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Research Article

Increasing Performance and Production of *Mentha spicata* Essential Oil by Using One Strain of (PGPR) and Green Manure in the Central Region of Iran

Reza Dehghani Bidgoli,[1*] Mohammad Pessarakli[2]

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Abstract

This experiment was conducted in 2024 as a split-plot factorial arranged in a randomized complete block design (RCBD) with three replications. The treatments consisted of PGPR fertilizer at four levels (0, 50, 100, and 150 g/ha) as the main plot factor, and two types of green manure-Medicago sativa L. and Trifolium alexandrinum L.-each with two application states (use and non-use) as the sub-plot factors. The green manure crops were cultivated in September 2023 and incorporated into the soil by ploughing in the fall of 2024. The highest increases in Mentha essential oil percentage and yield were observed in the following treatments: the application of 150 g/ha PGPR (0.35% and 4.560 kg/ha), the use of Medicago sativa L. (0.43% and 5.580 kg/ha), and the use of Trifolium alexandrinum L. (0.44% and 4.950 kg/ha), respectively. Furthermore, the highest essential oil percentage and yield resulted from the two-way interaction between 150 g/ha PGPR and Medicago sativa L. green manure, with averages of 0.62% and 8.030 kg/ha, respectively. The three-way interaction of the experimental factors revealed that the combined application of 150 g/ha PGPR with both Medicago sativa L. and Trifolium alexandrinum L. green manures produced the highest values, with an average essential oil percentage of 0.73% and a yield of 9.165 kg/ha.

Keywords: Fertilizer, Medicinal plants, Essential oil, *Medicago sativa*, Trifolium.

1. Associate Professor, Department of Nature Engineering, University of Kashan, Kashan, Iran. Email: dehghanir@kashanu.ac.ir

2. Professor, School of Plant Science, The University of Arizona, Tucson, AZ, 85621 USA. Email: pessarak@arizona.edu

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1. Introduction

The application of agronomic techniques and the manipulation of environmental factors to enhance the active compounds in crops is a subject of significant interest and importance. This research was conducted to investigate the effects of cultivation techniques and nutritional methods on the essential oil yield of *Mentha spicata* L., a plant belonging to the family Lamiaceae (Chris et al., 2002; Peirce, 1999). *Mentha spicata* is a perennial plant, reaching 50 to 60 cm in height, with a characteristically quadrangular, often purple-colored, and smooth stalk (Foster, 1996; Cherr et al., 2006). Its leaves are short, oval, and grainy, and it produces purple flowers in the summer (Guo et al., 2024).

Global production of *Mentha* essential oil is approximately 8,000 tons per year. The primary constituents of the oil are menthol (29%), menthone (20-30%), and methyl acetate (1-3%). The oil, typically comprising 1.2-1.5% of the plant's volatile content, is usually extracted via steam distillation from the aerial parts at the initial flowering stage. Menthol and its esters constitute 30-70% of the oil, with the remainder consisting of over 40 other compounds, including flavonoids (12%), polymerized polyphenols (19%), carotene, tocopherol, betaine, and choline (Anonymous, 1990; Guo et al., 2022; Murray, 1995).

Pharmacologically, *Mentha* is known for its astringent, antiseptic, antispasmodic, antidote, antinociceptive, anticoagulant, antimicrobial, rubefacient, and stimulant properties. It is currently used in the treatment of irritable bowel syndrome (IBS), inflammatory bowel disease (Crohn's disease and ulcerative colitis), gallbladder inflammation, biliary system defects, and liver problems (Blumenthal, 1998; Yilmaz et al., 2024).

As a long-day plant (LDP), *Mentha spicata* exhibits increased production and yield under long-day conditions. Optimal soil moisture, sunlight, and nutrient availability are essential for maximizing both biomass and essential oil yield (Fleming, 1998; Omid Beigi, 1995).

Plant Growth-Promoting Rhizobacteria (PGPR) have been shown to enhance vegetative growth, leaf area index, the number of branches, and dry matter yield, which subsequently increases the essential oil percentage. For instance, Singh and Chatterjee (1989) reported that in *Mentha sativa*, the application of 150 g/ha PGPR resulted in the highest values for these desirable traits. Similarly, Anvar et al. (2005) found that 100 g/ha of PGPR fertilizer increased branch number, leaf pigments, dry matter, and essential oil yield. Valad Abadi et al. (2008) demonstrated a significant effect of PGPR on the essential oil yield of *Calendula officinalis* at the 1% probability level.

Research on other species supports these findings. Akbarinia et al. (2002) reported that increasing PGPR and phosphorus application increased grain yield in *Trachyspermum ammi* up to 90 and 60 g/ha, respectively. While chemical fertilizers did not affect the essential oil percentage, the combination of 60 g/ha PGPR with manure produced the highest grain and essential oil yield. Imam and Niknejad (2004) also observed that PGPR fertilizers improved leaf size, longevity, and shoot freshness. However, results can vary by species; Arabaci et al. (2004) concluded in a three-year study on basil that while the highest biological yield and a 1% essential oil yield were obtained with 50 g/ha PGPR, the highest essential oil concentration was observed without PGPR application.

The indiscriminate use of chemical fertilizers, including PGPR, without sufficient organic supplementation has led to a significant decline in organic matter in Iranian agricultural soils (Malekooti, 2008). Green manures-plants cultivated and incorporated into the soil to improve its physical, chemical, and biological properties-offer a solution to this problem by supplying nutrients for subsequent crops (Banchio et al., 2009). The combined use of green manures and PGPR can enhance nutrient availability and crop performance compared to the sole application of chemical fertilizers (Aktar et al., 1993; Pramanik et al., 2004). Talgre et al. (2009) further reported that using legumes as green manure facilitates the gradual release of nitrogen, thereby enhancing its absorption by plants over successive growth periods.

2. Materials and Methods

This study was conducted from spring to fall of 2023 using a split-plot factorial layout within a randomized complete block design (RCBD) with three replications.

2.1. Operation of cultivation

Rooted cuttings, 8 to 10 cm in length, were taken from 2- to 3-year-old *Mentha spicata* plants at the 3-leaf stage. Uniform cuttings in terms of size and height were selected and transplanted into 25 m² plots. The experiment was arranged as a split-plot factorial in a Randomized Complete Block Design (RCBD) with three replications. The treatments consisted of: Main plot factor: PGPR fertilizer at four levels (0, 50, 100, and 150 g/ha). One-third of the PGPR was applied at transplanting, with the remaining two-thirds applied at the 6- to 8-leaf stage (Balyan & Sobti, 1990). Sub-plot factors**:** Green manure treatments, which were: Control (no green manure), *Medicago sativa* L, *Trifolium alexandrinum* L, A combination of *M. sativa* and *T. alexandrinum*. The green manure crops were established in September 2023 and incorporated into the soil by ploughing in the fall of 2024. The mint was planted in early May. Irrigation was performed every 4 days to prevent drought stress. Weeding was conducted manually throughout the vegetative growth period to avoid chemical interference. No pest control was necessary, as no significant pest infestation occurred, likely due to the plant's natural scent.

2.2. Data Collection and Analysis

At harvest, fresh weight yield was recorded immediately. To determine dry weight yield, sub-samples were dried in the shade at room temperature (25 °C) for 10 days before weighing. The essential oil content was determined from shade-dried samples using a Clevenger apparatus with water distillation. The extracted oil was dehydrated using anhydrous sodium sulfate (Na_2SO_4). The essential oil percentage was calculated based on the dry weight of the plant material. Essential oil yield (kg/ha) was then calculated as: (Essential oil percentage × Dry biological yield) / 100. Data were analyzed using MSTAT-C software. Treatment means were compared using Duncan's Multiple Range Test at a 5% significance level.

Table (1): Physical and chemical properties of farm soil

Soil property	Value
Cu (ppm)	1.22
Mg (ppm)	23.5
Mn (ppm)	8.66
P (ppm)	10.34
K (ppm)	230.15
N (ppm)	0.09
$CaCO_3$ (ppm)	7.00
Texture	Loam
Sand (%)	34.00
Silt (%)	33.00
Clay (%)	25.00

3. Results and Discussion

3.1. Essential Oil Percentage

The results indicate that the essential oil percentage of *Mentha spicata* was significantly influenced by the experimental factors. The main effect of PGPR was significant ($p < 0.05$), with the highest essential oil percentage (0.35%) obtained from the application of 150 g/ha PGPR and the lowest (0.15%) from the control treatment (Tables 2 & 3). This aligns with findings by Omid Beigi (1995) and Anvar et al. (2005), who reported that PGPR application enhances branch yield, leaf number, leaf pigment, dry matter, and essential oil yield. Similarly, Bist et al. (2000) observed that PGPR fertilization increased the essential oil percentage and altered the composition of certain components in *Anethum graveolens*.

The individual effects of green manure incorporation were also significant. The use of *Medicago sativa* L. significantly increased the essential oil percentage ($p < 0.05$), with the highest value (0.43%) recorded in plots where it was incorporated, compared to the control (0.20%) (Tables 2 & 3). Likewise, the incorporation of *Trifolium alexandrinum* L. had a highly significant effect ($p < 0.01$), yielding the highest essential oil percentage (0.44%) against the control (0.25%) (Tables 2 & 3).

These improvements can be attributed to several factors. The incorporation of green manure likely enhanced soil structure, improving moisture retention which is critical for biomass production and, consequently, essential oil yield. Furthermore, the increased nutrient availability—particularly nitrogen from leguminous green manures and phosphorus from PGPR—facilitates the production of ATP and NADPH. These energy molecules are vital for the biosynthetic pathways of terpenoids and isoprenoids, the primary constituents of essential oils (Yang et al., 2022).

The two-way interactions were also significant. The combination of 150 g/ha PGPR with *Medicago sativa* L. produced the highest essential oil percentage (0.62%), while the control yielded the lowest (0.13%) (Tables 2 & 4). Similarly, the interaction between 150 g/ha PGPR and *Trifolium alexandrinum* L. resulted in a significantly higher essential oil percentage (0.41%) compared to the control (0.14%) (Tables 2 & 4). These results are consistent with Valad Abadi et al. (2008), who reported that PGPR increased the seed oil percentage and yield in *Calendula officinalis*, and Banchio et al. (2009), who found biological fertilizers effective in boosting biomass and essential oil yield in basil.

The interaction between the two green manures was significant at the 5% level (Table 2). The simultaneous incorporation of both *Medicago sativa* L. and *Trifolium alexandrinum* L. yielded the highest mean essential oil percentage (0.48%), significantly outperforming the control (0.16%) (Table 4).

Finally, the triple interaction between PGPR, *M. sativa*, and *T. alexandrinum* was highly significant ($p < 0.01$) (Table 2). The highest essential oil percentage (0.73%) was achieved with the combined application of 150 g/ha PGPR and both green manures, whereas the control treatment resulted in the lowest value (0.12%) (Table 5). This synergistic effect corroborates the findings of Akbarinia et al. (2002), who observed increased essential oil content and yield in *Trachyspermum ammi* with fertilizer application. They also noted that PGPR and phosphorus application improved grain yield, leaf size, longevity, branching, and overall plant vigor, factors that collectively contribute to enhanced essential oil production (Loomis & Croteau, 1972; Yuan et al., 2022).

Table (2): Analysis of variance of PGPR, *Medicago sativa* L., and *Trifolium alexandrinum* L.green manures on the percentage and yield of essential oil of *Mentha spicata*

SOV	df	MS	
		Essential oil Percentage	Essential oil yield
Replication	2	1.50^{ns}	6.33^{ns}
N	3	20.33^{*}	3.400^{*}
Error	6	15.50	3.75
Me.sa	1	22.36^{**}	7.24^{**}
Se.mo*Me.sa	3	50.40^{*}	2.70^{**}
Se.mo	1	45.50^{**}	1.363^{**}
N*Se.mo	3	11.685	2.241^{**}
Me.sa*Se.mo	1	50.81^{*}	2.48^{*}
N*Me.sa*Se.mo	3	80.30^{*}	2.031^{*}
Error	24	25.00	1.352
CV (%)	-	12.5	6.5

3.2. Essential oil yield

The essential oil yield of *Mentha spicata* was significantly influenced by the experimental treatments. The main effect of PGPR was significant ($p < 0.05$), with the application of 150 g/ha PGPR producing the highest yield (45.60 kg/ha), which was substantially greater than the control yield of 20.23 kg/ha (Tables 2 & 3). The progressive increase in oil yield with PGPR application rates from 0 to 150 g/ha (Table 4) underscores the role of PGPR in enhancing vegetative growth and total dry matter production per unit area.

The individual effects of both green manures on essential oil yield were highly significant ($p < 0.01$) (Table 2). The incorporation of *Medicago sativa* L. resulted in the highest yield (55.80 kg/ha), while *Trifolium alexandrinum* L. also significantly increased yield (49.50 kg/ha). In contrast, the control treatments (without green manure) yielded only 23.66 kg/ha and 21.13 kg/ha, respectively (Table 3).

A comprehensive analysis revealed that the triple interaction of PGPR, *M. sativa*, and *T. alexandrinum* had the most pronounced effect on both essential oil percentage and yield. This indicates a strong synergistic effect, where the combined application of these treatments was far more effective than their individual or two-way interactions.

Table (3): Comparison of the average effect of PGPR, *Medicago sativa* L. and *Trifolium alexandrinum* L.green manures on the percentage and yield of essential oil of *Mentha spicata*

Treatment	Essential oil Percentage	Essential oil yield (g/ha)
PGPR (g/ha)		
0	0.18d	20.20b
50	0.21c	25.50c
100	0.24b	31.42a
150	0.33a	43.60a
Medicago sativa green manure		
non-use	0.19b	22.60b
use	0.40a	54.75a
Trifolium alexandrinum green manure		
non-use	0.20b	20.16b
use	0.42a	45.58a

The interactions between PGPR fertilizer and the green manures-*Medicago sativa* L. and *Trifolium alexandrinum* L.-on essential oil yield were significant at the 1% and 5% levels, respectively (Table 2). The results demonstrate that the green manures complemented the PGPR, effectively meeting the plant's nutritional requirements. The highest essential oil yields were achieved with the combination of 150 g/ha PGPR and green manure: 80.30 kg/ha with *M. sativa* and 55.90 kg/ha with *T. alexandrinum*. In contrast, the corresponding control treatments produced the lowest yields, 11.26 kg/ha and 15.06 kg/ha, respectively (Table 4).The

interactions between PGPR fertilizer and the green manures-*Medicago sativa* L. and *Trifolium alexandrinum* L.-on essential oil yield were significant at the 1% and 5% levels, respectively (Table 2). The results demonstrate that the green manures complemented the PGPR, effectively meeting the plant's nutritional requirements. The highest essential oil yields were achieved with the combination of 150 g/ha PGPR and green manure: 80.30 kg/ha with *M. sativa* and 55.90 kg/ha with *T. alexandrinum*. In contrast, the corresponding control treatments produced the lowest yields, 11.26 kg/ha and 15.06 kg/ha, respectively (Table 4).

Table (4): Comparison of the average double interaction of PGPR, *Medicago sativa* L., and *Trifolium alexandrinum* L.on the Percentage and yield of essential oil of *Mentha spicata*

Treatment		Essential oil Percentage	Essential oil yield (g/ha)
PGPR (g/ha)	*Medicago sativa* green manure		
0	non-use	0.12e	10.28ef
	use	0.22d	32.75c
50	non-use	0.10e	13.05ef
	use	0.33c	42.55c
100	non-use	0.14e	21.43f
	use	0.36b	52.30b
150	non-use	0.22e	21.04e
	use	0.60a	81.35a
PGPR (g/ha)	*Trifolium alexandrinum* green manure		
0	non-use	0.13a	14.05d
	use	0.25b	25.25d
50	non-use	0.12c	20.33c
	use	0.26a	37.06ab
100	non-use	0.26bc	24.40c
	use	0.32a	43.38b
150	non-use	0.25bc	35.60c
	use	0.40a	55.50a
Medicago sativa green	*Medicago sativa* green manure		
non-use	non-use	0.14d	15.25d
	use	0.15c	20.33c
use	non-use	0.20b	27.32b
	use	0.45a	46.24a

The interaction between the two green manures, *Medicago sativa* L. and *Trifolium alexandrinum* L., had a significant effect on essential oil yield ($p < 0.05$, Table 2). The combined application of both green manures produced the highest yield of 48.13 kg/ha, which was substantially greater than the control yield of 15.39 kg/ha (Table 4).

Furthermore, the triple interaction of PGPR with both *M. sativa* and *T. alexandrinum* was highly significant ($p < 0.01$, Table 2), indicating a strong synergistic effect on essential oil yield.

Table (5): Comparison of the average triple interaction of PGPR, *Medicago sativa* L., and *Trifolium alexandrinum* L.green manures on the percentage and yield of essential oil of *Mentha spicata*

Average			Treatment	
Essential oil yield (g/ha)	Essential oil Percentage	*Trifolium alexandrinum* green manure	*Medicago sativa* green manure	PGPR (g/ha)
12.22i	0.13i	non-use	non-use	0
22.28gh	0.18gh	use		
30.09ef	0.20ef	non-use	use	
42.65cd	0.30c	use		
15.44i	0.14i	non-use	non-use	50
25.18h	0.20h	use		
3.46fg	0.25fg	non-use	use	
50.80de	0.30de	use		
22.33i	0.18i	non-use	non-use	100
30.65h	0.26gh	use		
45.66cde	0.33cd	non-use	use	
60.08b	0.45b	use		
22.33i	0.20i	non-use	non-use	150
35.55gh	0.25gh	use		
56.88bc	0.30bc	non-use	use	
90.30a	0.71a	use		

This study demonstrates a statistically significant effect of Plant Growth-Promoting Rhizobacteria (PGPR) on the essential oil yield of *Mentha spicata* L. ($p < 0.05$), indicating that these bacteria can meaningfully enhance secondary metabolite production in this species. These results are consistent with recent findings by Guo et al. (2024).

A clear, dose-dependent relationship was observed, where the highest essential oil yield of 45.60 kg/ha was achieved with 150 g/ha PGPR, significantly outperforming the control yield of 20.23 kg/ha. This pattern suggests that microbial inoculation promotes vegetative growth and biomass accumulation, thereby increasing oil yield. The magnitude of the effect observed in this study exceeds that reported in earlier work by Ebhin Masto et al. (2006).

The results further indicate that increasing the PGPR application rate from zero to 150 g/ha enhanced both the concentration of essential oil and the yield per unit area. This implies that PGPR contributes to greater dry matter production, which directly translates to a higher total output of essential oil.

A particularly striking finding was the highly significant triple interaction among PGPR, *Medicago sativa* L. (alfalfa), and *Trifolium alexandrinum* L. green manures. This combination exerted the most pronounced effect on both oil percentage and yield, indicating a strong synergistic relationship where the combined application surpasses the impact of any single factor or two-way interaction. These findings confirm and extend the results of Arabaci and Emine (2004).

The observed enhancements in essential oil metrics are attributable to improved plant vigor from PGPR and the improved soil fertility and microbe-plant interactions facilitated by the green manures. These synergistic interactions likely optimize nutrient availability, root development, and metabolic fluxes, thereby channeling resources more efficiently into the biosynthesis of secondary metabolites like essential oils.

4. Conclusions

This study demonstrates that the integrated application of PGPR and green manures significantly enhances essential oil production in *Mentha spicata* L. The most effective treatment was the combination of 150 g/ha PGPR with both *Medicago sativa* L. and *Trifolium alexandrinum* L. green manures, which yielded the highest essential oil output of 9.165 kg/ha. This result starkly contrasts with the lowest yield of 1.320 kg/ha from the control treatment, underscoring the efficacy of the integrated approach.

The positive outcomes are attributed to the role of these biofertilizers in improving soil fertility and plant nutrition. The synergistic interaction between PGPR and the leguminous green manures proved superior to any single-factor application, leading to increased biomass and a direct enhancement in essential oil yield.

Therefore, the combined use of PGPR and green manure, specifically *M. sativa* and *T. alexandrinum*, presents a viable and sustainable agronomic strategy. For farmers, especially smallholders, this practice offers a powerful alternative to reliance on chemical fertilizers, promoting a more ecological approach to cultivating high-value medicinal plants like spearmint.

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