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Effect of Rhizobacteria and Microalgae Treatments on Some Physiological and Biochemical Parameters of Fenugreek (*Trigonella foenum-graecum* L.) Grown under Drought Stress

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ABSTRACT

Background: In this study, the effects of deficit irrigation (DI) (normal=control, ½ reduced and 3/4 reduced) and some beneficial rhizobacteria (*Azospirillum lipoferum, Bacillus megaterium*) and microalgae (*Chlorella saccharophilia*) on some physiological and biochemical parameters of fenugreek (*Trigonella foenum-graecum*) were investigated.

Methods: The experiment was carried out in a fully controlled climate cabinet with 4 replications in factorial order according to the completely randomized plot trial design. Fenugreek (*Trigonella foenum-graecum*) plant was used as material. The study aims to investigate the effects of different deficit irrigation treatments (normal (control), 1/2 reduced and 3/4 reduced) and some rhizobacteria (Control= R0, *Azospirillum lipoferum*= R1, *Bacillus megaterium*= R2) and microalgae (*Chlorella saccharophilia*= R3) on the growth and development of fenugreek plants.

Result: Relative water content and membrane durability index values of leaf tissues decreased due to deficit irrigation applications, while ion leakage in leaf tissues, MDA and total phenolic substance content increased in leaf tissues. However, it was determined that they had positive effects on ion leakage in leaf tissues, total phenolic and flavonoid substance amounts of rhizobacteria and microalgae applications, but, membrane durability index in leaf tissues and MDA contents were affected as negative compared to the control.

Key words: Drought stress, Rhizobacteria, Fenugreek, Physiological and Biochemical parameters.

INTRODUCTION

Drought is determined as an environmental factor that affects the fertility and productivity of plants (Bartels and Sunkar, 2005; Basheer-Salimia *et al.* 2021). Plant growth, crop productivity and development of plants are restricted as a result of drought and insufficient irrigation. Climatic change is worsening the severity of drought (Wu *et al.* 2017). On the earth, around 30% of the whole land is arid land and inadequate for crop production (Bray, 2004). Water deficiency is considered one of the main environmental factors that limit the improvement and growth of plants (Dudley, 1996; Jincya *et al.* 2021). Crop production and plants are strongly influenced by insufficient water which causes water deficit stress (Passioura, 1996). Water deficiency causes the reduction of tissue concentration of chlorophylls at plants (Jaleel *et al.* 2009). Bartels and Sunkar (2005) reported that water stress leads to changes in turgor pressure, cell volume and membrane shape and causes disruption of water potential gradients. Currently, many researchers spend their time and efforts on understanding plants' tolerance to drought conditions and the adaptation of plants to inadequate irrigation that influences plant development (Bray, 2004). Plant growth promoting rhizobacteria (PGPR) is a significant and environmentally friendly solution for sustainable agricultural production (Rashid *et al.* 2021; Sharma 2021). It has been reported that PGPR has the potential in increasing plant tolerance to water deficiency which is an abiotic stress (Sandhya *et al.* 2010).

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Fenugreek (*Trigonella foenum-graecum* L.), which is one of the significant medicinal plants, is native to India and Mediterranean region. It is in the Fabaceae family and cultivated as a spice, supplement for foods and vegetables. Leaves and seeds of fenugreek are preferred for medicinal purposes, especially for the treatment of diabetes (Sharma and Raghuram, 1990; Basch *et al.* 2003; Fernández-Aparicio *et al.* 2008).

Although fenugreek's significance, benefits and usage in the medicinal and other field have been searched by many, understanding its adaptation to abiotic stress and drought condition is among the major research objects. The aim of this study was to investigate the effects of different PGPR (*Azospirillum lipoferum, Bacillus megaterium, Chlorella saccharophilia*) inoculations and different deficit water levels (normal (control), 1/2 reduced and 3/4 reduced) and interactions of PGPR inoculations and deficit water levels on Fenugreek's (*Trigonella foenum-graecum* L.) tolerance to drought conditions and physiological and biochemical properties.

MATERIALS AND METHODS

The study was carried out in 2020 in the controlled climate room of the Field Crops Department of Van Yuzuncu Yil University, Faculty of Agriculture. In the research, Gürarslan cultivar were used as the seed material. The experiment was carried out in a factorial order with 4 replications according to the completely randomized design. In the research, three different irrigation levels (normal (control), 1/2 reduced and 3/4 reduced) and some bacterial species (*Azospirillum lipoferum* (1×10^6 cfu/ml) and *Bacillus megaterium* (1×10^5 cfu/ml)), which survive in the root zone of plants and are known to contribute to the growth and development of plants and protect the plants against environmental stress and an algae species (*Chlorella saccharophilia* (2×10^4 cfu/ml)), which lives in the water and has functions in macro-micro nutrient uptake in plant development, were used. Before sowing, seed surfaces were sterilized with 3% sodium hypochlorite. The seeds, except the sterilized control group, were treated separately for two hours in 10 ml/L doses of *Azospirillum lipoferum* and *Bacillus megaterium* rhizobacter solutions and 5% *Chlorella saccharophila* algae solution prepared. Treated and untreated seeds were sown as 6 each in 1-liter pots filled with 1/3 perlite, 1/3 peat and 1/3 garden soil by volume. After planting, the pots were placed in a climate room with a temperature of 25°C and a humidity of 65% in a light/dark photoperiod of 16/8 hours. The seeds started germinating homogeneously 4 days after sowing. Bacteria prepared at the rate of 10 ml/L and algae solutions prepared at the rate of 5% were given in the pots containing the seeds treated with bacteria and algae instead of irrigation water, 2 times with 5 days intervals, 3 days after germination. NPK was given as basic fertilization to all pots after two weeks from germination. The thinning process was done before the deficit irrigation application to remain one plant in each pot. Different irrigation regimes was started 30 days after planting and the experiment was ended 45^{th} day after planting.

NBI (Nitrogen balance index) and total chlorophyll ratio

Before the experiment was ended, nitrogen balance index (NBI) and total chlorophyll ratio were measured from leaves with Dualex scientific+ (FORCE-A, France) device.

MDA (Lipid Peroxidation)

In the study, the amount of malondialdehyde (MDA), the end product of lipid peroxidation; According to the methods of (Heath and Packer, 1968; Sairam and Saxena, 2000); 0.5 g leaf sample taken from the plant was homogenized with 10 ml of 0.1% trichloroacetic acid (TCA) and then the homogenate was centrifuged at 15000 g for 5 minutes. 1 ml of the centrifuged sample was taken from the supernatant and 0.5% thiobarbituric acid (TBA) dissolved in 4 ml of 20% TCA was added. After the mixture is kept in a 95°C water bath for 30 minutes, it is rapidly cooled in an ice bath and centrifuged at 10000 g for 10 minutes, the absorbance of the supernatant at 532 and 600 nm wavelengths were determined and the malondialdehyde (MDA) content was calculated.

Total phenolic substance amount (mg GA/100 g)

In the determination of the total amount of phenolic substance; The method developed by modifying the Folin-Cicaltea spectrophotometric method specified by (Obanda *et al.* 1997) was used. The Folin-Cicaltea solution was diluted at 1:3. Saturated sodium carbonate (35%) solution; 87.5 g of sodium carbonate was dissolved in distilled water, made up to 250 ml and left overnight and then filtered. Gallic acid stock solution (500 µg/ml); was prepared by dissolving 50 mg of gallic acid in 100 ml of distilled water. Gallic acid working solution; Each of 500 µg/ml gallic stock solution was prepared in 5 ml measuring balloons as 9 separate solutions with concentrations ranging from 0-55 µg/ml. 1 ml of these solutions was taken and mixed with 1 ml of Folin-Cicaltea solution. After waiting for 5 minutes, 2 ml of sodium carbonate was added and shaken and diluted with 2 ml of water. After this mixture was kept in the dark for 30 minutes, the absorbance value was read in the spectrometer at a wavelength of 700 nm. A calibration curve was obtained by graphing the absorbance values read against these different concentrations of gallic acid (r^2= 97.47).

Total flavonoid substance amount (mg QE/100 g)

Determination of total antioxidant activity; After weighing 2 g of fenugreek leaves adding 4 ml of methanol, the material passed through the homogenizer was centrifuged at 10000 rpm for 10 minutes, the supernatant remaining on top was taken. Also, after preparing 300 mM acetate buffer (pH 3.6), 10 mmol/L 2,4,6-tripyridyl-s-triazine (TPTZ) prepared by dissolving in 40 mM HCl and 20 mmol/L $FeCl_3.6H_2O$ solutions, FRAP reagent was prepared by mixing them at a ratio of 10:1:1, respectively. The mixture prepared for the analysis of 2850 µL of FRAP reagent and ABTS (2.2-Azinobis 3-ethyl-benzothiazoline-6-sulfonic acid) on fenugreek leaves was diluted 50 times with ethanol, then 150 µL of the sample was mixed and kept at room temperature for 30 minutes. The resulting ferrous tripyridyltriazine complex was measured at 593 nm in the spectrophotometer and the results were reported as mg Trolox/g (Lutz *et al.* 2011). Trolox concentration range has been studied as 0-500 ppm.

Total antioxidant activity (mg TE/g)

Determination of the total amount of flavonoid substances; Total flavonoid substance determination was specified according to the method that (Quettier-deleu *et al.* 2000) developed. 2 ml of 2% $AlCl_3$ was added to 2 ml of extract and left in the dark for 1 hour at room temperature. The total flavonoid contents of the extracts were measured with a spectrophotometer at a wavelength of 415 nm by performing 2 parallel studies in each sample and calculated in mg QE/ 100 g using the calibration curve prepared using standard quartetin.

Membrane durability ýndex, Ion leakage and relative water content in leaf tissues

Membrane strength index, ion leakage and relative water content in leaf tissues were determined according to the method of (Premachandra *et al.* 1990; Sairam *et al.* 1994).

Statistics 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 (Düzgüneş *et al.* 1987).

RESULTS AND DISCUSSION

Relative water content in leaf tissues (%)

According to the research data, the effect of rhizobacteria and rhizobacteria × deficit irrigation interaction on the relative water content in leaf tissues was not found statistically significant. The effect of deficit irrigation practices was found to be statistically significant at the rate of 1%. In rhizobacteria treatments, the ratio of relative water content in leaf tissues was between 68.73-70.21%. In deficit irrigation applications, DI1 had the highest relative water content in leaf tissues with 77.94%. However, it was observed that there was no statistically significant difference with the DI2 applications and it was determined in the same Duncan group. The lowest value was resulted in DI3 with 55.94% (Table 1). However, Rashid *et al*. (2021) indicated that PGPR inoculated (especially *Bacillus megaterium*) wheat increased relative water content in an arid/semi-arid place in Pakistan. Similarly, Sandhya *et al*, (2010) reported that the application of PGPR strains to maize improved the relative water content of leaves in a semi-arid climate condition in India.

Ion leakage in leaf tissues (%)

As a result of the research; The effect of deficit irrigation, rhizobacteria and R × DI interaction on the rate of ion leakage in leaf tissues was found to be statistically significant at 1%. In deficit irrigation applications, the highest ion leakage in leaf tissues was derived from DI3 treatments with 63.12%. However, it was determined that there was no statistically significant difference with the DI2 applications and it was in the same Duncan group. The lowest ion leakage in leaf tissues was observed as 51.85% from DI1. In terms of rhizobacteria applications, the highest value was obtained from R3 with 70.58%. However, it was determined that there was no statistically significant difference with the R2 applications and it was in the same Duncan group. Control plots had the lowest ion leakage in leaf tissues with 35.48%. The highest value in the interaction of rhizobacteria × deficit irrigation was obtained from interaction of DI2 and R3 applications with 79.07%. However, there was no statistically significant difference with the R2 × DI2 and R2 × DI3 interactions and they were in the same Duncan group (Table 1). Unlike our findings, it has been reported that *arabidopsis* plants applied PGPR showed lower Ion leakage than control plants under drought stress conditions (Zhou *et al.* 2017).

Membran durability index in leaf tissues (%)

The effect of deficit irrigation, rhizobacteria and rhizobacteria × deficit irrigation interaction on the membrane durability index in leaf tissues was resulted as statistically significant at a rate of 1%. In rhizobacteria applications, the highest value was obtained from R0 with 63.86% and the lowest value was 25.81% from application of R3. In deficit irrigation applications, the highest value was derived from DI1 treatments with 51.21% and DI3 had the lowest membran durability index value (25.39%). Interaction of DI2 and R0 showed the highest result with 92.40% (Table 1). It has been reported that in various environmental stress situations, the cell membrane is the first damaged structure in the plant, decreases in membrane permeability and stability occur due to the increase in stress and membrane durability can therefore be used to determine the level of stress (Bajji *et al.* 2002). In different studies conducted in echinacea, it was reported that drought and salt levels decreased membrane durability and bacterial application in rice increased membrane durability (Shukla *et al.* 2012; Kara *et al.* 2019; Bat *et al.* 2020). Our results are in agreement with previous studies.

Chlorophyll ratio (μg cm^{-1})

The effect of deficit irrigation, rhizobacteria and R × DI interaction on chlorophyll ratio did not cause a statistically significant difference. While the chlorophyll ratio in rhizobacteria applications varied between 41.95-48.1 μg cm^{-1}, the chlorophyll ratio in deficit irrigation applications was ranged between 43.56-47.04 μg cm^{-1} (Table 1). It has been reported that different drought doses in echinacea did not affect the chlorophyll ratio statistically (Bat *et al.* 2020) and drought treatments increased the amount of chlorophyll in sensitive soybean varieties but decreased the tolerant ones (Guzzo *et al.* 2021) and drought stress did not cause a change in the chlorophyll ratio in different apple varieties (Mihaljević *et al.* 2021). It has been observed that drought stress increases the chlorophyll content in different grape varieties and the chlorophyll ratio in plants may vary according to species and genotypes and environmental conditions (Rustioni and Bianchi, 2021). Our results are compatible with the literature.

MDA (nmol g^{-1})

According to the study data; deficit irrigation, rhizobacteria and rhizobacteria × deficit irrigation interaction had a statistically significant 1% effect on MDA. In rhizobacteria

Table 1: Effects of deficit irrigation and rhizobacteria-microalgae on some physiological and biochemical parameters of fenugreek.

Application		Relative water content in leaf tissues (%)	Ion leakage in leaf tissues (%)	Membran durability index in leaf tissues (%)	Chlorophyll ratio ($\mu g\ cm^{-1}$)	MDA ($nmol\ g^{-1}$)	Nitrogen balance index (Dualex index)	Total phenolic subtance amount (mg GA/100 g)	Total flavonoid subtance amount (mg QE/100 g)	Total antioxidant activity (mg TE/g)
Rhizobacteria (R)	Deficit Irrigation (DI)									
Control (R0)	DI1 (Control)	77.29	32.37 g	67.63 b	41.9	0.84 cd	61.46	45.14 b	24.54	56.02 cd
	DI2 (1/2 reduced)	73.99	20.53 h	92.40 a	42.6	1.19 b	57.88	48.05 b	19.62	51.68 cd
	DI3 (3/4 reduced)	59.36	53.55 ef	31.55 de	45.71	1.41 a	54.27	62.22 ab	25.95	114.28 a
Average		70.21	35.48 C	63.86 A	43.4	1.15 A	57.87	51.80 B	23.37 C	73.99
Azospirillum lipoferum (R1)	DI1 (Control)	78.14	54.70 def	61.03bc	44.28	0.66 d	62.23	73.47 ab	28.08	68.40 bc
	DI2 (1/2 reduced)	72.54	66.32 bc	34.22 de	42.52	0.78 cd	54.11	67.22 ab	28.37	71.55 bc
	DI3 (3/4 reduced)	55.5	59.13 de	28.49 ef	44.2	1.33 a	44.25	61.80 ab	25.88	56.55 cd
Average		68.73	60.05 B	41.25 B	43.67	0.92 B	53.53	67.49 A	27.44 AB	65.5
Bacillus megaterium (R2)	DI1 (Control)	79.91	49.70 f	46.03 cd	44.28	0.65 d	57.61	59.72 ab	22.6	59.44 cd
	DI2 (1/2 reduced)	73.41	77.43 a	34.21 de	42.52	0.79 cd	55.24	83.33 a	25.57	82.74 b
	DI3 (3/4 reduced)	54.25	77.79 a	10.81 g	44.2	1.44 a	57.92	59.58 ab	24.65	63.39 bc
Average		69.19	68.31 A	30.35 C	41.95	0.96 B	56.92	67.54 A	24.27 BC	68.52
Chlorella saccharophilia (R3)	DI1 (Control)	76.46	70.63 ab	30.16 def	47.17	0.73 cd	58.1	55.28 b	29.84	63.26 bc
	DI2 (1/2 reduced)	77.18	79.07 a	16.56 fg	43.68	0.89 c	50.8	70.00 ab	28.21	85.76 b
	DI3 (3/4 reduced)	54.68	62.03 cd	30.71 de	53.46	1.13 b	63.96	65.14 ab	26.6	65.89 bc
Average		69.44	70.58 A	25.81 D	48.1	0.92 B	57.61	63.47 A	28.21 A	71.63
Deficit irrigation average	DI1 (Control)	77.94 A	51.85 B	51.21 A	43.56	0.71 C	59.85	58.40 B	26.26	61.77
	DI2 (1/2 reduced)	74.28 A	60.83 A	44.34 B	42.22	0.91 B	54.5	67.14 A	25.44	72.93
	DI3 (3/4 reduced)	55.94 B	63.12 A	25.39 C	47.04	1.32 A	55.1	62.18 *AB*	25.76	75.02
Rhizobacteria (R)		ns	**	**	ns	**	ns	**	*	ns
Deficit irrigation (DI)		**	**	**	ns	**	ns	*	ns	ns
R×DI		ns	**	**	ns	**	ns	**	ns	**
CV		6.54	11.66	8.3	13.07	6.95	20.02	10.313	14.74	19.14

*P<0.05 level, **P<0.01 level significance; ns: Not significance.

applications, R0 amendments had the highest MDA value with 1.15 nmol g^{-1} and R1 and R3 had the lowest MDA value with 0.92 nmol g^{-1}. However, it was determined that there was no statistically significant difference with the R2 applications and it was in the same Duncan group. In deficit irrigation applications, the highest value was obtained from DI3 applications with 1.32 nmol g^{-1} and the lowest value was from DI1 (0.71 nmol g^{-1}). In the interaction of rhizobacteria × deficit irrigation, the highest MDA was observed from the interaction of DI3 and R2 treatments with 1.44 nmol g^{-1} (Table 1). Similarly, MDA content increased around 2.5-fold under hard drought stress (-7 bar) condition compared to the nonstressed control level (0 bar) in fenugreek in Iran (Zamani *et al.* 2020). It has also been reported that while severe drought stress increased MDA, arbuscular mycorrhizal fungus (*Glomus mosseae*) decreased MDA in evening primrose in plastic pots in a semi-arid house condition in Iran (Mohammadi *et al.* 2019).

Nitrogen balance index (Dualex index)

The effect of deficit irrigation, rhizobacteria and R × DI interaction on nitrogen balance index did not make a statistical difference. While the nitrogen balance index was between 53.53-57.87 (dualex indeks) in rhizobacteria applications, it was between 54.5-59.85 (dualex indeks) in deficit irrigation applications (Table 1). In a similar current study, opposite results were observed that PGPR inoculation to soybean and drought stress affected nitrogen balance index significantly in a fully controlled climate situation in Turkey (Oral *et al.* 2021).

Total phenolic substance amount (mg GA/100 g)

The effect of rhizobacteria and rhizobacteria × deficit irrigation interaction on the total phenolic substance amount varied significantly across treatments at $P<0.01$ while the effect of deficit irrigation applications was found to be significant at $P<0.05$. The highest value in rhizobacteria applications was derived from R2 amendments with 67.54 mg GA/100 g. However, it was determined that there was no statistically significant difference with the R1 and R3 applications and they were in the same Duncan group. It was observed that R0 had the lowest total phenolic substance amount with 51.80 mg GA/100 g. In deficit irrigation applications, the highest total phenolic substance amount was detected from DI2 treatments with 67.14 mg GA/100 g whereas the lowest value was obtained from DI1 with 58.40 mg GA/100 g. The highest value in the interaction of rhizobacteria × deficit irrigation was obtained from DI2-R2 interaction with 83.33 mg GA/100 g (Table 1). Plants take various protective measures to reduce the negative effects of stress situations. One of these is increasing the production of phenolic and flavonoid compounds (Jaafar *et al.* 2012). Phenolics and flavonoids are naturally produced in the cytoplasm and endoplasmic reticulum and they take part in the elimination of free radicals, both naturally produced and increased in adverse conditions (Ibrahim and Jaafar, 2011). Increases in the amount of polyphenol and flavonoid substances due to restricted irrigation in buckwheat (Siracusa *et al.* 2017) and deficit irrigation in sugar beet increased polyphenols (Alkahtani *et al.* 2021). Our study findings are in agreement with previous studies.

Total flavonoid substance amount (mg QE/100 g)

In the study; Total amount of flavonoid substances was not significantly different across restricted irrigation and interaction of rhizobacteria×restricted irrigation. The total amount of flavonoid substances varied significantly across rhizobacteria applications at $P<0.05$. In rhizobacteria applications, the highest total flavonoid substance content value was obtained from R3 applications with 28.21, the lowest total flavonoid substance amount was obtained from R0 with 23.37 mg QE/100g. In terms of deficit irrigation applications, the total amount of flavonoid substances was found to be between 25.44-26.26 mg QE/100 g (Table 1). It has been demonstrated that bacterial applications caused an increase in flavonoid substances in wheat grown under drought stress (Furlan *et al.* 2017) and similarly, bacterial applications caused flavonoid substance accumulation in the plant under drought stress (Saikia *et al.* 2018). Our study results show parallelism with the literature.

Total antioxidant activity (mg TE/g)

According to research data; Rhizobacteria and deficit irrigation applications did not have a statistically significant effect on total antioxidant activity. The effect of the R × DI interaction on the total antioxidant activity was significant at a rate of 1%. In rhizobacteria applications, total antioxidant activity values were between 65.5-73.99 mg TE/g. In deficit irrigation treatments total antioxidant activity values were between 61.77-75.02 mg TE/g. The highest rhizobacteria × deficit irrigation interaction was derived from DI3 applications of R0 applications with 114.28 mg TE/g (Table 1). Plants produce enzymatic or non-enzymatic antioxidant substances to reduce the damage caused by reactive oxygen derivatives (which they produce or increase in adverse environmental conditions) (Abdelaal *et al.* 2020). It has been reported that rhizobacteria used in drought stress conditions cause increases in antioxidant activity and rhizobacteria applied in corn plants grown under deficit irrigation conditions increase some antioxidant enzyme activities (Vardharajula *et al.* 2011). It has been published that there is a negative correlation in the amount of some antioxidant (SOD and GPX) substances due to drought stress in peas and a positive correlation with the CAT enzyme (Farooq *et al.* 2021). It was detected that drought stress did not statistically affect the total antioxidant activity, which is the total activity of many enzymatic antioxidants in lettuce (Shin *et al.* 2021). Enzymatic antioxidants can give direct and inversely proportional results in stress situations. In stress situations, total antioxidant activity may increase, decrease or give neutral results. For this reason, our research finding is supported by the literature.

CONCLUSION

According to the study data; it was determined that rhizobacteria and microalgae applications decreased membrane durability index in leaf tissues and MDA contents, while caused increases on the ion leakage in leaf tissues, total phenolic and flavonoid contents compared to control. According to the results of the research, it was concluded that drought stress caused decreases in phsysiological values such as relative water content and membrane durability index in leaf tissues, but it was increased the ion leakage in leaf tissues and total phenolic contents from the other research parameters. The use of beneficial soil bacteria, which can be applied in various ways, is of great importance in order to reduce the damage caused by adverse environmental conditions such as drought to plant growth and development. In the future, if different rhizobacteria and their combinations are applied to agriculturally produced plants and positive results are obtained, both the healthy growth and development of the plant and the damage caused by many negative environmental factors, especially drought to plant production will be minimized. As a result, ecological agriculture in harmony with nature will become sustainable.

Conflict of interest: None.

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