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Soil and Plant Enzymes Responses to Zinc Oxide Nanoparticles in Submerged Rice (*Oryza sativa* L.) Ecosystem

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Abstract

In the present study, the effects of zinc oxide nanoparticles (ZnO NPs) on rice (*Oryza sativa* L. cv. PB1509) plant growth were assessed in hydroponics (5, 10, 25, 50 mg L^{-1}) and soil microcosm (5, 10, 25 50 mg kg^{-1}) experiments. In both hydroponics and soil experiments, Zinc (Zn) accumulation in plant parts (roots, shoots and grains) was found to increase with increasing doses of ZnO NPs. Grains accumulated 29 mg kg^{-1} Zn at 50 mg kg^{-1} ZnO NPs in the soil experiment. In the hydroponics experiment, growth and photosynthetic pigments were induced by ZnO NPs up to 10 mg L^{-1}, while higher doses of 25 and 50 mg L^{-1} were toxic to plant growth. Antioxidant enzyme activities (SOD, CAT, APX and GPX) were mostly increased or unaffected by all ZnO NPs doses. In soil experiments, acid and alkaline phosphatase activities were increased at 5 mg kg^{-1} followed by a declining trend. However, a significant decrease occurred only at 50 mg kg^{-1}. Urease activity in soil was significantly increased at all doses of ZnO NPs, while the activity of dehydrogenase did not show any significant change up to 25 mg kg^{-1}. The length of plants and photosynthetic pigments did not show much toxicity except root length beyond 10 mg kg^{-1}; however, the biomass of plants including grains was significantly lower than control beyond 5 mg kg^{-1} dose. The activity of antioxidant enzymes (GPX, APX and CAT) showed a significant increase at all doses of ZnO NPs. The DTPA extractable Zn concentration in the soil was significantly elevated with increasing exposure concentrations of ZnO NPs. Based on hydroponics and soil experiments, this study suggests a dose of up to 10 mg L^{-1} or 10 mg kg^{-1} would be an appropriate dose for augmenting the growth of rice plants and Zn accumulation, and this can be practically utilized for rice plants growing in submerged conditions.

Keywords: Antioxidant enzymes, DTPA extractable Zn, Hydroponic and soil experiment, Plant growth, *Oryza sativa*, SEM, TEM, UV-visible spectrophotometry, XRD, Zn deficiency, Zinc oxide nanoparticle

Introduction

Nanotechnologies play with materials at the nanometre scale, which are developed either by scaling up from groups of atoms or by scaling down from bulk materials. Nanotechnologies have the potential to bring benefits in diverse areas such as water decontamination, pesticides, and fertilizers in agriculture, drug development, information and communication technology, fire safety, environmental remediation, plant pathogen protection, etc. [1-5]. The vast potential of nanotechnology emanates from the distinct and unique properties of nanoparticles (NPs) like large surface area and quantum size effects that are not observed with solid, liquid, gaseous, and plasma states. The number of products containing NPs as one of the components is increasing continuously [6]. The annual global production of metal-based NPs is about 267,000 to 318,000 tons [4,5] and is expected to increase in the coming years. Nanoparticles like nano-silica and fullerene have shown the ability to reduce the toxicity and uptake of toxic heavy metal(loid)s [7].

Zinc (Zn) is an essential element for both plants and animals [8]. Being the least available micronutrient worldwide, it becomes necessary to supply this nutrient exogenously in the form of different zinc fertilizers [9,10]. Singh *et al*. compared the effects of the nano-form of ZnO with the bulk form of ZnO on rice plants in hydroponic conditions and observed biomass accumulation to be higher in seedlings treated with nano-form of ZnO [11]. Dimpkpa *et al*. reported that under low NPK fertilization, ZnO NPs increased growth, nutrient absorption and Zn content of sorghum crops to a greater extent than that of $ZnSO_4$ [12]. Other studies also demonstrate better growth augmentation and Zn enrichment in plants with nano-Zn fertilizer in comparison to $ZnSO_4$ [13]. ZnO NPs have found application in diverse areas and products, such as skin care products [14], water remediation [15], as sensors for detecting pollutants [16,17], and so on. Zinc has a role in the function of several enzymes, and Zn deficiency in both plants and humans causes growth retardation [18]. The ZnO NPs, therefore, find wide application in the agricultural sector for the augmentation of growth and stress tolerance of crop plants [19]. ZnO NPs can enhance plant biomass, photosynthetic pigments [20], the activity of antioxidant enzymes [21], and induce molecular changes also [22].

Zinc is not a prevalent contaminant in paddy and other soil. However, agricultural fields nearer to smelters, mines, and fertilizers industries get incidentally contaminated by the discharges of smelter slags and wastes, mine tailings, coal and bottom fly ash, and the use of commercial products such as fertilizers and wood preservatives that contain Zn. Due to a wide range of applications, it is expected that ZnO NPs can be accidentally or unknowingly released into the environment [23]. This has raised concerns over the widespread usage of ZnO NPs as higher than required quantities of these NPs can pose toxicity to the ecosystem. Lin and Xing evaluated the phytotoxicity of 5 different NPs, including ZnO, on seed germination and root growth of 6 higher plant species, and ZnO NPs were observed to pose toxicity [24]. Song and Lee observed high doses of ZnO NPs (1,000 mg L^{-1}) to be toxic to aquatic plants, *Hydrilla verticillate*, and *Phragmites australis* [23]. The toxicity of NPs to plants also depends on the type of plant and environmental conditions. For example, Bai *et al*. found that C3 plants (wheat and rice) suffered from greater toxicity than C4 plants (amaranth and maize) upon exposure to different NPs [25].

Zinc oxide (ZnO) nanoparticles may provide a more soluble and plant available source of Zn in Zn fertilizers due to their greater reactivity compared to equivalent bulk ZnO and promote plant growth and yield [26]. For instance, the use of 10 mg kg^{-1} of ZnO NP enhanced the biomass and photosynthesis of lettuce grown in soil [27]. The foliar application of ZnO NPs increased plant defense mechanisms against biotic stress, mainly microbial pathogens, by developing a systemic defense in plants [28]. The priming of rice seeds with 30 % PEG containing ZnO NPs (0 - 750 mg L^{-1}) enhanced amylase activity and sugar content [29]. For the Zn nutrition in rice grain, ZnO NPs application significantly increased the Zn content of edible polished rice and promoted the relocation of Zn from the aleurone layer [30]. Foliar application of Zinc oxide nanoparticles (ZnONPs) and/or salicylic acid significantly overcome the toxicity of arsenic in rice plants [31]. Yang *et al*. reported that ZnO nanoparticles' phytotoxicity to rice plants is size and concentration dependent. They reported that the critical concentration and size of the ZnO nanoparticle for rice growth are 2,000 mg L^{-1} and < 50 nm [32]. The rice seed pre-conditioning with ZnONPs at a concentration of 25 ppm is helpful in increasing yield attributes and in alleviating drought induced damages [33]. Synergistic impact of green synthesized zinc oxide nanoparticles (ZnO-NPs) (5 - 10 mg/L) and *Bacillus* spp. (*Bacillus cereus* and *Lysinibacillus macroides*) have been observed in the improvement of physiological and biochemical activities of rice seedlings under heavy metals contaminated wastewater [34].

Due to the potential of NPs to affect the physiological and biochemical processes of both aquatic and terrestrial plants, their optimum dose needs to be identified for different plants, and studies on the toxicity of crop plants become necessary. The use of an optimum dose of ZnO NPs can positively regulate plant growth, metabolism and net productivity in a given environmental condition and soil geochemistry.

Rice (*Oryza sativa* L.) is cultivated in approximately 160 mha of areas worldwide. Rice is an important staple food feeding more than half of the world's population and thus, it contributes to the food security of the world with an estimated production of 513.02 million tons in 2021 - 2022. The global rice trade is projected to expand by 0.1 million tons in 2022 to reach 46.4 million tons [35]. India is one of the largest exporters of rice in the world and it is cultivated in large areas of the country by millions of farmers [36]. Therefore, rice cultivation is important for the economic security of Indian farmers. The growth, development, and production of rice plants are affected by several toxic chemicals and elements on 1 hand and by the deficiency of essential elements on the other [37,38]. Both zinc deficiency and toxicity have been observed in Indian soils [39]. There are studies on the effect of NPs on rice plant growth and development in stressed conditions [25,40].

Zinc fertilizer application through foliar and soil can increase the absorption and transformation of Zn to the edible segments of plants. Zinc application through conventional fertilizer is prone to be fixed and adsorbed by iron and aluminium oxides, clay minerals, and humus in the soil, reducing its effectiveness in the soil. ZnO NPs are non-saturable and can be integrated with other atoms and stabilized, exhibiting high chemical activity. As the particle size decreases, the surface area, surface energy, and surface binding energy of ZnO NPs particles increase rapidly [41]. Therefore, compared with traditional fertilizers, nano-zinc fertilizers are less affected by soil texture, structure and colloidal content and are easily absorbed and utilized by plants. Most ZnO NPs are combined with traditional fertilizers to develop new fertilizers or are used as seed dressing agents [42].

However, there is a need to optimize the dose of ZnO NPs for rice plants. This study, therefore, examined the effects of ZnO NPs on rice plants to optimize their dose for application. This is a comprehensive study that has been done in an anoxic rice ecosystem in 2 different mediums (soil and hydroponics) to see the effect of Zone NP on plant growth and rhizospheric enzyme activities. The hypothesis was that the effects of ZnO NPs would be dose-dependent and differential in hydroponics and soil experiments. This would enable the selection of the optimum dose of ZnO NPs for rice plant growth, which is grown in semi-aquatic conditions.

Material and methods

Characterization of nanoparticles

ZnO nanoparticles were purchased from Sigma-Aldrich, Saint Louis, MO 63103, USA, with a purity of 97.0 % and an average particle size of ≤ 50 nm. The ZnO nanoparticles used in the study were also characterized for their optical and nano-structural properties. The size distribution of ZnO NPs was evaluated by transmission electron microscopy, the JEOL JEM1011 (JEOL USA, Inc, Peabody, MA) [20]. The UV-VIS spectrum of ZnO dispersed in water was recorded using a UV-VIS spectrophotometer (JASCO double-beam UV-VIS spectrophotometer model V-530) with a resolution of 2 nm. The X-ray diffraction pattern for the ZnO NPs was recorded using an X-ray diffractometer (Philips X'pert Pro XRD unit) with Cu K_α radiation of wavelength $\lambda = 0.1541$ nm in the scan range $2\theta = 20 - 90$ °. The Attenuated total reflection infrared (ATR-IR) spectroscopy was employed to characterize ZnO NPs using the ALPHA-P model, Bruker Optics, Germany. The Scanning Electron Microscope - Energy Dispersive X-ray Spectrometry system (SEM-EDS) Hitachi SU 3,500 was used to record SEM images and for the elemental analysis.

Experimental site and crop selection

Soil microcosm pot and hydroponics experiments were set up on the premises of the glass house at the Centre for Environmental Science and Climate Resilient Agriculture, Indian Agricultural Research Institute, New Delhi. Rice (*Oryza sativa* L. cv. PB 1509) was selected for the research work because rice is a good indicator crop for Zn deficiency as well as toxicity. In experimental soil, total Zn content was 52.1 mg kg^{-1}, and Diethylenetriaminepentaacetic acid (DTPA) extractable Zn, which indicates the plant an available Zn, was 0.8 mg kg^{-1} (**Table 1**). We selected 0, 5, 10, 25 and 50 (mg L^{-1} for hydroponic and mg kg^{-1} for soil experiment uniformly). The critical limit of DTPA extractable Zn is 1.5 - 2 mg kg^{-1} for rice crops, which indicates the soil used in this study was deficient in Zn [43]. Zinc application is recommended when soils have available Zn level below the critical level. During flooding or inundation (anaerobic conditions), plant available Zn decreases because of the formation of insoluble compounds like $Zn(OH)_2$ and ZnS [44]. In this context, 4 fertilizer doses were chosen to see the response of ZnO NP fertilization to rice crops.

Table 1 Some physicochemical properties of experimental soil.

Sr. No.	Parameter	Value
1	Particle size analysis	
	Sand (%)	50
	Silt (%)	24
	Clay (%)	25
	Textural class	Sandy clay loam
2	pH (1:2.5)	7.9

Sr. No.	Parameter	Value
3	Electrical Conductivity (E.C.) (dS m^{-1})	0.370
4	Cation Exchange Capacity (C.E.C.) (C mol (p+) kg^{-1})	14.20
5	Organic Carbon (g kg^{-1})	3.568
6	Calcium carbonate (%)	3.60
6	Total N (%)	0.021
7	Available N (kg ha^{-1})	105
8	Total P (kg ha^{-1})	450
9	Available P (kg ha^{-1})	34.6
10	Available K (kg ha^{-1})	294.5
11	Total Zn (mg kg^{-1})	52.1
12	DTPA extractable Zn (mg kg^{-1})	0.8

Hydroponics experiment

Rice seeds were immersed in a 2.5 % sodium hypochlorite solution for 15 min for surface sterilization and grown in the sand for 10 days. After this, plants were grown in a 1 L plastic container (15 cm in height×10 cm in diameter) filled with modified Hoagland's solution with different concentrations of Zn by using ZnO NPs (0, 5, 10, 25, 50 mg L^{-1}). Initially, plants were maintained in a 1/4th strength of modified Hoagland's solution for 2 days and then transferred to a full strength modified Hoagland's solution for 21 days. Each container had 4 equal size rice plants. Each treatment was replicated 4 times with a control. The plants were provided with 14 h of artificial photoperiods, 60 - 70 % relative humidity, 25/20 °C day/night temperature, and a light intensity of 16,500 lx (lux) along with aeration to maintain proper mixing of the solution. At harvesting, whole seedlings were harvested and roots and leaves were separated for various analyses. All the analyses were performed with 4 biological replicates with each replicate representing 5 seedlings.

Soil experiment

A net house pot culture experiment was conducted to evaluate the different doses of ZnO NPs on rice during the *Kharif* season. The soil from 0 - 15 cm depth from an uncultivated field was collected, air-dried, and ground to pass through a 2-mm sieve. Some important physicochemical properties of experimental soil were analyzed following the standard procedures. The particle size distribution of the soils was determined by the international pipette method [45]. Soil pH was measured in a 1:2.5 soil: Water ratio. Soil electrical conductivity (EC) was measured at a soil-to-water ratio of 1:2.5. Soil organic carbon (OC) was determined by the wet digestion method as described by Walkley and Black [46]. The cation exchange capacity (CEC) was determined by the ammonium acetate method [45]. Available N, P, and K were determined by alkaline potassium permanganate [47], Olsen extraction [48], and neutral ammonium acetate [45], respectively.

Soil micronutrients were extracted by DTPA at pH 7.3 using a 1:2 soil: Solution ratio as outlined by Lindsay and Norvell [49]. The amount of extractable Zn was estimated by an atomic absorption spectrometer. Some physicochemical properties of experimental soil are presented in **Table 1**. The soil was spiked with ZnO NPs by using different concentrations of Zn, *viz.*, 0, 5, 10, 25, and 50 mg kg^{-1} in soil. The spiking was done 3 weeks before the experiment started, and the soil was continuously mixed every 2nd day to prepare homogenously spiked soil. Two kg of spiked soil were filled into a plastic pot (6 inches in diameter). Four rice plants were transplanted into each pot. All the pots were maintained in flooded conditions. All the treatments were done in 4 replicates. The experimental pots were under regular observation to find out if there were any toxicity symptoms. Plant samples were collected at the maturity stage. Roots, shoots (flag leaves) and grain samples were properly washed first with distilled water, then with 0.1 M HCl, and again with distilled water and dried with blotting paper. After, that plant samples were dried at 60 °C for 56 h in a hot air oven. After complete drying, the dry matter yield of roots, shoots and grains were recorded. All the analyses were performed with 4 biological replicates, with each replicate representing 5 plants.

Enzymatic activities in soil

The fresh soil sample was collected at the anthesis stage to estimate the activities of acid phosphatase, alkaline phosphatase, urease and dehydrogenase. The estimation of phosphatase activity in soils was done following the procedures described by Tabatabai and Bremner [50]. One g of soil was placed in a 50 mL Erlenmeyer flask and treated with 0.25 mL of toluene, 4.0 mL of modified universal buffer (MUB) at pH 6.5 for acid phosphatase and at pH 11.0 for alkaline phosphatase; 1 mL of p-nitrophenyl phosphate solution was made in the same buffer. After stoppering the flasks, the contents were mixed and incubated for 1 h at 37 °C. Then, 1 mL of 0.5 M $CaCl_2$ and 4 mL of 0.5 M NaOH were added. The soil suspension was filtered through a Whatman no. 42 filter paper and the absorbance was measured at 400 nm by a UV-Visible Spectrophotometer.

Urease enzyme activity was determined by the procedure described by Bremner and Douglas [51]. Five g of fresh soil was taken in a 125 mL polypropylene bottle and treated with 5 mL of urea solution followed by incubation at 37 °C for up to 5 h. Then, 50 mL of 2M KCl-PMA solution was added and shaken for 1 h and filtered through Whatman no. 42 filter paper. One mL aliquot of the extract containing up to 200 mg L^{-1} of urea was taken into a 50 mL volumetric flask and the volume was made up to 10 mL with 2M KCl-PMA solution. To this, 30 mL of colouring reagent was added and kept in a boiling water bath for 30 min. After cooling, the final volume was made to 50 mL, and the absorbance of the developed red colour was measured at 527 nm. Dehydrogenase enzyme activity was determined by the procedure described by Klein *et al*. [52]. To 1 g of air-dried soil taken in an airtight screw-capped test tube, 0.2 mL of 3 % TTC solution was added to saturate the soil. After this, 0.5 mL of 1 % glucose solution was added and the tubes were gently tapped at the bottom to drive out all trapped oxygen. The tubes were incubated at 28 °C for 24 h, followed by the addition of 10 mL methanol and allowed to stand for 6 h. The absorbance of pink formazan was determined using a spectrophotometer at 485 nm.

Estimation of available zinc in soil

Soil samples were collected at the physiological maturity of the crop for the analysis of available Zn in the soil. The estimation of available Zn in soils was done following the procedures described by Lindsay and Norvell [49]. Extraction of soil sample was performed by diethylenetriaminepenta acetic acid (DTPA; 0.005 M) - calcium chloride ($CaCl_2.2H_2O$; 0.01 M) - triethanolamine (TEA; 0.1 M). Each of the collected soil samples (10 g) was taken into a 100 mL conical flask and 20 mL of extracting reagent was added to each flask. The flasks were shaken at 120 rpm for 2 h on a mechanical shaker and then filtered through Whatman No. 42 filter paper. Filtrates were made up to 20 mL and used for the estimation of available Zn content using an Atomic Absorption Spectrophotometer (AAS, Shimadzu Model AA 7000).

Zinc analysis in plant samples

For Zn analysis, dried plant samples were grounded in a sample grinder to a smooth-textured powder for analysis. The samples were digested by the di-acid (nitric acid and perchloric acid in a 5:1 ratio) digestion method [53], and estimation of Zn in the acid digested samples was done with Atomic Absorption Spectro-photometer (AAS) (Shimazdu Model AA 7000).

Assay of photosynthetic pigments and antioxidant enzyme activities

The level of photosynthetic pigments was determined by the methods of Arnon [54], and Duxbury and Yentsch [55]. About 200 mg of plant samples from freshly harvested seedlings were used for enzyme extractions and assays. The sample was made to a fine powder with liquid nitrogen and enzymes were extracted in 0.1 M sodium phosphate buffer at pH 6.2. Then the homogenate was centrifuged at 10,000 rpm for 15 min at 4 °C and the supernatant was used for enzyme activity determinations. The antioxidant enzymes, i.e., superoxide dismutase (SOD), guaiacol peroxidase (GPX), ascorbate peroxidase (APX), peroxidase (POX), and catalase (CAT) were determined by using the following methods described by Singh *et at.* [56].

Statistical analysis

Data of 4 separate replications are expressed as the mean ± SD. One-way Analysis of Variance (ANOVA) and post-hoc Duncan's Multiple Range test (DMRT) analysis were performed to determine to know the variability among the treatments ($p \leq 0.05$) [57]. Microsoft Excel program was used to calculate various statistical parameters.

Results and discussion

Characterization of zinc oxide nanoparticles

The average particle size in XRD (**Figure 1**(**A**)) was determined using Scherrer's formula from the peak corresponding to the highest intensity was centered around 36.23 degrees belonging to the plane of ZnO [58]. It was approximately 18 nm (17.9 nm). The absorption spectrum of ZnO nanoparticles is shown in **Figure 1**(**B**). It exhibits a strong absorption at about 380 nm. The very sharp absorption of ZnO indicates the monodispersed nature of the nanoparticle distribution [59]. The TEM images (**Figures 1**(**C**) - **1**(**D**)) showed that individual primary particles are mainly spherical in shape, with a size between 15 and 20 nm, which was in good agreement with the particle sizes (18 nm) calculated from Scherrer's formula. The average zeta potential of ZnO NPs was 22.9 ± 1.2 mV. The microstructure study was conducted using scanning electron microscopy (SEM) (**Figure 1**(**E**)). Energy-dispersive X-ray analysis of the ZnO NP showed the presence of zinc and oxygen elements (**Figure 1**(**F**)). Therefore, from SEM-EDS spectra, it is clear that it is a pure ZnO nanoparticle and absent of traces of other elements. The ATR-IR analysis of zinc nanoparticles was performed to identify the various functional and characteristic groups associated with the synthesized nanoparticles (**Figure 1**(**F**)). It is inferred that the samples have absorption peaks in the range of 3,485.58, 2,554.18, 1,982.35, 1,487.58, 1,002.21, 871.93 and 663.45 cm^{-1}. The vibration mode associated with metal-oxygen (ZnO stretching vibrations) corresponds to the absorption peak at 663.45 cm^{-1}. The stretching vibration of the C-N bond in the primary amine or the stretching vibration of the C-O bond in the primary alcohol is responsible for the peak at 1,002.21 cm^{-1}. Alkyl and aromatic nitro compound vibration modes are attributed to the peak at 1,487.8 cm^{-1}. The stretching vibration of hydroxyl compounds is attributed to the peaks at 2,554.8 and 3,485.58 cm^{-1}.

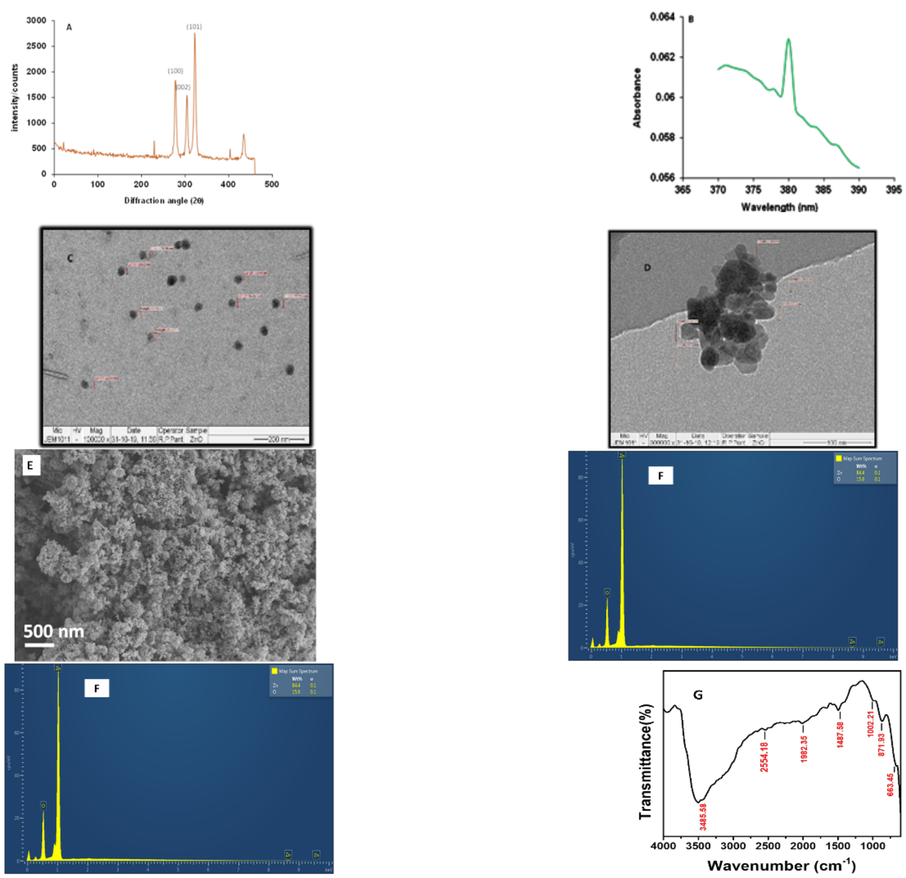

Figure 1 Characterization of ZnO NPs: (A) XRD results showing the peak at 2Θ, (B) uv-vis spectra of the particle, (C) TEM images of individual primary ZnO nanoparticles at a magnification of 100,000×, (D) TEM images of aggregated ZnO nanoparticles at a magnification of 300,000× seen under the transmission electron microscope, (E) Scanning Electron Microscopy (SEM) images of the ZnO NPs, (F) Energy-dispersive X-ray spectra (EDS) of ZnO NPs, and (G) ATR-IR spectrum of the ZnO NPs.

Zinc accumulation in hydroponics and soil experiments

Zinc accumulation was found to increase in plant parts with an increase in the concentration of ZnO NPs. The maximum Zn accumulation in both hydroponics and soil experiments was observed at the maximum dose of 50 mg L^{-1} and 50 mg kg^{-1} ZnO NPs, respectively. The maximum Zn in root and shoot was 54 and 26 mg kg^{-1} DW, respectively, in hydroponics (**Figure 2**(**A**)). The root-to-shoot translocation factor of Zn varied from 0.45 to 0.54, with the maximum being at 10 mg L^{-1}. In soil experiments, the maximum Zn accumulation in the root, shoot, and grain was 74, 71 and 29 mg kg^{-1} DW, respectively, at 50 mg kg^{-1} ZnO NPs (**Figure 2**(**B**)). The translocation factor for Zn for root-to-shoot and shoot-to-grain varied from 0.95 - 1.18 and 0.40 - 0.68, respectively. The translocation factor of Zn declined with an increasing dose of ZnO NP for both root-to-shoot and shoot-to-grain. The increase in Zn content in grains would be beneficial for the nutritive value of rice. Dimkpa *et al*. reported that the root surfaces of plants grown with ZnO NPs were whiter than the control roots, suggesting coverage of the root surface by the ZnO NPs [60]. Therefore, the supply of ZnO NPs is linked to the increased accumulation of Zn in plants, which may be attributed to the higher availability of Zn in the form of NPs. Lopez-Moreno investigated the uptake and accumulation of ZnO NPs by soybean (*Glycine max*) plants and found significantly high accumulation at 500 mg L^{-1} that was attributed to less aggregation of ZnO NPs, while at higher concentrations (1,000 - 4,000 mg L^{-1}), Zn accumulation declined due to the formation of agglomerates [61]. The agglomerated NPs reduce their movement through the pores of the cell walls. However, in the present study, Zn accumulation showed a continuous increase with the increasing dose of ZnO NPs in hydroponics and soil experiments. This appears to be due to the use of practically relevant doses of ZnO NPs. An increase in Zn content in rice plants has been observed in several studies in normal conditions as well in presence of another stressor element like arsenic [40,62]. The studies on other NPs like Ag and CuO have also reported a significant increase in the accumulation of elements (Ag, Cu and Ti) in rice and wheat [25].

Zinc concentration in rice grain (**Figure 2**(**B**)) is increasing with increasing ZnO NPs doses, however, the difference is not statistically significant. Zn accumulation in rice grain is dependent on the rice genotypes that determine the fate of Zn from the soil to the grain. This has implications for overcoming Zn translocation barriers between vegetative parts and grains and achieving grain Zn loading [63]. Therefore, in the present study rice accumulation in rice grain is not affected significantly by the increasing doses of ZnO NPs.

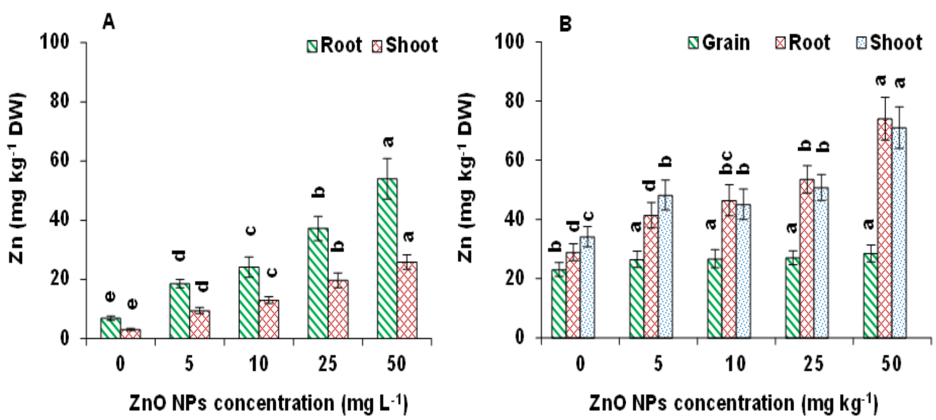

Figure 2 Effect of ZnO NPs on zinc accumulation in rice plants in root and shoot in hydroponics (A) and in grain, root and shoot in soil (B). All values represent the mean of 4 biological replicates ± S.D. One-way ANOVA was significant at $p \leq 0.05$. Different letters indicate significant differences among the treatments (DMRT at $p \leq 0.05$).

Effect of ZnO NPs on the activity of soil enzymes

The available content of Zn in the soil at the time of harvesting was recorded and it was found to be 5.3 at 50 mg kg^{-1} treatment. Soil samples were taken at the anthesis stage of rice, and the activities of soil enzymes were assayed. Soil enzymes are considered the major indicators of soil health. Dehydrogenase (in microbial respiration), urease (nitrogen cycle), and phosphatases (phosphorus cycle) are among the most important soil enzymes [64]. Dysfunction of the enzymatic activity of soils may disturb the biological equilibrium of soil, which may have ecological and economic consequences. Soil contamination with Zn changes the enzymatic activity of soil and the effect is dose-dependent [65]. In the present study, alkaline phosphatase and acid phosphatase activity in ZnO NP treatment was increased significantly at 5 mg kg^{-1}, which were 22 and 66 %, respectively as compared to control. At 10 and 25 mg kg^{-1}, there was no significant change in the activity while at 50 mg kg^{-1}, the activity of alkaline phosphatase (19 %) and acid phosphatase (23 %) decreased significantly as compared to control (**Figure 3**(**A**)). Urease activity (**Figure 3**(**B**)) in the soil in ZnO NP treatment increased significantly at all doses; however, the maximum increase of 45 % occurred at 10 mg kg^{-1} Zn in comparison to control. Dehydrogenase activity did not change significantly up to 25 mg kg^{-1} Zn and depicted a significant decline of 69 % at 50 mg kg^{-1} ZnO NPs as compared to control (**Figure 3**(**C**)). The activities of alkaline and acid phosphatases play important role in the phosphors cycle and solubilize the organic phosphorus and remobilize remobilization phosphate to help the plant cope with P-stressed conditions [66]. The urease enzyme is responsible for the hydrolysis of urea fertilizers applied to the soil into NH_3 and CO_2 [67]. The dehydrogenase enzyme activity is commonly used as an indicator of microbial activity in soils [68], and the dehydrogenase enzyme oxidizes soil organic matter by transferring protons and electrons from substrates to acceptors. In general, ZnO NPs have a greater influence on soil microbial activity than phytotoxic effects due to the immobilization and aggregation of ZnO NPs in the soil. The results of the present study demonstrated a stimulating effect of the NPs, especially at the lowest concentration. Zn ions released from ZnO NPs serve as a source of Zn nutrition to plant growth and the stimulatory effect of Zn on enzymes related to nutrient cycling in soil and microbial growth imparts a positive effect on plant growth. However, excess Zn ions released from the NPs may be responsible for the toxic effects. The generation of reactive oxygen species (ROS) by NPs may be a cause of toxicity [69]. ROS, causing oxidative stress in the cells of microorganisms, can cause their decay, which leads to a decrease in the production and secretion of soil enzymes at a higher level of ZnO NPs. According to Zhi-Xin *et al.*, catalase and urease activity in soil decreased as Zn concentration increased from 0 to 800 mg kg^{-1}; however, alkaline phosphatase activity was unaffected [70]. Soil dehydrogenase, β-glucosidase, and acid phosphatase activities were significantly reduced by ZnO NP as compared to control [71]. The toxicity of NPs like CeNPs on soil enzymes, urease and β-glucosidase, and soil microbial biomass has been noted in earlier studies [72,73].

There is no direct relationship between soil enzyme activity and Zn accumulation in plants that have been reported so far. However, soil enzyme activity is much sensitive to heavy metal pollution, hence regarded as sensors of soil environmental quality [74] and widely used as eco-indicators of the soils surrounding polluted sites [75].

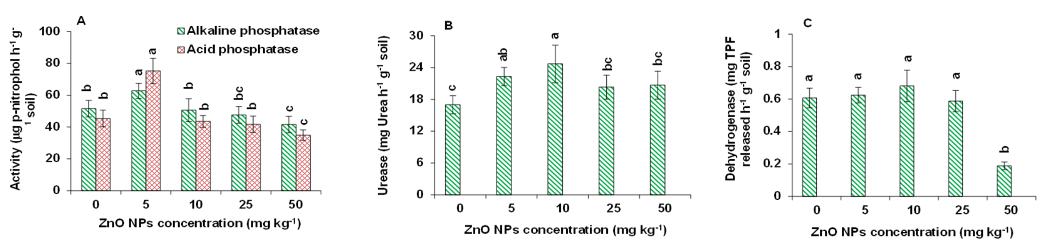

Figure 3 Effect of ZnO NPs on soil enzymatic activity: Acid and alkaline phosphatase (A), urease (B), and dehydrogenase (C). All values represent the mean of 4 biological replicates ± S.D. One-way ANOVA was significant at $p \leq 0.05$. Different letters indicate significant differences among the treatments (DMRT at $p \leq 0.05$).

Effect of ZnO NPs on plant growth parameters

The dry biomass of whole seedlings and length of roots and shoots showed a significant increase or no significant change up to 10 mg L^{-1} treatment. The maximum increase in biomass (12 %) was observed at 5 mg L^{-1}, while the maximum increase in the root (60 %) and shoot (47 %) length was noticed at 10 mg L^{-1} as compared to control. Plant growth was significantly reduced at higher doses of 25 and 50 mg L^{-1}. The maximum decline in biomass, root length and shoot length occurred at 50 mg L^{-1}, which was 82, 40 and 35 %, respectively, as compared to control (**Figures 4**(**A**) - **4**(**B**)). In the soil microcosm study, root length of plants showed a significant increase only at 5 mg kg^{-1} (17 %), while depicted significant decline at 25 and 50 mg kg^{-1} with the maximum decline being 53 % at 50 mg kg^{-1} as compared to control (**Figure 4**(**C**)). The shoot length of the plants, however, did not show any significant change in comparison to the control (**Figure 4**(**C**)). However, the dry weight of root, shoot and grains showed a significant decline beyond 5 mg kg^{-1} ZnO NP treatment. The maximum decline of 80, 78 and 87 % occurred in the root, shoot and grain biomass at 50 mg kg^{-1} in comparison to control (**Figure 4**(**D**)). NPs are reported to increase the growth of plants under normal as well as stressed conditions. Liu *et al*. [76] reported enhanced growth of rice plants upon nano-CuO treatment while nano-ZnO mediated improvement in biomass and growth of maize plants was demonstrated by Rizwan *et al*. [77]. Yoon *et al*. reported that ZnO NP negatively affected the soybean growth at developmental and reproductive stages and soybean biomass decreased with increasing Zn dose as ZnO NPs [78]. Kim *et al*. reported that biomass and root length of *Cucumis sativus* decreased in ZnO NP and Zn^{2+} treatment and the Zn accumulation in *C. sativus* was much lower in the ZnO NP treatment than those in the Zn^{2+} treatment [71]. Mahajan *et al*. studied the effect of ZnO NPs on the growth of mung (*Vigna radiata*) and gram (*Cicer arietinum*) seedlings and they found that significant inhibition of mung and gram seedlings' growth above 20 ppm ZnO NPs and 1 ppm ZnO NPs, respectively [79]. This was due to high accumulation of ZnO NPs by the roots. The changes in the growth pattern of plants (plant height, root and shoot fresh and dry weight) have also been detected in response to other NPs in cucumber [80] and sweet potato [81]. The growth changes in plants in response to NPs exposure might be linked to changes in their hormonal profile [81].

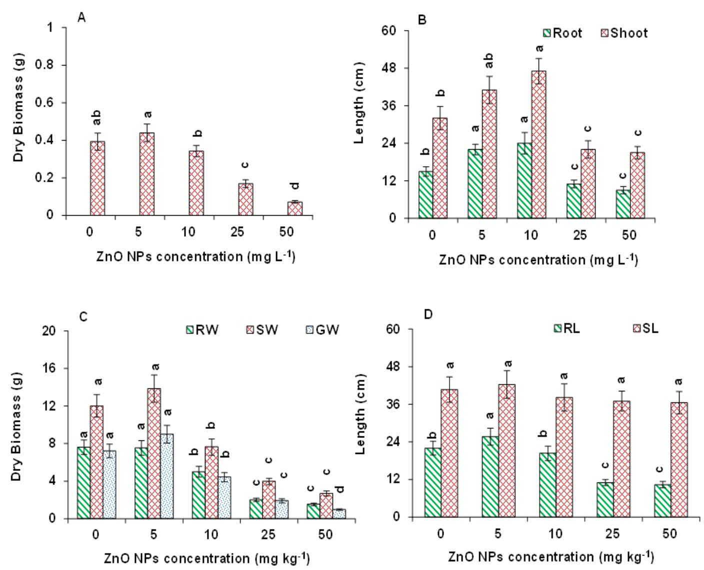

Figure 4 Effect of ZnO NPs on the biomass and length of rice plants in hydroponics (A, B) and soil (C, D) experiments. All values represent the mean of 3 biological replicates ± S.D. One-way ANOVA was significant at $p \leq 0.05$. Different letters indicate significant differences among the treatments (DMRT at $p \leq 0.05$).

Effect of ZnO NPs on photosynthetic pigments

In hydroponics set up, chlorophyll a, b, and total chlorophyll content also depicted a significant increase up to 10 mg L^{-1} with the maximum increases being 63, 53 and 56 %, respectively, as compared to control. Carotenoids showed a maximum increase of 53 % at a 5 mg L^{-1} dose as compared to control. However, at higher doses, photosynthetic pigments showed a significant decline with the maximum effect being at 50 mg L^{-1} (**Figure 5**(**A**)). In the soil experiment, chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid contents were significantly increased at 5 mg kg^{-1}, which were 11, 3, 14 and 11 %, respectively higher than control. Zinc is known to enhance the photosynthetic ability of plants by improving carbonic anhydrase activity [82]. Hence, the positive effects of Zn along with the improved level of photosynthetic pigments may improve the photosynthesis and biomass accumulation of rice plants. A declining trend in photosynthetic pigments was observed beyond 5 mg kg^{-1}. However, the levels of all pigments remained higher than control even at 50 mg kg^{-1} ZnO NP dose (**Figure 5**(**B**)). Priyanka and Venkatachalam also reported that ZnO NP treatment increased the photosynthetic pigment content; including chlorophyll a, b, and carotenoids in the leaves of the cotton plants, and the increased percentage of photosynthetic pigment content was directly proportional to the ZnO NP doses [83]. Excess concentrations of Zn^{2+} also decrease the levels of Chl *a* and *b* as well as the *a*/*b* ratio [84], by inhibiting PSII and/or PSI photochemical efficiency [85].

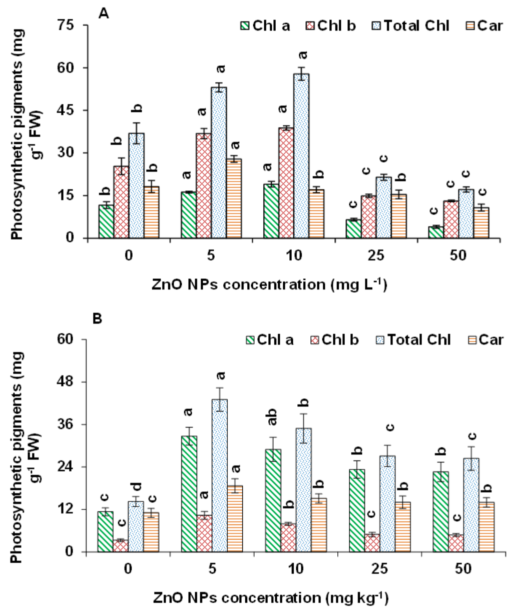

Figure 5 Effect of ZnO NPs on the photosynthetic pigments of rice plants grown in hydroponics (A) and soil (B). All values represent the mean of 3 biological replicates ± S.D. One-way ANOVA was significant at $p \leq 0.05$. Different letters indicate significant differences among the treatments (DMRT at $p \leq 0.05$).

Effect of ZnO NPs on the activity of the antioxidant enzyme

In the hydroponics experiment, SOD activity did not show any significant change in response to ZnO NPs in both root and shoot. However, a slight increase of 18 and 14 % was observed in SOD activity in root and shoot, respectively, at 10 mg L^{-1} as compared to control (**Figure 6(A)**). CAT activity depicted a significant increase at all doses of ZnO NPs in roots and up to 10 mg L^{-1} in the shoot. The maximum increase in CAT activity was found at 5 mg L^{-1} in both roots (53 %) and shoots (45 %) as compared to control (**Figure 6(B)**). APX activity showed a significant increase up to 25 mg L^{-1} in roots (16 %) and shoots (36 %) as compared to control (**Figure 6(C)**). GPX activity did not show a significant decline at any dose of ZnO NPs. However, the maximum increase in GPX activity was seen at 10 mg L^{-1}, which was about 6.4-fold in roots and 1.3-fold in shoots as compared to control (**Figure 6(D)**). In the soil experiment, SOD activity did not show any significant change, while the activity of GPX, CAT, and APX demonstrated a significant increase at all treatments (**Figures 6(E) - 6(F)**). The maximum activity of GPX and APX was 23 and 95 % higher than control, respectively at 50 mg kg^{-1} (**Figure 6(F)**). The maximum increase in CAT activity occurred at 25 mg kg^{-1}, which was 23 % higher than the control (**Figure 6(F)**). Enzyme activity decreases at higher concentrations of Zn due to the elevation of ROS due to disturbed harmony among various non-enzymatic and enzymatic systems and a rapid increase in ROS levels [48]. Antioxidant enzymes are well known to fine-tune stress-induced ROS levels in plants and a harmonized increase in various antioxidant mechanisms help plants tolerate the stress. Zinc is a component of antioxidant enzymes like SOD and therefore, Zn itself performs in ROS regulation [86]. SOD, however, did not depict a significant change in response to ZnO NPs while other enzymes showed a variable response in hydroponics and soil experiments with a declining trend at higher ZnO NPs in hydroponics. Thus, the coordination among various antioxidant enzymes was better at lower concentrations of ZnO NP but not at higher concentrations and this might be responsible for the observed toxicity. In sweet potato plants, the application of NPs was found to alter the activity of enzymatic and non-enzymatic antioxidants [81].

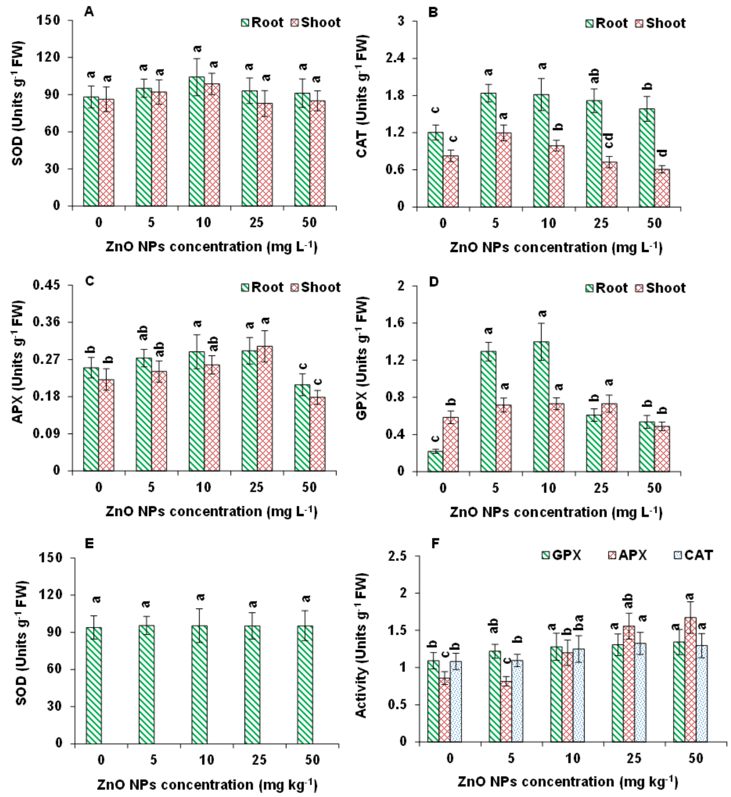

Figure 6 Effect of ZnO NP treatment on the activity of SOD (A), CAT (B), APX (C) and GPX (D) of rice plants grown in hydroponics and on SOD (E) and GPX, APX and CAT (B) activity of rice plants grown in soil. All values represent the mean of 3 biological replicates ± S.D. One-way ANOVA was significant at $p \leq 0.05$. Different letters indicate significant differences among the treatments (DMRT at $p \leq 0.05$).

Effect of ZnO NPs on DTPA-extractable soil Zn after the harvest of rice

Soil DTPA-extractable Zn concentrations after harvest of rice are presented in **Table 2**. Significantly higher DTPA-extractable Zn was observed in ZnO NPs treatments as compared to control and increased (35.8 to 141 %) as ZnO NPs dose increased. Our findings are also in agreement with those of earlier studies by Peng *et al.* who reported that the application of ZnO maintained significantly higher DTPA-extractable Zn content in the soil.

Table 2 DTPA extractable Zn content in the soil after the harvest of rice. Values are means of 4 replicates. Comparison between the mean of treatments was made by the least significance difference (LSD) test ($p \leq 0.05$).

Treatment	**DTPA-extractable Zn (mg kg^{-1} soil)**
control No Zn	0.852
5 ppm ZnO NP	1.157
10 ppm ZnO NP	1.481
25 ppm ZnO NP	1.652
50 ppm ZnO NP	2.054
LSD ($p \leq 0.05$)	0.121

Conclusions

In conclusion, the present work demonstrated that lower concentrations of ZnO NPs (up to 10 mg/L or 10 mg/kg in hydroponics and soil experiments) augmented plant growth. Plants depicted an increasing Zn accumulation with the increase in ZnO NP concentration. The toxicity of ZnO NPs at higher doses stimulated the activity of antioxidant enzymes. It is recommended that the application of ZnO NPs would be beneficial when applied in low dose (10 mg kg^{-1} or 10 mg L^{-1}) while higher doses might be toxic to plants. The DTPA extractable Zn concentration in the soil was significantly elevated with increasing exposure concentrations of ZnO NPs. In the future, field experiments should be conducted with the recommended dose of ZnO NPs at different locations so that ZnO NPs treatment can be taken to farmers for growth and yield improvements of rice.

This study has been performed in solid (soil) and aqueous (hydroponics) mediums. Soil is a natural medium whereas hydroponics is a synthetic medium and the availability of Zinc to plants from different sources is varied in both the medium. In the present study, results have been validated in both conditions. In soil, there is 2 different sources of nutrients one is native which is already present in the soil, and another one which is supplied as an external source of fertilizers. So, in the case of soil, nutrient availability to the plants could not be controlled, whereas in the case of hydroponics nutrient availability can be controlled. Both the systems are being used for commercial cultivation of the crop and the results from this study will be beneficial for both systems.

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