HEAVY METAL TOXICITY IN PLANTS: RECENT INSIGHTS ON PHYSIOLOGICAL AND MOLECULAR ASPECTS, VOLUME II

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HEAVY METAL TOXICITY IN PLANTS: RECENT INSIGHTS ON PHYSIOLOGICAL AND MOLECULAR ASPECTS, VOLUME II

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Editorial: Heavy metal toxicity in plants: Recent insights on physiological and molecular aspects, volume II

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Editorial on the Research Topic

Heavy metal toxicity in plants: Recent insights on physiological and molecular aspects, volume II

Metals with relatively high densities, atomic numbers, or atomic weights are regarded as heavy metals. Among them, some metals are considered essential nutrients (Fe, Co, Zn), or relatively harmless (Ru, Hg, In). However, they can be toxic in higher amounts. At higher levels, heavy metals have abilities to create severe toxic symptoms in crop plants, and therefore, their utilization and uptake are greatly controlled by the plant cells. It has been reported that both essential and non-essential metals produce common toxic symptoms on crop plants, such as chlorosis, low biomass accumulation, photosynthesis, inhibition of growth, and nutrient assimilation that ultimately cause plant death. To combat metal stress, plants have evolved different defense modifications viz., less uptake of metals, phytochelatins binding, and the activation of numerous antioxidants. The first strategy to combat heavy metals in plants is to activate their enzymatic (POD, SOD, APX, CAT, GR) and non-enzymatic (ASA, GSH) antioxidants. The second strategy is to activate their root exudates and cell wall. The third strategy is to utilize plants (phytoremediation) which can absorb the metal content from the soil at higher rates. Lastly, exogenous applications of growth regulators, nutrients, and organic amendments such as ALA, GABA, MeJA, AsA, NPs, MLE, and Se provide some kind of relief to crop plants to combat the hazardous effects of HMs. In a summary, all the amendments under metal/metalloid stress can help the plants to strengthen their defense mechanism and improve their growth in a metal toxic environment. Our Research Topic mainly focuses on plant abnormalities, like alteration in PG&D, nutrient unbalances, and enzyme dysfunctioning due the metal/metalloid toxicity. This Research Topic proved successful in collecting twelve research articles and two review articles based on the impacts of diverse HMs (Cd, Cr, Al, As, Hg) on the plants and alleviation with different organic and chemical amendments.

Cd-toxicity in plants: Its impact and alleviation

In plants, metal toxicity reduces the uptake and translocation of nutrients and water, and enhances oxidative damage, thus inhibiting plant growth. However, alleviation of different metal toxicity through different plant growth regulators, nutrient application, and organic amendments is an emerging technique. Thus, Yang et al. delineate the beneficial role of 5-aminolevulinic acid (5-ALA) to reduce Cd-toxicity in *Brassica pekinensis* L. In their hydroponic experiment, they prove that emerging plant growth regulator 5-ALA lowers the Cd stress by maintaining redox homeostasis, strengthening the photosynthetic machinery, and improving the expression of different genes related to Cd transport in *B. pekinensis* seedlings. Several studies have shown that the application of methyl Jasmonate (MeJA) induces a stimulatory effect on photosynthetic machinery and thus enhances plant morphology and growth. Manzoor et al. find that the application of MeJA improved the plant growth parameters in different pea varieties under Cd toxicity conditions, such as greater fresh and dry biomass of shoots and roots. Further, enhancement in root biomass was more significant after the application of MeJA as compared to other plant parts. Therefore, the stress-alleviating role of MeJA is attributed to its role in photosynthesis and redox balance of pea plants under Cd stress conditions. Higher Cd absorption has been found higher in wheat (*Triticum aestivum* L.) compared to other cereal crops which can cause high daily Cd intake, thus huge impact on public health. In this context, a pot experiment by Farhat et al. is carried out on wheat seedlings exposed to Cd-stress to explore physio-morphological changes by two bio-stimulants, i.e., ascorbic acid and moringa leaf extract. The results show that Cd-deposition predominantly occurs in roots compared with shoots of wheat seedlings, which is reduced after the application of AsA and MLE. In conclusion, MLE proved to be more efficient to alleviate Cd-induced toxicity compared with AsA treatment.

Further, Cd has been found to interact with plants at physio-biochemical levels, thus resulting in reduced plant growth. Accordingly, Ramzan et al. conduct research to explore the potential of *Moringa oleifera* leaf extract and zinc oxide nanoparticles in alleviating Cd-toxicity in linseed plants. They find that the application of *MLE* and ZnO NPs reduces the negative effects of Cd stress in linseed seedlings by enhancing the contents of antioxidant enzymes. Further, it has been found that amendments like biochar and thiourea have the ability to reduce Cd stress and uptake in crop plants. Therefore, Haider et al. have designed a research trial to evaluate the impact of 1) biochar made of three maize stalks, and 2) three thiourea foliar application rates in alleviating the negative impacts of Cd on the plant growth in maize seedlings. Their study concludes that the application of biochar and thiourea significantly enhanced maize plant growth under Cd-stressed conditions by decreasing Cd concentration in different plant tissues.

Cr-toxicity in plants: Antioxidants and photosynthetic responses

Chromium (Cr) toxicity in plants is a diverse phenomenon that decreases seed germination, reduces plant growth, inhibits enzymatic activities, and impairs photosynthetic machinery and oxidative imbalances. By keeping in mind the possible beneficial role of different bacteria and nanoparticles in reducing Cr stress in plants, Ma et al. have designed a pot trial to explore the beneficial role of cerium dioxide (CeO_2) nanoparticles and a bacteria named *Staphylococcus aureus* in alleviating the negative effects of Cr-toxicity in sunflower seedlings. The outcome of this study shows that CeO_2 nanoparticles and *S. aureus* application significantly ameliorated Cr-induced negative impacts in sunflower plants. In another study, Shah et al. attempt to explain the scarce information on the role of harzianopyridone (HZRP) in the alleviation of Cr stress in *Vigna radiata*. In this study, they primed HZRP at 1 and 2 ppm and find that the application of HZRP under Cr stress enhanced intercellular CO_2 concentration and photosynthetic rate with enhanced levels of antioxidant enzyme activities and reduced levels of chlorophyllase (Chlase) enzyme in *V. radiate*.

As and Hg-toxicity in plants: Impact on antioxidants, chlorophyll, and accumulation

Among all the pollutants, Hg and As are known to be among the top five key dangerous metals/metalloids (Hg, As, Cu, Pb, Cd). Though many amendments have been suggested for coping with metalloid toxicity, they are not practically suitable in crop production due to time taking, high cost, or operational complexity. However, the utilization of nanoparticles to potentially reduce metal toxicity in plants has increased in recent times. Therefore, an *in vitro* trial is conducted by Emamverdian et al. to test different levels of ZnO-NPs both alone and in combination with As and Hg in *Pleioblastus pygmaeus*. They find that the application of ZnO-NPs increased antioxidant activities, glycine betaine content, chlorophyll attributes, and eventually plant growth under As and Hg stress in *Pleioblastus pygmaeus.* Further, Nazir et al. have designed a study to investigate the positive role of CaO NPs in reducing As toxicity in two genotypes of barley differing in As tolerance. They state that CaO NPs application under As toxicity significantly enhanced Ca uptake, lowered ROS contents, and reduced As uptake and transportation.

Al and Pb-toxicity in plants: Impact on plant growth, ROS and transcriptome sequencing

Heavy metal toxicities, i.e., Pb and Al, have been found to cause inhibition in ATP production and enhance ROS and DNA damage. In addition, both metals significantly inhibit germination, root elongation, plant growth, and chlorophyll production, and disturbs transcriptome sequencing. In this context, Ashraf et al. conduct a research trial to evaluate the positive role of exogenously applied gamma-aminobutyric acid (GABA) in modulating the growth and physio-biochemical mechanisms in two genotypes of aromatic rice under Pb stress conditions. They suggest that the application of GABA in rice decreased Pb contents in both shoots and grains, and lowered ROS by enhancing antioxidant enzyme activities compared with Pb-exposed plants. Further, they also find that cultivar GXZ exhibited better performance than NX-18 under Pb-stressed conditions. In recent times, ultrasonic treatment has become an effective strategy to cope with stress under heavy metal toxicity. The study by Bao et al. consists of two treatments, control and ultrasonic seed treatment, in peanut plants. Both treatments were applied with $AlCl_3$ in hydroponic conditions. The results depict that plant growth increased significantly with ultrasonic treatment under Al stress. Moreover, a transcriptome study explains that plant signal hormone transduction and transcription factors are enriched significantly in the differentially expressed genes in ultrasonic treatment under Al-stress conditions.

Se supplementation to enhance crop abiotic stress tolerance

Chemical-assisted approaches, i.e. the application of different essential micronutrients like selenium (Se), is an emerging strategy to mitigate the adverse effects of heavy metals because of their biochemical nature. Hereby, Hasanuzzaman et al. present a review that describes the beneficial role of Se in improving plant stress tolerance under metals stress. They report the improvement in plant growth, and various physiological and biochemical attributes with the application of Se under metal stress. Further, they also suggest that photosynthetic attributes are enhanced by the supplementation of Se under metal stress.

Phytoremediation mechanisms of potentially toxic elements

The deposition of different toxic elements (Hg, Cd, Se, Cu, As, Pb, Cr) in nature draws significant negative impacts on plants and, ultimately, on humans. Different anthropogenic activities, e.g., gas industries, coal-burning power plants, and agricultural activities, are specific contributors that increase the amounts of toxic pollutants in the atmosphere. The review article by Alsafran et al. reviews different proteomic and molecular approaches to discover the mechanisms underlying plant stress tolerance and bioaccumulation of different pollutants within the plants. According to them, several researchers have elucidated the plants with high-stress tolerance, metal uptake, and metal deposition in different plant parts.

Response of ZnO NPS on developmental stages of *Phaseolus vulgaris*

Nowadays, extensive use of foliar-applied ZnO NPs has been found to down-regulate different plant growth mechanisms involving photosynthesis, chlorophyll synthesis, and nutrient uptake in crop plants. Similarly, Salehi et al. foliarly applied the ZnO NPs on bean (*Phaseolus vulgaris* L.) plants and find negative effects from ZnO NPs on the differential mechanism involved in the reproductive stage of the plants compared with the salt form.

Author contributions

BA and RG wrote the first draft of the article. Both authors contributed to the article and approved the submitted version.

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Selenium Supplementation and Crop Plant Tolerance to Metal/Metalloid Toxicity

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Selenium (Se) supplementation can restrict metal uptake by roots and translocation to shoots, which is one of the vital stress tolerance mechanisms. Selenium can also enhance cellular functions like membrane stability, mineral nutrition homeostasis, antioxidant response, photosynthesis, and thus improve plant growth and development under metal/metalloid stress. Metal/metalloid toxicity decreases crop productivity and uptake of metal/metalloid through food chain causes health hazards. Selenium has been recognized as an element essential for the functioning of the human physiology and is a beneficial element for plants. Low concentrations of Se can mitigate metal/metalloid toxicity in plants and improve tolerance in various ways. Selenium stimulates the biosynthesis of hormones for remodeling the root architecture that decreases metal uptake. Growth enhancing function of Se has been reported in a number of studies, which is the outcome of improvement of various physiological features. Photosynthesis has been improved by Se supplementation under metal/metalloid stress due to the prevention of pigment destruction, sustained enzymatic activity, improved stomatal function, and photosystem activity. By modulating the antioxidant defense system Se mitigates oxidative stress. Selenium improves the yield and quality of plants. However, excessive concentration of Se exerts toxic effects on plants. This review presents the role of Se for improving plant tolerance to metal/metalloid stress.

Keywords: abiotic stress, biofortification, heavy metals, phytoremediation, plant nutrition, oxidative stress, trace elements, xenobiotics

INTRODUCTION

As plants and environments are intensively connected, they face multifaceted stresses due to their sessile nature. Due to rapid industrialization and agricultural development, plenty of wastewater, fertilizers, and pesticides are discharged that result in toxic metal/metalloid contamination of soil and other environmental components (Feng et al., 2020). Exposure of plants to various metals including cadmium (Cd), chromium (Cr), lead (Pb), arsenic (As), copper (Cu), nickel (Ni), iron (Fe), mercury (Hg), antimony (Sb) etc. causes the alteration of physiological and biochemical processes by higher metal uptake and translocation, thereby hampering the normal growth and development of the plant. In addition, these unrest the cellular metabolic functions by binding with proteins, nucleic acids, and enzymes (Hasanuzzaman et al., 2019). The obvious effect of metal stress is the stimulation of reactive oxygen species (ROS) generation, which causes the oxidative stress by damaging cellular macromolecules including lipids, protein, and DNA. The ultimate extreme levels of metal/metalloid can lead to plant death (Hasanuzzaman et al., 2020). Contrarily, plants possess the defense tactics for keeping ROS at a nontoxic level to regulate its signaling roles through involving non-enzymatic and enzymatic antioxidants (Hasanuzzaman et al., 2019). Toxic metal contamination in the environment also causes threats to human health *via* the food chain (Feng et al., 2020).

Therefore, much attention should be given for inducing higher metal stress tolerances of plants for increasing worldwide crop production along with reducing health hazard. For aiming this, thirst of researchers is to develop technology or management practice, which will help to reduce metal uptake in plants in contaminated soil. Consequently, the use of chemical approaches, especially essential micronutrients like selenium (Se), has become one of the effective strategies to mitigate the toxic effects of metals because of its biochemical functions (Wang et al., 2020). It is beneficial in regulating plant growth and development by alleviating environmental stress-induced damage when applied at a low concentration (Ismael et al., 2019). Particularly, Se supplementation can restrict metal absorption by roots and translocation to shoots, which is one of the vital metals/metalloid stress tolerance mechanism (Hasanuzzaman et al., 2020). Besides, Se-induced improvement in cellular functions and membrane stability, mineral nutrition with upregulation of antioxidants response, and metabolites function and also reduction of oxidative stress in plants has been widely explored against metal stresses (Gupta and Gupta, 2017; Zhao et al., 2019; Wang et al., 2020). Such roles of Se at low dose in plant growth as antioxidants, stress alleviators, and uptake inhibitor of metals, including Cd (Luo et al., 2019), Cr (Ulhassan et al., 2019), Pb (Wu et al., 2016), As (Shahid et al., 2019), Cu (Trevisan et al., 2011), Hg (Tran et al., 2018), etc. have been reported already. Additionally, lower Se concentration is able to stimulate the biosynthesis of hormone-like auxin, which causes the remodeling of root architecture with higher root growth for resulting in lower metal uptake (Feng et al., 2020). Supplementation of Se causes an increase of pectin and hemicellulose contents as well as thickness of cell wall, which enhances the binding of toxic metals by the cell wall (Zhao et al., 2019). Selenium can regulate the subcellular distribution of metals. Moreover, exogenous Se application in various major crops including rice, lettuce, cucumber, *Brassica,* etc. significantly has been reported to reduce metal accumulation with growth improvement, which also ensures better crop productivity and health benefits to consumers (Pandey and Gupta, 2018). It is also reported that, excessive concentration of Se is also toxic for plants by causing chlorosis, growth reduction, and even oxidative stress (Molnár et al., 2018; Hasanuzzaman et al., 2020); that is why it is imperative to emphasize selecting the optimal dose of Se. Therefore, extensive and broad scale research is still required for determining the crucial dose and engagements of Se for attaining plant tolerance toward metals. In this review, information on the potentiality of Se for reducing metal toxicity in plants through various strategies, including restriction of metal uptake, regulation of ROS metabolism, responses of antioxidants, and ion homeostasis, which are associated with the improvement of plant physiology, growth, development, and yield has been gathered, which will be supportive to get insight into Se-induced metal tolerance in plants.

PLANT RESPONSES TO METAL/METALLOID TOXICITY

All the metals/ metalloids, either essential or nonessential, produce toxic effects on plants, which result in poor biomass accumulation and stunted growth whenever they present above their threshold levels. In *Zea mays*, Cd ($CdSO_4$, 6 mg kg^{-1}) phytotoxicity decreased plant height and above ground fresh weight (FW) by 21 and 22% compared with control (Liu et al., 2018). Upon exposure to Cd stress, growth attributes of *Brassica oleracea* including root FW, root dry weight (DW), shoot FW, and shoot DW decreased by 14, 41, 27, and 53%, respectively over the control due to the accumulation of Cd in plant root and shoot (Shah et al., 2020). Combination of Cd (100 μM, $CdCl_2$) and As (150 μM, $NaAsO_2$) reduced plant height, leaf area, shoot FW, and shoot DW in two cultivar of *Z. mays*. Among two cultivars, Dong Dan 80 showed higher tolerance to Cd and As stress compared with Run Nong 35 (Anjum et al., 2017). In *Cicer arietinum*, reduction of plant height (by 46%), plant FW (by 90%), plant DW (by 89%), number of primary branches (by 80%), and secondary branches (by 84%) were observed under Cr (120 μM, $K_2Cr_2O_7$) over the control (Singh et al., 2020).

Metal toxicity led to several deleterious effects on physiological attributes of plants including chlorophyll (chl) functioning, stomatal conductance (g_s), net photosynthetic rate (P_n), intercellular CO_2 concentration (C_i), transpiration rate (T_r), and photosynthetic enzyme activities (**Table 1**). Shah et al. (2020) stated that water potential and leaf osmotic potential in *B. oleracea* decreased in plants subjected to Cd stress and resulted in a sharp decline of leaf relative water content (RWC) by 46% in comparison with control condition. Cadmium toxicity along with leaf-Fe deficiency affects photosynthetic electron transport and reduced CO_2 fixation, maximal and actual efficiency of photosystem II (PS II), chl synthesis, etc. (Lešková et al., 2017). Reduction of C_i (by 20.33%), g_s (by 39%), and T_r (by 10%) under Cd stress was observed in *B. oleracea* (Shah et al., 2020).

Toxic metals reduce CO_2 fixation by downregulating the activity of ribulose-1,5-bisphosphatecarboxylase-oxygenase (RuBisCO) or by reacting with its thiol group. In Cd-treated (100 and 200 μM $CdCl_2$) plants, chloroplast structure is damaged due to the increased production of small starch grain, enlarged osmiophilic globules which led to condensation of grana and lamella (Guo et al., 2016). Reduction of P_n, T_r, g_s, and chl synthesis was higher in combined application of Cd and As treatments in sensitive cultivar Dong Dan 80 compared

TABLE 1 | Influence of metal/metalloid toxicity on different plants.

Species	Metal/metalloid treatment	Major effects	References
Zea mays	Cd (3 and 6 mg kg^{-1} $CdSO_4$), 60 d	Reduced plant height, plant fresh weight (FW)	Liu et al., 2018
Brassica oleracea	Cd (5 mg L^{-1}), 28 d	Reduced growth attributes, photosynthetic pigments, intercellular CO_2 concentration (C_i), stomatal conductance (g_s) and transpiration rate (T_r)	Shah et al., 2020
Triticum aestivum	Cd (100 μM $CdCl_2$), 10 d	Declined leaf water potential, chlorophyll (chl) content and maximum quantum yield (F_v/F_m)	Kaya et al., 2019
Fragaria ananassa	Cd (80 mg L^{-1}, $CdCl_2$) 7 d	Decreased plant height and plant biomass, chl and carotenoid content, average fruit weight, vitamin C and soluble sugar content	Zhang et al., 2020
Z. mays	As (150 μM $NaAsO_2$), 15 d	Reduction of plant height, stem diameter, shoot FW, shoot DW, no. of leaves plant^{-1}, leaf area, net photosynthetic rate ($P_{n)}$), g_s and T_r; C_i, grain yield and 100 grain weight	Anjum et al., 2017
T. aestivum	As (50 and 100 ppm), 84 d	Reduced growth attributes, water use efficiency, C_i, g_s, T_r, chl, flavonoid and anthocyanin content	Ali and Perveen, 2020
Oryza sativa	As (25 μM Na_3AsO_4), 10 d	Restriction of plant growth with higher chlorosis, membrane damage, production of H_2O_2 and malondialdehyde (MDA)	Banerjee et al., 2020
Vicia faba	As (5 μM Na_3AsO_4), 27 d	Inhibition of chl biosynthesis with higher chlorophyllase activity, gas exchange parameters including P_n, g_s and C_i as well as elevated reactive oxygen species (ROS) accumulation	Siddiqui et al., 2020
V. faba	As (5 μM Na_3AsO_4), 30 d	Decrease of chl content with higher chlorophyllase activity, electrolyte leakage, ROS accumulation, nitrate reductase and nitrite reductase activity	Siddiqui et al., 2021
Solanum melongena	As (25 μM Na_3AsO_4), 17 d	Reduction in seedling fresh and dry weight, root growth and P uptake, F_v/F_m, photosynthesis with higher ROS and cell death	Alamri et al., 2021
C. arietinum	Cr (30, 60, 90, 120 μM, $K_2Cr_2O_7$), 72 h	Reduced germination, germination index, root length and shoot length	Singh et al., 2020
B. Juncea	Ni (100, 200 and 400 μM $NiSO_4.6\ H_2O$), 7d	Reduced seed germination, root length, shoot length and seedling FW	Thakur and Sharma, 2016
O. sativa	Ni (50 and 200 μM $NiSO_4.6\ H_2O$), 9 d	Reduction of root length, shoot length, seedling FW and seedling DW and soluble protein	Rizwan et al., 2018
B. Juncea	Ni (50 and 100, 150 μM of $NiCl_2.6\ H_2O$), 14 d	Reduction of root length, shoot length, plant DW, leaf relative water content (RWC), total chl and carotenoid content, $T_{r,}$ C_i and increased g_s	Abd_Allah et al., 2019
Solanum melongena	Ni (100 mg kg^{-1} of $NiCl_2.6H_2O$), 14 d	Reduction of growth, leaf water status, pigment content, $g_{s,}$ ci and T_r	Shah et al., 2021
S. lycopersicum	Cu (10 and 100 mg kg^{-1} of $CuSO_4{\cdot}5H_2O$), 40 d	Alterations in root morphology, reduced chl content and photosynthetic capacity and stomata aperture	Nazir et al., 2019
O. sativa	Pb (1,200 mg kg^{-1} of $Pb(NO_3)_2$), 95 d	Reduction in pigments content, decreased filled grain percentage, grain yield and harvest index	Ashraf and Tang, 2017
T. aestivum	Pb (0.5 and 1 mM of $Pb(NO_3)_2$), 48 h	Reduction in plant height, FW and DW, leaf water status and pigment concentrations	Hasanuzzaman et al., 2018
Helianthus annuus	Pb (300, 600 and 900 mg kg^{-1}, $PbNO_3$), 105 d	Reduced root and shoot FW and DW, chl *a*, chl *b* and carotenoid content and yield plant^{-1}	Saleem et al., 2018
C. arietinum	Hg (15, and 30 μM) $HgCl_2$, 70 d	Reduction in root and shoot growth, reduced pigment concentration and leaf RWC	Ahmad et al., 2018
H. tuberosus	Hg (0.15, 1, 5 and 10 mg kg^{-1} of $HgCl_2$), 70 d	Delay in seedling time emergence, reduction of leaf area and growth and chl content	Lv et al., 2018
Allium sativum	Hg (3 mg L^{-1} $HgCl_2$), 185 d	Decreased growth attributes, P_n, g_s and T_r	Hu Y. et al., 2020

with more tolerant Run Nong 35 (Anjum et al., 2017). Nitrogen metabolism (N uptake, transfer, and assimilation) is also affected by toxic metals/metalloids due to the altered activity of N-assimilatory enzymes. Combined application of Cd (40 μM $CdCl_2$) and As (40 μM Na_2HAsO_4. $7H_2O$) in *Solanum tuberosum* significantly inhibited nitrate reductase (NR) and nitrite reductase (NiR) activities of leaves, roots, and stolons (Shahid et al., 2019). Metals also downregulated ammonia assimilation through reducing the activity of enzymes, namely glutamine, glutamate synthetase, and glutamate dehydrogenase (Hussain et al., 2020).

Under high metal/metalloids concentration, there is an increase in the synthesis of ROS such as superoxide ($O_2^{\bullet-}$) and hydroxyl ($OH^{\bullet}$) free radicals, or non-free radical species such as singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2). Methylglyoxal (MG) with a high cytotoxicity is also generated resulting in an imbalance in the antioxidant cell homeostasis. Consequently, the imbalance led to the lipids and protein oxidation, ion leakage, DNA injuries, and even the programmed cell death (Syed et al., 2018).

The exposure of plants to metal/metalloids causes oxidative stress which can affect the cellular homeostasis in different ways (i) inducing the generation of ROS, (ii) direct generation of ROS *via* Fenton like reactions and the Haber–Weiss cycle, and (iii) consumption of element of cellular redox homeostasis such as glutathione as direct chelator and/or as a precursor of phytochelatines (Sharma and Dietz, 2009). Metal/metalloids such as Cu, Cr, Fe, and Co are redox active, being able to generate ROS directly *via* Haber–Weiss and Fenton reactions, whereas Zn, Ni, Cd, Pb, and Al are non-redox reactive and participate in the synthesis of ROS through the reduction of glutathione content, promoting NADPH oxidase activity or with the initiation of calcium-dependent systems and participating in iron-mediated processes (**Figure 1**; Mahmud et al., 2018).

Metal/metalloid toxicity alters the physiological processes, which leads to stunted growth, reduced biomass, and yield loss. In *Triticum aestivum*, stress resulted in significant reduction of grain yield plant^{-1}, no. of seeds plant^{-1}, and 100 grain weight (Ali and Perveen, 2020). Responses of *Oryza sativa* to Cd toxicity depends on the storage of Cd in root and translocation through the root to stem and grain, stress doses, and cultivars variation. Different Cd levels ($CdCl_2$; 50, 100 and 150 mg kg^{-1}) were applied on five cultivars of *O. sativa*, and their effects on yield were studied as compared with control where grain yield reduced by 34, 43, and 64% for Meixianzhang-2, 10, 31, and 39% for Xiangyaxianzhang; 11, 24, and 40% for Guixianzhang; 12, 23, and 31% for Basmati, and 29, 46, and 48% for Nongxian 18, respectively. Highest panicle numbers, spikelet panicle^{-1}, 1,000-grain weight, seed setting, and grain yield were found in Guixiangzhan, followed by Meixianzhang-2, whereas lowest values of these parameters were observed in Nongxian 18 and Xiangyaxiangzhan (Kanu et al., 2017). Quality attributes of *O. sativa* including brown rice rate, milled rice rate, milling degree, head rice rate, moisture content, protein content, and 2-acetyl-1-pyrroline content decreased, whereas the chalkiness rate and chalkiness degree increased significantly under Cd ($CdCl_2$, 100 mg kg^{-1}) stress due to the increased oxidative damage (Imran et al., 2021). Exposure to Cd stress (80 mg L^{-1}, $CdCl_2$) inhibited growth with increased accumulation of Cd in leaf and fruit of strawberry; thus, reducing average fruit weight, vitamin C, and soluble sugar content in the fruit (Zhang et al., 2020). Application of 120 μM $K_2Cr_2O_7$ in *C. arietinum* significantly reduced yield and different yield-contributing parameters, including number of pods plant^{-1} and number of seeds pod^{-1} (Singh et al., 2020).

PROTECTIVE ROLE OF SELENIUM IN PLANT

The progress in unveiling the protective roles of Se in plants shows that Se can act as a vital component for plant growth, development, and induce tolerance toward abiotic stresses in the era of climate change (Hasanuzzaman et al., 2020). Selenium is reported to play a vital role in mitigation of metal stress (**Figure 1**; Feng et al., 2013). Selenium protects plants from toxic metals by reducing their uptake and transportation from roots to upper plant parts like shoots, leaves, and even grain (Gao et al., 2018). The excessive production of ROS is reported to decrease with Se supplementation by boosting antioxidant defense system under stress (Hasanuzzaman et al., 2020). Moreover, soil or foliar Se treatment (50 g ha^{-1}) boosted chl formation and photosynthesis and also increased the antioxidant capacity as well as the yield of cowpea (*Vigna unguiculata* L. Walp.) plants (Silva et al., 2018). In another work, Se application under normal conditions to quinoa improved growth, photosynthesis, proline (Pro), antioxidants, and reduced the ROS production (Khalofah et al., 2021). Selenium application also reported to influence osmolytes under various abiotic stresses. For instance, Khan et al. (2015) observed a decline in Cd-induced oxidative stress in wheat *via* altering Pro and glutathione production. In another work, Regni et al. (2021) reported a stabilized level of Pro in the leaves and a reduced loss of this osmolyte from the roots under salt stress in olive plants. Selenium application to plants is also observed to enhance nutrient uptake in plants under abiotic stresses. For instance, in salt-stressed bread wheat, application of Se (8 μM) improved Ca and K concentration and improved salt stress tolerance (Desoky et al., 2021). Selenium nanoparticles are also reported to play an important role in ameliorating the Cd-induced oxidative stress in *B. napus* (Qi et al., 2021). However, the high doses of Se causes phytotoxicity symptoms, such as cell plasma membrane disruption, chlorosis, senescence, and reduced yield (Ribeiro et al., 2016; Silva et al., 2018).

ROLE OF SE IN METAL/METALLOID TOLERANCE IN PLANTS

Se Minimizes Metal/Metalloids Uptake

Selenium causes the restriction of toxic metal ions uptake and their metabolism by rescuing their translocation from lower plant to upper parts (Alyemeni et al., 2018; Hasanuzzaman et al., 2020). For instance, such Se-induced lower Cd uptake by roots had been reported in Cd-stressed tomato seedlings along with reduced translocation into shoots and leaves (Alyemeni et al., 2018). Not only that, Se alters the translocation ability of cultivar through

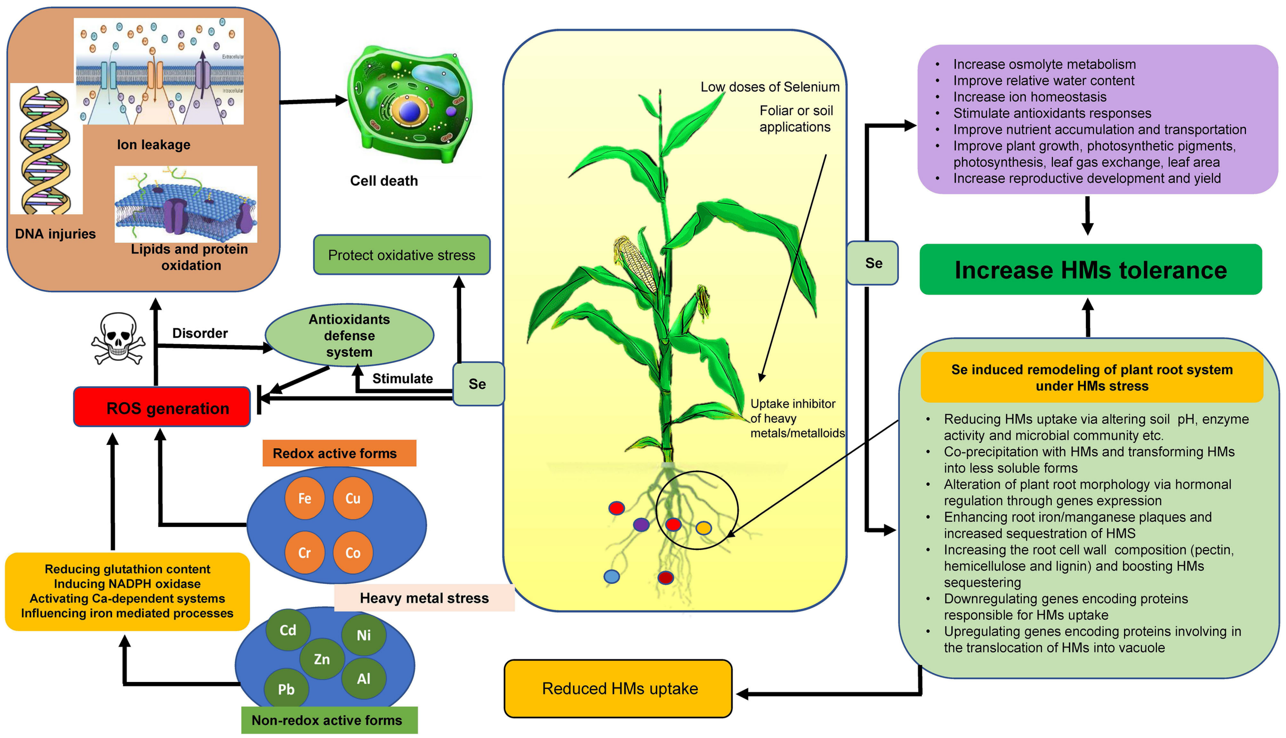

FIGURE 1 | ROS generation under heavy metal stress and protective roles of selenium.

lowering root to stem and stem to grain along with increasing stem to leaves translocation of different rice cultivars (Gao et al., 2018). Supplemented Se is able to enhance the binding of metal ions to the root cell wall by improving pectin and hemicellulose, and also changes the subcellular distribution in roots, for example metal contents are reduced in plastids and mitochondria, whereas enhanced in vacuolar sap and ribosome (Zhao et al., 2019). Moreover, protective role of Se is also involved in improving phytochelatin (PC) synthesis or nontoxic Se-metal complexes formation as well as reducing metals transportation from root to aerial plant parts for alleviating metal toxicity (Hawrylak-Nowak et al., 2014).

In *B. campestris* sp. *Pekinensis*, exogenous application of Se reduced the concentration and influx of Cr by altering root morphology (Zhao et al., 2019). Ulhassan et al. (2019) observed a decrease in Cr accumulation in plant tissues with the application of Se in *B. napus*. Hawrylak-Nowak et al. (2014) reported decrease in Cd accumulation and phytochelatins in roots of cucumber and an increased translocation toward shoot in Se-treated plants. Conversely, some studies have demonstrated that Se supplementation has no significant impact in the accumulation of Pb in the bean grown under field conditions (Mroczek-Zdyrska and Wójcik, 2012; Mroczek-Zdyrska et al., 2017). Pokhrel et al. (2020) reported a decrease in As uptake in rice. Se application influences the uptake of heavy metals in a time-dependent manner. For instance, in rice grown under Cd stress, supplementation of Se enhanced the uptake of Cd, but decreased this uptake on longer Cd exposure (Wan et al., 2016). Further Se-induced decrease in heavy metal uptake in plant varies even among the cultivars of the plant species. Gao et al. (2018) evaluated the influence of Si and Se individually and in combination on the uptake of Cd in three rice cultivars namely WYHZ, NJ5055, and ZF1Y. They observed that all the three forms of protectants applications (Si, Se, and Si + Se) decreased the uptake of Cd in WYHZ, but such influence was not noted in NJ5055 and ZF1Y.

Se Improves Plant Growth and Physiology Under Metal/Metalloid Toxicity

Selenium plays a crucial role in the protection of plants from the toxic impacts of metals/metalloids (Feng et al., 2020; Riaz et al., 2021). It is a common phenomenon that plants under the exposure of metals/metalloids tend to reduce their growth. A number of studies have unveiled that Se in different forms applied at different levels to crop plants grown under heavy metals/metalloids stress boost plant growth and improve physiological functions. Hawrylak-Nowak and Matraszek-Gawron (2020) evaluated the influence of 2 or 6 μM of different forms of Se (selenite IV and selenate VI applied as Na_2SeO_3 or Na_2SeO_4) on Ni (5 and 10 μM)-exposed lettuce plants. The authors observed that Se (IV) at 2 or 6 μM levels caused a reduction in leaf area, shoot (34 and 72%), and root (39 and 73%) FW under 5 μM Ni, whereas a decrease of 82–84% in shoot and root FW was observed in 6 μM Se (IV)-supplemented plants grown under 10 μM Ni. Contrary to this, 2 μM Se(VI) did not influence leaf area and biomass but improved root biomass (23%) under 10 μM Ni stress. Regarding physiological impacts, application of Se (IV) and (VI) improved photosynthetic pigments, except 6 μM Se (IV) (Hawrylak-Nowak and Matraszek-Gawron, 2020). In *B. juncea,* application of selenite (Se IV, 50 μM) not only enhanced germination by improving germination rate and seedling vigor, but also improved growth by reducing As-induced (As III, 300 μM) adverse effects in shoot and root length, fresh and dry weight by about 9, 12, 5, and 9%, respectively with improvement of root/shoot ratio by 9% under (Sahay et al., 2020). In another work, Handa et al. (2019) reported that in *B. juncea* grown under Cr (300 μM kg^{-1}), application of Se (4 μM kg^{-1}) recovered root and shoot length by 44 and 18%, respectively. Nano-Se is also reported to provide a relieving effect to crop plants grown in contaminated soils. For instance, supplementation of nano-Se to rice plants grown under Pb and Cd contaminated soil improved plant growth and photosynthesis and its related genes, proteins, and chl concentration (Wang et al., 2021). Alves et al. (2020) also reported that application of 1.0 μM of selenite or selenate resulted in an increase in photosynthesis and biomass of tomato grown under Cd stress (0.5 mM $CdCl_2$). In wheat, combined application of Se and Zn (10, 20, and 40 mg L^{-1}) alleviated the Cd-induced reduction in growth, photosynthesis, and photosynthetic pigments (Wu et al., 2020). Han et al. (2015) reported an increase in growth of *Nicotiana tabacum* under As stress (0–5 mg L^{-1}) supplemented with selenite ($\leq$ 1 mg L^{-1}), but it was inhibited under high Se and As (5 mg L^{-1}). In another work, Se (IV; 10 μM) boosted the growth of primary roots, but reduced the growth of lateral roots. Moreover, in the same work, Se application downregulated the expressions of genes of auxin and ethylene biosynthesis, and reduced the levels of these hormones in rice roots (Malheiros et al., 2019). Moulick et al. (2016) evaluated the influence of Se-based seed priming in rice under As stress and reported improved germination by 9%, and shoot-, root-length, and plant biomass by 1.6, 1.3, and 1.4-fold, respectively compared with non-treated As stressed plants.

Alves et al. (2020) demonstrated that Se improved photosynthesis and caused alteration in anatomical traits. In *B. napus*, Se application induced reduction in phytotoxicity, resulting in enhanced growth, biomass, photosynthetic pigments, leaf gas exchange, and F_v/F_m (Ulhassan et al., 2019). Khan et al. (2015) reported a decrease in Cd-induced oxidative stress following the application of Se in wheat plants. Sun et al. (2016) demonstrated that Se application caused a reduction in Cd-induced phytotoxic effects on cucumber plants by regulating stress response related proteins and pathways, such as glycolysis pathway and nitrate assimilation pathway (i.e., fructose bisphosphate aldolase 2, NiR), which may increase Se-induced Cd tolerance. Chauhan et al. (2020) reported a boosted activity of glutathione *S*-transferases (GST), peroxiredoxin, glutaredoxins, and heat shock proteins resulting in amelioration of As-induced oxidative stress in rice. In another work, combined application of 24-epibrassinolide (EBL) and Se under Cu stress ameliorated the negative impacts of Cu in *B. juncea via* altering Pro metabolism with increasing Pro accumulation (87%) through stimulating proline synthesizing enzymatic activity of pyrroline-5-carboxylate synthase (Yusuf et al., 2016). In addition, foliar application of both Se and EBL caused the higher activities of RuBisCO and carbonic anhydrase and also improved net photosynthetic rate and stomatal conductance under Cu toxicity.

Se Improves Ion Homeostasis Under Metal/Metalloid Toxicity

Although studies related to ion homeostasis in response to Se application under heavy metal stress are available, but the available ones have shown positive results regarding said trait in plants. For instance, Zhao et al. (2019) examined the impact of Se supplementation in in *B. campestris* sp. *Pekinensis* grown under Cr stress and observed that 0.1 mg L^{-1} of Se-induced enhanced uptake of nutrients including Na by 40%, Ca by 32%, Fe by 63%, Mn by 21%, Cu by 24%, and Zn by 30% under 1 mg L^{-1} of Cr stress due to alterations in root morphology. In another work, Ulhassan et al. (2019) tested the effect of 5 and 10 μM of Se supplementation on 100 and 200 μM of Cr in *B. napus*. The authors noted a decline in major (N, P, K) and micronutrients (Zn, Fe, Mn) uptake under Cr stress. Application of Se (5 and 10 μM) notably improved the mineral transportation under Cr contamination condition which was restricted in only stress condition (Ulhassan et al., 2019). The above discussed studies demonstrates the positive impact of Se application on ion homeostasis under heavy metals stress (**Table 1**). However, more research is needed to unveil the mechanisms involved in Se induced maintaining of ions homeostasis in plants.

Se Enhances Antioxidant Defense Under Metal/Metalloid Toxicity

The Se application in stressed plants results in a decrease in ROS *viz.* $OH^{\bullet}$, $O_2^{\bullet-}$, and H_2O_2 levels, which protects the plants from oxidative stress (Hasanuzzaman et al., 2020). One of the main functions of Se is the enhancement of GPX activity because selenocysteine is present at the catalytic site of this enzyme. Under metal/metalloids stress, plants suffer several damages in different organelles like chloroplast, which suffer modifications in their shape and organization. Nevertheless, the supply of appropriate levels of Se reduces the level of damages in chloroplast and enhances the photosynthetic process, which has been disrupted (Feng et al., 2013). There are several works reporting the positive effect of Se application in the photosynthetic apparatus (Chauhan et al., 2017), which can be associated with a higher capacity of Fe uptake and which have a crucial role in the electron transport chain (ETC), resulting in the generation of substrates to remain organized under high excitations of electronic levels (Feng et al., 2013). Moreover, Se is involved in a higher synthesis of PC or the generation of nontoxic Se metal complexes, which are responsible for the reduction of metals/metalloids resulting in toxicity (Hawrylak-Nowak et al., 2014). The Se application also has a positive effect in DNA structure avoiding methylation changes, which occurs in species grown under metal stress conditions (**Figure 2**; Filek et al., 2008).

Ulhassan et al. (2019) noted a reduction in the generation of ROS species such as H_2O_2, $O_2^{\bullet-}$, and MDA, a higher membrane integrity capacity associated with the increase of

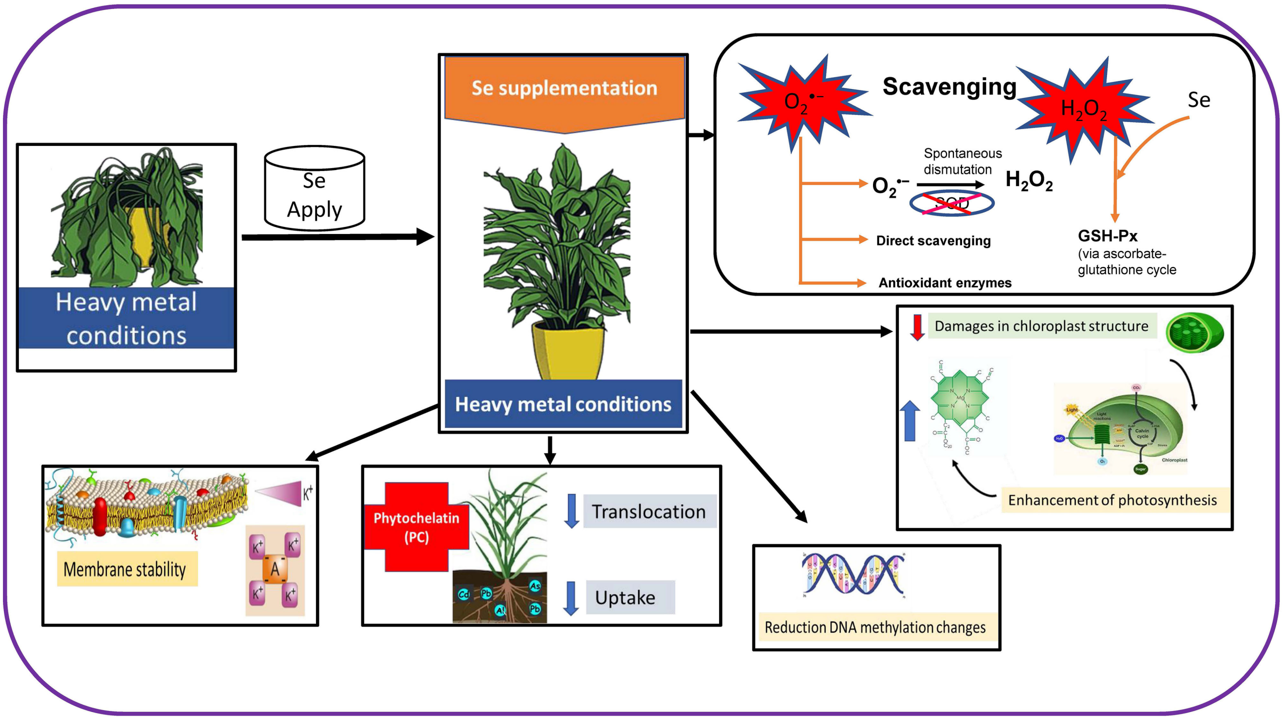

FIGURE 2 | Effects of Se supplementation in plants under heavy metal conditions.

GSH and AsA levels, and lower activities of SOD and CAT and enzymes involved in AsA–GSH cycle (ascorbate peroxidase, APX; monodehydroascorbate reductase, MDHAR; dehydroascorbate reductase, DHAR; and glutathione reductase, GR) under Cr stress. Moreover, the application of Se also resulted in a reduction of glyoxalase I (Gly I) and glyoxalase II (Gly II) enzyme activities, and consequently in a decline of MG contents. Hawrylak-Nowak et al. (2014) reported decrease in MDA in Se-treated cucumber plants. Qutab et al. (2017) demonstrated that Se supplementation to root system reduced Cd stress triggering a decrease in protein concentrations in the roots of maize exposed to different Cd levels. Sun et al. (2013) found that the application of exogenous Se reduced the toxicity to the different Cd levels. Similarly, in Cd-stressed rice, supply of Se significantly reduced Cd accumulation in rice grains (Hu et al., 2013). Wu et al. (2020) investigated the combined impact of Se and Zn on wheat grown under Cd stress. They observed an increase in photosynthesis and antioxidant enzyme activity, but a decrease in MDA in plant tissues. Zhang et al. (2019) discerned the interaction of different sites with natural presence of Se and Cd and reported that the high ratio of Se to Cd in soil has a beneficial effect on mitigation of Cd stress and its uptake in maize plant.

Reviewing previous literature, there are several references focused on the ameliorative effects of the supply of Se on the antioxidant defense of different species grown under metal/metalloid toxicity (**Table 2**). The effects of increasing supplies of Se as Na_2SeO_4 amendments (0, 2, 4 and 6 $\mu M\ kg^{-1}$) in *B. juncea* plants were tested in a pot experiment with soils containing increasing Cr concentrations (0 and 300 $\mu M\ kg^{-1}$). The levels of H_2O_2 and $O_2^{\bullet-}$ were reduced and there was a significant increase in SOD, CAT, MDHAR, DHAR, GR, and GPX activities as well as in non-enzymatic antioxidant *viz.* AsA and GSH due to the external supply of Se (Handa et al., 2019).

There are also studies about the ameliorative effects of Se in several species under Cd stress. The foliar spraying of Se (Na_2SeO_4) in the different growth stages in wheat plants grown under Cd-contaminated soil resulted in higher biomass accumulation and antioxidant enzyme activity. Lipid peroxidation decreased whereas leaf SOD, POD, CAT, and APX increased significantly with the supplies of Se (Wu et al., 2020). Presoaking sunflower seeds with Se (5, 10, and 20 μM) resulted in a reduction of the oxidative damage associated with Cd (20 μM of Cd) toxicity. Presoaking with Se induced an enhancement of CAT, APX, and GR activities (Saidi et al., 2014). Two varieties of wheat (soft and durum) showed a reduction of Cd toxicity due to the external supply of Se *via* root and foliar, where a reduction of lipid peroxidation and an increase in SOD and POD activities were observed (Zhou et al., 2021).

Regarding As toxicity, Singh et al. (2018) assessed the effects of Se application in rice plants. The chemical analysis reported an antagonism between both nutrients reducing the uptake of As under increasing Se concentrations. This antagonism also resulted in lower values of MDA concentration as well as the consequent rise of antioxidant enzymes such as SOD, CAT, and APX. Similarly, in wheat plants the supply of 10 μM of selenate in the nutrient solution resulted in lower oxidative damage in

TABLE 2 | Effects of selenium application on the antioxidant defense in several species under metal/metalloid toxicity.

Species	Doses of selenium	Mode of application	Metal/metalloid doses	Antioxidant defense	References
Lycopersicum escullentum	0, 1, 5 and 10 μM of Na_2SeO_3	Diluted in hydroponic solution	Cd; 0.5 mM $CdCl_2$	Decrease in lipid peroxidation and reduction in SOD, CAT and GR activities	Alves et al., 2017
L. escullentum	10 μM of Na_2SeO_3	Diluted in hydroponic solution	Cd; 150 mg L^{-1} $CdSO_4{\cdot}8H_2O$	SOD, CAT, APX and GR activities increased	Alyemeni et al., 2018
Triticum aestivum	0.4 and 0.8 mg Se^{6+} kg^{-1} soil	Supplied in soil	Pb; 50 and 100 mg Pb^{2+} kg^{-1} soil	GR and GPX activities increased	Balakhnina and Nadezhkina, 2017
Brassica napus	0, 1, 5, 10, 15, 20 mg kg^{-1} soil	Supplied in the substrate	Pb; 300 or 500 mg kg^{-1}	SOD and GPX activities decreased	Wu et al., 2016
B. napus	50 and 100 μM as Na_2SeO_4	Diluted in hydroponic solution	Cd; 0.5 and 1.0 mM $CdCl_2$	APX, GR and GPX activities increased	Hasanuzzaman et al., 2012
Capsicum frutescence	0, 3 and 7 μM Na_2SeO_3	Diluted in hydroponic solution	Cd; 0.25 and 0.5 mM $CdCl_2$	POD and CAT activities increased	Shekari et al., 2017
Raphanus sativus	2, 4 and 8 mg L^{-1} Na_2SeO_3	Diluted in hydroponic solution	Cd; 5, and 10 mg L^{-1} Cd SO_4	Lipid peroxidation reduced and GPX, CAT, APX activities increased	Amirabad et al., 2020
Cucumis sativus	0, 5 or 10 μM as Na_2SeO_4	Diluted in hydroponic solution	Cd; 25 or 50 μM, as $CdCl_2{\cdot}2.5\ H_2O$	Lipid peroxidation reduction	Hawrylak-Nowak et al., 2014
Oryza sativa	(0.0, 5, 10, 25 and 50 μM Na_2SeO_3	Diluted in hydroponic solution	As; 25 μM sodium arsenite (Na_2HAsO_2)	APX, CAT and GPX activities increased	Kumar et al., 2014
R. sativus	1, 3, 6, 12, and 24 mg Na_2SeO_4 kg^{-1} soil	Supplied in the substrate	As; 30 mg As(III) kg^{-1} soil	APX, GR and DHAR activities increased	Hu L. et al., 2020
Phaseolus aureus	2.5, 5.0 μM Na_2SeO_4	Diluted in hydroponic solution	As; 2.5, 5.0, 10 μM	SOD, CAT, and GR activities increased	Malik et al., 2012
B. juncea	Se (50 μM)	Diluted in hydroponic solution	Cd; 100. and 200 mg L^{-1} $CdSO_4{\cdot}8H_2O$	SOD, APX and GR activities increased	Ahmad et al., 2016

seedlings exposed to increasing As concentrations. Selenium has improved the growth of wheat plants, thereby increasing the activity of APX as well as GPX and GR (Ghosh and Biswas, 2017). In case of rice plants, Pandey and Gupta (2018) reported a clear reduction in the accumulation of H_2O_2 as well as $O_2^{\bullet-}$ and the increase in the activity of several antioxidant enzymes (CAT, SOD, POD, and GR) with an exogenous supply of Se in rice plants under As stress.

The damages caused by Pb stress can be ameliorated by external supplies of Se. For instance, Mroczek-Zdyrska and Wójcik (2012) reported that the supply of Se at concentrations of 1.5 and 6 μM as sodium selenite in a Hoagland nutrient solution alleviated Pb toxicity (50 μM) in *V. faba* roots. The alleviation was based on the reduction of $O_2^{\bullet-}$ generation in the apical parts of roots and enhanced the total sulfhydryl content and GPX activity. Moreover, the supply at low dosage enhanced cell viability, whereas at high levels it improved both lipid peroxidation and cell membrane injury. Zhou et al. (2017) investigated the positive role of Se in alleviating Hg stress. The supplies of increasing concentrations of Se (1, 2, 3, and 4 μM) under 0.5 mg L^{-1} Hg stress resulted in enhanced antioxidant activities (SOD, POD, CAT, and APX). Consequently, there is an increase in the scavenging of ROS and a reduction in lipid peroxidation.

Dai et al. (2019) assessed the positive role of Se in *B. campestris* sp. *Pekinensis* grown under Zn stress. They worked with two treatments (0.5 mg kg^{-1} Se + 30 mg kg^{-1} Zn and 1.0 mg kg^{-1} Se + 30 mg kg^{-1} Zn), and the data obtained reported that the antioxidant enzyme activities increased (POD, SOD, CAT, APX, GR) as well as Pro concentration under Se supplies. Cartes et al. (2010) tested the effects of Se supplies in ryegrass grown under Al stress in hydroponic conditions. They applied 0.2 mM Al and six levels of Se ($Na_2SeO_3{\cdot}5H_2O$) (0, 1.0, 1.5, 2.0, 5.0, and 10 μM) for 20 days. Selenium application increased POD activity whereas SOD showed a significant decrease. Hawrylak-Nowak and Matraszek-Gawron (2020) tested if there were differences between selenite and selenate application in lettuce exposed to Ni stress for 14 days. The data reported that selenate had a higher ameliorative effect compared with selenite and also a dose-dependent response. Zhu and Ma (2018) studied the influence of sodium selenite (Na_2SeO_3) on physiological characteristics of wine grape seedlings under Cu stress in a greenhouse pot experiment. Selenium treatment (1.0 mg kg^{-1} soil) enhanced pigment concentration, POD, and CAT activities and reduced the level of lipid peroxidation.

Se-induced Yield Improvement in Plant Under Metal/Metalloid Toxicity

With respect to yield, only limited number of studies have demonstrated the yield potential under Se-supplemented heavy metal stressed crop plants. Shekari et al. (2019) evaluated the impact of Se supplemented at different growth stages on cucumber (*Cucumis sativus* L.) grown under Cd and Pb stress. The authors observed that 4 and 6 mg L^{-1} of Se application induced an increase in flowering, reduction in flowering time, improved female to male flowers ratio, and induced significant enhancement in fruit yield and production time under 60 mM Pb and 100 mM Cd stress compared with the non-treated heavy metals stressed plants (Shekari et al., 2019). In a pot experiment under greenhouse conditions, Huang et al. (2017) tested the effect of Se application in three rice cultivars (Zhongjiu A (female parent) and Huazhan R (male parent), and their F1 hybrid) grown under Cd stress and observed that Se enhanced dry weight of the male parent of rice grain and F1 hybrid in a concentration-dependent manner at the same level of Cd stress. Mozafariyan et al. (2014) examined the impact of Se (0, 3 or 7 μM) under Cd (0, 0.25 or 0.50 mM) on peppers. The authors noted that under Cd stress (0.25 mM), Se applications at 3 or 7 μM while at 0.5 mM Cd level, 3 μM of Se enhanced fruit yield per plant through increasing photosynthetic pigments and improving antioxidant (Mozafariyan et al., 2014).

CONCLUSION AND OUTLOOK

It is now quite obvious that metal toxicity is not only harmful for plant growth, development, and yield but also a threat for human health as metal enters the human body *via* the food chain. Therefore, consequences of heavy metal stress to agriculture and environment are very alarming and hence demands great attention to search and develop approaches for plant tolerance. Currently, exogenous Se application has been implemented as a remedial strategy for managing metal toxicity because of its beneficial functions. In this review, Se-induced mechanisms in inhibiting the metal stress in plant, including reduction of metal uptake, and translocation to aerial parts, redistribution of subcellular metal distribution such as chelation and compartmentalization, improvement in plant nutrition as well as recovering photosynthesis and maintenance of osmoregulation, are discussed. Upon exposure to metal stress, Se induced the upregulation of antioxidants responses, lowering ROS generation in plant along with strengthening of cell membrane stability. Use of Se for increasing yield under metal contamination has been also demonstrated, which resulted in better production in plants and thus provides new hope to increase food production under the threat of heavy metals. However, for practical utilization, it is imperative to test optimal range of Se for a specific plant species in a particular growing media as the optimal range of Se is quite narrow. The functionality of Se also varies with soil type, treatment time, method of application, and experimental conditions. Testing of all these factors for attaining the best potential of Se against heavy metals is still at an infancy stage and therefore requires future research attention, especially under field conditions. Research must also be executed from a safety view point, as broad scale Se application in agriculture sector, especially at high concentration, may cause toxicity in plants and animals or even higher intake of Se may induce diseases in humans (Rizwan et al., 2020). From the discussion, the positive impacts of Se for increasing plant tolerance under metal stress are obvious. Not only that, more researches in including the supplemental Se effects on food value needs to be executed by which human health hazardous issues could be minimized. In addition, the interactive effects of Se to other metabolites, plant nutrients, hormone, and signaling molecules should be emphasized by in-depth studies for getting best approaches in increasing plant tolerance to metal toxicity with the reduction of residual effects in food crops. However, in-depth studies considering Se-induced mechanisms in plant at molecular and physiological levels need to be investigated further in field condition for economically important plant species.

AUTHOR CONTRIBUTIONS

MH conceived the idea, prepared the outline of the manuscript, guided the writing process, revised, and edited the manuscript. All authors have collected the literature, written the manuscript draft, read, and agreed to the published version of the manuscript.

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Aerially Applied Zinc Oxide Nanoparticle Affects Reproductive Components and Seed Quality in Fully Grown Bean Plants (*Phaseolus vulgaris* L.)

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The development of reproductive components in plant species is susceptible to environmental stresses. The extensive application of zinc oxide nanoparticles (nZnO) in various agro-industrial processes has jeopardized the performance and functionality of plants. To understand the response of the developmental (gametogenesis and sporogenesis) processes to nanoparticles (NPs) exposure, the aerial application of nZnO and their ionic counterpart of $ZnSO_4$ at four different levels were examined on bean plants (*Phaseolus vulgaris*) before the flowering stage. To evaluate the mentioned processes, briefly, flowers in multiple sizes were fixed in paraffin, followed by sectioning and optical analysis. The possibility of alteration in reproductive cells was thoroughly analyzed using both light and electron microscopes. Overall, our results revealed the histological defects in male and female reproductive systems of mature plants depend on NPs levels. Furthermore, NPs caused tapetum abnormalities, aberrations in carbohydrate accumulation, and apoptosis. The nZnO induced abnormal alterations right after meiosis and partly hindered the microspore development, leading to infertile pollens. The seed yield and dry weight were reduced to 70 and 82% at 2,000 mg L^{-1} nZnO foliar exposure, respectively. The sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis pattern showed the increased expression of two proteins at the molecular weight of 28 and 42 kDa at various concentrations of nZnO and $ZnSO_4$. Overall, our results provided novel insights into the negative effect of nano-scaled Zn on the differential mechanism involved in the reproductive stage of the plants compared with salt form.

Keywords: reproductive components, pollen dysfunction, seed productivity, food quality, nanotechnology

INTRODUCTION

Life cycle of flowering plants (angiosperms) translates into two generations of sporophyte and gametophyte, in which a haploid gametophyte (i.e., pollen grain and embryo sac) is formed from a diploid sporophyte (Yadegari and Drews, 2004). Due to the increasing trend of environmental pollution, the intrusion of contaminants in the plant reproductive life cycle (i.e., generative phase and gametophyte) is inevitable (Zinn et al., 2010; De Storme and Geelen, 2014; Paupière et al., 2014; Salehi et al., 2021a). Overwhelming studies have reported that the sexual reproduction system and seed productivity are severely affected by environmental stresses (Zinn et al., 2010; De Storme and Geelen, 2014; Paupière et al., 2014; Salehi et al., 2021a). For example, severe reproductive effects including disproportionate time intervals of gametophyte development, sporophytic anomalies, and cellular damages of gametes have been reported under temperature stress (Zinn et al., 2010). These alterations are difficult to track due to the short course of gametogenesis and fertilization within sporophytic tissues. Therefore, there is a lack of knowledge in the developmental phase to understand the biological response of the plant reproductive system under normal and stress conditions.

In modern agricultural practices, nanotechnology advancement has developed as a revolutionary breakthrough in food science and engineering. Nanomaterials are vital components of many achievements in agricultural food safety. Nanoparticles (NPs) have immense potential to develop highly precise and effective crop cultivation techniques. Zinc oxide NPs (hereafter named nZnO) possess unique properties, such as high surface area and adsorption capacity, easy operation, and cost-effective production (Khan et al., 2019). The applications of nZnO have been found to stimulate plant growth, chlorophyll synthesis, and nutritional fortification. For instance, some recent studies have shown the beneficial effects of nZnO on seed germination, shoot, and root growth in different plants including maize (López-Moreno et al., 2017) and wheat (Awasthi et al., 2017). Another recent study showed that cilantro (*Coriandrum sativum* L.) grown in soil amended with nZnO (400 mg kg^{-1}) promotes defense responses through improved photosynthetic pigments such as chlorophyll and decreased lipid peroxidation contents (Pullagurala et al., 2018). Recently, Dimkpa et al. (2019) reported that nZnO (up to 5 mg kg^{-1}) mitigate the adverse effect of drought stress in sorghum (*Sorghum bicolor* L.), which improved the total potassium acquisition and enhanced grain productivity. However, the phytotoxicological effects of nZnO on mature plants are highly neglected in the literature (Chen et al., 2016; Jahan et al., 2018; Singh et al., 2018). Impacts of foliar application of NPs have demonstrated a positive correlation with applied concentrations. However, the presence of nZnO associated with the release of reactive oxygen species (ROS) is a likely reason for inhibitory effects on plant performance (Zhao et al., 2013; Bandyopadhyay et al., 2015; Wang et al., 2016). The extent of ROS release depends on several factors, including NPs size and surface charge, plant species, and exposure time (Marslin et al., 2017). The applied NPs concentrations may expose potent effects that dominate all other effects induced by different environmental factors or by triggering them. Therefore, we hypothesized that the perturbation of pollen and embryo sac cells is due to growth inhibition caused by oxidative stresses and the negative effects of NPs on the plant reproduction system. To our best knowledge, there is no study on the consequences of nZnO on the reproductive phase of plant development, mainly in bean. In this study, we applied nZnO at four different levels to elucidate their effects on the reproductive phase and seed productivity of bean (*Phaseolus vulgaris* L.) plants. The main objective of this study was to identify the potential impacts of nZnO on reproductive organs, including pollen and embryo sac.

MATERIALS AND METHODS

Materials

The nZnO (10–30 nm 99% purity) was purchased from Nanosany Company (US-Nano), Mashhad, Iran. The NPs were fully characterized using transmission electron microscopy (TEM) (100 kV Philips, EM208, Sigma-Aldrich, Germany) and scanning electron microscopy (SEM) (JSM-840A, JEOL, Tokyo, Japan) images. Briefly, the specific surface area ranged from 20 to 60 m^2 g^{-1} with a density of 5.6 g/cm^3. A detailed analysis of NPs physiochemical properties is provided in **Supplementary Figure 1**. Zinc sulfate ($ZnSO_4.7H_2O$) was supplied by Sigma-Aldrich (Milan, Italy). The formaldehyde, glacial acetic acid, ethanol, acetocarmine, acid fuchsin, and orange G used at analytical grade were purchased from Sigma-Aldrich (Heidelberg, Germany).

Experimental Design

Pinto bean (*P. vulgaris*) seeds were purchased from Behineh Sazane Sabze Mehregan Company (Biotek Seeds, Iran). Approximately 100 seeds were sterilized in 5% sodium hypochlorite for 15 min and germinated in moist sand. After 4 days of germination, healthy seedlings were transplanted in pots (25 cm × 30 cm) containing local soil (20% clay, 10.6% silt, 69.4% sand, 2.3% organic matter, and pH 7.6). The growth condition was controlled in a greenhouse under a 16/8 day-night cycle at 30/16°C, and the average humidity was 70%. Among 3-week-old plants (Biologische Bundesanstalt, Bundessortenamt und CHemische Industrie (BBCH) 13-15; phenological stage: 3–5 true leaves), healthy plants were selected for further nZnO foliar treatments.

Foliar Application of nZnO

Foliar solutions of nZnO were prepared in four concentrations (250, 500, 1,000, and 2,000 mg L^{-1}) in ultrapure water (18Ω). The nZnO concentrations were selected based on previous studies reporting contradictory findings of their function in terms of both phytotoxicity and positive effectiveness (Torabian et al., 2016; García-Gómez et al., 2018; García-López et al., 2019). The nZnO dispersion was subjected to 30 min sonic bath at 25°C to ensure the homogenous dispersion of the NPs. As a positive control for the nZnO, the zinc sulfate ($ZnSO_4$) solution (normally applied as a foliar spray to the crop) at the aforementioned levels was prepared. All pots were divided into four groups with five replicates for each concentration, and the soil surface was thoroughly covered with plastic films to avoid exposure of NPs. The suspensions of NPs were manually sprayed on the leaves every 2 days at 9 a.m. for 2 weeks. A total volume of 200 ml of each concentration of either nZnO or $ZnSO_4$ was applied foliar. Next, treatments were subjected to air drying, and the pots were kept in the greenhouse under the aforementioned conditions (16/8 day-night cycle at 30/16°C, and the average humidity of 70%). The irrigation with tap water took place every 2 days until the termination stage.

Sample Harvest and Developmental Studies

At the termination stage, flowers through the growth transformation from young buds to mature flowers and pods were carefully collected with a blade. Immediately, the samples were stabilized in formalin aceto-alcohol (FAA_{70} composed of 3% formaldehyde, glacial acetic acid, and 70% ethanol at 2:1:17 proportions, respectively) for 24 h and were rinsed with deionized water five times to remove residual of the FAA solution and stored in 70% ethanol for further developmental analysis. The plant tissues were subjected to hydration in exposure to ethanol gradual series (70, 80, 90, and two times in 100%) and finally toluene. The hydrated tissues were placed into liquid paraffin and incubated in an oven at 65°C for 3 days. The embedded tissues in paraffin were cut to slices of $\sim$8.5 μm thickness using a rotary microtome (Micro DS 4055, Germany) and then fixed onto glass slides. The combination of hematoxylin and eosine solutions were used for staining internal organelles (Chehregani Rad and Salehi, 2016). The prepared slides were quantified using the optical microscope (LABOMED model LX50, Germany), and dynamic changes in male and female gametophytes were captured using LABOMED digital camera (Germany). At least ten samples of each developmental stage per pot and over 100 experimental slides were observed to confirm the possible changes compared with controls.

Pollen Viability Assay

To investigate the pollen viability, pollen and anthers were stained with acetocarmine (1% in acetic acid) and Alexander stains. Briefly, acetocarmine stained the viable pollens with regular shapes in reddish color, whereas non-viable pollen was pale. Alexander stain contained a mixture of 10 ml 96% ethanol, 10 mg malachite green, 50 ml distilled water, 25 ml glycerol, 5 g phenol, 5 g chloral hydrate, 50 mg acid fuchsin, 5 mg orange G, and 4% glacial acetic acid. For sample preparation, the dissected flowers that were about to open were selected and all tissues were removed except for anthers and then were placed into a 20 μl volume of Alexander staining solution on a microscopic slide and incubated for 3 h. After staining with Alexander, red or pink colors represented the viable pollen while the green-pale ones were non-viable. In the case of pollen viability, at least 20 slides per treatment were monitored. For additional characteristics, SEM (JSM-840A, JEOL, Tokyo, Japan) observation was carried out using a gold coating of dried pollen grains.

Measuring the Seed-Related Traits and Zn Accumulation

The final measurements on seeds were performed at the end of the life cycle of bean plants (120 days after planting). Briefly, the number of pods for each cultivar, the total number of produced seeds per plant and per pod, and seed dry weight were evaluated at the termination stage. The total Zn content in seeds was also quantified using atomic absorption spectrometry (Varian Inc, Applied Science Co., Iran), following our previously published work (Salehi et al., 2021b). Dry seeds were gently washed with distilled water to remove any potential contamination. The oven-dried seeds were mechanically grounded using a grinding machine. Approximately 1 g subsample of each treatment was weighted for further acid digestion. A mixture of 65% HNO_3 and 20% H_2O_2 at 5:1 proportion was used to digest the samples at 60°C for about 6 h. Samples were allowed to cool down to room temperature, and then each sample was diluted to 50 ml with deionized water and filtered using Whatman No. 42 filter paper (2.5 μ M).

Total Protein Content and Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Bradford assay was used to determine total protein content (Bradford, 1976). Initially, seeds were grounded in liquid nitrogen until fully homogenized. The total protein was extracted using 25 mM phosphate buffer (KH_2PO_4) at 4°C. After centrifuge, the supernatants were used to measure protein concentration using UV/Vis Spectrometer (Biowave II, England) at 595 nm.

In addition, the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) pattern of bean seed proteins was performed using the extracted total protein. Briefly, the seeds were grounded using a mixer machine, and then total protein was extracted using sodium phosphate buffer (pH = 7) at 4°C. The Bradford standard assay was used to determine total protein content at 595 nm. The SDS-PAGE analysis was performed as follows: an equal proportion of buffer was loaded (0.125 M Tris-HCl, 4% SDS, 20% glycerol, 10% mercaptoethanol, and 0.1% bromophenol blue) and protein extract was boiled at 100°C for 5 min, and then loaded into 12% polyacrylamide gel holes, and finally, electrophoresis was run at 90 V (constant voltage) (Salehi et al., 2013). Coomassie Brilliant Blue R-250 (Sigma-Aldrich, Germany) was used for gel staining. To compare the molecular weight, an SDS-PAGE protein marker was also loaded (CinnaGen, Iran).

Statistical Analysis

Data on the pollen irregularity, seed weight, and seed yield were analyzed using a one-way ANOVA followed by the Duncan test at $p \leq 0.05$ using PASW Statistics 18.0 (SPSS Inc., Chicago, IL,

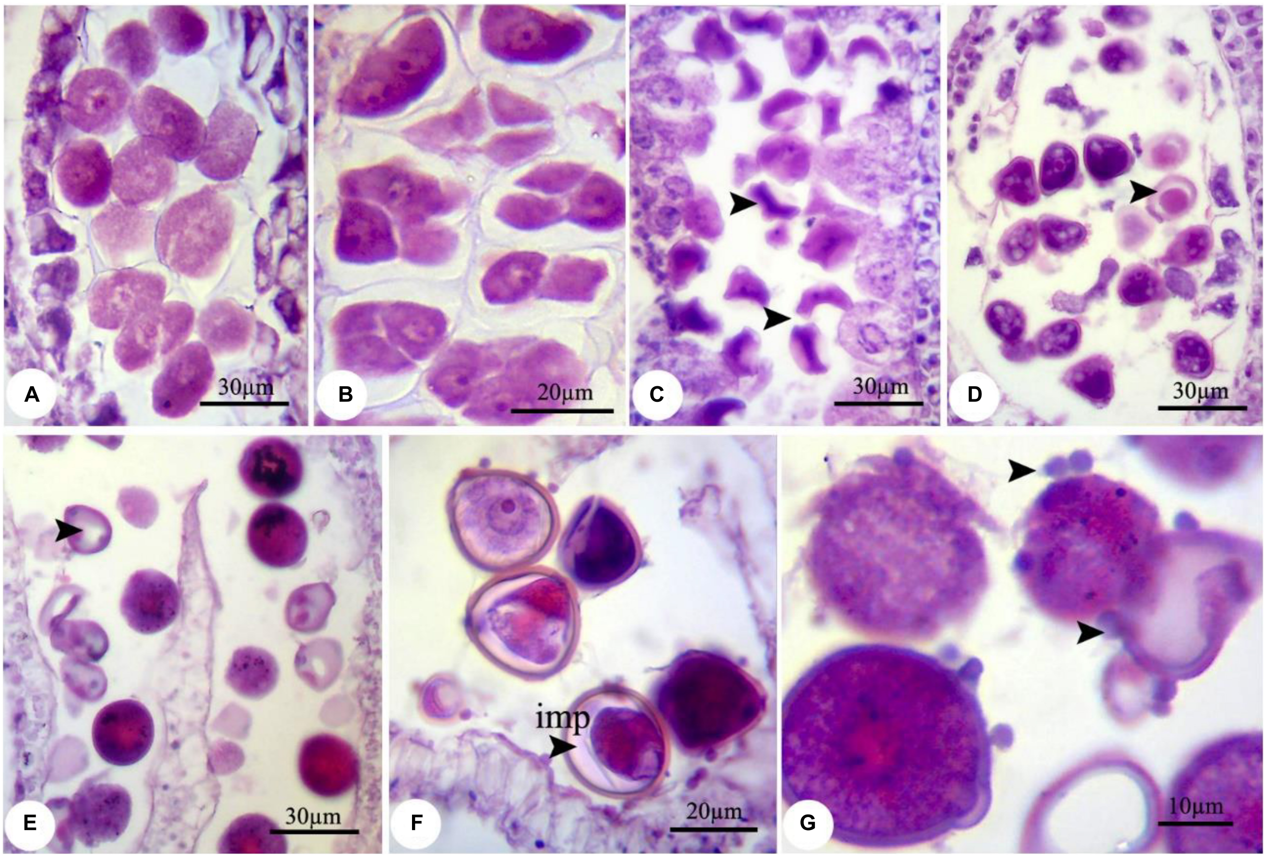

FIGURE 1 | Observed modifications of the microsporogenesis process in bean plants exposed to 250 mg L^{-1} nZnO. **(A)** Pollen mother cells in the prophase stage with varied sizes. **(B)** The irregular placing of microspores in tetrad and degenerating some of them. **(C)** Released microspores have unusual sizes (arrows), microspores, and cytoplasmic content have degenerated. **(D)** Pollen sac containing microspores with early degenerated cytoplasm. **(E)** Full cytoplasmic-degenerated and paled microspores (arrow). **(F,G)** Off-key microspores and emerging spherical bodies on exine surface of pollen grain wall (arrows). imp, immature pollen grain.

United States). The statistical differences between datasets of each treatment were processed by *post hoc* test on a mean basis. All experiments were performed in five replicates.

RESULTS

The Effect of nZnO and $ZnSO_4$ on the Male (Anther and Pollen Grain) Development

During the nZnO and $ZnSO_4$ exposure, no structural abnormalities were observed in anther shape and microsporocyte stage. However, from onset meiosis, unprecedented changes were realized. For example, reproductive structures were altered in both treatments, while the effects of nZnO were more prominent compared with other treatments; cellular dysfunction started at 250 mg L^{-1} of nZnO. The developmental stage was affected differently for each treatment depending on the applied doses. Under nZnO treatments, the initial phases of meiosis were affected, as at prophase I, the pollen mother cells (PMC) had various sizes, with some being degenerated (**Figure 1A**) compared with control plants (**Supplementary Figure 2**). Similarly, deformation of tetrads was observed at the tetrad stage, including the triads (languishing one of the cells) and irregular shapes (**Figure 1B**). In addition to tetrad irregularity, asynchronous in cytokinesis and wall formation in tetrads were visible. The released immature microspores were irregular and dominated by falciform or starlike (asteroid) (**Figure 1C**).

Despite the anther and microspore growth, the cytoplasmic content of microspores was degenerated (**Figures 1D,E**), leading to form amorphous and likely non-viable pollen grains, which were more evident at higher concentrations. There were some spherical, bubble-shaped bodies on the pollen grains surface, which seemed to be the cytoplasmic exudations (**Figures 1F,G**). At high concentrations (especially 2,000 mg L^{-1}), in most of the microspores, the nucleus and cytoplasm contents were plasmolyzed and agglomerated (**Figure 2A**). The other remarkable point was the premature disappearance of the tapetal cells in some pollen sacs (**Figure 2B**) or abnormally expanded and persisted tapetum cells in some others (**Figure 2A**), which may be leading to the disproportionate maturation of the microspores due to nutritional deficiency. Followed by pollen grains development, they were crushed and broken

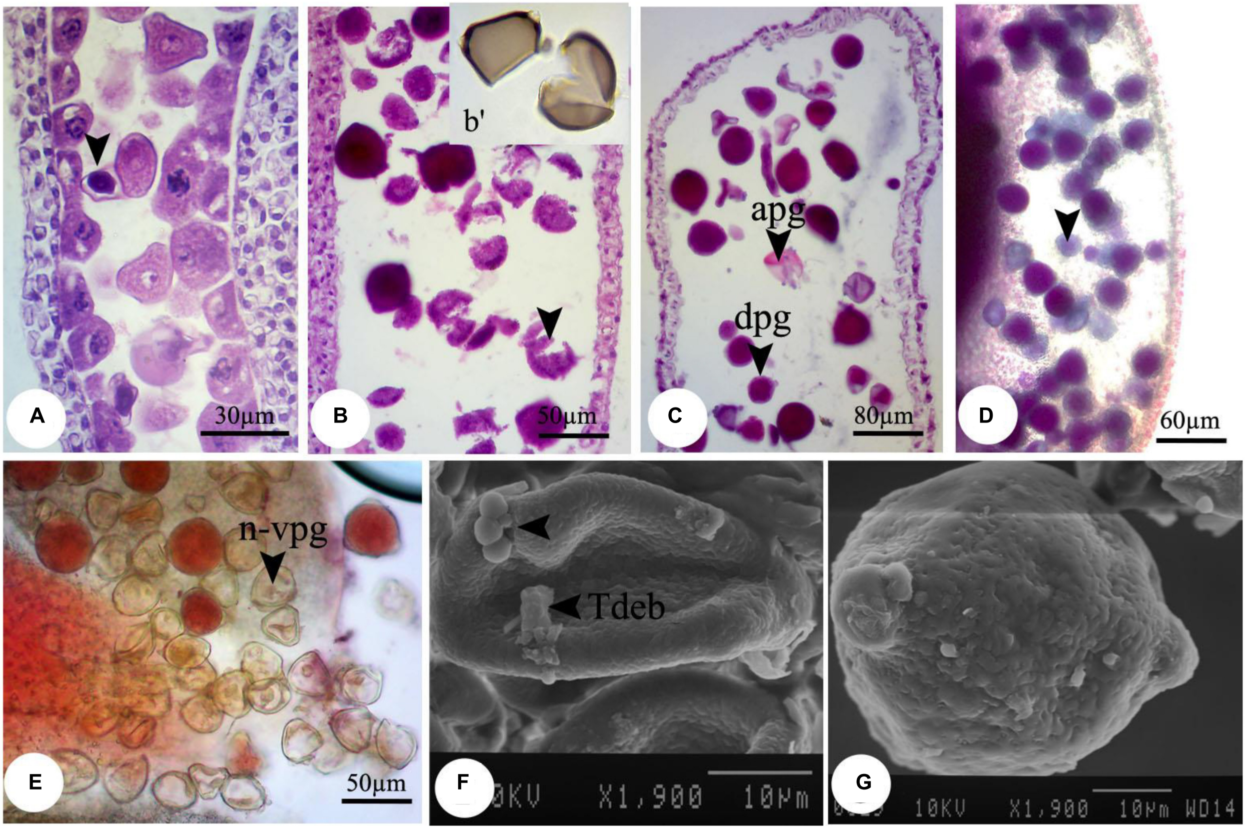

FIGURE 2 | Observed modifications of the microsporogenesis process in bean plants exposed to 2,000 mg L^{-1} nZnO. **(A)** Anther loculus contains immature microspores with different shapes; some have completely degenerated, and others have been crumpled and plasmolyzed (arrow). **(B,b′)** Brittle mature pollen grains. **(C)** Irregular mature pollen grains with different shapes. **(D)** Alexander staining of anther, viable pollen grains (arrow). **(E)** Stained pollen grains by acetocarmine. **(F)** SEM image of the immature pollen grain and cytoplasmic exudation on the pollen surface is observed. **(G)** Mature pollen grain with an uneven surface. apg, abrasived pollen grain; dpg, degenerated pollen grain; n-vpg, non-viable pollen grain; Tdeb, tapetum debris.

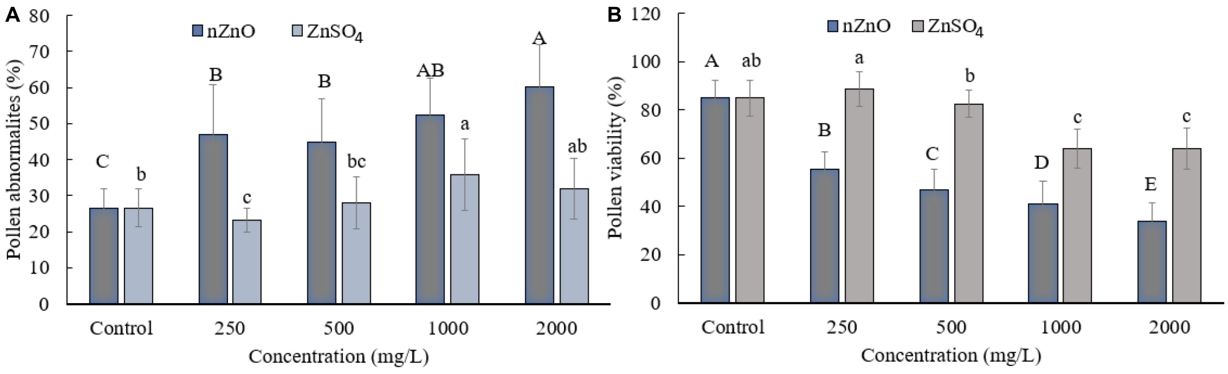

FIGURE 3 | Grain abnormality rate of pollen **(A)** and viability **(B)** at different concentrations of nZnO and $ZnSO_4$. The data are the average. Error bars represent mean ± SD. Different letters above the bar indicate the significant difference at $p < 0.05$.

(**Figure 2B**,b′), likely due to defects in wall formation, allowing the cytoplasmic exudations and probably producing non-functional pollen grains (**Figure 2C**).

Moreover, compared with control plants (**Supplementary Figure 3**), in the highest dosage of nZnO, pollen grain ornamentations and its surface were bumpy, and SEM images showed some spherical-shaped bodies on the surface (**Figures 2F,G**). Pollen viability was also considered to assess the male gametophyte developmental stage and particularly the fitness of reproductive organs. Staining with Alexander and

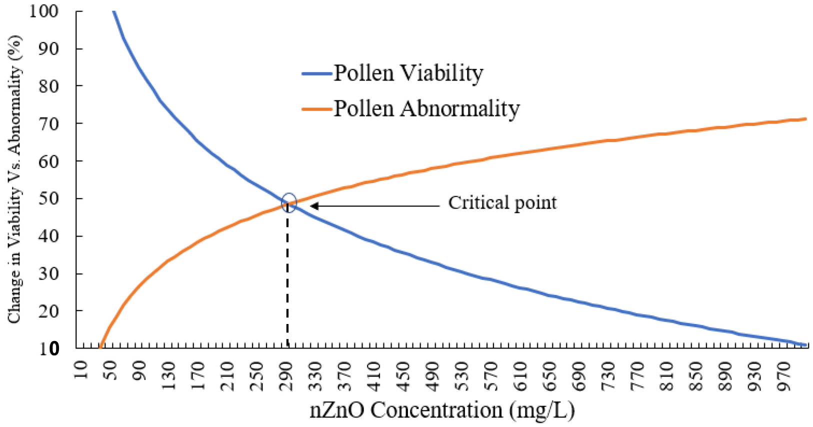

FIGURE 4 | The simulated behavior of both pollen viability and abnormality is based on experimental values. The critical points indicate the limiting doses of the nZnO that will lead to phytotoxicity effects.

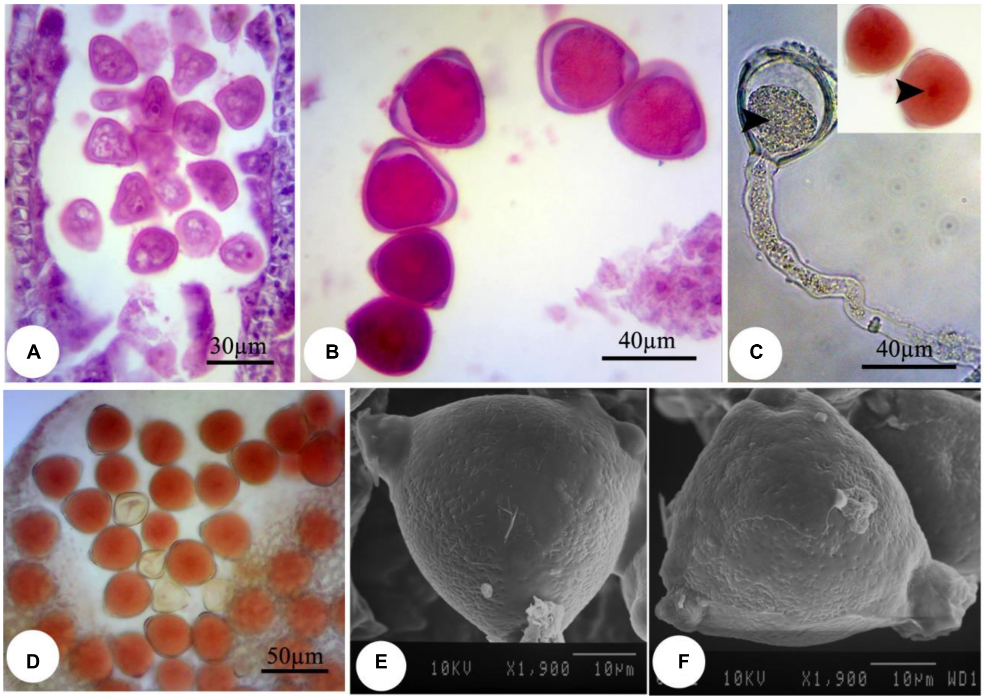

FIGURE 5 | Induced changes in the microsporogenesis process in bean plants exposed to 2,000 mg L^{-1} $ZnSO_4$. **(A)** Immature pollen grain. **(B)** Mature pollen grain. **(C)** Pollen grain along with pollen tube and its content. The central part of the pollen grain is more colorful (arrow). **(D)** Pollen grains staining with acetocarmine. **(E,F)** Scanning electron micrographs of the mature pollen grain.

acetocarmine showed most pollen grains have lost their coloring ability and were non-viable (**Figures 2D,E**) in comparison with pollen grains in control plants (**Supplementary Figure 3**). Interestingly, approximately 61% of mature pollen grains were found in irregular and abnormal structures when exposed to 2,000 mg L^{-1} of nZnO (**Figure 3**). **Figure 4** was derived from simulating the behavior of pollen abnormality and viability in exposure to a different range of nZnO.

The critical point can be seen in a range of 250–350 mg L^{-1} nZnO, which limits the pollen viability of the reproduction system. The pollen viability might be affected during different stages of the male development from early phases in anther to placing on the stigma. In this context, the low viability of pollens in nZnO treatment might be related to high dehydration and alternation in carbohydrates type and content.

Compared with nZnO, the effects of Zn ions on the deformation of male gametophyte development were insignificant (**Figures 5A–F**). However, further image analysis revealed that the cytoplasm tends to be more compressed in exposure to Zn ions compared with control plants, with a

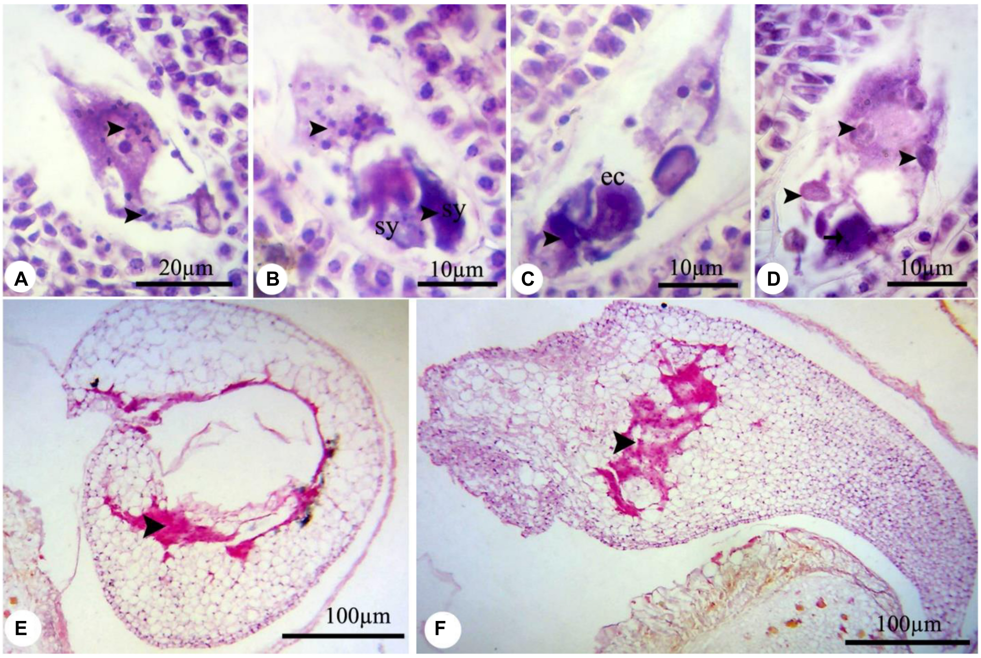

FIGURE 6 | The nZnO-induced modifications of *P. vulgaris* female gametophyte at different concentrations. **(A)** The apoptosis of egg apparatus (arrows). **(B)** Apoptosis of central cell (arrow) and degenerating of the nucleus, and excessive coloration of synergids. **(C,D)** Abnormal division of endosperm and degenerating of nuclei. **(E,F)** Destruction of embryonic tissue (arrow) and irregularity in placing of cells. sy, synergid; cc, central cell.

visible space between the cell wall and cytoplasmic content (**Figures 5B,C**). This observation was consistent with cellular acetocarmine staining (**Figures 5B–D**), which could be due to cytoplasm plasmolysis. However, no visible changes were observed in the pollen grains appearance compared with control plants using SEM images (**Figures 5E,F**).

Female (Ovule and Embryo Sac) Development Under nZnO and $ZnSO_4$ Exposure

In all treated plants, the female development responses were identical with the control plants (**Supplementary Figures 4–6**) until the functional megaspore division stage. The major alteration was observed at the eight-cell embryo sac stage. High concentrations of nZnO caused apoptosis of the oocyte apparatus (**Figure 6A**) and central cells (**Figure 6B**) as cellular pieces of degeneration processes were found in the embryo sac cytoplasm. Also, synergid cells seemed to bear serious damage in some ovules, resulting in their dysfunctionality (**Figure 6C**). Synergid cells govern pollen tube conducting, which such abiotic stresses may disturb this action and cause pollen grain infertility (Leshem et al., 2013). Furthermore, the induction of abnormalities in the endosperm division (**Figure 6D**) was noticed by dislocation and scattering of the nuclei of the nuclear endosperm in the cytoplasm. Compared with control plants (**Supplementary Figure 6**), embryo abortion occurred in most of the ovules at higher concentrations of nZnO (**Figures 6E,F**). Also, the interruption of embryonic tissue at different stages is likely due to cellular damage and fragility.

The Evaluation of Seed Productivity at the End of the Plant Growth Cycle

The seed-related parameters upon exposure to nZnO and $ZnSO_4$ are shown in **Table 1**. The corresponding images of pods and seeds are illustrated in **Supplementary Figure 6**. As shown in **Table 1**, both nZnO and Zn ions significantly altered the yield and weight of seeds ($P \leq 0.005$). As expected, in exposure to 2,000 mg L^{-1}, most of the ovules failed to transform into seeds. A negative correlation between the number of seeds and their productivity with nZnO levels was observed as approximately 70% of total seed reduction occurred at 2,000 mg L^{-1}, compared with control plants. Similarly, the weight of the total seeds was reduced by 82% at the highest nZnO level. Lower numbers of pods (6.60 compared with 13.3 in control plants) and affected seed physiology may be caused by smaller plant elongation and increased flower abscission rate during growth term. The microscopic images revealed that seed development and growth were inhibited at the early stages. Two mechanisms may be responsible for this phenomenon: first, the potential lack of successive divisions of endosperm as the embryonic nutritive tissue and, second, impairing the cellular tissue of the embryo. Notably, both mechanisms lead to the inhibition of the embryo and the development of cotyledons. It is worth mentioning that plants showed different responses to Zn ions treatment in the same concentrations. For example, exposure to 250 mg L^{-1} increased the number of pods per plant (by 12%), the number of seeds per plant (by 31%), and seed dry weight (by 22%) in comparison with control plants (**Table 1**). Respectively, the appearance of the seed improved, and the lowest mortality rate was recorded (**Figure 7**). However, at 2,000 mg L^{-1} $ZnSO_4$, the total yield and dry weight were insignificantly reduced by 12% and 11%, respectively.

The Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis Profile of Seeds

To investigate the effect of applied nZnO and Zn ions on protein content, the total protein was measured, followed by SDS-PAGE analysis. **Figure 8** shows a positive relationship between protein synthesis of the plant and an increasing dose of nZnO up to 1,000 mg L^{-1} compared with the control plants. The remarkable increase in width and intensity of 28 kDa protein band (**Figure 8**) may correspond to the pod storage protein (PSP), which acts as nutrient storage in plants exposed to stress. In agreement with this, we found the upregulation of PSP in leaves exposed to nZnO treatments compared with control plants as addressed in proteomics profiles. Similarly, a 42 kDa chitin-binding proline-rich protein was another upregulated protein in treatments exposed to nZnO, which is involved in plant-pathogen interaction (Salehi et al., 2021b). The limiting effects of nZnO on protein synthesis were observed at the highest applied dose (**Figure 8**). Moreover, the SDS-PAGE of this concentration was representative of reduced total protein as depicted in the intensity and width of bands (h pit, 2,000 mg L^{-1}). These results confirmed the phytotoxicity of nZnO on the productivity of bean plants at a concentration of less than 1,000 mg L^{-1}. As shown in **Figure 7**, the protein content changes in $ZnSO_4$ treatments were significant at $p \leq 0.05$ as the total protein seems to increase. However, there were no tangible SDS-PAGE protein pattern changes in $ZnSO_4$ treatments.

DISCUSSION

The progressing trend of foliar application of NPs agrees on their phytotoxicity at a higher dosage while observing a positive response on the growth at a lower level. A wide range of effects, from phenotypic symptoms to physiological and biochemical alternations involved in primary and secondary metabolism, have been reported (Salehi et al., 2018, 2020, 2021b; Sharifan et al., 2019). These alternations are often accompanied by plant yield and productivity (Gomez et al., 2020; Sharifan et al., 2020a). Our previous study has already shown both positive and negative effects of nZnO and $ZnSO_4$ on bean plant metabolism (Salehi et al., 2021b). In this study, we propounded another aspect of NPs-induced changes in *P. vulgaris* besides the aforementioned reports. Reproductive development in some crops is considered the most stress-sensitive stage after germination-related traits. Therefore, the risk of damage to this period of plant development is often critical (Ma et al., 2020b). The evaluation of male and female gametophytes showed that the development of these reproductive gametes is affected at specific stages, which could be considered as the specific sensitive stages to NPs treatments that seem distinctive from other materials in the micro style. These differences could be due to various factors, including smaller size, higher area surface, lower solubility, and controlled release systems of NPs forms (Khan et al., 2019).

Our results showed that each applied Zn species (NPs and ions forms) might induce different responses corresponding to certain stages of microsporogenesis and macrosporogenesis, which could appear as toxicity symptoms. From the point of the male development, the results confirmed higher toxicity of nZnO compared with their ionic counterpart. This difference is probably due to the physicochemical features of NPs and their ability to cross through plant barriers (Rossi et al., 2019; Sharifan et al., 2020b) and leads to the higher accumulation of ions in tissues. In this term, our previous study showed that higher amounts of Zn were accumulated in the plants exposed to nZnO compared with Zn ions (Salehi et al., 2021b), which could be a critical factor involved in the higher adverse effects on microsporogenesis processes due to the infirmity of performance of the bean plants during the vegetative stage.

The proper male gametogenesis (pollen grain development) is fundamental to plant sexual reproduction. To gain this importance, the main crucial steps in the meiotic cell cycle must be driven forward properly (Coimbra et al., 2009). The higher destructive effects of nZnO likely observed through early phases of meiosis and also the disintegration of tapetal cells caused abnormal PMC, tetrads, and finally, non-functional mature pollen grains compared with ion forms. This timely phenomenon is critical for proper microspore development and pollen maturation. In fact, the disordering of the function of tapetum cell may represent less sporopollenin production or its defective transmit to the cell wall, resulting in inhibition of generating exine and causing cell wall instability (Ariizumi and Toriyama, 2011). Therefore, defects and aberrations in the timing of tapetum development, including hypertrophy and premature as well as delayed degeneration, have been reported, which negatively affect the pollen number and viability (Harsant et al., 2013; De Storme and Geelen, 2014).

Since the tapetum cells produce some necessary metabolites for microspores, they serve as an essential nutrient supplier and provide energy for microspore development (Lei and Liu, 2020), and the interaction between the tapetum layer behavior and normal pollen development is inevitable. Similar results of premature or delayed degradation of the tapetum have been reported in several studies (Vizcay-Barrena and Wilson, 2006; Liu et al., 2018). Additionally, pollen morphology and architecture and its chemical composition were also affected, such as accumulating the starch. Similar findings have previously reported that micro and nano-based metals can be phytotoxic to reproductive organs like reduced pollen germination, pollen tube growth, and minimize the viability of pollen grains as fertilization tools (Kumbhakar et al., 2016; Marmiroli et al., 2021; Yoshihara et al., 2021). For instance, a decreased germination rate and tube elongation of pollen grains (*Lilium longiflorum*) exposed to 100 mg L^{-1} nZnO was observed in the *in vitro* condition (Yoshihara et al., 2021). The aforementioned modifications could alter pollen fitness by reducing the cytoplasmic content and essential materials for exine and intine cell wall production. The literature declared that most of the abnormalities in pollen development are associated with an abnormal tapetal function

TABLE 1 | The comparison of Zn content and seed-related parameters of bean plants exposed to different concentrations of nZnO and $ZnSO_4$ at the end of the growth period.

	Zn content (mg kg^{-1})	**Pods per plant**	**Seeds per pod**	**Seeds per plant**	**Seed dry weight (g/10 seeds)**
nZnO (mg L^{-1})					
0	45.5 ± 1.5*c*	13.3 ± 1.63*a*	5.2 ± 1.03*a*	67 ± 6.2*a*	3.19 ± 0.19*a*
250	52.3 ± 1.6*b*	12.0 ± 0.94*ab*	4.7 ± 0.94*ab*	55 ± 11.1*b*	2.60 ± 0.18*b*
500	58.9 ± 1.3*a*	12.1 ± 0.99*ab*	4.5 ± 0.84*ab*	53.6 ± 9.5*b*	2.07 ± 0.11*c*
1000	59.8 ± 0.4*a*	11.5 ± 2.27*b*	4.2 ± 0.91*bc*	47.3 ± 4.0*b*	1.68 ± 0.10*d*
2000	60.7 ± 0.3*a*	6.60 ± 1.83*c*	3.6 ± 0.69*c*	20.3 ± 2.5*a*	0.97 ± 0.11*e*
$ZnSO_4$ (mg L^{-1})					
0	45.5 ± 1.5*a*	13.3 ± 1.63*bc*	5.2 ± 1.03*a*	67 ± 6.2*b*	3.19 ± 0.19*b*
250	48.6 ± 1.4*b*	14.9 ± 1.28*a*	5.3 ± 0.94*a*	88.3 ± 4.1*d*	3.89 ± 0.30*a*
500	50.3 ± 1.6*b*	13.2 ± 1.68*bc*	5.2 ± 1.03*a*	65.6 ± 10.4*c*	3.13 ± 0.27*b*
1000	55.2 ± 0.1*c*	13.5 ± 1.26*b*	4.8 ± 1.13*a*	67.6 ± 6.0*b*	3.05 ± 0.08*b*
2000	57.3 ± 0.9*c*	12.0 ± 1.15*c*	4.6 ± 0.51*a*	58.3 ± 7.0*a*	2.83 ± 0.30*b*

Data represent mean ± SD. Different letters indicate statistical significance at $p < 0.05$.

(Deng et al., 2018; Chaban et al., 2020). As expected, diverse factors such as abiotic stresses influence different developmental stages of pollen grain (Barnabás et al., 2008; Smith and Zhao, 2016). However, there is a remarkable gap in information about the potential effects of NPs on the reproductive developmental stage, in particular, the processes of pollen formation and maturation. Previous studies investigated the developmental fitness under some other environmental risks, in which pollen viability is linked to dehydration and the composition of pollen cytoplasm (Firon et al., 2012; Pacini and Dolferus, 2019). A study showed that *Pennisetum typhoides* pollens containing 5% sucrose exhibited a higher level of non-viability than those containing 14% sucrose, likely due to the high dehydration rate (Hoekstra et al., 1989). Cellular tonicity plays a crucial role in pollen viability, specifically in the productivity of cereals. For example, water stress imposes detrimental effects on the pollen viability, abnormal exine, and intine layer (Pacini and Dolferus, 2019). In agreement with our previous report (Salehi et al., 2021b), the lower relative water content (RWC) in leaves of plants exposed to nZnO during vegetative stages may explain dehydration of pollen grains in the reproductive stage. Therefore, the observed unbalance fitness in pollen grains could be due to physiochemical alternations and also cytological aberrations during meiosis and mitosis processes, resulting in reduced pollen viability and consequently a higher mortality rate of seeds. In agreement with this, Marmiroli et al. (2021) has recently reported some regulated pollen-specific genes in zucchini (*Cucurbita pepo* L.) plants upon exposure to CuO nanoparticles. This study, along with data presented in this study, would strengthen the concept of NPs translocation into the floral parts and also trigger an NPs-specific response that has been proven somehow distinct from that induced by other counterparts (such as bulk and ion forms). Moreover, it has been shown that those genes related to cell wall composition, dynamic 3-dimensional structures (which fill the cytoplasm), and starch metabolism were the targets of NPs (Marmiroli et al., 2021). All of these abovementioned metabolic and genomic pathways are considered key aspects of pollen maturation.

Megasporogenesis (female gametophyte development) in the ovule of a flowering plant serves as the site of double fertilization (which later develops into the embryo and endosperm). This phenomenon is crucial for plant reproduction and world food production. Despite that, only a few reports are available on female gametophyte development under diverse environmental stresses (Klatt et al., 2016). Likewise, the number of studies upon exposure to NPs is almost zero. For example, a few studies have shown several abnormalities like reduced ovule size and its viability upon cold stress [reviewed by Zinn et al. (2010)]. In this trial, apoptosis of embryo sac cells, including egg cells and synergid cells, disturbing the endosperm division, embryonic tissues damage, and embryo abortion were among the traceable modifications. Therefore, the female gametophyte may not be affected as intensely as male development by such abiotic stresses. Nevertheless, given the interaction between these two reproductive constituents and the necessity of this interaction for pollen tube growth and guidance through the pistil, any damages to these reproductive cells could affect the fertilization (Klatt et al., 2016). For example, in our experiment, apoptosis of synergid cells may disturb the precise track of pollen tube growth to central cells and egg cells, leading to produce unfertilized seeds as observed at higher dosages of Zn treatments. Overproduction of ROS most likely causes the embryo-sac cellular apoptosis and oxidative stress induced by NPs (Rossi et al., 2018; Salehi et al., 2021b). The nZnO used in this study at higher concentrations have led to the upregulation of several proteins (i.e., peroxiredoxin, non-specific lipid transfer protein, and wound-induced basic protein) and metabolites (glucosinolates and pteridine-based metabolites), most likely due to response to oxidative stress in the leaves of exposed plants (Salehi et al., 2021b). The previously published data showed that exposure of bean plants to Zn species, especially nZnO, resulted in cumulative effects of ROS accumulation leading to the reduced level of antioxidant enzymes such as catalase (CAT), peroxidase (POX), and ascorbate peroxidase (APX). As a result, impaired male and female development is expected, such as premature pollen abortion, non-functional pollens, cellular apoptosis, and seed abortion.

FIGURE 7 | The images of sheath and seeds of treated and control plants. The sheath of control plant **(A,D)**, 2,000 mg L^{-1} nZnO **(B,E)**, and $ZnSO_4$ **(C)**. Furthermore, **(F–H)** are related to the control seeds, 250 mg L^{-1} $ZnSO_4$, and nZnO treatments, respectively. The characters of **(I,J)** represent seeds of plants exposed to 2,000 mg L^{-1} $ZnSO_4$ and nZnO treatments, respectively.

Zinc deficiency has been associated with pollen infertility and reduced seed productivity in wheat and lentil (Pandey et al., 2006; Cakmak, 2008; Zulfiqar et al., 2020). Unlike the high-level NPs (>1,000 mg L^{-1}), at lower levels, no visible toxic symptoms on cells or tissues of reproductive organs were observed. Nevertheless, Zn ions at low concentrations elevated the total seed yield and promoted the production of healthier seeds. It has been proven that Zn drives many biological mechanisms, including pollen development and seed yield. In this regard, our results suggested stimulating effects of Zn ions at low amounts on sporogenesis, gametogenesis, and reproduction. Previous studies have confirmed the plant uptake and distribution of Zn species either in the form of NPs or ions, which impact developmental stages from germination to seed production (Wang et al., 2018;

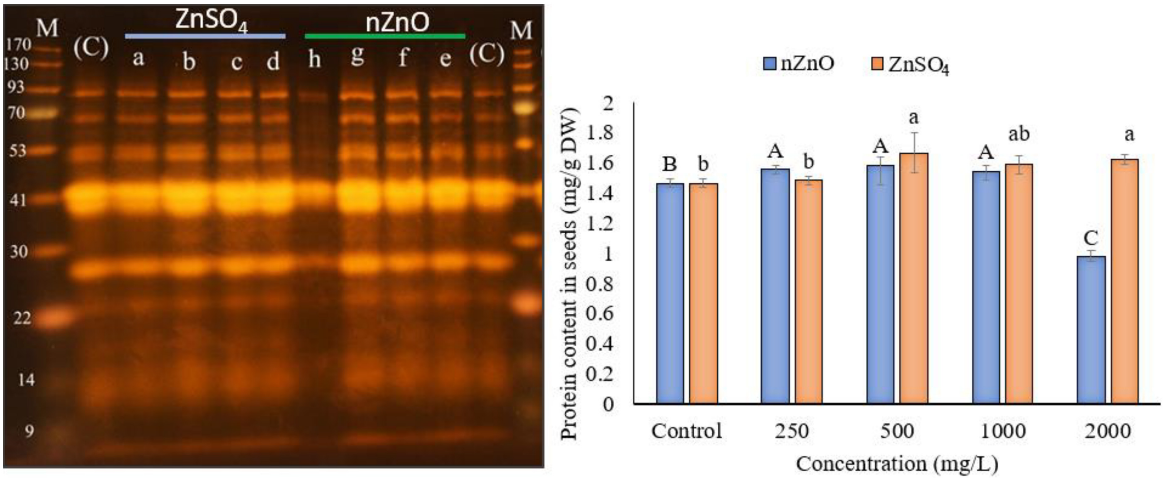

FIGURE 8 | The left image presents the SDS-PAGE analysis, where the a–d indicated the cultivar genome in treatments exposed to 250, 500, 1,000, and 2,000 $ZnSO_4$ (mg L^{-1}), and e–h for 250, 500, 1,000, and 2,000 nZnO (mg L^{-1}), respectively. The letters (C) and (M) indicate the control and marker plants, respectively. The plot on the right shows the protein content of dry seeds. The character above each bar indicates the statistical significance ($p < 0.05$).

Ma et al., 2020a,b). However, few studies have shown the positive or negative effects of nZnO on seed productivity. For example, the soybean plants exposed to 500 mg kg^{-1} ZnO NPs did not produce seeds (Yoon et al., 2013). In contrast, adverse effects on the yield of sweet potato (*Ipomoea batatas* L.) were only observed at 1,000 mg kg^{-1} nZnO concentration (Bradfield et al., 2017). Therefore, further studies are needed to elucidate their potential effects on the storage components of both pollen grains and seeds.

CONCLUSION AND FUTURE PERSPECTIVE

Given the critical role of Zn in entire plant development, there has been remarkable interest in its nanoscaled form application as a slow-release fertilizer. However, despite its proven phytotoxicity at the vegetative stage of development, there is no information on the reproductive perspective. Therefore, apart from the previous reports, this study investigated the impacts of foliar application of nZnO on plant reproductive development. Both male and female organs have been monitored from the earliest emergence until full maturity. The spray application of Zn ions at lower levels induced positive responses on sporogenesis and gametogenesis processes of male and female organs of bean plants, resulting in higher seed yield. However, nZnO impaired the normal developmental trend; particularly, two developmental processes (i.e., meiosis and pollen development) were distinctively affected depending on NPs dosages. In terms of male development, it was indicated that the most induced alternations occurred during microspore development. Moreover, tapetum dysfunction appears to be a critical factor involved in premature microspore and pollen abortion. The impaired reproductive and reduced seed productivity with higher NPs levels may be explained by a higher accumulation of Zn and reactivity in this form, oxidative stress induced by NPs, interrupting the pollen performance, and physiochemistry. In fact, increased non-viability of pollen was attributed to structural-induced defects during microgametogenesis. Overall, the results confirm that nZnO exerted stronger phytotoxicity on reproductive components fitness while an ion at a certain amount positively influences plant development and could lead to a higher yield. Since there is a complicated network between vegetative and generative stages of plant growth, a comprehensive omics analysis in both stages might uncover more mechanistically the distinct response of nZnO than its counterparts. Therefore, there is an urgent need to elucidate the underlying molecular mechanisms of nZnO and other nano-based materials on reproductive organs, especially pollen grains, and their safety in agro-sustainable application.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

HS and ACR conceived the idea. HS performed the work, analyzed the data, and wrote the manuscript. AR, HSh, and ACR reviewed and edited the first draft. ACR and RKV proofread and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.808141/full#supplementary-material

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Integrated Application of Thiourea and Biochar Improves Maize Growth, Antioxidant Activity and Reduces Cadmium Bioavailability in Cadmium-Contaminated Soil

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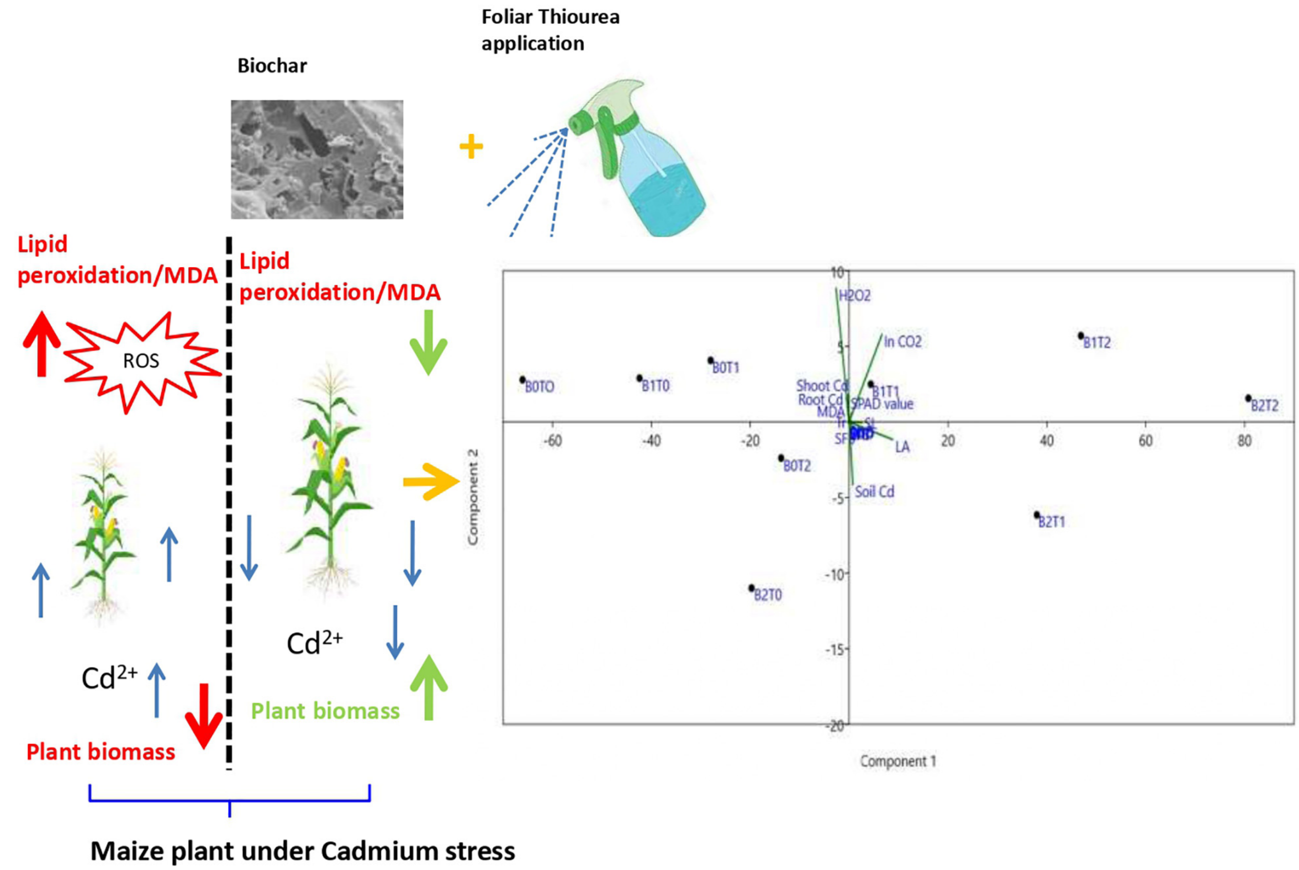
Cadmium (Cd) contamination of croplands jeopardizes sustainable crop production and human health. However, curtailing Cd transfer and mobility in the rhizosphere-plant system is challenging. Sole application of biochar (BC) and thiourea (TU) has been reported to restrain Cd toxicity and uptake in plants. However, the combined applications of BC and TU in mitigating the harmful effects of Cd on plants have not yet been thoroughly investigated. Therefore, this study attempts to explore the integrated impact of three maize stalk BC application rates [B_0 (0% w/w), B_1 (2.5% w/w), and B_2 (5% w/w)] and three TU foliar application rates [T_0 (0 mg L^{-1}), T_1 (600 mg L^{-1}), and T_2 (1,200 mg L^{-1})] in remediating the adverse effects of Cd on maize growth, development, and physiology. Results demonstrated that Cd concentration in soil inhibited plant growth by reducing leaf area, photosynthesis activity, and enhanced oxidative stress in maize. Nevertheless, BC and TU application in combination (B_2T_2) improved the fresh biomass, shoot height, leaf area, and photosynthesis rate of maize plants by 27, 42, 36, and 15%, respectively, compared with control (B_0T_0). Additionally, the oxidative stress values [malondialdehyde (MDA), hydrogen peroxide (H_2O_2), and electrolyte leakage (EL)] were minimized by 26, 20, and 21%, respectively, under B_2T_2 as compared with B_0T_0. Antioxidant enzyme activities [superoxide dismutase (SOD) and catalase (CAT)] were 81 and 58%, respectively, higher in B_2T_2 than in B_0T_0. Besides, the shoot and root Cd concentrations were decreased by 42 and 49%, respectively, under B_2T_2 compared with B_0T_0. The recent study showed that the integrated effects of BC and TU have significant potential to improve the growth of maize on Cd-contaminated soil by reducing Cd content in plant organs (shoots and roots).

Keywords: biochar, cadmium toxicity, thiourea, maize growth, antioxidant



Graphical Abstract |

HIGHLIGHTS

- Biochar (BC) application reduces the Cd uptake in maize plants.
- Thiourea (TU) application improves the plant growth and reduces the Cd uptake.
- The combined application of BC and TU could be profitable for Cd contamination.

INTRODUCTION

Cadmium (Cd) is a very toxic trace element found in soil. It has been identified as a carcinogenic element and poses severe threats to human health (Haider et al., 2021). The primary sources of Cd contamination of croplands are anthropogenic activities, including fertilization, mining, and sewage sludge application which require additional study to alleviate Cd pollution and assure food security for the ever-growing global population (Wu et al., 2015). Cd availability and translocation in the soil-plant system substantially affect soil ecosystem services, reducing crop growth, yield, and quality (Zhao, 2020). Accumulation of Cd in the grains and other edible parts is the primary source of human exposure to contaminated food. Therefore, reducing health risk, particularly for crops cultivated on Cd contaminated soils, has been recently focused on (Zhang et al., 2009). Cd enters into human food chains and creates complicated health problems after being readily transported from Cd-contaminated soils to plant tissues (Ahmad et al., 2016). Plants grown on Cd-contaminated soil are subjected to osmotic stress, causing physiological degradation due to a reduction in relative leaf water content, transpiration, stomatal conductance, and photosynthetic activity (Anjum et al., 2017). Cd accumulation in plants not only damages chloroplast tissues, membrane permeability, and cell necrosis (Rafique et al., 2019) but also enhances the formation of reactive oxygen species (ROS). Therefore, an appropriate approach to remedy the harmful effects of Cd contamination is to limit the bioavailability of Cd in the rhizosphere to reduce its uptake by plants (Wang et al., 2019). Reducing Cd uptake by plants is also imperative for ensuring food safety. The current focus of the study has been on the decrease of Cd availability to crop plants.

Due to its higher ability to accumulate high Cd concentration, maize is ranked first among cereal crops, earning Cd in most plant organs as one of the leading crops. Besides, maize is one of the significant cereal crops in China, and most maize planting regions in China are Cd contaminated (Kollárová et al., 2019). Minimizing the bioavailability of Cd in the rhizosphere and reducing the uptake and accumulation of Cd by plants could effectively reduce the risk to human health and increase crop growth and yield (Zhu et al., 2020). Biochar (BC) has attained significant interest as a soil amendment for increasing carbon sink (Virk et al., 2020), mitigating greenhouse gas emission, and minimizing the Cd bioavailability in the rhizosphere (Younis et al., 2016). In addition, the role of BC in improving plant water availability, soil base saturation, and corn yield and minimizing Cd bioavailability in the rhizosphere has been widely reported (AbdElgawad et al., 2020). This decrease in Cd bioavailability might be attributed to the high absorption capacity and higher surface area of applied BC. The physicochemical properties of BC, such as surface area, surface charge, and pore spaces, and BC production parameters, such as pyrolysis retention time, pyrolysis temperature, and feedstock type, are major variables (Younis et al., 2016) that determine the adsorption capacity of Cd in contaminated agricultural soils (Khanna et al., 2019a).

The exogenous application of growth regulators has the potential to enhance plant growth and reduce the plant capacity to accumulate heavy metals. Thiourea (TU), a plant growth hormone, is actively involved in a series of developmental, physiological (Perveen et al., 2015), and biochemical changes in plants. Exogenous application of TU can mitigate Cd toxicity. Seed priming or foliar application of TU can play an imperative role in Cd detoxification and minimize Cd levels in plant tissues (Sharma and Dietz, 2009). Sulfur, the constituent of TU, improves plant metabolism, biochemical reactions (Srivastava et al., 2017), and protein folding and curtail plant Cd uptake.

Moreover, sulfur is also an essential constituent of tripeptide glutathione (GSH), which plays a critical function in plant redox regulation found on TU. Application of TU under Cd toxicity was found to improve leaf area expansion, chlorophyll *b*, stomatal conductance, photosynthesis rate, carotenoids in maize, and Cd transport from the rhizosphere to plant tissues. Moreover, exogenous application of TU has also been documented to decrease the uptake of arsenic in rice (Younis et al., 2016; Srivastava et al., 2017).

Although, the application of BC or TU alone has been reported to mitigate trace-metal bioavailability and enhance maize productivity. However, to the best of our understanding, the simultaneous application of BC application and TU to reduce Cd bioavailability and plant Cd uptake has not been studied yet. To this end, this study hypothesizes that the integrated application of BC and TU could reduce Cd uptake by reducing Cd bioavailability in the rhizosphere and maintaining antioxidant activity in plants than BC/TU application alone. Thus, this experiment was intended to investigate the impacts of combined application of BC and TU on mitigating Cd uptake in maize by evaluating the shoot and root growth, plant metabolic, and redox balance responses in maize grown on Cd contaminated soil.

MATERIALS AND METHODS

Experiment Design and Management

A pot trial was performed using a complete randomized design (CRD) having factorial arrangement having three replications in the wirehouse of College of Resources and Environmental Sciences, Gansu Agricultural University, Lanzhou, China in the first week of July 2020 to mid-week of September 2020. The wirehouse was wrapped with a polythene sheet to protect the plants from rainwater. Metrological data, i.e., temperature, humidity, rainfall, and sunshine duration, were mentioned in **Supplementary Table 1**. Maize straw-derived BC was applied at three rates, namely (B_0) 0% w/w, (B_1) 2.5% w/w, and (B_2) 5.0% w/w, mixed in the soil before pot filling. The BC used in the current experiment was manufactured from maize (*Zea may* L) straw at a pyrolysis temperature of 550°C (Zhou et al., 2019). The chemical and physical properties of BC and soil are presented in **Table 1**. Additionally, three TU dose rates were applied, namely, (T_0) 0 mg L^{-1}, (T_1) 600 mg L^{-1}, and (T_2) 1,200 mg L^{-1} at 20 and 40 days after sowing (DAS). Before BC addition, well-mixed air-dried soil was spiked artificially with 30 ppm Cd using Cd nitrate as a source of Cd. Each containing 5 kg soil, experimental pots were randomly placed during the experiment to minimize location effects (Haider et al., 2021). Before sowing, maize cultivar Liyu-16 (LY-16) seeds were sterilized with 10% H_2O_2 solution (v/v) for 15 min and then washed with distilled water. The seeds of maize variety (LY-16) were acquired from Hebei Maohua Seed Industry Co., Ltd., Maohua, China. Two grains of maize were sown in each pot, and the latter one was uprooted to maintain one plant in each pot. Every pot was fertilized properly with 150 g of N, 100 g of P_2O_5, and 50 g of K_2O, using urea (CH4N2O), diammonium phosphate [$(NH_4)_2HPO_4$], and sulfate of potash (K_2SO_4), respectively. The optimum amount of water was applied to maintain the requirements of the growing plant.

Destructive Plant Sampling and Measurements

The maize plants were harvested at 70 DAS. The root and shoot lengths were determined using a measuring tape. Root

TABLE 1 | Physiochemical properties of biochar and soil used in the experiment.

Parameters	Maize straw biochar	Soil
pH	8.60	8.64
EC (mS cm^{-1})	3.78	2.94
Total N (g kg^{-1})	0.14	0.37
Total K %	17.30	–
Total P %	0.19	0.77
Total C (g kg^{-1})	6.70	3.71
Organic matter %	–	6.40
Available P mg kg^{-1}	–	161.08
Available K mg kg^{-1}	–	117.60
Total Cd mg kg^{-1}		0.61

EC, electrical conductivity; N, nitrogen; K, potassium; P, phosphorous; C, carbon; Cd, cadmium.

and fresh shoot biomass were selected *via* an electronic weighing balance. At the same time, shoot and dry root biomass were determined after putting shoot/root samples in the oven at 75°C till constant weight (Virk et al., 2020). Leaf area was manually calculated by measuring leaf length, width, and multiplying with a correction factor of 0.75.

Physiological Activities

Photosynthetic activity and chlorophyll content were recorded at 60 DAS. Chlorophyll content was measured using soil plant analysis development (SPAD) values of a fully expanded leaf with a SPAD meter (SPAD 502, Konica Minolta, Osaka, Japan). Crop photosynthetic activities including intercellular CO_2 (Ci), transpiration rate (Tr), photosynthetic rate (Pn), and stomatal conductance (Gs) were measured with a portable IRGA analyzer (Infrared gas analyzer, Portable Gas Exchange Fluorescence System GFS-3000, Walz Heinz GmbH, Eichenring, Effeltrich, Germany). These data were collected between 10:00 and 12:00 h from a representative plant leaf.

Oxidant and Antioxidant Activities

Activities of lipid peroxidation (MDA and H_2O_2) and electrolyte leakage (EL) were measured from fully developed leaves of the maize plant 60 DAS. EL of the maize leaves was measured according to the standard protocol. Prior to analysis, harvested leaves were washed with sterilized water to remove any trapped electrolyte and placed in glass tubes (with 10 ml of distilled water) and then incubated adequately for 6 h at 25°C on a shaker. Finally, the electrical conductivity (EC) was calculated (EC1). Subsequently, the samples were suitably placed for 2 h at 90°C and after reaching equilibrium at 25°C, the EC was determined (EC2) (Dionisio-Sese and Tobita, 1998). The percentage of EL was calculated using the following equation:

$$\mathrm{EL} = \frac{\mathrm{EC1}}{\mathrm{EC2}} \times 100$$

The MDA and H_2O_2 activities were analyzed using the standard procedure. In brief, 0.1% thiobarbituric acid (TBA), with a volume of 5.0 ml, was added to 0.25 g leaves of maize, and the supernatant was centrifuged (12,000 r/min) at 4°C for 10 min. Then, 4 ml of 20% trichloroacetic acid (TCA) containing 0.5% was added to 1 ml of supernatant and heated at 95°C for 30 min. The mixture was cooled, and the mixture was again centrifuged (10,000 r/min) for 10 min under 4°C (Heath and Packer, 1968). The mixture was analyzed by absorbance at 450, 532, and 600 nm using a spectrophotometer (V-5800 visible spectrophotometer, Mesh Shanghai Yuanxi Instrument Co., Ltd., Shanghai, China). To determine H_2O_2, phosphate buffer solution (pH 6.5, 50 mM) at 3 ml was added in 50 mg maize leaf tissues and centrifuged (12,000 r/min) at 4°C for 30 min. Subsequently, 1 ml titanium sulfate in 20% sulfuric acid was gently mixed in the solution and again centrifuged (12,000 r/min) at 4°C for 20 min. Then, the absorption of the liquid was measured at 410 nm. The extermination coefficient of 0.28 $\mu\mathrm{mol}^{-1}$ cm^{-1} was managed to record H_2O_2 contents (Dionisio-Sese and Tobita, 1998).

Antioxidant activities, i.e., catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD), were determined using a spectrophotometer (visible spectrophotometer V-5800, Mesh Shanghai Yuanxi Instrument Co., Ltd., Shanghai, China). Maize leaves were kept in liquid nitrogen. After standardization, maize leaves were ground with quartz sand (0.5 g), polyvinyl tyrosanone (PVP; 100 mg), and 0.05 M phosphate buffer (pH 7.8) in pestle and mortar. The supernatants were collected in centrifuged tubes and allowed to centrifuge (12,000 r/min) at 4°C for 10 min. CAT activity was determined according to Aebi (1984).

Cadmium Determination in Maize Shoot, Root, and Soil

Dried shoot, root, and soil samples were ground correctly and allowed to sieve through a 0.5-mm sieve. The samples were digested in 10 ml of perchloric acid ($HClO_4$) and nitric acid (HNO_3) (1:3 v/v) overnight (Ryan et al., 2001). Subsequently, contents of Cd in the soil, shoot, and root were observed *via* an atomic absorption spectrophotometer (Model 3200-C, S/N: KETC0478, Company Heinz Walz GmbH, 91090, Effecltrich, Germany).

Statistical Analyses

Collected data were subjected to ANOVA technique using SPSS statistical software (Version 25.0, SPSS Inc., Chicago, IL, United States). The average treatment means were compared using the least significant difference at $p < 0.05$. Graph Prism was used to create the graphical presentation. Pearson's correlation analysis was performed to find a linear association between Cd concentration and maize plant physiology.

RESULTS

Plant Morphology and Leaf Area

The root length, fresh root biomass, root dry biomass, shoot length, shoot fresh biomass, shoot dry biomass, and leaf area of maize were significantly altered by the application of BC and TU grown under Cd-contaminated soil (**Figures 1**, **2**). Results revealed that contamination of Cd significantly reduced the root length, root fresh biomass, root dry biomass, shoot length, shoot fresh biomass, shoot dry biomass, and leaf area of maize (**Figures 1**, **2**). However, incorporation of B_2 in Cd-contaminated soil significantly improved the root length, fresh root biomass, root dry biomass, shoot length, shoot fresh biomass, shoot dry biomass, and leaf area by 23, 17, 19, 18, 11, 14, and 15%, respectively, more outstanding in comparison with control. Similarly, alone foliar application of TU (T_2), enhanced the root length, fresh root biomass, root dry biomass, shoot length, shoot fresh biomass, shoot dry biomass, and leaf area by 31, 24, 24, 21, 13, 14, and 18%, respectively, more outstanding in comparison with control. Correspondingly, combined application of T_2 and B_2 tends to improve the root length, fresh root biomass, root dry biomass, shoot length, shoot fresh biomass, shoot dry biomass, and leaf area as they were 47, 41, 43, 42, 27, 30, and 36%, respectively, higher as compared with control.

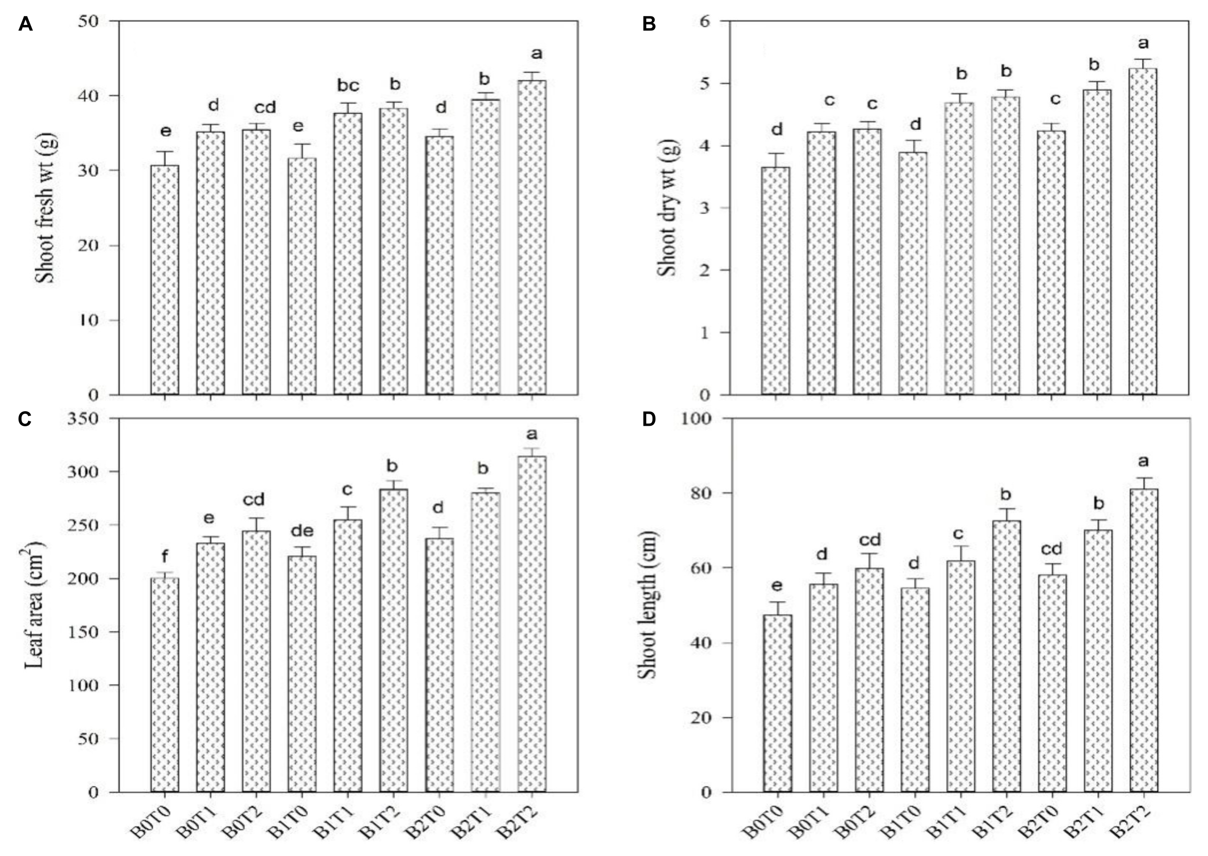


Figure 1 | Effect of combined application of TU and BC on **(A)** shoot fresh weight, **(B)** shoot dry weight, **(C)** leaf area, and **(D)** shoot length grown under cadmium (Cd) contamination. Values are averages ± SD (*n* = 3). Different statistical letters represent significant differences in values between treatments (using Tukey's multiple range test, $p < 0.05$). TU, thiourea; BC, biochar; T_0 = 0 mg L^{-1} thiourea; T_1 = 600 mg L^{-1} thiourea; T_2 = 600 mg L^{-1} thiourea; B_0 = 0% w/w BC; B_1 = 2.5% w/w BC; B_2 = 5.0% w/w BC.

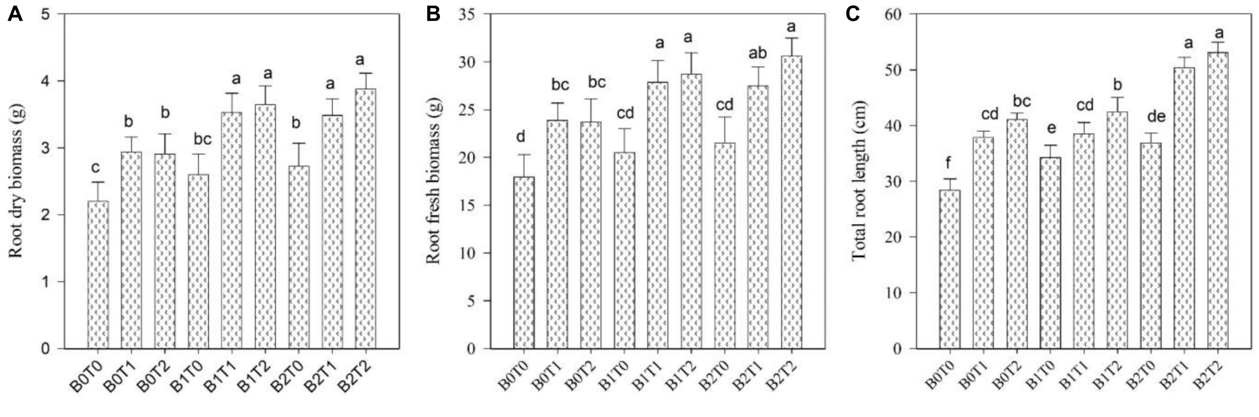


Figure 2 | Effect of combined application of TU and BC on **(A)** root dry biomass, **(B)** root fresh biomass, and **(C)** total root length grown under Cd contamination. Values are averages ± SD (*n* = 3). Different statistical letters represent significant differences in values between treatments ($p < 0.05$). TU, thiourea; BC, biochar; T_0 = 0 mg L^{-1}; T_1 = 600 mg L^{-1}; T_2 = 600 mg L^{-1}; B_0 = 0% w/w; B_1 = 2.5% w/w; B_2 = 5.0% w/w.

Oxidant and Antioxidant Activity

Oxidative stress values, i.e., MDA, H_2O_2, and EL, were significantly enhanced in maize leaves under Cd stress (**Figures 3**, **4**). Highest MDA, H_2O_2, and EL values in maize leaves were recorded in control (B_0T_0), i.e., with the mean values of 9, 153, and 69%, respectively. However, results revealed that incorporating B_2 in soil minimizes the MDA, H_2O_2, and EL values in maize leaves by 20, 13, and 13%, respectively, compared

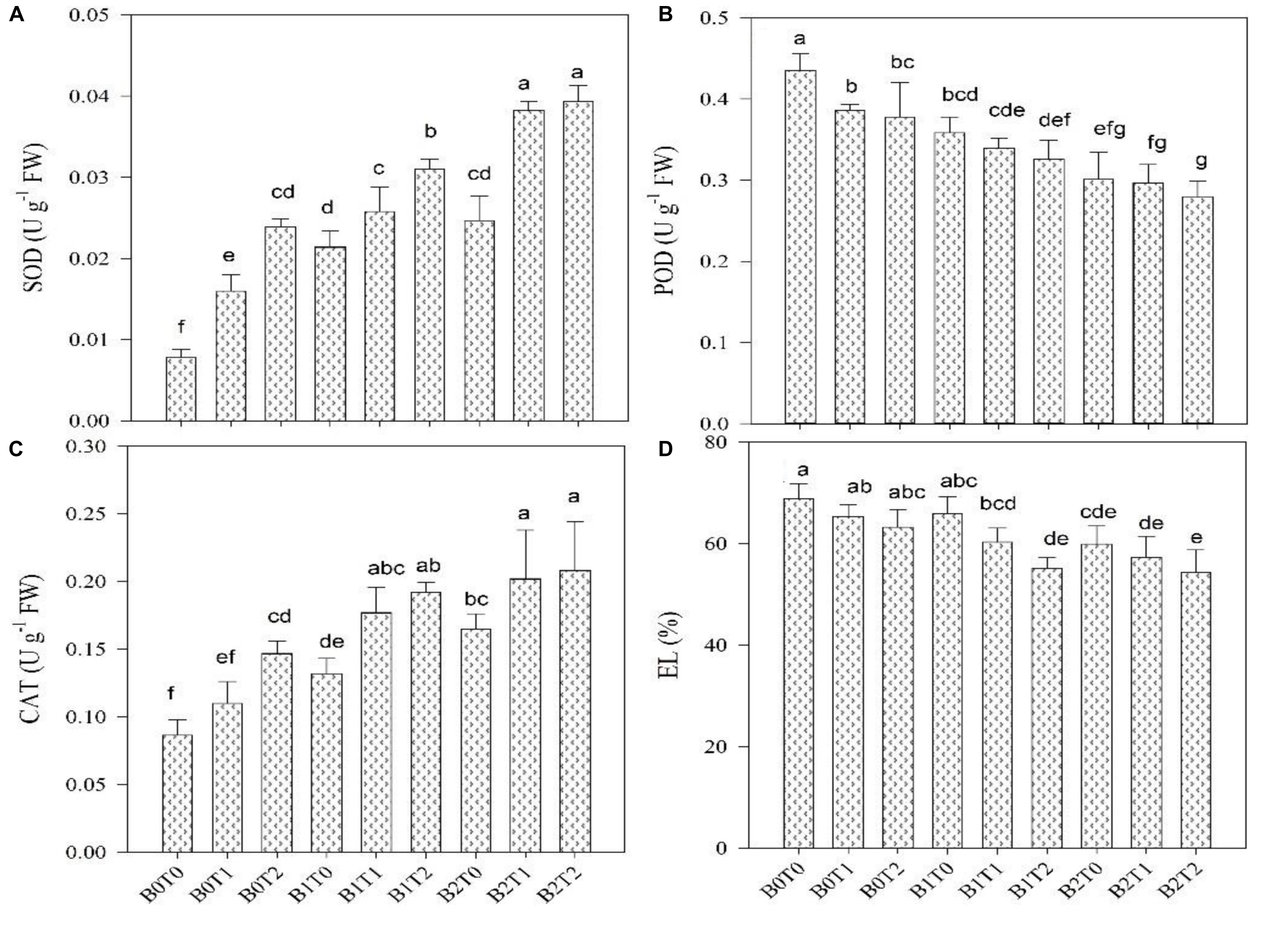


Figure 3 | Effect of combined application of TU and BC on **(A)** superoxide dismutase (SOD), **(B)** peroxidase (POD), **(C)** catalase (CAT), and **(D)** electrolyte leakage (EL), grown under Cd contamination. Values are averages ± SD (n = 3). Different statistical letters represent significant differences in values between treatments ($p < 0.05$). TU, thiourea; BC, biochar; T_0 = 0 mg L^{-1}; T_1 = 600 mg L^{-1}; T_2 = 600 mg L^{-1}; B_0 = 0% w/w; B_1 = 2.5% w/w; B_2 = 5.0% w/w.

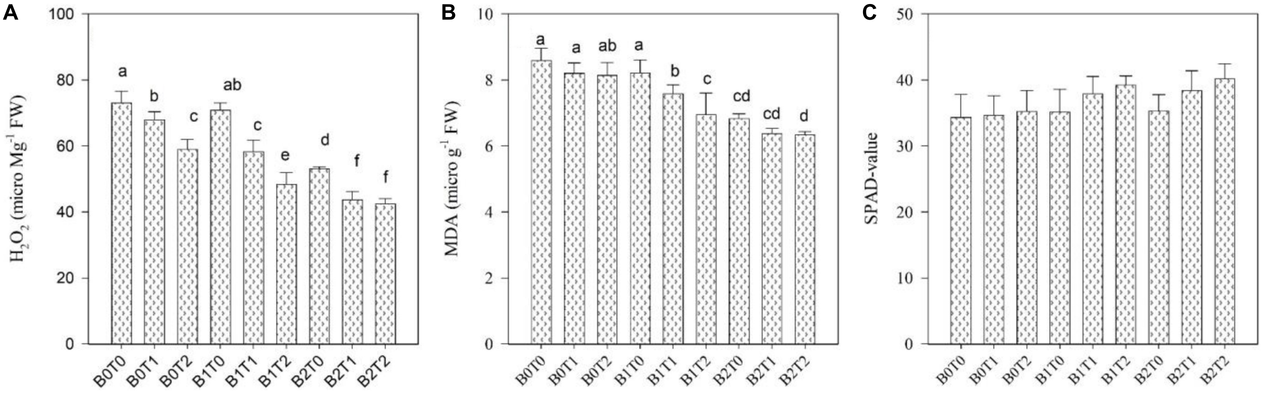


Figure 4 | Effect of combined application of TU and BC on **(A)** hydrogen peroxide (H_2O_2), **(B)** malondialdehyde (MDA), and **(C)** SPAD value grown under Cd contamination. Values are averages ± SD (n = 3). Different statistical letters represent significant differences in values between treatments ($p < 0.05$). TU, thiourea; BC, biochar; T_0 = 0 mg L^{-1}; T_1 = 600 mg L^{-1}; T_2 = 600 mg L^{-1}; B_0 = 0% w/w; B_1 = 2.5% w/w; B_2 = 5.0% w/w.

with control. Likewise, exogenous application of T_2 significantly minimized the MDA, H_2O_2, and EL values by 5, 9, and 8%, respectively, compared with control. Correspondingly, combined T_2 and B_2 reduced the MDA, H_2O_2, and EL values by 26, 20, and 21%, respectively, lower than B_0T_0. The antioxidant activities, i.e., SOD, CAT, and POD in maize leaves, were significantly

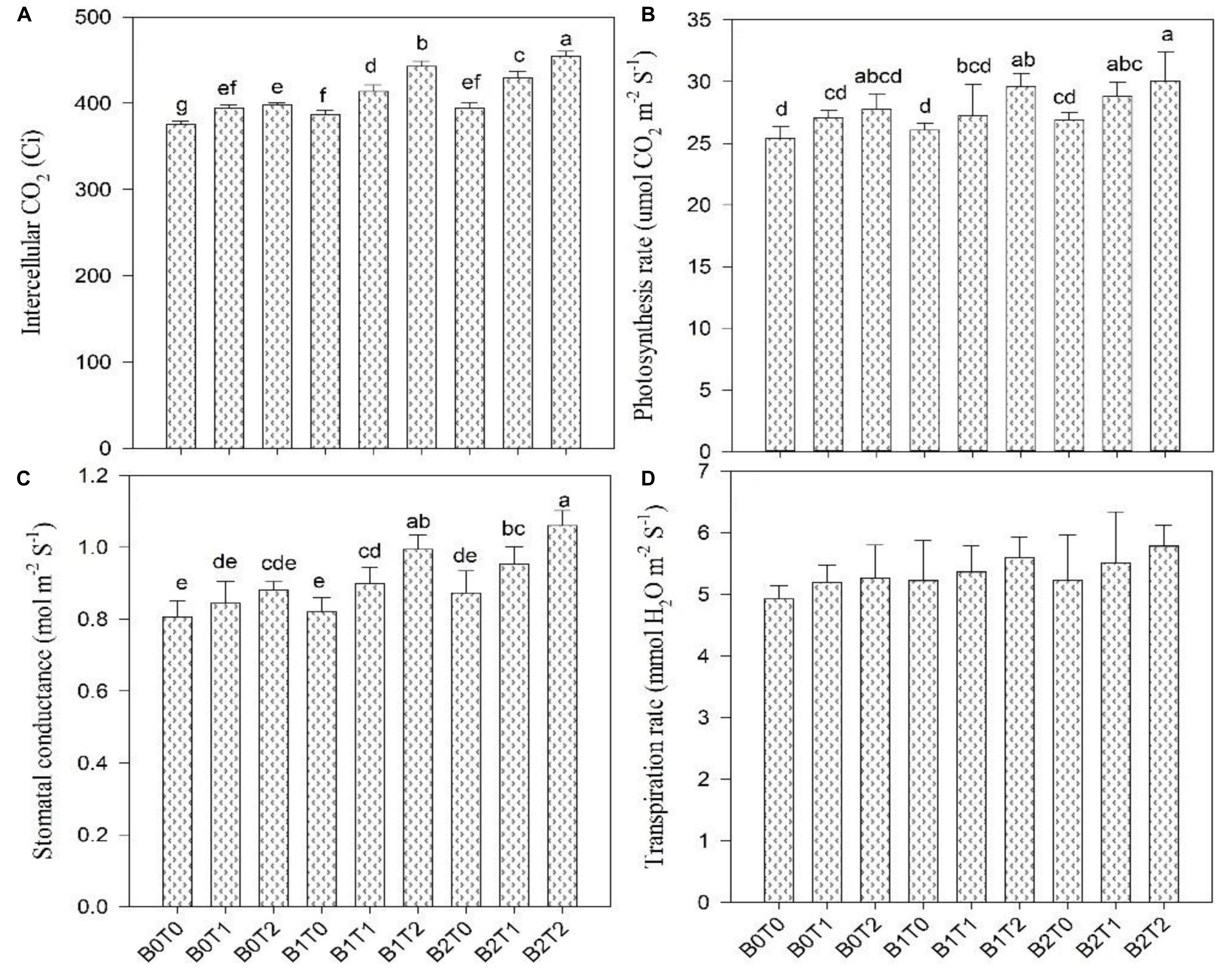


Figure 5 | Effect of combined application of TU and BC on **(A)** intercellular CO_2, **(B)** photosynthesis rate, **(C)** stomatal conductance, and **(D)** transpiration rate grown under Cd contamination. Values are averages ± SD ($n = 3$). Different statistical letters represent significant differences in values between treatments ($p < 0.05$). TU, thiourea; BC, biochar; $T_0 = 0$ mg L^{-1}; $T_1 = 600$ mg L^{-1}; $T_2 = 600$ mg $L^{-1;}$ $B_0 = 0\%$ w/w; $B_1 = 2.5\%$ w/w; $B_2 = 5.0\%$ w/w.

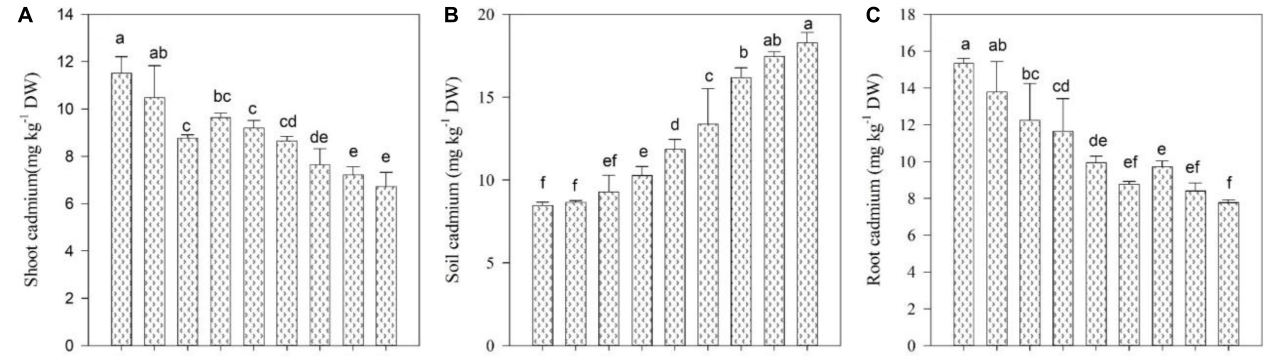


Figure 6 | Effect of combined application of TU and BC on **(A)** Cd concentration in shoot, **(B)** Cd concentration in soil, and **(C)** Cd concentration in root grown under Cd contamination. Values are averages ± SD ($n = 3$). Different statistical letters represent significant differences in values between treatments ($p < 0.05$). TU, thiourea; BC, biochar; $T_0 = 0$ mg L^{-1}; $T_1 = 600$ mg L^{-1}; $T_2 = 600$ mg $L^{-1;}$ $B_0 = 0\%$ w/w; $B_1 = 2.5\%$ w/w; $B_2 = 5.0\%$ w/w.

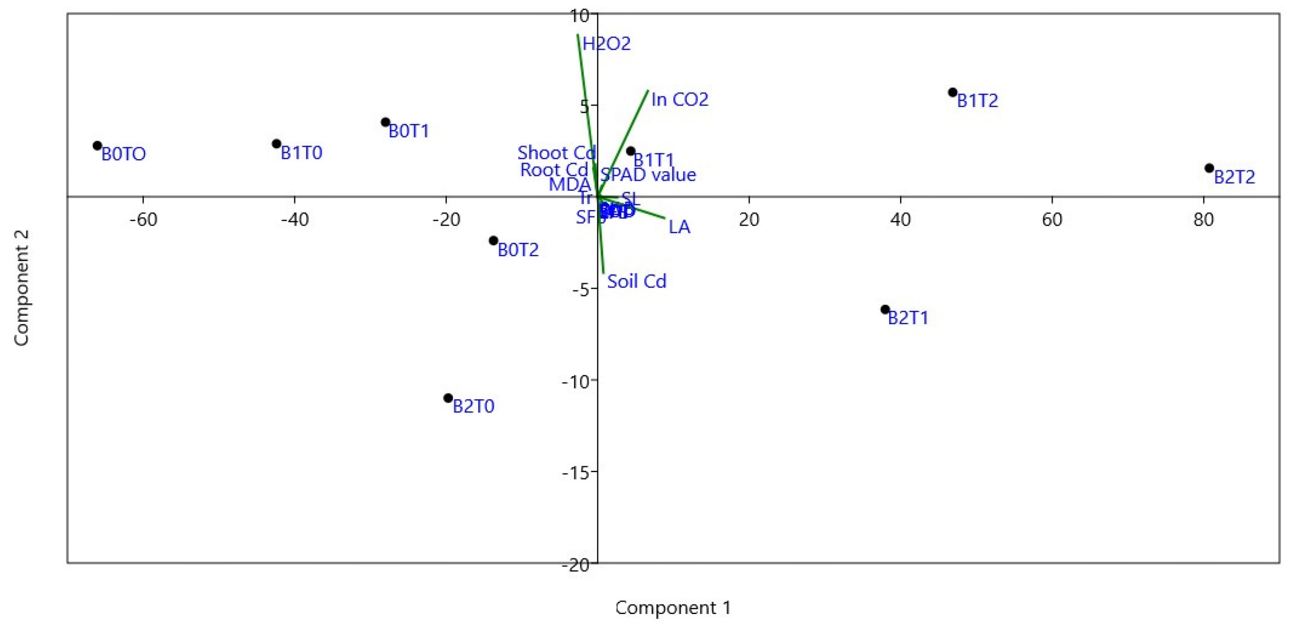


Figure 7 | Principal component analysis (PCA) of all parameters for treatment combination of TU and BC. T_0 = 0 mg L^{-1}; T_1 = 600 mg L^{-1}; T_2 = 600 mg L^{-1}; B_0 = 0% w/w; B_1 = 2.5% w/w; B_2 = 5.0% w/w. BC, biochar; TU, thiourea; RL, total root length; RFB, root fresh biomass; RDB, root dry biomass; SL, shoot length; SFB, shoot fresh biomass; SDB, shoot dry biomass; LAI, leaf area index; SOD, superoxide dismutase in leaves; POD, peroxidase in leaves; CAT, catalase in leaves; EL, electrolyte leakage in leaves; MDA, malondialdehyde; H_2O_2, hydrogen peroxide; Ci, intercellular CO_2 concentration; Pn, net photosynthesis rate; Gs, stomatal conductance; Tr, transpiration rate; SPAD, SPAD value; Soil Cd, cadmium concentration in soil; Shoot Cd, cadmium concentration in maize shoot; Root Cd, cadmium concentration in root.

altered under both TU and BC addition under Cd stress (**Figures 3**, **4**). The highest POD activity was recorded in the control treatment (B_0T_0), having a mean value of 0.44 μ g^{-1} FW. The combined exogenous application of T_2 and B_2 decreases the POD activity in maize leaves by 36% than that of the control. Likewise, SOD and CAT activities were significantly improved in maize leaves by exogenous foliar application of TU and BC incorporation in soil. The highest SOD (0.040 μ g^{-1} FW) and CAT (0.21 μ g^{-1} FW) activities were in the combined exogenous foliar application of BC and TU (B_2T_2), which were 81 and 59% higher in comparison with B_0 (0.0074 μ g^{-1} FW) and T_0 (0.087 μ g^{-1} FW).

Photosynthesis Activity, Soil Plant Analysis Development Value, and Gas-Exchange Parameters

Photosynthesis activity and gas-exchange parameters, i.e., stomatal conductance and intercellular CO_2 concentration, were significantly affected in maize leaves under Cd stress; however, the SPAD value and transpiration rate results were non-significant under Cd stress (**Figures 4**, **5**). Results revealed that contamination of Cd significantly reduced the photosynthesis activity, SPAD value, and gas-exchange parameters of maize. However, incorporating B_2 in Cd-contaminated soil significantly improved photosynthesis activity, SPAD value, and gas-exchange parameters of maize. Concerning BC, the highest photosynthesis activity, SPAD value, stomatal conductance, transpiration rate, and intercellular CO_2 concentration were recorded in B_2 that were 6, 3, 8, 6, and 5% higher in comparison with control (B_0T_0), respectively. Likewise, exogenous application of TU_2 enhanced the photosynthesis activity, SPAD value, stomatal conductance, transpiration rate, and intercellular CO_2 concentration by 9, 3, 9, 7, and 6%, respectively, higher as compared with control. The most increased photosynthesis activity, SPAD value, stomatal conductance, transpiration rate, and intercellular CO_2 concentration were recorded in the combined application of B_2T_2 that were 15, 15, 25, 15, and 17%, respectively, higher in comparison with control.

Cadmium Concentration in Different Plant Parts

Cadmium accumulation in soil and various maize components, i.e., shoot and root, significantly impacts the two-way association between BC and TU (**Figure 6**). With all forms of Cd contamination, the Cd concentration in soil was usually the highest relative to the Cd concentration in maize shoot and root. The plant-available Cd in maize shoot and root was significantly reduced after incorporating BC into the soil. The incorporation of BC reduced the Cd concentration in the shoot and root of maize plants by 33 and 37%, respectively, compared with B_0T_0. Likewise, exogenous application of TU minimized the Cd concentration in shoot and root of maize plants by 24 and 20%, respectively, than that of B_0T_0. Correspondingly, the combined application of B_2T_2 minimized the Cd concentration in shoot and root of maize plants by 42 and 49% compared with B_0T_0, respectively.

Correlation Analysis and Principal Component Analysis

Correlation analysis showed that root indicators are strongly interrelated with the growth parameters such as shoot length, shoot dry biomass, and leaf area index (**Table 2**). Most of the determined enzymatic activities, i.e., SOD, POD, EL, MDA, and H_2O_2, were negatively ($p < 0.05$) correlated with the maize growth indicators. In contrast, the values of CAT were positively correlated with maize growth parameters. Similarly, the correlation between stomatal conductance and POD (-0.71), EL (0.81), MDA (-0.69), and H_2O_2 (-0.78) were negatively linked at $p < 0.05$ statistical significance. Specifically, Cd concentration adversely affected ($p < 0.05$) enzymatic activities of maize plants, which eventually decreased plant growth. The principal component analysis (PCA) demonstrated that the combined application of BC and TU decreased plant Cd and increased soil Cd (**Figure 7**).

DISCUSSION

The plant growth is affected when agricultural land is contaminated with high levels of Cd. To reduce Cd toxicity in plants, it is essential to mitigate Cd uptake and accumulation. Maize plants exposed to Cd toxicity showed impaired growth (Tanveer and Shabala, 2022). Also, in Cd-toxicity situations, however, the incorporation of BC and TU alone or in combined form recovered these declines in growth attributes (**Figures 2**, **7** and **Table 2**). The addition of BC and TU in the combined form was found to be the most effective in reducing the adverse effects of Cd. This should be clarified in terms of their roles. Exogenous application of TU protects the degradation of chlorophyll contents under Cd stress. In addition, TU significantly minimized the Cd concentrations in various plant tissues of maize (**Figure 6**), which may be attributed to the function of TU in cell wall lignifications. Cd stress lowers leaf chlorophyll concentrations and causes oxidative stress to photosynthetically active cell development (**Figure 5**). The replacement of magnesium ions with Cd ions in chlorophyll molecules causes a decrease in chlorophyll contents since the proportion conversion of chlorophyll to pheophytin enhances with increased Cd stress. Carotenoids are known to protect chlorophyll and other essential macromolecules from free radical production by lowering the excited triplet state of chlorophyll (Dad et al., 2021). In this study, the synergistic effect of TU and BC induced maize growth enhancement due to greater chlorophyll concentrations and photosynthesis activity under Cd stress.

Lignification is a process comprised of lignin formation, where complex phenolic-heteropolymers are found in cell walls. In plants, these phenolic metabolites may connect to the plant cell wall, reducing the accumulation and assimilation of trace-metal contaminates in plant tissues (Khanna et al., 2019b). Moreover, root lignification can limit the Cd-apoplastic pathway and its translocation from the root zone to the plant aboveground part, i.e., stem, flowers, and leaves (Khanna et al., 2019c). Lignification is a multienzymatic mechanism mediated by various exogenous plant hormones in plants. TU contributes to regulating metabolic processes, including the phenolic pathways, during the lignification process (Perveen et al., 2015). BC is enriched with cations, i.e., Mg^{+2}, K^{+}, and Ca^{+2}. These cation ions might be embedded in the cell walls of plant tissues such as amorphous buffers, reducing Cd absorption, and uptake from the rhizosphere to plants (Younis et al., 2016).

Furthermore, plants can competitively absorb cations, Cd, Mg^{+2}, K^{+}, and Ca^{+2} compete with Cd for exchange sites in soils, and Cd absorption in soils can be decreased by the presence of competitive ions, i.e., Mg^{+2}, K^{+}, and Ca^{+2}. BC and TU had a synergistic effect, indicating that the two together were more effective at reducing the Cd toxicity than either one alone. Under Cd stress, maize treated with both BC and TU had significantly higher plant height, above, and below-ground biomass than maize treated with the Cd only (Xu et al., 2014).

Appropriate exogenous application of soil amendment or plant growth hormones reduces the health hazards and metal contamination in an environmentally sustainable manner (Chaoui and El Ferjani, 2005). The porous structure of BC improves the water holding capacity of the plant and maintains the photosynthesis activity of the plant under Cd stress. In addition, BC may also increase soil conditions by improving the plant absorption of macronutrients/micronutrients (Mg, Ca, P, K, Mn, Fe, and Cu) (Retamal-Salgado et al., 2017), rendering it an excellent agricultural fertilizer source. Increasing the content of macronutrients in plant tissues, TU can also increase the nutrient content in soil (Ge et al., 2019). These findings suggest that the combination of BC and TU could significantly improve the nutrient content and thereby reduce Cd uptake in root and shoot parts of maize. Nutrients essential for plants can affect Cd uptake, aggregation, translocation, bioavailability, and dispersal by adsorbing Cd on the surface, chelating or binding with Cd, thereby reducing Cd assimilation, uptake, and availability. Therefore, the improvement in maize growth observed in this research following the addition of BC and TU showed the combined effect of BC and TU in reducing Cd accumulation in maize. This research illustrates that exogenous application of BC and TU has a combined remediation effect on Cd-induced stress, implying that BC as a soil improver in conjunction with foliar application of maize with TU may be a potentially useful way to reduce Cd toxicity in agricultural development.

Under normal development circumstances, plants usually maintain a balance between ROS formation and elimination; however, this equilibrium is disrupted by Cd stress conditions. ROS release under a stressful situation causes lipid peroxidation, which contributes to cell membrane permeability, as demonstrated in this study. Cell membrane permeability is enhanced in response to Cd stress (**Figures 3**, **4**). The plasma membrane may be damaged by various mechanisms, including the oxidation reaction caused by ROS (Fahad et al., 2016). It was reported that Cd has a significant attraction to nitrogen

TABLE 2 | Pearson's association between .various traits in maize (n = 3) grown under Cd contamination

	RFB	RDB	SL	SFB	SDB	LAI	SOD	POD	CAT	EL	MDA	H_2O_2	Ci	Pn	Gs	Tr	SPAD	Soil Cd	Shoot Cd	Root Cd
RL	0.754**	0.759**	0.868**	0.870**	0.867**	0.890**	0.907**	–0.739**	0.760**	–0.715**	–0.732**	–0.864**	0.841**	0.668**	0.787**	0.451*	0.506**	0.747**	–0.794**	–0.746**
RFB	1	0.998**	0.808**	0.865**	0.897**	0.847**	0.752**	–0.539**	0.738**	–0.696**	–0.624**	–0.756**	0.855**	0.734**	0.744**	0.267	0.551**	0.560**	–0.636**	–0.740**
RDB		1	0.821**	0.868**	0.905**	0.857**	0.776**	–0.576**	0.765**	–0.716**	–0.653**	–0.774**	0.868**	0.732**	0.755**	0.281	0.566**	0.595**	–0.658**	–0.772**
SL			1	0.879**	0.897**	0.990**	0.877**	–0.724**	0.839**	–0.801**	–0.808**	–0.831**	0.955**	0.779**	0.873**	0.532**	0.580**	0.743**	–0.732**	–0.830**
SFB				1	0.992**	0.937**	0.850**	–0.665**	0.812**	–0.801**	–0.734**	–0.859**	0.904**	0.775**	0.835**	0.455*	0.608**	0.708**	–0.730**	–0.774**
SDB					1	0.948**	0.879**	–0.708**	0.850**	–0.823**	–0.769**	–0.877**	0.925**	0.776**	0.845**	0.448*	0.627**	0.745**	–0.758**	–0.828**
LAI						1	0.892**	–0.727**	0.853**	–0.822**	–0.808**	–0.861**	0.965**	0.797**	0.885**	0.522**	0.604**	0.753**	–0.751**	–0.837**
SOD							1	–0.811**	0.887**	–0.778**	–0.821**	–0.910**	0.856**	0.669**	0.794**	0.486*	0.575**	0.835**	–0.872**	–0.861**
POD								1	–0.754**	0.826**	0.788**	0.807**	–0.713**	–0.505**	–0.716**	–0.399*	–0.382*	–0.823**	0.850**	0.828**
CAT									1	–0.826**	–0.784**	–0.870**	0.803**	0.765**	0.747**	0.513**	0.490**	0.795**	–0.782**	–0.828**
EL										1	0.766**	0.835**	–0.827**	–0.753**	–0.811**	–0.489**	–0.428*	–0.718**	0.782**	0.791**
MDA											1	0.843**	–0.781**	–0.597**	–0.692**	–0.418*	–0.574**	–0.914**	0.801**	0.857**
H_2O_2												1	–0.851**	–0.676**	–0.788**	–0.393*	–0.532**	–0.866**	0.871**	0.825**
Ci													1	0.759**	0.918**	0.463*	0.665**	0.712**	–0.697**	–0.807**
Pn														1	0.796**	0.367	0.323	0.542**	–0.600**	–0.591**
Gs														1	0.796**	0.367	0.323	0.542**	–0.600**	–0.591**
Tr																1	0.372	0.464*	–0.305	–0.394*
SPAD																	1	0.523**	–0.437*	–0.623**
Soil Cd																		1	–0.832**	–0.829**
Shoot Cd																			1	0.831**
Root Cd																				1

***represents that results are significant at $p < 0.01$; *represents that results are significant at $p < 0.05$; RL, total root length; RFB, root fresh biomass; RDB, root dry biomass; SL, shoot length; SFB, shoot fresh biomass; SDB, shoot dry biomass; LAI, leaf area index; SOD, superoxide dismutase in leaves; POD, peroxidase in leaves; CAT, catalase in leaves; EL, electrolyte leakage in leaves; MDA, malondialdehyde; H_2O_2, hydrogen peroxide; Ci, intercellular CO_2 concentration; Pn, net photosynthesis rate; Gs, stomatal conductance; Tr, transpiration rate; SPAD, SPAD value; Soil Cd, cadmium concentration in soil; Shoot Cd, cadmium concentration in maize shoot; Root Cd, cadmium concentration in root.*

and sulfur-containing amino acids, ligands, and proteins. As a result, it binds to proteins, disrupting membrane ion channels and causing ion leakage (Dad et al., 2021). In contrast, the BC and TU application mitigated the harmful effects of Cd impact on lipid peroxidation and cell membrane permeability. Under Cd stress, BC and TU application decreased EL and MDA contents by 69 and 9%, respectively, compared with Cd stress alone (**Figures 3D**, **4B**), suggesting that TU-BC might enhance Cd tolerance in maize by retaining cell membrane permeability. BC may reduce oxidative stress by mitigating ROS generation and improving antioxidant activity (Fahad et al., 2016). When antioxidant activity rises in response to BC, it aids in the prevention of the start or expansion of oxidizing chain reactions, resulting in inhabitation or oxidation delays in plant cells, lipids, proteins, and other essential components (Abbas et al., 2017; Liu et al., 2018). Excessive ROS generation is regulated by antioxidant activation as an adaptive response; enhanced antioxidant activity does not imply better ROS detoxification. The activities of SOD and CAT were modulated in response to TU and BC application under Cd stress in the current investigation. TU and BC application increases CAT and SOD activities (**Figures 3A,C**), which was negatively connected with MDA levels and positively correlated with the improved shoot and root biomass accumulation under Cd stress (**Figure 4B**).

The foliar spray of TU causes Cd to accumulate in the root cell walls in rice, which is analogous to the positive effect of BC under Cd-stress agricultural soil. The contribution of plant cell walls in resistance against trace metal toxicity is imperative (Ahmad et al., 2016). As the first line of defense against toxic effects, the cell wall supplies a critical aptitude for Cd storage and accumulation in plants. It reacts effectively and rapidly to trace-metal contamination. The absorption of Cd in plants can be inhibited by the ability of the cell wall to compare Cd (Zhang et al., 2019; Wang et al., 2020). The critical components of the plant cell wall are matrix polysaccharides and cellulose. Polygalacturonic acids bind the majority of trace metals found in cell walls. In plants, cell walls may give functional groups, i.e., pectin, cellulose-microfibrils, and lignin, to unite Cd ions simultaneously and limit their bioavailability (Khanna et al., 2019a; Feng et al., 2020). TU can reduce Cd toxicity in cereals by enhancing hemicellulose and pectin content in root cell walls, improving the accumulation of Cd in cell walls of the root zone, and decreasing the accumulation of Cd in the below-aboveground fraction (Zhang et al., 2009). Cd translocation from the root zone to shoots is thought to be a key mechanism for increasing plant Cd tolerance. Our findings suggest that BC and TU are active in the deposition of Cd in cell walls or maybe the Cd-chelation reaction in maize plants, reducing Cd toxicity (**Figures 6**, **7**). Furthermore, the combined utilization of BC and TU could have the same improving behavior as other trace metals.

The use of BC and TU reduces the Cd content in plant tissues by minimizing the bioaccumulation of the metal in the plant and its translocation from the soil to the plant. Enrichment of BC can reduce the Cd content in the biosphere by increasing soil pH and by various mechanisms, i.e., complexation, physical sorption, precipitation, membrane filtration, ion exchange, and electrostatic interactions, thereby affecting metal speciation in the rhizosphere and reducing Cd availability (Sfaxi-Bousbih et al., 2010; Ali et al., 2013). Changes in Cd adsorption caused by BC and TU arbitrated variations in the nutrient contents in the soil can reduce the uptake of Cd by maize and its subsequent accumulation to stem and grain of maize (Ahmad et al., 2018). Likewise, exogenous foliar application of TU has been shown to improve rice Cd resistance by increasing hemicelluloses and pectin contents in the root cell wall (Perveen et al., 2015). BC also comprises micronutrients such as silicon (Si), and the wall-bound Si in the rice cell can inhibit the Cd uptake. Decreased Cd accumulation in maize, shoot, and root parts under combined application of B_2T_2 can be attributed to a non-availability and lower Cd uptake. Exogenous addition of BC or TU to cereal plants, i.e., wheat, rice, sorghum, maize, and barley, could alter the Cd distribution. In recent years, it was reported that BC incorporation suppresses the gene expression involved in Cd transport and Cd uptake, as well as apoplastic Cd translocation to shoots, potentially reducing Cd toxicity. Due to its high mobility and solubility, plants grown in Cd-contaminated soils can consume Cd easily (Abbas et al., 2017). Cd absorption causes photosynthesis inhibition, chlorophyll depletion, growth retardation, and most significantly, accumulation of Cd in maize edible parts such as grain, resulting in adverse and even lethal effects for the health of human beings *via* contaminating the food chain (Kamnev and Van Der Lelie, 2000). So, combining the application of BC and TU to maize could increase the maize growth and grain quality while also lowering the human health risk allied with consuming Cd-contaminated food. The utilization of BC and TU in combination could offer a cost-effective and environmentally safe way to reduce Cd toxicity. Furthermore, BC fertilizers are now inexpensive, and the quantity of TU added is lower, all of which are practical for practical applications.

CONCLUSION

Early identification of Cd translocation and accumulation is critical to deal with food-safety issues, and this necessitates the in-season measurement of Cd accumulation in plants. Overall, the experiment results verify our hypothesis that combined application of BC and TU decrease Cd concentration in plant organs and improve maize growth and development. Specifically, a higher application rate of B_2 (5% w/w) and T_2 (1,200 mg L^{-1}) significantly decreases Cd concentration in maize shoot and root. However, higher soil Cd was present under B_2T_2 due to lower plant uptake than B_0T_0. A higher application rate of TU and BC also improved crop growth by increasing root dry biomass, root fresh biomass, and total root length as compared with B_0T_0. The maize physiological activities, such as intercellular CO_2, photosynthetic rate, and stomatal conductance, were significantly improved under B_2T_2 and B_2T_1 treatments. Overall, our results suggest that the combined application of a higher level of B_2T_2 (B_2; 5% w/w, T_2; 1,200 mg L^{-1}) not only provides a simple and enumerate agronomic management strategy for reducing

Cd concentration in maize plant organs but also improves its physiological activities in Cd contaminated soil. Nevertheless, further farming studies should be seriously investigated in the future to conclude a more precise and effective strategy for reducing Cd bioavailability in the rhizosphere.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

FU, CL, AL, and MR: conceptualization. FU and AL: methodology. FU, AL, and MR: validation and formal analysis. NA, AL, and AS: investigation and data curation. FU, AL, MR, SA-U-K, CL, WS, MS, MB, and AS: writing—original draft preparation, review, and editing. CL, AL, and MR: supervision and project administration. All authors contributed to the article, read, and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.809322/full#supplementary-material

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Transcriptome Analysis Revealed the Mechanisms Involved in Ultrasonic Seed Treatment-Induced Aluminum Tolerance in Peanut

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Ultrasonic (US) treatment is an efficient method to induce crop tolerance against heavy metal toxicity; however, US-induced aluminum (Al) tolerance in peanuts was rarely studied. This study was comprised of two treatments, namely, CK, without ultrasonic treatment, and US, an ultrasonic seed treatment, for 15 min. Both treated and non-treated treatments were applied with Al in the form of $AlCl_3.18H_2O$ at 5 mmol L^{-1} in Hoagland solution at one leaf stage. Results depicted that plant height, main root length, and number of lateral roots increased significantly under US treatment. Transcriptome analysis revealed that plant hormone signal transduction and transcription factors (TFs) were significantly enriched in the differentially expressed genes (DEGs) in US treatment, and the plant hormones were measured, including salicylic acid (SA) and abscisic acid (ABA) contents, were substantially increased, while indole acetic acid (IAA) and jasmonic acid (JA) contents were decreased significantly in US treatment. The TFs were verified using quantitative real-time (qRT)-PCR, and it was found that multiple TFs genes were significantly upregulated in US treatment, and *ALMT9* and *FRDL1* genes were also significantly upregulated in US treatment. Overall, the US treatment induced the regulation of hormone content and regulated gene expression by regulating TFs to improve Al tolerance in peanuts. This study provided a theoretical rationale for US treatment to improve Al tolerance in peanuts.

Keywords: aluminum, peanut, growth, gene expression, transcription factor genes

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INTRODUCTION

As much as 40–50% of the potentially arable lands are acidic in the world, and more than 40% of the arable lands in China are acidic soil, distributed in South China, with a total area of 2.04×10^7 km, including Guangdong Province (Kochian et al., 2015; Li, 2019). Aluminum (Al) is the most abundant metal element in nature, accounting for 7.45% of the total weight of the crust of the earth, and exists in a dormant state. When the soil was acidified ($pH \leq 5.5$), Al^{3+} would be released. When the pH was <4.3, a large amount of Al^{3+} would be released, which would cause harmful effects on plants (Han et al., 2020). The soil types in southern China were mainly red soil, latosol soil, and yellow-brown soil (Yin, 2017). The total nitrogen content of the soil was generally 0.04–0.18%. The inorganic phosphorus was mainly iron phosphate and aluminum salt. The total phosphorus content of red soil was generally 0.01–0.03%, and the total potassium content was 0.9–0.4%, respectively. Most of the trace elements in the soil were in the form of inorganic salts (Zhu et al., 2005). As the desilication and aluminization process of red soil is a slow acidification process, it is also affected by acid rain, environmental pollution, and farming methods. In the second soil survey, the pH of the soil was mostly 6.0–6.5 in South China. The current soil fertility monitoring results showed that the pH of the soil had dropped by 0.2–0.5 pH units (Li et al., 2013). At the same time, the acidic red soil in this area was dominated by kaolin. The cation exchange capacity was low, and the acid buffer capacity was weak. In addition, the temperature in this area was relatively high, and organic matter was easily decomposed, which made less contribution to the acid buffer capacity (Li et al., 2013). Peanut (*Arachis hypogea* L.) is among the four major oil crops with great economic importance (Liu et al., 2020). The growth and development of peanuts had been impaired by Al toxicity for a long time in South China, and the average yield had been reduced by more than 20% which is the most important reason for low peanut yield in this region than the national average (Zhang et al., 2009; Zhang and Hu, 2020).

Aluminum toxicity limits crop production in those parts of the world. The effects of Al toxicity on crops were manifold, and it was generally believed that the main site of Al toxicity was the root tip (Wang Y. Q. et al., 2020). On the one hand, it was possible to influence the transport of molecules across the membrane by changing the properties of the cell wall, thereby causing a disorder of the intracellular metabolic process (Singh et al., 2017). On the other hand, it restricted the absorption of essential elements such as magnesium (Mg), phosphorus (P), and molybdenum (Mo), thereby affecting plant growth (Rahman et al., 2018). In addition, Al also inhibited the ductility of the leaves, stomata closure, and photosynthesis (Rahman et al., 2018; Singh et al., 2021). Research on Al in peanuts was relatively rare. Al stress caused the peroxidation of root tip cell membrane lipids and resulted in damage to the plasma membrane. After the injury, the plasma membrane was overloaded with Ca^{2+}, and cytochrome C was released into the cytoplasm to induce programmed death of root tip cells, thereby inhibiting root growth (Zhan et al., 2009; Yao et al., 2014). Generally, there are two mechanisms responsible for Al tolerance in plants. First, an exclusion mechanism involved the secretion of organic acids, phenolic compounds, phosphates, and so on for chelating Al^{3+} into non-toxic compounds, thus preventing them from entering into root cells (Liu, 2018; Fang et al., 2020; Chauhan et al., 2021). Second, the internal tolerance mechanism referred to that after Al^{3+} enters the cytoplasm, the Al^{3+} in the cells were transported to the vacuole through the transporter to isolate them from other organelles, and thus Al^{3+}-induced disruptions to the plant body were reduced (Zhang X. et al., 2019; Dai et al., 2020; Quimbaya et al., 2020). Two transporter families related to organic acid secretion, namely, multidrug and toxic compound extrusion (*MATE*), which regulates the secretion of citric acid, and aluminum-activated malate transporter (*ALMT*), which regulates the secretion of malate to chelate Al^{3+}, respectively. Al^{3+} entering the cytoplasm might be transported into the vacuole through the regulation of *ALS1* or *VALT1* genes (Zhang X. et al., 2019; Fang et al., 2020; Chauhan et al., 2021; **Figure 1**).

Ultrasonic (US) treatment involves low to medium frequency (20–100 kHz) sound waves and is considered a cheap, safe, simple, and environment-friendly technology (Chen Y. P. et al., 2013). The application of ultrasound in agriculture was one of the research topics of applying new technologies in physics to large-scale agricultural production in modern agriculture (Zheng et al., 2008; Zhu et al., 2008). A series of mechanical, high-pressure, thermal, electrochemical, photochemical, oxygen, enzyme, and biological effects caused by ultrasound on organisms could sterilize seeds, promote seed germination, and increase yields (Liu J. et al., 2016). The modifications in seed coats by US waves could enhance the seed germination rate and early growth of the plant (Sun et al., 2020). US treatment could also restore seed vigor by improving antioxidant activities, such as superoxide dismutase (SOD) and peroxidase (POD), and reducing malondialdehyde (MDA) content (Ali et al., 2013a,b; Huang et al., 2021). Moreover, US treatment had substantial regulatory effects in different crops, for instance, US treatment reduced lead (Pb) accumulation in brown rice (Rao et al., 2018) and improved cadmium (Cd) tolerance in oilseed rape (*Brassica campestris* L.) (Rao et al., 2019). Studies on lupin (*Lupinus polyphyllus*) and buckwheat (*Fagopyrum mill*) showed that US treatment not only promoted seed germination but also improved antioxidant defense system (Zhang et al., 2015; Guo, 2016). Thus, employing US technology to treat seeds could effectively enhance the ability of the crop to resist various abiotic stresses and could prove to be a quality agricultural seed treatment method in future (Ge and Ren, 2019).

In addition, plant hormones, i.e., salicylic acid (SA), jasmonic acid (JA), ethylene, abscisic acid (ABA), and indole acetic acid (IAA), play a crucial role in responding to Al stress (Shen et al., 2004; Yang et al., 2016; Ye et al., 2018; Wang Z. R. et al., 2020). ROS induced by Al stress affects the homeostasis of IAA (Panda et al., 2009; Yuan et al., 2013b; Wang M. et al., 2019). The accumulation of ROS induced by Al stress changed the intracellular redox balance and polar transport of auxin by regulating the expression of PIN protein (Chapman et al., 2019; Swarup and Bhosale, 2019). Al stress enhanced the expression of JA receptor *COI1* and JA signal regulator *MYC2* (Yang et al., 2017). Exogenous application of IAA, cytokinin, and ABA positively affect Al-induced root inhibition (Ranjan et al., 2021). Cytokinin and IAA participated in the regulation of the response process against Al stress synergistically (He et al., 2012). Furthermore, transcription factors (TFs) such as STOP1, ART1, and WRKY were involved in the mechanism of Al tolerance in crops (Yamaji et al., 2009; Godon et al., 2019; Li C. et al., 2020). Previously, effects of Al stress on various crops have been widely reported; however, US-induced modifications in internal mechanisms responsible for Al tolerance in peanut were rarely reported. Therefore, this study was conducted to get insights into the mechanisms involved in US treatment-induced Al tolerance in peanuts.

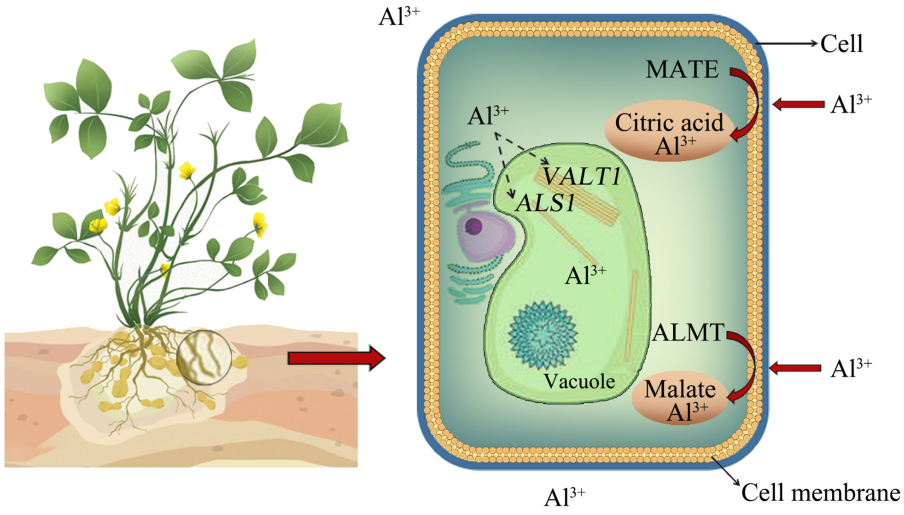

FIGURE 1 | External rejection mechanism of aluminum (Al) and internal tolerance mechanism. Organic acids could chelate Al3+ into non-toxic compounds, thereby preventing them from entering root cells. MATE regulates the secretion of citric acid, and ALMT regulates the secretion of malate to chelate Al3+. Al3+ entering the cytoplasm might be transported into the vacuole through the regulation of *ALS1* or *VALT1* genes (Zhang X. et al., 2019).

MATERIALS AND METHODS

Experimental Setup

The uniform seeds of peanut cultivar "Guihua58" were US treated for 15 min (at 20–40 kHz frequency) using a small tunnel-type plant seed dry method ultrasonic treatment machine (5ZCG-T6, Golden Rice Agricultural Science and Technology Co. Ltd., Guangzhou, China) regarded as US and non-US treated seed regarded as CK. The experiment was conducted at the Guangzhou Key Laboratory for Research and Development of Crop Germplasm Resources, Zhongkai University of Agriculture and Engineering, Guangzhou, China (23104 N, 113281 E). Three statistical replicates of each treatment and 100 g of seeds per treatment were used. After treatment, peanut seeds were placed on a wet filter paper for 3 days to germinate, at a room temperature of 26°C, and water was added to maintain moisture for 3 days. The seeds with uniform germination were selected and sown in a petri dish with twenty capsules in each petri dish. After culturing for 3 days, the seedlings were transferred to Hoagland nutrient solution containing plastic culture bowls. When the seedlings grew to one leaf and one heart stage (8 days of growth), the $AlCl_3.18H_2O$ (5 mmol L^{-1}) was added to the nutrient solution for Al stress treatment. The nutrient solution was replaced once in every 3 days. The experiment was conducted at room temperature with 12 h day/12 h night photoperiod. The seedlings were harvested 5 days after Al treatment for the determination of morphological traits, Al contents, as well as biochemical and molecular analyses.

Morphological Traits

Plant height and main root length were determined after harvesting of seedlings using a scale from all the treatments and averaged. The number of lateral roots of each seedling was counted from each treatment and averaged.

Determination of Aluminum Concentration

The dried sample (0.2 g) in powder form was digested with diacidic mixture of $HClO_4:HNO_3$ (1:4 v/v) for 4 h, and the volume was adjusted to 25 ml after filtration. The Al contents in the sample were measured using Atomic Absorption Spectrophotometer (AA6300C, Shimadzu, Japan) (Ashraf et al., 2017; Bao et al., 2021).

Transcriptome Sequencing

The purity, concentration, and integrity of RNA were tested using Nanodrop2000 (Thermo Fisher Scientific, Wilmington, DE). High-quality RNA was used to construct the cDNA library. The q-PCR method was used to accurately quantify the effective concentration (>2 nM) of the library. Transcriptome sequencing was performed on the Illumina platform with three biological replicates for each sample.

Bioinformatics Analysis

The clean reads were obtained from the original reads through quality control. The clean reads were compared with the genome sequence of the cultivated peanut Tifrunner to get the mapped reads.[1] After quantitative gene analysis, differentially expressed genes (DEGs) were screened for functional annotation and enrichment analysis. The amount of gene expression used fragment per kilobase million (FPKM) to indicate the level of gene expression. Fold change ≥ 2 and FDR < 0.01 were used as the screening criteria. Raw sequencing data have been uploaded

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[1] http://peanutbase.org/

in the NCBI Gene Expression Omnibus under the accession number PRJNA PRJNA753947.[2]

Determination of Jasmonic Acid, Abscisic Acid, Salicylic Acid, and Indole Acetic Acid Contents

Fresh peanut leaves (100 mg) were extracted with acetonitrile and centrifuged at 4°C at 12,000 rpm for 10 min and then used for LC-MS analysis (Vanquish, Thermo, United States). Waters HSS T3 (50 mm × 2.1 mm, 1.8 μm) liquid chromatography column, in which the injection volume was 2 μl and the column temperature was 40°C, with mobile phase A (0.1% acetic acid/acetonitrile) and mobile phase B (0.1% acetic acid/water) was used. The optimized mass spectrometry analysis conditions were as follows: sheath gas 40, auxiliary gas 10, ion spray voltage −2,800 V, temperature 350°C, and ion transfer tube temperature 320°C.

Quantitative Real-Time PCR

The TRIZOL method was used to extract total RNA and further synthesize cDNA (TaKaRa, Beijing, China). Based on the representative sequence of the sequence library required for sequencing, Primar5.0 was used to design qPCR primers (**Supplementary Table 1**). The peanut *actin* gene was used as the internal reference gene. There were three statistical replicates for each gene. The $\Delta\Delta$Ct analysis was used for gene expression analysis. The $2^{-\Delta\Delta Ct}$ method was used to calculate the amount of gene expression.

Statistical Analysis

The treatments were arranged in a completely randomized design (CRD). SPSS Statistics 20.0 (IBM, Chicago, United States) was used for one-way analysis of variance, and the Tukey's test at the 5% significance level was used to determine the difference among the treatments.

RESULTS

Ultrasonic Treatment-Induced Modulations in Morphological Traits and Aluminum Contents

Ultrasonic treatment substantially improved the plant height and main root length in peanuts under Al stress. Compared with CK, the plant height and main root length were increased by 2.31- and 1.6-fold, respectively, under US treatment in peanuts. Moreover, the Al contents were decreased by 53.07 and 18.8% in leaves and roots under US treatment than CK (**Figures 2A–C**).

Ultrasonic Treatment Regulated the Expression of *ALMT9* and *FRDL1*

Compared with CK, the expression of *ALMT9* and *FRDL1*was increased by 222.9 and 132.1% under US treatment (**Figures 3A,B**).

Sequencing Quality Analysis

Transcriptome sequencing of CK and US of "Guihua58" showed clean reads between 19383803 and 23820080. The GC content was between 44.7 and 45.1%, whereas Q30 was greater than 92.22%. Mapped reads were noted between 93.65 and 95.05%. Uniq Mapped reads were noted between 85.34 and 86.39%, while multiple mapped reads were noted between 8.31 and 8.8% (**Table 1**).

Differentially Expressed Genes and Pathway Analysis

Compared with CK, US treatment had 1,667 DEGs, of which 455 were upregulated and 1,212 were downregulated (**Figure 4A**). The volcano plot showed the similarity of gene expression and DEGs in CK vs. US (**Figure 4B**). The kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis showed that 55 DEGs (66% of all genes) were annotated into the KEGG pathway, and among them, 123 DEGs were significantly enriched (**Figure 5**). Among the up-annotated genes, the significantly enriched KEGG pathways were "peroxisome" and "plant hormone signal transduction." Among the down-annotated genes, the significantly enriched KEGG pathways were "endocytosis" and "plant hormone signal transduction."

The Effect of Ultrasonic Treatment on Salicylic Acid, Abscisic Acid, Indole Acetic Acid, and Jasmonic Acid Contents

Compared with CK, the SA and ABA contents were increased by 913.08 and 202.91% under US treatment (**Figures 6A,B**). In contrast, the JA and IAA contents were decreased by 33.25 and 53.06% under US treatment, as compared with CK (**Figures 6C,D**).

The Effect of Ultrasonic Treatment on Transcription Factors

Ultrasonic treatment affects the gene expression of TFs. The key TFs associated with US treatment in peanuts are exhibited in **Figure 5**. Among them, AP2 (28), bHLH (17), WRKY (11), MYB (8), and NAC (4) were differently expressed in both treatments (**Figure 7A**), where 15 TF genes were selected for qRT-PCR verification. The gene expression level was consistent with the trend of the FPKM value, indicating that the transcriptome data were reliable (**Figure 7B**). The PCA and PLS-DA showed that NAC had the greatest regulatory effect on the *ALMT9* and *FRDL1* in US treatment (**Figures 7C,D**).

DISCUSSION

In previous studies, US treatment could trigger the antioxidant defense mechanism, such as increasing the activity of POD, catalase (CAT), ascorbate peroxidase (APX), and glutathione (GSH) contents (Huang et al., 2021). US treatment could produce irreversible changes in plant cells, such as the enlargement of microchannels and intercellular spaces, and improve the activity of biologically active hydrolysates, including plant primary

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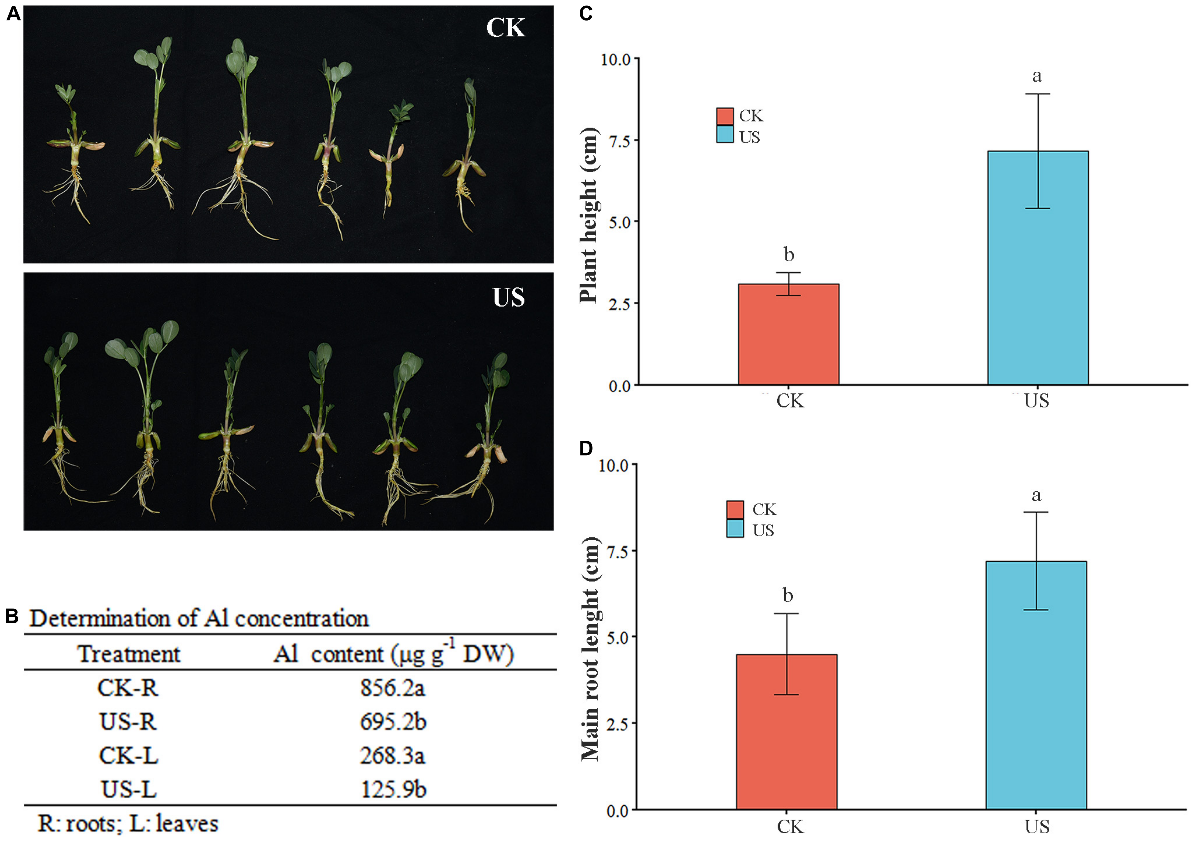
[2] http://www.ncbi.nlm.nih.gov/geo



B Determination of Al concentration

Treatment	Al content (μg g^{-1} DW)
CK-R	856.2a
US-R	695.2b
CK-L	268.3a
US-L	125.9b

R: roots; L: leaves

FIGURE 2 | Ultrasonic (US) treatment improved Al resistance in peanut. **(A)** Photography was performed after 5 days of Al treatment at room temperature. **(B)** Zn contents in leaves and grains in fragrant rice. Plant height **(C)** and main root length **(D)** were measured after 5 days of Al treatment at room temperature. Marking the same letters means $P \geq 0.05$ (LSD), and there is no significant difference; the difference between different letters means $P < 0.05$ (LSD), and the difference is significant.

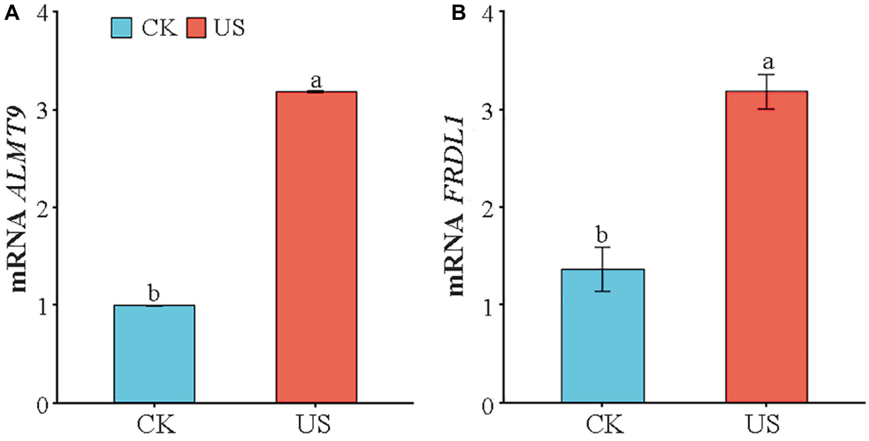


FIGURE 3 | Analysis of transcript levels of **(A)** *ALMT9* and **(B)** *FRDL1*. Marking the same letters means $P \geq 0.05$ (LSD), and there is no significant difference; the difference between different letters means $P < 0.05$ (LSD), and the difference is significant.

and secondary metabolites (Rajewska and Mierzwa, 2017). For example, US treatment could increase gamma-aminobutyric acid (GABA) content in rice, and GABA content was closely related to stress (Ding et al., 2018a). US treatment caused the temperature to rise, and the enzyme activity increased with the increase in temperature, and the invocation of enzymes was conducive to

TABLE 1 | Statistics of raw sequencing data results.

	Clean reads	GC content	%≥Q30	Mapped reads	Uniq mapped reads	Multiple map reads
CK-1	2,38,20,080	45.04%	92.92%	45,201,903 (94.88%)	41,009,840 (86.08%)	4,192,063 (8.80%)
CK-2	2,03,63,133	44.88%	92.57%	38,700,086 (95.02%)	35,306,767 (86.69%)	3,393,319 (8.33%)
CK-3	1,94,06,816	44.93%	92.93%	36,893,310 (95.05%)	33,610,053 (86.59%)	3,283,257 (8.46%)
US-1	1,93,83,803	45.10%	92.45%	36,643,216 (94.52%)	33,333,320 (85.98%)	3,309,896 (8.54%)
US-2	2,11,41,555	44.70%	92.31%	39,598,744 (93.65%)	36,084,199 (85.34%)	3,514,545 (8.31%)
US-3	2,03,92,853	44.98%	92.22%	38,208,017 (93.68%)	34,804,622 (85.34%)	3,403,395 (8.34%)

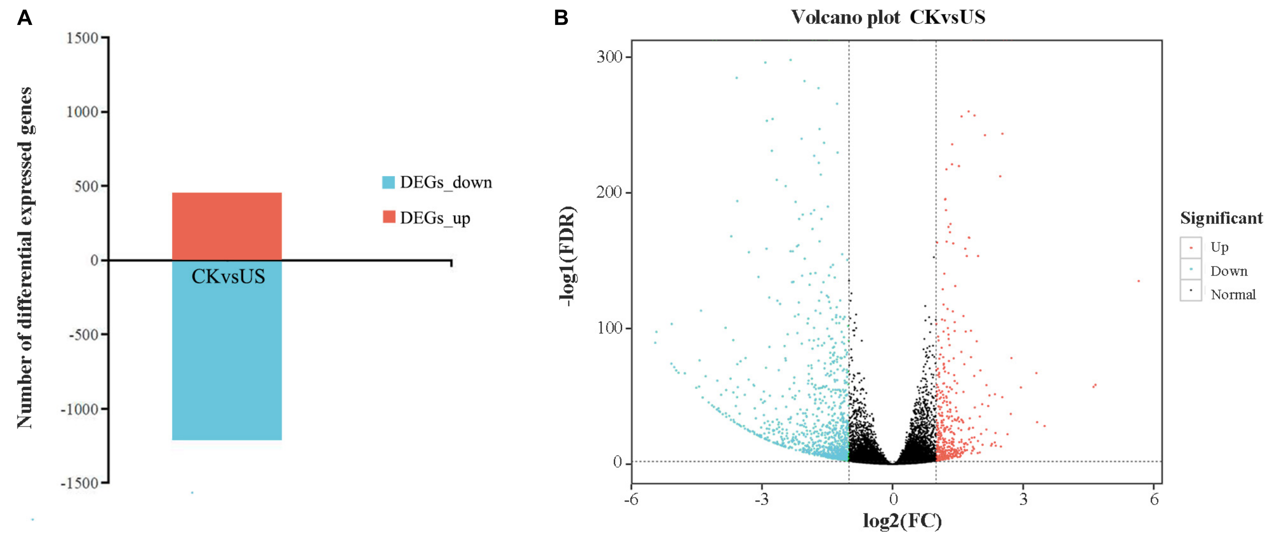


FIGURE 4 | Summary of differentially expressed genes (DEGs) and volcano plot analysis. **(A)** DEGs. **(B)** Volcano plot analysis. Red color indicates upregulated genes. Blue color indicates downregulated genes.

further growth and development (Ding et al., 2018a). Holes and cracks were found in the surface microstructure of the germinated brown rice grains after US treatment (Ding et al., 2018b). Plants have developed exclusion and internal tolerance mechanisms for heavy metal stress tolerance (Ali et al., 2014, 2015; Mwamba et al., 2016, 2020). Similarly, Al tolerance mechanisms have been explained in **Figure 1**. Generally, the root tip was an initial part of a plant that came in contact at first with Al^{3+} in soil solution and/or Al contaminated growing medium (Shen and Yan, 2001). High concentrations of Al toxicity could cause obvious morphological and structural changes in peanut root tip cells, which were manifested by inhibiting root elongation and causing programmed cell death (Zhan et al., 2008), such as *Panax ginseng* (Farh et al., 2017), *Zea mays* (Kidd et al., 2001), *Triticum aestivum* (Ma and She, 2006), *Allium cepa* (Achary et al., 2012), *Camellia sinensis* (Qu et al., 2021), *Glycine max* (Dos et al., 2018), *Oryza sativa* L. (Liu S. et al., 2016), *Arabidopsis* (Degenhardt et al., 1998), and *Citrus sinensis* (Yang et al., 2021). Al^{3+} entered the nucleus of wheat root cells to condense chromatin or bind DNA molecules, causing DNA damage in the rhizosphere (Ma and She, 2006). Al induced ROS to promote oxidative burst, causing cell death and DNA damage in *A. cepa* (Achary et al., 2012). Al stress significantly inhibited the growth of tea seedlings by reducing chlorophyll synthesis and reducing photosynthetic efficiency (Qu et al., 2021). After Al treatment, Al-tolerant corn cultivars not only secrete citric acid but also secrete a mixture of phenols including catechol, catechin, and bark to chelate Al, thereby improving the Al tolerance of corn (Kidd et al., 2001). However, there were relatively few studies on how to improve the Al tolerance mechanism of peanuts. In this study, it was found that US treatment substantially improved the plant height and main root length in peanuts under Al stress. Moreover, the Al contents were found to be decreased in roots and leaves under US treatment than CK (**Figure 2**). A large number of studies had found that US treatment mainly stimulates the internal material properties and physiological activities of plants through vibration. For example, it could promote the oxidation, reduction, decomposition, and synthesis of the internal substances of plant cells. By increasing the germination rate and germination potential of seeds, the yield and quality of crops could be improved (Fu et al., 2020).

The two transporter families MATE and ALMT play an essential role in improving the resistance to metal toxicity, counterion stability, and promoting the absorption of mineral elements in crops (Omote et al., 2006; Sharma et al., 2016). Through heterologous expression in *Xenopus oocytes*, it was found that the MATE gene in tobacco, wheat, and barley could promote citric acid efflux and enhance its Al tolerance

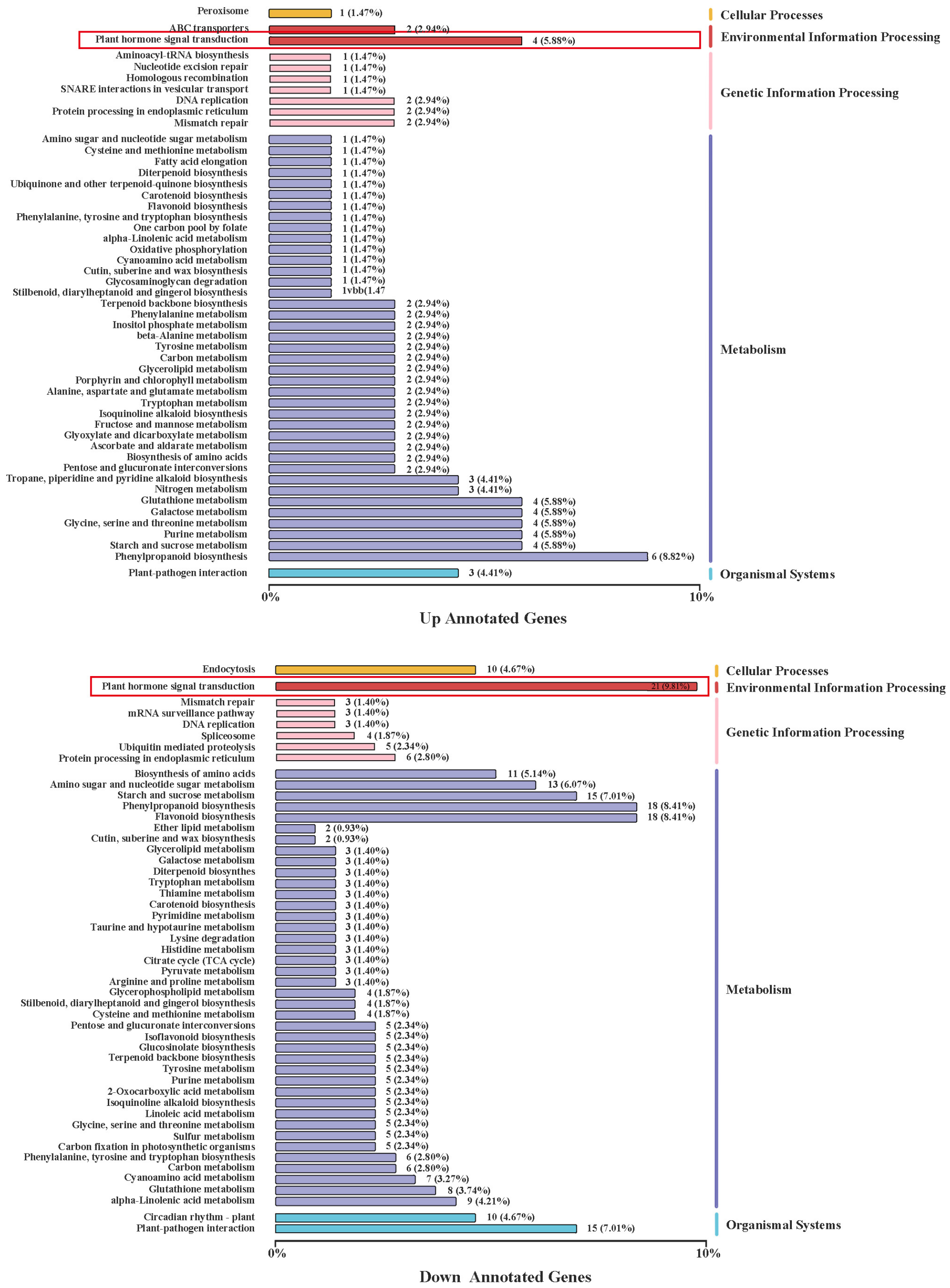


FIGURE 5 | Regulations of the KEGG pathway.

(Furukawa et al., 2007). *ALMT* encoded a malate transporter while also performing other important functions, such as maintaining the balance of malate and participating in cell osmotic regulation (Kochian et al., 2015). *ZmALMT1* was found to be involved in ion-selective transport in maize root tips (Sharma et al., 2016); *HvALMT1* was involved in the regulation of stomata opening and closing and root growth and development in barley (Xu et al., 2015). The *AhFRDL1* gene was cloned in peanuts, and the expression of the *AhFRDL1* gene was upregulated to improve the tolerance of roots to Al stress

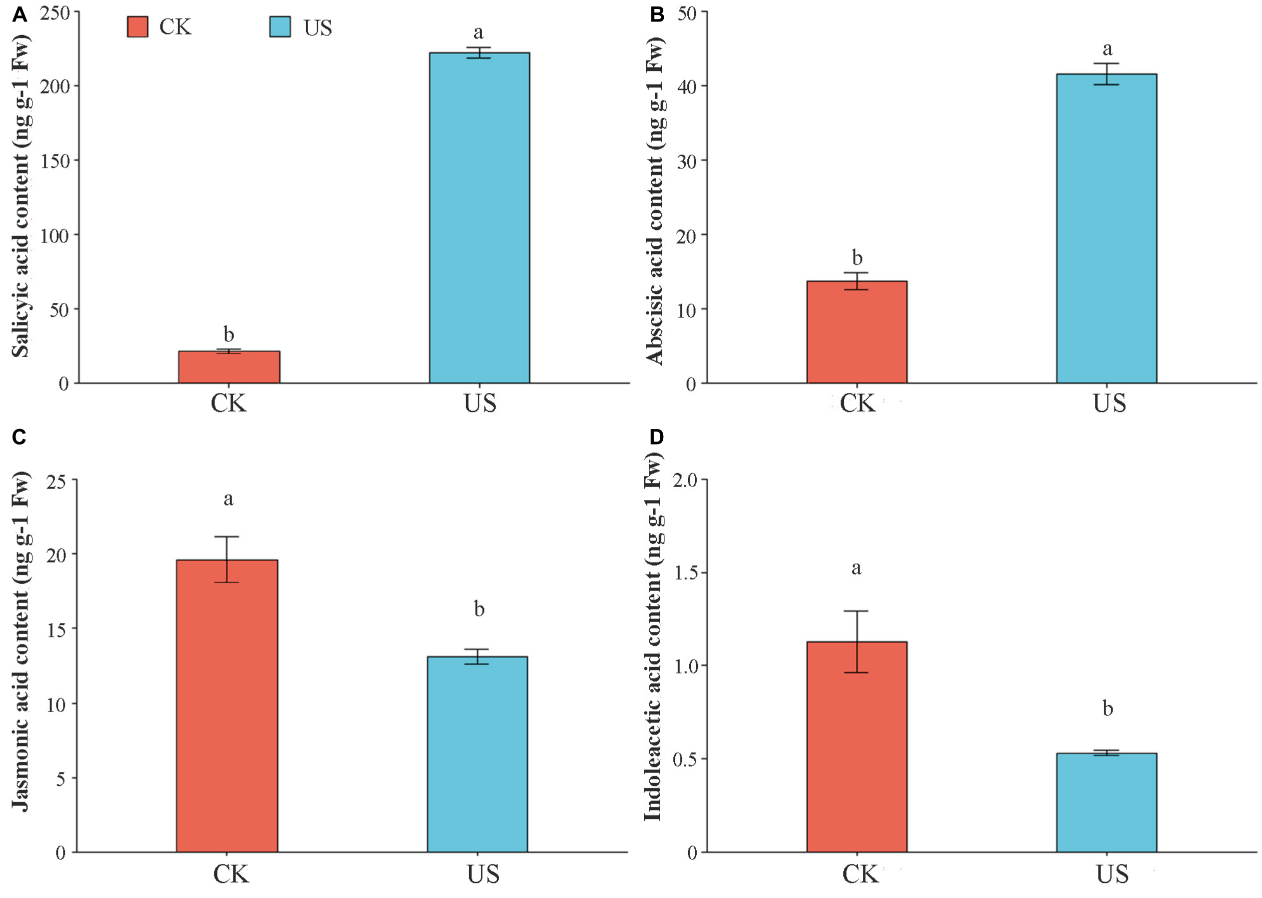


FIGURE 6 | The effect of ultrasonic treatment on salicylic acid (SA) **(A)**, abscisic acid (ABA) **(B)**, indole acetic acid (IAA) **(C)**, and jasmonic acid (JA) **(D)**. Marking the same letters means $P \geq 0.05$ (LSD), and there is no significant difference; the difference between different letters means $P < 0.05$ (LSD), and the difference is significant.

(Qiu et al., 2019). This study showed that the expression of the *FRDL1* gene was also upregulated after ultrasound treatment (**Figure 3B**). Furthermore, it was found that Al-tolerant cultivars of wheat (*T. aestivum* L.), soybean (*G. max*), and *Arabidopsis* (*Arabidopsis thaliana*) had higher *ALMT* gene expression than the sensitive cultivars (Liang et al., 2013). We have also found that the expression of the *ALMT9* gene was significantly upregulated under US treatment (**Figure 3A**), indicating that US treatment could increase the expression of the *FRDL1* and *ALMT9* genes to increase the external Al excretion mechanism of peanut.

Transcriptome analysis revealed that KEGG enrichment analysis showed that the DEGs of plant hormone signal transduction were significantly enriched (**Figure 5**). Plant hormones play an important role in the Al tolerance of crops. For instance, the SA induced the Al stress tolerance by inducing plant systemic resistance (SAR), cellular antioxidant mechanisms, and photosynthesis (Wang et al., 2016). Under Al stress, the accumulation of endogenous ABA content in soybeans played an essential role in Al tolerance (Hou et al., 2010). This study found that the SA and ABA contents were significantly upregulated after US treatment, indicating that US treatment could promote the perception stage of hormones and the signal transmission stage to promote their synthesis (**Figure 6B**). The endogenous JA in corn played an important role in the drought resistance response. The lack of endogenous JA could reduce water loss and improve the survival ability under drought conditions (Wang H. Y. et al., 2019). In this study, it was found that the JA content was also significantly reduced under US treatment (**Figure 6C**). Cytokinin and IAA participated in the regulation of the response process against Al stress synergistically (He et al., 2012). The interaction between IAA and cytokinin under Al stress was mainly linked by auxin-responsive transcription factor (ARF). Under Al or metal stress, ARF7 mediated the stalk between IAA and cytokinin and promoted the synthesis of isopentenyl transferases (IPT). The IPT-dependent cytokinin acted on the downstream of ARF7-mediated auxin signal and synergistically regulated the inhibition of root growth (Yang et al., 2017). However, this study found that IAA content was downregulated under US treatment (**Figure 6D**), which may be related to the regulation of IAA and cytokinin in a synergistic manner.

In addition, four main types of TFs were involved in the regulation of Al tolerance in peanuts. First, the expression of

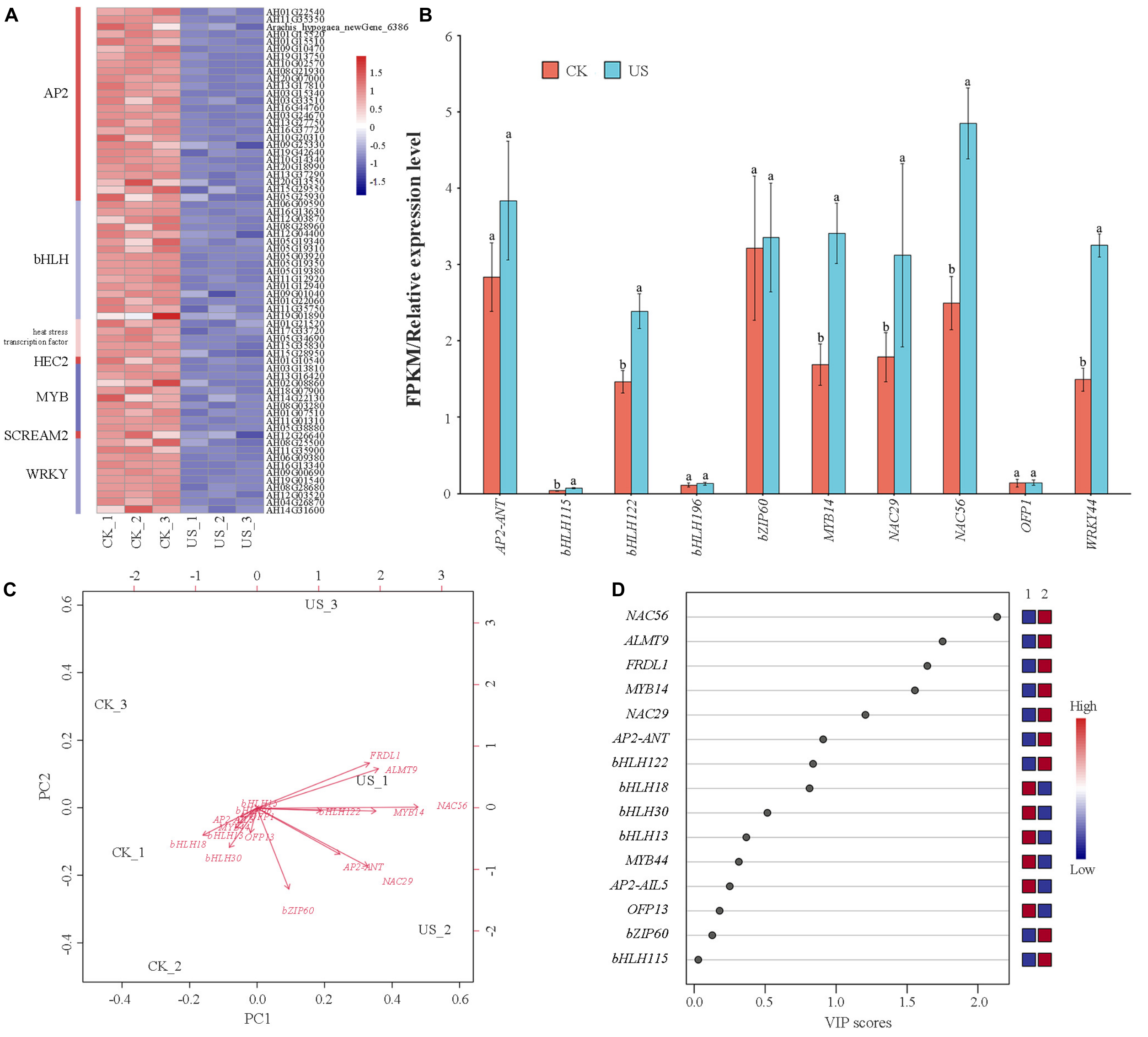


FIGURE 7 | Heatmap of known 33 transcription factors (TFs). **(A)** Red color indicates downregulation, and blue color indicates upregulation. **(B)** Comparison of the FPKM value obtained using RNA-seq analysis with the gene expression obtained using quantitative real-time (qRT)-PCR analysis. **(C)** Principal component analysis (PCA). **(D)** Partial least squares discriminant analysis (PLS-DA).

Al-tolerant genes increased with the copy number of genes in the genome (Daspute et al., 2017). Second, the transposon insertion at the front end of the Al-tolerant gene, i.e., insertion of the transposon could be used as the promoter of the subsequent gene and enhanced the expression of the subsequent gene (Ferreira et al., 2017). Third, the tandem repeats in the promoter region enhanced the expression level of resistance genes (Ryan et al., 2010). The expression level of the fourth resistance gene was related to the number of *cis*-acting elements of the TF ART1 (Chen Z. C. et al., 2013). In *Arabidopsis*, *AtSTOP1* could regulate the expression of downstream Al-tolerant genes, but it was not affected by Al stress at the transcription level. Studies have shown that *AtSTOP1* was regulated by the F-box protein RAE1 (AL-activated malate transporter expression 1) after transcription. RAE1 regulated the stability of *AtSTOP1* through the ubiquitin/26S proteasome pathway (Zhang Y. et al., 2019). Guo et al. (2020) showed that hyperrecombination protein 1 (HPR1) regulated the output of nuclear-cytoplasmic *STOP1* mRNA, thereby regulating the expression of genes downstream of *STOP1*. Fang et al. (2020) showed that the SUMO of STOP1 was involved in the regulation of Al tolerance. In rice, the homologous protein *OsART1* participated in rice Al tolerance by regulating the expression of downstream Al-tolerant genes (Yamaji et al., 2009). Members of the WRKY family-involved Al stress response have been found in both *Arabidopsis* and rice. In *Arabidopsis*, *WRKY46* was a transcriptional repressor. It promoted the expression of *AtALMT1* through the downregulation of its expression level, increased root tip malic acid secretion, and ultimately improved the Al tolerance of *Arabidopsis* (Ding et al., 2013). Recently, Li C. X. et al. (2020) identified another member of the WRKY family, *AtWRKY47*, which modulated the distribution of Al between apoplasts and symplasts by regulating genes related to cell wall modification, thereby increasing the Al tolerance of *Arabidopsis*. Lou et al. (2019) found that the NAC family was involved in the Al tolerance of *Vigna unguiculata*. This study found that the gene expression of many TFs was upregulated under US treatment (**Figure 7A**), and the expression of *ALMT9* and *FRDL1* genes were also upregulated. This may be due to the effect of US treatment on TFs related to the further regulation of gene expression; however, the mode through which TFs regulate gene expression requires further research. We speculated that US treatment may improve the external rejection mechanism and internal tolerance mechanism of peanuts by regulating the antioxidant defense system, enzyme activity, hormones, and TFs. Ultrasonic treatment, as a pollution-free physical treatment technology, had the value of popularization and application.

CONCLUSION

Ultrasonic treatment improved the morphological traits of peanuts under Al stress. US seed treatment regulates the expression of internal hormones, namely, SA, ABA, IAA, and JA, and TFs, which further regulate gene expression (*ALMT9* and *FRDL1*) to improve the Al tolerance in peanuts. There is no doubt that the US seed treatment induced the Al tolerance in peanuts, but it is necessary to optimize the seed treatment time and frequency for different peanut cultivars and further experiments in the field are required.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA753947.

AUTHOR CONTRIBUTIONS

YZ, GB, and XW designed the experiment. GB, QZ, SL, SH, AM, and ZC performed the experiment, data collection, lab analysis, and data analysis. QZ, SL, and SH contributed in providing chemicals, reagent, analyses, and tools. GB and QZ prepared the initial draft. GB and UA finalized the initial draft. XW was fully responsible for the distribution of all materials associated with this manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.807021/full#supplementary-material

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The Impact of Bio-Stimulants on Cd-Stressed Wheat (*Triticum aestivum* L.): Insights Into Growth, Chlorophyll Fluorescence, Cd Accumulation, and Osmolyte Regulation

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It has been established that wheat (*Triticum aestivum* L.) has a higher Cd absorption capacity than other cereal crops causing an excess daily Cd intake and a huge threat for public health. Therefore, the reduction of Cd accumulation in wheat from the soil is a crucial food-security issue. A pot trial was performed on Cd-stressed wheat seedlings to evaluate the morphological and physio-biochemical responses *via* foliage spray of two different bio-stimulants, i.e., ascorbic acid (AsA) and moringa leaf extract (MLE). Two wheat cultivars (Fsd-08 and Glxy-13) were exposed to cadmium ($CdCl_2.5H_2O$) stress (0, 500, and 1,000 μM), along with foliar spray of AsA (0 and 50 mM) and MLE (0 and 3%). The most observable growth reduction was documented in plants that are exposed to a higher Cd concentration (1,000 μM), followed by the lower Cd level (500 μM). The wheat growth attributes, such as number of leaves per plant, number of tillers per plant, biomass yield, shoot/root length, and leaf area, were greatly depressed under the Cd stress, irrespective of the cultivar. Under the increasing Cd stress, a significant diminution was observed in maximum photochemical efficiency (Fv/Fm), photochemical quenching (qP), and electron transport rate (ETR) accompanied with reduced gas exchange attributes. However, Cd-induced phytotoxicity enhanced the non-photochemical quenching (NPQ) and internal carbon dioxide concentration (Ci), which was confirmed by their significant positive correlation with Cd contents in shoot and root tissues of both cultivars. The contents of proline, AsA, glycine betaine (GB), tocopherol, total free amino acid (TFAA), and total soluble sugar (TSS) were greatly decreased with Cd stress (1,000 μM), while MLE and AsA significantly enhanced the osmolytes accumulation under both Cd levels (especially 500 μM level). The Cd accumulation was predominantly found in the root as compared to shoots in both cultivars, which has declined after the application of MLE and AsA. Conclusively, MLE was found to be more effective to mitigate Cd-induced phytotoxicity up to 500 μM Cd concentration, compared with the AsA amendment.

Keywords: gaseous exchange rate, secondary metabolites, Cd accumulation, ascorbic acid, moringa leaf extract, growth, chlorophyll fluorescence

INTRODUCTION

Heavy metal toxicity in the rhizosphere is a potential health menace to the growing population, as well as great anxiety for environmentalists globally (Ali et al., 2013; Sabir et al., 2020; Turan, 2021a). Cadmium (Cd) is generally released from industries due to various anthropogenic activities, excessive utilization of fertilizers (especially phosphate) in agricultural soils, and natural weathering of rocks and minerals (Ahanger et al., 2017; Turan et al., 2018; Wang et al., 2021; Xing et al., 2022). The rapid increase in industrialization, urbanization, and improper environmental planning has caused various limitations including severe yield reduction in various crop plants such as *Zea mays* L. (An et al., 2022), *Oryza sativa* L. (Sun et al., 2021), and *Triticum aestivum* L. (Hao et al., 2021).

It has been well-established that wheat (*Triticum aestivum* L.) has a higher Cd absorption capacity than other cereal crops (Zhou et al., 2021). In developing countries, more than 80% of wheat supply is used as a staple food, compared to developed countries that use less than 50% (Awika, 2011); thus, inhabitants may intake a high quantity of Cd through grains posing a high health risk to human beings (Abedi and Mojiri, 2020). Previous researchers found that the Cd in wheat is taken up through roots and then translocated to the aerial parts, thus, interfering with major metabolic processes to hinder normal growth and reproduction activities (Abedi and Mojiri, 2020). The photosynthetic processes are the chief modulators for the growth and development of plants (Maishanu et al., 2017). An elevated level of Cd in the rhizosphere imposed severe phytotoxic effects in plant tissues (Riaz et al., 2021b), which replicate itself by hampering plant's morphological, physiological, and biochemical processes (Wali et al., 2015), like destroying the leaf chlorophyll structure to deteriorate photosynthetic and gas exchange relations (Vieira Santos et al., 2001; Malik et al., 2021). The photosynthetic system is highly prone to any modulation in the surrounding environment. Any environmental constraints will directly damage the structural and functional capacities of the photosynthetic apparatus and may disturb plant growth and survival (Saleem et al., 2020).

It has been established that Cd induces toxic effects by reacting with sulfur and nitrogen atoms (Astolfi et al., 2004). These atoms are involved in the biosynthesis of certain important amino acids that in turn, inhibit the biosynthesis of vital osmolytes in crop plants (Dong et al., 2016). The increased accumulation of Cd in various plant tissue contributes toward the enhanced generation of free radical oxygen species, which induce oxidative stress in plants (Gill et al., 2012; Mwamba et al., 2016a,b; Riaz et al., 2021a). These radical oxidative species also destabilize plant development by distorting various signal transduction pathways like antioxidant systems, photosynthesis, and hormonal signaling in different manners. The inception of such stress-stimulated responses is to counteract the injury and develop a system of their survivability under adverse conditions (Noctor et al., 2018). Plants, in response to major abiotic threats, try to alleviate stress and enhance their physiological and cellular functioning through their innate mechanism (Ali et al., 2020b; Riaz et al., 2021b). Accumulating osmolytes, or compatible solutes, is one of the major strategies to avoid damage to cellular machinery and to interfere with normal metabolic processes (Fujita et al., 2006).

Natural bio-stimulants are the most promising and suitable strategy nowadays to address yield losses caused by various stresses, which are intensified by climate change. Biostimulants possess many diverse compounds, with positive effects in plants to increase growth, diminish stress-induced restrictions, and enhance yield (Yakhin et al., 2017). The moringa (*Moringa oleifera*) leaf extract (MLE) has been categorized as one of the novel and natural biostimulants that are enriched with higher nutrient contents, improved growth, and Cd tolerance in *Phaseolus vulgaris* L. (Howladar, 2014), *Zea mays* L. (Basra et al., 2011; Bashir et al., 2021), *Lepidium sativum* L. (Khalofah et al., 2020), and *Triticum aestivum* L. (Fozia et al., 2021). The MLE is a prolific source of antioxidants, plant growth-promoting substances, and macro- and micronutrients (Khan et al., 2017), which are critical for the regulation of physiological, biochemical, and yield parameters of plants that are under stressed and normal environmental conditions (Rady et al., 2013; Khan et al., 2021; Rashid et al., 2021). Ascorbic acid (AsA), which is a commonly found non-enzymatic antioxidant, has significant potential for scavenging reactive oxygen species (ROS) and regulates several biochemical processes of considerable importance both under stressed and normal conditions (Ali et al., 2020a; Parveen et al., 2020). Previously, it was reported that exogenous application of AsA could significantly increase endogenous production of AsA in wheat tissues which might be involved in the biosynthesis of some vital molecules to trigger a powerful response against Cd stress (Jung et al., 2020). Further, the AsA applications have been observed to improve the various physiological and biochemical processes in barley (*Hordeum vulgare* L.) (Yaseen et al., 2021) and wheat (*Triticum aestivum* L.) (Al-Hakimi and Hamada, 2011) grown under Cd stress. Therefore, it is interesting to compare the costs and benefits of using pure organic active compounds to increase yield under abiotic stress conditions (García-García et al., 2020).

The beneficial role of the combined application of MLE and AsA to mitigate Cd stress in the wheat plant is scarcely available. Therefore, the current study was based on the hypothesis that

MLE and AsA will enhance the tolerance of wheat seedlings by maintaining morpho-physiological responses and accumulation of secondary metabolites to reduce the impact of Cd. Our findings will provide an insight to guide further studies of the Cd resistance with MLE in wheat at the molecular level, as well as tolerance against other heavy metals in other plant species. The objectives of the current study were to: (i) investigate the impact of Cd on wheat growth attributes, chlorophyll fluorescence, antioxidant, and osmolyte performance, and (ii) explore valuable physiological and biochemical traits as a marker to induce Cd tolerance with exogenously applied MLE and AsA. The findings would further highlight the mechanisms involved to ensure tolerance against Cd in wheat seedlings, along with the provision of useful, innovative, and cheap techniques in the form of moringa to mitigate other abiotic stresses.

MATERIALS AND METHODS

Materials and Growth Environment

A pot investigation was performed in the wirehouse of the old botanical garden, University of Agricultural, Faisalabad, Pakistan (31.26" latitude, 73.06" longitude). The growing conditions in the wirehouse remained at 27/20°C day/night temperature, a 12-h illumination per day, and 65–70% moisture contents. The seeds of two wheat cultivars, i.e., Faisalabad-08 (Fsd-08) and Galaxy-13 (Glxy-13), were provided by Ayyub Agriculture Research Institute (AARI), Faisalabad, Pakistan. Healthy seeds were surface-sterilized by soaking in the solution of sodium hypochlorite for 5 min and washed with distilled water repeatedly. The plastic pots (32 × 22 cm) were filled with 5.5 kg dried and sieved sand. Later, the dried seeds were sown in pots for germination. All the pots were watered with a half-strength Hoagland nutrient medium once a week.

Experimental Design

Both wheat cultivars were allowed to grow up to the three-leaf stage, thinning was done to ensure 5 uniform seedlings/pot before application of Cd, ascorbic acid (50 mM), and moringa leaf extract (3%). The Cd was saturated in the rhizosphere by dissolving $CdCl_2.5H_2O$ in a full-strength Hoagland nutrient medium (Hoagland and Arnon, 1950), while AsA and MLE were applied as foliar treatment having 2 ml between -20 in each solution. The stock solution of Cd was prepared in mmol/L (mM), which was further diluted to micromole/L (μM) to maintain 500 and 1,000 μM Cd concentrations. Seven treatments were laid down in completely randomized design, and designated as Control, Cd_{500}, Cd_{1000}, Cd_{500} + AsA, Cd_{500} + MLE, Cd_{1000} + AsA, and Cd_{1000} + MLE, respectively. Moringa leaves were obtained from the forestry department, University of Agriculture, Faisalabad and authenticated by the local experts. Leaves were frozen at -80°C for 72 h and extract was made in a locally fabricated extract machine with water (10:1; fresh leaves: water). The concentrated extract was further diluted to 3% with distilled water before foliar application. To maintain the moisture level of plants up to 75–85%, pots were watered regularly, while Cd and foliar application were given once a week (6 weeks). To avoid nutrient deficiency, the nutrient solution was irrigated once a week as well. All the below-mentioned attributes were recorded 45 days after sowing (DAS), with and without treatment. All the measurements were performed according to the set criterion to reduce the difference caused by determination time.

Determination of Morphological and Gas Exchange Attributes

The shoot and root length and the fresh biomass were measured separately with the measuring tape (cm) after harvesting and subsequent weighing, respectively. Leaf area (cm^2) was calculated by taking the length and width of three randomly selected leaves/plant/replicate with a measuring tape. The number of tillers and the number of leaves were recorded by counting the respective trait.

The gaseous attributes, such as carbon dioxide assimilation rate (*A*), stomatal conductance (*Gs*), internal CO_2 concentration (*Ci,*), and transpiration rate (E), were assessed using fully turgid uppermost leaves with a portable photosynthesis system, Infra-Red Gas Analyzer (LCA-4 ACD, Analytical Development, Hoddesdon, United Kingdom) at a light-saturating intensity between 9:00 am and 12:00 pm. On the other hand, water-use efficiency was calculated by adopting the formula (*A/E*).

Determination of Photosynthetic Pigments and Chlorophyll Fluorescence Parameters

The concentrations of chlorophyll a or b, total chlorophyll, and carotenoid contents (mg g^{-1} FW) were examined by following the method devised by Arnon (1949). In brief, about.25 g of fresh leaf discs (45 days old) were dipped in 5 ml of 80% acetone solution. The mixture was placed in a dark place overnight. After overnight extraction, the optical density (OD) of the acetone extract was measured at 663, 645, and 480 nm. The obtained OD values were used to calculate chlorophyll a or b, total chlorophyll contents ($a + b$), and carotenoids by applying the respective formula.

The chlorophyll fluorescence (CF) parameters from the upper leaf surfaces of wheat seedlings were determined with an OS5p Modulator Fluorometer [ADC BioScientific Ltd., Great Amwell Herts, United Kingdom (Strasserf and Srivastava, 1995)]. Three seedlings from each treatment were randomly chosen and used to measure the CF parameters. After 30 min of the dark adaptation, the minimal fluorescence (F_0) was determined by a weak red light (< 0.1 μmol m^{-2} s^{-1}). Then, the maximum fluorescence (Fm) was determined using a saturating pulse (8,000 μmol m^{-2} s^{-1}) of.8 -s duration. The activity of photosystem II (PSII) was determined by F_V/F_M, when the leaves had been dark-adapted for 30-min, using the dark leaf clip. Quantum efficiency of photochemical transports used for photosynthesis (Φ_{PSII}), photochemical quenching (qP), quantum efficiency of thermal dissipation promoted by the photoprotective non-photochemical quenching *via* the xanthophyll cycle (Φ_{NPQ}), and electron transport rate (Φ_{ETR}) was calculated using 1,500 μmol m^{-2} s^{-1} actinic light.

Determination of Glycine Betaine, Ascorbic Acid, Proline, and Tocopherol Contents

For GB, a slightly modified method of Grieve and Grattan (1983) was adopted. Dry leaves (0.25 g) were extracted with.5% toluene. Extracts were kept at 4°C overnight. An extract of 1mL of supernatant was taken and mixed with 1mL $2NH_2SO_4$. From the mixture,0.5 ml was further mixed with.5 ml of KI_3. The mixture was cold at 90 min, then, was added a pre-chilled distilled water and 1,2-dichloroethane. It will form two layers, the upper layer will be discarded, and absorbance was taken from the spectrophotometer at 365 nm. Endogenous ascorbic acid was determined according to the standard protocol (Mukherjee and Choudhuri, 1983). Fresh leaf material (0.1 g) of each wheat replicate and treatment was extracted with 5 ml of 6% trichloroacetic acid. The extract was centrifuged at 12,000 rpm for 15 min. Two milliliters of the extract was thoroughly mixed with 1 mL of 2% dinitrophenyl hydrazine (in acidic medium), followed by the addition of one drop of 10% thiourea (in 70% ethanol). The mixture was placed in a water bath for 15 min and cooled at room temperature, then, 2.5 ml of 80% (v/v) H_2SO_4 was further added to the mixture at 0°C. The absorbance was read at 530 nm with a spectrophotometer.

The proline contents were measured using the protocol given by Bates et al. (1973). Briefly, 2 ml proline extract, 2 ml of glacial acetic acid, and 2 ml of acid ninhydrin were mixed, placed (1 h) in a boiling water bath, and finally put in an ice bath. Later, the absorbance (520 nm) of the reaction mixture was noticed on the Spekol Spectrocololourimeter, VEB Carl Zeiss. To estimate the final proline contents, a standard curve was drawn by using a known concentration of authentic proline. Tocopherol contents were estimated by the methodology devised by Backer et al. (1980). Fresh leaf (1 g) was homogenized with a mixture of petroleum ether and ethanol (2:1.6 v/v). The extract was centrifuged at 10,000 rpm for 20 min to gain clear supernatant. One milliliter of extract and 2% of 2,2-dipyridylethnolic solution was mixed, then the mixture was kept in dark for 5 min. The resulting color in the aqueous layer was used to estimate tocopherol at 520 nm.

Determination of Flavonoids, Total Free Amino Acid, Phenolics, and Total Soluble Sugars

Flavonoid contents were determined by following the method of Kim et al. (1999). Fresh leaves (0.2 g) were ground in 80% acetone and plant extract was filtered. Later, 1 ml of plant extract was mixed in a 10 ml volumetric flask having 4 ml distilled water. The further reaction mixture was mixed with 5% $NaNO_3$, 10% $AlCl_3$, and 1M NaOH with a break of 5 and 2 min, respectively. The reaction mixture was diluted with 3mL of distilled water. The absorbance was taken at 510 nm. The quercetin is used as standard. Hamilton and Van Slyke's (1943) method, with a slight modification of quantities to estimate total free amino acid in treated and non-treated wheat plant samples. Enzyme extract (0.5 ml) was mixed with a solution containing 10% pyridine (0.5 ml) and 20% ninhydrin solutions (0.5 ml). The reaction mixture was heated in a water bath by covering the tubes with aluminum foil at 50°C for 30 min; the volume is up to 25 ml with distilled water. Optical density was taken at 570 nm. Phosphate buffer is used as blank. Total soluble phenolic was analyzed according to Julkunen-Titto (1985). A known weight of fresh leaf sample was homogenized with 80% acetone followed by centrifugation for 15 min. at 1,000 g. Then, supernatant (0.1 ml) was mixed with 2 ml of water and 1 ml of Folin-Ciocalteau's phenol reagent and was shaken well. About 5 ml of 20% Na_2CO_3 was added with 10 ml of distilled water. Optical density (OD) was taken with a spectrophotometer (IRMECO 2020) at 750 nm. Total soluble sugar (TSS) contents were extracted by using fresh leaves (0.1 g) of both cultivars by cutting and boiling them in the distilled water (5 ml) for 1 h. The extract was filtered and the volume was up to 25 ml. The TSS was analyzed by using a reaction of 1 ml of an extract with 5 ml fresh anthrone reagent (150 mg anthrone, + 100 ml 72% H_2SO_4) (Handle, 1968). Thereafter, the reaction mixture was placed in a boiling water bath for 10 min. The absorbance of the sample and blank was determined at 620 nm using IRMECO 2020.

Determination of Cd Contents

The root and leaf samples were dried in a hot-air oven at 72°C for six days. Shoot and root dry samples (0.25 g) were separately placed in a 50 ml digestion flask and digested in 5 ml concentrated nitric acid (HNO_3) and kept at room temperature for 3 h. The samples were transferred to a heating block and digested at 300°C until samples turned black to tinted yellow. The samples were taken from the heating block, cooled, diluted with distilled water up to 25 ml, and were filtered. The acid extract of the samples was used to determine Cd concentration by atomic absorption spectrum (PerkinElmer, Waltham, MA, United States).

Statistics Analysis

All the attributes discussed above were measured by 45 DAS and subjected to statistical analysis using the SPSS (SPSS 21.0 statistical software package). For each treatment, the mean values with SE (± SE) are described in the figures and tables. The parameters were analyzed by multivariate ANOVA (MANOVA). The significant difference among the means was calculated by Tukey's test ($p < 0.05$).

RESULTS

Growth Responses With Foliar Spray of Bio-Stimulants Under Cd Stress

All growth attributes, such as shoot length, roots length, the number of tillers per plant, the number of leaves per plant, and leaf area, were significantly reduced under Cd stress, while significantly increased with foliar spray of MLE and AsA, except root length (**Table 1**). Compared to the control (NS) plants (0 μM Cd), the shoot length of the Fsd-08

TABLE 1 | Variation among morphological attributes under the foliar spray of ascorbic acid (AsA) and moringa leaf extract (MLE), along with no-spray (NS) in two wheat cultivars (Fsd-08 and Glxy-13) exposed to the cadmium (Cd) toxicity.

Amendment	Cd (μM)	Shoot Length (cm)		Root Length (cm)		Leaf area (cm^2)		No. of tillers (Per plant)		No. of leaves (Per plant)	
		Fsd-08	**Glxy-13**	**Fsd-08**	**Glxy-13**	**Fsd-08**	**Glxy-13**	**Fsd-08**	**Glxy-13**	**Fsd-08**	**Glxy-13**
NS	0	42.7 ± 2.4 b	40.5 ± 3.3 b	18.75 ± 2.2 b	16.5 ± 1.3 b	28.6 ± 3.1 a	16.6 ± 3.1 b	4.5 ± 0.6 b	4.0 ± 0.8 b	11.0 ± 0.8 b	11.5 ± 0.6 ab
	500	30.3 ± 1.7 d	26.2 ± 2.06 d	12.0 ± 2.3 c	12.5 ± 3.0 d	15.1 ± 0.6 c	14.1 ± 0.3 c	3.5 ± 0.6 b	3.2 ± 0.5 c	7.6 ± 0.9 c	8.8 ± 0.5 c
	1000	23.0 ± 2.9 e	23.0 ± 1.8 e	7.5 ± 0.6 d	7.5 ± 0.5 e	15.8 ± 1.9 c	10.6 ± 0.6 d	1.3 ± 0.5 d	1.0 ± 0.0 e	5.7 ± 0.9 c	5.0 ± 1.4 d
AsA	0	45.5 ± 3.5 a	42.7 ± 1.7 a	19.3 ± 1.97 a	20.0 ± 1.1 a	29.9 ± 2.0 a	20.4 ± 1.9 a	5.5 ± 1.0 a	4.8 ± 0.5 a	13.2 ± 0.9 a	12.6 ± 0.9 a
	500	35.0 ± 2.8 c	35.7 ± 4.1 c	12.5 ± 1.7 c	13.5 ± 2.4 cd	18.3 ± 0.9 b	14.4 ± 1.0 c	4.0 ± 1.1 b	3.8 ± 1.0 c	9.5 ± 0.5 b	11.0 ± 0.8 b
	1000	23.3 ± 1.0 e	26.0 ± 2.4	7.2 ± 1.7 d	6.8 ± 0.5 e	18.3 ± 1.1 b	11.6 ± 1.5 d	2.5 ± 0.6 c	2.3 ± 0.5 d	6.0 ± 1.2 c	5.7 ± 0.9 d
MLE	0	45.5 ± 4.0 a	45.5 ± 4.0 a	20.8 ± 2.8 a	21.4 ± 0.5 a	29.7 ± 3.6 a	22.5 ± 5.9 a	5.5 ± 0.6 a	5.5 ± 0.5 a	14.0 ± 0.8 a	13.3 ± 1.3 a
	500	37.5 ± 2.5 c	34.0 ± 4.6 c	13.0 ± 2.2 c	14.0 ± 1.6 c	19.6 ± 1.1 b	17.3 ± 3.2 b	4.3 ± 0.6 b	4.3 ± 0.5 b	10.8 ± 0.5 b	11.5 ± 0.6 ab
	1000	30.5 ± 3.0 d	28.7 ± 3.4 d	6.75 ± 1.3 d	7.3 ± 0.9 e	14.1 ± 2.9 c	14.3 ± 3.7 c	1.7 ± 0.5 d	2.0 ± 0.0 d	6.0 ± 1.4 c	6.0 ± 0.0 d

The means sharing different lowercase letters differ significantly as determined by LSD test at $p \leq 0.05$. The data presented is average of 4 replications ± SD. AsA and MLE were applied at the rate of (50 mM) and (3% w/v), respectively.

TABLE 2 | Variation among gaseous exchange attributes under the foliar spray of AsA and MLE, along with NS in two wheat cultivars (Fsd-08 and Glxy-13) exposed to the Cd toxicity.

Amendment	Cd (μM)	CO_2 assimilation rate (A) (mmol CO_2 m^{-2} s^{-1})		Net transpiration rate (E) (mmol H_2O m^{-2} s^{-1})		Water use efficiency (WUE) (mmol CO_2/mmol H_2O)		Stomatal conductance (Gs) (nmol CO_2 m^{-2} s^{-1})		Internal CO_2 concentration (Ci) (μmol CO_2 m^{-2} s^{-1})	
		Fsd-08	**Glxy-13**	**Fsd-08**	**Glxy-13**	**Fsd-08**	**Glxy-13**	**Fsd-08**	**Glxy-13**	**Fsd-08**	**Glxy-13**
NS	0	10.4 ± 0.5 c	9.7 ± 0.4 c	1.34 ± 0.04 b	1.37 ± 0.51 b	7.78 ± 0.43 d	7.72 ± 2.12 b	0.08 ± 0.01 b	0.09 ± 0.01 b	205.0 ± 5.7 d	203.3 ± 14.4 d
	500	7.4 ± 0.5 d	8.0 ± 0.1 d	1.63 ± 0.31 a	1.83 ± 0.09 a	4.66 ± 0.97 e	4.38 ± 0.18 c	0.07 ± 0.01 b	0.05 ± 0.01 b	225.8 ± 10.1 c	230.2 ± 10.5 c
	1000	5.7 ± 0.3 e	4.5 ± 0.4 e	0.78 ± 0.17 d	0.67 ± 0.09 d	1.98 ± 0.14 f	1.67 ± 0.08 d	0.08 ± 0.02 b	0.06 ± 0.01 b	324.6 ± 18.2 a	305.3 ± 17.8 a
AsA	0	13.9 ± 0.9 b	10.7 ± 1.1 b	1.28 ± 0.22	1.40 ± 0.53 b	11.03 ± 1.64 b	8.25 ± 2.24 a	0.11 ± 0.01 a	0.10 ± 0.02 a	186.4 ± 17.3 e	172.2 ± 30.8 f
	500	12.9 ± 0.6 b	11.2 ± 0.8 b	1.48 ± 0.53 b	1.22 ± 0.16 c	9.79 ± 3.89 c	9.29 ± 1.13 a	0.09 ± 0.01 b	0.07 ± 0.02 b	232.2 ± 14.3 c	229.5 ± 9.3 c
	1000	10.2 ± 1.2 c	10.3 ± 0.8 bc	1.04 ± 0.49 c	0.63 ± 0.23 d	4.62 ± 1.16	4.52 ± 1.39 c	0.08 ± 0.01 b	0.06 ± 0.01 b	284.7 ± 10.4 b	246.8 ± 1.72 b
MLE	0	14.2 ± 0.4 a	10.9 ± 0.6 b	1.10 ± 0.05 c	1.37 ± 0.33 b	12.91 ± 0.86 a	8.38 ± 2.20 a	0.12 ± 0.02 a	0.10 ± 0.02 a	189.1 ± 19.5 e	193.7 ± 17.3 e
	500	13.9 ± 1.6 b	12.2 ± 0.2.7 a	1.09 ± 0.05 c	1.65 ± 0.26 a	12.81 ± 1.48 a	7.44 ± 1.28 b	0.09 ± 0.02 b	0.07 ± 0.01 b	241.3 ± 6.6 c	221.4 ± 5.7 c
	1000	10.7 ± 1.3 c	9.7 ± 0.4 c	0.67 ± 0.04 d	0.55 ± 0.08 d	4.53 ± 0.69 e	4.44 ± 1.29 c	0.08 ± 0.01 b	0.06 ± 0.01 b	269.1 ± 5.5 b	257.9 ± 7.4 b

The means sharing different lowercase letters differ significantly as determined by LSD test at $p \leq 0.05$. The data presented is average of 4 replications ± SD. AsA and MLE were applied at the rate of (50 mM) and (3% w/v), respectively.

cultivar was reduced by 29 and 46% under 500 μM and 1000 μM Cd stress, respectively. Likewise, at the highest Cd concentration (1,000 μM), the leaf area, the number of leaves per plant, and the number of tillers per plant were reduced by 45 and 36%, 48 and 57%, and 71 and 75% in Fsd-08 and Glxy-13 cultivars, respectively (**Table 1**). However, exogenous application of AsA and MLE significantly improved (particularly at 500 μM Cd stress) growth traits except for root length. In comparison to the stressed plants (500 μM Cd), exogenous application of MLE exhibited a prominent improvement in shoot length (24 and 30%), leaf area (30 and 63%), number of tillers per plant (23% and 34%), and number of leaves per plant (42 and 31%) in Fsd-08 and Glxy-13 cultivars, respectively.

Gas Exchange Responses With Foliar Spray of Bio-Stimulants Under Cd Stress

The results exhibited that carbon dioxide assimilation rate (*A*), transpiration rate (*E*), water use efficiency (*WUE*), and stomatal conductance (G_s) in both wheat cultivars were deprived of the increasing levels of Cd stress, except internal CO_2 concentration (*Ci*) (**Table 2**). However, a highly significant impact of MLE and AsA was recorded to mitigate Cd stress up to 500 μM

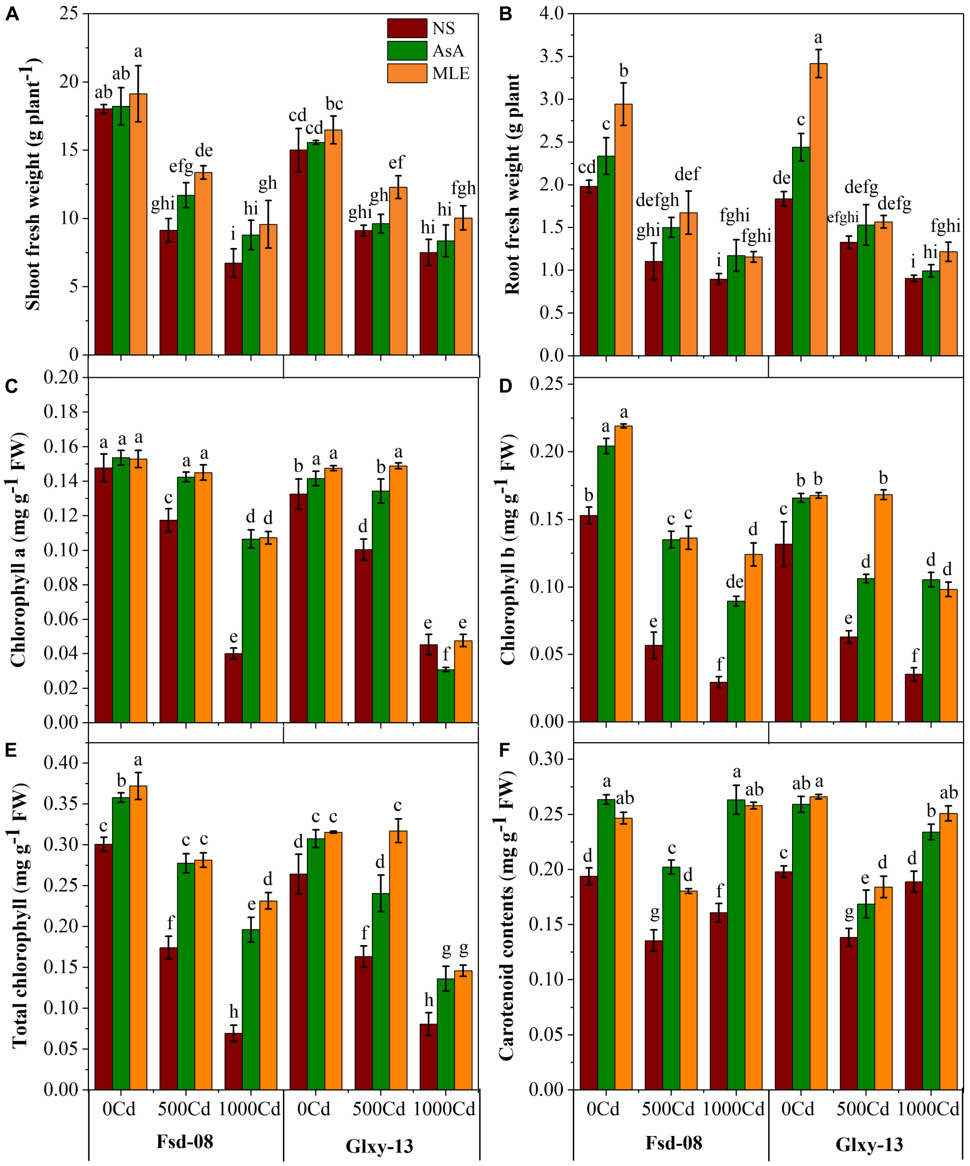

FIGURE 1 | Fresh biomass and photosynthetic pigment responses as **(A)** shoot fresh weight, **(B)** root fresh weight, **(C)** chlorophyll a, **(D)** chlorophyll b, **(E)** total chlorophyll, and **(F)** carotenoid contents, under the foliar spray of ascorbic acid (AsA) and moringa leaf extract (MLE), along with no-spray (NS) in two wheat cultivars (Fsd-08 and Glxy-13) exposed to the cadmium (Cd) toxicity. Different lowercase letters denote significant difference as determined by least significant difference (LSD) test ($p < 0.05$, $n = 4$). AsA and MLE were applied at the rate of 50 mM and 3% (w/v), respectively.

for all gaseous attributes. The CO_2 assimilation rate (*A*) was prominently increased with the application of both foliar stimulants, with and without Cd stress (**Table 2**). Both wheat cultivars showed a significant decline in transpiration rate € under 1,000 μM Cd toxicity, as compared to the non-stressed seedlings. However, the transpiration rate was maintained to normal with foliar spray of MLE and AsA under the lower Cd stress (500 μM). The water-use efficiency (WUE), though depressed with Cd stress in both wheat cultivars, was significantly enhanced after the foliage application of MLE and AsA. Overall, the Fsd-08 cultivar showed greater gaseous exchangeability compared with the Glxy-13 cultivar. The stomatal conductance of both wheat cultivars showed a non-significant difference in all the treatments. The intercellular CO_2 concentration (*Ci*) was increased under stress, while slightly decreased by MLE and AsA foliar treatments (**Table 2**).

Biomass Accumulation and Photosynthetic Pigments With Foliar Spray of Bio-Stimulants Under Cd Stress

A reduction in biomass accumulation and photosynthetic attributes governed by Cd stress was concentration-dependent; therefore, higher levels of Cd resulted in a higher loss in biomass

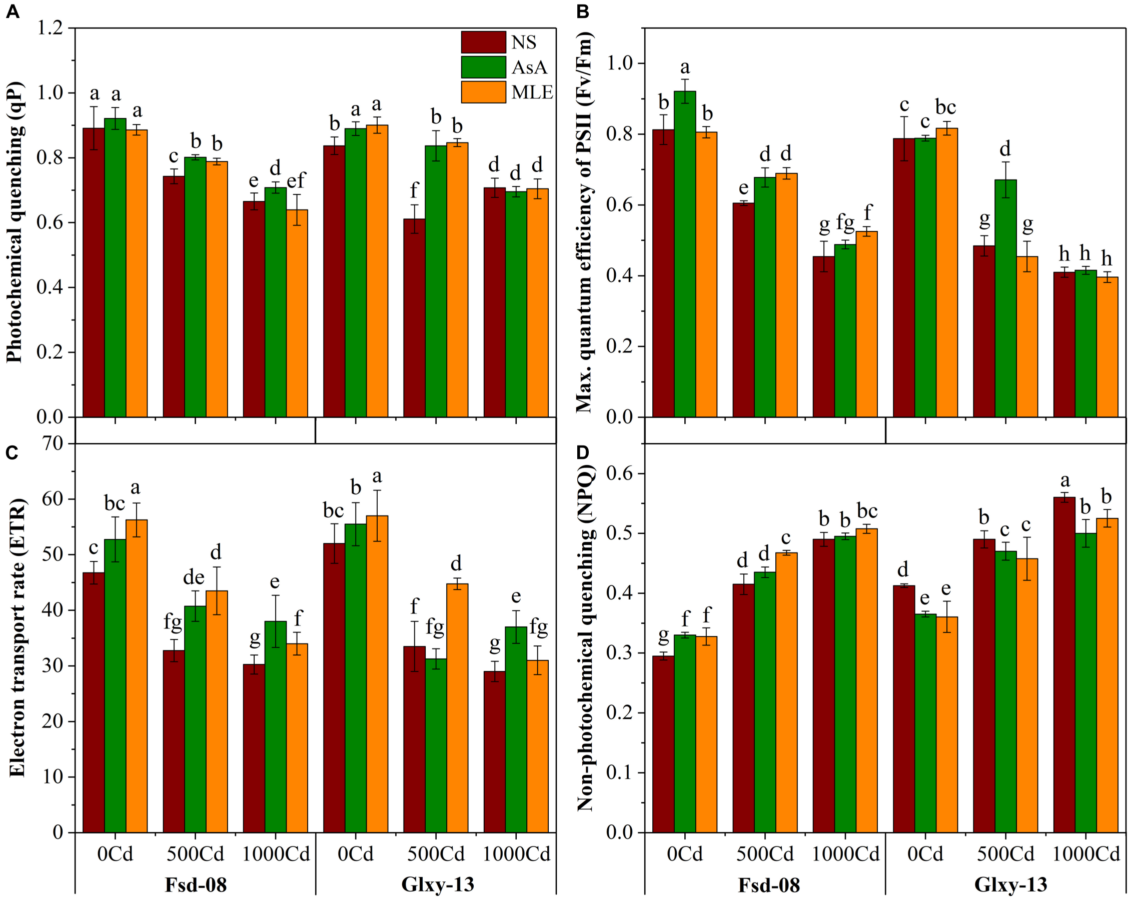

FIGURE 2 | Chlorophyll fluorescence responses as **(A)** photochemical quenching (qP), **(B)** maximum quantum efficiency of PSII (Fv/Fm), **(C)** electron transport rate (ETR), and **(D)** non-photochemical quenching (NPQ), under the foliar spray of AsA and MLE, along with N) in two wheat cultivars (Fsd-08 and Glxy-13) exposed to the Cd toxicity. Different lowercase letters denote significant difference as determined by LSD test ($p < 0.05$, $n = 4$). AsA and MLE were applied at the rate of 50 mM and 3% (w/v), respectively.

accumulation and chlorophyll contents in both wheat cultivars (**Figure 1**). The results exhibited that Cd toxicity significantly reduced the shoot and root fresh biomass of both wheat cultivars (Fsd-08 and Glxy-13). However, the application of MLE has highly improved the biomass accumulation (46 and 35%) and (52 and 18%) under the lower Cd stress (1,000 μM), respectively, compared to the relevant treatment without MLE application. Total chlorophyll contents were decreased by 76 and 69% in Fsd-08 and Glxy-13 under Cd stress (1000 μM), compared with normal growth conditions, respectively. However, this loss was minimized with the foliar application of MLE and AsA. Moreover, carotenoid contents increased in a parallel way with increasing Cd stress by utilizing both sources of foliar applications (**Figure 1**).

Chlorophyll Fluorescence Responses With Foliar Spray of Bio-Stimulants Under Cd Stress

A significant difference in chlorophyll fluorescence traits was recorded among all the treatments that are applied on the wheat cultivars (**Figure 2**). Photochemical quenching (qP), the maximum quantum efficiency of photosystem II (F_v/F_m), and electron transport rate have significantly declined with the increasing Cd stress in the rhizosphere, with one exception of non-photochemical quenching (NPQ), which increased significantly under the elevated level of Cd concentration in both wheat cultivars (**Figure 2**). Wheat seedlings grown under 1,000 μM Cd concentration showed relatively higher NPQ values compared to 500 μM Cd alone and in combination with AsA or MLE (**Figure 2D**). However, foliar application of AsA and MLE significantly enhanced the qP, *Fv*/Fm, and ETR values under 500 μM Cd stress, compared to the non-sprayed stressed seedlings of both wheat cultivars (**Figures 2A–C**).

Osmolytes Regulation in Wheat Leaves With Foliar Spray of Bio-Stimulants Under Cd Stress

According to the obtained results, ascorbic acid contents have decreased, while tocopherol, glycine betaine, and proline contents have increased under the Cd toxicity, particularly with

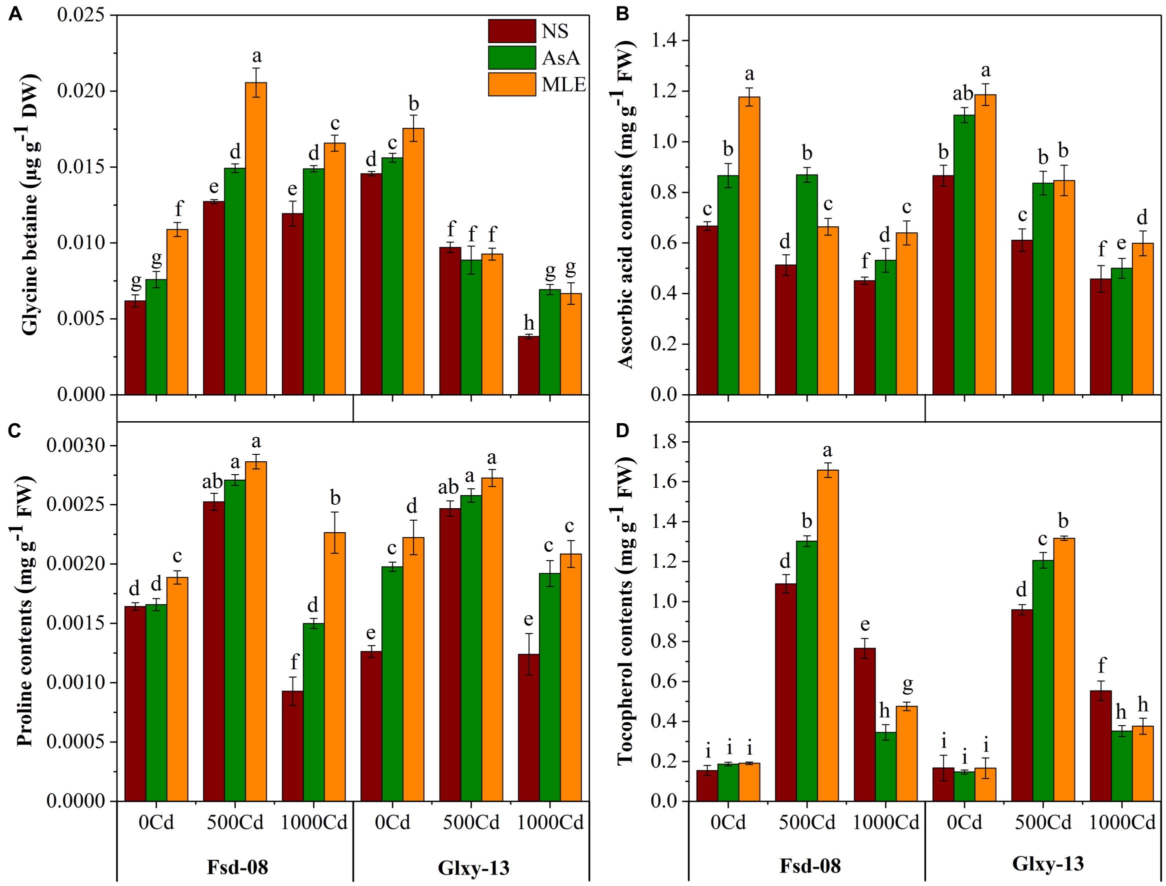

FIGURE 3 | Osmolyte accumulation responses as **(A)** glycine betaine, **(B)** AsA, **(C)** proline contents, and **(D)** tocopherol contents, under the foliar spray of AsA and MLE, along with NS in two wheat cultivars (Fsd-08 and Glxy-13) exposed to the Cd toxicity. Different lowercase letters denote significant difference as determined by LSD test ($p < 0.05$, $n = 4$). AsA and MLE were applied at the rate of 50 mM and 3% (w/v), respectively.

500 μM Cd concentration. Meanwhile, exogenous application of biostimulants modulated the innate mechanisms to boost the non-enzymatic antioxidant defense system of both wheat cultivars (**Figure 3**). Briefly, glycine betaine (GB) contents were enhanced significantly after applying MLE (62%) and AsA (17%) under Cd stress (500 μM) in Fsd-08 cultivar, compared to the corresponding treatments without MLE spray. However, Fsd-08 accumulated higher GB contents compared to the Glxy-13 under the Cd stress in combination with MLE or AsA. Furthermore, the experimental data expressed that AsA concentration in the leaves of Fsd-08 was decreased by 23 and 32% under 500 and 1,000 μM Cd stress, respectively, compared to the control. Meanwhile, foliar application of AsA mitigated the Cd stress (500 μM) by elevating *in vivo* the ascorbic acid pool by 70 and 37% in Fsd-08 and Glxy-13, respectively, compared with the relevant treatments without AsA application. Proline contents varied significantly ($p \leq 0.05$), with and without a foliar spray of MLE and AsA, in both cultivars exposed to Cd stress (**Figure 3**). The alpha-tocopherol contents in wheat cultivars also exhibited a significant ($p \leq 0.05$) escalation with the exposure to Cd stress. However, the effect of MLE and AsA on alpha-tocopherol contents was more obvious at 500 μM Cd stress compared to the 1,000 μM Cd level. After the supply of MLE and AsA, an increment in tocopherol contents was noticed by 52 and 20% in Fsd-08, while 37 and 26% in Glxy-13 cultivar exposed to 500 μM Cd stress, respectively, compared with the related treatments without a foliar spray of biostimulants.

Modulation in Biological Compounds With Foliar Spray of Bio-Stimulants Under Cd Stress

Various biological compounds, such as flavonoids, total free amino acid (TFAA), total phenolics, and TSS contents, decreased with the increasing Cd stress (**Figure 4**). Both wheat cultivars showed a significant ($p \leq 0.05$) reduction for flavonoid pool and total free amino acids at all levels of Cd stress. However, flavonoid contents displayed a positive response in Fsd-08 and Glxy-13 cultivars after the foliar spray of MLE (38% and 12%), in reaction with 500 μM Cd stress, respectively, relative to the similar treatments without MLE application. However, the mitigation of 1,000 μM Cd concentration was not obvious with the spraying of biostimulants and of flavonoid contents that are sharply diminished (**Figure 4A**). The seedlings exposed to

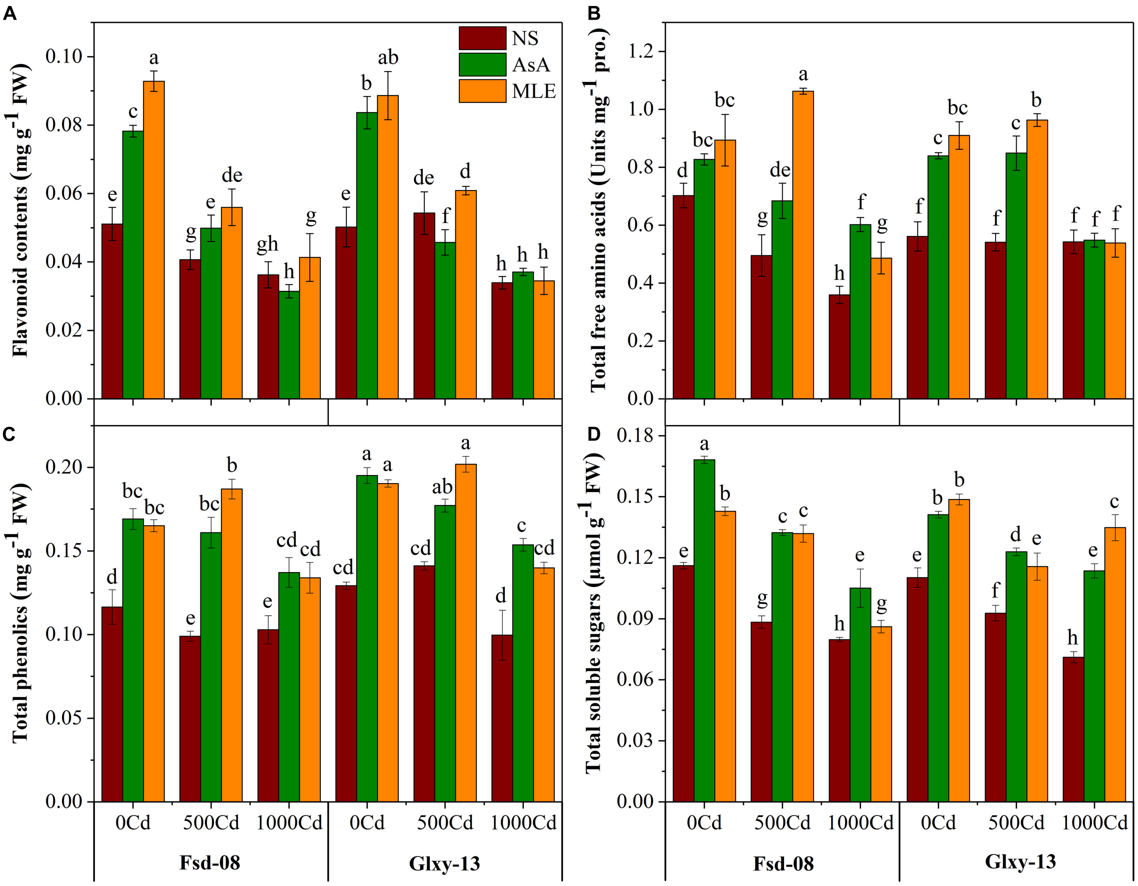

FIGURE 4 | Osmolyte accumulation responses as **(A)** flavonoid contents, **(B)** total free amino acid, total free amino acid (TFAA), **(C)** total phenolics, and **(D)** total soluble sugars, under the foliar spray of AsA and MLE, along with NS in two wheat cultivars (Fsd-08 and Glxy-13) exposed to the Cd toxicity. Different lowercase letters denote the significant difference as determined by LSD test ($p < 0.05$, $n = 4$). AsA and MLE were applied at the rate of 50 mM and 3% (w/v), respectively.

Cd showed severe reduction in TFAA contents up to 41–95% and 25–29% in Fsd-08 and Glxy-13, respectively, compared to the NS (no-spray) treatment. However, foliar spray of AsA and MLE alleviated the Cd (500 μM) stress and produced more TFAA contents (38% and 114%) in Fsd-08 cultivar, respectively, compared to the relevant treatment without biostimulants application (**Figure 4B**). Similarly, phenolic contents were declined drastically by 12% in Fsd-08 and 64% in Glxy-13 with the highest level of Cd (1,000 μM), compared to the control plants. However, in MLE-treated plants, phenolic activity was enhanced up to 30% and 40% in the leaves of Fsd-08 and Glxy-13 grown under 1,000 μM Cd stress, respectively. The application of MLE and AsA showed a better response under 500 μM Cd stress but AsA presented a slightly better behavior by elevating phenolic contents even under 1000 μM Cd stress in both wheat cultivars (**Figure 4C**). Likewise, TSS contents were also depressed due to the Cd stress in both wheat cultivars. On contrary to the negative effects of Cd, MLE application substantially increased the contents of TSS in the leaf tissues of wheat. Briefly, Cd tends to decrease TSS contents by 31–63%; however, foliar spray of biostimulants recovered its status up to 20–25%, compared to the no-spray (NS) plants (**Figure 4D**).

Cd Concentration Modulation in Wheat With Foliar Spray of Bio-Stimulants Under Cd Stress

The Cd concentrations in the different wheat tissues (shoot and root) under the foliar applications of the two biostimulants on two wheat cultivars are presented in **Figure 5**. The foliar application of AsA and MLE significantly ($p < 0.05$) reduced Cd accumulation in the shoot in root tissues of both wheat cultivars in comparison to the no-spray treatment. Cd concentration in the shoot reduced by 58–61% in Fsd-08 and 33–40% in Glxy-13 with the foliar spray of AsA followed by MLE under the lower Cd stress (500 μM).

Relationship of Cd Accumulation in Wheat Tissues With Measured Attributes of Wheat

A correlation analysis was performed to evaluate the linkage between the accumulated Cd concentration and various physio-biochemical attributes of Cd-stressed wheat cultivars after the amendment of MLE and AsA (**Figure 6**). There is a significantly positive relationship of Cd contents in root and

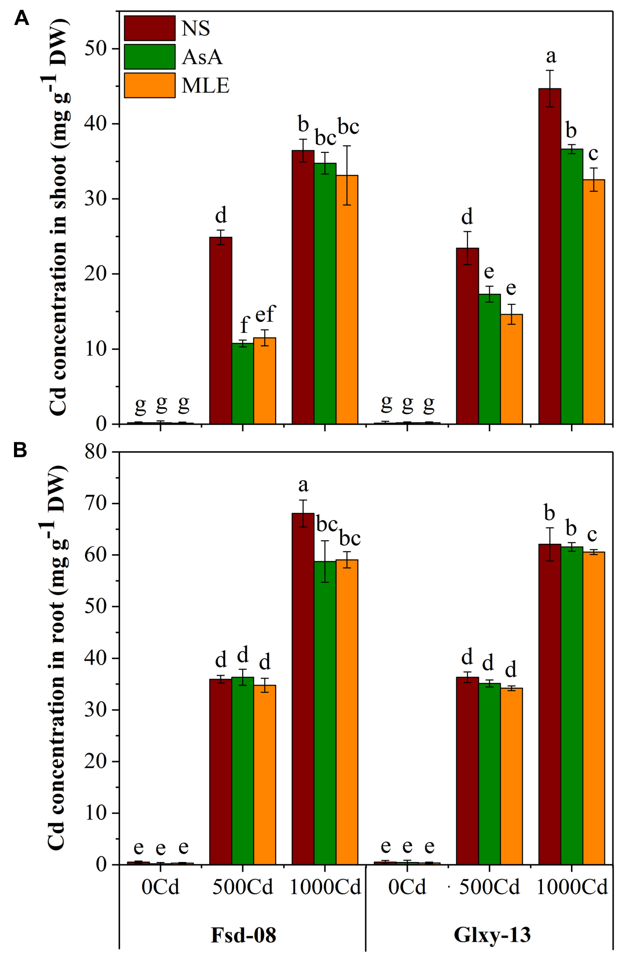

FIGURE 5 | Accumulation of Cd in shoot **(A)** and root **(B)**, under the foliar spray of AsA and MLE, along with NS in two wheat cultivars (Fsd-08 and Glxy-13) exposed to the Cd toxicity. Different lowercase letters denote the significant difference as determined by LSD test ($p < 0.05$, $n = 4$). AsA and MLE were applied at the rate of 50 mM and 3% (w/v), respectively.

shoot tissues, with intercellular CO_2 concentration (Ci) and non-photochemical quenching (NPQ), while a negative interaction was observed with other attributes of both wheat cultivars.

DISCUSSION

Minimization of Cd uptake in the wheat plant is key to decreasing the Cd toxicity, which ultimately ensures food safety (Ali et al., 2015; Wu et al., 2020). Crop health and vigor are addressed by its growth attributes. The detrimental effects of various heavy metals including Cd may be attributed to the modulation of the plant's morpho-physiological and biochemical pathways, which indirectly influence plant growth (Kamran et al., 2019b; Turan, 2020, 2021b). It has been well-established that heavy metal contamination may cause a reduction in plant growth and physiological characteristics (Ali et al., 2014a; Kamran et al., 2019a). On the other hand, AsA application could be involved in improving the essential nutrients, which are the key growth-limiting elements to mitigate the hostile effects of different ecological stresses (Arshad et al., 2016; Golubkina et al., 2019). The MLE is rich in zeatin and other growth-enhancing substances, which are directly involved in improving the growth-related features of wheat (Barciszewski et al., 2000). According to the previous findings, the use of MLE *via* foliage has already been proven to boost plant growth (Maishanu et al., 2017). Moreover, MLE blended with growth-promoted substances, enhances germination parameters and seedling performance in plants (Khan et al., 2017). The chlorophyll fluorescence attributes are useful indicators for growth, physiological response, and modulation of PSII in plants under abiotic and biotic stress. The current data suggested a direct relationship between the growth and the fluorescence parameters because the growth and chlorophyll fluorescence attributes were affected by all Cd levels (**Figures 1**, **2**). According to the findings of Yotsova et al. (2020), the reduced chlorophyll contents displayed an impaired PSII reaction center. A decrease in *qP* value may be attributed to the closed stomatal apertures and restrict the conversion of light energy into chemical energy (Gao et al., 2021), which was also evident from the significant reduction in *Fv/Fm* values (**Figure 2B**). Moreover, the application of AsA mitigated the adverse effects of Cd by enhancing the photoprotection ability. However, Plumb et al. (2018) reported that AsA is an essential growth-promoting substance, but is not involved in the photoprotection of *Arabidopsis thaliana*.

It was previously exhibited that Cd stress downregulated the gas exchange features in wheat seedlings (Khan et al., 2017). Moreover, Cd caused a considerable loss in photosynthetic pigments by degrading the chlorophyll apparatus (Ali et al., 2014b; Yan et al., 2021). The suppression in photosynthetic attributes, *i.e.,* decreased chlorophyll contents, gas exchange, and transpiration due to Cd, emerges as the main cause for the decline in growth and other associated characteristics (Aqeel et al., 2021; Rahman et al., 2021). According to Shah et al. (2019), wheat seeds treated with ASA maximized chlorophyll contents, tillers per plant, number of grains per spike, and 1,000-grain weight. This finding further elaborated that AsA could persuade the upregulation of SOD, POD, and CAT activities, thus, offsetting the adversities on wheat. The increase in chlorophyll contents may also depict a shielding role of AsA on the photochemical efficiency, which influenced the photochemical reactions. Our results conform with those related to the downturn of photochemical efficiency with Cd toxicity, and projection in photosynthetic attributes with the foliar application of AsA (Gaafar et al., 2020). The higher effectiveness of MLE for photosynthetic attributes in the recent trial is likely to be coupled with various allelochemicals and secondary metabolites, such as ascorbate, phenols, and zeatin, which are naturally present in the leaves of moringa (Foidl et al., 2001). Moreover, improvement in CO_2 assimilation rate and *Gs* due to MLE is a consequence of essential metabolites and antioxidants that are present in the moringa leaves (Mona, 2013). Similar findings have previously suggested that MLE was found to be a potential amplifier for growth attributes, chlorophyll *a* and *b*, stomatal conductance

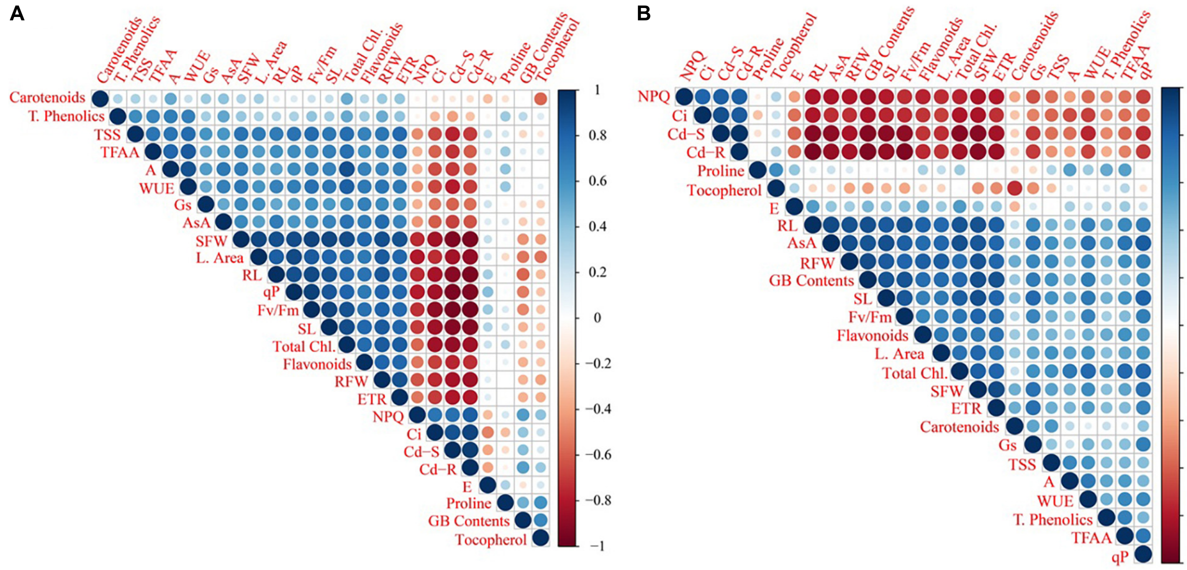

FIGURE 6 | The relative correlation analysis among growth and physio-biochemical attributes of two wheat cultivars, i.e., Fsd-08 **(A)** and Glxy-13 **(B)**, under the foliar spray of AsA and MLE, along with NS treatments. The blue and red colors indicate positive (+) and negative (-) correlation, whereas size of circle and color deepness indicates the intensity (stronger or weaker) of correlations at $P < 0.05$, respectively. The abbreviations are as following: qP: photochemical quenching; TFAA: total free amino acid contents; WUE: water use efficiency; TSS: total soluble sugars; ETR: electron transport rate; SFW: shoot fresh weight; RFW: root fresh weight; Fv/Fm: max. quantum efficiency of PSII; GB: glycine betaine contents; NPQ: non-photochemical quenching; RL: root length; SL: shoot length; AsA: ascorbic acid contents.

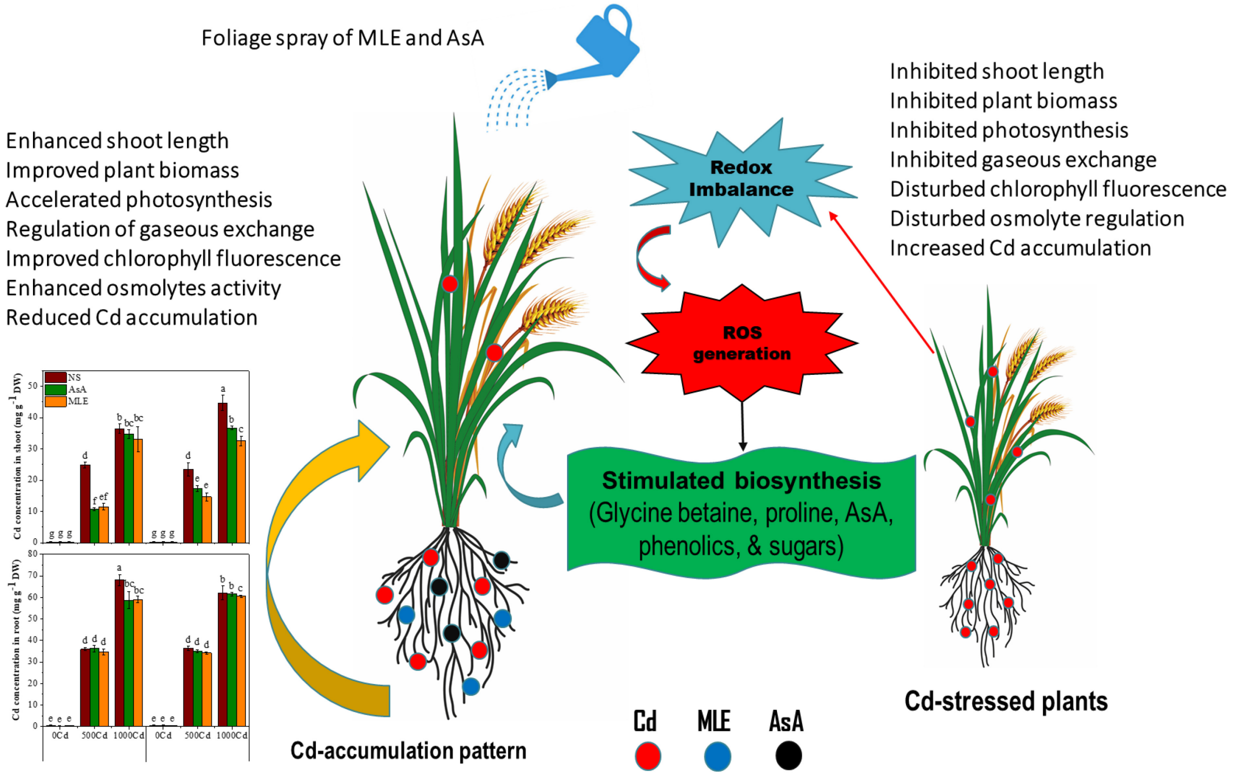

FIGURE 7 | The schematic diagram exhibiting the beneficial role of AsA and MLE against Cd-induced phytotoxicity in wheat seedlings.

(*Gs)*, total soluble protein, and ascorbic acid in the rocket plant (*Erusa vesicaria*) (Abdalla, 2012).

To rescue crop plants from oxidative injury caused by abiotic stresses, the antioxidant enzymes and other self-defense systems display an important role (Kamran et al., 2020, 2021). It has been suggested that the increment in Cd contamination can lead to increased levels of oxidative damage (H_2O_2), ionic leakage, and lipid peroxidation resulting in an escalation in the activities of non-enzymatic antioxidants, antioxidative enzymes, and enzyme gene expression (Alharby et al., 2021). Cd stress also mediates the osmolyte accumulation in various plants (Zhao et al., 2013). The accumulation and biosynthesis of bioactive, physiologically active, and low molecular weight osmolytes serve as defensive molecules to avoid protein decay and breakage of cell structures without hampering the normal metabolic activities in the plant (Dumanović et al., 2021). Different plant species, as well as compartments of the cells, have been specialized to synthesize varying concentrations of such organic molecules under environmental stresses (Lugan et al., 2010). It has also been demonstrated that upregulation of amino acid metabolism, alkaloid biosynthesis, and proline metabolism. Furthermore, various metabolic intermediates are involved in the biosynthesis of the antioxidant defense system, cell wall, and phytochelatin metabolism allied with other organic ligands, playing vital roles in creating the Cd tolerance in plants (Lu et al., 2021). It was reported that foliar spray of AsA enhanced the peroxidase (POD) activity, as well as the phenolic contents and under stressed conditions (Gaafar et al., 2020). Currently, it was reported that moringa leaves contain a varying concentration of phytohormones, associated with seasonal changes of temperature of day length (Brockman et al., 2020). This attribute of moringa leaves makes them ideal to utilize its extract against abiotic stresses in wheat and other plants.

The cascade of AsA biosynthesis under the heavy metal stress depends on the length of its exposure period in the plant and its sole properties. The accumulation of free AsA in plant cells is of enormous significance for scavenging ROS, which could also be involved in the activation of both non-enzymatic and enzymatic antioxidant systems (Xu et al., 2015). Ascorbic acid is the most vital antioxidant in moringa, having a super-active role in enduring non-biological stress conditions. Previous studies have confirmed that ascorbic acid contents in various tissues of *Malus domestica* (Bai et al., 2013) and *Brassica napus* L. (Naeem et al., 2012) have been increased when treated with *Moringa oleifera* extract under abiotic stresses. Along with ascorbic acid, some other osmoprotectants like glycine betaine (GB) have been reported earlier in participating in osmotic balance and maintenance of macromolecules concentration, hence, improving the tolerance to abiotic conditions (Ahanger et al., 2017). Ascorbic acid acts as a precursor by restoring α-tocopherol and zeaxanthin levels in the xanthophyll cycle (Dumanović et al., 2021). Meanwhile, AsA also serves as a cofactor that adjusts the mode of action of many enzymes through a synergistic activation (Foyer and Noctor, 2005). Proline is a well-recognized aliphatic, a cyclized amino acid that plays a significant role to detoxify Cd stress. Proline concentration synergistically increased with phenylalanine biosynthesis for activating defense signaling cascades (Maeda and Dudareva, 2012). Furthermore, MLE contains many phytohormones such as cytokinins and indole-3-acetic acid (Vieira Santos et al., 2001). Under the non-biological stresses, the accumulation of sugars such as mannitol and galactinol takes place and confers tolerance in transgenic plants (Anjum et al., 2014). In addition, total soluble protein concentration and antioxidant activities were upgraded, thus, counteracting Cd stress in maize (Golubkina et al., 2019). A significant increase was reported in the emergence of the potential phenolics accumulation in the maize seedlings when the seeds were pre-treated with MLE (Basra et al., 2011), which might be due to the higher contents of vitamin C in MLE (Moyo et al., 2011). Cd accumulation in shoot and root tissues was increased with increasing Cd levels, as reported previously (Bashir et al., 2020). However, Cd accumulation was higher in roots than those in shoots. Similar findings were observed in our previous experiments (Fozia et al., 2018, 2021).

CONCLUSION

In this study, Cd caused a significant reduction in growth, photosynthetic apparatus, and osmoprotectant activities in two wheat cultivars, *i.e.,* Fsd-08 and Glxy-13. The mitigation of Cd toxicity by foliar spray of MLE was the most fascinating feature of the present study. An exogenous spray of MLE enhanced the phenolic, flavonoid, tocopherol, ascorbate, GB, proline, TSS, and TFAA, which directly or indirectly reciprocated morpho-physiological and biochemical parameters in contrast to non-spray plants under Cd toxicity. For AsA, in most of the studied features, MLE surpasses AsA in improving wheat performance under Cd stress. In a nutshell, an exogenous application of easily available and inexpensive natural bio-stimulants (especially MLE) could be a useful strategy to enhance Cd tolerance in wheat plants by improving growth, reducing Cd uptake, and accumulation of metabolites in various tissues (**Figure 7**).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

FF: data curation, formal analysis, investigation, and writing – original draft. MA: formal analysis, investigation, and writing – original draft. XW, RI, and HT: data curation and writing – review and editing. AT: supervision, writing – original draft, and writing – review and editing. MK: data curation, investigation, resources, writing – original draft, and writing – review and editing. IT: investigation and writing – review and editing. FM-P: data curation, investigation, and writing – review and editing. AE-S: resources and writing – review and editing. HE: resources, data curation, and writing – review and editing. All authors contributed to the article and approved the submitted version.

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Corrigendum: The Impact of Bio-Stimulants on Cd-Stressed Wheat (*Triticum aestivum* L.): Insights Into Growth, Chlorophyll Fluorescence, Cd Accumulation, and Osmolyte Regulation

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Keywords: gaseous exchange rate, secondary metabolites, Cd accumulation, ascorbic acid, moringa leaf extract, growth, chlorophyll fluorescence

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The Impact of Bio-Stimulants on Cd-Stressed Wheat (*Triticum aestivum* L.): Insights Into Growth, Chlorophyll Fluorescence, Cd Accumulation, and Osmolyte Regulation
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In the published article, there was an error in affiliation "Fozia Farhat^{1,3}." Instead of "Fozia Farhat^{1,3}" it should be "Fozia Farhat^{1,2}."

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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Zinc Oxide Nanoparticles Improve *Pleioblastus pygmaeus* Plant Tolerance to Arsenic and Mercury by Stimulating Antioxidant Defense and Reducing the Metal Accumulation and Translocation

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The utilization of nanoparticles to potentially reduce toxicity from metals/metalloids in plants has increased in recent years, which can help them to achieve tolerance under the stressful conditions. An *in vitro* experiment was conducted to investigate five different levels of zinc oxide nanoparticles (ZnO-NPs; 0, 50, 100, 150, and 200 μM) both alone and in combination with 150 μM arsenic (As) and 150 μM mercury (Hg) in one-year-old *Pleioblastus pygmaeus (Miq.) Nakai* plants through four replications. The results demonstrated that As and Hg alone had damaging effects on the plant growth and development. However, the addition of various concentrations of ZnO-NPs led to increased antioxidant activity, proline (79%) content, glycine betaine (71%) content, tyrosine ammonia-lyase (43%) activity, phenylalanine ammonia-lyase (69%) activity, chlorophyll indices, and eventually plant biomass, while the lipoxygenase activity, electrolyte leakage, soluble protein, hydrogen peroxide content, and thiobarbituric acid reactive substances were reduced. We concluded that ZnO-NPs detoxified As and Hg toxicity in the plants through increasing antioxidant activity, reducing As and Hg accumulation, As and Hg translocation from roots to shoots, and adjusting stomatal closure. This detoxification was further confirmed by the reduction of the translocation factor of As and Hg and the enhancement of the tolerance index in combination with ZnO-NPs. However, there is a need for further investigation with different metals/metalloids.

Keywords: metal/metalloid toxicity, nanomaterials, tolerance index, ROS metabolism, translocation factor

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INTRODUCTION

In recent decades, with the unprecedented acceleration in industrialization and urbanization, contamination from heavy metals (HMs) and metalloids constitutes a considerable health hazard to humans around the world, tainting soil, air, and drinking water (Schlutow et al., 2021). Environmental contamination arising from HM has become an increasingly major dilemma, influencing the atmosphere, water, and soil (Hu et al., 2014). Among these contaminants, arsenic (As) and mercury (Hg) are known to be two of the five key toxic metal/metalloid pollutants (copper, Cu; cadmium, Cd; lead, Pb, As, and Hg) in China (Hu et al., 2014). Today, As is responsible for the pollution of a large volume of groundwater in many other countries (Naidu et al., 2006). According to World Health Organization (WHO) reports, the optimum As in drinking water is $10\,\mu g\,L^{-1}$, and they caution that 200 million people worldwide are exposed to the dangers of As toxicity (Abbas et al., 2018). The intensive mining, burning of fossil fuels, metal smelting, and the profligate use of herbicides, insecticides, as well as pesticides are the main sources of As in water, soil, and air (Awasthi et al., 2017). In plants, As leads to the production of oxidative stress with the generation of reactive oxygen species (ROS) components, such as hydrogen peroxide (H_2O_2), superoxide radicals ($O_2^{\bullet -}$), and hydroxyl radicals (•OH; Shahid et al., 2017). This generation of ROS occurs through the conversion of As (V) to As (III; Armendariz et al., 2016). Arsenic in plants leads to a reduction in their morphology, physiology, and growth process. Morphologically, As causes leaf senescence and necrosis, as well as defoliation, leading to a reduction in leaf number and chlorosis. Physiologically, As impacts antioxidant activity, photosynthesis inhibition, and stomatal conductance leading to ROS generation, which can cause DNA damage, lipid peroxidation, carbohydrate damage, and compromised chloroplast membrane. Regarding plant growth, As limits root extension and proliferation, as well as plant biomass, and reduces plant yield (Abbas et al., 2018). Mercury, as one of the natural components of the Earth's crust, pollutes a large area of land, including many agricultural regions (Montero-Palmero et al., 2014). The main anthropogenic activities that cause toxic release of Hg to the environment include; silver, gold, and Hg mining; fossil fuel combustion; and smelting of non-ferrous metals (Li et al., 2020; Arregui et al., 2021). It is reported that Hg causes contamination of the lands and irrigation water that produce approximately 100,000 tons of crops annually (Sengar et al., 2010). China is the largest anthropogenic source of Hg, with emissions of 500–1,000 t annually (Streets et al., 2005; Obrist et al., 2018). Mercury-stressed plants tend to have high levels of ROS, which induces oxidative stress, leading to cell membrane damage, disruption of membrane permeability, and eventually antioxidant enzymatic and nonenzymatic activation in the plants (Cargnelutti et al., 2006).

Nanoparticles with minute sizes of 1–100 nm can play a unique role in plant biotechnology and toxicology (Cele, 2020). Many studies have explored the prominent role of nanoparticles on nucleotides, plant chemicals, and proteins in various sites within plants (Rastogi et al., 2019). Nanoremediation is an emerging technology used to quickly clean up the environment, and it can be employed as an eco-friendly and environmentally safe material (Sanaeimehr et al., 2018). Among nanoparticles, the economic rationale for the nanoremediation use of zinc oxide nanoparticles (ZnO-NPs) is noteworthy, as they are shown to be more cost-effective than other nanoparticles such as titanium dioxide nanoparticles (TiO_2 NPs; Raja et al., 2018). ZnO-NPs, because of their chemical catalytic properties and strong physical adsorption, can be a good choice for use in environmental remediation (Jing et al., 2001). Bulk ZnO, due to its low reactivity and solubility, is absorbed with low efficiency by plants. However, ZnO-NPs with tiny size, high rate of dissolution, and optimal specific surface area enhance zinc (Zn) absorption by plants. As a result, they can solve the Zn deficiency problem in plants (Rastogi et al., 2019). Zinc is an essential element in plant metabolism and growth. It is involved in many processes, such as the biosynthesis of enzymes, proteins, and chlorophyll (Chl, Singh et al., 2018), for instance, Zn can reduce oxidative stress in plants by role-playing in chloroplastic and cytosolic Cu/Zn-SOD enzymes (Barker and Pilbeam, 2015). Or it is reported that metalloproteins of Zn are responsible for the regulation of gene expression in plants under oxidative stress (Barker and Pilbeam, 2015). The positive role of ZnO-NPs in the growth and development of plants is reported in many studies (Rizwan et al., 2019b; Adrees et al., 2021; Faizan et al., 2021a,b). It is reported that ZnO-NPs increase the production of secondary metabolites like phenolics in *Melissa officinalis* (Babajani et al., 2019). ZnO-NPs reduce the negative impact of ROS compounds in HM exposed plants by enhancing antioxidant capacity of plants (Ahmad et al., 2020; Faizan et al., 2021b), which is one of the main mechanisms in the amelioration of HMs by ZnO-NPs. However, there are few studies detailing the positive role of ZnO-NPs in decreasing HM toxicity in plants. This study can help to promote bamboo safety when used as a nutrient and economic source for regional populations in China and can introduce bamboo cultivation for use in phytoremediation technology, eventually increasing our understanding of how the mechanisms of ZnO-NPs are involved in the face of metal stress.

Bamboo (*Bambusoideae*) plants are one of the fastest-growing plants with high biomass, and they cover a remarkable area of Chinese forestland (Yao et al., 2020). It is reported that bamboo species cover 31.5 million ha of the world's forestland (Huang et al., 2020a). In China, bamboo species are classified into 48 genera and 500 species (Huang et al., 2016). Bamboo is an important economic source for the livelihood of the local people in southern and southwestern China (Hogarth and Belcher, 2013). Additionally, bamboo shoots because of their high dietary fiber, high-calorie contents, and low fat are consumed as a tasty nutrient-rich food source by local people in Asia and around the globe (Bal et al., 2012; Emamverdian et al., 2020b). Bamboo species can be particularly used in phytoremediation technology because of their fast vegetative growth and high biomass yields coupled with high metals ions extraction capacities in the roots (Bian et al., 2019). Very few hyperaccumulators possess such features all together. Ornamental plant species are shown to be tolerant to metal stress in urban areas. Therefore, they can be used in remediation schemes as they exhibit phytoremediation potentials (González-Chávez Mdel and Carrillo-González, 2013). Hence, in addition to the commercial purposes (beautification/gardening), they can be used as sustainable and eco-friendly solutions for cleaning up air pollution and removing sewage contamination in urban areas (Liu et al., 2008). *Pleioblastus pygmaeus* is known as an evergreen bamboo species with an average height of 30 cm–50 cm and has often been used for landscape purposes. It was introduced from Japan to China in the early 20th century. It has high tolerance to grow in basic (alkaline), acidic, and neutral soils throughout the year (Huang et al., 2020b) and is cultivated in many areas and provinces in China such as Jiangsu province. On the other hand, HMs are one of the important toxic factors in water and soil in the Southwest regions of China (Emamverdian et al., 2021a,b), which pose significant risks to human health. An early survey conducted on edible plants in some local markets in China showed that the content of As was remarkably high in the shoots of bamboo species (Zhao et al., 2006). On the other hand, it was recently reported that there are 4.1 × 104 metric tons of Hg in agricultural and forestry lands of China (Zhou et al., 2018). Therefore, these two particular metal/metalloids pose a significant threat to human health through the widespread cultivation and consumption of bamboo in China. Hence, it is essential to identify bio-nutrient factors for the removal or reduction of toxicity from the surrounding environment, agricultural, and forest lands. The study aims to improve bamboo plant tolerance under As and Hg toxicity by the application of different concentrations of ZnO-NPs and to pinpoint the mechanisms involved that allow ZnO-NPs detoxification. To our knowledge, this is the first study to investigate the role of ZnO-NPs on bamboo species under the influence of two important toxic metals/metalloids (As and Hg). This can be an important step in the application of ZnO-NPs in plants and environmental detoxification. We hypothesized that the exogenous application of ZnO-NPs can increase bamboo plant tolerance by reducing metal accumulation and the translocation of metals from roots to shoots, stimulating antioxidant activities and promoting closer stomatal regulation.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Single clone 1-year-old branches of *P. pygmaeus* were selected as plant materials, which have been growing since 1982 at Nanjing Forestry University, Bamboo Garden (Nanjing, Jiangsu, China). For shoot production and shoot expansion, long nodal (10 mm) explants were grown under tissue culture conditions conducted in medium of Murashige and Skoog (1962) containing 0.5 ml, 4 ml, 7–10 g L^{-1}, and 30 g L^{-1} kinetin, 6-benzyl amino purine, agar, and sucrose. In this regard, roots proliferated from young shoots that were used in glass petri dishes with a constant diameter (60 mm) to keep the MS medium containing 4 μM nicotinic acid, 0.6 mM myoinositol, 1.2 μM thiamine-HCl, 30 g L^{-1} sucrose, 3 μM pyridoxine, 7–10 g L^{-1} agar, and 1 mg L^{-1} Indole-3-acetic acid (IAA) as growth hormones.

In this study, five concentrations of ZnO-NPs (0, 50, 100, 150, and 200 μM) alone or in a combination with two types of toxic metals/metalloids (150 μM As and 150 μM Hg) were used in a completely randomized design (CRD) through four replications. The total duration of the experiment was 3 weeks. Thus, different treatments including ZnO-NPs and As/Hg in a form of powder were added to 1 L MS medium with 30 g of sucrose. The As was prepared from sodium arsenate heptahydrate powder ($Na_2HAsO_4.7H_2O$) and the Hg was from the salt of white Hg powder ($HgCl_2$). After the regulation of the MS medium in an appropriate pH (5.8 ± 0.1), 7–10 g L^{-1} agar was added. Then, the MS medium was transferred to a microwave oven (China Energy Label) for 30 min at an optimum temperature of 120°C. Sterilization of the MS medium was conducted by an autoclave (HiClave HVE-50; ZEALWAY). For the bamboo plantation, an inoculation hood (Air Tech), fluorescent white lamps, and UV light (wavelengths of 10–400 nm) were used at temperatures of 15 and 30°C. Finally, the planted bamboos were preserved in a special chamber room under controlled conditions for 3 weeks.

ZnO-NPs were provided by the Nanjing Jiancheng Company, Nanjing, Jiangsu Province, China. This material has the characteristics of a powder with high nano Zn purity and a diameter of less than 50 nm. In this study, the levels of ZnO-NPs and metals were selected according to the different ranges of bamboo tolerance obtained by our previous studies (Emamverdian et al., 2020a, 2021a,b).

At the end of the experiment, all bamboo samples were separated from the cleaned MS medium. For this study, antioxidant enzyme activities, thiobarbituric acid reactive substances (TBARS), H_2O_2 content, soluble protein (SP), proline (Pro), and glycine betaine (GB) content, total phenolics, flavonols, tocopherols, tyrosine ammonia-lyase (TAL) activity and phenylalanine ammonia-lyase (PAL) activity, electrolyte leakage (EL), lipoxygenase activity (LOX), Chl, carotenoid contents, ZnO-NPs, and metal accumulation in plant shoots, stems, and roots were measured carefully. The translocation factor (TF), shoot tolerance index, and root tolerance index (TI) were calculated. Then, a scanning electron microscopy (SEM) was used to observe the stomata in fresh leaves under metal stress. Finally, plant biomass indices such as root and shoot dry weight (DW) were measured.

Determination of Antioxidant Enzyme Activities

Bamboo leaves (0.5 g) were crushed in a mortar and pestle, ground into powder, mixed into liquid nitrogen, and kept at 2°C–8°C. The obtained powder was dissolved in 2 mg of phosphate buffer (pH 7.8) inside a test tube. The obtained homogenate was centrifuged at 2,000–3,000 *g* for 20 min at 4°C. After centrifugation, the obtained supernatant was used for the measurement of antioxidant activities.

The activity of superoxide dismutase (SOD; E.C. 1.15.1.1) was quantified based on the method of Beauchamp and Fridovich

(1971), which was achieved by the photoreduction obtained by nitroblue tetrazolium (NBT). Then, the supernatant (100 µl) was mixed with 50 mM phosphate buffer (pH 7.8), which was added to a solution containing 13 mM methionine, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 75 µM NBT, and 2 µM riboflavin. In the next step, the obtained solution was exposed to fluorescent lamps for 10 min. To determine the SOD, the absorbance was measured at 560 nm by a spectrometer. The activity of catalase (CAT; E.C. 1.11.1.6) was recorded by Urbanek et al. (1991). In this process, 0.1 ml of the extracted sample was dissolved in 3 ml phosphate buffer (pH 6.8) by using extinction coefficient of H_2O_2 in 39.4 $M^{-1} cm^{-1}$. CAT activity was recorded by measuring the decline in absorbance at 240 nm. The activity of ascorbate peroxidase (APX; E.C. 1.11.1.11) was determined according to the method of Nakano and Asada (1981). Therefore, in this measurement, the reaction buffer solution including 50 mM potassium phosphate (pH 7.0) was added to the extracted sample, and then 0.1 mM EDTA, 0.1 mM H_2O_2, and 0.5 mM ascorbate were added to the solution. Hence, APX antioxidant activity was obtained by recording the reduction in absorbance at 290 nm (the coefficient of absorbance at 2.8 mM^{-i} cm^{-1}). The activity of glutathione reductase (GR; E.C. 1.6.4.2) was measured by the method of Foyer and Halliwell (1976) with some modifications. For this index, the mixture consisted of extract samples which were added to 100 mM phosphate buffer (pH 7.8) and then mixed by 3.0 mM oxidized glutathione, 0.05 mM nicotinamide adenine dinucleotide phosphate (NADPH), 0.1 µM EDTA, 50 µl of the enzyme extract, and 1.0 ml NADPH oxidation was recorded in the absorbance of the 340 nm twice. The first time included the addition of H_2O_2, and the second time occurred 1 min later. The difference between the two data points was determined by the extinction coefficient of the NADPH molar (6.22 mM cm^{-1}). The activity of GR was obtained as U mg^{-1} protein.

Measurement of Tyrosine Ammonia-Lyase and Phenylalanine Ammonia-Lyase Activity

The activity of TAL was determined by the method of Berner et al. (2006). Therefore, 20 µl of the extracted sample was added to the solution that contained tyrosine (30 mM) and boric acid buffer (pH 8.5; 500 µl). Then, in the final step, the TAL activity was recorded by the absorbance at 310 nm after 30 min. p-Coumaric acid was used for the standard curve. PAL activity was measured according to the method of Berner et al. (2006). Therefore, 500 µl boric acid buffer (pH 8) was mixed in the 20 µl extracted samples, and the absorbance was recorded at 290 nm after 30 min. In this test, various levels of Ecinnamic acid were used as the standard curve.

Measurement of Hydrogen Peroxide, Lipid Peroxidation, Lipoxygenase Activity, and Electrolyte Leakage

The H_2O_2 content was determined by the method of Velikova et al. (2000). For this purpose, 0.1% (w/v) trichloroacetic acid (TCA; 5 ml) was homogenized with leaf samples (0.5 g). Then, it was centrifuged at 12,000*g* for 15 min. In the next step, supernatant (0.5 ml) was added to 10 mM potassium phosphate buffer (pH 7.0; 0.5 ml) and 1 M potassium iodide (1 ml). In final step, the absorbance at 390 nm was recorded. For the calculation of H_2O_2, one standard calibration curve was used. The estimation of lipid peroxidation was made by the TBARS content, which is used as a cell peroxidation indicator. In addition, it was used by the method of Cakmak and Horst (1991). In this experiment, 0.5 g of leaf samples were crushed in containers of 0.1% (w/v) TCA (5 ml). As for the next step, the mixture was centrifuged at 12,000*g* for 7 min. The obtained supernatant was added to 4 ml of 0.5% (w/v) TBA and 4 ml of 20% (w/v) TCA and kept at 90°C for 30 min. In the following step, the mixture was centrifuged at 10,000*g* for 5 min. Then, the TBARS content was recorded by absorbance at 532 nm. The TBARS content was determined as µM g^{-1} leaf FW. The lipoxygenase (LOX) activity was determined based on the methods of Grossman and Zakut (1979) and Sekha and Reddy (1982). Using these methods, 25 ml of the 0.1 M sodium tetraborate containing 0.1% Tween 20 was added to 10 µl linoleic acid. Then, 0.1 ml of the solution was added to an optimal amount (2.9 ml) of 0.1 M phosphate buffer of pH 4–5. EL was measured according to the method of Valentovic et al. (2006). Using this method, 0.3 g of leaf sample were added to 15 ml of deionized water. Then, the samples were preserved at 27°C for 2 h. Then, the EC_1 (electrical conductivity) of the solution was recorded. In the next step, the samples were kept in one autoclaved at 120°C for 17 min. Finally, (EC_2) electrical conductivity was recorded again. The EC was obtained as follows:

$$\mathrm{EC}(\%) = (\mathrm{EC}_1 / \mathrm{EC}_2) \times 100$$

Measurement of Glycine Betaine and Proline Contents

The content of GB was determined by the method of Grieve and Grattan (1983). The samples (leaves) were placed in an oven to dry at a controlled temperature (80°C). The dried samples were finely ground with deionized water at 110°C for 40 min. The GB concentration was recorded at 365 nm, which was obtained by the mixture of dry leaf powder after reaction with $KI–I_2$.

The proline content was measured based on the ninhydrin method (Bates et al., 1973). Therefore, 400 mg of the samples (leaf) were homogenized in sulfosalicylic acid. In the next step, 3 ml glacial acetic acid and 3 ml acid ninhydrin were added to the mixture. Then, the solution was heated at 110°C. The supernatant was extracted with toluene, and the free toluene was measured by absorption at 528 nm. In this experiment, the standard used L-proline.

Determination of Total Phenolics, Flavonols, and Tocopherols

Dry leaf samples (0.5 g) were mixed in 80% methanol (5 ml) and then centrifuged at 7,000*g* for 15 min. Methanolic extract was obtained for use in the experiment. The content of total phenolics was measured by the Akkol method (Akkol et al., 2008). For this purpose, 2.5 ml of 10% Folin–Ciocalteu reagent was added to 0.1 ml of methanolic extract. Then, the obtained mixture was neutralized by 7% sodium bicarbonate. In the final step, the content of total phenolics was recorded by absorbance at 765 nm. The results were expressed by using gallic acid calibration (mg GAE g^{-1} plant material). The flavonol content was determined according to the methods of Akkol (Akkol et al., 2008). In this test, 0.5 ml methanolic extract was homogenized with 1.5 ml of 5% sodium acetate and 2% 0.4 ml aluminum chloride. The obtained supernatant was preserved for 2.5 h at normal room temperature, and then flavonoid content was recorded by absorbance at 445 nm. The calibration for rutin was used for the calculations and the results were expressed as (mg RE/g of plant material). The content of tocopherol was determined by the method of Kayden et al. (1973). In this study, 0.1 g of leaves and samples were added to 3 ml ethanol, and then the mixture was transferred to a centrifuge machine and centrifuged at 10,000*g* for 10 min. Next, the ethanol extract (0.1 ml) was added to 0.2 ml ferric chloride (0.001 M), 0.2 ml bathophe-nanthroline (0.2%), and 0.2 ml phosphoric acid (1 mM). The tocopherol content was obtained by recording the absorbance at 534 nm. TAC was determined by comparison with the tocopherol acetate standard calibration curve. The amount of TAC was expressed for extract samples in mM tocopherol acetate equivalent/g plant material.

Assay of Chlorophyll and Carotenoid Contents

Chlorophyll pigments including Chl *a*, Chl *b*, total Chl, and carotenoid contents were recorded according to the Lichtenthaler method (Lichtenthaler and Buschmann, 2001). For this study, 0.5 g of bamboo samples (leaf) were exposed to liquid nitrogen in a mortar and then crushed. Then, the obtained powder was mixed with 20 ml of 80% acetone at a temperature of 0°C–5°C. The mixture was extracted and centrifuged at 7,000 × *g* for 15 min. Finally, the obtained supernatant was placed in a UV/vis spectrometer. Therefore, Chl a, Chl b, and carotenoid contents were measured by recording the absorbance at 663, 645, and 470 nm. To obtain the final data, the following formulae were used, which are displayed in units of mg g^{-1} fresh weight:

$$\text{Chl}\,a = 12.25A_{663} - 2.79A_{647}$$

$$\text{Chl}\,b = 21.50A_{647} - 5.10A_{663}$$

$$\text{Total Chl} = \text{Chl}\,a + \text{Chl}\,b$$

$$\text{Carotenoid} = 1{,}000A_{470} - 1.82\text{Chl}\,a - 95.15\,\text{Chl}\,b/225$$

Assay of ZnO-NP Contents and As and Hg Accumulation in Leaves, Stems, and Roots of Bamboo Species

The quantities of ZnO-NPs and As and Hg in leaves, stems, and roots of bamboo species were measured in Nanjing Forestry University lab, and the sample preparations were according to the method of Karimi et al. (2013) with some modification. The different plant organs, including leaves, stems, and roots, were carefully washed and then dried at an oven temperature of 110°C for 5–8 h. Then, nitric acid (70%) was added to the samples at a temperature of 80°C for 15 min. Then, this process was continued by centrifugation of the samples at 10,000*g* for 7 min. For the determination of ZnO-NP contents and As and Hg accumulation in leaves, stems, and roots, the recordings were performed by atomic absorption spectrometry (AAS), which was equipped with a Zeeman-effect background correction system and a graphite furnace (Analyst 800, Perkin Elmer). These instruments were used to determine the metal accumulation through analysis. Determination of metal standards was conducted based on nitric acid (2.5%) using a spectral scan. The machine calibration of the standard (Perkin Elmer), a standard including all of the elements in an inorganic target analyst list (TAL), was run at optimum intervals in an unattended automatic analysis run mode.

Determination of Plant Biomass

In the last step of the experiment, bamboo samples were separated from MS medium and divided into two types of roots and shoot samples. For the analysis of physiobiochemical treatments in the future, a tiny fraction of the fresh shoots was stored under cold conditions at −80°C. The other parts of the plant root and plant shoot were placed in an oven to dry at the optimum temperature of 70°C for 48 h. Then, the plant biomass, including shoot dry weight (SHDW) and root dry weight (RDW), were obtained after weighing the samples.

Translocation Factor and Tolerance Index Assays

To determine the performance of various concentrations of ZnO-NPs in coping with metal toxicity and to identify the involved mechanisms, the TF and TI were obtained. This was calculated based on the Souri method (Souri et al., 2020). The value was calculated by the following formulae:

$$\begin{aligned}\text{Translocation factor}\left(\text{TF}\right) = {} & \text{the concentrations of} \\ & \text{ZnO - NPs and metal / metalloid} \\ & \left(\text{As and Hg}\right)\text{ in the plant} \\ & \text{shoots}\left(\text{leaves}\right)\left(\mu g\,\text{g}^{-1}\right) \\ & /\text{the concentrations of} \\ & \text{ZnO - NPs and metal / metalloid} \\ & \left(\text{As and Hg}\right)\text{ in the plant} \\ & \text{roots}\left(\mu g\,\text{g}^{-1}\right)\end{aligned}$$

$$\begin{aligned}\text{Tolerance index}\left(\text{TI}\right)\text{ of shoots} = {} & \text{dry weight}\left(\text{DW}\right) \\ & \text{of plant shoots from} \\ & \text{ZnO - NP and metal / metalloid} \\ & \left(\text{As and Hg}\right)\text{ treatments}\left(\text{g}\right) \\ & /\text{dry weight}\left(\text{DW}\right)\text{ of the plant} \\ & \text{shoots from the control}\left(\text{g}\right)\end{aligned}$$

$$\begin{aligned}\text{Tolerance index}\left(\text{TI}\right)\text{ of root} = {} & \text{dry weight}\left(\text{DW}\right)\text{ of plant} \\ & \text{root from ZnO - NPs and} \\ & \text{metal / metalloid}\left(\text{As and Hg}\right) \\ & \text{treatment}\left(\text{g}\right) / \text{dry weight}\left(\text{DW}\right) \\ & \text{of the plant root from control}\left(\text{g}\right)\end{aligned}$$

Scanning Electron Microscopy

The bamboo leaves were observed based on method of Li et al. (2016b) with some corrections and modifications. The central section of fresh leaves of the bamboo species was used for SEM. For this purpose, after the leaves were cut into 6.0 mm × 6.0 mm parts, the pieces were transferred to an oven and dried at 70°C for 20 min. A gold anion sputter apparatus (Model E-1010 Hitachi Ion Sputter JEOL, Japan) operated at 16 mA for 60 s was used to coat the samples. The middle section of leaves was scanned using a scanning electron microscope (SEM) system (JSM-6380, JEOL, Tokyo, Japan) operated at a voltage of 15–25 kV.

Statistical Analysis

Data were analyzed by a two-way factorial design with four replicates. ANOVA was conducted using the R statistical software package. Tukey's test was used for comparison of the mean differences between treatments at the $p < 0.05$ probability level.

RESULTS

ZnO-NPs Increase the Antioxidant Activity Under As and Hg Toxicity

The ANOVA showed that there were significant differences between the various levels of ZnO-NPs alone and in combination with As and Hg and antioxidant activities (SOD, CAT, APX, and GR; $p < 0.001$). Therefore, ZnO-NPs could increase antioxidant enzyme activity under As and Hg (**Figure 1**). The greatest increase in antioxidant activity was related to high levels of ZnO-NPs (200 and 150 μM), with 69%, and 59% increases in SOD activity, 95%, and 85% increases in CAT activity, 84%, and 65% increases in APX activity and 69%, and 57% increases in GR activity in comparison with their control treatments, respectively. Additionally, the results indicated that the treatments with 150 μM As and 150 μM Hg produced the lowest stimulation of the antioxidants, as shown by the SOD, CAT, APX, and GR activities, which were reduced by 59, 52, 63, and 40% by As and 75, 68, 89, and 46% by Hg, respectively, compared with their control.

ZnO-NPs Increase Tyrosine Ammonia-Lyase Activity and Phenylalanine Ammonia-Lyase Activity Under As and Hg Toxicity

Tyrosine ammonia-lyase and PAL are two important antioxidants that were measured in this study. Therefore, the results showed that the addition of ZnO-NPs alone and in combination with 150 μM As and 150 μM Hg significantly increased both TAL and PAL activities ($p < 0.001$; **Figure 2**). In this study, the greatest increase in TAL and PAL activities was related to 200 μM ZnO-NPs, with 35%, and 46% increases in TAL and PAL activities, respectively, compared with their control treatment. The lowest one was related to a concentration of 150 μM As and 150 μM Hg with 32%, and 48% reduction in TAL activities, and 50 and 60% reductions in PAL activities in comparison with control treatments. Therefore, we suggested that ZnO-NPs have the ability to increase TAL and PAL activities under metal/metalloid stress (**Figure 2**).

ZnO-NPs Have a Positive Impact on the Reduction of H_2O_2 Content, Lipid Peroxidation, and Electrolyte Leakage Under As and Hg

The investigation of the effect of HMs on plant membrane and cell peroxidation is important. For estimation, the impact of ZnO-NPs on ROS compounds and lipid peroxidation and the rate of plant membrane injury, H_2O_2 contents, TBARS, LOX, and EL in bamboo species were measured. The results showed that there was a significant difference between the various levels of ZnO-NPs alone and in the form of combinations with As and Hg metals ($p < 0.001$; **Figure 3**). Therefore, the results revealed that ZnO-NPs can reduce oxidative stress and membrane injury caused by As and Hg (**Figure 3**). In this study, the greatest reduction in H_2O_2 content, TBARS and LOX activity, as well as EL percentage, was related to 200 μM ZnO-NPs with 52, 56, 62, and 73% reductions compared with their control treatments. This showed the ability of ZnO-NPs to reduce membrane injury. Therefore, the combination of ZnO-NPs with metal/metalloid has a strong ability to reduce oxidative stress in plants, which can be related to the stimulation of antioxidant activities by separate concentrations of ZnO-NPs.

ZnO-NPs Increase Nonenzymatic Antioxidants to Cope With As and Hg

According to the results, the total phenolic, flavonol, and tocopherol contents reacted with an increasing trend upon the addition of ZnO-NPs. Therefore, there was a significant difference between the various levels of ZnO-NPs alone and in the form of combinations with As and Hg ($p < 0.001$; **Table 1**). In this study, the greatest enhancement of total phenol, flavonol, and tocopherol contents was related to 200 and 150 μM ZnO-NPs with 81, and 73% increases in total phenol and 37%, and 27% increases in flavonol and 44%, and 38% increases in tocopherol content in comparison with their control treatments. According to the results, the addition of ZnO-NPs could significantly increase nonenzyme antioxidants in bamboo plants, which can help to reduce ROS compounds and oxidative stress in plants. On the other hand, the total phenolic, flavonol, and tocopherol contents showed 29, 42, and 36% reductions by 150 μM As and 40, 59, and 54% reductions by 150 μM Hg relative to their control, respectively. This shows the toxic role of metals in reducing nonenzyme activities in the present study.

ZnO-NPs Increased Proline and Glycine Betaine Content in Bamboo Species Under As and Hg Stress

Previously, it had been reported that Pro and GB can be suitable indicators to measure metal toxicity and plant defense mechanisms' abilities to respond to stress conditions. The results showed that ZnO-NPs significantly increased GB and Pro ($p < 0.001$), which indicates the role of ZnO-NPs in the reduction of As and Hg

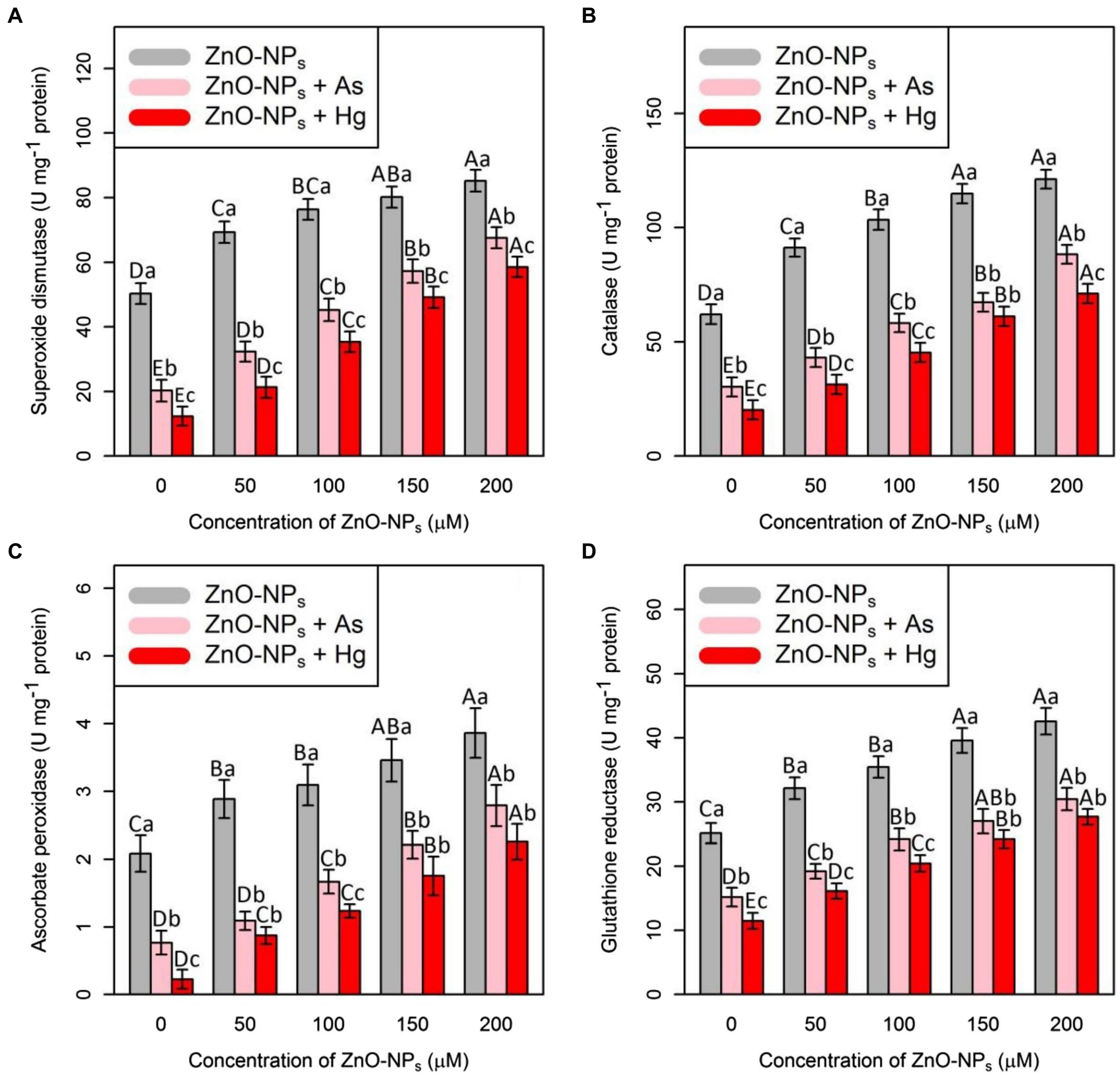

FIGURE 1 | The impact of zinc oxide nanoparticles (ZnO-NPs) concentrations on the antioxidant enzyme activities **(A)** superoxide dismutase (SOD), **(B)** catalase (CAT), **(C)** ascorbate peroxidase (APX), and**(D)** glutathione reductase (GR) in bamboo species (*Pleioblastus pygmaeus*) with 150 μM As and 150 μM Hg. The capital letters indicated statistically significant differences across various concentration of ZnO-NPs alone or in combination with 150 μM As and 150 μM Hg (the bars with the same colors), while the lowercase letters indicated statistically significant differences within each level of ZnO-NPs alone or in combination with 150 μM As and 150 μM Hg (the bars with different colors) according to Tukey's test ($p < 0.05$).

(**Table 1**). According to the obtained results, the greatest increase in Pro and GB was related to 200 μM ZnO-NPs, with 56%, and 55% increases relative to the control treatments, respectively. The lowest amount was related to 150 μM As and 150 μM Hg, with 46 and 59% reductions in Pro and 40 and 54% reductions in GB in comparison with their control treatment.

ZnO-NPs Improve Chlorophyll and Carotenoid Contents in Bamboo Species Under As and Hg Stress

To evaluate the impact of ZnO-NPs on plant photosynthesis and plant metabolism after exposure to As and Hg toxicity, Chl indices, including Chl *a*, Chl *b*, and total Chl as well as carotenoid contents, were measured. In this study, the data recorded a positive impact of various levels of ZnO-NPs on the quantity of Chl indices and carotenoid contents (**Figure 4**), which demonstrated a significant difference between the various levels of ZnO-NPs alone or in the form of combinations of As and Hg ($p < 0.001$). Therefore, the greatest enhancement of Chl *a*, Chl *b*, total Chl, and carotenoids was related to 200 μM ZnO-NPs with 16, 35, 25, and 57% increases compared with their control treatments, respectively. As expected, 150 μM As and 150 μM Hg had a deleterious impact on Chl and carotenoid indices, which reduced Chl *a*, Chl *b*, total Chl, and carotenoid contents by 23, 42, 32, and 59% by As and 32, 60, 46, and

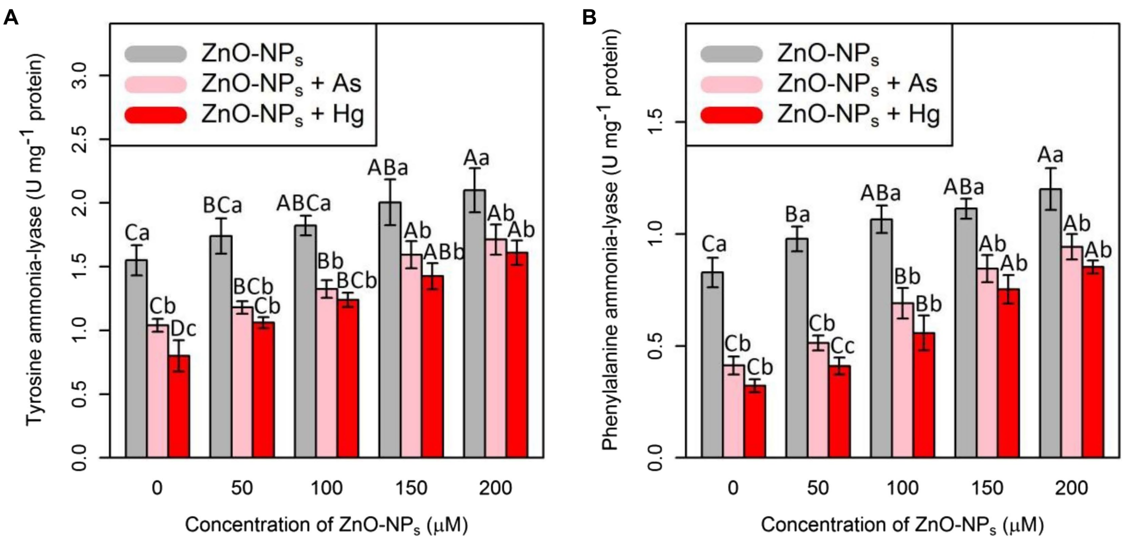

FIGURE 2 | The impact of ZnO-NPs concentrations on **(A)** tyrosine ammonia-lyase (TAL) activity and **(B)** phenylalanine ammonia-lyase (PAL) in bamboo species (*Pleioblastus pygmaeus*) with 150 μM As and 150 μM Hg. The capital letters indicated statistically significant differences across various concentration of ZnO-NPs alone or in combination with 150 μM As and 150 μM Hg (the bars with the same colors), while the lowercase letters indicated statistically significant differences within each level of ZnO-NPs alone or in combination with 150 μM As and 150 μM Hg (the bars with different colors) according to Tukey's test ($p < 0.05$).

43% reduction by Hg in comparison with their controls, respectively.

ZnO-NPs Lead to Increased Plant Growth and Biomass Indices in Bamboo Species Under As and Hg Toxicity

In this study, plant biomass indices were demonstrated as indicators of plant growth and development, including the bamboo shoot and root dry weight. According to the obtained data analyses, ZnO-NPs significantly increased the dry weight of shoots and roots in bamboo species under two As and Hg. Therefore, the greatest increase in shoots and roots DW was related to the high concentration of ZnO-NPs (150 and 200 μM) with 0.12 and 0.15 g increases in shoots DW and 0.38 and 0.54 g increases in DW of roots in comparison with their control treatments. However, the levels of ZnO-NPs in combination with 100 μM As and 100 μM Hg showed the ability to increase shoots and roots DW in bamboo species (**Figure 5**; **Table 2**).

Determination of ZnO-NP Accumulation as Well As and Hg Contents in Bamboo Species

The data analysis obtained by this study demonstrated that there was a significant difference between the various levels of ZnO-NPs alone and in the form of combinations with As and Hg ($p < 0.001$). Therefore, the various levels of ZnO-NPs in combination with 150 μM As and 150 μM Hg could reduce the accumulation of metal/metalloid in plant leaves, stems, and roots. The greatest reduction was related to 200 μM ZnO-NPs in combination with 150 μM As and 150 μM Hg, with 66 and 59% reductions in the leaves, 66 and 61% reductions in the stem, and 23 and 47% reductions in the root in comparison with their control, respectively (**Table 3**).

Determination of the Translocation Factor and Tolerance Index in Bamboo Species Under As and Hg Toxicity

In this study, to investigate the involved mechanisms in the reduction of metal/metalloid by ZnO-NPs, TF, and TI were calculated. According to the data from **Table 4**, the tested levels of ZnO-NPs did not significantly reduce the TF value for Hg (although a positive downward trend could be observed); in the case of As, there was a clear decrease in translocation in response to the use of ZnO-NPs, but no dose–response correlation was observed (no differences between the different levels of ZnO-NPs). On the other hand, the investigation of the tolerance index in shoots and roots demonstrated that ZnO-NPs remarkably increased plant tolerance in combination with As and Hg, and the results showed that ZnO-NPs in combination with As and Hg increased the tolerance index in bamboo plants by 7 and 4% enhancement in shoots and 37 and 18% enhancement in roots, respectively (**Table 4**).

Impact of ZnO-NPs on Stomatal Closure in the Leaf Epidermis

As shown in **Table 3**, Zn in the form of ZnO-NPs reduced As and Hg accumulation, with alterations in metal/metalloid translocation from the roots to the shoots of bamboo. This phenomenon impacted the opening and closing of stomata in the leaves. Thus, ZnO-NPs could regulate the opening and closing of stomata in plants under As and Hg, which might

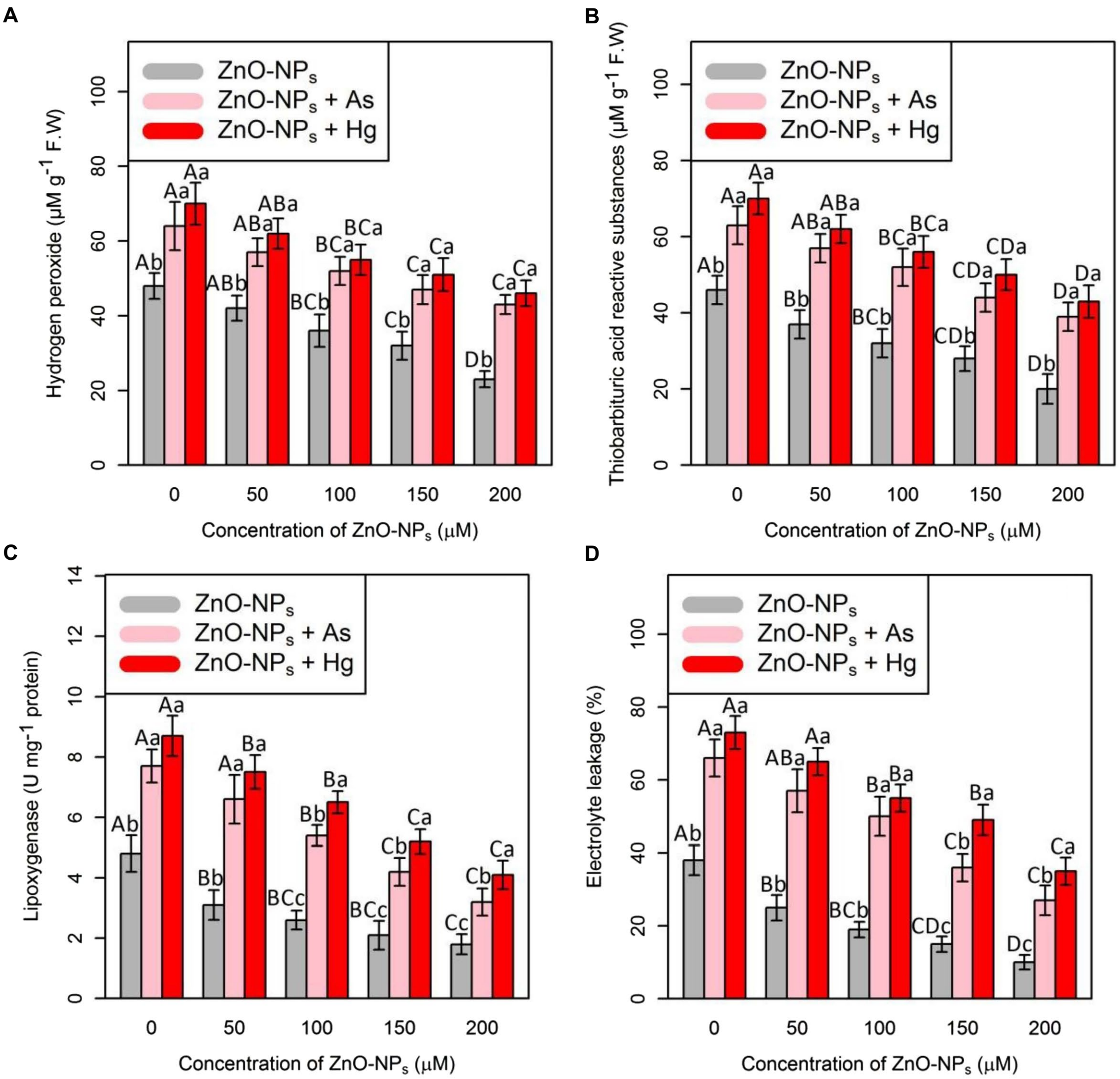

FIGURE 3 | The impact of ZnO-NPs concentrations on **(A)** hydrogen peroxide (H_2O_2) content, **(B)** thiobarbituric acid reactive substances (TBARS), **(C)** lipoxygenase activity (LOX), and **(D)** electrolyte leakage (EL) in bamboo species (*Pleioblastus pygmaeus*) with 150 µM As and 150 µM Hg. The capital letters indicated statistically significant differences across various concentration of ZnO-NPs alone or in combination with 150 µM As and 150 µM Hg (the bars with the same colors), while the lowercase letters indicated statistically significant differences within each level of ZnO-NPs alone or in combination with 150 µM As and 150 µM Hg (the bars with different colors) according to Tukey's test ($p < 0.05$).

be attributed to an enhancement in the antioxidant capacity in cells (**Figure 6**). We suggest that optimal levels of ZnO-NPs may regulate the function of stomatal pores *via* certain mechanisms, such as electrochemical and hydraulic adjustments in guard cells and osmotic pressure regulation. These processes may be related to the ability of ZnO-NPs to scavenge H_2O_2 and reduce the extent of lipid peroxidation by increasing the antioxidant capacity.

DISCUSSION

Nanoparticles can absorb and translocate HMs through their special surface area (Liu et al., 2020). Among them, Zn, in the form of ZnO-NPs, is used as a micronutrient that can help plant growth and development (Kloubert and Rink, 2015). ZnO-NPs can remove HMs from the surface with the exchange of HM ions in water solutions to improve plant growth (Venkatachalam et al., 2017a). It has been shown that excess HMs in plants lead to the generation of ROS compounds and oxidative stress in the plant (Gechev and Petrov, 2020; Wani et al., 2021), which cause damage that includes wall thickness, osmotic shock or stress, decreased water potential and transpiration, lipid membrane, protein, cell nuclear and DNA injury and, finally, the disturbance of plant photosynthesis, and plant growth and development (Akhtar et al., 2021). Therefore, plants adopt a different strategy in the form of antioxidant defense to scavenge ROS

TABLE 1 | Impact of the combination of ZnO-NPs with 150 μM As and 150 μM Hg on the non-enzymatic antioxidants (total phenolics, flavonols, and tocopherols), proline content and glycine betaine (GB) content.

ZnO NPs levels (μM)	As/Hg (μM)	Flavonols (mg RE g^{-1} F.w.)	Tocopherols (mM TAE g^{-1} F.w.)	Total Phenolics (mg GAE g^{-1} F.w.)	Proline (μg g^{-1} F.w.)	GB (μg g^{-1} F.w.)
0	0	270 ± 12.24^{Da}	622.50 ± 33.04^{Ca}	525.0 ± 45.09^{Ca}	320 ± 25.81^{Ba}	770.25 ± 34.47^{Ca}
0	150 μM As	156.50 ± 12.66^{Eb}	392.25 ± 24.70^{Eb}	182.50 ± 42.72^{Db}	170 ± 16.32^{Cb}	460 ± 39.15^{Eb}
0	150 μM Hg	110.5 ± 13.20^{Ec}	281.25 ± 22.86^{Dc}	72.50 ± 45.00^{Dc}	130 ± 18.25^{Db}	350 ± 29.43^{Ec}
50 μM	0	303.75 ± 12.20^{Ca}	782.75 ± 50.24^{Ba}	742.5 ± 70.88^{Ba}	430 ± 31.62^{Aa}	990.00 ± 52.91^{Ba}
50 μM	150 μM As	191.25 ± 11.44^{Db}	472.50 ± 23.90^{Db}	267.50 ± 26.29^{Db}	$230 \pm 21.60B^{Cb}$	620 ± 37.41^{Db}
50 μM	150 μM Hg	160.5 ± 12.71^{Dc}	423.00 ± 20.81^{Cb}	185.00 ± 34.15^{Cb}	$180 \pm 25.81C^{Db}$	520 ± 37.41^{Dc}
100 μM	0	331.50 ± 12.66^{BCa}	816.50 ± 58.22^{Aba}	800.0 ± 79.58^{ABa}	450 ± 33.66^{Aa}	1050.25 ± 81.65^{ABa}
100 μM	150 μM As	231.75 ± 13.54^{Cb}	560.00 ± 33.50^{Cb}	447.50 ± 49.91^{Cb}	270 ± 25.81^{Bb}	730 ± 35.59^{Cb}
100 μM	150 μM Hg	201 ± 11.86^{Cc}	480.25 ± 32.85^{Cb}	287.50 ± 26.29^{Bc}	$240 \pm 29.43B^{Cb}$	640 ± 39.15^{Cb}
150 μM	0	344 ± 14.30^{ABa}	862.50 ± 49.24^{ABa}	860.0 ± 87.55^{ABa}	480 ± 38.29^{Aa}	1110.0 ± 82.46^{ABa}
150 μM	150 μM As	270.25 ± 14.63^{Bb}	652.50 ± 33.04^{Bb}	572.50 ± 53.77^{Bb}	360 ± 29.43^{Ab}	850 ± 65.82^{Bb}
150 μM	150 μM Hg	241.5 ± 13.47^{Bc}	581.50 ± 29.99^{Bb}	492.50 ± 41.93^{Ab}	280 ± 34.64^{Bc}	750 ± 41.63^{Bb}
200 μM	0	371 ± 13.03^{Aa}	889.00 ± 68.31^{Aa}	898.0 ± 52.59^{Aa}	500 ± 41.63^{Aa}	1200.00 ± 109.84^{Aa}
200 μM	150 μM As	301.75 ± 12.68^{Ab}	771.00 ± 47.27^{Ab}	748.75 ± 28.97^{Ab}	420 ± 45.46^{Ab}	980 ± 43.96^{Ab}
200 μM	150 μM Hg	275.5 ± 11.73^{Ac}	675.00 ± 34.15^{Ab}	583.75 ± 60.19^{Ac}	370 ± 27.08^{Ab}	870 ± 34.64^{Ab}

Each data point is the mean ± SE of four replicates. The treatments included four levels of ZnO-NPs (50, 100, 150, and 200 μM) alone and in combination with 150 μM As and 150 μM Hg. The capital letters indicated statistically significant differences across various levels of ZnO-NPs alone or in combination with 150 μM As and 150 μM Hg, while the lowercase letters displayed statistically significant differences within each level of ZnO-NPs alone and in combination with 150 μM As and 150 μM Hg based on Tukey's test ($p < 0.05$). They are superscripted on top of the numbers.

compounds (Emamverdian et al., 2018). Antioxidants can ameliorate the toxicity of H_2O_2 by converting it into oxygen and water (Jiang et al., 2021). However, as reported in many studies, the efficiency of the antioxidant system depends on the severity, exposure time, and type of stress as well as plant species (Dawood et al., 2012; Hasanuzzaman et al., 2013, 2019). For instance, in a study on rice (*Oryza sativa*), the results showed that CAT activity decreased in the plants under aluminum (Al) toxicity due to the inhibited enzymes subunit assembly, leading to a significant reduction in enzyme synthesis (Sharma and Dubey, 2007). This shows that the severity of oxidative stress can have a negative impact on the synthesis of enzymes and diminish their functions. Our results demonstrated that ZnO-NPs can increase antioxidant activity, especially SOD and CAT, under metal toxicity, which has been evidentially confirmed in *Triticum aestivum* (Hussain et al., 2018), *Zea mays* (Rizwan et al., 2019a,b), and rice (Faizan et al., 2021b). SOD is the front line of the antioxidant defense system, converting O_2– to H_2O_2 with less toxicity, and CAT has completed this process by scavenging H_2O_2 to H_2O and O_2– (Venkatachalam et al., 2017b). Therefore, they play an essential role in the scavenging of ROS; a process that the present study suggests could be increased. However, all antioxidant activities (SOD, CAT, GR, and APX) were increased following the addition of ZnO-NPs under 150 μM As and 150 μM Hg.

Tyrosine ammonia-lyase and phenylalanine ammonia-lyase (PAL) are two key enzymes in phenolics compounds and biosynthesis pathways. Phenolics compounds include nonenzymatic antioxidants that are involved in ROS scavenging (Rezayian et al., 2020). According to our results, while 150 μM As and 150 μM Hg reduced the TAL and PAL activities, the addition of ZnO-NPs to metal concentrations increased TAL and PAL activities in bamboo species, which demonstrated the role of ZnO-NPs in the stimulation of phenolics content HMs. Therefore, the total phenolics, flavonols, and tocopherols contents were accounted for. Hence, in this study, the results showed that different concentrations of ZnO-NPs increased the phenolics content, including total phenolics, flavonols, and tocopherols, in bamboo species under metal stress. An increase in phenolics content has been reported in the previous studies (García-López et al., 2018; Rezayian et al., 2020). Therefore, we suggest that the combination of ZnO-NPs with increasing antioxidant enzymes capacities improves the plant defense mechanism under ROS conditions.

Many studies have reported that HMs lead to the generation of ROS compounds (H_2O_2 and O_2–; Ahmad et al., 2019; Bhat et al., 2019; Kaya et al., 2020), which finally leads to plant death caused by oxidative stress. However, the results obtained in our study indicated that ZnO-NPs reduced the accumulation and content of H_2O_2 and TBARS in the plants under As and Hg toxicity. A similar result was reported by other authors (Li et al., 2016a; Faizan et al., 2020, 2021a,b). Our study demonstrated the role of ZnO-NPs in the activation of plant antioxidant capacities. On the other hand, ZnO-NPs with adsorption mechanisms enhance the interaction between metal ions and NPs to scavenge ROS compounds in plants under metal stress (Maity et al., 2018).

Zinc can play an essential role in the biological regulation of the cell membrane *via* binding groups of sulfhydryl with phospholipids under critical situations such as stress conditions (Hafeez et al., 2013). In this study, LOX showed increasing lipid peroxidation with one important role in the oxidation of poly-unsaturated fatty acids. EL acts as an indicator of cell membrane damage. Our results showed that all concentrations of ZnO-NPs could reduce the EL and LOX contents under

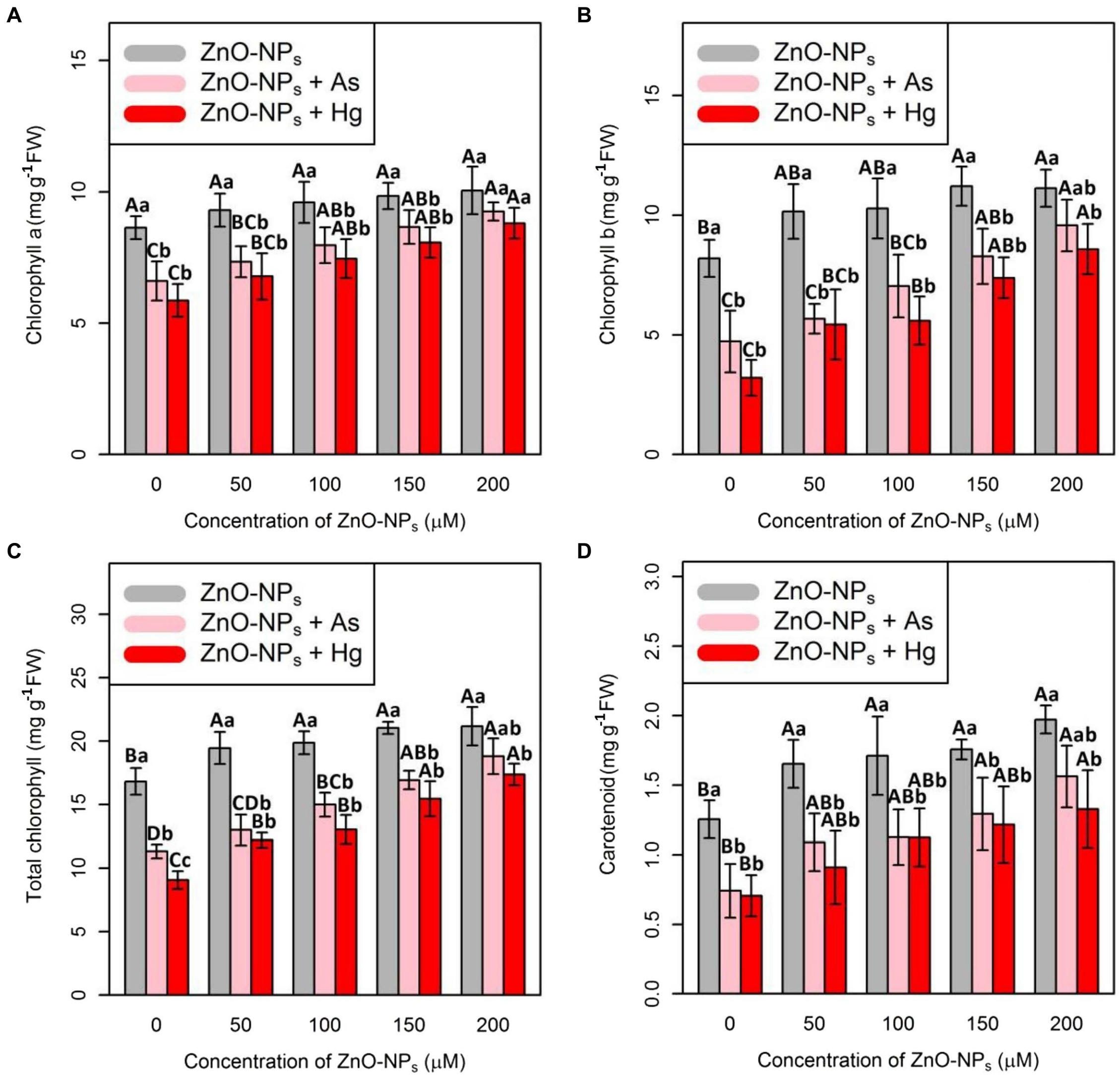

FIGURE 4 | Impact of the combination of ZnO-NPs with 150 μM As and 150 μM Hg on chlorophyll *a* **(A)**, chlorophyll *b* **(B)**, and total chlorophyll **(C)** as well as carotenoid contents **(D)**. Each data point is the mean ± SE of four replicates. The treatments included four levels of ZnO-NPs (50, 100, 150, and 200 μM) alone and in combination with 150 μM As and 150 μM Hg. The capital letters indicated statistically significant differences across various levels of ZnO-NPs alone or in combination with 150 μM As and 150 μM Hg, while the lowercase letters displayed statistically significant differences within each level of ZnO-NPs alone and in combination with 150 μM As and 150 μM Hg based on Tukey's test ($p < 0.05$). They are superscripted on top of the numbers.

150 μM As and 150 μM Hg. However, the high concentration of ZnO-NPs had the most impact on the reduction of EL and LOX. Therefore, we suggest that ZnO-NPs can protect the cell membrane under metal/metalloid toxicity, which is related to scavenging ROS compounds and improving the plant antioxidant capacity.

Proline and GB are two syntheses of osmolytes that can preserve plant cells from dehydration stress (Ahmad et al., 2019). Pro accumulation in plants plays an important role in plant growth regulation under stress conditions (Torabian et al., 2016), and the positive role of Pro has been revealed in ROS scavenging, membrane stabilization, and finally osmotic stress (Bandurska, 2001). In this study, our results showed that ZnO-NPs can increase the accumulation of Pro in bamboo species, which is related to the role of ZnO-NPs in the expression of genes involved in Pro biosynthesis (Faizan et al., 2021a,b). This result is confirmed by the findings of other studies (Helaly et al., 2014; Faizan et al., 2020). GB acts as an important osmoregulator in plants and can occur at various concentrations in different plant species. It can play a key role in the activation of glutathione (GSH), ascorbic acid (AsA), and glutathione reductase (GR) under HM stress (Ali et al., 2020). Our results showed that ZnO-NPs could increase the accumulation of GB in plants under As and Hg.

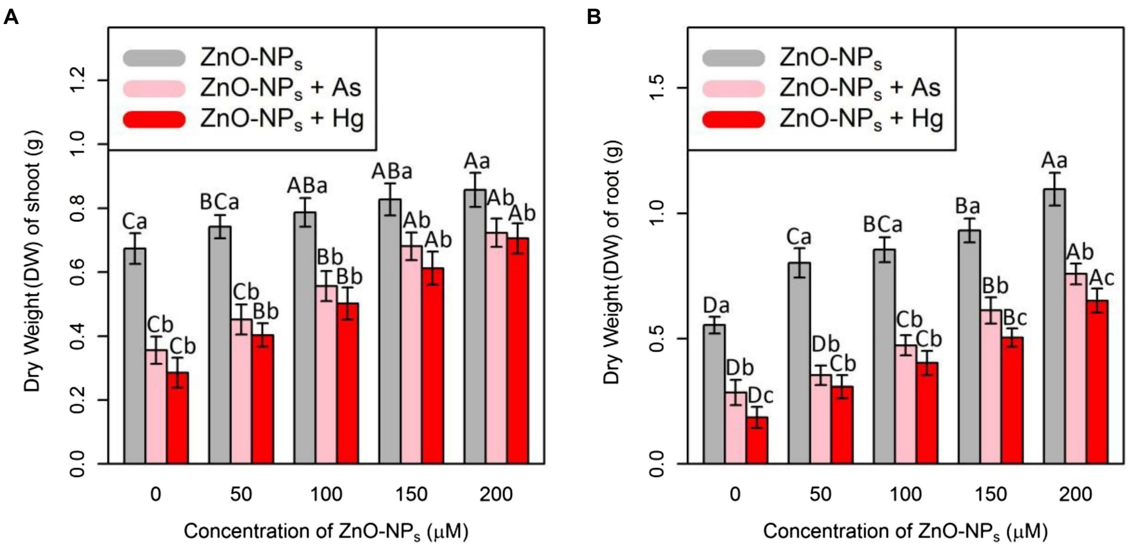

FIGURE 5 | The impact of ZnO-NPs concentrations on **(A)** dry weight (DW) of shoot and **(B)** dry weight (DW) of root in bamboo species (*Pleioblastus pygmaeus*) with 150 μM As and 150 μM Hg. The capital letters indicated statistically significant differences across various concentration of ZnO-NPs alone or in combination with 150 μM As and 150 μM Hg (the bars with the same colors), while the lowercase letters indicated statistically significant differences within each level of ZnO-NPs alone or in combination with 150 μM As and 150 μM Hg (the bars with different colors) according to Tukey's test ($p < 0.05$).

TABLE 2 | The percent changes of bamboo biomass in shoot and root dry weight at various levels of ZnO-NPs in combination with 150 μM As and 150 μM Hg compared to those of the control treatment (100%).

ZnO-NPs Levels (μM)	Metal/metalloid levels	Dry shoot weight	Dry root weight
0	150 μM As	47%↓	53%↓
0	150 μM Hg	58%↓	66%↓
50	0	10%↑	44%↑
50	150 μM As	32%↓	36%↓
50	150 μM Hg	40%↓	44%↓
100	0	16%↑	54%↑
100	150 μM As	17%↓	14%↑
100	150 μM Hg	25%↓	27%↓
150	0	23%↑	68%↑
150	150 μM As	1% ↑	10%↑
150	150 μM Hg	9% ↓	9% ↓
200	0	27% ↑	96%↑
200	150 μM As	7%↑	36%↑
200	150 μM Hg	4%↑	18%↑

It has been reported that ZnO-NPs are involved in the activation of the enzymatic activity of Rubisco and carbonic anhydrase (CA). Therefore, these enzymes, through the expression of particular genes, can increase the chemical energy in the photosynthetic system, which can improve plant photosynthetic properties (Rico et al., 2015). On the other hand, it has been reported that most GBs accumulate in chloroplasts, which can have a positive impact on the effectiveness of safeguarding in photosystem II (PSII) as well as the photosynthesis system in plants under stress. The enhancement in the content of Chl can be an indicator of photosynthesis performance in plants (Altaf et al., 2020). Our results indicated that ZnO-NPs increased the Chl content and carotenoids under 150 μM As and 150 μM Hg in our bamboo species. Therefore, the accumulation of GB can be one of the reasons for increasing Chl and carotenoids contents in plants under stress. This can also involve the activation of antioxidant enzyme activity, including SOD and CAT (Rasheed et al., 2017). Faizan reported that ZnO-NPs enhance Chl contents with a reduction in Cu toxicity in tomato and Cd toxicity in rice (Faizan et al., 2021a,b). This is consistent with the results obtained in the present study. Therefore, it can be suggested that ZnO-NPs can increase photosynthetic properties in plants under As and Hg.

According to SEM observation (**Figure 6**), our results revealed that ZnO-NPs could regulate stomatal aperture under the stress. So that ZnO-NPs were able to keep stomata open under 150 μM As and also maintained stomata in a semi-open and closed state under 150 μM Hg. Some mechanisms are proposed to explain the stomatal responses to the application of NPs under metal/metalloid stress. Of which, the removal of the As and Hg by NPs through the adsorption process is notable (Fu and Wang, 2011; Burakov et al., 2018) where Zn NPs dispersed on the surface of the leaves and the epidermis can sequester metal ions through adsorption. This has also been shown with different HMs, such as Cu (Mobasherpour et al., 2012), Cd, and tin (Sn; Ghahremani et al., 2017) using different types of nanoparticles. On the other hand, it is reported that ZnO-NPs could be accumulated below the stomata and consequently be transferred through the apoplast pathway. During this transfer, a fraction of the ZnO-NPs releases Zn cations through the dissolution in the apoplast, which

TABLE 3 | The concentrations of ZnO-NPs, As, and, Hg in bamboo leaves, stems, and roots.

ZnO-NPs level (μmol L^{-1})	As/Hg level (μmol L^{-1})	Corresponding As/Hg concentration (μg g^{-1})			Corresponding ZnO-NPs concentration (μg g^{-1})		
		Leaf	Stem	Root	Leaf	Stem	Root
0	0	nd	nd	nd	nd	nd	nd
0	150 μM As	18.65 ± 0.98^{Aa}	22.70 ± 0.70^{Ab}	15.70 ± 0.94^{Cb}	nd	nd	nd
0	150 μM Hg	20.50 ± 1.32^{Aa}	24.55 ± 0.94^{Aa}	27.85 ± 1.24^{Aa}	nd	nd	nd
50 μM	0	nd	nd	nd	13.60 ± 1.10^{Ba}	16.92 ± 1.11^{Ca}	17.30 ± 1.18^{Da}
50 μM	150 μM As	15.35 ± 0.98^{Ba}	18.40 ± 0.98^{Bb}	22.10 ± 0.97^{Ab}	5.22 ± 0.65^{Cb}	7.35 ± 0.70^{Db}	9.62 ± 0.95^{Cb}
50 μM	150 μM Hg	16.80 ± 0.82^{Ba}	20.52 ± 0.98^{Ba}	24.70 ± 1.21^{Ba}	3.30 ± 0.77^{Dc}	6.82 ± 0.90^{Cb}	7.52 ± 0.77^{Cc}
100 μM	0	nd	nd	nd	15.32 ± 1.12^{Ba}	18.75 ± 1.27^{BCa}	21.45 ± 0.98^{Ca}
100 μM	150 μM As	13.60 ± 0.90^{Ba}	14.65 ± 1.01^{Cb}	19.52 ± 1.23^{Bb}	7.37 ± 0.69^{Bb}	9.50 ± 0.90^{Cb}	10.50 ± 1.06^{Cb}
100 μM	150 μM Hg	14.62 ± 0.86^{Ca}	16.20 ± 1.12^{Ca}	21.70 ± 1.32^{Ca}	6.10 ± 0.76^{Cb}	8.50 ± 0.65^{Cb}	8.35 ± 0.88^{Cc}
150 μM	0	nd	nd	nd	17.72 ± 0.95^{Aa}	20.55 ± 1.28^{ABa}	25.52 ± 1.24^{Ba}
150 μM	150 μM As	11.55 ± 0.97^{Ca}	11.32 ± 0.99^{Db}	15.42 ± 0.98^{Cb}	8.50 ± 0.61^{Bb}	12.60 ± 1.02^{Bb}	13.62 ± 1.10^{Bb}
150 μM	150 μM Hg	11.80 ± 0.99^{Da}	13.62 ± 1.24^{Da}	18.47 ± 1.06^{Da}	7.60 ± 0.71^{Bb}	10.92 ± 0.87^{Bb}	11.50 ± 1.14^{Bb}
200 μM	0	nd	nd	nd	19.55 ± 1.25^{Aa}	22.42 ± 1.10^{Aa}	29.50 ± 1.56^{Aa}
200 μM	150 μM As	6.34 ± 0.79^{Da}	7.70 ± 0.87^{Eb}	11.95 ± 0.75^{Db}	10.60 ± 0.94^{Ab}	15.80 ± 0.98^{Ab}	16.45 ± 1.26^{Ab}
200 μM	150 μM Hg	8.30 ± 0.79^{Ea}	9.40 ± 1.00^{Ea}	14.50 ± 1.32^{Ea}	9.72 ± 0.57^{Ab}	13.55 ± 0.99^{Ac}	14.20 ± 1.09^{Ab}

Each data point is the mean ± SE of four replicates. The treatments included four levels of ZnO-NPs (50, 100, 150, and 200 μM) alone and in combination with 150 μM As and 150 μM Hg. The capital letters indicated statistically significant differences across various levels of ZnO-NPs alone or in combination with 150 μM As and 150 μM Hg, while the lowercase letters displayed statistically significant differences within each level of ZnO-NPs alone and in combination with 150 μM As and 150 μM Hg based on Tukey's test ($p < 0.05$). They are superscripted on top of the numbers.

nd, not detected.

TABLE 4 | The change in translocation factor (TF) and tolerance index (TI) in shoot and root at different concentrations of ZnO-NPs in combination with 150 μM As and 150 μM Hg compared to those of the control treatment.

ZnO-NPs levels	As/Hg level	Translocation factor (TF)	Tolerance index (TI; shoot)	Tolerance index (TI; root)
0	0	0.00 ± 0.00^{Cb}	1.00 ± 0.00^{Ca}	1.00 ± 0.00^{Ca}
0	150 μM As	1.21 ± 0.02^{Aa}	0.53 ± 0.10^{Db}	0.51 ± 0.10^{Db}
0	150 μM Hg	0.73 ± 0.07^{Ac}	0.42 ± 0.09^{Db}	0.33 ± 0.06^{Dc}
50 μM	0	0.78 ± 0.05^{Aa}	1.09 ± 0.05^{BCa}	1.45 ± 0.19^{Ba}
50 μM	150 μM As	0.64 ± 0.04^{Bb}	0.67 ± 0.07^{CDb}	0.63 ± 0.05^{CDb}
50 μM	150 μM Hg	0.62 ± 0.04^{Ab}	0.60 ± 0.09^{CDb}	0.55 ± 0.11^{CDb}
100 μM	0	0.71 ± 0.08^{ABa}	1.16 ± 0.07^{ABa}	1.54 ± 0.18^{Ba}
100 μM	150 μM As	0.70 ± 0.08^{Ba}	0.82 ± 0.10^{BCb}	0.85 ± 0.12^{BCb}
100 μM	150 μM Hg	0.69 ± 0.05^{Aa}	0.74 ± 0.10^{BCb}	0.73 ± 0.13^{BCb}
150 μM	0	0.69 ± 0.06^{ABa}	1.22 ± 0.08^{ABa}	1.68 ± 0.16^{ABa}
150 μM	150 μM As	0.69 ± 0.02^{Ba}	1.01 ± 0.07^{ABb}	1.10 ± 0.16^{ABb}
150 μM	150 μM Hg	0.64 ± 0.06^{Aa}	0.91 ± 0.11^{ABb}	0.91 ± 0.12^{Bb}
200 μM	0	0.66 ± 0.00^{Ba}	1.27 ± 0.10^{Aa}	1.97 ± 0.13^{Aa}
200 μM	150 μM As	0.59 ± 0.04^{Bb}	1.07 ± 0.13^{Aab}	1.37 ± 0.16^{Ab}
200 μM	150 μM Hg	0.62 ± 0.02^{Aab}	1.04 ± 0.06^{Ab}	1.18 ± 0.15^{Ab}

Each data point is the mean ± SE of four replicates. The treatments included four levels of ZnO-NPs (50, 100, 150, and 200 μM) alone and in combination with 150 μM As and 150 μM Hg. The capital letters indicated statistically significant differences across various levels of ZnO-NPs alone or in combination with 150 μM As and 150 μM Hg, while the lowercase letters displayed statistically significant differences within each level of ZnO-NPs alone and in combination with 150 μM As and 150 μM Hg based on Tukey's test ($p < 0.05$). They are superscripted on top of the numbers.

could be absorbed by cells of mesophyll and distributed in the lower and upper mesophyll tissues (Zhu et al., 2020) thus the stomatal opening is regulated as revealed in **Figure 6**.

Many studies have reported that ZnO-NPs reduce HM accumulation in plants (Skiba et al., 2020; Faizan et al., 2021a,b), which is related to the fact that ZnO-NPs precipitate the HM content on the surface of roots. This limits the absorption and translocation of HMs from roots to aerial organs (Venkatachalam et al., 2017b; Ahmad et al., 2019). In fact, Zn can be taken up by plant roots quickly and reduce the uptake of HMs by roots (Hasan et al., 2008). Alternatively, Zn can act as a physical barrier that prevents the translocation of metals from roots to shoots, which occurred in our study. Therefore, Zn accumulation in roots limits metal translocation from roots to shoots and reduces metal accumulation in shoots. Hence, the levels of toxic metal/metalloid in the shoots and stems were significantly lower than those in the roots in our present study. This result has been suggested by other studies (Vasiliadou and Dordas, 2009; Garg and Kaur, 2013). Our results indicated that ZnO-NPs can significantly reduce toxic metal/metalloid accumulation in plants under As and Hg toxicity, as displayed in **Table 3**. Therefore, the reduction in accumulation and limitation of toxic metal/metalloid translocation by levels of ZnO-NPs can be important mechanisms in the reduction of As and Hg in our bamboo species. Zn^{2+} in the form of ZnO-NPs is known as an essential micronutrient that can help plant growth and development (Liu et al., 2015). Zn also plays a role in the synthesis of auxin (IAA), which can help to improve cell expansion and cell division in plants (Begum et al., 2016). The results demonstrated that while 150 μM As and 150 μM Hg reduced the plant biomass (DW of shoots and roots), the addition of different concentrations of ZnO-NPs could help to increase plant biomass under As and Hg. On the other hand, excess HMs in plants lead to reduced cell viability in plant roots,

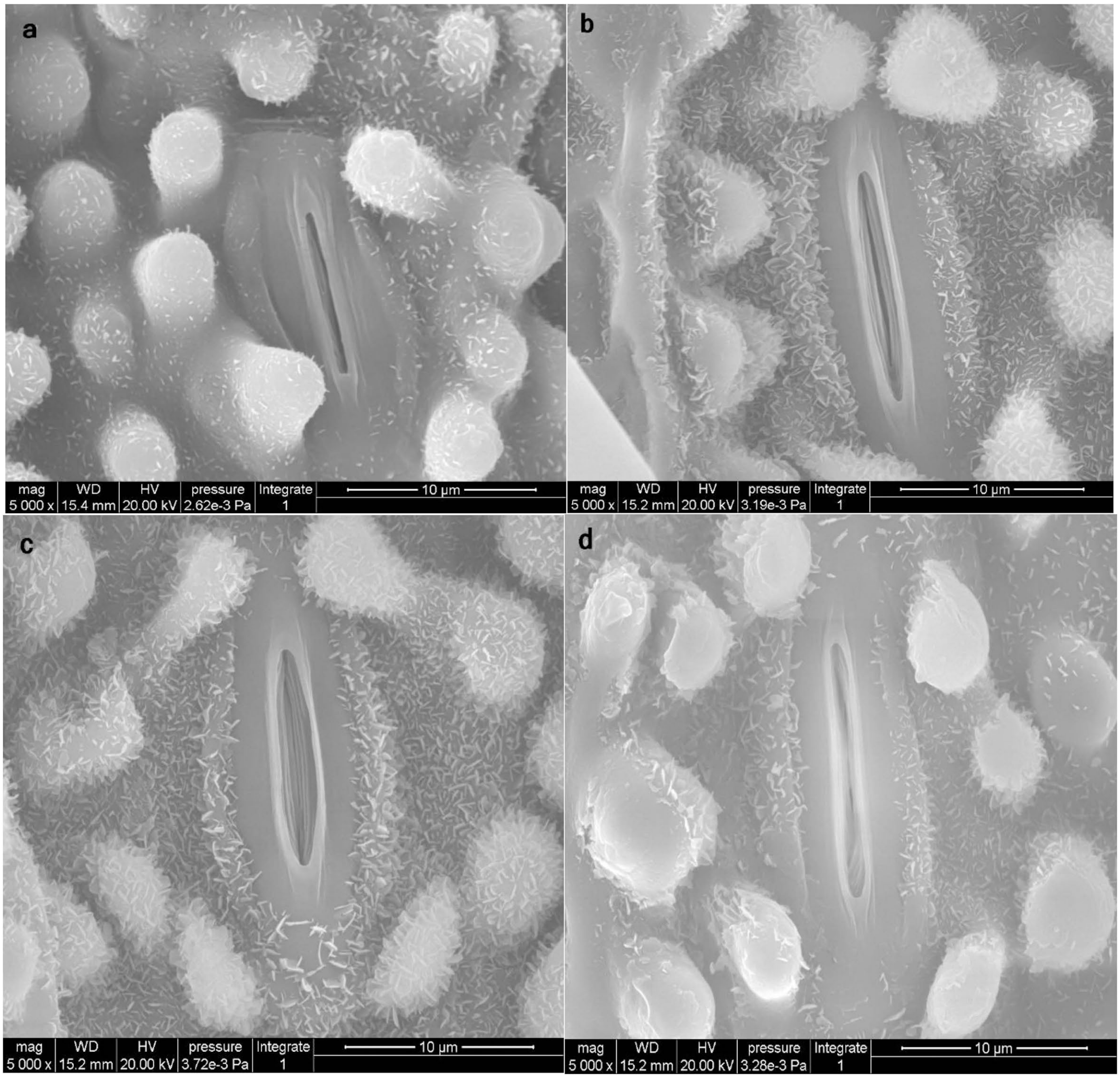

FIGURE 6 | Scanning electron microscopy (SEM) images of stomata closure (opening and closing of stomata) on the leaf surface of bamboo (*Pleioblastus pygmaea*). Image **(a)** displays the control treatment under normal conditions. Image **(b)** displays the condition of stomata in plants receiving 200 μM ZnO-NPs treatment. Images **(c,d)** display the condition of stomata receiving 150 μM As and 150 μM Hg (**c** = As, **d** = Hg) treatment in combination with 200 μM ZnO-NPs. The results suggest that ZnO-NPs kept stomata open when combined with 150 μM As and can also help maintain stomata in a semi open and closed state when combined with 150 μM Hg.

but ZnO-NPs increase the viability of cells in roots. This can help plant growth and development, which has been reported by other researcher (Rajapakse et al., 2017; Faizan et al., 2021a,b). We suggested that the addition of ZnO-NPs increased the plant biomass with the enhancement of plant root and shoot DW. Therefore, this enhancement can be related to improving photosynthesis indices by increasing antioxidant activity and GB accumulation in plants under 150 μM As and 150 μM Hg. There was one major question in this study: Can ZnO-NPs increase plant tolerance under metal/metalloid toxicity? Our results found that ZnO-NPs have the ability to increase TI in plants toxic metal/metalloid toxicity (As and Hg), as shown in **Table 4**. Therefore, it can be of considerable importance to use bamboo species in phytoremediation technology in the polluted areas. The mechanistic diagram to indicate the actual mechanisms/ role of ZnO NPs under metal/metalloid toxicity in bamboo species has been observed in **Figure 7** has shown.

CONCLUSION

It was concluded that As and Hg had a pernicious impact on the plant growth by increasing ROS compounds, reducing antioxidant activity, injuring the cell membrane, and depressing the plant photosynthesis. The addition of ZnO-NPs increased

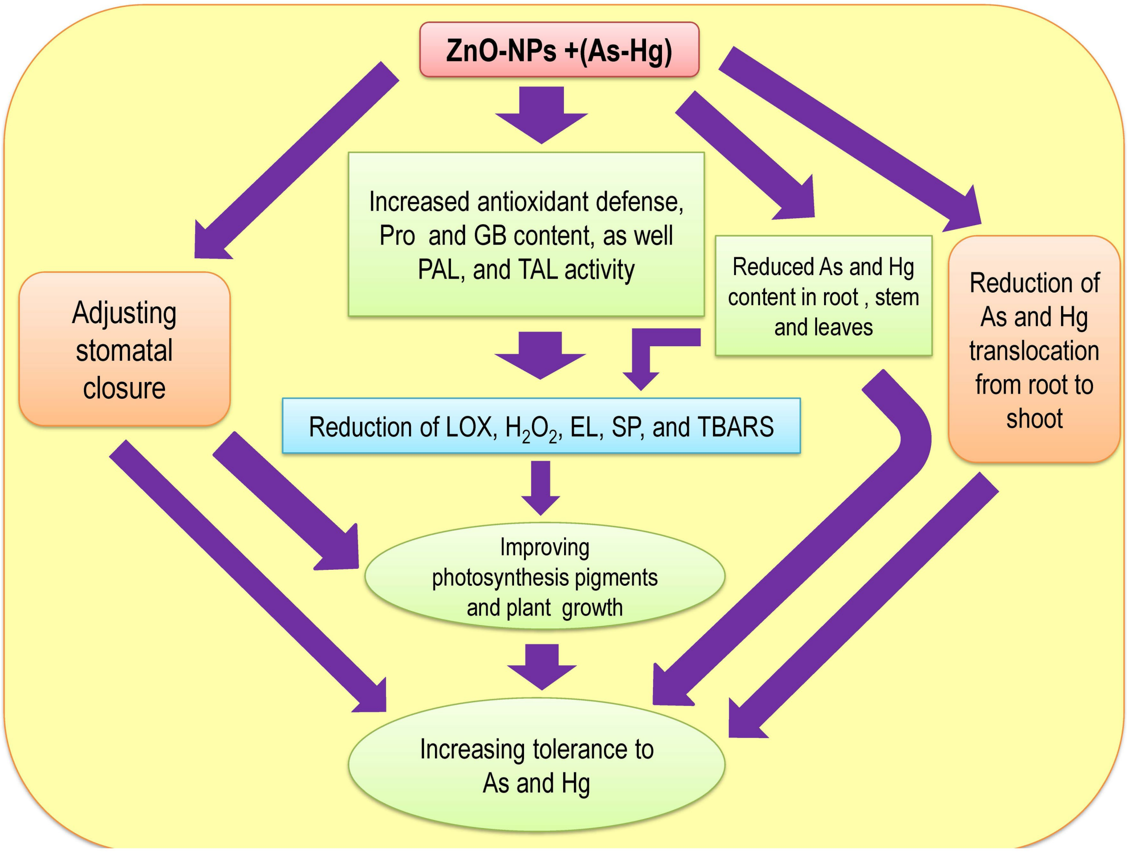

FIGURE 7 | The Mechanistic diagram to indicate the actual mechanisms of ZnO NPs under metal/metalloids toxicity in bamboo.

the plant photosynthesis by boosting antioxidant activities, GB, Pro, PAL, and TAL contents and reducing cell membrane injury *via* a reduction in H_2O_2, LOX, TBARS, and EL. Therefore, ZnO-NPs were able to successfully improve the plant biomass and growth under 150 µM As and 150 µM Hg. We suggest that ZnO-NPs can increase bamboo plant tolerance under As and Hg. This enhancement occurred by reducing metal/metalloid accumulation in the plant parts and decreasing toxic metal/metalloid translocation from the roots to the shoots. Consequently, it raises the potential of the bamboo plants to be used in phytoremediation technology in the polluted areas to clean the environment.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

AE, YD, MH, and JB: conceptualization. AE: statistical analysis. AE, YD, JB, FM, MH, and GL: writing original draft and revised preparation. AE and GL: investigation. AE, YD, and GL: supervision and funding acquisition. AE, YD, and MH: project administration. JB and FM: English editing. All authors contributed to the article and approved the submitted version.

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Calcium Oxide Nanoparticles Have the Role of Alleviating Arsenic Toxicity of Barley

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Arsenic (As) contamination in agricultural soils has become a great threat to the sustainable development of agriculture and food safety. Although a lot of approaches have been proposed for dealing with soil As contamination, they are not practical in crop production due to high cost, time-taking, or operational complexity. The rapid development of nanotechnology appears to provide a novel solution to soil As contamination. This study investigated the roles of calcium oxide nanoparticles (CaO NPs) in alleviating As toxicity in two barley genotypes (LJZ and Pu-9) differing in As tolerance. The exposure of barley seedlings to As stress showed a significant reduction in plant growth, calcium and chlorophyll content (SPAD value), fluorescence efficiency (*Fv/m*), and a dramatic increase in the contents of reactive oxygen species (ROS), malondialdehyde (MDA) and As, with LJZ being more affected than Pu-9. The exogenous supply of CaO NPs notably alleviated the toxic effect caused by As in the two barley genotypes. Moreover, the expression of As transporter genes, that is, *HvPHT1;1*, *HvPHT1;3*, *HvPHT1;4* and *HvPHT1;6*, was dramatically enhanced when barley seedlings were exposed to As stress and significantly reduced in the treatment of CaO NPs addition. It may be concluded that the roles of CaO NPs in alleviating As toxicity could be attributed to its enhancement of Ca uptake, ROS scavenging ability, and reduction of As uptake and transportation from roots to shoots.

Keywords: antioxidants, arsenic, barley, calcium oxide, nanoparticles

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INTRODUCTION

Arsenic (As) is a ubiquitous heavy metalloid that imposes severe toxic effects on living organisms (Zhang et al., 2021). As exists in the soil in the forms of arsenate (As^V) and arsenite (As^{III}; Kanwar and Bhardwaj, 2015). As^V, being a chemical analog of phosphorus (P), enters root cells *via* phosphate transporters (PHTs) located in the root epidermis (Zvobgo et al., 2019), while As^{III} utilizes aquaporin channels for its transportation to aerial plant parts (Clemens and Ma, 2016). Plants may accumulate a surplus amount of As from soil, causing phytotoxic effects, such as inhibition of plant growth, photosynthesis and biomass production, limitation of nutrient supply, cellular damage, and disturbance of cellular redox homeostasis (AbdElgawad et al., 2021b). As the arsenic level in soil and water systems has been increasing because of anthropogenic activities (Abbas et al., 2018), effective strategies for fighting against its toxicity should be performed to ensure sustainable development of agriculture production and food safety. Although there are a lot of approaches proposed for dealing with soil As contamination, they are impractical due to high capital investment, time-taking, or operational complexity (Alka et al., 2021).

Nanotechnology has emerged as a promising technique for achieving long-term agricultural sustainability due to its several advantages for the agro-ecosystem, including increasing nutrient utilization efficiency, alleviating the impacts of climate change, and remediating heavy metals/metalloids (Manzoor et al., 2021). Recently, calcium oxide nanoparticles (CaO NPs) have gained great attention worldwide because of their promising agricultural applications. The positive effects of calcium NPs on the growth of Bengal gram (*Cicer arietinum* L.; Gandhi, 2021), lettuce (*Lactuca sativa*), zucchini (*Cucurbita pepo*; Meier et al., 2020), and rice (*Oryza sativa*; Syu et al., 2020) have been reported. In a recent study, Ahmed et al. (2021a) revealed that the application of magnesium oxide NPs significantly reduced As uptake in roots and shoots by improving rice plant growth and cellular antioxidant content. Similarly, Ma et al. (2020) reported that zinc oxide NPs reduced As concentration in rice roots (39.5%) and shoots (60.2%), and the reduction was primarily due to the lowered inorganic As (III) and organic As species. Although various NPs have been used as nano-fertilizers to improve plant growth and development by reducing As toxicity, such as zinc oxide NPs (Yan et al., 2021) in rice, titanium oxide NPs in mung bean (Katiyar et al., 2020), and zinc oxide NPs in soybean (Ahmad et al., 2020), the roles of CaO NPs in alleviating As-induced crop toxicity are still elusive.

Barley (*Hordeum vulgare* L.) is one of the most important crops globally, with multiple uses (Zhang and Li, 2010; Han et al., 2020). Moreover, it is also an ideal crop for producing healthy food as barley grains contain high content of β-glucan, flavonoid, and bioactive compounds. Meanwhile, barley is considered as a classical model plant for physiological and genetic studies on cereal plants (Gürel et al., 2016). Barley is more sensitive to As toxicity in comparison with other cereal crops (Baghaie and Jabari, 2019). Therefore, it is imperative to reduce As uptake and accumulation in barley plants exposed to As-contaminated soils.

The objectives of the current study were to determine the beneficial effects of CaO NPs on growth and physiological traits of two barley genotypes differing in As tolerance under As stress. In addition, the roles of CaO NPs in reducing As bioavailability and acro-petal translocation through its regulation of *HvPHTs* was also examined, so as to determine if CaO NPs can be used as a nano-fertilizer to alleviate As toxicity and accumulation in barley.

MATERIALS AND METHODS

Characterization of CaO NPs

In the present study, CaO NPs (purity, 99.9%; size, 30–50 nm) was purchased from Chaowei Nanotechnology Co. (Shanghai, China). The surface morphology and size of CaO NPs were determined by scanning electron microscopy (SEM; SU-8010, Tokyo, Japan) and transmission electron microscopy (TEM; JEM-1230, Akishima, Japan). The samples were prepared on a carbon-coated Cu grid and aluminum stub for both SEM and TEM analysis, according to Ahmed et al. (2021a). X-ray diffraction (XRD) analysis was performed to characterize the crystalline structure of CaO NPs using an X-ray diffractometer (Bruker, Germany). The particle size of CaO NPs was calculated using Debye Scherrer's equation ($d = K\lambda/\beta \cos\theta$). Fourier transform infrared spectroscopy (FTIR, Bruker, Germany) was performed to analyze the functional groups of CaO NPs with a spectral range of 500–4,000 cm^{-1}. The metallic fractions and elemental compositions of CaO NPs were characterized by energy dispersive spectroscopy (EDS) at 20 KeV.

Plant Materials and Growth Conditions

Two barley genotypes, that is, Longjiangzao (LJZ; As high accumulator/sensitive) and Pu-9 (As low accumulator/tolerant), were used according to our previous screening experiment (data not shown). The seeds were surface-sterilized by soaking in 5% NaClO solution for 30 min and washed five times with sterile deionized water. For germination, the sterilized seeds were placed in petri dishes on wet filter paper for 24 h and then transferred to a 5 L box containing basic solution medium [BSM: 1 mM KNO_3 + 0.05 mM Ca $(NO_3)_2$] and covered with a plastic plate with 100 evenly spaced holes. After 3 days, healthy and uniform seedlings were selected and transplanted into new 5 L pots covered with plastic plates with 7 evenly spaced holes (2 plants/hole). Each pot was filled with barley nutrient solution containing ($mg\,l^{-1}$): 48.2 $(NH_4)_2PO_4$, 154.8 $MgSO_4.7H_2O$, 24.8 NaH_2PO_4, 101.1 KNO_3, 118.08 Ca $(NO_3)_2.4H_2O$, 7.6 EDTA Fe Na, 0.9 $MnCl_2.4H_2O$, 2.9 H_3BO_3, 0.015 $Na_2MoO_4.2H_2O$, 0.11 $ZnSO_4.7H_2O$ and 0.04 $CuSO_4.5H_2O$. pH was adjusted to 5.6 ± 1 using NaOH or HCl as required. Barley seedlings were exposed to different As concentrations (0, 25, 50, 100, 150 μm). The selection of As concentration for further experimentation was based on this primary experiment, which showed that 50 μM As caused the significant damage to both barley genotypes. The nutrient solution in each pot was continuously aerated with air pumps and renewed twice a week. At the 7th day after seedlings transplanting (14 plants/pot), As ($Na_2HAsO_4.7H_2O$) and CaO NPs were applied to the corresponding pots to form the 4 treatments: (1) control (CK); (2) 50 μM As; (3) 25 mg/l CaO NPs; (4) 50 μM As +25 mg/l CaO NPs. CaO NPs were sonicated for 1 h before use, which was beneficial for dispersing CaO NPs in hydroponic solution. The experiment was arranged in a complete randomized design with four replicates for each treatment.

At the 7th day after treatment, plants were sampled from each treatment, and plant height, root lengths, and fresh weights were measured immediately. Then metal ions possibly attached on the root surface were removed by soaking roots into the solution containing 20 mm EDTA (Ethylene Diamine Tetra Acetic acid) for 30 min, washing with distilled water, and dried with filter papers. Then these plants were divided into shoots and roots, and some of them were frozen in liquid N_2 and stored in a −80°C refrigerator for further use. Some of the plant tissues were dried in an oven (60°C) to constant weight for biomass determination.

Chlorophyll Content and Fluorescence

The chlorophyll content of barley leaves was measured by a portable device (SPAD-502+, Tokyo, Japan). Chlorophyll fluorescence (*Fv/m*) was measured using a portable fluorimeter (OS-30p+, Hudson, United States). The plants were placed in dark for 30 min before measurement, and quantum efficiency of photosystem II ($F_{v/m} = (F_m - F_o)/F_m$) was observed at solid-state light (660 nm) source with the intensity of 1,100 μmolm$^{-2}\cdot$s^{-1} as described by Cai et al. (2020).

As and Ca Content in Plant Tissues

Dry root and shoot samples (0.5 g) were digested in HNO_3 solution at 120°C for 1 h, followed by 140°C for 2 h in a dry thermos unit (DTU-2CN; Tokyo, Japan). Then the digested solution was diluted with double-distilled water to reach 10 ml as the final volume. As and Ca contents were measured using an inductively coupled plasma mass spectrometer (ICP-MS, iCAP RQ, Thermo scientific, United States). In addition, As accumulation and translocation factors were calculated according to Zhang et al. (2020).

Reactive Oxygen Species, Malondialdehyde, and Histochemical Analysis

This study measured reactive oxygen species in plant tissues, including hydrogen peroxide (H_2O_2) and superoxide-free radical ($O_2^{\bullet-}$). Hydrogen peroxide content was measured as described by Velikova et al. (2000). Briefly, barley shoot and root samples (0.2 g) were homogenized in trichloroacetic acid (0.1% TCA) and centrifuged at 13,000 rpm for 15 min. Then supernatant (50 µl), 1 M KI (100 µl), and 10 mM potassium phosphate buffer (50 µl-pH 7.0) were mixed and placed at a microplate reader (Synergy H1 Bio-Tec). H_2O_2 content was recorded at 390 nm wavelength.

Superoxide-free radical content was measured according to Schneider and Schlegel (1981). Briefly, 0.2 g of tissue samples was homogenized with 65 mm potassium phosphate buffer (pH 7.8) and centrifuged at 5,000 rpm for 10 min at 4°C. Afterward, 1 ml supernatant was mixed with 0.1 ml of 10 mm hydroxylamine hydrochloride and 0.9 ml of 65 mM potassium phosphate buffer (pH 7.8) and incubated at 25°C for 20 min. Then 1 ml of 17 mM 4-aminobenzenesulphonic acid ($C_6H_7NO_3S$) and 1 ml of 7 mm a-naphthylamine ($C_{10}H_7NH_2$) were added, gently shaked, and incubated again for an additional 20 min at 25°C. Finally, 3 ml trichloromethane ($CHCL_3$) was added to the samples and the absorbance was recorded at 530 nm.

Malondialdehyde (MDA) content was determined according to Morales and Munné-Bosch (2019). Briefly, 100 mg of plant tissues were homogenized with chilled potassium phosphate buffer (65 mm; pH 7.8) and centrifuged at 12,000 rpm at 4°C for 15 min. The reaction solution of 5% trichloroacetic acid (TCA) and thiobarbituric acid (TBA) was added to the resultant supernatant and incubated at 95°C for 25 min. Afterward, samples were placed on ice to stop the reaction and centrifuged at 4,800 rpm for 10 min, and specific and non-specific absorbance were recorded at 600 and 532 nm, respectively.

Accumulation of H_2O_2 and $O_2^{\bullet-}$ in plant tissues was identified by leaf or root staining with 3, 3-diaminobenzidine (DAB) and nitro blue tetrazolium (NBT), respectively, according to Romero-Puertas et al. (2004). The blotted leaves and roots were photographed using a digital microscope (Leica MZ-g5, Germany).

Antioxidant Enzymatic Activities

The root and shoot samples were homogenized in sodium phosphate buffer (pH 7.8), centrifuged at 13,000 rpm for 20 min at 4°C. Then superoxide dismutase (SOD) activity was measured spectrophotometrically as described by Zhang et al. (2008), at 560 nm by assessing the ability of each unit to inhibit 50% photochemical reduction of nitro blue tetrazolium chloride (NBT). Peroxidase (POD) activity was determined according to Zhou and Leul (1999), at 470 nm and the changes related to guaiacol were normalized with the activity constant ($\varepsilon = 26.6$ mm cm^{-1}). and catalase (CAT) activity was determined according to Aebi (1984).

RNA Extraction and Quantitative Real-Time PCR Assay

Total RNA was extracted from barley tissues (root and shoot) using the Steady Pure Universal RNA Extraction Kit (Accurate Biotechnology, Hunan, China) according to the manufacturer's instructions. The RNA quantity and quality were determined using a Nanodrop and 2% agarose gels, respectively. To synthesize the cDNA, an Evo M-MLV RT Kit (AG 11706, Accurate Biotechnology, Hunan, China) with gDNA Clean for qPCR was used following the manufacturer's instructions. The amplification reaction for qRT-PCR was performed by using 1 µl of cDNA, 0.8 µl of forward and reverse primers, 10 µl SYBR Green Pro Taq HS premix (AG 11701, Accurate Biotechnology, Hunan, China), and 7.4 µl RNA-free water to reach the final volume up to of 20 µl. All cDNA samples were performed in triplicate by qRT-PCR in a Light Cycler 480 II (Roche, Diagnostics system, Basel, Switzerland). The PCR profile was as follows: initial denaturation 95°C for 30 s, followed by 40 cycles and denaturation at 95°C for 5 s, annealing at 60°C for 30 s. Specific primers for PHT genes were designed using Primer-BLAST (**Supplementary Table S1**).[1] Barley ACTIN gene was used as a reference gene and subtracted from the threshold cycle (Ct) values for each sample. Quantitative relative gene expression levels were determined by following the $2^{-\Delta\Delta CT}$ method according to Schmittgen and Livak (2008).

Statistics Analysis

Data analysis was performed with the Statistics 8.1 software package. The significance between treatments was tested using two-way ANOVA and compared by LSD test at $p < 0.05$.

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[1] www.ncbi.nlm.nih.gov

RESULTS

Characterization of CaO NPs

The presence of capping agents on the CaO NPs surface was confirmed through FTIR analysis (**Figure 1A**). Multiple spectral bands (3,643, 3,424, 1,637, 1,413, 875 cm^{-1}) in the FTIR spectrum demonstrated the presence of diverse functional groups as capping agents around the surface of CaO NPs. The presence of strong spectral bands at 3,643 and 3,424 cm^{-1} confirmed the O-H stretching of the alcohol group. The medium peaks at 1,637, 1,413, and 875 cm^{-1} corresponded to C=C stretching, O-H bending, and C-H bending, respectively. The XRD spectrum of CaO NPs revealed the crystalline structure of CaO NPs. The various diffraction peaks of CaO NPs were observed at 32.2, 37.3, 53.8, 64.2, and 67.4 °, which corresponds to 111, 200, 202, 311, and 322 diffraction planes of CaO NPs (**Figure 1B**), respectively. The SEM and TEM analysis showed that CaO NPs have spherical shapes and variable sizes ranging from 10 to 24 nm (**Figure 1**). However, CaO NPs were not well dispersed and appeared in nanopowder as aggregated form. Furthermore, EDS spectra confirmed the existence of carbon (3.36%), oxygen (37.54%), calcium (58.54%), iron (0.01%), copper (0.36%) and zinc (0.38%) in CaO NPs (**Figure 1E**).

Plant Growth Traits

As treatment significantly reduced plant growth of both barley genotypes, with LJZ being more affected than Pu-9. As-treated plants showed a significant reduction in shoot length (33.4 and 28.2%), root length (27.9 and 23.4%), shoot fresh weight (36.3 and 30.8%), root fresh weight (25.6 and 20.6%), shoot dry weight (15.7 and 12.2%), root dry weight (11.2 and 9.8%) of LJZ and Pu-9, respectively as compared to control plants (**Figure 2**). The application of CaO NPs displayed a marked improvement in all growth parameters of the two genotypes, with Pu-9 showing greater improvement than LJZ compared to non-treated plants (**Figure 2**). Compared with As treatment, CaO NPs + As treatment showed much less inhibition in all plant growth traits.

Chlorophyll Content and Fluorescence

The toxic effects of As and its alleviation by CaO NPs on chlorophyll content and fluorescence of the two barley genotypes are presented (**Figures 2G,H**). The significant decline of both SPAD value and maximum quantum efficiency (*Fv/m*) was observed in the two barley genotypes, with LJZ being more affected than Pu-9 under As stress as compared to control. Compared to As treatment, the treatment of CaO NPs + As had significantly greater SPAD and *Fv/m* values for both barley genotypes.

Reactive Oxygen Species Contents and Antioxidant Enzyme Activities

As treatment significantly increased MDA, H_2O_2, and $O_2^{\bullet -}$ contents in the shoots and roots of the two barley genotypes compared with the control, with LJZ being more affected than Pu-9 (**Figure 3**). The addition of CaO NPs notably alleviated the oxidative stress in barley tissues caused by As stress, reflected by reduced MDA and ROS contents (**Figure 3**). In addition, histochemical staining confirmed the results that As stress caused more accumulation of ROS in shoots and roots of both LJZ and Pu-9 and the addition of CaO NPs could alleviate the oxidative stress (**Figure 4**). Moreover, LJZ had more and darker spots in both shoots and roots than Pu-9 after staining with NBT and DAB, respectively, indicating LJZ accumulated more H_2O_2 and $O_2^{\bullet -}$ than Pu-9.

For antioxidant enzyme activity, As treatment caused the significant increase of SOD, POD, and CAT in the leaves and roots of both barley genotypes compared with control, with Pu-9 showing more increase than LJZ (**Figure 5**). The treatment of As + CaO NPs showed significantly higher SOD, POD, and CAT activities in the leaves and roots of Pu-9 and LJZ, compared with As treatment alone.

Ca, As Content and Translocation

Without As addition in the culture solution (both control and CaO NPs treatment), As content was not detected in both root and shoots of the two genotypes (**Figures 6A,B**). There was a significant difference in As content between shoots and roots and the two barley genotypes in the As treatment alone. The As + CaO NPs treatment significantly reduced As content in shoots and roots compared with the As treatment alone, with Pu-9 showing more reduction than LJZ. Moreover, As treatment reduced calcium content in plant tissues of both genotypes, which are significantly increased by CaO NPs addition (**Figures 6D,E**). For As translocation factor (TF) from root to shoots, in As treatment, LJZ (40.8%) was larger than Pu-9 (31.1%). However, CaO NPs + As treatment caused a significant reduction of As TF in both genotypes, although LJZ had a significantly larger TF than Pu-9 (**Figure 6C**).

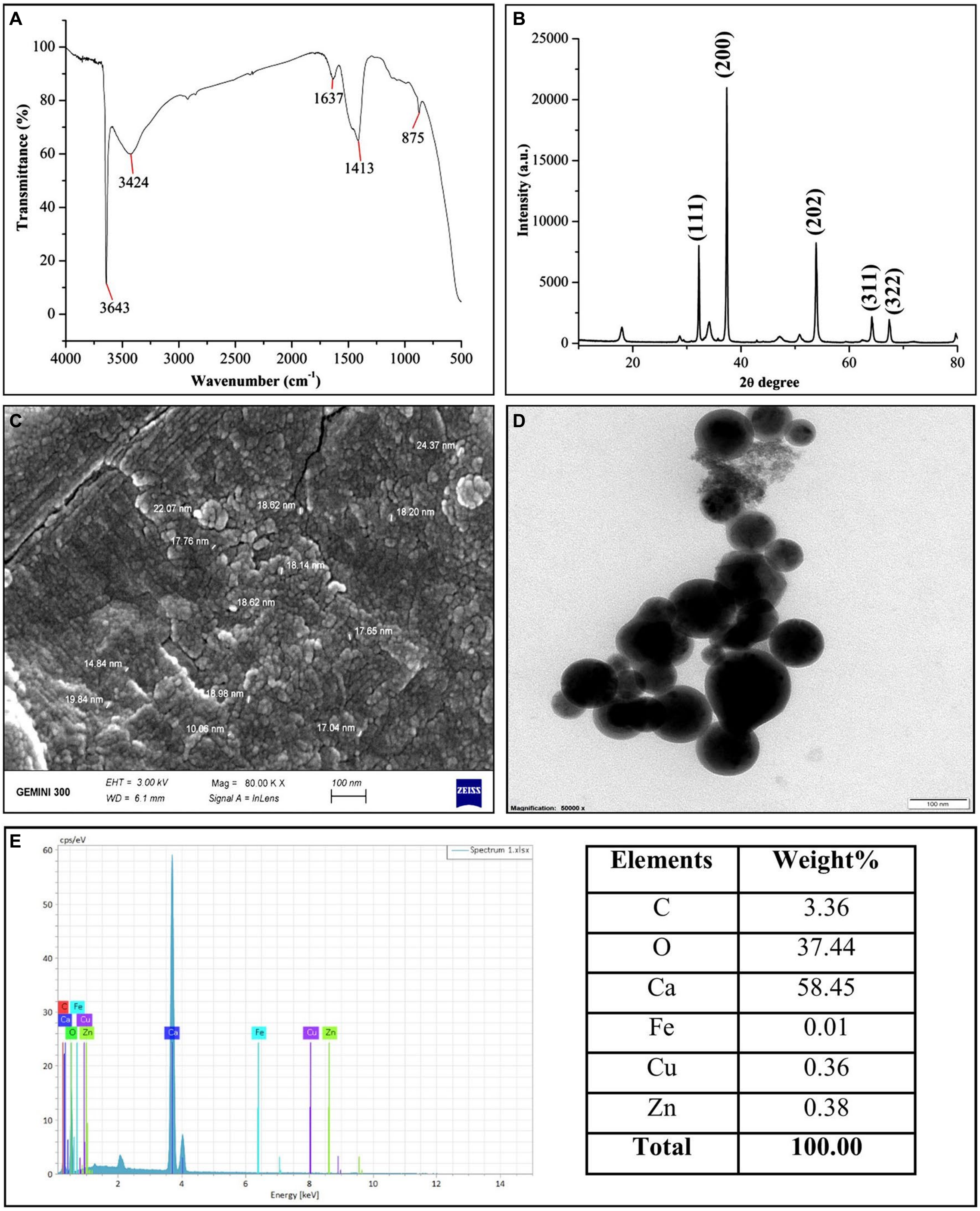
Relative Expression of As Transporter Genes

The relative expression of As transporter genes involved in As uptake are presented in **Figure 7**. In comparison with control, As treatment significantly upregulated the expressions of *HvPHT1;1, HvPHT1;3, HvPHT1;4 and HvPHT1;6* in the two barley genotypes; however, the greater expression was observed in LJZ as compared with Pu-9. The addition of CaO NPs in the As-containing solution caused the significant reduction in the expression of *HvPHT1;1*, *HvPHT1;3*, *HvPHT1;4* and *HvPHT1;6* in comparison with As treatment alone, although the expressions of these four transporter genes were still significantly higher than those in the control plants.

DISCUSSION

CaO NPs Alleviate Plant Growth Inhibition Caused by As Stress

As treatment caused a significant reduction in shoot and root lengths, and biomass, with LJZ being more affected than Pu-9, confirming the distinct difference in As stress tolerance between the two barley genotypes. The toxic effect of As stress on plant



Elements	Weight%
C	3.36
O	37.44
Ca	58.45
Fe	0.01
Cu	0.36
Zn	0.38
Total	**100.00**

FIGURE 1 | Characterization of CaO NPs. **(A)** FTIR; **(B)** XRD; **(C)** SEM (scale bar = 100 nm); **(D)** TEM (scale bar = 100 nm); **(D)** EDS.

growth has been intensively studied (Anawar et al., 2018; Singh et al., 2020). AbdElgawad et al. (2021a) reported that barley plants exposed to As stress showed the obvious decline in photosynthesis, which was closely associated with As accumulation in plant tissues and oxidative stress. In recent years, many studies demonstrated that applications of NPs could efficiently reduce As accumulation in plants (Ahmed et al., 2021b,c). In this study, we found CaO NPs significantly improved the growth of barley seedlings exposed to As stress. The alleviation of As toxicity on plant growth by CaO NPs could be attributed to reduce As accumulation in plant tissues and increased photosynthesis, reflected by increased chlorophyll content and fluorescence efficiency.

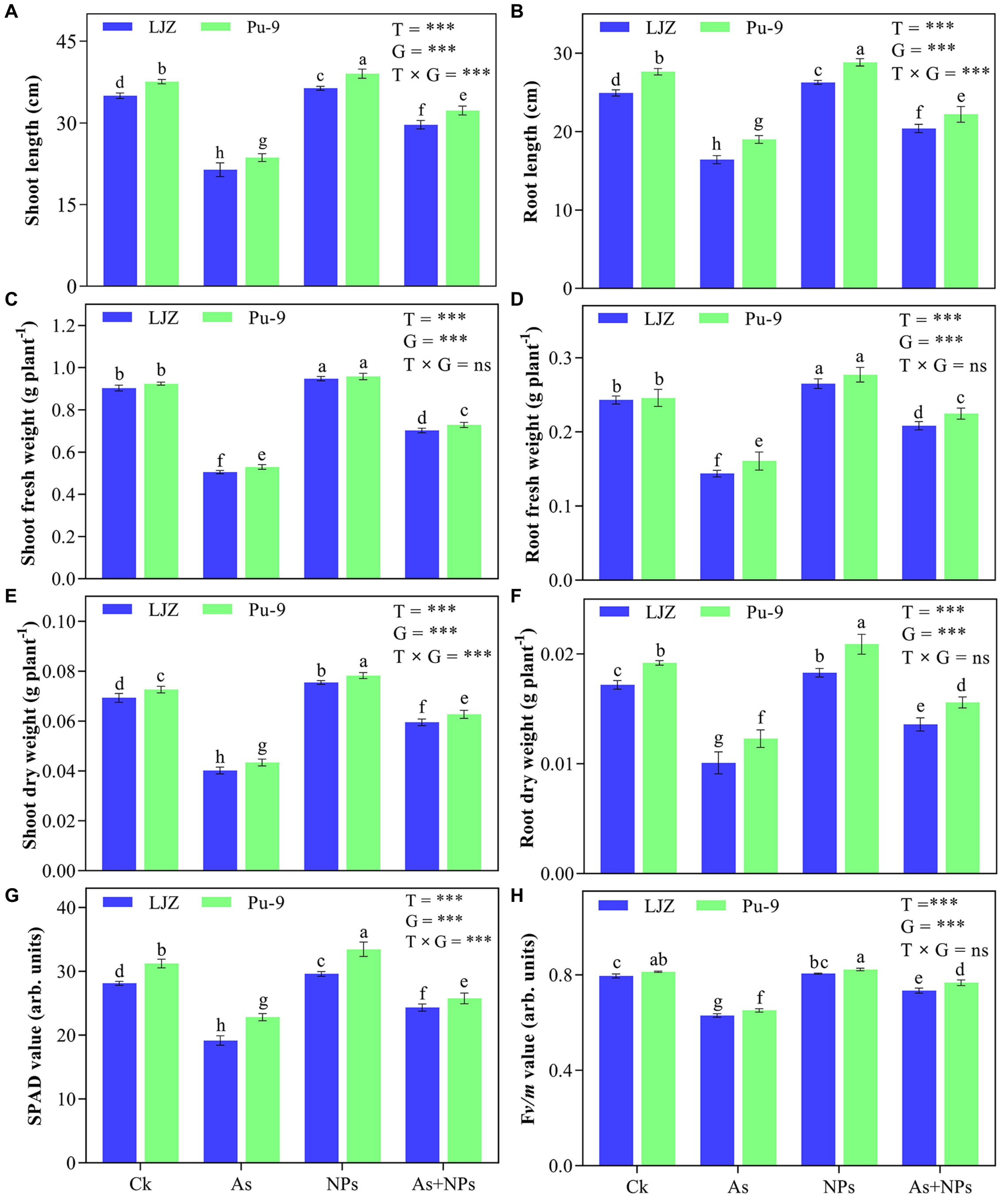


FIGURE 2 | Effects of different treatments on plant growth and chlorophyll contents. **(A,B)** shoot and root length; **(C,D)** fresh weight of shoots and roots; **(E,F)** dry weight of shoots and roots. **(G)**, SPAD value; **(H)**, maximum quantum efficiency (Fv/m) of photosystem II. Vertical bars represent the mean ± SD of four independent replicates. Different letters above error bars indicate the significant difference between treatments and genotypes at $p \leq 0.05$. T, treatment; G, genotype; T × G, the interaction between treatment and genotype.

CaO NPs Modulate Photosynthetic Efficacy Under Induced As Stress

Photosynthesis is an important indicator of plant adaptation to severe environmental conditions (Hussain et al., 2021). The current results showed that As stress notably reduced chlorophyll content (SPAD value) and photosynthetic efficiency (*Fv/m*) in both barley genotypes (**Figure 2**). Bidi et al. (2021) reported negative impacts of As on the working efficacy of photosynthetic pigments in rice plants. According to Patel et al. (2018), an increase in chlorophyllase activity and reduction in levels of chlorophyll synthesizing enzymes caused reduced chlorophyll content in As-exposed plants, while the low activity

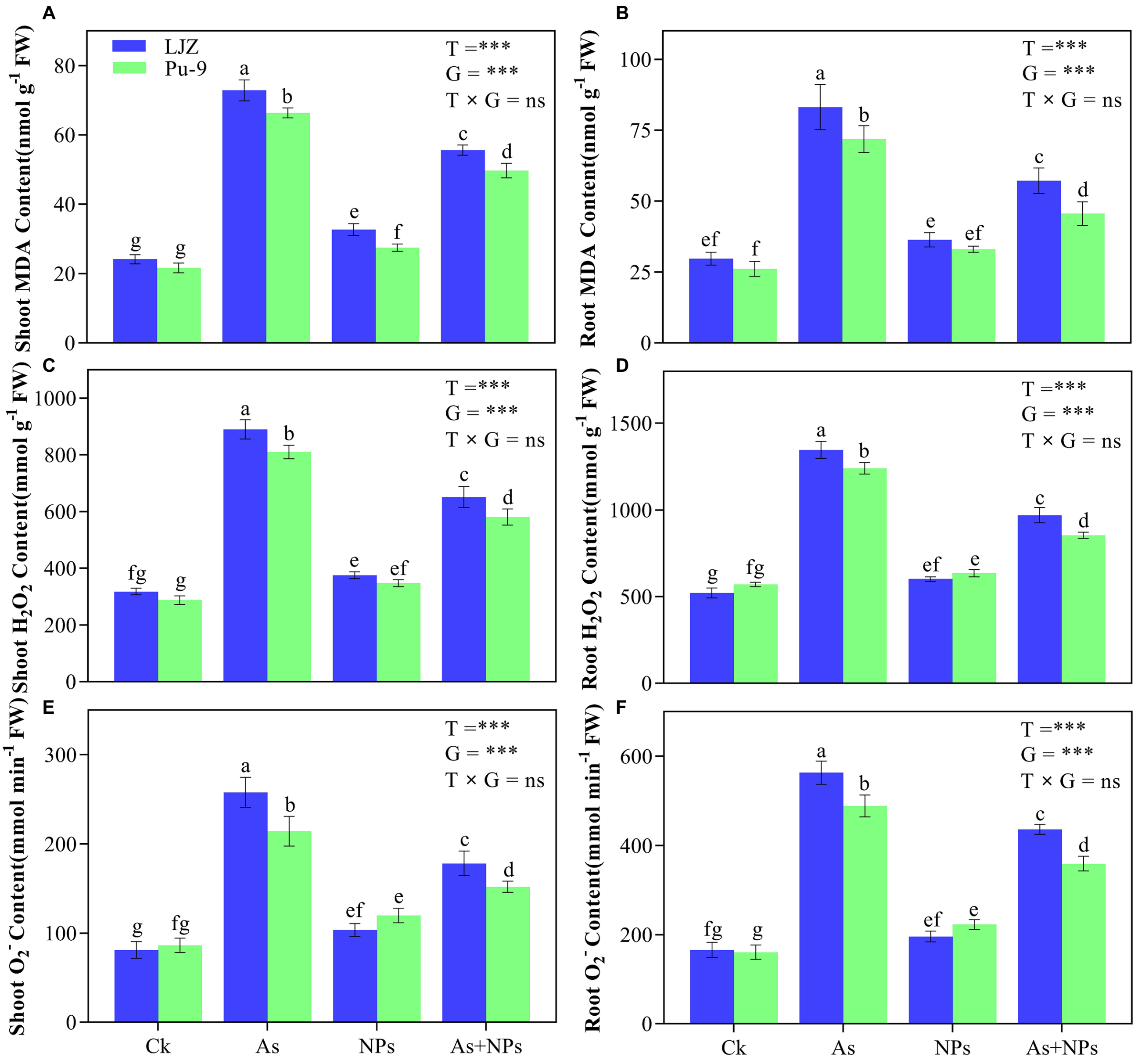


FIGURE 3 | Effects of the different treatments on MDA and ROS (H_2O_2 and $O_2^{\bullet-}$) contents. **(A,B)** MDA content in shoots and roots; **(C,D)** H_2O_2 content in shoots and root; **(E,F)** $O_2^{\bullet-}$ content in shoots and roots. Vertical bars represent the mean ± SD of four replicates. Different letters above error bars indicate the significant difference between treatments and genotypes at $p \leq 0.05$. T, treatment; G, genotype; T × G, the interaction between treatment and genotype.

of the photosynthetic apparatus resulted in less sugar production, finally reducing plant biomass (Yan et al., 2021). Obviously, the reduced SPAD and *Fv/Fm* values in the two barley genotypes exposed to As treatment could be attributed to As stress, reflected by higher As contents in the As-treated plants. In this study, exogenous CaO NPs application alleviated the negative impact of photosynthetic efficiency caused by As stress in both barley genotypes (**Figure 2**). Similarly, the application of ZnO NPs was found to improve the photosynthetic efficiency of wheat plants under cadmium (Cd) stress (Ali et al., 2019; Rizwan et al., 2019) and rice seedlings under As stress (Yan et al., 2021). Moreover, Gandhi et al. (2021) reported that CaO NPs could improve the photosynthesis activity in chickpea by reducing the damage of chloroplast thylakoids and maintaining cellular homeostasis under abiotic stress.

CaO NPs Reduce ROS Accumulation and Increase Antioxidative Enzyme Activity

Another reason for alleviating As toxicity on barley seedlings by CaO NPs is related to its ability to reduce oxidative stress

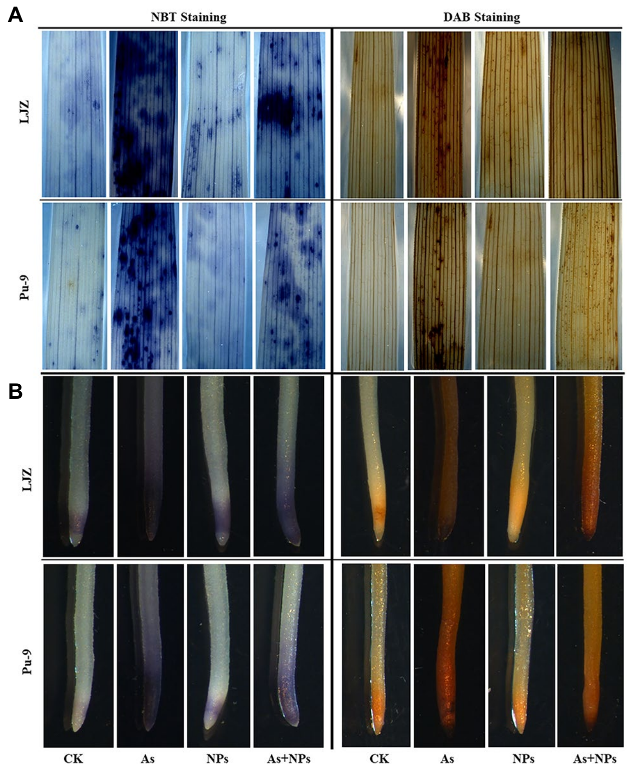


FIGURE 4 | *In situ* histochemical observation of $O_2^{\bullet-}$ through NBT and H_2O_2 by DAB staining. **(A)** shoot; **(B)** root.

by improving antioxidative capacity. As treatment significantly induced the extra generation of ROS, which will cause oxidative stress and membrane peroxidation, as reflected by increased MDA content (Zvobgo et al., 2019; Ahmed et al., 2021a). Indeed, we found more MDA, H_2O_2, and $O_2^{\bullet-}$ contents in the As-treated plants than control and As-tolerant Pu-9 than As-sensitive LJZ (**Figures 3, 4**). Normally, plants will strengthen their cellular antioxidant defense capacity for responding to oxidative stress, characterized by increased antioxidant enzyme activity. In the present study, As treatment increased SOD, POD, and CAT activities in barley seedlings, accompanied by the increase of MDA, H_2O_2, and $O_2^{\bullet-}$ contents. Obviously, the increase of these antioxidant enzyme activities is a self-defense response to oxidative stress and is not enough to scavenge the generated ROS. The exogenous application of CaO NPs enhanced SOD, POD, and CAT activities in plant tissues of both barley genotypes (LJZ and Pu-9), accompanied by a decrease in MDA, H_2O_2, and $O_2^{\bullet-}$ contents in plant tissues, indicating the alleviation of oxidative stress (**Figure 5**). It was reported that iron oxide NPs increased the activities of antioxidant enzymes (SOD and POD), resulting in a lower accumulation of MDA and H_2O_2 in Cd-exposed wheat tissues (Adrees et al., 2020).

Similarly, Ahmad et al. (2020) revealed that the application of zinc oxide NPs significantly improved antioxidative defense capacity and reduced As uptake in rice plants. Moreover, Wu et al. (2020) observed that seed priming with zinc oxide NPs could increase plant biomass, SOD, and CAT activities and reduce As uptake in rice seedlings. It may be suggested that these nanoparticles have the function of enhancing antioxidative stress in plants. However, the underlying mechanism is still elusive.

CaO NPs Reduce As Accumulation and Increase Ca^{2+} Content in Plant Tissues

In general, plant roots have a higher tolerance to heavy metals than shoots. Hence, less translocation of toxic metals accumulated in roots to shoots is a trait associated with higher tolerance (Cui et al., 2018; Tian et al., 2019; Basit et al., 2022). In this study, we quantified As translocation of roots to shoots in the two barley genotypes and the effect of CaO NPs on As translocation (**Figure 6C**). As expected, Pu-9 had a significantly smaller As TF than LJZ. Meanwhile, the addition of CaO NPs in the As-containing solution caused a significant reduction of As TF in the two barley genotypes. Hence, it is indicated that alleviation of As toxicity by CaO NPs is also attributed to its role in reducing As uptake and translocation from roots to shoots. A possible explanation is that CaO NPs might immobilize metal ions in the rhizosphere and protect roots by forming coats around the root surface, thus restricting As translocation from roots to shoots.

Furthermore, the possible roles of Ca^{2+} in the effect of CaO NPs in alleviating As toxicity of barley should be noted. Ca^{2+} ions play a vital role in improving plant abiotic stress tolerance by reducing heavy metal ions uptake and modulating the metal transporter genes (Bose et al., 2011). In the present study, we found that the addition of CaO NPs in the culture solution increased Ca^{2+} content in barley tissues, which should be beneficial for the alleviation of As toxicity (**Figure 5**). Siddiqui et al. (2020) reported the negative correlation between Ca^{2+} content and As metal uptake in *Vicia faba* plants, which was attributed to competing for inhibition of Ca^{2+} ions on As uptake. However, how much role Ca^{2+} ions play in alleviating As toxicity as a part of CaO NPs remains to be clarified.

CaO NPs Modulate Expression Levels of *HvPHTs* and Reduce Intraplant As Transport

It was reported that plant As toxicity was also associated with the expression levels of *HvPHT* genes, with sensitive genotypes showing higher expression than tolerant ones under As stress (Zvobgo et al., 2018). In the present study, the examined four PHT genes (*HvPHT1;1, HvPHT1;3, HvPHT1;4* and *HvPHT1;6*) were substantially upregulated under As stress in both genotypes as compared to control. However, mRNA transcripts of these PHT genes were relatively lower in Pu-9 than LJZ under As stress, which could account for less As uptake in Pu-9. The application of CaO NPs reduced the expression level of all PHT genes in As-exposed barley seedlings (**Figure 7**). Recently Zeshan et al. (2021) found that astaxanthin NPs could reduce the expression of Cd transporter genes in wheat plants under Cd stress conditions. In short, the current results showed that

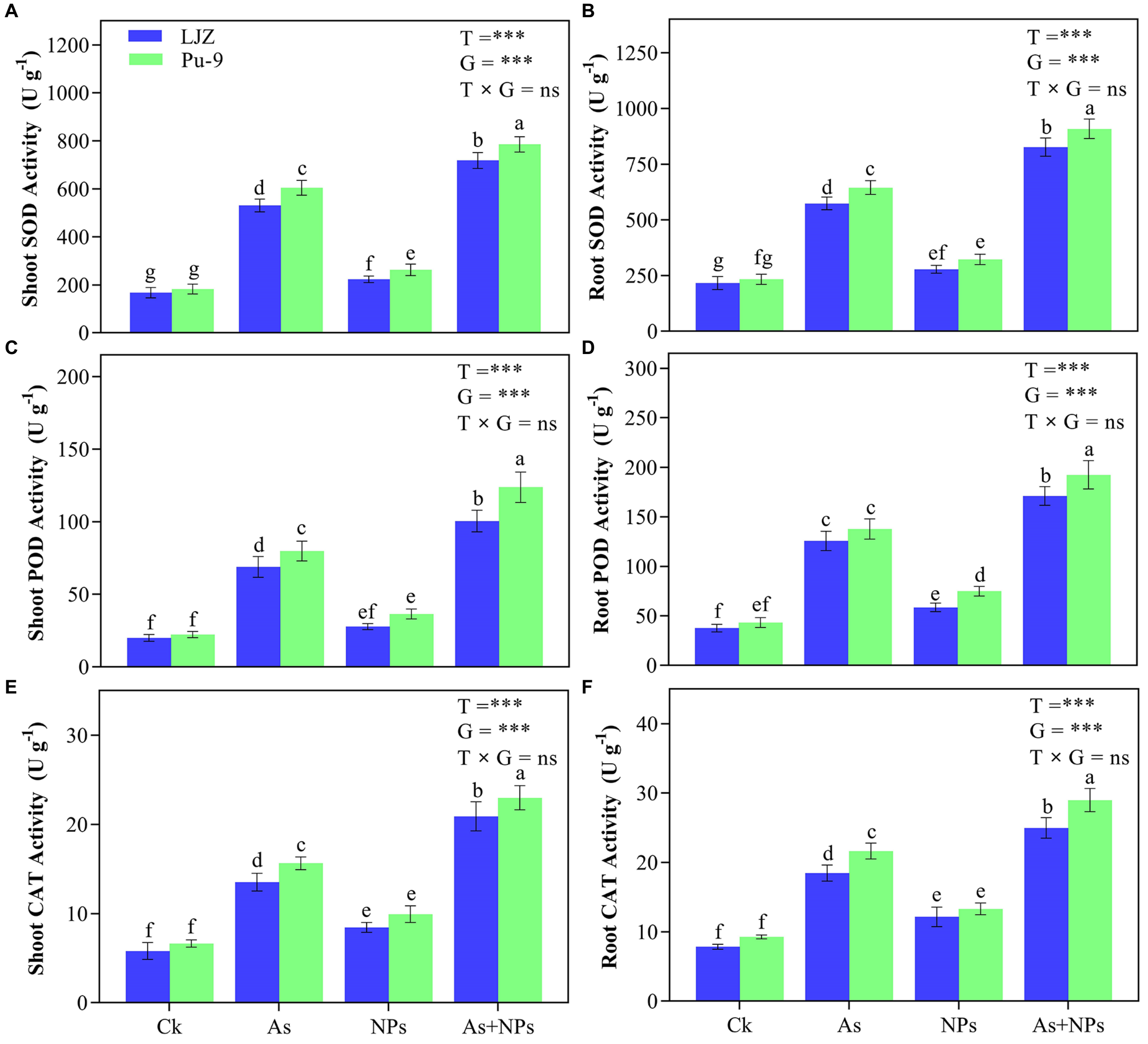


FIGURE 5 | Effects of the different treatments on antioxidant enzyme activities. **(A,B)** superoxide dismutase (SOD) activity in shoots and roots; **(C,D)** peroxidase (POD) activity in shoots and roots; **(E,F)** catalase (CAT) activity in shoots and roots. Vertical bars represent mean ± SD of four replicates. Different letters above error bars indicate the significant difference between treatments and genotypes at $p \leq 0.05$. T, treatment; G, genotype; T × G, the interaction between treatment and genotype.

CaO NPs could alleviate barley As toxicity by reducing As uptake and transportation and improving antioxidant capacity.

Taken together, our results show that CaO NPs alleviate As toxicity in barley by modulating different physiological and genetic pathways. More precisely, application of CaO NPs activates basal defense response by triggering enzymatic antioxidants, which subsequently reduce *in planta* ROS accumulation and rescue plants from As-induced oxidative stress. Further, CaO NPs application improves the photosynthetic efficacy by maintaining Ca^{2+} ions homeostasis. More interestingly, supplementation of CaO NPs downregulates expression levels of As transporters genes, and as a result ultimately reduce As mobilization and translocation within plant systems and protect barley seedlings from As-induced phytotoxicities. Conclusively, CaO NPs could protect barley seedlings from As stress by alleviating As-induced negative physiological alterations within plant systems (**Figure 8**).

CONCLUSIONS

This study investigated the roles of CaO NPs in alleviating As toxicity in two barley genotypes differing in As tolerance.

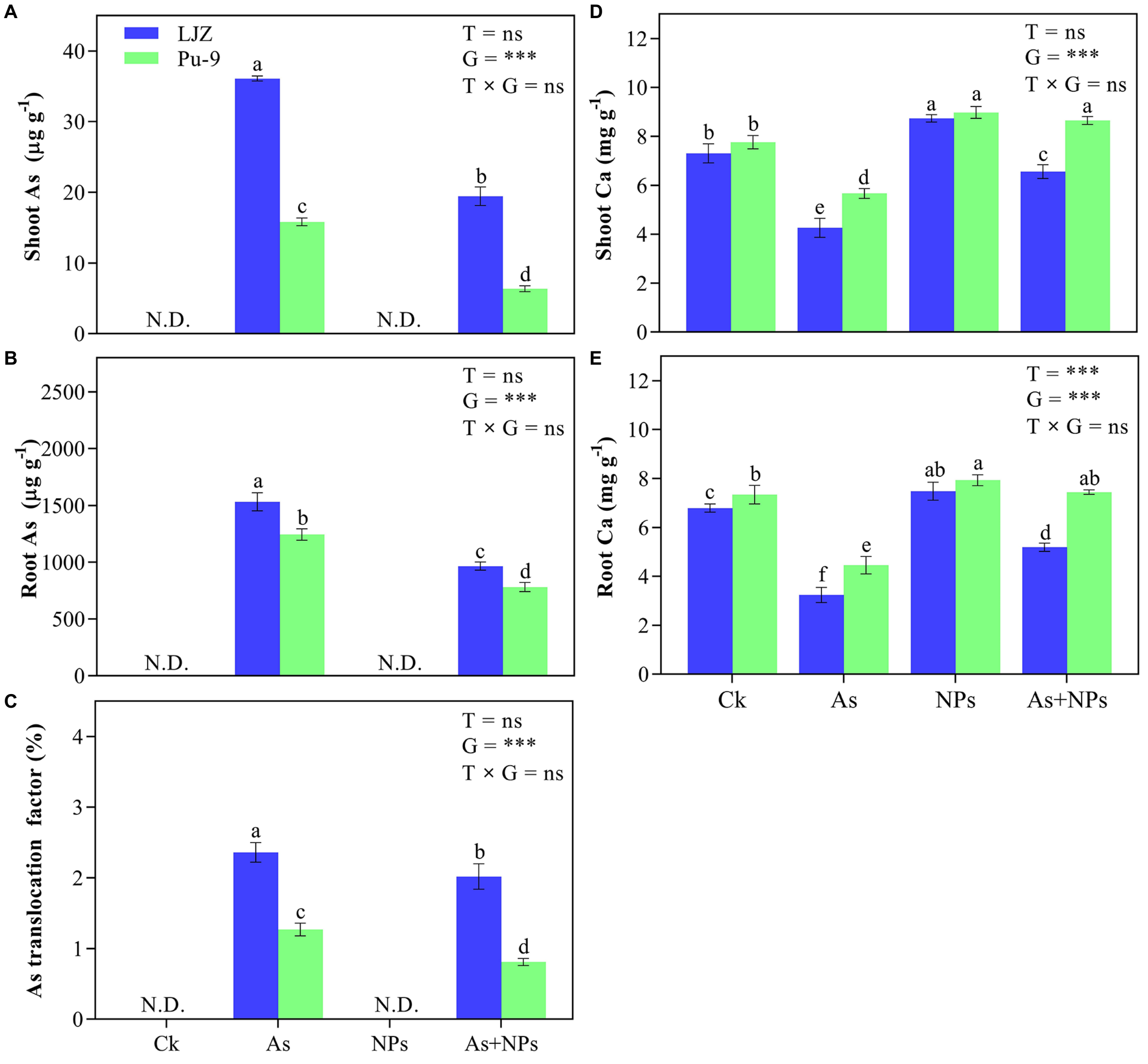


FIGURE 6 | Effects of the different treatments on Ca, As contents, and translocation factor in barley tissues. **(A, B)** As content in shoots and roots; **(C)** translocation factor; **(D, E)** Ca content in shoots nd roots. Different letters above error bars indicate the significant difference between treatments and genotypes at $p \leq 0.05$. T, treatment; G, genotype; T×G, the interaction between treatment and genotype.

The roles of CaO NPs in alleviating As toxicity could be attributed to its enhancement of Ca uptake and ROS scavenging ability and reduction of As uptake and transportation from roots to shoots. Moreover, CaO NPS restricted the uptake and translocation of As through downregulating expressions of As transporter genes (*HvPHT1;1, HvPHT1;3, HvPHT1;4* and *HvPHT1;6*) in roots of barley seedlings. In addition, our results showed that the effect of CaO NPs in alleviating As toxicity or growth inhibition is genotype-dependent, with LJZ being more affected than Pu-9. This study provided further evidence that the application of CaO NPs is a potential solution for As-contaminated soils. However, more studies are required to decipher the mechanisms underlying the roles of CaO NPs in alleviating As toxicity in plants.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

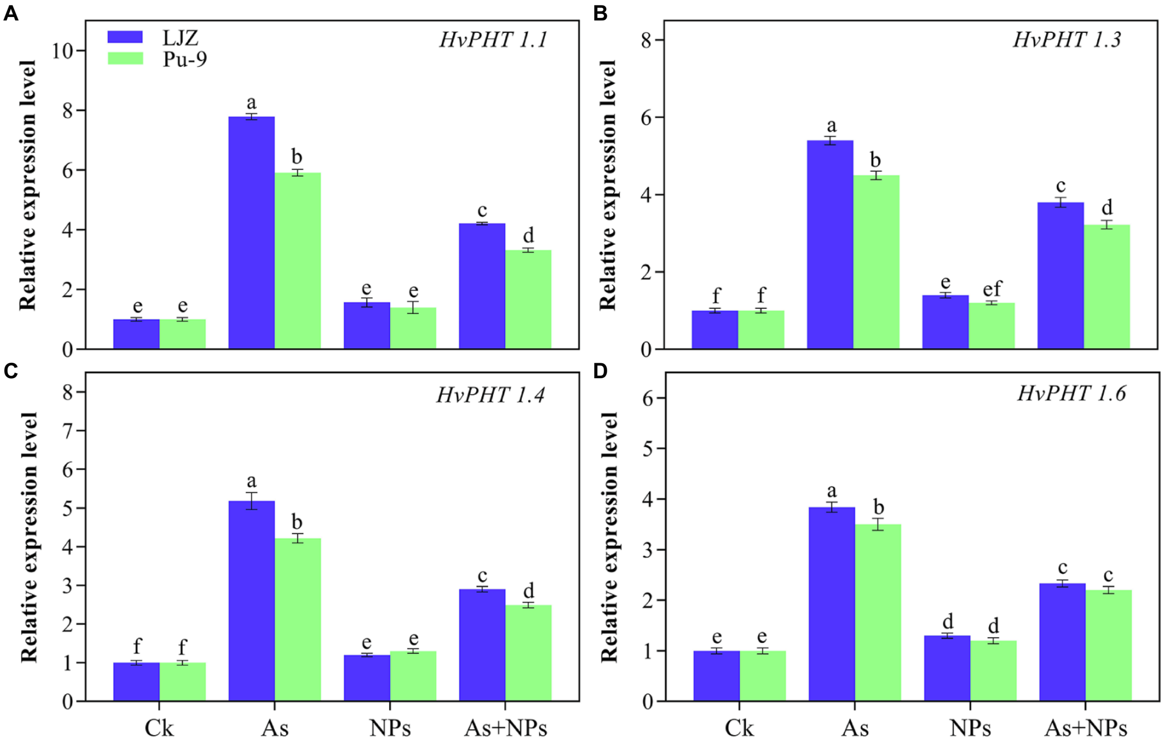


FIGURE 7 | Relative expression of barley phosphate transporter genes **(A)** *HvPHT1.1*, **(B)** *HvPHT1.3*, **(C)** *HvPHT1.4*, and **(D)** *HvPHT 1.6* in roots under As stress. Different letters above error bars indicate the significant difference between treatments and genotypes at $p \leq 0.05$.

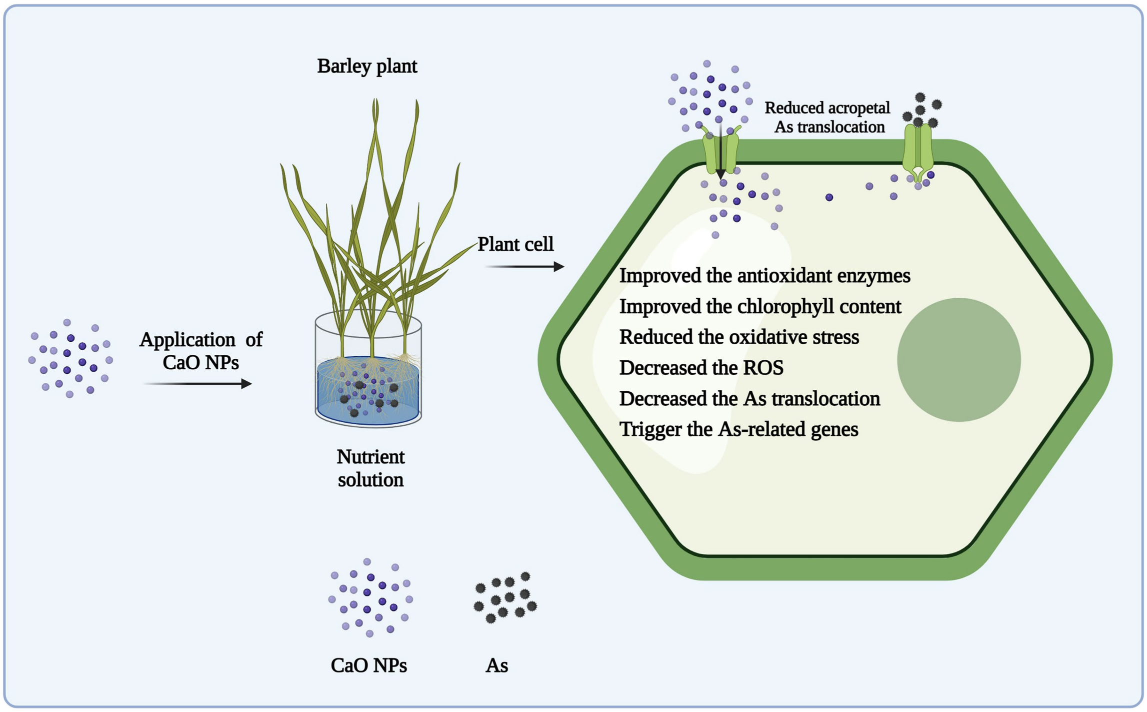


FIGURE 8 | Working model showing how CaO NPs supplementation reduce As-induced phytotoxicities in barley seedlings. Initially, CaO NPs, after entering plant body, activate enzymatic antioxidants (i.e., SOD, POD, and CAT), which in turn help barley seedlings to manage metal-induced oxidative stress. Further, CaO NPs application supports plant growth by improving Ca^{2+} ions homeostasis and photosynthesis. In addition, CaO NPs reduce uptake, mobilization, and acropetal translocation of As by decreasing the transcript abundance of As transporting genes (*HvPHTs*), which ultimately helps plant to reprogram its normal physiological and developmental processes.

AUTHOR CONTRIBUTIONS

MN: conceptualization, methodology, writing—original draft, validation, and writing—review and editing. QL: data collection, formal analysis, validation, and writing—review and editing. MN and ZU: formal analysis, validation, and writing—review and editing. SA and TA: validation and writing—review and editing. FZ: co-supervision, methodology, and investigation. GZ: conceptualization, supervision, project administration, writing—review and editing, and funding acquisition. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.843795/full#supplementary-material

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Methyl Jasmonate Alleviated the Adverse Effects of Cadmium Stress in Pea (*Pisum sativum* L.): A Nexus of Photosystem II Activity and Dynamics of Redox Balance

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The accumulation of cadmium (Cd) in leaves reduces photosynthetic capacity by degrading photosynthetic pigments, reducing photosystem II activity, and producing reactive oxygen species (ROS). Though it was demonstrated that the application of Methyl Jasmonate (MeJA) induces heavy metal (HM) stress tolerance in plants, its role in adjusting redox balance and photosynthetic machinery is unclear. In this study, the role of MeJA in modulating photosystem II (PSII) activity and antioxidant defense system was investigated to reduce the toxic effects of Cd on the growth of pea (*Pisum sativum* L.) cultivars. One-week-old seedlings of three pea varieties were subjected to Cd stress (0, 50, 100 μm), and MeJA (0, 1, 5, 10 μm) was applied as a foliar spray for 2 weeks. Cadmium stress reduced the growth of all three pea varieties. Cadmium stress decreased photosynthetic pigments [Chl a (58.15%), Chl b (48.97%), total Chl (51.9%) and carotenoids (44.01%)] and efficiency of photosystem II [Fv/Fm (19.52%) and Y(II; 67.67%)], while it substantially increased Cd accumulation along with an increase in ROS (79.09%) and lipid peroxidation (129.28%). However, such adverse effects of Cd stress varied in different pea varieties. Exogenous application of MeJA increased the activity of a battery of antioxidant enzymes [superoxide dismutase (33.68%), peroxidase (29.75%), and catalase (38.86%)], improved photosynthetic pigments and PSII efficiency. This led to improved growth of pea varieties under Cd stress, such as increased fresh and dry weights of shoots and roots. In addition, improvement in root biomass by MeJA was more significant than that of shoot biomass. Thus, the mitigating effect of MeJA was attributed to its role in cellular redox balance and photosynthetic machinery of pea plants when exposed to Cd stress.

Keywords: antioxidants, cadmium toxicity, methyl jasmonate, oxidative stress, photosystem II

INTRODUCTION

The excessive uptake and accumulation of heavy metals, including cadmium (Cd), inhibit plants' growth and development (Kranner and Colville, 2011; Haider et al., 2022). The presence of cadmium disturbs plant functions, such as uptake of water and mineral nutrients inhibition of photosynthetic machinery (Baryla et al., 2001). In addition, Cd stress causes the over a reduction of NADPH and thus cause an imbalance in electron transport from photosystem II (PSII) to photosystem I (PSI) and consumption of electrons in generating reducing equivalents, thereby resulting in ROS (reactive oxygen species) production. The generated ROS species at the PSI end can cause photoinhibition of PSII and PSI. Plants can avoid PSI photoinhibition by limiting the electron transport from PSII to PSI (lowering PSII activity) or re-routing electrons by providing alternative electron acceptors, such as cyclic electron transport (Taiz et al., 2015; Ogbaga and Athar, 2019; Shahzadi et al., 2021; Umer Chattha et al., 2021). These antioxidant enzymes scavenge ROS and protect the cellular structures (Bukhat et al., 2020; Askari-Khorasgani et al., 2021; Yasir et al., 2021). Photosystem II is more protected from ROS species than PSII by a battery of antioxidant enzymes (Taiz et al., 2015; Farid et al., 2018; Tikkanen and Grebe, 2018), which include superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT; Foyer et al., 2012; Foyer, 2018; Nazir et al., 2021). However, photoinhibition of PSII is protected by activating the xanthophyll cycle photoprotective component of non-photochemical quenching (NPQ). Sufficient evidence is available that demonstrates that various plant growth regulators, osmoprotectants, antioxidant signaling compounds can efficiently modulate nutrient uptake and transport, PSII activity, and antioxidants, thereby regulating plant growth under normal or stress conditions (Zhao et al., 2013; Athar et al., 2015; Ahmad et al., 2019; Ayyaz et al., 2021).

Jasmonate (JA) and methyl jasmonate (MeJA; methyl ester of JA) are well-known plant growth regulators that affect different biochemical and physiological processes (Yu et al., 2019; Bukhat et al., 2021), such as stomatal opening and photosynthetic activity (Yan et al., 2015). Several studies have shown that MeJA induces a stimulatory effect on photosystem II (PSII) photochemistry and photosynthetic pigments under normal conditions (Attaran et al., 2014; Qiu et al., 2020). While working with *Brassica juncea* Per et al. (2016) evidenced from transmission electron microscopy that MeJA protected the structure of chloroplast from cadmium toxicity. In addition, some studies showed that MeJA modulates the activities of some key antioxidant enzymes in different plants during heavy metal stress, such as in *B. juncea* (Per et al., 2016), Kandelia obovata (Chen et al., 2014), and *Brassica napus* (Farooq et al., 2018) Likewise, several studies showed that MeJA application in lower concentrations improved plant tolerance against abiotic stresses including Cd stress (Walia et al., 2007; Keramat et al., 2009; Per et al., 2016).

Pea (*Pisum sativum* L.) is one of the most crucial leguminous vegetable crop, whose yield is affected by Cd stress. Because of available information about MeJA, it is hypothesized that MeJA application might have improved the growth and yield of pea under cadmium stress. Although it is known that MeJA can change the PSII activity and activities of antioxidant enzymes, it is not yet known whether MeJA can also modulate solar energy absorption by PSII, and its distribution in electron transport or photochemistry and xanthophyll cycle under heavy metal stress conditions, including Cd stress. Since MeJA suppresses the growth and photosynthetic activity under stress conditions, when jasmonate signaling activates antioxidants enzymes and redirects metabolism from growth to defense, it is apt to assess up to what extent exogenously applied MeJA modulates the antioxidant mechanism and photosynthetic responses in pea plants to alleviate the adverse effects of Cd stress. The study's secondary objective was to assess genotypic variability for these responses in local pea germplasm.

MATERIALS AND METHODS

Plant Materials and Treatments

A pot experiment was conducted in a randomized block design with three local peas (*Pisum sativum* L.) cultivars (Meteor, S-Green, and Climax), four levels of cadmium (Cd) stress, and three levels of methyl jasmonate (MeJA) as a foliar spray with four replicates. Seeds of three pea cultivars were obtained from Ayub Agricultural Research Institute (AARI), Faisalabad, Pakistan. Seeds were disinfected with sodium hypochlorite before sowing. The experiment was conducted under controlled conditions (Light/Dark period 12/12 h, Humidity: 60%, Light intensity: 180–190 μmol m^{-2} s^{-1} and 20–25°C temperature) in growth room at the Institute of Molecular Biology and Biotechnology, Bahauddin Zakariya University, Multan, Pakistan. Pea seeds were sown in plastic pots filled with a mixture of sand and soil (3:1). Germinating seeds were supplemented with Hoagland nutrient solution. After 2 weeks of germination, healthy and homogenous plants were selected. Pea plants of the three cultivars were treated with different concentrations (0, 1, and 10 µm, foliar spray) of MeJA containing 0.01% Tween-20. Subsequently (72 h after MeJA treatment), plants were exposed to different levels of cadmium stress (0, 50, 100, and 200 µm $CdCl_2$). Cadmium stress was given from 50 µm and gradually increased to attain the required concentrations. Physiological and biochemical parameters were measured 48 h after the last cadmium treatment in four biological replicates.

Measurement of Biomass

After the completion of the duration of cadmium stress, pea plants were uprooted carefully, and plant parts were separated into shoots and roots. Plant parts were blotted dry, and their fresh weights (g) of shoots and roots were measured using a digital scale. Dry weights pg. shoots and roots of all the three cultivars of pea were recorded after drying the samples in the oven at 65°C for 1 week. The root length (cm) of each cultivar was measured using a ruler.

Determination of Photosynthetic Pigments and Photosystem II Photochemistry

Photosynthetic pigments were extracted from 0.5 g of fresh leaves samples using 80% acetone by placing them in the dark for 24 h. The absorbance of the extracted photosynthetic pigments (Chlorophyll a, b, total chlorophyll, and carotenoids) was measured at 480 nm, 663 nm, and 645 nm using a double beam spectrophotometer. The number of photosynthetic pigments was calculated using formulae as described elsewhere (Wildermuth and Fall, 1996). The maximum quantum yield of photosystem II (PSII) and distribution of absorbed solar energy in driving photochemistry and in processes other than photochemistry, such as a photoprotective component of non-photochemical quenching (NPQ), photoinhibition of PSII were evaluated using chlorophyll fluorescence induction analysis with DUAL-PAM 100 (Walz, Effeltrich, Germany). Briefly, plants were dark-adapted for 30 min, and then initial and maximum fluorescence (Fo, Fm) were investigated by applying week light (0.3 μmol photons/m^2/s^1 and then saturating light pulse of 0.8-s; 6,000 μmol photons/m2/s1). The steady-state PSII fluorescence yield was measured by exposing the leaf to red actinic light (635 nm). The actual efficiency of PSII, electron transport rate through PSII, non-photochemical quenching (NPQ), the fraction of photoprotective component of NPQ (Y-NPQ), and a fraction of NPQ due to photoinhibition of PSII were calculated following Schreiber and Klughammer (2008).

Determination of Lipid Peroxidation

Lipid peroxidation was measured to estimate the damaging effects of Cd in the cellular membrane. Approximately 0.25 g of fresh leaves were homogenized in 8 ml of 0.1% trichloroacetic acid (TCA) and was centrifuged for 30 min at 16000 rpm. After centrifugation, the supernatant was mixed with TBA (3 ml) prepared in a 20% solution of TCA. This mixture was placed at 95°C for 1 h in a water bath the following cooling on ice for almost 4–5 min. The absorbance of the mixture was taken at 600 nm with a double beam spectrophotometer (PerkinElmer Ltd., United Kingdome). MDA content was measured by an extinction coefficient of 155 mm^{-1} cm^{-1} (Dhindsa et al., 1981).

Estimation of Reactive Oxygen Species (H_2O_2)

Fresh leaves (0.25 g) were homogenized in a 3 ml solution of 0.1% TCA and centrifuged at 9000 × *g* for 15 min. The supernatant (0.1 ml) was mixed with potassium iodide (0.2 ml) and 50 mm potassium phosphate buffer (0.1 ml). The mixture was vortexed, and its optical density (OD) was measured at 390 nm (Velikova et al., 2000).

Determination of Antioxidant Activity

Enzymatic antioxidant activity was evaluated following the procedure described by Ananieva et al. (2004). Enzyme extract was prepared by homogenizing 0.25 g fresh leaves in 2 ml of 50 mm potassium phosphate buffer (pH = 7.8), which was then centrifuged for 20 min at 15000 × *g*. The supernatant was used as enzyme extract for assessing activities of catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD). CAT activity was measured by adding 0.1 ml H_2O_2 (300 mm), 3 ml phosphate buffer (pH = 7), and 0.1 ml enzyme extract in the reaction mixture (Miller and Rice-Evans, 1996). The samples were vortexed, and absorbance was measured at 240 nm for 1 min after 20 s. CAT activity was measured using the following formula: CAT activity = (activity*A * V/a)/(E·W). The reaction mixture for measuring SOD activity contained 75 μm nitroblue tetrazolium (NBT), 100 μm EDTA, 20 μm riboflavin, and 130 mm methionine and was placed in light for 1 h. After an hour, the color of the reaction mixture changed gradually, and this mixture was placed in the dark for 10 min to stop the reaction. The absorbance was taken at a wavelength of 560 nm, and its activity was evaluated using the standard curve of known concentrations of NBT (Armstrong, 1998). For POD activity, 300 mm H_2O_2 (0. 1 ml), 1.5% guaiacol (0.1 ml), enzyme extract (0.1 ml) and 50 mm potassium phosphate buffer (2.7 ml) were mixed. Its absorbance was measured for 2 min at 470 nm using a spectrophotometer (PerkinElmer Ltd., United Kingdome; Zhou and Leul, 1999).

Cadmium Quantification

Dried leaves (0.2 g) were digested in 5 ml of sulfuric acid overnight for cadmium determination. The samples were kept at 240°C on a hot plate until boiling and diluted with perchloric acid and nitric acid (1:5). This mixture was kept on a hot plate until it became transparent. Cd in this digested solution was quantified through atomic absorption spectrophotometer (240FS AA, Agilent Technologies, United States; Jackson, 2005).

Statistical Analysis

For statistical analysis, a three-way ANOVA (completely randomized block design) was performed using COSTATv.6.451 software (CoHort Software, California, United States). The least significant difference (LSD) was used for comparing the means of the different treatments if the interaction term was found significant.

RESULTS

Plant Growth

Increasing $CdCl_2$ (50, 100, and 200 μm) stress considerably declined both fresh and dry weight of roots and shoots of all the three pea cultivars. Foliar application of MeJA (1 and 10 μm) significantly increased the biomass of the three cultivars under normal and Cd-stressed conditions (**Table 1**). However, the application of 10 μm MeJA was more effective in improving the biomass of the three cultivars in Cd-stressed conditions (**Figures 1A–D**). In addition, this increasing effect of MeJA significantly varied in three pea cultivars. For example, at 10 μm MeJA application, the cultivar S-green showed a maximum increase in shoot fresh and dry weights by 115.16 and 118.86%, respectively, at the highest Cd stress. In contrast, cultivar Climax showed a minimum increase of 72.32 and 71.93%, respectively. The cultivar Meteor showed a maximum increase in root fresh and dry weight by 39.79 and 42.61%, respectively. Similarly, 10 μm MeJA resulted

TABLE 1 | Mean square values from ANOVA for root length, root & shoot fresh & dry weight, photosynthetic pigments, MDA, H_2O_2, antioxidant enzyme activities and PSII photochemistry of three cultivars of pea (*Pisum sativum* L.) plant treated with different methyl jasmonate concentrations under normal and Cd-stressed conditions.

Source of variation	*df*	Shoot fresh wt.	Shoot dry wt.	Root fresh wt.	Root dry wt.	Root length
Cd	3	12.481***	0.126***	1.711***	0.020***	230.261***
MeJA	2	16.760***	0.184***	2.068***	0.023***	1591.08***
Varieties	2	0.287***	0.002**	0.011 ns	0.000175 ns	25.974***
Cd*MeJA	6	0.069*	0.001**	0.005 *	0.0000553*	38.881***
Cd*Varieties	6	0.100**	0.001***	0.013 ns	0.0000759 ns	23.937***
MeJA*Varieties	4	0.094**	0.002***	0.008 ns	0.0001706 ns	0.856 ns
Cd*MeJA*Varieties	12	0.028 ns	0.00031 ns	0.002 ns	0.0000151 ns	5.634**
Error	72	0.02456	0.000308	0.00618	0.000069	2.0625
Source of variation	***df***	**Chl a**	**Chl b**	**Total Chl**	**Carotenoids**	**Cd Conc.**
Cd	3	35.617***	59.604***	184.819***	17.382***	6.001***
MeJA	2	36.492***	73.027***	214.793***	27.415***	3.722***
Varieties	2	1.529***	4.914***	2.421***	3.528***	0.014***
Cd*MeJA	6	0.101*	0.601**	0.919***	0.050*	0.413***
Cd*Varieties	6	0.890***	1.174***	2.727***	0.067 ns	0.004*
MeJA*Varieties	4	0.778***	0.138 ns	0.770**	0.053 ns	0.016***
Cd*MeJA*Varieties	12	0.222***	0.222 ns	0.394 ns	0.104 ns	0.003*
Error	72	0.05913	0.15738	0.21180	0.06454	0.00169
Source of variation	***df***	**ROS**	**MDA**	**POD**	**SOD**	**CAT**
Cd	3	0.201***	1140.435***	338.043***	628.65***	909.800***
MeJA	2	0.158***	9127.196***	50.177***	153.596***	113.147***
Varieties	2	0.059***	414.038***	37.459***	21.595***	133.714***
Cd*MeJA	6	0.00047*	782.336***	0.401*	0.589*	0.355*
Cd*Varieties	6	0.001*	95.114***	1.663***	3.112**	14.120***
MeJA*Varieties	4	0.001**	33.013**	0.179 ns	1.367 ns	1.538**
Cd*MeJA* Varieties	12	0.000286 ns	23.228**	0.319 ns	0.367 ns	0.704 ns
Error	72	0.000449	8.58802	0.23986	0.74955	0.42484
Source of variation	***df***	**Fv/Fm**	**Y (II)**	**ETR (II)**	**Y (NPQ)**	**Y (NO)**
Cd	3	0.089***	0.283***	408.960***	0.414***	1.071***
MeJA	2	0.061***	0.295***	372.751***	0.095***	0.269***
Varieties	2	0.0043***	0.033***	40.230***	0.00048 ns	0.0010 ns
Cd*MeJA	6	0.00017*	0.00041*	3.747**	0.004***	0.0023*
Cd*Varieties	6	0.00015 ns	0.006***	1.631 ns	0.009***	0.0053**
MeJA*Varieties	4	0.00043 ns	0.003**	2.231 ns	0.00025 ns	0.0015 ns
Cd*MeJA*Varieties	12	0.000039 ns	0.00023 ns	0.617 ns	0.000368 ns	0.0013 ns
Error	72	0.00027	0.000735	0.93815	0.000496	0.00156

*MeJA: Methyl Jasmonate; *, ** and *** show significance level at 0.05, 0.01 and 0.001, respectively, while ns = non-significant.*

in the maximum increase in root length and was observed at 200 µm $CdCl_2$ in Climax (120.93%; **Figure 1E**). Moreover, Cd content was found to be significantly higher in Cd-stressed plants compared to the control plants. However, applying both concentrations of MeJA remarkably reduced Cd accumulation in all Cd-stressed pea cultivars (**Table 1**). In addition, the supplementation of 10 µm MeJA remarkably reduced Cd accumulation in all cultivars under varying concentrations of $CdCl_2$ stress (50, 100, and 200 µm), particularly in Meteor by 66.57, 54.83, and 54.79%, respectively (**Figure 2E**).

Cd toxicity significantly reduced the photosynthetic pigments of all three pea cultivars under cadmium stress, while the MeJA application quite recovered photosynthetic pigments compared to typical and Cd-stressed plants (**Table 1**). Under the highest Cd stress, the supplementation of 10 µm MeJA effectively increased chl a, chl b, total chl and carotenoids in Meteor (153.92%), S-green (77.63%), Meteor (98.64%), and Climax (86.78%), respectively (**Figures 2A–D**).

Cd-induced considerable reduction in electron transport rate (ETRII) and the quantum yield of PSII [in terms of Fv/Fm and Y(II)] was improved by the application of MeJA (1 and 10 µm) under normal and Cd-stressed conditions (**Table 1**; **Figures 3A–C**). The more pronounced improvement in Fv/Fm, Y(II), and ETR(II) was observed by 10 µm MeJA at the highest cadmium stress in Climax (15.32, 139.6%) and Meteor (113.05%), respectively, compared to their respective control plants. Moreover, the quantum yield of non-photoprotective and photoprotective energy dissipation [Y(NO) and Y(NPQ)] was considerably increased with increasing cadmium concentrations in all pea plants. Although, the damage caused to PSII machinery by Cd toxicity was reduced by MeJA application in all pea cultivars compared to their respective controls (**Table 1**). From **Figures 3D,E**, it can be observed that at the highest cadmium stress, the supplementation of 10 µm MeJA considerably alleviated Y(NPQ) and Y(NO) parameters in S-green by 35.25 and 26.99%, respectively.

Cadmium toxicity considerably affected lipid peroxidation by increasing MDA and H_2O_2 contents in all Cd-stressed pea plants that were substantially reduced through the treatment

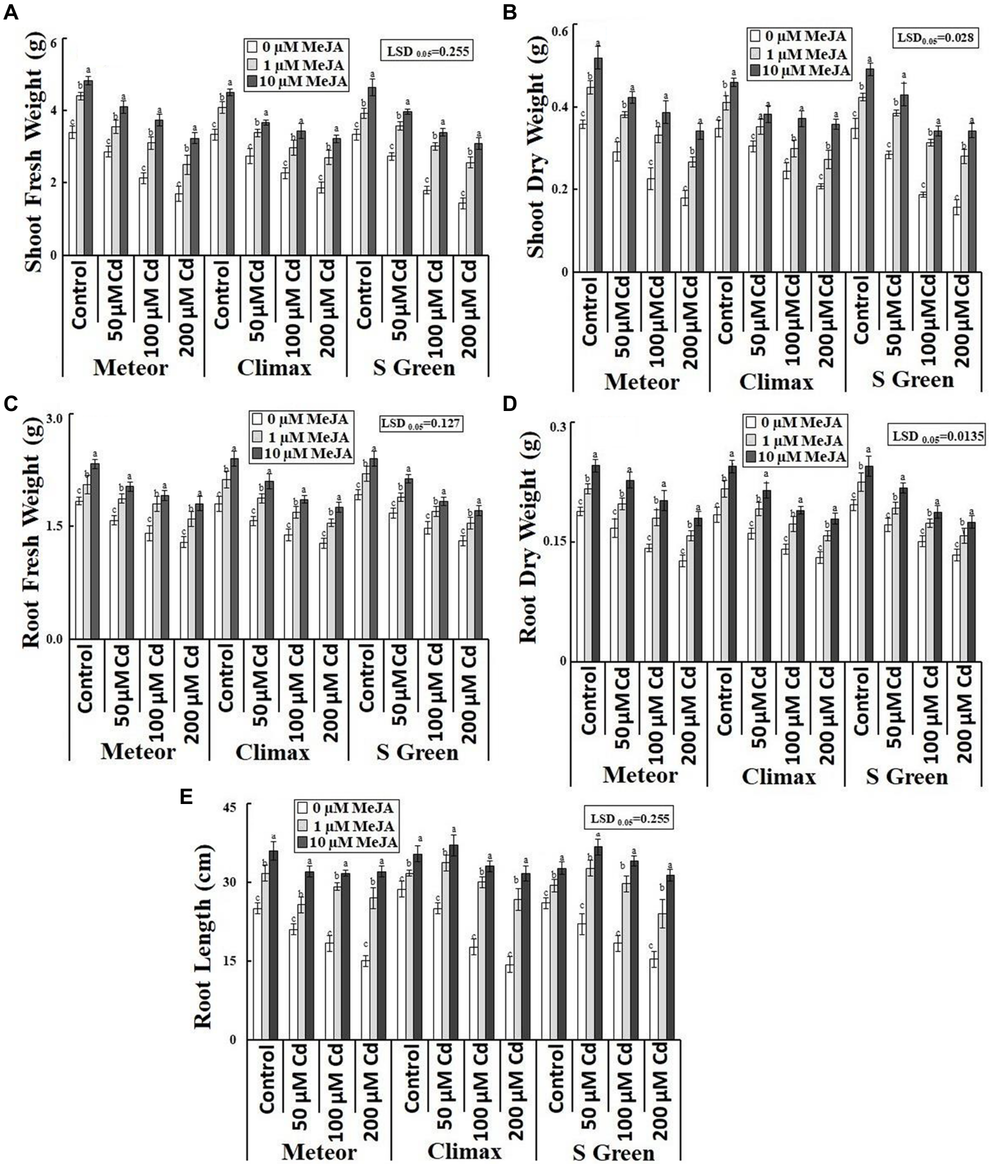

FIGURE 1 | Effect of CdCl2 (50, 100, and 200 µm) and MeJA (1 and 10 µm) on **(A)** Shoot fresh weight; **(B)** Shoot dry weight; **(C)** Root fresh weight; **(D)** Root dry weight; and **(E)** Root length of three pea cultivars. Bars show a mean of 3 three replicates ± standard deviation. Letters a, b, and c showed significant differences between means of different treatments.

of MeJA in typical and Cd-stressed plants under investigation (**Table 1**; **Figures 4A,B**). The exogenous application of 10 µm MeJA showed a more significant reduction of MDA and H_2O_2 contents at all cadmium concentrations in a cultivar-dependent way. For example, at 200 µm $CdCl_2$ stress, a considerable decrease in MDA and H_2O_2 contents was observed in Meteor (71.26%) and S-green (32.7%), respectively.

Cadmium stress also induced considerable changes in the activities of antioxidative enzymes among all pea cultivars, including POD, CAT, and SOD. The activities of all these enzymes increased significantly with increasing cadmium concentration; however, much increase was observed in Cd + MeJA treatment (**Table 1**; **Figures 4C–E**). The maximum increase in activities of all these enzymes was observed at 200 µm $CdCl_2$ plus 10 µm MeJA in all cultivars, particularly POD and CAT activity in S-green by 26.04 and 25.63%, respectively, while SOD activity in Climax by 17.3%.

DISCUSSION

Plants are susceptible to heavy metal toxicity, including Cd. Cd inhibits plant growth *via* interference with cell division, hormonal homeostasis, photosynthesis, nutrient uptake, and enhancing oxidative stress (Rizwan et al., 2018; Huang et al., 2020; Dobrikova et al., 2021). In this study, the exposure

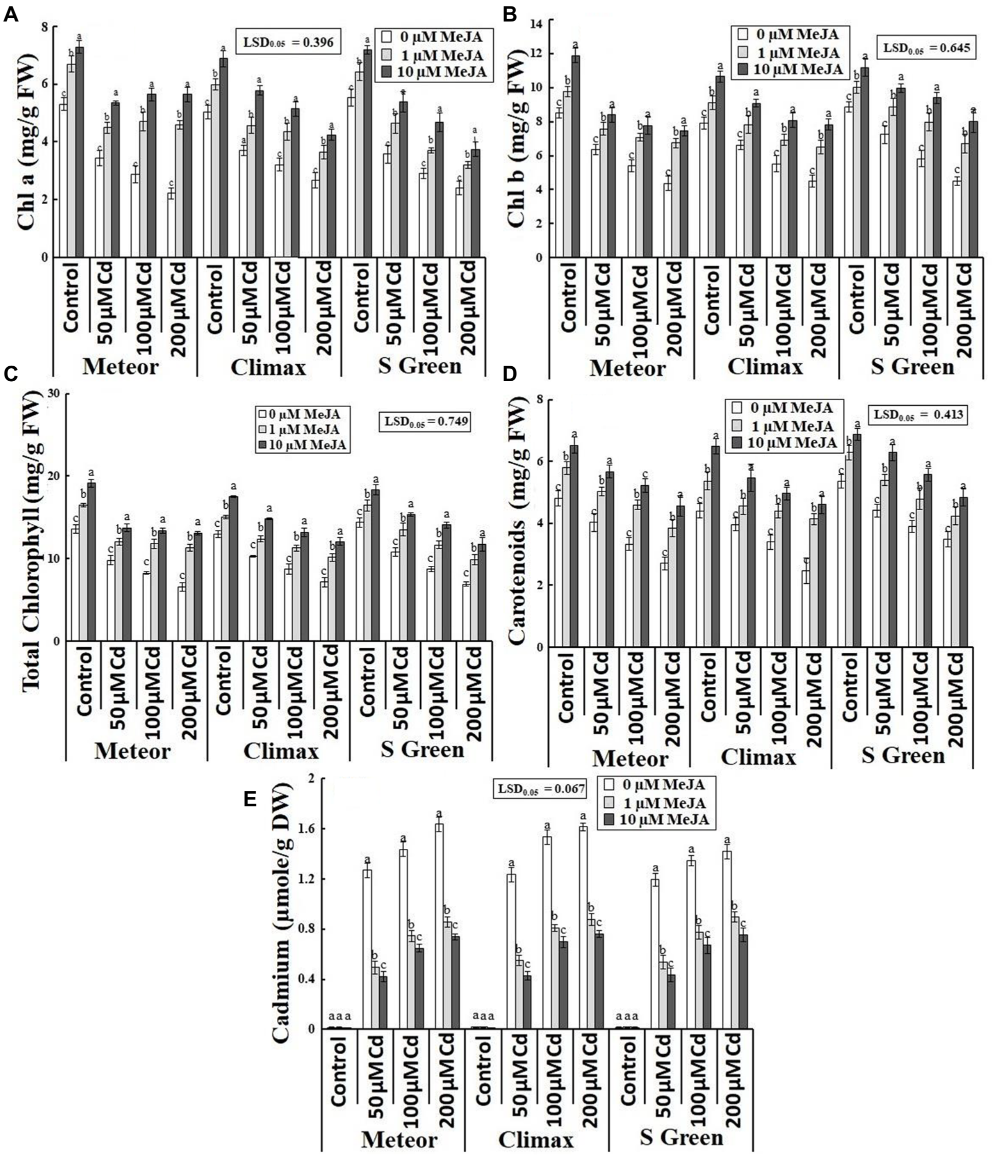

FIGURE 2 | Effect of CdCl2 (50, 100, and 200 µm) and MeJA (1 and 10 µm) on **(A)** Chl a; **(B)** Chl b; **(C)** Total Chl; **(D)** Carotenoids; and **(E)** Cadmium content of three pea cultivars. Bars show a mean of 3 three replicates ± standard deviation. Letters a, b, and c showed significant differences between means of different treatments.

of Cd considerably repressed the growth of pea plants, which is similar to earlier studies with different crop species (Huang et al., 2020; Shahzad et al., 2021; Wei et al., 2021). However, the toxic effects were compensated by the addition of MeJA. Previous research has shown that MeJA can modulate growth by altering antioxidant enzymatic activity increasing photosynthetic pigments and hormones under both biotic and abiotic stresses(Yan et al., 2015; Butt et al., 2019; Tayyab et al., 2020). In view of these reports, it is suggested that the ameliorative effect of MeJA on the growth of pea cultivars under Cd stress was possibly due to its impact on photosynthetic pigments, chloroplastic activity, and antioxidant enzymes.

Plant growth suppression caused by Cd is linked with changes in photosynthetic pigments, chloroplastic structures, and photosynthetic rate (Per et al., 2016; Qiu et al., 2020). In this study, Cd stress reduced the chl a, chl b, total chl, and carotenoids in pea leaves. The decrease in photosynthetic pigment content in three pea cultivars can be explained in view of some earlier studies in which it has been demonstrated that the high accumulation of Cd in leaves altered chlorophyll metabolism by affecting the activity of chlorophyll biosynthesis enzymes and chloroplast ultrastructure (Qiu et al., 2020). Application of MeJA increased photosynthetic pigments in pea cultivars. These results are analogous to earlier studies that demonstrated that MeJA promoted the accumulation of photosynthetic pigments

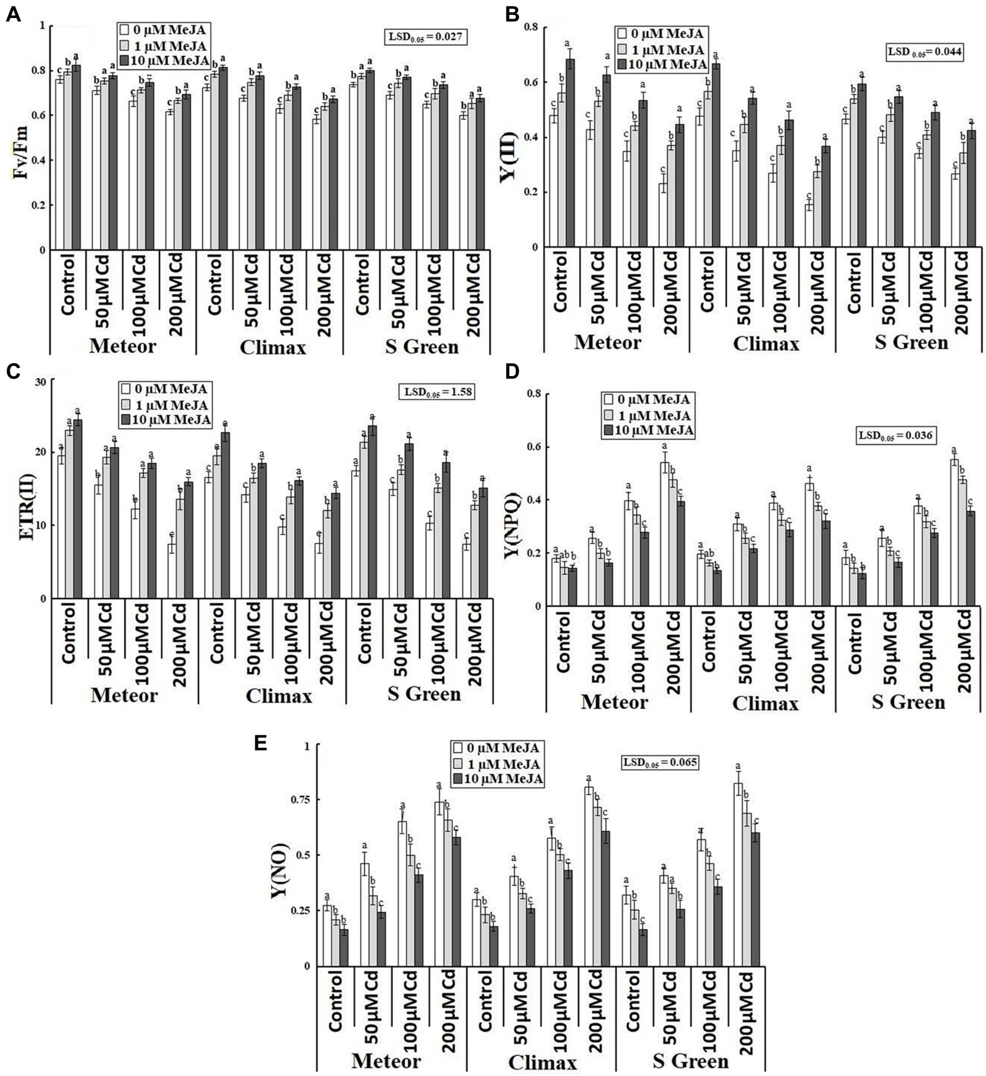

FIGURE 3 | Effect of CdCl2 (50, 100, and 200 µm) and MeJA (1 and 10 µm) on **(A)** Fv/Fm; **(B)** Y(II); **(C)** ETR(II); **(D)** Y(NPQ); and **(E)** Y(NO) of three pea cultivars. Bars show a mean of 3 three replicates ± standard deviation. Letters a, b, and c showed significant differences between means of different treatments.

in citrus (Qiu et al., 2020) and *Vicia faba* (Ahmad et al., 2017) exposed to metal stress.

The photosynthetic efficiency of plants is critical because it directly affects plant growth and productivity (Athar and Ashraf, 2005; Ogbaga et al., 2018). Cd stress significantly affected PSII structural and functional activity in all pea cultivars, which is in agreement with earlier research in which it was found that higher accumulation of Cd reduces the PSII structural ability and functional activity of Thellungiella salsuginea plants (Goussi et al., 2018). They explained it as Cd stress damages the PSII antenna and core resulting in reduced efficiency of PSII and impaired electron transport, as has been observed in this study. However, MeJA treatment enhanced the quantum yield [Y(II) and (Fv/Fm)] and electron transport rate ETR (II) of PSII. Several previous reports showed that MeJA improved photosynthesis-related attributes in Mentha arvensis (Zaid and Mohammad, 2018) and *Triticum aestivum* (Kaya et al., 2021) MeJA improved the parameters mentioned above.

On the other hand, increased energy dissipation in all Cd-stressed pea plants, as evidenced by elevated Y(NPQ) and Y(NO) values, indicates more energy loss along with reduced efficiency of PSII. This could be due to a reduction in carbon dioxide fixation in the Calvin cycle. Due to Cd stress, the production of excessive ROS denatures the D1 protein of PSII (Ruban et al., 2012). Usually, non-photochemical quenching gives a quick response and prevents ROS generation by dissipation of light energy in the form of heat through the antenna complex (Lambrev et al., 2012).

In plants, heavy metal toxicity usually causes oxidative stress due to excessive ROS production, including O^{2-}, H_2O_2, and $^{\cdot}OH$. These compounds are highly reactive and toxic; they cause membrane damage and oxidize macromolecules like

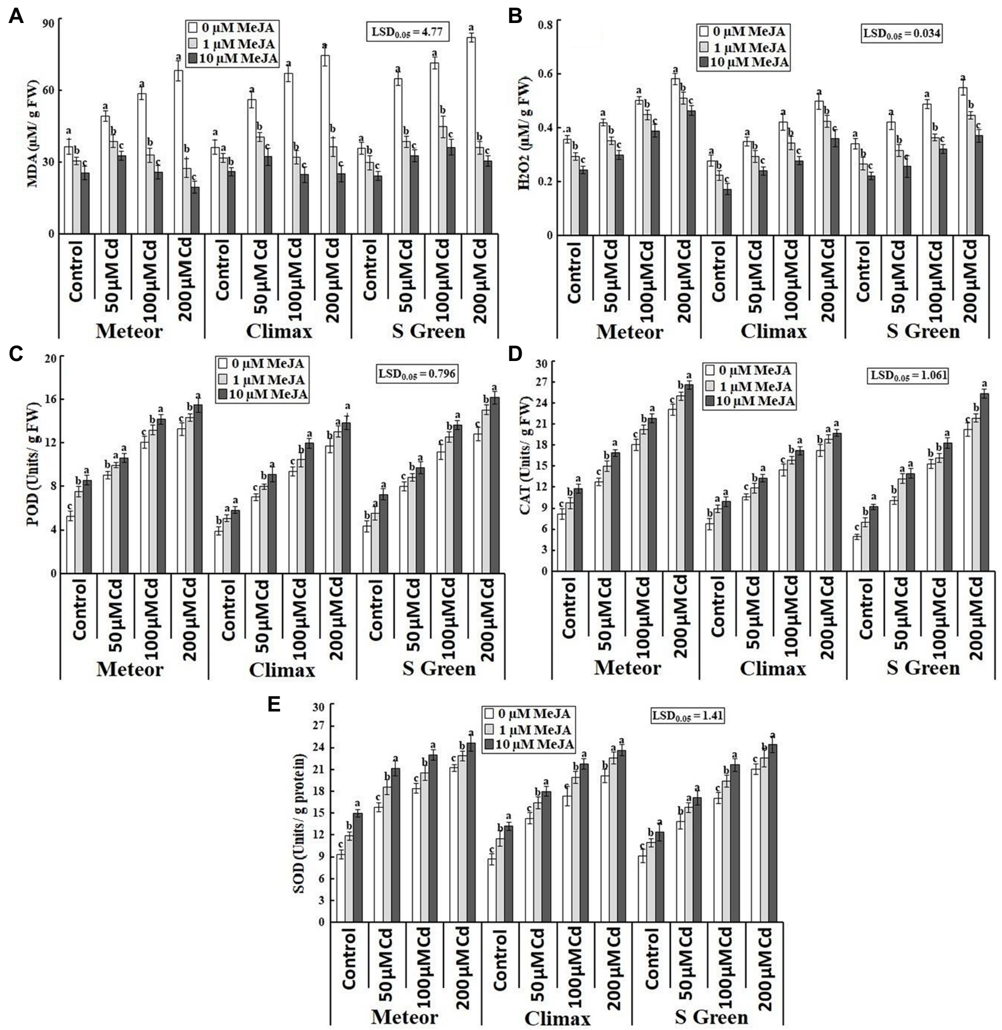

FIGURE 4 | Effect of CdCl2 (50, 100, and 200 μm) and MeJA (1 and 10 μm) on **(A)** Lipid peroxidation; **(B)** ROS; **(C)** POD; **(D)** CAT; and **(E)** SOD of three pea cultivars. Bars show a mean of 3 three replicates ± standard deviation. Letters a, b, and c showed significant differences between means of different treatments.

carbohydrates, proteins, lipids, and DNA (Gill and Tuteja, 2010). Cellular damage caused by heavy metal stress results in the increased production of H_2O_2 that ultimately enhances the peroxidation of membrane fatty acids leading to increased MDA content. MDA is commonly used for plants as the marker for lipid damage and lipid peroxidation that disrupts membrane fluidity, enhances electrolyte leakage, inhibits enzymes' activity, and interferes with protein channeling (Garg and Manchanda, 2009). In the present study, exposure of pea plants with Cd stress increased the H_2O_2 and MDA content. These results were similar to many studies conducted on *P. sativum* (Romero-Puertas et al., 2004) and Vigna radiata (Ahmad et al., 2011). The supplementation of MeJA to Cd-stressed pea plants significantly reduced the H_2O_2 and MDA levels indicating that MeJA has the potential to overcome oxidative stress found in intracellular membranes and cell membranes of all pea cultivars. A study supporting this conclusion showed that the application of MeJA enhanced the enzyme activities (APX, CAT, and SOD) of Bunium persicum plants exposed to Cd stress. These results were further supported by Chen et al. (2014), who demonstrated that supplementation of JA reduced MDA concentration in Kandelia obovate in response to Cd stress.

As discussed above, antioxidative machinery can scavenge ROS and protect plants against oxidative damage. In the present study, the increased antioxidant enzyme activities (CAT, POD, and SOD) correspond to those levels observed in *B. juncea* (Ahmad et al., 2016), Solanum lycopersicum (Cherif et al., 2011), and *P. vulgaris* (Rady, 2011). Moreover, in Capsicum frutescens plants, JA supplementation to Cd-stressed seedlings increased antioxidative enzyme activities and chlorophyll production (Yan et al., 2015). The application of JA also improved tolerance against Cd stress by increasing phytochelatin levels and activating defense-related genes (Maksymiec et al., 2007). Enzymatic activities enhanced by the JA supplementation can be attributed to the direct association with radicals, such as superoxide. It might be due to cells' improved ROS quenching capability by producing antioxidative enzymes (Hsu and Kao, 2007; Sirhindi et al., 2016). Overall, MeJA-mediated improvement against Cd toxicity in lipid membranes appears to be linked to increased antioxidant ability.

Furthermore, cadmium present in nutrient solution and soil can be easily absorbed by plants' roots and transferred to other plant tissues. The highest Cd content in leaves was found in this investigation, which was remarkably lowered by MeJA treatment. Heavy metals usually compete with Zn^{2+}, Ca^{2+}, Fe^{2+}, Mg^{2+}, and Mn^{2+} cations to access root cells through these cations transporters. It can be assumed that MeJA treatment enhanced the physiological parameters of all pea varieties and increased the uptake of valuable cations, decreasing Cd content and Cd^{2+} influxes in pea plants, similar to a previous study conducted on tomato plants (Wei et al., 2021). JA application reduced the uptake of Cd and improved antioxidant machinery in Kandelia obovata (Chen et al., 2014). However, the underlying mechanisms concerning how MeJA lowers the uptake of Cd are still unknown.

CONCLUSION

In the present study, Cd stress caused the higher Cd accumulation in pea plants, disturbing the metabolism for photosynthetic pigment biosynthesis and damaged photosynthetic machinery. Application of MeJA reduced the toxic effects of Cd on photosynthetic pigments and maintained the PSII activity by lowering uptake of Cd, downregulation of electron transport from PSII to PSI, increase in the photoprotective component of NPQ, and partial increase in activities of antioxidant enzymes. This helped the plants maintain cellular redox balance, thereby resulting in lower membrane damage. Thus, it can be inferred that the MeJA-induced growth improvement is related to (i) reduced Cd uptake by roots, (ii) optimization of the photosynthetic apparatus or photosynthetic rate, and (iii) enhanced antioxidant activity.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

AUTHOR CONTRIBUTIONS

SR: conceptualization and supervision. SR and HM: methodology. SR, H-u-RA, and SN: validation. MR and ZZ: formal analysis. Mehwish and SB: investigation. SR: resources. Mehwish: data curation. SB: writing—original draft preparation. MS, MB, WS, SR, HM, H-u-RA, MR and AE: writing—review and editing. SN and MR: visualization. HM: project administration. CCO: writing—review and editing. All authors contributed to the article and approved the submitted version.

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Application of Cerium Dioxide Nanoparticles and Chromium-Resistant Bacteria Reduced Chromium Toxicity in Sunflower Plants

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The continuous increase in the heavy metals concentration in the soil due to anthropogenic activities has become a global issue. The chromium, especially hexavalent chromium, is highly toxic for living organisms due to high mobility, solubility, and carcinogenic properties. Considering the beneficial role of nanoparticles and bacteria in alleviating the metal stress in plants, a study was carried out to evaluate the role of cerium dioxide (CeO_2) nanoparticles (NPs) and *Staphylococcus aureus* in alleviating the chromium toxicity in sunflower plants. Sunflower plants grown in chromium (Cr) contaminated soil (0, 25, and 50 mg kg^{-1}) were treated with CeO_2 nanoparticles (0, 25, and 50 mg L^{-1}) and *S. aureus*. The application of Cerium Dioxide Nanoparticles (CeO_2 NPs) significantly improved plant growth and biomass production, reduced oxidative stress, and enhanced the enzymatic activities in the sunflower plant grown under chromium stress. The application of *S. aureus* further enhanced the beneficial role of nanoparticles in alleviating metal-induced toxicity. The maximum improvement was noted in plants treated with both nanoparticles and *S. aureus*. The augmented application of CeO_2 NPs (50 mg l^{-1}) at Cr 50 mg kg^{-1} increased the *chl a* contents from 1.2 to 2.0, *chl b* contents 0.5 to 0.8 and mg g^{-1} FW, and decreased the leakage of the electrolyte from 121 to 104%. The findings proved that the application of CeO_2 nanoparticles and *S. aureus* could significantly ameliorate the metal-induced stress in sunflower plants. The findings from this study can provide new horizons for research in the application of nanoparticles in phytoremediation and bioremediation.

Keywords: bacteria, chromium, nanoparticles, phytoremediation, sunflower

INTRODUCTION

Chromium is naturally found in the soil, air, and water. The trivalent chromium (Cr^{+3}) and hexavalent chromium (Cr^{+6}) are two forms of chromium (Yoshinaga et al., 2018). The hexavalent chromium is considered the most toxic form that mainly exits as chromate or dichromate. Human activities and industrialization have increased the addition of chromium in the environment, and it has become a significant concern for plants, animals, and humans due to its lethal effects. The primary source of chromium pollution is the leather industry, followed by the electroplating industry textile and chromate mining (Belay, 2010; Tumolo et al., 2020). Chromium is a toxic and non-essential element for plants, and plants do not have a specific mechanism for its uptake (Shanker et al., 2005). Chromium is highly toxic for plants due to its detrimental effect on plants' growth and development. The most common effect of chromium on plants is reduced leaf and root growth, reduced yield, inhibition of enzymatic activity, and mutagenesis (Singh et al., 2013).

Sunflower (*Helianthus annuus* L.) is one of the essential vegetable oil sources, with an annual production of about 9 million tonnes. The sunflower oil is highly healthy due to its high concentration of mono-saturated and poly-saturated fatty acid and high contents of vitamin E (Kaya et al., 2012). Sunflower has been widely studied due to its unique ability to uptake and accumulate various heavy metals (Alaboudi et al., 2018; Chen et al., 2020). Sunflower can uptake and accumulate metals such as Pb, Cd, and Cr in its roots and shoots from contaminated soil (Chen et al., 2020). Due to its high biomass, sunflower is recommended to remediation soil contaminated with heavy metals. However, the high concentration of chromium has a detrimental effect on sunflower productivity and enzymatic activity (Fozia et al., 2008).

Phytoremediation is a green technology in which plants remove toxic pollutants and heavy metals from contaminated soil and water. In this technology, the potential of plants is applied to uptake, accumulate, and degrade pollutants from the contaminated medium (Hauptvogl et al., 2019). The ability of plants to remediate the pollutants depends upon the type of pollutants, concentration of pollutant and plant biomass production, and ability to accumulate metals (Ashraf et al., 2019). Hyperaccumulator plants have tremendous ability to accumulate particular metals and metalloids, hundred or thousand times greater than typical plants in the same environmental condition (Peer et al., 2005). These plants are most tolerant to high concentrations of heavy metals, can uptake these pollutants through their roots system, and accumulate high levels of these metals in their living tissue such as roots, stems, and leaves (Peer et al., 2005; Baker et al., 2020). Plants also have different metabolic processes to alleviate the metal stress, such as producing organic acids that can chelate the heavy metals and covert these metals into not toxic form and prevent the uptake of heavy metal by plants (Shahid et al., 2017, 2019). Other methods used by the plant to minimize the metal toxification include extracellular complexation and cytoplasmic complexation. The metal stress in the plant causes severe damage to the plant's metabolic process. To tolerate the metals stress, the plant should activate the antioxidant enzymes system and repair the oxidative stress and damage caused by metal stress (Sharma et al., 2016).

The microorganism can be applied as a biological tool to remove the heavy metals from the polluted medium due to their ability to concentrate and recover heavy metals (Devi et al., 2021). Bioremediation is the application of microorganisms to remediate polluted sites contaminated with heavy metals and other organic/inorganic pollutants (Shahid et al., 2020). Bacteria have evolved efficient mechanisms to bio-remediate heavy metals. These mechanisms include detoxification, adsorption, oxidation, and reduction of heavy metals (Kang et al., 2016; Naik and Kumar, 2020). The versatility of bacteria to degrade various pollutants and heavy metals has emphasized their application in the remediation of different types of soils. The bioremediation has shown promising results in the remediation of heavy metals such as lead, chromium, and cadmium, even in deficient concentrations in soil and water where other processes failed (Tiquia-Arashiro, 2018; Pushkar et al., 2021). The phytoremediation process can be boosted by applying selective bacteria resistant to specific metals. It has been widely proven that the application of bacteria in phytoremediation increases heavy metals such as lead, chromium, and iron uptake and removal from the contaminated soils (Kong et al., 2019; Ma et al., 2019).

Nanotechnology has grown remarkably in the last two decades with its wide application in agriculture and environmental remediation (Song et al., 2019; Zand et al., 2020). Nanoparticle-assisted phytoremediation has emerged as a reliable technology for removing contaminated soil with metals and metalloids. The nanoparticle has been widely applied from remediation of lead, cadmium, and arsenic from contaminated soil (Song et al., 2019; Zhu et al., 2019). The nanoparticle may improve the phytoremediation by a direct effect such as directly removing the pollutant from the soil by adsorption and immobilizing the metals, and ultimately reducing the concentration of metals in the soil (Omara et al., 2019). The nanoparticle can also improve phytoremediation by promoting plant growth, such as carbon nanotubes, Ag nanoparticles, and ZnO nanoparticles (Sabir et al., 2014). The nanoparticle may also increase the plant tolerance to abiotic stress by regulating the gene expression of enzymes, such as application of silicon nanoparticles improved the phytoextraction capacity of pea plant for chromium (VI; Tripathi et al., 2015). The applied nanoparticle may also improve plant growth by increasing the availability and absorption of nutrients and water (Kale and Gawade, 2016).

Considering the influential role of nanoparticles and bacteria, a field study was conducted to analyze the potential of Cerium Dioxide Nanoparticles (CeO_2 NPs) and a chromium-resistant bacteria *Staphylococcus aureus* in remediating the chromium from contaminated soil through sunflower plants. It was assumed that combined application of CeO_2 NPs and chromium-resistant bacteria will alleviate the chromium toxicity by their mutual role and enhance plant tolerance to metal toxicity. The augmented and individual role of CeO_2 NPs and *S. aureus* on the performance of chromium-stressed plants was evaluated by analyzing the changes in growth parameters, enzymatic activity, and oxidative stress. The finding from this research will provide valuable information about the application of nanoparticles and bacteria in remediating heavy metals from the contaminated medium.

MATERIALS AND METHODS

Soil Sampling and Analysis

The soil for this experiment was collected from the field of Govt. College University, Faisalabad from various points and depths from 0 to 20 cm to make a homogeneous mixture as recommended by previous researchers (Rehman et al., 2018; Rizwan et al., 2019). The collected soil sample was stored at 4°C in cooler box to protect from sunlight. All collected soil samples were air-dried, and the plant debris and large soil particles were removed by sieving the soil through a 2 mm sieve. The pH of the soil was analyzed by making the soil water paste and noted by calibrated pH meter. Similarly, the electrical conductivity (EC) of the soil was noted by calibrated EC meter. The collected soil was analyzed for metal contents by treating ammonium bicarbonate diethylene triamine penta acetic acid (AB-DTPA) at pH 6.7 (Soltanpour, 1991). The soil was artificially spiked with $K_2Cr_2O_7$ to make the chromium-contaminated soil just like the natural agricultural soil of district Qasur and Sialkot highly contaminated with chromium due to irrigation with tannery effluent.

Bacterial Inoculation of Seeds

The soil was collected, ground, and sieved through a 2 mm sieve after drying at 70°C. The sieved soil was autoclaved at 121°C for 20 min to remove bacterial contamination. Nutrient broth and Cr-resistant bacteria, *S. aureus*, were used to make the bacterial inoculum. The bacterial inoculum was shaken at 2000 rpm for 48 h at 30°C, then centrifuged for 10 min at 6000 rpm. The supernatant was collected and diluted with distilled water (Zulaika and Sembiring, 2014). The density of the bacterial isolate was measured using a hemocytometer. The population size of the centrifuged bacterial cells was set at 2.8×10^8 wet weight. Sunflower seeds were disinfected by inoculating them with a 10% sugar solution. The seeds were adequately coated with clay and an equal amount of peat moss (1:1) and placed overnight.

Sunflower Sowing and Harvesting

The experiment was performed in the pots at the botanical garden of Government College University, Faisalabad, at temperatures 20–25°C with 70% humidity. Each plastic container was filled with 5 kg of sieved soil mixed with three chromium concentrations (0, 25, and 50 mg kg^{-1}). Six uniform healthy sunflower seeds were rinsed with hydrogen peroxide solution (15% v/v) followed by tap and distilled water. Six sunflowers' seeds are sown in each pot, and after germination, only three healthy and uniform seedlings were kept to grow for further proceedings. A mixture of nitrogen, phosphorus, and potassium was applied at the rate of 120:50:25 kg ha^{-1} for the healthy growth of the plants. After 2 weeks of germination, the nanoparticle CeO_2 NPs were applied as a foliar spray in three concentrations (0, 25, and 50 mg L-1), while controls were treated with distilled water. Cerium (IV) oxide nano-powder (CeO_2-NPs) was of Alfa Aesar with size, purity, and surface area of 15–30 nm, 99.5%, and 30–50 m^2/g, respectively. The experiment was run for 4 months and when plants reached at maturity, the harvesting was done. The plants were harvested about 1 cm high from the soil, and plants were separated into different parts such as root, shoot, and leaves for further analysis. Fresh plant's samples were kept in a cooler box during transportation to the laboratory for analysis to prevent changes in biochemical properties. The plants shoot and roots were appropriately washed with distilled water and roots were washed with 1% HCl followed by filtered water to remove the soil, contamination, and acid. The samples were air-dried and then oven dried at 72°C for 72 h.

Plant Growth Parameter and Chlorophyll Contents

After harvesting, the plant height (cm) was measured by measuring tape; then, the shoot dry weight (g pot^{-1}) and roots dry weight (g pot^{-1}) were measured by weight balance. The number of leaves per plant was counted, and leaf area (cm^2) was measured. The chlorophyll a, b, total chlorophyll, and carotenoid contents were measured by extracting 0.2 g of fresh leaves in 0.5 ml of acetone (3% v/v). The supernatant was obtained through centrifugation for 10 min at 10,000 rpm, and absorption was noted through spectrophotometer for chlorophyll a (*chl a*) at 663 nm, chlorophyll b (*chl b*) 645 nm, and carotenoid at 470 nm (Lichtenthaler, 1987; Gohari et al., 2020).

Estimation of Antioxidant Enzymes and Reactive Oxygen Species Contents

The antioxidant activities of the enzymes (CAT, APX, POD, and SOD) in sunflower plants were determined using methods developed by Aebi (1984) for catalase (CAT), Nakano and Physiology (1981) for ascorbate oxidase (APX), and Zhang (1992) for peroxidases (POD) and superoxide dismutase (SOD; Nakano and Physiology, 1981; Aebi, 1984; Zhang, 1992).

The reactive oxygen species (ROS) were measured by analyzing the electrolyte leakage (EL), malondialdehyde (MDA) contents, and H_2O_2 in sunflower plants. The initial EC1 from the leaves was checked by extracting the supernatant by autoclaving the plants at 32°C for 2 h, then heating the sample at 121°C. The final EL contents were noted using the Dionisio-Sese and Tobita (1998) method (Dionisio-Sese and Tobita, 1998). The MDA and H_2O_2 contents were determined by preparing a supernatant solution by crushing a 0.1 g plant sample in liquid nitrogen and then in a 0.05 M phosphate buffer. This supernatant solution was prepared with TCA (0.1%) and TBA (0.5%) for MDA content, and absorbance was measured at 532 nm (Zhang and

Kirkham, 1994). The H_2O_2 content was determined by mixing the supernatant with phosphate buffer and measuring the absorbance at 410 nm (Jana and Choudhuri, 1981).

Metal Contents in Shoot and Roots

The concentration of Cr^{+6} and Cr^{+3} in plants roots and shoots sample was measured by acid digestion of root and shoot samples by the standard method (Apha, 2012). The roots and shoot samples of 1 g each were ground and acid digested using HNO_3 and $HClO_4$ (3:1) ratio. The metals contents in digested samples were determined by atomic absorption spectrophotometer.

Statistical Analysis

The data from the experimental research work are expressed as a mean value with a standard deviation. The replicated data were statistically analyzed using two-way variance (ANOVA) with Statistix 10.0 version software to recognize significant variance, and means were compared using Tukey's post-hoc test. At $p < 0.05$, values were considered significant for all treatments.

RESULTS

Effect on Growth Parameters

It is evident from **Figures 1A–F** that increasing the concentration of chromium from 0 to 50 mg kg^{-1} significantly decreased all plant growth parameters (plant height, shoot dry weight, root dry weight, number of leaves per plant, leaf area, and number of flowers per plant). At the highest concentration of chromium (Cr 50), the plant height decreased from 75 to 37 cm, and shoot dry weight decreased 19 to 9 g pot^{-1}. A similar reduction was observed at a concentration of Cr50 for other growth attributes. However, *S. aureus* and CeO_2 NPs significantly improved all plant growth parameters in sunflower plants under chromium stress. In plants with stress Cr50, the treatment with *S. aureus* increased the plant height up to 47 cm as compared to 37 in treatment without bacterial application. On the other hand, the application of CeO_2 NPs further boosted the role of bacteria in plants under chromium stress. At stress level Cr50, the combined application of *S. aureus* and CeO_2 NPs improved the plant height from 46 to 55 cm and shoot dry weight 11 to 13 g pot^{-1}.

Effect on Photosynthetic Parameters

Just like the effect of chromium stress on growth parameters, the high concentration of chromium severely reduced the photosynthetic rate, transpiration rate, stomatal conductance, and water use efficiency in sunflower plants (**Figures 2A–D**). The increasing chromium concentration from 0 to 50 mg kg^{-1} reduced the photosynthetic rate from 25.9 to 11.0 µmol CO_2 m^{-2} s^{-1} and water use efficiency from 0.4 to 0.1%. The application of *S. aureus* and CeO_2 NPs significantly alleviated the damage to photosynthetic parameters by increasing the photosynthetic rate from 15.4 to 19.0 µmol CO_2 m^{-2} s^{-1} and water use efficiency from 0.2 to 0.3% with increasing concentration of CeO_2 NPs from 0 to 50 mg L^{-1}.

Effect on Chlorophyll Contents

Under Cr stress, chlorophyll contents such as Chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids continued to decrease gradually with increasing concentration of chromium (**Figures 3A–D**). Whereas, the successful application of CeO_2 NPs and *S. aureus* individually and collectively enhanced the chlorophyll contents in the sunflower plants facing chromium stress. The maximum increase was observed by the combined application of CeO_2 NPs and *S. aureus*. In plants under chromium stress Cr 50, without *S. aureus*, CeO_2 NPs from 0 to 50 improved the *chl a* content from 0.3 to 1.2 mg g^{-1} FW. The combined application of *S. aureus* and CeO_2 NPs from 0 to 50 improved the *chl a* contents from 1.2 to 2.0 mg g^{-1} FW.

Effect on Enzymes Activity

The gradual decrease was depicted in enzymes activity (SOD, POD, CAT, and APX) in the sunflower plant with a gradual increase in the chromium concentration (0 to 50 mg kg^{-1}; **Figures 4A–D**). The treatment of plants with CeO_2 NPs alleviated the chromium stress and improved the plant's enzyme activity under chromium stress. For instance, the SOD activity in plants under chromium stress Cr50 improved from 35 to 48 with increasing CeO_2 NPs from 0 to 50 mg L^{-1}. The maximum improvement in the enzyme's activity in plants under the highest level of chromium stress (Cr50) was observed in plants treated with the combined application of *S. aureus* and CeO_2 NPs. POD contents were increased from 657 to 853 Units g^{-1} FW.

Effect on Oxidative Stress

The increasing concentration of chromium increased the oxidative stress, which was evident from the increasing level of electrolyte leakage, malondialdehyde contents, and H_2O_2 contents in the sunflower plants (**Figures 5A–C**). The increasing chromium concentration from 0 to 50 increased the EL contents by 78 to 137%, MDA contents of 11 to 20 µmol g^{-1} FW, and H_2O_2 contents of 105 to 189 µmol g^{-1} FW in treatments without application of *S. aureus* and CeO_2 NPs. In contrast, CeO_2 NPs significantly decreased oxidative stress by reducing the EL contents 137 to 116% in treatments with chromium stress Cr50 and without *S. aureus*.

Effect on Chromium Uptake and Accumulation

The increasing concentration of chromium (0–50 mg kg^{-1}) increased the accumulation of chromium Cr^{+6} and Cr^{+3} in plant roots and shoots (**Figures 6A–D**). The Cr^{+6} concentration increased from 0.4 to 31 and 0.2 to 16.2 mg kg^{-1} DW in the plant's roots and shoots. A similar trend was observed for Cr^{+3} in treatments without the application of *S. aureus* and CeO_2 NPs. The application of CeO_2 NPs decreased the accumulation of Cr^{+6} and Cr^{+3} in plant root, and shoots such as Cr^{+6} concentration in plant root decreased from 31 to 20.6 mg kg^{-1} DW and Cr^{+3} concentration decreased from 22 to 15.3 mg kg^{-1} DW in plant under stress level Cr50. However,

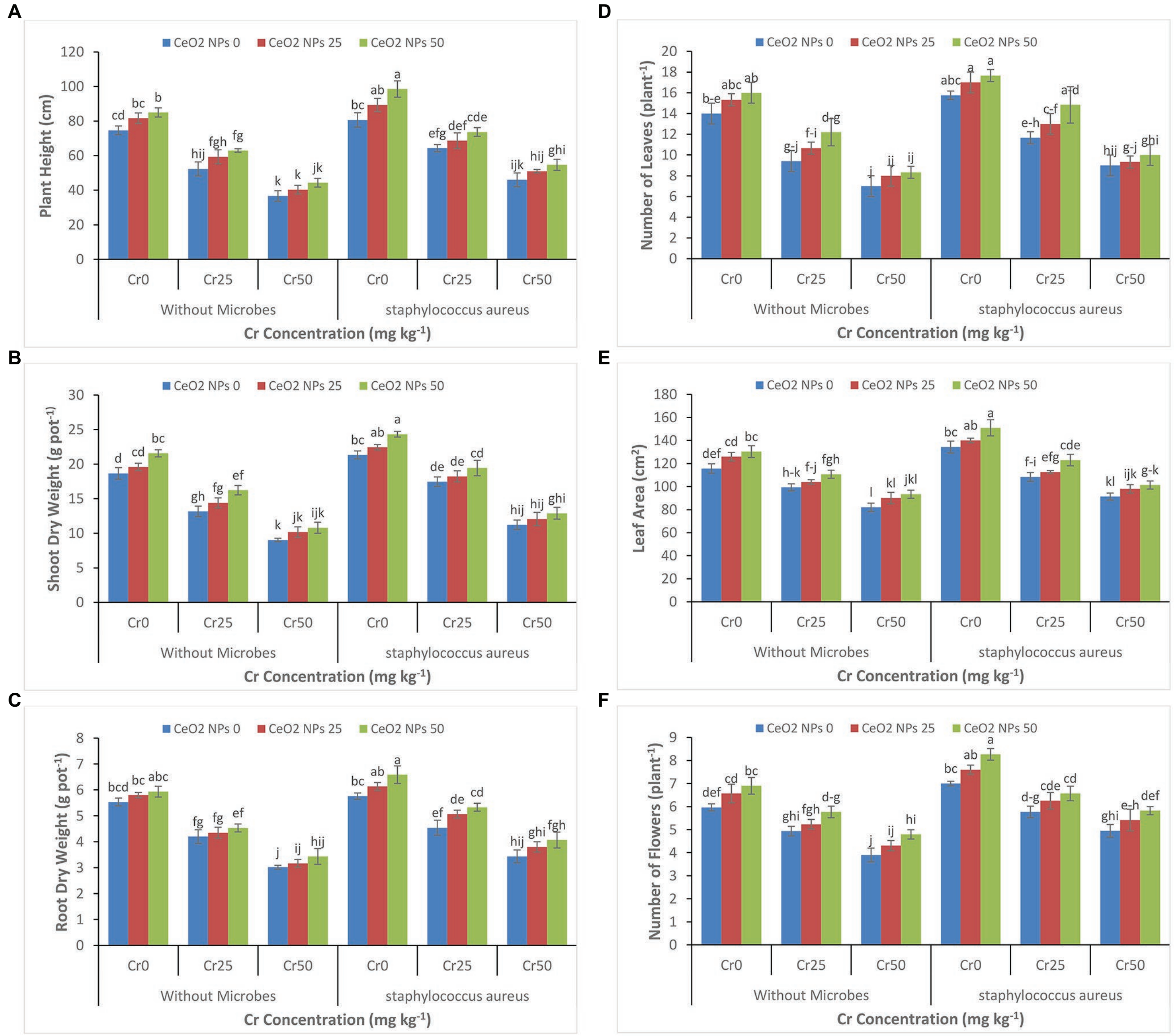

FIGURE 1 | (A–F): Effect of CeO_2 NPs and *Staphylococcus aureus* on plant height **(A)**, shoot dry weight **(B)**, root dry weight **(C)**, number of leaves **(D)**, leaf area **(E)**, and number of flowers **(F)** in sunflower plant under Cr stress. The given values are means of three replications with standard deviation. The alphabets represent the significant/non-significant difference at $p \leq 0.05$.

the application of *S. aureus* brought a dramatic change in the accumulation of chromium by facilitating the more accumulation of Cr^{+3} than Cr^{+6} in plant roots and shoot. In plants treated with *S. aureus*, the Cr^{+3} accumulation in roots was 8.0 mg kg^{-1} FW compared to Cr^{+6} concentration, which was 6.8 mg kg^{-1} FW and 7.9 Cr^{+3} as compared to 3.3 mg kg^{-1} DW Cr^{+6} in the shoot at stress level Cr50.

DISCUSSION

In this study, for the first time, we showed that the application of CeO_2 NPs and bacteria *S. aureus* could alleviate chromium-induced toxicity in the sunflower plants at an optimal concentration of chromium (Cr50). The combined application of CeO_2 nanoparticles and *S. aureus* efficiently ameliorated Cr-induced oxidative stress by promoting antioxidant enzymes activities and improved the photosynthetic activity by promoting chlorophyll contents in plants. In addition, CeO_2 NPs and *S. aureus* decreased the Cr accumulation in roots and shoots in plants tolerating the highest chromium concentration (Cr50). The high concentration of chromium negatively affects morphological and agronomical parameters of plants through altering the water use efficiency, stomatal conductance, and photosynthesis process (**Figures 1A–F, 2A–D**; Ertani et al., 2017). In the present study, we evaluated that

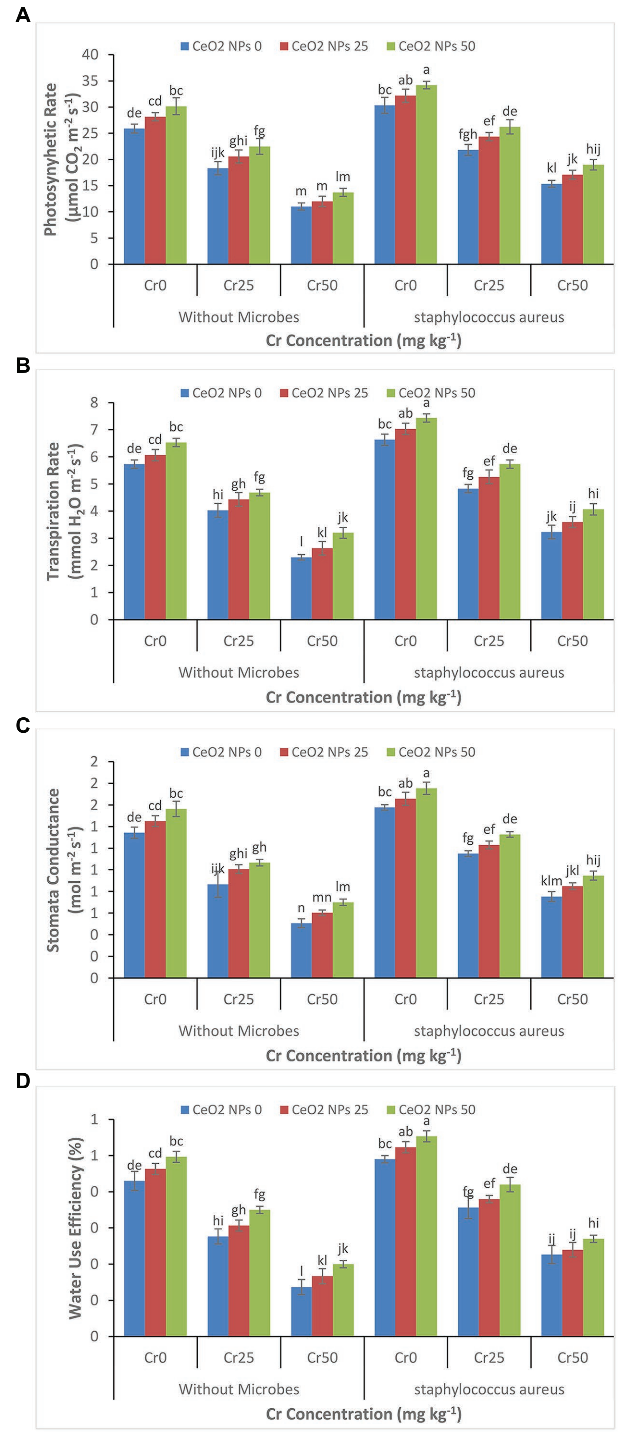

FIGURE 2 | (A–D): Effect of CeO_2 NPs and *Staphylococcus aureus* on photosynthetic rate **(A)**, transpiration rate **(B)**, stomatal conductance **(C)**, and water use efficiency **(D)** in sunflower plant under Cr stress. The given values are means of three replications with standard deviation. The alphabets represent the significant/non-significant difference at $p \leq 0.05$.

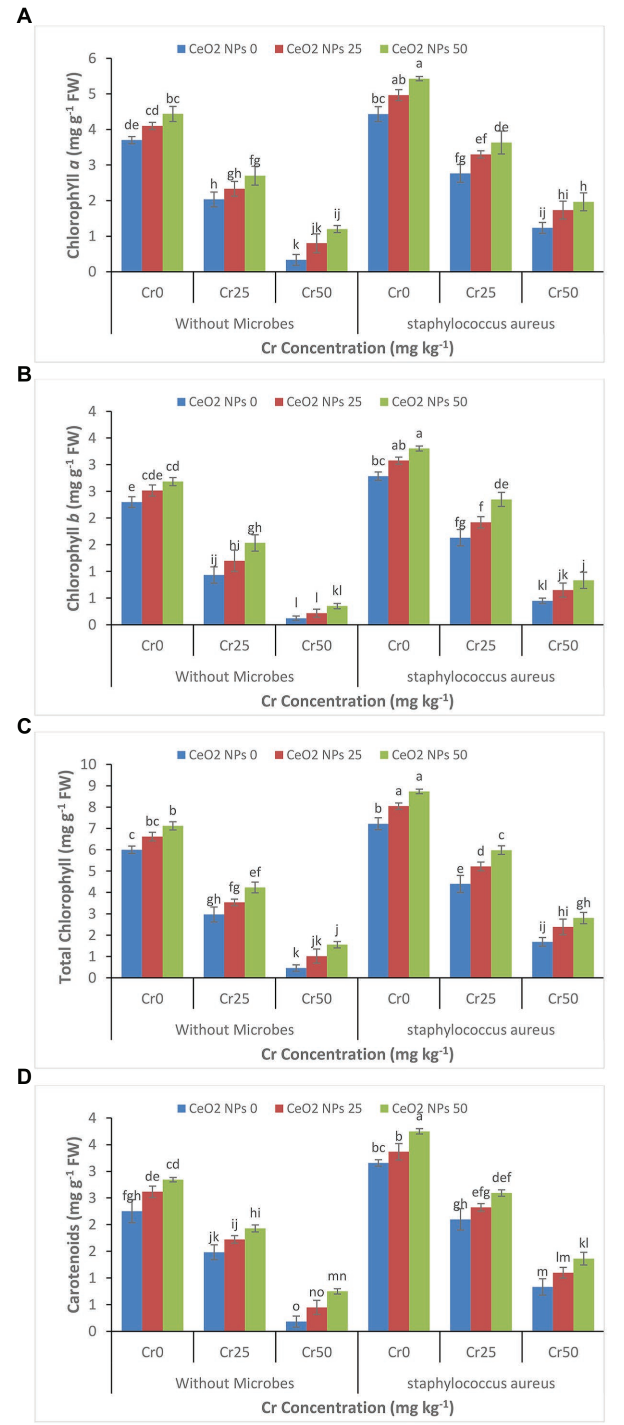

FIGURE 3 | (A–D): Effect of CeO_2 NPs and *Staphylococcus aureus* on chlorophyll *a* **(A)**, chlorophyll *b* **(B)**, total chlorophyll **(C)**, and carotenoid content **(D)** in sunflower plant under Cr stress. The given values are means of three replications with standard deviation. The alphabets represent the significant/non-significant difference at $p \leq 0.05$.

the application of CeO_2 NPs alleviated the adverse impacts of chromium and improved plant agronomic characteristics (**Figures 1A–F**). Similar findings were reported by Gohari et al. (2020), where TiO_2 NPs improved the agronomic traits in *Moldavian balm* under stress (Gohari et al., 2020). The large surface area and small size of the nanoparticles allow

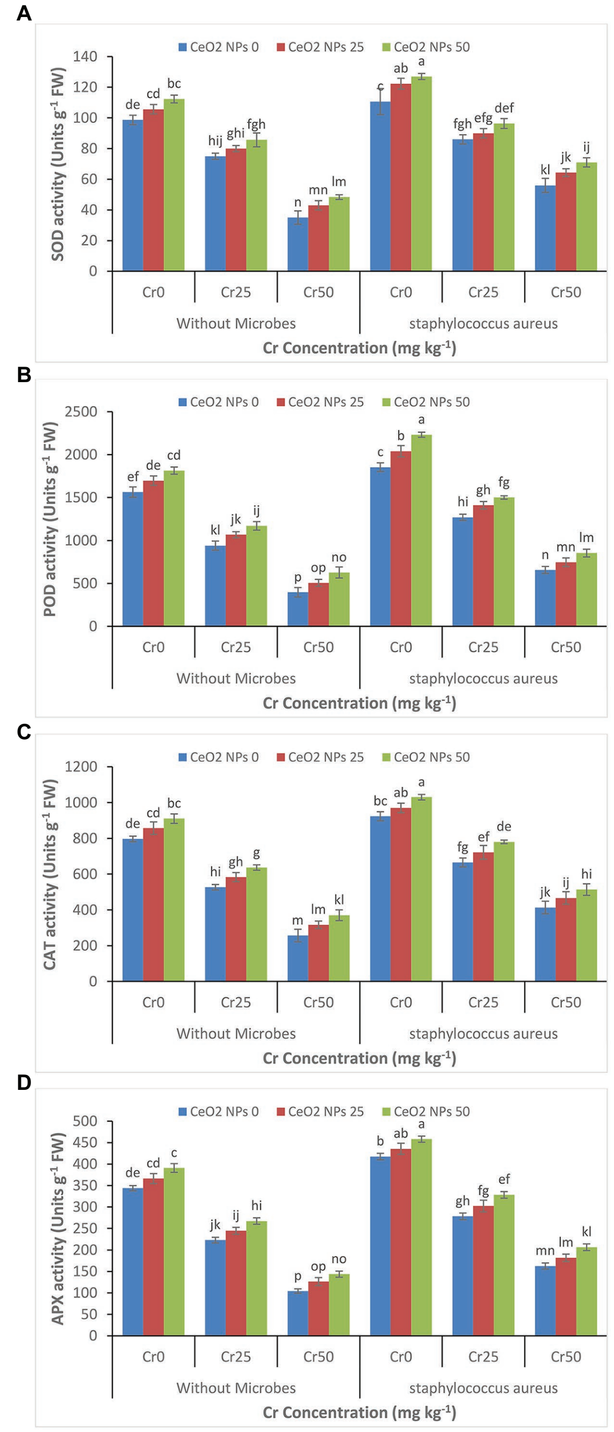

FIGURE 4 | (A–D): Effect of CeO_2 NPs and *Staphylococcus aureus* on SOD activity **(A)**, peroxidases activity **(B)**, catalase activity **(C)**, and ascorbate oxidase activity **(D)** in sunflower plant under Cr stress. The given values are means of three replications with standard deviation. The alphabets represent the significant/non-significant difference at $p \leq 0.05$.

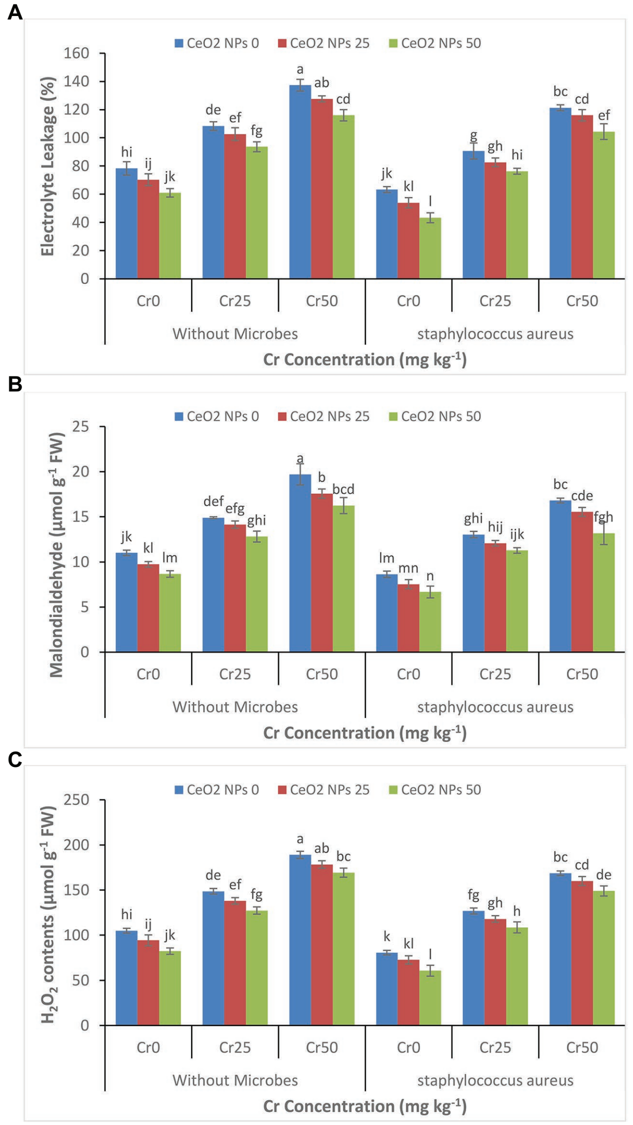

FIGURE 5 | (A–C): Effect of CeO_2 NPs and *Staphylococcus aureus* on electrolyte leakage **(A)**, malondialdehyde contents **(B)** and H_2O_2 contents in sunflower plant under Cr stress. The given values are means of three replications with standard deviation. The alphabets represent the significant/non-significant difference at $p \leq 0.05$.

nanoparticles to penetrate in plant cells and then alleviate the negative effect of metal stress by improving plant growth (Singh et al., 2019). This increase in plant growth might be attributed by increases in water use efficiency, transpiration rate, stomatal conductance, and photosynthetic rate (**Figures 2A–D**; Hezaveh et al., 2019). The combined application of *S. aureus* and CeO_2 NPs further improved the plant morphological and agronomic parameters as compared to the only application of NPs. It is well established that bacteria can increase essential nutrient availability to the plant (Ren et al., 2019). The bacteria also can degrade and transform the metals into less toxic forms and decrease the stress on the plant due to metal toxicity. Similarly, in this study, the application of *S. aureus* improved plant growth and biomass production due to its ability to detoxify the high concentration of chromium. The bacteria can improve plant nutrition through

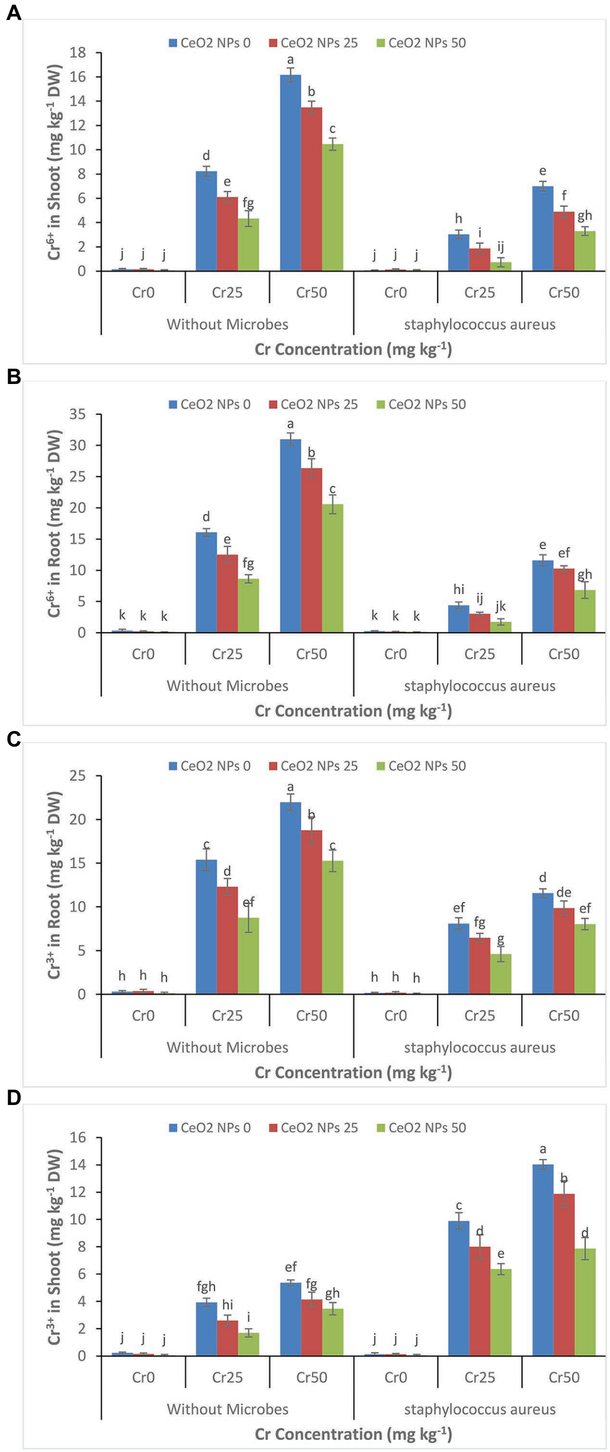

FIGURE 6 | (A–D): Effect of CeO_2 NPs and *Staphylococcus aureus* on bioaccumulation of Cr^{+6} in the shoot **(A)**, Cr^{+6} in roots **(B)**, Cr^{+3} in the shoot **(C)**, and Cr^{+3} in roots **(D)** of sunflower plant under Cr stress. The given values are means of three replications with standard deviation. The alphabets represent the significant/non-significant difference at $p \leq 0.05$.

phosphorus solubilization, nitrogen fixation, and secretion of hormones and enzymes essential for plant growth and ultimately enhance the phytoremediation potential of plants (Marciano Marra et al., 2012; Matse et al., 2020).

The increased metal stress in various plant species resulted in a decrease in chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid content, ultimately disrupting of whole photosynthesis process (Chandra and Kang, 2016; Sytar et al., 2019). The reduction in photosynthesis under metal stress is considered a sign of oxidative stress due to the rapid decrease in photosynthetic pigments and reduction in light absorbance capacity (Ashfaque et al., 2017). It was evident that the application of CeO_2 NP improved the chlorophyll *a*, chlorophyll *b,* and total carotenoid content in plants under chromium stress and reduced the oxidative stress (**Figures 3A–D**). The beneficial role of many nanoparticles in enhancing the chlorophyll contents and improving the plant photosynthetic activity has been reported by many researchers. Such as application of CeO_2 NPs acted as a catalyst in the production of *Chl a* and *Chl b* contents and increased the production of carotenoid contents in plant (Kataria et al., 2019; Etesami et al., 2021). Similarly, the foliar application of cerium oxide nanoparticles improved the photosynthesis pigment in *Calendula officinalis* (Jahani et al., 2019). The decline in antioxidant enzyme activity plays a crucial role in producing oxidative stress in plants suffering from chromium stress (Etesami, 2018). The significant increase in the chlorophyll contents and photosynthesis activity due to combined application of *S. aureus* and CeO_2 NPs might be attributed to the ability of bacteria to increase the availability of micronutrients, decrease in bioavailability of chromium, and increase the ability of the plant to tolerate metal stress (Rizwan et al., 2019; Etesami et al., 2021). Similar to our findings, the application of bacteria improved the chlorophyll *a*, chlorophyll *b*, and carotenoid contents in the sunflower plant under Pb stress (Saleem et al., 2018). In another study, the TiO_2 NPs improved the photosynthetic activity in soybean plants under Cd stress by entering the chloroplast and enhancing light adaption and electron transfer (Singh and Lee, 2016).

The increasing concentration of chromium declined the enzyme activity in the plants (**Figures 4A–D**). At the highest concentration of Cr50, the SOD activity, POD activity, CAT activity, and APX activity reduced sharply due to damage to the antioxidant enzymes metabolism induced by chromium toxicity. It is well reported that metal toxicity decreases the catalytic activities in the plant which ultimately decrease the activity of the antioxidant enzymes (Bhaduri and Fulekar, 2012). The production of ROS attacks antioxidants enzymes, consumes a huge concentration of antioxidant enzymes, and generates oxidative stress by disturbing the redox homeostasis in plants (He et al., 2017; Ahmad et al., 2019). This oxidative stress can cause severe damage to specific proteins and destruction to the cell structure and function (Ahmad et al., 2019). The application of nanoparticles has been reported to improve SOD, CAT, and POD activity without producing the hydroxyl radicals and improving the plants' overall growth.

The chromium stress in plants leads to the production and accumulation of toxic substances such as reactive oxygen species (ROS) that initiate the lipid peroxidation and damage to cell structure (**Figures 5A–C**; Ghori et al., 2019; Jahani et al., 2019). The production of ROS initiates the death of plant cells by damaging specific proteins, lipids, and nucleic acids and decreases the chlorophyll contents (Sytar et al., 2019). This damage can be identified by increased production and accumulation of MDA, and increased concentration of electrolytes leakage (EL) and high content of H_2O_2 (Mallhi et al., 2019). Under metals stress, the concentrations of MDA, EL, and H_2O_2 increased in the plants (Berni et al., 2019; Ghori et al., 2019). The nanoparticle has been found to reduce ROS production in plants under metal stress. TiO_2 NPs and Fe NPs have been found effective in reducing the abiotic stress in plants by reducing ROS production in *Zea mays* and grape, respectively (Mozafari and Ghaderi, 2018; Singh et al., 2021). In the current study, the increased production of antioxidants enzymes due to the positive effect of CeO_2 NPs ultimately decreased the production of EL, MDA, and H_2O_2 contents in plants under chromium stress. In the plants, ROS production is initiated in response to stress, and antioxidants act as a defense mechanism against stress condition and act as a front line to cope with and protect the plant cell from free radicals and minimize the damage initiated by ROS in response to stress (Kohli et al., 2017). Antioxidant enzymes scavenge the H_2O_2 contents APX and SOD cope with ROS and neutralize the superoxidase radicals. Our study showed that CeO_2 NPs increased the production of antioxidants enzymes, which resulted in decreased activity of EL, MDA, and H_2O_2 and alleviated the metal stress in the plant. Our findings agree with the aforementioned studies where the application of nanoparticles enhanced antioxidant enzyme activities in response to oxidative stress (Hussain et al., 2019b; Sun et al., 2019). The increased level of antioxidant enzyme activity might be linked to the antioxidant ability of nanoparticles, which enhances the plants' activities (Jahani et al., 2019). It was found that the increasing concentration of Ag NPs increased the catalase and peroxidase activity in the *Lycopersicon esculentum* (Karami Mehrian et al., 2016). Similarly, wheat plants treated with silver and gold NPs significantly improved the plant dry biomass and improved the SOD, CAT, and APX activity in plants under abiotic stress (Manaf et al., 2021). The increase in antioxidant enzymes activity and decrease in ROS contents with the application of *S. aureus* may be attributed to bacteria's ability to increase mRNA/gene expression of antioxidants in inoculated plants compared to non-inoculated plants (Gururani et al., 2013; Khan et al., 2022). The inoculation of bacteria enhanced the expression level of various ROS scavenging enzymes and increased the proline contents in potatoes plants under stress (Gururani et al., 2013).

The application of CeO_2 nanoparticles reduces the uptake and accumulation of Cr^{+6} and Cr^{+3} in the sunflower root and shoots despite increasing chromium concentration (**Figures 6A–D**). Many studies reported that exogenous application of nanoparticles could reduce the uptake of toxic metals by plants (Hussain et al., 2019b; Rizwan et al., 2019). The use of TiO_2 NPs in rice plants grown hydroponically reduced the concentration of Pb in roots and shoots and improved plant growth (Cai et al., 2017). Similarly, ZnO NPs decreased the arsenic concentration in the rice plat's roots and leaves suffering from arsenic stress (Wu et al., 2020). It is well reported that nanoparticles can absorb and transform the heavy metals in the soils by reducing their mobility and bioavailability such as Fe_3O_4 NPs reduced the mobility of Cd in the soil and reduced its bioavailability to the plants (Chen et al., 2018; Hussain et al., 2019a). Thus, the decreased accumulation of chromium in the sunflower plant can be attributed to the ability of the nanoparticle to decrease the bioavailability of chromium. Further, most of the NPs accumulate in the cell wall, bind with the heavy metals, make them unavailable, and hinder the migration of heavy metals in the plant (Molnár et al., 2020). The bacteria can tolerate metal toxicity and break down and remove the heavy metal through their metabolic process. Bacteria can reduce the bioavailability of heavy metals through biosorption, bioaccumulation, biotransformation, bio-precipitation, and bio-crystallization in contaminated soil (Medfu Tarekegn et al., 2020). In this study, the application of bacteria transformed the Cr^{+6} into Cr^{+3} through their metabolic process. It reduced the bioavailability of the chromium to the plant, which ultimately reduced its bioaccumulation in plant roots and shoots. Similarly, the application of *S. aureus* in wheat plants reduced the uptake and accumulation of chromium in plant roots and shoots grown in chromium-contaminated soil (Zeng et al., 2020). The nanoparticles can improve bacterial growth when applied in small amounts; for example, the use of TiO_2 NPs improved the performance of plant growth-promoting rhizospheric bacteria in plants under stress (Timmusk et al., 2018).

CONCLUSION

The application of nanoparticles is a promising approach that has the tremendous potential to protect the plant from metal-induced stress. The CeO_2 NPs improved the morphological, physiological, and biochemical properties and overall growth and biomass production of sunflower plants grown in the high chromium concentration. The CeO_2 NPs reduce the H_2O_2 stress, enhance the antioxidant enzymatic activities, and ultimately alleviate plants' oxidative stress due to chromium toxicity. Along with nanoparticles, bacteria also have a prominent role in alleviating the metal-induced toxicity through their metabolic process and reducing the bioavailability of Cr to the plant. The combined application of *S. aureus* and CeO_2 nanoparticles improved the metabolic process, triggered the activation of the enzymatic defense system, and thus enhanced the plant performance in sunflower plants under chromium stress. The use of nanoparticles and bacteria in combination could be a novel way to clean up contaminated soil and strengthen plants to withstand metal-induced stress. The novel application of nanoparticles in agriculture may aid in meeting rising food demand while also ensuring environmental sustainability. However, there is still needed to further explore about action mechanism of nanoparticles, permissible limit and ecotoxicity in edible crops.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

JM: conceptualization and project administration. HA: data curtain and investigation. MO: funding and resources allocation. YA: formal analysis and software. FC: methodology and data curtain. MA: formal analysis and project administration. AH: review and editing and formal analysis. SH: investigation and formal analysis. MS: writing an original draft, methodology, and review and editing. All authors contributed to the article and approved the submitted version.

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REVIEW
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Understanding the Phytoremediation Mechanisms of Potentially Toxic Elements: A Proteomic Overview of Recent Advances

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Potentially toxic elements (PTEs) such as cadmium (Cd), lead (Pb), chromium (Cr), and arsenic (As), polluting the environment, pose a significant risk and cause a wide array of adverse changes in plant physiology. Above threshold accumulation of PTEs is alarming which makes them prone to ascend along the food chain, making their environmental prevention a critical intervention. On a global scale, current initiatives to remove the PTEs are costly and might lead to more pollution. An emerging technology that may help in the removal of PTEs is phytoremediation. Compared to traditional methods, phytoremediation is eco-friendly and less expensive. While many studies have reported several plants with high PTEs tolerance, uptake, and then storage capacity in their roots, stem, and leaves. However, the wide application of such a promising strategy still needs to be achieved, partly due to a poor understanding of the molecular mechanism at the proteome level controlling the phytoremediation process to optimize the plant's performance. The present study aims to discuss the detailed mechanism and proteomic response, which play pivotal roles in the uptake of PTEs from the environment into the plant's body, then scavenge/detoxify, and finally bioaccumulate the PTEs in different plant organs. In this review, the following aspects are highlighted as: (i) PTE's stress and phytoremediation strategies adopted by plants and (ii) PTEs induced expressional changes in the plant proteome more specifically with arsenic, cadmium, copper, chromium, mercury, and lead with models describing the metal uptake and plant proteome response. Recently, interest in the comparative proteomics study of plants exposed to PTEs toxicity results in appreciable progress in this area. This article overviews the proteomics approach to elucidate the mechanisms underlying plant's PTEs tolerance and bioaccumulation for optimized phytoremediation of polluted environments.

Keywords: plants, proteomics, phytoremediation, toxic metals, pollution

INTRODUCTION

The continued accumulation of potentially toxic elements (PTEs), including cadmium (Cd), lead (Pb), chromium (Cr) and arsenic (As), copper (Cu), mercury (Hg), nickel (Ni), and selenium (Se) in the environment poses a significant danger to human health and undermines global environmental sustainability efforts (Habiba et al., 2019; Rizvi et al., 2019, 2020; Alsafran et al., 2021). Anthropogenic activities due to rapid industrialization, especially from oil and gas producing industries, infrastructural development, mining, foundries, smelters, coal-burning power plants, and agricultural activities, are significant contributors that enhance the elements of hazardous pollutants in the soil (Al-Thani and Yasseen, 2020; Yan et al., 2020; Usman et al., 2020b). While this is the case, bioaccumulation strategies and tolerance to higher concentrations of PTEs, thus sequestering of PTEs can be varied among different plant species as they faced diverse pollutant sources and other environmental conditions. Generally, PTEs sequestration mechanisms in plant tissues include exclusion, stabilization, removal, and transfer to the various parts such as roots, shoots, and stems. Of these, the removal and translocation of the elements to plant's aerial parts, the process also known as "phytoextraction," are known as the most efficient remediation strategy (Saleem et al., 2020a). Phytoextraction is inexpensive, the amount of waste material that must be disposed of is substantially decreased (up to 95%), and the disposal of hazardous material or biomass is not required (Usman et al., 2020b). Plant species demonstrating the capacity to remove and transfer PTEs to their aerials parts are categorized as metal hyperaccumulators (De Bellis and Aprile, 2020; Zhang et al., 2020; Usman et al., 2020b).

The PTEs are non-biodegradable and prone to ascend along food chains, making their environmental prevention a critical intervention (Wuana and Okieimen, 2011; Sharma and Pandey, 2014). Given the potential adverse effects of many remediation strategies, alternative technologies, including phytoremediation—the use of plants to remove PTEs from contaminated environments, are being explored for large-scale applications (Usman et al., 2019b). Phytoremediation is the direct use of living green plants and is an effective, cheap, non-invasive, and environmentally friendly technique used to transfer or stabilize all the toxic metals and environmental pollutants in polluted soil or ground water (Mosa et al., 2016). Phytoremediation is widely applicable for metal contaminated areas, with some long-term esthetic merits and it is famous due to its low cost and eco-friendly nature, so it is used on large-scale areas with high contents of toxics metals (Rascio and Navari-Izzo, 2011). Plants are sessile organisms, and therefore, could not escape from exposure to high concentrations of PTEs (Wiszniewska, 2021). However, several plant species (~450) are known to accumulate high concentrations of various PTEs (Rascio and Navari-Izzo, 2011). PTEs mainly enter plant systems from soil or water *via* passive or active transport. Following uptake that is facilitated by membrane-embedded ion channels, elemental ions translocate to aerial parts of plants (i.e., the stem and leaves) *via* xylem channels. In general, plants capable of accumulating PTEs in their tissues majorly bio-concentrate the elements in the root, followed by the stem, leaves, and in some species, even the seeds (Shamim, 2018; Dinu et al., 2020).

Biotechnologically, three main strategies are embarked upon to improve PTEs phytoextraction using different plants species: (i) utilizing the metal/metalloid transporters, (ii) enhancing metal/metalloid ligand production, and (iii) conversion of metal/metalloid into volatile and less detrimental forms (Mosa et al., 2016). The toxicity of PTEs primarily depends on various factors such as concentrations and chemical properties of toxic elements, their bioavailability, and plants' developmental stage. When exposed to PTEs, plant's basal tolerance mechanism becomes activated and enables them to cope with the stress (Gill et al., 2021). However, at elevated concentrations, these elements suppress the plant defense machinery and cause harmful effects to physiological processes, including photosynthesis, transpiration, and energy metabolism, thus reducing overall plant growth and development (Kumar et al., 2018; Gautam et al., 2020; Ahmad et al., 2020a; Usman et al., 2020a). Generally, PTEs stress symptoms on plants can be measured as it is similar to that of deficiency in essential nutrients that may be appeared in the forms of leaf necrosis, poor root development, and decreased fresh biomass (Usman et al., 2019a; Singh and Fulzele, 2021).

Recently, the "Omics" approaches emerge as valuable tools for understanding the changes in molecular mechanisms of plant's response to the PTEs during phytoremediation (Meena et al., 2017; Raza et al., 2021). The traditional characterization methods relating to physiological and biochemical assays seem insufficient, and therefore, further investigation especially on the response of whole-genome proteome to PTE can be a promising approach to coping with the potential threats posed by PTEs (Xie et al., 2019; Kosakivska et al., 2021). These changes are not only limited to the expression pattern but also protein quality and quantity. Transcriptomic approaches are used to target transcriptional changes at the mRNA level (i.e., changes in gene expression), which may differ from changes at the protein level (i.e., translational modifications). In a true sense, the mRNA/protein ratio is a factor of mRNA transcription rate and protein stability (Reimegård et al., 2021).

To alleviate PTEs stress and restore cellular homeostasis, plants develop antioxidative capacity, sophisticated and highly efficient regulatory mechanisms to help tolerate the uptake, accumulation, translocation, and eventual detoxification (El-Amier et al., 2019; Alsahli et al., 2020; Ahmad et al., 2020b; Bhat et al., 2021). To achieve this, the living system's functional molecules, the proteins, particularly metal chelators, transporters, and chaperones, play crucial roles in alleviating the negative impact of PTEs stress (Saleem et al., 2020b). Together, these proteins enable plants to tolerate PTEs, detoxify PTEs polluted environments and their system through binding, transport, and vacuolar sequestration (Peco et al., 2020; Dhir, 2021; Jogawat et al., 2021).

Proteins are crucial to regulating the cellular processes of plants; proteomics, comprising cellular protein roles, quantification, identification, the pattern of expression, modification, and interactions, all together provides an excellent strategy to assess stress impact on them. Because of the central roles of proteins, researchers in this area need to prioritize studies focusing on proteomics to gain further insights into the mechanisms of PTEs tolerance and detoxification in plants to improve the efficiency of PT Es removal from contaminated soil or medium.

Recent progress in plant proteomics could be possible due to new technological advancements in protein separation, quantification, mass spectrometry (MS), and bioinformatics. Mass spectroscopy is central to large-scale proteome analysis that enhances the resolution, sensitivity, and accuracy of proteins mass prediction (Cassidy et al., 2021). Due to these and the speed of analysis for large protein samples through released peptides after proteolytic digestion (bottom-up), shotgun proteomics is used to describe the process (Gutsch et al., 2019b). On the other hand, protein is partially digested to characterize co-existing post-translational modifications (PTMs; Sidoli et al., 2017). Following fractionation and tandem mass spectrometry (MS/MS) analysis, the bottom-up process indirectly measures proteins through tryptic digested peptides having amino acids approximately between 8 and 30 ($8 > aa > 30$). Proteins are inferred through identified peptides compared to MS/MS spectra previously generated from in-silico fragmented peptides in a protein database. **Figure 1** shows a schematic representation of typical steps in PTEs phytoremediation studies involving the use of shotgun proteomics.

In contrast to the bottom-up approach (analysis of digested proteins), the proteomics of characterizing intact proteins is another strategy termed "top-down" (**Figure 2**). Proteomic research has made significant progress, especially on model plants, *Oryza sativa*, and *Arabidopsis thaliana*. Essential proteins, such as metal ion transporters, binding proteins, phytochelatins (PCs), and metallothioneins (MTs), are notable in aiding PTEs sequestration in plants. PCs are induced by phytochelatins synthase (PCS), which is triggered when metal ions are present. PCs (oligomers of glutathione) bind to toxic metals to form a significant part of the detoxification mechanism, while MTs are gene-encoded, small, and cysteine-rich proteins (Jorrin-Novo et al., 2019; Usman et al., 2020b).

Due to the exponential increase in the number of studies and publications in the proteomics of plant abiotic stress, including PTEs, it is almost impossible to provide an extensive summary in one review. For further references within the last decade, readers are referred to some of the recent reviews (Ahsan et al., 2009; Hossain and Komatsu, 2013; Cvjetko et al., 2014; Kosová et al., 2018; Raza et al., 2020; Kosakivska et al., 2021; Sytar et al., 2021). This review aims to provide a non-exhaustive overview of plant proteomics and highlights its importance in understanding PTEs tolerance, uptake, and detoxification mechanisms in plants during phytoremediation when grown in metal contaminated soil. To the best of our knowledge, this review is among the few articles focused on the plant proteomics of trace and heavy metals.

PHYTOREMEDIATION

A combinatorial strategy involved the physiological and chemical properties, and biological processes adopted by plant species

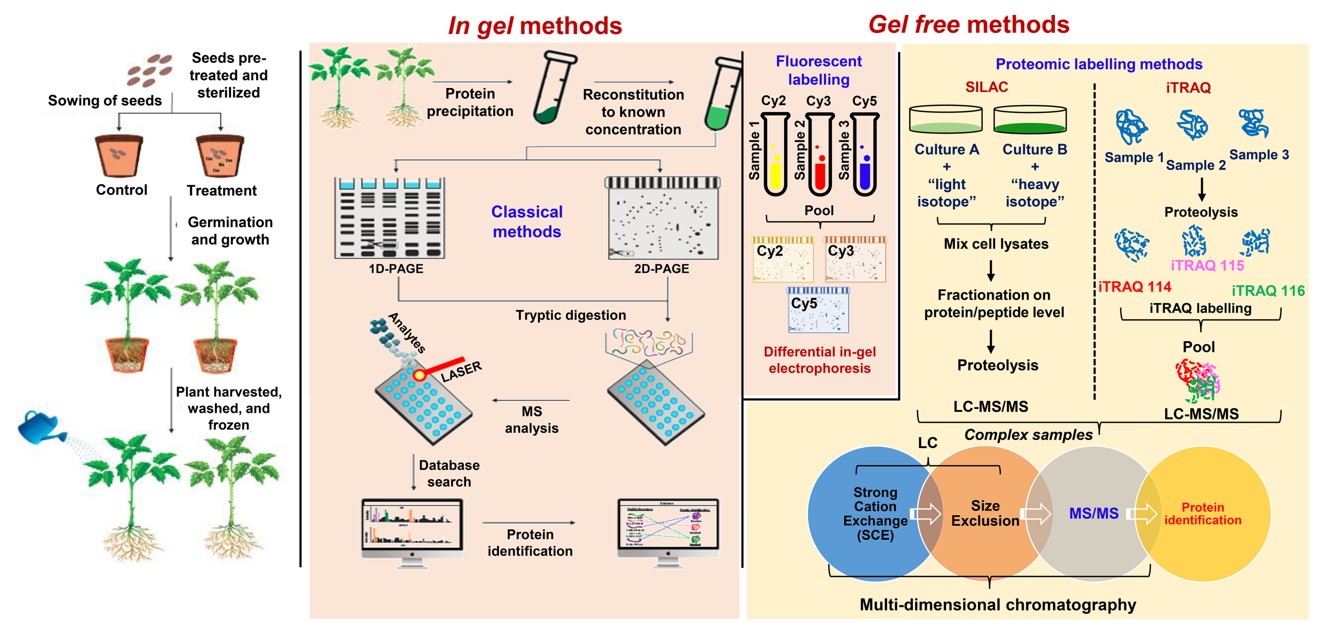

FIGURE 1 | A workflow illustrating the comparative proteomic methods that have been used to investigate the phytoremediation of potentially toxic elements. Classical in-gel proteomic methods include 1-D and 2-D polyacrylamide gel electrophoresis (PAGE) sometimes further developed by differential in-gel electrophoresis (DIGE) using fluorescent tags like cyanine 2 or 3 or 5 (Cy2, Cy3, and Cy5). Gel-free methods are advanced and used to overcome limitations in-gel proteomics and to study the heavy metal detoxification and phytoremediation mechanisms. These include novel gel-free methods with protein labeling such as Stable Isotope Labeling with Amino acids in Cell culture (SILAC) and Isobaric Tags for Relative and Absolute Quantitation (iTRAQ) techniques followed by multi-dimensional chromatography (MupPit).

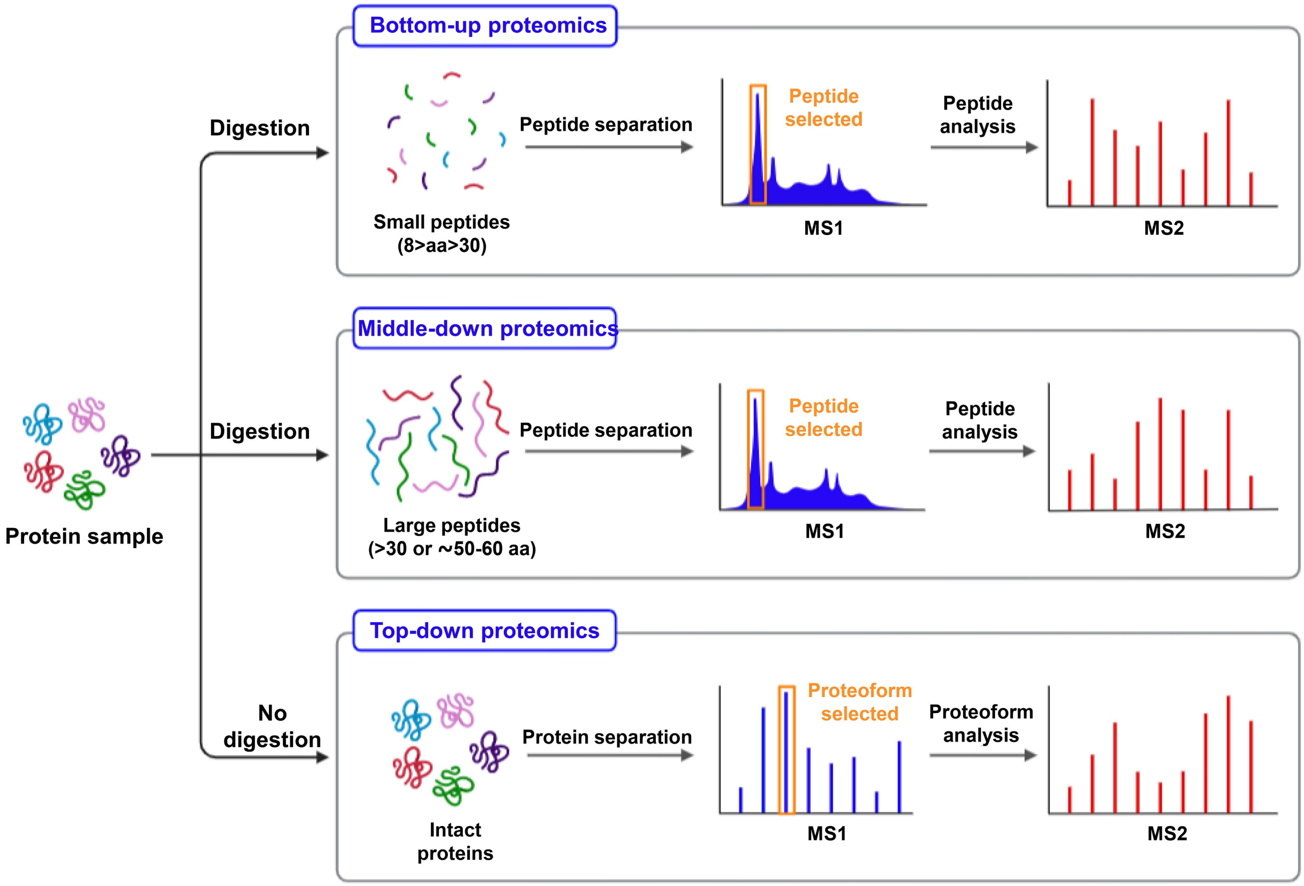

FIGURE 2 | A proposed workflow for protein digestion and MS analysis for the investigation of large (intact proteins), medium (peptides >30 aa), and small size (8–30 aa) protein molecules.

to clean up environmental pollutants (Baldwin et al., 2015; Hasegawa et al., 2016). Physical and chemical methods have several limitations such as non-economical, alterations in native soil flora, changes in the physicochemical properties of the soil, and need intensive labor (Shankar, 2017). PTEs are essentially immutable by any chemical or physical process short of nuclear fission and fusion, and thus, their remediation presents special scientific and technical problems. Because of this, new approaches for better treatment of PTEs polluted environment are essential. In this regard, the use of biological treatment strategies could be adopted that are cheaper and environmentally friendly. The promising one is phytoremediation which has gained increased attention in recent years since it is the most viable alternative. Phytoremediation takes advantage of plant ability to tolerate, accumulate, and translocate PTEs across their aerial tissues (Ludvíková and Griga, 2019). Phytoremediation is often referred to as "green remediation" or "botanical bioremediation" involving the use of plants to remove, transfer, or stabilize the PTEs (**Figure 3**) to clean up the environment and render the pollutants harmless (Suman et al., 2018; Adiloğlu et al., 2021). Moreover, this mechanism is a species-specific, effective, economical, eco-friendly, and scientifically accepted method. Generally, when there is an encounter with PTEs, plants activate their defense machinery by adopting one or several mechanisms simultaneously to safeguard themselves from unwanted physiological or molecular alterations induced by PTEs. Some of the most studied and common strategies are presented in **Table 1**.

Phytoremediation has a great potential for providing much-needed green technology. During phytoremediation, the plant's selection for the remediation strategy to neutralize PTEs may differ; the strategies used could be removal, transfer, degradation, immobilization, etc. (Hasanuzzaman et al., 2018). While hundreds of plant species have been identified as potential phytoremediators, identifying suitable hyperaccumulators is still a challenge (de Castro Ribeiro et al., 2018). Previously, Yıldız and Terzi (2016) studied *Brassica napus* under Cr stress and noticed through 58 proteins spots in two-dimensional electrophoresis (2-DE) that 58 proteins were differentially regulated by Cr (VI) stress (+S/+Cr), S-deficiency (−S/−Cr), and combined stress (−S/+Cr). The translocation capacity of plants (the ability to take up and accumulate toxic metal from the root to shoot parts) is a critical parameter considered in evaluating phytoremediation potential in plants (Meng et al., 2017). A translocation factor of 1 or more suggests a plant's ability

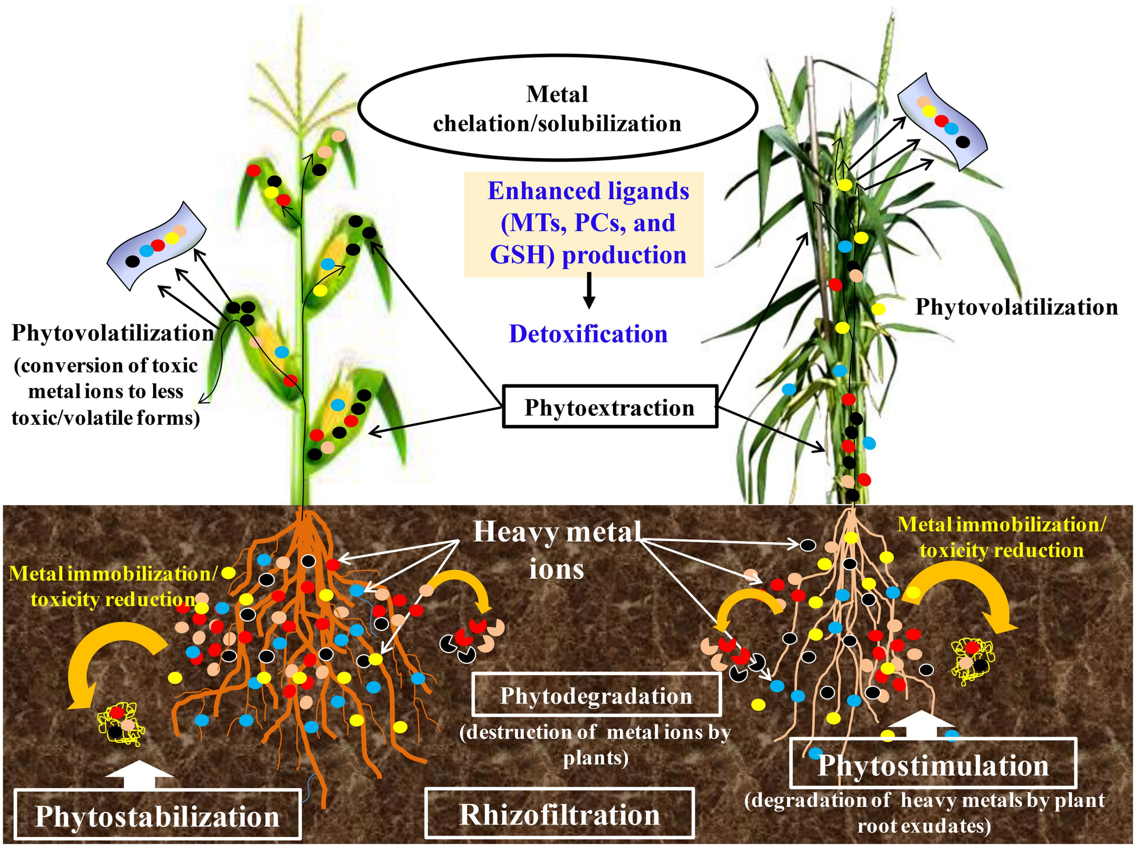

FIGURE 3 | An illustration on the phytoremediation strategies and general response of plants to potentially toxic elements stress.

TABLE 1 | Phytoremediation strategies adopted in response to PTEs.

S. No.	Strategies	Description	Crop	Family	References
1.	Phytoextraction	A low-cost technique by which PTEs are removed or concentrated by plants in different parts. This process produces plant biomass having PTEs that can be transported for disposal or recycling	*Calotropis procera*	Dogbanes	Singh and Fulzele, 2021
2.	Phytodegradation or Rhizodegradation	PTEs are degraded by proteins or enzymes produced by plants and associated microbes	*Phragmites australis*	Grasses	He et al., 2017
3.	Rhizofiltration	PTEs are absorbed by plant roots	*Carex pendula*	Sedges	Yadav et al., 2011
4.	Phytostabilization	PTEs are immobilized, and thus their bioavailability is reduced	*Juncus effusus*	Rushes	Najeeb et al., 2017
5.	Phytovolatization	Volatilization of PTEs by plants extracted from soils into the atmosphere	*Pteris vittata*	Brake	Sakakibara et al., 2010

to transfer metals to its aerial parts adequately. Various methods covered under phytoremediation strategies are discussed briefly in the following sections.

Phytoremediation Strategies

Plants employ different strategies during phytoremediation. The type of elements, their chemical properties, and bioavailability plays a crucial role in achieving PTEs remediation success (Usman et al., 2020b). The different phytoremediation strategies include phytoextraction, phytofiltration or Rhizofiltration, phytovolatilization, phytostabilization, and phytodegradation (**Figure 3**; Usman et al., 2018; Wei et al., 2021). Phytoextraction involves the use of plants to remove PTEs or organics from the soil by concentrating them in the harvestable parts (Kumar et al., 2017; Ali et al., 2018). PTEs accumulating plants are utilized to transport and concentrate contaminants (metals or organics) from the soil into the above-ground plant parts as shown in the example by Viana et al. (2021). Phytoextraction involves PTEs removal, accumulation, and translocation to plant's aerial parts (Viana et al., 2021). Often, phytosequestration, photoabsorption, and phytoaccumulation are used to refer to the same process. Several studies have reported plants demonstrating PTEs phytoextraction capacity. Phytoextraction is preferred over other techniques because toxic elements can be harvested from plant shoots in an extractable form (Jeyasundar et al., 2021). Several studies have reported different plants with varying capacities in the phytoextraction of PTEs. Examples are the Indian mustard, rapeseed, and sunflower plants (Shaheen and Rinklebe, 2015; Chowdhary et al., 2018; Surucu et al., 2020).

Phytofiltration or Rhizofiltration

Phytofiltration or Rhizofiltration involves the adsorption of PTEs *via* the root. It is a process most seen in aquatic plants (Meitei and Prasad, 2021). In rhizofiltration, plant roots are used to absorb and adsorb pollutants, mainly metals, from contaminated soils and aqueous waste streams. It is the removal of pollutants from metal-polluted soil/waters by precipitation, absorption, and accumulation into plant biomass (Mahajan and Kaushal, 2018). Phytofiltration is essential because it prevents toxic elements transmission to different environmental components, including underground water (da Conceição Gomes et al., 2016; Meitei and Prasad, 2021). However, phytofiltration is also demonstrated by terrestrial species, where metals are remediated with microbial bio-filter aid in the rhizosphere region (Wei et al., 2020). Previously, studies conducted on rhizofiltration by Yadav et al. (2011) in *Carex pendula* in Pb contaminated wastewater soil noticed that *C. pendula* accumulate a large amount of Pb in their roots and can be used to clean up the Pb contaminated environment in combination with proper biomass disposal alternatives.

Phytostabilization

Plants can reduce PTEs toxicity by converting them to a different form or changing their bioavailability. Thus, the bioavailability of PTEs in the environment is reduced using plant systems. Plants stabilize PTEs in soils, thus rendering them harmless, thereby reducing the risk of further environmental degradation by leaching of PTEs into the groundwater or by airborne spread. This is achieved by preventing surface runoff, erosion, and leaching (Yan et al., 2020). Phytostabilization is vital because it helps prevent PTEs transmission into the food chain. The element's chemical properties are some of the most critical determinants of whether potential plants can stabilize them (Hamidpour et al., 2020; Usman et al., 2020b). Although phytostabilization offers some advantages, it has limited use because metals are only temporarily immobilized and restricted, and therefore, unpopular compared to phytoextraction (Radziemska, 2018). It is commonly employed in emergencies for quick metal immobilization in plants' rhizosphere (Meng et al., 2017).

Phytotransformation or Phytodegradation

It is like phytostabilization, but pollutants are metabolically transformed into inactive forms (Bezie et al., 2021). The plant metabolic system employs the surrounding enzyme activities with the assistance of rhizosphere bacteria to reduce metal elements toxicity. Compared to other forms, phytotransformation is labor-intensive, often requires soil amendments, and is less reliable (Mishra et al., 2020; Bezie et al., 2021). Phytodegradation is commonly applicable against organic pollutants. However, it is less effective and rarely used, especially against inorganic contaminants, including PTEs.

Phytovolatilization

It involves converting metal contaminants into a gaseous form that is eventually released into the atmosphere (Aweng et al., 2018). In this process, PTEs are only transferred to other parts of the environment and could still be redeposited into the soil following precipitation. For this reason, phytovolatilization is less popular, especially compared to phytoextraction and phytofiltration (Nikolić and Stevović, 2015; Bisht et al., 2020).

THE MECHANISMS OF PTEs TOLERANCE AND BIOACCUMULATION

Plants with the enhanced potential of taking up PTEs and translocating them to their aerial parts are identified as metal hyperaccumulators, while those with limited metal translocation are known as non-hyperaccumulators (Maestri et al., 2010). Recently, the interest in proteomics studies of plant hyperaccumulators and their metal sequestration and detoxification mechanisms has increased (Visioli and Marmiroli, 2013; Kumar and Prasad, 2018; Raza et al., 2020). Proteomic studies of PTEs accumulators can make comparisons of differentially expressed proteins (DEPs) between different plant parts (**Table 2**). Many studies suggest that the hyperaccumulators including transporters and chelators showed enhanced DEPs compared to non-accumulating plants (Visioli and Marmiroli, 2013; Paape et al., 2016; Domka et al., 2020). During PTEs phytoremediation, plant tissues play essential roles.

The root is the first tissue to encounter metal stress, and therefore often witnesses dramatic proteomic changes. When comparing the root protein of two accessions, glycosyl hydrolase family 18 differed in abundance, affecting the plant's capacity to uptake metal; the variant that had a higher protein abundance had higher Ni and Cd accumulation (Lai et al., 2020; Raza et al., 2020). The proteome of a variety of plant species was studied, and several proteins that protect plants against various stresses, including oxidative, biotic, and abiotic stress conditions were identified (Fan et al., 2016; Goodin, 2018; Kumar et al., 2018). When comparing *Thlaspi caerulescens* proteomes that had variable tolerance to Cd and Zn, it was determined that the element's higher accumulation was due to the protein photosystem II (Paunov et al., 2018). Proteomic analysis of *Sorghum bicolor* has also shown that a total of 33 DEPs were found when plants were (**Table 2**) exposed to cadmium (Cd) stress (Roy et al., 2016). Examples of such proteins are glutathione S-transferase, ribulose bisphosphate carboxylase small chain, carbonic anhydrase, glyceraldehyde-3-phosphate dehydrogenase, and cytochrome P450, which are well characterized this far in historical literature. The less characterized contenders that were upregulated in *S. bicolor* include pentatricopeptide

TABLE 2 | Examples of PTEs phytoremediation studies involving the use of comparative proteomics from 2015 to date.

PTEs	Plant species	Plant parts	PTEs concentration/ exposure time/media	Technology used	Key findings	References
As	*Artemisia annua* L.	Shoot Root	100 µm $Na_2HAsO_4.7H_2O$/3 days Hoagland nutrient's solution	2-DE PAGE, MALDI-TOF-MS	Upregulation of secondary metabolites-related genes enhances as tolerance. Biomass, carotenoid, flavonoids were enhanced, whereas total chlorophyll pigment was reduced under As treatment.	Kumari and Pandey-Rai, 2018
	Brassica napus	Leaves	200 µmoll−1 $NaAsO_2$/ 7 days/ 50% Hoagland solution	LC–MS/MS, SEM, TOF-MS, qRT-PCR	Photosystem II (PSII) and photosystem I (PSI) proteins were upregulated. Secondary metabolites biosynthesis increased.	Farooq et al., 2021
	Oryza sativa L.	Leaves Root	$NaAsO_2$; 25 µM /7 d/ modified Hewitt's media	2-DE, MALDI-TOF-TOF	The sulfur treatment alleviates As stress by forming disulfide linkage in proteins involved in glycolysis, TCA cycle, energy metabolism, and photosynthesis.	Dixit et al., 2015
	Populus (deltoides cv. "zhonglin 2025" and euramericana cv. 'I-45/51')	Leaves Root	$Na_3AsO_4{\cdot}12H_2O$ 50, 100 µM/21 days/Hoagland's nutrient solution	MALDI-TOF/TOF MS, 2-DE, RT-PCR	Overexpression of photosynthetic and antioxidative responsive proteins in As tolerant cultivar	Liu et al., 2017
Cd	*Arabidopsis thaliana* L.	Leaves, Root	100 µm $CdCl_2$/7-days/1/2 MS solid media	2D-GE, MALDI-TOF/TOF-MS	The natural accession Chernobyl-07 (Che) has a higher Cd tolerance than normal accessions. This accession particularly changed the expression related to ROS protection and energy modulation proteins for obtaining tolerance.	Klimenko et al., 2019
	Brassica campestris	Root	50 µm CdCl2/1-day/ hydroponic	2D-GE, MALDI-TOF/TOF-MS	Hydrogen gas (H_2) and nitric oxide (NO) enhance the antioxidant capabilities of *B. campestris* seedlings in response to Cd toxicity.	Su et al., 2019
	Brassica napus	Xylem sap	10 µm $CdCl_2$/3-days/ hydroponic	LC–MS/MS	Cd stress-induced the overexpression of stress response-related proteins.	Luo and Zhang, 2019
	Medicago sativa	Stem	88.9 µm $CdSO_4$/4-months/ potted soil	2D-GE, MALDI-TOF/TOF-MS	Cd stress caused the differential expression of proteins involved in cell wall remodeling, defense response, carbohydrate metabolism, and promotion of the lignification process.	Gutsch et al., 2019a
	Microsorum pteropus	Leaves, Root	100, 250 and 500 µm $CdCl_2$/7-days/hydroponic	2D-GE, MALDI-TOF/TOF-MS	Different protein expression patterns were observed involving related functions of energy metabolism and antioxidant activity in the root, cellular metabolism, protein metabolism, and photosynthesis in leaves.	Lan et al., 2018
	Sorghum bicolor	Shoot	100 and 150 µm $CdCl_2$/5-days/semi hydroponic	2D-GE, MALDI-TOF/TOF-MS	Cd stress inhibits carbon fixation, ATP production, and the regulation of protein synthesis.	Roy et al., 2016
Cr	*Brassica napus* L.	Leaves	100 µm $K_2Cr_2O_7$/3-days/ hydroponics	2-DE, MALDI-TOF/ TOF MS	Increased abundance of defense-related proteins such as antioxidant enzymes, molecular chaperones involved in scavenging the excess ROS, and refolding of misfolded proteins under Cr stress.	Yıldız and Terzi, 2016
	Callitriche cophocarpa	Shoot	1 mm K_2CrO_4/3-days/liquid MS medium	SDS-PAGE, 2DE, MS/MS	Quinone dehydrogenase, FQR1 (NAD(P)H) newly identified to act as a detoxification protein by protecting the cells against oxidative damage.	Kaszycki et al., 2018
	Nicotiana tabacum	Shoot	100 µm $K_2Cr_2O_7$/5-days/ hydroponic	2D-GE, MALDI-TOF/TOF-MS	Twelve Cr-tolerance-associated proteins were identified. These include mitochondrial processing peptidase, dehydrin, superoxide dismutase, adenine phosphoribosyltransferase, and mitochondrial malate dehydrogenase proteins.	Bukhari et al., 2016
	Pteris alba	Leaves Root	146.7 ~ 261.5 mm Cr/4-years/waste landfill field	2D-GE, Nano HPLC MS/MS	ROS scavenging proteins assist poplar threes long-term adaptation to Cr polluted environments.	Szuba and Lorenc-Plucińska, 2018
Cu	*Agrostis capillaris* L.	Shoot	1–50 µm $CuSO_4$/90-days/ semi hydroponic	2D-GE, LC–MS/ MS	Overexpression of a Heat shock protein 70 (HSP70) may be pivotal for Cu tolerance by protecting protein metabolism.	Hego et al., 2016
	Hyoscyamus albus L.	Root	0, 0.1, 1, 20, and 200 µm $CuSO_4$/7-days/cell culture	MALDI-QIT-TOF-MS	High Cu levels enhanced respiration activity and propagated *H. albus* roots through the activation of the energy supply and anabolism. Increased abundance of proteins involved in carbohydrate metabolism, *de novo* protein synthesis, cell division, and ATP synthesis, and decreased proteasome.	Sako et al., 2016

(Continued)

TABLE 2 | Continued

PTEs	Plant species	Plant parts	PTEs concentration/ exposure time/media	Technology used	Key findings	References
	Triticum aestivum L.	Root Leaves	100 µm $CuSO_4$/3-days/ hydroponic	2D-GE, HPLC-Chip	Cu responsive network of 36 key proteins, most of which may be regulated by abscisic acid (ABA), ethylene, and jasmonic acid (JA). Exogenous JA application showed a protective effect against Cu stress and significantly increased glutathione S-transferase (GST) gene transcripts.	Li et al., 2013
Hg	*Paspalum distichum* L.	Root	1,115 µm Hg/ 60days/ contaminated soil in glass box	LC–MS/MS	Observed changes in the expression patterns of metal binding and transport protein. Increased accumulation of photosynthesis and energy metabolism, related proteins.	Ding et al., 2019
	Triticum aestivum L.	Root Shoot	25, 50, 100, 200 and 400 µm $HgCl_2$/3-days / hydroponic	2D-GE, LC–MS/ MS	49 abscisic acid (ABA) potentially regulated Hg-responsive proteins identified. Exogenous ABA application conferred protection against Hg stress and increased peroxidase enzyme activities, suggesting that it may be an important factor in the Hg signaling pathway.	Kang et al., 2015
Pb	*Cannabis sativa* L.	Leaves	$Pb(NO_3)_2$ 3 g/kg soil /40-days/Potted soil	LC-ESI-MS/MS. SWATH-MS	Adaptation to Pb stress by accelerating adenosine triphosphate (ATP) metabolism; enhancing respiration, light absorption, and light energy transfer; and eliminating reactive oxygen species.	Xia et al., 2019
	Chrysopogon zizanioides	Root Shoot	$Pb(NO_3)_2$ 400 mg/l, 800 mg/l and 1,200 mg/l/10-days/ hydroponic (half strength Hoagland solution)	LC–MS/MS	Increased levels of key metabolites including amino acids, organic acids, and coenzymes in response to Pb.	Pidatala et al., 2018
	Raphanus sativus L.	Root	1,000 mg/ L $Pb(NO_3)_2$/3-days/modified half-strength Hoagland nutrient solution	GC–MS	Pb exposure altered metabolites and divergent expression of enzymes which are responsible for profound biochemical changes, including carbohydrate metabolism, energy metabolism, and glutathione metabolism.	Pang et al., 2015
	Glycine max L.	Nodules	107.8 µm $PbCl_2$ or 1.84 µm $HgCl_2$/ 60-days /potted peat, perlite, and vermiculite (1:1:1)	2D-GE, MALDI-TOF MS/MS	Pb stress increased the abundance of defense, development, and repair-related proteins.	Baig et al., 2018
	Zea mays	Root	18,000 µm Pb (NO3)$_2$/12, 24 and 48 h/semi hydroponic	Nano-LC–MS/MS	Upregulation of stress, redox, signaling, and transport proteins, while proteins related to nucleotide metabolism, amino acid metabolism, RNA, and protein metabolism were down-regulated.	Li et al., 2016
Se	*Allium cepa* L.	Root	10 mg/l Se Na_2SeO_3/10-days/Hoagland's nutrient solution	Cap HPLC-ESI-QTOF-MS and MS/ MS, nano LC-ESI-Q Orbitrap-MS and MS/MS	Different abundances of proteins involved in transcriptional regulation, protein folding/ assembly, cell cycle, energy/carbohydrate metabolism, stress response, and antioxidant defense were identified in response to Se stress.	Karasinski et al., 2017
	Brassica oleracea L.	Florets Leaves	25 µm Na_2SeO_4/14-days/ Hoagland solution	UPLC–MS/MS, qRT-PCR, LC–MS/ MS	Glucosinolate reduction in broccoli leaves and florets is associated with negative effects on precursor amino acids (methionine and phenylalanine), biosynthesis, and glucosinolate-biosynthetic-gene expression in response to Se supplementation.	Tian et al., 2018
	Capsicum annuum L.	Shoot	100 ppm Na_2SeO_4/1-day	LC–MS/MS	Overexpression of heat shock and metabolism proteins. Others are involved in post-translational modification, protein turnover, chaperones, and protein processing in the endoplasmic reticulum.	Zhang et al., 2019
	Oryza sativa L.	Shoot Root	25 µM, $NaAsO_2$ and 25 µm Na_2SeO_3/15-days/Hewitt nutrient medium	MALDI-TOF/TOF, qRT-PCR, Western blot,	Differentially expressed proteins altered the gene expression related to abiotic and biotic stresses and defense responses such as ROS homeostasis, photosynthesis, energy metabolism, and transport and signaling.	Chauhan et al., 2020

repeat-containing protein, Zn finger CCCH domain-containing protein 14, flavonoid 3′,-5′ hydroxylase, aspartate aminotransferase 3 (chloroplastic), protein Brevis radix-like 1, bergaptol O-methyltranferase, and probable F-actin-capping protein subunit beta proteins under Cd stress (Roy et al., 2016). Physiologically, in *S. bicolor* plants, there is the suppression of carbon fixation, ATP production, and protein synthesis regulation in Cd-stressed plants (Roy et al., 2016). In fact, under 500 μm Cd stress, the fern *Microsorum pteropus* is capable of sequestering high amounts of cadmium in roots and dry matter of leaves (up to 4,000 mg/kg), while the water fern Azolla, widely seen in Asian rice fields, does not have the same capacity to phytoaccumulate Cd.

In a study performed on hemp cultivars (Xia et al., 2019), it was found that phytoremediation of Pb impacts the following key pathways: protein synthesis, transcription, transport, signal transduction, photosynthesis, energy metabolism, and protein storage, among other systems. Examples of proteins that are upregulated in Y1 cultivars of hemp include ones that optimize ATP generation using ATP synthase subunit a (P56758 and P56757), ATP synthase protein MI25 (Q04613), ATP synthase protein YMF19 (P93303), nucleoside diphosphate kinase III (O49203), pyruvate kinase (PKE; Q94KE3, Q9FNN1, and Q9FM97), and adenylate kinase 5 (ADK; Q8VYL1; Xia et al., 2019). Therefore, making more chemical energy appears to be a favorable development when exposed to high Pb stress. In particular, the pyruvate kinase that mediates pyruvate production for the Kreb's Cycle is a key protein that is upregulated. In the same cultivar (Y1) under Pb stress, the following proteins were upregulated for signal transduction and transport: Five water transport-related aquaporins (e.g., Q06611, P25818, and others), patellin (Q56ZI2 and Q56Z59), mitochondrial dicarboxylate/tricarboxylate transporter DTC (Q9C5M0), mitochondrial phosphate carrier protein 3 (Q9FMU6), mitochondrial carnitine/acylcarnitine carrier-like protein (Q93XM7), MD-2-related lipid recognition domain-containing protein/ML domain-containing protein (F4J7G5), and ras-related protein RABA2a (O04486; Xia et al., 2019).

Aquaporins on the contemporary are not seen solely as water transporters but can transport ammonia, boron, carbon dioxide, silicon, urea, and even PTEs such as As (Mosa et al., 2016). An Aqua1 gene from *Populus trichocarpa*, which has a very high number of aquaporins in its proteome, when expressed in a Zn-sensitive strain of yeast, was able to confer Zn-resistance. Furthermore, Aqua1 protein product was observed to co-localize with AtTIP1, a well-known *Arabidopsis* vacuolar marker (Ariani et al., 2019). The contenders for phytoremediation that are DEPs come in large datasets that it is difficult to describe in detail covering all proteins in one review article. There were 63 and 372 differently expressed proteins (≥1.5) in the tolerant (BM) and susceptible (Y1) cultivars of industrial hemp (Xia et al., 2019). A collection of 5,838 proteins were quantified in Poplar plants to check up- or down-regulation of proteins that play a role in phytoremediation in solely "Cd stressed" and "Cd stress remediated with nitrogen" groups (Huang et al., 2020). In the study, the differentially expressed proteins were in the high double digits and hundreds. The following pathways were also upregulated (in the process category) in Cd + N (nitrogen) plants compared to the Cd only group; inositol metabolic process, polyol biosynthetic process, polyol metabolic process, alcohol biosynthetic process, monosaccharide metabolic process, hexose metabolic process, and phospholipid biosynthetic process showcasing that nitrogen has the potential to recover phyto-destructive events (Huang et al., 2020). Furthermore, in the same study, there was upregulation of the following candidate proteins at both the proteome and phosphoproteome levels: heat shock protein 70 (HSP70), 14–3–3 protein, peroxidase (POD), zinc finger protein (ZFP), ABC transporter protein, eukaryotic translation initiation factor (elF), and splicing factor 3 B subunit 1-like (SF3BI). In fact, plant transport and absorption were optimized, with 11 binding proteins, seven transporter proteins, and five-storage proteins upregulated in the Cd + N treatment. The main transporters that were upregulated were ABC transporters, which represented 57.1% of total transporters that were upregulated in the Cd + N treatment (Huang et al., 2020).

Biotechnologically, three main strategies are embarked upon to improve the clean-up of PTEs (i) manipulating metal/metalloid transporters, (ii) enhancing metal/metalloid ligand production, and (iii) conversion of metal/metalloid into volatile and less detrimental forms (Mosa et al., 2016). For the first strategy, tinkering with aquaporins that are capable of As transport, as well as other metalloids, antimonite (SbIII), silicon (Si), and boron (B) can be one way forward. The As is known to be present in rice grains and contributes to As in the human body (Chowdhury et al., 2020). For the second strategy, cysteine-rich proteins such as metallothionein and glutathione S-transferase take precedence, and this is a well-researched area in phytoremediation (Mosa et al., 2016). For the third one, Se, which is an essential micronutrient that can have negative repercussions when consumed in excess, is seen as a contender for intervention to turn excess Se into volatile products, such as dimethyl selenide, that can be released into the air (Mosa et al., 2016).

Studies available to date report either the up- or down-regulation of a considerable number of proteins related to several cellular essential processes. A general observation cannot be made from these studies since the change in proteome profile may depend on many factors, including the type of metal, the concentration of metal, exposure duration, growth environment, and other biological or non-biological entities associated with the plant system. However, it can be suggested that the toxic outcome of PTEs lies in the profile of functional proteins subject to change by various parameters being major among them is the metal type/concentration. Some of the essential proteins/enzymes and their expression altered by PTEs in leaves and roots are presented in **Figures 4, 5**, respectively. Since there can be hundreds of proteins in a single type of plant tissue whose expression is changed by PTEs when comparative proteomics is performed, therefore, combining all under one umbrella is cumbersome. To understand the impact of specific PTE on a specific plant species, proteomic toxicity profiling of PTEs with respect to plant organs or tissue needs to be performed in future studies. Many hyperaccumulator species of Brassicaceae and Caryophyllaceae do not possess mycorrhizal networks in their roots. However, hyperaccumulator plants (for example, the genus *Thlaspi*) have been documented to possess mycorrhizae, although sparsely under field and experimental conditions (Ferrol et al., 2016). The

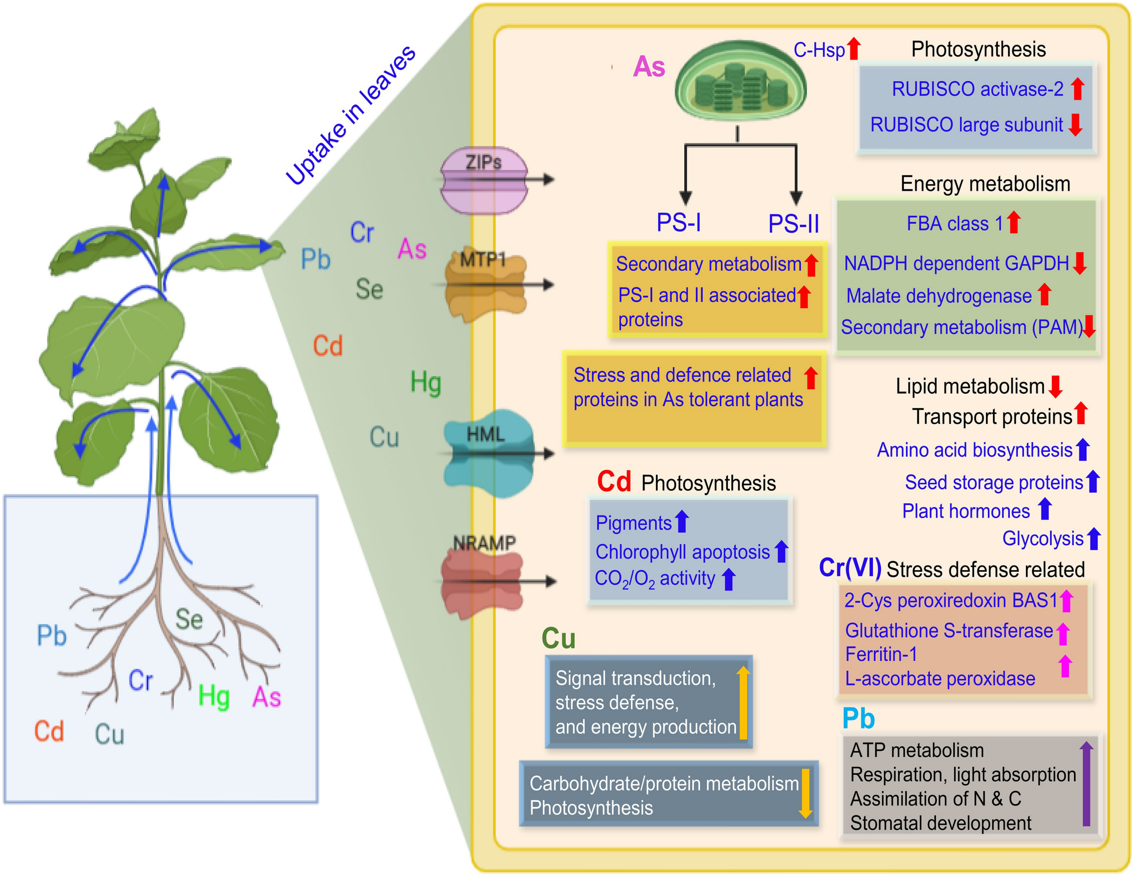

FIGURE 4 | An illustration of the uptake of potentially toxic elements and plant response in the leaves. Uptake of PTEs in plant leaf resulting in significant up- or down-regulation of several proteins as represented by up and down arrows, respectively. The fold change may vary with the metal species, exposure duration, and intercellular concentration. Up and down red arrows are for as, blue for Cd, pink for Cr(VI), yellow for Cu, and violet for Pb. Membrane-embedded channels show the metal transport inside the leaf cell. Abbreviations: RUBISCO, Ribulose bisphosphate carboxylase/oxygenase; FBA, Ructose-bisphosphate aldolase; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; PAM, Phenylalanine aminomutase; C-Hsp, Chloroplast heat shock proteins; ZIPs, zinc-iron permease; MTP1, Metal transport protein1; CDF, Cation diffusion facilitator; and NRAMP, Natural resistant associated macrophage protein.

inverse—mycorrhizae as determined by spore counts or root colonization has been significantly lower in soils rich in PTEs than non-metal rich soils—appears to claim that PTEs can have a detrimental effect on mycorrhizal survival (Ferrol et al., 2016).

However, mycorrhizal fungi never disappear from the ambient soil, suggesting that they can reform symbioses with plant roots under opportunistic conditions. AM fungi are capable of resisting PTEs by collective means that include cell wall binding to metals, chelation by glomalin, increased efflux to the exterior while diminishing uptake, cytosolic chelation, compartmentalization in the vacuoles, and upregulating antioxidant responses (Ferrol et al., 2016).

The success of exogenous nitrogen application suggests that the application of nitrogen-fixers (diazotrophs such as *Rhizobium* and *Azotobacter*) as biofertilizers can be an option to remediate polluted soils as well promote the capacity of plants to remediate PTEs such as Cd. In fact, metal-resisting Rhizobia can alleviate PTEs stress by production of phytohormones, nitrogen fixation, phosphorus solubilization, ACC deaminase synthesis, and siderophores (Brígido and Glick, 2015). The opulence in phenotypic and genotypic Rhizobial diversity makes it essential to choose the correct elite strains which can remediate soils that are not conducive to plant growth, such as N-deficient degraded lands.

CONCLUSION

The PTEs accumulation in the environment above threshold levels poses a high risk to biota health and significantly undermines global environmental sustainability efforts. Phytoremediation has proven to be one of the most efficient strategies to remediate PTEs polluted sites. However, the large-scale application and commercial success of phytoremediation are still to be demonstrated, partly due to the limited understanding of the PTEs sequestration mechanisms. Although several successes were recorded, the evolution of plant proteomics provides further opportunity to sufficiently elucidate PTEs phytoremediation mechanisms,

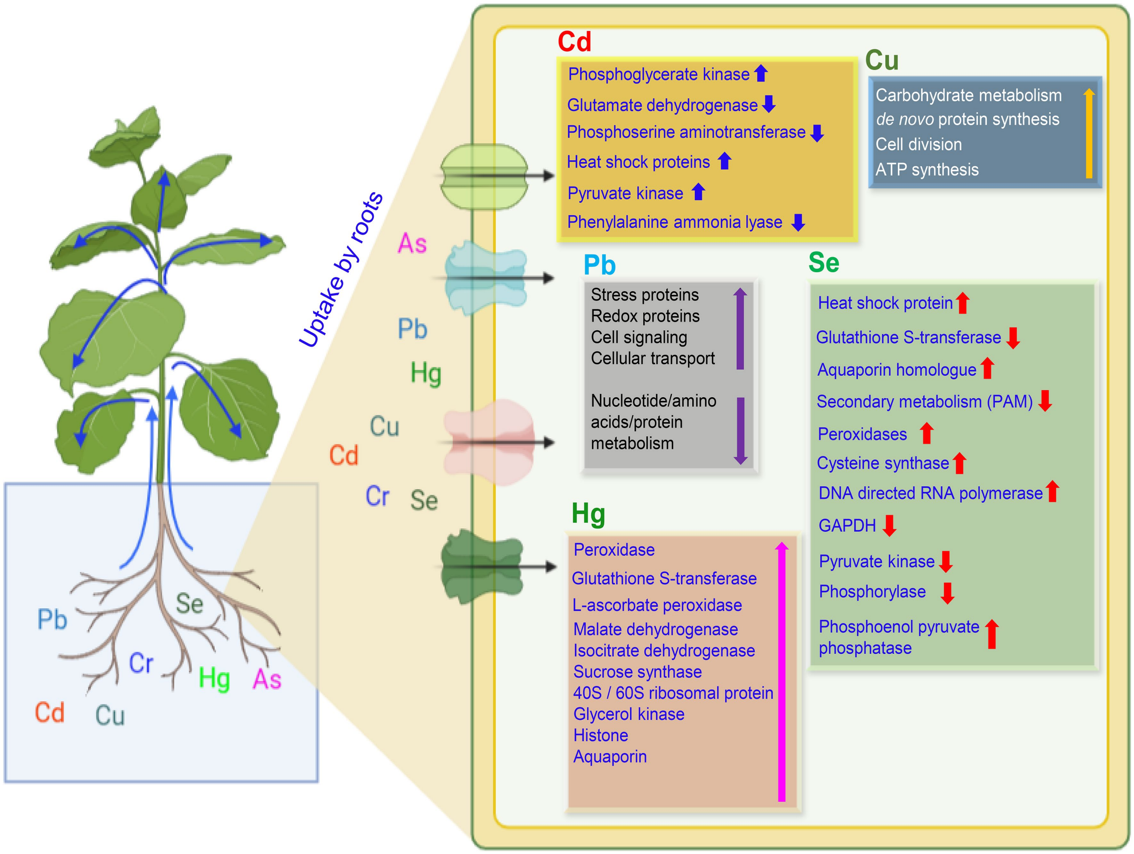

FIGURE 5 | A proposed model on the uptake of potentially toxic elements and plant response in the roots. Uptake of PTEs by plant roots causing significant up- or down-regulation of essential proteins or enzymes as represented by up and down arrows, respectively. Up and down blue arrows are for Cd, yellow for Cu, violet for Pb, red for Se, and pink for Hg. Abbreviations: GAPDH, Glyceraldehyde 3-phosphate dehydrogenase, and ATP, Adenosine triphosphate.

particularly in known high metal accumulating plants. This comprehensive review has demonstrated the potential of several PTEs accumulating plants and the active involvement of their proteome specific to the internal and/or external stimuli of applied PTEs. Various advanced tools and techniques both gel dependent and gel-free methods including qRT-PCR, western blotting, 2D-GE, LC-MS/MS, and MALDI TOF MS/MS have recognized the association of specific PTE with the enhanced expression of resulting proteome. The alteration of proteome expression (up- or down-regulation) in response to applied PTEs such as Cd, Cr, or Hg depends on the intracellular concentration of accumulated PTE, plant species, and the phytoremediation strategy being deployed by the plant. However, the PTE's concentration effectively mitigated by the plant species in a defined or local environment without reducing crop production still needs further investigation. The species-specific (both plant and PTE's species) knowledge of plant proteome changes under different growth conditions and growth phases such as from vegetation to flowering to fruiting stage indeed requires further investigation. A better understanding of PTEs-proteome relation will provide obvious benefits like (i) sustainable and effective decontamination of PTEs polluted sites while maintaining the plant growth and crop production and (ii) protection of soil biodiversity and quality. With an enhanced mechanistic understanding of the process, studies focusing on the engineering of the existing mechanisms of a plant's PTEs sequestration should be prioritized. This will enable the development of an increased number of transgenic plant species with enhanced PTEs tolerance, uptake, and detoxification capabilities.

AUTHOR CONTRIBUTIONS

KU and MA: conceptualization. KU and BA: software. KU, MA, and BA: formal analysis. MA and HA: resources and funding acquisition. KU: writing—original draft preparation. KU, MA, HA, MR, MS, and BA: writing—review and editing. MA, KU, and HA: project administration. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Physiological Mechanism of Exogenous 5-Aminolevulinic Acid Improved the Tolerance of Chinese Cabbage (*Brassica pekinensis* L.) to Cadmium Stress

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The 5-aminolevulinic acid (ALA), a new type of plant growth regulator, can relieve the toxicity of cadmium (Cd) to plants. However, its mechanism has not been thoroughly studied. In the study, the roles of ALA have been investigated in the tolerance of Chinese cabbage (*Brassica pekinensis* L.) seedlings to Cd stress. The results showed that Cd significantly reduced the biomass and the length of the primary root of seedlings but increased the malondialdehyde (MDA) and the hydrogen peroxide (H_2O_2) contents. These can be effectively mitigated through the application of ALA. The ALA can further induce the activities of antioxidant enzymes in the ascorbate-glutathione (AsA-GSH) cycle under Cd stress, which resulted in high levels of both GSH and AsA. Under ALA + Cd treatment, the seedlings showed a higher chlorophyll content and photosynthetic performance in comparison with Cd treatment alone. Microscopic analysis results confirmed that ALA can protect the cell structure of shoots and roots, i.e., stabilizing the morphological structure of chloroplasts in leaf mesophyll cells. The qRT-PCR results further reported that ALA downregulated the expressions of Cd absorption and transport-related genes in shoots (*HMA2* and *HMA4*) and roots (*IRT1, IRT2, Nramp1*, and *Nramp3*), which resulted in the low Cd content in the shoots and roots of cabbage seedlings. Taken together, the exogenous application of ALA alleviates Cd stress through maintaining redox homeostasis, protecting the photosynthetic system, and regulating the expression of Cd transport-related genes in Chinese cabbage seedlings.

Keywords: 5-minolevulinic acid, cadmium stress, Chinese cabbage, ascorbate-lutathione cycle, photosy nthesis

INTRODUCTION

Cadmium (Cd) is widely distributed in nature and is highly toxic to plants (Chen et al., 2018). The Cd-toxicity is a complex phenomenon that negatively impacts plant growth and development by inducing oxidative stresses and affecting element uptake (Xu et al., 2016). It also enhances the contents of reactive oxygen species (ROS) activity, which leads to lipid peroxidation

(María et al., 2007), disrupting the structure and function of proteins, and affecting the expression of genes that are encoding metal transcription factors (Giovanni et al., 2010). In response to heavy metal stresses, plants have evolved adaptive mechanisms, i.e., upregulating the antioxidant defense system. For example, Qin et al. (2018) reported that wheat (*Triticum aestivum* L.) seedlings were tolerant against Cd stress by increasing the activities of enzymes in the ascorbate-glutathione (AsA-GSH) cycle. The AsA-GSH cycle in plants plays a crucial role in combating Cd stress (Qin et al., 2018) and other stresses, such as drought (Kang et al., 2013), low light (Hu et al., 2019), salt stress (Wu et al., 2019), and chilling stress (He et al., 2021). The enzymes [dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), glutathione reductase (GR), ascorbic acid peroxidase (APX), and ascorbic acid oxidase (ASO)] and the antioxidants [ascorbic acid (AsA) and reduced glutathione (GSH) in AsA-GSH cycle can effectively scavenge ROS induced by the environmental stresses (Li et al., 2010)]. The high ratio of AsA/dehydroascorbic acid (DHA) and GSH/oxidized glutathione (GSSG) is essential for scavenging ROS, and the regeneration of AsA and GSH depends on the activities of DHAR, MDHAR, and GR (Niu et al., 2017).

Several families of metal transporters are found to play a key role in Cd absorption and transport in plants (Clemens, 2006). For example, the *iron-regulated transporter 1* (*IRT1*) and the *natural resistance-associated macrophage protein 1* (*Nramp1*) mediate Cd uptake in plant roots (Vert et al., 2002). The *Heavy metal ATPase 2* (*HMA2*) and *HMA4* can transfer Cd from the root to shoot through the xylem (Frederic et al., 2004; Takahashi et al., 2012). The over-expression of *OsHMA2* can reduce the Zn and Cd levels in rice grains (Takahashi et al., 2012). Studies have identified that the IRT and *Nramp* transporters participate in Cd accumulation and transport processes, which is important for plant tolerance to heavy metals (Alexander et al., 2011; Chen et al., 2019). The latest research found that the hydrogen-rich water reduced the Cd accumulation by downregulating *IRT1* gene expression (Wu et al., 2021).

The Cd stress can also be alleviated by 5-aminolevulinic acid (ALA) in plants (Wang F. J. et al., 2018; El-Amier et al., 2019). The ALA is a key precursor metabolic intermediate in plants, animals, and bacteria, which has been discovered as a new plant growth regulator (Wu et al., 2019). Recently, it was reported that ALA can improve plant growth, photosynthetic assimilation, and gas exchange capacity under Cd stress (Ali et al., 2013a,b). Recently, it was found that ALA was involved in the regulation of gene expression involved in Na^+ transporter in cucumber roots under salt stress, which reduces Na^+ upward transport (Wu et al., 2019). However, it remains unknown about the mechanism of ALA that is involved in the cabbage tolerance to Cd stress, and whether ALA is involved in the transcriptional regulation of Cd uptake and transporter genes.

Chinese cabbage (*Brassica pekinensis* L.) is one of the most widely grown vegetables in China. Its productivity and quality are considerably decreased under Cd stress. Therefore, reducing the accumulation of toxic metals in edible parts of vegetables by regulating the antioxidant enzyme system and metal ion absorption and transport genes is of great significance. Therefore, the aim of this study was to investigate the protective mechanism of ALA in the Cd tolerance of cabbage, and especially, the regulative mechanism involved in Cd transport and distribution.

MATERIALS AND METHODS

Plant Materials and Chemical Treatment

Chinese cabbage (*Brassica pekinensis* L. cv.) seeds (Huangfei) were purchased from Qingfeng Seed Service (Lanzhou, China). Seeds were surface-disinfected using 1.5% sodium hypochlorite for 15 min and washed five times with distilled water. Then, seeds were germinated in the dark for 48 h at 25 ± 1°C. At the two-leaf stage, the morphologically uniform seedlings were selected and plugged into plate holes on a plastic container (17 cm × 11 cm × 6 cm) containing half-strength of Hoagland's nutrient solution [25 mM KNO_3, 25 mM $Ca(NO_3)_2{\cdot}4H_2O$, 7.5 mM $MgSO_4{\cdot}7H_2O$, 5 mM $NaH_2PO_4{\cdot}2H_2O$, 0.04 μM EDTA·Na-Fe, 23 μM H_3BO_3, 4.75 μM $MnSO_4{\cdot}H_2O$, 0.4 μM $ZnSO_4{\cdot}5H_2O$, 0.15 μM $CuSO_4{\cdot}5H_2O$, and 0.008 μM $(NH_4)_6Mo_7O_2{\cdot}4H_2O$]. The seedlings were grown in the climate box at 26 ± 1/18 ± 1°C (day/night) with 75% humidity, 41.38 W/m^2 light intensity, and a 12 h/12 h light period.

Fourteen-day-old Chinese cabbage seedlings were treated for 7 days with 50 μM $CdCl_2$. At the same time, ALA (25 mg/L) was sprayed onto the leaves, and it was applied every 3 days. After treatment, the leaves and roots were frozen immediately in liquid nitrogen and stored at −80°C. At least 48 seedlings were used for each treatment and the experiments were repeated thrice.

Determination of the Biomass, Gas Exchange, and Chlorophyll Fluorescence Parameters

After treatment, 21-day-old seedlings were harvested and photographed. Primary root length, dry weight (DW), and fresh weight (FW) were measured from every treatment.

The content of chlorophyll was calculated according to the formar described by Lichtenthaler and Wellburn (1983). Samples (0.200 g each) of Chinese cabbage leaves were extracted using 80% buffered aqueous acetone for determining the chlorophyll content (Porra et al., 1989). The absorbance of the supernatant was determined at 646 and 663 nm. The *V* values indicate the dissolved volume of the determined solution; the FW values indicate the fresh weight of the sample.

$$\text{Chl } a\ (mg\ g^{-1}\ FW) = (12.21 \times OD_{663} - 2.81 \times OD_{646}) \times V/FW$$

$$\text{Chl } b\ (mg\ g^{-1}\ FW) = (20.13 \times OD_{646} - 5.03 \times OD_{663}) \times V/FW$$

Plant gas exchange parameters, including the net photosynthetic rate (Pn), intercellular CO_2 concentration (Ci), stomatal conductance (Gs), and transpiration rate (Tr), were measured using the portable photosynthesis system (CIRAS-2, PP System, United Kingdom) according to the method described by Pimentel et al. (1999). Before determination, the seedlings were acclimated to light for 10 min in the culture environment. Then, the fourth functional leaf was selected and placed into the leaf chamber for determination. The conditions for Pn measurement were set as follows: the photosynthetic photon flux density, 400 μmol m^{-2} s^{-1}; ambient CO_2 concentration, 380 μmol mol^{-1}; relative humidity, 70%, and leaf temperature, 25°C.

Chlorophyll fluorescence parameters of seedlings were measured using the modulated chlorophyll fluorescence imaging system (Maxi Imaging-PAM, Walz, Germany) (Gong et al., 2014). Before determination, the seedlings were adapted in darkness for at least 30 min. The fourth functional leaf of seedlings was selected for the determination. By applying a saturation pulse under 2,700 μmol m^{-2} s^{-1}, the fluorescence parameters of minimum fluorescence (Fo) and maximum fluorescence yield (Fm) were obtained from dark-adapted leaves. The actinic light was adjusted to 81 μmol m^{-2} s^{-1}, the leaves were light-adapted for 5 min, and opened every 20 s, lasting for 0.8 s. By applying actinic light, the indexes like minimum fluorescence (Fo'), steady chlorophyll fluorescence (Fs), and maximum fluorescence yield (Fm') could be calculated. The actual photosynthetic efficiency (Fv/Fm) was calculated as described by Genty et al. (1989). The coefficient of actinic light quenching (qP) was calculated according to the method of Klughammer and Schreiber (2008). The specific computational formulas were as follows:

$$\mathrm{Fv/Fm} = (\mathrm{Fm}' - \mathrm{Fs})/\mathrm{Fm}'$$

$$\mathrm{qP} = (\mathrm{Fm}' - \mathrm{Fs})/(\mathrm{Fm}' - \mathrm{Fo}') \times \mathrm{Fo}'/\mathrm{Fs}$$

Ultrastructural Analysis of Chinese Cabbage

The leaves and roots were fixed with a buffer containing 2.5% glutaraldehyde in 0.1 M phosphate buffer (PBS, pH 7.4) for 24 h at 4°C. The samples were washed thrice with 0.1 M PBS (pH 7.4) and fixed in 1% H_2OsO_4 for 5 h at 4°C. Afterward, the samples were washed thrice with 0.1 M PBS (pH 7.4), and dehydrated using graded ethanol solutions (50, 70, 80, 90, and 100%) for 15 min every time; following this, the samples were acetone-infiltrated and embedded in Epon 812 epoxy resin. Ultrathin sections were cut on a microtome (Leica EM UC6 ultra-microtome, Japan), and stained with uranyl acetate and lead citrate for 15 min. Ultrathin sections of cabbage leaf and root were examined and photographed with the transmission electron microscope (TEM, JEOL JEM-1230, Japan). The electron microscope sample was processed according to the method described by the electron microscope center of Lanzhou University.

Determination of Hydrogen Peroxide and Malondialdehyde Contents

The content of H_2O_2 was determined according to the method given by Gong et al. (2008). H_2O_2 content was measured using the ELISA kit (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) according to the instructions. The H_2O_2 content was calculated based on the standard curve.

The malondialdehyde (MDA) was determined according to the method of Zhang et al. (2007). The concentration of MDA was determined using the colorimetric method of thiobarbituric acid (TBA) with Micro MDA Assay Kit (BC0025; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) according to the manufacturer's instructions.

Determination of Antioxidant Enzyme Activities

Samples were homogenized in 5 ml of 50 mM PBS buffer (pH 7.8) at 4°C. The homogenate was centrifuged at 10,000 × *g* for 20 min at 4°C, and the supernatant was used to determine the enzyme activities (DHAR, MDHAR, GR, APX, and ASO). All the extraction methods of the enzyme have been slightly modified (Rao and Terry, 1989). The soluble protein concentration was determined by the method of Bradford (1976).

For dehydroascorbate reductase (DHAR) (EC 1.8.5.1) activity analysis, the reaction mixture included 50 mM PBS (pH 7), 2.5 mM GSH, and 0.1 mM DHA. The reaction was started by adding 50 μl of enzyme extract. The activity was measured using the decrease in absorbance at 265 nm in 1 min (Nakano and Asada, 1981).

For monodehydroascorbate reductase (MDHAR) (EC 1.6.5.4) activity analysis, the reaction mixture included 50 mM PBS (pH 7), 2 mM nicotinamide adenine dinucleotide phosphate (NADPH), and 2U ascorbate oxidase (AAO). The reaction was started by adding 90 μl of enzyme extract. The activity was measured using the decrease in absorbance at 340 nm in 1 min (Maria et al., 2000).

For glutathione reductase GR (EC 1.6.4.2) activity analysis, the reaction mixture included 0.1 M PBS (pH 7), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM GSSG, 0.2 mM NADPH, and 50 μl of enzyme extract. The reaction was started by adding GSSG, and the decrease in absorbance was recorded at 340 nm (Foyer and Halliwell, 1976).

For ascorbic acid oxidase (ASO) (EC 1.10.3.3) activity analysis, the reaction mixture included 50 mM PBS (pH 7), 1 mM AsA, 1 mM EDTA, 2% povidone, 0.25% triton X-100, and 0.15 ml of enzyme extract in a final volume of 3 ml. The reaction was started by adding H_2O_2. The activity was calculated from the recorded decrease in absorbance at 265 nm for 1 min (Esaka et al., 1990).

For APX (EC 1.11.1.11) activity analysis, the reaction mixture included 50 mM PBS (pH 7), 0.5 mM AsA, 0.1 mM EDTA, 0.1 mM H_2O_2, and 30 μl of enzyme extract in a final volume of 2 ml. The reaction was started by adding H_2O_2. The activity was calculated from the recorded decrease in absorbance at 290 nm for 1 min (Nakano and Asada, 1981).

Analysis of the Ascorbate-Glutathione Cycle

The total glutathione (GSH and GSSG) was extracted using an ELISA kit (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) according to the manufacturer's instructions. Either leaf or root (0.100 g) was homogenized and centrifuged at 7,100 × *g* (total glutathione) or 1,100 × *g* (GSSG) at 4°C for 10 min. The supernatant was collected to determine total glutathione and GSSG.

The contents of AsA and DHA were analyzed according to the method as described by Arakawa et al. (1981). The AsA and DHA contents were analyzed according to the following steps. The first extract reagent (5 mL) was added to the frozen samples (0.1 g), and it was homogenized in an ice bath. Then, the homogenate was centrifuged at 8,000 × *g* at 4°C for 20 min for AsA or 16,000 × *g* at 4°C for 20 min for DHA, respectively. The analysis was performed using the AsA ELISA kit and DHA ELISA kit, respectively.

Determination of Metal Concentrations

To determine the element contents, roots and leaves were separately harvested. The roots were washed thrice in distilled water. Then plant tissues were dipped in 20 mM EDTA for 30 s, following which they were washed with distilled water. The samples were then dried at 80°C to constant weight and grounded to a fine powder.

The content of Cd was measured according to the method as described by Wang F. J. et al. (2018). For determination of Cd content, the leaf and root samples (0.500 g) were put into a crucible on a hot plate until the initial smoke was observed and was burned to ash at 550°C in a muffle furnace. The ash samples were allowed to cool, and each was supplied with 10 ml of 6 M hydrochloric acid (HCl). Drops of 6 M HCl were added to dissolve the residues. Distilled water was then added to the mixture in a volumetric flask to the 50 ml mark. The Cd content was determined at 228.8 nm using the atomic absorption spectrophotometry method (ZEEnit 700P, Analytik Jena AG, Germany).

The contents of other elements were measured according to the method as described by Miria et al. (2016). For determination of other element contents, plant samples (0.500 g) were digested with 5 mL H_2SO_4 overnight. The samples were placed on a hot plate until the solution was brown. Ten drops of 30% H_2O_2 were slowly dropped into the solution. The operations were repeated thrice until the solutions were clear. Distilled water was then added to the mixture in a volumetric flask to the 50 mL mark. The concentrations of elements were determined using the atomic absorption spectrophotometer with the OD set as follows: 422.6 nm (Ca), 285.2 nm (Mg), 248.3 nm (Fe), 213.8 nm (Zn), and 278.4 nm (Mn).

Gene Expression Measurement

The total RNA was isolated from Chinese cabbage tissues using a total RNA kit (Tiangen Biotech Co., Ltd., Beijing, China). The DNA-free total RNA (5 μg) was used for the first-strand cDNA synthesis in a 20 μl reaction volume (Thermo Fisher Scientific, MD, Lithuania) according to the manufacturer's instructions. Real-time quantitative PCR (qRT-PCR) reactions were performed using the Bio-systems 7500 Real-Time PCR System (Applied Biosystems®, Foster City, CA, United States) with the SYBR Green intercalating dye fluorescence detection. The amplification program was as follows: 3 min at 95°C, 40 cycles of 5 s at 95°C, 10 s at 55°C. Relative gene expression was evaluated using the $2^{-\Delta\Delta Ct}$ method. The PCR primers were designed using Primer Premier 5 software (PREMIER Biosoft, Palo Alto, CA, United States), and is listed in **Table 1**. *Actin* (AF111812) was used as an internal control.

Statistical Analysis

Data are presented as the means ± SE. The data were analyzed by one-way ANOVA, which was conducted with the SPSS 19.0 (SPSS Inc., Armonk, NY, United States). Duncan's multi-range test was performed to compare significant difference between treatments at $P < 0.05$. All the figures are completed by using the SigmaPlot 12.5.

RESULTS

Effects of 5-Aminolevulinic Acid on the Biomass of Cabbage Under Cadmium Stress

To examine the effects of ALA on Chinese cabbage seedlings exposed to Cd stress, we investigated the changes in seedling growth, root length, FW, and DW. Changes in the phenotype following Cd and ALA treatments are shown in **Figure 1A**. Compared to the control, the Cd stress significantly caused a decrease in the FW of roots and shoots and the length of the primary root (**Figure 1**). The FW of roots and shoots was similarly reduced by about 50% (**Figures 1C,D**). Based on the concentration curve of ALA, the biomass of Chinese cabbage seedlings at 25 mg/L of ALA reached the maximum under 50 μM Cd treatment (data not shown). Thus, the 50 μM Cd and 25 mg/L ALA were selected for treatment in the study. The DW of roots and shoots was also markedly decreased (**Figures 1E,F**). Under ALA + Cd treatment, the root length was increased by about 31% compared to that under Cd treatment alone (**Figure 1B**). The application of ALA increased the DW of shoots (about 40%) compared to that from Cd treatment alone, but not in the DW of roots (**Figure 1F**). Under normal conditions, the application of exogenous ALA had no significant effects on primary root length, FW, and DW of Chinese cabbage seedlings.

Effects of 5-Aminolevulinic Acid on Chlorophyll Content and Photosynthetic Gas Exchange of Cabbage Seedlings

Effects of ALA and Cd on chlorophyll content and photosynthetic gas exchange attributes are delineated in **Figure 2**. Results showed that the content of chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl b) under Cd stress were reduced by 19 and 33%, respectively. Under ALA + Cd treatment, compared with the single Cd treatment, the content of Chl *a* and Chl *b* was increased by 13 and 29%, respectively. Under ALA treatment alone, there was no

TABLE 1 | The primer lists.

Gene name	Gene ID	5′→3′	3′→5′
IRT1	AY087095.1	TGGCATTCTTTTTCGCGGTG	GCCGAGCATGCATTGAGAAG
IRT2	BT025714.1	CTCGTCGACCTTCTGGCTAC	ACTTGGCGACGACAGACATT
Nramp1	AF165125.1	CCCCGAAGACCGTGCTAAAT	TACCCACCACGTTTCGTAGC
Nramp3	GC008629595.1	TCTTGATTGTTTCGTCTTC	TCCCATTGTAGCGATAAG
HMA2	NM119157.3	GAGGATGCCACATGGTTGGA	CTTTGGTACGGCGGAAGAGT
HMA4	AY096796	TTCCCCACAAGAATCGCTCC	CACTCGAACCTTCCACGTCA
Actin	AF111812	CCAGGAATCGCTGACCGTAT	CTGTTGGAAAGTGCTGAGGGA

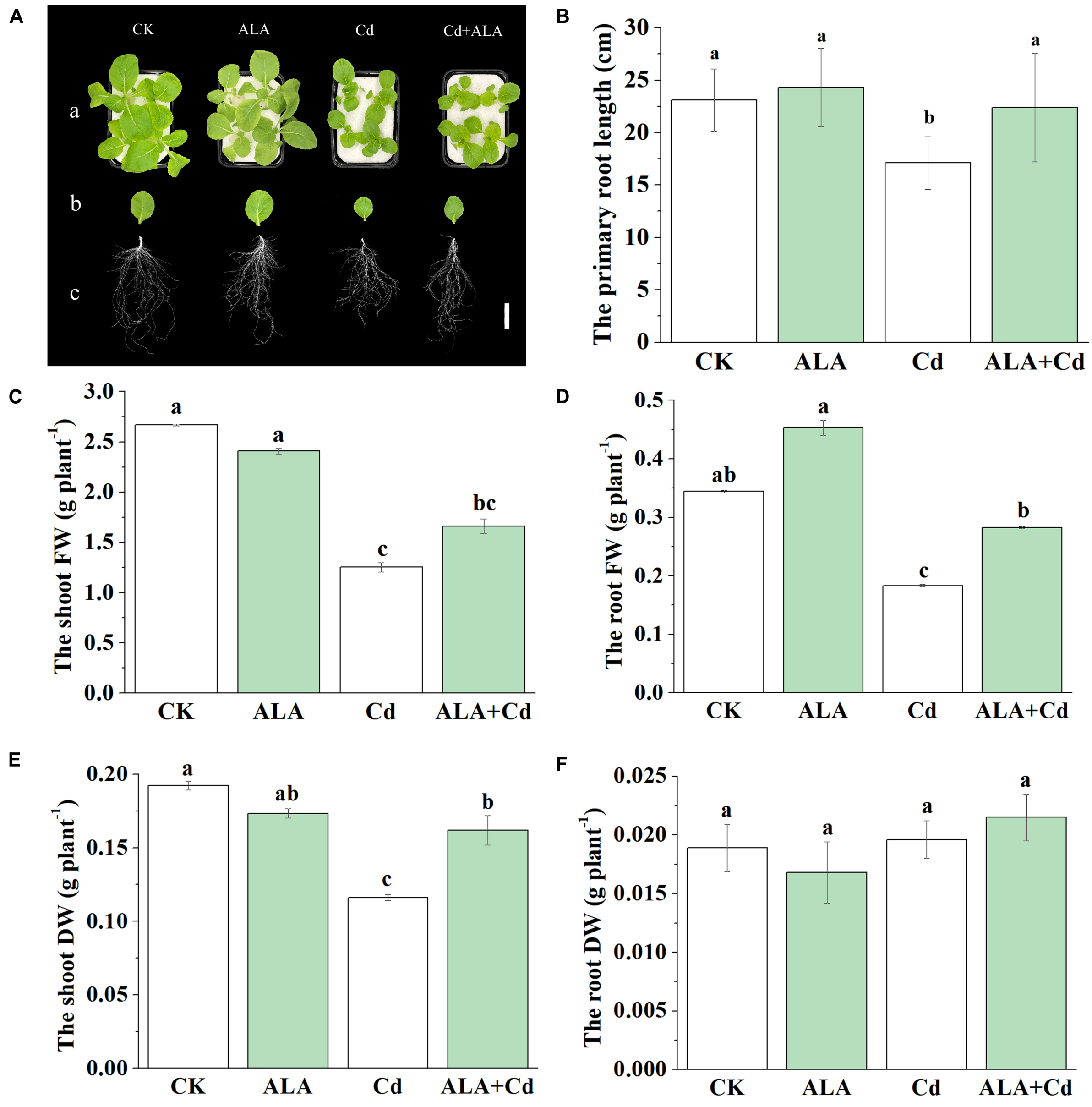

FIGURE 1 | Exogenous ALA alleviated Cd stress-induced growth inhibition in Chinese cabbage. **(A)** Phenotypes of individual. Bar = 5 cm. **(B)** Changes in primary root length. Fresh weight (FW) of shoots **(C)** and roots **(D)**. Dry weight (DW) of shoots **(E)** and roots **(F)** The 14-day-old cabbage seedlings were transferred to 1/2 Hoagland medium containing 50 μM Cd for 7 days. About 25 mg/L 5-aminolevulinic acid (ALA) was sprayed onto the leaves, and it was applied every 3 days under ALA+Cd as well as ALA treatments. The mean ± SE are shown ($n \geq 3$). Different letters indicate significant difference among the treatments ($P < 0.05$).

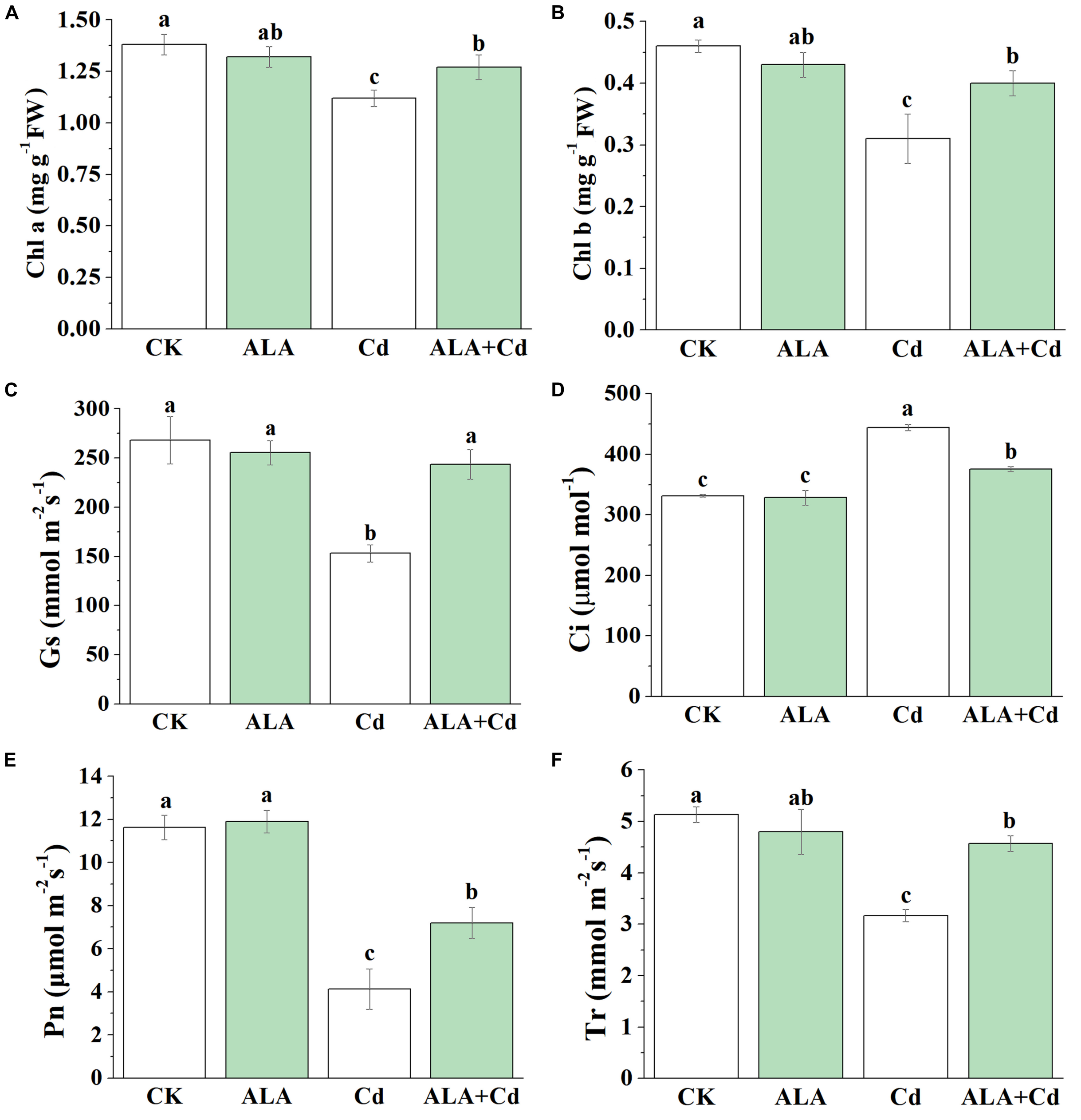

FIGURE 2 | Effects of ALA under Cd stress on Chlorophyll content and photosynthetic gas exchange parameters of cabbage. **(A)** Chl *a*, **(B)** Chl *b*, **(D)** intercellular CO_2 concentration (Ci), **(C)** stomatal conductance (Gs), **(E)** net photosynthetic rate (Pn), and **(F)** transpiration rate (Tr). The mean ± SE are shown ($n \geq 3$). The seedlings were treated as in **Figure 1**. Different letters indicate significant difference among the treatments ($P < 0.05$).

significant difference between Chl *a* and Chl *b* content compared with the control.

Under Cd treatment, the gas exchange parameters were significantly decreased. The Pn, Gs, and Tr were decreased by 65, 42, and 38%, respectively, while Ci was significantly increased by 34%. Upon adding ALA under Cd stress, Pn, Gs, and Tr increased significantly by 74, 59, and 44%, respectively, and the Ci value decreased significantly by 16%. There is no significant difference for the above parameters between the ALA treatment alone and control conditions. The results showed that exogenous ALA could improve Pn under Cd stress, which was independent of stomatal structure.

Effects of 5-Aminolevulinic Acid on Chlorophyll Fluorescence Parameters of Cabbage Under Cadmium Stress

Chlorophyll fluorescence is regarded as an internal probe to provide insights into the relationship between plant photosynthesis and the environment. The Fv/Fm reflects

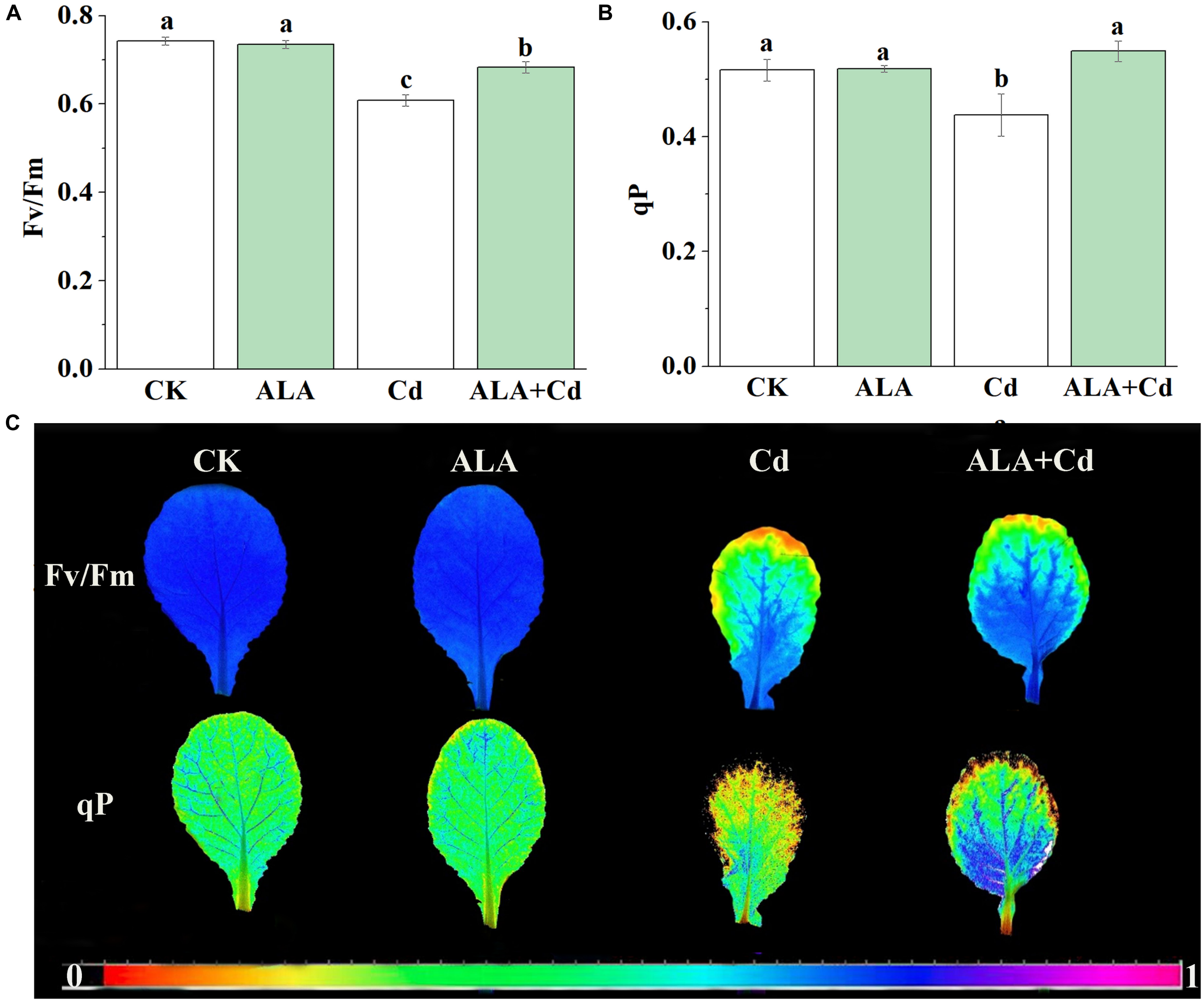

FIGURE 3 | Effects of ALA under Cd stress on chlorophyll fluorescence parameters of cabbage. **(A)** The maximum PSII quantum yield (Fv/Fm), **(B)** photochemical quenching coefficient (qP) and **(C)** Chlorophyll fluorescence imaging. The seedlings were treated as in **Figure 1**. The mean ± SE are shown (*n* ≥ 3). Different letters indicate significant difference among the treatments ($P < 0.05$).

the health status of the PSII system. The qP is the efficiency of light energy conversion and an important index for plants to absorb light energy, which reflects the rate of electron transfer in photosynthesis (Ivanov and Edwards, 2000). To further explore the mechanism by which ALA is involved in photosynthesis, the conversion efficiency of primary light energy of PS (Fv/Fm) and photochemical quenching coefficient (qP) were analyzed. Results stated that Fv/Fm and qP of cabbage leaves were decreased by 13 and 15%, respectively, under Cd stress (**Figure 3A**). They were significantly increased by 12 and 25%, respectively, under ALA + Cd co-treatment.

The fluorescence images of the Fv/Fm and qP are given in **Figure 2C**. The colors represent the absolute values of the ratio ranging from 0 (black) to 1 (purple). In the fluorescent image of qP, there were obvious orange spots in the serious stress area under Cd treatment, and the orange spots under co-treatment of ALA and Cd were less than those under Cd treatment alone. The color distribution of Fv/Fm under ALA and Cd co-treatment was more similar to the control treatment. The ALA alleviated the decrease of Fv/Fm under Cd stress, which indicated that ALA enhanced the light utilization of cabbage seedlings under Cd stress. The results showed that exogenous ALA could increase the electron transfer rate of PSII under Cd stress.

Effect of 5-Aminolevulinic Acid on Ultrastructural and Morphometric Images of Cabbage Leaves and Roots

To further investigate the effect of ALA on organelles such as chloroplast, ultrastructure and morphometric images were used. Changes in the whole mesophyll cells and chloroplasts are shown in **Figure 4**. Seedlings grown under normal conditions exhibited regular cell shape and typical chloroplast. There are smoothly arrayed grana lamellae and a small quantity of osmiophilic granules (**Figures 4A,a–d**). The cell morphological disturbance and plasmolysis occurred when seedlings were

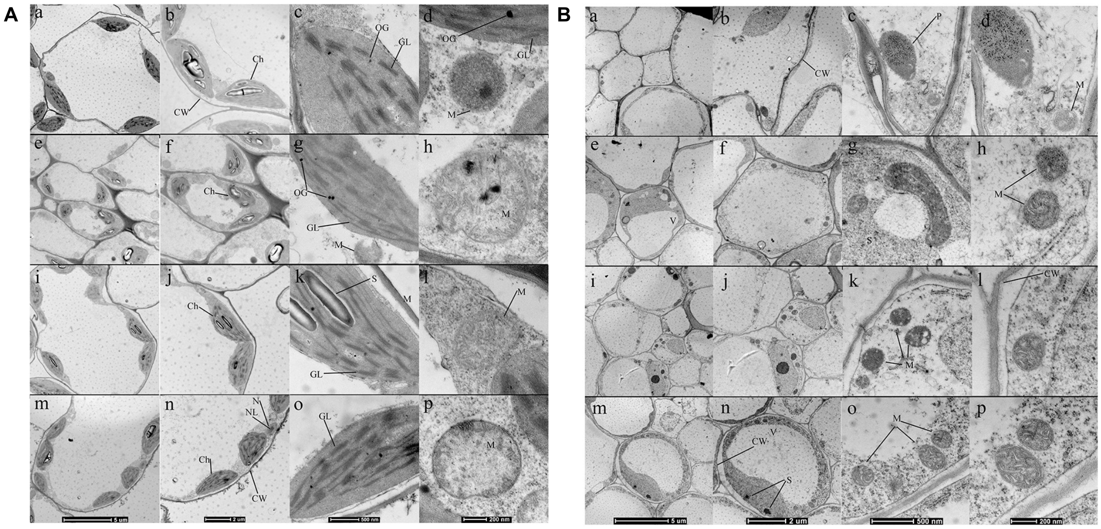

FIGURE 4 | Effects of ALA under Cd stress on the ultrastructural observation of mesophyll cell and chloroplast of cabbage shoots **(A)** and roots **(B)**. In **(A,B)**: **(a,e,i,m)** (×1,900), **(b,f,j,n)** (×2,900), **(c,g,k,o)** (×13,000), **(d,h,l,p)** (×23,000); **(a–d)** Seedlings grown in normal condition. **(e–h)** Seedlings sprayed 25 mg/L ALA only. **(i–l)** 50 μM $CdCl_2$ treated seedlings. **(m–p)** Seedlings simultaneously treated with 50 μM $CdCl_2$ and 25 mg/L ALA. The seedlings were treated as in **Figure 1**. CW, cell wall; Ch, chloroplast; OG, osmiophilic globules; GL, grana lamella; S, starch; M, mitochondria; N, nucleus; NL, nucleolus; P, plastid; S, starch; M, mitochondria; V, vacuole; ER, Endoplasmic reticulum; GA, Golgi bodies.

treated with 50 μM $CdCl_2$, but the number of mitochondria markedly increased (**Figures 4A,i–l**). The grana lamellae of thylakoid were loose, and the shapes of chloroplasts were severely swollen. Furthermore, there were plenty of osmiophilic granules in the chloroplast (**Figures 4A,i–l**). For the ALA-treated seedlings under Cd stress condition, although there was a little improvement in cell morphology, the shapes of chloroplast become typically fusiform (**Figures 4A,m–p**). Moreover, the chloroplasts contained more orderly grana lamellae and starch grains and fewer osmiophilic granules (**Figures 4A,p,l**). Under normal growth conditions, ALA-treated seedlings were very similar to those of the control (**Figures 4A,e–h**).

The ultrastructure of root tip cells with low and high magnifications is shown in **Figure 4B**. Seedlings grown in normal conditions are shown in **Figures 4B,a–d**, and the root cells had regular cell morphology. The cells contained endoplasmic reticulum and well-shaped mitochondria with large vacuoles in the center. The mitochondria had a clear inner membrane structure and were concentrated near the plastids. Under 50 μM Cd treatment, the cells exhibited obvious ultrastructural changes (**Figures 4B,i–l**). The cell wall was thickened, and plasmolysis was observed. The number of plastids and starch grains decreased; however, the number of mitochondria increased. The Cd existed in the form of small granules along the cell wall. The micrographs of root tip cells that were simultaneously treated with 25 mg/L ALA and 50 μM Cd were analyzed (**Figures 4B,m–p**). The large central vacuole was visible. The ion deposition of cytoplasm decreased, and the plastids and starch grains were few. However, the number of mitochondria increased. The ALA improved the cell structure of root tips, which showed various developed mitochondria. These results confirmed that ALA can ameliorate the effect of Cd stress. The micrographs of root tip cells under ALA treatment alone (**Figures 4B,e–h**) showed regular cell morphology and normal organelle structure, which were similar to that of the control group.

5-Aminolevulinic Acid Alleviated Oxidative Damage Under Cadmium Stress

The Cd stress inevitably induces H_2O_2 and MDA productions in plants (**Figure 5A**). Results showed that Cd stress significantly increased MDA content in the leaves and roots, and it reached 170 and 184% of the control, respectively. The application of exogenous ALA can reduce the MDA content of leaves and roots under Cd stress. While spraying ALA under normal conditions, the content of MDA in the leaves and roots is similar to the control level.

As shown in **Figure 5B**, the Cd stress significantly increased H_2O_2 content in cabbage leaves and roots, which reached 171 and 203% of the control, respectively. Exogenous application of ALA can significantly downregulate H_2O_2 content in the roots of stressed plants. It was decreased by 25 and 36% in the leaves and roots, respectively, compared to that under Cd treatment (**Figure 5B**). All these results showed that ALA can reduce the toxicity of Cd by reducing oxidative damage.

Effects of Exogenous 5-Aminolevulinic Acid on the Enzymes' Activities of Ascorbate-Glutathione Cycle in Cabbage

Under Cd stress, MDA and H_2O_2 content were dramatically increased, which caused oxidative stress in Chinese cabbage. The enzyme activities involved in the AsA-GSH cycle, which is the main way of scavenging H_2O_2, were analyzed in cabbage roots

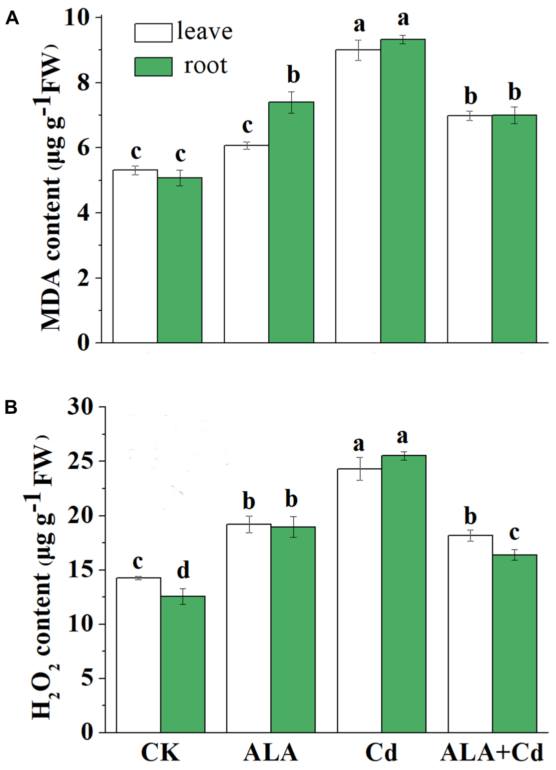

FIGURE 5 | Effects of ALA under Cd stress on contents of MDA **(A)** and H_2O_2 **(B)** of cabbage. The seedlings were treated as in **Figure 1**. The mean ± SE are shown ($n \geq 3$). Different letters indicate significant difference among the treatments ($P < 0.05$).

and leaves. Results showed that the activity of APX decreased slightly by 35 and 46% under Cd stress in leaves and roots, respectively (**Figure 6A**). The activity of ASO was also inhibited in leaves and roots and decreased by 40 and 33%, respectively (**Figure 6B**). Under the ALA + Cd treatment, the APX and ASO activities increased significantly by 52 and 108% in leaves and by 48% and 51% in roots, respectively.

Moreover, the Cd stress had no significant difference in the DHAR activity both in leaves and roots (**Figure 6C**). The activity of MDHAR was increased by 21 and 111% in leaves and roots, respectively, under ALA + Cd stress (**Figure 6D**). The GR activity can be inhibited by Cd stress (**Figure 6E**), and exogenous ALA treatment significantly increases GR activity, and it reached 129 and 153% in leaves and roots, respectively, of Cd treatment alone. The soluble protein content was significantly increased under Cd treatment alone as well as ALA + Cd treatment; however, there is no significant difference between Cd treatment alone and ALA + Cd treatment (**Figure 6F**).

Effects of 5-Aminolevulinic Acid on Ascorbic Acid and Glutathione Contents in Cabbage

As antioxidants, AsA and glutathione (GSH) play an important role in scavenging free radicals through the AsA-GSH cycle. To further verify the protective mechanism of ALA in cabbage tolerance to Cd stress, the AsA and GSH content were measured (**Figure 7**). The AsA content was significantly decreased by 51 and 60% in the leaves and roots of Chinese cabbage under Cd stress, respectively (**Figure 7**). The content of AsA was increased by approximately 53 and 54% in leaves and roots under the application of exogenous ALA under Cd stress, respectively. Contrary to the changes of AsA content, the DHA significantly increased about 46 and 65% in leaves and roots under Cd stress. The contents of DHA in ALA and Cd co-treatment were similar to that of Cd treatment. In addition, there was no difference in the DHA contents of the plant between control conditions and ALA treatment (**Figure 7B**).

Results showed that GSH content was significantly reduced by 33 and 44% in leaf and root, respectively, under Cd stress (**Figure 7D**). The change of GSSG content was opposite to that of GSH. The Cd stress markedly increased the GSSG content. However, the application of exogenous ALA reduced the GSSG content by 24.8 and 37.1% in leaves and roots under ALA + Cd co-treatment, respectively (**Figure 7E**).

Figure 7C showed that the ratio of AsA/DHA was significantly lower than that of CK condition under Cd stress. While the exogenous application of ALA can significantly improve AsA/DHA value. In addition, similar to the above results, the GSH/GSSG ratio was also decreased under Cd stress (**Figure 7F**), and spraying ALA under Cd stress could increase the ratio of GSH/GSSG.

Effects of 5-Aminolevulinic Acid on Accumulations of Mineral Elements in Cabbage

The Cd toxicity alters the absorption of mineral nutrition by plants (Khan et al., 2015). Foliar application of ALA not only significantly decreased Cd accumulation in leaves (by 40%) and roots (by 23%) of Chinese cabbage (**Figures 8A,B**) but also affected the uptake of other elements by plants. Compared to the control, Cd treatment significantly reduced the levels of the nutrient in plants (**Tables 2**, **3**). It has also reduced the contents of Ca, Mg, Fe, Zn, and Mn by 49, 60, 47, 39, and 53% in cabbage leaves, respectively. Additionally, Cd treatment reduced the content of Fe and Mn by 33 and 59% in roots, respectively, but there was no difference in levels of other elements in the roots. The contents of the mineral elements in the ALA and Cd co-treated plants were lower than those of control plants, but they were generally higher than that of plants treated with Cd alone. After the exogenous application of ALA, the contents of nutrient elements in leaves and roots were similar to those of control plants.

Effects of 5-Aminolevulinic Acid on the Expression of Genes Involved in Cadmium Transport

Cadmium is absorbed from the soil by the roots, loaded into the xylem, and transported to the ground by several transporters (Han et al., 2014). Iron-regulated transporter 1 (*IRT1*) and *IRT2* are the major transporters for Fe uptake

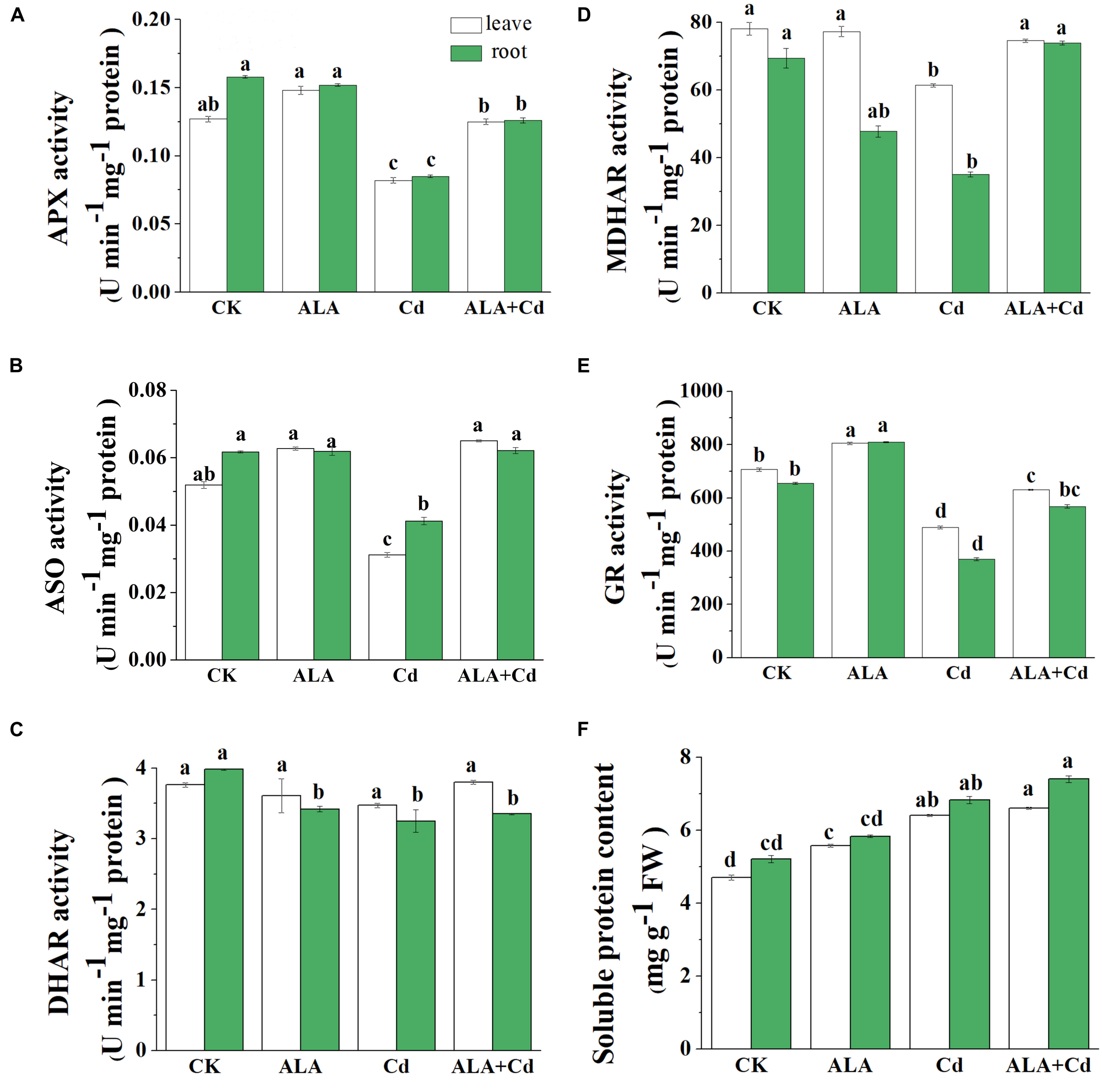

FIGURE 6 | Effects of ALA under Cd stress on the key enzyme activities of AsA-GSH cycle in cabbage. **(A)** APX; **(B)** ASO; **(C)** DHAR; **(D)** MDHAR; **(E)** GR; **(F)** Soluble protein. The seedlings were treated as in **Figure 1**. The mean ± SE are shown ($n \geq 3$). Different letters indicate significant difference among the treatments ($P < 0.05$).

(Vert et al., 2002), and they can also transport several other divalent metals, i.e., Fe, Zn, Mn, and Cd (Rogers et al., 2000). Both *Nramp1* and *Nramp3* participated in Cd transportation *via* the vacuole tonoplast (Lin and Aarts, 2012). The *HMA2* and *HMA4* are responsible for Cd translocation and distribution (Mill et al., 2003; Elif and Argüello, 2004). The effects of ALA on the ion uptake and the translocation-related genes under Cd stress were investigated. Using qRT-PCR technology, the expression level of *HMA2* and *HMA4* genes in the shoots of Chinese cabbage seedlings under Cd stress was significantly increased by 5.2 and 13.3 times, respectively, in the roots; only *HMA4* expression increased and *HMA2* expression showed no changes (**Figure 9**). The transcription level of *HMA4* genes in shoots and roots were decreased significantly under ALA + Cd treatment compared with that under Cd treatment, respectively, while *HMA2* decreased by 75% in shoots.

The expression level of the *IRT1* and *IRT2* genes in the roots of seedlings under Cd stress was significantly increased by 7.6 and 9.5 times, respectively, while in the shoots, only the *IRT1* expression increased, and *IRT2* expression showed no change. The transcription levels of *IRT1* and *IRT2* genes were significantly decreased by 1.4 and 2.5 times under ALA and Cd treatment, respectively, in the roots, while *IRT1* expression was decreased

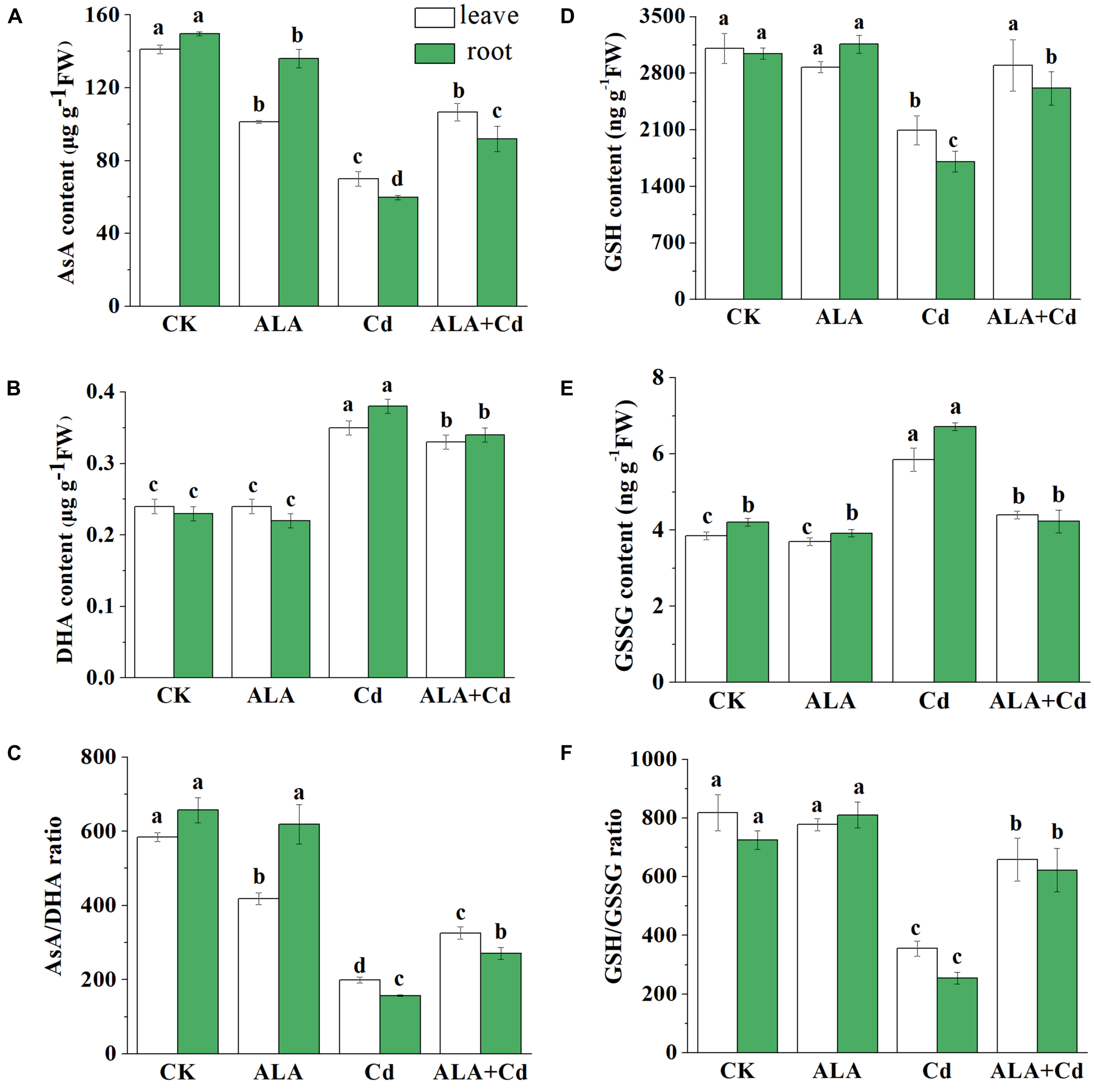

FIGURE 7 | Effects of ALA under cadmium stress on contents of AsA, DHA, GSH, and GSSG in cabbage. **(A)** AsA; **(B)** DHA; **(C)** AsA/DHA; **(D)** GSH; **(E)** GSSG; **(F)** GSH/GSSG. The seedlings were treated as in **Figure 1**. The mean ± SE are shown ($n \geq 3$). Different letters indicate significant difference among the treatments ($P < 0.05$).

by 2.6 times in shoots. Similarly, the Cd stress can upregulate the transcription level of *Nramp1* and *Nramp3* in the roots of Chinese cabbage, and *Nramp1* was also regulated in shoots. The transcription level of these genes was significantly increased under Cd stress (**Figures 9A,B**). The exogenous application of ALA can significantly decrease the expression of genes under Cd stress. Overall, there was no significant effect on these genes when the exogenous ALA was applied under the control condition. Most genes were significantly upregulated under Cd stress in shoots or roots. There were significantly downregulated in the ALA + Cd treatment compared with Cd treatment alone (**Figure 9**). These results indicated that ALA could affect Cd uptake and accumulation of Chinese cabbages by downregulating the transcription of Cd uptake and transfer-related genes.

DISCUSSION

The Cd can accumulate in the edible part of crops, and ultimately enter the food chain (Hu et al., 2013; Song et al., 2016; Rizwan et al., 2017). The Cd toxicity causes cell death, leaf roll, and chlorosis, and diminishes plant growth biomass and yield (Mombo et al., 2016). The Cd stress reduced the biomass of cabbage and shortened the primary root length (**Figure 1**). In the former studies, ALA has been proven as a new plant growth regulator due to its effects on plants at the physiological, biochemical, and molecular levels (An et al., 2019). The ALA can be effective against the harmful effects caused by abiotic stress in plants (Aksakal et al., 2017). In this study, we aimed to investigate the protective mechanism of ALA involved in Cd

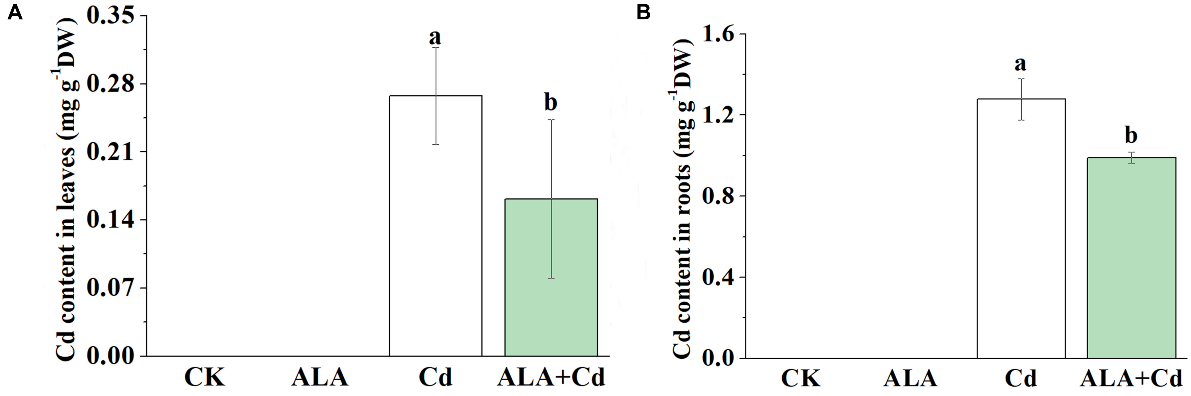

FIGURE 8 | Effects of ALA under Cd stress on content of Cd in leaves **(A)** and roots **(B)**. The seedlings were treated as in **Figure 1**. The mean ± SE are shown ($n \geq 3$). Different letters indicate significant difference among the treatments ($P < 0.05$).

stress in Chinese cabbage. In this study, Cd stress was found to decrease the Chl content in Chinese cabbage (**Figure 2**). However, ALA effectively alleviated Cd stress and increased the biomass, the length of primary roots, and the Chl contents of cabbage. The exogenous application of ALA increased the biomass and leaf Chl contents of *Brassica napus* L. seedlings under Cd stress (Xu et al., 2016). These results confirmed the protective effects of ALA in the tolerance of cabbage plants to Cd stress.

The Cd stress caused oxidative stress in vegetables by inducing the excessive accumulation of H_2O_2 (Gratão et al., 2015). The MDA is the main product of membrane system peroxidation, and it is an important index of membrane lipid peroxidation. In this study, Cd increased H_2O_2 and MDA content in cabbage leaves and roots. Exogenous application of ALA significantly decreased H_2O_2 and MDA contents to the control level (**Figure 5**). It has been reported that ALA can reduce MDA and H_2O_2 levels by enhancing the gene expressions of antioxidant enzymes in *Brassica napus* under Cd stress (Ali et al., 2013a, 2015; Wu et al., 2015, 2017). These findings provided evidence that ALA can improve the tolerance of cabbage to Cd by decreasing oxidative damage. Plants have enzymatic and non-enzymatic systems that respond to ROS in the cell when exposed to stresses, thus, reducing the damage to plant cells (Wang and Tam, 2018). The enzymatic and non-enzymatic systems can be triggered in response to Cd-induced oxidative stress, which involves a set of antioxidant enzymes and non-enzymes antioxidants. The AsA-GSH is an important active oxygen scavenging system in plants (Kaya et al., 2020). This study revealed that Cd toxicity decreased the activities of enzymatic antioxidants (APX, ASO, DHAR, MDHAR, and GR) in cabbage seedlings (**Figure 6**). Meanwhile, the application of ALA stimulated the activities of major enzymes in the AsA-GSH system. The AsA and GSH regulate plant metabolism by promoting the removal of ROS and relieving the oxidative damage on plants caused by stresses (Gill and Tuteja, 2010). It was reported that foliar application of ALA in sunflower hybrid can reduce drought-induced yield losses and improve oil content by improving the activities of catalase, superoxide dismutase, and ascorbate peroxidase (Sher et al., 2021). Under Cd stress, DHA and GSSG levels increased, but AsA and GSH levels decreased. The exogenous application of ALA promoted the exchange of DHA to AsA and increased the content of GSH and the GSH/GSSG ratio (**Figure 7**). Thus, the antioxidant capacity and redox balance of plants under stresses regulated by ALA is crucial. These results, along with previous findings (Wu et al., 2019) showed that ALA application could improve the tolerance of Chinese cabbage to Cd by increasing the non-enzymatic antioxidant level and the activity of antioxidant enzymes.

The structural integrity of the chloroplast is essential to maintaining normal growth and photosynthesis, and it can be easily subjected to oxidative stress under stress conditions. A Pn indicates the strength of organic matter accumulated through photosynthesis in plants (Lucas et al., 2013). Electron microscopy technology was used to analyze ultrastructural changes and investigate the mechanism of biosynthesis under Cd stress. Cell structure was damaged when treated with 50 μM Cd, for example, the cell wall, cell membrane intercellular spaces, and increased size and number of starch grains were observed in ruptured chloroplasts. The thylakoid membrane was dispersed and elongated under Cd treatment alone, and there was an increased number of lipid bodies (**Figure 4A**). Meanwhile, Cd stress caused Pn, Gs, and Tr to decrease and Ci to increase (**Figure 2**). The increase in starch grains under Cd stress might be due to nutrient deficiency (Daud et al., 2009). Chloroplast was pronounced and was visible upon ALA application, and there was less starch grain and plastoglobuli (**Figure 4A**). Exogenous ALA can alleviate the toxic effects of Cd in the mesophyll cell and improve the cell structure (Ali et al., 2015). Exogenous ALA can reduce the lipid bodies in the cucumber cytoplasm under salt stresses (Wu et al., 2018). The reduction in the number of lipid bodies and plastoglobuli in chloroplasts is an indication of lesser oxidative stress. Zhang et al. (2008) concluded that ALA reduced the lipid peroxidation of thylakoids by inducing the antioxidant system. The application of biological fertilizer [Arbuscular Mycorrhizal Fungi (AMF)] also decreased the cell and grain size and improved the tolerance of soybean to drought stress (Sheteiwy et al., 2021). In this study, we found that ALA

TABLE 2 | Effects of ALA on the concentrations of Fe^{2+}, Zn^{2+}, Mn^{2+}, Ca^{2+}, and Mg^{2+} in leaves under Cd stress.

Treatment mg g^{-1} DW	Fe	Zn	Mn	Ca	Mg
CK	0.1989 ± 0.0058a	0.3625 ± 0.0057a	0.3312 ± 0.0301a	2.4845 ± 0.0017a	3.8175 ± 0.0045a
ALA	0.1828 ± 0.0150a	0.3434 ± 0.0092a	0.2620 ± 0.0425a	2.5440 ± 0.0012a	3.6790 ± 0.0081a
Cd	0.1055 ± 0.0115b	0.2212 ± 0.0100b	0.1550 ± 0.0405b	1.2765 ± 0.0002c	1.5455 ± 0.0089c
Cd+ALA	0.1820 ± 0.0131a	0.3304 ± 0.0034a	0.2152 ± 0.0333a	2.2790 ± 0.0024ab	3.4285 ± 0.0029ab

Different letters indicate significant difference among the treatments.

TABLE 3 | Effects of ALA on the concentrations of Fe^{2+}, Zn^{2+}, Mn^{2+}, Ca^{2+}, and Mg^{2+} in roots under Cd stress.

Treatment mg g^{-1} DW	Fe	Zn	Mn	Ca	Mg
CK	0.7472 ± 0.0370a	0.6682 ± 0.0054a	0.3767 ± 0.0059a	0.6265 ± 0.0030a	1.1992 ± 0.0091a
ALA	0.7718 ± 0.0294a	0.5859 ± 0.0103a	0.3704 ± 0.0020a	0.6300 ± 0.0015a	1.2461 ± 0.0012a
Cd	0.5007 ± 0.0122b	0.6273 ± 0.0084a	0.1538 ± 0.0049c	0.6065 ± 0.0010a	1.0728 ± 0.0074a
Cd+ALA	0.6531 ± 0.0128a	0.6961 ± 0.0057a	0.2515 ± 0.0127ab	0.6395 ± 0.0035a	1.0497 ± 0.0484a

Different letters indicate significant difference among the treatments.

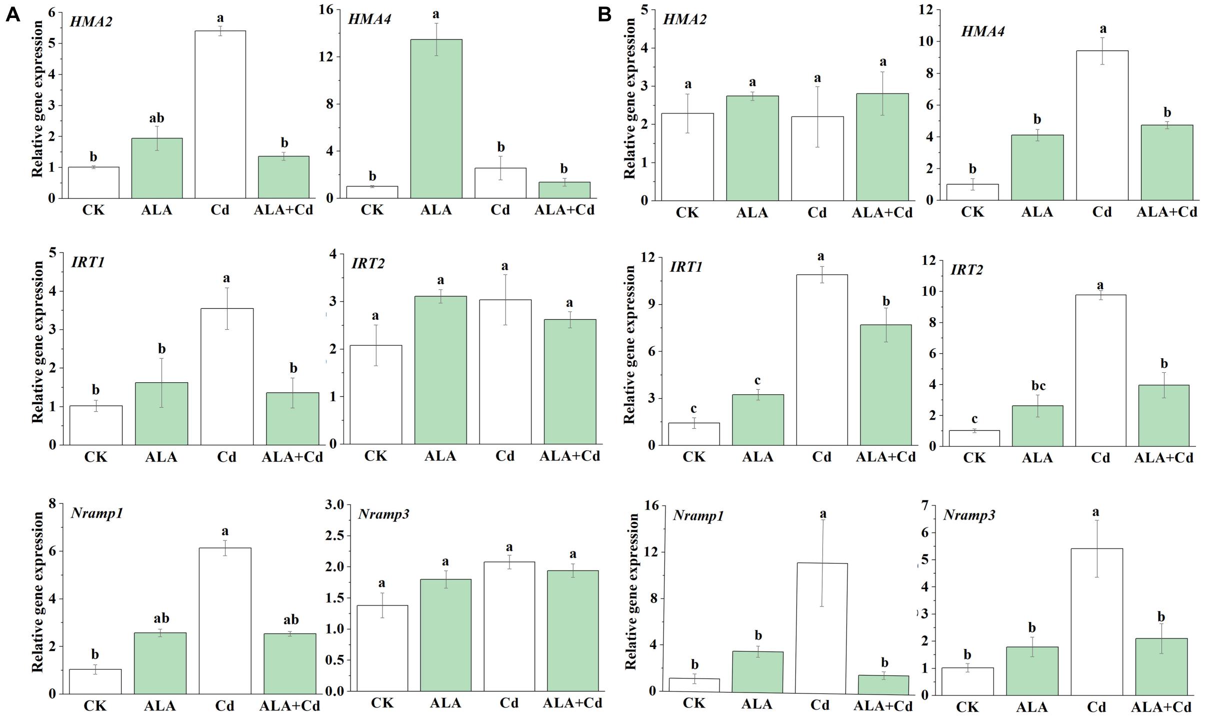

FIGURE 9 | The qRT-PCR analysis of the expression of genes involved in ion absorption and transport in Chinese cabbage seedlings in leaves **(A)** and roots **(B)**. The relative expression levels of genes are normalized to *Actin*. The seedlings were treated as in **Figure 1**. The mean ± SE are shown ($n \geq 3$). Different letters indicate significant difference among the treatments ($P < 0.05$).

improved cell form and chloroplast structure when treated either with ALA alone or in combination with Cd conditions. The ALA also significantly increased Pn, Gs, and Tr and reduced the Ci value in cabbage.

The content of Chl is directly related to the intensity of photosynthesis (Cao et al., 2013). The Cd significantly reduced Chl contents and Fv/Fm in Chinese cabbage plants (**Figure 3**). The decreased levels of Chl content and Fv/Fm might inhibit photosynthesis by affecting the regeneration and degradation of photosynthetic organs and reducing the photo energy conversion efficiency. These results are similar to the effects of low temperatures (Chen et al., 2017) or high temperatures (Wang D. Y. et al., 2018). The ALA treatment also enhanced Fv/Fm and qP under Cd stress, meanwhile, both Chl a and Chl b content were increased. The ALA can enhance Chl biosynthesis under stresses by upregulating gene expression of glutamyl-tRNA reductase (*HEMA1*), Mg-chelatase (*CHLH*), and protochlorophyllide oxidoreductase (*POR*) in the Chl synthesis

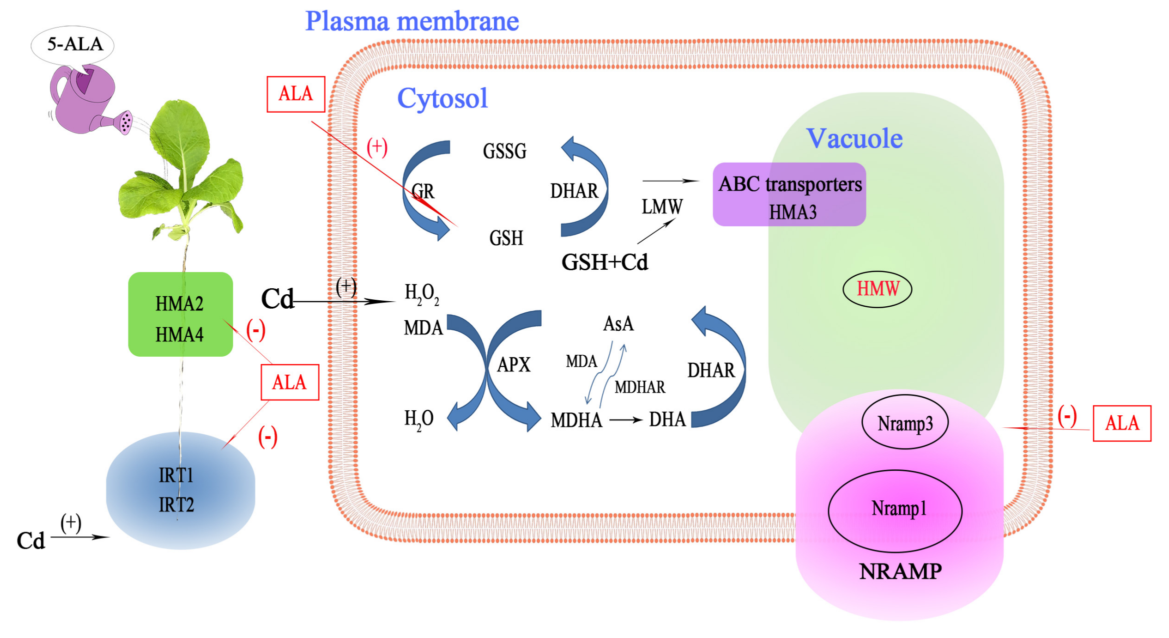

FIGURE 10 | A model illustrating the role of ALA in Chinese cabbage seedlings' tolerance to Cd stress. Cd stress increased the content of MDA and H_2O_2 in Chinese cabbage seedlings. The ALA can increase the enzyme activities of the antioxidant system, induce a large amount of GSH and AsA, and remove ROS and Cd ions in plants. Meanwhile, ALA downregulates the expression of the Cd absorption and transportation-related genes (*HMA2, HMA4, Nramp1,* and *Nramp3*), which ultimately leads to a decrease in Cd level and an increase in resistance to Cd stress. This process increases the tolerance of Chinese cabbage to Cd. (+) Positive regulation; (–) Negative regulation.

pathway (Wu et al., 2018). The ALA treatment significantly promoted the electron transfer activity of the PSII reaction center on the donor side, the reaction center itself, and the receptor site and increase the intensity of photosynthesis (Yang et al., 2021). From the above results, we concluded that exogenous ALA could repair the mesophyll cell structure, increase the utilization of CO_2, improve photosynthesis, and ultimately increase the biomass of cabbage.

The Cd stress may be attributed to the competition between nutrients and Cd for the same transporters, which inevitably disturbed the balance of mineral elements (Ramos et al., 2002). The Cd was found to impair the nutrient balance and accumulation (Matraszek et al., 2016; Wu et al., 2017). In oilseed rape and lettuce, Cd stress caused an imbalance of mineral elements (Xu et al., 2016; Dawuda et al., 2020). It was found that AMF could increase the nutrient absorption of Guar (El-Sawah et al., 2021). In this experiment, the Fe content significantly declined in leaves and roots of cabbage under Cd stress (**Tables 2, 3**). The ALA application increased Fe content in leaves and roots under Cd stress to the control level. The results showed that ALA had little effect on the contents of Zn, Ca, and Mg in the roots. Moreover, ALA improved the concentration of nutrients under Cd stress in *Brassica napus* (Xu et al., 2016). It has been found that ALA alleviated the decrease of N, P, Ca, Mg, Zn, and Fe content in rape (*Brassica napus*) under salt stress, but had no effect on Mn and Cu contents (Naeem et al., 2010). However, Liu et al. (2017) did not observe an increase in K, Fe, and Mg content alongside the improvement in alkaline tolerance of Swiss chard (*Beta vulgaris* L.) treated with ALA. Therefore, ALA can inhibit Cd uptake and improve Fe and Mn translocation from roots to leaves in cabbage. Thus, the effect of ALA on the content of mineral elements in plants may be related to species and types of stress.

The Cd-toxicity alternates the absorption of mineral nutrition for plants (Alexandre et al., 2012). The ALA could significantly reduce the Cd content in the roots and leaves of cabbage. Both *IRT1* and *IRT2* are expressed in the external cell layers of roots, specifically under Fe starvation (Vert et al., 2002). Wang reported that exogenous melatonin (MT) can decrease the expression of *IRT1* and Cd content (Wang et al., 2020). In this study, we found that ALA affected the absorption of Cd by decreasing the expression of *IRT1* and *IRT2*, thereby decreasing the content of Cd in the roots of Chinese cabbage (**Figure 8**). Similar results have been obtained in other studies. The exogenous hydrogen-rich water reduced Cd levels by suppressing the expression of *IRT1* in *Arabidopsis thaliana* (Wu et al., 2021). The *AtNramp1* can cooperate with *AtIRT1* to take up Fe^{2+} in Arabidopsis roots (Chen et al., 2019). It can be concluded that *Nramp* genes were indispensable for balanced element absorption in plants. It has been shown that overexpression of *HMA* plays a role in Cd accumulation (Takahashi et al., 2012). In this study, we showed that ALA could significantly inhibit the upregulation of *IRT1, IRT2*, *Nramp1*, *Nramp3, HMA2*, and *HMA4* transcription induced by Cd stress, which was consistent with the effect of ALA on Cd levels (**Figure 9**). Therefore, it can be inferred that spraying ALA on leaves may affect Cd uptake by downregulating the transcription of *IRT1* and *IRT2*, and inhibiting the activation of Cd in vacuole by affecting the transcription of *Nramp1* and *Nramp3*, thereby reducing the effective cytotoxic Cd concentration. The decrease in *HMA2* and *HMA4* transcription inhibits the transfer of Cd from root to shoot and improves the tolerance of Chinese cabbage to Cd stress.

CONCLUSION

In summary, exogenous ALA treatment can effectively reduce the Cd accumulation in Chinese cabbage by eliminating excess ROS and regulating Cd-induced transport genes. These results highlighted the potential function of ALA in combating Cd stress (**Figure 10**). According to the results, Cd treatment seriously inhibited the photosynthesis in cabbage, decreased antioxidant capacity, disturbed the nutrients balance, and resulted in oxidative stress. Exogenous ALA can promote the response of cabbage to Cd stress by enhancing the efficiency of light-to-energy conversion, improving Chl content, and maintaining high redox homeostasis by scavenging ROS and regulating the antioxidant enzyme activities in the AsA-GSH cycle. Moreover, ALA can also downregulate the expression of transport genes that mediate the binding to Cd, and, thus, reducing the Cd content. These changes in the physiological state and gene transcriptions reflect the adaptation of plant response to Cd stress under the exogenous ALA application. To study the precise mechanism of reduced Cd accumulation in cabbage due to ALA treatment, an environment-based design of soil research is required.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

LY, JL, and JY conceived and designed the research. LY and RW conducted the experiments. LY, XW, and ZT analyzed the data and prepared the figures and illustrations. LY wrote the manuscript. YW, SL, and LH read the manuscript and made valuable inputs. XW and BA read the manuscript and checked the language grammar. All authors read and approved the submission of the manuscript.

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Harzianopyridone Supplementation Reduced Chromium Uptake and Enhanced Activity of Antioxidant Enzymes in *Vigna radiata* Seedlings Exposed to Chromium Toxicity

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This study explains the scarce information on the role of harzianopyridone (HZRP) in the alleviation of chromium (Cr) stress alleviation in *Vigna radiata* (L.). To this end, *V. radiata* seedlings primed with HZRP at 1 and 2 ppm were exposed to 50 mg kg^{-1} Cr for 30 days. Cr stress reduced growth, chlorophyll (Chl) content, net photosynthetic rate, gas-exchange attributes along with enhanced oxidative damages, i.e., electrolyte leakage (EL), hydrogen peroxide (H_2O_2), and malondialdehyde (MDA). Application of HZRP enhanced intercellular carbon dioxide (CO_2) concentration, stomatal conductance, and net photosynthetic rate with decreased activity of the chlorophyllase (Chlase) enzyme in *V. radiata* seedlings exposed to Cr stressed conditions. To maintain Cr-induced oxidative damages, HZRP treatment increased the levels of antioxidant metabolites (phenolic and flavonoids) and the activity of antioxidative enzymes [superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD)] in *V. radiata* seedlings grown in normal and Cr-polluted potted soil. In addition to this, glycine betaine content was also increased in plants grown in Cr-contaminated soil. It is proposed the potential role of supplementation of HZRP in mitigating Cr stress. Further research should be conducted to evaluate the potential of HZRP in the mitigation of abiotic stresses in plants.

Keywords: mung bean, chromium, stress, growth, harzianopyridone

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INTRODUCTION

Excessive deposition of heavy metal toxicants in agricultural soils leads to growth retardation and hindrance of normal physiological processes in plants. Heavy metals accumulation in edible parts of plants poses various damages to animals and human health (Ihtisham et al., 2021; Zaynab et al., 2022). Chromium (Cr) is the 21st most abundant heavy metal and is regarded as one of the most potent pollutants in the environment. Cr is a transition metal and exists in two common oxidation states, i.e., hexavalent Cr (Cr^{6+}) and trivalent Cr (Cr^{3+}) (Singh et al., 2021). Cr^{6+} is more toxic, mobile, and exerts carcinogenic effects on living organisms (Bharagava and Mishra, 2018). Cr^{6+} uptake in plants is facilitated through sulfate and phosphate pathways and is easily transported to various parts of plants (Devi and Kumar, 2020). Chromates and dichromates are hexavalent Cr compounds and are mostly used in Cr stress tolerance mechanisms (Shah et al., 2020).

The accumulation of increased Cr content in soil and subsequently in different portions of plants affects plants and human health (Sharma et al., 2020). Moreover, increased Cr concentration in the soil affects the growth, photosynthesis, metabolism, biomass production, and yield of several crops (Anjum et al., 2017; Singh P. et al., 2020). For instance, higher Cr levels disturb physiological and biochemical changes in plants, leading to reduced yield and productivity (Anjum et al., 2017; Singh P. et al., 2020). In addition, the growth of mung beans is severely hampered by Cr stressed conditions (Jabeen et al., 2016; Husain et al., 2021). Cr^{6+} toxicity disturbs homeostasis in plants due to the enhanced accumulation of reactive oxygen species (ROS). Increased accumulation of Cr results in oxidative damage in plant tissues through enhanced production of hydrogen peroxide (H_2O_2), malondialdehyde (MDA) content, electrolyte leakage (EL), and ROS levels (Singh P. et al., 2020). ROS production causes oxidative stress in plants and results in oxidative modification of nucleic acids, proteins, and lipids (Huang et al., 2019). At the cellular level, Cr accumulation results in increased ROS production, as proved by an enhanced level of MDA, EL, and H_2O_2 in plants exposed to Cr toxicity (Yu et al., 2018; Patra et al., 2019). Cr (VI) toxicity causes more damage as compared with Cr (III) toxicity in plants (Beyersmann and Hartwig, 2008). Cr (VI) accumulation disturbs ROS homeostasis in plants grown in Cr polluted soil (Maqbool et al., 2018; Zaheer et al., 2019; Tirry et al., 2021). The accumulation of ROS enhances lipid peroxidation besides the oxidation of crucial biomolecules (Wakeel et al., 2019; Askari et al., 2021). ROS accumulation changes the morpho-physiology and architecture of plants facing Cr stressed conditions (Sharma et al., 2022).

To mitigate heavy metal-induced oxidative stress, plants regulate the activities of various enzymatic and non-enzymatic antioxidants. In the case of severe heavy metal toxicity, plant metabolomics is negatively affected resulting in disruption of some biomolecules, which leads to oxidative stress (Paithankar et al., 2021; Sarraf et al., 2022). However, Cr toxicity in plants depends on the concentration of Cr uptake from the rhizospheric region (Wakeel et al., 2018, 2019; Farid et al., 2019). This results in disturbed nutrient translocation in plants due to Cr binding with membranous H^+-ATPase and other carrier channels (Shahid et al., 2017). Antioxidant enzymes in plants reverse the deleterious effect of ROS produced by various mechanisms. Crucial antioxidant enzymes in plants include CAT, POD, and SOD (Zaheer et al., 2020). Cr stress affects the activity of antioxidant enzymes in plants (Zaheer et al., 2022).

Vigna radiata is a short-duration legume crop, cultivated predominately in Asia and other regions of the world (Nair et al., 2019). *V. radiata* is rich in nutritional content such as proteins, vitamins, dietary fibers, minerals, and a huge number of bioactive compounds (Hou et al., 2019).

Harzianopyridone (HZRP) is a *Trichoderma harzianum* secondary metabolite containing a penta-substituted pyridine ring system with a 2,3-dimethoxy-4-pyridinyl pattern. It is a volatile organic compound that has been reported to have active defensive mechanisms in plants and regulates growth in tomato, canola, and pea plants (Vinale et al., 2013; Stewart and Hill, 2014). In the study of Hermosa et al. (2012), HZRP may promote plant growth *via* auxin-like activity at low doses, but confer an antimicrobial effect at higher concentrations. Despite the utilization of HZRP as a promising metabolite to promote plant growth, its potential role in improving plant growth under heavy metal toxicity, e.g., Cr, is not yet evaluated. Thus, the current research was conducted to test the potential of HZRP in the alleviation of Cr toxicity and regulation of growth in *V. radiata* seedlings. To our knowledge, this study exploits the effect of HZRP on the growth and morpho-physiological characteristics of *V. radiata*.

MATERIALS AND METHODS

The experiment was conducted in the wirehouse of the Department of Botany, University of Education. A *V. radiata* cultivar, Inqalab Mung, was used during the experiment. Seeds of *V. radiata* were surface sterilized with 0.01% mercuric chloride for 5 min, followed by washing with double-distilled H_2O. For Cr toxification, $K_2Cr_2O_7$ was used during the experiment. Then, 50 mg kg^{-1} was added to the potting soil. This toxic Cr concentration refers to agricultural contaminated sites near District Lahore, Pakistan. Agricultural contaminated sites were irrigated with toxic effluents from the Hudiara drain. In the case of control (C) treatment, only distilled H_2O was added to the soil. HZRP was purchased from Sigma Aldrich. Two concentrations of HZRP were used during the experiment, i.e., 1.0 and 2.0 ppm. Seeds of *V. radiata* were primed in HZRP solutions for 2 h. A completely randomized design (CRD) was used during the experiment. After 3 weeks, the root and shoot length of *V. radiata* were determined.

Assessment of Photosynthetic Pigments

Photosynthesis pigment and other photosynthetic factors were determined. The chlorophyll (Chl) content of leaves was evaluated in a non-destructive manner throughout the experiment with a Chl meter, SPAD 402 PLUS (Minolta, Japan). Using an infrared gas analyzer (LI-6400XT, Portable Photosynthesis System, LI-COR, NE, United States), the net photosynthetic rate, intercellular carbon dioxide (CO_2) concentration, and stomatal conductance of the topmost fully developed leaves of *V. radiata* plants were recorded.

Determination of Reactive Oxygen Species Content

The methodology described by Velikova et al. (2000) was used to determine the H_2O_2 content in the leaves of *V. radiata* plants. Supernatant (0.5 ml) was mixed with 0.5 ml of 10 mM phosphate buffer (pH 7.0) having 1 ml potassium iodide following extraction in 5 ml of 1 M TCA (0.1 w/v) to determine H_2O_2. Using the extinction coefficient of 0.28 M1 cm1 and the content expressed as nmol g1 fresh weight (FW), the content of H_2O_2 was determined after taking the absorbance at 390 nm (FW).

Plant Extract and Enzyme Activity Assay

Fresh leaf samples from *V. radiata* plants were collected and mashed at 4°C in a pre-chilled pestle mortar for the enzyme assays. The 0.5-g powder was added to three volumes (w/v) of cold extraction medium buffer, which contained potassium phosphate buffer (100 mM) pH 7.0, 0.5% Triton X-100, and 1% polyvinylpyrrolidone. Following 20-min centrifugation at 15,000 g at 4°C, the supernatants were utilized in the experiments afterward. The extraction buffer was spiked with 2 mM ascorbate to estimate ascorbate peroxidase (APX). Filtered homogenates were centrifuged for 20 min at 4°C at 15,000 g. Protein and enzyme activity tests were done with the supernatants.

The activity of the enzyme chlorophyllase (Chlase) was evaluated according to the methods of McFeeters et al. (1971) and Fang et al. (1998). Using the method described by Jain and Gadre (2004), the activity of aminolevulinic acid dehydratase (ALAD) in *V. radiata* leaves was measured by quantifying the production of porphobilinogen (PBG) spectrophotometric value at 553 nm for 15 min against a zero-time control. Using this definition, one unit of enzyme activity was defined as 1 nmol of PBG produced per hour per gram (h^{-1} g^{-1}) of freshly harvested leaf weight (LW).

Spectrophotometric analysis at 560 nm was used to calculate the activity of SOD (Giannopolitis and Ries, 1977). The activity of APX was determined using the Nakano and Asada (1981) procedure.

Determination of Total Phenolic Content and Flavonoids

The Folin-Ciocalteau method was used for the estimation of total phenolic content (Ordon et al., 2006). Then, a 0.5-ml plant sample was mixed with Folin-Ciocalteau reagent (0.2 N) for 5 min and sodium carbonate (2.0 ml of 75 g/L). After 2 h, the absorbance of the reaction was carried out at 760 nm at room temperature.

The method of Sarker and Oba (2018) was used for the determination of flavonoid content in the leaf extract. Leaf extract (500 μl), methanol (1.5 ml), potassium iodide (1 M), and aluminum chloride (1 ml of 10%) were allowed to stand for half an hour in the test tube at room temperature. Absorbance was calibrated at 415 nm using a spectrophotometer (Hitachi, Tokyo, Japan).

Determination of Chromium Content

Root and shoot samples of *V. radiata* seedlings were dried in an oven and digested with the help of $HClO_4$. The Cr content in the study samples was determined with the help of an atomic absorption spectrophotometer (SpectraAA-220FS).

Statistical Analysis

The SPSS software version 20.0 was applied for the analysis of the variance of the obtained data. The mean values obtained during the research were compared by employing Duncan's multiple range test (DMRT) at $p \leq 0.05$. The data depicted are mean ± *SE*, where $n = 5$.

RESULTS

Harzianopyridone Increased Growth of *Vigna radiata* Grown Under Control and Chromium Stress Conditions

Table 1 shows that Cr stress reduced root and shoot length by 57 and 38%, respectively, as compared with C-treated *V. radiata* seedlings. In contrast, HZRP priming increased the root, and shoot length of treated seedlings in normal as well as Cr-polluted soil. In the case of soil spiked with Cr, HZRP, mainly at a concentration of 2 ppm, enhanced root and shoot length by 85 and 33% as compared to Cr-only treatment.

Harzianopyridone Reduced Chromium Accumulation in the Content in Root and Shoot of *Vigna radiata*

Harzianopyridone priming reduced Cr uptake in *V. radiata* seedlings exposed to Cr stress. A high Cr value was found in the roots of *V. radiata* seedlings exposed to Cr-alone treatment (**Table 2**). Priming with HZRP at 2 ppm reduced Cr content in the root and shoot of *V. radiata* seedlings by 80.7 and 78.9%, respectively, in comparison with Cr-treatment.

TABLE 1 | Effect of harzianopyridone (HZRP) on root and shoot length of *Vigna radiata* exposed to chromium (Cr) stress.

Treatments	Root length (cm)	Shoot length (cm)
C	11 ± 0.89bc	29 ± 1.76bc
Cr	7 ± 0.54d	21 ± 1.05d
HZRP1	14 ± 1.03ab	33 ± 2.18b
HZRP2	15 ± 0.72a	38 ± 2.89a
Cr + HZRP1	10 ± 0.38c	25 ± 1.27cd
Cr + HZRP2	13 ± 1.28b	28 ± 1.47c

Different letters indicate significant differences among the treatments ($p \leq 0.05$). Cr, 50 mg kg^{-1}, HZRP1, 1 ppm; HZRP2, 2 ppm.

TABLE 2 | Effect of HZRP on Cr content in the root and shoot of *V. radiata* exposed to Cr stress.

Treatments	Cr content in root ($\mu g\ g^{-1}$ DW)	Cr content in shoot ($\mu g\ g^{-1}$ DW)
C	ND	ND
Cr	1.97 ± 0.056a	0.34 ± 0.034a
HZRP1	ND	ND
HZRP2	ND	ND
Cr + HZRP1	1.17 ± 0.029b	0.21 ± 0.073ab
Cr + HZRP2	1.09 ± 0.062bc	0.19 ± 0.081b

Different letters indicate significant differences among the treatments ($p \leq 0.05$). Cr, 50 mg kg^{-1}, HZRP1, 1 ppm; HZRP2, 2 ppm.

Harzianopyridone Improved Chlorophyll Metabolism and Photosynthetic Reactions in *Vigna radiata*

Chromium stress reduced the net photosynthetic rate in *V. radiata* seedlings by 29% as compared with the control treatment. On the other hand, HZRP supplementation increased the net photosynthetic rate in *V. radiata* seedlings grown in normal and Cr-toxificated soil. In contrast, 2 ppm of HZRP increased the net photosynthetic rate by 47.05% in *V. radiata* seedlings grown in Cr-toxic soil, in comparison with Cr-only treatment. To understand the bases of increased photosynthesis, stomatal, and non-stomatal parameters were measured. At the stomatal level, HZRP at 2 ppm significantly enhanced stomatal conductance and intercellular CO_2 concentration in *V. radiata* seedlings grown in normal and Cr-toxificated soil, as compared with the Cr-only treatment (**Figure 1**). Regarding the non-stomatal parameters, Cr toxicity reduced Chl content in *V. radiata* seedlings. However, 1 and 2 ppm of HZRP enhanced Chl content by 35 and 45%, respectively. In the case of *V. radiata* seedlings grown in Cr-toxic conditions, HZRP (2 ppm) enhanced Chl content by more than onefold in comparison with Cr-only treatment. Contrarily, Cr stress significantly increased the Chlase activity in *V. radiata* seedlings. Both applied concentrations of HZRP decreased the Chlase activity in *V. radiata* seedlings grown in Cr-contaminated soil.

Harzianopyridone Mitigated Chromium Stress-Induced Oxidative Damage in *Vigna radiata*

Chromium can induce oxidative stress on the plant by the generation of ROS. Consequently, the destruction of membrane lipids under cobalt stress could increase the MDA content and EL. Here, Cr toxicity increased MDA content, EL, and H_2O_2 content by 36, 60, and 52%, respectively, as compared with control-treated *V. radiata* seedlings. Interestingly, HZRP supplementation reduced MDA content and EL in *V. radiata* seedlings grown in normal and Cr-exposed soil. A high concentration of HZRP (2 ppm) significantly reduced MDA content in *V. radiata* as compared with Cr-only treatment. In the case of *V. radiata* seedlings grown in Cr-toxificated soil, HZRP (2 ppm) treatment reduced MDA content by > 1-fold in comparison with Cr-only treatment. HZRP (2 ppm) treatment also reduced EL and H_2O_2 content in *V. radiata* seedlings grown in Cr-polluted soil (**Figure 2**).

Harzianopyridone Improved the Redox Status of Chromium Stressed *Vigna radiata* Seedlings

To cope with stress conditions, plants might induce antioxidants, which could play a role in mitigating the detrimental effects of heavy metal stress. In addition, HZRP might contribute to increasing the antioxidant metabolites levels and antioxidant enzyme activities to reduce the oxidative stress under Cr stress. Our results indicated that Cr stress escalated the activity of antioxidant enzymes (SOD, CAT, and POD) in *V. radiata* seedlings. The two priming concentrations of HZRP (1 and 2 ppm) significantly increased SOD, CAT, and POD activities in *V. radiata* seedlings grown in Cr-toxificated soil, in comparison to the Cr-only treatment. In the case of normal soil, priming of 2 ppm HZRP increases SOD activity by 42% in *V. radiata* seedlings grown in normal soil. It also increased SOD activity by 34% in *V. radiata* seedlings grown in Cr-polluted soil. Both the two priming concentrations of HZRP (1 and 2 ppm) also escalated POD activity in *V. radiata* seedlings grown in normal and Cr-contaminated potted soil. In the case of *V. radiata* seedlings grown in Cr-toxificated soil, HZRP increased the POD activity by 44% in comparison with the Cr-only treatment. Furthermore, they increased the activity of the CAT enzyme in *V. radiata* seedlings grown in normal and Cr-polluted potted (**Figure 3**).

At the antioxidant metabolic level, Cr stress decreased total phenolic content (33%) in *V. radiata* seedlings as compared to C-treatment. Contrarily, Cr stress enhanced flavonoid content by 51% as compared to *V. radiata* seedlings grown in the control treatment. Priming with 2 ppm of HZRP significantly enhanced total phenolic content in normal and Cr-contaminated soil as compared with C-treatment. It also increased flavonoid content by 56% in *V. radiata* seedlings grown in Cr-polluted potted soil. Similarly, priming with both concentrations of HZRP significantly increased glycine betaine content in *V. radiata* seedlings grown in normal and Cr-polluted potted soil (**Figure 4**).

DISCUSSION

Globally, Cr pollution in the environment is one of the key reasons for the deterioration in ecosystem sustenance. Cr is one of the toxic heavy metals with hazardous effects on plants and human health. During this study, the effect of Cr on growth and physicochemical parameters was also investigated. Amin et al. (2013) reported that Cr stress reduced seed germination in *Hibiscus esculentus* and other legume crops.

Chromium toxicity reduced growth, net photosynthetic rate, and gas exchange attributes in *V. radiata* seedlings. Alamri et al. (2020) also reported an increase in the activation of Chl degrading enzyme Chlase in tomato seedlings exposed to hexavalent Cr stress. During this study, **Figure 5** shows that Cr toxicity enhanced Chlase activity, which reduced Chl

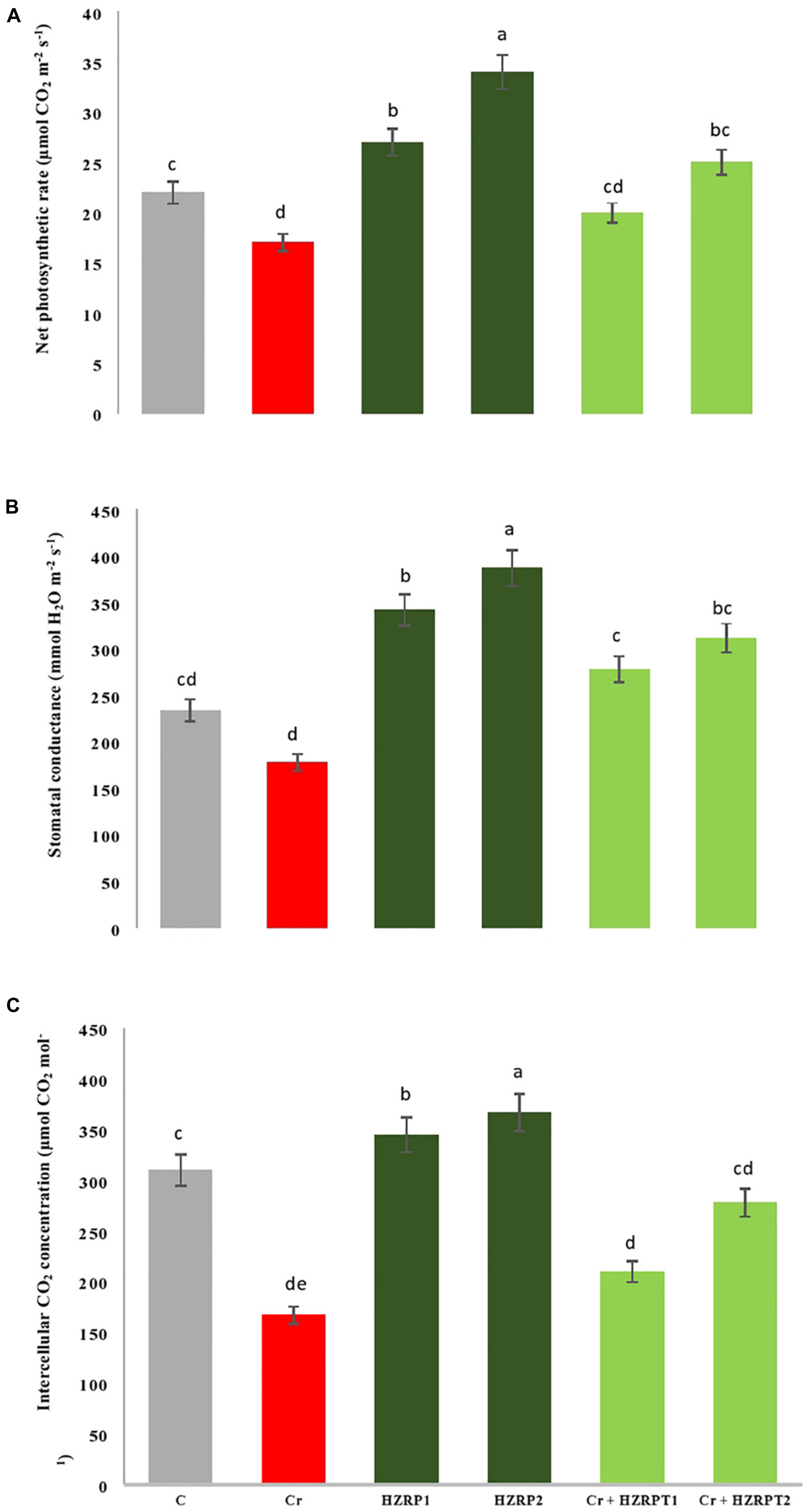

FIGURE 1 | Effect of harzianopyridone on net photosynthetic rate **(A)**, stomatal conductance **(B)** and intercellular CO_2 concentration **(C)** in *V. radiata* seedlings grown in Cr toxificated soil. Different letters indicate significant difference among the treatments ($p < 0.05$). Cr, 50 mg kg^{-1}, HZRP1, 1 p.p.m; HZRP2, 2 p.p.m.

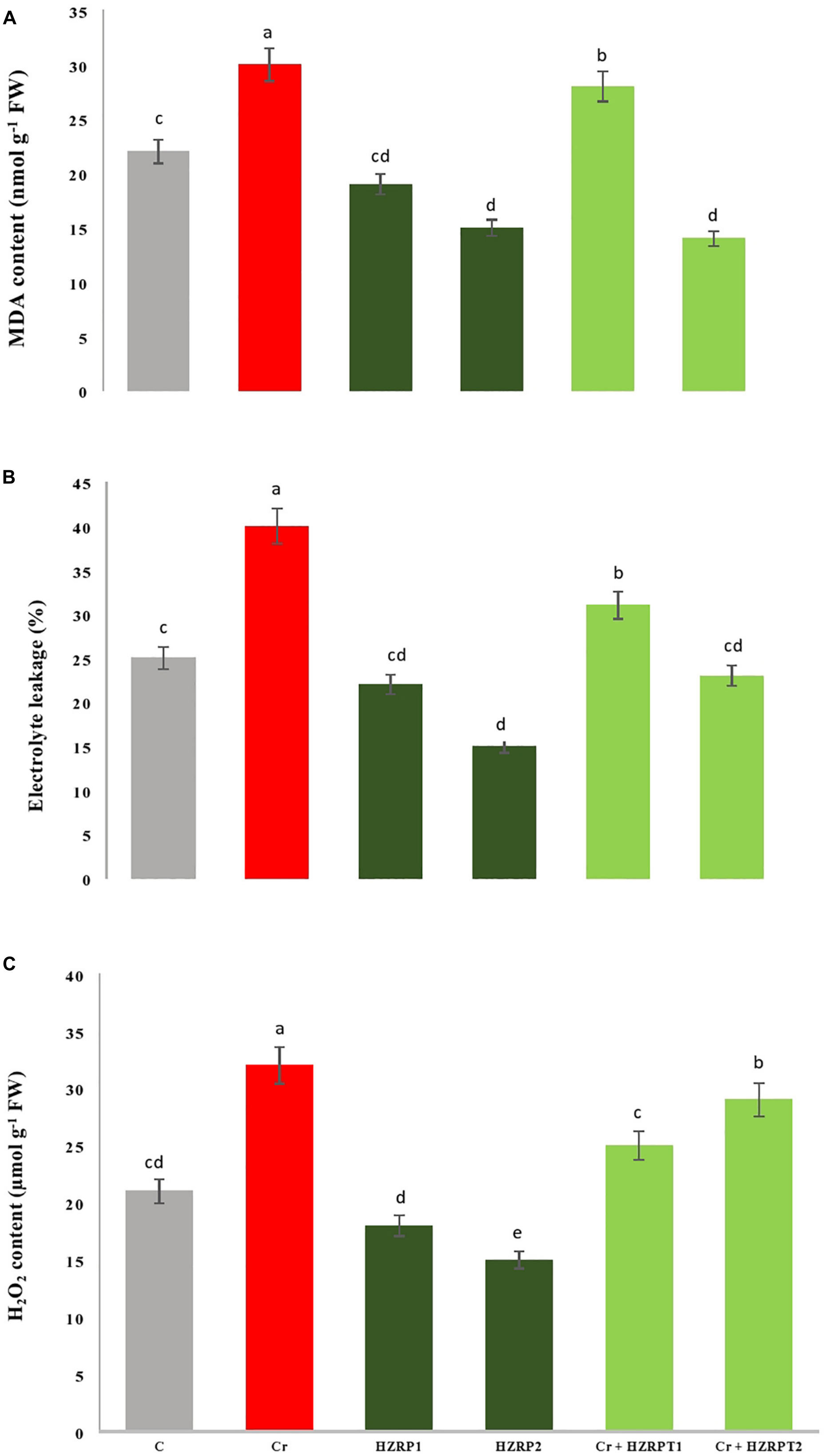

FIGURE 2 | Effect of HZRP on malondialdehyde content **(A)**, electrolyte leakage **(B)** and hydrogen peroxide content **(C)** in *V. radiata* seedlings grown in Cr toxificaled soil. Different letters indicate significant difference among the treatments ($p < 0.05$). Cr, 50 mg kg^{-1}, HZRP1, 1 p.p.m; HZRP2, 2 p.p.m.

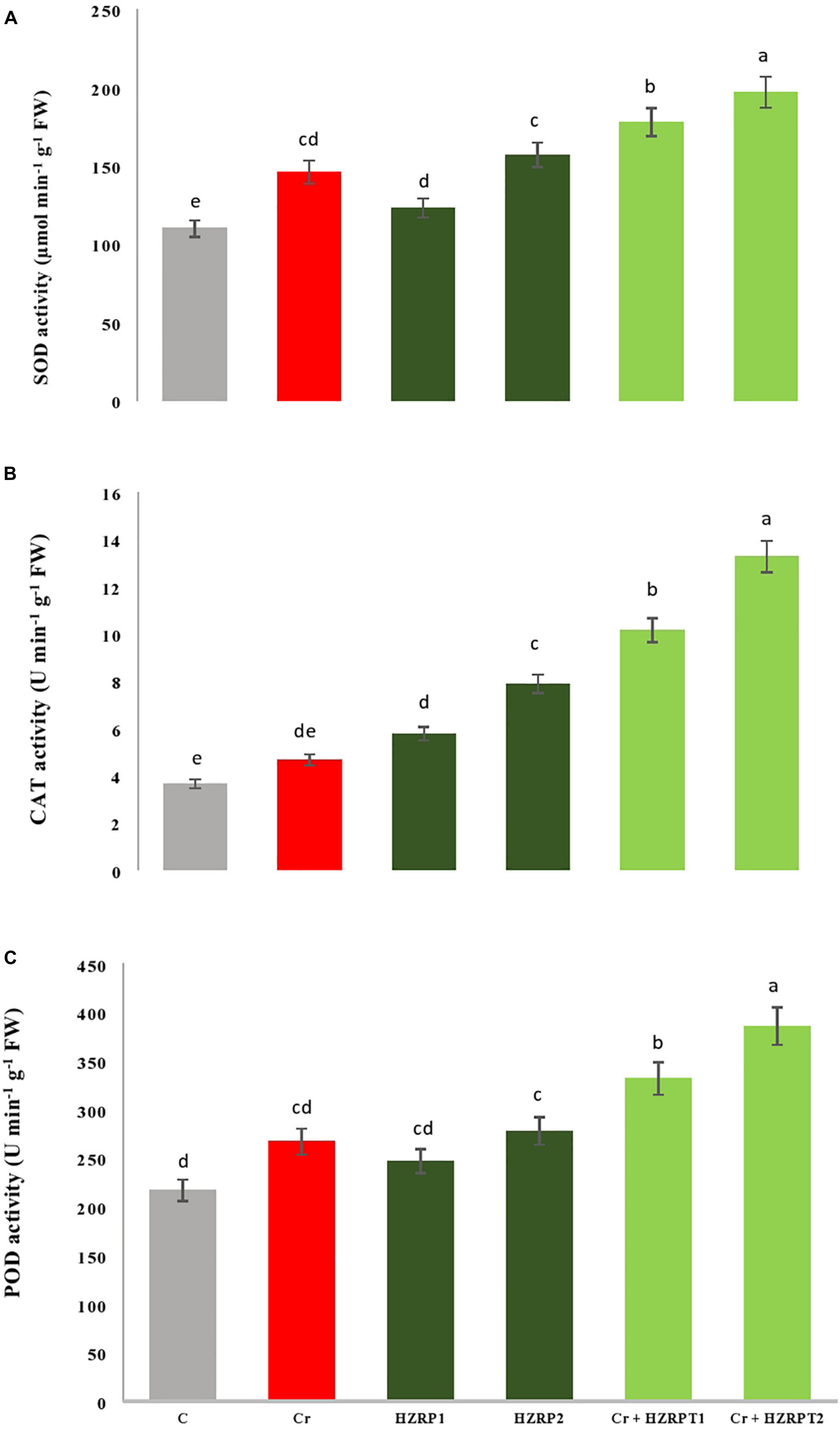

FIGURE 3 | Effect of HZRP on SOD **(A)**, CAT **(B)** and POD **(C)** activity in *V. radiata* seedlings grown in Cr toxificaled soil. Different letters indicate significant difference among the treatments ($p < 0.05$). Cr. 50 mg kg^1, HZRP1, 1 p.p.m; HZRP2. 2 p.p.m.

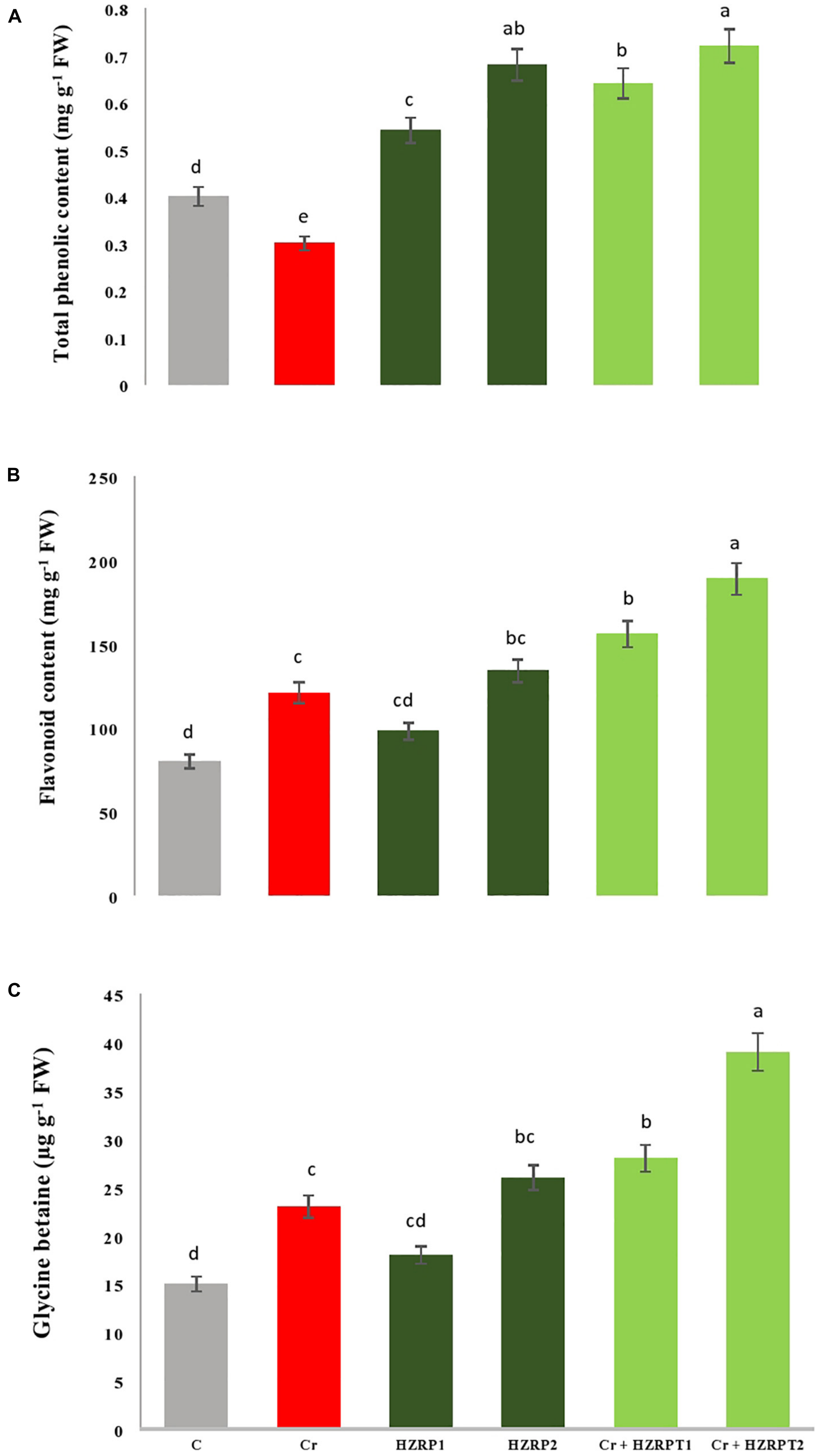

FIGURE 4 | Effect of HZRP on total phenolic content **(A)**, flavonoids **(B)** and glycine betaine **(C)** content in *V. radiata* seedlings grown in Cr toxificated soil. Different letters indicate significant difference among the treatments ($p < 0.05$). Cr, 50 mgkg^{-1}, H7.RP1, 1 p.p.m; HZRP2. 2 p.p.m.

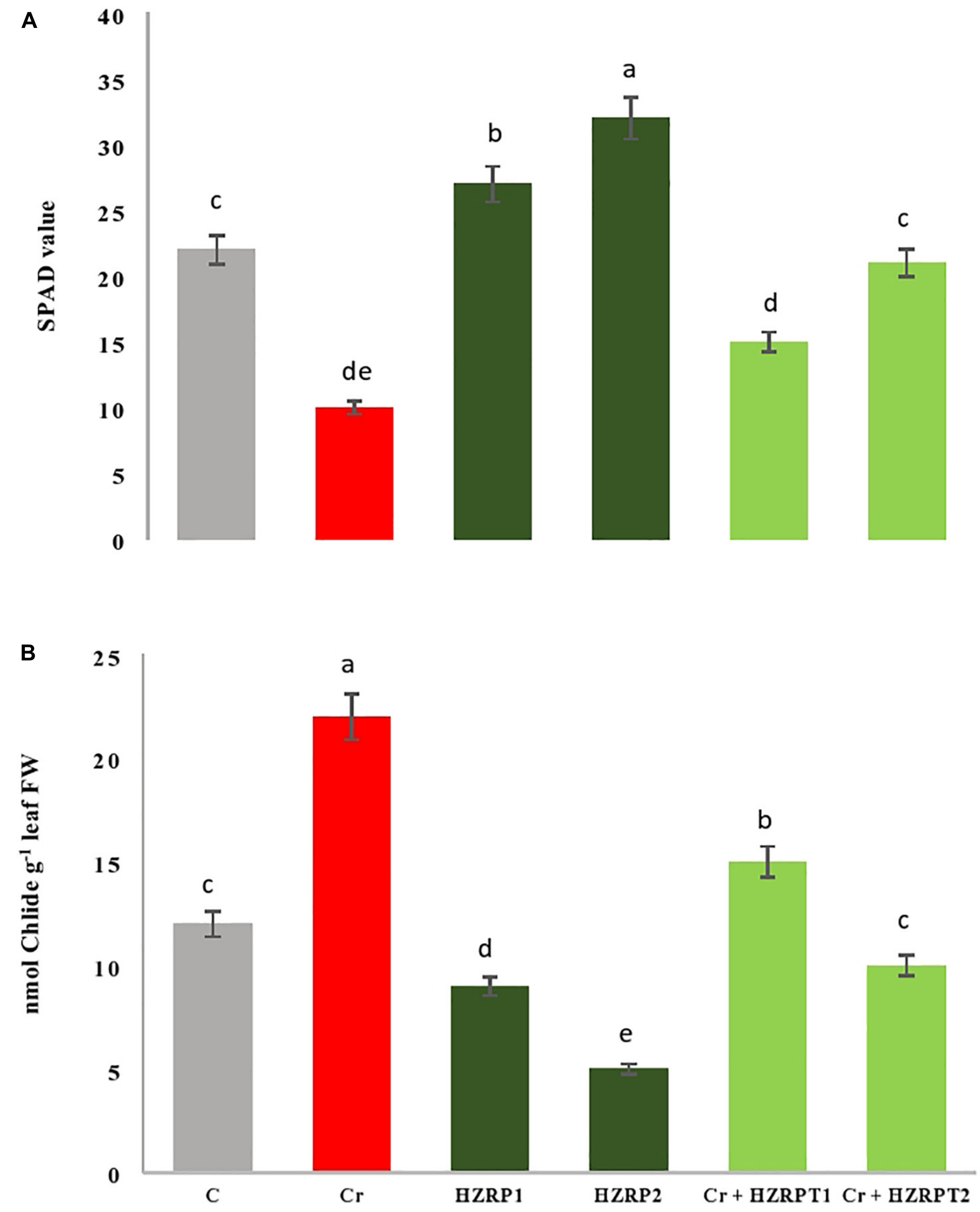

FIGURE 5 | Effect of harzianopyridone on Ch1 content **(A)** and chlorophyllase activity **(B)** in *Vigna radiata* seedlings grown in Cr toxificated soil. Different letters indicate significant difference among the treatments ($p < 0.05$). Cr, 50 mg kg^{-1}, HZRP1, 1 p.p.m; HZRP2, 2 p.p.m.

content, leading to a decrease in net photosynthetic rate and photosynthate production in *V. radiata* seedlings.

Increased levels of MDA, EL, and H_2O_2 advocated oxidative stress in mung beans. Increased content of these oxidative stress markers disturbed the equilibrium between the antioxidative defensive approach and ROS accumulation. Similar results are reported in *Zea mays* (Anjum et al., 2017), *Brassica napus* (Gill et al., 2016), and *Cicer arietinum* (Singh D. et al., 2020).

Accumulation of H_2O_2 in plants is a crucial stress marker that results in oxidative stress in plants (Sharma et al., 2012). At higher concentrations, H_2O_2 disturbs the crucial physiological processes in plants, such as photosynthesis, respiration, stomatal conductance, and cellular development (Noctor et al., 2002). Current research showed an increase in H_2O_2 content in *V. radiata* seedlings exposed to Cr stress. This increase in H_2O_2 content resulted in a disturbance in normal physiological processes in *V. radiata* seedlings. Contrarily, HZRP treated seedlings showed reduced H_2O_2 levels.

Figure 3 shows that HZRP treatment enhanced the activities of SOD, CAT, and POD in *V. radiata* seedlings in normal and Cr-toxic conditions. Exogenous application of 2 ppm HZRP significantly increased the activity of the antioxidant enzyme, which reduced MDA, EL, and H_2O_2 content in treated *V. radiata* seedlings. SOD is an important line of

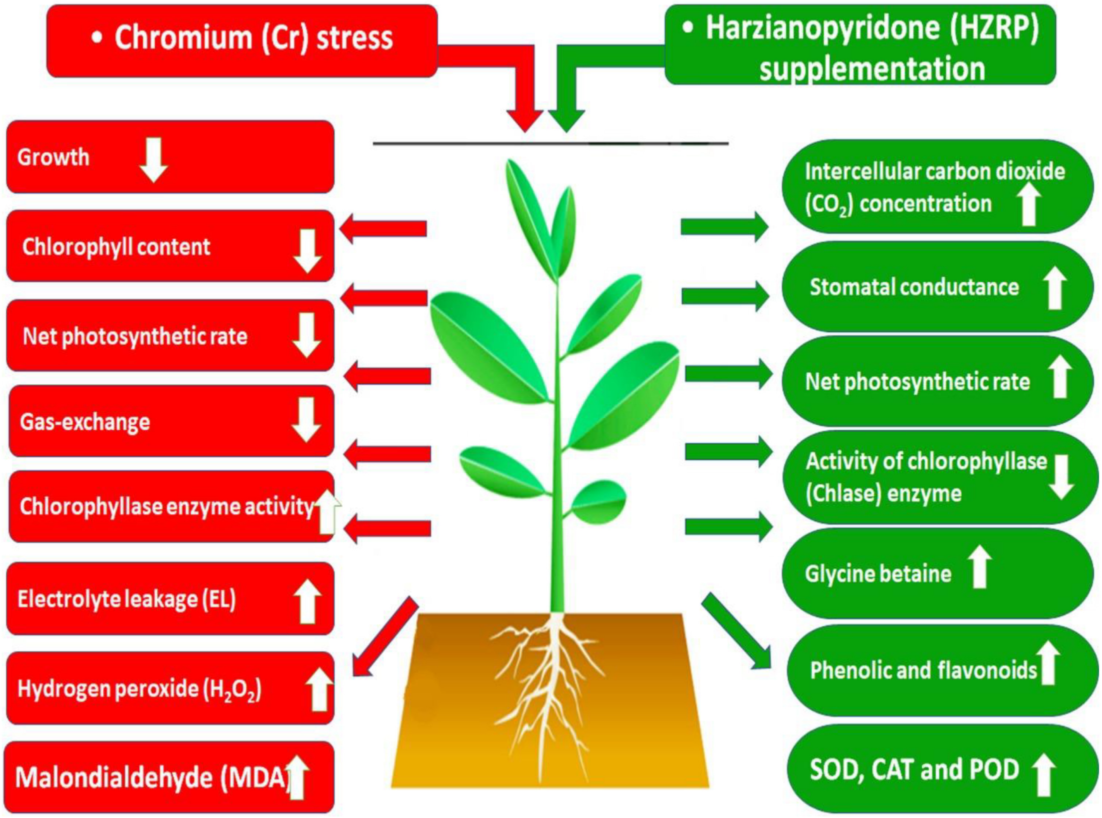

FIGURE 6 | Schematic model explaining the role of HZRP in alleviation of Cr stress in *V. radiata* seedlings.

defense in plants against stresses. SOD converts $O^{\cdot -}_2$ into O_2 and H_2O_2. This conversion reduces $OH^{\cdot}$ formation. The activity of SOD is found to be upregulated in plants facing stressed conditions (Das and Roychoudhury, 2014). Current research reveals that HZRP application increased the activity of antioxidative enzymes (SOD, CAT, and POD) in *V. radiata* seedlings exposed to Cr stress.

Flavonoids are crucial for stress responses in plants. Flavonoids are involved in the scavenging of ROS produced in plants exposed to stress (Di Ferdinando et al., 2012). These secondary metabolites are involved in the stabilization of photosynthetic apparatus in plants (Stefanov et al., 2021). Glycine betaine is a crucial organic osmolyte that plays a pivotal role in mediating osmotic balance in plants facing stressed conditions (Ashraf and Foolad, 2007). This study showed HZRP-treated seedlings showed an increase in flavonoid content, which might have reduced ROS, thereby leading to Cr stress alleviation in *V. radiata* seedlings exposed to Cr stress.

Glycine betaine is reported to alleviate numerous abiotic stresses in sorghum (Kumar, 2021). The present study revealed that HZRP treatment alleviated Cr toxicity in *V. radiata* seedlings. This alleviation in Cr stress might be due to a reduction in Cr uptake in treated seedlings. It means that MDA and oxidative stress markers were not increased, which maintained Chl content, net photosynthetic rate, stomatal conductance, and intercellular CO_2 concentration in *V. radiata* seedlings exposed to Cr stress. Aamer et al. (2018) reported that foliar application of glycine betaine alleviated Cd stress in *Spinacia oleraceae* through a reduction in Cd uptake and increased the activity of the antioxidative defensive system.

The plant has developed various adaptations to detoxify Cr content, such as Cr uptake (Shahid et al., 2017). Similar results have been reported in *Oryza sativa* (Chen et al., 2017), *Arabidopsis thaliana* (Wakeel et al., 2019), and *Brassica juncea* (Handa et al., 2018). Current studies depicted that HZRP supplementation reduced Cr uptake in *V. radiata* seedlings. This reduced Cr uptake regulated the growth and physiochemical properties of treated seedlings.

CONCLUSION

Chromium stress reduced the growth of *V. radiata* seedlings in potting soil. Cr stress decreased Chl content, net phostosynthetic rate, besides an increase in Chlase activity. Increased levels of EL, MDA, and H_2O_2 were observed in *V. radiata* seedlings exposed to Cr-toxificated soil. Contrarily, HZRP increased the activities of SOD, CAT, and POD in *V. radiata* seedlings. Apart from this, HZRP increased total phenolic content, flavonoids, and glycine betaine level in treated seedlings. Moreover, HZRP supplementation reduced Cr uptake in *V. radiata* exposed seedlings exposed to Cr stress. It is proposed that HZRP and other volatile organic compounds may be exploited for abiotic stress tolerance in plants (**Figure 6**).

DATA AVAILABILITY STATEMENT

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

AAS and ANS: experimentation and validation. MB: research design. AA and SJ: statistical analysis. MR: validation. SA, HA, and TJ: Research design and review and drafting. SSA, AT, and HMK: Funding acquisition and review and drafting. All authors contributed to the article and approved the submitted version.

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Exogenous Gamma-Aminobutyric Acid Application Induced Modulations in the Performance of Aromatic Rice Under Lead Toxicity

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Gamma-aminobutyric acid (GABA) is a non-protein amino acid and has a multi-functional role in abiotic stress tolerance. A pot experiment was conducted to assess the role of exogenous gamma-aminobutyric acid (GABA) application to modulate the growth, yield, and related physio-biochemical mechanisms in two aromatic rice cultivars, that is, Guixiangzhan (GXZ) and Nongxiang 18 (NX-18), under Pb toxic and normal conditions. The experimental treatments were comprised of Ck: without Pb and GABA (control), GABA: 1 mM GABA is applied under normal conditions (without Pb), Pb + GABA: 1 mM GABA is applied under Pb toxicity (800 mg kg^{-1} of soil), and Pb= only Pb (800 mg kg^{-1} of soil) is applied (no GABA). The required concentrations of GABA were applied as a foliar spray. Results revealed that Pb stress induced oxidative damage in terms of enhanced malondialdehyde (MDA), electrolyte leakage (EL), and H_2O_2 contents, while exogenous GABA application improved leaf chlorophyll, proline, protein and GABA contents, photosynthesis and gas exchange, and antioxidant defense under Pb toxicity in both rice cultivars. Moreover, glutamine synthetase (GS) and nitrate reductase (NR) activities were variably affected due to GABA application under Pb stress. The yield and related traits, that is, productive tillers/pot, grains/panicle, filled grain %, 1,000-grain weight, and grain yield were 13.64 and 10.29, 0.37% and 2.26%, 3.89 and 19.06%, 7.35 and 12.84%, and 17.92 and 40.56 lower under Pb treatment than Pb + GABA for GXZ and NX-18, respectively. Furthermore, exogenous GABA application in rice reduced Pb contents in shoot, leaves, panicle, and grains compared with Pb-exposed plants without GABA. Overall, GXZ performed better than NX-18 under Pb toxic conditions.

Keywords: antioxidants, GABA, lead, photosynthesis, rice

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INTRODUCTION

Environmental pollutants are well known due to their toxic effects on plants and the extent of these effects is generally dependent on their concentration, persistence, speciation, and toxicity potential (Ashraf et al., 2020; Aslam et al., 2021; Huang et al., 2021). Pollutants commonly enter into the plant systems *via* roots (from the soil) or direct depositions externally from the open environment Soil and/or air contamination with such pollutants are due to continued usage and waste emission from industries, contaminated wastewater or sewage sludge application to agricultural lands, mining activities, wide use of automobiles, and chemical usage in agriculture (Farid et al., 2020). Lead (Pb) is known as the most potent pollutant (Tian et al., 2014; Khan et al., 2016) and its eco-toxicological manifestations for plants are widely reported in previous literature (Cheng and Hu, 2010; Ashraf et al., 2015, 2017a). According to the (United States Geological Survey, 2009), the highest recoverable Pb from mining was generated by China followed by Australia and USA with 1,690, 516, and 400 thousand metric tons of annual production.

Lead (Pb) often has serious consequences for plants from morpho-physiological and genetic levels to growth, yield, and quality produce (Guedes et al., 2021). For example, Pb inhibits root/shoot growth and causes photosynthetic capacities, chloroplastic ultra-structures, chlorophyll biosynthesis, nutritional imbalance, and yield and quality traits (Maestri et al., 2010; Ali et al., 2014b,c; Ashraf and Tang, 2017). The presence of Pb in soil and its subsequent uptake by plants is substantially affected by plant and soil factors as well as forms of Pb in soil solution (Tangahu et al., 2011; Ashraf et al., 2015); however, the availability of free Pb^{2+} ions in the soil solution is often assessed by the participation of Pb in adsorption/desorption processes within the soil (Vega et al., 2010). Plant roots are the first organs that come into contact with soil Pb, which then transfers to above-ground plant parts and damages the physio-biochemical mechanisms as well as yield and food quality deterioration in plants (Uzu et al., 2010; Ashraf et al., 2020; Ma et al., 2021).

Rice is a major cereal crop in many Asian countries (Abid et al., 2015), of which fragrant rice is well-famous owing to have its special aroma and excellent cooking qualities (Singh et al., 2000; Bryant and McClung, 2011; Li M. et al., 2016); however, soil contamination with toxic heavy metals especially Pb may cause substantial reduction in growth and productivity of aromatic rice.

Gamma-aminobutyric acid (GABA) is often involved in signaling transduction mechanisms in plants under different abiotic stresses such as chilling, heat, osmotic, respiratory stress, and so on (Yang et al., 2011; Vergara et al., 2013; Nayyar et al., 2014; Vijayakumari and Puthur, 2016), as well as modulates numerous physio-biochemical responses that help plants to cope against these stresses. GABA-induced signaling against abiotic stresses is also associated with the activation of the antioxidative defense system, osmoregulation, pH maintenance, and buffering tool for C and N metabolism (Bouche and Fromm, 2004; Li W. et al., 2016). To the best of our knowledge, the GABA-induced regulations in physio-biochemical processes and yield of aromatic rice under Pb toxicity have never been studied. Therefore, the current study was conducted to assess the GABA-induced modulations in the performance of aromatic rice under normal and Pb toxic conditions.

MATERIALS AND METHODS

Experimental Setup

A pot experiment was conducted in a rain-protected net house at Experimental Research Area, College of Agriculture, South China Agricultural University, Guangzhou, China (23°09′ N, 113°22′ E, and 11 m above the sea level) from April to July 2016. Seeds of two aromatic rice cultivars, that is, Guixiangzhan (GXZ) and Nongxiang-18 (NX-18), were obtained from the College of Agriculture, South China Agricultural University, Guangzhou, China. The seeds were surface sterilized with $NaClO_4$ (5% sol.) for 10 min, rinsed with distilled water, and then dipped in tap water overnight to germinate. The germinated seeds were then sown in soil-filled plastic trays (specially designed for nursery raising), placed on a leveled field, and carefully covered with a plastic sheet. On the other hand, the plastic pots (height: 25 cm; diameter: 32 cm) were filled with 10 kg of air-dried soil having 79–80 mg kg^{-1} available N, 9.0–9.5 mg kg^{-1} available P, and 120–125 mg kg^{-1} available K, 20–22 g kg^{-1} organic matter, 5.58 soil pH (moderately acidic), and 45 ± 5 mg kg^{-1} soil Pb contents. Before transplanting, the $Pb(NO_3)_2$ salt was added in solution form into the soil at the required concentration (800 mg kg^{-1} of soil) and mixed (Ashraf and Tang, 2017) 10 days before transplanting. The pots were applied with water with 3–4 cm water layer above the soil till transplantation of rice seedlings. Uniformly grown and well-developed 25 days old rice seedlings were transplanted into the pots with five hills per pot and three to four seedlings per hill. The experimental treatments were comprised of Ck: without Pb and GABA (control), GABA: 1 mM GABA is applied under control conditions (without Pb), Pb + GABA: 1 mM GABA is applied under Pb toxicity (800 mg kg^{-1} of soil), and Pb= only Pb (800 mg kg^{-1} of soil) is applied (no GABA). The GABA at 1 mM was exogenously applied uniformly at the active tillering stage (3 weeks after transplanting) till the runoff through leaves. The optimized GABA levels are based on previous studies by Nayyar et al. (2014) who used a range of GABA concentrations (0.25–2 mM) and found 1 mM as the most suitable one for rice under stress conditions. Moreover, the NPK was applied at 2.30, 3.50, and 1.50 g to all pots, and water was applied regularly to keep the soil saturated with water.

Sampling and Data Collection

Plant leaves were sampled randomly at 7, 14, and 21 days after treatment (DAT) and stored at −80°C for plant physio-biochemical characters and chlorophyll contents, whereas for Pb quantification, the plants were sampled at late vegetative (VEG), panicle heading (PH) and maturity (MAT) stages, oven-dried, ground into powder form, and stored for digestion purpose.

Biochemical Assays

The contents of hydrogen peroxide (H_2O_2) were assessed according to Velikova et al. (2000) and the final H_2O_2 contents were denoted as μmol g^{-1} fresh weight (FW). The contents of malondialdehyde (MDA) were determined according to (Hodges et al., 1999) and the final contents were presented as μmol g^{-1} FW with the formula:

$$\text{MDA Contents (umol } g^{-1} \text{ FW)} = \{645\,(OD532 - 600) - (0.56OD450)\} \quad (1)$$

Electrolyte leakage (EL) was determined according to (Valentovic et al., 2006):

$$(\%) = \frac{EC1}{EC2} \times 10EL\,0 \quad (2)$$

Leaf chlorophyll contents, that is, Chl a, Chl b, and carotenoids were estimated according to (Arnon, 1949) and the absorbance was recorded at 665, 649, and 470 nm. Leaf proline contents were estimated according to Bates et al. (1973) by using ninhydrin whereas the protein contents in leaves were estimated according to the methods advised by (Bradford, 1976) using G-250. The GABA contents in leaves were estimated according to Zhao et al. (2009). In brief, fresh leaves (0.2 g) were homogenized in 5 mL of 60% ethanol and then put in an oscillator (HZS-H, China) for 4 h at 200 oscillations min^{-1}, centrifuged at 8,000 rpm for 5 min, and 1 ml of it was mixed with 0.6 ml of 0.2 mol l^{-1} sodium tetraborate, 2 ml of 5% phenol, and 1 ml of 7% sodium hypochlorite, and heated at 100°C and then cooled down in water bath. The absorption of the reaction mixture was read at 645 and the GABA contents were determined and expressed as μg g^{-1} FW.

Fresh leaves samples (0.3 g) were homogenized in 6 ml of 50 mM sodium phosphate buffer (pH 7.8) with pestle and mortar and the homogenate was centrifuged at 8,000 rpm for 20 min at 4 C. The supernatant was then used to estimate the antioxidant enzyme activities.

Superoxide dismutase (SOD, EC 1.15.1.1) was determined according to Zhang et al. (2008) by following the inhibition of photochemical reduction due to nitro blue tetrazolium (NBT). The SOD activity per unit was the amount of enzyme required to inhibit NBT photochemical reduction to 50% as an activity unit (U). The activity of peroxidase (POD, EC 1.11.1.7) was determined according to (Zhang, 1992) whereas one unit of POD activity was the amount of enzyme that caused the decomposition of 1 μg substrate at 470 nm. Catalase activity (CAT, EC 1.11.1.6) was determined according to the protocols of Aebi (1984), whereas one unit of enzyme activity (U) was the decomposition of 1 M H_2O_2 at A_{240} within 1 min in 1 g of fresh leaves samples. Ascorbate peroxidase (APX, EC 1.11.1.11) activity and reduced glutathione (GSH) contents were estimated by using kits purchased from Nanjing Jiancheng Bioengineering Institute, China.

Glutathione synthetase (GS) activity was determined according to (Li, 2006). The fresh leaves (0.2 g) were homogenized in 3 ml of Tris-HCI (pH 8.0) extraction buffer containing 1.5295 g Tris, 0.1245 g $MgSO_4{\cdot}6H_2O$, 0.1543 g DTT, and 34.25 g sucrose in 250 ml water. The homogenate was centrifuged at 10,000 rpm for 20 min, and 0.7 ml of supernatant was added to 1.6 ml of assay, a solution mixture containing 3.0590 g Tris, 4.9795 g $MgSO_4.7H_2O$, 0.8628 g glutamic acid-Na, 0.6057 g cysteine, 0.1920 g amino polycarboxylic acid, and 1.3898 g hydroxylamine hydrochloride in 250 ml water and 0.7 ml ATP (0.1210 g ATP) in 5 ml water. The mixture was incubated for 30 min at 25°C. Subsequently, the reaction was stopped after adding 1 ml of color reagents [3.3176 g trichloro acetic acid (TCA), 10.1021 g acidic $FeCl_3.6H_2O$ dissolved in water, and added 5 ml HCl, reached to 100 ml]. The samples were centrifuged at 4,000 rpm for 15 min, and the absorbance was read at 540 nm. The blank group contained 1.6 ml of an assay mixture containing 3.0590 g Tris, 4.9795 g $MgSO_4.7H_2O$, 0.8628 g glutamic acid-Na, 0.6057 g cysteine, and 0.1920 g aminopolycarboxylic acid. The GS activity was measured as: change in absorption ($\Delta A \times Vt$)/($Vs \times FW \times t$), where ΔA = Change in absorbance; Vt = total volume; Vs = sample volume; FW = fresh weight; and t = reaction time.

Nitrate reductase (NR) activity was determined according to Yu and Zhang (2012) with some modifications. Fresh leaves (0.25 g) were precisely homogenized with 4.0 ml extraction buffer containing 0.1211 g cysteine and 0.0372 g EDTA-Na_2 in 100 ml phosphate buffer (pH 7.5). The homogenate was clarified by centrifugation at 4,000 rpm for 15 min at 4°C. The supernatant was collected and assayed. Crude enzyme (0.4 ml) extract was added 1.2 ml KNO_3 buffer (8.8640 g $Na_2HPO_4.12H_2O$ and 0.0570 g $K_2HPO_4.3H_2O$ in 1,000 ml water, pH 8.7) and 0.4 ml NADH [2 mg NADH in 1 ml phosphate buffer (pH 7.5)]. The mixture was kept for 30 min at 25°C. For control, 0.4 ml phosphate buffer was used, whereas the reaction was stopped after adding 1.0 ml of 1% each of 4-aminobenzene sulfonic acid and 0.2% 1-naphthylamine and left for 30 min for color development at 30°C. The tubes were then centrifuged at 4,000 rpm for 10 min and the absorbance was noted at 540 nm immediately. NR activity is expressed in Units h^{-1} g^{-1} fresh weight (FW), which refers to the amount of enzyme required to produce NO_2 in 1 h by 1 g of FW.

Photosynthesis and Gas Exchange

Net photosynthetic rate (A) and gas exchange attributes were determined in flag leaves of four plants from each treatment at the vegetative stage by using a portable photosynthesis system (LI-6400, LI-COR, USA) at 09:00–11:30 am according to (Pan et al., 2016).

Determination of Pb Contents

Plant samples in powder form (0.2 g) of each part were digested with HNO_3:$HClO_4$ (4:1 *v/v*) on an electric digestion system and the resultant solutions were diluted to 25 ml and filtered. The Pb contents in different plant parts were estimated by using Atomic Absorption Spectrophotometer (AA6300C, Shimadzu, Japan).

Determination of Yield and Related Attributes

Tillers bearing panicles were counted in each pot to get productive tillers per pot. Rice panicles of both rice cultivars were threshed manually to estimate the grains per panicle and filled grain percentage. At maturity, all remaining pots were harvested with sickle and manually threshed and the grains were sun-dried. Grain samples were taken randomly from a filled seed lot and weighed to get 1,000-grain weight. Grain yield pot^{-1} was the total grain weight from each pot.

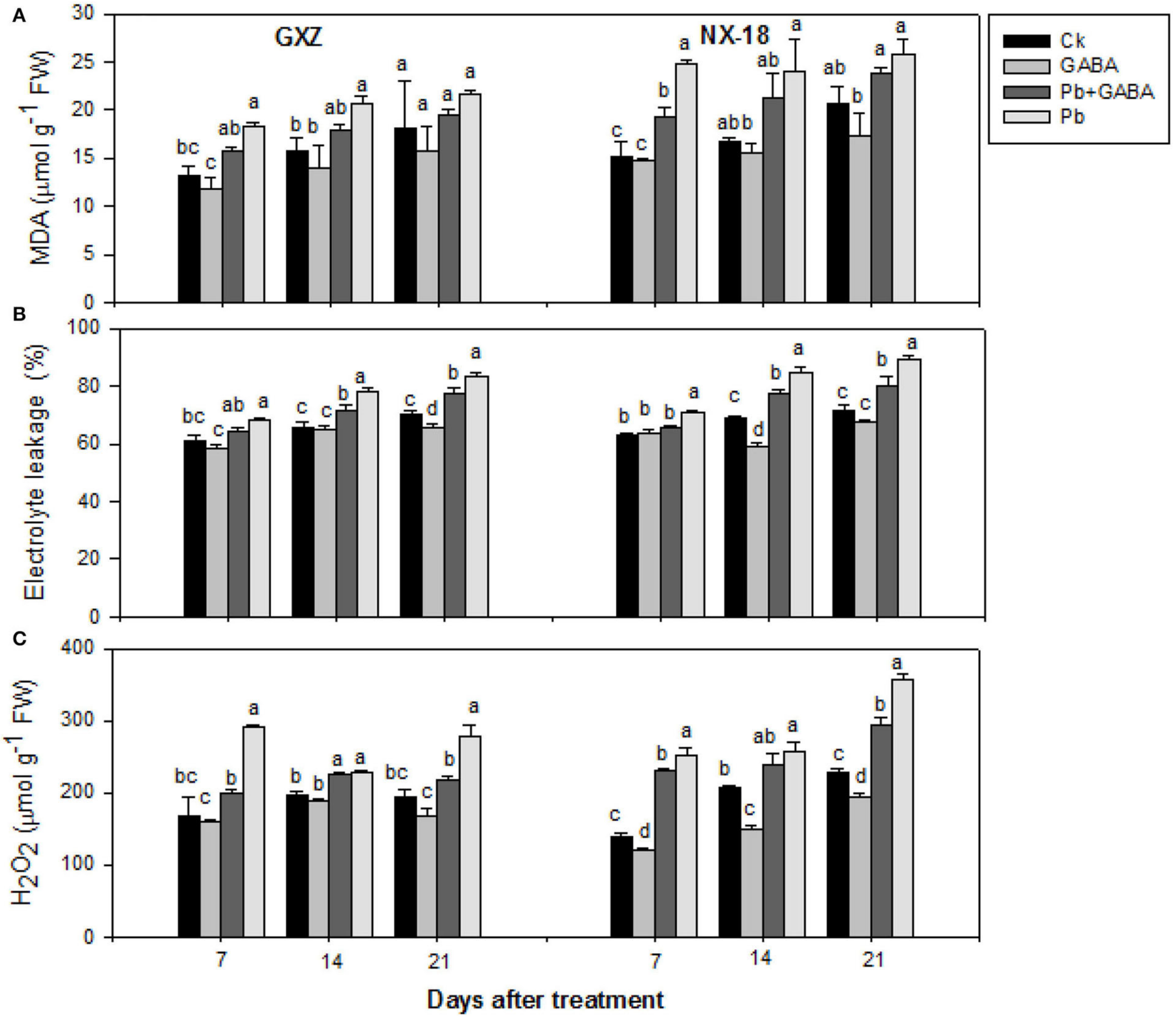

FIGURE 1 | Influence of exogenous GABA on **(A)** malondialdehyde (MDA), **(B)** electrolyte leakage (EL), and **(C)** hydrogen peroxide (H_2O_2) contents in fragrant rice under Pb toxicity at 7, 14, and 21 days after treatment. Vertical bars are means ± S.E. Vertical bars sharing a letter in common do not differ significantly at $P < 0.05$. Ck: without Pb and GABA (control); GABA: 1 mM GABA; Pb + GABA: 1 mM GABA + 800 mg of Pb kg^{-1} of soil; Pb = 800 mg kg^{-1} of soil. GXZ, Guixiangzhan; NX-18, Nongxiang-18.

Experimental Design and Statistical Analysis

Pots were arranged in a completely randomized design with 10 pots per treatment, whereas the dataset was analyzed by "Statistix 8" (Analytical Software, Tallahassee, Florida, USA) while the least significant difference (LSD) ($P_{0.05}$) was used to calculate the difference among treatment means. SigmaPlot 9.0 (Systat Software Inc., San Jose, CA, USA) was used to generate graphs.

RESULTS

Oxidative Damage

Pb stress enhanced MDA, EL, and H_2O_2 contents in both rice cultivars while exogenous GABA application protects against oxidative stress. Compared with Ck, the MDA contents were 39.64, 31.13, and 19.82% higher in GXZ and 63.44, 43.25, and 24.94% higher in NX-18 at 7, 14, and 21 DAT, respectively. The lowest MDA contents were found in GABA-treated rice plants without Pb followed by GABA + Pb. GABA also protected membrane permeability (reduced EL values) in both rice cultivars but the values were higher for NX-18 than GXZ. The EL values in Pb were almost 2-fold than Pb + GABA in both rice cultivars at 7, 14, and 21 DAT. Similarly, GABA was also found effective against H_2O_2 production in both rice cultivars. The H_2O_2 contents in Pb + GABA treatment were 46.39, 1.35, and 5.82% lower for GXZ as well as 9.65, 8.04, and 21.06% lower for NX-18 than only Pb treatment at 7, 14, and 21 DAT, respectively. Overall, the degree of oxidative damage was comparatively higher in NX-18 than in GXZ (**Figure 1**).

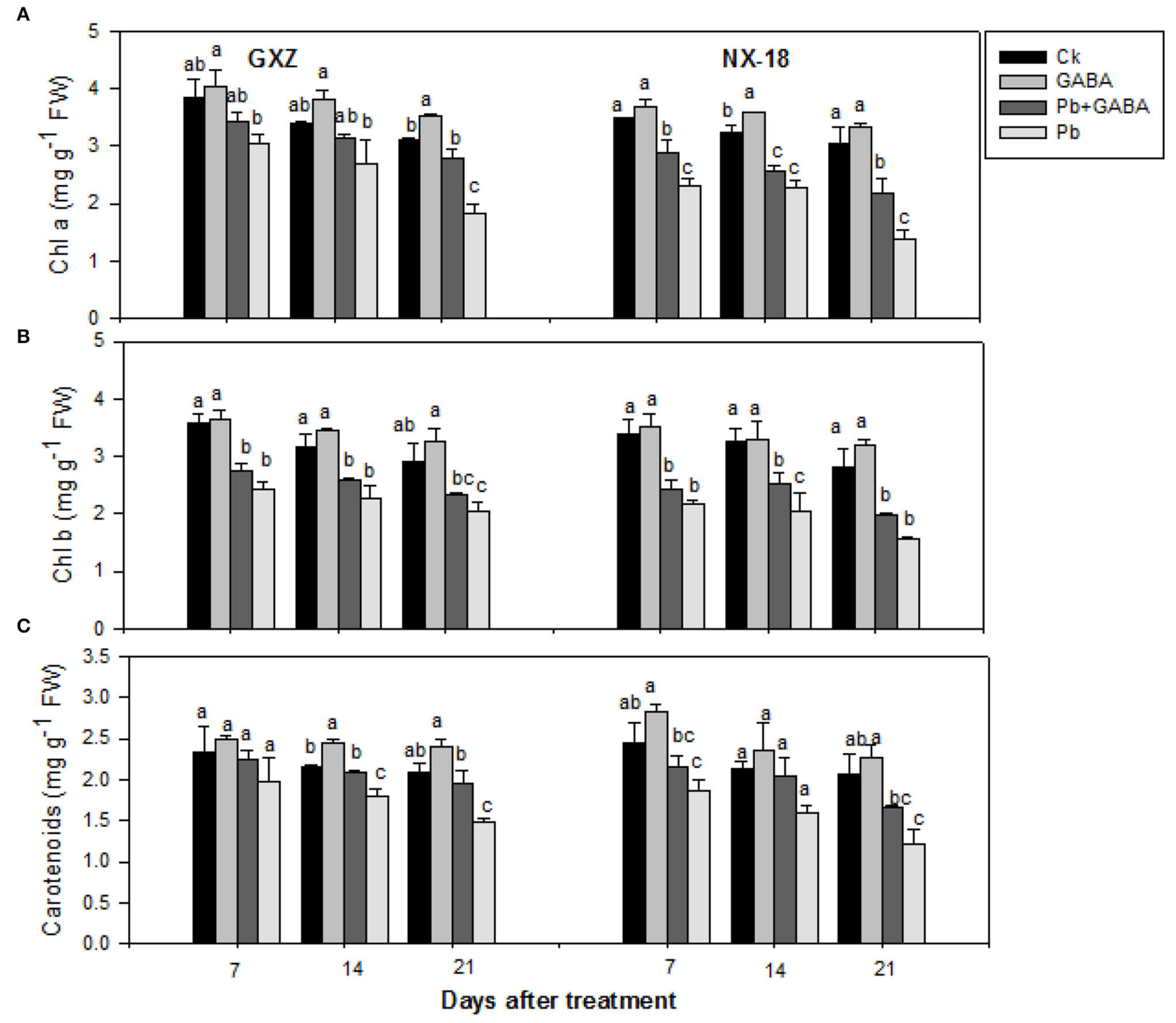

FIGURE 2 | Influence of exogenous GABA on **(A)** chlorophyll a (Chl a), **(B)** chlorophyll b (Chl b), and **(C)** carotenoids (Car) contents in fragrant rice under Pb toxicity at 7, 14, and 21 days after treatment. Vertical bars are means ± S.E. Vertical bars sharing a letter in common do not differ significantly at $P < 0.05$. Ck: without Pb and GABA (control); GABA: 1 mM GABA; Pb + GABA: 1 mM GABA + 800 mg of Pb kg^{-1} of soil; Pb = 800 mg kg^{-1} of soil. GXZ, Guixiangzhan; NX-18, Nongxiang-18.

Chlorophyll Contents and Carotenoids

Significant reductions ($P < 0.05$) were noted in Chl a, Chl b, and carotenoids in both cultivars under Pb toxicity while exogenous GABA application was found effective to confer inhibitory effect of Pb on leaf chlorophyll contents. There was 21.28, 20.67, and 41.44% reduction in Chl a, 32.45, 28.55, and 29.45% in Chl b, and 15.82, 16.92, and 28.97% in carotenoids in GXZ and 33.80, 29.31, and 54.29% reduction in Chl a, 36.06, 37.18, and 44.09% in Chl b, and 23.65, 24.89, and 41.11% in carotenoids, which were recorded in NX-18 at 7, 14, and 21 DAT, respectively. Although Pb inhibited photosynthetic pigments in both rice cultivars, the foliar GABA application improved photosynthetic pigments in both rice cultivars. Maximum Chl a, Chl b, and carotenoids were recorded in GABA-treatment followed by Ck and Pb + GABA while least in Pb-treatment. In addition, the carotenoids were reduced but remained statistically similar ($P > 0.05$) in GXZ at 7 DAT under all treatments (**Figure 2**).

Proline, Protein, and GABA Contents

Significant improvements ($P < 0.05$) were noticed in leaf proline, protein, and GABA contents in both GXZ and NX-18 under GABA application, while Pb stress reduced protein contents dramatically. Maximum proline contents were recorded in Pb + GABA treatment which was 67.04, 74.18, and 1.41% higher than Ck at 7, 14, and 21 DAT, respectively, in GXZ, whereas proline contents in NX-18 were remained statistically similar

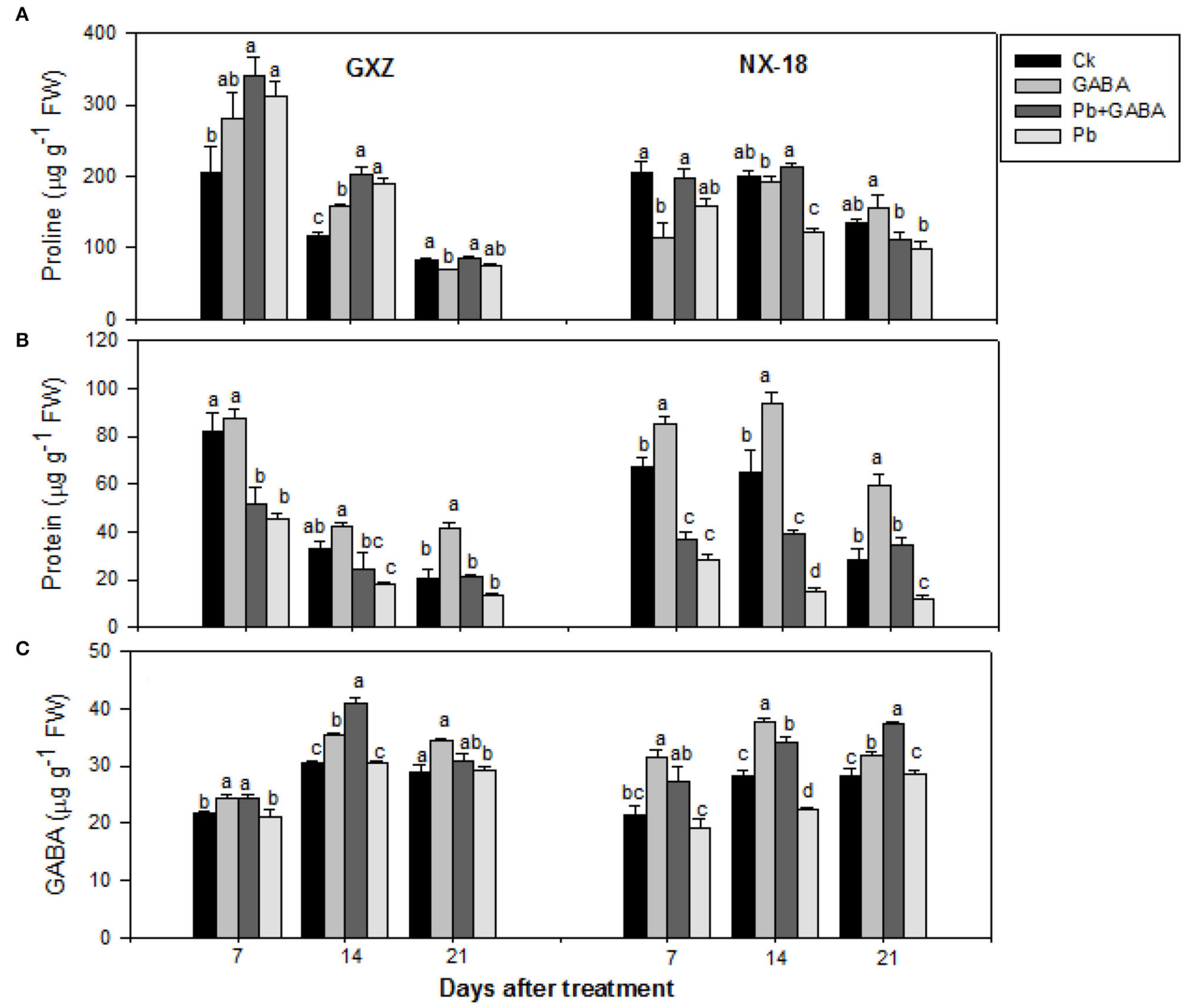

FIGURE 3 | Influence of exogenous GABA on **(A)** proline, **(B)** protein, and **(C)** GABA contents in fragrant rice under Pb toxicity at 7, 14, and 21 days after treatment. Vertical bars are means ± S.E. Vertical bars sharing a letter in common do not differ significantly at $P < 0.05$. Ck: without Pb and GABA (control); GABA: 1 mM GABA; Pb + GABA: 1 mM GABA + 800 mg of Pb kg^{-1} of soil; Pb = 800 mg kg^{-1} of soil. GXZ, Guixiangzhan; NX-18, Nongxiang-18.

($P > 0.05$) for Ck and Pb + GABA at 7 and 14 DAT but Ck and only GABA at 21 DAT, respectively. In contrast, exposure to Pb severely reduced protein contents in both rice cultivars; however, the effects were more apparent in NX-18 than GXZ. In both rice cultivars, the maximum protein contents were recorded in GABA treatment (without Pb) followed by Ck, Pb + GABA, and Pb. Compared with Ck, the GABA application enhanced protein contents by 6.15, 26.19, and 102.80% in GXZ and 27.23, 44.50, and 91.10% in NX-18 at 7, 14, and 21 DAT, respectively. Compared with Ck, the values of the percentage decrease in protein contents were higher in NX-18 (57.47, 76.97, and 56.90%) than in GXZ (44.52, 44.40, and 35.01%). In addition, exogenous GABA application substantially improved leaves GABA contents. The highest GABA contents, that is, 24.43, 40.88, and 34.34 µg g^{-1}, were noted in GABA, Pb + GABA, and GABA treatment in GXZ, 31.37 and 37.71 µg g^{-1} under GABA treatment, and 37.41 µg g^{-1} under Pb + GABA in NX-18 at 7, 14, and 21 DAT, respectively. Moreover, the lowest GABA contents were noticed under Pb treatment without GABA application in both rice cultivars (**Figure 3**).

Net Photosynthesis and Gas Exchange Attributes

Pb toxicity led to a significant reduction in net photosynthesis, stomatal conductance, intercellular CO_2, and transpiration rate against Ck; however, exogenous GABA application improved net photosynthesis and gas exchange attributes significantly.

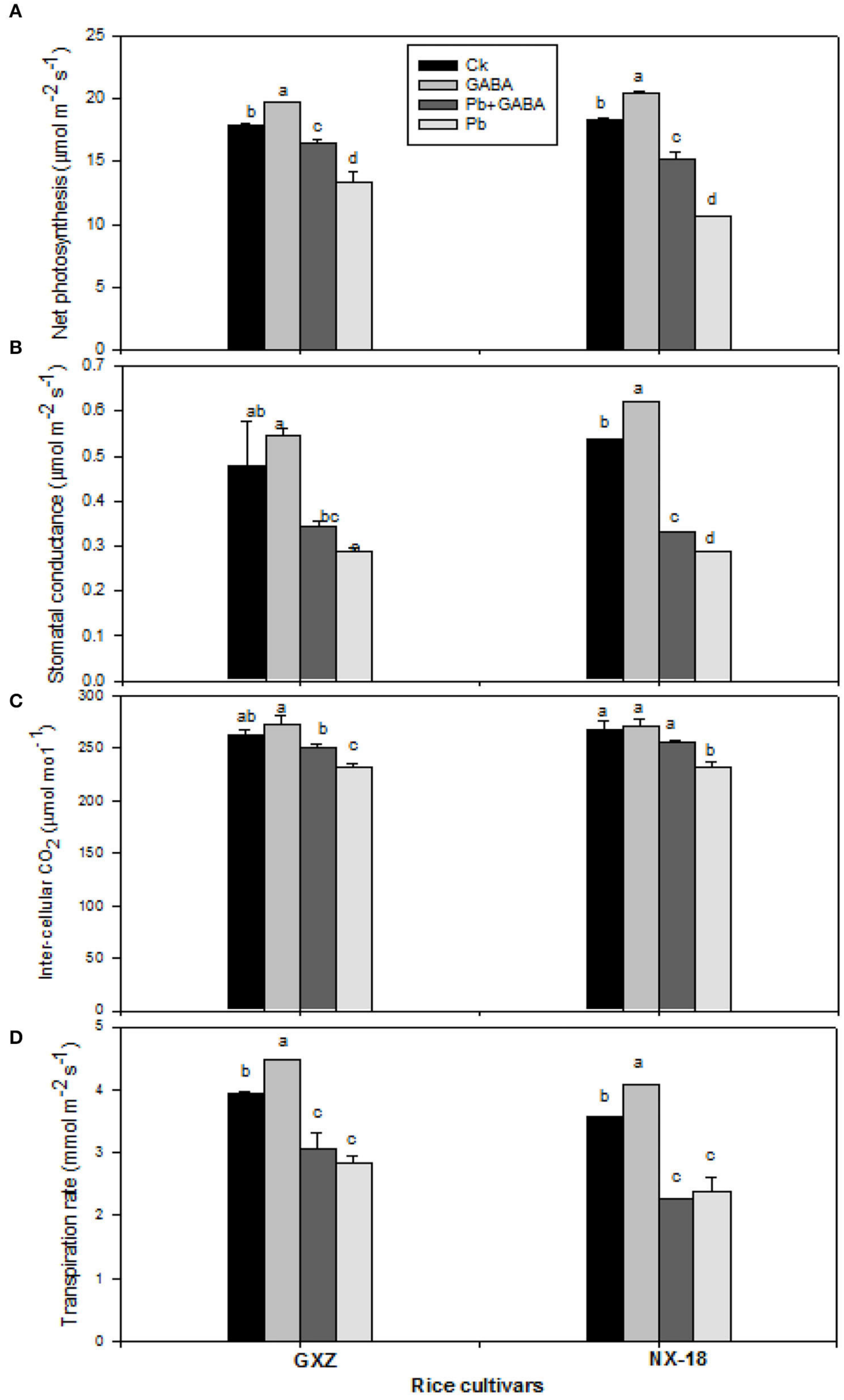

FIGURE 4 | Influence of exogenous GABA on **(A)** net photosynthesis, **(B)** stomatal conductance, **(C)** inter-cellular CO_2, and **(D)** transpirational rate in fragrant rice under Pb toxicity at 7, 14, and 21 days after treatment. Vertical bars are means ± S.E. Vertical bars sharing a letter in common do not differ significantly at $P < 0.05$. Ck: without Pb and GABA (control); GABA: 1 mM GABA; Pb + GABA: 1 mM GABA + 800 mg of Pb kg^{-1} of soil; Pb = 800 mg kg^{-1} of soil. GXZ, Guixiangzhan; NX-18, Nongxiang-18.

The GABA application enhanced net photosynthesis (9.86 and 11.18%), stomatal conductance (13.81 and 15.51%), intercellular CO_2 (4.04 and 0.76%), and transpiration rate (13.41 and 3.58%) than Ck in GXZ and NX-18, respectively. Moreover, the GABA application under Pb treatment (GABA + Pb) enhanced the net photosynthesis by 19.46 and 29.93%, stomatal conductance by 15.74 and 13.27%, and intercellular CO_2 by 7.69 and 9.23% for both GXZ and NX-18, respectively, and transpiration rate 7.92% (in GXZ only) as compared with Pb (**Figure 4**).

Antioxidative Defense

Exogenous GABA application modulated the antioxidant activities, that is, SOD, POD, CAT, APX, and GSH contents significantly under Pb toxicity. For instance, SOD activities were found the highest in Pb + GABA treatment at all sampling intervals in both GXZ and NX-18, while significantly higher POD activities were recorded in Ck at 7 and 14 DAT and in Pb + GABA at 21 DAT in GXZ. In NX-18, except at 7 DAT, where POD activity was significantly higher in Pb + GABA than in other treatments, the POD activity remained statistically similar ($P > 0.05$) for all the treatments at 14 and 21 DAT. Furthermore, in GXZ, the CAT and APX activities were substantially higher in plants under Pb + GABA at all sampling stages (except CAT at 7 DAT, where the highest CAT activity was recorded in Ck). In NX-18, reduced CAT activities were recorded in Pb-exposed plants even with GABA application except for CAT at 7 DAT (maximum CAT activity under GABA-treated plants) than in Ck. The APX activities were found higher in Pb-exposed plants even with or without GABA treatment than Ck at 7, 14, and 21 DAT; however, the activity of APX in Ck was marginally higher than APX activity in Pb treatment at 21 DAT. Likewise, GABA promoted the GSH contents in both rice cultivars under Pb stress. At 7 DAT, the maximum GSH contents were recorded in GABA-applied plants without Pb, while at 14 and 21 DAT, the maximum GABA contents were found in the Pb + GABA treatment. Furthermore, GABA application also enhanced GSH contents in NX-18 but the effects remained only significant at 14 DAT, whereas, at 7 and 21 DAT, the GSH contents remained statistically similar ($P > 0.05$) for all the treatments (**Figure 5**).

GS and NR Activities

Both GS and NR activities were variably affected due to GABA application in both rice cultivars under Pb stress. For example, in GXZ, the highest GS activities were recorded in Ck, Pb, and Pb + GABA treatments at 7, 14, and 21 DAT, respectively, while NR activity was found higher in GABA-treated plants (with or without Pb) but statistically similar ($P > 0.05$) for all the treatments at all sampling stages. On the other hand, for NX-18, the maximum GS activity was recorded in GABA treatment at 7 DAT, while in Ck at 14 and 21 DAT, but the lowest in Pb-treatment without GABA application. Similarly, Pb reduced the NR activity by 25.79, 58.58, and 36.33% in NX-18 than in Ck, while the highest NR activity was recorded in Pb + GABA treatment at 7 and 21 DAT, and in Ck at 14 DAT. In general, the reduction in GS and NR activities was more severe in NX-18 than GXZ under Pb stress (**Figure 6**).

Pb Contents in Different Plant Parts

Both rice cultivars showed differential behavior regarding Pb accumulation in different plant parts; however, the highest Pb contents were recorded in roots followed by stems, leaves (at VEG), ears (at PH), and grains (at MAT) stages in both GXZ and NX-18. Exogenous application of GABA in plants under Pb toxicity significantly reduced Pb contents in different plant parts compared with Pb exposed plants without GABA. For example, at the VEG stage, the Pb contents 27.37 and 29.70% (in roots), 16.39 and 21.70% (in stems), and 62.50 and 35.01% (in leaves) were lower in GABA + Pb than only in Pb-exposed plants of GXZ and NX-18, respectively. Similarly, the Pb contents in roots, stems, leaves, and ears under Pb + GABA-treated plants at the PH stage were 36.07 and 34.15%, 53.50 and 32.30%, 113.58 and 84.98%, and 60.01 and 58.81% lower in Pb + GABA for GXZ and NX-18, respectively, compared with Pb treatment, while 25.65 and 26.58% lower Pb contents in roots, 18.18 and 23.36% in stems, 34.04 and 50.94% in leaves, and 67.06 and 38.38% in grains were recorded in GXZ and NX-18, respectively, under Pb + GABA than only in Pb-exposed plants. Overall, Pb + GABA showed substantially lower Pb accumulation in both rice cultivars than Pb-exposed plants without GABA application (**Table 1**).

Yield and Related Components

Yield and related traits of both rice cultivars were severely affected by Pb toxicity but some of the yield-related traits, that is, filled grain % and 1,000 grain weight (in GXZ) and grains per panicle (in NX-18) were remained statistically at par ($P > 0.05$) for all the treatments. However, exogenous GABA application improved yield and yield components considerably as the percentage reduction under Pb-exposed plants (without GABA) was higher than only GABA-applied plants. For example, Pb treatment led to a reduction in productive tillers/pot (13.64 and 10.29%), grains/panicle (0.37 and 2.26%), filled grain % (3.89 and 19.06%), 1,000-grain weight (7.35 and 12.84%), and grain yield (17.92 and 40.56) as compared with Pb + GABA. Overall, the yield reduction was more severe in NX-18 than GXZ, while the highest yield and yield components were recorded in plants only under GABA application without Pb (**Table 2**).

DISCUSSION

Exogenous GABA-induced regulations on physiological, biochemical, and yield of two fragrant rice cultivars were assessed in this study. It was found that Pb significantly elevated the lipid peroxidation, electrolyte leakage, and H_2O_2 contents, while the exogenous GABA application reduced the oxidative stress in both rice cultivars. Comparatively, the degree of oxidative damage was higher in NX-18 than in GXZ even with or without GABA (**Figure 1**). Plants produced reactive oxygen species (ROS) as signaling molecules for any kind of stress; however, under acute stress conditions, the rate of ROS production was accelerated enough that ROS starts to oxidize and/or degrade essential cellular structures and distort cell normal functionality. Enhanced MDA contents showed that lipid peroxidation and electrolyte leakage are the loss of membrane integrity, whereas H_2O_2 is a potential ROS (at higher levels) that reacts with micro

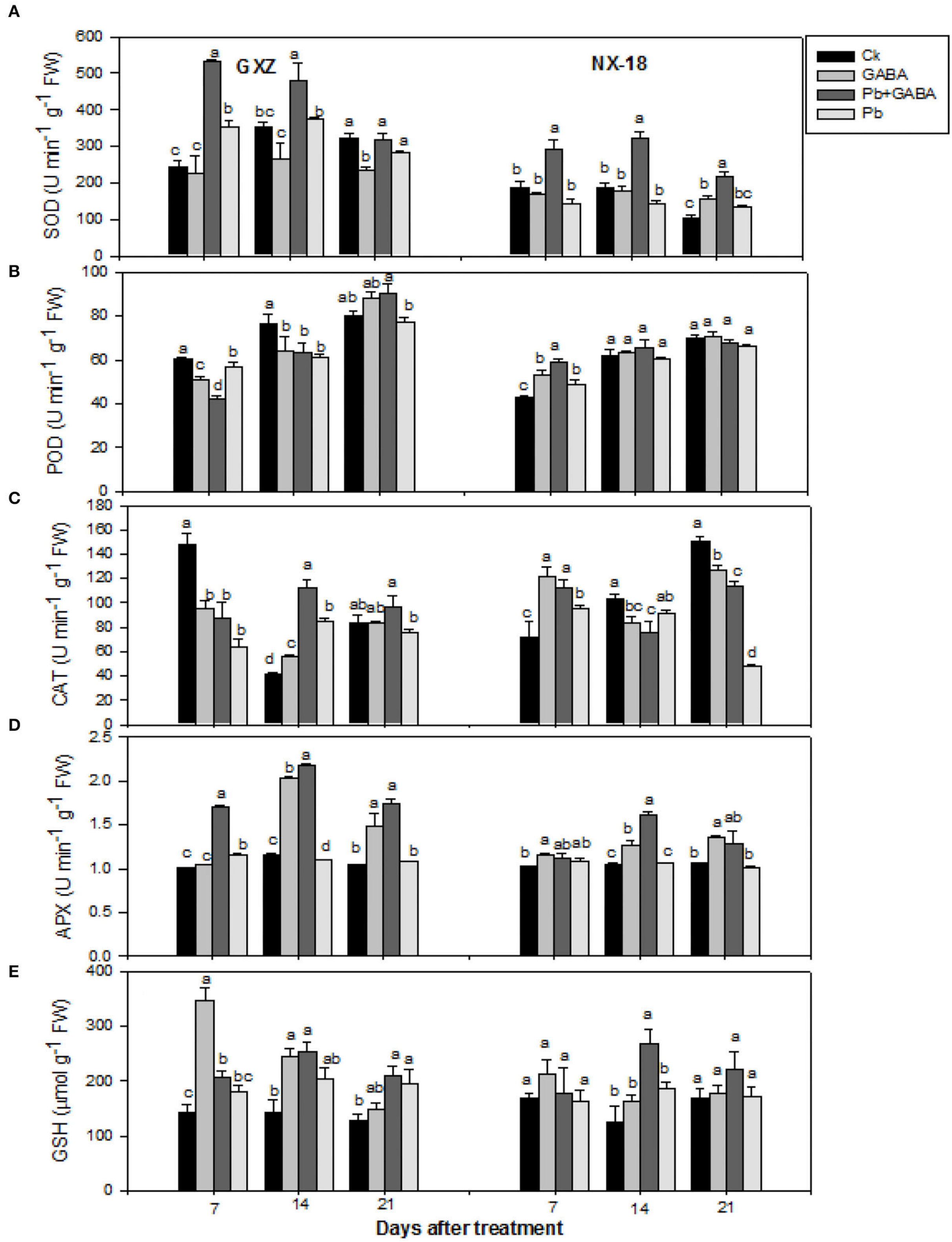

FIGURE 5 | Influence of exogenous GABA on **(A)** superoxide dismutase (SOD), **(B)** peroxidase (POD), **(C)** catalase (CAT), **(D)** ascorbate peroxidase (APX) activities, and **(E)** reduced glutathione (GSH) contents in fragrant rice under Pb toxicity at 7, 14, and 21 days after treatment. Vertical bars are means ± S.E. Vertical bars sharing a letter in common do not differ significantly at $P < 0.05$. Ck: without Pb and GABA (control); GABA: 1 mM GABA; Pb + GABA: 1 mM GABA + 800 mg of Pb kg^{-1} of soil; Pb = 800 mg kg^{-1} of soil. GXZ, Guixiangzhan; NX-18, Nongxiang-18.

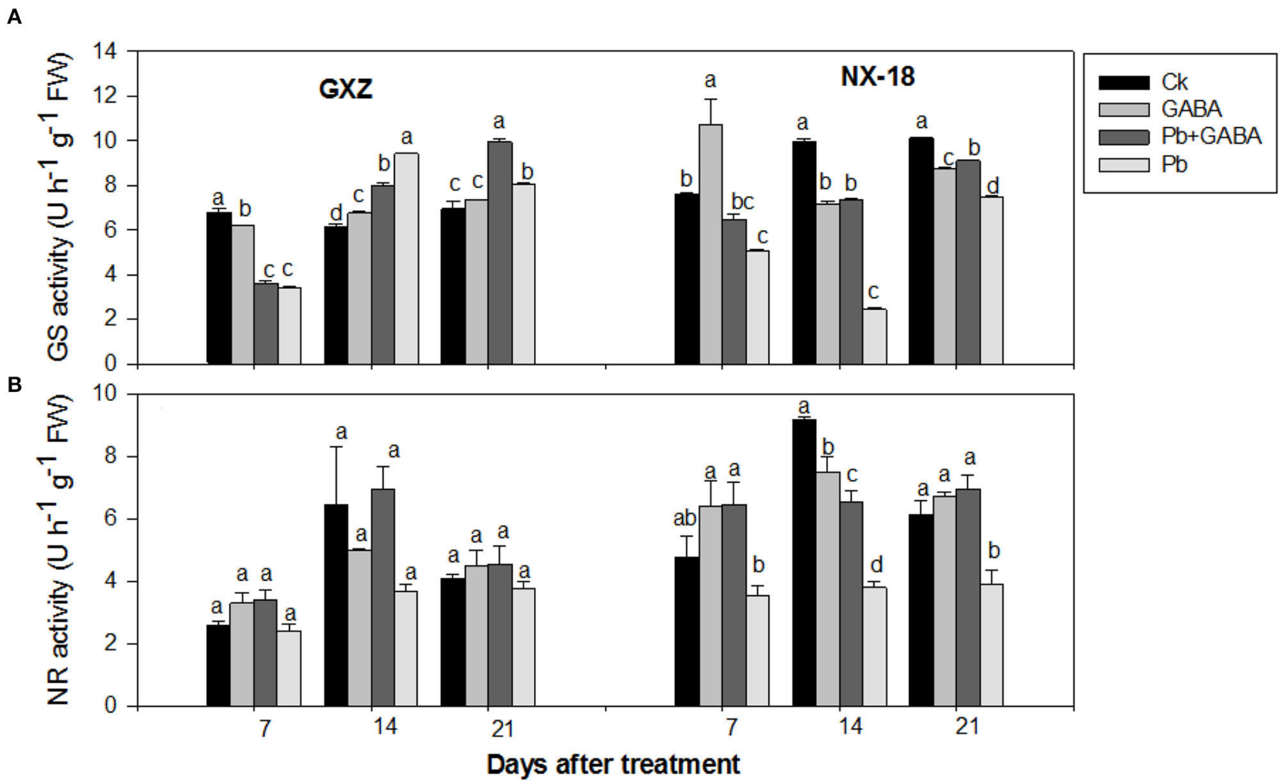

FIGURE 6 | Influence of exogenous GABA on **(A)** glutamine synthetase (GS) and **(B)** nitrate reductase (NR) activities in fragrant rice under Pb toxicity at 7, 14, and 21 days after treatment. Vertical bars are means±S.E. Vertical bars sharing a letter in common do not differ significantly at $P < 0.05$. Ck: without Pb and GABA (control); GABA: 1 mM GABA; Pb + GABA: 1 mM GABA + 800 mg of Pb kg^{-1} of soil; Pb = 800 mg kg^{-1} of soil. GXZ, Guixiangzhan; NX-18, Nongxiang-18.

TABLE 1 | Influence of exogenous gamma-amino butyric acid (GABA) application on the accumulation of Pb contents (μg g^{-1}) in different plant parts of fragrant rice.

Cultivars	Treatments	Tillering			Panicle heading				Maturity			
		Root	Stems	Leaves	Root	Stems	Leaves	Ears	Root	Stems	Leaves	Grains
GXZ	Ck	28.99 c	6.02 c	4.97 c	23.40 c	6.36 c	5.58 c	0.80 c	25.02 c	4.96 c	2.97 c	0.20 c
	GABA	25.69 c	6.19 c	3.29 c	26.00 c	8.05 c	5.03 c	0.63 c	26.52 c	6.10 c	3.38 c	0.16 c
	Pb + GABA	1,547.94 b	98.26 b	21.40 b	1,758.70 b	85.10 b	29.00 b	8.05 b	2,075.13 b	106.31 b	75.71 b	1.96 b
	Pb	1,971.64 a	114.37 a	34.77 a	2,393.11 a	130.63 a	61.93 a	12.89 a	2,607.47 a	125.64 a	101.48 a	3.27 a
	LSD$_{0.05}$	164.49	4.78	5.97	171.18	9.16	6.82	3.42	116.88	4.51	8.58	0.58
NX-18	Ck	17.25 c	6.60 c	4.54 c	18.63 c	6.80 c	5.78 c	0.97 c	22.33 c	5.26 c	5.49 c	0.51 c
	GABA	18.46 c	7.25 c	4.35 c	16.40 c	8.05 c	4.97 c	0.72 c	21.10 c	6.29 c	5.02 c	0.40 c
	Pb + GABA	1,616.88 b	111.15 b	38.98 b	1,853.22 b	116.11 b	42.89 b	17.93 b	1,906.83 b	128.07 b	85.37 b	6.43 b
	Pb	2,097.16 a	135.31 a	52.63 a	2,486.02 a	153.61 a	79.33 a	28.47 a	2,413.66 a	157.98 a	128.87 a	8.90 a
	LSD$_{0.05}$	202.24	4.97	6.20	171.18	11.01	5.22	2.14	203.10	6.68	4.81	1.50

Values are the means of three replicates S.E. Values sharing a letter in common within the same column do not differ significantly at the 5% probability level according to the least significant difference (LSD) test. Ck: without Pb (control), GABA: 1 mM GABA, Pb + GABA: 1 mM GABA + 800 mg of Pb kg^{-1} of soil, Pb = 800 mg kg^{-1} of soil. GXZ, Guixiangzhan; NX-18, Nongxiang-18.

and macro biological molecules and disturbs their structures and functions. Meanwhile, plants treated with GABA showed comparatively less oxidative stress than non-treated plants, which depicts that GABA plays some role in scavenging ROS to reduce oxidative damage in fragrant rice under Pb toxicity, whereas the high oxidative stress in NX-18 than GXZ showed that NX-18 is sensitive to Pb stress. The GABA may have some ROS scavenging and/or regulatory roles in plants (Shi et al.,

TABLE 2 | Influence of exogenous gamma-amino butyric acid (GABA) application on yield and related components of fragrant rice under Pb toxicity.

Rice cultivars	Treatments	Productive tillers/pot	Grains/panicle	Filled grain %	1000-grain weight (g)	grain yield/pot (g)
GXZ	Ck	30.41 ± 0.41 a	167.17 ± 1.69 ab	79.45 ± 3.75 a	20.05 ± 0.36 a	69.54 ± 3.68 ab
	GABA	31.53 ± 0.87 a	176.90 ± 3.55 a	78.14 ± 2.95 a	20.04 ± 0.17 a	75.58 ± 3.41 a
	Pb + GABA	26.63 ± 0.86 b	163.87 ± 2.19 b	72.76 ± 0.21 a	19.82 ± 0.10 a	62.34 ± 1.31 b
	Pb	23.00 ± 1.15 c	163.27 ± 6.14 b	69.93 ± 5.19 a	18.37 ± 0.38 a	51.17 ± 2.03 c
	Means	27.89	167.80	72.27 3.89	19.57	64.66
	$LSD_{0.05}$	3.17	12.55	10.64	1.08	10.49
NX-18	Ck	26.83 ± 1.17 a	162.80 ± 6.40 a	81.87 ± 5.73 a	20.02 ± 1.03 a	57.46 ± 2.12 b
	GABA	27.48 ± 1.06 a	151.20 ± 1.94 a	81.82 ± 1.35 a	20.48 ± 0.35 a	63.39 ± 1.68 a
	Pb + GABA	22.67 ± 1.45 b	147.33 ± 5.21 a	70.83 ± 1.89 ab	19.96 ± 1.13 a	48.28 ± 2.71 c
	Pb	20.33 ± 1.45 b	144.00 ± 6.66 a	57.33 ± 5.58 b	17.40 ± 0.60 b	28.70 ± 0.40 d
	Means	24.33	151.33	75.32	19.47	49.46
	$LSD_{0.05}$	3.91	20.89	15.94	1.36	4.46

Ck: without Pb (control); GABA: 1 mM l^{-1} GABA; Pb + GABA: 1 mM GABA + 800 mg of Pb kg^{-1} of soil; Pb = 800 mg kg^{-1} of soil. GXZ, Guixiangzhan; NX-18, Nongxiang-18.

2010), as the GABA shunt pathway provides succinate and nicotinamide adenine dinucleotide (NADH) during respiration, hence regulations in GABA might have a direct role in regulating the ROS/RO intermediates under oxidative stress (Bouche and Fromm, 2004). The reduced oxidative stress in GABA-treated plants under metal toxicity was also reported previously (Nayyar et al., 2014; Kumar et al., 2019). Generally, the GABA rapidly accumulates in plants under abiotic stress conditions, thus regulations in the endogenous GABA levels substantially affect the plant morphogenesis. The GABA-induced stress tolerance is associated with the expression of genes responsible for the biosynthesis of phytohormones, plant signaling, redox reactions, transcriptional regulation, and polyamine metabolism. Hence, associations of GABA with polyamines and phytohormones could improve the plant performance under stress conditions (Podlešáková et al., 2019).

The GABA also improved chlorophyll and carotenoids (**Figure 2**), proline, protein, and GABA contents (**Figure 3**), net photosynthesis, and gas exchange attributes (**Figure 4**), as well as modulating the antioxidative activities (**Figure 5**) in fragrant rice under Pb toxicity, whereas such improvements were prominent in GXZ than NX-18. The reduction in the leaf chlorophyll contents is the distinctive character of Pb-induced oxidative stress as a consequence of chlorophyll degradation due to enhanced chlorophyllase activity and/or photo-oxidation (Hegedus et al., 2001) of ROS. This reduction in chlorophyll contents might lead to reduced photosynthesis and gas exchange in both rice cultivars. The GABA provided partial protection to the photosynthetic pigments, by scavenging the degenerative ROS activities *via* proline accumulation and/or activation of the antioxidative defense system (Khanna et al., 2021). These inter-linked physiological mechanisms are modulated by GABA to improve rice performance under Pb stress. The GABA-induced modulations in photosynthetic machinery, especially "photosystem I and II," were noted in *Piper nigrum* Linn (Vijayakumari and Puthur, 2016). Alternatively, non-significant improvements due to GABA application were noted in chlorophyll contents and chlorophyll fluorescence, except for photochemical quenching coefficient, and actual photochemical efficiency in hydroponically cultured tomato seedlings under salt stress (Luo et al., 2011). In addition to cytosolic adjustments, proline is involved actively in scavenging ROS and protecting proteins and thylakoid membrane under stress conditions (Hayat et al., 2012). The Pb stress led to a significant increase in proline contents in aromatic rice cultivars (Ashraf et al., 2017b), whereas Li W. et al. (2016) reported substantial enhancements in proline contents in maize under GABA application. GABA application promoted SOD, POD, CAT, and APX activities either in Pb stressed and non-stressed rice plants or in Pb stress only (without GABA). Both the SOD and CAT play a significant role in cleansing ROS; for example, SOD detoxifies super-oxide anion to O_2 and then H_2O_2, which, later, is further reduced by CAT and POD enzymes (Alscher et al., 2002). The APX and GSH are involved in the ascorbate-glutathione cycle that detoxifies the harmful effects of ROS (Ali et al., 2014a). The lower antioxidant activities in plants under Pb stress without GABA showed that the production rate of ROS may be too high to be scavenged by antioxidants activities and/or reduced ROS scavenging efficiencies; however, exogenous application of GABA improved the antioxidant activities by lowering ROS production and enhancing scavenging abilities of antioxidants. Gupta et al. (2010) also stated that antioxidants have a principal role to quench the Pb-caused production of ROS. GABA-induced maintenance in energy status and enhanced chilling tolerance due to modulations in enzymatic and non-enzymatic antioxidants were previously noted in peach fruit under chilling stress (Yang et al., 2011). Our findings are further concomitant with Shi et al. (2010) who found significant modifications in antioxidants to remove ROS in *Caragana intermedia* plants due to GABA application. Pb stress reduced the GS and NR activity in both GXZ and NX-18; however, their activities were found higher in GABA-treated plants than in non-GABA under Pb stress. The reduction in GS and NR activities was comparatively higher in NX-18 than in GXZ (**Figure 6**). Generally, GABA contributes to maintaining the C-N equilibrium by regulating N-metabolism and/or by controlling the transport and storage of nitrogen

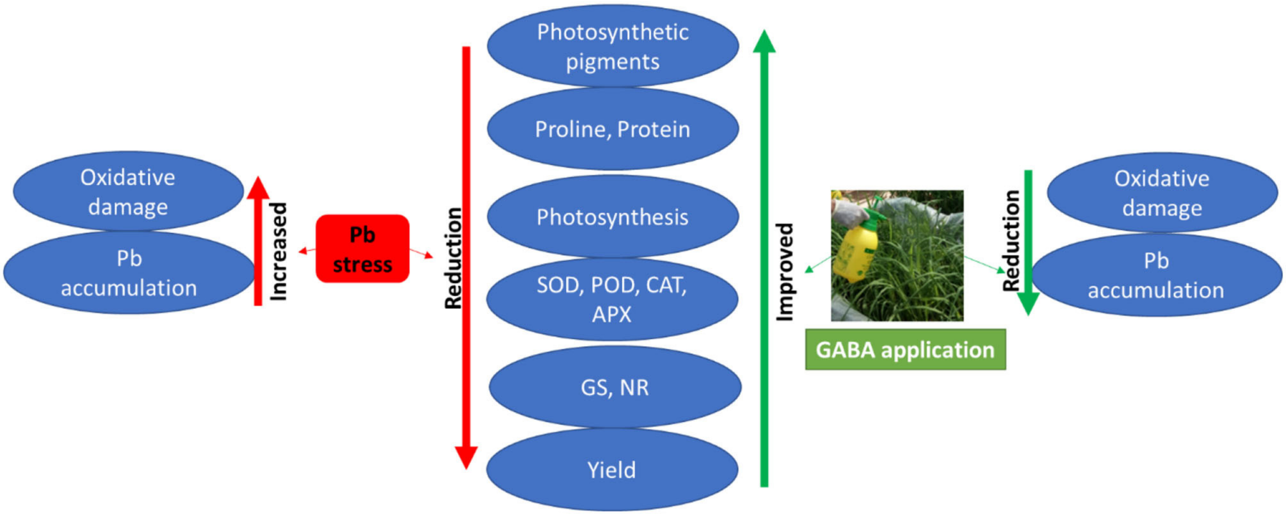

FIGURE 7 | Effects of exogenous GABA application on physio-biochemical attributes and yield as well as Pb accumulation in fragrant rice under Pb stress.

within plant organs. The GABA shunt pathway also regulates C:N fluxes by actively assimilating the glutamate-generated carbons (Bouche and Fromm, 2004). Positive correlations were also observed between NR activity and nitrate influx (higher nitrate uptake) in growing rape plants (Beuve et al., 2004). Regulations in the activities of NR and GS were also observed in *Arabidopsis thaliana* seedlings under GABA application (Barbosa et al., 2010). The GABA application improved the Cd tolerance in maize by upregulating the antioxidant enzyme activities and polyamine biosynthesis-responsive genes, that is ornithine decarboxylase and spermidine synthase (Seifikalhor et al., 2020). Hence, GABA helped rice plants to maintain GS and NR activity under Pb stress, while, comparative to GXZ, reduced GS and NR activities in NX-18, depicting that Pb toxicity badly affected the C-N metabolism in this cultivar. Previously, modulation in GS and NR activities in maize (Li M. et al., 2016; Li W. et al., 2016) was noted due to GABA, which shows the involvement of GABA in C:N metabolism; however, the mechanism and extent of its involvement in C-N metabolism are still not clear.

Exogenous GABA application improved the yield and related components, while it reduced Pb uptake and accumulation in stems, leaves, and grains under Pb stress. The yield loss was more severe in NX-18 (with more Pb contents in upper plant parts) than in GXZ (**Tables 1**, **2**). Reductions in yield-related components might lead to reduced grain yield in both rice cultivars. Previously, Liu et al. (2003) declared that relative changes in spikelets per panicle under Pb toxicity have significant and positive correlations with the relative changes in rice grain yield, whereas effects of Pb toxicity were more severe for spikelets per panicle and rice grain weight. In general, relative changes in rice grain yield were related to productive tillers, grain per panicle, and 1,000-grain weight under Pb toxic conditions (Ashraf et al., 2020). Improve chlorophyll contents, net photosynthesis, proline accumulation, antioxidative defense mechanism, and balanced GS and NR activity due to GABA application possibly led to improved yield and yield components in both GXZ and NX-18; however, the improvements were more prominent in GXZ that might be due to its tolerant behavior against Pb. GABA application-induced improvements in yield and quality attributes of fragrant rice were also reported by (Xie et al., 2020).

In addition, Pb uptake in plants is largely affected by different plant and soil factors (Bharwana et al., 2014); however, less Pb contents in GABA-treated plants may be due to the protective role of GABA and maintenance of cell integrity, thus less Pb intake into the plants. Moreover, the signaling role of GABA could also help plants to respond effectively against Pb stress. Ramesh et al. (2015) found that GABA-induced Al tolerance was due to aluminum-activated malate transporters (ALMT) in wheat; nevertheless, the exact mechanism of GABA involvement to reduce Pb contents in upper plant parts and molecular basis of Pb tolerance due to GABA application are yet to be explored. Overall, the positive effects of exogenous GABA application on rice under Pb stress have been summarized in (**Figure 7**).

CONCLUSION

In conclusion, Pb toxicity adversely affected the physiology and grain productivity of both rice cultivars; however, exogenous GABA application reduced oxidative damage, improved chlorophyll contents and photosynthesis, and regulated the antioxidant defense mechanism as well as GS and NR in both rice cultivars. The GABA application improved yield and related components in both GXZ and NX-18 under Pb stress; nevertheless, the yield reduction was higher in NX-18 than in GXZ. The Pb contents in different plant parts were recorded as root > stems > leaves > ears (at panicle heading) > grains (at maturity); nonetheless, the concentrations were substantially lower in respective organs under GABA treatments. Hence, rather than just acting as a signaling molecule, the GABA may also be applied exogenously as an "osmolyte" to induce Pb stress tolerance in rice due to its multi-functional role.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

UA and XT designed the experiment. UA and ZM investigated the traits and lab analyses. UA, SM, and SAA analyzed the data and wrote the manuscript. FR, RNA, JI, and XT revised and edited the manuscript. All authors read and approved the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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Synergistic Effect of Zinc Oxide Nanoparticles and *Moringa oleifera* Leaf Extract Alleviates Cadmium Toxicity in *Linum usitatissimum*: Antioxidants and Physiochemical Studies

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Among heavy metals, cadmium (Cd) is one of the toxic metals, which significantly reduce the growth of plants even at a low concentration. Cd interacts with various plant mechanisms at the physiological and antioxidant levels, resulting in decreased plant growth. This research was conducted to exploit the potential of synergistic application of zinc oxide nanoparticles (ZnO NPs) and *Moringa oleifera* leaf extract in mitigation of Cd stress in linseed (*Linum usitatissimum* L.) plants. The main aim of this study was to exploit the role of *M. oleifera* leaf extract and ZnO NPs on Cd-exposed linseed plants. Cd concentrations in the root and shoot of linseed plants decreased after administration of MZnO NPs. Growth parameters of plants, antioxidant system, and physiochemical parameters decreased as the external Cd level increased. The administration of MZnO NPs to the Cd-stressed linseed plant resulted in a significant increase in growth and antioxidant enzymes. Furthermore, the antioxidative enzymes superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX) exhibited a considerable increase in the activity when MZnO NPs were applied to Cd-stressed seedlings. The introduction of MZnO NPs lowered the levels of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) in the linseed plant grown in Cd-toxic conditions. The NPs decreased electrolyte leakage (EL) in Cd-stressed linseed leaves and roots. It was concluded that synergistic application of ZnO NPs and *M. oleifera* leaf extract alleviated Cd stress in linseed plants through enhanced activity of antioxidant enzymes.

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It is proposed that role of MZnO NPs may be evaluated for mitigation of numerous abiotic stresses.

Keywords: ZnO-NPs, cadmium, linseed, antioxidant, plants

INTRODUCTION

Heavy metal contamination in the ecosystem has been studied extensively and has attained a lot of attention due to the substantial health risks that these metals pose to terrestrial and marine ecosystems (Ali et al., 2014; Mwamba et al., 2016). Toxic effects of heavy metals to humans include nervous disorder, vascular damage, birth defects, and kidney dysfunction. Lead (Pb), cadmium (Cd), and chromium (Cr) are the most dangerous nonessential metals (Sebastiani et al., 2004; Ali et al., 2019). Cd is a "priority contaminant" among these, causing potential hazards not just to public health but also to a broad ecosystem (Campbell, 2006; Ali et al., 2015). It is another extensively distributed heavy metal whose transport into the atmosphere and plant-soil natural environment is mostly caused by industrial operations, mining operations, solid wastes, municipal wastewater, and use as fertilizer in farm fields (Rao et al., 2010).

Cadmium poisoning raises the oxidative stress while deteriorating chloroplast structures, reducing the rate of photosynthesis in damaged plants. Higher reactive oxygen species (ROS) levels induce nucleotide breakdown, protein oxidation, and lipid peroxidation (Zaheer et al., 2018; Mwamba et al., 2020). Cd is promptly absorbed by the roots and can be transported into the xylem for transfer to the leaves. The quantity of Cd deposited in roots or translocated to leaves varies greatly between species (Ali et al., 2013a,b). Most plants are susceptible to low Cd concentrations, which restrict root and shoot growth due to changes in the photosynthetic rate, macronutrient absorption, and micronutrient distribution (Shi et al., 2005).

Nanotechnology is a contemporary scientific discipline concerned with nanoscale materials. It is an emerging field that has been rapidly evolving and works in everyday life as a result of the significant impact that its vast applications have in all aspects of human life (Khan et al., 2020; Zhang, 2020). Nanomaterials, which are molecular and atomic aggregates with a diameter of lower than 100 nm, are the focus of nanotechnology. These are modified analogs of essential elements created by changing their molecular and atomic characteristics (Kato, 2011). Metal-based nanoparticles (NPs) are the most researched nanomaterials among the many nanomaterials (Lin et al., 2009). Nanomaterials increase soil fertility by chelating various ions and salts available in the soil, regulating soil pH, and interacting with soil microbes, indirectly assisting better growth of plants (Fraceto et al., 2016). Furthermore, in the agricultural field, NPs are used as a quick diagnostic tool to check different challenges of environment such as drought and salinity and to determine the level of soil nutrients and heavy metals (Alghuthaymi et al., 2015).

Zinc is a micronutrient that is required for humans, wildlife, and crop production. The deficiency of zinc can result in stunted growth in plants and loss of yield (Merchant, 2010). Zinc oxide (ZnO) NPs are widely employed in medical fields and other applications due to their antibacterial qualities, with roughly 528 tons produced globally each year (Gümüş et al., 2014). In current years, application of micro and macronutrients in the form of NPs has been regarded as a viable method for enhancing the crop growth and yield in most crops (Rizwan et al., 2017a,b, 2019a,b). These NP supplements may assist to prevent nutrient loss and increase crop output in a sustainable way (Dimkpa et al., 2017). Several scientists investigated the effect of ZnO NPs on various crop plants, and their findings indicate that ZnO NPs have a good effect on plant growth. It has also been found that the harmful effects of ZnO NPs on agricultural crops are substantially lower than that of Zn^{2+} or bulk particles (i.e., ZnO) (Kouhi et al., 2015). When compared with the ionic form (i.e., Zn^{2+}) of the equivalent metal and micro (macro) particles, foliar-applied ZnO NPs concentrations can have both favorable and harmful effects on the development of plants (Mousavi Kouhi et al., 2014).

Moringa oleifera belongs to the monogeneric Moringaceae family and is also known as sohanjana, drumstick, and horseradish tree (Marrufo et al., 2013). *Moringa oleifera* a tiny, rapidly growing ornamental tree found throughout Africa and Asia's tropical climates (Sreelatha et al., 2011). Moringa leaves has been observed to be a rich source of carotene, protein, vitamin C, calcium, and potassium, as well as a good source of natural antioxidants (Moyo et al., 2012). In recent years, extracts of *M. oleifera's* leaves, seeds, and roots have been extensively researched for a variety of possible applications, such as wound healing, antihepatotoxicity, antifertility, hypotensive, and analgesic action (Karadi et al., 2006).

Linseed, generally known as common flax, is a fibrous crop and dicotyledonous plant in the Linaceae family with economic values (Feller et al., 2019). It is a white or a blue-flowered plant that has traditionally been used for fiber and food in cooler parts of the world. It is an annual herbaceous plant. Linseed has been farmed for 5,000 years in China and India and 10,000 years ago in Egypt and Samaria (Akhtar et al., 2013; Wiszniewska et al., 2016). Flaxseeds provide numerous nutritional benefits and a higher proportion of short-chain omega-3 fatty acids. To determine whether *M. oleifera*-stabilized ZnO NPs can minimize Cd toxicity in the linseed plant. The study was conducted to evaluate the potential for synergistic application of ZnO NPs and *M. oleifera* in alleviation of Cd stress in linseed.

MATERIALS AND METHODS

Preparation of *Moringa oleifera* Extract

Cholistan Institute of Desert Studies (CIDS)/The Islamia University of Bahawalpur (IUB) provided the powder form of washed and dried *M. oleifera* leaves. To prepare the leaves extract, a 2.8 g of *M. oleifera* powder was added to 100 ml of deionized water and then heated at 90°C for ∼1 h using a hotplate under continuous stirring condition. After being cooled at room temperature, the extract was filtered using the Whatman No. 1 filter paper to remove the residues. The filtrate was stored in a glass bottle at 4°C for further usage. The as-prepared *M. oleifera* green extract contains various biological elements such as alkaloids, flavonoids, phenols, gallic acid, and amino acids. These elements play a vital role in the preparation and stability of *M. oleifera*-reduced ZnO NPs (Patel et al., 2012).

Synthesis of *Moringa oleifera*-Stabilized Zinc Oxide Nanoparticles

For *M. oleifera* extract-mediated synthesis of ZnO NPs, deionized H_2O was used as a solvent throughout the procedure. On a laboratory hot plate, 4.5 g of zinc acetate dehydrate/$Zn(CH_3CO_2)_2.2H_2O$ (Sigma Aldrich, 99.99% purity) was dissolved in 100 ml of deionized water with continuous stirring to make a 0.25 M Zn stock solution. The obtained *M. oleifera* leaves extract was then added to the zinc acetate stock solution, which was continuously stirred with a magnetic stirrer while heating at 70°C for 2 h. The resultant solution was evaporated on a hot plate, and the leftover solid sample or paste-like substance was placed in a ceramic crucible cup and calcined for 2 h at 200°C in a muffle furnace to eliminate impurities. Following that, changes in the color of the sample indicate the formation of *MO*-ZnO NPs (Mahendiran et al., 2017). A yellowish-brown-colored solid product was obtained, which was ground in a sterilized mortar/pestle system to produce powdered *M. oleifera*-ZnO NPs. Several early experiment trails were performed to get this optimal concentration of zinc acetate and plant extract (**Figure 1**).

Characterization

To confirm the morphology and stability of *M. oleifera*-stabilized ZnO NPs, the absorption spectrum of the as-prepared sample was obtained using an Epoch Microplate Spectrophotometer in 300–700 nm. The X-ray diffraction (XRD) for this sample was performed through the Bruker-D8 Advance X-ray Diffractometer with Cu $K\alpha$ radiation ($\lambda = 1.54$ Å), operated at 35 mA and 40 keV. The machine scanned the sample at room temperature from 20° to 85° for its structural analysis. To examine the surface morphology of *M. oleifera*-stabilized ZnO NPs, a JEOL SEM (made in Japan) was used. An X-ray analysis option, energy-dispersive X-ray (EDX) equipped with the scanning electron microscope (SEM), was utilized to determine the elemental/quantitative compositional information of the as-synthesized sample.

Experiment Design

Linseed (*Linum usitatissimum)* was selected for the experiment. Viable seeds were selected and used for greenhouse experiment. Cd was purchased from the Sigma International Company. Different concentrations of Cd were prepared for further experimentation. This experiment was carried out at the Department of Botany and Wire house of Horticulture Department, the Islamia University of Bahawalpur. The soil was collected from the local Nursery of Bahawalpur for the greenhouse experiment. The Bhal soil was washed *via* a 2 mm steel sieve after being acquired from a local Bahawalpur nursery. Cd in the form of Cd chloride ($CdCl_2$) was introduced into the test soil. For Cd contaminations, soil was spiked with 100 mg/kg Cd, whereas noncontaminated soil was used as control. To achieve a balance between the soil's solid phase and liquid phase, contaminated soils were stored in the shade for 1 month prior to seed sowing. The experiment was carried out in a completely randomized design (CRD). Each treatment composed of 3 replicates. Each pot contained 3 plants. Greenhouse conditions during the experiment were 30°C average temperature, 16 h day/8 h night photoperiod, 80% relative humidity, and 200 μmol m^{-2} s^{-1} light intensity.

Application of *Moringa oleifera* Leaf Extract and Zinc Oxide Nanoparticles on Linseed

Three concentrations of NPs, namely, 100 mg/L, 500 mg/L, and 1 g/L, were dissolved in 1 L (1,000 ml) of distilled water and mixed well and sprayed on 1 month seedling of linseed. *Moringa oleifera*-grinded leaves extract was also sprayed on linseed. In total, six to eight linseed seeds were sown at equal distances in 1.5 kg of soil. Plastic containers were used to propagate the seeds. After 1 week, the germinating plants were thinned. The plants were irrigated regularly to maintain a moisture content of 40% throughout all containers. All plants were studied until constant heights, and those plants with constant heights were harvested after 3 months. Shoots were taken from the roots, and the roots were cleaned with distilled water to eliminate particles of soil. The plant tissue and soil were oven dried at a temperature of 400°C. The fresh and dry plant root and shoot weights were measured and reported as root and shoot biomass.

Estimation of Plant Growth Attributes

Using the measuring scale, morphological parameters were determined. Shoot and root fresh weight (FW) and dry weight were computed in grams, while root and shoot length was measured in cm. The dry mass of shoots and roots was recorded from 9-day-old seedlings after keeping them in a scientific oven at 70°C for 75 h.

Estimation of Chlorophyll and Carotenoids Contents

The concentration of photosynthetic pigment (i.e., chlorophyll a, b, and total chlorophyll) was quantified using the Lichtenthaler method (1987). The pigment extract was quantified against a blank of acetone at wavelengths of 646 nm and 663 nm for chlorophyll assays and 470 nm for carotenoids, and all pigments were expressed as mg g^{-1} FW leaf.

Estimation of Leaf Relative Water Content

The completely expanded leaves specimens were immersed in 100 ml of sterilized distilled water for 24 h in a dark area at 10°C. These turgid leaf samples were quickly wrapped in blotting paper for 2 min before being weighed. Samples of leaves were oven dried at 75°C for 2 days, and LRWC was calculated using the Smart and Bingham (1974) method.

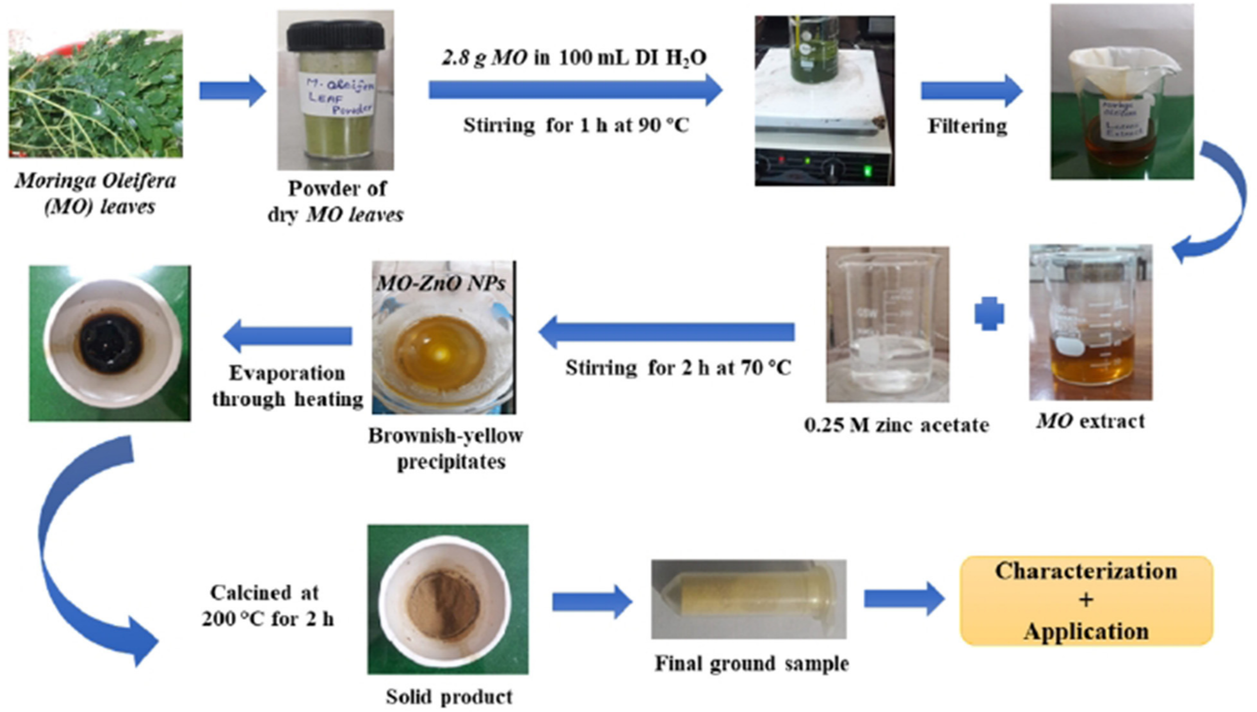

FIGURE 1 | Schematic of the synthesis of *Moringa Oleifera* (*MO*) leaves-stabilized ZnO NPs.

Estimation of Antioxidant Enzyme

To evaluate SOD activity, a piece of 0.2 g fresh leaves was put in an ice bucket and crushed with 1% polyvinylpyrrolidone (PVP) in the presence of phosphate buffer at pH 7.0. This mixture was centrifuged at 4°C for 25 min at 15,000 *g*. The supernatant superoxide dismutase (SOD) characteristic was examined by Giannopolitis and Ries (1977) by measuring the enzyme that generated 50% suppression of nitroblue tetrazolium chloride in the presence of riboflavin at 560 nm.

Fresh leaf specimens (1 g) were well agitated with a 4 ml extractor buffer comprising 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% PVP, 1 mM ethylenediaminetetraacetic acid (EDTA), and 50 mM phosphate buffer at pH 7. Upadhyaya et al. (1985) used the resultant supernatant to test peroxidase (POD) operation after centrifugation at 15,000 rpm for 30 min at 4°C. Guaiacol oxidation enhanced the absorbance values measured at 420 nm.

For catalase (CAT) analysis, 0.5 g of fine powder from dried oven leaves was mixed in an ice-cold pestle and mortar with 1 mM EDTA, 1 mM ascorbic acid, 2% PVP (w/v), and 0.05% Triton X-100 (w/v) in 50 mM potassium phosphate buffer (2 ml) at pH 7. According to Aebi (1983), after centrifuging the solution at 1,000 rpm for 20 min at 4°C, the resultant supernatant was utilized to assess the CAT enzyme activity. The lowered absorbance rate of H_2O_2 was spectrophotometrically measured at 240 nm to determine the CAT behavior. As a result, for 2 min, 10.5 mM H_2O_2 and 50 mM potassium phosphate buffer at pH 7 were added to the 25°C enzyme extract.

For APX estimation, 0.2 ml of the solution was mixed with 0.1 mM EDTA, 0.25 mM ascorbic acid, and 25 mM phosphate buffer at pH 7. The first reading for ascorbate oxidation was obtained after the addition of 1 mM H_2O_2, and the second reading was obtained at 290 nm after 1 min interval using a spectrophotometer. The extinct value distinguished the differences in ascorbate oxidation values.

Estimation of Electrolyte Leakage in Leaves

The completely developed leaves (2) were sliced into 0.5 cm pieces and immersed in 7 ml sterilized water in a glass container containing leaf segments before being placed in a rotatory shaker at 120°C for 30 min. The reading for primary leaf conductivity (EC-i) was derived by autoclaving the sample for 30 min at 120°C and then cooling at room temperature (maximum value) or final conductivity (EC-f) by formula (Li et al., 2018),

$$\mathbf{EL = (EC - i)/(EC - f) \times 100} \tag{1}$$

Evaluation of Hydrogen Peroxide

The fresh leaf extract (0.25 g) of treated plants was homogenized with 5% trichloroacetic acid (TCA, 3 ml) in the presence of activated charcoal (0.1 g) at 0°C, followed by centrifugation at 12,000 rpm for 15 min. The supernatant was then mixed with 1 M potassium iodide (0.75 ml) and 10 mM potassium phosphate buffer (0.5 ml) at pH 7.0. The spectrophotometric values of the solution were measured at 390 nm and correlated with known H_2O_2 concentration (Velikova et al., 2000).

Measurement of Malondialdehyde Content

The malondialdehyde content was determined using the thiobarbituric acid reaction reported by Rao and Sresty (2000). A fresh 0.5 g specimen of prewashed leaves was vortexed with 10 ml of trichloroacetic acid (0.1% w/v) and centrifuged for 15 min at 4°C in an ice bath. The supernatant (2 ml) and 2 ml thiobarbituric acid (0.67% w/v) solution were produced and stored at 100°C. Following 0.5 h, the supernatant was transferred to the ice bath. The precooled solution (4°C) was centrifuged at 10,000 *g* for 30 s, followed by absorbance measurement of the supernatant at 532 nm. Nonspecific absorption was removed from the 600 nm reading. The MDA concentration was determined using the MDA molar extinction coefficient.

Estimation of Proline Content

Shaking for 30 min at 150 rpm, the freeze-dried powder (0.1 g) was extracted in 3 ml of 3% sulfosalicyclic acid dihydrate. The supernatant was centrifuged at 2,400 g for 1 min and then passed through a 0.2 m syringe filter with a 1 ml syringe and stored. The supernatant was then diluted with 3% sulfosalicyclic acid dehydrate, and the proline content was determined with a spectrophotometer using the method published by Bates et al. (1973).

Estimation of Total Soluble Protein Content

The protein content was assessed using a standard curve established by distinct concentrations of bovine serum albumin (BSA). Furthermore, 1 ml of sample plant leaf extract was placed in a test tube. Phosphate buffer was mounted at 1 ml with pH 7.0 in log. The test tubes containing the reagents were held at room temperature for 1 min. A 0.5 ml of Folin-phenol reagent was combined and incubated for 30 min. The optical range was read by a spectrometer at 620 nm.

Estimation of Total Soluble Sugar

The Yemm and Willis (1954) method was used to determine the maximum soluble sugars. In 25 ml test tubes, 0.1 ml of plant extract was collected. Anthrone reagent (6 ml) was used in each tube, which was then immersed in a boiling bath for 10 min to heat up. The test tubes were solidified at room temperature for 10 min before being incubated for 20 min. The spectrophotometric value was determined at 25nm.

Statistical Analysis

The data were entered into the computer software in this investigation. The arithmetic mean and standard error were computed. To compare different treatments, a one-way ANOVA test was used. To make multiple comparisons between averages of different treatments, the one-way ANOVA test was used followed by a *post-hoc* test using the Duncan's test.

RESULTS

To estimate the dimensions and shape of *M. oleifera*-stabilized ZnO NPs, the SEM analysis is performed and the micrograph is shown in **Figure 2A**. It is depicted that the sample is incorporating small granules of sizes in the nano-regime. It seems that the NPs sample could not be fine-ground before the SEM analysis. The size estimate of these nano-granules falls below 700 nm.

Figure 2B shows corresponding EDX spectroscopic analysis of *M. oleifera*-stabilized ZnO NPs, noticeably indicating the peaks for Zn and oxygen elements. The EDX spectrum of our sample obtained from the SEM-EDX analysis shows that the sample prepared by using *M. oleifera* leaves extract has pure ZnO phases, and the reaction product comprises of high-purity ZnO NPs. The approximate atomic ratio of Zn to oxygen is found to be 3.14:96.86, as shown in **Figure 2B**.

Figure 2C shows the UV-Vis absorption spectrum of *M. oleifera*-stabilized ZnO NPs. Usually, pristine ZnO does not show any absorption peak in the visible region, but an equitable absorption band can be observed in the UV region, which may be attributed to the band edge absorption of hexagonal wurtzite crystalline structure of ZnO material (Alenezi et al., 2013). **Figure 2D** shows the XRD pattern of *M. oleifera*-stabilized ZnO NPs, where the diffraction peaks are obvious to locate at 2θ values of 31.852, 34.511, 36.331, 47.62, 56.66, 62.917, 66.435, 67.99, and 69.175° corresponding to (100), (002), (101), (102), (110), (103), (200), (112), and (201) crystalline planes, respectively. This is in agreement with the previously reported literature being indexed to the hexagonal wurtzite type of ZnO material (JCPDS 36-1451) (Wang et al., 2017). The perception of the XRD pattern reflects the crystalline quality of *M. oleifera*-stabilized ZnO NPs. No other diffraction peaks have been observed in this pattern, indicating the absence of any secondary phases of our sample.

Estimation of Plant Growth Attributes

As demonstrated in **Table 1**, fresh shoot weight (FSW) did not decrease much in stressed plants treated for Cd stress. The application of 2.8 g/100 ml *Moringa* leaf extract (MLE) on the plant decreased the FSW compared with control and Cd-stressed plants, whereas under Cd stress, *M. oleifera* leaf extract increased the FSW. The application of NP1 (100 mg/L) alone significantly increased the FSW, whereas other levels of MZnO NPs, i.e., NP2 (500 mg/L) and NP3 (1 g/L), did not significantly increase the FSW in nontreated plants. Dry shoot weight (DSW) did not decrease much in stressed plants treated to Cd stress as shown in **Table 1**. The application of NP1 (100 mg/L) alone significantly increased the DSW in nonstressed plants, whereas other levels of MZnO NPs NP2 (500 mg/L) and NP3 (1 g/L) moderately increase the DSW. Fresh root weight declined in Cd-stressed plants as compared with control. The application of 2.8 g/100 ml *Moringa oleifera* leaves (M.L) powder decreased the FRW and DRW compared with control and Cd-stressed plants. The application of MZnO NPs alone significantly increased the fresh root weight, while an increased level of MZnO NPs (1 g/L) decreased the fresh root weight. The application of 2.8 g/100 ml *M. oleifera* leaf extract alone on the nontreated plant decreased the shoot length compared with the control and Cd-stressed plants. In contrast, under Cd stress, MLE increases the shoot length. The root length decreased at zero concentration of MZnO NPs (0 mg/L). Supplementation of *M. oleifera* leaf extract under

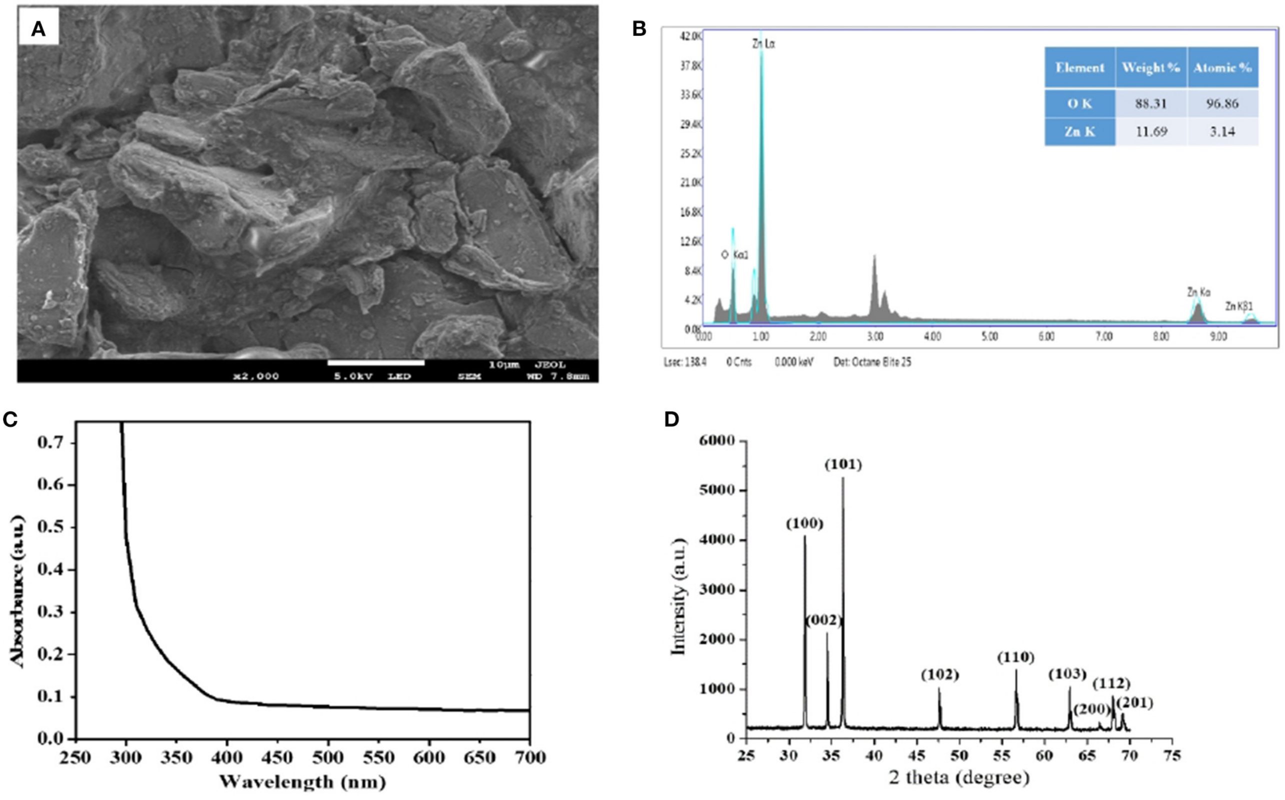

FIGURE 2 | (A) SEM image of *MO*-stabilized ZnO NPs exhibiting nano-granules accumulated on the bulk, **(B)** EDX map clearly showing traces of Zn and oxygen elements, **(C)** corresponding UV-Vis absorption spectrum and **(D)** XRD pattern of *MO*-stabilized ZnO NPs.

Cd stress increased the root length. The application of NPs NP1 (100 mg/L) alone significantly enhanced root length followed by NP2 (500 mg/L).

Evaluation of Chlorophyll a, b and Carotenoids in Linseed Shoots Under Cadmium Stress

In this study, MZnO NPs and *M. oleifera* leaf extract significantly impacted the Chl *a* parameter compared with control as shown in **Table 2**. The lower concentration of Chl *a* and *b* was observed in Cd-alone-treated seedlings. The application of NP1 (100 mg/L) to Cd-stressed plants enhanced the Chl *a* in contrast to those plants treated with Cd alone. The treatments (500 mg/L and 1 g/L) of MZnO NPs also enhanced Chl *a* over those in Cd-alone-treated plants. In this study, MZnO NPs and *M. oleifera* leaf extract significantly impacted the Chl *b* parameter compared with control. The treatment (500 mg/L and 1 g/L) of MZnO NPs also enhanced total Chl over those in Cd-alone-treated plants. The results of this study depicted that carotenoid amount significantly increased in control seedlings over Cd-treated seedlings. Nonsignificant carotenoids amount was found in the 2.8 g/100 ml *M. oleifera* leaf extract supplemented seedlings over Cd-stressed plants. However, among NPs treatments, it was found that NP2 (500 mg/L) enhanced carotenoids in nonstressed plants.

Evaluation of Leaf Relative Water Content in Linseed Under Cadmium Stress

Leaf water content decreased in Cd-stressed plants compared with control as shown in **Table 2**. However, levels of leaf water content decreased when plants were treated with *M. oleifera* leaf extract (2.8 g/100 ml). The application of MZnO NPs (500 mg/L) enhanced leaf water content in plants. Under Cd stress, MZnO NPs increased the leaf relative water content.

Evaluation of Soluble Sugar in Linseed Root and Shoots Under Cadmium Stress

The levels of total soluble sugar in Cd-treated plants and without stressed plants were found to be declined when they were treated with 2.8 g/100 ml *M. oleifera* leaf extract only in contrast to those plants subjected to Cd alone (**Figure 3**). The supplementation of alone MZnO NPs does not show any significant difference in total soluble sugar amount of root. The amount of total suspended solid (TSS) in shoots enhanced in nonstressed plants in contrast to Cd-stressed plants as shown in **Figure 2**. The levels of total soluble sugar in Cd-treated plants and without stressed plants were found to be declined when they were treated with 2.8 g/100 ml MLE in contrast to those plants treated with Cd only. The amount of total soluble sugar increased under Cd stress compared to that in the M.L-supplemented plants.

TABLE 1 | Effects of MZnO nanoparticles (NPs) on shoot length, root length, fresh shoot weight, fresh root weight, dry shoot weight, and dry root weight under Cd stress in linseed.

Treatments		**Growth traits**				
	Shoot length (cm)	**Root length (cm)**	**FSW (g plant^{-1})**	**FRW (g plant^{-1})**	**DSW (g plant^{-1})**	**DRW (g plant^{-1})**
C	32 ± 0.13bc	2 ± 0.3d	0.15 ± 0.09d	0.018 ± 0.13d	0.04 ± 0.08e	0.02 ± 0.06b
Cd	28.3 ± 0.2de	1.4 ± 0.02e	0.173 ± 0.04cd	0.013 ± 0.8e	0.042 ± 0.09f	0.003 ± 0.03de
M.L	24.1 ± 0.09f	2 ± 0.19dc	0.124 ± 0.11e	0.012 ± 0.20f	0.02 ± 0.11c	0.002 ± 0.31f
Cd + M.L	32.7 ± 0.11bc	2.2 ± 0.13d	0.152 ± 0.14*d*	0.014 ± 0.15e	0.04 ± 0.14a	0.003 ± 0.09de
NP1	35.2 ± 0.07b	4.1 ± 0.4a	0.48 ± 0.01a	0.031 ± 0.20c	0.112 ± 0.10de	0.008 ± 0.16c
NP2	41.5 ± 0.12a	3.4 ± 0.14c	0.24 ± 0.05b	0.064 ± 0.15a	0.093 ± 0.15de	0.008 ± 0.21c
NP3	30.2 ± 02a	2 ± 0.21dc	0.181 ± 0.25c	0.012 ± 0.11*f*	0.052 ± 0.23d	0.013 ± 0.15a
Cd + NP1	35 ± 0.12b	3.6 ± 0.09b	0.13 ± 0.15f	0.016 ± 0.03ef	0.054 ± 0.17b	0.003 ± 0.04de
Cd + NP2	25 ± 0.11e	1.8 ± 0.11f	0.05 ± 0.41g	0.036 ± 0.08b	0.034 ± 0.20e	0.004 ± 0.06d
Cd + NP3	30 ± 0.03d	4.2 ± 0.01a	0.091 ± 0.07g	0.009 ± 0.21g	0.033 ± 0.04e	0.003 ± 0.06de

Different letters indicate significant difference between the treatments. Data are means ± SE (n = 5). Nonidentical letters specify significant difference at P ≤ 0.05. C, control; Cd, 100 mg/kg Cd; M.L, Moringa oleifera leaves; NP1, 100 mg/L MZnO NPs; NP2, 500 mg/L MZnO NPs; NP3, 1 g/L MZnO NPs.

TABLE 2 | Effects of MZnO nanoparticles (NPs) on Chl *a*, Chl *b*, total chl, carotenoids, and leaf relative water content under Cd stress in linseed.

Treatments	**Chl *a* (mg/g FW)**	**Chl *b* (mg/g FW)**	**Total Chlorophyll**	**Carotenoids**	**LRWC**
C	0.312 ± 0.021c	0.185 ± 0.04b	0.497 ± 0.06c	0.415 ± 0.3a	30 ± 0.06d
Cd	0.256 ± 0.014d	0.134 ± 0.02f	0.391 ± 0.03e	0.328 ± 0.031b	20 ± 0.09e
M.L	1.339 ± 0.09a	0.214 ± 0.07g	0.553 ± 0.01a	0.252 ± 0.14de	13.1 ± 0.21g
Cd + M.L	1.315 ± 0.09b	0.198 ± 0.04a	0.513 ± 0.40b	0.215 ± 0.31a	5 ± 0.05a
NP1	0.312 ± 0.02bc	0.178 ± 0.08cd	0.49 ± 0.07c	0.255 ± 0.19de	13.7 ± 0.12g
NP2	0.296 ± 0.03d	0.165 ± 0.01c	0.461 ± 0.04cd	0.274 ± 0.17d	52.9 ± 0.07b
NP3	0.312 ± 0.01c	0.174 ± 0.03cd	0.486 ± 0.11cd	0.231 ± 0.22e	11.5 ± 0.15h
Cd + NP1	0.312 ± 0.06c	0.185 ± 0.02b	0.497 ± 0.07c	0.254 ± 0.41e	15.8 ± 0.30f
Cd + NP2	0.274 ± 0.04e	0.153 ± 0.04d	0.427 ± 0.02d	0.232 ± 0.32f	56 ± 0.03a
Cd + NP3	0.235 ± 0.019f	0.142 ± 0.08e	0.377 ± 0.08f	0.315 ± 0.02c	47.8 ± 0.06c

Different letters indicate significant difference between the treatments. Data are means ± SE (n = 5). Nonidentical letters specify significant difference at P ≤ 0.05. C, control; Cd, 100 mg/kg Cd; M.L, Moringa oleifera leaves; NP1, 100 mg/L MZnO NPs; NP2, 500 mg/L MZnO NPs; NP3, 1 g/L MZnO NPs.

Evaluation of Total Soluble Protein in Linseed Root and Shoots Under Cadmium Stress

The levels of total soluble protein in roots increased when plants were supplemented with 2.8 g/100 ml *M. oleifera* leaf extract. The amount of protein is different among all the MZnO NPs levels. However, enhancement in the level of protein was observed in NP2 (500 mg/L) MZnO NPs, under Cd stresses compared with that in the plants supplemented with MZnO NPs alone. The amount of total soluble protein in shoots enhanced in control in relation to Cd-stressed plants as shown in **Figure 3**. However, the levels of total soluble protein increased when plants were supplemented with 2.8 g/100 ml *M. oleifera* leaf extract. The amount of total soluble protein is not significantly different among all the MZnO NPs levels. However, enhancement in the level of protein was observed in NP2 (500 mg/L) MZnO NPs under Cd stresses compared with that in the plants supplemented with alone MZnO NPs.

Evaluation of Proline Content in Linseed Shoots Under Cadmium Stress

The amount of proline in roots enhanced in control plants in contrast to Cd-stressed plants as shown in **Figures 3**, **4**. However, the levels of proline decreased when plants were supplemented with 2.8 g/100 ml *M. oleifera* leaf extract. The amount of proline is not significantly different among all the MZnO NPs levels. However, enhancement in the level of proline was observed in NP3 (1 g/L) MZnO NPs under Cd stress compared with that in the plants supplemented with MZnO NPs alone.

The amount of proline in shoots increased in control plants in contrast to Cd-stressed plants as shown in **Figure 4**. However, the levels of proline decreased when plants were supplemented with 2.8 g/100 ml *M. oleifera* leaf extract. The amount of proline is not significantly different among all the MZnO NPs levels. However, enhancement in the level of proline was observed in NP1 (100 mg/L) MZnO NPs under Cd stress compared with that in the plants supplemented with MZnO NPs alone.

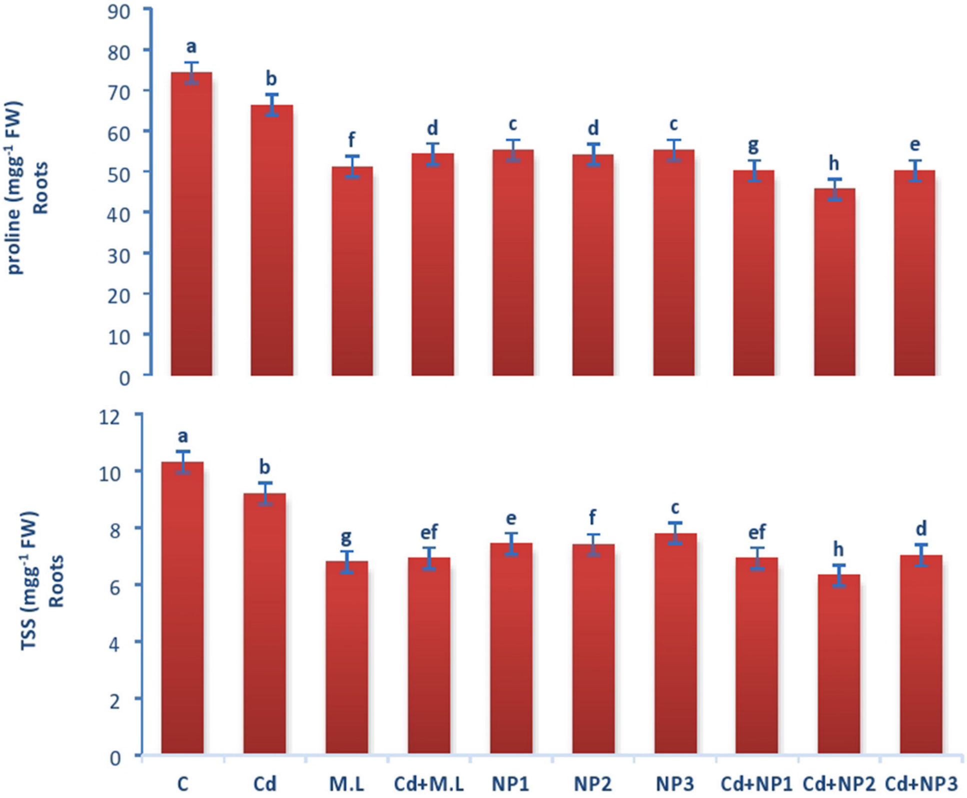

FIGURE 3 | Effects of MZnO–NPs on protein, proline, and TSS under Cd stress in Linseed roots. Different letters indicate significant difference between the treatments. Data are means ± SE ($n = 5$). Non-identical letters specify significant difference at $P \leq 0.05$. C, Control; Cd, 100 mg kg^{-1} Cd; M. L, *Moringa oleifera* leaves; NP1, 100 mg L^{-1} MZnO-NPs; NP2, 500 mg L^{-1} MZnO-NPs; NP3, 1 g L^{-1} MZnO-NPs.

Evaluation of Catalase Enzyme Activity in Linseed Roots and Shoots Under Cadmium Stress

The activity of CAT enzyme in untreated plants was found to be increased compared with Cd-treated plants roots (**Figure 5**). However, levels of CAT were declined when they were supplemented with alone *M. oleifera* leaf extract, relative to untreated and Cd-stressed plants alone. While in case of NPS application alone, no significant difference was observed among the all levels of MZnO NPs. The activity of CAT enzyme in shoot tissues of untreated plants was increased relative to Cd-treated seedlings as shown in **Figure 6**. The application of *M. oleifera* leaf extract alone exhibits declined value of CAT enzyme activity compared with controlled and Cd-stressed seedlings, while in case of NPs application alone, no significant difference was observed among all the levels of MZnO NPs.

Evaluation of Superoxide Dismutase Activity in Linseed Root and Shoots Under Cadmium Stress

Superoxide dismutase performance in untreated plants was found to be increased compared with Cd-treated plants roots (**Figure 5**). However, the levels of SOD were declined when they were supplemented with *M. oleifera* leaf extract alone, relative to untreated and Cd-stressed plants. While in case of NPs application alone, no significant difference was observed among the all levels of MZnO NPs. SOD performance in shoot tissues of untreated plants was increased relative Cd-treated plants seedlings in case of shoots as shown in **Figure 6**. The application of *M. oleifera* leaf extract alone exhibits declined value of SOD compared with controlled and Cd-stressed seedlings, while in case of NPs application alone, no significant difference was observed among the all levels of MZnO NPs.

Evaluation of Peroxidase Dismutase Activity in Linseed Root and Shoots Under Cadmium Stress

The activity of POD in untreated plants was found to be increased compared with Cd-treated plants roots (**Figure 5**). However, levels of POD were declined when they were supplemented with *M. oleifera* leaf extract alone, relative to untreated and Cd-stressed plants. While in case of NPs application alone, no significant difference was observed among the all levels of MZnO NPs. The activity of POD in shoot tissues of untreated plants was

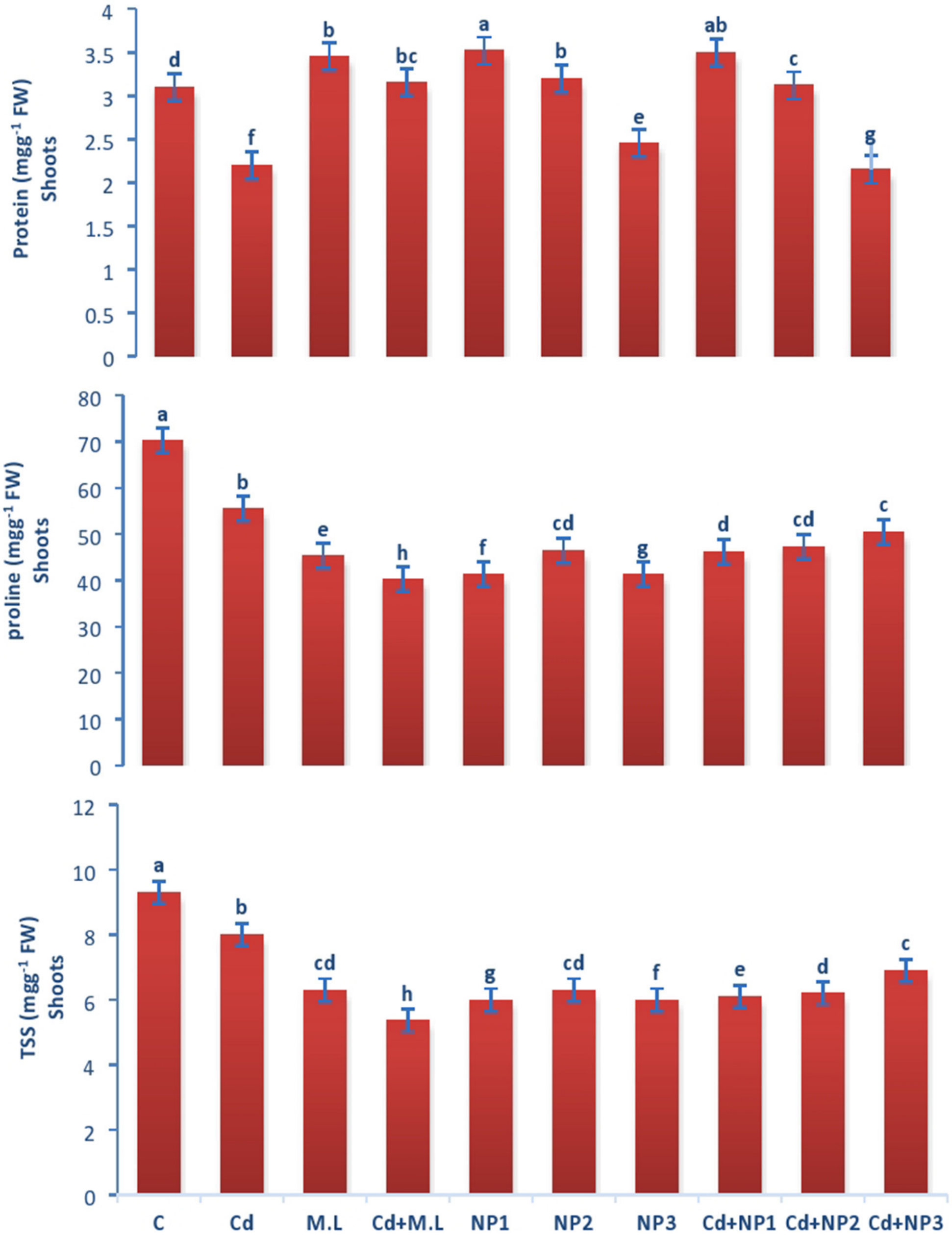

FIGURE 4 | Effects of MZnO–NPs on protein, proline, and TSS under Cd stress in Linseed shoots. Different letters indicate significant difference between the treatments. Data are means ± SE ($n = 5$). Non-identical letters specify significant difference at $P \leq 0.05$. C, Control; Cd, 100 mg kg^{-1} Cd; M. L, *Moringa oleifera* leaves; NP1, 100 mg L^{-1} MZnO-NPs; NP2, 500 mg L^{-1} MZnO-NPs; NP3, 1 g L^{-1} MZnO-NPs.

increased relative to Cd-treated seedlings (**Figure 6**). The application of *M. oleifera* leaf extract alone exhibits declined value of POD compared with controlled and Cd-stressed seedlings, while in case of NPs application alone, no significant difference was observed among the all levels of MZnO NPs.

Evaluation of Ascorbate Peroxidase Activity in Linseed Shoots Under Cadmium Stress

The activity of APX in untreated plants was found to be increased compared with Cd-treated plants roots (**Figure 5**). However, the levels of APX were declined when they were supplemented with

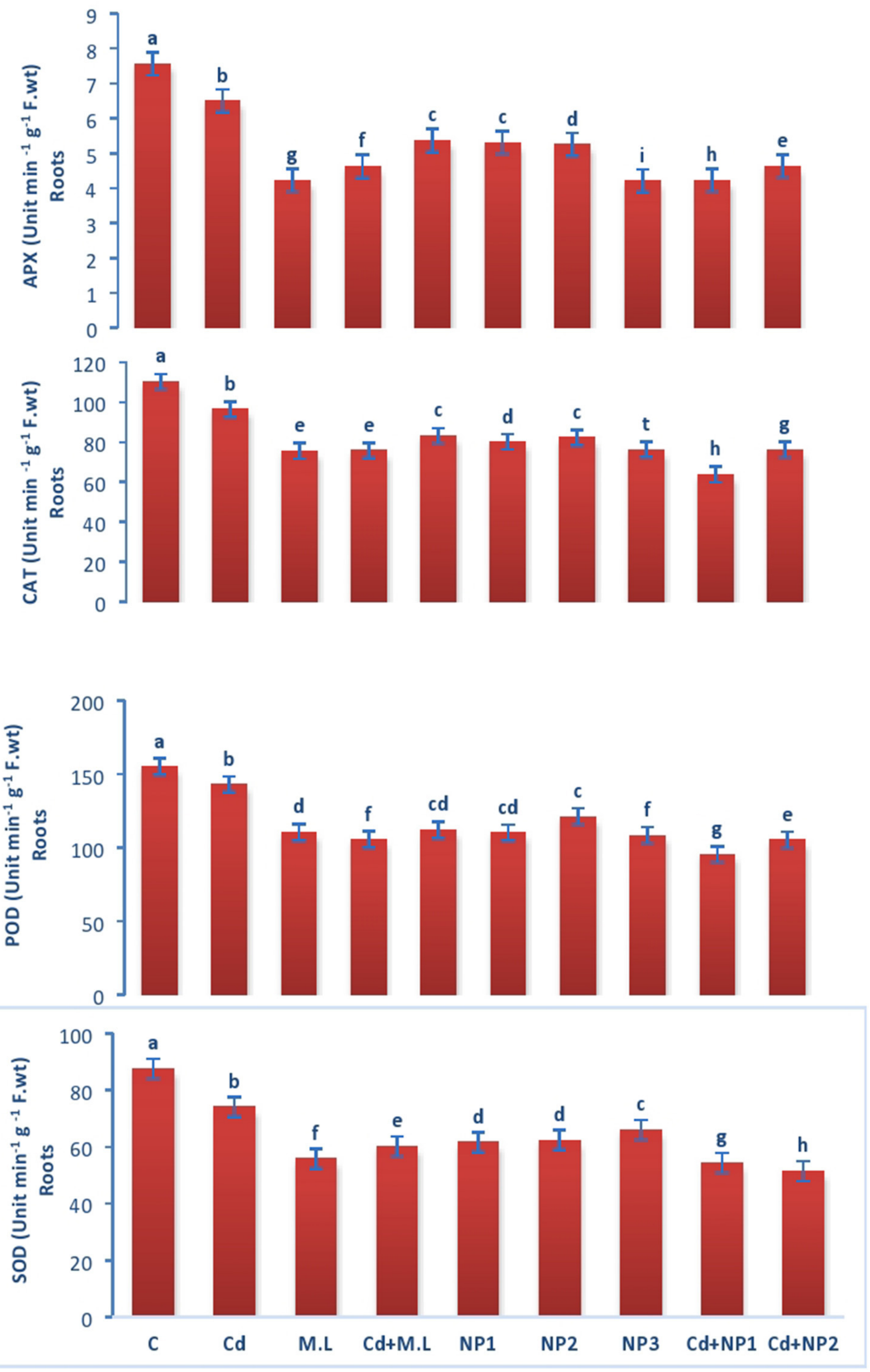

FIGURE 5 | Effects of MZnO–NPs on APX, CAT, POD, and SOD under Cd stress in Linseed roots. Different letters indicate significant difference between the treatments. Data are means ± SE ($n = 5$). Non-identical letters specify significant difference at $P \leq 0.05$. C, Control, Cd, 100 mg kg^{-1} Cd, M. L, *Moringa oleifera* leaves; NP1, 100 mg L^{-1} MZnO-NPs; NP2, 500 mg L^{-1} MZnO-NPs; NP3, 1 g L^{-1} MZnO-NPs.

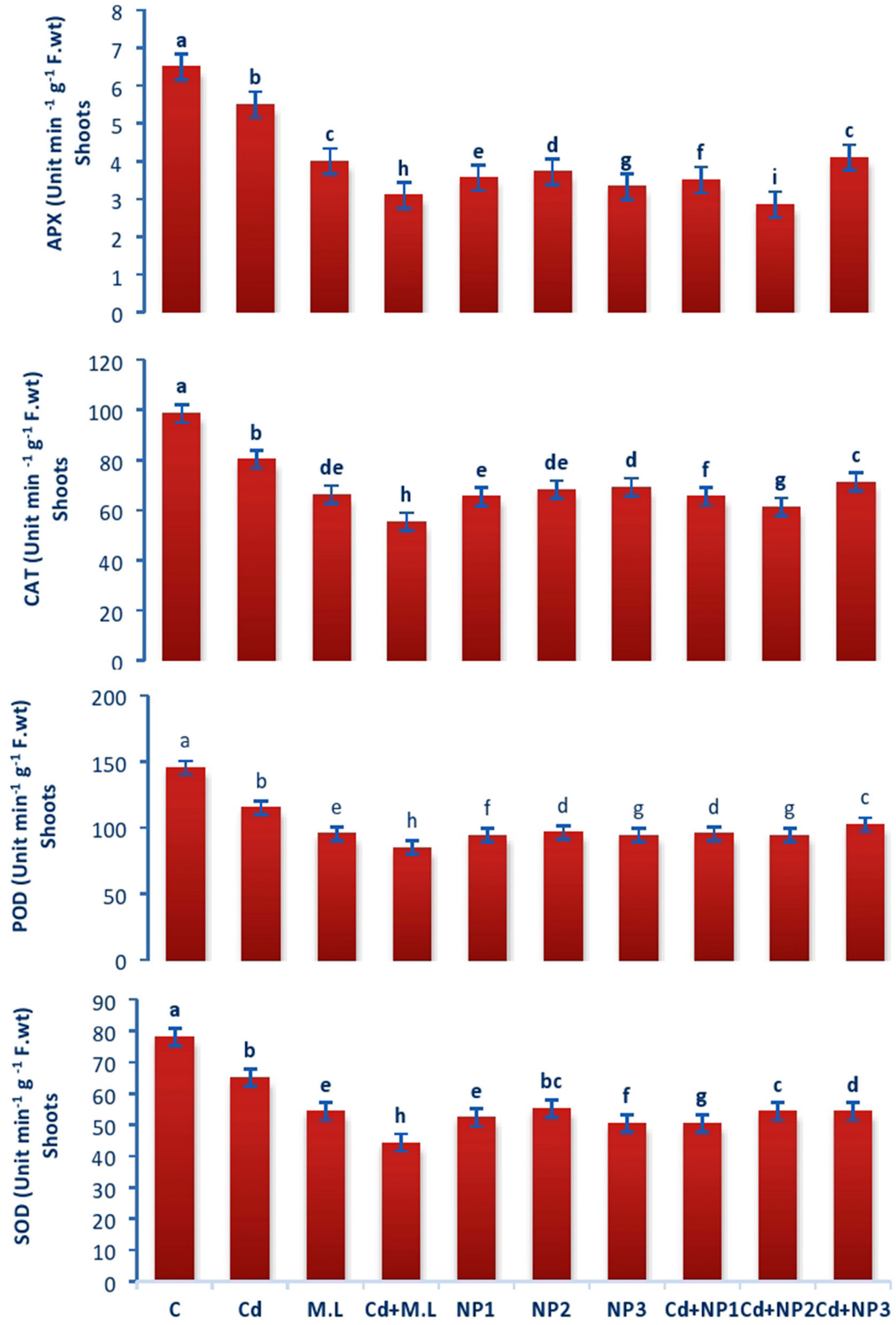

FIGURE 6 | Effects of MZnO–NPs on APX, CAT, POD, and SOD under Cd stress in Linseed shoots. Different letters indicate significant difference between the treatments. Data are means ± SE ($n = 5$). Non-identical letters specify significant difference at $P \leq 0.05$. C, Control; Cd, 100 mg kg^{-1} Cd; M. L, *Moringa oleifera* leaves; NP1, 100 mg L^{-1} MZnO-NPs; NP2, 500 mg L^{-1} MZnO-NPs; NP3, 1 g L^{-1} MZnO-NPs.

alone M.L extract, relative to untreated and Cd-stressed plants. While in case of NPs application alone, no significant difference was observed among the all levels of MZnO NPs. The activity of ascorbate peroxidase (APX) in shoot tissues of untreated plants was increased relative to Cd-treated seedlings (**Figure 6**). The application of *M. oleifera* leaf extract alone exhibits declined value of APX compared with controlled seedlings. While in case of NPs application alone, no significant difference was observed among the all levels of MZnO NPs. APX activity increased under all Cd levels when supplemented with NP2 (500 mg/L) and NP3 (1 g/L).

Evaluation of Electrolyte Leakage in Linseed Root and Shoots Under Cadmium Stress

The electrolyte leakage declined in nontreated plants compared with Cd-treated plants as shown in **Figure 7**. Exogenous application of NPs alone without Cd stress increased the EL at NP1 (100 mg/L) level while decreased the EL at NP3 (1 g/L) level. Under Cd stress, exogenous application of MZnO NPs deceased the EL relative to those plants treated with Cd alone.

Evaluation of Malondialdehyde Acetate Content in Linseed Root and Shoots Under Cadmium Stress

Malondialdehyde content in shoots increased with Cd over the control as shown in **Figure 8**. The application of *M. oleifera* leaf extract (2.8 g/100 ml) shows accumulation of MDA content with the treatment of Cd levels over the alone *M. oleifera* leaf extract supplemented root and shoot. However, exogenous application of NP2 (500 mg/L) enhanced MDA content only in non-Cd-treated plants as compared with those of plants subjected to NP1 (100 mg/L) and NP3 (1 g /L) alone.

Evaluation of Hydrogen Peroxide in Linseed Root and Shoots Under Cadmium Stress

In case of roots, H_2O_2 content increased with Cd levels over the control as shown in **Figure 7**. The application of *M. oleifera* leaf extract (2.8 g/100 ml) shows accumulation of H_2O_2 content with the treatment of Cd (**Figure 7**). The application of *M. oleifera* leaf extract (2.8 g/100 ml) shows accumulation of H_2O_2 content with the treatment of Cd levels over the *M. oleifera* leaf extract-supplemented plants alone. However, exogenous application of NP3 (1 g/L) enhanced H_2O_2 content only in nonstressed plants as compared with those of plants subjected to NP1 (100 mg/L) and NP2 (500 mg/L) alone.

DISCUSSION

Heavy metals and metalloids are extremely poisonous to all species, including humans, causing major problems with normal metabolism and cellular functioning. Cd is continuously introduced to the soil from many sources, and because it is an analog of phosphate fertilizer, it is easily translocated by plants (di Toppi and Gabbrielli, 1999; Rao et al., 2010; Dixit et al., 2011). Excessive usage of Cd-rich sewage sludge and fertilizer has had a negative influence on human health; it accumulates extensively in edible plant parts (Karthik et al., 2017). The recent study investigated to examine the potentiality of exogenous MZnO NPs application in alleviation of Cd uptake and growth in linseed. In this study, Cd declined growth significantly, but exogenously applied MZnO NPs was effective in mitigating the Cd-induced negative effects and improving growth under normal, as well as Cd-induced stress circumstances. Similar to our findings, Paparella et al. (2015) and Rizwan et al. (2018) found that ZnO NPs and Fe NPs promoted wheat growth and development.

Another possible cause for higher plant growth with nanomaterials is that nutrients like Zn encourage plant chlorophyll production (Briat et al., 2007). The key component of chloroplast, i.e., chlorophyll, is positively associated to the photosynthetic rate of plants. Cd and many other metals have a considerable effect on leaf chlorophyll concentration, which is the principal signs of metal toxicity in plants (Rizwan et al., 2016). Variation in chlorophyll concentration represents plants growth as well as plant reaction to environmental change. Cd revealed in the results, there was enhancement in chlorophyll content and photosynthetic parameters in linseed leaves by Moringa-stabilized ZnO NPs (1 g/L) as shown in **Table 2**. Rizwan et al. (2018) revealed that zinc oxide and Fe NPs both boost chlorophyll content in Cd-stressed plants.

Same findings were found by Mahakham et al. (2017), who discovered that a rise in chlorophyll concentrations in NPs-primed plants can be associated to increased water and nutrient absorption, resulting in increased physiological activity of the plants. Some previous studies have revealed the efficacy of NPs in improving photosynthesis in plants, with dose-dependent and plant species-dependent responses (Sreelatha et al., 2011; Rizwan et al., 2017b). In our research, EL was decreased in roots and shoots tissues after supplementation of MZnO NPs, whereas leaf water content was also increased in these seedlings. This could be attributed to increased water and mineral element absorption in the presence of ZnO NPs (Pervaiz et al., 2020).

Cadmium stress decreases the content of essential osmolytes, particularly proline, total soluble sugar, and protein in this study. However, MZnO NPs supplemented Cd-stressed seedlings increased these osmolytes. Similar findings were found to be in agreement with Ahanger and Agarwal (2017a,b), who indicated that these osmolytes are significant in mitigating stress-induced damage. As a result, an enhancement in their content under hazardous state is a natural defensive mechanism in plants. According to Choudhury et al. (2011), Cd stress increases proline accumulation in soybean. Siddiqui et al. (2015) found that Cd stress increased proline accumulation in *Withania somnifera* and *Oryza sativa*. They stimulate proline buildup, and GB preserves biochemical processes, enhances ROS scavenging, and regulates redox homeostasis and the activities of several enzymes (Kaya et al., 2020).

The growing severity of As stress in soybean plants resulted in elevated amounts of H_2O_2, MDA, and EL. Other plants cultivated under other conditions have likewise shown increased H_2O_2 and MDA acetate buildup, as well as enhanced EL (Ahanger and Agarwal, 2017a,b; Ahmad et al., 2018; Kaya et al., 2020).

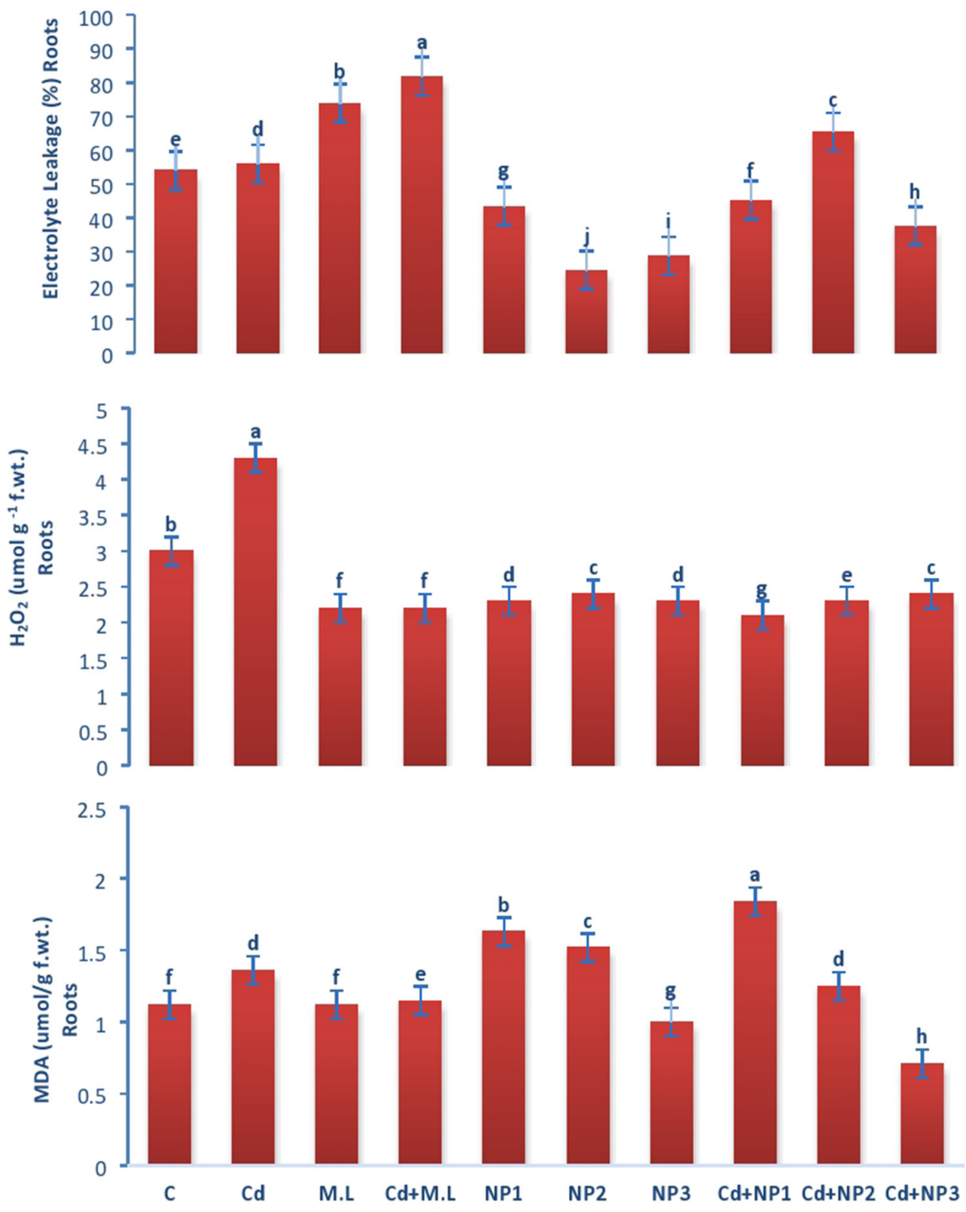

FIGURE 7 | Effects of MZnO–NPs on EL, H_2O_2, and MDA under Cd stress in Linseed roots. Different letters indicate significant difference between the treatments. Data are means ± SE ($n = 5$). Non-identical letters specify significant difference at $P \leq 0.05$. C, Control; Cd, 100 mg/kg Cd; M. L, *Moringa oleifera* leaves; NP1, 100 mg/L MZnO-NPs; NP2, 500 mg/L MZnO-NPs; NP3, 1 g/L MZnO-NPs.

Supplementation of MZnO NPs (1 g/L) in our research lowered the accumulation of MDA and H_2O_2 contents, and our results correlate with the findings of some earlier reports with different plant species (Hussain et al., 2016; Rizwan et al., 2017a,b, 2019a,b; Venkatachalam et al., 2017; Pervaiz et al., 2020).

Plants have an antioxidant system containing enzymatic and nonenzymatic antioxidants, the latter of which include ascorbic acid (ASA), glutathione (GSH), alpha-tocopherols, and phenolic chemicals (Ahanger and Agarwal, 2017a). Cd stress increased the activity of antioxidant enzymes, which is consistent with the results of Talukdar (2013), Yadav and Srivastava (2015), and Siddiqui et al. (2015). Under Cd stress, Lu et al. (2019) found increased activity of SOD, CAT, POD and APX in tartary buckwheat. The level of NPs increased after MznO NPs administration and antioxidant enzyme activation.

Various studies reported similar findings; López-Moreno et al. (2010) and Ghosh et al. (2016) both noticed an enhancement in antioxidant defense system under ZnO NPs exposure, which is a result of gene expression (Nair and Chung, 2014). Many other plant species react similarly to ZnO NPs by increasing the effectiveness of antioxidant systems and the functions of peroxidase, CAT enzyme, and superoxide dismutase (Singh et al.,

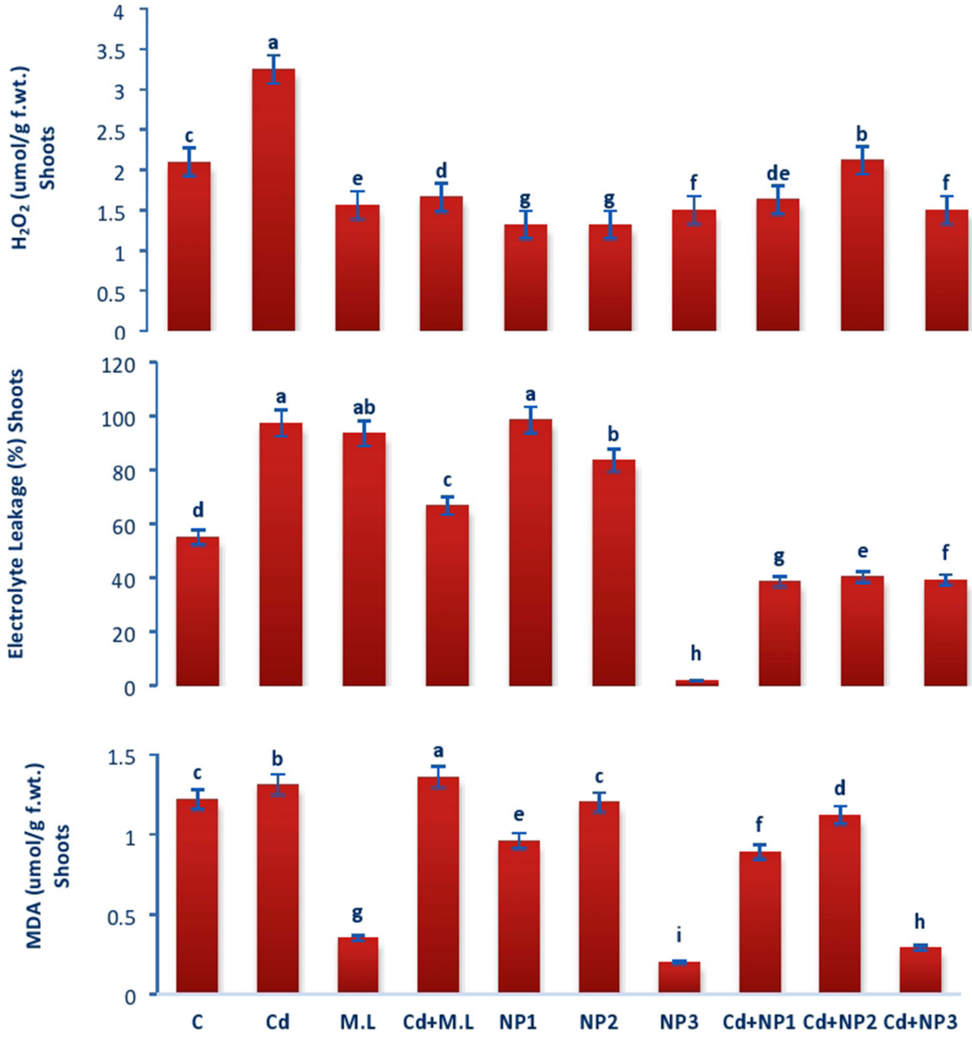

FIGURE 8 | Effects of MZnO–NPs on EL, H_2O_2, and MDA under Cd stress in Linseed shoots. Different letters indicate significant difference between the treatments. Data are means ± SE ($n = 5$). Non-identical letters specify significant difference at $P \leq 0.05$. C, Control; Cd, 100 mg kg^{-1} Cd; M. L, *Moringa oleifera* leaves; NP1, 100 mg L^{-1} MZnO-NPs; NP2, 500 mg L^{-1} MZnO-NPs; NP3, 1 g L^{-1} MZnO-NPs.

2013; Soliman et al., 2015; Abdel Latef et al., 2017; Rizwan et al., 2017a,b, 2019a,b; Kantabathini et al., 2018; Wang et al., 2018). Many authors have found elevated SOD, CAT, APX, and GR activities produced through ZnO–NP in various plants, including Tripathi et al. (2015) in pea, Hernandez-Viezcas et al. (2011) in *Prosopis juliflora*, and Krishnaraj et al. (2012) in *Bacopa monnieri*. These increased antioxidant functions aided the plants in scavenging the additional ROS produced in response to stressful signals.

CONCLUSION

Cadmium stress reduced growth and physiochemical properties of linseed plants. However, application of MZnO NPs reduced Cd accumulation in linseed. The MZnO NPs raised Zn concentrations, indicating that NPs might be employed in the bio-fortification of cereals while lowering Cd levels. As a result, this technology could be a greener alternative to traditional methods used to lower Cd levels in plants and, eventually, in human beings. Synergistic application of *M. oleifera* leaf extract and ZnO NP protects photosynthesis and ameliorates the deleterious effects on the osmolyte component by restricting the accumulation of Cd thereby minimizing the chance of oxidative stress. Furthermore, investigations in real-world field circumstances and with different plants are required to validate these findings. Synergistic application of *M. oleifera* leaf extract and ZnO NP increased the activity of antioxidant enzymes, providing additional strength to reduce ROS generation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

FA: experimentation. MR: conceptualization. RS: formatting and software. AAS: review and formatting, statistical analysis. ANS: drafting. GN: formatting and review. AM: formatting. AT: statistical analysis and funding acquisition. HK: funding acquisition and writing-review and editing. ED: formal analysis and funding acquisition. HE: statistical analysis and funding acquisition. All authors contributed to the article and approved the submitted version.

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