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Alleviation of cadmium stress and improved growth performance of periwinkle (*Catharanthus roseus* L.) by foliar application of zinc oxide nanoparticles

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A B S T R A C T

Cadmium (Cd) is a nonessential and very toxic element that confines the growth of ornamental plants worldwide. Nanoparticles (NPs) have been used as a novel approach to act as nano-fertilizers and directly alleviate the Cd stress. However, its defensive mechanisms are not well understood in ornamental/medicinal plants, especially *Catharanthus roseus* (periwinkle). The objectives of the current experiment are intended to examine the impacts of zinc oxide nanoparticles (ZnO—NPs; 25 mg l^{-1}) on the reduction of oxidative stress by enhancing antioxidant activities, physiological improvements, and elemental status in periwinkle plant parts under Cd stress (0.5 mM). The periwinkle plants were transplanted and exposed to Cd stress one week later by soil drenching, whereas, ZnO—NPs were applied after 14 days by foliar supplementation. The Cd toxicity significantly reduced the morphological traits, negatively affected the pigments and photosynthetic apparatus, abridged antioxidant enzyme activities, and Cd accrual in periwinkle plants. However, exogenous ZnO—NPs supplementation produced elevated plant height, flower numbers, root length, plant fresh and dry biomass, chlorophyll, and carotenoid contents in Cd-stressed plants. Likewise, the ZnO—NPs have also regulated the gaseous exchange rates, antioxidant enzymes (SOD, CAT, APX), soluble protein, and free proline contents in Cd-polluted periwinkles. The application of ZnO—NPs also considerably reduced the hydrogen peroxide (−25 %) and malondialdehyde (−47 %), activated by Cd stress. Furthermore, the Zn and Cd contents were also elevated and reduced, respectively, in ZnO—NPs supplied Cd-stressed plants. The present experiment recommends that the exogenous supplementations of ZnO—NPs are a feasible and sustainable strategy for enhancing the growth attributes and reducing the Cd levels in periwinkle plants in metal-hoarded soil conditions.

1. Introduction

Heavy metals (HMs) contamination in soil has emerged as one of the global environmental concerns of the greatest importance. Amongst different HMs, cadmium (Cd), chromium (Cr), arsenic (As), lead (Pb), and mercury (Hg) are the most injurious and toxic to plant life at all pollution levels (Angon et al., 2024). The over-standard soil contamination rate is 16.1 %, whereas the over-standard rates of Cd are 7 %, As 2.7 %, Cr 1.1 %, Hg 1.6 %, and Pb 1.5 % (Zhao et al. 2022). Cd can be very effortlessly captivated by the plant roots due to its fluidity, water solubility, and toxicity, and can modify the phenotypic properties of the plants and inhibit growth and root development (Zhao et al. 2021; Ahmad et al. 2022). Cd can impede photosynthesis and the movement of water and gas exchange in the plants, which leads to a weakening in plant metabolism (Naveed et al. 2020; Imran et al. 2021). Furthermore, Cd can decline the plant biomass, disturb the synthesis of photosynthetic pigments, and affect the

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antioxidative defense system by elevating the reactive oxygen species (ROS) production in affected plants (Sun et al. 2023b). Cd is absorbed by the roots of the plants via symplastic and apoplastic pathways and then translocated to shoots through long-distance transport, redistribution of phloem, and xylem loading (Luo and Zhang, 2021). In plant shoots, Cd is sequestered, and detoxification occurs in plant vacuoles and cell walls (Luo and Zhang, 2021). Various experiments have found the lethal impacts on plants by Cd at different concentrations (Chen et al. 2020; Haider et al. 2022; Sattar et al. 2023). Therefore, it is essential to reduce the accumulation of Cd concentrations in plants.

The use of sustainable and environmentally safe additives for the restoration of soils and water sources contaminated with metals has gained a lot of interest in recent years (Ma et al. 2020; Ajmal et al. 2022; Sun et al. 2023b). Nanotechnology is becoming more and more common among these methods for cleaning up contaminated water and soil (Rizwan et al. 2019; Mahamood et al., 2023). Applications of nanotechnology are highly valuable for the sustainable development of agriculture (Lawry et al. 2019). Amongst these, zinc oxide nanoparticles (ZnO—NPs), have been utilized as an effective nanofertilizer because of the global zinc shortage in agricultural soils (Li et al., 2021b). Because of their chemical, optical, chemical, and biomedical characteristics, they can also be applicable in medicine, electronics, photocatalyst, and solar cells (Wang et al. 2018). It has been found that crop plants can receive Zn from ZnO—NPs in minuscule amounts (Faizan et al. 2021b). Various studies reported that ZnO—NPs augmented growth and reduced oxidative damage due to abiotic stresses in chickpeas (Mahajan et al. 2011), cotton (Venkatachalam et al. 2017), wheat (Hussain et al. 2018), rice (Faizan et al. 2021b), and tomato (Sun et al. 2023b). Verma et al. (2023) reported that ZnO—NPs can decrease oxidative stress by enhancing the integrity of the membrane, stimulating cell division, scavenging ROS produced by stress, moderating concentrations of carbohydrates, protein metabolism, osmoregulation, amino acids, and photosynthetic pigments. The ZnO—NPs possess properties such as smaller size, easy diffusion, and dissolution, letting plants speedily absorb the Zn than traditional ZnO fertilizers, as a result, the plant gets nutritive requirements and encourages growth and development (Geremew et al. 2023). Nazneen and Sultana (2024) stated that ZnO—NPs have diverse physical and chemical behaviors depending on several materials or the pathways utilized for the synthesis. Various studies also showed the harmful impacts of ZnO—NPs due to the dissolution of Zn^{2+} ions, which could disrupt the organization of chloroplast, decrease chlorophyll concentrations and decreased the number of grana and thylakoid (Rao and Shekhawat, 2014; Mousavi et al., 2015). Rajput et al. (2021) reported that ZnO—NPs could restrict nutrient and water transport in tissues of above-ground plant parts. Recently, researchers have found that supplementation of ZnO—NPs has effectively ameliorated the Cd-stress in numerous crops by elevating their growth by serving as an excellent substitute to avert micronutrient deficiency in plants (Liu et al. 2022; Sun et al. 2023b). Hussain et al. (2024) proposed ZnO—NPs (below 50 mg l^{-1}) as an ideal candidate to alleviate Cd-toxicity for safe agricultural practices. Regarding sustainability in agriculture, utilizing ZnO NPs as a novel approach to enhance crop yields has to be pursued as a potentially effective method to guarantee ecological sustainability (Sun et al., 2023a). The ZnO—NPs contents that are unsafely discharged into the soil and plant tissues give rise to negative environmental impacts (Sheteiwy et al. 2021). Furthermore, Zn toxicity rises with its bioavailability in the plants. It depends on the pH and organic matter of the soil, microbial communities, root exudate, and plant types that encourage or limit its bioavailability (Balafrej et al. 2020). Scheid et al. (2017) found that Zn toxicity induced a reduction in the root superficial area, which influences growth and decreases the capability of nutrient and water uptake. Thus, the safety concerns associated with Zn supplementations in plants and soil should be addressed.

Catharanthus roseus (commonly called periwinkle) belongs to the Apocynaceae family and is a highly medicinal and colorful ornamental bedding and ground cover plant, native to the Indian Ocean Island of Madagascar (Kaur et al. 2021). Periwinkle plants are traditionally used in traditional medicines used for constipation, malaria, diuretics, and hypertension (Dhyani et al. 2022). All parts of *C. roseus* contain saponins, tannins, and different types of anti-cancer alkaloids like vincristine (VCR), vinblastine (VLB), and leurosin (Nejat et al. 2015). The *C. roseus* plants are commonly used as an ornamental crop due to the versatility of different colors such as red, pink, purple, and white corolla (Nejat et al. 2015; Ali et al. 2021). Pandey et al. (2007) reported that *C. roseus* is resistant to Cd stress (up to 500 μM) concerning germination but higher contents of Cd may lead to reduced germination and biomass, encouraging sterility and reducing total alkaloid level. Sun et al. (2023b) documented that foliar spray of ZnO—NPs up to 50 mg l^{-1} enhanced Cd tolerance by improved antioxidant efficiency and photosynthetic capacity, whereas, decreased Cd accrual by 41 % and enhanced Zn uptake by 79 %.

The information regarding foliar supplementation of ZnO—NPs in medicinal and ornamental plants especially, *C. roseus,* under Cd-stress and its uptake in different plant parts are not properly reported. In this experiment, we hypothesized that ZnO—NPs can ameliorate Cd-mediated stress in periwinkle by elevating its growth, improving photosynthetic apparatus, and positively regulating the antioxidative enzymatic activities as well as Zn contents whereas dropping Cd level in plants. Assessing the influence of NPs on important medicinal and ornamental plants of *C. roseus* with Cd toxicity will deliver innovative understandings of the utilization of nanoparticles in metal-hoarded growing media.

2. Materials and methods

2.1. Planting material, nanoparticle preparation, and experimental setup

An experiment was conducted during the early spring to early winter season in natural outdoor conditions at the experimental site of the Islamia University of Bahawalpur, Pakistan. The potting media utilized in this study was sandy clay loam (sand 46 %, silt 28 %, clay 26 %) while pH and electrical conductivity were 7.7 and 3.11 dSm^{-1} respectively. Macronutrients in the soil were: nitrogen, 87 g kg^{-1} of soil, phosphorus, 9.87 g kg^{-1} of soil, and potassium 167.1 g kg^{-1} of soil. The day and night average temperatures were 40 and 32 °C respectively with relative humidity of 75 %.

Catharanthus roseus cv. Titan, nurseries at three leaf stage seedings were obtained from Greenview nursery and shifted to clay pots (14 cm diameter with 30 cm depth). *C. roseus* was selected for this study due to its extensive use in landscape for aesthetic beautification and wide adaptability in harsh climatic conditions of Cholistan desert regions of Bahawalpur in the subtropical climate with extremely hot summers (above 46 °C; March to September) and mild winters (10 °C; December to February). The pots were filled with 3.75 kg of soil (each pot for a single plant). The pots were grouped according to a completely randomized design with a two-factor factorial setup. After two weeks of being transplanted into pots, the plants were treated directly into the soil with Cd stress ($CdCl_2$; 0.5 mM). The exogenous foliar spray of ZnO—NPs (25 mg l^{-1}) was given after one week of Cd stress to plants (six sprays on alternate days). The concentration of $CdCl_2$ viz. 0.5 mM and ZnO—NPs viz. 25 mg l^{-1} was selected based on our previous experiments because the influence of ZnO—NPs on different species of the plant differs noticeably. The ZnO—NPs, measuring 30 ± 10 nm in size, having a 99.9 % purity and density of 5.103, were acquired from Sigma-Aldrich (St. Louis, Missouri, USA). The zeta potential and mean hydrodynamic size of ZnO—NPs were 3.11 ± 0.72 mV and 277.13 ± 12.71 nm, respectively. An adequate volume of NPs was prepared at 25 mg l^{-1} by mixing the requirable number of NPs in double distilled water in a 100 mL flask with 10 mL volume, stirred for 2 h, and standardized by ultrasonication for 60 min at 40 kHz until the NPs were distributed evenly and making volume by addition of double distilled water as proposed by Zou et al. (2022). With appropriate grinding, the anticipated morphology of NPs was formed and characterized by X-ray powder diffraction examination of the materials by utilizing Cu, K1 radiation on a Rigaku diffractometer. Furthermore, the size of the ZnO—NPs was assessed with the help of scanning electron microscopy (SEM; JSM5910, JEOL). Tween 20 (0.05 % level), as a surfactant, in the solution, was added for the uniform coverage and retention of the solution on the leaves (Hofmann et al., 2020). In each treatment, there were seven plants which were replicated four times, for a total of 28 plants per treatment. All the pots were categorized into four groups: without any spray as control (I), foliar spray of ZnO—NPs at 25 mg l^{-1} (II), the addition of Cd at 0.5 mM (III), the addition of Cd at 0.5 mM + ZnO—NPs at 25 mg l^{-1} (IV).

2.2. Measurement of morphological attributes and plant biomass

Plant aesthetic characteristics were measured during the final days of the experiment. After being carefully pulled out, eight plants were divided into their roots and shoots and cleaned with tap water. The leaf as well as flower numbers per plant were noted whereas by using a metric scale, the overall height of the plant and its root length were measured. A computerized electric balance was used to assess the biomass of fresh plants; the fresh samples were then dried in an oven for 48 h at 70 °C to estimate the dry biomass (Ahsan et al. 2023).

2.3. Estimation of leaf chlorophyll and gas exchange attributes

Leaf Chl-a and Chl-b contents were recorded by adopting the methodology suggested by Takaichi et al. (1995). Carotenoid concentrations were measured by following Arnon (1949) methodology. A fresh leaf sample (about 0.15 g) was obtained for this purpose, and it was homogenized in acetone (80 %). A spectrophotometer (Jenway, Staffordshire, UK) was used to measure the samples' absorption at wavelengths of 645, 663, and 480 nm. An infrared gas analyzer (Li-COR 6400, Li-COR Inc., USA) was used to measure the gaseous exchange parameters, such as photosynthetic rate (*A*), transpiration rate (*E*), and stomatal conductance (*gs*). Assuming entirely functional plants at that time, these characteristics were measured between 10 and 11am in bright sunlight (Faizan et al., 2021a).

2.4. Determination of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA)

Fresh leaf material (1.0 g) was acquired and crushed in an ice-cooled mortar with 0.1 % of 5 mL (w/v) trichloroacetic acid (TCA) to assess the H_2O_2 contents. For 15 min, the mixture was centrifuged at 12,000 × *g*. After vortexing, the blend's optical density (OD) was assessed at 390 nm, and the H_2O_2 content was determined using the Velikova et al. (2000) approach. The MDA levels were ascertained by immersing a 0.5 g leaf sample in 1.0 % trichloroacetic acid (w/v), as recommended by Li et al. (2010).

2.5. Estimation of defense enzymes

The antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX), were determined using a spectrophotometer. According to Dixit et al. (2001), a fresh leaf sample (1.0 g) was standardized in 50 mM phosphate buffer (pH 7.0) and dithiothreitol (DTT) and centrifuged at 4 °C for 20 min at 12,000 rpm. The supernatant was removed, and readings for activities of antioxidant enzymes at different wavelengths were noted by using a spectrophotometer (Jenway, Staffordshire, UK). Giannopolitis and Ries (1997) recommendations were used to measure SOD activities which were observed at 560 nm, whereas Zhang et al. (2012) methodology was used to quantify POD and CAT activities and absorbance was recorded at 470 nm and 240 nm, respectively. The process recommended by Cakmak (1994) was followed in determining the APX value at an absorbance of 290 nm.

2.6. Quantification of proline and total soluble proteins

A sample (1.0 g) of freshly collected leaves was combined with 10 mL (3 %) sulfosalicylic acid to measure the free proline content of periwinkle. The incubation process lasted 15 min at 90 °C. Then, 4 mL of ninhydrin and 4 mL of glacial acetic acid were mixed and heated to 90 °C for one hour. After cooling it down, 8 mL of toluene was added. At 520 nm, the absorbance was measured (Bates et al. 1973). The total protein content from the fresh leaf sample was assessed with the help of a spectrophotometer at 595 nm absorbance by following the procedure of Bradford (1976).

2.7. Measurement of Zn and Cd levels in plants

For the measurement of Zn and Cd levels in the lower and upper ground parts (four samples of each part) of periwinkle plants, the dried sample (about 1.0 g each part) was digested with HNO_3_$HClO_4$ (3:1, *v:v*), to oxidize the organic material, with the help of hot plate (at 550 °C for 2 h) as documented by Kanee et al. (2001). After heating, the flask contents were treated with H_2O_2 (10 mL), heated gently and reflux was continued for a further 1 h When the volume of the contents was reduced to half, the watch glasses were taken from the flask tops, and the heating process was continued. The digests were filtered through filter paper (Whatman No 42) after being diluted to 100 mL with the distilled water. An atomic absorption spectrophotometer (iCE 3500 FAAS, Thermo Scientific, USA) was utilized to quantify the levels of Zn and Cd in the processed samples of the plants.

2.8. Statistical analysis

An analysis of variance (ANOVA) was done using the complete investigational data with the help of STATISTIX (computer software, version 8.1). At a 5 % probability level, treatment means were compared using the least significant difference test.

3. Results

3.1. Effect of ZnO-NPs on physical traits of periwinkle

Cadmium stress (0.5 mM) remarkably abridged the morphological attributes like height of the plant (HP), number of leaves (NL), number of flowers (NF), and root length (RL) of periwinkle by 54 %, 63 %, 89 %, and 62 %, respectively, compared with unstressed plants without any spray (control). The foliar application of ZnO—NPs showed improved performance of these physical properties viz., HP, NL, NF, and RL by 23 %, 31 %, 36 %, and 34 % in Cd-stressed plants, whereas, 19 %, 6 %, 15 %, and 25 %, respectively, with non-stressed periwinkle plants (Fig. 1a,b,c,d).

3.2. Impact of ZnO-NPs on plant biomass

Foliar supplementation of ZnO—NPs improved periwinkle fresh weight by 8 % in controlled conditioned plants and by 26 % in Cd-subjected plants (Fig. 2a). Similarly, there was a reduction in plant dry weight of Cd-stressed plants by 48 % in comparison with control treatment, but foliar supplementation of ZnO—NPs enhanced dry plant biomass by 6 % in normal-conditioned plants and by 35 % in Cd-stressed periwinkle plants (Fig. 2b).

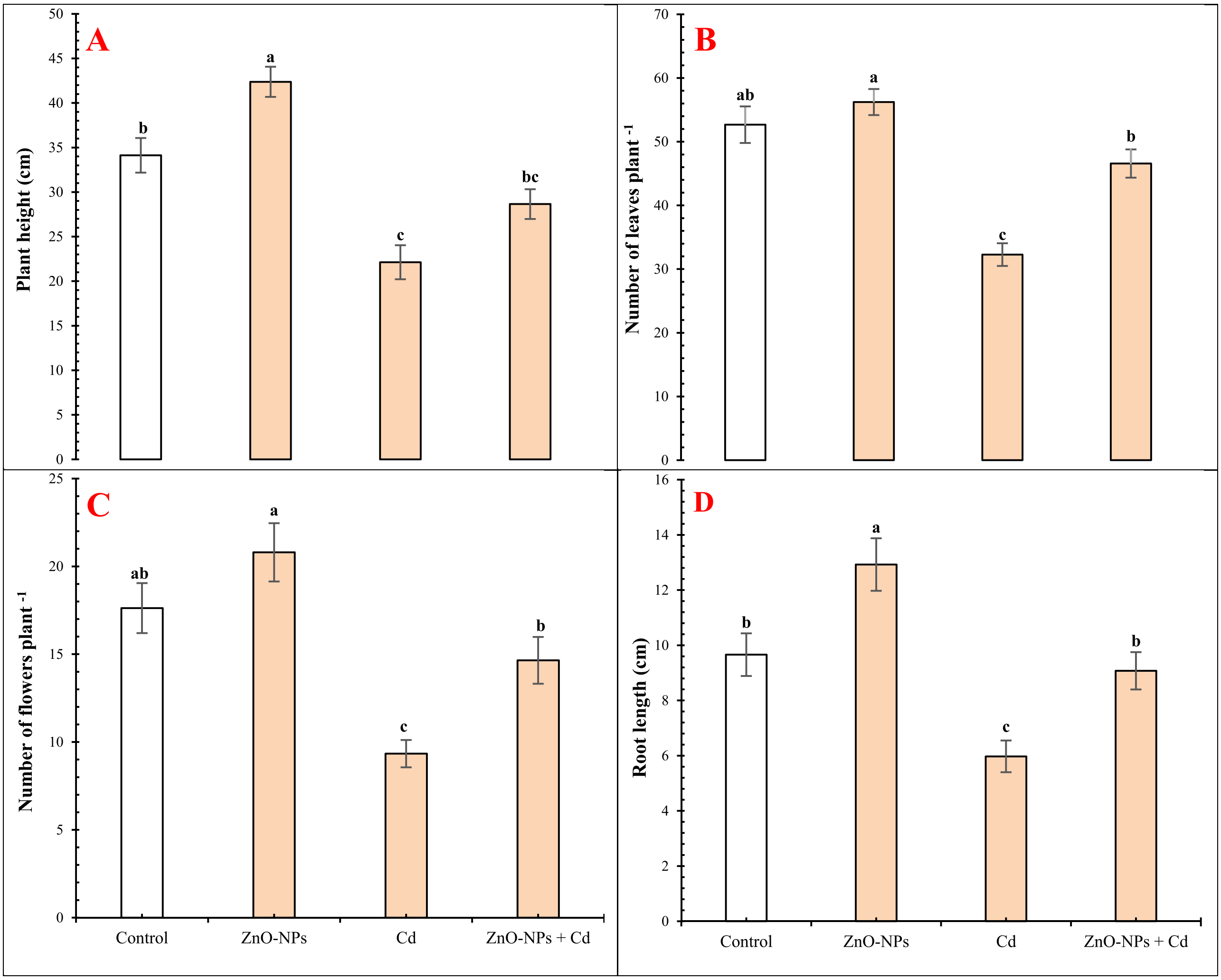

Fig. 1. Regulatory role of ZnO—NPs under Cd stress on plant height (**A**), number of leaves (**B**), flower numbers (**C**), root length (**D**) of *Catharanthus roseus*. All the data are means of seven plants with four replications (n = 4) and the erect bars illustrate the standard error (±SE). Dissimilar alphabets indicate statistically significant variations. ZnO—NPs: zinc oxide nanoparticles (25 mgL^{-1}); Cd: cadmium (0.5 mM); ZnO—NPs + Cd: zinc oxide nanoparticles (25 mgL^{-1}) + cadmium (0.5 mM).

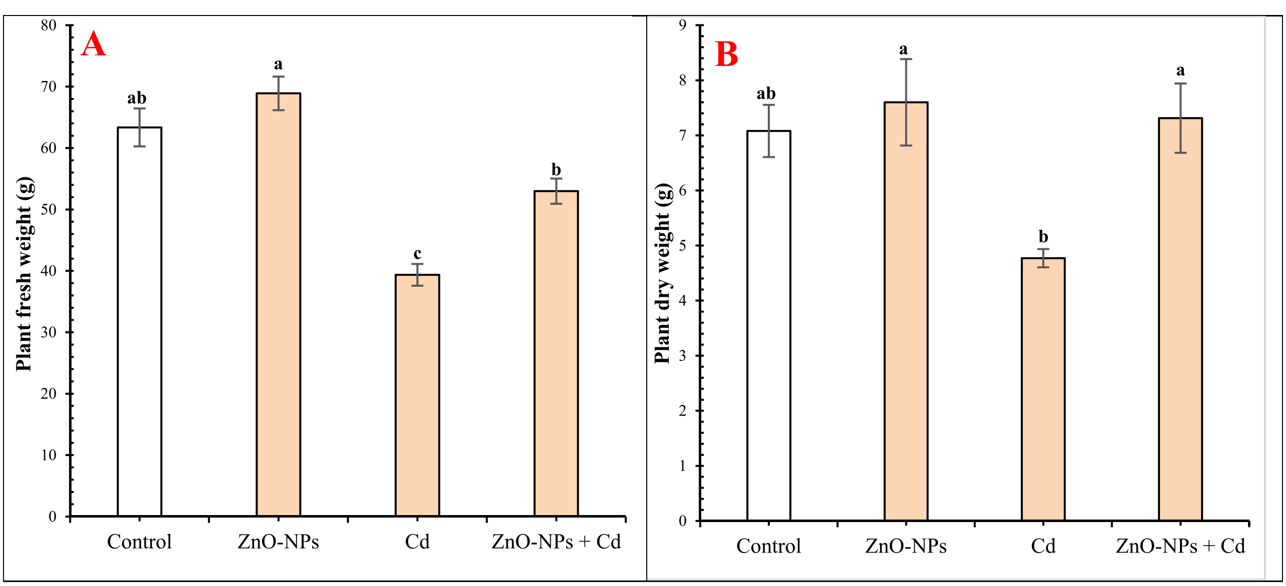

Fig. 2. Regulatory impact of ZnO—NPs under Cd stress on plant fresh weight (**A**), and plant dry weight (**B**) of *Catharanthus roseus*. All the data are means of seven plants with four replications (n = 4) and the erect bars illustrate the standard error (±SE). Dissimilar alphabets indicate statistically significant variations. ZnO—NPs: zinc oxide nanoparticles (25 mgL^{-1}); Cd: cadmium (0.5 mM); ZnO—NPs + Cd: zinc oxide nanoparticles (25 mgL^{-1}) + cadmium (0.5 mM).

3.3. Influence of ZnO-NPs on pigments and gas exchange traits of periwinkle

The Cd-stress significantly ($p < 0.05$) reduced the concentration of Chl-a (−103 %), Chl-b (−70 %), and carotenoid (−83 %) contents, compared with non-treated periwinkle plants (control). However, foliar spray of ZnO—NPs to periwinkle plants showed enhancement by 33 %, 36 %, and 29 % in Chl-a (Fig. 3a), Chl-b (Fig. 3b), and carotenoid (Fig. 3c) contents, respectively, compared with Cd-stressed without foliar supplemented plants. A similar trend was recorded with improved *A*, *E*, and *gs* in periwinkle leaves. There was an increment of 12 %, 21 %, and 17 %, in non-stressed plants due to foliar supplementation of ZnO—NPs, and an elevation of 38 %, 24 %, and 19 % in Cd-stressed plants for *A*, *E*, and *gs* levels of *C. roseus* plants, respectively (Fig. 3d,e,F).

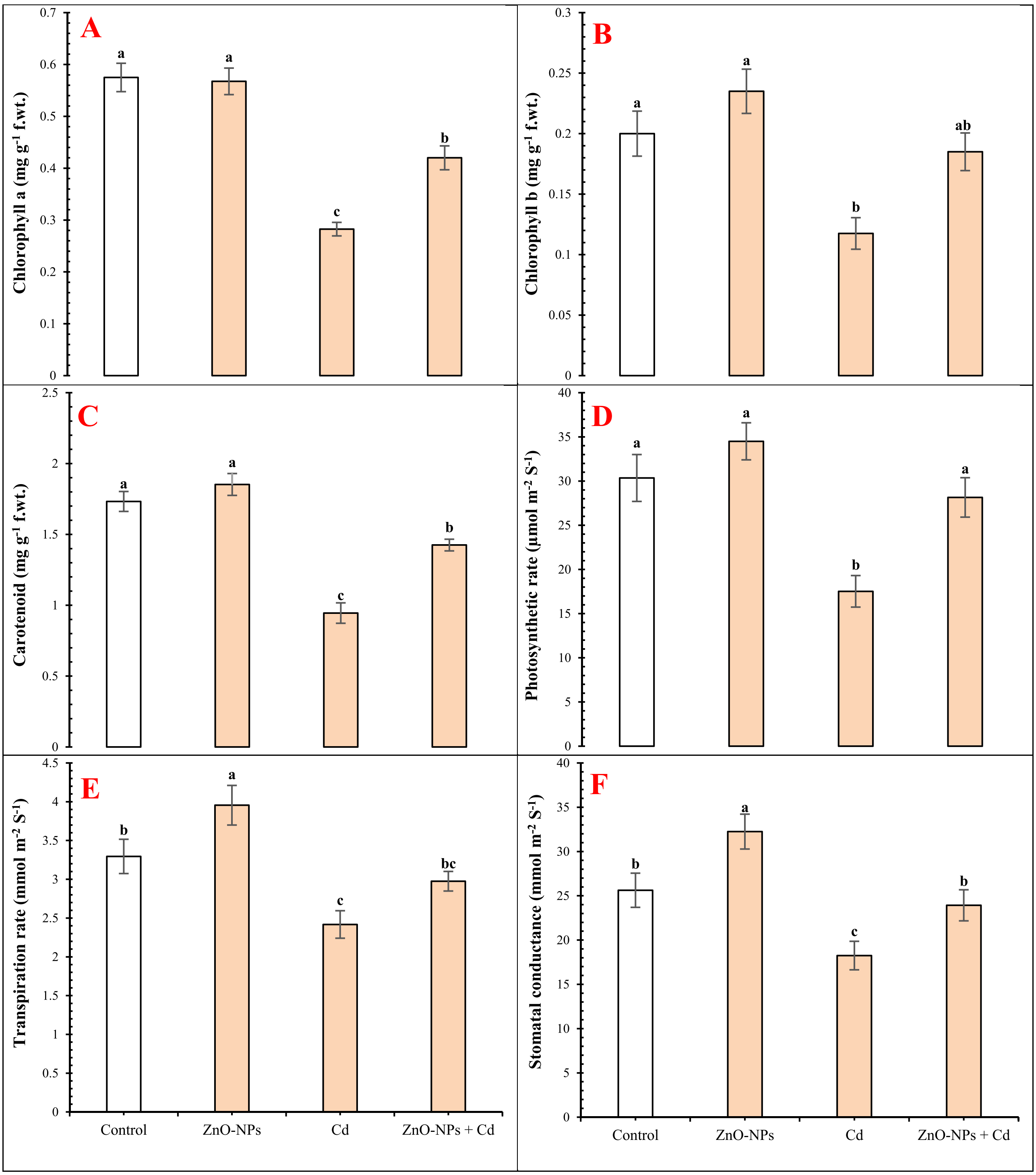

Fig. 3. Regulatory role ofZnO—NPs under Cd stress on contents of chlorophyll a (**A**), chlorophyll *b* (**B**), carotenoid (**C**), photosynthetic rate (**D**), transpiration rate (**E**), and stomatal conductance (**F**) of *Catharanthus roseus*. All the data are means of seven plants with four replications ($n = 4$) and the erect bars illustrate the standard error (±SE). Dissimilar alphabets indicate statistically significant variations. ZnO—NPs: zinc oxide nanoparticles (25 mgL^{-1}); Cd: cadmium (0.5 mM); ZnO—NPs + Cd: zinc oxide nanoparticles (25 mgL^{-1}) + cadmium (0.5 mM).

3.4. Impact of ZnO-NPs on H_2O_2 and MDA

The Cd stress significantly ($p < 0.01$) enhanced the H_2O_2 and MDA concentration in Cd-stressed periwinkle plants. There was an accrual of 49 %, and 52 % in H_2O_2 and MDA contents, respectively, in comparison with control plants. Foliar supplementation of ZnO—NPs remarkably reduced the levels of H_2O_2 by 24 % and MDA by 47 % in Cd-stressed plants compared with Cd-subjected non-treated (without ZnO—NPs application) periwinkle plants (Fig. 4a,b,c).

3.5. Influence of ZnO-NPs on antioxidant enzyme activities of periwinkle

Statistically, ZnO—NPs highly significantly ($p < 0.01$) stimulated the activities of antioxidant enzymes under controlled and Cd-stressed conditions. The Cd toxicity enhanced SOD by 30 %, while, supplementations of ZnO—NPs further stimulated SOD by 21 % under Cd-polluted environments (Fig. 5a). The POD contents were elevated due to ZnO—NPs spray by 3 % under controlled-conditions and reduced by 31 % in Cd-stressed periwinkle plants, whereas, 34 % increment in POD level was recorded in Cd-stressed untreated plants (Fig. 5b). The activities of CAT and APX were enhanced by 32 % and by 77 % in Cd-stressed plants. The supplementation of ZnO—NPs further stimulated CAT by 17 % and APX by 20 % in Cd-toxicity conditions in comparison with untreated Cd-stressed plants (Fig. 5c,d)

3.6. ZnO—NPs influence free proline and soluble protein levels

The supplementation of ZnO—NPs significantly ($p < 0.05$) elevated free proline contents by 34 % in Cd-stressed plants which was already increased (35 %) in Cd-subjected without ZnO—NPs applied periwinkle plants compared with control conditioned plants (Fig. 6a). Total soluble protein level was remarkably reduced by 108 % with Cd-stressed plants. However, ZnO—NPs foliar treatment increased protein levels by 44 %, and 21 %, in Cd-stressed and without Cd-subjected periwinkle plants, respectively (Fig. 6b).

3.7. Effect of ZnO-NPs on contents of Zn and Cd in periwinkle plant parts

Experimental data showed that ZnO—NPs application remarkably enhanced Zn levels in periwinkle plant parts. Cd-stressed untreated plants noticeably reduced Zn contents by 53 % (in roots), 47 % (in shoots), 25 % (in leaves), and 70 % (in flowers). Foliar spray of ZnO—NPs increased Zn contents in roots of periwinkle plants by 30 % and by 58 %; in shoots by 62 % and by 77 %; in stems by 46 % and by 52 %; and in flowers by 35 % and by 62 %, in controlled-conditioned plants and Cd-stressed plants, respectively (Table 1). Cadmium drenching in soil noticeably enhanced Cd levels by 100 % (in roots), 95 % (in shoots), 110 % (in leaves), and 91 % (in flowers), compared with controlled plants. The ZnO—NPs foliar spray efficiently alleviated Cd stress in all examined plant parts, which showed the phytoremediation tendency of periwinkle plants for Cd-polluted soils. The foliar application of ZnO—NPs reduced Cd accumulation by 32 % in roots, by 20 % in shoots, by 30 % in stems, and by 35 % in flowers, compared with Cd-stressed non-treated (without ZnO—NPs spray) periwinkle plants (Table 1).

4. Discussion

ZnO—NPs, with their minute size (1 to 100 nm), are commonly used in the nanotechnology field (Lv et al., 2022). Due to their large specific surface area and higher solubility rate, they are effortlessly engrossed by different plat parts (Liu et al., 2022). Beig et al., (2023) found that translocation of Zn from root to above-ground parts is higher with the supplementation of $ZnSO_4$ (Zn^{++} ions) in comparison with ZnO—NPs, due to the high solubility of $ZnSO_4$ than the ZnO—NPs, that presents steady and slow availability of dissolved Zn. Subsequently, the speedy release of Zn^{2+} can positively regulate morphological, physio-biochemical, and nutrient uptake that finally impact the plant growth performance (Xie et al., 2022; Wang et al., 2023). The findings of this experiment presented that ZnO—NPs heightened the morphological traits and total biomass of *C. roseus* under Cd-polluted soil (Fig. 1 and 2). Cd-property for growth retardation is also found in this study for periwinkle plants as previously documented in mung bean (Aqeel et al., 2021) and tomato (Hédiji et al., 2015). The ZnO—NPs supplementation positively regulated plant length, leaf number, root length, and plant biomass of wheat in Cd-stressed conditions (Hussain et al., 2018). These results are very similar to the current experiment. These findings illustrated that ZnO—NPs not only improved the growth of *C. roseus* but also enhanced periwinkle tolerance to Cd toxicity. Hussain et al., (2024) reported that foliar spray of ZnO—NPs on leaf surface plays a key role in preserving and defending structure of cell membrane ultimately

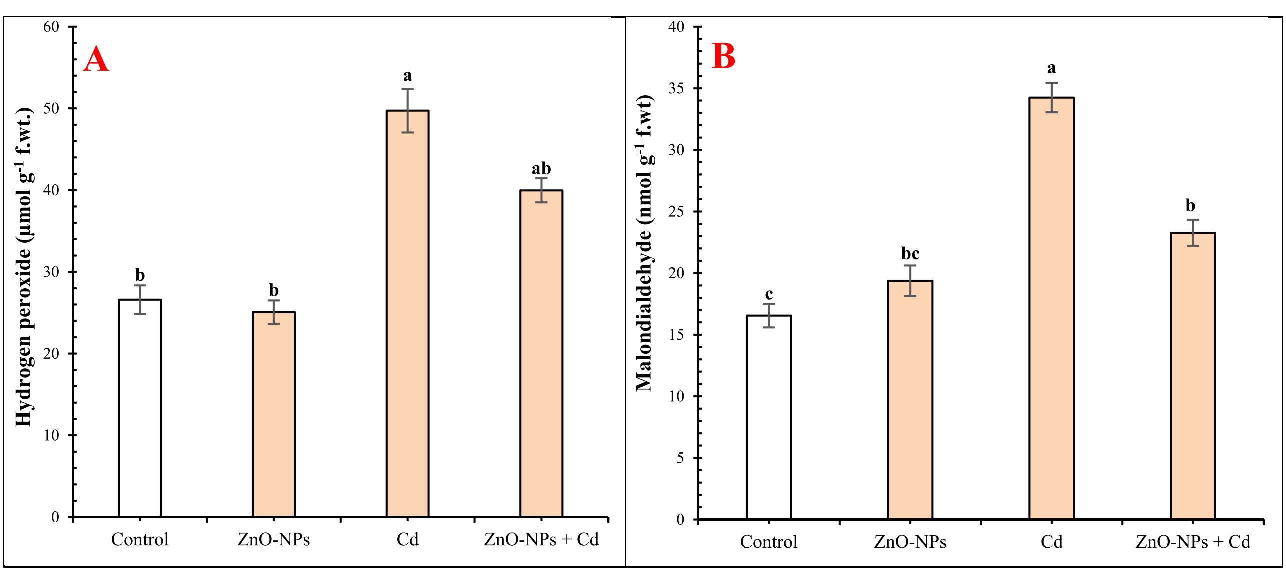

Fig. 4. Regulatory effect of ZnO—NPs under Cd stress on concentrations of hydrogen peroxide (**A**), and malondialdehyde (**B**) of *Catharanthus roseus*. All the data are means of seven plants with four replications ($n = 4$) and the erect bars illustrate the standard error (±SE). Dissimilar alphabets indicate statistically significant variations. ZnO—NPs: zinc oxide nanoparticles (25 mgL^{-1}); Cd: cadmium (0.5 mM); ZnO—NPs + Cd: zinc oxide nanoparticles (25 mgL^{-1}) + cadmium (0.5 mM).

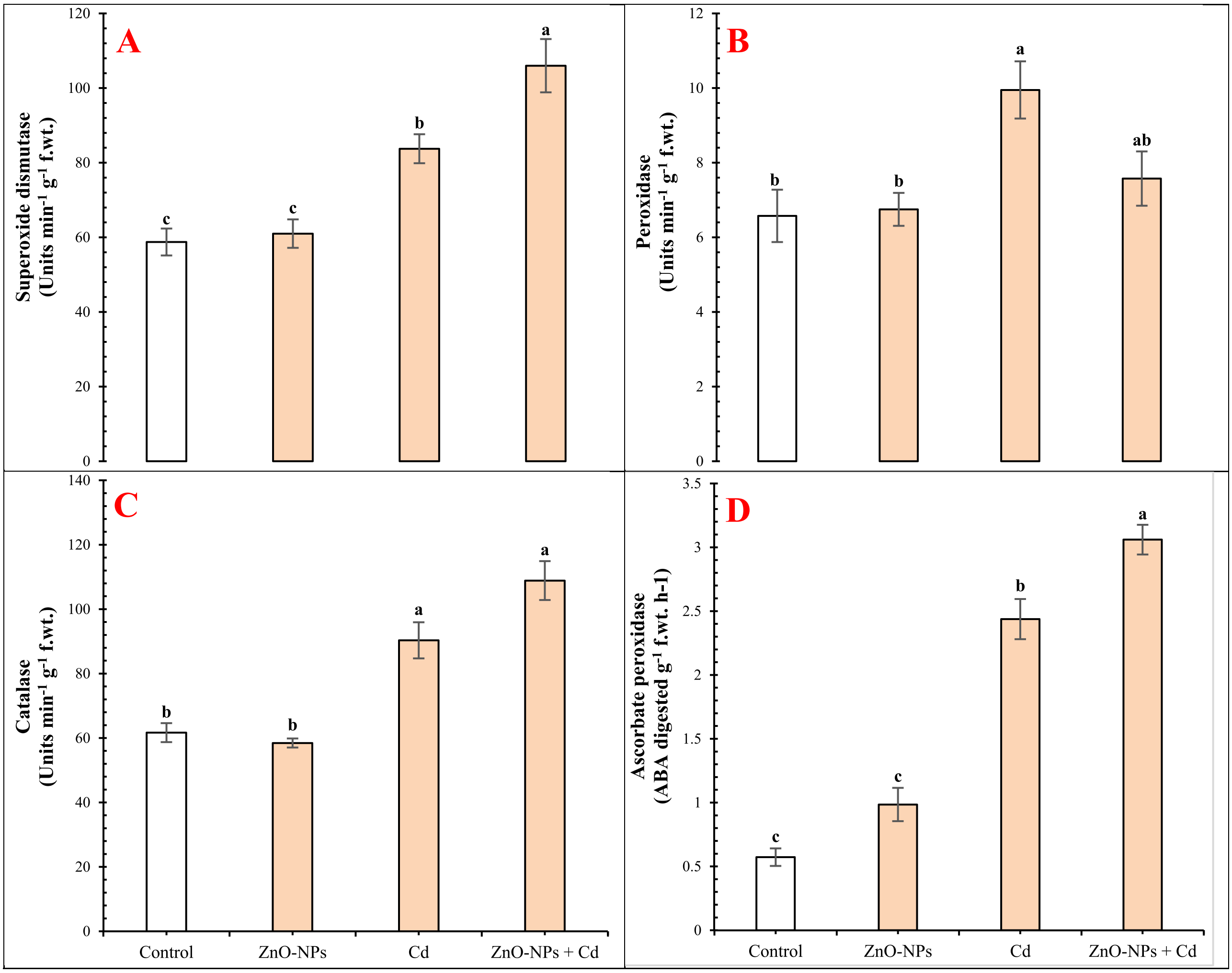

Fig. 5. Regulatory role of ZnO—NPs under Cd stress on antioxidant activities of superoxide dismutase (**A**), peroxidase (**B**), catalase (**C**), and ascorbate peroxidase (**D**) of *Catharanthus roseus*. All the data are means of seven plants with four replications ($n = 4$) and the erect bars illustrate the standard error (±SE). Dissimilar alphabets indicate statistically significant variations. ZnO—NPs: zinc oxide nanoparticles (25 mgL^{-1}); Cd: cadmium (0.5 mM); ZnO—NPs + Cd: zinc oxide nanoparticles (25 mgL^{-1}) + cadmium (0.5 mM).

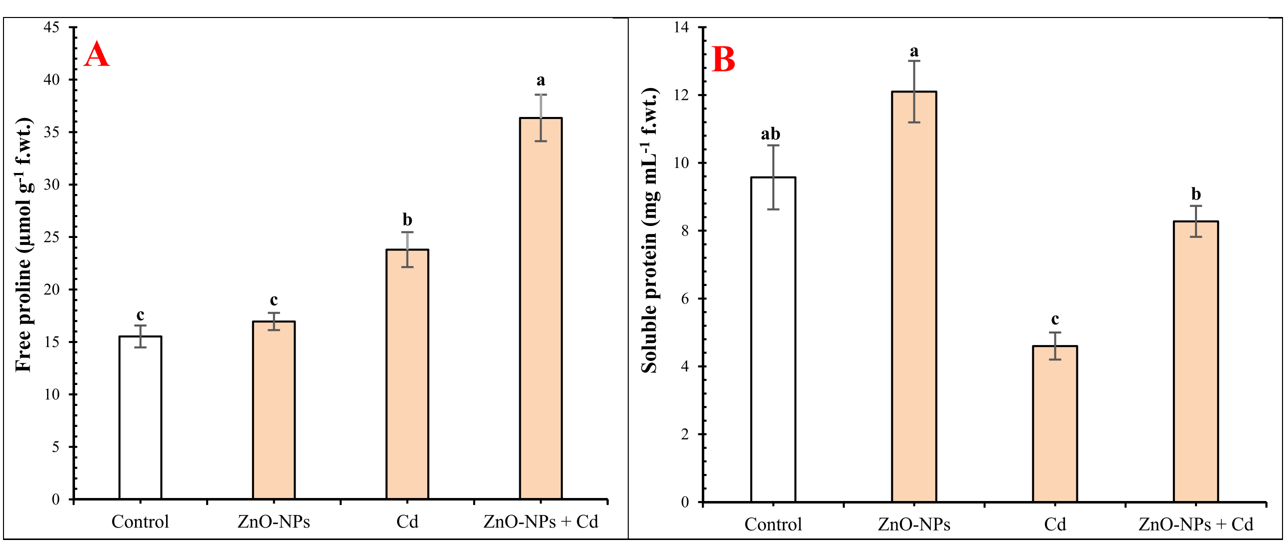

Fig. 6. Regulatory role of ZnO—NPs under Cd stress on concentrations of free proline (**A**), and soluble protein (**B**) of *Catharanthus roseus*. All the data are means of seven plants with four replications ($n = 4$) and the erect bars illustrate the standard error (±SE). Dissimilar alphabets indicate statistically significant variations. ZnO—NPs: zinc oxide nanoparticles (25 mgL^{-1}); Cd: cadmium (0.5 mM); ZnO—NPs + Cd: zinc oxide nanoparticles (25 mgL^{-1}) + cadmium (0.5 mM).

Table 1
Concentration of zinc and cadmium uptake and accrual in different plant parts of *Catharanthus roseus*.

Treatments	Zn concentrations (mg kg^{-1})				Cd concentrations (mg kg^{-1})			
	Roots	Shoots	Leaves	Flowers	Roots	Shoots	Leaves	Flowers
Control	36.45±3.7^b	5.30±0.8^b	18.53±2.3^b	14.70±2.1^b	nd	nd	nd	nd
ZnO—NPs	52.18±2.2^a	13.98±1.5^a	34.35±2.2^a	22.90±1.9^a	nd	nd	nd	nd
Cd	23.8 ± 2.4^b	3.57±0.7^b	14.83±1.5^b	8.65±0.7^b	2.10±0.21^a	1.32±0.15^a	1.81±0.19^a	0.95±0.17^a
ZnO—NPs + Cd	57.53±4.1^a	16.08±1.2^a	30.88±1.9^a	21.93±2.2^a	1.59±0.18^b	1.09±0.14^a	1.40±0.12^b	0.71±0.12^b

All the data are means of seven plants with four replications (n = 4) and the standard error (±SE). Dissimilar alphabets indicate statistically significant variations. ZnO—NPs: zinc oxide nanoparticles (25 mgL^{-1}); Cd: cadmium (0.5 mM); ZnO—NPs + Cd: zinc oxide nanoparticles (25 mgL^{-1}) + cadmium (0.5 mM); nd: not detected.

helpful in Cd-stress tolerance, synthesis of protein, cell enlargement, functioning of the membrane by increasing antioxidant defenses and reducing oxidative damages by increasing activities of antioxidant enzymes. Foliar treatment of ZnO—NPs increased activities of nitrate reductase, and enhanced accrual of proline, sugar, and amino acid contents which are linked to the efficiency of foliar supplementation of ZnO—NPs in plants (Hussain et al., 2024). The higher plant biomass with foliar supplementation might be due to elevated Zn contents in plants (Table 1). This enhanced Zn enrichment in different plant parts may decrease the Cd stress because of different mechanisms like Zn and Cd co-precipitation in metabolically sedentary parts and decreasing the Zn deficiency which leads to healthier plant growth (Wang et al., 2023). Furthermore, longer roots and enhanced plant growth as a result of NP feeding that stimulates the redox process and manufacture of photosynthetic pigments in plants may have a favorable impact on plant growth (Hussain et al., 2018).

In plants, heavy metal-mediated stress may be evaluated by determining the chlorophyll contents (Wang et al., 2023). The major component of chloroplast is chlorophyll which is completely associated with the photosynthetic rate of plants. The Cd stress significantly influences the chlorophyll concentrations in plant leaves and is the primary symptom of metal stress in plants (Rehman et al., 2018). Therefore, an alteration in the contents of chlorophyll may portray the plant's healthiness and response to the variation in the environment. The present study documented that photosynthetic pigments and exchange of gas attributes were enhanced in periwinkle leaves by ZnO—NPs supplementation than Cd-stressed plants (Fig. 3a-F). The decrease in methionine and cysteine concentrations (key components of chloroplast target proteins) during heavy metal stress, may be connected to this decrease in chlorophyll pigments (Ledda et al., 2019). The elevated chlorophyll levels in periwinkles might be linked to reduced plant Cd levels (Faizan et al., 2021b). Hussain et al., (2018) also reported elevated photosynthetic attributes by alleviating Cd stress in tomatoes. In addition, with ZnO—NP treatment, there is an association between decreased oxidative stress and Cd engagement with the safeguarding of chlorophyll and total photosynthesis (Rizwan et al., 2019).

The most profound impact of Cd stress is the production of excessive ROS, which produces oxidative stress to lipids, proteins, and some plant molecules (Faizan et al., 2021b). Inhibition of electron transfer due to Cd stress prevents the photo-activation of photosystem II (Gutsch et al., 2019). So, Cd toxicity indirectly contributes to ROS production via disturbance in the chloroplasts of leaves and the mitochondrial electron transfer chain (Farooq et al., 2016). The present study revealed, elevated MDA and H_2O_2 levels in tissues of the plants under stress of Cd (Fig. 4a—c). MDA has been regarded as a scale of oxidative damage and is the producer of lipid peroxidation in plants (Ahsan et al., 2022). Nevertheless, foliar supplementation of ZnO—NPs considerably reduced MDA and H_2O_2 in Cd-subjected plants and, consequently, defend the cell membrane integrity. Furthermore, reduction in Cd accrual in plant tissues in ZnO—NPs treated plants of periwinkle leads to amelioration of ROS-mediated damage (Adrees et al., 2021). To detoxify the ROS, the plants have established a well-organized mechanism for defense (Garcia-Gomez et al., 2018). The ascorbate-glutathione (AsA, GSH) pathway encompasses four enzymes i.e. glutathione reductase, ascorbate peroxidase, dehydroascorbate reductase and monodehydroascorbate reductase except AsA and GSH, play a key role in ROS detoxification in plants under heavy metal (Hasanuzzaman et al., 2019). AsA-GSH pathway also interrelates with plant defense system and defends the plants from Cd-mediated damages (Hasanuzzaman et al., 2017). Plant enzymes triggered by Zn are responsible for the metabolism of carbohydrates, maintenance of cellular membrane integrity, synthesis of protein, and regulation of auxin production (Jabri et al., 2022). Most plant species possess antioxidant enzymes that abolish the accrual of ROS. The present experiment showed elevated activities of SOD and CAT but decreased POD contents in *C. roseus* leaves due to foliar spray of ZnO—NPs (Fig. 5a-d). These findings strengthen the reports of Mahamood et al. (2023), who documented that SOD changes the O_2^- to less deadly H_2O_2, which makes the top defense line in the antioxidant mechanism of plants; to do this, CAT scavenges H_2O_2 (Rizwan et al., 2019), whereas, POD acts as catalysis of H_2O_2 (Su et al., 2019). Similarly, other antioxidant enzymes such as APX also function as ROS scavenging in different plants under abiotic stress (Khoshbakht et al., 2018). Nevertheless, depending on the type of plant, the kind of soil, the quantity of NPs, and the length of exposure, ZnO—NPs respond differently to antioxidative enzymatic activities (Faizan et al., 2021b).

Plants under various abiotic stresses accumulate solutes such as proline (Srivastav et al., 2021). Proline performs functions in membrane stability, smooth water absorption, and exclusion of extra ROS (Li et al., 2021b). The present experiment revealed higher free proline levels after supplementation of ZnO—NPs in Cd-subjected plants (Fig. 6a). Similarly, an increment of free proline level by ZnO—NPs foliar supplementation was noted in cucumber (Li et al., 2021a). Proline accrual under abiotic stress in numerous plant species has been associated with stress tolerance. The concentration of proline has been increasing in stress-tolerant plants compared to stress-sensitive plants (Zahedi et al., 2023). Faizan et al. (2021b) stated that ZnO—NPs have great potential to increase proline level mediated expression of genes for proline biosynthesis. In plants, the soluble protein concentrations differ from one plant species to another (Kaur et al., 2017). Total soluble protein level was drastically decreased in this study in Cd-stressed plants (Fig. 6b). These findings verify the results of Faizan et al. (2021b), who showed a reduction in protein synthesis under Cd toxicity in wheat. Enhanced activities of protease destroy the structure and activity of the protein, which in turn results in augmented denaturation of protein under abiotic stress (Chanu and Upadhyaya, 2019). Cd-linked damage to DNA includes the destruction of nucleic acid and cell membrane, reduced synthesis of protein, and photosynthetic protein impairment (Abbas et al., 2018). In severe cases, oxidative stress due to Cd-toxicity can damage proteins, nucleic acids, and lipids leading to inhibition of growth and even death of cells (Loix et al., 2017). So, the application of ZnO—NPs ameliorates Cd-mediated degradation of protein in the periwinkle plants, as has been documented in this study. This increment in soluble protein levels might be one of the chief mechanisms for the extenuating Cd-stress damage by NPs (Bala et al., 2019).

Stress brought on by Cd toxicity in plants can result in nutritional deficiencies, which may be related to Cd and certain other divalent ions' struggles to be absorbed by the plants (Konate et al., 2017). Presence of Cd ions in the soil can affect absorption of roots and successive transportation and distribution of nutrients in plants (Haider et al., 2021). Cadmium stress remarkably reduces N, Ca, Mn, K, P, B, and Zn contents and interact with storage and utilization of these elements and plant water uptake (Kinay, 2018; Zhang et al., 2019). The current study's findings demonstrated a considerable decrease in Zn accumulation in all examined plant parts under Cd-stressed conditions (Table 1). However, the supplementation of ZnO—NPs noticeably recovered this reduction of Zn contents in Cd-stressed periwinkle plants, and similar reports were found in tomatoes (Sun et al., 2023b) and wheat (Rizwan et al., 2019). Generally, toxicity or deficiency does not occur; though, they both harmfully affect crop growth and development (Suganya et al., 2020). The ZnO—NPs have been shown to enhance the bioavailability of Zn in the soil and its contents in several plant parts compared to control plants (Munir et al., 2018). Saleem et al. (2022) reported that there are two possible mechanisms of Zn uptake by the plants. Mechanism-I involves solvent efflux, H^+ ions, and organic acids, which enhance Zn-complexes absorption and release of Zn^{2+} ions for root epidermal cells absorption. While, mechanism-II involves the discharge of phyto-metallophones, that produce complex compounds with Zn and then uptake by epidermal cells of the roots. Kareem et al., (2022) showed that Zn ions in ZnO—NPs-supplemented plants were heterogeneously dispersed than $ZnSO_4$-sprayed plants. ZnO—NPs cross the epidermis of the leaf through stomata after foliar spray, accumulate, and release Zn-ions in the apoplast, and are absorbed by the mesophyll cells (Sperdouli et al., 2022). It has been observed that Zn has been shown to have a hostile impact on the uptake of Cd by plants and lessened the harmful effects of Cd to plants (Qaswar et al., 2017). According to Hussain et al., (2018), adding ZnO—NPs to the soil may limit the amount of Cd there and reduce the amount that plants can absorb from it. Supplementation of Zn^{2+} in Cd-stressed soils enhanced the energy fraction utilized for photochemistry (Φ_{PSII}) and maximized the PSII efficacy, reduced lipid peroxidation, and showed less production of ROS (Sperdouli et al., 2022). Plant roots are directly exposed to dangerous metals such as Cd, and their concentration in the roots is higher than in any other part of the plant (Faizan et al., 2021b). Rassaei et al., (2020) stated that Zn declined the Cd contents in the fractions of carbonate and organic matter in sandy clay loamy soils and enhanced the iron and magnesium oxides as well as water soluble and exchangeable forms, so, residual forms were not affected. Certainly, the present experiment also found the greatest Cd contents in periwinkle roots compared with other above-soil surface plant parts under Cd stress conditions (Table 1). Supplementation of ZnO—NPs diminishes Cd accrual in all plant parts. Furthermore, root apoplast (regarded as a natural fence) prevents the Cd uptake in vascular plants (Siemianowski et al., 2014). Lignification of roots (by NaCl) may also remarkably decrease the Cd absorption in tobacco plants (Yang et al., 2020). Increment in various antioxidant enzymes especially POD is also responsible for morphological and physiological modifications that reduced the efficacy of apoplastic transport for heavy metal ions and macromolecules which ultimately decreased Cd absorption (Yang et al., 2024). Present findings suggested that ZnO—NPs slowed down the Cd transportation in the root-shoot direction; hereafter, ZnO—NPs affected the movement and absorption of Cd in *C. roseus* in a parallel manner, as formerly testified by Bhuyan et al., (2020). Chai et al., (2024) found that ZnO—NPs activated the expression of stress signal transduction-associated genes, afterward upholding the downstream target genes expression linked with the synthesis of secondary metabolites, cell wall, and transport of metals via transcription factors. This molecular mechanism activation contributed to increasing Cd-toxicity tolerance in plants. Henceforward, all the data of this experiment revealed that ZnO—NPs certainly normalize the Cd-mediated stress tolerance in *C. roseus* by regulating the numerous phenotypic and biochemical attributes.

5. Conclusion

In the current study, we studied the ameliorating role of ZnO—NPs on the growth performance of *Catharanthus roseus* under Cd stress. The findings of our experiment revealed that ZnO—NPs may play a key role in enhancing the Cd tolerance to *C. roseus* plants. Positive regulatory effects of foliar-supplied ZnO—NPs on morphological traits (plant height, number of leaves and flowers, root length) and plant biomass were found to be associated with ZnO—NPs-induced improvements in physiological mechanisms via enhancing the photosynthetic pigments and rates of gaseous exchange. Foliar supplementations of ZnO—NPs reduced oxidative stress by lowering the hydrogen peroxide and malondialdehyde levels resulting in improved antioxidant enzymatic activities, elevating Zn accumulation, and lowering uptake and accrual of Cd in below and above-ground plant parts. Cd-stressed plants sternly decreased total soluble protein levels, but the supplementation of ZnO—NPs might ameliorate such antagonistic effects. So, exogenous foliar application of ZnO—NPs averts the lethal impacts of Cd stress and permits the periwinkle plants to endure in stressed conditions. For future research, it is strongly suggested that the role of ZnO—NPs against Cd toxicity in field settings be appreciated, as the effect of ZnO—NPs is mostly studied under limited or controlled conditions. An extensive molecular approach is necessary, to explore and unveil the mechanisms of ZnO—NPs-mediated enhanced tolerance to Cd toxicity in plants. Furthermore, research on plant-based nanoparticles is projected to increase tremendously in the future due to their potential applications in the cosmetics, pharmaceutical, and food industries.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Muhammad Ahsan: Conceptualization, Data curation, Formal analysis, Supervision, Validation, Visualization, Writing – original draft. **Emanuele Radicetti:** Data curation, Investigation, Methodology, Visualization, Writing – original draft, Supervision, Writing – review & editing. **Roberto Mancinelli:** Visualization, Writing – review & editing. **Hayssam M. Ali:** Validation, Visualization, Writing – review & editing. **Adnan Younis:** Conceptualization, Data curation, Investigation, Validation, Visualization, Writing – original draft. **Mateen Sajid:** Validation, Visualization, Writing – original draft. **Abdul Manan:** Investigation, Methodology, Writing – original draft. **Sajjad Ali:** Conceptualization, Investigation, Writing – original draft. **Mohammad Valipour:** Methodology, Validation, Visualization, Writing – review & editing.

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