



Role of Zinc Oxide Nanoparticles in Countering Negative Effects Generated by Cadmium in *Lycopersicon esculentum*

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Received: 21 May 2019 / Accepted: 6 December 2019 / Published online: 5 February 2020
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Abstract

Nanotechnology now plays a revolutionary role in many applications; nanomaterials have experienced significant importance in both basic and applied sciences as well as in bio-nanotechnology. Zinc oxide nanoparticles (ZnO-NPs) have become one of the most important metal oxide NPs in biological applications due to their beneficial impacts. The purpose of this study was to explore the effects of ZnO-NPs in reducing Cd toxicity by studying the growth, photosynthesis reactions, antioxidant system, oxidative stress, and protein content in *Lycopersicon esculentum* (tomato). ZnO-NPs induced an upregulation of antioxidative enzymes which protect the photosynthetic apparatus in plants. Seeds of tomato were sown to create nursery. At 20 days after sowing (DAS), seedlings were transferred to soil pots. Varied concentrations (0.4, 0.6 or 0.8 mM) of Cd were applied to the soil after 24 and 25 DAS. Zinc (Zn; 50 mg/L) and ZnO-NPs (50 mg/L) treatments were given continuously for 5 days from 31 to 35 DAS and sampling took place at 45 DAS. The results indicate that a Cd-generated oxidative burst in the form of elevated hydrogen peroxide (H_2O_2) levels resulted in a decline in cell viability through enhanced activity of the antioxidant system and proline content; the data increased on follow-up treatment with ZnO-NPs. Foliar application of ZnO-NPs significantly enhanced plant height, fresh, and dry weight of plant, leaf area, SPAD chlorophyll, photosynthetic attributes, i.e., net photosynthetic rate (P_N), transpiration rate (E), internal CO_2 concentration (C_i), and stomatal conductance (g_s). Application of ZnO-NPs reduced the adverse effects generated by Cd and increased protein content, activities of nitrate reductase and carbonic anhydrase over the control in both stressed and non-stressed plants. Additionally, microscopic studies showed a marked increase in stomatal aperture after ZnO-NPs treatment in the presence or absence of Cd. This was associated with decrease in malondialdehyde and superoxide radical (O_2^-) levels. The present study suggests that ZnO-NPs can be effectively used to reduce the toxicity of Cd in tomato plants and may also be suitable for testing on other crop species.

Keywords Nanotechnology · Oxidative burst · Photosynthetic attributes · Upregulation

Introduction

In recent decades, significant concerns have arisen regarding the adverse impacts of environmental contaminants on agricultural production; this includes both biotic and abiotic stresses. According to Godfray et al. (2010) and Lutz and Samir (2010), food production must be increased by 70 to

100% to meet the nutritional demands of the growing global population, which is expected to reach 9 billion by 2050.

Cadmium (Cd) is one of the most important heavy metals in an environmental and public health context, as it induces a suite of toxic responses to biota and raises the risk of food contamination from consumption of plants (Irfan et al. 2014). According to the report of the International Agency for Research on Cancer (IARC), Cd and its associated compounds are carcinogenic to humans (Wang et al. 2018). Zhao et al. (2015) confirmed that Cd is one of the most widespread inorganic pollutants globally.

Plants take up Cd and translocate it to various parts, with potential negative effects to organisms consuming them. High Cd concentration in soil adversely affects cell functions that lead to plant death (Gratao et al. 2006; Liu et al. 2007; Hasan et al. 2009). Cd is considered an antimetabolite which

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causes enzyme inactivation and dysfunction of various plant physiological and biochemical processes (Hayat et al. 2012). Excess Cd in plants reduces growth and increases oxidative stress by the production of reactive oxygen species (ROS) including H_2O_2 , MDA, and O_2^- in plants (Perez-Chaca et al. 2014; Ali et al. 2015; Rizwan et al. 2016).

Nanotechnology has recently emerged as a new scientific discipline and is considered by many as an ideal solution for overcoming the destructive impact of metals on agricultural productivity. In recent years, the use of zinc oxide nanoparticles (ZnO-NPs) has received great attention due its purported ability to increase nutrient accumulation by plants for enhancing the quality of the food crop (Hussain et al. 2018; Rizwan et al. 2019). Zinc (Zn) is an essential micronutrient and plays an important role in the activities of enzymes like dehydrogenases, tryptophan synthetase, aldolases, isomerases, transphosphorylases, superoxide dismutase, and DNA and RNA polymerases (Auld 2001; Broadley et al. 2007). ZnO-NPs are now widely employed in agriculture; as components of solar cells, sunscreens, wall paints, and ceramics; and in catalysis and biomedical applications (Suliman et al. 2007; Santhoshkumar et al. 2017). The optical, physical, and antimicrobial activities of ZnO-NPs offer positive impacts to plants (Liu and Lal 2015; Hussain et al. 2016; Tripathi et al. 2017) and serves to overcome the toxic effects of heavy metals such as Cd (Saxena et al. 2016; Rizwan et al. 2019). By virtue of their small size and massive surface area, ZnO-NPs easily penetrate contaminated zones of plant and possess a strong affinity to Cd (Khan et al. 2017). Wang et al. (2018) revealed that exogenous application of ZnO-NPs reduced Cd concentration in *Sorghum bicolor*. Similarly, in *Triticum aestivum*, ZnO-NPs decreased Cd concentration and oxidative stress (Hussain et al. 2018). Furthermore, Venkatachalam et al. (2017) reported similar observations in *Gossypium hirsutum*.

In plants, production of ROS is a natural consequence of oxygen metabolism and holds a promising role in cell signaling and homeostasis (Ray et al. 2012). However, an excess of ROS creates oxidative stress, damages DNA, lipids and proteins, and finally leads to cell death (Tripathy and Oelmüller 2012). To overcome the toxic effects of oxidative stress, plants activate non-enzymatic (proline) and enzymatic (POX, SOD, and CAT) antioxidants (Sewelam et al. 2016). These enzymes are the key components of the plant defense system (Andre et al. 2010).

Tomato is one of the most important ‘protective foods’ widely known for its outstanding antioxidant, antidiabetic, and anticancerous properties. The fruit contains large quantities of water, calcium, and niacin, all of which are significant in human metabolic function. Tomato is a highly nutritious plant food and contains vitamins A, C, and E, and minerals that protect the body against disease (Beecher 1998). Tomato not only has substantial nutritional value but also

offers medicinal properties as it is a rich source of lycopene, carotenoids, flavonoids, and potassium (Beecher 1998; Leonardi et al. 2000).

The present work was designed with the aim of investigating the effects of foliage-applied ZnO-NPs on the physiological and biochemical parameters of Cd-stressed *Lycopersicon esculentum* plants.

Materials and Methods

Plant Material

Tomato seeds, cv. PKM-1, procured from the National Seed Corporation Ltd., New Delhi, India, were surface sterilized with 0.01% mercuric chloride solution followed by repeated washings with double distilled water (DDW). The sterilized seeds were sown to create the nursery under natural conditions in the net house of the Department of Botany, Aligarh Muslim University, Aligarh India.

Nanoparticle Preparation

ZnO-NPs were purchased from Sigma-Aldrich Chemicals Pvt. Ltd., India. An adequate volume (50 mg/L) of ZnO-NPs was prepared by dissolving the required amounts of ZnO-NPs in 10 mL DDW in a 100 mL volumetric flask, and making to volume.

Experimental Design and Procedure

The experiment was carried out in a simple randomized block design with 27 earthen pots (6 inch diameter) filled with a sandy loam soil and mixed with farmyard (cattle) manure in a ratio of 6:1. A uniform basal starter dose of inorganic fertilizers (urea, single superphosphate and muriate of potash) was added at a rate of 40, 138, and 26 mg, respectively, per kg of soil to maintain fertility. At 20 DAS, seedlings were transplanted to pots from the nursery. The 27 pots were divided into nine sets of three pots each (replicates) representing one treatment. The treatments were as follows:

- | | |
|---------|--|
| Set I | control (-Cd and -NPs) at 31–35 DAS foliage was sprayed with DDW only. |
| Set II | at 31–35 DAS the foliage was sprayed with 50 mg/L of Zn. |
| Set III | at 31–35 DAS foliage was sprayed with 50 mg/L of ZnO-NPs |
| Set IV | at 24 and 25 DAS the plants were exposed to 0.4 mM of Cd solution, via soil. |
| Set V | at 24 and 25 DAS plants were exposed to 0.6 mM of Cd solution, via soil. |

- Set VI at 24 and 25 DAS plants were exposed to 0.8 mM of Cd solution, via soil.
- Set VII a combination of sets III and IV (50 mg/L ZnO-NPs + 0.4 mM of Cd).
- Set VIII a combination of sets III and V (50 mg/L ZnO-NPs + 0.6 mM of Cd).
- Set IX a combination of sets III and VI (50 mg/L ZnO-NPs + 0.8 mM of Cd).

Each plant was sprayed three times and the nozzle of the sprayer was adjusted so that it pumped out 1 mL of Zn or ZnO-NPs in one spray. Plants from each treatment were uprooted at 45 DAS to assess selected parameters.

Measurement of Morphological Parameters

Plants were removed from the pots along with soil and dipped in tap water. Soils were removed gently and shoot and root lengths were measured using a meter scale. The plants were oven-dried at 80 °C for 24 h and then weighed for dry biomass. Leaf area was calculated using a leaf area meter (ADC Bio scientific, Hoddesdon, UK).

Determination of Chlorophyll Content (SPAD Value)

A chlorophyll meter (SPAD-502; Konica, Minolta sensing, Inc., Japan) was used to measure the SPAD values of chlorophyll in the leaves.

Evaluation of Gas Exchange

Gas exchange parameters (P_N , g_s , C_i , and E) were measured in entirely expanded leaves using a portable photosynthesis system (LI-COR 6400, LI-COR, Lincoln, NE, USA). Air temperature, relative humidity, CO₂ concentration, and photosynthetic photon flux density were maintained at 25 °C, 85%, 600 ppm, and 800 $\mu\text{mol mol}^{-2} \text{s}^{-1}$, respectively. All measurements were made between 11:00 and 12:00 h under clear sunlight.

Biochemical Determinations

Nitrate Reductase (NR) Activity

Activity of NR was computed by employing the method of Jaworski (1971). A mixture of newly formed leaf (0.1 g), phosphate buffer (pH 7.5), KNO₃, and isopropanol was placed in a incubator at 30 °C for 2 h. Sulfanilamide and N-1-naphthylethylenediamine hydrochloride mixture were then added to the incubated mixture. Absorbance was read on a UV–visible spectrophotometer (Spectronic 20D; Milton Roy, USA) at 540 nm.

Carbonic Anhydrase (CA) Activity

CA level in leaves was measured with the method of Dwivedi and Randhawa (1974). Leaves were cut into small pieces and transferred to a cysteine hydrochloride solution. They were blotted and transferred to a test tube containing 4 cm³ of phosphate buffer (pH 6.8), followed by addition of 0.2 M NaHCO₃, bromothymol blue, and methyl red indicator. 0.5 N HCl was used for titrating.

Determination of Proline Content

The method of Bates et al. (1973) was adopted for identification of proline content in leaves.

Protein Analysis

Protein in leaf samples was analyzed by the method of Bradford (1976).

Determination of Activities of Antioxidant Enzymes

Antioxidant enzymes (CAT, POX, and SOD) were analyzed by the method proposed by Khan et al. (2015). Glutathione reductase (GR, 1.8.1.7) activity was estimated as per the method of Smith et al. (1988).

Oxidative Stress Markers

Determination of Lipid Peroxidation, Hydrogen Peroxide (H₂O₂) and Superoxide Radical (O₂⁻)

The rate of lipid peroxidation and concentrations of H₂O₂ and O₂⁻ were followed as described in Siddiqui et al. (2018).

Histochemical Detection of Lipid Peroxidation, H₂O₂ and O₂⁻

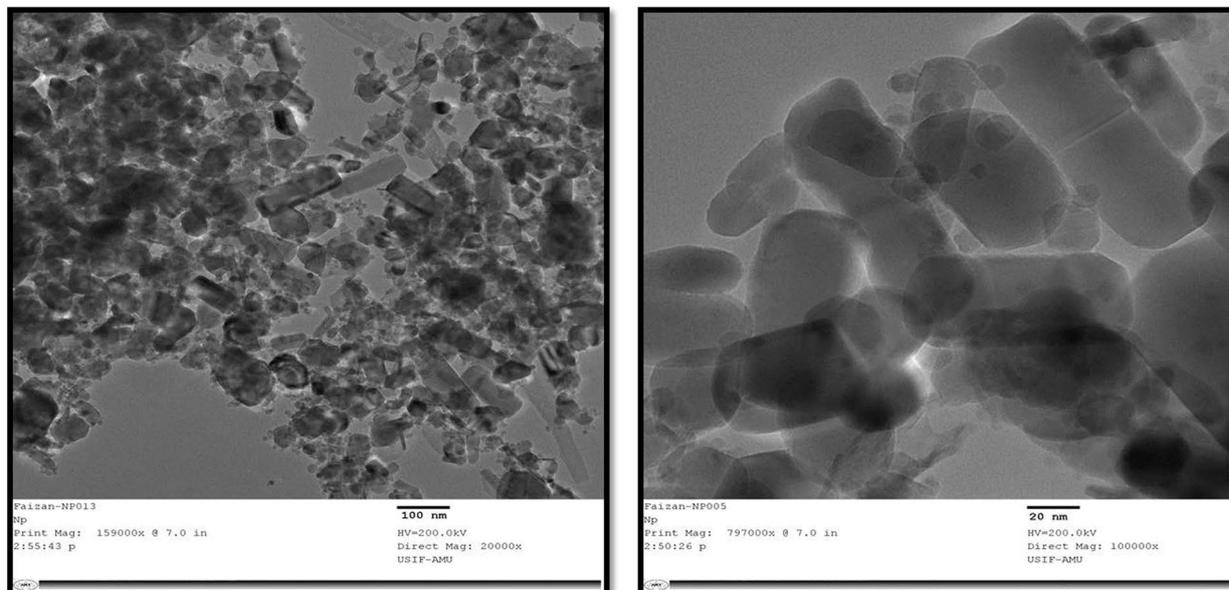
Histochemical detection of lipid peroxidation, H₂O₂, and O₂⁻ was followed as described in Fareen and Hayat (2019).

Stomatal Studies

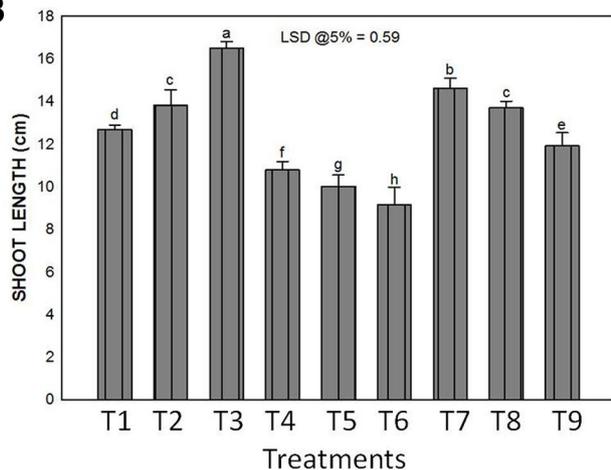
Scanning Electron Microscopy

Stomatal apertures on the abaxial surface of plant leaves were observed under a scanning electron microscope (JEOL, JSM 6510). Fresh leaves were collected and fixed with 2% paraformaldehyde, 2.5% glutaraldehyde, and 0.1 M sodium cacodylate buffer (pH 7.3) for 2 h. The leaves were transferred to Petri plates to run through the ethanol graded series (50, 70, 80, 90, and 100%). After dehydrating, samples

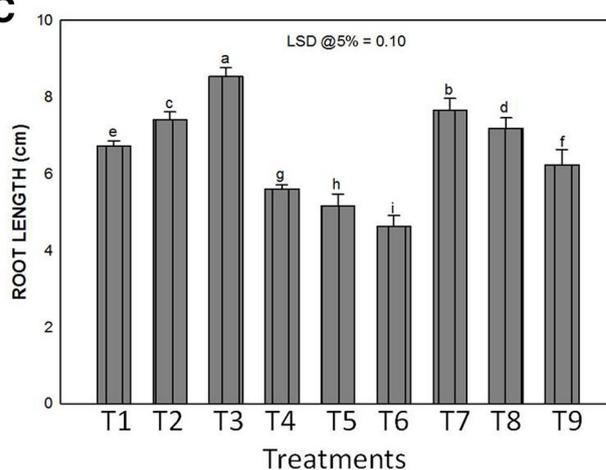
A



B



C



D

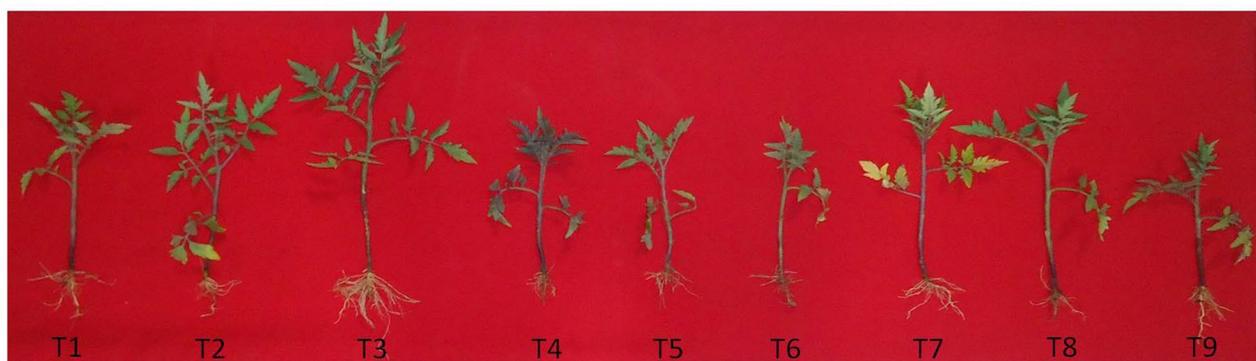


Fig. 1 Transmission electron microscope (TEM) images of ZnO-NPs at 100 nm and 20 nm (**a**); effect of zinc and zinc oxide nanoparticles on cadmium-induced changes in **b** shoot length, **c** root length and **d** growth (image) of *Lycopersicon esculentum* at 45 days. All data are means of 3 replicates ($n=3$); vertical bars show standard

error (\pm SE). Letters indicate a significant difference between control and treatment ($p < 0.05$). T1 control, T2 Zn (50 mg/L), T3 ZnO-NPs (50 mg/L), T4 Cd (0.4 mM), T5 Cd (0.6 mM), T6 Cd (0.8 mM), T7 Cd (0.4 mM)+ZnO-NPs (50 mg/L), T8 Cd (0.6 mM)+ZnO-NPs (50 mg/L), T9 Cd (0.8 mM)+ZnO-NPs (50 mg/L)

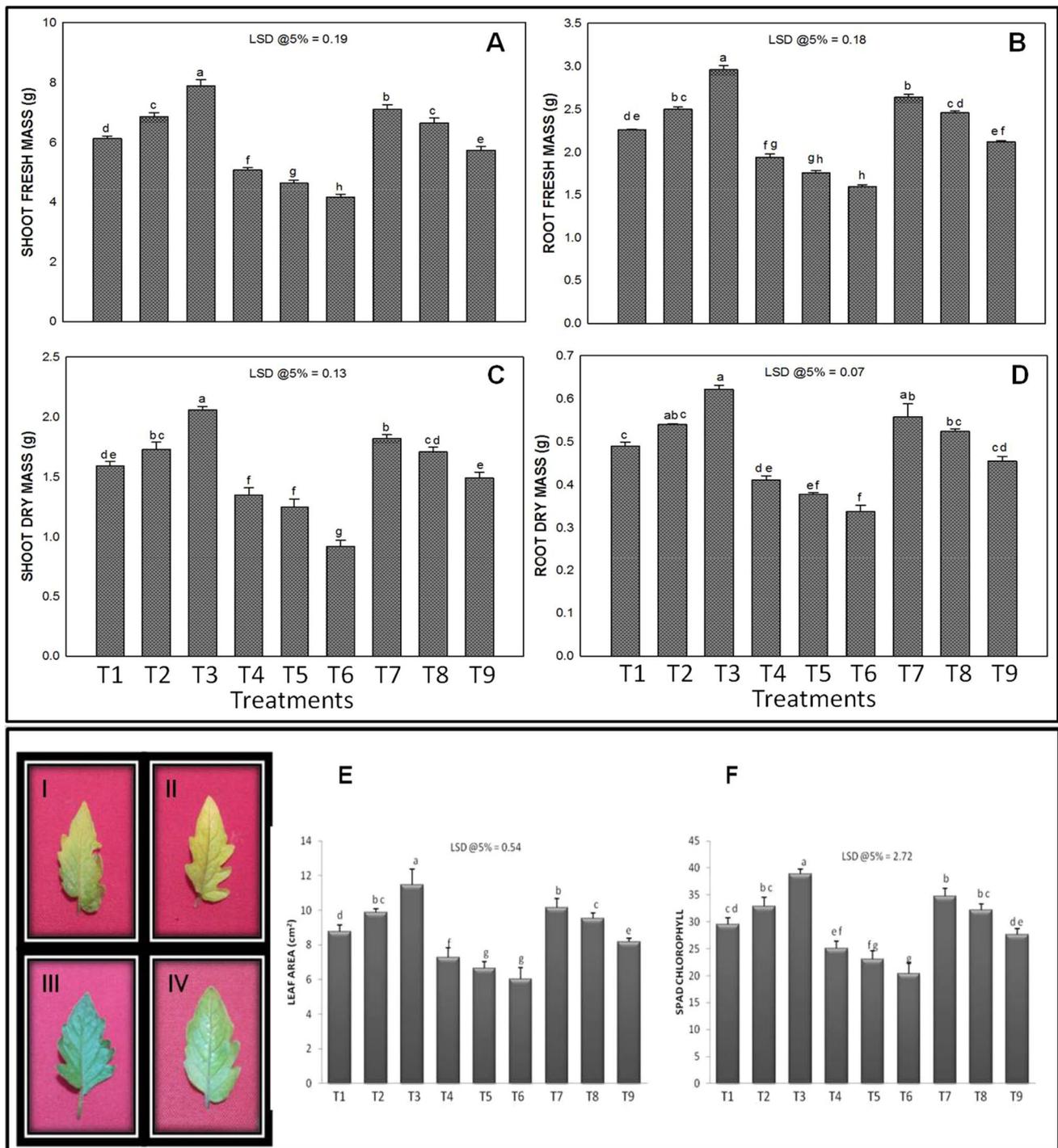


Fig. 2 Effect of zinc and zinc oxide nanoparticles on cadmium-induced changes in **a** shoot fresh mass, **b** root fresh mass, **c** shoot dry mass, **d** root dry mass, **e** leaf area, **f** SPAD chlorophyll of *Lycopersicon esculentum* at 45 days. (I), (II), (III), and (IV) represent control, cadmium (0.4 mM), ZnO-NPs (50 mg/L), and Cd (0.4 mM)+ZnO-NPs (50 mg/L) images, respectively. All data are

means of 3 replicates ($n=3$) and vertical bars show standard error (\pm SE). Letters indicate a significant difference between control and treatment ($p<0.05$). T1 control, T2 Zn (50 mg/L), T3 ZnO-NPs (50 mg/L), T4 Cd (0.4 mM), T5 Cd (0.6 mM), T6 Cd (0.8 mM), T7 Cd (0.4 mM)+ZnO-NPs (50 mg/L), T8 Cd (0.6 mM)+ZnO-NPs (50 mg/L), T9 Cd (0.8 mM)+ZnO-NPs (50 mg/L)

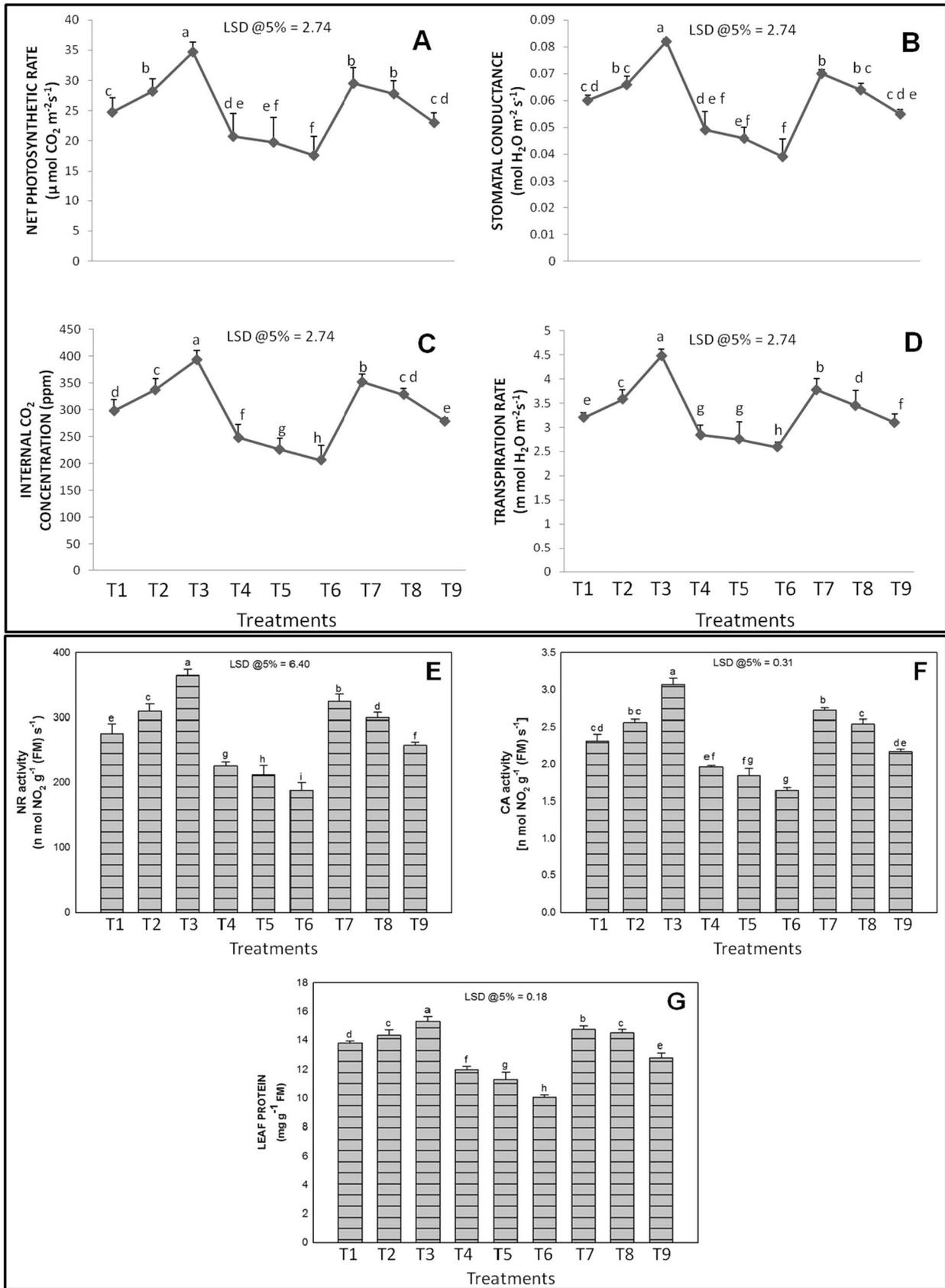


Fig. 3 Effect of zinc and zinc oxide nanoparticles on cadmium-induced changes in **a** net photosynthetic rate, **b** stomatal conductance, **c** internal CO₂ concentration, **d** transpiration rate, **e** nitrate reductase activity, **f** carbonic anhydrase activity, **g** leaf protein content in *Lycopersicon esculentum* at 45 days. All data are means of 3 replicates (*n*=3) and vertical bars show standard error (±SE). Letters indicate a significant difference between control and treatment (*p*<0.05). T1 control, T2 Zn (50 mg/L), T3 ZnO-NPs (50 mg/L), T4 Cd (0.4 mM), T5 Cd (0.6 mM), T6 Cd (0.8 mM), T7 Cd (0.4 mM)+ZnO-NPs (50 mg/L), T8 Cd (0.6 mM)+ZnO-NPs (50 mg/L), T9 Cd (0.8 mM)+ZnO-NPs (50 mg/L)

were coated with gold–palladium in a sputter coater (JEOL JFC-1600).

Compound Microscopy

Compound microscopy was used for detection of stomatal activity (Li et al. 2013). At least three leaves were collected per treatment and dipped in a NaOH (30%) solution

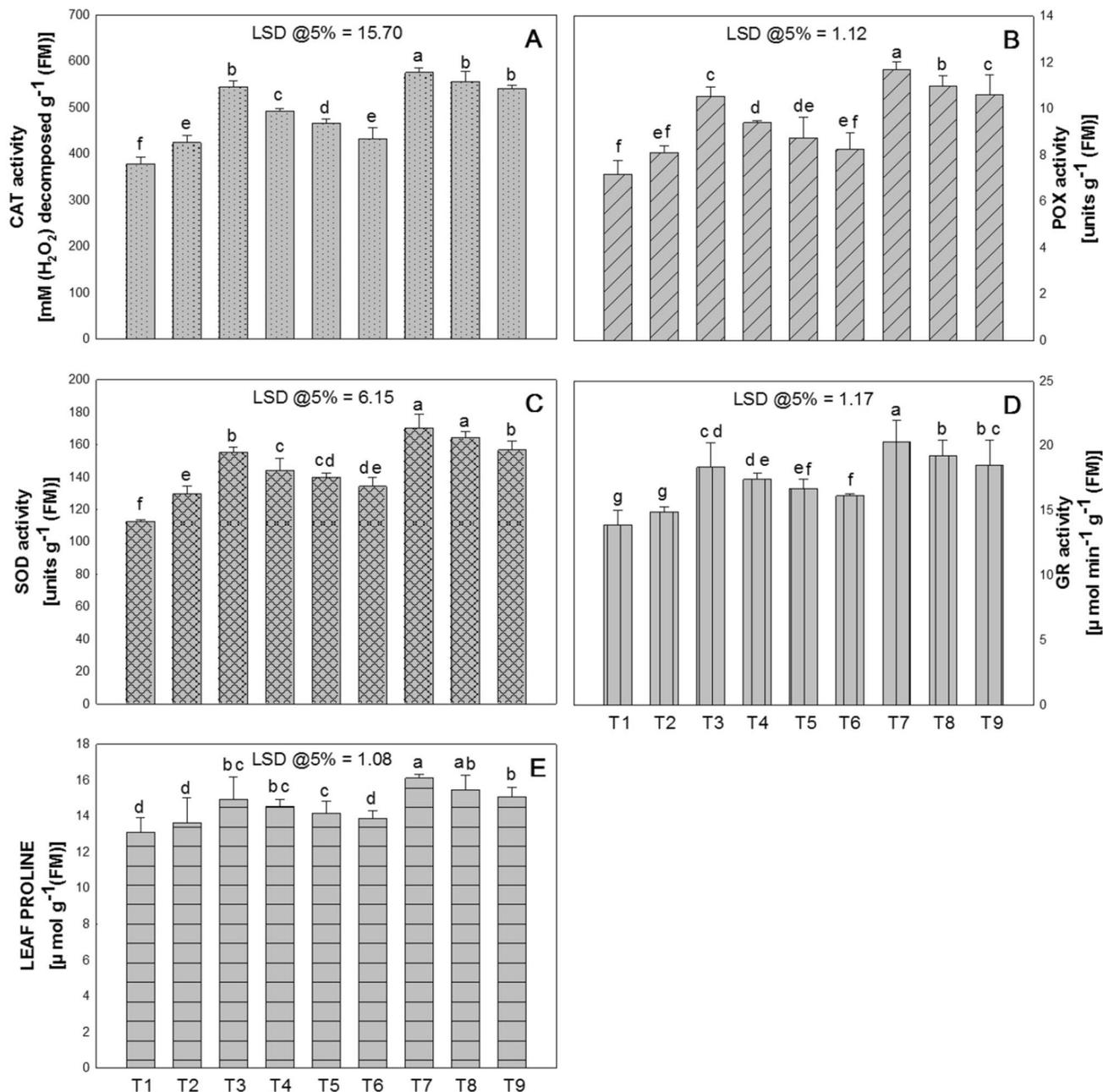


Fig. 4 Effect of zinc and zinc oxide nanoparticles on cadmium-induced changes on the activity of **a** catalase, **b** peroxidase, **c** superoxide dismutase, **d** glutathione reductase, **e** leaf proline content of *Lycopersicon esculentum* at 45 days. All data are means of 3 replicates (*n*=3) and vertical bars show standard error (±SE). Letters indicate a significant difference between control and treatment (*p*<0.05). T1 control, T2 Zn (50 mg/L), T3 ZnO-NPs (50 mg/L), T4 Cd (0.4 mM), T5 Cd (0.6 mM), T6 Cd (0.8 mM), T7 Cd (0.4 mM)+ZnO-NPs (50 mg/L), T8 Cd (0.6 mM)+ZnO-NPs (50 mg/L), T9 Cd (0.8 mM)+ZnO-NPs (50 mg/L)

Letters indicate a significant difference between control and treatment (*p*<0.05). T1 control, T2 Zn (50 mg/L), T3 ZnO-NPs (50 mg/L), T4 Cd (0.4 mM), T5 Cd (0.6 mM), T6 Cd (0.8 mM), T7 Cd (0.4 mM)+ZnO-NPs (50 mg/L), T8 Cd (0.6 mM)+ZnO-NPs (50 mg/L), T9 Cd (0.8 mM)+ZnO-NPs (50 mg/L)

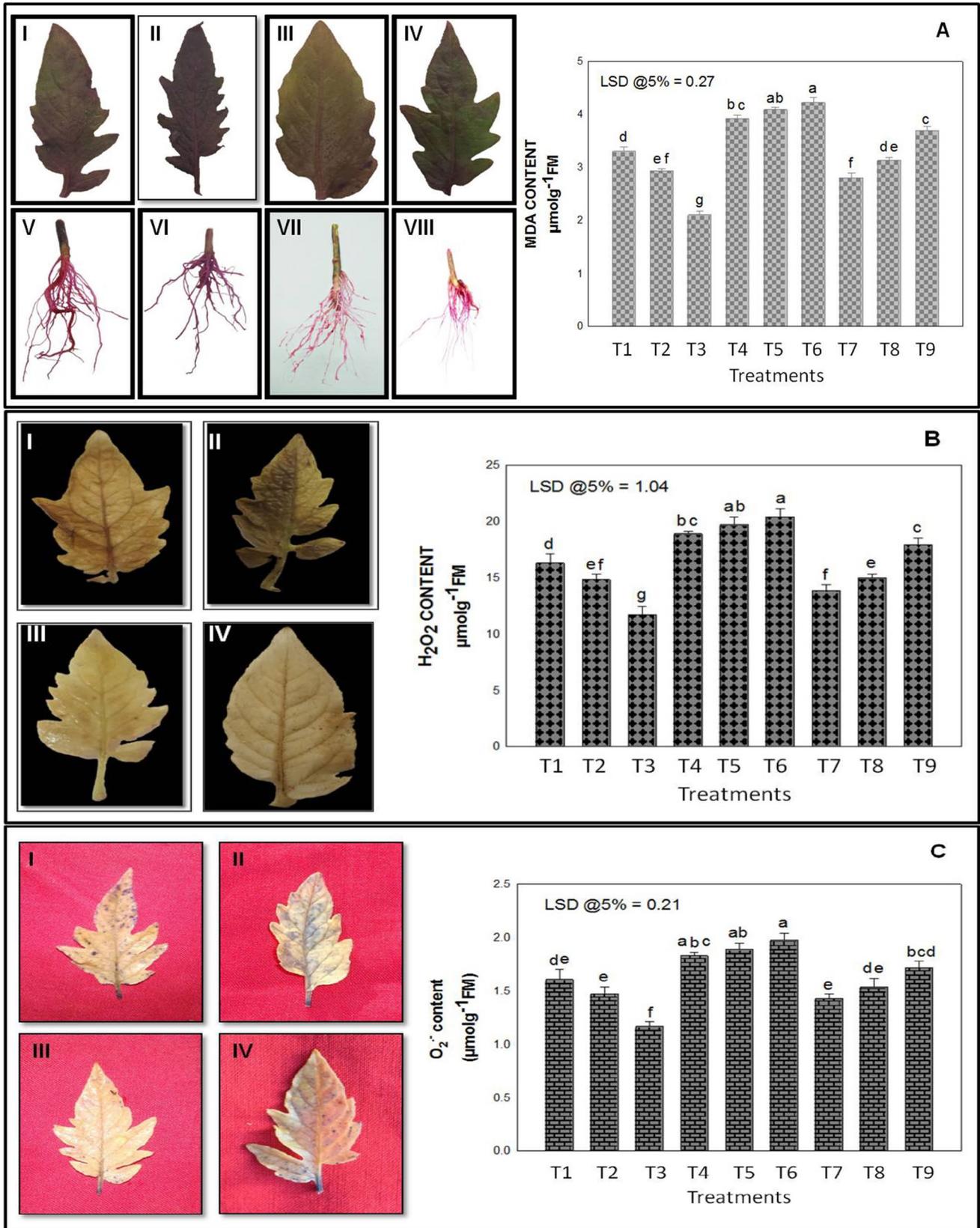


Fig. 5 Histochemical detection of a lipid peroxidation with Schiff's reagent; the intensive pink color indicates a specific reaction for lipid peroxidation, **b** peroxide localization; stereomicroscopic images depicting peroxide localization as brown spots on leaf surface, **c** superoxide localization; stereomicroscopic images depicting superoxide localization as blue spots on leaf surface in *Lycopersicon esculentum* at 45 days. (I), (II), (III), and (IV) represent control, treated with cadmium (0.4 mM), ZnO-NPs (50 mg/L) and Cd (0.4 mM)+ZnO-NPs (50 mg/L) leaf images, respectively, and (V), (VI), (VII), and (VIII) represent roots of the above treatments. Data presented in the graph shows the effect of Zn and ZnO-NPs on cadmium-induced changes on **a** MDA, **b** H₂O₂, **c** O₂⁻ content. The data are means of 3 replicates ($n=3$) and vertical bars show standard error (\pm SE). Letter indicate a significant difference between control and treatment ($p<0.05$). T1 control, T2 Zn (50 mg/L), T3 ZnO-NPs (50 mg/L), T4 Cd (0.4 mM), T5 Cd (0.6 mM), T6 Cd (0.8 mM), T7 Cd (0.4 mM)+ZnO-NPs (50 mg/L), T8 Cd (0.6 mM)+ZnO-NPs (50 mg/L), T9 Cd (0.8 mM)+ZnO-NPs (50 mg/L)

(for ease in removal of the epidermis). The abaxial surface was exposed under the cover slip to view in the microscope (Nikon ECLIPSE Ci-E) interfaced with a Nikon digital camera (DS-Filc).

Transmission Electron Microscopy

Particle size and shape of ZnO-NPs were recorded using TEM (Fig. 1a). The phase formed was reconfirmed using transmission electron microscopy (TEM) (JOEL/TEM2100).

Confocal Laser Scanning Microscopy

Cell viability tests were conducted using the method of Rattan et al. (2017). Roots were dipped in a solution containing 25 μ M propidium iodide for 10 min. The samples were then washed twice with DDW and placed on glass slides for viewing in a confocal laser scanning microscope (Zeiss, LSM 780) at $\times 20$ magnification with maximum excitation of 535–617 nm.

Statistical Analysis

Data were computed to calculate analysis of variance (ANOVA) using SPSS, 17.0 for Windows (SPSS, Chicago, IL, USA). Least significant difference (LSD) was calculated to separate means.

Experimental Results

Growth Biomarkers

Compared to the control, Cd (0.4, 0.6 or 0.8 mM) significantly reduced plant growth (shoot length, root

length, shoot and root fresh mass, dry mass and leaf area) (Figs. 1b, c and 2a–e). Moreover, the highest concentration of Cd (0.8 mM) was most toxic and decreased the shoot length (28%), root length (31%), shoot fresh mass (32%), root fresh mass (29%), shoot dry mass (28%), root dry mass (31%), and leaf area (32%), respectively, compared to the control. However, plants grown with ZnO-NPs experienced better growth over the control and also successfully countered the damaging effects of Cd completely at 0.4 and 0.6 mM and partially at 0.8 mM.

SPAD Chlorophyll

Exogenously applied ZnO-NPs significantly increased SPAD chlorophyll levels over the control (Fig. 2f). However, the presence of Cd caused a marked reduction in SPAD values. The foliar spray of ZnO-NPs reduced Cd toxicity in a concentration-dependent manner. The highest Cd concentration (0.8 mM) decreased the SPAD chlorophyll by 31% in comparison to the control.

Gaseous Exchange Parameters

Photosynthetic attributes (P_N , C_i , E , and g_s) declined significantly in the plants exposed to Cd (Fig. 3a–d). However, application of ZnO-NPs to leaves increased values of P_N (40%), g_s (38%), C_i (32%), and E (41%) over the non-treated plants (Fig. 3a–d).

Biochemical Determinations

The activity of NR and CA increased with the application of ZnO-NPs; however, Cd significantly reduced their levels in a concentration-dependent manner (Fig. 3e, f). The toxicity generated by Cd was totally or partially eliminated by application of ZnO-NPs.

Leaf Protein Content

Exposure of the seedlings to Cd resulted in a significant loss in leaf protein content (Fig. 3g). However, protein levels increased by application of ZnO-NPs, both in stressed and non-stressed plants. The plants subjected to ZnO-NPs showed subtle increase of protein content (11%) in comparison to control.

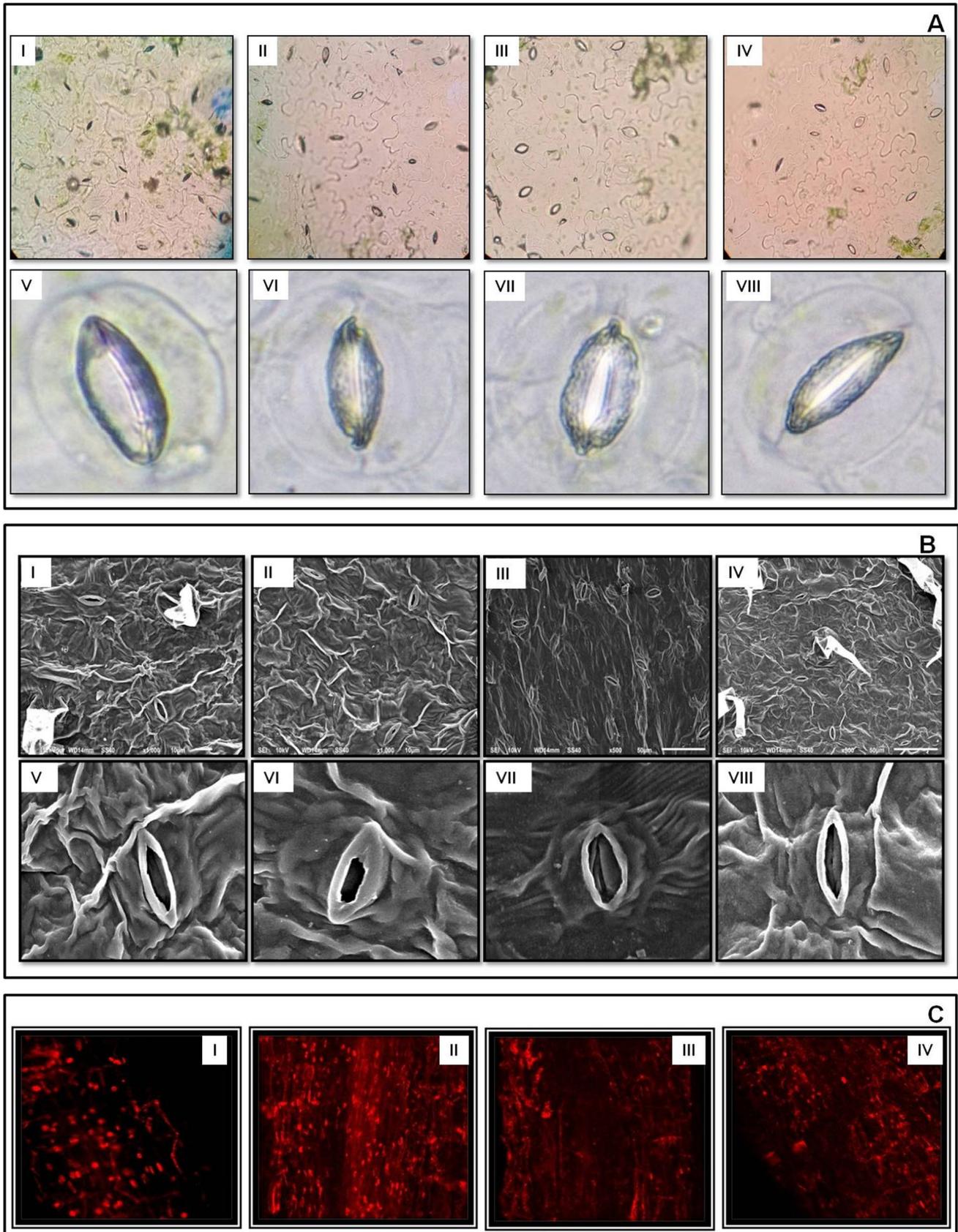


Fig. 6 Figure represents **a** compound microscopic, **b** scanning electron microscopic images of stomata, **c** confocal micrographic images of root cell of 45-day-old plant of *Lycopersicon esculentum*; (I), (II), (III), and (IV) represent control, treated with cadmium (0.4 mM), ZnO-NPs (50 mg/L) and Cd (0.4 mM) + ZnO-NPs (50 mg/L) images, respectively, and (V), (VI), (VII), and (VIII) represent their respective magnified images

Effect of ZnO-NPs on Antioxidant Metabolism of Tomato Plants Under Cd Stresses

CAT activity in plants treated with ZnO-NPs was enhanced by 44% over the control (Fig. 4a). However, Cd (0.4, 0.6 or 0.8 mM) decreased CAT activity in a concentration-dependent manner. Follow-up treatment of the Cd-stressed plants with ZnO-NPs increased CAT levels in comparison to control as well as in plants supplemented with ZnO-NPs alone. The maximum increase (51%) in CAT activity occurred in plants treated with Cd (0.4 mM) and amended with foliar spray of ZnO-NPs. Application of ZnO-NPs increased POX activity by 47% (Fig. 4b). The ZnO-NPs had an additive effect on POX activity in Cd-stressed plants; therefore, 63% higher enzyme activity was recorded in plants dosed with 0.4 mM Cd in association with the NPs over the control. SOD activity followed a rising pattern in response to both ZnO-NPs and Cd, in comparison to control plants (Fig. 4c). Cd-stressed plants (0.4, 0.6 or 0.8 mM) whose foliage also received ZnO-NPs as a follow-up treatment had 51, 46, or 39% more SOD activity over control plants.

Application of ZnO-NPs significantly increased GR activity in both stressed as well as non-stressed plants (Fig. 4d). Maximum level (46% greater than control) was recorded in plants dosed with Cd (0.4) along with ZnO-NPs. Compared to the control, the proline content improved in plants treated with Cd or ZnO-NPs (Fig. 4e). Moreover, the cumulative effect of Cd + ZnO-NPs triggered maximum levels of proline (23, 18, or 15% with the three respective levels of Cd, compared with the control).

Oxidative Stress Markers

On the basis of histochemical observations of leaves and roots, accumulation of MDA, H_2O_2 , and O_2^- content increased with dose of Cd. However, significant reduction in concentration (31% in MDA, Fig. 5a; 28% in H_2O_2 , Fig. 5b; and 31% in O_2^- , Fig. 5c) was determined in plants treated with ZnO-NPs.

Stomatal Response

Analysis of stomatal aperture revealed a clear-cut difference in response to ZnO-NPs, both in the presence and/or absence of Cd (Fig. 6a). Availability of Cd to plants reduced the

opening of the stomata; however, foliar application of ZnO-NPs alone and combined with Cd enlarged the stomatal orifice (Fig. 6a). SEM images of stomata also show somewhat similar results (Fig. 6b).

Confocal Microscopy

Cell viability could be examined visually by observing nucleic acid staining. Viability shows antagonistic effects with stained nuclei. In the present study, Cd retards the viability of the cell as compared to control (Fig. 6c). However, exogenous application of ZnO-NPs in the presence and/or absence of Cd increase the cell viability through staining lesser number of nuclei (Fig. 6c).

Discussion

The purpose of this study was to test the possibility of overcoming the adverse effects of a notorious heavy metal (Cd) in *Lycopersicon esculentum* by employing ZnO-NPs as a foliar spray. Plants treated with Cd experienced poor growth (shoot length, root length, shoot and root fresh mass as well as dry mass and leaf area) in a concentration-dependent manner (Figs. 1b, c and 2a–e). Several studies have revealed that Cd is toxic to plants and reduces growth and development (Abbas et al. 2017; Baycu et al. 2017; Qayyum et al. 2017). The presence of Cd in soil causes water scarcity by disturbing the water balance, which is considered the prime factor associated with loss of growth (Ekmekci et al. 2008). ZnO-NPs impart positive effects on plant growth up to a certain concentration (Pullagurala et al. 2018; Faizan et al. 2018). ZnO-NPs provide Zn^{2+} , an important micronutrient, which supports plant growth (Liu et al. 2015). The observed toxic effects of Cd can be neutralized by speeding the uptake of Zn and decreasing uptake of Cd in plants treated with ZnO-NPs (Sun et al. 2005; Hasan et al. 2008; Garg and Kaur 2013). Rizwan et al. (2019) reported that exogenously applied ZnO-NPs enhanced height and biomass of *Triticum aestivum* under Cd stress. Similar observations have been reported by Wang et al. (2018) and Hussain et al. (2018) in sweet sorghum and wheat, respectively.

The present investigation revealed that improved plant growth is the result of efficient photosynthetic machinery and enhanced chlorophyll content (Figs. 2f and 3a–d). Photosynthesis is one of the most important plant metabolic processes and is highly sensitive to environmental stresses (Khan et al. 2017). The presence of heavy metals (including Cd) imparted negative impacts on photosynthetic attributes and chlorophyll content, which are among the key symptoms of heavy metal toxicity in plants (Sharma and Dubey 2005; Malar et al. 2014; Manikandan et al. 2015). These symptoms may be caused by excessive production of H_2O_2 (Fig. 7c) or

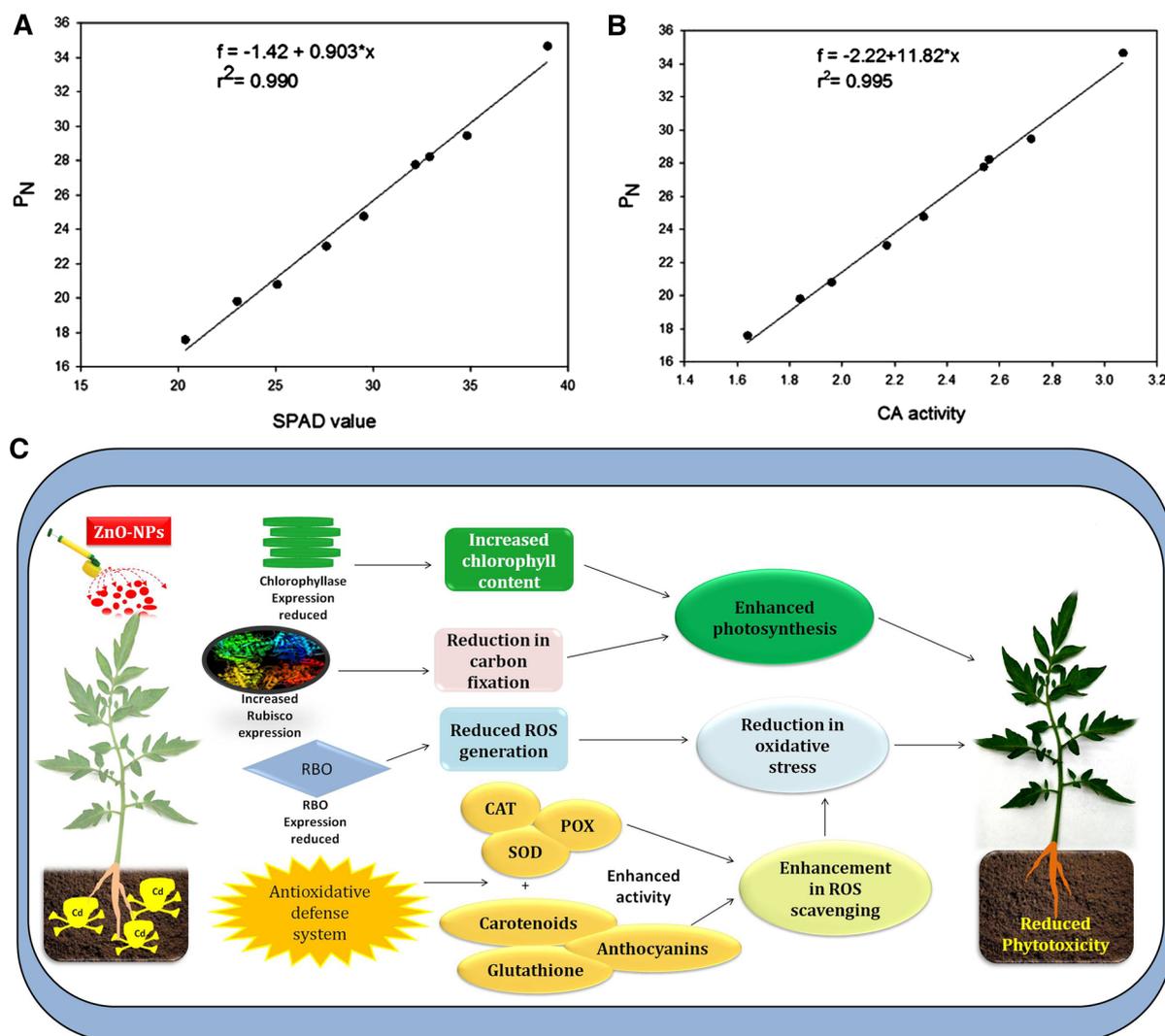


Fig. 7 Correlation coefficient values of net photosynthetic rate (P_N) with **a** chlorophyll content, **b** carbonic anhydrase activity, **c** describes various processes regulated by ZnO-NPs application for overcoming metal toxicity in plants. *RBO* respiratory burst oxidase, *ROS* reactive

oxygen species, *MDA* malondialdehyde, H_2O_2 hydrogen peroxide, O_2^- superoxide anion, *CAT* catalase, *POX* peroxidase, *SOD* superoxide dismutase

speeding up the destruction of chlorophyll by chlorophyllase (Hasan et al. 2011). Exogenous application of ZnO-NPs improved chlorophyll concentration in several plant species under stresses imparted by different metals (Venkatachalam et al. 2017; Hussain et al. 2018; Pullagurala et al. 2018; Rizwan et al. 2019). The findings of this study showed increased activity of the photosynthetic machinery along with chlorophyll content after ZnO-NPs exposure in stressed and stress-free plants (Figs. 2f and 3a–d). NPs enhance photosynthetic rate by accelerating the photolysis of water and the electron transport chain (Pradhan et al. 2013). According to Gao et al. (2006), activity of ribulose-1, 5-bisphosphate carboxylase/oxygenase enhanced after NP exposure which

was associated with increased photosynthetic rate. Our findings are consistent with those of Rajiv et al. (2018), Latef et al. (2017), and Venkatachalam et al. (2017). A positive correlation between P_N with SPAD chlorophyll and CA activity further supported the regulation of photosynthesis by varied factors (Fig. 7a, b).

The presence of Cd in soil significantly reduced the activity of NR and CA in *Lycopersicon esculentum* in a concentration-dependent manner (Fig. 3e, f). CA is a ubiquitous Zn metalloenzyme which catalyzes reversibly the conversion of CO_2 to HCO_3^- in plants. CA activity depends primarily on availability of Zn, hormonal signaling, concentration of CO_2 , photon flux density and the regulation of genetic

expression of the transcripts (Xin-Bin et al. 2001; Tiwari et al. 2005). The stress generated by Cd reduced intercellular CO₂ concentration (Fig. 3c) and reduce the availability of Zn that retard the CA activity (Fig. 3f). In contrast, ZnO-NPs increased stomatal conductance and intercellular CO₂ concentration, both in the presence and/or absence of Cd (Fig. 3b, c), favoring higher CA activity (Fig. 3f) (Siddiqui and Al-Whaibi 2014). Nitrate reductase catalyzes the NAD(P)H-mediated reduction of nitrate (NO₃⁻) to nitrite (NO₂⁻) (Campbell 1999) to ensure sufficient supply of nitrogen to plants for healthy growth (Srivastava 1995). Our observations demonstrate that ZnO-NPs enhanced activity of NR both in stressed and stress-free plants (Fig. 3e). Dubchak et al. (2010) demonstrated that, due to their high surface area, NPs interact with cellular biomolecules and speed up several biochemical pathways.

In the current study, Cd supplied to tomato plants resulted in reduced protein concentration (Fig. 3g). This could be due to a reduction in protein synthesis under Cd stress and the degradation of proteins by protease activity, leading to an enhanced degree of protein denaturation (Palma et al. 2002; Balestrasse et al. 2003). ZnO-NPs as foliar spray increased protein content compared with control plants and also overcome the loss caused by the presence of Cd (Fig. 3g). Zinc is known to increase the capacity of ionic interconversions, which favors nitrogen uptake leading to higher protein content (Lawre and Raskar 2014). Similar observations have been reported earlier (Krishnaraj et al. 2012; Tripathi et al. 2015).

Normal plant metabolism of O₂ generates ROS [superoxide (O₂⁻), singlet oxygen (¹O₂), hydroxyl radical (HO[•]), and H₂O₂] as byproducts which hold promising roles in cell signaling and homeostasis (Ray et al. 2012). Therefore, in an oxidizing environment (particularly photosynthesis and respiration) ROS are produced continuously in peroxisomes, mitochondria, and chloroplasts. However, an excess in ROS causes oxidative stress, damage to DNA, proteins and lipids, and finally cell death (Tripathy and Oelmüller 2012). To overcome the toxic effects of oxidative stress plants activate enzymatic (CAT, POX, SOD, and GR) and non-enzymatic (proline) antioxidants (Tripathy and Oelmüller 2012; Sewelam et al. 2016) and (Fig. 4a–e) which comprise key components of the plant defense mechanism (Andre et al. 2010). ZnO-NPs decreased the oxidative stress in *Leucaena leucocephala* plants under metal stress (Venkatachalam et al. 2017). In the current study, application of ZnO-NPs to foliage in both stressed and stress-free environments enhanced the activity of antioxidant enzymes and increased proline content (Fig. 4). Lopez-Moreno et al. (2010) and Ghosh et al. (2016) also observed an improvement in the antioxidant defense system under ZnO-NPs treatment, which is a consequence of the expression of genes (Nair and Chung 2014) or its involvement in different oxidative processes

(Hossain et al. 2015). Other plants respond similarly to ZnO-NPs by elevating efficacy of antioxidant systems and activities of CAT, POX, and SOD (Singh et al. 2013; Soliman et al. 2015; Latef et al. 2017; Kantabathini et al. 2018; Wang et al. 2018; Rizwan et al. 2019).

Levels of MDA, H₂O₂, and O₂⁻ are key parameters which indicate the rate of oxidative damage in plants exposed to adverse environmental conditions. Increases in MDA, H₂O₂, and O₂⁻ levels were observed in the Cd-doped plants (Figs. 5a–c and 7c) which indicates that the metal causes cellular metabolic imbalance by increasing lipid peroxidation through formation of free radicals (Mahajan and Tuteja 2005). An important finding in the present study is that application of ZnO-NPs to foliage of stressed plants reduced MDA, H₂O₂, and O₂⁻ content (Figs. 5a–c and 7c). This appears to be a reflection of the elevated activity of CAT, POX, and SOD (Figs. 4a–c and 7c). It may, therefore, be concluded from the above discussion and from the reports of Burman et al. (2013) and Pullagurala et al. (2018) that ZnO-NPs relieve tomato plants from severe damage from Cd stress.

Conclusion

Foliar application of ZnO-NPs reduces the negative impacts of Cd to tomato plants by enhancing production of photosynthetic pigments, adjusting osmoregulation and decreasing contents of H₂O₂, MDA, and O₂⁻. Furthermore, protection under Cd stress was achieved through successfully modified biochemical pathways and a robust antioxidant system. However, studies at the field level using different crops, soil types, and ZnO-NPs of various sizes are needed before recommendations can be confirmed.

Acknowledgement M. Faizan gratefully acknowledges the Chairman, Department of Botany, Aligarh Muslim University, for providing all necessary facilities in carrying out this work. Authors are thankful to Prof. John Pichtel, Ball State University, USA for correcting the manuscript for English grammars.

Funding This research did not receive any grant from agencies in the public, commercial, or not-for-profit sectors.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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