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Additive effects of arbuscular mycorrhizae and TiO2 nanoparticles on growth and essential oils enhancement of peppermint

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ABSTRACT

Peppermint is an important medicinal plant, particularly regarding its high essential oil (EO) content. The present study assesses the effects of two Arbuscular mycorrhiza fungi (AMFs) and three concentrations of Titanium dioxide nanoparticles (TiO_2 NPs) (i.e., 100, 150, and 200 mg L^{-1}) on growth characteristics, EO constituents, phenolic content and anti-oxidative enzymatic activities of peppermint. Applying a mixed treatment of *Glomus intraradices* and TiO_2 NPs (150 mg L-1) enhanced peppermint's shoot-fresh and dry weight by 69% and 158%, respectively, compared with controls. Also, membrane stability index (MSI) and leaf relative water content (RWC) exhibited a maximum enrichment of 69.85% and 39%, respectively, over control. Some other plant physiological characteristics were also positively affected by applying either the single or combined treatment of *G. intradices* and TiO_2 NPs (150 mg L^{-1}). In this regard, total phenolic content increased by 300%, DPPH radical scavenging ability by 164%, catalase by 103%, and ascorbate peroxidase activities by 124% in mixed treatment of *Glomus intraradices* and TiO_2 NPs (150 mg L^{-1}). Moreover, AMF symbiosis and the implementation of TiO_2 NPs intervention augmented the amount of peppermint EO compounds such as menthol, menthyl acetate, and 1, 8 cineole. The highest amount of menthol production (52.04%) was obtained by adding a mixture of *G. intraradices* and TiO_2 NPs (150 mg L^{-1}), which had more positive effects on the other studied characteristics. Overall, AMF symbiosis and TiO_2 NPs application seem suitable for promoting plant growth, improving antioxidant activity, and incrementing major components of EOs in medicinal plants.

1. Introduction

Medicinal plants are known as phytochemical assets containing active compounds with well-recognized exceptional properties worldwide (Gupta et al., 2015). Peppermint, as an important medicinal plant, has been used in traditional medicine to reduce appetite, cough, fever, nausea, headache, and colon inflammation (Shah and Mello 2004). The medicinal importance of this plant is mainly attributed to its essential oils (EOs) (Ahmad et al., 2019). Peppermint EO mainly consists of menthol, menthone, menthofuran, menthyl acetate (Singh and Misra 2001), and compounds such as limonene and cineole, isomentone, isopolegol, polegon, and carvone (Soleymani et al., 2017). Research has shown that EO compounds biosynthesis is mainly influenced by biotic and abiotic factors (Maffei et al., 2011; Caser et al., 2016; Ghanbarzadeh et al., 2019).

Some researchers have reported the effects of mycorrhization and nanoparticles (NPs) on producing terpenoids (e.g., menthol, carvone, menthone, pulegone, thymol, and carvacrol) in aromatic plants (Freitas et al., 2004; Pandey et al., 2018; Ahmad et al., 2018). In most of these studies, increasing the terpenoids could be owing to phosphorous (P) availability and transcription of the genes responsible for terpenoid biosynthetic pathways (Torelli et al., 2000; Kapoor et al., 2002a; Krishna et al., 2005). AMF symbiosis may alter phytohormones levels (gibberellic acid (GA3), jasmonic acid (JA), and cytokinins) and induce the plastidic methyl erythritol phosphate (MEP) path with upregulating DXR (1-deoxy-D-xylulose-5-phosphate reductoisomerase) and DXS (1-deoxy-D-xylulose-5-phosphate synthase). Overall, this symbiosis increased the production of precursors, including isopentenyl pyrophosphate (IPP) and dimethylallyl diphosphate (DMAPP), for terpenoid biosynthesis (Kapoor et al., 2017).

Nanoparticles (NPs) could influence plants morphologically and physiologically (Agrahari and Dubey 2020) and have recently been used in agriculture due to their positive effects on the yield and growth of

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Abbreviations:

AM	arbuscular mycorrhizal
NM	non-arbuscular mycorrhiza
APX	ascorbate peroxidase
CAT	catalase
DDW	double distilled water
DAP	days after planting
DPPH	2,2-diphenyl-1-picrylhydrazyl
GC-MS	gas chromatography-mass spectrometry
EO	essential oil
NPs	nanoparticles
ROS	reactive oxygen species
OD	optical density
TPC	total phenol content
TiO_2 NPs	titanium dioxide nanoparticles
VOCs	volatile organic compounds

plants (Ahmad et al., 2018). TiO_2 NPs are acknowledged for their important role in increasing antioxidant enzymes activity, and EO contents (Ahmad et al., 2018). The remarkable role of TiO_2 NPs in producing EO has been reported in the plants such as *Mentha piperita* L. (Lamiaceae) (Ahmad et al., 2019), *Salvia officinalis* L. (Lamiaceae) (Ghorbanpour, 2015), *Rosmarinus Officinalis* L. (Lamiaceae) (Golami et al., 2018), and *Vetiveria zizanioides* L. (Poaceae) (Shabbir et al., 2019). According to the beneficial effects of TiO_2 NPs on different medicinal plants, an attempt was made to investigate the effect of the NPs on peppermint growth, biochemical traits and content of EO constituents. Despite some credible studies on the plants-AM fungi interaction, the roles of AM fungi in TiO_2 NPs-plant interactions have not been well examined. Therefore, considerable knowledge gaps have remained regarding the potential of TiO_2 NPs for peppermint, especially in the presence of AM fungi. Accordingly, it seems that more profound research is required to distinguish the interactive effects between TiO_2 NPs and AM fungus on plant growth, and secondary metabolite production. In this respect, the present study aims to investigate the combined effects of AM inoculation and TiO_2 NPs application on growth, anti-oxidative enzymatic activities, and EO constituents of peppermint.

2. Materials and methods

2.1. Plant material and growth condition

The rhizomes of peppermint were obtained from the Faculty of Agriculture, Ferdowsi University of Mashhad, Iran. The experiment was conducted from May to September of 2019 in the study field of Hakim Sabzevari University, Iran. Each experimental unit included a plastic pot (22 cm diameter × 20 cm height) filled with an autoclaved mixture (0.11 MPa, 120 °C, 1 h) of Coco peat:Vermicompost:Perlite:Soil (1:1:1:1, v/v). Table 1 shows the chemical characteristics of the examined soil. The plastic pots were arranged randomly, and the rhizomes were planted under natural conditions (an open greenhouse). It had a relative humidity ranging from 50 to 70%, and average night and day temperatures were maintained at 17 and 32 °C, respectively. First, the rhizomes were planted in the pots, then the 30-day-old seedlings transported to the plastic pots. Each treatment was duplicated in three pots, and three seedlings were included in each pot. Watering the plants was performed daily.

Table 1
Chemical properties of the experimental soil.

pH	EC (dS/m)	N (%)	P (mg/kg)	K (mg/kg)	Fe (mg/kg)	Zn (mg/kg)	Mn (mg/kg)
7.25	1.2	1.4	120	1250	842.8	36.6	62.8

2.2. Arbuscular mycorrhiza preparation and inoculation

The inoculum was an AM fungus inoculum comprising external mycelium, spores, and mycorrhizal roots, provided by the Organic Plant Health Company (Hamedan, Iran). Mycorrhizal-bearing soil (*G. intraradices and G. mosseae*) with a ratio of 100 g per 1 kg of soil was added to some pots (12,000 to 15,000 spores per 1 kg of soil).

2.3. TiO_2 NPs

TiO_2 NPs were pepared from the US Research Nanomaterials (USA), with their features explained in our former work (Ghorbani, 2020). Briefly, XRD analysis showed an anatase structure at $2\theta = 25.3°$, 37.8°, and 48.1° with a four-faced tetragonal structure. The mean crystallite size of NPs was estimated to be 13 nm. Also, the hydrodynamic diameter distribution profile of TiO_2 NPs was measured to be between 12 and 25 nm using a dynamic light scattering (DLS) device. In addition, the zeta potential of TiO_2 was 10 ± 30 mV. These characteristics were considered for further application and analysis of TiO_2 NPs in our experiments. TiO_2 solution was made at different concentrations (0, 100, 150, and 200 mg L^{-1}) with filtered double-distilled water (DDW). Different TiO_2 NPs concentrations were applied to the 30-days-old seedling through foliar spraying. DDW was used as a control treatment. Treatments were used at intervals of 7-day (7 times) using a hand sprayer. Aggregation was avoided by sonicating the TiO_2 solutions when required.

2.4. Experimental design and treatments

The experiment was conducted using a 3 × 4 factorial arranged in a completely random design with three levels of AM fungi (inoculated with *G. mosses*, *G. intraradices* and non-inoculated plants (NM)) and four concentrations of TiO2 NPs (0, 100, 150, and 200 mg L-1). Each experimental unis was repeated for three times.

2.5. Growth characteristics

The plants were harvested two months after treatments, and morphological features such as plant height (H) and aerial biomass were determined. The mentioned parameters were determined by monitoring the three plants per pot. The aerial fresh and dry weight (g) were determined, and the specimens were dried at 70 °C in an oven for 48 h.

2.6. Leaf relative water content (RWC)

Leaf relative water content was determined from the upper fully extended young leaves according to a method described by Whetherley (1950), as follows:

$$RWC\ (\%) = ((FW - DW)\ /\ (SW - DW)) \times 100$$

FW = Fresh weight
DW = Dry weight
SW = Saturated weight

2.7. Membrane stability index (MSI)

The MSI was evaluated as described by Barrs and Weatherley (1962), using eight-leaf discs for each treatment. Then, completely prolonged young leaf specimens were rinsed 3 times with deionized water to eliminate surface-adhered electrolytes. Leaf samples were put in closed vials comprising 50 mL of deionized water and were incubated at 40 °C for 24 h. Afterward, the first electrical conductivity of the solution (EC1) was determined by an EC meter. Finally, the specimens were autoclaved

at 100 °C for 60 min and the second electrical conductivity (EC2) was determined (Barrs and Weatherley 1962).

The MSI was computed according to the following formula:

$$MSI\% = [1 - (EC1 \ / \ EC2)] \times 100$$

2.8. Estimation of root colonization

The AMF colonization was visualized by clearing roots by boiling in 10% KOH for 4 min, further rinsing three times with tap water, and staining with 5% ink/household vinegar (= 5% acetic acid) solution based on the procedure described by Vierheilig et al. (1998). Eventually, the root colonization percentage was determined based on the method proposed by Newman (1966).

2.9. Biochemical analysis

2.9.1. Sample preparation

To measure biochemical traits, fresh leaves from each treatment were separately placed in aluminum foil and stored in a freezer at −80 °C after putting them in liquid nitrogen.

2.9.2. Total phenolic content (TPC)

The TPC was determined by a spectrophotometer (Analytik Jena, SPECORD 210, Germany) based on the method proposed by Sadasivam and Manickam (2008). The leaf specimens (500 mg) were ground, accompanied by a 5-time volume of 80% ethanol using a pestle and mortar. The homogenate was centrifuged for 10 min at 4 °C at 10,000 rpm to save the supernatant. In the end, it evaporated to dryness after adding 5 mL of DDW. Then, 0.5 mL of the Folin-Ciocalteau reagent and 2 mL of 20% Na_2CO_3 were added to each tube. The solution OD was measured at 650 nm versus a reagent blank. The findings were expressed using equivalents of mg Gallic acid (GAE) g^{-1} of fresh weight with gallic acid considered the standard (Sadasivam and Manickam 2008).

2.9.3. Determination of rosmarinic acid

Dry plant powder (0.1 g) was ground with 10 mL of 80% methanol and put in a shaker at 70 °C for 90 min. The resulting extracts were filtered using filter paper. The absorption of the methanolic extracts at 333 nm was read by a spectrophotometer (Analytik Jena, SPECORD 210, Germany). Then, the concentration of rosmarinic acid was determined using a standard curve (Lopez-Arnaldos et al., 1995).

2.9.4. Determination of caffeic acid

The samples were ground on ice with 80% methanol at a ratio of 1.5 w/v until obtaining a homogeneous solution. The resulting homogenate was stirred at 40 °C for 3 h and then centrifuged at 45 rpm for 45 min, and the obtained supernatant was employed to determine caffeic acid.

Next, 1 mL of 80% methanolic extracts was taken, followed by adding 1 mL of the ARNO reagent (containing 10% sodium molybdate and 10% sodium nitrate), 1 mL of sodium hydroxide 1 M, and 1 mL of hydrochloric acid 0.1 M. After vortex operation, the mixture absorbance was immediately measured at 490 nm by a spectrophotometer (Analytik Jena, SPECORD 210, Germany). In the control sample, 1 mL of 80% methanol was added instead of the extract (Sauvesty et al., 1992).

2.9.5. DPPH-radical scavenging activity

The antioxidant activity was presented as the DPPH radical scavenging activity percentage. This criterion was defined based on the technique clarified by Fernández-Agulló et al. (2013). The dried plant leaves (100 mg) were crushed with 2 ml methanol, and the mixture was centrifuged at 3500 rpm for 10 min. Subsequently, the solution received a 1.5 mL methanolic solution of DPPH. The mixture was placed in the dark to measure the mixture's absorbance at 517 nm after 30 min using a spectrophotometer (Analytik Jena, SPECORD 210, Germany), and antioxidant activity was calculated according to the following formula (Fernández-Agulló et al., 2013).

$$\text{DPPH radical scavenging activity } (\%) = \frac{A_C - A_S}{A_{cC}} \times 100$$

A_C = absorbance in control
A_S = absorbance in sample

2.9.6. Antioxidant enzyme activities

In liquid nitrogen, the fresh leaves (100 mg) were ground and homogenized in 1 mL phosphate buffer (pH = 7) comprising 0.1 mM of EDTA and 1% (w/v) of Polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 13,000 rpm for 30 min at 4 °C, and the supernatant was used for the following enzyme activity assay.

Catalase enzyme activities were measured using the protocol offered by Alici and Arabaci (2016) after the H_2O_2 consumption. The reaction mixture included 50 µL enzyme extract, 3 mL of 50 mM phosphate buffer, and 100 µL hydrogen peroxide (15 mM). The extracts were added to the reaction mixture, and the absorbance was read immediately at 240 nm at 0, 15, 30, 45, and 60 s. The enzyme activity was spectrophotometrically determined through monitoring the decrease in absorbance.

Ascorbate peroxidase (APX) activity was determined based on the protocol proposed by Nakano and Asada (1981). The reaction mixture (1 mL) comprised 50 mM potassium phosphate buffer (pH = 7), 10 mM ascorbate, 0.1 mM H_2O_2. The reaction was initiated by adding 100 µL of the crude enzyme to the mixture. Ascorbate oxidation was read using a spectrophotometer for 1 min by measuring the reduction in absorbance at 290 nm at 10 s intervals (Analytik Jena, SPECORD 210, Germany). Eventually, the activity was determined using the extinction coefficient of 2.8 $mM^{-1}cm^{-1}$ (Nakano and Asada 1981).

2.10. EO extraction and analysis

Fresh leaves from various treatments were chopped and blended. The hydrodistillation method was used to extract EO content in the leaves for 3 h using the Clevenger apparatus. Over anhydrous sodium sulfate, the EO was dried and kept in sealed glass vials at 4 °C for the gas chromatography-mass spectrometry (GC-MS) analysis.

The GC-MS analysis was conducted via Agilent 7890 B gas chromatograph linked with a mass detector (Model 5977 A, Agilent Technologies, USA). The gas chromatograph was equipped with an HP-5MS capillary column (30 m × 0.25 mm ID 0.25 µm, Phenyl methyl siloxane, Agilent Technologies). The injector temperature was 270 °C, and the oven temperature was programmed from 60 °C (10 min) to 200 °C at a rate of 5 °C/min. Helium was selected as the carrier gas. In this process, the flow rate was set to injection volume (1 µL) and 1 mL/min. Also, the mass range was 35–500 m/z, and the mass ionization energy was 70 eV. The interface temperature was adjusted to 280 °C. The compounds were recognized by comparing the retention times (RT) with those kept in the National Institute of Standards and Technology (NIST) and the Wiley library.

2.11. Statistical analysis

Two-way analysis of variance (ANOVA) was performed via IBM SPSS V.23.0, and Duncan's multiple range test was performed to define the statistical differences at $P < 0.05$. The test was carried out in triplicate, and the results were stated as the mean ± standard error (SE).

3. Results

3.1. Growth features

The outcomes of two-way ANOVA revealed that Mycorrhiza, TiO_2 NPs, and the treatment interaction significantly affected all evaluated

parameters (Table 2). In general, shoot length and shoot weight (dry and fresh) increased significantly in plants inoculated with *G. intraradices* compared to the plants inoculated with *G. mossea* in all TiO_2 NPs concentrations. The results demonstrated that using 150 mg L^{-1} TiO_2 NPs amd *G. intraradices* enhances plant growth, which was manifested as increased shoot length (76.16%), shoot dry weight (160%), and shoot fresh weight (69.76%) compared to control.Our findings further revealed that applying 200 mg L^{-1} of TiO_2 NPs significantly reduced the aerial biomass of AM plants (Fig. 1A–C).

3.2. Relative water content (RWC)

Using TiO_2 NPs (150 mg L^{-1}) increased RWC significantly in AM plants (Fig. 1D). The highest RWC (67.68%) was found in plants inoculated with *G. intraradices* with TiO_2 NPs (150 mg L^{-1}) application, which increased by 69% compared to the control. The RWC rate in plants inoculated with *G. intraradices* × TiO_2 NPs (various concentrations) was higher than in plants inoculated with *G. mosseae*.

3.3. Membrane stability index (MSI)

AM plants showed greater MSI than non-inoculated plants (NM plants), regardless of TiO_2 NPs treatments. The maximum MSI (70.25%) was found in plants colonized by *G. intraradices* with 150 mg L^{-1} TiO_2 NPs. The results demonstrated that the inoculation with *G. mossea* and *G. intraradices* separately and in combination with TiO_2 NPs (100 and 150 mg L^{-1}) significantly increase MSI compared to control plants. Foliar spray of 200 mg L^{-1} TiO_2 NPs significantly decreased the MSI (Fig. 1E).

3.4. Root colonization

The root colonization percentage was significantly higher in plants inoculated with *G. intraradices* than *G. mossea* in all TiO_2 NPs concentrations (Fig. 1F). The highest root colonization percentage (66.6%) was found in plants inoculated with *G. intraradices* and foliar application of TiO_2 NPs (150 mg L^{-1}).

3.5. Total phenolic contain (TPC)

Analysis of variance showed that, TiO_2 NPs, mycorrhiza, and their interaction had significant effects on all measured biochemical features (Table 3). Our findings showed that TPC in both NM and AM plants increased significantly with the application of TiO_2 NPs (except 200 mg L^{-1}) (Fig. 2A). Besides, the highest amount of TPC was obtained in NM plants treated with 150 mg L^{-1} TiO_2 NPs (40% higher than control). Overall, the use of *G. intraradices* could have a more significant effect on the promotion of TPC compared to *G. mossea*.

3.6. Rosmarinic acid and caffeic acid

Based on the results of Fig. 2B and C, the highest content of rosmarinic acid (102.61 ± 0.4304 mg. g^{-1} dry weight) and caffeic acid (81.4 ± 1.1758 mg. g^{-1} dry weight) belonged to the application of *G. intraradices* and 150 mg L^{-1} TiO_2 NPs (62% and 85% higher than the control (without elicitation), respectively). In general, the exogenous application of AMF fungus significantly increased rosmarinic and caffeic acids in both TiO_2 NPs-treated plants and without TiO_2 NPs plants.

3.7. DPPH-radical scavenging activity

The antioxidant activities of the extracts were reported as the percentages of DPPH radical scavenging activities. Using TiO_2 NPs and AM fungus enhanced the DPPH-scavenging activity significantly more than the control (Fig. 2D). DPPH-scavenging activity in *G. intraradices* × 150 mg L^{-1} TiO_2 NPs treatment was more than 2.5% compared to the control. Also, the free radical scavenging activity of DPPH was 58.38% in plants colonized with *G. intraradices* × 150 mg L^{-1} TiO_2 NPs treatment versus 22.05% in the control treatment.

3.8. CAT and APX activities

Generally, the CAT activity in AM and NM plants considerably increased when applying 100 and 150 mg L^{-1} of TiO_2 NPs (Fig. 2E). The CAT and APX activity was the highest in plants inoculated with *G. intraradices* with 150 mg L^{-1} TiO_2 NPs application. As expected, APX activity in plants inoculated with *G. intraradices* was higher significantly than *G. mosseae* in all TiO_2 NP concentrations (Fig. 2F).

3.9. The EOs content

According to the results (Table 4), using AM fungus (*G. mossea* and *G. intraradices*) and TiO_2 NPs, except for a few cases, could increase EOs constituents compared to the control.

GC-MS analyses revealed that 53 constituents were obtained in the EOs of peppermint, comprising menthol (24.21–52.04%) as the major component, isomenthone (0.94–4.3%), menthyl acetate (2.63–19.58%), and 1, 8-cineole (0.86–7.58%). The percentage of menthol in AM and NM plants treated with TiO_2 NPs exerted a significant increase in comparison to the control. The highest menthol percentage was observed in *G. intraradices* × 150 mg L^{-1} TiO_2 NPs treatment (52.04%), i.e., 114% more than the control (24.24%). In comparison, plants exposed to 200 mg L^{-1} TiO_2 NPs did not show an increase in menthol content. Moreover, other constituents in EO demonstrated significant fluctuations with AM fungus and TiO_2 NPs in comparison to the control. The GC-MS analyses indicated that the content of menthofuran represented a considerable decline with AM fungus (*G. mossea* and *G. intraradices*), while it increased with the application of TiO_2 NP (100 and 150 mg L-1). Table 4 illustrates the impact of AM fungus, and TiO_2 NPs on the EO contents of peppermint. Further, the EO chromatogram for some treatments is showed in supplementary file.

4. Discussion

4.1. Growth characteristics

The results showed that AM fungus, especially *G. intraradices*, significantly improved some morph physiological characteristics such as shoot dry weight, shoot length, MSI, and RWC. The ameliorating effects of AMF seem to be related to the improved nutritional status of the host plant (Koschier et al., 2007) especially phosphorus (Begum et al., 2019). In current study, AM plants have a better water supply (RWC) than NM

Table 2
Analysis of variance for different growth properties of *Mentha piperita* L. plants.

Source	df	Shoot length (cm)	Fresh weight (g)	Dry weight (g)	WRC (%)	MSI (%)	Root colonization (%)
Mycorrhiza	2	80.123*	2.996*	0.478*	786.016*	257.058*	9416.983*
TiO_2 NPs	3	150.942*	2.809*	0.699*	354.956*	288.378*	48*
Mycorrhiza* TiO_2 NPs	6	38.967*	0.407*	0.197*	16.656*	71.327*	8.406*
Error	24	0.277	0.008	0.000	0.000	11.045	3.091

Note. *: Significant at $P \leq 0.05$. TiO_2 NP: Titanium dioxide nanoparticle.

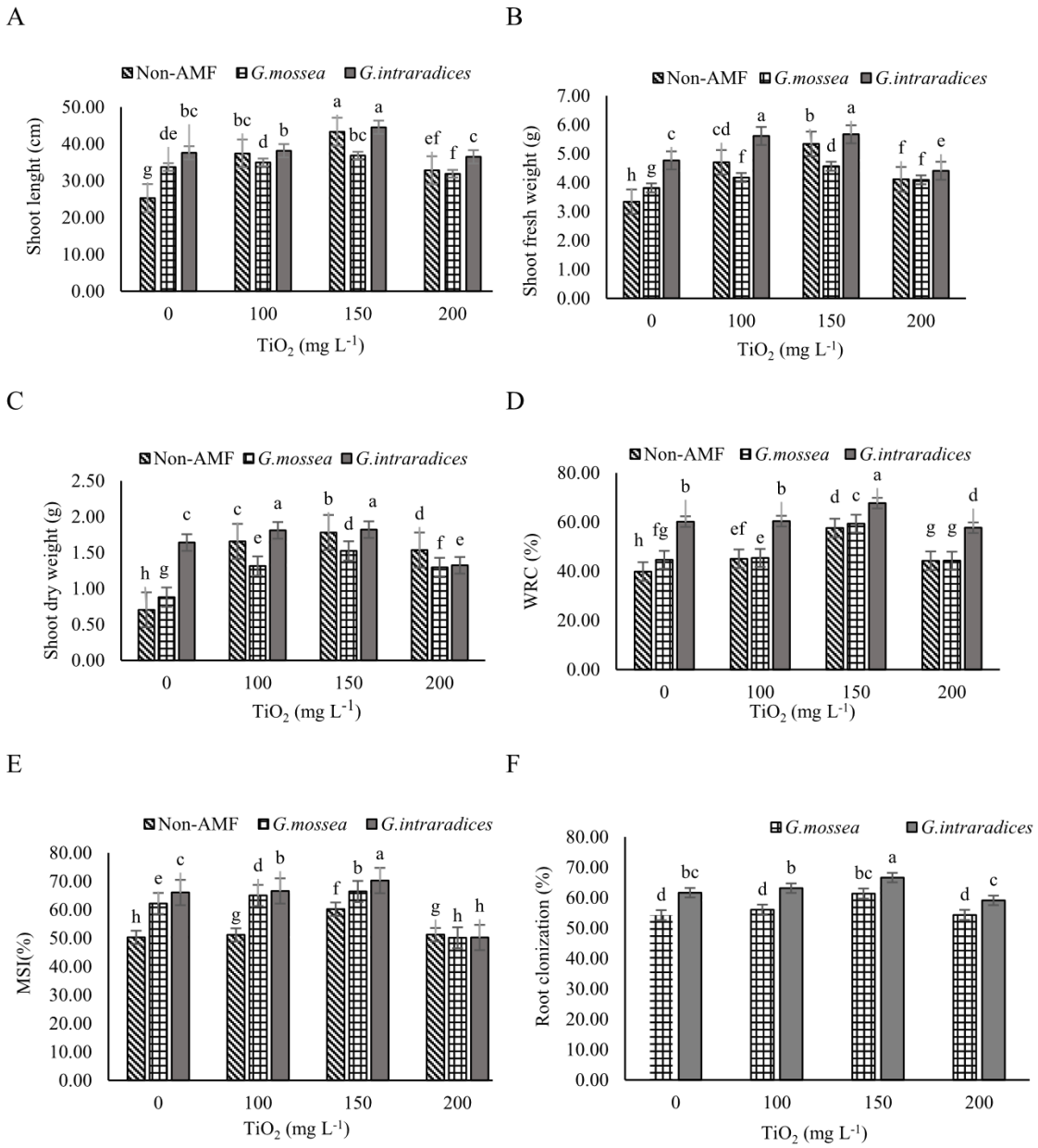

Fig. 1. Effects of AMF and TiO_2 NPs application on Shoot length(A), Shoot fresh weight (B), Shoot dry weight (C), Water Relative Content (WRC) (D), Membrane Stability Index (MSI) (E), Root colonization (%)(F). In each figure, means with the same letter(s) are not significantly different according to Duncan's test at $P < 0.05$. Means ± S.D from the three replications. Error bars (⊤) show SE.

Table 3
Analysis of variance for the biochemical characteristics of *Mentha piperita* L. plants.

Source	df	Total Phenolic contain (mg g^{-1} Fw)	Rosmarinic acid (mg. g^{-1} dry weight)	Caffeic acid (mg. g^{-1} dry weight)	DPPH (%)	Catalase activity (unit min^{-1} g^{-1}Fw)	Peroxidase activity (unit $min^{-1}g^{-1}$ Fw)
Mycorrhiza	2	358.792*	2451.367*	685.112*	561.906*	0.005*	0.059*
TiO_2 NPs	3	509.057*	513.305*	190.556*	421.666*	0.005*	0.019*
Mycorrhiza* TiO_2 NPs	6	136.727*	27.852*	87.175*	129.326*	0.001*	0.001*
Error	24	0.174	0.825	0.604	0.065	0.000	0.000

Note. *: Significant at $P \leq 0.05$. TiO_2 NPs: Titanium dioxide nanoparticle; DPPH: 2-diphenyl-1-picrylhydrazyl.

plants. This difference might result from, more efficient water absorption via external hyphae and elevating root hydraulic conductivity (Amiri et al., 2017; Le Pioufle and Declerck 2018) and intracellular water transport through promotion in the expression of water channel proteins (Aroca et al., 2007; Ruiz-Lozano et al., 2009). Furthermore, AM in a symbiotic relationship could have substantial effects on the root physiology of host plants, especially in glutamine synthetase, arginase, and urease activity. These enzymes play key roles in nitrogen transport from mycelium to the root and lead to encourage root growth (Dutta and Neogm, 2016).

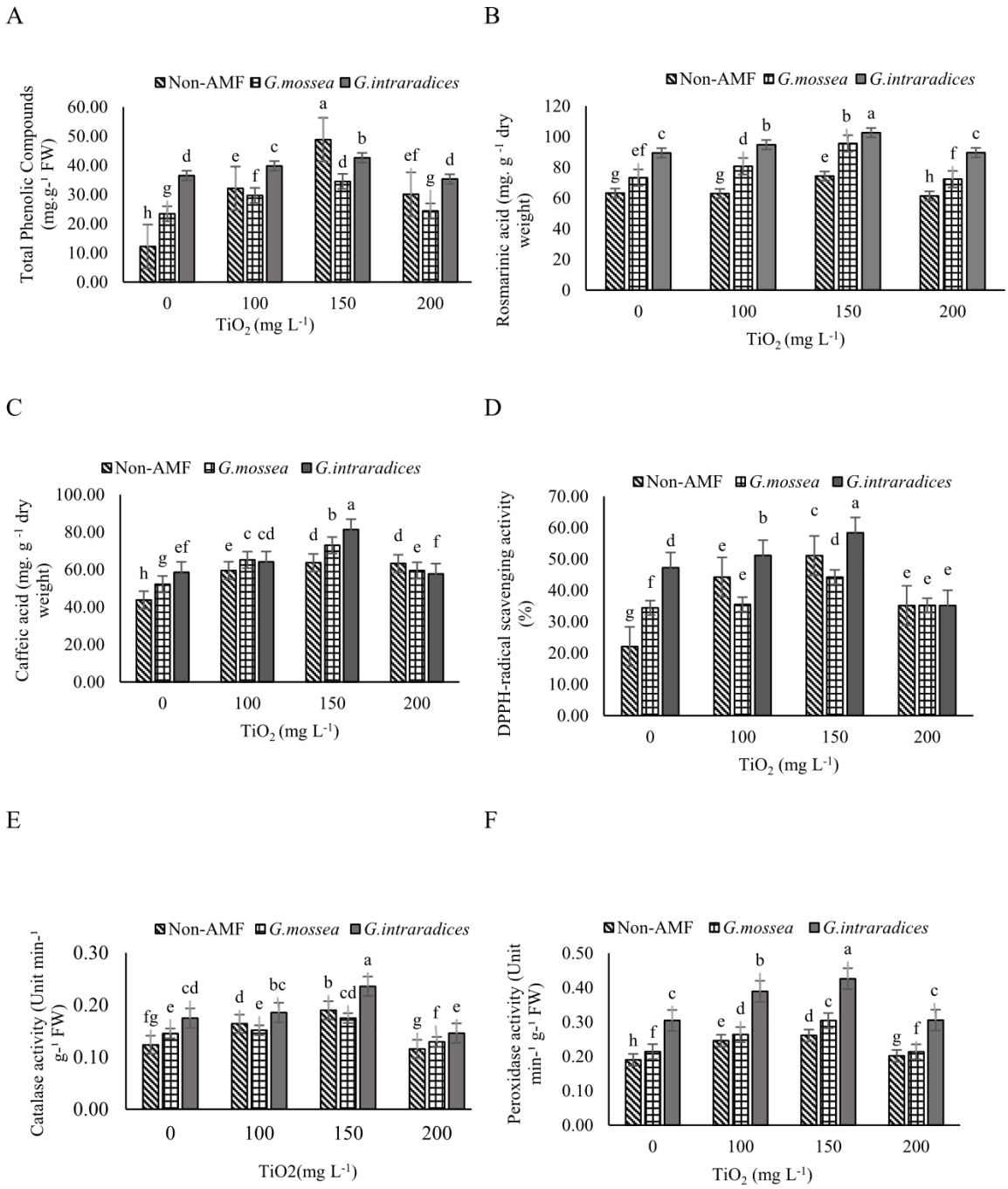

Fig. 2. Effects of AMF and TiO_2 NPs application Total Phenolic Compounds(A), Rosmarinic acid (B), Caffeic acid (C), DPPH-radical scavenging activity (D), Catalase activity (E), Ascorbat peroxidase activity (F). In each figure, means with the same letter(s) are not significantly different according to Duncan's test at $P < 0.05$. Means ± S.D from the three replications. Error bars (⊤) show SE.

Researchers believe that some parts of the hyphae can induce the plant root system by increasing the cytokinin content. Consequently, they lead to the foundation system expansion and increase water uptake and plant height (Wu and Xia 2006). In a study, the increase in plant height of AM plants contributed to the increase in mitotic activity of stem cells (Marschner 1995). The beneficial effects provided by AM fungus have been reported in previous studies for Basil (Gupta et al., 2009), *Cucurbita pepo* L. (Sensoy et al., 2013), and *Salvia officinalis* (Tarraf et al., 2017).

Our result showed that TiO_2 NPs enhanced the growth characteristics of plants inoculated with AM. Increased biomass production in AM plants exposed to TiO_2 NPs may be related to the promoted absorption of minerals (Chen et al., 2018), especially nitrogen and magnesium, and nitrogen metabolism acceleration. In another study, Feizi et al. (2012) indicated that TiO_2 NPs had a positive effect on wheat shoot length compared to control. Gao et al. (2008) showed that spinach seeds treated with TiO_2 NPs increased spinach's dry and fresh weights by 61% and 71%. They found that TiO_2 NPs could significantly improve RWC in plants. It is believed that TiO_2 NPs can make new pores in the plant cells and facilitate water uptake inside the cells (Singh et al., 2016). Accordingly, the higher reactivity of TiO_2 NPs might extend root pores or lead to more increased water flow in roots (Subramanian et al., 2006; Larue et al., 2012).

In our study, the MSI was higher in AM plants. It seems that AM fungi

Table 4
Effect of AMF (*G.mossea* and *G.intraradices*) inoculation and TiO_2 NPs application on percentage composition of *Mentha piperita* EOs.

No	Compound	RT	Concentration (%)											
			Non-mycorrhiza				*G.mossea*				*G.intraradices*			
			0	100	150	200	0	100	150	200	0	100	150	200
1	α-Pinene	4.15	0.93	0.55	0.6	0.92	–	0.57	–	1	0.55	0.58		
2	Comphene	4.46	–				–		–	0.31				
3	Sabinene	4.88	1.2	0.51	0.51	0.53	–	0.65	–	0.96				
4	β-Pinene	5	1.97	0.84	0.95		–	1.1	0.57	1.87	0.85	0.99		0.6
5	Ethanone,1-cyclohexyl	5.13	–				–			0.37				
6	O-Cymene	5.99	0.55				–			0.48			0.53	
7	Limonene	6.09	1.3	1.1	1.05		–	1.55	0.73	1.28	0.85	1.26		
8	1,8Cineol	6.15	2.41	5.35	5.65	2.87	1.81	0.86	4.27	3.98	3.18	7.58	2.4	4.35
9	4,7,dimethyl, undecane	6.22	0.35	3.02	1.3					0.53				7.05
10	4,4-dimethyl undecane	6.62	–				2.82						2.35	
11	cis sabinene hydrate	7.07	–	0.92			–							
12	1-octen-1-ol acetate	7.97	1.15				–	0.87					0.98	
13	3-octyl acetate	8.2	0.31				–							
14	l-menthone	9.21	–	3.5	4.24	0.94	–		2.66	0.94	2.13	2.47		4.3
15	Menthofuran	9.43	5.39	27.36	22.71		–	0.86		3.05	10	5.09		3.01
16	P-mentone	9.45	–			0.56	–		1.87					
17	Isoneomenthol	9.61	–		2.6	0.86	–		2.95	1.63	2.99	4.15		3.11
18	Isomenthol	9.62	–	2.67			–							
19	Menthol	9.84	24.24	39.03	39.31	25	31.82	40.02	45.31	33	45.27	49.1	52.04	46.62
20	Neomenthl	10.1	–				–			0.53				0.69
21	dl-menthol	10.14	–	0.73	0.78		–		0.75			1.43	1.23	
22	Cumaldehyde	10.82	1.89				1.47						1.01	
23	1-H-Benzimidazole-5-methoxy	10.87	–			1.69	–	2.13						
24	Pulegon	11.39	5.59	1.22	1.19	0.45	0.73	7.94	1.43	5.88	0.69	1.37	1.32	1.33
25	2,3,4,trimethyl, Decane	11.98	0.41				–							0.86
26	2,2,6,trimethyl, octane	11.99	–			0.26	2.18			0.35				
27	Neomenthyl acetate	12.26	–				–				0.72	0.63		
28	4,6,-dumethyl-dodecane	12.31	–				4.92			0.84			3.96	
29	Bornyl acetate	12.57	2.68				–	1.82		2.54				
30	Isomenthol acetate	12.72	–				–						2.51	
31	Menthyl acetate	12.73	15.25	7.99	10.48	2.63	6.18	12.17	12.12	19.58		12.15		13.03
32	Neomenthol acetate	13.13	–				–				16.01	0.49		
33	phenol-3-isopropoxy-5-methyl	13.99	–				–					0.85		
34	Orcinol, monoacetate	14.04	2.05				–	1.27		0.5				
35	Menthofourolactone	14.28	5.78	0.62	1.94	6.31	–	11.65	2.9	2.13	2.73	2.38	1.42	1.74
36	Amol	14.61	–			3.86	–							
37	Furfuryl isothiocyanate	15.17	–			0.43	–							
38	Caryophylene	16.09	7.54	1.82	0.91		–	4.22	1	6.6	1.8	1.95	1.08	
39	3H-2-1-Benzoxathiole-3chloro-1-1-dioxide	16.21	–			0.86	–							
40	Acetophenone,2-hydroxy-5-methoxy	16.24	0.69				–							
41	3-octen-2-one-3-butyl	16.37	–			5.6	–							
42	Cis-Trans-1,6-Dimethylspiro(4,5)decane	16.68				1.51		1.73		0.45		0.76	0.66	
43	Humulene	16.98	0.78							0.62				
44	3,3-Dimethylhexane	17.1	0.45				3.24			0.36			2.82	
45	Cis murrola 4(14)-diene	17.61	0.63							0.56				
46	Germacrene D	17.62	–	1.88			1.96							
47	hexadecane	17.75	0.83	0.4			6.74			0.82	0.7		5.06	
48	Anisol	17.94	1.02				–							
49	Mintfuranone	18.01	1.26		1.29	9.16	–					1.15		
50	Mintlactone	18.05	–				–	3.88	3.93	2	0.86			1.28
51	3-Ethyl-3-methylheptane	18.86	0.33				3.48	1.2		0.42		1.92	2.03	
52	7-a-Hydroxymintlactone	19.67	1.49		1.11	16.13	–	2.14	4.77		1.68	2.08	1.16	3.77
53	Caryophylene oxide	20.03	3.3			0.64	–	1.4	1.73	4.53	0.78	1.09		1.24
54	Viridiflorol	20.36	–	0.48			–	–	1.07		0.83			
55	tau-cadinol	21.44	–				–	–		1.55				
56	3-8dimethyl undecane	22.66	0.62				5.08						3.47	0.93

Note. RT: Retention time; 0, 100, 150 and 200 = 0, 100, 150 and 200 mg L^{-1} TiO_2 NPs; TiO_2 NP: Titanium dioxide nanoparticles.

supported plants in maintaining membrane integrity and structure. Lower electrolyte leakage in a plant with fungal symbiosis may be related to a reduction in cellular membranes and improved proactive defense mechanisms' performance in the cytosol (Khalvandi et al., 2019). Consistent with our results, Feng et al. (2002) reported higher membrane stability due to increasing the phosphorus uptake, cell water content, or activity of enzymatic and non-enzymatic antioxidants. Our results approved that using TiO_2 NPs reduces electrolyte leakage and improves membrane stability.

4.2. Total phenolic content

The present study showed that AM fungus inoculation increases total phenolic content in peppermint plants. The higher accumulation of total phenolic content in AM plants may be due to improves the host nutritional condition, especially inorganic phosphorus (P_i) and calcium content, transported by the fungus' extra radical hyphae to the root cells (Heidari and Nazari Deljou 2014). Sharifi et al. (2011) stated that TPC in AM inoculated basil was significantly higher compared to the NM plants. In AM plants, the accumulation of secondary metabolites like phenolic compounds may be attributed to the activation of metabolic routes

(Lohse et al., 2005) and alterations in the activity of key enzymes, such as Phenylalanine ammonialyase (PAL), responsible for producing these compounds in response to symbiosis (Dutta and Neogm, 2016). Levels of transcripts encoding PAL revealed transient rises in *Oryza sativa* L. colonized by *Glomus mosseae* and *Medicago truncatula* Gaertn (Fabaceae) inoculated by *Glomus versiforme* (Blilou et al., 2000; Harrison and Dixon 1993). Probably, higher production of phenolic compounds in AM colonized peppermint can be related to a defensive reaction. This reaction forms the peltate glandular trichomes (site of oil synthesis) and increases the absorption of mineral nutrients, particularly phosphorus (Khalvandi et al., 2019). The potential of AM fungus to induce changes in phytohormones such as cytokinin and gibberellin (Tarraf et al., 2017) is another mechanism that explains the TPC accumulation in response to mycorrhizal symbiosis. Certainly, further assessment is needed to confirm the exact efficacies of these mechanisms).

This study revealed an increased phenolic content in plants treated with TiO_2 NPs. Our findings are consistent with those of Oloumi et al. (2015) about *Glycyrrhiza glabra* L. (Fabaceae). In line with our results, Li et al. (2015) reported a positive correlation between TPC and antioxidant activity in medicinal and aromatic plants.

4.3. Antioxidant enzymes activity

Results of the present study represented a considerable increase in CAT and APX activity following the addition of different concentrations of TiO_2 NPs except for 200 mg L^{-1}. Metal oxide nanoparticles can induce reactive oxygen species (ROS) generation (Ma et al., 2015). Oxidative stress occurs when the balance between ROS and antioxidant defense is disturbed. The activity of antioxidant enzymes may be altered depending on the concentration and type of NPs (Movafeghi et al., 2018). For example, Servin et al. (2013) reported increased SOD, CAT, and POD activities and a reduction in ROS accumulation when plants were treated with TiO_2 NPs. Furthermore, a 750 mg/kg dose of TiO_2 NPs increased CAT activity, and 500 mg/kg of TiO_2 NPs decreased APX activity in cucumber plants (Servin et al., 2013). Also, SOD, CAT, APX, and GPX activity in spinach leaf chloroplasts increased with NPs application (Lei et al., 2008).

The current study showed a significant reduction in CAT and APX activities when using 200 mg L^{-1} of TiO_2 NPs. This reduction may be due to the overproduction of ROS and damage to the plant defense system (Hazani et al., 2013).

In our study, TiO_2 NPs and AM fungus treatments significantly enhanced the DPPH-scavenging activity. These results are consistent with those reported earlier by Ceccarelli et al. (2010), who stated antioxidant activity in *Cynara scolymus* L. was markedly increased in AM plants. The activity of antioxidant enzymes could be changed depending on the concentration and type of nanoparticles. ROS overproduction in plants exposed to TiO_2 NPs could induce the antioxidant system to scavenge ROS. Moreover, the ROS levels under stress conditions can be controlled by antioxidant enzymes like catalase, peroxidase, and superoxide dismutase (Kardavan Ghabel and Karamian 2020).

4.4. The EOs content

In the present study, the content of EOs, such as terpenoids, was significantly influenced by the implementation of AM fungus and TiO_2 NPs. Our results regarding AM fungal inoculation on the EO content are consistent with those of Ostadi et al. (2020). According to these authors, the cytological changes of plastids and mitochondria in the roots of AM plants led to the activation of the plastidial methyl-erythritol-phosphate (MEP) path of isopentenyl pyrophosphate biosynthesis.

Isopentenyl diphosphate is the central precursor of all isoprenoids. Besides, EOs such as terpenoid compounds and their constituent units (isoprenoids) like isopentenyl pyrophosphate and dimethylallyl pyrophosphate (DMAP) require ATP and NADPH (Ostadi et al., 2020). Therefore, more accumulation of EOs in current study may be related to the increase in available P in AM plants (Tarraf et al., 2017) and overexpression of genes involved in terpenoid biosynthetic pathways (Kapoor et al., 2017; Ahmad et al., 2018). The effect of AM fungi inoculation in improving the EO compounds has already been reported; e.g., geraniol and linalool in *Coriandrum sativum* L. (Apiaceae) (Kapoor et al., 2002b), limonene and carvone in *Anethum graveolens* L. (Apiaceae) and thymol in *Trachyspermum ammi* (L.) Sprague (Apiaceae) (Kapoor et al., 2002a), limonene, 1,8 cineole, carvone, eugenol, and methyl cinnamate in *Mentha viridis* L. (Lamiaceae) (Karagiannidis et al., 2011), and bornyl acetate, 1,8-cineole, α-thujones, and β-thujones in *Salvia officinalis* (Geneva et al., 2010).

In the present research, TiO_2 NPs had a positive effect on the important components of EOs. This effect could be related to the hypothesis that TiO_2 nanoparticles can increase the expression of enzymes included in the monoterpenes biosynthesis. For instance, in an experiment, Ahmad et al. (2018) found that increasing menthol concentration could probably be due to increased expression of the menthone-menthol reductase (MMR) enzyme, which reduces the menthone-to-menthol ratio. However, further studies are needed to confirm these results. The incremented oil glands density has also been attributed to the application of TiO_2 NPs, for instance the positive effects of TiO_2 NPs on EOs production were reported in *Rosmarinus Officinalis* (Golami et al., 2018), *Salvia officinalis* (Ghorbanpour 2015), and *Vetiveria zizanioides* (Shabbir et al., 2019). According to Ahmed et al. (2018), menthol, menthone, and menthyl acetate concentrations increased with the foliar applied TiO_2 NPs compared to control.

5. Conclusion

The physicochemical properties of NPs enable them to elicit different responses in plants. We revealed that the foliar spray with TiO_2 NPs increased the growth, biochemical properties, and percentage yield of EO compounds. Also, AM fungus in a symbiosis relationship enhanced the production of major EO compounds (i.e., menthol, menthone, and methyl acetate) when TiO_2 NPs was applied. Nevertheless, this result was not achieved in some cases, such as caryophyllene and sabinene. In conclusion, applying these elicitors (TiO_2 NPs, *Glomus mossea,* and *Glomus intraradices*) individually or in combination positively affected the growth, enzyme activities, and major compounds of EOs synthesis compared to the control. The current results suggest the beneficial effects of the optimal doses of TiO_2 NPs and the effective type of AM fungi for improving performance and increasing peppermint's qualitative and quantitative yield.

Declaration of competing interest

The authors of this study declare that they have no conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rhisph.2022.100659.

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