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Faculty of Agriculture

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Research Article

Effect of Humic Acid Applications on Physiological and Biochemical Properties of Soybean (*Glycine max* L.) Grown under Salt Stress Conditions

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Keywords

Glycine max L.,
Humic Acid,
Salt stress,
Soybean.

Abstract: In the study, humic acid was applied to soybean (*Glycine max* L.), which has high economic value and importance, to determine the tolerance level of the plant against salt stress, and physical and chemical changes in the plant were observed. The study was carried out in the climate room of Van Yuzuncu Yil University Faculty of Agriculture, Department of Field Crops in 2019. In the research, İlksoy soybean variety was used. The experiment was carried out in 4 factorial orders according to the factorial experiment was designed based on Completely Randomized Design. In the research, four different Humic acid doses (0, 500, 1000 and 2000 ppm) and 3 different NaCl salt doses (0, 125 and 250 mM) were used. In the study, root length, stem length, root fresh weight, stem fresh weight, root dry weight, stem dry weight, leaf area, chlorophyll content, ion leakage in leaf tissues, lipid peroxidation level (MDA), relative water content and membrane resistance index in leaf tissues were determined. Properties such as index were also examined. As a result of the study, the longest root was 38 cm for the control plots that salt and humic acid didn't apply to the plants. The highest root fresh weight was 2.08 g and the stem fresh weight was 1.87 g of the plots where 500 ppm humic acid dose applied. In addition, the plants with the highest chlorophyll ratio was 51.05 under 250 mM salt applied without humic acid application.

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1. Introduction

Soil salinity creates stress on the plant and causes many changes. Most of the world's water resources (70%) are salty. Therefore, there are yield losses in aquaculture in areas with high salt content (Celik and Karakurt, 2022). Salinity; It is one of the leading sources of abiotic stress that threatens the life of plants (Toprak and Tunçtürk, 2018). Therefore, the effects of stress on different plants grown in the same saline soil differs. While some plants can initiate various morphological, physiological, biochemical and molecular changes depending on the increase in salinity in the environment in which they are grown, and minimize the damages of stress and continue their lives, the mechanisms that can

tolerate stress are not sufficient in some plants and their chances of survival decrease (Çulha and Çakırlar, 2011).

Humic acid which facilitates the uptake of water and nutrients by plants is a group of molecules and significantly increase productivity. Humic acid is a commercial product contains many elements which improve the soil fertility and increase the availability of nutrients and consequently increase plant growth and yield. It particularly is used to enhance or reduce the negative effect of salt stress. Many investigators reported that humic acid applications led to a significant increase in soil organic matter which is improves plant growth and crop production (SoilBiotics, 2019).

Glycine max L. is one of the most important oil plants. Soybean (*Glycine max* L.) is the most valuable oilseed crop in human nutrition as a good protein feed and biofuel raw material, and as fodder for detailed livestock and aquaculture. World soybean production is 75.5 million tons and its area is 176.6 million tons of beans. It is a product grown by irrigation. It is also grown in warm conditions in tropical, subtropical and temperate climates (FAO, 2019).

The objectives of this study were: to investigate the integrated effects of humic acid fertilizer on soybean growth and nutrient uptake; to understand the mechanism underlying soybean salt tolerance; to establish the effective-ness of preparations, made on the basis of humic acids on the yields of crops in case of different methods and amounts used; to study plant growth parameters and physiological and biochemical changes in soybean seedlings under salt stress.

2. Materials and Methods

In the present study, Ilksoy soybean variety obtained from Trakya Agricultural Research Institute was used as seed material in the experiments. Experiment was carried out in 48 plastic pots with 500 cc capacity. The factorial experiment was designed based on Completely Randomized Design with four replications, with four different humic acid doses (0, 500, 1000 and 2000 ppm) and three different NaCl salt doses (0, 125 and 250 mM). In the study, 3 seeds were planted in each pot and after germination of the seeds only one the best healthy plant was left and the other two were removed. The seeds were sterilized with 5% sodium hypochlorite for 15 minutes and thoroughly washed with pure water, and then they were ready for planting. The growing media of the seeds was 1/3 perlite and 2/3 soil mixture. After planting, the pots were placed in a 16/8 hour light/dark photoperiod, under 25° C temperature and 65% humidity in a chamber. Plants were applied 100 mg kg^{-1} nitrogen, 45 mg kg^{-1} phosphorus and 75 mg kg^{-1} potassium per plant as basic fertilization from planting (Ertürk, 2011). The experiment conducted in the climate room of the Department of Field Crops, Faculty of Agriculture, Van Yuzuncu Yil University in 2019.

Humic acid doses was mixed into the soil before planting, and 300mg kg^{-1}nitrogen, 150 mg kg^{-1} phosphorus and 200 mg kg^{-1}potassium were applied to each pot as basic fertilization. The salt stress applications were started when the plants reached a certain growth stage (about 1 month later). The application of salt was made by adding the solution prepared with different salt doses as irrigation. At the stage where physiological problems occurred in the plants, the experiment ended and plants were harvested for the necessary analyzes.

Root length (cm), from the most extreme part of the root part of the plants up to the root neck was found by measuring. Stem height (cm), the height of the plants was measured from the soil level to the highest point of the plant. Root fresh weight (g), after separating the root part of the plants representing the applications, root fresh weight was determined using a sensitive balance.

Stem fresh weight (g), after the plants representing the applications were cut at soil level, the fresh stem weight was determined using a sensitive balance. Root dry weight (g), after the harvest, plant samples were kept in the oven at 70 ºC for 48 hours and the root dry weight was calculated. Stem dry weight (g), after the harvest, plant samples were stored in the oven at 70 ºC for 48 hours and the stem dry weight was calculated.

Relative water content in leaf tissues (RWC) (%), to determine the proportional water content of the plants, 4 discs were cut and wet weights was weighed for each leaf immediately after harvest. Leaf discs weighed in ultrapure water at 25ºC for 2 hours, and turgor weights were weighed. The samples were then dried at 110° C for 24 hours to record their weight. Arora et al. (1998) equation was used for calculating.

$$RWC\ (\%) = [(\text{fresh weight-oven dry weight})/(\text{turgor weight - oven dry weight})] \times 100 \quad (1)$$

Determination of lipid peroxidation levels (MDA): Lipid peroxidation in plants is expressed as malondialdehyde (MDA) content. 0.5 g of the leaf sample was homogenized with 10 ml of 0.1% trichloroacetic acid (TCA) and the homogenate was centrifuged at 15000 g for 5 minutes. 1 ml of the supernatant portion was removed and 0.5% thiobarbituric acid (TBA) dissolved in 4 ml of 20% TCA was added. After the mixture was kept in a 95 °C water bath for 30 minutes, it was rapidly cooled in ice bath and centrifuged at 10000 g for 10 minutes (Sairam and Saxena 2000).

$$MDA\ (nmol\ ml^{-1}) = [(A532\text{-}A600) / 155\ 000]\ 10^6 \quad (2)$$

Determination of ion leakage in leaf tissues (%): The wet leaf samples (0.1 g) were taken before harvest and washed with tap water and then with pure water. The plant samples were kept in 10 ml of purified water at 40° C for 30 minutes, this was (C1). The EC were measured again in the sample held in a hot water bath at 100° C for 10 minutes (C2) and ion leakage or membrane permeability in leaf tissues were calculated by the following equation (Sairam, 1994).

$$\text{Ion Leakage in Leaf Tissues} = (C1 / C2) \times 100 \quad (3)$$

Membrane endurance index in leaf Tissues (%): First of all, leaf samples (0.1 g) were washed with tap water and then purified with pure water and the plant samples were kept in 10 ml of pure water for 30 minutes at 40° C and the EC was measured (C1), in the water bath which is kept at 100° C for 10 minutes, the EC was measured again (C2) and the membrane stability index or membrane stability index calculated in the leaf tissues with the following equation (Sairam 1994).

$$\text{Membrane Endurance Index in Leaf Tissues (\%)} = [1- (C1 / C2)] \times 100 \quad (4)$$

Leaf area: The leaves selected as representative of plant saplings were placed on A4 paper and photographed with android device. The leaf area was determined using the Easy Leaf Area program

Chlorophyll content: The chlorophyll content we determined by the portable chlorophyll meter device (Minolta SPAD-502, Osaka, Japan), which indirectly measures the chlorophyll content in the leaf.

Data were subjected to analysis of variance (ANOVA) using COSTAT statistical (version 6.3) package program according to the factorial experiment was designed based on Completely Randomized Design. The means compared with Duncan Multiple Range Test (DMRT) at α=0.05. Also, correlation analysis was done using IBM SPSS statistics (version 22.0) program (IBM Corp., 2013).

3. Findings and Discussion

The effects of humic acid (HA) were determined in controlled growth chamber via applying different concentrations of HA on soybean seedlings grown under different concentrations of salt stress through measuring some plant growth, physiological and biochemical properties.

In the study, while the effect of salt doses on root length, relative water content in leaf tissues, membrane resistance index of leaf tissues, leaf area and total chlorophyll content parameters was not statistically significant, its effect on other parameters was found to be significant.The effect of humic acid applications on root fresh weight and membrane resistance index of leaf tissues was found to be insignificant.The effect of S x HA interaction has statistically influenced on all other parameters, except features such as stem height, relative water content in leaf tissues, MDA, ion leakage in leaf tissues and membrane resistance index of leaf tissues.

Root length values of the soybean plants as a result of different salt dosage applications were determined as 29.7 - 30.0 cm. Although, salt doses negatively affected root length, this effect was not statistically significant. Growth and development are generally negatively affecting plants under salt stress, and in some cases the plant dies as an effect of the salt effect (Erdal et al., 2000). In many similar studies (Turkmen et al., 2008; Tunçtürk et al., 2011a; Kalyoncu, 2013), it was reported that increased salt concentrations had a negative effect on the root length values of the plant. Kondetti et al. (2012),

found that root seedling decreased linearly when the salt concentrations increased. The effect of different dosage of humic acid application on the average soybean plant root length grown under salt stress varied between 27.8 and 31.3 cm. In this study, the longest root length (31.3 cm) was obtained from HA_2 application. But, there is no statistical difference between control and HA_3 applications. The lowest (27.8 cm) was determined in HA_1 application. The results indicate that increasing humic acid dosage had a positive effect on the plant root length. Kalyoncu (2013) reported that increasing humic acid doses positively affects the root length of mung bean plants, which is similar to the findings of this study. Furthermore, Başalma (2014), Malik and Azam (1985) reported that application to humic acids to wheat increases root length.

Table 1. The effect of humic acid applications on some morphological parameters in salt stressed soybean.

Salt Doses (mM)	Humic Acid Doses (ppm)	Root Length (cm)	Stem Height (cm)	Root Fresh Weight (g)	Stem Fresh Weight (g)	Root Dry Weight (g)	Stem Dry Weight (g)
S_0	HA_0	38.0 ^{a}	30.5	1.28 ^{bcd}	1.37 ^{ab}	0.18 ^{b}	0.18 ^{b}
	HA_1	29.7 $^{a-c}$	27.3	2.08 ^{a}	1.87 ^{a}	0.24 ^{ab}	0.24 ^{ab}
	HA_2	26.7 ^{bc}	26.5	1.56 $^{a-d}$	1.45 ^{ab}	0.22 ^{ab}	0.22 ^{ab}
	HA_3	28.3 $^{a-c}$	23.7	1.23 ^{cd}	0.93 ^{bc}	0.27 ^{ab}	0.27 ^{ab}
S_0 Average		**30.7**	**27.0 A**	**1.54 AB**	**1.41 A**	**0.23 B**	**0.31 A**
S_1	HA_0	28.3 ^{bc}	26.3	1.78 ^{ab}	1.28 ^{b}	0.27 ^{ab}	0.27 ^{ab}
	HA_1	23.7 ^{d}	23.5	1.18 ^{d}	0.87 ^{c}	0.25 ^{ab}	0.25 ^{ab}
	HA_2	32.5 ^{ab}	23.5	1.12 ^{d}	1.28 ^{b}	0.27 ^{ab}	0,27 ^{ab}
	HA_3	34.5 ^{ab}	24.7	1.78 ^{ab}	0.86 ^{c}	0.29 ^{a}	0.29 ^{a}
S_1Average		**29.7**	**24.5 ABC**	**1.46 B**	**1.07 B**	**0.27 A**	**0.26 B**
S_2	HA_0	26.3 $^{b-d}$	28.0	1.72 ^{abc}	1.27 ^{b}	0.23 ^{ab}	0.23 ^{ab}
	HA_1	30.0 $^{a-c}$	20.7	1.46 ^{bcd}	1.19 b	0.28 ^{ab}	0.28 ^{ab}
	HA_2	34.5 ^{ab}	23.0	1.87 ^{a}	1.39 ^{ab}	0.31 ^{a}	0,31 ^{a}
	HA_3	29.3 $^{a-c}$	20.7	1.81 ^{a}	0.99 ^{b}	0.27 ^{ab}	0.27 ^{ab}
S_2Average		**30.0**	**23.1 C**	**1.72 A**	**1.21 B**	**0.27 A**	**0.27 AB**
Humic Acid Doses (ppm)	**HA_0**	30.8 A	28.3 A	1.59	1.31 A	0.23 B	0.34 A
	HA_1	27.8 B	23.8 B	1.57	1.31 A	0.26 AB	0.33 A
	HA_2	31.3 A	24.3 B	1.52	1.37 A	0.26 A	0.22 B
	HA_3	30.7 A	23.1 B	1.61	0.93 B	0.28 A	0.23 B
C.V (%)		**9.579**	**9.469**	**16.925**	**17.497**	**13.196**	**17.208**
S		ns	**	*	**	**	*
HA		*	**	ns	**	**	**
S x HA		**	ns	**	**	*	**

S: Salt Doses, HA: Humic asit, HA0: 0 (Control), HA1:500 ppm, HA2: 1000 ppm, HA3: 2000 ppm, S0: 0 (Control), S1: 125 mM, S2: 250 mM, * P <0.05; ** P <0.01 ns: non-significant.
There is nonsignificant difference between values with the same letter in the same column.

The effect of salt stress was significantly high on the soybean plants and control application (0 mM NaCl) produced taller plants. Average stem height was 27.0 cm, and the shortest plants were obtained from 250 mM salt application as 23.1 cm. However, the control and 125 mM salt concentration were within the same statistical mean group. Tunçturk et al. (2008 and 2011b) findings were similar, and they suggested that salt stress negatively affected on the stem height. The results from Table 1 shows that HA_0 (control) produced plants with the highest value of stem height as 28.3 cm, while all the other application doses (500, 1000 and 2000 g) were in the same comparison group. Furthermore, the plants were shorter than that of the control application, (23.8, 24.3 and 23.1 cm) respectively. Several previous researches support the results of this experiment's findings. El-Shafey and Zen El-Dein (2016), reported that the lowest values of stem height and ear height were recorded when maize intercropped with soybean and fertilizer by foliar humic acid in the two experimental seasons. Dawood et al. (2019), found that stem height was reduced with the increase of HA doses.

The average root fresh weight obtained from different salt applications varied between 1.46 g and 1.72 g. The highest root fresh weight (1.72 g) determined in the 250 mM NaCl applications, while the lowest root fresh weight (1.46 g) was obtained from 125 mM NaCl application. The highest value of root fresh weight for the HA treatment was 1.61 g obtained from the application of HA_3, and the lowest value was 1.52 g obtained from HA_2. However, the effect of the HA different doses was

statistically non-significant on root fresh weight. Basalma (2014), studied safflower varieties and humic acids levels and found that there were no significant effect the HA in terms of fresh root weight among the varieties, as well as humic acids doses, the highest root weight was achieved 5.189 g and 5.179 g respectively, from cv. Dinçer and 180 g of humic acids treatment. The S x HA interaction gave the highest value of rot fresh weight (2.08 g) under 0 mM NaCl with HA_1 treatment. The lowest value was 1.121 g obtained from the 125 mM NaCl with HA_2.

The different salt concentrations had a significant effect on the stem fresh weight. The highest weight was 1.41 g obtained from the control treatment 0 mM NaCl, while the lowest stem fresh weight was 1.07 g obtained from application of 125 mM NaCl. It was same group with 250 mM NaCl applications. In the study, different salt concentration applications are adversely affected by the stem fresh weight values compared to the control application. Tunçturk and colleagues, (2009), reported that salt stress was detrimental to stem fresh weight in soybean, weight of plants under salt stress at final harvest were significantly reduced compared with those of plants in the control treatment. Another work by Tunçturk et al., (2011a), suggested the same findings but on several canola (*Brassica napus* L.) cultivars. The effect of HA doses was significant on the stem fresh weight. The highest stem fresh weight was 1.37 g obtained from applying HA_2, and the lowest value was 0.93 g from the HA_3 dose. However, the control and HA_1 applications were in the same group with the HA_2, and the value of the stem fresh weight was 1.31 and 1.31 g respectively. In terms of S x HA interaction, the plants which received HA_1 with 0 mM NaCl, gave the highest value of stem fresh weight 1.87 g, and the lowest value was 0.86 g from HA_3 with 125 mM NaCl. These findings are similar to Dawood et al. (2019) suggestions for faba bean plants. Humic acid application caused increases in stem fresh weight.

The different salt concentrations had a significant effect on the root dry weight. The highest root dry weight was 0.27 g obtained from 125 and 250 mM NaCl application, while the lowest value was 0.23 g from the control applications. These results are similar to what Kondetti et al. (2012) found. They reported that root dry weight production of *Phaseolus mungo* for all the studied varieties decreased from 12.10 mg to 0.55 mg as salt concentrations increased from 0-300 mM NaCl. Tunçturk et al. (2008, 2011b) findings were similar; they suggested that salt stress affects negatively on soybean stem dry weight. In terms of HA, the highest root dry weight was 0.28 g obtained from the application of HA_3, and it was same group with HA_1 and HA_2 with 0.26 g dry root weights. The lowest value was from the control with 0.23 g root dry weight. Basalma (2014), finding was close to these results. There was variation in safflower seedling root dry weight, different cultivars were grown under different HA dosages, and the control application produced plants with lower root dry weight and the highest value was from higher doses of HA. In another experiment by Boogar et al. (2014), the effect of humic acid on the measured traits of betonia hybrid root weight did not show a statistically significant difference between humic acid treatments, but there was significant statistical difference between HA and the control. They found that increase in fresh and dry weight of roots was observed with HA applications. The interaction of S x HA results showed that plants received 250 mM NaCl with HA_2 had the highest value of root dry weight, 0.31 g, and those received 0 mM NaCl (control) with HA_0 (control) HA had the lowest value of root dry weight, 0.18 g.

In this study, salt applications negatively affected stem dry weight averages. The highest stem dry weight was 0.31 g obtained from 0 mM NaCl (control) applications, while the lowest stem dry weight was 0.26 g obtained from the 125 mM NaCl application. It was same Duncan group with 250 mM NaCl applications. The HA had a significant effect on stem dry weight. The highest value was 0.34 g obtained from the control and the lowest stem dry weight value was 0.22 g from the HA_2. For the interaction of S x HA, the highest stem dry weight value was 0.31 g obtained from the 250 mM NaCl with HA_2, and the lowest value was 0.18 g obtained from control 0 mM NaCl with HA_0. This result is similar to the findings of Tunçtürk et al. (2011b) on Canola, salt stress caused a significant decrease in the stem dry weights. Furthermore, Kondetti et al., (2012) studied *Phaseolus mungo* under salt and observed that dry weight of the seedling decreased with increasing NaCl.

The Relative water content in leaf tissues (RWC) was not statistically affected by salt doses (Table 2), the RWC values determined between 63.86-71.85%. The results indicate that increasing humic acid dosage had a positive effect on the average RWC, the highest value of RWC was 74.83% and the lowest value was 60.88% obtained from HA_1 and HA_3 respectively. RWC in leaf tissues of pepper cultivars at different salinity levels was investigated by Hand et al., (2017). The increased RWC

values in salt-tolerant cultivars suggest that, accumulation of osmolytes makes the surplus of water uptake possible.

The highest MDA value obtained from different salt applications was 0.78 nmol g^{-1} F.W obtained from 250 mM NaCl application, while the lowest value was 0.58 nmol g^{-1} F.W obtained from 0 mM NaCl application (control). In a conducted experiment on the effect of salt stress on soybean plant by Kumari et al., (2015), they found that MDA values increases with the increase of salt stress. The same result was discovered on other crops (Sairam and Srivastava, 2002; Porcel et al.,2003; Yildirim et al., 2004; Han and Lee, 2005; Shukla et al., 2012; Yolci et al. 2021). HA had a significant effect on the soybean plants for MDA. The HA_0 (control) had the highest MDA value 0.73 nmol g^{-1} F.W, and the MDA content in the HA_3 application was the lowest 0.66 nmol g^{-1} F.W. Similar results was discovered by Chen and Aviad (1990) and Kıran et al. (2019) , they documented that the application of HA on plants under stress reduces the MDA significantly.

Table 2. The effect of humic acid applications on some physiological parameters in salt stressed soybean

Salt Doses (Mm)	Humic Acid Doses (ppm)	Relative Water Content in Leaf Tissues (%)	MDA (nmol g^{-1} F.W)	Ion Leakage in Leaf Tissues (%)	Membrane Resistance Index of Leaf Tissues (%)	Leaf Area (cm^2)	Total Chlorophyll Content (SPAD)
S_0	HA_0	65.49	0.63	2.40	88.51	12.10 ^{cd}	43.55 $^{c-e}$
	HA_1	74.92	0.53	2.11	85.66	16.65 ^{ab}	46.45 $^{a-d}$
	HA_2	68.78	0.61	3.49	89.59	18.97 ^{a}	46.23 $^{b-d}$
	HA_3	46.27	0.56	3.69	91.38	17.31^{ab}	40.83 ^{de}
S_0 Average		**63.86**	**0.58 C**	**2.93 B**	**88.78**	**16.21**	**44.26**
S_1	HA_0	68.35	0.75	1.88	90.27	11.06 ^{d}	48.13 $^{a-c}$
	HA_1	61.99	0.68	4.44	86.75	18.65 ^{a}	43.75 ^{cd}
	HA_2	67.36	0.67	5.67	89.08	14.66 ^{bc}	43.65 ^{cd}
	HA_3	67.18	0.64	7.03	85.51	16.45^{ab}	45.03 ^{cd}
S_1 Average		**66.22**	**0.68 B**	**4.75 A**	**87.90**	**15.20**	**45.14**
S_2	HA_0	65.38	0.81	1.96	87.15	14.47 ^{bc}	51.05 ^{a}
	HA_1	87.58	0.78	2.65	91.47	16.64 ^{ab}	48.40 ^{ab}
	HA_2	65.26	0.75	6.61	89.04	17.76 ^{a}	40.83 ^{e}
	HA_3	69.20	0.78	2.49	94.04	15.75 ^{b}	42.23 ^{de}
S_2 Average		**71.85**	**0.78 A**	**3.43 AB**	**90.42**	**16.15**	**45.63**
Humic Acid Doses (ppm)	HA_0	66.40 AB	0.73 A	2.08 C	87.96	12.54 B	47.57 A
	HA_1	74.83 A	0.66 B	3.07 B	89.24	17.25 A	46.2 A
	HA_2	67.13 AB	0.67 AB	5.26 A	89.24	17.13 A	43.56 B
	HA_3	60.88 B	0.66 B	4.41 AB	90.31	16.49 A	42.69 B
C.V (%)		**13.26**	**9.96**	**11.21**	**8.99**	**8.894**	**5.186**
S		**ns**	**	*	ns	ns	ns
HA		*	**	**	ns	**	**
S x HA		**ns**	ns	ns	ns	**	**

S: Salt, HA: Humic asit, HA0: 0 (Control), HA1:500 ppm, HA2: 1000 ppm, HA3: 2000 ppm, S0: 0 (Control), S1: 125 mM, S2: 250 mM, ns: Non-significant. * P <0.05 significant. ** P <0.01 high significant, n.s: non-significant.
There is nonsignificant difference between values with the same letter in the same column.

The highest leakage in leaf tissues obtained from different salt treatments applied to soybean plant seedlings was 4.75 % obtained from 125 mM NaCl application, and the lowest value was obtained from control application with 2.93 %. At the end of the study, it was determined that the ion leakage in the leaf tissues increased in the plants applied salt source according to control applications. In terms of HA doses, the highest value of this parameter was 5.26 % obtained from the HA_2 application, and the lowest value was 2.08 % obtained from the application of HA_0 (control).

Membrane resistance index of leaf tissues obtained as a result of different salt applications varied between 87.90-90.42 %. The results of the application of HA on soybean, the mean membrane resistance index of plant leaf tissues varied between 87.96-90.31 %. The effect of soybean applications with HA on membrane resistance index of leaf tissues in plant was positive and the rate increased as the doses increased. Sairam and Srivastava (2002), in the study of the effects of salt stress on antioxidant

properties of long-term salt applications in wheat plants in the study of salt membrane stability index of the study reported that the reduction of the membrane shows a parallel with this study.

The leaf area varied between 15.20 and 16.21 cm^2 in terms of salt doses. However, there was no significant differences when the data were statistically analyzed. The effect of HA was significant, the highest value of leaf area was 17.25 cm^2 obtained from the HA_1 applications, and the lowest value 12.54 cm^2 was obtained from the control. But, HA_1 application was same group with HA_2 and HA_3 applications. The interaction of S x HA was significant; the highest value of leaf area was 18.97 cm^2 obtained from the 0 mM NaCl with HA_2 applications. However, this treatment was with the same group with 125 mM NaCl with HA_1 and 250 mM NaCl with HA_2 applications with values of 18.65 and 17.76 cm^2 respectively. Yasar (2003), stomata of plants containing salt stress to close the leaf area is reported to be reduced by reducing transpiration rates. Our findings were in parallel to the results of these studies and the results of our research. El-Shafey and Zen El-Dein (2016) results on soybean plant experiment showed similar effect on leaf area.

In study, there was no significant differences when the data were statistically analyzed in terms of salt doses. It was obtained between 44.26-454.63 SPAD values. The effect of HA was significant, the highest value of total chlorophyll ratio was 47.57 obtained from the control HA applications. But, it was same group with HA_1 application. The lowest value 42.69 was obtained from the HA_3. There aren't differences statistically with HA_2 applications. The S x HA interaction showed significant effect. The highest value was obtained 51.05 from the 250 mM NaCl with HA_0, and the lowest value was obtained 40.83 from 250 mM NaCl with HA_2. Sairam et al. (2000), reported that chlorophyll content in plants was negatively affected as a result of salt applications. Sairam and Srivastava (2002) observed that salt stress in wheat genotypes reduced total chlorophyll content in leaf tissue. Turan and Aydin (2005), examined the effect of different salts on some physiological properties of corn plant in a study, determined that the plant growth and chlorophyll content decreased as the applied salt concentration increased. Turhan et al. (2006), salt stress due to the negative effects of chlorophyll in sunflower found. Turan (2007), salt stress in the lentil plant as a result of increased salt applications reported that the total chlorophyll content significantly decreased compared to control.

4. Conclusion

Soybean plant has become one of the most important plants in the world with the increasing usage areas in recent years. In the study, physiological and biochemical changes occurring in the plant under stress conditions were observed by applying different salt doses on soybean plants along with the application of different humic acid doses. In the research, by applying different humic acid doses and different salt doses to soybean plant, some growth parameters (root length, stem length, root fresh weight, stem fresh weight, root dry weight and stem dry weight) and some biochemical properties (RWC, MDA, membrane resistance index in leaf tissues, ion leakage in leaf tissues total chlorophyll content, and leaf area) were determined. The results of the experiment showed that; root fresh and dry weight, stem fresh and dry weight, stem length, and lipid peroxidation level (MDA), among the properties examined with salt applications, were statistically affected. The application of different humic acid doses, had statistically affected the root and stem length, leaf area and chlorophyll content. The effect of salt and humic acid doses applied in the study on relative water content, membrane resistance index and ion leakage properties in leaf tissues was not found statistically significant. According to the results obtained from the research; it can be recommended that humic acid applications is preferable in terms of minimizing the stress factors on plants that are adversely affected by salt stress conditions. In addition, it is thought that more positive results can be obtained on the physical and biochemical properties of the plant by applying humic acid applications before the stress effects are seen in the plant.

Ackonowledge

This study titled *"*Effect of Humic Doses Applications on Physiological and Biochemical Properties of Soybean (*Glycine max* L.) Grown Under Salt Stress Conditions*"* is the summary of Noor Maiwan BAHJAT master's thesis.

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Research Article

Practical Application and Influence of "Avigen duck" Immunomodulator to White Pekin Ducks Humoral Immune Factors

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Abstract: Factors of non-specific immunity are responsible for protecting birds from a number of viruses and bacteria. Bactericidal proteins are played here lysozyme and complement, interferon, as well as beta-lysine. Adding appropriate active compounds to the bird's diet to activate the innate immune response are subject to studies by many authors. The practical application and influence of the "Avigen Duck". immunomodulator in the cultivation of White Pekin ducks aims to clarify the dynamics of the indicators. It was found that the ducks treated with the immunomodulator had higher non-specific protection expressed in the values of the observed indicators. Complement activity for the experienced group (529.45 ± 17,85 CH50) is significantly higher than that of untrained birds (308.56 ± 10.19 CH50), on the 30th day of their lives (P <0.001). Lysozyme values for the experienced group (6.34 ± 0.86 mg L^{-1}) is more than twice as high as its concentration in control birds - 2.52 ± 0.59 mg L^{-1}. At 30 days of age, the mean concentration of IFN γ in the control group of ducks was 108.86±6.12 pg ml^{-1}, and in the experimental group treated with immunomodulator - 518.06±12.80 pg ml^{-1}, accompanied by an increase in the values of IL-2 and IL-6 and beta-lysine activity. These data show that, despite the short life of the ducks, the concentrations of the investigated factors of non-specific immunity increase significantly and statistically reliably, even after a single treatment with immunomodulator "Avigen Duck". The practical application of the immunomodulator in the duck diet has a strong effect on their non-specific immunity.

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1. Introduction

Modern poultry farming requires fast-growing periods, high food conversion ratios, and maintenance of health status at the lowest possible cost. One of the biggest challenges is the high density of the bird population, raised in relatively small spaces, which increases the risk of spontaneous disease outbreaks (Linares and Martin, 2010). If the first two necessities are influenced by the nutritional

properties of the food provided. The third requirement has a far more complex nature where animal selection and the introduction of specific food supplements play a crucial role. However, targeting a high immune response against a specific pathogen is not always the best option. In several cases, farmers face subclinical infections from a well-known cocktail of locally presented infectious agents. This category of diseases has a massive impact on animals' productivity and well-being. The unpredictable nature of such processes requires prominent levels of non-specific immune response factors among all animals (Al-Mansour et al., 2011).

Two major factors of natural humoral immunity are the complement activity and the lysozyme values in the blood serum. The complement system and the two major pathways of its activation are one of the oldest mechanisms for protection against pathogenic bacteria and play a crucial role in both specific and nonspecific immune responses. The system consists of several proteins, produced in an inactive form (Karakolev, 2014). Its activation results in a cascade of consecutive reactions aimed at eliminating a specific infectious agent. The activation of the complement in the absence of a specific antigen is an alternative variant of classic activation and does not require the formation of an antigen-antibody complex. This makes it one of the very first defense mechanisms against a wide range of pathogens. Its key role remains further evidenced by the fact that this mechanism was the first to evolve in evolution (Zewde et al., 2016). The triggering of the alternative mechanism complement system is quite effective against different gram-negative bacteria, viruses, neoplastic cells, etc. (Cortes et al., 2013). Lysozyme is an antimicrobial enzyme that has essential functions in the innate immune response. Muramidase is found in egg white, saliva, blood serum, etc. The serum lysozyme is predominantly produced by the macrophages (Besarabov, 2013) and is effective against Gram-negative bacteria as well as some large viruses, such as *Avipoxvirus* (Zhang et al., 2017). Breed, sex, age, and species variations in both parameters have been observed (Koynarski et al., 2018; Koynaski and Sotirov, 2013). Given the large number of genes encoding different protein fractions for both parameters of interest, marker-assisted selection does not provide a useful solution. Moreover, poultry farming has always focused more on productive parameters than health and welfare (Bahmanimehr, 2012). In the past, fighting pathogens favored using antibiotics, including some with nutritional effects. Nowadays trends for the production of antibiotic-free animal products motivate the use of different probiotics, symbiotics, immunostimulants, and vaccines (Jiang et al., 2015; Wang et al., 2016). Many of these substances have pathogen inhibition, growth performance, and welfare properties (Chen et al., 2017). At present, the market offers several immunomodulators of herbal origin, but their effect is limited (Georgieva et al., 2013). Given that triggering molecules for APCA and lysozyme actions are lipopolysaccharides found in the bacterial cell membrane and viral envelopes, using an immunomodulator based on the same concept seems promising. Beta-lysine is part of the thermostable bactericidal fractions in serum active against bacilli that do not require the presence of complement to perform their function (Weinert, 2013). Their influence affects both non-specific resistance and homeostasis. Interferon γ are products of T-lymphocytes and natural killer cells in birds and participate in all immune reactions, and IFN α/β are mainly responsible for carrying out antiviral immunity (Masuda et al., 2012, Karakolev, 2014, Swaggerty et al. 2015). Bacterial lipopolysaccharides are known to be a powerful inductor of interferon (Lalev et al., 2015). IL-2 and IL-6 in birds play an important role as regulation factors and mediators of the immune response (Fernando et al., 2015).

The major focus of this study was given to the impact of the "Avigen Duck" on some innate immune factors among White Pekin ducks. Since the product is based on lipopolysaccharide components of the thermostable endotoxin of Gram-negative bacteria from *Enterobacteriaceae* we could assume its stimulating effect on parameters of the innate immune response.

2. Material and Methods

2.1. Birds

White Pekin ducks of two flocks - experienced and controlled, grown under the same production conditions. The birds of the experienced flock received an immunomodulator from the 1st to the 10th day of the fattening period. The birds of the control flock did not receive an immunomodulator. Both flocks were treated with the preparations "Aspivit C" and "Bioxan", which played an auxiliary role. The first has an anti-stress effect, and the second increases the permeability of the mucous intestinal surfaces.

2.2. Method of treatment

Polybacterial immunomodulator "Avigen Duck" is a concentrated form of lipopolysaccharide, extracted from the Enterobacteriaceae family. The immunomodulator was administered in liquid form, containing 3 000 doses (ten days) in 1000 ml. It is used orally through drinking water, using the available farm dispensers.

2.3. Sampling

The experimental and control flocks numbered 14 000 birds each. From the birds treated with the immunomodulator in the experimental flock, as well as from the control one, 45 birds were randomly selected, from which blood was taken for testing. On the 20th and 30th day of the life of the birds (the 10th and 20th day after treatment with the immunomodulator) we took blood from the axillary vein. The separated blood serum was stored at 4°C. Serum testing was not later than 24 hours after sampling.

2.4. Determination of interferon-γ, interleukin-2 and interleukin-6

The interferon concentration was determined by enzyme-linked immunosorbent assay. We used DUCK Interferon (IFN γ) ELISA kit, DUCK Interleukin ELISA kit (MYBIOSOURCE). We added the following standards to the wells of the plate with concentrations: 0, 15.6, 31.2, 62.5, 125, 250, 500, and 1000 pg ml^{-1} for IFN γ, for IL-2 we prepared standards of 0, 6.25, 12.5, 25, 50, 100, 200 and 400 pg ml^{-1}, and for IL-6 - 6.25, 12.5, 25, 50, 100, 200 pg ml^{-1}, respectively. Extinctions were measured at a wavelength of 450 nm. We have calculated concentrations based on a standard curve.

2.5. Determination of complement activity and lysozyme concentrations

Complement activation was evaluated by the method of (Sotirov et al., 2005). One hundred microliters of each serum sample were diluted with 350 µl veronal-veronal Na buffer (in final concentrations: 146 mM NaCI, 1.8 mM 5.5-diethylbarbi-turic acid sodium salt; 3.2 mM 5.5-diethylbarbituric acid; 1 mM EGTA and 0.8 mM $MgCl_2$). Using U bottomed plates, 7 other dilutions from each diluted serum were again prepared in veronal-veronal Na buffer, so the final serum dilutions were 8/45, 7/45, 6/45, 5/45, 4/45, 3/45, and 2/45, respectively. Subsequently, each well was supplemented with 100 µl of 1% rabbit erythrocyte suspension. Samples were incubated for 1 hour at 37°C statically and then centrifuged at 150 g for 3 minutes at room temperature. Thereafter, 150 µl of each supernatant was placed into a flat-bottomed plate for measurement of optical density at 540 nm using 'Sumal-PE2' ELISA reader (Karl Zeiss, Germany). The final APCA activity was calculated using a dedicated software developed at Trakia University (Bulgaria) and expressed as CH50 units (corresponding to 50% of complement-induced hemolysis of applied erythrocytes).

Blood serum lysozyme concentrations were analyzed by the method of (Sotirov and Koynarski, 2003). The method consists of mixing 20 ml of 2% agarose dissolved in phosphate buffer (0.07 M $NaHPO_4$ and NaH_2PO_4) with 20 ml suspension of a 24-hour culture of *Micrococcus lysodeicticus* at 67°C. While still warm the mixture is poured into a 14-cm Petri dish. After solidifying at room temperature, 5 mm wells are made. Each well is filled with 50 µl of undiluted serum. Eight standard lysozyme dilutions (from 0.025 to 3.125 µg/ml) are prepared and pipetted into eight wells. The plate is then incubated for 20 hours at 37°C. The final lysozyme concentration is calculated by dedicated software developed at Trakia University (Bulgaria), comparing the lytic zone of each sample with the standard lysozyme dilutions.

2.6. Determination of beta-lysine activity.

The beta-lysine activity of blood serum was determined by a spectrophotometric method of Bucharin et al. (1977), modified by Karakolev and Nikolov (2015). The research was performed in flat-bottomed plates. We used a pre-prepared spore suspension of Bacillus subtilis ATCC 6633. We added the controls with an automatic pipette - 80 µl of saline + 80 µl of spore suspension in each of the first 4 wells. We then instilled the experimental sera with an automatic pipette - 80 µl serum + 80 µl spore suspension in each of the following wells, according to the number of samples tested. We homogenized it with a plate shaker. Optical density measurements were performed using a BioTek L80 spectrophotometer at a wavelength of 630 nm, before incubation. We incubated the plate in a plate

incubator with a timer (37°C for 2 hours). Immediately after incubation, we again measured the optical densities at the same wavelength. Since the optical densities of the controls did not change for 2 hours in the incubator, we performed the calculations by taking the changes in the optical densities of the samples, for each well separately, according to the formula:

$$\% \text{ of lysis} = OD1 - OD2 / OD1 \times 100 \quad (1)$$

where OD1 is the optical density of the sample before incubation and OD2 is the optical density of the sample after incubation.

2.7. Statistical analysis

Obtained data have been processed by independent t test with the fixe defect model using the Data analysis tool pack, Microsoft Excel 2016, Microsoft Corporation Ltd., at a level of significance $P < 0.001$.

3. Results

3.1. Values of lysozyme, complement and beta-lysine activity in blood serum

As can be seen from the Table. 1 the lysozyme values in experimental birds was higher than in the control flock ($P<0.001$). Lysozyme concentrations varied from 6.58 mg L^{-1} to 6.34 mg L^{-1} in experimental ducks. In untreated birds, these levels were significantly lower and ranged from 3.08 mg L^{-1} to 2.52 mg L^{-1}. In experimental flock, significantly higher APCA activity (529.45 CH 50) was found while in control chickens this value was lower (308.56CH50; $P <0.001$) on the 30th day of the life.

Table.1. Serum lysozyme concentrations, complement activity (APCA) and beta-lysine activity.

Groups	Lysozyme (mg mL^{-1})	APCA (CH50)	Beta-lysine activity (%)
White Pekin ducks, 20th day of the life			
Experimental	6.58±0.94***	506.52±18.34***	32.58±1.55***
Control	3.08±0.68	311.40±15.50	12.52±1.40
White Pekin ducks, 30th day of the life			
Experimental	6.34±0.86***	529.45±17.85***	34.89±0.95***
Control	2.52±0.59	308.56±10.19	12.60±1.72

*** $P < 0.001$.

Beta-lysine activity in blood serum was also higher than measured in birds untreated with the immunomodulator – from 32.58% to 34.89% in experimental ducks against from 12.52% to 12.60% in the control flock ($P<0.001$).

3.2. Values of interferon-γ, interleukin-2 and interleukin-6 in blood serum

Interferon γ and interleukins concentrations in birds are presented in Table 2. In birds treated with "AVIGEN DUCK", significantly higher concentrations of IFN γ were found compared to the control flock ($P <0.001$). These values fluctuate between 540.45 pg mL^{-1} and 518.06 pg mL^{-1}, and although they slightly decrease by day 30, remain much higher than the concentrations of controls.

The concentration of interleukins also responded in a positive direction as a result of the administration of the immunomodulator. In control birds, the levels of IL-2 hesitated between 6.30 pg mL^{-1} and 6.54 pg mL^{-1}, respectively on the 20th and 30th day of the life of the birds, while in the experimental group, there was an increase in these values. We have received similar data for the levels of IL-6. In birds treated with "Avigen Duck", the concentration of IL-6 in the blood serum reached 95.10 pg mL^{-1}, while in the control groups, it remained at low values – from 8.65 to 9.22 pg mL^{-1}.

Table 2. Concentrations of interferon-γ, interleukin-2 and interleukin-6 in blood serum

Groups	IFN γ (pg mL^{-1})	IL-2 (pg mL^{-1})	IL-6 (pg mL^{-1})
White Pekin ducks, 20th day of the life			
Experimental	540.45±10.20***	10.46±0.58***	70.25±7.40***
Control	119.22±6.58	6.30±0.90	8.65±2.72
White Pekin ducks, 30th day of the life			
Experimental	518.06±12.80***	10.98±0.66***	95.10±8.19***
Control	108.86±6.12	6.54±0.75	9.22±2.50

*** $P < 0.001$.

4. Discussion

In all our tests in blood serum, we found a higher concentration of lysozyme, higher complement activity and beta-lysine activity in birds from the experimental flock that received an immunomodulator. Other non-specific humoral indicators - interferon γ, interleukins 2 and 6, also showed increased values in the experienced flock, compared to the control. These indicators are likely to respond of stimulating mucosal membranes with lipopolysaccharides of polybacterial immunomodulator, as well as the auxiliary action of the preparations "Aspivit C" and "Bioxan", with the latter helping to improve the superficial absorption of the intestinal mucosa. In addition, the application of the anti-stress preparation "ASPIVIT C" provides additional equalization in the conditions of the experiments that were conducted under production conditions. This makes it possible to exclude the influence of some technological stressors and to believe with a high degree of confidence that the increase in lysozyme, complement, beta-lysine activity, IFN γ, IL 2, and IL 6 in experimental birds is due to the effect of the immunomodulator "AVIGEN DUCK". In any case, the results obtained from the control herds and reflected in Tables 1 and 2 confirm this judgment. Several studies on germ-free animals show that symbiotic bacteria and/or bacterial molecules (lipopolysaccharides, β-glucan and peptidoglycans) can completely cause a non-specific immune response (Gensollen et al., 2016; Ganalvonarburg et al., 2016; MacPherson et al., 2017), as the intestinal mucosa has its leading meaning in the initial activation of the innate immunity and influence its regulation and maturation.

Lipopolysaccharides of polybacterial immunomodulators are powerful inductors of lysozyme, complement, interferon, and cytokines (Karakolev et al., 2014). The current experiments also establish their effect on beta-shade activity, which is part of the non-specific immune factors in the blood serum. Obviously, lipopolysaccharides are one of the earliest factors stimulating congenital immunity, and their additional activity can be induced by lipopolysaccharides by enterobacteria included in the drinking water of birds, as our experiments show. According to Cellak and Babacanoglu (2022) on the blood biochemical parameters of broiler chicks, can be influenced very early by the injection of leptin in the yolk sac of the embryo.

In our previous studies, the physiological values of beta-lysine in the blood serum of broiler breeders and broilers were monitored. Unlike broilers, in parent flocks, there is a strong stress factor associated with the onset of laying and accompanying hormonal and immune rearrangement in the body (Karakolev, 2015; Karakolev and Nikolov, 2015). Nevertheless, the concentration of beta-lysine may be influenced by some factors that have a beneficial effect on the activity of complement, lysozyme and other indicators of the natural (innate) immune response. A number of authors (Ganalvonarburg et. al., 2016; Zemskov et al., 2018; Bozakova et al., 2018) consider the factors that may influence the mechanisms of nonspecific, innate resistance and emphasize that polysaccharide substances are one of the most effective for this purpose. Hung et al. (2013) consider that AblAs from methanoarchaea are lysine 2,3-aminomutases that may function as potential biocatalysts for the synthesis of β-lysine *in vivo* and *in vitro*. Okanishi et al. (2013), Zhang et al. (2013), Weinert et al. (2013), also developed genetic methods for the biosynthesis of lysine for biotechnological purposes. From the present experiments, it is clear that lipopolysaccharides from enterobacteria contained in concentrated form in the immunomodulator "AVIGEN DUCK" have a positive effect on the activity of beta-lysin fractions in the blood serum as well as other observed indicators. Increased activity of IFN and IL-4, IL-6, and IL-15 monitors Liao et al. (2021) after the treatment of Muskovy ducklings with *Astragalus polysaccharide*

(APS). The results showed that APS significantly affects intestinal injuries of villi length and wall thickness of the small intestine infected with Muscovy duck reovirus, subsequently increasing sIgA and all the cytokine productions at most time points, suggesting that APS pretreatment can effectively stimulate mucosal immune function by improving intestinal morphology. Similar data in broilers reported Sotirov et al. (2021), which compare the effect of the application of *Schizochytrium limacinum* on some indicators of natural immunity in broilers. The authors found an increase in the activity of beta-lysine in the blood serum in experimental group III, accompanied by a slight decrease in the values of lysozyme and complement. Bozakova et al., (2018) followed the influence of the immunomodulator "Immunobeta" on innate humoral immunity in laying hens and also found changes in beta-lysine activity. In conditions of temperature stress (Bozakova et al., 2018) studied the effect of the immunomodulator "Immunobeta" on innate humoral immunity in turkeys and laying hens, which supports our data on the special role of beta-lysine in stressful situations. The results we have obtained are in line with the established by other modern authors about the ability of polysaccharides of different origins, including vegetables, to regulate the immune response in fattening ducks and broiler chickens (Liu et al., 2016; Wang et al., 2016; Li et al., 2018).

Following the administration of the "AVIGEN DUCK" immunomodulator, the activity of nonspecific immune factors in the blood serum of White Pekin ducks increases well above the physiological boundary observed in the control flock grown under the same conditions. In this way, the nonspecific resistance of ducks, which is of a lasting character, is of particular importance for their health status.

Conclusion

The experimental data we receive, allow the conclusion to be made that the „Avigen Duck" immunomodulator containing lipopolysaccharides of enterobacteria and administered with drinking water at a suitable dose causes an increase in nonspecific resistance factors in White Pekin ducks and is suitable for use, in view of their health status when growing for meat.

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Research Article

An Approach for Developing a Simple and Quick Method for Separation of Asiatic Acid and Asiaticoside Rich Fraction From *Centella Asiatica* and Simultaneous Determination by Reversed-Phase High-Performance Liquid Chromatography

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Centella asiatica,
Extraction,
Isolation

Abstract: In Asian countries, *Centella asiatica is* exploited for abundant types of pharmacological activities due to the presence of opulent phytochemicals. Asiaticoside, madecassoside, and their sapogenin triterpene acids such as asiatic acid and madecassic acid are the most noticeable triterpenes present in *C. asiatica.* To date, numerous techniques/methods are used to extract and isolate the different kinds of phytoconstituents from *C. asiatica.* Still, most methods require some special requirements, and some procedures are monotonous and time-consuming. Meanwhile, previously reported methods used for the extraction and isolation were not validated for large-scale production, yield, and purity. The study's primary goal is to develop the methodology for extracting and isolating the Asiaticoside and asiatic acid from *C. asiatica* at the minimum time with the highest yield and purity. Asiaticoside and asiatic acid extraction and isolation involved the acid hydrolysis method and recovered in alcohol. The Reverse Phase-High Performace Liquid Chromatography (RP-HPLC) method was developed and validated as per ICH guidelines for quantifying both compounds. The obtained results indicate that the developed method produces asiaticoside and asiatic acid with good purity. As per the ICH guidelines, the RP-HPLC was developed and validated. The proposed method can be used to isolate the asiaticoside and asiatic acid from *C. asiatica.* Some modification in this method leads to the large-scale production of highly pure asiatic acid and asiaticoside for their versatile application in the area of cosmetics and phytopharmaceuticals.

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1. Introduction

Traditionally, in Asian countries, the *Centella asiatica* (Gotu kola) (Family-Apiaceae) is exploited, especially in Ayurveda and Unani, for memory enhancement, healing wounds, reducing

anxiety and stress, leprosy treatment, fever, syphilis, acne, allergies, eczema, psoriasis, etc. types of pharmacological activities. It also produces well-documented neuroprotective effects. Such types of activity/effects produce by *C. asiatica* due to the presence of opulent phytochemicals. To date, more than seventy compounds have been extracted and identified from *C. asiatica.* The majority of phytoconstituents belong to the triterpenes, flavonoids, and essential oils categories. Asiaticoside, madecassoside, and their sapogenin triterpene acids such as asiatic acid and madecassic acid are the most noticeable triterpenes present in *C. asiatica.* (Idris et al., 2021; Lu et al., 2021; Nouri et al., 2021; Ribeiro et al., 2021; Songvut et al., 2021; Sabaragamuwa et al., 2022) Polypharmacological properties of asiatic acid and asiaticoside sparked interest in the research community. Numerous researchers throughout the globe reported the different pharmacological properties of the active phytoconstituents such as anti-inflammatory, antioxidant, hepatoprotective properties, anti-diabetic properties, analgesic, antibacterial, cytotoxic, etc. (Lu et al., 2021; Pingyod et al., 2021; Ribeiro et al., 2021; Thong et al., 2021; Mohammed et al., 2022). These pharmacological activities associated with the asiatic acid and asiaticoside has been thoroughly studied and well documented.

Idris and Nadzir summarised the various techniques/methods (conventional and modern methods) used to extract and isolate the different kinds of phytoconstituents from *C. asiatica* to date. Techniques such as maceration, distillation, Soxhlet, ultrasound-assisted extraction, microwave-assisted extraction, vacuum microwave-assisted extraction, solvent-free microwave extraction, enzymatic pretreatment microwave extraction, and subcritical water extraction were enlisted for the extraction of constituents from *C. asiatica.* Most researchers used ethanol, methanol, water, and a mixture of water with ethanol or methanol to extract the desirable phytoconstituents. (Idris et al., 2021)

Ongoing through the literature on methods for extracting and isolating the phytoconstituents from *C. asiatica*, it was observed that most methods were targeted for extraction of asiaticoside, madecassoside, asiatic acid, and madecassic acid. The main reason behind such target-specific research is the diverse and effective pharmacological activities of these constituents from *C. asiatica.* Global demand for plant extracts with a high percentage of pure phytoconstituents has significantly increased but has not commensurate increased the supply. This urgency has attracted researchers from different communities to develop the extraction and isolation methodology for the essential constituents of *C. asiatica.*

The past decade has seen an increase in the use of plant-based phytoconstituents to prevent or treat various diseases. The versatile use of both constituents states the importance of extraction and isolation in phytopharmaceuticals. All the developed methodologies in the literature are not suitable for large-scale production due to the limitation of each method. Such as, many researchers use column chromatography to isolate desirable constituents from *C. asiatica.* But the column chromatography has its limitations, such as being time-consuming, tedious, and laborious. The reproducibility for the column chromatography was significantly less. Therefore, column chromatography cannot be used for the large-scale production of asiatic acid or asiaticoside. Other methods where sophisticated instruments used for the separating such as supercritical fluid extraction, flash chromatography, etc led to an increase in the cost of the final product. The use of such kinds of appliances produces expensive products. Majorities of phytopharmaceutical industries need an economical method with higher yield and purity. The previously reported methods used for the extraction and isolation were not validated for large-scale production, yield, purity, and economical method. Therefore, there is a need for a simple and economical method for extracting and isolating both constituents from *C. asiatica.*

The availability of these constituents is challenging. We have strongly focused on addressing these obstacles related to the availability of these materials at low cost. This is the essential motivation behind this research. The main purpose of this study was to develop a generic, simple and accurate approach to the extraction and isolation of asiatic acid and asiaticoside from *C. asiatica.* Specifically, we aim to investigate the yield along with purity. To assist the purity, we perform the HPLC analysis of asiatic acid and asiaticosides. We developed the HPLC method and validate it as per International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines.

2. Material and Methods

2.1 Plant material

Dried powder of plant material was purchased from the local crude herbal drugs market, Mumbai. The voucher specimen (ICT/MNPRL/2021/CA-02) was deposited at the Medicinal Natural Products Research Laboratory, Institute of Chemical Technology, Mumbai.

2.2 Reference standards and chemicals

Laboratory reagent-grade solvents were used for extraction and isolation. All the solvents were obtained from Finar Limited. HPLC grade acetonitrile, *o*-phosphoric acid, and water were purchased from Finar Limited. Asiatic acid (Purity ≥ 97% by HPLC) was purchased from Sisco Research Laboratories Pvt Ltd, (SRL) Mumbai, India. Asiaticoside (Purity ≥ 95 % by HPLC) was procured from Sunpure Herbal extract Pvt Ltd, Delhi.

2.3 Method of isolation

In the Soxhlet apparatus, approximately 1000 g of dried powder of *Centella asiatica* was subjected to solvent extraction using petroleum ether (4.5 L) for 8 hr. The obtained extract was collected and stored for further studies. The powdered plant material was dried and further subjected to acid hydrolysis. The defatted material was macerated with 5 % hydro-alcoholic solution (70:30) of sulphuric acid for 6 hr. After hydrolysis, the material was separated using simple filtration. In the obtained filtrate, water was added to precipitate the product (10-12 g). The obtained precipitates were separated and washed with water till neutral for the litmus test. The obtained material was kept for drying under a vacuum. The dried product was further dissolved in alcohol, and activated charcoal was added to remove insoluble impurities. The charcoal containing alcoholic solution was filtered and water was added to the filtrate to precipitate the product. The obtained product was further recrystallized using methanol. The recrystallized product (7-8 g) was dried and used for further analysis. HPLC studies were performed to assess the sample's purity.

2.4 Instrument and chromatographic conditions

RP-HPLC analysis was performed on Thermo Vanquish HPLC System equipped with quaternary pump F, a variable wavelength UV-visible detector, Vanquish column compartment (Oven temperature range 5 ºC to 90 ºC), and an autosampler. The HPLC System is controlled by Chromeleon 7 software. The analysis takes place on the RP-C18 column (Zodiac C_{18}, 4.6 x 250 mm i.d, particle size 5 µm). The appropriate separation of compounds was achieved in the isocratic mode. Acetonitrile and 0.2 % orthophosphoric acid in water (85:15 v/v) with a flow rate of 0.6 mL/min was used as the mobile phase. The UV detector was set at 210 nm for the detection. The run time was 45 min for the analysis. Analysis was performed at room temperature.

2.5 Preparation of stock solutions for RP-HPLC

The standard stock solution of asiatic acid and asiaticoside was prepared by dissolving 10 mg of each in 10 mL methanol to give a 1 mg ml $^{-1}$ concentration. Further calibration levels (2-12 µg ml $^{-1}$) were prepared by diluting the standard stock solution of each standard with methanol to obtain appropriate concentrations. The mixture of both standards was also prepared as per above mention method. Standard solutions were stored at 4 °C. These prepared standards were used for the RP-HPLC analysis.

2.6 RP-HPLC analysis

The RP-HPLC method was `developed and validated as per ICH guidelines (Q2 (R1)). The method validation was performed for the linearity, accuracy, precision, specificity, selectivity, and sensitivity ((limit of detection (LOD) and limit of quantification (LOQ)).

2.6.1 Optimization of method

2.6.1.1 Selection of wavelength

Due to the different absorption maxima of both compounds, the selection of scanning wavelength was essential for detecting both compounds. The UV detection was carried out at a lower wavelength due to the absence of strong chromophores in both compounds. Therefore 210 nm was selected for the scanning because scanning at 210 nm gave the best sensitivity with minimum noise detected for both compounds.

2.6.1.2 Selection of stationary phase

C_{18} RP columns from different makers were tried during the method development. Zodiac C_{18} RP-HPLC column (4.6 * 250 mm, 5 µm) was selected for further studies.

2.6.2 Validation of method

The developed HPLC method was validated as per the ICH guidelines (Q2 (R1)).

2.6.2.1 Linearity

Linearity of both compounds was performed using six different concentrations ranging from 2 µg/mL to 12 µg/mL. All the measurements took triplicate and were plotted using linear regression of the mean peak area versus concentration. The obtained linear regression equation was utilized for further calculations.

2.6.2.2 Accuracy (recovery study)

Accuracy or recovery study of the method was performed using the standard addition method. The analysis was carried out in triplicates. In the recovery experiments, by spiking (Adding) a known amount of both standards at three different levels (spike level-1 (50 %), spike level-2 (100 %), and spike level-3 (150 %)) to a standard of known concentration. Calculate the percentage recovery for each spiked level.

2.6.2.3 Precision

To determine the method's precision, intraday and interday precision of standard solutions were performed. Intraday precision was measured in replicates (n = 6) of both standard solutions on the same day, while interday precision was achieved over six consecutive days (n = 6). The precision results were expressed in terms of % relative standard deviation (RSD) ≤ 2%. Values with % RSD ≤ 2% for peak area responses were accepted.

2.6.2.4 Sensitivity (limit of detection and limit of quantification)

As per the ICH guidelines, the limits of detection and quantification of the developed method were calculated from the standard deviation of the response and slope of the calibration curve of markers using the following formulas:

Limit of detection = $3.3 \times \sigma / S$,

Limit of quantification = $10 \times \sigma / S$,

where σ is the standard deviation of the response and S is the slope.

2.6.2.5 Specificity and selectivity

The specificity of the developed method was estimated by studying the blank non-interference and comparing the retention time of target analyte peaks from the sample analyst with the reference standard. No difference was found in the peaks and spectra of reference standards and the sample analyzed. The peak purity tool was used to evaluate the peak purity of the samples. Hence the developed method demonstrates specificity and selectivity.

3. Results and Discussion

3.1. HPLC method development and validation

3.1.1 Optimization of chromatographic conditions

A novel RP-HPLC method was developed to quantify asiatic acid and asiaticoside in the extract and isolated fractions/compounds/material. Maintaining the optimum chromatographic condition throughout the experimentation is very important for the detection and quantification of asiatic acid and asiaticoside in the extract and isolated compounds/fractions. During the method development, various experimental trials were performed to obtain an accurate, rapid, precise, and sensitive RP-HPLC method with a high level of specificity. In the RP-HPLC method, various chromatographic conditions were considered, such as selecting the appropriate stationary phase, suitable type and mobile phase ratio, flow rate, and detection wavelength. Optimization of each chromatographic condition is described below. A standard solution of asiatic acid and asiaticoside was used for the method development.

3.1.2 Selection of stationary phase

Numerous provisional runs were performed using C_{18} RP columns from different makers. Initially, we used the Agilent RP column (Agilent Zorbax Bonus-RP; 4.6 * 250 mm, 5 µm) to separate the asiatic acid and asiaticoside. Both compounds were not adequately resolved using this column, and the retention time (RT) of both compounds was found to be more than 10 min. We rejected the Agilent RP column because the carbon load has approx. 9.5 % and surface area is around approx. 180 m^2/g. Due to its specifications, it's not suitable for our analysis. The important objective of the method development was to reduce the cost and time of quantifying both compounds. To satisfy the purpose of the method development, we change the stationary phase. For further trials, we select, Cosmosil C_{18} (Cosmosil $5C_{18}$-MS II; 4.6 * 250 mm, 5 µm) column. In this case, fairly resolved symmetrical peaks of both compounds were observed, along with RT of both compounds was found to be around 7-9 min. The results obtained from this stationary phase were also not satisfactory. We rejected the Cosmosil C_{18} column because the carbon load and surface area are around approx. 16 % and 300 m^2/g respectively. These specifications are also not suitable for the analysis of both compounds. Again, there was a need to select a suitable stationary phase that produces a good separation and peak symmetry and less than 10 min RT. To achieve the set goal, we performed the trials with Zodic C_{18} RP-HPLC column (4.6 * 250 mm, 5 µm). Both compounds separated properly, and the RT of both compounds was less than 8 min. The carbon load on Zodic C_{18} RP-HPLC column is approx. 24% and surface area approx. 440m^2/g. The column has a high carbon load and surface area as compared to the previous stationary phases as well as specifications of these columns were suitable for the analysis of both compounds. Therefore, for further study, this stationary phase was selected.

3.1.3 Influence of mobile phase and organic modifier

The reported literature suggested the use of methanol, acetonitrile, water, phosphoric acid, and acetic acid as a mobile phase for the chromatographic separation of both compounds. (Kaur et al., 2016; Monton et al., 2019) Initially, gradient elution of the mobile phase was tried for the separation. During the experimentation, the baseline was not stable as well as the RT of both compounds was very high. Therefore, the gradient elution method was rejected. To obtain the optimum separation, isocratic mixtures of several mobile phases were tried. The use of methanol instead of other organic solvents is the cost-effective approach for the quantification of several compounds. But in the case of asiatic acid and asiaticoside, methanol is not a suitable organic solvent for chromatographic separation. To ensure the proper chromatographic separation of both compounds, the organic content of the mobile phase was also considered. Substituting methanol with acetonitrile led to the improvement of chromatographic conditions. The effect of acetonitrile concentration in the acetonitrile: water mobile phase on the retention time of both compounds was studied. The water concentration in the mobile phase increased the retention time of both compounds (due to higher hydrophobic interaction between the stationary phase and both compounds). To improve the chromatographic conditions in water, *o*-phosphoric acid was added. The effect of *o*-phosphoric acid on the retention time of both compounds was investigated. Based on all performed trials, a combination of acetonitrile: water with 0.2 % *o*-phosphoric acid led to good chromatographic conditions. The expected results, i.e. optimum resolution with a short analysis

time, were achieved using this mobile phase combination. So, acetonitrile: water with 0.2 % *o*-phosphoric acid was selected as the mobile phase throughout the experiments.

3.1.4 Influence of flow rate

The flow rate of the mobile phase significantly affects the separation of the desirable constituents. To obtain a suitable flow rate for separating both compounds, we tried a range of different flow rates from 0.5 to 1.5 ml/ min of the mobile phase. An increase in the flow rate during the experiments led to faster elution of the compounds, but no proper separation of the desired product was observed. A sharp and symmetric peak was observed at the mobile phase's 0.6 ml/min flow.

3.1.5 Influence of detection wavelength

The absorption maxima for both compounds were different. Selecting an appropriate scanning wavelength was crucial for the experiments to detect both compounds. Several scanning wavelengths were tried to obtain good sensitivity and minimum noise during method development. Initially, we selected a few scanning wavelengths mentioned in the literature, such as 200 nm, 205 nm, and 206 nm. The strong chromophores are absent in both compounds therefore UV detection is carried out at lower wavelengths (200–210 nm). During the analysis of the experimental trials, it was observed that there was a single unwanted peak observed between the asiaticoside and asiatic acid at scanning wavelengths of 200 nm, 205 nm, and 206 nm. To find out the unknown peak, several experiments were repeated (trials with a single compound analysis and a mixture of both compounds). Finally, a blank run of Methanol (without any compound) was performed with the developed chromatographic conditions. The obtained results suggested that the methanol produces the peak at selected scanning wavelengths. Therefore, the unwanted peak observed during the analysis was confirmed as methanol because the samples were dissolved in the methanol. So, to minimize the area of the methanol from the chromatograph, we tried slightly higher scanning wavelengths for detection. Scanning at 210 nm gave the best sensitivity with minimum noise detected for both compounds, and the unwanted peak of methanol was also reduced (shows the minimum area in the chromatograph).

Finally, the effect of the stationary phase, mobile phase, organic modifier, flow rate, and detection wavelength was investigated to develop the RP-HPLC method for quantification of the asiaticoside and asiatic acid. The Zodic C_{18} RP-HPLC column was selected as a stationary phase for the chromatographic analysis. Acetonitrile in combination with 0.2 % OPA in water (ratio of 85: 15 (v/v) respectively) was selected as the mobile phase with 0.6 ml/min flow rate. At 210 nm, the samples were analyzed. The RT for Asiaticoside and Asiatic acid were found to be at 4.1 min and 5.9 min respectively.

The developed RP-HPLC method was validated as per the ICH guidelines. The observations of Calibration curves, Repeatability, Recovery Studies, and Sensitivity of Asiaticoside and Asiatic acid are reported in Table 1, Table 2, Table 3, and Table 4, respectively.

Table 1. Parameters of calibration curves for two quantified compounds (Asiaticoside and Asiatic acid)

Compound	Regression Equation	R^2	Linear Range (µg ml $^{-1}$)
Asiaticoside	y = 0.4721x - 0.3732	0.9997	2-12
Asiatic acid	y = 1.0697x - 0.5505	0.9995	2-12

Table 2. Repeatability of Asiaticoside and Asiatic acid

Compound	Repeatability RSD (%) (n = 6)
Asiaticoside	0.2301
Asiatic acid	0.3695

Table 3. Recovery Studies of Asiaticoside and Asiatic acid

Compound	Level (%)	Initial Amount (µg ml $^{-1}$) (n =3)	Added (µg/ml) (n =3)	Found (µg ml $^{-1}$) (n =3) ± SD	Recovery (%) (n =3)	RSD (%)
Asiaticoside	50	4	2	5.98 ± 0.065	99.66	1.098
	100	4	4	8.00 ± 0.017	100.08	0.216
	150	4	6	10.04 ± 0.019	100.40	0.191
Asiatic acid	50	4	2	5.99 ± 0.007	99.92	0.129
	100	4	4	8.02 ± 0.047	100.28	0.596
	150	4	6	9.99 ± 0.086	99.90	0.870

Table 4. Sensitivity Studies of Asiaticoside and Asiatic acid

Compound	LOD (µg/ml)	LOQ (µg/ml)
Asiaticoside	0.5049	1.5302
Asiatic acid	0.6931	2.1003

3.2. Extraction method development

Various methods were tried to optimize the isolation process to extract the Asiatic acid and asiaticoside from the *Centella asiatica*. During the development process, methods already mentioned in the literature were also attempted. Acid hydrolysis and basic hydrolysis methods were utilized for the procedure (Table 5). Based on various observations, the acid hydrolysis method was selected during the process development, and the yield obtained from this method was high compared to the other methods.

3.2.1 Basic hydrolysis method trials

Firstly, the plant material was subjected to the basic hydrolysis method. The aqueous solution of 2% potassium hydroxide was used for basic hydrolysis. The mixture of the plant material and the basic solution was kept for 5 hr. for hydrolysis. After completing the basic hydrolysis, the material was separated, and in the filtrate (dark green solution) addition of the dilute hydrochloric acid (5 %) took place. Adding hydrochloric acid leads to the formation of the precipitate of the crude triterpenoids. The obtained precipitated was washed till neutral to litmus paper. After the washing, the residue was kept for drying and used further for recrystallization. For the recrystallization process, crude triterpenoids were dissolved in the ethanol; some water was added in the ratio of 1:5 and kept in a cooling condition. After a few hours, there was the formation of the buff white color precipitate takes place. The precipitate was separated, identified, and analyzed for purity.

During the trials with the basic hydrolysis method, various concentrations of potassium hydroxide (aqueous as well as alcoholic) such as from 2% to 10 %, and for hydrochloric acid, the range of 2% to 10 % were also tried. The crude triterpenoids obtained from the basic hydrolysis method were sticky and challenging the processing to obtain the desired product. For the recrystallization process, ethanol, methanol, and ethyl acetate were used. But, ethanol gives better yield and purity than the other solvents. The results did not demonstrate a direct correlation between purity and the yield of the desired product.

After the basic hydrolysis, the solution was very difficult for separation/filtration. The plant material absorbed a large amount of solution/solvent, and laboratory-scale filtration takes time for the separation of absorbed solvent/solution. Therefore, we used the mechanical method to separate the basic solution from the plant material. The developed method performs the worst in terms of the filtration process, nature of the material (sticky material obtained), purity, and yield.

We are left with the conclusion that this approach was limited in terms of purity and yield of the desired product. A low performance was observed with this approach; therefore, the method was rejected.

3.2.2 Acid hydrolysis

For the acid hydrolysis method development, hydrochloric acid and sulphuric acid were used. At initial trials, various concentrations of hydrochloric acid were used, but the yield and purity of the product were obtained with the sulphuric acid trials.

3.2.2.1 With hydrochloric acid

Initially, the plant material was kept for acid hydrolysis with 10 % hydro-alcoholic solution (70:30) of hydrochloric acid for 12 hr. After the hydrolysis, the plant material was separated by filtration, and the filtrate was processed for further experimentation. The filtrate's volume was reduced to 30 % to 50 % from its initial concentration (to remove the alcohol). The concentrated filtrate was kept aside for a while to sediment some residue of crude triterpenoids. The formed precipitate was separated, dried, and processed for recrystallization with the ratio of methanol and water. Diethyl ether, methanol, ethanol, and ethyl acetate were used as the solvent for recrystallization. The product was obtained in the portion of methanol and water. The obtained product was further processed for the HPLC analysis. Based on HPLC analysis, it was observed that the method needs modifications concerning the yield and purity of the final product.

To enhance the yield and purity of the product, the following modifications were made in the previous acid hydrolysis methodology such as there a range of 2 to 10 % solution of hydrochloric acid was tried as well as alcohol and methanol were used as a solvent along with water at various concentrations from a range of 50 % to 90 %. During the method development of the acid hydrolysis with hydrochloric acid, it was observed that the yield was significantly less for processing the sample for further studies. In the final product, the green color was observed, which may be due to various impurities. The purity of the obtained product was also less compared with the desired standards.

3.2.2.2 With sulphuric acid

The same methodology was utilized with sulphuric acid instead of hydrochloric acid. Various trials with modifications were performed to obtain more yield and purity of the product. Change in the concentration of acid, change in the solvent (methanol and alcohol), change in the proportion of the water concerning solvent, variation time of the hydrolysis, etc. modification was tried with the sulphuric acid hydrolysis method. The material was recrystallized using methanol, alcohol, diethyl ether, and ethyl acetate. The yield of crude triterpenoids was more as compared to hydrochloric acid hydrolysis. The obtained results show that the product's purity and yield are satisfactory compared to the previous acid hydrolysis and basic hydrolysis methods. The results conclude that no apparent advantage exists in utilizing our method for the industrialization of the laboratory to a large scale.

Table 5. Few experimental trials for the extraction of Asiaticoside and Asiatic acid

Sr No	Experimental Conditions	Observation	Conclusion
	Basic Hydrolysis Methods		
1.	Plant material --» Defatting with Petroleum ether (Pet ether) --» Defatted plant material kept for basic hydrolysis with 2 % aqueous KOH (5 hr) --» Dark green basic solution obtained --» Addition of 5 % aqueous HCl solution --» Formation of Precipitae of Crude triterpenoids (CTT) --» Wash PPT till neutral to litmus --» Crude triterpenoids subjected to Recrystallization using various solvents. Recrystallization using Methanol Recrystallization using Alcohol Recrystallization using Ethyl acetate	➢ The alcohol recrystallized product was sticky. The product was difficult for the separation. The obtained product was dark green. ➢ Methanol recrystallized product was not sticky. The obtained product was light gray. Methanol recrystallization product yields more yield as compared to alcohol recrystallized product. ➢ The ethyl acetate recrystallization process does not yield any product.	HPLC results are not favorable to the process. Need to change the process or recrystallization process. Because there is green coloration was observed in the product.
2.	Plant material --» Kept for basic hydrolysis (2 % Aq. KOH) (5 hr) --» Dark green basic solution obtained --» Addition of 10 % aqueous HCl solution --» Formation of CTT --» Wash PPT till neutral to litmus --» Crude triterpenoids subjected to Recrystallization using methanol-water combination (1:5 ratio) at cooling conditions	The obtained crude triterpenoids were sticky. No product was obtained from the recrystallization method.	The method was rejected because no product was obtained during the recrystallization. As well as the obtained crude Triterpenoids are sticky.
3.	Plant material --» Defatting with Pet ether --» Defatted plant material kept for extraction with methanol --» Methanolic extract --» Addition of water --» Formation of CTT --» Rejected due to the sticky nature of the product	The obtained crude triterpenoids were sticky.	The obtained material was sticky therefore the method was rejected.

Table 5. Few experimental trials for the extraction of Asiaticoside and Asiatic acid (continue)

Sr No	Experimental Conditions	Observation	Conclusion
	Acid Hydrolysis Methods		
4.	Plant material --» Kept for Acid hydrolysis (HCl) (15 hr.) (70:30 Alcoholic Solution) 10 % of acid --» Addition water --» Kept aside for formation of CTT --» Crude Triterpenoids --» Subjected to Recrystallization Using Ethyl acetate at the cooling condition	Greenish PPT obtained.	Need to change the method for the better results.
5.	Plant material --» Kept for Acid hydrolysis (H_2SO_4) (15 hr.) (70:30 Alcoholic Solution) 10 % of acid --» Addition water --» Kept aside for formation of CTT --» Crude Triterpenoids --» Subjected to Recrystallization Using Ethyl acetate at the cooling condition	The H_2SO_4 method produces the cruder triterpenoids as compared to the HCl method. Therefore for further studies, we select H_2SO_4.	Need to change the method for the better results.
6.	Defatted plant material --» Kept for Acid hydrolysis (H_2SO_4) (70:30 Alcoholic Solution) 10 % of acid --» Addition water --» Kept aside for formation of CTT --» Crude Triterpenoids --» Subjected to Recrystallization Using Ethyl acetate at the cooling condition	No Compound was observed in the recrystallization process.	Need to change the recrystallization process.
	Acid-Base Titration Methods		
7.	Defatted plant material --» Kept for basic hydrolysis with 2 % Aqueous KOH --» Filter --» In the filtrate addition of 5 % Dil. HCl --» Formation of CTT --» Wash the PPT till neutral to litmus --» Dry the PPT & Dissolved in Methanol --» Addition of water in the Methanolic solution of PPT --» Formation of precipitae --» Subjected to Recrystallization	No Compound was observed in the recrystallization process.	Need to change the recrystallization process.
	Final Developed Method		
8.	Defatted plant material --» Kept for Acid hydrolysis (H_2SO_4) (70:30 Alcoholic Solution) 10 % of acid --» Filter --» Addition water --» Kept aside for formation of CTT --» Crude Triterpenoids --» Separate the obtained PPT and dried under vacuum --» Dried product dissolved in methanol/alcohol --» addition of activated charcoal and filter --» In filtrate addition of water --» Formation of PPT --» Separated and dried --» Used for further analysis.	The obtained material was light brown with a good yield.	HPLC analysis confirms the presence of asiatic acid and asiaticoside.

3.2.3 Final developed method

The plant material was defatted with the pet ether to remove the wax, lipids, and phytosterols. These phytoconstituents create interfere with the product yield as well as purity. The defatted plant material was kept for acid hydrolysis with 10 % hydro-alcoholic solution (70:30) of sulphuric acid. After the hydrolysis, the plant material was separated by a simple filtration method. Now, in the filtrate, the addition of water takes place to precipitate the product. (Change of pH from 1 to 3 – 4) The obtained precipitate was separated by simple filtration and dried under a vacuum. The dried product was the other process for recrystallization with alcohol and methanol. Before the recrystallization, the material was dissolved in alcohol or methanol, and activated charcoal was added to absorb the impurities. Filter the solution and in the filtrate addition of water takes place. After the addition of water, there was the formation of a precipitate takes place. The formed precipitate was separated and dried for further analysis.

After the analysis, it was observed that the purity of the obtained product concerning the desired product was around 65-70 % only. To enhance the purity, some modifications in the previously developed methodology take place. Up to the addition of the charcoal, the process was repeated, and the precipitate was formed by the addition of water into the filtrate. The formed precipitate was dissolved in methanol and concentrated in the methanolic solution. The concentrated solution was kept aside for some time to form the desired product. After some time, a buff white color product was obtained (7 - 8 gm). The practical yield of the method was 0.7 - 0.8 % with respect to the purity of the product. The obtained material was subjected to analysis. The RP-HPLC analysis of the obtained material shows the presence of asiatic acid around 12-15 % and asiaticoside 65-68 % (Figure 1). The obtained material is the rich fraction of both compounds. The results surpass the earlier work in this area regarding purity and yield (Table 5).

This method produces more crude triterpenoids, asiatic acid yield, and purity was also high compared to the previously developed methodologies. This method has demonstrated a marked improvement in the quality of desirable compounds. In all of our experiments, we found that the proposed solution was better than the previous methods presented in the literature.

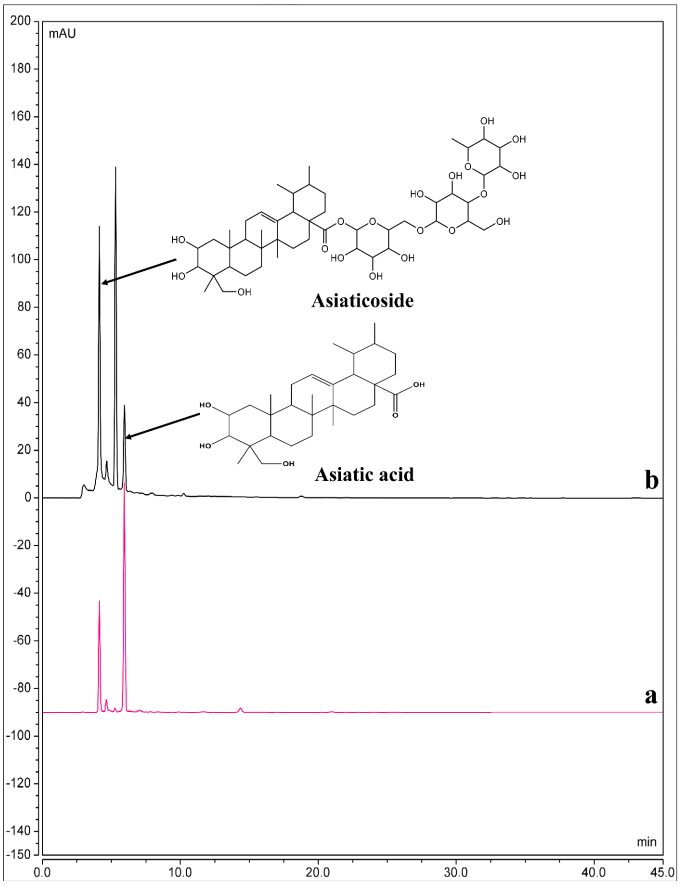

Figure 1. Overlaid HPLC Chromatograph of a) Standard of Asiaticoside and Asiatic acid b) extracted rich fraction of Asiaticoside and Asiatic acid.

Conclusion

The outcome of various experiments led to the conclusion that the developed method is simple enough to be applied for the extraction of a rich fraction of the asiatic acid and asiaticoside. It is also producing a good quality product with significant yield and purity as compared to the previous methodologies. This has been regarded as a useful method for the large-scale production of both compounds. The developed method presents some practical advantages, especially in the case of large-scale production because the materials (Chemicals) which are we used during the extraction method development are accepted by the industries for the pilot-plant setups. Overall, the methodology produces good results for both compounds and can be applied to industries.

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Research Article

Total Phenolic, Flavonoids Contents, and Antioxidant Activities in The Stems and Rhizomes of Java Cardamom as Affected by Shading and N Fertilizer Dosages

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Abstract: Java cardamom is an herbal medicinal plant known as the "queen of spices." This research aims to determine the influence of shading and nitrogen fertilizer dose on the total phenolics, flavonoids, and antioxidant activity of Java cardamom stems and rhizomes. The study employed a split-plot design with two factors: the level of shading (0, 25, 50, and 75%) as the main plot and the dosage of nitrogen (N) fertilizer (0, 0.9, and 1.36 g polybag^{-1}) as the subplots. Twelve months after planting, the rhizome and stem dried powder were extracted using the sonication-maceration technique with ethanol as the solvent. The 75% shading affected the more outstanding production of total phenolics (1.65 ± 0.59 mg GAE g^{-1} DW), DPPH antioxidant (4.95 ± 0.50 µmol TEAC g^{-1} DW), and FRAP antioxidant (8.94 ± 2.56 µmol TEAC g^{-1} DW) activities of the rhizomes cultivated with 0 g/polybag N in comparison to the stems of the plants. Contrary to phenolics and antioxidant activities, total flavonoids cultivated at 0% shading with 1.36 g polybag^{-1} N of the stems increased concentration than the rhizomes. The results indicated that the 75% shading affected the Java cardamom rhizome's phenolic content and antioxidant activities.

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Footnote: This study includes a part of the magister thesis prepared by the first author.

1. Introduction

Cardamom is a tropical and subtropical plant from the Zingiberaceae family and is one of the Indonesia's export commodities with the third-highest economic value after saffron and vanilla (Rani et al., 2018). Cardamom is a cooking ingredient known as the 'Queen of spices,' which comes from 3 genera: Elettaria, Amomum, and Aframoum (Silalahi, 2017; Hartady et al., 2020). Cardamom grows and develops in the Asian continent, such as Indonesia, Sri Lanka, Nepal, Tanzania, Guatemala, and India (Garg et al., 2016; Abu-Taweel et al., 2018). Qonita et al. (2018) reported that traditionally Java cardamom is often used as a spice in certain dishes, herbal medicines, health drinks, and aromatherapy. The use of herbal medicine and cooking spices from Java cardamom is related to the secondary metabolite content in the essential oil of cardamom (Silalahi, 2017). Pujiarti and Kusumadewi (2020) reported that the availability of essential oils in cardamom provides many benefits, including anti-

inflammatory, antibacterial, therapeutic, and aromatherapy agents. Phytochemical compounds in cardamom consist of flavonoids, sterols, tannins, starch, terpenoids, proteins, and phenols (Moulai-Hacene et al., 2020).

Java cardamom is a possible source of natural bioactive substances containing secondary metabolites such as polyphenol antioxidants. Light intensity, light quality, and photoperiodism influence the accumulation of nearly all forms of secondary metabolites in plants (Zhang et al., 2021). However, the polyphenol content of each type of plant, both sun, and shade plants, showed different results (Idris et al., 2018). Cardamom plants are C3 plants that require 50% sunlight intensity during the day for optimal growth (Alagupalamuthirsolai et al., 2018). The shading will restrict the amount of light plants receive, resulting in a decrease in air temperature and an increase in humidity, both of which are conducive to the development and growth of cardamom. Bhuiyan et al. (2012) reported that the height growth of turmeric and ginger plants in the shade was reported to be more fertile and stronger than those living in the open. Different shading levels were followed by changes in morphology and physiological characteristics of plants that will affect secondary metabolites such as phenolic compounds (Ghasemzadeh and Ghasemzadeh, 2011). Nitrogen is the primary essential nutrient for plants and is required in relatively significant quantities (Leghari et al., 2016). Nitrogen plays a role in the metabolism of protein and chlorophyll, influences the growth and development of vegetative components, and encourages root growth (Leghari et al., 2016).

Several previous studies on the content of secondary metabolites and the bioactivity of cardamom plants have been reported. Winarsi et al. (2013) explained that cardamom leaf extract could control blood glucose levels, which may be antiatherogenic in diabetic rats. In addition, Asra et al. (2019) have tested the phytochemical and antioxidant activity of cardamom leaf extract showing that cardamom leaf extract contains phenolic compounds, flavonoids, tannins, and saponins and may have antioxidant activity. Tmušic et al. (2021) reported that *Melissa officinalis* L. produced higher total phenolic content under shaded conditions than treatment without shade. Ekawati (2018) also reported the same thing, reporting that her administration had a higher total flavonoid content than the shade of *Talinum triangulare* (Jacq.) Willd).). The effect of shading and nitrogen fertilizer dose on the total phenolic, flavonoid, and antioxidant activities of the stems and rhizomes of Java cardamom has not been established. This study aimed to examine the effect of shading and nitrogen fertilizer dose on the phenolic content, total flavonoids, and antioxidant activity of the stems and rhizomes of the Java cardamom plant. Therefore, this study can provide scientific information regarding enhancing the polyphenols of the Java cardamom plant as an antioxidant herbal medicine.

2. Material and Methods

2.1. Plant material and sample preparation

Java cardamom plants are cultivated in the field of Tropical Biopharmaca Research Center, Bogor Agricultural University, West Java, Indonesia, at a latitude of –6.54713°, east longitude 106.71665°, and an altitude of 141 m above sea level. This study used a split-plot design with two factors, namely providing shade as the main plot with four levels: 0 (control) and 25%, 50%, 75% (treatment). The N fertilizer dosage was a subplot with three levels: 0 (control) and 0.90 g polybag-1, 1.36 g polybag-1 (treatment). Therefore, there were 12 combinations of treatments. The treatment was carried out in 3 replications with the plant spacing 50 cm x 50 cm. Java cardamom plants were treated for seven months and harvested at 12 months. The stems and rhizomes were prepared by first washed and cut into small sizes. Next, the stems and rhizomes were sundried for ± 3 days, then milled using a grinding machine into a dried powder with a size of 100 mesh.

2.2. Sample extraction

Dried powders of the samples of stems and rhizomes were extracted based on modifications by Nurcholis et al. (2021a). Briefly, 2 g of stems and rhizomes dried powder were extracted twice using a 10 mL pro-analytical ethanol solvent in a sonicator (Decon Ultrasorics Ltd., England) at a dark-room temperature for 30 min. The homogenate was centrifuged (Kitman-T24, Tomy Kogyo CO. Ltd., Tokyo) for 15 min at 4℃ at 10000 g. The supernatant was then concentrated using a rotary evaporator

(Hahnvapor HS-2005V, Korea) at 50°C and calibrated to 10 mL. The supernatant with a concentration of 0.2 g mL^{-1} was collected and used to determine total phenolics, flavonoids, and antioxidant activity.

2.3. Total phenolic (TPC) and flavonoid content (TFC)

Total phenolic content (TPC) was calculated using the Folin-Ciocalteu method, as described by Khumaida et al. (2019). In a 96-well microplate, 20 µL of ethanol extract of the sample was mixed sequentially with 120 µL of Folin-Ciocalteu (10%) and incubated for 5 min in the darkroom. Then, 80 µL of 10% Na_2CO_3 solution was added, and the mixture was incubated for 30 min at room temperature in the dark. At a wavelength of 750 nm, the absorbance of each treatment was measured using a nano-spectrophotometer (SPECTROstar^{Nano} BMG LABTECH). The TPC was reported as mg equivalent gallic acid per gram of dry weight (mg GAE/g DW).

Total flavonoid content (TFC) was assessed using a modified colorimetric approach employing an aluminum chloride ($AlCl_3$) reagent, as described by Calvindi et al. (2020). A 96-well microplate was filled with 10 µL of ethanol extract, 50 µL of ethanol pro analysis, 10 µL of 10% aluminum chloride, 10 µL of glacial acetic acid, and 120 µL of distilled water. The mixture was then homogenized and incubated for 30 min at room temperature and in the dark. At a wavelength of 415 nm, the absorbance of each treatment was measured using a nano-spectrophotometer (SPECTROstar^{Nano} BMG LABTECH). TFC was reported as mg equivalent quercetin per gram of dry weight (mg QE g^{-1} DW).

2.4. Antioxidant analysis

Two *in-vitro* methods were used to measure antioxidant activity. The free radical scavenging activity was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method (Nurcholis et al., 2016), while the reducing power antioxidant was evaluated using the ferric reducing antioxidant power (FRAP) procedure (Calvindi et al., 2020).

DPPH antioxidant activity was determined using a nano-spectrophotometer (SPECTROstar^{Nano} BMG LABTECH) based on Nurcholis et al. (2016) with a slight modification. A 96-well microplate was loaded with 100 µL of ethanol extract and 100 µL of 125 µM DPPH solution (in ethanol pro analysis). Afterward, the mixture was homogenized and incubated for 30 min in the dark. Finally, the nano-spectrophotometer (SPECTROstar^{Nano} BMG LABTECH) measured the absorbance at 515 nm. The DPPH result is expressed in µmol of Trolox equivalent antioxidant capacity per g of dry weight (µmol TEAC g^{-1} DW).

Analysis of antioxidant activity using the FRAP method was determined using a nano-spectrophotometer (SPECTROstar^{Nano} BMG LABTECH) according to Calvindi et al. (2021) with a modification. In a row, 10 µL of ethanol extract samples were added with 300 µL of FRAP reagent (made by mixing acetate buffer pH 3.6 with 10 µM TPTZ solution (in 40 µM HCl) and 20 µM $FeCl_3$ (in distilled water) in a v/v/v ratio 10:1:1) was put into microplate-96 wells. Afterward, the mixture was homogenized and left to sit at room temperature for 30 min. At a wavelength of 593 nm, a nano-spectrophotometer was used to detect the absorbance of each sample. The final unit is measured in µmol of Trolox equivalent antioxidant capacity per g of dry weight (µmol TEAC g^{-1} DW).

2.5. Data analysis

By utilizing the IBM SPSS 25 statistical tool, we analyzed the variance (ANOVA). In addition, Tukey's range test was used to compare the data.

3. Results

3.1. Total phenolic content (TPC)

Applying shading levels and nitrogen fertilizer dosages were intended to increase the phenolic component content of the ethanol extract of the stems and rhizomes of Java cardamom. The total phenolics were determined using the Folin-Ciocalteu technique with gallic acid as the phenolic compound standard. The primary group of secondary metabolites in plants that play a significant role in antioxidant activity is phenolics (Rahman et al., 2021). Table 1 depicts the total phenolics of the ethanol extract of the stems and rhizomes.

Table 1. Total phenolic content of ethanol extract of Java cardamom stems and rhizomes

Part of plant	Shade	Total fenolik Content (mg GAE g^{-1} DW)		
		Fertilizer Dosage (g $polybag^{-1}$)		
		0	0.9	1.36
Stems	0%	0.40 ± 0.00bB	0.47 ± 0.04abB	0.68 ± 0.04aA
	25%	0.29 ± 0.04bA	0.37 ± 0.06bA	0.30 ± 0.01aA
	50%	0.62 ± 0.14abA	0.67 ± 0.12abA	0.64 ± 0.05aA
	75%	1.54 ± 0.35aA	1.35 ± 0.30aA	1.30 ± 0.45aA
Rhizomes	0%	0.33 ± 0.00aA	0.41 ± 0.02aA	0.37 ± 0.04aA
	25%	0.66 ± 0.13aA	0.78 ± 0.15aA	0.59 ± 0.05aA
	50%	0.55 ± 0.07aA	1.01 ± 0.32aA	0.95 ± 0.19aA
	75%	1.65 ± 0.59aA	0.70 ± 0.07aA	1.25 ± 0.64aA

Each value is presented as mean ± standard error mean (SEM); numbers followed by different lowercase letters (a-b) showed significant differences ($p<0.05$) in the same column and the same plant parts; Numbers followed by capital letters (A-B) that differ in the same row show significantly different values at ($p<0.05$).

The ethanol extract of Java cardamom rhizomes had a higher total phenolic content in the combination of 75% shading treatment and the nitrogen fertilizer dosage of 0 g $polybag^{-1}$ than in Java cardamom stems ethanol extract. The ethanol extract of Java cardamom stems contains the highest total phenolic content, obtained at 75% shade treatment and the nitrogen fertilizer dosage of 0 g $polybag^{-1}$, which has 1.54 ± 0.35 mg GAE g^{-1} dry weight. On the other hand, the ethanol extract of stems contains the lowest total phenolic content at 25% shade treatment and the nitrogen fertilizer dosage of 0 g $polybag^{-1}$, which is 0.29 ± 0.04 mg GAE g^{-1} dry weight. Meanwhile, in the ethanol extract of the Java cardamom rhizomes, the highest total phenolic content was obtained in the 75% shading and the nitrogen fertilizer dosage of 0 g $polybag^{-1}$, which has 1.65 ± 0.59 mg GAE g^{-1} dry weight. Conversely, the lowest total phenolic content was obtained in the 0% shading and the nitrogen fertilizer dosage of 0 g $polybag^{-1}$, which has 0.33 ± 0.00 mg GAE g^{-1} dry weight. In this study, it can be seen (Table 1) that each shading level with the nitrogen fertilizer dosage of 1.36 g $polybag^{-1}$ did not have a significant effect ($p>0.05$) on the phenolic content of the ethanol extract of the stems and rhizomes of Java cardamom. The combination treatment of 75% shading and the nitrogen fertilizer dosage of 0 g $polybag^{-1}$, both in the ethanol extract of stems and rhizomes, obtained the highest total phenolic content compared to other treatments.

3.2. Total flavonoid content (TFC)

The total flavonoid content was evaluated using the aluminum chloride ($AlCl_3$) reagent and quercetin as a standard for flavonoid compounds. The highest total flavonoid content in the ethanol extract of Java cardamom stems and rhizomes can be seen in Table 2.

The ethanol extract of Java cardamom stems had a higher total phenolic content in the combination of 0% treatment and the nitrogen fertilizer dosage of 1.36 g $polybag^{-1}$ compared to the ethanol extract of Java cardamom rhizomes and other treatments. The ethanol extract of the stems contains the highest total flavonoid content, obtained in the combination treatment of 0% shade (without shade) and the nitrogen fertilizer dosage of 1.36 g polybag-1, which was 0.89 ± 0.13 mg QE g^{-1} dry weight. Conversely, the ethanol extract of the stems contains the lowest total flavonoid content, obtained in the combination treatment of 25% shading with the nitrogen fertilizer dosage of 0 g $polybag^{-1}$, which was 0.62 ± 0.06 mg QE g^{-1} dry weight. The ethanol extract of rhizomes contains the highest total flavonoid content, obtained from the combination of 25% shading and the nitrogen fertilizer dosage of 0.9 g $polybag^{-1}$ with a value of 0.47 ± 0.01 mg QE g^{-1} dry weight. In contrast, the ethanol extract of rhizomes contains the lowest total flavonoid content, obtained in the combination treatment of 75% shade and the nitrogen fertilizer dosage of 1.36 g $polybag^{-1}$ with a value of 0.28 ± 0.01 mg QE g^{-1} dry weight. Based on Table 2, each shading level with the nitrogen fertilizer dosage of 0 g $polybag^{-1}$ shows no significant effect ($p>0.05$) on the total flavonoid content of the ethanol extract of the stems and rhizomes of Java cardamom. TFC showed that the combination of 0% shade treatment and the nitrogen fertilizer dosage of 1.36 g $polybag^{-1}$ had higher yields than the shading treatment of the ethanol extract

of the stems. In comparison, the ethanol extract of the rhizomes, combination treatment of 25% shading with the nitrogen fertilizer dosage of 0.9 g polybag^{-1} showed a higher total flavonoid content than the treatment without shade with each dosage of nitrogen fertilizer.

Table 2. Total flavonoid content of ethanol extract of Java cardamom stems and rhizomes

Part of plant	Shade	Total flavonoid Content (mg QE g^{-1} DW)		
		Fertilizer Dosage (g polybag^{-1})		
		0	0.9	1.36
Stems	0%	0.76 ± 0.08aA	0.71 ± 0.06aA	0.89 ± 0.13aA
	25%	0.62 ± 0.06aA	0.68 ± 0.04aA	0.69 ± 0.05aA
	50%	0.85 ± 0.11aA	0.80 ± 0.04aA	0.75 ± 0.01aA
	75%	0.75 ± 0.03aA	0.79 ± 0.04aA	0.81 ± 0.01aA
Rhizomes	0%	0.45 ± 0.02aA	0.44 ± 0.06aA	0.45 ± 0.10aA
	25%	0.39 ± 0.06aA	0.47 ± 0.01aA	0.45 ± 0.04aA
	50%	0.42 ± 0.02aA	0.41 ± 0.02aA	0.37 ± 0.05aA
	75%	0.38 ± 0.03aA	0.35 ± 0.03aAB	0.28 ± 0.01aB

Each value is presented as mean ± standard error mean (SEM); numbers followed by different lowercase letters (a-b) showed significant differences (p<0.05) in the same column and the same plant parts; Numbers followed by capital letters (A-B) that differ in the same row show significantly different values at (p<0.05).

The ethanol extract of Java cardamom stems had a higher total phenolic content in the combination of 0% treatment and the nitrogen fertilizer dosage of 1.36 g polybag^{-1} compared to the ethanol extract of Java cardamom rhizomes and other treatments. The ethanol extract of the stems contains the highest total flavonoid content, obtained in the combination treatment of 0% shade (without shade) and the nitrogen fertilizer dosage of 1.36 g polybag^{-1}, which was 0.89 ± 0.13 mg QE g^{-1} dry weight. Conversely, the ethanol extract of the stems contains the lowest total flavonoid content, obtained in the combination treatment of 25% shading with the nitrogen fertilizer dosage of 0 g polybag^{-1}, which was 0.62 ± 0.06 mg QE g^{-1} dry weight. The ethanol extract of rhizomes contains the highest total flavonoid content, obtained from the combination of 25% shading and the nitrogen fertilizer dosage of 0.9 g polybag^{-1} with a value of 0.47 ± 0.01 mg QE g^{-1} dry weight. In contrast, the ethanol extract of rhizomes contains the lowest total flavonoid content, obtained in the combination treatment of 75% shade and the nitrogen fertilizer dosage of 1.36 g polybag^{-1} with a value of 0.28 ± 0.01 mg QE g^{-1} dry weight. Based on Table 2, each shading level with the nitrogen fertilizer dosage of 0 g/polybag shows no significant effect (p>0.05) on the total flavonoid content of the ethanol extract of the stems and rhizomes of Java cardamom. TFC showed that the combination of 0% shade treatment and the nitrogen fertilizer dosage of 1.36 g polybag^{-1} had higher yields than the shading treatment of the ethanol extract of the stems. In comparison, the ethanol extract of the rhizomes, combination treatment of 25% shading with the nitrogen fertilizer dosage of 0.9 g polybag^{-1} showed a higher total flavonoid content than the treatment without shade with each dosage of nitrogen fertilizer.

3.3. Antioxidant activity

The antioxidant activity test was aimed at measuring the total antioxidant capacity of the stems and rhizomes of the Java cardamom plant. The antioxidant activity of stem and rhizome samples was measured using DPPH and FRAP assays, with Trolox as the standard antioxidant compound. The results of the measurement of antioxidant activity using the DPPH and FRAP methods are presented in Table 3 and Table 4, respectively.

Table 3. DPPH antioxidant activity of ethanol extract of Java cardamom stems and rhizomes

Part of plant	Shade	DPPH antioxidant capacity (μmol TEAC g^{-1} DW)		
		Fertilizer Dosage (g $polybag^{-1}$)		
		0	0.9	1.36
Stems	0%	0.20 ± 0.08aB	0.51 ± 0.04aA	0.62 ± 0.01aA
	25%	0.22 ± 0.08aA	0.36 ± 0.17aA	0.23 ± 0.02bA
	50%	0.42 ± 0.17aA	0.48 ± 0.11aA	0.54 ± 0.07aA
	75%	0.61 ± 0.01aA	0.61 ± 0.00aA	0.61 ± 0.00aA
Rhizomes	0%	3.35 ± 0.68aA	3.06 ± 0.04aA	2.32 ± 0.01aA
	25%	3.26 ± 0.38aA	3.58 ± 0.34aA	3.62 ± 0.25aA
	50%	3.36 ± 0.41aA	3.91 ± 0.43aA	4.45 ± 0.30aA
	75%	4.95 ± 0.50aA	3.67 ± 0.38aA	4.03 ± 1.24aA

Each value is presented as mean ± standard error mean (SEM); numbers followed by different lowercase letters (a-b) showed significant differences ($p<0.05$) in the same column and the same plant parts; Numbers followed by capital letters (A-B) that differ in the same row show significantly different values at ($p<0.05$).

Table 4. FRAP antioxidant activity of ethanol extract of Java cardamom stems and rhizomes

Part of plant	Shade	FRAP antioxidant capacity (μmol TEAC g^{-1} DW)		
		Fertilizer Dosage (g $polybag^{-1}$)		
		0	0.9	1.36
Stems	0%	1.91 ± 0.10bA	2.40 ± 0.15aA	3.29 ± 0.62bA
	25%	1.88 ± 0.15bA	2.25 ± 0.32aA	1.76 ± 0.10bA
	50%	2.89 ± 0.57bA	3.12 ± 0.71aA	2.77 ± 0.35bA
	75%	8.75 ± 1.25aA	4.85 ± 0.82aA	8.02 ± 1.66aA
Rhizomes	0%	1.27 ± 0.00bB	1.72 ± 0.03aA	1.32 ± 0.12aAB
	25%	4.35 ± 0.70abA	5.50 ± 1.18aA	4.18 ± 0.22aA
	50%	2.27 ± 0.45abA	5.54 ± 1.42aA	5.84 ± 0.85aA
	75%	8.94 ± 2.56aA	4.28 ± 0.58aA	4.71 ± 1.85aA

Each value is presented as mean ± standard error mean (SEM); numbers followed by different lowercase letters (a-b) showed significant differences ($p<0.05$) in the same column and the same plant parts; Numbers followed by capital letters (A-B) that differ in the same row show significantly different values at ($p<0.05$).

Based on the DPPH antioxidant measurement results (Table 3), the rhizome ethanol extract showed higher antioxidant activity than the stem ethanol extract in the DPPH method. The combination treatment of 0% shading and the nitrogen fertilizer dosage of 1.36 g $polybag^{-1}$ showed the highest DPPH antioxidant activity compared to other combinations of stem ethanol extract, which was 0.62 ± 0.01 TEAC g^{-1} dry weight. In comparison, the lowest antioxidant activity was shown in a combination treatment of 0% shade and a nitrogen fertilizer dosage of 0 g $polybag^{-1}$, which was 0.20 ± 0.08 mol TEAC g^{-1} dry weight. In the ethanol extract of rhizomes, the combination treatment of 75% shading and the nitrogen fertilizer dosage of 0 g $polybag^{-1}$ showed the highest DPPH antioxidant activity of 4.95 ± 0.50 mol TEAC g^{-1} dry weight. Conversely, the lowest DPPH antioxidant activity was obtained in the combination treatment of 0% shade and the nitrogen fertilizer dosage of 1.36 g $polybag^{-1}$, which is 2.32 ± 0.01 mol TEAC g^{-1} dry weight. In Table 4, the ethanol extract of the rhizome showed higher FRAP antioxidant activity than the stem ethanol extract. In the ethanol extract of stems, the combination treatment of 75% shade and the nitrogen fertilizer dosage of 0 g $polybag^{-1}$ showed the highest FRAP antioxidant activity, which was 8.75 ± 1.25 mol TEAC g^{-1} dry weight. In comparison, the lowest antioxidant activity resulted from the combination treatment of 25% shading and the nitrogen fertilizer dosage of 1.36 g $polybag^{-1}$, which is 1.76 ± 0.10 mol TEAC g^{-1} dry weight. In the ethanol extract of the rhizome, the highest FRAP antioxidant activity was obtained in a combination of 75% shade treatment and the nitrogen fertilizer dosage of 0 g $polybag^{-1}$, which was 8.94 ± 2.56 mol TEAC g^{-1} dry weight. Conversely, the lowest FRAP antioxidant activity was obtained in a combination of 0% shade treatment and the nitrogen fertilizer dosage of 0 g $polybag^{-1}$, which is 1.27 ± 0.00 mol TEAC g^{-1} dry weight.

4. Discussion

Phenolic compounds contain one (phenol) or many (polyphenol) phenol rings, notably hydroxy groups that link to aromatic rings to make them easily oxidizable by giving hydrogen atoms to free radicals (Dhurhania and Novianto, 2018). The ability of phenolic compounds to form stable phenoxy radicals in oxidation reactions makes them highly effective antioxidants; Natural phenolic compounds are typically polyphenols that form ether compounds, esters, or glycosides from flavonoids, tocopherols, tannins, coumarins, cinnamic acid derivatives, lignins, and polyfunctional organic acids (Dhurhania and Novianto, 2018). Meanwhile, flavonoids are part of phenolic compounds with a molecular weight. Flavonoid compounds are plants' most prominent family of polyphenolic compounds; more than 6000 flavonoids have been identified (Ghasemzadeh dan Ghasemzadeh, 2011). They are widespread in almost every part of the plant that functions as plant protection from insects and pests, and environmental adaptation (Thakur et al., 2018). Phenolic and flavonoid compounds are secondary metabolites produced by plants in specific quantities under stressed conditions (Kusbiantoro and Purwaningrum, 2018).

The phenolic content of the ethanol extract of the stems and rhizomes of Java cardamom, the combination treatment of 75% shading, and the nitrogen fertilizer dosage of 0 g polybag^{-1}, both in the ethanol extract of stems and rhizomes, give the highest total phenolic content compared to other treatments (Table 1). Busaifi (2017) states that shading conditions will provide stress for plants which can increase secondary metabolite compounds in plants to respond to environmental stress; plants will produce various phytochemicals. The phytochemicals play a vital role in plants' incredible growth and development under stress conditions (Khurshid et al., 2020). In contrast to that reported by Alagupalamuthirsolai et al. (2018), the highest phenolics were obtained in the treatment without shading of the leaf extract of the *E. cardamom* plant. This result is inversely proportional to that obtained in this study; this is due to several factors, namely the type of cardamom used as a sample, climatic factors, growing conditions, physiological conditions, plant age, as well as the cardamom plant parts used in the study, which will also affect the secondary metabolite content in plants. Deepa et al. (2013) reported that the total phenolic content in *A. cardamom* seeds was 1.25 mg GAE g^{-1} (methanol extract) and 0.55 mg GAE g^{-1} (water extract). Tmuši´c et al. (2021) also reported that *Melissa officinalis* L. produced higher total phenolic content under shaded conditions than treatment without shading. Phenolic compounds are secondary metabolites synthesized as a form of defense mechanism for plants (Jain et al., 2017), which are produced in response to changes in UV radiation, temperature, salinity, pathogens, and drought that function as signaling molecules, allelopathic compounds, and detoxifying agents (Karimi et al., 2013).

TFC showed that the combination of 0% shading and the nitrogen fertilizer dosage of 1.36 g polybag^{-1} had higher yields than the shading treatment of the Java cardamom stem ethanol extract. In comparison, for the ethanol extract of the rhizomes, a combined treatment of 25% shade with the nitrogen fertilizer dosage of 0.9 g polybag^{-1} showed a higher total flavonoid content than without shading with each dosage of nitrogen fertilizer. The difference in the total flavonoid content in the stems and rhizomes with shade treatment and fertilizer dosage is because flavonoids are the phenolics most easily produced in epidermal plant cells exposed to high light intensity, which act as a protective response against oxidative damage. However, Karimi et al. (2013) reported that the content of flavonoids and other secondary metabolites in plants are not evenly distributed throughout the plant tissue because the concentration and distribution of secondary metabolites are affected by genetics and environmental factors such as light, moisture, and soil fertility. Nurcholis et al. (2021a) reported that the total flavonoid content in *A. compactum* fruit ranged from 0.19 to 2.26 mg QE g^{-1} DW, indicating that cardamom fruit without shading treatment has higher total flavonoids because environmental light conditions give more effect on flavonoid accumulation. However, Ghasemzadeh and Ghasemzadeh (2011) reported that 60% of shading affected the flavonoid content in *Zingiber officinale* R. plant leaf extract compared to treatment without shade. Solar radiation will increase the accumulation of flavonoids in fruit plants but will reduce the accumulation of flavonoids in *Heliophytes* species and some medicinal plants give the highest total flavonoids in the rhizome sample under shading treatment (Idris et al., 2018). Mir et al. (2017) the low light intensity state showed higher flavonoid productivity (2.5 mg g^{-1} dry weight) when compared to the high sunlight intensity condition in *A. amygdalina* plants. However, Yolci and Tunçtürk (2022) reported was the total content of flavonoids and antioxidant activity, with the best results obtained in the NP0 treatment (without nitrogen and phosphorus fertilizers) on safflower (*Carthamus tinctorius*

L.). This is due to differences in plant species, geography, and environmental factors that cause differences in total flavonoid content and antioxidant activity.

Antioxidants suppress oxidation reactions caused by free radicals, which can cause degenerative diseases by damaging cell wall membranes, unsaturated fatty acids, alkaline DNA, blood vessels, and tissue lipids (Ramadhan et al., 2020). Free radicals are atoms or molecules with unpaired electrons, including superoxide (O_2), hydroxyl (OH^-), peroxyl (RO_2), and hydroperoxyl (HO_2) (Halliwell and Gutteridge, 2015; Nurcholis et al., 2017). Internal enzymes such as glutathione peroxidase (GPX), superoxide dismutase (SOD), glutathione (GSH), and catalase (CAT), tocopherol, and beta-carotene provide the body with antioxidants. The measurement results show that the overall antioxidant activity of FRAP is higher than that of DPPH; this is related to the different reaction mechanisms that occur in the ethanol extract of stems and rhizomes, which are more dominant using the single electron transfer (SET) mechanism than hydrogen atom transfer (HAT). Nurcholis et al. (2021b) reported that the antioxidant activity value of the FRAP method was higher than the DPPH method on cardamom fruit water extract. In addition, Nurcholis et al. (2021a) also reported that the antioxidant activity value of the DPPH method was lower than that of the CUPRAC method. The value of antioxidant activity varies between plant treatments, indicating that the content of compounds that act as antioxidants varies (Zhang et al., 2018). Özyürek et al. (2011) reported that the DPPH method provides the lowest antioxidant activity value compared to other methods because the reagent can influence the stability of DPPH radicals, so high and low antioxidant activity of DPPH is also influenced by several factors, namely the reagents that can be damaged when exposed to light, oxygen, high temperatures, and drying.

Plants can synthesize non-enzymatic antioxidants; however, under stress conditions caused by biotic and abiotic factors, the formation of reactive oxygen species (ROS) rises in plants, resulting in the induction of oxidative stress. In response to increased oxidative stress, plants produce and accumulate more antioxidants (Kasote et al., 2015). Zaini et al. (2021) reported that the antioxidant activity of kencur rhizomes was higher in the 25% shade than in the 50% shade. However, Khusni et al. (2018) reported that the provision of 70% shade was the best shade for producing the highest antioxidant activity compared to other treatments on red spinach (*Alternanthera amoena* Voss.), This difference indicates that the types of plants will give different responses to the effects of both the application and the dosage of nitrogen fertilizer on the secondary metabolite content in plants. Each of the ethanol extracts of the stems and rhizomes of Java cardamom contains flavonoid compounds that act as antioxidants. However, Suhendra et al. (2019) stated that the antioxidant activity value of a sample is not always directly proportional to the total flavonoid content, so there may be other compounds besides phenolic compounds and flavonoids that act as antioxidants. The measurement results show that both cardamom stem and rhizome may act as antioxidants and can be used as candidates for antioxidant herbal medicines. Each part of the plant will produce a different secondary metabolite content based on the function of the plant's organ.

Conclusion

The results presented that the treatment of 75% shade with 0 g polybag^{-1} N improved the phenolic content and antioxidant activity of Java cardamom. The stems contain higher flavonoids than the rhizome part of the plants. Nevertheless, the rhizomes have more potent antioxidant activity than the stems of Java cardamom, as measured by the DPPH and FRAP assays.

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Research Article

Effects of Vermicompost, Compost and Animal Manure on Vegetative Growth, Physiological and Antioxidant Activity Characteristics of *Thymus vulgaris* L. under Water Stress

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Abstract: This study investigated the effect of organic fertilizers on vegetative growth and the physiological and antioxidant activity characteristics of thyme plants grown under stress. A factorial experiment was conducted according to randomized complete block design with 12 combinations and 3 replications in the 2018 growing season. The experiment factors were the implementation of organic fertilizers at 4 levels (vermicompost, manure compost, animal manure, and control) and irrigation regime at 3 levels (Irr1, Irr2, and Irr3, respectively, irrigation after 60, 90, and 120 mm evaporation from A pan). The results showed that With delayed irrigation, the chlorophyll a and b contents, total chlorophyll, and carotenoid decreased, while the application of low water stress enhanced the amount of oil and the oil yield with the respective highest values of 2.61% and 3.68 g/m under mild stress conditions. Nonetheless, higher values for the aforementioned properties were noted with the application of vermicompost. Water deficit decreased nutrient uptake (K, P, and N) and relative water content, biological yield, and seed yield of thyme, indicating that thyme was sensitive to drought, and organic fertilizers application improved nutrient uptake (K, P, and N) and relative water content, biological yield and seed yield of the plant within irrigation levels. The activities of catalase, superoxide dismutase and ascorbate peroxidase were reduced under organic fertilizers such as vermicompost and manure compost as compared with control under drought stress. The plants of thyme showed a good response to organic fertilizers under water deficit circumstances, with vermicompost being the most effective.

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1. Introduction

The thymus genus, consists of 215 species, belongs to the Lamiaceae (Labiatae) family and is one of the most prominent medicinal herbs that are commercially grown in the Mediterranean region

(Azadi et al., 2021). *Thymus vulgaris* L. is a perennial shrub commercially cultivated in several northern countries of the Western Mediterranean region (Kosakowska et al., 2021). Its volatile components are often used as flavors, herbal tea, as well as several medicinal purposes. Also, its blossoms, stems, and leaves are known as digestive, anti-inflammatory, carminative, anti-spasmodic, and expectorant remedies (Hossain et al., 2022).

Thyme production is significantly affected by water stress conditions. The water stress (drought stress) normally decreases the water supply in the soil by evapotranspiration. As water stress is a key factor in agricultural productivity, improving water efficiency by increasing crop yield per unit area is very important in water deficit conditions (Abd Elbar et al., 2019; Raza et al., 2021). Water stress can adversely affect host plants at the molecular, physiological, biochemical, and morphological levels (Arpanahi et al., 2020). A study on biochemical traits in stress conditions would be useful to understand the adaptation mechanisms (Mohammadzadeh and Pirzad, 2020). Reduction in leaf senescence, as well as the increase in photosynthetic capacity, are the key indices of drought stress, which adversely impacts crop growth (Mohammadzadeh and Pirzad, 2020; Nasar et al., 2021). Photosynthesis, particularly under water stress conditions, leads to increase electron leakage by producing several types of reactive oxygen species (ROS) including hydroxyl radicals, superoxide, hydrogen peroxide as well as oxygen radicals (Heydarzadeh et al., 2022).

Intensive use of chemical fertilizers affected non-target organisms, altered biological ecosystems, and influenced soil microorganisms (Maddahi et al., 2021). Organic farming, which involves the cultivation with preservation of soil health could be considered as an alternative to the current farming systems which are mainly dependent on chemical application (Heidarzadeh et al., 2022). Organic fertilizers have been considered as eco-friendly approaches to improving soil fertility, and improving production, resulting in minimizing chemical fertilizers application (Khosravi Shakib et al., 2019; Maddahi et al., 2021).

Vermicompost (VC) is a nutrient-rich organic matter, which is a microbiologically active compound produced by the interaction of microorganisms with earthworms (Celikcan et al., 2021). It could be used in sustainable agriculture for improving soil porosity, thus increasing nutrient availability (Ievinsh et al., 2020). Vermicompost is rich in microorganisms that release several organic acids, including oxalic acid, and increase the solubility of elements, especially potassium and phosphorus (Celikcan et al., 2021).

Manure compost (MC) is also important in the sustainable farming system by improving soil porosity, increasing water consumption efficiency as well as nutrient availability (Ievinsh et al., 2020). Composting is widely regarded as a possible alternative for untreated manure and is used for the production of high-quality organic fertilizer due to stabilizing organic matter and destroying pathogens in raw manure in high temperature conditions (50-60 ºC) (Jiang et al., 2021).

Animal manures (AM) are organic sources of nutrients for plants' sustainable production. Also, the increase of organic matter/root improves fertility, structure, and water and air permeability of soils, so, improves host plant growth and yield (Rahimi et al., 2019). In Iran, rainfall fluctuations, as well as pollution with industrial fertilizers and pesticides are the two major challenges in the production of medicinal and aromatic plants, which have resulted in a significant reduction in farm products. Introducing such organic fertilizers, as well as providing the protocols of their application, can be useful for farming under water stress conditions. Thus, the current study aims to study the effect of organic fertilizers on the physiological, antioxidant, and yield parameters of *T. vulgaris* L. under water stress conditions.

2. Material and Methods

2.1. Field experiment

Field experiments were conducted in 2018 at Urmia University, Iran, which is located 45°10' E, 37°44' N, at an altitude of 1338 m. The study aimed to assess the effect of organic fertilization on the physiological, antioxidant, and yield characteristics of Thyme Garden (*T. vulgaris* L.). The physiochemical characteristics of the soil used in this study are presented in Table 1.

Table 1. Average physicochemical properties of the soil sample used in the study

ppH	EEC	Organic carbon	Total N	Olsen-P	Available K	$CaCO_3$	Sand	SSilt	Clay
	(dS m^{-1})	(%)	(%)	(mg kg^{-1})	(mg kg^{-1})	(%)	(%)	(%)	(%)
77.33	00.06	1.14	0.03	9.02	282	115.71	444	332	224

The study adopted a factorial experiment with 12 treatments and three replications based on a Randomized Complete Block Design. The first factor was assigned to the irrigation regime at three levels, including irrigation after 60, 90, and 120 mm evaporation from A pan. The second factor was assigned to the application of organic fertilizers at sowing time at four levels: vermicompost (VC), manure compost (MC), animal manure (AM), and control, in which no fertilizer was applied. Experimental treatments included cow manure at 30 ton ha^{-1}, compost at 20 ton ha-1 and vermicompost at 10 ton ha^{-1}, and control (no fertilizer application). Physiochemical characteristics of vermicompost, manure compost, and animal farm manure used in this study are presented in Table 2. The water required during watering to resupply the soil moisture deficit and restore field capacity is known as irrigation water needed before watering (VN). According to Walker (1984), the value of VN was calculated (Eq 1).

$$VN=[(FC-WP)\times BD\times D\times(1-ASM)\times A]/100 \quad (1)$$

Table 2. Physiochemical characteristics of vermicompost, compost and animal manure

Parameter	Vermicompost	Compost	Animal manure
EC (dS m^{-1})	3.81	7	4.21
pH	7.2	7.6	7.4
Organic carbon (%)	30	29	35
Available K (%)	1.85	0.75	1.25
Available P (%)	2.3	1.1	0.94
Total N (%)	1.45	1.25	2.27

Where FC is field capacity (%), VN is the irrigation water in m^3 needed before watering, WP is wilting point (%), Dis root zone depth (m), BD is bulk density (g cm^{-3}), A is the field area (m^2), and ASM is available soil moisture before watering (a fraction). The respective amounts of irrigation for 60, 90, and 120 mm evaporation from A pan water were 2 150, 1 850, and 1 550 m^3 ha^{-1}. The data on rainfall and temperature of the research area is given in Figure 1.

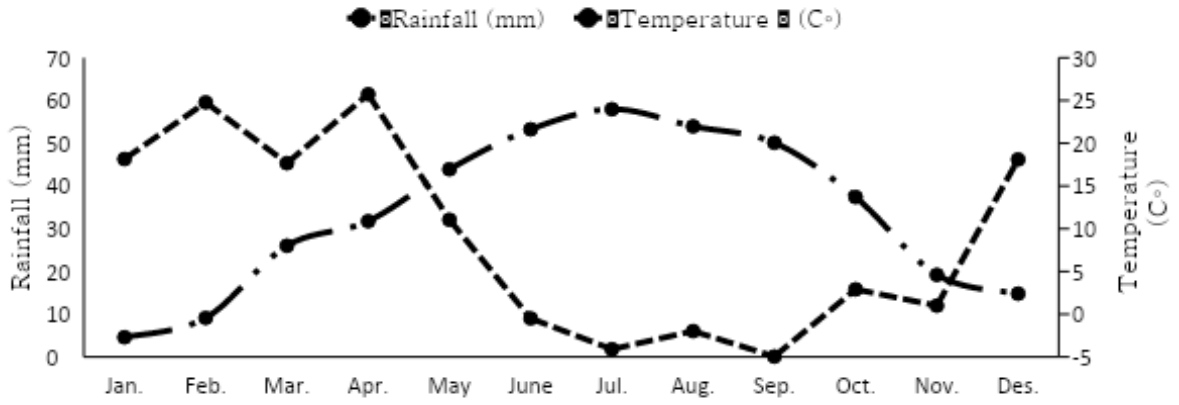

Figure 1. Monthly average air temperature and total precipitation for the years 2018-2019.

In January 2018, the seeds were sown in seedling trays containing a mixture of perlite: soil (1:2, v: v). After about 90days, the seedlings were relocated to the farm's experimental site. Each experimental

unit comprised eight rows of planting with a length of four meters and 15 cm tall thyme seedlings planted at a spacing of 50 × 25cm (Figure 2). Seedlings of thyme were irrigated immediately after planting; the subsequent irrigations were done on a weekly basis. Weeds were controlled manually when required. The treatments were sampled at full flower state. They were individually stored in nitrogen tanks and stored in a -80°C freezer. All cultivation methods were carried out uniformly for all experimental treatments.

Figure 2. The cultivated *T. vulgaris* L. in the experimental field.

2.2. Measurement

2.2.1. Plant growth characteristics

All experimental treatments were harvested individually after reaching full maturity of growth, paying attention to grain yield and biological yield from 10 plants per plot. The plant samples were oven-dried to a constant weight at40°C.

2.2.2. Plant pigment contents

About 0.5 g of fresh leaves was milled in liquid nitrogen, blended with 10 mL of 80% acetone, and homogenized by centrifuge at 4000 rpm for 15 minutes. The chlorophyll a and b contents and carotenoid were measured by using a spectrophotometer at respective wavelengths of 645, 662, and 470 nm according to the following equations 2,3,4,5 (Lichtenthaler and Wellburn, 1987) where A is the absorbance of light at 662, 645, and 470 nm.

$$\text{Chlophyll } a = 11.24 \text{ x } A_{662} - 2.04 \text{ x } A_{645} \quad (2)$$

$$\text{Chlophyll } b = 20.13 \text{ x } A_{645} - 4.19 \text{ x } A_{662} \quad (3)$$

$$\text{Total chlophyll} = 7.05 \text{ x } A_{662} + 18.09 \text{ x } A_{645} \quad (4)$$

$$\text{Carotenoid} = \frac{1000\ x\ A470 - 1.90\ x\ chlorophyll\ a - 63.14\ x\ chlorophyll\ b}{214} \quad (5)$$

2.2.3. Relative water content

Relative water content on leaf samples was measured by the method of [(fresh weight – dry weight)/ (turgid weight– dry weight)] × 100 (Khosravi Shakib et al., 2019).

2.2.4. Nutrients of (N, P, and K)

To determine the nutrient content of leaves' samples, dried leaves samples were milled, digested, and analyzed with combustion (4 h at 500 °C) of the leaf sample. On the 5 mg ash samples were added 1 ml of 2 N HCl, and the extracts obtained were filtered. After the samples were then filtered, the phosphorus (P) content of samples was detected calorimetrically by the vanado-molybdate method based on the yellow color of the unreduced vanado-molybdo-phosphoric heteropoly acid suspended in an HNO_3 medium. The amount of potassium (K) was measured by a flame photometer (Edward et al.,1999). The total concentration of nitrogen (N) in the plant leaves was measured by the Kjeldahl method (Schuman et al.,1973).

2.2.5. Antioxidant enzyme activity

For quantification of antioxidant enzyme activity, fresh plant material (100 mg) was ground in 2 mL of 0.1 M potassium phosphate with containing 5% polyvinylpyrrolidone (PVP) and buffered at a pH of 6. Then the extracts were centrifuged at 3°C for half an hour at 15.000 rpm, and the activity of the enzymes was estimated from the clear supernatant (Tejera et al., 2004). Catalase activity was determined at 240 nm based on the variation in concentration of hydrogen peroxide (H_2O_2). The reaction mixture contained 1.9 mL of 50 mM K_3PO_4, which was buffered at a pH of 710, mM H_2O_2, and 0.2 mL of enzyme extract. Enzymatic activity was then determined in 60 seconds per mg of protein based on absorption variations (Aebi, 1984). Superoxide dismutase activity was assessed at 560 to minimize the loss of nitroblue tetrazolium (NBT) photochemical (Beyer and Fridovich, 1987). In this study, one unit of Superoxide dismutase (SOD) was taken as the quantity of enzyme that inhibits a 50% decrease in NBT. By employing the Nakano and Asada (1987) method, ascorbate peroxidase activity (APX) was measured with a reaction mixture containing 1mL of 0.5 mM ascorbic acid, 1 mL 100 mM potassium phosphate-buffered at a pH of 7, 100 µL enzyme extract, and0.1 mL H_2O_2 0.1 mM. The absorption was then determined at 290 nm.

2.2.6. Essential oil and essential oil yield

Essential oil extraction was carried out by distillation using a Clevenger apparatus. For extraction (which was done for 3 hours), 10 g of dried leaves were transferred into a 1 000 ml balloon, followed by the addition of 100 ml of distilled water. By use of a Clevenger machine (Adams, 2007), the volatile compounds were extracted with water vapor as characterized by the formation of a distinct layer on the surface of the water in the graduated tube after cooling. Essential oil yield was estimated using Eq. 6.

$$\text{Essential oil yield} = \text{dry matter yield of the plant} \times \text{the percentage of essential oil} \quad (6)$$

2.3. Statistical analysis

The results were expressed as mean ± SE then analysis of variance was accomplished by ANOVA procedure, after which significant differences were computed using SAS (version 9.1.3) software as per Duncan's multiple range tests ($p < 0.05$).

3. Results and Discussion

3.1. Characteristics of thyme

ANOVA data showed that the content of chlorophyll a, b, a+b and carotenoid, essential oil, and essential oil yield of thyme were significantly affected by the effect of irrigation and fertilizer treatments. While RWC, the content of nutrients (N, P, K), the activity of the CAT, APX, and SOD enzymes, the biological yield, and seed yield of thyme were significantly affected by the interaction of irrigation and fertilizer treatments (Table 3).

Table 3. Analysis of variance of some traits of *T. vulgaris* L. medicinal plant under the influence of irrigation regime and organic fertilizers

Source of variation	df	Chl a	Chl b	Chl a+b	Car	RWC	N	P	K	CAT	SOD	APX	EEO	EEOY	BY	SY
Repetition	2	0.00007	0.006	0.005	0.0007	3.27	6.0006	0.00001	0.002	0.007	5.65	0.00008	0.008	0.01	328.57	2.04
Irrigation (Irr)	2	0.42**	0.04**	0.76**	0.03**	663.80**	0.36**	0.02**	0.15**	7.55**	4300.39**	0.72**	1.22**	4.64**	53787.84**	1729.47**
Fertilizer (F)	3	0.74**	0.24**	1.67**	0.02**	143.77**	0.37**	0.009**	0.03**	0.29**	51.52**	0.08**	2.67**	10.44**	27980.43**	1754.13**
Irr × F	6	0.00001^{ns}	0.0001^{ns}	0.0001^{ns}	0.00001^{ns}	6.35*	0.01*	0.0005**	0.002*	0.04**	16.98**	0.009**	0.0001^{ns}	0.11^{ns}	2947.29**	68.78*
Error	22	0.02	0.002	0.01	0.0009	1.80	0.005	0.00001	0.0007	0.0005	0.59	0.0001	0.017	0.06	37.50	23.93
CV (%)		12.59	0.73	0.73	8.90	2.13	3.53	1.32	2.09	0.93	1.36	1.21	5.75	7.75	1.51	3.53

*, ** and ns signify significant at 5% and 1% levels of probability and non-significant, respectively. Chl a and b, chlorophyll a and b content; Car, carotenoid; RWC, relative water content; N, nitrogen; P, phosphorus; K, potassium; CAT, catalase activity; SOD, superoxide dismutase activity; APX, ascorbate peroxidase activity; EO, essential oil; EOY, essential oil yield; BY, biological yield; SY, seed yield; ns, non-significant.

3.2. Photosynthesizing pigments

The average comparison revealed that the chlorophyll a and b contents, total chlorophyll, and carotenoid decreased significantly with the delay in irrigation (Table 4). On the other hand, based on the plants that had been subjected to organic fertilizer, these properties were significantly higher compared to the control plants (Table 4). So that the highest chlorophyll a and b contents, total chlorophyll and carotenoid were 1.47, 0.86, 2.33, and 0.42 mg g^{-1} FW, respectively, obtained from plants that were treated with VC fertilizer. While the lowest chlorophyll a and b contents, total chlorophyll and carotenoid were 0.86, 0.49, 1.35, and 0.29 mg g^{-1} FW, respectively, observed in the control treatment (Table 4). The content of chlorophyll in living plants is a key factor in maintaining photosynthetic efficiency. Increased degradation or loss of synthesis of these pigments (chlorophyll and carotenoids), in addition to the deterioration of enzyme activity that is in charge of the synthesis of photosynthesizing pigments, cause a photosynthetic deficiency in plants exposed to drought stress, resulting in reduced assimilation and production declines (Ashrafi et al., 2018; Khosravi Shakib et al. 2019). In peanuts, salt stress decreased chlorophyll content compared to the control (Yolci et al., 2021). Carotenoid and chlorophyll content increased with the application of AM, VC, and MC to the substrate (Table 4). Organic fertilizers secure the N requirement of photosynthesizing pigments and plant amino acids, and at the same time, N wastage (by leaching, sublimation, or fixation) is reduced (Rahimi et al., 2019), thereby affecting the amount of these pigments in the crops. In addition, the plants exhibited not only higher sunlight absorption capacity and photosynthate synthesis but also improved growth and yield (Khosravi Shakib et al., 2019). This combination of traits increases plant productivity.

3.3. Relative water content

The highest RWC (75.85%) was obtained in the application of VC fertilizer and irrigation after 60 mm of evaporation from A pan. While the lowest one (50.55%) was obtained in severely stressed plants and without the application of organic fertilizer (Figure 3). It has been reported that RWC of Marigold and melons decreases with increasing water stress (Kusvuran et al., 2011; Khosravi Shakib et al., 2019). The first impacts of water deficit can be a reduction in relative water content in leaves and a reduction of turgor in plant tissues, which can naturally impact cell growth and final size (Amirnia et al. 2019). Organic fertilizers increase water absorption and enhance water connections in the host plant, most likely by altering root morphology and improving the host plant's root system, as well as by increasing the absorption region (Ashrafi et al., 2018; Bistgani et al., 2018).

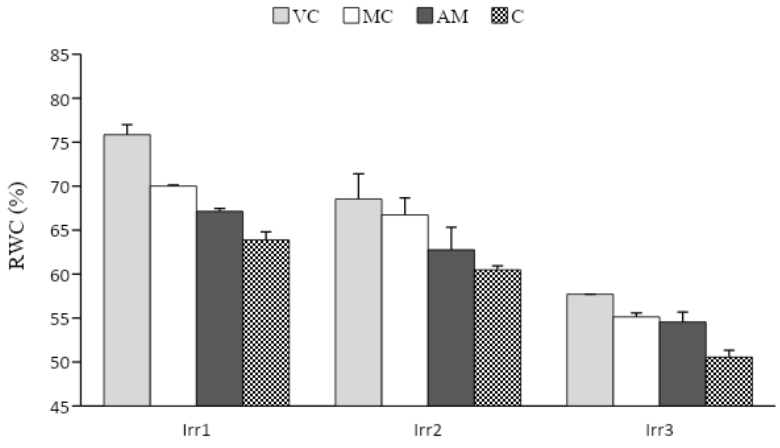

Figure 3. Comparing of the impact of irrigation regime and organic fertilizers implementation on RWC, relative water content ($P \leq 0.05$ by LSD test). VC, vermicompost; MC, manure compost; AM, animal manure; C, Control; Irr1, Irr2, and Irr3, respectively, irrigation after 60, 90, and 120 mm evaporation from A pan.

3.4. Nutrients of P, K, and N

According to means comparison results, the amount of P, K, and N was increased under the use of organic fertilizers compared to the control treatment (no fertilizer application) in all irrigation levels. The highest P, K, and N were 0.36, 1.48, and 2.48% respectively (Figure 4), which were surveyed in plants under well-watered circumstances (Irr1) conditions and the application of VC. Whereas the lowest ones 0.20, 1.11, and 1.67%, respectively, were obtained under the most restrictive irrigation regime and without the application of fertilizer. However, the amount of P, K, and N in well-watered plants were considerably higher than in plants in deficit irrigation appearances (Figure 4). When the water supply in the soil is decreased, its uptake is limited. Furthermore, from a physiological perspective, the reduced water uptake results in not only reduced photosynthesis but also transpiration (Nyawade et al., 2021; Alavi-Samani et al., 2015). Under such conditions, active mobilization systems are equally disrupted for the purpose of saving biological energy consumption. These combine to cause a substantial loss of root absorbability hence reducing the nutrient uptake capacity (Bistgani et al. 2018). Under water deficit conditions, plants are exposed to both water deficit and osmotic stress due to a decrease in the soil matrix potential resulting in ionic imbalance and nutrient deficiency (Rahimi et al., 2022). In these conditions, organic fertilizers extend the root network and improve nutrition uptake; it also provides improved conditions for water uptake by plants and, so, better thriving conditions for the crop (Khosravi Shakib et al. 2019; Ievinsh et al., 2020). Besides, organic fertilizers enrich the nutrition of the plants, improving their vegetative growth (Maddahi et al., 2021).

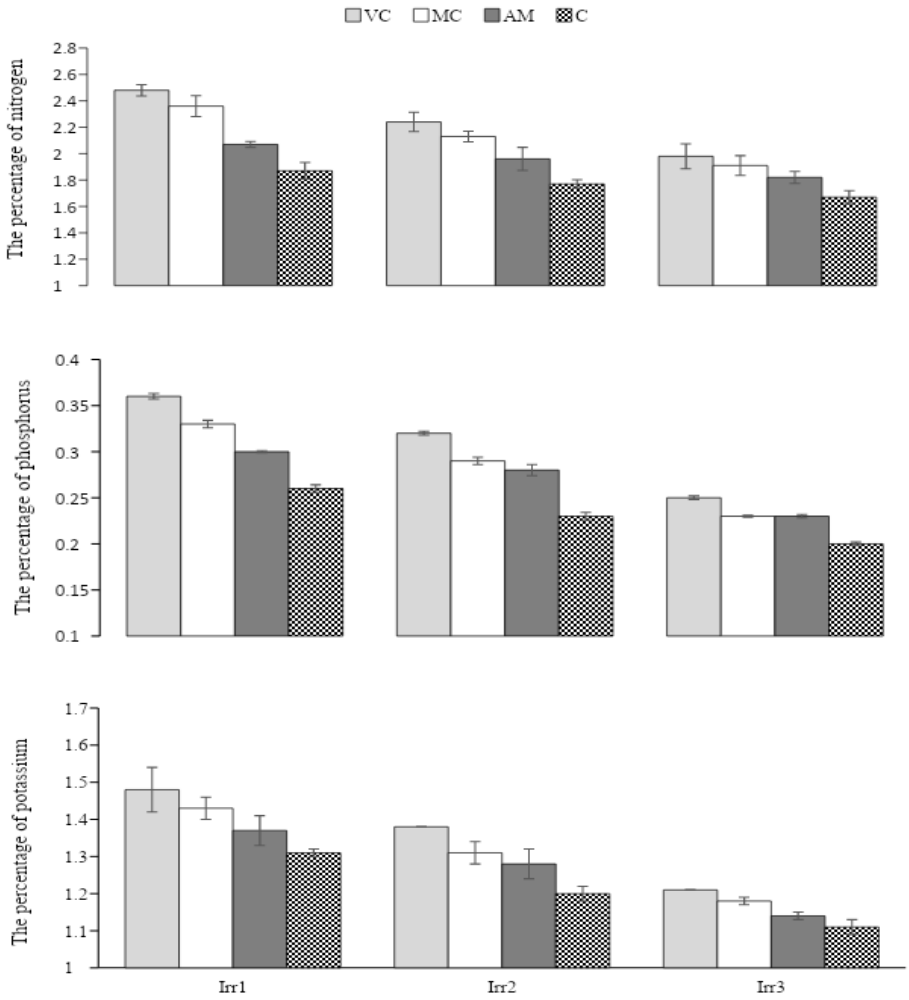

Figure 4. Comparing for impact of irrigation regime and organic fertilizers implementation on the percentage of nitrogen (a), phosphorus (b) and potassium (a). ($P \leq 0.05$ by LSD test). VC, vermicompost; MC, manure compost; AM, animal manure; C, Control; Irr1, Irr2 and Irr3, respectively, irrigation after 60, 90, and 120 mm evaporation from A pan.

Table 4. Means a comparison of the effect of irrigation regime and organic fertilizers on some traits of *T. vulgaris* L.

		Chlorophyll a (mg g^{-1} FW)	Chlorophyll b (mg g^{-1} FW)	Chlorophyll a+b (mg g^{-1} FW)	Carotenoid (mg g^{-1} FW)	Essential oils (%)	Essential oils yield (g m^{-1})
	Irr1	1.37±0.12a	0.69±0.03a	2.06±0.11a	0.40±0.02a	2.29±0.13b	3.51±0.20a
	Irr2	1.18±0.12b	0.62±0.04b	1.80±0.09b	0.34±0.02b	2.61±0.11 a	3.68±0.22a
	Irr3	0.99±0.12c	0.56±0.04c	1.56±0.09c	0.29±0.02c	1.97±0.11c	2.53±0.19b
Organic fertilizers							
	VC	1.47±0.25a	0.86±0.10a	2.33±0.15a	0.42±0.03a	2.96±0.19a	4.45±0.33a
	MC	1.35±0.06a	0.58±0.02b	1.94±0.08b	0.34±0.00b	2.48±0.10b	3.64±0.22b
	AM	1.02±0.09b	0.56±0.04b	1.59±0.07c	0.33±0.01b	2.01±0.09c	2.70±0.15c
	C	0.86±0.09c	0.49±0.00c	1.35±0.08d	0.29±0.05c	1.71±0.11d	2.08±0.11d

The same letters are shown statistically non-significant at $P \leq 0.05$ by LSD test. VC, vermicompost; MC, manure compost; AM, animal manure; C, Control; Irr1, Irr2, and Irr3, respectively, irrigation after 60, 90, and 120 mm evaporation from A pan, irrigation after evaporation from Class A pan.

3.5. Antioxidant enzymes activity

According to the results, the SOD, CAT, and APX in water-deficit stress conditions were higher than in well-watered plants (Figure 5). The utmost SOD, CAT, and APX of 80.04, 3.48, and 1.32 µmol g^{-1}, respectively (Figure 5), were observed in plants under water impairment stress and without the implementation of fertilizer, whereas the lowest ones (33.82, 1.5, and 0.75 µmol g-1) was obtained from plants with well-watered and treated with VC (Figure 5). Water stress increased CAT, APX, and SOD activity. In some plants, damage emanating from water stress causes oxidative stress resulting in the production of toxic oxygen species and their accumulation preventing respiration and photosynthesis, hence negatively affecting plant growth (Keshavarz Mirzamohammadi et al., 2021).

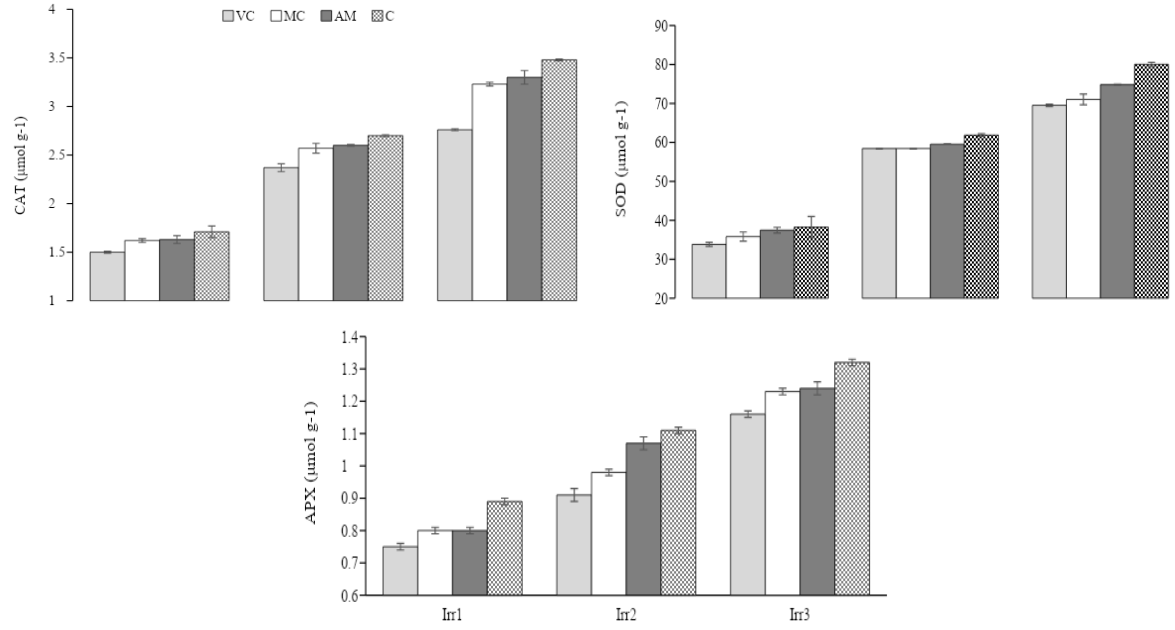

Figure 5. Comparing the interactive impact of irrigation regime and organic fertilizers implementation on CAT, catalase activity (a); SOD, superoxide dismutase activity (b) and APX, ascorbate peroxidase activity (c). ($P \leq 0.05$ by LSD test). VC, vermicompost; MC, manure compost; AM, animal manure; C, Control; Irr1, Irr2, and Irr3, respectively, irrigation after 60, 90, and 120 mm evaporation from A pan.

3.6. Essential oil and essential oil yield

The results of mean comparisons showed that the highest essential oil (2.61%) and essential oil yield (3.68 g m^{-1}) were obtained under mild stress conditions (irrigated plants after 80 mm of evaporation from A pan). Also, there was no significant effect on essential oil yield in mild stress conditions

compared to the well-watered plants (Table 4). The highest essential oil (2.96%) and essential oil yield (4.45 g m^{-1}) were obtained from plants treated with VC, while the lowest ones (1.71% and 2.08 g m^{-1}) were obtained from plants without the use of fertilizers (control) (Table 4). It has been reported that the application of organic fertilizers can enhance the essential oils of canola by increasing nutrient absorption and the resulting improvement in CO_2 absorption and photosynthetic efficiency (Bistgani et al., 2017b; Bistgani et al., 2017a). Likewise, the application of vermicompost, compost, and animal manure increased the essential oil percentage and yield of Marigold (Khosravi Shakib et al., 2019). The results established that thymus' essential oil increases under slight drought stress conditions, with severe drought stress reducing the percentage of essential oil, and it is ascribed to essential oil storage, reduction in leaf area, and antioxidant power, which results in higher oil glands density. As drought level increases, the percentage of essential oil decreases, leading to not only protein degradation but also cell and plant death (Rahimi et al., 2022). Organic fertilizers stimulate the metabolite process and plant growth hence enhancing the production of medicinal plants (Saki et al., 2019). Nitrogen is fundamental in the promotion of cell division, suggesting that such organics can improve essential oil synthesis in plants and especially medicinal ones(Amooaghaie and Golmohammadi, 2017; Heidarzadeh et al., 2022). A decrease in essential oil content and biological yield in the plant can reduce the essential oil yield (Rahimi et al., 2022). Nonetheless, essential oil yield improved with the application of VC, MC, and AM. This might be attributed to improvement in soil cation exchange capacity (CEC) besides an increase in the availability of some elements such as nitrogen, which together could have decreased nutrient leaching, leading to not only higher total dry matter but also increased phytochemical concentration of plants (Saki et al., 2019; Heidarpour et al., 2019).

3.7. Biomass yield and seed yield

The biomass yield and seed yield of the plants that had been subjected to severe stress (irrigated after 120 mm of evaporation from A pan) were significantly lower compared to the irrigated plants after 60 mm of evaporation from A pan (Irr1). The highest biomass and seed yields were 570.14 and 169.36 g m^{-1}, respectively, were found to be related to well-watered plants and the application of VC, whereas the lowest ones (291.17 and 113.92 g m^{-1}) were obtained in severely stressed plants and without the use of fertilizers (control) (Figure 6). Cell growth is among the most key processes that are affected by water deficit due to lower turgor pressure (Das et al., 2017). When leaf size is reduced, the capacity to intercept light decreases, which subsequently decreases the total photosynthesis capacity (Das et al., 2017). Additionally, drought stress resulted in smaller stomatal apertures, leading to a significant decrease in the CO2 exchange rate. Such reduction, therefore, resulted in lower photosynthesis and, consequently, reduced plant growth and performance (Amirnia et al., 2019; Nasar et al., 2021). Organic fertilizers (vermicompost and compost) can enhance plant growth and photosynthesis assimilation by increasing leaf area and photosynthetic activity capacity during the pre-flowering phase. As a consequence, increased re-mobilization of this organic matter from source to sink enhances growth regulators' characteristics during the post-flowering cycle (Khosravi Shakib et al., 2019). Organic fertilizers can have a direct effect on plant growth by increasing nitrogen absorption, phytohormone synthesis, and mineral solubility, all of which can have a significant impact on plant production (Goswami et al., 2017; Khosravi Shakib et al., 2019). Manure application may improve growth, biomass production, and seed yield by increasing mineral uptake, as well as reduce the negative effects of water scarcity on plants by improving leaf water efficiency, photosynthetic efficiency, transpiration rate, and water uptake of host plant roots (Askary et al., 2018). As a result, it leads to high leaf and stem dry weight as well as high total dry weight per plant.

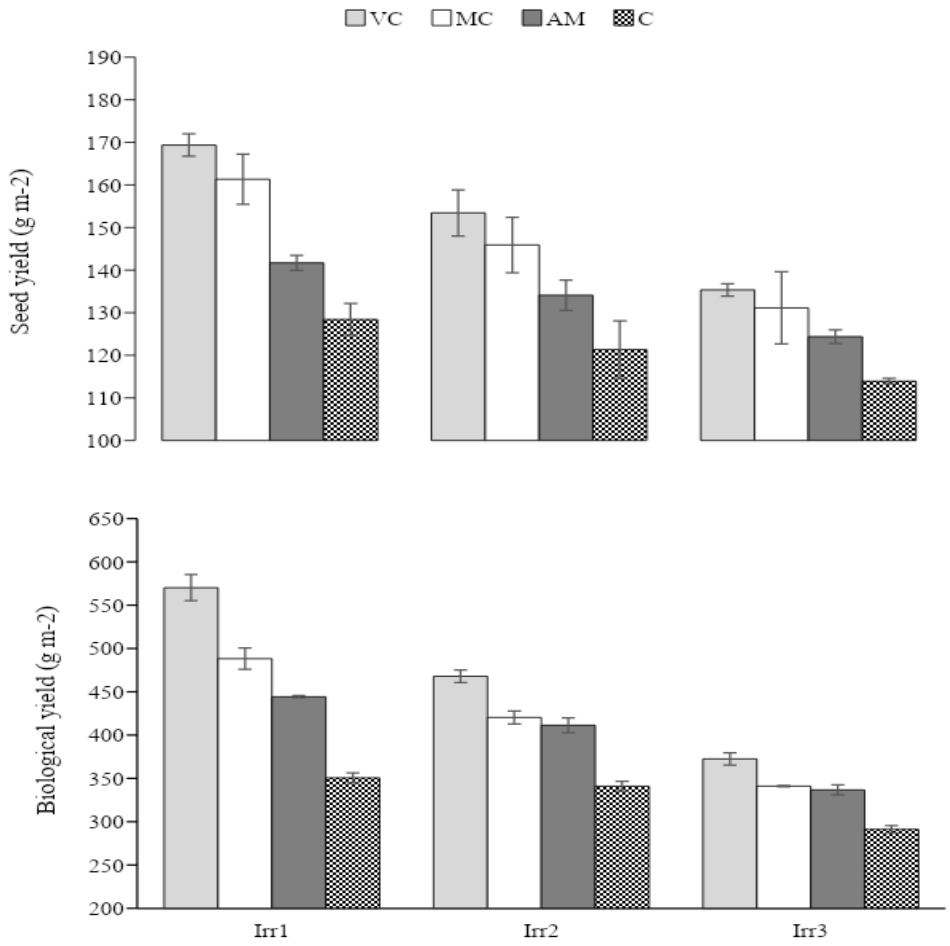

Figure 6. Comparing irrigation regime and organic fertilizers implementation on seed yield (a) and biological yield (b). ($P \leq 0.05$ by LSD test). VC, vermicompost; MC, manure compost; AM, animal manure; C, Control; Irr1, Irr2, and Irr3, respectively, irrigation after 60, 90, and 120 mm evaporation from A pan.

4. Conclusions

The results showed maximum contents of carotenoid chlorophyll a and b, and total chlorophyll of thyme were attained in well-watered conditions, while essential oil and essential oil yield were maximized under mild stress conditions. The highest chlorophyll a and b contents, total chlorophyll, carotenoids, essential oil, and essential oil yield were achieved in the application of VC. Under each irrigation regime, the application of VC fertilizer is more effective in improving nutrient uptake (K, P, and N) and RWC, biological yield, and seed yield of thyme. Water stress increases antioxidant activity by activation of enzymes (SOD, APX, and CAT), which enhances the protection of plants against MDA (lipid peroxidation). Hence, the application of organic fertilizers through increased water efficiency under water deficit conditions could improve antioxidant activity and thyme production in order to move towards sustainable agriculture.

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Research Article

First Report of '*Candidatus* Phytoplasma australasia' Strain Related to Witches'-Broom of Tomato in Türkiye

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Abstract: Phytoplasmas are dangerous bacteria severely infecting agricultural production worldwide. In the present study, the identification of phytoplasmas infecting tomato plants showing symptoms such as small leaves, flower abnormalities, stunting, witches' broom, and reddening was performed. Five plants, two symptomatic and three asymptomatic, were tested to verify phytoplasma infection. Total DNA isolated from 5 leaf samples was used as a template for PCR reactions. The phytoplasma agents were confirmed in the two symptomatic samples. BLASTn search of 16S rRNA of two sequences shared identity similarity of 99.84% with '*Candidatus* Phytoplasma australasia'. Computer-simulated virtual RFLP profiles show that the 16S rRNA sequences is identical to the reference pattern of the 16SrII-D subgroup, with a similarity coefficient of 1.00. Based on BLAST, virtual RFLP, and phylogenetic dendrogram, the identified phytoplasma strains are enclosed in the 16SrII-D subgroup. This is the first report of tomato witches' broom disease related to 16SrII-D subgroup phytoplasma strains in the Antalya province of Türkiye.

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1. Introduction

Tomatoes are one of the most popular vegetables in the world, with high economic value and health benefits. At a country level, China is the largest producer in 2019 and accounted for 62.7 million tons in production and Türkiye ranks third with 12.8 million tons. In Türkiye, tomato production is made mainly in Antalya, Bursa, Manisa, and Izmir provinces (CIA, 2017). Tomato (*Solanum lycopersicum* L.) is a basic host of many pathogenic microorganisms, including fungi, viruses, bacteria, viroids, and phytoplasmas. Among them, phytoplasmas were first evaluated as viruses or virus-like diseases, then named mycoplasma-like organisms (MLO) in 1967, and finally called 'Phytoplasma' in 1992. Up to now, these pathogens are categorized into 33 taxonomic groups that rely on the 16Sr ribosomal RNA gene, each comprising a different number of subgroup members (Bertaccini and Lee, 2018). Phytoplasmas, belonging to the '*Candidatus* Phytoplasma' genus, are the smallest living microorganisms and are devoid of the cell wall. Some basic genes are missing from the small genome with a low G+C content (Oshima et al., 2013).

Their infection in over 700 plants involving annual and perennial plants, fruit and forest tree, and ornamental plants is a huge constraint on agricultural production, which leads to a significant loss of quality and quantity (Zibadoost et al., 2016; Venkataravanappa et al., 2017). As it damages the vegetative and generative organs, product losses because of infection in cultivated plants such as cucumber, tomato, and eggplant can reach up to 100% in distinct ecological zones (Rao and Kumar 2017; Kumari et al., 2019). Microscopic techniques have been used mainly in the diagnosis of phytoplasmas, they do not allow for adequate identification (Namba 2019). Currently, nucleic acid-based techniques (16S rRNA, SecA, SecY, Tuf gene) are a reliable tool for identifying this pathogen and ensuring the group/subgroup separation to which it belongs (Venkataravanappa et al., 2017; Usta et al., 2021). A total of seven main 'Ca. Phytoplasma' species have so far been identified from tomato: '*Ca*. P. lycopersici' and '*Ca*. *P. asteris*' in Bolivia and Poland (Arocha et al., 2007; Krawczyk et al., 2010), '*Ca*. P. aurantifolia' and '*Ca*. P. australasia' in China and India (Singh et al., 2012; Dong et al., 2013), '*Ca*. P. pruni' in Brazil (Amaral-Mello et al., 2006), '*Ca*. P. trifolii' and '*Ca*. P. solani' in Türkiye (Usta et al., 2018), '*Ca*. P. ulmi' in central Italy (Del Serreno et al., 2001).

Phytoplasmas are related to metabolic and phenotypic alterations in their hosts and affect the fecundity of the plant. The associated with all phytoplasmas symptoms infecting tomatoes are close to each other. The characteristic ones appearing are yellowing, lateral shoots, little young leaves, fruit abnormalities, reddening, over-longed calyx, purple leaves, and witches' broom (Xu et al., 2013).

Greenhouse vegetable cropping has been one of the important livelihoods in Kaş (Antalya, Türkiye). In this district, tomato plants are grown in heated greenhouses in an area devoted to protected-culture vegetable production (Gül and Özenç, 2020). The symptoms of tomato-phytoplasma disease have been identified in different regions of Turkey (Güller and Usta, 2020; Çağlar and Şimşek, 2022). However, there is no scientific report related to the Antalya province. The objective of this study is to identify the phytoplasma presence and identity of diseased tomato plants from greenhouse-grown tomatoes in the Antalya province of Turkey.

2. Material and Methods

2.1. Source of tomato plants

In the spring of 2020, 5 tomato samples (2 symptomatic and 3 symptomless) were collected from three greenhouses in the Kaş district (Antalya, Turkey). All plant samples were transported to the virology laboratory of Van Yuzuncu Yil University for molecular analyses and stored at -20 °C prior to molecular tests.

2.2. DNA Extraction and molecular amplification (PCR)

Total DNA extraction was done from 0.5 g of tomato leaf tissue using a commercial plant DNA extraction kit (Thermo Fisher, USA) according to manufacturer's guidance by grounding through a ball-bearing homogenizer. The purified DNA was subjected to PCR to provide amplification of the 16S rRNA gene by direct PCR and nested PCR steps. PCR-targeting universal primers (R16mF2/R16mR1 for d-PCR and R16F2n/R16R2 for n-PCR) amplifying a segment of 1.8 kb and 1.25 kb, for both steps were adopted as specified by Lee et al., (1993) and Gundersen and Lee (1996), respectively. The reaction parameters and temperature cycles were applied as described by Usta et al., (2018). To ensure the reliability of the PCR assays, DNA of '*Ca*. P. solani' (KX977570) was used as a positive control, as well as, DNA from symptom-free plants and DNA-free mix were used as negative controls. The d-PCR yields were diluted 1:30 ratio and employed as templates for re-amplification in n-PCR. Following n-PCR, amplified DNAs of 20 µl and DNA ladder (3.0 kb) as size standard were electrophoretically fractionated in agarose gel stained with EtBr (Ethidium Bromide) buffered in 1×TAE (Tris Acetic EDTA) and visualized under UV light with a digital imaging device (SYNGENE).

2.3. Cloning, sequence identity, and identification of phytoplasma

Amplified products were gel-purified using a kit (Thermo Fisher, USA) from the supplier. Standard cloning protocol was used to transfer the eluted product (1µl) into a bacterial cloning vector (pGEM T- Easy) (Promega, Madison, USA).

Recombinant plasmids were electroporated into *E. coli* JM109 competent cells. White recombinant colonies consisting of insert DNA were selected from solid medium containing ampicillin (1%) and cultured in a liquid LB medium containing the same amount of antibiotics. To detect the relevant gene sequence of the pathogen, recombinant plasmids were purified from the cell suspension and sequenced by next generation sequencing (NGS) (Sentebiolab/Ankara/Turkey). The sequence identities were analyzed using BLASTn queries in the GenBank database.

2.4. Consensus tree and in silico RFLP Analysis

*i*PhyClassifier program was used to calculate the similarity coefficient and to determine the group and subgroup of sequences of R16F2n/R16R2-primed n-PCR yields. For further characterizations, *in silico* digestion was also conducted using seventeen key digestion enzymes (*Alu*I, *Bam*HI, *Bfa*I, *Bst*UI (*Tha*I), *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI (*Mbo*I), *Mse*I, *Rsa*I, *Ssp*I, and *Taq*I by pDRAW32program (Zhao *et al.,* 2013). The restriction profiles of '*Ca*. P. australasia' Antalya strains were compared with that of the reference strain (Access no: Y10096). A phylogenetic dendrogram was constructed from Antalya strains along with a set of 27 available 16S rRNA sequences archived into NCBI. *Acholeplasma palmae* (Accession no: L33734) was selected as the outsource to ensure a valid rooting. The consensus tree was constructed by the maximum likelihood algorithm using CLC Main workbench software. Tree branches were bootstrapped with 1000 replicates to assess the accuracy of the concluded clades and sub-clades.

3. Results

3.1. Visual assessment

Symptoms were usually conspicuous in infected tomatoes with floral and leaf symptoms. The most commonly observed symptoms on greenhouse-grown tomato plants ranged from mild to severe floral sterility and abnormalities, no leaves on the branch tips, adventitious shoot, hypertrophied calyx, witches' broom, curled and purplish leaves, and yellowing and small deformed young leaves (Figure 1).

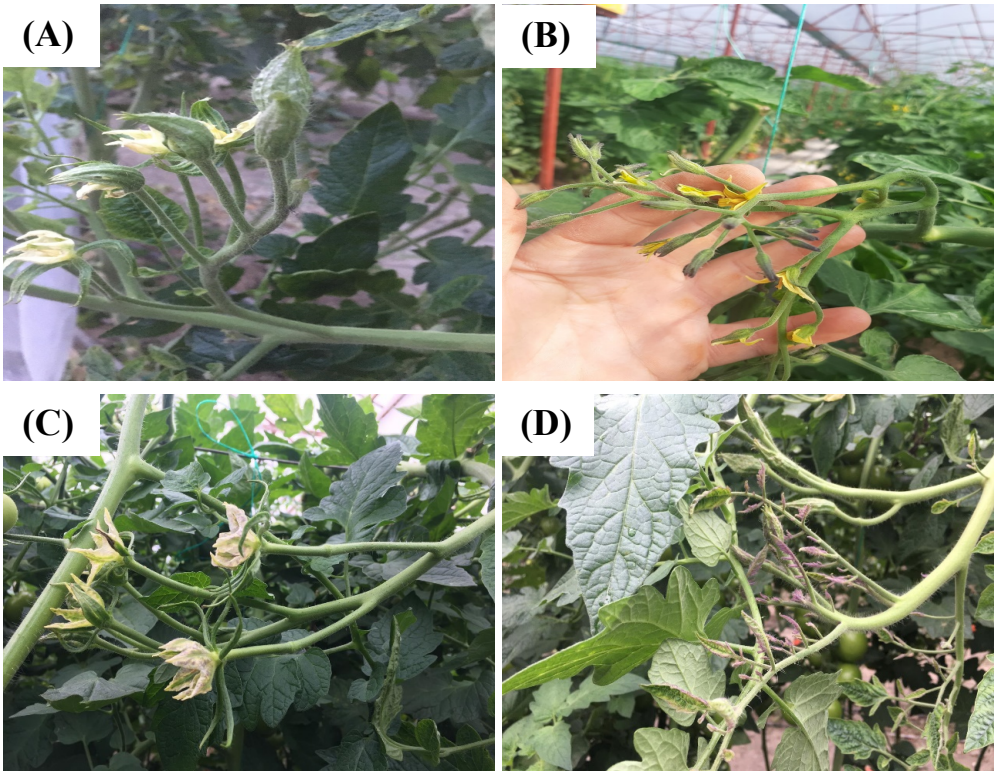


Figure 1. **A-** Heavily phytoplasma infected tomato plants growing in different greenhouses in Kaş district. Big bud, **B, C-** floral sterility and abnormalities, **D-** purplish small sized leaves due to '*Ca*. P. australasia' infections.

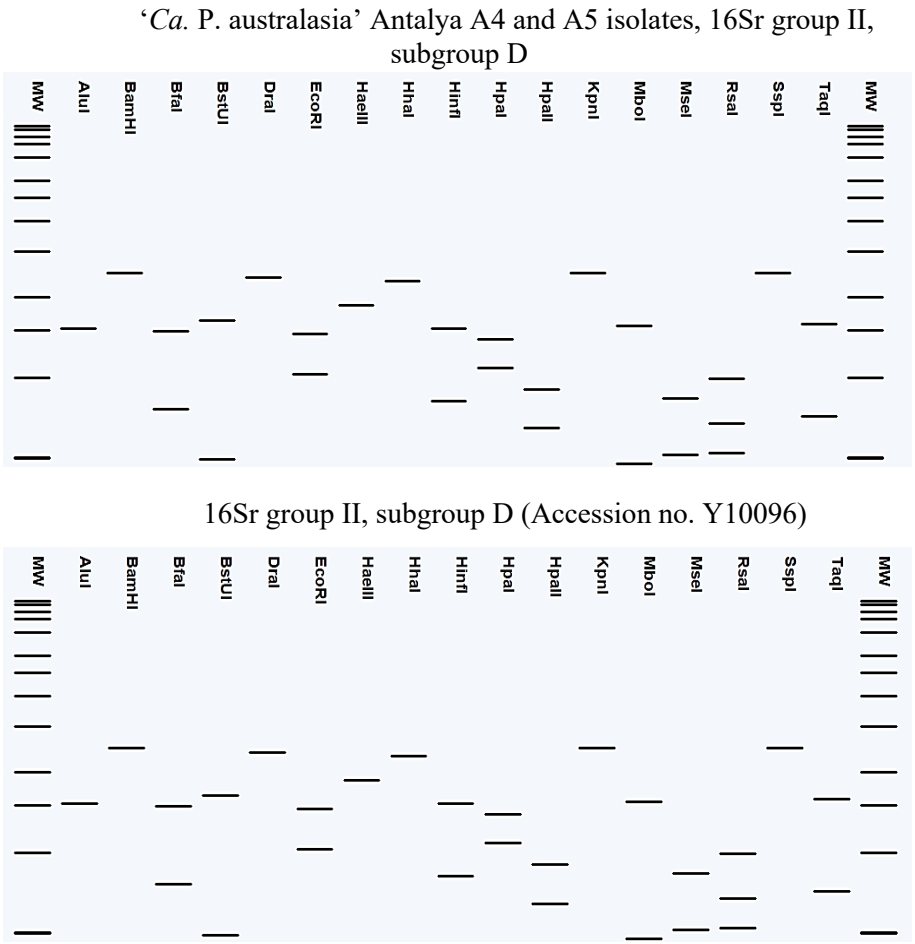
3.2. Detection of phytoplasma, sequence identity and similarity coefficients

In direct PCR tests, the universal R16mF2/R16mR1 primer pair generated DNA fragments of 1.8 kb from positive control and two symptomatic tomato samples (data not shown), but not from symptomless samples and negative control. The occurrence of phytoplasmas was identified in 2 out of 5 analyzed, resulting in an expected size of 1.25 kb nested-PCR amplicons primed with R16F2n/R16R2, corresponding to the 16S rRNA gene of the pathogen. Two cloned obtained 16S rRNA sequences displayed 99.92% nucleotide identity with that of reference strains '*Ca*. P. australasia' (GenBank accession no: Y10096).

A sequence BLAST search of F2nR2 fragments proved that the two tomato strains from this study have a 99.84% identity with many phytoplasma members 16SrII '*Ca*. P. australasia' strains (Accession no. OM416010, OM416008, OL306323, OK644503, OM415996, i.e.) and '*Ca.* P. aurantifolia' (Accession no. OK625583, MZ348527). Based on *i*Phyclassifier software, the OM513906 and OM616883 sequences, identified in this study, were '*Ca*. P. australasia' (16Sr II-D subgroup, Peanut witches' broom (PnWB)), with a 1.00 similarity coefficient.

3.3. Virtual RFLP and phylogenetic analysis

Virtual RFLP studies also confirmed the groups to which tomato-related phytoplasmas belong. The cut profile of the two '*Ca*. P. australasia' sequences were identical with (1.00 similarity coefficient) the reference strain of '*Ca*. P. australasia' (Accession no.Y10096), demonstrating no genetic diversity (Fig. 2). The present finding pointed out a minor difference in the 16S rRNA gene sequence of 16Sr group II, subgroup D compared to that of the 16Sr group II, subgroup B reference isolate(Access no. U15442). This assignment was also corroborated by the clustering assays with other '*Ca*. Phytoplasma' strains in primarily 16SrII and other groups retrieved from the NCBI (Figure 2).



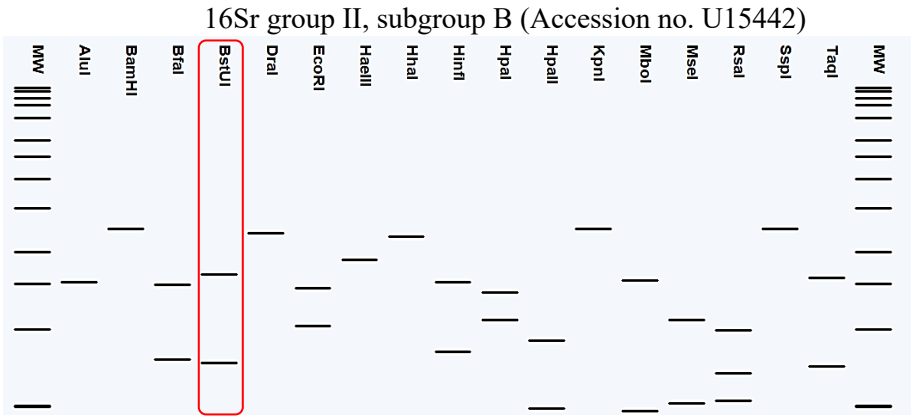


Figure 2. Virtual restriction endonuclease digestion patterns of tomato-associated '*Ca*. P. australasia' A4 (Accession no.OM513906) and A5 (Accession no.OM616883) isolates. No differences have been identified in restriction patterns of two isolates (A4 and A5) and the reference strain of 16SrII-D subgroup (Accession no. Y10096). However, a slight difference has been identified in electrophoretic mobility between the members of 16SrII-D subgroup and the reference strain in 16SrII-B subgroup (Accession no. U15442) when digested with *Bst*UI (shown in box). M: 100 bp DNA size standard.

The phylogenetic tree of the aligned 16S rRNA sequences showed that tomato phytoplasmas from Kaş (Antalya) clustered with '*Ca*. P. australasia' strains belonging to the Peanut witches'-broom phytoplasma group, more specifically with the 16SrII-D subgroup (Figure 3).

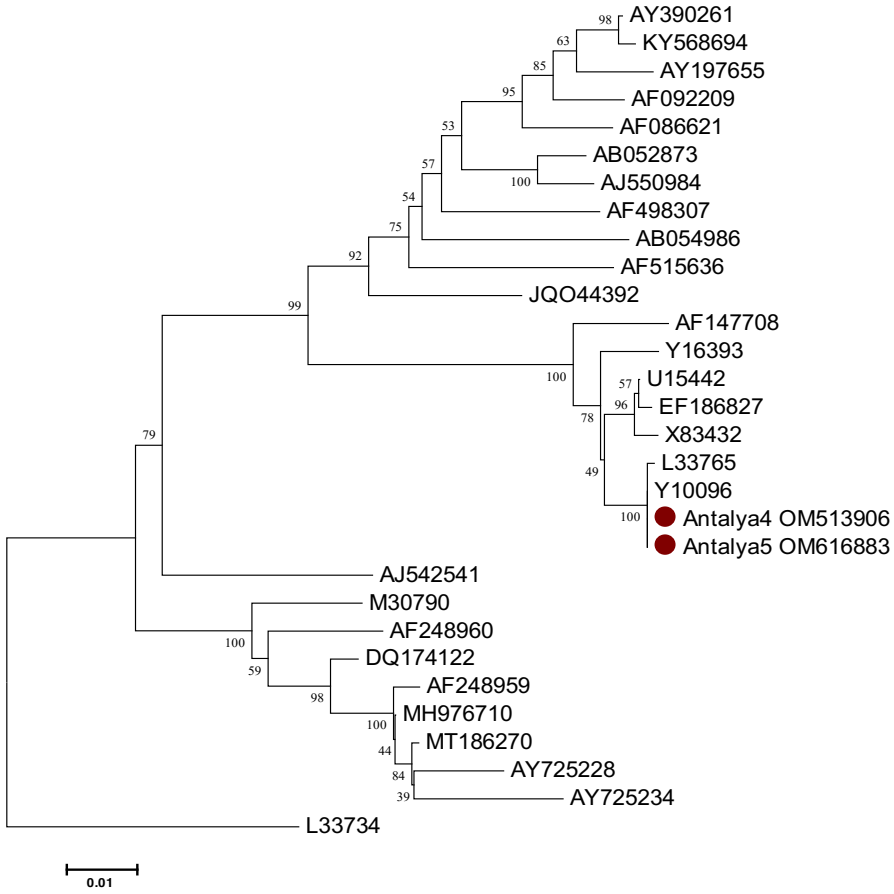


Figure 3. Phylogenetic dendrogram of 16Sr RNA gene sequences of 'Ca. P. australasia' A4 and A5 strains constructed with maximum likelihood algorithm using the reference strains and other 'Ca. Phytoplasma' species and isolates. L33734 (*Acholeplasma palmae* 16S rRNA gene) was added as outgroup. The bootstrap score of 1000 replicates as percent are shown on branches.

4. Discussion

Phytoplasma-infected tomato plants from various 16S ribosomal groups have been described in various countries (Contaldo et al., 2021; Davoodi et al., 2019; Khalil et al., 2019; Oksal, 2020). In the present study, the '*Ca*. P. australasia' (16SrII-D subgroup) was identified in phytoplasma-symptomatic tomato plants in Turkey, which is the first record nationally (Fig 2). In Turkey, phytoplasmas belong to eight 16Sr groups which are I, IX, and X (Canik et al., 2011, 2019), II (Ayvaci et al., 2020), V (Sertkaya et al., 2004), VI and XII (Usta and Güller, 2018), and XIV (Çağlar et al., 2013) have been identified in different hosts, including ornamental plants, orchards, weeds, and cultured crops.

'*Ca*. P. australasia' (16SrII-D subgroup) is associated with a multitude of plant diseases. The new data is important for the locality in terms of agent/disease transmission. In Turkey, Ayvaci et al., (2020), Özdemir and Cagirgan (2015), and Ozdemir et al., (2014) described this pathogen in alfalfa (*Medicago sativa*), jute (*Corchorus olitorius*) plants exhibiting symptoms such as little leaves, leaf yellowing, and witches' broom and in Orosius orientalis, respectively. The molecular characterization of this strain in tomato affirmed the 'Ca. P. australasia' identity and represented a new epidemiological host about this agent in Turkey. Because of the prevalence of tomato cultivation, this pathogen has a potential to cause epidemics in other crop plants when the insect vector(s) and alternative hosts are available. Similar morphological symptoms triggered by the same phytoplasma in tomato plants were described in India (Singh et al., 2012), in Egypt and Iraq (Omar and Foissac, 2012; Alkuwaiti et al., 2017), in Iran (Salehi et al., 2016).

The 16SrII-D ribosomal subgroup of phytoplasmas has a diverse spectrum of hosts with geographical prevalence, including *Petunia violacea*, *Calendula officinalis*, alfalfa, squash, pomegranate, and parsley in Iran (Salehi et al., 2014; Esmailzadeh Hosseini et al., 2015, 2018; Hemmati et al., 2019); *Cycas revoluta* and *Phoenix dactylifera* in Oman (Hemmati et al., 2020a; 2020b); periwinkle, onion, and *Opuntia abjecta* in Egypt (El-Sisi et al., 2017); sweet potato, pale purple coneflower, *Echinacea pallida* and papaya in Australia (Pearce and Scott 2017; White et al., 1998), tomato, eggplant and mallow in Iraq (Alkuwaiti et al., 2017); *Daucus carota* and sesame in India (Venkataravanappa, 2017); eggplant in China (Li et al., 2019); sesame, faba bean, and chickpea in Pakistan and Sudan (Akhtar et al., 2009; Alfaro-Fernández et al., 2012).

Based on this, a rapid and accurate diagnosis of phytoplasma infection in economically important plants is important for the surveillance and management of the disease. In the past, the identification and taxonomy of phytoplasmas were based on the sensitivity of the test plants, their induced symptoms, and their relationship with insect hosts (Shiomi and Sugiura 1984; Chiykowski 1991). Today, RFLP methods of the conserved 16S rRNA marker gene sequence and DNA hybridization method have been a basic tool adopted by most researchers for precise detection and differentiation of phytoplasma infections (Zelyut et al., 2022). In this study, we employed the 16S rRNA gene region, which is widely used in phytoplasma taxonomy. Although this sequence is valuable for revealing interspecies taxonomic levels, a multilocus analysis should be performed using the rp, secY, secA, cpn60, and tuf gene locus to distinguish closely related phytoplasma strains in the same 16Sr groups and gain advantages over single gene analysis (Davis et al., 2013; Dumonceaux et al., 2014; Serçe and Yılmaz 2019; Valiunas et al., 2019).

Management of phytoplasma diseases is extremely troublesome. Many methods have been tried and no single effective method has been developed. Chemicals such as chloramphenicol, tetracycline and salicylic acid, antimicrobial compounds, and developing resistant cultivars are sufficiently ineffective because of both costly and no long-term protective ways, although they provide symptom remission (Bertaccini, 2021; Upadhyay, 2016). Therefore, the most plausible approach strategically is that prophylactic methods restricting the expansion dissemination of these agents may be more usable and sustainable. Control of weeds or insects is important for inoculum formation, its transmission and disease development (Cagirgan et al., 2014). Further studies are needed to determine the distribution, inoculum sources and insect vectors of '*Ca*. P. australasia' in these areas where protected edible crops are grown. In this way, it can be ensured that the agent is brought under control before it reaches the threshold of economic threat.

Conclusion

In the present study, the etiology and epidemiology of phytoplasma bacteria, one of the dangerous pathogens of agriculture and production, are evaluated. In Turkey, the new host of the tomato plants, '*Ca*. Phytoplasma australasia' has been detected using PCR-based RFLP approaches and phylogenetic analyzes based on the 16s rRNA gene of the relevant pathogen. This is the first report of '*Ca*. P. australasia' (16SrII group/D subgroup) associated with Witches'-Broom of tomato in Turkey. The outputs of this study will shed light on the epidemiology of phytoplasma diseases in Turkey.

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Research Article

Heat Stress Response of Bread Wheat Genotypes Under High and Low Rainfall Environments

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Abstract: The research was carried out to determine the yield, yield components, quality, and stability of the bread wheat *(Triticum aestivum* L.*)* genotypes in heat-stressed and water-limited environments for two years. ANOVA and GGE biplot analysis were applied to determine the differences and relationships of investigated traits belonging to 10 different wheat varieties. While grain yield (GY) is associated with thousand-grain weight (TGW) and test weight (TW); protein ratio (PR) was found to be positively correlated with heading time (HT) and the number of spikes per square meter (SN). Besides, spike weight (SW) was found to be negatively correlated with PR, HT, and (SN). The biplot graph showed that PC1 explained 83.67% of the variability and the proportion attributed to PC2 was 16.33%. The cultivar of Kate A-1 was the most stable genotype, according to the biplot graph and it could be visually determined from the biplot graph in PC1 and PC2 together to explain 100% of the variability. In terms of the quality characteristics examined, Tahirova 2000 passed other varieties. It was concluded that Kate A-1 and Anapo varieties can be used for grain yield-oriented breeding studies, while Tahirova 2000 and Karatopak varieties can be used as parents for quality purposes.

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1. Introduction

Since wheat is of great importance in human nutrition worldwide, one of the most important targets of wheat breeders is to increase the grain yield (GY) obtained per unit area (Aktas, 2016; Barutcular et al., 2017; Yildirim et al., 2018; Kizilgeci et al., 2019a). Optimizing agronomic applications and developing varieties with high yield capacity are two main factors in increasing GY (Aktas, 2016; Güngör and Dumlupınar, 2019; Yüce et al., 2022). Different statistical methods are used in the evaluation of genotypes according to the data obtained in breeding programs. The responses of genotypes to different environmental conditions may be different. The GGE (genotype, genotype x

environment) biplot model used to determine the response of genotypes under different environmental conditions provides an advantage in interpreting data with visual graphics.

Genotype x environment interaction (GEI) is one of the important arguments to evaluate the stability of genotypes for plant breeders. Especially, determining the relationship between the performance of genotypes and ecological factors has been an important research subject of plant breeders and geneticists (Yan, 2001). Information about GEI is obtained through trials established in different ecological conditions. In addition, the yield performance of genotypes in different ecological conditions was determined by stability analyses (Kilic et al., 2003; Aydemir et al., 2019).

Yield is a complex feature that is directly or indirectly linked to many agricultural characteristics and is significantly affected by environmental factors. Therefore, growers prefer high-yielding and also stable varieties to ensure yield (Kendal and Dogan, 2015; Aydemir et al., 2019). The grain quality obtained from the unit area in bread wheat is also greatly important as well as the GY. Protein ratio (PR) and protein-related quality characteristics are among the important factors that make up the quality of wheat. PR is considered very high for 14-17%, 11-14% high, and 10-12% medium according to the change limits. In addition, it has been reported that PR is significantly affected by environmental effects (Grausgruber et al., 2000).

This study aims to test some different bread wheat varieties in terms of adaptability in high rainfall and drought-stressed conditions, and to determine the varieties that are superior in terms of GY, stability and other (Tables 4 and 5) examined characteristics.

2. Material and Methods

This research was carried out in the 2012-2013 and 2013-2014 growing seasons under the condition of Diyarbakır province of Türkiye (Figure 1).

Figure 1. Map of Türkiye showing the experiment area.

In the research, winter (Pehlivan and Tanya), alternative (Kate A-1), and spring type (Karatopak, Ceyhan-99, Nurkent, Cemre, Anapo, Tahirova 2000 and Dariel) bread wheat *(Triticum aestivum* L.*)* varieties were used.

Experiments were designed according to randomized complete blocks design in three replications using 10 bread wheat varieties. Seeds were planted (in the first week of november) with a 6-row parcel drill and 450 seed/m^2 sowing norm. Each parcel was sowed as 7.2 m^2 (1.2 m x 6 m) and 6 m^2 was harvested (1.2 m x 5.0 m). Harvest processes were done between 10-25 June.

In the trials, 60 kg ha^{-1} nitrogen (N) + 60 kg ha^{-1} phosphorus (P_2O_5) fertilizer was applied over the pure material at the planting, and 60 kg ha^{-1} N top fertilizer over the pure substance during the tillering period. A spraying procedure was done to control weeds (narrow and broadleaf weeds) and harvest was carried out with Hege 140 parcel combine harvester.

2.1. Climate properties of the experiment area

Growing day and degree day, precipitation, average temperature and maximum average temperature values based on growth stages for the 2012-2013 and 2013-2014 growing seasons, are given in listed (Table 1).

Defining the climatic conditions that occurred during the years of the experiment according to the critical plant development periods will facilitate the evaluation of the genotype performances. The average precipitation in the wheat development period in the first year is above the average for long years. The average temperatures were higher than the average for long years except for the tillering and-stem elongation stages. The first year of the experiment was defined as a high-temperature stress environment since the average maximum temperatures were also high during the entire development period. Since drought stress was not observed in any growing stage, genotypes were exposed to high heat stress this year. High temperature is one of the most important climatic factors affecting the yield and growth of the plant, and the exposure of field crops (wheat, barley, corn, etc.) to fluctuating high temperatures significantly affects plant metabolism (Saleh et al., 2007; Gürsoy et al., 2012).

Table 1. Rainfall and temperature values according to plant growth stages during two years of experiment

	Number of days		Total of Temperature (degree-day)		Precipitation (mm)			Average Temperature (°C)			Average Max Temperature (°C)		
Plant growth stages	**2013**	**2014**	**2013**	**2014**	**2013**	**2014**	**Long Years**	**2013**	**2014**	**Long Years**	**2013**	**2014**	**Long Years**
Sowing-Emergence (GS00-10)	20.0	21.0	170.1	156.2	66.8	37.4	61.6	7.7	7.1	6.6	12.6	7.4	6.4
Emergence-Tillering (GS10-20)	18.5	38.9	77.1	25.8	94.0	30.8	69.7	4.5	0.7	2.9	8.8	3.7	2.6
Tillering- Stem elong. (GS20-30)	47.5	43.5	192.2	292.2	147.2	96.6	68.4	3.9	6.1	4.5	8.7	12.6	5.3
Stem elong.-Heading (GS30-50)	67.5	36.0	698.2	434.3	79.4	44.0	68.3	10.4	11.4	8.5	16.2	17.9	11.2
Heading- Phys. Matur (GS50-90)	57.5	50.5	1253.3	984.6	100.8	88.7	55.0	21.2	18.2	19.8	29.5	26.0	19.8
Total Average	211.0	189.0	2390.9	1893.1	488.2	297.5	323.0	9.54	8.70	9.04	15.16	13.52	9.08

GS: determined according to the Zadoks growth scale (Zadoks *et al*. 1974).

In the second year, it is seen that the total precipitation in the development period is a little lower than the average precipitation for long years. Precipitation after the tillering stage is sufficient and higher than average for long years. The low rainfall from the sowing to the end of tillering stage shows that plants are exposed to early drought stress. When the plant loses water for any reason (drought, etc.), turgor pressure drops suddenly at first. Since drought stress will have a negative effect on the growth cells of the plant, there will be losses in germination ability due to the dispersion of membrane proteins and the decrease in chlorophyll content (Jaleel et al., 2009; Gürsoy et al., 2012). High temperatures occurring between the heading and physiological maturity period indicate that high-temperature stress is experienced in this period, in which grain yield is mainly determined. Long-term exposure to minus temperatures from emergence to starting of tillering indicates that cold and frost stress are experienced. In the second year, sudden frost damage was experienced between stem elongation and the heading stage and it caused necrosis in plants. When the plant is exposed to cold stress, the permeability of stem cell membranes is impaired. Even if there is water in the soil, water cannot be taken from the soil by the plant due to its low or lack of fluidity. If cold stress conditions continue for a long time, the leaves turn yellow as the first symptom and then the plants die (Taulavuori et al., 2005; Gürsoy et al., 2012).

When the second year is evaluated in general, it is seen as an environment where drought and cold stress are experienced in the early growing stages and moderately high-temperature stress is experienced in the later stages. The first year of the experiment was evaluated as high rainfall and high temperature stressed environment in the period when the yield and yield factors were determined, and the second year was considered a drought and cold stress environment for early growing stages (Table 1). On the other hand, the results of the analysis of the soil samples belonging to the field experiment

area are given in listed (Table 2). The soil structure of the experiment area is clay-loam and slightly alkaline and has low organic material.

Table 2. Soil traits properties of the research experimental area

Soil Class	Total Salt (%)	pH	Lime $CaCO_3$ (%)	Phosphorus P_2O_5 (kg/da)	Organic Matter (%)	Saturation with Water (%)
Clay loam	0.245-0.246	7.75-7.81	6.26-8.50	1.28-1.30	0.676-0.680	77-65

Source: Anonymous (2014).

2.2. Data collection procedures for the investigated traits

GY of each parcel was determined by weighing the wheat grains obtained after harvesting the whole parcel on a 0.01 g scale and converting it to kg ha^{-1} (Pask et al., 2012). Heading time (HT) is determined as the days between the emergence and half of the spike (GS55) visible in 70% of the plants in each plot (Bell and Fischer, 1994). As a method in yield components, Kirtok et al. (1988)'s method has been taken into account. The number of spikes per square meter (SN): It was determined by counting the spike per square meter in each plot. The number of grains per spike (GN): The grains in 10 spikes taken from each plot were counted separately and averaged. Spike weight (SW): 10 spikes taken from each plot after physiological maturity were weighed separately on a 0.001 g scale. Then, the average weight of the spike was determined. Thousand grain weight (TGW): 1000 wheat grains representing each parcel were determined by weighing with a precision scale of 0.001 g (Pask et al., 2012).

Test weight (TW) and protein ratio (PR) were determined using the Near Infrared Model 6500 device (Anonymous, 1990).

2.3. Statistical analysis

The effects of genotype and environment on investigated traits were tested by using ANOVA. Genotype-traits and stability biplot graphics in visual properties were created using the GenStat 12th Edition program (GENSTAT, 2009). The differences between the means for each trait were examined by the least significant difference (LSD) test ($p < 0.01$ and $p < 0.05$) (Gomez and Gomez, 1984).

3. Results and Discussion

The ANOVA analysis revealed statistically significant differences in the mean GY and other traits ($p \leq 0.01$ and $p \leq 0.05$) (Table 3).

Table 3. ANOVA results for investigated traits, mean squares and significance levels of each variable

		Mean Squares							
Resources	DF	HT	SN	GN	SW	GY	TW	TGW	PR
Y	1	4352**	1100^{NS}	376**	18.7**	2138560**	214.4**	2325**	676**
R[Y]	4	3.8	1654	46.8	0.1	11313	0.5	6.3	1.8
C	9	49.4*	5855**	118.9**	0.3*	12245.7**	16.3**	45.7**	3.2^{NS}
Y x C	9	1.7^{NS}	4788**	58.1*	0.2^{NS}	4279.3^{NS}	3.5^{NS}	3.2^{NS}	0.9**
CV(%)		0.7	5.1	10.7	12.6	15.1	1.8	5.9	6.6
LSD (0.05)		1.2	44.7	5.8	0.4	73.9	1.7	2.2	1.1

R: Replication, Y: Year, C: Cultivar, CV: Coefficient of variation, DF, Degree of freedom, HT: Heading time, SN: Number of spike per square meter, GN: Number of grain per spike, SW: Spike weight, GY: Grain yield, TW: Test weight, TGW: Thousand-grain weight, PR: Protein ratio, NS: not significant, * Significant level, P<0.05, ** Significant level, P<0.01.

Genotype x environment interaction has been reported to be effective on grain yield, test weight, and thousand-grain weight in studies of bread wheat (Beleggia et al., 2013; Rozbicki et al., 2015; Sakin et al., 2015). Although different results were obtained in this study, it was seen that the YxC interaction was significant in the yield components (SN and GN) and the PR. This shows that cultivars are affected at different levels by environmental conditions in terms of SN, GN, and PR properties.

Table 4. Mean values of the first and second year of trial for the trait of HT, SN and GN

Cultivars	HT (day)			SN (spike m^{-2})			GN (grain $spike^{-1}$)		
	2013	2014	Mean	2013	2014	Mean	2013	2014	Mean
Karatopak	136.67	119.67	128.17	470.00	456.67	463.33	53.20	44.80	49.00
Ceyhan-99	136.00	117.67	126.83	446.67	443.33	445.00	48.50	47.50	48.00
Nurkent	135.33	116.67	126.00	371.67	441.67	406.67	47.70	49.70	48.70
Pehlivan	136.67	120.33	128.50	403.33	463.33	433.33	37.20	35.50	36.30
Cemre	136.67	121.33	129.00	445.00	391.67	418.33	48.50	42.90	45.70
Anapo	128.67	112.00	120.33	352.33	396.67	374.50	46.00	40.50	43.20
Tanya	138.67	121.33	130.00	486.67	428.33	457.50	49.80	39.30	44.60
Tahirova 2000	138.67	122.33	130.50	450.00	341.67	395.83	44.50	46.00	45.30
Dariel	134.67	118.33	126.50	393.33	365.00	379.17	49.50	47.20	48.30
Kate A-1	135.67	117.67	126.67	431.67	436.67	434.17	62.30	43.80	53.00
Means	135.77	118.73	127.25	422.85	416.50	420.78	48.7	43.7	46.20
CV (%)	0.6	0.9	-	9.1	8.9	-	10.2	11.3	-
LSD (0.05)	1.6**	1.8**	-	67**	63.9**	-	8.5**	NS	-

HT: Heading time, SN: Number of Spike per square meter, GN: Number of grains per spike, ** Significant level, P<0.01.

The heading time in the first year was prolonged due to the high precipitation during the tillering-heading stages and also because tillering- stem elongation period was cooler than the mean of long years. In the study, it was determined that the earliest cultivar was Anapo, and Tahirova 2000 was the latest heading time. In early growing genotypes, because of the long grain filling time, more dry matter accumulates in the grain and causes to increase in GY (Sharma, 1994).

Temperature stress in the location where the experiment is conducted is one of the important abiotic stress factors that limit GY. Early heading genotypes have the advantage of providing stable GY through the stress-escaping mechanism in the years when the high-temperature stress is observed. Due to the above-mentioned reasons, the high yield potential of the "Anapo" cultivar may be due to the earliness feature. Although there were differences in precipitation and temperature in both years during the development stage in which the number of the tiller is determined, the total number of the spike was similar. This case showed that there was a high genetic effect in the formation of spike numbers related to tillering. It had been reported that the number of spikes per square meter was influenced by genetic and environmental factors (Sakin et al., 2015).

The number of spikes per square meter ranged between 374.50-463.33 spikes m^{-2}. Karatopak cultivar had the highest number of ears per square meter (463.33 spikes m^{-2}). It had been reported that spike density per unit area in wheat was one of the most important yield components that determine GY (Kadum et al., 2019). The number of grains in wheat is potentially determined starting from the stem elongation and reaches its final limit during the flowering period. The decline in grain number potential in the second year was caused by drought during stem elongation and heading stages and the shortening of these stages (Table 4). To guarantee the number of grains, which are important yield factors, it will be beneficial to make irrigation when drought occurs in the pre-heading period. Exposure of plants to chilling stress due to sudden temperature drop at the booting stage in the 2nd year of the experiment was able to damage the flower primordium and decrease the number of grains.

The GN varied from 36.30 to 53.00 among genotypes and Kate A-1 had the highest GN. GN ranged from 31.20 to 44.90 in a study conducted in Türkiye (Aydogan and Soylu, 2017). Although the varieties used in our study were different from theirs, close values were obtained in GN. Depending on the drought season in the 2nd year, a drastic decrease in GN of Kate A-1, Tanya, and Karatopak cultivars negatively affected the yield potential. Pehlivan and Ceyhan-99, which had stable grain yield in the environment in which the study was carried out, had similar GN in both years, indicating that grain number is important in genotype stability.

There was a significant difference between the varieties for SW and it ranged between 2.00-2.70 g, while the Cemre cultivar (2.70 g) had the highest value (Table 5). Spike weight (SW) was found to be significantly higher in the first year due to high precipitation and humidity. In the first year, the time between stem elongation and physiological maturity was as long as 40 days compared to the 2nd year, which led to both good developments of spike before heading time and high dry matter

accumulation at grain filling duration. Despite the high temperature occurring throughout the whole season in the first year, the high production of the dry matter showed that wheat in high rainy conditions turned the temperature increase into an advantage.

In the second year, in which the growing stages were shortened and early period drought stress occurred, SW decreased by approximately 40%. The highest reduction in SW was observed in the Kate A-1 cultivar with 50%. The most resistant cultivar was the Nurkent with a 22% reduction. Test weight (TW) ranged from 77.00-82.47 kg hl^{-1} and the highest test weight was obtained from Tahirova 2000 (82.47 kg hl^{-1}) cultivar. In the study conducted by Karaman et al. (2017) with bread wheat at the same location as our study, similar results were obtained with this study and it was reported that TW varied between 78.2 and 82.7 kg hl^{-1}. TGW, ranged from 28.91-38.37 g among cultivars, and Pehlivan (38.37 g) had the highest TGW (Table 5). Considering that TGW is largely under the control of genetic factors, it would be more useful to examine the decreases in the stressful year instead of grading the varieties from large to small. In the 2nd year, there was less reduction in TGW in comparison to SW and the last decrease was in the Nurkent cultivar. In the second year, the Nurkent cultivar had the lowest decrease for SW and GN characteristics as well as TGW, and it can be accepted as a strong stable cultivar for spike characteristics.

Table 5. Mean values of the first and second year of trial for the trait of SW, TW, TGW and PR

Genotypes	SW (g)			TW (kg hl^{-1})			TGW (g)			PR (%)		
	2013	2014	Mean	2013	2014	Mean	2013	2014	Mean	2013	2014	Mean
Karatopak	2.80	1.87	2.30	83.19	79.46	81.33	35.42	22.67	29.04	11.45	18.28	14.87
Ceyhan-99	3.00	1.54	2.30	82.74	75.77	79.26	38.08	25.00	31.54	10.45	17.26	13.86
Nurkent	2.90	2.27	2.60	82.81	79.65	81.23	37.67	26.67	32.17	10.05	15.97	13.01
Pehlivan	2.70	1.81	2.20	82.80	81.02	81.91	45.92	30.83	38.37	10.75	16.93	13.84
Cemre	3.20	2.20	2.70	82.95	79.53	81.24	39.83	25.75	32.79	10.86	17.82	14.34
Anapo	3.00	1.75	2.40	83.95	80.44	82.19	38.75	26.00	32.38	10.28	15.97	13.13
Tanya	2.50	1.46	2.00	79.57	74.43	77.00	34.00	23.83	28.91	10.59	18.15	14.37
Tahirova2000	2.80	1.94	2.40	83.55	81.39	82.47	38.33	27.00	32.67	11.00	19.20	15.10
Dariel	3.20	1.85	2.50	82.24	77.40	79.82	35.42	23.17	29.30	10.94	17.64	14.30
Kate A-1	3.40	1.71	2.60	82.10	78.98	80.54	36.92	24.92	30.90	9.96	16.28	13.12
Means	2.95	1.84	2.40	82.59	78.81	80.70	38.03	25.58	31.81	10.63	17.35	13.99
CV (%)	9.5	17.6	-	0.8	2.5	-	5.5	6.5	-	8.8	5.3	-
LSD(0.05)	0.4*	NS	-	1.1**	3.3**	-	3.6**	2.9**	-	NS	1.6**	-

SW: Spike weight, TW: Test weight, TGW: Thousand grain weight, PR: Protein ratio, Av.: Average, NS: not significant, ** Significant level, $P<0.01$.

TGW is one of the important technological quality parameters as well as being the main component of GY. Our TGW findings were in line with the study, which ranged from 25.49 g to 37.51 g, and were carried out under similar conditions (Kizilgeci et al., 2019b). The PR is one of the important quality parameters for flour and pasta making. The PR of cultivars ranged from 13.01 to 15.10% and Tahirova 2000 cultivar had the highest value (15.10%). The PR of a study conducted in Diyarbakir conditions of Türkiye had been reported to vary between 14.36 and 16.48% (Kizilgeci et al., 2019b). Their results are similar to those obtained in our study. Although the PR has generally high heritability (Mckendry et al., 2011), this feature is highly affected by environmental factors such as soil nitrogen content, location of the field and climate components. Significant differences were observed for GY, between the varieties at $p\leq0.01$ level and it changed between 3414-4638 kg ha^{-1} (Fig 2). Kate A-1 cultivar gave the highest GY (4638 kg ha^{-1}). The GY of bread wheat is a complex feature and is under the influence of genetic and environmental conditions. GY is considered the most basic feature used directly in the selection of genotypes in breeding programs (Forgone, 2009; Kizilgeci et al., 2019b).

3.1. Evaluation of the features examined with The GGE biplot model

The relationship between cultivars and examined traits is given visually (Figure 3). The figure is interpreted as follows; there is a positive correlation if the angle between the vectors of the two traits

is less than 90^0, negative if greater than 90^0, and no relationship if equal to 90^0. In addition, if the vector of one trait is longer than the other vectors, the variation between the genotypes is high in this traits, and if the vector is short, the variation among the genotypes is low (Yan et al., 2000; Yan and Tinker, 2006; Kendal et al., 2019). According to this explanation, there is a strong positive relationship among GY, TW and TGW; among PR, SN and HT and a negative relationship between TGW and GN (Figure 3). The long vectors of TGW, SW and GN mean that there is a large variation between the varieties for these properties (Figure 3). Variation between varieties is low in GY, SN, TW, PR and HT properties with short vectors. The relationship between variety and traits represents 60.67% of the total variation (Figure 3 and 4). Kate A-1, Pehlivan, Anapo and Nurkent varieties were associated with GY, Pehlivan with TGW, Tahirova 2000, Karatopak and Tanya with PR (Figure 3 and 4).

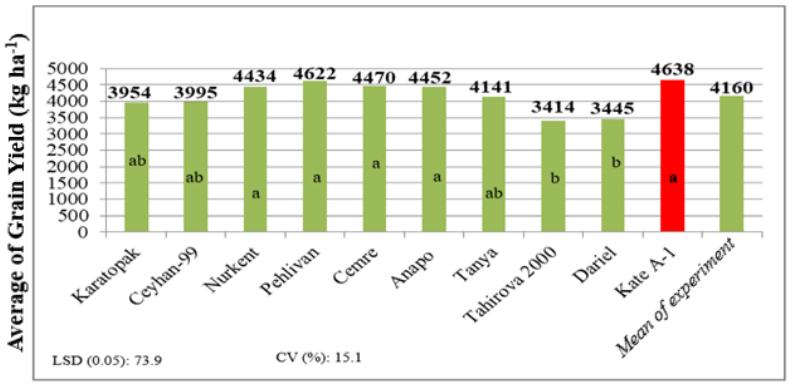

Figure 2. Average grain yield of varieties over two years of experiment results.

3.2. Polygon view of the GGE biplot

Genotypes at the top of the sectors (on the diagonal of the polygon) in the GGE biplot polygon graph are the most preferred genotypes of that sector (Yan and Tinker, 2006; Erdemci, 2018).

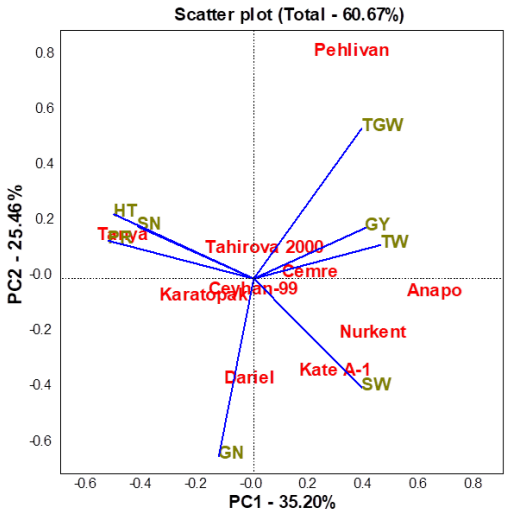

Figure 3. GGE biplot graph showing the cultivar traits relationship.

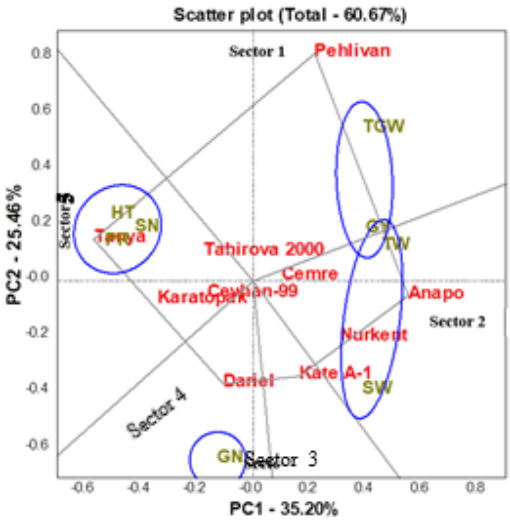

Figure 4. Which-won-where for cultivar and traits.

The cultivars in the diagonal of the polygon in the sector are considered to be a good cultivars in terms of properties close to the diagonal. According to the rule of being on the diagonal of the polygon; Pehlivan was the best variety for TGW and GY properties, Anapo varieties for GY and TW properties, and Tanya varieties were the best varieties for HT, PR and SN properties (Figure 4). According to the GGE biplot graph, five different sectors were created and they are grouped in a blue circle (Figure 4). The traits in the same sector are positively related to each other. According to the sectoral evaluation, TW contributed the most to the increase in grain yield.

3.3. Mean performance and stability of genotypes

In this study, according to the ranking biplot graph, PC1 represented 83.67% of the total variation, PC2 16.33% and both (PC1 + PC2) 100% (Figure 5). Genotypes showing high PC1 and low PC2 (close to zero) had been reported to be highly efficient and stable genotypes (Yan and Hunt, 2001). Accordingly, the biplot graph (Figure 5 and 6), consisting of PC1 and PC2 components, were sufficient to clearly determine the grain yield stability of cultivars and ideal genotypes. If a genotype is to the right of the PC1 axis, the grain yield is above the experimental mean, whereas the grain yield of the genotypes to the left of the axis is lower than the mean of the experiment. In addition, while the varieties close to the stability line are evaluated as stable varieties, the varieties that are far from the stability line are considered as a variable (unstable) for grain yield (Figure 5). According to this evaluation, Kate A-1 had both high grain yield and high stability (Figure 5).

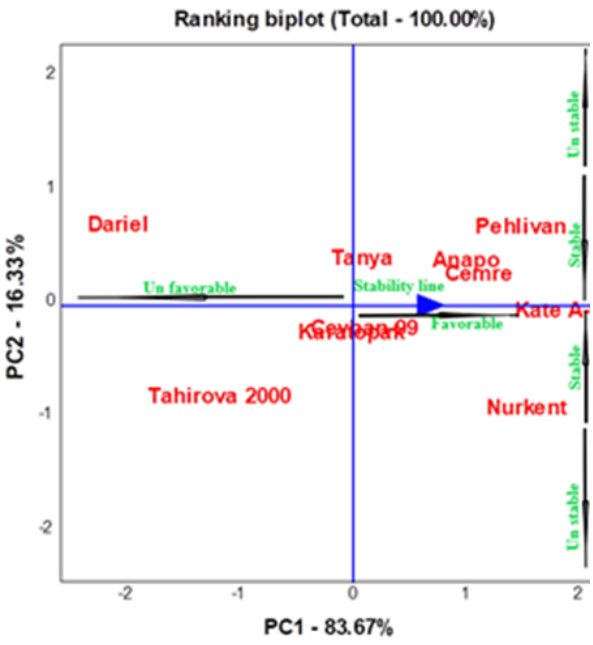

Figure 5. Grain yield stability graph with a ranking biplot.

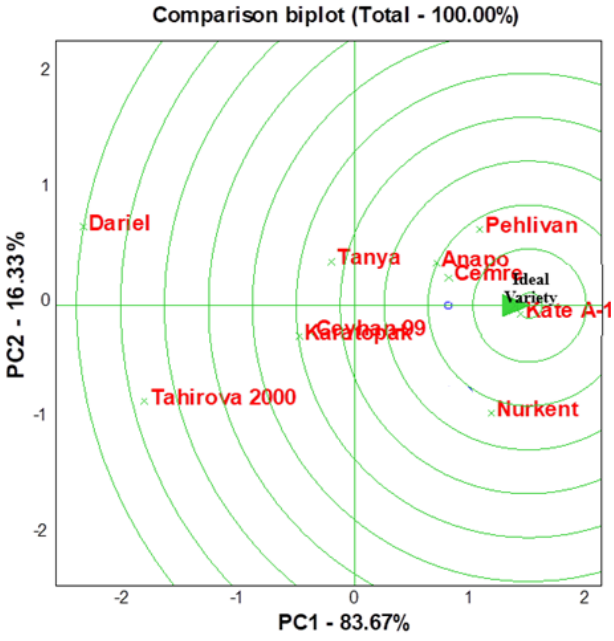

Figure 6. Presentation of the ideal genotype with the comparison biplot graph.

Different methods are used by researchers to determine the stability of genotypes. However, it is stated that the GGE biplot model shows the adaptation of genotypes to different environments easily and more understandably (Hassanpanah, 2011; Mortazavian et al., 2014). In addition, the grain yields of Tanya, Ceyhan-99, Karatopak, Tahirova 2000, and Dariel varieties were below the experiment mean and classified as undesired varieties.

3.4. Evaluation of genotypes based on the ideal genotype

The ideal genotype is defined as the most stable cultivar that has the highest grain yield in test environments and whose yield does not vary much from environment to environment (Yan and Kang, 2003). In Figure 6, there are many circles with the same centers. The cultivar in the smallest circle in the center of these circles is the ideal cultivar. Therefore, the varieties close to where the ideal cultivar is located are considered desired varieties. On the contrary, the suitability of varieties for any environment decreases as they move away from the central circle. When Figure 6 was evaluated, it was seen that the Kate A-1 cultivar was located very close to the center of the first circle. This showed that Kate A-1 was the most ideal cultivar compared to other varieties in terms of grain yield.

Conclusion

It was determined that Kate A-1, Pehlivan, Anapo, and Nurkent varieties had high yield potential. In addition to the highest grain yield, Kate A-1 was the most stable cultivar. Anapo cultivar was suitable for environments where terminal heat stress is experienced due to the mechanism of escaping from heat caused by earliness. Although the grain yield of Tahirova 2000 was below the experiment mean, it had the highest grain quality. It has been concluded that Kate A-1 will be the most suitable cultivar for the producer in terms of grain yield, and it will be beneficial to use Kate A-1 and Tahirova 2000 varieties as a parent in breeding programs for grain yield and quality improvement, respectively.

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Research Article

Land Quality Index for Paddy (*Oryza sativa* L.) Cultivation Area Based on Deep Learning Approach using Geographical Information System and Geostatistical Techniques

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Precision agriculture

Abstract: Türkiye has ideal ecological conditions for growing rice, and its yield per hectare is often higher than the average worldwide. However, unbalanced fertilization, nutrient deficiency, and irrigation problems negatively affect paddy production when soil characteristics are not considered. The present study was conducted on a 1763-hectare field (652000-659000E-W and 4528000-4536000N-S) in 2019. This study's primary goal was to categorize land quality for rice production using 15 different physicochemical parameters and a GIS (Geographical Information Systems) and deep learning (DL) technique. Using these parameters soil types were classified and regression analysis was performed by DL. Different soil parameters as network outputs used in this study caused different performance levels in models. Therefore, different models were suggested for each network output. The R^2 values indicated a respectable level for parameter prediction, and an accuracy of 88% was attained when classifying "class" data. The findings of the study demonstrated that deep learning may be used to forecast soil metrics and distinguish between different land quality classes. Additionally, a field investigation was used to validate the indicated land quality classifications. Using statistical techniques, a substantial positive link between rice yield and land quality classes was discovered.

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1. Introduction

Rice, a warm climate grain (*Oryza sativa* L.) is the main food source for 50% of the world's population (Sirat et al., 2012; Akay et al.,2017). Although rice cultivation is conducted in all

geographical regions in Türkiye, 56.0% of the total rice area is in the Thrace-Marmara region, 36.5% in the Black Sea region, and 7.5% in other regions (Meral and Temizel 2006; Garris et al., 2005)

Considering different scenarios derived from climate change models, food security is the most pressing issue in densely populated developing countries (Jagadish et al., 2007). A great effort has been exerted to meet the nutritional needs of the growing population in developing countries in terms of achieving consistently high yield rates (Araus and Cairns, 2014). In addition, the specification and classification of plant diseases are one of the most vital methods for early-stage intervention to increase yield (Shrivastava et al., 2019). It also ensures the ecological sustainability of soil types, which is one of the important components in both the economic and terrestrial ecosystem, as well as the use of produce considering land needs, its management, yield, and increase in quality. Therefore, important studies have been conducted on soil and land quality index approaches in recent years. The main characteristics of the alluvial land and soils which are widely classified as fluvent sub order of the Entisol order often show large variations in their features such as textural or organic matter distribution over short distances (Dengiz 2010). Identifying land quality is actually a difficult process. The reason for the complex relationship is between the physical, chemical, and biological properties of the soil and other factors. Many studies have been conducted to search the relationships between physical, chemical, and biological properties of soil types and yield (Dengiz 2013; Li et al., 2018; Dedeoğlu and Dengiz, 2019; Mwendwa et al., 2019; Rezaee et al., 2020). While it is possible to assess land quality by directly conducting land testing, several modal approaches such as comparative assessment, dynamic assessment, and land quality index (LQI) can be used indirectly. Since direct approaches are generally expensive, labor-intensive, and time-consuming, modal approaches are used more often (Dengiz 2020). Land Quality Index(LQI) approach was used in the rice land assessment. In this approach, the land quality index assessment process, which usually starts with the creation of a data set, is graded by giving score ratios according to the severity of limiting plant growth on indicators with different units. In addition, the possibility of the deep learning system, which had never been used before in rice land quality studies, was investigated in this study.

Deep learning is a modern and popular technique for image processing and data analysis with promising results and great potential. Deep learning, which has been successfully applied in various fields, has recently been used in precision agriculture applications (Kamilaris 2018). To give an example of these studies, computer-aided diagnosis (CAD) systems, using AI (Artificial Intelligence), were used in order to accurately identify diseases and pests affecting small farmers' production and also to help understand the severity of symptoms, as well as allowing any farmer with access to a smartphone to benefit from expert knowledge in a practical and cost-effective manner (Esgario et al., 2020).

Azizi et al. (2020) used a convolutional neural network (CNN), a deep learning method, to classify soil clusters while they used VggNet16, ResNet50, and Inception-v4 trained models to train CNN. Esgario et al. (2020) used deep learning to classify biotic stress in coffee and to estimate its severity. The ResNet50 produced a high accuracy rate of 95.24%. Padarian et al. (2019) used deep learning to predict soil properties from regional spectral data and this study in which they used CNN showed that it could be reduced by 87% compared to predicting soil properties (PLS), a traditional method. in deeper soil layers with a high accuracy rate.

The decrease in land quality due to intensive rice cultivation threatens the sustainability of rice agriculture in the Çorum-Osmancık region of Türkiye. In the present study, we focused on identifying detailed rice land quality classes and mapping their spatial distributions in order to perform sustainable rice agriculture practices. The possibility of using the deep learning technique, accompanied by geographic information systems and geostatistics to determine the land quality classes for rice production has been investigated in this study. The identification of land quality classes has also been validated with data collected from field studies.

2. Material and Methods

2.1. Study Area

The study area is located within the boundaries of Çorum- Osmancık district, in the Kızılırmak Valley of the Western Black Sea region, and between the coordinates 652000-659000E-W and 4528000-

4536000N-S (WGS84, Zone 36 UTM-m). The study area covers approximately 1,763 ha and is between 399-480m above sea level (Figure 1).

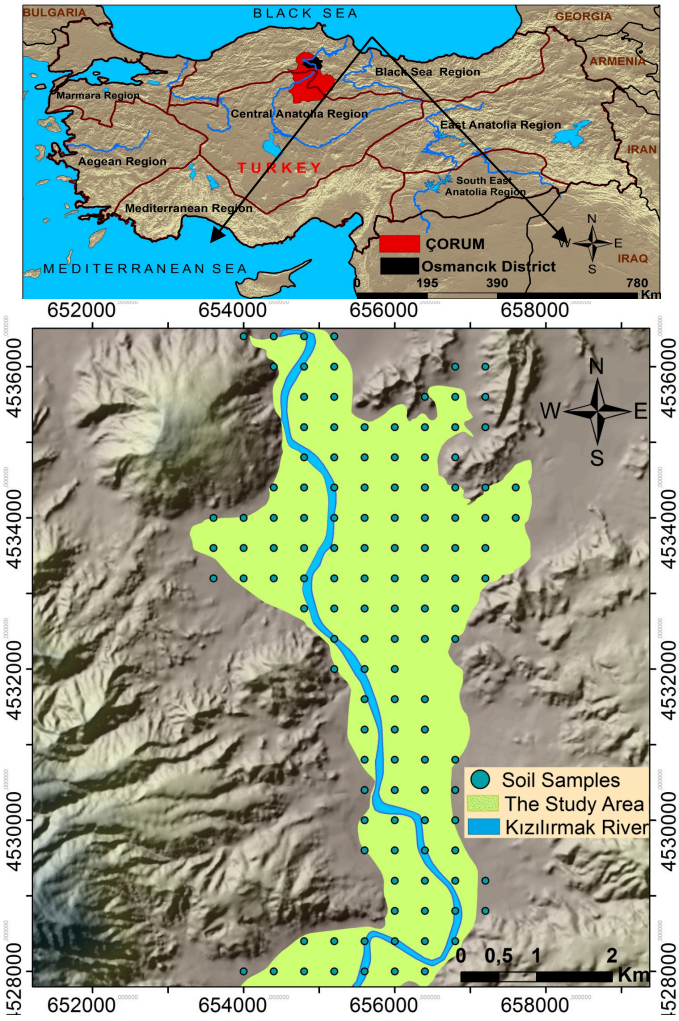

Figure 1. Soil sample pattern and location map of the study area.

The study area is located in a transition area between the Black Sea and Central Anatolian climate regimes and falls into the semi-arid climate class. The physico-chemical properties of the study area were assessed in terms of the coefficient of variation (CV) which clearly indicated that the soil properties were highly variable.

The region is surrounded by Ilgaz Mountains, which extends through the east-west direction, from the west, and by its extensions and Koroglu Mountains from the south. The geological structure of these mountainous areas in the region is generally composed of Paleozoic metamorphic rocks. The wide valley bottom plains through which Kizilirmak (the Halys river) flows, make up alluvial deposits belonging to the Quaternary period. Generally, rice is grown on soil formed on these alluvial deposits. The study area is mostly flat or slightly inclined (0.0-2.0%). A total of eight soil series have been identified in the study area. Dengiz et al., (2009), defined 29 mapping units according to the digital soil map they created (Figure 2).

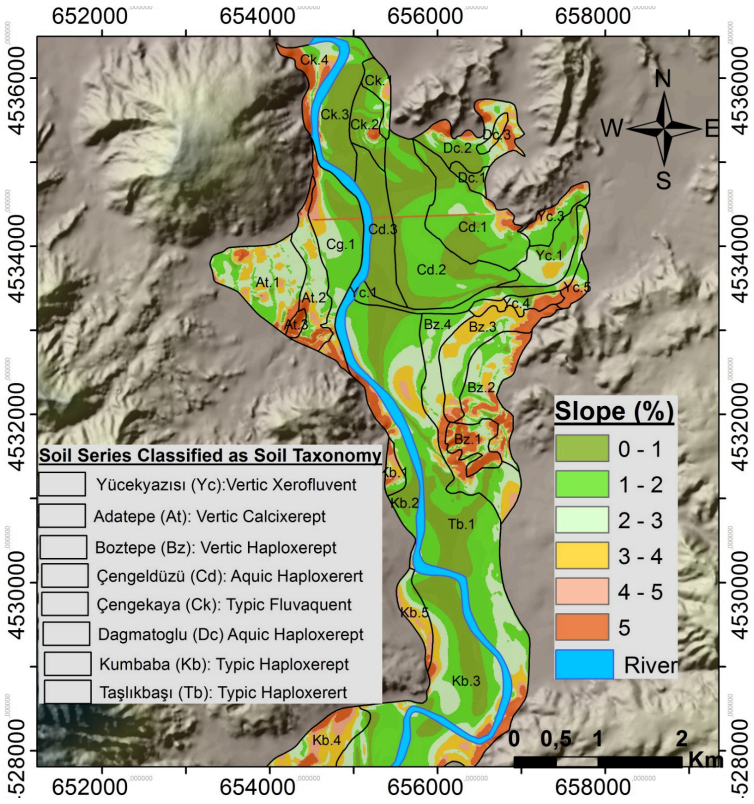

Figure 2. Slope and soil map of the study area.

2.2. Soil sampling and indicator selection

In this study, a total of 246 soil samples, disturbed and undisturbed sampling from the surface (0-30 cm), with distributed soil types of Vertiso, Entisol, and Inceptisol were collected from the grid points (400 m x 400 m) created. Soil samplings were conducted especially in the autumn after the harvest, in order to avoid the effects of soil management processes such as fertilization and irrigation during the rice-growing period. Each mapping unit (land mapping units) defined with its unique soil and land properties significantly affects the suitability of the determined land utilization type to the land. Therefore, it is necessary to identify the land needs of each land utilization type for a successful and sustainable agricultural practice.

The land utilization type investigated in this study is rice. Some literature sources were examined in order to identify the land needs of rice and soil physicochemical and topographic indicators required for the model (FAO, 1983 and 1985; Sys et al., 1993, Mongkolsawat et al., 2002, Bunting, 1981; Dengiz, 2013; Sezer and Dengiz, 2014; Dengiz et al., 2015; Nath et al., 2016). The development of the rice plant depends on the physical and chemical conditions of the soil type that affect the plant's root system and affects ability to grow efficiently. Therefore, Moron (2005) stated that the indicators used in soil quality identification should be sensitive enough to track changes and be easily measured and interpreted. Fourteen quality parameters fall into two different main categories for the rice land quality index model (LQIR). These are i-(Nutrient Availability Index (NAI) (including nitrogen, phosphorus, potassium, and zinc content in the soil), ii-) soil quality index (SQI) (including slope, soil depth, bulk density, clay, silt, sand, hydraulic conductivity (HC), organic matter, electrical conductivity (EC), lime-CaCO3, and soil reaction-pH (Table 1). Table 1 shows the analytical protocols used.

Table 1. Analytical Protocol measurements for indicators

Indicators	Unit	Protocol	Reference
Soil quality indicators			
Soil Depth	cm	From soil map	Dengiz et al. (2009)
Slope	%	From DEM	Dengiz et al. (2009)
BD	gr cm^{-3}	Undisturbed condition	Blacke and Hartge, 1986
HC	cm h^{-1}	Undisturbed and saturated condition	Oosterbaan (1994)
Texture (Clay, Silt and Sand)	%	hydrometer method	Soil Survey Staff (1996)
OM	%	wet oxidation method (Walkley-Black) with potassium dichromate (K2Cr2O7)	Nelson and Sommers 1982
pH	1:2.5	(w:v) soil-water suspension	Soil Survey Staff (1996)
EC	dS m^{-1}	(w:v) soil-water suspension	Soil Survey Staff (1996)
CaCO3	%	Scheibler calsimeter	Soil Survey Staff (1993)
Nutrient availability indicators			
NaHCO3–P	mg kg^{-1}	the molybdophosphoricblue method	Kacar B (2016)
Total N	%	Kjeldahl	Bremner and Mulvaney (1982)
NH4OAC–K,	mg kg^{-1}	Ammonium acetate extraction, flame spectrometry detection	Soil Survey Staff (1992)
DTPA–Zn	mg kg^{-1}	DTPA extraction, AAS detection	Kacar B (2016)

2.3. Land quality index and rating assignment

The rice plant likes soil that is deep, clayish, and rich in plant nutrients and organic matter, as well as being medium resistant to salt (Özkan et al., 2019).

Land quality indicators were used in the study area (Table 2). The identification of the rice land quality index consists of the nutrient availability index and soil quality index. The formula used to identify the nutrient availability index (Dengiz, 2013) is given below.

$$NAI(\text{Nutrient Availability Index} = N(\%) \times P(mg\ kg^{-1}) \times K\ (mg\ kg^{-1}) \times Zn(mg\ kg^{-1}) \quad (1)$$

It is used to identify soil quality index (SQI) (Gupta and Abrol, 1993) is shown below.

$$SQI = Cy \times Si \times Sa \times D \times F \times P \times G \times S \times K \times H \quad (2)$$

Where; Cy is clay, Si is silt, Sa is sand, D is soil depth, F is slope, P is bulk density, G is hydraulic conductivity, S is exchangeable sodium percentage (ESP), K is (CaCO3) content, and H is pH.

Table 2. Rating factors for indications of land quality for paddy cultivation

Land quality indicator			**Factor rating**			
	Diagnostic Factor	**Unit**	1.0	0.8	0.5	0.2
			NAI= N x P x K x Zn			
I. Nutrient Availability Index (NAI)	TN	%	>0.2	0.1-0.2	<0.1	-
	P	mg kg^{-1}	>25	10-25	<10	-
	K	mg kg^{-1}	>60	30-60	<30	-
	Zn	mg kg^{-1}	>0.7	0.7-0.5	<0.5	-
II. Soil Quality Index (SQI)			SQI = Cy x Si x Sa x D x F x P x G x S x K x H			
Texture	Clay-Cy	%	> 50	40-50	25-40	<25
	Silt-Si	%	<25	25-40	40-50	>50
	Sand-Sa	%	<30	30-45	45-50	>50
Depth (D)		cm	> 50	25-50	15-25	< 15
Topography (F)	Land form or Slope	%	Flood plain or 0-2%	Low terrace or 2-4%	Middle terrace or 4-6%	High terrace/ mountain or >6%
Bulk Density (P)	BD	gr cm^{-1}	<1,40	1,40-1,45	1,45-1,60	>1,60
Hydraulic conductivity (G)	HC	cm h^{-1}	< 0.5	0.5-2.0	2.0-6.25	> 6.25
Electric Conductivity (S) or ESP	EC	dS/m (%)	0-3.1 10	3.2-4 10-20	4.1-5 > 20	> 5.1 > 20
Organic matter (O)		%	>3	3-1,5	1,5-0,5	<0,5
Lime (K)	CaCO3	%	0-5	5-15	15-20	> 20
Soil reaction (H)	pH	-	5.5-7.3	7.4-7.8 5.1-5.5	7.9-8.4 4.0-5.0	>8.4 < 4.0

Each indicator was scored with a ratio value between 0.2 and 1.0. The results of the analysis on the indicator take a value of 1.0 if it has the most suitable condition for rice cultivation, and 0.2 if it has the most unfavorable condition. The indicator takes a value between 0.2 and 1.0 according to the severity of limiting rice growth. The spatial information of both descriptive indicators on the NAI and descriptive indicators on the SQI were obtained from land mapping units and surface soil samples. In order to identify the land quality index value for rice, the following formula was used (Dengiz, 2013; Sezer and Dengiz, 2014).

$$\text{LQIR (land quality index)} = \text{NAI} \times \text{SQI} \quad (3)$$

The above-mentioned formula was applied to each soil sample. As a result, the higher the point value is, the higher the suitability of land is for the specified Land Utilization Type. Rice land quality classification according to Dengiz (2013) is given in Table 3.

Table 3. Land quality index value for rice cultivation

Definition	Suitability Class	Land Quality Index Value
Highly Suitable	S1	1.00-0.250
Moderate Suitable	S2	0.250-0.100
Marginally Suitable	S3	0.100-0.025
Unsuitable	N	< 0.025

For the purpose of model verification, for each quality class in the study area, random blocks field trial pattern using 12 paddy varieties (Sumnu, Osmancik-97, Gonen, Beser, Duragan, Halilbey, 7721, Karadeniz, Kizilirmak, Koral, Negis, and Aromatik) was carried out for two years. In the experiment where the strewing planting method was applied, parcel yields were obtained by removing the edge effect so that the plot size was 4 x 4 = 16 m² and the harvest area was 3 x 4 = 12 m² (Sezer et al., 2017). ANOVA and LSD0.05 were performed for the grain yields. In addition to that, in order to gain values of basic descriptive statistics parameters, IBM SPSS Statistics 23v. program was used (IBM, 2015).

2.4. Deep learning and algorithms

Classification and estimation are skills that a person has learned and used multiple times throughout their life. Previously used neural networks only had one or two hidden layers; however, deep models may have a hundred layers (Goodfellow et al, 2016). These layers are used to classify pre-tagged input data or to perform numerical prediction (Kamilaris, 2018). Multiple linking between layers generates a large number of parameters. These parameters are usually initialized with random values.

2.5. Architecture of deep learning and tools

Despite the differences in deep learning architectures with their unique features, they all share the same aimwhich is to reduce the complexity of the model and increase its accuracy. (Esgario, 2020). Our model in the present study is trained on Google Colaboratory (2020), a free Jupyter notebook environment operating on the cloud. Keras (2020) backend (Python Deep Learning library) is used as a deep learning package with Tensorflow. Python 3 programming language was used to implement the deep model. In addition to many libraries required to implement deep learning algorithms, Numpy, Pandas, and MatPlotlib libraries were used. Feedforward Neural Networks (FNN), a basic deep learning method was used (Goodfellow et. al, 2016). In prepared feedforward neural network (FNN) layers, the Sequential model, which provided a flat layer stack, the most common model type in which each layer had one input tensor and one output tensor, was used (Chollet 2020).

Table 4. Deep learning regression (RM1, RM2, RM3) and classification models (CM1, CM2)

Models	Input Layer	Hidden Layer	Output Layer	Trainable parameters
RM1	Dense: 15	Dense: 30 Dropout: 20% Dense: 45 Dropout: 20%	Dense: 1	1.921
RM2	Dense: 15	Dense: 64 Dropout: 20% Dense: 128 Dropout: 40%	Dense: 1	9.473
RM3	Dense: 15	Dense: 64 Dropout: 20% Dense: 64 Dropout: 20% Dense: 128 Dropout: 20%	Dense: 1	13.633
CM1	Dense: 15	Dense: 64 Dropout: 20%	Dense: 4	5.444
CM2	Dense: 15	Dense: 32 Dropout: 20% Dense: 64 Dropout: 30% Dense: 64 Dropout: %40	Dense: 4	7.044

Fifteen different physicochemical properties (pH, EC, lime, OM, depth, slope, HC, BD, clay, silt, sand, N, P, K, and Zn) of soil types investigated in the study were chosen as input layer parameters in the deep learning system. The ReLU (Rectified Linear Unit) activation function, which is a widely used system in identifying the activation status of neurons in models as well as offering a computational advantage, was used in the study. RMSprop, based on gradient descent, was used as the optimization method. The learning rate was chosen as 0.001. In order to eliminate the uncertainty caused by network randomness, fixed seed data were input at the beginning of the program.

2.6. Training, Test, and Validation

In the present study, the dataset was divided into education (80%) and test (20%) sets. In addition, 20% of the training set was chosen as validation data. In deep neural networks, the learning is based on a gradient descent algorithm and back propagation approach. The cross entropy cost function was used for classification evaluation. After the cost function was calculated, the derivative of this function was assessed on weights. While performing regression, MSE (Mean squared error) loss function was used.

2.7. Performance metrics

During network training, the cases where the models provided the minimum cost function value for the validation set (weight set) were recorded. Then, these recorded models were assessed using the test dataset. In the classification study, the results were compared in terms of Confusion Matrix and Accuracy (ACC). In the regression study, results were assessed in terms of RMSE and R2. To evaluate the proposed deep learning algorithms, the accuracy metric was used as shown in equation 4:

$$\text{Accuracy} = (TP + TN)/(TP + TN + FP + FN) \quad (4)$$

Where TP, TN, FP, and FN are truly positive, true negative, false positive, and false negative, respectively (Aggarwal and Agrawal, 2012).

2.8. Interpolation Analyses

Interpolation techniques are used in expressing and mapping the changeability of values on investigated properties depending on the distance (Goovaerts, 1999; Mulla and McBratney, 2000).

IDW is the most commonly used interpolation models in identifying the spatial distribution of rice land quality index (LQIR) value for each point defined within the study area. The RBF (spline)

deterministic and stochastic models (also known as Kriging) models such as ordinary, universal, and simple Kriging models were also used. A total of 15 models used for forming a spatial distribution map of LQIR on the interpolation were (Inverse Distance Weighting-IDW; 1, 2, 3, Radial Basis Function-RBF; Thin Plate Spline-TPS; Completely Regularized Spline (CRS); Spline With Tension (ST), and Ordinary, Simple, and Universal Kriging models. The method that provided the lowest square-root-mean-error-value was assessed as the most suitable method. The following formula was used to calculate the square-root-mean-error.

$$\boldsymbol{RMSE} = \sqrt{\frac{\sum(\boldsymbol{z_{i*}} - \boldsymbol{z_i})^2}{\boldsymbol{n}}} \quad (5)$$

Zi: refers to the estimated value, measured value, and the number of samples.

3. Results and Discussion

3.1. Soil physico-chemical characteristics

The descriptive statistics of some physico-chemical properties of soil samples are shown in Table 5. Wilding et al (1994) and Mulla and McBratney (2000) classified the variability as low if the CV is less than 15%, moderate if the CV is between 15% and 35%, and high if the CV is greater than 35%. In this sense, variables of pH had low CV. On the other hand, the variables of HC, sand, EC and AvP, AvK, AvZn, and OM content showed a high level of variability. In this study, clay, silt, sand, BD, HC, pH, EC, and CaCO3 showed normal data distribution.

Table 5. Descriptive statistics of some physicochemical properties of soil samples

Indicators	Mean	SD	(%) CV	Variance	Min.	Max.	Skewness	Kurtosis
Clay (%)	53.34	1..21	25.23	181.04	23	67	-0.11	-0.84
Silt (%)	27.15	0.53	21.82	35.08	21	43	1.71	1.41
Sand (%)	19.52	1.26	71.94	197.13	6	55	1.09	1.32
BD (gr cm^{-1})	1.35	0.02	15.42	0.04	0.88	1.88	0.63	-0.31
HC (cm h^{-1})	1.28	0.17	144.6	3.43	0.11	5.93	1.47	1.69
pH	7.76	0.04	5.37	0.42	7.14	8.82	1.79	1.33
EC (dS/m)	6.62	0.46	78.02	26.65	1.14	17.15	-0.83	0.74
$CaCO_3$ (%)	9.73	0.27	30.47	8.79	3.04	15.97	1.02	-0.19
OM (%)	2.57	0.16	69.34	3.18	0.81	8.08	5.56	2.60
TN (%)	0.13	0.004	31.07	0.002	0.054	0.337	8.86	2.15
P (mg kg^{-1})	18.88	0.69	40.98	59.88	6.67	52.22	2.38	1.19
K (mg kg^{-1})	38.19	2.306	67.25	659.81	11.9	257.15	44.77	5.80
Zn (mg kg^{-1})	2.36	0.26	122.7	8.42	0.31	19.65	13.45	3.29

3.2. Regression with deep learning (dnn) on randomly selected data, independent of soil classes

Parameters on the dataset are clearly grouped into different soil classes. During deep learning, training, and testing dataset were randomly selected without considering class information. the regression estimation of the "index" parameter using DNN is conducted (Figure 3). The R2 value of 86.07% was achieved for RM2 after 1,500 epochs on the test dataset. The R2 values on the test conducted for other network models: RM1 77.77%, and RM3 85.61% The number of network parameters in Model 1 was insufficient, the number of network parameters in RM2 was at the optimum level, and the large number of network parameters in RM3 caused overfitting. Therefore, higher estimation was achieved with the network trained using RM2. The error rate decreased as the number of epochs increased. There was not much change after approximately 250 epochs.

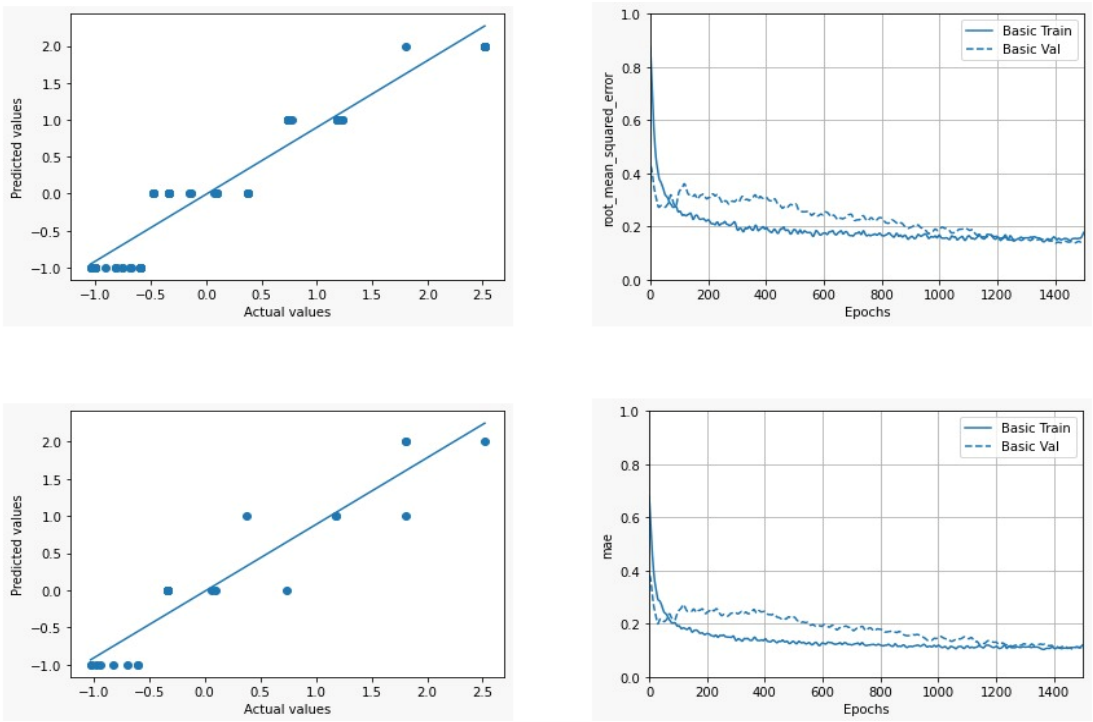

Figure 3. R2 and error (MAE and RMSE) graphics obtained from RM2 network used for training and test data on the “index” parameter.

According to results obtained in regression estimation using DNN on the "yield" parameter in Figure 4, an R2 value of 86.61% was obtained on the dataset after 1,000 epochs for RM3. The R2 values for other network models on the testRM1 81.88%, and RM2 79.61%. A high accuracy rate in estimation is obtained as the number of parameters increases in the network. Therefore, RM3 showed the highest R2 value. The error rate decreased as the number of epochs increased. After approximately 50 epochs, the training error continues to decrease; however, the validation error decreased in a slower trend.

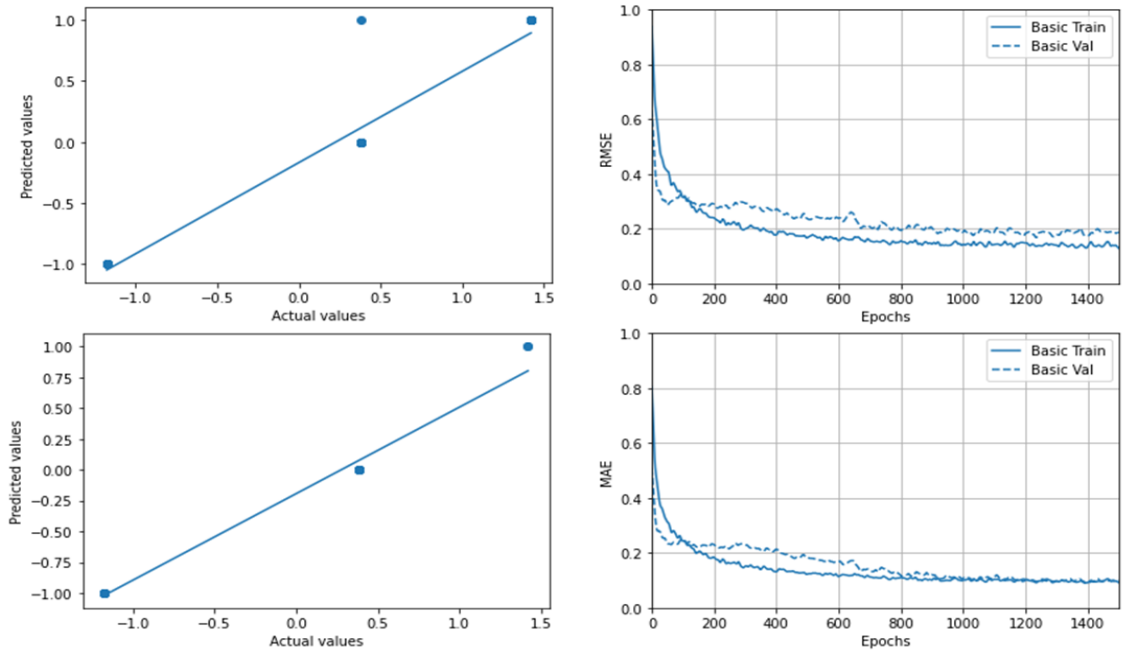

Figure 4. R^2 and error (MAE and RMSE) graphics obtained from RM3 network used for training and test data on the "yield" parameter.

According to results obtained in regression estimation using DNN on the "NAI" parameter in Figure 5, an R2 value of 84.53% was obtained on the dataset after 1000 epochs for RM2. The R2 values for other network models on the test were obtained as RM1 81.74%, and RM3 81.07%. The number of parameters in RM2 provided the best estimation success rate for NAI. It also provided a high accuracy rate in the NAI estimation in the other two models. The error rate decreased as the number of epochs increased. There was not much change after approximately 200 epochs.

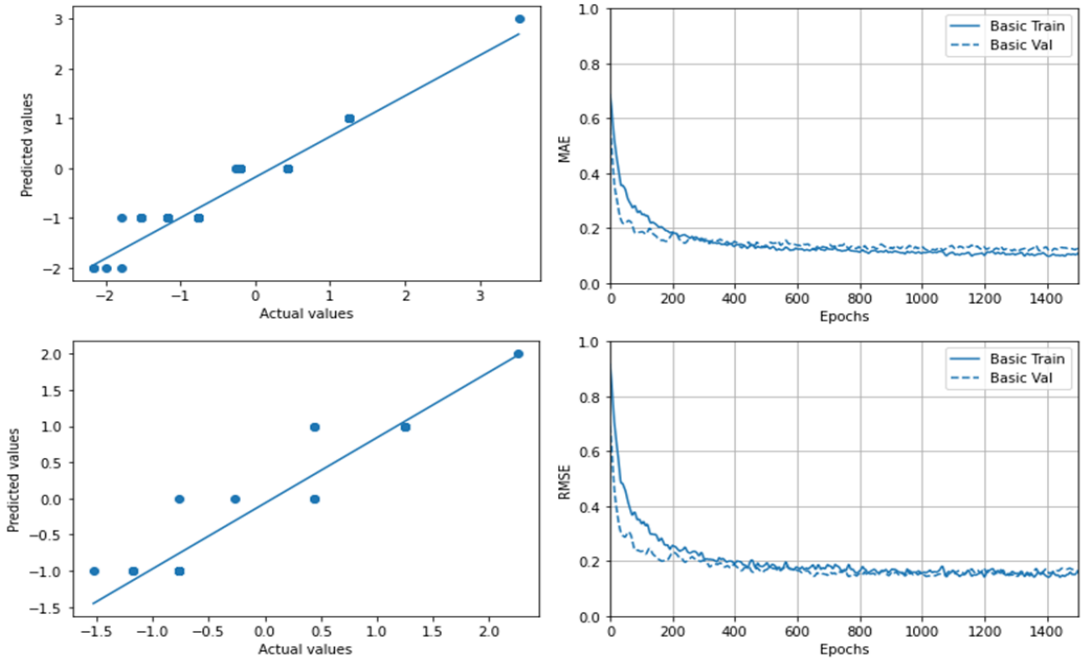

Figure 5. R^2 and error (MAE and RMSE) graphics obtained from RM1 network used for training and test data on the "NAI" parameter.

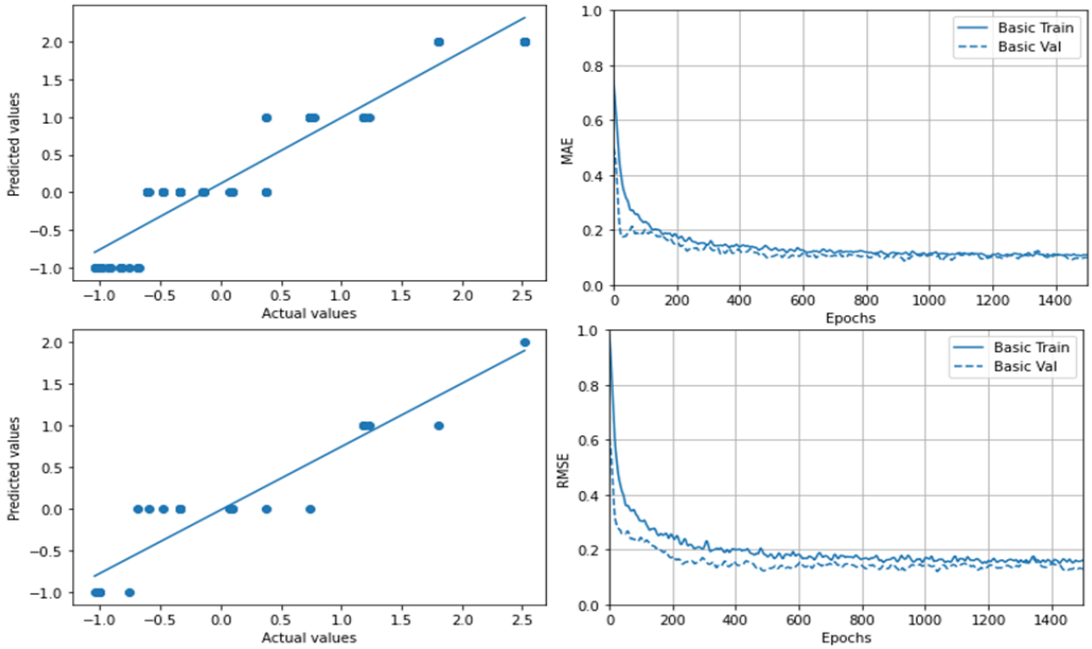

Figure 6. R^2 and error (MAE and RMSE) graphics obtained from RM3 network used for training and test data on "SQI" parameter.

According to results obtained in regression estimation using DNN on the "SQI" parameter in Figure 6, an R^2 value of 87.80% was obtained on the dataset after 1500 epochs for RM3. The R^2 values for other network models on the test were shown to be 83.83% for both RM1 and RM2. Therefore, RM3 indicated the highest R^2 value. The error rate decreased as the number of epochs increased. There was not much change after approximately 200 epochs.

The study found that using the index, efficiency, NAI, and SQI soil characteristics as network outputs led to varying levels of model performance. As a result, various models were recommended for each network output. All of the R^2 values that were obtained for estimating the index, yield, NAI, and SQI parameters were within acceptable bounds.

3.3. Regression using deep learning (DNN) on randomly selected data, dependent on soil classes

During deep learning, the training and test dataset were randomly selected depending on the soil class information. The results obtained in this way are given in Table 6.

Table 6. R^2 results of deep learning on randomly selected data dependent/independent of soil classes

	train		test		model	
	(%) Independent	(%) Dependent	(%) Independent	(%) Dependent	Independent	Dependent
Index	88.70	91.49	86.07	91.14	RM2	RM2
Productivity	87.23	88.29	86.61	87.50	RM3	RM3
NAI	89.02	89.00	81.74	87.54	RM2	RM1
SQI	89.50	91.09	87.80	87.54	RM3	RM2
Mean	88.61	89.97	85.56	88.43		

In this sense, selecting samples, considering class information, yields healthier results.

3.4. Soil classification using deep learning

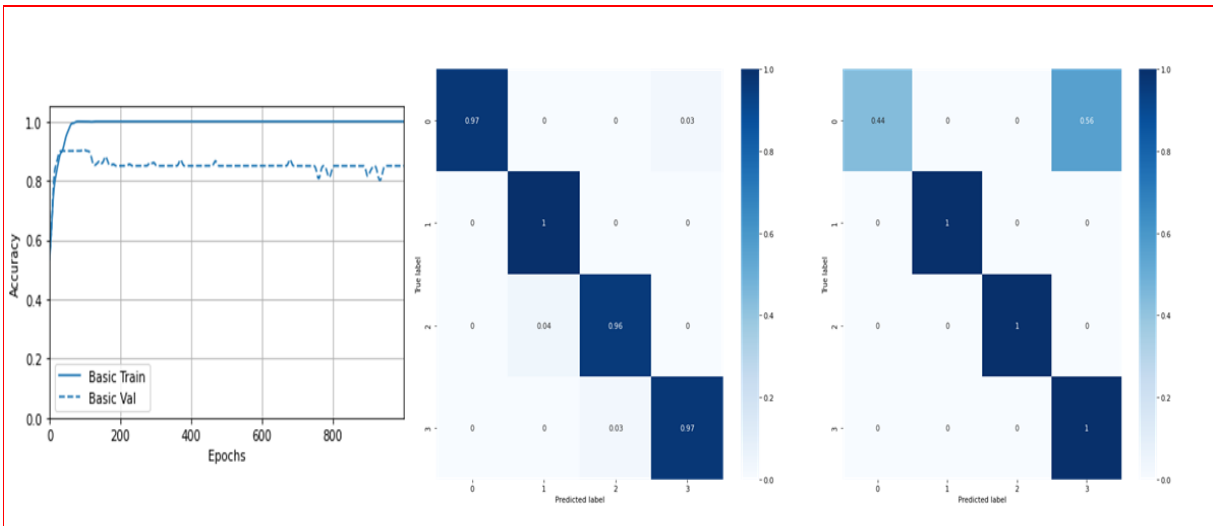

Figure 7. Graphic of Accuracy and Confusion matrix for training and test data for CM1.

In Figure 7, the results obtained from 1,000 epochs are given when CM1 is used to classify the "class" information. A performance rate of 96.97% for training and 80.00% for testing was achieved. The classifying properties generated an error in Class 0. Around 56% of Class 0 samples are classified as Class 3 errors.

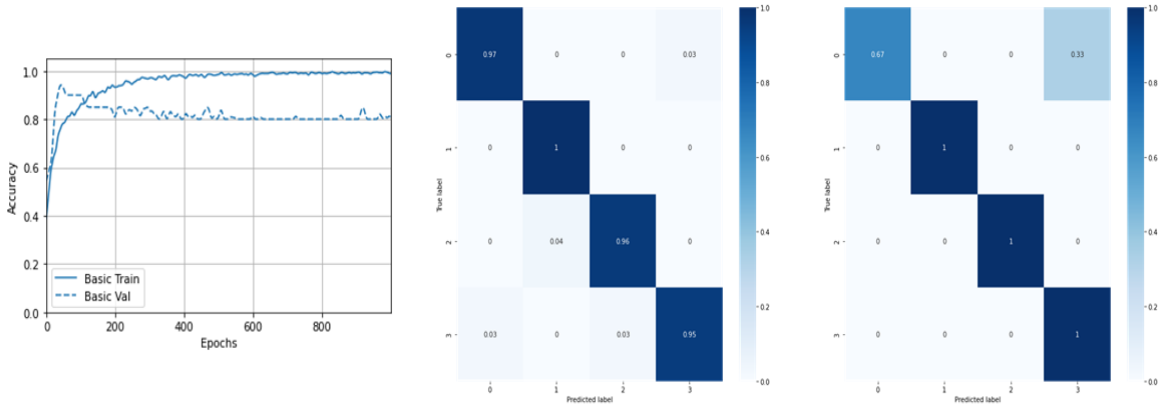

Figure. 8. Graphic of Accuracy and Confusion matrix for training and test data for CM2.

In Figure 8, the results obtained from 1,000 epochs are given when CM2 is used to classify the "class" information. A performance rate of 95.96% for training and 88.00% for testing was achieved. The classifying properties generated an error in Class 0. Around 33% of Class 0 samples are classified as Class 3 errors. The accuracy rate obtained on the test dataset was higher in CM2. Therefore, CM2 should be used in soil classification studies.

3.5. Land quality assessment and model verification

In order to form a distribution map of LQIR values for each point identified by the deep learning system, a total of 15 semi-variogram models were applied and the model comparison obtained for RMSE values is given in Table 7. In Table 7, the lowest RMSE value was found to be 0.1095 and the Completely Regularized Spline model, belonging to the Radial Basis Function, was identified. Moreover, in Table 3, a distribution map of the LQIR map, consisting of 4 classes, was created. (Figure 9).

Table 7. Cross validation according to different interpolation models

Criteria	Inverse Distance Weighing IDW			Radial Basis Function RBF		
	1	2	3	**TPS**	**CRS**	**ST**
LQI_R	0.1171	0.1120	0.1098	0,1169	0.1095	0.1096

Criteria	Kriging								
	Ordinary			Simple			Universal		
	Gau.	**Exp.**	**Sph.**	**Gau.**	**Exp.**	**Sph.**	**Gau.**	**Exp.**	**Sph.**
LQI_R	0.1109	0.1101	0.1101	0.1123	0.1114	0.1109	0.1109	0.1102	0.1101

TPS: Thin Plate Spline, CRS: Completely Regularized Spline, ST: Spline with Tension; Gau.: Gaussian, Exp.: Exponential, Sph.: Spherical.

According to results obtained in the study, it was found that 64.9% of the total land was distributed between suitable (S1) and medium-suitable (S2) classes for rice cultivation while 26.5% was in the marginal class (S3). In addition, a very small part of this land (8.6%) was found to be unsuitable for paddy cultivation. The lands that were found to be unsuitable for rice cultivation were the At.1 mapping unit, belonging to the Adatepe soil series that are classified as Vertic Calcixerept, with shallow soil depth and high slopes, Boztepe (Bz.1) classified as Vertic Haploxerept, and Bz.2 soil series. The marginal suitability class in terms of land quality is on Dağmatoğlu, Çengeldüzü, Yücekyazısı, and Kumbaba soil series which are respectively classified as Aquic Haploxerept, Vertic Xerefluvent, and Typic Haploxeret including mapping units Dc.2, Dc.3, Cd.1, Yc.3 and Kb.1 mapping units which were respectively classified as Aquic Haploxerept, Vertic Xerefluvent, and Typic Haploxeret on Dağmatoğlu, Çengeldüzü, Yücekyazısı, and Kumbaba soil series. The most important limiting feature of these soils is their salinity and coarse texture.

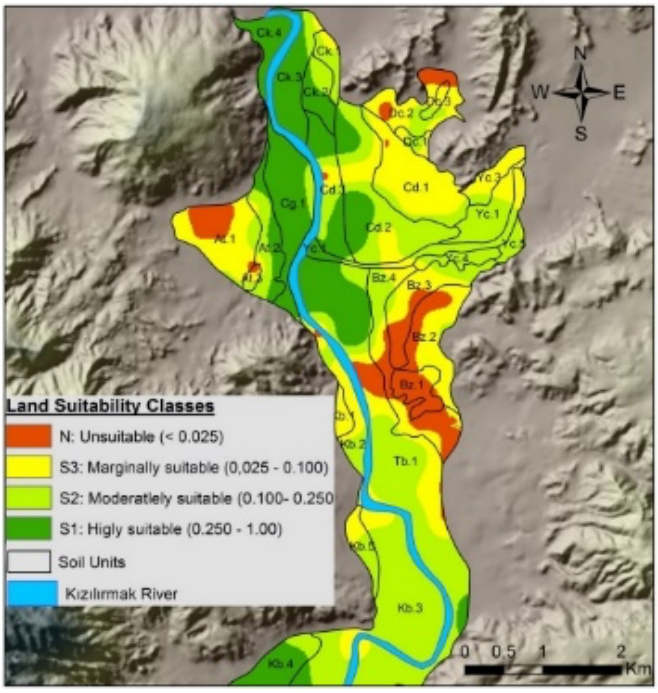

Figure 9. Spatial distribution map of the LQI_R.

Table 8. Grain yields (kg ha^{-1}) of paddy varieties cultivated in the Çorum-Osmancık (LSD0.05 = 2.07)

Suitability Classes	Variety	Max.	Min.	SD	Mean*
S1	Osmancık-97	8100	8053	17	8078 a
	Neğiş	6135	6015	50	6060 e
	Aromatik	7740	7536	77	7652 b
	Beşer	8365	8002	133	8166 a
	7721	7400	7190	88	7269 c
	Halilbey	7897	7500	165	7748 b
	Gönen	6695	6490	91	6558 d
	Karadeniz	6800	6695	43	6759 d
	Kızılırmak	7840	7368	208	7679 b
	Koral	6310	6140	70	6245 e
	Durağan	7185	6922	100	7036 c
	Şumnu	7383	6940	177	7116 c
S2	Osmancık-97	6150	6045	42	6087 b
	Neğiş	4345	3830	173	4085 h
	Aromatik	2780	2280	210	2595 ı
	Beşer	6260	6230	11	6247 ab
	7721	5730	5605	53	5650 c
	Halilbey	6560	6525	53	6480 a
	Gönen	4800	4550	108	4712 g
	Karadeniz	5485	5140	128	5332 d
	Kızılırmak	4135	3825	110	3990 h
	Koral	4955	4870	36	4923 fg
	Durağan	5405	5030	134	5232 de
	Şumnu	5130	4950	72	5058 ef
S3	Osmancık-97	4475	4240	100	4390 b
	Neğiş	2505	2305	78	2423 g
	Aromatik	1805	1645	68	1703 h
	Beşer	4357	3830	185	4079 cd
	7721	4318	4063	87	4187 bcd
	Halilbey	5930	5463	179	5662 a
	Gönen	3830	3275	212	3513 f
	Karadeniz	4515	4155	149	4291 bc
	Kızılırmak	1657	1244	174	1505 ı
	Koral	4063	3873	73	3954 de
	Durağan	4075	3650	166	3827 e
	Şumnu	3375	3302	25	3339 f

*Means followed by the same subscripted letters are not significantly different.

In order to test the model verification, a field trial study was conducted for two years in classes belonging to different rice land quality indices identified within the study area. The yield values of all rice cultivars were affected by their location. The average yield values for S1-class, S2-class, and S3-class were found to be 7,197, 5,032, and 3,572 kg ha^{-1} respectively. The difference between S1 and S3 was found to be 3,624 kg ha^{-1}. The highest yield was in S1 in the class Beser and Osmancik-97 varieties with 8,166 and 8,078 kg ha^{-1} respectively while the lowest yield was obtained in the S3 suitability class in Kızılırmak variety with 1,505 kg ha^{-1} (Table 7). According to statistical analysis, the grain yields were significantly affected by LSC and it also affected varieties differently (ANOVA, $P < 0.001$).

The results of the LSD test are shown in Table 8. For the S1 class, the ranking of paddy varieties for decreasing grain yield was Beser > Osmancik > Halilbey > Kizilirmak > Aromatik > 7721 > Sumnu > Duragan > Karadeniz > Gonen > Koral > Negis. As for the S2 class, Kizilirmak was also observed to have the lowest grain yield for the S3 class. According to grain yield, Beser, Osmancik-97, and Halilbey were the 3 best varieties. The worst varieties are Aromatik, Kizilirmak, and Negis. According to the results, the most suitable class was determined as S1 for growing high grain yield, followed by S2 and S3 classes.

Conclusion

Considering the land quality distribution for rice, most of the land (64.9%) was found to be suitable for rice cultivation while very few (8.6%) were found to have low land quality, and unsuitable for rice cultivation. The decrease in soil quality due to intensive rice cultivation threatens the sustainability of rice agriculture in the Çorum-Osmancık region. Land quality classes, which are an important factor in agricultural production, have been prioritized in this study, and different physicochemical soil properties have been chosen as input parameters in order to conduct a regression analysis and classification using deep learning. It was found that the selection of training and test samples in the dataset, considering class information, produced high-performance results in estimating soil parameters and identifying land quality classes for rice. In addition, field trials were conducted in order to identify the accuracy levels of defined land quality classes, and results showed that the data were statistically significant according to obtained test results.

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Research Article

Exploring Adaptation Abilities of Barley Genotypes in Van Growing Conditions for Biomass and Grain Yield

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Abstract: Discovering the variation among genotypes is an important criterion for selecting the suitable cultivar for a certain environment. The study aimed to explore the genetic variation among 17 genotypes of barley based on grain yield and some related traits. Plants were grown under field grown conditions in the 2019-2020 and 2020-2021 growing seasons, and plant height (PH), spike per square meter (SSM), spike length (SL), spikelets per spike (NSS), seed per spike (SPS), biological yield (BY), grain yield (GY), and thousand grain weight (TGW) were measured. Results indicated that PH ranged (51.7 to 81.33 cm) and (58.20 to 79.90 cm), SSM (374 to 582) and (418 to 701), SL (7.10 to 9.63 cm) and (6.87 to 9.13 cm), NSS (9 to 15) and (8 to 17), SPS (21 to 49) and (21 to 51), BY (3466.7 to 5905.3 kg h^{-1}) and (3731.7 to 6080 kg h^{-1}), GY (1442 to 2192 kg h^{-1}) and (811.8 to 1763.7 kg h^{-1}), TGW (34 to 55.67 g) and (33.47 to 52.63 g) for the first and second year of experiment respectively. The advanced lines measurement values were higher in the second year of the experiment. It can be concluded that the advanced lines Anka-08 and Anka-11 are promising in most of the parameters. Some of the old and new cultivars still preserve their yield potential.

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1. Introduction

Barley is one of the oldest cereal crops still in use today. It is ranked among the important cereal crops around the world, it comes after maize, wheat, and rice in production quantity and cultivation area (FAO, 2022). World barley production was more than 157 million tons in 2020, about 7.73 million tons was produced in Turkey (FAO, 2022). Various morphological variations of the crop, such as two-row or six-row and diverse colours are currently available (black, blue, purple or yellow). Plants can also be different in hulls, with or without, which can be used to distinguish varieties (Biel et al., 2020). As reported by Sullivan et al. 2013, the whole grain of barley contains about 70% starch, protein 10 to 20%, 2 to 3% lipids, about 2.5% minerals, soluble dietary 3 to 20%, and 11 to 34% dietary fibres. Thus, barley

come to be an essential ruminant cereal grain feed around the world, directly for meat cattle and after processing to increase milk production (Güney, 2019).

Based on the growth habits of the crops, barley classified into three categories; winter, spring, and alternative (or facultative). Winter barley requires to be vernalized for a period of time to initiate flowering, also sensitive to photoperiod which delays flowering after cold conditions pass (Cuesta-Marcos et al, 2016). Winter barley may be grown in double-cropping or intercropping systems with a summer annual cash crop like soybeans since it is often harvested sooner than winter wheat in some regions. The capacity of winter barley to assist the economy and the environment depends on how well it can withstand the winter and deliver adequate amount of high-quality grain (Zhong et al., 2019). Spring types do not need vernalization to start flowering and sown in spring. Facultative genotypes, on the other hand, do not require vernalization but can still survive under cold temperatures, and can be planted in spring or autumn (Cockram et al., 2015).

Considering climate change, the development of sustainable agriculture and food security still depends on efforts to find suitable barley cultivars carrying yield-improving traits in varied climates. Therefore, it was aimed in this experiment, to evaluate some barley cultivars, and advanced lines of barley under rainfed conditions to explore adaptability to Van climate for biomass and grain yield potential.

2. Material and Methods

2.1. Site, plant material, and experimental design

During the growing season of 2019-2020, 2020-2021, barley genotypes were planted at the experimental field of the Field Crops Department, Van Yuzuncu Yil University (38°33'46.3" N 43°17'54.7" E, 1725 a.s.l). As a plant material, 13 varieties and 4 advanced lines were used, a brief description of the genotypes is presented in Table 1.

Table 1. Description of the genotypes

Genotype		Row type	Growth habit	Producer	Registration year
Akar	G1	2 rows	winter-alternative	CRIFC-2012	CRIFC-2012
Anka-06	G2	2 rows	winter	CRIFC-2019	CRIFC-2019
Anka-08	G3	2 rows	alternative	CRIFC	-
Anka-09	G4	2 rows	winter	CRIFC	-
Anka-10	G5	2 rows	winter	CRIFC	-
Anka-11	G6	2 rows	alternative	CRIFC	-
Asil	G7	2 rows	winter	CRIFC-2019	CRIFC-2019
Avci 2002	G8	6 rows	winter	CRIFC-2002	CRIFC-2002
Aydanhanim	G9	2 rows	winter	CRIFC-2002	CRIFC-2002
Bozlak	G10	2 rows	winter	CRIFC-2018	CRIFC-2018
Burakbey	G11	2 rows	alternative	CRIFC-2013	CRIFC-2013
Cacabey	G12	2 rows	winter	CRIFC-2019	CRIFC-2019
Cetin 2000	G13	6 rows	winter	CRIFC-2000	CRIFC-2000
Larende	G14	2 rows	alternative	BDIARI-2006	BDIARI-2006
Olgun	G15	6 rows	winter	EAARI-2011	EAARI-2011
Tarm-92	G16	2 rows	alternative	CRIFC-1992	CRIFC-1992
Tosunpasa	G17	2 rows	winter	CRIFC-2016	CRIFC-2016

BDIARI: Bahri Dağdaş International Agricultural Research Institute, CRIFC: Central Research Institute of Field Crops, EAARI: Eastern Anatolia Agricultural Research Institute.

The meteorological data of the experiment location is described in Table 2. The trial was established as a randomized complete block design with three replications and plots of 6 m^2 (6 seed rows 5 m long and 1.2 m wide). The sowing rate was adjusted based on thousand grain weight of each genotype to achieve plant population of 500 plants per square meter. Fertilizers were added to each plot at rate of 80 kg h^{-1} P_2O_5 and 100 kg h^{-1} N.

Table 2. Meteorological data of trial site

Month	Average Temperature (°C)		Total Rainfall (mm)		Average Humidity (%)	
	2019-2020	2020-2021	2019-2020	2020-2021	2019-2020	2020-2021
September	18.8	20.1	0.8	5.6	42.7	41.3
October	13.4	13.3	24.1	1.8	32.9	53.0
November	5.2	6.7	22.9	12.8	48.2	65.4
December	3.0	1.4	46.7	30.7	51.3	71.5
January	-1.7	-0.7	31.1	15.7	59.5	67.2
February	-1.5	0.8	21.3	18.0	63.8	73.3
March	2.7	3.7	24.4	53.6	63.4	66.9
April	7.0	11.7	36.2	12.1	56.1	48.8
May	15.2	16.7	15.3	22.7	51.9	46.4
June	21.0	21.6	7.2	18.6	45.4	32.0
Total			229.9	191.6		

2.2. Trait measuring and data analysis

Represented sample of each genotype was taken at maturity from the plots' middle rows. The measured traits were Plant height (PH cm), Number of spikes per square meter (SSM), Spike length (SL cm), Number of spikelets per spike (NSS), Number of seeds per spike (SPS), Biological yield (BY t h^{-1}), Grain yield (GY t h^{-1}), and Thousand grain weight (TGW g). Separate (Table 3) and combined (Table 4) statistical analyses of the years were performed with COSTAT. Analysis of Variance and significance of mean values were tested by least significant difference test (LSD) using at significance level of $p < 0.05$.

3. Results and Discussion

Analysis of main effects of genotypes (G), year (Y), and two-way interactions of genotype x year (G x Y) showed significant differences in some parameter (Table 3, 4). A wide variation in the examined genotypes was detected for most of the studied traits, PH, SL, NSS, SPS, BY, GY, and TGW were significant in both experiment years, only SSM was significant in the second year of experiment. Furthermore, across year combined analysis of variance for studied traits across years indicates significant G x Y interaction on the SL, NSS, and BY.

Table 3. First and second year mean squares for studied traits

Source of variance	df	Mean squares							
		PH		SSM		SL		NSS	
		2019-20	2020-21	2019-20	2020-21	2019-20	2020-21	2019-20	2020-21
Blocks	**2**	1226.57***	33.04 ns	5870.13 ns	536.01 ns	0.29 ns	0.11 ns	1.58 ns	2.33 ns
Genotypes	**16**	179.23***	140.73***	7037.14 ns	17605.54***	1.59***	1.86 ***	7.96***	21.54***
Error	**32**	44.59	35.67	5183.65	3876.29	0.31	0.42	0.7	1.67
CV (%)		9.71	8.31	15.03	11.77	6.74	7.71	6.67	11.57

Source of variance	df	Mean squares							
		SPS		BY		GY		TGW	
		2019-20	2020-21	2019-20	2020-21	2019-20	2020-21	2019-20	2020-21
Blocks	**2**	16.34 ns	23.91 ns	34655.21***	9984.37 ns	11778.60***	933.55 ns	237.80***	2.26 ns
Genotypes	**16**	237.80***	176.09***	9999.88***	21286.37**	1728.84*	2712.35***	103.91***	92.91***
Error	**32**	21.13	11.43	2491.42	5930.81	832.99	499.36	4.75	5.82
CV (%)		16.11	11.16	11.36	15.98	15.69	17.47	4.67	5.48

PH: plant height, SSM: number of spike per square meter, SL: spike length, NSS: number of spikelets per spike, SPS: number of seeds per spike, BY: biological yield, GY: grain yield, TGW: thousand grain weight (g), CV (%): coefficient of variation.

Table 4. Mean square combined analysis data for variance of studied traits in barley genotypes

Source of Variance	Mean squares								
	df	PH	SSM	SL	NSS	SPS	BY	GY	TGW
Genotypes	**16**	267.54***	19112.65***	2.56***	23.40***	406.06***	19287.68***	2934.38**	176.64***
Year	**1**	251.16 ns	63450.35***	0.43 ns	55.07***	78.88*	46408.53**	80161.31***	184.27***
G x Y	**16**	52.42 ns	5530.04 ns	0.89 **	6.09***	7.82 ns	11998.58*	1506.81 ns	20.18 ns
Error	**68**	74.82	4451.92	0.35	1.23	16.51	5276.3362	1000.8766	12.03
CV (%)		12.3	13.24	7.15	8.34	13.81	15.77	20.29	7.65

PH: plant height, SSM: number of spike per square meter, SL: spike length, NSS: number of spikelets per spike, SPS: number of seeds per spike, BY: biological yield, GY: grain yield, TGW: thousand grain weight (g), CV (%): coefficient of variation.

3.1. Plant height (PH)-cm

Genotypes had a significant effect on PH in both years of experiment, while the G x Y interaction was non-significant (Table 3). The average value of PH ranged 54.63 cm (Anka-09) to 79.78 cm (Olgun) for both years (Table 7). Plants of the varieties Olgun and Tosunpasa were the tallest (81.33 cm) in the first year, whereas the advanced line Anka-08 (79.90 cm), the varieties Akar (79.40 cm) and Aydanhanim (78.37 cm) produced the tallest plant in the second year. Anka-09 had the lowest value of plant height in both years (51.07 cm and 58.20 cm) (Table 6). Most of the varieties had tall plants in the second year compare to the first year. The mean value of the parameter's variation of both years was 3.14 cm (Table 7). The highest variation was detected in Anka-08 (12.57 cm) whereas the lowest variation was in Cetin 2000 (1.03 cm). Variation among barely genotypes in PH was reported by a number of previous studies. The genotypes showed variation in PH. Ahmadi et al. (2016) mentioned a significant effect of genotypes and G x Y interaction on PH, and the barley advanced lines were superior.

3.2. Number of spike per square meter (SSM)

Genotypes had a significant effect on SSM only in the second year, whereas the G x Y interaction was non-significant (Table 3). The advanced line Anka-08 and the variety Asil had the highest mean value of SSM (593 and 592 spikes), respectively, Anka-06 had the lowest mean value of SSM (396 spikes) (Table 7). The variety Asil had the highest SSM in the first year (582 spikes) and Anka-08 had 701 spikes in the second year. The lowest SSM was from the advance line Anka-06 (374 and 418 spike) in both years, respectively. SSM value was higher in the second year compare to the first year for all the genotypes except Akar and Calabey (Table 5). The yearly variation for genotypes was 49.88 spikes. The highest variation was observed in Anka-08 (215 spikes) and the lowest variation in SSM was 6 spikes form Calabey (Table 7). Mirosavljević et al. (2020) found a significant effect of G, Y, and G x Y interaction. The highest value they obtained range from 406-442. Lower values of SSM were gained by Dorostkar et al. (2015), 95.25 to 233.00.

3.3. Spike length (SL)-cm

A significant effect of genotypes and G x Y interaction on SL was detected in both years of experiment (Table 3). The yearly SL mean value of the variety Asil was the highest (9.38 cm) and the highest value among the advanced lines was from Anka-06 (9.00 cm), whereas the lowest yearly SL mean value was 7.10 cm from Avci 2002(Table 7). The tallest spikes were produced by Asil (9.63 cm) in the first year, and in the second year most of the genotypes were within the same statistical group at the range of 8.53 cm (Anka-08 and Anka-10) to 9.17 cm (Tosunpasa). The minimum SL value was 7.10 cm from Olgun in the first year and 6.87 cm from Avci 2002 in the second year (Table 5). The variation in SL for both years was 0.31 cm. Experiment year had the most effect on the genotype Anka-08 (0.84 cm) and the lowest effect was 0.01 cm from the genotype Akar (Table 7). Our results were similar to Güngör et al. (2022) who obtained a significant effect of G, Y, and G x Y interaction on SL. Moreover, the SL value recorded in Mirosavljević et al. (2016) experiment was close to our findings, 9.9 cm. The range of SL reported by Ahmadi et al. (2016) was 5 to 12.30 cm with a mean value of 8.14 cm, which is lower than the SL in our experiments.

3.4. Number of spikelets per spike (NSS)

NSS was affected significantly by genotypes and the G x Y interaction in both years of the experiment (Table 3). The mean value for both years and it was in the range of 9 (Avci 2002) to 15 (Akar, Anka-06, Asil, Aydanhanim, Bozlak, and Burakbey) (Table 7). The heights NSS value was 15 for Asil and Burakbey in the first year and 17 for Aydanhanim in the second year. The lowest NSS value was from the genotype Avci 2002 (9 and 8 spikelets) for both years, respectively (Table 5). Differences among the genotypes in both years for NSS was 1.53, the genotypes Calabey, Catin 2000 and Tusunpasa were most affected genotypes by years (4 spikelets), whereas the genotype Burakbey was not affected by years (Table 7). Güngör et al. (2022) stated a significant effect of G, Y, and their interaction. The value of NSS they obtained was higher than our result, ranged from 16.4 to 20.3.

3.5. Number of seed per spike (SPS)

SPS was affected by genotypes in both years, whereas the G x Y interaction was non-significant (Table 3). The SPS was in the range of 21 (Anka-9) to 50 (Olgun) SPS (Table 7). In the first year, the genotype Olgun had the highest SPS (49 seeds) and it was in the same statistical group with Avci 2002 (46 seeds) and Catin 2000 (44 seeds), the genotype Olgun had the highest value of the parameter in the second year (51 seeds). The minimum obtained SPS was 21 seeds from Anka-09 and Tarim-92 in the first year, and 21 seeds obtained from Anka-09 in the second year (Table 5). The yearly variation was 1.88 seeds, the genotypes Avci 2002, Aydanhanim, Calabey, and Larende were the most affected (4 seeds), whereas Anka-09 and Burakbey showed no variation (Table 7). Mirosavljević et al. (2020) found a significant effect genotypes on SPS, with a range value of 48.3-53.1 to 49.8-54.5. However, their research indicated a significant effect of year and G x Y interaction on SPS. Ahmadi et al. (2016) obtained a mean value of 36.79 and Güngör et al. (2022) obtained 34.2 to 59.6 SPS, which are close to our findings.

Table 5. Year 1 and year 2 values of Plant height (cm), Spikes per square meter, Spike length (cm), Number of spikelets per spike, and Number of seeds per spike of barely genotypes

Genotypes	PH (cm)		SSM		SL (cm)		NSS		SPS	
	Y1	Y2	Y1	Y2	Y1	Y2	Y1	Y2	Y1	Y2
AKAR	74.73 ab	79.40 a	486	452 fgh	8.96 abcd	8.97 a	14 ab	16 ab	27 bc	28 c
ANKA-06	62.23 cd	71.77 abcde	374	418 h	9.07 abc	8.93 a	14 ab	16 a	27 bc	29 c
ANKA-08	67.33 bcd	79.90 a	486	701 a	7.69 efgh	8.53 a	12 cdef	14 bc	22 bc	25 cd
ANKA-09	51.07 e	58.20 g	466	513 cdefgh	7.41 fgh	7.10 c	11 efg	12 c	21 c	21 d
ANKA-10	59.93 de	64.70 efg	497	592 bcd	8.17 cdefg	8.53 a	12 efg	15 ab	22 bc	25 cd
ANKA-11	67.33 bcd	73.20 abcde	509	532 cdefg	8.37 cde	8.33 ab	13 abc	15 ab	25 bc	27 cd
ASIL	72.23 abc	75.73 abcd	582	602 abc	9.63 a	9.13 a	15 a	16 ab	29 b	30 c
AVCI 2002	67.13 bcd	59.07 fg	422	465 efgh	7.34 gh	6.87 c	9 h	8 d	46 a	42 b
AYDANHANIM	74.47 ab	78.37 a	459	513 cdefgh	8.39 cde	8.87 a	14 ab	17 a	26 bc	30 c
BOZLAK	74.60 ab	68.37 bcdef	448	460 efgh	8.31 cdef	8.73 a	14 ab	16 ab	26 bc	28 c
BURAKBEY	69.87 bcd	71.57 abcde	538	562 bcde	9.33 ab	8.37 ab	15 a	15 ab	27 bc	27 cd
CALABEY	68.80 bcd	75.03 abcd	501	495 defgh	8.07 defg	8.73 a	12 defg	16 ab	24 bc	28 c
CETIN 2000	67.13 bcd	66.10 defg	433	443 gh	8.91 abcd	7.33 bc	13 bcd	9 d	44 a	44 b
LARENDE	59.75 de	67.60 cdefg	452	503 cdefgh	7.72 efgh	9.10 a	12 defg	15 ab	22 bc	26 cd
OLGUN	81.33 a	78.23 ab	460	553 cdef	7.10 h	7.15 c	11 g	10 d	49 a	51 a
TARM-92	68.67 bcd	77.57 ab	508	658 ab	7.77 efgh	9.10 a	11 fg	14 bc	21 c	26 cd
TOSUNPASA	81.33 a	76.50 abc	520	527 cdefg	8.49 bcde	9.17 a	12 cde	16 a	26 bc	29 c
%CV	9.70	8.30	15.00	11.70	6.70	7.70	6.69	9.20	16.10	11.10

PH: plant height, SSM: number of spike per square meter, SL: spike length, NSS: number of spikelets per spike, SPS: number of seeds per spike. Numbers with no letter indicated non-significant differences. The same letter means non-significant differences between groups, whereas different letters indicate significant differences between groups.

3.6. Biological yield (BY)-t h^{-1}

The effect of genotypes and the G x Y interaction on BY was significant in the first and second year (Table 3). The maximum yearly BY value was obtained from Olgun (5.91 t h^{-1}), whereas the minimum value was form Anka-09 (3.47 t h^{-1}) (Table 8). In the first year, Olgun produced the highest

BY (5.91 t h^{-1}), while in the second year Asil (6.08 t h^{-1}), Anka-11 (6.00 t h^{-1}), Olgun (5.86 t h^{-1}) and Calabey (5.73 t h^{-1}) gave the highest BY value (Table 6). The lowest BY was 3.47 t h^{-1} obtained from Anka-09 and 3.73 t h^{-1} obtained from Avci 2002, for year one and two, respectively. BY was varied between years (0.43 t h^{-1}), Ails was the most affected by years with a value of 2.44 t h^{-1} variation in BY, while the variation was 0.05 t h^{-1} in Olgun (Table 8). Dorostkar et al. (2015) found variation among the tested genotypes, the values of BY ranged 523.3 to 770 g m^2. In addition, Saroei et al. (2017) results were 0.40 to 2.10 t h^{-1} BY from 42 different barley varieties.

3.7. Grain yield (GY)-t h^{-1}

GY was significantly affected by genotypes, while the G x Y interaction was non-significant (Table 3). GY average of years was in the range of 1.21 t h^{-1} (Anka-09) to 1.97 t h^{-1} (Akar) (Table 8). In the first year, Akar (2.19 t h^{-1}) and Tarim-92 (2.17 t h^{-1}), produced the highest grain, and Anka-06 (1.76 t h^{-1}), Akar (1.76 t h^{-1}), and Olgun (1.71 t h^{-1}) in the second experiment year. The lowest GY was 1.44 t h^{-1} produced from the genotype Catin 2000, and 0.81 t h^{-1} from Anka-09 in the first and second year, respectively (Table 6). Variation in GY for both years was 0.56 t h^{-1}, the genotype Tarim 92 was the most affected (1.22 t h^{-1}), and Asil GY had the lowest reduction (0.08 t h^{-1}) (Table 8). Our results suggested that GY of the varieties were higher than the advanced lines. In contrast, Beşer et al. (2019) mentioned that the GY of the advanced lines they studied were higher than the standard varieties. Furthermore, Erol et al. (2017) concluded that some barely lines obtained from CIMMYT were promising in terms of grain yield compare to varieties. The yield of Tarim-92 was higher than other varieties as Ertuş (2021) indicated when comparing barley varieties. The values ranged from 1.82 to 2.43 t h^{-1}. In contrast, Kılıç et al. (2010) reported higher GY obtained from Aydanhanim (4.56 t ha^{-1}).

Table 6. Year 1 and year 2 values of Biological yield (t h^{-1}), Grain yield (t h^{-1}), and Thousand grain weight (g) of barely genotypes

Genotypes	BY (t h^{-1})		GY (t h^{-1})		TGW (g)	
	Y1	Y2	Y1	Y2	Y1	Y2
AKAR	5.21 ab	5.42 ab	2.19 a	1.76 a	49.67 bcde	46.93 bcde
ANKA-06	4.55 bcd	5.19 abc	1.89 abcde	1.76 a	48.00 de	50.10 ab
ANKA-08	4.43 bcde	4.87 abcd	2.10 ab	1.24 bcde	50.17 bcd	52.63 a
ANKA-09	3.47 f	3.80 d	1.61 cde	0.81 f	47.17 de	42.57 fg
ANKA-10	4.50 bcd	3.96 cd	1.72 abcde	0.87 ef	48.17 cde	48.07 bcd
ANKA-11	4.11 cdef	6.00 a	1.75 abcde	1.23 cde	55.67 a	44.07 def
ASIL	3.64 ef	6.08 a	1.46 de	1.53 abc	42.50 f	36.23 hi
AVCI 2002	4.47 bcd	3.73 d	1.60 cde	1.30 bcd	35.50 gh	39.43 gh
AYDANHANIM	3.80 def	5.09 abc	1.68 bcde	1.25 bcd	47.00 de	44.23 def
BOZLAK	4.58 bcd	3.97 cd	1.78 abcde	1.12 def	48.33 cde	45.30 cdef
BURAKBEY	4.15 cdef	3.99 cd	1.82 abcde	1.07 def	46.33 e	42.37 fg
CALABEY	4.47 bcd	5.73 a	1.97 abc	1.31 bcd	51.67 bc	49.67 ab
CETIN 2000	4.15 cdef	4.36 bcd	1.44 e	1.14 def	38.33 g	34.10 i
LARENDE	4.08 cdef	3.76 d	1.93 abcd	1.07 def	52.50 ab	48.63 abc
OLGUN	5.91 a	5.86 a	2.14 ab	1.71 a	34.00 h	33.47 i
TARM-92	4.86 bc	4.80 abcd	2.17 a	0.96 def	49.67 bcde	46.80 bcde
TOSUNPASA	4.30 cde	5.31 ab	2.01 abc	1.61 ab	48.83 cde	43.20 efg
%CV	1.10	1.60	1.60	1.70	4.60	5.40

BY: biological yield, GY: grain yield, TGW: thousand grain weight (g). Numbers with no letter indicated non-significant differences. The same letter means non-significant differences between groups, whereas different letters indicate significant differences between groups.

3.8. Thousand grain weight (TGW)-g

The effect of genotypes was significant on TGW, whereas the G x Y interaction was non-significant (Table 3). The mean TGW was in the range of 33.73 (Olgun) to 51.40 g (Anka08) for both years (Table 8). The maximum TGW in the first year was from Anka-11 (55.67 g) and Anka-08 (52.63 g) in the second year. The minimum value was 34 g from Olgun in the first year, and 33.47 g from Olgun and 34.10 g from Cetin 2000 in the second year (Table 6). TGW was affected by years, 2.69 g, the Anka-

11 showed reduction in TGW by 11.6 g, while the reduction was 0.1 g in Anka-10. These indicates that TGW of modern varieties was higher in the new varieties compare to old varieties of barley (Table 8). Kılıç et al. (2010) stated that only genotypes had an effect on TGW, and Y and G x Y effect is non-significant. Vasilescu et al. 2022 examined varieties of three periods starting from 1952 until 2019. They reported that TGW mean value of the new varieties was higher, 44.14 g, compared to the old varieties, 42.01 g. Moreover, Ay et al. (2018) stated a genotypic, environment and their interaction effect TGW, and the highest value was 65.75 g which is higher than our TGW values.

Table 7. Year 1 and year 2 mean values and year variation of Plant height (cm), Spikes per square meter, Spike length (cm), Number of spikelets per spike, and Number of seeds per spike of barely genotypes

Genotypes	PH		SSM		SL		NSS		SPS	
	Y1-Y2 average	Year variation	Y1-Y2 average	Year variation	Y1-Y2 average	Year variation	Y1-Y2 average	Year variation	Y1-Y2 average	Year variation
AKAR	77.07	4.67	469.00	-34.00	8.96	0.01	15	2	28	1
ANKA-06	67.00	9.54	396.00	44.00	9.00	-0.14	15	2	28	2
ANKA-08	73.62	12.57	593.00	215.00	8.11	0.84	13	2	23	3
ANKA-09	54.63	7.13	490.00	47.00	7.25	-0.31	12	1	21	0
ANKA-10	62.32	4.77	544.00	95.00	8.35	0.36	13	3	24	3
ANKA-11	70.27	5.87	520.00	23.00	8.35	-0.04	14	2	26	2
ASIL	73.98	3.50	592.00	20.00	9.38	-0.50	15	1	29	1
AVCI 2002	63.10	-8.06	444.00	43.00	7.10	-0.47	9	-1	44	-4
AYDANHANIM	76.42	3.90	486.00	54.00	8.63	0.48	15	3	28	4
BOZLAK	71.48	-6.23	454.00	12.00	8.52	0.42	15	2	28	2
BURAKBEY	70.72	1.70	550.00	24.00	8.85	-0.96	15	0	27	0
CALABEY	71.92	6.23	498.00	-6.00	8.40	0.66	14	4	26	4
CETIN 2000	66.62	-1.03	438.00	10.00	8.12	-1.58	11	-4	44	0
LARENDE	63.67	7.85	478.00	51.00	8.41	1.38	13	3	24	4
OLGUN	79.78	-3.10	507.00	93.00	7.13	0.05	10	-1	50	2
TARM 92	73.12	8.90	583.00	150.00	8.44	1.33	12	3	23	5
TOSUN PASA	78.92	-4.83	523.00	7.00	8.83	0.68	14	4	27	3
Mean		3.14		49.88		0.13		1.53		1.88

* The (-) indicates a reduction in the parameter value.

Table 8. Year 1 and year 2 mean values and year variation of Biological yield (t h^{-1}), Grain yield (t h^{-1}), and Thousand grain weight (g) of barely genotypes

Genotypes	BY		GY		TGW	
	Y1-Y2 average	Year variation	Y1-Y2 average	Year variation	Y1-Y2 average	Year variation
AKAR	5.31	0.21	1.97	-0.44	48.30	-2.74
ANKA-06	4.87	0.64	1.83	-0.13	49.05	2.10
ANKA-08	4.65	0.43	1.67	-0.86	51.40	2.46
ANKA-09	3.64	0.34	1.21	-0.79	44.87	-4.60
ANKA-10	4.23	-0.54	1.30	-0.85	48.12	-0.10
ANKA-11	5.05	1.88	1.49	-0.52	49.87	-11.60
ASIL	4.86	2.44	1.49	0.08	39.37	-6.27
AVCI 2002	4.10	-0.74	1.45	-0.30	37.47	3.93
AYDANHANIM	4.44	1.29	1.46	-0.43	45.62	-2.77
BOZLAK	4.28	-0.61	1.45	-0.66	46.82	-3.03
BURAKBEY	4.07	-0.15	1.45	-0.75	44.35	-3.96
CALABEY	5.10	1.26	1.64	-0.66	50.67	-2.00
CETIN 2000	4.26	0.21	1.29	-0.30	36.22	-4.23
LARENDE	3.92	-0.31	1.50	-0.86	50.57	-3.87
OLGUN	5.88	-0.05	1.92	-0.43	33.73	-0.53
TARM 92	4.83	-0.06	1.56	-1.22	48.23	-2.87
TOSUN PASA	4.81	1.01	1.81	-0.41	46.02	-5.63
Mean		0.43		-0.56		-2.69

* The (-) indicates a reduction in the parameter value.

4. Conclusion

Analysis of variance indicates the existence of variation among the genotypes, and the effect of growing season on the yield. The examined genotypes varied in most of the studied parameters. Some of the advanced lines showed promising data. Anka-11 biological yield was among the highest group with compare to the variety Olgun which had the highest biological yield among the varieties. Thousand grain weight of the advanced line Anka-08 was higher than all other genotypes, and its grain yield was among the highest genotypes. Even though, the total rainfall in the second year was lower by 38.3 mm, the genotypes, especially the advance lines, showed an increase in some of the studied traits; NSS, SPS, BY, GY, and TGW. Thus, further studies on water requirement of the genotypes are necessary.

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Research Article

The Effects of Different Fertilizer Applications on Some Morphological Traits in Fresh Bean

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Abstract: The study was conducted to determine the morphological changes caused by different fertilizer applications on the fresh bean, in the Göllü Village of Tusba District of Van Province according to the randomized blocks experimental design in 2019 and 2020. Sazova 1949 dwarf bean variety was used as plant material in the study. The field experiment was carried out with 4 replications and 4 different fertilizer applications (chemical, organomineral, cattle, and vermicompost) except for the control. In the study, plant height, stem diameter, node number, internode length, flower bud length, flower bud width, flower stem length, number of flowers per cluster, bract length, number of nodes with the first flower, middle leaflet length, number of leaves, first pod height, pod length, pod width, number of pods per bunch, pod weight, pod thickness, number of seeds per pod, chlorophyll (SPAD value) and leaf color L*, a*, b, Chroma°, and hue° values were investigated. As a result of the study; it was determined that different fertilizer applications gave significantly different results in terms of the traits examined, higher results were obtained from organomineral and vermicompost fertilizers compared to the control group, and generally equivalent or better results were obtained than chemical fertilizers. It was concluded that some organic fertilizer applications in bean cultivation might be used as an alternative to chemical fertilizer applications in terms of a sustainable world.

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1. Introduction

Beans (*Phaseolus vulgaris* L.) (2n = 22) are among the predominantly self-pollinated legume group vegetables (Ferreira et al., 2000). The domesticated bean had multiple origins in the New World and is one of the oldest cultivated plants (Beebe et al., 2013). Bean, which has an important place in terms of agricultural production, also has an important place in human nutrition because it contains protein, vitamins, minerals, complex carbohydrates, and micronutrients (Ekincialp and Şensoy, 2018). Because beans are evaluated in many ways such as fresh, canned, pickled, and dried, it is very valuable to know the effects of quality-enhancing practices in agriculture.

It is important that the practices carried out do not harm human health, guarantee long-term income for the producer, protect the ecological system and biodiversity, and aim to leave better quality

and efficient products to future generations. In this context, the importance of sustainable agricultural practices is increasing day by day. The concept of sustainable agriculture is an approach that aims to balance agronomic, environmental, social, and economic dimensions in agricultural production. Its aim is to keep the economy alive in the short and long term, to increase the quality of life of those engaged in agriculture and to develop practices for this purpose, while maintaining productivity in agriculture on the one hand, reducing the damage to the environment on the other hand (Turhan, 2005). Considering the nutritional value of organic agricultural products included in sustainable agriculture, they may contain more vitamins and mineral matters in addition to containing less nitrate and heavy metals than conventional agricultural products (Girgel et al., 2018). It has been reported that inorganic fertilization causes nitrate accumulation three times more in lettuce and salad compared to organic fertilizer (Özgen et al., 2011). Organic fertilizers, which are the main inputs of organic farming systems that have become widespread with people's interest in organic products, are offered to the use of producers under various names and contents in the market (Okur et al., 2007). Among these fertilizers, vermicompost, farmyard, sheep, cattle, poultry manure, and organic fertilizer-reinforced chemical fertilizers are the most commonly used fertilizers, and different effects on the growth and development of many plant species have been examined in different studies by different researchers (Şensoy et al., 1996; Toy, 2015; Yeşilbaş 2015; Tunçtürk et al., 2016; Dumlupınar, 2017; Ata, 2018; Kabay et al., 2018; Müjdeci et al., 2020). The idea that yield-oriented wrong fertilizer applications in the production of vegetable species that have an important place in human nutrition damage the ecological system and that biodiversity is gradually disappearing has awakened the awareness that the natural balance should be regained all over the world.

Therefore, studies to ensure the sustainability of the ecological system and to protect biodiversity have great importance. In this study, it was aimed to determine the effects of some organic and conventional fertilizer applications on some morphological properties of fresh bean and to determine the fertilizers that can be an alternative to chemical fertilizers.

2. Materials and Methods

In the present study, the dwarf standard fresh bean variety Sazova 1949, which was obtained from the original seed, whose field and laboratory controls were made by the Geçitkuşağı Agricultural Research Institute, was used as the bean variety in the study. Morphological measurements were used to determine the difference between organic and conventional fertilizer applications in fresh bean cultivation. The experiment was established in the village of Göllü with latitude 38.7202 and longitude 43.3124 coordinates within the borders of Tuşba district of Van for 2 years, according to the randomized blocks experimental design with 4 replications and 4 different fertilizer applications except for the control. Total of 6 kg N, 7 kg P_2O_5, and 8 kg K_2O were applied as fertilizer per decare. The 6 kg of N:P:K per decare was given as the compound fertilizer of 18:18:18 (NPK). The rest of the phosphorus and potassium were completed as triple superphosphate and potassium sulfate, respectively. For other fertilizers, 54.50 kg organomineral fertilizer, 300 kg vermicompost and cattle manure per decare in 2019, 54.56 kg organomineral fertilizer, 290 kg vermicompost and 923 kg cattle manure per decare were applied to the parcels in 2020, approximately equivalent to chemical fertilizers. The distances between the plots were 2 m, the row spacing within the plot was 25 cm, and the intra-row spacing was 50 cm, and the size of each plot corresponded to 4m x 3.5m. The side effect was applied to the plots and there were a total of 112 plants from each plot, and 80 plants in the middle of these were used for analysis, measurement, and observations. The irrigation process in the plots was carried out by drip irrigation method.

2.1. Morphological traits

IPGRI (International Plant Genetic Resources Institute) and EU CPVO (European Community Plant Variety Office) data (Anonim, 2009) and some agronomic traits included in the technical instruction of the Ministry of Agriculture Turkey for the measurement of agricultural values were used for morphological observations (Erdinç et al., 2013). Moreover, the necessary measurements were made on 10 randomly selected plants and pods in each plot. All the morphological traits used are listed below.

Plant height (cm) (at physiological maturity): It was measured from the root crown to the tip of the stem with the help of a meter.
Stem diameter (mm): The stem diameter was measured with the help of digital caliper.
The number of nodes on the stem at physiological maturity: It was determined by counting the nodes on the main stem.
Internode length (mm): Nodes located in the middle of the main stem were measured with the help of a digital caliper.
Flower bud length (mm): It was measured with the help of a digital caliper during 50% flowering.
Flower bud width (mm): It was measured with the help of a digital caliper during 50% flowering.
Flower stem length (mm): It was measured with the help of a digital caliper at 50% flowering.
The number of flower buds per cluster: The flower buds on the first cluster were counted.
Bract length (mm): It was measured with the help of a digital caliper.
The number of nodes with the first flower: The node with the first flower was determined by counting.
The length of the middle leaflet (mm): It was measured with the help of a digital caliper.
The average number of leaves per plant: The leaves over the 1 plant were determined by counting total in 10 plants.
First pod height (cm): The height of the first pod to the soil surface was determined with the help of a digital caliper.
Pod length (mm): Measured with the help of a digital caliper.
Pod width (mm): Measured with the help of a digital caliper.
The number of pods per bunch: The pods in the bunch were determined by counting.
Average pod weight (g): The weight of each harvested pod was determined with the help of precision scales by taking the average of 10 plants.
Pod thickness (mm): It was measured with the help of a digital caliper.
The number of seeds per pod (piece): The seeds in the pods were measured by shelling.
Leaf chlorophyll content (SPAD value): It was determined with the help of an SPAD meter (Minolta SPAD-502, Osaka, Japan).
Leaf color (L, a*, b, Chroma° and hue°):* Determined by a Colorimeter (CR-400 Minolta).

2.2. Statistical analysis

The data obtained in the study were evaluated according to the degree of significance with one-way analysis of variance according to the randomized blocks experimental design. Means that were found to be statistically significant in the analysis of the data were grouped according to the "Duncan Multiple Comparison Test". Descriptive statistics were expressed as mean and standard error. The statistical significance level was taken as 5% in the calculations. The “SPSS version 20.0” statistical package program was used for the calculations.

3. Results and Discussion

In the present study, plant height, stem diameter, number of node, internode length, flower bud length, flower bud width, flower stem length, number of flower buds per cluster, bract length, number of nodes with first flower, middle leaflet length, number of leaves, first pod height, pod length, pod width, number of pods per bunch, pod weight, pod thickness, number of seeds per pod, chlorophyll (SPAD) and leaf color L*, a*, b, Chroma°, and hue° values were investigated (Table 1-7).

When the obtained values are analyzed statistically; The difference among the fertilizers was found to be significant in terms of plant height both in 2019 and in years average (Table 1). For plant height; it was also observed that there was a difference between years in terms of organomineral and vermicompost fertilizers. In the values obtained from the stem diameter; in terms of organomineral fertilizer, the difference between years was significant. In the data obtained from the number of nodes, the difference between fertilizers for both 2019 and 2020 was found to be significant. For the internode lengths; while the difference between fertilizers was found to be significant in 2019, the difference between years was determined to be significant in all applications. Çiftçi and Şehrali (1984) reported that plant height in bean varies between 17.0-164.0 depending on the variety and environmental conditions. In a study investigating the effects of sowing dates and potassium fertilizer rates on bean yield, it was reported that plant height, stem diameter, and potassium content increased with potassium

application (Badawy et al., 2019). In a study conducted for the performance evaluation of fresh bean genotypes for yield and related traits, it was reported that stem diameter values ranged between 3.00-5.70 cm (Yohannes et al., 2020). Sepetoğlu (1992) stated that the number of nodes in dwarf bean cultivars is 3 to 10 per plant, and they reported that this difference may vary depending on genetic structure and growing conditions. In another study conducted in Southern Ethiopia for the performance evaluation of fresh bean genotypes for yield and related traits, internode length values were reported to vary between 4.00 to 7.00 cm (Yohannes et al., 2020).

Table 1. The effects of fertilizers on plant height (cm), stem diameter (mm), number of node and internode length (mm)

2019	**Plant height**		**Stem diameter**		**Number of nodes**	**Internode length**	
Control	36.84 ± 1.80d		7.15 ± 0.24		5.03 ± 0.28 b	63.48 ± 4.69 ab	B
Chemical	42.53 ± 1.40 bc		7.56 ± 0.24		5.35 ± 0.19 b	58.34 ± 0.76 b	B
Organomineral	49.11 ± 1.15 a	A	7.49 ± 0.15	B	6.49 ± 0.43 a	72.10 ± 3.62 a	B
Cattle manure	39.79 ± 2.03 cd		7.58 ± 0.42		4.88 ± 0.18 b	62.73 ± 4.85 ab	B
Vermicompost	47.30 ± 1.74 ab	A	7.28 ± 0.25		5.90 ± 0.42 ab	61.94 ± 1.41 ab	B
Mean	43.11 ± 1.24		7.41 ± 0.12		5.53 ± 0.19	63.72 ± 1.74	
$p_{fertilizer}$	0.001		0.748		0.015	0.131	
2020							
Control	36.10 ± 3.07		7.83 ± 0.25		3.50 ± 0.17 b	75.20 ± 0.53	A
Chemical	41.70 ± 1.12		8.07 ± 0.28		4.15 ± 0.21 a	76.14 ± 0.64	A
Organomineral	38.65 ± 0.64	B	8.36 ± 0.20	A	4.15 ± 0.24 a	77.00 ± 0.88	A
Cattle manure	41.70 ± 1.37		8.08 ± 0.20		4.05 ± 0.10 ab	77.02 ± 0.26	A
Vermicompost	39.90 ± 2.14	B	8.00 ± 0.34		4.00 ± 0.22 ab	75.53 ± 0.51	A
Mean	39.61 ± 0.89		8.07 ± 0.11		3.97 ± 0.09	76.18 ± 0.29	
$p_{fertilizer}$	0.232		0.705		0.148	0.162	
			Years average				
Control	36.47 ± 1.65 b		7.48 ± 0.20		4.26 ± 0.32	69.34 ± 3.11	
Chemical	42.11 ± 0.84 a		7.81 ± 0.19		4.75 ± 0.26	67.24 ± 3.39	
Organomineral	43.88 ± 2.06 a		7.92 ± 0.19		5.31 ± 0.49	74.54 ± 1.95	
Cattle manure	40.74 ± 1.19 ab		7.82 ± 0.23		4.26 ± 0.18	69.87 ± 3.51	
Vermicompost	43.60 ± 1.89 a		7.64 ± 0.23		4.95 ± 0.42	68.73 ± 2.66	
$p_{fertilizer}$	0.015		0.633		0.269	0.503	

Small letters show the difference among the applications for the same year and years average. Capital letters show the difference between the years for the same application and parameter ($p<0.05$). Data are expressed as ± standard error.

When the obtained values are analyzed; it was observed that there was a difference between years in terms of all fertilizer applications for flower bud length (Table 2). In the data on flower bud width; it was observed that there was a difference between years in terms of organomineral fertilizer, cattle manure, and vermicompost applications. In the number of flower buds per cluster; It was observed that there was a difference between years only in terms of the control group. Karahan (1997), in a study in which the effects of bacterial inoculation and five nitrogen doses in dwarf bean cultivars in Thrace conditions were determined; reported that the number of flowers in the cluster varies between 3.1 and 6.2. In another study, it has been reported that the flower bud length is 8.51-17.05 mm, the flower bud width is 3.04-5.51 mm, the flower stem length is 2.72-10.35 mm, and the number of flower buds in a cluster varies between 1.20 and 10.40 (Erdinç et al., 2013).

When the obtained values are analyzed; The difference between the fertilizers was found to be significant in terms of both the year 2020 and the average of years in terms of bract length (Table 3). In addition, for the bracket length; it was observed that there was a difference between years in terms of cattle manure. For the number of nodes with the first flower; the difference between fertilizers was found to be significant in terms of both the year 2019 and the average of the years. In the data obtained from the length of the middle leaflet; the difference between fertilizers was found to be significant for both 2019 and year averages. In the values of the number of leaves; the difference between years was determined to be significant in all applications. It was stated that plant height, number of branches, number of leaves, and number of fruits are important factors affecting yield in beans (Ayanoğlu et al., 1995). In a study carried out to determine the flower and seed characteristics of various bean (*Phaseolus vulgaris* L.) genotypes, the length of the bract was 3.98-6.19 mm in the first year, the number of nodes with the first flower was 3.50-7.50; In the second year, it was determined that the length of the bract

varies between 4.31-7.07 mm and the number of nodes with the first flower varies between 2.67-7.17 (Çiftçi et al., 2012). In another study, the bract size is 3.64-8.10 mm, the number of nodes with the first flower is 1.75-7.0, the length of the middle leaflet is 55.21-120.39 mm, and the presence of leaves at physiological maturity varies between 1 and 7 (Erdinç et al., 2013). In a study carried out to determine the effects of vermicompost and mycorrhiza use on plant growth and yield in beans and onions, when the values of the number of leaves were examined, it was found that for beans it varied between 5.66 and7.00, the highest result was found in vermicompost application (7.00), and the lowest value was obtained in the application of vermicompost fertilizer + mycorrhiza (5.66) (Uluğ, 2018).

Table 2. The effects of fertilizers on flower bud length (mm), flower bud width (mm), flower stem length (mm) and number of flower buds per cluster

2019	**Flower bud length**		**Flower bud width**		**Flower stem length**	**Number of flower buds per cluster**	
Control	8.04 ± 0.38	B	4.10 ± 0.21		3.70 ± 0.06	3.15 ± 0,15	B
Chemical	7.80 ± 0.33	B	4.43 ± 0.17		3.93 ± 0.11	3.22 ± 0.27	
Organomineral	8.04 ± 0.54	B	4.21 ± 0.14	B	3.91 ± 0.21	4.15 ± 0.41	
Cattle manure	7.78 ± 0.21	B	4.30 ± 0.07	B	4.03 ± 0.19	3.55 ± 0.31	
Vermicompost	7.71 ± 0.35	B	4.35 ± 0.07	B	4.08 ± 0.25	3.97 ± 0.22	
Mean	7.87 ± 0.15		4.28 ± 0.06		3.93 ± 0.08	3.61±0.14	
p $_{fertilizer}$	0.949		0.545		0.608	0.101	
2020							
Control	11.10 ± 0.99	A	4.97 ± 0.54		3.96 ± 0.10	3.40 ± 0.40	A
Chemical	13.09 ± 0.60	A	5.49 ± 0.43		4.20 ± 0.16	3.50 ± 0.13	
Organomineral	12.85 ± 0.86	A	6.00 ± 0.47	A	4.36 ± 0.21	3.45 ± 0.50	
Cattle manure	12.70 ± 0.51	A	5.97 ± 0.31	A	4.48 ± 0.09	3.50 ± 0.31	
Vermicompost	12.39 ± 0.41	A	5.87 ± 0.39	A	4.40 ± 0.13	3.75 ± 0.15	
Mean	12.43 ± 0.32		5.66 ± 0.19		4.28 ± 0.07	3.52 ± 0.13	
p $_{fertilizer}$	0.339		0.438		0.161	0.952	
Years average							
Control	9.57 ± 0.76		4.54 ± 0.31		3.83 ± 0.07	3.28 ± 0.20	
Chemical	10.44 ± 1.05		4.96 ± 0.29		4.07 ± 0.11	3.36 ± 0.15	
Organomineral	10.44 ± 1.02		5.11 ± 0.41		4.13 ± 0.16	3.80 ± 0.33	
Cattle manure	10.24 ± 0.96		5.13 ± 0.35		4.25 ± 0.13	3.53 ± 0.20	
Vermicompost	10.05 ± 0.92		5.11 ± 0.34		4.24 ± 0.14	3.86 ± 0.13	
p $_{fertilizer}$	0.963		0.711		0.621	0.234	

Small letters show the difference among the applications for the same year and years average. Capital letters show the difference between the years for the same application and parameter ($p<0.05$). Data are expressed as ± standard error.

Table 3. The effects of fertilizers on bract length (mm), number of nodes with first flower, middle leaflet length (mm) and number of leaves

2019	**Bract length**		**Number of nodes with first flower**	**Middle leaflet length**		**Number of leaves**	
Control	3.70 ± 0.05		1.33 ± 0.06b	78.25 ± 1.21c	B	16.23 ± 0.95	B
Chemical	3.93 ± 0.11		1.40 ± 0.06b	92.27 ± 1.10ab		16.33 ± 1.27	B
Organomineral	3.90 ± 0.20		1.83 ± 0.09a	88.94 ± 2.24b		16.25 ± 0.65	B
Cattle manure	4.02 ± 0.19	B	1.55 ± 0.15ab	87.99 ± 1.15b		16.30 ± 0.66	B
Vermicompost	4.08 ± 0.24		1.60 ± 0.04ab	93.82 ± 1.22a		16.70 ± 0.92	B
Mean	3.92 ± 0.07		1.54 ± 0.05	88.25 ± 1.37		16.36 ± 0.37	
p $_{fertilizer}$	0.608		0.010	0.001		0.996	
2020							
Control	3.96 ± 0.10 b		1.45 ± 0.10	88.36 ± 1.17	A	20.70 ± 1.24	A
Chemical	4.20 ± 0.16 ab		1.30 ± 0.06	94.37 ± 2.00		22.50 ± 0.79	A
Organomineral	4.36 ± 0.21 ab		1.50 ± 0.24	94.14 ± 4.83		22.95 ± 0.87	A
Cattle manure	4.47 ± 0.09 a	A	1.70 ± 0.13	93.45 ± 3.36		22.25 ± 0.85	A
Vermicompost	4.40 ± 0.13 ab		1.60 ± 0.12	91.72 ± 3.41		21.95 ± 0.98	A
Mean	4.28 ± 0.07		1.51 ± 0.06	92.41 ± 1.38		22.07 ± 0.42	
p $_{fertilizer}$	0.161		0.367	0.668		0.552	
Years average							
Control	3.83 ± 0.7b		1.39 ± 0.06bc	83.30 ± 2.06b		18.46 ± 1.11	
Chemical	4.06 ± 0.10ab		1.35 ± 0.04c	93.32 ± 1.13a		19.41 ± 1.36	
Organomineral	4.13 ± 0.16ab		1.66 ± 0.13a	91.54 ± 2.65a		19.60 ± 1.36	
Cattle manure	4.25 ± 0.13a		1.63 ± 0.10ab	90.72 ± 1.94a		19.28 ± 1.23	
Vermicompost	4.24 ± 0.14a		1.60 ± 0.06bc	92.77 ± 1.72a		19.33 ± 1.17	
p $_{fertilizer}$	0.146		0.031	0.006		0.973	

Small letters show the difference among the applications for the same year and years average. Capital letters show the difference between the years for the same application and parameter ($p<0.05$). Data are expressed as ± standard error.

When the obtained data are analyzed; the difference between years in terms of the first pod height was found to be significant in all applications (Table 4). The difference between the applications was found to be significant for both the years and the average of the years in the values of the pod length. In addition, the difference between years was found to be significant for the control group for pod length, and for chemical and organomineral fertilizers. In the data obtained from the width of the pod; For 2020, the difference between fertilizers and between years for all applications was found to be significant. In bean cultivation, pod characteristics have an important place and there can be great differences between varieties (Gündüz et al., 2000; Balkaya and Odabas, 2002). It has been determined that the variety, cultivation technique (such as sowing density, fertilization, etc.) and environmental conditions have a significant effect on the height of the first pod (Önder and Şentürk, 1996). It was emphasized that the first pod height, one of the parameters examined in the study, is the most important criterion for mechanical harvesting in beans (Odabaş and Gülümser, 2001). In a study investigating the effects of different nitrogen-based fertilizers on yield and yield components in beans, the researchers stated that fertilizer doses increased the first pod height and length. In a study investigating the effects of different boron doses applied from leaves and soil on yield and yield components in bean, it was determined that increasing fertilizer doses caused an increase in the first pod height compared to the control group, and the highest value was 22.13 cm at 1.5 kg ha^{-1} boron application (Gülümser et al., 2005). In a study carried out to determine the effects of vermicompost and mycorrhiza use on plant growth and yield in beans and onions, when the pod length and width values were examined, it was found that the pod length in beans varied between 10.89 cm and 12.76 cm, and the pod width ranged between 12.82 mm and 15.07 mm, and the highest results were obtained with vermicompost (Uluğ, 2018). In a study conducted to determine the yield and some quality factors of some dwarf fresh bean cultivars in Konya conditions, it was determined that the average pod length varies between 128.7 mm and 146.2 mm and the average pod width varies between 13.9 mm and 15.3 mm (Seymen et al., 2010).

Table 4. The effects of fertilizers on first pod height (cm), pod length (mm) and pod width (mm)

2019	**First pod height**		**Pod length**		**Pod width**	
Control	7.93 ± 0.55	A	121.00 ± 2.27ab	A	9.78 ± 0.25	B
Chemical	7.53 ± 0.34	B	126.00 ± 2.74ab	A	10.09 ± 0.17	B
Organomineral	8.42 ± 0.38	A	130.00 ± 2.92a	A	10.09 ± 0.28	B
Cattle manure	8.41 ± 0.34	B	117.50 ± 4.56b		9.66 ± 0.20	B
Vermicompost	7.86 ± 0.43	B	121.25 ± 1.65ab		9.87 ± 0.17	B
Mean	8.03± 0.18		123.15 ± 1.55		9.90 ± 0.09	
$p_{fertilizer}$	0.516		0.073		0.544	
2020						
Control	7.50 ± 0.52	B	109.37 ± 1.47c	B	11.26 ± 0.11b	A
Chemical	8.75 ± 0.63	A	113.87 ± 2.32bc	B	11.47 ± 0.28ab	A
Organomineral	8.15 ± 0.73	B	114.72 ± 0.84bc	B	11.57 ± 0.01ab	A
Cattle manure	8.55 ± 0.43	A	119.55 ± 0.41b		11.42 ± 0.28ab	A
Vermicompost	8.40 ± 0.39	A	127.98 ± 3.58a		12.10 ± 0.23a	A
Mean	8.27 ± 0.24		117.10 ± 1.67		11.56 ± 0.11	
$p_{fertilizer}$	0.566		0.001		0.112	
			Years average			
Control	7.71 ± 0.36		115.19 ± 2.53b		10.52 ± 0.31	
Chemical	8.14 ± 0.39		119.93 ± 2.83ab		10.78 ± 0.30	
Organomineral	8.29 ± 0.38		122.36 ± 3.21ab		10.83 ± 0.31	
Cattle manure	8.48 ± 0.26		118.53 ± 2.15ab		10.54 ± 0.37	
Vermicompost	8.13 ± 0.29		124.61 ± 2.22a		10.98 ± 0.44	
$p_{fertilizer}$	0.613		0.131		0.861	

Small letters show the difference among the applications for the same year and years average. Capital letters show the difference between the years for the same application and parameter ($p<0.05$). Data are expressed as ± standard error.

When the data is analyzed; the difference between fertilizers in the number of pods in a bunch was found to be significant in 2019 (Table 5). In terms of pod weight; while the difference between fertilizers was found to be significant for 2020, it was observed that the difference between years was significant in all applications. The difference between fertilizers was found to be significant in terms of

pod thickness in 2020. In addition, in the thickness of the pod; the difference between years was found to be significant in chemical and cattle manure. The difference among the fertilizer was found to be significant in terms of the number of seeds in the pod in 2020. In addition, the number of seeds in the pod; The difference between years was found significant in control group, chemical, and cattle manure fertilizer. In a study carried out to determine the effects of vermicompost and mycorrhiza use on plant growth and yield in beans and onions, when the values of the pod weight and the number of seeds in the pod were examined, the weight of the pod in bean varied between 5.84 g and 8.94 g, the number of seeds in the pod varied between 4.87 and 5.71 and it was determined that the highest pod weight and the number of seeds in the pod were in the application of vermicompost (Uluğ, 2018). In a study carried out to determine the yield and some quality factors of some dwarf fresh bean cultivars in Konya conditions, the average number of pods per plant was between 13.5 and 33.4, the pod thickness was between 6.7 mm and 7.9 mm, the number of seeds per pod ranged from 6.7 to 7.5 and it was determined that there were significant differences among them (Seymen et al., 2010).

Table 5. The effects of fertilizers on the number of pods per bunch (piece), pod weight (g), pod thickness (mm) and number of seeds per pod (piece).

2019	Number of pods per bunch	Pod weight (g)	Pod thickness (mm)	Number of seeds per pod
Control	3.15 ± 0.15b	5.65 ± 0.22 B	2.61 ± 0.25	6.00 ± 0.00ab B
Chemical	3.22 ± 0.27ab	6.27 ± 0.24 B	2.78 ± 0.10 B	6.25 ± 0.25ab B
Organomineral	4.15 ± 0.41a	5.90 ± 0.52 B	2.95 ± 0.07	6.75 ± 0.25a
Cattle manure	3.55 ± 0.31ab	5.90 ± 0.50 B	2.57 ± 0.25 B	5.50 ± 0.29b B
Vermicompost	3.97 ± 0.22ab	5.65 ± 0.14 B	2.90 ± 0.12	6.50 ± 0.50a
Mean	3.61 ± 0.14	5.87 ± 0.15	2.76 ± 0.08	6.20 ± 0.16
p fertilizer	0.101	0.728	0.461	0.085
2020				
Control	3.40 ± 0.40	7.19 ± 0.10c A	2.75 ± 0.20b	6.70 ± 0.13b A
Chemical	3.50 ± 0.12	8.03 ± 0.04a A	3.05 ± 0.07ab A	7.20 ± 0.14a A
Organomineral	3.45 ± 0.49	8.01 ± 0.08a A	2.89 ± 0.12ab	7.25 ± 0.22a
Cattle manure	3.50 ± 0.31	7.52 ± 0.09b A	3.19 ± 0.10a A	7.15 ± 0.10ab A
Vermicompost	3.75 ± 0.15	7.69 ± 0.09b A	3.18 ± 0.06a	7.40 ± 0.14a
Mean	3.52 ± 0.13	7.69 ± 0.08	3.01 ± 0.06	7.14 ± 0.08
p fertilizer	0.952	0.001	0.087	0.052
		Years average		
Control	3.27 ± 0.20	6.42 ± 0.31	2.68 ± 0.15	6.35 ± 0.15
Chemical	3.36 ± 0.15	7.15 ± 0.35	2.91 ± 0.08	6.73 ± 0.22
Organomineral	3.80 ± 0.32	6.96 ± 0.47	2.92 ± 0.06	7.00 ± 0.18
Cattle manure	3.52 ± 0.20	6.71 ± 0.38	2.88 ± 0.17	6.33 ± 0.34
Vermicompost	3.86 ± 0.13	6.67 ± 0.39	3.04 ± 0.08	6.95 ± 0.29
p fertilizer	0.234	0.710	0.299	0.178

Small letters show the difference among the applications for the same year and years average. Capital letters show the difference between the years for the same application and parameter ($p<0.05$). Data are expressed as ± standard error.

When the data were analyzed, the difference between fertilizers for chlorophyll (SPAD value) in 2019 and 2020 was found to be significant (Table 6). In addition, the difference between years for SPAD value was found to be significant in all applications. In the data of leaf color L* values, the difference between applications for 2020 was found to be significant. The difference between years was also found to be significant for leaf color L* in terms of the control group, organomineral, and vermicompost. In the data obtained from the leaf color a* parameter; the difference between fertilizers and between years for all applications was found to be significant in 2020. In a study investigating the effect of different nitrogen sources at different doses on the yield and amount of chlorophyll in the leaf; It has been reported that increasing doses of fertilizers affect the chlorophyll a and b content (Odabaş and Gülümser, 2001). Abou El-Yazied (2011) reported that the average leaf chlorophyll content varied between 35.4 and 46.4 SPAD values in another study in which the effects of leaf chlorophyll content on bean growth, biochemical components, physiological parameters, and yield were determined. The L* value determines the light-darkness coordinates of the color (Çavuşoğlu and Gökçenay, 2018). The a* value from the color parameters expresses the color changes from red (positive) to green (negative) (Kibar et al., 2020). In a study in which the morphological characteristics of some fresh bean cultivar

candidates and commercial cultivars were determined, it was reported that the leaf color varies between light green and very dark green (Balkaya and Yanmaz, 2003). It has been reported that the L* and a* values of boron applications at different times were higher than the control, the leaf color L* value varied between 35.38 and 52.90, and the leaf color a* value varied between (-1.93) and (-11.92) values in the bean genotypes (Akoğlu, 2013). In a study in which the effects of salt and putrescine applications on germination and seedling growth in beans were determined, the leaf color L* value varied between 39.15 and 41.92, and the leaf color a* value varied between (-8.60) and (-11.74) (Kibar et al., 2020).

Table 6. The effects of fertilizers on chlorophyll (SPAD value), and leaf color values, (L*) and (a*)

2019	**SPAD**		**L***		**a***	
Control	31.46 ± 0.58b	B	45.74 ± 0.46	A	-15.48 ± 0.12	B
Chemical	33.46 ± 0.30a	B	44.62 ± 2.10		-15.78 ± 0.43	B
Organomineral	32.45 ± 0.60ab	B	44.44 ± 1.56	A	-15.73 ± 0.35	B
Cattle manure	32.81 ± 0.44ab	B	44.43 ± 0.80		-15.63 ± 0.22	B
Vermicompost	32.46 ± 0.41ab	B	43.97 ± 0.61	A	-14.98 ± 0.27	B
Mean	32.53 ± 0.24		44.64 ± 0.52		-15.52 ± 0.14	
p $_{fertilizer}$	0.110		0.892		0.359	
2020						
Control	37.84 ± 2.15b	A	40.31 ± 0.29b	B	-11.69 ± 0.63a	A
Chemical	39.92 ± 0.30b	A	41.10 ± 0.77b		-12.17 ± 0.34ab	A
Organomineral	44.45 ± 0.23a	A	41.04 ± 0.43b	B	-12.70 ± 0.37ab	A
Cattle manure	41.03 ± 0.28ab	A	43.08 ± 0.90a		-14.25 ± 0.24c	A
Vermicompost	40.94 ± 1.80ab	A	40.98 ± 0.52b	B	-13.22 ± 0.22bc	A
Mean	40.83 ± 0.70		41.30 ± 0.33		-12.80 ± 0.26	
p $_{fertilizer}$	0.032		0.063		0.003	
			Years average			
Control	34.65 ± 1.59		43.03 ± 1.06		-13.58 ± 0.77	
Chemical	36.69 ± 1.24		42.86 ± 1.23		-13.97 ± 0.73	
Organomineral	38.45 ± 2.29		42.74 ± 0.99		-14.22 ± 0.62	
Cattle manure	36.92 ± 1.57		43.75 ± 0.61		-14.94 ± 0.30	
Vermicompost	36.70 ± 1.81		42.48 ± 0.67		-14.10 ± 0.37	
p $_{fertilizer}$	0.661		0.901		0.592	

Small letters show the difference among the applications for the same year and years average. Capital letters show the difference between the years for the same application and parameter ($p<0.05$). Data are expressed as ± standard error.

Table 7. The effects of fertilizers on the values of leaf color (b), Chroma° and hue°

2019	**b***		***Chroma*°**		***hue*°**	
Control	29.11 ± 0.58	A	33.08 ± 0.50	A	118.46 ± 0.54	B
Chemical	27.66 ± 2.45	A	31.93 ± 2.33	A	120.16 ± 1.56	
Organomineral	27.13 ± 1.90	A	31.40 ± 1.84	A	120.46 ± 1.08	
Cattle manure	27.74 ± 0.55	A	31.88 ± 0.55	A	119.65 ± 0.38	
Vermicompost	26.14 ± 0.78	A	30.26 ± 0.80	A	120.09 ± 0.56	B
Mean	27.55 ± 0.63		31.71 ± 0.60		119.76 ± 0.40	
p $_{fertilizer}$	0.710		0.723		0.601	
2020						
Control	20.34 ± 1.24b	B	23.97 ± 1.36b	B	121.44 ± 0.97	A
Chemical	20.20 ± 1.01b	B	23.64 ± 1.01b	B	121.50 ± 0.82	
Organomineral	20.42 ± 0.40b	B	24.08 ± 0.51b	B	122.28 ± 0.15	
Cattle manure	24.21 ± 1.17a	B	28.14 ± 1.10a	B	120.97 ± 0.95	
Vermicompost	21.45 ± 0.59ab	B	25.23 ± 0.61ab	B	121.81 ± 0.31	A
Mean	21.33 ± 0.51		25.01 ± 0.54		121.60 ± 0.31	
p $_{fertilizer}$	0.042		0.029		0.772	
			Years average			
Control	24.73 ± 1.77		28.52 ± 1.85		119.95 ± 0.76	
Chemical	23.93 ± 1.87		27.78 ± 1.96		120.83 ± 0.85	
Organomineral	23.77 ± 1.55		27.74 ± 1.64		121.37 ± 0.61	
Cattle manure	25.97 ± 0.89		30.01 ± 0.91		120.31 ± 0.54	
Vermicompost	23.79 ± 0.99		27.74 ± 1.06		120.95 ± 0.44	
p $_{fertilizer}$	0.800		0.804		0.588	

Small letters show the difference among the applications for the same year and years average. Capital letters show the difference between the years for the same application and parameter ($p<0.05$). Data are expressed as ± standard error.

When the data is analyzed; in terms of leaf color b* and Chroma° parameters, the difference between fertilizers and between years in all applications for 2020 was found to be significant (Table 7). In the data obtained from the leaf hue° parameter; the difference between years was found to be significant for the control group and vermicompost applications. While the color b* value indicates the color changes from yellow (positive) to blue (negative), the C* value determines the saturation of the color increases as the value increases (Kibar et al., 2020). The hue (h) value, one of the color criteria in the study, is used to express the whole ratio of basic colors (Çavuşoğlu and Gökçenay, 2018). In a study in which the effects of salt and putrescine applications on germination and seedling growth in beans were determined, it was found that the b* value of leaf color varied between 16.62 and 23.22, Chroma (C*) value between 18.71 and 25.62, and hue° color values between 116.83 and 118.29 (Kibar et al., 2020). Leaf color b* values in all genotypes of boron applications at different times were found to be lower than the values in control applications, and an increase was observed in the leaf color C* values of the genotypes in parallel with increasing boron concentrations. It has been reported that the color b* value varies between 12.39 and 21.78, the C* value varies between 15.62 and 22.36, and the hue value varies between 96.28 and 128.13 (Akoğlu, 2013).

Conclusion and Recommendations

When the average of years is examined in the results obtained in the current study; it was determined that plant height, stem diameter, the number of nodes, flower bud length, flower bud width, stem length, the number of flower buds per cluster, bract length, middle leaflet length, the number of leaves, first pod height, pod length, pod width, the number of pods per bunch, pod weight, pod thickness, and chlorophyll (SPAD) values increased compared to the control group for all fertilizer applications. Organomineral and vermicompost fertilizers were equivalent to chemical fertilizers in plant height, stem diameter was slightly higher than other studies due to the variety difference. The number of internodes did not show any change in cattle manure compared to the control group, and chemical and vermicompost fertilizers for internode length decreased compared to the control group. In the number of nodes with the first flower, all fertilizer applications except chemical fertilizer increased compared to the control group. In the number of seeds in the pod, all fertilizer applications increased compared to the control group, except for cattle fertilizer application. While all fertilizer applications except cattle manure for leaf color L* were compared to the control group, it was determined that all fertilizer applications were lower than the control group. All fertilizer applications were greener than the control group because the negative a* value indicated greener color. When the leaf color b*, Chroma° and hue° results obtained from the study are examined; it was determined that different results were obtained according to fertilizer applications and years. Moreover, the data obtained from these parameters were in agreement with previous studies. it has been determined that this agreement is within the value range obtained from the results of previous studies and that bean genotypes may differ from each other in terms of variety characteristics. So, organic and organic mixed fertilizers could give equivalent or better results than chemical fertilizers. In the bean plant with different fertilizer applications, the best results were found in vermicompost fertilizer and organomineral fertilizer, which is chemical mineral fertilizers with organic matter, and it was determined that they had equivalent or better results than chemical fertilizers. In addition, it is important to test organomineral and vermicompost fertilizers with different ratios and combinations in future studies, where the best results are obtained from fertilizer applications. In addition, it is thought that these fertilizer applications will be beneficial in terms of determining the best fertilizer combination that can be offered as an alternative to chemical fertilizer in bean cultivation with the results to be obtained with different combinations of micronutrient additives.

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Research Article

Influence of Different Stocking Densities on Some Blood Parameters in Laying Hens

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Abstract: The aim of the scientific work is to study the changes in the hematological indicators of chickens when they are kept at a high stocking density. In the conditions of the current complex for the formation of eggs, 4 groups of laying hens were formed. The results showed that high stocking density to 24.0 birds m^{-2} was accompanied by an increase in their blood of leukocytes by 12.2%, heterophils – by 1.8%, and a decrease in thrombocytes' concentration by 4.0%. Provided that the planting density is increased to 25.3 birds m^{-2}, there was an increase in the content of leukocytes by 13.7%, heterophils – by 3.1%, and a decrease in thrombocytes concentration by 10.8% with a decrease in their volume by 9.2%. Further increase in stocking density to 26.7 birds m^{-2} caused an increase in the blood content of leukocytes by 22.7%, heterophils – by 13.5%, and a decrease in thrombocytes concentration by 69.0% with a decrease in their volume by 18.6%. Thus, the high stocking density of laying hens is shown by changes in their hematological parameters, which is reflected in the increase of leukocytes, due to an increase in the number of heterophils, and a decrease in thrombocytes.

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1. Introduction

The effect of technological stressors, such as increased planting density, the formation of new microclimate conditions of production premises, the conditions of keeping hens, and the composition of the diet, vaccination, transportation, and movement reduce the level of the immunological reactivity of poultry, which causes a productivity decrease (Hall et al., 2014; Stoyanovskyy et al., 2018; Zhuchaev et al., 2019). It is impossible to avoid the influence of stressors in the conditions of intensive production, as increased stocking density is one of the ways to save resources in the poultry industry and is often used to produce more products per 1 m^2 (Sakhatsky et al., 2020). However, the effect on the body of hens of high stocking density has not been studied enough. It should be noted that under the influence of stress, the activity of all physiological structures that are responsible for the process of adaptation to changed life factors is undermined. The reason for the spread of the stress reaction is an increase in the dynamics of the work of the endocrine glands, in particular on the axis of the hypothalamus-anterior lobe of the pituitary gland-adrenal cortex (Olubodun et al., 2015).

An important place in the development of stress is occupied by the cortex of the adrenal glands, because they, due to the influence of the pituitary gland, contribute to the development of secreted steroid hormones, which in turn play an important role during adaptation (Berger et al., 2019). Based on this, it is appropriate to note that the main tools for the development and maintenance of stress in a living organism are the sympathoadrenal and hypothalamic-pituitary-adrenocorticotropic systems. Thus, this process is generally affected by adaptive reactions that appear during the appearance of non-specific environmental conditions. This process can be implemented at the expense of the hypothalamic-pituitary-adrenal axis, as well as the sympathoadrenal system, based on catecholamines (Infante et al., 2017). The latter can implement such a process as the transition of the body from a state of rest to a state of excitement. Such a function is embedded in their biological actions, respectively, catecholamines contribute to ensuring the necessary condition for the body. At the same time, one should not forget about the possibility of increasing and deforming metabolic processes in immunocompetent poultry tissues, which is possible due to their implementation of physiological reactions, as well as the use of hormones of the medulla of the adrenal glands and mediators of the sympathoadrenal system (Stoyanovskyy et al., 2018; Zhanabayeva et al., 2021).

Recent studies showed that acute stress caused significant changes in the quantitative and qualitative composition of hens' leukocytes (Weimer et al., 2019; Nwaigwe et al., 2020). The leukemoid reaction of hens blood can be caused by different stressful factors such as starvation, temperature, light, contamination by microorganisms, transportation, shackling, and others (Huth and Archer, 2015; von Eugen et al., 2019; Hofmann et al., 2021; Hussein, 2021; Cellak and Babacanoğlu, 2022; Noaman et al., 2022). However, the vast majority of studies are concerned about the effects of acute stress on hens. The issue of transformations that occur in the structure of chickens under chronic stress caused by high density has not been studied enough.

The purpose of the research is to study the impact of high stocking density on the physiology and other parameters of the hens' bodies.

2. Material and Methods

2.1. Study sample

The research was based on the study of such living organisms as industrial laying hens Hy Line W-36. The main feature in the selection of hens for research was the intensity of egg laying. Hens were kept for 34 weeks in cages of multilevel batteries at different stocking densities: one group by European standards (13.3 birds m^{-2}) and three groups with increasing stocking densities – to 24.0; 25.3 and 26.7 birds m^{-2}. Laying hens were individually placed in appropriate conditions that resembled an aviary in terms of area (2640 m^2), in which 12-tier cage accumulators "Salmet" were placed (Table 1). Thus, this structure included 18,144 cages with an area of 7,506 cm^2 (120 x 62.55 cm). Experiments with animals were based on principles defined by the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (1986).

Table 1. The structure of the appearance of changes caused by an increase in stocking density on the hematological indicators of laying hens in systems of multi-tier accumulators

Characteristic	Group of laying hens			
	I	II	III	IV
The number of hens in the cage	10	18	19	20
Stocking density, birds m^{-2}	13.3	24.0	25.3	26.7
Provision of area, cm^2 per bird	750.6	417.0	395.1	375.3
Feeding front, cm	12.0	6.7	6.3	6.0

2.2. The environmental conditions

The stocking density of hens of the I group met the European norms and requirements of the manufacturer (Guide to the…, 2019) – 13-20 birds m^{-2} (area of 490-750 cm^2 per bird and feeding less than 7.0 cm per bird), the II group – Ukrainian standards (VNTP-APK-04.05…, 2005) – 22-25 birds m^{-2} (area – 400-450 cm^2 per bird), hens of the III and IV groups were held quite tightly. The housing density was regulated by the number of hens in the cage, which led to different feeding. During the experiment,

laying hens were given drinking water, as well as compound feed (Table 2), and kept in accordance with the requirements (Guide to..., 2019). The windows of the henhouse face the south side, and the door faces the east side. This is necessary in order for enough light to enter throughout the day. Because, due to the long daylight hours, the egg-laying capacity of hens increases. Artificial lighting is used early in the morning and at sunset. The henhouse has effective ventilation. The optimal temperature for keeping laying hens is +12 °C. The relative humidity of the air is 70%. Perches and nests are at a height of at least 60 cm above the floor. The floor is lined with sawdust litter in several layers. This serves not only to maintain hygiene but is also an additional source of heat.

Table 2. The content of the structure of special feed intended for laying hens in the production stage,%

The nutrient content of the rations of experimental hens	Dynamics of egg laying, %			
	95-100	93	88	85
Wheat	20.418	19.336	12.000	10.566
Corn	37.053	45.399	54.330	52.334
Sunflower meal	20.754	22.278	18.166	23.533
Soybean meal	7.000	0.000	3.000	0.000
Soybean oil	0.959	0.661	0.000	0.500
Shell 0-3 mm	10.701	9.922	10.25	11.088
Salt (NaCl)	0.210	0.200	0.200	0.210
Monocalcium phosphate	1.193	0.811	0.805	0.532
Sodium sulfate	0.160	0.117	0.120	0.095
Methionine	0.186	0.105	0.088	0.076
Lysine sulfate	0.637	0.585	0.516	0.579
Threonine	0.127	0.095	0.057	0.065
100	0.000	0.010	0.000	0.000
Globamax 1000	0.100	0.000	0.000	0.000
Proactive	0.000	0.000	0.150	0.150
Enteronormin Detox	0.150	0.150	0.000	0.000
Mastersorb	0.150	0.130	0.130	0.000
Mycocide Pro	0.000	0.000	0.000	0.090
Choline chloride	0.050	0.050	0.040	0.035
Cronozyme	0.000	0.000	0.000	0.011
Carophyll yellow	0.003	0.003	0.003	0.003
Carophyll red	0.003	0.003	0.003	0.003
Mineral complex	0.100	0.100	0.100	0.100
Vitamin complex	0.033	0.030	0.030	0.030
Total	100.000	100.000	100.000	100.000

Note: The content of the vitamin complex: Vitamins A, D, E, C, K, vitamins of group B; the content of the mineral complex: phosphorus, calcium, sulfur, manganese, and sodium chloride).

2.3. Randomization method

At the beginning of the study, at the age of 14 weeks, and at the end, namely at 52 weeks, 30 blood samples were taken from the studied living organisms. In this case, 1.0-1.5 ml of the substance was taken from the axillary vein into a test tube with EDTA (ethylenediaminetetraacetic acid). The following indicators were determined: the content of erythrocytes, leukocytes, and thrombocytes – change of impedance in conductometric method; hemoglobin – spectrophotometric method; hematocrit – integer integration; the average volume of erythrocytes, the average content of hemoglobin in the 1 erythrocyte, the concentration of hemoglobin in erythrocytes, with the distribution of erythrocytes, the average volume of thrombocytes – calculation based on the saved results of direct measurements; trough speed (the content of monocytes, lymphocytes, eosinophiles, basophils, and heterophiles – change of impedance by the conductometric method. The ratio of heterophils to lymphocytes was determined using the action of a division, in which the divisor was the number, and the divisor was the number of lymphocytes. Reference values of hens' hematological parameters were determined by N.C. Jain (1993).

2.4. The laying performance and egg quality parameters

Hematological parameters of hens were determined on a Micros 60 hematology analyzer (Horiba Ltd. (Privat,e limited company)) in the "Bald" stations (certificate No. LB/02/2016). Trading assets from Horiba Ltd. (United States of America) were used for the determination of hematological parameters, namely the reagent for diluting "ABX Minidil LMG", lysing reagent "ABX Minilyse LMG", reagent for washing "ABX Cleaner", "ABX Miniclair" deproteinizer, "ABX Minipack LMG" reagent container and a set of controls 2N, 1H, 1L "Para 12 Extend".

Eggs were weighed and the optimal quality parameters were determined: the egg weight was 53.9 g; egg length was 5.1 cm; egg width was 4.09 cm; yolk weight was 16.3 g; yolk height was 1.2 cm; albumen weight was 33.1 g; albumen height was 4.8 mm; shell thickness was 0.3 mm; yolk colour was 1.1; yolk index was 39.1; egg shape index was 0.9; albumen index was 20.8; specific gravity was 1.3; haugh unit was 67.1.

2.5. Data analysis

One-way analysis of variance (ANOVA) and Tukey-Kramer multiple comparison testing were used to establish and investigate group differences. The latter instrument was applied in the context of a posthoc test tool. Information placed in Tables 1; 2 was presented as M ± SEM (mean ± standard error of the mean). Assessment of the distribution of sample materials for normality was carried out using the Kolmogorov-Smirnov criterion. In this case, if the data distribution was significantly different from normal, the Mann-Whitney U-test was applied. Thus, the concept of normal differences meant those that reached a value of $p<0.05$. The statistical analysis data were produced by Microsoft Excel.

3. Results and Discussion

The hematological indicators of the chickens of all objects of the research objects at its initial stage corresponded to the physiological qualities of the indicators. Therefore, no fundamental distinguishing features of the studied categories were found. According to the results of studies (52 weeks of life), regardless of the stocking density the hemoglobin, erythrocytes, and hematocrit parameters in the blood of hens were within the physiological values (Table 3), while the content of leukocytes increased in proportion to the stocking density and in the IV group of hens the physiological norm exceeded. In general, the content of leukocytes in the blood of the IV group of birds significantly 22.7% higher than the I group, 9.3% higher than the II group, and 7.9% greater than the III group of hens. It should be noted that the structure of leukocytes in the blood of the population of groups I-III corresponded to the norm of biological values, but reached the upper limit. The content of leukocytes in the blood birds of the II group was significantly better by 12.2%, and the III – by 13.7% compared to the I group. There was no significant difference in the content elements in the blood of the research subject of II and III groups.

Table 3. Hematological parameters of laying hens depending on stoking density (n = 30 per group)

Parameters	Category of laying hens				Reference indicators
	I	II	II	IV	
Leukocytes, thousand per µl	34.4 ± 1.07^{a}	38.6 ± 0.61^{b}	39.1 ± 0.28^{b}	42.2 ± 1.16^{c}	20-40
Hemoglobin, g dl^{-1}	10.8 ± 0.33^{ab}	12.3 ± 0.12^{a}	11.0 ± 0.12^{b}	11.1 ± 0.23^{ab}	7-13
Hematocrit, %	31.7 ± 0.87	31.9 ± 0.46	31.1 ± 0.26	30.6 ± 0.54	22-35
Erythrocytes, million per mm^3	3.0 ± 0.08	3.1 ± 0.07	3.0 ± 0.01	2.9 ± 0.06	2.5-3.5
Thrombocyte, thousand per mm^3	46.3 ± 0.25^{a}	44.5 ± 0.09^{b}	41.8 ± 0.92^{c}	27.4 ± 0.35^{d}	32-100

Note: $^{a, b, c, d}$ – express traits that were fundamentally different in one row of the table ($P<0.05$).

There was also a decrease in thrombocytes in hens` blood with increasing stocking density. The lowest content of thrombocytes was established in the IV group of poultry – by 69.0%; 62.4% and 52.6% less in contrast to categories I, II, and III. The structure of thrombocytes in the I-III groups of hens`

blood decreased with increasing stocking density but was within the physiological values. Thus, in the blood of hens of the II group, fewer thrombocytes were found by 4.0% than in the I group, and in the III group of hens – by 10.8% and 6.5% than in the I and II groups. Erythrocyte and thrombocyte indices were within the physiological values in all groups of hens (Table 4). At the same time, there was a noticeable increase in the level of the proportion of hemoglobin in one erythrocyte within the physiological value at a high planting density. Thus, the highest content of hemoglobin in one erythrocyte was established in the IV group of hens, and the lowest – was in the first group.

Table 4. Erythrocytes and thrombocytes indices of laying hens depending on stoking density (n = 30 per group)

Parameters	Category of laying hens				Reference indicators
	I	II	II	IV	
Average erythrocyte volume, mkm^3	106.0 ± 0.96^a	98.2 ± 0.91^b	104.2 ± 0.59^a	105.4 ± 0.73^a	90-140
The average content of hemoglobin in the 1 erythrocyte, pg.	34.0 ± 0.34^a	34.4 ± 0.44^a	36.6 ± 0.38^b	38.4 ± 0.37^c	33-47
Concentration of hemoglobin in erythrocytes, g dl^{-1}	34.1 ± 0.32	34.9 ± 0.11	34.4 ± 0.26	34.1 ± 0.34	26-35
Erythrocyte distribution width, %	7.8 ± 0.13^a	7.9 ± 0.09^a	7.9 ± 0.07^a	8.2 ± 0.06^b	10-15
Average volume of thrombocyte, mkm^3	8.3 ± 0.30^{ab}	8.7 ± 0.15^a	7.6 ± 0.12^b	7.0 ± 0.17^c	7-10

Note: $^{a, b, c}$ – express traits that were fundamentally different in one row of the table ($P<0.05$)

There was also a decrease in the average volume of thrombocytes within the physiological values with an increasing stocking density of hens. The average volume of platelet in the IV group of birds was at the lower limit of the physiological values, which is less by 18.6%; 24.3% and 8.6% compared to the I, II, and III groups. In addition, no fundamental distinguishing features were established between the hens of the I and II groups, while the thrombocyte of hens of the III group had a smaller volume compared to the I group by 9.2%, and from the II – by 14.5%. In groups with a high density of planting, indicators were established that characterized the dynamic development of elements such as heterophils in the blood of hens (Table 5). Thus, in the IV group of hens, the number of heterophils exceeded the physiological values by 5.8% and in comparison with other groups – by 13.5; 11.7, and 10.4% higher than in the I, II, and III groups. The content of heterophils increased in I-III categories of birds, according to physiological indicators. In particular, in the II categories of hens` blood the content of heterophils was higher by 1.8%, and in the III group – by 3.1 compared to the I group.

Table 5. Leukogram of laying hens depending on stoking density, % (n = 30 per group)

Parameters	Category of laying hens				Reference indicators
	I	II	II	IV	
Monocytes	8.4 ± 0.18^a	6.2 ± 0.06^b	5.2 ± 0.23^c	3.4 ± 0.42^d	5-10
Lymphocytes	63.8 ± 0.52^a	62.0 ± 0.18^b	61.4 ± 0.42^b	52.2 ± 1.11^c	45-70
Eosinophils	2.5 ± 0.18^a	4.7 ± 0.26^b	5.0 ± 0.65^b	5.6 ± 0.58^b	1.5-6.0
Basophils	3.0 ± 0.25	3.0 ± 0.09	3.0 ± 0.22	3.0 ± 0.53	1-3
Heterophils	22.3 ± 0.70^a	24.1 ± 0.15^b	25.4 ± 1.34^b	35.8 ± 2.03^c	15-30

Note: $^{a, b, c, d}$ – express traits that were fundamentally different in one row of the table ($P<0.05$).

The content of eosinophils increases within the limits of physiological permissible indicators and in the IV group of hens reached its upper limit and was higher by 3.1% in contrast to the 1st group. The difference between the II and III groups was not statistically significant. At the same time, the content of eosinophils in blood was higher in the II group of hens by 2.2%, and in the III group – by 2.5% compared to the I group. The increase in the content of heterophils and eosinophils occurred due

to a decrease in the share of other types of leukocytes, namely lymphocytes, and monocytes. The number of lymphocytes in the IV categories of hens` blood was lower by 11.6; 9.8 and it was more than in the I, II, and III groups by 9.2%. The number of monocytes in the IV group of hens was 5.0% lower compared to the I group and 2.8% and 1.8 % compared to the II and III groups. In the II group of hens, it was found few monocytes by 2.2% compared to the I group, and in the hens of the III group – by 3.2 and 1.0% compared to the I and II groups. A significant crowding of laying hens was accompanied by the development and spread of the concentration of leukocytes in the blood. In particular, the concentration of leukocytes in the blood increased by 12.2% with high crowding of hens to 24.0 birds m^{-2}; up to 25.3 birds m^{-2} – by 13.7%; for increasing the stocking density to 26.7 birds m^{-2} was characterized by an increase in their number by 22.7%. The obtained data agree with the results of research by other scientists, who describe the dynamics of the distribution of leukocytes in the blood of laying hens, broiler chickens, and ducks at high stocking density (Kang et al., 2018; Nwaigwe et al., 2020; Xiong et al., 2020).

The increase in the content of leukocytes is a characteristic response of immunocompetent tissues to the action of glucocorticoids and catecholamines, the concentration of which in the blood of hens increases under the influence of various stressors (Jiang et al., 2017; Gryshchenko et al., 2019). Thus, the basis for the development of leukocytosis is an increase in the heterophil content in the blood, as noted in these studies (Table 5). According to a number of authors (Dhabhar et al., 2012; Wirths et al., 2014), the increase in the content of leukocytes due to heterophils occurs due to hypercortisolemia and hypercatecholamania, which lead to an increase in their mobilization in the blood. The increase in circulating heterophiles is the result of the body's preparation for a protective response to possible damage (Liew and Kubes, 2019; Vashchyk et al., 2020). The obtained results confirm this assumption. According to the results of research, it is shown that high crowding of hens to 24.0 birds m^{-2} leads to an increase in heterophils by 1.8%, to 25.3 birds m^{-2} – by 3.1%; to 26.7 birds m^{-2} causes an increase of their content by 13.5%. The obtained data on the increase in the content of heterophils coincide with the results obtained in similar experiments with overcrowding of laying hens, as well as in broiler chickens during stress, and stress from starvation, heat, and immobilization stress (Kang et al., 2018; Li et al., 2019; Nwaigwe et al., 2020; Gul et al., 2021).

Increased levels of heterophils in the blood can be explained by two assume mechanisms: slowing of the transition of heterophils from the blood to peripheral tissues caused by an increase in blood corticosteroid hormones due to stressors, as well as redistribution of heterophils in the vascular bed by reducing parietal and increasing circulating pools caused by hormones such as adrenaline, noradrenaline, and cortisol. At the same time, it is known that the rapid mobilization of the parietal pool of heterophils is due to adrenaline under acute stress, and the mobilization of the bone marrow pool occurs under the influence of corticosteroids during chronic stress (Ince et al., 2019; Nykonov et al., 2019). Therefore, it can be assumed that the increase in the level of heterophils in the blood during prolonged compaction of hens is due to the mobilization of the bone marrow pool of heterophils and the slowing of their transition from blood to peripheral tissues. There was also a decrease in thrombocyte concentration, with a high stocking density of hens. In particular, when creating tight conditions for laying hens up to 24.0 g m^{-2}, there was a decrease in thrombocyte content by 4.0%, to 25.3 birds m^{-2} – by 10.8%; to 26.7 birds m^{-2} was accompanied by a decrease in their concentration by 69.0%. The decrease in the volume and in the concentration of thrombocytes in the hens` blood – to 25.3 birds m^{-2} – by 9.2%, and to 26.7 birds m^{-2} – by 18.6%. The obtained data confirm a reduction in the number of platelets in the blood of broiler chickens in response to heat stress.

Conclusion

Changes in hematological parameters of laying hens due to factors caused by increased stocking density were analyzed. It was established that the high stocking density of laying hens is accompanied by changes in their blood system, which is reflected in the increase in its content of leukocytes, due to an increase in the number of heterophils, and a decrease in thrombocyte count. The decrease reduction in the number of thrombocytes in the blood may be explained by the high level of activity of lysosomal enzymes of heterophils in plasma, the activity of which increases due to increased heterophils.

In particular, an increase in the density of chickens to 24.0 birds m^{-2} was accompanied by a 12.2% increase in leukocytes in their blood. Heterophils – by 1.8% and a decrease in thrombocytes'

concentration by 4.0%. With a further increase in the density of planting up to 25.3 birds m^{-2}, there was an increase in the content of leukocytes by 13.7%, heterophils – by 3.1%, and a decrease in thrombocytes concentration by 10.8% with a decrease in their volume by 9.2%. An increase in the density of planting to 26.7 birds m^{-2} was characterized by the development of leukocytes in the blood by 22.7% (5.5%>normal), heterophils – by 13.5% (19.3%>normal), and a decrease in the concentration of platelets. by 69.0% (14.4%<normal) with a decrease in their volume by 18.6%.

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Research Article

Comparison of Some Biochemical Properties in the Seeds and Juice of Grapevine Cultivars (*Vitis vinifera* L.)

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Proanthocyanidin,
Seed

Abstract: This study revealed some biochemical properties in the seeds and the fruit juice of twelve local grapevine cultivars- Karrod, Siyah gozane, Tayifi, Resealya, Heseni, Boga, Binetati, Beyaz sinciri, Askar, Emiri, Duvrevi, and Cicikenator. The colored and white grapes were categorized separately, and it was evaluated whether the skin color affected the biochemical content of the seed, and the compositions of the seeds were compared with the parallel samples taken from the juice. Total phenolic contents, proanthocyanidin contents, and total antioxidant capacities were also determined in the samples taken from the seeds and fruit juice for comparison purposes. The obtained results were mostly statistically significant [(P<0,01), (P<0,05)]. Considering the highest values detected in the seeds; the highest phenolic content was determined in cv. Binetati (87.30 GAE mg $100g^{-1}$); antioxidant capacity was in cv. Gozane (1344.86 mg g^{-1}) and Proanthocyanidin value was in cv. Cicikenator (34.19 mg CE g^{-1}).

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1. Introduction

Grapevine, which is known to have been cultivated in Anatolian lands for at least 8 thousand years, is among the most important cultural plants for all civilizations that have existed in these lands. In addition to being consumed as fresh and dried grapes, it can be converted into many products such as wine, vinegar, molasses, fruit pulp, and several other sweets (kofter, sucuk, bastik, etc.) obtained by processing the must (Gazioglu Sensoy and Tutus, 2017; Unal, 2022). Around 4.2 million tons of grape production, which is 91 million tons worldwide, is made in Türkiye. Among the world countries, Türkiye ranks 5th with its vineyard area of 405 439 ha and 6th in terms of production amount. It has been reported that a total of 4.2 million tons of grapes are produced in the country, 2.2 million tons of which are table grapes, 1.5 million tons for dried, and about 0,5 million tons of wine (Anonymous, 2020).

Although studies on grapevines are generally concentrated in fruit juice, few studies have shown that the nutrient content in the seed is approximately four times higher than in fruit juice (Pantelić et al., 2016; Kamaladdin et al., 2020). Because most of the total grape production in the world is processed into wine, approximately 10 million tons of grape pulp is produced in the world in a few weeks of the

harvest campaign. This pulp remaining from wine and grape juice production contains significant levels of bioactive compounds (Demirtas et al., 2013; Barba et al., 2016; Gazioglu Sensoy, 2019). Grape seeds constitute an important part of this pulp, 38-52% by dry weight. While many wineries did not consider grape pulp as recyclable waste until recent years, these pulps, which have a very high nutritional value, have started to be sold as raw materials for factories producing grape seed oil or for sectors producing cosmetics and pharmaceuticals due to their high antioxidant content (Teixeira et al., 2014; Rombaut et al., 2015; Machado and Perles, R. 2017; Sevindik and Selli, 2020). It has been reported that the annual average amount of grape seed production in Türkiye is 25 000- 30 000 tons (Anonymous, 2014).

The most significant byproducts from the wine and grape juice industries are grape seeds, and proteins (6.3-11%), fiber (40%), oil (11.6-16.5%), complex carbohydrates (29.2%), proanthocyanidin, phenolic compounds, fatty acids, minerals, tocopherols, and other vitamins are all present in large amounts in grape seeds (Molva and Baysal 2015; Tangolar et al. 2019).

A survey study conducted in the province of Siirt, where the plant material is supplied, states that although the region has a very high potential in the field of viticulture, this potential is not used sufficiently, especially in terms of the amount of product obtained from the unit area (Gazioglu Sensoy et al, 2020). The present study aimed to provide a better understanding of the importance and value of both grape seeds and grape juice in these genetic sources. Revealing the valuable compositions of the seeds might help to prevent the seed leftovers from the production of foods made from molasses and grape juice, which are parts of this ancient culture, as waste, and to bring these valuable wastes into production. It is thought the present study might constitute an important reference for studies on the determination of chemical properties of other grapevine cultivars. The data within the scope of the study were categorized based on the white and colored grapevines to determine the effect of grape skin color on the studied traits.

2. Material and Methods

2.1. Plant materials and sampled vineyards

In the present study, twelve local grape cultivars are grown in the Siirt province of Türkiye colored grapes (Karrod, Siyah Gozane, Emiri, Tayifi, Resealya, and Boga) and white grapes (Askar, Duvrevi, Binetati Cicikenator, Haseni, and Sinciri) were studied as the plant materials. All of the cultivars were not grafted and grown on their roots. They all have produced similar conditions; in rainfed conditions with no fertilization. Goble applications are traditional training systems for 10-15 years old vineyards.

2.2. Methods

2.2.1. Sample preparation

The width, length, and weight measurements of the seeds and the number of seeds were determined in the samples taken from the local cultivars chosen from the vineyards with similar cultivation conditions. The above-mentioned measurements, counts, and weighing were carried out in 3 replications for each cultivar and 10 berry samples (taken as mixed from the middle parts of the clusters) per replication (using two clusters randomly taken from 5 different vines). Grape berries were harvested at full maturity in the 2020 production season and stored at -20 °C until analysis, and fresh grape seeds were used in the analysis. (Duran, 2014; Demiray, 2019). Total phenolic compounds, total antioxidant capacity, and proanthocyanidin in the seeds and grape juice of the cultivars were studied.

2.2.2. Total phenolic content

Total phenolic content was determined by spectrophotometer by modifying the Folin-Ciocaltaeu calorimetric method (Swain and Hillis, 1959). About 50 g of grape berry flesh was fragmented and 1 ml of grape juice from each sample was transferred to centrifuge tubes. About 5 g of grape seeds were fragmented and 1 g from each sample was transferred to centrifuge tubes. Then, 5 ml of methanol was added and centrifuged at 10 000 rpm for 10 minutes, and the supernatant remaining on top was taken. 150 µl of supernatant is taken from the part, 2400 µl of distilled water, 150 µl of Folin cioucelta (1:10 solution) are added, 3-4 seconds vortex is made, 300 µl of 20% sodium carbonate is added, and it is kept in the dark at room temperature for 60 minutes, then the absorbance of the resulting solution was read

spectrophotometrically at 725 nm wavelength and the total amount of phenolic substance was expressed as gallic acid equivalent (GAE) mg 100 g^{-1} fresh weight (FW). The analyses were carried out in 3 replications for each cultivar.

2.2.3. Total antioxidant capacity

About 50 g of grape berry flesh was fragmented and 1 ml of grape juice from each sample was transferred to centrifuge tubes. About 5 g of grape seeds were fragmented and 1 g from each sample was transferred to centrifuge tubes. Then, 5 ml of methanol was added and centrifuged at 10 000 rpm for 10 minutes, and the supernatant remaining on top was taken. The FRAP (Ferric Reducing Antioxidant Power) reagent was prepared with a 300 mmol l^{-1} acetate buffer (pH 3.6), 20 mmol/L ferric chloride ($FeCl_3.6H_2O$), and 10 mmol/L TPTZ (2,4,6-tripyridyl-s-triazine in 40 mmol/L hydrochloric acid) at a ratio of 10:1:1. The mixture prepared for ABTS analysis with 2850 µl of FRAP reagent for samples was diluted 50 times with ethanol, then 150 µl of the sample was mixed and left at room temperature for 30 minutes. The resulting ferrous tripyridyl triazine complex was measured at 593 nm in the spectrophotometer and the results were reported as µmol Trolox equivalent (TE) g^{-1} FW (Lutz et al., 2011). Trolox concentration range has been studied as 0-500 ppm.

2.2.4. Proanthocyanidin content

About 50 g of grape berry flesh was fragmented and 1 ml of grape juice from each sample was transferred to centrifuge tubes. About 5 g of grape seeds were fragmented and 1 g from each sample was transferred to centrifuge tubes. After the samples were centrifuged, the supernatant remaining on top was removed, first passed through coarse filter paper and then through a 0.45 µm membrane filter twice. The obtained extraction was kept at -20°C until readings were taken. The total proanthocyanidin content of the must and seeds of the cultivars was determined using the dimethylaminocinnamaldehyde (DMACA) method. A 1% (w/v) DMACA solution in a cold mixture of methanol and HCl (4:1) was prepared just before analysis. It was diluted with methanol (1/10, v/v) and 1.5 mL of acidified DMACA solution was added to 30 µL of extraction. The mixture was allowed to react for 10 minutes at room temperature. The samples were read against a methanolic blank solution at 640 nm absorbance in the spectrophotometer. The amount of proanthocyanidin was standardized according to a catechin curve expressed as mg catechin (CE) equivalent/gram (mg (CE) g^{-1}) (Nayak et al., 2018). The amount of proanthocyanidin was standardized according to a catechin curve expressed as mg catechin (CE) equivalent g^{-1} (mg CE g^{-1}). The calibration curve was formed by diluting the stock solutions with methanol to give the standard concentration for catechin [(+) Catechin Sigma Aldrich] in the range of 1-250 mg l^{-1}. The analyses were carried out in 3 replications for each cultivar.

2.2.5. Statistical analysis

The measurement, weighing, and laboratory analysis results obtained in the study were subjected to a completely randomized design, and the differences among the grape cultivars were analyzed with the One-way ANOVA in the SPSS package program (IBM SPSS Statistics 21.0). Duncan's multiple comparison test was used to determine the differences between the means according to $P<0.01$ and $P<0.05$ levels. Significance levels, mean values, and ± standard error values are indicated in the tables (Eckstein, 2013).

3. Results

3.1. Seed traits

The difference in the number of seeds of the cultivars was statistically significant ($P<0.01$), and the maximum number of seeds per berry was 3.00 in cv. Emiri with black skin color and the least seed per berry was 1.47 in cv. Heseni with white skin color. It was seen that the number of seeds of the colored cultivars was higher and the average number of seeds according to the colors was also significant ($P<0.01$). (Table 1). There were significant ($P<0.01$) differences for each grape color. Accordingly, the highest value was found to be 0.12 g in cv. Cicikenator and the lowest value was 0.06 g in cv Karrod, cv. Boga and cv. Askar. Seed weights were not statistically different between the two grape skin colors (Table 1). The seed widths of each cultivar were statistically significant ($P<0.01$), but the difference

between grape skin colors was insignificant. The highest value was determined as 4.83 mm in cv. Siyah Gozane and the lowest value was found as 3.20 mm in cv. Beyaz Heseni (Table 1). There was a significant (P<0.01) difference, and the highest value was 9.77 mm in the white cv. Cicikenator and the lowest value was 4.60 mm in black cv. Emiri (Table 1). The seed size was also found to be significantly different (P<0.01), with the highest value being 44.18 mm in white cv. Cicikenator variety and the lowest 14.63 mm in the black cv. Emiri variety (Table 1).

Table 1. Some seed traits in the studied grape cultivars

Grape skin color	Grape cultivars	Seed number	Seed weight (g)	Seed width (mm)	Seed length (mm)	Seed size (width x length)
White	Askar	1.60±0.12B-D**	0.06±0.00 C**	3.90±0.06 B**	7.33±0.09BC**	28.61±0.77 C**
	Binetati	2.73 ±0.07 A	0.07± 0.00 B	3.70±0.15 B	7.70±0.21 B	28.55±1.92 C
	Sinciri	1.73 ±0.18 BC	0.11± 0.01 A	4.43±0.15 A	7.90±0.23 B	34.96±0.15 B
	Heseni	1.47 ±0.07CD	0.08± 0.00 B	3.20±0.06 C	6.33±0.03 D	20.27±0.46 D
	Cicikenator	1.20± 0.12 D	0.12± 0.01 A	4.53±0.19 A	9.77±0.62 A	44.18±2.56 A
	Duvrevi	2.00 0.20 B	0.09± 0.00 B	3.80±0.12 B	6.47±0.17 CD	24.57±0.96 CD
	Mean	**1.79±0.13 B****	**0.09±0.01 ns**	**3.93±0.12 ns**	**7.58±0.29 A****	**30.19±1.92 ns**
Colored	Boga	2.80±0.12AB**	0.06±0.00C**	4.50±0.00 AB**	7.27±0.19 ns	32.70±0.84 A**
	Karrod	1.60±0.23 C	0.06±0.01 B	3.37±0.17 C	6.50±0.12	21.85±0.75 BC
	Tayifi	1.67±0.24 C	0.09±0.00 A	3.83±0.19 BC	7.13±0.19	27.31±1.11 AB
	Siyah gozane	2.93±0.24 AB	0.10±0.01 B	4.83±0.38 A	6.97±0.12	33.76±3.28 A
	Emiri	3.00±0.12 A	0.09±0.00 A	3.33±0.23 C	4.60±1.95	14.63±6.11 C
	Resealya	2.33±0.13 B	0.10±0.00 B	3.73±0.18 C	6.60±0.15	24.66±1.45 AB
	Mean	**2.39±0.15 A**	**0.08±0.00**	**3.93±0.16**	**6.51±0.35 B**	**25.82±1.88**
	General mean	**2.0889±0.11**	**0.09±0.00**	**3.93±0.10**	**7.05±0.00**	**28.00±1.38**

** Values within the same column for white or colored grapevine cultivars not followed by the same letter indicate a significant difference (P<0.01); ns: non-significant.

3.2. Biochemical Content

3.2.1. Total phenolic content

It was found to be significantly varied (P<0.05) in varieties with white grapes- the lowest amount was in cv. Cicikenator, but insignificant in colored grapes. Moreover, there were significant differences in the total phenolic contents in the grape juices of both white and colored cultivars; the white cultivars Askar and Sinciri and black cv. Emiri stands out in terms of total phenolic content. It was determined that the amount in the seed was considerably higher (about twice) than the fruit juice (Table 2).

Table 2. Total phenolic matter contents

Grape skin color	Grape cultivars	Phenolic matter in the seed (GAE mg $100g^{-1}$)	Phenolic matter in the grape juice (GAE mg $100g^{-1}$)
White	Askar	86.19± 5.28 A*	59.99±15.87 A*
	Binetati	87.31± 2.44 A	28.37±7.54 AB
	Sinciri	85.64± 3.01 A	47.31±15.31 A
	Heseni	78.19 ±0.99 AB	10.41±0.52 B
	Cicikenator	72.77 ±0.32 B	27.95±4.89 AB
	Duvrevi	81.61 ±3.90 AB	42.26±9.54 AB
	Mean	**81.95± 1.65**	**36.05±5.27**
Colored	Boga	77.07 ±3.50 ns	34.76±5.44 B*
	Karrod	76.01 ±0.40	18.97±3.18 B
	Tayifi	77.68 ±1.00	33.65±12.69 B
	Siyah gozane	80.18 ±5.09	42.21±5.75 AB
	Emiri	83.56± 3.70	61.29±6.28 A
	Resealya	81.70 ±3.54	31.66±5.17 B
	Mean	**79.37 ±1.31**	**37.09±3.94**

* Values within the same column for white or colored grapevine cultivars not followed by the same letter indicate a significant difference (P<0.05); ns: non-significant.

3.2.2. Total antioxidant capacity

It is seen that the antioxidant capacity of the seed is much higher than the fruit juice. The difference between the cultivars in terms of total antioxidant capacity was significantly varied in both white ($P<0.05$) and colored cultivars ($P<0.01$) (Table 3). The white cultivars except for cv. Sinciri and black cultivars Siyah gozane and Resealya stand out in terms of total phenolic content in the seeds. Moreover, white cv. Duvrevi and black cv. Emiri had the highest total phenolic content in grape juice.

3.2.3. Proanthocyanidin contents

It is also seen that the proanthocyanidin content of the seed is much higher than the fruit juice. The difference between the cultivars in terms of total proanthocyanidin content was significantly varied in both white and colored cultivars ($P<0.01$) (Table 4). The white cv. Cicikenator and black cultivars Siyah gozane and Tayifi stand out in terms of proanthocyanidin content in the seeds. Moreover, white cv. Askar and black cv. Emiri and cv. Tayifi had the highest proanthocyanidin content in the grape juice.

Table 3. Total antioxidant capacity

Grape skin color	Cultivar	Antioxidant capacity in the seed (µmol (TE) g^{-1})	Antioxidant capacity in the grape juice (µmol (TE) g^{-1})
White	Askar	750.57± 165.79 A*	1.22±0.14 B**
	Binetati	786.29± 154.21 A	1.28±0.66 B
	Sinciri	296.29± 86.15 B	1.02±0.23 BC
	Heseni	749.14± 138.23 A	0.07±0.01 C
	Cicikenator	957.71± 121.78 A	0.81±0.19 CD
	Duvrevi	516.29± 129.58 AB	4.48±0.19 A
	Mean	**676.05± 69.379**	**1.48±0.36**
Colored	Boga	609.14± 124.47 B**	2.67±0.63 B**
	Karrod	419.43± 95.74 B	0.87±0.24 C
	Tayifi	689.14± 95.86 B	2.89±0.27 B
	Siyah gozane	1344.86 ±161.33 A	2.86±0.00 B
	Emiri	607.71 ±128.74 B	4.29±0.15 A
	Resealya	1193.43± 262.26 A	2.61±0.51 B
	Mean	**810.62± 97.617**	**2.70±0.27**

Values within the same column for white or colored grapevine cultivars not followed by the same letter indicate a significant difference **($P<0.01$); *($P<0.05$).

Table 4. Proanthocyanidin contents

Grape skin color	Cultivar	Proanthocyanidin in the seed (mg CE g^{-1})	Proanthocyanidin in the grape juice (mg CE g^{-1})
White	Askar	25.55±6.05 B**	0.28±0.06 A**
	Binetati	14.84±1.99 CD	0.04±0.02 BC
	Sinciri	7.31±0.64 D	0.05±0.01 BC
	Heseni	12.77±0.73 CD	0.01±0.01 C
	Cicikenator	34.19±1.23 A	0.03±0.00 C
	Duvrevi	17.52±0.36 BC	0.13±0.03 B
	Mean	**18.69±2.33**	**0.09±0.02**
Colored	Boga	16.38±1.54 B**	0.09±0.01 B**
	Karrod	13.02±1.41 B	0.01±0.00 C
	Tayifi	25.52±1.50 A	0.17±0.01 A
	Siyah gozane	22.65±2.04 A	0.09±0.02 B
	Emiri	12.89±2.04 B	0.15±0.03 A
	Resealya	17.58±0.91 B	0.07±0.02 B
	Mean	**18.01±1.27**	**0.09±0.01**

** Values within the same column for white or colored grapevine cultivars not followed by the same letter indicate a significant difference ($P<0.01$).

4. Discussion

4.1. Seed traits

Approximately 20% of the grapes consist of seeds. In the wine and fruit juice industry, grape seed emerges as a secondary product. It is known that approximately 15-25% of grapes are produced after processing the grapes. It has been determined that grape pomace is composed of approximately 33-45% of seeds (Yu & Ahmedna, 2013; Dwyer, 2014). Kupe et al. (2021) studied different clones of the Turkish grape cultivar 'Karaerik' and the seed traits given for common cultivars used in both studies show parallelism. In the present study, the highest and the lowest seed number values were determined between 1 to 4. In a study examining the sizes of 12 grape varieties grown in Southeastern Anatolia, seed properties ranged in: length, 5 - 8.5 mm; width, 2.5 - 5.5 mm; and thickness, 2 - 4.7 mm (Levent and Demir, 2020). In a study comparing the morphological features and chemical components of the seeds of 6 table grape varieties, the number of seeds per berry was 2-5; the average weight of the seeds was 82-315 mg; the weight of the seeds was 17-73 mg; the length of the seed was 1.4 - 9.3 mm; and the seed width was 1.9-5.8 mm (Elagamey, 2013). Dogan and Uyak (2022), in their study on some table grape varieties, found that the total antioxidant capacity of varieties with red-black skin color varied between 6.45-8.21 μmol TE g^{-1}; and they reported that the total antioxidant capacity of cultivars with green-yellow peel color varied between 4.27-6.20 μmol TE g^{-1} during the harvest period. Aydın (2015) reported the lowest total antioxidant capacity as 2.06 μmol TE ml^{-1} for cv. Sari kokulu and 9.33 μmol TE ml-1 for cv. Civek. In the present study, the seed samples of 12 local grape varieties grown in Siirt province in terms of various seed traits were as: the number of seeds ranges from 1.20 to 3.00; seed weight varied from 0.07- 0.12 g; seed width ranged from 3.20 to 4.83 mm; and seed length varied as 6.47-9.77. The white varieties were significant in all traits ($P<0.01$); and colored cultivars were significant ($P<0.01$), except for the seed size value. When evaluated according to the color of the seed, it was seen that the average number of seeds was higher in colored varieties, and the seed weight and seed length were higher in white varieties.

4.2. Phenolic matter content

In the present study, the distribution of the total phenolic contents of the seeds based on white cultivars varied from 72.77 to 87.31 mg GAE $100g^{-1}$; and it was found in the range of 76.01 to 83.56 mg GAE $100g^{-1}$ in colored cultivars. Moreover, the distribution of the total phenolic contents of the grape juice based on white cultivars varied from 10.41 to 59.99 mg GAE 100 g^{-1}, while the values of colored cultivars ranged from 18.97 to 61.29 mg GAE $100g^{-1}$. The phenolic contents of the seeds of the white cultivars were higher than the seeds of the colored cultivars. Grape juice total phenolic content values were found to be quite close to each other in white and colored cultivars. As a result of the study, it was observed that the ratio of total phenolic content in the seed was quite high compared to fruit juice.

Kupe et al. (2021) studied 9 different clones of the Karaerik grape cultivars and found that the highest FRAP values were expressed from seeds. The seed extract from FRAP values between are varied 52460 μmol Trolox 100 g^{-1} FW and 39880 μmol Trolox 100 g^{-1} FW. In fruit pulp, this value was found between 128 μmol Trolox 100 g^{-1} FW and 77 μmol Trolox 100 g^{-1} FW. In a study by Pantelić et al., (2016), the total phenolic concentration in grape seed samples ranged between 102.98 and 38.02 mg GAE g^{-1}, and grape juice samples ranged between 0,20-0,07 mg GAE g^{-1}, respectively. In a study of fresh grapes, the total phenolic content was 3.35 GAE mg g^{-1} in cv. Gros noir, 1.21 mg GAE g^{-1} in cv. Cardinal, 3.04 mg GAE g^{-1} in cv. Muscat noir, 1.82 mg GAE g^{-1} in cv. Victoria, and 1.58 mg GAE g^{-1} in cv. Muscat blanc (Derradji et al., 2014). In another study, in which the total phenolic content of 33 different grapevines was determined, the average phenolic contents of black, green, and red grapes were expressed as 77.00, 44.56, and 31.42 mg GAE $100g^{-1}$ (Chen et al., 2014). In a study evaluating the effects of whole grape pomace flour, seedless pomace flour, and seed flour on the quality of cookies, it was determined that the total phenol and antioxidant activities of cookies containing 10% seed flour were found to be higher than other additives, thus increasing the nutritional value the most (Acun and Gul, 2014). Gül et al. (2013), in their study on Narince and Okuzgozu cultivars, determined the total phenolic content of seed samples to be 563.27 g GAE kg^{-1} and 552.10 g GAE kg^{-1}, respectively. In another study in which phenolic compounds were determined in the seeds of *Vitis labrusca* B., the mean value was determined as 2.41 mg GAE l^{-1} (Ghafoor et al., 2012). In a study in which 7 standard cultivars

and 5 wild vine genotypes were examined, the total phenolic content of grape seed extracts was determined as between 1694 and 1136 mg GAE 100 g FW^{-1} (Yegin and Uzun, 2018). Karateke et al., (2022), stated that the total phenolic content in grapes taken from control vines was 254.80 mg GAE $100g^{-1}$. In a study by Doshi et al. (2015), the total phenolic content of the Pusa navarang variety was determined as 95.8 mg ml^{-1}. The results of the present study do not fully agree with the studies mentioned in this paragraph; it is thought that the difference is due to different effects such as varietal traits, ecological differences, phenological periods from which the samples were taken, growing conditions, as well as the difference in the method used. On the other hand, Çakır et al. (2021) stated that some quality criteria may vary from year to year as well as the grape variety used, the way the grape is evaluated, climate, rootstock used, soil, aspect, cultural processes and altitude groups. In the study conducted by Gazioglu Sensoy et al. (2018), the average phenolic content was 73.60 mg GAE $100g^{-1}$ in the seed; 58.73 mg GAE $100g^{-1}$ in the skin, and 40.52 mg GAE $100g^{-1}$ in the fleshy part of the grape. The results expressed by the literature are generally compatible with the present study.

4.3. Total antioxidant capacity

Significant differences were observed among the values obtained for the antioxidant capacity in the seed parts of the studied cultivars; The highest value was measured with 1344.86 µmol TE g^{-1} in cv. Siyah gozane, which is a black variety, and the lowest value was measured in the variegated cv. Karrod with 242.00 µmol TE g^{-1}. In grape juice, the black cv. Duvrevi had the highest value with 4.48 µmol TE g^{-1} and cv. Heseni variety with white skin color had the lowest value with 0.07 µmol TE g^{-1}. The values were determined to be high in the seed and very low in the grape juice compared to the seed. In general, it was determined that the average values of the colored cultivars were higher than those of the white cultivars. It was seen that the rate was higher in colored varieties. Researchers stated that the antioxidant content of grapes is mostly in the skin and seeds. It was also reported that the antioxidant capacity of the extracts obtained from grape pomace was high. In the present study, it was determined that the antioxidant content in grape seeds was significantly higher when compared to grape juice. In the study conducted by Gazioglu Sensoy et al., (2018), the average antioxidant capacity was 1009.85 µmol TE g^{-1} in the seed; It was expressed as 310.92 µmol TE g^{-1} in the skin and 204.39 µmol TE g^{-1} in the pulp.

In a study conducted on grape juice, it was reported that the average antioxidant capacity of black grapes was 94.30 µmol TE 100 ml^{-1}, and that of white grapes was 31.10 µmol TE 100 ml^{-1} (Keskin-Šašić et al., 2012). In another study, the average antioxidant capacities of black, green, and red grapes were 3.34, 2.75, and 1.36 µmol TE g^{-1}, respectively (Chen et al., 2014). Pantelić et al., (2016) determined the total antioxidant content of the cultivars as 1039.92 and 481.69 µmol (TA) g^{-1}, respectively. Sochorova et al., (2020) determined that average antioxidant activity values using the FRAP method vary according to the cultivars in the years as it was 12,217 µg g^{-1} GAE in 2015; 13,724 µg g^{-1} GAE in 2016; and 14,807 µg g^{-1}GAE in 2017. Liu et al. (2018), in a study examining the FRAP values of 30 separately measured in grape pomace, stated that many researchers stated that the differences in antioxidant capacity in different grapes could be very large. Gazioglu Sensoy (2012) determined that the antioxidant activity values of the cultivars Kis kirmizisi, Okuzgozu, Agin beyazi, Ercis uzumu, and Silfoni; the highest antioxidant content was determined in cv. Kis kirmizisi as 5.74 mmol TE L^{-1}, and the lowest value was found in Ercis uzumu as 2.29 mmol TE L^{-1}. These values were largely similar except for a few varieties. The antioxidant capacity obtained by the FRAP method of several local grape varieties grown in Elazığ and Malatya provinces by Duran (2014) ranged from 2.64 mg TE L^{-1} to 31.47 mg TE L^{-1}. Sanyürek et al. (2018) conducted a study on several local cultivars grown in Tunceli province and determined the antioxidant capacity in the range from 32.30 µg TE ml^{-1} to 56.20 µg TE ml^{-1}. Tahmaz and Soylemezoglu (2019) studied the antioxidant contents of the grape skin and seeds separately by the HPLC method, and the antioxidant capacity of the seeds in all varieties was higher than the skins, and the highest content in the grape skin was measured in cv. Bogazkere as 544.3 µmol TE g^{-1} and the lowest in cv. Calkarası as 60.1 µmol TE g^{-1}. The highest antioxidant content in the seeds was measured in cv. Okuzgozu with 1133.00 µmol TE g^{-1}, and the lowest in cv. Bogazkere with 573.1 µmol TE g^{-1}. It was determined that the antioxidant content of the seeds compared with the fruit juice content was 99% higher than that of the grape juice. Most of the studies have found similar antioxidant values to the present study. Uyak et al. (2020) examined grape varieties that have similar ecology and maintenance conditions and observed statistical differences in terms of organic acid and phenolic compound contents;

therefore, it was reported that the variety was an effective factor in the chemical composition. Similarly, in the present study, it was determined that the results differed according to the cultivars.

4.4. Proanthocyanidin content

Proanthocyanidins, which are found in high amounts in grape seeds, regulate lipid homeostasis; and balance hyperlipidemia, one of the main causes of cardiovascular disease (Margalef, et al., 2014; Nunes et al., 2016). Numerous types of research utilizing both human and animal models have found that proanthocyanidins offer considerable health advantages. These have anti-protective effects against oncogenic events, metabolic diseases, and cardiovascular illnesses. Proanthocyanidins are therefore anticipated to be viable medicinal treatments for such discrepancies. (Nie and Stürzenbaum, 2019; Rauf et al, 2019). It has been stated that the grape seed is a very valuable product that cannot be seen as a waste, as it contains approximately 90% of proanthocyanidins and is rich in unsaturated fatty acids (Aktan and Kalkan, 2000). Mattivi et al. (2009) reported that proanthocyanidins in the structure of grape seeds vary according to the grape variety and ripening stages, but the largest amount of proanthocyanidins is localized in the seed. In the study in which the differences in the amount and structure of extractable skin and seed tannins between red grape varieties were revealed, the catechin equivalent value in the seed content was found to be the highest in cv. Pinot Noir with 232.9 mg (CA) kg^{-1} and the lowest was detected in cv. Syrah variety, 16.8 mg (CA) kg^{-1}. It is seen that the majority of the studies on proanthocyanidins are in the basic field of medicine. The amount of proanthocyanidin contained in the seed and fruit juice is proportionally quite different, and the proanthocyanidin content of the seed is much higher than the fruit juice. The difference between the cultivars in terms of total proanthocyanidin content was significantly varied in both white and colored cultivars. It was also seen that the proanthocyanidin content in the seed was quite high; in both, the proanthocyanidin content did not have a significant relationship with the grape skin color; It was determined that the color of the fruit skin did not make a difference in the proanthocyanidin content of the fruit juice, as in the seed. It is also seen that the majority of the studies on proanthocyanidin are in the basic field of medicine.

5. Conclusion

The present study is important in terms of determining some seed and grape juice traits of this grapevine germplasm, which is in danger of extinction. Considering the phenolic amounts, it was seen that the black varieties had higher amounts than the white varieties. It was also determined that the total antioxidant amounts were quite high in the seeds compared to the grape juice. It was determined that the total proanthocyanidin amounts were higher in the seed than in the grape juice. In the present study, it is seen that the biochemical properties of grapes are higher in the grape seeds than those of the grape juice and the skin, and this study is parallel when compared with other studies. The present study re-emphasized the fact that grape seeds could be used for both health and different evaluation forms increasing the value of this product.

There are not enough studies on grape seeds, and the total phenolic and antioxidant capacity are generally emphasized in the literature; It is seen that there are fewer studies dealing with proanthocyanidin content. Grape seed, both an important agricultural and industrial product, has bioactive components beneficial to human health. Its content has positive effects on human health supported by the literature in medicine, pharmacy, and food processing. In addition to the valuable phenolic substances, antioxidants, and proanthocyanidin contents were revealed in the present study. The present study has revealed again how valuable the grape seed is. Conscious consumption will be ensured thanks to the determination of the chemical compounds in the grape, especially in its seeds, and it has been determined that this important product can be used in many areas from cosmetics to the pharmaceutical industry, from the food industry to animal nutrition. By intensifying research on grape seed, this product, which is seen as a waste material, will be brought to different industries and new evaluation methods will be introduced. For this reason, researches on proanthocyanidin components, and phenolic and antioxidant contents of grape seeds provide important contributions to the reproduction and economy of this product, which is at risk of being lost without value.

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Research Article

The Effect of Some Endophytic Bacteria on Seedling Growth and Physiological Properties of *Salvia officinalis* L.

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Abstract: In order to meet the demand for medicinal sage (*Salvia officinalis* L.), which is an important economic product, harvesting from nature has economic value. However, it may not always be of the desired standard and quality. Also, the harvesting from nature endangers their natural population causing their genetic base to decline. For this reason, it is important to produce it in an agrosystem and to increase yield in a sustainable way. In this study, the effects of eleven endophyte bacteria (EB) isolates applications on the development, morphology, and physicochemical properties of *Salvia officinalis* L. were investigated by climate chamber experiments. Peat+perlite+soil (1:1:2) mixture was used as the growing medium and EB was applied two times by soaking method. Effects of EB applications on shoot/root length, root/stem fresh and dry weight, Dualex values (Nitrogen balance index (NBI), flavonol, anthocyanin, and chlorophyll), leaf area, leaf temperature and color values (L*, a) *, b * C and Hue° were examined. All EB applications increased the plant height and leaf area. Also, the majority of EB isolates enhanced the root dry weight. The effect of EB applications on flavonol and chlorophyll content was not found statistically significant. However, there was a statistically significant increase in the nitrogen balance index (NBI). It was also observed that EB applications caused changes in plant color. According to the results obtained, it has been seen that it is possible to produce environmentally friendly and sustainable medicinal sage with appropriate plant-bacteria combinations..

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1. Introduction

Türkiye, which is the gene center of many plants cultivated in world agriculture, is home to a total of 92 *Salvia sp.* (Lamiaceae), 47 of which are endemic (Altındal ve Akgun, 2015). The essential oil and leaves of sage, are widely used in alternative or traditional medicine as an additive, herbal medicine, food, cosmetics, and perfumery (Elmas, 2021). In 2020, approximately 2 176 tons of sage,

which is one of the important export products, was exported and 8 155 503 dollars were brought to the economy (Elmas, 2021). The trade of sage is carried out by cultivation in different provinces and collecting from nature. It is difficult to obtain products of desired standards and quality by collecting from nature. In addition, unconscious and over-collecting sage populations lead to unsustainable extinction, overexploitation, and the narrowing of their genetic base. For this reason, in order to ensure its sustainable use, it is necessary to expand the production of sage, which is in the standards and quantities required by the world markets. Studies have shown that yield and quality in sage production differ according to the climate and soil conditions of the region where it is grown, harvest periods, drying conditions, and agricultural practices (Elmas, 2021). Although collecting from nature is economical, it makes it difficult to obtain the product of the desired standard and quality. For this reason, in order to meet the demands of industrialists and consumers, sage production has been started in different provinces of our country in recent years in the quality required by the world markets. (Bayraktar et al., 2017).

The excessive and unconscious use of chemical fertilizers and pesticides negatively affects the environment and human health. It is important to avoid the use of chemical fertilizers and pesticides in the cultivation of medicinal and aromatic plants such as sage. (Egamberdieva and Teixeira da Silva, 2015). Nowadays, symbiotic or free-living microorganisms, which are one of the environmentally friendly sustainable agriculture approaches, have an important potential to increase the development of plants, their nutrient uptake, and their tolerance to biotic and abiotic stress conditions (Altunlu et al., 2019).

Beneficial bacteria to plants are named in general as "Plant growth promoting rhizobacteria/bacteria" (PGPR-PGPB). PGPBs live or colonize the plant as endophytes (inside the plant) and/or epiphytes (on the plant surface). Endophyte bacteria (EB) are defined as microorganisms that can spread throughout the plant without harming the plant and spend at least a part of their life in the internal tissues of the plant (Hallmann 1997; Hardoim et al., 2008; Hardoim, 2011). EB can affect the growth and development of plants directly and indirectly (Imriz et al., 2014; Ucar and Akkopru, 2022). EB contributes directly to the plants in ways such as fixing nitrogen, dissolving phosphorus, supporting the uptake of iron and other nutrients, produce phytohormones (Grobelak et al., 2015). They can make an indirect contribution by providing protection against harmful organisms by activating antibiosis, competition, hyperparasitism, and activated plant-induced resistance or tolerance (Glick, 2014). Endophytic bacteria have some advantages over epiphytic bacteria. Since they live in the internal tissues of plants, the metabolites they produce can be taken directly by plants (Romano et al., 2020; Akkopru et al., 2021). EB can reach and affect all tissues in the plant thanks to the transmission systems of plants (Hardoim et al., 2008; Hardoim, 2011; Mercado-Blanco and Lugtenberg 2014; Romano et al., 2020).

This study aimed to investigate the effects of endophytic bacteria on seedling growth and some physiological parameters of medicinal sage (*Salvia officinalis* L.), an important medicinal and aromatic plant with high market value.

2. Material and Methods

2.1. Plant material and running of the experiment

The seeds of the *S. officinalis* were obtained from Van Yüzüncü Yıl University Medicinal and Aromatic Plants Garden. The study was carried out in the Van YYU Faculty of Agriculture, Field Crops Department, Climate room, Physiology laboratory, and Plant Protection Department laboratory. The seeds were sown in a growth medium consisting of peat + perlite + soil (1:1:2) 500 cc pots. After sowing, the plants were grown in a dark/light photoperiod of 8/16 hours, in a controlled climate chamber at 25°C and 65% humidity. The experiment was carried out in seven replications according to the Randomized Complete Plots Experimental Design. The hoagland nutrient solution was applied to supply the nutritional needs of the seedlings after the cotyledon leaves emerged (Hoagland ve Arnond 1950).

2.2. Endophyte bacteria and their application

Endophyte bacteria isolates used in the study, which were previously isolated from plants belonging to the Poaceae family in the Van Lake basin, were obtained from the bacteriology laboratory stocks of Van Yüzüncü Yıl University, Faculty of Agriculture, Department of Plant Protection. The

isolation and detection of some characteristics of the EBs were carried out within the scope of the project supported by the Scientific Research Projects Department of Van Yüzüncü Yıl University (Grant no: FBA-2020-8551) (Table 1). For this purpose, EB suspensions with a density of 10^8 CFU/mL were prepared from 48-hour cultures grown in King B medium. The EB suspension was applied to 15 mL/plant by drenching method (Ucar and Akkopru, 2022).

Table 1. Data on the endophytic bacteria (EB) isolates used and some of their characteristics that may contribute to plant health and growth

	EB Isolate Code	Gram React.*	IAA	ACCd	Nitrogen fix.	" P " solubilization (EI)	Sid. (EI)
1	G58S1	-	0.938	+	-	1.69	1.47
2	G129K1-1	-	1.419	-	+	2.12	1.75
3	G113Y1	+	0.024	-	-	1.60	1.51
4	G43K2	-	0.430	-	+	1.89	1.28
5	G106Y1	+	0.941	-	-	1.57	-
6	G91S2	+	0.554	-	+	6.75	1.33
7	G21Y1	-	1.472	+	-	1.12	2.04
8	G100Y2	-	0.740	+	-	3.94	1.33
9	G118K1T	-	0.045	-	-	6.91	2.30
10	G59S2	-	0.655	-	+	8.13	2.42
11	G104Y1	-	1.108	+	-	2.61	1.89

*Gram React.: Gram reaction according to KOH test, IAA: indole acetic acid production, ACCd: 1-aminocyclopropane-1-carboxylate deaminaze Nitrogen fix.: Nitrogen fixation ability, P solubilization (EI): ability to dissolve phosphorus in vitro (enzyme index), Sid. (EI): Siderophore production ability (enzyme Index).

2.3. Determination of plant growth parameters

The study was terminated after 8 weeks. The plant was cut from the root collar and, the roots and shoots were washed separately. Roots were placed between blotting papers for the washing water to come out. Root and stem lengths were measured by the ruler as centimeters. Fresh and dry weights of root and stem were determined with the help of precision scales. The shoots and roots were then dried at 60°C for 72 hours and their dry weights were weighed. Leaf surface temperatures (Spectrum Technologies Inc.) were measured with the help of a portable infrared thermometer using the "Easy Leaf Area" program (Tuncturk et al., 2020). Nitrogen balance index (NBI), chlorophyll, flavonol, and anthocyanin content in leaves were measured in real-time and non-destructively on the leaf with Dualex scientific+ (FORCE-A, France). Color values were determined by (Minolta CR-400) brand colorimeter as L*, a*, b* C, and Hue° angle values. L* lightness ((L*=0 black and L*=100 white), a* red/green (+a* red, – a* green), b* yellow/blue (+b* yellow, -b* blue), Chroma is the brightness or opacity, Hue is the perceived color and the values that determine the name of the color (Anonim, 2022).

2.4. Analysis of Data

Statistical analyzes of the obtained data were made using the COSTAT (version 6.03) package program and multiple comparison tests were performed according to the Duncan test.

3. Results

The effects of EB isolates on morphological and physiological development and color parameters (L*, a*, b*, Chroma, and Hue) in medicinal sage were investigated. The obtained values are given in Tables 2, 3, and 4. The effects of endophyte bacteria were found to be statistically significant on all parameters except shoot dry weight. All EB treatments provided a significant increase in plant height compared to the control group. While the most successful application was obtained from the G43K2 isolate with 27.64 cm, it was observed that it was in the same statistical group with G129K1, G106Y1, and G59S2 isolates. In addition, it was determined that the difference between the applications was statistically significant at the level of 1% on the fresh weight of the shoots. Among the bacterial applications, the highest shoot fresh weight was obtained using the G104Y1 (5.11 g), while six bacterial isolates were found to be in the same statistic group. The lowest value was obtained with G58S1 isolate

(3.37 g). The control group, G129K1, G43K2, and G118K1T isolates are in the same statistical group. There was no statistically significant effect of experimental factors on shoot dry weight, which varies between 0.58-1.05 g observed (Table 2).

The effects of bacterial applications on means of the root length were statistically significant at the level of 5% level. While the highest plant root length was measured with G113Y1 (30.42 cm) application group, it was included in the same statistical group with seven different bacterial applications. The lowest root length was obtained from the G129K1 (22.14 cm) application, and also it is in the same statistical group as the control and other three isolates (Table 2).

The effect of EB on means of plant root fresh and dry weight was statistically significant at the 1% level. While the highest root fresh weight of 1.55 g was obtained from the application of G91S2 isolate, the lowest value was obtained with the use of G129K1 and G43K2 isolates with 0.76 g and 0.94 g, respectively, and they are in the same statistical group. While the lowest value in terms of root dry weight was determined from the control group (0.14 g), it was statistically found in the same group as G59S2, G129K1, G129K1, and G104Y1 isolates. The highest root dry weight was observed with 0.27 g from the G91S2 isolate (Table 2).

Table 2. Effects of EB treatments on the morphological parameters of *S. officinalis*

	Plant Shoot	Shoot weight (g)		Plant Root	Root weight (g)	
Applications	height (cm)	Fresh	Dry	length (cm)	Fresh	Dry
Control	14.71^{f}	3.77^{de}	0.88	23.57^{cd}	0.99^{cd}	0.14^{d}
G58S1	19.35^{e}	3.37^{e}	0.85	24.85^{bcd}	1.00^{cd}	0.20^{bc}
G129K1	24.35^{abc}	4.01^{cde}	0.87	22.14^{d}	0.76^{e}	0.15^{d}
G113Y1	22.61^{cde}	4.25^{a-d}	0.91	30.42^{a}	1.17^{bc}	0.22^{b}
G43K2	27.64^{a}	4.07^{b-e}	0.92	24.42^{abcd}	0.94^{de}	0.20^{bc}
G106Y1	25.57^{abc}	4.92^{ab}	0.98	28.14^{abc}	1.20^{bc}	0.22^{b}
G91S2	19.64^{e}	4.83^{abc}	1.05	29.00^{ab}	1.55^{a}	0.27^{a}
G21Y1	22.57^{cde}	4.77^{abc}	1.03	29.78^{ab}	1.00^{cd}	0.20^{bc}
G100Y2	19.35^{e}	4.56^{a-d}	0.89	28.64^{abc}	1.26^{b}	0.19^{bc}
G118K1T	20.57^{de}	4.15^{b-e}	0.90	28.33^{abc}	1.16^{bc}	0.19^{bc}
G59S2	26.42^{ab}	4.61^{a-d}	0.85	23.71^{cd}	1.00^{cd}	0.14^{d}
G104Y1	23.68^{bcd}	5.11^{a}	1.05	28.02^{abc}	1.29^{b}	0.17^{cd}
Average	22.20	4.37	0.93	17.46	1.11	0.19
F value	7.909**	2.794**	1,001ns	2.200*	7.464**	8.70**
LSD	3.66	0.87	0.20	5.14	0.21	0.03
CV (%)	15.50	18.73	20.90	18.09	17.93	17.78

**P < 0.01 level; * P < 0.05 level significance: ns: no significance.

The effect of bacterial isolates on NBI content was significant at the 1% level. While the lowest NBI value was obtained from the control group as 26.32 dualex index, the highest value was determined as 45.98 dualex index with the G129K1 isolate. It was also determined that G129K1 was in the same statistical group with G100Y2 and G59S2 isolates (Table 3).

The effects of bacterial applications on chlorophyll and flavonol content were statistically insignificant (Table 3). In the study, chlorophyll content was determined between 21.32-25.44 dx and flavonol content between 0.54-0.71 dx. On the other hand, anthocyanin content was found to be significant at the 1% level. While the highest anthocyanin content was detected in the control group (0.074 dx), it was in the same group with five different bacterial isolates. The lowest anthocyanin content (0.050 dx) was determined from the G21Y1 application, but there was no statistically significant difference between them and the three bacterial isolates (G58S1, G113Y1, G100Y2).

The effect of the treatments on leaf area was significant at the 1% level. The highest leaf area with 14.79 cm2 was obtained from the G21Y1 treatment, while it was found to be in the same statistical group with seven different bacterial isolates. The lowest leaf area was determined as 8.82 cm^2 in the control group and the G118K1T (10.92 cm2) isolate (Table 3).

The effect of EB on plant temperature was found to be significant at the 1% level. The highest plant temperature was obtained as 27.31°C, 27.30°C, 27.27°C and 26.68°C from G129K1, control, G58S1, and G106Y1 treatments, which are in the same statistical group, respectively. The lowest plant

temperature (24.25°C) was determined from G100Y2. However, it is in the same statistical group as G43K2 (25.58 °C), G113Y1 (25.45 °C), and G59S2 (25.42 °C) isolates (Table 3).

Table 3. Effect of endophytic bacterial applications on some biochemical parameters of *Salvia officinalis*

Applications	NBI (Dualex index)	Chlorophyll (Dualex index)	Flavonol (Dualex index)	Anthocyanin (Dualex index)	Leaf area (cm^2)	Plant temperature (°C)[§]
Control	26.32f	21.32	0.71	0.074a	8.82d	27.30a
G58S1	39.47bcd	25.35	0.64	0.058cde	12.17bc	27.27a
G129K1	45.98a	22.10	0.54	0.065a-d	14.54ab	27.31a
G113Y1	34.50e	24.64	0.70	0.055de	14.20ab	25.45d
G43K2	35.94de	22.91	0.65	0.061bcd	12.86abc	25.58d
G106Y1	41.15bc	23.48	0.57	0.061bcd	14.28ab	26.68ab
G91S2	36.62de	23.50	0.65	0.071ab	13.73ab	26.15bcd
G21Y1	41.09bc	25.44	0.66	0.050e	14.79a	26.41bc
G100Y2	43.78ab	24.11	0.57	0.057cde	14.58ab	24.25e
G118K1T	37.29cde	23.41	0.60	0.072a	10.92cd	25.66cd
G59S2	41.56abc	23.50	0.59	0.067abc	12.57abc	25.42d
G104Y1	39.23cd	24.65	0.63	0.065a-d	12.34bc	25.68bcd
Average	38.58	23.70	0.63	0.063	12.98	26.10
F value	10.360**	0.690ns	1.384ns	3.825**	4.295**	10.643**
LSD	4.45	4.15	0.12	0.01	2.41	0.80
CV (%)	10.82	16.46	18.60	15.82	17.47	2.90

**P < 0.01 level; * P < 0.05 level significance: ns: no significance.

It was noted that the difference of color measurements between of EB tretment groups was statistically insignificant. The lowest and highest values were determined as L*; 41.94 - 47.49, a*; (-14.18)-(-12.03), b*; 18.85-24.63, Chroma; 22.50-28.59 and Hue; 121.06-122.97 (Table 4).

Table 4. Effect of EB applications on color values of *Salvia officinalis*

Uygulamalar	L*	a*	b*	Chroma	Hue
Control	41.94b[§]	-12.03a	18.85b	22.50b	122.97
G58S1	44.14ab	-13.58ab	21.68ab	25.59ab	122.37
G129K1	44.00ab	-14.18b	22.20ab	26.35ab	122.86
G113Y1	46.08ab	-13.19ab	21.17ab	24.97ab	122.28
G43K2	45.98ab	-13.35ab	21.68ab	25.47ab	121.91
G106Y1	43.80ab	-13.56ab	20.97ab	24.98ab	122.94
G91S2	46.42a	-13.49ab	21.66ab	25.54ab	122.42
G21Y1	46.15a	-14.83b	23.25a	27.59a	122.81
G100Y2	46.33a	-14.38b	23.05ab	27.20a	122.46
G118K1T	47.49a	-14.45b	24.63a	28.59a	121.06
G59S2	45.76ab	-14.82b	23.47a	27.77a	122.42
G104Y1	45.33ab	-13.43ab	21.32ab	25.22ab	122.42
Average	45.29	-13.77	21.99	25.98	122.40
F value	1.147[ns]	1.963[ns]	1.014[ns]	1.134[ns]	0.443[ns]
LSD	4.16	1.66	4.30	4.49	2.31
CV (%)	5.45	7.17	11.60	10.25	1.12

**P < 0.01 level; * P < 0.05 level significance: ns: no significance.

4. Discussion

Secondary metabolites of medicinal plants such as *S. officinalis* are the main source of bioactive products and are considered as important pharmaceuticals. Medicinal sage is grown commercially in many countries around the world since it is difficult to obtain products of the desired standard and quality with pick up from nature (Phillipson, 2001; Bayraktar et al., 2017). In agricultural production, many chemical inputs are used in order to increase yield and protect plants from diseases and pests

(Egamberdieva and Teixeira da Silva, 2015). In our study, the effects of 11 EB isolates (Table 1) on some morphological, physiological, and color properties of medicinal sage were investigated.

It was determined that the obtained data were compatible with the relevant literature. Many studies have shown that growth-promoting bacteria (EB or PGPR) can increase plant growth. PGPRs can contribute to plant growth by increasing the availability of mineral phosphate and other nutrients (Hayat et al., 2010; Akkopru and Ozaktan, 2018), by biological fixation of nitrogen (Ardakani et al., 2010); by the production of IAA (Mishra et al., 2010; Etesami et al., 2015). In addition, they contribute to plant growth by reducing the amount of ethylene in the roots of plants under stress through the production of ACC deaminase (Glick, 2014). It is known that some of the EB isolates we used in our study have different properties such as nitrogen fixation, IAA, ACC deaminase, phosphate solubilization, and siderophore production (Table 1).

It was determined that each of the EB isolates had different; positive, negative, or neutral effects on the plants in terms of the investigated parameters. It was observed that all EB applications significantly increased plant height and leaf area (except G118K1T). Rahimi et al., (2013) reported that bacteria with nitrogen fixation ability increase the height of the basil plant (*Ocimum bacilicum* L.). Similarly, Anbi et al., (2020) reported that bacteria of different characteristics increase plant height and leaf area at different levels. It was shown that nitrogen and phosphorus have an important role in increasing the number and surface area of leaves by affecting the cell division process and chlorophyll production as a growth factor (Larimi et al., 2014). Zhang et al., (2019) revealed that there is a positive correlation between root and stem length, secondary root number, and leaf area index in plants inoculated with bacteria. It was indicated that PGPR (*Bacillus pumilus* TV-67C, *Bacillus subtilis* TV-13B, and *Bacillus megaterium* TV6D + *Brevibacillus choshiensis* TV-53D + *Pantoea agglomerans* RK-92+) application decreased damage caused by stress at the cabbage plant (Samancıoglu et al., 2016). Tuncturk et al., (2021), in their study on soybean, showed that rhizobacteria applications increased the leaf area index compared to the control. It was shown that the results of the researchers and our findings are similar.

Ghorbanpour and Hatami (2014) and Ghorbanpour et al., (2016) stated that the increase in shoot fresh and dry weights and root length in *S. officinalis* may be related to IAA production of the PGPR isolates used. In our study, isolates that contributed positively to root and stem development had the ability to produce high or low levels of IAA (Table 1). Also, these EB isolates are also successful in terms of "1-aminocyclopropane-1-carboxylate deaminase" (ACCd) production, nitrogen fixation, and siderophore production ability, and they can contribute in this way. Therefore, the effects of these characters should not be ignored tooThe results of the study conducted by Samani et al. (2019) using the plant *S. officinalis* support our findings.Anbi et al., (2020) determined that bacteria that can fix nitrogen and have the ability to dissolve phosphorus increased the chlorophyll content of *S. officinalis*. Researchers conducted studies on garlic (Esringu et al., 2016), broccoli (Yıldırım et al., 2011), cabbage (Turan et al., 2014), calendula (Selem et al., 2022), and broad beans (Cirka et al., 2022) revealed that different bacterial applications are effective on the increasing amount of chlorophyll in plants.

It is known that the temperature increases in plants due to stress. It was revealed that the temperature increases in the plants are in a negative correlation with the yield (Blum, 2009). In the study carried out, it was indicated that the temperature in the plants treated with bacteria was lower than the control group and that the bacterial applications had positive effects on plant growth in terms of temperature parameters.

All the EB applications increased the nitrogen balance index (NBI) in leaves significantly compared to the control group. G129K1-1 isolate, which produced the most successful application, appears to have nitrogen fixation ability (Table 2). It was determined that bacteria with nitrogen fixation ability increased the leaf nitrogen content of Salvia spp. more than other phosphorus-dissolving bacteria (Anbi et al., 2020). Researchers stated that stress reduces nitrogen use efficiency and NBI values (Shakiba et al., 2010). The nitrogen balance in the sorghum plant decreases with stress and causes early leaf maturation (Chen et al., 2015). Similarly, Oral et al., (2021), in their study on soybean, showed that plant growth-promoting bacteria increased the NBI value in the plant compared to the control group. According to the findings obtained from our experiments, the lowest NBI value was obtained from the control group as a dualex index of 26.32, and it was determined that it was compatible with the relevant literature.

Flavonols are low molecular weight polyphenolic compounds found in many plants. They have important roles in flowering in plants. During pollination, they produce red-blue or yellow pigments that serve to attract insect and bee populations (Birman, 2012). In this study, no significant difference was found between the plants in the control group and the plants treated with PGPR in terms of flavonol content. Anthocyanins are water-soluble compounds of various colors such as flavonols. They are found in the cavities within the epidermal and mesophyll tissue in the plant. It creates a secondary defense mechanism in plants by providing color change in various organs against abiotic factors such as drought stress (Aztekin and Kasım, 2016). Studies have shown that there is an increase in the amount of anthocyanins under stress (Hanson et al., 2011). In our study, however, the highest anthocyanin value was found in the control group with a dualex index of 0.074. The amounts of anthocyanins in bacterial treatment groups were found to contain lower than the control.

The values of the color characteristics, L* (brightness), a* (red-green), b* (yellowish), Chroma, and Hue (color tone), of sage treated by EB the difference between them, were not statistically significant. In the study performed on the basil plant, the color values were determined as L*(48.60), a*(-14.18), and b*(25.19) (Inan, 2010). In the study performed on the basil plant, the color values were determined as L*(48.60), a*(-14.18), and b*(25.19). The hue angle is defined as a color circle and it has been observed in the study that it corresponds to the range of 90 to 180. Positive a* values are obtained in red, while negative a* values are obtained in green. In our study, it was observed that a* values were negative and the highest values were obtained in bacterial applications. The positive b* value represents yellow, while a negative value represents blue. In the study, the b* value was positive and the lowest value was determined as 18.85 in the control group. The researchers found that the effects of compost and chicken manure application in lettuce and tomatoes, respectively on color values were found insignificant (Sonmez et al., 2017 and 2019). It has been reported that Chroma (C) value expresses vividness-opacity and Chroma values are high in vivid colors and low in dull colors (Ozbay, 2021). It has been reported that the Chroma value in the spinach plant, where different fertilizer applications are applied, varies between 21.48 and 24.86 (Demir and Sonmez, 2019). In the study carried out, the lowest Chroma value was found to be 22.50 in the control group, while the highest was determined as 28.59 (Table 4). It was concluded that the plants treated with bacteria were brighter.

Conclusion

Due to the fact that high yield is the priority in agricultural production, producers tend to use excessive fertilizers and the majority of these fertilizers negatively affect the environment and human health. Alternative methods have been developed in terms of reducing fertilizer applications to a low level, and PGPRs, which positively affect plant growth, have come to the fore. It was concluded that the bacteria used in the study made positive contributions to plant growth parameters. The appropriate plant-bacteria combinations will enable the production of environmentally friendly and sustainable *S. officinalis*. The studies to be carried out with different bacterial strains and sage varieties can make significant contributions to our country's agriculture.

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Research Article

The Boron Application Effects on Germination and Seedling Parameters of Sorghum Cultivars [*Sorghum bicolor* (L.) Moench] in Drought

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Abstract: The aim of study was to investigate the effects of boron on germination and seedling parameters of sorghum [*Sorghum bicolor* (L.) Moench] under drought stress conditions. The experiment was conducted in a factorial trial in a randomized plot design with four replication in a growth chamber. In this study, three different sorghum cultivars were used. Drought conditions were performed at three different levels using PEG (0.-0.4 MPa and -0.8 MPa). Four boron doses (0-5-10-15 mM B) solutions are formed as boric acid (H_3BO_3). Parameters measured in Gözde 80 were superior to other cultivars under drought stress conditions. The maximum mean germination time, seedling viability index, shoot and root length, shoot and root fresh weight, and total biomass, were detected as 4 days, 74%, 10 cm, 13 cm, 63 mg, 21 mg, and 80 mg, respectively. Differences were noted in the response of different sorghum cultivars to drought stress, and significant decreases were observed as the drought level increased. Low boron applications generally increased germination and seedling parameters compared to control under drought conditions. The boron effects applied to alleviate the drought stress effects have been noticeably positive. Compared to control conditions, the best results were also generally observed in the application of 5 mM B at a drought stress dose of -0.4 MPa. It was concluded that high doses of boron applications caused double stress with drought and were even lower than drought applications alone. It was recorded that due to the reasons listed above, careful attention should be paid to the boron doses to be applied.

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1. Introduction

Sorghum (*Sorghum bicolor* L.) appears as a product with great potential, therefore it indicates the fifth most considerable grain yield worldwide (Dahlberg et al., 2012; Avila et al., 2021). This plant is a cereal family product with plural uses for food, forage crop, and energy. The feed quality and energy stature of sorghum are similar to maize. Furthermore, sorghum is valued as silage, direct grazing, and fresh and dry fodder (Rad et al., 2021). Sorghum has high genetic variability and germplasm sources to settle new cultivars into varied ecoregions (Qadir et al., 2015; Al-Naggar et al., 2018; Erdurmuş et al., 2021).

Sorghum is a model plant for a more suitable crop development program in agriculture to use marginal areas to supply the energy and food requirements that may increase in the immediate future (Bibi et al., 2012; Qadir et al., 2015). Compared to other cereal crops, sorghum is extremely tolerant of abiotic stresses such as drought, flooding, and salinity. (Ali et al., 2011; Turner et al., 2016). The plants' response to abiotic stress is attached to genetic and environmental conditions. Drought stress reduces sorghum production, and deterioration is more vigorous when lack of water consists of the pre-flowering stage of development (Kothari et al., 2020).

It is estimated that the world population, which was about seven billion in recent years, will reach nine billion by 2050, and to support such a population, food security is more threatened by different kinds of environmental stresses (Khan et al., 2020). In recent years, with the effect of global warming, being an environmental problem, the importance of water has begun to be felt with agricultural drought (Khanna-Chopra and Selote, 2017; Rafique et al., 2022).

Plants grow tolerance mechanisms that provide physiological, biochemical, and molecular responses to drought stress and adapt to environmental conditions (Mut et al., 2010; Franks, 2011). Due to the sensitivity of plant species to drought, which can affect physiological processes such as photosynthesis, nutrient uptake, and transport during the growth phase is water restriction (Cai et al., 2020). Effect of drought stress on plant growth (Yao et al., 2009; Kamran et al., 2018) and yield; It varies depending on the developmental period in which the stress occurs and the severity and duration of the stress (Aykanat et al., 2009). Polyethylene glycol 6000 is the best chemical known for applying water stress, reflecting the kind of stress seen in drying soil (Verlues and Bray, 2004). Gholami et al. (2021) reported that the germination percentage and germination rate tended to decrease with increasing drought stress induced by PEG.

Boron (B) is an essential microelement and is constantly requisitioned by vascular plants to compose varied basic physiological processes in the life cycle, such as carbohydrates and RNA metabolisms (Tanaka and Fujiwara, 2008), the cell wall integrity and plasma membrane, meristematic tissues elongation (Herrera-Rodriguez et al., 2010), the growth and germination of the pollen tube, the flowers fertility formation, anther (Parry et al., 2016) and seed improvement (Iqbal et al., 2017; Nadeem et al., 2019).

Lack of boron can therefore affect and reduce vigor against abiotic stresses such as drought (Möttönen et al., 2001). Nevertheless, a few research has identified the availability of B in drought tolerance in plants. Considering these issues, the aim of this study is to investigate the effects of different boron doses on the germination and seedling growth of sorghum [*Sorghum bicolor* (L.) Moench] under drought stress conditions.

2. Material and Methods

The aim of this experiment is to investigate the curative effects of different boron doses on the germination and seedling growth of sorghum [*Sorghum bicolor* (L.) Moench] plant under drought stress conditions. This experiment was conducted at the forage crops laboratory, Department of Field Crops, Akdeniz University, Turkey during the spring of 2022. The experiment was carried out in four replications with the factorial arrangement according to the randomized plot design. The sorghum seeds were provided from Bati Akdeniz Agricultural Research Institute. Three selected cultivars, Erdurmuş, Uzun, and Gözde 80, were used as genetic materials. Petri dishes used were sterilized and incubated as a general protocol procedure. Twenty seeds from each cultivar were chosen and placed in 9 mm petri dishes, two Whatman filter papers were lined in. Tree drought stress levels causing 0, -0.4MPa, and -0.8 MPa were calculated by the equation of Michel and Merril (1973) using PEG 6000 concentration. Boron was applied as H_3BO_3 at 0-5-10-15 mM. 10 ml of solution was used for moistening in each application. Petri dishes were wrapped with parafilm to prevent water loss by evaporation. The petri dishes were settled in a growth chamber at 20ºC under photoperiodic conditions of 16 hours light and 8 hours dark. Observations were recorded daily. The study ended on the eleventh day.

Germination tests were carried out according to ISTA rules (2017). The seed of germination (MGT) was calculated using formulas described by Majda et al. (2019). The germination rate (GR) was calculated according to Xia et al. (2019). The germination index (GI) and seedling vigor index (SVI) were counted by the method of Xia et al. (2019). The root/shoot ratio (R/S ratio) was calculated as the

following equation (Shtaya et al., 2021). The calculation of stress tolerance indices formulas as described by Nawaz et al. (2014).

$$\text{MGT (day)}=\sum \frac{number\ of\ seeds\ germinated\ on\ the\ i^{th}\ day}{number\ of\ days\ to\ count\ the\ n^{th}day} \quad (1)$$

$$\text{GR}(\%)=\frac{\text{number of germinated seed/}}{\text{total number of seeds tested}}*100 \quad (2)$$

$$\text{GI}=\sum \frac{\text{the number of germinated seeds in day}}{\text{day of counting seed germination}} \quad (3)$$

$$\text{SVI}=\frac{\text{germination percentage}*\text{average seedling length}}{100} \quad (4)$$

$$\text{R/S ratio}=\frac{\text{roots length}}{\text{shoot length}} \quad (5)$$

$$\text{SLSI (\%)}=\frac{\text{Shoot lenght of stressed plant}}{\text{shoot length of control}}*100 \quad (6)$$

$$\text{RLSI (\%)}=\frac{\text{Root lenght of stressed plant}}{\text{root length of control}}*100 \quad (7)$$

$$\text{SFSI (\%)}=\frac{\text{Shoot fresh weight of stressed plant}}{\text{Shoot fresh weight of control}}*100 \quad (8)$$

$$\text{RFSI (\%)}=\frac{\text{Root fresh weight stress of stressed plant}}{\text{Root fresh weight of control}}*100 \quad (9)$$

Data determined for the study subjected to analysis variance using R (ANOVA) and means were compared by one-way ANOVA and Duncan's post hoc test in the agricolae, which differed significantly at 0.05 levels. (4.3.19) package.

3. Results and Discussion

Based on the statistical analysis, the plant growth parameters of sorghum cultivars influenced by different boron applications and drought conditions were given in Table 1 and Table 2.

Table 1. Results of variance analysis on growth parameters of boron doses and drought stress levels in sorghum cultivars

SV	df	MGT	GR	GI	SVI	SL	RL	R/S
C	2	0.08	100.52**	738.33**	1 708.97**	28.91**	38.42**	0.03**
DL	2	0.90**	1 338.02**	1 575.97**	595.85**	28.28**	23.77**	0.01
B	3	0.36**	2 000.93**	2 280.07**	881.45**	131.34**	223.31**	0.62**
C*DL	4	0.11*	65.10**	70.50**	45.25**	4.58**	0.97*	0.07**
C*B	6	0.02	83.39**	85.27**	58.48**	0.93**	3.75**	0.05**
DL*B	6	0.04	66.03**	63.51**	28.21**	1.81**	8.05**	0.08**
C*DL*B	12	0.06	8.39	10.08	3.95	0.83**	2.14**	0.04**

*Significant at the 0.05 probability level.**Significant at the 0.01 probability level. (Cultivar: C, Drought level: DL, Boron: B, Mean germination time: MGT, Germination Rate: GR, Germination index: GI, Seedling Vigor index: SVI, Shoot length: SL, Root length: RL, Root/shoot rate:R/S)

Except for mean MGT and RFSI, the growth parameters, studied in the experiment, were significantly ($p<0.01$) influenced in each cultivar. Similarly, drought conditions adversely affected the seedling growth parameters except for the R/L rate ($p<0.01$). Increasing boron application caused a significant ($p<0.01$) effect on parameters. MGT, RL, and RFW were statistically influenced by cultivar and drought interaction ($p<0.05$) and the others were also affected by interaction, significantly except RFSI. The interaction of cultivar, drought, and boron applications had a meaningful effect ($p<0.01$) on the plant growth parameters except for MGT (Table 1 and Table 2).

Table 2. Results of variance analysis on growth parameters and stress tolerances of boron doses and drought stress levels in sorghum cultivars

SV	df	SFW	RFW	TB	SLSI	RLSI	SFSI	RFSI
C	2	166**	163.81**	657.10**	3063**	913**	4573.00**	65
DL	2	1 179**	81.92 **	1 872.90**	113 971**	127 135**	116 172**	110 496**
B	3	4 999**	540.10**	8 792.70**	567**	1 588**	973.00**	5 204**
C*DL	4	260**	4.82*	205.80**	786**	260.00**	1 218**	57.00
C*B	6	75**	39.53**	184.20**	361**	1 104**	864**	1 423.00**
DL*B	6	99**	28.09**	223.10**	200**	578**	293**	1 658.00**
C*DL*B	12	57**	7.97**	64.50**	129**	516.**	297**	524.00**

*Significant at the 0.05 probability level.**Significant at the 0.01 probability level. (Cultivar: C, Drought level: DL, Boron: B, Mean germination time: MGT, Germination Rate: GR, Germination index: GI, Seedling Vigor index: SVI, Shoot length: SL, Root length: RL, Root/shoot rate:R/S)

Generally, the maximum growth parameters were realized with Gözde 80. Nevertheless, the MGT and GI were determined at the highest level in Erdurmuş. When the results were examined on the basis of cultivars, MGT was found as 3.74, 3.71, and 3.65 days at Uzun, Gözde, and Erdurmuş, respectively. A higher germination time indicates late emergence. The TB was observed as 40.60, 46.02, and 47.76 mg with Erdurmuş, Uzun, and Gözde 80, respectively (Table 3). In this study, the different botanical characteristics caused the differences in the effect of drought conditions (Ulukapi & Nasircilar, 2021) and boron doses. On the other hand, it was thought that some cultivars grown enriched with boron and drought conditions have developed resistance to stress.

Table 3. The effects of boron doses on growth parameters of sorghum cultivars exposed to drought stress level

Growth Parameters	Erdurmuş	Uzun	Gözde
MGT (day)	3.65±0.23b	3.74±0.24a	3.71±0.25ab
GR (%)	89.17±7.32a	86.77±7.89b	86.56±10.78b
GI (%)	96.40±7.91a	95.09±8.64b	89.05±11.21c
SVI (%)	52.16±4.28c	55.01±5.00b	63.62±7.92a
SL (cm)	5.82±2.04c	6.34±1.84b	7.35±1.81a
RL (cm)	5.01±2.02c	5.91±2.61b	6.79±2.67a
R/S	0.85±0.15b	0.89±0.19a	0.90±0.15a
SFW (mg)	35.21±12.43b	38.07±13.40a	38.70±10.52a
RFW (mg)	5.45±2.02c	7.95±4.79b	9.06±5.06a
TB (mg)	40.60±14.19c	46.02±17.86b	47.76±15.12a

Different letters next to values indicate statistically different means at $p<0.05$ level, and $p<0.01$ levels.

In this experiment, the lowest growth parameters were obtained in -0.8 Mpa DL and the highest were achieved in non-DL. The drought levels increased as the growth declined. The SL was determined as 7.33, 6.40, and 5.80 cm at increasing DL, respectively (Table 4). Drought stress conditions reduce plant growth (Gökkaya, 2016; Gulser et al., 2019) and cell division (Farah, 1981). As a result, this situation leads to the shrinking organs of plants proportionally. As it is known, if the plant height is getting higher, it increases surface evaporation and also the loss of water. Similarly, several researchers reported that drought stress has inhibitory effects on plant growth and decreases the shoot and root fresh weight (Sattar et al., 2021, Rafique et al., 2022).

Table 4. The growth parameters of sorghum cultivars exposed to drought stress levels

Growth Parameters	0 MPa	-0.4 MPa	-0.8 MPa
MGT (day)	3.57±0.19c	3.69±0.22b	3.84±0.23a
GR (%)	92.50±6.01a	88.02±7.20b	81.98±9.55c
GI (%)	99.01±6.75a	93.96±7.94b	87.57±10.90c
SVI (%)	60.23±7.01a	57.34±7.53b	53.22±6.90c
SL (cm)	7.33±211a	6.40±1.77b	5.80±1.81c
RL (cm)	6.66±3.11a	5.78±2.33b	5.27±1.87c
R/S	0.87±0.21	0.88±0.17	0.90±0.11
SFW (mg)	42.41±13.83a	37.09±10.66b	32.50±9.83c
RFW (mg)	8.92±5.70a	7.16±3.92b	6.37±2.85c
TB (mg)	51.33±18.37a	44.24±14.30b	38.87±12.44c

Different letters next to values indicate statistically different means at $p<0.05$ level, and $p<0.01$ levels.

Water deficit decreased seed GR and seed germination percentage. Germination can be affected by water absorption of seeds at strongly negative water potentials, especially at the inhibition beginning, and this event cannot reverse the germination process. Osmotic pressure and increased PEG concentration, cause a decrease in water potential, inhibiting water absorption, delaying germination time, and reducing the germination rate as reduced absorption may inhibit the germination metabolic process (Jamil et al., 2006, Dawadi et al., 2019). Similar to previous studies, it was determined that PEG-induced drought stress was negatively associated with germination (Khodarahmpour, 2011).

Some effects of drought stress on plant growth are enhanced energy demand due to an increase in plant respiration (Moud and Maghsoudi, 2008) and a decrease in the level of photosynthesis (Abdel-Motagally and El-Zohri, 2016). This causes an increase in the stiffness of the cell wall, reducing the rate of cell division, expansion, and elongation (Baek et al., 2005, Sadak et al., 2020). RL and SL and SFW decreased with increasing PEG levels (Bilgili et al., 2019). As a result, seedling growth was inhibited in sorghum (Table 4).

Plant roots hold a key role in uptaking the water and nutrients (Wu et al., 2019). Various studies have shown that root water uptake is related to root morphology (Gao et al., 2010; Yang et al., 2012; Li et al., 2015). In addition, the development of plants is one of the criteria for evaluating drought tolerance and is directly affected by the degree of root development (Chen et al., 2011). Many researchers have reported that plant roots exposed to a lack of water develop better than roots that grow without water restriction (Almaghrabi, 2012; Liu et al., 2017). However, in our study, increasing PEG levels decreased the RL. The increase in RL appears to be due to uptake from the water source, and we experienced drought stress in aqueous solutions of PEG in our study, so both cultivars had reduced roots (Table 4).

Results obtained in our experiment, TB showed significantly decreased under drought stress in cultivars (Table 4). These results are the same those determined by Sadiq et al. (2018) and Dawood et al. (2019). The drought's adverse effects on fresh shoot biomass may be due to reduced photosynthesis rates under drought conditions (Haq et al., 2014).

Table 5. The effects of boron doses on growth parameters of sorghum cultivars

Growth Parameters	0 mM	5 mM	10 mM	15 mM
MGT (day)	3.66±0.25b	3.62±0.19b	3.67±0.22b	3.85±0.23a
GR (%)	89.86±3.04b	95.14±4.70a	87.64±6.03c	77.36±8.22d
GI (%)	96.28±4.19b	101.57±5.83a	93.52±6.34c	82.68±10.40d
SVI (%)	58.50±5.06b	61.92±5.53a	57.16±5.00c	50.15±5.74d
SL (cm)	7.24±1.08b	8.59±1.47a	6.13±0.90c	4.08±0.92d
RL (cm)	6.99±1.46b	8.72±1.97a	4.91±0.80c	2.99±0.79d
R/S	0.96±0.11b	1.02±0.16a	0.81±0.12c	0.74±0.10d
SFW (mg)	41.12±7.31b	51.13±8.99a	33.88±5.02c	23.19±4.35d
RFW (mg)	8.55±2.83b	12.35±4.88a	5.65±1.15c	3.38±0.78d
TB (mg)	49.67±9.22b	63.48±12.42a	39.53±5.20c	26.57±4.83d

Different letters next to values indicate statistically different means at $p<0.05$ level, and $p<0.01$ levels.

The highest means were reported at 5 mM boron level. It was indicated that low boron increases the parameters, moreover, the others decrease the growth (Table 5). Also, Muhammad et al. (2013) and Habtamu et al. (2014) determined that high boron doses decreased the germination percentage. These results correlate with our study. The GI of seeds was directly obtained by boron levels like other properties (Xia et al., 2019). The highest MGT was realized at 15 Mm B with 3.85 days (Table 5). Memon et al. (2013) and Xia et al. (2019) have similar results. Excessive boron doses could not provide sufficient energy for seeds in time to complete their germination and seedling growth (Deb et al., 2010; Chen and Arora, 2013; Iqbal et al., 2017). Low concentration of boron had positive effects on meristematic growth (Khan et al., 2006) and SVI (Table 5, Farooq, 2011; Xia et al, 2019). The SVI frequently reflected the establishment ability of the seedling during plant growth (Xia et al., 2020) In this experiment SL was reduced by 52.50 % and RL decreased by 65.71 % (Table 5). The main reason for the reduction in RL is that higher doses of boron inhibit root growth (Habtamu et al., 2014), primarily by limiting cell protraction and cell division (Brown et al., 2002). Increasing boron doses above 5 mM significantly reduced the FW of plants (Ayvaz et al., 2012; Mohammed et al. 2013; Habtamu et al., 2014). Compared to the control application, there was a 27.80% increase in TB at the 5 mM boron, whereas higher doses caused 20.41% and 46.50% reduction, respectively (Table 5).

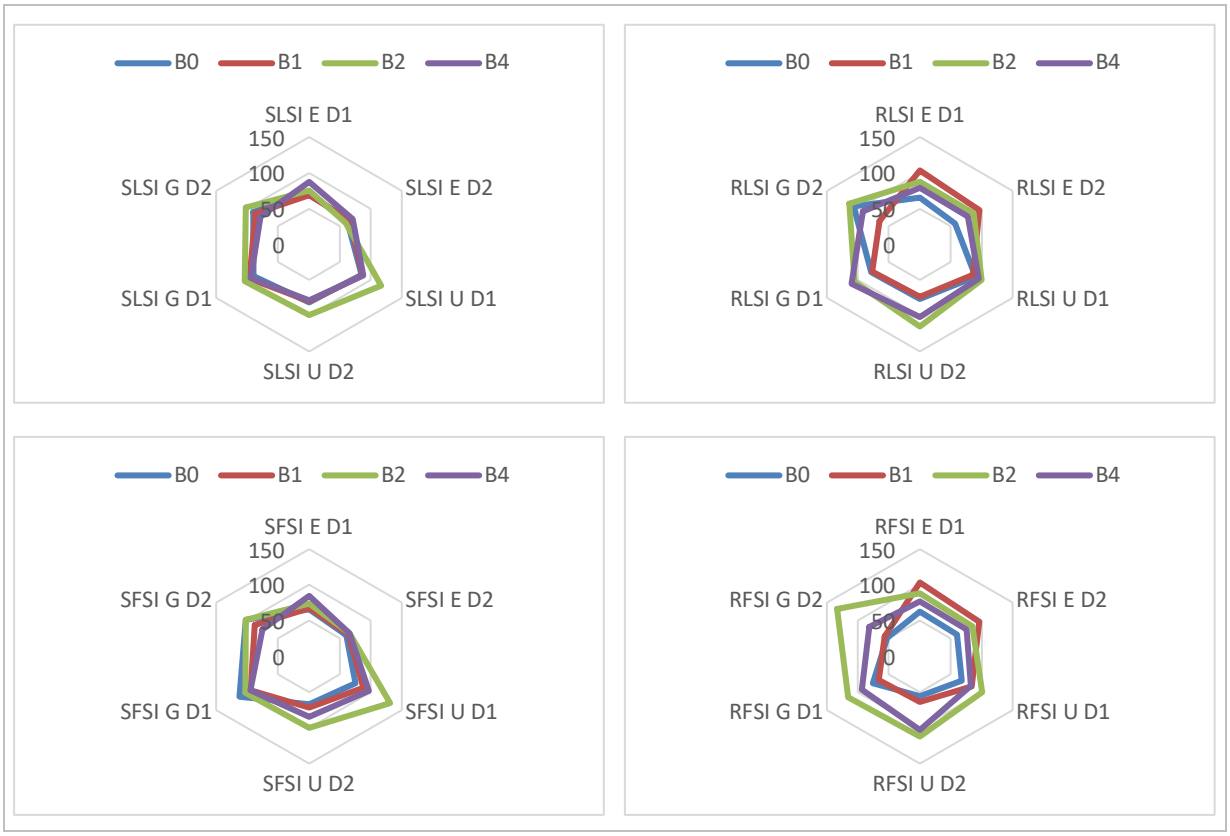

Figure 1. Effects of boron doses on SLSI, RLSI, SFSI, and RFSI of sorghum cultivars under drought conditions (E: Erdurmuş, U: Uzun, G: Gözde 80, D1: -0.4 MPa, D2:-0.8 MPa, B0: 0 mM B, B1 5 mM B, B2: 10 mM B, B3: 15 mM B, Shoot length stress tolerance index: SLSI, Root length stress tolerance index: RLSI, Shoot fresh weight stress tolerance index: SFSI, Root fresh weight stress tolerance index: RFSI).

When it was noticed cultivar x drought x boron interactions, the lowest and highest SL and SI have obtained as 59.79 % and 116.64 % in -0.8 MPa and 10 mM B application in Erdurmuş and -0.4 MPa and 10 mM B application in Uzun, respectively. While the lowest RLSI was determined as 57.10 % (Erdurmuş) in -0.8 MPa-0 Mm B application, the highest was in -0.8 MPa-10 Mm B application at Uzun (115 %). The highest SFSI was found as 131.07 % by -0.4 MPa- 10 mM B application in Uzun. The lowest was detected as 59.96 % (Erdurmuş) in -0.8 MPa- 0mM B applications. The lowest and highest RFSI belong Gözde 80 were realized as 52.15 % and 133.52 % in -0.8 MPa 0 Mm B and 10 Mm

B applications, respectively (Figure 1). The process of cell wall synthesis and elongation in plants is highly sensitive to water restriction, and the reduction in growth may be due to decreased turgor pressure of cells (Mohammadkhani and Heidari, 2008). In this study, it was observed that drought levels decreased sorghum plant growth. But low boron applications had significant and positive effects on stress tolerances.

Conclusion

As a result of this study, a decrease in germination and seedling parameters was obtained at different drought levels created with PEG. It has been observed that different species give different responses. In addition, it was determined that low-level boron applications caused an increase in these parameters in drought conditions created with PEG. The results of different drought levels determined in this study and the effects of boron applications on germination and seedling are thought to be useful for further studies.

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Research Article

Different POST-em Herbicide Programs for Weed Management in Lowland Flooded Rice System in North Macedonia

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Abstract: The field experiments were carried out during 2017 and 2018 on commercial rice field in Kochani region to assess different POST-em herbicide programs for weed management in lowland flooded rice system in North Macedonia. In addition, herbicide selectivity and impact on rice grain yield were estimated. POST-em herbicide treatments were used in early-(EPOST-em), mid-(MPOST-em) and late-(LPOST-em) rice growth stages (BBCH 26; 29 and 32-34, respectively). Weed control varied among herbicide treatments, herbicide programs, and weeks after treatments (WAT). All herbicides applied EPOST-em controlled *Echinochloa crus-galli* (ECHCG) and *Scirpus maritumus* (SCMA) 91-100%. At MPOST-em treatment, herbicides showed control of ECHCG between 93 and 97%. However, all herbicides applied LPOST-em controlled ECHCG 79-88%. SCMA control was less than 88 and 85% with MPOST-em and LPOST-em treatments, respectively, perhaps as a consequence of progressive growth stage of SCMA (BBCH 40). Control level of *Cyperus rotundus* (CYPRO) and *Heteranthera reniformis* (HETRE) was high in all POST-em treatments (between 90-100%, and 95-100%, respectively). EPOST-em and MPOST-em application of any herbicide resulted no phytotoxicity to rice plants. LPOST-em treatments caused rice phytotoxicity by cyhalofop-butyl + penoxsulam, cyhalofop-buthyl + bentazon, and profoxidim + bentazon which were ranged from 8-20%. Unlike rice yield at LPOST-em treatments was 6235 kg ha^{-1}, all EPOST-em and MPOST-em used herbicides has impact in rice yield 6685 and 6610 kg ha^{-1}, respectively which, but there were no statistically significant differences with the weed free control 6710 kg ha^{-1}.

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1. Introduction

Rice is considered as one of the greatest cereal crops and the staple food for the majority of the world's population (Jiang et al., 2013). However, worldwide, rice is challenging with several problems and beside harmful biological agents and the environmental damage the climate is the main factor as stressor that can cause failure in rice production (Heriansyah et al., 2022). The favourable environment

in rice-production countries, including Macedonia, not only provides valuable conditions for the cultivation of rice but also furthermore offers a suitable climate for many weed species. In some countries, such as in Turkey, researches have been carried out to establish the land suitability classes of rice lands (Dengiz et al., 2022). The weed species in rice are frequently composed of species that are not found as weeds of terrestrial crops, and therefore, rice weed communities are highly different and composed mainly of aquatic plants (McConnell and Barrett, 1985; Pinke et al., 2014). Grasses, like barnyard grass, broadleaf weeds, like mud plantains, and sedges, nutsedges, and bulrushes are dominant weeds in lowland flooded rice systems in North Macedonia (Pacanoski and Glatkova, 2009; Pacanoski, 2015).

Echinochloa crus-galli (L.) P. Beauv. (ECHCG) occurs with high frequency and distribution in all rice-growing areas and is one of the dominant weeds infesting paddy fields in the world (Dowler, 1997; Andres et al., 2007). ECHCG is a strong competitor with rice as a consequence of its adjustment to submerged conditions, high reproductive capacity, quick increment, and C4-photosynthetic mechanism (Marambe and Amarasingle, 2002). Globally troublesome weeds, *Heteranthera limosa* (Sw.) Willd. (HETLI) and *Heteranthera reniformis* Ruiz & Pav. (HETRE), C3 species are the most frequently reported aquatic weeds and a serious problem in lowland flooded rice (Chandler, 1981; Ferrero 1996; Vescovi et al., 1996; Vasconcelos et al., 1999). *Cyperus difformis* L. (CYPDI), *Scirpus maritimus* L. (SCPMA), and *Scirpus mucronatus* L. (SCPMU) are some of the most frequently encountered sedges in rice fields. *Cyperus rotundus* L. (CYPRO) is considered one of the worst weeds in the world (Holm et al., 1991). It is widely spread throughout the tropics and subtropics, and well adapted to lowland flooded environments (Rao, 2000; Pena-Fronteras et al., 2009). CYPRO lately has been reported that occurs in 21 countries where rice is cultivated (Rao et al., 2007). SCPMA, perennial sedge is a serious problem in lowland rice fields in several countries (Caton et al., 2010). The weed is more competitive than other lowlands weeds because its top growth elongates rapidly and nutrient uptake is rapid during its early growth stages (Bernasor and De Datta, 1986).

In North Macedonia, some POST-em herbicides are few herbicides for ECHCG control, and they may be useful in controlling broadleaf weeds and sedges, as well. Cyhalofop and profoxydim are POST-em herbicides, and inhibitors of acetyl-CoA carboxylase (Monadjemi et al., 2012; Kanatas, 2020). Cyhalofop at 200 g ai ha^{-1} controlled ECHCG at least 88% when applied EPOST as well as LPOST (Ntanos et al., 2000). Profoxydim applied at 200 g/ha provided 95-100% control of two ECHCG accessions (Vidotto et al., 2007; Kaloumenos et al., 2013). Similarly, Matzenbacher et al., (2013) reported that ALS-resistant biotypes of ECHCG were successfully controlled by profoxydim and cyhalofop-butyl. Penoxsulam as a triazolopyrimidine sulfonamide inhibits the acetolactate synthase (ALS) enzyme (Lassiter et al., 2004). It is a broad-spectrum herbicide registered for weed control in rice. It provides effective control of *Echinochloa* spp., sedges *Cyperus* spp. and *Scirpus* spp., and numerous broadleaf weeds, including, mud plantain *Heteranthera* spp. (Walton et al., 2005; Lassiter et al. 2006). Bentazon is a benzothiadiazole herbicide, an inhibitor of a photosystem II (Fleming et al. 1988; Bradshaw et al. 1992; Han and Wang, 2002). It is a POST-em herbicide commonly used to control broadleaf weeds and sedges in rice (Nyarko and De Datta, 1991). Bentazon effectively controlled SCPMA (Bernasor and De Datta, 1986) and CYPRO (Pathak et al., 1989), when applied at the six-eight leaf stage, respectively.

Taking into account that for weed management in lowland flooded rice in North Macedonia only POST-em herbicides are registered, and that period of weed germination and growth in rice crops is under substantial alterations, especially in environmental conditions, the reliability of POST-em weed-control programs is fluctuating and greatly determined by the floristic composition of weed population and environmental condition. Hence, the aim of this investigation was to estimate different POST-em herbicide programs for successful weed management and optimal rice yield in lowland flooded rice systems in North Macedonia.

2. Material and Methods

The field experiments were carried out in 2017 and 2018 on commercial rice fields in the Kochani region in North Macedonia. The type of soil was a vertisol with 3.5% coarse, 9.1% coarse sand, 30.0% sand, 60.3% silt + clay, 2.4% organic matter, and pH 7.2. The rice seedbed was arranged by moldboard plowing in the autumn. Two passes with a field cultivator were done in the spring. The

fertilizers with the content of potassium and phosphorus were added before rice sowing at a rate of 80 and 60 kg ha^{-1} as potassium sulphate (48% K_2O) and superphosphate (15.5% P_2O_5), respectively. Additionally, the supplementary fertilizer, 150 kg nitrogen fertilizer/ha^{-1} as ammonium nitrate (33.5% N) was applied at 2/3 and 1/3 doses at the beginning of tillering stage (BBCH 21) and the panicle initiation (green ring) stage (BBCH 30), respectively. Usual water management applications were utilized, so the plots were flooded 2 days before the sowing of rice. Italian rice variety "Gloria" was used in the field trials, which was drill-seeded in a well-prepared seedbed at a seeding rate of 200 kg ha^{-1} on May 1st, 2017, and May 5th, 2018.

The experiment was set in a randomized block design with four replications comprising three POST-em herbicide programs. POST-em herbicides were applied in early-(EPOST-em), mid-(MPOST-em), and late-(LPOST-em) rice growth stages, i.e. on June 10th, 17th and 24th in 2017, and June 12th, 20th and 27th in 2018, respectively. In the POST-em weed control investigation were included four herbicide treatments: penoxsulam at 1.5 L ha^{-1} + bentazon at 4.0 L ha^{-1}, cyhalofop-buthyl at 1.5 L ha^{-1} + bentazon at 4.0 L ha^{-1}, cyhalofop-buthyl at 1.5 L ha^{-1} + penoxsulam at 1.5 L ha^{-1} and profoxidim at 1.0 L ha^{-1} + bentazon at 4.0 L ha^{-1}. The used herbicides were applied with a CO_2-pressurized backpack sprayer calibrated to distribute 300 L ha^{-1} aqueous solution at 220 kPa in drained plots, which were re-flooded two days after treatment (DAT). Untreated and weed-free controls were included in the studies, as well. The control plots were not treated with herbicides during the entire experimental period. In weed-free control, weeds were removed by hand. Hand-weeding was started at weeds emergence and continued as required to maintain weed-free plots. Weed and rice growth stages during different POST-em herbicide applications are presented in table 1.

Table 1. Weeds and rice growth stages during POST-em herbicide applications

		Growth stages (BBCH)		
		EPOST-em	MPOST-em	LPOST-em
Weeds	**ECHCG**	BBCH 21-23	BBCH 29	BBCH 32-34
	SCPMA	BBCH 30-32	BBCH 37-39	BBCH 40
	CYPRO	(BBCH 11-12)	BBCH 13-15	BBCH 17-19
	HETRE	BBCH 12-14	BBCH 14-16	BBCH 16-18
Crop	**Rice**	BBCH 26	BBCH 29	BBCH 32-34

The efficacy of weed control was estimated 2 and 4 Weeks After Treatment (WAT) by the weed plants for 1m^2 within each plot, at both localities during a two-year experimental period, while the herbicide efficacy was calculated by equitation (Chinnusamy et al., 2013):

$$WCE = \frac{Wup - Wtp}{Wup} \times 100 \quad (1)$$

Where:
WCE - weed control efficiency
Wup- number of weeds in the untreated plots
Wtp- number of weeds in the treated plots

Rice phytotoxicity was visually assessed based on a ranking scale of 0-100%, where 0 is not any phytotoxicity to rice plants, and 100 is complete death of rice plants (Frans et al., 1986). Visual assessments of percent rice phytotoxicity were assessed one and 3 WAT, based on leaf chlorosis and necrosis for each replication.

A cutting survey was conducted to measure the grain yield of rice in the October harvest season, for both years. The yield of rice grain was assessed from 1m^2 for each repetition t ha^{-1}, and yield was measured after the harvest of grain that contained 13% moisture.

The data were tested for homogeneity of variance and normality of distribution (Ramsey and Schafer, 1997) and were log-transformed as needed to obtain roughly equal variances and better symmetry before ANOVA was performed. Years, replication (nested within years), and all interactions containing either of those effects were considered random effects; herbicide program and DAT were

considered fixed effects. Based on the mixed procedure used, all data were pooled over years. Finally, data were transformed back to their original scale for presentation. Means were separated by using the LSD test at 5% of probability.

3. Results and Discussions

3.1. Weed control

The site was naturally infested with a high population of ECHCG, SCPMA, CYPRO, and HETRE. Weeds number in the non-treated control plot was 191 and 232 plants/m^2 in 2017 and 2018, respectively. POST-em herbicide program and WAT main effects were identified, hence, data are presented individually by POST-em herbicide program averaged over years and WAT (Table 2), and by WAT averaged over years and herbicide program (Table 3).

3.2. *Echinochloa crus-galli*

ECHCG control varied among POST-em treatments, herbicide programs, and WAT. At EPOST treatment, all herbicides controlled ECHCG 91-100%. Nevertheless, the greatest control was achieved with penoxsulam + bentazon and cyhalofop-butyl + penoxsulam (98-100%). The efficacy of cyhalofop-butyl in ECHCG control is acceptable if application follows the early phenological phases (2-4 leaves) (Kalsing et al., 2017). At MPOST-em treatment, herbicides assured control of ECHCG between 93-97%. However, all herbicides applied LPOST-em controlled less ECHCG 79-83%, except cyhalofop-butyl + penoxulam which controlled ECG 88% (Table 2). Averaged ECHCG control over different POST-em herbicide programs was 95-92% at EPOST-em and MPOST-em treatments at 2 WAT, and 98-99% at 4 WAT, respectively. Substantially poorer efficacy was achieved in LPOST-em treatment (84% and 80%) at both assessment periods (Table 3). Inadequate ECHCG control in LPOST-em treatment probably is a consequence of the progressive weed growth stage (stem elongation stage - BBCH 32-34). For this reason, herbicides should be applied at early growth stages of ECHCG (maximum tillering stage-BBCH 29) to achieve the most effective control. Regarding the phenology effect on profoxydim effectiveness, the study of Kanatas (2020) revealed a higher ECHCG control at the earlier growth stage (BBCH 13) for 15-50% than at the late growth stages (BBCH 22 and 30). In addition, it is reported by Ntanos et al. (2000) that cyhalofop-butyl applied EPOST at 150 g ai ha^{-1} controlled ECHCG between 85 and 95% in drained plots 30 DAT. Cyhalofop-butyl applied LPOST at the same rate provided only 75% control of ECHCG. Penoxsulam applied alone in EPOST-em and MPOST-em periods controlled ECHCG nearly 100% (Ottis et al., 2003). In the investigation of Pacanoski (2015) ECHCG control across POST-em herbicide programs (penoxulam, cyhalofop-buthyl, azimsulfuron, and profoxidim) was 99-92% at EPOST-em and MPOST-em treatments at 14 DAT, and 99-98% at 28 DAT, respectively. Substantially poorer efficacy was achieved in LPOST-em treatment (87% and 81%) at both assessment periods in investigated localities.

3.3. *Scirpus maritumus*

SCPMA control varied among POST-em treatments, herbicide programs, and WAT. The used EPOST-em herbicides suppressed SCPMA 96-99%. However, SCPMA control was less than 88 and 85% with MPOST-em and LPOST-em treatments, respectively. Between the MPOST-em and LPOST-em, only penoxsulam + bentazon controlled SCPMA was statistically greater in comparison to other assessed herbicides (Table 2). Averaged across POST-em herbicide programs, SCPMA control was 96-99% at EPOST-em treatments at 2 and 4 WAT, respectively. This efficacy was perhaps due to the better activity of the herbicides applied to younger weed growth stages, which was not the case in MPOST-em and LPOST-em herbicide programs. Significantly lower efficacy was provided in these POST-em treatments (between 81% and 76%) at both estimation periods (Table 3). This indicates the regrowth of SCPMA plants affecting weak control as the season evolved. Single penoxsulam treatment was applied at 20, 30, and 40 g a.i. ha^{-1} controlled SCPMA between 50-80%, but the combination of penoxsulam (30 g a.i. ha^{-1}) and bentazon (960 g a.i. ha^{-1}) provided complete (100%) control of SCPMA (Kogan et al., 2011).

3.4. *Cyperus rotundus*

The efficacy of POST-em herbicides for the control of CYPRO varied amongst applied herbicides, herbicide programs, and WAT, as well. EPOST-em treatments provided control of CYPRO >92%, but the highest control was attained with cyhalofop-butyl + penoxsulam and penoxsulam + bentazon (99-100%). Similar efficacy was noted at the MPOST-em program. Although all herbicides provided control of CYPRO higher than 92%, cyhalofop-butyl + penoxsulam and penoxsulam + bentazon showed statistically higher efficacy in their control compared to other herbicides. The high herbicide efficacy was recorded at LPOST-em treatment when herbicides controlled CYPRO between 90-96% (Table 2).

Averaged CYPRO control over different POST-em herbicide programs ranged between 92-95% at 2 WAT. At 4 WAT, control of CYPRO increased to 97 and 95% in EPOST-em and MPOST-em treatments, respectively, but it was the same at LPOST-em applied herbicides (92%) (Table 3). Mahajan and Chauhan, (2013) reported that pendimethalin alone applied PRE-em and penoxsulam applied POST-em poor controlled CYPRO (66%). Similarly as in previous research, in the Philippines, penoxsulam + cyhalofop applied POST-em provided poor control of CYPRO in direct-seeded rice (Chauhan and Opeña 2012).

3.5. *Heteranthera reniformis*

The control of HETRE differed among POST-em herbicides, but no differences were observed among herbicide programs and DAT, respectively. Control of HETRE by EPOST-em treatments was above 95%; penoxsulam + bentazon and cyhalofop-butyl + penoxsulam provided excellent control (100%). Cyhalofop-buthyl + bentazon and profoxidim + bentazon increased MPOST-em control of HETRE compared to their EPOST-em application by 3 and 1%, respectively (Table 2). MPOST-em penoxsulam + bentazon and cyhalofop-butyl + penoxsulam provided a similar level of control of HRE as EPOST-em treatment. A negligible decrease in HETRE suppression was recorded at LPOST-em penoxsulam + bentazon and cyhalofop-butyl + penoxsulam application. Opposite, LPOST-em profoxidim + bentazon increased control of HRE by 2% in comparison with their MPOST-em application. LPOST-em cyhalofop-buthyl + bentazon achieved the same level of HETRE control as MPOST-em treatment (Table 2). Nonsignificant differences were observed among herbicide programs and DAT. HETRE efficacy averaged across all POST herbicide programs was 96-100% at 2 and 4 WAT, respectively. Consistent HETRE control was probably due to younger weed growth stages during all POST-em herbicide programs.

Table 2. ECHCG, SCPMA, CYPRO, and HETRE control with EPOST-em, MPOST-em and LPOST-em herbicide treatments, respectively in lowland flooded rice in Kochani region in 2017 and 2018, averaged over years and WAT

Treatments	Rate (L ha^{-1})	ECHCG			SCPMA			CYPRO			HETRE		
		EPOST-em (%)	MPOST-em (%)	LPOST-em (%)	EPOST-em (%)	MPOST-em (%)	LPOST-em (%)	EPOST-em (%)	MPOST-em (%)	LPOST-em (%)	EPOST-em (%)	MPOST-em (%)	LPOST-em (%)
Non-treated control	-	0	0	0	0	0	0	0	0	0	0	0	0
penoxsulam + bentazon	1.5+4.0	98^{ab}±0.65	96^{a}±0.96	79^{b}±1.68	99^{a}±0.29	88^{a}±1.29	85^{a}±1.65	100^{a}±0.00	99^{a}±0.25	96^{a}±0.63	100^{a}±0.25	100^{a}±0.25	97^{b}±0.82
cyhalofop-buthyl + bentazon	1.5+4.0	95^{c}±0.95	93^{b}±1.11	83^{ab}±2.21	97^{ab}±0.91	80^{b}±1.85	74^{b}±1.25	94^{b}±1.25	92^{b}±1.49	91^{bc}±1.11	97^{b}±0.41	100^{a}±0.29	100^{a}±0.25
cyhalofop-butyl + penoxsulam	1.5+1.5	100^{a}±0.00	97^{a}±0.85	88^{a}±1.38	96^{b}±1.11	78^{b}±2.20	75^{b}±1.75	99^{a}±0.25	98^{a}±0.85	94^{ab}±0.85	100^{a}±0.50	100^{a}±0.25	97^{b}±1.11
profoxidim + bentazon	1.0+4.0	96^{bc}±0.48	95^{ab}±1.11	79^{b}±1.80	98^{ab}±0.48	75^{b}±1.75	72^{b}±1.29	92^{b}±1.68	93^{b}±1.47	90^{c}±1.49	95^{c}±0.85	96^{b}±1.44	98^{ab}±0.71
LSD (0.05)		2.06	2.49	6.59	2.09	5.40	4.83	3.51	3.20	3.76	1.37	2.23	2.78

Abbreviations: EPOST-em-early-posteemergence; MPOST-em-mid-postemergence; LPOST-em-late-postemergence.
EPOST-em treatments were applied at rice BBCH 26, ECHCG BBCH 21-23, SCPMA BBCH 30-32, CYPRO BBCH 11-12, and HETRE BBCH 12-14.
MPOST-em treatments were applied at rice BBCH 29, ECHCG BBCH 29, SCPMA BBCH 37-39, CYPRO BBCH 13-15 and HETRE BBCH 14-16.
LPOST-em treatments were applied at rice BBCH 32-34, ECHCG BBCH 32-34, SCPMA BBCH 40, CYPRO BBCH 17-19 and HETRE BBCH 16-18.
Weed control efficacy was estimated 2 and 4 WAT.
Means followed by the same letter within a column are not significantly different according to Fisher's Protected LSD at P<0.05.

Table 3. Control of ECHCG, SCPMA, CYPRO, and HETRE with different POST-em herbicide programs at different WAT in lowland flooded rice in Kochani region in 2017 and 2018, averaged over years herbicide program

	Control									
POST-em Programs	ECHCG (%)		SCPMA (%)		CYPRO (%)		HETRE (%)		Total for all Weeds (%)	
	2 WAT	4WAT	2 WAT	4 WAT	2 WAT	4 WAT	2 WAT	4 WAT	2 WAT	4 WAT
EPOST-em	95^{a}±1.89	99^{a}±0.58	96^{a}±0.82	99^{a}±0.48	95^{a}±2.38	98^{a}±1.50	96^{a}±2.02	100^{a}±0.50	96^{a}±0.29	99^{a}±0.41
MPOST-em	92^{a}±1.03	98^{a}±0.85	81^{b}±1.03	80^{b}±2.95	94^{ab}±1.93	97^{a}±1.89	98^{a}±1.41	100^{a}±0.50	92^{ab}±2.72	94^{ab}±3.64
LPOST-em	87^{b}±1.87	80^{b}±2.86	78^{b}±2.78	76^{b}±2.95	92^{b}±1.03	92^{b}±2.17	96^{a}±0.75	99^{a}±0.48	88^{b}±3.04	87^{b}±3.82
LSD (0.05)	4.16	5.67	4.17	5.53	2.90	2.25	4.83	1.50	5.93	8.92

Herbicide programs included penoxsulam at 1.5 L ha^{-1} plus bentazon at 4.0 L ha^{-1}, cyhalofop-buthyl at 1.5 L ha^{-1} plus bentazon at 4.0 L ha^{-1}, cyhalofop-buthyl at 1.5 L ha^{-1} plus penoxsulam at 1.5 L ha^{-1} and profoxidim at 1.0 L ha^{-1} plus bentazon at 4.0 L ha^{-1} applied EPOST-em, MPOST-em and LPOST-em.
Means followed by the same letter within a column are not significantly different according to Fisher's Protected LSD at P<0.05.

3.6. Rice phytotoxicity

Rice phytotoxicity of cyhalofop-butyl + penoxsulam, cyhalofop-buthyl + bentazon, and profoxidim + bentazon was more serious and ranged from 8-20%. Phytotoxicity caused by cyhalofop-butyl + penoxsulam and cyhalofop-buthyl + bentazon significantly reduced by 1 and 3 WAT (Table 4). However, rice phytotoxicity of profoxidim + bentazon was still evident at 3 DAT. The LPOST-em treatment caused rice phytotoxicity, particularly by treatments that contained cyhalofop-buthyl and profoxidim, probably related to the high temperature and advanced rice growth stage. During LPOST-em application temperature was about 30ºC and rice was at the stem elongation stage (BBCH 32-34).

Rice phytotoxicity has been confirmed with ACCase-inhibiting herbicides (Carey et al., 1992; Baltazar and Smith, 1994; Buehring et al., 2006). For example, the 0.4 kg ha^{-1} rate of cyhalofop-butyl caused phytotoxicity in rice and a significant impact on grain yield (Ntanos et al., 2000). Although excellent outcomes in the control of *Echinochloa* spp. were found with profoxidim, it showed phytotoxicity over all tested indica type cultivars (Marchesi, 2012). Opposite, 10 rice cultivars showed tolerance to penoxsulam as proved by plant height, number of days to 50% heading, and rice grain yield (Bond et al., 2007).

3.7. Rice grain yield

Rice grain yields for each treatment in both years mostly revealed overall weed control and crop phytotoxicity (Table 4). Evaluation of non-treated and weed-free controls showed that weeds reduced rice grain yield by 60% averaging across both experimental years (Table 4). Similarly, many authors estimated that average yield losses in rice attributed to weed competition are between 40 and 96% (Johnson et al., 2004; Ekeleme et al., 2009; Mahajan et al., 2009); Chauhan and Johnson, 2011). Particularly large reductions in the rice yield caused ECHCG (Ottis and Talbert, 2007; Wilson et al., 2014; Shabbir et al., 2019), SCPMA (Lieffers and Jennifer, 1982; Mamun et al., 2013), CYPRO (Rabbani et al., 2011; Chauhan and Opeña, 2012; Donayre et al., 2015) and HETRE (Schiele, 1988; Ferrero, 1996).

Averaged across both experimental years all EPOST-em and MPOST-em used herbicides resulted in rice yield (6685 and 6610 kg ha^{-1}, respectively) which was statistically in pair with rice yield in the weed-free control (6710 kg ha^{-1}). Contrary, rice yield at LPOST-em treatments (6235 kg ha^{-1}) was significantly weak in comparison with the rice yield in the weed-free control. The LPOST-em herbicides showed lower weed control and affected rice phytotoxicity, and yield was lower in all replications treated with LPOST-em herbicides, particularly in plots treated with profoxidim + bentazon (-690 kg ha^{-1}). However, statistical differences were observed among profoxidim + bentazon and other LPOST herbicides treatments, as well (Table 4).

In the investigation of Sekhar et al. (2020) herbicide combination penoxsulam + cyhalofop butyl resulted in higher grain yield than the sole application of cyhalofop-butyl. The application of this herbicide combination increased the grain yield by 28-60% more than the sole application of cyhalofop-butyl. Similarly, the application of penoxsulam + cyhalofop butyl at its higher dose (130 + 135 g a iha^{-1}) recorded the highest grain yield of 8.46 t/ha (Sheeja and Syriac, 2015). Tagour et al. (2010) reported that the mixture bentazon + penoxsulam has a higher impact in increasing the number of productive tillers, number of panicles m^{-2}, 1000 grain weight, and grain and straw yield. Similarly, plots treated with penoxsulam + bentazon achieved the highest yield 9.17 t/ha (Kogan et al., 2011).

Table 4. Effect EPOST-em, MPOST-em and LPOST-em applied herbicides at different WAT in rice plant phytotoxicity and grain yield in lowland flooded rice in Kochani region in 2017 and 2018, averaged across years

Treatments	Rate (L ha^{-1})	Rice phytotoxicity (%)						Grain yield (kg ha^{-1})		
		EPOST-em		MPOST-em		LPOST-em		EPOST-em	MPOST-em	LPOST-em
		1 WAT	3 WAT	1 WAT	3 WAT	1 WAT	3 WAT			
Non-treated control*	-	-	-	-	-	-	-	2670±97.21	2670±97.21	2670±97.21
Weed-free control	-	-	-	-	-	-	-	6710^{ab}±40.21	6710^{a}±40.21	6710^{a}±40.21
Penoxsulam + bentazon	1.5+4.0	0	0	0	0	2	0	6740^{a}±44.91	6660^{a}±35.00	6370^{b}±50.41
Cyhalofop-buthyl + bentazon	1.5+4.0	0	0	0	0	18	8	6680^{ab}±39.16	6630^{ab}±52.18	6250^{b}±47.87
Cyhalofop-butyl + penoxsulam	1.5+1.5	0	0	0	0	16	5	6700^{ab}±42.30	6610^{ab}±50.39	6290^{b}±57.93
Profoxidim + bentazon	1.0+4.0	0	0	0	0	20	18	6620^{b}±44.04	6530^{b}±49.05	6020^{c}±61.56
Average yield of herbicide treatments								6685	6610	6235
LSD 0.05								102.14	111.70	140.77

*Non-treated control was excluded from the analysis of variance in order to detect the significant differences between the herbicide treatments
Means followed by the same letter within a column are not significantly different according to Fisher's Protected LSD at P<0.05

4. Conclusion

The use of POST-em herbicides in rice, depending on the time, has an effect on the control of weeds, but at the same time also has phytotoxic effects. The weed control level for all herbicides differed among herbicide programs and WAT. EPOST-em application of any herbicide evaluated in this study provided overall weed control of 96-99% at 2 and 4 WAT, respectively. A non-significantly lower efficacy was provided in MPOST-em treatment (92% and 94%) at both assessment periods. The lowest efficacy (88 and 87%, respectively) was recorded in LPOST-em applied herbicides at 14 and 28 DAT.

EPOST-em and MPOST-em application of any herbicide evaluated in this study resulted in no phytotoxicity to rice plants averaged over years at one and 3 WAT, respectively. Phytotoxicity was evident only in LPOST-em treatment. At one WAT phytotoxicity, between 2 and 20% was detected in all LPOST-em herbicides.

In general, rice yields are a result of the differences in weed control; the crop yields increase as control with the different POST-em treatments increased. The reason each LPOST-em herbicide assured weak weed control and some of them caused rice phytotoxicity, in plots where LPOST-em herbicides were applied the results showed significant yield reduction.

Based on the results on the efficacy of herbicides, time of use, their impact on yield, as well and rice plant phytotoxicity it is recommended the use the herbicides penoxsulam + bentazone and cyhalofop-butyl + penoxsulam EPOST-em application in rice crop.

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