

Effects of Bio-Fertilizers and Different Levels of Phosphorus on Biochemical Traits, Essential Oils, and Antioxidant Enzymes in Lavender (*Lavandula Angustifolia*) Under Water Stress

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Research Article

Keywords: Drought stress, Thiobacillus, Pseudomonas, Relative water content, flower wet weight

Posted Date: March 15th, 2024

DOI: <https://doi.org/10.21203/rs.3.rs-3934553/v1>

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Additional Declarations: No competing interests reported.

Abstract

To investigate the effects of biological fertilizers and phosphorus on the effects of reduced irrigation in medicinal plant lavender, a split-split plot experiment based on randomized complete block design (RCBD) with three replications was conducted during two cropping years of 2018/2019 and 2019/2020 at The Research Station, Islamic Azad University of Tabriz. The studied treatments in this research included irrigation levels (irrigation after 60, 90, and 120 mm of class A evaporation pan) as the main factor, four levels of seed inoculations (inoculation with *Thiobacillus*, inoculation with *Pseudomonas*, combined inoculation (*Thiobacillus* + *Pseudomonas*) and control (non-inoculation) as a sub-factor and the amount of phosphorus chemical fertilizer (0 and 50 kg/ha) was as a sub-sub-factor. The experiment assessed several traits, including dry weights of flowers, the quantity of cell membrane leakage, leaf relative water content, soluble sugars content, proline content, superoxide dismutase, catalase, and peroxidase activities. This two-year experiment measured the following traits: Wet weight of the flower, quantity of cell membrane leakage, leaf relative water content, proline, superoxide dismutase, catalase, and peroxidase activities. The wet weight of flowers increased compared to the control during the first year of the investigation due to all irrigation levels, phosphorus application, and inoculation with integrated biological fertilizers. Results showed that in the first year of measuring, all irrigation levels, phosphorus application, and integrated biological fertilizer inoculation increased the wet weight of the flower compared to the control. The greatest amount of proline, catalase, peroxidase, and superoxide dismutase enzymes was obtained at 60 ml of evaporation with a consumption of 50 kg of phosphorus per hectare and applying *Thiobacillus* + *Pseudomonas*. This study also showed that the interactions between irrigation levels, phosphorus fertilizer, and biological fertilizer on the characteristics of cell membrane leakage rate and leaf-relative water content were not significant. The results of the second-year experiment showed that the highest amount of catalase, superoxide dismutase, and peroxidase was obtained as a result of the irrigation combination after 120 mm of evaporation pan and the application of *Thiobacillus* + *Pseudomonas* and phosphorus fertilizers and proline content (5.48 mg/g wet weight) were determined by irrigation treatment after evaporation of 120 mm and without application of bacteria and phosphorus fertilizers. It appears that plant growth-promoting bacteria and phosphorus increased the yield of lavender flowers by reducing the negative effects of drought stress.

1 Introduction

The genus *Lavandula* belongs to the Lamiaceae family and includes 39 species which the most cultivated species are English lavender (*Lavandula angustifolia*), Portuguese lavender (*Lavandula latifolia* Medik.), Spanish lavender (*Lavandula stoechas* L.) and lavandin (*Lavandula x intermedia* Emeric ex Loisel.) (Crisan et al., 2023). Lavender essential oil may contain over 100 terpenoid and phenolic compounds (Sharma et al., 2019). Monoterpenes in lavender oil, such as limonene and perillyl alcohol, are effective anticancer agents. In addition, their known antibacterial properties depend on phenolic compounds in their composition, such as carvacrol, eugenol, and thymol; some terpenes are also useful for preventing cardiovascular diseases (Panda et al., 2020). The main compounds of its essential oil include 1,8-cineole, camphor, borneol, α -pinene, β -pinene, trans-pinocarol, nerol, linalool, propionic acid, butyric acid, valeric acid, germabiol, free linalool, flavonoids, tannin, phenol and monoterpene compounds (Sharma et al., 2019). Its essential oil is commonly used in veterinary medicine as a disinfectant for wounds, burns, and insect bites and as a sedative and analgesic (Darzi et al., 2020; de Melo Alves Silva et al., 2023). Plants are constantly exposed to abiotic stresses such as drought during their life cycle. Drought is the most common environmental stress worldwide, affecting plant growth, especially in arid and semi-arid regions. Therefore, drought has become the main obstacle to crop production (Bahodur et al., 2019). Furthermore, drought stress has caused plant structural, physiological and biochemical changes (Ghosh et al., 2017). Reactive oxygen species (ROS) generated under water-deficit stress can degrade proteins, lipids, carbohydrates, and nucleic acids (Hussain et al., 2020). Plants have developed various mechanisms to reduce the oxidative effect of ROS (Cruz de Carvalho, 2008). Enzymatic and non-enzymatic antioxidant systems are one of the mechanisms for eliminating ROS. In the antioxidant defense system, the enzymes superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) are key enzymes, and the main non-enzymatic antioxidant compounds glutathione, α -tocopherol and ascorbic acid (Dumanović et al., 2021). CAT breaks down hydrogen peroxide directly, and POX breaks down hydrogen peroxide by using electron-donating phenolic compounds. While SOD produces hydrogen peroxide by changing the shape of superoxide anions (Gaber, 2010). Changes in enzyme activity under water stress have been documented well. Synthesis of antioxidant enzymes under stress conditions can prevent further yield loss (Seyd Sharifi and Seyed Sharifi, 2019). When O_2^- and H_2O_2 increase under stress conditions, these compounds will be controlled by the activity of the enzymes peroxidase, catalase, and superoxide dismutase (Niliwajski and Sklodowska, 2021). Plants accumulate a range of osmolytes to regulate their intracellular osmotic potential in stress conditions. Osmolytes are known to stabilize protein structures and are ubiquitous in living organisms. Major organic osmolytes include amino acids (e.g., proline), methylamines (e.g., betaine and trimethylamine N-oxide), polyamines (e.g., spermidine), sugars (e.g., trehalose), and polyols (e.g., sorbitol) (Ozturk et al., 2021; Singh et al., 2021). Among these osmolytes, proline is required as a structural unit of proteins and as an osmolyte for osmoprotection in plants (Moukhtari et al., 2020; Alvarez et al., 2022; Spormann et al., 2023). When plants are subjected to osmotic stress, proline accumulates in the cytoplasm and acts as an osmotic buffer, stabilizing cell membranes and maintaining turgor pressure. It helps plants avoid damage to the cell components and allows plants to keep growing and continue reaching their maximum yield despite the stress. Accumulation of proline in stressed plants is caused by proline synthesis and inactivation of its degradation. The increase in proline content under stress conditions protects the cell membrane, proteins, and cytoplasmic enzymes, prevents reactive oxygen species, and destroys free radicals (Mukhtari et al., 2020). However, proline accumulation alone cannot induce tolerance but other activated pathways under different abiotic stresses are linked with effects on plants (Chun et al., 2018; Ozturk et al., 2021). One of the essential solutions to adapt or reduce the effect of water stress on crops is using different types of biological fertilizers (bacteria and beneficial soil-borne fungi) (Marulanda Aguirre et al., 2008). These fertilizers include beneficial soil microorganisms which provide absorbable nutrients to plants (Chen, 2006). *Thiobacillus* bacteria are the most important sulfur oxidizers in farmlands, providing absorbable soil sulfur for plants (Chgazrdi et al., 2013). In addition, through sulfur oxidation, these bacteria increase the solubility of trace elements in the soil, provide plants with the necessary sulfate, and reduce the soil's acidity around the roots. Increasing the ability to absorb microelements and macro elements of the soil leads to better plant growth (Besharaty, 2001). Phosphorus (P) is a macronutrient that affects plant growth and development. However, the limited phosphorus availability in the soil seriously challenges crop productivity, especially when plants are exposed to abiotic stresses such as drought, salinity, and extreme temperatures (Khan et al., 2023). P availability will also be influenced by soil microorganisms that can compete with plants for P or make beneficial relationships, such as mycorrhizae, to increase the efficiency of P uptake (López-Arredondo et al., 2014). The

ineffectiveness of phosphorus fertilizers depends on their properties. It has been caused that only 15–25% is absorbed by plants, and the amount of debris remaining is washed away and degrades the soil (Johnston et al., 2014).

2 Materials and Methods

A field experiment was conducted in the cropping year 2018–2019 at Agricultural Research Station, Faculty of Agriculture, Islamic Azad University, Tabriz branch, located 5 kilometers from Tabriz with latitude 46° and 17° E and longitude 38° and 5° N and 1360 meter altitude. Based on Dumarten's climate classification method, the region has a cold and semi-arid climate. Before starting the experiment, samples were taken to measure the chemical and physical properties of the soil of this region. The samples were sent for analysis at the soil science laboratory. The results are presented in Table 1.

Table 1
Soil properties of the studied site at a depth of 0–30 cm

Soil texture	Clay	Loam	Sand	Potassium	Phosphorus	Acidity	EC	Organic matter	Organic carbon
	——(%)——				mg kg ⁻¹		dS m ⁻¹	——(%)——	
Loamy-Sand	14	18	68	600	48	8.7	56.1	2.9	1.7

This experiment was conducted as a split-split plot based on a randomized complete block design with three replications. The irrigation regime was considered as the main plot at three levels (irrigation after 60, 90, and 120 mm evaporation from class A evaporation pan) and the bio-fertilizer inoculation as the sub-plot at four levels (inoculation with *Thiobacillus*, inoculation with *Pseudomonas*, combined inoculation (*Thiobacillus*+ *Pseudomonas*) and no inoculation (control)) and the phosphorus fertilizer application as the sub-sub-plot at two levels (0 and 50kg/ha). Before planting, the growing media was prepared by deep plowing, disking, leveling, and plotting based on the crop mapping. From April 29 to May 1, 2018, lavender seedlings (provided by Agricultural Jihad Management of Tabriz) were transplanted to the main field at the 4–6 leaf stage. Plants were grown in rows of 1m width while the space between seedlings within each row was 1m. The number of experimental plots was 72; each plot was 12m long and 7m wide and consisted of three planting rows. The main plots were spaced 2m apart. There were 1m unplanted rows between sub-plots and sub-sub-plots. Sari Agricultural Sciences and Natural Resources University provided the bacteria used in this study. Before transplanting the main field, seedling roots were immersed for 30 min in the inoculum (water ratio was 1:10) according to the manufacturer's instructions. Before planting, according to the test plan, triple superphosphate fertilizer was applied to the soil at 50 kg per hectare in specified plots. All seedlings were watered immediately after planting. Irrigation was carried out every two to three days until the establishment of seedlings and once a week after the establishment of seedlings. With the appearance of the first flower in each experimental plot, drought stress levels were applied according to the experimental treatments. For this purpose, irrigation intervals were based on class A pan evaporation and involved irrigation after every 60 ± 5, 90 ± 5, and 120 ± 5mm evaporation from a class A evaporation pan. The irrigation treatment after evaporation of 60 ± 5 mm from the class A pan, depending on the climatic conditions of the region and soil physical properties, as well as the determination of the weight percentage of soil moisture, was considered the control treatment. The amount of daily cumulative pan evaporation after reaching the required amount for each treatment (60, 90, and 120 mm evaporation from pan evaporation) determines the irrigation time. The irrigation amount for each plot was measured based on the total water requirements measured by a random sampling method from three different parts of each plot. The weight percentage of soil moisture was determined approximately 24 hours before irrigation.

Two equations, $ET_c = K_c \times ET_0$ and $ET_0 = K_{pan} \times E_p$ were used to calculate the crop coefficient. The crop evapotranspiration $ET_c = K_c \times ET_0$, where K_c is the crop coefficient and ET_0 = reference crop evapotranspiration (Fazeli Rostampour et al., 2013). To control weeds, hand weeding was done at required times. The field was dominated by weeds, which were melde, amaranth, and alfalfa. At the flowering completion, biochemical and physiological traits were measured in all experimental plots. The same farm operation remained in the second year of the same farm, and the whole process was repeated as in the first year. The lavender aerial parts were collected in the first year (October 25, 2018) and the second year (October 7, 2019). Leaf Relative Water Content (the last leaf of the tallest branch) in the early morning was determined according to the following formula:

The relative water content of a leaf (RWC) is calculated from the following parameters (Levitt, 1980).

$$RWC = \left(\frac{FW - DW}{TW - DW} \right) * 100$$

DW = leaf dry weight

FW = leaf fresh weight

TW = leaf turgit (re-saturated) weight

At the same time, the leakage rate of the cell membrane was determined by measuring the electrical conductivity of five immersed terminal leaves in distilled water. After the completion of flowering, the yield of fresh weight of flowers per hectare was obtained by removing marginal effects. -80°C stored leaf samples were used to measure antioxidant enzyme activity. Catalase activity was measured by the described method (Aebi, 1984; Ezhilmathi, 2007). The measurement of superoxide dismutase enzyme (SOD) was carried out according to the method of (Giannopolitis et al., 1977 and Ries), based on enzyme units per milligram of wet weight. The amount of malondialdehyde (MDA) was measured using the (Aston and Sidney, 1987) method. The peak of MDA was identified with a spectrophotometer at 532 nm wavelength and measured based on the area under the curve. Soluble sugar content was measured using the

youngest leaf and the (Omokolo et al., 1996) method. Proline content was also measured in the youngest leaf according to the (Bates et al., 1973) method. The percentage of essential oil of lavender flowering tops was measured, and the essential oil yield per area unit was calculated by the Clevenger apparatus at the laboratory of Islamic Azad University, Tabriz. To determine the amount of essential oil in flowering lavender tops, after harvesting the flowering tops and drying them in the shade, a 50-gram sample was prepared from each experimental plot, and after grinding, the essential oil was extracted by using water distillation method by Clevenger apparatus that lasted three hours. The percentage of essential oil has also been calculated after dehydration with sodium sulfate. The percentage of essential oil was calculated using the following equation: the following relation was also used to measure essential oil yield:

$$\text{Essential oil \%} = \frac{\text{mass of essential oil}}{\text{mass of flowering top}} \times 100$$

Essential oil yield = Essential oil percentage $\times$ flowering top yield (kg/ha)

SAS statistical software was used to measure ANOVA of the data, MSTAT-C software was used to compare means, and Excel software was used to draw figures. Also, because treatments were not randomized over two years, composite analysis was not carried out due to the lack of independence of treatments. The initial assumption of ANOVA is non-randomization and independent errors caused by the violation.

3 Results and Discussions

The main and interaction effects of experimental factors on the studied traits in 2018 and 2019 have been presented in Tables 2 and 4.

3.1 Wet Weight of Flowers

In 2018, the highest wet weight of flowers was obtained (281 kg/ha) at the irrigation level of 60 mm evaporation from the evaporation pan and the phosphorus application at the rate of 6 kg/ha and the combined inoculation of *Thiobacillus* + *Pseudomonas* bacteria and the lowest wet weight of flowers (45 kg/ha) was achieved under severe stress conditions (irrigation after 120 mm evaporation from evaporation pan) and no-inoculation with bacteria. Bacterial treatments improved the wet weight of flowers compared to the control at all irrigation levels (Table 3). The mean results of the comparison of the three-way interaction effect of the experimental treatments in the second year showed that the highest wet weight of flowers was obtained (415 kg/ha) with irrigation after 60mm evaporation from the evaporation pan and the combined inoculation of *Thiobacillus* + *Pseudomonas* bacteria and the phosphorus application at the rate of 50 kg/ha and the lowest one (35 kg/ha) was obtained from severe stress and lack of bacteria and phosphorus (Table 5). The investigation of the changes in the wet weight of lavender flowers under the influence of irrigation level showed that the wet weight of flowers decreased with increasing water stress intensity at all bacterial levels. The lowest reduction intensity was achieved by treatment with the bacterium *Thiobacillus*; the highest reduction intensity was obtained when the bacteria were not consumed. Water deficit stress reduced the wet weight of flowers in lavender plants. It can be said that the reduction of photosynthetic materials due to the transfer of assimilated materials to the flowers and the reduction of the leaf surface has caused a decrease in their yield and weight (Shubhra et al., 2004). This issue may be because nutrients are available to all bushes. Thus, competition between plants to absorb nutrients is reduced, ultimately increasing yield per unit area. Phosphorus is necessary for medicinal plants' natural growth, especially in the reproductive organs, and is one of the main components of plant protein (Saiadi et al., 2015). In the condition of phosphorus deficiency, the allocation of carbohydrates to the roots will be increased, which is simultaneous with the differentiation in the lateral roots and leads to the delicate formation of the root system (Lopes, 1996). The results of different investigations show that the application of phosphorus fertilizer in plants whose flowers are apart from their economic yield is very beneficial and directly affects flowering continuity, yield of dry and wet weight of flowers, and direct impacts on essential oil yield.

Table 2
Analysis of variance of studied traits of the lavender plant under the influence of experimental treatments in the first year.

Mean square										
Source of Variatrom	df	Fresh weight of flower	Leaf relative water content	Cell membrane leakage	Proline	Catalase	Super oxide dismutase	Peroxidase	Essential oil percentage	essential oil yield
Block	2	187.09*	2.23 ^{ns}	31.514 ^{ns}	0.006**	.000279*	0.003 ^{ns}	0.000035 ^{ns}	0.007 ^{ns}	0.015 ^{ns}
Levels of irrigation	2	39758.72 ^{ns}	5.08 ^{ns}	11.55 ^{ns}	2.406**	0.001**	0.013*	0.000089 ^{ns}	*0.005	*0.182
experimental error1	4	1.014	31.65	26.45	0.000316	0.000021	0.001	0.000168	0.001	0.016
Bio-fertilizer	3	23887.53**	114.65	371.55**	2.955**	0.001**	0.032**	0.001**	*0.024	^{ns} 0.009
Irrigation × Bio-fertilizer	6	1402.96**	178.92*	23.00 ^{ns}	0.819**	0.00001**	0.013**	0.000067**	*0.054	**0.123
experimental error2	18	1.04	65.51	91.213	0.002	0.000146	0.001	0.0000023	0.003	0.006
phosphorus fertilizer	1	48932.34**	191.95 ^{ns}	6.72 ^{ns}	0.074**	0.000013 ^{ns}	0.002 ^{ns}	0.000051*	0.006 ^{ns}	0.995 ^{ns}
Irrigation × phosphorus fertilizer	2	8034.05 ^{ns}	24.42 ^{ns}	42.88 ^{ns}	0.975**	0.001**	0.009**	0.0000014**	*0.002	0.033**
× Bio-fertilizer × phosphorus fertilizer	3	5240.97**	128.55 ^{ns}	14.87 ^{ns}	0.461**	0.000489**	0.005*	0.002**	**0.023	*0.039
Irrigation × Bio-fertilizer × phosphorus × fertilizer	6	438.29**	187.03 ^{ns}	14.92 ^{ns}	0.816**	0.000279**	0.010**	0.000024**	**0.024	**0.048
experimental error3	24	1.43	80.54	18.97	0.001	0.000002	0.001	0.000003	0.002	0.008
CV (%)		17.16	10.69	23.83	1.4	18.16	6.69	9.6	9.8	22.68

Table 3
Comparing the mean of the three-way effect of experimental treatments on the mean of studied traits of lavender in the first year.

Irrigation regime	Bio-fertilizer	Phosphorus fertilizer	Fresh weight of flower	Proline (mg per gram wet weight)	Catalase (absorbance changes at µg protein per minute)	Superoxide dismutase (absorbance changes at µg protein per minute)	Peroxidase (absorbance changes at µg protein per minute)	Essential oil percentage	essential oil yield (kg per hectare)
	No inoculation	0	64.5e	2.047 o	0.071i	0.386 i	0.072l	0.6cde	4.1d
	No inoculation	6	108.4de	2.161 h	0.0736hi	0.4157ghi	0.081k	0.7cde	4.5d
60mm	T	0	101bcd	2.193 mn	0.07433ghi	0.414hi	0.08067k	1.1cd	6.8c
	T	6	173b	2.251 lm	0.076ghi	0.419ghi	0.08467jk	1.1cd	7.1bc
	P	0	102.2cde	2.203 mn	0.0743ghi	0.451fghi	0.08367jk	0.8bc	6.9bc
	P	6	190bc	2.562 j	0.08167fghi	0.4967c-g	0.084jk	1.1abc	7.5bc
	T + P	0	130 bc	2.311 l	0.08267e-i	0.4467fghi	0.08633ij	1.0abc	8.02b
	T + P	6	281a	2.634 ij	0.08433d-i	4880d-h	0.08967ghi	1.2 a	9.06a
	No inoculation	0	53cde	2.076 o	0.081fghi	0.4683efgh	0.08933hi	0.5cde	3.5ef
	No inoculation	6	61e	2.41k	0.08633c-i	0.4713d-h	0.09067fgh	0.70cde	4.1d
	T	0	73de	2.483 k	0.0885c-i	0.5017b-f	0.09167efgh	0.84bc	5.9dc
	T	6	144cde	2.724 h	0.08933b-h	0.537a-e	0.092efgh	1.0bc	6.5bc
90 mm	P	0	85de	2.603 j	0.08633c-i	0.503b-f	0.09167efgh	0.74bc	6.4bc
	P	6	124cde	2.874 fg	0.09067b-h	0.5447a-e	0.09333d-h	0.85bc	6.7bc
	T + P	0	105cde	2.557 j	0.09067b-h	0.553abcd	0.094defg	0.75bc	7.4bc
	T + P	6	197cde	2.944 ef	0.1003a-e	0.6047a	0.096de	1.0abc	7.9b
	No inoculation	0	45e	3.02 de	0.09167b-h	0.505b-f	0.09433def	0.4ef	2.1g
	No inoculation	6	41e	3.38 bc	0.1023abcd	0.5123b-f	0.09533de	0.51ef	3.2ef
	T	0	51de	3.027 d	0.092b-g	0.522b-f	0.097	0.6cd	4.7def
	T	6	57de	3.187 c	0.103abc	0.581ab	0.1027c	0.8cde	5.6dc
120 mm	P	0	52e	3.028 d	0.09433b-f	0.552a-e	0.09533de	0.6cde	5.3dc
	P	6	58de	3.267 c	0.1067ab	0.6133a	0.1093b	0.7bc	5.4dc
	T + P	0	69de	3.935 b	0.09733a-f	0.5733abc	0.1043c	0.6cd	6.5bc
	T + P	6	121cde	4.828 a	0.1147a	0.6173a	0.1193a	0.9bc	6.9bc

Table 4
Analysis of variance of studied traits of the lavender plant under the influence of experimental treatments in the second year.

Mean square										
Source of Variatrom	df	Fresh weight of flower	Leaf relative water content	Cell membrane leakage	Proline	Catalase	Super oxide dismutase	Peroxidase	Essential oil percentage	Essential oil performance
Block	2	1858.16 ^{ns}	24.069 ^{ns}	19.847 ^{ns}	*0.009	0.000037*	0.003 ^{ns}	0.0000016 ^{ns}	0.006 ^{ns}	0.022 ^{ns}
Levels of irrigation	2	42343.16 ^{ns}	75.807 ^{ns}	18.764 ^{ns}	8.631**	0.006**	0.017**	**0.02	*0.004	0.001
experimental error1	4	9.2	35.073	27.889	0.029	0.000054	0.001	0.0000011	0.001	0.003
Bio-fertilizer	3	61810.77	37.68 ^{ns}	299.256**	0.38**	0.000377**	0.038**	0.002**	*0.012	^{ns} 0.027
Irrigation × Bio-fertilizer	6	1048.11**	167.53 ^{ns}	23.208 ^{ns}	0.571**	0.000349**	0.016**	0.002**	*0.012	0.022
experimental error2	18	9.02	18.46	16.505	0.036	0.000075	0.002	0.00003	0.002	0.009
phosphorus fertilizer	1	57573.55	26.28 ^{ns}	8.000 ^{ns}	6.963**	0.004**	0.002 ^{ns}	0.016**	0.006 ^{ns}	^{ns} 0.004
Irrigation × phosphorus fertilizer	2	193.05**	*147.16	49.292*	0.688**	0.000068**	0.007**	0.000056**	*0.001	0.090**
× Bio-fertilizer × phosphorus fertilizer	3	10985.29**	55.55 ^{ns}	11.370 ^{ns}	0.372**	0.001**	0.008*	0.002**	**0.024	**0.065
Irrigation × Bio-fertilizer × phosphorus × fertilizer	6	1175.18**	**172.21	9.329 ^{ns}	0.895**	0.000238**	0.014**	0.002**	**0.013	**0.040
experimental error3	24	6.76	40.47	11.764	0.045	0.000105	0.002	0.0014	0.004	0.007
CV (%)		11.81	7.63	18.74	5.68	9.56	8.8	5.75	8.1	23.63

Table 5
comparing the mean of the three-way effect of experimental treatments on the mean of studied traits of lavender in the second year.

Irrigation regime	Bio-fertilizer	Phosphorus fertilizer	Fresh weight of flower	Relative humidity percentage of leave	Proline (mg per gram wet weight)	Catalase (absorbance changes at µg protein per minute)	Super oxide dismutase (absorbance changes at µg protein per minute)	Peroxidase (absorbance changes at µg protein per minute)	Essential oil percentage	Essential oil performance
60mm	No inoculation	0	250 c-f	74.2cd	3.257jk	0.07633l	0.3937j	0.01267p	0.7cde	4.5d
	No inoculation	6	336bcde	76.1bcd	2.9n	0.07867kl	0.4137hij	0.01967o	0.9cde	5.1d
	T	0	341abcd	82.88bcd	3.26jk	0.08233jk	0.407ij	0.02133o	1.2cd	7.2c
	T	6	410ab	88.95abcd	3.113lm	0.08367jk	0.414hij	0.03n	1.4cd	7.8bc
	P	0	367abc	83.09bcd	3.193j-l	0.08733j	0.4473ghij	0.033mn	1.1bc	7.4bc
	P	6	412abc	85.5abcd	3.033m	0.08833j	0.451ghij	0.031n	1.3bc	8.1bc
	T + P	0	380abc	85.6abcd	3.153kl	0.09433i	0.4717f-j	0.03633lm	1.2abc	8.7b
	T + P	6	415a	95.75a	2.78n	0.096i	0.4933efgh	0.03967kl	1.7 a	9.4a
	No inoculation	0	160ghij	74.1cd	4.433d	0.09767i	0.477e-i	0.041jk	0.62cde	4.01ef
	No inoculation	6	300defg	75.8bcd	4.187ef	0.1063h	0.5023defg	0.04367ijk	0.85cde	4.5d
	T	0	340f-i	81.4bcd	4.22ef	0.1067h	0.5067c-g	0.04467ij	1.0bc	6.5dc
	T	6	386bcde	87.5abcd	3.443i	0.1077gh	0.5227c-g	0.04667hi	1.2bc	7.1bc
90mm	P	0	335e-h	82.1bcd	4.133f	0.111gh	0.507c-g	0.04933h	0.8cd	6.9bc
	P	6	363c-f	84.4bcd	3.29j	0.1133g	0.5377b-f	0.06114gh	1.1bc	7.3bc
	T + P	0	361d-g	84.5 bcd	3.873g	0.119f	0.5093c-g	0.09067f	0.9bc	7.8bc
	T + P	6	352ab	93.1ab	3.273j	0.1193ef	0.5387b-f	0.09633e	1.4abc	8.2b
	No inoculation	0	105j	72.6d	5.483a	0.124def	0.511c-g	0.083g	0.54ef	2.8g
	No inoculation	6	261ij	73.1cd	3.197j-l	0.1263d	0.554a-f	0.094ef	0.62ef	3.7ef
120mm	T	0	287ij	80.3bcd	5.233b	0.1233def	0.5563a-e	0.09667e	0.7cd	5.3def
	T	6	320ij	86bcd	3.087lm	0.1253de	0.587abc	0.1147b	0.8cde	6.1dc
	P	0	280ij	81bcd	4.64c	0.1233def	0.5787abcd	0.106d	0.71cde	5.9dc
	P	6	315fghi	83.5bcd	3.727h	0.1267d	0.615ab	0.1113bc	0.9bc	6.1dc
	T + P	0	322ij	82.8bcd	4.678c	0.139b	0.607ab	0.108cd	0.7bc	7.0bc
	T + P	6	338fghi	89.7abcd	4.273e	0.1463a	0.6243a	0.1233a	1.1bc	7.6bc

3.2 Leaf Relative Water Content

In 2018, the highest amount of leaf relative water content (89.93%) was obtained from the irrigation level (irrigation after 60 mm evaporation from a Class A evaporation pan) and application of *Thiobacillus* and *Pseudomonas* bacteria and The lowest one (77.15%) was associated with severe stress conditions (120 mm evaporation) and no bacterial inoculation. The trend of changes in the leaf relative water content decreased with increasing water deficit stress intensity in all studied treatments; the highest rate of decline was observed when inoculated without bacteria, and the lowest rate of decline was observed when inoculated with two bacteria. However, the observed decreases were not statistically significant (Fig. 1).

In 2018 (Table 4), the leaf relative water content of the interaction effect of irrigation level and phosphorus fertilizer became significant at the 5% probability level. Three-way effects of experimental treatments were significant at the 1% probability level. According to Table 5, the relative leaf growth will be reduced by increasing the irrigation intervals and irrigation levels. Still, the application of bio-fertilizer and phosphorus positively affected this trait and increased it at the irrigation levels. The highest percentage of relative humidity of leaf (95.75%) was obtained from irrigation levels (60 mm evaporation from class A evaporation pan) and combined application of bio-fertilizers *Thiobacillus*+ *Pseudomonas* and phosphorus fertilizer and the lowest one (72.6%) was obtained from the irrigation level (120 mm of evaporation from the evaporation pan of class A) and without inoculation, and phosphorus application. The relative water content accurately estimates plant water status regarding cellular water deficit (Lu et al., 2004). It has been reported that drought stress causes severe damage to the cellular membranes of leaves of soybean cultivars due to a reduction in leaf relative humidity and leaf-surface relative humidity of soybeans (Munns, 2002). It appears that bio-fertilizer consumption helps keep more water content of lavender leaves by increasing water absorption (Ghonjalipour Goshki et al., 2021).

3.3 Proline Content

In 2018, the amount of proline increased with the increase in water deficit stress intensity. The use of phosphorus and bio-fertilizers caused different responses to the amount of this substance in the lavender plant. The lowest amount of proline content (2.047 mg/g wet weight) was obtained from not using inoculation and not applying phosphorus and irrigating after 60 mm evaporation from pan and the highest proline content (4.828 mg/g wet weight) was achieved from the irrigation (after 120 mm evaporation from the evaporation pan) and the application of phosphorus fertilizer and *Thiobacillus*+*Pseudomonas* bacteria which allowed to increase the proline content by 57.6% compared to the control (Table 3). Comparing the mean of the experimental treatments on the mean proline content in 2019 shows that the amount of proline increases with the increase in the water deficit stress intensity. However, the phosphorus and bio-fertilizer treatment reduced this matter by reducing the negative effects of drought stress in the lavender plant. The lowest proline content (2.047 mg/g wet weight) was obtained without inoculation of bio-fertilizers and without application of phosphorus and at irrigation level (irrigation after 60mm evaporation from class A pan (control) and the highest proline content (4.828 mg/g wet weight) was obtained from irrigation level (irrigation after 120mm evaporation from class A pan) and application of *Thiobacillus*+*Pseudomonas* with phosphorus fertilizer which increased by 57.6% compared to the control (Table 5).

In the present study, drought stress increased the concentration of proline while phosphorus and bio-fertilizer treatment decreased the concentration of this amino acid. Proline content change is one of plants' most commonly reported phenomena induced by salinity and drought stress. It has often been proven to be involved in stress tolerance mechanisms. The increase in proline content during stress may result from protein breakdown and reduced use due to a decrease in plant growth (Ashraf and Foolad, 2007). Deficit irrigation actually reduces soil water potential. In such conditions, to continue water absorption, the plant reduces its osmotic potential by accumulating osmotic compounds such as soluble carbohydrates and proline; in other words, osmotic regulation will be performed. In osmoregulation, turgescence and its related processes continue under water deficit conditions; hence, osmotic regulation contributes to cellular development and plant growth under water stress (Ashraf and Foolad, 2007). Proline is one of the most important substances that increase their accumulation in plant cells under water deficit stress. This amino acid is increased under stress conditions and effectively maintains osmotic potential (Zafari et al., 2012; Per et al., 2017). the present study also showed that with increasing irrigation regime levels, the plant's proline content has increased. Plants exposed to the water deficit stress, instead of spending large amounts of nitrogen resources and plant carbon to maintain turgor pressure of their cells, produce osmotic regulators, including proline (Aranjuelo et al., 2011). It has been generally accepted that reduction in turgescence is the primary factor of proline accumulation under deficit irrigation stress. The reduction of turgescence activates a complex sequence of adaptive processes related to plant stress tolerance. The primary effect of bio-fertilizer is that it affects the biosynthesis of gibberellin, abscisic acid, and sterols. These secondary substances indirectly affect the starch percentage so that proline amino acids will be increased under stress conditions (Sawan, 2008). Other researchers have reported that plant proline accumulation results from increased drought stress (Zhu and Gong, 2005; Nayyar, 2002).

Increased proline during stress may result from the breakdown of proteins and the reduction of their application due to plant growth reduction (Ashraf and Foolad, 2007). The plant reduces its osmotic potential in water-deficit conditions by accumulating osmotic compounds, including soluble carbohydrates and proline, to maintain water absorption. In other words, osmotic regulation will occur. Drought stress increases the proline in plants in two pathways: first, by increasing the synthesis of enzymes that stimulate proline production, and second, by inhibiting the action of enzymes that degrade proline (Rontaine et al., 2002). Application of phosphorus element at 50 kg/ha increased proline amount compared to non-applied ones. Phosphorus, as an important and necessary element, increases plant growth. Finally, more prerequisites for proline are produced, and as a result, a larger amount of this amino acid will be produced (Marschner, 2002). Application of bio-fertilizers under environmental stress conditions such as drought stress not only increases drought stress tolerance in plants, but it also increases granular soil-structure water-holding capacity, increases enzymatic and microbial activities, and releases soil colloid nutrients and finally compensates for missed soil-borne microorganisms. Plants inoculated with these fertilizers have better nutritional and water relations than plants without inoculation and can temporarily escape from water deficit stress conditions and suffer less damage. Therefore, the amount of soluble sugars and proline increases less than in plants without inoculation. In addition to reducing the effects of stress, it increases the absorption of elements such as nitrogen, increases the amount of carbon and nitrogen that plants need to produce other amino acids, and reduces the breakdown of proteins (Aghaei et al., 2020). On the other hand, proline production is related to the production of soluble sugars because glutamate is one of the pathways of proline production. With the increase in the production of soluble sugars, the amount of glutamate production increases, and the synthesis of proline will be intensified (Alikhani and Mahmudi zarandi, 2019).

3.5 Antioxidant Enzyme

3.4 Catalase

The highest amount of catalase enzyme activity in 2018 (absorbance changes of 0.1147 µg protein per minute) and 2019(absorbance changes 0.1463 µg protein per minute) was obtained from the combined application of *Thiobacillus* + *Pseudomonas* and phosphorus fertilizer at irrigation level (120 mm evaporation from evaporation pan) and the lowest one in the first year (absorbance changes 0.071 µg protein per minute) and the second year (absorbance changes 0.07633 µg protein per minute) was obtained from no phosphorus and no inoculation at irrigation level(60 mm evaporation from evaporation pan). The amount of catalase enzyme activity has increased with increasing irrigation levels and the use of phosphorus and bio-fertilizers. Catalase is an enzyme whose activity inhibits the hydrogen peroxide produced in photorespiratory pathways inside the peroxisomes (Mittler, 2006). This enzyme also belongs to the iron-containing proteins and oxidoreductase enzyme group. It can directly convert hydrogen peroxide into oxygen and water and eliminate the toxicity of this oxygen-free radical (Sarvajeet and Narendra, 2010; Nayyar and Gupta, 2006). The results of this study are consistent with the research of Sarvari et al. 2017 to increase catalase activity under water deficit stress. PGB bacteria effectively reduce oxidative stress damage and reactive oxygen species in plants under stress by increasing the activity of some antioxidant enzymes (Saravanakumar et al., 2010). It can be said that the production of metabolites by these bacteria, including growth-promoting hormones, plays an important role in expressing and stimulating antioxidant enzyme proteins. Oxidative stress caused by increasing reactive oxygen species in the cell is one of the most important undesirable effects on plants and important plant physiological processes.

Considering the key role of peroxidase and catalase enzymes in removing the toxic radical of hydrogen peroxide under stress conditions, increasing the activity of catalase as a result of bacterial inoculation can be an effective factor in removing reactive oxygen species and ultimately increasing the resistance of plants to all types of stress. PGB bacteria applications reduce enzyme and fatty acid damage and the destructive effects of stress by scavenging free oxygen radicals (Belimove et al., 2015). An increase in catalase activity has been reported in PGPB-inoculated beets compared to plants under stress conditions (Gururani et al., 2012). Generally, the bacterium *Pseudomonas* has acted as catabolism and has increased the activity of enzymes such as catalase and superoxide (Bianco and Defez, 2009). Research (Rahbari et al., 2020) has shown that inoculation with *Pseudomonas* bacteria, particularly strain 150 in dill plants, significantly increases peroxidase and catalase enzyme activity.

3.6 Superoxide Dismutase

The highest amount of this enzyme activity in 2018 (absorbance changes 0.6173 µg protein per minute) and 2019 (absorbance changes 0.6243 µg protein per minute) was obtained from the combined application of *Thiobacillus + Pseudomonas* and phosphorus fertilizer at irrigation level (120 mm evaporation from evaporation pan) and the lowest one in the first year (absorbance changes 0.386 µg protein per minute) and in the second year (absorbance changes 0.3937 µg protein per minute) was obtained without inoculation of bio-fertilizers and without application of phosphorus and at irrigation level (irrigation after 60mm evaporation from evaporation pan (Table 3,5). Superoxide dismutase activity increased with increasing irrigation levels and applying phosphorus and bio-fertilizers. The enzyme superoxide dismutase forms the cell's first line of defense against active oxygen radicals (Alscher et al., 2002). They also catalyze the regeneration of superoxide radicals into hydrogen peroxide and molecular oxygen. In the next step, the resulting hydrogen peroxide is purified using the enzymes catalase, ascorbate peroxidase, and peroxidase (Zeid and Shedeed, 2006). In this study, superoxide dismutase enzyme activity has been increased due to the water deficit stress. Other researchers have reported similar results (Mittler, 2002; Yang et al., 2006). Increased activity of this enzyme correlates with increased protection against damage from different environmental stress (Pang et al., 2005). In general, antioxidant enzymes play an important and key role in controlling free oxygen radicals and peroxides at the cellular level (Apel and Hirt, 2004). According to the results of this study, the application of phosphorus fertilizer and bio-fertilizer under water deficit stress conditions, especially in vegetative and reproductive growth phases, can prevent some stress-induced damage in lavender plants by increasing the superoxide dismutase enzyme activity to some extent, which is consistent with the results of (Hashmsti et al., 2016) on safflower.

3.7 Peroxidase

The highest amount of this enzyme in 2018 (absorbance changes 0.1193 µg protein per minute) and in 2019 (absorbance changes 0.1233 µg protein per minute) was obtained from the combined application of *Thiobacillus + Pseudomonas* and phosphorus fertilizer at irrigation level (120 mm evaporation from evaporation pan) and the lowest one in the first year (absorbance changes 0.072 µg protein per minute) and the second year (absorbance changes 0.01267 µg protein per minute) was obtained from not using inoculation and not applying phosphorus and irrigating after 60 mm evaporation from evaporation pan. The amount of superoxide dismutase enzyme activity increased with increasing drought stress intensity and the use of phosphorus and bio-fertilizers (Tables 3, 5). Peroxidase is an important enzyme in oxide oxidation, and peroxidases protect plants by converting hydrogen peroxide into water. This enzyme is sensitive to environmental fluctuations and is considered an indicator of tolerance of abiotic stresses in plants. The results of this research represent that the increase in peroxidase enzyme activity can be a reason for the induced defence compounds by bacteria which is consistent with the results of other researchers (Harish et al., 2008).

3.8 Essential Oil Percentage

The essential oil percentage decreases with the increase of water deficit stress intensity, and applying phosphorus fertilizer and bio-fertilizer improved the essential oil percentage in lavender plants. The lowest essential oil percentage in the first year (0.4%) and the second year (0.54%) was obtained from no bio-fertilizer inoculation and no phosphorus application at the irrigation level (120 mm evaporation from evaporation pan), and the highest one in the first year (1.2%) and the second year (1.7%) was obtained from the application of *Thiobacillus + Pseudomonas* and phosphorus application at irrigation level (60 mm evaporation from evaporation pan (Tables 3, 5). According to the results of bacterial inoculation on the lavender essential oil percentage, it was affected under different drought stress levels.

3.9 Essential Oil Yield

The essential oil yield decreases with the increase in water deficit stress intensity, and applying phosphorus and bio-fertilizer improved the lavender essential oil yield. The lowest essential oil yield in the first year (1.2 kg/ha) and the second year (2.8 kg/ha) was achieved from no bio-fertilizer inoculation and no phosphorus application at the irrigation level (120 mm evaporation from evaporation pan) and the highest one in the first year (9.06 kg/ha) and second year (9.4 kg/ha) was achieved from application of *Thiobacillus + Pseudomonas* and phosphorus application at irrigation level (60 mm evaporation from evaporation pan) (Table 3,5). In the first year, the irrigation after 60, 90, and 120 mm evaporation from the evaporation pan and the application of phosphorus and bio-fertilizers increased essential oil yield by 54%, 55%, and 69%, respectively. In the second year, the irrigation after 60, 90, and 120 mm evaporation from the evaporation pan and the application of phosphorus and bio-fertilizers increased the essential oil yield by 52%, 51%, and 63%, respectively. The report of Ping and Boland (2004) represents that PGP bacteria are considered bio-stimuli in synthesizing secondary metabolites.

4 Conclusion

The present research found that during the two years of the study, increasing irrigation levels and applying drought stress had a negative effect on the trait wet weight in lavender medicinal plants. Drought stress decreased the wet weight of flowers, essential oil yield, and percentage and relative humidity of leaf water. On the other hand, the application of drought stress increased proline content, antioxidant enzyme activity, and cell membrane leakage. In this research, using bio-fertilizers under water deficit stress conditions could greatly reduce the effects of malnutrition in the lavender medicinal plant. The drought tolerance mechanisms of this plant include accumulation of proline, soluble sugars, and increased antioxidant enzyme activity in this plant under water deficit stress.

The combined use of *Thiobacillus* and *Pseudomonas* bacteria led to better results than using these bacteria alone. Therefore, if this plant's water deficit stress intensity is not high, it will be possible to reduce the effects of water deficit stress by using bio-fertilizers.

Declarations

Author Contribution

All authors reviewed the manuscript

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Figures

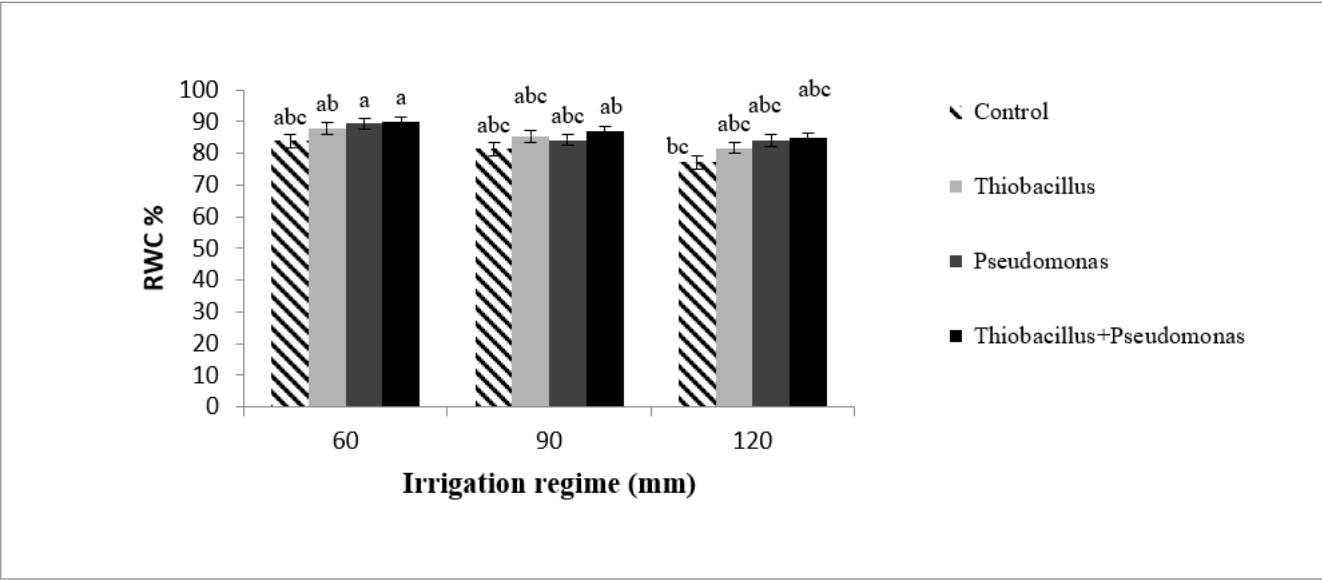


Figure 1
compares the mean of the interaction effects of bio-fertilizer and irrigation regime treatment on the mean of leaf relative water content.