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Original Article

Evaluation of Inoculation *Pseudomonas fluorescens* and Arbuscular Mycorrhizal Fungus on Growth, Morphological Characteristics and Essential Oil Percentage of *Thymus kotschyanus*

Ali Salehnia Sammak[1], Masoumeh Anvari[1*], Mohammad Matinizadeh[1, 2] and Mehdi Mirza[1, 2]

[1]Department of Microbiology, Rasht Branch, Islamic Azad University, Rasht, Iran
[2]Research Institute of Forests and Rangelands, Agricultural Research, Education and Extension Organization, Tehran, Iran

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ABSTRACT

To investigate the inoculation effect of *Pseudomonas fluorescens* and native mycorrhizal fungus on growth, morphological characteristics and essential oil percentage of *Thymus kotschyanus* Boiss. & Hohen., an experiment was conducted in a Randomized Complete Block Design with 5 treatments and 3 replications in Research Institute of Forests and Rangelands. Understudied treatments were included as *Pseudomonas fluorescens*, *P. fluorescens* and *Rhizophagus clarus*, *P. fluorescens* and *Funneliformis badium*, *P. fluorescens* and *Acaulospora laevis*, and control. According to the results, all treatments had a positive effect on thyme growth and *P. fluorescens* treat had the most essential oil percent. The results showed that all treatments had a significant effect on root volume, with the highest one in the treatment of *F. badium* and *P. fluorescens* (36.25 ml). The highest mean weight of dry and fresh root and dry plant belonged to *A. laevis* and *P. fluorescens* ($p \leq 0.05$) (, 13 g, 6.9 g and 32.25 g). This result confirms the synergistic relationship between *P. fluorescens* and *A. laevis*. The most amount of colonization was observed in *R. clarus* and *P. fluorescens* (94.9%). The results indicated that the synergistic intraction between *P. fluorescens* and native mycorrhizal fungus has different effects on morphological traits of this medicinal plant and these findings can be used to enhance the growth and yield of *T. kotschyanus* for organic production.

INTRODUCTION

The positive effects of symbiosis between plants and microorganisms have been confirmed in many sources [1]. Inoculation of growth-promoting rhizosphere microorganisms with medicinal plants increases the growth and production of secondary metabolites by increasing nutrients and moisture, suppressing pathogens, stress tolerance, and increasing phytochemical synthesis. The use of growth-promoting bacteria and mycorrhizal fungi reduces the need for chemical fertilizers and pesticides for medicinal and aromatic plant species. Many studies have shown the effect of mycorrhizal fungi and growth-promoting bacteria on increasing the growth and synthesis of medicinal compounds in plants [2].

Thyme is one of the most important medicinal plants of the Mentha family that has wide applications in the pharmaceutical and food industries [1,3]. The active ingredient is an essential oil, of which thymol and carvacrol are the main components. Thyme essential oil is one of the ten most famous essential oils in the world and has antibacterial, antifungal, antioxidant, natural preservative and food flavoring properties [4].

Thymus kotschyanus is one of the most widely used medicinal plants in Iran and its application in a wide range, especially in the pharmaceutical, food and biological control industries has a special place. Plant growth-promoting rhizobacteria (PGPR) is a term introduced by Kloepper in the late 1970s to refer to a group of bacteria that enhance plant growth through various mechanisms, including phosphorus

*Corresponding author: Department of Microbiology, Rasht Branch, Islamic Azad University, Rasht, Iran
Email Address: anvari@iaurasht.ac.ir

solubilization, nitrogen fixation, and the production of iron-chelating siderophores Balancing plant hormones, synthesizing volatile organic compounds (VOCs), transmitting quorum sensing signals to pathogens, and cause plant resistance to biotic and abiotic stresses [5].

However, some reports indicate that rhizobacteria inhibit the maximum growth of some plants by producing hydrogen cyanide. Research has shown that growth-promoting soil bacteria can increase plant growth and mineral uptake. Facilitate even in stressful situations [6].

There have been numerous reports of stimulant effects of these bacteria in the production of more valuable plant chemicals and medicinal metabolites. Jaleel *et al*. (2007) reported that a significant increase in ajmalicin was recorded by the application of non-native *P. fluorescens* on Vinca seedlings [7,8].
P. fluorescens is able to produce fluorescence pigments that enable them to show fluorescence in the face of ultraviolet light (245nm) especially in iron deficiency conditions. These pigments are fluorescents and soluble in water from the group of siderophores. They increase the plant's access to absorbable iron in the rhizosphere and subsequently play an important role in improving plant growth in terms of quantity and quality. Pseudomonas also through various mechanisms such as stimulating the production of plant hormones such as auxin, cytokine and gibberellin and also inhibiting the production of ethylene, increasing the solubility of inorganic and organic phosphate, producing microbial siderophores to increase plant access to absorbable iron, nitrogen fixation in symbiotic or non-symbiotic in stimulating and improving plant growth in terms of quantity and quality [9].

The effect of rhizobacteria such as *P. fluorescens* on *Origanam majorana* was tested and observed that indicators such as yield, essential oil, plant length, shoot weight, number of leaves, number of nodes and root dry weight showed significant differences compared to control. Arbuscular mycorrhizal fungi, in addition to their role in improving the growth and establishment of medicinal plants in habitat ecosystems, increase the biosynthesis of secondary metabolites in these specific plants. PGPR and mycorrhizal fungi can improve the quality of medicinal and aromatic products [9,10].

Gupta *et al*. (2002) in a study on inoculation of mycorrhizal fungi with *Mentha arvensis* observed that significantly increased oil content and yield compared to non-mycorrhizal plants [8,11].
Since inoculation effect of native mycorrhizal fungi and *P. fluorescens* on growth and their impact on medicinal plants rarely studied in Thymus genus, in this study co-inoculation effect of arbuscular mycorrhizal fungi and *P. fluorescens* due to the mentioned benefits of these microorganisms in absorbing plant nutrients and usability in organic biofertilizers in order to evaluate and influence the growth and morphological characteristics of *T. kotschyanus* were measured.

MATERIAL AND METHODS

In this study to investigate Inoculation of *P. fluorescens* and native mycorrhizal fungus on growth, morphological characteristics and percentage of essential oil of *T. kotschyanus*, an experiment was conducted in Randomized Complete Block Design with five treatments and three replications at Alborz Research Complex, Research Institute of Forests and Rangelands.

Inoculum preparation for arbuscular mycorrhizal fungi

Inoculation of mycorrhizal fungi was prepared in the laboratory of forest ecophysiology and biotechnology of the National Forest Rangeland Research Institute. To identify symbiotic fungi based on their spore morphology, soil samples were collected from a depth of 10 to 30 cm of each native thyme plant from the area of Alamut to identify and extract arbuscular mycorrhizal fungi. To separate the spores using Gerdman and Nicholson method method [12,13] and under the microscope, the observed heterogeneous spores were separated from others and propagated, stored separately in the trap plant. Identification of genus and species based on International Code of Nomenclature for Algae, Fungi, and Plants (ICN) nomenclature rules and morphological characteristics such as presence or absence of sporocarp, shape and type of arbuscle, color change during different spore growth periods, size of walls and layers, septumlocation and number of hyphae According to the identification keys in the sources [14,28] and INVAM site information. The mycorrhizal fungi *Rhizophagus clarus*, *F. badium*, and *Acaulospora laevis* were identified.

Bacterial preparation for inoculation

The standard bacterial strain of *P. fluorescens* (R-169) was obtained from the Soil and Water Research Institute. To prepare the bacterial inoculum with a population of 10^8 cfu/mL, equivalent to the McFarland turbidity, physiologic serum in a ratio of 1:9 was added to a 100-ml Erlenmeyer to reach a population of 10^7 cfu/mL and kept at room temperature (25 °C) on a shaker at 120 rpm. After 48 hours, the inoculum was ready to be used.

Greenhouse planting

For this purpose, the peat soil was sterilized with fine perlite in a volume ratio of 2:1 for 3 hours in an autoclave at 121 °C. five cultivation trays were prepared for the cultivation of *T. kotschyanus* for five biological treatments including 1-*P. fluorescens*, 2-*P. fluorescens* and *R. clarus* 3- *P. fluorescens* and *F. badium*, 4- *P. fluorescens* and *A. laevis*, 5 - Control or without inoculation.

AMF (25 mL) mixed with thyme seeds were inoculated into the cultivation tray per pot. For the bacterial treatment, the thyme seeds were placed in sterile Petri dishes containing bacterial liquid suspension with a population of 10^7 cfu/mL for three hours to inoculate the seeds of thyme. Then the seeds were placed on the cultivation tray to a depth of about 2 cm from the soil surface. *P. fluorescens* suspension, containing 5 mL of bacterial treatment and mycorrhizal treatment, was added to the cultivation tray.

The trays were irrigated by daily spraying for 10 minutes to maintain soil moisture. After two weeks of growth, thyme thinning was performed three times in one month, until eventually one better-grown plant remained in each pot.

Planting in the field

After sufficient growth and rooting of the seedlings, five planting trays were transferred to the field located at the Alborz Research Complex, Research Institute of Forests and Rangelands. Thyme planting was linear with a 50-cm planting interval. The distance between each line was 50cm. The planting operation consisted of five lines with three replications,a total of 225 Thyme plants, with biological microorganisms and control treatments. The amount of fungal inoculum for each plant was 50 ml and a diluted suspension of *P. fluorescens* (50 ml), with a population of 10^7 cfu/ml, was added in contact with the plant root and rhizosphere; drip irrigation was then immediately done to establish seedlings. Irrigation was performed one day for up to three weeks and then twice a week for three months. Hand weeding was also performed during the growing season. Also No fertilizers or chemical pesticides were applied in this experiment.

Harvesting and measuring of morphological features

For Harvesting After three months of growth, five samples were randomly harvested from a height of five cm on each cultivation line to determine growth factors and characteristics of *T. kotschyanus.* The marginal effect was removed by removing half a meter from the beginning and end of each line. For this purpose, after reaching the 50% flowering stage, two diameters, the tallest shoot length, and the plant dry weight were measured, flowering shoots and thyme leaves were cut from a height of 5 cm above the ground and put in the shade and dried for one week. Also, some of the thyme roots were randomly extracted from the soil to measure dry and fresh root weight, and the root volume was measured by a graduated cylinder.

Colonization percentage

To measure the coexistence of roots with arbuscular mycorrhizal fungi, their roots, especially capillary and thin roots, were sampled. The roots were then rinsed with distilled water and FAA solution (Formalin Acetic Acid Alcohol) was used to fix the roots. The roots were stained by the Phillips and Hayman method [15]. The mycorrhizal infection percentage was determined by the gridline intersection method [16].

Essential oil extraction

Samples containing 50% Thyme branches were taken from a height of 5 cm above the ground and shoots were dried in shade, crushed and prepared for essential oil extraction. Essential oil extraction was performed by distillation method using the Clevenger apparatus for four hours.

Statistical Analysis

For data analysis, SPSS software was used and the means were compared by Tukey's test. Excel software was used to draw graphs.

RESULTS

The results showed that inoculation of *P. fluorescens* and different species of arbuscular mycorrhizal fungi had positive effects on some growth indices. The effect of *P. fluorescens* with different species of native arbuscular mycorrhizal fungi isolated and identified from thyme root have different effects on the morphological traits of thyme (Fig. 1).

Different treatments of arbuscular mycorrhizal fungi with *P. fluorescens* had a significant effect on root dry and fresh weight, root volume plant and dry weight. Moreover, the effect of *P. fluorescens* alone on the root volume and root fresh weight of *T. kotschyanus* was significant compared to the control group (p-value< 0/5) (Table 1).

In this study, the highest Thyme shoot height was obtained from the treatment of *A. laevis* and *P. fluorescens* with an average of (24.75 cm), and the shortest shoot height was related to the control (20 cm). However, no significant difference was found among treatments. The highest vegetation cover, calculated by a formula with two plant diameters, belonged to *R. clarus* and *P. fluorescens* treatments with an average of (192.075 cm) and the lowest vegetation cover belonged to the control group with an average of (165.075 cm), indicating a synergistic effect between *R. clarus* and *P. fluorescens* on the size of the vegetation cover. The highest and lowest number of branches belonged to the *A. laevis and P. fluorescens* treatment with an average of (31.25) and the control group with an average of (18.75), respectively. However, no significant differences were observed between treatments. The results of the analysis of variance showed that all treatments had a significant effect on root volume so that the *F. badium* and *P. fluorescens* treatment with an average of (36.25 ml) had the highest root volume compared to control (18 ml). It was also shown that the highest mean root dry and fresh weight and plant dry weight were related to the *A. laevis* and *P. fluorescence* treatment with an average of 6.9 g, 13g and 32.25 g, compared to the control group with an average of 3.45 g, 8.5 g, and 20 g, respectively, indicating a significant difference and a synergy between *P. fluorescens* and *A. laevis.* All treatments had a significant effect on root volume and the greatest effect was in the treatment of *F. badium* and *P. fluorescens* with an average of 36.25 ml, which was significant at the level of p-value< 0.0001, followed by *A. laevis* and *P. fluorescens* 35 ml and was significant at the level of p-value < 0.001.

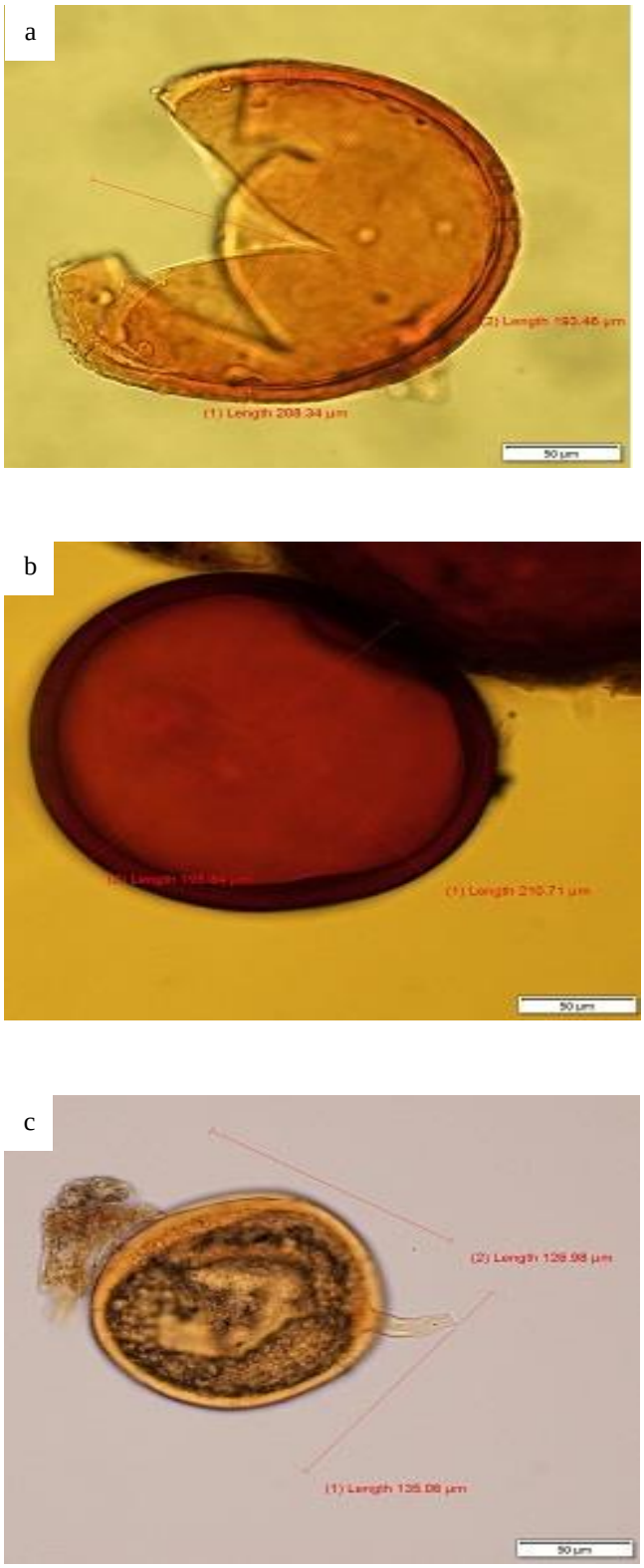

Fig. 1 Microscopic images of arbuscular mycorrhizal fungi isolated from the roots *T. kotschyanus*: A) *A. laevis*, B) *F. badium*, C) *R. clarus*

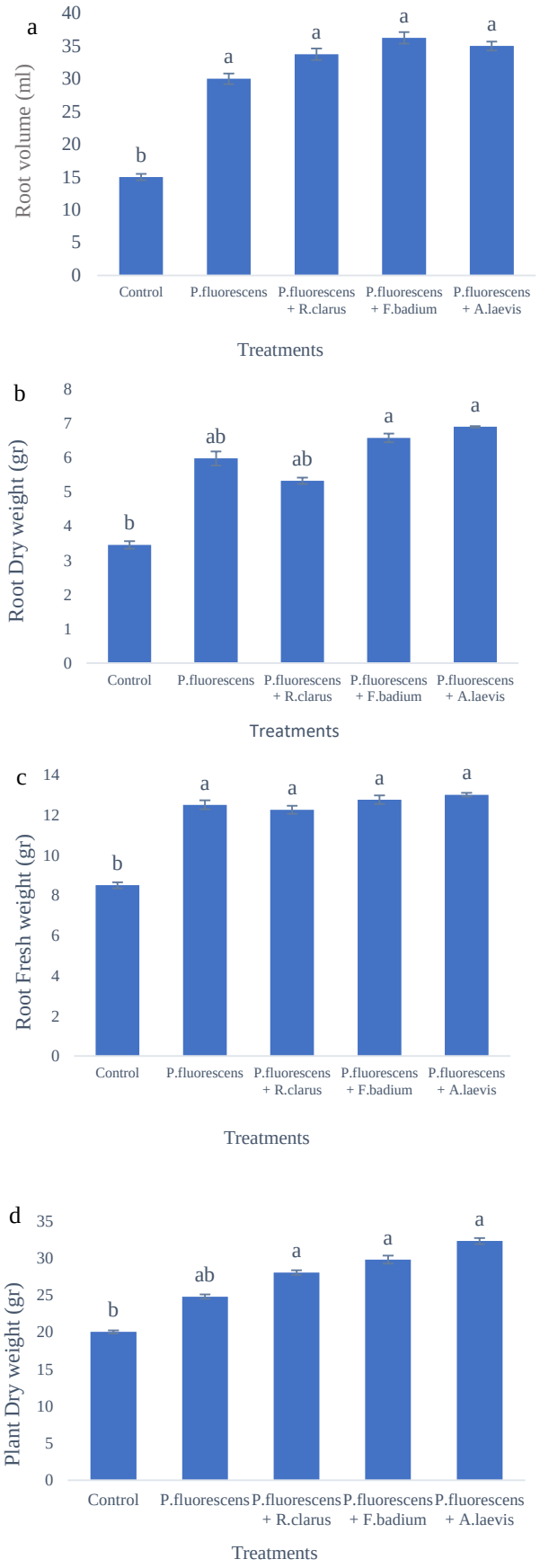

Fig. 2 Comparison between treatments on root volume (a) root dry weight (c) root fresh weight (c) plant dry weight (d). The average of 3 replicates is ± SE. Identical letters indicate no significant difference using the Tukey test.

Also, *R. clarus* and *P. fluorescens* treatment with an average of 33.75 ml and *P. fluorescens* treatment with an average of 30 ml were significant at the level of p-value< 0.01 and p-value < 0.05, respectively, compared to the control group. The effect of *A. laevis* and *P. fluorescens* treatments with an average of 6.9 g and *F. badium* and *P. fluorescens* with an average of 6.5 g on root dry weight were significant at the level of p-value< 0.01 and p-value< 0.05, respectively.

Colonization percentage, hyphae, vesicles and arbuscules

In this study, Analysis of variance showed that all treatments except *P. fluorescens* had a significant effect on mycorrhizal root infection. The highest root infection percentage was observed in the *Rhizophagus clarus* and *P. fluorescens* treatment with an average infection of (94.9%) and the lowest with an average of (46.6%) in the control group, showing a significant difference and the synergistic effect of *Pseudomonas fluorescens* and *R. clarus* mycorrhizal fungi on the root colonization percentage (Table 2).

Essential oil percentage

According to the results, the highest essential oil percentage per 100 g of plant dry weight belonged to *P. fluorescens* treatment with 1.74% and the lowest percentage in the control group with 0.85.

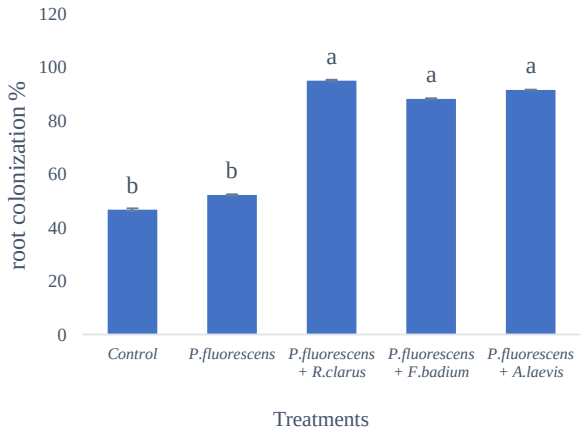

Fig. 3 Effect of different AMF and *P. fluorescens* treatments on root colonization percentage of *T. kotschyanus* Boiss. & Hohen

Table 1 Results of analysis of variance and sum of squares of morphological traits and growth indices of *T. kotschyanus* Boiss. & Hohen. under different treatments of AMF and *P. fluorescens* compared to control group

S.O.V	DF	Bheight		No.branch		Root.vol (ml)		Ro.D.w (gr)		Root.f.w (gr)		Dri.w (gr)	
		MS	F	MS	F	MS	F	MS	F	MS	F	MS	F
Treat	4	26.68	1.45 ^{ns}	86.75	2.59 ^{ns}	303.12	7.13 **	7.46	6.39 **	13.9	5.57 **	90.1	8.77 **
Error	15	18.37		33.53		42.5		1.16		2.5		10.2	

p-value <0.05 *, <0.10 **, ^{ns}: no significant

Treatment	Bheight (cm)	Vegetation cover	No. branch	Ro.vol (ml)	Ro.D.w (gr)	Root.f.w (gr)	Dri.w (gr)
R.clarus & *P.fluorescens*	24.7 a	192.07 a	28.2 a	33.75 a	5.3 ab	12.25 a	28 ab
F.badium & *P.fluorescens*	27 a	185.32 a	26.7 a	36.25 a	6.51 a	12.75 a	29.76 ab
A.laevis & *P.fluorescens*	24.71 a	184.63 a	31.2 a	35.00 a	6.92 a	13 a	32.25 a
P.fluorescens	25 a	188.45 a	25.0 a	30.00 a	5.96 a	12.5 a	24.73 bc
Control	20 a	165.07 a	18.7 a	15.00 b	3.45 b	8.53 b	20 c

Within each column means followed by the same letter are not significantly different based on Tukey's test.

Table 2 Results of analysis of variance and sum of squares for *T. kotschyanus* Boiss. & Hohen. root colonization percentages under different AMF and *P.fluorescens* treatments compared to the control group

Source	DF	Hyphe		Vesicule		Arbuscule		Total colonization	
		MS	F	MS	F	MS	F	MS	F
Treat	4	759.96	244.08 ***	81.247	40.65 ***	103.84	38.37 ***	2097.3	309.35 ***
Error	15	3.11		1.998		2.71		6.8	

p-value < 0.05 *, < 0.10 **, <0.001 ***, < 0.0001 ****. ^{ns}: no significant

Treatment	Hyphe	Vesicule	Arbuscule	Total colonization%
R. clarus & *P. fluorescens*	49.65 a	27 a	18.32 ab	94.97 a
F. badium & *P. fluorescens*	46.65 a	23.52 b	17.95 b	88.12 b
A. laevis & *P. fluorescens*	46.82 a	23 b	21.6 a	91.42 ab
P. fluorescens	23.27 b	16.65 c	12.2 c	52.12 c
Con	22.02 b	16.9 c	9 c	47.92 c

Within each column means followed by the same letter are not significantly different based on Tukey's test.

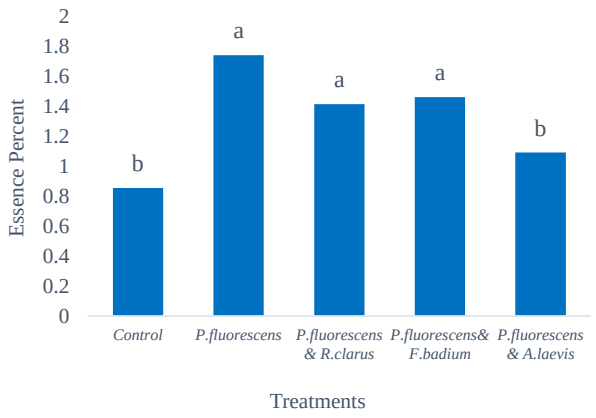

Fig. 4 *T. kotschyanus* Boiss. & Hohen. essential oil percentage under the influence of AMF and *P. fluorescens* treatments

This indicates the role of this bacteria in increasing the production of plant secondary metabolites and essential oils in *T. kotschyanus*, which was higher than the co-inoculation with AMF. Moreover, *P. fluorescens* and *F. badium*, *R.clarus* and *P. fluorescens*, *A. laevis* and *P. fluorescens* respectively had 1.46%, 1.41% and 1.09%, EO percent. The lowest percentage of essential oil belonged to the control group with 0.85%.

CONCLUSIONS

In this study, it was shown that incoculation of *P. fluorescens* with arbuscular mycorrhizal fungi of native thyme had positive and different effects on growth and morphological indices of *T. kotschyanus*,

so it had a significant effect on fresh and dry root weight, plant dry weight and root volume. *P. fluorescens* is one of the important phosphate solubilizing bacteria (PSB), and acts as mycorrhiza helper bacteria by increasing root colonization. Research has shown that growth-promoting soil bacteria can increase plant biomass and facilitate mineral uptake even under stressful conditions [17], which is consistent with the results of the present study.

Also in this study, inoculation of *Pseudomonas fluorescence* by liquid suspension method with seeds, roots and rhizosphere of the plant had a positive effect on morphological characteristics and percentage of *T. kotschyanus* essential oil, which had a significant effect on some characteristics. The increased fresh and dry weight of roots in comparison with other traits such as branch height treated by *Pseudomonas fluorescens* and AMF can be associated with the stimulation of plant hormone production by these microorganisms. They affected root growth and produce dense and branched roots [18] these results are consistent with the reports of other researchers. In this study, it was found that co-inoculation of *F. badium* and *P. fluorescens* had the greatest effect on root volume, which could be the result of synergism between these two microorganisms in the spread of mycelium and increase the induction of plant root growth.

It was also shown that co-inoculation of *A. laevis* and *P. fluorescens* had a significant effect on the dry and fresh weight of roots and dry weight of the plant, which showed a significant difference. According to other results, some growth-promoting bacteria increase growth rate, root length and weight, accelerate root elongation, and increase the number of lateral roots. The production of plant growth regulators, especially auxin, gibberellin, and cytokinin, can be the cause of these effects [19], and the results of this study are consistent with the findings of other researchers.

Also, in a study of Banchio [20], the effect of rhizobacteria such as *P. fluorescens* on *O. majorana* was examined and observed that root dry weight showed a significant increase compared to the control, which is consistent with the results of this study.

This study indicated that co-inoculation of *A laevis* and *P. fluorescens* with average (24.75 cm) on branch height was observed, which could be due to the induction of growth hormones in the plant by these microorganisms Which is related to the results of other researchers. Hazarika *et al*. (2000) reported that the application of phosphate-solubilizing bacteria significantly increased plant height in tea compared to the control. Ratti [21], also showed an increase in plant height due to mycorrhizal coexistence on lemongrass, which is consistent with the results obtained in this study.

Growth promoting bacteria help AMF fungi by promoting spore germination and mycelial proliferation. *Pseudomonas fluorescens* as a phosphate-solubilizing bacteria (PSB) in synergy with arbuscular mycorrhizal fungi with an increasing percentage of root colonization as mycorrhizal helper bacteria. These microorganisms produce specific signals by stimulating and producing specific molecules in the plant, and affect gene expression and the production of specific enzymes [22], which is consistent with the findings of this study.

In this study, it was shown that the combined application of *P. fluorescens* and arbuscular mycorrhizal fungi had a greater influence on growth characteristics, which could be facilitated by the movement of nutrients from the soil, easier absorption of phosphorus and iron, as well as the spread of fungal mycelia.

YK Karishma [23] showed that application of *G. mosseae*, *A. laevis* and *P. fluorescens* had a significant effect on the percentage of root colonization of Gerbera plant and according to the results obtained by the synergistic effect of *Pseudomonas fluorescens* as a helper of mycorrhizal fungi. In root colonization, it is similar to the present study.

Bahadori [24] indicated that the combination of AMF and *B. subtilis* and the combined treatment of *G. moseae* and *P.fluorescens* both decreased the percentage of colonization, which contradicts the results of this study. This difference could be due to the use of native arbuscular mycorrhizal fungi of *T. kotschyanus* and consequently a positive interaction with *P. fluorescens* in root colonization.

In a study on *Origanam majorana*, the effect of *P. fluorescens* were tested and found that the yield of essential oil showed a significant increase compared to control plants [25,20], which is also consistent with our study. In the present study, the use of *P. fluorescence* increased the amount of essential oil in

T. kotschyanus, which is consistent with the results of the study.

Since the effect of these microorganisms on *T. kotschyanus* has been studied for the first time, the results of this study showed that co-inoculation of growth-promoting bacteria, especially *Pseudomonas fluorescens* with native mycorrhizal fungi of *T. kotschyanus*, has a significant effect on plant growth and morphological characteristics. Also, the synergistic effect of *P. fluorescence* with AMF increased the percentage of Root colonization due to facilitating the reception of nutrients for plant growth and a positive effect on the morphological traits of *T. kotschyanus* growth due to nutrient exchange between fungi and bacteria and plants. It is predicted that the use of commercial bio-inoculum in the future could be the use of PGPR and AMF in organic production and exploitation of medicinal plants.

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