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The Effect of Mycorrhizal Fungi Glomus spp. on the Quality and Quantity of Medicinal and Defensive Compounds of two Medicinal Plants: Salvia mirzayanii and Salvia macrosiphon

Abstract

Today, agriculture is moving towards producing organic products. Mycorrhizal fungi play a key role in the production of such products. In this survey, the effects of inoculation with mycorrhizal Fungi on the qualitative and quantitative traits of two medicinal plants: Salvia mirzayanii and Salvia macrosiphon, have been investigated. Here, four separate pot experiments were carried out using a fully randomized design with 4 treatments of mycorrhizal fungi (Geosporum, Etunicatum, and the habitat soil of both species) and the absence of inoculation of mycorrhizal fungus as a control in three replications on the two plants. The analysis of gene expression of linalool showed that in the treatment of Salvia macrosiphon and Geosporum, there is a significant difference compared to the control group, and the p-value is less than 0.0001($p<0.0001$). The analysis of gene expression of 1.8-Cineole showed that in the treatment of Salvia macrosiphon and Geosporum, there is a significant difference compared to the control group, and the p-value is less than 0.0001($p<0.0001$). It became clear that the highest level of peroxidase enzyme(0 .68 µmol/min/mg protein) belonged to the plants sown in the bed containing Glomus Geosporum and the amount of catalase in Salvia macrosiphon(0.63 µmol/min/mg protein) was significantly higher than that in the Salvia mirzayanii(0.56 µmol/min/mg protein). The highest amount of the catalase (0.75 µmol/min/mg protein) belonged to the plants sown in the soil as the control group. In other treatments, the level of catalase is reduced significantly.

Keywords: *mycorrhizal fungi, Salvia macrosiphon, Salvia mirzayanii, gene expression*

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Introduction

From long ago, medicinal plants have had a special place in the traditional system of Iran, and using them as medicines to prevent and treat diseases has been emphasized by traditional medicine specialists. The growth and quality of medicinal plants are important to improve because they are used to treat diseases. Sowing medicinal plants have become more important in recent years because of the great potential in traditional and modern medicine (Bhale, 2013).

Since a few decades ago, using chemical inputs in agricultural lands has caused many problems for the environment, including the pollution of water sources, the decline in the quality of agricultural products as well as the reduction of soil fertility. In this sense, mycorrhizal fungi can be a helpful substitute for some chemical fertilizers, especially for phosphatic fertilizers in various ecosystems. Mycorrhizal fungi are a type of strong biological fertilizer which create physiological and morphological changes in the host in order to control and reduce the harmful effects (Mathew & Malathy, 2006). A symbiosis occurs between many of the vascular plants and mycorrhizal fungi in the soil. These fungi belong to three groups: basidiomycetes, ascomycetes, and zygomycetes(Kristek, 2005). Most of the plants in various habitats can form mycorrhizal systems. In general, 83% of dicots and 79% of monocots are of this ability(Zeng et al. 2013), but a few agricultural plants do not have this ability, many of which are the members of the cabbage family, including Brassica and Sienna, the beta spinach family, and the polygonum family genus Fagopyrum(Lekberg, 2015).

The herbal species of the Salvia mirzayanii belongs to the branch of flowering plants (*Magnoliophyte*), family *Lamiaceae*, subfamily *Stachyoideae*, genus Salvia and species *mirzayanii.* This plant is stable, bushy, thick-stemmed, in dull green color, covered with wool-like white fluffs, tuberous, and with folded fluffs, and 20-40 cm high(Soltanipour, 2019). The research carried out has told that the presence of some compounds in the essential oils of these plants, such as Thujene, cineol, and camphene, are antimicrobial, antioxidant, and anti-cancer properties (Izadi et al. 2020).

Salvia is a common medicinal plant that is used widely in food sources and pharmaceuticals. A greenhouse experiment was carried out using a completely randomized design with three replications in the Department of Biology, Faculty of Sciences, Urmia University, to investigate the effect of the arbuscular mycorrhiza fungi (AMF) on the growth and physiology of the salvia as a medicinal plant. The experimental treatments include three species of arbuscular mycorrhizal fungi (*Glomus versiform, G. etunicatum*, and *G. intraradices*) and plants in the control group. According to the results, mycorrhizal symbiosis increases growth and improves the physiological traits of salvia as a medicinal plant. (Habibi et al., 2014). A study was carried out to evaluate the growth and content of salvia essential oil inoculated with two species of arbuscular mycorrhizal fungi (AMF) (*Rhizophagus clarus* and

Claroideoglomus etunicatum) at different levels of phosphorus (P). The findings showed that the essential oil content of the plant increased with the inoculation of *R. Clarus* in the substrate with higher P. The conclusion was that inoculation with *R. clarus* while adding phosphorus at the time of sowing can increase the growth of salvia and the essential oil content of this medicinal plant.

A study was carried out entitled "evaluating the role of mycorrhizal species and phosphorus levels on the traits of the leaves and the production of peppermint essential oil in different aquatic conditions." The results showed that applying a mixture of mycorrhizal species while reducing the consumption of phosphorus chemical fertilizer to 57% of the recommended dose can improve the growth and yields of the essential oil as well as some physiological traits of the plant in both deficit and full irrigation conditions (Eslamifar et al. 2018). The research entitled " the effect of the rhizospheric bacteria stimulating the plant growth and arbuscular mycorrhizal fungi on the morphological traits concentration of essential elements of the medicinal peppermint (*piperitaL Mentha*)" was performed under greenhouse conditions. The obtained results showed that using these bacteria and arbuscular mycorrhizal fungi plays an effective role in improving the growth traits and nutrient concentration of peppermint. Mahmoudzadeh and colleagues (2015) carried out research entitled "the effect of mycorrhizal fungi on the growth and yield of bell pepper (*L.annum Capsicum*) under drought stress conditions." Their results showed that the symbiosis with mycorrhizal fungi under irrigation conditions of 75% of the water requirement of the plant is suitable for achieving good performance and saving water consumption in the production of this plant (Engili et al. 2018).

The results obtained from research entitled "the effect of the symbiotic relationship of five endomycorrhiza fungi Glomus spp. on induction of resistance in potato plants against Rhizoctonia solani" showed that Glomus species individually or in combination increased shoot plant length, fresh and dry weight, and fresh and dry root weight, tuber number and weight. Shahabi and colleagues (2014) carried out research entitled " improving sunflower growth by arbuscular mycorrhizal fungi under drought conditions." Their results showed that *Rhizophagus irregularis, Rhizophagus aggregatus,* and *Glomus hoi* had the best AMF for improving the growth and performance of the sunflower. In addition, *Glomus etunicatum* had less effect on the growth of this plant compared to the other glomus (Nacoon et al., 2021).

The results obtained from various experiments have shown that how to manage of fertilizer is a key factor in successfully sowing medicinal plants. In fact, increasing the production of valuable medicinal plants without using harmful chemical inputs is the most important point in their breeding. The fungal symbiosis receives carbohydrate materials mainly in the form of sucrose from the plant and provides nutrients (especially phosphorus) to the plant; in this way, food elements from the arbuscular membrane are actively provided to the plant through the membrane carriers, which act with the proton gradient. In this process, first, the carbohydrate materials in the phloem vessel of the plant are converted into glucose and fructose by the fungus, and then they are attracted by the carriers (Ismailpour et al., 2016). Temperature, humidity, and pH traits should be desirable for the photosynthesis of the plant so that the plant can meet the nutritional needs of mycorrhizae (carbohydrates) (Ozonido et al., 2015). Thus, this paper is to investigate the effect of mycorrhizal fungi glomus spp. On the quality and quantity of medicinal and defensive compounds of two medicinal plants: Salvia mirzayanii and Salvia macrosiphon.

Materials and Methods

1. Time and Location of the plan

The present research was conducted in a completely randomized factorial design with three replicates and eight treatments using a greenhouse pot form. For this purpose, Salvia macrosiphon and Salvia mirzayanii were collected from an area in Fars province, Dehkoyeh city, in the spring season of 2019 during sowing. Both plants were treated at the three - to -four leaves stage (4 weeks after growth). First, the plants sown in pots were classified into two groups. In the first group, 100 g mycorrhizal inoculum was used, and in the second group (control), no inoculum was used. Mycorrhizal fungi treatments used in this research were obtained from Propagule fungi extract. The treatments were done separately with the seedbed. The soil for this research was collected from a garden located in Larestan, and general soil tests were done on it. Table 1 shows the chemical and physical properties of the soil, respectively. The treatments were done using a 20 g soil substrate containing mycorrhizal fungi per kilo of soil used in the seedbed. Then the pots were arranged randomly with three repetitions.

Table 1: chemical and physical properties of the soil

Property	Unit	Size
Copper		1.09
Iron		3.55
Magnesium		8.21
Zinc	Mg/kg	0.59
Available phosphorus		21.6

Available potassium		227	
Total nitrogen		0.062	
Organic carbon		.0663	
Acidity		7.55	
Electrical conductivity	S/m	2.020	
Physical properties			
percentage of sand	**percentage of celite**	**percentage of clay**	**Soil texture**
69.5	22.5	8	Loamy sand

2. Measuring the vegetative traits of the plant

Vegetative traits of the Salvia mirzayanii and Salvia macrosiphon, including the number of leaves, number of flowers, wet and dry weight of aerial and ground parts, and the plant height, were measured in order for the effect of inoculation of mycorrhizal fungi to be evaluated.

3. Measuring the photochemical traits of the plant

Antioxidant phytochemical traits, including total phenol, total flavonoid, anthocyanin, and antioxidant capacity, were measured in order for the phytochemical traits of the plants to be evaluated.

The aerial and flowering branches of the Salvia mirzayanii were collected in May 2021 and dried in the open air away from direct sunlight for the essential oil to be extracted. The Agricultural and Natural Resources Research Center of Fars Province identified the species. Different parts and the whole plant were dried under the shade and at room temperature. For the percentage of essential oil to be determined, 30 g of different parts of the plant were ground, and essential oil was extracted by water distillation using the Clevenger device according to the British Pharmacopoeia and for 1.5 hours in the medicinal plant laboratory with three repetitions. Then, the essential oil yield of different parts was read based on volume to weight percentage. The essential oil was separated from the device column using a special syringe, and then it was dehydrated by anhydrous sodium sulfate and kept in the refrigerator until injection into the GC-MS machine.

In the next stage, a Raman spectrum was used for the analysis of the essential oil sample of the Salvia macrosiphon. Then, the compound of linalool one of the basic components of Salvia macrosiphon was purchased to test verification. The Raman spectrum was used for them and the Raman spectrum of the combination of linalool was compared with the Raman spectrum of essential oils.

The total phenolic content was measured using Folin–Ciocalteu reagent, and the results were expressed as mg of GA/g of extract. The total flavonoid content was measured using the aluminum chloride colorimetric method. In this method, 0.5 ml of extract solution was mixed with 1.5 ml of 95% ethanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate, and 2.8 ml of distilled water. The samples were kept at room temperature for 30 minutes. After this time, a spectrophotometer (Kerry model 100, Germany) was used to read the absorbance of the mixture at a wavelength of 415 nm. Quercetin was applied to draw a standard curve, and the results were expressed as mg of quercetin/ g of extract (Wolf et al., 2003). 800 mL of 0.1 mM DPPH solution were added to measure the antioxidant capacity of 200 mL of the extract from the samples, and after 30 minutes, the absorbance was read using a spectrophotometer (Kerry model 100, Germany) at a wavelength of 517 nm(Lee et al., 2003).

4. Evaluating the expression pattern of selected genes with the Real-time - PCR method

The expression patterns of linalool and 1,8 cineol were investigated using the Real Time-PCR method. First, 50 to 100 mg of the plant sample was dissolved in 1 cc of RiboexT solution, and the final solution was incubated for 5 minutes at room temperature. The solution was centrifuged at 12000 g for 10 min. Using a Refrigerated Microcentrifuge and the supernatant was transferred to a new microtube. 0.2 cc of chloroform was added to the solution per 1 cc of RiboexT. The solution was shaken for 15 seconds and kept at room temperature after 2 minutes. Then 0.5 cc of isopropyl alcohol per 1 cc of RiboexT was added to the solution, the solution was shaken 3 to 5 times to dissolve, and the samples were incubated for 10 minutes at room temperature. After 10 minutes, the supernatant was discarded, and 1 cc of 70% ethanol per 1 cc of RiboexT was added to wash the RNA sediment. The new solution was centrifuged at a speed of 7500 g for 5 min. And the supernatant was slowly separated. Then, the RNA sediment was exposed to dry air for 5 minutes.

In the next step, RNA has dissolved in DEPC water or 0.5% SDS solution, and incubating was done for 10-15 minutes at 56^0C. To analyze the sample as quickly as possible, the extracted RNA was stored at 4°C.

5. Making cDNA

Fermentas K1621 was used to make cDNA

6. Analyzing the PCR product of cDNA production control in the 2% agarose gel process

DNA sample of PCR product (5 microliter volume) was mixed with 1uL of 6x loading dye into the wells on 2% agarose gel. The electrophoresis process was performed for 45 minutes at 95 volts. After taking a photo of the gel in the gel dock machine, the correct step for the cDNA production was checked by observing the desired clear band in the appropriate place on the gel. The materials needed to perform Real-Time RT-PCR are presented in Table 2. The final volume of PCR was 15 mL.

Table 2: Materials and concentrations needed to perform Real-Time RT-PCR

Material	**Amount(mL)**
SYBR Green Master Mix	7.5
Primer (5 pmol) F	0.4
Primer (5 pmol) R	0.4
cDNA	5
Nuclease-free water	1.7

The relative expression of linalool and cineole was obtained in 16 samples using the $2^{-\Delta\Delta ct}$ formula, and it was analyzed using SPSS software by performing the Anova test.

7. Determining the number of defensive enzymes

The enzyme assay was done using the methods introduced by Banshi and Mohammadi (2015) and Amiri and colleagues (2015). For this purpose, half a gram of fresh leaves of two species of Salvia was crushed in liquid nitrogen, and the mixture was homogenized in 5 ml of K-phosphate buffer solution. At final, it was centrifuged at 4°C at 12,000 rpm for 30 min. The supernatant was used to measure catalase. Peroxidase enzyme activity was evaluated using the method introduced by Kar and Mishra(1976). Proline was evaluated based on the amount of Trans-cinnamic acid formed in one minute per mg of protein at a wavelength of 290 nm against a blank (control). For this purpose, catalase and proline were measured based on the given protocols.

SAS software, version 9, was used to analyze the data, and the results were compared with the results of Duncan's multiple range test. The data obtained from the gene expression were analyzed using the ct Δ model corrected with replication efficiency and TEST test.

Findings

1. The results of the effect of mycorrhizal fungi on growth traits of the plants

Table 3: Variance analysis of the effect of mycorrhizal fungi on the growth traits of Salvia mirzayanii and Salvia macrosiphon

Sources of variations	Degree of freedom	Height (cm)	Number of flowers	Number of leaves	Fresh weight of aerial parts(g)	The dry weight of aerial parts(g)	Fresh weight of the root(g)	The dry weight of the root(g)
Salvia macrosiphon								
Treatment	4	160.72	213.56	3436.39	1230.25	62.40	233.55	49.85
Error	16	52.89	19.72	134.56	25.53	3.56	8.58	1.20
Coefficient of variation	-	7.74	21.86	15.98	11.68	20.09	17.53	16.12
Salvia mirzayanii								
Treatment	4	371.56	12.06	190.41	1159.97	122.95	2477.18	316.83
Error	16	17.02	1.2	15.37	57.76	3.87	58.96	13.03
Coefficient of variation	-	8.78	19.8	18.34	18.69	18.19	18.33	22.43

Data from variance analysis showed that the effect of mycorrhizal fungi on the height(p= 0.05), the number of flowers, number of leaves, fresh weight of the root, dry weight of root, fresh weight of the aerial parts, and dry weight of the aerial parts of the Salvia macrosiphon was significant. Moreover, the effect of mycorrhizal fungi on the growth traits of Salvia mirzayanii was significant. Height, number of leaves, fresh weight of aerial parts, dry weight of aerial parts, fresh weight of roots, and dry weight of roots were affected by the

mycorrhizal fungus(p= 0.1) and the number of flowers(p=0.05) (Table 3).

2. **The results of the effective compounds of Salvia macrosiphon and Salvia mirzayanii**

Table 4: Variance analysis of the effect of mycorrhizal fungi on the phytochemical traits of leaves and stems of Salvia mirzayanii and Salvia macrosiphon

Sources of variations	Degree of freedom	Total phenol	Total flavonoids	Anthocyanin	Antioxidant capacity
Salvia macrosiphon					
Treatment	4	191.28	0.00072	0.315	92.38
Error	16	2.81	0.00008	0.027	3.75
Coefficient of variation	-	2.19	7.84	6.48	2.93
Salvia mirzayanii					
Treatment	4	389.77	0.002	0.167	8.60
Error	16	28.02	0.00009	0.01	0.85
Coefficient of variation	-	8.68	12.88	19.91	1.36

The effect of mycorrhizal fungi on the phytochemical traits of the leaves and stems of the Salvia macrosiphon was measured according to the results of variance analysis on all phytochemical traits, and it was found to be significant for the concentration of total phenol, total flavonoid, anthocyanin and antioxidant capacity(P = 0.01). Data from variance analysis showed that the effect of mycorrhizal fungi on the phytochemical traits of the leaves and stems of the Salvia mirzayanii was significant. This effect on total phenol content, anthocyanin content, antioxidant capacity (p=0.01), and total flavonoid (p=0.05) was significant.

Table 5: Variance analysis of the effect of mycorrhizal fungi on the phytochemical traits of flowers of Salvia mirzayanii and Salvia macrosiphon

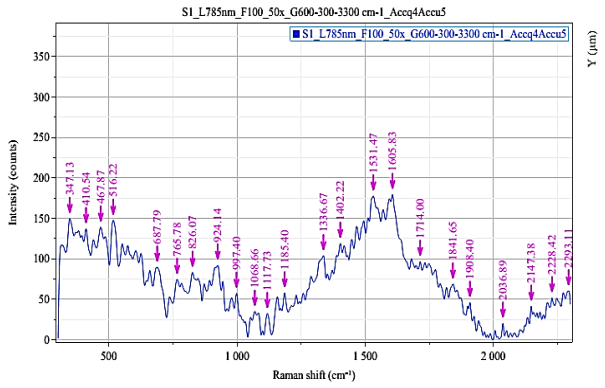
Sources of variations	Degree of freedom	Total phenol	Total flavonoids	Anthocyanin	Antioxidant capacity
Salvia macrosiphon					
Treatment	4	1286.49	0.00084	0.0048	180.40
Error	16	21.41	0.00039	0.014	25.28
Coefficient of variation	-	5.32	6.71	13.77	7.63
Salvia mirzayanii					
Treatment	4	20.41	0.000018	0.05	5.60
Error	16	2.34	0.000005	0.014	1.14
Coefficient of variation	-	7.18	22.18	14.7	1.58

Data from variance analysis showed that the effect of mycorrhizal fungi on the concentration of the total phenol and flavonoid of the Salvia macrosiphon(p=0.01) and the amount of the anthocyanin and antioxidant capacity (p=0.05) was significant. In addition, data from variance analysis showed the effect of mycorrhizal fungi on the concentration of the total phenol and flavonoid of the Salvia mirzayanii (p=0.01) and the amount of the anthocyanin and antioxidant capacity (p=0.05) was significant.

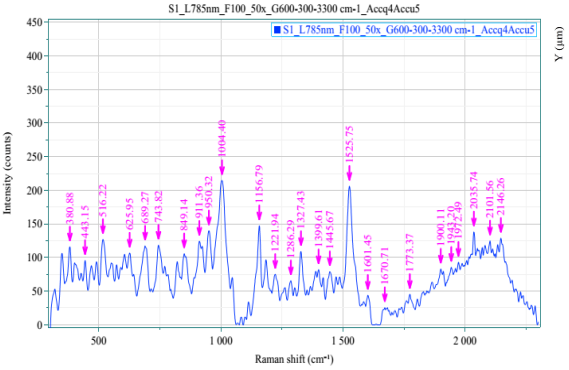
3. The results of the Raman spectrum

The results from FT-Raman spectra of standard compounds and essential oils are shown in Figure 1. There are some differences between the standard spectrum and the spectrum of essential oils with dominant components, which belong to different classes of compounds. Each terpene in the

Raman spectrum is a trait of the molecule structure. As a result, FT-Raman spectra of the essential oils, which were dominated by one component, showed similar traits. In graph 1(a), spectra of the essential oils, which were dominated by one component, showed similar traits. In graph (1a), the most important active ingredient was linalool in the range of 1643-1382 as the strongest peak in the range of 1605 and 1531, which indicates the presence of linalool as an active ingredient in Salvia macrosiphon.



a



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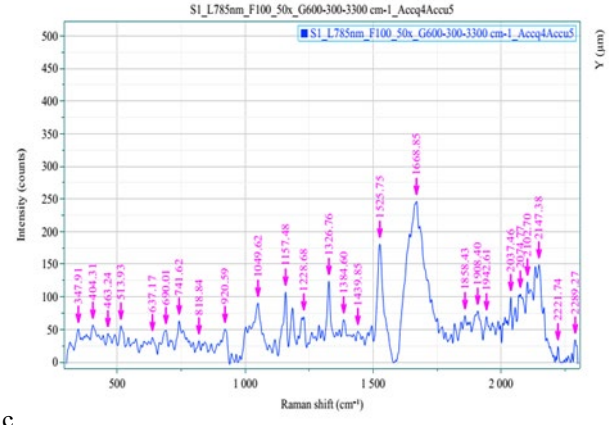

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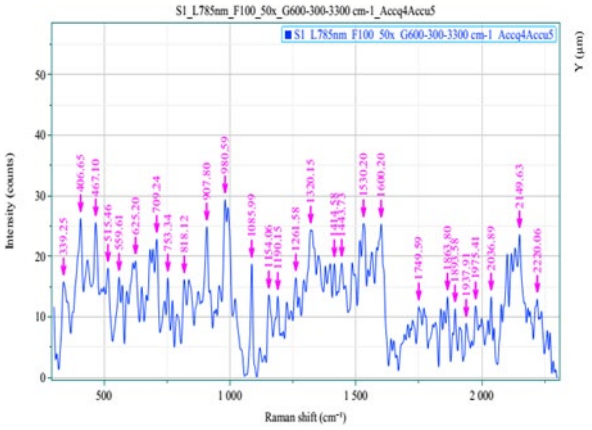


d

Graph 1: a) The results obtained from the infrared spectrum of Salvia macrosiphon and Geosporum, b) The results obtained from the infrared spectrum of Salvia macrosiphon and soil (control), c) The results obtained from the infrared spectrum of Salvia macrosiphon and soil of habitat and d) The results obtained from the infrared spectrum of Salvia macrosiphon and Etunicatum

4. Comparing the main percentage of the main compounds of essential oils of Salvia mirzayanii in the different seedbed

According to the results obtained from the variance analysis, the percentage of the essential compounds of the Salvia mirzayanii was determined. Using mycorrhizal fungi had a significant effect on the Bicyclogermacrene, alpha-terpinyl-acetate, Linalyl acetate, 1,8-cineole, alpha-Gurjunene and delta- Cadinene(p=0.01) and on the beta-pinene and alpha-pinene(p=0.05). It had no significant effect on the myrcene compounds(Table 6).

Table 6: Variance analysis of the percentages of the main compounds of essential oils of Salvia mirzayanii

Sources of variations		Average of squares				

	Degree of freedom	Bicyclogermacrene	α_Terpinyl_acetate	Linalyl_acetate	1,8- Cineole	Sabinene
mycorrhizal fungi	3	13.13	33.79	642.50	742.79	10.72
Error	8	0.75	1.00	0.98	1.01	0.75
Coefficient of variation	-	15.42	9.61	3.67	4.39	13.84
Sources of variations	Degree of freedom	Average of squares				
		β_Pinene	α_Pinene	Myrcene	α_Gurjunene	δ_Cadinene
mycorrhizal fungi	3	5.17	2.45	1.09	9.05	43.38
Error	8	0.75	0.70	1.00	0.57	0.25
Coefficient of variation	-	4.07	17.16	13.33	16.46	14.36

According to the results obtained from this experiment, the highest amount of Bicyclogermacrene belonged to the plants sown in the soil of the habitat and the control soil, and they had no significant difference from each other. The lowest amount of Bicyclogermacrene belonged to the plants sown in the bed of Glomus Geosporum (Table 7).

Table 7: comparing the average percentage of the essential compounds of the Salvia mirzayanii in the bed of Glomus Geosporum

Evaluated traits/ mycorrhizal fungi	the average percentage of the essential compounds of the Salvia mirzayanii			
	Control	Soil of habitat	Glomus geosporum	Glomus etunicatum
Bicyclogermacrene	4.23 ab	5.82 a	0.96 c	2.61 b
α_Terpinyl_acetate	10.99 b	10.47 b	5.99 c	14.14 a
Linalyl_acetate	26.34 c	43.84 a	8.34 d	30.23 b
1,8- Cineole	37.59 a	19.34 c	7.68 d	26.45 b
Sabinene	2.69 b	2.85 b	0.05 c	4.64 a
β_Pinene	2.77 a	2.23 a	0.31 b	3.33 a
α_Pinene	1.99 a	1.54 ab	0.24 b	2.29 a
Myrcene	1.92 a	2.02 a	2.08 a	3.21 a
α _Gurjunene	1.38 bc	3.00 ab	0.61 c	4.51 a
δ_Cadinene	0.11 b	0.18 b	7.69 a	0.23 b

Averages with the same letters in each row show no significant difference at the 5% probability level
of Duncan'stest

5. The results obtained from investigating defensive enzymes

According to the results obtained from the analysis of variation of the data, it was highlighted that the effect of different species of mycorrhizal fungi on catalase enzyme was significant (p=0.01). In addition, different species of Salvia showed a significant difference in the amount of catalase enzyme (p= 0.01). The mutual effect of mycorrhizal fungus × of Salvia macrosiphon on the amount of catalase enzyme was significant (p=0.01) (Table 8).

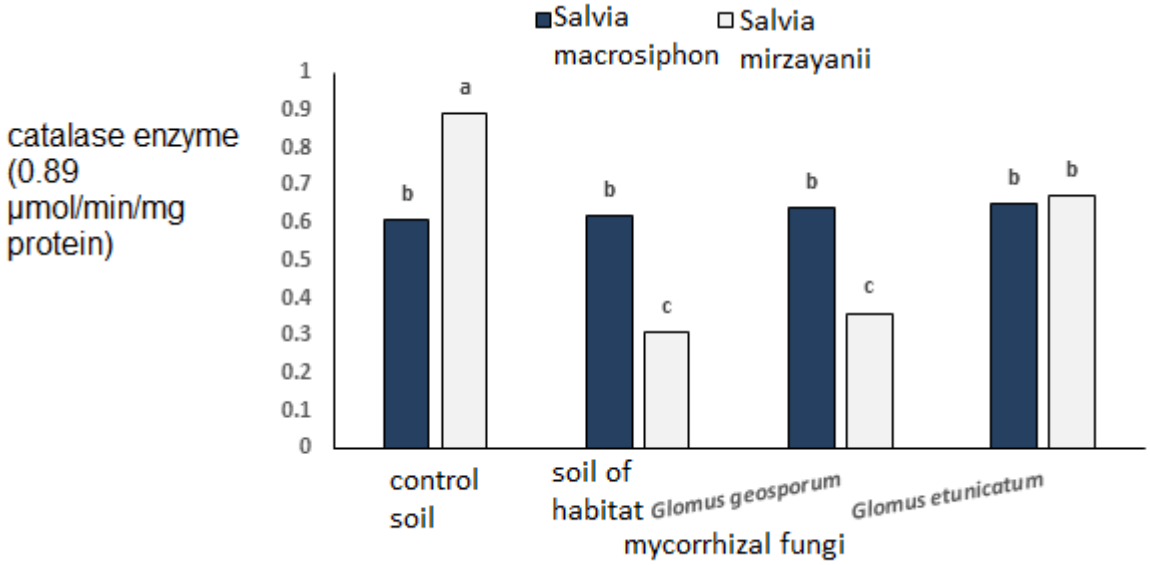
Table 8: Variance analysis of the catalase enzyme in Salvia macrosiphon

Sources of variations	Degree of freedom	Catalase enzyme		
		Mean of squares	F Statistic	Pr>F
mycorrhizal fungi(A)	3	0.10	49.40	0.0001

Salvia macrosiphon spp.(B)	1	0.03	15.85	0.001
Mutual effect AxB	3	0.11	54.70	0.0001
Error	16	0.002	-	-
Coefficient of variation	7.76			

From the results of the present research, it was found that the highest amount of catalase enzyme (0.75 micromol/min/mg protein) belonged to the plants sown in the control soil.

The results from comparing the mutual effect of mycorrhizal fungus x of Salvia macrosiphon spp. Highlighted that the highest amount of catalase enzyme (0.89 µmol/min/mg protein) belonged to Salvia mirzayanii sown in the control soil. In other treatments, the amount of catalase enzyme was significantly lower than that treatment. The lowest amount of catalase enzyme belonged to the Salvia mirzayanii sown in the soil of the habitat or the bed containing Glomus geosporum and no significant difference was observed among them (Graph 2)



Graph 2: Mutual effect of the mycorrhizal fungi, Salvia macrosiphon, and Salvia mirzayanii on the amount of the catalase enzyme

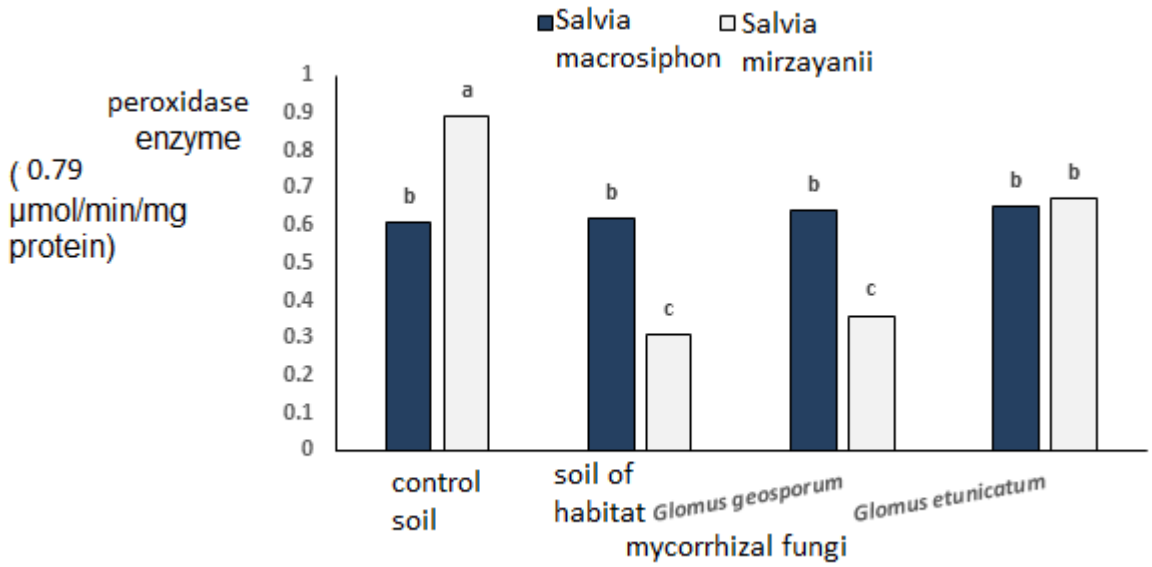
The effects of different species of mycorrhizal fungi on the peroxidase enzyme were found to be significant (p=0.05). In addition, different types of salvia had a significant difference in terms of peroxidase enzyme (p=0.05). The mutual effect of mycorrhizal fungus × of Salvia species on the amount of peroxidase enzyme was significant (p=0.05) (Table 9).

Table 9: Variance analysis of the peroxidase enzyme in Salvia macrosiphon

Sources of variations	Degree of freedom	Catalase enzyme		
		Mean of squares	F Statistic	Pr>F
mycorrhizal fungi(A)	3	0.05	7.14	0.05
Salvia macrosiphon spp.(B)	1	0.08	11.42	0.03
Mutual effect AxB	3	0.08	11.41	0.03
Error	16	0.007	-	-
Coefficient of variation	15.78			

The results from comparing the mutual effect of mycorrhizal fungus x of Salvia macrosiphon spp highlighted that the highest amount of peroxidase enzyme (0.79 µmol/min/mg protein) belonged to Salvia mirzayanii sown in the bed containing Glomus geosporum. Moreover, sowing salvia macrosiphon in control soil, habitat soil, or beds containing mycorrhizal fungus Glomus geosporum or containing mycorrhizal fungus Glomus etunicatum showed no difference

significantly from that treatment, and all were in the same statistical group. In other treatments, the amount of peroxidase enzyme was lower than in these treatments (Graph 3).



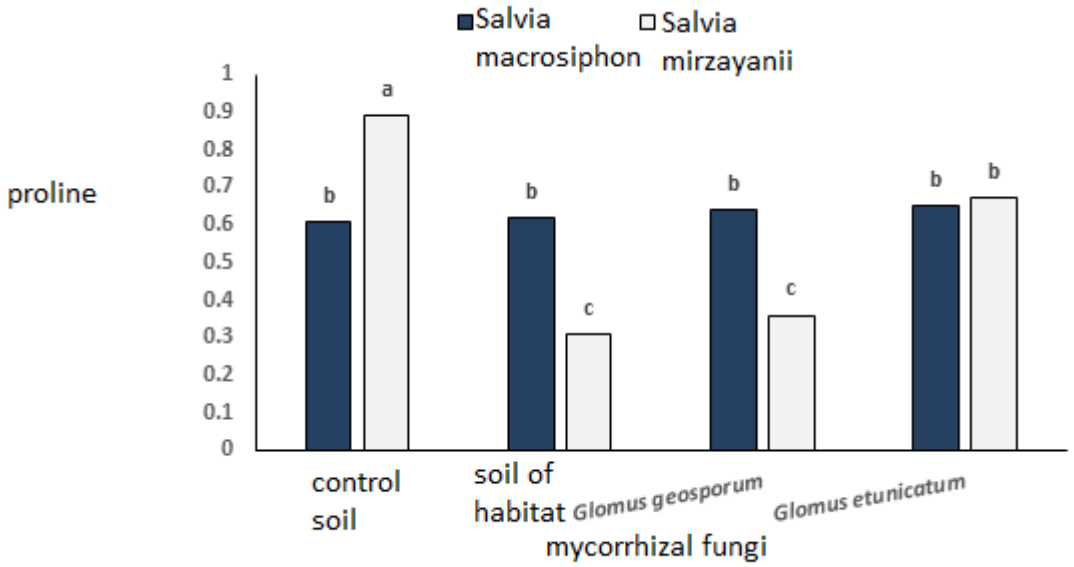
Graph 3: Mutual effect of the mycorrhizal fungi, Salvia macrosiphon, and Salvia mirzayanii on the amount of the peroxidase enzyme

According to the variance analysis, it was highlighted that the effect of different species of mycorrhizal fungi on the amount of proline was significant (p=0.05), and different types of salvia showed a significant difference in terms of proline content (p=0.05). The mutual effect of mycorrhizal fungus × of the salvia macrosiphon on the amount of proline was significant (p=0.01) (Table 10)

Table 10: Variance analysis of proline in Salvia macrosiphon

Sources of variations	Degree of freedom	Catalase enzyme		
		Mean of squares	F Statistic	Pr>F
mycorrhizal fungi(A)	3	0.34	68.31	0.0001
Salvia macrosiphon spp.(B)	1	0.02	4.80	0.04
Mutual effect AxB	3	0.05	10.85	0.0004
Error	16	0.005	-	-
Coefficient of variation	15.78			

The results from comparing the mutual effect of mycorrhizal fungus x of Salvia macrosiphon spp highlighted that the highest amount of proline(0.79 µmol/min/mg protein) belonged to Salvia mirzayanii sown in the control soil or the bed containing Glomus geosporum and all were in the same statistical group. In lowest amount(0.17 µmol/min/mg protein) belonged to Salvia mirzayanii sown in the soil of the habitat(Graph 4)



Graph 4: Mutual effect of the mycorrhizal fungi and Salvia macrosiphon on the amount of the peroxidase enzyme

6. evaluating the results from the Real-time PCR test

In this section, the results obtained from the Real-Time PCR test are reviewed. Linalool gene for Salvia macrosiphon, 1,8-cineole for Salvia mirzayanii, and s18 gene as a housekeeping gene were investigated in 4 samples of Salvia macrosiphon and 4 samples of Salvia mirzayanii. The principle of experiment repetition is of great importance in biological experiments. Thus, the experiments were done in 3 repetitions in a real-time reaction. In this analysis, groups included control, soil, G.etanicatum, and G.geosporum. ΔΔCt was used to compare gene expression between the treatment group and control group.

Table 11: Two by two comparison of the groups

Tukey.test	mean.diff.	Summary	p.value	fold.change	Description
soil vs. control	0.680	ns	0.23858	1.602	upregulated:1.6021fold
G.etanicatum vs. control	2.445	***	0.00033	5.445	upregulated:5.4453fold
G.geosporum vs. control	3.565	****	0.00002	11.835	upregulated:11.8351fold
G.etanicatum vs. soil	1.765	**	0.00289	3.399	upregulated:3.3987fold
G.geosporum vs. soil	2.885	***	0.00010	7.387	upregulated:7.3871fold
G.geosporum vs. G.etanicatum	1.120	*	0.03674	2.173	upregulated:2.1735fold

In Table 11, all the groups were compared with each other two by two. The amount of the fold change is significant if the level of p-value is less than or equal to 0.05.

Table 12: Statistical analysis and the interpretation of the effect of the groups on the expression of Linalool

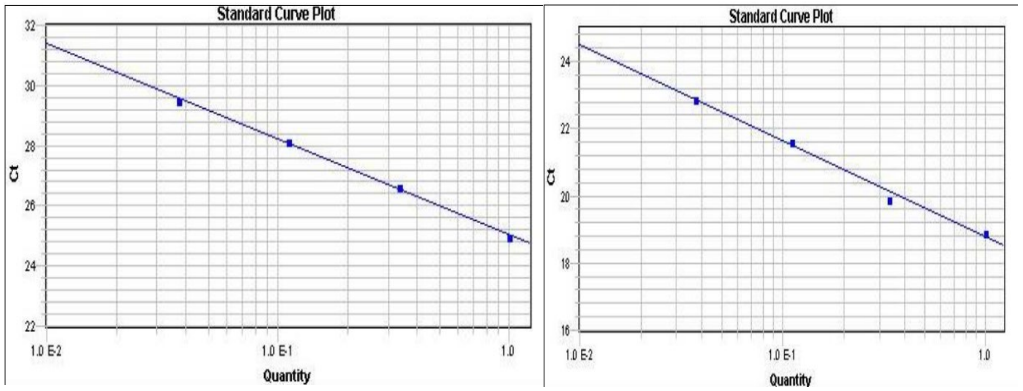
Tukey.test	mean.diff.	Summary	p.value	fold.change	Description
soil vs. control	2.315	***	0.00021	4.976	upregulated:4.976fold
G.etanicatum vs. control	2.250	***	0.00026	4.757	upregulated:4.7568fold
G.geosporum vs. control	3.495	****	0.00001	11.275	upregulated:11.2746fold

G.etanicatum vs. soil	-0.065	ns	0.99579	0.956	downregulated:-1.0461fold
G.geosporum vs. soil	1.180	*	0.01575	2.266	upregulated:2.2658fold
G.geosporum vs. G.etanicatum	1.245	*	0.01173	2.370	upregulated:2.3702fold

In Table 12, all the groups were compared with each other two by two. The amount of the fold change is significant if the level of p-value is less than or equal to 0.05.

8. **The results from standard curves of Cineole, Linalool, and 1,8-cineole**

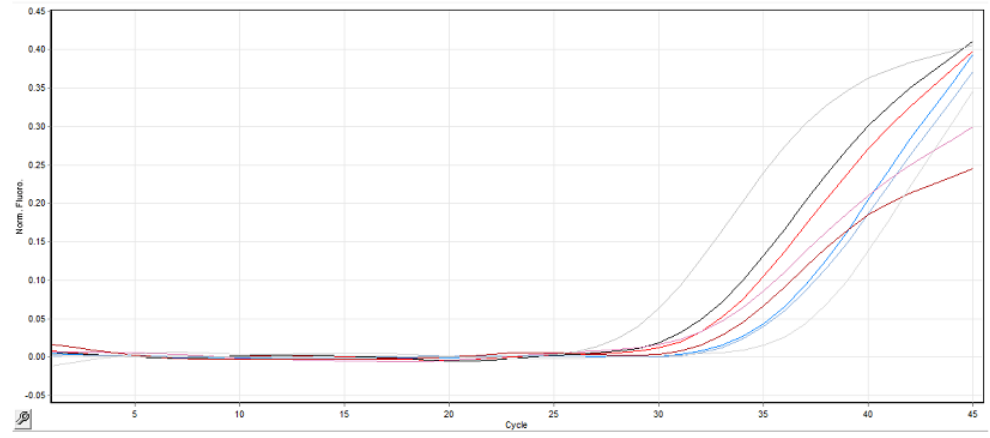
When the standard curves were dawned, the yield of the Linalool and Cineole primer pair was found to be 105.3%. Thus, according to graph (8), the yield of primers is in an acceptable range.



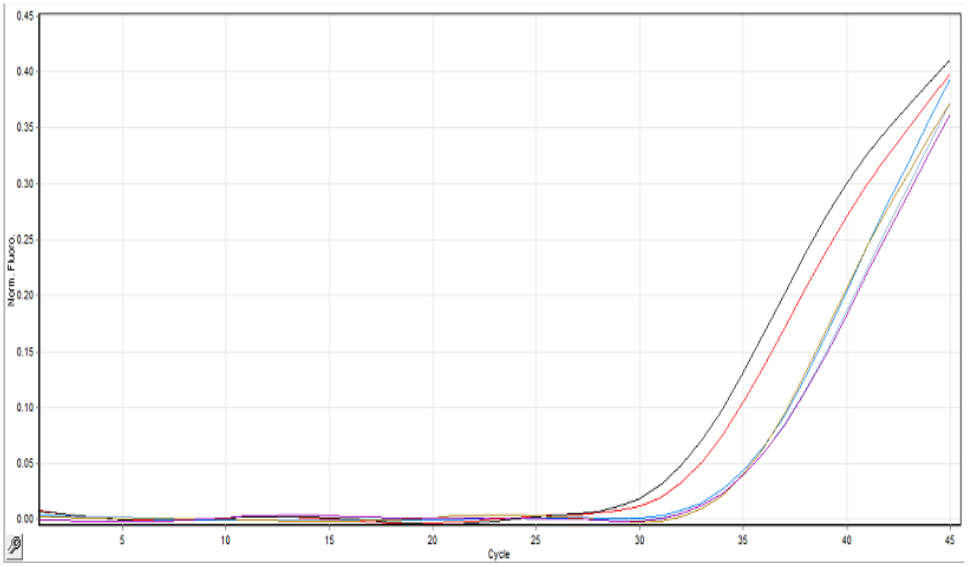
a b

Graph 5: The standard curves of Cineole, Linaloole, and 1,8-cineole

Considering diagram 6, the proliferation curve of proline and Cin genes and the proliferation of curve of the 18S gene have been drawn. In both figures, the Threshold line and the graph related to the NTC^{-1} were shown.



a



b

Diagram 6- a) Cin gene amplification curve in Real-Time PCR, b) 18S gene amplification curve in Real-Time PCR

Discussion

As the results show, the use of mycorrhizal fungi in the sowing Salvia mirzayanii and Salvia macrosiphon is useful for boosting the measured traits, however; the intensity and quantity of such traits are different depending on the genus and species of the mycorrhizal fungi. The results from the present research are consistent with all other results from other studies on this topic, including studies on the hyssop, six genotypes of mint, garden thyme, artichoke, and savory(Kouchaki et al. 2017, Azimi et al. 2013, Ghasemnejad and et al. 2019, Islamifar and et al. 2019).

The reason for the better performance of these medicinal plants in this situation is that mycorrhiza is one of the most common communities among soil microorganisms and organic plants. The performance of all mycorrhizal systems depends on the ability of the symbiotic fungi to absorb available mineral or organic substances from the soil. Mycorrhiza is of great importance since it has a high ability to increase the growth and yield of the plant in certain conditions.

It has been reported that Mycorrhiza facilitates host plants to grow better and faster. The main reason is the symbiotic fungi to absorb available minerals such as phosphorus. The content of phosphorous affects the physiological parameters of the plant, including the photosynthesis process, because it plays a key role as an energy carrier during this process (Kapoor et al., 2002). Mycorrhizal fungi can increase nitrogen fixation in the plant, which, in turn, makes nitrogen available so that the absorption of this substance increases. In alkaline soils, the increase in potassium absorption depends strongly on the species of symbiotic fungi and species of the plant. The difference between mycorrhizal and non-mycorrhizal plants in terms of the concentration of total iron and absorbed iron is dependent on the efficiency of mycorrhizal hyphae in absorbing it and transferring it to the symbiotic plant and other traits, including the type of root, which indirectly affects the absorption of the iron (Datta & Kulkarni 2012). Mycorrhizal fungi can transfer nutrients, especially carbon and phosphorus, from plant roots to other plants in mycorrhizal hyphae. This action is very beneficial for the growth and stability of young seedlings and can increase their chance of survival. Thus, using the method of mycorrhization of the medicinal plants in the soils where they are sown, pastures and forests can increase the range of ecological distribution of these plants (Ho et al., 2010). In this way, the hydraulic conductivity of the root in mycorrhizal plants will increase, and water will be transferred more efficiently. As a result, mycorrhizal plants produce more root biomass (Shah Hosseini et al., 2013).

Berg and colleagues (2012) have reported an increase in auxin content in mycorrhizal roots. It can add to the growth of the root and, as a result, lead to its volume compared to plants that were not inoculated with mycorrhizae. The exophytic roots increase phosphorus absorption by the plant through secreting organic acids that dissolve insoluble phosphates such as malic acid so that the function of aerial parts, the amount of phosphorus in aerial parts as well as the function of underground parts increase if mycorrhizal fungi are used. The increase in the performance of aerial parts and ground parts are, respectively, associated with the increase in foliage and material absorption. Phosphorus, as one of the three elements needed by plants, increases biological performance because it plays a key role in cell division by regulating plant hormones. On the other hand, it has a role in the production of photosynthetic materials and energy in the plant (Aliabadi and Valadabadi 2010).

Mycorrhizal fungi lead to the adhesion of soil grains and the creation of fine soil grains by producing a substance of

glycoproteins called glomalin. Thus, mycorrhizal fungi in compacted soils improve plant growth and increase its function by improving the soil structure and increasing ventilation and the water-holding capacity in the soil (Bedini et al., 2009). On the other hand, the roots of mycorrhizal plants are branched and the diameter of the subsidiary roots decreases, and the length of the root increases. These factors lead to the bonding of roots with the soil and absorbing water faster from the soil (Cameron et al., 2013).

The mechanism of the increase in the height, number of leaves, and fresh and dry weight of aerial parts and roots is that some of the fungal filaments enter the root and lead to a decrease in the concentration of Abscisic acid and an increase in the number of cytokinins. This, in turn, expands the roots and increases the absorption of water and nutrients. It is assumed that exophytic mycelium can increase phosphorus absorption by releasing organic acids that dissolve insoluble phosphates such as malic acid into the rhizosphere (Azimi et al., 2012), and this, in turn, leads to the increasing the quantitative and qualitative growth index in the medicinal plants.

The results showed that the use of mycorrhizal fungi was effective in increasing the measured traits compared to not using them in the sowing Salvia mirzayanii and Salvia macrosiphon; however, their intensity and quantity were different depending on the various factors such as the genus of the plants and mycorrhizal fungus species. The results obtained were consistent with the results of the other research on the same topic, including *Catharanthus roseus*, green and purple basil, and sweet-scented geranium (Harborne & Williams, 2000).

The reason why there is an increase in the measured phytochemical traits of these plants according to the measured indicators is that many of the secondary plant metabolites play important ecological roles in a plant. Phenolic compounds, as a group of secondary metabolites, are of a diverse chemical structure and a wide distribution in plants, which play a major role in the chemical defense of plants against microbes. According to the evidence, phenols act as signals in plant growth and plant-microbe interactions. An increase in the accumulation of phenols in the plants treated with the fungus can be due to the increase in the activity of the polyphenol oxidase enzyme. The increase in the enzyme activity of polyphenol oxidase and peroxidase in the plant has to do with the increase in total phenol content in the samples inoculated with the fungus(Harborne & Williams, 2000).

Of the most important phenolic compounds found in plants are anthocyanins, which have antioxidant properties. These compounds may be induced by several environmental factors, such as the symbiosis of plants with mycorrhizal fungi (Ghorbanian et al., 2014).

As studies show, Effective factors in the increase in the production of dry matter may affect the production of primary and secondary metabolites and lead to an increase in the biosynthesis of secondary metabolites. Thus, it appears that the significant improvement in plant biomass depends on the availability of nutrients for the biosynthesis of secondary metabolites. Moreover, the increase in phenolic concentration can be the result of the improvement of the nutritional status of the plant. The production of secondary metabolites is associated with the increase of reactive oxygen species as the by-products of biotic and abiotic stresses. The increase observed in the concentration of phenolic compounds can be a primary defense response to fungal colonization(Reis, 2013).

The increase in the content of total phenol in mycorrhizal plants compared to the control shows that, on the one hand, These compounds involve the production of symbiosis and, on the other hand, mycorrhizae stimulate their production. In a study on the leaves and roots of a jujube species treated with 6 species of mycorrhizal fungi, it was observed that the numbers of the phenols in all fungal treatments were increased, and there was a positive correlation between total phenol accumulation and polyphenol oxidase enzyme activity in this plant species(Ghorbanian et al., 2013).

The increase in the enzyme activity of the plant has to do with the total phenol content in the samples inoculated with the fungus. Although there is doubt about the role of flavonoids as necessary signaling compounds in the formation of vesicular-arbuscular mycorrhiza, it has been confirmed that certain flavonoids in mycorrhizal plants can increase the germination percentage of mycorrhizal fungi. The increase in the amount of anthocyanin in plants treated with mycorrhizal compared to the control group can boost the hypothesis of the role of this class of compounds in signaling (Zhang et al., 2013). The results obtained from examining the expression of two reference genes, linalool, and 1,8 cineole, and the housekeeping gene S18 in this research showed that in all three tests, S18 had more transcripts than 1,8 cineole, and in other words, its Ct values were lower, which was consistent with the results obtained by Gopesh and George(2012). The expression of both genes showed a significant change among the eight examined samples. The expression of the cineole gene in the Salvia mirzayanii increased effectively in the treatment of Geosporum fungus. This fungus can be used for increasing the effective compound of cineol, which is of many medicinal properties. Moreover, G. etunicatum with a lower percentage can increase the expression of the cineol gene. At final, it can be concluded that the use of mycorrhizal fungi, Geosporum, and etunicatum has effectively increased the expression of the gene.

Geosporum in Salvia macrosiphon effectively increased the expression of the linalool gene, but etunicatum showed no

increase. In general, from the results of the RT-PCR, it can be said that Geosporum mycorrhizal fungus is considered the best symbiont of Salvia mirzayanii and Salvia macrosiphon.

In addition, according to the results of this spectroscopy, the presence of linalool substance in Salvia macrosiphon was confirmed, and the linalool gene was selected for investigation of the gene expression.

The results obtained from the catalase enzyme showed a significant difference; however, the highest amount of catalase was recorded in the control soil for both plant species. The amount of the catalase enzyme in Salvia macrosiphon was higher than that in Salvia mirzayanii. The highest amount of catalase enzyme belonged to the treatment of Salvia mirzayanii and the control soil. The highest amount of peroxidase enzyme belonged to the substrate containing Geosporum.

All in all, the amount of peroxidase enzyme in Salvia macrosiphon was higher than that of Salvia mirzayanii. The highest amount of peroxidase enzyme belonged to the treatment of Salvia mirzayanii and Geosporum.

Conclusion

In this research, it was concluded that the inoculation of the seeds of medicinal plants with mycorrhizal fungi has a significant effect on the absorption efficiency of nutrients and subsequently stimulates their growth. This can lead to an increase in the measured parameters of the plants treated with mycorrhizal fungi, including height, number of flowers, number of leaves, fresh weight of aerial parts, dry weight of aerial parts, and fresh weight of roots and dry weight of roots. It was also observed that inoculation with mycorrhizal fungi significantly increased the phytochemical properties investigated in plants, including total phenol, total flavonoid, anthocyanin, antioxidant capacity, and gene expression in the plant, and they showed better quantity and quality.

Acknowledgments

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Conflict of interest

None.

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Ethics Statement

None.

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