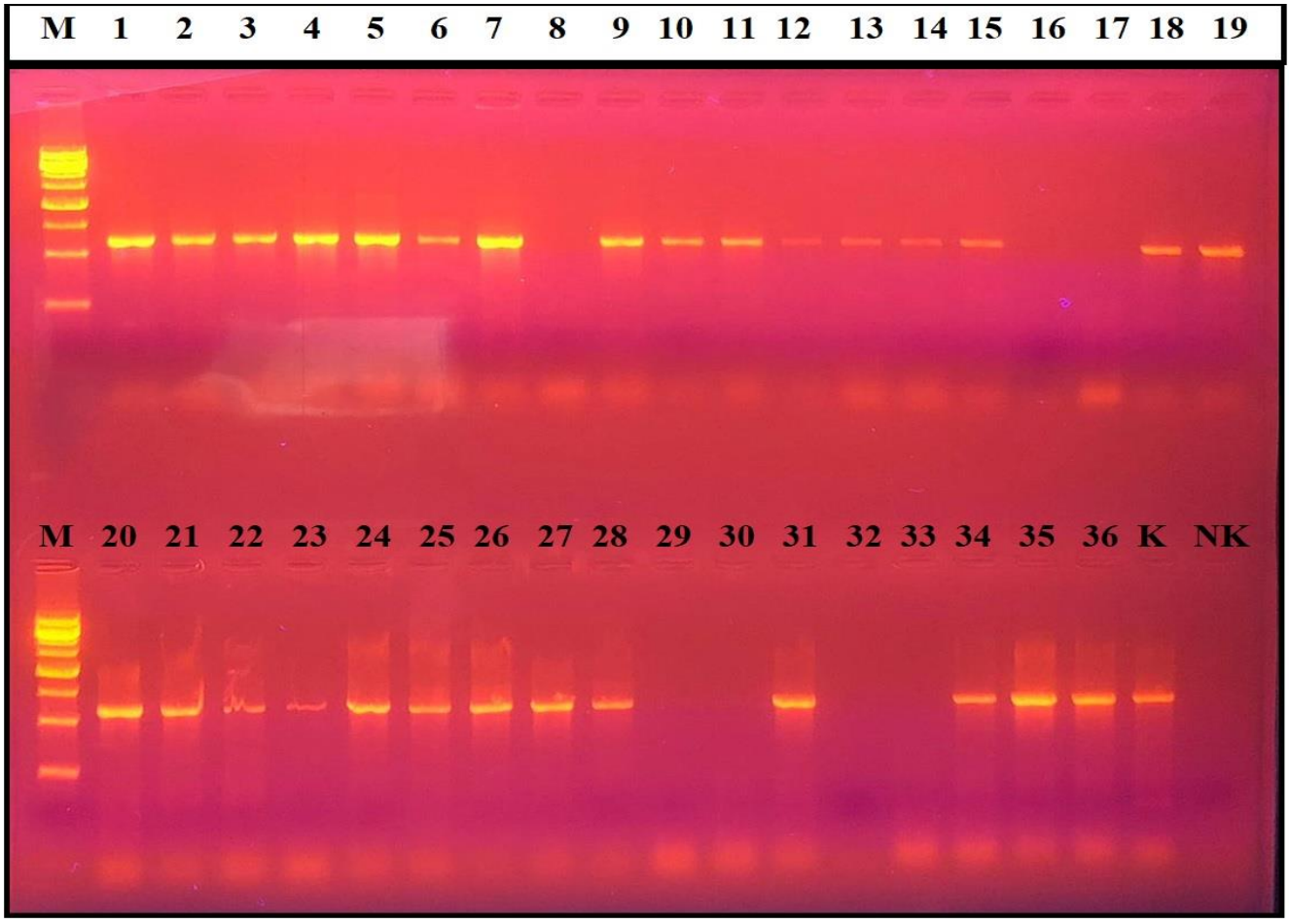

Figure 2- The agarose gel screen of *agg*2 gene positive enterococcal strains isolated from Urfa cheese samples (M; Marker, K; positive control, NK; negative control, *agg*2 positive strains; 1-7, 9-15, 18-19, 20-28, 31, 34-36)

Although many enterococci are the endogenous and beneficial microbial part of fermented foods, certain strains are recognized as a nosocomial pathogen and may carry virulence genes and antibiotic resistance characteristics. Misused and uncontrolled antibiotic treatment in human health and animal husbandry result in an increase in the development of antibiotic resistance among commensal bacteria of animal-sourced foods, especially in raw milk and dairy products (Hammad et al. 2015; Mrkonjic Fuka et al. 2017, Özdemir & Tuncer 2020).

In a study conducted by Zdolec et al. (2016), it was reported that enterococcal strains isolated from the milk samples of drug-treated udders and healthy cow udders were found to be resistant to tetracycline, chloramphenicol, and erythromycin with equal distribution. It was thought that this could be the result of animal cohabitation and cross-contamination. Although food-borne enterococci are not a direct cause of resistant enterococci in humans, they may transfer resistance determinants to bacteria in human microbiota; therefore, raw milk consumption without thermal process is considered to be a potential health risk for the public.

In another study carried out by Bouymajane et al. (2019), 150 raw cow's milk samples obtained from street traders in Meknes city, Morocco were analyzed for the identification of *Enterococcus* spp. and the antimicrobial susceptibility of the isolates was determined. It was found that ampicillin, streptomycin, and tetracycline resistance in *Enterococcus* spp. strains and as well as the multiple antibiotic resistance (MAR) index were higher than 0.5 in most of *Enterococcus* spp. The researchers emphasized that these findings may be indicated as a risk for public health.

Sanlibaba & Senturk (2018) isolated and identified *E. faecalis* (n=125) and *E. faecium* (n=88) strains from 215 traditional Turkish cheese samples such as White, Kasar, Tulum, Ezine, Lor, Orgu, and Civil. The isolates were found to be resistant against nalidixic acid (100%), kanamycin (98.6%), rifampicin (78.4%), ampicillin (48.8%), ciprofloxacin (45.5%), erythromycin (18.8%), tetracycline (11.7%), penicillin G (5.6%), chloramphenicol (4.2%), gentamycin (3.8%) and streptomycin (1.4%) in phenotype, and none of them were resistant to vancomycin. The antibiotic resistance levels of *E. faecium* strains were higher than those of *E. faecalis* strains. Moreover, it was determined that *E. faecium* (100%) and of *E. faecalis* (88.8%) strains were resistant to multiple drugs.

In another study about the antibiotic resistance of enterococci in traditional Turkish cheese varieties such as Tulum, Ezine, Antep, Civil, White, Sülk, Lor, Dil, Van Otlu, Kasar, and Orgu, it was determined that there was a resistance against lincomycin (88.5%), kanamycin (84.2%), gentamycin (51.1%), rifampin (46.8%), tetracycline (33.8%), high levels of gentamycin (2.2%) and streptomycin (5.8%), and low levels of ciprofloxacin, erythromycin and chloramphenicol. It was suggested to establish and monitor a quality control system for dairy products from farm to retail in antimicrobial resistance among emerging food-borne pathogens (Kürekçi et al. 2016).

Silvetti et al. (2019) investigated the antibiotic resistance incidence of 40 *E. faecalis* isolated from 10 Italian raw milk cheeses. While tetracycline, rifampicin, chloramphenicol, and erythromycin resistance were determined in the isolates, vancomycin resistance was not observed. It was concluded that *E. faecalis* strains from raw milk cheese may be a source for transferring antimicrobial resistance and other pathogenic characteristics to humans.

Calonico et al. (2018) reported drug resistance against vancomycin, chloramphenicol, ampicillin, tetracycline, linezolid and teicoplanin with a higher prevalence of *E. faecalis* than *E. faecium* in cheese samples. It was also reported that the patterns of resistance feature varied over the years for both *E. faecalis* and *E. faecium* and that the number of antibiotic-resistant and multidrug-resistant strains increased from 2002 to 2015.

The study conducted by Camara et al. (2020) analyzed the antibiotic resistance characteristics of 28 autochthonous *Enterococcus* isolates from Pico cheese and reported that tetracycline, rifampicin, erythromycin, and chloramphenicol resistance was found in the isolates. They emphasized the importance of evaluating the safety of enterococcal isolates from artisanal cheeses.

It was reported that enterococcal strains isolated from artisan Istrian raw milk cheese showed multidrug resistance (83.72%). They were found to be resistant against clindamycin (63.07%), streptomycin (82.00%), rifampicin (72.35%), chloramphenicol (28.41%), tetracycline (17.99%), erythromycin (29.35%), and vancomycin (23.48%) (Mrkonjic Fuka et al. 2017).

It was reported that there was very few knowledge about the passage of virulent and/or multidrug enterococcal strains from fresh raw milk cheese to the human gastrointestinal tract (Hammad et al. 2015). They isolated enterococci from Egyptian raw milk cheese and karish cheese, and detected some *E. faecalis and E. faecium* strains that carried one or more virulence genes, including *gel*E, *asa*1, *cyl*A, *esp*, and *hyl*. It was concluded that the potential reservoir of virulent and antibiotic-resistant enterococci may have a risk for public health.

Templer & Baumgartner (2007) tested the virulence genes (*gel*E, *agg*, *esp*, *cyl*, *efaAfs*, *efaAfm*, *cpd*, *ccf* and *cob*) in enterococci isolated from artisanal raw milk cheese (Schabziger and Appenzeller) produced in Switzerland. They reported that all tested strains contained at least 2 of the 9 virulence genes analyzed and that Schabziger and Appenzeller cheeses may be the

source of some antibiotic resistance and virulence determinants. All virulence genes found in the strains analyzed were also present in human clinical isolates.

4. Conclusions

Traditional Urfa cheese, which is produced using raw milk, may carry enterococci as a dominant part of the autochthonous microbiota. Different studies showed that *Enterococcus faecium*, *E. faecalis* and *E. durans* were the most frequently isolated species from Urfa cheese samples. The antibiotic resistance, which is a rising concern nowadays, has become a serious public health issue even for the commensal bacterial member of foods. A high-level tetracycline, streptomycin and erythromycin resistance, and multiple antibiotic resistance were found in most of the enterococcal strains isolated from Urfa cheese. Besides, *agg*2 and *gel*E genes, which were related to pathogenicity, were detected from the isolates in high rate. These results indicated that consuming unpasteurized milk and milk products may cause important health risk for humans and environment.

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The Effect of Neutral Electrolyzed Water on the Microbial Population and Quality of Dried Figs (*Ficus carica* L.) During Storage

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ABSTRACT
Fig is one of economically the most important agricultural products and preserving its quality is of crucial importance. In this study, the efficiency of Neutral Electrolyzed Water (NEW) in the controlling of the microbial load of dried figs was researched. For this reason, it was used three different fig groups. The first group was washed with 10% NEW, the second group was washed with 6% salty water at 50 °C for 1 min, and finally, the third group did not wash as control group. The samples were taken at the beginning of the storage and in monthly periods through 6 months. Initial average amount of mesophilic aerophilic bacteria (MAB) was 3.89 log cfu g^{-1}. This amount was reduced to 0.73 log cfu g^{-1} after 6 months storage in the group washed with NEW and 2.52 log cfu g^{-1} in the group washed with salty water. The yeast-mold number was reduced from 3.08 log cfu g^{-1} to 0.96 log cfu g^{-1} in the group washed with NEW and 1.9 log cfu g^{-1} in the group washed with salty water at the end of the 2nd month. It was determined that both applications in comparison with the control group did not cause an important change in physical and chemical parameters such as colour and pH of dried figs. These results show that NEW can be used as a more safe and effective method in reducing the microbial load in comparison with the standard application.

Keywords: *Ficus carica* L., Colour and pH of dried figs, Microbial load, Neutral electrolyzed water (new), Salty water

1. Introduction

Fig (*Ficus carica* L.) is an economically valuable and highly valued fruit. Its growing area is mostly the Mediterranean region (Stover et al. 2007) in which mild winters and hot dry summers are typical seasonal characteristics. In Turkey, it mainly grows in the Aegean region. With the yearly production of fresh figs of 306.499 tons (TUİK, 2018/2019) and dried fig of 72.000 tons (FAO, 2016/2017), Turkey ranks the first in the World in fig production.

Figs are generally dried on racks under the sun and among critical foods for microbiological contamination at the stages of harvesting, drying, storing, shipment and processing. They are generally contaminated with *Escherichia coli*, *Bacillus cereus* and its spores, *Penicillium spp*. and *Aspergillus spp*. (Frazier & Westhoff 1988; Boudra et al. 1994). The number of the microorganisms in the dried figs can reach up to 10^7–10^8 cfu g^{-1} under poor storage conditions and especially in inadequate storage conditions. Inadequate storage conditions increase aflatoxin (AF), ochratoxin A, and fumonisin risk which can cause hepatocellular carcinoma and cancer and immunosuppression for humans. Boudra et al. (1994) reported that the highest level of AF levels of dried figs was determined 6 times higher than fresh frigs and also, 30 times higher than unripe ones.

In routine Turkish plants, dried figs are first washed with salty (3-6% NaCl) high-temperature water (60-80 ºC) to reduce surface contamination and soften them.

One of the feasible alternatives to salty hot water can be electrochemical disinfection including acid electrolyzed water (AEW) and neutral electrolyzed water (NEW). Electrolyzed water is generated in electrolysis chamber, through anodes and cathodes separated by an ion-permeable membrane, using sodium chloride water solution. This process produces chlorine compounds such as hypochlorous acid (HOCl), hypochlorite ions (ClO^-) and chlorine (Cl_2) (Guentzel et al. 2010). These compounds are responsible for the effectiveness of electrolyzed water. It has been well documented that AEW has a strong bactericidal and antifungal effect against various microorganisms, attached to fruit and vegetables, including *Escherichia coli*, *Bacillus cereus*, and *Bacillus subtilis* (Ileri et al. 2006; Ding et al. 2011; Graça et al. 2011; Hao et al. 2011). Although having potential antimicrobial activity, AEW has a relatively low pH of 2-3 at which Cl_2 is easy to volatilize into the air which consequently poses threat to health. Moreover, AEW may be corrosive to organic materials due to its high acidity, which ultimately limits its application (Rahman et al. 2010).

In theory, the main types of chlorine in electrolyzed water are HOCl and ClO^- at pH between 6.0 and 9.0 (White 1998). NEW has a neutral pH of 7–8, resulting in less corrosion of contact surfaces and greater stability of chlorine agents (Len et al. 2002). It has been shown that NEW has strong antibacterial or antifungal, or anti-mycotoxigenic effect against *Yersinia enterocolitica*, *E. coli*, *Candida lambica* in fresh-cut vegetable washing water (Lehto et al. 2017), *Colletotrichum fructicola* on strawberry (Hirayama et al. 2016), indigenous flora of cabbage and carrot (Lee et al. 2014), *Alycyclobacillus acidoterrestris* spores on apples (Torlak 2014), mesophilic bacteria, psychrophilic bacteria, enterobacteria, yeast and molds in broccoli (Navarro-Rico et al. 2014), *E. coli* O157:H7, *Salmonella enteretidis* and *Listeria monocytogenes* on the surface of tomatoes (Deza et al. 2003), *E. coli*, spores of *Bacillus cereus*, *Aspergillus flavus*, *Penicillium expansum* isolated of fig fruit (Yamaner et al. 2016), and aflatoxin-contaminated Maize (Jardon-Xicotencatl et al. 2015).

The aim of this study is to assess the efficiency of NEW as a disinfectant in reducing total number of mesophilic aerophilic bacteria (MAB), yeasts and mold on dried figs stored at room temperature for 6 months by comparison with salty water which is a routine process in Turkey. This is the first study about NEW effects on microbial load and physicochemical properties of dried figs.

2. Material and Methods

2. 1. The preparation of neutral electrolyzed water

NEW was obtained by electrolysis of a mixture of NaCl (20 g L^{-1}) and tap water using STEL-10H-120-01 generator (STEL - 10H- 120-01, Russia) at 40.0 V, 9.0 A and a rate of 250 mL/22 sec. NEW dilutions were prepared by using sterile tap water, prepared by autoclaving at 121 °C for 15 min, at rates of 10%. The concentration of NEW and the time of exposure to the washing treatments was selected based on previous studies (Yamaner et al. 2016). Analytical indices [Oxidation Reduction Potential (ORR), pH and available chlorine concentration (ACC)] of the treated solutions were measured immediately after 10% NEW preparation. The pH was measured with a pH meter (HI 2211-02, HANNA, USA), and ORP was measured with an ORP meter (HI98120, HANNA, USA). The pH meter was calibrated using commercial standard buffers at pH 4.0 and 7.0 (Merck Ltd., Germany). The ACC was measured on the basis of the iodometric method reported by Dychdala (1983).

2. 2. Washing the figs and storing the samples

In this study, while one fig group was washed with 10% NEW, the other group was washed with 6% salty water which is the standard practice. Samples in the third group were stored as control group without any processing. The washing processes were performed for 1 min at 50 °C in a laboratory setting. After washing, each group was stored from November to May.

2. 3. Determining microbial load of samples

Ten fig samples from each group were randomly chosen at the beginning of the storage and in monthly periods for determining microbial load. By taking small parts of 1 g from the exterior surface of each of these samples under aseptic conditions, a total of 10 g sample was prepared. This sample was transferred into sterile peptone water (0.1 w/v%) and stirred in a magnetic stirrer for 5 minutes to homogenize the sample (Öztekin et al. 2006). Ten-fold dilutions were prepared from homogenate. 1 mL was taken from each dilution rate and inoculations were performed by using surface spread method to Nutrient Agar (NA) (Merck KGaA, Germany) for the total MAB count and to Potato Dextrose Agar (PDA) (Merck KGaA, Germany) (its pH was reduced to 3.5 with 10% lactic acid) for the yeast and molds count. The NA plates were incubated at 30 °C for 48 h, while PDA plates were incubated at 25 °C for 3-5 days (Messer et al. 2000). The inoculations were carried out with 3 parallels in 3 repetitions. After incubation, the colonies of the microorganisms were counted and results were recorded. Count of totally MAB and yeast-mold was calculated as log colony forming unit per gram (log cfu g^{-1}).

2. 4. Physicochemical analyses

pH values of the dried figs were determined with a pH meter (HI 2211-02, HANNA, USA), according to AOAC (1990) method 970.21. The colour of the samples was measured with a Minolta chromameter (Minolta, model CR310, Osaka, Japan) (Cemeroğlu 2007). Using L*, a*, b* values according to Mohammadi et al. (2008), the browning index (B.I) was calculated. Moisture contents of the samples were determined using dried fruit moisture tester meter - Type A series (DFA of California, PO Box 270A, Santa Clara). The conductivity values obtained were converted into % moisture content from the device's conversion table. For water activity measurement, fruit at room temperature were minced. The pulps were placed in the TESTO 650 reference measurement device and their water activity was read (Cemeroğlu 2007). All physicochemical analyses were carried out with 10 fig samples from each group.

2. 5. Statistical analysis

All experiments were of complete randomised design and carried out with 3 parallels in 3 repetitions. Results were analysed by One-way ANOVA using the Duncan's multiple range test ($P<0.05$) to determine differences in effects of treatments on microbial

inactivation. The statistical analyses were performed with SPSS (version 15.0).

3. Results and Discussion

3. 1. Physicochemical properties of NEW solution.

To facilitate the NEW usage for fig process, 10% NEW concentrations were prepared by tap water. The ORP, pH and ACC for the treated solutions (NEW) are shown in Table 1.

Table 1- Physicochemical properties of 10% NEW solution

Concentrations (%)	10
pH	7.65
EC (mS)	2.5
ORP (mV)	855
ACC (ppm)	71.4

ACC composes of Cl_2 gas, HOCl and ClO^-. Composition of ACC (%) in EW changes depending to pH values. At lower pH (0-2.0), chlorine exists in gas form. Cl_2 proportion decreases as pH value increases. At pH 5.0-6.5, HOCl concentration is the highest amount. As pH increases, HOCl dissociates into ClO^- (Rahman et al. 2016). In this study, 10% NEW was produced which has 7.65 pH and 71.4 ppm ACC values. Therefore, predominant composition of ACC in this solution consists of HOCl and ClO.

3. 2. Microbiological analysis results

In this study, MAB and yeast-mold loads of samples were determined before and after the washings for understanding the effectiveness of NEW on microbial loads (Tables 2 and 3). Dried figs wait between 3-6 months until they reach the consumer from the post-harvest producer warehouses, wholesaler warehouses and finally the warehouses of the fig processing plants. Considering these storage periods, the study is established on a 6-month storage period.

Table 2- Efficacy of treatment solutions (10% NEW and 6% salty water) against MAB load on surface of dried figs during the storage time*

Treatments	*Storage Time (Month)*							
	Before washing	*After washing*	1	2	3	4	5	6
	MAB count (log cfu g^{-1})		*MAB count (log cfu g^{-1})*					
Control	3.89 ± 0.05^{Ca}	3.89 ± 0.05^{Cc}	3.35 ± 0.00^{Bc}	3.33 ± 0.05^{Bc}	3.24 ± 0.07^{Bc}	3.25 ± 0.07^{Bc}	3.04 ± 0.03^{Ab}	3.27 ± 0.11^{Bb}
6% salty water	3.87 ± 0.03^{Ea}	3.32 ± 0.14^{Db}	2.61 ± 0.14^{BCb}	2.31 ± 0.28^{Ab}	2.82 ± 0.17^{Cb}	2.58 ± 0.09^{ABCb}	2.66 ± 0.05^{BCb}	2.52 ± 0.17^{ABb}
10% NEW	3.89 ± 0.03^{Da}	2.71 ± 0.16^{Ca}	2.15 ± 0.22^{BCa}	1.88 ± 0.11^{BCa}	1.94 ± 0.17^{BCa}	1.38 ± 0.40^{ABa}	1.79 ± 0.36^{BCa}	0.73 ± 1.26^{Aa}

^{AB}: Values followed by the same letter in the same row are not significantly different ($P < 0.05$); ^{ab}: Values followed by the same letter in the same column are not significantly different ($P < 0.05$); *: Data are reported as mean ± standard deviation

Table 3- Efficacy of treatment solutions (10% NEW and 6% salty water) against yeast-mold load on surface of dried figs during the storage time*

Treatment Solution	*Storage Time (Month)*							
	Before washing	*After washing*	*1*	2	3	4	5	6
	Yeast-mold count (log cfu g^{-1})		*Yeast-mold count (log cfu g^{-1})*					
Control	3.34 ± 0.47^{Ba}	3.36 ± 0.46^{Bb}	3.01 ± 0.29^{ABb}	2.81 ± 0.60^{ABc}	2.78 ± 0.40^{ABb}	2.82 ± 0.30^{ABb}	2.47 ± 0.53^{ABb}	1.95 ± 1.25^{Aa}
6% salty water	3.07 ± 0.04^{Ea}	2.89 ± 0.20^{DEab}	2.48 ± 0.10^{CDEa}	1.9 ± 0.25^{BCb}	2.06 ± 0.63^{BCab}	2.34 ± 0.22^{CDab}	1.47 ± 0.42^{ABab}	0.97 ± 0.66^{Aa}
10% NEW	3.08 ± 0.03^{Ea}	2.6 ± 0.28^{DEa}	2.10 ± 0.21^{CDa}	0.96 ± 0.26^{ABa}	1.5 ± 0.49^{BCa}	1.32 ± 0.86^{ABCa}	0.77 ± 0.67^{ABa}	0.5 ± 0.69^{Aa}

^{AB}: Values followed by the same letter in the same row are not significantly different ($P < 0.05$); ^{ab}: Values followed by the same letter in the same column are not significantly different ($P < 0.05$); *: Data are reported as mean ± standard deviation

The number of MAB was decreased by 81% in the group washed with 10% NEW after 6 months of storage. In the same period, the number of MAB was decreased by 35% in the group washed with 6% salty water (Table 2). The yeast-mold count was decreased by 69% in the group washed with 10% NEW after 2 months storage. In the same period, the yeast-mold count was decreased by 38% in the group washed with 6% salty water (Table 3).

The rate of decrease in yeast-mold load in fig samples washed with 10% NEW in the 2nd month was 69%, but this rate decreased in the third month. The reason for this decrease is that the storage conditions are not controlled and the water activities of the stored fig samples increase from aw 0.69 to 0.78 due to seasonal changes (Figure 1). Therefore, since the 2nd month of the storage, there have been fluctuations in the yeast-mold number of the stored figs due to the fluctuation in water activity. The fluctuation in water activity and yeast-mold count of fig samples washed with 10% NEW was also observed in fig samples stored under the same conditions and washed with 6% salty water. In general, the lowest water activity required for bacterial growth is aw 0.85, while this value for fungi is aw 0.65 (Manolopoulou et al. 2017). Therefore, the fluctuations between aw 0.6 and 0.8 of the water activities values of figs in storage conditions did not affect the MAB load as much as the yeast mold number. As a result, 10% NEW is significantly ($P<0.05$) more effective than 6% salty water at all the storage periods for all microorganisms.

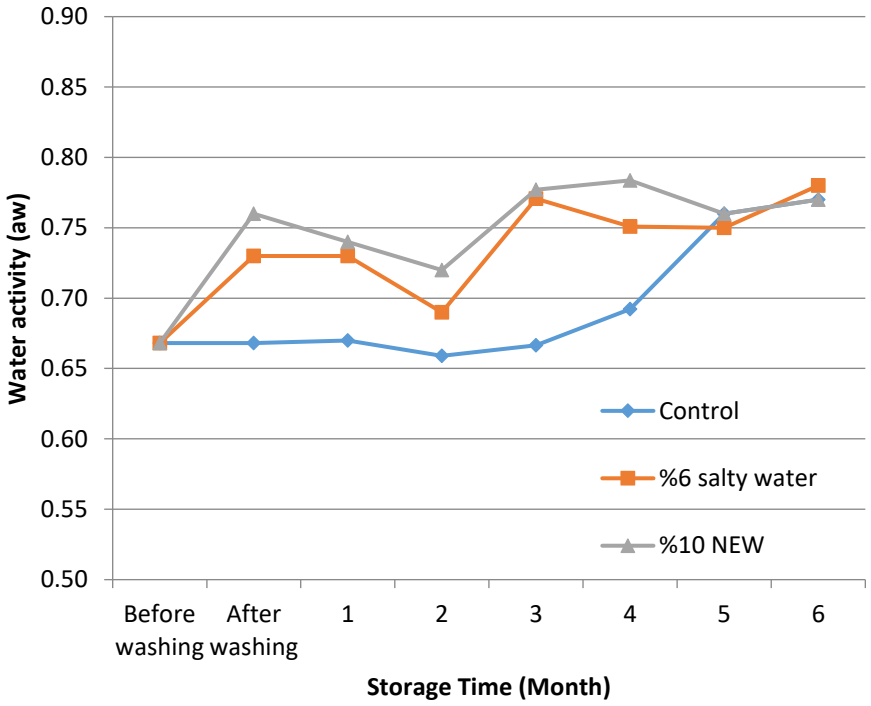

Figure 1- Water activities changes on threated figs during the storage period

Deza et al. (2003) reported a more reduction than 4 log for *Escherichia coli* O157:H7, *Salmonella enteritidis* and *Listeria monocytogenes* on tomato surfaces exposed to NEW (pH: 7.99-8.15, ORP:745-771 mV, Cl:86-93 mg L^{-1}) for 1 min. Graça et al. (2011) explored sanitizing effect of NEW (pH: 8.39, ORP:753 mV, Cl:50 mg L^{-1}) containing 50 mg L^{-1} of active chlorine for *Escherichia coli, Listeria innocua* and *Salmonella choleraesuis* on fresh-cut apples. They reported that all microorganisms tested on apple slices were reduced by about 1.50 log in 5 min. Torlak (2014) observed a reduction of 1.98 log spore counts of *Alicyclobacillus acidoterrestris* on apples surfaces exposed to NEW (pH: 7.52, ORP:770 mV, Cl:50 mg L^{-1}) for 1 min. In all of three studies using NEW above, the materials and bacteria that were tested and NEW exposure durations, are different. Therefore, the low bacterial inactivation rate in the study that has longer NEW treatment duration from these three studies may arise from difference of the materials and bacteria that were tested. Graça et al. (2011) reported that the structure of surfaces of materials that were tested can likely affects resistance of microorganism to detachment by washing agents and to inactivation by antimicrobial agents. Since the surface of figs used in our study is not smooth like that of tomato, the inactivation power of 10% NEW may have decreased. Graça et al. (2011) also indicated that chlorine reacts with organic matter and some ingredients from tissues of cut fruit surfaces, diminishing its efficiency which does not occur in vegetable). If fig had been a smoother surface and less organic matter on its surface, it would have been obtained a more microbial reduction than 3.16 log for MAB and ≥2.1 log for yeast and mold.

In this study, the microbial inactivation power of 10% NEW may also be affected from its application temperature (50 °C). Yamaner et al. (2016) reported that while NEW (5%, 33.9 mg L^{-1}) application for 1 min led a reduction of 0.5 log cfu mL^{-1} on spores of *A. flavus* at 22 °C, the numbers of spores of *A. flavus* were reduced more than 6 log cfu ml^{-1} by NEW (33.9 mg L^{-1} ACC) application for 1 at 50 °C. It has been showed that a higher temperature helped EW to more effectively inactive microorganisms. Consequently, the duration and way of application (spray, dip, or others), pH, ACC, ORP, temperatures of NEW are just some variables that can be combined to find the better results of NEW application (Athayde et al. 2018).

Although there is no study on antimicrobial effect of electrolyzed water for fig, there are some studies on disinfection of fig by ozone exposure. Öztekin et al. (2006) exposed the dried figs to 10 ppm ozone for 5 h. While the total MAB count was decreased from 2.57 to 1.59 log cfu g^{-1}, the yeast-mold count was reduced from 1.46 to 0.40 log cfu g^{-1} for 5-hour ozone exposure. They reported that as the ozone dosage and exposure duration were reduced, microbial inactivation rate also decreased. In addition, up to 2 log reductions in the number *B. cereus* spores were obtained after 360 min-ozonation exposure above 1.0 ppm (Akbas et al. 2008). Given the processing and packaging duration of dried figs in an enterprise, 5-6 hours ozone treatment is too long and unsuitable for dried fig enterprises. As a result, NEW has shorter application duration than ozone for disinfection, more antimicrobial activity than salty water, low production and application cost. Also, it is an all-natural, organic, non-toxic, non-irritant, environmentally and ecologically safe sanitizing and disinfecting solution (Rahman et al. 2016), so 10% NEW should be used in fig enterprises. Also, the results show that NEW should be used together with suitable high temperatures.

3. 3. Physicochemical characteristics of stored figs

Water activities (a_w) of washed figs (for 1 min at 50 ºC) just after 10% NEW and 6% salty water treatment increased from 0.67 to 0.76 and 0.73, respectively (Figure 1). In parallel with the a_w value increasing as a result of the washing the dried figs, moisture content (%), too, increased from 17.4 up to 23 just after 10% NEW and 6% salty water treatment (Figure 2). It was detected that a decrease (from aw 0.76 to 0.72 for samples washed with NEW, from aw 0.73 to 0.69 for samples washed with 6% salty water) in water activity during storage up to 2nd month and an increase from 2nd to 6th month exist. It was determined that the % humidity rate of the washed samples varied between 23% and 24.5%. The changes in water activity and % humidity may have resulted from ordinary storage conditions of the samples and seasonal changes. The a_w and moisture values of figs in all three groups including the control group (which was not washed) showed slight fluctuations due to seasons changes in the storage period (Figures 1 and 2).

Water activity is an important parameter for the toxigenic fungus and mycotoxin formation during the harvest and processing of figs. Heperkan (2006) reported that *Aspergillus* section Nigri existed at a rate of 100% in samples obtained from storage. The main ochratoxin A producers in figs in Turkey are *A. carbonarius* and *A. niger* (Karbancioglu-Güler & Heperkan 2008). Also, it was reported that *A. niger* in dried figs was a fumonisin producer (Daskaya & Heperkan 2010). The drying process of fig can lead to a selective and suitable environment for *A. niger* that are xerotolerant, for while the moisture content of fig diminishes, the sugar content increases (Heperkan et al. 2012). Consequently, the washing dried figs with 10% NEW to reduce the *A. niger* load in dried figs is important for human health.

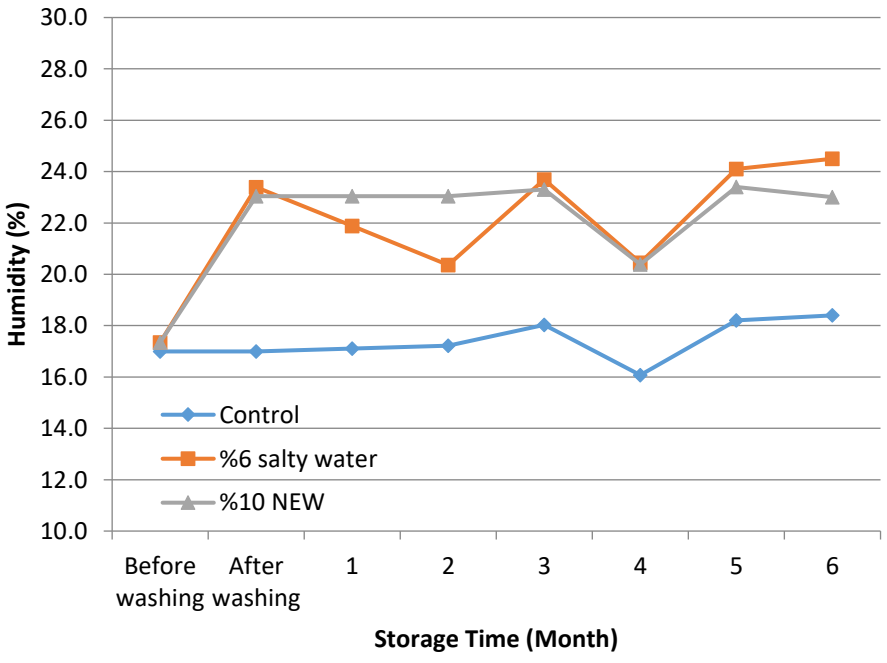

Figure 2- Changes in the moisture levels (%) on dried figs during storage

Colour is one of the most important quality parameters for the consumers. Colour values were expressed as L (whiteness, brightness /blackness), a (redness/ greenness) and b (yellowness/blueness) (Table 4). It is seen that while the L-value of the figs in the control group is 63.23, L-value of the dried figs washed with 10% NEW is 61.45 and L value of figs washed with 6% salty water is 62.39. The browning index of the control group, the samples washed with 6% salty water and 10% NEW were detected to vary between 85.52 - 76.43; 90.35 - 76.76 and 97.44 - 75.81, respectively during storage. The Browning index values obtained in this study correspond with the sun-dried fig browning index values reported by Manolopoulou et al. (2017). Also, in this study,

B.I of figs washed with 10% NEW is parallel to those of figs washed with 6% salty water, which is the standard practice. L *, a *, b * and B.I values of all three groups are given in Table 4.

Table 4- Changes in the colour values during the storage period after the washing of dried figs

Storage time	*Control*				*6% Salty Water*				*10% NEW*			
	*L**	*a**	*b**	*B.I*	*L**	*a**	*b**	*B.I*	*L**	*a**	*b**	*B.I*
0.Month*	63.23	9.77	33.95	85.52	62.39	9.55	35.07	90.35	61.45	10.36	36.31	97.44
1. Month	61.49	9.54	31.86	81.99	59.72	9.39	33.24	89.57	59.44	9.59	34.05	93.16
2. Month	61.94	9.61	29.98	75.73	60.32	9.82	32.11	85.26	60.45	9.84	32.72	87.01
3. Month	59.51	8.97	28.76	75.24	57.4	8.49	28.29	76.76	55.38	8.49	27.82	78.92
4. Month	58.71	8.48	27.89	73.32	56.83	8.39	27.28	74.46	57.43	8.33	27.88	75.22
5. Month	62.2	8.1	28.84	70.24	60.18	7.79	27.98	70.40	57.94	7.84	26.45	69.40
6. Month	57.71	9.005	28.14	76.44	54.81	8.69	26.77	76.76	53.48	8.16	25.97	75.81

*: The sample taken after the washing, before the storage.

4. Conclusions

In this study, the efficiency of 10% NEW and 6% salty water on microbial load on the surface and physicochemical properties of dried fig samples during the storage for six months was compared. Microbiological and physicochemical results show that washing figs with 10% NEW for 1 min at 50 °C is better than 6% salty water for disinfection of figs. By its neutral pH level, electrolyzed water usage became a safer and easier alternative for food industry. The NEW is thought to be more effective alternative for the salty water used in fig plants. According to our current information, this study is the first report on the applicability of NEW in the controlling of the microbial population of dried figs and provides a scientific background for further experiments

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Genetic Diversity and Population Structure of Barley Cultivars Released in Turkey and Bulgaria using iPBS-retrotransposon and SCoT markers

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ABSTRACT
To improve quantitative traits, it is essential to acknowledge genetic structure and diversity of the crop plants. In this study, 54 barley cultivars released from 1963 to date by different institutes in both Turkey and Bulgaria were screened with 18 iPBS and four SCoT markers to evaluate population structure and genetic diversity. According to the results, while total polymorphic band numbers was identified as 560, the polymorphic ones were found as 530 (438 and 92 amplified bands for iPBS and SCoT markers, respectively). In addition, the average polymorphic band number was found as 24.09. While the average polymorphism information content (PIC) value was 0.48, the average PIC value was 0.48 for iPBS and 0.48 for SCoT markers. The highest PIC value was determined as 0.50. The highest effective number of alleles, Shannon's information index, and Nei's genetic diversity were detected from the iPBS2271 marker at 1.61, 0.52 and 0.35, respectively among the iPBS markers while the highest values were obtained from SCoT-71 marker as 1.55, 0.32 and 0.48, respectively. As a result of a distribution of the 530 amplified bands in 54 barley cultivars, structure analysis showed that the subpopulations in the barley cultivars as a value of k=5. The average expected heterozygosity and fixation indices were identified as 0.234 and 0.322, respectively. Based on DICE similarity index, Martı and Zahir cultivars were found the most similar barley cultivars with 75% genetic similarity, whereas Özdemir and Karatay 94 and Tosunpaşa and Konevi cultivars were found 73% similar. On the other hand, Bayrak and Avcı-2002 were found the most diverse cultivars with 19.9% genetic similarity. As a result, the barley cultivars released in Turkey and Bulgaria were found varying and, the genetic diversity and statistics index analysis indicated that iPBS and SCoT markers are powerful markers to perform genetic diversity analysis.

Keywords: Barley, Genetic diversity, iPBS-retrotransposons, SCoT marker, Structure analysis

1. Introduction

Plant genetic diversity (PGD) is the major component for plant's life and crop improvement. In addition, genetic diversity becomes more crucial in response to climatic change and different biotic and abiotic stresses in nature. Intra and inter-specific differences in plant genetic resources (PGR) are the basic requirement for all crop improvement programs as well as providing preferred traits for farmers. Genetic diversity is affected primarily by sexual recombination as well as evolutionary forces such as genetic drift, mutation, migration etc. and domestication or artificial selection (Bhandari et al. 2017). Barley (*Hordeum vulgare*), derived from its wild progenitor *Hordeum vulgare* ssp. *spontaneum*, is vital crop for the malting and brewing industries (about 20% of global production) and it constitutes as an important animal feed (about 75% of global production). It is also using as a staple food in different parts of the world (5%) owing to adaptation to drought, soil salinity, and high altitudes (Baik & Ullrich 2008; Blake et al. 2011). Barley is a diploid crop (2n=14) with a genome size of 5.1 gigabases (Gb) and its genome contains 26.159 'high-confidence' genes (The International Barley Genome Sequencing Consortium, 2012).

Molecular (DNA) markers can be used for analyses of molecular variations that resulted by deletion, duplication, inversion, and/or insertion in the genomes (Govindaraj et al. 2015). Start Codon Targeted polymorphism (SCoT) is a dominant marker system and has been effectively employed to detect genetic variations. Single 18-mer primers are used to anneal short conserved region neighboring the ATG translation initiation (or start) codon. This method provides high polymorphism and reproducible markers related with characters of biological interest (Collard & Mackill 2009). SCoT marker system has been

successfully applied in various crops such as Tunisian citrus species (Mahjbi et al. 2015), landrace chickpea (Pakseresht et al. 2013), durum wheat (Etminan et al. 2016), cultivars of Egyptian wheat (Abdel-Lateif & Hewedy 2018), and 20 barley genotypes (Dora et al. 2017). Retrotransposons are repetitive and mobile sequences and observed in virtually all known eukaryotic genomes (Flavell et al. 1992). Particularly, retrotransposons are abundant in plant genomes and play important roles in genome evolution. In many cases, retrotransposons in plants cover more than 50% of the genome (Kumar & Bennetzen 1999). Retrotransposons are classified into two groups like long terminal repeat (LTR) and non-LTR retrotransposons. The inter-primer binding site (iPBS) method is a PCR-based marker system as a reverse transcriptase primer-binding site (PBS) based on complement of tRNA in LTR retrotransposons (Kalendar et al. 2010).

Shannon's diversity index (I), and Nei's genetic diversity (h) were used to evaluate the information of the markers including iPBS and SCoT markers by the researches. Diversity of the statistics index values of the markers with a value of zero or below zero than couldn't be used in the analysis, because of lower level of knowledge for genetic diversity. The highest value of the diversity index tends to a greater level of polymorphism of the primers and, thus, assisted to select the best marker loci in the genetic separation analysis (Tahir et al. 2019).

The iPBS markers have been used in numerous crops including Turkish okra (Yıldız et al. 2015), common bean (Nemli et al. 2015), pea, lens (Baloch et al. 2015a & 2015b, respectively) and tobacco (Yaldız et al. 2018). Thus, in the present study it was aimed to evaluate the genetic diversity of 37 Turkish and 17 Bulgarian barley genotypes using SCoT and iPBS-retrotransposon markers for the first time.

2. Material and Methods

2.1. Plant material

The plant materials used in the study consisted of 54 barley cultivars developed by Aegean Agricultural Research Institute Turkey (AARI), Bahri Dağdaş International Agricultural Research Institute Turkey (BDIARI), Field Crops Central Research Institute Turkey (FCCRI), Trakya Agricultural Research Institute Turkey (TARI), Transition Zone Agricultural Research Institute Turkey (TZARI) and Institute of Agriculture Karnobat, Bulgaria (IAK). Cultivar name, origin, developing institute, spike type, release date and pedigree of the cultivars are given in Table 1.

2.2. DNA isolation

Genomic DNA of the barley cultivars was isolated from single seeds. Three seeds from each cultivar were planted in a 10.5 cm pot and at two leaves stage one seedling per pot left. The leaves were collected from the seedling and fixed in 2 ml micro centrifuge tubes for DNA extraction. Genomic DNA of the cultivars was extracted using a cetyl trimethyl ammonium bromide method, indicated by Oliver et al. (2010).

Table 1- Pedigree, origin and spike type of the barley genotypes used in the study

No	*Cultivar*	*Origin*	*Institute*	*Spike Type*	*Release Date*	*Pedigree*
1	Bozhin	Bulgaria	IAK	Six-Rowed	1994	H280 - 7/ NaN_3 - mutant
2	Zemela	Bulgaria	IAK	Six-Rowed	2016	F2 2012/01 x Кт 2152 200 Gy - mutant
3	Aheloy 2	Bulgaria	IAK	Six-Rowed	1994	Hemus x №102/121
4	Zagoretz	Bulgaria	IAK	Two-Rowed	2008	4943 - 41 x 1023K-7
5	Alekssan	Bulgaria	IAK	Six-Rowed	2017	K-2169-01 x Кт 2145
6	Odisey	Bulgaria	IAK	Two-Rowed	2011	Obzor x Nutans 85242/64/ x Nutans 85242/64 (5)
7	Bul Perun	Bulgaria	IAK	Two-Rowed	1996	Alpha x Jet
8	Sladoran	Turkey	TARI	Two-Rowed	1998	Inrtoduced from Yugoslavia
9	Kıral-97	Turkey	BDIARI	Six-Rowed	1997	ADAIR/SL//WA1094-67
10	Erginel 90	Turkey	TZARI	Six-Rowed	1990	Escourgeon Hop 2171 (Fransa)
11	Tarm-92	Turkey	FCCRI	Two-Rowed	1992	Tokak / local population no 4875
12	Çetin 2000	Turkey	FCCRI	Six-Rowed	2000	Star (Iran) /line 4875
13	Tokak 157/37	Turkey	FCCRI	Two-Rowed	1963	Selection from Landraces
14	Avcı-2002	Turkey	FCCRI	Six-Rowed	2002	Sci/3Gi-72AB58, F1//WA1245141
15	Burakbey	Turkey	FCCRI	Two-Rowed	2013	Coss/OWB 71080-44-1H//Obruk 86
16	Ayrancı	Turkey	BDIARI	Two-Rowed	2018	Osk4.197/12-84//HB854/Astrix/3/Rod/4/Slad/3/Vict//Yrm/Lhfm
17	Akhisar 98	Turkey	AARI	Six-Rowed	1998	GEM*4/PİAST SEA-2636-4S-3S-2S-1S-0S
18	Aydanhanım	Turkey	FCCRI	Two-Rowed	2002	GK Omega / Tarm 92
19	Çıldır 02	Turkey	TZARI	Two-Rowed	2002	3896/28//284/28/3/Cum-50/4/624/682/5/WBQT12
20	Hazar	Turkey	TARI	Six-Rowed	2016	Osk4.39/2-84//Barbe-Rousse
21	Emon	Bulgaria	IAK	Two-Rowed	1998	137HS-21/M-21-H/3/Malta/M-20-H/M-21-H/4/111G-65
22	Sancak	Turkey	AARI	Six-Rowed	2014	1861112/ROBUR/7/ HLLA/EH 21B/6/MAN/HUIZ//M69.69/3/APAM/RL//H 272/4/CP/BRA/5/JOSO
23	Egebeyi	Turkey	AARI	Six-Rowed	2019	CEN-B/2*CA-I92//VIRINGA/3/ATACO/4/ Harma-02//11012-2/Cm67/3/Market semple Marageh /5/ ROHADES//TB//CHZO/3/GL/COPAL/3/BAR/RHODES//GL/COME
24	Martı	Turkey	TARI	Six-Rowed	2009	Flam/WM/5/Yky387/3/Api/Cm67//Manc/4/Yrm/Lhfm
25	Zahir	Bulgaria	IAK	Two-Rowed	2016	K10 x Кт 1206
26	Hasat	Turkey	TARI	Two-Rowed	2014	Rod/Scala
27	Asparuh	Bulgaria	IAK	Two-Rowed	2009	2119У-75 x Korten
28	Deviniya	Bulgaria	IAK	Two-Rowed	2011	Tamara x Aster
29	Vesletc	Bulgaria	IAK	Six-Rowed	1994	№102/121 x Karnobat
30	Kuber	Bulgaria	IAK	Two-Rowed	2009	2119У-41 x 2119У-165
31	Dariya	Bulgaria	IAK	Two-Rowed	2016	CRT 059 x Lambic
32	Orfej	Bulgaria	IAK	Two-Rowed	2007	Kjfi x Nutans 8486/40
33	IZ Bori	Bulgaria	IAK	Six-Rowed	2010	K 280-7 NaN3 - mutant
34	Konevi	Turkey	BDIARI	Two-Rowed	1998	CO55/OWB 710-80 (WBCB)
35	Tosunpaşa	Turkey	FCCRI	Two-Rowed	2016	Atlas/Zarjou
36	Zeynel Ağa	Turkey	FCCRI	Two-Rowed	2003	Antares/Ky63-1294//Lignee131
37	Yesevi 93	Turkey	FCCRI	Two-Rowed	1993	Tokak / local population 4857
38	Bülbül 89	Turkey	FCCRI	Two-Rowed	1989	13GTH / local population
39	IZ Sayra	Bulgaria	IAK	Two-Rowed	2010	Alfa x Nutans 85242/76 / x Yubileĭ 100
40	İnce-04	Turkey	TZARI	Two-Rowed	2004	4671/Tokak//4648/p12-119/3/WBCB-4
41	Bilgi-91	Turkey	TZARI	Two-Rowed	1991	Selection
42	Vamikhoca 98	Turkey	AARI	Six-Rowed	1998	GEM*3/3/CR 115/POR//BLANCO MA
43	Hilal	Turkey	AARI	Two-Rowed	2010	Melusine/Aleli/3/Matico/Jet//Shyri/4/Canela/5/Arupo/K8755//Mora/3/Canela CBSS 96M00698D-P-5M-1Y-1M-0Y
44	Kalaycı-97	Turkey	TZARI	Two-Rowed	1997	Erginel 90//364 TH / Tokak
45	Orza 96	Turkey	FCCRI	Two-Rowed	1996	Tokak 157-37/4857
46	Bolayır	Turkey	TARI	Two-Rowed	2007	Osk 4.197/12-84//HB854/Astrix/3/Alpha/Durra
47	Akar	Turkey	FCCRI	Two-Rowed	2012	Alpha/Durra//Antares/KY-63-1294/3/Tarm 92
48	Özdemir	Turkey	TZARI	Two-Rowed	2005	CUM/4060//P12-62/P169-2
49	Karatay 94	Turkey	BDIARI	Two-Rowed	1996	VONTAGE/GÜZAK//TAPLANİ/3/REKAL/CUM50/RIGIC
50	İmbat	Turkey	AARI	Six-Rowed	2020	80.5064//BOLDO/MJA/3/GEM
51	Larende	Turkey	BDIARI	Two-Rowed	2006	ALM(4652)/TOKAK//342TH/P12-119/3/W.BELT22
52	Ünver	Turkey	TZARI	Two-Rowed	2013	YEA389-3/YEA475-4//97-98DH8
53	Cumhuriyet 50	Turkey	TZARI	Two-Rowed	1973	No:28 (Kayseri) / Mansholt's-2 Rijige (Holland)
54	Bayrak	Turkey	AARI	Six-Rowed	2014	ARRAYAN/OLMO//LEO-B/3/Lignee527/Aths//Aths/Lignee686

AARI: Aegean Agricultural Research Institute Turkey; BDIARI: Bahri Dağdaş International Agricultural Research Institute Turkey; FCCRI: Field Crops Central Research Institute Turkey; TARI: Trakya Agricultural Research Institute Turkey; TZARI: Transition Zone Agricultural Research Institute Turkey; IAK: Institute of Agriculture Karnobat, Bulgaria

2.3. iPBS and SCoT genotyping

A set of iPBS (18) markers reported by Kalendar et al. (2010) and SCoT (4) markers by Collard et al. (2009) and Luo et al. (2010) were used for diversity analysis in this study.

The information about the markers is shown in Table 2.

Table 2- DNA primers used in molecular characterization of the barley genotypes

Primer name	*Primer sequence (5'–3')*	*Tm (°C)*	*G/C ratio*
iPBS 2075	CTCATGATGCCA	50	50
iPBS 2083	CTTCTAGCGCCA	50	58.3
iPBS 2095	GCTCGGATACCA	44.8	58.3
iPBS 2219	GAACTTATGCCGATACCA	50	44.4
iPBS 2222	ACTTGGATGCCGATACCA	55	55.6
iPBS 2230	TCTAGGCGTCTGATACCA	50	50
iPBS 2244	GGAAGGCTCTGATTACCA	53.3	50
iPBS 2255	GCGTGTGCTCTCATACCA	57.1	50
iPBS 2271	GGCTCGGATGCCA	57.4	69.2
iPBS 2276	ACCTCTGATACCA	50	46.2
iPBS 2375	TCGCATCAACCA	45.1	50
iPBS 2378	GGTCCTCATCCA	44.2	58.3
iPBS 2387	GCGCAATACCCA	50	58.3
iPBS 2388	TTGGAAGACCCA	43.4	50
iPBS 2391	ATCTGTCAGCCA	48	50
iPBS 2394	GAGCCTAGGCCA	51.3	66.7
iPBS 2400	CCCCTCCTTCTAGCGCCA	57.4	66.7
iPBS 2415	CATCGTAGGTGGGCGCCA	50	66.7
SCoT-18	ACCATGGCTACCACCGCC	50	67
SCoT-39	CAATGGCTACCACTAGCG	50	56
SCoT-71	CCATGGCTACCACCGCCG	50	72
SCoT-74	CCATGGCTACCACCGGCA	50	67

Polymorphism information content (PIC) values were calculated for each iPBS and SCoT markers using the formula described by Weir (1996) using a web based (https://www.gene-calc.pl/pic) software. PIC=1-$\sum P_i^2$, where P_i is the frequency of the i^{th} allele in the 54 barley cultivars in the research.

Polymerase chain reactions (PCR) for the iPBS and SCoT markers were completed in a total volume of 20 µL, including iPBS and SCoT markers 10 µL primers (1 µM forward and reverse), 5 µL (150 ng) of genomic DNA, 5 µL of master mix (0.1 µL MgCl, 0.2 µL Taq polymerase enzyme, 2 µL reaction buffer, 1.2 µL dNTP mix (A+T+G+C) and 1.5 µL ddH_2O).

The reactions were conducted in Sensoquest Thermocycler (Labcycler) with a first denaturing (94 °C, 5 min.), then 40 cycles of denaturing (94 °C, 1 min), annealing (44.2-57.4 °C, 1 min, gradient) and extension (72 °C, 1 min) afterwards a final extension (72 °C, 10 min). Products obtained from the PCR were fragmented by 3% agarose gel [100 ml 1xTBE {1 Lt H_2O + 10.8 g Tris + 5.5 Boric acid + 0.5 M 4 mL EDTA (pH: 8)} with 3 g agarose] electrophoresis in 1X TBE buffer after adding 5 µL loading dye to 10 µL PCR product and running for approximately 2.5 hours at 120V to 130V. The gels were stained with 1 µl/mL ethidium bromide. Gel images were captured using a Bio-Rad ChemiDoc (California, USA) gel documentation system and fragment sizes were determined by comparison with a 1 kb DNA ladder (Thermo Scientific Gene Ruler).

2.4. Data analysis

The presence or absence of iPBS and SCoT marker amplicons were scored as "1" or "0" to produce binary matrix data. The genetic similarity of the barley genotypes was calculated by Dice index (Dice 1945). A dendrogram was created based on an unweighted pair-group mean average (UPGMA) tree using NTSYSpc (Rohlf 1998) and effective allele numbers (ne), Nei's

genetic diversity (Kimura & Crow 1964) and Shannon's information index (Lewontin 1972) were calculated using POPGEN32 software (v3.2 Microsoft Windows-Based Freeware for Population Genetics Analysis) (Yeh et al. 2000).

A Bayesian model-based clustering algorithm named population structure was completed using STRUCTURE ver. 2.3.4 with almost default parameters to figure out the population structure of the barley cultivars. The admixture model (the ancestry and allele frequency model) which provides allele frequency correlations against a set of K genetics and shared allele frequencies were chosen to detect the populations numbers (K) with the range of 1 to 10, which are measured best in cases of complex population structure and the analysis repeated six times (Falush et al. 2003; Montilla-Bascon et al. 2013). The admixture alpha degree set to 1000 with 100000 Markov chain Monte Carlo (MCMC) and ten neutral simulations per K value were performed as indicated by Montilla-Bascon et al. (2013) and Earl & vonHoldt (2012). The ΔK method was implemented by STRUCTURE HARVESTER v0.6.94 web based software was used to determine K value that best fit the data (Evanno et al. 2005).

3. Results and Discussion

Molecular markers were used to determine genetic diversity and population structure for several plant species (Pasam et al. 2014; Dumlupinar et al. 2016; Güngör 2019; Hossein-Pour et al. 2019; Yıldız et al. 2020). Kalendar et al. (2010) indicated that retrotransposon-based molecular markers may be efficiently used to locate developmental issues, at the intragenus or intraspecific scale, as for their addition into the genome results polymorphic DNA regions. Among the retrotransposan markers, iPBS markers are used universally due to ease of usage, high reproducibility and relatively inexpensive compared to other technologies and they do not require prior sequence knowledge (Yıldız et al. 2020). Among the PCR-based gene target technologies, SCoT markers reported as low in cost and effective to use besides, highly reproducibility and high polymorphic bands per reaction (Luo et al. 2010). Barley cultivars released from 1963 to date by different institutes in both Turkey and Bulgaria were evaluated for their genetic diversity and structural backgrounds. Total and polymorphic band numbers was identified as 560 and 530, respectively. Also, the average polymorphic band number was found as 24.09. The polymorphism ratios of the iPBS and SCoT markers was identified as 93.9%and 97.8%, respectively. In addition, the highest ne, h and I values were obtained from iPBS2271 marker (Table 3).

Table 3- PIC values and total polymorphic band numbers of DNA markers

No	*Primer Name*	*Amplified Band Number*	*Polymorphic Band Number*	*Polymorphism Rate (%)*	*PIC Value*	*Effective number of alleles (ne)*	*Nei's genetic diversity (h)*	*Shannon's information index (I)*
1	iPBS 2075	24	23	95.83	0.49	1.51	0.29	0.44
2	iPBS 2083	23	23	100	0.49	1.48	0.31	0.48
3	iPBS 2095	15	15	100	0.40	1.51	0.29	0.44
4	iPBS 2219	20	18	90	0.47	1.46	0.28	0.43
5	iPBS 2222	29	27	93.1	0.50	1.42	0.26	0.40
6	iPBS 2230	23	18	78.26	0.49	0.95	0.12	0.21
7	iPBS 2244	27	27	100	0.50	1.37	0.25	0.41
8	iPBS 2255	26	26	100	0.50	1.48	0.28	0.43
9	iPBS 2271	28	28	100	0.50	1.61	0.35	0.52
10	iPBS 2276	33	33	100	0.48	1.57	0.32	0.48
11	iPBS 2375	22	20	90.9	0.48	1.34	0.22	0.35
12	iPBS 2378	26	20	76.92	0.50	1.33	0.20	0.31
13	iPBS 2387	26	26	100	0.50	1.54	0.31	0.48
14	iPBS 2388	22	21	95.45	0.48	1.27	0.22	0.36
15	iPBS 2391	22	20	90.9	0.48	1.29	0.21	0.34
16	iPBS 2394	25	23	92	0.50	1.45	0.29	0.44
17	iPBS 2400	39	34	87.17	0.40	1.38	0.26	0.40
18	iPBS 2415	36	36	100	0.44	1.39	0.25	0.39
19	SCoT-18	20	20	100	0.47	1.50	0.30	0.46
20	SCoT-39	30	30	100	0.49	1.54	0.31	0.47
21	SCoT-71	24	22	91.66	0.49	1.55	0.32	0.48
22	SCoT-74	20	20	100	0.47	1.53	0.31	0.47
Average		25.45	24.09	94.65	0.48	1.43	0.27	0.42
Total		560	530	-	-			

Five hundred thirty polymorphic bands obtained from by iPBS (438 bands) and SCoT (92 bands) markers were used to create a dendrogram (Figure 1) and also used to calculate effective allele numbers (ne), Nei's genetic diversity and Shannon's information index for DNA markers (Table 3) and cultivars used in the study (Table 4). In current study, SCoT and iPBS markers confirmed useful in determination genetic diversity and population structure of barley cultivars. A ratio of 94.6% polymorphism was obtained from both SCoT and iPBS markers and the polymorphic band number was found as 24.09. In a recent study, Yildiz et al. (2020) reported 92% of polymorphic bands and 8.6 bands per iPBS marker reaction as 20 iPBS markers used and 158 polymorphic bands were generated and Hossein-Pour et al. (2019) stated 3.16 average band number per primer in iPBS markers. On the other hand, Luo et al. (2010) indicated 8.27 bands per SCoT primer and 76.19% polymorphism rate with 208 total polymorphic bands and Khodayari et al. (2012) reported 8.1 allele per locus in an Iranian barley landrace panel. Pasam et al. (2014) indicated a 5.74 average allele number in SSR markers in a spring barley set, while Elakhdar et al. (2018) indicated four allele number/locus in barley for SSR and SNP markers.

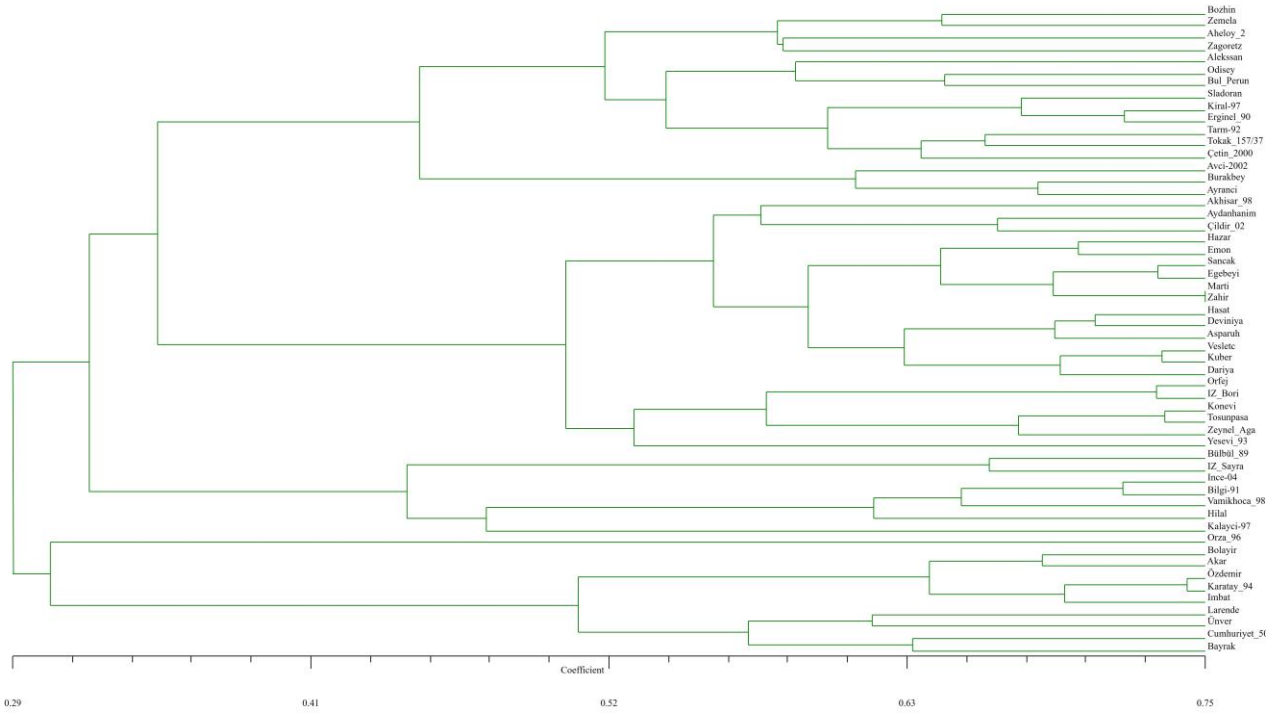

Figure 1- Dendrogram of 54 barley genotypes based on data of iPBS and SCoT markers according to UPGMA method with the Dice similarity index

Table 4- Summary statistics for 54 Barley genotypes assessed with DNA primers used in molecular characterization

No	*Genotypes*	*Effective number of alleles (ne*)*	*Nei's genetic diversity (h*)*	*Shannon's information index (I*)*
1	Bozhin	1.50	0.33	0.52
2	Zemela	1.55	0.35	0.54
3	Aheloy 2	1.43	0.30	0.47
4	Zagoretz	1.57	0.36	0.55
5	Alekssan	1.50	0.33	0.51
6	Odisey	1.44	0.30	0.48
7	Bul Perun	1.55	0.35	0.54
8	Sladoran	1.63	0.38	0.57
9	Kıral-97	1.65	0.39	0.58
10	Erginel 90	1.65	0.39	0.58
11	Tarm-92	1.59	0.37	0.55
12	Çetin 2000	1.57	0.36	0.55
13	Tokak 157/3	1.62	0.38	0.57
14	Avcı-2002	1.62	0.38	0.57
15	Burakbey	1.57	0.36	0.55
16	Ayrancı	1.59	0.37	0.55
17	Akhisar 98	1.59	0.37	0.55
18	Aydanhanım	1.53	0.34	0.53
19	Çıldır 02	1.56	0.36	0.54
20	Hazar	1.59	0.37	0.55
21	Emon	1.61	0.38	0.56
22	Sancak	1.67	0.40	0.59
23	Egebeyi	1.55	0.35	0.54
24	Martı	1.61	0.38	0.56
25	Zahir	1.55	0.35	0.54
26	Hasat	1.62	0.38	0.57
27	Asparuh	1.57	0.36	0.55
28	Deviniya	1.61	0.38	0.57
29	Vesletc	1.55	0.35	0.54
30	Kuber	1.61	0.38	0.56
31	Dariya	1.49	0.33	0.51
32	Orfej	1.56	0.36	0.54
33	IZ Bori	1.57	0.36	0.55
34	Konevi	1.56	0.36	0.54
35	Tosunpaşa	1.55	0.35	0.54
36	Zeynel Ağa	1.53	0.34	0.53
37	Yesevi 93	1.50	0.33	0.51
38	Bülbül 89	1.46	0.31	0.49
39	IZ Sayra	1.48	0.32	0.50
40	İnce-04	1.48	0.32	0.50
41	Bilgi-91	1.52	0.34	0.52
42	Vamikhoca 9	1.42	0.30	0.47
43	Hilal	1.48	0.32	0.50
44	Kalaycı-97	1.49	0.33	0.51
45	Orza 96	1.64	0.39	0.58
46	Bolayır	1.60	0.37	0.56
47	Akar	1.56	0.36	0.54
48	Özdemir	1.58	0.36	0.55
49	Karatay 94	1.52	0.34	0.52
50	İmbat	1.46	0.31	0.49
51	Larende	1.50	0.33	0.52
52	Ünver	1.49	0.33	0.51
53	Cumhuriyet 5	1.40	0.28	0.46
54	Bayrak	1.34	0.25	0.42
Average		1.54	0.35	0.53

PIC values were also calculated for each DNA markers and shown in Table 3. The average PIC value was 0.48 and the highest PIC value was 0.50, while the lowest one was 0.40. The highest ne, h, and I were obtained from the iPBS2271 marker at 1.61, 0.35 and 0.52, respectively, in contrast the lowest ones were found in the iPBS2230 marker at 0.95, 0.12 and 0.21, respectively. In addition, the total average ne, h, and I values were found as 1.43, 0.27 and 0.42, respectively (Table 3). A brief statistical results for each of the 54 barley genotypes was presented in Table 4. The highest ne, h, and I were obtained from Sancak cultivar at 1.67, 0.40 and 0.59, respectively, though the lowest values were observed in the Bayrak cultivar at 1.34, 0.25 and 0.42, respectively. In addition, the total average ne, h, and I were identified as 1.54, 0.35 and 0.53, respectively. Polymorphism information content is a measure of the primers used in a set of genotypes. In current study, the average PIC

value was 0.48 and PIC values of the iPBS markers ranked from 0.40 to 0.50 obtained from iPBS markers, while 0.46 to 0.49 for the SCoT markers. In earlier studies, Moragues et al. (2007) indicated an average PIC value 0.24 for AFLP and 0.70 for SSR markers in a durum wheat panel. Khodayari et al. (2012) determined a PIC value of 0.65 for SSR markers in barley landraces. Pasam et al. (2014) stated a 0.54 average PIC value for SSR markers in spring barley. Elakhdar et al. (2018) also reported a PIC value of 0.49 in barley genotypes. Hossein-Pour et al. (2019) reported an average PIC value with 0.20. Güngör (2019) reported an average PIC value of 0.72 on durum wheat cultivars. In addition, Kiraz et al. (2019) determined a PIC value of 0.79 in bread wheat mutant lines. Aydemir et al. (2020) calculated an average PIC value as 0.98 in a durum wheat population for DNA markers.

Based on a distribution of the 530 bands in 54 barley cultivars, STRUCTURE analysis was conducted with K=10. The subpopulations in the barley cultivars supported a value of k=5 (Figure 2) and the amount of admixture of each cultivar in the related subpopulation detected five barley subpopulations by STRUCTURE analysis is shown in Figure 3. According to STRUCTURE data, sub-population A, B, C, D and E indicated an admixture with 16.6%, 38.8%, 24%, 12.9% and 7.7% of the genotypes, respectively (Figure 3). Despite the maximum ΔK value was conducted at K=10, clusters at K=5 were best identified in terms of genotypic data. The sub-populations of the barley cultivars were grouped regardless to country, spike type and pedigree. In earlier works in different barley accessions the genetic diversity and population structure described two (Elakhdar et al. 2016), three (Elakhdar et al. 2018), five (Munoz- Amatriain et al. 2014), seven (Pandey et al. 2006), eight (Zhang et al. 2014) and 10 (Pasam et al. 2014) subpopulations.

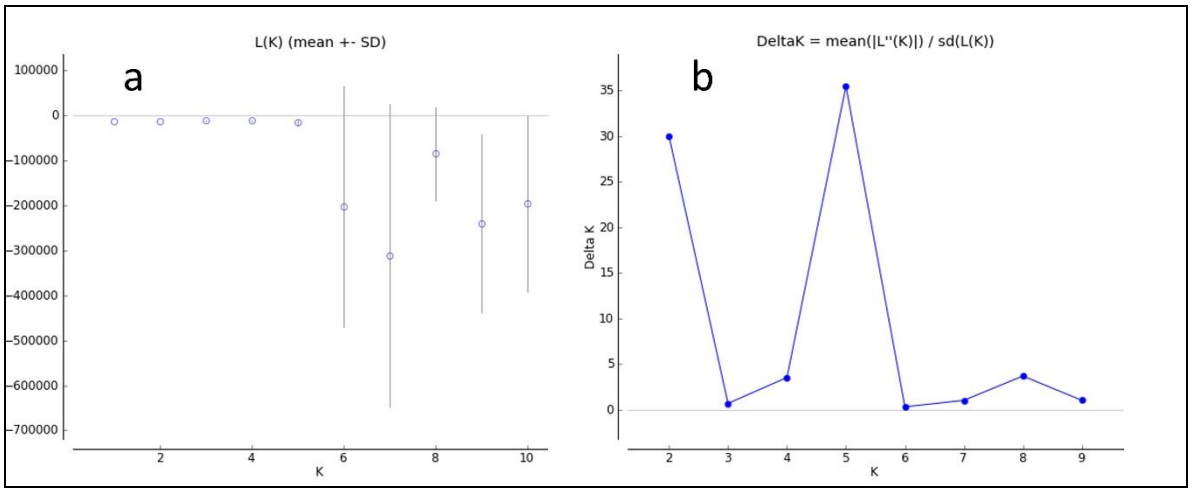

Figure 2- Admixture model of structure of Ln P (D) and ΔK for *Barley* subpopulations. a; Mean value of the statistic Ln P (D) b; DK

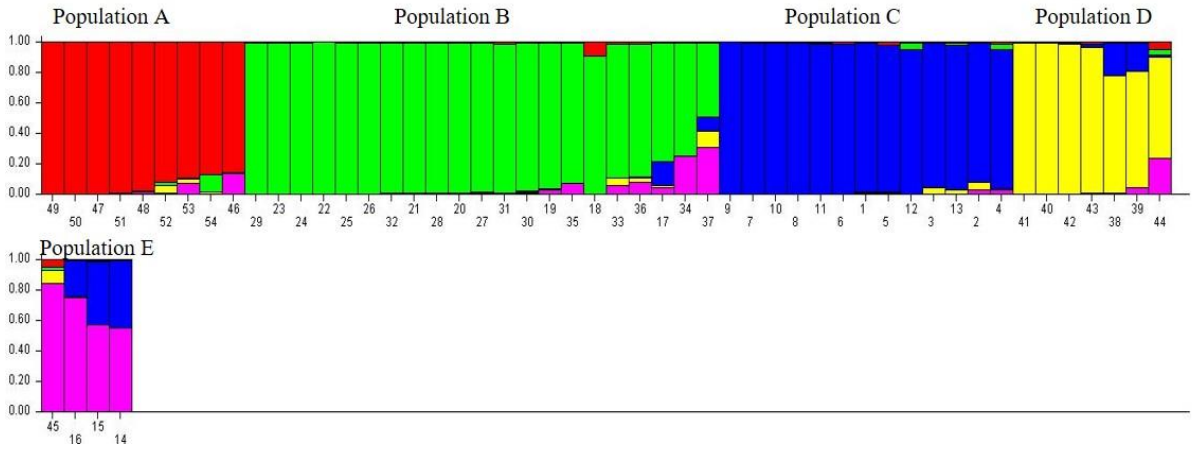

Figure 3- Population structure analysis of barley genotypes

To acknowledge about the genetic population structure of cultivars derived from different pedigrees. A Bayesian clustering modelling conducted in STRUCTURE software using 560 DNA markers. The number of groups (K) was arranged against ΔK to examine the most appropriate value of K. The highest ΔK value was detected at K = 5 (ΔK = 35.4787), including five sub-populations. At this K, most of the genotypes were linked to population B (Figure 1).

Expected heterozygosity (He) and fixation indices (Fst) values of sub-populations are shown in Table 5 and genetic differentiation measurement based on Fst values among sub-populations are indicated in Table 6. The barley sub-populations varied for Fst, determined to observe the relation within alleles drawn at varying scales of a hierarchically sub-divided population, however it was primarily modeled to measure the quantity of allelic fixation due to genetic alteration. The average He and Fst were found as 0.2335 and 0.3216, respectively (Table 5). The highest He was calculated from sub-population E with 0.3435, while the highest Fst value was obtained from sub-population A with 0.4697. Genetic differentiation based on Fst values among five barley sub-populations, sub-population A and B was found the most diverse populations with a value of 0.1225 (Table 6). The highest ne shows the existence of a high genetic variation suggesting the alleles capable to proceed in next-generation (Kimura 1965; Romero et al. 2019). It is concluded that ne is an important measure to assess markers with a high addition to variations in germplasms. In current study, the average ne was found 1.42 which was higher than a recent study reported as 1.26 by Barut et al. (2020) similar with a study for iPBS markers (Hossein-Pour et al. 2019). Shannon's information index is an important model to evaluate the diversity in a given set as it distinguishes the genetic variation in a germplasm combining plenty and equality (Yıldız et al. 2020). Shannon's information index and Nei's genetic diversity brought out the presence of satisfactory amount of genetic diversity in current germplasm. The average Shannon's information index was 0.42, which was consistent with previous works using different molecular markers (Barut et al. 2020). The average gene diversity was found 0.27 using SCoT and iPBS marker technologies in consistent with Hossein-Pour et al. (2019). In the study the average ne, h, and I of the cultivars were also calculated to evaluate cultivars with high ne, h and I. The average ne was 1.54, h was 0.35 and I was 0.53 (Table 4). Karagöz et al. (2020) reported the ne, I and h values for Oregano by iPBS marker respectively as 1.61, 0.37 and 0.55. Hossein-Pour et al. (2019) reported that ne, h and I values for quinoa by iPBS marker respectively as 1.52, 0.32 and 0.49. The expected heterozygosity values indicate the diversity amount of the primers, as those values are high; the primers variability is high (Pompanon et al. 2005). In previous works, He was reported as 0.28 in a barley set (Elakhdar et al. 2018) which was similar with our results, while (Zhang et al. 2014) indicated a value of 0.52 for a 80 wild and 16 commercial Tibetan barley genotypes. Elakhdar et al. (2018) explain the differences in expected heterozygosity as primer problems such as deletion of alleles and occurrence of inadequate alleles at annealing spots. Nevertheless, it is also concluded that heterozygote deficiencies might be due to Technical limitations (Elakhdar et al. 2018), while cleistogamy in barley may reduce the heterozygosity that flower sheds its pollen before opening makes it almost completely autogamous (Wang et al. 2013). In the research, the average Fst was 0.32. Elakhdar et al. (2018) reported an Fst value of 0.57 in an Egyptian barley set using SSR and SNP markers and stated that the difference between populations was higher than variation among population, which was in harmony with our findings.

Table 5- Heterozygosity and Fst values of 5 barley sub-populations

Sub-population (K)	*Expected heterozygosity (He)*	F_{ST}
A	0.1903	0.4697
B	0.2085	0.3560
C	0.2224	0.3294
D	0.2030	0.4429
E	0.3435	0.0098
Average	0.2335	0.3216

Table 6- Genetic differentiation based on Fst values among five Barley sub-populations identified by population structure analysis

Sub-populations (K)	*Sub-Pop A*	*Sub-Pop B*	*Sub-Pop C*	*Sub-Pop D*
Sub-Pop A	-			
Sub-Pop B	0.1225	-		
Sub-Pop C	0.1067	0.0981	-	
Sub-Pop D	0.1109	0.1033	0.0886	-
Sub-Pop E	0.0582	0.0456	0.0371	0.0459

The dendrogram generated by UPGMA clustered into two groups. The first group was consisted of 10 cultivars and the second one which was the major one included 44 cultivars. The first small group consisted of two sub-groups with 9 members in the first and one cultivar in the second one. The major group firstly divided into two and the first group contained 7 cultivars, the second sub-group clustered in two groups with 20 and 16 cultivars in each group, respectively. Martı and Zahir cultivars were found the most similar barley cultivars with 75% genetic similarity, whereas Özdemir and Karatay 94 and Tosunpaşa and Konevi cultivars were found 73% similar. On the other hand, Bayrak and Avcı-2002 were found the most diverse cultivars with 19.9% genetic similarity. Genetic diversity studies with 530 polymorphic bands obtained from four SCoT and 18 iPBS markers recommended that the commercial barley cultivars had high genetic variation due to different sources. The existence of genetic variation in a population may be described originated from the amount of various alleles, their position, the impacts they have on accomplishment and the complete characteristics of observable phenotypes of desired the ones that construct the population (Hamrick et al. 1992). Expanding the genetic base is one of the main goals of the breeders while cultivation is mostly done by genetically uniform cultivars thus raising the concerns about narrowing the genetic base. A dendrogram was created using genotypic data obtained from iPBS and SCoT markers. A wide genetic diversity

was observed on the commercial barley cultivars derived from different institutes of Turkey and Bulgaria. The genetic diversity of the barley cultivars ranked from 19.9% to 75%. As it is shown in Figure 1, the barley cultivars obtained from IAK were mostly grouped together (Bozhin, Zemela, Aheloy 2, Zagoretz, Alekssan, Odisey, Bul Perun) with the exceptions. Although the most similar cultivars Martı and Zahir derived from different institutes (TARI and IAK, respectively), they are neighbors and the most closest institutes geographically. Bayrak and Avcı-2002 were the most diverse cultivars obtained from AARI and FCCRI, even they are both six-rowed. Besides their different origination, the high dissimilarity might be caused by their growth habit since Bayrak is a spring barley, while Avcı-2002 is a winter type. In earlier studies, genetic diversity of the barley accessions was revealed using different molecular marker technologies. Orabi et al. (2009) indicated a high genetic diversity in wild barley accessions and barley landraces was nearly high as well. Khodayari et al. (2012) stated a genetic diversity in Iranian barley landraces and defined Iranian gene pool as a valuable source of new alleles for crop improvement. Pasam et al. (2014) indicated a genetic diversity in a wide barley accession including both two and six-rowed barleys. Bengtsson et al. (2017) reported a higher genetic diversity in two-rowed (SSR: 0.431; SNP: 0.305) barley lines compared to six-rowed ones (SSR: 0.386; SNP: 0.225). Our findings are in confidence with the previous works.

4. Conclusions

In the current study, 54 barley cultivars were characterized by 18 iPBS and four SCoT markers and 530 polymorphic bands were produced. Population structure analysis conducted with genotypic data revealed five subpopulations in the barley cultivars. Diversity analysis showed that cultivars clustered with regardless to the releasing institutes and country origins. However, Martı and Zahir cultivars originated from Trace region with different country origins were found the most similar barley cultivars with 75% genetic similarity. Nevertheless, Bayrak and Avcı-2002 were found the most diverse cultivars with 19.9% genetic similarity. The average effective number of alleles, Shannon's information index, and Nei's genetic diversity were found 1.43, 0.27 and 0.42, respectively. Structure analysis of barley cultivars derived from different pedigrees resulted in five sub-populations. In addition, the average expected He and Fst values were determined as 0.234 and 0.322, respectively. These results showed that the iPBS and SCoT markers are polymorphic, may be used for diversity analysis of barley.

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Employing Barcode High-Resolution Melting Technique for Authentication of Apricot Cultivars

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ABSTRACT
Fast, accurate and affordable identification of food products is important to ensure authenticity and safety. There are various apricot (*Prunus armeniaca* L.) cultivars are being produced in Turkey. Each cultivar differs in quality and purpose of use. In this paper, we aimed to develop an easy and reliable method, Barcode High-Resolution Melting (Bar-HRM), to distinguish apricot cultivars. We designed and tested novel Bar-HRM primer sets HRM-ITS1 and HRM-ITS2, targeting the most popular barcoding region ITS1 and ITS2, specific to apricot cultivars. According to the results, HRM analysis distinguished 31 cultivars of 35 for ITS1, and 35 for ITS2. We recommend using ITS2 barcode region, amplified with using HRM-ITS2 primer set, for Bar-HRM analysis of different apricot cultivars.

Keywords: *Prunus armeniaca*, Internal Transcribed Spacer, Bar-HRM, Molecular Identification

1. Introduction

Apricot (*Prunus armeniaca* L.) is an important drupe fruit with a rich gene pool that makes the plant capable to adapt from Siberia to North Africa and Greece to China (Mehlenbacher et al. 1991). Turkey leads to world apricot production with 677,000 tons on average (FAOSTAT 2020). Apricot production is specialised especially in Malatya, Erzincan and Iğdır regions in Turkey (Ercisli 2004). Because apricot has been cultivated in Inner Anatolia since ancient times, some cultivars adapted to different climatic areas of the country i.e. Hacıhaliloğlu in Malatya, Hasanbey in Erzincan and Şalak in Iğdır (Güleryüz et al. 1997). According to the Turkish Apricot Research Institute data, there are 28 registered cultivars and numerous genotypes in Turkey including the Protected Designation of Origin (PDO) cultivars Hacıhaliloğlu, Hasanbey, Kabaaşı, Soğancı, Çataloğlu, Çöloğlu, Şalak (Anonymous 2021).

The economic importance of the PDO products is higher than the common products. Both producers and consumers must ensure PDO authenticity against adulteration. This makes “tracing the original food product” is crucial.

DNA barcoding, a method based on comparing nucleotide sequences of a specific DNA fragment, is a widely used tool for identifying the species, reconstructing the phylogenetics, assessing the biodiversity since the early 2000s (Cheng et al. 2016). Different DNA regions have been using as DNA barcodes for various plant groups such as *rbc*L, *mat*K, *ycf*1, *LEAFY* and the most used Internal Transcribed Spacer (ITS) region (Won & Renner 2005; Chase et al. 2007; Kress & Erickson 2007; Hollingsworth 2011; Li et al. 2011; Dong et al. 2015). It is also confirmed that ITS barcode region distinguishes apricot cultivars successfully (Hürkan 2020). Although the DNA barcoding technique is very useful for species identification, it prolongs the workflow, increases the expenses due to post-PCR sequencing analysis, and needs a bioinformatics background for the researcher. Recently, DNA barcoding technique supported with other techniques to overcome these disadvantages. In this paper, we urged on the High Resolution Melting (HRM) technique coupled with DNA barcoding region to develop a cost-effective method to discriminate closely related apricot cultivars.

High Resolution Melting analysis is a technique that used for genotyping by discriminating DNA sequence differences of Single Nucleotide Polymorphisms (SNPs) and sequence length polymorphisms in PCR products (Zhou et al. 2005). In this technique, the shape of the melting transitions of the PCR products is being acquiesced continuously. HRM is a more powerful, cheaper and easier technique than other approaches since requires neither post-PCR processing nor bioinformatics skills. Moreover, it is faster and more economical since being a sequencing-free method. Although HRM technique has majorities over conventional methods, it had some disadvantages that could directly influence the results. During the early stages of the HRM,

SYBR® Green was used as dsDNA binding dye, which inhibits the DNA polymerase. Therefore, SYBR™ Green dye does not allow discriminating closely related genotypes that have small sequence variations on HRM (Reed et al. 2007). However, saturation dyes e.g. LC Green™, SYTO9™ or Eva Green™, which is selected for this study, do not affect the DNA polymerase performance even at high concentrations (Vossen et al. 2009). The discriminating power of HRM directly related to the markers used in the analysis. In recent researches, there are two marker types are being used for HRM analysis, microsatellites and DNA barcoding regions. Both have advantages and disadvantages. While microsatellites are usually organism-group specified and need more experience to design and mine the primers, DNA barcoding regions are universal.

Combining the discrimination success of DNA barcoding and the easiness of HRM results in successful identification of food products such as PDO cheese (Ganopoulos et al. 2013), codfish species (Shi et al. 2020), poisonous plants (Thongkhao et al. 2020), medicinal plants (Li et al. 2016; Sun et al. 2017; Mishra et al. 2018), and bean crops (Madesis et al. 2012). In a recent study, 16 SSR markers from the literature were used to characterise the wild apricot genotypes from Nevşehir region (Turkey) (Bakır et al. 2019).

Because of the advantages above, herein, we developed and tested a Bar-HRM based method, using specific primer sets targeting the most used barcoding region ITS1 and ITS2, to rapid, affordable, reliable and quantitative identification of apricot cultivars and apricot products.

2. Material and Methods

2.1. Samples collection and DNA extraction

The Republic of Turkey, Ministry of Agriculture and Forestry Apricot Research Institute (Malatya, Turkey) kindly provided fresh leaf samples of the 35 apricot cultivars available in April 2020 (Table 1).

Table 1- Samples information, Cycle Threshold (C_q) values, average Melting Temperature (T_m) and genotyping confidence percentages

Sample Code	*Colour*	*Cultivar Name*	*C_q±SD*		*Average T_m (°C)±SD*		*Confidence (%)*[2]	
			ITS1	*ITS2*	*ITS1*	*ITS2*	*ITS1*	*ITS2*
AS		Aprikoz (Şalak)	9.49±0.21	11.66±0.12	87.70±0.06	90.56±0.04	100.00[3]	100.00[3]
AY		Alyanak[1]	9.55±.018	11.47±0.85	87.34±0.06	90.20±0.15	69.06	63.17
AZ		Aprikoz[1]	12.77±0.04	14.70±0.55	87.60±0.06	90.26±0.08	93.91	85.67
CG		Çekirge 52	9.21±1.02	11.72±0.45	87.40±0.10	90.26±0.06	91.66	81.27
CT		Çataloğlu[1]	9.08±0.71	10.95±0.38	87.56±0.10	90.10±0.10	86.42	63.11
EA		Erken ağerik	9.99±0.11	11.53±0.65	87.36±0.06	90.14±0.06	66.64	68.92
EB		Ethembey[1]	10.00±0.07	12.12±0.47	87.34±0.10	90.16±0.12	74.15	62.24
HB		Hasanbey[1]	9.76±.065	11.22±0.55	87.40±0.10	90.04±0.04	96.69[3]	59.31
HG		Hungarish Beste	8.57±1.07	10.70±0.48	87.50±0.06	90.26±0.06	91.67	78.07
HH		Hacıhaliloğlu[1]	8.98±0.66	10.62±0.11	87.50±0.06	90.16±0.10	85.92	76.57
HK		Hacıkız	10.27±0.41	12.22±0.09	87.50±0.10	90.10±0.18	94.19	63.78
HR		Hırmanlı Dırağı	9.82±0.94	12.18±0.16	87.40±0.06	90.10±0.05	96.54[3]	75.19
IA		İsmailağa	9.49±0.37	11.23±1.14	87.66±0.06	90.04±0.06	85.07	52.55
IB		İri bitirgen[1]	9.40±0.98	11.27±0.87	87.44±0.10	90.14±0.06	86.14	56.94
IH		İmrahor[1]	9.60±1.22	11.68±0.61	87.26±0.10	90.20±0.10	21.91	40.45
KA		Kabaaşı[1]	9.52±1.18	11.97±1.55	87.50±0.10	90.10±0.08	98.64[3]	80.55
KB		Karacabey[1]	9.15±0.57	11.36±0.78	87.60±0.06	90.26±0.10	71.48	73.32
KK		Kuru Kabuk[1]	9.93±.098	10.93±0.08	87.50±0.10	90.24±0.11	20.54	30.47
PB		Precose de Boulbon	9.76±0.19	11.65±0.41	87.36±0.11	90.04±0.08	27.56	11.76
PL		Polenais	10.81±0.48	13.16±0.68	87.96±0.08	90.16±0.06	81.46	78.88
PM		Paşa Mişmişi	10.92±0.98	12.58±0.49	87.44±0.14	90.10±0.10	58.15	81.00
PV		Paviot	9.58±1.54	11.71±1.24	87.56±0.08	90.30±0.14	78.72	88.71
RK		Rakowsky	10.46±0.64	12.98±0.34	87.46±0.06	90.24±0.10	48.14	45.63
RY		Royal	9.82±0.15	11.86±1.23	87.60±0.14	90.24±0.09	77.44	73.40
SC		Soğancı[1]	9.99±0.45	12.10±0.24	87.90±0.10	90.20±0.18	63.38	82.10
SE		Stark Early Orange[1]	10.01±0.11	12.40±0.42	88.06±0.06	90.36±0.04	78.00	81.35
SK		Şekerpare[1]	10.48±1.02	12.95±0.35	87.64±0.12	90.50±0.10	77.76	75.21
SM		Şam[1]	10.52±0.12	12.67±0.57	87.70±0.02	90.46±0.06	86.07	94.84
TE		Tokaloğlu (Konya Ereğli)	10.12±0.98	12.54±1.74	87.80±0.13	90.44±0.06	49.92	70.13
TF		Turfanda (Eski Malatya) [1]	10.32±0.41	12.52±0.35	87.84±0.17	90.36±0.08	45.72	87.63
TK		Tekeler	10.16±0.48	12.70±0.54	87.90±0.09	90.34±0.10	29.86	82.66
TO		Tokaloğlu (Erzincan)	12.07±0.94	14.58±0.38	87.90±0.06	90.54±0.12	45.60	39.47
TY		Tokaloğlu (Yalova)	10.75±0.89	13.06±0.68	88.00±0.14	90.84±0.06	44.10	59.89
WD		Wilson Deliciouse	10.93±0.87	13.90±1.56	88.16±0.05	91.10±0.06	1.35	5.90
YN		Yeğen (Eski Malatya)	11.23±0.14	14.18±0.34	88.40±0.06	91.30±0.14	0.30	1.06

[1]: Cultivars are registered to the Republic of Turkey, Ministry of Agriculture and Forestry Apricot Research Institute (Malatya, Tukey); [2]: Confidence percentage calculated regarding AS; [3]: Confidence value >95% infers no genotyping success, <95% successful genotyping

To extract DNA, we used ~100 mg of fresh leaf tissues and followed the modified CTAB protocol described in the literature (Aydın et al. 2018). The DNA concentration was measured by NanoDrop (Maestrogen) and integrity was confirmed on agarose gel electrophoresis. We normalised the concentration of all the DNA samples to 10 ng μL^{-1}. The DNA samples were stored at -20 °C for further analyses.

2.2. Primers mining

Since there is no available nucleotide sequence of the studied cultivars available on GenBank, we designed the primer sets for ITS1 and ITS2 barcode regions according to the GenBank apricot nucleotide sequence records with the accession number MT072696, EF211085, EF211084, EF211083 and MG735482. We downloaded the GenBank formatted files with annotations, imported them to the Geneious R8 software (Kearse et al. 2012) and evaluated them for quality and variable characters. Sequence characteristics were also analysed using MEGA Version X (Kumar et al. 2018). Then, the sequences were aligned (Geneious Alignment Tool) and the novel primer sets were designed by considering the variable positions, GC content, expected amplicon size and melting temperature (T_m) in the same software (Table 2). We in silico confirmed the specificity of the primers on the Primer-BLAST tool in the National Center for Biotechnology Information (NCBI).

Table 2- Primers information

Primer Name	*Target Region*	*Sequence (5'→3')*	*T_m (°C)*	*GC Content (%)*	*Expected (Detected) Amplicon Size (bp)*
HRM-ITS1	ITS1	F: TCGAAACCTGCCTAGCAGAA	59.0	50.0	121 (110)
		R: CGCGCTCTCTCGTTCAAGTT	61.0	55.0	
HRM-ITS2	ITS2	F: GAAGCCATTAGGCCGAGGG	60.2	63.2	144 (136)
		R: GGTTTCGCAACCACCGATTG	60.4	55.0	

Detected amplicon sizes by sequencing were given in parenthesis; T_m: Melting temperature; bp: Base pair

2.3. HRM analysis and sequencing of PCR products

We performed the HRM amplifications using Rotor-Gene-Q 5plex thermal cycler (Qiagen, USA) with a 72-well carousel. The HRM reaction mix was prepared as 5 µL Luminaris Colour HRM Master Mix (Thermo Scientific, USA), 0.5 µL of 10 mM each primer (Sentebiolab, Turkey), 10 ng template and nuclease-free water to 10 µL total volume. The cycling protocol was 95 °C 10 min initial denaturation followed by 40 cycles of 95 °C 10 s denaturation, 60 °C 30 s annealing, and 72 °C 30 s extension. Data acquiesced following each extension step. We added 95 °C 30 s and 50 °C 30 s steps for heteroduplex formation to the end of the cycle. We performed HRM immediately after the amplification in increments of 0.1 °C s^{-1} from 75 °C to 95 °C and data acquiesced continuously. All the reactions were performed as triplicates and no template control (NTC) was included in the reactions.

For HRM data analysis, we used the Rotor-Gene Q 2.3.5 (Qiagen, Germany) software. We calculated the Cycle Threshold (C_q) values by comparative quantification method of the software, Melting Temperatures (T_m), and normalised the HRM curves by removing the background fluorescence. The difference plots were generated regarding AS. Then, the software calculated the Genotype Confidence Percentage (GCP) for each cultivar. We set the confidence threshold to 95%.

To validate HRM analysis results, four ITS1 PCR products for the cultivars AS, HB, HR, KA which could not be separated by HRM (confidence percentage >95% threshold) and five ITS2 PCR products for the cultivars AS, AZ, PV, SM, TF which were separated by HRM (confidence percentage >85%). We sent the PCR products to the direct sequencing (Macrogen Europe) were performed in both directions using the same primers used for HRM amplifications by on ABI 3730xl System. We used Geneious R8 (Kearse et al. 2012) software for assembling the sequences and generating the consensus sequences. The sequences were aligned by the Geneious Aligner algorithm in the same software.

3. Results

3.1. DNA extraction and data mining

We obtained a sufficient amount of DNA from the samples with the followed extraction protocol. The DNA concentrations ranged from 29.69 to 187.29 ng μL^{-1}, and the A260/230 ratio ranged from 1.540 to 2.000.

We first analysed the sequences of ITS1 and ITS2 regions from the cultivars retrieved from GenBank to generate an initial comparison of the characteristics (Table 3). The length of the ITS1 region was identical among the cultivars as 215 bp. Ten variable sites (4.65%), 205 conserved sites (95.35%), and one parsimony informative sites were observed for the ITS1 region. ITS2 region was relatively longer (277.8±0.4 bp) and three folds by the variable sites (37 sites equals 13.32%) compared to ITS1. The conserved sites were lower 241 (86.75%) than ITS1, and there was no parsimony-informative site on ITS2. The average GC contents were almost the same for both regions (Figure 1). We would like to include the coding 5.8S region in the comparison

table to demonstrate why this coding region is not suitable for any genotyping analysis for apricot since all the sequence was conserved and there was no variable site available.

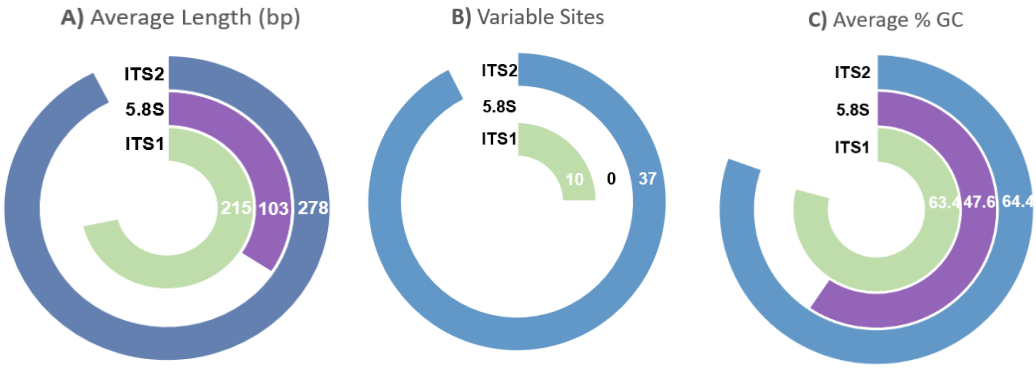

Figure 1- Comparison of average length (A), variable sites (B) and average GC per cent (C) of apricot cultivar ITS1, 5.8S and ITS2 sequences retrieved from GenBank.

Table 3- Characteristics of sequences retrieved from GenBank and sequencing of the HRM-PCR products

	GenBank			*HRM Amplicons*	
Statistics	***ITS1***	***5.8S***	***ITS2***	***ITS1***	***ITS2***
Average ungapped length (bp)+SD	215±0	103±0	277.8±0.4	110±0	136±0
Variable sites	10	0	37	0	6
Conserved sites	205	103	241	110	130
Parsimony informative sites	1	0	0	0	1
Average GC content (%)	63.4	47.6	64.4	60.9	61.8

3.2. HRM and sequencing results

ITS1 primer set amplified the expected size products, approximately 160 bp long. The software calculated the threshold cycles (C_q) and melting temperatures (T_m) of the amplicons (Table 1). The C_q values for ITS1 ranged from 8.57 to 12.77, and this marker generated 31 different, 4 identical (AS-IA, HB-PB, HR-RY and EA-SC) C_q values. The T_m values of the amplicons ranged from 87.26 to 88.40, and the software calculated 20 different T_m values. Although only 20 different T_m calculated of the 35 cultivars, HRM analysis successfully distinguished 32 cultivars (Figure 2). The cultivars HB, HR and KA could not be distinguished, and the GCPs were above 95% (96.69, 96.54 and 98.64, respectively). Figure 1 displays the HRM results utilizing difference and normalised plots. All the cultivars, except HB, HR and KA, yielded unique HRM profiles. So that, the software could distinguish the 32 cultivars by GCP calculations.

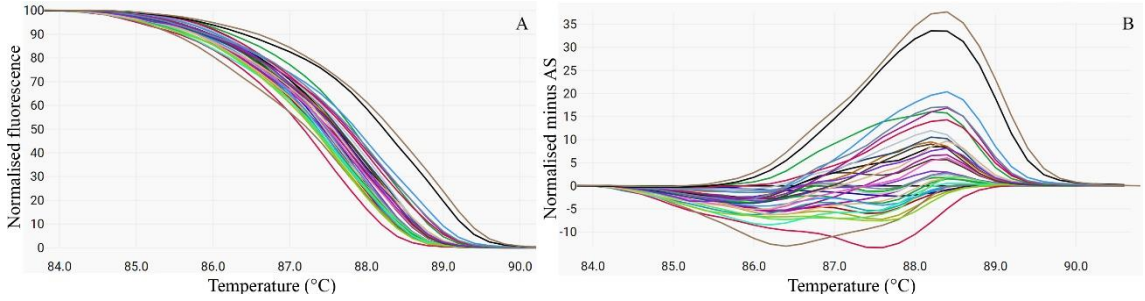

Figure 2- HRM analysis result of the apricot cultivars with the ITS1 nuclear marker. A) Normalised difference plot of 35 cultivars. B) Assigned genotypes plot drawn as AS control. Cut off value for Genotype confidence is 95%. Colour codes of the genotypes were indicated in Table 1. Normalisation ranges were adjusted to 84 °C (leading) and 90 °C (trailing).

ITS2 marker yielded better results than the ITS1. ITS2 primer set amplified approximately 170 bp long PCR products, as expected. The C_q values were ranged from 10.62 to 14.70, and each value was unique (Table 1). The T_m values ranged from 90.04 to 91.30, and the amplicons had 18 unique T_m values. HRM analysis of ITS2 distinguished all the cultivars by melting shapes (Figure 3), as we aimed. In contrast to conventional Tm difference analysis, the normalisation of the fluorescence by the software distinguished all the cultivars by melting curve shapes for ITS2.

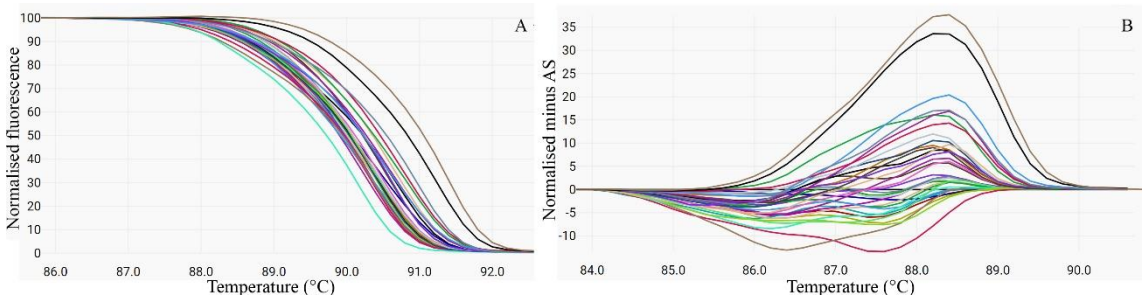

Figure 3- HRM analysis result of the apricot cultivars with the ITS2 nuclear marker. A) Normalised difference plot of 35 cultivars. B) Assigned genotypes plot drawn as AS control. Cut off value for Genotype confidence is 95%. Colour codes of the genotypes were indicated in Table 1. Normalisation ranges were adjusted to 86.6 °C (leading) and 92.2 °C (trailing).

We analysed the amplicon sequences to validate the results of the HRM analysis. After trimming the primer binding sites, we obtained 110 bp sequence for ITS1 and 136 bp sequence for the ITS2 region (Table 2). Sequencing results showed while ITS1 sequences of the AS, HB, HR, KA were identical, six variable sites were detected for ITS2 sequences of AS, AZ, PV, SM, TF (Figure 4, Figure 5 and Table 3).

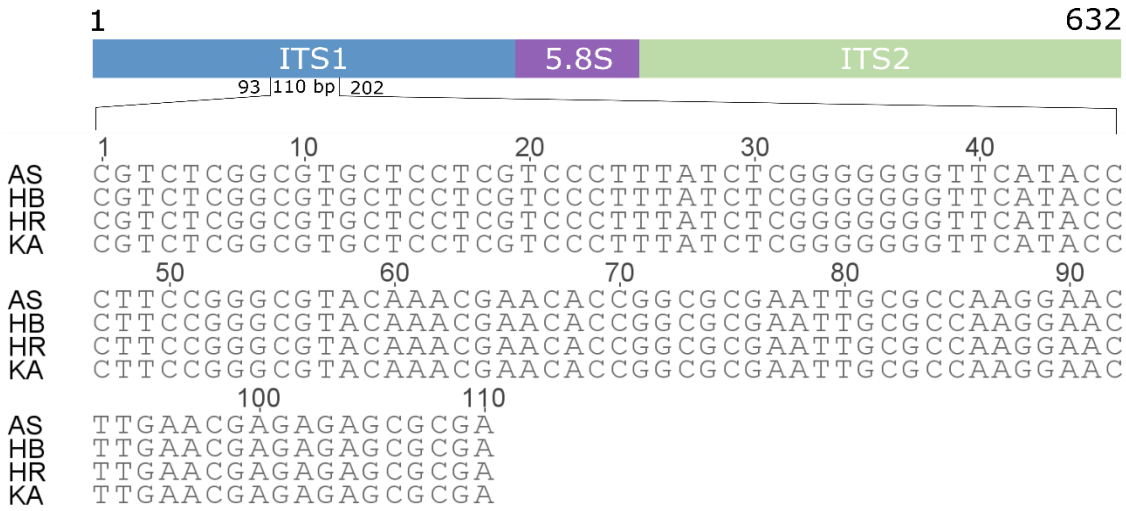

Figure 4- Alignment of the ITS1 target region for Bar-HRM analysis. The amplicon is 110 bp long. There are no nucleotide variable sites. The numbers indicate the position of the amplicon according to the complete ITS region.

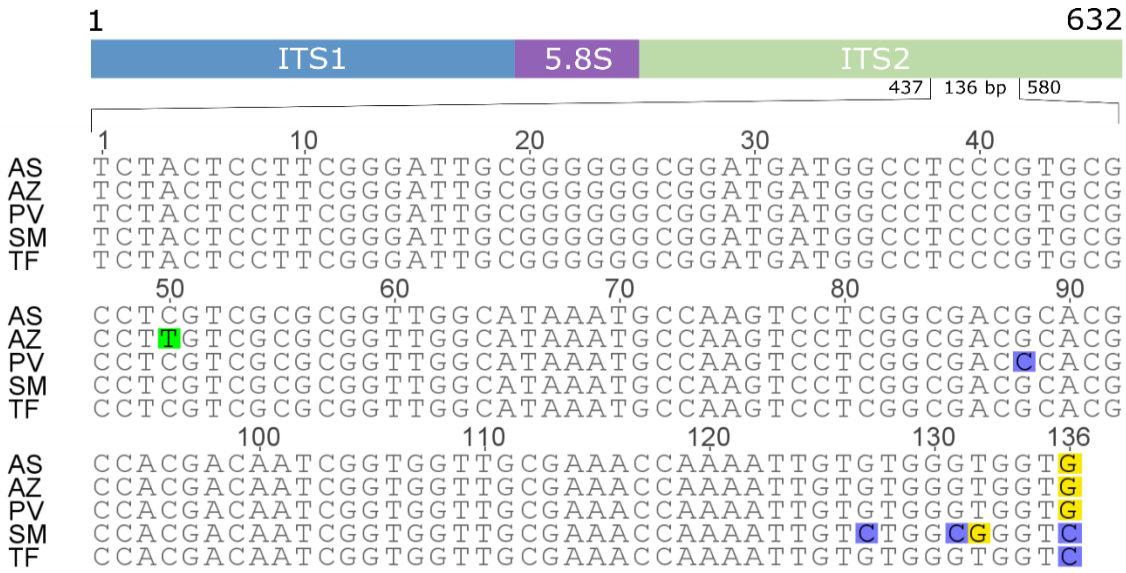

Figure 5- Alignment of the ITS2 target region for Bar-HRM analysis. The amplicon is 136 bp long. Nucleotide variable sites are indicated with colours. The numbers indicate the position of the amplicon according to the complete ITS region.

4. Discussion

The ITS region is a suitable DNA barcoding region for plants (Kress et al. 2005), but this region has some problems such as duplications, paralogue copies and causes pseudogenes on some plant groups (Chase et al. 2007). The success of the ITS as a barcoding region and an HRM marker was reported for apricot cultivars (Hürkan 2020), Fabaceae (Gao & Chen 2009), *Artemisia* spp. (Song et al. 2016), and *Medicago lupulina* and *Trifolium pratense* (Ganopoulos et al. 2012). The ITS region consists of two non-coding, ITS1 and ITS2, and one coding, 5.8S, parts (Cheng et al. 2016). Systematic researches have proposed the ITS2 could be the core DNA barcode due to the region has high interspecific divergence (Xin et al. 2013). In this study, the basic comparison of the ITS parts showed parallel results to the literature as ITS2 was the most variable (13.22%) region followed by ITS1 (4.65%) for the studied apricot cultivars. Although the 5.8S gene coding region was well characterised for inter-specific level, the region has very limited nucleotide variations in deeper levels since it is a conserved region (Hershkovitz & Lewis 1996). Supporting the literature, the 5.8S region showed no variable regions for the studied apricot cultivars.

The optimal amplicon length for HRM analysis should be shorter than 300 bp (Reed & Wittwer 2004). Shorter amplicons emphasize Single Nucleotide Polymorphisms (SNPs) in HRM analysis. Both ITS1 and ITS2 primers worked fine for each studied cultivar and yielded approximately 160 and 170 bp amplicons, respectively, which were in the "ideal" range for HRM analysis. The melting temperature of the PCR products depends on both sequence length and nucleotide content. However, solely T_m values are not reliable for discrimination the organisms as seen in the results. ITS1 primer yielded 20 unique T_m values, and ITS2 was only 18. During the HRM reaction, following the PCR amplification, the thermal cycler applies temperature increment to PCR products to denature the double-strand DNA, while a detector continuously tracking the fluorescence change. Thus, the software considers not only T_m values but also the melting shapes of the amplicons. This provides the software with better discrimination ability (Reja et al. 2010, Reed & Wittwer 2004). After the normalisation of the melting shapes on the software, HRM analysis distinguished 32 cultivars of the 35 for ITS1, and 35 for ITS2. This result supports the comparison of the variable sites (Table 3 and Figure 1), and the literature (Ganopoulos et al. 2012; Song et al. 2016; Pereira et al. 2018; Mishra et al. 2018). ITS2 region had three times more variable sites than the ITS1 and yielded better discrimination result.

The sequencing results were parallel to both comparisons of the sequences retrieved from GenBank (Table 3) and the HRM analysis results. Although obtained amplicon lengths were shorter than expected, we found the variable sites for ITS2, which was necessary to distinguish the cultivars. Sequencing results showed ITS2 (six variable sites) was the more variable region than ITS1 (no variable sites). The lack of variable sites on ITS1 sequences for the cultivars, which have a confidence percentage higher than 95%, clearly explains why HRM analysis could not distinguish the cultivars. The identical sequences resulted in similar melting profiles. In contrast to ITS1, ITS2 had six variable sites on the sequences. All these variations we detected were transversion mutations. There was no insertion or deletion. The selected ITS2 samples for sequencing were the samples that have more than 85% confidence percentage. The sequencing result of ITS2 validated the sensitivity and reliability of the Bar-HRM analysis by distinguishing genetically closely related cultivars.

Standard DNA barcoding based Sanger-sequencing is relatively expensive. In this study, we used a commercial HRM kit and a single HRM reaction was costed 0.31 USD, while single direction Sanger-sequencing was costed 5.5 USD. Next-generation sequencing (NGS) based genotyping is a comparable level on cost. However, it needs sophisticated workflow e.g. pre-sequencing library preparation, high-level computing for post-sequencing assembly of the reads. It also needs experienced and expert researcher on bioinformatics. Therefore, we believe a well-designed Bar-HRM assay would be a fast, robust and cost-effective way to genotype organism groups.

5. Conclusions

Bar-HRM is a cost-effective, fast and robust identification method. Moreover, no specialisation is needed for handling the data. In this study, ITS based Bar-HRM has proven for the identification of the 35 apricot cultivars. ITS2 primers set had the highest discrimination rate and can be used for the identification of various apricot cultivars. ITS2 marker can also be used for identification of *Prunus* species, authentication of apricot products.

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Use of MARS Data Mining Algorithm Based on Training and Test Sets in Determining Carcass Weight of Cattle in Different Breeds

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ABSTRACT
This research was carried out with the purpose of estimating hot carcass weight by using parameters such as race, carcass weight and age with Multivariate Adaptive Regression Spline (MARS) algorithm. To achieve this goal, 700 cattle data belonging to the years 2017-2018, which were taken in equal numbers from 7 different breeds, were used. A total of 700 data were used, taking equal numbers of data from each breed. In order to test the accuracy of the model created in the research, the data set was divided into two data subsets as training and test subsets. In order to test the compatibility of these separated subsets with the MARS model, a new package program named "ehaGoF" which estimates 15 goodness of fit criteria was used. According to the analysis results, the MARS model with the smallest SD_{RATIO} (0.157, 0.130) and the highest determination coefficient (R^2) (0.975, 0.983) of the training and test sets, respectively, was determined. Looking at the other fit values, it is seen that the training and test set are quite compatible. In terms of hot carcass weight among the breeds, it was determined that the Limousine race performed higher than the other breeds. As a result, the implementation of the MARS algorithm can allow livestock breeders to obtain effective clues by using independent variables such as breed, age, and body weight in estimating hot carcass weight.

Keywords: Carcass weight, MARS algorithm, Multiple regression analysis, Beef cattle, ehaGoF, K-fold Cross Validation

1. Introduction

Beef, Turkey as well as to people all over the world in an adequate and balanced nutrition emerge as one of the most important resources. Although there are many cattle breeds in the world, most of the meat and milk production is met from certain breeds. native cattle breed in Turkey, is reported to be low meat yield in conventional farming conditions and the fattening end weight varied between 186-387 kg, and the daily live weight gain of 673-973 g (Kumlu 2000; Sak & Duru 2017). The carcass obtained from cattle is affected by many parameters. Among these, there are many factors such as the breed, sex, fattening period, care and nutrition of the cattle (Sak & Duru 2017; Seker et al. 2017). In the study conducted to determine the fattening performance and carcass characteristics of Simental, Aberdeen-Angus, Hereford, Limousine and Charolais breeds, it was reported that while Charolais performed better than others for carcass weight, the Simental breed was higher for daily weight gain (Sak & Duru 2017). Galiç & Takma (2019) have conducted on the determination of live weight and the genetics factors affecting this situation. In addition, it was stated in the studies that subjective and objective methods can be used in estimating the carcass composition in live animals. At the same time, the fact that the devices used to estimate the carcass composition in live animals are very expensive is seen as the most important obstacle in front of the studies. Therefore, since the most important thing in animal production is the production of animal products economically, it may be important to do different researches on this subject (Kor & Ertuğrul 2000). There are many scientific studies written for this purpose. However, in the breeding practice, it is very important to estimate the properties of the independent variables that the researcher should use economically. Such forecasts can assist the farmer in the decision process regarding herd management. Rather, these situations can be decided by looking at the physical characteristics of the animals subjectively in farm conditions. However, one way of producing such estimates, especially when the number of data is large, can be obtained by using statistical methods such as data mining. These methods include, among others, artificial neural networks (ANNs), decision trees, and Multivariate Adaptive Regression Spline (MARS) (Kibet 2012; Eyduran et al. 2018; Orhan et al. 2018; Eyduran et al. 2019). With the MARS algorithm used in this study, linear models are explained by dividing nonlinear multivariate models with more than one independent variable. As is known, regression analysis investigates the relationship between two or more variables with a cause-effect relationship. The main purpose of chains MARS analysis, which is an application of the techniques popularized by Friedman (1991) to solve regression type problems; estimating the result variable or the value of a continuously dependent variable with the set of independent variables. MARS offers the opportunity to be explained with linear models by breaking down multivariable nonlinear models (Sevimli 2009; Eyduran et al. 2017a; Eyduran et al. 2018; Orhan et al. 2018; Eyduran et al. 2019). As in other scientific fields, the selection of

breeds gives effective results in determining the weight of the carcass, which is one of the main issues within the scope of animal breeding. To obtain these results, powerful statistical methods, ie data mining algorithms, are required. MARS, one of these algorithms, is a statistically significant tool that can capture the relationship between dependent and independent variables. There are other studies that use the MARS algorithm in agriculture and animal husbandry (Aksoy et al. 2018; Aytekin et al. 2018; Celik &Yılmaz 2018; Celik et al. 2020; Canga & Boga 2019; Canga et al. 2019; Eyduran et al. 2019). The research also included goodness of fit values and the comparative use of training and test sets. Here, the training data set is a sample data set used for learning and created in accordance with the parameters of a classifier. The test data set is a data set that is independent of the training data set, but follows the same probability distribution as the training data set. For this, the estimation equation was created based on all the values in the train set, and then the accuracy of the values created with the test set was compared with the values in the train set.

When the literature is reviewed, estimation of economically determined variables such as estimation of live carcass weight has not been investigated by MARS algorithm yet. Therefore, the use of live weight and carcass weight of animals used for meat production in Turkey and there is a gap in terms of increasing productivity. This research was carried out to estimate the hot carcass weight efficiency using the MARS algorithm. Therefore, the aim is to draw attention to this issue and to contribute to the literature by leading more comprehensive research in this field.

2. Material and Methods

2.1. Materials

In the research, data belonging to the cattle belonging to the year 2017-2018 brought to the slaughterhouse in the open prison in Niğde province for slaughter from the provinces other than Niğde and Niğde were used. In the data used in the study, a total of 700 male calf data, 100 from each of the Aberdeen-Angus, Simmental, Limousine, Holstein–Friesian, Charolais, Zebu and Hereford breeds, were used. Live weight, age and breed were used as independent variables in the estimation of hot carcass weight determined as the dependent variable.

The animals were slaughtered between 2017-2018 in line with the observational decisions of the technical staff in the slaughterhouse belonging to the enterprise. For this, first of all, descriptive statistics values of the data are shown in Table 1.

Table 1- Descriptive statistics of the explanatory variables studied

Descriptive Statistics						
	N	*Minimum*	*Maximum*	*Mean*	*Std. Error*	*Std. Deviation*
AGE (year)	700	1	3	1.63	0.025	0.650
LIVEWEIGHT (kg)	700	440	1020	696.520	4.299	113.749
CARCASSWEIGHT(kg)	700	237	515	353.390	2.178	57.611

2.2. Methods

MARS method, which is one of the non-parametric regression methods, was developed by the statistician Jerome H. Friedman in the early 1990s (Tunay 2001; Mukhopadhyay & Iqbal 2009; Sevimli 2009). This regression method is designed for both continuous and binary response variables. MARS is a nonparametric regression method that makes no assumptions about the underlying functional relationships between dependent and independent variables (Kibet 2012; Oguz 2014; Eyduran et al. 2019). The main purpose of this method is to predict the values of the result variable or continuous variable regardless of the set of independent variables. The biggest advantage of the MARS model is that it defines both the individual effects of independent variables and their interactions in the model and presents them graphically (Chou et al. 2004; Sevimli 2009; Orhan 2018).

2.2.1. Mars model

Regions and spline functions are formed, and these regression extensions, which are regional, are called the basic functions (Put et al. 2004). The basic functions that occur are in a piecewise linear relationship with the dependent variable. Fundamental functions and model parameters (estimated by least squares method); consists of the results of the determinants that gave the data entries. Basic functions (BF) are mechanisms used in generalized searches for nodes. BF is a set of functions used to represent information contained within one or more variables. The structural model created with MARS uses the piecewise linear basis functions expansion, shown in the form of $(x - t)_+$ and $(t - x)_+$. The "+" subscript used here indicates the positive part and indicates that the basic function will take the result of zero when the desired condition is not met, and the formation process of the BF is defined as follows (Friedman 1991; Deconinck et al. 2005; Kayri 2010).

$$BF_1(x) = |x-t|_+ = \max(0, x-t) = \begin{cases} x-t, x > t \\ 0, \ x \le t \end{cases} \quad (1)$$

$$BF_2(x) = |t-x|_+ = \max(0, t-x) = \begin{cases} t-x, x < t \\ 0, \ x \ge t \end{cases} \quad (2)$$

Here "t" is the node value and each function is linear piecewise at the value of "t". The fundamental functions $(x - t)_+$ and $(t - x)_+$ (linear extensions) are also called a reflected pair, denoting the right and left regions of the node "t", respectively (Hastie et al. 2001; Sevimli 2009; Oğuz 2014; Eyduran et al. 2017b; Eyduran et al. 2018; Orhan et al. 2018).

2.2.2. MARS model selection criteria

How to measure the accuracy of the model is the most important issue in regression problems. For this purpose, the generalized cross validation (GCV) value developed by Craven & Wahba (1979) in the selection criteria of the most suitable MARS model measures the accuracy of the mean squares errors (Sevimli 2009). As a result, GCV is a form of regulation that transforms model complexity into goodness of fit. With the GCV approach, BF that have the least contribution to the model are thrown into the model, preventing the addition of excessive number of extension functions in the final model. The GCV criterion, which is the goodness of fit criterion, can be defined as follows (Xu et al. 2006; Grzesiak et al. 2010; Ali et al. 2015; Zhang & Goh 2016; Celik & Yılmaz 2018; Çanga & Boga 2019; Eyduran et al. 2019; Zaborski et al. 2019; Celik & Boydak, 2020; Eyduran & Gulbe 2020).

$$GCV(M) = \frac{1}{N}\sum_{i=1}^{N}\left([y_i - \hat{f}_M(x_i)]^2 \Big/ \left[1 - \frac{C(M)}{N}\right]^2\right) \quad (3)$$

Where; *N*, number of observations; *C(M)*, constant basic function; *C(M) = M + dM*, d is the smoothing parameter. Studies have shown that the best value for d is between *2 ≤d ≤ 4* (Friedman, 1991; Salford 2001). The most appropriate MARS model is the value with the smallest GCV measurement (Xu et al. 2004). The quality of the MARS model in the study was evaluated using the following criteria (Grzesiak et al. 2010; Ali et al. 2015; Zhang & Goh, 2016; Çelik &Yılmaz, 2018; Çanga & Boga, 2019; Eyduran et al. 2019; Eyduran et al. 2019; Zaborski et al. 2019; Celik & Boydak 2020). Pearson's correlation coefficient between actual values and predicted values (*r*).

1. Akaike Information Criteria (*AIC*):

$$AIC = n.ln\left[\frac{1}{n}\sum_{i=1}^{n}\left(y_i - y_{ip}\right)^2\right] + 2k \ , if; \frac{n}{k} > 40 \quad (4)$$

2. Root-mean-square error (*RMSE*):

$$RMSE = \sqrt{\frac{1}{n}\sum_{i=1}^{n}\left(y_i - y_{ip}\right)^2} \quad (5)$$

3. Mean error (*ME*)):

$$ME = \frac{1}{n}\sum_{i=1}^{n}\left(y_i - y_{ip}\right) \quad (6)$$

4. Absolute mean deviation (*MAD*):

$$MAD = \frac{1}{n}\sum_{i=1}^{n}\left|y_i - y_{ip}\right| \quad (7)$$

5. Standard deviation rate (SD_{ratio}):

$$SD_{ratio} = \frac{S_m}{S_m} \tag{8}$$

6. Relative approximate error rate (*RAE*):

$$RAE = \sqrt{\frac{\sum_{i=1}^{n} \left(y_i - y_{ip}\right)^2}{\sum_{i=1}^{n} {y_i}^2}} \tag{9}$$

7. Average absolute error percentage (*MAPE*):

$$MAPE = \frac{1}{n} \sum_{i=1}^{n} \left| \frac{y_i - y_{ip}}{y_i} \right| .100 \tag{10}$$

8. Performance index (ρ):

$$\rho = \frac{\sqrt{\sum_{i=1}^{n} \left(y_i - y_{ip}\right)^2}}{(1+r)\frac{1}{n}\sum_{i=1}^{n} y_i} .100 \tag{11}$$

Where; *n*, the number of observations in the data set; *k*, Number of model parameters (selected terms); y_i, i. dependent variable value of the observation; y_{ip}, i. dependent variable estimation value of the observation; S_m, Standard deviation of model error terms and S_d, Standard deviation of the dependent variable expresses (Eyduran et al. 2020).

2.3. Cross Validation process for training and test set

Cross validation is any of the model validation techniques used to evaluate how statistical analysis results will be generalized to an independent data set.

The purpose of cross validation is to test the model's ability to predict new data that is not used in its prediction, to mark problems such as selection bias, and to give an idea of how the model will generalize a generalization method. In a cross validation process, the analysis is first divided into complementary subsets of a data sample. Then, the analysis is performed in a subset called the training set and the analysis is verified in the other subset called the test set. To reduce variability, in most methods, multiple rounds of cross validation are performed using different parts. That is, cross validation combines fit measures in predictions to get a more accurate estimate of model forecast performance (Geisser 1993; Ripley 1996; Salford 2001).

Cross validation is done in a predetermined number of k. In the literature, 10-fold expression of cross-validation is also common. The data is divided into k pieces of equal size and evaluated k times. When doing cross validation, researchers first divide the data sets into two sets, training and test sets. Next, they choose X% of the training data set as the actual test set and the remaining (100-X) % as the verification set, where X is a constant number. If we define x as 80, the 80% model is repeatedly trained and verified on these different sets. There are multiple ways to do this and it is commonly known as cross validation. K-fold cross validation is the most used cross validation method (Devijver & Kittler 1982; Kohavi 1995). K-fold cross validation is a special case of cross validation where we iterate over a dataset k times. In each round, the data set is split into k parts and one split part is used for validation, then the remaining k - 1 parts are combined into a training subset for model evaluation. Figure 1 below shows the step-by-step cross-validation process (Anonymous 2021):

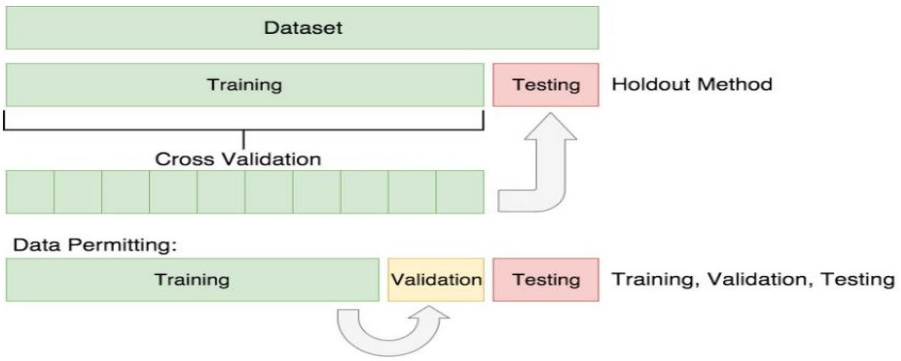

Figure 1- Visual representation of train/test split and cross validation

Some descriptive statistical values in the analysis were performed using IBM SPSS 23.0 software, and the MARS model was developed using the R software "*earth"* package and "*ehaGoF*" (Milborrow 2011; R Core Team 2014; Milborrow 2018; Eyduran et al. 2019; Eyduran & Duman 2020; Eyduran & Gulbe 2020). Among the observed and predicted values of carcass weight in the study, the smallest *GCV, SDRATIO, RMSE, MAPE, MAD, AIC, AICc* and the MARS model with the highest determination coefficient (R^2) and Pearson coefficient (r) were accepted as the best.

3. Results and Discussion

In the research, the carcass yield was analyzed with the MARS method. Graphics of hot carcass weight, breeds, body weight and age, which are the dependent variables for better understanding of the data, are given in Figure 2.

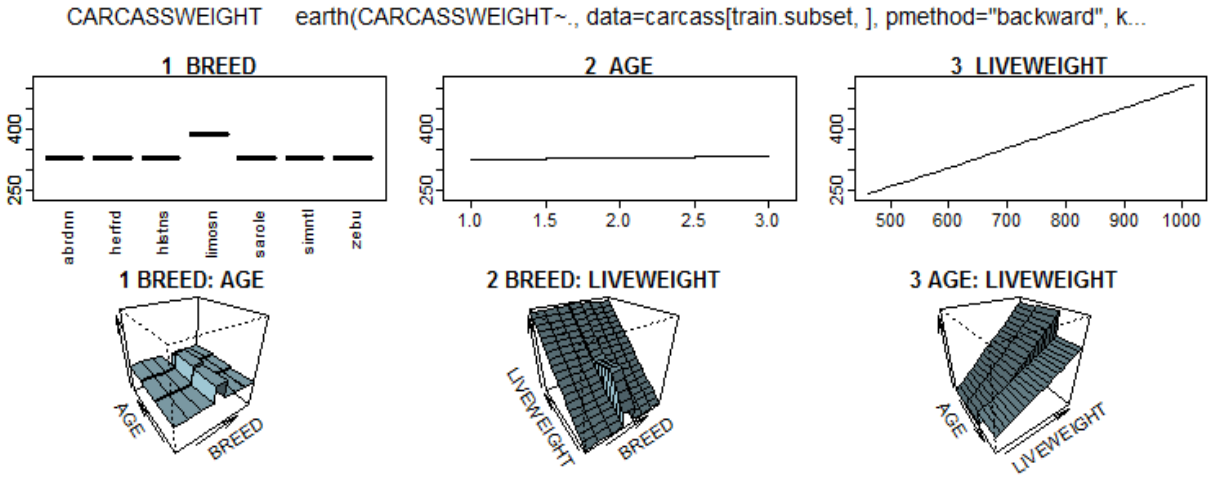

Figure 2- Distribution of breeds (1), age (2), live weight (3) by carcass weight

When looking at the data obtained from 700 cattle beef cattle from 7 different breeds of different ages; it is seen that the carcass weight is in the animals belonging to the highest limusin race, while the other breeds are very close to each other (Figure 2). Duru & Sak (2017) aimed to determine the fattening performance and carcass characteristics of Simmental, Aberdeen-Angus, Hereford, Limousine and Charolais breeds. In this study, 606 male cattle aged 10-12 months imported from Uruguay and France in 2015 were used. All animals were fed unlimitedly with the same ration during the fattening period of about 7-9 years. Daily weight gain (DWA) Simmental 1362.9; Aberdeen-Angus 1275.9; Hereford 1214.2; Limousine 1266.9; Charolais was obtained as 1101.1 g. This situation was found to be similar to that the limusin breed in our study was more than the charolais and hereford breeds. Differences with other breeds indicate that it can also be affected by different factors such as environment, care and feeding. The age of animals with a carcass weight between 300 and 350 varies between 1 and 3 years (Figure 2). According to the research data, it is seen that as the body weight increases, the carcass weight increases positively (Figure 2). Age is one of the important factors affecting the fattening performance in beef cattle. The development of culture breeds and their hybrids continues until maturity. This period is 18 months. In domestic breeds, it is 2.5-3 years old (Yıldız 2020). Looking at Figure 3, it is seen that there is a positive relationship between the real and predicted values on the training and test set, respectively, according to the results of MARS analysis (r = 0.994).

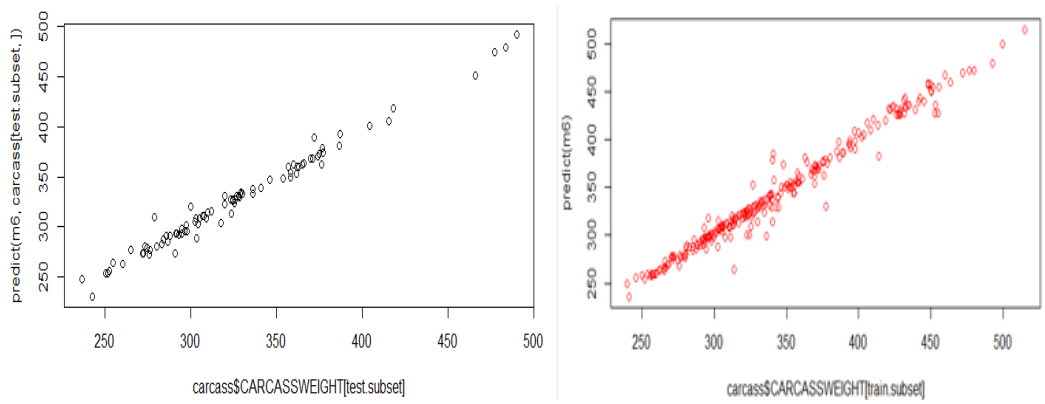

Figure 3- Relationship between real and predicted values for test set and training set

The prediction model formed as a result of MARS analysis is mathematically written as follows:

CARCASSWEIGHT = 296.807 + 0.492 * max (0, LIVEWEIGHT-585) - 0.444 * max (0,585-LIVEWEIGHT) -9.481 * BREEDLimousine + 0.554 * BREEDLimousine * max (0,737.22-LIVEWEIGHT) - 99.854 * BREEDLimousine * max (0, AGE-2) +147.262 max (0, AGE-2) -6.151 max (0,2-AGE) -0.921 * BREEDLimousine * max (0,585-LIVEWEIGHT) -0.218 * max (0, AGE-2) * LIVEWEIGHT.

This prediction function is represented in terms of basic functions as follows:

CARCASSWEIGHT = 296.807 +0.492 * BF1 - 0.444 * BF2- 9.481 * BF3 + 0.554 * BF4 - 99.854 * BF5 + 147.262 * BF6- 6.151 * BF7 - 0.218 * BF8.

Here y is defined as the carcass weight. BF is defined as the basic function. When looking at the MARS estimation model by considering equation 2 and equation 3; For the fundamental function BF1 max (a, b) = a, a> b otherwise the result will be b. So, when looking at the equation, there are two nodes, 585 and 737. Thus, the two nodes in 585 and 737 divide the interval into three intervals in which different linear relationships are determined (Canga & Boga 2019; Eyduran et al. 2019; Celik & Boydak 2020). The results of the MARS algorithm for carcass yield are presented in Table 2. As can be seen, all coefficients for the carcass yield were statistically significant (P<0.001). In interpretation, for example, when the body weight is less than 585; max (0, LIVEWEIGHT-585) = 0, so the effect of the MARS term number 1 is masked in the carcass yield. Likewise, when the age of the animal is less than 2; max (0, AGE-2) = 0, i.e. the effect of MARS terms 5^{th}, 6^{th}, and 9^{th} is masked on carcass yield (Celik & Yılmaz 2018; Celik et al. 2020; Canga & Boga 2019; Canga et al. 2019; Eyduran et al. 2019; Sevgenler 2019; Zaborski et al. 2019; Canga & Boga 2020; Sengul et al. 2020).

When LIVEWEIGHT is> 585 cm (BF1), the carcass yield is expected to be higher and when LIVEWEIGHT is greater than 585 cm, the carcass yield will be above BF2, so it is masked. However, when the breed is Limousine, LIVEWEIGHT has a positive effect on carcass weight in live animals with <737.22 cm (BF4, P <0.001). In addition, when the breed is Limousine, LIVEWEIGHT has a negative effect on carcass weight in live animals with <585 cm (BF7, P <0.01). When AGE> 2 there is a positive high effect on the carcass weight for the BF5 coefficient (147,262). In addition, the negative effect of the BF6 coefficient (-6.151) on the carcass weight for animals with AGE> 2 was masked. Also, for animals with AGE <2, the effect of LIVEWEIGHT on carcass weight was masked by age, considering BF8 (first order interaction term). However, for animals with AGE> 2, LIVEWEIGHT has a negative effect on carcass weight (BF8, P <0.001)

In the study, the cross validation coefficient (CVR^2) was found to be 0.959, while the highest Pearson correlation coefficient (r) between the real and predicted values was 0.997 and the results were accepted as the best model. The suitability of this model was evaluated with the criteria for the GCV to be minimum and R^2 to be maximum (Celik & Yılmaz 2018; Celik et al. 2020; Canga & Boga, 2019; Canga et al. 2019; Eyduran et al. 2019; Eyduran et al. 2019; Sevgenler 2019; Zaborski et al. 2019; Sengul et al. 2020).

Table 2- Coefficients of the MARS model and results of MARS analysis

	Basis Functions (BF_i)	*Coefficients*	*P*
	Intercept	296.807	<2e-16 ***
BF1	max (0, LIVEWEIGHT-585)	0.492	<2e-16 ***
BF2	max (0,585-LIVEWEIGHT)	-0.444	<2e-16 ***
BF3	BREEDlimousine	9.481	4.00e-09 ***
BF4	BREEDlimousine* (0,737.22-LIVEWEIGHT)	0.554	<2e-16 ***
BF5	BREEDlimousin*max(0,AGE-2)	-99.854	<2e-16 ***
BF6	max(0,AGE-2)	147.262	1.94e-06 ***
BF7	max(0,2-AGE)	-6.151	1.69e-08 ***
BF8	BREEDlimousine*max(0,585-LIVEWEIGHT)	-0.921	4.05e-16 ***
BF9	max(0,AGE-2)*LIVEWEIGHT	-0.218	1.55e-05 ***
	GCV: 88.2 RSS: 24531 GRSq: 0.972 RSq:0.975 CVRSq:0.959		

***: P<0.001

As can be seen from Table 3, it is seen that the live weight value has the highest relative importance (100%) in the estimation of carcass weight, both the GCV value and the RSS criterion (Eyduran et al. 2019; Sevgenler 2019; Canga & Boga 2020; Celik & Boydak 2020; Eyduran & Duman 2020; Sengul et al. 2020).

Table 3- Relative importance of independent variables in the model

Variables	*Nsubsets*	*GCV*	*RSS*
LIVEWEIGHT	9	100.00	100.00
BREEDLimousine	7	22.3	22.5
AGE	6	18.5	18.7

3.1. Reducing model bias by creating training and test subsets

Model bias will be reduced with the training and test sub-sets created in the researc4. Therefore, in this study, the significance of the model was tested as follows. The model is divided into two parts as training and test set. Also, like most other things in machine learning, the split ratio in the training / test set is highly specific to your use case, making it easier to master the situation while developing more models. Here, this ratio (75:25) has been chosen; This means that 75% of the observations of the model are included in the training data set, while 25% of the observations of the model are included in the test data set. First, a model was obtained with the training data set, and then the reliability of this model was sent to the test set and the resulting values were compared. Thus, all values were checked by performing the validity process. While performing the analysis, the test set is not used until all studies related to the model are completed, it is used to test this model after the final model is decided. Using the test set more than once increases the model bias. In this case, it will not make sense to do such a partition. In other words, the training data set used in the research is the data sample used to provide an unbiased assessment of a final model that fits the data set, and the test data set provides the gold standard used to evaluate the model. It is used only after the model has been fully trained. The R codes created for the training and test sub-sets in the research and which will reduce the model bias are given in Appendix (Eyduran & Duman 2020).

The most suitable model was determined by testing the goodness of fit values of the carcass weight, which was determined as the dependent variable in the study, with the training and test set. To estimate the goodness of fit between the training set and the test set, was used in the R program of the "EhaGof" package. The most important aspect of this newly created package is that all 15 different goodness of fit criteria are calculated at the same time. After all these calculations, a comparative representation of the goodness of fit values on the training and test set of the most suitable model is given in Table 4 (Zhang & Goh 2016; Eyduran et al. 2019; Zaborski et al. 2019).

Table 4- Goodness of fit criteria for training set MARS and test set MARS algorithms

	Criterias	*Train-Set MARS results*	*Test- set MARS results*
1	Root mean square error (RMSE)	77.877	47.382
2	Relative root mean square error (RRMSE)	2.571	2.049
3	Standard deviation ratio (SDR)	**0.157**	**0.130**
4	Coefficient of variation (CV)	**2.570**	**2.060**
5	Pearson's correlation coefficients (PC)	**0.998**	**0.992**
6	Performance index (PI)	1.294	1.029
7	Mean error (ME)	0.000	0.391
8	Relative approximation error (RAE)	0.001	0.000
9	Mean relative approximation error (MRAE)	0.001	0.002
10	Mean absolute percentage error (MAPE)	**1.587**	**1.533**
11	Mean absolute deviation (MAD)	5.436	4.984
12	Coefficient of determination (Rsq)	**0.975**	**0.983**
13	Adjusted coefficient of determination (ARsq)	**0.974**	**0.981**
14	Akaike's information cCriterion (AIC)	1391.866	425.114
15	Corrected Akaike's information criterion (CAIC)	1392.590	427.455

By comparing the goodness of fit criteria of the model for the data belonging to the training and test set, it is ensured that bias is reduced by performing cross validation. In this case, goodness of fit criteria such as R^2 and *RMSE* were used. As can be seen from Table 4, the MARS model with the smallest SD_{RATIO} (0.157, 0.130) and the highest determination coefficient (R^2) (0.975, 0.983) of the training and test sets, respectively, was determined. In the model, it can be said that the bias of the model is low since the goodness of fit criteria of the training and test sets are very close to each other (Eyduran & Duman 2020). Some authors reported that the standard deviation ratio of the structured model, which fits well for regression-type problems, should be less than 0.20.

Seven different cattle breeds were used in the study, and only Limousine race among these breeds was found to be statistically significant in determining the carcass yield (P<0.001). In the report, the average obtained for hot carcass weight in Limousine breed is 319.3 ± 3.5 kg. This value is 386.76 in this study, Zahrádková et al. (2010) and Anonymous (2020) can be said to be an average value. As is known, the reliability of the obtained results depends on the selection of effective independent variables and strong statistical approaches (Eyduran et al. 2018). This study provides good evidence with the resulting results regarding the superiority of the MARS data mining algorithm. Grzesiak et al (2010); In his study on MARS analysis, he identified cows with artificial insemination difficulty using statistical and machine learning methods (classification functions, logistic regression, artificial neural networks and MARS). He also showed that the best results were obtained with artificial neural networks (ANN) and MARS methods in his study and stated that ANN and MARS gave more accurate results compared to other statistical methods in the detection of cows with artificial insemination differences with the test set. In agricultural sciences, t-test, one-way ANOVA, two-way ANOVA, multiple linear regression analysis is widely used (Efe et al. 2000; Agaoglu et al. 2007). In addition, more complex approaches, namely data mining, have recently been adopted (Grzesiak et al 2010; Aksoy et al. 2018; Akin et al. 2020). Although there are many studies on the carcass yield, no studies have been found investigating the use of the MARS algorithm in connection with the carcass yield characteristics. Therefore, no further discussion could not be made on the subject.

4. Conclusions

In the research, in order to estimate the carcass yield, MARS prediction models with first order interaction effects have been developed using the MARS algorithm. With the help of the comparison of the goodness of fit criteria of the model belonging to the data belonging to the training and test set, it was ensured that the bias was reduced by performing cross validation. It has been determined that MARS algorithms are a good determinant for the relationship between the properties used and the carcass yield. Estimating the carcass weight by data mining; It causes the determination of the animal's carcass yields in a shorter time. In this way, it is possible to have information about whether an economic animal husbandry can be done. In addition, with the development of such practices, it will be possible to access the carcass data of the animals to be obtained from slaughterhouses by looking at parameters such as race, live weight and age, and it will be possible to slaughter animals more economically. For such reasons, estimating the carcass data with the MARS method will be an important reference for the breeders.

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Reducing Ammonia Volatilization from Urea Fertilizer Applied in a Waterlogged Tropical Acid Soil *via* Mixture of Rice Straw and Rice Husk Biochars

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ABSTRACT
Nitrogen deficiency frequently occurs in agricultural soil because of ammonia volatilization to the environment, resulting in low urea-N use efficiency by plants. A laboratory incubation experiment was conducted to assess the effect of rice straw and rice husk biochar's on ammonia volatilization, soil pH, exchangeable ammonium, and available nitrate in comparison to the urea without additives under waterlogged conditions. Application of rice straw and rice husk biochar's mixture at application rate 5-10 t ha^{-1} had significantly minimized ammonia volatilization by 30.86% - 38.61% over T1 (175 kg ha^{-1} urea). T2 (5 t ha^{-1}) and T3 (10 t ha^{-1}) also had significantly increased retention of ammonium by 79% - 95% and nitrate ions by 49% - 51% over control. The treatments amended with biochar had successfully improved soil pH compared to T0 (soil only) and T1. Hence, the findings suggest that urea amended with rice straw and rice husk biochar's altered the nutrients level in the soil by minimizing ammonia loss to enhance nitrogen availability in waterlogged conditions.

Keywords: Ammonia volatilization, Biochar, Nitrogen, Urea, Ammonium

1. Introduction

Nitrogen (N) is a vital soil nutrient essential for good and abundant plant growth (Hajdu 2020). The main source of N for the plant comes from the external input application. Currently, urea (46% N) is being used in rice field as a main N contributor due to its abundant availability and relatively low price compared to other N fertilizers. However, there is a major concern in using urea as a N source because it is easily hydrolyzed and volatilized to the environment (Soares et al. 2012; Sunderlage & Cook 2018). Urea hydrolyzes upon contact with water and forms ammonia gas (NH_3) which is susceptible to atmospheric loss via volatilization process. Around 60% of urea volatilized in the form of NH_3 to the environment (Sommer et al. 2004; Rochette et al. 2009). The emission of NH_3 gas to the environment triggers air pollution, which harms living things. Besides, during the urea hydrolysis process, the formation of inorganic ammonium ion (NH_4^+) speeds up. Retention of NH_4^+ ion in the soil is relatively very poor due to the lack of binding-adsorptive agent. The NH_3 volatilization and poor retention of inorganic-N ions become very problematic for both farmers and plants. Deficient of N to plants cause farmers to increase the application of urea fertilizer, whereby this practice is not economical, efficient and reliable for long term use since it creates an environmental problem and costly.

Hence, an organic amendment such as biochar is necessary to minimize NH_3 loss to the environment. Biochar is a porous carbonaceous solid produced by charring or pyrolysis method of organic materials under oxygen-depleted environment (Lehmann & Joseph 2015; Ding et al. 2016). Biochar can be produced by utilizing agricultural wastes that are easily accessible and abundant. Mansor et al. (2018) stated that rice residues such as rice straw and rice husk are being produced annually more than 7,518,073 tonnes and 926,886 tonnes, respectively. The wastes are abundant and being burnt continuously. Burning wastes is hazardous for both the environment and human. So, turning the rice residues waste into biochar could be a promising approach to achieve sustainable waste management and benefits agronomy.

Biochar has a huge potential to improve soil fertility either by direct supply of nutrients or by fixing nutrients from the external source followed by slow release of the adsorbed nutrients (Unger 2008). The porosity and larger surface area of the biochar helps in nutrients absorption from the soil, which directly improves soil fertility (Lehmann & Joseph 2015). Biochar's surface area plays an essential role in binding cations, and anions (Atkinson et al. 2010; Chan & Xu 2009) which directly increases nutrient retention in biochar amended soil (Gai et al. 2014). Biochar capable to adsorb ammonium (NH_4^+) and (nitrate) NO_3^- onto its surface, thus increase the presence of these ions in the soil for plant uptake. Besides, biochar has been said to increase the formation of NH_4^+ and NO_3^- over NH_3. Eventually, this reduces NH_3 volatilization from applied urea fertilizer.

Yeboah et al. (2009) reported that an increase in soil nutrient retention due to the application of biochar results in the reduction of total fertilizer requirements in agricultural soil. Moreover, the alkaline nature of biochar modifies the soil acidity. Yuan et al. (2011), stated that biochar could act as an alternative liming agent to modify the soil pH in a way that it fits the crop growth.

Previous researchers demonstrated that biochar comes in different properties based on feedstock, charring condition, and activation. This agrees with Spokas et al. (2012) who also stressed the need for further research on biochar's economic and agronomic benefits. Additionally, there is also a scarcity of information on green feedstock biochar in amending soil fertility by preventing N loss either in rice, cash crops or other agricultural fields. It is crucial to know the properties of rice husk biochar and its ability to retain nutrients. It is hypothesized that the use of biochar produced from rice straw and rice husk wastes can create a pool of negative charges to retain and chelate the positively-charged NH_4^+ ions to prevent it from loss through NH_3 volatilization. Over time, the sorbed NH_4^+ ions on biochar will be gradually released and become available to plants and microorganisms. Biochar can also induce microbial immobilization of N into the soil. Hence, this study was carried out to determine the effect of mixing urea with mixtures of rice straw and rice husk biochar on NH_3 volatilization, exchangeable soil NH_4^+, and available NO_3^- as compared to the application of urea alone under a waterlogged condition.

2. Material and Methods

2.1. Soil sampling, preparation and characterization

The soil used in this study was Renggam sandy clay loam (*Typic Paleudult*). It was sampled at 0-30 cm from a land at the Agro Techno Park in University Malaysia Kelantan Jeli Campus, Malaysia (5^0 44' 69.55" N latitude and 101^0 51' 83.89" E longitude) has not been cultivated since 2007. The collected soil was air-dried, crushed, and sieved to pass through a 2-mm sieve for initial soil characterization. Soil pH was measured in a ratio of 1:10 (soil:water) using a digital pH meter (Peech 1965). Soil texture was determined using the hydrometer method (Bouyoucos 1962). Total organic matter content, ash content, and total organic carbon were determined using the loss-on ignition method (Tan 2005).

Total N was determined using the Kjeldahl method (Bremner 1965). The double acid method described by Mehlich (1953) was used to extract soil available P and exchangeable cations (Ca, Mg, K, and Na), after which the cations were determined using an Atomic Absorption Spectrophotometer (AAS) (Analyst 800, Perkin Elmer, Norwalk, USA). Soil available P was determined using molybdenum blue method (Murphy & Riley 1962). The developed blue color was analyzed using a UV-VIS spectrophotometer (Thermo Scientific Genesys 20, USA) at 882 nm wavelengths. Soil cation-exchange capacity (CEC) was determined by the ammonium acetate leaching method (Cotteinie 1980). The exchangeable acidity and exchangeable aluminum (Al^{3+}) were determined by the acid-base titration method described by Rowell (1994). The method described by Keeney & Nelson (1982) was used to extract exchangeable NH_4^+ and available NO_3^-, after which the ions were determined *via* steam distillation (Tan 2005).

2.2. Biochar production, activation and characterization

Rice husk was collected from Pasir Puteh Rice Mill whilst rice straw was collected from Kemubu granary area, Kota Bharu, Malaysia. Two cylindrical kilns, a 200 L drum with removable chimney caps and an airtight 110 L drum were constructed for biochar production. The rice husk and rice straw were bulked separately inside the 110 L drum then, closed and placed in the middle of the 200 L drum, where the fire was kindled starting from the bottom of the drum. The residence time was 4 hours with the temperature ranging from 300 - 400 °C and left for cooling for 2 hours. The temperature inside the kiln was measured using Extech TM100 K/J (Single Input Thermometer, Waltham, Massachusetts, United States). Later, the pile of biochar sample was spread out for cooling. After this, the enrichment of biochar was carried out by soaking with 5% chicken slurry for 7 days which later was dried and stored in a big container for further use. The enrichment of biochar with chicken slurry was crucial to further increase the nutrient content, alter the surface area, and increase the pore size (Selvarajh et al. 2021a). The enriched biochars were analyzed for pH (Peech 1965), CEC and total N (Bremner 1965). The single dry ashing method (Tan 2005) was used to extract nutrients from rice husk and rice straw biochar for analysis of Ca, Mg, Na, P, and K using an AAS (Analyst 800, Perkin Elmer, Norwalk, USA), while total P content was determined using the molybdenum blue method (Murphy and Riley 1962), after which the blue color developed was analyzed using a UV-VIS Spectrophotometer (Thermo Scientific Genesys 20, USA) (Murphy and Riley 1962). Total C was determined using the loss on ignition method (Tan 2005). Additionally, microanalysis through Scanning Electron Microscopy-attached with Energy Dispersive X-ray Spectroscopy analysis (SEM-EDX JEOL JSM-6400) was carried out analyze the surface morphology of enriched rice husk and rice straw biochar.

2.3. Ammonia volatilization laboratory incubation study

For laboratory-scale NH_3 volatilization study, the actual amount of urea applied was 0.7 g, scaled down from the 175 kg ha^{-1} application rate. The rice husk and rice straw biochar (1:1 ratio) actual application for 100 g of soil, scaled-down from 5, 10, 15, and 20 t ha^{-1} was 2.8 g, 5.5 g, 8.3 g, and 11.1 g, respectively. The treatments evaluated were listed in Table 1. Soil, urea, and biochar were mixed well before deposited into 250 mL conical flask, after which water was added to create a waterlogged condition. The water level was maintained 3 cm above the soil throughout the study. This set up was done to depict the

waterlogged condition in the actual rice field. The system was set to be a closed dynamic airflow system, and the NH_3 loss from urea was measured daily (Siva et al. 1999; Ahmed et al. 2006a, 2006b). The system includes a 250 mL conical flask exchange chamber containing soil mixture and a trap 250 mL conical flask chamber containing 75 mL of boric acid, which were stoppered and fit with inlet/outlet pipes. The chamber inlet containing the water was connected with an aquarium air pump and outlet connected with pipe tubing to the trap containing boric acid solution. Air was passed through the chambers at a rate of 2.75 L^{-1} min^{-1} $chamber^{-1}$. This setup was done to create soil aeration and trap NH_3 loss *via* volatilization process. The released NH_3 was captured in the trapping solution containing 75 mL of boric acid with colour indicator. The incubation chambers Boric acid-indicator traps were replaced every 24 h and back, titrated with a size of 0.01 M HCl, to estimate the NH_3 released. Measurement was continued until the loss declined to 1% of the N added with urea (Ahmed et al. 2008) After the NH_3 volatilization was evaluated, the soil samples were used for pH, exchangeable NH_4^+ and available NO_3^- determinations.

Table 1- Treatments evaluated in ammonia volatilization incubation study

Treatments	*Description*
T0	Soil only
T1	Soil + 175 kg ha^{-1} urea
T2	Soil + 175 kg ha^{-1} urea + 2.5 t ha^{-1} rice husk biochar + 2.5 t ha^{-1} rice straw biochar
T3	Soil + 175 kg ha^{-1} urea + 5 t ha^{-1} rice husk biochar + 5 t ha^{-1} rice straw biochar
T4	Soil + 175 kg ha^{-1} urea + 7.5 t ha^{-1} rice husk biochar + 7.5 t ha^{-1} rice straw biochar
T5	Soil + 175 kg ha^{-1} urea + 10 t ha^{-1} rice husk biochar + 10 t ha^{-1} rice straw biochar

2.4. Statistical analysis

The experiments were arranged in a completely randomized design with three replicates. The effect of different rice husk and rice straw biochar addition rates was subjected to one-way analysis of variance (ANOVA). Statistical analysis for all the data was performed using SPSS software version 24.0 (SPSS Inc, US). Significant differences among treatments were separated by Tukey's HSD test and considered significant at $P \leq 0.05$.

3. Result and Discussion

3.1. Selected soil physico-chemical properties

The selected soil physico-chemical properties used in this study are presented in Table 2. The soil showed a sandy clay loam texture with pH of 5.5. The soil showed relatively high Al (1.14 $cmol_c$ kg^{-1}) concentrations and Fe (0.091 $cmol_c$ kg^{-1}) due to low soil pH. This condition correlates to the lesser P availability (0.385 ppm) in soil because of P fixation by Al and Fe. Exchangeable K, Ca, Mg, and Na was low in the soil due to the soil's lower CEC (5.4 $cmol_c$ kg^{-1}). Lower CEC of the soil leads to inefficient nutrient holding and retention capacity of basic cations. The content of N (0.07 %), NH_4^+ (89 ppm), and NO_3^- (30 ppm) in the soil were low because of soil acidity, which slows down the mineralization process. Khalil et al. (2005) stated that acidic soil causes N immobilization instead of N mineralization. The soil used in this study needs amelioration to improve the soil quality and fits for crop growing.

Table 2- Selected soil physico-chemical properties

Property	*Value obtained*
pH	5.5
EC (dS m^{-1})	0.022
Texture	Sandy clay loam
Soil organic matter (%)	6.24
Total Carbon (%)	3.62
Ash content (%)	6.4
Cation exchange capacity ($cmol_c$ kg^{-1})	5.4
Ammonium (ppm)	89
Nitrate (ppm)	30
Total N (%)	0.07
Available P (ppm)	0.385
Exchangeable K ($cmol_c$ kg^{-1})	0.084
Exchangeable Ca ($cmol_c$ kg^{-1})	0.10
Exchangeable Mg ($cmol_c$ kg^{-1})	0.082
Exchangeable Fe ($cmol_c$ kg^{-1})	0.091
Exchangeable acidity ($cmol_c$ kg^{-1})	0.7
Exchangeable Al ($cmol_c$ kg^{-1})	1.14

3.2. Characterization of rice husk and rice straw biochars

The surface morphological characteristics of rice husk biochar and rice straw biochar are shown in Figure 1 and Figure 2. Both rice straw and rice husk biochar composed of numerous pores and comes with a high surface area. This property is highly beneficial for agronomical practices in terms of increasing nutrient retention and boosting crop growth. Biochar's porous structure and bigger surface area help in binding ions from soil and external inputs. Lin et al. (2012) stated that biochar's high porous structure might have extractable humic-like and fluvic-like substances that act as chelators. Humic and fluvic acid restrict toxins in the soil by reducing harmful substances to reach the crop roots. Besides, biochar has a high surface area with a strong affinity to attract inorganic ions (Schmidt et al. 2015). This would be a great advantage in the agriculture field to bind nutrients from the soil and release it slowly as it degrades. The capability of biochar to bind nutrients also related to the higher CEC value, where rice straw biochar and rice husk biochar CEC is 75.6 $cmol_c$ kg^{-1} and 66.6 $cmol_c$ kg^{-1}, respectively (Table 3). Biochar with higher CEC value adsorb more nutrients onto its surface and minimize volatilization. Not only that, the alkaline nature of both biochars (pH>9) can act as natural liming agent to reduce the acidity of soil at a certain application rate. The biochar also inherently packed with nutrients such as N, P, K, Ca, Mg, and Na. Eventually, the nutrients in biochar will be released to the soil for effective utilization by plants.

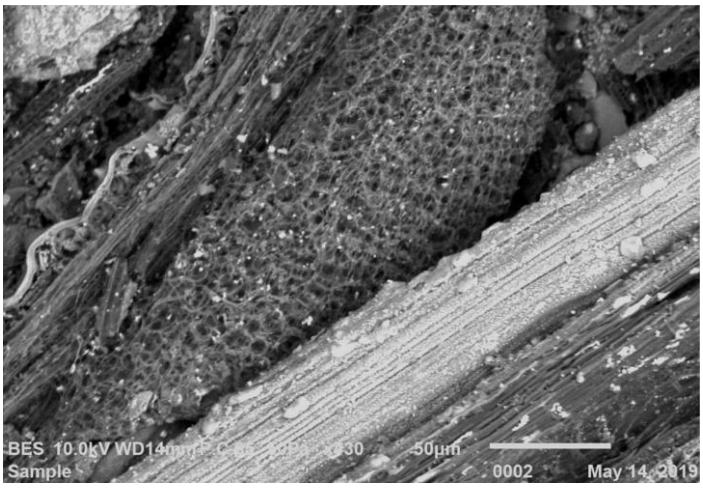

Figure 1- Rice husk biochar surface at 550x, magnification under SEM

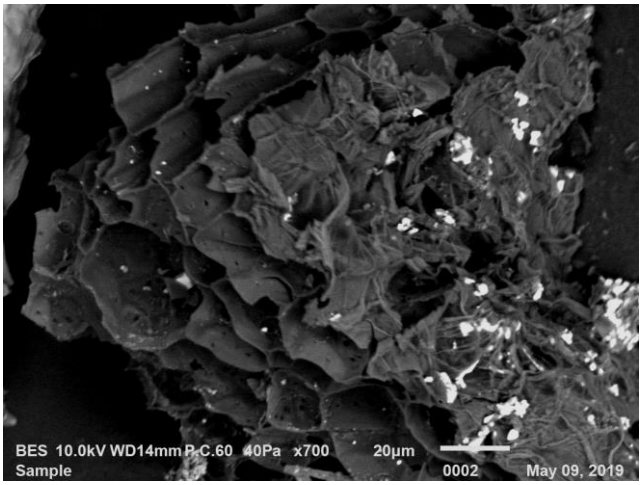

Figure 2- Rice straw biochar surface at 730x, magnification under SEM

Table 3- Selected physico-chemical properties of rice straw and rice husk biochar

Property	*Rice straw biochar*	*Rice husk biochar*
pH (water)	9.2	9.1
CEC (cmol kg^{-1})	75.6	66.6
Total Nitrogen (%)	0.45	0.33
Available P (ppm)	14.3	14.3
Exchangeable Ca ($cmol_c$ kg^{-1})	0.98	0.21
Exchangeable Mg ($cmol_c$ kg^{-1})	0.58	0.27
Exchangeable K ($cmol_c$ kg^{-1})	7.68	2.51
Exchangeable Na ($cmol_c$ kg^{-1})	0.04	0.05

3.3. Combined effect of rice husk and rice straw biochars on NH_3 volatilization

The daily NH_3 volatilization from urea fertilizer during the incubation study over 28 days is presented in Figure 3. NH_3 loss started on day 2 of incubation in treatment T1, on day 7 for T2 and T3, day 6 for T4 and T5, while no loss was found for T0. The delayed loss upon urea application shows the efficacy of added rice straw and rice husk biochars as an organic amendment in minimizing NH_3 formation. The NH_3 loss from urea can be delayed for 3-6 days with the addition of organic materials (Omar et al. 2010). The maximum NH_3 loss for T1 occurred on day 5, T2 on day 12, T3 on day 13, T4 on day 11, and T5 on day 13. The graph shows that NH_3 loss peaks up and reduces gradually up to 28th day until N added as urea ceases up to 1%. Rapid NH_3 loss in T1 was probably due to increased soil pH as urea hydrolysis leads to hydrogen ions (H^+) from the soil solution. However, in treatment amended with rice straw and rice husk biochar, the NH_3 loss was minimal due to the increased formation of NH_4^+ over NH_3 in the soil. Besides, Dougherty et al. (2017) stated that biochar's addition minimizes NH_3 volatilization by increasing the NH_3 adsorption at the oxygen-containing surface functional group or biochar micropores.

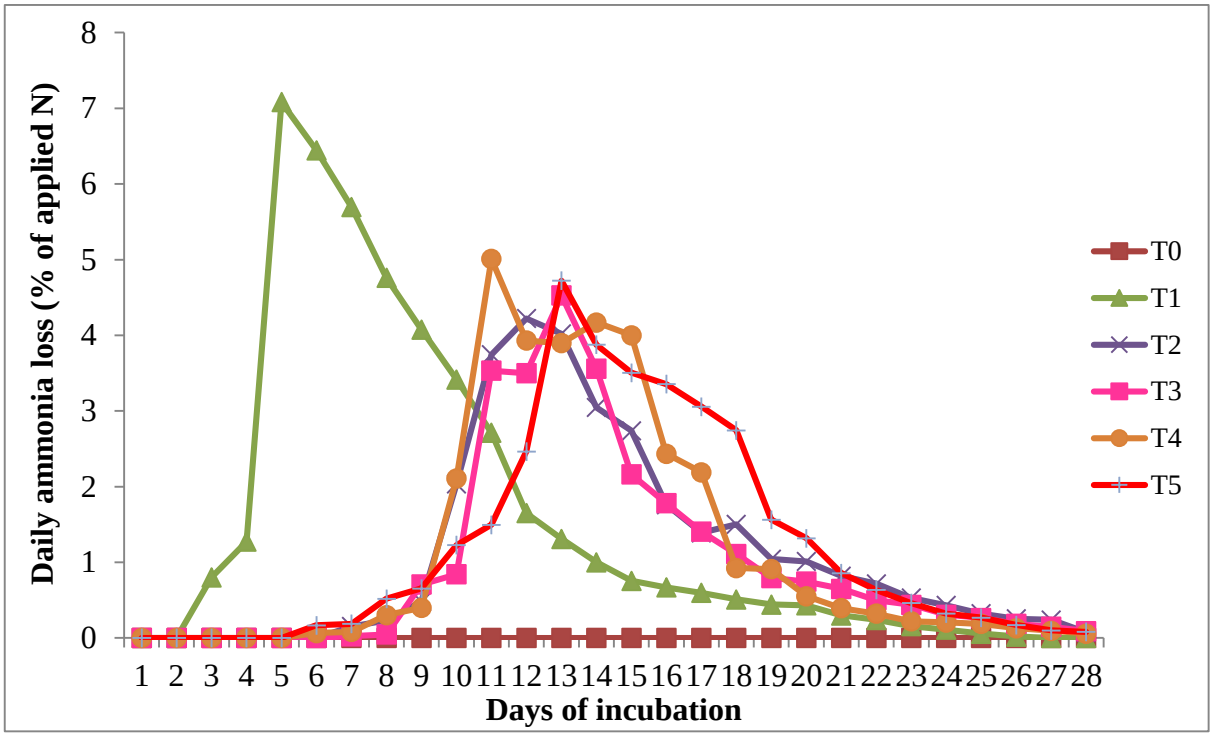

Figure 3- Ammonia volatilization over 28 days of incubation under waterlogged condition

The treatments with biochar as an additive (T2, T3, T4, and T5) had significantly minimized NH_3 loss compared to urea without additives (T1) (Figure 4). The total amounts of NH_3 lost at the end of the incubation period as a percentage of urea-N added were 0, 44.52, 30.79, 27.33, 32.62, and 33.66% for T0, T1, T2, T3, T4, and T5, respectively. Noticeably, T2 and T3 were significantly effective in minimizing NH_3 loss over T1. Irrespective of the application rate, all the treatments with biochar as an additive had effectively reduced NH_3 loss compared to T1. Addition of porous biochar with larger surface area delayed and minimized NH_3 loss due to its capability to bind more NH_4^+ and NO_3^- ions (Figures 6 and 7). This was in agreement with a study conducted by Chen et al. (2013). Besides, biochar increases the soil volume and pore size and stabilize the soil aggregate (Burrell et al. 2016). Since the volume of soil increased with which urea is mixed, it will also increase the time required for complete urea hydrolysis (Fan & Mackenzie 1993). Delays in urea hydrolysis due to the biochar application can minimize N loss, which will benefit plants in the agricultural field.

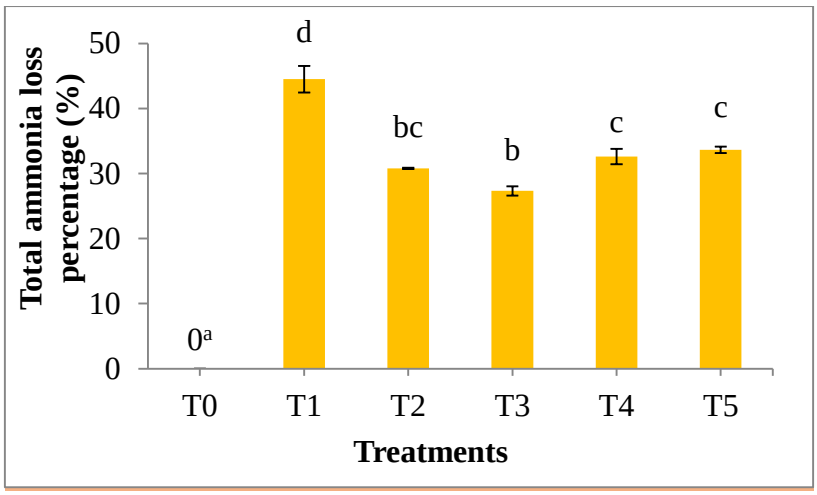

Figure 4- Total NH_3 losses from incubation study under waterlogged conditions.

Besides, biochar has alkaline nature where it can act as a liming agent to reduce soil acidity. Treatments with biochar had improved soil pH (Figure 5). Ch'ng et al. (2016) and Tang et al. (1999) stated that an increase in the soil pH was due to the rapid proton exchange between soil and biochars. The increase in soil pH is also related to the release of anions from rice straw and rice husk biochar, where anions undergo decarboxylation and exchange of protons in soil. In the previous study, it has been reported that NH_3 volatilization speeds up in soil with higher pH (Sun et al. 2019), but contrastingly in this study, the added biochars minimized the volatilization. This is due to the nutrients' adsorptive capability of the rice straw and rice husk biochars. Selvarajh et al. (2021b) also stated that increased soil pH due to biochar's addition does not significantly trigger ammonia loss.

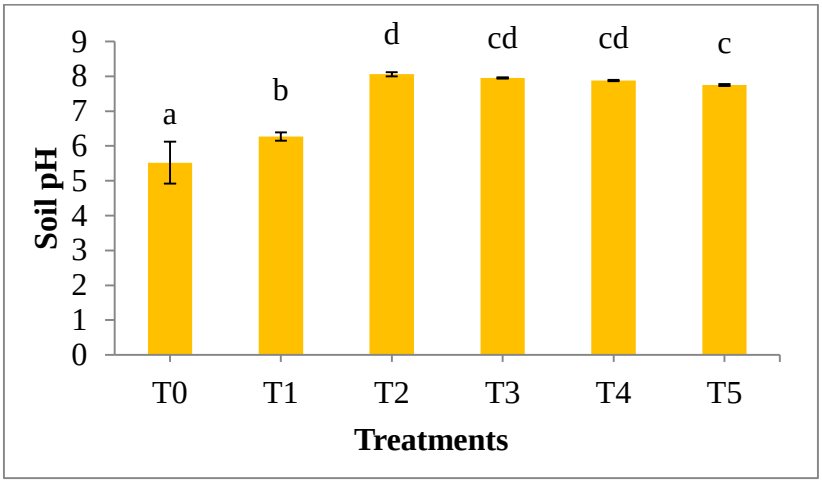

Figure 5- Soil pH after incubation study

Additionally, biochar had successfully sorb nutrients from the soil. T2, T3 and T4 had shown significant NH_4^+ retention in soil over T0, T1, and T5 (Figure 6). T2 and T3 had retained the highest amount of NH_4^+ by 95% and 79% respectively over T1, followed by T4 and T5, which is 54% and 12%. This shows that the biochar had increased the formation of NH_4^+ ions over NH_3. Besides, the nitrate ions in the soil are found to be higher. The T2 and T3 had retained more amount of NO_3^- by 51% and 49%, respectively, compared to T1, followed by T4 and T5 which is 23% and 9% (Figure 7). Biochar retains more charged ions because it has zwitterions properties that bind ions on its surface (Waters et al. 2010). Another reason for the higher retention of NH_4^+ could be associated with the higher CEC of rice husk and rice straw biochar, 66.6 $cmol_c\ kg^{-1}$ and 75.6 $cmol_c\ kg^{-1}$, respectively absorbs the ions and releases it slowly. This was in agreement with Omar et al. (2010). The higher content of NH_4^+ and NO_3^- suggest that the inclusion of combined biochar of rice husk and rice straw had improved the presence of nutrients in the soil for uptake by plants.

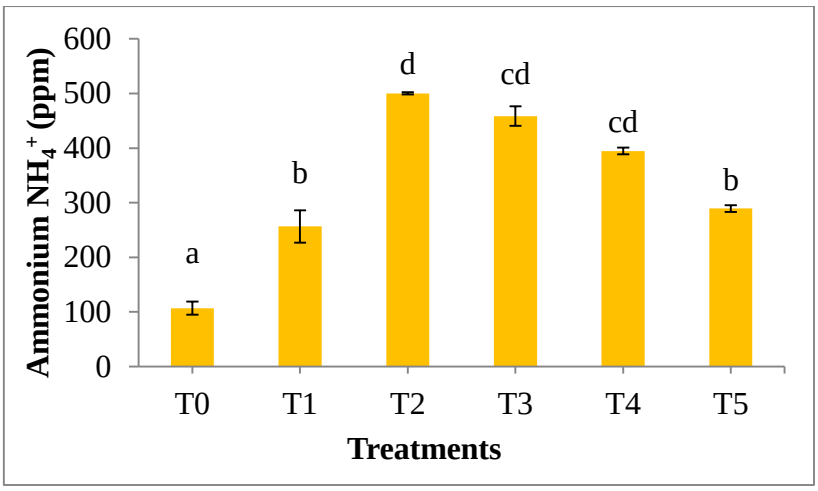

Figure 6- Ammonium (NH_4^+) retention in soil after incubation study.

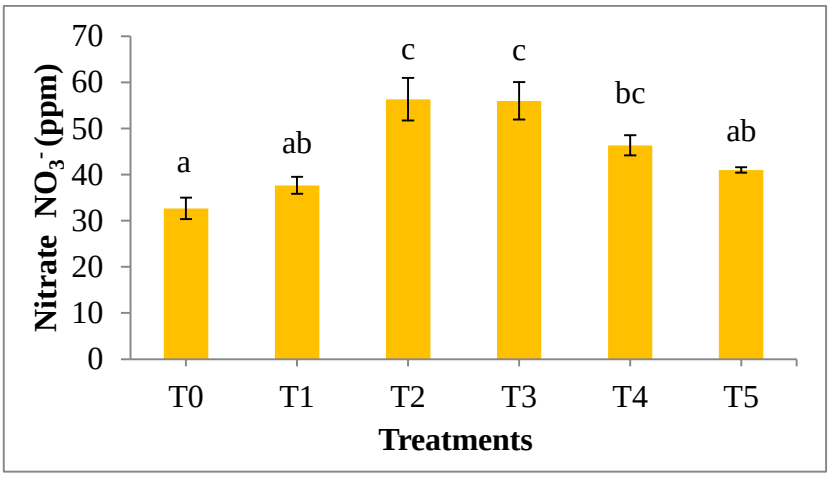

Figure 7- Nitrate (NO_3^-) retention in soil after incubation study.

Note: Mean values with different letter(s) indicate significant difference between treatments by Tukey's test at P≤0.05. Bars represent the mean values ± SE.

4. Conclusions

The result of this study suggests that the application of urea with a mixture of rice straw and rice husk biochars at the rates of 5 t ha^{-1} and 10 t ha^{-1} offers a significant advantage over urea alone. The biochar mixtures have effectively retained more NH_4^+ and NO_3^- ions in the soil by minimizing conversion to NH_3 even at increased soil pH levels. This leads to a significant reduction in NH_3 released into the atmosphere. The addition of a mixture of rice straw and rice husk biochars retained more inorganic N in the soil. Eventually, this will lead to sustainable N management in rice production and prevent greenhouse NH_3 gas emissions.

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Multi-Environment Analysis of Grain Yield and Quality Traits in Oat (*Avena sativa* L.)

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ABSTRACT
Oat is used for food, in animal feeding and non-food products. Twenty-five oat genotypes were evaluated at six different environments to determine high-yielding, good-quality and stable genotypes. Experiments were conducted in randomized blocks design with 4 replications. Grain yield, plant height, test weight, thousand-grain weight, screening percentage, groat percentage, protein, β-glucan and starch contents were evaluated for 25 oat genotypes. Genotype, environment and genotype × environment interaction had extremely important effects on yield and quality of oat grains. The additive main effects and multiplicative interactions analysis disclosed important genotype and environmental effects in addition to genotype by environmental interaction according to grain yield. Using AMMI analysis, three promising oat genotypes (G1, G3 and G7) were defined in comparison to the cultivars and these genotypes had 4.03, 3.77 and 3.70 t ha^{-1}, respectively. AMMI-2 biplot revealed that E6 was the most discriminating environment for grain yield of oat genotypes. Genotype-by-trait (GT) biplot explained 54.9% of total variation. Grain yield were positive associated with all traits except plant height. G1, G3 and G7 genotypes, which showed the best performance and higher stability, also had good quality traits.

Keywords: AMMI, Biplot, β-glucan, Grain yield, Oat, Protein

1. Introduction

Oat (*Avena sativa* L.) is a multi-purpose product used in both human nutrition and animal feeding. It is quite less selective in soils and climate, thus can reliably be grown in infertile soils and cool and humid climates (Sánchez-Martín et al. 2014). World oat cultivation is usually practiced between 35˚ and 65˚ North latitudes. From 1978 to 2018, world oat production decreased from 48.3 million tons to 23.1 million tons (FAO 2018). Despite decreasing production quantities, there is a growing interest in oat production just because of positive impacts on human health. Therefore, oat breeders focus not only on yield and morphological traits, but also on nutritional and industrial quality traits in selection of superior oat genotypes. Oat grains have greater nutritional values than many other unfortified cereals and also quite rich in protein, β-glucan, carbohydrate, fat, vitamins, minerals, some antioxidants and anti-carcinogenic substances, thus have several positive impacts on human health (Michels et al. 2020).

Oat grains to be used for animal feeding and human nutrition may exhibit diverse variation in grain quality parameters. Price is mostly designed by quality parameters. However, recent researches have mostly focused on herbage yield, stress tolerance and disease resistance rather than grain quality. Thousand-grain weight, test weight, screenings percentage, groat percentage, protein content, fat content and β-glucan content are the most important physical and chemical parameters designating the purpose of use of the grains. Besides grain yield, these quality attributes are also significantly influenced by the genotype, environmental conditions and the interaction of these two components (Doehlert et al. 2001; Peterson et al. 2005; Mut et al. 2018; Mohammadnia et al. 2021). Genotype-environment interactions (GEI) reason genotypes to realize differently in diverse environments (Crossa 1990; Blagojević et al. 2017). The aim of the breeders is not only to quantify GEI, but also to find the most suitable growing environment for their genotype (Yan & Tinker 2006). An ideal stable genotype is one that performs for agronomic and quality in an extensive range of environments resulting well achievement regardless of changes in environmental conditions. Zobel et al. (1988) reported that to predict phenotypic responses to environmental changes of studied genotypes, genotype × environment interaction is frequently evaluated by the AMMI (additive main effects and multiplicative interaction) model. This model combines that the analysis of variance (ANOVA) with additive parameters and the principal component analysis (PCA) with multiplicative parameters in only one analysis. The AMMI biplot display together both main and interaction effects for environments and genotypes. In addition, provides a single analysis of the genotype by environment interaction (Gauch & Zobel 1996).

The objectives of the study were (I) to evaluate grain yield and important quality traits of different oat genotypes at six locations, (II) to select stable and high yielding oat genotypes for the grain yield.

2. Material and Methods

2.1. Plant material, location and experimental design

In present study, 23 advanced lines and two registered cultivars were used as the plant material (Table 1). Experiments were conducted during 2015-2016 growing season at 6 different locations (Amasya - 40° 50' N, 35° 27' E, elevation 610 m; Çorum- 39° 54' N, 34° 04' E, elevation 801 m; Tokat - 40° 27' N, 36° 50' E, elevation 365 m; Sinop- 41° 24' N, 34° 50' E, elevation 417 m; Samsun-Central - 41° 21' N, 36° 11' E, elevation 194 m; Samsun-Bafra- 41° 33' N, 35° 52' E, elevation 22 m) in the Central Black Sea Region of Turkey. Location, climate and soil parameters of the experimental sites are provided in Table 2. Climate data were obtained from the Turkish State Meteorogical Services in the period (Anonymous 2016).

Table 1- Code genotype / pedigrees of 25 oat studied in six environments

Code	*Genotype /pedigree*
G1	IA B605 X//Dane/Newdak
G2	Morton/IL99-8803
G3	LA966BIB77/TX96M1398
G4	Horizon 321/LA9819IBI-75-2-B
G5	LA966BIB119-1(FL9595MEO29/TX93M2107)/UPF94H1400-6-1-1
G6	FL03224 F1 (UPF94174-1/FL9605-A6-B4(FLLA89104-U1-G7/GA8702-C13-4-7))/FL03021 F1 (UFRGS 02B6193-9-4/OA 1039-1)
G7	Sesqui/WIX7571-1//ND010426
G8	FL0206FSB-34-S2/FL06033 F1(Horizon 474/IL 3555)
G9	LA02018SBSBS5-B-S1 / MN02124
G10	UFRGS16/UPF16
G11	LA99017SBSBSB-275-B-S2)
G12	TAMO405/LA99016
G13	833-99AB118*2/LA604
G14	UFRGS 028152-1/LA97006GSB-59-2-4-SBS1
G15	FL03001BSB-S7(LA9339/Bw3996 /FL0740 F1(FL0105FSBSBS20-B-S2 (TX97C1168/IA 91462-45-1-6)/IL 2838)
G16	SD030888-19//SD030888-19/ND030349
G17	FL03129-Ab3 /Bw 4899/UPF96146-5-7-2/FL0846 /FL0742 /IL 2838
G18	Stallion//OA1021-1/SD97575-29-115
G19	TAMO386ERB/TX83Ab2923
G20	UPF90H400/UFRGS16
G21	ND000811/ND980671
G22	WIX7535-9/WIX7395-4
G23	LA02029SBSBS48/UFRGS 028153-2
G24	Seydişehir (Check)
G25	Kahraman (Check)

Table 2- Location, soil and climate traits of testing environments

	Environment	*Amasya*	*Çorum*	*Tokat*	*Sinop*	*Samsun1*	*Samsun2*
Location traits	*Code*	*E1*	*E2*	*E3*	*E4*	*E5*	*E6*
	Latitude (N)	40° 50'	39° 54'	40° 27'	41° 24'	41° 21'	41° 33'
	Longitude (E)	35° 27'	34° 04'	36°50'	34° 46'	36° 11'	35° 52'
	Altitude (m)	610	801	365	417	194	22
	Soil type	Sandy loam	clay loam	clay loam	clay loam	clayey	Sandy loam
Soil Traits	Organic matter (%)	0.99	1.87	2.89	3.11	1.45	0.98
	Salinity (%)	0.25	0.05	0.45	0.02	0.35	0.04
	pH	7.82	7.88	8.11	5.98	7.22	8.11
Climate traits	Mean temperature (˚C)	11.7	11.8	7.7	13.1	15.5	14.2
1 October 2015-	Total rainfall (mm)	419.7	529.1	439.2	661.3	800.6	811.6
31 July 2016	Relative humidity (%)	65.6	66.2	74.6	66.5	66.1	82.0

Samsun1: Samsun- Center; Samsun2: Samsun- Bafra

Experiments at all environments were conducted in randomized complete block design with four replications. Sowings were performed between 1 October and 15 November the year 2015. Each genotype was grown in a six row plot of 7.2 m^2 with a 0.20 m row distance and a sowing density of 500 seeds m^{-2}. For all plots in locations were fertilized the 31.3 kg N and 80.0 kg P per ha (as di-ammonium phosphate) with sowing. And then 28.7 kg N ha^{-1} (as ammonium sulphate) was added to complete 60 kg ha^{-1} of N. Finally, from urea fertilizer was applied as 60 kg N per ha at the beginning of stem elongation stage. Herbicides (Tribenuran-metil (DF) 75%) were used for weed control throughout the experiments and plants were grown under rain-fed conditions without irrigation. Samples of all the plots were hand harvested close to the ground by hand using with a sickle in July at all locations. Then these samples were threshed by plot threshing machine.

2.2. Grain yield, plant height, physical and chemical analyses

Following the harvest and threshing, grains of each plot were weighed and resultant values were converted into grain yield (GY) per hectare (ton). The Plant Height (PH) of each genotype was measured from the soil surface to panicle end: the mean measurement of 10 randomly plants were taken for each plot, excluding border plants. Test Weight (TW) was determined in accordance with 55-10 Approved Methods (AACC 2000). Thousand-grain weight (TGW) was determined by weighing 1000 seeds counted with seed counting device (Chopin technologies-Numigral). For screenings percentage (SP), 50 g samples were shaken over 2 mm sieve for 20 seconds and the grains passed through sieve openings were weighed and proportioned to total grain weight. For groat percentage (GP), 20 g grain sample was manually dehulled and dehulled grains were weighed and proportioned to total weight.

Oat grains separated for chemical analyses were freed of any foreign materials, ground in a hammer mill as to pass through 0.5 mm sieve. Ground samples were preserved in a fridge at +4 ºC until the analyses. All determinations were conducted in duplicate. Protein (%, Nx6.25) contents were determined according to AACC International Methods 46-30.01, respectively (AACC 2000). β-glucan, and starch contents of samples were determined with the aid of enzymatic test kits (Megazyme International, Bray, Ireland) according to AACC Approved Methods 32-23.01 and 76-13.01, respectively (AACC 2000). Results were expressed as the mean on dry weight basis.

2.3. Statistical analysis

In variance analysis, each location was considered as an environment and combined analysis was conducted over six environments. To determine the size of the main effects of variation and their interaction on each trait was applied a two-way fixed effect model. Tukey post-hoc test was conducted to indicate the statistically different means. In the study, the tested environments were abbreviated as E1-E6, which were E1: Amasya, E2: Çorum, E3: Tokat, E4: Sinop, E5: Samsun-Center, E6: Samsun-Bafra, respectively. ANOVA analysis for the all traits data of 25 genotypes in 6 environments and AMMI analysis for the grain yield were calculated using SAS (1998).

For grain yield, AMMI model was applied to determine high yielding and stable genotypes, and specific and wide adaptation (Gauch 1993). The AMMI analysis was applied in based on the following model (Crossa 1990) (Equation 1):

$$Yij = \mu + \text{gi} + \text{ej} + \sum_{k=1}^{n} \lambda k \alpha ik \gamma jk + eij \quad \text{(Eq. 1)}$$

Where: *Yij*, is the yield of the *i*th genotype in the *j*th environment; μ, is the general mean; *gi*, is the *i*th genotype mean deviation; *ej*, is the *j*th environment mean deviation; λk, is the square root of the eigen value of the PCA axis *k; αik* and *γjk*, are the principal component scores for PCA axis *k* of the *i*th genotype and the *j*th environment, respectively and *eij* is the residual (Zobel et al. 1988).

As described by Purchase et al. (2000) the AMMI stability values (ASVs) were used to compare the stability of genotypes (Equation 2):

$$ASV = \sqrt{\left[\frac{SS_{IPCA1}}{SS_{IPCA2}} \times (IPCA_1)\right]^2 + (IPCA_2)^2} \quad \text{(Eq. 2)}$$

Where: *SS*, was the sum of squares; IPCA1 and IPCA2, were the genotypic scores in the AMMI model. The higher the IPCA score, either negative or positive, the more particularly adapted a genotype is to specific environments. Lower the AMMI stability values show more stable genotype across environments. The AMMI stability value was the distance from zero in a two-dimensional scatter plot of IPCA1 and IPCA2 scores. Based on the rank (R) of mean grain yield of genotypes (Yi), denoted (RYi) across environments and the rank of AMMI stability value (RASVi), Genotype Selection Index (GSI) was calculated for each genotype According to Farshadfar & Sutka (2003), genotype selection index combines both mean yield and stability in a single criterion (Equation 3). Genotypes with low this value have high average yield and high stability.

$$GSI_i = RASV_i + RY_i \quad \text{(Eq. 3)}$$

Biplot graphs were generated to assess genotypes, environments and GEI, and to put forth the most stable genotypes with the prominent traits in each environment. The Figure 1A introduced by AMMI analysis based on grain yield and the Figure 1B introduced that Biplot analysis of GEI based on AMMI 2 model for the first two interactions principal component scores. Data were also graphically analyzed by the genotype × trait biplot method (Figure 1C) by Yan & Tinker (2006). Significance levels in graphs were identified based on vector angles of biplot graphs. Principle component analysis was composed of two principle components (PC1 and PC2). Total variance explanation levels close to 100% indicate high variance explanation coefficients for these parameters (Yan & Tinker 2006).

3. Results and Discussion

Results of combined variance analysis over 6 environments are provided in Table 3. The effects of genotype, environment and their interactions on grain yield, plant height and all quality traits were highly significant (P<0.01).

Table 3- Combined analysis of variance for investigated traits of 25 oat tested across six environments

Paremeter	*Source (df)*								
	Genotypes (G) (24)		*Environments (E) (5)*		*Rep/ E (18)*	*G × E (120)*		*Error*	*CV*
	MS	*TSS%*	*MS*	*TSS%*	*MS*	*MS*	*TSS%*		
GY	6.82**	16.7	66.83**	51.3	0.57	1.74**	32.0	0.11	10.14
PH	2471.91**	42.4	7046.59**	31.3	83.31	272.27**	26.3	42.65	7.77
TW	70.92**	17.8	693.60**	55.6	17.09	19.12**	26.6	5.78	5.72
TGW	116.99**	18.1	327.33**	17.7	5.13	48.85**	64.2	8.77	8.91
SP	245.90**	25.0	429.92**	10.9	14.94	87.65**	64.1	6.93	3.11
GP	33.32**	14.6	642.67**	79.4	5.12	5.26**	6.0	3.26	2.4
PC	3.11**	4.2	195.30**	88.2	0.24	0.95**	7.6	0.28	4.46
βC	0.45**	10.9	10.52**	80.1	0.05	0.12**	9.0	0.07	6.72
SC	52.87**	10.1	943.85**	65.8	3.51	42.94**	24.1	18.96	5.48

**: Significant at the P<0.01 probability level; MS: Mean squares; TSS%: percent of total sum of squares; CV: Coefficient of variation; df: degree of freedom; GY: Grain yield; PH: Plant height; TW: Test weight; TGW: Thousand-grain weight; SP: Screenings percentage; GP: Groat percentage; PC: Protein content; βC: β-glucan content; SC: Starch content

The ANOVA also indicated that out of the total sum of squares, 16.7, 51.3 and 32.0% for grain yield, 42.4, 31.3 and 26.3% for plant height, 17.8, 55.6 and 26.6% for test weight, 18.1, 17.7 and 64.2% for thousand grain weight, 25.0, 10.9 and 64.1% for screening percentages, 14.6, 79.4 and 6.0% for groat percentage content, 16.8, 56.0 and 27.2% for ash, 4.2, 88.2 and 7.6 % for protein content, 10.9, 80.1 and 9.0% for β-glucan content, 10.1, 65.8 and 24.1% for starch content were attributable to genotype, environment and GEI effects, respectively (Table 3).

Among the agronomic traits, grain yield has the most complex heredity and it is quite hard to achieve genetic progress in this issue. To measure the yield potential of a genotype, it should be experimented in more than one location with different climate and soil characteristics and/or in more than one year. Genotype yields varied between 1.97 (G22) - 4.06 (G7) ton ha^{-1} with an average value of 3.24 ton ha^{-1} (Table 4). The means for grain yield ranged from 2.21 ton ha^{-1}in environment E2 to 4.43 ton ha^{-1} in environment E5 (Table 5). In E5 and E6 environments with high grain yields, total precipitation and average temperature throughout the growing season were greater than the other environments. Such a case indicated that differences in genotypes, environments and genotype x environment interactions were mostly resulted from differences in soil characteristics, precipitation and temperature-like environmental factors (Table 2) and differences between the genotypes. Besides genetic factors, biotic and abiotic stress factors also result in different reactions of the genotypes to different environments. Low or high precipitations, high or low temperatures also influence interactions. Previous studies were reported that genotypes (Peterson et al. 2005; Mut et al. 2018), environmental factors (Doehlert et al. 2001) and agronomic practices (Finnan et al. 2019) were effective on grain yield.

There were significant differences in plant heights indicating a large genetic variation between the genotypes in all environments (Table 3). Plant height varied between 68.20 cm (G19) – 108.91 cm (G24) with an average value of 84.03 cm. Significant differences were also observed in plant heights of the environments (Table 4). The highest plant height was observed in E5 environment (93.31 cm) and the lowest plant height was seen in E2 environment (73.35 cm) (Table 5). Plant heights are largely influenced by water, temperature, nutrients, day light-like environmental factors (Coffman & Frey 1961) and plant genetics (Buerstmayr et al. 2007). In present study, plant heights generally varied based on total precipitations (Table 2). Oat is also a desirable feature, as short plant heights and resistance to lodging attributes facilitate harvest processes (Erbaş Köse et al. 2021).

According to combined variance analysis, test weights of the genotypes varied between 40.99 kg (G24) and 48.84 kg (G5) (Table 4). The lowest test weight was observed in E6 environment (43.28 kg) and the greatest in E3 environment (50.39 kg) (Table 5). Test weights generally vary with the genotypes, environmental conditions and cultural practices (Mut at al. 2018). High temperatures, drought or location-induced environmental stress reduce test weights of the cereals (Mut et al. 2018). In present study, E5 and E6 environments with greater average temperatures (Table 2) had lower test weights. Test weight designates the quality of oat grains, thus designate market price of oat. It is also used to identify quantity of grains damaged by adverse environmental conditions, diseases or cultural practices. Since greater test weights indicate greater groats percentages and less hull ratios, breeders generally try to select the genotypes with high test weights (Doehlert 2002). Because of high hull ratio and grain shape, oat generally has lower test weights than the other cereals and values vary between 40-60 kg. In previous studies, test weights of oat genotypes were reported as between 40.3 to 55.6 kg (Mut et al. 2018).

Table 4- ASV and GSI values for grain yield of 25 oat genotypes grown in different environments and average values of all characteristics of the genotypes studied in these environments

Genotypes	*GY*					*PH*	*TW*	*TGW*	*SP*	*GP*	*PC*	*βC*	*SC*
	Mean (t ha^{-1})	*RGY*	*ASV*	*RA*	*GSI*	(cm)	(kg)	(g)	(%)	(%)	(%)	(%)	(%)
G1	4.03^{ab}	2	0.52	14	16	81.22^{cd}	47.96^{a}	28.70^{i}	85.67^{c-g}	75.42^{b-g}	12.28^{ab}	3.72^{f}	45.46^{a-e}
G2	3.69^{bcd}	6	0.91	19	25	81.52^{cd}	47.16^{ab}	28.89^{i}	87.05^{bcd}	75.58^{a-f}	11.20^{fg}	4.00^{a-f}	44.65^{b-g}
G3	3.77^{abc}	3	0.73	15	18	76.51^{de}	48.36^{a}	36.28^{ab}	89.48^{ab}	75.14^{b-g}	11.74^{b-f}	3.93^{b-f}	46.77^{abc}
G4	3.70^{bcd}	5	0.51	12	17	79.39^{cd}	48.75^{a}	35.92^{abc}	86.09^{cde}	73.62^{ghi}	11.93^{b-e}	4.08^{a-d}	44.43^{c-g}
G5	2.66^{jkl}	21	0.38	7	28	80.59^{cd}	48.84^{a}	31.23^{ghi}	82.93^{gh}	75.07^{b-g}	12.17^{abc}	3.99^{a-f}	46.98^{ab}
G6	3.59^{cde}	8	0.47	9	17	85.78^{bc}	47.35^{ab}	32.22^{e-h}	83.66^{efg}	72.86^{hi}	11.86^{b-e}	3.89^{b-f}	44.15^{d-g}
G7	4.06^{a}	1	0.90	18	19	80.14^{cd}	47.86^{a}	33.01^{c-h}	79.85^{ij}	75.88^{a-e}	12.27^{ab}	4.12^{abc}	43.32^{e-h}
G8	3.22^{f-i}	14	0.51	11	25	91.05^{b}	47.14^{ab}	35.55^{a-d}	84.86^{d-g}	74.81^{b-g}	11.88^{b-e}	3.93^{b-f}	43.46^{e-h}
G9	3.15^{ghi}	15	0.90	17	32	80.44^{cd}	46.75^{ab}	33.12^{c-h}	82.91^{gh}	73.95^{f-i}	12.04^{a-d}	3.85^{c-f}	44.93^{a-f}
G10	3.09^{ghi}	16	0.88	16	32	76.55^{de}	47.69^{ab}	32.65^{d-h}	83.50^{e-g}	75.13^{b-g}	11.49^{d-g}	4.17^{ab}	45.11^{a-f}
G11	3.01^{hij}	19	0.52	13	32	85.92^{bc}	47.38^{ab}	35.21^{a-e}	85.86^{c-f}	76.73^{ab}	12.04^{a-d}	4.26^{a}	45.25^{a-f}
G12	3.29^{e-h}	13	0.94	20	33	71.01^{ef}	48.30^{a}	30.20^{hi}	83.17^{fgh}	72.64^{i}	12.12^{abc}	4.01^{a-e}	43.51^{e-h}
G13	3.05^{hi}	18	0.29	4	22	84.94^{bc}	47.54^{ab}	33.68^{a-g}	85.93^{c-f}	75.63^{a-f}	11.74^{b-f}	4.00^{a-f}	43.72^{e-h}
G14	3.59^{cde}	9	0.42	8	17	85.55^{bc}	48.27^{a}	31.79^{f-i}	89.35^{ab}	74.60^{c-h}	12.18^{abc}	4.11^{abc}	45.20^{a-f}
G15	3.56^{c-f}	10	1.22	22	32	88.55^{b}	48.13^{a}	34.08^{a-g}	85.05^{d-g}	77.26^{a}	11.64^{c-f}	3.82^{def}	46.44^{a-d}
G16	3.71^{abc}	4	1.40	24	28	86.16^{bc}	47.03^{ab}	32.10^{e-h}	84.92^{d-g}	76.36^{abc}	11.50^{d-g}	3.79^{ef}	44.46^{c-g}
G17	2.42^{l}	23	0.50	10	33	80.73^{cd}	46.81^{ab}	35.71^{a-d}	88.23^{bc}	74.38^{d-i}	11.80^{b-e}	4.11^{abc}	42.30^{gh}
G18	3.43^{c-g}	11	0.14	2	13	75.05^{def}	46.98^{ab}	34.13^{a-g}	86.13^{cde}	76.24^{a-d}	11.66^{c-f}	4.01^{a-e}	45.14^{a-f}
G19	3.36^{d-h}	12	0.35	6	18	68.20^{f}	46.96^{ab}	34.40^{a-f}	84.58^{d-g}	74.17^{e-i}	11.90^{b-e}	4.04^{a-e}	43.65^{e-h}
G20	2.88^{ijk}	20	0.11	1	21	76.89^{de}	47.10^{ab}	33.24^{b-h}	86.12^{cde}	76.08^{a-e}	11.84^{b-e}	3.98^{a-f}	47.42^{a}
G21	3.07^{hi}	17	0.33	5	22	89.12^{b}	46.87^{ab}	34.53^{a-f}	80.81^{hi}	75.14^{b-g}	12.53^{a}	4.16^{ab}	42.91^{fgh}
G22	1.97^{m}	25	1.50	25	50	103.14^{a}	43.27^{cd}	30.15^{hi}	77.89^{j}	75.50^{a-g}	10.98^{g}	3.78^{ef}	41.45^{h}
G23	2.59^{kl}	22	1.36	23	45	107.67^{a}	45.14^{bc}	32.92^{c-h}	79.20^{ij}	74.89^{b-g}	11.65^{c-f}	3.78^{ef}	44.77^{b-g}
G24	2.41^{l}	24	0.99	21	45	108.91^{a}	40.99^{d}	34.33^{a-g}	79.72^{ij}	74.42^{d-i}	11.40^{efg}	4.01^{a-e}	44.99^{a-f}
G25	3.69^{bcd}	7	0.14	3	10	75.76^{de}	48.36^{a}	36.72^{a}	91.94^{a}	77.09^{a}	12.23^{abc}	4.00^{a-f}	46.81^{abc}

The values followed by common letters at each column are not significant at P>0.05 level of probability using the Tukeys test. GY: Grain yield; ASV: AMMI stability values; GSI: Genotype selection index; PH: Plant height; TW: Test weight; TGW: Thousand-grain weight; SP: Screenings percentage; GP: Groat percentage; PC: Protein content; βC: β-glucan content; SC: Starch content

Although thousand-grain weight is a cultivar-specific trait, it varies with the years and climate factors. In present study, thousand-grain weights of the genotypes varied between 28.70 (G1) and 36.72 g (G25) with an average value of 33.23 g. The means for thousand-grain weight ranged from 30.75 g in environment E3 to 35.56 g in environment E5 (Table 4). Genotype x environment interactions was found to be significant for thousand-grain weight. It was thought that greater precipitation and temperatures of E4, E5 and E6 environments than of E1, E2 and E3 environments (Table 2) positively influenced oat growth and thousand-grain weight. Peterson (1992) reported greater impacts of environment on thousand-grain weights than on the other quality traits. In previous studies, thousand-grain weights of oat genotypes were reported as between 18.55 - 47.11 g (Buerstmayr et al. 2007; Mut et al. 2018; Mut et al. 2021).

Based on combined results of the environments, screening percentage of the genotypes varied between 77.89 (G22) and 91.94% (G25) with an average value of 84.60% (Table 4). The greatest screening percentage was observed in E6 environment (88.12%) and the lowest value in E3 environment (81.70%) (Table 5). Because of differences in panicle lengths and number of grains per spikelet, oat grains generally have different sizes (Doehlert 2002). Screening percentage is influenced by environmental factors as much as genotypes. Screening percentages are closely related to grain size and the genotypes with greater thousand-grain weights also have greater screening percentages (Kahraman et al. 2017). Grain size generally designates the purpose of use in oat grains. Homogeneous grain size is preferred in flour industry. Hulls should be removed in oat meals. Therefore, larger grains are preferred as to have greater groats percentages. Small grains generally fractured while dehulling process and such a case then reduces milling performance. Thus, oat grains are size-classified in milling industry and very small and weak grains are generally used in animal feeding (Doehlert 2002). Screening percentages of hulled-oat genotypes were reported as between 17.3-95.5% by Kahraman et al. (2017).

Table 5- Average values of the investigated properties of the environments

Env.	GY					PH	TW	TGW	SP	GP	PC	βC	SC
	Mean (t ha^{-1})	RGY	ASV	RA	GSI	(cm)	(kg)	(g)	(%)	(%)	(%)	(%)	(%)
E1	3.16^{c}	3	1.27	2	5	81.59^{c}	46.85^{c}	31.71^{cd}	85.52^{b}	70.62^{d}	12.44^{b}	4.22^{b}	40.33^{e}
E2	2.21^{f}	6	1.10	1	7	73.35^{d}	49.27^{b}	32.77^{c}	84.05^{b}	76.50^{b}	13.84^{a}	3.99^{c}	42.70^{d}
E3	2.94$_{d}$	4	1.33	3	7	75.10^{d}	50.39^{a}	30.75^{d}	81.70^{c}	75.92^{b}	12.31^{b}	3.95^{c}	45.00^{c}
E4	2.75^{e}	5	1.46	4	9	90.19^{b}	47.64$_{c}$	34.25^{b}	84.17^{b}	77.91^{a}	11.96^{c}	3.64^{d}	49.27^{a}
E5	4.43^{a}	1	2.35	6	7	93.31^{a}	45.05^{d}	35.56^{a}	85.00^{b}	75.78^{b}	10.48^{d}	3.63^{d}	46.43^{b}
E6	3.94^{b}	2	1.77	5	7	90.15^{b}	43.28^{e}	34.33^{b}	88.12^{a}	74.10^{c}	10.02^{e}	4.46^{a}	44.42^{c}
Mean	3.24					84.03	47.08	33.23	84.60	75.14	11.84	3.98	44.69

The values followed by common letters at each column are not significant at P>0.05 level of probability. The values followed by common letters at each column are not significant at P>0.05 level of probability using the Tukeys test. GY: Grain yield; ASV: AMMI stability values; GSI: Genotype selection index; PH: Plant height; TW: Test weight; TGW: Thousand-grain weight; SP: Screenings percentage; GP: Groat percentage; PC: Protein content; βC: β-glucan content; SC: Starch content

Oat groats (caryopsis) are covered with the hull at harvest. Such grains should be dehulled to use in food industry. Groat percentage of the genotypes varied between 72.64 (G12) and 77.26% (G15) with a general average of 75.14% (Table 4). The lowest groat percentage was observed in E1 environment (70.62%) and the greatest value in E4 environment (77.91%) (Table 5). Buerstmayr et al. (2007) indicated that environment had greater effects on groat percentage than the genotypes. Doehlert et al. (2001) indicated almost identical effects of genotype and environment on groat percentage. Groat percentage could be increased by >10% by selecting an appropriate growing site. Groat percentage is an important quality indicator of oat grains. Greater hull ratios limit the use of oat grains in animal feeding. Also, greater groat percentage and lower hull ratios are desired for oat grains to be used in human nutrition. Groat percentage of oat genotypes was reported as between 63.7 - 91.7% by Doehlert et al. (2001) and between 70.1 - 73.6% by Mut et al. (2018).

Protein content of oat grains is an important quality parameter. Protein contents of the genotypes varied between 10.98 (G22) and 12.53% (G21) with a general average of 11.84% (Table 4). Peterson et al. (2005) reported protein content of oat genotypes as between 10.0 - 18.0%. Significant differences were reported in protein content of different oat genotypes (Mut et al. 2018). In this study, the means for protein content ranged from 10.02% in environment E6 to 13.84% in environment E2 (Table 5). Acar et al. (1995) and Peterson et al. (2005) indicated that although protein contents were largely depended on genotypes, it was also largely influenced by environment. Mut et al. (2018) reported that protein contents were influenced both by environment and genotypes. Similar to the results obtained in this study, Peltonen-Sainio & Peltonen (1993) reported that the differences in protein content of genotypes could be attributed to the reduction in starch accumulation as a result of the troubles occurring in the grain filling period. According to tested environments, decreasing protein contents were observed (Table 5) with increasing precipitations of the environments (Table 2). Dryer vegetation periods in E1, E2 and E3 environments resulted in reduced grain weight and increased crude protein contents.

The β-glucan is an important naturally dissolving dietary fiber and oat grains are quite rich in β-glucan. β-glucan contents of the genotypes varied between 3.72 (G1) and 4.26% (G11) with a general average of 3.98% (Table 4). For β-glucan contents of the environments, the greatest value was observed in E6 environment (4.46%) and the lowest value in E5 environment (3.63%) (Table 5). It was reported that β-glucan boosted the immune system, reduced blood cholesterol and glucose levels (Peterson et al. 2005). β-glucans are also used in cosmetics, food and pharmaceutical industries. High β-glucan contents are desired in oat grains to be used in human nutrition and animal feeding. It was reported in previous studies that β-glucan contents were influenced by genotypes (Doehlert et al. 2001; Mut et al. 2017) environments and cultural practices (Mut et al. 2018).

Based on combined results of the environments, starch contents of the genotypes varied between 41.45 (G22) and 47.42% (G20) with a general average of 44.69%. The means for starch content ranged from 40.33% in environment E1 to 49.27% in environment E4. Starch is composed of amylose and amylopectin and starch of oat grain is located in endosperm covered with bran layers rich in β-glucan and protein (Punia et al. 2020). Starch is the primary digestible carbohydrate of the plants, thus offers important source energy in human nutrition and animal feeding. In this study, greater starch contents were observed in environments with greater total precipitations (Table 2, 4 and 5). Doehlert et al. (2001) indicated highly significant effects of environments on starch contents. Mut et al. (2018) indicated that starch contents were influenced both by environment and genotypes and reported starch contents of oat genotypes as between 42.7 - 49.6%.

AMMI analysis for the grain yield indicated variation among E, G and G×E showed highly significant different at level P< 0.01, indicating the existence of differential responses of genotypes to different environments (Table 6). In the analysis of variance, the sum of squares for environment main effect accounted for 51.3% of the general sum. The differences between genotypes explained 16.7 % of the total variation, while the effects of GE interaction explained 32.0%.

Table 6- AMMI analysis for grain yield of 25 genotypes evaluated in 6 environments

Source	*DF*	*SS*	*MS*	*F Ratio*	*Explained (%)*
Model	152	711.00	4.67	38.75**	
Environment	5	334.15	66.83	553.57**	51.3
Genotypes	24	163.92	6.82	56.57**	16.7
G × E	120	209.98	1.74	14.49**	32.0
IPCA 1	28	97.75	3.49	28.92**	46.55
IPCA 2	26	38.53	1.52	12.59**	18.82
IPCA 3	24	34.90	1.45	12.05**	16.62
IPCA 4	22	22.47	1.02	8.47**	10.71
IPCA 5	20	15.32	0.77	6.34**	7.29
Pooled error	447	53.96	0.12		
Total	599	794.67			

Results of AMMI analysis showed that the first five interaction principal component axes (IPCA) for grain yield were highly significant (P<0.01). IPCA1, IPCA2, IPCA3, IPCA4 and IPCA5 respectively explained for 46.55%, 18.82%, 16.62%, 10.71%, and 7.29% of the variation formed by interaction (Table 6). The AMMI with IPCA1 and IPCA2 is the best predictive model for to explain interpretable patterns of the yield variation explained by the GEI (Gauch & Zobel 1996). The model was adequate enough to explain the total GEI component (Yan & Tinker 2006). In Figure 1A shows the stability of genotypes and environments, as well as association between genotypes and environments (Gauch and Zobel 1996). G16, G15, G12, G19, G7, G18, G3, G1, G6, G25, G14, G4 and G2 had above mean grain yield in the favourable environments, while G9, G10, G21, G5, G20, G8, G13, G11, G24 and G23 were below the average grain yield in the unfavourable environments. When environments suitability are also classified according their position found in the quadrant, E5 and E6 were favourable environments while E1, E2, E3, E4 and E7 were considered as unfavourable environments for grain yield (Figure 1A). Genotype stability is considered as non-significant response to changing environmental conditions, agricultural practices, climate conditions, multiple stresses. In this study, weather conditions were the source of this variation component.

According to AMMI stability value (ASV), genotypes with lower ASV values are considered more stable than the genotypes with higher ASV (Purchase et al. 2000). According to this model, G18, G20 and G25 were the most stable while G22 were the most unstable. In terms of environments, the smallest ASV values were E2, E1, E3, E4, E6 and E5, respectively (Table 5). The GSI, which incorporates both stability and yield, pointed out G25, G18, G1, G4, G6, G7, G14, G3 and G19 as the best genotypes (Table 4).

Biplot graph (Figure 1B and 1C) offer visual assessment of the relationships among the traits, to define positive or negative relationships among the investigated traits and to define traits to be used in indirect selection of another trait. Biplot graph also presents strong and weak traits of the genotypes (Yan & Tinker 2006). The length of an environmental vector is a forecast of discriminating power of the environment (Yan et al. 2016). In this study, the environments E6, E3 and E1 largely contributed to the G × E interaction. With the longest vectors from the origin, environment E6 was the most discriminating of the genotypes while E4 provided little information about the genotype differences. The genotypes G5, G14, G15 and G18 interacted positively with the E1 and E2 environments (Figure 1B). The genotypes G3, G12, G23, G24 and G25 interacted positively with the E3, E4 and E5 environments, but negatively with the E6. The genotypes G1, G2, G6, G17, G19 and G21 interacted positively with the E6 environment.

According to Figure 1C, multivariate relationships between the environments and investigated traits of 25 oat genotypes were assessed through PCA. And then PC1 and PC2 values were compared to generate biplot graphs (Figure 1C). PC1 explained 38.2% and PC2 explained 16.7% of total variation. These two components constituted more than half of total variation (54.9%). In biplot graph, vector angles of less than 90˚ indicate genotype performance of higher than the average, vector angles greater than 90˚ indicate genotype performance of lower than the average and finally vector angles equal or close to 90˚ indicate genotype performance of close to average (Yan & Tinker 2006). For 25 oat genotypes, grain yield had positive correlations with all characteristics except plant height (<90˚). The G25 cultivar had greater than the general average in terms of all traits except plant height. The G22, G23 and G24 genotypes had the greatest plant heights. The genotypes positioned closer to the center were prominent for more than one trait and generally averages were lower than the values of genotypes prominent for a single trait (Figure 1C). Similar with the present findings, Yan et al. (2016) reported positive relationships between grain yield and β-glucan content while they reported negative correlations between β-glucan and groat percentage. Buerstmayr et al. (2007) showed that the positive association of grain yield with thousand kernel weight and screenings percentage.

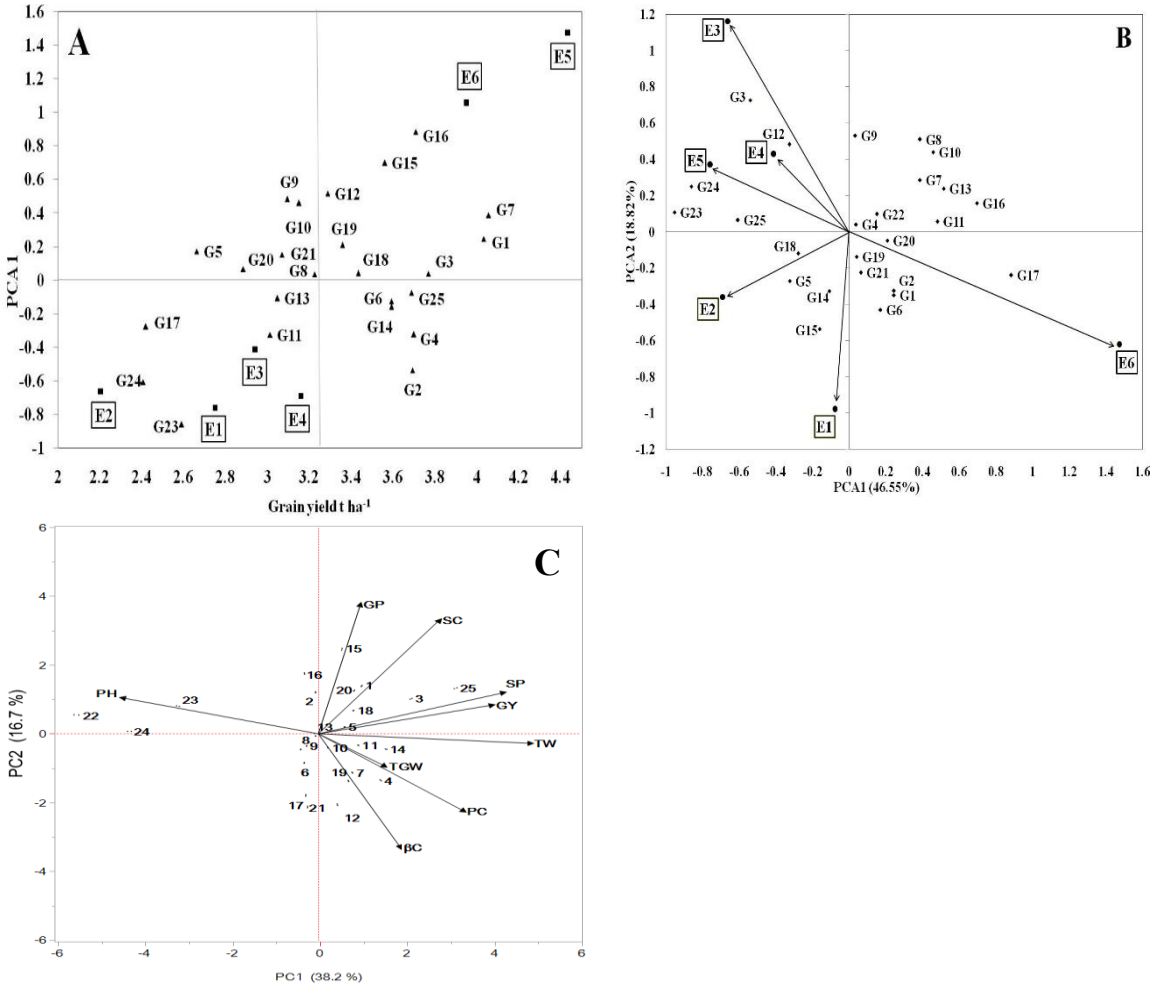

Figure 1- The AMMI model based on grain yield of genotypes (G) in 6 environments (E) (A), Biplot analysis of GEI based on AMMI 2 model for the first two interactions principal component scores (B), Genotype × Trait biplot for investigated traits of oat genotypes (C)

4. Conclusions

In this study, grain yield and quality traits were significantly influenced by genotype, environment and their interactions. Based on genotype selection index (GSI), G25, G18, G1, G4, G6, G7, G14, G3 and G19 were considered as most stable genotypes. The G1, G3 and G7 numbered advanced lines among these genotypes had higher yields than standard cultivars (G24 and G25). In addition, these genotypes showed above average values for most of the quality traits. These advanced lines could be advised for wider growing environments. In addition, these genotypes as parents can be used in oat breeding programs.

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Competition-Productivity Relationship Between Some Common Grasses and Forbs Plant Species in High Altitude Rangelands

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ABSTRACT
Facilitation or competition occurs among plant species when one species alter ameliorates the environment for another species or when one species needs the same sources of another species. The objective of this study was to determine to facilitative or competitive effect on *Bromus variegatus, Festuca ovina, Koeleria cristata* of growing with isolated, *Medicago papillosa, Astragalus microcephalus, Thymus parviflorus,* and *Hypericum scabrum,* and arranged in completely randomized design. Plant height, above-and belowground biomass, and above-and belowground Relative Neighbour Effect (RNE) significantly affected main, some first- and second-order interaction depending on the year, grasses species, surrounding. Aboveground biomass increased in grasses, which grow in interaction with legume species, and significant differences were determined between the grass species and years. While average belowground biomass was 26,66 g/plant, the belowground biomass of the grass species grown in interaction with *M. papillosa* and *A. microcephalus* was positively affected and it was 31,58 and 34,99 g/plant, respectively. *A. microcephalus* had a facilitative effect on above ground RNE of the grass species and the other species had a competitive effect. All plant species had a competitive effect on belowground RNE.

Keywords: Facilitation, Competition, Plant-plant interaction, Plant environment

1. Introduction

Rangelands are an important component of farming systems in the Eastern Anatolia region of Turkey for centuries. The majority of studies on rangelands are focused on grazing effect, botanical composition, carrying capacity, and rangeland condition. The species relationships in these areas, consist of many different species, did not mostly take into account. Plant species can decrease or increase abundance on the rangelands depend on grazing, climatic change, and plant-plant interactions. Biotic interactions play an important role in the botanical composition of rangelands, influencing the abundance of species. The botanical composition consists of abundant and sparse species as a result of combinations of biotic interactions (Brooker 2006; Erkovan et al. 2008a; Grant et al. 2014). Biotic interactions among species are described by positive (facilitation), negative (competition), and neutral effect (Oksanen et al. 2006; Lutscher & Iljon 2013). Consequently, vegetation structure and composition alter depending on changing these relationships (Tilman 1987).

Rangelands have a lively and dynamic structure consisting of many species. The details of these interactions are still not clear, particularly concerning the effects of the forbs, shrubs, and woods on grass growth and species composition. Plant facilitates other plants especially N_2 fixing plants both directly and actively by ameliorating harsh environmental conditions, by altering soil properties, or by increasing availability of resources (Kurokawa et al. 2010), and also some plant species could be altered in their surrounding microsites of soil environment by inhibiting nitrification process (Ehlers et al. 2014). Plant species improve the micro climatic condition, organic matter content, microorganism, and water availability in the neighbour plant species-environment (Forey et al. 2010; Castanho et al. 2015). As a result of these processes, plant competition intensity modified and altered the community structure. Relative frequency of facilitation and competition will inversely proportional gradients of ecosystem productivity (Bertness & Callaway 1994).

Plant-plant interaction and neighbour or target plants are explained by the stress gradient hypothesis (SGH). The balance of facilitation, competition, and neutral effect may be influenced to plant density, physiology, life stage, and invasion properties of species (Grant et al. 2014). Among plant species have reciprocal competition and have contrasting growth forms. Because of different root niches, plant species are assumed to be able to valuable water and mineral nutrition from lower soil layers than grasses (Cramer et al. 2010; Koc et al. 2013a). Negative effects include suppression of seed germination, seedling growth, and survival, but these effects change the life stages of plant species (Callaway & Walker 1997). For example, adult plant species facilitate seedling but compete with other adult plant species as a result of these effects, target plants have a facilitative,

competitive, or neutral effect on growth and yields (Castanho et al. 2015). Environmental and physical conditions restrict resource acquisition and the effect of the neighbour on the target plant becomes more severe, and competitive (Callaway 2007; Koc et al. 2008). Competition increase monotonically with increasing environmental severity. These complex interactions among plant species determine whether positive or negative and composition changes depending on the effect of direction. This complex effect contributes to the development of vegetation by affecting the existence, invasive nature, and production of species in vegetation.

Understanding the role of grasses and forb interactions in the alpine zone is important for botanical composition and rangeland management strategies. Therefore, we tested the effect of dominant plant species that two legumes forb and two the other families species forb interaction dominate grasses species in the alpine rangelands.

2. Material and Methods

The experiment was carried out on the Palandoken Mountain rangelands, where has been protected from grazing for afforestation by the Erzurum Sky Centre Office for more than 30 years, during 2013 and 2014. It has a 10% slope, north aspect, and elevation of 2200 m. The mean annual temperature and mean annual precipitation were 5.1 °C and 391.6 mm, respectively. The average temperature was 5.3 and 7.7 °C and total annual precipitation were 284.2 and 419.5 mm in 2013 and 2014, respectively (Table 1). The soil characteristics are taken around isolated, *M. papillosa*, *A. microcephalus*, *T. parviflorus*, and *H. scabrum* were determined to soil texture, sandy, clay, loam, electrical conductivity (EC), pH in soil saturation extract, Olsen phosphorus (P) contents, organic matter (OM), $CaCO_3$, and aggregate stability (AS) as described by Soil Survey Laboratory Staff (1992) and presented in Table 2. The soil properties had differed greatly depending on isolated, *M. papillosa*, *A. microcephalus*, *T. parviflorus*, and *H. scabrum*.

Table 1- Some meteorological data of the Erzurum province during in the experiment years 2013, 2014 and long term average (LTA)

Month	*Monthly Total Precipitation (mm)*			*Monthly Air Temperature (°C)*		
	2013	*2014*	*LTA*	*2013*	*2014*	*LTA*
January	28.7	11.3	16.5	-9.5	-10.1	-10.6
February	28.5	8.0	20.3	-7.4	-4.5	-9.1
March	30.9	47.1	35.5	-0.8	2.7	-2.5
April	36.3	34.0	58.1	7.2	7.7	5.4
May	36.3	115.9	67.5	11.6	11.7	10.5
June	32.3	24.5	40.9	15.0	15.9	14.9
July	25.1	44.7	25.3	19.4	21.2	19.2
August	7.8	4.8	14.1	19.5	22.2	19.4
September	13.6	47.7	21.0	13.6	15.4	13.9
October	16.8	51.6	43.3	6.0	8.8	7.7
November	19.6	17.2	27.4	2.3	1.3	0.0
December	8.3	12.7	21.8	-13.4	-0.2	-7.2
Total/Av.	284.2	419.5	391.6	5.3	7.7	5.1

Table 2- General physical and chemical properties of the experimental soils

Plants	*Texture*	*Sandy (%)*	*Clay (%)*	*Loam (%)*	*EC (µmhos/cm)*	*pH*	*P_2O_5 (kg/da)*	*OM (%)*	*$CaCO_3$ (%)*	*AS (%)*
Isolated	Loamy	42.25	35.61	22.15	225.82	6.20	5.96	5.77	0.39	79.61
M. papillosa	Loamy	44.14	36.09	19.78	257.48	6.14	12.73	6.61	0.38	81.70
A. microcephalus	Loamy	41.52	37.42	21.08	269.00	6.22	10.96	7.69	0.35	83.00
T. parviflorus	Loamy	45.26	34.72	20.03	241.75	6.14	11.28	7.03	0.44	83.38
H. scabrum	Loamy	43.59	36.05	20.38	226.07	6.13	6.72	5.85	0.40	84.32
Average		43.35	35.98	20.68	244.02	6.17	9.47	6.59	0.39	82.40

EC: Electrical conductivity, OM: organic matter, AS: aggregate stability

The area covered by shortgrass steppe vegetation, of which common plant species with more than 1% in the botanical composition are *Bromus variegatus*, *Festuca ovina*, *Koeleria cristata*, *Phleum montanum*, for Poaceae; *Astragalus microcephalus*, *Astragalus lineatus*, *Medicago papillosa* for legumes; *Alyssum desertorum*, *Dianthus multicaulis*, *Galium verum*, *Hypericum scabrum*, *Plantago lanceolata*, *Thymus parviflorus* and *Ziziphora persica* for the other families. At the beginning of the growing seasons, three wide-spread types of grass species (*F. ovina*, *B. variegatus*, and *K. cristata*) were chosen and marked to investigate their relation with *M. papillosa*, *A. microcephalus*, *T. parviflorus*, and *H. scabrum* which are common forbs in the rangelands. Ten grass and forb for every species were matched grown in 10 cm periphery. There was no other plant closer than 0.5 m to avoid inter-and intra-specific interaction. If there were more plants belong to investigated species in 0.5 m peripheries were removed by clipping the ground. To understand the intra-specific interaction between investigated species, ten sole grown plants for each grass species were selected. While selecting grasses plants are considered

as possible as similar age and canopy size. Consequently, the experiment was arranged completely randomized design with 10 replications.

When investigated grasses reached full flowering stage (beginning of July), firstly plant height was measured, thereafter, the plants were excavated using trench method, with the peripheral trench being 25 cm in width and 30 cm in depth where 90% of the root mass was produced (Manning et al. 1989; Gokkus & Koc 1996). The soil on the roots was separated by hand, thereafter, transported laboratory with paper bag, plant roots were separated from soil by washing off under tap water. Thereafter, plants were separated into above- and belowground parts by cutting at crown level. The samples were dried in the oven at 65 °C to constant weight and weighed. Relative neighbour effect (RNE) was determined from the dry mass both above- and belowground biomass following equations by using (Oksanen et al. 2006).

RNE= (Wr-Wc)/max(Wr,Wc)

Where; RNE, is the relative neighbour effect ($-1 \leq RNE \leq +1$), Wr is the performance of manipulated plants; Wc, is the performance of isolated. This index compares the above- and belowground biomass of *F. ovina, B. variegatus,* and *K. cristata* with that grown as isolated and in the *M. papillosa, A. microcephalus, T. parviflorus,* and *H. scabrum*. Negative and positive values indicate respectively competition and facilitation by neighbours.

The same procedure was also repeated in the second experimental years. We performed the analysis of variance considering based on General Linear Model (GLM) for a completely randomized design using StatView package (SAS Institute 1998). Multiple comparisons with Bonferroni/Dunn were used to determine the effect of grasses species, year and growing isolated and interaction within under *M. papillosa, A. microcephalus, T. parviflorus,* and *H. scabrum* plant species on the above- and belowground biomass and RNE.

3. Results

Under natural conditions, the plant height of grasses species changed significantly depending on years, grasses, and surroundings. It was higher in 2013 than in 2014 (Table 3 Among the grasses species, plant height varied from 33.35 cm to 52.30 cm and B. *variegatus* had the highest plant height (Table 3). The grasses species growing with A. *microcephalus* had higher plant height than the others (P<0.001). Except for *B. variegatus*, plant height of *F. ovina* and *K. cristata* varied significantly according to years, and both growing surrounding. For example, the height of *F. ovina* growing with *H. scabrum* was higher in 2013, but it was the lowest in 2014. While plant height of *K. cristata* growing with *H. scabrum* was the lowest in 2013, the highest in 2014. Hence, Y x S and Y x G x S interactions were significant for the plant height (Figure 1).

Table 3- Plant height, above- and belowground biomass, above- and belowground RNE the means of grasses species growing with isolated and forb plant species and their ANOVA results

Treatments	*Plant Height*	*Aboveground Biomass*	*Belowground Biomass*	*Aboveground RNE*	*Belowground RNE*
Years (Y)					
2013	45.07 A	9.91 A	34.51 A	0.006 A	0.089 A
2014	36.96 B	3.67 B	19.37 B	-0.207 B	-0.320 B
Grasses (G)					
F. ovina	33.35 C	7.76 B	29.02 B	-0.006 A	-0.166
B. variegatus	52.30 A	11.91 A	47.58 A	-0.223 B	-0.040
K. cristata	37.40 B	0.84 C	3.39 C	-0.073 A	-0.140
Surrounding (S)					
Isolated	41.18 BC	6.63 AB	24.02 B	-	-
M. papillosa	41.38 B	7.38 A	31.58 AB	-0.102 AB	-0.050a
A. microcephalus	45.25 A	8.31 A	34.99 A	0.023 A	-0.030 a
T. parviflorus	39.58 BC	5.51 B	21.34 C	-0.191 B	-0.150 ab
H. scabrum	37.68 C	6.34 AB	21.38 C	-0.135 B	-0.230 b
Average	41.02	6.83	26.66	-0.180	-0.120
Y	**	**	**	**	**
G	**	**	**	**	ns
S	**	**	**	**	*
Y x G	ns	**	**	**	**
Y x S	*	**	**	ns	ns
G x S	ns	**	**	**	**
Y x G x S	*	*	*	*	*

Values followed by small and capital in a column shows significantly differences at P< 0.05 and P< 0.01 levels, respectively

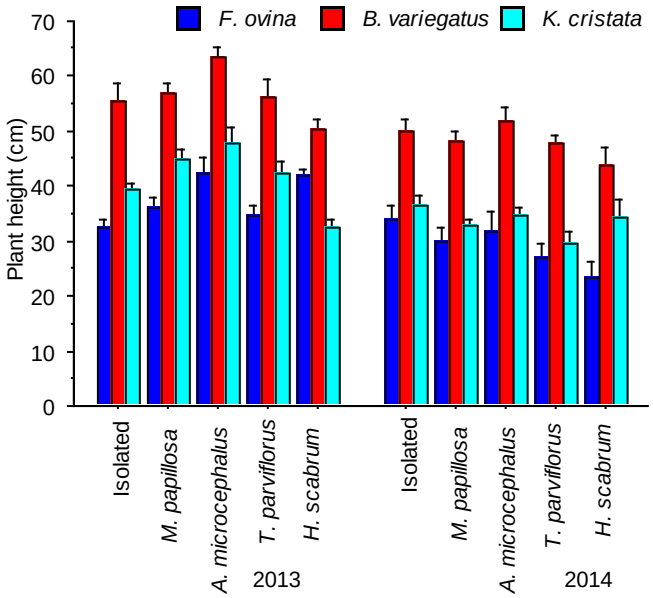

Figure 1- Growing with isolated and forb plant species first and second order interaction on plant height of grasses species

In the experiment, the main effect of year, grasses species (*F. ovina*, *B. variegatus*, and *K. cristata*), surrounding (isolated, *M. papillosa*, *A. microcephalus*, *T. parviflorus*, and *H. scabrum*), and all first second-order interaction was significant for above- and belowground biomass (Table 3). Above- and belowground biomass of grasses species decreased in the 2014 year compared to 2013 (Table 3). Above- and belowground biomass of *F. ovina*, *B. variegatus*, and *K. cristata* were above- 7.76, 11.91, 0.84 g/plant, and belowground 29.02, 47.58, and 3.39 g/plant, respectively (Table 3). The average aboveground biomass of grasses species was 6.83 g/plant, and growing with isolated, *M. papillosa*, *A. microcephalus*, *T. parviflorus*, and *H. scabrum* were 6.63, 7.38, 8.31, 5.51, and 6.34 g/plant, respectively (Table 3). Belowground biomass was higher for growing with *A. microcephalus* than the other but there was no difference among *M. papillosa* (Table 3). Both aboveground and belowground biomass showed strong variation among years, grasses species, and growing surrounding. As a result of this variation, all the main effects as well as the first and second-order interaction were significant (Figures 2 and 3).

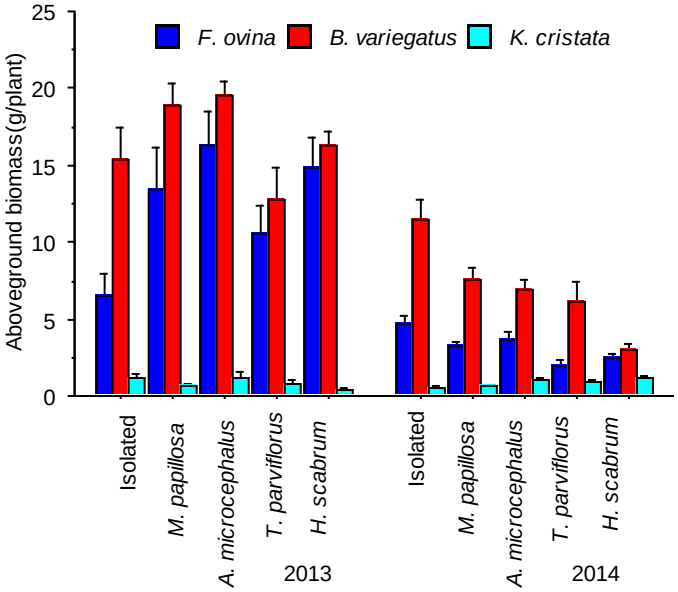

Figure 2- Growing with isolated and forb plant species first and second order interaction on aboveground biomass of grasses species

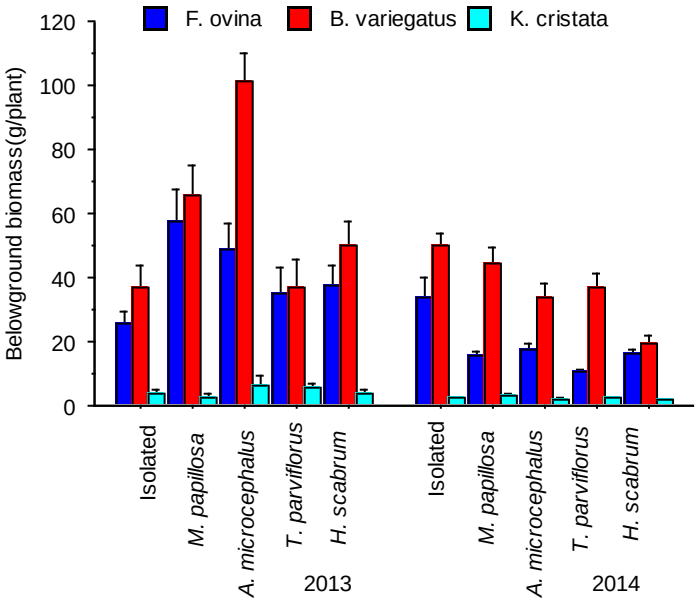

Figure 3- Growing with isolated and forb plant species first and second order interaction on belowground biomass of grasses species

Grasses species were significantly facilitated by neighbour plants in 2013, but this effect turned to competition in 2014 on both above- and belowground RNE (Table 3). Grasses species had negative above- and belowground RNE values and aboveground RNE was significant but belowground RNE was not significant (Table 3). RNE on aboveground growth of grasses species was significantly competed by neighbour plants, the highest negative RNE value was recorded in *B. variegatus*. RNE on belowground growth of grasses species was not significantly affected by neighbour plants, but all grasses species had negative RNE values. Grasses species growing with isolated, *M. papillosa*, *A. microcephalus*, *T. parviflorus*, and *H. scabrum* significantly affected both above- and belowground RNE. Grasses species were facilitated on aboveground RNE by *A. microcephalus* and competed by the others but belowground RNE competed for all of them (Table 3). Except for Y x S interaction, the other first and second-order interactions were significant for above- and belowground RNE. Both above- and belowground RNE varied greatly according to the years, grasses species, and surrounding (Figures 4 and 5). For example, *B. variegatus* aboveground RNE was facilitated by *M. papillosa*, *A. microcephalus*, and *H. scabrum* but competed by *T. parviflorus* in 2013, thereafter, it competed for all of in 2014 and a similar result was also observed belowground RNE (Figures 4 and 5). Hence, first and second-order interactions were significant for above- and belowground RNE, except for Y x S interaction.

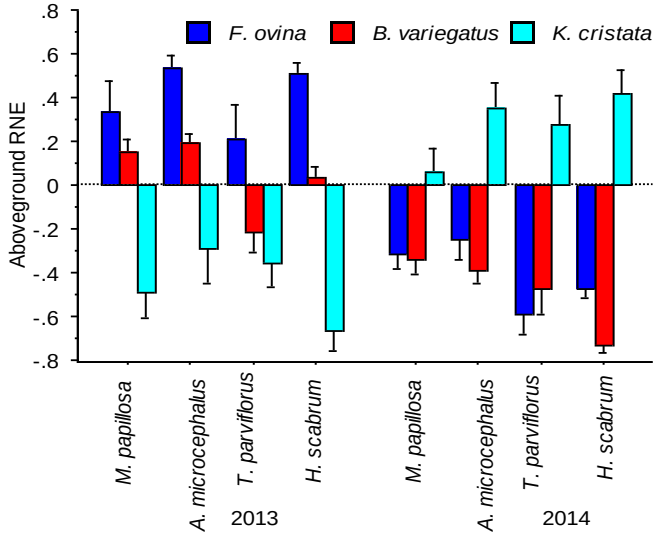

Figure 4- Growing with *M. papillosa, A. microcephalus, T. parviflorus* and *H. scabrum* first and second order interaction on aboveground RNE of grasses species

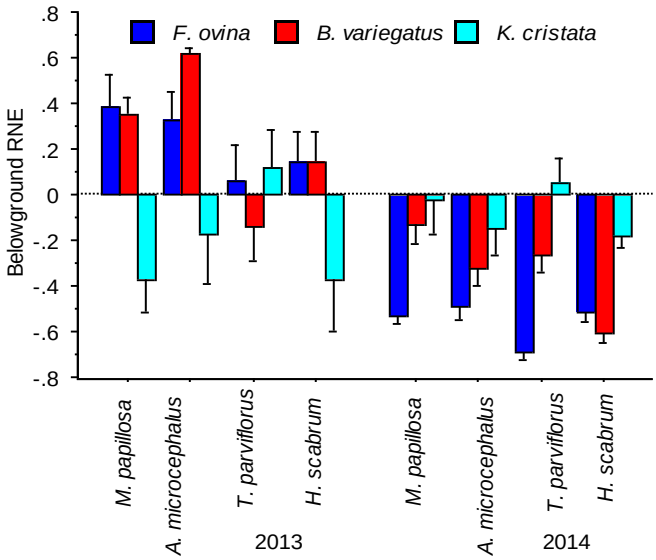

Figure 5 - Growing with *M. papillosa, A. microcephalus, T. parviflorus* and *H. scabrum* first and second order interaction on belowground RNE of grasses species

4. Discussion

Plant height, above- and belowground biomass of the grasses species was affected significantly by years, grasses species, and surrounding (isolated or interaction within under *M. papillosa, A. microcephalus, T. parviflorus,* and *H. scabrum* plant species). In the main effects, the differences among grass species will not be discussed ongoing text because the main reason for these differences stemmed from their genetic differences. Cool-season grasses, which need vernalization for reproduction showed a good response preceding autumn precipitation (Koc 2001). Preceding autumn of the first year received plenty of precipitation (41,7 mm only in October; non-presented data) and this precipitation support autumn regrowth of grasses. On the other hand, late autumn and winter precipitation accumulated in the soil in the cool climate, and it support plant growth during the following growing season. Hence, grasses produced more biomass in the first experimental year, which dried than in the second year. Since the first experimental year autumn received limited precipitation and it did not support the regrowth of grasses, therefore, grasses produced less biomass in the second year. Plant height results also support the above-mentioned interpretation.

Plant species can be affected by grasses in a facilitative or competitive way depending on the associated plant. The facilitative effect occurs especially in dry environments rather than humid or wet environments and it is more pronounced if associated plants have the nitrogen-fixing ability (Eldridge et al. 2011; Blaser et al. 2013; Sitters et al. 2013). Above- and belowground production results showed that *A. microcephalus* and *M. papillosa* have a facilitative effect on the neighbour grasses. This situation must be originated from these two neighbours of the grasses being nitrogen-fixing ability because the plant has the ability of nitrogen fixation provide a facilitative effect on neighbour plants under favourable condition (Table 2) (Erkovan et al. 2008b; Eldridge et al. 2011; Blaser et al. 2013; Sitters et al. 2013). The relation was neutral with *H. perforatum* but competitive with *T. parviflorus* with respect to above- and belowground production. Competitive effects occurred in *T. parviflorus* and neighbour grasses must probably be originated form it's allelopathic effect because *T. parviflorus* have a strongly allelopathic effect, especially, it causes nitrogen deficiency due to decreasing microbial activity of nitrogen released microorganism (Abdul-Rahman & Habib 1989; Small et al. 1990).

The grasses did not show the same relation trend with neighbour plants in both years. The main reason for these differences should be originated from the discrepancy in climate pattern between the years because preceding autumn and winter received plenty of precipitation in the first year which supports the growth of the plants at the beginning of the growing season while received lower precipitation in the same period in the second years. In the experiment, the neighbour plants are broadleaved and have taproot systems. This root system used efficiently the moisture stored in the deep soil layer which is carried upper layer by capillarity than fibrous root system (Kiaer et al. 2013; Liu et al. 2013), consequently, since ceased moisture transport from the deeper layer to the upper layer, the grasses grown with broadleaved neighbour plants must have lived serious moisture stress than grown without broadleaved neighbour (Ludwig et al. 2003; Li et al. 2006; Herben et al. 2007; Wang et al. 2014). Thus, the grasses have grown together broadleaved neighbours produced less above- and belowground biomass than isolated grasses. Hence, the interaction related to years was significant in the experiment. Although it was not clear as in above- and belowground production, a similar shift was observed also plant height (Figure 1).

Environmental factors, especially climate, significantly affect plant-environment relations (Koc et al. 2013b; Grant et al. 2014). In the first year, RNE was about neutral for aboveground growth while it was slightly positive for belowground growth. Whereas it was negative for both properties in the second year. This incompatible situation must be originated the differences in the soil water regime of the beginning of the growing season between the years. Because the precipitation preceding period of the first year's growing season provides sufficient water storing in the root zone, it was not limited due to insufficient precipitation preceding the second year's beginning of the growing season. Broadleaved plants used available water from deeper soil layer before reaching grasses root zone by capillarity (Ludwig et al. 2003; Torres & Montana 2015; Soliveres et al. 2015), hence, grasses lost competitive advantage due to water stress and consequently, RNE for both above- and belowground growth were negative in the second year (Table 3).

Aboveground competition performance was more severe for *B. variegatus* than the other. The main reason for this situation must be originated from water deficiency at the beginning of the growing season in the second year because the plant showed partly positive competition performance in the first year (Figure 4). Because this plant has large habitus and need more water during the life cycle than the others and competitive effect of the plants alters with changing environmental condition. Water shortage at the beginning of the growing season in the second year causes the loss of the competitive advantage of *B. variegatus*. This result implied that *B. variegatus* can be eliminated from natural vegetation in the future if moisture deficiency stress increases due to global warming. This opposite response of *B. variegatus* with respect to aboveground competition to years also causes interactions including years. Another reason is this interaction was related to *K. cristata*'s response to years. This plant has small habitus and maturates earlier (Arnow 1994), therefore, avoids drought easily. Under this condition, the plant loses competition advantage under a longer growing season, whereas, this growing trait provides a competitive advantage under a short growing season. For this reason, *K. cristata* showed a different competitive performance than the other two grass species. Consequently, these different responses of the grasses to both year and neighbour plants caused two and three-way interactions for aboveground growth.

The belowground competition was more vigorous than the aboveground (Wang et al. 2014). Thus, belowground competition, except for *B. variegatus*, the grasses more negatively than that of aboveground (Table 3). These effects were more pronounced in the second year due to the dry beginning of the growing season but the grasses were affected differently by neighbour plants and years. This situation was the main reason of two and three was interactions and were originated mainly changing of responses of the plants to changing environmental condition such as soil, water, neighbour plant, etc. (Garcia-Cervigon et al. 2013).

Competitive effect of neighbour broadleaves plants on the grasses changes among species. *A. microcephalus*, which is a member of Fabaceae, had an overall positive effect on the aboveground growth of grasses. This relation was positive for *F. ovina* and *B. variegatus* and was negative for *K. cristata* in the first year and these relations reversed back in the second year (Figure 4). *A. microcephalus* provide nitrogen to growing media, therefore the plant lived together with them can use this nitrogen, hence, aboveground growth of *F. ovina* and *B. variegatus* were affected positively in the first year since the plants absorbed enough water during the growing period. Whereas this two grasses species showed negative competitive performance concerning aboveground growth in the second year. This situation must be originated from water deficiency during the advanced period of the growing season because microbial nitrogen availability increases with the advanced season due to warming soil temperature (Erkovan 2007; Novoplansky & Goldberg 2001; Anthelme & Michalet, 2009), whereas, since water limitation restricts to plant growth, plants do not use available nitrogen because plants uptake nutrients by using water. As plants complete life cycle without stress under current condition, the plants complete their life cycle later suppress the plant which is complete life cycle earlier (Fabbro & Prati 2015). Thus, *K. cristata*, which complete the growth cycle earlier, was affected negatively by other plants' competition performance with respect to aboveground growth. Whereas, the other plants encountered water stress during the growing season in the second year than did not showed normal growth performance but *K. cristata* complete the life cycle before the drought period. Consequently, *K. critata* showed positive competitive performance under dry conditions while it showed negative competitive performance under normal conditions. These results implied that increase in botanical composition in the future if precipitation patterns do not change positively under global warming conditions. Similar trends also occurred in the belowground competition performance of the plants (Figure 4).

M. papillosa also a member of Fabaceae but its positive effect on neighbour grasses was not clear as was *A. microcephalus* (Table 3, Figures 3 and 4) and its effect was generally negative due to its allelopathic properties (Abdul-Rahman & Habib 1989; Small et al. 1990).

In conclusion, the dominant species of rangeland vegetation are species included in the Poaceae family. However, depending on the climate, environment, and usage conditions, a significant increase can be observed in Fabaceae or other families, and even these species may become dominant. The interactions between the species of Poaceae, Fabaceae, and other families that make up the rangeland vegetation affect the species composition and richness. For example, species included in the Fabaceae family can positively affect other plant species with nitrogen fixation, taproot, and root excretions. Another positive relationship between plants is stress tolerance. These biotic relationships between plants change according to climate, environment, and usage characteristics and this situation can change the structure and functions of pasture vegetation. There is a continuous interaction between the plant species, and this interaction can affect the plant's neighbour positively, negatively,

or neutral. The relationship between plant species in the rangeland vegetation will undoubtedly provide important benefits in terms of fulfilling the functions of rangelands. Grasses species, their different reactions over the years, and interaction with other plant environments have been the main source of first and second-order interaction. Also, similar results have been recorded in other studies (Cavieres & Sierra-Almeida 2012; Blaser et al. 2013; Ehlers et al. 2014; Grant et al. 2014).

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Determination of Morphological, Pomological and Molecular Variations among Apples in Niğde, Turkey using iPBS Primers

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ABSTRACT
In addition to morphological and pomological techniques, the molecular analysis produces more information for diversity studies. Recently, the iPBS marker system is one of the techniques and a new marker system for apple studies. In this study, morphological, pomological, and molecular characteristics of local apples were investigated in 48 different samples from 29 different rural areas with varying altitudes between 1125-1726 m in Niğde, Turkey. Fruit size, fruit weight, the color of fruit peel, total soluble solids content, fruit flesh firmness characteristics are important in terms of yield, quality, storage, transportation and attractiveness. According to the pomological results from these traits, CKR2, DMR3, CLL, HCB2, YSL, ULG, ELM1, ICM have been found to superior among genotypes. In order to molecular results, the similarity of the samples varies between 0.61-1.00, under the light of this result, molecular data differentiated all individuals used in the study except one pair. Molecular data displayed that these differences were caused by genotypic differences as well as environmental conditions. This study has contributed further information about the usage of iPBS primers on apple. To protect the plant material used in the study, a collection orchard was established with genotypes. To conclude, the findings are expected to shape future breeding studies.

Keywords: *Malus domestica*, Genetic resource, Genetic diversity, Molecular analysis, Polymorphism

1. Introduction

Apple (*Malus domestica* Borhk.), which is cultivated all over the world, has great diversity and the number of known cultivars are more than 6500 and, particularly 600 cultivars in Turkey (Hancock 2012). Central Asia, Caucasus, and Turkey were the center of origin for apples. Kayseri and Ağrı provinces are two major diversity points in Turkey (Ozbek 1978). According to FAO 2020 stats, Turkey has an important position in world apple production, ranked fourth amongst China, United States, and Poland. According to TUIK 2020 stats, Niğde is one of the three major provinces in Turkey's apple production and 81.0% of Niğde's apple production is covered by Central and Bor districts. Niğde has important advantages for apple production as follows: the flat land structure of the province is suitable for establishing orchards, land prices are cheaper than other provinces producing apples in Turkey, fewer diseases have been seen such as black spots by low humidity and better-colored fruits by the temperature difference between day/night (Anonymous 2014).

The key to high yields is application of modern agricultural techniques (Demir & Doğan 2020). Although new and modern apple orchards have started to establish in Niğde recently, a lot of established orchards that are using old agricultural practices for many years. In this case, the size and color of fruits are non-uniform. The local non-registered cultivars that are widely cultivated in the province are 'Amasya', 'Orak Apple', 'Demir Apple', 'Tavşanbaşı', 'Arapkızı' and 'Hüryemez'. Also, in recent years, popular cultivars such as 'Granny Smith', 'Fuji', 'Red Chief', 'Mondial Gala', 'Super Chief', 'Scarlet Spur' have been cultivated in the newly established orchards by a dwarf and semi-dwarf rootstocks.

Morphological and pomological characteristics are environmentally affected due to the nature of it. This information is not enough to effectively differentiate individuals. On the other hand, genetic characteristics supply more stable information for this purpose. After the development of molecular techniques and utilization of plant breeding, the researcher got a chance to clarify their phenotypic data with genetic data. Thus, researchers enhance the accuracy of their studies.

The selection of parents is always an important process for plant breeders and genetic similarity data helps to make more precise decisions about this step. Numerous DNA marker systems are available to show genetic variations among plants. Simple sequence repeat (SSR) (Hokanson et al. 1998; Hokanson et al. 2001; Zhang et al. 2007, Bakır et al. 2019), inter-simple sequence repeat (ISSR) (Smolik & Krzysztoszek 2010), RAPD (random amplified polymorphic DNA) (Dunemann et al. 1994; Zhou & Li

2000) and amplified fragment length polymorphism (AFLP) (Kenis & Keulemans 2005) are just some of them. AFLP and SSR systems are considered as a substantial way to indicate genetic variations in plants but these methods demand more costly instruments, usage of advanced steps in the process, and a significant amount of time compared to others.

Inter-primer binding sites (iPBS) retrotransposon marker system was developed by Kalendar et al. (2010) for plants and as well as animal kingdoms. The importance of this marker system is not to require sequence knowledge about plant/animal of interest. Retrotransposons can relocate themselves to the genome via copying. This mechanism usually ended up with different outcomes but the main result, expansion of genome size as well as genetic variation. For this reason, retrotransposons are accepted as valuable tools among other molecular marker systems.

Other studies on fruits have been carried out using iPBS. Guo et al. (2013), conducted a study with 35 grape varieties to evaluate for their molecular diversity associated with iPBS markers. In their experiment, 99 polymorphic DNA bands were produced with 15 iPBS primers. They indicated iPBS markers suitable for genetic diversity studies on grapes. Rovna et al. (2020), carried out a study with Rosa canina fruits to determine their genome size, iPBS profiles as well as antioxidant and antimicrobial actives. Their results suggested that iPBS markers provide favorable techniques for evaluating the genetic variability of Rosa canina.

Kuras et al. (2013), performed an experiment with 5 different DNA marker techniques to distinguish five different apple cultivars and their spots. According to their results, iPBS primers produced many polymorphic DNA output that has been able to distinguish five progenitor cultivars however not many polymorphic bands were sport specific. Correct utilization of iPBS marker system has been shown the power to identify different apple cultivars.

In general, the iPBS marker system is economic compared to other marker systems (which is an important feature especially for some countries), including screening huge part of plant genomes, usage for a different living organism, and user friendly to researchers (Kuras et al. 2013; Demirel et al. 2018; Milovanov et al. 2019).

Apple has already a growing market in Turkey. The current situation can be improved by eliminating known issues and evaluating the potential of local cultivars. This is the first study conducted in Niğde with these local apple genotypes since the study of Eltez & Kaska (1985). This study aims to evaluate the situation in the manner of genetic diversity to apple on the region of interest. For archive to this goal, iPBS markers utilized as a main tool and results also shown a convenient method to apple. Some studies were conducted on apple cultivars & mutants with iPBS markers, but this is the first study to the utilization of iPBS marker system on apple genotypes. This study is also the first step of the future breeding program in the region.

2. Material and Methods

The study was carried out with 48 different apple trees in 29 rural areas of Niğde, Turkey in 2018-2019 (Table 1). Altitude values of trees vary between 1125-1726 m (Table 1). ‘Super Chief’, ‘Fuji’, ‘Granny Smith’ cultivars taken from Niğde Ömer Halisdemir University Faculty of Agricultural Sciences and Technologies Research and Application Orchard was used as a control group for pomological analysis. In addition to pomological control groups ‘Golden Delicious’, ‘Scarlet Spur’ cultivars taken from the application orchard and known local apples called ‘green sour apple’, ‘sour summer apple’, ‘red sour apple’, ‘golden seed’, ‘rabbit head’, ‘bowl apple’, ‘red summer apple’ also used in the control group of molecular analysis.

Table 1- Information about plant materials (apple trees) and locations

Tree codes	*Name of locations*	*GPS data*	*Elevation (meter)*
KMR	Kemerhisar	37°49'56.9"N 34°35'29.3"E	1125
BHC	Bahçeli	37°50'06.7"N 34°36'39.5"E	1147
SZL	Sazlıca	37°54'04.3"N 34°38'34.8"E	1211
HLC	Halaç	37°49'39.0"N 34°41'19.3"E	1297
KRC	Karacaören	37°48'04.1"N 34°43'36.9"E	1487
KLV	Kılavuz	37°47'53.8"N 34°46'06.7"E	1571
HVZ	Havuzlu	37°46'38.0"N 34°37'59.1"E	1213
PST	Postallı	37°43'46.9"N 34°45'17.0"E	1394
DGR	Değirmenli	38°02'54.4"N 34°54'06.4"E	1494
DND	Dündarlı	38°05'28.7"N 35°09'54.4"E	1326
CKR1	Çukurbağ	37°50'09.6"N 35°03'25.8"E	1484
CKR2	Çukurbağ	37°50'08.7"N 35°03'33.2"E	1493
CKR3	Çukurbağ	37°49'60.0"N 35°03'27.7"E	1499
CKR4	Çukurbağ	37°50'07.1"N 35°03'21.4"E	1480
CKR5	Çukurbağ	37°50'07.2"N 35°03'10.9"E	1455
BDM1	Bademdere	37°55'04.7"N 35°04'14.8"E	1601
BDM2	Bademdere	37°55'01.5"N 35°04'18.1"E	1595
BDM3	Bademdere	37°54'58.9"N 35°04'24.5"E	1586
BDM4	Bademdere	37°54'53.9"N 35°04'24.2"E	1582
BDM5	Bademdere	37°54'47.8"N 35°04'26.2"E	1576
PNR1	Pınarbaşı	37°53'43.7"N 35°05'00.8"E	1574
PNR2	Pınarbaşı	37°53'36.7"N 35°05'15.9"E	1569
PNR3	Pınarbaşı	37°53'26.4"N 35°05'35.5"E	1572
PNR4	Pınarbaşı	37°53'15.0"N 35°06'02.0"E	1562
DMR1	Demirkazık	37°51'41.0"N 35°05'31.5"E	1577
PNR5	Pınarbaşı	37°53'06.4"N 35°06'24.2"E	1598
DMR2	Demirkazık	37°51'32.2"N 35°05'16.6"E	1558
DMR3	Demirkazık	37°51'28.7"N 35°05'04.8"E	1545
DMR4	Demirkazık	37°51'28.4"N 35°04'50.9"E	1556
DMR5	Demirkazık	37°51'25.4"N 35°04'43.4"E	1560
CLL	Celaller	37°48'34.6"N 34°56'09.5"E	1687
BRC	Burç	37°48'12.9"N 34°59'11.4"E	1445
ELG	Elekgölü	37°46'18.5"N 35°00'59.3"E	1365
KVL1	Kavlaktepe	37°59'29.8"N 35°05'34.0"E	1671
KVL2	Kavlaktepe	37°59'00.8"N 35°05'34.9"E	1726
HCB1	Hacıbeyli	38°07'17.7"N 35°09'19.9"E	1280
HCB2	Hacıbeyli	38°07'05.3"N 35°09'28.9"E	1283
DKL	Dikilitaş	38°06'56.9"N 35°04'25.3"E	1435
YSL	Yeşilova	38°03'31.3"N 34°49'58.3"E	1388
ULG	Uluağaç	38°02'34.6"N 34°50'20.2"E	1435
GMS	Gümüşler	37°59'56.2"N 34°45'59.7"E	1344
HMM	Himmetli	38°02'08.8"N 34°56'32.7"E	1552
ELM1	Elmalı	38°01'52.1"N 34°57'41.6"E	1603
ELM2	Elmalı	38°01'12.8"N 34°58'29.0"E	1605
KCP	Kocapınar	38°01'37.2"N 35°05'43.2"E	1571
EYN	Eynelli	37°53'51.3"N 35°03'46.9"E	1531
ICM	İçmeli	38°03'24.2"N 35°05'49.6"E	1519
YLT	Yelatan	37°40'51.6"N 35°01'14.0"E	1320

2.1 Morphological analysis

Morphological analysis was conducted before harvest (Tijskens et al. 2007). Tree habit (Upright, spreading, drooping, weeping), tree trunk diameter (Measured from 15 cm above to ground) and height of tree trunk (Measured from grafting point to first branches) (cm), one-year-old shoot length (cm), leaf blade attitude in relation to shoot (Upwards, outwards, downwards), leaf blade length and width (mm), leaf blade incisions of margin (Crenate, bicrenate, serrate type 1, serrate type 2, biserrate), petiole length (mm) and fruit general shape (Cylindrical waisted, conic, ovoid, cylindrical, ellipsoid, globose, obolid) measurements was collected for morphological analysis (UPOV 2005). All measurements except tree habit, tree trunk diameter and height of tree trunk, held with 3 repeats, each repeat subjected to 5 related plant materials in total 15.

2.2 Pomological analysis

The pomological analysis was carried out for 3 repeats, each repeat contains 5 fruit in total 15 ripe fruits for each tree. Fruit height and diameter (mm), fruit weight (g), depth of stalk cavity (mm), fruit skin color (Measured by KONICA MINOLTA CM-700d Spectrophotometer), the color of flesh (White, cream, yellowish, greenish, pinkish, reddish), number of seeds, the aperture of locules (Closed or slightly open, moderately open, fully open), firmness of flesh (Measured by handheld fruit penetrometer) (kg/cm^2), pH (Measured by VWR pHenomenal 1000L digital ph meter) and total soluble solids content (Measured by KRÜSS AR2008 digital refractometer) measurements were collected for pomological analysis (UPOV 2005).

2.3 Molecular analysis with iPBS primers

DNA extraction was conducted by the CTAB method from 3-5 young leaves taken from each tree (Dellaporta et al. 1983). The concentrations of the DNAs were then determined by the Quawell Q5000 UV-Vis Spectrophotometer and diluted to 5 ng /uL. IPBS primers were used in molecular marker analyzes (Table 4).

In total 60 apple genotypes (12 of them belong to the control group) were evaluated with 15 iPBS markers developed by Kalendar et al. (2010) to show the genetic diversity of these apple genotypes (Table 4). Diluted DNAs was amplified by PCR. The PCR was performed in a 25 µL reaction mixture containing 5 µL DNA (5ng/µL), 2.5 µL 10X DreamTaq PCR buffer, 0.375 µL dNTPs, 3 µL primer for 18 bp primers & 5 µL primer for 12-13 bp primers, 0.2 µL DreamTaq DNA polymerase.

Following initial denaturation at 95 °C for 3 min, PCR was conducted in order of amplification for 35 cycles with denaturation at 95 °C for 15 s annealing at 50-63 (specific to primers in Table 4) for 60 s and extension at 72 °C for 2 min. Lastly, the final extension was completed in a stage of 72 °C for 7 min. Products of PCR electrophoresed at 60 volts for 2.5 hours on a 1.8% agarose gel prepared with 1X TAE, stained with ethidium bromide for 30 minutes, and then viewed with Bio-Rad Gel Doc™ XR + gel imaging system. PCR or electrophoresis process repeated if it requires to get a clearer image of gel that suitable for scoring.

2.4 Statistical analysis

The SAS program was used for statistical analysis of pomological data (SAS 2005). The Duncan's Multiple Range Test was used to differentiate the mean values of the significant values ($P<0.05$). In the evaluation of the data obtained as a result of molecular analysis, the result file was created in a binary number system according to whether the molecular markers used in the gel images were shown as (1) or not (0). From these results, a similarity matrix was created with the appropriate Jaccard method and then data clustering and TKoA analyzes were applied by using the NTSYS program (Rohlf 1998). As a result of the clustering analysis, the dendrogram was generated by the UPGMA method. Mantel's matrix correspondence test was used to test the agreement of the dendrogram with the similarity matrix. Polymorphism information content (PIC) was calculated according to the formula given by Hinze et al. (2015) due to the iPBS makers system is a dominant marker. Principal Coordinates Analysis (PCoA) was performed using PAST 4.03 software (Hammer et al. 2001).

3. Results

3.1 Morphological results

Duncan's Multiple Range Test was applied to quantitative data of morphological observations. According to the morphologic results, no 'upright' types were found in any tree habit characteristics, and 'drooping' is the most common type 20 times. The highest values (significant in statically) found in tree trunk diameter was KVL2 (46.63), the height of tree trunk was BDM1 (148.5) cm, one-year-old shoot length was BDM4 (114.22 cm). 'Downwards' type was not found in leaf blade attitude in relation to shoot characteristics and the 'upwards' type was the most common feature with 33 times. The highest values (statically significant) found in leaf blade length was ULG (87.57 cm), leaf blade width was KCP (47.59 cm). Among leaf blade incisions of margin characteristics 'crenate' and 'bicrenate' type was not found, 'serrate type 2' is the most common type with 25 times. The highest values (statically significant) found in petiole length were KVL1 (36.65cm). Results of fruit general shape indicated that only 'globose' and 'obloid' features were founded among the genotypes and 'obloid' was the most common type with 33 times (Table 2).

The results obtained from the morphological analyzes revealed the expected characteristics and values of the local Niğde, Turkey apples, and provided a basis for further characterization studies.

Table 2- Morphological analysis results

Tree codes	*Tree habit*	*Tree trunk diameter*	*Height of tree trunk*	*One year old shoot length*	*Leaf blade attitude in relation to shoot*	*Leaf blade length*	*Leaf blade width*	*Leaf blade incisions of margin*	*Petiole length*	*Fruit general shape*
KMR	Spreading	32.79	83	51.56	Upwards	71.8	45.08	Serrate type 2	30.91	Globose
BHC	Drooping	40.33	106.5	60.32	Upwards	68.25	39.58	Serrate type 2	33.51	Obolid
SZL	Spreading	13.05	92.5	30	Upwards	58.27	32.44	Serrate type 2	24.79	Obolid
HLC	Drooping	15.37	100.3	70.28	Upwards	63.52	40.1	Biserrate	27.25	Obolid
KRC	Weeping	32.75	79	83.12	Upwards	62.71	35.79	Biserrate	27.27	Obolid
KLV	Weeping	27.28	112	51.82	Upwards	70.72	40.93	Serrate type 1	33.3	Obolid
HVZ	Weeping	33.01	108	73.94	Outwards	66.56	40.87	Biserrate	31.6	Obolid
PST	Weeping	27.95	84	53.98	Upwards	74.84	38.82	Serrate type 2	26.64	Obolid
DGR	Drooping	38.13	125.7	63.8	Upwards	73.75	40.67	Serrate type 2	31.37	Obolid
DND	Drooping	22.38	108.5	44.82	Upwards	72.15	40.02	Serrate type 2	29.51	Globose
CKR1	Drooping	20.18	123	49.52	Upwards	64.56	39.09	Biserrate	32.31	Obolid
CKR2	Drooping	20.72	74.3	38.66	Upwards	70.03	36.28	Serrate type 1	27.81	Obolid
CKR3	Drooping	39.57	82.2	107.5	Outwards	71.21	36.41	Biserrate	29.17	Obolid
CKR4	Weeping	23.62	132.5	43.08	Outwards	77.86	44.15	Biserrate	35.01	Obolid
CKR5	Spreading	26.74	116	60.46	Outwards	80.93	42.16	Biserrate	34.02	Globose
BDM1	Spreading	30.46	148.5	41.24	Upwards	66.83	36.59	Serrate type 1	31.8	Obolid
BDM2	Drooping	33.61	132	71.36	Upwards	73.79	44.8	Serrate type 2	30.17	Obolid
BDM3	Drooping	36.06	50.4	65.78	Upwards	78.49	39.8	Biserrate	29.93	Globose
BDM4	Weeping	20.21	64	114.22	Outwards	73.55	41.28	Serrate type 2	34.17	Obolid
BDM5	Weeping	23.87	122	62.94	Outwards	72.47	40.4	Serrate type 2	36.21	Obolid
PNR1	Spreading	33.9	123	85.24	Upwards	69.58	41.89	Serrate type 2	29.73	Globose
PNR2	Drooping	24.67	112.4	51	Upwards	72.11	44.26	Serrate type 2	35.48	Obolid
PNR3	Spreading	28.74	97.4	81.44	Upwards	76.93	46.23	Serrate type 2	35.02	Obolid
PNR4	Drooping	32.79	83	51.56	Upwards	71.8	45.08	Biserrate	30.91	Obolid
PNR5	Weeping	40.33	106.5	60.32	Upwards	68.25	39.58	Biserrate	33.51	Obolid
DMR1	Weeping	13.05	92.5	30	Upwards	58.27	32.44	Serrate type 1	24.79	Obolid
DMR2	Weeping	15.37	100.3	70.28	Outwards	63.52	40.1	Biserrate	27.25	Obolid
DMR3	Weeping	32.75	79	83.12	Upwards	62.71	35.79	Serrate type 2	27.27	Obolid
DMR4	Drooping	27.28	112	51.82	Upwards	70.72	40.93	Serrate type 2	33.3	Obolid
DMR5	Drooping	33.01	108	73.94	Upwards	66.56	40.87	Serrate type 2	31.6	Globose
CLL	Drooping	27.95	84	53.98	Upwards	74.84	38.82	Serrate type 2	26.64	Obolid
BRC	Drooping	38.13	125.7	63.8	Outwards	73.75	40.67	Serrate type 2	31.37	Obolid
ELG	Spreading	22.38	108.5	44.82	Upwards	72.15	40.02	Serrate type 2	29.51	Obolid
KVL1	Weeping	36.48	87.3	60.7	Upwards	69.19	38.95	Serrate type 1	36.65	Obolid
KVL2	Drooping	46.63	88.1	55.76	Upwards	71.65	45.84	Serrate type 2	32.17	Globose
HCB1	Weeping	30.81	92.3	51.92	Upwards	79.4	43.73	Serrate type 1	33.44	Globose
HCB2	Drooping	40.14	79.7	57.96	Upwards	71.13	43	Serrate type 1	29.84	Globose
DKL	Spreading	35.81	101.3	61.32	Outwards	68.43	40.78	Biserrate	30.12	Globose
YSL	Drooping	23.65	62.1	67.14	Outwards	79.09	38.87	Biserrate	33.82	Obolid
ULG	Spreading	31.83	79.2	57.68	Outwards	87.57	47.36	Serrate type 2	33.48	Globose
GMS	Weeping	33.3	79.4	41.06	Outwards	80.33	44.98	Biserrate	30.49	Obolid
HMM	Spreading	21.74	60.4	76.16	Upwards	65.47	38.25	Serrate type 2	23.81	Globose
ELM1	Spreading	31.26	73.6	45.64	Outwards	69.21	41.26	Serrate type 2	23.5	Globose
ELM2	Drooping	37.97	88.6	54.36	Outwards	77.43	40.96	Serrate type 2	33.58	Globose
KCP	Spreading	33.84	120	62.08	Upwards	87.44	47.59	Serrate type 2	36.2	Globose
EYN	Spreading	38.67	98.7	71.08	Outwards	80.77	47.49	Serrate type 2	34.69	Obolid
ICM	Drooping	32.47	121.7	72.78	Upwards	82.24	40.48	Biserrate	30.66	Obolid
YLT	Spreading	35.91	117.6	81.38	Upwards	83.9	46.46	Serrate type 1	35.18	Obolid

3.2 Pomological analysis

According to the Duncan's Multiple Range Test, the highest values (significant in statically) was found in fruit diameter CKR2 (72.64 mm), YSL (72.17 mm), ULG (72.07 mm), HCB2 (71.40 mm); fruit height ULG (66.40 mm); fruit weight ULG (154.44 g), YSL (147.50 g), HCB2 (145.50 g), CKR2 (144.04 g); depth of stalk cavity HCB2 (17.76 mm); the number of seeds SZL (10.33 pieces); firmness of flesh ELM1 (9.10 kg/cm^2); fruit skin color L value DMR3 (65.62), a value ICM (37.07), b value DMR3 (30.15), CLL (29.859). The most common types in flesh color were white with 31 samples and greenish with 7 samples. It was not found yellowish, pinkish, and reddish color flesh. The most observed type in the aperture of locules closed or slightly open with 28 samples and the fully open with the least common two samples. The highest value for pH was found in DMR5

(3.88) and the lowest value was in DMR3 (3.05). The highest amount of total soluble solids content was seen in ICM (14.60%) and the lowest value was found in HCB1 (10.30%) (Table 3).

Table 3- Pomological analysis results

Genoype & cultivar	FHE	FDI	FWE	DSC	FSC-dL	FSC-da	FSC-db	CFE	NSE	ALO	FFE	pH	TSSC
Fuji	71.92	58.12	152.15	14.24	50.91	17.56	18.51	2	9.07	1	10.04	3.40	13.00
Granny Smith	74.12	65.73	175.17	15.64	61.67	8.70	41.40	4	7.87	2	9.67	3.24	13.28
Super Chief	75.05	66.78	181.38	16.39	38.39	27.39	13.31	2	5.73	2	6.08	3.51	14.70
KMR	62.05	52.59	93.99	13.09	50.40	26.83	14.30	1	8.80	1	6.86	3.74	11.40
BHC	60.86	46.44	81.71	13.13	66.91	16.39	26.02	1	9.00	1	6.68	3.61	11.00
SZL	59.55	49.13	83.61	11.13	62.27	14.38	22.51	1	10.33	1	6.70	3.65	11.00
HLC	66.20	57.76	112.19	13.71	56.16	20.24	19.18	1	7.00	1	6.99	3.51	12.60
KRC	63.05	56.03	100.50	13.21	53.64	21.37	17.63	1	8.60	1	7.13	3.50	13.30
KLV	61.46	52.43	88.67	11.81	59.66	19.70	21.95	1	8.60	1	7.15	3.71	13.70
HVZ	59.60	47.78	79.16	12.35	56.20	24.00	18.19	1	8.47	1	7.64	3.50	13.80
PST	57.52	49.49	78.10	11.43	61.34	12.78	22.89	1	8.33	1	6.82	3.59	11.40
DGR	59.17	51.17	86.41	12.02	59.64	18.15	19.66	4	8.47	1	6.58	3.65	11.10
DND	58.05	51.24	81.72	11.41	63.64	9.83	24.00	1	6.87	1	6.95	3.55	10.80
CKR1	72.65	62.85	144.05	14.79	62.25	11.24	27.42	4	7.80	2	5.38	3.65	13.20
CKR2	63.33	53.22	98.21	12.94	55.20	21.17	19.75	1	6.93	2	6.97	3.61	12.40
CKR3	59.48	54.05	82.71	10.21	61.85	10.26	25.50	1	8.07	1	7.67	3.65	13.40
CKR4	62.09	52.86	91.88	16.51	58.57	19.37	20.69	1	8.47	1	7.17	3.56	11.00
CKR5	59.53	50.42	84.80	11.48	57.29	17.06	20.86	1	8.67	2	8.03	3.55	11.30
BDM1	64.12	56.40	103.81	13.14	50.67	27.50	14.59	1	9.53	1	7.32	3.59	12.30
BDM2	68.01	62.89	126.44	16.31	50.37	28.00	15.33	1	8.67	2	7.41	3.57	13.80
BDM3	58.56	50.71	81.83	12.02	59.89	16.80	21.16	1	8.20	1	7.25	3.41	12.40
BDM4	61.35	50.00	87.12	12.66	53.50	27.11	15.82	1	8.67	1	7.07	3.48	12.40
BDM5	58.21	50.06	80.69	11.84	54.94	23.58	17.54	1	10.27	1	7.52	3.50	11.40
PNR1	62.69	52.89	96.39	13.21	55.90	19.05	19.69	1	7.73	1	7.63	3.53	12.20
PNR2	65.03	55.70	103.93	13.01	52.92	26.32	16.23	1	7.33	1	7.49	3.43	13.60
PNR3	63.60	55.56	103.04	13.39	56.03	23.38	18.32	1	8.67	1	7.07	3.46	13.20
DMR1	63.62	55.73	103.78	12.94	52.31	27.68	20.96	1	8.00	1	6.76	3.76	13.30
PNR5	63.47	54.60	99.96	13.83	40.90	35.47	17.95	1	7.73	1	7.42	3.42	11.40
DMR2	67.33	59.32	124.54	13.92	53.66	21.52	23.55	1	8.60	3	6.49	3.42	13.80
DMR3	63.45	56.90	104.16	15.47	65.63	14.18	30.16	1	8.27	1	7.16	3.05	13.10
DMR4	63.10	56.01	104.91	13.03	46.94	30.96	17.12	1	9.47	1	6.83	3.52	13.10
DMR5	60.65	53.41	95.71	11.86	54.06	21.63	22.13	4	8.07	1	6.24	3.88	11.80
CLL	52.49	45.32	58.86	9.44	58.97	12.30	29.86	4	8.93	2	7.80	3.62	14.20
BRC	58.85	50.47	82.65	13.14	50.72	28.06	18.88	1	7.87	1	6.55	3.57	13.90
ELG	68.67	57.47	119.44	14.53	50.17	27.67	18.27	1	8.53	1	6.16	3.42	12.20
KVL1	66.14	56.47	110.46	14.36	53.73	18.25	25.96	2	6.73	1	6.65	3.35	13.60
KVL2	67.94	58.10	126.55	13.36	54.24	16.66	26.16	2	6.87	2	6.36	3.65	13.40
HCB1	60.32	53.73	92.63	12.92	51.90	25.35	20.11	1	6.73	1	6.90	3.47	10.30
HCB2	71.41	61.94	145.51	17.77	43.83	33.90	13.92	1	8.53	1	5.44	3.44	12.20
DKL	65.77	62.93	125.60	15.00	45.31	30.67	16.58	1	6.07	1	6.74	3.66	14.00
YSL	72.18	63.64	147.50	16.30	42.91	29.89	16.45	2	8.67	2	6.31	3.56	13.60
ULG	72.07	66.40	154.44	16.56	52.50	21.04	22.15	2	6.40	2	5.83	3.73	12.80
GMS	63.03	54.97	106.98	12.65	51.79	19.06	22.06	4	8.07	2	6.72	3.51	11.00
HMM	63.36	57.24	108.67	12.19	55.26	11.45	27.58	4	8.80	2	8.09	3.47	13.80
ELM1	52.75	48.59	66.20	8.51	53.45	17.56	24.83	4	7.40	2	9.11	3.56	14.40
ELM2	66.94	59.96	121.23	14.33	48.71	25.21	19.74	1	6.47	2	7.82	3.53	12.30
KCP	65.24	60.97	116.30	14.68	59.36	18.02	26.52	2	6.53	2	7.23	3.63	13.20
EYN	64.80	57.19	108.34	12.10	53.39	26.24	21.18	2	9.67	2	7.78	3.57	13.10
ICM	63.02	54.07	100.94	12.41	43.40	37.07	15.77	2	8.53	2	7.87	3.71	14.60
YLT	62.97	54.26	100.55	13.51	56.43	12.81	24.89	2	8.60	3	7.29	3.63	12.40

FHE: fruit height; FDI: fruit diameter; FWE: fruit weight; DSC: depth of stalk cavity; FSC-dL FSC-da FSC-db: fruit skin color; CFE: the color of flesh; NSE: number of seeds; ALO: aperture of locules; FFE: firmness of flesh; TSSC: total soluble solids content; CFE: white=1, cream=2, yellowish=3, greenish=4, pinkish=5, reddish=6; ALO: closed or slightly open=1, moderately open=2, fully open=3

3.3. Molecular results

Fifteen different iPBS primers were used in the study (Table 4) to the determination of total of 60 samples for molecular analysis. Gel images of the iPBS 2392 which one of the primers used in the study, are given in Figure 1. As a result of molecular analyzes, 143 polymorphic bands were obtained.

Table 4- Description of used iPBS primers with names, sequence, annealing temperature, polymorphism rates and PIC values

Primer names	Primer sequences	Temperatures of annealing (°C)	Polymorphism rate (%)	PIC value
2075	CTCATGATGCCA	50°C	94%	0.34
2381	GTCCATCTTCCA	50°C	79%	0.28
2382	TGTTGGCTTCCA	50°C	88%	0.34
2400	CCCCTCCTTCTAGCGCCA	50°C	52%	0.24
2398	GAACCCTTGCCGATACCA	51°C	71%	0.29
2252	TCATGGCTCATGATACCA	52°C	71%	0.39
2277	GGCGATGATACCA	52°C	55%	0.23
2375	TCGCATCAACCA	52°C	60%	0.16
2392	ATCTGTCAGCCA	52°C	88%	0.36
2085	ATGCCGATACCA	53°C	47%	0.21
2095	GCTCGGATACCA	53°C	15%	0.11
2232	AGAGAGGCTCGGATACCA	55°C	17%	0.13
2237	CCCCTACCTGGCGTGCCA	55°C	17%	0.10
2079	AGGTGGGCGCCA	60°C	13%	0.10
2081	GCAACGGCGCCA	63°C	5%	0.10

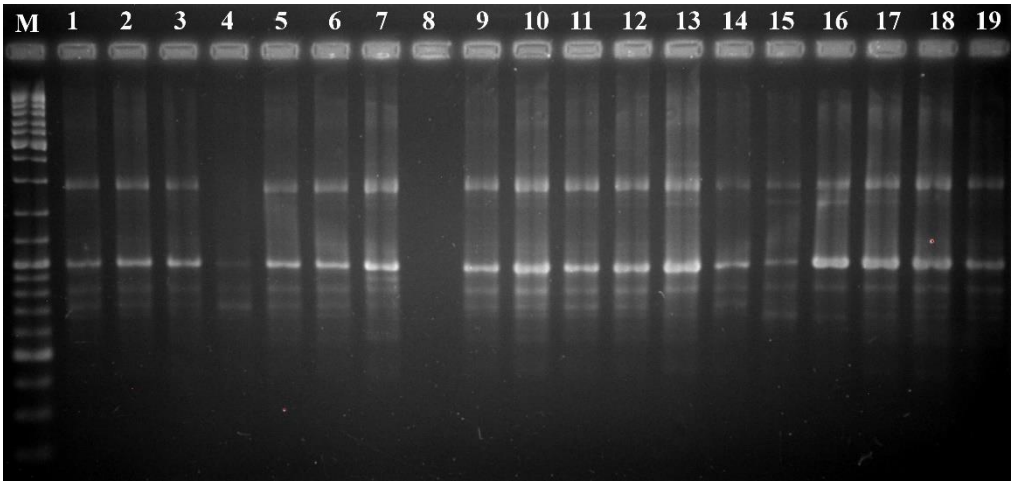

Figure 1- Gel image of iPBS 2392 primer, samples 1-19. 'M' stand for ladder (1 kb DNA ladder)

The main 48 samples were divided into 4 main branches and the similarity rates varied between 0.61-1.00 (Figure 2), with control groups these results extend to 7 main branches and the 0.54-1.00 similarity rates. Polymorphism rates of primers vary between 94%-5% and their PIC values vary between 0.10-0.34 (Table 4). The results of PCoA were similar to those of the cluster analysis. The first, second and third dimensions explained 23.6, 14.3 and 11.1% of the total variation making a total of 49% (Figure 3).

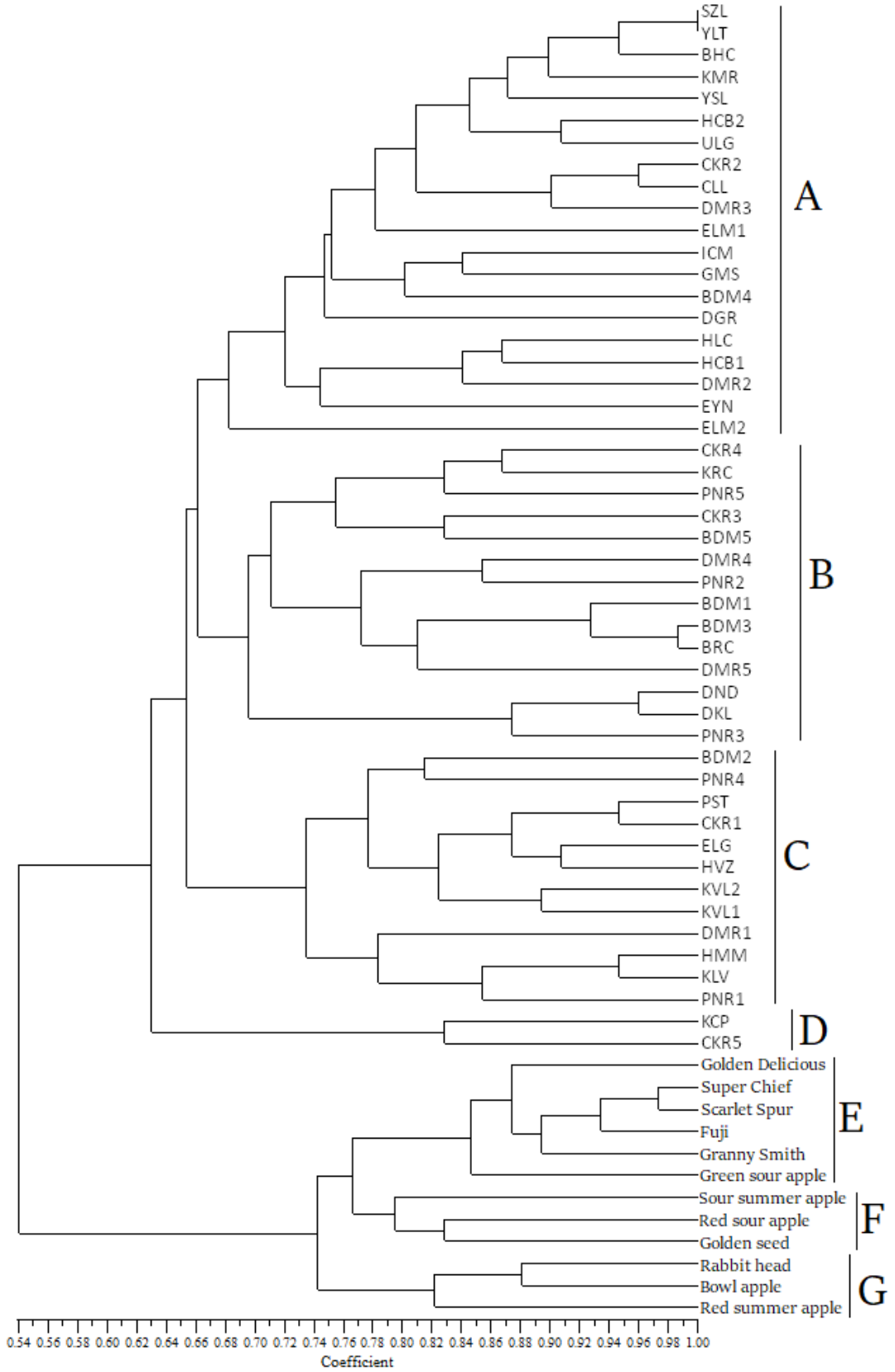

Figure 2- UPGMA dendrogram that shows the genetic diversity of samples with control groups using iPBS primers

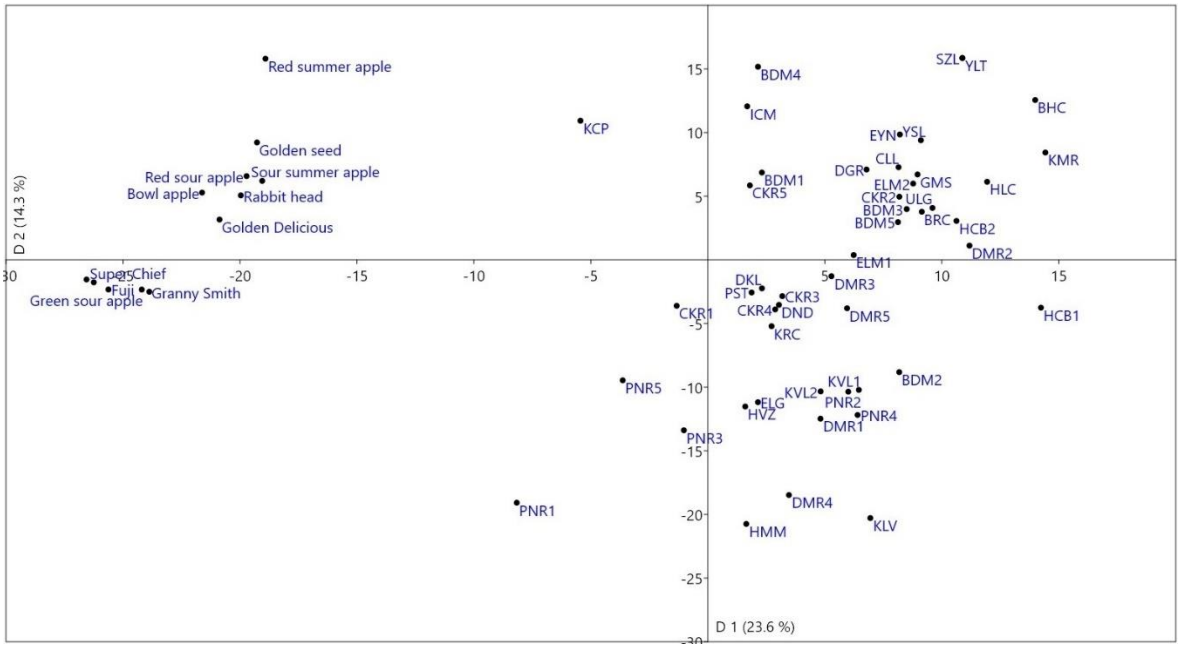

Figure 3- PCoA scatter plot showing the genetic diversity of a total 60 sample on first 2 dimension

4. Discussion

Eltez & Kaska (1985) stated that fruit length 56.25-31.30 mm, fruit width 60.25-34.25 mm, fruit weight 180.20-55.45 gr, depth of stalk cavity 19.00-4.66 mm, total soluble solids content 16.65-11.20, firmness of flesh of 10.05-6.48 kg/cm^2, the number of seeds 10.60 in their study with Amasya apples in Niğde. Although compared with the results of this study were shown prominent values among samples of the fruit diameter and fruit length, the overall values of the samples overlap with the value range. This is especially important for showing the consistency of the results, as the location and material of the two studies are similar. Under this information and with molecular results of our study, the diversity of area clarified. Coskun & Askın (2016) stated that fruit length 64.70-53.90 mm, fruit width 76.60-64.90 mm, fruit weight 184.30-96.90 gr, total soluble solids content 14.20-11.30, firmness of flesh 8.73-6.43 kg/cm^2 in their study on local apple cultivars in Eğirdir. In this study, prominent samples were seen as fruit flesh hardness when pomological results compared with the previous study. Fruit flesh hardness important for crispness (De Belie et al. 2000). Although the two studies carried out different locations as materials both apples local to their location. Senyurt et al. (2015) stated that fruit weight of Amasya cultivar 96.43 g, fruit length 54.95 mm, fruit diameter 61.73 mm, stalk pit depth 8.86 mm, fruit flesh hardness 7.92 kg/cm^2, the weight of fruit 210.60-72.50 g, fruit width 79.70-57.40 mm, fruit length 71.15-50.68 mm, fruit flesh hardness 10.00-6.80 kg / cm^2, total soluble solids content % 14.20- 11.90, pH 4.24-3.08 values in their study in Egirdir different Amasya types. Compared to the result with earlier studies, although the samples in the total soluble solids content are prominent, the values overlapped.

The fruit size and weight are important for higher yield in production and also a wanted feature for most of the consumers. The color of the fruit skin is from the consumer's initial assessment and appeal of the product. Fruit flesh firmness is important to less damage during storage and transportation and also important for the crunchiness which is a trait consumer seeks when eating apples. Total soluble solids content was used in the selection and comparisons were made based on pomological results in this study because of its unique taste, sensation, and saturation (Arıkan et al. 2015). Since the color of the fruit skin increases the attractiveness of the product for the consumer and high total soluble solids content value increases the taste of fruit and saturation of the fruit, it is important that the situation of samples used in the study show superiority (Chagne et al. 2014). Pomological results show that CKR2, DMR3, CLL, HCB2, YSL, ULG, ELM1, ICM samples stand out among other samples according to fruit size, fruit weight, fruit shell color, fruit flesh hardness, total soluble solids content.

Günes & Durgac (2018) stated that the similarity rate between 0.39-0.72 as a result of the analysis using RAPD markers on local apples in the Gülnar region. Kaya et al. (2015) stated that the similarity ratio between 0.38-0.79 as a result of the analysis using RAPD markers on local apple sources in Lake Van Basin. Masum et al. (2014) stated that the highest similarity between Marmara and Black Sea Region samples was 92.4%, the lowest similarity between Black Sea-Central Anatolia region samples was 70.5% in the study conducted with local apples belonging to Marmara, Black Sea, Aegean, and Central Anatolian regions at Atatürk Central Horticultural Research Institute.

In this study, phenotypic results indicated diversity among collected genotypes as well as their distinction to the control group. But some of these results may be influenced environmentally due to genotypes collected from 29 different rural areas. Under these circumstances, the most coherent way to validation of phenotypic results is the utilization of molecular techniques

to achieve more correct information about samples. In this study, iPBS is the chosen technique to manage this goal. The similarity ratio of this study 0.61-1.00 was compared with the previous studies and most of the samples shown higher similarities probably caused by location's effects on diversity but the results of molecular analysis have shown a variation among collected genotypes as well as their distinction to the control group. Although environmental influences can't be denied, both phenotypic and molecular results consistent with each other. Likewise, each sample that distinguishes the other can be used as a genetic source.

The study was conducted in an area where the total coverage approximately 1 853 km^2. Locations of BDM, PNR, DMR and CKR are relatively close to each other and contain 10 of 14 genotypes that under braches 'B'. On the other hand, this kind of phenomenon couldn't be seen for other main branches. Especially branches 'A' spread the whole area as well as 'B' on the UPGMA dendrogram. Branches 'D' only contain two genotypes and the distance between these two genotypes 26.6 km. In account of the most distance between two genotypes 59.72 km (HBC1 and HVZ), 26.6 km nearly the half of the distance between most distance genotypes. The location where the genotypes collected may affect to structure of braches on UPGMA dendrogram in some cases but the only reason underlying diversity among genotypes (Figure 2).

5. Conclusions

Morphological, pomological, and molecular differences were due to both location and genetics sources as the trees were found at varying heights in the elevation range of 1125-1726 m and sampled from the different parts of the province. Currently, limited studies have been carried out on apple with iPBS markers. Some studies were conducted on apple cultivars and mutants as well as other fruit species but this study is the first utilization of the iPBS markers system on apple genotypes. This research also supplied valuable information about this field via expanding the information about it. The current status can be improved by increasing and improving apple production in these rural areas with the use of modern agricultural techniques in production and storage stages, establish orchards with dwarf or semi-dwarf rootstocks and narrow plant spacing, perform agricultural practices on time with correct methods. According to information gathered from local peoples, these local apples in the province have decreased from the past and they are in danger of disappearing in the future. To preserve the plant material used in the study, 4 scions were taken from all of the trees and grafted on MM106 rootstocks and have been established a collection orchard in Niğde Ömer Halisdemir University Faculty of Agricultural Science and Technologies Faculty. Thus, in addition to the conservation of these resources, it will be possible to make controlled breeding for future studies.

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Identification of Rice Varieties Using Machine Learning Algorithms

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ABSTRACT
Rice, which has the highest production and consumption rates worldwide, is among the main nutrients in terms of being economical and nutritious in our country as well. Rice goes through some stages of production from the field to the dinner tables. The cleaning phase is the separation of rice from unwanted materials. During the classification phase, solid ones and broken ones are separated and calibration operations are performed. Finally, in the process of extraction based on color features, the striped and stained ones other than the whiteness on the surface of the rice grain are separated. In this paper, five different varieties of rice belonging to the same trademark were selected to carry out classification operations using morphological, shape and color features. A total of 75,000 rice grain images, including 15,000 for each varieties, were obtained. The images were pre-processed using MATLAB software and prepared for feature extraction. Using a combination of 12 morphological, 4 shape features and 90 color features obtained from five different color spaces, a total of 106 features were extracted from the images. For classification, models were created with algorithms using machine learning techniques of k-nearest neighbor, decision tree, logistic regression, multilayer perceptron, random forest and support vector machines. With these models, performance measurement values were obtained for feature sets of 12, 16, 90 and 106. Among the models, the success of the algorithms with the highest average classification accuracy was achieved 97.99% with random forest for morphological features. 98.04% were obtained with random forest for morphological and shape features. It was achieved with logistic regression as 99.25% for color features. Finally, 99.91% was obtained with multilayer perceptron for morphological, shape and color features. When the results are examined, it is observed that with the addition of each new feature, the success of classification increases. Based on the performance measurement values obtained, it is possible to say that the study achieved success in classifying rice varieties.

Keywords: Color features, Image processing, Morphological features, Rice classification, Shape features

1. Introduction

Looking at the production values of grain products around the world, the most important product after wheat and corn is rice. Rice is a grain product that is quite rich in carbohydrates and starch. In addition, it is of great importance in human nutrition in Turkey as it is in the world in terms of being nutritious and economical, and it is also widely used in the industrial field. Different quality criteria are available for rice varieties produced in Turkey. These criteria are physical appearance, cooking features, taste and aroma features, as well as efficiency (Tipi et al. 2009). Determining physical features from quality criteria can be expensive and unreliable when performed with traditional manual rice seed classification processes. Because human decisions are inconsistent, subjective and slow. Machine vision systems can be an alternative to automated systems, which is a non-destructive, cost-effective, fast and accurate technique.

Through studies in recent years using machine vision systems and image processing techniques on grain products, it is seen that the products are examined in terms of many physical features such as color, texture, quality, and size. Studies on grain varieties in the literature are examined and summarized below.

The studies without using color features summarized as follow. Digital image analysis of ground rice was performed by Yadav & Jindal (2001) to check whiteness and determine the percentage of broken seeds. The length, perimeter and shape features of the rice grain were extracted and their quantities were calculated. Dubey et al. (2006) used 45 morphological features for artificial neural network-based classification. An increase in the number of features in the classification has been seen to increase the success rate. They have achieved approximately 88% accuracy for all grains as classification accuracy. Zapotoczny et al. (2008) mentioned the utility of morphological features in the classification of five different barley varieties. In the study, they extracted 74 morphological features of each barley variety. They used principal component analysis (PCA), linear discriminant analysis (LDA) and nonlinear discriminant analysis (NDA) as classification methods. As a result, they concluded that the method in which they used morphological features could be successfully used to identify barley varieties. They also stated that the LDA is the best method in the classification methods. Aggarwal & Mohan (2010) performed aspect ratio analysis using image processing technique for grain quality of rice. The aim of the analysis was to examine the mixtures by taking samples

from three different classes (full, semi and broken) sold in markets and priced according to their size and to determine the reference aspect ratio in the market. An automatic image-receiving tape system was designed by OuYang et al. (2010) using image processing technique to distinguish between five different rice seeds. Using the back propagation classification (BP-ANN), they obtained an average accuracy rate of 86.65% for five different rice varieties. Abirami et al. (2014) used image processing and neural network pattern recognition techniques to classify Basmati rice grains. Various morphological features were extracted from the images taken with the help of the camera and the neural network was classified by pattern recognition. As a result of the classification, they achieved an accuracy rate of 98.7%. Sethy & Chatterjee (2018) classified the geometric and texture features of 6 pieces rice varieties using the multi-class support vector machine (M-SVM) algorithm. As a result of the classification, they achieved 92% accuracy. Chen et al. (2019) developed a machine vision system to study broken, calcareous, stained and defective rice grains using morphological features on images of red Indica rice. They used support vector machine (SVM) for classification and achieved results of 99.3%, 96.3% and 93.6%, respectively, as the accuracy of recognizing broken, calcareous, stained and defective rice grains. Koklu & Ozkan (2020) performed classification operations using morphological and shape features in images of seven different dry bean varieties. Classification models have been created using multi-layer perceptron (MLP), SVM, k-nearest neighbors (kNN) and decision tree (DT) machine learning methods. The SVM model achieved the highest classification accuracy of 93.13%.

The studies with using color features summarized as follow. Visen et al. (2003) using image processing techniques and an artificial neural network, they obtained color images of five types of grains: barley, oats, rye, wheat, and durum wheat. Also, they developed algorithms to analyze these images. They developed an artificial neural network-based classifier to identify unknown grains through more than 150 color and textural features of the resulting images. They have achieved over 90% accuracy rate for all grain types in identifying unknown grains. Demirbas & Dursun (2007) aimed to determine the morphological features of 13 different wheat varieties using image processing technique. Images were evaluated using UTHSCSA Image Tool version 3.0 as image processing program. As a result, due to the close proximity of the measurement results obtained by manual and image processing, they stated that image processing technique can be used to determine some of the physical features of wheat grains. Silva & Sonnadara (2013) used an artificial neural network to classify rice varieties. In the study, they developed an algorithm to extract 13 morphological features, 6 color features and 15 texture features using images from 9 different rice varieties. For these features, they have made different classifications, separately and together. As a result of classification, it was observed that texture features, rather than morphological and color features, provide a higher success rate in separate classifications. As a result of the classification, which is a combination of all features, the accuracy rate was achieved as 92%. Kaur & Singh (2013) have studied on a machine algorithm for rice classification using multi-class support vector machines. They have classified rice grains using their shape features, percentages and opaque state and have achieved an accuracy rate of more than 86%. Digital images of 13 rice varieties in Iran in three different forms were analyzed by Abbaspour-Gilandeh et al. (2020) with pre-processing and segmentation using the MATLAB application. Ninety two features were extracted for each rice variety, including 60 colors, 14 morphological and 18 texture features. The least significant difference (LSD) test was performed to obtain a more accurate comparison between varieties. PCA has been used to reduce data sizes and focus on the most effective components. Using discriminant analysis (DA), they achieved classification accuracy of 89.2%, 87.7% and 83.1% for paddy, brown rice and white rice, respectively.

In the literature, it has been studied to obtain product features using morphological features as well as shape and color features using various image processing techniques in images obtained from different grain products. In addition, classification processes were carried out using different machine learning methods with the help of these features. In this study, morphological, shape and color features were extracted for non-destructive, fast and accurate classification of rice varieties. The resulting features were used as inputs to perform classification operations with machine learning methods. In order to see the effect of the resulting features on the classification result, these features were combined, respectively, and the results were examined in detail. The contribution of the features obtained in this way to the classification processes has been interpreted.

2. Material and Methods

The aim of this study is to extract morphological features, shape features and color features by obtaining images from 5 different rice varieties. It is also to perform classification operations of the obtained features using various artificial intelligence techniques. Figure 1 shows the classification flow chart.

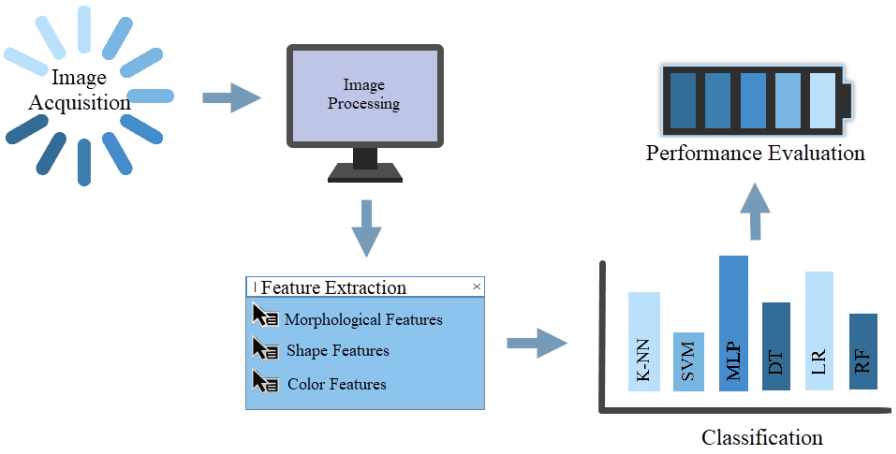

Figure 1- The classification flow chart

2.1. Image acquisition

In order to obtain images of the rice used in the study, the mechanism given in Figure 2 was used. A camera with an Ikegami brand CCD imaging sensor was used to capture the image. The camera used for study has 2.2 megapixels, 2048 × 1088 resolution and full resolution at a maximum frame rate of 53.7 fps. Features such as white balance and backlight correction are available. It is powered by 12V DC voltage and has power consumption below 4.5 W (Ikegami 2020).

The camera used in the study was placed on a closed box with a lighting device inside and a structure to prevent light from receiving from the external environment. Box background color is selected as black for easy processing of the image. The box sizes were designed so that images can be captured from an area 14 cm wide and 18 cm length. The height of the camera was set to 15 cm. The resulting images were recorded by transferring them to computer.

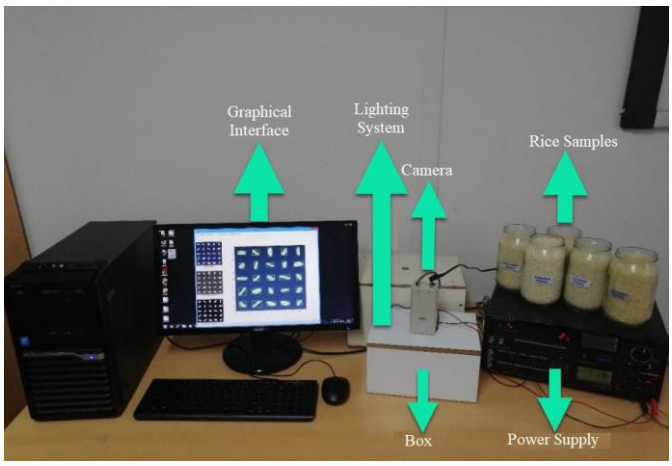

Figure 2- Mechanism used to obtain images

2.2. Image processing

In order to perform feature extraction and classification operations in the most accurate way during image processing phase, preprocessing operations related to images were described. Image processing was carried out with the help of MATLAB software. Images taken from the camera are primarily converted to grayscale images. It was then converted to a binary image using the global threshold level of the grayscale image with the help of the otsu method (Kurita et al. 1992). Unwanted objects on the resulting binary images have been removed and prepared for the feature extraction stage by applying the open process. Figure 3 shows the stages of image preprocessing.

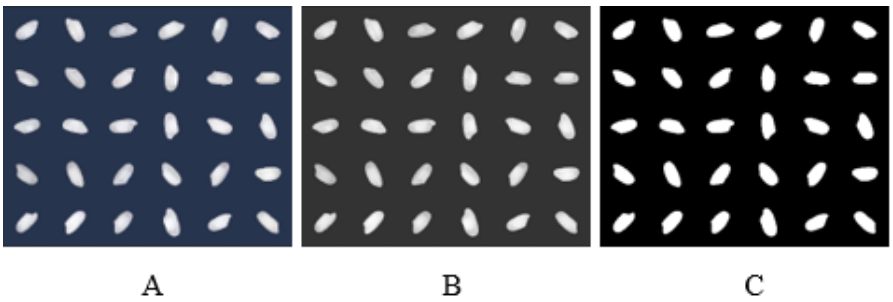

Figure 3- Image preprocessing stages; (A) Color image (B) Grayscale image (C) Binary image after pre-processing

2.3. Feature extraction

In the study, 12 morphological features using MATLAB software, 4 shape features obtained using morphological features and 90 color features obtained using five different color spaces were extracted.

Morphological and shape features were obtained using MATLAB regionprops function components. Shape features are calculated using area, major axis, and minor axis lengths from morphological features. The resulting feature values refer to the number of pixels of each rice grain. List of morphological features is given in Table 1 and list of shape features in Table 2 (Pazoki et al. 2014).

Table 1- Morphological features

No	*Feature*	*Explanation*	*No*	*Feature*	*Explanation*
1	Area (A)	The number of pixels within the boundaries of the rice grain area.	**7**	Solidity (S)	Is the ratio of pixels in the convex body to pixels in the rice grain region. In Equation 2, the calculation formula is given. $S = \frac{A}{CA}$ (2)
2	Perimeter (P)	The grain of rice gives the perimeter boundary length of.	**8**	Convex_Area (CA)	The number of pixels in the smallest convex polygon that can accommodate the rice grain area.
3	Major_Axis_Length (L)	The longest line that can be drawn on a grain of rice.	**9**	Extent (Ex)	The ratio of pixels in the bounding box to pixels in the rice grain region.
4	Minor_Axis_Length (l)	The longest line on a grain of rice that can be drawn perpendicular to the major axis.	**10**	Aspect_Ratio (AR)	It is calculated by dividing the the major axis length by the the minor axis length. The calculation formula is given in Equation 3. $AR = \frac{L}{l}$ (3)
5	Eccentricity (E)	It gives the eccentricity of the circle, which has the same moments as the region.	**11**	Roundness (R)	It is calculated by using area and perimeter. The calculation formula is given in Equation 4. $R=\frac{4xAx\pi}{P^2}$ (4)
6	Equivalent_Diameter (ED)	It is the diameter of a circle with the same area as the area of the rice grain. The calculation formula for the equivalent diameter is given in Equation 1. $\mathrm{ED} = \sqrt{\frac{4xA}{\pi}}$ (1)	**12**	Compactness (Co)	It is calculated by dividing equivalent diameter by the major axis length. The calculation formula is given in Equation 5. $Co = \frac{ED}{L}$ (5)

Table 2- Shape features

No	*Feature*	*Explanation*
1	Shape_Factor_1 (SF1)	It is calculated by dividing the major axis length by the area. The calculation formula is given in Equation 6. $SF1 = \frac{L}{A}$ (6)
2	Shape_Factor_2 (SF2)	It is calculated by dividing the minor axis length by the area. The calculation formula is given in Equation 7. $SF2 = \frac{l}{A}$ (7)
3	Shape_Factor_3 (SF3)	The calculation formula is as given in Equation 8. $SF3 = \frac{A}{\left(\frac{L}{2}\right)^2 x\ \pi}$ (8)
4	Shape_Factor_4 (SF4)	The calculation formula is as given in Equation 9. $SF4 = \frac{A}{\frac{L}{2} x \frac{l}{2} x\ \pi}$ (9)

Color (RGB) images of rice grains used in the study were converted from RGB color spaces to HSV, L*a*b*, YCbCr and XYZ color spaces using MATLAB software. Conversion formulas and explanation are given in Table 3 (Chaudhary et al. 2012; Pazoki et al. 2014).

Table 3- Color conversions and formulas

No	*Explanation*	*Formula*
1	*RGB-HSV Conversion;* The HSV color space consists of three parameters: color essence-tone (H), saturation (S), and value (V).	$Max = Max(R, G, B)$ (10) $Min = Min(R, G, B)$ (11) $V = Max$ (12) $S = \frac{Max - Min}{Max}$ (13) $H = \begin{cases} \frac{1}{6}\frac{G - B}{Max - Min}, & V = R \\ \frac{1}{6}\frac{B - R}{Max - Min} + \frac{1}{3}, & V = G \\ \frac{1}{6}\frac{R - G}{Max - Min} + \frac{2}{3}, & V = B \\ (\text{If } H < 0 \rightarrow H = H + 1) \end{cases}$ (14)
2	*RGB-L*a*b* Conversion*; In the L*a*b* color space, the value L* denotes lightness, 0 denotes black and 100 denotes white. The value a* refers to red and green. (+) a* indicates red, and (-) a* indicates green. The value b* refers to yellow and blue. (+) b* indicates yellow, and (-) b* indicates blue.	$L = 0.2126\ x\ R + 0.7152\ x\ G + 0.0722\ x\ B$ (15) $A = 1.4749\ x\ (0.2213\ x\ R - 0.3390\ x\ G + 0.1177\ x\ B) + 128$ (16) $B = 0.6245\ x\ (0.1949\ x\ R + 0.6057\ x\ G - 0.8006\ x\ B) + 128$ (17)
3	*RGB-YCbCr Conversion*; The YCbCr color space consists of brightness (Y), blue difference (Cb), and red difference (Cr) components.	$Y = 0.299\ x\ R + 0.587\ x\ G + 0.114\ x\ B$ (18) $Cb = -0.168\ x\ R - 0.331\ x\ G + 0.500\ x\ B$ (19) $Cr = 0.500\ x\ R - 0.418\ x\ G - 0.081\ x\ B$ (20)
4	*RGB-XYZ Conversion*; In the XYZ Color Space, X denotes red, Z denotes blue, and the Y component also denotes brightness.	$X = 0.4124\ x\ R + 0.3576\ x\ G + 0.1805\ x\ B$ (21) $Y = 0.2126\ x\ R + 0.7152\ x\ G + 0.722\ x\ B$ (22) $Z = 0.0193\ x\ R + 0.1192\ x\ G + 0.9505\ x\ B$ (23)

After the conversion process, the features of the color spaces are duplicated using the average density (MeanIntensity) and the pixel value (PixelValue) components using the regionprops function in MATLAB. Using RGB, HSV, L*a*b*, YCbCr and XYZ color spaces, a total of 90 color feature were extracted with the components of mean, standard deviation, skewness, kurtosis,

entropy and wavelet decomposition for each color channel (Arefi et al. 2011; Kaya & Saritas 2019). Explanations of the components applied to the color features are given in Table 4, and the list of the resulting color features is given in Table 5.

Table 4- Explanations of the components applied to the color features

No	*Feature*	*Explanation*
1	Mean (M)	The mean density value. (N variable vector, represent X input data). The calculation formula is given in Equation 24. $M = \frac{1}{N}\sum_{i=1}^{N} Xi$ (24)
2	Standard Deviation (SD)	Returns the standard deviation of pixel values. The standard deviation is a square root of the variance (V). The calculation formulas are given below. $V = \frac{1}{N-1}\sum_{i=1}^{N}(Xi - M)^2$ (25) $SD = \sqrt{V}$ (26)
3	Skewness (Sk)	Returns the skewness value of the pixel values. The calculation formula is given in equation 27. $Sk = \frac{\frac{1}{N-1}\sum_{i=1}^{N}(Xi - M)^3}{SD^3}$ (27)
4	Kurtosis (K)	Returns the kurtosis value of pixel values. The kurtosis calculation formula is given in Equation 28. $K = \frac{\frac{1}{N-1}\sum_{i=1}^{N}(Xi - M)^4}{SD^4} - 3$ (28)
5	Entropy (E)	Returns the entropy of pixel values. Entropy is a statistical measurement used to characterize the image texture. The entropy calculation formula is given in Equation 29. $E = -\sum_{i=1}^{m} p_i \log_2 p_i$ (29)
6	Wavelet Decomposition	Using the two-dimensional wavelet, it returns the wavelet separation level of the matrix from the pixel value. The WaveDec2 function has been used and the wavelet order DB4 has been selected.

Table 5- List of features obtained from color spaces

Color Space	*Mean*	*Standard Deviation*	*Skewness*	*Kurtosis*	*Entropy*	*Wavelet Decomposition*
RGB	Mean_RGB_R	StdDev_RGB_R	Skewness_RGB_R	Kurtosis_RGB_R	Entropy_RGB_R	Daub4_RGB_R
	Mean_RGB_G	StdDev_RGB_G	Skewness_RGB_G	Kurtosis_RGB_G	Entropy_RGB_G	Daub4_RGB_G
	Mean_RGB_B	StdDev_RGB_B	Skewness_RGB_B	Kurtosis_RGB_B	Entropy_RGB_B	Daub4_RGB_B
HSV	Mean_HSV_H	StdDev_HSV_H	Skewness_HSV_H	Kurtosis_HSV_H	Entropy_HSV_H	Daub4_HSV_H
	Mean_HSV_S	StdDev_HSV_S	Skewness_HSV_S	Kurtosis_HSV_S	Entropy_HSV_S	Daub4_HSV_S
	Mean_HSV_V	StdDev_HSV_V	Skewness_HSV_V	Kurtosis_HSV_V	Entropy_HSV_V	Daub4_HSV_V
L*a*b*	Mean_LAB_L	StdDev_LAB_L	Skewness_LAB_L	Kurtosis_LAB_L	Entropy_LAB_L	Daub4_LAB_L
	Mean_LAB_A	StdDev_LAB_A	Skewness_LAB_A	Kurtosis_LAB_A	Entropy_LAB_A	Daub4_LAB_A
	Mean_LAB_B	StdDev_LAB_B	Skewness_LAB_B	Kurtosis_LAB_B	Entropy_LAB_B	Daub4_LAB_B
YCbCr	Mean_YCbCr_Y	StdDev_YCbCr_Y	Skewness_YCbCr_Y	Kurtosis_YCbCr_Y	Entropy_YCbCr_Y	Daub4_YCbCr_Y
	Mean_YCbCr_Cb	StdDev_YCbCr _Cb	Skewness_YCbCr_Cb	Kurtosis_YCbCr_Cb	Entropy_YCbCr_Cb	Daub4_YCbCr_Cb
	Mean_YCbCr_Cr	StdDev_YCbCr_Cr	Skewness_YCbCr_Cr	Kurtosis_YCbCr_Cr	Entropy_YCbCr_Cr	Daub4_YCbCr_Cr
XYZ	Mean_XYZ_X	StdDev_XYZ_X	Skewness_XYZ_X	Kurtosis_XYZ_X	Entropy_XYZ_X	Daub4_XYZ_X
	Mean_XYZ_Y	StdDev_XYZ_Y	Skewness_XYZ_Y	Kurtosis_XYZ_Y	Entropy_XYZ_Y	Daub4_XYZ_Y
	Mean_XYZ_Z	StdDev_XYZ_Z	Skewness_XYZ_Z	Kurtosis_XYZ_Z	Entropy_XYZ_Z	Daub4_XYZ_Z

2.4. Cross validation

In data mining and artificial intelligence techniques, where model development data is scarce, the most common procedure that can be used to check the model's generalization ability is the k-fold cross validation method (Singh & Panda 2011).

Cross validation is an error estimation method developed to improve the reliability of classification. Cross validation works by dividing the dataset so that it is random into the number of subsets set for training and testing. One of the subsets is accepted as a test set and the system is trained with the remaining sets. This process is repeated up to the number k and the system is tested (Browne 2000). Figure 4 shows the working logic of cross validation.

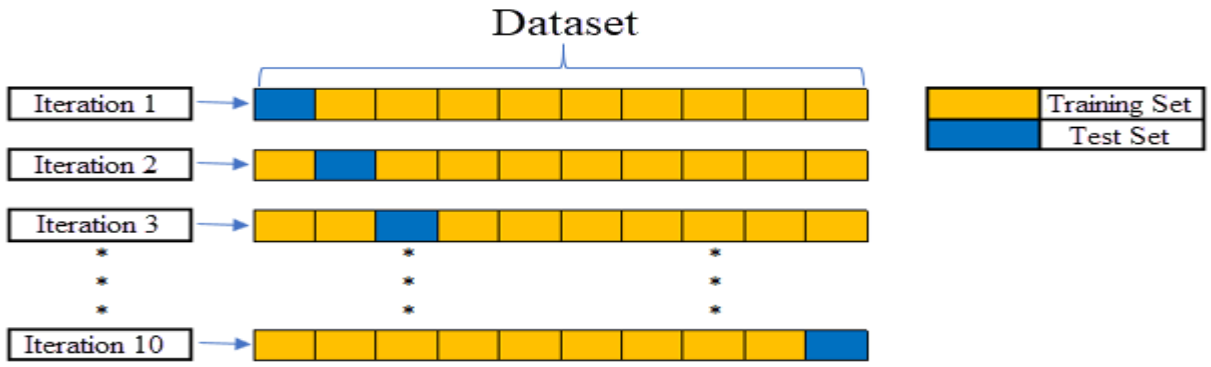

Figure 4- The working logic of cross validation

In the example given in Figure 4, the number of iterations (k) was selected as 10. In this example, the dataset was divided into 10 sections. Nine sections were taken sequentially as training data, and one was used as test data. The process repeats for all subsets and the system test was completed (Berrar 2019).

2.5. Kappa test

The Kappa test is a statistical method used to measure reliability by looking at harmony between two or more observers (Kilic 2015). Kappa coefficient values can vary between -1 and + 1. It can be interpreted as being completely compatible for harmony between observers when the value is +1, depending on luck when it is 0, and completely inverse of harmony when it is -1. In Table 6, the interpretation table of the kappa coefficient value ranges is given (Landis & Koch 1977; Kilic 2015).

Table 6- The interpretation table of the kappa coefficient value ranges

Kappa Value Range	*Explanation*
<0.00	Reverse / Bad Harmony
0.01-0.20	Trivial Harmony
0.21-0.40	Poor Harmony
0.41-0.60	Moderate Harmony
0.61-0.80	Good Harmony
0.81-1.00	Very Good Harmony

2.6. Rice dataset

A total of five different varieties of rice were used in the study. Rice varieties were selected as Arborio, Basmati, Ipsala, Jasmine and Karacadag, sold under Metro Chef Brand. Rice varieties used in the study are given in Figure 5 and technical information about the varieties is given in Table 7.

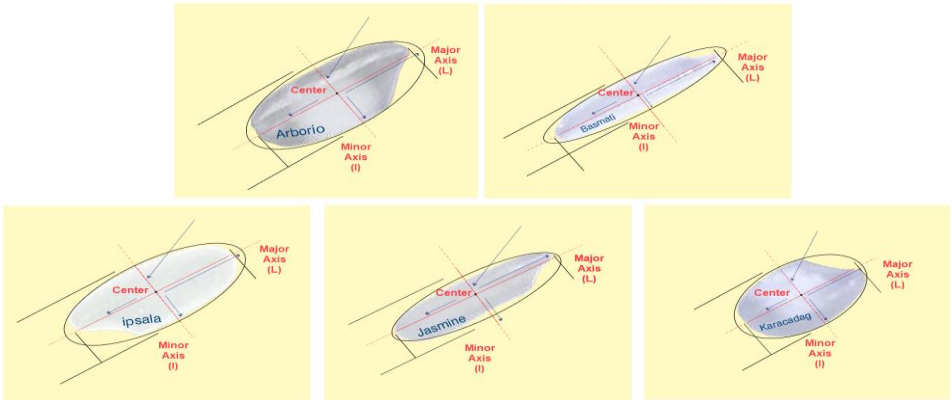

Figure 5- Rice varieties used in study

Table 7- Technical information of rice varieties

Variety Name	*Grain Length (mm)*	*Grain Width (mm)*	*Number of grains corresponding to 10 gr*
Arborio	6-7.5	3-4	303
Basmati	8.5-11.5	3.5-4.5	507
Ipsala	9-11	4-5.5	425
Jasmine	6.5-10	2.5-3.5	547
Karacadag	4.5-6	3-4	513

In our study, 15,000 images of rice grains belonging to each rice variety were obtained. In total, studies were carried out on data belonging to 75,000 grains of rice (Cinar 2019).

2.7. Performance evaluation

Creating a new model required for classification problems or using existing models and achieving success on this model was calculated by the number of accurate estimates. This affects the accuracy of the classification rather than the estimation of whether the model is good or not. The confusion matrix is therefore used to explain predictive assessments of classification. It is matrix confusion matrix that provides information about actual classes with predicted classes performed by a classification model on test data (Cataloluk 2012; Cinar & Koklu 2019). In Table 8, the confusion matrix used for binary classification is given, and in Table 9, the confusion matrix used for multiclass classification is given (Hossin & Sulaiman 2015).

Table 8- Confusion matrix used for binary classification

		Prediction Class	
		Positive	*Negative*
Actual Class	Positive	True positive (tp)	False negative (fn)
	Negative	False positive (fp)	True negative (tn)

Table 9- Confusion matrix used for multiclass classification

		Prediction Class				
		C_1	C_2	C_3	…	C_n
Actual Class	C_1	**T_1**	F_{12}	F_{13}	…	F_{1n}
	C_2	F_{21}	**T_2**	F_{23}	…	F_{2n}
	C_3	F_{31}	F_{32}	**T_3**	…	F_{3n}
	…	…	…	…	…	…
	C_n	F_{n1}	F_{n2}	F_{n3}	…	**T_n**

(C: Class, T: True, F: False)

The accuracy of a classification can be evaluated by calculating the number of correctly recognized (true positives) class instances, the number of correctly recognized instances that do not belong to the class (true negatives), and instances that are incorrectly assigned to the class (false positives) or are not recognized as class (false negatives) instances (Sokolova & Lapalme 2009).

Calculation formulas for success criteria such as accuracy, error rate, recall, specificity, precision and F1 score, were calculated using the confusion matrix for binary classification performance measurements, and are given in Table 10 (Hossin & Sulaiman 2015).

Table 10- Performance measurements and calculation formulas for binary classification

No	*Performance Metrics*	*Explanation*	*Formula*	
1	Accuracy	It is used to measure the ratio of true prediction on all samples included in the assessment.	$\frac{tp+tn}{tp+fp+tn+fn}x100$	(30)
2	Error Rate	It is used to measure the ratio of false prediction on all samples included in the assessment.	$\frac{fp+fn}{tp+fp+tn+fn}x100$	(31)
3	Recall	It is used to measure the ratio of correctly classified positive values.	$\frac{tp}{tp+fn}x100$	(32)
4	Specificity	It is used to measure the ratio of correctly classified negative values.	$\frac{tn}{tn+fp}x100$	(33)
5	Precision	It is used to measure the ratio of accurately classified positive samples to estimated total positive samples.	$\frac{tp}{tp+fp}x100$	(34)
6	F1-Score	Gives the harmonic mean of the measurements of recall and sensitivity.	$\frac{2x\frac{tp}{tp+fp}x\frac{tp}{tp+tn}}{\frac{tp}{tp+fp}+\frac{tp}{tp+fn}}x100$	(35)

Calculation formulas for average accuracy, average recall, average accuracy, average error rate, and average F1-score were calculated using the confusion matrix for multi-class classification performance measurements, and are given in Table 11 (Hossin & Sulaiman 2015).

Table 11- Performance measurements and calculation formulas for multi-class classification

No	*Performance Metrics*	*Explanation*	*Formula*	
1	Averaged Accuracy	It is used to measure the average effectiveness of all classes in the classification.	$\frac{\sum_{i=1}^{1}\frac{tp_i+tn_i}{tp_i+fn_i+fp_i+tn_i}}{l}x100$	(36)
2	Averaged Error Rate	It is used to measure the average error rate of all classes in classification.	$\frac{\sum_{i=1}^{1}\frac{fp_i+fn_i}{tp_i+fn_i+fp_i+tn_i}}{l}x100$	(37)
3	Averaged Precision	It is used to measure the precision average for each class.	$\frac{\sum_{i=1}^{1}\frac{tp_i}{tp_i+fp_i}}{l}x100$	(38)
4	Averaged Recall	It is used to measure the recall average for each class.	$\frac{\sum_{i=1}^{1}\frac{tp_i}{tp_i+fn_i}}{l}x100$	(39)
5	F1-Score	It is used to measure the F1-score average for each class.	$\frac{2x\frac{\sum_{i=1}^{1}\frac{tp_i}{tp_i+fp_i}}{l}x\frac{\sum_{i=1}^{1}\frac{tp_i}{tp_i+fn_i}}{l}}{\frac{\sum_{i=1}^{1}\frac{tp_i}{tp_i+fp_i}}{l}+\frac{\sum_{i=1}^{1}\frac{tp_i}{tp_i+fn_i}}{l}}x100$	(40)

3. Classification Models

3.1. K-Nearest neighbor (K-NN)

The K-NN method is a nonparametric learning algorithm. K-NN uses the euclidean distance as a parameter in the name of classification of the dataset, where K represents the number of neighbors, to calculate the distance between the data (Kumar et al. 2019).

K-NN is intended to classify sample data whose class is unknown. For this reason, the distance to the sample data is calculated with the pre-classified data set in the training set. Given that there is a certain amount of data to be tested, the test data is processed with all the existing data individually. The test data will have many neighbors that are close to it in terms of all the measured

features. For this reason, K pieces of data closest to the test data are selected. As a result, it is said that the tested data belongs to that class if there is more data belonging to which class than the selected data (Richman 2011; Beyaz & Ozturk 2016). For this study, the K value was set to 10.

3.2. Decision tree (DT)

DT is one of the first classification methods that comes to mind along with neural networks in data mining. If DT is generally thought as a tree diagram, it branches so that it has a classification query on each of its branches and nodes (Safavian & Landgrebe 1991).

DT's features in dealing with complex problems and their inferences in logical classification rules are seen as advantages (Amor et al. 2006). In addition, DT's integration into databases is easy and their reliability is high, making it stand out among other classification models.

3.3. Logistic regression (LR)

LR is one of the commonly used statistical models. In LR, the dependent variable is estimated from one or more variables. LR clarifies the relationship between dependent variables and independent variables. There is no need to create normal distribution of variables in LR. Because the values envisaged in the LR are probabilities, LR is limited to 0 and 1. This is because LR predicts its probability, not itself, in the results. (Cruyff et al. 2016; Kalantar et al. 2018).

3.4. Multilayer perceptron (MLP)

Today, many artificial neural network models have been developed for use for specific purposes, and MLP is one of the most used of these models. In MLP, the sequence of neurons is in layers, and there is a hidden layer between them, along with two main layers. MLP can contain more than one hidden layer. The input layer, which is the first of the main layers, is the layer where the data is read and contains information about the problem that needs to be solved. The output layer, which is the second main layer, is the layer where classes are defined and outputs are received for information processed in the network. The hidden layer is the layer where intermediate operations are performed on the data between the main layers (Sabanci 2016).

MLP has as many neurons as the number of features, and the data is provided by a flow of data in one direction from the input layer to the output layer. In addition, it is possible to monitor and modify the network structure during the training period (Arora 2012). In this study, there are 4 hidden layers and also the sigmoid activation function was used.

3.5. Random forest (RF)

RF is a classifier consisting of multiple DT's. To make a new classification, each DT provides a classification for the inputs. After that, RF evaluates the classifications and selects the estimate that with the most votes. RF has the ability to manage a large number of variables in a dataset. It is also quite successful at predicting incomplete data. The biggest drawback of RF is its lack of repeatability. Also, the final model and subsequent results are difficult to interpret. This is also due to the fact that it contains many independent decision trees (Oshiro et al. 2012).

3.6. Support vector machine (SVM)

SVM is a kernel-based method that creates a hyper plane for classification and regressions. Different kernel functions are used in SVM models. In this study, classification was made using the polynomial kernel function.

SVM has the ability to classify data in the form of linear in two-dimensional space, planar in three-dimensional space and hyper plane in multidimensional space with separation mechanisms (Abhang et al. 2016). SVM performs the classification process by finding the best hyper plane that separates the data belonging to the classes.

SVMs have features similar to other classification algorithms. It is especially similar to neural networks, but more similar to the K-NN algorithm. Like the K-NN algorithm, SVM determines its neighbors based on sample data presented to the algorithm and assumes that estimates are made for new data (Shi et al. 2011; Abhang et al. 2016).

4. Results

From a total of 75,000 images of rice grains belonging to the rice varieties used in the study, 12 morphological features were extracted from the features found in the list of morphological features given in Table 1. Classification operations were performed using K-NN, DT, LR, MLP, RF and SVM algorithms on the data of 12 features obtained. In addition to 12 morphological features to increase classification accuracy, 4 shape features given in Table 2 were added and classification operations were performed using K-NN, DT, LR, MLP, RF and SVM algorithms on data belonging to a total of 16 features. Given the results we have

obtained when we examine the studies conducted in the literature, it is thought that success will increase even more when morphological features, shape features and color features are evaluated together. For this reason, 90 color features have been extracted from the color feature list given in Table 5. Color features were evaluated primarily independently of morphological and shape features, and classification operations were performed using K-NN, DT, LR, MLP, RF and SVM algorithms on data belonging to 90 features. Then, a total of 106 features were extracted, which evaluated the morphological, shape and color features obtained together, and classification operations were performed using k-NN, DT, LR, MLP, RF and SVM algorithms.

In the study, the confusion matrix and performance measurement values of classification results obtained from algorithms for 12 morphological, 16 morphological and shape, 90 colors and 106 morphological, shape and color feature data are given in Table 12, respectively.

Table 12- Confusion matrix obtained using morphological features of all algorithms

		Algorithm	*Prediction Class*				
			Arborio	***Basmati***	***Ipsala***	***Jasmine***	***Karacadag***
Actual Class	**Arborio**	K-NN	**14,313**	1	21	49	616
		DT	**14,459**	0	12	30	499
		LR	**14,463**	0	8	32	497
		MLP	**14,387**	0	14	51	548
		RF	**14,492**	0	11	36	461
		SVM	**14,411**	0	2	48	539
	Basmati	K-NN	0	**14,620**	0	380	0
		DT	1	**14,688**	0	311	0
		LR	0	**14,634**	0	365	1
		MLP	0	**14,671**	0	329	0
		RF	1	**14,713**	0	286	0
		SVM	0	**14,323**	0	677	0
	Ipsala	K-NN	27	0	**14,920**	53	0
		DT	31	0	**14,909**	60	0
		LR	26	0	**14,937**	37	0
		MLP	26	7	**14,927**	40	0
		RF	22	0	**14,929**	49	0
		SVM	38	0	**14,910**	52	0
	Jasmine	K-NN	47	380	61	**14,511**	1
		DT	33	250	31	**14,685**	1
		LR	20	260	25	**14,694**	1
		MLP	28	553	31	**14,387**	1
		RF	20	250	32	**14,697**	1
		SVM	32	458	23	**14,486**	1
	Karacadag	K-NN	500	0	0	1	**14,499**
		DT	374	0	0	1	**14,625**
		LR	358	0	0	1	**14,641**
		MLP	334	0	0	0	**14,666**
		RF	341	0	0	1	**14,658**
		SVM	365	0	0	0	**14,635**

When Table 12 was examined, 72,863 pieces from K-NN algorithm, 73,366 pieces from DT algorithm, 73,369 pieces from LR algorithm, 73,038 pieces from MLP algorithm, 73,489 pieces from RF algorithm and 72,765 pieces from SVM algorithm rice grains were correctly classified.

For all algorithms used in the study, accuracy, error, precision, recall and F1-score average performance measurements and kappa coefficient values obtained by evaluating only morphological features using confusion matrix are given in Table 13.

Table 13- Average performance measurements and kappa coefficient values of all algorithms used in classification for morphological features

Performance Metrics	*Random Forest*	*Logistic Regression*	*Decision Tree*	*Multi Layer Perceptron*	*k-Nearest Neighbor*	*Support Vector Machine*
Accuracy (%)	97.99	97.83	97.82	97.38	97.15	97.02
Error (%)	2.01	2.17	2.18	2.62	2.85	2.98
Precision (%)	98.00	97.80	97.80	97.40	97.20	97.00
Recall (%)	98.00	97.80	97.80	97.40	97.20	97.00
F1-Score (%)	98.00	97.80	97.80	97.40	97.20	97.00
Kappa coefficient	0.975	0.973	0.973	0.967	0.964	0.963

When the average performance measurement values given in Table 13 are examined, it is seen that the classification accuracy for all algorithms is above 97%. It seems that the best classification accuracy belongs to the random forest algorithm with 97.99%. The lowest classification accuracy belongs to the support vector machine algorithm with 97.02%.

For random forest algorithm with the best classification accuracy, the confusion matrix in which morphological features are evaluated is given in Table 12. When the table is examined, the accuracy rates of Arborio, Basmati, Ipsala, Jasmine and Karacadag rice varieties are respectively, 96.61%, 98.09%, 99.53%, 97.98%, and 97.72%. Figure 6 shows the accuracy rates of classification algorithms derived from morphological features for rice varieties used in the study.

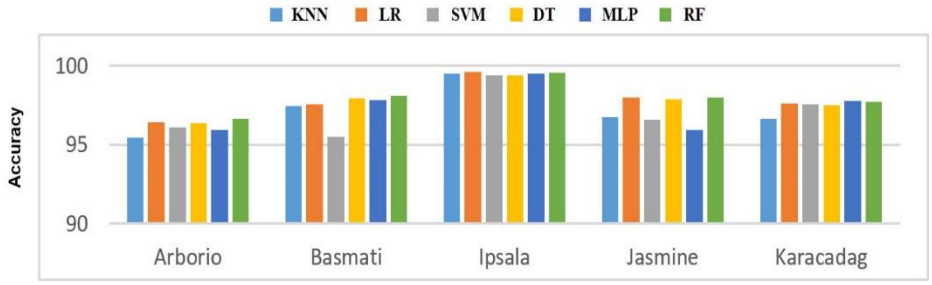

Figure 6- Accuracy rates of classification algorithms obtained from morphological features for all rice varieties used in the study

In the random forest algorithm, Ipsala rice variety, which has the highest accuracy rate among varieties, also reaches the highest accuracy rates in other algorithms. The Arborio variety, on the other hand, has a lower accuracy rate than other varieties.

Table 14- Confusion matrix obtained using morphological and shape features of all algorithms

		Algorithm	*Prediction Class*				
			Arborio	*Basmati*	*Ipsala*	*Jasmine*	*Karacadag*
Actual Class	**Arborio**	K-NN	**14,324**	1	18	44	613
		DT	**14,484**	0	13	35	468
		LR	**14,470**	0	8	31	491
		MLP	**14,455**	0	11	36	498
		RF	**14,508**	0	11	31	450
		SVM	**14,426**	0	3	46	525
	Basmati	K-NN	0	**14,634**	0	366	0
		DT	1	**14,677**	0	322	0
		LR	0	**14,631**	0	368	1
		MLP	0	**14,706**	0	294	0
		RF	0	**14,716**	0	284	0
		SVM	0	**14,488**	0	512	0
	Ipsala	K-NN	30	0	**14,925**	45	0
		DT	27	0	**14,935**	37	1
		LR	23	0	**14,941**	36	0
		MLP	25	2	**14,941**	32	0
		RF	22	0	**14,937**	41	0
		SVM	39	0	**14,922**	39	0
	Jasmine	K-NN	29	388	44	**14,538**	1
		DT	25	267	31	**14,676**	1
		LR	18	268	29	**14,684**	1
		MLP	36	506	76	**14,381**	1
		RF	20	255	30	**14,694**	1
		SVM	33	355	22	**14,589**	1
	Karacadag	K-NN	496	0	0	0	**14,504**
		DT	412	0	0	1	**14,587**
		LR	356	0	0	0	**14,644**
		MLP	351	0	0	0	**14,649**
		RF	326	0	0	1	**14,673**
		SVM	358	0	0	0	**14,642**

When Table 14 was examined, 72,925 pieces from K-NN algorithm, 73,359 pieces from DT algorithm, 73,370 pieces from LR algorithm, 73,132 pieces from MLP algorithm, 73,528 pieces from RF algorithm and 73,067 pieces from SVM algorithm rice grains were correctly classified.

For all algorithms used in the study, accuracy, error, precision, recall and F1-score average performance measurements and kappa coefficient values obtained by evaluating together of morphological and shape features using confusion matrix are given in Table 15.

Table 15- Average performance measurements and kappa coefficient values of all algorithms used in classification for morphological and shape features

Performance Metrics	*Random Forest*	*Logistic Regression*	*Decision Tree*	*Multi Layer Perceptron*	*Support Vector Machine*	*k-Nearest Neighbor*
Accuracy (%)	98.04	97.83	97.81	97.51	97.42	97.23
Error (%)	1.96	2.17	2.19	2.49	2.58	2.77
Precision (%)	98.00	97.80	97.80	97.50	97.40	97.20
Recall (%)	98.00	97.80	97.80	97.50	97.40	97.20
F1-Score (%)	98.00	97.80	97.80	97.50	97.40	97.20
Kappa coefficient	0.976	0.973	0.973	0.969	0.968	0.965

When the average performance measurement values given in Table 15 are examined, it is seen that the classification accuracy for all algorithms is above 97%. It seems that the best classification accuracy belongs to the random forest algorithm with 98.04%. The lowest classification accuracy belongs to the k-nearest neighbor algorithm with 97.23%.

For random forest algorithm with the best classification accuracy, the confusion matrix in which morphological and shape features are evaluated is given in Table 14. When the table is examined, the accuracy rates of Arborio, Basmati, Ipsala, Jasmine

and Karacadag rice varieties are respectively, 96.72%, 98.11%, 99.58%, 97.96%, 97.82%. Figure 7 shows the accuracy rates of classification algorithms derived from morphological and shape features for rice varieties used in the study.

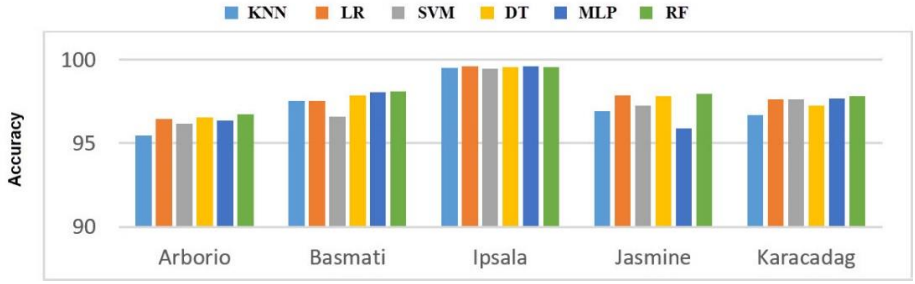

Figure 7- Accuracy rates of classification algorithms obtained from morphological and shape features for all rice varieties used in the study

Looking at the classification accuracy rates obtained from morphological and shape features, it seems that the Ipsala rice variety has the highest accuracy rate among the varieties. The Arborio variety, on the other hand, has a lower accuracy rate than other rice varieties.

Table 16- Confusion matrix obtained using color features of all algorithms

		Algorithm	*Prediction Class*				
			Arborio	***Basmati***	***Ipsala***	***Jasmine***	***Karacadag***
Actual Class	**Arborio**	K-NN	**14,712**	9	0	262	17
		DT	**14,664**	46	3	234	53
		LR	**14,842**	8	1	134	15
		MLP	**14,803**	8	0	177	12
		RF	**14,784**	8	0	190	18
		SVM	**14,749**	5	0	237	9
	Basmati	K-NN	43	**14,590**	1	37	329
		DT	50	**14,450**	2	39	459
		LR	16	**14,859**	0	22	103
		MLP	15	**14,836**	1	16	132
		RF	29	**14,681**	0	17	273
		SVM	39	**14,677**	0	76	208
	Ipsala	K-NN	1	0	**14,999**	0	0
		DT	2	3	**14,992**	2	1
		LR	0	0	**15,000**	0	0
		MLP	0	1	**14,999**	0	0
		RF	0	0	**15,000**	0	0
		SVM	1	0	**14,999**	0	0
	Jasmine	K-NN	219	8	0	**14,747**	26
		DT	195	29	4	**14,718**	54
		LR	104	18	0	**14,865**	13
		MLP	130	10	1	**14,839**	20
		RF	101	11	0	**14,867**	21
		SVM	121	70	0	**14,800**	9
	Karacadag	K-NN	57	297	0	63	**14,583**
		DT	51	421	0	68	**14,460**
		LR	17	95	0	13	**14,875**
		MLP	21	79	0	11	**14,889**
		RF	44	228	0	33	**14,695**
		SVM	31	196	0	69	**14,704**

When Table 16 was examined, 73,631 pieces from K-NN algorithm, 73,284 pieces from DT algorithm, 74,441 pieces from LR algorithm, 74,366 pieces from MLP algorithm, 74,027 pieces from RF algorithm and 73,929 pieces from SVM algorithm rice grains were correctly classified.

For all algorithms used in the study, accuracy, error, precision, recall and F1-score average performance measurements and kappa coefficient values obtained by evaluating only color features using confusion matrix are given in Table 17.

Table 17- Average performance measurements and kappa coefficient values of all algorithms used in classification for color features

Performance Metrics	*Logistic Regression*	*Multi Layer Perceptron*	*Random Forest*	*Support Vector Machine*	*k-Nearest Neighbor*	*Decision Tree*
Accuracy (%)	99.25	99.15	98.70	98.57	98.17	97.71
Error (%)	0.75	0.85	1.30	1.43	1.83	2.29
Precision (%)	99.30	99.20	98.70	98.60	98.20	97.70
Recall (%)	99.30	99.20	98.70	98.60	98.20	97.70
F1-Score (%)	99.30	99.20	98.70	98.60	98.20	97.70
Kappa coefficient	0.991	0.989	0.984	0.982	0.977	0.971

Looking at the average performance measurement values given in Table 17, it seems that the best classification accuracy belongs to the 99.25% logistic regression algorithm. The lowest classification accuracy belongs to the decision tree algorithm with 97.71%.

For logistic regression algorithm with the best classification accuracy, the confusion matrix in which color features are evaluated is given in Table 16. When the table is examined, the accuracy rates of Arborio, Basmati, Ipsala, Jasmine and Karacadag rice varieties are respectively, 98.95%, 99.06%, 100%, 99.10%, 99.17%. Figure 8 shows the accuracy rates of classification algorithms derived from color features for rice varieties used in the study.

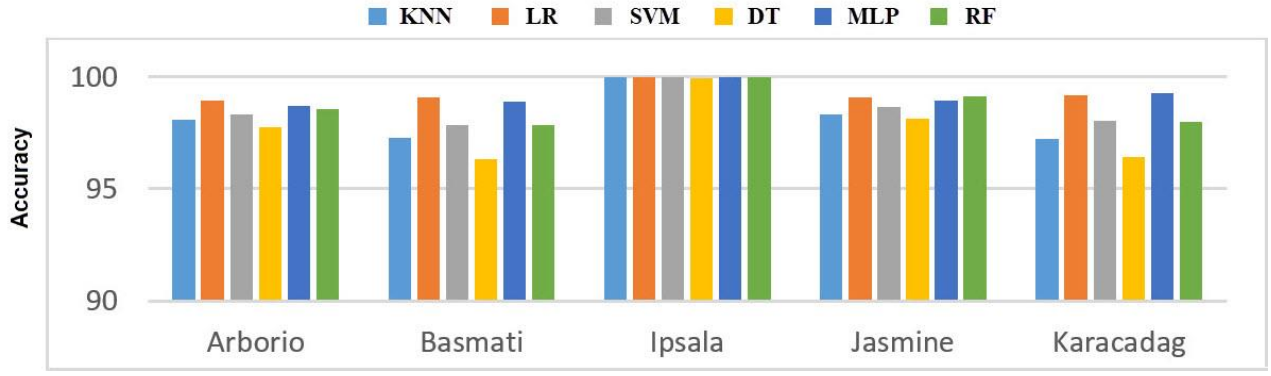

Figure 8- Accuracy rates of classification algorithms obtained from color features for all rice varieties used in the study

In logistic regression algorithm, Ipsala rice variety, which has 100% accuracy rate among varieties, also reaches the highest accuracy rates in other algorithms. Arborio and Basmati varieties, on the other hand, have a lower accuracy rate than other varieties.

Table 18- Confusion matrix obtained using morphological, shape and color features of all algorithms

		Algorithm	*Prediction Class*				
			Arborio	*Basmati*	*Ipsala*	*Jasmine*	*Karacadag*
Actual Class	**Arborio**	K-NN	**14,947**	0	0	34	19
		DT	**14,924**	0	4	38	34
		LR	**14,952**	4	1	31	12
		MLP	**14,971**	0	0	18	11
		RF	**14,969**	0	0	21	10
		SVM	**14,973**	0	0	18	9
	Basmati	K-NN	0	**14,963**	0	37	0
		DT	0	**14,954**	1	45	0
		LR	4	**14,975**	0	21	0
		MLP	0	**14,985**	0	15	0
		RF	0	**14,981**	0	19	0
		SVM	0	**14,946**	0	54	0
	Ipsala	K-NN	0	0	**15,000**	0	0
		DT	5	1	**14,990**	4	0
		LR	2	1	**14,995**	2	0
		MLP	0	0	**15,000**	0	0
		RF	1	0	**14,999**	0	0
		SVM	0	0	**15,000**	0	0
	Jasmine	K-NN	23	3	0	**14,973**	1
		DT	24	38	5	**14,932**	1
		LR	30	24	0	**14,945**	1
		MLP	9	4	0	**14,986**	1
		RF	16	3	0	**14,981**	0
		SVM	8	12	0	**14,979**	1
	Karacadag	K-NN	32	0	0	1	**14,967**
		DT	33	0	0	1	**14,966**
		LR	19	0	0	2	**14,979**
		MLP	12	0	0	0	**14,988**
		RF	21	0	0	0	**14,979**
		SVM	12	0	0	1	**14,987**

When Table 18 was examined, 74,850 pieces from K-NN algorithm, 74,766 pieces from DT algorithm, 74,846 pieces from LR algorithm, 74,930 pieces from MLP algorithm, 74,909 pieces from RF algorithm and 74,885 pieces from SVM algorithm rice grains were correctly classified.

For all algorithms used in the study, accuracy, error, precision, recall and F1-score average performance measurements and kappa coefficient values obtained by evaluating morphological, shape and color features together using confusion matrix are given in Table 19.

Table 19- Average performance measurements and kappa coefficient values of all algorithms used in classification for morphological, shape and color features

Performance Metrics	*Multi Layer Perceptron*	*Random Forest*	*Support Vector Machine*	*k-Nearest Neighbor*	*Logistic Regression*	*Decision Tree*
Accuracy (%)	99.91	99.88	99.85	99.80	99.79	99.69
Error (%)	0.09	0.12	0.15	0.20	0.21	0.31
Precision (%)	99.90	99.90	99.80	99.80	99.80	99.70
Recall (%)	99.90	99.90	99.80	99.80	99.80	99.70
F1-Score (%)	99.90	99.90	99.80	99.80	99.80	99.70
Kappa coefficient	0.999	0.999	0.998	0.998	0.997	0.996

When the average performance measurement values given in Table 19 are examined, it is seen that the classification accuracy for all algorithms is above 99%.

It belongs to multi layer perceptron algorithm with best classification accuracy 99.91%. The lowest classification accuracy belongs to the decision tree algorithm with 99.69%. Looking at the parameters of performance measurements, it seems that the multi-layer perceptron algorithm has the best values. In classification algorithms, the high F1-score value is proof that the algorithm performs well in terms of classification accuracy.

For multi layer perceptron algorithm with the best classification accuracy, the confusion matrix in which morphological, shape and color features are evaluated with together is given in Table 19. When the table is examined, the accuracy rates of Arborio, Basmati, Ipsala, Jasmine and Karacadag rice varieties are respectively, 99.81%, 99.99%, 100%, 99.91%, 99.92%. Figure 9 shows the accuracy rates of classification algorithms obtained for rice varieties used in the study.

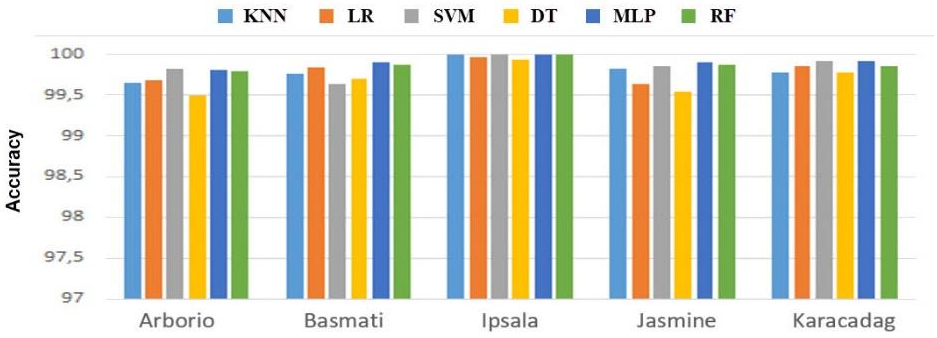

Figure 9- Accuracy rates of classification algorithms obtained from morphological, shape and color features for all rice varieties used in the study

In the multi-layer perceptron algorithm, Ipsala rice variety, which has a 100% accuracy rate among varieties, also reaches the highest accuracy rates in other algorithms. Arborio and Basmati varieties, on the other hand, have a lower accuracy rate than other varieties.

5. Conclusions

In this study, a total of 75,000 images of rice grains were obtained from 5 different rice varieties for the classification of rice grains. These images were pre-processed with the help of MATLAB software and were cleared of unwanted materials that may be present on the image and prepared for the feature extraction stage.

Firstly, 12 morphological features were extracted on the images that had been pre-processed before the classification phase. Afterwards, in addition to morphological features, 4 shape features were added and a total of 16 morphological and shape features were obtained. In addition, a total of 90 color features obtained from 5 different color space were extracted and a total of 106 features were obtained where morphological, shape and color features were evaluated together. Morphological features were obtained in MATLAB software using regionprops components, and shape features were obtained using these morphological features. RGB, HSV, L*a*b, YCbCr and XYZ color spaces were used for color features. With MATLAB software, conversion operations were performed to other color spaces using pixel values for each RGB image. After color conversion, a total of 90 color features were obtained for 5 color spaces using mean, standard deviation, skewness, kurtosis, entropy and wavelet decomposition components.

K-NN, DT, LR, MLP, RF and SVM algorithms, which are the most commonly used artificial intelligence techniques, were used for classification. Confusion Matrix inferences of algorithms were made and performance evaluation was performed. The number of cross-validation iteration folds used to control the generalization ability of algorithms was selected as 10.

The classification accuracy belonging to algorithms are given in Table 20 for morphological features, shape features evaluated with morphological features together, color features, and finally, shape and color features evaluated with morphological features together.

Table 20- Average classification accuracy percentages obtained from feature sets and algorithms used in the study (%)

Feature Sets	*Random Forest*	*Logistic Regression*	*Multi Layer Perceptron*	*Decision Tree*	*k-Nearest Neighbor*	*Support Vector Machine*
Morphological	**97.99**	97.83	97.38	97.82	97.15	97.02
Morphological and Shape	**98.04**	97.83	97.51	97.81	97.23	97.42
Color	98.70	**99.25**	99.15	97.71	98.17	98.57
Morphological, Shape and Color	99.88	99.79	**99.91**	99.69	99.80	99.85

Over 97% success was achieved in all algorithms by evaluating only morphological features. The highest classification accuracy belongs to the random forest algorithm with 97.99%. In order to increase the success obtained from morphological features, 16 pieces morphological and shape features were extracted by adding shape features. 98.04% accuracy was achieved by random forest algorithm as the highest classification accuracy. When the studies carried out in the literature were examined, it is thought that the success will increase even more after the classifications performed by adding color features in addition to morphological and shape features. For this reason, 90 color features were extracted from the color images of rice grains. 99.25% accuracy was obtained with logistics regression algorithm as the highest classification accuracy. Finally, a total of 106 features of morphological, shape and color features together were extracted, resulting in a classification accuracy of over 99% in all algorithms. The highest classification accuracy belongs to the multi-layer perceptron algorithm with 99.91%. F1-score values obtained from classification algorithms appear to be high. This is proof that the algorithms used perform well in terms of classification accuracy. In addition, when examining the kappa coefficient values used to measure the reliability of classification algorithms, it is possible to reach an interpretation that a very good level of compliance is achieved. In our classification results, it can be seen that the increase in the quality and number of features used in the study contributed positively to the success of classification. When the confusion matrix and performance measurement values of the algorithms used were examined, it was seen that the Ipsala variety reaches the highest performance values in all feature sets. The Arborio variety, on the other hand, has a lower accuracy rate than other varieties. The results show that this study can be successfully used to classify various varieties of rice.

6. Discussion

With 106 features used in the study, feature extractions can be made on other rice varieties. Using the data obtained, a machine can be designed that can perform calibration operations or separation of unwanted materials from varieties by designing an automatic image-taking system to distinguish rice varieties.

In the 106 features used in the study, the classification process can be performed with these features by identifying the features that are decisive. A database can be created for the features of rice by increasing the number of rice varieties. This database can be adapted to a mobile application and made available in the field of Agriculture. Through this application, determination of rice varieties, determination of physical features, etc. information can be accessed instantly.

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Resistance Response of Drought and Heat Tolerant Spring Wheat Lines against the Cereal Cyst Nematode, *Heterodera filipjevi*

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ABSTRACT
Wheat (*Triticum aestivum* L.) is extremely affected by several abiotic and biotic stress factors. Drought and/or heat alongside the parasitism of cereal cyst nematodes of the *Heterodera* genera can have a combined destructive impact on wheat. Solely, the cereal cyst nematode species *Heterodera filipjevi* can cause wheat yield losses of up to 50%. Several control measures have been implemented, yet the most economical and convenient control strategy is the use of resistant hosts. Therefore, the main aim of this study was to evaluate the resistant response of 257 spring wheat lines obtained from the International Maize and Wheat Improvement Centre to *Heterodera filipjevi* that might contain novel sources of resistance and be added as genetic resources for future breeding programs. Also, provide a base for future research to understand the relationship between nematode resistances and drought and heat tolerance. The results indicated that 11 wheat lines (4%) and 36 wheat lines (14%) were resistant and moderately resistant, respectively. High frequency of susceptible and highly susceptible lines and low frequency of resistant lines within this set was also recorded. The linear regression analysis between the number of cysts formed and the resistance response grouping showed a strong, positive, linear correlation. Log-linear regression analysis showed that there is a weak positive correlation between the yield of heat tolerant wheat lines and their resistance to the cyst nematodes as these lines showed tolerance, while there was a weak negative correlation of formed cyst nematodes on the yield of drought tolerant lines. This study was able to add new genetic sources of resistance to *Heterodera filipjevi* for upcoming breeding programs.

Keywords: Biotic stress, Breeding, Cereal crops, Plant-parasitic nematodes, Resistant source

1. Introduction

Bread wheat (*Triticum aestivum* L.) is a vital crop for many countries, contributing nearly to one-third of the total global food grain production (FAOSTAT 2020). Abiotic and biotic stress factors can interact together to adversely affect wheat yield and production worldwide (Lichtenthaler 1996; Afzal et al. 2015). In the wheat gene pool, there is an adequate genetic variation that can ensure continuous improvement of wheat adaptation to these stress factors (Lawlor & Cornic 2002; Trethowan & Mujeeb-Kazi 2008).

Climate change is a challenge facing humanity and its effects have been harmful to the agricultural industry. It is projected that countries near the equator will have a reduction in food production (Droogers & Aerts 2005). The International Water Management Institute (IWMI) study forecasts that wheat production in South Asia will decline by 50% by 2050 (De Fraiture et al. 2007). Studies indicated that increasing temperatures have negative effects on wheat yields in numerous regions of the world (Parry et al. 2004; Asseng et al. 2015; Zhao et al. 2017). It is going to be a challenge to increase or at least maintain the world's production of wheat to provide future generations with food needed to satisfy the demands of the increasing population. Also, it is a current and future challenge to find ways to reduce the impact of stress factors that decreases the yield of wheat such as plant-parasites (i.e., plant-parasitic nematodes) along with environmental factors (i.e., drought and heat).

Plant-parasitic nematodes that are the cause of agriculture production reduction, are regarded to be alarming. Despite their widespread compared to other pests, plant-parasitic nematodes are commonly very dangerous, stealthy, and costly to be managed and there is not enough detailed information or data on their economic impact (Webster 1987; Vaish 2017). Handoo (1998) valued the losses of international crop production due to nematode infection was around US$ 80 billion and McCarter (2009) estimated that the global economic loss due to nematode infection to be US$ 118 billion per year. The widespread of plant-parasitic nematodes on a majority of the vital crops, especially cereal cyst nematodes (CCNs) on wheat and their effect that

dramatically reduces crop yields has caught the awareness of governments and international organizations to find methods of management.

Nicol et al. (2011) reported that environmental conditions influence losses that are caused by CCNs and may exceed 90%. In association with other biotic and abiotic factors such as fungal pathogens, water stress, and heat, CCNs can have a synergistic destructive impact (Nicol et al. 2006). The species of CCN *avenae* complex *H. avenae*, *H. filipjevi* and *H. latipons* (Rivoal & Cook 1993; Nicol & Rivoal 2008; Akar et al. 2009; Dababat et al. 2015; Seid et al. 2021) are considered the most economically important species in West Asia, North Africa, and the Mediterranean (Nicol et al. 2011; Dababat et al. 2021). *H. filipjevi* can be found generally in China, Germany, India, Iran, Norway, Poland, Russia, Spain, Syria, Sweden, Tajikistan, Turkey, and the USA (Nicol et al. 2006). In Turkey, *H. filipjevi* causes yield losses of up to 50% in winter wheat under rainfed conditions (Nicol et al. 2006; Imren et al. 2019). While Hajihasani et al. (2010) reported that in Iran *H. filipjevi* causes approximately 48% yield losses in winter wheat under rainfed conditions. Additionally, Karimipour Fard et al. (2018) reported that *H. filipjevi* in field conditions significantly had a negative effect on grain yield (23% reduction) and growth parameters (plant height, number of tillers, root dry weight, root height and aerial shoot dry weight) in all of the tested three cultivars.

Cereal cyst nematodes management methods have been mainly attained by rotation with non-host crops, such as legumes and moderately resistant cultivars. Due to the multi-year survival nature of the cyst that protects the eggs, a crop rotation period of at least 2 years is needed to maintain population densities below the economic damage threshold (Bridge & Starr 2007). This might be considered a non-feasible and non-profitable way of management due to its time and cost in cultivating practices during crop rotation. Host resistance is a desirable alternative because it is less expensive, easy to be used once identified, and it has no environmental toxicity like nematicides, despite the successful use of nematicides to control nematodes (Williamson & Kumar 2006, Dababat & Fourie 2018). Dababat et al. (2014) stated that globally, the evolution of cultivars with genetic resistance plus genetic tolerance has been accomplished. Screens (resistant tests) have been established for almost every crop to present phenotypic data for their particular breeding program. These screens aim to find and identify new sources of resistance and also the identification of resistant progeny in segregating populations. Miniaturized screening test either by using the test tube method is considered to show the greatest accuracy regarding phenotypic reaction distinction (Blok et al. 2018).

The study aims to screen and evaluate a set of drought and heat tolerant international spring wheat lines for the resistance response to *H. filipjevi*. It is believed that this set is a unique set for CCN resistance because of their drought and heat tolerant nature, which is believed to have a relation with CCN resistance in general but needs other in-depth studies to establish this theory and our study is considered to be a starting point. It is also believed that this study would provide new resistant spring wheat lines against *H. filipjevi*, which might contain novel sources of resistance and be added as genetic resources for future breeding programs. Another primary expectation of the research is to provide a base for future research to try to understand the relationship between nematode resistances and drought and heat tolerance.

2. Material and Methods

2.1. Plant Materials

A set of 257 spring wheat lines that originated from various countries were obtained from the International Maize and Wheat Improvement Center (CIMMYT) in Mexico **(Supplementary Table 1).** This set has been screened and genotyped for drought and heat tolerance traits by CIMMYT–Mexico. The set was tested for cereal cyst nematode resistance response at the Transitional Zone Agricultural Research Institute (TZARI) in Eskisehir, Turkey (39° 46' 1.2612" N, 30° 24' 10.8282" E) and has been repeated in two independent experiments. Four well-known check lines for their resistance response to *H. filipjevi* were used as reference: 2 susceptible cultivars (Bezostaya and Kutluk-94) and 2 moderate resistant cultivars (Katea-1 and Sonmez-2001).

2.2. Nematode inoculum: collection, hatching, and identification

Soil samples were collected from a wheat field historically known to be infested with *H. filipjevi* in Çiçekdağı district in the province of Kırşehir, Turkey (39° 63' 80" N; 34° 46' 72" E). Cysts were extracted using Cobb's decanting and sieving method (Cobb, 1918). Cysts were handpicked from the organic matter residue under a dissecting microscope (Olympus SZ61). Then the cysts were surface sterilized with NaOCl (0.5%) for about 10 minutes and rinsed several times with distilled H_2O before being transferred onto a fine mesh (45 µm) placed in a glass petri dish and stored at 4 °C to enhance hatching. Freshly hatched second-stage juveniles (J2s) were used as an inoculum source. Species identification was previously validated by CIMMYT-Turkey using molecular methods from random individual cysts and identified as *H. filipjevi* (Pariyar et al. 2016a). To reinsure that there were no other species, morphological identification was done by using a light microscope (LEICA DM5500 B) along with imaging software Leica Application Suite (LAS V4.12) in the labs of the Faculty of Agricultural Sciences and Technology of Nigde Omer Halisdemir University (37° 56' 36.2" N; 34° 37' 42.4" E), as the fenestra of the cyst was the main aspect of species determination. The morphological measurements and characteristics were compared to previously published data of Siddiqi (2000); Handoo (2002); Subbotin & Baldwin (2010).

2.3. Assessment of wheat lines

Three representative spikes of a single wheat line were selected and threshed. About 15-20 similar and healthy seeds per line were selected and germinated on a moist filter paper in a Petri dish for 3 days at 22 °C and 70-80% of relative humidity. A single germinated seed of each line with identical sized radicles was selected and transplanted into RLC4-pine tubes (25 mm × 160 mm Ray Leach Cone-tainer™; Stuewe & Sons, Inc., USA) containing 100 g of a sterilized growing mixture containing sand, organic matter, and field soil (70:29:1, v/v/v). The tubes were placed in a 200-cell tray (RL200; Ray Leach Cone-tainer™; Stuewe & Sons, Inc., USA) with 3 replications per line arranged in a randomized block design. One day after transplanting, each tube was inoculated with 250 freshly hatched J2s suspended in 1 ml of water and injected into 3 holes of 2 cm depth made by a thin plastic rod around the stem base. Plants were kept under controlled conditions (25 °C, 70% RH and a photoperiod of 16 h) at TZARI. Fourteen weeks after nematode inoculation, plants were uprooted and cysts were extracted from both roots and soil of each plant as per Dababat et al. (2014). The resistance response of the screened wheat lines was determined and classified into five groups based on the mean number of cysts and females per plant (Dababat et al. 2016). The following grouping was used: 1) R = Resistant (fewer cysts and females/plant than the moderately resistant checks). 2) MR = Moderately resistant (as few cysts and females/plant as the moderately resistant checks). 3) MS = Moderately susceptible (significantly more cysts and females/plant than in the moderately resistant check, but not as many as in the susceptible checks). 4) S = Susceptible (as many cysts and females/plant as in the susceptible check and the number of cysts per root system considered damaging). 5) HS = Highly susceptible (more cysts and females/plant than in the susceptible check).

2.4. Statistical analysis

Descriptive statistical parameters (mean, standard error (SE) and standard deviation (SD)) of the number of cysts and females per line were calculated and compared with the check cultivars for their resistant response evaluation. Regression analysis was conducted to assess the correlation between the mean number of cysts and females and the sorting of the resistance response grouping and to calculate the best fitting equation, a polynomial regression analysis was used. Log-linear regression analysis was conducted to assess the correlation between the yield of drought and heat tolerant wheat lines and the mean number of formed cysts.

3. Results and Discussion

3.1. Assessment of wheat lines

The results of the screening evaluation of the 257 spring wheat lines showed that 11 lines (4.28%) were resistant, 36 lines (14%) were moderately resistant, 72 lines (28.02%) were moderately susceptible, 79 lines (30.74%) were susceptible and 59 lines (22.96%) were highly susceptible, as shown in Figure 1 which also indicates a high frequency of susceptible and highly susceptible lines and low frequency of resistant lines within this set of drought and heat tolerant lines and represents a histogram distribution of the mean number of cysts formed on the root systems and estimated kernel density plot of the mean number of cysts formed per plant.

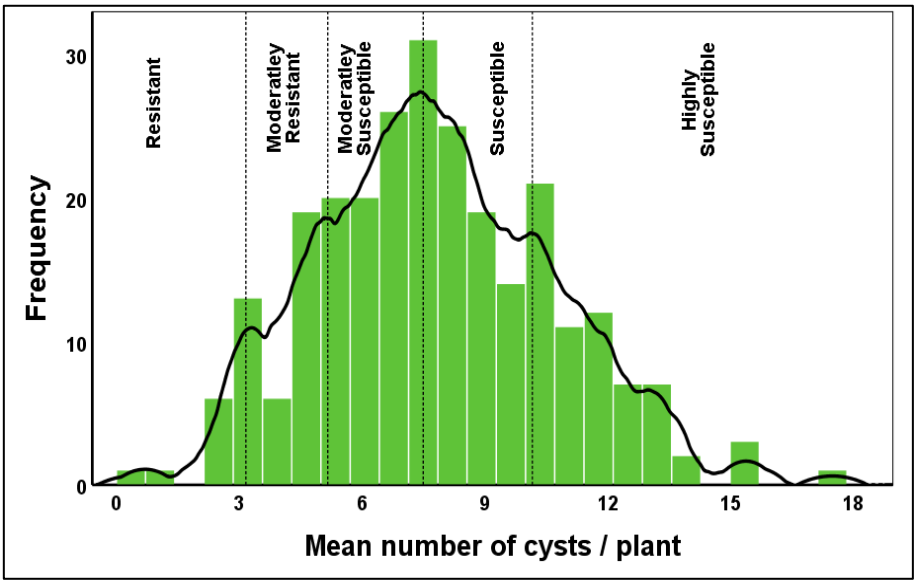

Figure 1- Histogram distribution and estimated kernel density plot of the mean number of cysts formed per plant, Frequency represents the number of wheat lines that cysts have formed on their root system between 0 – 18

3.2. Wheat lines resistant response grouping

To further assess the correlation between the mean number of cysts and females and the resistance response grouping of the wheat lines, linear regression analysis was used ($P < 0.05$, $R^2 = 0.958$). The data points to a strong, positive, linear correlation between the number of cysts and females formed and the resistance response grouping (Figure 2), which shows that the increase of the number of cysts formed leads to the categorization of the wheat lines from resistant to highly susceptible.

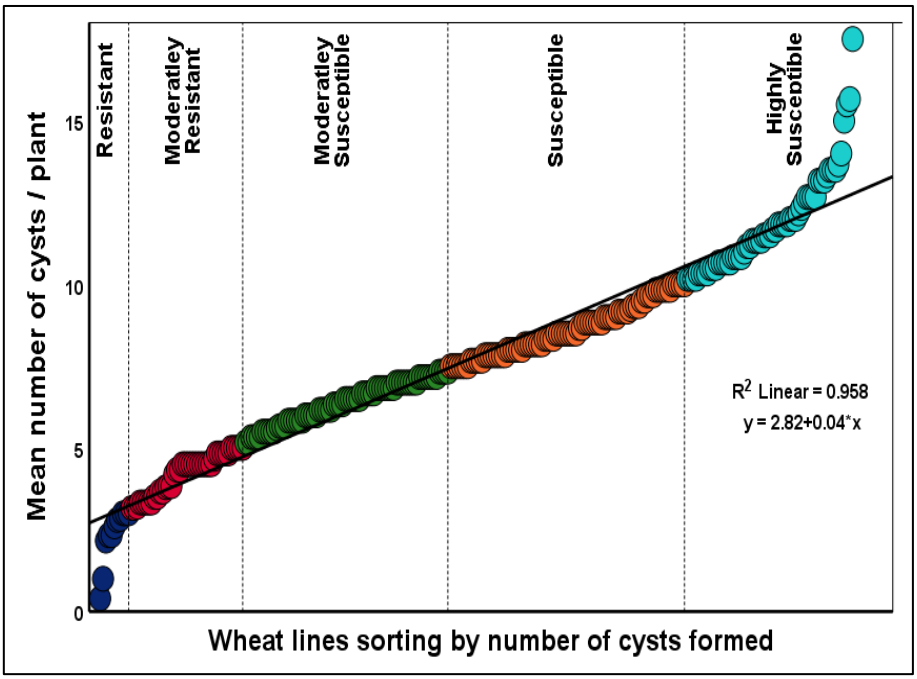

Figure 2- Linear regression of the mean number of cysts formed per plant and wheat lines sorting. Values are a mean of 2 trials, each with 3 replications. (P<0.05)

3.3. Source of resistance

After examining and comparing the parent materials of the wheat lines that have shown resistant and moderately resistant responses to all the spring wheat lines within this set, it has been found that there is no coherent pattern or indication that one parent tends to pass down resistance traits to their pedigree over another. As the parent material of the resistant and moderately resistant lines can be also found in the moderately susceptible, susceptible, and highly susceptible lines. So, the source of resistance of this set is due to different factors other than the dominant traits of a certain parent in resistant and moderately resistant lines (data not presented). Further analysis is required to be conducted to get a better idea of what the resistant sources are.

3.4. Effect of cyst nematodes on yield

In order to assess the effect of drought and heat on the relation between wheat yield and the mean number of formed cysts, Log-linear regression analysis was conducted (Figure 3). A negative relationship was noted between the number of formed cyst nematodes and the yield of drought tolerant wheat lines ($R^2 = 0.11$), while heat tolerant wheat lines seem to be more tolerant to the effect of cyst nematode formation as wheat yield values that were positively related to the number of formed cysts ($R^2 = 0.15$). Moreover, despite the noted negative effect of the number of formed cysts, the yield of drought tolerant lines was considered to be higher than the heat tolerant lines.

Breeding wheat for resistance against CCNs started in the 1970s (Brown & Ellis 1976) and has become one of the most effective and desirable methods of control especially against *H. filipjevi* to prevent yield losses. The use of resistant lines is desirable and the only enduring method present to control CCNs due to its low cost, being user-friendly and is acknowledged to not be harmful to the environment (Dababat et al. 2014; Williamson & Kumar 2006). Different control methods can be used but have limitations and there are very few reports related to wheat-nematode interaction. Goverse & Smant (2018) pointed out that the complete mechanism of resistance is still an enigma with incomplete knowledge on plant immunity to plant-parasitic nematodes. Specific genes for the resistance against *H. filipjevi* are yet to be identified despite some of the *Cre* genes have shown certain degrees of success against the nematode such as; *Cre8* and *CreR* which showed some levels of resistance (Imren et al. 2012). Toktay et al. (2012) screened resistant wheat lines containing the *Cre1* gene which showed different resistance responses to *H. filipjevi*. It has been recognized that some of the identified 12 *Cre* genes that are known as a resistant source in wheat to *H.*

avenae can show resistance to *H. filipjevi* and have shown success against other CCNs (Blok et al. 2018). To date, 16 different resistance genes to *H. avenae*, including 12 *Cre* genes; *Cre1*, *Cre2*, *Cre3*, *Cre4*, *Cre5*, *Cre6*, *Cre7*, *Cre8*, *Cre9 CreR*, *CreY* and *Cre3S* in wheat and its wild relatives and *Ha1*, *Ha2*, *Ha3*, *Ha4* genes in barley were reported (Bakker et al. 2006; Zhai et al. 2008; Smiley & Nicol 2009; Moens et al. 2018; Cui et al. 2020), which also might have resistance to *H. filipjevi*. Kimber & Feldman (1987) mentioned that wheat varieties showing resistance or tolerance responses have shown to provide resistance against a wide range of biotic and abiotic stress factors. There are some assumptions that there might be a strong connection between drought and heat tolerance with CCN resistance and is yet to be proven. Dababat et al. (2018) has addressed this matter, as it was mentioned that water stressed crops grown in arid and semi-arid regions that have the ability to secure adequate amounts of water can be severely weakened due to the effect of nematodes on the crops root system. Correspondingly, with our current findings, it has been found that there is a weak positive correlation between wheat lines with heat tolerance and the number of formed cysts (resistance response), as these lines showed more tolerance without negatively affecting their yield. Our findings also indicated that there is a weak negative correlation between drought tolerant wheat lines and number of formed cysts. This is most likely due to the genetic background of these lines.

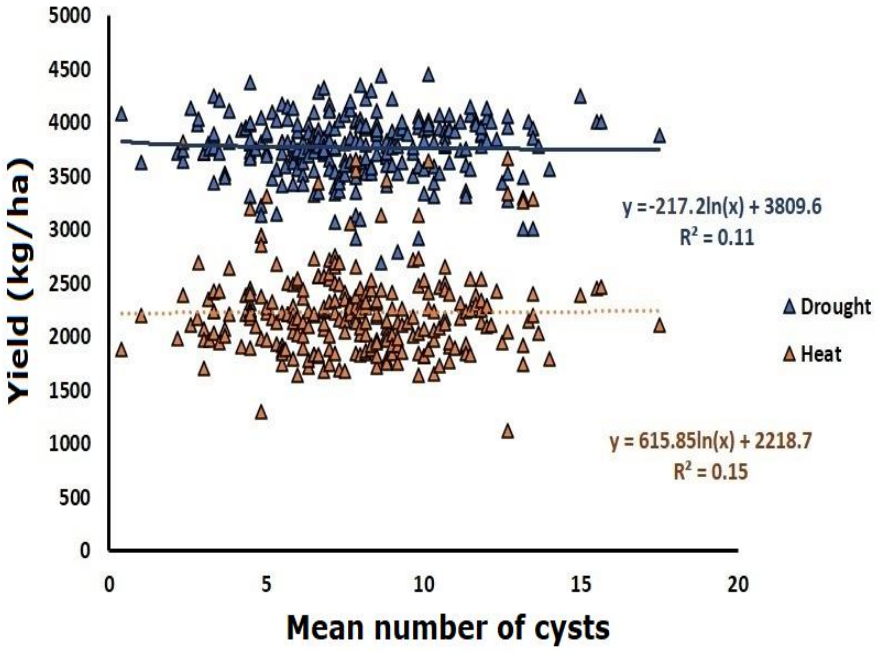

Figure 3- Log-linear regressions of wheat yield and mean number of formed cysts in 2 experiments. All R^2 values were significant at P<0.05. Values are means of 1 years, each with 6 replicates per line. The link represents the predicted logarithmic regression model. Equations were represented on kg ha^{-1}

The study has managed to find 11 resistant and 36 moderately resistant spring wheat lines, noting that the screened wheat set originates from a diverse genetic background. Different screening studies that have been done on wheat accessions originating from different sources have shown resistance to *H. filipjev*i (Toktay et al. 2012; Dababat et al. 2014; Pariyar et al. 2016a, b; Yavuzaslanoglu et al. 2016; Dababat 2019).

There is a difficulty to truly compare the result of this study to other similar or related studies despite using the same experimental setup, as there are a lot of variables between the experiments. One of the main reasons regarding the difficulty in comparison is due to different categorizing of the resistant response groups that rely on the average number of formed cysts and females on the root system per plant and comparing them to the check wheat cultivars with known resistance response. Pariyar et al. (2016b) and Yavuzaslanoglu et al. (2016) have used a different arrangement of the average number of formed cysts and females/plant assigned to the resistant groups with reliance on the check wheat cultivars response. Zhang et al. (2012) even used a different method of sorting which relied on the relative resistance index (RRI); RRI = [1 – (the mean number of white females per plant on a tested line/the mean number of white females per Wenmai 19 check plant).

Also, a point of difference is the experiment setup conditions, Dababat (2019) conducted his experiment under field conditions, Hajihasani et al. (2010) experiment was conducted in pots under field conditions, while Zhang et al. (2012) conducted his experiment in greenhouse conditions. Toktay et al. (2012) and (Pariyar et al. 2016a, b) conducted their study in a growth chamber under a controlled condition and with resembling methodology, so this can be a point of similarity to compare the results.

Generally, when screening wheat accessions for their resistant response almost all studies have obtained a low percentage of resistant accessions from the total screening. This study managed to find a total of 4.28% resistant lines from 257 lines, Dababat

(2019) tested 35 resistant lines that were obtained from the previous screening of thousands of wheat accessions. Pariyar et al. (2016a) found only 1% resistant accessions from a total of 161 accessions and in another study by Pariyar et al. (2016b) found only 1% resistance of wheat accessions from a total of 291 accessions.

In this type of study, it is expected to find a very low percentage of resistance among the screened accessions. When comparing the results of this study in terms of the percentage of resistance accessions found with the other studies, this study is considered to have a noticeably high percentage of success.

A possible reason for our current finding is the possibility that resistant and moderately resistant lines may have a relationship with the drought and heat tolerant QTLs as assumed. The screened set may also contain a source of *Cre* genes; like *Cre1* as in Toktay et al. (2012) study, *Cre8* or *CreR* in Imren et al. (2012) study, *Cre5* as in Dababat et al. (2014) study, or the same QTLs Pariyar et al. (2016a) has identified or due to the presence of new sources of resistance. This matter cannot be confirmed in this study but future analysis is required to obtain a clearer idea of why these specific lines showed a resistant response to *H. filipjevi.*

4. Conclusions

It is notable that despite the abundance of screening studies related to nematode resistance, this study is the first to evaluate the resistant response of *H. filipjevi* against wheat genotyped with drought and heat tolerance. It has been managed to add 11 wheat lines with resistance and 36 lines with moderate resistance to *H. filipjevi* as genetic resources for future wheat breeding programs. This might be good for helping advance resistance studies to CCNs in general but specifically to improve resistance against *H. filipjevi*. It is expected that the study can provide supplementary data with previous work by Pariyar et al. (2016a) for future studies concerned with finding resistant genes to *H. filipjevi*.

Although resistant and moderately resistant lines to *H. filipjevi* were found, it should be noted that further assessment of these lines is recommended to fully verify their resistant and moderately resistant status. Also, it is recommended that these lines should be screened for resistance response to other *Heterodera* species, mainly *H. avenae* and *H. latipons* to obtain wheat lines with resistance to more than one *Heterodera* species.

This study supports Dababat et al. (2018) remarks, statistically, that there is a correlation between drought and heat tolerance with CCN resistance despite being a weak one but establishes a foundation for more detailed studies.

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Supplementary Table1: List of Drought and Heat Tolerant Spring Wheat lines and their Resistant Response to the CCN Heterodera filipjevi. (SD) Standard Deviation, (SE) Standard Error, (CID) Cross ID

ENTRY NUMBER	*CID*	*PEDIGREE (CROSS)*	*SELECTION HISTORY*	*(MEAN) CYSTS AND FEMALES*	*SD*	*SE*	*RESISTANT RESPONSE*
265	620759	PERSIA-88/3/PBW343*2/KUKUNA*2//FRTL/PIFED/4/QUAIU #1	SDSS13Y00190T-0B-0Y-0M-0Y-0B-71Y	0.40	0.55	3.11	R
210	620687	CROC_1/AE.SQUARROSA (436)//KACHU/3/BAJ #1	SDSS13Y00118T-0B-0Y-0M-0Y-0B-113Y	1.00	0.89	0.37	R
70	620629	H-1546/NELOKI/3/ATTILA*2/PBW65//MURGA	SDSS13Y00060T-0B-0Y-0M-0Y-0B-31Y	2.17	1.33	1.14	R
203	620683	H-1601/NAVJ07//KACHU	SDSS13Y00114T-0B-0Y-0M-0Y-0B-106Y	2.33	1.21	0.84	R
264	620749	IG 122743/NAVJ07//KACHU	SDSS13Y00180T-0B-0Y-0M-0Y-0B-33Y	2.33	0.82	0.33	R
54	620613	IG 1505/4/PRL/2*PASTOR//PBW343*2/KUKUNA/3/ROLF07/5/NELOKI	SDSS13Y00044T-0B-0Y-0M-0Y-0B-31Y	2.60	2.41	2.56	R
49	620609	DOY1/AE.SQUARROSA (447)/4/PRL/2*PASTOR//PBW343*2/KUKUNA/3/ROLF07/5/NELOKI	SDSS13Y00040T-0B-0Y-0M-0Y-0B-43Y	2.80	2.28	2.83	R
263	620748	IG 122741/NAVJ07//KACHU	SDSS13Y00179T-0B-0Y-0M-0Y-0B-28Y	2.83	1.72	1.14	R
42	620600	H-1357/8/CNDO/R143//ENTE/MEXI_2/3/AEGILOPS SQUARROSA (TAUS)/4/WEAVER/5/PICUS/6/TROST/7/TACUPETO F2001/9/KAUZ//ALTAR 84/AOS/3/PASTOR/4/MILAN/CUPE//SW89.3064/5/KIRITATI	SDSS13Y00031T-0B-0Y-0M-0Y-0B-41Y	3.00	1.79	1.32	R
251	620729	IG 122139/NAVJ07//KACHU	SDSS13Y00160T-0B-0Y-0M-0Y-0B-38Y	3.00	1.90	1.18	R
254	620736	IG 122627/6/KAUZ//ALTAR 84/AOS/3/PASTOR/4/MILAN/CUPE//SW89.3064/5/KIRITATI/7/SW89.5277/BORL95//SKAUZ/3/PRL/2*PASTOR/4/HEILO	SDSS13Y00167T-0B-0Y-0M-0Y-0B-38Y	3.00	1.41	1.51	R
128	620641	68.111/RGB-U//WARD RESEL/3/STIL/4/AE. SQUARROSA (630)/5/BORL14/6/COPIO	SDSS13Y00072T-0B-0Y-0M-0Y-0B-58Y	3.17	1.60	0.65	MR
KATEA (MR)		KHEBROS/BEZOSTAYA-1		3.17	2.71	1.11	MR
81	620631	H-1699/NELOKI/3/ATTILA*2/PBW65//MURGA	SDSS13Y00062T-0B-0Y-0M-0Y-0B-23Y	3.20	2.28	2.76	MR
69	620629	H-1546/NELOKI/3/ATTILA*2/PBW65//MURGA	SDSS13Y00060T-0B-0Y-0M-0Y-0B-30Y	3.33	1.97	0.80	MR
157	620652	PERSIA-7/COPIO/3/KACHU #1/KIRITATI//KACHU	SDSS13Y00083T-0B-0Y-0M-0Y-0B-65Y	3.33	2.07	2.03	MR
225	620690	GAN/AE.SQUARROSA (206)//KACHU/3/BAJ #1	SDSS13Y00121T-0B-0Y-0M-0Y-0B-83Y	3.33	1.51	1.41	MR
250	620729	IG 122139/NAVJ07//KACHU	SDSS13Y00160T-0B-0Y-0M-0Y-0B-34Y	3.33	2.07	1.58	MR
257	620746	IG 122738/3/PBW343*2/KUKUNA*2//FRTL/PIFED/4/QUAIU #1	SDSS13Y00177T-0B-0Y-0M-0Y-0B-54Y	3.33	2.16	0.88	MR
57	620615	IG 122145/4/PRL/2*PASTOR//PBW343*2/KUKUNA/3/ROLF07/5/NELOKI	SDSS13Y00046T-0B-0Y-0M-0Y-0B-38Y	3.50	2.35	1.41	MR
256	620736	IG 122627/6/KAUZ//ALTAR 84/AOS/3/PASTOR/4/MILAN/CUPE//SW89.3064/5/KIRITATI/7/SW89.5277/BORL95//SKAUZ/3/PRL/2*PASTOR/4/HEILO	SDSS13Y00167T-0B-0Y-0M-0Y-0B-43Y	3.50	1.64	1.28	MR
208	620687	CROC_1/AE.SQUARROSA (436)//KACHU/3/BAJ #1	SDSS13Y00118T-0B-0Y-0M-0Y-0B-101Y	3.67	4.97	2.03	MR
261	620748	IG 122741/NAVJ07//KACHU	SDSS13Y00179T-0B-0Y-0M-0Y-0B-22Y	3.67	2.50	1.23	MR
SONMEZ (MR)		BEZOSTAYA-1//BEZOSTAYA-1/TEVERE/3/KREMENA/LOVRIN-29/4/KATYA-1[3669]		3.80	2.17	0.97	MR
87	620633	IG 131672/3/ATTILA*2/PBW65//MURGA/4/BORL14	SDSS13Y00064T-0B-0Y-0M-0Y-0B-60Y	3.83	2.23	1.68	MR
227	620693	D67.2/PARANA 66.270//AE.SQUARROSA (506)/3/KACHU/4/BAJ #1	SDSS13Y00124T-0B-0Y-0M-0Y-0B-49Y	3.83	1.47	0.60	MR

Supplementary Table2(Continue): List of Drought and Heat Tolerant Spring Wheat lines and their Resistant Response to the CCN Heterodera filipjevi. (SD) Standard Deviation, (SE) Standard Error, (CID) Cross ID

ENTRY NUMBER	*CID*	*PEDIGREE (CROSS)*	*SELECTION HISTORY*	*(MEAN) CYSTS AND FEMALES*	*SD*	*SE*	*RESISTANT RESPONSE*
174	**620655**	**CETA/AE.SQUARROSA (872)/3/KACHU #1/ KIRITATI//KACHU/4/PBW343*2/KUKUNA*2//FRTL/PIFED**	**SDSS13Y00086T-0B-0Y-0M-0Y-0B-58Y**	**4.20**	**2.49**	**3.58**	**MR**
143	620646	IWA8611400/BORL14//COPIO	SDSS13Y00077T-0B-0Y-0M-0Y-0B-112Y	4.33	2.66	1.09	MR
219	620689	CROC_1/AE.SQUARROSA (176)//KACHU/3/BAJ #1	SDSS13Y00120T-0B-0Y-0M-0Y-0B-87Y	4.33	2.73	1.63	MR
127	620641	68.111/RGB-U//WARD RESEL/3/STIL/4/AE. SQUARROSA (630)/5/BORL14/6/COPIO	SDSS13Y00072T-0B-0Y-0M-0Y-0B-57Y	4.50	2.95	1.54	MR
144	620646	IWA8611400/BORL14//COPIO	SDSS13Y00077T-0B-0Y-0M-0Y-0B-113Y	4.50	3.02	1.89	MR
145	620647	T.DICOCCON PI94624/AE.SQUARROSA (454)// COPIO/3/KACHU #1/KIRITATI//KACHU	SDSS13Y00078T-0B-0Y-0M-0Y-0B-71Y	4.50	2.74	1.69	MR
168	620653	PERSIA-21/COPIO/3/KACHU #1/KIRITATI//KACHU	SDSS13Y00084T-0B-0Y-0M-0Y-0B-75Y	4.50	2.88	1.61	MR
169	620653	PERSIA-21/COPIO/3/KACHU #1/KIRITATI//KACHU	SDSS13Y00084T-0B-0Y-0M-0Y-0B-77Y	4.50	2.81	1.96	MR
189	620665	GARZA/BOY//AE.SQUARROSA (281)/3/ PBW343*2/KUKUNA*2//FRTL/PIFED/4/QUAIU #1	SDSS13Y00096T-0B-0Y-0M-0Y-0B-62Y	4.50	2.81	1.34	MR
199	620680	IG 41243/NAVJ07//KACHU	SDSS13Y00111T-0B-0Y-0M-0Y-0B-58Y	4.50	2.88	1.18	MR
239	620699	IG 41506/3/PBW343*2/KUKUNA*2//FRTL/ PIFED/4/QUAIU #1	SDSS13Y00130T-0B-0Y-0M-0Y-0B-71Y	4.50	3.15	1.28	MR
247	620718	IG 43238/NAVJ07//KACHU	SDSS13Y00149T-0B-0Y-0M-0Y-0B-5Y	4.50	2.88	1.48	MR
273	620785	IWA8614378/NAVJ07//KACHU	SDSS13Y00216T-0B-0Y-0M-0Y-0B-119Y	4.50	2.35	0.96	MR
SONMEZ (MR)		BEZOSTAYA-1//BEZOSTAYA-1/TEVERE/3/ KREMENA/LOVRIN-29/4/KATYA-1[3669]		4.50	2.07	0.85	MR
160	620652	PERSIA-7/COPIO/3/KACHU #1/KIRITATI//KACHU	SDSS13Y00083T-0B-0Y-0M-0Y-0B-71Y	4.67	2.34	0.95	MR
170	620653	PERSIA-21/COPIO/3/KACHU #1/KIRITATI//KACHU	SDSS13Y00084T-0B-0Y-0M-0Y-0B-81Y	4.83	3.37	1.92	MR
215	620687	CROC_1/AE.SQUARROSA (436)//KACHU/3/BAJ #1	SDSS13Y00118T-0B-0Y-0M-0Y-0B-137Y	4.83	4.45	1.82	MR
229	620694	INDIA-59/KACHU//BAJ #1	SDSS13Y00125T-0B-0Y-0M-0Y-0B-79Y	4.83	2.56	1.05	MR
246	620710	IG 41735/NAVJ07//KACHU	SDSS13Y00141T-0B-0Y-0M-0Y-0B-78Y	4.83	4.79	1.96	MR
278	620786	IWA8612701/6/KAUZ//ALTAR 84/AOS/3/PASTOR /4/MILAN/CUPE//SW89.3064/5/KIRITATI/7/SW89.5277/BORL95//SKAUZ/3/PRL/2*PASTOR/4/HEILO	SDSS13Y00217T-0B-0Y-0M-0Y-0B-75Y	4.83	2.32	0.95	MR
165	620652	PERSIA-7/COPIO/3/KACHU #1/KIRITATI//KACHU	SDSS13Y00083T-0B-0Y-0M-0Y-0B-79Y	5.00	2.76	1.53	MR
166	620652	PERSIA-7/COPIO/3/KACHU #1/KIRITATI//KACHU	SDSS13Y00083T-0B-0Y-0M-0Y-0B-81Y	5.00	2.76	1.13	MR
221	620689	CROC_1/AE.SQUARROSA (176)//KACHU/3/BAJ #1	SDSS13Y00120T-0B-0Y-0M-0Y-0B-100Y	5.00	3.03	1.81	MR
241	620699	IG 41506/3/PBW343*2/KUKUNA*2// FRTL/PIFED/4/QUAIU #1	SDSS13Y00130T-0B-0Y-0M-0Y-0B-75Y	5.00	5.14	2.10	MR
KATEA (MR)		KHEBROS/BEZOSTAYA-1		5.00	2.58	1.29	MR
20	620583	H-1624/BAJ #1//SUP152	SDSS13Y00014T-0B-0Y-0M-0Y-0B-32Y	5.17	2.64	1.97	MS
29	620588	H-1311/3/FRET2*2/SHAMA//KACHU/4/ HUW234+LR34/PRINIA*2//KIRITATI	SDSS13Y00019T-0B-0Y-0M-0Y-0B-4Y	5.17	2.32	0.95	MS
30	620588	H-1311/3/FRET2*2/SHAMA//KACHU/4/ HUW234+LR34/PRINIA*2//KIRITATI	SDSS13Y00019T-0B-0Y-0M-0Y-0B-7Y	5.33	3.14	1.61	MS
114	620640	68.111/RGB-U//WARD RESEL/3/STIL/4/AE.SQUARROSA (628)/5/BORL14/6/COPIO	SDSS13Y00071T-0B-0Y-0M-0Y-0B-46Y	5.33	3.61	1.48	MS
137	620645	IWA8612416/BORL14//COPIO	SDSS13Y00076T-0B-0Y-0M-0Y-0B-46Y	5.33	3.01	1.91	MS
244	620710	IG 41735/NAVJ07//KACHU	SDSS13Y00141T-0B-0Y-0M-0Y-0B-67Y	5.33	2.80	1.15	MS
148	620648	T.DICOCCON PI94625/AE.SQUARROSA (372)// COPIO/3/KACHU #1/KIRITATI//KACHU	SDSS13Y00079T-0B-0Y-0M-0Y-0B-102Y	5.50	3.15	1.61	MS
159	620652	PERSIA-7/COPIO/3/KACHU #1/KIRITATI//KACHU	SDSS13Y00083T-0B-0Y-0M-0Y-0B-69Y	5.50	3.78	1.54	MS
184	620662	YAV79//DACK/RABI/3/SNIPE/4/AE.SQUARROSA (477)/5/KACHU #1/KIRITATI//KACHU/6/PBW343*2/ KUKUNA*2//FRTL/PIFED	SDSS13Y00093T-0B-0Y-0M-0Y-0B-87Y	5.50	6.47	2.64	MS

Supplementary Table3(Continue): List of Drought and Heat Tolerant Spring Wheat lines and their Resistant Response to the CCN Heterodera filipjevi. (SD) Standard Deviation, (SE) Standard Error, (CID) Cross ID

ENTRY NUMBER	*CID*	*PEDIGREE (CROSS)*	*SELECTION HISTORY*	*(MEAN) CYSTS AND FEMALES*	*SD*	*SE*	*RESISTANT RESPONSE*
262	**620748**	**IG 122741/NAVJ07//KACHU**	**SDSS13Y00179T-0B-0Y-0M-0Y-0B-27Y**	**5.50**	**2.59**	**1.06**	**MS**
271	620783	IWA8612134/NAVJ07//KACHU	SDSS13Y00214T-0B-0Y-0M-0Y-0B-88Y	5.50	3.78	1.89	MS
110	620638	LOCAL RED/AE.SQUARROSA (223)//BORL14/3/COPIO	SDSS13Y00069T-0B-0Y-0M-0Y-0B-62Y	5.60	3.85	3.83	MS
47	620603	IG 42147/6/KAUZ//ALTAR 84/AOS/3/PASTOR/4/MILAN/CUPE//SW89.3064/5/KIRITATI/7/SW89.5277/BORL95//SKAUZ/3/PRL/2*PASTOR/4/HEILO	SDSS13Y00034T-0B-0Y-0M-0Y-0B-48Y	5.67	3.20	1.31	MS
181	620661	YAV79//DACK/RABI/3/SNIPE/4/AE.SQUARROSA (460)/5/KACHU #1/KIRITATI//KACHU/6/PBW343*2/KUKUNA*2//FRTL/PIFED	SDSS13Y00092T-0B-0Y-0M-0Y-0B-99Y	5.67	3.56	1.45	MS
234	620695	INDIA-107/KACHU//BAJ #1	SDSS13Y00126T-0B-0Y-0M-0Y-0B-108Y	5.67	3.83	1.56	MS
88	620633	IG 131672/3/ATTILA*2/PBW65//MURGA/4/BORL14	SDSS13Y00064T-0B-0Y-0M-0Y-0B-70Y	5.83	3.76	1.92	MS
99	620635	INDIA-50/3/ATTILA*2/PBW65//MURGA/4/BORL14	SDSS13Y00066T-0B-0Y-0M-0Y-0B-62Y	5.83	3.71	1.51	MS
216	620687	CROC_1/AE.SQUARROSA (436)//KACHU/3/BAJ #1	SDSS13Y00118T-0B-0Y-0M-0Y-0B-139Y	5.83	4.17	1.70	MS
248	620726	IG 107128/4/PRL/2*PASTOR//PBW343*2/KUKUNA/3/ROLF07/5/NELOKI	SDSS13Y00157T-0B-0Y-0M-0Y-0B-47Y	5.83	3.60	1.68	MS
270	620777	IWA 8602098/NAVJ07//KACHU	SDSS13Y00208T-0B-0Y-0M-0Y-0B-46Y	5.83	5.95	2.43	MS
272	620785	IWA8614378/NAVJ07//KACHU	SDSS13Y00216T-0B-0Y-0M-0Y-0B-116Y	5.83	4.02	2.32	MS
33	620594	INDIA-223/7/SHA7/VEE#5/5/VEE#8//JUP/BJY/3/F3.71/TRM/4/2*WEAVER/6/SKAUZ/PARUS//PARUS/8/CNDO/R143//ENTE/MEXI_2/3/AEGILOPS SQUARROSA (TAUS)/4/WEAVER/5/PICUS/6/TROST/7/TACUPETO F2001	SDSS13Y00025T-0B-0Y-0M-0Y-0B-24Y	6.00	6.51	2.66	MS
41	620600	H-1357/8/CNDO/R143//ENTE/MEXI_2/3/AEGILOPS SQUARROSA (TAUS)/4/WEAVER/5/PICUS/6/TROST/7/TACUPETO F2001/9/KAUZ//ALTAR 84/AOS/3/PASTOR/4/MILAN/CUPE//SW89.3064/5/KIRITATI	SDSS13Y00031T-0B-0Y-0M-0Y-0B-40Y	6.00	6.39	2.61	MS
67	620629	H-1546/NELOKI/3/ATTILA*2/PBW65//MURGA	SDSS13Y00060T-0B-0Y-0M-0Y-0B-23Y	6.00	8.27	3.38	MS
202	620683	H-1601/NAVJ07//KACHU	SDSS13Y00114T-0B-0Y-0M-0Y-0B-104Y	6.00	4.15	2.35	MS
222	620690	GAN/AE.SQUARROSA (206)//KACHU/3/BAJ #1	SDSS13Y00121T-0B-0Y-0M-0Y-0B-54Y	6.00	3.95	1.61	MS
204	620684	MEX94.30.10/NAVJ07//KACHU	SDSS13Y00115T-0B-0Y-0M-0Y-0B-98Y	6.17	3.37	1.38	MS
206	620685	ARLIN/AE.SQUARROSA (283)//KACHU/3/BAJ #1	SDSS13Y00116T-0B-0Y-0M-0Y-0B-68Y	6.17	4.26	1.74	MS
218	620688	AE.SQUARROSA (1029)/DVERD_2//KACHU/3/BAJ #1	SDSS13Y00119T-0B-0Y-0M-0Y-0B-69Y	6.17	3.97	2.06	MS
220	620689	CROC_1/AE.SQUARROSA (176)//KACHU/3/BAJ #1	SDSS13Y00120T-0B-0Y-0M-0Y-0B-92Y	6.17	4.17	1.70	MS
275	620785	IWA8614378/NAVJ07//KACHU	SDSS13Y00216T-0B-0Y-0M-0Y-0B-123Y	6.17	3.31	1.94	MS
34	620594	INDIA-223/7/SHA7/VEE#5/5/VEE#8//JUP/BJY/3/F3.71/TRM/4/2*WEAVER/6/SKAUZ/PARUS//PARUS/8/CNDO/R143//ENTE/MEXI_2/3/AEGILOPS SQUARROSA (TAUS)/4/WEAVER/5/PICUS/6/TROST/7/TACUPETO F2001	SDSS13Y00025T-0B-0Y-0M-0Y-0B-27Y	6.33	6.47	2.64	MS
60	620620	IG 122193/4/PRL/2*PASTOR//PBW343*2/KUKUNA/3/ROLF07/5/NELOKI	SDSS13Y00051T-0B-0Y-0M-0Y-0B-71Y	6.33	4.23	1.73	MS
78	620630	H-1694/NELOKI/3/ATTILA*2/PBW65//MURGA	SDSS13Y00061T-0B-0Y-0M-0Y-0B-48Y	6.33	4.32	1.76	MS
130	620641	68.111/RGB-U//WARD RESEL/3/STIL/4/AE.SQUARROSA (630)/5/BORL14/6/COPIO	SDSS13Y00072T-0B-0Y-0M-0Y-0B-62Y	6.33	3.44	1.89	MS
23	620583	H-1624/BAJ #1//SUP152	SDSS13Y00014T-0B-0Y-0M-0Y-0B-43Y	6.50	4.55	2.69	MS
64	620621	IG 122196/4/PRL/2*PASTOR//PBW343*2/KUKUNA/3/ROLF07/5/NELOKI	SDSS13Y00052T-0B-0Y-0M-0Y-0B-88Y	6.50	8.02	3.27	MS
77	620630	H-1694/NELOKI/3/ATTILA*2/PBW65//MURGA	SDSS13Y00061T-0B-0Y-0M-0Y-0B-39Y	6.50	4.51	1.84	MS
155	620650	IG 41620/COPIO/3/KACHU #1/KIRITATI//KACHU	SDSS13Y00081T-0B-0Y-0M-0Y-0B-64Y	6.50	1.05	0.43	MS
158	620652	PERSIA-7/COPIO/3/KACHU #1/KIRITATI//KACHU	SDSS13Y00083T-0B-0Y-0M-0Y-0B-66Y	6.50	4.51	2.40	MS

Supplementary Table4(Continue): List of Drought and Heat Tolerant Spring Wheat lines and their Resistant Response to the CCN Heterodera filipjevi. (SD) Standard Deviation, (SE) Standard Error, (CID) Cross ID

ENTRY NUMBER	*CID*	*PEDIGREE (CROSS)*	*SELECTION HISTORY*	*(MEAN) CYSTS AND FEMALES*	*SD*	*SE*	*RESISTANT RESPONSE*
274	**620785**	**IWA8614378/NAVJ07//KACHU**	**SDSS13Y00216T-0B-0Y-0M-0Y-0B-121Y**	**6.50**	**3.73**	**1.52**	**MS**
124	620641	68.111/RGB-U//WARD RESEL/3/STIL/4/ AE.SQUARROSA (630)/5/BORL14/6/COPIO	SDSS13Y00072T-0B-0Y-0M-0Y-0B-51Y	6.67	3.67	1.50	MS
146	620647	T.DICOCCON PI94624/AE.SQUARROSA (454)// COPIO/3/KACHU #1/KIRITATI//KACHU	SDSS13Y00078T-0B-0Y-0M-0Y-0B-85Y	6.67	3.72	2.17	MS
211	620687	CROC_1/AE.SQUARROSA (436)//KACHU/3/BAJ #1	SDSS13Y00118T-0B-0Y-0M-0Y-0B-123Y	6.67	6.65	2.72	MS
249	620726	IG 107128/4/PRL/2*PASTOR//PBW343*2/KUKUNA /3/ROLF07/5/NELOKI	SDSS13Y00157T-0B-0Y-0M-0Y-0B-50Y	6.67	8.89	3.63	MS
277	620786	IWA8612701/6/KAUZ//ALTAR84/AOS/3/PASTOR/4/ MILAN/CUPE//SW89.3064/5/KIRITATI/7/SW89.5277/ BORL95//SKAUZ/3/PRL/2*PASTOR/4/HEILO	SDSS13Y00217T-0B-0Y-0M-0Y-0B-63Y	6.67	4.41	1.80	MS
39	620595	CHIH95.4.6/7/SHA7/VEE#5/5/VEE#8//JUP/BJY/3/F3.7 1/TRM/4/2*WEAVER/6/SKAUZ/PARUS//PARUS/8/C NDO/R143//ENTE/MEXI_2/3/AEGILOPS QUARROSA (TAUS)/4/WEAVER/5/PICUS/6/TROST/7/TACUPETO F2001	SDSS13Y00026T-0B-0Y-0M-0Y-0B-52Y	6.83	7.55	3.08	MS
82	620631	H-1699/NELOKI/3/ATTILA*2/PBW65//MURGA	SDSS13Y00062T-0B-0Y-0M-0Y-0B-26Y	6.83	4.40	2.14	MS
109	620638	LOCAL RED/AE.SQUARROSA (223)//BORL14/3/COPIO	SDSS13Y00069T-0B-0Y-0M-0Y-0B-56Y	6.83	7.33	2.99	MS
139	620645	IWA8612416/BORL14//COPIO	SDSS13Y00076T-0B-0Y-0M-0Y-0B-49Y	6.83	3.87	2.21	MS
217	620687	CROC_1/AE.SQUARROSA (436)//KACHU/3/BAJ #1	SDSS13Y00118T-0B-0Y-0M-0Y-0B-145Y	6.83	3.43	1.40	MS
236	620698	IG 41474/NAVJ07//KACHU	SDSS13Y00129T-0B-0Y-0M-0Y-0B-46Y	6.83	4.31	2.14	MS
253	620736	IG 122627/6/KAUZ//ALTAR 84/AOS/3/PASTOR/4/ MILAN/CUPE//SW89.3064/5/KIRITATI/7/SW89.5277/ BORL95//SKAUZ/3/PRL/2*PASTOR/4/HEILO	SDSS13Y00167T-0B-0Y-0M-0Y-0B-37Y	6.83	5.04	2.06	MS
18	620583	H-1624/BAJ #1//SUP152	SDSS13Y00014T-0B-0Y-0M-0Y-0B-29Y	7.00	3.69	2.11	MS
45	620600	H-1357/8/CNDO/R143//ENTE/MEXI_2/3/AEGILOPS SQUARROSA (TAUS)/4/WEAVER/5/PICUS/6/TROST /7/TACUPETO F2001/9/KAUZ//ALTAR 84/AOS/3/ PASTOR/4/MILAN/CUPE//SW89.3064/5/KIRITATI	SDSS13Y00031T-0B-0Y-0M-0Y-0B-50Y	7.00	4.69	2.38	MS
51	620612	IG 41489/4/PRL/2*PASTOR//PBW343*2/ KUKUNA/3/ROLF07/5/NELOKI	SDSS13Y00043T-0B-0Y-0M-0Y-0B-40Y	7.00	4.52	1.84	MS
52	620612	IG 41489/4/PRL/2*PASTOR//PBW343*2/ KUKUNA/3/ROLF07/5/NELOKI	SDSS13Y00043T-0B-0Y-0M-0Y-0B-54Y	7.00	2.97	2.50	MS
55	620613	IG 41505/4/PRL/2*PASTOR//PBW343*2/ KUKUNA/3/ROLF07/5/NELOKI	SDSS13Y00044T-0B-0Y-0M-0Y-0B-34Y	7.00	4.82	3.40	MS
56	620615	IG 122145/4/PRL/2*PASTOR//PBW343*2/ KUKUNA/3/ROLF07/5/NELOKI	SDSS13Y00046T-0B-0Y-0M-0Y-0B-36Y	7.00	4.00	1.63	MS
62	620620	IG 122193/4/PRL/2*PASTOR//PBW343*2/ KUKUNA/3/ROLF07/5/NELOKI	SDSS13Y00051T-0B-0Y-0M-0Y-0B-75Y	7.00	3.10	1.26	MS
97	620635	INDIA-50/3/ATTILA*2/PBW65//MURGA/4/BORL14	SDSS13Y00066T-0B-0Y-0M-0Y-0B-60Y	7.00	3.22	1.32	MS
84	620631	H-1699/NELOKI/3/ATTILA*2/PBW65//MURGA	SDSS13Y00062T-0B-0Y-0M-0Y-0B-30Y	7.17	4.45	1.82	MS
90	620634	INDIA-38/3/ATTILA*2/PBW65//MURGA/4/BORL14	SDSS13Y00065T-0B-0Y-0M-0Y-0B-48Y	7.17	7.36	3.00	MS
98	620635	INDIA-50/3/ATTILA*2/PBW65//MURGA/4/BORL14	SDSS13Y00066T-0B-0Y-0M-0Y-0B-61Y	7.17	4.62	2.18	MS
106	620638	LOCAL RED/AE.SQUARROSA (223)//BORL14/3/ COPIO	SDSS13Y00069T-0B-0Y-0M-0Y-0B-40Y	7.17	3.87	1.58	MS
197	620678	H-1491/ROLF07//NAVJ07	SDSS13Y00109T-0B-0Y-0M-0Y-0B-73Y	7.17	2.32	0.95	MS
245	620710	IG 41735/NAVJ07//KACHU	SDSS13Y00141T-0B-0Y-0M-0Y-0B-68Y	7.17	8.45	3.45	MS
255	620736	IG 122627/6/KAUZ//ALTAR 84/AOS/3/PASTOR/4/ MILAN/CUPE//SW89.3064/5/KIRITATI/7/SW89.5277/ BORL95//SKAUZ/3/PRL/2*PASTOR/4/HEILO	SDSS13Y00167T-0B-0Y-0M-0Y-0B-41Y	7.17	3.31	1.89	MS
74	620629	H-1546/NELOKI/3/ATTILA*2/PBW65//MURGA	SDSS13Y00060T-0B-0Y-0M-0Y-0B-49Y	7.33	4.89	2.68	MS
83	620631	H-1699/NELOKI/3/ATTILA*2/PBW65//MURGA	SDSS13Y00062T-0B-0Y-0M-0Y-0B-28Y	7.33	3.61	1.48	MS
154	620648	T.DICOCCON PI94625/AE.SQUARROSA (372)// COPIO/3/KACHU #1/KIRITATI//KACHU	SDSS13Y00079T-0B-0Y-0M-0Y-0B-113Y	7.33	4.50	2.33	MS

Supplementary Table5(Continue): List of Drought and Heat Tolerant Spring Wheat lines and their Resistant Response to the CCN Heterodera filipjevi. (SD) Standard Deviation, (SE) Standard Error, (CID) Cross ID

ENTRY NUMBER	*CID*	*PEDIGREE (CROSS)*	*SELECTION HISTORY*	*(MEAN) CYSTS AND FEMALES*	*SD*	*SE*	*RESISTANT RESPONSE*
267	620768	H-1659/3/PBW343*2/KUKUNA*2//FRTL/PIFED/4/QUAIU #1	SDSS13Y00199T-0B-0Y-0M-0Y-0B-39Y	7.33	3.14	1.28	MS
131	620642	D67.2/PARANA 66.270//AE.SQUARROSA (1085)/3/BORL14/4/COPIO	SDSS13Y00073T-0B-0Y-0M-0Y-0B-73Y	7.50	5.24	2.14	S
136	620645	IWA8612416/BORL14//COPIO	SDSS13Y00076T-0B-0Y-0M-0Y-0B-40Y	7.50	4.81	1.96	S
205	620684	MEX94.30.10/NAVJ07//KACHU	SDSS13Y00115T-0B-0Y-0M-0Y-0B-102Y	7.50	3.78	1.54	S
223	620690	GAN/AE.SQUARROSA (206)//KACHU/3/BAJ #1	SDSS13Y00121T-0B-0Y-0M-0Y-0B-59Y	7.50	3.39	1.38	S
226	620692	D67.2/PARANA 66.270//AE.SQUARROSA (448)/3/KACHU/4/BAJ #1	SDSS13Y00123T-0B-0Y-0M-0Y-0B-122Y	7.50	2.59	1.06	S
243	620699	IG 41506/3/PBW343*2/KUKUNA*2//FRTL/PIFED/4/QUAIU #1	SDSS13Y00130T-0B-0Y-0M-0Y-0B-83Y	7.50	4.76	1.95	S
BEZOSTAJA (S)		LUT17/SRS2		7.50	2.65	1.32	S
37	620595	CHIH95.4.6/7/SHA7/VEE#5/5/VEE#8//JUP/BJY/3/F3.71/TRM/4/2*WEAVER/6/SKAUZ/PARUS//PARUS/8/CNDO/R143//ENTE/MEXI_2/3/AEGILOPS QUARROSA (TAUS)/4/WEAVER/5/PICUS/6/TROST/7/TACUPETO F2001	SDSS13Y00026T-0B-0Y-0M-0Y-0B-24Y	7.67	2.80	1.15	S
38	620595	CHIH95.4.6/7/SHA7/VEE#5/5/VEE#8//JUP/BJY/3/F3.71/TRM/4/2*WEAVER/6/SKAUZ/PARUS//PARUS/8/CNDO/R143//ENTE/MEXI_2/3/AEGILOPS QUARROSA (TAUS)/4/WEAVER/5/PICUS/6/TROST/7/TACUPETO F2001	SDSS13Y00026T-0B-0Y-0M-0Y-0B-34Y	7.67	4.32	1.76	S
119	620641	68.111/RGB-U//WARD RESEL/3/STIL/4/AE.SQUARROSA (630)/5/BORL14/6/COPIO	SDSS13Y00072T-0B-0Y-0M-0Y-0B-43Y	7.67	8.87	3.62	S
201	620683	H-1601/NAVJ07//KACHU	SDSS13Y00114T-0B-0Y-0M-0Y-0B-100Y	7.67	5.13	3.12	S
231	620695	INDIA-107/KACHU//BAJ #1	SDSS13Y00126T-0B-0Y-0M-0Y-0B-87Y	7.67	4.23	1.73	S
4	620575	DOY1/AE.SQUARROSA (488)//BAJ #1/3/SUP152	SDSS13Y00006T-0B-0Y-0M-0Y-0B-57Y	7.83	4.26	1.74	S
9	620577	DVERD_2/AE.SQUARROSA (333)//BAJ #1/3/SUP152	SDSS13Y00008T-0B-0Y-0M-0Y-0B-55Y	7.83	8.16	3.33	S
11	620577	DVERD_2/AE.SQUARROSA (333)//BAJ #1/3/SUP152	SDSS13Y00008T-0B-0Y-0M-0Y-0B-61Y	7.83	3.76	1.54	S
149	620648	T.DICOCCON PI94625/AE.SQUARROSA (372)//COPIO/3/KACHU #1/KIRITATI//KACHU	SDSS13Y00079T-0B-0Y-0M-0Y-0B-105Y	7.83	3.54	1.45	S
182	620661	YAV79//DACK/RABI/3/SNIPE/4/AE.SQUARROSA (460)/5/KACHU #1/KIRITATI//KACHU/6/PBW343*2/KUKUNA*2//FRTL/PIFED	SDSS13Y00092T-0B-0Y-0M-0Y-0B-100Y	7.83	5.38	2.20	S
212	620687	CROC_1/AE.SQUARROSA (436)//KACHU/3/BAJ #1	SDSS13Y00118T-0B-0Y-0M-0Y-0B-124Y	7.83	2.40	0.98	S
228	620694	INDIA-59/KACHU//BAJ #1	SDSS13Y00125T-0B-0Y-0M-0Y-0B-73Y	7.83	5.04	2.80	S
242	620699	IG 41506/3/PBW343*2/KUKUNA*2//FRTL/PIFED/4/QUAIU #1	SDSS13Y00130T-0B-0Y-0M-0Y-0B-78Y	7.83	5.04	2.06	S
31	620588	H-1311/3/FRET2*2/SHAMA//KACHU/4/HUW234+LR34/PRINIA*2//KIRITATI	SDSS13Y00019T-0B-0Y-0M-0Y-0B-16Y	8.00	3.29	1.34	S
40	620598	IG 122727/8/CNDO/R143//ENTE/MEXI_2/3/AEGILOPS SQUARROSA (TAUS)/4/WEAVER/5/PICUS/6/TROST/7/TACUPETO 2001/9/KAUZ//ALTAR 84/AOS/3/PASTOR/4/MILAN/CUPE//SW89.3064/5/KIRITATI	SDSS13Y00029T-0B-0Y-0M-0Y-0B-55Y	8.00	4.86	1.98	S
76	620630	H-1694/NELOKI/3/ATTILA*2/PBW65//MURGA	SDSS13Y00061T-0B-0Y-0M-0Y-0B-36Y	8.00	10.79	4.40	S
150	620648	T.DICOCCON PI94625/AE.SQUARROSA (372)//COPIO/3/KACHU #1/KIRITATI//KACHU	SDSS13Y00079T-0B-0Y-0M-0Y-0B-106Y	8.00	5.06	2.70	S
178	620657	CETA/AE.SQUARROSA (895)/3/KACHU #1/KIRITATI//KACHU/4/PBW343*2/KUKUNA*2//FRTL/PIFED	SDSS13Y00088T-0B-0Y-0M-0Y-0B-120Y	8.00	5.37	3.59	S
196	620678	H-1491/ROLF07//NAVJ07	SDSS13Y00109T-0B-0Y-0M-0Y-0B-54Y	8.00	4.60	1.88	S
27	620586	INDIA-101/3/FRET2*2/SHAMA//KACHU/4/HUW234+LR34/PRINIA*2//KIRITATI	SDSS13Y00017T-0B-0Y-0M-0Y-0B-61Y	8.17	5.49	2.24	S
58	620616	IG 122146/4/PRL/2*PASTOR//PBW343*2/KUKUNA/3/ROLF07/5/NELOKI	SDSS13Y00047T-0B-0Y-0M-0Y-0B-71Y	8.17	4.71	3.03	S
108	620638	LOCAL RED/AE.SQUARROSA (223)//BORL14/3/COPIO	SDSS13Y00069T-0B-0Y-0M-0Y-0B-55Y	8.17	5.53	3.12	S

Supplementary Table6(Continue): List of Drought and Heat Tolerant Spring Wheat lines and their Resistant Response to the CCN Heterodera filipjevi. (SD) Standard Deviation, (SE) Standard Error, (CID) Cross ID

ENTRY NUMBER	*CID*	*PEDIGREE (CROSS)*	*SELECTION HISTORY*	*(MEAN) CYSTS AND FEMALES*	*SD*	*SE*	*RESISTANT RESPONSE*
200	620680	IG 41243/NAVJ07//KACHU	SDSS13Y00111T-0B-0Y-0M-0Y-0B-64Y	8.17	5.71	3.24	S
207	620685	ARLIN/AE.SQUARROSA (283)//KACHU/3/BAJ #1	SDSS13Y00116T-0B-0Y-0M-0Y-0B-83Y	8.17	5.64	2.30	S
209	620687	CROC_1/AE.SQUARROSA (436)//KACHU/3/BAJ #1	SDSS13Y00118T-0B-0Y-0M-0Y-0B-111Y	8.17	5.04	3.07	S
240	620699	IG 41506/3/PBW343*2/KUKUNA*2//FRTL/PIFED/4/QUAIU #1	SDSS13Y00130T-0B-0Y-0M-0Y-0B-74Y	8.17	3.37	1.38	S
2	620575	DOY1/AE.SQUARROSA (488)//BAJ #1/3/SUP152	SDSS13Y00006T-0B-0Y-0M-0Y-0B-52Y	8.33	3.08	3.09	S
48	620604	IG 42152/6/KAUZ//ALTAR 84/AOS/3/PASTOR/4/MILAN/CUPE//SW89.3064/5/KIRITATI/7/SW89.5277/BORL95//SKAUZ/3/PRL/2*PASTOR/4/HEILO	SDSS13Y00035T-0B-0Y-0M-0Y-0B-60Y	8.33	4.89	1.99	S
102	620635	INDIA-50/3/ATTILA*2/PBW65//MURGA/4/BORL14	SDSS13Y00066T-0B-0Y-0M-0Y-0B-67Y	8.33	5.65	2.65	S
118	620641	68.111/RGB-U//WARD RESEL/3/STIL/4/AE.SQUARROSA (630)/5/BORL14/6/COPIO	SDSS13Y00072T-0B-0Y-0M-0Y-0B-42Y	8.33	4.68	1.91	S
16	620580	GARZA/BOY//AE.SQUARROSA (695)/3/BAJ #1/4/SUP152	SDSS13Y00011T-0B-0Y-0M-0Y-0B-44Y	8.50	5.32	2.17	S
32	620592	68.111/RGB-U//WARD RESEL/3/STIL/4/AE.SQUARROSA (390)/7/SHA7/VEE#5/5/VEE#8//JUP/BJY/3/F3.71/TRM/4/2*WEAVER/6/SKAUZ/PARUS//PARUS/8/CNDO/R143//ENTE/MEXI_2/3/AEGILOPS SQUARROSA (TAUS)/4/WEAVER/5/PICUS/6/TROST/7/TACUPETO F2001	SDSS13Y00023T-0B-0Y-0M-0Y-0B-60Y	8.50	5.09	2.08	S
53	620613	IG 41505/4/PRL/2*PASTOR//PBW343*2/KUKUNA/3/ROLF07/5/NELOKI	SDSS13Y00044T-0B-0Y-0M-0Y-0B-28Y	8.50	5.32	3.00	S
113	620640	68.111/RGB-U//WARD RESEL/3/STIL/4/AE.SQUARROSA (628)/5/BORL14/6/COPIO	SDSS13Y00071T-0B-0Y-0M-0Y-0B-45Y	8.50	9.03	3.69	S
134	620642	D67.2/PARANA 66.270//AE.SQUARROSA (1085)/3/BORL14/4/COPIO	SDSS13Y00073T-0B-0Y-0M-0Y-0B-80Y	8.50	5.92	3.29	S
162	620652	PERSIA-7/COPIO/3/KACHU #1/KIRITATI//KACHU	SDSS13Y00083T-0B-0Y-0M-0Y-0B-73Y	8.50	4.55	2.46	S
167	620653	PERSIA-21/COPIO/3/KACHU #1/KIRITATI//KACHU	SDSS13Y00084T-0B-0Y-0M-0Y-0B-74Y	8.50	5.89	2.40	S
224	620690	GAN/AE.SQUARROSA (206)//KACHU/3/BAJ #1	SDSS13Y00121T-0B-0Y-0M-0Y-0B-79Y	8.50	5.68	2.72	S
142	620646	IWA8611400/BORL14//COPIO	SDSS13Y00077T-0B-0Y-0M-0Y-0B-106Y	8.67	5.43	2.78	S
269	620768	H-1659/3/PBW343*2/KUKUNA*2//FRTL/PIFED/4/QUAIU #1	SDSS13Y00199T-0B-0Y-0M-0Y-0B-44Y	8.67	5.50	3.07	S
22	620583	H-1624/BAJ #1//SUP152	SDSS13Y00014T-0B-0Y-0M-0Y-0B-37Y	8.83	5.12	2.52	S
36	620594	INDIA-223/7/SHA7/VEE#5/5/VEE#8//JUP/BJY/3/F3.71/TRM/4/2*WEAVER/6/SKAUZ/PARUS//PARUS/8/CNDO/R143//ENTE/MEXI_2/3/AEGILOPS SQUARROSA (TAUS)/4/WEAVER/5/PICUS/6/TROST/7/TACUPETO F2001	SDSS13Y00025T-0B-0Y-0M-0Y-0B-48Y	8.83	5.64	2.77	S
59	620616	IG 122146/4/PRL/2*PASTOR//PBW343*2/KUKUNA/3/ROLF07/5/NELOKI	SDSS13Y00047T-0B-0Y-0M-0Y-0B-74Y	8.83	5.38	3.29	S
75	620629	H-1546/NELOKI/3/ATTILA*2/PBW65//MURGA	SDSS13Y00060T-0B-0Y-0M-0Y-0B-51Y	8.83	5.04	2.06	S
115	620640	68.111/RGB-U//WARD RESEL/3/STIL/4/AE.SQUARROSA (628)/5/BORL14/6/COPIO	SDSS13Y00071T-0B-0Y-0M-0Y-0B-47Y	8.83	5.71	2.33	S
135	620645	IWA8612416/BORL14//COPIO	SDSS13Y00076T-0B-0Y-0M-0Y-0B-35Y	8.83	5.81	2.94	S
252	620736	IG 122627/6/KAUZ//ALTAR 84/AOS/3/PASTOR/4/MILAN/CUPE//SW89.3064/5/KIRITATI/7/SW89.5277/BORL95//SKAUZ/3/PRL/2*PASTOR/4/HEILO	SDSS13Y00167T-0B-0Y-0M-0Y-0B-34Y	8.83	5.85	2.99	S
17	620581	IG 42134/BAJ #1//SUP152	SDSS13Y00012T-0B-0Y-0M-0Y-0B-54Y	9.00	5.40	2.70	S
129	620641	68.111/RGB-U//WARD RESEL/3/STIL/4/AE.SQUARROSA (630)/5/BORL14/6/COPIO	SDSS13Y00072T-0B-0Y-0M-0Y-0B-60Y	9.00	5.90	3.34	S
171	620654	CETA/AE.SQUARROSA (850)/3/KACHU #1/KIRITATI//KACHU/4/PBW343*2/KUKUNA*2//FRTL/PIFED	SDSS13Y00085T-0B-0Y-0M-0Y-0B-62Y	9.00	5.55	2.27	S
183	620662	YAV79//DACK/RABI/3/SNIPE/4/AE.SQUARROSA (477)/5/KACHU #1/KIRITATI//KACHU/6/PBW343*2/KUKUNA*2//FRTL/PIFED	SDSS13Y00093T-0B-0Y-0M-0Y-0B-82Y	9.00	5.51	2.68	S

Supplementary Table7(Continue): List of Drought and Heat Tolerant Spring Wheat lines and their Resistant Response to the CCN Heterodera filipjevi. (SD) Standard Deviation, (SE) Standard Error, (CID) Cross ID

ENTRY NUMBER	*CID*	*PEDIGREE (CROSS)*	*SELECTION HISTORY*	*(MEAN) CYSTS AND FEMALES*	*SD*	*SE*	*RESISTANT RESPONSE*
276	620785	IWA8614378/NAVJ07//KACHU	SDSS13Y00216T-0B-0Y-0M-0Y-0B-125Y	9.00	5.93	2.42	S
43	620600	H-1357/8/CNDO/R143//ENTE/MEXI_2/3/AEGILOPS SQUARROSA (TAUS)/4/WEAVER/5/PICUS/6/TROST /7/TACUPETO F2001/9/KAUZ//ALTAR 84/AOS/3/ PASTOR/4/MILAN/CUPE//SW89.3064/5/KIRITATI	SDSS13Y00031T-0B-0Y-0M-0Y-0B-44Y	9.17	4.62	1.89	S
153	620648	T.DICOCCON PI94625/AE.SQUARROSA (372)//COPIO/3/KACHU #1/KIRITATI//KACHU	SDSS13Y00079T-0B-0Y-0M-0Y-0B-112Y	9.17	5.71	3.00	S
177	620657	CETA/AE.SQUARROSA (895)/3/KACHU #1/ KIRITATI//KACHU/4/PBW343*2/KUKUNA*2//FRTL/ PIFED	SDSS13Y00088T-0B-0Y-0M-0Y-0B-115Y	9.17	4.96	2.02	S
266	620759	PERSIA-88/3/PBW343*2/KUKUNA*2//FRTL/PIFED /4/QUAIU #1	SDSS13Y00190T-0B-0Y-0M-0Y-0B-89Y	9.17	4.45	1.82	S
KUTLUK (S)		KRASNODARSKAYA//INIA-66/LILIFEN/3/ CALIBASAN		9.25	8.62	4.31	S
12	620577	DVERD_2/AE.SQUARROSA (333)//BAJ #1/3/SUP152	SDSS13Y00008T-0B-0Y-0M-0Y-0B-64Y	9.33	5.79	2.36	S
19	620583	H-1624/BAJ #1//SUP152	SDSS13Y00014T-0B-0Y-0M-0Y-0B-31Y	9.33	3.44	2.75	S
193	620670	LOCAL RED/AE.SQUARROSA (222)/3/PBW343*2/ KUKUNA*2//FRTL/PIFED/4/QUAIU #1	SDSS13Y00101T-0B-0Y-0M-0Y-0B-81Y	9.33	4.68	1.91	S
141	620646	IWA8611400/BORL14//COPIO	SDSS13Y00077T-0B-0Y-0M-0Y-0B-92Y	9.50	5.13	2.09	S
KUTLUK (S)		KRASNODARSKAYA//INIA-66/LILIFEN/3/CALIBASAN		9.50	4.73	2.36	S
147	620647	T.DICOCCON PI94624/AE.SQUARROSA (454)// COPIO/3/KACHU #1/KIRITATI//KACHU	SDSS13Y00078T-0B-0Y-0M-0Y-0B-86Y	9.67	6.71	2.74	S
190	620667	DOY1/AE.SQUARROSA (415)/3/PBW343*2/ KUKUNA*2//FRTL/PIFED/4/QUAIU #1	SDSS13Y00098T-0B-0Y-0M-0Y-0B-69Y	9.67	5.24	2.14	S
233	620695	INDIA-107/KACHU//BAJ #1	SDSS13Y00126T-0B-0Y-0M-0Y-0B-103Y	9.67	6.65	2.72	S
13	620577	DVERD_2/AE.SQUARROSA (333)//BAJ #1/3/SUP152	SDSS13Y00008T-0B-0Y-0M-0Y-0B-65Y	9.83	6.52	3.50	S
25	620586	INDIA-101/3/FRET2*2/SHAMA//KACHU/4/ HUW234+LR34/PRINIA*2//KIRITATI	SDSS13Y00017T-0B-0Y-0M-0Y-0B-43Y	9.83	6.62	3.11	S
46	620600	H-1357/8/CNDO/R143//ENTE/MEXI_2/3/AEGILOPS SQUARROSA (TAUS)/4/WEAVER/5/PICUS/6/ TROST/7/TACUPETO F2001/9/KAUZ//ALTAR 84/ AOS/3/PASTOR/4/MILAN/CUPE//SW89.3064/5/KIRITATI	SDSS13Y00031T-0B-0Y-0M-0Y-0B-51Y	9.83	5.49	2.24	S
186	620662	YAV79//DACK/RABI/3/SNIPE/4/AE.SQUARROSA (477)/5/KACHU #1/KIRITATI//KACHU/6/PBW343*2/ KUKUNA*2//FRTL/PIFED	SDSS13Y00093T-0B-0Y-0M-0Y-0B-97Y	9.83	6.62	2.70	S
198	620678	H-1491/ROLF07//NAVJ07	SDSS13Y00109T-0B-0Y-0M-0Y-0B-74Y	9.83	4.54	1.85	S
235	620696	IG 41242/3/PBW343*2/KUKUNA*2//FRTL/ PIFED/4/QUAIU #1	SDSS13Y00127T-0B-0Y-0M-0Y-0B-75Y	9.83	6.08	2.48	S
5	620577	DVERD_2/AE.SQUARROSA (333)//BAJ #1/3/SUP152	SDSS13Y00008T-0B-0Y-0M-0Y-0B-47Y	10.00	5.18	3.04	S
72	620629	H-1546/NELOKI/3/ATTILA*2/PBW65//MURGA	SDSS13Y00060T-0B-0Y-0M-0Y-0B-44Y	10.00	6.07	3.31	S
105	620638	LOCAL RED/AE. SQUARROSA (223)//BORL14/3/ COPIO	SDSS13Y00069T-0B-0Y-0M-0Y-0B-39Y	10.00	10.18	4.16	S
230	620694	INDIA-59/KACHU//BAJ #1	SDSS13Y00125T-0B-0Y-0M-0Y-0B-91Y	10.00	6.72	3.59	S
BEZOSTAJA (S)		LUT17/SRS2		10.00	4.24	2.12	S
14	620579	D67.2/PARANA 66.270//AE.SQUARROSA (677)/3/BAJ #1/4/SUP152	SDSS13Y00010T-0B-0Y-0M-0Y-0B-35Y	10.17	5.95	3.44	HS
79	620630	H-1694/NELOKI/3/ATTILA*2/PBW65//MURGA	SDSS13Y00061T-0B-0Y-0M-0Y-0B-49Y	10.17	3.97	1.62	HS
103	620637	TXL92.8.1/3/ATTILA*2/PBW65//MURGA/4/BORL14	SDSS13Y00068T-0B-0Y-0M-0Y-0B-65Y	10.17	5.64	2.91	HS
258	620746	IG 122738/3/PBW343*2/KUKUNA*2// FRTL/PIFED/4/QUAIU #1	SDSS13Y00177T-0B-0Y-0M-0Y-0B-59Y	10.17	6.68	3.09	HS
92	620634	INDIA-38/3/ATTILA*2/PBW65//MURGA/4/BORL14	SDSS13Y00065T-0B-0Y-0M-0Y-0B-54Y	10.33	6.53	3.02	HS
172	620654	CETA/AE.SQUARROSA (850)/3/KACHU #1/ KIRITATI//KACHU/4/PBW343*2/KUKUNA*2//FRTL/ PIFED	SDSS13Y00085T-0B-0Y-0M-0Y-0B-64Y	10.33	4.23	1.73	HS
188	620665	GARZA/BOY//AE.SQUARROSA (281)/3/PBW343*2/ KUKUNA*2//FRTL/PIFED/4/QUAIU #1	SDSS13Y00096T-0B-0Y-0M-0Y-0B-58Y	10.33	5.54	2.26	HS

Supplementary Table8(Continue): List of Drought and Heat Tolerant Spring Wheat lines and their Resistant Response to the CCN Heterodera filipjevi. (SD) Standard Deviation, (SE) Standard Error, (CID) Cross ID

ENTRY NUMBER	*CID*	*PEDIGREE (CROSS)*	*SELECTION HISTORY*	*(MEAN) CYSTS AND FEMALES*	*SD*	*SE*	*RESISTANT RESPONSE*
192	620669	LOCAL RED/AE.SQUARROSA (220)/3/PBW343*2/ KUKUNA*2//FRTL/PIFED/4/QUAIU #1	SDSS13Y00100T-0B-0Y-0M-0Y-0B-87Y	10.33	6.44	3.36	HS
21	620583	H-1624/BAJ #1//SUP152	SDSS13Y00014T-0B-0Y-0M-0Y-0B-34Y	10.50	5.89	2.40	HS
120	620641	68.111/RGB-U//WARD RESEL/3/STIL/4/AE.SQUARROSA (630)/5/BORL14/6/COPIO	SDSS13Y00072T-0B-0Y-0M-0Y-0B-45Y	10.50	3.15	1.28	HS
122	620641	68.111/RGB-U//WARD RESEL/3/STIL/4/AE.SQUARROSA (630)/5/BORL14/6/COPIO	SDSS13Y00072T-0B-0Y-0M-0Y-0B-48Y	10.50	5.24	3.25	HS
3	620575	DOY1/AE.SQUARROSA (488)//BAJ #1/3/SUP152	SDSS13Y00006T-0B-0Y-0M-0Y-0B-56Y	10.67	6.74	2.75	HS
65	620625	IG 122795/4/PRL/2*PASTOR// PBW343*2/KUKUNA/3/ROLF07/5/NELOKI	SDSS13Y00056T-0B-0Y-0M-0Y-0B-96Y	10.67	6.31	2.58	HS
95	620634	INDIA-38/3/ATTILA*2/PBW65//MURGA/4/BORL14	SDSS13Y00065T-0B-0Y-0M-0Y-0B-60Y	10.67	6.65	2.72	HS
125	620641	68.111/RGB-U//WARD RESEL/3/STIL/4/ AE.SQUARROSA (630)/5/BORL14/6/COPIO	SDSS13Y00072T-0B-0Y-0M-0Y-0B-52Y	10.67	6.98	2.85	HS
175	620655	CETA/AE.SQUARROSA (872)/3/KACHU #1/ KIRITATI//KACHU/4/PBW343*2/KUKUNA*2//FRTL/ PIFED	SDSS13Y00086T-0B-0Y-0M-0Y-0B-64Y	10.67	6.77	3.13	HS
35	620594	INDIA-223/7/SHA7/VEE#5/5/VEE#8//JUP/BJY/3/ F3.71/TRM/4/2*WEAVER/6/SKAUZ/PARUS//PARUS/ 8/CNDO/R143//ENTE/MEXI_2/3/AEGILOPS SQUARROSA (TAUS)/4/WEAVER/5/PICUS/6/ TROST/7/TACUPETO F2001	SDSS13Y00025T-0B-0Y-0M-0Y-0B-36Y	10.83	2.04	0.83	HS
50	620611	CETA/AE.SQUARROSA (391)/4/PRL/2*PASTOR// PBW343*2/KUKUNA/3/ROLF07/5/NELOKI	SDSS13Y00042T-0B-0Y-0M-0Y-0B-6Y	10.83	6.15	3.89	HS
63	620621	IG 122196/4/PRL/2*PASTOR//PBW343*2/ KUKUNA/3/ROLF07/5/NELOKI	SDSS13Y00052T-0B-0Y-0M-0Y-0B-80Y	10.83	6.62	2.70	HS
180	620661	YAV79//DACK/RABI/3/SNIPE/4/AE.SQUARROSA (460)/5/KACHU #1/KIRITATI//KACHU/6/PBW343*2/ KUKUNA*2//FRTL/PIFED	SDSS13Y00092T-0B-0Y-0M-0Y-0B-97Y	10.83	6.62	2.70	HS
191	620669	LOCAL RED/AE.SQUARROSA (220)/3/PBW343*2/ KUKUNA*2//FRTL/PIFED/4/QUAIU #1	SDSS13Y00100T-0B-0Y-0M-0Y-0B-79Y	11.00	7.40	3.74	HS
111	620638	LOCAL RED/AE.SQUARROSA (223)//BORL14/3/ COPIO	SDSS13Y00069T-0B-0Y-0M-0Y-0B-65Y	11.17	6.40	3.29	HS
176	620657	CETA/AE.SQUARROSA (895)/3/KACHU #1/ KIRITATI//KACHU/4/PBW343*2/KUKUNA*2//FRTL/ PIFED	SDSS13Y00088T-0B-0Y-0M-0Y-0B-110Y	11.17	7.00	2.86	HS
71	620629	H-1546/NELOKI/3/ATTILA*2/PBW65//MURGA	SDSS13Y00060T-0B-0Y-0M-0Y-0B-33Y	11.33	7.09	3.38	HS
138	620645	IWA8612416/BORL14//COPIO	SDSS13Y00076T-0B-0Y-0M-0Y-0B-48Y	11.33	6.56	2.68	HS
173	620654	CETA/AE.SQUARROSA (850)/3/KACHU #1/ KIRITATI//KACHU/4/PBW343*2/KUKUNA*2//FRTL/ PIFED	SDSS13Y00085T-0B-0Y-0M-0Y-0B-69Y	11.33	7.39	3.90	HS
232	620695	INDIA-107/KACHU//BAJ #1	SDSS13Y00126T-0B-0Y-0M-0Y-0B-101Y	11.33	7.39	3.02	HS
94	620634	INDIA-38/3/ATTILA*2/PBW65//MURGA/4/BORL14	SDSS13Y00065T-0B-0Y-0M-0Y-0B-58Y	11.50	7.87	4.01	HS
107	620638	LOCAL RED/AE.SQUARROSA (223)//BORL14/3/ COPIO	SDSS13Y00069T-0B-0Y-0M-0Y-0B-52Y	11.50	7.29	2.97	HS
117	620640	68.111/RGB-U//WARD RESEL/3/STIL/4/ AE.SQUARROSA (628)/5/BORL14/6/COPIO	SDSS13Y00071T-0B-0Y-0M-0Y-0B-53Y	11.50	5.92	3.50	HS
96	620635	INDIA-50/3/ATTILA*2/PBW65//MURGA/4/BORL14	SDSS13Y00066T-0B-0Y-0M-0Y-0B-59Y	11.67	7.31	3.38	HS
140	620645	IWA8612416/BORL14//COPIO	SDSS13Y00076T-0B-0Y-0M-0Y-0B-50Y	11.67	8.02	3.27	HS
6	620577	DVERD_2/AE.SQUARROSA (333)//BAJ #1/3/SUP152	SDSS13Y00008T-0B-0Y-0M-0Y-0B-51Y	11.83	8.04	3.28	HS
26	620586	INDIA-101/3/FRET2*2/SHAMA//KACHU/4/HUW234+LR34/P RINIA*2//KIRITATI	SDSS13Y00017T-0B-0Y-0M-0Y-0B-54Y	11.83	6.37	2.60	HS
66	620627	68.111/RGB-U//WARD/3/FGO/4/RABI/5/ AE.SQUARROSA (890)/6/NELOKI/7/ATTILA*2/ PBW65//MURGA	SDSS13Y00058T-0B-0Y-0M-0Y-0B-43Y	11.83	6.62	3.48	HS
80	620630	H-1694/NELOKI/3/ATTILA*2/PBW65//MURGA	SDSS13Y00061T-0B-0Y-0M-0Y-0B-55Y	11.83	6.65	2.71	HS
121	620641	68.111/RGB-U//WARD RESEL/3/STIL/4/ AE.SQUARROSA (630)/5/BORL14/6/COPIO	SDSS13Y00072T-0B-0Y-0M-0Y-0B-47Y	12.00	8.02	3.28	HS

Supplementary Table9(Continue): List of Drought and Heat Tolerant Spring Wheat lines and their Resistant Response to the CCN Heterodera filipjevi. (SD) Standard Deviation, (SE) Standard Error, (CID) Cross ID

ENTRY NUMBER	*CID*	*PEDIGREE (CROSS)*	*SELECTION HISTORY*	*(MEAN) CYSTS AND FEMALES*	*SD*	*SE*	*RESISTANT RESPONSE*
132	620642	D67.2/PARANA 66.270//AE.SQUARROSA (1085)/3/ BORL14/4/COPIO	SDSS13Y00073T-0B-0Y-0M-0Y-0B-77Y	12.00	7.51	3.07	HS
185	620662	YAV79//DACK/RABI/3/SNIPE/4/AE.SQUARROSA (477)/5/KACHU #1/KIRITATI//KACHU/6/ PBW343*2/KUKUNA*2//FRTL/PIFED	SDSS13Y00093T-0B-0Y-0M-0Y-0B-91Y	12.00	4.82	1.97	HS
156	620650	IG 41620/COPIO/3/KACHU #1/KIRITATI//KACHU	SDSS13Y00081T-0B-0Y-0M-0Y-0B-78Y	12.17	5.12	2.09	HS
260	620747	IG 122740/3/PBW343*2/KUKUNA*2//FRTL/ PIFED/4/QUAIU #1	SDSS13Y00178T-0B-0Y-0M-0Y-0B-47Y	12.33	8.12	3.87	HS
152	620648	T.DICOCCON PI94625/AE.SQUARROSA (372)// COPIO/3/KACHU #1/KIRITATI//KACHU	SDSS13Y00079T-0B-0Y-0M-0Y-0B-108Y	12.50	6.12	3.59	HS
116	620640	68.111/RGB-U//WARD RESEL/3/STIL /4/AE.SQUARROSA (628)/5/BORL14/6/COPIO	SDSS13Y00071T-0B-0Y-0M-0Y-0B-51Y	12.67	7.81	4.82	HS
133	620642	D67.2/PARANA 66.270//AE.SQUARROSA (1085)/3/ BORL14/4/COPIO	SDSS13Y00073T-0B-0Y-0M-0Y-0B-79Y	12.67	7.26	2.96	HS
161	61665	EMPTY PLOT		12.67	8.04	3.28	HS
213	620687	CROC_1/AE.SQUARROSA (436)//KACHU/3/BAJ #1	SDSS13Y00118T-0B-0Y-0M-0Y-0B-129Y	12.67	7.84	3.20	HS
85	620633	IG 131672/3/ATTILA*2/PBW65//MURGA/4/BORL14	SDSS13Y00064T-0B-0Y-0M-0Y-0B-50Y	13.17	6.37	3.79	HS
104	620637	TXL92.8.1/3/ATTILA*2/PBW65//MURGA/4/BORL14	SDSS13Y00068T-0B-0Y-0M-0Y-0B-67Y	13.17	5.64	2.30	HS
151	620648	T.DICOCCON PI94625/AE.SQUARROSA (372)// COPIO/3/KACHU #1/KIRITATI//KACHU	SDSS13Y00079T-0B-0Y-0M-0Y-0B-107Y	13.17	7.76	3.99	HS
194	620674	JAL95.4.3/VORB//ROLF07	SDSS13Y00105T-0B-0Y-0M-0Y-0B-29Y	13.33	9.14	4.91	HS
15	620579	D67.2/PARANA 66.270//AE.SQUARROSA (677)/3/BAJ #1/4/SUP152	SDSS13Y00010T-0B-0Y-0M-0Y-0B-47Y	13.50	8.31	3.39	HS
89	620634	INDIA-38/3/ATTILA*2/PBW65//MURGA/4/BORL14	SDSS13Y00065T-0B-0Y-0M-0Y-0B-46Y	13.50	9.31	4.88	HS
187	620663	GARZA/BOY//AE.SQUARROSA (278)/3/PBW343*2/ KUKUNA*2//FRTL/PIFED/4/QUAIU #1	SDSS13Y00094T-0B-0Y-0M-0Y-0B-47Y	13.50	7.69	4.06	HS
126	620641	68.111/RGB-U//WARD RESEL/3/STIL/4/ AE.SQUARROSA (630)/5/BORL14/6/COPIO	SDSS13Y00072T-0B-0Y-0M-0Y-0B-56Y	13.67	8.26	3.37	HS
101	620635	INDIA-50/3/ATTILA*2/PBW65//MURGA/4/BORL14	SDSS13Y00066T-0B-0Y-0M-0Y-0B-66Y	14.00	8.37	3.42	HS
100	620635	INDIA-50/3/ATTILA*2/PBW65//MURGA/4/BORL14	SDSS13Y00066T-0B-0Y-0M-0Y-0B-63Y	15.00	8.49	3.46	HS
7	620577	DVERD_2/AE.SQUARROSA (333)//BAJ #1/3/SUP152	SDSS13Y00008T-0B-0Y-0M-0Y-0B-53Y	15.50	8.34	3.40	HS
91	620634	INDIA-38/3/ATTILA*2/PBW65//MURGA/4/BORL14	SDSS13Y00065T-0B-0Y-0M-0Y-0B-53Y	15.67	10.75	6.02	HS
8	620577	DVERD_2/AE.SQUARROSA (333)//BAJ #1/3/SUP152	SDSS13Y00008T-0B-0Y-0M-0Y-0B-54Y	17.50	10.01	5.15	HS

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Elimination of Plant Pathogenic Bacteria by Solar Ultraviolet Radiation in Hydroponic Systems

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ABSTRACT

Removing plant pathogens with the sun as a free, available, clean, and sustainable source of energy is interesting. However, there is no data for disinfecting major plant pathogenic bacteria such as *Pseudomonas syringae* and *Clavibacter michiganensis* subsp. *michiganensis* by solar ultraviolet radiation. To obtain the required time for killing these bacteria at different temperatures, a bacterial suspension of active growing cells (approximately 10^7 CFU mL^{-1}) was prepared and subjected to heat inside a water bath. The minimum required time for killing both of the bacteria was achieved 420, 45, and 15 min at 50, 55, and 60 °C, respectively. To examine the effect of solar ultraviolet radiation, the bacteria suspensions inside a quartz tube were exposed to the sun on a horizontal surface at the constant temperature of 50 °C within the water bath (water depth: 0.1 m). Both of the bacteria were killed after one hour by receiving 95.481 kJ m^{-2} ultraviolet and 2.79315 MJ m^{-2} solar radiation doses. The synergy of heat and solar UV could considerably reduce the killing time of the bacteria (7 to 1 hours) at 50 °C. The recommended solar UV dose is 95.481 kJ m^{-2} for this condition.

Keywords: Disinfection, Heat, Renewable energy, Soilless culture, UV, Water treatment

1. Introduction

Worldwide predictions state that approximately 70,000 km^2 annually enter the classification of the desert Şen (2015). “Over the last 25 years, droughts covered more than 37% of the EU territory and affected more than 100 million people” (Andreu et al. 2015). Population growth and climate change will increase water shortage for different purposes such as drinking water and irrigation. Therefore, reusing reclaimed water is essential (Fatta-Kassinos et al. 2016). The hydroponic system is one of the proper methods in dry regions to increase water usage efficiency. Also, reusing hydroponic water drainage can reduce 20 to 30 percent of water consumption (Tripanagnostopoulos & Rocamora 2008). However, disease control is a key factor in hydroponic systems since plant pathogens rapidly spread in irrigation water. Thus, water disinfection is unavoidable.

Various methods have been applied for water disinfection. Heat and ultraviolet (UV) radiation are physical methods without a significant level of by-products versus chemical materials (Bolton & Cotton 2008). Hence, cleaner agricultural products can be supplied without chemical residues. Soil and water will not be contaminated by fungicides and other chemical materials.

One of the best disinfectants is the sun. The sun has thermal radiation, and its ultraviolet rays can kill pathogens (Aniruddha Bhalchandra & Jyoti Kishen 2013). Furthermore, solar energy is a renewable, clean, free, and sustainable resource. Therefore, using solar radiation for disinfection has all benefits together.

In this study, Pseudomonas sp. and Clavibacter michiganensis subsp. michiganensis as important plant pathogenic bacteria in hydroponic systems were considered for disinfection with heat, UV, and solar radiation.

Table 1 briefly shows previous researches in this field. Most studies in the field of ultraviolet have focused on UVC (100-280 nm) since 240-280 nm effectively inactivates microorganisms and irreparably damages nucleic acid (Aniruddha Bhalchandra & Jyoti Kishen 2013). Maximum relative ultraviolet absorption by DNA pertains to the wavelength of 260 nm. Since low-pressure lamps produce a narrow band of UV light peaking near 254 nm, these lamps are the most efficient source of germicidal UV light (Wolfe 1990).

Table 1- A research list about the disinfection of some plant pathogenic bacteria by heat and UV

Pathogens	*Conditions*	*Reference*
Clavibacter michiganensis subsp. *michiganensis*	30 min at 56 °C	(Fatmi et al. 1991)
Clavibacter michiganensis subsp. *michiganensis*	113 mJ cm^{-2}, UV (254 nm)	(Scarlett et al. 2016)
Pseudomonas aeruginosa (wild type)	5 min at 55 °C	(Spinks et al. 2006)
Pseudomonas chlororaphis	10 min at 60 °C	(Tu & Zhang 2000)
Pseudomonas corrugata Strains	30 min at 60 °C	(Bella et al. 2002)
Pseudomonas syringae	24h at 40 °C	(Hao et al. 2012)
Pseudomonas syringae	4 h sunlight at 25-27 °C	(Miller et al. 2001)
Xanthomonas campestris	24h at 48 °C	(Hao et al. 2012)
Xanthomonas campestris pv. *malvacearum*	20 min at 65 °C	(Honervogt & Lehmann-Danzinger 1992)
Xanthomonas fragariae	60, 15 min at 52, 56 °C	(Turechek & Peres 2009)
Xanthomonas sp.	Solar UV-B	(Gunasekera & Paul 2007)

Considered treatments in some studies relate to seeds, fruits, and leaves. However, these data are not applicable for water in hydroponic systems, surface water, and irrigation water, since the contamination form is a suspension in these cases.

Killing pathogens at lower temperatures is desirable for conventional solar collectors due to radiation limitations and heat losses. Moreover, the required energy for heating can be declined and much water will be disinfected when a goal temperature is low. Therefore, the effect of lower temperatures on pathogens is considered in this research.

The synergistic effect of heat and UV on some pathogens has been proved (Tyrrell 1976; Petin et al. 1997; Kim et al. 2001; Maktabi et al. 2011), but there are not data about some plant pathogens, particularly for solar UV.

This paper aims to obtain the required solar UV dose for killing two major plant pathogenic bacteria (Pseudomonas syringae and Clavibacter michiganensis subsp. michiganensis) at a low temperature in hydroponic systems using the sun as a clean and sustainable source of energy. Safe agricultural production and the reduction of chemical controls are the importance of this study.

2. Material and Methods

Pseudomonas syringae (Pss) and Clavibacter michiganensis subsp. michiganensis (Cmm) were selected as important plant pathogens, particularly in irrigation water (Lamichhane & Bartoli 2015; Scarlett et al. 2016). Their isolates were supplied from the Laboratory of Plant Pathology, Urmia University. Clavibacter michiganensis subsp. michiganensis was isolated from tomato plants with bacterial canker symptoms, a widespread and destructive disease of tomato plants in Urmia, West Azarbaijan, Iran, and its pathogenicity was confirmed on healthy tomato plants based on Koch's postulates. Pseudomonas sp. was isolated from tomato plants with tomato pith necrosis symptoms, a disease that is commonly found in tomato fields and causes economic losses and its pathogenicity was confirmed on tomato plants. The inoculum concentration used for all experiments was 10^7 CFU mL^{-1}. One pathogenic strain was used for the experiments for both pathogenic bacteria.

A suspension of active growing cells of the pathogens (approximately 10^7 CFU mL^{-1}) was prepared (Petin et al. 1997; Wolf & Beckhoven 2004; Berney et al. 2006) and subjected to heat treatments at different lapses of time to determine the minimum lethal time at each temperature and pathogen. The temperatures and time were selected according to pre-tests and the results of previous researches (Fatmi et al. 1991; Grondeau et al. 1992; Toben & Rudolph 1997; Hao et al. 2012). The pathogens inside a tube were submerged in a water bath (Figure 1). The water temperature was controlled by an electronic board (WX-101W, Shenzhen Eshinede Technology Co.), a heater, and a water pump. The maximum variation of water temperature was ±0.25 °C. As the temperature of suspensions before submerging inside the water was room temperature (25-30 °C), delay time, 2-3 min (at 50 to 60 °C), was added for temperature balance according to the temperature measurements of water in the tubes.

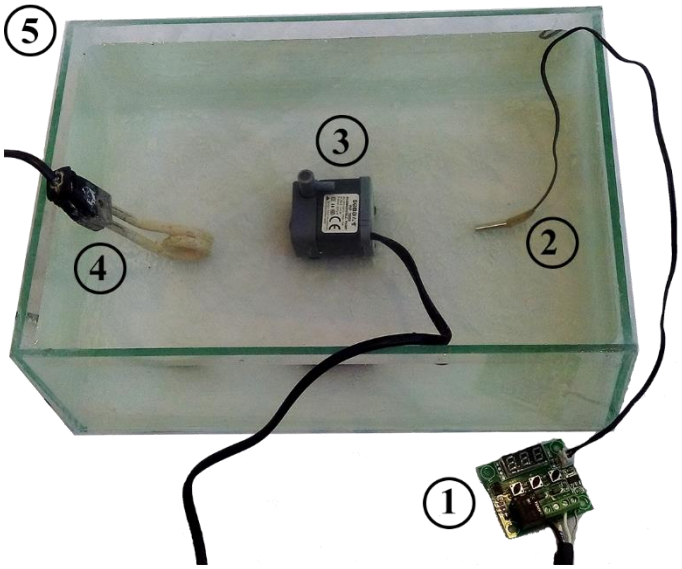

Figure 1- The water bath for temperature control, 1: Electronic board, 2: Temperature sensor, 3: Pump for water circulation, 4: Electric heater, 5: Glass container

The bacteria viability was tested at each temperature and exposure time (Table 2) with a completely randomized design in three repetitions. Temperatures lower than 50 °C were not considered because disinfection time was very long, for example, removing Pseudomonas syringae needs 24 h at 40 °C (Hao et al. 2012). The suspension of pathogens was cultured on Nutrient Agar (NA) medium after exposure to heat, and viable cells were counted after 72 h at 25 °C (Sholberg et al. 2005; Rai et al. 2006).

To examine the effect of solar ultraviolet radiation on disinfection, the pathogen suspension (approximately 107 CFU mL^{-1}) was spilled inside a quartz tube and was exposed to the sun on a horizontal surface. The quartz glass has a high transmittance of UV radiation (Gross et al. 2015). The mentioned water bath (water depth: 0.1 m) was used to control water temperature. Solar radiation was recorded on a horizontal surface during the experiment by a solar power meter, model ST-1307 (Standard, Hong Kong). Solar ultraviolet radiation was measured by the ML8511 UV sensor (LAPIS Semiconductor Co. 2013). This sensor is sensitive to UVA and UVB (Figure 2). Terrestrial solar ultraviolet radiations contain UVA and UVB (Caldwell et al. 2007). The frequency of data recording was 15 min.

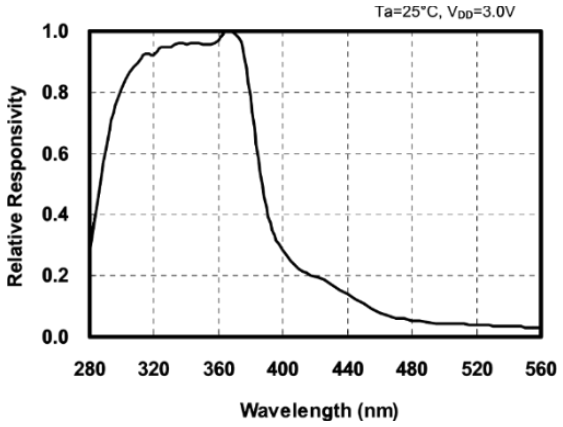

Figure 2- The spectral responsibility characteristics of the ML8511 UV sensor (LAPIS Semiconductor Co. 2013)

The fluence (UV dose) during the experiment was calculated by Equation 1 (Bolton & Cotton 2008).

$$UVdose\ (\mathrm{J\,m^{-2}}) = \int_{t_{start}}^{t_{end}} I_{UV}\,\mathrm{d}t \qquad (1)$$

Where: I_{UV}, Ultraviolet radiation (W m^{-2}); t, time (s)

Since the suspension of pathogens was submerged in the water bath, the received dose had a lower value compared to Equation 1. On the other hand, the solar UV dose reported in this research is slightly higher than the actual value. However, to control the temperature, applying the water bath was necessary.

The effect of solar radiation on the bacteria was tested on three different days in August 2016 in Urmia University campus (37° 39' 30.33" N, 44° 58' 43.94" E), and the minimum lethal solar ultraviolet dose was reported. Another experiment (August 21, 2016) was conducted to examine shorter disinfection times (15, 30, and 45 minutes at 50 °C) for the solar ultraviolet test. The viable cell counting was used as described previously to evaluate solar radiation effects.

Burch & Thomas (1998) reported that disinfection time changed exponentially with temperature. Hence, a regression equation can be in the form of Equation 2.

$$time = a_1 \times e^{(b_1 \times temperature)}, \quad (2)$$

Where: a1 and b1, are constant. By taking natural logarithm of Equation 2, the following expression could be derived:

$$temperature = a + b \times Ln(time), \quad (3)$$

Where: a and b, are constant; If let $x = Ln(time)$, Equation 3 is converted to the following equation:

$$temperature = a + b \times x \quad (4)$$

Equation 4 is related to a straight line. Therefore, Pearson's correlation coefficient test can be used to certify the statistical significance of temperature and time dependency. IBM SPSS Statistics software was used for statistical analysis.

3. Results and Discussion

Table 2 shows the results of experiments for different temperatures. Pss and Cmm had the same response. Cases with 100% survival were completely similar to control treatment, and zero survival cases had very clear surfaces without any colony (Figure 3).

Table 2- Disinfection by only heat for *Pseudomonas syringae* (Pss) and *Clavibacter michiganensis* subsp. *michiganensis* (Cmm)

Temperature (°C)	***Time (min)***	***Survival (%)*** *Pss and Cmm*	***Temperature (°C)***	***Time (min)***	***Survival (%)*** *Pss and Cmm*	***Temperature (°C)***	***Time (min)***	***Survival (%)*** *Pss and Cmm*
50	45	100	**55**	15	100	**60**	10	100
	60	100		20	100		15	0
	75	100		30	100		20	0
	90	100		45	0		30	0
	120	100		60	0		45	0
	180	100		120	0		60	0
	240	100		180	0		90	0
	300	100		240	0			
	360	100						
	420	0						
	480	0						
	540	0						
	600	0						
	660	0						

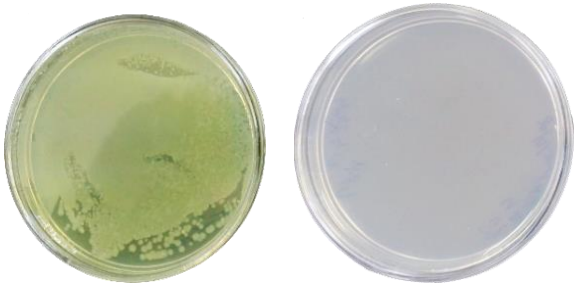

Figure 3- Cmm control (left), completely clear without any bacterial colony (right)

Tu & Zhang (2000) reported that Pseudomonas chlororaphis needed 10 min at 60 °C to be killed. This condition is close to the results of Pss (15 min at 60 °C).

A logarithmic regression between the lethal temperature and time was fitted (Figure 4) according to the results presented in Table 2. The logarithmic regression was used since a linear relation was reported between the logarithm of time and disinfection temperature (Burch & Thomas 1998; Tang 2007). Root mean square error (RMSE) and coefficient of determination (R^2) were obtained 0.8932 and 0.9628 for the regression, respectively. This certifies that the logarithmic regression presented in Figure 4 is proper, and it is consistent with the results of Burch & Thomas (1998), and Tang (2007). Therefore, it was confirmed that the results showed a linear relation between disinfection temperature and the logarithm of time. Hence, Pearson's correlation coefficient can determine the statistical significance of this relation. Table 3 presents the result of Pearson's correlation coefficient test. The correlation coefficient was obtained 0.981 showing there is a statistically significant relation between disinfection temperature and the logarithm of time. The minus sign in Table 3 shows that increasing temperature reduces the killing time.

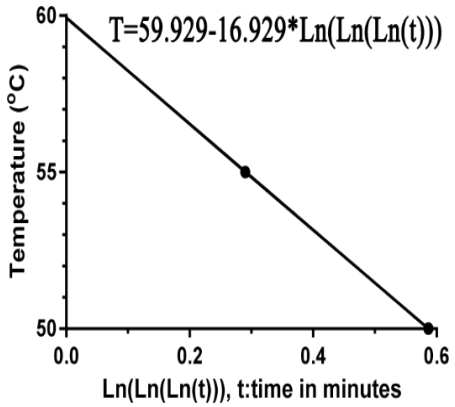

Figure 5- Linear regression for temperature and time by a natural logarithm, (R^2=1, RMSE=0.0106)

Table 3- Pearson's correlation coefficient test for the lethal temperature and the natural logarithm of time (each temperature had three repeats)

Factors		*Temperature*	*Ln(time)*
Temperature	Pearson Correlation	1	-0.981**
	Sig. (2-tailed)		0.000
	N	9	9
Ln(time)	Pearson Correlation	-0.981**	1
	Sig. (2-tailed)	0.000	
	N	9	9

**: The correlation is significant at the 0.01 level (2-tailed)

If a natural logarithm is reused for Figure 4 data, regression errors will be lower, and the estimation will be better (Figure 5).

The equation between temperature and time (Figure 4) can be rearranged so that time becomes an independent variable and temperature becomes a dependent variable (Equation 5).

$$time = e^{\left(\frac{67.092 - temperature}{2.889}\right)} \quad (5)$$

This equation can predict the required disinfection time for a specific temperature.
The derivative of the time respect to the temperature is:

$$d(time)=\frac{-1}{2.889}e^{\left(\frac{67.092-temperature}{2.889}\right)}d(temperature) \quad (6)$$

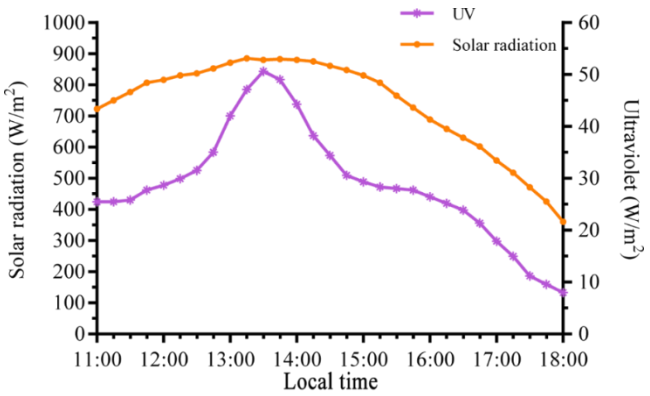

Figure 6- Solar and UV radiation on 17 August 2016 in Urmia University campus.

This equation determines the sensitivity of disinfection time to temperature variations. On the other hand, if the temperature varies one-Celsius degree, Table 4 shows variations in time, based on Equation 6. The minus sign in Table 4 indicates that the time decreases with increasing the temperature. The time variations of lower temperatures are considerably higher than the temperature of 60 °C. This issue is confirmed by comparing the result of Hao et al. (2012) for Pss (24h at 40 °C, Table 1) with the disinfection time of 50 °C (7 h, Table 2). Changing 10 °C in temperature led to the difference of 17 h in disinfection time.

Table 4- The time variation when the temperature increased by 1 degree Celsius at each temperature

Temperature (°C)	*d(time)(min)*
50	-128.4
55	-22.8
60	-4.0

When temperature increases from 55 to 56 °C, the disinfection time decreases 22.8 minutes (Table 4). Therefore, the disinfection time is estimated 22.2 min (45-22.8=22.2) at 56 °C for Cmm (Table 2) approaching Fatmi et al. (1991) report (30 min at 56 °C, Table 1).

Killing Pathogens are not always proportional to temperature-time product (Tang 2007). As Table 5 shows, the temperature-time product is considerably variable at each temperature, and it shows that temperature-time product cannot be a valid criterion for killing Cmm and Pss.

Table 5- Temperature-time product for Cmm and Pss disinfection

Temperature (°C)	*Time (min)*	*Time*temperature (min*°C)*
50	420	21000
55	45	2475
60	15	900

Figure 6 and Table 6 present solar and ultraviolet radiation data on 17 August 2016. This day had a minimum lethal UV dose among three different days. As Table 6 shows, a shorter time and lower UV dose may exist to kill the pathogens. However, the result of the experiment on August 21, 2016 showed that Cmm and Pss were survived at 45 min with 84.665 kJ m^{-2} and 2.3510 MJ m^{-2} ultraviolet and solar radiation, respectively. Therefore, lethal UV dose and time at 50 °C were obtained 95.481 kJ m^{-2} and 1 h, respectively.

Table 6- Bacteria survival after exposing to solar ultraviolet radiation on a horizontal surface on 17 August 2016 in Urmia University campus

Temperature (°C)	*Time (min)*	*UV dose (kJ m^{-2})*	*Solar radiation (MJ m^{-2})*	*Survival (%)*
				PSS, CMM
50	Control	0	0	100
	60	95.481	2.79315	0
	120	214.151	5.8203	0
	180	385.106	8.99145	0
	240	511.092	12.087	0
	300	611.834	14.83965	0
	360	695.142	17.10225	0
	420	738.914	18.78795	0

Comparing Table 2 (temperature: 50 °C, heat only) with Table 6 (heat + UV) shows that the required time for killing bacteria was decreased from 7 h to 1 h by adding the solar ultraviolet radiation (UV dose: 95.481 kJ m^{-2}). It confirms that there is a synergistic effect between solar UV and heat at 50 °C for Cmm and Pss.

Since no research was found that directly matched with the conditions of this research in solar radiation tests, similar cases were investigated.

In a study, the culturability reduction of Pss was reported at least 1000-fold when it was exposed to sunlight for 4 h at the temperature range of 25 to 27 °C (Miller et al. 2001). Results presented in Table 6 shows that 1 h was enough to remove Pss. However, the treatment temperature was 50 °C. This certifies again that there is a synergistic effect between solar UV and heat. Gunasekera & Paul (2007) estimated 24.8 MJ m-2 solar radiation to reduce Xanthomonas sp. population on tea leaves (LOG10(CFUs cm-2 leaf) ≈0). Table 6 shows that 2.79 MJ m-2 is required to eliminate Pss and Cmm. Apart from the bacterium type, this difference can be due to a synergistic effect. Moreover, Xanthomonas sp. bacteria attended in an environment with 18-19 °C temperature, while Cmm and Pss were exposed to heat at 50 °C.

In another research, inactivation time for Pseudomonas aeruginosa was reported 180 min when it was exposed to natural sunlight (Forte Giacobone & Oppezzo 2015). This time was higher than the inactivation time for Pss (60 min) due to the temperature difference. Pss was exposed to heat at 50 °C while the sample temperature for Pseudomonas aeruginosa was 29-31 °C.

A tilted surface with a proper angle can receive more solar radiation than a horizontal surface (Duffie & Beckman 2013). Thus, the reported effect of solar UV can be higher on a tilted surface and solar collectors. Also, Additives (Casado et al. 2021) and photocatalysts (Roshith et al. 2021) are other methods to enhance disinfection power and decrease disinfection time and solar UV dose.

4. Conclusions

Killing active growing cells of Pseudomonas syringae and Clavibacter michiganensis subsp. michiganensis by heat (50, 55, and 60 °C) and solar UV showed:

1) Solar UV and its synergy with heat could considerably reduce disinfection time (7 to 1h) at 50 °C. Thus, it can nicely recoup increasing time at lower temperatures,

2) The required solar UV dose was 95.481 kJ m^{-2} for killing Pss and Cmm at 50 °C temperature,

3) Governing equations between heat and time were logarithmic. Therefore, treatment time had lower sensitivity to temperature variations at higher temperatures,

4) Temperature-time product was not a valid criterion for killing Cmm and Pss.

Heat treatment at a lower temperature is desirable for solar collectors since more water will be disinfected with constant energy when the intended temperature is lower. Therefore, applying solar UV beside heat can enhance disinfection capability.

Future studies can precisely estimate the reported solar UV dose because it was overestimated due to the absorption of tube glass and water height (0.1 m). Moreover, considering the cumulative effects of solar UV and preheat will improve disinfection capacity.

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