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Phytotoxicity of Zinc-oxide Nanoparticles on Seedling Growth and Antioxidant Activity of Red gram (*Cajanus cajan L.*) Seed

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Abstract

The present study is aimed at investigating the effects of zinc oxide nanoparticles (ZnO NPs) on *Cajanus cajan* L. seeds. Seeds were exposed to five different concentrations of ZnO NPs (5, 10,15,20,25 mg/100ml). The results show that at higher concentrations (20, 25 mg/100ml ZnO NPs) there is reduction in the seed germination percentage, root length, shoot length, number of leaves, width of leaves and fresh and dry weight of plant. ZnO NPs had an increased effect on Glutathione Reductase **(**GR) and Guaiacol peroxidase (GPX), Hydrogen peroxide (H_2O_2) and ascorbic acid content, had a decreased effect on Catalase (CAT) activity. Transmission Electron Microscopy studies showed aggregated nanoparticles around the cell wall of the seed, which clearly indicates the absorption of nanoparticles by *Cajanus cajan* L. seed and shows negative effects on seedling growth. Reactive oxygen species (ROS) stress was caused by ZnONPs in seed and leaf. This study shows that direct exposure to ZnONPs causes significant phytotoxicity. This highlights the necessity for safe disposal of wastes containing nanoparticles.

Keywords: Zinc oxide nanoparticles, *Cajanus cajan* L., Seed germination, Antioxidant activity, Transmission Electron Microscopy

1. Introduction

Recent advances in nanotechnology facilitated finding practical applications in biomedical, electronics, agriculture, renewable energy and green technologies (Ma *et al.,* 2015). The unrestricted use of nanoparticles in many aspects of daily life has led researchers to consider the problems and consequences of their environmental impact (Gottschalk *et al.,* 2015). Nanoparticles enter into the environment through effluents, agricultural application, atmospheric deposition, surface runoff or other pathways and will ultimately accumulate in the soil. Exposure modelling indicated that the concentrations of nanoparticles in soil are higher than those in water or air. So soils may be the main source of nanoparticles released into the environment (Gottschalk *et al.,* 2009). Nanoparticles in the soil will interact with plants accumulate in plant biomass, pass through the food chain and accumulate in higher trophic levels of consumers (Zhu *et al.,* 2008). Zn is an important molecule of key enzymes like Cu-Zn SOD (Broadley 2007), metabolism of carbohydrates and protein (Singh *et al.,* 2013). Plants can absorb ZnO nanoparticles (Zhao *et al.,* 2012), and accumulate Zn. They have both positive and negative effects on organisms (Salah and Naif, 2016).

In recent years, research in this area has been focused on the interaction between plants and nanoparticles. The effect of nanoparticles on germination depends on concentrations of nanoparticles and varies from a plant to another. De la Rosa *et al.* (2013) applied different concentrations of ZnONPs on cucumber, alfalfa and tomato, and found that only cucumber seed germination was enhanced. Zhang *et al.* (2015) reported that corn exposed to ZnONPs showed no significant negative physiological effects. Most studies showed that nanoparticles could produce toxic effects above a certain concentration (Rico *et al.,* 2011). Therefore, a better understanding of behavior and impact of nanoparticles on the health of plants and hence on environment is highly demanding (Chow *et al.,* 2005). This study can be helpful in understanding the effects of environmentally released ZnO NPs on *Cajanus cajan L.*

2. Materials and Methods

Zinc oxide nanoparticles of mean size of 100 nm diameter (Sigma Aldrich) was used in the study. Different concentrations of ZnO NPs solutions were prepared by directly suspending the nano-particles in deionised water and magnetic bars were placed in the suspensions for stirring before use to avoid aggregation of the particles.

Cajanus cajan L. (Red gram) was collected from local market. Seeds were surface sterilized with 0.1% sodium hypochlorite for 5 min to prevent any fungal contamination. After that, the seeds were washed with deionised water and dried between two paper towels. The seeds were then soaked in 5, 10,15,20,25 mg/100ml of ZnO NPs solution, deionised water (control) for 8 hours. *Cajanus cajan L.* seeds that are soaked in ZnO NPs solution were sown in pots (20 cm × 40 cm) filled with red soil and a layer of coco peat.

The effect of ZnO NPs on *Cajanus cajan L.* was determined by studying growth (germination percentage,

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root and shoot length, leaf length, leaf width, no. of leaves, fresh weight, dry weight) and biochemical (Total Sugars, Reducing Sugars, Protein, chlorophyll**)** parameters on 20th, 40th, 60th and 80th day of experiment. The number of seeds germinated was counted and the germination percentage was calculated by using the following formula:

Germination percentage = (number of seeds germinated / number of seeds sown) *100.

The physiological parameters of the seedlings were analysed in terms of root, shoot length, leaf length and leaf width with the help of standard scale, fresh weight and dry weight with the help of electrical balance. After the fresh weight was taken, the seedlings were kept in a hot air oven at 60 ° C for 48 hrs then the weight of dry matter was recorded.

Protein content was determined by the method of (Lowry et al., 1951) Total Sugars, Reducing Sugars and chlorophyll were estimated by the recommended methods of (AOAC, 2000). Experiments were carried out in triplicate.

2.1. Antioxidants Enzymes

Antioxidant potential was investigated through monitoring the activity of different antioxidative enzymes including, catalase (CAT), Guaiacol Peroxidase (GPX), Glutathione Reductase (GR) and Hydrogen Peroxide (H_2O_2), Ascorbic acid.

2.2. Enzyme extraction

0.2g of sample (seed and leaf) was homogenized in 2 ml of extraction buffer having 50mM sodium phosphate with pH 7.0, 0.1% phenyl methyl sulfonyl fluoride (PMSF) and 0.1% EDTA. The homogenate was centrifuged at 12000g for 25 minutes and the supernatant was used as enzyme source.

2.3. Giaiacol peroxidase (GPX)

Guaiacol peroxidase was assayed following (Chance and Maehly, 1955) method. A reaction mixture consisting of 50mM phosphate buffer (pH 7.0) 20mM guaiacol, 10mM H_2O_2 and 100 µl enzyme extract was used to measure POX activity. Guaiacol peroxidase activity was measured by the increase in absorbance at 470nm (A_{470}) due to guaiacol oxidation. One unit of POX is defined as the amount of enzyme need to convert 1 µmol. Of H_2O_2 min^{-1} at 25°C. It is expressed as µ mol. Guaiacol/min-1g-1 FW or activity U/g FW.

2.4. Catalase (CAT)

Catalase enzyme activity was measured by following (Aebi, 1984) method. A reaction mixture having 50mM Sodium Phosphate buffer (pH 7.0) and 50 µl of enzyme extract was utilized to this mixture. 10mM H_2O_2, was added gradually and its consumption was measured for 2 minutes to measure CAT activity. CAT activity was assayed by following the decline in absorbance of H_2O_2 at 240nm (A_{240}). One unit of activity is defined as the amount of enzyme that catalyses the oxidation of 1 µmol. Of H_2O_2 min^{-1} under the assay conditions. It is expressed as µ mol. H_2O_2 / mg protein min^{-1} or activity U/g FW.

2.5. Glutathione Reductase (GR)

Glutathione reductase was assayed using (Carlburg and Mannervik, 1985) method. 50mM Tris-HCl buffer (pH 7.5), 3mM MgCl2, 0.5mM GSSG, 0.2mM NADPH and 250µl of enzyme extract was taken and made up to 1.5ml reaction mixture. GR activity was determined by monitoring the oxidation of NADPH at 340nm (A_{340}).

2.6. Hydrogen Peroxide (H2O2)

Hydrogen peroxide activity was determined by the (Velinkovar *et al.*, 2000) method. 500mg leaf sample weighed and homogenized in an ice cold bath wih 5 ml of 0.1% (w/v) trichloroacetic acid. The homogenate was centrifuged at 10,000g for 15 minutes and 0.5ml of the supernatant was added to 0.5 ml of 10mM potassium phosphate buffer (pH 7.0) and 1 ml of 1M KI. The absorbance of the supernatant was measured at 390nm.

2.7. Ascorbic acid

Ascorbic acid activity was determined using (Oser, 1979) method. 0.1g leaf was homogenized in 6% TCA. From the homogenate, 4ml was taken and to this 2ml of 2%DNPH, 1 drop of 10% thiourea was added. The content was boiled for 15 minutes in water bath and cooled. After cooling, 5ml of 80% (v/v) H_2SO_4 was added. The absorbance was read at 530nm. 2% DNPH (2,4 dinitrophenyl hydrazine) was prepared by dissolving 2g of DNPH in 100ml of 0.5 N H_2SO_4. 10% thiourea was prepared by dissolving 10g in 100ml of 70% ethanol.

2.8. TEM analysis

In 25mg/100ml ZnO NPs solution, red gram seeds were soaked for 8 hrs and later fixed in 2.5% gluteraldehyde in 0.1M phosphate buffer(PH 7.2). The samples were fixed for 24 h at 4°C, and rinsed with PBS for 4 times each sample and then fixed in 1% aqueous Osmium Tetroxide, later rinsed for 6 times with distilled water. This was followed by dehydration in series of graded alcohols and later infiltrated with in araldite 6005 resin or spur resin(spur,1969) . For polymerization samples were incubated for 72h at 80°C. Ultra-thin (60 nm) sections were made with a glass knife on ultra-microtome (Leica Ultra cut UCT-GA-D/E-1/00), mounted on copper grids and stained with saturated aqueous Urenyl acetate (UA) and counter stained with Reynolds lead citrate (LC) **(**John J, 1998**)**. The samples were viewed and photographed under TEM(Hitachi, H-7500 from JAPAN) at required magnifications.

2.9. Statistical Analysis:

Data recorded from three replications were subjected to single way analysis of variance (ANOVA) and critical differences were calculated at p=0.05 level.

3. Results

The desired concentrations (5, 10 ,15,20 and 25mg/100ml ZnONPs) used to testify their effect on growth parameters of *Cajanus cajan* L. Seedlings were selected based on a preliminary test on seed germination. The seedling growth parameters have been considered as primary indicator for the phytotoxicity of ZnONPs, especially in 80 days with higher concentrations. Significant decrease in the germination percentage, root length, number of leaves, width of leaves and increase in fresh and dry weight of plant was observed upon exposure to higher concentrations of ZnONPs. On the other hand, the results showed that the exposure of red gram seeds upto 15mg/L of ZnONPs exhibited a significant increase in

the growth of root and shoot length and number, length and width of leaves as compared to higher concentrations (25 mg/Lof ZnONPs). The results indicated that nano-ZnO in an appropriate concentration promoted root and shoot length of red gram(Fig.1-4).

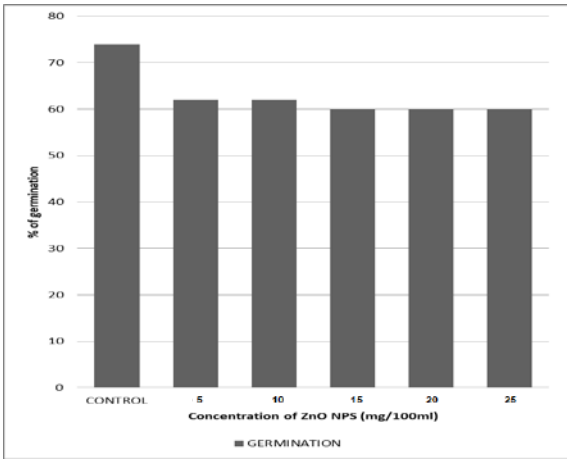

Figure 1. Germination % of *Cajanuscajan L.* grown in ZnO NPs

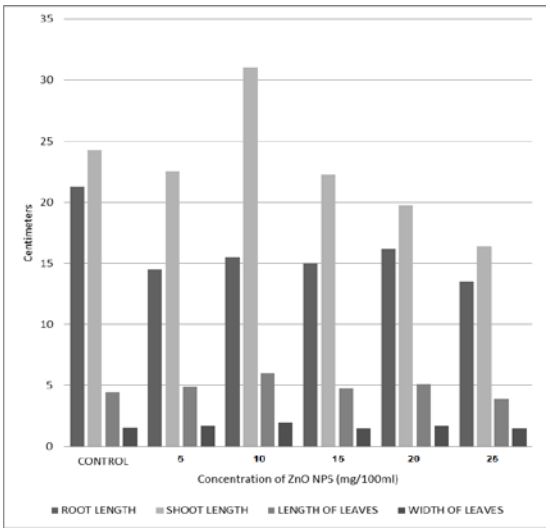

Figure 2. Lengths of Roots, Shoots and Leaves, and Widths of Leaves of *Cajanuscajan L.* grown in ZnO NPs

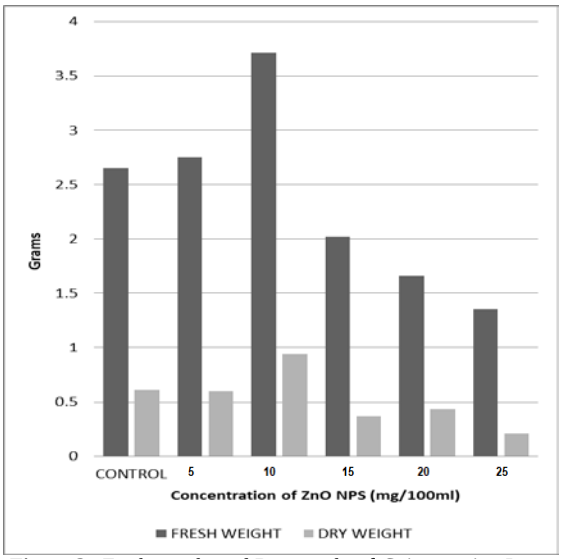

Figure 3. Fresh weight and Dry weight of *Cajanuscajan L.* grown in ZnO NPs

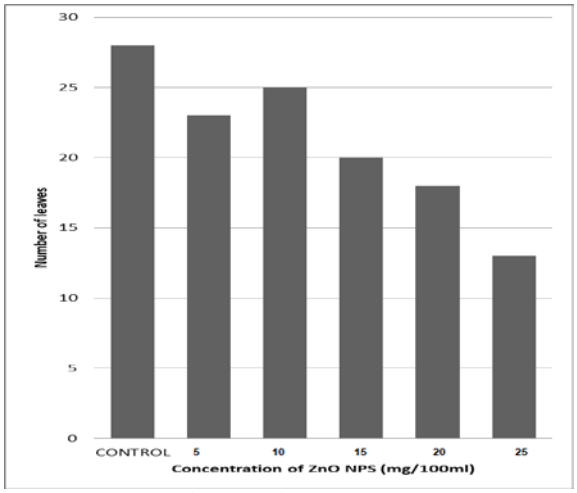

Figure 4. Number of Leaves of Cajanuscajan L. grown in ZnO NPs

In this study, a significant increase in carbohydrate, protein and chlorophyll of red gram seeds was found in response to the application of ZnONPs. The highest reducing sugars and protein content was recorded for seeds treated with 10 mg/100ml ZnONPs (Fig.5). Compared with the control, the chlorophyll content was found to be significantly increased in the plants grown with the 25 mg/100ml ZnO NPs treatment (Fig.6).

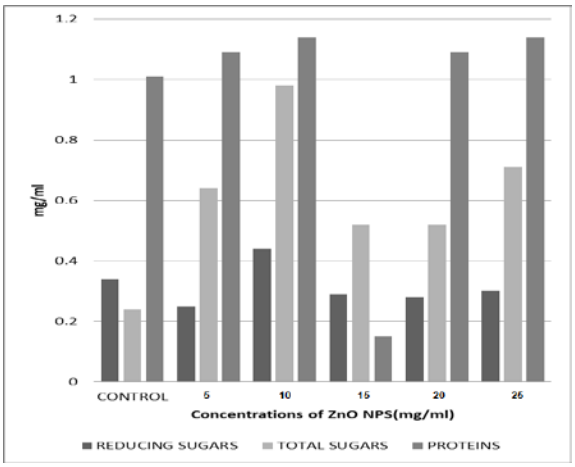

Figure 5. Reducing Sugar, Total sugar and Protein content of *Cajanuscajan L.* grown in ZnO NPs

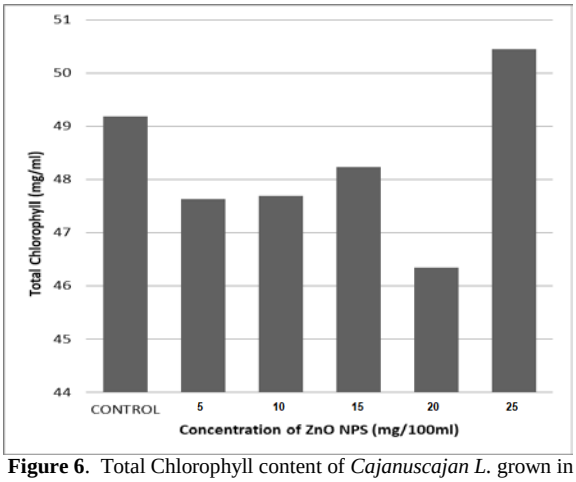

Figure 6. Total Chlorophyll content of *Cajanuscajan L.* grown in ZnO NPs

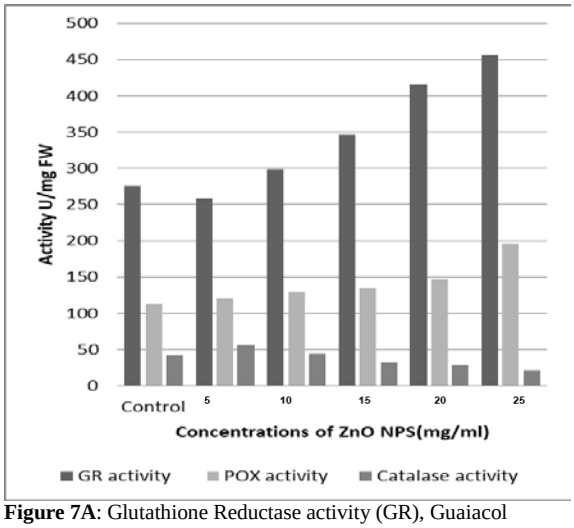

Figure 7A: Glutathione Reductase activity (GR), Guaiacol peroxidase (POX) activity, Catalase activity of *Cajanuscajan L.* Seed grown in ZnO NPs.

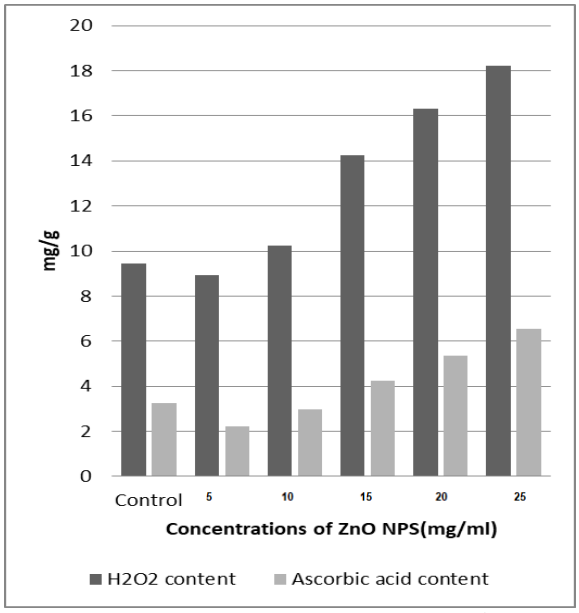

Figure 7B. H_2O_2 content and Ascorbic Acid content of *Cajanuscajan L.* Seed grown in ZnO NPs.

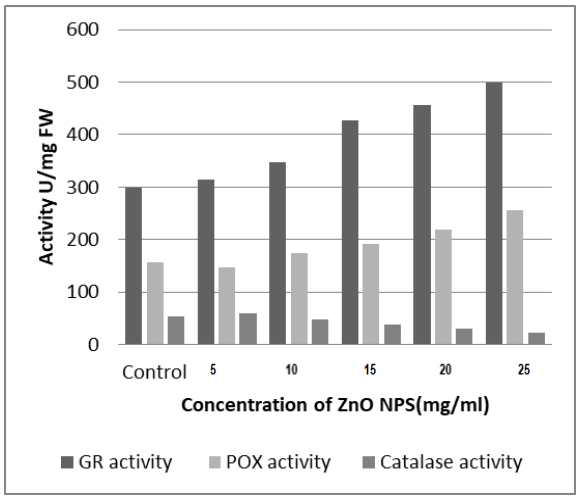

Figure 8A. Glutathione Reductase activity(GR), Guaiacol peroxidase (POX) activity, Catalase activity of *Cajanuscajan L.* Leaf grown in ZnO NPs

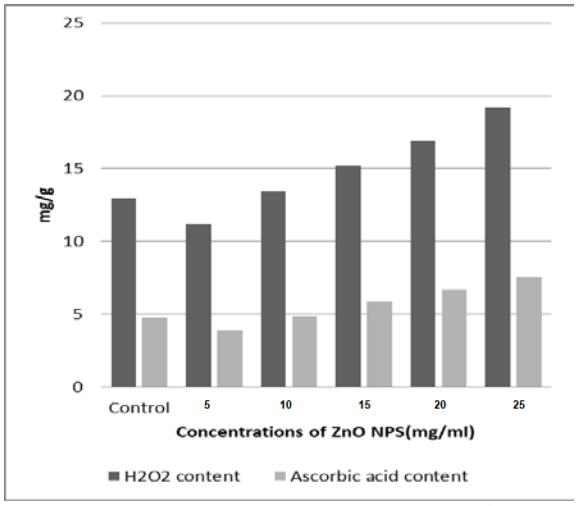

Figure 8B. H_2O_2 content and Ascorbic Acid content of *Cajanuscajan L.* Leaf grown in ZnO NPs.

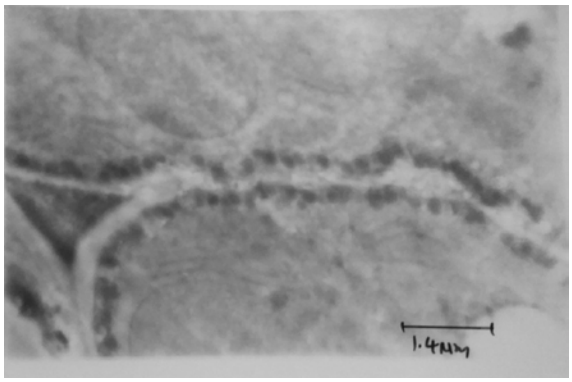

Figure 9. TEM image of red gram seed (Magnification: 13510X)

The activity of key antioxidant enzymes including, GR, GPX,CAT, Hydrogen Peroxide and Ascorbic acid in *Cajanus cajan L.* seeds that were grown in the presence of zinc oxide nanoparticles were analyzed. The activity of GR, POX and Hydrogen Peroxide was increased up to 25 mg/100 in seed compared to the control, whereas activities of CAT and Ascorbic acid were increased for concentrations up to 10 mg/100ml and were decreased with increasing NPs concentration. As shown in (Fig.7A-7B). GR, POX, Hydrogen Peroxide and Ascorbic acid activity in *Cajanus cajan L.* leaf in higher concentrations was significantly higher in comparison to the control. This is contrary to CAT activity. CAT activity is significantly decreased in the presence of NPs, except for 10 mg/100ml treatment. It should be noted that at the highest concentration, the activity for every antioxidant enzymes was significantly increased (Fig.8A-8B).

TEM analysis of the *Cajanus cajan L.*seeds treated with 25 mg/ml ZnONPs revealed clusters of nanoparticles along the cell wall of seed cells (Fig.9). Nanoparticles of different sizes were found inside the cell, however average size of 1.4 µm were predominantly recorded.

4. Discussion

Objectives of the present study were to investigate the effects of ZnONPs on the growth of red gram. On exposure to ZnONPs, our results had shown significant impact on the seed germination and seedling growth of red gram. (Lin and Xing, 2007) reported that ZnO NPs

inhibited seed germination in *Lolium multiflorum* and *Zea mays*. The damage to the structure of roots at higher concentration may be the reason for the germination of the seeds (Kwang *et al.*, 2018). Lee e*t al.* (2008) also found that the seedling growth parameters of mung bean and wheat plants were significantly decreased due to the presence of copper nanoparticles. Zhongzhou Yang *et al.* (2015) reported that there is no inhibition effect of ZnONPs on root and shoot length of rice. Mechanism of metal oxide NP phytotoxicity was related to the type of nanoparticles, test plants, concentration, chemical composition, surface modification and particle size (Brunner *etal.*, 2006). Sharma *et al.* (2012) also observed that AgNPs increased plants growth profile and biochemical attributes of common bean and corn.

ZnONPs treated red gram showed improvement in reducing sugars, total sugars, protein and chlorophyll content. Tarafdar (2013) reported that ZnONPs induced a significant improvement in *Cyamopsis tetragonoloba* chlorophyll and protein synthesis. But significant reduction of total chlorophyll and total protein contents were observed under the higher concentrations of AgNPs (Asma *et al.*, 2018). Mechanism of action of different kinds of nanoparticles is unclear in different plants (Mohamed *et al.*, 2014). The increase in biochemical parameters illustrate the importance of zinc in protein synthesis, chlorophyll production and activation of many enzymes on the biosynthetic pathway of secondary metabolites (Sasan and Sevedeh, 2017).

With increased concentration of ZnONPs, the activities of antioxidative enzymes increased in the seed and leaf except catalase and H_2O_2. Such increase in antioxidative enzyme activity may be due to the stress over the seedlings of *Cajanus cajan L.* Metal stress commonly generates toxic free radicles and superoxides and activates the production of anti-oxidative stress enzymes. These enzymes scavenge the ROS by reduction reaction and protect cells from any damage (Gao *et al.*, 2013; Garcia-Sanchez *et al.*, 2015). GPX, GR activity increased in Faba bean *(Vicia faba L)* seedling treated with ZnONPs (Salah M. H. Gowayed and Naif M. Kadasa, 2016). Repression of catalase might be caused by the accumulation of H_2O_2 (Yi *et al*., 2003). H_2O_2 can play an important role in the induced tolerance against oxidative stress by activation of the plant antioxidant system in a dose dependent manner. Reduced catalase activity can be compensated by alternative H_2O_2-scavenging mechanisms such as increased ascorbate peroxidase and glutathione peroxidase levels (Willekens *et al*., 1997).

The micrographs of the seed sections clearly show that the cell wall acts as primary accumulation sites for NPs. These results strongly point toward possible transformations and/or aggregation of ZnO NPs during or after their internalization. The TEM image clearly shows the transport of nanoparticles into the seeds, which is responsible for decrease in growth parameters and enhanced antioxidant activity.

5. Conclusion

ZnONPs caused significant inhibition on *Cajanus cajan.* L. seed germination, shoot and root growth, ZnONPs caused significant inhibition on *Cajanus cajan.L.* seed germination, shoot and root growth, number of leaves, fresh and dry weight of plants upon exposure to higher concentrations. But sugars, protein and chlorophyll content increased with higher concentrations. We also observed increase in Glutathione Reductase and Peroxidase activity, H_2O_2 and ascorbic acid content. But upon exposure to higher concentrations of ZnONPs, decrease in catalase activity of seeds and leaves of *Cajanus cajan.L.* was observed. TEM image of red gram clearly showed translocation of ZnONPs into the seed. According to our results, we can conclude that inhibition of seedling growth and antioxidant activity differ significantly with the nanoparticle concentration. It can be speculated that the toxic effects of ZnONPs on plants is due to formation of ROS, following the NPs uptake. Since red gram is the most staple food, NPs can find their way into the human body and interact with the cells with unexpected consequences. Therefore, more investigations are needed to determine the positive or negative effects of ZnONPs on red gram/ plants.

Acknowledgements

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