

The combination of nanoparticles (NPs) and endophytes boosts Thyme (*Thymus vulgaris* L.) resistance to drought by elevating levels of phenolic compounds, flavonoids, and essential oils

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Abstract

To assess the growth and biochemical responses of *Thymus vulgaris* to the application of iron oxide nanoparticles (Fe_2O_3 NPs) and endophytes under drought stress, a factorial experiment was designed in a completely randomized design (CRD). Experimental treatments included 4 irrigation levels (100, 75, 50, and 25% FC), 4 levels of Fe_2O_3 NPs (0, 0.5, 1, and 1.5 mgL^{-1}), and 3 levels of endophytes (control, bacteria and fungi). Drought stress had a detrimental impact on total dry matter (TDM). Inoculation of plants with endophytes and foliar Fe_2O_3 NPs spraying played a positive role in preserving and increasing the phenolic and flavonoid contents of thyme under drought-stress conditions. The highest total phenolic content ($2.86 \text{ mg g}^{-1} \text{ FW}$) and total flavonoid content ($4.54 \text{ mg g}^{-1} \text{ FW}$) were observed in plants treated with bacteria along with $1 \text{ mgL}^{-1} \text{ Fe}_2\text{O}_3$ NPs and fungal treatment with $0.5 \text{ mgL}^{-1} \text{ Fe}_2\text{O}_3$ NPs, respectively, under 25% FC irrigation. Exposure to moderate and severe drought stresses increased the predominant phenolic compounds (p-coumaric acid, caffeic acid, and ferulic acid) in the methanolic extract of thyme. During moderate stress conditions (50% FC), bacterial endophytes exerted a more substantial influence on the elevation of p-coumaric acid compared to fungal endophytes. In response to reduced irrigation levels, the essential oil percentage increased in thyme plants, while the predominant constituents of the essential oil, namely thymol and carvacrol, decreased. Endophytes and Fe_2O_3 NPs positively influenced the percentage of essential oil and the concentrations of thymol and carvacrol in the essential oil.

Background

Studying drought stress in plants is crucial because it significantly affects both the quantity and quality of plant functioning. Drought stress can lead to reduced crop yield and productivity, impacting plant performance in terms of quantity [1]. Moreover, it can negatively affect the nutritional value and chemical composition of plant products, thereby compromising overall performance quality. To mitigate the harmful effects of drought stress on plants, it is essential to comprehend its impact. This knowledge can facilitate the development of drought-resistant crop varieties, the implementation of efficient irrigation techniques, and the adoption of sustainable farming methods.

The importance of iron oxide nanoparticles in influencing plant growth under drought stress circumstances lies in their ability to maximize iron intake, enhance plant resistance to drought stress, and promote overall plant growth and development. Numerous studies have demonstrated that the use of iron oxide nanoparticles improves the nutritional status of plants, increases their resistance to drought stress, and fosters overall growth and development. When applied to drought-stressed plants, iron oxide nanoparticles act as a source of iron, thereby enhancing the plants' nutrient levels. This, in turn, helps preserve vital physiological functions such as germination, root activity, and photosynthesis, ultimately enhancing the plants' ability to withstand drought stress. Additionally, research has shown that iron oxide nanoparticles play a crucial role in strengthening the antioxidant defense system of plants during drought stress, leading to reduced oxidative damage and the preservation of cellular

homeostasis. Ultimately, these effects contribute to improved plant growth and development [2]. Endophytes, comprising bacteria and fungi, reside within plant tissues without causing harm to their hosts [3]. When plants face drought stress, endophytes play a significant role in enhancing plant development and survival [4]. They support the growth, well-being, and development of host plants by providing defense against both biotic and abiotic challenges [5]. Bacterial endophytes are particularly beneficial for plant growth during drought stress as they produce bioactive compounds that stimulate the growth of host plants [6]. This involves facilitating the uptake of primary and secondary nutrients, such as phosphate solubilization and atmospheric nitrogen fixation. Moreover, bacterial endophytes aid plants in adapting to and thriving in dry conditions by promoting osmotic adjustment and controlling stomatal function, as stated by Dang et al. (2020) [7]. Fungal endophytes also play a crucial role in promoting plant growth during drought stress by protecting plants from abiotic factors like drought, salt, and high metal toxicity. They achieve this by synthesizing growth regulators and plant hormones that enhance plant development and yield in harsh environmental conditions [8]. In conclusion, the significance of bacterial and fungal endophytes in influencing plant growth in drought-stressed environments cannot be overstated. They provide essential nutrients, facilitate nutrient uptake, and assist in osmotic adjustment and stomatal regulation, enabling plants to adapt and thrive in challenging drought conditions.

The pharmaceutical industry plays a crucial role in promoting human health through its dedicated efforts in researching, developing, and manufacturing medical products. *T. vulgaris*, due to its numerous therapeutic applications, holds significant importance for the pharmaceutical industry [9]. Thymol, a bioactive compound found in this plant, exhibits antiviral, antiseptic, antimicrobial, and antifungal properties. These qualities make *T. vulgaris* a valuable ingredient in pharmaceutical products, aiding in the treatment and prevention of various illnesses and infections. Thymol, specifically, has been extensively studied for its potential health benefits. Research suggests that it possesses anti-inflammatory and antioxidant properties, making it a valuable component in the development of medications for conditions like rheumatoid arthritis and diseases associated with oxidative stress, such as neurological disorders and cardiovascular diseases [10]. The objective of this study was to examine the biochemical and growth responses of *T. vulgaris* under drought stress conditions when exposed to iron oxide nanoparticles and Lamiaceae endophytes.

Results

Total Fresh Matter (TFM) and Total Dry Matter (TDM)

Overall, drought stress had a significant negative effect on both the total fresh matter (TFM) and total dry matter (TDM) of thyme plants. The lowest TFM and TDM (25.36 and 9.73 g, respectively) were observed under irrigation at 25% FC without endophytes and Fe₂O₃ NPs (Fig. 3). Application of endophytes and Fe₂O₃ NPs through foliar spraying led to a notable increase in TFM and TDM. The highest TFM (45.45 g) and TDM (21.56 g) were achieved with the combined application of a bacterial endophyte and 1 mg.L⁻¹ Fe₂O₃ NPs under irrigation at 100% FC. Additionally, TDM increased by 44.44% and 43.78% under

irrigation at 50% and 25% FC, respectively, with the combined application of bacterial endophyte and 1 mg.L⁻¹ Fe2O3 NPs compared to no endophyte and Fe2O3 NPs application (Fig. 3).

PPO and PAL Activities, and Phenolic and Flavonoid Compounds

A decrease in irrigation levels to 50% and 25% FC resulted in an increase in PPO activity. Bacterial and fungal endophytes, along with Fe2O3 NPs, played a significant role in reducing PPO activity under water-deficit stress (Fig. 4).

The activity of the PAL enzyme, crucial in phenols biosynthesis, was significantly affected by irrigation levels and endophytes. However, Fe2O3 NPs foliar spray did not impact PAL activity. The highest PAL activity (1.88 µmol mg⁻¹ pro. h⁻¹) was observed with 25% FC irrigation and no endophyte application. Bacterial and fungal endophytes reduced PAL activity by 13.29% and 15.42%, respectively, under severe drought stress (Fig. 5).

An increase in total phenolic and flavonoid contents was observed in plants with reduced irrigation levels. Endophytes and Fe2O3 NPs played a positive role in preserving and enhancing phenolic and flavonoid contents under drought stress conditions. The highest total phenolic content (2.86 mg g⁻¹ FW) and total flavonoid content (4.54 mg g⁻¹ FW) were seen in plants treated with bacterial and fungal endophytes along with Fe2O3 NPs under 25% FC irrigation (Table 2).

Table 1
Physicochemical characteristics of the soil used

Soil texture	pH	ECe	CEC	SP	Organic matter	N	K	P	Zn	Fe
Sandy loam	7.38	dS m ⁻¹	Cmc kg ⁻¹	%	g kg ⁻¹	(%)	mg kg ⁻¹			
		1.17	16	32	7.4	0.11	77	9	3.2	2.87

Table 2

Effect of irrigation levels, bacterial and fungal endophytes and Fe₂O₃ nanoparticles on total phenolic and flavonoid contents of *T. vulgaris* L.

Endophyte	Fe ₂ O ₃ NPs (mg.L ⁻¹)	Irrigation (%FC)	Total phenolic content (mg g ⁻¹ FW)	Total flavonoid content (mg g ⁻¹ FW)
0	0	100	^w 1.58 ± 0.05	^u 2.52 ± 0.17
		75	^{o-s} 1.83 ± 0.04	^{n-r} 2.99 ± 0.15
		50	^{lm} 2 ± 0.06	^{p-t} 2.93 ± 0.25
		25	^{ef} 2.42 ± 0.02	^{f-j} 3.65 ± 0.03
	0.5	100	^{uv} 1.68 ± 0.03	^{stu} 2.6 ± 0.13
		75	^{r-v} 1.75 ± 0.05	^{stu} 2.61 ± 0.17
		50	^{ijk} 2.15 ± 0.03	^{l-p} 3.21 ± 0.21
		25	^d 2.58 ± 0.03	^{cd} 4.08 ± 0.05
	1	100	^{r-v} 1.76 ± 0.07	^{stu} 2.62 ± 0.09
		75	^{p-t} 1.79 ± 0.06	^{q-u} 2.74 ± 0.06
		50	^{hi} 2.23 ± 0.04	^{g-k} 3.58 ± 0.21
		25	^{cd} 2.65 ± 0.03	^{cd} 4.08 ± 0.29
	1.5	100	^{tuv} 1.71 ± 0.03	^{tu} 2.59 ± 0.03
		75	^{tuv} 1.73 ± 0.07	^{q-u} 2.74 ± 0.15
		50	^{hi} 2.24 ± 0.02	^{h-l} 3.42 ± 0.33
		25	^d 2.59 ± 0.01	^{cde} 4.04 ± 0.22
Fungi	0	100	^{vw} 1.67 ± 0.02	^{n-r} 2.98 ± 0.29
		75	^{lm} 2 ± 0.06	^{k-o} 3.3 ± 0.14
		50	^{kl} 2.09 ± 0.02	^{g-k} 3.56 ± 0.17
		25	^{ef} 2.42 ± 0.08	abc 4.21 ± 0.30
	0.5	100	^{s-v} 1.74 ± 0.04	^{q-u} 2.79 ± 0.09
		75	^{nop} 1.88 ± 0.03	^{stu} 2.61 ± 0.2

Endophyte	Fe ₂ O ₃ NPs (mg.L ⁻¹)	Irrigation (%FC)	Total phenolic content (mg g ⁻¹ FW)	Total flavonoid content (mg g ⁻¹ FW)
	1	50	gh 2.29 ± 0.03	d-g 3.85 ± 0.13
		25	d 2.6 ± 0.04	a 4.54 ± 0.24
		100	o-s 1.83 ± 0.02	m-q 3.04 ± 0.06
		75	o-r 1.84 ± 0.02	k-o 3.29 ± 0.26
		50	fg 2.35 ± 0.03	f-k 3.62 ± 0.17
		25	bc 2.73 ± 0.07	ab 4.47 ± 0.15
	1.5	100	mno 1.92 ± 0.06	i-m 3.34 ± 0.1
		75	opq 1.86 ± 0.04	h-l 3.41 ± 0.11
		50	e 2.47 ± 0.12	bcd 4.14 ± 0.09
		25	cd 2.64 ± 0.08	cde 4.04 ± 0.09
Bacteria	0	100	o-r 1.84 ± 0.07	r-u 2.66 ± 0.08
		75	jk 2.13 ± 0.04	f-i 3.68 ± 0.84
		50	hij 2.21 ± 0.02	j-n 3.30 ± 0.16
		25	d 2.58 ± 0.16	e-h 3.71 ± 0.25
	0.5	100	r-u 1.76 ± 0.07	q-u 2.71 ± 0.05
		75	q-t 1.78 ± 0.04	o-s 2.95 ± 0.09
		50	ef 2.44 ± 0.08	h-l 3.48 ± 0.25
		25	ab 2.82 ± 0.03	bcd 4.15 ± 0.12
	1	100	kl 2.08 ± 0.03	q-u 2.76 ± 0.07
		75	mn 1.96 ± 0.04	n-r 2.98 ± 0.10
		50	e 2.46 ± 0.04	f-k 3.62 ± 0.08
		25	a 2.86 ± 0.05	c-f 3.96 ± 0.28
	1.5	100	ijk 2.17 ± 0.02	q-u 2.85 ± 0.13

Endophyte	Fe ₂ O ₃ NPs (mg.L ⁻¹)	Irrigation (%FC)	Total phenolic content (mg g ⁻¹ FW)	Total flavonoid content (mg g ⁻¹ FW)
		75	^{hij} 2.21 ± 0.02	^{m-r} 3.01 ± 0.14
		50	^d 2.63 ± 0.05	^{c-g} 3.87 ± 0.18
		25	^{ab} 2.77 ± 0.11	^{cd} 4.09 ± 0.17
† Means followed by the same letter are not significantly different according to the least significant difference (LSD) test at P ≤ 0.05.				

The predominant phenolic compounds in thyme were p-coumaric acid, caffeic acid, and ferulic acid. Moderate and severe drought stress increased the levels of these compounds in plants. Endophytes enhanced the presence of p-coumaric acid, while Fe₂O₃ NPs did not affect its level. Endophytes and Fe₂O₃ NPs increased caffeic acid levels, with bacterial endophytes showing a greater impact under moderate stress and fungal endophytes under severe stress. Ferulic acid content was influenced by endophytes and Fe₂O₃ NPs, with Fe₂O₃ NPs reducing its level in plants (Fig. 6–8).

Essential oil, Thymol and Carvacrol Percentage

The percentage of thyme essential oil increased with decreasing irrigation levels. Fungal endophytes had a greater influence on essential oil percentage compared to bacterial endophytes. Foliar application of Fe₂O₃ NPs increased essential oil percentage under moderate and severe drought stresses. Thymol and carvacrol were the predominant compounds in thyme essential oil, with their levels not influenced by drought, endophytes, or Fe₂O₃ NPs interaction (Fig. 9–11).

Correlation and principal component analysis

HCA analysis

This clustered heatmap illustrates the various treatment groups, including endophyte at three levels (control (E0), fungal endophyte (E1), and bacterial endophyte (E2)), iron nanoparticles at four levels (F0 - F3), and drought at three levels (D0 - D3). The colors and clustering lines indicate that similar treatments are closer to each other. It also shows the different measured traits, which include PAL, phenolic, essential, ferulic, PPO, flavonoid, Caffeic acid, p-coumaric, fresh weight (FW), dry weight (DW), thymol, and carvacrol. The clustering lines indicate that similar traits are closer to each other. The numerical scale ranges from - 3 (dark blue) to + 3 (dark red), showing the extent of each trait, where red indicates a high value and blue indicates a low value. This heatmap reveals how each trait changes under different treatments. For example, the PPO trait is very high in the E0F3D0 treatment (dark red), but it might be lower in other treatments. Additionally, treatments with similar responses are shown closer together, such as E1F2D0 and E1F2D1, which have similar responses and are clustered closely on the map. This

type of heatmap is very useful for understanding how plants respond to different conditions and helps researchers better grasp the patterns in plant responses (Fig. 12).

Pearson's correlation analysis

In the correlation matrix, blue colors denote robust positive correlations among specific traits, while red colors signify strong negative correlations between these traits. The size of the data points (bullets) within the matrix indicates the variable values displayed on each axis: larger bullets represent higher variable values, whereas smaller bullets denote lower values. For instance, there exists a strong positive correlation among traits such as PAL enzyme activity, phenolic compounds, flavonoids, and caffeic acid, indicating these characteristics are concurrently influenced by factors like endophytes and drought stress. The strongest correlation was observed between fresh weight and dry weight, as well as the activity of PPO. Additionally, there was a strong correlation between dry weight and the levels of thymol and carvacrol. Conversely, a negative correlation is evident between traits such as PPO enzyme activity and basic compounds, suggesting that increased PPO activity could potentially reduce essential plant compounds. Traits like fresh weight, dry weight of plants, and compounds such as thymol and carvacrol exhibit weak correlations with other traits, implying their susceptibility to influences beyond endophytes and drought stress. Moreover, iron nanoparticles (F0 - F3) and varying levels of drought stress (D0 - D3) significantly impact most measured traits, discernible from the diverse color patterns in the matrix (Fig. 13).

Principle component analysis (PCA)

A biplot is a two-dimensional plot used to display multivariate data. In this biplot (a), the F1 and F2 axes explain 58.01% and 23.19% of the data variance, respectively, together accounting for 81.20% of the total variance. In the second plot (b), the horizontal axis (D1) explains 42.04% and the vertical axis (D2) explains 39.86% of the total variance of the data. Together, these two axes cover 81.90% of the variance. This significant proportion indicates that these two principal components effectively capture the variations in the data. The F1 axis explains the most variance and has the greatest impact on separating the different combinations, while the F2 axis covers the variance not explained by F1. The blue points represent different combinations of endophytes (E0, - E2), iron nanoparticles (F0 - F3), and drought levels (D0 - D3), with points closer together indicating combinations with similar effects on the measured traits. The red lines represent the direction and magnitude of the impact of the measured traits, with the length of the lines indicating the strength of each trait's influence on the combinations. The direction of the lines shows which combinations have the greatest effect on each trait. Longer lines indicate stronger impacts, such as the long red line for "essential," indicating this trait has a significant influence on the combinations. The direction of the lines also indicates the relationship between the trait and the combinations; for example, combinations in the direction of the "flavonoid" line have high levels of flavonoids. Red lines for PAL and phenolic pointing to the top right of the plot indicate that combinations in this area (E1F2D0 and E1F3D0) have high levels of these traits. Similarly, red lines for thymol and carvacrol pointing to the bottom right indicate that combinations in this area (E2F3D0 and E2F2D0) have high levels of these traits. The red line for ferulic acid pointing to the top left shows that combinations in

this area (E0F2D0 and E1F1D0) have high levels of ferulic acid. Combinations that are close to each other share similar characteristics, and the active variables with longer red lines have stronger impacts on the combinations. The direction of the red lines indicates which combinations have the most influence on a specific trait, helping researchers identify the optimal combinations to enhance desired plant traits (Fig. 14).

Discussion

In times of drought stress, plants have adaptive mechanisms that increase the production of phenols and flavonoids. These mechanisms involve activating specific genes in the biosynthetic pathway of these compounds to counteract oxidative stress caused by water deficiency [11]. Phenols and flavonoids act as antioxidants, protecting plant cells from damage by neutralizing reactive oxygen species. These compounds are crucial not only for protecting cellular structures but also for maintaining the integrity of the photosynthetic apparatus, which is particularly vulnerable under drought conditions [12]. In the present study, inoculation of plants with endophytes and foliar Fe_2O_3 NPs spraying played a positive role in preserving and increasing the phenolic and flavonoid contents of thyme under drought-stress conditions. Iron nanoparticles have a significant impact on plant physiology under drought stress, increasing the production of phenols and flavonoids. The exact mechanisms by which iron nanoparticles enhance these compounds are still being studied, but it is believed that they may boost enzyme activity involved in their biosynthesis. Additionally, iron nanoparticles may improve nutrient uptake and overall plant performance, helping plants withstand drought [13, 14].

Fungal and bacterial endophytes also enhance the synthesis of phenols and flavonoids under drought stress, acting as antioxidants to protect plant cells [15]. This mutualistic interaction benefits both the endophytes and plants, strengthening the plant's defense system and resilience to drought. Endophytes may also stimulate the synthesis of phytohormones, promoting plant growth [8]. Reduced irrigation levels increase the percentage of essential oil in thyme plants under drought stress, with changes in essential oil components attributed to plant defense mechanisms [16]. The increase in essential oil production is a protective response to combat oxidative stress, with elevated enzyme activity contributing to this increase [17]. However, drought stress can also lead to a decrease in thymol percentage in essential oil due to factors like reduced plant biomass and oxidative damage [18]. Endophytes and iron oxide nanoparticles positively impact essential oil production in plants under drought stress, potentially by enhancing plant metabolism and defense mechanisms. Endophytes may stimulate bioactive compound synthesis, while iron oxide nanoparticles can improve water regulation and nutrient uptake, leading to increased essential oil production. One mechanism involves the activation of plasma membrane proton pump ATPases, increasing stomatal aperture and improving water regulation [19]. Overall, bacterial and fungal endophytes play a crucial role in increasing essential oil levels in plants under drought stress, potentially by enhancing plant defense mechanisms and nutrient absorption. These mechanisms collectively contribute to the observed increase in essential oil content in plants facing drought stress conditions [20]. In HCA analysis, the similarities and differences in plant responses to various treatments depend on multiple factors. Common defense mechanisms, such as

the activity of fungal and bacterial endophytes, can be activated against environmental stresses and lead to similar responses [21]. Additionally, different treatments may have similar effects on plant metabolism, such as the stimulation of enzyme activity by iron nanoparticles and fungal endophytes [21, 22]. Synergy between treatments can also enhance similar defensive responses. Conversely, the differences are influenced by the specific characteristics of each treatment, such as the production of free radicals by iron nanoparticles, and varying levels of stress, such as severe and mild drought. Interactions between treatments can also produce different effects; for instance, the combination of drought and iron nanoparticles may result in reduced plant growth. Overall, cluster analysis shows that similarities arise from common defense mechanisms, similar effects of treatments, and their synergy, while differences depend on the specific characteristics of each treatment, the level of stress, and the interactions between treatments. This analysis helps improve our understanding of plant responses to environmental stresses and optimize agricultural practices. In PCA analysis the dry weight of plants serves as an indicator of their total biomass and physiological development, directly influencing the production and accumulation of secondary metabolites such as essential oils. Plants thriving under favorable growth conditions and exhibiting higher dry weights likely allocate more resources to synthesize secondary metabolites like thymol and carvacrol. This resource allocation is a strategic response by plants to optimize their defense mechanisms and increase their chances of survival in challenging environments. thymol and carvacrol, known for their antimicrobial and antioxidant properties, are produced by plants as a response to environmental stresses such as pathogens or drought [23]. The negative correlation between these compounds and PPO enzyme activity can be explained by several factors: PPO enzyme plays a role in the oxidation of phenols, including thymol and carvacrol, which may lead to chemical changes that reduce the levels of these compounds. Additionally, the high activity of PPO enzyme might increase the plant's consumption of nutrients and energy, potentially reducing the available resources for synthesizing thymol and carvacrol [24]. Moreover, the negative correlation observed between the fresh and dry weight of plants with the percentage of essential oils and the levels of thymol and carvacrol could indicate competition for resources between essential oil synthesis and plant growth. Fresh weight represents the available resources and energy in plants, which may not adequately support the production of essential oils if resources are limited for synthesizing thymol and carvacrol. In conclusion, these correlations reflect the complex strategies plants employ to adapt to environmental challenges, where resource allocation and metabolic processes play critical roles in determining their chemical profiles and overall health. In PCA-Biplot analysis the presence of fungal (E1) and bacterial (E2) endophytes significantly impacts the measured traits, with combinations involving E1 and E2 aligned along the axes of PAL, phenolic compounds, and essential oils. This strong influence, shown by long red lines, suggests that endophytes boost the production of these compounds due to their symbiotic relationship with plants, enhancing secondary metabolite production as a defense mechanism. Adding iron nanoparticles (F0 - F3) also affects plant traits. Higher levels of nanoparticles (F2 and F3) are linked to increased levels of flavonoids, thymol, and carvacrol, likely because iron acts as a cofactor for enzymes crucial in synthesizing these compounds [25]. The biplot highlights synergistic effects of combining endophytes and iron nanoparticles. For example, the combination E1F2D0 (fungal endophyte, moderate iron nanoparticles, no drought) shows high levels of

essential oils and phenolic compounds. This synergy indicates that a multifaceted approach is more effective in enhancing plant traits than single-factor treatments, due to complementary roles in nutrient uptake and enzyme activation. These findings are significant for sustainable agriculture. By understanding these interactions, strategies can be developed to enhance crop resilience and productivity. Inoculating plants with specific endophytes and supplementing with iron nanoparticles can boost valuable secondary metabolites, improving plant health and market value [26]. In conclusion, the biplot analysis provides a comprehensive overview of how endophytes and iron nanoparticles interact to influence plant traits, highlighting the potential of integrated agricultural practices to enhance productivity and resilience, offering a pathway to more sustainable crop management. The process of screening strains plays a pivotal role in advancing biological research and enhancing industrial processes. A critical criterion in this study was the strains' capability to endure dry conditions. Examination of the findings revealed that the selected strains exhibit substantial potential for further exploration and application in agricultural and biotechnological contexts. Depositing the genetic sequences into GenBank and acquiring accession numbers facilitates global access to the genetic information of these strains, thereby significantly contributing to future research endeavors.

Conclusion

Here we have reported the activity of the PAL enzyme, as the principal enzyme in the biosynthesis of phenols, was significantly influenced by irrigation levels and endophytes. However, the activity of this enzyme was not affected by foliar application of Fe_2O_3 NPs. Inoculation with endophytes, coupled with the foliar application of Fe_2O_3 NPs, positively impacted the phenolic and flavonoid contents of thyme under drought stress. The predominant phenolic compounds in the methanolic extract of thyme were p-coumaric acid, caffeic acid, and ferulic acid. The application of moderate and severe drought stresses increased the levels of these compounds in the plants. The application of endophytes significantly elevated the content of p-coumaric acid in plants, contrasting with the negligible impact of foliar Fe_2O_3 NPs spray on its levels. Under moderate stress conditions (50% FC), bacterial endophytes had a more significant impact on the increase of p-coumaric acid compared to fungal endophytes. However, under severe stress (25% FC), fungal endophytes exhibited a more pronounced effect. The application of endophytes and Fe_2O_3 NPs increased the amounts of caffeic acid in the tested plants, both under moderate and severe drought stress conditions, according to the results. Bacterial and fungal endophytes elevated the amounts of caffeic acid in thyme plants grown under 25% FC irrigation. The treatment of endophytes and Fe_2O_3 NPs affected the amount of ferulic acid in thyme plants. Ferulic acid levels rose after bacterial endophyte inoculation under 25% FC irrigation. In the face of diminished irrigation levels, thyme plants exhibited an augmentation in the essential oil percentage, accompanied by a reduction in the predominant constituents of the essential oil, specifically thymol and carvacrol. Endophytes, along with Fe_2O_3 NPs, exerted a positive impact on the essential oil percentage and the concentrations of thymol and carvacrol in the essential oil. Across all irrigation levels, the fungal endophyte exhibited a more pronounced effect in augmenting the percentage of essential oil compared to the bacterial endophyte. Nonetheless, no significant distinction was observed in the impact of

bacterial and fungal endophytes on the concentrations of thymol and carvacrol. The findings of this study indicate the positive impact of bacterial and fungal endophytes as well as Fe₂O₃ NPs on enhancing the resistance of thyme plants to drought stress and improving quantitative and qualitative characteristics under stressful conditions.

Materials and Methods

Collection of Plant Samples

Plants from the Lamiaceae family, including *Salvia officinalis*, *Salvia mirzayanii*, *Zhumeria majdae*, *Teucrium polium*, *Zataria multiflora*, *Lavandula angustifolia*, *Origanum majorana*, *Thymus daenensis*, and *Ziziphora clinopodioides*, were collected on October 31, 2022, from Mount Genow in Hormozgan Province, Iran, at an elevation of 2347 meters above sea level.

Endophyte Isolation and Identification

Bacterial Endophytes

Plant samples were cultured on Nutrient Agar (NA) medium for morphological identification of bacterial endophytes. Physiological identification methods and 16S rDNA sequencing were used for further identification. Bacterial strains were sprayed on *T. vulgaris* plants, and drought tolerance was evaluated (Fig.1a).

Fungal Endophytes

Plant samples were grown on Potato Dextrose Agar (PDA) medium for fungal endophyte isolation. Microscopic evaluation and genomic DNA extraction were conducted for identification. Fungal isolates were sprayed on *T. vulgaris* plants, and drought resistance was assessed.

Plant Material

Azospirillum lipoferum and *Aspergillus oryzae* isolated from *Salvia mirzayanii* were used for treatment. *T. vulgaris* seeds were sourced from Hatem Agricultural Growth Company. Seeds were sterilized and inoculated with endophytes before planting in germination trays. Plants were then transferred to main pots for further growth and inoculation.

Drought Tolerance Test

Isolates were tested for tolerance to different levels of dryness using PEG concentrations. Water potentials were calculated to determine the level of dryness tolerance [27].

$$QS = (1018 \times 10^{-2}) C - (1018 \times 10^{-4}) C^2 + (2067 \times 10^{-4}) CT + (8039 \times 10^{-6}) C^2 T,$$

QS = Osmotic potential (bars), T = The Temperature (°C), C = The PEG concentration (grL⁻¹)

Enzyme Activities

Polyphenol oxidase (PPO) and phenylalanine ammonia-lyase (PAL) activities were measured in plant leaves [28].

Total Phenolic and Flavonoid Contents

Total phenolic and flavonoid contents [29] were determined using spectrophotometric methods [30].

2.8. Essential Oil Percentage

Identification of Polyphenols

HPLC analysis was conducted to identify phenolic fractions in thyme leaf extract [30].

Essential Oil Percentage

Essential oils were extracted from thyme plants and analyzed using GC-MS and GC [31].

Analysis of Essential Oil

Volatile components of essential oils were analyzed using GC-MS and GC.[32].

Statistical analysis

Data was analyzed using SAS version 9.4, and significant differences were determined using the LSD test. Parametric tests were used for data analysis. three replications' mean values plus standard deviation ($M \pm SD$) were displayed as the results. The analysis, conducted using XLSTAT software and the Shapiro-Wilk test, revealed a P-value ≥ 0.05 , indicating that our data adhere to a normal distribution. Consequently, parametric tests were employed for data analysis [33].

Principal Component Analysis (PCA)

Principal Component Analysis (PCA) was performed to visualize the relationships between the bacterial isolates and various attributes of *T. vulgaris* under control and drought stress conditions. Cluster analysis and The PCA was conducted using XLSTAT program, version 2020 (www.xlstat.com, Addinsoft SARL). The PCA-biplot was generated to display the distribution of samples and the loading of each attribute in the principal component space.

Heatmap and Correlation Analysis

HCA was conducted using SRPLOT (on line) [34].

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Correspondence and requests for materials should be addressed to D.S

Competing interests

The authors declare no competing interests.

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Author Contributions

Methodology, A.K; Software, A.K; Validation, A.K., D.S; Formal analysis, A.K.; Investigation, D.S, N.A. and A.B.; Resources, A.K., D.S; Writing-original draft, A.K; Writing-review & editing, A.K and D.S.; Supervision, D.S.; Project administration, A.K., DS; Data curation, A.K., DS; Conceptualization, A.K, D.S and N.A. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Figures

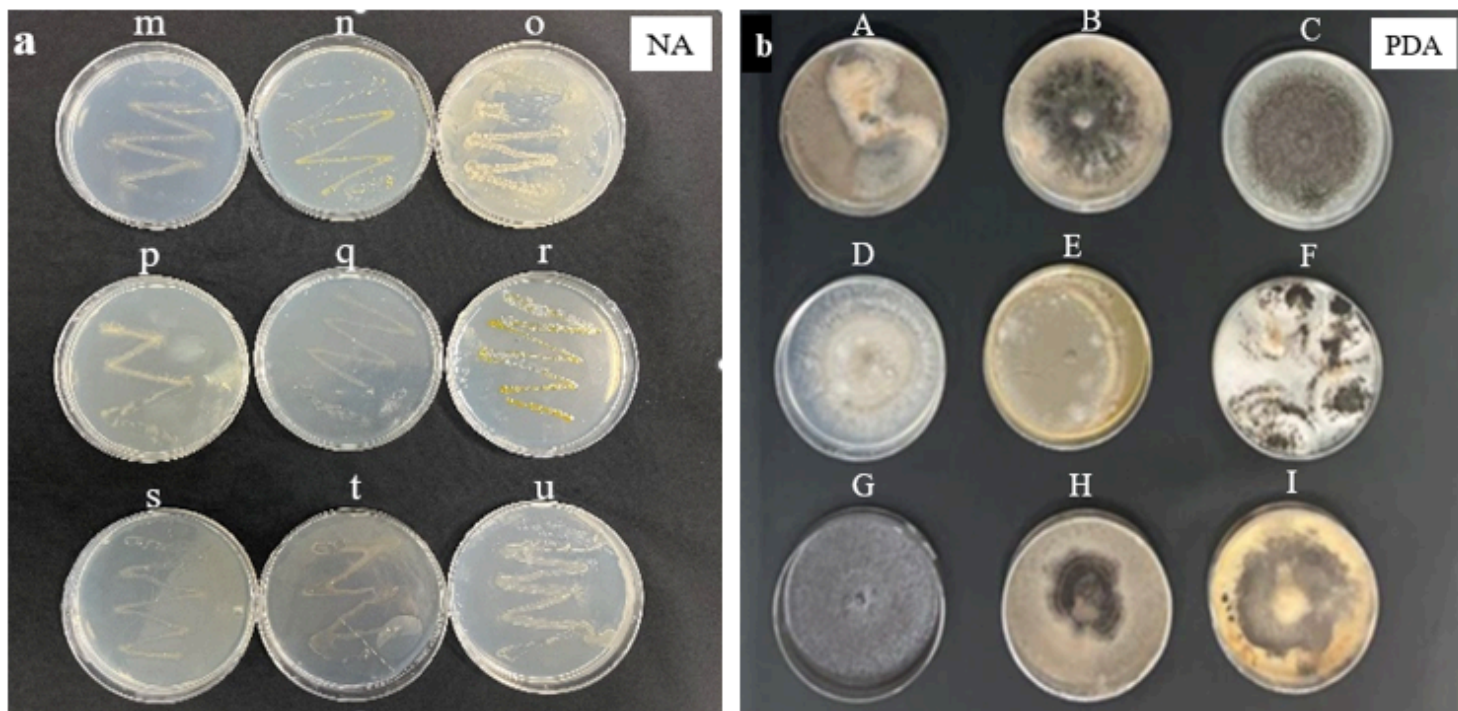


Figure 1

Assessment of drought tolerance for bacterial **(a)** and fungal **(b)** endophyte Isolates in controlled laboratory environments

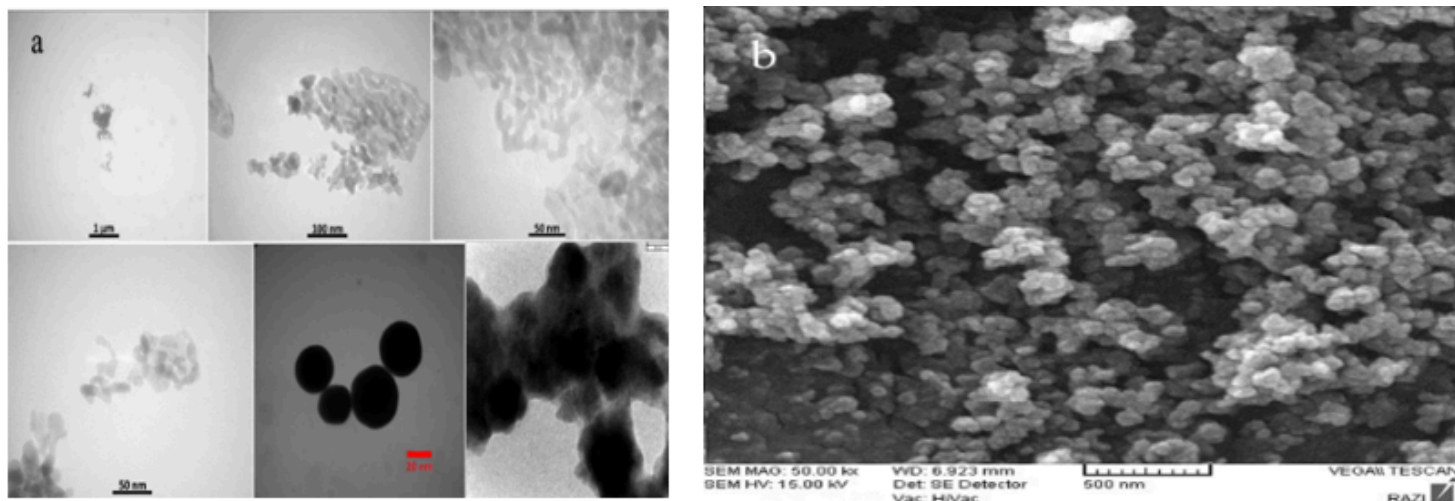


Figure 2

The TEM image of Fe_2O_3 NPs **(a)** The SEM image of Fe_2O_3 NPs **(b)**

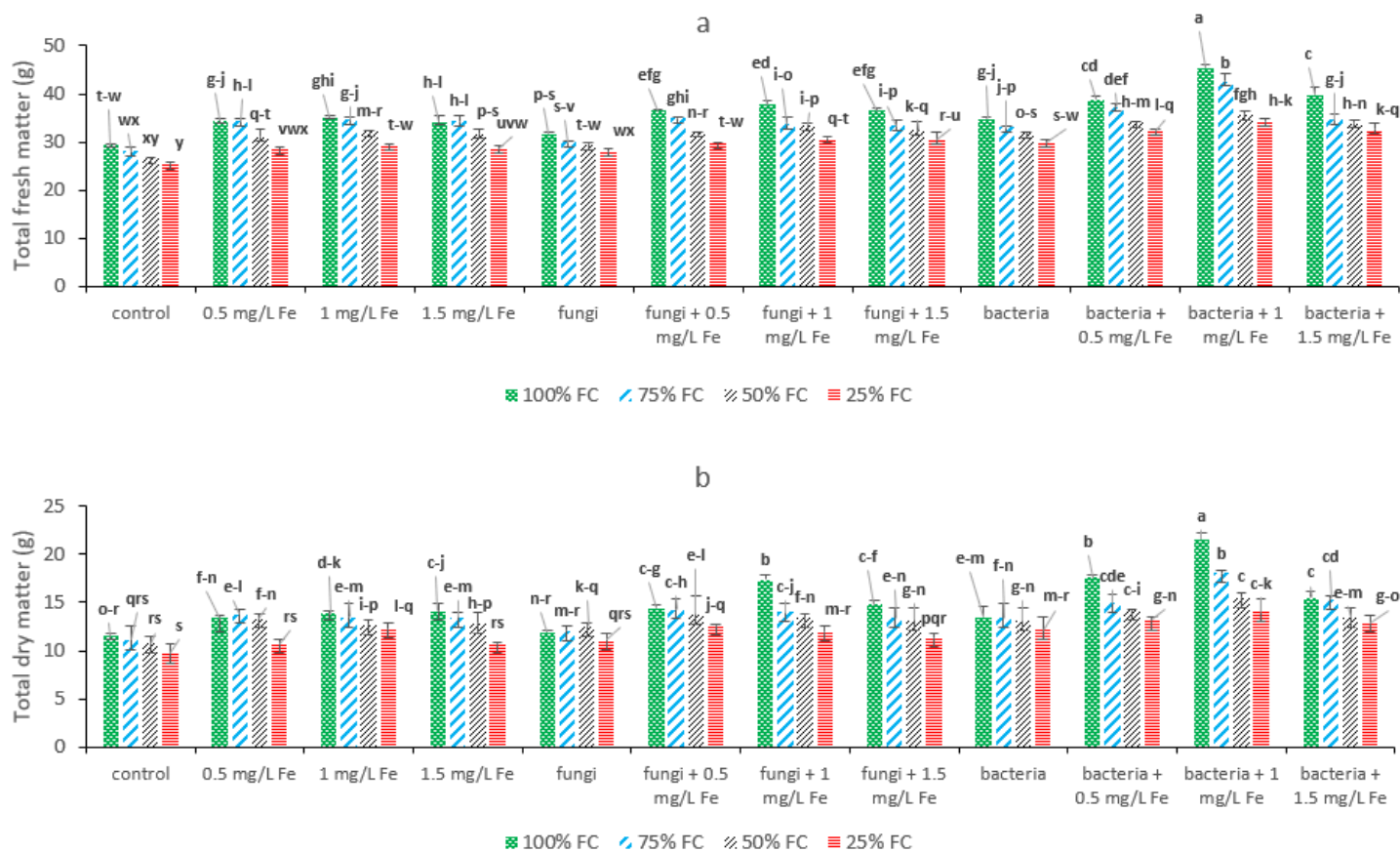


Figure 3

Effect of drought stress, bacterial and fungal endophytes and Fe_2O_3 nanoparticles on total fresh matter (a) and total dry matter (b) of *T. vulgaris* L.

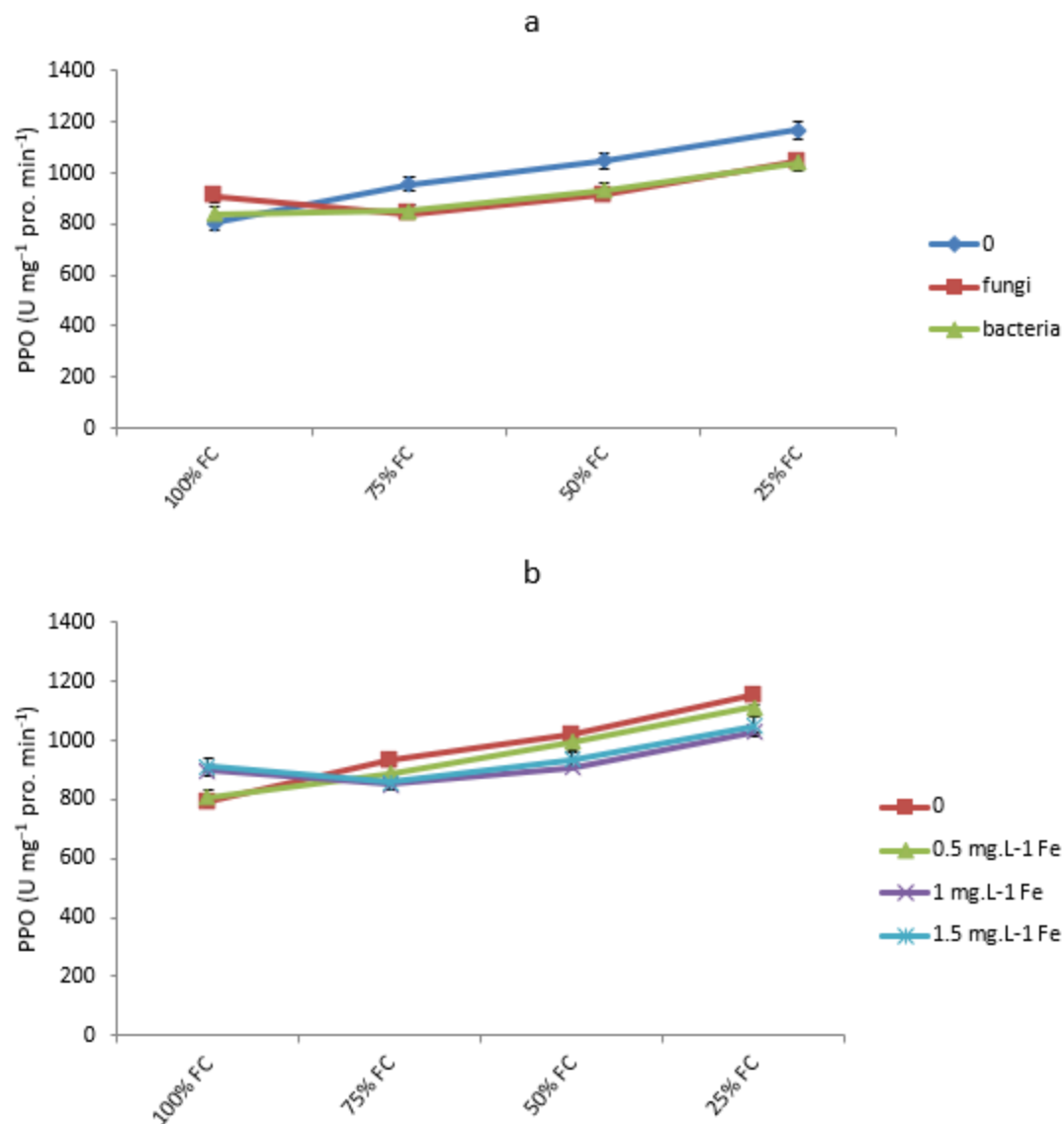


Figure 4

Effect of bacterial and fungal endophytes (**a**) and Fe₂O₃ nanoparticles (**b**) on PPO activity in the leaves of *T. vulgaris* L. under four different irrigation levels

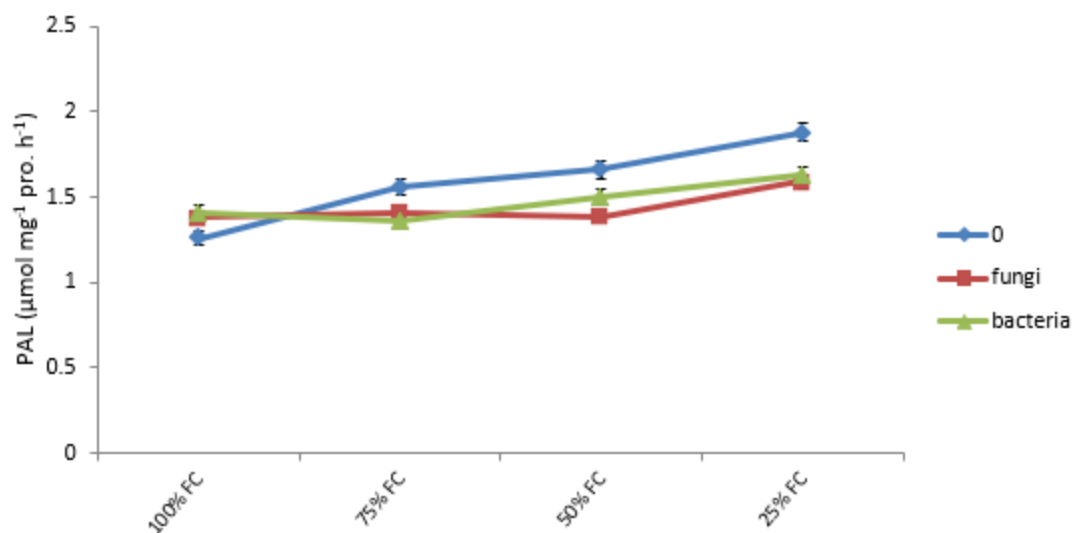


Figure 5

Effect of bacterial and fungal endophytes on PAL activity in the leaves of *T. vulgaris* L. under four different irrigation levels

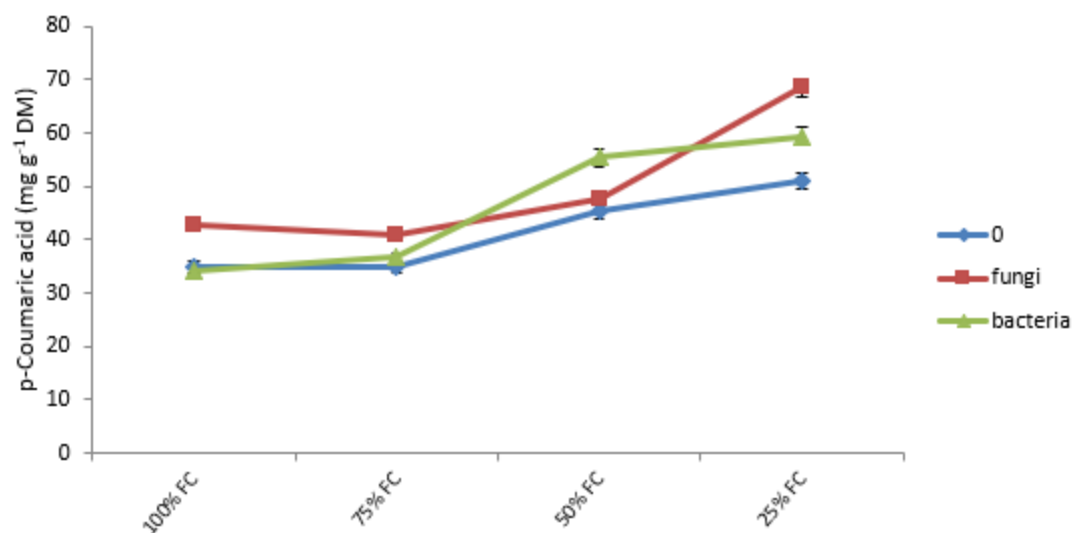


Figure 6

Effect of bacterial and fungal endophytes on p-Coumaric acid content in the leaves of *T. vulgaris* L. under four different irrigation levels

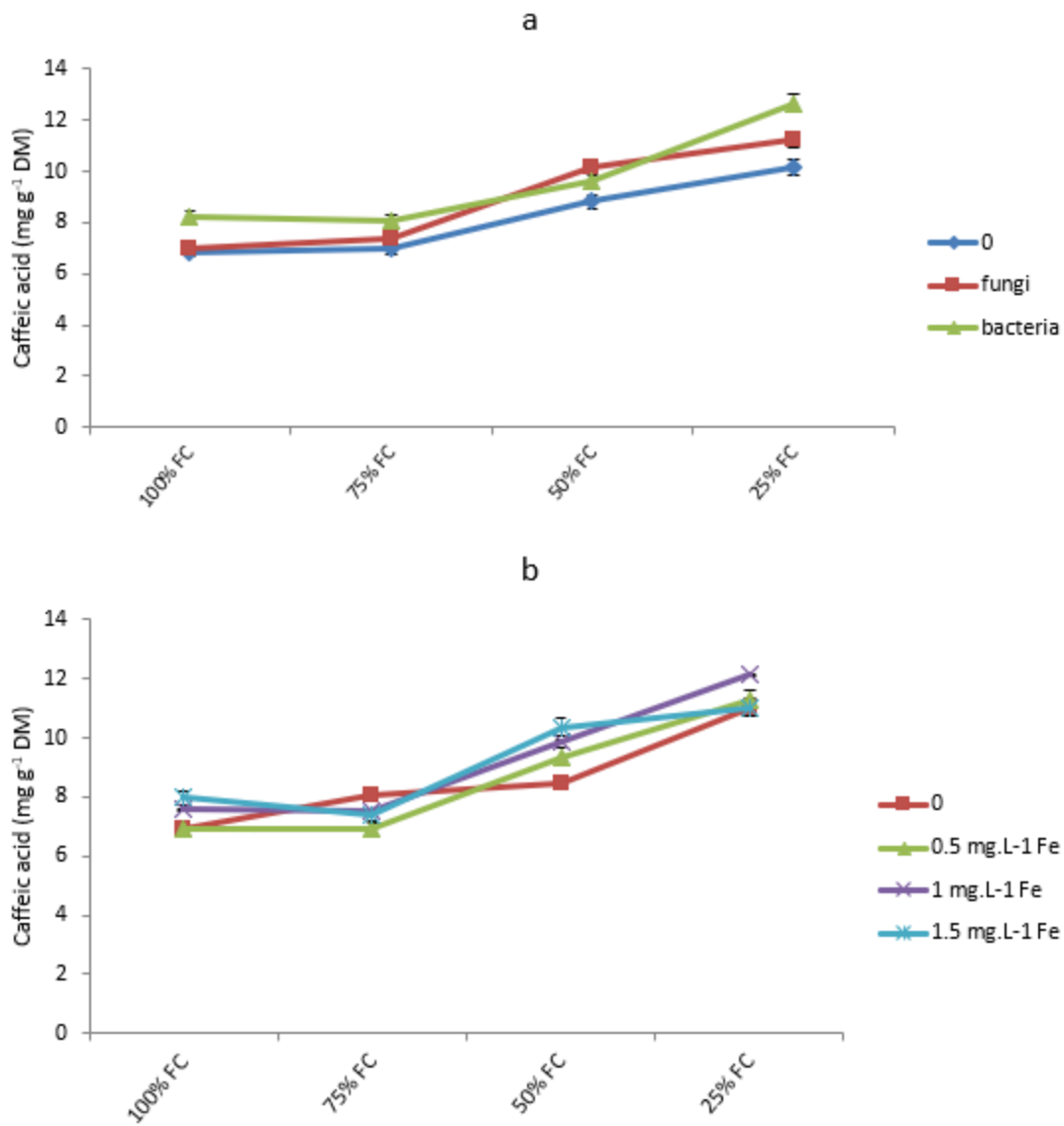


Figure 7

Effect of bacterial and fungal endophytes (**a**) and Fe₂O₃ nanoparticles (**b**) on caffeic acid content in the leaves of *T. vulgaris* L. under four different irrigation levels

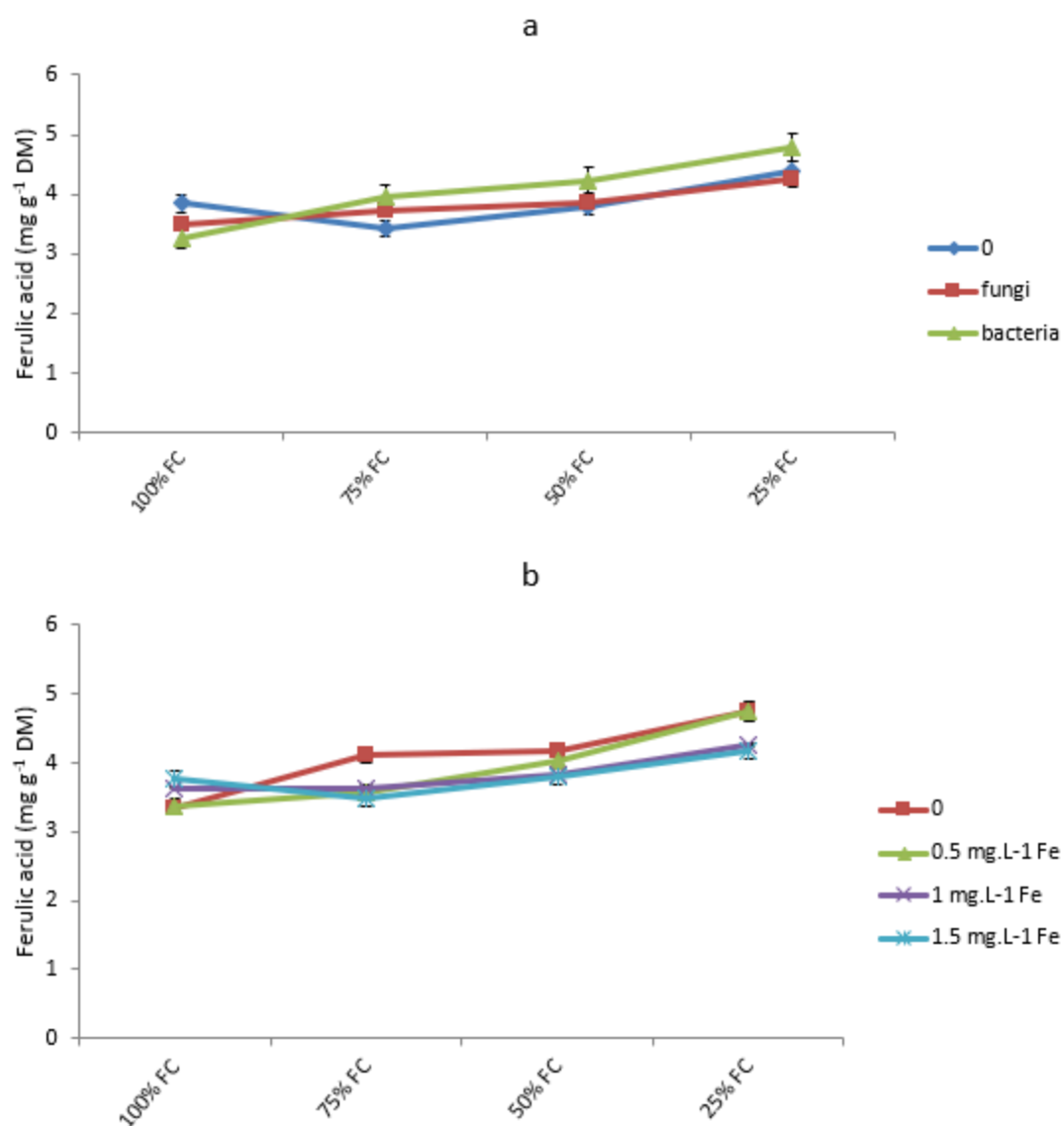


Figure 8

Effect of bacterial and fungal endophytes (**a**) and Fe₂O₃ nanoparticles (**b**) on ferulic acid content in the leaves of *T. vulgaris* L. under four different irrigation levels

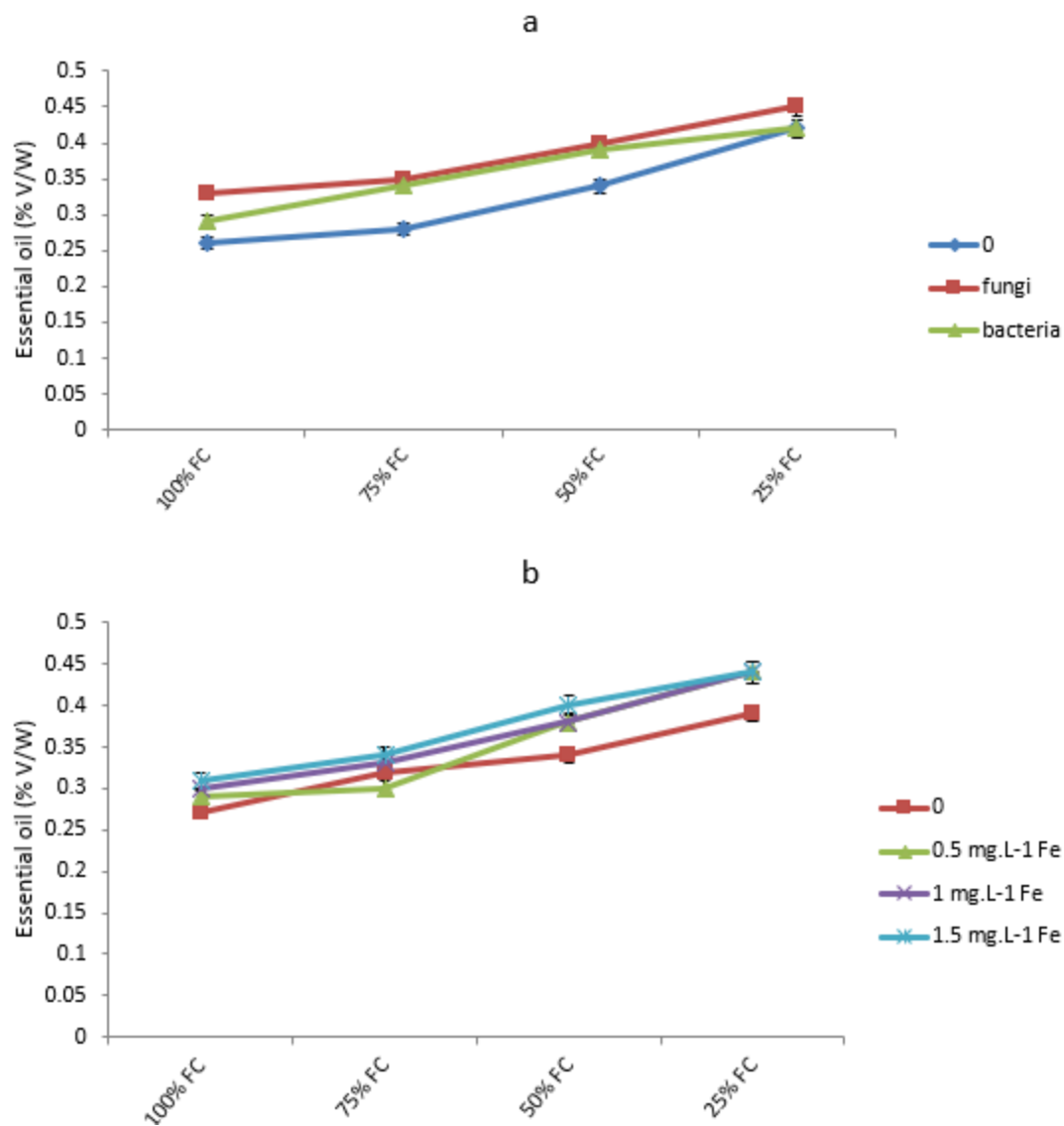


Figure 9

Effect of bacterial and fungal endophytes (**a**) and Fe_2O_3 nanoparticles (**b**) on essential oil percentage of *T. vulgaris* L. under four different irrigation levels

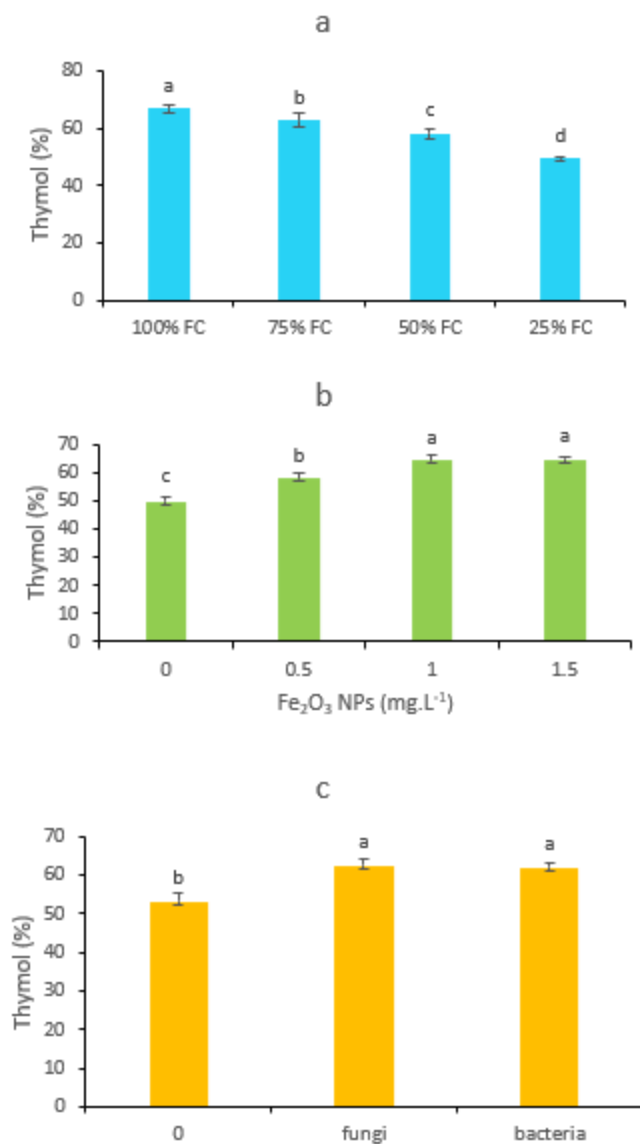


Figure 10

Effect of irrigation levels (**a**), Fe_2O_3 nanoparticles (**b**) and bacterial and fungal endophytes (**c**) on the percentage of thymol in essential oil of *T. vulgaris* L.

† Means followed by the same letter are not significantly different according to the least significant difference (LSD) test at $P \leq 0.05$.

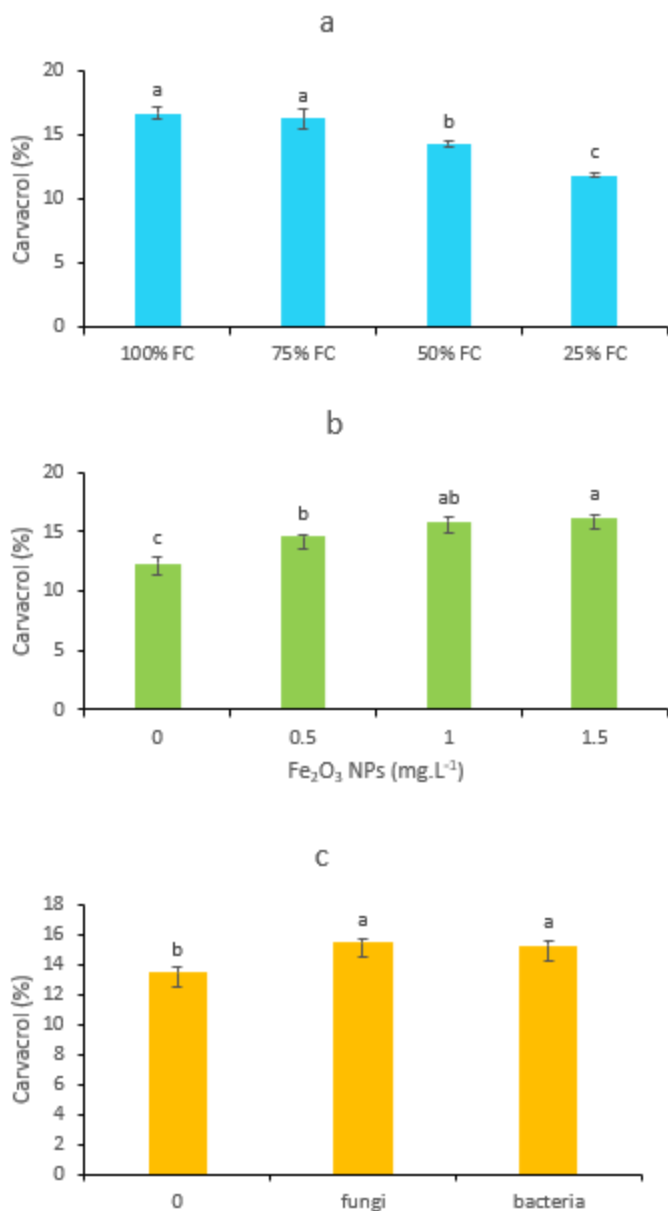


Figure 11

Effect of irrigation levels (**a**), Fe_2O_3 nanoparticles (**b**) and bacterial and fungal endophytes (**c**) on the percentage of carvacrol in essential oil of *T. vulgaris* L.

† Means followed by the same letter are not significantly different according to the least significant difference (LSD) test at $P \leq 0.05$.

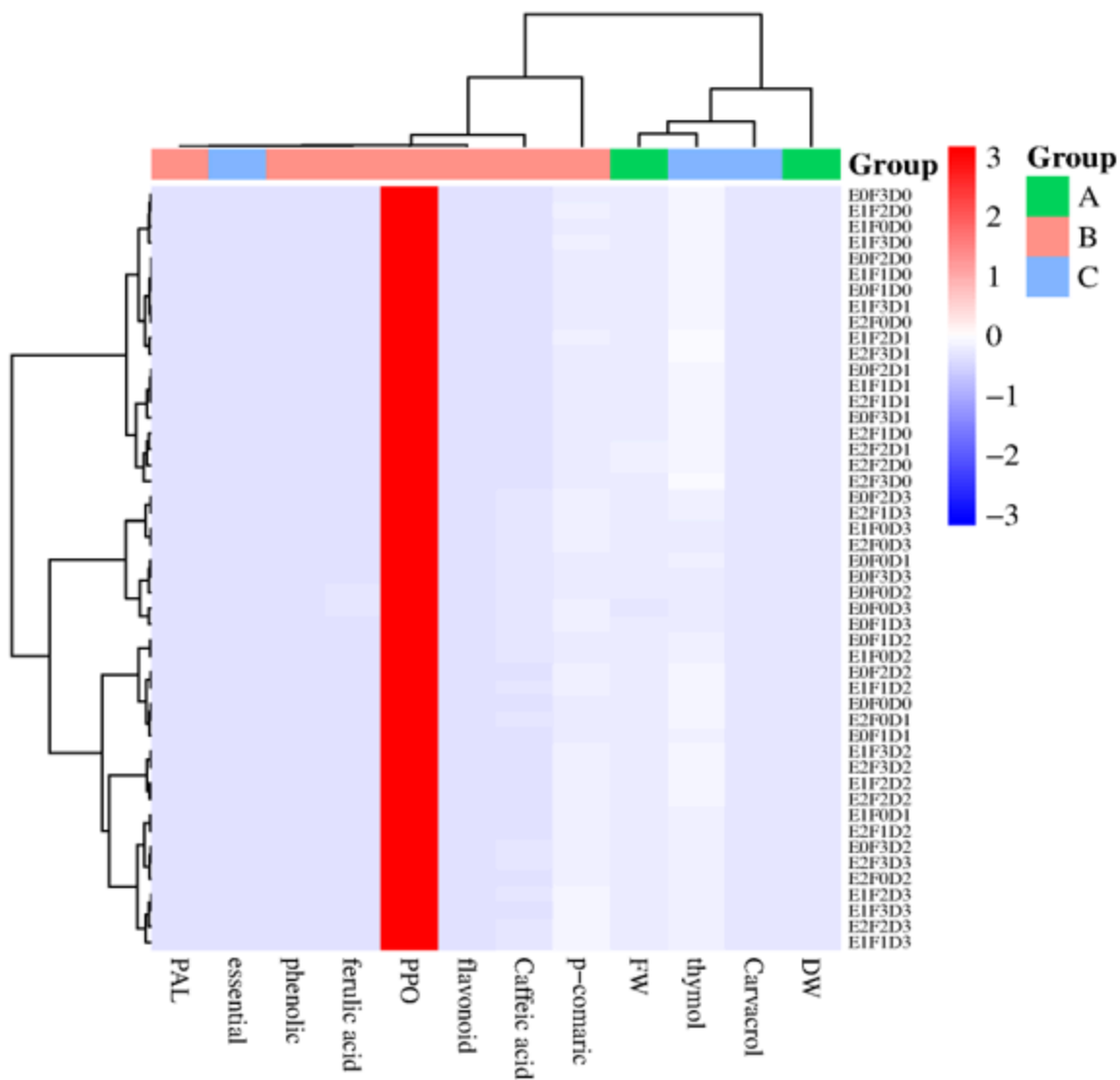


Figure 12

Using a heatmap of the Pearson correlation coefficients (r values) for the variable characteristics, hierarchical clustering analysis (HCA) was used to examine the correlations between the treatments, drought levels, and variable trait relationships in *T. vulgaris*. The r coefficient values (r = -3 to +3) are displayed on a colored scale, indicating positive (red) and negative (blue) correlations, respectively. *T. vulgaris* inoculated with various endophytic fungal (E1), endophytic bacteria (E2), and iron nanoparticles (F), under varying drought stress (D0-D3) conditions (100, 75, 50, and 25% FC).

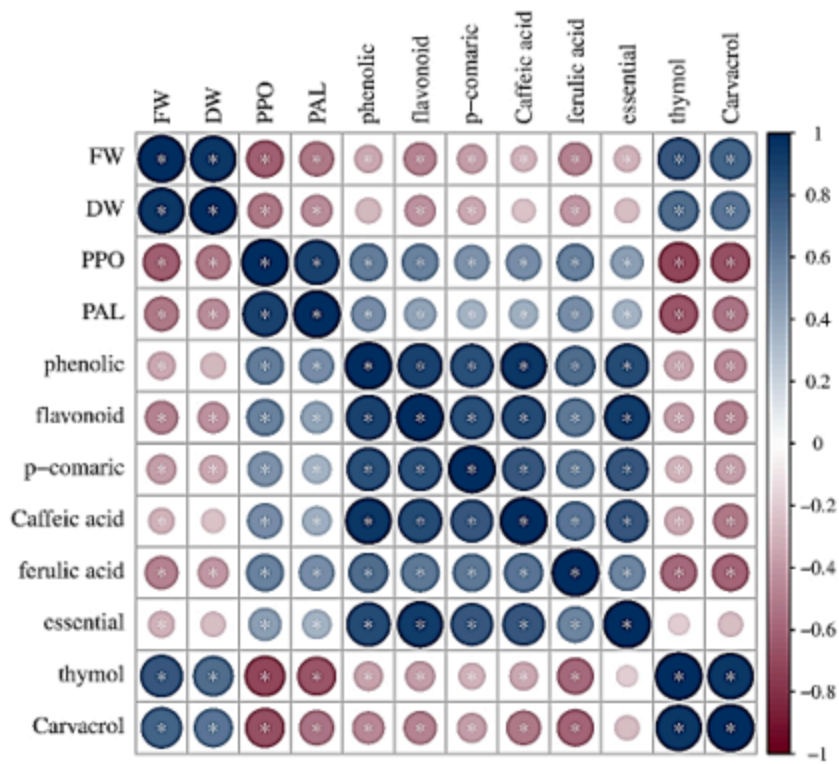


Figure 13

Pearson correlation between measured parameters of *T. vulgaris* under control and drought stress

