

Effect of mycorrhizal inoculation and phosphorus supply on morphological traits of rosemary under greenhouse conditions

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Abstract

Rhizobium inoculation increases nutrients uptake by modification of root characteristics. This experiment was conducted in 2015 at Zabol university research farm (Chah-Nimeh) in a completely randomized design based on factorial arrangement with three replications. The first factor was five levels of phosphate: 100, 75, 50, 25 and 0 (control) kg ha⁻¹ and the soil inoculation consisted of two arbuscular mycorrhizal: *Glomus intraradices* and *G. mosseae*. The measured traits include number of leaves, stem dry weight, root fresh weight, shoot dry weight, stem diameter, root length, plant height, SPAD readings, root and shoot nitrogen content, essential oil percentage and essential oil yield. Results indicated that using of *G. intraradices* and *G. mosseae* have no significant effects on rosemary essential oil yield. The highest and lowest essential oil percentage rate of 2.2 and 1.6 %, respectively were as a result of taking ammonium phosphate 100 kg ha⁻¹ and in the control (no ammonium phosphate). On the other hand, higher shoot (1.17) and root (1.96) nitrogen percentage and were recorded followed by interaction between *G. mosseae* species and the control, respectively. The SPAD readings of rosemary increased significantly by the application of fertilizer in levels. On interaction effects, *G. intraradices* (M1) and application of 75 kg ha⁻¹ ammonium phosphate treatments had the best SPAD readings. The results of this study indicated that the inoculation of arbuscular mycorrhizal fungi in soil with optimal fertilizer application greatly improved rosemary growth and nutrient uptake and the effect was greater under greenhouse conditions.

Key words: Essential oil, fertilizer, Glomus, root, shoot, SPAD value.

Introduction

Rosemary (*Rosmarinus officinalis* L.) has long been considered as an important plant because of its essential oil used in perfumes and medicine as well as an important spice and antioxidant in processed foods. It is also a delightful herb with ornamental value that may stretch beyond the herb garden, either being used as a standard Armitage, or as a holiday pot plant at Christmas (DeBaggio, 1987; Armitage 1997). For these reasons and other reasons, it has been grown since ancient times (Simon *et al.*, 1984).

Even Shakespeare's Ophelia pays tribute to rosemary in Hamlet. Rosemary is a member of the mint family, Lamiaceae. It has opposite, simple, entire, evergreen leaves up to two inches long and an eighth of an inch wide. The leaf margins are revolute and the leaves are a shiny green on top and whitish beneath due to a dense collection of very fine hairs (Dirr, 1990). The plant begins to flower in late winter and continues through spring (Armitage, 1997). Despite the plant popularity and its many uses, there is very limited published research on the growing criteria. (Boyle et al., 1991) found out that rosemary does not respond well to high levels of fertilizer, but the ideal fertilization concentration was not determined. For this reason, the global research focus is on the production of medicinal plants under sustainable farming systems through various management techniques. One of the most important management techniques is to increase the use of biofertilizers and reduce the chemical inputs in the soils, especially

in arable land under cultivation of medicinal plants.

Mycorrhiza is a term coined to describe the interaction of soil fungi and plant roots. In general, these soil fungi evolved from the symbiotic association with plant roots. Both plants and fungi gained chemical, physical, biological, and physiological benefits. The management of this association showed an increase in agricultural and natural plant growth. Colonization of plant roots by arbuscular mycorrhizal fungi can greatly increase the plant uptake of phosphorus and nitrogen. The most prominent contribution of arbuscular mycorrhizal fungi to plant growth is as a result of the uptake of nutrients by extra radical mycorrhizal hyphae. Quantification of hyphal nutrient uptake has become possible by using soil boxes with separated growing zones for roots and hyphae. Many (but not all) tested fungal isolates increased phosphorus and nitrogen uptake of the plant by absorbing phosphate, ammonium, and nitrate from the soil. However, when compared with the nutrient demand of the plant for growth, the contribution of arbuscular mycorrhizal fungi to the plant phosphorus uptake is usually much larger than the contribution to the plant nitrogen uptake.

The two pathways by which arbuscular mycorrhizal plants absorb phosphorus involve different cell types, (1) different Pi transporters (PiTs) and (2) P access from different regions and volumes of the soil. Direct uptake by root epidermis (including root hairs when they are formed), accesses Pi in the soil solution close to the roots. Expression of genes encoding high-affinity PiTs in these cells are in its maximum in the root apex and root hairs (Gordon-Weeks et al., 2003) and declines in more mature regions. Expression is often reduced by high phosphorus supply and arbuscular mycorrhiza colonization (Javot et al., 2007). These reductions will lead to lower direct uptake in older regions of the root, but its relative importance is not clear. Arbuscular mycorrhiza colonization and the potential operation of the arbuscular mycorrhiza pathway occur behind the root apex. Arbuscular mycorrhiza fungi grow extensively in soil to form a well-developed hyphal network that absorbs Pi (via fungal highaffinity PiTs) from the soil to several centimeters from the root surface and can markedly extend the depletion zone. Phosphorus is translocated rapidly to the roots (probably as polyphosphate), by overcoming the slow diffusion that occurs in the soil solution. The individual fungal hyphae have much smaller diameters than the roots, thereby allowing access to narrower soil pores and hence increasing the soil volume explored (Drew et al., 2003; Schnepf et al., 2011). These factors are the major cause of increased phosphorus uptake and positive arbuscular mycorrhiza growth responses. Specialized arbuscular mycorrhiza fungus-plant interfaces develop within root cortical cells, in association with complex fungal structures known as arbuscular and also with coiled hyphae (Smith and Read, 2008). These structures are completely enveloped by the plant plasma membrane, such that the interfaces are bound by specialized membranes of the plant and fungus with an apoplectic region between them. This organization is important with respect to the control of nutrient transfers between the symbionts (Smith and Smith, 1990). The aim of this paper was studying the effect of mycorrhizal inoculation and phosphorus supply on early establishment and morphological traits of Rosemary under greenhouse conditions.

Material and methods

The effects of mycorrhizae and phosphorus fertilizer on early establishment and morphological traits of rosemary under greenhouse condition were studied at the Agricultural Research Institute, University of Zabol, Iran in 2015. This greenhouse is located at latitude 30° 54′ N, longitude 61° 41′ E, and an altitude of 481 m above mean sea level.

Table 1. Soil physical and chemical properties

Electrical con- ductivity	pН	N (ppm)	P (ppm)	K (ppm)	Zn (ppm)
1.46	8	6.3	9.2	125	2.8
0 1		0.11	C1	I	a
Organic carbon	Organic matter	Silt	Clay	Sand	Soil texture
(%)	(%)	(%)	(%)	(%)	
0.47	0.81	20.4	32.6	45	clay loam

A completely randomized design was used based on factorial arrangement with three replications. The first factor consisted of five levels of phosphorus: 100, 75, 50, 25 and 0 (control) kg per ha was applied as ammonium phosphate fertilizer, and the second factor was the inoculation of soil with two mycorrhizae fungi species: G. intraradices (M1) and G. mosseae (M2). On the 5th of March, before planting rosemary seedlings, chemical fertilizers were mixed with soil in pot. After fertilization, the seedlings were planted in each pot and then all pots were placed for 16 weeks in a greenhouse at temperature 30±2 °C and 65 % relative humidity. Urea was applied at 3 stages (early planted, early vegetative and early reproductive growth). Irrigation and weed control was carried out in the pots during the growing season. The SPAD value (SPAD 502 made by Minolta Co., Ltd.) representing green degree of a leaf was measured and the total nitrogen content of the rosemary was also determined by combustion N analysis and/or Kjeldahl N analysis (AOCS, 1990). Traits were measured, including number of leaves, stem dry weight, root fresh weight, shoot dry weight, shoot fresh weight, stem diameter, root length, plant height, SPAD readings, root nitrogen content, shoot nitrogen content, essential oil yield and essential oil percentage. Essential oil content in the plant was determined by laboratory distillation in a Clevenger's apparatus. Moisture was determined by drying a weighed sample of the plant in an oven at 35°C for 48 hours and the dry matter (plant) was weighed. The dried samples were preserved for nutrient analysis. Oil yields were calculated from dry matter and oil content was calculated on a dry basis. Data were analyzed by analysis of variance (ANOVA) using SAS version 9 (SAS institute, Inc., Cary, N.C.).

Results and discussion

Plant height: The results of the analysis of variance showed that the impact of the mycorrhizae and fertilizer treatment and interaction between mycorrhizae and different levels of fertilizer on this trait was significantly different at 1 % (Table 2). According to the compared data,

TAULE 2. INICAL	n phrai		at articitization OI	TOSCIIIAI Y UL	INT INTRODUTIN	740 AUN 10101		21						
SOV	DF	Plant	Root	Stem	Sho	ot	Root	Number of	Stem	Essential	Essential	Nitroger	i content	
		neignt	lengun	alameter -	Fresh weight	Dry weight	nresn weight	leaves per plant	ary weight	011 (%)	yield	Shoot	Root	2
Rep	7	318.40ns	56.59ns	0.009ns	0.014ns	1.590ns	0.006ns	26.13ns	0.010ns	0.153ns	0.066 ns	0.059**	0.026ns	0
Mycorrhizae		1325.74**	2470.85**	0.057ns	0.237ns	0.672ns	0.006ns	192.53**	0.203^{**}	0.003ns	0.020ns	0.973**	2.092**	
Fertilizer	4	2609.28**	5958.48**	0.097ns	1.713*	3.406^{**}	0.921**	391.91**	0.640^{**}	0.424**	0.079*	0.160^{**}	0.308^{**}	40
M×F	4	1151.24**	334.43ns	0.040ns	0.463ns	0.154ns	0.083**	125.95**	0.392^{**}	0.040ns	0.053ns	0.019^{**}	0.042*	-
Error	18	145.7	179.18	0.042	0.59	0.61	0.006	13.91	0.009	0.08	0.019	0.003	0.013	
CV %		8.8	9.9	11	9.7	11	11	11.5	8.7	14.8	13.4	8.2	8.4	
**, * significa	nt at P:	=0.05 and P=	=0.01, respectiv	vely. ns: not s	significant									

9.97ns 185* 9.23** 51.97* 14.46 11.8

PAD tdings the highest 195.1 mm and lowest 110.1 mm plant height were obtained, respectively from G. mosseae and application of 100 kg ha⁻¹ ammonium phosphate treatment and G. mosseae and application of 0 kg ha⁻¹ ammonium phosphate treatment (Table 4). Researchers reported that with increasing in mycorrhizae fungi rate, the plant height of acacia (Acacia holosericea L.) also increased (Duponnois et al., 2005). Mycorrhizal fungi are beneficial micro-organisms closely associated with the roots of most plants. These fungi enable plants to absorb more nutrients, such as phosphorus, zinc, and copper from many soils than the corresponding non-mycorrhizal plants. Mycorrhizal fungi may also increase water uptake. In this way, mycorrhizal fungi increase the efficiency of fertilization. Also, Ghorbanian et al. (2011) reported that mycorrhizal fungi, by extending their root absorbing area through their mycelium network and changing unavailable phosphorus to available form and translocate to root system cause increase in plant height and growth parameters. The highest values in the plant heights of Zea mays were observed when G. mosseae was used (Abdelmoneim et al., 2014). Martinetti et al. (2003) mentioned that the highest plant height of rosemary was observed when 200 mg plant⁻¹ N, 40 mg plant⁻¹ P₂O₅ and 200 mg plant⁻¹ K₂O were combined. The results of Rahimi et al. (2016) suggested that partial replacment of phosphorus chemical fertilizers by biological sources increased the flower yield of Calendula officinalis L. using both biofertilizers (Glomus intraradices, G. mosseae and G. hoi) and chemical P.

Root length: Analysis of variance showed that the effect of mycorrhizae and different levels of fertilizer treatments on root length was significant at 1 % (Table 2) and the root length did not vary due to the interaction between mycorrhizae and different levels of fertilizer. Also, the use of more fertilizer increased the root length, and this increase was significant. The highest root length 174.9 mm was obtained from using 100 kg ha⁻¹ ammonium phosphate, whereas the lowest root length 88.9 mm was obtained when ammonium phosphate (control) was not applied (Table 3). The recorded higher root length was 144.1 mm, followed by G. intraradices species 126 mm and the least recorded root length in G. mosseae species was 96.4 mm (Table 3). The results of Bhartia et al. (2016) showed that the bio-inoculants (Dietzia natronolimnaea and G. intraradices) improved Ocimum basilicum growth under salinity stress in both glasshouse and field conditions.

Stem diameter: The effect of experimental treatments on this trait was not significant (Table 2). It seems that in rosemary, the stem diameter is more influenced by the plant genetics. Interaction between mycorrhizae fungi and different levels of Table 3. Mean of some characteristics of rosemary affected by Mycorrhizae and fertilizer treatments

fertilizer treatments on the stem diameter was not significant (Table 2). The addition of nitrogen, phosphorus and potassium (NPK) treatments increased the leaf area, stem diameter, number of leaves, fresh weight and dry weight of *Cucurbita moschata* and all the NPK treatments had significantly broader leaf area, stem diameter, number of leaves, fresh and dry weight than the control (Okonwu and Mensah, 2012).

Fresh and dry weight of shoot: The results of this research showed that the fresh and dry weight of shoot was not affected by mycorrhizae species (Table 2). The effect of different levels of fertilizer treatment on these traits was significant at 5 and 1 %, respectively (Table 2) and the use of more fertilizer increased the fresh and dry weight of the shoot. There was a significant difference between control (without ammonium phosphate) and first level of fertilizer 100 kg ha⁻¹. Higher shoot dry weight 8.04 g was obtained by using 75 kg ha⁻¹ fertilizer and the highest shoot fresh weight 8.6 g was in 75 kg ha⁻¹ fertilizer treatment. No significant difference was observed between the application of 100 kg ha⁻¹ ammonium phosphate treatment and the second level of fertilizer 75 kg ha⁻¹. Also, the lowest shoot fresh weight was obtained without applying fertilizer (control) (Table 3). The effect of the interaction between mycorrhizae species and different levels of fertilizer treatments on the fresh and dry weight of shoot was not significant (Table 2). Martinetti et al. (2003) reported that the highest fresh yield on rosemary was obtained by applying the combination of 200 mg plant⁻¹ N and 40 mg plant⁻¹ P₂O₅. Researchers reported the highest and lowest levels of shoot dry weight of wheat (Triticum spp.) under greenhouse conditions in bio-phosphor and control, respectively (Forouzandeh et al., 2014).

Root fresh weight: Regarding this trait, the effect of fertilizer treatment and interaction between mycorrhizae and different levels of fertilizer was significant (P=0.01) (Table 2). Based on the comparison of the means, the highest fresh root weight 1.59 g was obtained as a result of first level of ammonium phosphate fertilizer 100 kg ha⁻¹ with *G.mosseae*, and the lowest fresh root weight 0.21 g was obtained as a result of the control treatment 0 kg ha⁻¹ with *G.mosseae* (Table 4). This data is in agreement with the results of (Rezvani *et al.*, 2010) in *Medicago sativa* L. Habibzadeh (2015) reported that colonization percentage of *G. mosseae* was more than *G. intraradices* and was less reduced with increasing phosphorus levels.

Stem dry weight: The effects of treatments on this trait were significant at 1 % (Table 2). The interaction effect between and fertilizer treatments

Treatment	Root length	Stem diameter	Shoot fresh	Shoot dry weigh	t Root fresh weight	Essential oil	Essential oil yield
	(mm)	(mm)	weight (g)	(g)	(g)	(%)	(mg per pot)
Mycorrhizae							
G. intraradices	144.1A	1.83A	7.83A	6.90A	0.73A	1.9A	1.01A
G. mosseae	126.0B	1.92A	8.00A	7.20A	0.76A	1.9A	1.07A
Fertilizer							
100	174.90A	2.06A	8.22A	7.53AB	1.40A	2.25A	1.09A
75	138.35BC	1.91AB	8.59A	8.04A	0.70B	2.21A	1.14A
50	147.65B	1.88AB	7.94AB	7.02BC	0.66B	2.02AB	1.09A
25	125.61C	1.73B	7.63AB	6.38C	0.61B	1.71B	1.03A
0	88.95D	1.78B	7.20B	6.27C	0.34C	1.68B	0.85B
Maana fallowed by	aimilar lattara in	angh galumn ara na	t significantly di	fforant at $P=0.05$	GIM multiple renges	tost	

Means followed by similar letters in each column are not significantly different at P=0.05, GLM multiple ranges test

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mycorrhizae fungi and fertilizer was observed on stem dry weight. *G. mosseae* and application of 75 kg ha⁻¹ ammonium phosphate treatment recorded higher mean (1.81 g) (Table 4). Rahimzadeh *et al.* (2011) showed that chemical fertilizer increased the dry matter yield of *Dracocephalum moldavica*.

Number of leaves per plant: Analysis of variance showed that the number of leaves per plant was significantly affected at 1 % level by mycorrhizae fungi, fertilizers and M×F treatments (Table 2). Interaction effects show that the G. mosseae species at 100 kg ha⁻¹ fertilizer treatment has the highest number of leaves per plant (45) among other treatments (Table 4). This shows that higher ammonium phosphate rates enhanced the vegetative growth of the rosemary and increased the source capacity of the plants by the number of leaves produced per plant. Nitrogen and phosphorus are the two elements that have positive effect on uptake and impact of biochemical interplay. In other word, by increasing nitrogen, phosphorus uptake by plant and its effect on plant metabolic activity increases and with increasing soil phosphorus, nitrogen uptake and physiological effects of the plant increases. Phosphorus that is a major factor in the storage and transfer of energy within the plant can be used as a major factor in the stored energy for the metabolic processes of the plant (Mahmoudi and Hakimian, 2000). Also, the root system of plants inoculated with AMF were often more finely divided and thus have more absorptive surface area (Okon et al., 1996). Pal et al. (2016) showed that N, P and K fertilizations positively affect the development of Thymus and the highest yield was obtained in the highest rate of N (150 kg urea ha⁻¹), P (250 kg phosphorus. ha⁻¹) and K (150 kg potash ha⁻¹) fertilizers.

Essential oil percentage: The results of this research indicated that the effect of the fertilizer levels on essential oil percentage was not significant (P<0.01) and the effect of mycorrhizae type and interaction M×F on this trait was also not significant (Table 2). The highest 2.2 % and lowest 1.6 % essential oil percentage was obtained in ammonium phosphate (100 kg ha⁻¹) and in control (no ammonium phosphate), respecively, and mean values showed no significant difference between first 100 kg ha⁻¹ and second 75 kg ha⁻¹ levels of fertilizer (Table 3). Bahonar *et al.* (2016) cited that inoculation of rosemary with mycorrhizal fungi had no significant effect on the essential oil percentage. Many other investigations reported that the use of mineral fertilizers would increase essential oil content in medicinal plants (Tawfeeq *et al.*, 2016; Abdollahi *et al.*, 2016). By adding phosphorus, photosynthesis and respiration

increases, but when nitrogen is too high in the soil, excessive application of phosphorus (or *vice versa*) increases respiration and the quantitative and qualitative yield or essential oil is adversely affected. Most of the medicinal plants are used for extraction of active substances. Therefore, the use of fertilizers for plants, especially herbs require careful application. Hosseini Valiki *et al.* (2015) showed that manure treatment (N = 150 and P = 150 kg ha⁻¹), had significant effect on essential oil and essential oil yield of rosemary compared to other fertilizers. Also results showed that application of 200 kg ha⁻¹ nitrogen and 250 kg ha⁻¹ of phosphorus fertilizers reduced essential oil and essential oil yield compared with other chemical treatments.

Essential oil yield: The effect of the fertilizer levels on the essential oil yield was not significant (P < 0.05) and the effect of mycorrhizae type and interaction M×F on essential oil yield were not significant (Table 2). The highest $(1.14 \text{ mg pot}^{-1})$ and the lowest (0.85 mg pot⁻¹) essential oil yield was obtained in ammonium phosphate (75 kg ha⁻¹) and control (no ammonium phosphate), respectively. Comparing the mean values showed no significant difference between first 100 kg ha-1, second 75 kg ha⁻¹, third 50 kg ha⁻¹ and fourth 25 kg ha⁻¹ levels of fertilizer (Table 3). The results of Bahonar et al. (2016) showed that the inoculation of mycorrhiza fungi (G. intraradices) improved the morphological and phytochemical traits of rosemary positively under different levels of salinity. Essential oil is a terpenoid compound and its components (isoprenoids) such as Isopantyl pyrophosphate (IPP) and Dimethyl Alyl pyrophosphate (DMAPP) highly demand NADPH, ATP, and the fertilizers such as nitrogen and phosphorous are required for production of the secondary compounds (Ghazi Manas et al., 2013). Ozguven et al. (2008) concluded that by increasing level of nitrogen fertilizer, essential oil content of Artemisia (Artemissia annua L.) increased.

Shoot and root nitrogen content: Regarding these traits, the effect of the mycorrhizae and fertilizer treatment was significant at 1 % level, but the interaction between mycorrhizae and different levels of fertilizer on shoot and root nitrogen content was significant at 1 and 5 %, respectively (Table 2). The highest shoot (1.17 %) and root (1.96 %) nitrogen was recorded in interaction between *G. mosseae* species and control (Table 4). Martinetti *et al.* (2003) reported that the highest nitrogen uptake with mean 10.1 g kg⁻¹ dry matter in rosemary was obtained by the application of 80 mg plant⁻¹ P₂O₅.

al., 2016). By adding phosphorus, photosynthesis and respiration Results of Soleymani and Pirzad (2016) showed the order Table 4. Interaction between Mycorrhizae and fertilizer on some characteristics of rosemary

Mycorrhizae	Fertilizer	Plant heigh	Root fresh	Stem dry weight	Number of	Nitrogen co	ntent	SPAD
		(mm)t	weight (g)	(g)	leaves per plant	Shoot (%)	Root (%)	readings
M1	100	147.02BC	1.21B	1.37B	37B	0.38F	0.87E	61.16B
	75	124.96DC	0.71C	0.92DC	36B	0.42FE	0.95E	78.83A
	50	121.66D	0.59DC	1.29B	25.33C	0.47DFE	0.91E	53.53BC
	25	127.79DC	0.65C	0.71E	37B	0.58DCE	1.24D	48.43BC
	0	129.54DC	0.48D	0.83DE	13.66D	0.64DC	1.50BC	52.30BC
M2	100	195.10A	1.59A	1.65A	45A	0.64DC	1.63B	57.10B
	75	159.21B	0.69C	1.81A	35.66B	0.67C	1.37CD	57.70B
	50	130.08DC	0.62DC	0.76DE	40AB	0.86B	1.56BC	53.46BC
	25	122.89DC	0.68C	0.72E	29C	0.95B	1.59B	42.93C
	0	110.17D	0.21E	1.01C	24.66C	1.17A	1.96A	58.23B

Means followed by similar letters in each column are not significantly different at P=5 %, GLM multiple ranges test

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of highest colonization of Hyssop root was *G. mosseae*, *G. intraradices*, *G. fasiculatum*, *G. claroideum*, respectively. Also, any increase in the ammonium phosphate levels would reduce shoot and root nitrogen content.

SPAD readings: The SPAD value of rosemary was affected by mycorrhizae and interaction of M×F at P=0.05 and by fertilizer P=0.01 levels (Table 2). On interaction effects, *G. intraradices* (M1) and the application of 75 kg ha⁻¹ ammonium phosphate treatment had the best SPAD value (Table 4). In addition, Habibzadeh and Abedi (2014) reported that inoculation of mung bean with *G. intraradices* and *G. mosseae* had no effect on the SPAD value. The effect of arbuscular mycorrhiza on the SPAD value in this experiment is similar to the results reported by other researchers (Mathur and Vyas, 2000; Srivastava *et al.*, 2002).

The findings of the present study suggested that mycorrhizal fungi significantly increase plant growth and yield of rosemary, and could be replaced for chemical fertilizer. Application of both phosphorous and mycorrhizae could affect growth characteristics. In general, overall results indicated that positive impact of mycorrhizal symbiosis on rosemary was not related to fungi species and it seems phosphorous application at 75 kg ha⁻¹ would be appropriate for rosemary production.

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