Plant biostimulants (*Funneliformis mosseae* and humic substances) rather than chemical fertilizer improved biochemical responses in peppermint

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Abstract

Plant biostimulants such as arbuscular mycorrhizal (AM) fungi and humic substances (HS) can be used as an appropriate alternative to chemical fertilizers, as regards to environmental problems of chemicals. The effects of *Funneliformis mosseae* as an AM fungus, HS (foliar spray and topdressing application), and chemical fertilizer (NK), separately or interacting, on biochemical responses in *Mentha piperita* L. plants were investigated under field conditions. Results revealed that the mentioned three factors appear as valid practicable method for improving growth and metabolites of peppermints cultivated in the field even when root colonization of AM fungus does not achieve high rates. The triple interaction between mycorrhizal inoculation, HS application (especially foliar spray), and NK fertilizer treatment induced the maximum accumulation of photosynthetic pigments, starch, soluble sugars, total proteins, proline, total phenolics, and the antioxidants in leaves. However, positive effects of *F. mosseae* and HS were more than those of the chemical fertilizer. We propose that *F. mosseae* and foliar spray of HS, particularly in combination can be used as suitable plant biostimulants in peppermint plants under field conditions, which in turn will improve soil health and reduce environmental problems.

Keywords: chemical fertilizer; humic substances; mycorrhizal fungi; peppermint; plant biostimulants

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Introduction

The continuous and comprehensive use of the chemical and synthetic fertilizers, due to the increase in population and the growing demand for food has caused environmental problems like degradation of the soil, increased soil salinity, loss of crop genetics and microbial diversity, contamination of groundwater, and pollution of the atmosphere (Kaur et al., 2008; Chaudhry et al., 2009). As a viable alternative, the practicable strategies (such as application of plant biostimulants) have been proposed to improve productivity with a reduction in production costs and increased efficiency of inputs, without compromising environmental sustainability (Bettoni et al., 2016) resulting in the reduced chemical fertilizer application, and protecting the

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environment and soil health. Plant biostimulants are various substances and microorganisms that are applied to plants to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits, regardless of their nutrients content (Du Jardin, 2015). The global market for biostimulants has been projected to reach $2.241 million by 2018 and to have a compound annual growth rate of 12.5 % from 2013 to 2018 (Anonymous, 2013). The use of plant biostimulants or biofertilizers such as fertilization with humic substances (HS) and inoculation with arbuscular mycorrhizal fungi (AMF) are important for reducing the use of mineral and synthetic fertilizers. HS are the natural molecules that are composed of humic acids (HA), fulvic acids (FA), and humins derived from microbial decomposition and chemical degradation of dead soil biological material such as lignin, cellulose, hemicelluloses, sugars, and amino acids (Schiavon et al., 2010). HS increase the capacity of moisture retention in the soil or its substrate (Khaled and Fawy, 2011) and exert physiological influences on plant growth (Nardi et al., 2002). The most commonly reported effects of HS on plants are hormone-like effects (Nardi et al., 2002; Zandonadiet al., 2007), influence of photosynthetic process (Ertani et al., 2011), stimulation of beneficial soil microorganisms (Linderman and Davis, 2001), enhancement of plant leaf and root growth and nutrient uptake such as Fe and Zn (Chen et al., 2004; Ertani et al., 2011), and increase in chlorophyll content (Ertani et al., 2011). Also, HS reduce the need for chemical fertilizer application because they can help formation of complexes and chelates (Zhang et al., 2013). Also, as essential contributors to soil fertility, HS can improve soil quality and health such as aggregation, aeration, permeability, water holding capacity, microbial growth, organic matter mineralization, and solubilization and availability of micro- and macro-elements (Tahir et al., 2011; Du Jardin, 2015).

The adaptation of soil microbial structure and function to the environment is inevitable for sustainable agricultural production (Maji et al., 2017). AMF are important soil microorganisms forming beneficial symbiotic associations with most of the vascular plant species including crop and horticultural plants. Inoculation of plants with AMF can enhance plant water and nutrient uptake especially P and hence increase their tolerance to the stresses, due to the production of a very extensive network of fungal hypha in the soil (Gosling et al., 2006; Miransari et al., 2008). AMF brings various benefits to plants such as increased growth and photosynthesis (Wu and Xia, 2006), improving soil fertility and stabilization of soil structure (Charles et al., 2006) and enhanced hydrolytic enzyme activities (Adriano-Anaya et al., 2006). Due to the multiple benefiting features of AM symbiosis, there is an increasing interest for the use of mycorrhiza to promote sustainable agriculture (Du Jardin, 2015).

Peppermint (*Mentha × piperita*) is a hybrid mint (a cross between watermint and spearmint) from the Lamiaceae family that is cultivated worldwide. Its production has increased over the past few decades due to its valuable essential oil, fragrances, and pharmaceutical compounds (Lawrence, 2006). Peppermint demonstrates antioxidant, antitumor, antimicrobial, antiallergenic, anti-convulsion, digestive, and antiseptic properties (Lv et al., 2012; Pytlakowska et al., 2012).

This work was conducted in response to a growing demand for plant biostimulants, and the need to pay attention to the effect of plant biostimulants on various crop characteristics such as biochemical and physiological features under field conditions. We investigated the influence of AM fungus symbiosis and HS treatment (in the soil or topdressing treatment, and foliar exposure) as two plant biostimulants, and chemical fertilizer (NK) on peppermint response in the field. Comparisons in growth, pigments content, and some biochemical changes such as contents of chlorophyll, starch, total soluble sugar, total soluble protein, and proline attributed to mycorrhizal establishment, HS application, and chemical fertilizer addition are discussed.

Materials and Methods

Trial location and soil properties

This study was performed from May to July 2016 at the Research Station of University of Maragheh, Iran (46°27′ E, 37°37′ N). The experimental location experiences cold and semi-arid climate (Peel et al., 2007). During the field

study, the mean maximum monthly temperature ranged between 22.9 °C (in May) and 36.0 °C (July) and the mean minimum monthly temperature was between 11.2 °C (May) and 23.8 °C (July). The total rainfall during the experiment was 21.4 mm, with the highest recorded monthly rainfall (15 mm) in May. The experiment soil contained 52% sand, 31% silt, 17% clay, 1.2% organic matter, 0.05% total N, 21.4 mg/kg available P, 350 mg/kg available K, 1.۴ mg/kg Zn, and 11.5 mg/kg Fe, having pH of 7.4 and 0.69 ds/m EC.

Experimental setup and growth condition

Mentha piperita L. var. Vulgaris seeds (obtained from the Pakan Bazr Esfahan Co., Esfahan, Iran) were germinated in polystyrene trays filled with vermiculite. Trays were kept in a greenhouse with sprinkler irrigation every four hours. When seedlings had 10 cm of height were transplanted to field plots. Five rows of plants per plot (4 × 2.5 m with 50 cm boundaries) were grown, with 40 cm of row spacing and 10 cm of distance between plants in the same row. The experimental design was completely randomized in a factorial 2 × 2 × 3 with four replications for each treatment. The treatments included (1) mycorrhizal inoculation (M) or without mycorrhizal inoculation (nM), (2) application with (C) or without (nC) chemical fertilizer, and (3) humic substances treatments including foliar application (fH), topdressing application or addition of HS in the soil (sH), and without application (nH). The AM species used for our experiment was *Funneliformis mosseae* (formerly known as *Glomus mosseae*), obtained from the Touran Biotech Co. under the supervision of Semnan Science and Technology Park, Iran. *F. mosseae* inoculum consisted of spores, soil, hyphae, and infected maize root fragments and the inoculated dosage was 50 g of inoculum per plant containing approximately 20 spores/g soil. *F. mosseae* inoculum was placed 2 cm below the seedlings at sowing time. Nitrogen fertilizer as urea was applied according to a dose of 200 kg N/ha in three equal splits (0, 30, and 60 days after transplanting), to avoid leaching. Potassium fertilizer was added before planting according to a dose of 100 kg K/ha in the form of potassium sulfate. Also, chemical fertilizer was added to the soil according to the results of soil testing. The original commercial solution of HS had 12% humic acid, 4% fulvic acid with 49% C, 5.6% H, and 4.7% N. Dose of humic substances was 1.5 mL/L in both applied methods. For foliar spraying, plants were first sprayed 15 days after transplanting and afterwards, they were treated every 15 days (a total of four times). Plants were thoroughly sprayed with HS until the excess solution was dripping. For HS topdressing, the plants received 50 mL of HS solution together with the treatment of foliar spray. Plants were irrigated twice every week to ensure adequate soil moisture. Final harvest of all plants was carried out on day 90th after sowing.

Determination of shoot dry matter and root colonization

At harvest time, plants of each plot (1 m^2) were taken for determination of shoot dry weight. Shoot dry weight was determined after drying plant material in a shade place until the weight was constant. Root samples were cleared and stained (Phillips and Hayman, 1970) and root colonization was determined according to the gridline intersection method described by Giovannetti and Mosse (1980). In this technique, the percentage of root colonization per plant was determined by dividing the total number of colonized root fragments (either with arbuscules, vesicles, or hyphae) by the total number of root pieces examined × 100.

Proline and total soluble phenolic concentration measurement

The plant tissues (1 g) were ground in liquid nitrogen and then the powder was suspended in 10 mL sulfosalicylic acid (10%) for 30 min. The supernatant was used for proline analysis as described by Bates et al. (1973) after centrifugation at 12000 rpm for 15 min at 4 °C. Total phenolic compounds were extracted according to Chapuis-Lardy et al. (2002) with some modifications. 0.5 g of leaf sample was pulverized and homogenized in 20 mL of 80% methanol at room temperature for 1 min. After filtration, 0.5 mL of the sample was mixed with 10 mL of distilled water. The total phenolic content was determined

from aqueous solutions by spectrophotometric analysis at 760 nm with Folin Ciocalteu reagent.

Determination of total protein, starch and total soluble sugar contents

Leaf sample (0.5 g) was homogenized with a pre-chilled mortar and pestle under ice cold condition in 1 mL of extraction buffer, containing 0.1 M sodium phosphate buffer (pH 7.0) with 1% polyvinyl pyrrolidone (PVP). The homogenate was centrifuged at 10,000 rpm for 20 min at 4 ˚C and the protein content was measured by the method of Bradford (1976) using bovine serum albumin as standard. For starch content measurement, 0.1 g of leaf tissue was extracted in 1 mL potassium phosphate buffer (50 mM, pH 7.5) and then was filtered through four cheesecloth layers and centrifuged at 38,720 × g for 10 min at 4 °C. The pellet was used for starch determination (Jarvis and Walker, 1993). Total sugar content was estimated by the phenol-phosphoric acid method (by Du Bois et al., 1956) with some modifications. 0.5 g of leaf sample was homogenized in 80 % methanol and made to 10 mL. 0.5 mL of aliquot was taken and volume made to 3 mL with the distilled water followed by 0.5 mL distilled phenol and mixed thoroughly. Five mL of H_2SO_4 was carefully added at the side of the tube. Test tubes were kept for 30 min at room temperature. The absorbance was measured at 485 nm. The content was calculated with the help of a reference curve prepared from D-glucose as standard.

Pigments content determination

The concentrations of chlorophyll *a*, *b*, and total chlorophyll as well as total carotenoids, were spectrophotometrically determined (Shimadzu, Japan) in an acetone (100%) extract solution using the re-evaluated equations of Lichtenthaler (1987).

Determination of antioxidant power

Leaf tissue (1 g) was mashed with a cool mortar and pestle using 9 mL cool 0.1 M phosphate buffer (pH 7.6, containing 0.1 mM EDTA). This mixture was filtered through a filter paper and centrifuged at 15.000 rpm for 10 min. The supernatant was used to measure the antioxidant power of peppermints leaf by the Ferric Reducing Ability of Plasma (FRAP; Benzie and Strain, 1996). The absorbance was read at 593 nm. The antioxidant potential of the extract was calculated against standard curve of ferrous sulfate equivalent.

Statistical Analysis

Three-way ANOVA was performed on all experimental data using IBM SPSS version 19 software (Chicago, USA). Differences between means were determined using Duncan's Multiple Range Test at 0.05 and 0.01 probability level. Data were expressed as the means of replicates ± standard error (SE).

Results

Root colonization and shoot dry matter

Results from our study in Table 1 revealed that percentages of root colonization in mycorrhizal inoculated plants ranged from 21.25% in peppermint amended with NK and HS (C+sH) to 62.71% in peppermint not amended with NK and HS (nC+nH). Application of HS and NK significantly reduced root colonization in comparison with plants not supplied with HS and NK (Table 1). There was no significant difference between foliar application and topdressing treatment of HS on root colonization (Table 1). Shoot dry biomass was increased after applying HS, inoculating AMF, and exposing plants to NK fertilizer. However, increases induced by chemicals were less than those induced by HS addition and AMF inoculation (Table 1). Also, the maximum and minimum values of shoot dry weight (351 and 275 g/m^2, respectively) were achieved under treatments of M + C + fH and nM + nC + nH, respectively (Table 1).

Table 1
Effects of mycorrhizal fungus (*F. mosseae*), chemical fertilizer (NK), and humic substances (HS) treatments on root colonization, shoot dry matter, proline, and total soluble phenolics contents in leaves of peppermint.

Fungal treatment	Chemical treatment	Humic treatment	Root colonization (%)	Shoot dry matter (g/m^2)	Proline (µmol/g FW)	Total soluble phenolics (mg gallic acid/g FW)
nM	nC	nH	ND	275.0 ± 6.0 c	20.62 ± 0.81 f	20.33 ± 8.41
nM	nC	sH	ND	298.0 ± 4.4 bc	25.07 ± 1.99 e	33.33 ± 7.53
nM	nC	fH	ND	296.5 ± 6.0 bc	23.04 ± 0.93 ef	34.66 ± 2.90
nM	C	nH	ND	293.9 ± 4.4 bc	22.44 ± 0.52 ef	37.66 ± 11.46
nM	C	sH	ND	316.2 ± 2.8 b	26.68 ± 0.81 de	37.66 ± 7.17
nM	C	fH	ND	319.1 ± 4.4 b	37.01 ± 0.98 bc	64.33 ± 9.02
M	nC	nH	62.71 ± 3.65 a	310.7 ± 4.6 bc	29.72 ± 2.02 d	106 ± 23.12
M	nC	sH	40.42 ± 1.05 b	323.9 ± 4.0 b	30.51 ± 0.93 d	97 ± 14.73
M	nC	fH	39.96 ± 7.92 b	348.5 ± 6.0 a	40.88 ± 0.64 ab	58.33 ± 7.62
M	C	nH	27.76 ± 2.92 c	317.5 ± 8.1 b	35.50 ± 1.80 c	83.33 ± 9.20
M	C	sH	21.25 ± 1.30 d	340.7 ± 1.5 a	39.25 ± 2.09 abc	89.33 ± 1.33
M	C	fH	23.42 ± 1.25 de	351.0 ± 1.6 a	42.68 ± 1.41 a	123.33 ± 8.41
Main effect						
nM			ND b	296.9 ± 3.1 b	25.81 ± 5.74 b	37 ± 3.84 b
M			35.92 ± 3.66 a	331.8 ± 3.5 a	36.42 ± 3.04 a	93.88 ± 6.42 a
	Main effect					
	nC		23.85 ± 6.18 a	308.2 ± 4.7	28.31 ± 7.02 b	59.27 ± 8.12 b
	C		12.07 ± 3.00 b	323.1 ± 2.6	34 ± 4.32 a	71.61 ± 8.12 a
		Main effect				
		nH	22.61 ± 7.83 a	299.7 ± 3.4 b	27.07 ± 1.89 c	51.41 ± 8.36 b
		sH	15.42 ± 5.10 b	319.4 ± 5.6 a	30.37 ± 1.78 b	63 ± 9.47 b
		fH	15.84 ± 5.37 b	328.5 ± 3.8 a	35.90 ± 2.36 a	78.33 ± 11 a
ANOVA						
M			**	**	**	**
C			**	ns	**	*
H			**	**	**	**
M × C			**	**	ns	ns
M × H			**	ns	ns	ns
H × C			*	ns	ns	ns
M × C × H			*	**	**	ns

nM: non-inoculated (control), M: inoculated with *F. mosseae*, nC: non-amended, C: amended with chemical fertilizer (NK), nH: non-amended, sH: amended with topdressing treatment of humic substances (HS), fH: amended with foliar spray of HS, ND: not detected. Values are mean ± SE, n = 4. The same letter within each column indicates no significant difference among treatments using Duncan's Multiple Range Test. ns: not significant, **P* < *0.05*, ***P* < *0.01*.

Proline and total soluble phenolic compounds contents

Data from Table 1 show that the interactions between the three factors (M × C × H) had a significant effect on proline content whereas this triple interaction had no significant influence on total soluble phenolics. However, the main effects of all three factors on proline and total phenolics were significant (Table 1). Inoculation of AMF and application of NK and HS (either as foliar application or as topdressing treatment) significantly increased proline content in peppermint plants ($p<0.01$) so that the lowest (20.62 µM/g FW) and highest (42.68 µM/g FW) levels of proline were found in nM + nC + nH and M + C + fH plants, respectively.

Similar to proline, the minimum (20.33 mg gallic acid/g FW) and maximum (123.33 mg gallic

Table 2
Effects of mycorrhizal fungus (*F. mosseae*), chemical fertilizer (NK), and humic substances (HS) treatments on Chl. *a*, Chl. *b*, total Chl, and carotenoids contents in leaves of peppermint

Fungal treatment	Chemical treatment	Humic treatment	Chlorophyll *a* (mg/g FW)	Chlorophyll *b* (mg/g FW)	Total chlorophyll (mg/g FW)	Carotenoids (mg/g FW)
nM	nC	nH	0.73 ± 0.18 f	0.28 ± 0.08	1.01 ± 0.26 h	0.44 ± 20
nM	nC	sH	1.40 ± 0.23 ef	0.72 ± 0.02	2.12 ± 0.21 g	0.88 ± 0.45
nM	nC	fH	2.27 ± 0.30 de	0.77 ± 0.18	3.04 ± 0.46 efg	1.09 ± 0.15
nM	C	nH	2.0 ± 0.26 de	0.63 ± 0.16	2.63 ± 0.40 fg	0.86 ± 0.23
nM	C	sH	2.37 ± 0.71 de	0.90 ± 0.22	3.27 ± 0.93 fg	1.31 ± 0.04
nM	C	fH	2.6 ± 0.24 cd	1.11 ± 0.31	3.71 ± 0.20 de	1.30 ± 0.40
M	nC	nH	2.32 ± 0.04	0.97 ± 0.18	3.29 ± 0.14 ef	1.39 ± 0.25
M	nC	sH	2.87 ± 0.32 cd	1.26 ± 0.13	4.13 ± 0.26 cd	1.37 ± 0.20
M	nC	fH	3.6 ± 0.23 bc	1.22 ± 0.05	4.82 ± 0.25 bc	1.23 ± 0.20
M	C	nH	2.33 ± 0.37 de	1.01 ± 0.31	3.34 ± 70 def	1.53 ± 0.47
M	C	sH	4.25 ± 0.27 b	1.35 ± 0.20	5.60 ± 0.18 b	1.89 ± 0.65
M	C	fH	6.12 ± 0.61 a	1.81 ± 0.36	7.93 ± 0.96 a	2.10 ± 0.63
Main effect						
nM			1.89 ± 0.20 b	0.73 ± 0.14 b	2.62 ± 0.32 b	0.98 ± 0.17 b
M			3.58 ± 0.34 a	1.27 ± 0.15 a	4.58 ± 0.48 a	1.58 ± 0.21 a
	Main effect					
	nC		2.20 ± 0.24 b	0.87 ± 0.16 b	3.07 ± 0.40 b	1.07 ± 0.18 b
	C		3.28 ± 0.38 a	1.13 ± 0.20 a	4.41 ± 0.57 a	1.50 ± 0.25 a
		Main effect				
		nH	1.85 ± 0.22 c	0.77 ± 0.19 c	2.62 ± 0.41 c	1.06 ± 0.30 b
		sH	2.72 ± 0.36 b	1.06 ± 0.17 b	3.78 ± 0.50 b	1.36 ± 0.27 a
		fH	3.65 ± 0.48 a	1.23 ± 0.25 a	4.88 ± 0.71 a	1.43 ± 0.30 a
ANOVA						
M			**	**	**	**
C			**	**	**	**
H			**	**	**	*
M × C			ns	ns	ns	ns
M × H			*	ns	ns	ns
H × C			ns	ns	ns	ns
M × C × H			**	ns	*	ns

nM: non-inoculated (control), M: inoculated with *F. mosseae*, nC: non-amended, C: amended with chemical fertilizer (NK), nH: non-amended, sH: amended with topdressing treatment of humic substances (HS), fH: amended with foliar spray of HS. Values are mean ± SE, n = 4. The same letter within each column indicates no significant difference among treatments using Duncan's Multiple Range Test. ns: not significant, $*P < 0.05$, $**P < 0.01$.

acid/g FW) levels of total soluble phenolics were recorded in nM + nC + nH and M + C + fH plants, respectively. Also, foliar treatment of HS produced higher levels of proline and soluble phenolics than topdressing HS except for nM + nC plants for proline, and M + nC peppermints for soluble phenolics (Table 1).

Chlorophyll (Chl) *a*, Chl *b*, total Chl and carotenoids concentrations

Table 2 shows that triple interaction effect of mycorrhizal colonization, application of chemical fertilizer, and humic substances treatment on contents of Chl *a* ($p<0.01$) and total Chl ($p<0.05$) was significant. The main effects of all three factors on all studied pigments contents were significant (Table 2). Inoculation of AM fungus and application of humic substances and chemical fertilizer produced higher amounts of Chl *a*, Chl *b*, total Chl, and carotenoids in peppermint leaves compared to control plants (Table 2). Maximum levels of Chl *a* (6.12 mg/g FW), Chl *b* (1.81 mg/g FW), total Chl (7.93 mg/g FW), and carotenoids (2.1 mg/g FW) were observed in leaves of peppermints grown under inoculation of

Table 3
Effects of mycorrhizal fungus (*F. mosseae*), chemical fertilizer (NK), and humic substances (HS) treatments on starch, total soluble sugar, total soluble protein and antioxidant power (FRAP) contents in leaves of peppermint

Fungal treatment	Chemical treatment	Humic treatment	Starch (mg/g FW)	Total soluble sugar (mg/g FW)	Total soluble protein (mg/g FW)	Antioxidant power (μM Fe(II)/g FW)
nM	nC	nH	11.68 ± 1.19	7.38 ± 3.86 e	20.55 ± 1.33	52.7 ± 8
nM	nC	sH	12.27 ± 0.97	8.75 ± 0.67 e	24.05 ± 2.08	68.5 ± 3.4
nM	nC	fH	14.20 ± 0.97	13.11 ± 6.46 bc	28.48 ± 6.88	81.26 ± 13.6
nM	C	nH	11.58 ± 0.57	8.81 ± 5.48 e	19.57 ± 6.68	48.5 ± 5.6
nM	C	sH	13.51 ± 0.30	11.33 ± 5.54 d	31.04 ± 1.92	72.93 ± 6.3
nM	C	fH	15.54 ± 0.81	13.19 ± 1.30 bc	35.01 ± 3	86.03 ± 4.9
M	nC	nH	15.68 ± 0.83	12.17 ± 7.10 cd	33.46 ± 2.80	119.35 ± 9.2
M	nC	sH	16.45 ± 0.82	13.13 ± 2.25 bc	45.74 ± 1.65	116.3 ± 3.3
M	nC	fH	17.38 ± 1.07	13.85 ± 1.26 b	53.7 ± 3.43	243.34 ± 10
M	C	nH	11.56 ± 0.32	12.20 ± 6.41 cd	36.88 ± 1.01	112.1 ± 8.8
M	C	sH	16.72 ± 0.39	13.72 ± 5.71 bc	51.17 ± 4.70	122.75 ± 5.7
M	C	fH	17.83 ± 0.11	15.35 ± 5.90 a	60.28 ± 4.26	291.7 ± 2.7
Main effect						
nM			13.13 ± 0.43 b	10.43 ± 5.74 b	26.45 ± 1.75 b	68.32 ± 4.1 b
M			15.94 ± 0.56 a	13.41 ± 3.04 a	46.87 ± 2.70 a	167.6 ± 17.1 a
	Main effect					
	nC		14.61 ± 0.57	11.40 ± 6.12 b	34.33 ± 3.14 b	113.57 ± 14.2
	C		14.46 ± 0.64	12.44 ± 5.26 a	39 ± 3.48 a	122.33 ± 17.7
		Main effect				
		nH	12.63 ± 0.64 c	10.14 ± 6.70 c	26.62 ± 2.68 c	83.16 ± 10.2 c
		sH	14.74 ± 0.64 b	11.73 ± 6.10 b	38 ± 3.74 b	95.12 ± 7.5 b
		fH	16.24 ± 0.54 a	13.87 ± 3.54 a	44.37 ± 4.28 a	175.58 ± 28 a
ANOVA						
M			**	**	**	**
C			ns	*	*	ns
H			**	**	**	**
M × C			*	**	ns	ns
M × H			ns	ns	ns	**
H × C			*	ns	ns	*
M × C × H			ns	*	ns	ns

nM: non-inoculated (control), M: inoculated with *F. mosseae*, nC: non-amended, C: amended with chemical fertilizer (NK), nH: non-amended, sH: amended with topdressing treatment of humic substances (HS), fH: amended with foliar spray of HS. Values are mean ± SE, n = 4. The same letter within each column indicates no significant difference among treatments using Duncan's Multiple Range Test. ns: not significant, $*P < 0.05$, $**P < 0.01$.

fungus (M) and fertilized with chemical fertilizer (C), and HS (fH). In contrast, the minimum levels of these pigments (0.73, 0.28, 1.01, and 0.44 mg/g FW for Chl *a*, Chl *b*, total Chl and carotenoids contents, respectively) were recorded in plants non-inoculated with *F. mosseae* (nM), without chemical fertilizer (nC), and humic substances (nH) addition.

Starch, total soluble protein, and total soluble sugar concentrations

The triple interaction effect of M × C × H was significant on total soluble sugar at $p<0.05$ whereas this triple interaction was not significant on starch and total soluble protein in leaves (Table 3). Although, inoculation of fungus and HS treatment had positive significant effects on starch content, but chemical fertilizer had no significant influence on this parameter (Table 3). There were positive effects of different factors, separately or in interaction, on the concentrations of total soluble sugar and total soluble protein in peppermints (Table 3). Maximum levels of starch

(17.83 mg/g FW), total soluble protein (60.28 mg/g FW), and total soluble sugar (15.35 mg/g FW) were found in mycorrhizal plants amended with the chemical fertilizer and humic substances as foliar application (M + C + fH). In addition, the minimum concentration of total soluble sugar (7.38 mg/g FW) in leaves corresponded to plants grown under no mycorrhizal inoculation (nM) without neither the fertilizer (nC) nor HS application (nH). In the case of starch and total soluble protein, the lowest contents (11.58 and 19.57 mg/g FW, respectively) were observed in M + C + nH and nM + C + nH plants, respectively (Table 3).

Antioxidant power (FRAP) content

Data from Table 3 showed that main effects of the presence of AMF and application of HS on FRAP content were significant ($p<0.01$) whereas the triple interaction of M × C × H on the FRAP was not significant. Foliar application of HS and inoculation of AMF significantly increased FRAP content in peppermint leaves compared to the control; however, addition of the chemical fertilizer (C) had no significant influence on FRAP (Table 3). Also, the maximum and minimum levels of FRAP (291.7 and 48.5 µM Fe(II) /g FW, respectively) were obtained under M + C + fH and nM + C + nH treatments, respectively (Table 3).

Discussion

As shown in Table 1, the exposure to the chemical fertilizer (C) substantially reduced root colonization in peppermints in comparison with plants not treated with the chemical fertilizer (nC). Cheeke et al. (2010) showed that the application of chemical fertilizer inhibits the establishment of AMF symbiosis in two maize cultivars. Exposure to chemical fertilizers is usually reported to decrease mycorrhizal colonization in agricultural crops, as a lower AM fungal activity was observed in conventional agricultural systems with high inputs of inorganic fertilizers than in organic crop systems (Douds et al., 1993). Rajesh Kannan et al. (2011) concluded that higher concentrations of chemical fertilizer in the soil may be harmful to microbes such as AMF because chemicals may enter into the fungus cells and disturb their metabolism and affect their population. On the other hand, in our study, HS application significantly decreased root colonization in peppermints (Table 1). In laurel roots, higher concentrations of humic acids had a depressing effect on the percentage of root length infected by *G. mosseae* (Vallini et al., 1993). Addition of the humic acid has been reported to increase soil P availability (Tahir et al., 2011) and it seems that HS application caused an increase in P availability for peppermint plants, resulting in a reduction for root colonization.

The three factors applied in our study (*F. mosseae*, HS, and chemical fertilizers) promoted shoot growth of peppermints when applied separately. Also, there were additive effects between different factors on shoot biomass (Table 1). However, positive effects of *F. mosseae* and HS on shoot dry matter were more than those of the NK fertilizer. Hoseinzade et al. (2016) reported that AM fungi significantly increased r growth of the rice plants compared with the chemical fertilizer. Our findings are in accordance with the studies of Bettoni et al. (2014), and Linderman and Davis (2001) who reported that mycorrhizal inoculation and application of HS interacted synergistically and better enhanced plant growth in combination than separately. The positive effect of HS on plant growth is probably related to their auxin-like activity (Nardi et al., 2002). Auxins activate the H^+-ATPase of the plasma membrane, acidifying the apoplast and activating enzymes that act directly on the cell wall, allowing greater plasticity and leading to cell elongation (Aguirre et al., 2009). Besides, the positive effect of AMF on shoot growth can be attributed to the improvement of P nutrition, the uptake of water by hyphae, and the increase of root length density (Wu and Xia, 2006).

The proline levels were increased in peppermint leaves fertilized with HS and chemicals, and such enhancements were more evident under inoculation with AMF than at non-inoculated plants (Table 1). HS addition and AMF inoculation enhanced the accumulation of proline, having additive effects of both treatments in onion seedlings (Bettoni et al., 2014). Also, Anjum et al. (2011) found that HS application (as fulvic acid) elevated the proline content in maize leaves under both well-watered and drought conditions. Excess N supply caused an increased proline level in sugar

beet so that maximum proline level was recorded when nitrogen in plants was the highest (Monreal et al., 2007). Proline is the most common compatible solute that occurs in a wide variety of plants especially under stress conditions. The physiological functions such as osmoregulation, a sink for energy and nitrogen, a signal of senescence, and an indicator of stress sensor have been attributed to proline. Besides, the highest content of total phenolics was found in mycorrhizal plants that received HS and were exposed to chemicals (Table 1). Phenolic compounds, as a group of secondary metabolites that have diverse chemical structures, act as a signal in plant development and interactions between plants and microorganisms. The application of mycorrhizal fungi had significant effects on the accumulation of total phenolic compounds in 6-month-old plantlets of licorice and increased them up to fourfold (Orujei et al., 2013). Bettoni et al. (2014) reported that AMF and HS treatments significantly elevated total soluble phenolics in onion leaves under greenhouse condition. On the other hand, in the present work, there was a positive relationship between the contents of proline and soluble phenolics in leaves (Table 1). In this case, Cheynier et al. (2013) hypothesized that the synthesis of proline is accompanied by the oxidation of NADPH and an increased $NADP^+$/NADPH ratio may enhance activity of the oxidative pentose phosphate pathway providing precursors for phenolic biosynthesis via the shikimic acid pathway.

Results from our work revealed that the chemical fertilizer and HS application produced higher amounts of Chl *a*, Chl *b*, and total Chl than that of control plants, and foliar spray of HS had a positive effect on these pigments rather than the top dressing HS (Table 2). Similarly, the content of chlorophyll in the leaves of chrysanthemum treated with the foliar humic acid fertilizer was significantly higher than that of the control and the plants treated with chemical fertilizers (Fan et al., 2014). Also, Anjum et al. (2011) reported that foliar HS application elevated the chlorophyll contents in maize under both deficient and optimal water regimes. In this study, inoculation of *F. mosseae* (in solitary or together with other treatments) increased chlorophyll content (Chl *a*, Chl *b*, and total Chl) in comparison with non-inoculated plants. Shahabivand et al. (2012) reported that the presence of *G. mosseae* significantly increased chlorophyll contents in wheat plants. These results indicate the positive influence of this plant-microbe interaction as well as chemical and HS fertilizers on photosynthetic apparatus and higher rates of photosynthesis in peppermint plants.

Data from Table 3 revealed that there were positive effects of different factors (chemical and HS fertilizers and AMF inoculation), separately or in interaction, on the contents of starch and total soluble sugar in peppermint leaves. Increased content of non-structural sugars (starch and soluble sugars) in leaves of mycorrhizal plants and plants treated with HS have been previously found (Baslam et al., 2011; Bettoni et al., 2014). This enhancement can be a consequence of photosynthesis promotion with increased chlorophyll content and Rubisco activity (Ertani et al., 2011). Apart from carbohydrates, leaves of peppermints fertilized by chemicals and HS, and inoculated with AMF accumulated the highest content of soluble proteins (Table 3). Khalid (2012) found that accumulation of protein in leaves of anise, coriander and sweet fennel plants was promoted by applying various levels of chemical fertilizers. Onion plants fertilized by immersion and further foliar pulverization with HS accumulated the highest content of soluble proteins, which indicates that HS influenced N cell metabolism (Bettoni et al., 2016). Maximum content of the soluble protein was recorded in leaves of onion seedlings inoculated with AMF, fertilized with HS, and grown under elevated CO_2 (Bettoni et al., 2014). It has been demonstrated that increased activity of the enzymes glutamine synthetase (GS) and glutamate synthase, which act in the availability of NH_4^+ could enhance N organic compounds in plants (Ertani et al., 2011). Bettoni et al. (2014) found that improved uptake and translocation of N from roots to shoots in onion seedlings that received HS and/or were inoculated with AMF would explain the enhanced protein content found in leaves.

Several procedures are known to measure the total antioxidant capacity (or antioxidant power) of biological samples. In the present study, the FRAP assay was used to measure the reduction of ferric tripyridyltriazine (Fe(III)-TPTZ) complex to

the ferrous tripyridyltriazine (Fe(II)-TPTZ) by a reductant at low pH (Szôllôsi and Varga, 2002). So, the reduction capacity was considered as an indicator of antioxidant power. Data from Table 3 showed that the foliar application of HS and inoculation with AMF significantly increased FRAP content in peppermint leaves compared to plants not treated with HS and also non-inoculated plants. Mycorrhizal inoculation and addition of HS enhanced the antioxidant capacity in onion seedlings (Bettoni et al., 2014). The higher total antioxidant capacity of leaves in plants treated with mycorrhizae and HS (particularly foliar spray method) would help peppermint plants in dissipating the photosynthetically produced electrons and in alleviating probabilistic oxidative damage.

In conclusion, results of the present work demonstrated that the presence of AM fungus *F. mosseae,* application of HS, and NK treatment interacted synergistically and promoted plant growth, and enhanced contents of pigments, biochemical metabolites, and antioxidants more in combination than separately in peppermints under the field condition. The increases induced by the chemical fertilizer were smaller than those induced by mycorrhizal inoculation and HS addition. Also, foliar spray of HS improved the amount of abovementioned parameters compared with topdressing treatment of HS. Therefore, we suggest using *F. mosseae* and HS foliar application as plant biostimulants, particularly in combination, which will not only improve growth and biochemical response of peppermint but also reduce the environmental problems in sustainable agriculture.

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