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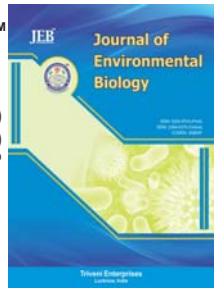

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Effect of zinc nanoparticles on antioxidative system of potato plants

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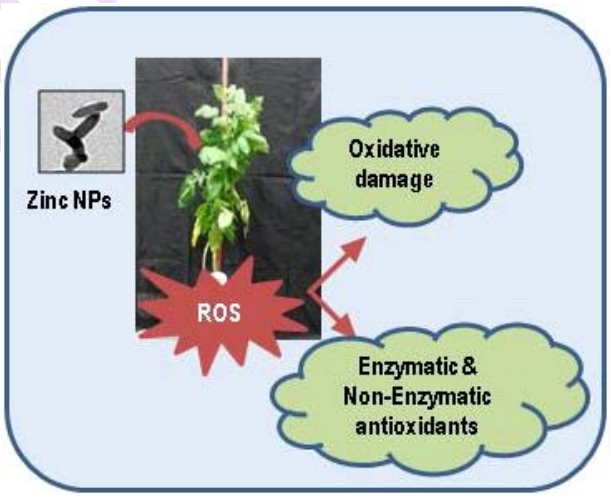
Abstract

Aim: Nanotechnology has the potential to change the entire agriculture sector but there might be several risks which may affect the environment. Raw materials such as heavy metals used to synthesize metal nanoparticles have significant negative effect on various growth, physiological and biochemical characteristics of plants. The present study was carried out to assess the effect of zinc nanoparticles on some metabolic activities of potato plants.

Methodology: Potato plants were grown in growth chambers. Two foliar sprays of zinc nanoparticles of 100, 300 and 500 ppm were given on potato plants. First spray was given after 25 days and the second after 45 days of planting. Enzymatic antioxidants (catalase and peroxidase), non enzymatic antioxidants (total phenolics and anthocyanins) and carbohydrates (starch and total soluble sugars) were analysed.

Results: The activity of enzymes was high in zinc nanoparticle treated plants compared to control plants. There were 142, 109 and 212% increase in catalase activity in plants treated with 100, 300 and 500 ppm concentration of nanoparticles, respectively. Increase in peroxidase activity was significant at 500 ppm concentration. Treatment with zinc nanoparticles increased the total phenolics and anthocyanin content significantly at 300 and 500 ppm concentrations. There were 15, 29 and 259% increase in the starch content due to 100, 300 and 500 ppm concentration of nanoparticles, respectively.

Interpretation: The increase in enzymatic and non-enzymatic antioxidants clearly indicated that the zinc nanoparticles lead to oxidative stress and caused toxicity to potato plants.



Introduction

Now-a-days, looking into the potentiality of nanotechnology in the areas of electronics, pharmaceuticals, medicine, etc., nanoparticles are also being used in agriculture field to enhance the yield of a given crop. Nanoparticles have received considerable attention in agriculture field due to their high rate of uptake and penetration in plants. Moreover, extremely small size, structure and surface characteristics of nanoparticles result in unique physico-chemical properties (Prasad *et al.,* 2012).

Although nanotechnology has potential to change the entire agriculture sector and food industry chain, but there might be several risks which may affect the plant health and subsequently reduce the yield. Nanotechnology industry used metal based nanoparticles such as silver, zinc oxide, titanium oxide, iron oxide etc., on large scale. With ever increasing use of nanoparticles, soil gets contaminated affecting the growth and yield of food crops. Nanoparticles have high surface area, more catalytic efficiency, abundant reactive sites and high absorption rate (Desai *et al.,* 2015). It has been reported to have negative effects on growth of ryegrass, mung bean, wheat, radish, rape, lettuce, corn, cucumber and stevia (Lin and Xing 2008, Lee *et al.,* 2008, Menard *et al.,* 2011, Desai *et al.,* 2015,). With release of nanoparticles in the environment, plants are exposed to additional stress besides biotic and abiotic stresses.

Plants require many nutrients for their growth and development, among them zinc is an essential micro nutrient and 2nd most abundant transition metal in organisms after iron. Zinc is required for all the enzyme classes viz. oxidoreductase, transferases, hydrolases, lyases, isomerases and ligases (Prasad *et al.,* 2012). Trace amount of zinc is required for normal growth of plants and its deficiency causes several physiological disorders. It is also known to regulate the functioning of stomata by retaining the potassium content (Laware and Raskar, 2014).

Potato is one of the most important non-grain food crops and is the fourth most important food crop of the world. However, the global average yield of potato is far low as compared with its potential yield, mainly due to biotic and abiotic stresses (Gangadhar *et al.,* 2014). Potato being an underground modified stem has high chances of direct uptake of nanoparticles from soil and there are gaps in the current knowledge of understanding the impact of zinc nanoparticles (Zn-NPs) on the growth and metabolism of potato plants. The stress induced by nanoparticle in plants can be assessed by estimation of antioxidative enzymes such as catalase, peroxidase, superoxide dismutase, ascorbate peroxidase, and glutathione reductase, as well as secondary metabolites such as phenolics, terpenes and nitrogen and sulphur containing compounds (Goswami, 2013). Therefore, in the present study, the effect of zinc nanoparticles on enzymatic and non-enzymatic antioxidants and carbohydrate fractions of potato plants were estimated which indirectly represents the health status of the plant.

Materials and Methods

Growth conditions : Sprouted tubers of cultivar Kufri Chandramukhi were used in the study. Tubers were planted in pots filled with sterilized soil (treated with 0.2% Bavistin solution) and kept in growth chamber (Conviron, Model E-15, Canada). Potato plants were grown in growth chambers for 30 days at non-tuberisation conditions (24°C day/night temperature and 24 hr photoperiod), so that no tuberization stimulus was formed. After 30 days of plantation, favourable conditions for tuberization *i.e.,* 24°C/18°C day/night temperature and 12 hr photoperiod were created.

Preparation of solutions and transmission electron microscopy : Commercially available zinc oxide nanoparticles <100 nm size were purchased from Sigma (USA) and suspensions of Zn-NPs were prepared with varying concentrations viz. 0, 100, 300, 500 ppm in deionized water and ultrasonicated for 30 min to avoid aggregation. Before spraying Zn-NPs on potato plants, Zn-NPs were observed with the help of transmission electron microscopy (120 kV, FEI make). The suspensions of Zn-NPs were sonicated for 30 min and a drop of representative suspension was dropped on carbon coated copper grids and air dried. The air dried grids were later stained with 2% uranyl acetate and viewed under TEM.

Treatment : The plants were sprayed with graded concentrations of Zn-NPs after 25 and 45 days of planting in three replicates. The water spray without nanoparticles was kept as control.

Biochemical analysis : Control and Zn-NP treated samples for biochemical analysis were collected after three days of foliar application of nanoparticles. Third and fourth leaves were used for studying catalase, peroxidase activity and total phenolics, anthocyanin content, starch and total soluble sugars content.

Catalase activity : Catalase activity was determined following the method of Aebi (1983). Fresh leaf sample (0.5 g) was homogenized in 5 ml of ice cold 0.05 M phosphate buffer (pH 7.5). Samples were centrifuged at 10,000 rpm for 20 min at 4°C and supernatant was collected. The activity was carried out in a reaction mixture containing 1 ml H_2O_2 solution (0.2 ml H_2O_2 diluted to 50 ml with sodium phosphate buffer, pH 7.5) and 1.8 ml sodium phosphate buffer, pH 7.5. To this 0.1 ml enzyme extract was added and absorbance was immediately recorded continuously for up to 2 min at 15 sec interval at 240 nm on UV-Vis spectrophotometer (UV 1102 spectrophotometer).

Peroxidase activity : Peroxidase activity was estimated following the method of Shannon *et al.* (1966). Leaf sample (1 g) was homogenized in 5 ml of ice cold 0.1M chilled phosphate buffer (pH 6.5) and centrifuged at 10,000 rpm for 20 min at 4°C.

Supernatant was collected and volume was made upto 3 ml with extraction buffer. In spectrophotometric cuvette, 3 ml 0.05 M guaiacol containing 0.1 M phosphate buffer (pH 6.5) and 0.1 ml enzyme extract was taken. Reaction was initiated by adding 0.1 ml of 0.8 M hydrogen peroxide and reading was immediately recorded for 3 min at 15 sec interval at 470 nm (UV 1102 spectrophotometer).

Starch : Starch content was determined according to the modified method of McCready *et al.* (1958). Leaf samples (100 mg) were extracted in 5 ml of 80% ethanol and centrifuged thrice to remove sugars from the sample. The residues were suspended in 6.5 ml of 52% perchloric acid and 5 ml of distilled water. The samples were mixed thoroughly and centrifuged and residue was extracted with 6.5 ml of 52% perchloric acid and centrifuged again. Both the supernatants were combined and final volume was made to 50 ml with distilled water. A five ml of extract and 4.5 ml of distilled water was boiled in the presence of 10 ml of anthrone-sulphuric acid reagent (200 mg anthrone in 100 ml chilled concentrated sulphuric acid). Boiling was done for 8 min and samples were cooled to room temperature. Optical density was recorded at 620 nm (T60 Visible Spectrophotometer, PG Instruments, India).

Total soluble sugars : Total soluble sugars were determined from leaf samples following the method of Dubois *et al.* (1956). Leaf sample (100 mg) was extracted with 5 ml of 80% ethanol and centrifuged. Residue was again extracted with 3 ml of 80% ethanol twice and centrifuged. All the supernatants were pooled. One ml of extract was mixed with 1 ml of 5% phenol and incubated for 5 min at room temperature. Five ml of chilled sulphuric acid (96%) was added to the samples with continuous stirring. Optical density was recorded at 490 nm (T60 Visible Spectrophotometer, PG Instruments, India).

Total phenolics : Total phenolics content was estimated from leaf samples following the method described by Oloumi *et al.* (2015) with some modifications. Leaf sample (100 mg) was extracted in 5 ml of 95% ethanol and centrifuged for 5 min at 8000 rpm. Supernatant was mixed with 0.5 ml of Folin-Ciocalteu reagent prepared in 1:1 ratio with distilled water. Five percent of sodium carbonate solution (1.5 ml) was added to the samples and absorbance was measured at 725 nm (T60 Visible Spectrophotometer, PG Instruments, India) after 1 hr incubation at room temperature.

Anthocyanins : Anthocyanin content was measured from leaf samples following the standard method (Oloumi *et al.,* 2015)**.** Leaf samples (100 mg) were extracted in 10 ml of acidified methanol [methanol: HCl 99:1 (v/v)]. Samples were incubated at 25°C in dark for 24 hrs. The samples were centrifuged at 4000 g for 5 min at room temperature. The absorbance of the supernatant was measured at 550 nm (T60 Visible Spectrophotometer, PG Instruments, India). Anthocyanin content was calculated by using 33000 mol^{-1} cm^{-1} extinction coefficient.

Statistical analysis : All the analysis was performed in three replicates using complete randomization block design (CRBD). The data were analyzed using MSTAT 4.0C software following the method of Gomez and Gomez (1984). One way ANOVA was performed at 5% level of significance.

Results and Discussion

The solutions prepared from commercially available Zn-NPs were viewed under TEM and the results of TEM images (Fig. 1) showed that the particles were in slight aggregated form with different shapes like capsule, cylindrical and few uneven shapes of the particles at 11000x magnification. Prasad *et al.* (2012) and Dimpka *et al.* (2012) also reported zinc nanoparticles in slightly aggregated form under HRTEM and AFM, respectively. After confirming the particles in TEM the same solution was used for further studies.

Catalase activity was low in control plants as compared to Zn-NP treated plants. There was significant increase in catalase activity with Zn-NP treatments (Table 1). There were 142, 109 and 212% increase in catalase activity in potato plants treated with 100, 300 and 500 ppm Zn-NP concentration, respectively. Catalase activity was high at 100 and 500 ppm and low at 300 ppm. Similar trend in catalase activity was reported by Desai *et al.* (2015) where high catalase activity was reported in stevia at 50 ppm Zn-NP as compared to 100 ppm and again increase in activity from 200 to 1000 ppm Zn-NP. During oxidative stress, catalase activity is reported to increase manifolds probably due to activation of defense mechanism. Increase in catalase activity leads to more scavenging of reactive oxygen species and therefore, protecting the cells from damage due to these species (Dipierro and Leonardis, 1997, Delaplace *et al.,* 2009).

Peroxidase activity showed increasing trend with increase in Zn-NPs concentration (Table 1). Peroxidase activity was 0.513 µmoles.$min^{-1}g^{-1}$ f.wt. in control plants. Peroxidase activity showed similar trend as that of catalase. Activity increased upto 7 and 75% at 100 and 500 ppm Zn-NPs concentration, respectively. However, 300 ppm of Zn-NPs decreased the activity to 42%. Increase in peroxidase activity was significant after 500 ppm concentration of Zn-NP application. The results of peroxidase is in accordance with the result of Desai *et al.* (2015). Who reported high peroxidase activity at 50 and 100 ppm, whereas activity was low at 200, 400 and 1000 ppm in *Stevia rebaudiana*. Activity of both the enzymes was the maximum at 500 ppm concentration of Zn-NPs in *Stevia rebaudiana*. Sharma *et al.* (2014) reported that 300ppm zinc concentration in soil produced stress conditions in *Beta vulgaris* and led to increased lipid peroxidation.

On contact of nanoparticles to cell, lipid membrane peroxidation increases due to generation of reactive oxygen

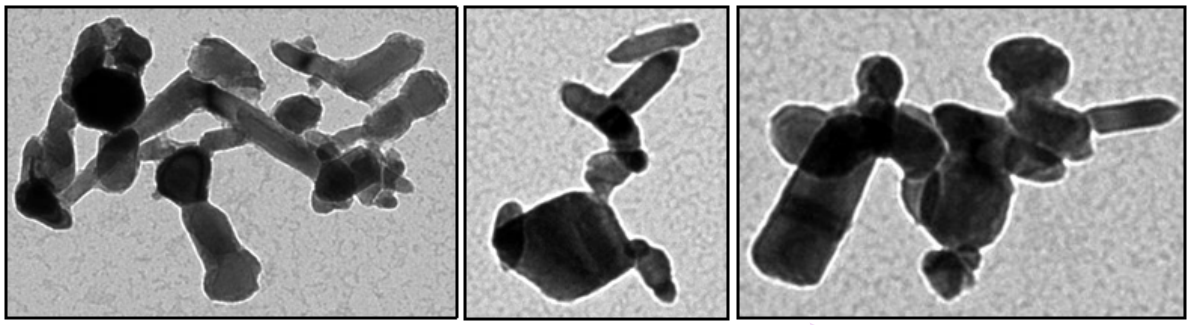


Fig. 1 : Transmission electron microscopic images of commercially procured zinc nanoparticles which are in slight aggregated form with different shapes of the particles at 11000x magnification

Table 1 : Effect of different concentrations of Zn-NPs on carbohydrates and enzymatic and non-enzymatic antioxidants

Treatments	Catalase (μmoles min^{-1} g^{-1} f.wt.)	Peroxidase (μmoles min^{-1} g^{-1} f.wt.)	Total phenolics (μg g^{-1} f.wt.)	Anthocyanin (mg 100mg^{-1} f.wt.)	Starch (mg g^{-1} f.wt.)	Total soluble sugars (mg g^{-1} f.wt.)
Control	0.106	0.513	81.5	0.423	1.98	9.847
100 ppm	0.256	0.551	82.2	0.395	2.28	8.859
300 ppm	0.221	0.297	97.8	0.488	2.55	8.379
500 ppm	0.331	0.896	99.5	0.624	7.11	12.086
SE (0.05) at 8df	0.026	0.026	1.89	0.026	0.506	1.489

species (Nel *et al.,* 2006). Plant increases the level of its antioxidative enzymes such as catalase and peroxidase to scavenge the reactive oxygen species. Researchers have reported that zinc nanoparticles affect the metabolic activity by generating reactive oxygen species and cause oxidative stress and decrease chlorophyll content in shoots and increase the glutathione content (Chang *et al.,* 2012, Dimpka *et al.,* 2012). The results are in accordance to the earlier reports. A high activity of catalase and peroxidase indicates high rate of reactive oxygen species in potato plants treated with Zn-NPs. Therefore, Zn-NPs may be assumed as toxic to potato plants.

Amongst the non-enzymatic antioxidants, total phenolics and anthocyanin content was assessed in control and Zn-NP treated potato plants. Total phenolic content was 81.5 $\mu g.g^{-1}$ f.wt in control plants. Nanoparticle treatment increased total phenolics in plants and increase was 1, 20 and 22%, respectively, at 100, 300 and 500 ppm concentration (Table 1). Treatment of potato plants with 300 and 500 ppm nanoparticles increased total phenolics significantly. Phenolic content was high in zinc nanoparticle treated licorice plants and the content increased with increase in concentration of zinc nanoparticles (Oloumi *et al.,* 2015). Reports are available on accumulation of phenolics in response to metal in toxicity (Santiago *et al.,* 2000, Lavid *et al.,* 2001). Lavid *et al.,* (2001) reported accumulation of metals tissues along with accumulation of polyphenols in waterlily plants grown on heavy metal contaminated medium.

Anthocyanin content was 0.423 mg 100mg^{-1} f.wt. in control plants (Table 1). Treatment of potato plants with 100 ppm concentration of Zn-NPs decreased the anthocyanin content non-significantly, whereas significant increase in anthocyanin content was observed after treatment of plants with 300 and 500 ppm concentration of Zn-NPs. The results of anthocyanin content in the present study is contradictory to those reported by Oloumi *et al.* (2015) where decrease in anthocyanin content was observed with increase in zinc nanoparticles concentration. This could be due to difference in tolerance level of different plants to oxidative stress. Nine times increase in anthocyanin content was reported in Azolla plants incubated with aluminium sulphate (Ayala-Silva and Al-Hamdani, 1997). Anthocyanin content increased with increase in severity of salt stress in tomato and red cabbage (Eryilmaz, 2006).

Starch content was 1.98 $mg.g^{-1}$ f.wt. in leaves of control plants. Starch accumulation increased with increase in concentration of Zn-NPs and this increase was significant with 500 ppm treatment (Table 1). There were 15, 29 and 259% increase in starch content after treatment of plants with 100, 300 and 500 ppm nanoparticles. Accumulation of starch in leaves of potato plants was due to oxidative stress caused by Zn-NPs. Changes in the ultrastructure of chloroplast has been reported due to oxidative stress caused by excess copper, which resulted in deterioration of grana structure and accumulation and swelling of starch granules in the stroma (Bouazizi *et al.,* 2010).

Total soluble sugars were 9.847, 8.859, 8.379 and 12.086 mg g^{-1} f.wt. in control, 100, 300 and 500 ppm nanoparticle treated plants, respectively (Table 1). Zn-NPs at 100 and 300 ppm concentration decreased the total soluble sugars non-

significantly. Whereas, significant increase was reported after treatment of plants with 500 ppm concentration of Zn-NPs. Reducing sugars increased in zinc nanoparticle treated Licorice plants as compared to control plants (Oloumi *et al.,* 2015). Starch, as well as total soluble sugars increased significantly after treatment of potato plants with 500 ppm Zn-NPs. Carbohydrates are known to accumulate in response to oxidative stress. Carbohydrates have several functions in plants from energy storage to signaling and plant adapt to environmental stresses by using several carbohydrate based strategies (Anderson and Kohoron, 2001). This could be the reason for accumulation of high carbohydrate fractions in potato leaves at high concentration of zinc nanoparticles.

In the present study, Zn-NPs were found to increase the enzymatic antioxidants (catalase and peroxidase), non-enzymatic antioxidants (total phenolics and anthocyanin) and carbohydrates (starch and total soluble sugar) in potato plants. These contents increased due to oxidative stress caused by Zn-NPs. Therefore, exposure of potato plants to Zn-NPs was found to have negative effect on potato plant. However, further studies are needed on this line to see the effects of Zn-NPs on growth and productivity of plants.

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