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EFFECT OF ZINC OXIDE NANOPARTICLES ON STRESS ALLEVIATION IN RICE PLANTS GROWN IN LEAD CONTAMINATED WATER

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Abstract

Lead toxicity is harmful to plants, animals, and humans, as photosynthesis is inhibited, mineral nutrition and water balance are upset, growth is stunted, causes root chlorosis and blackening, affects hormonal status, and alters membrane structure and permeability. This study explored the ZnO (zinc oxide) nanoparticles role in reducing lead stress in rice plants, impacting agriculture in lead-contaminated areas. ZnO nanoparticles were proposed as a potential solution towards alleviating lead stress in plants due to zinc's role as a cofactor for various plant enzymes, chlorophyll formation, starch-to-sugar conversion, and auxin production. To determine the effects of ZnO nanoparticles on rice growth under lead stress (500 ppm and 1000 ppm), we experimented with an investigation into the physiological, biochemical, and antioxidant responses of ZnO-treated and non-treated rice variety Dir-97 in hydroponic culture. For treatment, seeds were primed in a 5 ppm ZnO nanoparticle solution for 24 hours in the dark. When the lead concentration was raised, a pronounced drop in biomass was seen. Further, with increasing lead stress, we observed a stress response in the form of an increase in metabolic products, including total soluble sugars, total soluble proteins, and glycine betaine, along with antioxidants like catalase, ascorbate peroxidase, peroxidase, superoxide dismutase, and malonyl dialdehyde content in rice. In comparison, plants treated with nanoparticle solution showed a decrease in these contents. Our results showed that the nanoparticle solution alleviated lead stress and positively affected plant development and growth by inducing physiological and morphological changes. However, high concentrations of zinc can increase membrane leakiness, and disturb the detoxification of reactive oxygen species, gene expression, and normal enzymatic activity. Therefore, we recommend using a low ppm nanoparticle solution for optimal benefits, while high ppm solutions should be avoided due to their negative role.

Key words: Lead toxicity, Nanoparticles, Nano-priming, Lead Stress, Rice, ZnO.

Introduction

Cereals are one of the most important nutrients for growing the world population of mankind. Almost 50% of calories consumed by people are produced by wheat, rice, and maze (Acevedo-Pacheco & Serna-Saldívar, 2016). Rice remains the second most important grain crop in the planted area, it is an incalculable food source for Asian countries, including South East Asian countries (Shiferaw *et al.*, 2011). Rice provides about 21% energy and 15% total protein for humans thus its growth and quality yield require serious attention as it is also an essential food in most countries (Vega-Gálvez *et al.*, 2010). Asia, where 60% of the global population lives, produces and consumes about 90% of the world's produced rice. Rice is cultivated at 154 million hectares per year, which is equal to 11% of the worldwide cultivated land as of 2007, and its production is more than 750 million tons per year (Arunakumara *et al.*, 2013). Globally, there are two most cultivated species of rice; *Oryza sativa,* cultivated in Asia and grown around the world, and *O. glaberriama*, which is mainly grown in Africa.

As a result of the industrial revolution, human civilization has faced the problem of environmental pollution, which also includes the release and continuous increase in the concentration of heavy metals in an open environment. Although heavy metals are intrinsic constituents of the earth's crust, most environmental contamination and human exposure to metals and metal compounds are caused by anthropogenic activities that include mining, metallurgy, industrial production, and farming (Okereafor *et al.*, 2020). Sources of lead emissions are variable from one area to another. At a broader level, the main sources of lead in air are the releases from metal ores, as well as piston engines operating on leaded fuel. Lead is stable in the environment, and it can be added to sediments and soils by precipitating lead from sources of air pollution. Other resources of lead in the ecosystem include the direct discharge of wastewater streams into water bodies from mining sites. In the world, lead (Pb) is used mostly to produce lead-acid batteries for cars, which account for more than three-quarters of all lead consumption. Furthermore, lead also appears in a wide variety of products, for example, paints, pigments, stained glass, lead crystal dishes, ammunition, ceramic glaze, jewelry, cosmetics, and some medicines (Dignam *et al.*, 2019). An elevated level of Pb in the environment causes a decrease in reproductivity and growth in plants and animals, adversely affects the morphological, biochemical, and physical properties of plants, also has adverse neurological effects in vertebrates (Manisalidis *et al.*, 2020). Particularly in rice plants, the toxicity of lead prevents the seeds' germination and affects the length of the roots and shoots, growth, and final productivity. It also reduces nutrient absorption through roots, destroys the chloroplast's ultrastructure and permeability of cell membrane, causes changes in the respiratory activity of the leaves, induces reactive oxygen species (ROS) production, and increases the enzymatic as well as non-enzymatic production of antioxidants (Ashraf *et al.*, 2015).

Nanotechnology is the study and manipulation of matter at sizes in the range of 1 to 100 nanometers, where certain unique phenomena allow its use (Hulla *et al.*, 2015). Nanotechnology also includes visualization, measurement, modeling, and manipulation of matter in this scale of length.

Nanomaterials are commonly used in semiconductors, memory and storage devices, photonic and optical technology, biotechnology, energy, and health care products, in addition, nanotechnology is now increasingly being used as an environmental technology to mitigate pollution and its effects in order to protect the environment (Yata *et al.*, 2017). ZnO nanoparticles are frequently produced metal oxide manufactured nanoparticles that are used in a wide variety of applications, including photocatalysis, solar cells, electrodes, biosensors, and sunscreens, to name a few. ZnO nanoparticles are also increasingly being recognized as a significant advancement in the field of plant science. They demonstrate considerable potential for enhancing plant growth and yield by reducing plant stress, particularly from environmental contaminants such as heavy metals. This development is particularly crucial in light of the rapidly expanding global population, as it offers a viable solution to meet the escalating demand for increased agricultural productivity (Thounaojam *et al.*, 2021).

Among numerous strategies to alleviate heavy metal toxicity, the priming of plant seeds with nanoparticles, typically known as nano-priming, is recognized as a potent method that triggers alterations in seed metabolism. This process beneficially impacts the initial phases of plant growth and later influences the entire plant life cycle (Del Buono *et al.*, 2022). It is applied commercially in lots of seeds and is widely used by seed technologies to increase the capability of seed germination and increases tolerance towards stress. Priming can also be useful for carrier bank operators who need an improved protocol for germplasm collections that can be conserved ex-situ (harvest and local species) (Shahzad *et al.*, 2017). Many priming methods can be applied according to the plant type, the seed physiology, and the plant's morphology, all of which lead to the so-called pre-interacting metabolism of the plant (Rhaman *et al.*, 2020). As part of this physiological process, the antioxidant mechanisms and DNA repair pathways are activated in order to ensure proper germination of the seed during early seed implants thereby protecting the integrity of the genome (Ventura *et al.*, 2012). The application of ZnO nanoparticles in seed priming has significantly mitigated cobalt stress and cadmium toxicity in maize and rice, respectively. It is hypothesized that zinc competes with certain heavy metals, thereby diminishing their toxicity levels within the seed (Li *et al.*, 2021, Salam *et al.*, 2022). Contrarily, certain studies have reported adverse effects, predominantly phytotoxicity, associated with nano-priming techniques. The observed negative effects of nano-priming were largely dependent on factors such as their chemical structure, the concentration applied, and the particle size, with the extent of these effects varying across different genotypes. Moreover, both a lack and excess of zinc nanoparticles can be harmful to plants, necessitating the provision of an accurate quantity (Thounaojam *et al.*, 2021; Lee & Kasote, 2024).

The unwanted exposure to heavy metals, such as lead, is detrimental to the environment and causes stress in crop plants, particularly when present in large quantities. However, nano-remediation is an environmentally friendly approach that aids in mitigating and eliminating such pollutants from the environment. The aim of this study is to examine the impact of lead stress on a specific rice variety (Dir-97) and explore the potential of ZnO nano-priming as a remedial measure. The study involves a comparative investigation of various biochemical, physiological, metabolic, and oxidative processes in rice plants under lead stress, both with and without the application of ZnO nano-priming. This comprehensive approach offers a holistic understanding of the problem and its potential solution.

Material and Method

Seeds collection and sterilization: Seeds of a local rice variety Dir-97 were collected from the Agricultural University Peshawar, Pakistan. Small and broken seeds were discarded. Healthy seeds were immersed in a 3.5% sodium hypochlorite (NaOCL) solution for 5 min. for sterilization. After which, the sterilized seeds were removed from the solution and rinsed 5 times with distilled water.

Nanoparticles solution and nano-priming of seeds: ZnO nanoparticles, procured from the Pir Mehr Ali Shah (PMAS) Arid Agriculture University, Rawalpindi, Pakistan, were synthesized utilizing the co-precipitation method, following the modified protocol of (Hussain *et al.*, 2017). These nanoparticles were subsequently characterized using the Field Emission Scanning Electron Microscopy (FESEM), Transmission Electron Microscopy (TEM), Fourier Transform Infrared Spectroscopy (FTIR), and X-ray Diffraction (XRD) (Sohail *et al.*, 2019). The size of the synthesized ZnO nanoparticles was determined to be within the range of 30 to 50 nm (Akhtar *et al.*, 2022). 5 mg/l of ZnO nanoparticles were used for making nanoparticle solution (5 PPM) using deionized water, which was sonicated for 20 minutes for complete mixing and then stored in air-tight, cold, and dark conditions until further use. Sterilized seeds were then nano primed for 24 hrs by soaking them in the ZnO nanoparticle solution along with continuous shaking. Besides this, hydro-primed seeds, which were primed using distilled water only in the same way, were taken as control samples.

Seeds germination and development of plants under Pb stress: The experiment was conducted in triplicate. The primed seeds were transferred to petri plates having double layers of wet filter paper and were placed into an incubator in dark conditions at 37°C for 5 days. The seeds were then relocated to an open environment under normal room temperature and light conditions for the next 5 days, during which they were regularly irrigated with distilled water. Following that, the young seedlings were shifted to hydroponic culture having Hoagland solution (Hoagland & Arnon, 1950) and were put in an incubation chamber for the next 21 days, during which the Hoagland solution was replaced weekly with a new one. After 21 days, the plants were separately irrigated with lead acetate solutions of 500 and 1000 ppm concentration, and lead acetate + ZnO solution of 500 + 5 and 1000 + 5 ppm concentration, respectively, for 5 days before harvesting.

Harvesting and biomass determination: After stress treatment, some leaves of rice plants were gleaned randomly, liquid nitrogen-freeze-dried, powdered using pestle and mortar, and stored at -40ºC for metabolic, molecular, and antioxidant analysis. Shoots and roots lengths were measured. The young plants were harvested, their roots and shoots were separated, and their fresh weight was measured. Following that, 2 hours of hot air on the roots and shoots were oven dried at 80ºC, and their dry weight was measured. Further, they were also crushed and stored at -40ºC for biochemical analysis.

Cell membrane stability: To analyze the stability of the cell membrane, fresh leaves from every treatment group were taken and cut into twenty 1 cm strips. Those fine strips were separately placed into tubes containing 20 ml of distilled water, warmed at 25ºC, and placed in an incubator for 24 hrs. A Initial electrical conductivity (C_1) of the solution was then determined. Following autoclaving for 40 minutes, the final electrical conductivity (C_2) of the tubes was determined with the help of a conductivity meter.

Analysis of chlorophyll a, b, and carotenoids: To analyze the Carotenoids, Chlorophyll a, and Chlorophyll b content, a protocol developed by Lichtenthaler & Wellburn, (1983) was adopted. Twenty-five mg of dried biomass was taken in falcon tubes, to which 25 mg of MgO was added and vortexed. Following that, 5 ml methanol was added and tubes were placed on a shaker for 2 hours at the speed of 200 rpm for complete homogenization and extraction of photosynthetic pigments. Next, the samples were centrifuged for five minutes at 400 rpm. Afterward, the optical densities of 3 ml of the supernatant at 3 different wavelengths, 470, 653, and 666 nanometers were determined with the help of a spectrophotometer, while pure methanol was taken as a blank. Calculations of chlorophyll a (C_a), chlorophyll b (C_b), and carotenoids (Car) in µg/ml were performed in accordance with the following formulas:

$$C_a = 15.65\, A_{666} - 7.340\, A_{653}$$
$$C_b = 27.05\, A_{653} - 11.21\, A_{666}$$
$$Car = (1000\, A_{470} - 2.860\, C_a - 129.2\, C_b) / 245$$

where “A” represents the absorbance at specific wavelengths.

Lead content: To measure lead content, the plant material was first digested by taking 100 mg of dried plants biomass into a beaker, to which nitric acid and sulfuric acid were added, and a hot plate was used to heat the mixture. Once the solution had cooled down to room temperature, the distilled water (20 ml) was used to dilute it, which was then filtered using syringe filters, followed by the lead quantification through an atomic absorption spectrophotometer.

Proline: Proline is a proteinogenic amino acid which is used in the biosynthesis of proteins. Its concentration was measured according to the procedure described by Bates *et al*., (1973). To start with, 100 mg of fresh root sample was thoroughly mixed in 5 ml of 3% sulfosalicylic acid dihydrate and subjected to centrifugation for 30 minutes at 4000 rpm to remove the residues. A mixture of 20 ml of 6M phosphoric acid along with 30 ml of anhydrous acetic acid was used to incubate the 1 ml homogenized tissues for 1 hour at 100°C with acid ninhydrin (1 ml). Following that, 2 ml toluene was introduced to the solution, and it was mixed thoroughly to form two distinct layers. Toluene was used as a blank to measure the optical density of the top aqueous color phase's absorbance using a spectrophotometer at 520 nanometers. Using the absorbance value, the quantity of proline was found by using the following equation.

$$\text{µmole proline/g fresh weight} = [(\text{µg Proline /ml} \times 2/115.5)] / (0.1/5)]$$

Analysis of total soluble sugars (TSS): The modified phenol-sulfuric acid technique of Chow & Landhäusser, (2004) was utilized to quantify the total soluble sugars. In order to create an extract, fresh roots (50 mg) were crushed in 3 ml of slightly pre-heated ethanol. After that, the composite was incubated for 1 hr at 70°C. A 30-minute incubation period was then followed by the addition of 1 ml carbolic acid (5%) and 5 ml sulfuric acid to the solution. At 485 nm, the mixture's absorbance was measured, while distilled water served as a control. A pre-made calibration curve of glucose was employed to estimate the soluble sugar content.

Quantification of total soluble proteins (TSP): Bradford assay (Bradford, 1976) was used to measure the quantity of total soluble proteins. To start with, 500 mg of fresh root biomass was thoroughly mixed with 10 ml phosphate buffer solution at pH 7.8 and subsequently subjected to centrifugation for 20 minutes at 14000 rpm. After that, in the reaction solution, 20 µl of the root protein extract was added, shaken thoroughly, and left for a short period at room temperature for complete homogenization. Following this, the absorbance at 595 nm was measured against distilled water, which was taken as a blank. BSA (bovine serum albumin) standard curves were used to quantify the protein concentration (Du *et al*., 2022).

Determination of glycine betaine: Glycine Betaine, also known as Trimethylglycine is an osmolyte, that helps plants to cope with these stress conditions by stabilizing membranes, proteins, and DNA structure. 50 mg of dried roots were crushed and mixed in 4 ml distilled water and 1 ml 2N H_2SO_4, followed by the mixture being stirred well, filtered, and cooled down for 60 mins. After that, the solution was added with 0.2 ml of potassium triiodide (KI_3), vortexed for 5 mins, and incubated for 16 hours at -4ºC. The mixture was then subjected to centrifugation for 15 minutes at the speed of 1000 rpm at 0ºC, which was proceeded by the addition of 9 ml of 1,2-dichloroethane to dissolve the crystals. After giving 2 and a half hours for the reaction to complete, the optical density of the mixture was observed at 365 nm to determine the amount of glycine betaine.

Analysis of antioxidants and superoxide dismutase: To analyze the antioxidant content, 500 mg of fresh root biomass was crushed and mixed with 10 ml phosphate buffer, followed by the super centrifugation for 20 minutes at 20000 rpm at 4°C, as per the method established by (Du *et al*., 2022).

The protocol of Beauchamp & Fridovich, (1971) was utilized to estimate the quantity of superoxide dismutase (SOD), a key enzyme that protects against reactive oxygen species (ROS). As substratum, riboflavin, nitro blue tetrazolium chloride (NBT), methionine, and edetate disodium (Na EDTA) were dissolved together. A reaction composite of 3 ml was then made, comprising of deionized water, enzyme extract, and reaction substrate and was placed under light conditions for 20 min at 4000 lux. The optical density was then obtained at 560 nm, as previously established by Verma & Dubey, (2003) to determine the SOD content.

Measurement of peroxidase activity: Using the procedure developed by Kumar & Khan, (1983) the peroxidase enzyme activity was measured. Three milliliters of the reaction mixture was made, consisting of 0.1 ml of guaiacol (1.5%), 0.1 ml of plant enzyme extract, 0.1 ml of 0.4% hydrogen peroxide, and 2.7 ml of tri-potassium orthophosphate buffer. The resulting solution was allowed to completely react and the optical density was determined at 470 nm. Distilled water was taken as a blank.

Catalase analysis: To determine the content of catalase, a mixture was prepared by adding 0.1 ml enzyme extract, 2.8 ml tri-potassium orthophosphate buffer, and 0.1 ml of hydrogen peroxide. In total, 3 ml of the mixture was prepared. The optical density of the mixture was then observed at 240 nanometers.

Analysis of ascorbate peroxidase: Ascorbate Peroxidase (APX) is a photosynthetic enzyme that is crucial in modulating organellar and cellular levels of H_2O_2, a key signaling molecule for plant stress responses. To determine APX activity, 0.1 millimolar Na EDTA, 0.66 millimolar hydrogen peroxide, 100 millimolar phosphate buffer (pH 7), and 0.3 millimolar ascorbic acid, were thoroughly mixed with 100 ml of enzyme extract and the optical density at 290 nm was measured.

Determination of malondialdehyde: Malondialdehyde (MDA) is a fatty acid oxidation by-product, used as an oxidative stress marker, and plays a key role in abiotic stress responses. To calculate MDA contents, a reaction substrate was prepared. In 500 ml of distilled water as a solvent, 25 mg of trichloroethanoic acid salt was added. From the mixture, 2.5 ml was taken, and 2.5 mg of 2-mercaptobarbituric acid salt was added to it. Further, 1.5 ml of enzyme extract was also added, and the thoroughly mixed solution was placed for 15 minutes at 95°C in a hot water bath. Following that, the mixture was instantly cooled down with the help of an ice bath for 35 minutes and then vortexed for 5 minutes. After centrifuging for 10 minutes at 1529 rpm, the absorbance at 532 nm was measured.

Results

Physiological parameters

Seed germination: The treatments exhibited varying patterns of seed germination. The stress treatment reduced seed germination significantly, with a reduction seen at 500 ppm and 1000 ppm stress levels compared to control. In contrast, the nano-primed seeds showed an improvement in seed germination compared to the stress treatment applied, as depicted in (Fig. 1).

Root and shoot length: A lead stress treatment resulted in a reduction of 1.94 cm and 3.07 cm in root and shoot length, correspondingly, in comparison to the control, where the length was 8.26 cm and 8.63 cm. However, the nano priming enhanced root and shoot lengths in plants under stressful conditions. At 500 ppm+5 ppm (NP), the root and shoot lengths were recorded as 6.01 cm and 4.47 cm, respectively, while at 1000 ppm+5 ppm (NP), the recorded length was 5.01 cm and 3.75 cm (Fig. 2).

Fresh weight: Compared to the control, the stress treatment lowered the fresh weight of roots and shoots both. At 1000 ppm, the fresh weight was recorded as 9.22 mg and 13.52 mg, while at 500 ppm, it was 11.03 mg and 17.06 mg, respectively, whereas the control fresh weight was 23.06 mg and 29.35 mg. However, Nano priming alleviated the stress, resulting in an improvement in fresh weight. At 500 ppm+5 ppm (NP), the fresh weight roots and shoots were 15.13 mg and 22.21 mg, and at 1000 ppm+5 ppm (NP), it was improved to 13.08 mg and 21.07 mg, respectively (Fig. 3).

Dry weight: The dry weights of both roots and shoots were reduced as a result of the stress treatment. At 1000 ppm, the dry weight was 12 mg and 9.6 mg, while at 500 ppm, it was 14.3 mg and 11.1 mg, compared to the control, which was 20.7 mg and 19.85 mg, respectively. However, Nano priming alleviated the stress and improved the dry weight. At 1000 ppm+5 ppm (NP), the weight of the roots and shoots were 15.3 mg and 13.4 mg, and at 500 ppm+5 ppm (NP), the dry weight was 16.1 mg and 14.25 mg, respectively (Fig. 4). Overall, the stress treatments reduced seed germination, root and shoot length, and fresh and dry weight of both roots and shoots. However, nano priming with ZnO nanoparticles improved these parameters under Pb stress conditions.

Cell membrane stability: The stress treatment increased cell membrane stability compared to the control. At 1000 ppm, root cell membrane stability was 48.15 $\mu S/cm^{2,}$ and shoot cell membrane stability was 44.64 $\mu S/cm^2$. At 500 ppm, shoot cell membrane stability was 35.87 $\mu S/cm^2$ and root cell membrane stability was 34.80 $\mu S/cm^2$. This showed that cell injury was increased in roots compared to shoots, in contrast to the control where root cell membrane stability was 17.03 $\mu S/cm^2$ and shoot cell membrane stability was 19.99 $\mu S/cm^2$. However, nano priming improved the results and decreased cell membrane stability. At 500 ppm+5 ppm (NP), shoot cell membrane stability was 26.95 $\mu S/cm^2$ and root cell membrane stability was 28.47 $\mu S/cm^2$. At 1000 ppm+5 ppm (NP), shoot cell membrane stability was 32.90 $\mu S/cm^2$, while root cell membrane stability was 28.12 $\mu S/cm^2$ (Fig. 5).

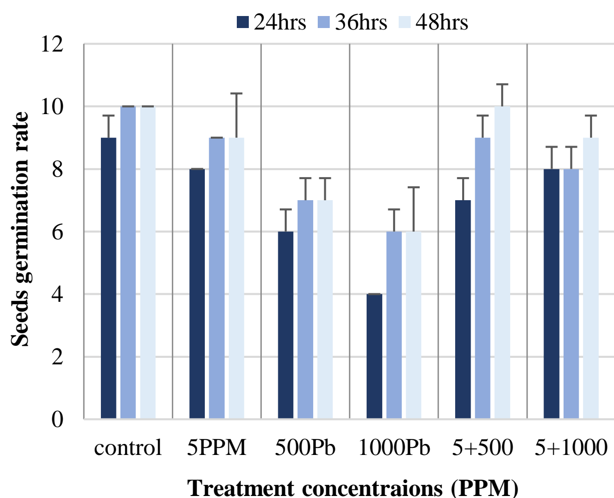

Fig. 1. Impact of stress and nano-priming on seed germination rates: Stress decreases germination, while nano-priming enhances it.

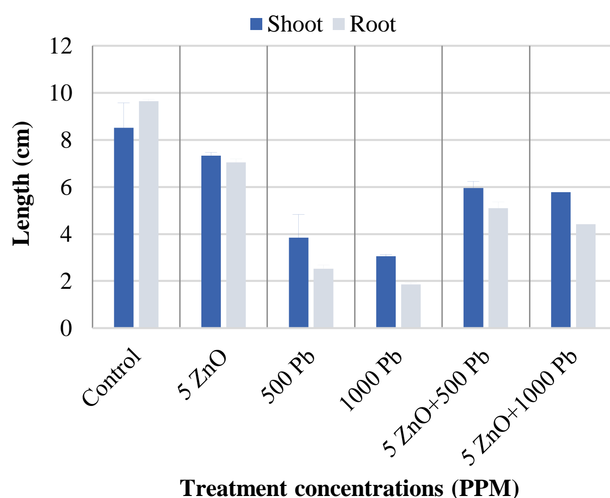

Fig. 2. Graph showing Root and Shoot length. Stress treatment reduced the roots and shoots length while nano priming improved them.

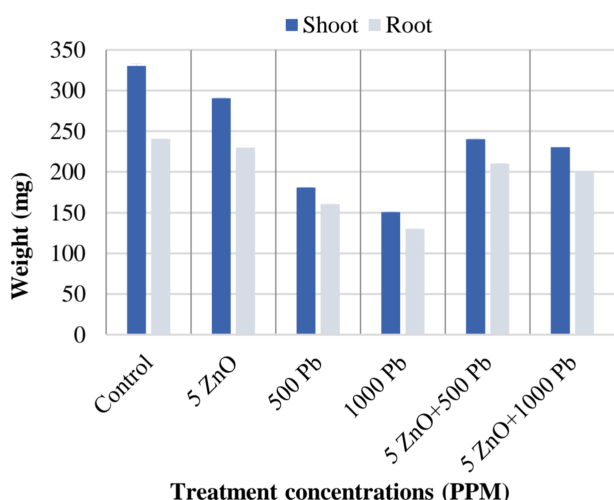

Fig. 3. Graph presenting that stress treatment reduced the fresh weight of roots and shoots while nano priming improved the overall fresh weight of both.

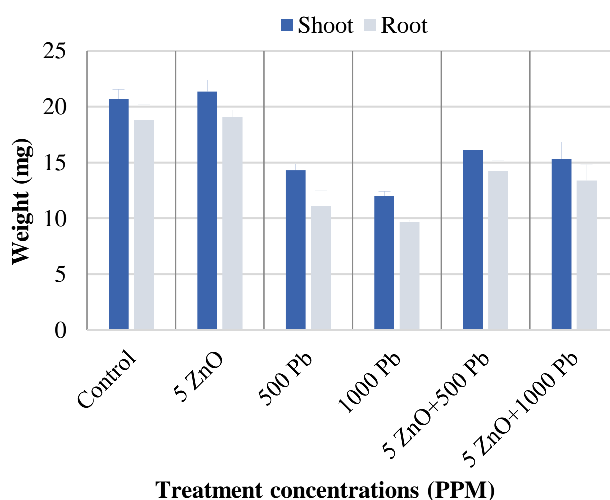

Fig. 4. Graph showing the dry weights of shoots and roots, the stress treatment reduced the overall dry weight while nano priming improved it.

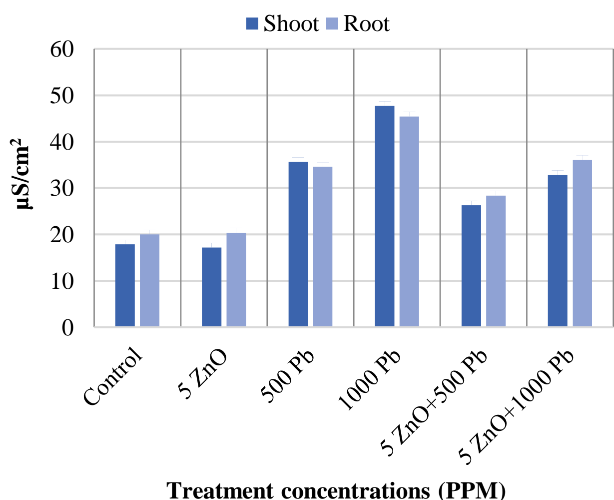

Fig. 5. Graph showing the cell membrane stability. The cell membrane stability increases with respective stress, the nano priming reduced the cell membrane stability.

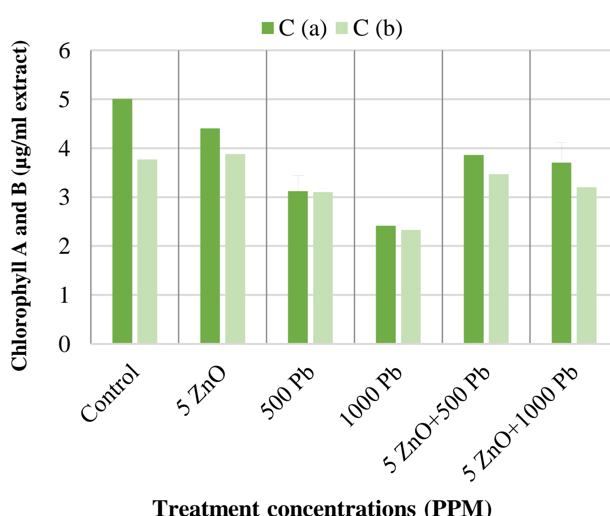

Fig. 6a. Graph representing the chlorophyll contents.

Chlorophyll and carotenoid analysis: There was a drop in the content of chlorophyll a and chlorophyll b following the stress treatment. At 1000 ppm, the quantity of chlorophyll a and b was recorded as 2.413 and 3.881, respectively, and carotenoids were 215.83. Similarly, at 500 ppm stress, the chlorophyll a and b concentrations were recorded as 3.102 and 3.906, respectively, and carotenoids were 288.74. In comparison, the control had a chlorophyll a and b content of 5.401 and 4.259, respectively, and the carotenoid content of 355.76. However, in addition to improving chlorophyll a and b quantity, the nano priming also increased carotenoid content. At 1000 ppm+5 ppm (NP), the content was improved to 3.044 and 3.319 for chlorophyll a and b, correspondingly, while the carotenoid amount was determined as 315.42. Similarly, in the case of 500 ppm+5 ppm (NP), the chlorophyll a, b, and carotenoids concentrations were found to be 3.861, 3.401, and 331.4, respectively (Figs. 6a & 6b).

Lead content determination: Lead levels in plants increased as a result of stress. At 1000 ppm, the lead content was recorded as 0.2757 ppm in shoot and 0.281 ppm in root. Similarly, at 500 ppm, the lead content was 0.112 ppm in the shoot and 0.124 ppm in the root. In comparison, the lead content was much lower in the control group, where it was 0.0115 ppm in shoot and 0.0125 ppm in root. However, nano priming significantly reduced the lead content (Fig. 7). At 500 ppm+5 ppm (NP), the lead concentrations in roots and shoots were 0.0705 ppm and 0.088 ppm, respectively. Similarly, at 1000 ppm+5 ppm (NP), the lead concentrations in roots and shoots were 0.09 ppm and 0.091 ppm, correspondingly.

Metabolic tests

Proline: With the application of stress, the shoots and roots became more proline rich. At 1000 ppm, the proline quantity in the root and shoot was recorded as 21.79 and 24.75, respectively, while at 500 ppm, the content was found to be 16.41 and 17.43, respectively, as compared to the control, where it was 8.96 and 9.12 in roots and shoot, respectively. However, nano priming reduced the content of proline, and at 1000 ppm+5 ppm (NP), it was recorded as 14.74 and 16.74 in root and shoot, respectively. Similarly, at 500 ppm+5 ppm (NP), it was found to be 12.14 and 10.35 (Fig. 8).

Total soluble sugars (TSS): In response to stress, TSS concentrations were increased. At 1000 ppm, the TSS content was recorded as 330.12, and at 500 ppm, it was 278.87. In comparison, the control had a TSS content of 127.62. However, nano priming was effective in reducing the level of TSS. At 1000 ppm+5 ppm (NP), the TSS content was found to be 278.75, and similarly, at 500 ppm+5 ppm (NP), the TSS content was found to be 240.12 (Fig. 9).

Glycine betaine (GB): The GB content in both root and shoot was increased with stress treatment. At 1000 ppm, the GB content was recorded as 1.1832 in root and 1.428 in the shoot, while at 500 ppm, it was 1.1682 in root and 2.062 in the shoot, in contrast with the control the GB concentration was 0.9732 in roots and 1.038 in the shoots. However, nano-priming reduced the GB content. At 1000 ppm+5 ppm (NP), the GB content was found to be 0.7932 in the shoot and 1.108 in the root. Similarly, in 500 ppm+5 ppm (NP), the content was recorded as 0.9832 in the shoot and 0.988 in the root (Fig. 10).

Total soluble proteins (TSP): The TSP content increased with stress treatment. At 1000 ppm, it was recorded as 61.44, and at 500 ppm, it was found to be 82.32, compared to the control, which was 54.63. Nano-priming reduced the TSP level, with 66.6 recorded at 1000 ppm+5 ppm (NP) and 53.75 at 500 ppm+5 ppm (NP) (Fig. 11). In summary, stress treatment increased proline, total soluble sugars (TSS), glycine betaine (GB), and total soluble proteins (TSP) in both roots and shoots. Notably, nano priming effectively reduced these levels under stress conditions.

Antioxidant analysis

Super oxide dismutase (SOD): Upon treatment with stress, root and shoot SOD contents increased. At 1000 ppm, the recorded SOD content in roots and shoots was 181.22 and 179.61, respectively. Under 500 ppm stress, roots and shoots contained 149.35 and 139.87 ppm of SOD, respectively, in comparison with control, in roots and shoots it was at 112.08 and 108.32 ppm. Nanopriming reduced the SOD content; at 1000 ppm+5 ppm (NP), it was recorded in roots and shoots as 136.91 and 144.75, respectively. Similarly, at 500 ppm+5 ppm (NP), the SOD content was recorded in roots and shoots as 124.3 and 126.27 (Fig. 12).

Ascorbate peroxidase (APX): The APX content was elevated in roots and shoots with the treatment of stress. At 1000 ppm, the APX content was recorded as 2.22 and 1.637, and at 500 ppm, it was increased up to 1.11 and 1.145, as compared to the control, which was 1.11 and 1.145 in roots & shoots, respectively. Nano-priming decreased the APX content. In 1000 ppm+5 ppm (NP) treatment, the APX content in root and shoot was 1.0185 and 1.27. In the case of 500 ppm+5 ppm (NP), the APX content was 0.915 and 0.955 in shoot and root, respectively (Fig. 13).

Peroxidase (POD): The POD content was increased in roots and shoots with increasing stress treatment. At 1000 ppm, the POD content was found to be 110.31 and 120.54 in roots and shoots, correspondingly. Upon application of 500 ppm Pb stress, the POD content was observed as 90.64 and 91.85, compared to the control, which was 82.82 and 84.35 in roots and shoots. Nano priming decreased the POD activity, in the 1000 ppm+5 ppm (NP) treatment, the recorded activity in shoot and root was 91.04 and 92.37, respectively. Similarly, in the 500 ppm+5 ppm (NP) treatment, the POD content was 85.04 and 84.14 in the shoots and roots, respectively (Fig. 14).

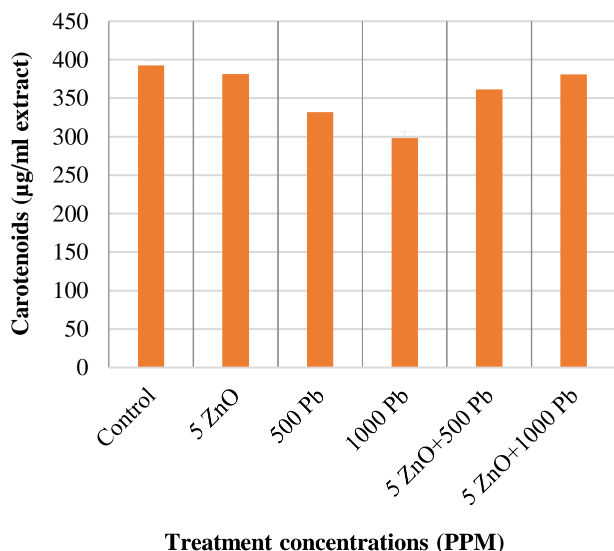

Fig. 6b. Graph depicting the carotenoid contents.

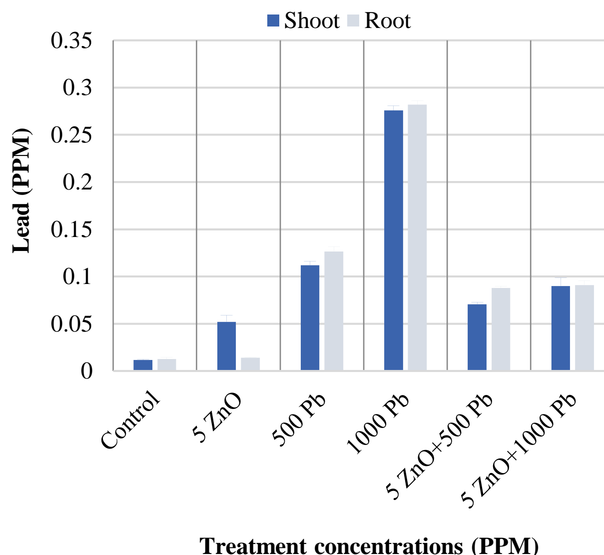

Fig. 7. Graph revealing the lead content in plant material.

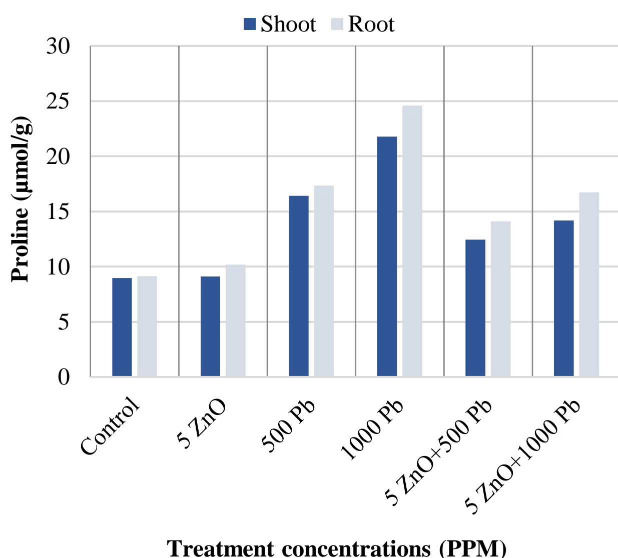

Fig. 8. Graph indicating the proline content in root and shoot.

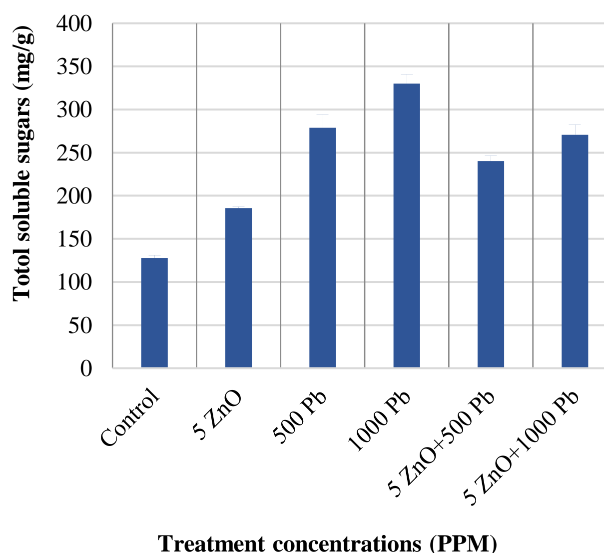

Fig. 9. Graph portraying the TSS content.

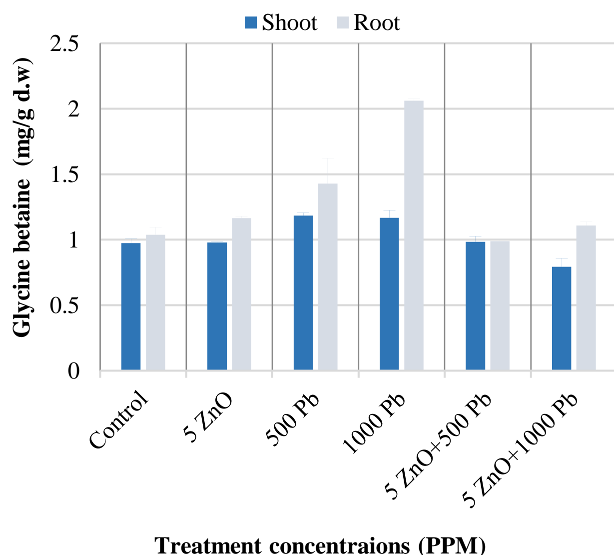

Fig. 10. Graph indicating the GB content in root and shoot.

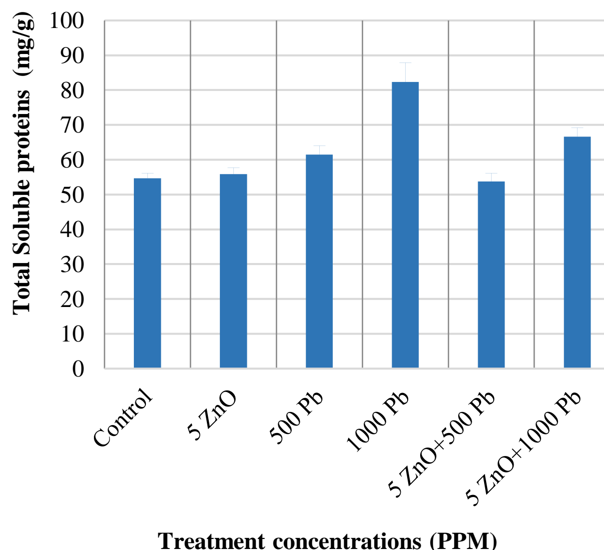

Fig. 11. Graph representing the TSP content.

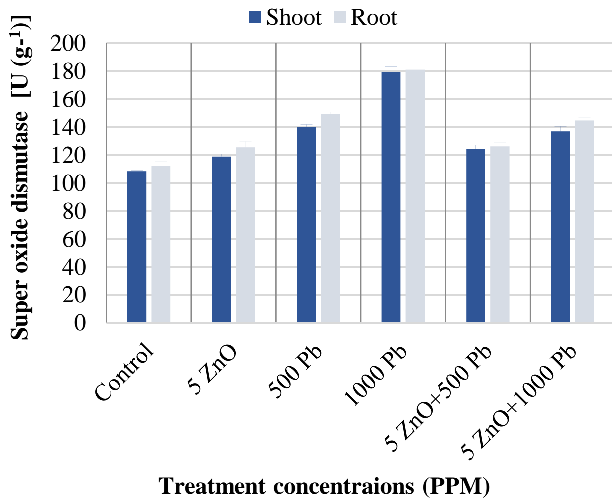

Fig. 12. Graph showing SOD content in shoot and root.

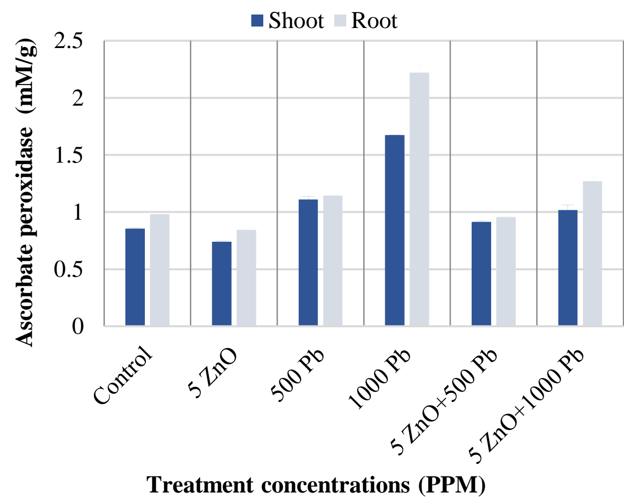

Fig. 13. APX content in root and shoot.

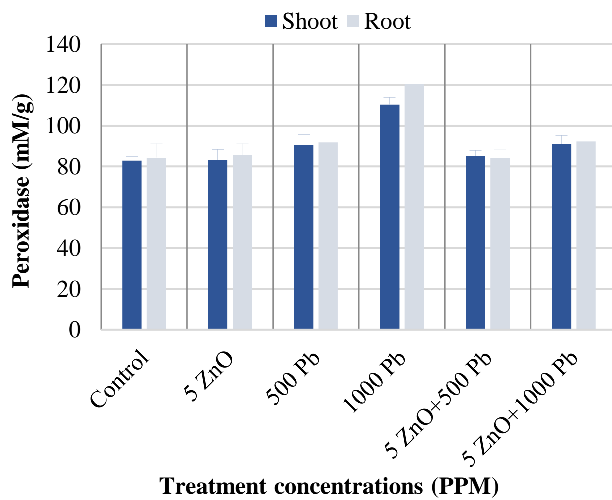

Fig. 14. Graph depicting the POD content in root and shoot.

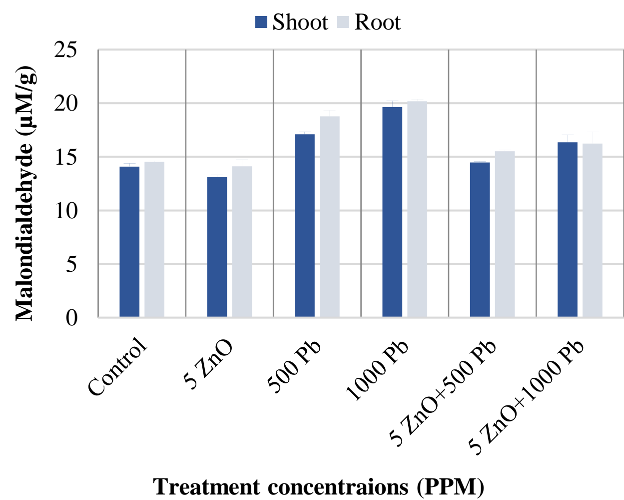

Fig. 15. Graph demonstrating the MDA content in root and shoot.

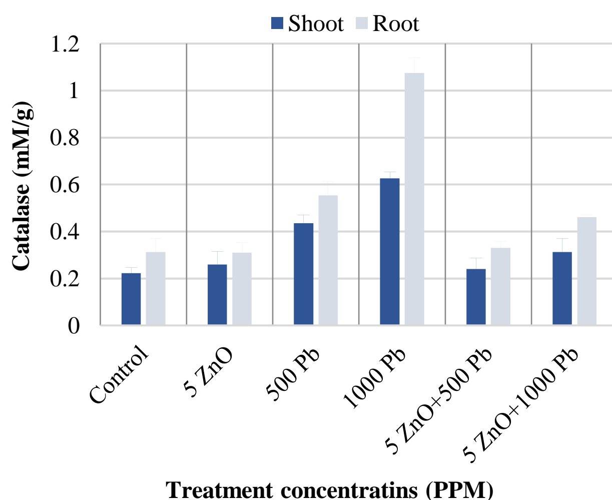

Fig. 16. Graph presenting the CAT content in root and shoot.

Malondialdehyde (MDA): The MDA content was increased in roots and shoots with increasing stress treatment. At 1000 ppm, the MDA content was 19.634 and 20.174 in shoot and roots, respectively. In the case of 500 ppm stress, the content in roots and shoots was 17.103 and 18.764 compared to control in which the MDA content was 14.077 and 14.116 in shoot and root respectively. Nano priming decreased the MDA content. In 1000 ppm+5 ppm (NP) treatment, the MDA content was found to be 16.358 and 16.235 in shoots and roots, respectively. Similarly, in 500 ppm+5 ppm (NP), the MDA content was 14.474 and 15.519 in roots and shoots, respectively (Fig. 15).

Catalase (CAT): The CAT activity increased in roots and shoots with treatment. At 1000 ppm, the CAT content was recorded as 0.6258 and 1.0743 in shoot and root, while at 500 ppm, the CAT activity was 0.4347 and 0.554 in shoot and root. Nano-priming reduced the CAT activity in 1000 ppm+5 ppm (NP), which was 0.3129 and 0.4609 in shoot and roots. Similarly, in 500 ppm+5 ppm (NP), the CAT activity was found to be 0.2406 and 0.3306 in the shoot and root, respectively (Fig. 16). In brief, the application of Pb stress led to an increase in the levels of Superoxide Dismutase, Ascorbate Peroxidase, Peroxidase, Malondialdehyde, and Catalase in both roots and shoots. Notably, these levels were effectively diminished under stress conditions through the use of nano priming.

Discussion

According to the study, seed germination was strongly affected by increased lead stress concentrations, as the seed germination decreased with increasing lead concentration. This is because the water content decreases as the concentration of lead increases, leading to damage to enzyme activity, cell walls, and the rate of photosynthesis. Lead stress causes cell death by increasing active oxygen species and oxidizing lipid and protein membranes. It also alters metabolic enzymes such as acid invertases, proteases, and amylases, which delay seed germination (Alpaslan & Yukselen, 2002). The comparable results were documented by Saha *et al*., (2010) indicating that the use of nanoparticle solutions for promoting seed growth is safe for both the environment and plants. Nano-priming of seeds proved to be particularly advantageous for growth and germination under lead stress. Moreover, nano-primed seeds demonstrated a significantly higher percentage of germination compared to those that were hydro-primed under lead acetate stress.

In the previous studies (Zhang *et al*., 2021; Singh *et al*., 2023), ZnO nanoparticles were reported to promote rice seed growth, increasing their seed germination rate as well as enhancing the growth and length of roots and shoots. This alleviation of stress is achieved by reducing seed dormancy and activating compounds such as nitrates, cyanides, and nitric oxides, which enhance seed germination. A critical function of cyanide is to alleviate seed dormancy, while nitrogen oxides act in a similar way in encouraging seed germination (Baruah *et al*., 2010).

The results demonstrated that the fresh and dry root and shoot weights were decreased by lead stress in the rice variety Dir-97, ultimately leading to a reduction in growth. However, nano-priming of seeds in nanoparticle solution alleviated the lead stress, increasing both fresh and dry roots and shoots' weight. This was because zinc, which was present in the nanoparticle solution, was involved in producing auxins and gibberellins, the growth-promoting hormones (Tymoszuk & Wojnarowicz, 2020).

The integrity of the cell membrane is a crucial issue, and high concentrations of lead stress can lead to cell injury in plants. Electro leakage, which damages the cell membrane, has been observed in maize roots during times of stress. The hydroxyl group of phospholipids and the sulfhydryl groups of proteins can interact with heavy metals to displace calcium that is anchored to the cell membrane. This can cause changes in the cell membrane and oxidation in the cross-link of thiol protein, leading to an inhibition of plasma membrane ATPase. As a result of all these events, the non-specific permeability of the membrane increases. However, nano-primed seeds showed a positive response and prevented seepage in Dir-97 during stress conditions (Janicka-Russak *et al*., 2008).

Lead toxicity also negatively affects the root and shoot lengths of plants. In the subject rice variety Dir-97, increasing lead stress reduces the length of roots and shoots. This is because of the reduction in cell division at the tips of roots and a decline in nitrate reductase activity, which inhibits root growth (Oaks *et al*., 1977). As reported by Yang *et al*., (2000), elevated concentrations of lead also inhibited shoot elongation, disturbed hormones, water balance, mineral permeability, and cell membrane stability, as well as decreased the rate of photosynthesis and inhibited shoot growth.

When rice plants are exposed to lead stress, their chlorophyll content decreases, which results in a decreased rate of photosynthesis. This happens because the Calvin cycle enzymes are disturbed, there is a decrease in CO_2 amount because of the closure of stomata, the chain of electron transport is affected, and the chloroplast structure is damaged (Melis *et al*., 1992). Lead interferes with the -SH group, inhibiting aminolevulinic acid dehydratase (ALAD) activity, which results in the decrease in chlorophyll production (Zulfiqar *et al*., 2019). In addition, nutrients are being up taken in an imbalanced manner, like absorption of more Mg^{2+} that causes the degradation of chlorophyll, as well as an imbalance of chlorophyllase and pheophytinase (Khan *et al*., 2018). Lead also decreases the levels of chlorophyll a and b due to the impaired supply of Fe^{+2} and Mg^{+2}, as well as the impaired synthesis of chlorophyll through enzymes like protochlorophyllide reductase and aminolevulinic acid dehydratase (Zhu *et al*., 2017). Furthermore, lead affects the accepting and donor sites of PS II, as well as those of PS I and the cytochrome complex (Ashraf *et al*., 2015).

There is an increase in total soluble sugars and total soluble protein in rice plants under lead stress, as previously indicated by Talha *et al*., (2023). The increase in TSP and TSS is a defense mechanism the plant uses against stress, which helps in maintaining osmoregulation and energy-soluble proteins, while also preventing the catalytic activity of lead (Anjum *et al*., 2016). However, nano-priming can alleviate lead stress and prevent any adverse effects on the metabolism of the plant.

Glycine betaine, which is abundantly synthesized in chloroplasts, serves as an essential component in the maintenance of thylakoid membrane defense and the regulation of photosynthesis (Kumar *et al*., 2017). In response to stress, glycine betaine levels rose compared to the control, acting as an osmo-protectant to protect the plant from environmental stressors and influencing the enzymes and cell membrane integrity (Siddiqui *et al*., 2021). However, nano-priming decreased the concentration of glycine betaine needed to mitigate the impacts of lead stress, indicating the effectiveness of nano-priming in reducing the impact of lead stress.

In order to guard themselves against the oxidative harm resulting from the reactive oxygen species (ROS), plants have established an antioxidant defense mechanism consisting of both non-enzymatic and enzymatic components. This defense system functions by releasing, neutralizing, and eliminating ROS (Verma & Dubey, 2003). In rice plants, as a response to Pb exposure, superoxide activity was enhanced. Enzymatic protein de novo synthesis is accountable for the rise in superoxide activity in response to stress. Further, our findings are also supported by Sharma & Dubey, (2005), who found that lead stress raised SOD levels in rice seedlings. According to Fecht-Christoffers *et al*., (2006), peroxidases are polyfunctional enzymes that are capable of detoxifying as well as generating hydrogen peroxide as part of separate

cycles. In our study, it was found that in response to a higher level of Pb stress, POD content was increased. A similar trend was documented by Zou *et al*., (2011), who demonstrated that *Athyrum wardii* species showed increased peroxidase activity when subjected to 800 mg kg^{-1} soil of lead in the pot study. Results depicted that nano-priming mitigated lead stress and reduced superoxide and peroxidase quantities. Catalase is an important enzyme that reduces harmful peroxidases. Our results also confirmed a significant rise in CAT as lead content increased in both root and shoot. Verma & Dubey, (2003) also discovered a rise in CAT quantity due to lead toxicity. Nano-primed seeds exhibited a decrease in CAT content, indicating alleviation of lead stress.

The enzyme ascorbate peroxidase (APX) is also critically important in the antioxidant defense mechanism, which directly scavenges molecular oxygen and hydrogen peroxide. An essential part of the ascorbate glutathione pathway in photosynthetic organisms is APX, which utilizes ascorbate as an electron donor to scavenge hydrogen peroxide and stabilize the cell's redox state. This enzyme is produced in the chloroplast and other organelles of the photosynthetic cell (Shigeoka *et al*., 2002). As a result of stress conditions, APX content increases, as indicated by Verma & Dubey, (2003). Similarly, our findings also demonstrated a boost in APX concentration during lead stress in both root and shoot. However, nano-priming of rice seeds led to a decrease in APX content under lead stress.

MDA is the second to last product of polyunsaturated fatty acid oxidation and is considered a marker of lipid peroxidation. The plant cell membrane contains phospholipids and glycolipids, and the peroxidation reaction is initiated by the hydroxyl radical, forming unstable lipid hydroperoxides. These hydroperoxides can enter a Fenton-like reaction in the presence of metals, resulting in the production of aldehydes such as malonaldehyde (MDA) (Frankel, 1984). High lead concentration increases the MDA content, and the amount is greater in leaves than in roots. However, when rice seeds are nano-primed, the stress is alleviated, and the MDA content is reduced. Our results aligned with the study of Singh *et al*., (2023), who demonstrated that ZnO nanoparticles reduced the MDA concentration in rice plants under the stress induced by increased salinity because nanoparticles aided in cell membrane restoration, thereby enhancing plant vitality and minimizing stress-induced damage.

Conclusion

Due to its toxicity, lead can cause oxidative stress and damage to rice plants. The study concluded that lead stress can cause stunted growth, an upswing in reactive oxygen species (ROS), lipid peroxidation, and antioxidant enzymatic activity among rice plants. However, nano-priming with ZnO has been found to alleviate lead stress and reduce the harmful effects associated with it. A ZnO nano-priming process has resulted in improving the germination rate, plant development, and biomass accumulation while reducing the concentration of lead and ROS in the plant tissues. This study suggests that the use of ZnO nano-priming can improve the quality of agriculture in contaminated soils and mitigate the detrimental impacts of heavy metals contamination in the environment. There are potential future applications of ZnO nano-priming such as the development of more cost-effective and efficient methods of nano-priming on a larger scale, as well as further research into the mechanisms by which nano-priming reduces lead stress in plants.

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