# Melatonin alleviates cadmium phytotoxicity through regulation of growth, photosynthesis and antioxidant potential in two pepper genotypes

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# Abstract

Heavy metal contamination has severely hampered worldwide crop productivity. Cadmium (Cd) pollution is one of the most important environmental and agricultural soil challenges facing the world today, and it is mostly caused by human activity. The current research examines the potential effect of melatonin (ME) in alleviating Cd toxicity in pepper (*Capsicum annuum* L) seedlings. Cd stress reduced the growth traits, pigments molecules, and gas exchange elements, but increased phenolic content, hydrogen peroxide ($H_2O_2$), malondialdehyde (MDA) content and activities of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD). Conversely, exogenously applied ME promoted growth characteristics, chlorophyll content, and leaf gas exchange parameters in treated seedlings. Furthermore, ME supplementation further enhanced protein, total phenolics, and antioxidant enzyme activity. The increased activity of antioxidant scavengers played a central role in reducing $H_2O_2$ and MDA content in seedlings under Cd toxicity. According to the present research, seedlings pretreatment with ME decreases Cd-toxicity and allows pepper to grow in Cd-contaminated environments. Future research may aid in the understanding of the ME-assisted stress reduction mechanism in pepper crops. In addition, study into the potential of melatonin for stress relief in various horticultural and agronomic crops would aid in agricultural production development.


# Introduction

The anthropogenic activities in recent times have increased the matter of concern toward the enhancement of heavy metals in soil and water bodies (Altaf et al., 2022a). The goal of global agricultural and environmental research is to eliminate heavy metal pollution in agricultural soil and heavy metal toxicity in plants. In recent years, the area of soil polluted by heavy metals has grown due to the continual transportation, accumulation, and transformation of heavy metals within the environment (Li and Yan, 2021; Altaf et al., 2021a). Cadmium (Cd) has become more well-identified as a hazardous heavy metal because of its adverse effects on agricultural yield and the possible health risks connected with food chain contamination (Xiang et al., 2019). Cd is a ubiquitous environmental hazard because of its prolonged half-life in soil, higher solubility in water, and increasing human activity worldwide (Lin et al., 2012). Excess Cd in the soil system restricts growth, physiological, morphological, anatomical, and metabolic abnormalities in plants (Lin and Aarts, 2012). However, the detrimental effects of Cd might be prolonged to subsequent plant generations, decreasing the germination and viability of seeds from plants growing in Cd-contaminated soils (Carvalho et al., 2018). Cd toxicity is commonly related to its concentration in plant tissues, it is tempting to speculate that differences in Cd absorption, transportation, and accumulation may account for the varying tolerance level of various cultivars of a similar species when exposed to Cd (Altaf et al., 2022b).

Plants have developed efficient mechanisms for regulating Cd uptake and mitigating the damage caused by Cd toxicity (Gallego et al., 2012). The roots are the first organs to be harmed by Cd toxicity (Sigel et al., 2013). The reactive oxygen species (ROS) accumulation caused by Cd stress might have a dual purpose in plant cells, functioning as a harmful byproduct of metabolism and as important regulators of growth,

development, and protection mechanisms (Piqueras et al., 1999; Sharma and Dietz, 2009). For instance, ROS may cause lipid peroxidation and oxidative damage to other macromolecules and cell death (Foy et al., 1978; DalCorso et al., 2008). Plant cells have enzymatic antioxidant defense mechanisms responsible for ROS scavenging to maintain redox balance. To mitigate the negative effects of Cd toxicity, a promising way may be to employ phytohormones, which may successfully boost plant resilience to damaging stresses.

Melatonin (ME) is known as a bio-promoter due to its multiple physiological effects on plants, which include the suppression of leaf senescence, improved seed germination, increased root and shoot development, enhanced mineral homeostasis, and increased tolerance to abiotic stress (Tiwari et al., 2021; Nawaz et al., 2016; Liang et al., 2018). Plant scientists have recently examined the physiological function of ME in plants employing synthetic ME molecules or plants with increased endogenous melatonin levels (Kaya et al., 2019). ME has also been discovered to improve plant tolerance to a plethora of abiotic stresses, including salinity, temperature (low and high), drought, heavy metals (vanadium, nickel, boron, Cd), and UV radiation (Nawaz et al., 2018, Devi et al., 2021; Altaf et al., 2022c). Hasan et al. (2015) reported that ME supplementation mitigates Cd toxicity by recovering biomass production leaf photosynthesis and antioxidant machinery in tomato seedlings. In a recent study, Altaf et al., (2022b) observed that ME application significantly enhanced root system architecture and mineral nutrient homeostasis in tomato genotypes under Cd toxicity. In addition, ME application efficiently reduced Cd transportation from root to shoot in tomato. ME increased Cd tolerance in *Arabidopsis* seedlings via reducing Cd content in root tissue and maintaining redox balance (Gu et al., 2017). Furthermore, ME pretreated pepper seedlings subjected to boron toxicity showed significant enhancement in growth, gas exchange elements, pigment molecules, and mineral nutrient homeostasis (Sarafi et al., 2017).

Pepper (*Capsicum annuum* L.) is one of the most widely consumed vegetables in the solanaceous family (Korkmaz et al., 2021). Due to its nutritional and economic significance, it is regarded as a globally important cash crop. In terms of nutrients, pepper fruit is a rich source of antioxidants, vitamins, proteins, carbs, lipids, and phenolic compounds (Howard et al., 2000). Pepper plants are moderately sensitive toward metal stress such as vanadium, boron, and Cd (Sarafi et al., 2017; Kaya et al., 2020; Altaf et al., 2021b). Numerous studies have been conducted on the multifaceted effects of melatonin on the reduction of environmental stressors in a wide range of agricultural crops. However, to the best of our knowledge, the impact of ME in alleviating Cd stress in pepper still has to be investigated. As a result of the above considerations, the present study was intended to see whether ME might increase Cd tolerance and its responses in pepper plants by modulating the antioxidant system.

# Material And Methods

### Experimental setup

The seeds of pepper cultivars (Super Shimla and Ganga) were collected from a seed store located in the vegetable seed market in Multan, Pakistan. Melatonin (ME) and cadmium ($CdCl_2$)were purchased from

Dua enterprises, Multan, Punjab, Pakistan.

The present study was conducted in the Bahauddin Zakariya University (Bahauddar sub-campus Layyah) Multan, Pakistan. Seeds of pepper genotypes were sown in seedling trays filled with vermiculite. After thirty days of sowing, uniform-sized seedlings were removed from seedling trays and transferred into the black plastic pot with a top diameter of 12 cm, a height of 8.5 cm, and a bottom diameter of 10 cm). Pots were filled with vermiculite (80 g). After the transplant, seedlings were raised under normal growth conditions for twelve days to adapt to new conditions. This study was carried out under a controlled-conditioned greenhouse having (average day/night temperatures of 24/13 °C, with relative humidity of 65-85 %, and PPFD 350 µmol $m^{-2}$ $s^{-1}$ and a 15-h photoperiod). Then root application of ME (1 µM; 200 ml per plant) was done after every third day and continued for 12 days. After 12 days of ME pretreatment, the solution of 0.1 mM $CdCl_2$ (200 ml per plant) was applied to seedlings. Control (CK) plants were irrigated normally with Hoagland nutrient solution (pH 5.5±0.1) without adding $CdCl_2$ or ME. Each treatment contained ten plants, and each treatment was reproduced three times. The plants were treated with cadmium for four weeks, and then they were sampled to determine various growth and physiological factors. After harvesting, samples were immediately treated with liquid nitrogen and stored at -80 °C for analysis. The dose of Cd and ME was selected for this experiment from previous literature (Korkmaz et al., 2021; Kaya et al., 2020). The treatments were as follows: (1) CK (control); (2) Cd stress; (3) ME treatment + Cd stress.

## Plant Growth

After four weeks of Cd application, various plant growth determinants, including fresh and dry weights of leaves and roots, were determined. Fresh weight was immediately taken after uprooting the plants using an electronic weighing balance, and for dry weight, samples were oven-dried at 80 °C up to the constant weight (Altaf et al. 2019).

## Photosynthesis parameters

Leaf chlorophyll content was determined by homogenizing fresh leaves (0.5 g) in cold acetone (80%, 10 ml) followed by centrifugation at 12000 rpm for 10 minutes. To measure chlorophyll a, chlorophyll b, and carotenoid content, absorbance of the supernatant was taken at 645, 663, and 449, respectively at UV-Vis spectrophotometer (WE, 6000, China) (Lichtenhaler and Wellburn, 1983). Relative leaf chlorophyll content of fully opened new leaves was determined using SPAD-502 meter (Minolta, Japan). To determine the net photosynthetic rate (PN), transpiration rate (E), stomatal conductance (gs), and intercellular $CO_2$ concentration (ci) of fully expanded leaves, an infrared gas analyzer (ADC, Bio Scientific Ltd. UK) was used. These photosynthetic measurements were carried out under leaf temperature of 25 ± 2 °C, a RH of 65 ± 5 %, a PPFD of 800 µmol $m^{-2}$ $s^{-1}$, and a $CO_2$ level of 360 µmol $mol^{-1}$ (Jahan et al., 2020).

## Determination of total soluble proteins (TSP) and total phenolic content (TPC)

A modified method by Sambrook and Russell (2001) was adopted to extract total soluble proteins from pepper leaves and roots. Whereas TSP content was measured using the methodology of Bradford (1976). extracted sample (200 µL) was added in Coomassie blue dye (20 µL) and deionized water (780 µL). The absorbance of this 1 mL aliquot was checked at 595 nm in a UV-Vis spectrophotometer (WE, 6000, China). The unit to express total soluble proteins (TSP) and total phenolic content (TPC) was mg $mL^{-1}$.

Total phenolic content of pepper leaves was estimated using the procedure of Singleton and Rossi's (1965). 0.5 g of plant material was extracted in a 70:29:1 mixture of ethanol, deionized water, and glacial acetic acid. After filtration, the extract (1 mL) was mixed with Folin–Ciocalteu's reagent (1 mL) and distilled water (20 mL). The resultant mixture was kept at room temperature for 10 minutes before adding 7.5% sodium carbonate (10 mL) solution. After adding this, tubes were placed in dark conditions for 2 h and then absorbance was measured at 750 nm. Different concentrations of Gallic acid were used as the standard to estimate the actual value of total phenolic content in pepper samples.

### Determination of $H_2O_2$ and MDA content

$H_2O_2$ content in pepper leaves and roots was determined using the methodology of Velikova et al. (2000). The plant sample (0.1 g) was extracted in 0.1% TCA solution (1 mL) followed by centrifugation at 12000 rpm for 10 minutes. The supernatant (500 µL) was mixed with 1 M KI (1000 µL) and 10mM potassium phosphate buffer (500 µL). A UV-Vis spectrophotometer (WE, 6000, China) was used to measure absorbance at 390 nm. Commercial $H_2O_2$ was used as a standard to compare values of absorbance values of samples, and $H_2O_2$ content was expressed as µmol $g^{-1}$

The MDA content in pepper leaves and roots was determined using Thiobarbituric acid (TBA) test developed by (Heath and Packer 1968). The previously extracted sample for $H_2O_2$ measurement was used for MDA content determination. An amount of (500 µL) supernatant was added in 1 mL of 0.5% (w/v) thiobarbituric acid (TBA) in 20% TCA. The tubes were placed in a water bath maintained at 100 °C for 30 minutes and then immediately placed in the ice bath to stop the reaction followed by centrifugation at 12000 rpm for 5 minutes. The absorbance was then checked at 600 and 532 nm separately in a UV-Vis spectrophotometer (WE, 6000, China). To calculate the MDA content, absorbance taken at 600nm was subtracted from absorbance taken at 532 nm, and results were expressed as reactive substances of thiobarbituric acid (RSTBA), which represent malondialdehyde equivalents as µmol $g^{-1}$ DW.

### Determination of Antioxidant enzymes

Antioxidant enzymes were determined from freshly harvested pepper leaves and roots. After harvesting, plant samples were immediately crushed in liquid nitrogen to stop the enzymatic activity. For analysis purposes, the crushed sample (0.3 g) either leaf or root was completely homogenized in 50-mM sodium phosphate buffer (3 mL) with pH 7.8 and transferred to an Eppendorf tube. After that, these were

centrifuged at 12000 rpm for 10 minutes. The supernatant was further used to determine enzymatic antioxidant activities.

For SOD measurements, a reaction mixture was prepared by adding 0.5 mL ethylenediaminetetraacetic acid (EDTA) (75 mM), 1-mL nitroblue tetrazolium (NBT) (50 mM), 950-µL sodium phosphate buffer (50 mM), 1-mL riboflavin (1.3 µM), and 0.5-mL methionine (13 mM). This mixture was reacted with sample extract (50 µL), and tubes were placed under a fluorescent lamp for 5 minutes. Afterwards, a UV-Vis spectrophotometer was taken at 560 nm (WE, 6000, China).

CAT activity was determined by adopting the (Chance and Maehly 1955) methodology. A reaction mixture was formulated by mixing 900 µL of $H_2O_2$ (5.9 mM) into 50 mM phosphate buffer (2 mL) in a glass tube. The supernatant of the plant extract (100 µL) was added to the reaction mixture and the absorbance of the aliquot was taken at 240 nm every 30 s for up to 5 minutes.

POD activity was checked using the modified method of (Chance and Maehly 1955). The reaction mixture contained 20 mM guaiacol (0.4 mL), 50 mM sodium phosphate buffer (2-mL), and 40mM $H_2O_2$ (0.5 mL). The enzyme extract (0.1 mL) was added to this mixture The absorbance of the resultant aliquot was taken at 470 nm every 20 s up to 5 minutes. The variation in absorbance was used to determine the POD activity.

### Statistical analysis

Collected data were statistically analyzed using Origin Pro 2021 software. Duncan's multiple range tests (DMRT) were used to estimate the significant differences among treatments and cultivars. Means were also compared by applying the DMR test. Mean values with the corresponding standard error were calculated for biological triplicate per treatment.

## Results

### Plant Growth

To examine the effects of exogenous supplementation of ME on Cd stress in two pepper genotypes, the nutrient solution with ME supplementation was irrigated to roots for 12 days before Cd treatment. The results (Fig. 1) show a clear difference is visualized among pepper seedlings under Cd, with and without ME pretreatment. It was noted that fresh shoot weight, dry shoot weight, fresh root weight, and dry root weight were reduced by 60.89%, 56.45%, 63.41%, and 62.51%, respectively, in Super Shimla and decreased by 56.42%, 55.45%, 59.69%, and 61.27, respectively, in the Ganga seedlings grown under Cd-stress conditions, when compared with CK plants. Conversely, ME application enhanced the fresh shoot weight (64.66% and 50.38%), dry shoot weight (66.20% and 57.41%), fresh root weight (72.23% and 63.51%), and dry root weight (66.66% and 58.94%), in Super Shimla and Ganga seedlings, respectively, compared to the plants grown under Cd-stress environment (Fig. 2).

## Leaf Photosynthesis parameters

The results of leaf photosynthetic related elements (Chlorophyll a, Chlorophyll b. Carotenoids, SPAD index, net photosynthetic rate (Pn), stomatal conductance (Gs), intercellular $CO_2$ (Ci), and transpiration (Tr) compared are indicated in Fig. 3 and Fig. 4. The current findings revealed that Cd treatment dramatically decreased the pigments content of pepper genotypes (Super Shimla and Ganga) (Fig. 3A-C). Surprisingly, ME supplementation combined with Cd (ME+Cd) noticeably improved the chlorophyll an (81.71% and 59.26%), chlorophyll b (69.55% and 64.37%), and carotenoids (63.63% and 39.81%), in the Super Shimla and Ganga seedlings, respectively, compared to the plants grown under Cd treatment (Fig. 3A-C). As shown in Fig. 3D, after four weeks of Cd treatment, leaves of pepper genotypes significantly reduced SPAD index (relative chlorophyll content). The SPAD index was reduced by 59.64% and 56.51%; whereas ME application enhanced the SPAD index by 64.61% and 55.02%, respectively, in Super Shimla and Ganga seedlings (Fig. 3D).

The Pn, Gs, Ci, and Tr results are shown in Fig. 4. The present results revealed that gas exchange elements were considerably affected due to Cd toxicity. The Pn (65.56% and 62.55%), Gs (54.59% and 53.38%), Ci (53.46% and 64.51%), and Tr (59.17% and 67.11%) were decreased under Cd stress in the Super Shimla and Ganga plants, respectively, compared CK group. Nonetheless, ME (1 μmol $L^{-1}$) application enhanced the Pn, Gs, Ci, and Tr by 95.81%, 64.01%, 49.68%, and 89.31%, respectively, in the Super Shimla cultivar and reduced by 75.87%, 55.81%, 75.21%, and 98.22%, respectively, in Ganga variety, compared to the seedlings grown in the Cd environment (Fig. 4).

## Phenolic and protein content

The TPC effectively increased in the leaves/roots of Super Shimla and Ganga seedlings, respectively, when compared with CK group. Conversely, ME application and Cd treatment efficiently enhanced the level of phenols 25.46/224.51% in the leaves/root of Super shimla seedlings and increased by 22.35/31.41% in the leaves/root of Ganga plants, respectively, compared with Cd group. (Fig. 5A-B). Cd-treated plants showed a significant reduction in protein content 46.53/38.69% and 45.49/34.44% in the leaves/roots of super Shimla and Ganga, respectively, compared to CK seedlings. On the other hand, ME pretreatment enhanced the protein content of leaves/roots of pepper plants compared to Cd-treated pepper seedlings (Fig. 5C-D).

## $H_2O_2$ and MDA content

The current results revealed that Cd-treated pepper seedlings considerably increased $H_2O_2$ and MDA content in pepper genotypes' tissues (leaves and root) (Fig. 6). The findings also indicated that ME application reduced $H_2O_2$ and MDA content in the leaves/root of pepper cultivars. In detail, under Cd toxicity, the level of $H_2O_2$ (46.01/23.08% and 54.79/48.26%) and MDA (70.14/79.71% and 48.19/40.71%) were increased in the leaves/roots of Super Shimla and Ganga seedlings, respectively, as compared to Cd-treated seedlings (Fig. 6). Conversely, Under Cd toxicity, ME application effectively

reduced the content of $H_2O_2$ 11.88/17.21% and 19.37/18.93% in the leaves and roots of Super Shimla and Ganga plants. Also, it decreased the level of MDA 21.16/20.39% and 25.19/20.97% in the leaves/root of Super Shimla and Ganga seedlings, respectively, compared to Cd treatment (Fig. 6).

### Antioxidant enzymes

The activities SOD, CAT, and POD were also measured under Cd medium with or without the application of ME to the two pepper genotypes, as shown in Fig. 7. Antioxidant enzymes activities increased when plants were grown under Cd toxicity, with maximum increases in the activities of SOD, CAT, and POD of 53.04/42.21 %, 63.15/36.33 %, and 42.28/22.01 % in the leaves and roots of Super Shimla plants, and 76.95/35.16 %, 66.51/28.26 %, and 37.11/17.02 % in the leaves and roots of Ganga plants, Nonetheless, it was also observed that ME application further increased the activities of SOD, CAT, and POD by 47.09/45.36%, 39.75/26.09%, and 34.61/33.45%, respectively, in the leaves/roots of Super Shimla plants and also by 38.53/45.11%, 35.85/16.21%, and 24.52/33.81%, respectively, in the leaves/roots of Ganga seedlings, compared to Cd treatment.

# Discussion

Environmental research on heavy metals in agriculture has been focused on reducing heavy metal pollution in agricultural soils and its toxicity in plants. Several recent studies on agricultural soil pollution have revealed that these contaminants are heavy metals (vanadium, lead, nickel, and Cd). Cd is a non-essential and hazardous element that hinders plant growth and development amongst heavy metals (Altaf et al., 2022). Earlier research revealed that heavy metal stresses tolerance in many plant species might be enhanced by ME, which provides a novel method for eliminating heavy metal toxicity in plants (Nawaz et al., 2016; Jahan et al., 2020; Ahammed et al., 2020). However, ME has only been confirmed in a few investigations to prevent plant growth against Cd stress. ME was reported to reduce Cd toxicity in tomato, strawberry, and rose seedlings. (Hasan et al., 2015; Tang et al., 2015; Nabaei et al., 2019; Wu et al., 2021). In our study, the potential of ME in decreasing Cd toxicity in pepper seedlings was investigated. Melatonin's effect on growth, photosynthesis and antioxidant pool in Cd-stressed pepper seedlings was examined.

The pepper plants exposed to Cd treatment considerably reduced growth traits. However, ME application efficiently improved the growth characteristics of pepper (Fig. 1). Our results were concordant with the earlier research where it was validated that growth attributes were remarkably hampered by metal toxicity, including vanadium, copper, and arsenic in tomato, cucumber, and fava bean, respectively. Conversely, the growth elements were effectively reinforced by ME supplementation (Cao et al., 2019; Nawaz et al., 2016; Siddiqui et al., 2020). Exogenous application ME efficiently enhanced growth traits of cucumber under low and high iron stress (Ahammed et al., 2020). Furthermore, ME improved growth of wheat (Al-Huqail et al., 2020), radish (Tang et al., 2016) and tomato (Hasan et al., 2018), under boron, aluminum, and low-sulfur stress. An earlier report by Li et al., 2016 suggested that both selenium (Se) and melatonin inhibit cadmium (Cd) absorption in plants and ameliorate Cd toxicity. In addition, Se and melatonin supplements

dramatically enhanced Cd tolerance in plants, as shown by lower growth inhibition, photoinhibition, and electrolyte leakage (EL). Moreover, pretreatment with various forms of Se greatly stimulated the manufacture of melatonin and its precursors (tryptophan, tryptamine, and serotonin), with selenocysteine having the greatest impact on melatonin biosynthesis (Li et al., 2016). These studies suggest that ME and the combination of other nutrients might be used as an effect strategy to reduce the toxic effect of heavy metals.

One of the most important metabolic processes in plants is photosynthesis. Chlorophyll is the most important component of photosynthesis (Jahan et al., 2021). In this work, Cd-treated pepper seedlings dramatically reduced gas exchange elements (Pn, Ci, Gs, and Tr) of both pepper genotypes (Super Shimla and Ganga). Nonetheless, ME pretreated pepper seedlings significantly enhanced these characteristics under Cd toxicity (Fig. 4A-D). Siddiqui et al. (2019) revealed that ME supplementation enhanced the growth and photosynthesis of tomato plants under lanthanum toxicity. Similar, in another study, application of ME increased the photosynthetic assimilation rate of pear (Liu et al. 2019). In addition, ME efficiently recovered leaf photosynthesis in watermelon under vanadium toxicity, in tomato under nickel toxicity, and in cucumber under iron toxicity (Nawaz et al., 2018; Jahan et al., 2020; Ahammed et al., 2020). Furthermore, pigments molecules and SPAD index were decreased under Cd-toxicity. Conversely, pepper plants subjected to Cd toxicity, ME-pretreated plants revealed an enhancement in pigments content and SPAD index, compared with Cd-treated plants (Fig. 3). Previous literature revealed that ME pretreatment effectively enhanced pigments content of many crops under abiotic stress (Zhang et al. 2017; Chen et al., 2018; Manafi et al., 2021; Altaf et al., 2022c). Our results were concordant with the recent report that revealed that the Cd treatment decreased foliar photosynthetic pigment concentrations in both Cd-exposed apple rootstocks but to a higher extent in the Cd-sensitive *M. micromalus* 'qingzhoulinqin'. Exogenous melatonin ameliorated the deleterious effects of Cd. These characteristics were more prominent in *M. micromalus* 'qingzhoulinqin' (Cd-susceptible) than in *M. baccata* (Cd-tolerant) (He et al., 2020). Cd-treatment significantly reduced chlorophyll a and chlorophyll b content in strawberry seedlings. However, ME supplementation efficiently enhanced chlorophyll a and chlorophyll b in the leaves of strawberries under Cd toxicity (Wu et al., 2021). Similarly, Siddiqui et al. (2020) observed that arsenic toxicity considerably reduced gas exchange elements and chlorophyll content in *Vicia faba*. But, ME application improved leaf gas exchange parameters and the photosynthesis process.

Phenols are secondary metabolites with antioxidant properties that serve as a second line of defense against free radicals. The TPC in leaves and roots under Cd toxicity was improved the CK group in both genotypes (Super Shimla and Ganga) (Fig. 5). ME application along under Cd toxicity further enhanced the concentration of TPC in both the cultivars. ME in pepper plants exposed to boron toxicity has been found to have similar properties, which confirm to our findings (Sarafi et al., 2017). The exogenous melatonin reduces the absorption and toxicity of cadmium in apple rootstocks by enhancing soluble phenolics in the roots, which has a higher concentration of glutathione in both root and leaves (He et al., 2020). Furthermore, Jahan et al. (2020) reported that ME efficiently enhanced the phenolic content in the leaves of tomato seedlings under nickel toxicity. Plants' protein content is also controlled by photosynthetic processes when they are exposed to stressful conditions (Simkin et al. 2019). Additionally,

we observed a positive connection between net photosynthetic rate and photosynthetic pigments and protein in pepper plants pretreated with ME and subjected to Cd toxicity. Similar results were also reported in rice under vanadium toxicity (Altaf et al., 2022a).

The plants were subjected to environmental stresses, resulting in excessive ROS production, interfering with the metabolic processes. Excessive ROS production, which is toxic to cell parts like, protein and lipids, can cause membrane damage and cell death in plants (Tiwari et al., 2020). Cd caused an increase in ROS production by disrupting the balance between ROS generation and detoxification in plants. Cd-treated pepper plants accumulated high $H_2O_2$ and MDA content in leaves and roots. These compounds accumulated excessively in pepper seedlings, causing oxidative damage, which was line with the findings in pepper (Altaf et al., 2021b) and wheat (Al-Huqail et al., 2020). However, ME application in Cd-stressed plants considerably reduced $H_2O_2$ and MDA content in leaves and roots. Similarly, ME helped to decrease the $H_2O_2$ and MDA content have been identified in watermelon under vanadium toxicity (Nawaz et al., 2018), cucumber under low and high iron stress (Ahammed et al., 2020), and tomato under nickel toxicity (Jahan et al., 2020). A previous report on mallow (*Malva parviflora*) also showed that exogenous melatonin ameliorates cadmium-induced phytotoxicity and promotes plant development. Cd was applied to mallow plants that had been pretreated with 15, 50, and 100 M of melatonin. Melatonin, particularly at concentrations of 15 and 50 M, had positive effects on Cd tolerance, including a considerable increase in growth, photosynthetic pigments, and soluble protein content. Additionally, reduced melatonin concentrations decreased Cd translocation to the shoots. Melatonin significantly boosted the activities of catalase (CAT), superoxide dismutase (SOD), and guaiacol peroxidase (GPX), as well as the formation of phenols (Tousi et al., 2020). The antioxidant system in plants is critical in the reduction of ROS and the induction of metal tolerance (Imtiaz et al., 2015).

ME is well known powerful antioxidant (Arnao and Hernández-Ruiz et al., 2019). ME has been reported to enhance enzyme activity in a wide range of plant species when they are exposed to metals (Park et al., 2021; Debnath et al., 2021; Tiwari et al., 2020; Zhao et al., 2021). Yadu et al. (2018) observed that ME remarkably promoted activity of antioxidant enzymes and decreased ROS production. Under nickel toxicity, exogenous ME supplementation further enhanced antioxidant enzymes in the leaves and roots of tomato plants (Jahan et al., 2020). Furthermore, Ahammed et al. (2020) and Nawaz et al. (2018) also reported that ME application boosted antioxidant enzymes system and reduced overproduction of ROS in cucumber and watermelon seedlings. The present results noticed that in pepper plants under Cd toxicity, ROS overproduction was controlled and activity of antioxidant enzymes was increased by ME (Fig. 7). In addition, ME supplementation markedly improved activity of antioxidant enzymes under boron toxicity (Sarafi et al., 2017), under aluminum toxicity (Tang et al., 2016), and under arsenic toxicity (Siddiqui et al. (2020) in pepper, radish and *Vicia faba*, respectively. Many studies have demonstrated the importance of ME in the plant defense system, and exogenous ME can ameliorate any stress-induced oxidative stress. Exogenous ME is useful in agriculture, for impeding the decline of stress-induced crop damage. ME is a low-cost, stable, environmentally friendly, and easily accessible molecule that can protect plants from environmental hazards by reducing contaminant availability, particularly heavy metals.

# Conclusion

The goal of this study was to demonstrate the devastating effects of Cd toxicity on pepper plants and the protective role of ME in promoting plant growth. Cd application noticed severe declines in growth, photosynthesis, and excessive ROS accumulation. However, ME supplementation effectively circumvented the Cd-induced phytotoxicity via increased growth traits, improved pigments molecules, enhanced leaf gas exchange elements and promoted phenolics content. Melatonin-induced metabolic alterations in response to Cd stress are likely responsible for the improvement in growth and chlorophyll content. Furthermore, ME application impaired ROS production and enhanced the antioxidant profile of both pepper genotypes (Super Shimla and Ganga). Under Cd stress, however, exogenous melatonin had complicated and significant impacts on plant development, ROS scavenging, and antioxidant capacity. The exogenous melatonin treatments may minimize the intake risk of Cd-contaminated plants. In order to enhance the performance of plants under Cd stress and produce nutritious food, it appears vital to investigate the effect of melatonin on lower Cd root-to-shoot translocation in greater depth. To summarise, ME application efficiently alleviated Cd-induced phytotoxicity in pepper genotypes, resulting in increased Cd stress tolerance. According to the findings of this study, the protective effects of ME against other environmental contaminants such as chromium, nickel, vanadium, and copper must be investigated in various plant species.

# Declarations

**Ethics approval and consent to participate:**

Not applicable

**Consent for publication:**

Not applicable

**Availability of data and materials:**

The datasets are available on reasonable request.

**Competing interests:**

No competing interests have been declared by the authors.

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# References


1. Ahammed GJ, Wu M, Wang Y, Yan Y, Mao Q, Ren J, Ma R, Liu A, Chen S (2020) Melatonin alleviates iron stress by improving iron homeostasis, antioxidant defense and secondary metabolism in cucumber. Sci Hortic 265:109205
2. Al-Huqail AA, Khan MN, Ali HM, Siddiqui MH, Al-Huqail AA, Al Zuaibr FM, Al-Muwayhi MA, Marraiki N, Al-Humaid LA (2020) Exogenous melatonin mitigates boron toxicity in wheat. Ecotoxic Environ Safe 201:110822
3. Altaf MA, Shahid R, Altaf MA, Ren MX, Tan K, Xiang WQ, Qadir A, Shakoor A, Altaf MM (2019) Effect of NPK, organic manure and their combination on growth, yield and nutrient uptake of chilli (*Capsicum annum* L.). Horticult Int J 3:217–222
4. Altaf MA, Shahid R, Ren MX, Naz S, Altaf MM, Khan LU, Lal MK, Tiwari RK, Shakoor A (2022b) Melatonin Mitigates Cadmium Toxicity by Promoting Root Architecture and Mineral Homeostasis of Tomato Genotypes.J Soil Sci Plant Nutr1–17
5. Altaf MA, Shahid R, Ren MX, Naz S, Altaf MM, Khan LU, Tiwari RK, Lal MK, Shahid MA, Kumar R, Nawaz MA (2022c) Melatonin improves drought stress tolerance of tomato by modulating plant growth, root architecture, photosynthesis, and antioxidant defense system. Antioxidants 11:309
6. Altaf MA, Shu H, Hao Y, Zhou Y, Mumtaz MA, Wang Z (2021b) Vanadium toxicity induced changes in growth, antioxidant profiling, and vanadium uptake in pepper (*Capsicum annum* L.) seedlings. Horticulturae 8:28
7. Altaf MM, Diao XP, Shakoor A, Imtiaz M, Altaf MA, Khan LU (2021a) Delineating Vanadium (V) Ecological Distribution, Its Toxicant Potential, and Effective Remediation Strategies from Contaminated Soils.J Soil Sci Plant Nutr1–19
8. Altaf MM, Diao XP, Wang H, Khan LU, Shakoor A, Altaf MA, Farooq TH (2022a) Salicylic Acid Induces Vanadium Stress Tolerance in Rice by Regulating the AsA-GSH Cycle and Glyoxalase System.J Soil Sci Plant Nutr1–17
9. Arnao MB, Hernández-Ruiz J (2019) Melatonin: a new plant hormone and/or a plant master regulator. Trends Plant Sci 24:38–48
10. Cao YY, Qi CD, Li S, Wang Z, Wang X, Wang J, Ren S, Li X, Zhang N, Guo YD (2019) Melatonin alleviates copper toxicity via improving copper sequestration and ROS scavenging in cucumber. Plant Cell Physiol 60:562–574

11. Carvalho MEA, Piotto FA, Franco MR, Borges KLR, Gaziola SA, Castro PRC, Azevedo RA (2018) Cadmium toxicity degree on tomato development is associated with disbalances in B and Mn status at early stages of plant exposure. Ecotoxicol 27:1293–1302
12. Chen YE, Mao JJ, Sun LQ, Huang B, Ding CB, Gu Y, Yuan M (2018) Exogenous melatonin enhances salt stress tolerance in maize seedlings by improving antioxidant and photosynthetic capacity. Physiol Plant 164:349–363
13. DalCorso G, Farinati S, Maistri S, Furini A (2008) How plants cope with cadmium: staking all on metabolism and gene expression. J Integr Plant Biol 50:1268–1280
14. Debnath B, Sikdar A, Islam S, Hasan K, Li M, Qiu D (2021) Physiological and molecular responses to acid rain stress in plants and the impact of melatonin, glutathione and silicon in the amendment of plant acid rain stress. Molecules 26:862
15. Devi R, Behera B, Raza MB, Mangal V, Altaf MA, Kumar R, Kumar A, Tiwari RK, Lal MK, Singh B (2021) An Insight into Microbes Mediated Heavy Metal Detoxification in Plants: a Review.J Soil Sci Plant Nutr1–23
16. Foy CD, Chaney RT, White MC (1978) The physiology of metal toxicity in plants. Annual Rev Plant Physiol 29:511–566
17. Gallego SM, Pena LB, Barcia RA (2012) Unravelling cadmium toxicity and tolerance in plants: insight into regulatory mechanisms. Environ Exp Bot 83:33–46
18. Gu Q, Chen Z, Yu X, Cui W, Pan J, Zhao G, Xu S, Wang R, Shen W (2017) Melatonin confers plant tolerance against cadmium stress via the decrease of cadmium accumulation and reestablishment of microRNA-mediated redox homeostasis. Plant Sci 261:28–37
19. Hasan M, Ahammed GJ, Yin L, Shi K, Xia X, Zhou Y, Yu J, Zhou J (2015) Melatonin mitigates cadmium phytotoxicity through modulation of phytochelatins biosynthesis, vacuolar sequestration, and antioxidant potential in *Solanum lycopersicum* L. Front Plant Sci 6:601
20. Hasan M, Liu CX, Pan YT, Ahammed GJ, Qi ZY, Zhou J (2018) Melatonin alleviates low-sulfur stress by promoting sulfur homeostasis in tomato plants. Sci Rep 8:1–12
21. He J, Zhuang X, Zhou J, Sun L, Wan H, Li H, Lyu D (2020) Exogenous melatonin alleviates cadmium uptake and toxicity in apple rootstocks. Tree Physiol 40:746–761
22. Howard LR, Talcott SY, Brenes CH, Villalon B (2000) Changes in phytochemical and antioxidant activity of selected pepper cultivars (*Capsicum* sp.) as influenced by maturity. J Agric Food Chem 48:1713–1720
23. Imtiaz M, Rizwan MS, Xiong S, Li H, Ashraf M, Shahzad SM, Shahzad M, Rizwan M, Tu S (2015) Vanadium, recent advancements and research prospects: a review. Environ Int 80:79–88
24. Jahan MS, Guo S, Baloch AR, Sun J, Shu S, Wang Y, Ahammed GJ, Kabir K, Roy R (2020) Melatonin alleviates nickel phytotoxicity by improving photosynthesis, secondary metabolism and oxidative stress tolerance in tomato seedlings. Ecotoxicol Environ Saf 197:110593
25. Jahan MS, Shu S, Wang Y, Hasan MM, El-Yazied AA, Alabdallah NM, Hajjar D, Altaf MA, Sun J, Guo S (2021) Melatonin pretreatment confers heat tolerance and repression of heat-induced senescence in

tomato through the modulation of ABA- and GA-mediated pathways. Front Plant Sci 12:650955

26. Kaya C, Ashraf M, Alyemeni MN, Ahmad P (2020) The role of nitrate reductase in brassinosteroid-induced endogenous nitric oxide generation to improve cadmium stress tolerance of pepper plants by upregulating the ascorbate-glutathione cycle. Ecotoxicol Environs Safe 196:110483
27. Kaya C, Okant M, Ugurlar F, Alyemeni MN, Ashraf M, Ahmad P (2019) Melatonin-mediated nitric oxide improves tolerance to cadmium toxicity by reducing oxidative stress in wheat plants. Chemosphere 225:627–638
28. Korkmaz A, Değer Ö, Szafrańska K, Köklü Ş, Karaca A, Yakupoğlu G, Kocacinar F (2021) Melatonin effects in enhancing chilling stress tolerance of pepper. Sci Hortic 289:110434
29. Li C, Liang B, Chang C, Wei Z, Zhou S, Ma F (2016) Exogenous melatonin improved potassium content in *Malus* under diferent stress conditions. J Pineal Res 61:218–229
30. Li L, Yan X (2021) Insights into the roles of melatonin in alleviating heavy metal toxicity in crop plants. Phyton 90:1559
31. Liang C, Ma C, Zhang Z, Wei Z, Gao T, Zhao Q, Ma F, Li C (2018) Long-term exogenous application of melatonin improves nutrient uptake fluxes in apple plants under moderate drought stress. Environ Exp Bot 155:650e661
32. Lichtenhaler K, Wellburn AR (1983) Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. Biochem Soc Trans 11:591–592
33. Lin L, Zhou W, Dai H (2012) Selenium reduces cadmium uptake and mitigates cadmium toxicity in rice.J Hazard Mater235343–3
34. Lin YF, Aarts MGM (2012) The molecular mechanism of zinc and cadmium stress response in plants. Cell Mol Life Sci 69:3187–3206
35. Manafi H, Baninasab B, Gholami M, Talebi M (2021) Effect of Melatonin and Sodium Nitroprusside Foliar Application on Improving High-Temperature Stress Tolerance of'Kurdistan'Strawberry. Iran J Hortic Sci Technol 22:383–398
36. Nabaei M, Amooaghaie R (2019) Nitric oxide is involved in the regulation of melatonin-induced antioxidant responses in *Catharanthus* roseus roots under cadmium stress. Botany 97:681–690
37. Nawaz MA, Huang Y, Bie Z, Ahmed W, Reiter RJ, Niu M, Hameed S (2016) Melatonin: current status and future perspectives in plant science. Front Plant Sci 6:1230
38. Nawaz MA, Jiao Y, Chen C, Shireen F, Zheng Z, Imtiaz M, Bie Z, Huang Y (2018) Melatonin pretreatment improves vanadium stress tolerance of watermelon seedlings by reducing vanadium concentration in the leaves and regulating melatonin biosynthesis and antioxidant-related gene expression. J Plant Physiol 220:115–127
39. Park HS, Kazerooni EA, Kang SM, Al-Sadi AM, Lee IJ (2021) Melatonin enhances the tolerance and recovery mechanisms in *Brassica juncea* (L.) Czern. under saline conditions.Front Plant Sci12
40. Piqueras A, Olmos E, Martinez-Solano JR, Hellin E (1999) Cd-induced oxidative burst in tobacco BY2 cells: time course, subcellular location and antioxidant response. Free Radical Res 31:33–38

41. Sarafı E, Tsouvaltzis P, Chatzissavvidis C, Siomos A, Therios I (2017) Melatonin and resveratrol reverse the toxic effect of high boron (B) and modulate biochemical parameters in pepper plants (*Capsicum annuum* L.). Plant Physiol Biochem 112:173–182
42. Sharma SS, Dietz KJ (2009) The relationship between metal toxicity and cellular redox imbalance. Trends Plant Sci 14:43–50
43. Siddiqui MH, Alamri S, Alsubaie QD, Ali HM, Ibrahim AA, Alsadon A (2019) Potential roles of melatonin and sulfur in alleviation of lanthanum toxicity in tomato seedlings. Ecotoxicol Environ Safe 180:656–667
44. Siddiqui MH, Alamri S, Khan MN, Corpas FJ, Al-Amri AA, Alsubaie QD, Ali HM, Kalaji H, Ahmad P (2020) Melatonin and calcium function synergistically to promote the resilience through ROS metabolism under arsenic-induced stress. J Hazard Mater 398:122882
45. Sigel A, Sigel H, Sigel RK (eds) (2013) Cadmium: from toxicity to essentiality, vol 11. Springer, New York
46. Simkin AJ, López-Calcagno PE, Raines CA (2019) Feeding the world: improving photosynthetic efciency for sustainable crop production. J Exp Bot 70:1119–1140
47. Tang Y, Lei F, Zhang E, Li H (2016) July. Soaking Seeds with Exogenous Melatonin Improve Photosynthetic Characteristics of Radish under Aluminum Stress. In 2016 2nd International Conference on Advances in Energy, Environment and Chemical Engineering (AEECE 2016) (pp. 64–67). Atlantis Press
48. Tang Y, Li J, Li H (2015) December. Effects of exogenous melatonin on photosynthetic characteristics of eggplant (Solanum melongena L.) under cadmium stress. In International Conference on Manufacturing Science and Engineering (ICMSE 2015)
49. Tiwari RK, Lal MK, Kumar R, Chourasia KN, Naga KC, Kumar D, Das SK, Zinta G (2021) Mechanistic insights on melatonin-mediated drought stress mitigation in plants. Physiol Plant 172:1212–1226
50. Tiwari RK, Lal MK, Naga KC, Kumar R, Chourasia KN, Subhash S, Kumar D, Sharma S (2020) Emerging roles of melatonin in mitigating abiotic and biotic stresses of horticultural crops. Sci Hortic 272:109592
51. Tousi S, Zoufan P, Ghahfarrokhie AR (2020) Alleviation of cadmium-induced phytotoxicity and growth improvement by exogenous melatonin pretreatment in mallow (*Malva parviflora*) plants. Ecotoxicol Environ Safe 206:111403
52. Wu S, Wang Y, Zhang J, Gong X, Zhang Z, Sun J, Chen X, Wang Y (2021) Exogenous melatonin improves physiological characteristics and promotes growth of strawberry seedlings under cadmium stress. Hortic Plant J 7:13–22
53. Xiang G, Lin L, Liao MA, Tang Y, Liang D, Xia H, Wang J, Wang X, Sun G, Zhang H, Zou Y (2019) Effects of melatonin on cadmium accumulation in the accumulator plant Perilla frutescens. Chem Ecol 35:553–562
54. Yadu B, Chandrakar V, Meena RK, Poddar A, Keshavkant S (2018) Spermidine and melatonin attenuate fuoride toxicity by regulating gene expression of antioxidants in *Cajanus cajan* L. J Plant

Growth Regul 37:1113–1126

55. Zhang R, Sun Y, Liu Z, Jin W, Sun Y (2017) Effects of melatonin on seedling growth, mineral nutrition, and nitrogen metabolism in cucumber under nitrate stress. J Pineal Res 62:12403
56. Zhao YQ, Zhang ZW, Chen YE, Ding CB, Yuan S, Reiter RJ (2021) Melatonin: a potential agent in delaying leaf senescence. Crit Rev Plant Sci 40:1–22

# Figures

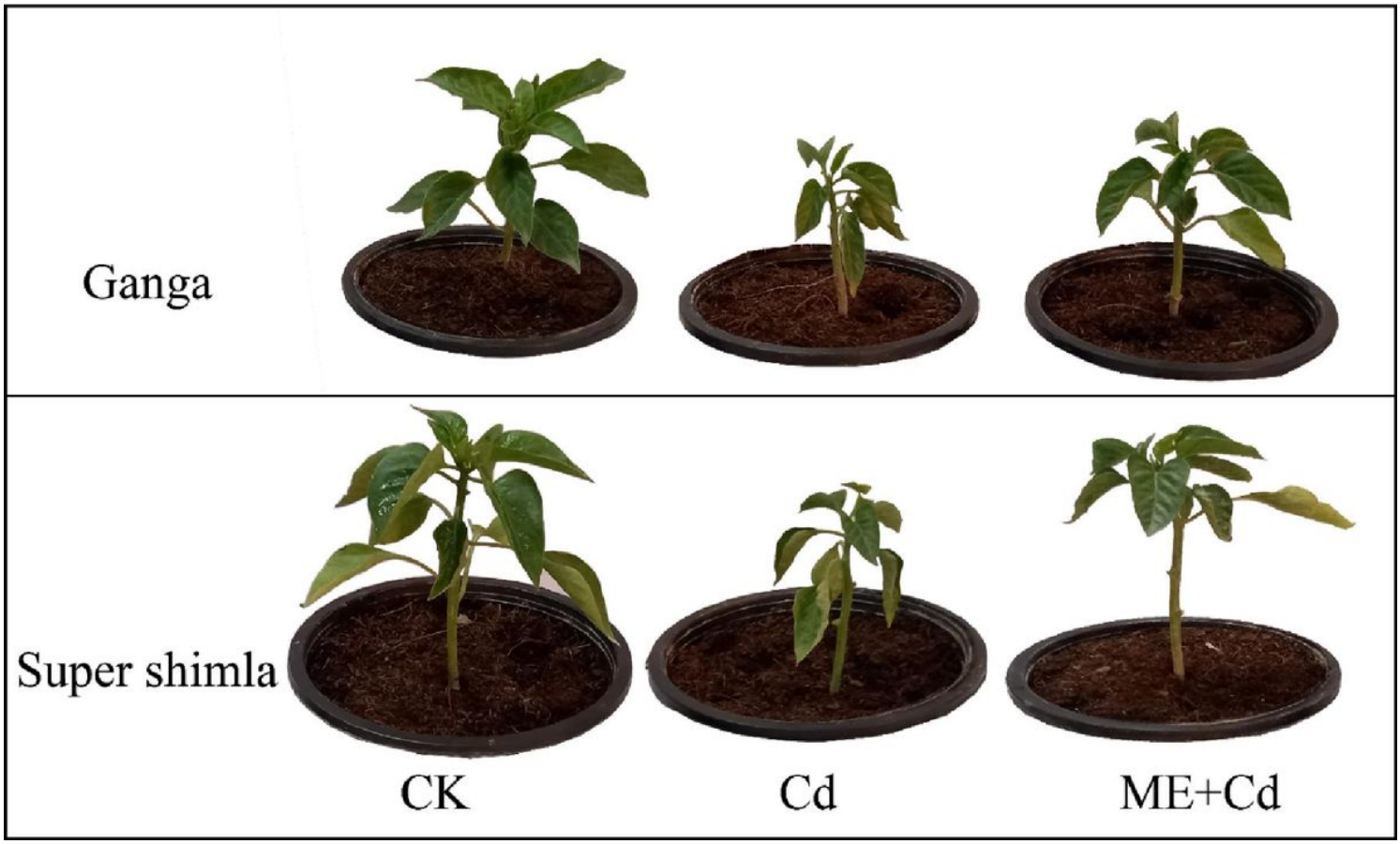



**Figure 1**

The effect of exogenous melatonin application on pepper genotypes subjected to cadmium toxicity

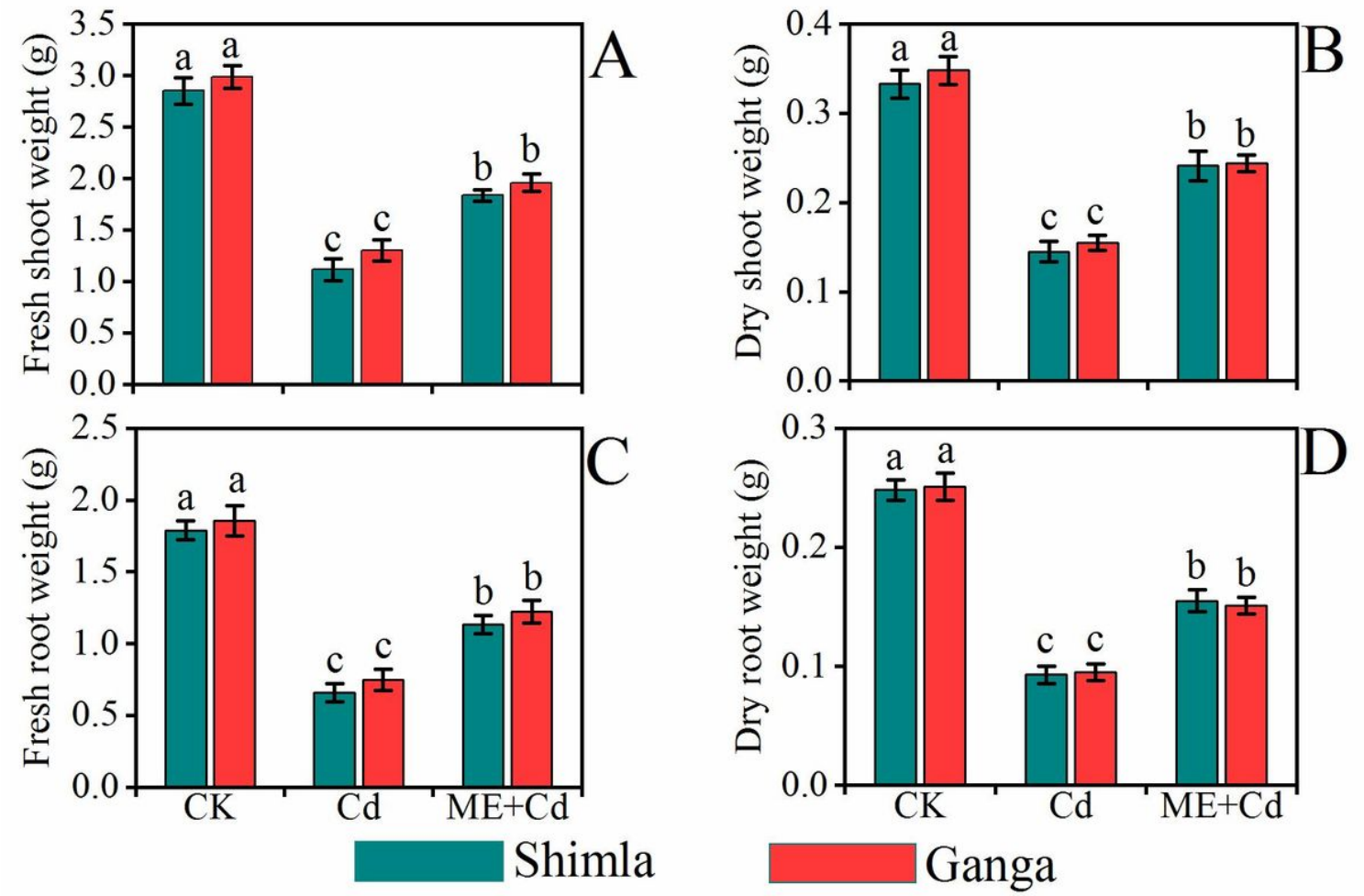



**Figure 2**

Effect of cadmium, alone or in combination with melatonin, in two pepper genotypes on growth characteristics. Results are means ± standard error for n=3. Moreover, lowercase letters exhibit significant differences at $P < 0.05$ (DMRT).

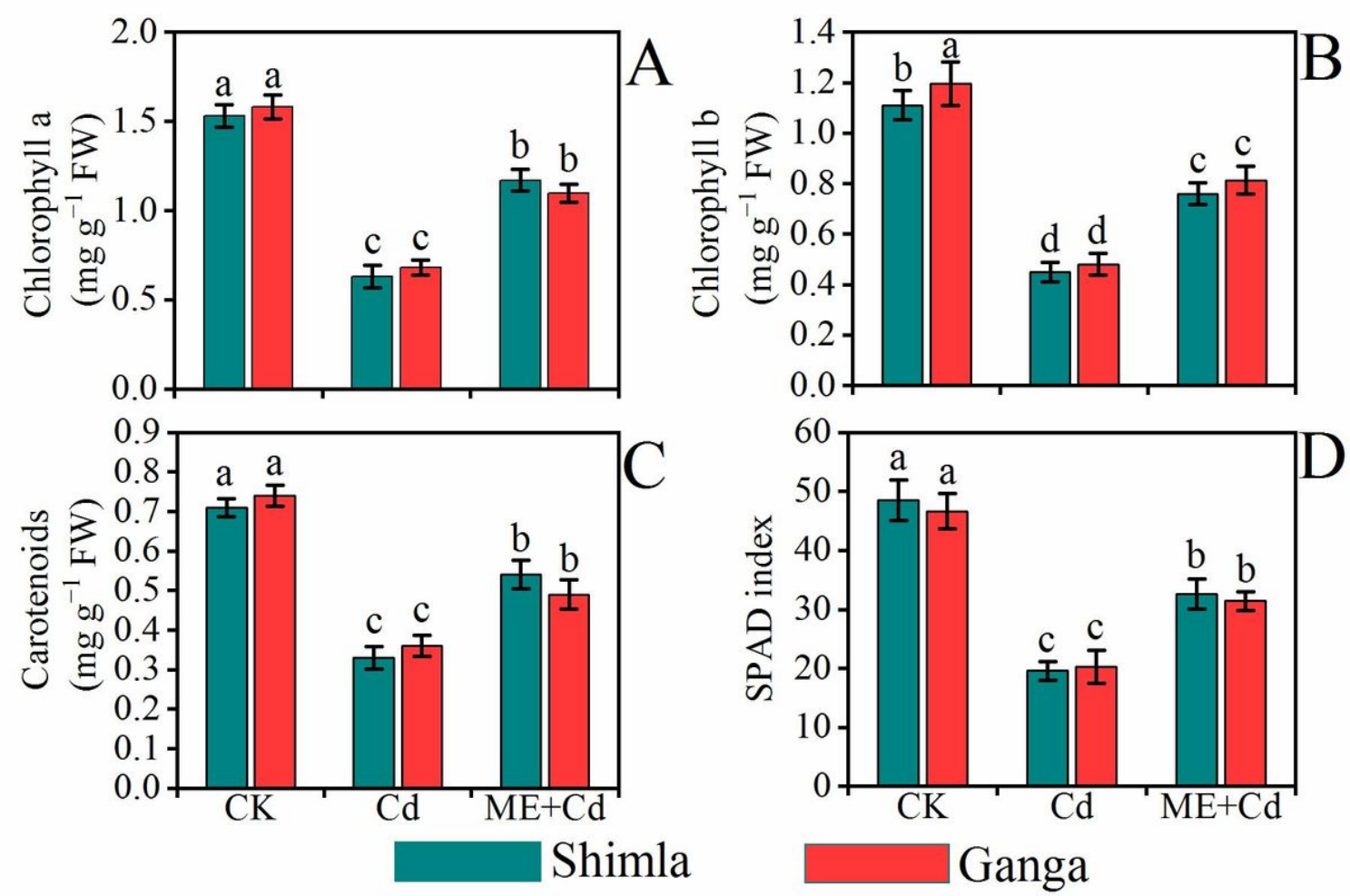



**Figure 3**

Effect of cadmium, alone or in combination with melatonin, in two pepper genotypes on Pigments content and SPAD index. Results are means ± standard error for n=3. Moreover, lowercase letters exhibit significant differences at $P < 0.05$ (DMRT).

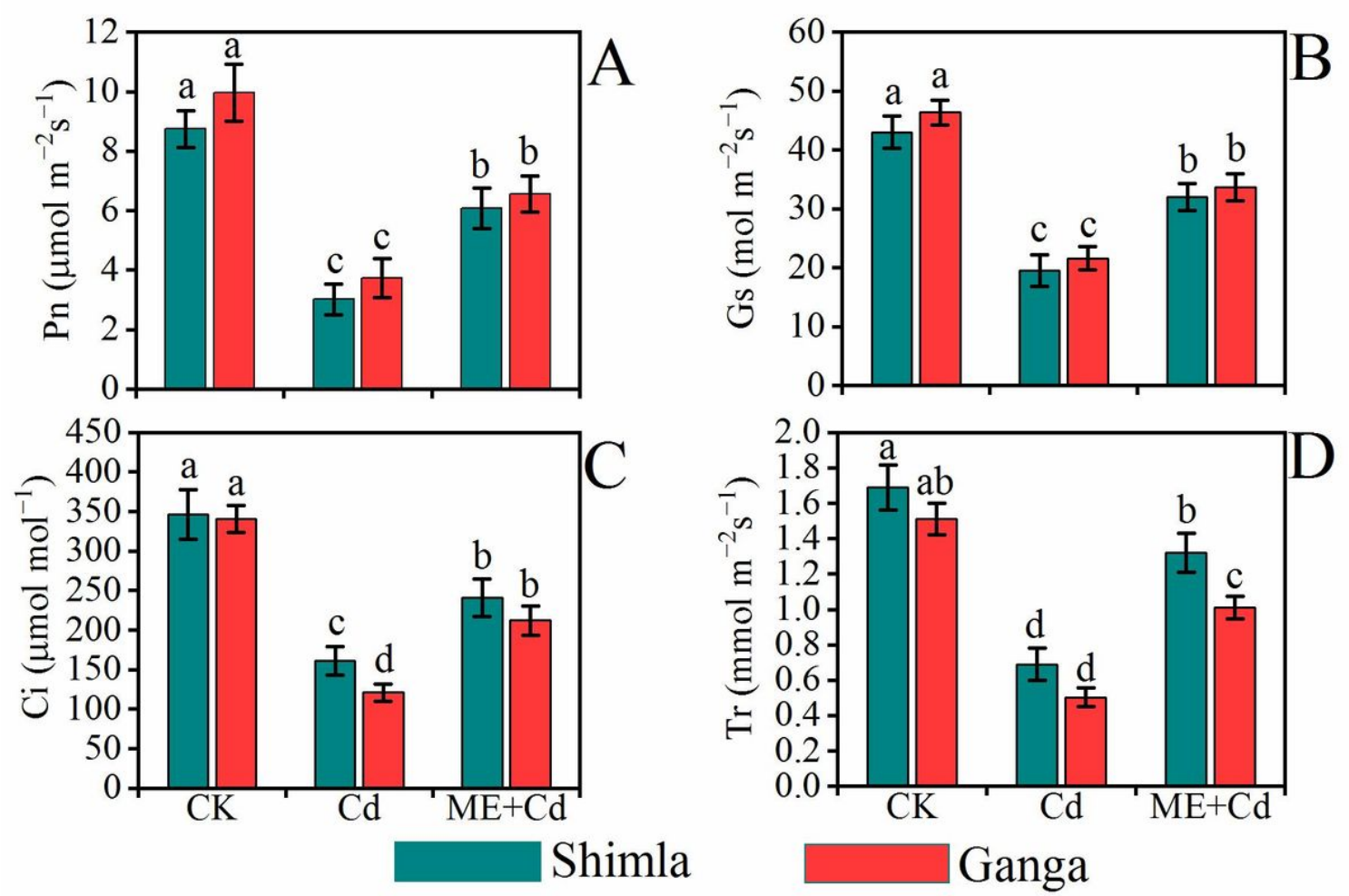



**Figure 4**

Effect of cadmium, alone or in combination with melatonin, in two pepper genotypes on leaf gas exchange elements. Results are means ± standard error for n=3. Moreover, lowercase letters exhibit significant differences at $P < 0.05$ (DMRT).

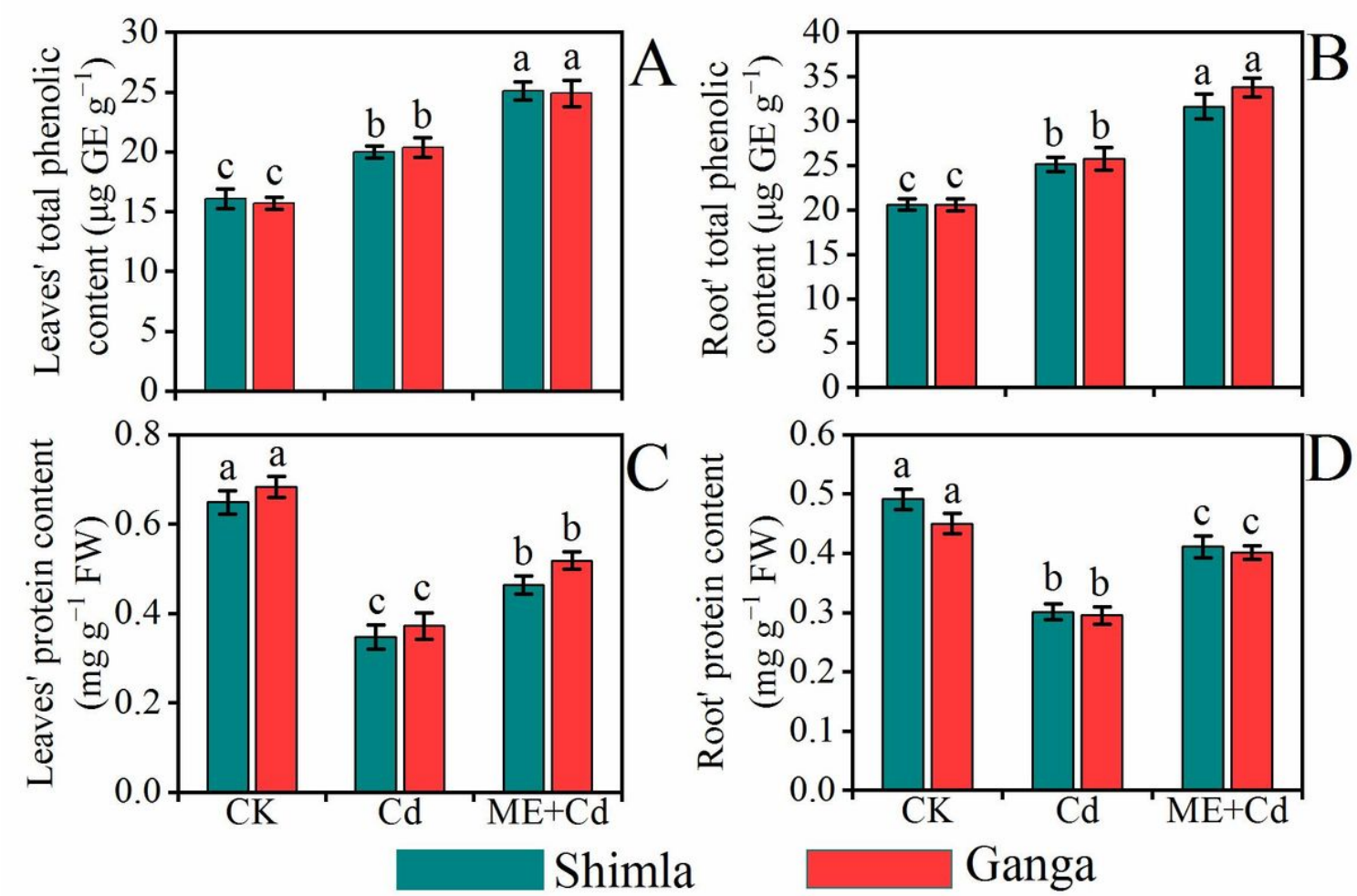



**Figure 5**

Effect of cadmium, alone or in combination with melatonin, in two pepper genotypes on total phenols and protein content. Results are means ± standard error for n=3. Moreover, lowercase letters exhibit significant differences at $P < 0.05$ (DMRT).

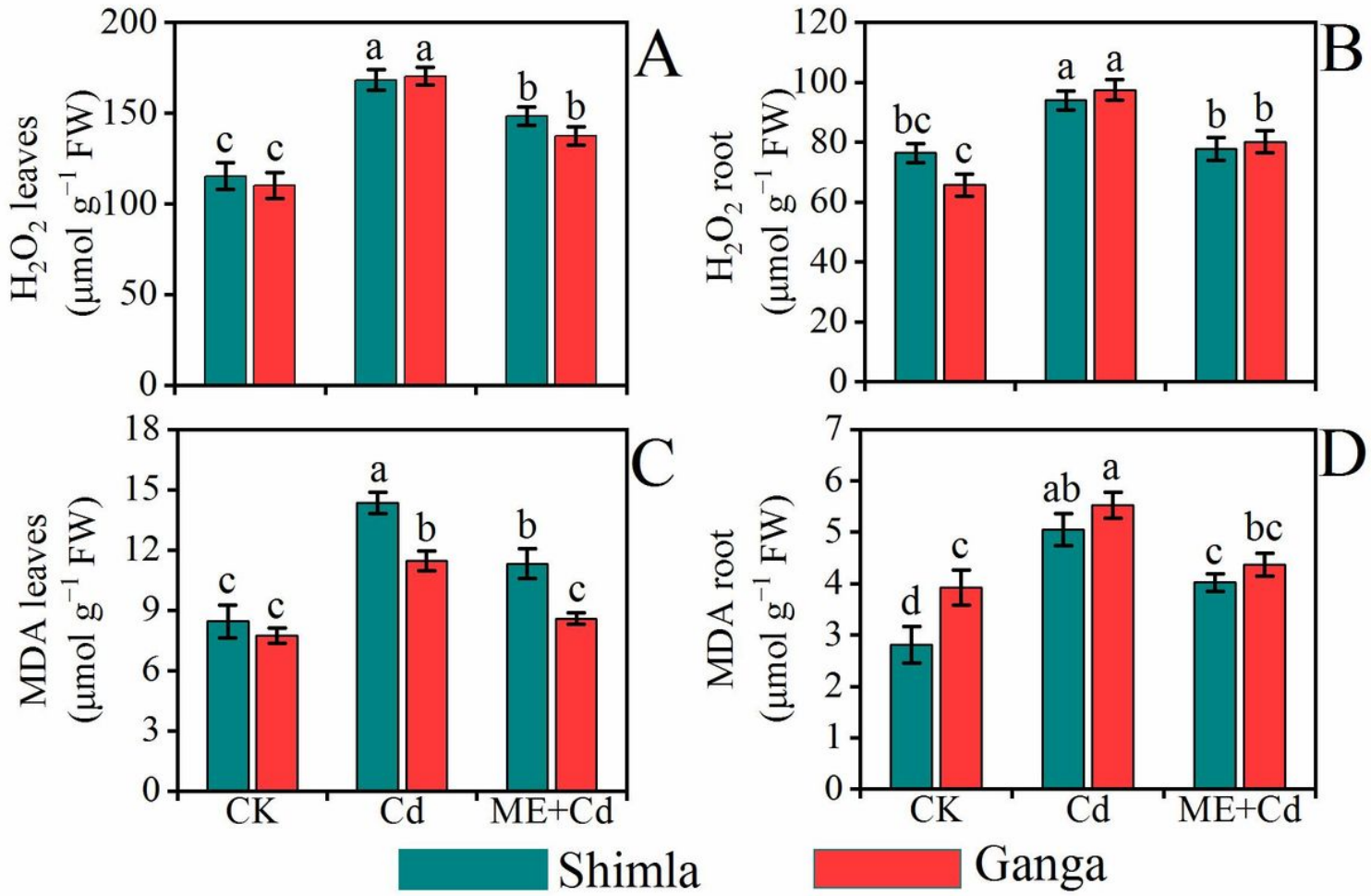
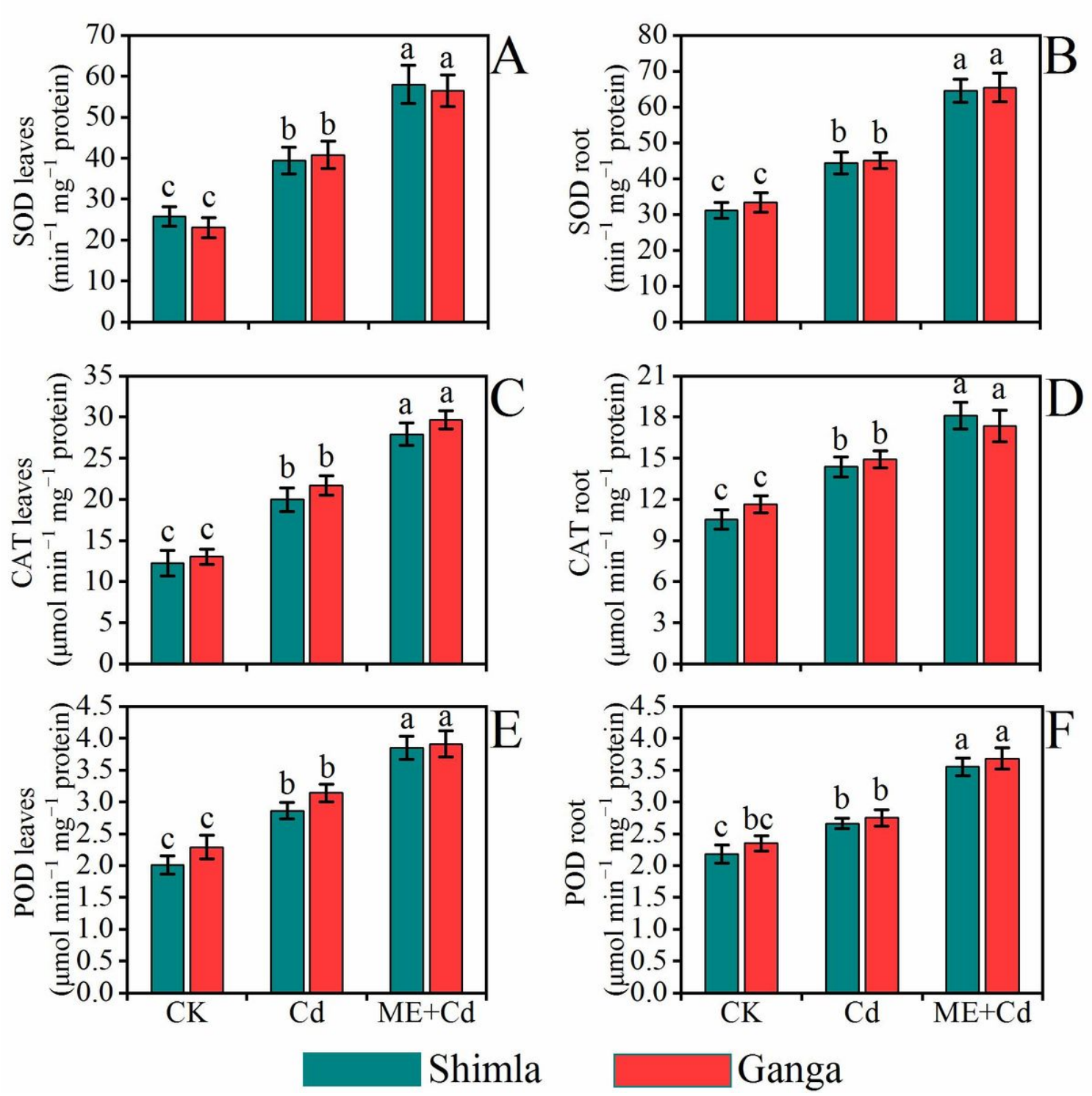



**Figure 6**

Effect of cadmium, alone or in combination with melatonin, in two pepper genotypes on $H_2O_2$ and MDA content. Results are means ± standard error for n=3. Moreover, lowercase letters exhibit significant differences at $P < 0.05$ (DMRT).




## Figure 7

Effect of cadmium, alone or in combination with melatonin, in two pepper genotypes on SOD, CAT, and POD content. Results are means ± standard error for n=3. Moreover, lowercase letters exhibit significant differences at $P < 0.05$ (DMRT).