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Unraveling the influence of TiO₂ nanoparticles on growth, physiological and phytochemical characteristics of *Mentha piperita* L. in cadmium-contaminated soil

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Among the metals contaminants, cadmium (Cd) is one of the most toxic elements in cultivated soils, causing loss of yield and productivity in plants. Recently, nanomaterials have been shown to mitigate the negative consequences of environmental stresses in different plants. However, little is known about foliar application of titanium dioxide nanoparticles (TiO₂ NPs) to alleviate Cd stress in medicinal plants, and their dual interactions on essential oil production. The objective of this study was to investigate the effects of foliar-applied TiO₂ NPs on growth, Cd uptake, chlorophyll fluorescence, photosynthetic pigments, malondialdehyde (MDA) and hydrogen peroxide (H₂O₂) contents, total phenols, anthocyanins, flavonoids, antioxidant enzymes (SOD, CAT and POD) activity and essential oil content of *Mentha piperita* L. (peppermint) under Cd stress. For this purpose, plants were grown in Cd-contaminated (0, 20, 40, and 60 mg L⁻¹) soil, and different concentrations of TiO₂ NPs (0, 75, and 150 mg L⁻¹) were foliar sprayed at three times after full establishment until the beginning of flowering. Exposure to TiO₂ NPs significantly ($P < 0.01$) increased shoot dry weight (37.8%) and the number of lateral branches (59.4%) and decreased Cd uptake in plant tissues as compared to the control. Application of TiO₂ NPs increased the content of plastid pigments, and the ratio *Fv/Fm* (13.4%) as compared to the control. Additionally, TiO₂ NPs reduced the stress markers, MDA and H₂O₂ contents and enhanced the activity of the phenylalanine ammonia-lyase (PAL) enzyme (60.5%), total phenols (56.1%), anthocyanins (42.6%), flavonoids (25.5%), and essential oil content (52.3%) in Cd-stressed peppermint compared to the control. The results also demonstrated that foliar spray of TiO₂ NPs effectively improved the growth and chlorophyll fluorescence parameters and reduced Cd accumulation in peppermint, which was mainly attributed to the reduction of oxidative burst and enhancement of the enzymatic (SOD, CAT, and POD) antioxidant defense system due to the uptake of NPs. The findings provide insights into the regulatory mechanism of TiO₂ NPs on peppermint plants growth, physiology and secondary metabolites production in Cd-contaminated soil.

Soil pollution with certain metals, especially in agricultural soils, has reached to the levels that can threaten food security through plant uptake^{1,2}. Cadmium (Cd) is a major concern among other metals due to its high toxicity to animals and humans. Cadmium readily accumulates in plant-accessible parts of the soil, posing a threat to human health through food chains. Cadmium is a naturally occurring element, and its presence has been identified in over 1000 species of aquatic and terrestrial plants and animals^{3,4}. The bioaccumulation of this toxic metal in the biosphere has significantly increased since the beginning of the industrial revolution, and Cd accumulation in soil and water is currently a primary environmental and human health concern⁵. Literature reveals that Cd adversely affects plant growth, development and metabolism⁶. Exposure to Cd reduces the plant productivity

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by decreasing the seed germination, seedling vigor as well as biomass accumulation through changing multiple morpho-physiological and structural attributes such as chlorosis/necrosis in leaves and inhibition of lateral roots⁷. It triggers osmotic stress in plant cells by decreasing leaf relative water content (RWC), stomatal conductance, chlorophyll content, and transpiration rate⁸. Cd toxicity causes excessive generation of reactive oxygen species (ROS), which damages the enzymatic and non-enzymatic antioxidant systems in plants and break the necessary molecules (e.g., proteins, nucleic acids, and lipids) of the biochemical reactions of different related enzymes⁹.

Peppermint (*Mentha piperita* L.), commonly known as mint, is one of the Lamiaceae family's most essential aromatic and perennial medicinal plants that has attracted the attention of many researchers due to its multiple therapeutic attributes including antibacterial, antifungal, anti-inflammatory, anti-flatulent, anti-inflammatory, and relieving the pain of irritable bowel syndrome. These properties are largely attributed to peppermint essential oil, which is a part of secondary metabolites¹⁰. Peppermint essential oil is obtained from the flowering tops of the plant and has a sharp taste. The essential oil of peppermint contains a high percentage of menthol, as well as menthone and carboxyl esters, especially methyl acetate, which are among its active components¹⁰. The essential oil also contains small compounds, including limonene, caryophyllene, and pinene¹⁰. Peppermint is rich in terpenoids and flavonoids such as eriocitrin, hesperidin, and kaempferol¹¹.

Advancements in agricultural technologies are the most significant factors in creating modern agriculture. In this regard, nanotechnology has provided a suitable platform for producing food and agricultural products. Moreover, the expansion of nano-tools and various nanomaterials has led to new applications in plant biotechnology and agriculture. Studies show that nanotechnology plays a crucial role in improving crop management methods. The National Nanotechnology Initiative in the United States describes nanotechnology as "research and development aimed at understanding and manipulating matter at the atomic and molecular levels"¹². Nanotechnology as an interdisciplinary science can have a wide range of advantages in the agricultural sector, including increasing crop yields, reducing pesticides and fertilizers, prolonging the shelf life of agricultural products, and potentially revolutionizing all stages, inputs, and agricultural tools for improvement¹³. Among mineral oxides, titanium dioxide (TiO₂) is highly regarded due to its chemical stability, non-toxicity, and low cost¹⁴.

Heavy metal stress is one of the most significant non-biological stresses that limit plant productivity. Plants can respond and adapt to stress through changes in their cellular metabolism and various defense mechanisms¹⁵. Reducing the detrimental effects of heavy metal stress or making plants more resistant to stress can be crucial in overcoming barriers to agricultural productivity. Titanium is the ninth most abundant element and the second most abundant transition metal in the Earth's crust, with an approximately 6.32 ppm concentration. The most acute effects of titanium compounds on plants include increasing the yield of various crops (~ 10 to 20%), improving specific essential element contents in plant tissues, enhancing the activity of peroxidase (POD), catalase (CAT), and nitrate reductase (NR) enzymes in plant tissues, and increasing chlorophyll content in red pepper (*Capsicum annuum*) and green algae (*Chlorella pyrenodosa*)¹⁶. It has been suggested that NPs can substitute for plant defense management as these compounds induce plant defense mechanisms¹⁷. TiO₂ NPs have a strong oxidative reaction and can easily target organic compounds¹⁸. Studies have shown that exposure to TiO₂ NPs promote plant root and shoot growth¹⁹. Additionally, another study found that the application of TiO₂ NPs at specific concentrations can induce physiological and biochemical responses in the medicinal plant linseed²⁰. A specific study determined that nanoparticles stimulate growth and secondary metabolites, especially the essential oil of the scented geranium plant²¹. It has been acknowledged that seed priming with TiO₂ NPs mitigate Cd toxicity in *Coriandrum sativum* L. through modulating antioxidant machinery, stress markers and reducing Cd uptake²². At the field levels, foliar exposure to chemically and green synthesized TiO₂ NPs decreased the Cd content in wheat grain below the maximum Cd acceptable limit for cereals²³. Moreover, Singh and Lee²⁴, reported that application of TiO₂ NPs may decrease Cd stress on growth and biomass production, restricts Cd toxicity by improving the photosynthetic rate, and increase Cd uptake in soybean plants. However, the toxicity mechanism of TiO₂ NPs-Cd on rice (*Oryza sativa* L.) via transcriptomics and metabolomics analyses revealed that application of NPs to combat the phytotoxicity of heavy metals need to pay more attention²⁵.

Like all living organisms, plants have evolved mechanisms to control the absorption and accumulation of essential and non-essential metals and respond to them. These mechanisms include chelation and metal displacement by specific ligands and, in some cases, the coding of the ligand-metal complex in vacuoles. Plants respond to environmental metal concentrations through three main strategies: (1) excluder plants restrict metal movement to shoots as a tolerance strategy, maintaining low and constant metal concentrations in their shoots up to the critical soil threshold, (2) indicator plants reflect internal metal concentrations that mirror external metal levels, (3) hyper-accumulator plants accumulate high metal levels at very low external metal concentrations^{26,27}. Hyper-accumulator describes a plant that primarily accumulates metals in its shoots and maintains low metal concentrations in its roots²⁶. Hyper-accumulator plants are found in metal-contaminated soils, such as calamine (with high levels of Zn, Pb, and Cd) and serpentine soils (with high levels of Ni, Cr, and Co)²⁷.

Researchers have primarily used NPs directly in growing media. Most of the research in exploring the use of TiO₂-NPs has focused on aqueous media. However, some studies have also investigated the potential of TiO₂-NPs as a seed priming agent to alleviate heavy metal stress in plants grown in soil media or hydroponic systems²². To achieve maximum biomass and ensure high production of essential oil in Cd-contaminated soils, it is important to have a good understanding of the effects of foliar-applied TiO₂ NPs on Cd uptake in essential oil-bearing plants and the possible mechanisms involved. Our study's findings may provide new insights into the use of nano-TiO₂ to resist Cd toxicity by stimulating essential oil biosynthesis. Given the importance of peppermint as a medicinal plant in ensuring community health and the effects of the heavy metal stress of Cd on its growth and physiological and phytochemical parameters, an experimental study was designed with the following objectives: (1) investigating morphological and physiological responses of peppermint to Cd stress treatments, (2) examining the effects of TiO₂ NPs on the growth and phytochemical parameters of peppermint, (3) evaluating physiological responses and essential oil production of peppermint to the exogenous application of TiO₂ NPs.

Materials and methods

TiO₂ NPs and their characteristics

In the present study, TiO₂-NPs were purchased from Pishgaman Nanomaterials Company, Iran.

The below details were reported from the manufacturer; diameter of the TiO₂ NPs: 10–25 nm, purity: >99%, APS: 10–25 nm, SSA: 200–240 m²/g, color: white, bulk density: 0.24 g/cm³, true density: 3.9 g/cm³, PH: 6–6.5, loss of weight in drying: 4.17%, loss of weight on ignition: 8.24%. The transmission electron microscopy (TEM) image and XRD spectrum of TiO₂ NPs used in this study are represented in Fig. 1A,B, respectively.

Plant materials, treatments and experimental setup

To investigate the effects of TiO₂ NPs and Cd metal stress on peppermint's growth, physiological parameters, and phytochemical characteristics, a factorial experiment based on a completely randomized block design with three replicates was conducted under greenhouse environment. The first factor included different concentrations of Cd metal (0, 20, 40, and 60 mg L⁻¹), and the second factor included foliar application of TiO₂ NPs at different concentrations (0, 75, and 150 mg L⁻¹). The experimental treatments were applied after the complete establishment of seedlings until the harvest stage (beginning of flowering). In the present study, the following traits were measured: shoot dry weight, chlorophyll fluorescence using a fluorimeter, content of photosynthetic pigments, total phenol content, flavonoids, anthocyanins, and essential oil yield.

After separating the aerial parts of the plants from the pots, they were dried at a temperature of 70 °C for 48 h in an oven. The dried samples were weighed with a precision of 0.0001 and considered to be the dry weight of the plants. The uptake and distribution of TiO₂ NPs inside plants were detected using Scanning electron microscopy (SEM) and energy-dispersive X-ray (EDX) techniques³⁸.

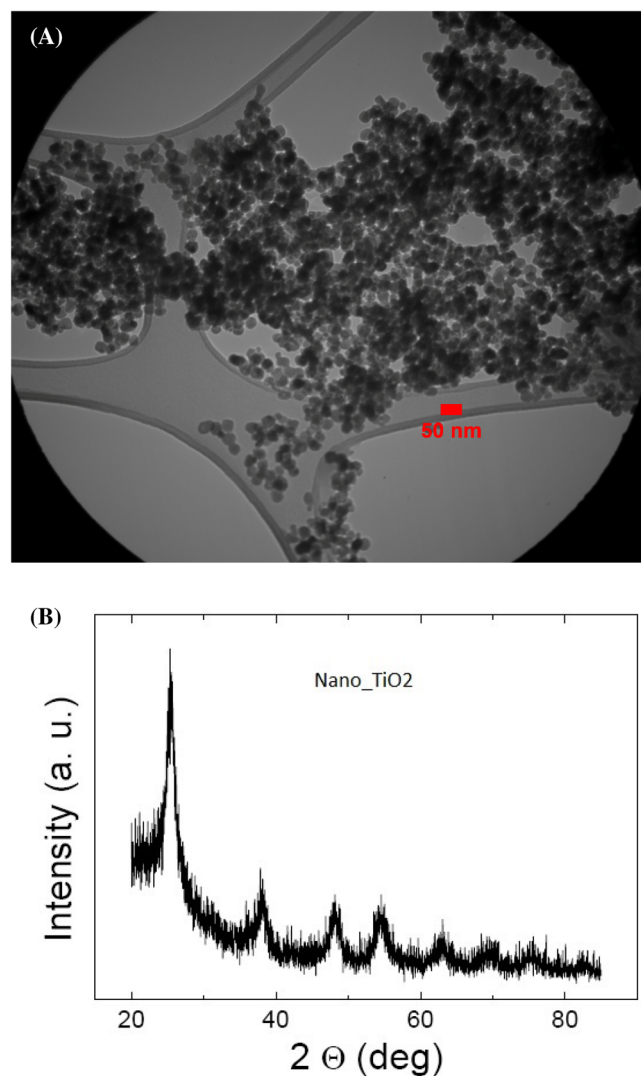


Figure 1. (A) Transmission electron microscopy (TEM) image of TiO₂ NPs, and (B) XRD spectrum of TiO₂ NPs used in this study.

Photosynthetic pigments

Several plants were randomly selected from each pot immediately after the treatments to measure chlorophyll. The aerial parts were wrapped in aluminum foil and transferred to the laboratory on ice. Then, 0.1 g of these samples were weighed using a precision balance. The samples were placed in a mortar and homogenized with 80% acetone. The homogenized mixture was filtered with filter paper No. 2. The mortar, pestle, and remaining plant materials were rinsed with 80% acetone, and the final volume was noted after the filtered solution was transferred. The absorption of light by the solution at wavelengths of 663 nm and 645 nm was measured. The readings obtained from the spectrophotometer were converted to micrograms per milliliter using the nomogram, representing the concentration of chlorophyll a or b or the total of both, which was finally expressed as milligrams per gram of fresh weight of the leaf material²⁹. To estimate the number of carotenoids, the absorption of the samples at a wavelength of 470 nm was considered based on the prepared extract above, and the concentration was determined in milligrams per gram of fresh weight³⁰.

Cd contents

Concentrated nitric acid was added to measure the accumulation of Cd in the crushed and dried samples, and the solution was allowed to rest for 12 h. Then, in the next step, it was heated in a water bath at 90 °C for 3 to 4 h. Afterward, hydrogen peroxide was added to the extracts to obtain a clear solution, and the extracts were transferred to filter paper No. 2. Finally, the solutions were brought to the desired volume with distilled water. The concentration of elements in the samples was measured using a flame atomic absorption spectrometer (Varian 240).

MDA and H₂O₂ contents

The thiobarbituric acid test (TBAT) was used for determination of lipid peroxidation in the leaf membranes of plants. Frozen (in liquid nitrogen) samples were weighed (0.1 g) and placed in a mortar, then crushed and homogenized with liquid nitrogen. The samples were transferred to tubes, and 1.0% trichloroacetic acid (TCA) (1.5 mL) was added to each tube. The tubes were centrifuged at 12,000g for 10 min at 4 °C. Then, 0.5 mL of the supernatant was taken, and 1.0 mL of 20% TCA containing 0.5% thiobarbituric acid was added. The mixture was incubated in a water bath at 95 °C for 30 min. After that, the mixture was immediately placed in an ice bath to cool down and centrifuged at 10,000g for 10 min. The absorbance of the supernatant was measured at 532 nm, and the non-specific absorption at 600 nm was subtracted. The malondialdehyde concentration was calculated using a correction coefficient of 155 mM⁻¹ cm⁻¹ and expressed in nmol per gram of fresh weight³¹.

To measure the hydrogen peroxide (H₂O₂) content, 0.1 g of powdered leaves were mixed with 1.5 mL of 0.1% trichloroacetic acid (TCA) and centrifuged at 10,000g for 20 min at 4 °C. Then, 500 µL of phosphate buffer and 1000 µL of 1 M potassium iodide were added to the supernatant, and the OD was recorded at 390 nm³².

Phenol contents

The collected samples were taken to the laboratory after removing the plants from the greenhouse to evaluate their morphological traits. The samples were examined in a pollution-free environment under shade and indirect sunlight in the laboratory. The samples were dried and stored in small, dark-colored glass containers at 4 °C to evaluate phytochemical parameters and essential oil yield.

The plant extract will be obtained using the ultrasonic method. A 25-mL Erlenmeyer flask containing 0.5 g of finely ground dried plant material, to which 10 mL of methanol–water solvent was added, will be placed in an ultrasonic bath for 30 min. Then, the contents of the Erlenmeyer flask will be filtered through filter paper, and the obtained extract will be stored in small dark-colored glass vials at a temperature of 4 °C until the analysis time.

The total phenolic content was measured using the Folin-Ciocalteu method³³. Briefly, 0.1 g of the samples was rubbed with 5 mL of 80% methanol containing 1% hydrochloric acid for 2 h on a shaker at room temperature to obtain a complete extraction. After that, the resulting mixture will be centrifuged at 8000g, and the supernatant will be used to determine the total phenolic compounds. In the next step, 100 µL of the supernatant will be mixed with 750 µL of Folin-Ciocalteu reagent and kept at room temperature for 5 min, and then 750 µL of 6% sodium carbonate will be added to the mixture. After 90 min, the absorption of each sample at 725 nm will be measured. A gallic acid standard curve will be plotted to calculate the concentration of total phenolic compounds, and the concentration of phenolic compounds will be reported in milligrams per gram of dry weight.

Flavonoid contents

The method described by Chang et al.³⁴, will be used to measure the flavonoid content. For this purpose, 0.5 mL of the methanol extract will be mixed with 1.0 mL of 10% aluminum chloride and 0.1 mL of 1 M potassium acetate. Finally, 2.8 mL of distilled water will be added. After 30 min, the absorbance of the flavonoids will be measured at 415 nm, and the flavonoid content equivalent to quercetin per gram of dry plant powder will be determined based on a standard curve of quercetin concentration.

Anthocyanin contents

The pH differential method will be used to determine the anthocyanin content, and the results will be reported in milligrams per gram³⁵.

PAL enzyme activity

PAL was extracted from 100 mg mint leaves; plant material was homogenized with liquid nitrogen using a mortar and pestle containing appropriate buffer solution (50 mM potassium phosphate and 1 mM EDTA, pH 7.8) and

1% PVP (polyvinylpyrrolidone) and then filtered through a 0.20 mm nylon filter into a centrifuge tube. The tissue extract was centrifuged at 12,000g for 40 min at 4°C. The supernatant to be used for enzymatic activity determination was stored at 4°C. Protein concentration was determined by the method described by Bradford³⁶. PAL activity was assayed following the method described by Beaudoin-Eagan and Thorpe³⁷ by measuring the amount of trans-cinnamic acid formed at 290 nm. The reaction mixture consisted of 100 mL of enzyme extract, 900 mL 6 mM of L-phenylalanine and 500 mM Trise HCl buffer solution (pH 8). The mixture was placed in a water bath at 37 °C for 70 min, and the reaction was stopped by the addition of 50 mL of 5 N HCl. Trans-cinnamic acid (1 mg mL⁻¹) was used as a standard, and PAL activity was expressed as mmol transcinnamic acid min⁻¹ mg⁻¹ protein.

Antioxidant enzymes (SOD, CAT and POD) activity

Briefly, 0.1 g of powdered leaf samples were added to 1.5 mL of ice-cold phosphate buffer (0.5 mM EDTA, pH 7.5) and after centrifugation at 15,000g for 30 min, the supernatant was used as an enzyme extract. For the superoxide dismutase (SOD) assay, 100 µL of the enzyme extract was mixed with 2000 µL of solution A (containing 50 mM phosphate buffer, 50 mM sodium carbonate, 13 mM methionine, 75 µM nitro blue tetrazolium chloride), and 100 µL of solution B (1 µM riboflavin). Thereafter, the reaction mixture was placed inside the glass sample tube, for 10 min under a cool white fluorescent lamp (15 W) at a distance of 35 cm. The reaction was stopped after turning off the lamp, and its OD was noted at 560 nm using a spectrophotometer. A sample tube containing the reaction mixture (solutions A and B) was used as a blank. One unit of SOD activity was considered as the amount of enzyme that leads to 50% inhibition of the photoreduction of nitro blue tetrazolium³⁸. For CAT assay, 30 µL of enzyme extract was mixed with 2970 µL of phosphate buffer (2 mM H₂O₂) and the absorbance was recorded at 240 nm. Enzyme activity was calculated using the Beer-Lambert law and extinction coefficient (40 µM⁻¹ cm⁻¹) as described by Badihi et al.³⁹. For POD assay, 50 µL of enzyme extract was mixed with 700 µL of phosphate buffer and 700 µL of H₂O₂. The changes of OD were recorded at 470 nm for 2 min. Cold phosphate buffer was used as a blank/control sample⁴⁰.

Extraction and yield of essential oils

Hydro-distillation method was performed using a Clevenger apparatus to extract the essential oil. To enhance the contact surface of the leaf with distilled water, the dried sample needed to be ground into powder. The powdered sample was then placed in a flask, and distilled water, nine times the weight of the dry sample, was added to the flask. The extraction process was repeated for 4 h. At the end of the process, the weight of the dry matter used was determined. The obtained essential oil and water were collected in a graduated tube, and the water was returned to the distillation flask, completing the distillation cycle. The essential oil was poured into glass microtubes and weighed. The yield of essential oil was calculated by multiplying the percentage content of the essential oil by the weight of the dry plant material.

Statistical analysis

Finally, the data obtained from the experiment will be analyzed using SAS software version 9.1. The mean comparison test will be performed using the Duncan test, and the graphs will be plotted using Excel software version 2007.

Permission to collect fruit samples

The permission for collection of peppermint plants was acquired from Agricultural and Natural Resources Ministry of Iran

Statement on experimental research and field studies on plants

The *Mentha piperita* L. plants sampled comply with relevant institutional, national, and international guidelines and domestic legislation of Iran.

Results and discussion

Leaf uptake of TiO₂ NPs and growth traits

The SEM images of leaf tissues in plants exposed to TiO₂ NPs and control group are presented in Fig. 2A, C, E and G. The EDX analysis revealed the uptake and presence of TiO₂ NPs in leaf tissues of treated plants at a bending energy of 4.5 keV (Fig. 2B, D, F and H). When NPs are sprayed on the leaves of a plant, they can be absorbed through various routes such as stomata, trichomes, cuticles, lenticels, hydathodes, etc. Once absorbed, they can move into the plant's vascular tissues through either the apoplast or symplast pathways. TiO₂-NPs can also enter plant cells either through plasmodesmata or by passing via ion channels⁴¹. As a result, TiO₂-NPs can interact directly or indirectly with receptor-binding sites on the cell membrane surface. When an elicitor recognizes these sites, it triggers a complex signaling mechanism that includes ion fluxes, activation of MAPKs cascades, and generation of ROS. The plant's first response to NP exposure is likely the exchange of active ions across the plasma membrane. Calcium (Ca²⁺) is an important signaling messenger that regulates various cellular functions fundamental to the elicitation mechanism⁴².

The comparison of the data mean showed that the heavy metal stress of Cd in all three levels, especially 60 mg L⁻¹, led to a decrease of 40 and 66.33% in the number of lateral branches and shoot dry weight, respectively, compared to the control (Tables 1, 2). Foliar spraying with TiO₂ NPs in both levels, especially at 150 mg L⁻¹, increased the number of lateral branches (59.46%) and shoot dry weight (37.86%) under 60 mg L⁻¹ of Cd stress compared to the respective control (Table 2). Studies have shown that Cd stress through damage to the

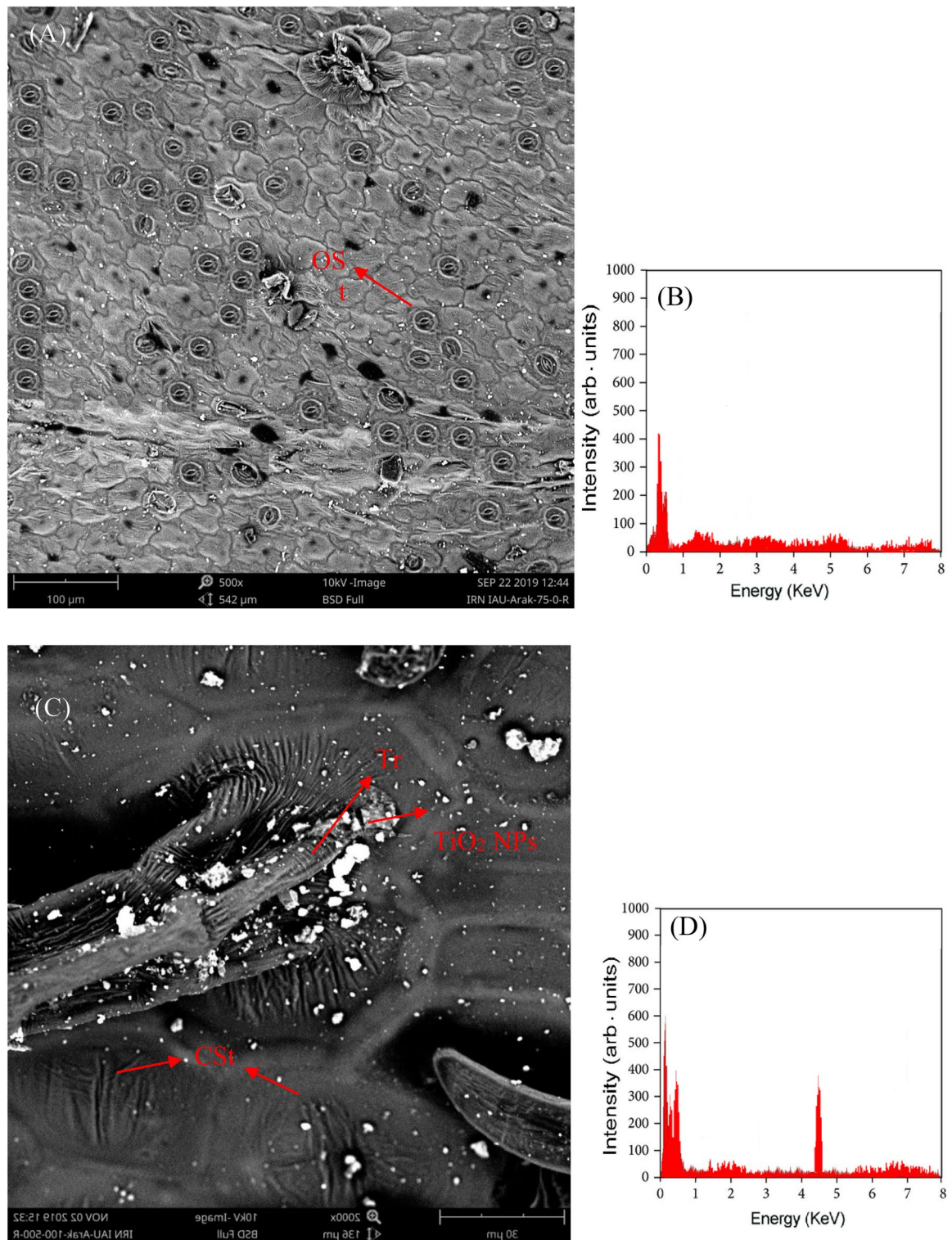


Figure 2. The SEM images of (A) leaf sample from control plants, (C) plants treated with TiO_2 NPs at 75 mg L^{-1} , and (E) plants exposed to 150 mg L^{-1} in no Cd-polluted soil. (G) SEM micrograph of leaf sample from plants treated with TiO_2 NPs at 150 mg L^{-1} in a Cd-polluted soil (20 g/l). The presence of TiO_2 NPs in leaf tissues was further confirmed by the EDX spectrum at 4.5 keV (B, D, F and H). OST: open stomata, CST: closed stomata, Tr: trichome. Accumulation of TiO_2 NPs around stomata and trichomes were shown by arrows.

thylakoid membrane leads to a severe decrease in photosynthesis and, as a result, a reduction in cell growth and division⁴³. Also, other studies show that Cd reduces chlorophyll synthesis by affecting nutritional elements

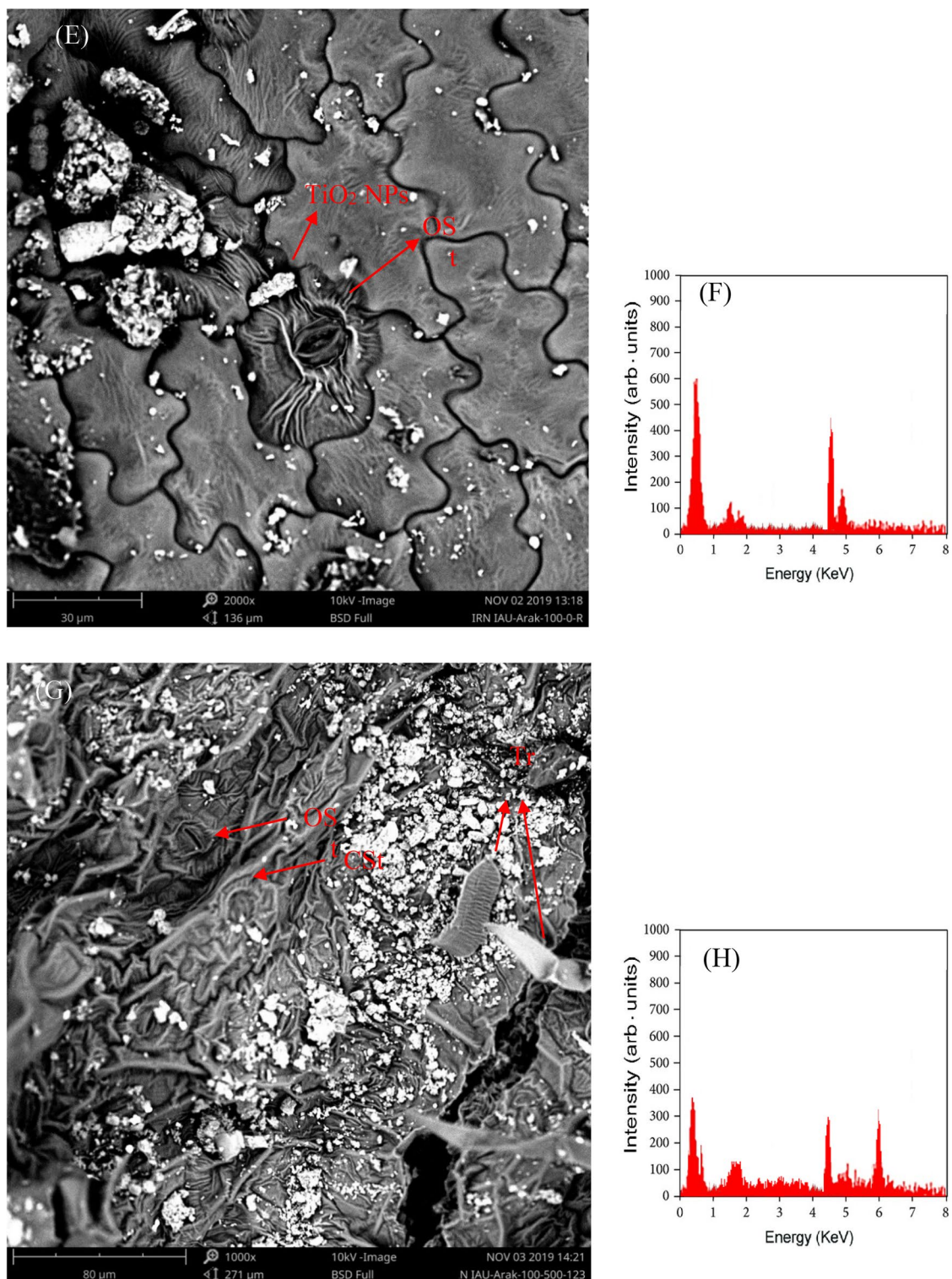


Figure 2. (continued)

and the lack of iron, magnesium, and calcium elements, decreasing photosynthesis and growth⁴⁴. The research results show that spraying with TiO₂ NPs increases plant growth parameters by improving the plant's defense mechanism against Cd stress⁴⁵.

Cd content

The interaction between Cd stress and TiO₂ NPs foliar spraying significantly affects the 1% level of Cd content in the roots and shoots (Table 1). The comparison of means showed that the Cd absorption in the roots and shoots

Source of variation	Df	Mean square					
		No. lateral branches	Shoot dry weight	Fv/Fm	Root Cd content	Shoot Cd content	MDA content
Block	2	0.44ns	1.03*	0.0002ns	0.019ns	0.001**	0.002ns
A	3	62.25**	75.46**	0.056**	27.079**	0.018**	7.217**
B	2	325.69**	189.32**	0.011**	4.925**	17.878**	0.599**
a × b	6	5.80**	7.36**	0.002**	1.302**	2.267**	0.063**
Error	22	0.41	0.09	0.0001	0.012	0.519	0.0008

Table 1. Analysis of variance (ANOVA) for the studied traits in *Mentha piperita* L. plants under different cadmium stress (Factor a) with nanoparticles of TiO₂ sprayed (Factor b). ns, Not significant. *Significant at the 0.05 probability level. **Significant at the 0.01 probability level.

Cd stress (mg/l)	Nano-TiO ₂ application (mg/l)	Shoot dry weight	No. lateral branches	Fv/Fm	Root Cd content (mg/g DW)	Shoot Cd content (mg/g DW)	MDA content (nmol/g)
0	0	8.99 ^h ± 0.21	8 ^{ef} ± 0.33	0.78 ^b ± 0.01	0 ⁱ	0 ^c	0.06 ⁱ ± 0.01
	75	15.53 ^d ± 0.29	15 ^c ± 0.58	0.79 ^{ab} ± 0.02	0 ⁱ	0 ^c	0.05 ⁱ ± 0.02
	150	19.87 ^a ± 0.34	22 ^a ± 0.53	0.90 ^a ± 0.01	0 ⁱ	0 ^c	0.02 ⁱ ± 0.01
20	0	8.60 ^{hi} ± 0.16	7 ^g ± 0.31	0.71 ^d ± 0.01	1.63 ^f ± 0.05	1.36 ^{cd} ± 0.01	0.75 ^e ± 0.03
	75	13.78 ^c ± 0.23	13 ^d ± 0.35	0.73 ^c ± 0.01	1.36 ^g ± 0.03	1.09 ^{de} ± 0.02	0.39 ^g ± 0.02
	150	17.00 ^b ± 0.28	19 ^b ± 0.34	0.73 ^c ± 0.01	1.08 ^h ± 0.04	0.93 ^{de} ± 0.03	0.26 ^h ± 0.03
40	0	7.75 ^j ± 0.18	6 ^g ± 0.32	0.63 ^g ± 0.02	3.69 ^b ± 0.06	3.06 ^{ab} ± 0.04	1.33 ^d ± 0.02
	75	11.88 ^f ± 0.22	12 ^d ± 0.3	0.68 ^e ± 0.03	2.88 ^c ± 0.05	2.60 ^{bc} ± 0.06	0.79 ^f ± 0.04
	150	16.48 ^c ± 0.27	16 ^c ± 0.29	0.70 ^d ± 0.01	2.08 ^c ± 0.04	1.88 ^{bcd} ± 0.05	0.72 ^f ± 0.05
60	0	5.96 ^k ± 0.12	5 ^h ± 0.02	0.52 ^h ± 0.01	5.55 ^a ± 0.17	4.12 ^a ± 0.07	2.45 ^a ± 0.08
	75	8.42 ⁱ ± 0.19	9 ^e ± 0.28	0.64 ^{fg} ± 0.02	3.73 ^b ± 0.07	3.05 ^{ab} ± 0.03	1.98 ^b ± 0.05
	150	9.60 ^g ± 0.25	12 ^d ± 0.34	0.65 ^f ± 0.01	2.60 ^d ± 0.09	2.26 ^{bcd} ± 0.04	1.92 ^b ± 0.1

Table 2. Effect of different Cd stress with foliar Nano-TiO₂ application on morpho-physiological traits of *Mentha piperita* L. plants. *Means followed by the same letter(s) in each column are not significantly different based on Duncan's multiple range test (n = 3).

also increased with increasing Cd concentration. However, applying TiO₂ NPs reduced Cd content, especially in the shoots (Table 2). Studies have shown that Cd is a non-essential and toxic element for plants, and its accumulation in plant tissues significantly inhibits root growth through its effects on photosynthesis and other biochemical processes⁴⁶. Investigations indicate that the use of nanoparticles can reduce Cd accumulation in various plant species^{47,48}, and other studies have shown that TiO₂ NPs can reduce Cd transfer to the shoot, possibly through the modulation of gene expression in plants, thus reducing metal translocation from the root to the shoot^{49,50}. The reduction of Cd content in mint plants treated with TiO₂-NPs is confirmed by the role of these NPs in the process that limits the uptake and translocation of Cd in plant tissues.

Fv/Fm

Simultaneous Cd stress and TiO₂ NPs foliar spraying positively affected the Fv/Fm ratio (Table 1). The comparison of means revealed that the Fv/Fm ratio significantly decreased with increasing Cd concentrations up to 60 mg L⁻¹. However, foliar-applied TiO₂ NPs, especially at 150 mg L⁻¹, increased the ratio of Fv/Fm by 13.4% compared to the control (Table 2). The reduction in photosynthesis under Cd stress conditions, and consequent growth inhibition have been observed in previous studies⁴⁹, however, application of TiO₂ NPs in soybean plants under Cd stress has been shown to improve photosynthesis²⁴. In a study by Tripathi et al.⁵¹, it was also found that exposure to NPs improved chlorophyll fluorescence parameters under heavy metal stress.

MDA and H₂O₂ contents

The interaction between Cd stress and TiO₂ NPs significantly affected the stress markers, MDA (Table 3) and H₂O₂ (Table 7) contents. Cadmium treatment, especially at higher concentrations, led to an increase in MDA content, while the application of TiO₂ NPs resulted in a decrease in MDA level (Table 4). The maximum content of H₂O₂ (8.09 μmol g⁻¹ FW) was observed in plants grown at 60 mg L⁻¹ Cd stress without exposure to TiO₂ NPs (Fig. 3A). However, TiO₂-NPs treatment (at all levels) to Cd subjected plants significantly ($P < 0.01$) decreased the H₂O₂ content compared to the respective control.

Investigations indicate that the main toxicity of Cd in plants is attributed to the production of reactive oxygen species (ROS), which leads to membrane lipid peroxidation and damage to proteins and lipids⁵². A study by Rizwan et al.⁴⁹, found that using TiO₂ NPs reduced MDA as an indicator of ROS in rice leaves. Another

Source of variation	df	Mean square				
		Chl a	Chl b	Total Chl	Carotenoid	PAL activity
Block	2	0.186*	0.111*	0.585*	0.043*	0.003*
a	3	4.402**	0.828**	8.820**	0.241**	92.560**
b	2	0.436**	0.019**	0.615**	0.109**	19.00**
a × b	6	0.058**	0.005**	0.063**	0.010**	8.953**
Error	22	0.001	0.001	0.003	0.0002	0.015

Table 3. Analysis of variance (ANOVA) for the studied traits in *Mentha piperita* L. plants under different cadmium stress (Factor a) with nanoparticles of TiO₂ sprayed (Factor b). ns, Not significant. *Significant at the 0.05 probability level. **Significant at the 0.01 probability level.

Cd stress (mg/l)	Nano-TiO ₂ application (mg/l)	Chl a (mg/g)	Chl b (mg/g)	Total Chl (mg/g)	Carotenoid (mg/g)	PAL activity (Unit/mg protein)
0	0	1.89 ^e ± 0.07	1.51 ^b ± 0.05	3.40 ^e ± 0.12	0.41 ⁱ ± 0.02	5.97 ^h ± 0.04
	75	2.02 ^b ± 0.09	1.62 ^a ± 0.07	3.64 ^b ± 0.17	0.41 ⁱ ± 0.02	6.17 ^{gh} ± 0.06
	150	2.42 ^a ± 0.08	1.58 ^a ± 0.05	4.00 ^a ± 0.13	0.65 ^f ± 0.04	6.30 ^g ± 0.05
20	0	1.81 ^d ± 0.1	1.36 ^c ± 0.06	3.18 ^d ± 0.15	0.69 ^e ± 0.03	7.12 ^f ± 0.07
	75	1.89 ^e ± 0.1	1.50 ^b ± 0.07	3.39 ^c ± 0.16	0.79 ^c ± 0.05	8.36 ^d ± 0.03
	150	1.94 ^e ± 0.09	1.51 ^b ± 0.06	3.45 ^c ± 0.14	0.81 ^{bc} ± 0.06	8.66 ^c ± 0.06
40	0	0.99 ^g ± 0.06	0.97 ^e ± 0.04	1.96 ^g ± 0.08	0.81 ^{bc} ± 0.04	7.57 ^e ± 0.08
	75	1.11 ^f ± 0.07	1.00 ^e ± 0.03	2.12 ^f ± 0.09	0.82 ^b ± 0.03	12.86 ^b ± 0.14
	150	1.62 ^c ± 0.08	1.06 ^d ± 0.06	2.69 ^c ± 0.14	0.90 ^a ± 0.07	14.96 ^a ± 0.07
60	0	0.43 ⁱ ± 0.03	0.99 ^e ± 0.08	1.41 ⁱ ± 0.11	0.45 ^h ± 0.02	4.04 ^j ± 0.04
	75	0.60 ^h ± 0.05	0.96 ^e ± 0.07	1.56 ^h ± 0.12	0.53 ^g ± 0.04	4.34 ⁱ ± 0.06
	150	0.64 ^h ± 0.04	0.98 ^e ± 0.08	1.61 ^h ± 0.13	0.74 ^d ± 0.05	4.51 ⁱ ± 0.1

Table 4. Effect of different Cd stress with foliar Nano-TiO₂ application on physiological traits of *Mentha piperita* L. plants. *Means followed by the same letter(s) in each column are not significantly different based on Duncan's multiple range test (n = 3).

study demonstrated that applying TiO₂ NPs increased the activity of antioxidant enzymes and consequently reduced lipid peroxidation²². Here, similar to the previous studies^{53,54}, H₂O₂ amplified in the leaf tissue of *M. piperita* plants under employed Cd treatments in a concentration-dependent manner. Excessive generation of ROS enhances lipid peroxidation process which in return elevated MDA content in Cd-stressed plants⁵². However, the declined production/and accumulation of cellular MDA and H₂O₂ portrayed that TiO₂-NPs assuaged Cd toxicity in peppermint plants.

Photosynthetic pigments

The interaction between Cd stress and the application of TiO₂ significantly affected the photosynthetic pigments' content (Table 3). Cadmium stress reduced chlorophyll b, a, and total chlorophyll, especially at a concentration of 60 mg L⁻¹, while the content of chlorophyll improved (Table 4). Studies have shown that the content of photosynthetic pigments decreases under heavy metal stress conditions³⁴, and the application of nanoparticles improves the photosynthetic apparatus and chlorophyll fluorescence parameters, leading to an improvement in the content of photosynthetic pigments (Table 4). The content of carotenoids increased under Cd stress up to a concentration of 40 mg L⁻¹. However, it decreased with further increases in concentration up to 60 mg L⁻¹, while applying TiO₂ NPs improved the content of carotenoids at all Cd stress levels (Table 4).

The increase in carotenoid synthesis under Cd stress is likely due to the photoprotective role of carotenoids in photosynthesis, as they act as antioxidants and protect lipids from photooxidation and oxidative stress⁵⁵. TiO₂ NPs can also be influential in improving membrane structure and preventing oxidative stress by increasing carotenoid content⁵⁶.

Phenol contents

The mutual effect of Cd and TiO₂ application positively affected the total phenolic content (Table 5). A comparison of the mean data showed that Cd stress up to 40 mg L⁻¹ increased the total phenolic content. However, concentration of 60 mg L⁻¹ Cd led to a decrease in the total phenolic content. However, applying TiO₂ NPs improved the total phenolic content at all Cd stress levels (Table 6). Studies have shown that secondary metabolites, such as phenols, play an essential role in plant interactions with the environment for adaptation and defense against stress⁵⁷. A study found that the production of secondary metabolites increases under heavy metal stress conditions⁵⁸. Additionally, a study by Karamian et al.⁵⁹, revealed that treatment with TiO₂ NPs

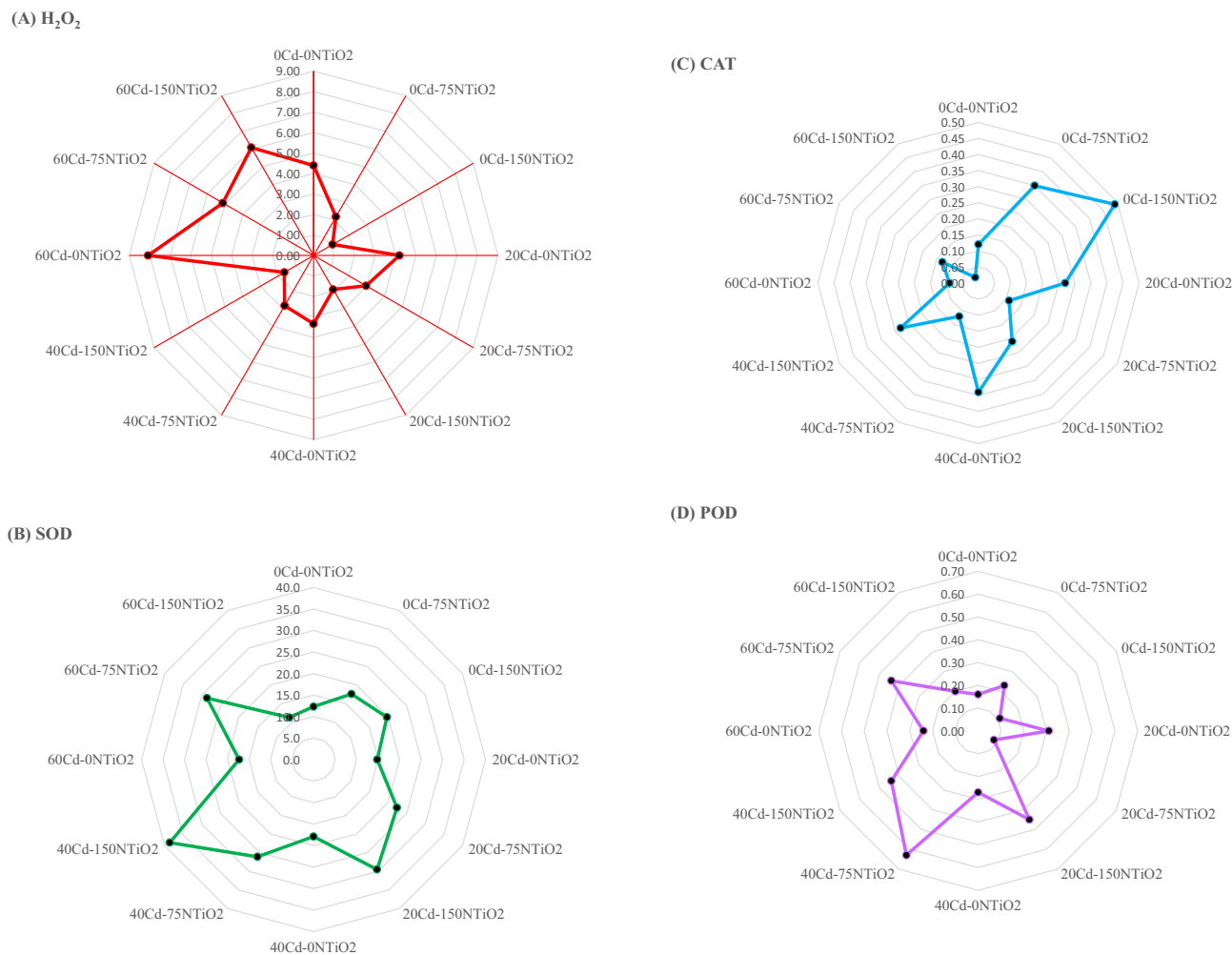


Figure 3. Radar plots for the effects of different cadmium (Cd) levels (0, 20, 40, and 60 mg L⁻¹) with foliar Nano-TiO₂ particles application (0, 75, and 150 mg L⁻¹) on H₂O₂ content (A), and antioxidant enzymes [SOD (B), CAT (C), and POD (D)] activities in *Mentha piperita* L. plants.

Source of variation	Df	Mean Square			
		Phenol	Flavonoid	Anthocyanin	Essential oil content
Block	2	128.83**	6.19**	0.054**	0.008ns
a	3	4325.77**	38.42**	1.234**	0.874**
b	2	955.13**	11.38**	0.260**	0.485**
a × b	6	192.85**	0.49**	0.025**	0.088**
Error	22	2.48	0.02	0.0006	0.002

Table 5. Analysis of variance (ANOVA) for the studied traits in *Mentha piperita* L. plants under different cadmium stress (Factor a) with nanoparticles of TiO₂ sprayed (Factor b). ns, Not significant. *Significant at the 0.05 probability level. **Significant at the 0.01 probability level.

improved the shikimate metabolism, leading to an increase in phenolic compounds. Plants use osmoregulators to adjust osmosis and regulate the configuration of organelles and macromolecules. In a study by Khan⁶⁰, tomato (*Solanum lycopersicum*) plants treated with TiO₂-NPs exhibited a higher amount of phenolics, photosynthetic content, antioxidative potential, and yield under saline conditions. This suggests that the higher accumulation of osmoregulators and improved activity of antioxidant enzymes can help reduce the toxicity induced by Cd in plants⁶¹.

Flavonoid content

The mutual effect of Cd and TiO₂ NPs significantly affected the flavonoid content (Table 5). The flavonoid content increased with increasing Cd stress at 20 and 40 mg L⁻¹ but showed a decreasing trend at a 60 mg L⁻¹

Cd stress (mg/l)	Nano-TiO ₂ application (mg/l)	Total phenol (mg gallic acid/g DW)	Total flavonoid (mg QE/g DW)	Total anthocyanin (mg/g)	Essential oil content (% w/w)
0	0	39.42 ^a ± 1.15	10.12 ^c ± 0.34	0.97 ^c ± 0.03	0.82 ^b ± 0.01
	75	40.77 ^a ± 1.20	12.12 ^c ± 0.41	1.28 ^d ± 0.04	0.91 ^{ef} ± 0.02
	150	46.92 ^f ± 1.44	12.27 ^c ± 0.38	1.36 ^c ± 0.04	1.06 ^d ± 0.03
20	0	54.48 ^e ± 1.46	11.15 ^d ± 0.37	1.28 ^d ± 0.03	0.88 ^g ± 0.02
	75	78.83 ^c ± 2.25	12.81 ^b ± 0.47	1.54 ^b ± 0.04	1.35 ^c ± 0.03
	150	85.15 ^b ± 2.46	12.95 ^b ± 0.48	1.57 ^b ± 0.02	1.47 ^b ± 0.02
40	0	61.75 ^d ± 2.34	11.29 ^d ± 0.44	1.28 ^d ± 0.05	0.97 ^c ± 0.01
	75	85.65 ^b ± 3.41	13.50 ^a ± 0.54	1.58 ^b ± 0.06	1.35 ^c ± 0.04
	150	89.98 ^a ± 3.6	13.59 ^a ± 0.49	1.69 ^a ± 0.07	1.72 ^a ± 0.1
60	0	33.82 ^b ± 1.10	7.75 ^e ± 0.3	0.69 ^f ± 0.02	0.64 ^h ± 0.02
	75	34.40 ^b ± 1.13	8.41 ^f ± 0.33	0.70 ^f ± 0.01	0.66 ^h ± 0.03
	150	36.47 ^b ± 1.22	8.46 ^f ± 0.29	0.72 ^f ± 0.02	0.67 ^h ± 0.01

Table 6. Effect of different Cd stress with foliar Nano-TiO₂ application on biochemical traits of *Mentha piperita* L. plants. *Means followed by the same letter(s) in each column are not significantly different based on Duncan's multiple range test (n = 3).

concentration. The application of TiO₂ NPs also increased the flavonoid content at all stress levels (Table 6). In a study by Ali et al.⁶², it was found that under heavy metal stress conditions, the induction of the activities of glucose-6-phosphate dehydrogenase, shikimate dehydrogenase, cinnamyl alcohol dehydrogenase, and the accumulation of phenolic compounds and flavonoids occurred. Another study by Kruszka et al.⁶³, showed that applying TiO₂ NPs led to an increase in flavonoids and anthocyanins in the flower of *Hypericum perforatum* L.

Anthocyanin content

The mutual effect of Cd and TiO₂ NPs significantly affected anthocyanin content (Table 5). A comparison of the mean data showed an increasing trend in anthocyanin content under moderate Cd stress but a decreasing trend at high-stress levels (60 mg L⁻¹ Cd). However, applying TiO₂ NPs improved the anthocyanin content at all stress levels (Table 6). The accumulation of anthocyanins under heavy metal stress conditions has been observed to have a detoxification role⁵⁸. In a study by Faizan et al.⁶⁴, it was also found that using NPs increased the anthocyanin content in *Solanum lycopersicum*. Nanoparticle treatment of plants has been shown to have numerous beneficial effects. These include the stimulation of seed germination and seedling emergence, increased biomass accumulation, net photosynthetic rate, pigments content, and nutrient uptake. The specific effects vary depending on the species of plant, concentration of nanoparticles used, and method of application⁶⁵.

PAL enzyme activity

The mutual effect of Cd and TiO₂ NPs significantly affected PAL enzyme activity (Table 5). A comparison of the mean data showed an increasing trend in PAL enzyme activity under moderate Cd stress, and the application of TiO₂ NPs improved the activity of this enzyme (Table 6). Studies have shown that plants under environmental stresses use enzymatic antioxidant compounds such as PAL, CAT, and SOD, etc., to detoxify various reactive oxygen species⁶⁶. In a study by Kovacic et al.⁶⁷, it was found that under oxidative stress induced by Cd and Cu, PAL enzyme and phenolic compounds play a crucial role in heavy metal detoxification. A study by Rajendran et al.⁶⁸, revealed that applying NPs improved the content of secondary metabolites by increasing the PAL enzyme and polyphenol oxidase activity. PAL is an essential enzyme for producing many phenolic secondary metabolites. However, its activity as part of the immune system is highly dependent on both the plant growth stage and environmental factors⁶⁹. Previous research has shown that the foliar spray of Ag NPs and Cu NPs can increase the activity of PAL enzyme in *Annona muricata* L. plant⁷⁰.

Antioxidant enzymes activity

The double interaction between Cd stress and TiO₂ NPs significantly ($P < 0.01$) affected the antioxidant enzymes activity (Table 7). As shown in Fig. 3B, it was found that the specific activity of SOD (EC 1.15.1.1) was significantly increased in TiO₂-NPs treated plants (at both levels) under all Cd concentrations except 60 mg L⁻¹ compared to the respective control (Fig. 3B). The SOD activity in the highest TiO₂ NPs concentration-treated plants under moderate Cd stress (40 mg L⁻¹) was significantly higher (by 68.1%) compared to the control. CAT (EC 1.11.1.6) activity was increased (by 75.4%) in TiO₂ NPs-treated plants at 150 mg L⁻¹ without Cd stress compared to the control (Fig. 3C). At 75 mg L⁻¹ TiO₂ NPs treatment under moderate Cd stress, the POD (EC 1.11.1.7) activity increased to the maximum levels (Fig. 3D).

Cadmium has been shown to negatively impact the antioxidative system of plants, resulting in an increased accumulation of ROS and MDA^{71,72}. However, plants have the ability to scavenge ROS through a variety of antioxidative mechanisms, including the activities of antioxidative enzymes. Specifically, the activity of the enzyme SOD has been shown to be strongly correlated with increased stress tolerance, as it converts O₂⁻ to a less toxic form (H₂O₂), providing the first step of defense in the antioxidative machinery of plants⁷³. Additionally, the

Source of variation	df	Mean square			
		H ₂ O ₂	SOD	CAT	POD
Block	2	10.96**	3.99**	17.51**	9.19**
a	3	31.25**	10.89**	8.48**	108.40**
b	2	29.83**	10.14**	9.95**	38.09**
a × b	6	6.46**	8.91**	0.24**	3.76**
Error	22	0.26	0.11	0.05	0.27

Table 7. Analysis of variance (ANOVA) for the studied traits in *Mentha piperita* L. plants under different cadmium stress (Factor a) with nanoparticles of TiO₂ sprayed (Factor b). ns, Not significant; H₂O₂, hydrogen peroxide; SOD, superoxide dismutase; CAT, catalase; POD, peroxidase. *Significant at the 0.05 probability level. **Significant at the 0.01 probability level.

higher levels of H₂O₂ are scavenged through CAT activity, which converts it to water in peroxisomes, effectively neutralizing its harmful effects⁷⁴. During the present study, the up-regulated activity of SOD, CAT, and POD in TiO₂-NPs treated plants certainly mitigated Cd stress. However, SOD mainly functions in the outside defense system against oxidative injuries by detoxifying ROS. Therefore, it may be established that the least level of H₂O₂ witnessed in case of 150 mg L⁻¹ TiO₂-NPs was meticulously connected to the enhanced activities of SOD, CAT, and POD in the same samples. The decontamination of ROS by TiO₂-NPs application may be linked to the assuaged composition of cells and upgraded integrity of cellular membranes. Although the lower dose of TiO₂-NPs alleviated heavy metal toxicity in *Lolium perenne* plants, a higher concentration of TiO₂-NPs further enhanced stress in treated plants⁷⁵. Therefore, a higher concentration of TiO₂-NPs may have caused phytotoxicity and was not much effective in mitigating stress.

Essential oil content

The mutual effect of Cd and TiO₂ NPs significantly ($P < 0.01$) affected the essential oil content (Table 5). A comparison of the mean data showed an improvement in the essential oil content under moderate Cd stress, but it decreased in plants grown under high-stress level (60 mg L⁻¹) of Cd. However, the application of TiO₂ was efficient in increasing the essential oil content at all stress levels (Table 6). Studies have shown that essential oil production increases under heavy metal stress conditions and upon application of NPs^{58,76}. TiO₂ can stimulate plant growth by activating different defense mechanisms that are involved in plant tolerance against various abiotic stress agents⁷⁷. However, the effects of TiO₂ may vary based on the concentration applied and the specific plant species or environmental conditions⁷⁸. In a study, TiO₂ NPs were applied to plants growing under moderate Cd stress (40 mg L⁻¹) and it resulted in a remarkable increase in essential oil percentage. The maximum content (1.72%) was obtained at a concentration of 150 mg L⁻¹. This increase in essential oil content is consistent with the findings of Ahmad et al.⁷⁹, and Ghorbanpour⁸⁰, who reported similar results for *M. piperita* and *Salvia officinalis* plants, respectively. The observed increase in essential oil content could potentially be explained by the observed increase in growth, photosynthesis, expression of secondary metabolite enzymes and size and distribution of oil glands following NP application⁷⁹. The actual mechanism by which NP application modulates plant secondary metabolites is not yet fully understood. However, TiO₂ NPs have been found to improve the performance of secondary metabolites by increasing photosystem II (PSII) photoreduction and chloroplast photophosphorylation activity, releasing oxygen, changing the activity of rubisco, nitrate reductase, CAT, and POD enzymes, as well as increasing the content of some essential elements in plant tissues. These secondary metabolites serve as antioxidant molecules and play vital role in mitigating oxidative/nitrosative stresses and maintaining cellular homeostasis.

Conclusions

The results of the study revealed that Cd stress had adverse effects on the growth of peppermint plants, leading to a decrease in lateral branches, shoot dry weight, and chlorophyll fluorescence parameter (*Fv/Fm*). In addition, exposure to Cd caused an increase in stress markers (MDA and H₂O₂) content, lipid peroxidation, and photosynthetic pigments. The total phenolic content, anthocyanins and flavonoids values increased under moderate Cd stress, but as Cd concentration increased to 60 mg L⁻¹, there was a decrease. The PAL enzyme activity showed an increasing trend under moderate Cd stress but decreased with an increase in Cd concentration. Cadmium stress led to more Cd accumulation in the root and aerial parts, with a more significant effect in the roots. However, foliar application of TiO₂ NPs under Cd stress conditions improved growth by reducing lipid peroxidation, enhancing photosynthetic pigments, increasing PAL enzyme activity, and enhancing the accumulation of non-enzymatic antioxidants (phenolic compounds/total phenols, flavonoids, and anthocyanins) as well as enhancement of the enzymatic (SOD, CAT, and POD) defense system. Furthermore, TiO₂ NPs application improved the quantity of essential oil in peppermint plants under Cd stress. It also reduced Cd uptake in the roots, especially reducing its transfer to the aerial parts. The study demonstrates that applying TiO₂ NPs could be a sustainable approach to enhancing the quantitative and qualitative growth of peppermint plants under Cd stress by improving the defense system and immobilizing the heavy metal Cd.

Data availability

All the data generated/analyzed during the study are available with the corresponding author on reasonable request.

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Author contributions

Z.K. performed the research and contributed to sample collection. H.M. and A.A. designed and supervised the research, contributed to data analysis and wrote the manuscript. S.H. and M.G. advised the research and revised the manuscript critically. R.G.D. helped to technical analysis. All authors read and approved final version of the manuscript. All authors have agreed to submit the manuscript in its current form for consideration and possible publication in “Scientific reports”.

Competing interests

The authors declare no competing interests.

Additional information

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