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Additive effects of *Trichoderma harzianum* and TiO_2 nanoparticles on growth, cadmium stabilization and macroelements absorption of *Mentha piperita* L. under cadmium stress

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ABSTRACT

Purpose: Nowadays, heavy metal pollution of soils has become a serious environmental concern and a potential threat to human health. Peppermint (*Mentha piperita* L.) is a medicinal and aromatic plant belonging to Lamiaceae family that its essential oil is used in different pharmaceutical industries. This study focused on the effects of arbuscular mycorrhizal fungi (AMF) and different concentrations of titanium dioxide nanoparticles (TiO_2 NPs) addition on the growth, absorption of macronutrients, migration and bioaccumulation of cadmium in soil- peppermint system. **Research Method:** A factorial experiment was conducted in a completely randomized design with three factors. The first factor was TiO_2 NPs (0, 200, and 300 mg/kg), the second factor included Cd-spiked soil (0 and 50 mg/kg soil) and third *Trichoderma harzianum*. **Findings:** Our results showed cadmium caused negative effects on the growth of peppermint except for shoot and root length. The concentration of all the nutrients assessed (nitrogen and phosphorus) decreased in the presence of cadmium. Mycorrhization under cadmium stress conditions significantly increased nitrogen concentration in roots and shoots by 15% and 3%, respectively, respect to Cd treatment group. Following mycorrhizal inoculation under cadmium stress conditions, phosphorus concentration significantly increased in roots and shoots by 33% and 12%, respectively, respect to Cd treatment group. Similarly, application of TiO_2 nanoparticles at 200 mg/Kg and 300 mg/Kg concentrations alone and in combination with mycorrhizal soil amendments in stress as well as non-stress conditions effectively enhanced nutrient acquisition. With the addition of TiO_2 NPs and mycorrhiza to soil, the phytoavailability and uptake of Cd by peppermint roots increased. **Research limitations:** There was no limitation. **Originality/Value:** Our research demonstrates that applying AMF and TiO_2 NPs could be a sustainable approach to enhancing growth parameters of peppermint plants under Cd stress by improving nutrient acquisition and immobilizing the heavy metal Cd. The results obtained here can provide scientific data for the use of *Mentha piperita* L. in the remediation of Cd-contaminated soil.

Keywords:
Heavy metals, Inoculation, Medicinal plants, Phytoremediation

INTRODUCTION

Peppermint (*Mentha pip*erita L.) is an important medicinal and aromatic plant that belongs to the Lamiaceae that has more than 4000 species in 200 genera. Peppermint is a perennial herb and is obtained by natural hybridization between spearmint and watermint. The essential oil of peppermint possesses significant antimicrobial and antiviral capacity and also contains antioxidant, antitumor and anti-allergic activities (Loolaie et al., 2017). With the development of industrialization and urbanization, the abundance of heavy metals in the environment has increased enormously during the past decades, which raised significant concerns throughout the world (Suman et al., 2018; Ashraf et al., 2019). Heavy metals are non-degradable by any biological or physical process and are persistent in the soil for a long period, which pose a long-term threat for the environment (Suman et al., 2018). Cadmium, though a non-essential metal, is rapidly absorbed by plant roots (Pagani et al., 2012). The accumulation of the Cd threatens environmental quality, food safety, and public health when it is far beyond the cleaning capabilities of the soil ecosystem itself (Rizwan et al., 2017; Lata & Mishra, 2019). Some recent studies have focused on the interaction between mineral nutrient metabolism processes and Cd phytotoxicity, as well as the influence of this interaction on Cd absorption, translocation, accumulation and detoxification (Shanying et al., 2017; Lai et al., 2020a; Khaliq et al., 2019).

Phytoremediation is a plant-based approach, which involves the use of plants to extract and remove elemental pollutants or lower their bioavailability in soil (Berti & Cunningham, 2000). Plants have the abilities to absorb ionic compounds in the soil even at low concentrations through their root system. Plants extend their root system into the soil matrix and establish rhizosphere ecosystem to accumulate heavy metals and modulate their bioavailability, thereby reclaiming the polluted soil and stabilizing soil fertility (Jacob et al., 2018).

Several fungal genera, including *Trichoderma*, *Penicillium*, *Aspergillus*, and *Phoma*, have been reported to involve in bioremediation of metal contaminated sites (Talukdar et al., 2020). In recent agricultural practices, *Trichoderma* species have gained special recognition for their ability to enhance plant growth, suppress plant pathogens, and improve nutrient acquisition (Hermosa et al., 2012) via the release of siderophores (Syed et al., 2023), phosphate solubilizing enzymes (Bononi et al., 2020) and phytohormones (Vinale et al., 2008). The release of glomalin by AM fungi plays a crucial role in binding toxic metals, forming stable complexes that further reduce their uptake by roots (Dhalaria et al. 2020). AMF induce the production of metallothioneins (MTs) and activate antioxidant enzymes, thereby enhancing plant resistance to HM pollution (Zhan et al., 2018).

Chang (2018) showed an improvement in biomass and nutritional status of plants after inoculation with arbuscular mycorrhizal fungi (AMF) under Cd and lanthanum stress (N, P and K uptake increased between 20.1 % and 76.8 %) and the alleviation of heavy metal toxicity were associated with reduced uptake of the heavy metals by plant organs. The expansion of the extra-radical mycelium and the increase in the surface absorbing capability of host roots can effectively enhance the absorption of nutrients and improve plant yield (Wagg et al., 2015). In addition, the higher nutrient uptake by inoculation of AMF could be due to the increasing soil acidity around the rhizosphere by the release of H^+ ions, leading to solubility of macro- and micro-nutrients (Begum et al., 2019). AMF induces tolerance in plants indirectly by alleviating oxidative stress produced by heavy metals by altering the root morphology, resulting in an increased in above-ground biomass through improved water and mineral nutrient absorption (Riaz et al., 2021). AMF by increasing soil health parameters (soil moisture, fertility levels, and soil quality), nutrients uptake and regulation of Aquaporin gene

(AQP), ABA-responsive gene, phytohormone biosynthesis pathways, and transcription factors were improved stress tolerance (Begum et al., 2019). AMF can enhance phosphorus and nitrogen uptake in alfalfa by collaborating with N-fixing bacteria and P-solubilizing microorganisms Jach-Smith and Jackson (2020). AMF hyphae can chelate Cd ions in the soil, making them inaccessible to plant roots. Additionally, AMF colonization can upregulate the transcription of Cd transporters in root cells, enhancing Cd sequestration and its accumulation in vacuoles, which reduces Cd translocation to shoots. This detoxification process through sequestration contributes to maintaining homeostasis within plant tissues (Li et al., 2023). Janousková et al. (2006) reported that inoculation of *Glomus intraradices* with *Nicotiana tabacum* cultivated in Cd contaminated soil decreased Cd toxicity to the plants due to Cd immobilization in soil.

Among different nanoparticles, titanium dioxide nanoparticles (TiO_2 NPs) have shown to increase nutrient uptake, improve chlorophyll content, and promote light capture in chlorophylls (a and b). When the content of $TiO_{2\ NPs}$ was relatively high (100 and 500 mg/kg), it significantly increased the Cd content in rice seeds (Zhang et al., 2020a, 2020b). Enhanced metal accumulation may be due to Cd adsorption on the surface of TiO_2 NPs and co-absorption with TiO_2 NPs by the root system, resulting in increased Cd uptake by the plants.

Studies have found that low concentrations of TiO_2 NPs can stimulate plant growth and antioxidant capacity, while improving plant tolerance to abiotic stresses (Emamverdian et al., 2021). TiO_2 NPs may decrease Cd content in plants by inhibiting its transmission from roots to shoots and fixing it within the root cell walls of *Coriandrum sativum* L. (Sardar et al., 2022). TiO_2 NPs have been found to reduce Cd content in cowpeas (Ogunkunle et al., 2020a, 2020b, 2020c), soybean (Singh & Lee, 2016) and bamboo (Emamverdian et al., 2022). The authors assumed that TiO_2 nanoparticles could strengthen the root apoplastic barriers and their impermeability. In addition, TiO_2 NPs exhibit a high adsorption capacity and act as efficient binders for metal ions. Consequently, this effectively restricts the movement of heavy metals within the extracellular or intercellular parts of the roots, thereby preventing the translocation of heavy metals from the root to the shoot (Emamverdian et al., 2022). TiO_2NPs enhances nutrient accessibility by regulating enzyme activity involved in nitrogen metabolisms including nitrate reductase, glutamine synthase, etc. (Shah et al., 2021). It has been reported that titanium dioxide can increase the absorption of micronutrients and macronutrients, which can be a major factor in plant growth and biomass increase (Ze et al., 2011). TiO_2 NPs have been found to reduce Cd content in cowpeas (Ogunkunle et al., 2020a, 2020b, 2020c) and bamboo (Emamverdian et al., 2022). The Cd^{2+} absorbed by plants is accumulated in the roots. This accumulation of cadmium underground reduces the damage of heavy metals to the shoots. Compared with the Cd group, Cd-AM treatment significantly reduced cadmium ion concentration in *Medicago truncatula* (Li et al., 2023). Khoramivafa et al. (2012) studied the potential of microbial associations to the uptake of Cd (50 mg/ kg) in *Mentha piperita* L. It was found that applications of microbes resulted in a significant additive effect on the uptake of Cd in roots.

This study focused on the effects of mycorrhizal fungi and different concentrations of TiO_2 NPs addition on the growth, macroelements absorption and migration & bioaccumulation of Cd in soil- peppermint system. On the whole, our research will provide insights into the responses, protection and potential application of AMF and TiO_2 NPs in Cd contaminated soils.

MATERIALS AND METHODS

Site and growing conditions

The entire experiment took place in a glass greenhouse of the Urmia University from 21 June to 20 September in 2024.

Experimental design and treatments

In order to investigate the effect of titanium dioxide nanoparticles (TiO_2 NPs) supplementation and mycorrhiza inoculation on cadmium stabilization, macronutrients absorption and growth parameters of *Mentha piperita* L. under cadmium stress, a factorial experiment was conducted in a completely randomized design. The experiment included three factors. The first factor was titanium dioxide nanoparticles (0, 200, and 300 mg/kg), the second factor included Cd-spiked soil (0 and 50 mg/kg soil) and third mycorrhiza (inoculated and non-inoculated). Each treatment had three replications with two plants per replicate pot. To prevent loss of nutrients and Cd from the pots, plastic trays were placed under each pot and the collected leachates were put back in their respective pots. To obtain the final soil, sand, garden soil, perlite and peat moss mixed together and transferred to the pots and finally prepared for planting. The soil was thoroughly mixed with cadmium nitrate [Cd $(NO3)_2$] serving as Cd source (56.5 mg kg^{-1}), and was then incubated at room temperature for one month for metal stabilization before executing pot experiment according to Irfan et al. (2013). For Cd treatment, the experimental pots were filled with Cd-affected soil (1 kg soil per pot). Bioavailable Cd was about 50 mg kg^{-1}.

The tested AMF strain (*Trichoderma harzianum*) was provided by the Royan Tison (Tehran, Iran). *Trichoderma harzianum* concentration was 10^7 CFU / ml.

Titanium dioxide nanoparticles were obtained from Nano Sadra Company (Mashhad). The nanoparticles were in crystalline form with a size of 15 nm. The color of the nanoparticles was white and spherical in morphology, and the purity of these nanoparticles was 99 %.

Measurement of soil parameters

The selected soil was free of heavy metals and the physical and chemical properties of the tested soil examined in the soil analysis laboratory and the results reported (Table 1). Soil available nitrogen was determined by the alkali-hydrolysis diffusion method (Jackson, 1973) and soil available P was extracted by the diacid method and determined by molybdenum-antimony colorimetry (Olsen, 1954). The content of soil N was determined by H_2SO_4–H_2O_2 extraction and semi-micro Kjeldahl method (Hess, 1990) and the content of soil P was determined by H_2SO_4–H_2O_2 extraction and molybdenum-antimony colorimetry (Zhang et al., 1999). Soil texture and pH were determined by the methods (Bouyoucos, 1997) and (Richards, 1954), respectively.

Table 1: Physical and chemical traits of the studied soil.

Sand (%)	Silt (%)	Clay (%)	pH	Total Cadmium (ppm)	Total Nitrogen (%)	Available Phosphorus (ppm)
60	13	27	7.4	nd	0.06	60.2

(nd): No detected. Values represent the average of three samples.

Soil samples of the pots were also taken after harvest for extraction of 1M $NH_4NO_3^-$ extractable Cd concentrations. These Cd concentrations are considered the main source of phytoavailable metal in the soil (Langer et al., 2009).

Measurement of macronutrient and Cd concentrations of roots and shoots

The nitrogen (N) concentrations of the shoots and roots were determined, according to Nelson and Sommers (1980). The content of phosphorus was determined by a molybdenum antimony colorimetric method (Ghazanshahi, 2006). For Cd extraction from plant biomass, 2.0 g aliquots of ground shoots or roots were digested in 30 mL of HNO_3, $HClO_4$, and H_2SO_4 mixture (40:4:1) followed by 20 mL of deionized water (Gupta, 2000). The plant and soil extracts were analyzed for their Cd by atomic absorption spectroscopy (Perkin-Elmer AA-800).

Measurement of vegetative growth parameters

After 8 weeks of growth, the plants were harvested by carefully removing the soil from the roots. The plant samples were collected and washed three times with deionized water. Measurement of growth factors: Growth indices (root and shoot length, fresh weight of leaves, shoots and roots; also, dry weight of leaves, shoots and roots) calculated. To obtain the dry weight of the biomass, the plants will be dried in an oven at 70 °C for 48 hours.

Estimation of translocation Factor (TF) and bioconcentration factor (BCF)

On the basis of the Cd concentrations, we calculated the Cd bioconcentration factor (BCF) and translocation factor (TF). Biological Concentration Factor (BCF) was calculated as metal concentration ratio of plant roots to soil given in equation 1 (Yoon et al., 2006; Embrandiri et al., 2017). Translocation Factor (TF) was described as ratio of heavy metals in plant shoot to that in plant root given in equation 2 (Cui et al., 2007).

$$BCF = [Cd]\ root / [Cd]\ soil \quad \ldots\ldots\ldots\ldots\ldots\ldots\ (1)$$

$$TF = [Cd]\ shoot / [Cd]\ root \quad \ldots\ldots\ldots\ldots\ldots\ldots\ (2)$$

Fungal staining

For staining fungi organs, fine roots were cut into 1-cm-long segments, cleared by soaking in 10 % KOH at 90 °C for 1 h, and stained with Aniline blue (0.05 % w/v) in lactoglycerol (lactic acid–glycerol–water, 14:1:1 v/v) at 90 °C for 1 h. Stained roots were rinsed with deionized water and destained in lactoglycerol (Phillips & Hayman, 1970). Finally, stained root samples were studied microscopically and were taken images.

Statistical analysis

Before ANOVA, data were checked for normality by the Shapiro–Wilk test and the homogeneity of variance. These tests did not reveal restrictions for processing the data by ANOVA. The significance of the difference among means was determined based on the post hoc Duncan test at a significance level of $p \leq 0.05$ (n=3). All statistical analyses were performed with IBM.SPSS statistics, Version 23. The data in tables and graphs are reported as mean ± SE (Standard Error).

RESULTS

Figure 2 shows the organs of the fungus *Trichoderma harzianum* in the roots of peppermint (*Mentha piperita* L.). Based on the results of analysis of variance (ANOVA) (Table 2), cadmium, cadmium plus fungus, and cadmium plus fungus plus nanoparticles treatments had no significant effect on root fresh weight ($p>0.05$). Also, fungus treatment and combined application of fungus & cadmium had no significant effect on shoot fresh weight ($p>0.05$). Cadmium treatment has no significant effect on shoot length ($p>0.05$). Application of cadmium and TiO_2 NPs alone had no significant effect on leaf fresh weight ($p>0.05$) (Table 2).

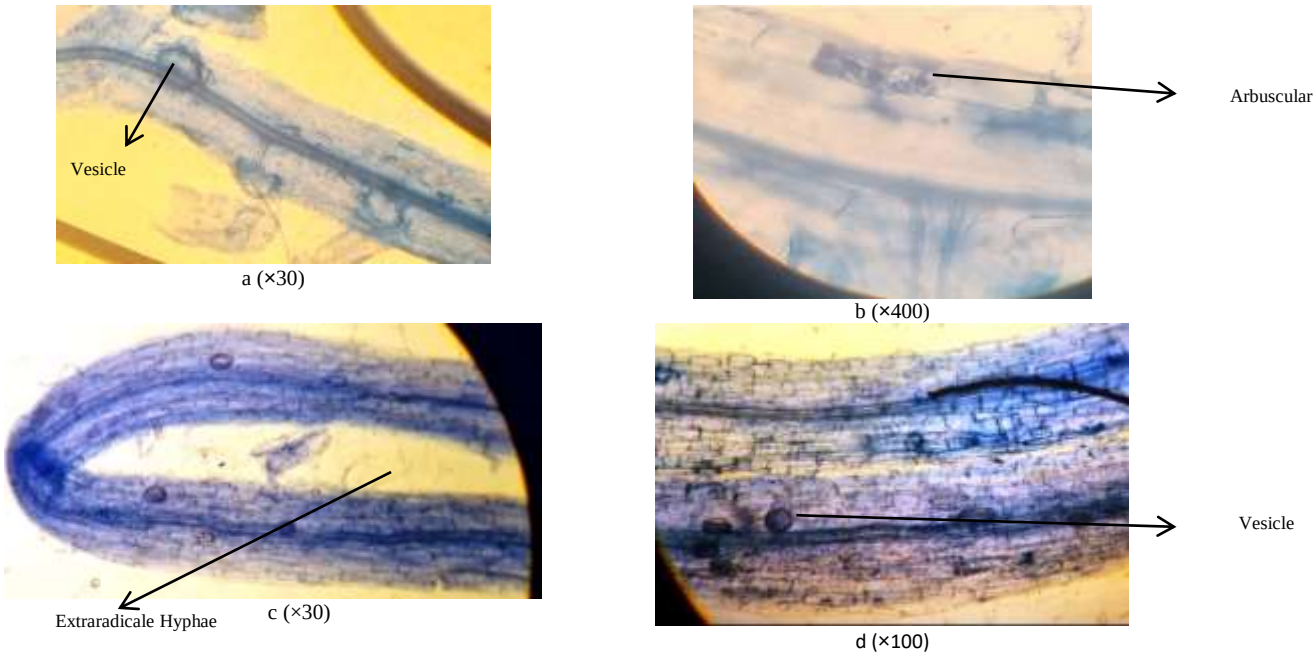

Fig. 1. Organs of the fungus *Trichoderma harzianum* in the roots of peppermint (*Mentha piperita* L.).

Growth characteristics

The effect of titanium dioxide nanoparticles and mycorrhiza on the length of peppermint shoots

Soil contamination with cadmium did not have a significant effect on the shoot length of peppermint (Table 2). Mycorrhization increased shoot length (29 %) compared to the control. Addition of TiO_2 NPs at 200 and 300 mg/kg soil concentrations significantly increased shoot length compared to the control (23 % and 26 %, respectively). There was no significant difference in shoot length between two TiO_2 NPs treatments. Addition of TiO_2 NPs under cadmium stress did not have a significant effect on shoot length compared to Cd treatment condition (Table 2). However, when TiO_2 NPs was combined with AMF under cadmium stress caused a significant increase in shoot length compared to Cd treatment group ($p<0.05$). Therefore, the interaction effect of TiO_2 NPs x AMF showed an increase of 16 % and 18 % at 200 and 300 mg/kg concentrations indicating an additive effect of mycorrhiza and nanoparticles treatments (Table 2).

Table 2. Summary of the ANOVA for growth parameters of *Mentha piperita.*

Mean squares

Source of Variation	df	Growth parameters								
		Leaf			Root			Shoot		
		Number	Fresh weight	Dry Weight	Length	Fresh Weight	Dry Weight	Length	Fresh Weight	Dry Weight
Ti	2	156.36*	0.12n	0.00*	5.14*	2.44*	0.04*	5.14*	8.45*	0.33*
TH	1	205.44*	1.71*	0.00*	38.39*	7.84n	0.11*	38.39*	6.13n	0.19*
K	1	658.77*	4.82n	0.001*	7.30n	6.66*	0.09*	11.78n	44.8*	1.76*
Ti×TH	2	28.52*	0.01*	0.00*	38.39*	0.77*	0.004*	5.78*	2.35*	0.62*
Ti×K	2	8.52*	0.01*	0.00*	5.01*	1.08*	0.009*	5.01*	3.42n	0.06*
TH×K	1	21.77*	0.72*	0.0004*	3.59*	0.29n	0.010*	3.59*	1.64*	0.03*
K×Ti×TH	2	1.69*	0.01*	0.00*	2.78*	0.30n	0.005*	2.78*	0.69*	0.03*
Error	24	0.47	0.001	0.00002*	0.48	0.13	0.001	0.48	0.21	0.001

n, *: Non – significantly difference and significantly difference at the 5 % probability levels, respectively.
(TH: *Trichoderma harzianum,* K*: cadmium, Ti:* titanium dioxide nanoparticles (TiO_2 NPs)

The effect of titanium dioxide nanoparticles and mycorrhiza on the root length of peppermint

Titanium dioxide nanoparticle treatment caused 13% and 20% increase at 200 and 300 mg/kg soil concentrations, respectively, compared to the control. Cd addition had no significant effect on plant root growth compared to control plants. Supplementation with TiO_2 NPs under Cd stress had no significant effect on root growth compared to the control. Mycorrhization caused 14 % increase in root length compared to the control. Inoculation with AMF under Cd stress showed non-significant effect on root length compared with treatment containing Cd alone (Table 3). Simultaneous treatment with mycorrhizal fungi and titanium dioxide increased root growth compared to cadmium-contaminated soil, such that 17 and 19 percent increase in root growth was observed at concentrations of 200 and 300 mg in the mycorrhizal treatment under cadmium stress (Table 3).

Table 2. (***Countinued***) Summary of the ANOVA for macronutrients concentration and cadmium concentration of *Mentha piperita.*

Mean squares

Source of Variation	df	Macronutrients concentration				Cadmium concentration	
		Root		Shoot			
		Number	Phosphorus	Nitrogen	Phosphorus	Root	r
Ti	2	0.55*	0.02*	0.73*	0.009*	1702.6*	6.97*
TH	1	4.16*	0.13*	0.39*	0.0004*	5801.36*	8.86*
K	1	8.89*	0.004*	43.71*	0.03*	230.02*	3.15*
Ti×TH	2	0.05*	0.02*	0.02*	0.00*	7245.86*	5.21*
Ti×K	2	0.09*	0.01*	0.14*	0.008*	1155784.02*	26.16*
TH×K	1	3.36*	0.008*	0.34*	0.0004*	330816.69*	2.07*
K×Ti×TH	2	0.08*	0.01*	0.006*	0.0002*	391017.19*	31.72*
Error	24	0.01	0.00	0.00	0.00	3.55	0.002

The effect of titanium dioxide nanoparticles and mycorrhiza on the fresh weight of the studied peppermint organs

The results of the present study showed that TiO_2 NPs and mycorrhiza treatments significantly increased fresh weight traits of shoots, roots and leaves compared to the control. Cd exposure significantly decreased the fresh weight of shoots, roots and leaves by 18%, 15% and 16%, respectively, compared to the control. Mycorrhization under cadmium stress conditions increased the fresh weight of shoots, roots and leaves by 3%, 9% and 55%, respectively, compared to cadmium stress conditions (Table 3).

The effect of titanium dioxide nanoparticles and mycorrhiza on the dry weight of the studied peppermint organs

Cd exposure significantly decreased dry weight of shoots, leaves and roots of peppermint plants compared to the control (17%, 11% and 11%, respectively). The addition of TiO_2 NPs at both concentrations significantly increased dry weight of the shoots and roots of peppermint compared to the control (Table 3). Mycorrhization of the plant under Cd stress caused approximately 11%, 19% and 9% increase in dry weight of shoots, leaves and roots compared to treatment containing Cd alone (Table 3).

The effect of titanium dioxide nanoparticles and mycorrhiza on leaf number

The cadmium treatment group showed significantly decrease in number of leaves by 27% compared to control (Fig. 2). Mycorrhization of the plant under Cd stress caused approximately 30 % increase in number of leaves compared to Cd alone treatment. Application of TiO_2 nanoparticles at 200 mg/Kg and 300 mg/Kg concentrations alone and in combination with mycorrhizal soil amendments in stress as well as non-stress conditions effectively increased number of leaves (Fig. 2).

Table 3. Effect of TiO_2 NPs and Mycorrhiza association on studied parameters of *Mentha piperita* L. under cadmium stress.

	Shoot length	
Treatments	Cd 0 mg/kg soil	Cd 50 mg/kg soil
Control	27.00±0.00e	26.13±0.08e
TiO_2 NPs 200	33.33±0.33b	26.20±.11e
TiO_2 NPs 300	34.06±0.06b	26.4±0.05e
M	35.00±0.5a	29.33±0.6d
TiO_2 NPs 200 + M	35.3±0.05a	31.33±0.3c
TiO_2 NPs 300 + M	35.76±0.03a	32.00±0.00c
	Root length	
Control	15.13±0.08d	14.20±0.11d
TiO_2 NPs 200	17.13±0.06c	15.06±0.06d
TiO_2 NPs 300	18.30±0.15b	15.10±0.05d
M	17.33±0.30c	15.30±0.05d
TiO_2 NPs 200 + M	18.66±0.60a	16.66±0.80c
TiO2 NPs 300 + M	19.16±0.40a	17.00±0.50c
	Shoot fresh weight	
Control	6.40±0.36d	5.30±0.05f
TiO_2 NPs 200	8.93±0.27b	6.20±0.006de
TiO_2 NPs 300	10.43±0.15a	6.29±0.006d
M	6.51±0.59d	5.47±0.03f
TiO_2 NPs 200 + M	7.63±0.03c	5.63±0.03ef
TiO2 NPs 300 + M	7.87±0.30c	5.65±0.3ef
	Root fresh weight	
Control	4.60±0.006g	3.92±0.02j
TiO_2 NPs 200	4.82±0.02e	4.02±0.02i
TiO_2 NPs 300	6.60±0.02a	4.36±0.03h
M	5.55±0.02c	4.31±0.01h
TiO_2 NPs 200 + M	5.33 ±0.03d	4.69±0.05f
TiO2 NPs 300 + M	5.82 ±0.03b	4.79±0.02e
	Leaf fresh weight	
Control	2.41±0.01f	2.03±0.03j
TiO_2 NPs 200	2.56±0.03e	2.16±0.01i
TiO_2 NPs 300	2.72±0.02d	2.18±0.003i
M	3.16±0.01c	3.16±0.01c
TiO_2 NPs 200 + M	3.33±0.01b	2.27±0.01h
TiO_2 NPs 300 + M	3.39±0.01a	2.34±0.01g
	Shoot dry weight	
Control	1.41±0.01g	1.18±0.02i
TiO_2 NPs 200	1.54±0.02e	1.31±0.01h
TiO_2 NPs 300	2.08±0.003a	1.42±0.002fg
M	1.74±0.02d	1.32±0.01h
TiO_2 NPs 200 + M	1.92±0.03c	1.39±0.006g
TiO2 NPs 300 + M	2.01±0.03b	1.45±0.01f
	Root dry weight	
Control	1.31±0.10f	1.11±0.04j
TiO_2 NPs 200	1.40±0.04d	1.21±0.01h
TiO_2 NPs 300	1.51±0.03c	1.27±0.02g
M	1.12±0.02i	1.21±0.01g
TiO_2 NPs 200 + M	1.78±0.003b	1.31±0.006f
TiO2 NPs 300 + M	1.86±0.003a	1.38±0.01e
	Leaf dry weight	
Control	1.31 ±0.00b	0.98±0.01f
TiO_2 NPs 200	1.14±0.10de	1.10±0.005e
TiO_2 NPs 300	1.29±0.01b	1.11±0.005e
M	1.20±0.006c	1.20±0.006c
TiO_2 NPs 200 + M	1.29 ±0.02b	1.19±0.00cd
TiO2 NPs 300 + M	1.35 ±0.007a	1.19±0.005cd

Values presented in the table are means of three replicates ± standard errors.

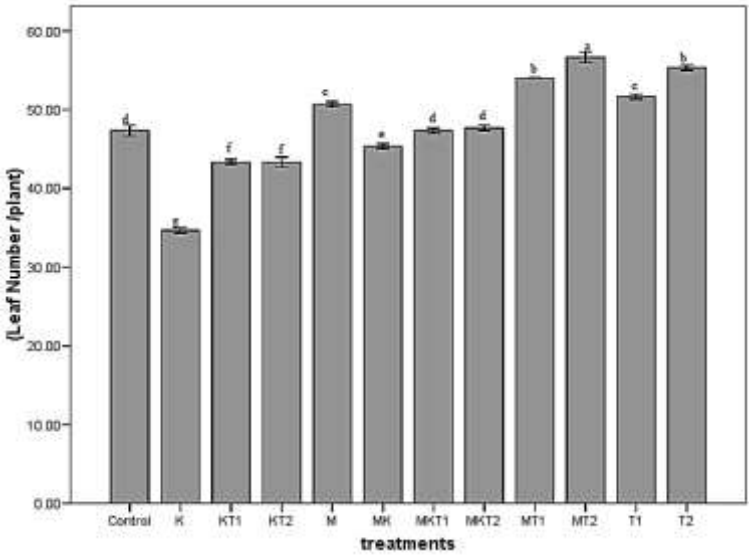

Fig. 2. Effect of TiO_2 NPs and AMF on leaf number of peppermint grown in Cd-contaminated soil. Plants were cultivated in soil spiked with 50 mg Cd/kg soil, Mycorrhiza and 200 & 300 mg TiO_2NPs/kg soil. K: Cadmium treatment, KM: Cd+M treatment, KMT1: Cd+M+TiO_2NPs200 treatment, KMT2: Cd+M+TiO_2NPs300 treatment, M: AMF treatment, MT1: M+ TiO_2NPs200 treatment, MT2: M+ TiO_2NPs300 treatment, T1: TiO_2NPs200 treatment, T2: TiO_2NPs300 treatment.

Effect of AMF inoculation and TiO_2 NPs supplementation on the Cd^{2+} distribution and content of peppermint organs

The effect of AMF and TiO_2 NPs on cadmium uptake and bioaccumulation in peppermint grown in Cd-contaminated soil is shown in table 11. Under Cd^{2+} treatment, the content in peppermint shoot and roots were 3.03 and 531 mg/Kg, respectively (Table 4). In mycorrhizal plants under cadmium stress, cadmium content of shoot and root were 2.36 mg/kg and 603.33 mg/kg, respectively, which significantly lower than the Cd group ($p \leq 0.05$) (Table 4). The highest root bioconcentration factor (RBCF) was observed in Cd+ TiO_2 NPs 300+M group (13.24). The translocation factor (TF), decreased markedly from 0.0050 to 0.0039 in Cd and 200 mg/Kg TiO_2 NPs+ Cd treatments (Table 4).

Table 4. Cd distribution, BCF and TF traits in studied treatments.

Treatments	Cadmium concentration (mg/Kg dry weight)		RBCF	TF
	Root	Shoot		
Cd alone	531.0±0.57e	3.03±0.03a	10.62±0.01e	0.0050±0.00006a
Cd+M	603.33±0.33d	2.36±0.03b	12.06±0.06d	0.0040±0.00004b
Cd+ TiO_2 NPs 200	604.0±0.02d	2.40±0.00b	12.08±0.04d	0.0039±0.00001b
Cd+ TiO_2 NPs 300	633.0±0.52c	2.16±0.03c	12.66±0.03c	0.0034±0.000005c
Cd+ TiO_2 NPs 200+M	651.33±0.66b	1.96±0.02d	13.02±0.01b	0.0030±0.00005d
Cd+ TiO_2 NPs 300+M	662.32±0.2a	1.82±0.09e	13.24±0.02a	0.0027±0.00002e

TiO_2 NPs 200 (200 mg/Kg TiO_2 NPs), TiO_2 NPs 300 (300 mg/Kg TiO_2 NPs), M (Mycorrhiza inoculation), RBCF: Root Bioconcentration Factor, TF: Translocation Factor, The same letters show no significant difference ($p \leq 0.05$) in each column and between levels of treatments. Mean pairs followed by different letters are significantly different ($p \leq 0.05$) by Duncan's test.

Table 5. Effect of Cadmium stress, AMF inoculation and TiO_2 NPs addition on nutrient uptake of *Mentha piperita* L.

Treatments	Macronutrients	Macronutrients in roots	Macronutrients in shoots
Control	Nitrogen (N) (%)	1.08±0.00f	3.01±0.006f
	Phosphorus (P) (%)	0.13±0.001d	0.11±0.008g
TiO_2 NPs 200	Nitrogen (N) (%)	1.26±0.03e	3.23±0.018e
	Phosphorus (P) (%)	0.16±0.001b	0.16±0.001c
TiO_2 NPs 300	Nitrogen (N) (%)	1.41±0.01d	3.41±0.016c
	Phosphorus (P) (%)	0.17±0.001a	0.18 ±0.005b
K	Nitrogen (N) (%)	0.70±0.006j	0.90±0.00l
	Phosphorus (P) (%)	0.06±0.00h	0.08±0.00i
KT1	Nitrogen (N) (%)	0.91±0.01h	1.21±0.008j
	Phosphorus (P) (%)	0.08 ±0.26g	0.09±0.003h
KT2	Nitrogen (N) (%)	0.98±0.0g	1.52±0.01g
	Phosphorus (P) (%)	0.09±0.00f	0.12±0.003f
M	Nitrogen (N) (%)	2.02±0.006c	3.36±0.003d
	Phosphorus (P) (%)	0.14±0.01c	0.13±0.003e
MK	Nitrogen (N) (%)	0.81±0.01i	0.93±0.018k
	Phosphorus (P) (%)	0.08±0.00g	0.09±0.29h
MKT1	Nitrogen (N) (%)	0.99±0.00g	1.33±0.01j
	Phosphorus (P) (%)	0.09 ±0.27f	0.12±0.005f
MKT2	Nitrogen (N) (%)	1.01±0.006g	1.41±0.01h
	Phosphorus (P) (%)	0.11 ±0.00e	0.14±0.003d
MT1	Nitrogen (N) (%)	2.76±0.01b	3.80±0.00a
	Phosphorus (P) (%)	0.14±0.00c	0.18±0.03b
MT2	Nitrogen (N) (%)	2.84±0.02a	3.71±0.008b
	Phosphorus (P) (%)	0.16±0.06b	0.21 ±0.03a

Values presented in the table are means of three replicates ± Standard Errors.
K: Cadmium treatment, KM: Cd+ Mycorrhiza treatment, KMT1: Cd+M+TiO_2 NPs200 treatment, KMT2: Cd+M+TiO_2 NPs300 treatment, M: Mycorrhiza treatment, MT1: M+ TiO_2 NPs200 treatment, MT2: M+ TiO_2 NPs300 treatment, T1: TiO_2 NPs200 treatment, T2: TiO_2 NPs300 treatment.

Nitrogen and phosphorus nutrient concentration

Treatment containing Cd alone, significantly decreased nitrogen (N) and phosphorus (P) concentrations in both the shoot and root of peppermint ($p \leq 0.05$) (Table 5). However, mycorrhization and supplementation with TiO_2 NPs alone and under cadmium stress significantly ($p \leq 0.05$) increased the nitrogen and phosphorus content of roots and shoots compared to a single application of Cd (Table 5). Mycorrhization under cadmium stress conditions significantly increased nitrogen concentration in roots and shoots by 15 % and 3 %, respectively, respect to Cd treatment group. Following AMF inoculation under cadmium stress conditions, phosphorus concentration significantly increased in roots and shoots by 33% and 12%, respectively, respect to Cd alone treatment. Similarly, application of TiO_2 nanoparticles at 200 mg/Kg and 300 mg/Kg concentrations alone and in combination with mycorrhizal soil amendments in stress as well as non-stress conditions effectively enhanced nutrient acquisition (Table 5).

DISCUSSION

Cadmium stress can substantially inhibit crop growth and development, primarily by reducing nutrient absorption, which affects overall crop growth (Castillo-Michel et al., 2009; Tanwir et al., 2015). Amirmoradi et al. (2011) showed that cadmium stress (100 ppm) caused a decrease in plant height of *Mentha piprita*. Growth inhibition is a common phytotoxic effect of Cd, resulting from oxidative damage as well as interference with essential nutrients and hormones (Mancini et al., 2016). Our results showed that Cd caused negative effects on the growth parameters of peppermint except for shoot and root length. Conversely, in sorghum, Liu et al. (2011) observed decreased root activity when Cd was added to the soil at concentrations of 50 and 100 mg/kg, and concluded that Cd decreased primary root elongation and lateral root

growth. Meanwhile, Dong et al. (2005) reported that the addition of 1 and 10 mmol/L Cd in the nutrient solution of tomato decreased plant height by 18.9% and 46.4%, respectively, root length by 25.8% and 41.1%, respectively, and root volume by 45.2% and 63.7%, respectively, compared to the control.

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 & Xia, 2006). 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 results showed that AM fungus inoculation significantly improved growth characteristics. AMF have the capability to boost the uptake of inorganic nutrients in almost all plants, specifically of phosphate (Nell et al., 2010). The ameliorating effects of AMF seem to be related to the improved nutritional status of the host plant (Koschier et al., 2007). Similar results have been reported by Klaus et al., 2013, Anjum et al., 2016. Increased biomass helps plants better cope with cadmium toxicity, thereby improving stress resistance. The production and secretion of the phosphatase enzyme by mycorrhizal hyphae causes insoluble and established phosphate in the soil is inverted into the soluble form and can be absorbed by the roots (Gonzales-Chavez et al., 2004). 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%. Our results are consistent with these results. Accordingly, with our results, increased biomass production in AM plants exposed to TiO_2 NPs may be related to the promoted absorption of minerals (Chen et al., 2018; Shenavaie Zare et al., 2022).

Cadmium accumulates preferentially in the root, and is sequestered in the vacuole of the cells, while only a small fraction is transported to the plant shoot, concentrating in descending order in the stems, leaves, fruits and seeds (Chan & Hale, 2004). Metal excluder plants prevent metal from entering their aerial parts or maintain low and constant metal concentrations over a broad range of metal concentration in the soil. Likewise, shoot Cd concentrations decreased in cucumber (Lee & George, 2005), sweet pepper (Jidesh and Kurumthottical, 2000) and soybean (Mane et al., 2010). Accordingly, in our study, the order of Cd accumulation in peppermint was as follows: roots > shoots. The Mycorrhizal pepper plants reduced Cd accumulation in shoot compared to NM (Non-Mycorrhizal) plants grown in the corresponding Cd Treatment (Abdel Latef, 2013). Our results agree with these findings.

Nitrogen (N) is an essential macronutrient and an important component of many structural, genetic and metabolic compounds in plants (Hassan et al., 2005). Nitrogen content was higher in shoots (0.90% to 3.80%) than in roots (0.70% to 2.84%) for all Cd treatments (Table 12). The higher N content seen in the shoots than in the roots was probably related to the maintenance of protein synthesis, electron transference in photosynthesis, and respiration processes (Wiedenhoeft, 2006). Moreover, increases in Cd accumulation in the shoots would lead to increased N contents there as N is required to synthesis Cd-detoxifying chelator molecules such as glutathione and phytochelatins (Gojon & Gaymard, 2010). Nazar et al. (2012) reported that high Cd levels in the soil alter the uptake and translocation of nutrients, which leads to nutrient deficiencies, oxidative stress and decreased plant growth and development. In the case of the present study, the concentration of all the nutrients assessed (Nitrogen and Phosphorus) decreased in the presence of Cd. Cadmium concentrations have been found to be related to decreases in nitrate reductase (NR) activity in plants, leading to decreases in photosynthetic rates and chlorophyll contents (Campbell, 1999; Hernandez et al., 1997), or to increases in enzymatic break down induced by active oxygen species generated

during their exposure to stress (Hassan et al., 2008). Correspondingly, our data indicates that Cd had influence on nitrogen absorbance in peppermint.

AMF improve plant nutrition by increasing the availability as well as translocation of various nutrients (Rouphael et al., 2015). AMF improve the quality of soil by influencing its structure and texture, and hence plant health (Zou et al., 2016; Thirkell et al., 2017). It is evident that AMF inoculation can enhance the concentration of various macro-nutrients and micro-nutrients significantly, which leads to increased photosynthate production and hence increased biomass accumulation (Chen et al., 2017; Mitra et al., 2019; Kuang et al., 2025). Recent studies have shown that AM fungal symbiosis with plants under Cd stress can enhance nutrient acquisition and plant growth, improving plant resistance to heavy metals (Li et al., 2016; Chang et al., 2018; Huang et al., 2018; Liu et al., 2018). Similar patterns have been observed in Japanese honeysuckle (*Lonicera japonica*), reed (*Phragmites australis*), and rice when inoculated with AM fungi under Cd stress (Luo et al., 2017; Huang et al., 2018; Jiang et al., 2018). This effect may be due to AM fungi increasing the root absorption area through hyphal networks and activating soil nutrients, thus enhancing nutrient uptake and partially alleviating Cd stress. AM fungi representing an effective strategy for mitigating Cd stress in crops.

The application of increasing doses of TiO_2 NPs (250, 500, and 750 mg kg^{-1}) to the soil was rising the accumulation of K and P concentration in cucumber fruit (Servin et al., 2013). The addition of silicon nanoparticles (Si NPs) alone increased the concentration of potassium and phosphorus in the roots and leaves of chickpea (*Pisum sativum*) seedlings and improved the uptake of these elements even in conditions of exposure to Cr (VI) (Tripathi et al., 2015). Mechanisms involved in plant resistance against cadmium include two strategies of inhibition of metal absorption or its accumulation in plant organ (Zhou & Song, 2004). In accumulation strategy (TF $\geq$ 1), higher values of cadmium are transferred to shoot and only a small amount is stored in root. In absorption inhibition strategy (TF$\leq$1), higher amounts of metal are stored in the root (Sun et al., 2009). The present study showed TF$\leq$1 for peppermint. Therefore, peppermint can be considered as a cadmium stabilizer. According to the results gained by the present study, *Trichoderma harzianum* inoculation and TiO_2 NPs supplementation decreased cadmium translocation to shoots and consequently a great amount of cadmium accumulates in root (Table 4), which is due to the ability of fungal hyphae in attaching to heavy metals inside and outside the roots which restrict their transfer to the upper parts as it also was shown in the findings of Hu et al. (2013). Also, the present study results are consistent with the results of Rizwan et al. (2019a) on *Oryza sativa* and Sardar et al. (2022) on *Coriandrum sativum* plants.

CONCLUSION

This study showed that under Cd stress, the combined application of *Trichoderma harzianum* and TiO_2 NPs enhanced the stress resistance of *Mentha piperita* through a tripartite mechanism involving a "physical barrier-nutrient absorption- growth promoters' strategy". This synergistic approach didn't promote all of plant growth parameters but facilitated Cd accumulation. The results obtained in this investigation can provide valuable information for choosing *Mentha piperita* as one of the phytoremediation species for Cd-polluted soils. Our results showed supplementation with TiO_2 NPs and AMF inoculation increased the uptake of Cd by roots of peppermint. Thus, demonstrates their potent ability to improve Cd phytostabilization. There are two different aspects of peppermint utilization; as an edible vegetable and another is chosen for its phytostabilization property on Cd. Due to the low translocation factor of Cd, edible consumption of peppermint grown in Cd contaminated soil will have lower apparent risk. Our study demonstrates that applying AMF and TiO_2 NPs could

be a sustainable approach to enhance growth parameters of peppermint plants under Cd stress by improving nutrient acquisition and immobilizing the heavy metal Cd. The results obtained here can provide scientific data for the use of *Mentha piperita* in the remediation of Cd-contaminated soil.

Conflict of interest

The authors have no conflict of interest to report.

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