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ORIGINAL ARTICLE

Physiological And Biochemical Effects Of Zinc Oxide Nanoparticles On Rice (*Oryza sativa* L.)

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ABSTRACT
Cereal grains are found to be deficient in micronutrient especially zinc and iron leading to severe malnutrition and deficiency disorders in humans as well as plants. Growth stimulatory effect of commercially available zinc oxide nanoparticle on rice variety Jaya is reported. The physical characterization of ZnO NPs using Dynamic Light Scattering (DLS) and Nanoparticle Tracking Analysis (NTA) revealed its size 20.65 ± 0.07 nm, zeta potential 41.09 mv and number of particles 32.02 x 10^8 per ml, respectively. Rice seeds were treated with different nano and bulk particle concentrations (0, 100, 200 and 400 ppm) for assessing their effect on morpho-physiological parameters such as shoot and root length, fresh and dry weight, total chlorophyll and protein content alongwith seedlings antioxidant enzyme status superoxide dismutase, catalase and peroxidase. Nanoparticle treatments at lower concentration enhanced all the morpho-physiological as well as biochemical parameters significantly over control and compared to its other bulk treatments. From the present investigation, it can be concluded that the nanoparticle has immense potential for its utilization as nanofertilizer if applied at optimal concentration.
Keywords: *Zinc oxide nanoparticles, rice, nanofertilizer, DLS, NTA*

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INTRODUCTION

Staple food crops comprising rice, wheat and maize constitute principal component of human diet and are responsible for shaping human civilization for thousands of years. Daily caloric requirement of more than 50% for billions of people are supplied by consuming cereal grains [1]. Among the three major cereal crops, rice (*Oryza sativa* L.) belonging to Graminace family holds a unique position in most of the vegetarian part of the world. Zinc (Zn) deficiency is among one of the most important nutritional constraints to rice growth across the globe (2). To reach the 30% of human estimated average requirement (EAR), polished grain of rice should contain 28μg g^{-1}Zn. However, the final polished grains of most popular rice varieties grown worldwide have Zn concentration of approximately 16μg g^{-1} [3]. Conventional breeding strategies combined with genetic engineering can overcome this issue by developing zinc deficiency tolerant as well as accumulator varieties. However, the major problem is the Zn content in the soil which hampers the uptake by plants. Most of the Indian soil has been found to have from moderate to high Zn deficiency which can only be overcome by foliar application of fertilizers containing Zn [4].
With the advent of nanotechnological interventions in different scientific fields results into rapid growth and utilization of a number of engineered nanoparticles of different sizes and physicochemical properties [5]. Zinc oxide nanoparticles (ZnO NPs) are one of the most widely used nanomaterials extensively utilized in personal care products, paints and also as anti-microbial agents [6,7].
Zinc (Zn) is a trace element needed in small but critical concentrations and if the amount available is not adequate, plants will suffer from physiological stress brought about by the dysfunction of several enzyme

systems and other metabolic functions in which Zn plays a major part as a cofactor [8]. Application of fertilizers in form of zinc sulphate for overcoming the Zn deficiency is a costly input as Zn can rapidly form insoluble complexes in the soil, limiting their availability for plant uptake. Overcoming this issue is possible only with the genotypes with an improved capability to access these soil bound nutrients [9].
Enhanced efficiency, economical and environment friendly nano-based fertilizers, pesticides and other foliar formulations for agriculture purpose can boost the way for sustainable agriculture development [10]. Several researchers have already demonstrated the positive effect of NPs which includes germination rate and seedling growth enhancement by carbon nanotubes (11), zinc oxide nanoparticles [12], silver nanoparticles [13], nanoanatase TiO_2 [14], alumina NPs [15], cerium oxide NPs [16], iron oxide NPs (17) and hydroxyapatite nanophosphorous (18). Nanomaterials especially nanoparticles of various metals could be effectively utilized for overcoming the hidden hunger of plants as well as have immense possibilities for reducing the dosage of applications owing to their enhanced catalytic activity at nanoscale.
In view of this, the present study was aimed at evaluating the effects of zinc oxide nanoparticles on rice. This study characterizes the effects of ZnO NPs on most widely grown cereal crop rice variety Jaya to shed light on the effects of NPs on morpho-physiological and biochemical response over a three different concentrations (100, 200 and 400 ppm) under hydroponically grown *in vitro* conditions. Nano-phytotoxicity assessment carried out under the study highlights growth stimulation of rice seedlings exposed to zinc oxide nanoparticles..

MATERIALS AND METHODS

Preparation of zinc oxide nanoparticles (ZnONPs) suspension

Commercially available zinc oxide nanoparticle dispersion (Catalog no. 721077) were procured from Sigma Aldrich, USA and used as supplied without any modification. This product has a reported particle size of<100 nm measured by dynamic light scatting (DLS) and an average particle size of <35 nm measured using an aerodynamic particle sizer (APS) spectrometer. To check the uniformity of the supplied nanoparticles, characterization of ZnONPs for DLS based distribution of size, intensity and poly dispersity index were carried out using Malvern Zetasizer (Model: ZS90) and zeta potential was analyzed using NTA based Nanosight, (Model: NS500) (Malvern Instruments, Herrenberg, Germany). The ZnONPs suspensions were prepared at 0, 100, 200 and 400 ppm in Millipore water and sonicated in ultra-sonicator (QSonica, USA) for 20 min before mixing the nanoparticles with hydroponic solution.

Plant materials

Seeds of rice variety Jaya supplied by Main Rice Research Station, Anand Agricultural University, Nawagam was used in the experiment. The variety was selected as it shows susceptibility towards zinc deficiency in early stages as well as it is still prefer variety for sowing by the rice farmers of Gujarat state, India.

Hydroponic study

The hydroponic solution for rice was prepared except the source of Zn is replaced with ZnONPs (100, 200 and 400 ppm) and the ionic strength was also maintained (data not shown) (19). The seeds were surface sterilized using 0.1%$HgCl_2$ for 10 min, followed by washing three times with autoclaved Millipore water and placed in Borosilicate test tube (Size: 25 x 20 mm). The seeds were immersed in the hydroponic solution over a paper towel support. The test tubes were kept under illumination chamber with 14-h photoperiod, 25/20°C day/night temperature and 65% relative humidity. The germination rate was measured when 65% of control roots were 5 mm long while ungerminated seeds were removed to avoid bacterial or fungal growth in the media and the seedlings were allowed to grow for 10 days under light (340 µmol m^{-2} s^{-1}). A single treatment is represented by five seeds per test tube with five replications in a completely randomized design. Morpho-physiological observations like total chlorophyll content (mg g^{-1} of fresh weight), shoot and root length (cm), fresh and dry weight (g) and biochemical observations like total protein content (mg g^{-1} of fresh weight), anti-oxidant enzyme activities like peroxidase (µmole mg $protein^{-1}$ min^{-1}), super-oxide dismutase (unit mg^{-1} protein) and catalase (µmole H_2O_2 mg $protein^{-1}$ min^{-1}) has been carried out on completion of incubation period i.e. on 10^{th} day.

Enzyme extraction and activity assay

On tenth day of incubation, rice seedlings (0.5 g fresh wt.) were grinded with four ml of 0.1 M phosphate buffer (pH 6.0) prechilled at 4°C containing 0.1 mM EDTA, 03% (w/v) Triton X-100 and 4% (w/v) PVP for SOD assay or containing 4mM DTT, 2mM EDTA and 2% (w/v) PVP for CAT and POX assay. The mixture was homogenized at 4°C and centrifuged at 10,000*g* for 15 min. The supernatant was collected for its downstream processing. SOD activity was assayed using photochemical NBT method. POX was assayed at

470 nm using guaiacol as a substrate and catalase was assayed at 240 nm using H_2O_2 as a substrate [20]. Total protein content were determined by Lowry method using BSA as the standard[21].

Statistical analysis

The morpho-physiological and biochemical results are represented as mean ± SE (n = 5). Nanoparticle suspension analysis was carried out using in built analysis software (A) For Malvern ZS 90 – version 7.04 and (B) For Nanosight NS 500 –version 2.3.

Results

Characterization of ZnONPs

Size and polydispersity index

DLS based hydrodynamic size and polydispersity index (pdi) measurement was carried out using Malvern ZetasizerNanoZS90. Fig. 1A represents the intensity size distributions for ZnONPs suspension obtained through DLS measurements at 25°C with each measurement repeated ten times for ten seconds each. The hydrodynamic diameters of ZnONPs was 20.65 ± 0.07 nm. The perfect correlogram (Fig. 1B) ZnONPs nanoparticles clearly suggests monodisperse particle solution with pdi of 0.32 (Fig. 1C) for ZnONPs.

Zeta potential (mV)

Surface charge of ZnO NPs were measured following standard operating procedures using Nanosight (NS 500) and the data analyzed using NTA software version 2.3. Zeta potential measurements specifies the electrokinetic potential of a colloidal system (22). This is a physical property which is exhibited by any particle in a suspension. Magnitude of the zeta potential gives the net charge at the diffuse boundary of a particle in a suspension which will ultimately indicate the potential stability of a colloidal system. The zeta potential of these particles was found to be 41.09 mV which is a strong zeta values (Fig. 2A) for zinc oxide nanoparticles and therefore, these particles were able to remain suspended in solution without forming aggregation. The zeta potential measurement supports the monodisperse behaviour of these particles in the solution. ***Concentration of particle (per ml)***

Determination of particles per ml is also one of the most important criteria while working with the nanomaterial. The concentration of particles can significantly effects both the aggregation as well as the zeta potential of the solution. In the present investigation zinc nanoparticle concentration was obtained using Nanosight (NS 500). The particles concentration per mL was found to be 32.02 x 10^8 for ZnO NPs (Fig. 2B).

Effect of ZnONPs on morpho-physiological parameters

Exposure of rice seedlings to zinc nanoparticles after 10^{th} day of incubation in hydroponic media showed positive effects on plant growth for shoot and root length (cm) and fresh and dry weight (gm). Among the treatments, seedlings exposed to 100 ppm zinc oxide nanoparticles showed maximum mean shoot length (9.12 cm).Seedlings treated with zinc oxide nanoparticles showed positive effects for root length with maximum root length in 100 ppm zinc oxide nanoparticles (Table 1).

Significant variation in fresh weight of seedlings exposed to different particle concentrations was observed after ten days of treatments. Increase in fresh weight (gm) of seedlings was found to be more in 100 ppm of zinc oxide (0.16 gm) nanoparticles compare to other treatments and found significantly higher than control (0.06 gm).The increase in dry weight for zinc particles was found to be more in the seedlings treated with 100 ppm zinc oxide nanoparticles (0.11 gm) compared to 400 ppm $ZnSO_4$ (0.10 gm) and control (0.03 gm) (Table 1). The morpho-physiological observation for nano and bulk particles of zinc showed completely reverse trend for concentration i.e. with increasing concentration of zinc oxide nanoparticles all the studied parameters showed inhibition of growth while all the zinc sulphate treated seedlings exhibits growth enhancement as the concentration is increase from 100 to 400 ppm.

Maximum total chlorophyll content was recorded in leaves treated with 200 ppm of zinc oxide nanoparticles (5.30 mg g^{-1} FW), whereas, the minimum total chlorophyll content was recorded in control (2.10 mg g^{-1} FW) (Fig. 3). Total chlorophyll content increase significantly in all the seedlings exposed to nanoparticles showing higher accumulation of chlorophyll content per gram of fresh samples compared to bulk particles.

Effect of ZnONPs on total protein and anti-oxidants status of rice seedlings

Total protein content was found to be higher in leaves treated with 200 ppm zinc oxide nanoparticles (61.07 mg g^{-1} FW), whereas, least total protein content was recorded in control leaves (29.20 mg g^{-1} FW) (Fig. 4). Total protein content increase significantly in all the treatment with nanoparticle treated leaves showing higher accumulation of protein content per gram of fresh samples compared to control plants.

Superoxide radical is one of the most deleterious reactive oxygen species that attacks membranes and induces peroxidation of lipids (23). SOD activity in the rice leaves sample significantly increased in all nanoparticle treatments. Fig. 5 depicts SOD activity in leaves collected from 400 ppm of $ZnSO_4$ (2.25 fold) showed maximum activity among all the treatments and significantly higher than control. However, increase in SOD activity was found to be at par with 200 ppm of zinc oxide nanoparticles (2.23 fold). Among the bulk and nanoparticle treatments, increase in SOD activity was more profound in leaves treated with different concentrations of nanoparticle.

POX activity in flag leaves collected from 400 ppm ZnO NPs (3.35 fold) showed maximum activity among all the treatments and significantly higher than control. Among the nanoparticle treatments, increase in POX activity was found to be more compare to other treatments and control (Fig. 6).

Increase in CAT activity in seedlings was observed among all the treatment and concentration based increment clearly suggest the effectiveness of applied treatment. Highest CAT activity was recorded in the leaves treated with 200 ppm of zinc oxide nanoparticles (2.31 fold) followed by 100 ppm ZnO NPs (2.19 fold) compare to control. Overall response of POX activity in seedlings was higher for nano compare to bulk particle treatments with significantly higher than control (Fig. 7).

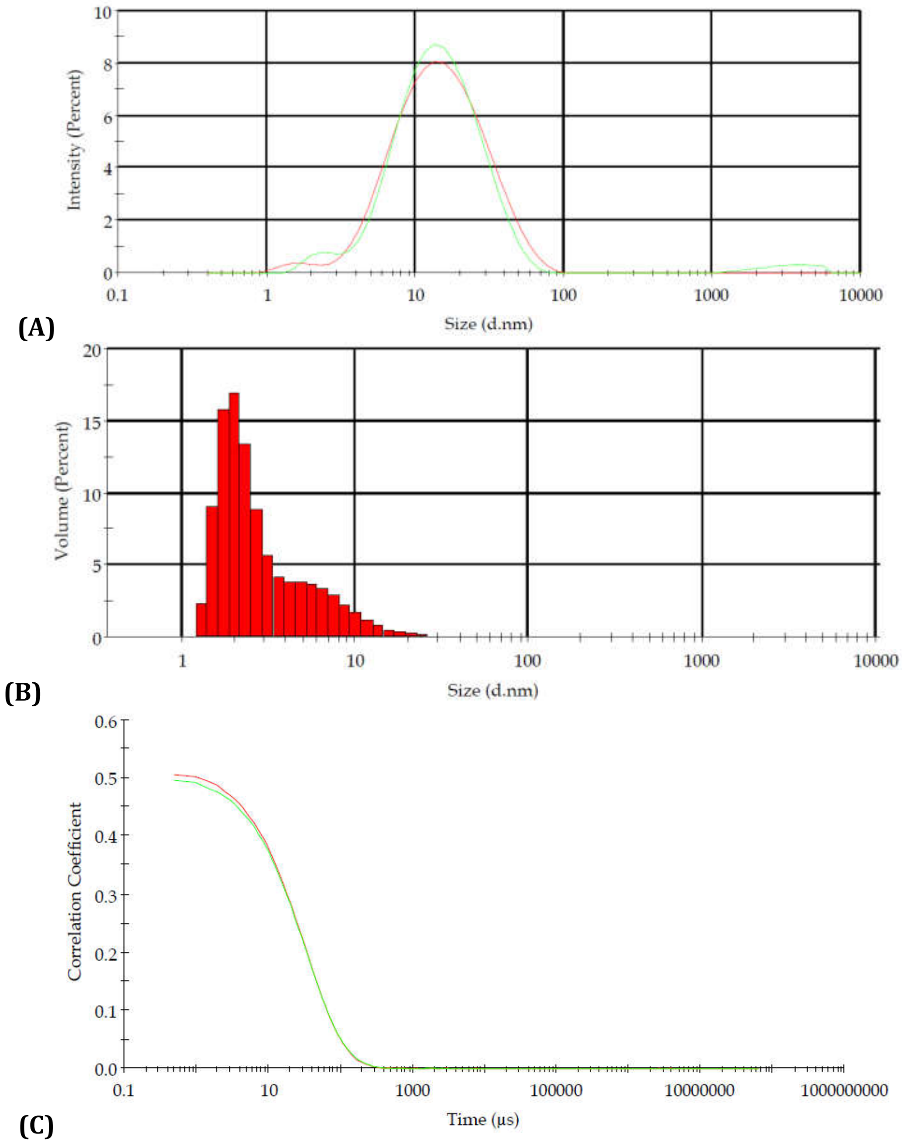
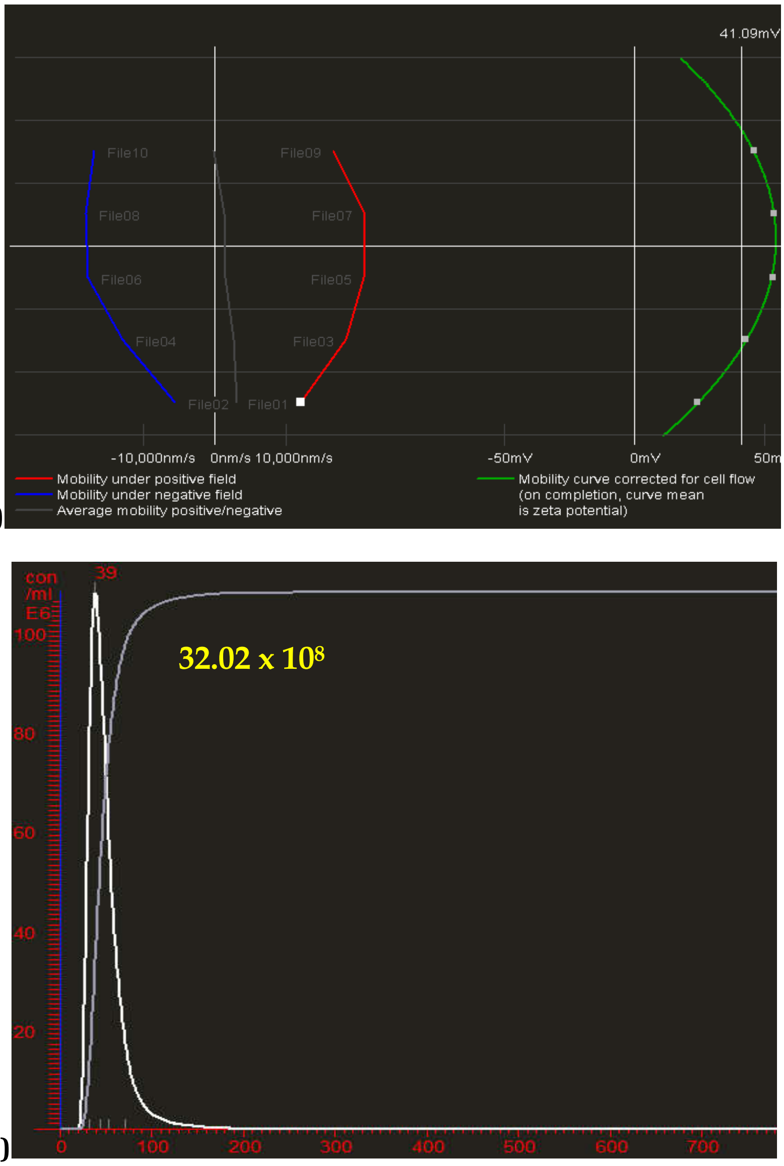


Fig. 1: Characterization of commercially available zinc nanoparticles using dynamic light scattering

(A) – Size Distribution by Intensity
(B) – Size Distribution by Volume
(C) – Correlogram



(A)

(B)

Fig. 2: (A) Zeta potential (mV) and (B) particles per ml measurement of commercially available zinc nanoparticles

Table 1: Effects of bulk and nano zinc particles on rice seedlings variety Jaya after ten days for shoot and root length (cm), fresh and dry weight (gm)

Treatment	Shoot length (cm)	Root length (cm)	Fresh weight (gm)	Dry weight (gm)
Control	3.59 ± 0.38	0.9 ± 0.12	0.06 ± 0.01	0.03 ± 0.00
100 ppm ZnONP	9.12 ± 0.52	2.1 ± 0.07	0.16 ± 0.01	0.11 ± 0.02
100 ppm $ZnSO_4$	6.77 ± 0.36	1.1 ± 0.11	0.13 ± 0.00	0.07 ± 0.01
200 ppm ZnONP	8.10 ± 0.54	1.7 ± 0.14	0.13 ± 0.01	0.09 ± 0.01
200 ppm $ZnSO_4$	8.33 ± 0.85	1.2 ± 0.04	0.14 ± 0.01	0.09 ± 0.01
400 ppm ZnONP	7.55 ± 0.68	1.5 ± 0.18	0.13 ± 0.01	0.08 ± 0.01
400 ppm $ZnSO_4$	8.85 ± 0.59	1.7 ± 0.07	0.15 ± 0.01	0.10 ± 0.01
S. Em.	0.56	0.098	0.008	0.005
C.D.	1.51	0.28	0.02	0.015
C.V.%	13.84	13.57	12.99	13.15

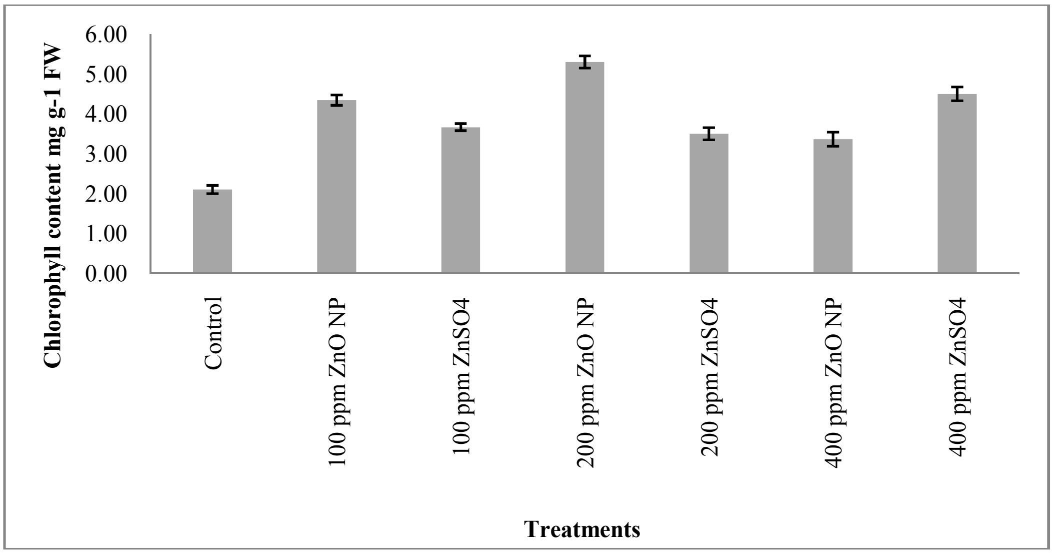


Fig. 3: Chlorophyll content (mg g^{-1}) in leaves of rice harvested at 10^{th} day

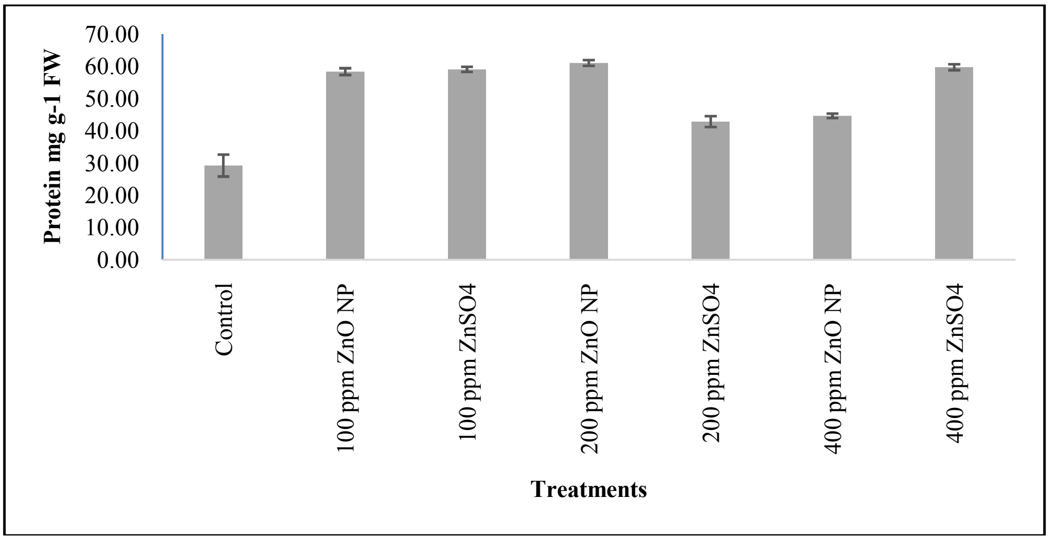


Fig. 4: Total protein content (mg g^{-1}) leaves of rice harvested at 10^{th} day

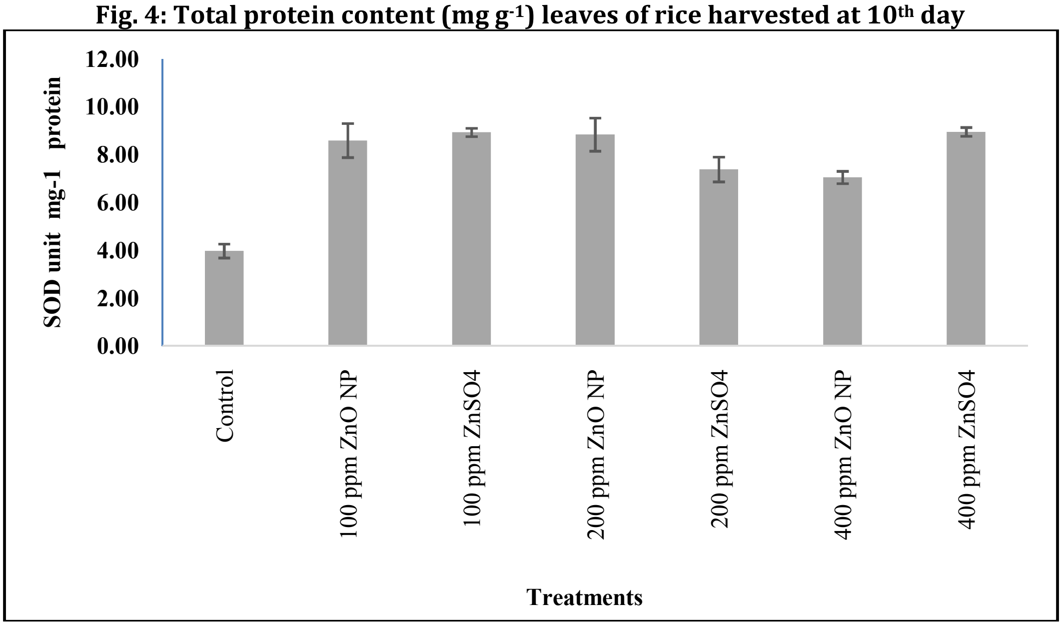


Fig. 5: Superoxide dismutase activity in leaves of rice harvested at 10^{th} day

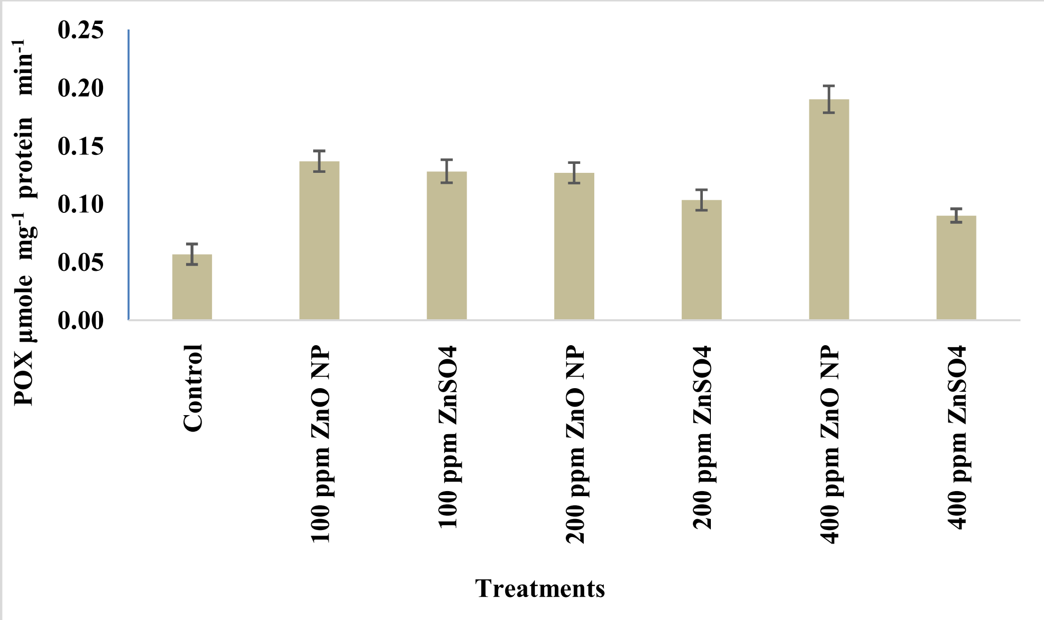


Fig. 6: Peroxidase activity in leaves of rice harvested at 10th day

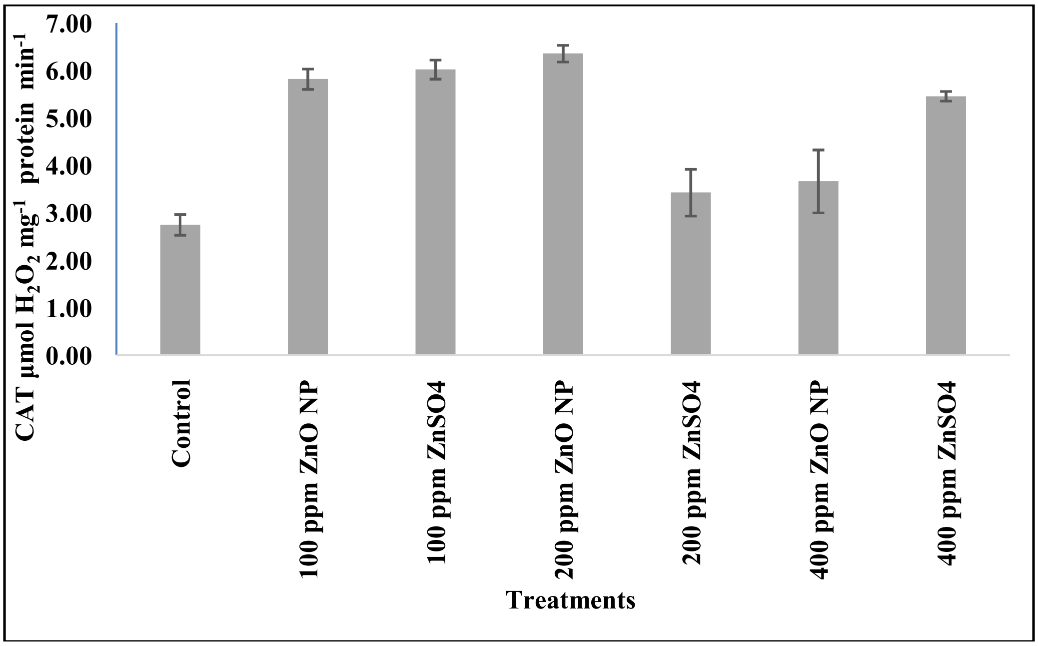


Fig. 7: Catalase activity in leaves of rice harvested at 10th day

DISCUSSION

Nutritional food security is one of the major concern the World is facing today. Ample amount of food grains are produced to feed the hunger of growing population, however, the biggest challenge is to overcome the hidden hunger for micronutrients especially zinc and iron. Foliar application of zinc in form of zinc sulphate is one of the alternative to overcome and enhance the zinc content in food grains. With ever-growing demand and increase in cost of cultivation, the application of zinc sulphate as per recommendation for a specific crop is difficult to achieved. Nanotechnology can enhance the uptake and reduce the foliar application of zinc fertilizers to a greater extent. However, till date there are many reports published with regard to negative impact of nanoparticles on various plants. The present investigation was aimed at assessing the impact of zinc oxide nanoparticles on rice seedlings. In vitro hydroponic system can be effectively utilized for evaluation of harmful effects of these nanoparticles on overall growth of rice seedlings.

In the present investigation, commercially available zinc oxide nanoparticles were characterized using DLS and NTA. Nanoparticle solution stability is depended mainly upon the size, zeta potential and number of particles present in the sample. The results of physical characterization reveals good stability of zinc oxide nanoparticles dispersion at different concentrations and hence could be utilized for similar nanophyto-toxicity studies. Similar results were reported by Kouhi and co-workers, (2014) wherein size and zeta potential measurement of bulk and nano forms of zinc was assessed and found to be optimum which further inhibits aggregation of particles.

Pokhrel and Dubey, [25] evaluated maize and cabbage seedlings against silver and zinc nanoparticles and reported similar increase in fresh weight at lower concentration. The enhancement in morpho-physiological parameters may be attributed to accumulation of PGRs like cytokininins and gibberlins which plays an important role in cell division and elongation, respectively. Zheng *et al.,* [26] have also reported that nano-TiO_2 could promote photosynthesis and improve spinach growth. The results indicates that more number of reaction centers are in an 'open state' to carry out light reaction. Presence of higher number of open or oxidized electron acceptors in PS-II decreases the probability of generation of reactive radicals [2].

Suriyaprabha *et al.,* [28] carried out field trial and reported similar increase in protein content using silica nanoparticles in maize leaves. Zinc is a major co-factor essentially required for optimum activity of a number of enzymes. Hence, the increase in protein content with zinc oxide nanoparticles may be attributed due to metabolic balance between inductions of proteins. Isoenzymes of super oxide dismutase (SOD) catalyse the dis-mutation of the super oxide anion, producing hydrogen peroxide (H_2O_2), which is educed to water by either catalases (CAT) [29]) or peroxidases (POX). Similar increase in activity was observed by Vannini *et al.,* (2013) for *Erucia sativa* leaves exposed to silver nanoparticle and silver nitrate. CAT and POX enzymes are known to be involved in the detoxification of H_2O_2 by converting the H_2O_2 to water and oxygen (31). The increase in CAT and POX activity in all the nanoparaticle treated seedlings suggest that the ZnO NPs could effectively modify these enzyme activity under hydroponically grown rice seedlings. Similar enhancement in CAT activity was observed by Zhao *et al.,* [32] in 10 day old maize plants.Our results for POX enzyme contradicts the observation reported by Mukherjee *et al.,* [33] while working on green peas exposed to different forms zinc particles which includes bulk, nano and coated zinc particles. The increase in activity clearly suggest that nanoparticle treatment could stimulate the peroxidase activity and helps the plant to overcome the abiotic stresses. Similar increment in growth parameters at lower concentration of ZnO NPs has been reported by Liu and co-workers, [18] in maize. The growth enhancement by ZnO NPs may be attributed to release of Zn^{2+} ions more for plant growth.

CONCLUSION

From the physical characterization of commercially available zinc oxide nanoparticles, it can be concluded that these nanoparticles could be utilized for assessing their effects on plants as these particles do not form aggregates and remain in suspended form due to good zeta potential value. Further, the physiological and biochemical studies conducted in the present investigation could also be utilized for evaluating the growth inhibitory or promontory effect of metallic nanoparticles. Finally, from the study it can be concluded that nanoparticles if applied at optimum concentration can enhance the growth of plants both morpho-physiologically as well as biochemically.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS CONTRIBUTION

M. Mankad designed and conducted the experiment and wrote prepared the manuscript with the assistance of A. Patel and P. Mankad; R. S. Fougat and Subhash N., mentor the whole experiment and G. Patil checked and corrected the manuscript.

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