Foliar Spray of Zinc Oxide Nanoparticles Improves Salt Tolerance in Finger Millet Crops under Glasshouse Condition

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Abstract
Nanomaterials improve the plant's resistance to abiotic stress with reduced environmental impact compared to traditional approaches. In the present study, nano-sized zinc oxide particles were synthesized by simple precipitation method. The structure, size and optical properties of ZnO NPs were characterized by UV-vis spectroscopy, FTIR, DLS, TEM and XRD. UV-Vis spectrum of ZnO NPs showed a characteristic peak at 380 nm. DLS analysis of ZnO NPs exhibited the size range of 50 nm and the average zeta potential as -30 mv which indicate the stability of nanoparticles. TEM analysis revealed the size of ZnO NPs as 30-50 nm. XRD analysis exhibited the crystalline nature of nanoparticle. The impact of ZnO NPs (0.05%, w/v) on seedling of *Eleusine coracana* (L.) Gaertn. was investigated under in vitro saline condition. Seedling bioassay showed increased shoot growth (106.6%) and biomass (125%) in treated seeds compared to control and salinity, whereas the root growth was negatively affected. Foliar spray of ZnO NPs to finger millet revealed a higher level of antioxidant enzymes (30-40%), and a decreased level of protein (50%) content. MDA (2-8 fold) and proline content (three-fold) was higher in plants under salt stress compared to control. Increased photosynthetic pigments (~one-fold) promoted the growth of finger millet under glass-house condition. All these results suggest the potential involvement of ZnO NPs in improving plant tolerance to salinity and opens up the possibility for sustainable finger millet production.

Keywords
ZnO NPs, Foliar spray, Glasshouse, Salt stress, Plant growth, Antioxidant enzymes

Introduction
Abiotic stresses are major constraints which adversely affect the crop productivity and plant growth. Among abiotic stresses, salinity affects the plant growth, physiology and biochemistry causing osmotic stress and ionic toxicity [1]. Nanotechnology opens a large scope for diverse applications in the field of agricultural biotechnology particularly in agricultural production. Applications of nanomaterial enhances plant germination, production of improved plant tolerance to abiotic and biotic stresses, efficient nutrient utilization and increased plant growth, with reduced environmental impact compared to traditional approaches. In recent years, a considerable improvement in salinity tolerance has been achieved in some vegetable crop species through the application of nanoparticles [2]. Reynolds [3] demonstrated that micro-nutrients in the form of nanoparticles (NPs) can be used extensively in crop production to increase the yield.

Zinc (Zn) is an important micronutrient required for the optimum growth and development of plants which carries vital metabolic reactions within the plants to promote growth and development. Despite its role in the growth and development, it also plays a vital role in reducing toxic heavy metal uptake by plants, thereby prevents the plants from heavy metal toxicity [4]. It also plays an active role in regulating various mechanisms involved in recognition and response to abiotic stresses in plants. In addition, zinc scavenges the reactive oxygen species (ROS) and helps in protecting the plant cells against oxidative stresses [5].
Zinc oxide nanoparticles (ZnO NPs) are non-toxic, eco-friendly, biosafe and bio-compatible making them an ideal candidate for biological applications [6,7]. Several studies have shown that antioxidant enzymes are increased in plants such as tomato, Plantago maritima, radish and wheat in response to salt stress [8-11]. Zinc oxide NPs treatment lead to an increase in contents of free radicals, including reactive oxygen and nitrogen species (ROS/RNS) and hydrogen peroxide (H₂O₂) in duckweed [12]. Foliar application of ZnO NPs on chickpea enhanced seed germination, seedling growth and biomass production [13]. There are few reports about the positive effect of nanoparticles on crops such as groundnut [14], pea [15], spinach [16] and cotton [17]. However, limited work has been done to evaluate their role and effect of zinc oxide nanoparticles (ZnO-NPs) in regulating physiological and biochemical processes in response to salt induced stress. In this context, the present study was undertaken to examine the effect of foliar spray of ZnO-NPs in improving salt tolerance in finger millet crops.

Materials and Methods

Synthesis and characterization of ZnO-NPs

ZnO NPs were synthesized by direct precipitation method using zinc sulphate and sodium hydroxide as precursors [18]. To the aqueous zinc sulphate (heptahydrate), sodium hydroxide was added drop-wise in the molar ratio (1:2) at room temperature under vigorous stirring for 12 hrs. The white precipitate obtained was centrifuged at 5000 rpm for 20 min and washed three times with deionized double distilled water. The obtained product was calcined at 100 °C in air atmosphere for 3 hrs, ground to fine powder and used for further studies.

The synthesized ZnO NPs was monitored by UV-VIS spectrum (Model 2202, Systronics Ltd.) at room temperature ranging from 200 nm to 500 nm for the confirmation of nanoparticle formation. The IR spectrum of dried ZnO NPs was recorded by Nicolet 560 FT-IR spectrometer in a range of 400-4000 cm⁻¹ using KBr pellet method. The mean particle size, polydispersity index (PDI) and zeta potential of synthesized nanoparticles was performed by DLS on a Zetasizer (Malvern Instruments, UK) at 25 °C at a scattering angle of 90° in triplicate. To determine the size of the ZnO NPs, Transmission Electron Microscopy was performed using TEM (TECNAI G2 SPIRIT TWIN, The Netherlands). The diffraction intensities of nanoparticles were recorded from 10° to 80° 2θ angle using Rigaku II X-ray diffractometer.

Seedling bioassay

Finger millet seeds (Eleusine coracana (L.) Gaertn). obtained from local farm was used to determine the efficacy of ZnO NPs on seedling growth using standard methods with some modifications [19]. Briefly, finger millet seeds were surface sterilized by immersing in 0.01% (w/v) sodium hypochlorite solution for 10 min and then rinsed three times with deionized double distilled water. The sterilized seeds were placed in Petri plates separately having filter paper with deionized water (control), 100 mM NaCl solution (salinity) and combination of ZnO NPs (0.05%, w/v) and 100 mM NaCl (treated). Each treatment was performed in triplicates with 30 seeds in each plate. The Petri plates were maintained at 28 ± 2 °C in a growth chamber for 10 days. Data were recorded for seed germination percentage, mean seedling length and fresh weight at different intervals (2, 4, 6, 8 & 10 d). Seedling vigor index (SVI) was calculated using the formula described by Abdul-Baki and Anderson [20]. Seed vigor index = (germination %) × (seedling length).

Foliar application of ZnO NPs

In pot experiment, NaCl (100 mM) was incorporated with soil in all the experimental pots (salinity, treated) except control consisting of three experimental groups. The surface sterilized finger millet seeds were sown in all the pots (10 seeds/pot) under glass-house condition. ZnO NPs (0.05%, w/v; 2 ml/plant) were applied to 30-day-old plants (treated) by the foliar spray method at a regular interval of 10 days.
up to 50 days (3 sprays performed at 30th, 40th & 50th day). Water was sprayed to control and salinity plants. For each experiment, 30 plants were used and replicated thrice. The leaves of finger millet plant were removed at regular intervals (35th, 45th & 55th d) and used for further studies.

**Leaf protein analysis**

Leaves (0.5 g) of finger millet plants (control, saline, ZnO NP treated) at different intervals (35th, 45th, 55th d) was taken, homogenized with potassium phosphate buffer (0.02 M, pH 7.6) and centrifuged for 10 min at 4 °C. The clear supernatant obtained was used as a source of protein/enzymes. Protein content was determined by Bradford [21] method using BSA fraction V (Sigma Chem. Co., USA) as standard.

**Determination of photosynthetic pigments**

For Chlorophyll (chl a, b) and carotenoid extractions, (0.5 g) finger millet leaves of above mentioned intervals was ground with 0.5 ml of acetone (80%, v/v). For pigment measurements, the absorbance was read at 480, 645 and 663 nm in a spectrophotometer. Pigment contents (mg g⁻¹ FW) were calculated using the formula described by Barnes, et al. [22].

**Estimation of lipid peroxidation and proline content**

The level of lipid peroxidation in leaves of finger millet plants was determined according to Heath and Packer [23]. The leaf samples (0.5 g) were homogenized with 2 ml of trichloroacetic acid (5%, v/v) and centrifuged. To 2 ml of supernatant, 2 ml of 0.6% thiobarbituric acid was added, placed in boiling water bath for 30 min. The absorbance of the reaction mixture was read at 532 NM. The MDA (malondialdehyde) content was expressed as μmol g⁻¹ FW.

The proline content was determined using D-Proline as a standard [24, 25]. The leaves (0.01 g/0.5 ml) were homogenized with 3% (v/v) aqueous sulfosalicylic acid and centrifuged. To this, 1 ml of acid-ninhydrin, 1 ml of glacial acetic acid was added and maintained at 100 °C for 1 hr. After cooling, 2 ml of toluene was added, mixed vigorously and the chromophore-containing toluene was read at 520 nm using toluene as blank.

**Antioxidant enzyme assays**

The activity of ascorbate peroxidase (APX; EC 1.11.11) was determined by the method of Jimenez, et al. [26] with slight modifications. The reaction mixture consisted of 0.5 mM ascorbic acid, 250 mM H₂O₂, and enzyme extract equivalent to 50 μl of protein. The enzyme activity was recorded as a decrease in absorbance at 290 nm for 1 min. One unit of ascorbate-peroxidising activity was defined as the oxidation of 1 μM of ascorbate (min mg⁻¹) under the assay condition.

Catalase (CAT; EC 1.11.1.6) activity was determined by recording the disappearance of H₂O₂ as described by Aebi [27]. The reaction mixture consisted of 15 mM H₂O₂ and 20 μl of enzyme extract. The enzyme activity was measured by recording the decrease in absorbance at 240 nm for 1 min. One unit of CAT activity was defined as the amount of enzyme required to decay 1 μmol of hydrogen peroxide (min mg⁻¹) protein under the assay condition.

Peroxidase (POX; EC 1.11.17) activity was determined by the method of Lee and Kim [28] with some modifications. The assay mixture consisted of 40 mM phosphate buffer (pH 6.8), 15 mM guaiacol, and 5 mM H₂O₂. The reaction was started by the addition of H₂O₂ and the increase in absorbance at 470 nm was measured for 1 min under the assay condition. One unit of POX activity was defined as the estimated consumption of 1 μmol of hydrogen peroxide (min mg⁻¹) protein under the assay condition.

**Statistical analysis**

All the data were subjected to one-way analysis of variance to determine the
significance of individual differences in p < 0.01 and 0.05 levels. All statistical analysis was conducted using SPSS 16 software support.

**Results and Discussion**

UV-visible spectra exhibited surface plasmon resonance (SPR) band at 380 nm, which is a typical plasmon resonance band of ZnO NPs (Figure 1). As in Figure 2, FTIR analysis showed the major characteristic peaks of the ZnO NPs: 3438.76 cm⁻¹ (N-H amine stretching), 2076.77 cm⁻¹ (C-H alkyl methylene stretch), 1637.58 cm⁻¹ (C=O aldehyde stretch), 1385.13 cm⁻¹ (C=C aromatic stretch) and 1109.71 cm⁻¹ (C-N amine stretch). The peak in the region 668.68 cm⁻¹ was allotted to Zn-O [29]. DLS analysis showed the average particle size of 50 nm with PDI value of 0.3 (Figure 3a). The zeta potential was observed as -30 mv which indicate the colloidal stability of ZnO nanoparticles (Figure 3b). The TEM micrograph of ZnO NPs revealed the particle size range of 30-50 nm as depicted in Figure 4. As shown in Figure 5, ZnO NPs showed a single phase with different diffraction peaks indicating the crystalline nature. The XRD peaks at 2θ- 100, 102, 110, 112 were in accordance with Raliya and Tarafdar and Tarafdar, et al. [30,31].

The development of low-cost nanomaterials, which are non-toxic to plants, is
highly essential in crop improvement relating to superior plant growth with outstanding yield. In order to access the plant growth stimulating role of ZnO NPs in finger millet under salinity, the physiological characters in seedling bioassay such as...
plant growth in terms of shoot, root length and biomass was calculated. The impact of ZnO NPs on plant growth (MSL & MRL) and biomass (MFW) was depicted in Table 1. The results showed that shoot growth, total biomass were enhanced whereas the root growth was negatively affected. The accumulation of NPs in the root tissue might be the cause of the reduced root length. Our results are in accordance with reports on root inhibition of radish, rape, rye-grass, lettuce, cucumber, and Arabidopsis [32-36]. However, the inhibitory effect may vary depending on the plant species, size and shape of NPs; adherence potential to root surface, translocation capability from root to shoot and dissolution, and release of metallic ions in the surrounding medium [37].

The seedling vigour index (SVI) was significantly higher in ZnO NP treated plants with respect to control and saline treatment (Figure 6). Increase in germination vigor results in vigorous growth and crop establishment. It has been reported that nanoparticles promote the growth of cluster bean [30], pearl millet [31], soybean [38], tomato [39], and coriander seedlings [40]. It is noteworthy that seedling bioassay showed no phytotoxicity symptoms besides the decreased root growth in NP treated seedlings under salt stress.

To determine the salt tolerance, the level of MDA content, photosynthetic pigments, total proteins and antioxidant enzyme activities (APX, CAT, and POX) were investigated. ZnO NP exposure under salt stress decreased the protein content (50%) over control, whereas saline treatment recorded increased (37%) protein content as compared to NP treatment (Figure 7). Zinc is used for protein synthesis, membrane function, and tolerance to environmental stresses [41]. The up-regulated expression of proteins in saline treatment could be attributed to reduced ionic leakage, thereby alleviate the cell damage caused by salt stress [42]. ZnO NP treatment increased the level of photosynthetic pigment content (one-fold) including chlorophyll a, b and
carotenoids in the leaves of finger millet plants (Table 2). Application of ZnO NPs increased the protein and photosynthetic pigments in cluster bean, pearl millet, soybean, Cyamopsis, tomato and green pea which ultimately promoted the growth rate and biomass [30,31,38,43,44]. These studies indicated that ZnO nanoparticles were absorbed and incorporated into the plant transport system. Although ZnO NPs treatment showed decreased protein content, it promoted the growth of NP treated plants in glasshouse condition (data not shown).

MDA is a good indicator of oxidative damage to membrane lipids [45]. MDA content was lower with ZnO NP exposure under salinity (Figure 8a). Salt treatment exhibits a higher level of MDA control in comparison with control and treated plants.

Table 2: Variation of chlorophyll (Chl a, Chl b) and carotenoid contents of control and treated finger millet plants under salinity; Data represent Mean ± Standard Error.

<table>
<thead>
<tr>
<th>Age of the plant (Days)</th>
<th>Treatment</th>
<th>Chl a</th>
<th>Chl b</th>
<th>Carotenoid (mg/g FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>Control</td>
<td>1.461 ± 0.25a</td>
<td>0.85 ± 0.67b</td>
<td>0.962 ± 0.02a</td>
</tr>
<tr>
<td></td>
<td>Salinity</td>
<td>1.927 ± 0.5a</td>
<td>1.19 ± 0.03b</td>
<td>1.91 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>Salinity + ZnO NPs</td>
<td>2.224 ± 0.78a</td>
<td>1.35 ± 0.01c</td>
<td>2.008 ± 0.9a</td>
</tr>
<tr>
<td>45</td>
<td>Control</td>
<td>1.261 ± 0.4a</td>
<td>0.9 ± 0.01a</td>
<td>0.8 ± 0.50</td>
</tr>
<tr>
<td></td>
<td>Salinity</td>
<td>1.675 ± 0.36a</td>
<td>1.1 ± 0.02c</td>
<td>1 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>Salinity + ZnO NPs</td>
<td>2.012 ± 0.004c</td>
<td>1.4 ± 0.99a</td>
<td>2.1 ± 0.24a</td>
</tr>
<tr>
<td>55</td>
<td>Control</td>
<td>1.9 ± 0.04a</td>
<td>1 ± 0.04a</td>
<td>1.1 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>Salinity</td>
<td>2.1 ± 0.89a</td>
<td>1.2 ± 0.33c</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Salinity + ZnO NPs</td>
<td>2.9 ± 0.77a</td>
<td>1.8 ± 0.39a</td>
<td>2.1 ± 0.5</td>
</tr>
</tbody>
</table>

a,b,c,d show significance at P < 0.05.
Decreased level of MDA content implies the absence of excess ROS in NP-exposed plants. An increase in proline content was observed upon exposure to salt stress with respect to control (Figure 8b). NP treatment recorded lower proline content compared to salinity, which might be due to the mitigation of stress caused by salinity. Similar findings have been documented that nano-silica is able to reduce the amount of proline in plants under salt stress [34].

The CAT and POX are the notable antioxidant defense enzymes involved in detoxification of H$_2$O$_2$ by converting free radicals to water (H$_2$O) and oxygen (O$_2$) [46]. The positive influence of ZnO NPs on POX, CAT and APX enzyme activity in the leaves of finger millet under salt stress was shown in Figure 9a, Figure 9b and Figure 9c. The SOD removes superoxide anion (O$_2^-$) free radicals, accompanied by the formation of hydrogen peroxide (H$_2$O$_2$), which is then detoxified by CAT and POX [47]. Zinc has been reported to increase the biosynthesis of antioxidant enzymes in the duckweed, Spirodelea polyrhiza [48].

The abiotic stress conditions over crop systems accelerate the formation of ROS resulting in the oxidative damage at the cellular level. In the present study, SOD and APX seem to play a major role in modification of salinity effects. Moreover, increased levels of antioxidant enzymes in ZnO-NP-treated plants under salinity show the ability to nullify ROS-mediated oxidative-stress-induced damage in finger millet plants. Induced antioxidant enzymes in NP treated plants under salt stress were positively correlated with low MDA content which could offer protection from cell damage during salt stress.

**Conclusion**

Our findings suggest that the foliar application of ZnO NPs induced physiological and biochemical changes by activating multiple antioxidant defense pathways which alleviate the salt stress in finger millet plants. However, further studies under field condition is necessary to conclude the role of ZnO NPs in mediating the stress response in plants in order to improve the ability of finger millet to withstand stresses in a wide range of environments.
References


