Industrial Crops & Products 141 (2019) 111777

Contents lists available at ScienceDirect

Industrial Crops & Products

journal homepage: www.elsevier.com/locate/indcrop

Improving growth, phytochemical, and antioxidant characteristics of peppermint by phosphate-solubilizing bacteria along with reducing phosphorus fertilizer use

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ARTICLE INFO

Keywords:
Mentha piperita L.
Phosphate-solubilizing bacteria
Pseudomonas putida
Pantoea agglomerans
Menthol

ABSTRACT

Phosphorus is a vital nutrient for plant growth and development. Its deficiency in farmlands is obviated by the phosphate fertilizer application. Excessive consumption of phosphate fertilizers harms to human health and the environment. Some soil bacteria are capable to increase phosphate fertilizers efficiency. Two-year field experiment was conducted to evaluate the effect of phosphate-solubilizing bacteria inoculation with phosphorus fertilizer on growth, antioxidant status, and phytochemical features of peppermint (*Mentha piperita* L.). The treatments were three amounts triple superphosphate (0, 50, and 100 kg ha^{-1}) and PSB strains (*Pseudomonas putida* and *Pantoea agglomerans*) which were arranged as factorial based on randomized complete block design. The results showed that phosphorus supplying by chemical fertilizer or PSB improved peppermint growth features included stem number, leaf length, leaf number, and dry weights of leaf, stem, and plant. The contents of photosynthetic pigments increased by chemical or biological phosphorus fertilizer application. The application of PSB with triple superphosphate reduced soluble carbohydrates and increased protein. The antioxidant enzymes activities including catalase, peroxidase, ascorbate peroxidase, and polyphenol oxidase were increased by the PSB inoculation. In contrast, activities of these enzymes were reduced by the triple superphosphate application. The highest EO yield was obtained by *Pseudomonas putida* and *Pantoea agglomerans* inoculation as phosphate-solubilizing bacteria with 50 kg ha^{-1} triple superphosphate. The menthol content was increased in response to the triple superphosphate and the PSB inoculation. Therefore, PSB inoculation increased phosphate fertilizer efficiency which leads to reducing phosphate fertilizer consuming and increasing the plant biomass as well as EO yield.[1]

1. Introduction

Phosphorus (Pi) is a vital nutrient for plant growth and development and forms 0.2% of the plant's dry weight (Toth et al., 2014). This element interferes in many biochemical processes, energy supplying and signal transduction in plant cells (Azziz et al., 2012, Tak et al., 2012). In addition, phosphorus is a structural component of essential biomolecules such as proteins, phospholipids, DNA, RNA, and etc. (Plaxton and Lambers, 2015). Therefore, its deficiency reduces plant yield and growth (Marschner, 2012). Souza et al. (2014) reported that shoot dry weight of menthol mint (*Mentha arvensis* L.) was increased along enhancement Pi concentration in the nutrition solution. In contrast, menthol content was reduced with increasing Pi concentrations, while p-cymene and pulegone content were increased.

Pi makes 0.05% of the soil weight and is present in both inorganic and organic forms. However, plants often uptake it in inorganic forms ($H_2PO_4^{-1}$ and HPO_4^{-2}). Phosphorus is found abundant in the soil to an insoluble form. Because it is intensive susceptible to soil pH and forms a complex with aluminum, iron oxides, carbonates, and organic matter (Pereira and Castro, 2014, Alori et al., 2017, Ingle and Padole, 2017). Soil Pi deficiency in farmlands is obviated by the phosphate fertilizer application (Toth et al., 2014). Inorganic phosphorous fertilizers due to fixation in the soil are consumed over plants demand, which leads to environmental problems such as groundwater pollution, soil fertility

Abbreviations: Pi, phosphorus; P, triple superphosphate; PSB, phosphate-solubilizing bacteria; ROS, reactive oxygen species; EO, essential oil; GC/MS, gas chromatography coupled to mass spectrometry; FID, flame ionization detector

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https://doi.org/10.1016/j.indcrop.2019.111777
Received 13 May 2019; Received in revised form 31 July 2019; Accepted 9 September 2019

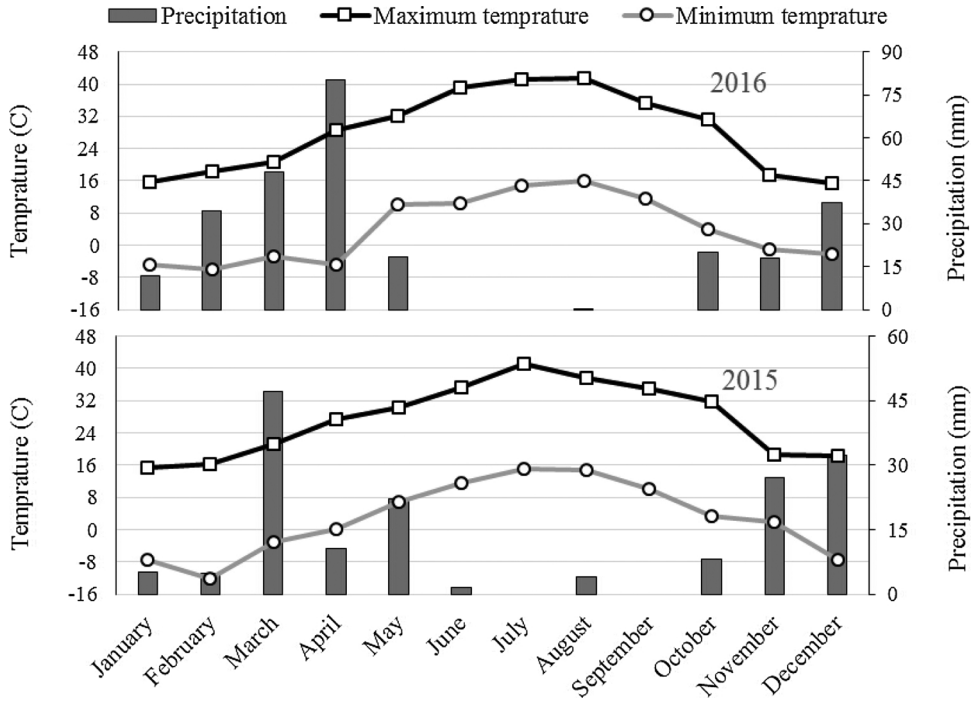

Fig. 1. Monthly variations of minimum and maximum temperatures and precipitation at 2015 and 2016.

decline, eutrophication, and toxic elements accumulation in the soil like selenium and arsenic (Kang et al., 2011, Plaxton and Lambers, 2015). Hence, implement management strategies for increase Pi fertilizer efficiency in order to improve crop yield and reducing environmental contaminations is necessary.

Some soil bacteria included *Pseudomonas* spp., *Agrobacterium* spp., *Bacillus* spp., and etc. are capable to increase solubilizing of soil Pi (Plaxton and Lambers, 2015). These microorganisms by secretion organic acids or enzyme phosphatase transformed insoluble Pi in mineral and organic compounds to soluble and absorbable forms for plants (Sharma et al., 2013, Ingle and Padole, 2017). There are numerous reports of increasing phosphate fertilizer efficiency when used with PSB (Richardson, 2001, Rehm and Lamb, 2009; Baas et al., 2016). Alzoubi and Gaibore (2012) reported that superphosphate fertilizer efficiency was increased by using *Bacillus megaterium*.

Several rhizospheric bacteria, in addition to mineral nutrition supplying, can promote plant growth by synthesizing phytohormones (such as auxins, gibberellins, and cytokinins), siderophores, antibiotics, vitamins, and etc. (Sokolova et al., 2011, Plaxton and Lambers, 2015). Ahmad et al. (2008) were expressed that synthesized phytohormones by rhizospheric bacteria are the main responsible factor in the bacteria interactions with the host plant. Sokolova et al. (2011) were found that new strains of *Azotobacter chroococcum*, *Bacillus megaterium*, and *Bacillus mucilaginosus* are able to increase indole-3-acetic acid (IAA) and cytokinins in cucumber seedling.

Oxidative stress occurred in many plants under P-deficiency via the reactive oxygen species (ROS) production (Bargaz et al., 2013, Israr et al., 2016). ROS are normal products of cellular activities in the mitochondria, chloroplast, cell membrane, and peroxisome. They are deleterious when being present in excess of plant antioxidant potency and can reduce plant growth and yield (Kandlbinder et al., 2004, Ishizawa et al., 2017). However, plants possess the enzymatic and non-enzymatic defense system to scavenge ROS (Masood et al., 2012, Israr et al., 2016). Moreover, it is reported that antioxidants up-regulation may have a negative effect on plant growth via interferences between developmental and stress-response networks (Cabello et al., 2014). Therefore, the highest levels of plant production are achieved when ROS and antioxidants are maintained at a low level. Apel and Hirt (2004) suggested oxidative stress may play a key role in determining the rhizobacteria beneficial and adverse effects on the plant. Thus, identifying the oxidative stress role in plant-bacteria interactions can help predict the rhizobacteria community effect on plant growth.

Peppermint (*Mentha piperita* L.) is a natural hybrid of water mint (*Mentha aquatic* L.) and spearmint (*Mentha spicata* L.) which cultivated all over the world (McKay and Blumberg, 2006, Sun et al., 2014). A main phytochemical compound of the peppermint is EO (Rita and Animesh, 2011). Menthol is the most important peppermint oil constituents, which creates a coolness sense in the mouth due to inhibit the TRPM8 channel in the neurons (Pan et al., 2012, McKemy, 2013). Clinical research has indicated the peppermint possesses an improving effect on upper gastrointestinal disorders, irritable bowel syndrome, muscle spasm, and respiratory problems (Kliger and Chaudhary, 2007, Adel et al., 2015). Peppermint requires a sufficient amount of mineral nutrition such as nitrogen and phosphorus to produce a maximum oil yield (Zheljazkov and Astatkie, 2012, Camen et al., 2017). It has been reported that peppermint absorbs about 20–40 kg phosphorus (P2O5), 170 kg potash, and 90–135 kg nitrogen from the soil during the growing season (Court et al., 1993).

This study aimed at the peppermint yield increasing through PSB inoculation. In addition, antioxidant status induced by PSB inoculation and its association with peppermint growth and phytochemical features was investigated.

2. Materials and methods

2.1. Filed location and weather information

A two-year field experiment was conducted as a factorial experiment based on randomized complete block design in the research farm of Medicinal Plants Institute, ACECR, Karaj, Iran (56° 35′ N and 50° 58′ E, 1500 m above sea level) during 2015 and 2016. Monthly variations of minimum and maximum temperatures and precipitation in 2015 and 2016 are presented in Fig. 1.

Table 1
Soil physicochemical characteristics of experimental farm since 2014–2016.

Year	Texture			K	P	N	Fe	Mn	Zn	Cu	pH	EC
	Clay (%)	Silt (%)	Sand (%)	(ppm)	(ppm)	(%)	(ppm)	(ppm)	(ppm)	(ppm)		(ds/m)
2014	16	22	62	177.4	9.2	0.075	4.81	4.9	0.6	0.7	7.2	1.2
2015	18	18	64	163.4	8.4	0.07	4.1	2.28	0.68	0.74	7.7	0.66
2016	19	20	61	195.6	11.3	0.08	6.4	5.42	0.89	0.85	7	1.72

2.2. Transplant preparation and field practices

Peppermint transplants obtained from three-node rhizomes which were cultured in a pot (12 × 14 cm) containing peat moss, vermiculite, and perlite (1:1:1). The pots were stored in a greenhouse for 30 days with 16/8 h light/dark photoperiod, 200 μM m^{-2} s^{-1} light intensity, temperature 28/20 °C day/night, and 60% relative humidity. All pots were fed twice a week with a Hoagland nutrient solution (510 ppm KNO_3, 1180 ppm $Ca(NO_3)_2$, 490 ppm $MgSO_4.7H_2O$, 80 ppm NH_4NO_3, 68 ppm KH_2PO_4, 0.1 mM Fe-EDDHA, 2.86 ppm H_3BO_3, 1.81 ppm $MnCl_2.4H_2O$, 51 ppb $CuSO_4.5H_2O$, 220 ppb $ZnSO_4.7H_2O$, 90 ppb $H_2MoO_4.4H2O$; pH 7.0). The transplants transported to the main farm on 4 April 2014. Each plot consisted of 7 cultivating rows with 40 cm distance from the other. Distance between the cultivated plants in the rows was 20 cm. A drip irrigation system was installed after the second year. Soil physicochemical characteristics were determinate before the preparation of the experimental plots as well as at the beginning of each year (Table 1). Also, 50 kg ha^{-1} urea, 150 kg ha^{-1} potash, 100 kg ha^{-1} iron sulfate, 30 kg ha^{-1} zinc sulfate, 40 kg ha^{-1} manganese sulfate, and 50 kg ha^{-1} magnesium sulfate were added to the soil each year at the beginning of the growing season. In addition, 75 kg ha^{-1} urea is used as top-dressing on 20 May 2014.

2.3. Bacterial strains culture, treatments application, and sampling

Treatments were included triple superphosphate (0، 50 and 100 kg ha^{-1}) and PSB, *Pseudomonas putida* and *Pantoea agglomerans*, inoculation. The PSB purchased from the Green Biotech Company in Iran. The bacterial species are confirmed by histochemical and morphological studies. Bacterial inoculums were cultured in liquid LB medium. It was held in an incubator at 28 °C and until reaching the exponential phase (24 h) was shaken with 120 rpm speed. Then, the medium culture was centrifuged (4500 × g, 10 min, 4 °C) and the pellet washed twice by sterile water, and adjusted to an ultimate concentration of ~10^9 (CFU)/mL. For the application of the treatment at 2015 and 2016, a groove along each row created and then chemical fertilizers and the bacterial inoculums (20 mL diluted in 2 liters sterile water) were poured into the groove. Sampling was carried out randomly at the beginning of flowering.

2.4. Plant growth evaluation

Plant height, lateral stem number, leaf length, leaf width and leaves number were measured after transfer the sample to the laboratory. Also, plant, leaf, and stem dry weight were measured after drying under the shade at room temperature.

2.5. Leaf chlorophyll and carotenoid contents

The chlorophyll and carotenoid contents were measured by Arnon (1949) method. 1 g frozen leaf was homogenized by a mortar and pestle with 80% acetone. The homogenate solution was filtered in 25 mL volumetric flask and was washed to form the colorless residual. The solution absorbance was recorded by a spectrophotometer at wavelengths of 663, 645, 510, and 480 nm, respectively. The chlorophyll and carotenoid content were calculated by the following equations and was expressed as mg g^{-1} fresh weight:

$$C_a = 12.7\ (A_{663}) - 2.69\ (A_{645}) \times V/1000W$$

$$C_b = 22.9\ (A_{645}) - 2.69\ (A_{663}) \times V/1000W$$

$$C_T = 20.2\ (A_{645}) + 8.02\ (A_{663}) \times V/1000W$$

$$Carotenoid = 7.6\ (A_{480}) - 14.9\ (A_{510}) \times V/1000W$$

2.6. Soluble carbohydrates

Soluble carbohydrate content was determinate according to Dubois et al. (1956) method. 0.2 g frozen leaf was extracted with distilled water (3 mL) by a mortar and pestle at 4 °C. The homogenate solution was filtered and its volume was brought to 10 mm. This solution (1 mL) was mixed with 5% aqueous solution of phenol (1 mL) in a test tube. Then concentrated sulfuric acid (3 mL) was added and the mixture was stirred for 30 s. The mixture solution was cooled in a water bath up to room temperature and then its absorbance was read at 480 nm. Carbohydrate content was calculated by the glucose standard curve and was expressed as mg g^{-1} fresh weight.

2.7. Protein and antioxidant enzyme

2.7.1. Extraction

0.5 g frozen tissue was crushed with liquid nitrogen via a mortar and pestle. Then, 5 mL extraction buffer (including 100 mM phosphate buffer, 0.1 mM EDTA, 1 mM ascorbate, and 2% PVP, pH 7) was added to the mixture and completely homogenized. The extract was centrifuged at 13,000 rpm and 4 °C for 20 min. The supernatant was used to determine soluble protein content and enzymatic activities (Matamoros et al., 2009).

2.7.2. Soluble protein

Soluble protein was determined according to Bradford (1976). The reaction mixture consisted of 50 μL extract, 5 mL Bradford reagent and 950 μL distilled water to 6 mL volume. The mixture absorption was read at 595 nm. The soluble protein concentration was determined using the bovine albumin standard curve and was expressed as mg g^{-1} fresh leaf weight.

2.7.3. Catalase (EC 1.11.1.6)

The Catalase activity was measured by Cakmak and Horst (1991) method. The reaction mixture was included 25 mM phosphate buff ;er (pH 6.8), 10 mM H_2O_2, and 200 μL extracted enzyme. H_2O_2 decomposition was recorded by absorbance reducing at 240 nm. The enzyme activity was expressed as μM H_2O_2 (ε = 43.6 mM^{-1} cm^{-1}) per minute per mg protein.

2.7.4. Ascorbate peroxidase (EC 1.11.1.11)

The reaction mixture was contained 50 mM phosphate buffer (pH 7), 0.5 mM ascorbate, 1 mM H_2O_2, 1 mM EDTA, and 200 μL extracted enzyme to 3 mL total volume. The reaction initiated with H_2O_2 adding, and the absorption was measured at 290 nm after 30 s. The enzyme

activity was expressed as μM oxidized ascorbate ($\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$) per minute per mg protein (Foyer et al., 1997).

2.7.5. Peroxidase (EC 1.11.1.7)

Peroxidase enzyme activity was measured according to the methods of Putter (1974) and Malik and Singh (1980). The reaction mixture was included 0.25 mL raw enzyme, 0.25 mL guaiacol (20 g/L), and 5.2 mL phosphate buff ;er 100 mM (pH 6.8). The reaction was beginning by adding 25 mL H_2O_2 (5 mM). Absorption increasing was recorded at 470 nm against a blank (0.25 mL guayacol 0.5%, 25 mL H_2O_2, 25 mL distilled water, and 2.25 mL phosphate buff ;er). The enzyme activity was expressed as μM tetraguaiacol ($\varepsilon = 26.6\ mM^{-1}\ cm^{-1}$) per minute per mg protein.

2.7.6. Polyphenol oxidase (EC 1.10.3.1)

The Polyphenol oxidase activity was measured by Waite (1976) method. The reaction mixture was included 1.45 mL phosphate buff ;er 100 mM (pH 6.8), 0.4 mL guaiacol 100 mM, and 50 μL extracted enzyme. The absorbance was read at 412 for 3 min every 30 s. The enzyme activity was expressed as μM quinine ($\varepsilon = 1010\ mM^{-1}\ cm^{-1}$) per minute per mg protein.

2.8. EO analysis

The EO was extracted by the hydro-distillation method in a Clevenger-type apparatus according to the European Pharmacopoeia (1997). A sufficient amount (200 g) of each dried sample well crushed and was boiled with water into a balloon until no more EO was obtained. The EO after cooling was collected by a syringe and its water removed by adding sodium sulfate. Then, the EO was stored at 4 °C until analyzed by GC–MS instruments. The experiment was repeated three times and their mean was reported as EO percent on the dried plant.

GC/MS analysis was performed on an Agilent instrument coupled with a 5973 Mass system equipped with flame ionization detector (FID) and a BPX5 capillary column (30 m × 0.25 mm; 0.25 μm film thicknesses). Temperature program includes oven temperature held for 2 min at 50 °C and was enhanced to 130 °C with 2 °C per min rate. Then, temperature enhancement was programmed up to 270 °C as 5 °C per min rate and this temperature held for 3 min. Other operating conditions include: carrier gas was He with a flow rate of 1 mL min^{-1}; injector and detector temperatures were 280 °C, and split ratio, 1:10. Mass spectra were taken at 70 eV. The mass spectra and retention indices of EO components were identified by comparison to published literature and presented the MS computer library (Adams, 2001, McLafferty, 1989).

2.9. Statistical analysis

All data were subjected to variance analysis by SAS 9.4 software. Means of main factors were compared by Duncan's multiple range tests at a 5% confidence interval and their interaction compared by Lsmeans procedure. Variance homogeneity was evaluated by the Bartlett test.

3. Results and discussion

3.1. Peppermint growth characteristic

Phosphorus fertilizer and phosphate solubilizing bacteria (PSB) had a significant effect on peppermint growth characteristics. The leaf characteristics included leaf number, leaf length, leaf width, and leaf dry weight improved with Pi supplying by the triple superphosphate or PSB (*Pseudomonas putida* and *Pantoea agglomerans*) inoculation (Table 2). The PSB inoculation with triple phosphate application had a synergistic effect on the peppermint leaf characteristics. The highest amount of the leaf number per plant (219.8), leaf length (4.41 cm), leaf width (2.04 cm), and leaf dry weight (575.2 kg ha^{-1}) was observed in the PSB use with 50 kg ha^{-1} triple phosphate (Table 2). The leaf characteristics response to increasing the P application was followed by a linear equation (Table 3). The application of 100 kg ha^{-1} P was caused to increment 24% dry leaf weight, 23% leaf number, 7.17% leaf length, and 8.15% leaf width compared with the control. In contrast, the leaf features response to use of the PSB with P was followed by a quadratic equation (Table 3). Hence, there was no a significant difference between the PSB + 50 kg ha^{-1} P with the PSB + 100 kg ha^{-1} P for the leaf number, leaf length, leaf width, and leaf dry weight (Table 2). Several studies reported Pi accessibility increment through chemical or biological fertilizer resulted in increasing leaf size and weight (Wang et al., 2010, Zayed, 2012; Tahami et al., 2017, Pagnani et al., 2018). The leaves number per plant and individual leaf size reduced in Pi deficiency condition. Also, leaf initiation in the apical meristem and its lateral expansion are adjusted with cell division activities (Chiera et al., 2002, Assuero et al., 2004; Tian et al., 2018). This may be related to the key role of Pi in the cell division program (Balemi and Negisho, 2012).

Plant height and lateral stem number were increased in response to the triple superphosphate. Also, the PSB inoculation had not significant synergistic effect on these traits. The highest amount of plant height was obtained in 2015 (57.6 cm) and 2016 (57.5 cm) at 100 and 50 kg ha^{-1} P, respectively.

The maximum number of the lateral stem was acquired in 100 kg ha^{-1} P (15.07). In contrast, stem dry weight had more increment when 50 kg ha^{-1} P used with the PSB inoculation (661.2 kg ha^{-1}) (Table 2). Likely, stem diameter increase plus plant height and stem number enhancing had an impress in stem dry weight improvement under P use with the PSB inoculation. The plant dry weight linearly increased in response to the P using (Table 3). Therefore, its greatest was obtained in 100 kg ha^{-1} P (1214.7 kg ha^{-1}). The PSB inoculation alone (1057.8 kg ha^{-1}) increased the plant dry weight compared with the control (954.8 kg ha^{-1}). The PSB inoculation with 50 kg ha^{-1} P raised 13.16% the plant dry weight compared with 50 kg ha^{-1} P without the PSB inoculation (1092.5 kg ha^{-1}). Several studies reported that increasing soil Pi accessibility through chemical or biological fertilizer resulted to increment plant dry weight (Pereira and Castro, 2014, Israr et al., 2016; Ishizawa et al., 2017). Positive correlation was found between plant dry weight with leaf number ($r = 0.83$; $p \leq 0.01$), leaf length ($r = 0.61$; $p \leq 0.05$), photosynthesis pigments such as chlorophyll *a* ($r = 0.89$; $p \leq 0.01$), chlorophyll *b* ($r = 0.81$; $p \leq 0.01$), total chlorophyll ($r = 0.78$; $p \leq 0.01$), carotenoids ($r = 0.92$; $p \leq 0.01$), and lycopene content ($r = 0.82$; $p \leq 0.01$). Therefore, it can be concluded that soil Pi supplying resulted to the increment leaf area and help to reception solar radiation. In addition, phosphorous supplying due to increasing photosynthetic pigments and subsequently raising the photosynthesis rate caused to improve dry matter accumulation and increasing peppermint dry weight. In addition, phosphate-solubilizing bacteria can promote plant growth via increase availability of trace elements such as Fe and Zn, plant growth regulators synthesis, and etc. (Israr et al., 2016).

3.2. Photosynthesis pigments

The P and PBS using have a significant positive effect on the content of chlorophyll *a*, b, total chlorophyll, carotenoid, and lycopene. PSB inoculation was more effective in raising chlorophyll b content (14% increase for chlorophyll b compared with 7.4% enhancing for chlorophyll *a*) and in contrast, P was more impressive in elevating chlorophyll *a* content (on average, 21.5% augmentation for chlorophyll *a* as compared with 16.3% enhancing for chlorophyll b) (Table 4). The content of total chlorophyll increased in response to use of 50 (1.43 mg g^{-1} FW) and 100 kg ha^{-1} (1.56 mg g^{-1} FW) P compared with the control (1.24 mg g^{-1} FW). Single the PSB inoculation had no significant difference with the control in total chlorophyll content, but when it was used with 50 kg ha^{-1} P enhanced this trait (Fig. 2a). Triple superphosphate increased total carotenoid and lycopene content than the control (0.23 and 0.11 mg g^{-1} FW, respectively). In compared with

Table 2
Means comparison of peppermint growth characteristic at different P amounts and the PSB inoculation.

PSB	P	LN	LL	LW	PH (Cm)		SN	LDW	SDW	PDW
			(Cm)	(Cm)	2015	2016		(kg ha^{-1})	(kg ha^{-1})	(kg ha^{-1})
0	0	167.5^{d}	4.04^{d}	1.84^{d}	49.7^{b,c}	53.1^{c}	12.5^{b,c}	454.3^{c}	500.5^{d}	954.8^{c}
0	50	188.9^{c}	4.23^{c}	1.93^{b,c}	52.6^{a,b}	57.5^{a}	14.2^{a,b}	512.3^{b}	580.2^{b,c}	1092.5^{b}
0	100	206.1^{b}	4.33^{a,b}	1.99^{a,b}	57.6^{a}	53.9^{b,c}	15.07^{a}	563.7^{a}	651^{a}	1214.7^{a}
1	0	189.6^{c}	4.27^{b,c}	1.92^{c}	45.5^{c}	55^{b}	11.47^{c}	497.5^{b}	560.3^{c}	1057.8^{b}
1	50	219.8^{a}	4.41^{a}	2.04^{a}	54^{a,b}	52.8^{c}	13.83^{a,b}	575.2^{a}	661.2^{a}	1236.3^{a}
1	100	215^{a,b}	4.34^{a,b}	2^{a}	54^{a,b}	52.3^{c}	13.64^{a,b}	560.7^{a}	630^{a,b}	1190.7^{a}

Means in each column with same letters have no significant difference at $p \leq 0.05$.
LN: Leaf number LL: Leaf length; LW: Leaf width; PH: Plant height; SN: Stem number; LDW: Leaf dry weight; SDW: Stem dry weight; PDW: Plant dry weight.

Table 3
The leaf characteristics response to use the various P amounts alone and along with the PSB inoculation.

Traits	P	P + PSB
LN	$y = 148.9 + 19.3x$; $r^2 = 0.99$	$y = 189.62 - 0.007x^2 + 0.93x$; $r^2 = 0.91$
LL	$y = 3.91 + 0.145x$; $r^2 = 0.97$	$y = 4.14 - 0.0001x^2 + 0.012x$; $r^2 = 0.93$
LW	$y = 1.77 + 0.075x$; $r^2 = 0.98$	$y = 1.81 - 0.0001x^2 + 0.013x$; $r^2 = 0.94$
LDW	$y = 400.7 + 54.7x$; $r^2 = 0.99$	$y = 508.25 - 0.016x^2 + 1.993x$; $r^2 = 0.97$
SDW	$y = 426.73 + 75.25x$; $r^2 = 0.99$	$y = 575.25 - 0.036x^2 + 3.91x$; $r^2 = 0.92$
PDW	$y = 827.43 + 129.95x$; $r^2 = 0.99$	$y = 1083.5 - 0.054x^2 + 6.23x$; $r^2 = 0.93$

LN: Leaf number; LL: Leaf length; LW: Leaf width; LDW: Leaf dry weight; SDW: Stem dry weight; PDW: Plant dry weight.

Table 4
Means comparison of photosynthesis pigments at different P amounts and the PSB inoculation.

Phosphorus		Chl a (mg g^{-1} FW)	Chl b (mg g^{-1} FW)
P	0	0.86^{b}	0.55^{b}
	50	1.06^{a}	0.63^{a}
	100	1.03^{a}	0.65^{a}
PSB	+	1.02^{a}	0.65^{a}
	–	0.95^{b}	0.57^{b}

Chl a: Chlorophyll *a*.; Chl b: Chlorophyll *b*.

the control, total carotenoid significantly increased by the PSB inoculation alone (12.17%) but lycopene content had not a significant difference. The PSB inoculation associated using 50 kg ha^{-1} P was the highest content of carotenoid and lycopene (0.3 and 0.24 mg g^{-1} FW, respectively) (Fig. 2b and c).

Han and Lee (2005) showed that total chlorophyll content of lettuce (*Lactuca sativa* L.) was increased by two PGPR strains inoculation, *Serratia* sp. and *Rhizobium* sp., under different soil salinity conditions. The chlorophyll content augmentation in response to bacterial strains inoculation could be due to increment mineral nutrition accumulation. (Mathivanan et al., 2017). Israr et al. (2016) were found a high positive correlation between chickpea (*Cicer arietinum* L.) chlorophyll content and the increase mineral uptake like N, Pi, and Ca after *Pseudomonas putida* inoculation.

3.3. Soluble carbohydrate and protein

Use of the P and PSB inoculation had a significant effect on the soluble carbohydrates and protein content. As compared with the control, the PSB inoculation alone reduced the content of soluble carbohydrate in 2015 (27.8%) and 2016 (16.2%). Changes in protein content by the PSB inoculation were dependent on the year effect. Thus this trait significantly increased in 2015 (10.9%) and reduced at 2016 (8.7%) as compared with the control (Table 5). The chemical and biological phosphate fertilizer reduced peppermint soluble carbohydrate and, on the contrary, increased its protein content. Only 100 kg ha^{-1} P significantly reduced the soluble carbohydrate and increased the protein content. The PSB inoculation alone had no significant impact on protein content. However, the PSB inoculation combined with 50 kg ha^{-1} P had a synergic impress on enhancement the protein content and reduction the soluble carbohydrate content (Fig. 3). Moreover, it has been shown that Pi has a key role in protein structure and its synthesis (Marschner, 2012). Zhang et al. (2017) reported phosphorous application increased the protein concentration in wheat grain but overuse phosphorus fertilizer reduces the protein content. Maybe for that reason, the content of peppermint protein decreased to 11.1% when 100 kg ha^{-1} P was applied with the PSB inoculation.

The results showed that the P availability in soil resulted to decrease soluble carbohydrate content in peppermint, and its protein content increased. These finding confirmed by other studies (Erel et al., 2016, Kurokura et al., 2017; Mathivanan et al., 2017). It is demonstrated that increasing cytosolic Pi concentration lead to activate phosphate-triose phosphate transporter located on the chloroplast membrane. After transfer glyceraldehyde 3-phosphate to the cytoplasm, soluble carbohydrates like glucose and fructose are formed. Sucrose is then synthesized from soluble carbohydrates and transferred to the sink after loading into the phloem (Marschner, 2012). In this study a negative correlation was obtained among soluble carbohydrate content with leaf dry weight ($r = -0.8$; $p \leq 0.01$) and plant dry weight ($r = -0.77$; $p \leq 0.05$). These may be indicated that the soluble carbohydrates expended for biomass production.

3.4. Antioxidant enzymes

Antioxidant enzymes include catalase, peroxidase, ascorbate peroxidase, and polyphenol oxidase significantly affected by the P and PSB inoculation (Table 6). Catalase activity in 2016 was more than 2015 (16.06%), on the contrary, peroxidase activity in 2015 was more than 2016 (52.34%). The results showed antioxidant enzymes activity include catalase, peroxidase, ascorbate peroxidase in 2015, and polyphenol oxidase significantly raised by The PSB inoculation. These findings are similar to some studies (Israr et al., 2016, Ishizawa et al.,

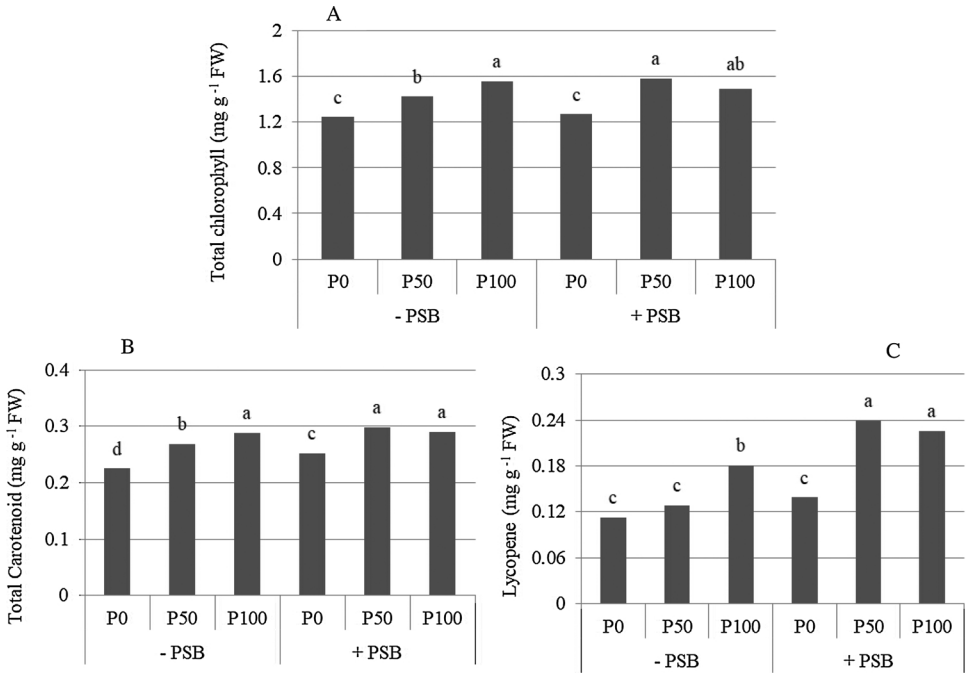

Fig. 2. Effect of different P amounts and the PSB inoculation on peppermint photosynthetic pigments. A: Total chlorophyll; B: Total carotenoid; C: Lycopene.

Table 5
Means comparison of soluble carbohydrate and protein content at different years and the PSB inoculation.

year	PSB	Soluble carbohydrate (mg.g^{-1} FW)	Protein (mg g^{-1} FW)
2015	–	42 ^{a}	61.5 ^{c}
	+	30.32 ^{b}	68.2 ^{ab}
2016	–	39.5 ^{a}	68.9 ^{a}
	+	33.1 ^{b}	62.9 ^{bc}

Means in each column with same letters have no significant difference at $p \leq 0.05$.

Table 6
Means comparison of peppermint leaf biochemical characteristic at different P amounts and the PSB inoculation.

PSB	P	CAT	PPO	APX		POX (μM mg^{-1} Pro min^{-1})	
		(μM mg^{-1} Pro min^{-1})	(μM mg^{-1} Pro min^{-1})	2015	2016	2015	2016
0	0	37.8^{b}	4.48^{b}	120.3^{b}	206^{a}	3.6^{b,c}	2.7^{b,c}
0	50	17.75^{c}	3.5^{d}	107^{b,c}	140^{b}	2.4^{c,d}	2.3^{c,d}
0	100	15.62^{c}	2.97^{e}	63.3^{c}	77.3^{c}	1.6^{d}	2.2^{d}
1	0	53.35^{a}	5.55^{a}	379.3^{a}	210^{a}	11.7^{a}	4.03^{a}
1	50	23.78^{c}	3.95^{c,d}	111.3^{b,c}	144.3^{b}	3.8^{b}	3^{b}
1	100	37.4^{b}	4.3^{b,c}	144.3^{b}	165.3^{c}	4.2^{b}	3.7^{a}

Means in each column with same letters have no significant difference at $p \leq 0.05$.
CAT: Catalase; PPO: Peroxidase; APX: Ascorbate peroxidase; POX: Polyphenol oxidase.

2017). Ishizawa et al. (2017) showed bacterial strains, *Aquitalea magnusonii* H3 and *Pseudomonas otitidis* M12, caused to increase $O_2^{\cdot -}$ content in inoculated plants. One of the plants immune reaction to microorganisms detection is production $O_2^{\cdot -}$ through microbe-associated molecular patterns (MAMPs) such as chitin or flagellins (Smith et al., 2015). Furthermore, it has been shown $O_2^{\cdot -}$ production from the

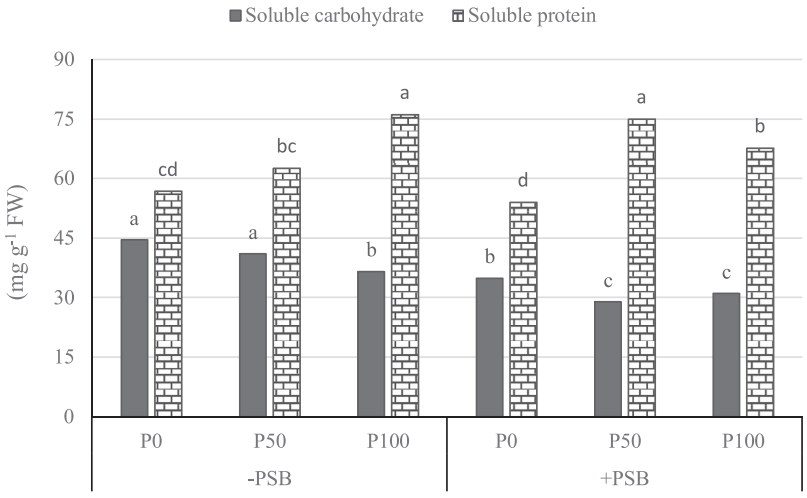

Fig. 3. Effect of different P amounts and the PSB inoculation on peppermint soluble carbohydrates and protein content.

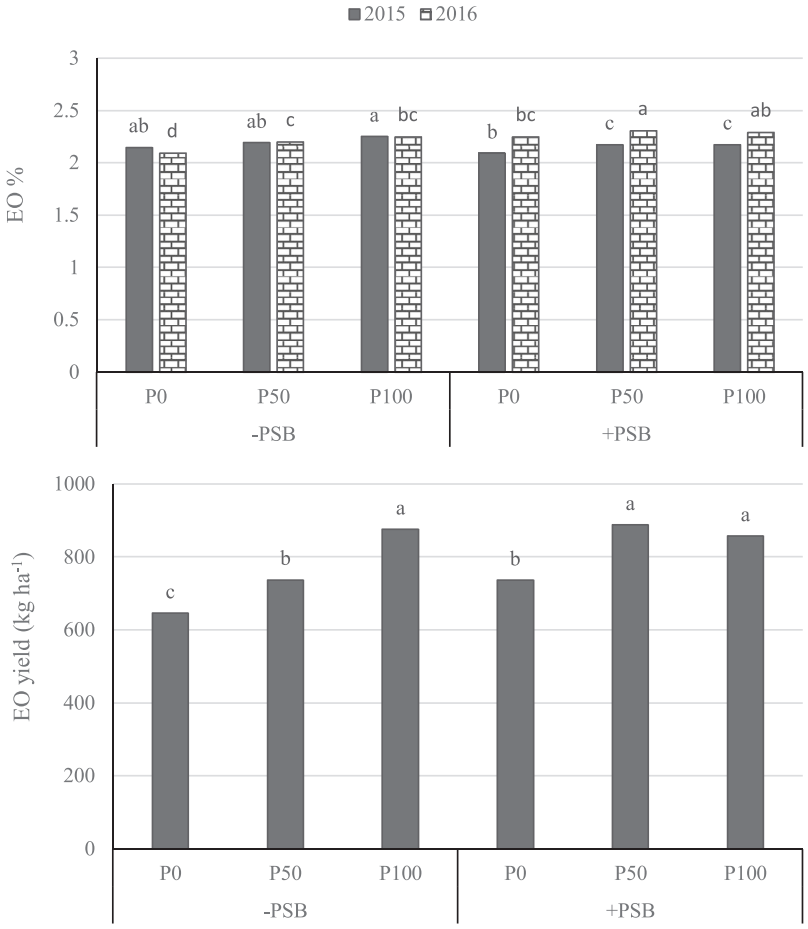

Fig. 4. Effect of different P amounts and the PSB inoculation on peppermint EO content and yield.

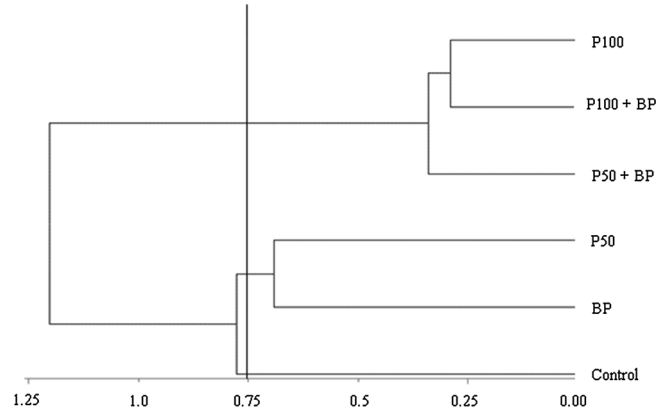

Fig. 5. Dendrogram obtained by cluster analysis based on euclidean distance performed on traits.

Table 8
Means comparison of EO components at different P amounts and the PSB inoculation.

PSB	P	1, 8- Cineole	Menthone	Menthol	Menthyl acetate	
					(%)	
		(%)	(%)	(%)	2015	2016
0	0	6.75 ^{bc}	20.3 ^{a}	34.67 ^{d}	2.82 ^{b}	3.05 ^{c}
0	50	7.31 ^{a}	18.05 ^{bc}	36.43 ^{bc}	3.12 ^{a}	3.26 ^{c}
0	100	7.17 ^{ab}	16.16 ^{e}	37.9 ^{ab}	3.32 ^{a}	4.52 ^{ab}
1	0	7.1 ^{abc}	18.9 ^{b}	35.68 ^{cd}	2.65 ^{b}	4.3 ^{ab}
1	50	6.58 ^{c}	16.76 ^{ed}	39.49 ^{a}	3.22 ^{a}	4.88 ^{a}
1	100	7.18 ^{ab}	17.25 ^{cd}	37.75 ^{b}	3.22 ^{a}	4.04 ^{b}

Means in each column with same letters have no significant difference at $p \leq 0.05$.

NADPH oxidase activity is a signal molecule for the establishment of plant-bacterial symbiosis (Nanda et al., 2010). Therefore, increasing the antioxidant activity in plants inoculated with PGPR is an immune response for ROS scavenging (Table 6).

Table 7
Means comparison of EO components at different years.

Year	Limonene (%)	Pulegone (%)	Menthone (%)	Menthol (%)	Menthyl acetate (%)	Germacrene D (%)	Caryophyllene (%)
2015	2.5 ^{b}	0.65 ^{b}	25.16 ^{a}	33.77 ^{b}	3.06 ^{b}	3.27 ^{a}	3.2 ^{a}
2016	3.78 ^{a}	2.8 ^{a}	10.64 ^{b}	40.21 ^{a}	4.01 ^{a}	1.84 ^{b}	2.04 ^{b}

Means in each column with same letters have no significant difference at $p \leq 0.05$.

Table 9
Means comparison of EO components at different years and the PSB inoculation.

Year	P	Limonene (%)	Pulegone (%)
2015	0	2.6 [a]	0.78 [a]
	50	2.63 [a]	0.6 [b]
	100	2.23 [b]	0.6 [b]
2016	0	3.97 [a]	3.72 [a]
	50	3.42 [a]	2.27 [b]
	100	3.96 [a]	2.39 [b]

Means in each column with same letters have no significant difference at $p \leq 0.05$.

The lowest antioxidant enzymes activity was obtained in 100 kg ha^{-1} P using (Table 6). Plant nutrition deficiency such as Pi is responsible to increase ROS concentrations and antioxidant enzymes activity (Chen et al., 2015). Israr et al. (2016) reported peroxidase activity increased under Pi deficiency, but in contrast, superoxide dismutase activity was not significant difference with Pi full access. Hence, increase use of the P in this study may be by increasing Pi accessibility caused to reduce the activity of all antioxidant enzymes as compared with the control. Also, we found a negative correlation among the activity of catalase (r = - 0.69; $p \leq$ 0.05) and polyphenol oxidase (r = - 0.66; $p \leq$ 0.05) with the total chlorophyll content. Rezaei-Chiyaneh et al. (2018) obtained similar results in black cumin (*Nigella sativa* L.). They have reported a negative relation between chlorophyll content and antioxidant enzymes activity. The antioxidant enzymes activity increased subsequent of ROS generation and it has been demonstrated that ROS generation in leaf tissues reduces chlorophyll and carotenoid content (Kiani et al., 2008, Chéour et al., 2014).

3.5. EO yield

EO content in 2015 had not a significant change by using the P and the PSB as compared with the control (2.15%). In contrast, the P application and the PSB inoculation in 2016 significantly increased EO content. The PSB use with different P amounts decreased the EO content in 2015 and increased in 2016 (Fig. 5). The positive impact of the P using and the PSB inoculation in 2016 may be related to increasing the soil soluble phosphorus. Soil soluble Pi in 2016 was 34.5% more than in 2015 (Table 1). It can be due to the use of phosphate fertilizer in consecutive years and the environmental and climatic factors impact. Also, the increment soil Pi availability in 2016 resulted to more improve growth characteristics than 2015.

Phosphorous plays an important role in the biosynthesis of EO compounds. Isopentenyl diphosphate and dimethylallyl pyrophosphate is a precursor to terpenoid compounds which have high energy phosphate bonds. Phosphorus is present in ATP and NADPH structure which these molecules required for the terpenoids biosynthesis. In addition, phosphorus availability plays a key role in terpenoids storage and reduced their emissions. Cell membrane stability and integrity reduced in Pi deficiency conditions due to phospholipids reduction. In this situation, isoprene emission compensates this damage. The isoprene compounds bonded to the phosphorus of membrane phospholipids and increase the membrane stability (Ormeño and Fernandez, 2012). Therefore, increase Pi accessibility by using the triple superphosphate and the PSB inoculation lead to increase in EO synthesis and accumulation. Esmaeil Zade et al. (2019) reported *Streptomyces* strains improved peppermint EO content. Also, similar results obtained from marigold (*Tagetes minuta*) inoculated with *Pseudomonas fluorescens* and *Azospirillum brasilense* (Cappellari et al., 2013).

Essential oil yield linearly increased in response to the P application. Single the PSB inoculation increased 14.1% EO yield as compared with the control (646.2 kg ha^{-1}). The PSB used with 50 kg ha^{-1} P (737.3 kg ha^{-1}) significantly increased EO yield than the non-inoculated plants. However, EO yield in plant treated by the PSB inoculation plus 100 kg ha^{-1} P was not a significant difference with the plant fertilized by 100 kg ha^{-1} P (Fig. 4). Similar to these results Bhandari et al. (2015) expressed the use of phosphate fertilizer at least 30 kg ha^{-1} increased the content and yield of basil (*Ocimum basilicum*) EO. Said-Al Ahl and Hussien (2016) reported using 30 kg ha^{-1} phosphate fertilizer increased winter savory (*Satureja montana* L.) dry weight and EO content. It is obvious that increased plant dry weight and EO content by using triple superphosphate and the PSB inoculation is responsible for increase EO yield. Hence, a positive correlation was found between EO yield and leaf dry weight (r = 0.94; $p \leq$ 0.01), leaf number per plant (r = 0.92; $p \leq$ 0.01), leaf length (r = 0.8; $p \leq$ 0.01), leaf width (r = 0.72; $p \leq$ 0.01), and plant dry weight (r = 0.90; $p \leq$ 0.01). Moreover, oil content had a positive correlation with leaf number per plant (r = 0.72; $p \leq$ 0.01), leaf length (r = 0.67; $p \leq$ 0.01), leaf width (r = 0.59; $p \leq$ 0.05), and leaf dry weight (r = 0.59; $p \leq$ 0.05).

3.6. EO components

Limonene, pulegone, menthol, and menthyl acetate content in 2016 was more than in 2015. In contrast, menthone, Germacrene D, and caryophyllene amount significantly reduced in 2016 (Table 7). Some studies reported EO composition change in different years due to environmental and climatic changes. Yesil and kara (2016) reported that spearmint (*Mentha spicata* L.) EO composition was different in various years. So that pulegone, 1, 8- cineole, and 4-terpineol contents were higher in 2011 and the carvone and piperitone were reduced in that year.

The results showed various P amounts increased 1, 8- Cineole, menthol, and menthyl acetate, but reduced menthone and pulegone content (Tables 8 and 9). Inverse to the results, phosphate fertilizer application in spearmint (*Mentha spicata* L.) reduced 1, 8- cineole amount and increased pulegone content (Yesil and kara, 2016). Ghaedi Jeshni et al. (2017) reported 150 kg ha^{-1} phosphate fertilizer increased chamazulene and bisabolo oxide B content in chamomile (*Matricaria recutita* L.).

Table 10
Pearson correlation coefficients between some EO components and leaf dimensions.

	LW	LL	EO	Limonene	Menthone	Menthol	Pulegone	MA
LL	0.97 **							
EO	0.59 *	0.67 *						
Limonene	0.73 **	0.61 *	0.26					
Menthone	−0.92 **	−0.86 **	−0.58 *	−0.88 **				
Menthol	0.92 **	0.90 **	0.78 **	0.73 **	−0.94 **			
Pulegone	0.64 *	0.48	−0.10	0.85 **	−0.72 **	0.49		
MA	0.79 **	0.81 **	0.85 **	0.63 *	0.80 **	0.89 **	0.27	
Menthofuran	0.86 **	0.76 **	0.41	0.93 **	−0.97 **	0.84 **	0.82 **	0.69 *

*Significant at $p \leq 0.05$, ** significant at $p \leq 0.01$.
LL: Leaf length; MA: Menthyl acetate; LW: Leaf width.

Menthyl acetate increased in 2016 (48.2%) by the PSB inoculation, however, menthone amount reduced (6.9%) as compared with the control. Also, menthofuran content reduced in plant inoculated with the PSB (6.02%) than non-inoculated plant (7.37%). The PSB inoculation increased twofold pulegone content in 2015. However, it has reduced (43.1%) in 2016 compared with the non-inoculated plant. Esmaeil Zade et al. (2019) were reported that some *Streptomyces* strains increased menthol, menthone, isomenthone, and piperitone in peppermint. Together use the P with the PSB inoculation had a positive effect on menthol and menthyl acetate increment content. Plant treated with 50 kg ha^{-1} P and the PSB inoculation had the highest menthol (39.49%) and menthyl acetate (3.22% in 2015 and 4.88% in 2016) amounts (Table 8).

Menthone had a negative correlation with menthol, menthofuran, and pulegone (Table 10). Menthol pathway after pulegone is divided into two directions which included menthofuran and menthone biosynthesis. Reports have shown that menthol content reduced by the increasing menthofuran amount because it has a competitive effect on the pulegone reductase enzyme and prevents the menthone biosynthesis (Rios-Estepa et al., 2008). The results of this study showed that menthol increasing was along with the enhancement of menthofuran. It is possible due to increasing menthofuran after the middle stage of leaf development. Because after this stage the menthone content that is the maximum amount, reduced by converting to menthol (Rios-Estepa et al., 2008). Therefore, menthone biosynthesis prevention by menthofuran accumulation leads to its content decline and increasing menthol and menthofuran contents. The PSB inoculation in peppermint reduced menthofuran production and increased menthone conversion to menthol.

Menthol and menthyl acetate had a positive and significant correlation with leaf length and width (Table 10). Other studies have been shown menthol content was higher in adult and larger leaves (Turner et al., 2000, Rios-Estepa et al., 2008). Therefore, leaf size can be used as an indicator for determining the amount of menthol and menthyl acetate.

3.7. Cluster analysis of treatments based on the PSB and P impact on traits

The cluster analysis results showed the treatments placed on three groups. The PSB inoculation alone and single-use 50 kg ha^{-1} P were placed on a cluster. 100 kg ha^{-1} triple superphosphate, 50 kg ha^{-1} P use with the PSB inoculation, and 100 kg ha^{-1} P use with the PSB inoculation were placed on a cluster (Fig. 5). These results showed the PSB inoculation is able to reduce phosphate fertilizer application to half.

4. Conclusion

Phosphorus supplying by chemical fertilizer or PSB improved peppermint growth characteristics. Photosynthetic pigments increased by usage of chemical or biological phosphorus fertilizer. The PSB inoculation increased antioxidant enzymes activity and triple superphosphate reduced them. Understanding of PSB microorganism and phosphate fertilizer effect on peppermint EO components needs more physiological and molecular evaluation. However, the highest quantitative and qualitative of EO yield in this study was obtained by *Pseudomonas putida* and *Pantoea agglomerans* inoculation with 50 kg ha^{-1} triple superphosphate. Therefore, it can conclude that combined PSB inoculation with phosphate fertilizer caused to reduce phosphate fertilizer consuming by increasing fertilizer efficiency.

Acknowledgement

We thank the Medicinal Plants Research Centre, Institute of Medicinal Plants, ACECR, Karaj, Iran for laboratory facilities and equipment availability.

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