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Research Article

Combined Effect of Zinc Oxide Nanoparticles and Bacteria on Osmolytes and Antioxidative Parameters of Rice (*Oryza sativa* L.) Plant Grown in Heavy Metal-Contaminated Water

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With the advancement in nanotechnology, the use of nanoparticles has been enhanced dramatically in biomedical, agriculture, and industrial processes. However, the combined effect of nanoparticles and bacteria on plant growth in heavy metal (Cd, Cr, Cu, and Pb)-contaminated wastewater is greatly limited. Therefore, the recent work was designed to determine the synergistic impact of green synthesized zinc oxide nanoparticles (ZnO-NPs) (5-10 mg/L) and *Bacillus* spp. (*Bacillus cereus* and *Lysinibacillus macroides)* on the physiological and biochemical activities of rice seedlings under heavy metal- (HM-) contaminated water. The results revealed that germination percentage (36%), root-shoot length (5.11 and 3.41 cm), fresh shoot-root weight (0.05 and 0.011 g), dry shoot-root weight (0.008 and 0.009 g), Chl a, Chl b, and carotenoid (5.4, 3.2 mg/g, and 4.3 μg/g), total soluble sugar (TSS) (26.44 mg/g), and total soluble protein (TSP) (21.99 mg/g) content considerably reduced in the plant tissues while combined impact of bacteria and ZnO NPs alleviates HM stress in contaminated water and improved seed germination (70%), root-shoot length (9.93 and 11.82 cm), fresh shoot-root weight (0.125 and 0.131 g), dry shoot-root weight (0.0532 and 0.042 g), Chl a, Chl b, and carotenoid (18.8, 13.9 mg/g, and 17.1 μg/g), TSS (57.651 mg/g), and TSP (47.990 mg/g) content. Lipid peroxidation induced by HM stress increased the amount of thiobarbituric acid reactive substances (TBRAS) (17.321 nM/mg) and hydrogen peroxide (H_2O_2) content (14.5 μM/g), stress markers such as glycine betaine (GB) (40.731 mg/g) and proline (Pro) (38.812 μmol/g) and antioxidant enzymes (SOD, POD, CAT, and APX) (180.87 U/mg, 450.677, 0.1066, and 0.631 μm/min/mg) under HM stress while the combined effect of ZnO NPs and bacteria reduced TBRAS (5.431 nM/mg), H_2O_2 content (2.25 μM/g), stress markers such as GB (24.731 mg/g) and Pro (18.811 μmol/g), and SOD, POD, CAT, and APX (187.53, 194.88, 0.061, and 0.271 μm/min/mg) contents. The present study suggested a potential role of combined impact of nanoparticles and bacteria in remediation of heavy metals from wastewater by improving plant growth.

1. Introduction

Seedling growth has an important role in plant development. Metabolic and antioxidative changes during growth are strongly related to the survival of seedlings which consequently affect plant yield and quality [1]. Abiotic stresses potentially affect growth through many factors including a reduction in water availability, imbalance in growth hormones, and effect on the metabolic and antioxidative activity [2]. Hydrogen peroxide accumulation and associated oxidative damages cause a decrease in antioxidant mechanisms that may suppress plant growth [3]. Bacteria-mediated seed priming is aimed at controlling seed hydration by way of decreasing outside water potential or shortening the hydration period at some stage in pressure. Several species of microorganism which include *Bacillus* spp. were said to solubilize the zinc ions in the course

of the growth by way of forming the complex with protons [4] and satisfy the requirement of nutrients by means of the use of zinc ions as a cofactor in their metabolic mechanism [5].

Bacteria can also increase the growth by utilizing minerals consisting of iron, phosphate, and nitrogen and prevent the plant from disease [6]. Conventional fertilizers were used to fulfil the demand of a rapidly growing population, but many fertilizers stay unavailable due to leaching, degradation by photolysis, hydrolysis, and decomposition [7]. Therefore, it is necessary to lower the nutrient losses in fertilization and improve plant growth [8]. Zinc oxide nanoparticles are used as nonfertilizers that act as important adsorbents for remediation, due to the fact that they carry various functional hydroxyl groups and protons on their surfaces [9]. ZnO nanoparticles (ZnO NPs) alter synthesis of many hormone, chlorophyll, and carbohydrate metabolism in the course of plant growth [10]. It has been shown that zinc can work as micronutrient and cofactor for metabolic processes, activating enzymes and organic solutes such as osmolytes or compatible solutes, amides, or betaine and amino acids [11]. Furthermore, zinc protects the plant cellular organelles from reactive oxygen species (ROS) and acts as a defense agent [4]; Various studies have shown that HMs can cause metabolic and antioxidative changes during plant growth [5]. Biopriming of seeds (seed priming with bacterial inoculation) grown in zinc oxide nanoparticle solution is an emerging, simple, and easily adaptable strategy to mitigate stress and improve the germination of seeds. Bacterial priming enhances the seed growth by improving gibberellin hormones that increase the amylase enzymes for development [12, 13].

Biopriming of seeds is not enough to increase the seed growth in toxic surroundings.It has been observed that solubilized form of HMs in water which cannot be removed by microorganism, so heavy metals easily bind with adsorbents of higher efficacy to bind with metals and increased the seed growth. Bio-nanotechnology has developed a relation between microorganism and nanoparticles, so nanoparticle at lower concentration improved the resistance level of microorganism against the toxic metals at low cost and high efficiency. Bacterial priming gave the protective coat around the seeds to prevent the entry by (1) producing auxin (IAA) hormones and activating cell division, (2) stabilizing the biomembrane integrity, (3) phospholipid formation, (4) increasing the protein synthesis, (5) remediating the oxidative stress, (6) transformation of nutrients towards new cells, and (7) lowering the uptake [4]. Furthermore, ZnO NPs at lower concentrations are more stable so dissolute into Zn^{+2} ions, which might be solubilized by bacterial cells by using secreting certain metabolites and organic acid in media [9]. These zinc ions also played a significant role in plant growth by stabilizing the membrane, macromolecules, extraordinary steroid receptors, and carbohydrate metabolism [10], further remediating the toxic effect of HMs from water. It was also reported [12, 13] that hormones such as auxin and IAA which synthesized nanoparticles also enhanced the growth of roots against stress as compared with plants grown in control. The hormone-stabilized nanoparticle has accumulated in the treated area and slowly gets oxidized to release rooting hormone and ion, which facilitates prolonged root promoting activity and protects root inhibition activity from soil inoculated phytopathogens. Individual effect of nanoparticles and bacteria under heavy metals has been studied extensively, but no data is available about the synergistic effect of ZnO NP- and bacteria-primed seeds on the improvement of growth, metabolic, and antioxidative parameters of rice under contaminated water. Therefore, it was hypothesized that the synergistic treatments of bacteria and nanoparticles may significantly improve plant growth in the toxic environment and further enhance the metabolic and antioxidative mechanism of plant. The current study investigated the significance of combined treatments of bacteria and nanoparticles on plant growth by alleviating the toxicity on rice plant by enhancing their metabolic and antioxidative activity.

2. Material and Methods

Wastewater samples were collected from the Hayatabad industrial estate (HIE) present near the suburban town of Hayatabad Peshawar, Pakistan. The samples were collected in clean bottles, stored at 4°C until analyzed for the physicochemical properties. Physicochemical properties and heavy metal (Pb, Cd, Cr, and Cu) concentration of wastewater were analyzed to investigate the toxic metals and then compared it with the standard value of the National Environmental Quality Standards (NEQS, 2000) [14] (Table 1). NEQS is the uniform standard applicable to all kinds of industrial and municipal effluents. The stock solution of nanoparticles was stabilized at pH 10 to maintain their activity. Rice (*Oryza sativa* L. cv Super Basmati) seeds were obtained from Islamabad, Pakistan. Seeds were surface sterilized with 1% solution of sodium hypochlorite (NaOCl) and rinsed thoroughly with deionized distilled water.

ZnO nanoparticles were collected from Pir Mehr Ali Shah (PMAS) Arid Agriculture University, Pakistan. It was synthesized by coprecipitation method by Amara et al. [15]. ZnO nanoparticles were prepared by mixing 0.05 M zinc acetate Zn (NO_3) $2.6H_2O$ (25 mL) solution with 4 mL of plant extract. Solution was heated and continuously stirred. The solution was centrifuged at 12,000 rpm for 15 min. Supernatant was discarded, and the isolated pellet was again suspended in deionized water. The solution was again centrifuged for 5 min and repeated the process three times to remove impurities. Synthesized ZnO NPs were white in color. The stock solution of nanoparticles was stabilized at pH 10 to maintain their activity. ZnO NPs were characterized by field emission scanning electron microscopy (FESEM), transmission electron microscopy (TEM), Fourier transform infrared spectroscopy (FTIR), and X-ray diffraction (XRD). ZnO NPs ranged from 30 to 50 nm in size [16].

2.1. Biopriming of Seeds and Bacteria Growth. Two heavy metal-resistant bacteria strains *Bacillus* strains (*B. cereus* (PMBL-3) and *L. macroides* (PMBL-7)) were previously isolated from Gadoon industrial estate's effluent [17] and were collected from the Plant and Microbial Biotechnology Lab, Kohat University of Science & Technology (KUST), Kohat, Pakistan. Strains were cultured for 24 hours at 37°C to

Table 1: Physicochemical parameters of water collected from Hayatabad industrial estate (HIE). The water was analyzed to evaluate the pollution load and heavy metal concentration.

Physicochemical parameters	HIE wastewater standard	Standard (NEQS)*
Temperature	24.5°C	40.0°C
pH	7.23 pH	6-10 pH
EC	682 (μS/m)	500 (μS/m)
Odor	Bad	Bad
TSS	400 mg/L	150 mg/L
TDS	4485 mg/L	3500 mg/L
BOD	250 mg/L	80 mg/L
COD	400 mg/L	150 mg/L
Lead	2.84 mg/L	0.5 mg/L
Cadmium	2.26 mg/L	1.0 mg/L
Chromium	2.40 mg/L	1.0 mg/L
Iron	1.19 mg/L	0.2 mg/L
Manganese	1.36 mg/L	1.5 mg/L
Nickle	1.83 mg/L	1.0 mg/L

*NEQS (National Environment Quality Standard) for industrial effluents.

increase cells up to 10^7 to 10^9 CFU/mL in cell suspension. After that, rice seeds were primed with *B. cereus* and *L. macroides* strains by adding 2% sucrose to bind seeds with bacteria and placed it for 10 hours in dark condition [18].

2.2. Experimental Work. Bacterial primed seeds were germinated in Petri plates supplemented with 5 and 10 mg/L of ZnO NP solution [18]. The young seedlings were exposed to sunlight after 5 days. Subsequently, after 10 days, young seedling were immediately transplanted in trays (3 L volume) containing one-fourth strength Hoagland solution [19]. Trays were covered with packing material to keep the root area dark [12, 13]. The experiment consisted of 9 treatments such as control (distilled water grown seedlings) seeds primed with *B. cereus* and *L. macroides* grown seedlings, seedlings grown in 5 and 10 mg/L ZnO NPs, and their combined treatments (*B.cereus*+5 mg/L ZnO NPs, *B.cereus* +10 mg/L ZnO NPs, *L.macroides*+5 mg/L ZnO NPs, and *L.macroides*+10 mg/L ZnO NPs). After 21 days, seedlings were then transferred from Hoagland solution to a new tray, which was filled with solutions containing 5 and 10 mg/L ZnO NPs along with HM-contaminated wastewater (w.w) of Hayatabad industrial estate (HIE). ZnO NPs at 5 and 10 mg/L have been used in several previous studies without showing any phytotoxic effect on plant seedlings [14, 15]. The hydroponic system was used to inhibit the adsorption of ZnO NPs to the soil surface and ensure that NPs and HMs are fully available [16]. Plants were grown in three replicates for each treatment and control. Plants were rotated and relocated to ensure equal light exposure during the exposure period. After the HM/ZnO NP exposure, the pH of the growth medium was measured with a pH meter. Seedlings were uprooted and analyzed for biochemical analysis. The synergistic data were subjected to one-way ANOVA by using statistic 9 software version (v.10) (Informer Technologies, Inc., United States).

2.3. Oxidative Damage to the Membrane

2.3.1. Cell Injury. Cell injuries in rice (root, shoot, and leaf) were examined by following the protocol of Hamim et al. [20]. Briefly, fresh plant materials were cut in 20 fine strips (1 cm each) and incubated at 25°C. After incubation, the first electrical conductance (C_1) was measured with an Electro Conductive (EC) meter (BMS EC-4001), and then, these mixtures were warmed for 40 min in a hot oven (121°C) to measure the electrical conductivity (C_2).

2.3.2. Malondialdehyde (MDA). Malondialdehyde (MDA) contents were determined by using the protocol of Velikova et al. [21]. Reaction mixture was made in 500 mL deionized water by adding trichloroacetic acid (TCA) (25 mg) and thiobarbituric acid (TBA) (2.5 mg). Enzyme extract (1.5 mL) was added in reaction substrate (2.5 ml) and incubated at 95°C for 15 min in water bath. Reaction was terminated on ice bath (35 min) and then vortexed for 10 minutes. Solution was centrifuged at 1300 rpm (15 min) until the clear solution formed. Record the absorbance at 532 nm while the (nonspecific) absorbance was measured at 600 nm. MDA-TBA value was calculated by coefficient 155 nM^{-1} cm^{-1}.

2.3.3. Hydrogen Peroxide (H_2O_2). Hydrogen peroxide contents were determined by using the protocol of Nankano and Asada [22]. Make the substrate (3 mL) by adding PBS buffer (1 mL), potassium iodide (KI) (2 mL), and enzyme extract (1 mL). Hydrogen peroxide content was then determined by the absorbance at 390 nm.

2.4. Photosynthetic Pigment. Photosynthetic pigments were determined as discussed previously by Li et al. [23]. In a falcon tube, the equal amount of plant materials (25 mg) and magnesium oxide (25 mg) was taken. Methanol (5 mL) was added in the mixture and shake at 200 rpm for 2 hours for pigment extraction. The sample was centrifuged at 400 rpm for 5 min to extract the supernatant. After that, the supernatant (3 mL) was added in cuvette. The absorbance was recorded at 470, 653, and 666 nm by using a spectrophotometer (BMS, Biotechnology Medical Services).

2.5. Osmolyte Contents

2.5.1. Proline (Pro) and Glycine Betaine (GB) Content. The proline (Pro) content was measured by following the protocol of Bates et al. [24]. Sample (100 mg) was mixed by adding 3% solution of 5 mL sulfosalicylic acid and then centrifuged at 4000 rpm for 30 min. Afterwards, 1 mL mixture was mixed with 1 mL of acid ninhydrin followed by addition of 30 mL of glacial acetic acid along with 6 M phosphoric acid (20 mL) and heated at 100°C for 60 minutes. The two layers were separated by adding toluene (2 mL) in reaction mixture, and then, toluene was calculated at 520 nm in a spectrophotometer. Proline content was calculated by following formula:

$$\frac{((\mu\text{gproline/mL}) \times (2/115.5))}{(0.1/5)}. \quad (1)$$

Glycine betaine (GB) content was determined by using the methodology of Shtisarnit et al. [25]. Plant material (50 mg) was crushed and mixed with 4 mL of deionized water. Sample was mixed and then filtered by filter paper to isolate the extract. After filtration, extract (1 mL) was mixed with 2 N H_2SO_4 (1 mL) and kept the solution on ice for 1 hour. After that, potassium triiodide (Kl_3) was added and then cooled the solution with occasional shaking (2 hours). Centrifuge the mixture and two layers were separated. The upper phase contains crystals which were then dissolved in 1,2-dicholorethane. Glycine betaine (GB) content was estimated by measuring the absorbance at 365 nm by using the glycine betaine (GB) standard curve.

2.5.2. Total Soluble Sugars (TSS) and Total Soluble Protein (TSP). The phenol-sulfuric acid method was followed to find the total soluble sugars (TSS) [26]. Plant sample (50 mg) was digested in 3 mL prewarmed ethanol solution (90%) and then incubate the solution for 1 hour at 80°C. Extracted plant mixture (1 mL) was mixed with 1 mL of 5% phenol solution. Concentrated 5 mL of sulfuric acid was added in the mixture and then added 10 mL of distilled water, mixed vertically and heated for 30 min. Measure the absorbance at 485 nm by taking distilled water (DW) as blank. The glucose-soluble sugar standard curve was used to find the TSS in sample. Bradford assay was used to determine the total soluble protein [27]. Fresh plant material was grinded in liquid nitrogen and mixed with 10 mL of potassium phosphate buffer (PPB) (pH 7.8) solution. The mixture was centrifuged at 14,000 rpm for 20 minutes (4°C) to extract the protein. The reaction mixture was taken in a falcon tube by adding enzyme extract (20 μL), Bradford reagent (500 μL), and DW (2 mL). The mixture was shaken well and placed at 37°C. Absorbance was recorded at 595 nm by using DW as blank. Protein was estimated by known protein (bovine serum albumin) BSA curve [28].

2.6. Extraction of Protein and Antioxidant Determination. Antioxidative parameters were determined as previously described by Vasconcelos et al. [28]. Briefly, fresh plant material (500 g) was crushed in liquid nitrogen and mixed with 10 mL precooled phosphate buffer (50 Mm potassium phosphate buffer). The extract was centrifuged at 20,000 rpm for 20 min. Total protein content was estimated by Bradford assay using bovine serum albumin (BSA) as standard.

2.6.1. Antioxidant Enzymes. Super oxidase (SOD) enzymes were determined by using the protocol as described earlier [29]. In a reagent bottle (250 mL), substrate solution was prepared by adding NBT (nitro-blue-tetrazolium) (15.5 mg) along with 2 mg of riboflavin, Na-EDTA (100 mg), and methionine (485 mg). After that, reaction solution (3 mL) was prepared by adding 2.725 mL of substrate, 25 mL of hydrogen peroxide, and 0.025 mL of enzyme extract. Place the mixture at 4000 lux under light for 20 minutes and then observe at 560 nm. Peroxidase (POD) content was measured by using the protocol of Liu et al. [23]. Substrate (3 mL) was prepared by mixing enzyme extract (1 mL), guaiacol (1.5%) along with PBS (2.7 mL) and 4% of hydrogen peroxide (0.1 mL). After 2 min, record the absorbance at 470 nm. Catalase (CAT) content was determined by the methodology as described by Aebi [30]. Reaction mixture (3 mL) was formed by adding 25 mM of potassium phosphate buffer (2.8 mL), enzyme extract (100 μL), and 30 Mm of H_2O_2 (100 μL) and recorded the absorbance at 240 nm. APX content was determined by using the protocol of Sofo et al. [31]. Briefly, reaction mixture was made by adding 100 mM potassium phosphate buffer (PPB) (pH 7.0), sodium EDTA (ethylenediaminetetraacetic acid) (1 mM), ascorbic acid (3 mM), H_2O_2 (hydrogen peroxidase) (0.06 mm), and enzyme extract (100 μL) and recorded the absorbance at 290 nm.

2.7. Statistical Analysis. The data were subjected to one-way ANOVA by using statistic 9 software version (v.10) (Informer Technologies, Inc., United States). Difference between means was determined by the least significance difference (LSD) at $P \leq 0.05$.

3. Results

3.1. Germination Percentage and Rate. Results indicated that seeds grown in the Petri plate under stress showed significantly decreased percentage (36%) than distilled water (60%) (Figure 1(a)). Synergistic treatment of bioprimed seeds with *B. cereus* and *L. macroides* along with ZnO nanoparticle solution improved the germination percentage at 5 mg/L (90 and 86%) and 10 mg/L (76 and 70%) in distilled water and 5 mg/L (70 and 63%) and 10 mg/L (62 and 30%) in wastewater as compared with control distilled water and wastewater (60% and 36%), respectively. Synergistic effect of bioprimed seeds with *B. cereus* and *L. macroides* along with ZnO nanoparticles showed enhanced germination rate ($1/t_{50}$) at 5 mg/L (0.342 and 0.321) and 10 mg/L (0.29 and 0.28) in distilled water and 5 mg/L (0.275 and 0.273) and 10 mg/L (0.264 and 0.253) in wastewater as compared with control distilled water and wastewater (0.21 and 0.143), respectively (Figure 1(b)).

3.2. Seedling Growth. Combined impact of ZnO NPs along with seeds primed with *B. cereus* and *L. macroides* significantly increased the length of shoot (Supplementary Figure 1) at 5 (14.21 and 12.5 cm) and 10 mg/L (11.73 and 9.745 cm) in distilled water and 5 (11.82 and 9.75 cm) and 10 mg/L (9.485 and 6.375 cm) in wastewater as compared with control distilled water and wastewater (7.7 and 3.4 cm) grown plant, respectively (Figure 2). Results showed that root length was significantly increased (Supplementary Figure 1) in bioprimed seed treatments with *B. cereus* and *L. macroides* at 5 mg/L (12.57 and 12.45 cm) and 10 ppm (11.17 and 9.995 cm) in distilled water and 5 mg/L (9.93 and 9.45 cm) and 10 mg/L (8.27 and 8.59 cm) in wastewater as compared with control distilled water and wastewater (8.72 and 5.1 cm), respectively (Figure 2). Result showed that fresh shoot and root weight were significantly enhanced in *B.*

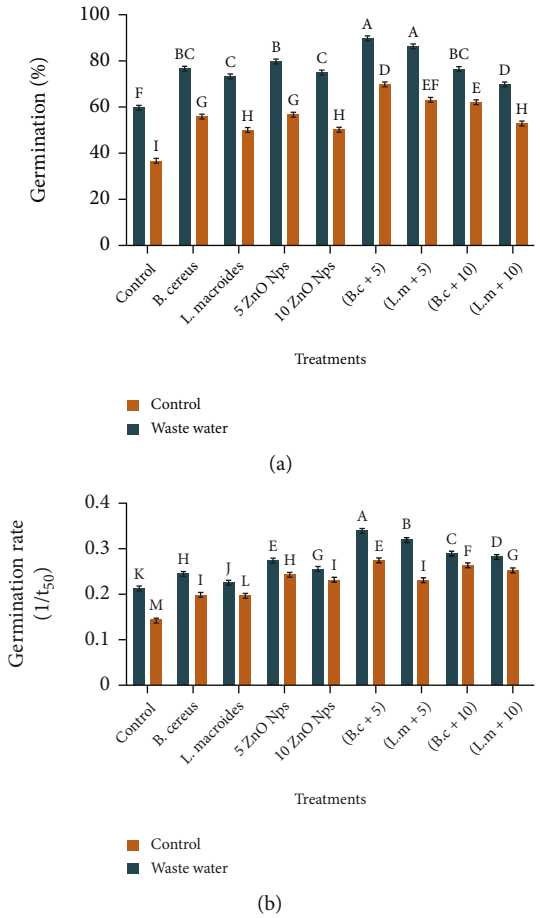

Figure 1: Synergistic effect of bacterial strains (*Bacillus cereus* and *Lysinibacillus macroides*) and ZnO NPs (5 and 10 mg/L) treatments on (a) germination % and (b) germination rate of rice grown in heavy metal-contaminated water. Error bars showed means of standard error (±SE) of three replicates ($n = 3$) followed by different alphabetic letters showing statistical significance at 5% probability level. Control (distilled water), B.C+5 (*Bacillus cereus* +5 mg/L ZnO NPs), L.M+5 (*Lysinibacillus macroides*+5 mg/L ZnO NPs), B.C+10 (*Bacillus cereus*+10 mg/L ZnO NPs), and L.M +10 (*Lysinibacillus macroides*+10 mg/L ZnO NPs).

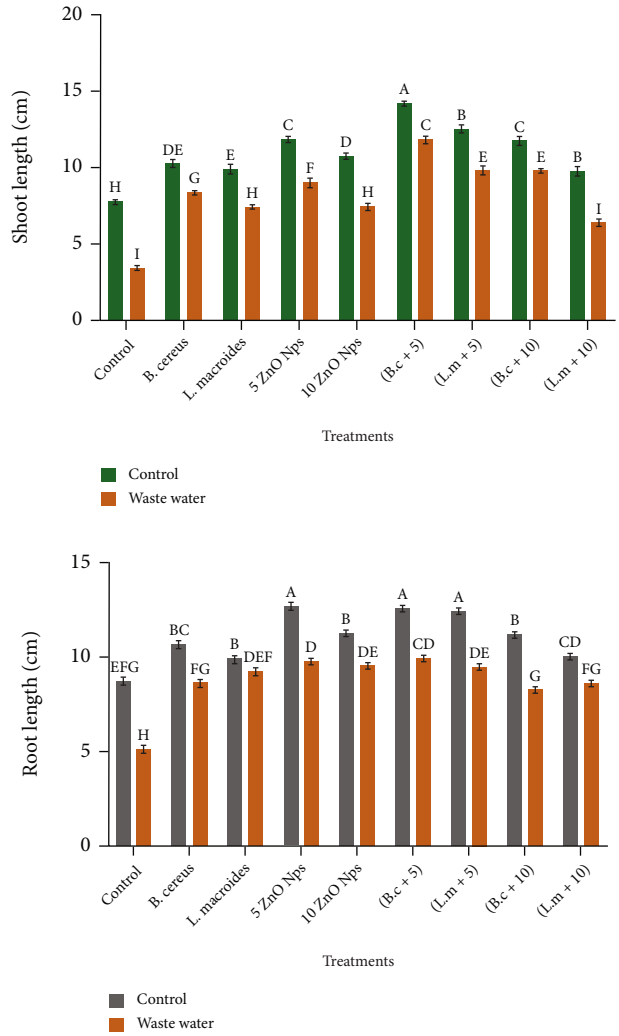

Figure 2: Synergistic effect of bacteria strain *Bacillus cereus* and *Lysinibacillus macroides* and ZnO NP (5 mg/L and 10 mg/L) treatments on shoot and root length of rice grown in heavy metal-contaminated water. Error bars showed means of standard error (±SE) of three replicates ($n = 3$) followed by different alphabetic letters showing statistical significance at 5% probability level. Control (distilled water), B.C+5 (*Bacillus cereus*+5 mg/L ZnO NPs), L.M+5 (*Lysinibacillus macroides*+5 mg/L ZnO NPs), B.C+10 (*Bacillus cereus*+10 mg/L ZnO NPs), and L.M+10 (*Lysinibacillus macroides*+10 mg/L ZnO NPs).

cereus and *L. macroides* primed seed treatments along with ZnO nanoparticles (Supplementary Figure 2) at 5 mg/L (0.147, 0.131, 0.031, and 0.028 g) and 10 mg/L (0.133, 0.115, 0.026, and 0.022 g) in distilled water and 5 mg/L (0.125, 0.131, 0.031, and 0.028 g) and 10 mg/L (0.106, 0.105, 0.026, and 0.022 g) in wastewater than control distilled water (0.065 and 0.0145 g) and wastewater (0.05 and 0.011 g), respectively (Figures 3 and 4). Results showed that dry shoot and root weight increased due to the synergistic effect of bioprimed seeds with *B. cereus* and *L. macroides* along with ZnO NP treatments at 5 mg/L (0.064, 0.0532, 0.0637, and 0.616 g) and 10 mg/L (0.0415, 0.035, 0.052, and 0.044 g) in distilled water and 5 (0.0532, 0.042, 0.051, and 0.047 g) and 10 mg/L (0.033, 0.031, 0.033, and 0.315 g) in wastewater than distilled water (0.024 and 0.042 g) and wastewater (0.012 and 0.014 g), respectively (Figures 4(c) and 4(d)).

3.3. Oxidative Damage to the Membrane

3.3.1. Cellular Injury. In addition to seedling growth, it was found that maximum cell injury (62.651) was measured in wastewater treatment, than control (distilled water)-treated plant (53.812 μS/cm). Surprisingly, the synergistic effect of bioprimed seeds with *B. cereus* and *L. macroides* along with 5 mg/L ZnO nanoparticle solution in wastewater showed lower cell injury (33.561 and 38.22 μS/cm) as compared with only bioprimed seeds (46.441 and 52.661) and 5 mg/L ZnO nanoparticles (54.702 μS/cm) (Figure 4).

3.3.2. Malondialdehyde (MDA) and Hydrogen Peroxide (H_2O_2). Current findings revealed that MDA content was

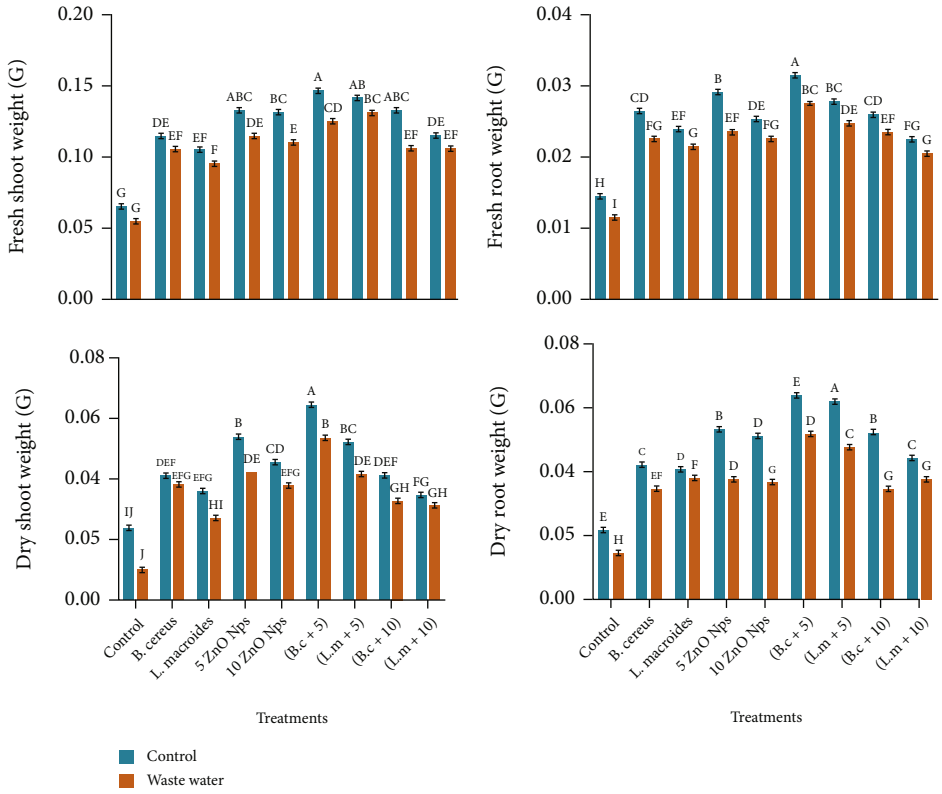

Figure 3: Synergistic effect of bacteria strains (*Bacillus cereus* and *Lysinibacillus macroides*) and ZnO NP (5 mg/L and 10 mg/L) treatments on (a) fresh shoot weight (FSW), (b) fresh root weight (FRW), (c) dry shoot weight (DSW), and (d) dry root weight of rice grown in heavy metal-contaminated water. Error bars showed means of standard error (±SE) of three replicates ($n = 3$) followed by different alphabetic letters showing statistical significance at 5% probability. Control (distilled water), B.C+5 (*Bacillus cereus*+5 mg/L ZnO NPs), L.M+5 (*Lysinibacillus macroides*+5 mg/L ZnO NPs), B.C+10 (*Bacillus cereus*+10 mg/L ZnO NPs), and L.M+10 (*Lysinibacillus macroides*+10 mg/L ZnO NPs).

enhanced in leaf, shoot, and root as compared to control (distilled water) (Figure 5(e)). Under stress, the MDA contents in leaf, shoot, and root were decreased in bioprimed seeds (4.096 and 5.368) and 5 mg/L ZnO nanoparticles (4.75 μm/g f.w). Significant decrease was observed in MDA content by synergistic effect of bioprimed seeds with *B. cereus* and *L. macroides* along with 5 mg/L ZnO nanoparticles under contaminated water in leaf, shoot, and root (2.241 and 2.71). H_2O_2 content was also increased in leaf, shoot, and root (6.34 μm/g f.w) than control (distilled water) (4.26 μm/g f.w) (Figure 5(f)). In addition, our results revealed that under stress, the MDA contents in leaf, shoot, and root were decreased in bioprimed seeds (3.41 and 4.54) and 5 mg/L ZnO nanoparticles (3.16 μm/g f.w). Maximum H_2O_2 content was observed in synergistic treatments of bioprimed seeds with *B. cereus* and *L. macroides* along with 5 mg/L ZnO nanoparticles under stress in leaf, shoot, and root (2.22 and 2.24 μm/g f.w).

3.4. Photosynthetic Pigments. Mean data revealed that chlorophylls (a, b, total pigments) and carotenoid contents were lowered in wastewater (5.4, 3.2, and 8.6) 4.3 μg/g than plant germinated in DW (12.4, 5.6, and 18.1 mg/g) 8.5 μg/g. Combined impact of *B. cereus* and *L. macroides* bioprimed seeds along with 5 mg/L ZnO NPs showed significant increase in photosynthetic pigments 21.2 (25.3, 16.3, and 41.6) and 17.2 (22.3, 14.3, and 36.7) as compared with alone bioprimed seeds 7.3 (9.3, 5.5, and 14.8) and 7.3 (8.1, 5.1, and 13.2) and 5 mg/L ZnO nanoparticles 14.3 μg/g (19.2, 11.2, and 30.2 mg/g) (Table 2).

3.5. Osmolyte Contents

3.5.1. Proline and Glycine Betaine. Higher contents of proline were observed in wastewater-treated leaf, shoot, and root (26.311 μmol/g) as compared with plant grown in control (distilled water) (12.881 μmol/g) (Figure 6(c)). In contaminated water, proline was decreased in *B. cereus* and *L. macroides* primed seeds (15.387 and 15.387) and 5 mg/L ZnO nanoparticles (11.890 μmol/g). Additionally, our results showed significant decrease in proline content in contaminated water when treated with *B. cereus* and *L. macroides* along with 5 mg/L ZnO nanoparticles in leaf, shoot, and root (4.315 and 6.735 μmol/g). The glycine betaine content was inecreased in plant tissues grown in wastewater while it was decreased in synergistic treatments of bacteria and ZnO nanoparticles (Figure 6(d)). GB content was enhanced in contaminated wastewater in leaf, shoot, and root (23.735 mg/g) than plant grown in control (distilled water) (10.041 mg/g). In

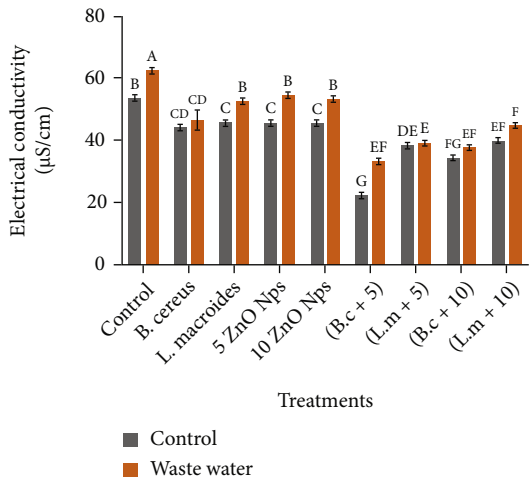

FIGURE 4: Synergistic effect of bacteria strains (*Bacillus cereus* and *Lysinibacillus macroides*) and ZnO NP (5 mg/L and 10 mg/L) treatments on cellular injury of rice grown in heavy metal-contaminated water. Error bars showed means of standard error (±SE) of three replicates ($n = 3$) followed by different alphabetic letters showing statistical significance at 5% probability level. Control (distilled water), B.C+5 (*Bacillus cereus*+5 mg/L ZnO NPs), L.M+5 (*Lysinibacillusmacroides*+5 mg/L ZnO NPs), B.C+10 (*Bacillus cereus*+10 mg/L ZnO NPs), and L.M+10 (*Lysinibacillusmacroides*+10 mg/L ZnO NPs).

contaminated water, glycine betaine was decreased in *B. cereus* and *L. macroides* primed seeds (14.725 and 13.618) and 5 mg/L ZnO nanoparticles (14.631 mg/g). Significant decrease in glycine betaine content was observed in synergistic treatments of *B. cereus* and *L. macroides* primed seeds along with 5 mg/L ZnO nanoparticles under treatments in leaf, shoot, and root (9.543 and 13.618 mg/g).

3.5.2. Total Soluble Sugar (TSS) and Total Soluble Protein (TSP). Total soluble sugar (TSS) content was significantly reduced (9.984 mg/g) in contaminated water as compared with plant grown in control (distilled water) (16.415 mg/g) (Figure 6(a)). In contaminated water, TSS was increased in *B. cereus* and *L. macroides* primed seed treatments (17.91 and 17.575 mg/g) and 5 mg/L ZnO nanoparticles (17.575 mg/g). Subsequent increase in TSS was determined in seeds treated with *B. cereus* and *L. macroides* along with 5 mg/L ZnO nanoparticles under contaminated water (32.311 and 31.251 mg/g). Similarly, TSP was significantly decreased (2.845 mg/g) in contaminated water as compared with (control) distilled water (2.845 mg/g), respectively (Figure 6(b)). In contaminated water, TSP was increased in combined treatment of *B. cereus* and *L. macroides* primed rice seeds (11.890 and 10.890) and 5 mg/L ZnO nanoparticles (10.890 mg/g). Maximum total soluble protein was determined in combined treatment of bioprimed seeds with *B. cereus* and *L. macroides* along with 5 mg/L ZnO nanoparticles under contaminated water (23.790 and 25.835 mg/g).

3.6. Antioxidant Enzymes. Synergistic effect of bioprimed seed and ZnO NPs on antioxidant enzymes was also tested. Data showed that superoxide dismutase (SOD) activity was increased in contaminated water (248.09 μg/g f.w) as compared with control (distilled water) (115.62 μg/g f.w) (Figure 5(a)). Similarly, our result revealed that under stress, the SOD content in leaf, shoot, and root was decreased in bioprimed seeds (63.73 and 158.05) and 5 mg/L ZnO nanoparticles (95.2 μg/g f.w). Maximum reduction in SOD content was observed in combined impact of bioprimed seeds with *B. cereus* and *L. macroides* along with 5 mg/L ZnO nanoparticles under stress (144.78 and 74.26 μg/g f.w). Result showed that POD content in leaf, shoot, and root was increased in contaminated water (197.23) as compared with control (distilled water) (126.32 μg/g f.w) (Figure 5(b)). Under stress, the POD content in leaf, shoot, and root was decreased in bioprimed seeds as compared with alone bioprimed seeds (100.21 and 112.5) and 5 mg/L ZnO nanoparticles (96.22 μg/g f.w). Significant reduction in POD content was observed in combined *B. cereus* and *L. macroides* along with 5 mg/L ZnO nanoparticles in contaminated water (62.07 and 68.03). Catalase activity (CAT) was increased in contaminated water (0.05 μg/g f.w) as compared with control (distilled water) (0.078 μg/g f.w), respectively (Figure 5(c)). It was observed that under stress, the CAT content in leaf, shoot, and root was decreased in bioprimed seeds (0.0431 μg/g f.w, 0.0431 μg/g f.w) and 5 mg/L ZnO nanoparticles (0.0404 μg/g f.w). Maximum reduction of CAT content was observed in bioprimed seeds with *B. cereus* and *L. macroides* along with 5 mg/L ZnO nanoparticles under stress in leaf, shoot, and root (0.0227 μg/g f.w, 0.0237 μg/g f.w). Ascorbate peroxidase (APX) activity in leaf, shoot, and root increased in plant grown in HMs (0.541) as compared with control (distilled water) (0.333 μg/g f.w) (Figure 5(d)). Additionally, we showed that under stress, the APX content in leaf, shoot, and root was decreased in bioprimed seeds (0.287 μg/g f.w, 0.172 μg/g f.w) and 5 mg/L ZnO nanoparticles (0.254 μg/g f.w). APX content was decreased in synergistic treatments of bioprimed seeds with *B. cereus* and *L. macroides* along with 5 mg/L ZnO nanoparticles in leaf, shoot, and root (0.152 μg/g f.w, 0.172 μg/g f.w) as compared in contaminated water.

4. Discussion

Plants are strongly influenced by metabolic and antioxidative mechanisms. Seed priming is an efficient strategy applied to improve plant growth and metabolic processes under stressful conditions. In the past, seed treatments with bacteria and application of NPs have been described as efficient strategies to improve plant growth under stress [32]. However, the synergistic treatments of ZnO NPs and *Bacillus* spp. on plant development have not been reported yet. Therefore, the present study was initiated to reveal the combined impact of ZnO NPs and bacteria on metabolic and antioxidative parameters of rice plant in heavy metal-contaminated water. *Bacillus* spp. release amino cyclopropane (1-carboxylic acid (ACC)) deaminase and bioactive metabolites (surfactant and lipopeptides) that covered the seed toxicity [33]. Furthermore, Zn^{+2} ions are essential for bacterial enzymes (dehydrogenase, thiol peroxidase, and glutathione reductase) that enhanced the growth by uptake

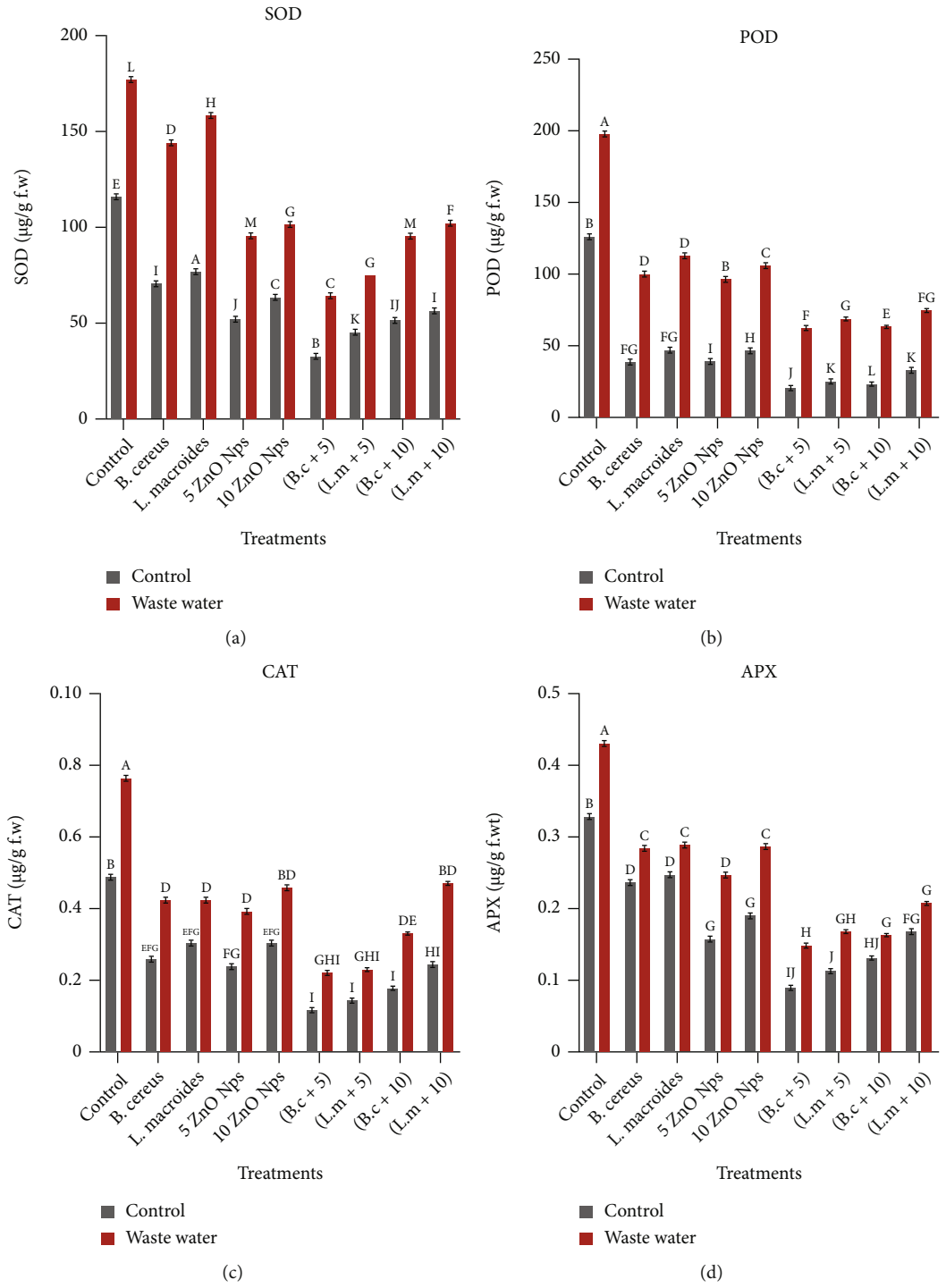

Figure 5: Continued.

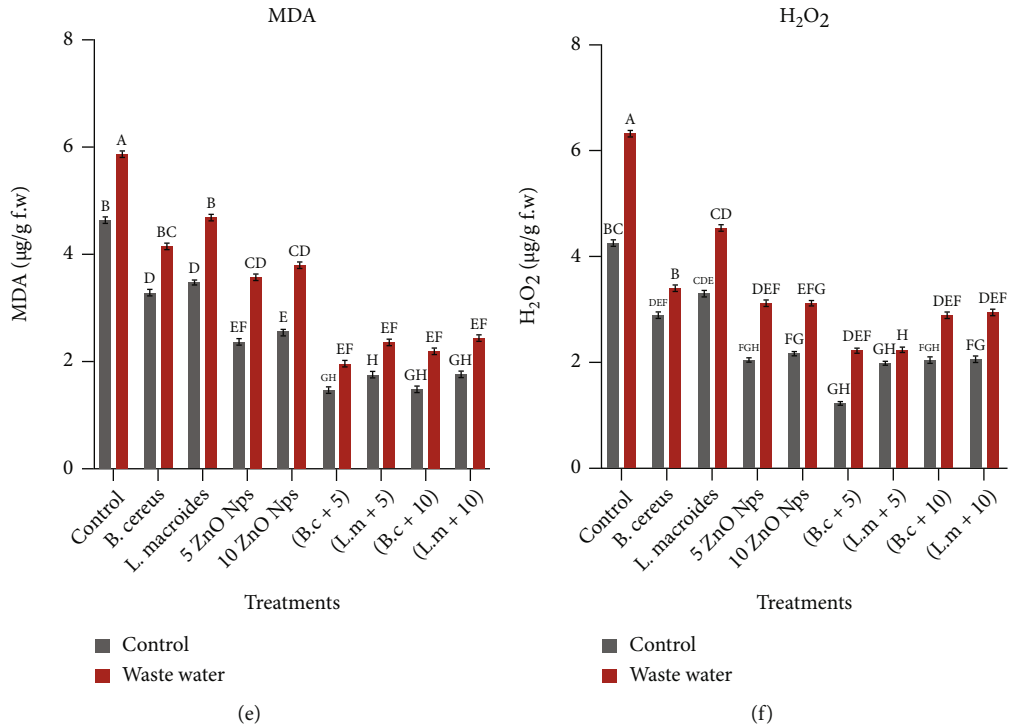

Figure 5: Synergistic effect of bacteria strains (*Bacillus cereus* and *Lysinibacillus macroides*) and ZnO NP (5 mg/L and 10 mg/L) treatments on (a) superoxide dismutase, (b) peroxidase, (c) catalase, (d) ascorbate peroxidase, (e) malondialdehyde, and (f) hydrogen peroxide on rice grown in heavy metal-contaminated water. Error bars showed means of standard error (±SE) of three replicates ($n = 3$) followed by different alphabetic letters showing statistical significance at 5% probability level. Control (distilled water), B.C+5 (*Bacillus cereus*+5 mg/L ZnO NPs), L.M+5 (*Lysinibacillus macroides*+5 mg/L ZnO NPs), B.C+10 (*Bacillus cereus*+10 mg/L ZnO NPs), and L.M+10 (*Lysinibacillus macroides* +10 mg/L ZnO NPs).

of nutrient reserves and metabolic and antioxidative activities [34]. Zinc ions also increased the cell stability by the nutrition uptake, water molecules, and translocation in plant tissues and enhanced the resistance level of plant against toxicity [35].

In this study, it was observed that stress reduced the germination of seeds by lowering the water potentials and nutrient uptake in seeds (Figure 1). Heavy metals inhibit seed germination by reduction in water availability and growth hormones and stop the metabolic and antioxidative activities [8]. These results are similar with other studies [32] in which they have shown that HMs create oxidative stress in the rice seed coat thus leading to the changes in microtubules during growth. The combined impact of bacteria and nanoparticles showed significant increase in germination under contaminated water (Figure 1). Overall, these can partially be attributed to the facts that ZnO NPs can potentially act as nanofertilizers at lower concentrations (<50 ppm) significantly enhanced seed germination [36]. These results are followed by other researcher data [12] that biopriming of rice seeds with bacteria enhances germination under heavy metal stress, while lower concentrations of ZnO NPs improved germination. It was observed that [32] the interaction of Si and PGPB seems an auspicious technique and eco-friendly approach to enhance metal tolerance in crop plants. Yan et al. [35] documented that zinc oxide nanoparticles improved the germination of wheat seeds at lower concentrations. It was reported that ZnO nanoparticles at lower concentrations act as a micronutrient and fertilizer for mung bean (*Vigna radiata*) chickpea (*Cicer arietinum*), ryegrass [37] (*Lolium perenne*), buckwheat (*Fagopyrum esculentum*), wetland plant (*softstem bulrush*, *Schoenoplectus tabernaemontani*) [1], and *Cucumis sativus* under drought stress [38]. Recently, it was reported [7] that the use of ZnO NPs (60 and 50 ppm) increased biochemical and nutritional quality of red radish plant.

Heavy metals affected the seedling biomass under toxic metals while combined impact of bacteria and ZnO nanoparticles enhanced the germination (Figures 2 and 3). The current study showed that bacteria primed seeds germinate in harsh environment; subsequently, ZnO NPs at lower concentrations act as micronutrients and remediate the HMs for better growth of the plant. Heavy metals drastically affect the seedling growth of rice [38]. Rizwan et al. [39] observed that phytohormonal seed priming protects the plant from toxicity by enhancing the tolerance mechanism such as causing the ROS detoxification, disrupting the photosynthetic activity and protein homeostasis. Seed priming also regulates the molecular mechanism by changing the stress inducible gene expression, transcriptional factors, and posttranslational modification. The current research [40] provides a novel insight into the potential mechanism of *B. megaterium* OSR-3 and putrescine in mitigation of hydrocarbon stress in *N. tabacum* plants. Moreover, these findings are further supported by earlier study [36] that ZnO NPs at lower concentration enhanced the enzyme production, synthesis of

Table 2: Synergistic effect of primed seeds with bacteria strains (*Bacillus cereus* and *Lysinibacillus macroides*) along with ZnO NP (5 and 10 mg/L) treatments on photosynthetic pigments of rice grown in heavy metal-contaminated water.

Treatments	Chl (a) (mg/g)	Chl (b) (mg/g)	Chl (a)+Chl (b) (mg/g)	Carotenoids (μg/g)
Distilled water	$12.4 + 0.350^{ab}$	$5.6 + 0.503^{cd}$	$18.1 + 3.212^{ab}$	$8.5 + 0.423^{d}$
Wastewater (w.w)	$5.4 + 0.173^{a}$	$3.2 + 0.432^{cd}$	$8.6 + 2.12^{ac}$	$4.3 + 0.321^{ad}$
Bacillus cereus	$18.8 + 1.043^{abc}$	$11.3 + 1.102^{abc}$	$30.1 + 1.231^{abc}$	$14.3 + 0.543^{cd}$
(B.C+w.w)	$9.3 + 0.157^{ab}$	$5.5 + 0.231^{cd}$	$14.8 + 1.32^{ab}$	$7.3 + 0.231^{abc}$
Lysinibacillus macroides	$16.2 + 0.321^{ab}$	$9.2 + 0.321^{ab}$	$25.4 + 1.43^{bc}$	$12.4 + 0.432^{ab}$
(L.M+w.w)	$8.1 + 1.173^{ab}$	$5.1 + 0.153^{ab}$	$13.2 + 1.21^{abc}$	$7.3 + 0.221^{ab}$
5 mg/L ZnO NPs	$22.2 + 0.323^{ab}$	$15.4 + 0.421^{cd}$	$37.6 + 3.231^{de}$	$17.2 + 0.543^{abc}$
(5+w.w)	$19.2 + 0.132^{bc}$	$11.2 + 0.231^{cd}$	$30.2 + 1.03^{a}$	$14.3 + 0.543^{ab}$
10 mg/L ZnO NPs	$25.4 + 0.432^{d}$	$19.2 + 0.421^{cd}$	$44.6 + 3.12^{ab}$	$20.1 + 0.231^{ab}$
(10+w.w)	$21.23 + 0.132^{bc}$	$15.4 + 0.142^{ab}$	$36.6 + 1.21^{a}$	$17.2 + 0.312^{abc}$
B.C+5 mg/L ZnO NPs	$27.6 + 0.243^{b}$	$19.2 + 1.12^{abc}$	$46.8 + 2.32^{bd}$	$22.3 + 0.432^{ab}$
(B.C+5+w.w)	$25.3 + 0.121^{bcd}$	$16.3 + 0.134^{ab}$	$41.6 + 1.05^{ab}$	$21.2 + 0.321^{ac}$
L.M+5 mg/L ZnO NPs	$24.9 + 0.32^{abc}$	$18.2 + 0.321^{ab}$	$43.1 + 2.12^{bc}$	$20.4 + 0.32^{abd}$
(L.M+5+w.w)	$22.3 + 0.102^{ab}$	$14.2 + 0.121^{abc}$	$36.5 + 1.01^{ac}$	$17.2 + 0.243^{ad}$
B.C+10 mg/L ZnO NPs	$32.5 + 0.341^{bc}$	$25.2 + 0.342^{bd}$	$57.7 + 1.23^{abc}$	$24.2 + 0.212^{ac}$
(B.cereus+10+w.w)	$23.4 + 0.121^{ab}$	$12.3 + 0.123^{abc}$	$35.7 + 1.11^{ab}$	$19.2 + 0.254^{ab}$
L.M+10 mg/L ZnO NPs	$30.4 + 0.231^{ab}$	$21.2 + 0.321^{ab}$	$51.5 + 1.11^{a}$	$25.3 + 0.341^{abc}$
(L.M+10 w.w)	$21.3 + 0.142^{abc}$	$13.3 + 0.113^{ab}$	$34.2 + 1.21^{ab}$	$18.2 + 0.232^{ac}$

Control (distilled water), B.C+5 mg/L (*Bacillus cereus*+5 mg/L ZnO NPs), L.M+5 mg/L (*Lysinibacillus macroides*+5 mg/L ZnO NPs), B.C+10 mg/L (*Bacillus cereus*+10 mg/L ZnO NPs), and L.M+10 mg/L (*Lysinibacillus macroides*+10 mg/L ZnO NPs). Data are statistically analyzed by statistic 9 software, and the numbers are mean of three replicates ($n = 3$) followed by standard deviation showed as (±) sign and analyzed by ANOVA test. Subsequently, different alphabetic letters appeared on each number showing statistical significance at 5% probability level and analyzed by Duncan's multiple range test (DMTR).

proteins, biomembrane stability, and growth regulation. A significant increase was observed in the root and shoot of the wheat plant by applying the different concentrations of ZnO nanoparticles [41]. It is also clear from the current findings that lower concentrations of ZnO NPs had no inhibitory effect on plant weight. These results were further supported by other experiments [35]; they showed that silver nanoparticles significantly increased the biomass and fresh and dry weight of plants. An earlier study showed that shoot-root elongation, fresh-dry weight was influenced in application of various doses of ZnO nanoparticles [42].

Plant cell membrane stability has a significant role in plant development while plant growth by bacteria and ZnO nanoparticle solution showed lowered cell injury than individual effect (Figure 4). Bioprimed treatment protects the seeds from toxic effect by using mineral (such as iron, phosphate, and nitrogen) from surrounding environment and enhanced the germination while ZnO NPs removed the HMs from water which caused cell stability. It has been shown previously that toxicity causes cellular deformation in plant tissues and damages the membrane structure [42]. ZnO NPs at lower concentration have an important function in enzyme productions, protein synthesis, biomembrane stability, and growth regulation in the plant [1]. Zinc ions enhanced the cell membrane stability by directly improving the nutrition uptake, water molecule conduction from roots to upper parts of the plant and vascular bundle tissue cells [36].

Photosynthetic pigments are bioindicators in plants for stress, for example, heavy metals change the sulfa-hydroxyl group, chlorophyllase, and pheophytinase enzymes by disturbing the nutrition uptake activities and Mg^{+2} ion [3]. HM contents increased the MDA contents and affect the membrane-bound organelles (chloroplast) and photosynthetic pigments, damage the chloroplast structure by closing the stomata [43, 44], and change the chloroplast, electron transport chain (ETC), and metabolic functions of the plant [8]. It was revealed that plants germinated by bacteria nanoparticle interaction increased the photosynthetic pigments than the individual treatments of bioprimed seeds and 5 mg/L ZnO nanoparticles (Table 2). Bacteria-nanoparticle combined treatment increased the photosynthesis process in plants by enhancing different enzymes involved in photosynthesis. These findings are confirmed by other reports [18] that chlorophyll pigments were enhanced in leaves of *Leucaena leucocephala* under ZnO NP treatments because Zn ions enhanced the chlorophyll enzyme function and carbohydrate production. Moreover, ZnO NP treatments have a significant impact on the chlorophyll content, plant height, and fresh weight of peanut plants [35]. Furthermore, bacteria priming of seeds has also been shown to improve the photosynthesis of *faba* bean plant under cadmium stress [45].

Total soluble sugar (TSS) and protein (TSP) content have function in osmoregulation, energy production, and stress resistance. Heavy metal stress increased the catalytic activity and protein hydrolysis in plants (Figure 6). Plants stabilize the sugar and protein level by balancing the osmotic potential and degradation of biomolecules and membranes. ZnO NPs act as zinc ions which subsequently increase the sugar content, protein metabolism and hormones, and growth and biomass [46]. ZnO nanoparticles have been

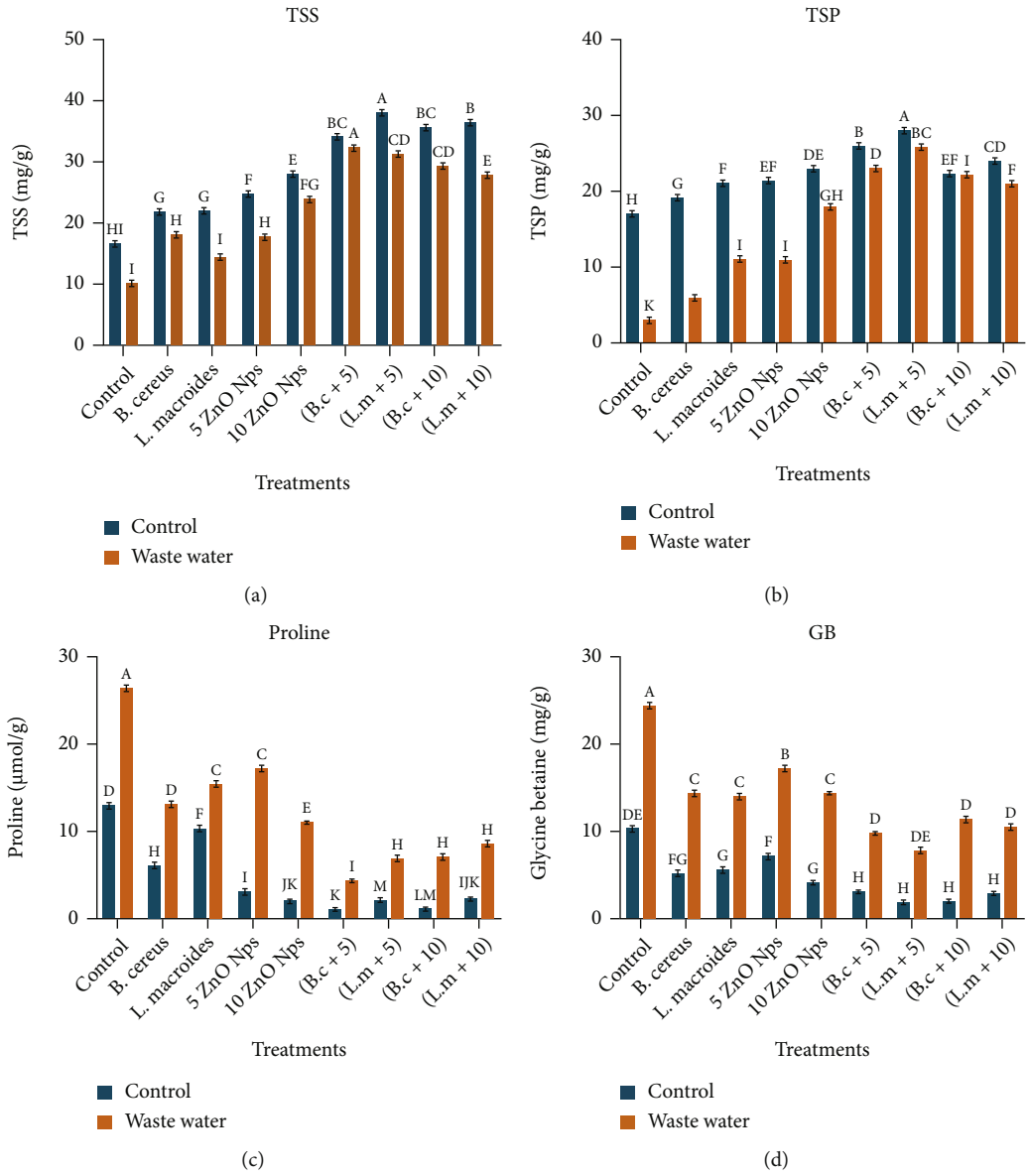

Figure 6: Synergistic effect of bacteria strains (*Bacillus cereus and Lysinibacillus macroides*) and ZnO NP (5 mg/L and 10 mg/L) treatments on (a) total soluble sugar, (b) total soluble protein, (c) total proline, and (d) glycine betaine content of rice grown in heavy metal-contaminated water. Error bars showed means of standard error (±SE) of three replicates ($n = 3$) followed by different alphabetic letters showing statistical significance at 5% probability level. Control (distilled water), B.C+5 (*Bacillus cereus*+5 mg/L ZnO NPs), L.M+5 (*Lysinibacillus macroides*+5 mg/L ZnO NPs), B.C+10 (*Bacillus cereus*+10 mg/L ZnO NPs), and L.M+10 (*Lysinibacillus macroides*+10 mg/L ZnO NPs).

shown to increase the protein content in cabbage followed by the increase in chlorophyll and carotenoids [47]. It was observed by Ahmad [14] that ZnO NPs lowered down the arsenic (As) stress in soybean plant by the synthesis of osmolytes, enzymatic and nonenzymatic antioxidant, enhanced the glyoxalase system in plants.

Proline (Pro) and glycine betaine (GB) are osmolytes that play important role in osmotic adjustment and growth of seeds. Proline has a specific role in protein denaturation and lipid peroxidation and scavenges the reactive oxygen species [48] while glycine betaine act as a phytoprotectant and its amount is increased in the plant under stress [35]. Bacterial treatments enhanced the sugar and protein content in seeds while zinc oxide nanoparticles give nutrients and remove the toxic metals, so proline and glycine betaine content was decreased in plants (Figure 6(c)). It has been noticed that ZnO NPs activate GB pathways and prevent the toxicity in *L. leucocephala* [18]. Similarly, it was revealed that silicon NPs increased the proline content in pea seedlings and protect the binding of HMs to functional groups of root cell wall [35].

Oxygen (O_2) is the basic element of metabolic processes in plant while oxidative stress caused many abnormalities in plants and damaged the biomolecules (proteins, lipids,

enzymes, and nucleic acid) [46]. Antioxidative enzymes in plant were enhanced under HM stress while the combined treatment of bacteria-nanoparticles lowered down the antioxidant enzymes (Figure 5). Superoxidase (SODs) is a multimeric metalloenzyme and in stress condition removed the superoxide free radicals and scavenges H_2O_2 into O_2. These results were further supported by other scientists [47]. He observed that the superoxidase level in rice seedlings increased by enzymatic protein de novo synthesis under stress condition. It was observed that in *Pisum sativum* plants, SOD activity increased under stress [48]. Bacteria priming may activate the oxidative stress resistance mechanism in seeds while zinc oxide nanoparticles may interact with the metals by cell surface metal retention and decreasing their permeability in the plant. These results were supported by another study that SOD level in rice seedlings increased under stress by enzymatic protein de novo synthesis [49]. In *Pisum sativum* plants, it was noticed that SOD activity increased under stress [35]. Foliar spray of zinc oxide nanoparticles in sunflower plants under salt stress showed an enhanced SOD activity [50]. It was also observed that combined application of ZnO-NPs and *B. fortis* IAGS-223 considerably changes the activity of antioxidant enzymes besides upgradation of the biochemicals and growth parameters of Cd-stressed plants. In cauliflower, enhanced concentration of ZnO nanoparticles (9.0 M) has been observed to stabilize the SOD, CAT, and sugar content [43]. Plant contains some metabolites and nonenzymatic components such as ascorbic acid and glutathione that act as ROS scavengers. These components are involved in many cellular processes and cause the detoxification of ROS due to its ability to donate electrons in a wide range of enzymatic and nonenzymatic reactions.

POD enzymes dismutase the H_2O_2 content under HM stress in plants by gaining electrons. Bacteria removed the HMs from media by making complex with metals and decreased the amount of SOD and POD while the low content of ZnO NPs changed into zinc ions that act as micronutrients for plant growth and essential cofactor for enzymes in the metabolic process. Increased activities of superoxidase and peroxidase have been documented in pigeon pea under stress [46]. Investigation [26] revealed that under Pb stress (800 mg kg^{-1} soil), *Athium wardii* specie, soybean, and rice seedlings showed an increase in POD content. It was recently reported that ZnO NPs reduced drought-related damages to cell organelles, increased melatonin content, and decreased antioxidative enzymes under drought stress in maize plant [47]. It is further confirmed [48] that seed priming of pearl millet plant with silver NPs enhances salinity tolerance by increasing physiological and biochemical responses in plants. Silver NPs reduced the oxidative stress by improving antioxidant enzyme, decreased the sodium (Na^+), and increased the potassium (K^+), total phenolic, and flavonoid content, so priming with NPs increased the crop production in salt-contaminated lands. It was reported [51] that the combined application of Ca and Bd could effectively relieve individual and combined Cd stress and DDT toxicity in *B. alboglabra*.

It has been documneted [51] that a high amount of CAT content in biomass may be due to scavenging of H_2O_2 and toxic peroxidase under metal stress. Higher CAT content was measured in plant *Jatropha curcas* cotyledons at a high concentration of metals [35]. The result showed that the synergistic effect of bacteria and zinc oxide nanoparticles may remove the stress by decreasing the oxidative stress in plant and generates lower activity of CAT enzymes (Figure 5). *Bacillus* spp. increased the lignin production and activated defense mechanisms and decreased the CAT, SOD, and guaiacol peroxidase level [46]. It was observed that ZnO NPs change into zinc ions which is necessary for metabolic enzymes [52]. Ascorbate peroxidases (APX) scavenge the harmful effect of H_2O_2 in the plant by utilizing the ascorbic acid and maintain the ROS of chloroplast and other organelles. These results showed similarity with findings of other report [49], who observed increased content of APX in *Allium cepa* and rice plants at higher concentrations of metals. It was reported that *Wolffia arrhiza* and *Talinum triangulare* plant roots under Pb and Cd stress showed enhanced activities of reduced nicotinamide adenine dinucleotide (NADH), CAT, APX, glutathione, and ascorbate [49]. Bacteria reduced the level of HMs from water by increasing the level of APX and reducing the oxidative stress while ZnO nanoparticles remediate metals from water and lower the antioxidative enzymes. These findings are confirmed by other studies [39] that bacterial treatments increased the plant germination in oxidative stress. ZnO NPs act as a cofactor for different enzymes used in metabolic mechanism in plants [10] It was also recently reported that higher concentrations of zinc oxide nanoparticles such as 50 and 100 mg/L in hydroponics culture cause the upregulation of genes encoding antioxidant enzymes [14]. The current research [32] reveals that Si and K may improve gladiolus growth by decreasing the oxidative stress and Cd uptake and by increasing the activity of antioxidant defense enzymes and the quantity of secondary metabolites and plant nutrition.

ROS generate hydroxyl radicals in the plant and produce lipid hydrogen peroxide by Fenton-like reaction and formed aldehydes (malondialdehyde) during stress condition. H_2O_2 content in plants binds with thiol-containing proteins and generates various signaling pathways, activating gene expression, cell cycle, and transcription of protein [39]. These findings are confirmed by other researchers [14], who revealed that under Cr stress the MDA content was increased in leaf and other parts of *Kandelia obovata* and *Acanthus ilicifolius* plant. The synergistic effect of bioprimed seeds with microbes along with ZnO NPs enhanced the growth of rice and lowered the MDA and H_2O_2 content by remediating the metals from media and enhanced the photosynthetic pigments. It was observed [53] that seed inoculation with *B. siamensis* removes the oxidative stress under Cd stress in wheat plants, by decreasing the MDA level, increasing the antioxidant enzyme activities and nitrogen and mineral nutrition by reducing the uptake of Cd metal and ultimately increasing the growth of plants. In plants, zinc prevents the cell organelles from ROS and acts as a defense system for cell organelles [9]. These results are

confirmed by other scientist [54],they revealed higher H_2O_2 content under HM stress. It was reported that priming of seeds with salicylic acid (SA) decreased the stress of metals [47]. Biopriming with microbes along ZnO nanoparticles treatments showed decreased H_2O_2 content by alleviating the stress from media. It was reported [1] that ZnO NPs remediate the toxic stress which reduced H_2O_2 content and enhanced the growth. However, melatonin combined with nitric oxide scavenger increased MDA and H_2O_2 and decreases antioxidative enzymes. Hydrogen peroxide accumulation and associated oxidative damages decreased metabolic and antioxidative changes during seed germination [49]. Similar findings were observed by Akhtar et al. [55]; they confirmed the importance of the combined effect of *Bacillus* spp. and ZnO NPs on protein and gene profiles of rice plants under HM stress.

5. Conclusions

Current findings revealed that HM-contaminated wastewater significantly lowered down the plant biomass, chlorophyll content, soluble sugar, and protein contents and enhanced antioxidative metabolism and stress biomarkers (proline and glycine betaine content). However, ZnO NPs and bacteria individually had low potential to enhance the biochemical aspects of plants in HM-contaminated water. Importantly, the combined impact of bacteria-nanoparticles enhanced the plant growth, chlorophyll contents, total soluble sugar, and protein (TSP). Moreover, the synergistic effect of both factors has lowered antioxidative parameters than the individual effect. Subsequently, the notion that ZnO NPs may be used at lowered content acting as nanofertilizer is helpful to enhance the metabolic and antioxidative activities in HM-contaminated water. It is highly recommended to check the effect of ZnO NPs with bacteria in field condition which are ultimately required for sustainable environmental protection. Further research is needed to elaborate the actual mechanism of toxicity in plants at the molecular level and to determine the different cell signaling pathways involved in the alleviation of HM stress in rice plants.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors have no potential conflicts of interest.

Supplementary Materials

Figure 1: synergistic effect of bacteria strains (Bacillus cereus and Lysinibacillus macroides) primed seeds along with ZnO NP (5 and 10 mg/L) treatments on shoot and root length in heavy metal-contaminated water. Figure 2: hydroponic culture experiment of plant grown from seeds primed with bacteria strains (Bacillus cereus and Lysinibacillus macroides) in 5 and 10 mg/L of ZnO NPs alone or in combination with heavy metal-contaminated water. *(Supplementary Materials)*

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