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QUANTITY AND QUALITY YIELD OF ESSENTIAL OIL FROM *Mentha × piperita* L. UNDER FOLIAR-APPLIED CHITOSAN AND INOCULATION OF ARBUSCULAR MYCORRHIZAL FUNGI

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ABSTRACT

Peppermint (*Mentha × piperita* L.) is cultivated for its benefits in pharmaceutical, medicinal, and cosmetic industries. The well-known essential oil of *Mentha × piperita* L. is widely produced and used all over the world. The aim of present study was to evaluate the impacts of different concentrations of chitosan on the quality and quantity of the essential oil from the aerial parts of peppermint under inoculation of the rhizomes of peppermint seedlings with arbuscular mycorrhizal fungi. Experimental treatments were arranged as factorial design in a completed random block design. The highest essential oil yield (2.4 mL 100 g^{-1} dry matter) was obtained from the peppermint plants under foliar sprayed at 5 g L^{-1} chitosan along the inoculum with arbuscular mycorrhizal fungi. For evaluation of phytochemical characteristics, the contents of the main constituents of the peppermint essential oils such as menthol, menthone, etc. (oxygenated monoterpenes and monoterpenes hydrocarbons) under different treatments were analyzed by GC-FID and GC/MS. Results indicated that using chitosan foliar meaningfully raised the amount of menthol, as the major constituent and quality index (>60% v/w), in the essential oil from the peppermint plants inoculation with arbuscular mycorrhizal, however, the plants under the foliar spray of chitosan (without inoculum) revealed the highest amounts of menthone and limonene. In conclusion, we found that the foliar-applied chitosan along inoculation with arbuscular mycorrhizal fungi can be improved quantity and quality active substances of *Mentha × piperita* L. such as the contents of essential oil, menthol, and balance menthol/menthone.

Key words: *Mentha × piperita* L., essential oil, chitosan, plant regulators, brassinosteroids, mycorrhizal fungi

Abbreviations: GC-FID – gas chromatography–flame ionization detector, GC/MS – gas chromatography/mass spectrometry, AMF – arbuscular mycorrhizal fungi

INTRODUCTION

Peppermint (*Mentha × piperita* L.) belonging to the family Lamiaceae has many economical values for its valuable impacts in various types of industrial productions [Toghyani et al. 2010]. This medicinal and aromatic plant has four-sided stem and is a perennial, which can reach 100 cm in height. The leaves are stalked opposite and toothed. The flowers are irregular in shape, they are pinkish or purplish [Bupesh et al. 2007]. The aerial parts of the herb have between 0.5 to 4% (v/w) essential oil. The major components of essential oil from the aerial parts of the herb are menthol and menthone [Samber et al. 2015]. In many studies, the fungicidal [Desam et al. 2017], antitumor [Lazutka et al. 2001], antibacterial [İşcan et al. 2002], antiox-

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idant [Dragland et al. 2003], antiviral [Minami et al. 2003], and antiallergenic [Satsu et al. 2004] activities as well as hepatic and renal actions [Vo et al. 2003] of secondary metabolites of this herb have been reported.

With respect to the healthy advantageous, the demand of essential oils for different industries is anticipated to grow [Aziz et al. 2018]. Breeding and genetic studies have been initiated to enhance the quality and quantity of essential oils [Kong et al. 2019]. However, these activities are so time consuming, costly, and in some cases not ecofriendly. Other strategy in order to increase essential oil in medicinal and aromatic plants under various environmental conditions is exogenous application of inexpensive synthetic and natural chemicals such as chitosan, etc. [El-Din and El-Wahed 2005, Aşcı et al. 2018, Vosoughi et al. 2018]. Chitosan is a polysaccharide, relatively reactive and could be constructed in a large number of forms, including fiber, paste, powder, film, etc. [Hafner et al. 2011]. Commercially available chitosan has an average molecular weight ranging between 3800 and 20,000 Daltons [Chen et al. 2013]. This product is taken from naturally occurring sources, that is the exoskeleton of fungi, crustaceans and insects which have been demonstrated to be biodegradable and biocompatible [Dash et al. 2011]. Furthermore, chitosan and chitin have been contrasted with other biomolecule-based active films applied as packaging materials and the observed consequences revealed that and chitin have more benefits due to their biological activities and bivalent minerals chelating abilities [Aider 2010].

The colonization of medicinal and aromatic plants with mycorrhizal fungi can also affect the production of essential oil in aromatic herbs [Urcoviche et al. 2015]. In addition, mycorrhizal fungi in exchange for photosynthetic productions, provide the host plant with water and mineral nutrients [Reinhart et al. 2017]. This coexistence with modifying physiological characteristics in host plants can improve the storage of products such as secondary metabolites [Daei et al. 2009]. Under stress, mycorrhizal plants are capable to adsorb more quantities of nutrients and water and accordingly raise their tolerance to different environmental stresses [Miransari M. 2014]. Therefore, this study aimed to provide new perspective about effect of foliar spray of chitosan on essential oil yield and chemical composition of the essential oil of *Mentha* × *piperita* under inoculum of arbuscular mycorrhizal fungi.

MATERIALS AND METHODS

Plant material and experimental site description. The rhizomes of *Mentha* × *piperita* were obtained from Zarringiah Co. (West Azerbaijan Province of Iran). At the spring 2017, the rhizomes were transplanted at experimental greenhouse of Islamic Azad University, Shiraz, South-central of Iran (longitude 52°36'E, latitude 29°32'N, altitude 1484 m above sea level). The climate of region study (Shiraz) was classified as a mid-latitude steppe/ semi-arid cool climate. The Köppen Climate Classification subtype for this climate is "BSk" (Tropical and Subtropical Steppe Climate). The average temperature for the year in Shiraz is 18.9°C. The average amount of precipitation for the year is 338 mm. The month with the least precipitation on average is June with an average of 0 mm.

Prior to planting, soil samples were taken at 0 to 30 cm depth and were tested for selected physical and chemical properties. The soil of plots was filled with soils C.L. at pH of 7.23; containing 1.07% O.C comprised of 0.12% total N, 174.7 mg kg^{-1} P (available), 207 mg kg^{-1} K (available), and salinity level E.C.: 2.01 dS m^{-1}. The soil samples air-dried, and tested for pH, EC, organic carbon (through sulfuric acid method), soil texture (hydrometer assay), total N (Kjeldahl assay), available P (Olsen procedure) and available K after extraction with ammonium acetate. The number of native arbuscular mycorrhizal fungi spores (~12 per 10 g soil) was determined in the field soil before planting.

At site experimental, plots (1.5 × 2 m) were separated each other by 2 m width and the distance between each row was 0.5 m and the distance between each plant in the rows was 0.15 m (plant density = 40 plants $plot^{-1}$). During the entire experiment, the plants were irrigated using trip irrigation weekly, weed control was done manually and no systemic pesticide and chemical fertilizers were used.

Experimental design and treatments. Experimental was laid out as a factorial (foliar-spraying of chitosan or factor A = three levels, inoculation of mycorrhizal fungi or factor B = two levels, JA or factor B = three levels, and irrigation regimes or factor

C = three levels) in a randomized complete block design with three replications during May to October 2017. Factor A were the foliar application by solvent for chitosan (positive control), foliar application at 5.0 g L^{-1} chitosan, and foliar application at 10 g L^{-1} chitosan. Chitosan (Merck Co., Darmstadt, Germany) was dissolved in acetic acid 1%, diluted in distilled water. The pH value of solution was adjusted to 5.4 with NaOH. These solutions were sprayed at dew point (approximately 100 mL $plant^{-1}$) with a hand sprayer (untreated plants were sprayed with an equivalent volume of distilled water). The solutions were sprayed three times, i.e. 90 (19 August), 110 (8 September), 120 (27 September) days after planting. After thrice spraying of chitosan, the aerial parts of the herb were harvested in 5 October 2017.

Factor B included inoculum and non-inoculum of arbuscular mycorrhizal fungi. Before planting, the rhizomes were inoculated with mycorrhizal fungi according to methodology by Soil Biology Research Division, Institute of Soil and Water Research, Tehran, Iran (a mixed of 27% *Glomus mosseae*, 27% *G. intraradices*, 26% *G. hoi*, 3% *G. etunicatum*, 3% *G. clarideum*, 3% *G. versiforme*, 3% *G. fasciculatum*, 3% *G. acaulospora longula*, and 2% *G. margarita*). Five mL of mycorrhizal spore suspension contained about 120 spores/ 1 mL with min 50 living spores/ 1 mL. The plants were inoculated with arbuscular mycorrhizal fungi at the rate of 5 g per plant placed in a 4–5 cm deep hole beneath and in contact with the roots.

Essential oil extraction. The aerial parts of *Mentha × piperita* were dried in the shade and room temperate (22 ±3°C) for ten days. In order to extract the essential oil, 200 g of dried plant was submitted to hydro-distillation using a Clevenger-type apparatus. Then, the essential oil was dried and stored in dark glass bottle and stored at 4°C until analyzed [Bajalan and Ghasemi Pirbalouti 2014].

Essential oil analysis and compound identification. Compositions of the essential oils were determined by GC-FID and GC-MS analysis was done on an Agilent Technologies 7890. GC/MS analyses of essential oils were performed on an Agilent Technologies 7890 gas chromatograph coupled to Agilent 5975 C MSD. An apolar HP-5 capillary column (30 m × 0.25 mm, 0.25 µm film thicknesses) coated with 5% phenyl, 95% methylpolysiloxane were used. The temperature was programmed from 60 to 280°C at 4°C min^{-1} ramp rate. The carrier gas was helium (99.999% purity) at a flow of 0.8 mL min^{-1}. The split ratio was 100 : 1 and 0.1 µL essential oils were injected manually. Constituents were identified by comparison of their KI (Kovats index) relative to C_5-C_{24} *n*-alkanes obtained on a nonpolar HP-5MS column with those reported in the literature [Adams 2007], and by comparison of the obtained mass spectra with those recorded in the NIST 08 and Willey MS libraries. The percentage composition (average of three independent analyses) was computed from the GC peak areas without using any correction factors.

Statistical analyses. Data were performed using $SAS_{ver.\ 9.2}$. Traits were considered significantly different when $p \leq 0.05$ (according to Duncan's multiple range test). Values were expressed as standard deviation (±SD).

RESULTS AND DISCUSSION

Essential oil yields. The essential oils extracted from the aerial parts of peppermint produced a clear, yellow liquid. Results of this study revealed that the application of experimental treatments significantly changed the essential oil yield of *Mentha × piperita* L. (Fig. 1), which range of these yields were from 1.41 to 2.38 mL 100 g^{-1} dry matter. The highest amount of essential oil yield was obtained for peppermint plants grown under the spraying at 5.0 g L^{-1} chitosan along inoculum with arbuscular mycorrhizal fungi. Elicitation of plants with chitosan increased essential oil content of peppermint. Similarly, the results of previous studies [EmamiBistgani et al. 2017, Ghasemi Pirbalouti et al. 2017, Vosoughi et al. 2018] indicted that the essential oil yields of some medicinal and aromatic plants such as two basil species (*Ocimum ciliatum* and *O. basilicum*), Iranian thyme (*Thymus daenensis*) and sage (*Salvia officinalis*) increased under the foliar application of chitosan. It has been demonstrated that signal molecules such as chitosan are very potential elicitors for induction of plant secondary metabolites [Zhao et al. 2005]. In addition, Ahmad et al. [2017] showed that in comparison with control, application of chitosan significantly increased the essential oil yield of peppermint which was in agreement with the results

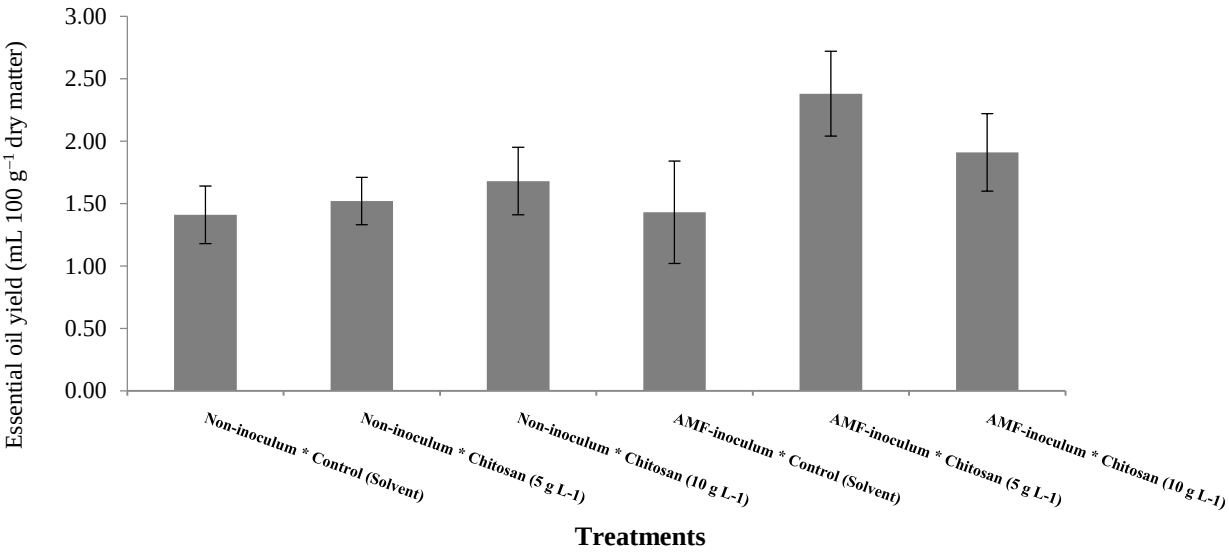

Fig. 1. Effects of the foliar spray of chitosan on essential oil yield of peppermint under non-inoculum and inoculum with arbuscular mycorrhizal fungi

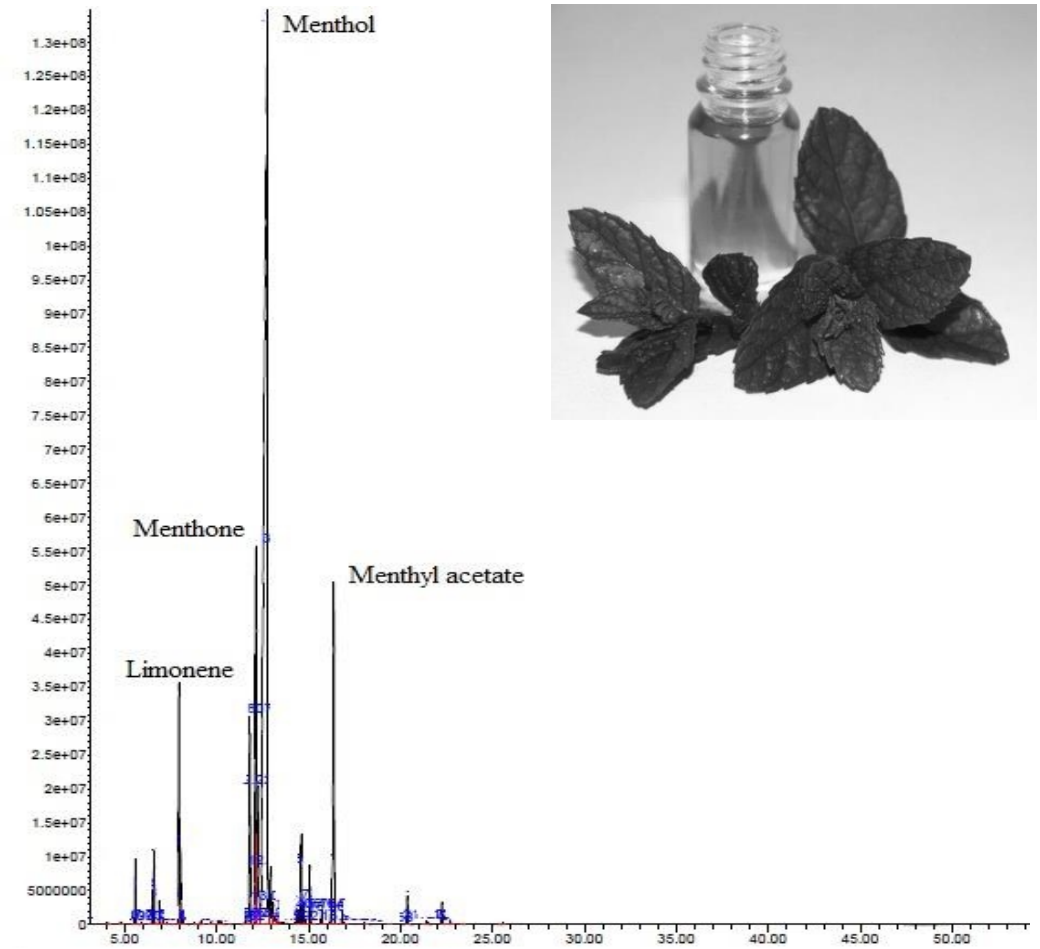

Fig. 2. TLC – Chemical compositions of the essential oil from peppermint under experimental treatments

of present study. Foliar spraying of chitosan increased essential oil yield, and possibly the material acted as a potent inducer for improving the biosynthesis of secondary metabolites. Other report also indicated that use of chitosan increased total terpene content in some medicinal and aromatic plants such as sage [Vosoughi et al. 2018], basil [Ghasemi Pirbalouti et al. 2017], Greek oregano [Yin et al. 2011], and thyme [Bistgani et al. 2017]. The modification in the essential oil yield by the spraying of chitosan may be due to the increase of CO_2 assimilation. The leaves are the most important metabolic organ, so the foliar application is the best way to influence on metabolism of plants [Vosoughi et al. 2018].

Results of an investigation by Arango et al. [2012] indicated that the inoculation with different mycorrhizal fungi such as *Glomus mosseae*, *G. intraradices*, and *G. intraradices* increased the essential oil content of peppermint. Similarly, Gupta et al. [2002] found that inoculation with mycorrhizal fungi increased the essential oil yields of different cultivars of menthol mint (*Mentha arvensis*). Karagiannidis et al. [2011] reported that inoculum with mycorrhizal fungi increased the essential oil of *Mentha viridis* and oregano (*Origanum onites*). Urcoviche et al. [2015] demonstrated that inoculated with mycorrhizal fungi enhanced the essential oil yield of *Mentha crispa*. The beneficial impacts of arbuscular mycorrhizal fungi on the nutrition, growth and development of herbs have been demonstrated since the extra-radical mycelium surrounding the roots of plant [Augé 2001]. Insemination also simplifies the accumulation of further dry matter and impresses the qualitative and quantitative profile of plant metabolites [Miransari 2014, Tarraf et al. 2017].

Generally, the enhancement of essential oil yield of plants under treated with chitosan and inoculated with mycorrhizal fungi is related to an increase in photosynthetic efficiency and production of biomass and secondary metabolites [Arango et al. 2012, Bistgani et al. 2017].

Essential oil components. Overall, several phytochemical investigations have revealed that the essential oil components are a group of secondary metabolites in peppermint with various chemical classes and considerable qualitative and quantitative variation in chemical composition [Andoğan et al. 2002, İşcan et al. 2002, Soković et al. 2009, Kizil et al. 2010]. There are some variations in qualitative and quantitative essential oil which are mainly attributed to genotype and ecological and agronomic management conditions under which plants were grown. In this study, results of essential oils analyses by GC–MS/FID showed that the main classes of components of the essential oils from peppermint were oxygenated monoterpenes (65–85%), monoterpenes hydrocarbon (6–20%), and sesquiterpenes hydrocarbon (0.5–8%) represented 91 to 96% of the total essential oil, which the major components were menthol, menthone, limonene, and menthyl acetate (Tab. 1, Fig. 2). These finding have been reported approximately in all other studies [Andoğan et al. 2002, İşcan et al. 2002, Soković et al. 2009, Kizil et al. 2010]. However, Sartoratto et al. [2004] reported that main components of *M. piperita* essential oil cultivated in Brazil were linalool, carvone, and 3-octanol.

Essential oils and treatments. Our results indicated that the experimental treatments significantly ($p \leq 0.05$) changed the main constituents of the essential oil from *Mentha × piperita* L. The highest concentrations for the major compounds of were generally observed for plants cultivated under non-inoculum with mycorrhizal fungi (Tab. 1). Results presented in Table 1 show that the highest (61.6%) and lowest (39.3%) amounts of menthol, the most abundant component in the peppermint essential oil, were obtained from the peppermint plants sprayed by chitosan under inoculum mycorrhizal fungi and non-inoculum, respectively (Tab. 1). But, the inoculum of the plants with mycorrhizal fungi had not benefit effect on the amount menthone in the essential from peppermint under foliar spraying of chitosan (Tab. 1). The maximum percentages of menthone, (+)-4-carene, limonene, and β-caryophyllene were observed from the foliar-applied chitosan under non-inoculation with arbuscular mycorrhizal fungi (Tab. 1). For other constituents, the highest concentrations of 1,8-cineole and menthyl acetate were obtained for the peppermint plants sprayed with solvent (positive control) and chitosan at 10 g L^{-1} under inoculation with arbuscular mycorrhizal fungi (Tab. 1). Similar to our results, results of an investigation by Khaosaad et al. [2006] showed that the colonization the root of *Origanum vulgare* by arbuscular mycorrhizal fungi had significant effect on the chemical components of the essential oil. However, in many studies the impacts of colonization with arbuscular

Table 1. Chemical compositions of peppermint essential oil grown under different treatments

No.	Components	RI	Non-inoculum			Inoculum with arbuscular mycorrhizal fungi			*ANOVA*
			Control (Solvent)	Chitosan (5 g L^{-1})	Chitosan (10 g L^{-1})	Control (Solvent)	Chitosan (5 g L^{-1})	Chitosan (10 g L^{-1})	
1	α-Pinene	933	0.24	0.77	0.75	0.81	0.84	0.59	
2	β-Pinene	979	0.58	1.17	1.19	1.18	0.94	0.68	
3	Limonene	1030	9.01 ±0.8* b	7.24 ±0.37 c	12.45 ±1.24 a	8.36 ±0.90 b	1.02 ±0.13 d	0.84 ±0.28 d	$P \leq 0.01$
4	*cis*-Sabinene hydrate	1063	0.45	0.21	0.11	0.46	0.9	0.14	
5	(+)-4-Carene	1064	4.99 ±0.67 b	6.12 ±0.93 a	5.36 ±0.84 ab	4.83 ±0.75 b	4.01 ±0.52 b	3.98 ±0.49 b	$P \leq 0.05$
6	α-Terpinolene	1087	0.36	0.5	0.31	0.35	0.01	0.11	
7	1,8-Cineole	1033	3.79 ±0.41 b	2.32 ±0.34 bc	1.93 ±0.18 c	6.34 ±0.74 a	3.49 ±0.34 b	3.04 ±0.23 b	$P \leq 0.05$
8	Linalool	1101	1.32	0.07	0.05	1.34	0.08	0.09	
9	Menthone	1159	10.43 ±1.77 b	17.71 ±3.11 a	17.22 ±2.93 a	9.95 ±2.01 b	8.93 ±1.55 b	11.21 ±2.03 b	$P \leq 0.01$
10	Menthofuran	1166	2.85	2.75	0.08	2.66	3.63	0.11	
11	Menthol	1177	49.87 ±7.01 b	41.05 ±6.52 bc	39.34 ±4.38 c	46.46 ±6.06 b	61.57 ±8.09 a	61.47 ±7.57 a	$P \leq 0.01$
12	Terpinene-4-ol	1181	0.26	0.8	1.12	0.26	1.08	0.67	
13	*neo*-Menthol	1185	0.31	0.64	0.92	0.37	0.51	0.74	
14	Menthyl acetate	1292	0.71 ±0.03 c	6.54 ±1.13 b	5.12 ±0.52 b	0.77 ±0.43 c	6.62 ±1.03 b	9.14 ±1.76 a	$P \leq 0.01$
15	β-Caryophyllene	1421	4.65 ±0.73 b	4.61 ±0.81 b	6.05 ±1.04 a	4.70 ±0.67 b	0.02 ±0.00 c	0.34 ±0.09 c	$P \leq 0.05$
16	Germacrene-D	1483	2.82	1.35	1.17	2.89	0.48	0.69	
	Monoterpenes hydrocarbons		15.63	16.01	20.17	15.99	7.72	6.34	
	Oxygenated monoterpenes		69.54	71.88	65.78	68.15	86.91	86.47	
	Sesquiterpene hydrocarbons		7.47	5.96	7.22	7.59	0.48	1.03	
	Total		92.64	93.85	93.17	91.73	95.11	93.84	
	Menthol/menthone		4.78 b	2.32 c	2.28 c	4.66 b	6.89 a	5.48 ab	$P \leq 0.01$

*Data are mean ±SD (standard deviation)

mycorrhizal fungi on the chemical constituents of the essential oil of various medicinal and aromatic plants have been reported. For instance, Karagiannidis et al. [2011] demonstrated that the mint plants inoculated with mycorrhizal fungi significantly increased the concentrations of limonene, 1,8-cineole, carvone, eugenol and (*E*)-methyl cinnamate, while in their study the quantity of linalool in plants treated with mycorrhizal fungi was lower than control. Copetta et al. [2006] reported that the arbuscular mycorrhizal fungi improved some main constituents of the essential oil from *Ocimum basilicum* like α-terpineol. Generally, soil microorganisms such as mycorrhizal fungi display an essential link between mineral nutrients in the soil and plants [Reinhart et al. 2017]. Thus, the positive impact of mycorrhizal fungi symbiosis may relies on the alleviating effects of mycorrhizal fungi on the absorption and forwarding water and an ameliorated absorption of nutrients from soil, specially phosphorus and other immobile minerals, leading to the hydration of plant tissues, a sustainable physiology and a clear promotion of growth [Rapparini and Peñuelas 2014].

Ahmad et al. [2019] also found that the application of chitosan and GA_3 increased menthol and menthone contents of *M. piperita* essential oil. In another study, Ahmad et al. [2017] reported that the exogenous application of chitosan at 80 mg L^{-1} increased most of the essential oil components of peppermint, specially menthol content, while the amounts of menthone and menthyl-acetate in this concentration of chitosan decreased. It seems that the functionality of chitosan is based on developmental stage of the plant, the concentration and structure of chitosan, environmental and

agronomic management conditions, and plant species [Li et al. 2008].

Chitin and its derivatives have many positive effects on growth and development of plants and these elicitors also can be improved secondary metabolites of the medicinal herbs [Chakraborty et al. 2009]. Several reports indicated that chitosan positively affected growth and development, ion uptake and transport, and transpiration rate of various plant species and secondary metabolite accumulation in plants [Malekpoor et al. 2016, EmamiBistgani et al. 2017, Vosoughi et al. 2018]. For example, some investigators [EmamiBistgani et al. 2017, Vosoughi et al. 2018] reported that the foliar spray of chitosan improved biosynthesis of phenols in Iranian thyme and sage. They stated the application of chitosan elicitor can to some degree compensate the negative impact of deficit irrigation on its biomass, essential oil yield, some secondary metabolites, and antioxidant activity. Kim et al. [2005] demonstrated that application of chitosan enhanced eugenol and l-linalool contents in the essential oil of *O. basilicum*. Lei et al. [2011] showed that the foliar application of chitosan increased some major constitutes in *Artemisia annua* L. essential oil. Chitosan also has been exploited in fruit, leaf, vegetable, and seed coating to protect herbs against microorganisms [El-Sawy et al. 2010, Pandey et al. 2018].

According of results of effects of the foliar spray of chitosan and inoculation with arbuscular mycorrhizal fungi on balance menthol/menthone in the essential oils from the peppermint plants under experimental treatments indicated that this qualitative feature improved in the foliar spray of chitosan (5 g L^{-1}) along inoculation with arbuscular mycorrhizal fungi in comparison with control and other treatments (Tab. 1).

Generally, the highest menthol concentration in the peppermint essential oil was observed from the plants inoculation with mycorrhizal fungi in this investigation, while the mycorrhizal fungi inoculation had not benefit effect on the menthone concentration in the peppermint essential oil under the foliar application of chitosan (Tab. 1). The most important components of peppermint essential oil are menthol and menthone [Fejéra et al. 2018]. Menthol [5-methyl-2-(1-methylethyl)cyclohexanol] belongs to the group of terpenes. It is used in confectionery, perfumery as well as liqueurs, cigarettes, nasal inhalers and cough drop production. In addition, menthol is used as a component of anaesthetic, antiseptic, and gastric sedative drugs [Ligor and Buszewski 1999]. Because of the wide range of applications of this compound in food and drugs production, the determination of it is important not only for consumers but also for analytical chemist. High quality essential oil should content high amounts of menthol, less menthone and few amounts of pulegone and menthofurane. For commercial purposes, the essential oil should contain very low amounts of pulegone and menthofuran. Therefore, experimental treatments can improve the quality essential oil of peppermint.

CONCLUSIONS

This investigation provides new information about the effects of exogenous foliar of chitosan on some phytochemical features of *Mentha* × *piperita* L. under the inoculation of plants with arbuscular mycorrhizal fungi. In conclusion, the foliar application of chitosan increased essential oil yield and some of the main components of peppermint under the inoculation of plants with arbuscular mycorrhizal fungi. Whereas, chitosan in absence of mycorrhizal fungi significantly increased the percentages of some constitutes in the essential oil such as menthone, limonene, and menthyl acetate. Indeed, chitosan along mycorrhizal fungi significantly increased the amount of menthol, as a quality index for peppermint essential oil. In final, results of this study can be used in cultivation programs in order to further improvement of quantity and quality yields of the essential oil from peppermint.

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