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Safe utilization of heavy metals pollution in soils for healthy food

Edited by
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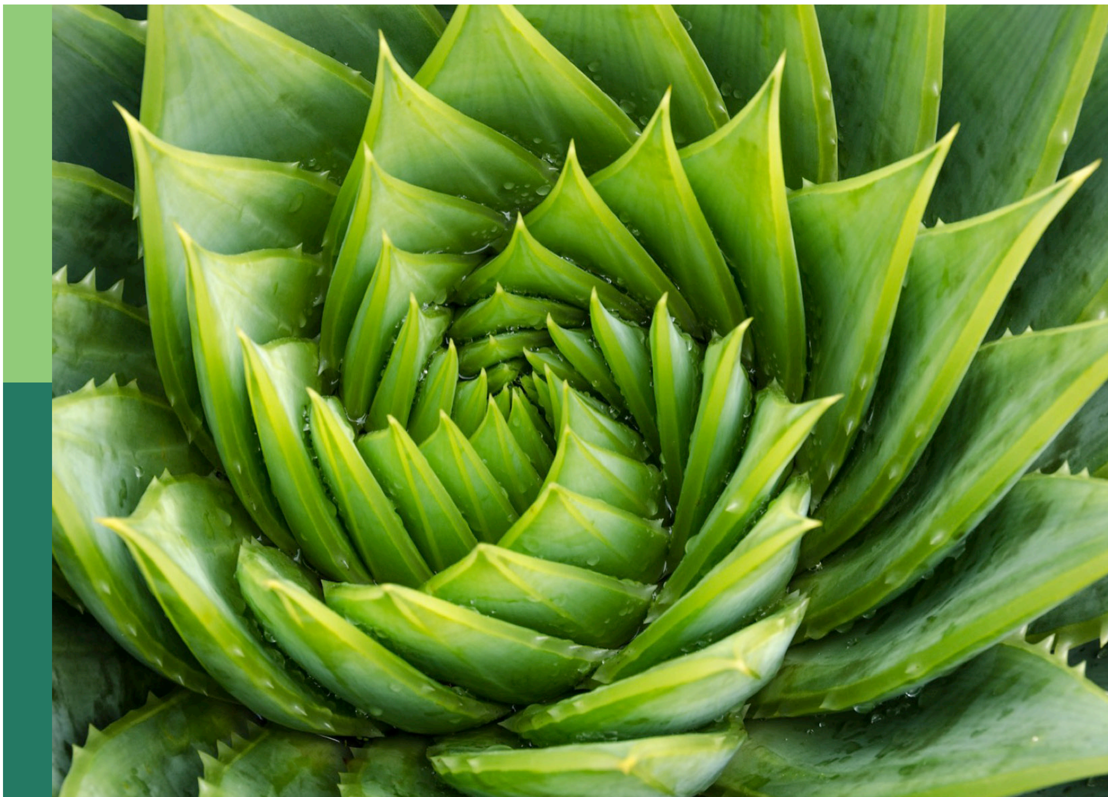

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Safe utilization of heavy metals pollution in soils for healthy food

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Editorial: Safe utilization of heavy metals pollution in soils for healthy food

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Editorial on the Research Topic
Safe utilization of heavy metals pollution in soils for healthy food

Heavy metal contamination affects more than five million soil locations globally. Heavy metals can be found in the soil through natural processes or as a result of human activity. Over the past few decades, a number of factors have contributed to the deposition of heavy metals in the global soil system: they include fast industrial growth, air pollution, irrigation using contaminated water, sewage sludge application, excessive pesticide usage, and the overuse of inorganic fertilizers. On the one hand, these toxic heavy metals in the soil damage photosynthesis, respiration, transpiration and other metabolic processes in plants resulting in retardation of plant growth or reduced crop yield. On the other hand, heavy metals enter the human body through the food chain, which leads to kidney diseases, liver diseases, central nervous system disorders, and insomnia. Therefore, it is imperative to develop strategies for the safe utilization of heavy metal-polluted soils. Nevertheless, environmentally-friendly, cost-effective, efficient and sustainable strategies to promote the phytoremediation efficiency of heavy metal-polluted soils or to suppress toxic heavy metal accumulation in edible organs of crops are still limited. Thus, this Research Topic highlights recent developments, current knowledge and perspectives on phytoremediation or mitigation of heavy metal stress in plants for the safe utilization of heavy metal-polluted soils.

A total of two review articles are included in the current Research Topic. Modern techniques for the analysis of heavy metals in plants have been reported by He et al., along with a discussion of their advantages and disadvantages. Hao et al. showed that arbuscular mycorrhizal fungi (AMF) inoculation reduced arsenic (As) accumulation in plants in a dose-dependent manner, and single AMF inoculation reduced As in plants more than mixed inoculation by using a meta-analysis. Since cadmium (Cd) is of utmost significance in polluted soil, the majority of the articles published in this Research Topic focus on the phytoremediation of Cd-polluted soil or the amelioration of Cd stress in plants.

Developments in the phytoremediation of Cd-polluted soils by stimulating Cd accumulation in plants are also presented. According to Wang et al., inoculating maize roots with a single AMF or dark septate endophytic fungus (DSE) significantly increased the concentration of Cd, whereas inoculating maize roots with DSE significantly enhanced the accumulation of Cd in both the shoots and the roots. Significant positive interactions between AMF and DSE on photosynthetic rate (Pn), root glutathione metabolism, and endogenous hormone contents suggest that AMF and DSE inoculation confers Cd tolerance in maize. Hu et al. reported that exogenous silicon (Si) application increased biomass, Cd translocation factor and Cd concentrations in shoots of *Sedum alfredii* Hance, resulting in a significant increase in Cd accumulation in this Cd hyperaccumulating plant. Moreover, Si application elevated chlorophyll content, improved antioxidant capacity, enhanced cell wall components, increased secretion of low molecular weight organic acids, and regulated expression levels of *SaNramp3*, *SaNramp6* and *SaCAD*, which are involved in Cd detoxification. Therefore, Si facilitates the phytoremediation of Cd by *S. alfredii*. Xie et al. elucidated that Cd acidified and improved organic carbon accumulation in the rhizosphere soil of *Siegesbeckia orientalis* L. contributing to active Cd bioavailability. Active organic carbon accumulation further reversed Cd-attenuated microbial and enzymatic activities in the rhizosphere soil. These findings provide new insights for improving the phytoremediation efficiency of the Cd-polluted soil.

The role of exogenous agents in reducing Cd uptake, transport, distribution and accumulation in plants is also explored. Tang et al. demonstrated that applying 100-500 mg L^{-1} salicylic acid (SA) increased lettuce growth and decreased Cd concentrations in lettuce shoots. SA promoted photosynthetic capacity, reduced Cd-induced oxidative stress, and downregulated expression levels of *Nramp5*, *HMA4*, and *SAMT*, which were the reasons for reducing Cd toxicity in lettuce. Hira et al. found that combining biochar application, *Rhizobium leguminosarum* inoculation and *Vigna radiata* intercropping promoted mustard growth rates by 65%–90% in the early growth stage and by 70%–90% in the mature stage. The combination remarkably reduced Cd translocation in mustard plants by 70%–95%. El-Sappah et al. discovered that foliar spraying of silymarin and clove fruit extract acted as a potent novel biostimulator that improved growth, photosynthetic efficiency, non-enzymatic and enzymatic antioxidants, grain yield, and limited Cd accumulation in wheat. Zhuang et al. revealed that higher contents of soil organic matter, cation exchange capacity, clay and silt, and lower sand contents accumulated less Cd in apple trees by enhancing antioxidant activities and downregulating expression levels of genes related to Cd absorption and transport.

Mutual effects of Cd with other environmental factors on plant growth and adaptation are discussed in this Research Topic. Shi et al. showed that original polyethylene microplastics absorbed more Cd from solution, thus increasing Cd accumulation in sweet potato tissues and exacerbating Cd toxicity. Correspondingly, Cd facilitated polyethylene microplastics in the cortex of roots but reduced their accumulation in shoots of sweet potato. Feng et al. demonstrated that a relatively high temperature of 27°C and medium/high CO_2 levels ameliorated the reduced plant growth of rice caused by Cd and salinity stress. The highest amounts of Cd were found in rice at 27°C and mild CO_2 levels. The distribution and accumulation of Cd in plants may be affected either positively or negatively by the combined environmental factors.

In summary, inoculation with microbes such as AMF or DSE, enhancement of Cd detoxification capacity by exogenous Si supply, and activation of soil Cd bioavailability by adjusting soil organic carbon are strategies supported by this Research Topic to improve phytoremediation of the Cd-polluted soils. Exogenous application of SA, biochar-*Rhizobium leguminosarum* inoculation-leguminous plants intercropping combination, and silymarin-clove fruit extract compounds are strategies provided by this Research Topic for decreasing Cd contamination in crops. Furthermore, factors such as microplastics, temperature, atmospheric CO_2 and salinity are worthy of consideration as they commonly co-exist with Cd pollution in the soil.

Author contributions

JW: Writing – review & editing, Writing – original draft, Conceptualization. QT: Writing – review & editing. MK: Writing – review & editing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Exogenous silicon promotes cadmium (Cd) accumulation in *Sedum alfredii* Hance by enhancing Cd uptake and alleviating Cd toxicity

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Soil Cadmium (Cd) pollution has become a serious environmental problem. Silicon (Si) plays key roles in alleviating Cd toxicity in plants. However, the effects of Si on mitigation of Cd toxicity and accumulation of Cd by hyperaccumulators are largely unknown. This study was conducted to investigate the effect of Si on Cd accumulation and the physiological characteristics of Cd hyperaccumulator *Sedum alfredii Hance* under Cd stress. Results showed that, exogenous Si application promoted the biomass, Cd translocation and concentration of *S. alfredii*, with an increased rate of 21.74-52.17% for shoot biomass, and 412.39-621.00% for Cd accumulation. Moreover, Si alleviated Cd toxicity by: (i) increasing chlorophyll contents, (ii) improving antioxidant enzymes, (iii) enhancing cell wall components (lignin, cellulose, hemicellulose and pectin), (iv) raising the secretion of organic acids (oxalic acid, tartaric acid and L-malic acid). The RT-PCR analysis of genes that involved in Cd detoxification showed that the expression of *SaNramp3*, *SaNramp6*, *SaHMA2* and *SaHMA4* in roots were significantly decreased by 11.46-28.23%, 6.61-65.19%, 38.47-80.87%, 44.80-69.85% and 33.96-71.70% in the Si treatments, while Si significantly increased the expression of *SaCAD*. This study expanded understanding on the role of Si in phytoextraction and provided a feasible strategy for assisting phytoextraction Cd by *S. alfredii*. In summary, Si facilitated the Cd phytoextraction of *S. alfredii* by promoting plant growth and enhancing the resistance of plants to Cd.

KEYWORDS

silicon, antioxidant enzyme activity, cell wall, organic acids, hyperaccumulator

1 Introduction

Soil heavy metal contamination caused by various anthropogenic activities has become a serious global environmental problem. Among the heavy metals, cadmium (Cd) is regarded as no known biological functions but highly harmful to the organisms (Gul et al., 2021). Due to the persistence of Cd in soil, Cd has been shown to strongly affect the growth and biomass development and cause plant poisoning (Gallego et al., 2012). In addition, Cd is easily absorbed by grain due to the high mobility of Cd, which will eventually affect animal and human health through the food chain (Żukowska and Biziuk, 2010; Xie et al., 2020). Therefore, effective methods are needed to eliminate Cd contaminated soil for assuring the environmental health and food security.

In the past few decades, various chemical and physical techniques have been developed for the remediation of Cd-contaminated soils. However, these methods usually display high costs, disturbance of soils and have environmental risks (Gul et al., 2021; Gavrilescu, 2022). Phytoextraction is currently considered an eco-friendly and low cost approach to remove heavy metals from soil. This technology based on the capacity of plants to uptake heavy metals from soil and then accumulate them in aboveground parts of the plant, without adverse effects on soil structure, fertility and biological activity (Kurade et al., 2021; Gavrilescu, 2022). The success of phytoextraction depends largely on the uptake and accumulation of metals by hyperaccumulators. However, their characteristics of slow growth and small biomass under metal stress severely affect the phytoextraction efficiency, which has become an obstacle for the large-scale application of phytoextraction (Feng et al., 2018). Therefore, from the point of view of increasing plant biomass to alleviate the toxicity of Cd for plants, a strategy for improving the removal efficiency may provide an effective technology for solving this setback of phytoextraction.

Silicon (Si), the second most abundant element in the earth crust after oxygen, is an important element for plants (Emamverdian et al., 2018; Bhat et al., 2019), which playing an important role in the survival of plants under heavy metal stress. After being absorbed by the plants, Si was able to reduce the harmful effects of heavy metal toxicity through various mechanisms, including stimulating the antioxidant defense system (Huang et al., 2019; El-Saadony et al., 2021), reducing metal uptake and transport (Gao et al., 2018; Chen et al., 2019; Gheshlaghpour et al., 2021), complexing (Yamaji et al., 2008; Keller et al., 2014; Bhat et al., 2019), compartmentalization (Richmond and Sussman, 2003; Gu et al., 2011) and altering the components of the cell wall or forming a bilayer cell structure (Fan et al., 2016). In addition, Si was also able to reduce the metal phyto-toxicity at the molecular level by elevating transcription of genes encoding metal transporters and phyto-chelatine (Vaculík et al., 2020). In general, the current research have demonstrated that Si addition is an effective strategy to alleviate toxicity caused by heavy metals. However, most of these studies only focused on the effects of Si on non-hyperaccumulating plants. To the best of our knowledge, there is also a research gap in the effects of Si on the mitigation of Cd toxicity as well as the uptake and accumulation of Cd by hyperaccumulators.

Sedum alfredii Hance, originally discovered in an old mining site in Southeast China, has been reported to be a naturally occurring Cd hyperaccumulator with a great potential in Cd phytoextraction (Yang et al., 2019). And *S. alfredii* has been used as a model hyperaccumulator to investigate Cd phytoextraction owing to its strong ability of Cd uptake and accumulation (Xu et al., 2023). To date, synergistic effects of Si on mitigation of Cd toxicity and accumulation of Cd by hyperaccumulators have not been reported. Hence, in this study, the physiological response of *S. alfredii* to exogenous Si application under Cd stress was comprehensively studied by a hydroponic experiment. The objectives were to: 1) determine the influence of Si on growth and Cd accumulation by *S. alfredii*; 2) investigate the effects of Si on the mitigation of plant Cd stress by measuring the root exudation of organic acids, cell wall constituents, level of lipid peroxidation, chlorophyll content, and antioxidant enzymes; 3) explore the response to Cd uptake and subsequent expression of genes encoding specific transporter (*SaNramp3*, *SaNramp6*, *SaHMA2*, *SaHMA3* and *SaHMA4*) and cell wall synthetase (*SaCAD*) under Cd stress. The results of this study will improve our understanding of role of Si in phytoextraction of Cd by hyperaccumulators.

2 Materials and method

2.1 Plant material and experimental design

The seedlings of *S. alfredii* were obtained from a mine area in Zhejiang Province, China. The plants were grown in uncontaminated soil for more than 3 generations to eliminate the influence of the previously accumulated heavy metals in the plants. *S. alfredii* plants with consistent growth conditions were pre-cultured in Hoagland nutrient solution (pH 5.8), which contained KH_2PO_4 100μM, Fe-EDTA 20μM, CuSO4·5H_2O 0.2μM, $Ca(NO_3)_2·4H_2O$ 2000μM, $ZnSO_4·7H_2O$ 0.5μM, $(NH_4)6Mo_7O_{24}$ 0.01μM, H_3BO_3 10μM, $MnSO_4·H_2O$ 0.5μM, $MgSO_4·7H_2O$ 500μM, K_2SO_4 700μM, KCl 100μM. *S. alfredii* was grown in a greenhouse at 26/20°C with a day/night cycle of 16h/8h, and the humidity was kept at about 70%. The nutrient solution was replaced every 3 days and continuously aerated. After 21 days of pre-culture, healthy seedlings (the seedling height is 6~7cm and the root length is 3~4cm) were subsequently chosen for the later hydroponic experiment.

The hydroponic experiment was conducted in a controlled greenhouse. During the experiment, *S. alfredii* was grown in a greenhouse at 26/20°C with a day/night cycle of 16h/8h, and the humidity was kept at about 70%. Four uniform plants were selected and transferred to each pot (diameter 15.5 cm and height 10 cm). All treatments contained 0.15 mM Cd, which was supplied as $CdCl_2$. Sodium metasilicate ($Na_2SiO_3·9H_2O$, SiO_2 21%) was applied as a source of Si. Na_2SiO_3 at different concentrations (0, 0.5, 1.0, 1.5 and 2.0 mM) were dissolved in nutrient solution, marked CK, T1, T2, T3 and T4, respectively. The HCl was used to neutralize the alkalinity of Na_2SiO_3, and the difference of Na^+ and Cl^- among each treatment were made up with NaCl. Each treatment was set up with three replications with a total of 15 pots. The nutrient solution was continuously aerated with an aquarium air pump and renewed every 3 days, and the pH value was adjusted to 5.8.

2.2 Plant harvesting and Cd/Si concentrations analysis

After 28 d growth, plants were harvested for analysis of morphological, physiological and molecular characteristics. After harvest, *S. alfredii* plants were fully washed with deionized water and divided into shoot and root. The root length, plant height and fresh weight of plants were measured by ruler and electronic balance, respectively. The plant samples were dried to constant weight and then recorded dry weight. The dried plant material was then ground to a fine powder for the determination of metal concentrations. To measure Cd concentrations of plants, the powdered plant material (0.1g) was digested with a mixture of HNO_3 and $HClO_4$ (5:1) on an electric heating plate. The concentration of Cd in the digested solution was determined by using ICP-MS (PerkinElmer NexION 300X). A standard plant material (GSB–11) was used throughout all the digestions and ICP analysis processes as the quality control. The translocation factors (TF) for Cd were calculated according to the following formula:

$$TF = \textit{Metal concentration in plant shoots}/\textit{Metal concentration in plant roots}$$

The Si content was measured by Si molybdenum blue colorimetry (Yang, 1995). Briefly, the oven-dried root and shoot samples (0.1g) were grinded with the help of a mortar. The samples were then placed on a high-temperature electric resistance furnace and heated at 720 ℃ for 10 min. Later on, the samples were mixed with 0.3 $mol \cdot L^{-1}$ H_2SO_4, 5% ammonium molybdate, 5% oxalic acid, and 0.5% ascorbic acid. Then the Si content was photometrically measured at a wavelength of 700 nm.

2.3 Analysis of antioxidant enzyme activities, lipid peroxidation and chlorophyll contents

To evaluate the antioxidant enzyme activities of plants, fresh leaves (0.2g) were reacted with potassium phosphate buffer (pH 7.8). The homogenate was centrifuged at 8000×g for 10 minutes was stored at 4 ℃ for the enzyme activity determination. The SOD (superoxide dismutase) was assayed in 200 µl of reaction mixture containing 10 µl enzyme extract, 160 µl phosphate buffer (pH 7.8) and 30 µl WST-8. Then the samples were measured by using a spectrophotometer at 450nm wavelength. Finally, the enzyme activity was calculated using SOD activity determination kit (Sino Best Biological Technology Co., Ltd, Shanghai, China). The CAT (catalase) was determined according to a previous method (Aebi, 1984). The reaction mixture (3mL) was comprised of 100µl enzyme extract, 100 µl H_2O_2 and 2.8mL phosphate buffer with EDTA (pH 7.0). The absorbance of the reaction mixture at 240nm decreases with the reaction time, and the CAT activity can be calculated according to the change rate of absorbance. The POD (peroxidase) was analyzed based on a previous method (Farooq et al., 2013). The assay mixture (245µl) was consisted of 5µl enzyme extract, 120µl guaiacol, 30µl H_2O_2 (300 mM), 30µl phosphate buffer with EDTA (pH 7.0) and 60µl distilled water. The absorbance of guaiacol oxidation products was determined at 470nm wavelength.

The level of lipid peroxidation in the leaf tissue was measured in terms of malondialdehyde (MDA, a product of lipid peroxidation) content. Fresh leaves (0.2g) were selected for the evaluation of MDA content by the method of Li et al. (2000). Simply, plant leaves were homogenized in phosphate buffer saline (PBS), then 2 ml of 2-thiobarbituric acid was added. Later, the MDA content was measured at different wavelengths such as 600 nm, 532 nm, and 450 nm respectively. Chlorophyll a, b and carotenoids were determined spectrophotometrically.

2.4 Analysis of cell wall composition

Cell wall was isolated by the method of Yang et al. (2011). Lignin was measured at 280 nm wavelength after sample (3mg) acetylated by mixing phenolic hydroxyl with the reaction solution in boiling water (Moreira-Vilar et al., 2014). The plant sample (0.3g) was reacted with ethanol and acetone to obtain a coarse cell wall, and the cell wall substance was dehydrated and subsequently condensed with anthrone under acidic conditions to generate furfural derivatives. The cellulose content was measured by spectrophotometer at 620 nm wavelength. After the plant sample (0.02g) was treated by acid, hemicellulose was converted into reducing sugar, which formed a reddish-brown substance with 3,5-Dinitrosalicylic acid. The absorbance at 540 nm reflected the hemicellulose content. The dry sample (0.1g) reacted with the extract to obtain total pectin, and after reacting with strong acid, its pectin content was measured at 530 nm absorbance.

2.5 Collection and determination of organic acids in root exudates

Four plants from each treatment were selected to collect root exudate. Plants were washed with sterilized water and retained in beakers containing 200 mL $CaCl_2$ solution (0.5 mM) for 24 h. Plant roots were cleaned with 50 mL of $CaCl_2$ solution every 6 hours and water was combined with the preceding 200 mL of $CaCl_2$ solution to make a combined 250 mL of root exudate volume. Furthermore, the filtered sample solutions (20 µL) were injected into a 250*4.6 mm reverse phase column with a flow rate of 0.9 $mL \cdot min^{-1}$ at 35℃ and detected at 210 nm by HPLC to analysis the low molecular weight organic acids (Muhammad et al., 2020).

2.6 RNA-seq and gene relative expression analysis

Total RNA was isolated from frozen fresh *S. alfredii* roots by using the TRUE script RT MasterMix (for real time PCR) (DF Biotech., CHENGDU, China) according to the requirements of the instructions (Ge et al., 2021). An aliquot (2 mg) of total RNA was used for first-strand cDNA synthesis using the TransScript First-Strand cDNA Synthesis kit (AiDLAB Biotech, Beijing, P.R. China).

Gene expression level was determined by Quantitative Real-Time PCR Thermal Cycler (Analytik Jena AG, Factory at Konrad-Zuse-Str.1, D-07745 Jena, Germany) with the primer shown in Table 1 (Zhang et al., 2014). Gene primers were designed according to the sequences obtained from the transcriptome. Results using ACTIN as internal reference, the relative gene expression of each sample and each group was calculated by $2^{-\triangle\triangle Ct}$. All measurements were conducted in triplicate.

2.7 Statistical analysis

In this study, all the data were presented as the average ± standard deviation of the mean based on the three replicates. The significance of the differences between different treatments was subjected to a One-way ANOVA analysis at $P<0.05$ level by using IBM SPSS 19.0. The figures were made by Origin 2018 and R 4.2.1.

3 Results

3.1 Exogenous Si promoted the growth and Cd accumulation of *S. alfredii*

The data in Table 2 showed the effects of different Si treatments on the fresh and dry weight of *S. alfredii* after 28 d growth. The fresh and dry weight of shoot and root of *S. alfredii* in Si treatments were significantly increased by 22.67-52%, 21.74-52.17%, 10.71-28.57% and 40-80%, respectively, compared with CK. Moreover, both fresh and dry weights showed the highest value under 2 mM Si treatment. Similarly, Si also significantly ($P< 0.05$) increased the plant height and root length of *S. alfredii* by 10.75-23.26% and 11.90-31.44%, respectively (Figure 1).

TABLE 1 Sequences of specific-primers used for qRT-PCR.

Gene	Sequence (5'to3')	TM
ACTIN-F	TGTGCTTTCCCTCTATGCC	60
ACTIN-R	CGCTCAGCAGTGGTTGTG	
Nramp3-F	AAGAAGCAGCTCATGGGTGT	60
Nramp3-R	TAAGCTGCGGTGAAGGTTGA	
Nramp6-F	TACCTGATAAGACGAGTTGGAA	60
Nramp6-R	GCCACCAATATGATCCACAATA	
HMA2-F	CTCAAAATGCTGCGAAGCGAAG	60
HMA2-R	CTACGCTCTTACAACCATGCCTCG	
HMA3-F	CTGTTGCGGACATTGAGA	60
HMA3-R	TAGGAGGAGCAGGTTCAG	
HMA4-F	GATGCACCAGCACTAGCTAC	60
HMA4-R	TCACCAACAAACACGTCCCA	
CAD-F	GTACCTGTGTTCTGGTGCTTA	60
CAD-R	AGACGAAAGAAATGCTGGAGT	

The Cd concentration and extraction in the shoot of *S. alfredii* was enhanced by the increasing of Si levels (Figure 2). It can be found that Si treatment significantly ($P< 0.05$) increased the Cd concentration and extraction in the shoot of *S. alfredii* by 39.52-68.44% and 412.39-621.00%, respectively. Moreover, the highest value in concentration and accumulation of Cd in the shoot of *S. alfredii* was found under T4 treatment. Furthermore, exogenous Si significantly ($P< 0.05$) increased Cd extraction in the root of *S. alfredii*, and it increased with higher Si concentrations, but Si showed a negative role on enhancing Cd concentrations in the root of *S. alfredii*. Exogenous Si remarkably increased the Si concentration and accumulation of *S. alfredii* by 25.92-126.61% and 346.02-899.65%, respectively (Figure 2). And both the Si concentration and the accumulation displayed the highest values under T4 treatments which was 2.27-fold and 10.00-fold higher than CK treatments.

To determine whether Si was able to promote the Cd extraction efficiency of *S. alfredii*, we analyzed the plant Cd translocation factor (TF) (Figure 3). The application of Si under the T3 and T4 treatments increased the TF of *S. alfredii* significantly in comparison to the control. The 2 mM Si was effective in improving the TF by 1.72-fold compared to CK. These results indicated that the T4 treatment can significantly ($P< 0.05$) enhance Cd extraction efficiency of *S. alfredii*.

3.2 Photosynthetic pigments, lipid peroxidation and antioxidant enzymes activities

Photosynthetic pigments (chlorophyll a, and b) and other photosynthetic pigments (carotenoids) were analyzed in *S. alfredii* leaves (Figure 4). The results showed that exogenous Si reduced Cd toxicity by increasing the total chlorophyll a content and chlorophyll b content by 75.55-95.94% and 90.06-134.04%, respectively. However, exogenous Si had no significant impacts on the carotenoid content of *S. alfredii*. Exogenous Si significantly ($P< 0.05$)decreased the MDA content in Cd stressed plants when compared to the control and the T2, T3 and T4 treatments significantly ($P< 0.05$) decreased the MDA content by 39.62%, 40.94% and 40.94%, respectively (Figure 5). These indicated that Si has a positive role on Cd-induced ROS stress.

Antioxidative enzymes play positive impacts in oxidative stress management. The changes of antioxidant enzyme activity in *S. alfredii* under Si treatment were displayed in Table 3. The maximum values of POD, SOD and CAT activities were found under T4 treatment. Compared with the control, the T4 treatment significantly ($P< 0.05$) enhanced POD, SOD and CAT activities by 34.92%, 14.57% and 19.63%, respectively. The T1, T2 and T3 treatment also significantly ($P< 0.05$) increased POD, SOD and CAT activities. These results showed that 2mM Si had the best effect in relieving Cd stress of *S. alfredii*.

TABLE 2 Effect of different Si treatments on weight, height and root length of *S. alfredii*.

Treatment	Fresh weight ($g·plant^{-1}$)		Dry weight ($g·plant^{-1}$)		Plant height (cm)	Root length (cm)
	shoot	Root	Shoot	Root		
CK	1.50 ± 0.02e	0.28 ± 0.01c	0.23 ± 0.02c	0.05 ± 0.01c	14.23 ± 0.07e	5.63 ± 0.09d
T1	1.84 ± 0.04d	0.31 ± 0.01b	0.28 ± 0.01b	0.07 ± 0.00b	15.76 ± 0.06d	6.30 ± 0.29c
T2	1.89 ± 0.01c	0.32 ± 0.01b	0.29 ± 0.02b	0.08 ± 0.00b	16.02 ± 0.17c	7.05 ± 0.05b
T3	2.18 ± 0.02b	0.34 ± 0.02a	0.35 ± 0.01a	0.09 ± 0.00ab	16.29 ± 0.14b	7.18 ± 0.08ab
T4	2.28 ± 0.03a	0.36 ± 0.01a	0.35 ± 0.01a	0.09 ± 0.01a	17.54 ± 0.14a	7.40 ± 0.10a

CK: control treatment, T1, T2, T3 and T4 were the Si concentrations of 0.5, 1.0, 1.5 and 2.0 $mmol·L^{-1}$, respectively. Values are mean ± SD and columns denoted with different letters indicate significant difference among treatments at $P < 0.05$.

FIGURE 1
Effects of different Si treatments on the growth of *S. alfredii*. CK: control treatment, T1, T2, T3 and T4 were the Si concentrations of 0.5, 1.0, 1.5 and 2.0 $mmol•L^{-1}$, respectively.

3.3 Effects of Si on cell wall components of *S. alfredii*

After 28 d growth, the cell wall components of *S. alfredii* were clearly different between Si treatments (Figure 6). We found that the cell wall components (pectin, cellulose, lignin, and hemicellulose) and the content of Si in leaves changed greatly. For instance, compared to the CK, Si significantly increased the lignin content, cellulose content, hemicellulose content and pectin content of *S. alfredii* by 0.41-20.43%, 10.37-19.39%, 0.73-17.70% and 8.15-23.30%, respectively.

3.4 Organic acids in root exudates of *S. alfredii* influenced by Si treatments

Significant differences ($P < 0.05$) in organic acid concentrations were found under different Si treatments (Table 4). The organic acid standard solutions are oxalic acid, tartaric acid, L-malic acid, acetic acid, citric acid and D-malic acid in the peak order in Figure 7. Tartaric acid, oxalic acid and L-malic acid were detected in the root exudates of *S. alfredii* in this study. The different Si application resulted in significant increases in excretion of root exudates by *S. alfredii* (Figure 7). The maximum values for tartaric acid, oxalic acid and L-malic acid were all found under 2.0 mM Si. The T4 treatment significantly ($P < 0.05$) promoted the excreted concentrations of oxalic acid, tartaric acid and L-malic acid by 114.78%, 136.83% and 203.08%, respectively, compared with CK (Table 4).

3.5 Gene expression of *S. alfredii* under different Si treatments

To test whether Si could regulate the symplastic transport routine of Cd, the expression of genes, including *SaNramp3*, *SaNramp6*, *SaHMA2*, *SaHMA3*, *SaHMA4* and *SaCAD*, potentially involved in *S. alfredii* Cd transport and cell wall synthesis was investigated (Figure 8). After 28d growth, the gene expression profile of *S. alfredii* was clearly different between treatments (Figure 8). Compared to the control, the Si application significantly down-regulated the expression of *SaNramp3*, *SaNramp6*, *SaHMA3*, *SaHMA2* and *SaHMA4* by 11.46-28.23%, 6.61-65.19%, 38.47-80.87%, 44.80-69.85% and 33.96-71.70% in the root of *S. alfredii*, respectively. While the Si treatments significantly increased the *SaCAD* expression, and the highest level was found under T4 treatment which was 1.46-fold higher than the CK.

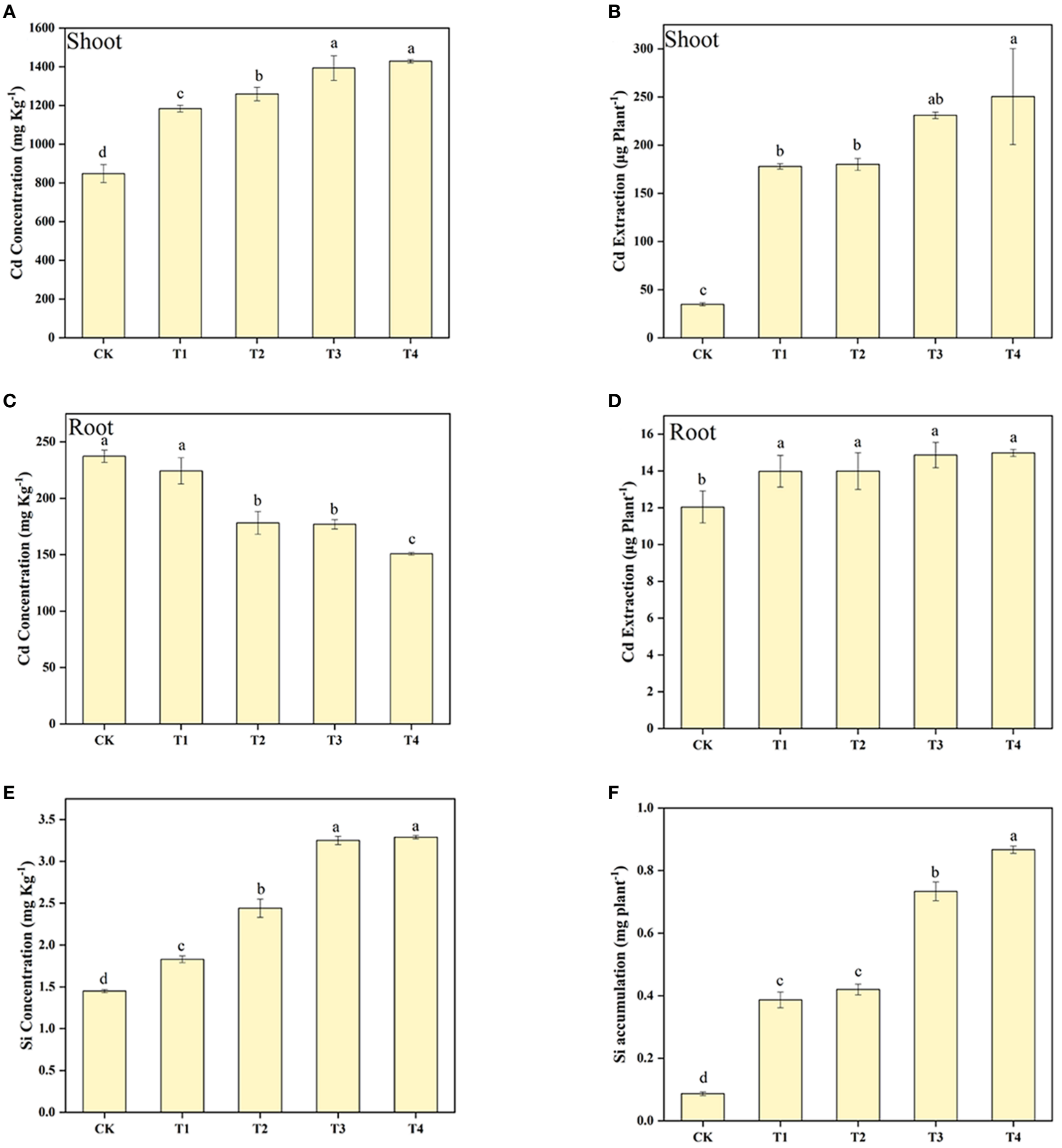

FIGURE 2
Shoot Cd concentration **(A)**, Shoot Cd accumulation **(B)**, root Cd concentration **(C)**, root Cd accumulation **(D)**, and Si concentration **(E)**, Si accumulation **(F)** of *S. alfredii* under different Si treatments. CK: control treatment, T1, T2, T3 and T4 were the Si concentrations of 0.5, 1.0, 1.5 and 2.0 mmol•L^{-1}, respectively. Values are mean ± SD and columns denoted with different letters indicate significant differences between treatments at *P*< 0.05.

4 Discussion

4.1 Influences of Si on growth and Cd accumulation of *S. alfredii*

This study aims to explore the feasibility of Si in enhancing the efficiency of phytoextraction by *S. alfredii*. According to the results obtained here, exogenous Si application significantly promoted Cd accumulation by *S.alfredii*, indicating Si enhanced the efficiency of phytoextraction. The success of phytoextraction depends largely on the plant biomass as well as the metal concentrations in plants (Eissa, 2017). Our results showed that Si promoted growth of *S. alfredii* and significantly enhanced the biomass of *S. alfredii* (Table 2). On the one hand, Si might improve mineral nutrition by reducing Cd toxicity of *S. alfredii* (Mukarram et al., 2022), and Si was shown to accumulate in the leaf apoplast as polymer and thereby act as a defense line preventing Cd from entering cells (Silva et al., 2017; Rahman et al., 2021). In addition, Si has been shown to alleviate the lipid peroxidation of membranes and promote the synthesis of chlorophyll, thus enhancing photosynthesis that is ultimately responsible for plant biomass production (Rastogi et al., 2021). On the other hand, Si altered the biosynthesis, structure, and function of the cell wall by regulating the production of lignin, pectin, cellulose and hemicellulose, and subsequently promote the absorption of nutrients by *S. alfredii* (Sheng and Chen, 2020). It is also possible to prevent plant root cells from being poisoned by Cd by increasing transcription of Cd transport genes (Figure 8), thus promote the development of

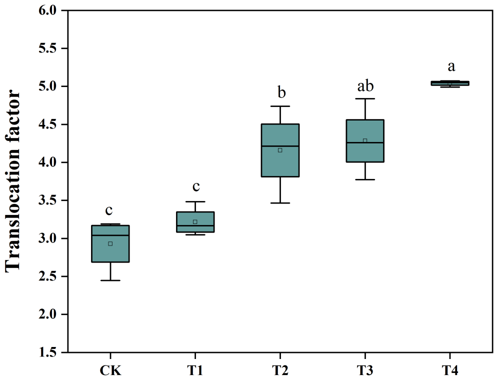

FIGURE 3
The Cd translocation factor of *S. alfredii*. CK: control treatment, T1, T2, T3 and T4 were the Si concentrations of 0.5, 1.0, 1.5 and 2.0 mmol•L^{-1}, respectively. Values are mean ± SD and columns denoted with different letters indicate significant difference among treatments at $P< 0.05$.

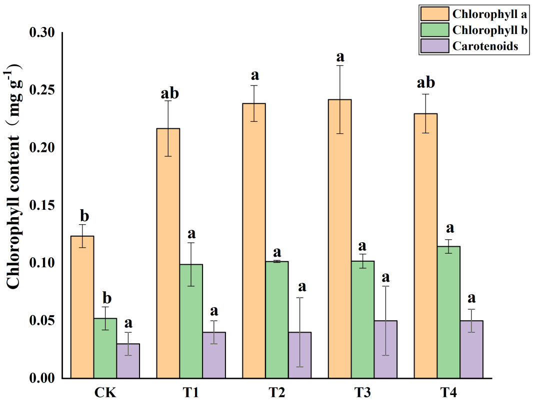

FIGURE 4
Effects of different Si treatments on chlorophyll content of *S. alfredii*. CK: control treatment, T1, T2, T3 and T4 were the Si concentrations of 0.5, 1.0, 1.5 and 2.0 mmol•L^{-1}, respectively. Values are mean ± SD and columns denoted with different letters indicate significant differences between treatments at $P< 0.05$.

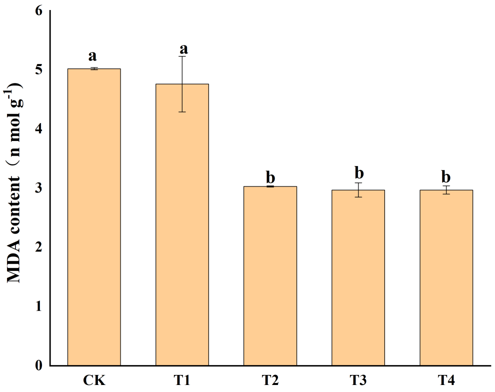

FIGURE 5
Effects of different Si treatments on MDA content of *S. alfredii*. CK: control treatment, T1, T2, T3 and T4 were the Si concentrations of 0.5, 1.0, 1.5 and 2.0 mmol•L^{-1}, respectively. Values are mean ± SD and columns denoted with different letters indicate significant differences between treatments at $P< 0.05$.

TABLE 3 Effects of different Si treatments on antioxidant enzymes activities of *S. alfredii*.

	SOD (Ug^{-1})	CAT (Ug^{-1})	POD (Ug^{-1})
CK	143.28 ± 2.57d	515.2 ± 14.92c	1.89 ± 0.06d
T1	145.83 ± 2.00d	523.93 ± 3.19c	1.91 ± 0.02d
T2	150.41 ± 1.21c	531.75 ± 8.86bc	2.08 ± 0.10c
T3	155.54 ± 3.83b	547.89 ± 12.28b	2.36 ± 0.04b
T4	164.16 ± 2.09a	616.84 ± 10.81a	2.55 ± 0.02a

CK, control treatment; T1, T2, T3 and T4 were the Si concentrations of 0.5, 1.0, 1.5 and 2.0 mmol·L^{-1}, respectively. Values are mean ± SD and columns denoted with different letters indicate significant differences between treatments at $P< 0.05$.

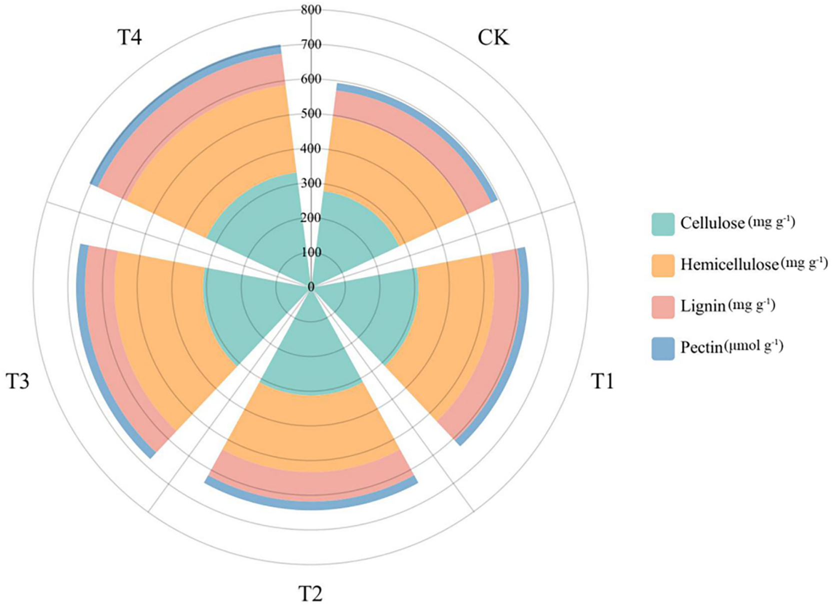

FIGURE 6
Cell wall components of *S. alfredii* under different Si treatments. CK: control treatment, T1, T2, T3 and T4 were the Si concentrations of 0.5, 1.0, 1.5 and 2.0 mmol•L^{-1}, respectively.

roots, and then increase the absorption of nutrients, which is beneficial to the growth of plants (Ma and Yamaji, 2006; Ma and Yamaji, 2015).

Another important factor that determines phytoextraction efficiency is the metal concentration in the hyperaccumulators. In this study, Si significantly increased Cd concentrations of *S. alfredii* (Figure 2). Several reasons may contribute to this. Firstly, the increased organic acids excretion, induced by Si application, contributes to the uptake and accumulation of Cd by *S. alfredii* (Tao et al., 2019). Previous studies had shown that oxalic acid, malic acid and tartaric acid all has the ability to make Cd complexes, and participate in various physiological metabolic processes in plants, including the uptake, transport, storage and detoxification of heavy metals (Ma et al., 2001). They have been shown to contribute to Cd accumulation in *S. alfredii* probably by releasing Cd and simultaneously complexing Cd, thus improving the Cd phytoextraction efficiency by *S. alfredii* (Tao et al., 2019). Besides, hyperaccumulators were reported to activate and chelate heavy metals by secreting a series of chelators such as low molecular weight organic acids (Qiao et al., 2022). In agreement with previous results, an increase in organic acids is beneficial for the transportation of Cd in the shoots of *S. alfredii*. Which was supported by the increasing of the TF (Figure 3). Secondly, the expression of Cd transporter gene was down-regulated by exogenous Si application in the root of plant, which was also beneficial for accumulation of Cd in the shoot (Figure 8). In summary, our study demonstrated that the presence of Si increased biomass and Cd concentrations in *S. alfredii*, which helps in enhancing Cd extraction efficiency.

4.2 Exogenous Si alleviated the Cd toxicity of *S. alfredii*

One of the most important mechanisms that hyperaccumulators reduce Cd damage is by improving photosynthesis and maintaining membrane stability. Photoreaction site is one of the most sensitive sites to heavy metal stress (Ashfaque et al., 2017; Bayçu et al., 2018;

TABLE 4 Effects of different Si treatments on the organic acids concentrations in root exudates of *S. alfredii*.

	oxalic acid (mg·L^{-1}h^{-1})	tartaric acid (mg·L^{-1}h^{-1})	L- malic acid (mg·L^{-1}h^{-1})
CK	2.03 ± 0.03e	5.05 ± 0.04d	0.65 ± 0.01e
T1	2.13 ± 0.06d	4.66 ± 0.44d	0.76 ± 0.02d
T2	2.49 ± 0.06c	8.93 ± 0.08c	1.57 ± 0.03c
T3	3.29 ± 0.01b	9.75 ± 0.02b	1.75 ± 0.04b
T4	4.36 ± 0.03a	11.96 ± 0.15a	1.97 ± 0.03a

CK, control treatment; T1, T2, T3 and T4 were the Si concentrations of 0.5, 1.0, 1.5 and 2.0 mmol·L^{-1}, respectively. Values are mean ± SD and columns denoted with different letters indicate significant differences between treatments at P< 0.05.

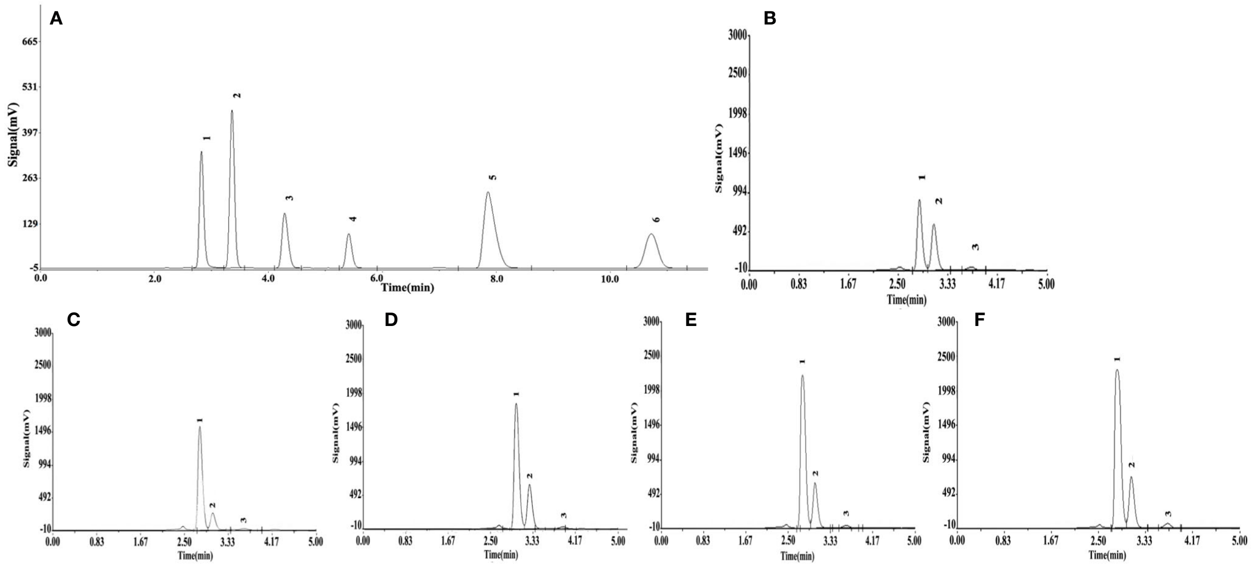

FIGURE 7
Effects of different Si treatments on organic acids content in root exudates of *S. alfredii*. Organic acid standard solutions **(A)**, root exudates of *S. alfredii* under different Si treatments (B~F). **(B)**: control treatment, **(C, D, E, F)** were the Si concentrations of 0.5, 1.0, 1.5 and 2.0 mmol•L^{-1}, respectively.

Rusinowski et al., 2019). Previous studies have confirmed that heavy metals could inhibit the photosynthetic pigments of plants (Araújo et al., 2017; Haque et al., 2021). In our study, Si increased the chlorophyll a and b content of *S. alfredii* (Figure 4). A possible explanation may be that Si is able to modulate expression of photosynthetic genes or promote plants to absorb more mineral nutrients, thus prevent damage to chloroplast membrane and thylakoids under metal stress (Feng et al., 2010; Song et al., 2014; Jia et al., 2021; Zhao et al., 2022). In addition, Si promoted the uptake of nutrients involved in photosynthetic process of plants (Rengel, 1999), which may also explain our results. Moreover, Si application has been shown to decrease ROS production or increase enzymatic antioxidant activity which ultimately reduce oxidative stress, thus increasing the chlorophyll contents (Kim et al., 2017). MDA is an indicator to measure membrane lipid peroxidation, and its level can reveal the damage degree of membrane structure and function (Gheshlaghpour et al., 2021). Previous studies have suggested that Cd stress can lead to the accumulation of MDA in plants (Muhammad et al., 2020; Pandian et al., 2020; Yuan et al., 2023). In our study, exogenous Si significantly minimized the content of MDA in *S. alfredii* (Figure 5). These results indicate that Si helps to eliminate various ROS species caused by Cd stress, thus ensuring the cell membranes not damaged. Similarly, Farooq et al. (2013) also confirmed that Si treatment helped to maintain the normalization of cell membrane.

Toxicity of Cd is also usually manifested by overproduction of toxic ROS (Vaculík et al., 2021). Previous research reported that excessive ROS accumulated in plants under Cd stress was able to lead to plant growth retardation (Zhou and Qiu, 2005), inhibition of photosynthetic pigment synthesis (Wahid et al., 2008; Farooq et al., 2013), cell wall looseness during cell expansion (Berni et al., 2019), and enzyme inactivation (Farooq et al., 2013). The antioxidant enzymes (POD, CAT and SOD) are considered as the first barrier against ROS (Zhang et al., 2009; Gong et al., 2019; Zulfiqar and Ashraf, 2022). Improving antioxidant enzyme activity is an important way to support hyperaccumulator to alleviate metal

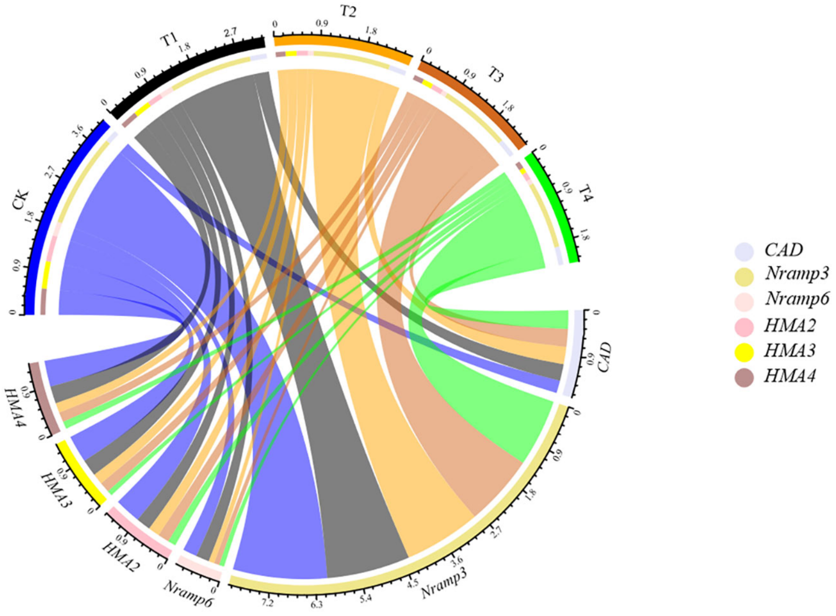

FIGURE 8
The expression level of related genes (*SaNramp3*, *SaNramp6*, *SaHMA2*, *SaHMA3*, *SaHMA4* and *SaCAD*) in the root of *S. alfredii* under different Si treatments. CK: control treatment, T1, T2, T3 and T4 were the Si concentrations of 0.5, 1.0, 1.5 and 2.0 mmol•L^{-1}, respectively.

toxicity (Asghari et al., 2020). Numerous studies have confirmed that the activity of antioxidant enzymes was increased due to Cd toxicity (Ahmad et al., 2016; Huang et al., 2019; Rahman et al., 2020; Zhou et al., 2022). In this study, it was found that Si application significantly increased the activity of antioxidant enzymes (Table 3). Ranjan et al. (2021) and Bhat et al. (2019) also reported that the relief of metal toxicity by Si was mainly due to a significant increase in enzymatic antioxidants. Our results demonstrated that Si might protect *S. alfredii* under Cd stress by enhancing the activity of defense enzymes.

Cell wall deposition is an important mechanism for plants to alleviate Cd toxicity. Research showed that Cd in deposited in the cell wall accounts for the majority of Cd in plants under high Cd stress (Yu et al., 2023). And Si has also been shown to protect cells of the plant against heavy metals toxicity by increasing the thickness and strength of the cell wall (Gall et al., 2015). Therefore, the tolerance and mitigation of Cd in *S. alfredii* might largely depend on cell wall deposition. The fixation of Cd by the plant cell wall was shown to be mainly dependent on various compounds displaying electronegative coordination provided by macromolecules such as cellulose, hemicellulose, lignin and pectin (Fu et al., 2011; Liu et al., 2014; Zhao et al., 2019; Wei et al., 2021; Yu et al., 2023). Chemically, the cell wall is mainly composed of three layers with structural components including pectin, cellulose, lignin and hemicellulose (Sajad et al., 2020). It is reported that Cd has negative effects on biosynthesis of pectin, lignin, cellulose and hemicellulose (Loix et al., 2017; Luyckx et al., 2021). In this study, we found that Si application remarkably increased the amount of lignin, pectin, cellulose and hemicellulose in *S. alfredii* (Figure 6), which were positively correlated to the increasing Si concentrations in plants (Figure 2). This was agreement with previous study which demonstrated that the content of lignin, pectin, cellulose and hemicellulose in plants were positively correlated to the content of Si (Zhang et al., 2015). There is evidence that Si enhances the antioxidant defense system of plants, alleviates the damage of ROS to cell wall, and promotes the synthesis of cell wall components (Xiong et al., 2010), such as the influence of POD activity on lignin biosynthesis and metabolism (Liu et al., 2019). Moreover, numerous studies have confirmed that the increase in cell wall components related to the gene expression, protein synthesis and transcription that caused by Si (Shafi et al., 2015; Haddad et al., 2019; Ranjan et al., 2021). Therefore, the influence of Si on the cell wall components is one of the important internal mechanisms for Si to reduce Cd toxicity of *S. alfredii*.

Another important mechanisms of Si in relieving Cd toxicity of *S. alfredii* is due to stimulating production and excretion of organic acid exudate from plants to subsequently chelate Cd^{2+} (Bhat et al., 2019). Tao et al. (2019) reported that oxalic acid, malic acid and tartaric acid are the main organic acids secreted by *S. alfredii*, which was similar to our results (Figure 7). One possible explanation may be that under Cd stress, exogenous Si reduced the production of excessive ROS, improved the photosynthetic efficiency, and then enhanced the secretion of organic acids in plant roots (Muhammad et al., 2020). Furthermore, organic acids are an middle step of photosynthesis through the tricarboxylic acid cycle (TCA) of the plant, thus, they may be involved with many physiological processes (Ubeynarayana et al., 2021). And previous studies showed that the contents of malic acid, tartaric acid and oxalic acid secreted by the roots of *S. alfredii* under Cd stress were increased (Chen et al., 2014; Muhammad et al., 2020), which may be a spontaneous adaptative mechanism of *S. alfredii* under Cd stress (Muhammad et al., 2020). The metal-organic acid anions-complex formed from this interaction reduced the toxicity of Cd to plants (Barcelo et al., 1993; Tanwir et al., 2015; Fan et al., 2016). In our study, exogenous Si promoted an increase in malic acid, tartaric acid and oxalic acid secreted by roots (Table 4). Therefore, we speculate that Si helps to activate various enzymes in the TCA cycle of plants, thus increasing the production of organic acids secreted by the roots and then alleviate the Cd toxicity (Rengel, 2002).

4.3 Root genes regulated tolerance and transportation of Cd were affected by Si

The expression levels of selected genes that regulate tolerance and transportation of Cd in roots of *S. alfredii* were analyzed. *SaNramp3* was reported to play an essential role in conferring tolerance and accumulation of Cd (Feng et al., 2018). *SaNramp6* was a Cd transporter located in the vesicular-shaped endomembrane compartment (Cailliatte et al., 2009). *HMA3* is a transport gene located in vacuole membrane, and participates in metal detoxification by isolating Cd into the vacuole (Chaudhary et al., 2016). *HMA2* and *HMA4* are proteins, which were shown to help excreting Cd^{2+} (Wu et al., 2015). The cinnamyl alcohol dehydrogenase of *S. alfredii* (*SaCAD*) is an essential enzyme in regulating lignin biosynthesis, which plays an critical role in enhancing Cd fixation on the cell wall (Qiu et al., 2018). It was reported that one of the strategies of *S. alfredii* in alleviating heavy metal stress in the root systems was expression of *SaNramp3* and *SaHMA3*, with their gene products being able to transport heavy metals to the vacuoles and subsequently store them in vacuoles (Yang et al., 2022). In this study, the expressions of *SaNramp3* and *SaHMA3* were down-regulated in the root of *S. alfredii* (Figure 8), which indicated that other mechanisms contributing more essentially and importantly than vacuolar sequestration might evolve (Peng et al., 2017). Besides, we found that the expression of *SaCAD* involved in plant lignin synthesis was significantly increased (Figure 8). The overexpression of *SaCAD* changed the cell wall composition of *S. alfredii* and might induce more Cd deposition in *S. alfredii* cell wall. In consistent with our results, Lu et al. (2020) also confirmed that *SaCAD*-overexpressing plants were capable of retaining more metal in the cell wall. Similarly, the down-regulation of *SaHMA2*, *SaHMA4* and *SaNramp6* will also discharge Cd from roots and accumulation of Cd in roots, so that the roots have a better environment for growth, which may help reduce the toxicity of Cd to root cells of plant and promote *S. alfredii* growth (Hanikenne et al., 2008; Chen et al., 2016; Zhang et al., 2016).

The down-regulation of genes encoding heavy metal transporters was not only alleviate the Cd stress of plants, but also explain the increase of Cd concentrations in the shoot of *S.alfredii*. *SaNramp3*, *SaHMA3* and *SaHMA2* are considered to be important transporters promoting root-to-shoot translocation of *S.alfredii*. Since the root-to-shoot translocation of Cd has been shown to be controlled by processes such as xylem loading and vacuolar sequestration, the down-regulation of *SaNramp3*, *SaHMA3* and *SaHMA2* indicated that the vacuole-isolated metal in the root of *S. alfredii* decreased, which resulting in an increased flux of metals towards the xylem, thus contribute to an increased transportation and accumulation of Cd in shoots (Mendoza-Cózatl et al., 2011; Ueno et al., 2011; Takahashi et al., 2012; Wu et al., 2015; Lu et al., 2020; Ge et al., 2021). Thus, the low expression of *SaNramp3*, *SaHMA3* and *SaHMA2* in roots could contribute to the high Cd accumulation in the shoot of *S. alfredii* (Figure 8). Our results indicates that the change in transport gene expression mediated by Si is beneficial to alleviate the growth inhibition of *S. alfredii* under Cd stress and increase the Cd accumulation by *S. alfredii*.

5 Conclusions

Our study revealed that exogenous Si application (mainly at 2 mM level) significantly promoted the biomass, Cd translocation factor and concentration in the shoots of *S. alfredii*, which led to a significantly increase in the Cd accumulation. In addition, this study suggested that Si application alleviated Cd toxicity of *S. alfredii* by: (i) increasing chlorophyll contents, (ii) improving antioxidant enzyme and lipid peroxidation of membrane, (iii) enhancing cell wall components, (iv) raising the secretion of low molecular weight organic acids. Moreover, the regulation of genes that involved in Cd detoxification such as *SaNramp3*, *SaNramp6* and *SaCAD* may also involve in the enhanced Cd resistance of *S. alfredii* after Si application. Our results demonstrated that Si facilitated Cd accumulation by *S. alfredii*. However, future research needs to corroborate the results of the present work in field phytoextraction trial.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. The raw sequence data in this study have been deposited in the NCBI database (https://www.ncbi.nlm.nih.gov/sra/) with the dataset accession number: SRR23460268. Further inquiries can be directed to the corresponding authors.

Author contributions

YH: Investigation, Methodology, Writing-original draft. XZ: Formal analysis, Investigation, Data curation. AS: Methodology, Formal analysis. YY: Data curation, Software. CR: Writing – review & editing. TZ: Conceptualization, Resources, Writing – review & editing. SX: Conceptualization. WY: Supervision, Project administration, Funding acquisition, Writing – review & editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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An integrated remediation approach using combinations of biochar, *Rhizobium leguminosarum*, and *Vigna radiata* for immobilizing and dissipating cadmium contaminants from the soil–mustard plant system

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Cadmium (Cd) contamination of soils is an environmental concern, as cadmium harms food crops and can therefore impact human health. The use of combinations of biochar (seeded with *Rhizobium leguminosarum*) and *Vigna radiata* (as an intercrop) has the potential to reduce the mobilization of Cd from soil *via* mustard plants (*Brassica juncea*). Mustard plants are grown as a food and oil production crop that is consumed worldwide. However, this plant has the property of hyperaccumulation; thus, it bioaccumulates Cd in its tissues, which in turn, if eaten, can become part of the human food chain. Hence, reducing Cd bioaccumulation in mustard plants is crucial to making these plants a reliable and safe source of food for consumption. To improve soil sorption capacity and immobilization efficiency, biochar (in the form of wheat husk) was mixed with *R. leguminosarum* and intercropped (using *V. radiata)* with mustard plants for further investigation. Sampling was performed at an early growth stage (i.e., at 30 days) and at maturity (i.e., at 60 days) to determine the impact of Cd on a plant's morphophysiological attributes. Data were analyzed in two ways: first by analysis of variance (ANOVA) and then by the *post hoc* Tukey's honestly significant difference (HSD) test. The statistical analysis concluded that combinations effectively improved plant traits by 65%–90% in the early growth stage and by 70%–90% in the maturity stage. The T6 treatment combination [i.e., biochar + *R. leguminosarum* + *V. radiata* (BC + RL + VR)] provided the most effective results in terms of growth, biomass, pod yield, and pigmentation content. In addition, this combination reduced the translocation of Cd in mustard plants by 70%–95%. The combination of BC + RL + VR effectively reduced Cd contamination of mustard tissue and provided a suitable growing environment for the plants. A post-harvesting soil analysis using X-ray diffraction (XRD) found that Cd was undetectable in soil. This provides clear confirmation that these approaches can lead to Cd soil remediation. Moreover, this study provided insight into the responses of different morphophysiological attributes of mustard plants to Cd stress and could aid in developing Cd stress tolerance in mustard plants.

KEYWORDS

cadmium remediation, mustard plant, phytoremediation, biochar, intercropping, plant growth-promoting rhizobacteria (PGPR)

1 Introduction

Rapid industrialization has harmful environmental effects resulting from the production of pollutants, including heavy metals (HMs). HMs are not essential elements for plant growth; consequently, HMs harm the growth and development of plants (Anjum et al., 2012). Cadmium (Cd) is one HM that can disturb the mechanisms of the whole plant, which in turn leads to plant death, and is considered the seventh most toxic HM in food crops. Cd persists in soil for long periods of time because of its simple structure and molecular weight. A soil Cd concentration ranging from > 5 to 10 mg/kg is considered toxic for plant growth (Mutlu et al., 2012). Cd travels through the xylem and accumulates in the aerial parts of plants. As leaves are considered the functional factories of plants, the accumulation of Cd in leaves disturbs all plant processes, including water and nutrient uptake and the manufacture of photosynthetic pigments (Da-lin et al., 2011). On a morphological level, the toxic effects of Cd manifest as reductions in plant growth, biomass, and yield quantity, increasing root length, and structural changes in the roots (Khan et al., 2007; DalCorso et al., 2008; Hossain et al., 2009; Hossain et al., 2010; Da-lin et al., 2011; Rascio and Navari-Izzo, 2011; Villiers et al., 2011).

Specific plants that have the strategies to adapt to Cd stress and have the ability to continue to grow and survive in this type of stress are known as hyperaccumulators. *Brassica juncea* (mustard plant) belongs to the family Brassicaceae (also known as Cruciferae) and is known as a hyperaccumulator plant. It is an important vegetable and oil crop, containing significant quantities of antioxidant and anti-inflammatory compounds, vitamins, and nutrients (Vélez, 2017). Mustard plants have the property of being able to accumulate Cd in their leaves, which in turn causes stunted growth (Kapoor et al., 2014). Green strategies (i.e., phytoremediation) also use mustard plants to bioremediate Cd-contaminated soils. Unfortunately, the green strategy also has some limitations, as most hyperaccumulating plants show growth retardation, low yield, and minimal economic value. Mustard plants that are used for phytoremediation programs, which have low yield quality, are not acceptable for food and oil production; however, these plants can be converted into beneficial products, such as biochar and biofuel, and be used in fermentation and ethanol production processes (Ye-Tao et al., 2012; Dhiman et al., 2016; ur Rehman et al., 2017).

Various plants and crops cannot survive under Cd stress; for this reason, scientists are working to develop biological approaches that can effectively overcome Cd toxicity, including intercropping, bioremediation using microbes, and soil amendments using biochar (Tang et al., 2020; Houssou et al., 2022; Taye et al., 2022). Intercropping is a method of cultivation of two or more crop species in the same field, and it can enhance plant productivity and biodiversity and provide biocontrol against pests and pathogens. Intercropping is also a suitable agricultural management practice for improving the phytoremediation process in brassicaceous plants (Cao et al., 2019). The selection of plant species for intercropping is key to the successful implementation of strategies that enhance the translocation of Cd from the roots to the shoots in mustard plants (Wang et al., 2019). Leguminous plants solubilize insoluble phosphorus and fix nitrogen through biological processes in the soil (Etemadi et al., 2019). Couëdel et al. (2018) confirmed that leguminous plants accumulate nitrogen in plant tissues under intensive HM stress. Mung beans (*Vigna radiata* L.) belong to the legume family “Fabaceae” and this plant species is known to be able to survive and grow under Cd stress. Wu et al. (2019) confirmed that intercropping of mung bean with tamarillo (*Cyphomandra betacea*) plants increases the defense mechanism in plants grown under Cd stress. However, to our knowledge, no extensive study on the intercropping of mung beans has been reported and conducted until now.

Many plant-beneficial soil microbes have been used for the bioremediation of Cd-contaminated soil. The rhizosphere plant root system closely interacts with soil microbes (Berg and Smalla, 2009). Plant–microbe interactions have provided potential evidence against Cd stress in various studiesSeveral studies have provided evidence that plant–microbe interactions can potentially mitigate the effects of Cd stress (Lay et al., 2018; Cordero et al., 2020; Taye et al., 2020). The *Rhizobiaceae* bacterial family is known for its symbiotic relationship with leguminous plants, which are capable of nitrogen fixation. A study by Noel et al. (1996) shows that *Rhizobium leguminosarum*’s symbiotic interaction with *Brassica napus* significantly enhances root length. Taye et al. (2022) confirmed the positive influence of *R. leguminosarum* on root architecture, plant health, and productivity of *B. napus*. Despite the importance of *B. juncea* root architecture, root growth dynamics and the relationship between root system architecture and microbe–cadmium interactions are still unknown. This was also a major focus of our study.

Different biological techniques have been investigated as a means to reduce Cd toxicity in agricultural land. The use of biochar (BC) has been widely reported as a means to increase the tolerance of crops to HM stress (Ahmad et al., 2016). BC products have a porous structure with high surface areas and absorption properties; consequently, these products enhance the ability of the plant to absorb and bioaccumulate various HMs and organic contaminants from the surrounding soil. BC also provides nutrients and minerals to plants, resulting in the enhancement of plant growth parameters (Younis et al., 2016). However, very little information is available regarding the effect of BC products on the morphophysiological and biochemical attributes of the Brassicaceae family, especially when grown under Cd stress.

The primary objective of this study was to find the best combination of BC product, co-planting (i.e., intercropping plant species), and bacterial strain that would improve soil structure to reduce the immobilization of Cd and confer CD tolerance on mustard plants while at the same time enhancing mustard morphophysiological traits. For this purpose, the variety BARD1 of *B. juncea* was selected based on the yield traits, fast growth progression, and high Cd tolerance. Thus, to prevent Brassica Juncea crop growth and yield quantity under Cd stress. Some co-combination techniques have been adapted to analyze their mutual benefits comprising toward the crop, including the bacterial strain of species (Rhizobium leguminosarum), biochar produced from (Wheat husk), and Co-planting with legume plant (Vigna radiata). This study also provides data to investigate the root architecture of plants grown under Cd stress (Nedjimi and Daoud, 2009; Huang et al., 2010; Houssou et al., 2022).

2 Materials and methods

2.1 Seed collection and germination

Mustard plant seeds were collected from the Oil & Seed Research Program, National Agriculture Research Center (NARC), Pakistan. This department preserves the germplasm of oilseed crops and, using hybridization, evaluates oil plant varieties for enhancement of yield; heat, pest, and drought resistance; and nutritional qualities. Four varieties of mustard plants were used for the germination rate test: BARD1, Canola, Bahawalpur, and Super-Raya. BARD1 was selected as this variety showed an 85% germination rate (Sharma and Bhardwaj, 2007). Experiments using the selected variety were conducted at Atta ur Rehman School of Applied Bioscience, NUST, Pakistan. Seeds were surface sterilized with 70% ethanol for 1 min, followed by washing with distilled water. Seeds were spread on filter paper inside a safety cabinet for maximum ethanol evaporation. UV-sterilized germination paper was used for seed germination in a box. The box was wrapped with aluminum foil to minimize light penetration. The box was placed in a dark place at 25°C–28°C for 48 hours to break seed dormancy (Alvarado and Bradford, 2005).

2.2 Seedling growth and experimental design

Soil with pH 7.78, which was autoclaved at 121°C for 1 hour, was used to fill the pots. Each pot contained 1.2 kg of soil. A 10 mmol/kg cadmium chloride ($CdCl_2$) solution was added to each pot. Cadmium was added to the soil by watering it with the $CdCl_2$ solution for 10 days. A total of 12 g of biochar (produced from wheat husk) was mixed into each pot, and 1 mL of the bacterial inoculum of *R. leguminosarum* was transferred to the soil 3 days before the sowing of the seedlings. The glasshouse chamber had a 16-h day/8-h night photoperiod, with a light intensity of 300/μmol/m^2/s. The temperature was set at 20°C, with a relative humidity of 50%–60%. Seedlings with a constant growth rate were planted in pots after the 10th day of treatment with the cadmium solution. The treatment design was as follows: negative control (NC), a single mustard plant; positive control (PC), mustard plant + cadmium; T1, *R. leguminosarum* (RL) inoculation only; T2, biochar only (BC); T3, *R. leguminosarum* + biochar; T4, *V. radiata* only (var. MN-11) (VR); T5, *V. radiata* + *R. leguminosarum*; T6, *V. radiata* + *R. leguminosarum* + biochar. The experimental pots were based on a completely randomized design with seven replicates, and data analysis was performed on six replicates. Plants were harvested for data analysis at two different time points, on day 30 day (flower/early-stage harvesting) and day 60 (pod/maturity stage).

2.3 Water-holding capacity

To determine the water-holding capacity of the soil, we added 1.2 kg of soil to each of the six pots: three contained plain soil (control group), and three contained a biochar mixture (experimental group). The three control pots were placed in one tub of water and the experimental pots in the other. Water was added to all six pots until it started dripping from the bottom. After 4 hours, pots were weighed with an electrical balance (W_1). The soil was dried in the oven for 2 hours at 100°C, after which the soil was again weighed (W_a). Air-dried pots were kept at room temperature and their weight was defined as W_b. W_2 was defined as $W_2 = W_a + W_b$. Water-holding capacity (WHC) was measured by the following formula (Wang et al., 2014):

$$100\% \ \ WHC = \frac{W_1 + \ W_2}{W_2} * 100$$

2.4 Morphological traits

Root, shoot, leaves, and pods [length and fresh and dry weights (g)] were measured during the first period (i.e., early stage of growth), on the 30th day, and at the end of the second period (i.e., mature stage), at harvesting, on the 65th day. Root and shoot length (cm) were measured manually using a measuring tape. The fresh weight was measured with a digital balance immediately after harvesting. The dry weight was collected by washing the roots, shoots, and leaves with deionized water, then the tissues were air dried in an air dryer at 70°C for 2 hours to remove moisture. The tissues were then weighed to determine the dry weight of the samples (Aina et al., 2019). Leaves and roots were scanned with ImageJ software (National Institutes of Health, Bethesda, MD, USA), leaves area was calculated in ImageJ software. However, for the measurement of various roots parameters GiARoots (general image analysis of roots) software was used for further analysis (Schneider et al., 2012). Fresh leaf samples from each plant were collected to calculate relative water content. Leaf samples were kept in water for 24 hours to allow them to regain turgidity. The samples were then weighed to determine the turgid weight. Leaves were then oven-dried at 70°C for 4 hours to determine the dry weight. The relative water content (RWC) was measured by using the formula described by Fariñas et al. (2019):

$$RWC = \frac{Fresh\ \ weight - dry\ \ weight}{turget\ \ weight - dry\ \ weight} \times 100$$

2.5 Root structure analysis

The root scanning method is considered effective for analyzing different parameters of roots. The plant root samples were washed with deionized water and then dried on filter paper until the overlapping of root hairs was observed. Sample roots were aligned on the Plastic sheet and scanned with a scanner. The images collected from the scanner were analyzed using the GiA Roots software (Tripathi et al., 2021).

2.6 Photosynthetic pigment analysis

The following method was used to measure the chlorophyll and carotenoid contents (Arnon, 1949). A 0.5-g sample of fresh plant tissue was homogenized with 5 mL of 80% acetone. The homogenized sample was centrifuged at 13,000 rpm for 20 min at 4°C. The supernatant of plant extract was collected for analysis. The absorbance of the supernatant was measured at 645, 663, and 470 nm for chlorophyll a, chlorophyll b, and carotenoid content, respectively. In the equations below, *A* represents the absorbance at a specific wavelength, *V* is the final volume of the chlorophyll extract in 80% acetone, and *W* is the fresh weight of the tissue extracted.

$$Chlorophyll\ \ A = \frac{12.7(A_{663}) - 2.69(A_{645}) \times V}{1000} \times W$$

$$Chlorophyll\ \ B = \frac{22.9(A_{645}) - 4.68(A_{645}) \times V}{1000} \times W$$

$$Total\ \ Chlorophyll\ \ = \frac{20.2(A_{645}) - 8.02(A_{663}) \times V}{1000} \times W$$

$$Carotenoid\ \ = \frac{7.6(A_{470}) - 1.49(A_{510}) \times V}{1000} \times W$$

2.7 Field-emission scanning electron microscopy and energy-dispersive X–ray spectrometric analysis for Cd

The surface morphology of dry root and leaf samples was monitored using field-emission scanning electron microscopy (FESEM) (model JSM 6490A; JEOL Ltd, Tokyo, Japan). The elemental composition of the tissues was analyzed using energy-dispersive X–ray (EDX) spectrometry (model JSM 6490A; JEOL Ltd). For the analysis, the following conditions were used: acceleration voltage, 15 kV; and emission of current, 12 µA.

2.8 X-ray diffraction analysis of Cd in soil

X-ray diffraction (XRD) was performed for crystalline soil after the plant post-harvesting process to detect the total quantity of Cd left in the soil. During XRD, the sample was placed on an XRD grid and spectra were recorded at 40 keV and 30 mA, with Cu K-α radiation.

2.9 Statistical analysis

Statistical analysis of univariate data, including morphological and physiological characteristics of plants, was performed in RStudio software (version 4.2; The R Foundation for Statistical Computing, Vienna, Austria). Sharpio test (default function in R) was used for data normality. Normalized data were analyzed by constructing a two-way ANOVA, which was followed by the *post hoc* Tukey's honestly significant difference (HSD) test (Tukey's HSD, $p< 0.005$). Correlation analysis among different variables was performed with RStudio by identification of means, medians, and standard deviations (SDs). Following ANOVA and Tukey analysis, the data bars were labeled with mean and SD using the RStudio package "multcompLetters4"). Pearson's correlation analysis was performed for a root architecture parameter and for analyzing morphophysiological parameters at maturity and at the early growth stage (i.e., early stage). XRD peak analysis was performed using X'Pert HighScore (version 3.0; Malvern Panalytical Ltd, Cambridge, UK) and graphs were made in RStudio software.

3 Results

3.1 Combinations enhanced growth and biomass under Cd stress

The *post hoc* Tukey's HSD test ($p< 0.005$) was used to analyze plant growth and biomass data. The results show that Cd stress significantly reduced shoot, root, and total plant length. Compared with normal conditions (i.e., the NC treatment), shoot, root, and total plant length in the PC treatment were, respectively, 59.33%, 52.79%, and 57.95% lower at maturity and 67.27%, 41.87%, and

57.21% lower in the early growth stage (see Figures 1A–C). However, different treatment combinations reduced or eliminated the effect of Cd stress and enhanced plant growth. Treatment T1 [i.e., *R. leguminosarum* only (RL)] reduced Cd stress, enhancing shoot, root, and total plant length by 48.78%, 36.24%, and 46.88%, respectively, at maturity, and by 46.38%, 2.85%, and 31.90%, respectively, in the early stage of growth. Treatment T2 [i.e., biochar only (BC)] was less effective than the other treatments, increasing shoot, root, and total plant length by only 26.81%, 29.54%, and 27.4%, respectively, at maturity, and by 3%, –12%, and –2%, respectively, in the early stage of plant growth (see Figures 1A–C).

The T3 treatment [i.e., the combination of *R. leguminosarum* (RL) and biochar (BC)] increased shoot, root, and total plant length under Cd stress by 49.62%, 46.38%, and 49.35%, respectively, at maturity, and by 42.89%, 27.36%, and 38.45%, respectively, in the early growth stage (see Figures 1A–C).

The T4 treatment [i.e., *V. radiata* only (VR)] enhanced shoot, root, and total plant length under Cd stress in the maturity stage by 49.8%, 37.51%, and 48.02%, respectively, and in the early stage of growth by 32.9%, 20.4%, and 29.3%, respectively. The T5 treatment (i.e., the combination of RL and VR) improved the growth of plants under Cd stress compared with the positive control (PC) in the maturity stage by 50.9%, 46.1%, respectively, and 50.4%, and in the early growth stage by 46.7%, 27.7%, and 41.14%, respectively (see Figures 1A–C). In contrast, the T6 treatment (i.e., the combination of VR, BC, and RL) enhanced plant growth to the same extent as the negative control (NC). This combination eradicates the effect of Cd stress, with shoot, root, and total plant length being, respectively, 92%, 98%, and 94.0% of that achieved under normal conditions in the maturity stage and 91%, 99%, and 97.7% of normal, respectively, in the early growth stage. This shows that the T6 treatment (i.e., the combination of VR, BC, and RL) effectively reduces Cd stress and improves plant growth (see Figure 1).

The presence of Cd significantly reduced plant fresh and dry biomass in the PC treatment, by 74.82% and 73.90%, respectively, in the mature stage and by 75.61% and 76.76%, respectively, in the early growth stage. However, exposure of mustard plants under Cd stress to different treatment combinations helps to increase the biomass of plants and decrease the toxic effect of Cd (see Figures 1D, E). The T1 treatment increased fresh and dry plant biomass by 21.35% and 19.10%, respectively, at maturity, and by 36.90% and 41.85%, respectively, in the early growth stage. The T2 treatment resulted in an increase in fresh and dry biomass of 21.74% and 21.72%, respectively, in the maturity stage, and of 19.30% and 19.49%, respectively, in the early growth stage. The results of T3 treatment were approximately 3% better than those of T2, with increases in fresh and dry biomass of 25.54% and 24.46%, respectively, being

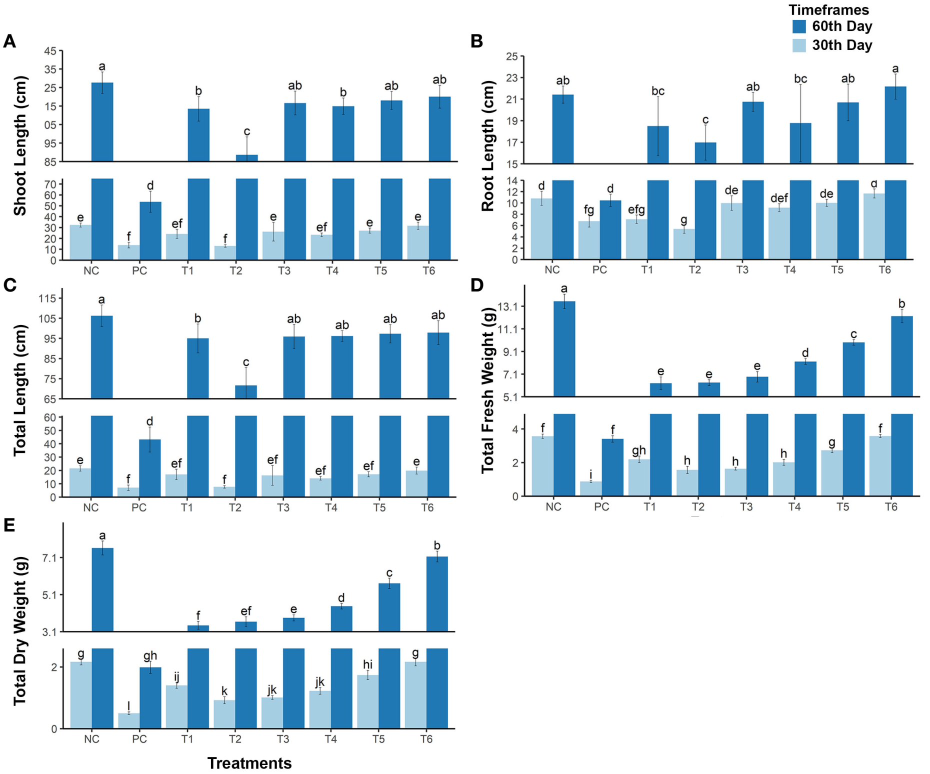

FIGURE 1
The growth rate of mustard plants grown under cadmium stress . The plants were analyzed at maturity and at an early stage of plant growth. **(A)** shoot length; **(B)** root length; **(C)** total length of the plant; **(D)** total fresh weight of the plant; and **(E)** total dry weight of the plant. The treatments are as follows: NC, negative control—mustard plant only; PC, positive control—mustard plant + cadmium; T1, treatment 1—Rhizobium leguminosarum (RL); T2, treatment 2—biochar (BC); T3, treatment 3—RL + BC; T4, treatment 4—Vigna radiata (VR); T5, treatment 5—VR + RL; T6, treatment 6—VR + BC + RL. Data present the interaction between plant growth and the applied cadmium stress. Significance was calculated using two-way ANOVA under the Tukey's HSD post hoc test for normally distribution data (honestly significant difference, $p < 0.005$). HSD, Tukey's honestly significant difference. The lower case letters show the significance difference between treatments.

recorded in the maturity stage, and of 21.30% and 23.61%, respectively, in the early growth stage (see Figures 1D, E). The T4 treatment (i.e., VR co-planting) increased fresh and dry plant biomass by 35.53% and 32.63%, respectively, at the maturity growth stage, and by 31.93% and 33.55%, respectively, at the early growth stage. Even better results were observed with the T5 treatment (i.e., the combination of RL + VR) in both the mature (fresh and dry biomass increased by 47.97% and 48.90%, respectively) and early growth stages (increases of 52.11% and 57.25%, respectively). The greatest biomass enhancement was noted in the T6 treatment (i.e., the combination of RL + VR + BC). This treatment increased the fresh and dry biomass of mustard plants at maturity by 65.18% and 67.87%, respectively, and in the early stages of plant growth by 75.74% and 76.98%, respectively (see Figures 1D, E).

In addition, some other morphological traits were analyzed (see Table 1), namely dry and fresh weights of leaves, roots, and shoots in the early and maturity stages of growth. Differences in these traits between the negative control (NC) and the T6 treatment were also non-significant, although a significant difference between the positive control (PC) and other treatments was found. Therefore, the results confirm that a combination of bacteria, biochar, and co-planting improves plant biomass and significantly diminishes Cd toxicity.

3.2 The outcome of combinations on plant physiological parameters

Physiological parameters of the mustard plant were analyzed, and differences were considered significant at $p< 0.005$. The parameters measured included the number of leaves, leaf area, chlorophyll a content, chlorophyll b content, total water content, and carotenoid content. These parameters were significantly reduced in treatment PC (under Cd stress), by 63.06%, 46.89%, 67.90%, 55.67%, 39.93%, and 16.36%, respectively, in the mature growth stage and by 51.19%, 50.95%, 89.47%, 63.30%, 40.57%, and 52.34%, respectively, in the early growth stage (see Figures 2A–F). However, different treatment combinations resulted in dramatic increases in these physiological parameters under Cd stress. For example, compared with the positive control (PC), the T1 treatment increased these physiological attributes by 18.47%, 20.66%, 42.81%, 16.46%, 22.40%, and 11.74%, respectively, at the mature growth stage and by 27.90%, 18.86%, 38.23%, –7.29%, 19.57%, and 42.86%, respectively, at the early growth stage. The T2 treatment, like other treatment combinations, was associated with a positive response to Cd stress, increasing the listed parameters by 42.03%, 41.29%, 17.32%, 27.32%, 23.78%, and 11.38%, respectively, at the mature growth stage and by 25.57%, 50.95%, 28.26%, –5.54%, –9.48%, and 50.85%, respectively, at the early stage of plant growth. However, the T3 treatment (i.e., the combination of RL with BC) increased the number of leaves, leaf area, chlorophyll a content, chlorophyll b content, total water content, and carotenoid content by 45.85%, 28.12%, 22.83%, 31.66%, 30.65%, and 7.47%, respectively, at maturity and by 51.14%, 26.07%, 43.95%, –3.26%, 25.31%, and 37.54%, respectively, in the early stage of growth. The T4 treatment (i.e., co-planting with VR) enhanced these physiological parameters under Cd stress by 36.94%, 28.16%, 24.01%, 35.66%, 23.78%, and 13.64%, respectively, at the mature growth stage and by 39.52%, 31.05%, 31.95%, –1.93%, 8.96%, and 47.30%, respectively, at the early growth stage. The same trend was observed in treatment T5 (i.e., the combination of RL + VR), which increased these

TABLE 1 Characteristics of mustard plants grown under cadmium stress in the early and maturity stages of growth.

Treatment	Growth stage	LFW (g)	LDR (g)	RFW (g)	RDW (g)	SFW (g)	SDW (g)
NC	*Maturity*	3.97 ± 0.19^{a}	1.93 ± 0.13^{a}	4.37 ± 0.5^{a}	2.62 ± 0.15^{a}	4.44 ± 0.19^{a}	2.64 ± 0.24^{a}
	Early	1.34 ± 0.1^{d}	0.81 ± 0.06^{d}	0.85 ± 0.07^{e}	0.57 ± 0.05^{f}	1.58 ± 0.07^{de}	0.79 ± 0.03^{cd}
PC	*Maturity*	2.49 ± 0.36^{c}	0.51 ± 0.09^{ef}	1.28 ± 0.24^{d}	0.87 ± 0.15^{e}	1.1 ± 0.11^{f}	0.57 ± 0.06^{de}
	Early	0.67 ± 0.09^{d}	0.14 ± 0.03^{h}	0.26 ± 0.03^{g}	0.17 ± 0.02^{i}	0.42 ± 0.03^{h}	0.2 ± 0.02^{f}
T1	*Maturity*	3.35 ± 0.34^{ab}	1.03 ± 0.11^{c}	1.49 ± 0.06^{d}	1.13 ± 0.08^{d}	1.91 ± 0.11^{bcd}	0.95 ± 0.05^{c}
	Early	0.84 ± 0.05^{d}	0.57 ± 0.06^{e}	0.55 ± 0.05^{efg}	0.43 ± 0.07^{fgh}	0.83 ± 0.13^{fg}	0.41 ± 0.06^{ef}
T2	*Maturity*	3.52 ± 0.76^{ab}	1.10 ± 0.12^{c}	2.39 ± 0.11^{c}	1.53 ± 0.17^{c}	1.98 ± 0.21^{bc}	0.99 ± 0.11^{bc}
	Early	0.71 ± 0.09^{d}	0.32 ± 0.04^{fgh}	0.46 ± 0.03^{g}	0.28 ± 0.05^{hi}	0.65 ± 0.18^{gh}	0.33 ± 0.09^{ef}
T3	*Maturity*	2.92 ± 0.77^{bc}	1.62 ± 0.10^{b}	1.49 ± 0.06^{d}	1.16 ± 0.03^{d}	1.45 ± 0.36^{e}	0.72 ± 0.18^{cd}
	Early	0.66 ± 0.08^{d}	0.31 ± 0.07^{gh}	0.51 ± 0.04^{fg}	0.36 ± 0.03^{ghi}	0.68 ± 0.06^{gh}	0.34 ± 0.03^{ef}
T4	*Maturity*	2.84 ± 0.52^{bc}	1.71 ± 0.13^{b}	2.33 ± 0.14^{c}	1.52 ± 0.11^{c}	1.74 ± 0.14^{cde}	0.87 ± 0.07^{c}
	Early	0.81 ± 0.08^{d}	0.46 ± 0.1^{efg}	0.47 ± 0.02^{g}	0.32 ± 0.02^{hi}	0.89 ± 0.15^{fg}	0.44 ± 0.08^{ef}
T5	*Maturity*	3.79 ± 0.41^{a}	1.95 ± 0.10^{a}	3.28 ± 0.07^{b}	2.18 ± 0.09^{b}	2.22 ± 0.11^{b}	1.23 ± 0.25^{b}
	Early	1.11 ± 0.13^{d}	0.77 ± 0.1^{d}	0.54 ± 0.03^{efg}	0.42 ± 0.07^{fgh}	1.09 ± 0.05^{f}	0.55 ± 0.02^{de}
T6	*Maturity*	3.87 ± 0.29^{a}	1.98 ± 0.06^{a}	3.40 ± 0.23^{b}	2.32 ± 0.15^{b}	4.33 ± 0.27^{a}	2.5 ± 0.28^{a}
	Early	1.18 ± 0.17^{d}	0.82 ± 0.05^{d}	0.83 ± 0.05^{ef}	0.55 ± 0.12^{fg}	1.58 ± 0.07^{de}	0.79 ± 0.04^{cd}

LFW, leaf fresh weight; LDR, leaf dry weight; NC, negative control; PC, positive control; RFW, root fresh weight; RDW, root dry weight; SFW, shoot fresh weight; SDW, shoot dry weight. Different lowercase letters in the same column indicate the significant difference between maturity and early stages of plant growth in the various treatments ($p< 0.005$).

parameters by 36.94%, 26.85%, 29.01%, 37.44%, 29.28%, and 6.72%, respectively, at the maturity stage of growth and by 41.85%, 18.36%, 49.05%, 10.75%, 39.19%, and 42.86%, respectively, at the early stage of growth. In plants subjected to treatment T6 (i.e., the combination of VR, BC, and RL), all parameters showed the same trends as of the negative control (NC) group, without Cd stress. These results show that these combinations significantly improve plant growth parameters in comparison with normal growth conditions. Under Cd stress, the T6 treatment (i.e., the combination of VR, BC, and RL) resulted in a remarkable increase in the number of leaves, leaf area, chlorophyll a content, chlorophyll b content, total water content, and carotenoid content, by 63.05%, 43.16%, 45.00%, 45.22%, 30.65%, and 10.14%, respectively, at the mature stage of growth and by 41.85%, 46.22%, 89.67%, 11.34%, 40.57%, and

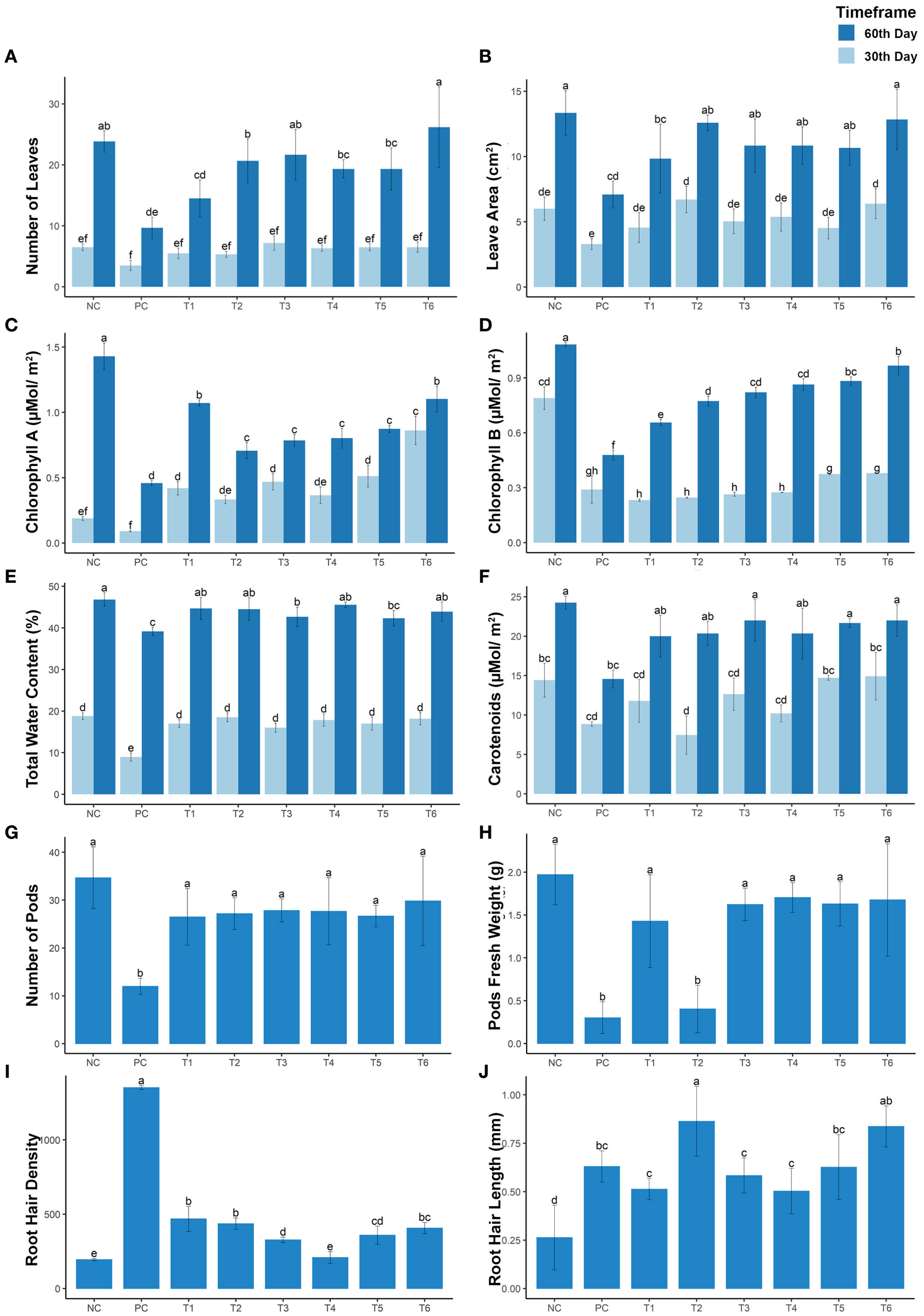

FIGURE 2
The growth rate of mustard plants grown under cadmium stress. The plants were analyzed at maturity and at an early stage of plant growth. The graphs present the following: **(A)** number of leaves; **(B)** leaf area; **(C)** chlorophyll a content; **(D)** chlorophyll b content; **(E)** total water content; **(F)** carotenoid content; **(G)** number of pods; **(H)** pod fresh weight; **(I)** root hair density; and **(J)** root hair length. The treatment are as follows: NC, negative control—mustard plant only; PC, positive control—mustard plant + cadmium; T1, treatment 1—Rhizobium leguminosarum (RL); T2, treatment 2—biochar (BC); T3, treatment 3—RL + BC; T4, treatment 4—Vigna radiata (VR); T5, treatment 5—VR + RL; T6, treatment 6—VR + BC + RL. Data present the interaction between plant growth and the applied cadmium stress. Significance was calculated using two-way ANOVA under the Tukey's HSD post hoc test for normally distribution data (honestly significant difference, $p < 0.005$). HSD, Tukey's honestly significant difference.

49.07%, respectively, at the early stage of growth. Thus, the T8 combination treatment demonstrated the equivalent enhancement in plant attributes as compared with the negative control (NC). This result demonstrates that the combination of VR, BC, and RL significantly impacts plant growth and physiological parameters (see Figures 2A–F).

3.3 Fresh pod yield and root hair density

In the current study, the different treatment combinations provide a sustainable enhancement in yield quantity and weight. These parameters were increased by 41.82% and 57.19%, respectively, under treatment T1, by 43.75% and 5.16%, respectively, under treatment T2, and by 45.67% and 67.09%, respectively, under treatment T3. Treatment T4 increased quantity and growth by 45.19% and 71.24%, respectively. whereas treatment T5 (i.e., the combination of RL + VR) increased the yield and growth by 42.31% and 67.43%, respectively. However, treatment T6 increased the yield and growth of plants grown under Cd stress by 51.44% and 69.80%, respectively. These results corroborate the effectiveness of combinations of VR, BC, and RL in improving growth and yield enhancement when plants are grown under Cd stress (see Figures 2G, H).

Plant roots are the first organs that interact with Cd in the soil. Cd stress induces significant changes in mustard plant roots by increasing the length and density of the root hairs. In this study, large increases in root hair length and density were observed in PC plants at the early growth stage. The rate of enhancement varied between treatments. Root hair growth and density were reduced by 28.95% and 20.26%, respectively, by the T1 treatment, by 69.49% and 17.90%, respectively, by the T2 treatment, by 37.06% and 9.86%, respectively, by the T3 treatment, by 27.79% and 1.01%, respectively, by the T4 treatment, and by 42.08% and 12.14%, respectively, by the T5 treatment. The T6 treatment (i.e., the combination of VR, BC, and RL) reduced the root hair length and density by 66.40% and 15.71%, respectively. This result concludes that these combinations provide potential to plant for its suitable growth under Cd stress (see Figures 2I, J).

3.4 Cd detection in leaves and roots with SEM/EDX

The analysis of dried leaves and root samples by EDX confirmed the presence of Cd ions in the leaves and roots of plants in the maturity stage (Figure 3). Table 2 shows the percentage by weight of different macroelements, including Cd, in the mustard plants. The results not only confirm the presence of Cd in root and leaf tissue but also show that different treatment combinations help to reduce Cd uptake, thereby increasing plant growth and nutrient uptake. The EDX results show that Cd in roots and leaves reduces nutrient absorption (see Table 2). The presence of high concentrations of Cd can interfere with a plant's uptake of essential minerals, leading to reduced growth and yield.

In addition, Cd toxicity may also cause damage to other organs, leaves. Table 2 shows that treatment T6 was associated with the lowest Cd accumulation in leaves and roots (3.37% and 0.2%, respectively). However, no changes were observed in the morphophysiological features of the plants. In the comparison of roots and leaves, the EDX analysis of all treatments demonstrated that Cd accumulation in leaves was higher than in roots. This result confirms that different combinations reduce Cd around the roots in soil. In addition, plants subjected to treatment T2 (i.e., BC only) showed considerable Cd at the root level. This might be because the absorption of Cd by BC was lower when BC was used alone than when it was part of combination treatments. A comparison of morphophysiological parameters in Figures 1, 2 shows that the results of treatment T2 were not as good as those obtained with the other treatments.

The effect of Cd on leaf stomata, mesophyll cells, and primary roots was studied *via* SEM on the 60th day of growth. Figure 4 shows that Cd in plants exposed to the PC treatment caused a significant number of stomata to be closed. Stomatal closure prevents water transpiration, which in turn leads to a reduction in nutrient loss from the plant, thus making more nutrients available in the long term. Some irregular structures were observed in mesophyll cells and attributed to Cd accumulation. In addition, the presence of Cd affected the internal and external structures of the primary roots. Cd accumulation reduces the translocation of other minerals *via* root hairs and inhibits the growth of the germ tissue region. We observed thinning and breakage of the root cell compartments, and an excessive number of pores on root hairs (see Figures 4A–D).

3.5 Identification of Cd in soil samples after harvesting

Soil samples were collected after harvesting plants at maturity for the analysis of abandoned Cd traces in the soil. XRD was performed and the analysis was within a scanning range of 10°–50° (2θ). Five distinct diffraction peaks were found, at 20.84°, 26.62°, 29.42°, 36.46°, 39.44°, and 42.47°, in all eight treatments, with indexed as the planes 100, 101, 104, 110, 102, and 113, respectively, as shown in Figure 5. Three different chemicals were observed in the XRD analysis: SiO_2, $CaCO_3$, and Al_2O_3. However, no evidence of $CdCl_2$ was found in the post-harvested soil. This confirms that *B. juncea* finished the phytoremediation process and cleaned the soil. In contrast to the EDX results, Cd was detected in roots and leaves. However, with different co-combination treatments Cd concentration in the root and leaves of the mustard plant indicates that co-combination treatments inhibit the translocation of the Cd as compared with positive control (PC). The results confirmed that the T6 treatment (i.e., the combination of BC, VR, and RL) helps to reduce Cd uptake in mustard plants and performs a phytoremediation function. In addition, as the mustard plant performs phytoremediation, the combination reduces the translocation of Cd in the plant. These treatments will be effective for non-hyperaccumulating plants.

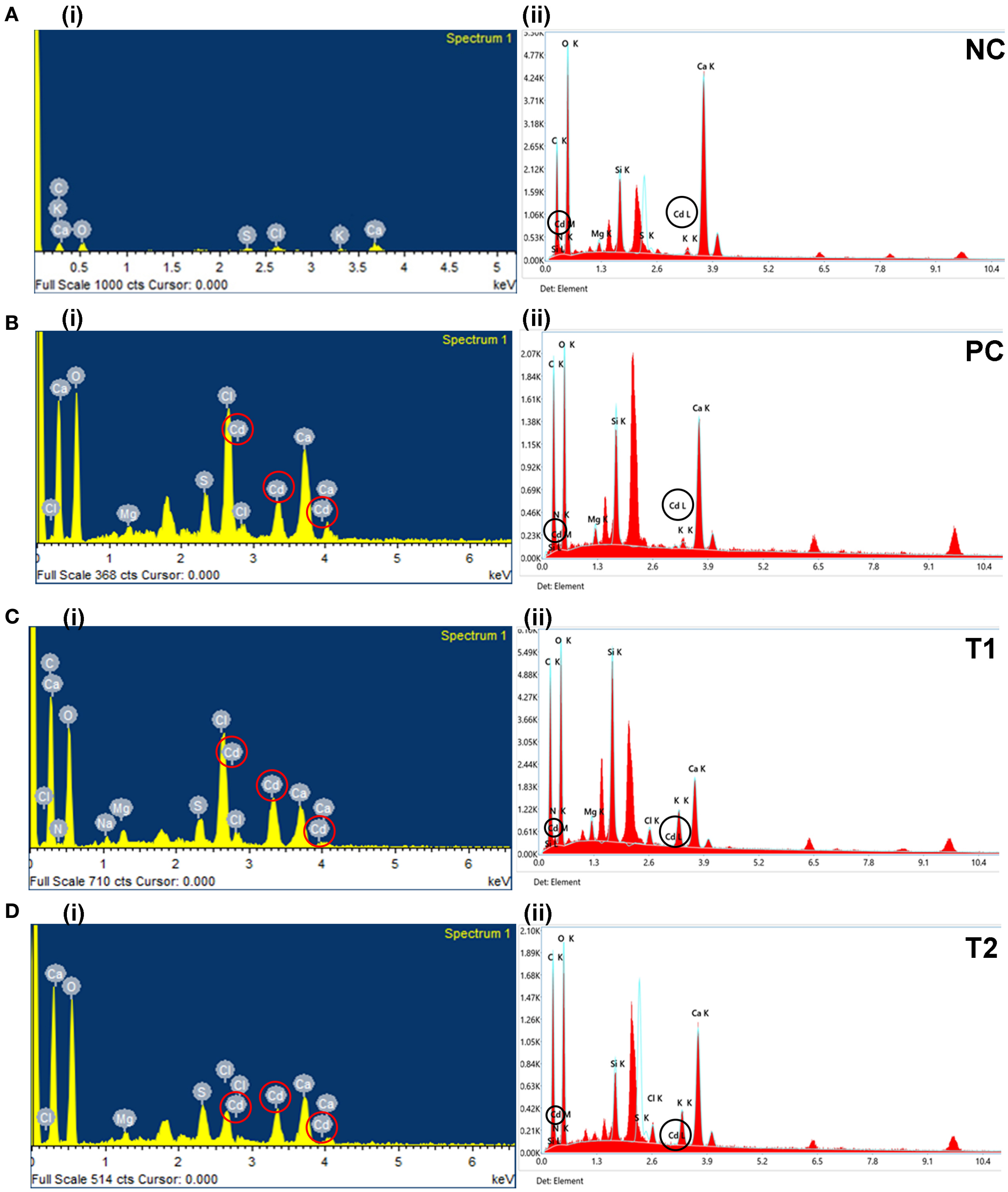

FIGURE 3 (Continued)

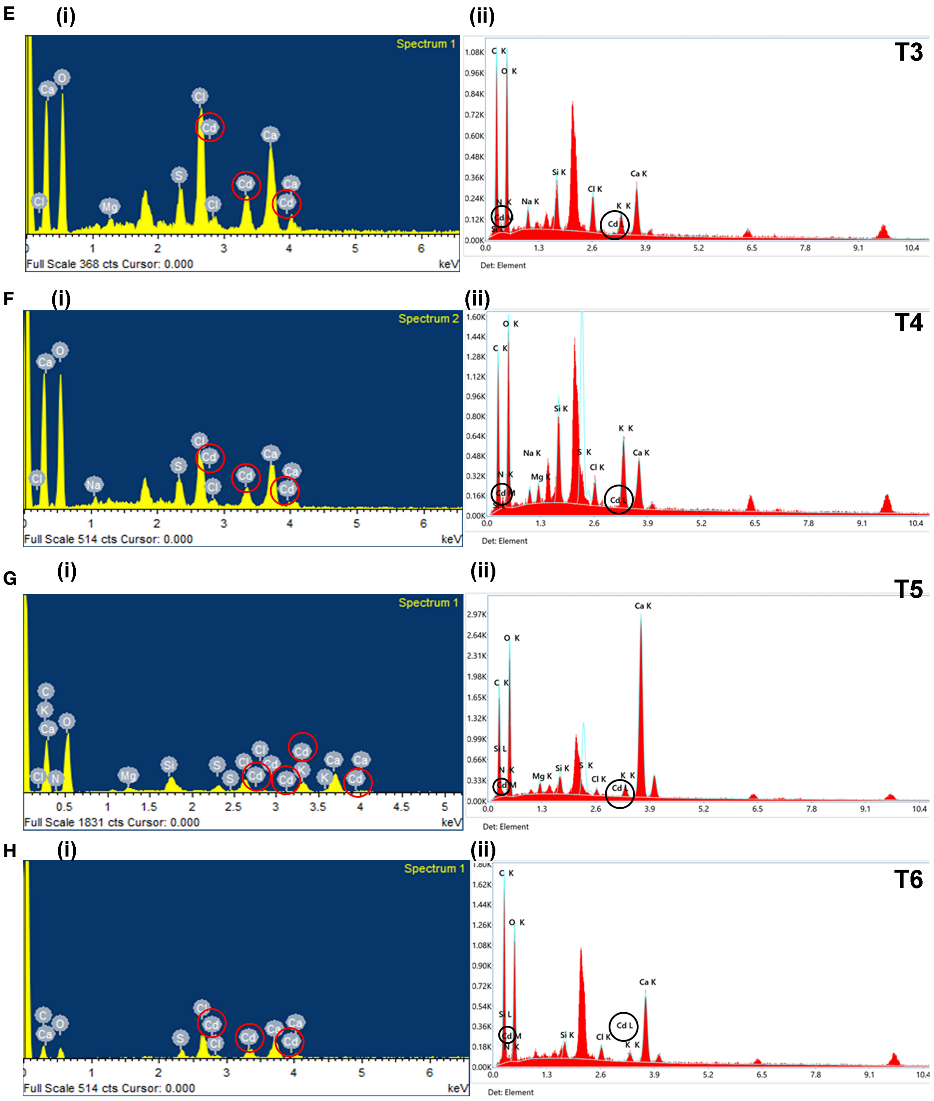

FIGURE 3
The EDX graphs of different treatment shows as: A-NC, B-PC, C-T1, D-T2, E-T3, F-T4, G-T5, and H-T6 whereas, (i)- for leafs sample and (ii)- represent roots EDX. The treatments are as follows: NC, negative control—mustard plant only; PC, positive control—mustard plant + cadmium; T1, treatment 1—*Rhizobium leguminosarum* (RL); T2, treatment 2—biochar (BC); T3, treatment 3—RL + BC; T4, treatment 4—*Vigna radiata* (VR); T5, treatment 5—VR + RL; T6, treatment 6—VR + BC + RL. EDX, energy-dispersive X-ray.

3.6 Correlation of root traits and architecture

Root architecture traits were evaluated under Cd stress in the early growth stage of the plant (i.e., at 30 days). The root architecture data were analyzed through the GiA Roots software to determine the different root traits. Data were further analyzed through Pearson's correlation analysis in RStudio to examine the positive and negative correlation among the different root traits. The correlation of (Cd-R), NS, NP, MNR, CC, MedNR, and RHD show a negative correlation with SRL, ND, MEA-9, EAR, MEA-13, NCA, NW, NWDR, NSA, NLD, and NB (Figure 6). We confirmed that the following traits:- NS, NP, MNR, CC, MedNR, RHD, SRL, ND, MEA-9, EAR, MEA-13,NCA, NW, NWDR, NSA, NLD, and NB decrease when plants are grown in the presence of Cd stress. Cd-R shows a positive correlation with NV, NA, NL, RHL, RHD, Av-RW, MedNR, CC, MNR, and NP, confirming that Cd enhanced these traits in the root system. However, NP, MNR, CC, MedNR, and RHD showed a positive correlation with NV, NA, NL, RHL, RHD, and other parameters. This demonstrates that Cd presence enhances some root traits and reduces others.

3.7 Correlation of early and maturity stages of plant

Pearson's correlation analysis was used to investigate the relationship between various morphophysiological parameters of

TABLE 2 The weight percent (W%) and atomic percent (A%) of macroelements present in dried leaf and root samples of different treatments.

Element	NC				PC				T1				T2			
	Leaves		*Root*		*Leaves*		*Root*		*Leaves*		*Root*		*Leaves*		*Root*	
	W%	*A%*	*W%*	*A%*	*W%*	*A%*	*W%*	*A%*	*W%*	*A%*	*W%*	*A%*	*W%*	*A%*	*W%*	*A%*
C	30.22	39.9	16.5	25	40.1	53	23.5	32.7	45	55.8	31	41.1	–	–	31	42.3
N	8.5	9.62	4.2	5.3	2.3	2.6	5.1	6.1	1.7	1.81	5	5.7	–	–	3.6	4.3
O	43.42	43	50.2	56	38.4	38	48.2	50.2	38.9	36.3	43.5	43.3	72.66	87	41.1	42.2
Na	–	–	–	–	–	–	–	–	0.45	0.29	–	–	–	–	–	–
Mg	–	–	0.8	0.6	–	–	1.4	1	0.81	0.5	1.4	0.9	1.55	1.22	–	–
Si	–	–	4	2.5	–	–	6.7	4	–	–	8.2	4.6	–	–	3	1.8
S	2.29	1.13	4.5	2.5	–	–	–	–	1.44	0.67	–	–	5.07	3.03	7.5	3.8
Cl	4.24	1.9	–	–	8.05	3.6	–	–	7.99	3.36	1.1	0.5	5.62	3.04	0.2	–
K	1.81	0.73	0.4	0.2	–	–	0.5	0.2	–	–	2.9	1.2	–	–	1	0.5
Ca	9.52	3.68	19.3	8.5	4.72	1.9	14.1	5.9	3.25	1.21	6.8	2.7	10.33	4.93	2.4	1
Cd	0.3	0	0.1	0	6.44	0.9	0.5	0.1	0.45	0.06	0.1	0	4.76	0.81	10.2	4.2

	T3				**T4**				**T5**				**T6**			
	Leaves		***Root***		***Leaves***		***Root***		***Leaves***		***Root***		***Leaves***		***Root***	
	W%	*A%*	*W%*	*A%*	*W%*	*A%*	*W%*	*A%*	*W%*	*A%*	*W%*	*A%*	*W%*	*A%*	*W%*	*A%*
C	–	–	34	44	71.7	86	33.3	45	42.8	53.1	18.9	28.3	41.17	56.3	34.9	44.7
N	–	–	5.2	5.7	–	–	4.9	5.7	2.89	3.08	3.1	3.9	–	–	4.4	4.8
O	63.2	80.4	44.6	43	–	–	36.4	36.9	46.4	42.1	46.9	52.7	31.87	32.7	46.4	44.7
Na	–	–	2.7	1.8	1.56	1.3	1.3	0.9	–	–	–	–	–	–	–	–
Mg	1.02	0.85	–	–	–	–	1	0.6	0.55	0.34	1.1	0.8	–	–	–	–
Si	–	–	2.9	1.6	–	–	3.6	2.1	1.51	0.8	1.3	0.8	–	–	1.7	0.9
S	3.96	2.51	–	–	4.13	2.5	9.1	4.6	0.95	0.44	4.8	2.7	2.79	1.43	–	–
Cl	15.47	8.88	2.8	1.2	9.82	5.3	1.3	0.6	2.21	0.93	0.5	0.3	10.97	5.08	1.4	0.6
K	–	–	1.8	0.7	–	–	4.6	1.9	2.02	0.77	1	0.5	–	–	1.1	0.4
Ca	13.63	6.92	5.4	2.1	10.5	5	4.1	1.7	0.13	0.02	22.2	9.9	9.83	4.03	9.9	3.8
Cd	2.72	0.49	0.4	0.1	2.4	0.4	0.2	0	4.23	1.57	0.2	0	3.37	0.49	0.2	0

The overall W% and A% are equivalent to 100 in each column.
NC, negative control; PC, positive control.

mustard plants grown under Cd stress and different combinations of biochar, *V. radiata*, and *R. leguminosarum* (Figure 7). The correlation analysis was carried out at two time points, that is, at maturity (65 days) and at an early stage of growth (30 days). At both time points, Cd concentrations in the root (Cd-R) and leaves (Cd-L) show a negative correlation with TL, SL, RL, SFW, RFW, LFW, TFW, Cd-L, SDW, RDW, LDW, TDW, No-L, L-A, Chl-A, Chl-B, Carot, and TWC. Only Cd-R and Cd-L were positively correlated with each other. In addition, all physiological characteristics were positive correlated with each other. Hence, these results reveal a close connection between the presence of Cd and a reduction in morphophysiological characteristics.

4 Discussion

The increased prevalence of Cd contamination in soils is an emerging worldwide concern (Gill, 2014). Cd is a heavy metal that causes adverse effects in crops. In developing countries, the presence of Cd causes serious disturbances in the food chain. Food contaminants also negatively impact human and animal health (Haider et al., 2021). The main purpose of this study was to evaluate the combination of biochar, intercropping (*V. radiata)*, and *R. leguminosarum* on crops grown in Cd-contaminated soil. For this purpose, the mustard plant was selected because of its recognizable hyperaccumulation properties (Rizwan et al., 2016a). The mustard

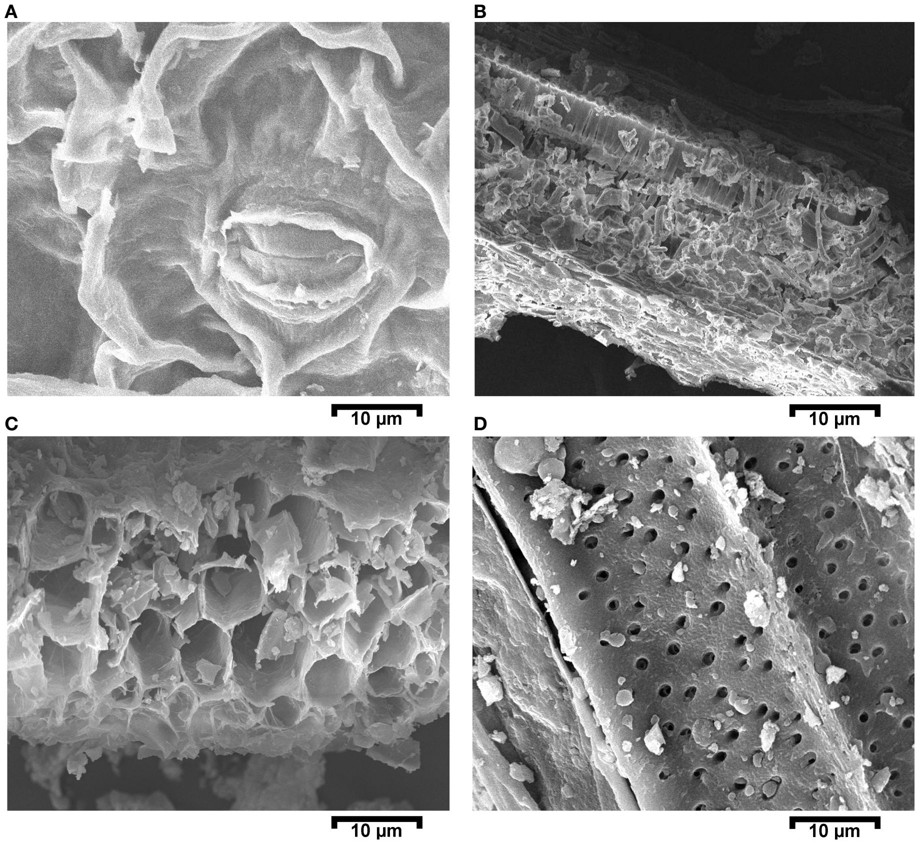

FIGURE 4
Scanning electron micrograph images for the positive control (PC) (i.e., a mustard plant grown under cadmium stress. Images were captured on a 10-µm scale. **(A)** Leaf with a closed stomata; **(B)** cross-sectional view of a leaf; **(C)** cross-sectional view of a primary root; and **(D)** upper structural primary root.

plant is highly consumed as a source of food and is of economic importance throughout the world. This plant is the third-largest oil production plant after palm oil and soybean (FAO, 2017). In the current study, Cd stress decreased plant growth, biomass, photosynthesis pigmentation, and yield; however, several studies reported in the literature have shown similar results. The observed reduction in growth, biomass, and photosynthesis indicates that Cd decreases plant nutrient uptake and alters the plant's cellular structure (see Figures 1, 2) (Rizwan et al., 2012; Ehsan et al., 2014; Rizwan et al., 2016b).

The use of biochar improves the stability of plant growth, photosynthesis, and yield, and induces defense mechanisms in plants grown under Cd stress (Pandit et al., 2018). The T2 (BC), T3 (BC + RL), and T6 (BC + RL + VR) treatments resulted in increased biomass, photosynthesis, and yield. Biochar contains a sustainable source of nutrients that helps to mitigate the effects of Cd and provides beneficial growth conditions to plants (Jang et al., 2018). The T2 treatment might also increase soil physiochemical properties to generate a better environment for plant growth (Sun et al., 2013). A study by Younis et al. (2016) found that 3% biochar enhances plant biomass and photosynthesis in *Spinacia oleracea* when it is grown under Cd stress. In our study, 1.2% biochar (i.e., T2 treatment) increased biomass growth by 19% at the early stage and by 21% at the mature stage. The photosynthesis pigmentation improved under the influence of biochar by 60% compared with the positive control (PC) (see Figure 1). In addition, the T2 treatment reduced fresh and dry weight loss compared with the PC. However, even more positive results were achieved using combinations of biochar and the intercropping plant *V. radiata* and *R. leguminosarum* (RL).

Inoculating plants with plant growth-promoting rhizobia (i.e., *R. leguminosarum*) stimulates growth and improves nutrient uptake in Cd-contaminated soils (Hao et al., 2014). *R. leguminosarum* forms a symbiotic relationship with the mustard plant. These bacteria developed a nitrogen-fixing symbiotic relationship with plants in sandy soil and enhance nutrient uptake. The bacteria repressed Cd translocation and promoted different defense mechanisms in the mustard plants that helped the plants to overcome Cd stress. A previous study, by Safronova et al. (2006), reported that pea plant biomass increased under Cd stress with the presence of *R. leguminosarum*. In our study, plant growth, biomass, photosynthetic pigments, yield quantity, and root parameters improved with *R. leguminosarum* inoculation in treatments T1, T3, T5, and T6 (see Figure 2). However, Cd and *R. leguminosarum* interact directly with the plant roots in the rhizosphere. The root is the tissue that directly interacts with bacteria, biochar,

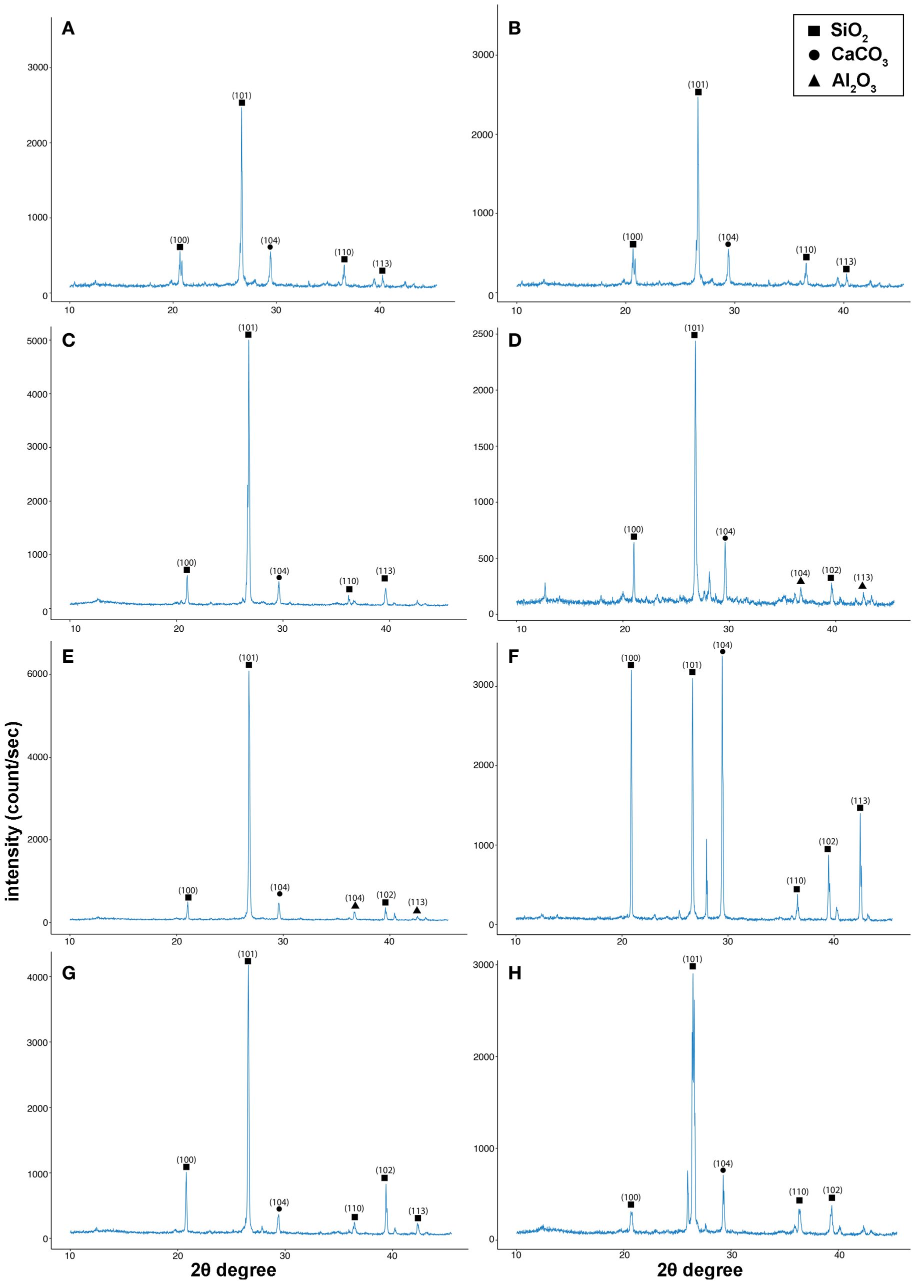

FIGURE 5
X-ray diffraction (XRD) graphs for soil analysis identifying the different compounds present in the soil. **(A)** negative control group (NC), **(B)** positive control group (PC), **(C)** as T1, **(D)** as T2, **(E)** as T3, **(F)** as T4, **(G)** as T5, and **(H)** as T6. The treatments are as follows: NC, negative control—mustard plant only; PC, positive control—mustard plant + cadmium; T1, treatment 1—*Rhizobium leguminosarum* (RL); T2, treatment 2—biochar (BC); T3, treatment 3—RL + BC; T4, treatment 4—*Vigna radiata* (VR); T5, treatment 5—VR + RL; T6, treatment 6—VR + BC + RL.

intercropping plants, and Cd. The roots of mustard plants grown in Cd-contaminated soil in the presence of *R. leguminosarum* did not show any significant change. Thus, *R. leguminosarum* might provide favorable conditions for root growth and development. A study conducted by Taye et al. (2022) found that the length and diameter of roots of *Brassica napus* plants grown in Cd-contaminated soil in the presence of *R. leguminosarum* increased until the flowering stage, then started to decrease until the plants reached maturity. In our study, significant changes in root length and diameter were not observed at the early stage of growth (i.e., at the 30th day), but were noted at the maturity stage (i.e., at the 60th day) (see Figures 2I, J). This confirms that the Brassicaceae family shows no changes in growth in response to Cd stress until the flowering stage, after which root growth becomes stunted (see Figure 1B). Nguyen (2009) and Taye et al. (2022) reported that the abundance of bacteria present has a positive correlation with root length. This might be attributable to causal symbiotic relationships between plants and bacteria. Rhizosphere microbes

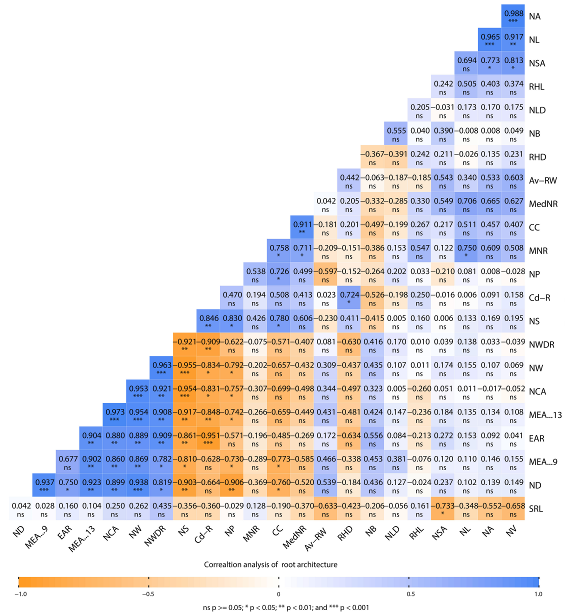

FIGURE 6
Pearson's correlation performed on root architecture attributes of Brassica juncea grown in cadmium-contaminated soil. Av-RW, average root width (diameter); NB, network bushiness; CC, connected components; ND, network depth; EAR, ellipse–axes ratio; NLD, network length distribution; MEA-13, major ellipse axis; MNR, maximum number of roots; NW, network width; MedNR, median number of roots; MEA-9, minor ellipse axis; NA, network area; NCA, network convex area; NP, network perimeter; NS, network solidity; SRL specific root length; NSA, network surface area; NL, network length; NV, network volume; NWDR, network width-to-depth ratio; Cd-R, cadmium in the root; RHD, root hair density; RHL, root hair length.

could bring about change in plant root architecture by providing phytohormones (Asghar et al., 2004). Our results show that *R. leguminosarum* (included in treatments T1, T5, and T6) promotes root growth in *B. juncea* in both the early and mature stages of growth (see Figures 1, 2).

Cadmium inhibits root growth and development in many plants (Ďurčeková et al., 2007). *B. juncea* shows tolerance to Cd, as a result of its hyperaccumulation properties, and Dhaliwal et al. (2022) found that root dry biomass in plants grown under Cd stress was higher in *B. juncea* than in other *Brassica* species (e.g., *B. rapa* and *B. napus*). This suggests that *B. juncea* root is more Cd tolerant than other species. A previous study, conducted by Meyers et al. (2008), reported that *B. juncea* has a highly significant potential to sequester different HMs in the root tissue. In contrast, we found that root biomass was 30% lower in the positive control (PC) than in the negative control (NC). Although large increases in root biomass were observed in other treatments, confirming the positive impact of the combination treatments on the mustard plant. A similar trend toward a reduction in yield and biomass has been observed in different *Brassica* species (Dhaliwal et al., 2020). This plant tends to escape from stress by enhancing its root rhizosphere, and, therefore, root diameter, root hair density, and root hair length increase in plants grown under Cd stress (Dhaliwal et al., 2022). However, the analysis of different attributes of root architecture concluded that some attributes were enhanced by Cd contamination, whereas others were reduced (see Figure 6) (Staňová et al., 2012; Kuijken et al., 2015; Cordero et al., 2020).

A study carried out by Yuan et al. (2017) found that the intercropping of *B. rapa* with *Clinopodium confine* increased root, stem, leaf, and shoot biomasses, in contrast to our study, intercropping between *B. juncea* with *V. radiata* resulted in significant increases in biomass. Additionally, Wu et al. (2019) reported that *V. radiata* shows significant growth enhancement when intercropped with *C. betacea* under Cd stress. Therefore, it might be possible that *V. radiata* enhances intercropped plant growth by providing metabolic or symbiotic help to the co-plant (Figures 1, 2).

The SEM/EDX results confirmed that Cd translocation takes place in leaf and root tissues (see Figure 3). This is due to the hyperaccumulation properties of the mustard plant, which stores heavy metal ions in its aerial parts. However, soil analysis *via* XRD confirms that the post-harvesting soil was free from Cd contamination (see Figure 5). The Cd concentration under different treatments varies in comparison with the positive control (PC), showing that different combinations help to prevent the translocation of Cd in the mustard plant to differing extents (see Table 2). The correlation analysis also proves the negative impacts of Cd on different morphophysiological attributes of mustard plants and root traits (see Figures 6, 7). A study conducted by Saleem et al. (2022) reported similar results.

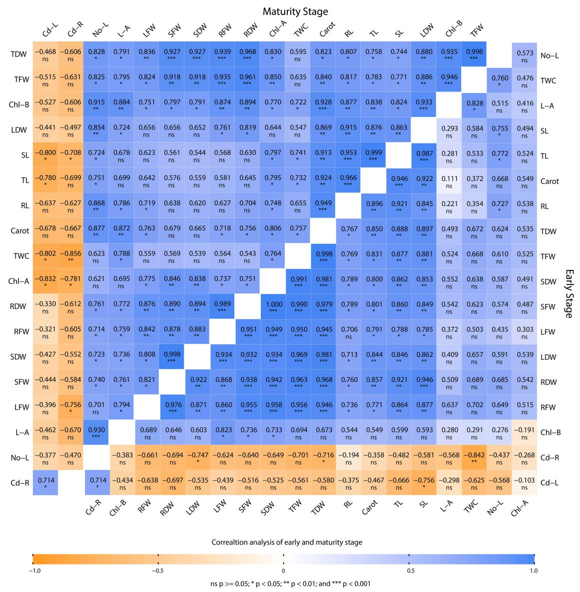

FIGURE 7
Pearson's correlation performed on the various morphophysiological attributes of Brassica juncea grown in Cd-contaminated soil. The plants were analyzed at maturity and at an early stage of plant growth. TL, total length; SL, shoot length; RL, root length; SFW, shoot fresh weight; RFW, root fresh weight, LFW, leaf fresh weight; TFW, total fresh weight; Cd-L cadmium in leaves; SDW, shoot dry weight; RDW, root dry weight; LDW, leaf dry weight; TDW, total dry weight; Cd-R, cadmium in root; No-L, number of leaves; L-A, leaf area; Chl-A, chlorophyll a content; Chl-B, chlorophyll b; carot, carotenoid content; TWC, total water content.

Our results show that the toxic effects of Cd on all morphophysiological attributes were reduced by combinations of treatments, especially a combination of *R. leguminosarum*, *V. radiata*, and biochar (treatment T6). This combination gives us a high percentage of yield, biomass, growth, pigmentations, and root traits at both time points (see Figures 1, 2). Moreover, our results showed that treatment T6 (i.e., a combination of RL, VR, and BC) decreased the uptake of Cd in the roots and leaves of the mustard plant (see Figures 5–7). This might be due to the restriction of Cd translocation (by the combination of RL, VR, and BC) and a decrease in the concentration of free Cd ions in the rhizosphere (Seth et al., 2008; Younis et al., 2016; Tang et al., 2020).

5 Conclusion

From the current study, it can be concluded that mustard plants readily absorb Cd through their roots and that CD is subsequently translocated to the aerial parts of the plant, where it accumulates. Cd prevents and reduces the quality of other mineral nutrients in the root tissue, which in turn causes disturbance in different processes within the plant. Accumulation of Cd in the leaves disturbs plant photosynthesis activity by reducing the quantity of chlorophyll a, chlorophyll b, and carotenoids, thus causing a reduction in the value of soil plant analysis development (SPAD) and photosynthesis activity. In addition, in the positive control treatment, SEM images show Cd toxicity, closed stomata, disturbed spongy mesophyll cells in the leaves, thinning and breakage in the root cell compartments, and an excessive number of pores on the root hairs. The closing of stomata in the presence of Cd restricted water translocation and CO_2 uptake from the environment, and t disrupted the respiration and photosynthesis mechanisms of the plants. These findings prove that combinations of *V. radiata, R. leguminosarum*, and biochar, as in treatment T6 (BC + RL + VR), reduce the negative impact of Cd toxicity in plants, leading to increased growth, weight, photosynthesis pigment contents, yield quality, yield quantity, and water content, while reducing the translocation of Cd. Moreover, EDX confirmed that the T6 treatment (BC + RL + VR) reduced Cd translocation compared with the positive control (PC). The T6 treatment (BC + RL + VR) significantly prevented Cd accumulation in the plant's root zone, reducing the further uptake of Cd ions and enhancing nutrient uptake. Furthermore, the present study identified that the T6 treatment combination alleviated Cd toxicity at an advanced stage of growth and improved root structure, nutrient mobility, photosynthesis, and growth. The T6 treatment (BC + RL + VR) reduced Cd toxicity at both time points, particularly at the 60 days maturity stage. We can conclude that treatment T6 (BC + RL + VR) influences plant growth temporarily during the early stages of plant growth (i.e., at 30 days), but that the response is permanent at the maturity stage. Plant morphophysiological attributes were enhanced by the T6 treatment, by up to 70%–95%. The T6 treatment resulted in a growth rate equivalent to that of the negative control (NC). Thus, the T6 treatment has been found to be the most effective combination to mitigate the effects of Cd contamination and should be used in the future. However, in future work, different intercropping plants, biochar products, and beneficial microbe combinations could be tested. In addition, long-term field studies should be conducted, and a parallel analysis of plants, root structures, and nutrient mobility under different HM stresses carried out, to gain further insights into the mechanisms underlying of the effects of these treatment combinations.

Data availability statement

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

Author contributions

RAm and TS designed the project and scheme of work. Q-u-AH, MM, RAz, and RAm conducted the research. Q-u-AH collected and analyzed the data. Q-u-AH wrote the manuscript draft for submission. RAm, FM, AG, and AH reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The original polyethylene microplastics inhibit the growth of sweet potatoes and increase the safety risk of cadmium

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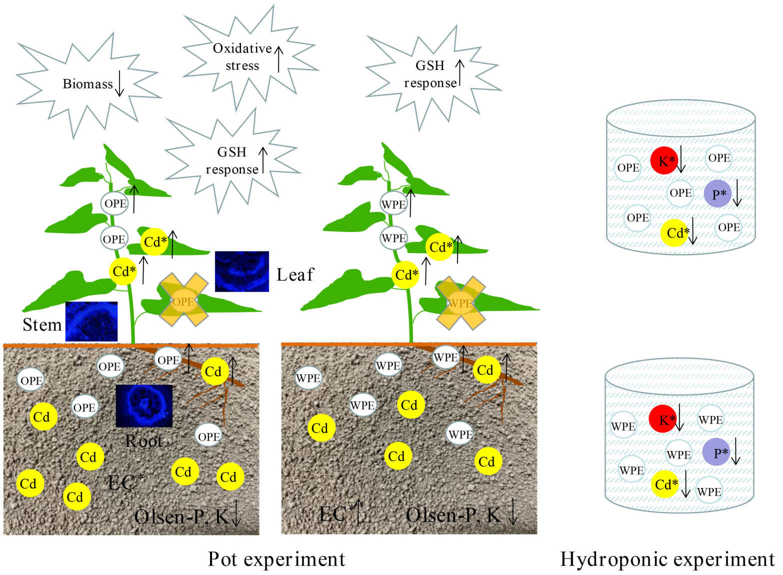
Microplastics (MPs) and heavy metals (HMs) co-exist in sweet potato fields of China. As the main component of agricultural field mulch and one of the most polluting and harmful HMs, the effects of polyethylene microplastics (PE MPs) and cadmium (Cd) on sweet potato and soil environment are remains unclear. Here, pot and hydroponic experiments are used to explore the effects of original and weathered PE MPs on growth and Cd uptake of sweet potatoes. The results of pot experiments reveal that compared with the control (0%), 5% of weathered PE MPs can significantly increase soil electrical conductivity (EC); both 5% of the original PE MPs and weathered PE MPs can significantly reduce the concentration of Olsen phosphorus (P) and Olsen potassium (K) in soil, inhibit plant growth, but significantly increase Cd accumulation and glutathione (GSH) level in tissues of sweet potatoes, and also induce membrane lipid peroxidation. In addition, compared to 5% weathered PE MPs, 5% original PE MPs significantly reduce soil EC, growth and peroxidase level of sweet potatoes, but significantly increase Cd concentration in leaves and stems. The results of hydroponic experiment show that original PE MPs significantly increase the P, K, and Cd adsorption compared with weathered PE MPs, and Cd increases the original PE MPs accumulation in the root cortex but decrease PE MPs accumulation in shoots. To sum up, our study investigates the differences and reasons of the effects of original and weathered PE MPs on growth and Cd absorption of sweet potatoes.

KEYWORDS

cadmium, polyethylene microplastics, root cortex, sweet potato, weathered



GRAPHICAL ABSTRACT
OPE: Original polyethylene (PE) microplastic; WPE: Weathered PE microplastic; K: Potassium; P: Phosphorus; Cd: Cadmium; EC: Electrical conductivity. ↑: The values in original or weathered PE treatment are significant higher than that of control (0 % PE). ↓: The values in original or weathered PE treatment are significant lower than that of control.

1 Introduction

The pollution of microplastics (MPs) in terrestrial environments has received increasing attention worldwide (Rillig et al., 2019). Being small in size with surface area, some MPs have negative effects on germination, shoot length and biomass of plants, reducing earthworm biomass and changing soil physical and chemical properties, such as by causing soil moisture loss and reducing soil porosity (Boots et al., 2019; Mbachu et al., 2021). In addition, MPs can be weathered in nature under the influence of several factors. During the weathering process, the particles are decreasing in size, surfaces are gradually becoming rougher, and various function groups are produced (Jahnke et al., 2017; Ding et al., 2020). As little is currently known regarding the changes in toxicity of MPs in soil over time, it is important to explore the impact of weathered MPs on plants and also the soil environment.

Cadmium (Cd) is one of the most toxic heavy metals (HMs) and it has been a major contaminant in agricultural soils. In China, approximately 1.3 $\times 10^4$ ha of cultivated soil is contaminated by Cd and 5.0×10^4 t of Cd-contaminated rice is produced every year (Zou et al., 2021). Cd could cause various health hazards, such as nephrotoxicity, osteoporosis, neurotoxicity, carcinogenesis, genotoxicity, and teratogenicity. For non-smokers, 90% of Cd exposure occurs *via* dietary intake (Tóth et al., 2016). In plants, Cd toxicity inhibit the carbon fixation and photosynthetic activity. Cd in soil can also lead to osmotic stress by reducing the relative water content, stomatal conductance, and transpiration of leaves. Over-production of reactive oxygen species (ROS) can be induced by Cd and cause damage to organelles. Cd interferes with the transportation of mineral elements (P, Mg, K, Mn) (Nazar et al., 2012; Haider et al., 2021). Therefore, the problem of food safety risks caused by the accumulation of Cd in crop cannot be ignored.

MPs can also adsorb several pollutants (Jacky et al., 2021), and serve as a vectors of these pollutants to organisms (Barnes et al., 2009; Andrew et al., 2020; Merga et al., 2020; Aghilinasrollahabadi et al., 2021). Considerable efforts have been devoted to investigate the interaction between metal and MPs (Oz et al., 2019). The types of metallic elements that can be adsorbed by MPs include HMs, such as Cd, as well as some beneficial elements, such as Zinc (Zn), allowing improved bioavailability of these beneficial elements to plants (Hodson et al., 2017). For example, the addition of polyester microfibers did not significantly change the level of Cd in leaves of lettuce (Zeb et al., 2022). Wang et al. (2020) found that polylactic acid caused higher Cd bioavailability than polyethylene microplastics (PE MPs), but no alterations in plant Cd content. However, in general, the adsorption of HMs to MPs requires continuous attention to prevent the accumulation of MPs, and harm from other pollutants can be further aggravated by adsorption to MPs (Aghilinasrollahabadi et al., 2021). In addition, the adsorption mechanism of different pollutants to MPs and influencing mechanism for the adsorption and desorption of HMs in agricultural soils are still unclear (Guo and Wang, 2019).

Sweet potato as an important food crop worldwide ranks third in production and fifth in calorifc contribution to human diet among all crops globally (Wang et al., 2021). China is the biggest cultivator country of sweet potato in the world, and the sweet potato production area in China about 6.6 million ha, accounted for 70% of total area under sweet potato cultivation of world. In addition, the output of sweet potato in China, about 100 million metric tons, accounted for 84.4% of total world output (Gao et al., 2000; Wang et al., 2021). Because sweet potato is also the main source of starch, it can be used as a staple food and can be an important raw material for alcohol production. Therefore, relevant food safety issues cannot be ignored (Bovell-Benjamin, 2007; Ma et al., 2012; Xie et al., 2018).

Plastic films are used in approximately 20 million hectares of farmland all over the world, and the largest proportion (~90%) are found in China (Steinmetz et al., 2016). The plastic films can be degraded or weathered into micro-sized particles after been left in fields (Astner et al., 2019). Therefore, long-term accumulation of MPs in farmland may pose a considerable threat to plant, animal growth and food security (Ramos et al., 2015). However, there are no reports about the effects of PE (the main component of the field film) MPs on sweet potato growth. In addition, Cd is one of the most seriously polluted HMs in soil of China, and nearly 33.54% and 44.65% of sites in farmland and urban soils were polluted with Cd (Yuan et al., 2021). Indeed, some edible-types sweet potato cultivars can accumulate more Cd and lead (Pb) than starch-type cultivars in flesh (Huang et al., 2020a). For instance, sweet potato with larger underground biomass shows high accumulation of Uranium (1.68-5.16 mg kg^{-1}) and Cd (0.78-2.02 mg kg^{-1}), and would pose a health risk if consumed (Lai et al., 2020). Therefore, in order to solve the problem that PE MPs may increase the consumption risk of Cd through sweet potato ingestion, the objectives of this study is (1) whether PE MPs can increase the Cd accumulation in sweet potato and cause more severe damage in plants; (2) the differences in the effects of original PE MPs and weathered PE MPs on sweet potato and soil properties. According to the above research objectives, the detailed research contents including: (1) the effect of PE MPs (original and weathered PE MPs) on physicochemical properties of Cd contaminated soil; (2) the effect of PE MPs (original and weathered PE MPs) on the growth and the absorption characteristics of sweet potato to Cd; and (3) reveal the effect of original PE MPs on the uptake and distribution of PE MPs in tissues of sweet potato. Therefore, (1) the micron particles of original and weathered PE MPs (the main component of plastic film in field) are used in pot and hydroponic experiments to simulate the effect of PE MP particles (which transformed from plastic film) on sweet potato growth and soil environment; (2) inductively coupled plasma-optical emission spectroscopy (ICP-OES) and cryo-scanning microscope are used to determinate Cd concentration and explore PE distribution in leaves, stems and roots of sweet potatoes respectively.

2 Materials and methods

2.1 Effect of PE MPs and Cd on physiological indexes and Cd absorption of sweet potato (pot experiment)

2.1.1 Original and weathered PE MPs

In this experiment, polyethylene (PE), the main component of agricultural mulch film and also the main MPs pollutant in farmland, was selected as the basic material of the MPs. Two types of PE MPs were used: (1) PE MPs powder (fragments with an average diameter of 5 μm and purity is 99.5%); (2) monodisperse blue fluorescent PE MPs (Spherical with an average diameter of 5 μm and purity is 99.7%), which purchased from Shaanxi Xinyan Bomei Biotechnology Co., Ltd. For monodisperse blue fluorescent PE MPs, the maximum excitation wavelength is 400 nm, and the maximum emission wavelength is 450 nm.

Referring to the aging method of MPs used in our previous study (Shi et al., 2022), after oxidizing the 5 μm MPs with Folin phenol weathering agent (Folin phenol weathering agent formula: 15 mL of 200 mM $FeCl_2$ solution, add 970 mL of distilled water, mix well and adjust the pH to 4.0, then add 15 mL of 30% H_2O_2 solution) for 7 days, distilled water were added to repeatedly rinse the MPs. After the oxidant on the surface of MPs was rinsed, the MPs were dried and stored at 60 °C for the next step.

2.1.2 Experimental soil

Experimental soil was collected from a sweet potato field in Ezhou, Wuhan, China (114°3' E, 30°34' N). The abundance of MPs in the soil was 9,072 items/kg soil, the Cd concentration was 0.025 mg/kg, and the soil pH was 5.76. One hundred milliliter $CdCl_2$ mother liquor (441 mg/L) was added to 10 kg soil by spraying to make the final concentration of Cd^{2+} in the soil is 2.7 mg/kg.

According to the method of Fei et al. (2020), a total of five treatment soils were prepared: (1) control (Cd-contaminated soil without PE MPs); (2) Cd-contaminated soil with 1% (10 g/kg) original PE MPs; (3) Cd-contaminated soil with 5% (50 g/kg) original PE MPs; (4) Cd-contaminated soil with 1% (10 g/kg) weathered PE MPs; (5) Cd-contaminated soil with 5% (50 g/kg) weathered PE MPs. After been preparation, soil was dried and wet balanced for 1 month to carry out the pot experiment.

2.1.3 Sweet potato variety

The sweet potato variety (*Ipomoea batatas*) used in this experiment was 'Fucai 18' (purchased from the Jiangsu Academy of Agricultural Sciences, Nanjing, China). The stem tips (10 cm) of the fresh 'Fucai 18' were cut off, and the extra leaves were removed from the cut stem tips, leaving only 2-3 new leaves. Then, they were cultivated with distilled water, which was changed every day. After root development, the distilled water was changed to 1/4 Hoagland nutrient solution and the sweet potato was cultivated for 3 days, and finally changed to 1/2 Hoagland nutrient solution and further cultivated for 3 days. Subsequently, excess leaves were trimmed off and stem tips of the sweet potato with the same weight were selected and transferred in a pot (Diameter = 15 cm; Height = 20 cm), which filled with 500 g of soil. Three sweet potato seedlings were planted in each pot, and three repetitions were set for each treatment. The sweet potatoes were treated for 15 days.

2.1.4 Determination of soil physical and chemical properties

After 15 days of sweet potato planting, the soils in the pots were mixed well and air-dried for physical and chemical properties determination. The methods to determinate pH, Electrical conductivity, available phosphorus, and available potassium were referred to Bao (2000).

2.1.5 Determination of Cd in plants and soils

Referring to the method of Huang et al. (2021b), the sweet potatoes were gentle collected from the soil after been treated for 15 days. Damage to the root systems was avoided, and the soil on the surfaces of the roots was rinsed off repeatedly with distilled water, and the lower halves of the roots and stems were immersed in 20 mM EDTA-2Na solution for 30 min to remove Cd adsorbed on the surfaces of the sweet potatoes. Then, the remaining EDTA-2Na on the root surfaces was rinsed with distilled water. Finally, the roots, stems, and leaves of the samples were dried in an oven at 60°C to a constant weight.

A dried plant sample was accurately weighed at 0.2500 ± 0.0050 g and put in digestion tube, and 3 mL of nitric acid-perchloric acid (87:13, v/v) mixed acid was also added to the tube. After digestion in an electric digester, 10 mL of 2.5% nitric acid was added and then shaken for reconstitution. After being kept for overnight, the supernatant was passed through a 0.45 μm filter membrane, and the filtrate was stored at 4°C. Inductively coupled plasma-optical emission spectroscopy (ICP-OES, Optima 8000, Shanghai Perkin Elmer, Co., Ltd) was used to determine the Cd concentration in the stems, roots, and leaves of the sweet potatoes. For soil samples, 0.2000 ± 0.0050 g of the dried soil sample was accurately weighed and put in a digestion tube, and 10 mL of nitric acid-perchloric acid (4:1, v/v) mixed acid was added to the tube. After digestion in an electric digester, 10 mL of 2.5% nitric acid was added to the tube and samples were shaken for reconstitution. After overnight incubation, the supernatant solutions were passed through a 0.45 μm filter membrane, and the filtrate was stored at 4°C. ICP-OES (Optima 8000, Shanghai Perkin Elmer, Co., Ltd) was used to determine the Cd concentration in the soil.

2.1.6 Biomass of ' Fucai 18' sweet potato

The sweet potatoes were carefully removed from the soil to maintain the integrity of the roots after being treated for 15 days. Then, the soil on the surfaces of the roots, stems, and leaves was rinsed off with distilled water repeatedly. After being rinsed, the plant samples were dried in an oven at 60°C to a constant weight, and the dry weight of each tissue was measured and recorded by one-thousandth balance.

2.1.7 Antioxidant index determination

For enzyme extracts and assays (Li et al., 2013), the fresh leaves (0.1 g) were ground in liquid nitrogen, and then suspended in 0.9 mL solution containing 10 mM phosphate buffer (pH 7.4). The homogenate was centrifuged at 4°C, 2500 rpm for 10 min and the resulting supernatant was collected for determination of the activities of superoxide dismutase (SOD, A001-1-1), peroxidase (POD, A084-3-1), and glutathione (GSH, A006-1-1) using commercial assay kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

The activity of SOD was determined by measuring the inhibiting rate of the enzyme to $O_2^-\cdot$ produced by the xanthine morpholine with xanthine oxidase using the SOD assay kit. Each endpoint assay was detected the red substances of the reaction system by absorbance at 550 nm after 40 min of reaction time at 37°C. And one unit SOD activity was defined as the quantity of SOD required to produce 50% inhibition of reduction of nitrite in 1 mL reaction solution by measuring the change of absorbance at 550 nm.

The POD activity was measured based on the change of absorbance at 420 nm by catalyzing H_2O_2. One unit was defined as the amount of enzyme which was catalyzed and generated 1 μg substrate by 1.0 g fresh tissues in the reaction system at 37°C. POD activity was calculated as the formula according to POD assay kit.

The activity of GSH was measured based on the absorbance of reaction products between sulfhydryl compounds and 5,5'-Dithiobis (2-nitrobenzoic Acid) at 420 nm. GSH activity was calculated as the formula according to GSH assay kit.

Lipid peroxidation was determined as the amount of malondialdehyde (MDA) based on the spectrophotometer measurement of a red-complex produced during the reaction of thiobarbituric acid (TBA) with MDA. For MDA determination, fresh leaves (0.1 g) were ground in liquid nitrogen, and then suspended in 0.9 mL extracted solution containing 50 mM phosphate buffer (pH 7.4) and 1% polyvinylpyrrolidone (PVP). Then the homogenate was centrifuged at 4°C, 3500 rpm for 10 min to collect the supernatant, which was further processed following the instruments of assay kit (MDA, A003-1-1) purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Finally, the absorbance of red TBA-MDA complex was measured using a microplate reader (SpectraMax M5, USA) at 532 nm and the MDA content was calculated according to detailed instructions of the MDA assay kit.

2.2 Effect of PE MPs on P, K, Cd adsorption and PE MPs distribution in sweet potato (Hydroponic experiment)

2.2.1 Adsorption experiment

Referring to the concentration of Cd in experimental soil, it was necessary to add available P and K to the soil, preparing a mixed solution. $CdCl_2$ was used to prepare the solution and the final Cd concentration was 2.70 mg/L. First, a 54.8 mg/L $CdCl_2$ solution was prepared and 50 mL of this solution was poured out and added an appropriate amounts of KCl and NaH_2PO_4 were added to adjust the pH to 5.76 ± 0.52, and the above solution was diluted to 500 mL. The concentrations of P and K in the solution were kept to about the original available P (600 mg/kg) and K (400 mg/kg) concentrations in the soil, respectively.

Referring to the method of Peng et al. (2019), five treatments were applied: (1) the original mixed solution; (2) 1% (10 g/L) original PE MPs mixed solution; (3) 5% (50 g/L) original PE MPs mixed solution; (4) 1% (10 g/L) weathered PE MPs mixed solution; (5) 5% (50 g/L) weathered PE MPs mixed solution. Each treatment was set three repetitions. After the mixed solutions were shaked at 150 r/min for 7 days in a shaker at 25°C, the solution was filtered with a 0.45 μm filter membrane and the P, K, and Cd concentrations in the filtrate were determined by ICP-OES (Optima 8000, Shanghai Perkin Elmer, Co., Ltd).

2.2.2 Fluorescent PE MPs distribution in sweet potato tissues

Referring to the method of Li et al. (2019), the stem tips (10 cm) of the fresh 'Fucai 18' were cut off, and the extra leaves were removed from the cut stem tips, leaving only 2-3 new leaves. Then, they were cultivated with distilled water, which was changed every day. After root development, the sweet potato seedlings were transferred in 198 mL of 1/4 Hoagland nutrient solution (including 5 mg/L Cd) and mixed with 2 mL of 1/4 Hoagland nutrient solution (0, 2 or 6 mg of 5 μm-PE blue fluorescent MPs suspension) for 7 days. Roots, stems, and leaves were washed repeatedly with distilled water and respectively cut and embedded in cryogen. Then, 100 μm-thick slices with cryostat were placed on a glass slide and covered with a glass lid. The excitation and emission wavelengths of the biological fluorescence microscope (Axiovert 40 CFL, Zeiss, Shanghai, China) were adjusted the to 400 and 450 nm, respectively, to observe the fluorescence in the roots, stems, and leaves. After that, the samples were stored at 4°C.

For PE MPs distribution in the root, stem, and leaf sections, the pretreating methods were the are same as those used by Li et al. (2019), and the fresh root, stem, and leaf samples were rinsed repeatedly with distilled water and frozen in liquid nitrogen. A cryo-scanning microscope (SU8010, Hitachi, Shanghai, China) was then used to observe the presence of MPs in the tissues.

2.3 Statistical analysis

The data were processed using Microsoft Excel 2010, and the figures were drawn by GraphPad prism software 8.0. One- and two-way ANOVA was conducted by SPSS software 21.0. The Tukey's test was used to explain the significant differences between various treatments at $p < 0.05$.

3 Results

3.1 Effect of PE MPs on the physicochemical properties of Cd-contaminated soil

Figure 1 shows that, compared with the control group (0% PE MPs), different concentrations of original or weathered MPs only slightly affected the pH and EC of the Cd-contaminated soil, except for 5% weathered PE MPs significantly increasing EC compared with original PE MPs. As shown in Figures 1C, D, 1% original or weathered PE MPs did not significantly affect the concentrations of available P and K in soil compared with that of the control group (0% PE MPs), but 5% of original or weathered PE MPs significantly decreased available P and K. Compared with the control group, 5% of original PE MPs reduced the concentration of available P and K by 5.14% and 6.17%, respectively, and 5% of weathered PE MPs reduced the concentration of available P and K by 5.76% and 5.35%, respectively.

3.2 Effect of PE MPs on Cd accumulation in plants

After the sweet potato seedlings were treated for 15 days, the Cd concentration in stems and roots exceeded the threshold of leafy vegetable safety standard (0.2 mg/kg, GB 2762-2017). In addition, compared with weathered PE MPs, original PE MPs significantly increased the Cd accumulation in stems. For leaves, the Cd concentration in control (0% PE MPs) leaves were lower than the safety standard, but 5% original or weathered PE MPs significantly increased Cd concentration in leaves, and exceeded the threshold of leafy vegetable safety standard. Furthermore, compared to the weathered PE MPs, the original PE MPs can bring higher food safety risks (Figure 2).

3.3 Effect of PE MPs on P, K, and Cd adsorption in solution

Figure 3 shows that all PE MPs treatments significantly decreased the P, K, and Cd concentration in solution compared with control group (0% PE MPs), and with the increase in original PE MPs concentrations, the Cd adsorbtion ability of PE MPs significantly increased (Figure 3C). However, the adsorption of P, K, and Cd by weathered PE MPs between 1% and 5% treatments was not significantly different. Compared with weathered PE MPs, the original PE MPs at the concentration of 1% and 5% significantly reduced the K and P, Cd concentration in solution respectively. Therefore, compared with weathered PE MPs, the original PE MPs had the stronger ability to adsorb Cd.

3.4 Effect of PE MPs on growth and oxidative damage of sweet potato planting in Cd contaminated soil

As shown in Figure 4 and Table 1, when the concentrations of original and weathered PE MPs increased, the dry weight of sweet potato decreased. Overall, 5% original PE MPs significantly reduced the dry weight of sweet potato, and 1% original PE MPs also reduced the biomass of sweet potato by 12.18% compared with control (0% PE MPs). However, 1% and 5% weathered PE MPs reduced the dry weight of sweet potato by 7.36% and 11.68%, respectively, but did not show significant differences compared with the control (0% PE MPs).

Figure 5 shows that compared with the control group (0% PE MPs), 1% original PE MPs significantly increased the concentration of SOD, POD, and GSH enzymes, but did not significantly affect the MDA concentration. However, 5% original PE MPs significantly increased the MDA concentration. Weathered PE MPs did not significantly affect the concentrations of SOD and

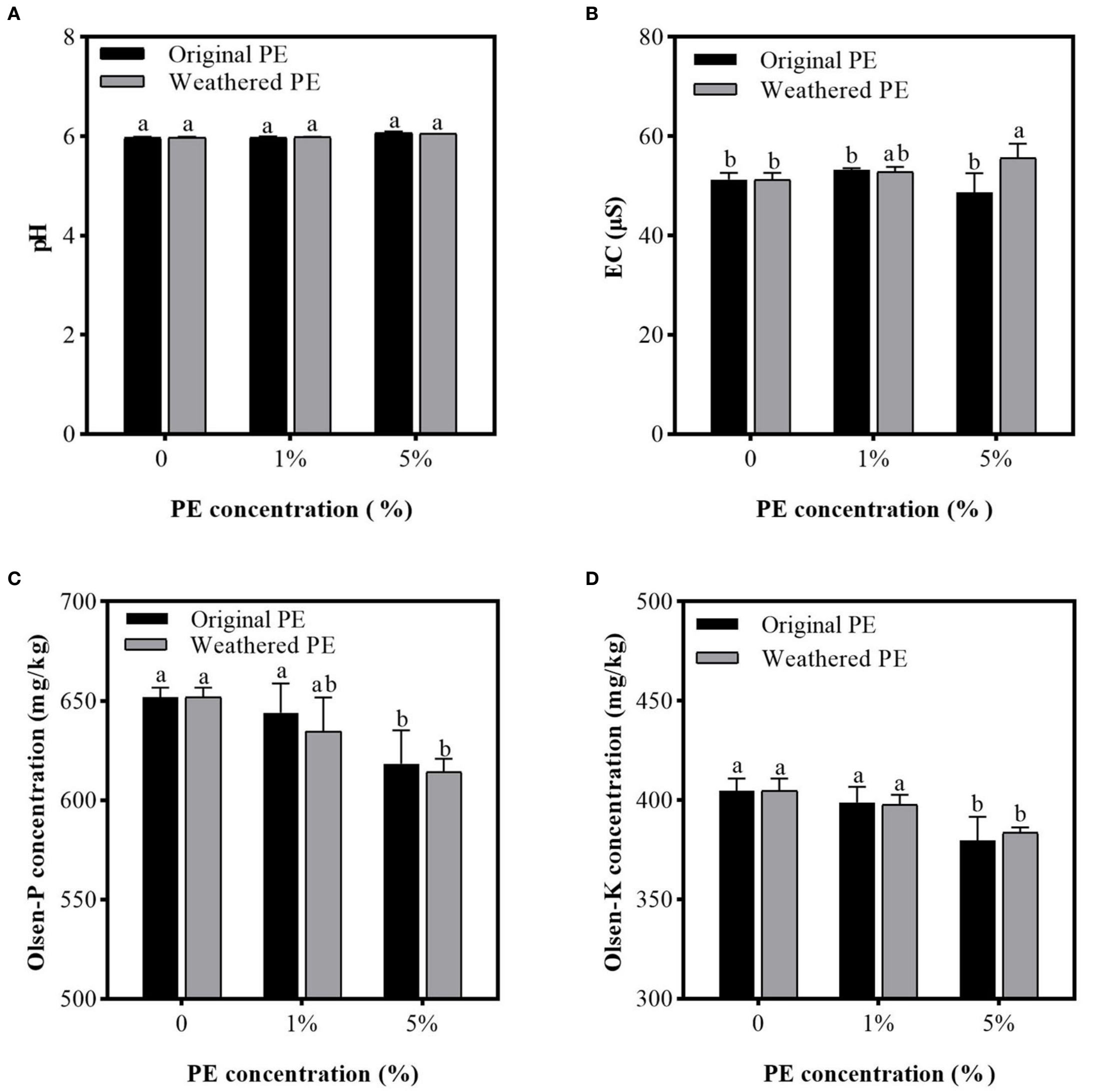


FIGURE 1
pH **(A)**, EC **(B)**, Olsen-P **(C)**, and olsen-K concentration **(D)** in PE MPs and Cd compound-contaminated soil. Soil samples are collected after sweet potatoes are treated by original or weathered PE MPs (0, 1% and 5%) for 15 days. Different letters in each figure indicate significant ($p < 0.05$) differences according to Tukey's test.

MDA, but 5% weathered PE MPs significantly increased the concentrations of POD and GSH compared with control group (0% PE MPs).

3.5 Distribution of PE MPs in the roots, stems, and leaves of sweet potato

Figure 6 shows that there no PE MPs were found in the leaves of sweet potatoes under the five different treatments (Figures 6A–M). Compared with the control (0% PE MPs), higher PE MPs concentration treatment resulted in higher PE MPs accumulation in the roots, and Cd increased the PE MPs accumulation in the cortex of the root (Figures 6B–N). In addition, we did not find any significant differences in PE MPs accumulation in the shoots of sweet potatoes between 1% and 3% treatments (Figures 6F–O). However, Cd significantly reduced PE MPs accumulation in the shoots (Figures 6F–O).

4 Discussion

Despite the fact that MPs like High-density polyethylene (HDPE) have the power to decrease soil pH through the cation/proton exchange in soil (Boots et al., 2019), the soil buffering capacity and plant root exudates are able to mediate soil pH collectively. Therefore, this is the possible explanation for the no difference in soil among different treatments. Compared with 5% original PE MPs treatment, the reason for the increase in EC caused by 5% weathered PE MPs treatment may be the lower leaf transpiration rate and higher soil bulk density (Lian et al., 2021).

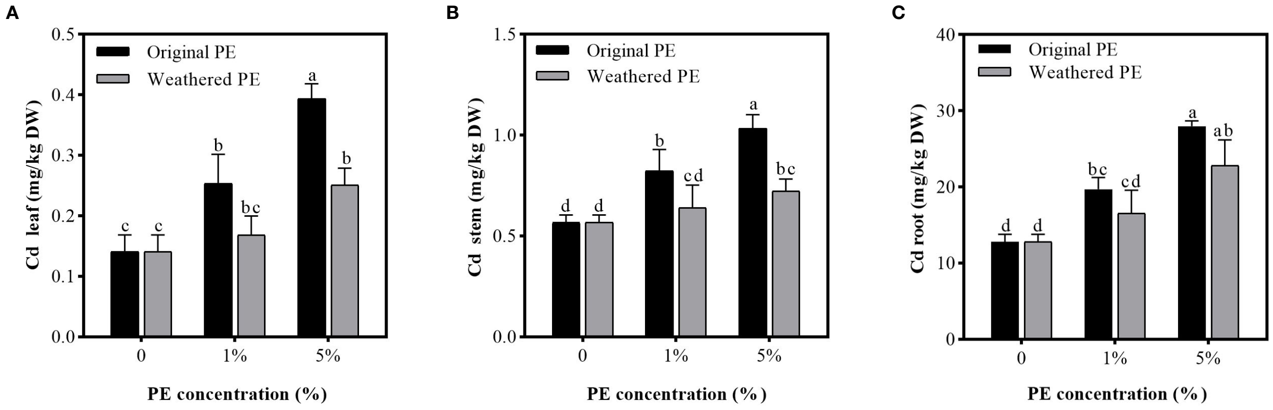


FIGURE 2
Cd concentration in the leaf **(A)**, stem **(B)**, and root **(C)** of sweet potato planting in PE MPs and Cd compound-contaminated soil. Plant samples are collected after been treated by original or weathered PE MPs (0, 1% and 5%) for 15 days. Different letters in each figure indicate significant ($p < 0.05$) differences according to Tukey's test.

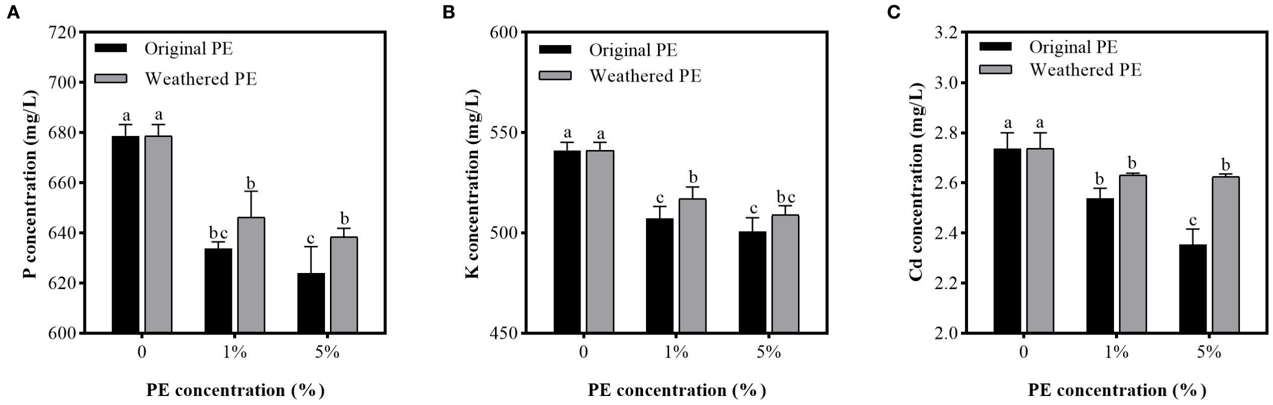


FIGURE 3
P **(A)**, K **(B)** and Cd **(C)** concentrations in PE MPs treated solutions. Solution samples are collected after been treated by original or weathered PE MPs (0, 1% and 5%) for 7 days. Different letters in each figure indicate significant ($p < 0.05$) differences according to Tukey's test.

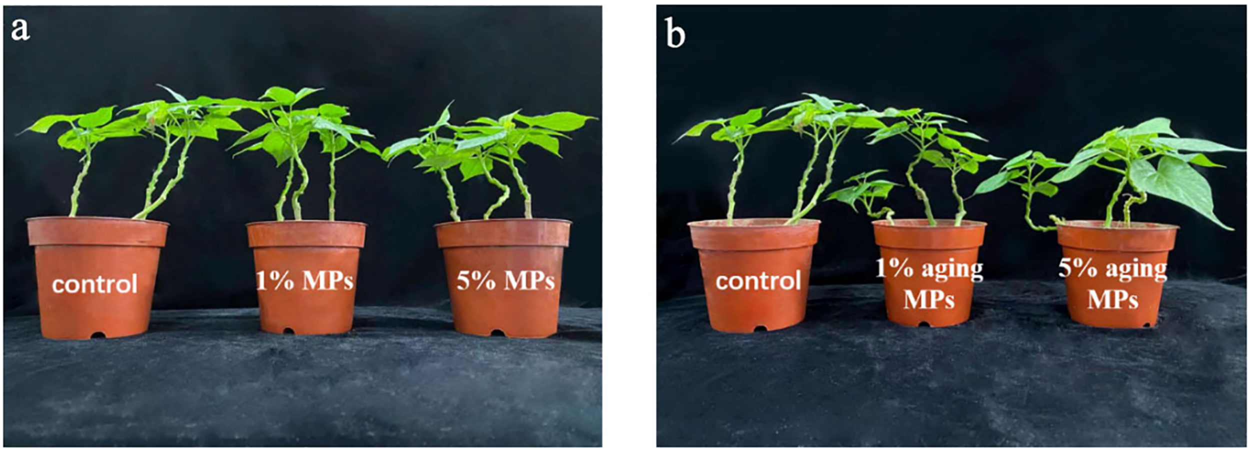


FIGURE 4
Growth of sweet potatoes planting in PE MPs and Cd compound-contaminated soil. Plant samples are collected after been treated by original **(A)** or weathered **(B)** PE MPs (0, 1% and 5%) for 15 days. Different letters in each figure indicate significant ($p < 0.05$) differences according to Tukey's test.

TABLE 1 Biomass of original or weathered PE treated sweet potato planting in Cd contaminated soil.

Style	PE concentration (%)	Dry weight (g)	Dry weight proportion (%)
Control	0	1.313 ± 0.04 ^{a}	100.0% ^{a}
Original PE	1% 5%	1.153 ± 0.05 ^{ab} 1.070 ± 0.13 ^{b}	87.82% ± 0.04 ^{bc} 81.47% ± 0.10 ^{c}
Weathered PE	1% 5%	1.217 ± 0.09 ^{ab} 1.160 ± 0.05 ^{ab}	92.64% ± 0.07 ^{ab} 88.32% ± 0.04 ^{bc}

Different letters in the same column represent significant differences.

MPs that accumulate gradually on the ocean surface or land can be absorbed by plants (Li et al., 2019) or consumed by animals (Zhou et al., 2020a). However, the greater threat of MPs is that they can act as carriers, accumulating and combining toxic chemicals (HMs and organic pollutants) to cause a range of damage to organisms through the food chain (Angelo and Andrew, 2017; Wang et al., 2019; Huang et al., 2021a). Therefore, the co-existence of PE MPs and Cd may increase the food safety risk of sweet potato. The reason why the original PE MPs have stronger adsorption capacity for Cd compared to weathered PE MPs was that original MPs may form various groups on the surface after been weathered, and the static electricity carried by themselves were neutralized, thus, reducing the adsorption capacity (Zhou et al., 2020b). In MP and Cd combined pollution, when the Cd concentration was

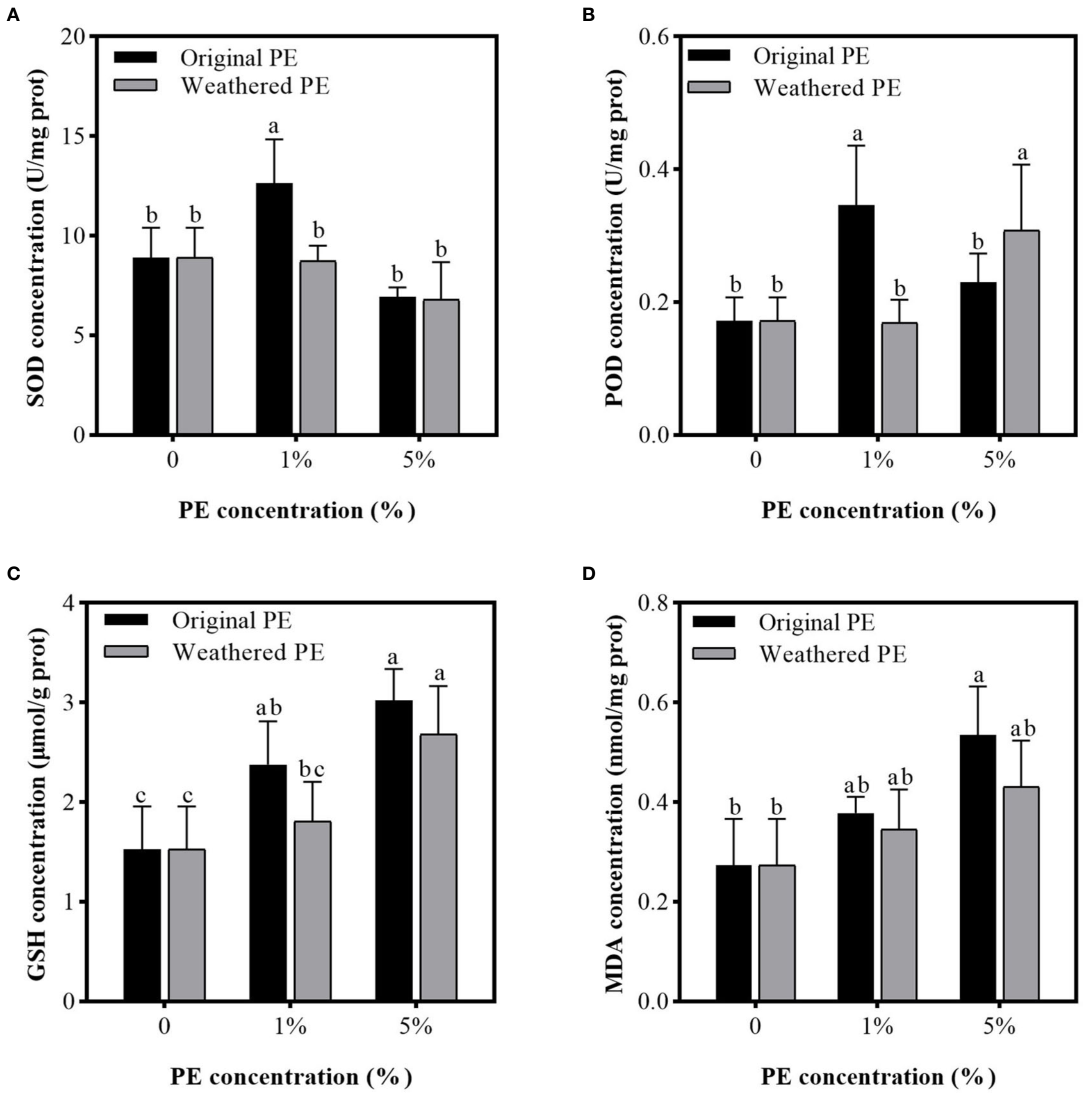


FIGURE 5
SOD **(A)**, POD **(B)**, GSH **(C)**, and MDA **(D)** concentrations in the leaves of sweet potatoes planting in PE MPs and Cd compound-contaminated soil. Plant samples are collected after been treated by original or weathered PE MPs (0, 1% and 5%) for 15 days. Different letters in each figure indicate significant ($p < 0.05$) differences according to Tukey's test.

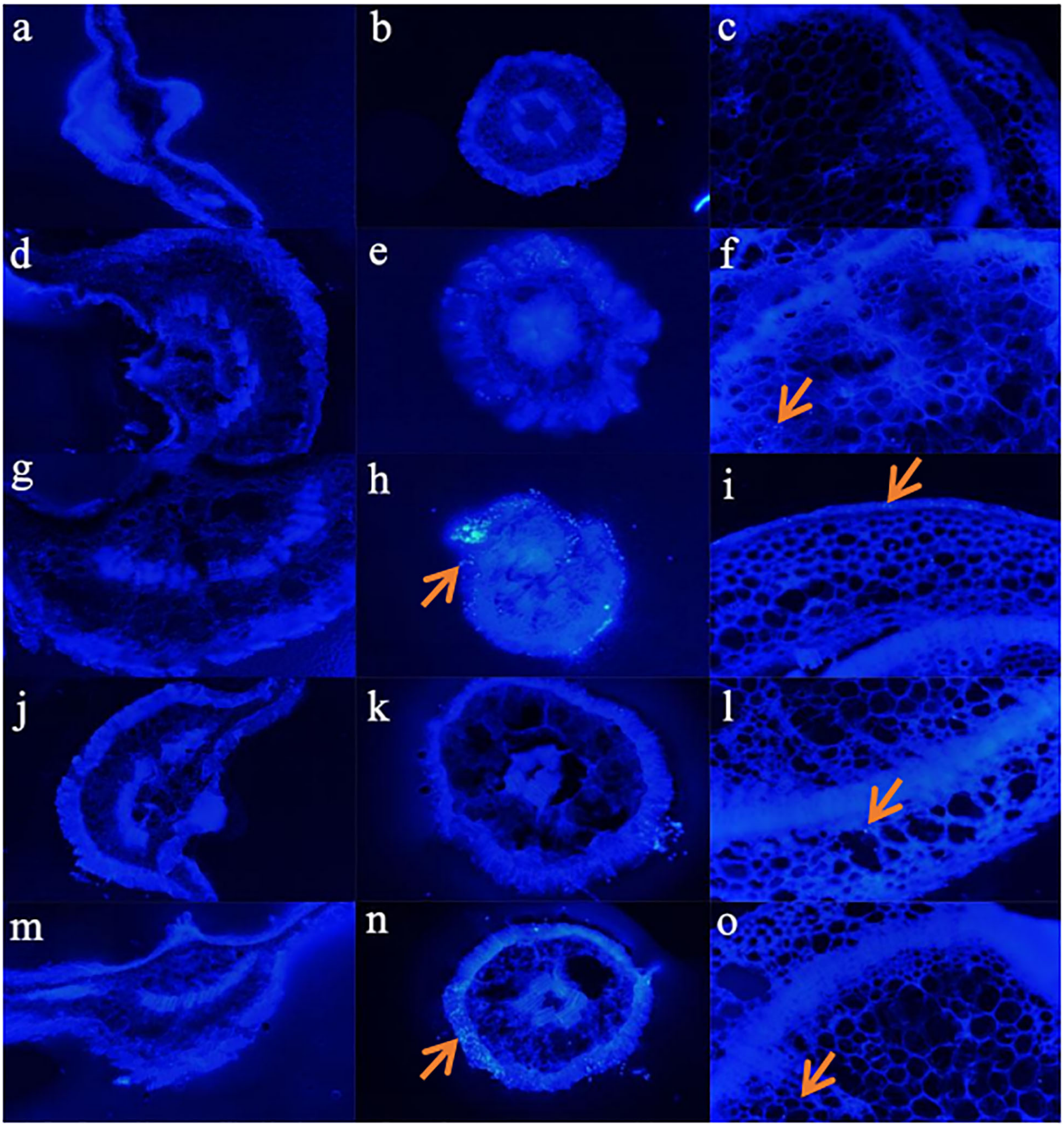


FIGURE 6
Distribution of PE fluorescent MP spheres in transverse sections of leaves **(A)**: Control, **(D)**: 1% PE MPs, **(G)**: 3% PE MPs, **(J)**: 1% PE MPs + Cd, **(M)**: 3% PE MPs + Cd), roots **(B)**: Control, **(E)**: 1% PE MPs, **(H)**: 3% PE MPs, **(K)**: 1% PE MPs + Cd, **(N)**: 3% PE MPs + Cd) and stems **(C)**: Control, **(F)**: 1% PE MPs, **(I)**: 3% PE MPs, **(L)**: 1% PE MPs + Cd, **(O)**: 3% PE MPs + Cd) of sweet potatoes. Sweet potatoes are treated with 5 μm of monodisperse blue fluorescent PE MPs and Cd for 7 days and are checked using a biological fluorescence microscope. The positions of the PE MPs are marked with red arrows.

constant, the increasing concentration of MPs can lead to more Cd are adsorbed by MPs and greater the damage to organisms (Zhang et al., 2020). These can explain our results in soil and hydroponic adsorbtion experiments that when the concentration of PE MPs was 5%, the available P and K concentration in soil and solution were the lowest; the Cd concentration in all organs of sweet potato was highest, the biomass of the sweet potato decreased to the maximum extent; and the degree of oxidative damage was also at the highest level among all of treatments (Table 1, Figures 2–5). In addition, because original PE MPs can adsorb more Cd and make plants more poisonous, which lead to the higher membrane lipid peroxidation damage compared with weathered PE MPs (Figure 5D). However, there were no significant differences between the effects of original and weathered PE MPs on MDA concentration in plants, may be due to the stronger ability of P and K adsorption by original PE MPs compared with weathered PE MPs (Figures 3A, B), through which providing more nutrient for plants and increasing their tolerance to Cd.

Ali et al. (2013) found that Cd increased the level of MDA, H_2O_2 and Cd accumulation in the shoots of oilseed rape (*Brassica napus* L.). In addition, plants have evolved antioxidative enzyme defense system consisting of SOD, POD, catalase (CAT), ascorbate peroxidase (APX), etc (Najeeb et al., 2011), which can reduce the MDA production (Najeeb et al., 2011; Xu et al., 2016). In our study, compared with the control (0%), although the activities of SOD, POD and GSH increased significantly under the treatment of the original PE MPs at a concentration of 1%, the MDA level did not significantly reduced compared with control. It may be due to the low Cd accumulation in leaves which was induced by addition of 1% PE MPs (Figure 2A). However, the high Cd accumulation in leaves was induced by addition of 5% PE MPs (Figure 2A), and the oxidative stress in plants led to a significant increase in MDA level (Figure 5D). However, due to the excessive Cd accumulation, the activities of SOD and POD under 5% original PE MPs treatments were significantly inhibited compared under 1% original PE MPs group (Figures 5A, B). Besides, we found that POD activity in 5% weathered PE MPs treatments were significantly higher than that of 1% weathered PE MPs treatments. It was due to the lower Cd accumulation in weathered PE MPs group compared with original PE MPs group (Figure 2). In addition, the POD activity in the tissues under the 5% weathered PE MPs treatment was significantly higher than that of the 5% original PE MPs and control treatments (Figure 5B). This may be another reason why plants were subjected to lower oxidative stress under 5% weathered PE MPs treatment than under 5% original PE MPs treatment. However, the GSH concentration were positively correlated with the concentration of original or weathered PE MPs, means it involved in Cd induced oxidative stress. However, since the original PE MPs and weathered PE MPs have no significant difference in the effects of GSH concentration in plant tissues, GSH may not be the main factor affecting the differences in plant growth and oxidative stress between the original and weathered PE MPs treatments. Obviously, the higher concentration of original or weathered PE MPs application, the greater the damage of Cd to the sweet potato. However, there were no paper has reported about the effect of HMs on MP accumulation in plant tissues.

In our study, Cd increased the PE MPs accumulation in the cortex of roots (Figures 6E–N), and reduced PE MPs accumulation in shoots (Figures 6F–O). This may be because, in extreme environments or under nutrient-deficient conditions, the transporters of nutrients with poor selectivity than those that operate at the normal nutrient conditions, and when transporting nutrients, they can also transport HMs such as Cd into the plant (Thomas, 2021). As a result, in extreme environments more nutrient elements and Cd adsorbed on the MPs and more MPs were absorbed into the roots. Plants can tolerate HMs at low levels by sequestering it safely into vacuoles in their cells. It is an important mechanism for plants to detoxify these toxic elements, and it is also a key step to limit their transport to the edible parts of crops (Zhao et al., 2022). Therefore, although the accumulation of Cd in roots, stems and leaves of sweet potato under the co-treatment of Cd and PE MPs was higher than that under the single treatment of Cd, the transport factor of Cd was reduced, thus reducing the accumulation of PE MPs in the shoot (Figure 6). On the other hand, this may be because, the combined effect of HMs and MPs tends to change from an additive effect in root to an antagonistic effect in shoot (Yuan et al., 2000). In addition, the coexistence of Cd and PE MPs aggravated oxidative stress and inhibited root activity, and also reduce the transport factor of Cd and MP, affecting the absorption of MPs by plants (Kim et al., 2019; Dong et al., 2020; Wang et al., 2020).

5 Conclusion

The 5% of weathered PE MPs can significantly increase soil EC compared with control (0% PE MPs) and 5% of original PE MPs. The original PE MPs can significantly adsorb more Cd from solution, and increase Cd accumulation in sweet potato tissues, which lead to higher membrane lipid peroxidation damage in plants and lower biomass compared with weathered PE MPs treatment. No PE MPs were found in the leaves, and higher concentration of PE MPs treatment make higher PE MPs accumulation in roots and stems. Cd increases the PE MPs accumulation in cortex of roots but reduce PE MPs accumulation in shoots. The coexistence of original PE MPs and Cd poses a greater edible safety risk of sweet potato.

Data availability statement

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

Author contributions

LS conceived the project, involved in planting of sweet potato and wrote paper. ZC involved in planting of sweet potato and analyzed the data. YH and JL carried out oxygen measurements and enzymatic assays. ZS wrote, reviewed and edited the paper. YC is responsible for conceptualization, funding acquisition, review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Activation and tolerance of *Siegesbeckia Orientalis* L. rhizosphere to Cd stress

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This experiment investigated the changes of rhizosphere soil microenvironment for hyperaccumulation-soil system under Cd stress in order to reveal the mechanism of hyperaccumulation and tolerance. Thus, Cd fractions, chemical compositions, and biochemical characteristics in rhizosphere soil of *Siegesbeckia orientalis* L. under Cd stress conditions of 0, 5, 10, 25, 50, 100, and 150 mg kg-1 were investigated through a root bag experiment, respectively. As a result, Cd induced the acidification of *S. orientalis* rhizosphere soil, and promoted the accumulation of dissolved organic carbon (DOC) and readily oxidizable organic carbon (ROC), which increased by 28.39% and 6.98% at the maximum compared with control. The percentage of labile Cd (acid-soluble and reducible Cd) in soil solution increased significantly ($P < 0.05$) from 31.87% to 64.60% and from 26.00% to 34.49%, respectively. In addition, rhizosphere microenvironment can alleviate the inhibition of Cd on soil microorganisms and enzymes compare with bulk soils. Under medium and low concentrations of Cd, the rhizosphere soil microbial biomass carbon (MBC), basal respiration, ammonification and nitrification were significantly increased ($P < 0.05$), and the activities of key enzymes were not significantly inhibited. This suggests that pH reduction and organic carbon (DOC and ROC) accumulation increase the bioavailability of Cd and may have contributed to Cd accumulation in *S. orientalis*. Moreover, microorganisms and enzymes in rhizosphere soils can enhance *S. orientalis* tolerance to Cd, alleviating the nutrient imbalance and toxicity caused by Cd pollution. This study revealed the changes of physicochemical and biochemical properties of rhizosphere soil under Cd stress. Rhizosphere soil acidification and organic carbon accumulation are key factors promoting Cd activation, and microorganisms and enzymes are the responses of Cd tolerance.

KEYWORDS

Cd, rhizosphere, bioavailability, *Siegesbeckia orientalis*, phytoremediation, hyper accumulator, phytoremediation

1 Introduction

Cadmium (Cd) is one of the primary heavy metal (HM) pollutants in soils due to its toxicity and non-biodegradability (Lu et al., 2015; Niu et al., 2020). The concentration of Cd in arable soil in China is seriously disturbed by human. Regionally, the maximum Cd concentrations in arable soil around mining and smelting activities, irrigation area by wastewater, urban and suburban area, and remote areas was 152.95, 54.05, 3.15, 2.04 mg kg-1 respectively (Zhang et al., 2015b). Cd in the soil can be easily absorbed by plant roots and then enters the food chain, causing a threat to living creatures and human beings *via* biomagnification and amplification (Salt and Wagner, 1993; Xin et al., 2015). In the past decades, significant progress has been made in the remediation techniques on Cd-contaminated soils (Liu et al., 2021). Among them, phytoextraction technology based on HMs accumulators or hyperaccumulators has been widely used to remediate contaminated soils due to its cost-effective and eco-friendly characteristics (Huang et al., 2018; Moreira et al., 2019). These accumulators can effectively mobilize metals mainly by changing the bioavailability of HMs surrounding roots, enhancing the uptake, and translocation of HMs in plants (Bolan et al., 2011; Zhan et al., 2018). Therefore, the root-soil system is considered to be the key process for phytoextraction, and is increasingly attracting the attention of scholars (Antoniadis et al., 2017; Zhan et al., 2018; Li Y. et al., 2021).

The rhizosphere effect and soil chemical processes that are located within the root-soil interface directly affect the uptakes/exclusions of HMs by plants (Antoniadis et al., 2017). Researches have shown that the chemical conditions of rhizosphere soil were different from those of bulk soil. These chemical conditions can lead to variations of soil compositions, and bioavailability changes of HMs in soil (Chaignon et al., 2002). Solubility and bioavailability of trace metals in the root region and microbial exudates can be adjusted by dissolved organic carbon (DOC) and pH by Rhizosphere effect (Shen et al., 2017; De Conti et al., 2018). Rehman et al. (2017) and Yang et al. (2017) have proved that the decrease of soil pH through rhizosphere effect is the most important single process affecting the availability of HMs. Cd ions absorbed by soil colloids can be exchanged by H^+ leading to the increase of concentration of Cd^{2+} in root-soil system. In addition, some researches have indicated that rhizosphere effect is induced by metal complexations of DOC. However, rhizosphere effect is very complicated because different results can be obtained even though the conditions are the same. Therefore, it is necessary to further elucidate the mechanisms for activating soil HMs in different plants.

The decrease of pH causes more H+ to exchange with Cd adsorbed by soil colloid and increase the content of Cd2+ in root-soil system, thus increasing Cd mobility (Li Y, et al., 2021). It has been reported that the pH of hyperaccumulators grown in Cd contaminated soil has a lower rhizosphere soil pH than that grown in uncontaminated soil, which increases the uptake of Cd by plants (Gonzaga et al., 2009; Li et al., 2011). Acidification may be ascribed to the secretion of some organic matters including DOC induced by hyperaccumulators (Rehman et al., 2017). DOC, which DOC is a part of soil activated carbon component, which includes low molecular weight organic acids that activate HMs in soil by forming soluble complexes with them (Luo et al., 2017). Soil active organic carbon components are composed of oxidized carbon (ROC) and water-soluble organic carbon (WSOC). ROC is a very abundant active organic carbon component, including easily decomposed humus and polysaccharides (Jia et al., 2015). Humus contains a large number of carboxyl and phenolic hydroxyl acid groups (Bai et al., 2018). These functional groups can change the solution and sorption equilibrium of HMs in soil, and react with metal ions in soil forming soluble complexes. This phenomenon promotes the absorption of metal elements by plants (Ondrasek et al., 2018). WSOC has positive effects on soil microorganisms under HMs stress (Epelde et al., 2010), but there are few studies on the relationship between WSOC and HMs activation. Although rhizosphere acidification and increase of DOC content play an important role in HM activation and accumulations in plants (Lux et al., 2011; Li et al., 2014; Xin et al., 2017). However, how rhizosphere processes help hyperaccumulators tolerance and accumulation of HMs have not been fully explained, and whether ROC and WSOC affect the activation of HMs in rhizosphere is rarely reported.

Soil biochemical properties are ecologically relevant indicators of soil quality, and are often used to evaluate the ecological status of soil under HMs stress (Yang et al., 2017). The researches indicate that microorganisms and enzymes in Pakchoj, black locust, and other nonaccumulators are inhibited by Cd contaminations (Shentu et al., 2014; Xian et al., 2015; Huang et al., 2016; Zhou et al., 2017). However, a completely different phenomenon is now observed in hyperaccumulators. Liu et al. (2020) found that the microorganisms counts and microbial metabolic activity of the hyperaccumulator *Trifolium repens* increased with the increase of Cd supply; a similar phenomenon was also found in Yang's work (Yang et al., 2017; Niu et al., 2020). Liu et al. (2020) suggested that the relative abundance of plant growth promoting bacteria (*Kaistobacter* and *Flavisolibacter*) and the utilization of difficultly metabolized compounds in rhizosphere would increase under HM stress, which may help alleviate the damage of heavy metals on hyperaccumulators. Yang et al. (2017) found that the remediation process using *S. alfredii* favored Gram-negative bacteria growth more than the Gram-positive bacteria. Niu et al. (2020) under HM stress, rhizosphere soil CAT activity increased in Indian mustard and tall fescue, which accelerated the release of hydrogen peroxide and led to the increase of bacterial 16S rRNA abundance. Therefore, it is necessary to further study which microbial composition and enzyme activity indicators play a key role in the newly discovered hyperaccumulators or different species.

Siegesbeckia orientalis L. is promised as an ideal material plant for *in situ* restoring Cd contaminated soils due to its high biomass and Cd concentrating ability in the aboveground (Zhang et al., 2013). Although previous works have investigated roots actively responded to Cd stress and the regulatory mechanisms of HM detoxification(Xu et al., 2020), how to explain that Cd is activated by *S. orientalis* from the root-soil interface and rhizosphere microenvironment is tolerated by Cd stress is still a challenge.

This study hypothesized that HMs were activated by rhizosphere effect originated from change of soil physicochemical properties, while the increase of rhizosphere soil microbial activity and enzyme activity is to enhance *S. orientalis* tolerance to Cd. Therefore, it was assumed that the physicochemical properties in rhizosphere soil will change under the stress of Cd, thus affecting the effectiveness of Cd and the absorption of Cd by *S. orientalis*. Enzymatic activity and microbial activity are also enhanced to maintain normal plant physiological functions. Therefore, the aim of this study was to (1) assess the capacity of *S. orientalis* to uptake Cd from soil; (2) investigate the effects of physicochemical properties rhizosphere of soil on Cd activation; (3) determine the effects of Cd stress on enzyme activities and microbial characteristics of rhizosphere soil.

2 Material and methods

2.1 Experiment design

S. orientalis seeds were harvested from a Pb-Zn mine area in South-West of Chengdu, Sichuan, China (102°46′E, 26°40′N). Seeds were pre-germinated at a plate and kept moist for further treatment. After germination, the seedlings were placed in sandy soil uncontaminated with heavy metal and watered with 1/2 Hoagland solution. After 4 weeks of sand culture, seedling of equal height, health, and leaf number (5 to 6 leaves and a height of about 6 cm) were selected for further treatment study in Cd stressed soil and control study (Xu et al., 2018).

The experiment was carried out in the greenhouse of Sichuan Agricultural University, Chengdu (103°52′E, 30°43′N), with an average air temperature of 27°C during the day and 18°C at night, and humidity of 75-80%. The physicochemical properties of soil were shown in Table 1. Stones and plant residues were removed after air-dried grinding and passed through a nylon sieve with a particle size of 4 mm. Each pot (25 cm×20 cm) was filled with 6.0 kg of soil and 4.2 g of compound fertilizer (N:P2O: K2O=17:17:17) prepared before and was mixed with Cd in solution (prepared by dissolving analytical grade CdCl2·2.5H2O) at 0 (control), 5, 10, 25, 50, 100, and 150 mg kg-1, respectively (Figure 1). Cd was added to the soil in the form of CdCl2·2.5H2O solution at one time, and the soil was stirred every day to ensure that the concentration and fraction of Cd reached a balanced state. After thoroughly mixing the soil with CdCl2·2.5H2O, the soil sample was stabilized for 40 days and then used for all subsequent experiments. 300-mesh nylon bags about 15 cm in diameter were selected as root bags and filled with prepared Cd-contaminated soil inside and outside. Three plants were cultivated in each pot, and three pots were set in each treatment. Soil moisture maintained at 80% by timely replenishing water during the plant cultivation.

TABLE 1 The physicochemical properties of soil.

Physicochemical properties of soil	Content
Clay	25.3%
Silt	40.1%
sand	34.6%
pH	6.35
available P	20.27 g/kg
total N	103 mg/kg
available P	15.36 mg/kg
available K	148.60 mg/kg
Cd	0.21 mg/kg

After 60 days, plants and soils were carefully harvested from pots. The rhizosphere soil and the non-rhizosphere soil were separated with nylon bags. A non-rhizosphere soil is composed of five samples from 2 cm away from the root bag, the remained soil adhered to the root hairs was collected as the rhizosphere soil (Su et al., 2009).

2.2 Soil property analysis

To evaluate the status of the soil environment, several target indices such as soil pH, soluble dissolved organic carbon (DOC), water-soluble organic carbon (WSOC), and readily oxidizable carbon (ROC), the collected soil was sieved through a 2 mm sieve to ensure homogeneity. The pH of rhizosphere and bulk soils was determined by a glass electrode with an l:2.5 soils: water ratio. DOC, WSOC and ROC in soil were extracted with 50 mL1 mol L^{-1} KCl, deionized water and 200 mmol L^{-1} KMnO4 solution in turn, and measured with total organic carbon instrument (TOC-VCPH, Shimadu, Japan) following the study of Li et al. (2016).

2.3 Fraction and determination of Cd

There are 4 different forms of Cd in soils that were analyzed in this study. The method of improved European Community Bureau of Reference (BCR) sequential extraction was used to determine the metal partitioning)(Liu et al., 2020). The first is to extract the acid-soluble Cd: 40 mL 0.11 M CH_3COOH (16 h, room temperature). The second step is to extract the reducible Cd: 40 mL 0.5 M $NH_2OH·HCl$ (16 h, room temperature). The third step is to extract the oxidizable Cd: 10 mL 8.8 M H_2O_2 + 10 mL 8.8 M H_2O_2 + 50 mL 1 M NH_4OAc (1 h, 85 C; 1 h, 85 C; 16 h, room temperature). After each extraction, the supernatant was taken for determination, and the residue was cleaned with deionized water before the next step. Residual: digested by 10 mL HF + 10 mL HNO_3 + 3 mL $HClO_4$, and dissolve with 1 mL solution (HNO_3: H_2O =1: 1. The Cd concentrations in all the extracts were analyzed with AAS (MKII, M6, Thermo Electron Corporation, USA).

2.4 Soil enzyme and bacterial analysis

Soil urease, phosphatase, and catalase activities were determined by conventional methods (Ma et al., 2015). Referring to (Vance et al., 1987), soil microbial biomass carbon (MBC) was

FIGURE 1
The growth of *S. orientalis* in potted plants under Cd stress.

analyzed by the method of chloroform fumigation- K_2SO_4 extraction. Basal respiration was determined by following (Wardle et al., 1993). The metabolic quotient of soil microorganisms was calculated by the ratio of soil basal respiration intensity to MBC. Soil ammonification activities were expressed as mg NH_4^+-N per kg dry soil per day, and the content of NH_4^+-N was determined by distillation and Kjeldahl method. Soil nitrification were expressed as the percent reduction after inoculation. The specific test steps refer to the method of Zhang et al. (2021).

2.5 Data and statistical analysis

The data were analyzed by one-way analysis of variation (ANOVA). T test was used to compare the significant difference between rhizosphere and non-rhizosphere in the same treatment, and the multiple comparison between treatments was performed by Duncan's new multiple range methods ($P < 0.05$). Correlation regression analysis was used for some indexes. Redundancy analysis was used to examine the relationships between soil enzyme activity, soil microorganism quantity, and main soil properties using Canoco 4.5. Some statistical graphs were drawn by Origin 9.0.

3 Result

3.1 Concentration of Cd fractions in rhizosphere soil

Increased concentrations of all four different Cd forms in rhizosphere and bulk soil can be found when higher Cd concentration were added (Figure 2). With the increase of Cd application, the proportion of acid-soluble Cd in rhizosphere soil obviously increased ($P < 0.05$) from 31.87% to 64.60%, while the value in bulk soil changed from 23.53% to 66.30%. For reducible Cd in rhizosphere soil, it accounted for 26.00 - 34.49 % in rhizosphere soil and 29.55%-38.29% in bulk soil when the added Cd concentration increased from 0 to 150 mg kg^{-1}. There was no significant difference in the proportion of oxidizable Cd among all Cd treatments ($P > 0.05$) in rhizosphere and bulk soil. The proportion of residual Cd in total Cd significantly decreased ($P < 0.05$) from 40.36% to 2.25% for rhizosphere soil, and from 36.13% to 2.39% for bulk soil.

3.2 Variations of soil properties

The variation trend of pH in rhizosphere and bulk soil of *S. orientalis* has an obvious difference (Figure 3A). The Cd stress did not change the pH in the bulk soil of all treatments, while it led to lower pH (0.2 units on average) in the rhizosphere soil of *S. orientalis*. The pH value of rhizosphere soil within the range of 10 – 150 mg kg^{-1} Cd use concentration was significantly decreased than those values at the

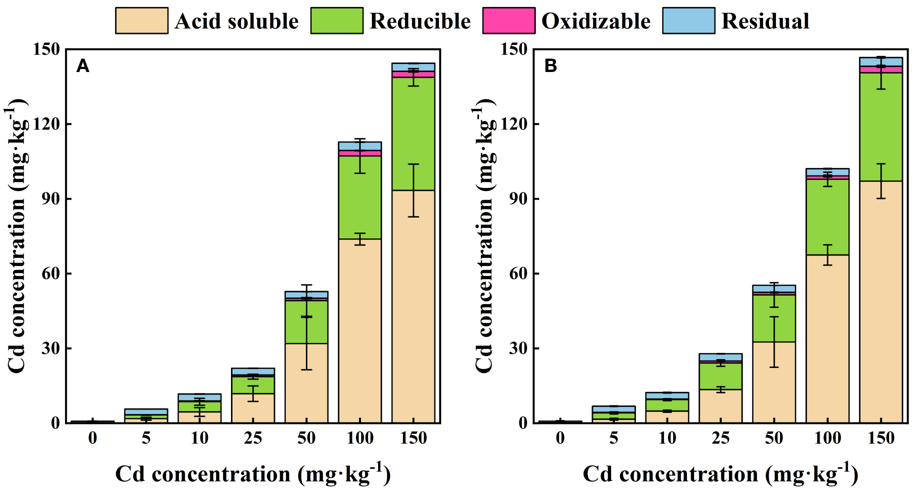

FIGURE 2
Proportion of Cd speciation in the rhizosphere **(A)** and bulk **(B)** soil of *S. orientalis*.

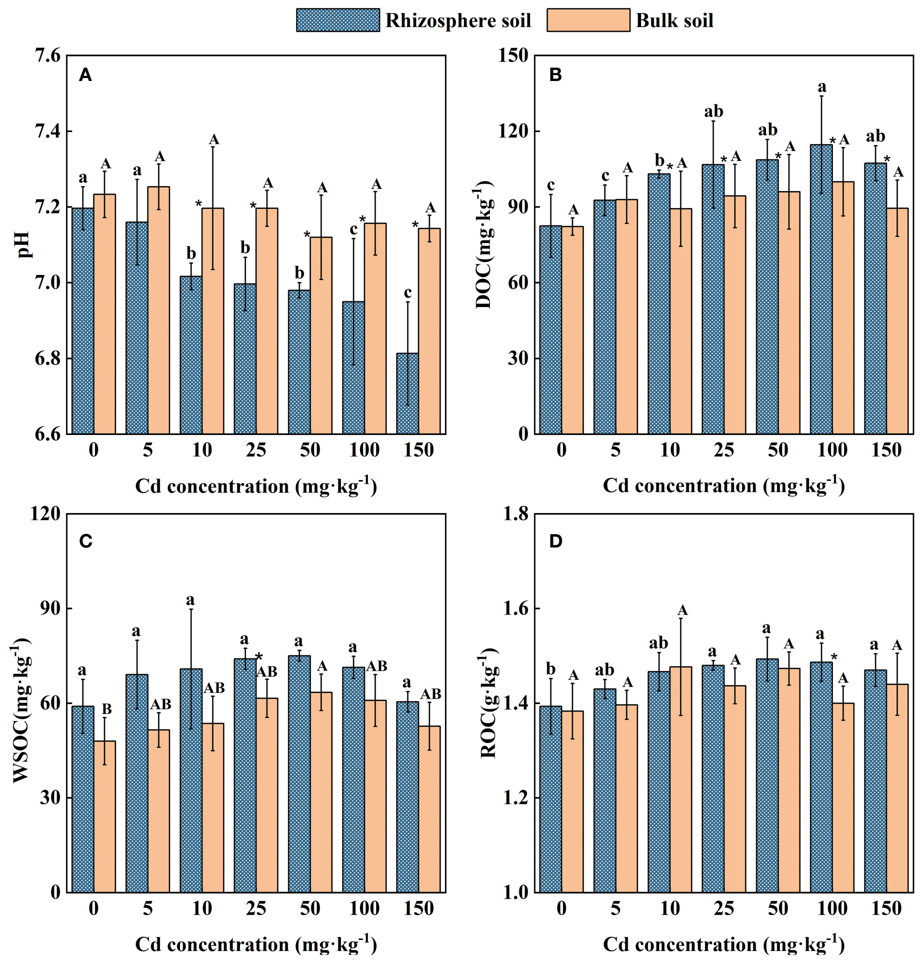

FIGURE 3
Effects of Cd on pH **(A)**, DOC **(B)**, WSOC **(C)**, ROC **(D)** in the rhizosphere and bulk of *S. orientalis*. Error bar represents standard deviation (n=3). * indicate significant difference at P < 0.05 level in the same treatment between rhizosphere and bulk soil, different lower case letter (upper case letter) at the column indicate significant difference at P < 0.05 level in the rhizosphere soil (in the bulk soil) according to ANOVA and Duncan tests, the same as follows.

Cd use concentration of 0 mg kg^{-1} and the bulk soils ($P < 0.05$). It can be found that the pH value of rhizosphere soil gradually decreased with the increase of added Cd concentration.

As shown in Figure 2B, a low concentration of external Cd (5 mg kg^{-1}) had no significant effect on DOC content in rhizosphere soil ($P > 0.05$), while the continuous increase of Cd supply (Cd ≥ 10 mg kg^{-1}) significantly increased the DOC content of rhizosphere soil ($P < 0.05$). The increase of external Cd concentration had no significant influence on DOC content in bulk soil ($P > 0.05$). Under 100 mg kg^{-1} Cd treatment, DOC content in rhizosphere soil reached the maximum value of 114.66 mg kg^{-1}, which was 28.39% higher than that of the control.

Similarly, the increase of external Cd concentration had no significant impact on bulk soil ROC content ($P > 0.05$). A low concentration of Cd (5-10 mg kg^{-1}) had no significant effect on the ROC of rhizosphere soil ($P > 0.05$). When Cd concentration is larger than 25 mg kg^{-1}, higher ROC content in rhizosphere soil can be seen from Figure 3 ($P < 0.05$). At the Cd use concentration of 50 mg kg^{-1}, ROC content in rhizosphere soil reached the maximum value of 1.49 g kg^{-1}, which was 6.98% higher than that of the control.

Different from changes in soil DOC and ROC, Cd stress had little effect on WSOC in both rhizosphere and bulk soil. Except for the Cd use concentration of 25 mg kg^{-1} and 50 mg kg^{-1}, there was no significant difference in WSOC concentration between rhizosphere and bulk soil in all treatments (Figure 2C). Meanwhile, the WSOC content of rhizosphere soil maintained small difference in these Cd use concentrations ($P > 0.05$).

3.3 Soil microorganism

As shown in Figure 4A, the concentration of MBC in both the rhizosphere and bulk soil of *S. orientalis* increased at first and then decreased with the Cd use concentration raised. The maximum concentrations of MBC in rhizosphere soil (80.88 mg kg^{-1}) and in bulk soil (72.11 mg kg^{-1}) both occurred at the Cd use concentration of 10 mg kg^{-1}, and increased by 50.56% and 51.11% compared with the control. When the Cd use concentration reached 150 mg kg^{-1}, the concentration of MBC in rhizosphere and bulk soil both dropped down to the lowest value. And the MBC concentration

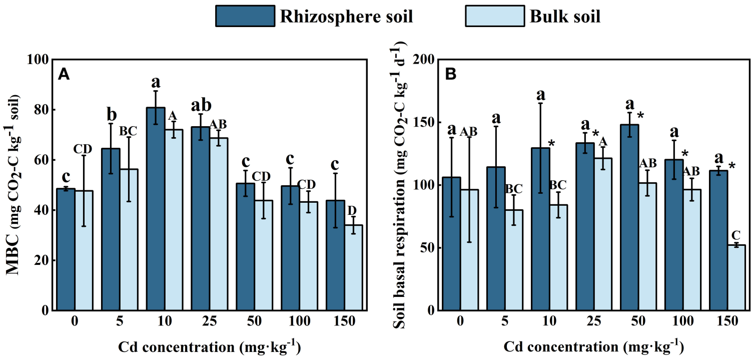

FIGURE 4

Effects of Cd on MBC **(A)** and soil basal respiration **(B)** in the rhizosphere soil and bulk soil of *S. orientalis*. Error bar represents standard deviation (n=3). * indicate significant difference at P < 0.05 level in the same treatment between rhizosphere and bulk soil, different lower case letter (upper case letter) at the column indicate significant difference at P < 0.05 level in the rhizosphere soil (in the bulk soil) according to ANOVA and Duncan tests, the same as follows.

in rhizosphere soil decreased by only 9.74% compared with the control, while in bulk soil was significantly lower than the control ($P < 0.05$), decreased by 28.67%.

The BR intensity of all rhizosphere soil was higher than that of bulk soil (Figure 4B). In comparison to the control, Cd stress did not significantly inhibit BR in the rhizosphere of *S. orientalis* ($P < 0.05$), and each treatment results increased by 7.65%-39.35%. More complex variation occurred in bulk soil. The maximum value of bulk soil basal respiration intensity occurred at the Cd use concentration of 25 mg kg^{-1}, which increased by 25.0% compared with the control treatment. And then the BR intensity in bulk soil decreased gradually with the increase of Cd added content. The minimum value was decreased by 45.79% compared with the control.

As shown in Figure 5, the intensity of ammonification and nitrification in both rhizosphere soil and bulk soil of *S. orientalis* significantly increased within the range of 5 – 50 mg kg^{-1} Cd concentration. The maximum intensity of both ammonification and nitrification was achieved at Cd use concentration of 25 mg kg^{-1}. With the continued increase of Cd use concentration from 50 mg kg^{-1}, both of them showed a decreasing trend in rhizosphere and bulk soil. The intensity of nitrification and ammonification in rhizosphere soil was significantly higher than that in bulk soil.

3.4 Soil enzyme activity

Urease activity in rhizosphere and bulk soil increased at first at low concentrations of external Cd treatment (0-10 mg kg^{-1}), subsequently reduced at high concentrations of Cd treatment (50-150 mg kg^{-1}). At Cd use concentration of 5 mg kg^{-1}, no significant effect on urease activity can be found. However, with the increase of external Cd concentration (10-100 mg kg^{-1}), the activity of urease in rhizosphere soils was significantly higher than that in bulk soils ($P < 0.05$). The maximum rhizosphere soil urease activity of 0.40 mg ammonia g^{-1} d^{-1} was achieved at the Cd use concentration of 10 mg kg^{-1}, and the value were increased by 13.21% compared with the control.

The changing trend of phosphatase was the same as that of urease. Phosphatase activity in rhizosphere soil was significantly

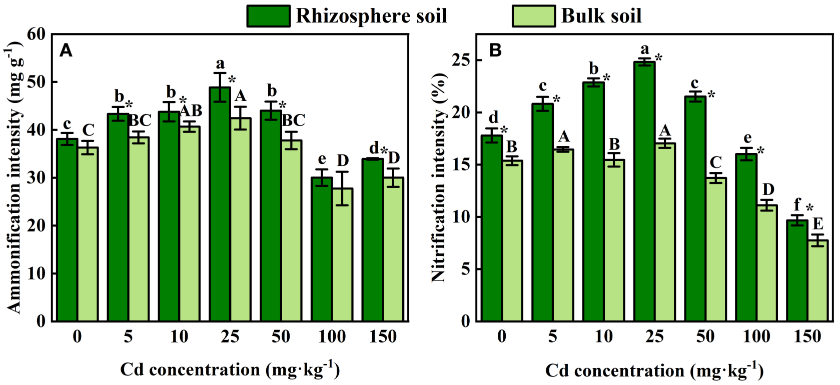

FIGURE 5

Error bar represents standard deviation (n=3). * indicate significant difference at P < 0.05 level in the same treatment between rhizosphere and bulk soil, different lower case letter (upper case letter) at the column indicate significant difference at P < 0.05 level in the rhizosphere soil (in the bulk soil) according to ANOVA and Duncan tests, the same as follows.

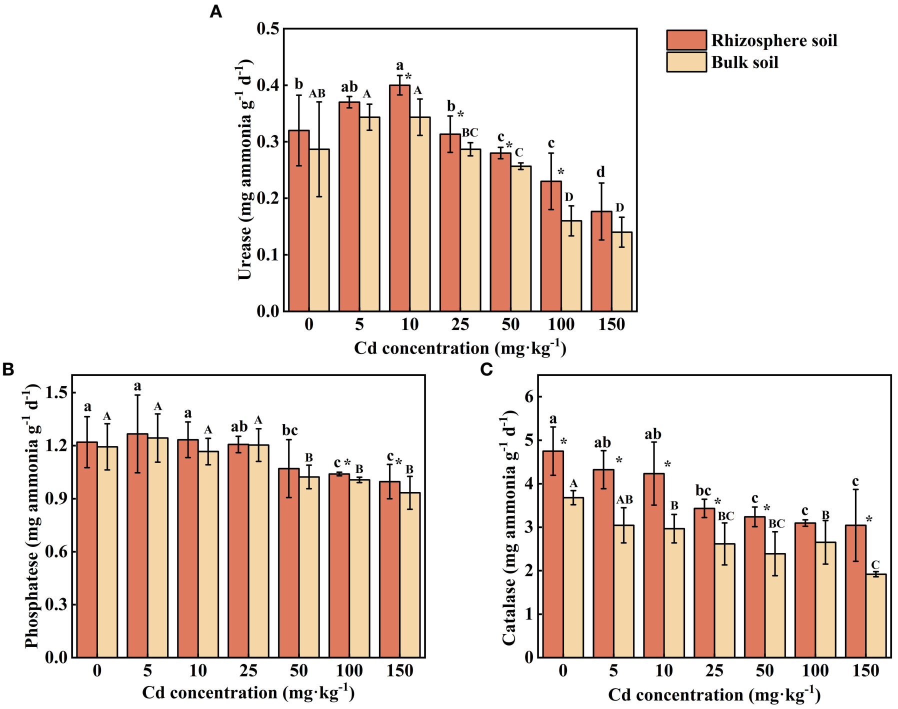

FIGURE 6
Effects of Cd on activity of urease **(A)**, phosphatase **(B)** and catalase **(C)** in the rhizosphere soil and bulk soil of *S. orientalis*. Error bar represents standard deviation (n=3). * indicate significant difference at P < 0.05 level in the same treatment between rhizosphere and bulk soil, different lower case letter (upper case letter) at the column indicate significant difference at P < 0.05 level in the rhizosphere soil (in the bulk soil) according to ANOVA and Duncan tests, the same as follows.

higher than that of bulk soil under the treatment of the two highest concentrations of Cd ($P < 0.05$), while there was no significant difference in phosphatase activity between rhizosphere and bulk soils treated with low and medium concentrations of Cd (5-50 mg kg^{-1}) (Figure 6B, $P > 0.05$). The maximum phosphatase activity in rhizosphere soil (1.27 mg phenol g^{-1} d^{-1}) and in bulk soil (1.24 mg phenol g^{-1} d^{-1}) occurred at the Cd use concentration of 5 mg kg^{-1}. As the supply of Cd increased, the phosphatase activity decreased significantly ($P < 0.05$) 9.56%-17.76% in rhizosphere soil and 14.25%-21.79% in bulk soil compared with the control.

The catalase activity increased in both rhizosphere and bulk soil with increasing Cd use concentration in all samplings (Figure 6C). The activity of catalase in rhizosphere soil was significantly higher than that in bulk soil except for one condition (100 mg Cd kg^{-1}, $P < 0.05$). Compared with the control, low Cd use concentrations (5-10 mg kg^{-1}) did not significantly inhibit the catalase activity of rhizosphere soil ($P > 0.05$), while high and medium Cd use concentrations (≥25 mg kg^{-1}) can decrease the catalase activity, which is decreased by 27.72%-35.93%.

4 Discussion

4.1 Rhizosphere acidification and organic carbon accumulation drives Cd activation

Cd activation refers to the process by which Cd in soil changes from stable fractions (acid-soluble and reducible Cd) to labile fractions (oxidizable and residual Cd), and the bioavailability of Cd increases during this process. We followed the objective to measure the related indexes and Cd morphological changes of soil. Relevant hypothesis was confirmed by the change of soil physical and chemical properties caused by rhizosphere effect. These changes are important to activate HMs. BCR extraction procedures were used to evaluate Cd availability. According to the BCR sequential extraction method, HMs in soils can be classified into acid-soluble, reducible, oxidizable, and residual fractions, among which the acid-soluble fraction has the highest bioavailable and the residual fraction has the lowest bioavailability (Anju and Banerjee, 2010; Yang et al., 2017; Huang et al., 2020). In general, the mobility of HMs in the soil is relatively weak, and the fractions with high bioavailability, such as acid-soluble fraction, are not high (Li et al., 2011). *S. orientalis* can absorb large amounts of Cd from the soil, so there must be an activation process to change the morphology and bioavailability of Cd. As can be seen from Figure 2, with the increase of the concentration of Cd, the proportion of weak-acid extracted Cd from the rhizosphere increased gradually, the proportion of reducible and oxidizable Cd changed little, while the proportion of residue Cd with the lowest bioavailability decreased significantly. This is consistent with the research results of *R. globose* under Cd treatment (Wei and Twardowska, 2013). The concentration of acid-soluble fraction in rhizosphere soil was lower than that in bulk soil under all treatments (Figure 2) because Cd absorption rate was higher than the activation rate. Some reports hold that the mobility and bioavailability of HMs in soil are mainly influenced by physicochemical properties such as soil pH and organic matter content (Bravin et al., 2012; Seshadri et al., 2015; Huang et al., 2020).

Rhizosphere soil pH can significantly impact the bioavailability and toxicity of HMs in soil, which could result in the desorption of HMs from soil particles by reducing the pH (Niu et al., 2021; Ning et al., 2022). These effects will further promote the uptake of HMs by plants (Li et al., 2014; Mimmo et al., 2014). In this study, external Cd induced a reduction of pH in the rhizosphere (Figure 3A), as Zhan et al. (2018) observed in *A. wardii*. Redundancy analysis (RDA) also showed that the pH of rhizosphere is closely related to Cd fractions, and rhizosphere acidification could increase Cd concentrations in four forms (Figure 7). Rhizosphere acidification can be attributed to the secretion of organics including organic acid and carbohydrates by hyperaccumulator (Kushwaha et al., 2015; Niu et al., 2020). Rhizosphere acidification is a soil chemical process occurring in the root-soil interface (Antoniadis et al., 2017). Consequently, acidification of the rhizosphere leads to the transformation of Cd from low bioavailable (reducible) to high bioavailable (acid-soluble) (Figures 2A).

In addition to rhizosphere acidification, the concentration of active organic carbon components can play important role in activation of HMs in soil solution (Clemente and Bernal, 2006; Kim et al., 2010). We divided the activated carbon components into DOC, ROC, and WSOC. These organic compounds are derived from the mineralization of soil organic matter and root exudation (Pii et al., 2015). DOC can be used as a carrier for accelerating hyperaccumulator uptake of HMs (Huang et al., 2020). Many studies have confirmed the activation effect of DOC on HMs (Wenzel et al., 2003; Li et al., 2011; Li Z, et al., 2021). In this study, DOC induced an increase in high bioavailability Cd fractions (acid-soluble and reducible Cd) (Figure 7). DOC can form soluble organometallics complexes with HMs, or substitute HMs for preferentially adsorbed on the soil surface, to reduce the adsorption of HMs on the soil surface and improve its bioavailability (Cornu et al., 2011; Bravin et al., 2012; Welikala et al., 2018). Therefore, DOC was one of the important factors promoting the activation and hyperaccumulation of Cd in *S. orientalis* under Cd stress.

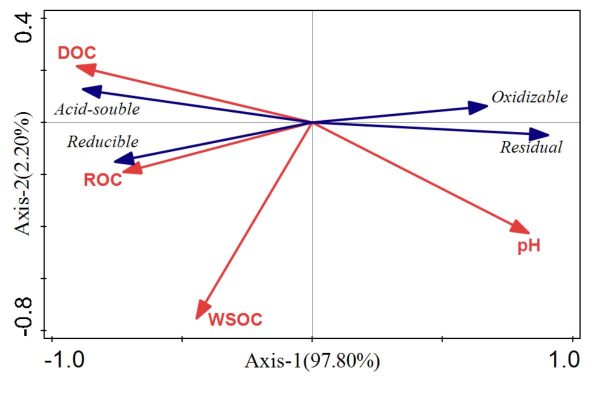

FIGURE 7
RDA ranking of Cd form and soil properties in rhizosphere soils.

Compared to DOC, ROC accounted for a higher proportion of labile soil organic carbon components and had a faster turnover, so it was more sensitive to soil environmental changes (Zhang et al., 2020). ROC is organic carbon that is easily oxidized by potassium permanganate, and its content is easily affected by anthropogenic activities (Jiang M, et al., 2022). In our study, the supply of exogenous Cd could increase the content of ROC in rhizosphere soil, especially at medium and high concentrations (Cd ≥ 25 mg kg^{-1}) significantly increased ($P < 0.05$) (Figure 2D). Combined with the results that ROC content in the bulk soil had no significant effect ($P > 0.05$), we can conclude that the ROC variation is caused by the root system. The elevated level of ROC may be due to Cd toxicity caused by a declining trend of soil pH with the increasing of Cd stress increases (Figure 2A), which may damage the roots and increase root exudation (Li et al., 2015). This has been confirmed in previous studies (Hurisso et al., 2014). At present, few studies have focused on the relationship between the change of ROC content in rhizosphere soil and the activation of HMs. However, our RDA showed that the content of ROC is closely related to the Cd availability (Figure 7). ROC is a large order of magnitude of active organic carbon components, mainly originate from the mineralization of microbial and plant root exudates (De Conti et al., 2018; Li G, et al., 2021; Jiang O, et al., 2022). Concerning the latter, root exudates included amino acids, low molecular weight organic acids, and soluble sugars, and their release was beneficial to the accumulation of ROC (Xiao et al., 2018; Liu et al., 2020; Li G, et al., 2021). From the composition of ROC, these substances play an important role in improving the mobility and the absorption of HMs by plants (Kim et al., 2010; Madej, 2016; Jiang M, et al., 2022). Consequently, the increase of rhizosphere secretions of *S. orientalis* under Cd stress promotes the accumulation of ROC, thus increasing the bio-availability of Cd and facilitating the hyperaccumulation of Cd by *S. orientalis*. However, some limitations are worth noting. Although our hypotheses were supported statistically, our samples did not analyze the specific composition of root exudates. Future work should include the effect of specific root exudate composition on Cd morphology.

WSOC is the most mobile and susceptible fraction of soil organic carbon (Li et al., 2015). Previous studies showed that WSOC content was not significantly affected by plant planting systems and soil depth, but was mainly controlled by precipitation leaching (Sharma et al., 2014; Guo et al., 2018). In this study, the content of WSOC was not significantly affected by Cd stress (Figure 3C), and there was no significant correlation between WSOC and the form of Cd in rhizosphere soil (Figure 7; Table 2). It can be inferred that WSOC played a limited role in Cd activation.

4.2 The increase of microorganism and enzyme activity is the mechanism of *S. orientalis* tolerance to Cd

In addition to accumulating high concentrations of HMs, hyperaccumulators also have the mechanism of tolerating and sustaining highly toxic HMs (Clemens, 2017; Pasricha et al.,

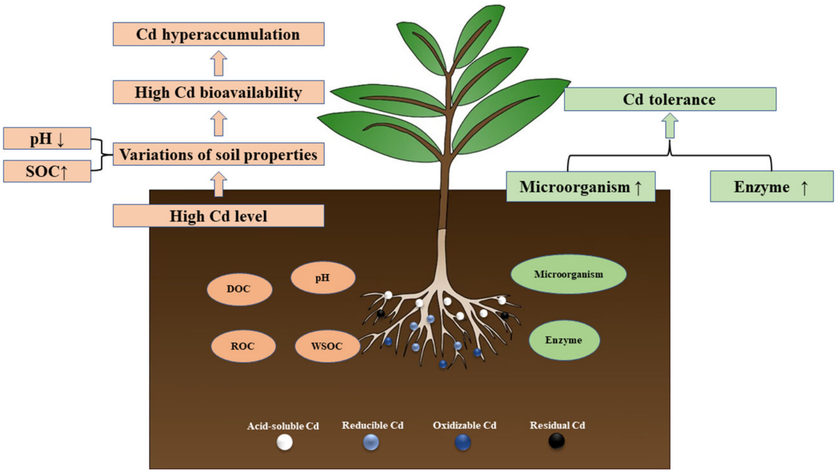

FIGURE 8
Schematic of the mechanism of the activation and tolerance of *S. orientalis* Cd.

2021). Hypertolerance is the key property that allows plants to avoid HM poisoning (Chaney et al., 1997). The properties can be divided into *in vivo* and *in vitro* according to the site of action. In vivo, chelation/sequestration deal with the toxicity of accumulated metal ions, while the anti-oxidative defense system of plants is used to cope with HM-induced oxidative damage (Manara, 2012; Macnair et al., 2000; Yan et al., 2020; Feng et al., 2021). These indicators have been thoroughly studied in previous researches (Lux et al., 2011; Pasricha et al., 2021; Yaashikaa et al., 2022), but the role of rhizosphere in plant tolerance is often ignored.

In vitro, soil enzyme activity is an important index reflecting soil quality and vitality (Zhang et al., 2015a). Their activity is related to the number of soil microorganisms, soil conditions, and plant growth (Jaiswal and Pandey, 2018; Gao et al., 2020). Therefore, soil enzyme activity can accurately reflect the response of soil microorganisms to HMs exposure during phytoremediation (Cao et al., 2020). In our study, with the increase of Cd supply, the enzyme activities showed a decreasing trend (Figure 6). Cd can reduce enzyme activity by denaturing the enzyme protein, binding with the enzyme substrate complex, and inhibiting microbial activity (Ma et al., 2015; Cao et al., 2020). However, medium and low concentrations of Cd (Cd ≤ 10 mg kg^{-1}), *S. orientalis* rhizosphere maintained the activity of rhizosphere soil enzymes so that they are not significantly inhibited. The role of soil microorganisms in detoxifying pollutants, facilitating nutrient cycling, and producing essential compounds for both microorganisms and plants has been demonstrated (Ma et al., 2015). Urease catalyzed hydrolysis of urea is one of the important sources of plant nitrogen, catalase can effectively remove the hydrogen peroxide toxicity caused by stress environment, and phosphatase can help maintain a good supply of phosphorus in Cd-contaminated soil (Weng et al., 2014; Ma et al., 2015; Sturikova et al., 2018; Wang et al., 2018). This suggests that rhizosphere plays an important role in maintaining nutrient supply and detoxification of plants in Cd-contaminated soils through soil enzymes. To sum up, the rhizosphere environment had a complex effect on soil enzyme activities, and the enhancement of plant rhizosphere soil enzyme activities improved the conversion of nitrogen, phosphorus, and organic matter in Cd contaminated soil, maintained soil fertility, and was conducive to promoting the growth of *S. orientalis*, and also promoted the absorption of Cd by plant roots to a certain extent (Figure 8).

TABLE 2 Correlations between partial soil properties and Cd speciation in the rhizosphere and non-rhizosphere of *S. orientalis* across all samples.

	Soil properties	Acid soluble	Reducible	Oxidizable	Residual
Rhizosphere soil	pH	-0.695**	-0.567**	0.502*	0.705**
	DOC	0.741**	0.718**	-0.644**	-0.780**
	WSOC	0.299	0.427	-0.393	-0.349
	ROC	0.280	0.449*	-0.353	-0.351
Bulk soil	pH	-0.409	-0.102	0.184	0.411
	DOC	0.261	0.316	-0.290	-0.314
	WSOC	0.470*	0.402	-0.505*	-0.483*
	ROC	0.304	0.317	-0.270	-0.356

Values are Pearson correlation coefficients, *indicated the correlations were significant at $P < 0.05$, **indicated the correlations were significant at $P < 0.01$.

Microorganisms in rhizosphere soils have been proven to detoxify of HMs, and promote nutrient cycling and transfer of soil energy (Guo et al., 2019). Therefore, rhizosphere microorganisms play an important role in improving the efficiency of phytoremediation (Cao et al., 2020). MBC is an active carbon reservoir in the soil micro-ecosystem, which can be a sensitive indicator of soil microbial biomass and soil quality, its enhancement of MBC may help increase crop productivity and sustainability (Liu et al., 2011; Zhan et al., 2018). Soil basal respiration was accepted as a sensitive indicator of environmental stresses on soil microbial community microbial stress, and microorganisms can decompose soil organic carbon and produce energy through soil basal respiration (Bian et al., 2015; Zhou et al., 2017). These indicators are often used to characterize environmental and microbial changes. In general, high concentrations of HM pollution can negatively affect the number and activity of microorganisms in the soil (Pan and Yu, 2011). Our results showed that both MBC content and soil basal respiration in rhizosphere soil increased in all Cd treatments compared with the control these in the control treatment (Figure 4). At the same time, the rhizosphere soils were always higher than bulk soils. This may be ascribed to the exudates of *S. orientalis* rhizosphere under Cd stress promoting the improvement of soil enzyme activities and was beneficial to the nutrient cycling in the soil and the survival of microorganisms (Li Z, et al., 2021). However, as the concentration increased, the bioavailability and mobility of Cd also increased (Zhan et al., 2018). This phenomenon leads to a gradual increase in the inhibition of microbial activity. Similar results have been found in other studies (Bérard et al., 2016; Jaiswal and Pandey, 2018). These results confirm that a series of rhizosphere activities maintain the microbial activity of rhizosphere soil and promote Cd tolerance to *S. orientalis*.

Ammonification and nitrification are two basic processes of N cycling in soil, which convert N into two main nitrogen forms, ammonium (NH_4^+) and nitrate (NO_3^-), that are efficiently taken up by plant roots (Salsac et al., 1987; Beeckman et al., 2018). Moreover, root growth is stimulated by ammonium and nitrate that provide sufficient nutrients (O'Brien et al., 2016; Xuan et al., 2017). Ammonification and nitrification of soil microorganisms are also important factors to maintain plant growth and alleviate heavy metal toxicity in plants(Li et al., 2019; Wang et al., 2021). Our results showed that ammonification and nitrification were inhibited when Cd concentration is greater than 100 mg kg^{-1}, whereas enhanced at Cd concentration of 5-50 mg kg^{-1}. And they were significantly higher in rhizosphere soil than these in bulk soil (Figure 5). The intensities of ammonification and nitrification were mainly influenced by the microhabitats. In addition, high concentrations of Cd will lead to microbial inactivation affecting the intensity (Wang et al., 2021). The rhizosphere exudates improved the microbial activity and alleviated the stress of Cd to *S. orientalis* when the Cd supply was low. These results indicate that ammonification and nitrification of rhizosphere soil play an important role in promoting tolerance of *S. orientalis*.

5 Conclusion

Cd activation refers to the process by which Cd in soil changes from stable fractions (acid-soluble and reducible Cd) to labile fractions (oxidizable and residual Cd), and the bioavailability of Cd increases during this process. Meanwhile, compared with the treatment without Cd, exogenous application of Cd resulted in rhizosphere acidification and accumulation of organic carbon, which induces Cd activation. In turn, accumulation of active organic carbon alleviated the inhibition effects on microbial and enzymatic activities in rhizosphere soil. These improvements presented great benefit for *S. orientali* tolerance in Cd-contaminated soils. Our findings provide new insights into the hyperaccumulation and detoxification of HMs by hyperaccumulator root-soil systems and provide possibilities for improving the phytoremediation efficiency of contaminated soils.

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

JYX: data curation; investigation; writing-original draft. XX: funding acquisition; supervision; writing-review and editing. SZ: project administration; writing-review and editing. ZY: software; writing-review and editing. GW: resources; writing-review and editing. TL: resources; writing-review and editing. YP: validation; writing-review and editing. WZ: validation; writing-review and editing. CX: project administration; writing-review and editing. GL: resources; writing-review and editing. ZC: visualization; writing-review and editing. JRX: software; writing-review and editing. ZP: validation; writing-review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Individual and mutual effects of elevated carbon dioxide and temperature on salt and cadmium uptake and translocation by rice seedlings

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Plant kingdoms are facing increasingly harsh environmental challenges marked by the coexposure of salinity and pollution in the pedosphere and elevated CO_2 and temperature in the atmosphere due to the rapid acceleration of industrialization and global climate change. In this study, we deployed a hydroponics-based experiment to explore the individual and mutual effects of different temperatures (low temperature, T1: 23°C; high temperature, T2: 27°C) and CO_2 concentrations (ambient CO_2: 360 ppm; medium CO_2: 450 ppm; high CO_2: 700 ppm) on the uptake and translocation of sodium chloride (NaCl, 0.0, 0.2, 0.6, and 1.1 g Na/L) and cadmium nitrate ($Cd(NO_3)_2{\cdot}4H_2O$, 0.0, 0.2, 1.8, and 5.4 mg Cd/L) by rice seedlings. The results indicated that Cd and Na exposure significantly ($P < 0.05$) inhibited plant growth, but T2 and medium/high CO_2 alleviated the effects of Cd and Na on plant growth. Neither significant synergistic nor antagonistic effects of Cd and Na were observed, particularly not at T1 or high CO_2. At increasing temperatures, relative growth rates increased despite higher concentrations of Cd and Na in both rice roots and shoots. Similarly, higher CO_2 stimulated the growth rate but resulted in significantly lower concentrations of Na, while the Cd concentration was highest at medium CO_2. Coexposure experiments suggested that the concentration of Cd in roots slightly declined with additional Na and more at T2. Overall, our preliminary study suggested that global climate change may alter the distribution of mineral and toxic elements in rice plants as well as the tolerance of the plants.

KEYWORDS

***Oryza sativa*, CO_2, climate change, salinization, phytotoxicity**

1 Introduction

Elevated atmospheric CO_2 concentrations are the driver of global climate change. The CO_2 "fertilization effect" was observed by enhancing vegetation productivity (Sperry et al., 2019; Song et al., 2022). In fact, elevated CO_2 stimulates the growth of C_3 plants in terms of plant biomass, water and nutrient use efficiency, and the rate and intensity of photosynthesis (Poorter et al., 2022). Ample evidence has shown that the optimal atmospheric CO_2 concentrations for crop photosynthesis range from 700 to 1000 ppm, while a deficiency in the CO_2 supply under natural conditions is a limiting nutritional factor for crops to produce enough C-containing biomass. However, more emissions of CO_2 from various sources might cause unexpected consequences, wherein warmer temperatures and changing precipitation patterns caused by greenhouse effects are major concerns, which will change the distribution and availability of freshwater, essentially alter tillage patterns and reduce crop yields in some regions (IPCC, 2019).

Another concern is salinization of soils, particularly in combination with irrigation in agricultural land (Corwin, 2021), since the vapour pressure of water increases exponentially with increasing temperature (Clausius-Clapeyron Law), leading to a higher water vapour deficit and more evaporation from soil surfaces. Therefore, the area at risk of salinization is projected to increase in the future (IPCC, 2019). It has been reported that 16% of agricultural land (831 million hectares) is affected by salinization worldwide (FAO, 2011; Thiam et al., 2021), wherein 99.13 million hectares of land are saline in China (Liu and Wang, 2021). It is known that above a certain plant-specific threshold, soil salinity leads to toxic effects and to declining yields (Syed et al., 2021; Yan et al., 2021). Terrestrial plants maintain their internal osmotic pressure at homeostatic levels (Zhao et al., 2021) and regulate their cytosolic K^+ to Na^+ ratio by molecular cation transporters (Cosco et al., 2019). Overloading of these pumps leads to toxic effects (Isayenkov and Maathuis, 2019), while exclusion of Na at the root increases salinization (Wei et al., 2022). Production efficiency (carbon use efficiency) increases at higher growth temperatures (Collalti et al., 2020), but adverse effects of soil salinity intensify (Silva et al., 2013); hence, there is no linear relationship between temperature and salt stress (Gadallah, 1996). Therefore, minimizing water use by regulating the transpiration rate is a positive strategy that can help to conserve water and reduce Na loading in plant materials (Xue et al., 2021). In fact, at elevated CO_2, plants typically use less water and use it more efficiently, and saving water under salt stress is beneficial (Bertolino et al., 2019). Although many studies have been conducted, the current knowledge about the interaction between elevated CO_2/temperature and salinity remains insufficient for predicting the impact of climate change on plant growth and production.

Today, heavy metal contamination has become a serious and growing problem for agricultural production and food safety (Fei et al., 2022; Zhang et al., 2022; Feng et al., 2023a). Rice (*Oryza sativa* L.) is one of the most important staple food crops worldwide, especially in eastern Asian countries. Over the last century, farmland deterioration due to metal contamination has become increasingly serious. Many anthropogenic activities, including mineral fertilizers, mining operations, manufacturing industries and landfills of industrial or domestic sludge, have resulted in an increasing amount of agricultural land being contaminated with heavy metals throughout the world (Dai et al., 2019). There are more than 20 million hectares of agricultural land contaminated with various species of heavy metals in China (Peng et al., 2019). Such amounts of heavy metals in soils make their entry into the food chain likely and pose a threat to humans and wildlife. It was estimated that the economic damage due to cadmium (Cd) contamination is at least 798 billion CNY in China alone (Yang et al., 2022). Cadmium is a nonessential element for plants (Abbas et al., 2022) and toxic at low concentrations. In fact, Cd can distribute and damage nutrient uptake, photosynthetic and respiratory activities, and hormone balance to decrease and/or repress plant growth (Rasafi et al., 2020). Although the uptake and translocation of Cd in different species of plants have been intensively studied from laboratory tests to field trials, the interaction between elevated CO_2/temperature and Cd is currently not available.

The uptake of heavy metals has been observed at elevated CO_2, mainly focusing on plant growth and development (Guo et al., 2015). For example, elevated CO_2 was able to enhance the uptake rate of heavy metals by stimulating root development, enriching root phenotype traits, and increasing metal availability in soils (Jia et al., 2018). Additionally, elevated CO_2 stimulated an increase in the fresh weight of soybean in the presence of Cd exposure by positively regulating flavonoid content and antioxidant defense capacity (Gong et al., 2023). Elevated concentrations of heavy metals and salt (NaCl) often occur together (e.g., in wastewater or in fertilizers, Tornabene et al., 2020), and several studies have addressed their mutual interactions on uptake and toxicity. Several physiological interactions between NaCl and Cd were observed in *Mesembryanthemum crystallinum* L. (Nosek et al., 2020). Combined, salt and Cd had a stronger negative effect on the growth and chlorophyll content of conocarpus (*Conocarpus erectus* L.) than each alone (Rehman et al., 2019). Elevated salinity intensified the effects of Cd in a study with quinoa (*Chenopodium quinoa* Willd.) and reduced the shoot and root growth of experimental plants by more than 50% (Abdal et al., 2021).

Because many biotic and abiotic factors are involved, it is difficult to quantify future trends. Present findings remain inconclusive, and very few studies have identified the effect of multiple stressors on terrestrial vegetation under the combined variation of temperature and CO_2 concentrations. Until now, the simultaneous effects of changing temperatures and CO_2 concentrations in air on the uptake, translocation, and toxicity of Na and Cd by rice seedlings have not been available. Therefore, the main objectives of this study were (1) to estimate the effects of temperature and CO_2 concentrations on the plant growth of rice seedlings under salt (measured as Na) and Cd stress alone and in combination; (2) to investigate the individual and mutual effects of temperature and CO_2 concentrations on the uptake and translocation of Na and Cd alone and in combination; and (3) to analyse the individual and mutual effects of temperature and CO_2

concentrations on the metal distribution in rice tissues under Na and Cd stress alone and in combination.

2 Methods and materials

2.1 Rice cultivars and exposure solutions

This experiment was conducted at Guilin University of Technology on the Yanshan campus, Guangxi, China (25.2895 N, 110.3178 E). The seeds (*Oryza sativa* L. cv. XZX 45, Feng et al., 2023b) of rice were cultivated in sandy soils in a climate-controlled chamber (temperature: 25 ± 0.5°C, relative humidity: 60 ± 2%, ambient CO_2: 360 ppm). The modified 8692 nutrient solution was prepared in 1.0 L of water with KNO_3 (285.2 mg/L), $MgCl_2{\cdot}H_2O$ (12 mg/L), $CaCl_2$ (18 mg/L), $MgSO_4$ (15 mg/L), KH_2PO_4 (33.46 mg/L), $NaHCO_3$ (150 mg/L), H_3BO_3 (0.1855 mg/L), $MnCl_2$ (0.415 mg/L), Fe-EDTA (4.83 mg/L), $NaMoO_4$ (7 mg/L), $CuSO_4$ (6.25 mg/L), $ZnSO_4$ (2.99 mg/L), and $CoCl_2$ (1.5 mg/L) (Feng et al., 2019). After 16 d of growth, ten young seedlings of similar size were collected and incubated with a modified ISO 8692 nutrient solution for 12 h. After acclimatization, the seedlings were cleaned with ionic removal buffer solution [1 mM $CaCl_2$ + 2 mM MES-Tris (pH 6.0)] for 4 h (Ling et al., 2021). Finally, the pretreated seedlings were used for the subsequent experiments. In detail, three different solutions with Cd and Na alone or combined were used as follows:

***Solution A* (*Cd stress*)**: Selected seedlings were exposed to a 50 mL Erlenmeyer flask spiked with 50 mL of Cd at nominal concentrations (0.0, 0.2, 1.8, and 5.4 mg Cd/L);

***Solution B* (*Na stress*)**: Selected seedlings were exposed to a 50 mL Erlenmeyer flask spiked with 50 mL of Na at nominal concentrations (0.0, 0.2, 0.6, and 1.1 g Na/L);

***Solution C* (*Cd+Na stress*)**: Selected seedlings were exposed to a 50 mL Erlenmeyer flask spiked with 50 mL of Cd+Na at nominal concentrations (0.0, 0.2 mg Cd/L+ 0.2 g Na/L, 1.8 mg Cd/L+ 0.6 g Na/L, and 5.4 mg Cd/L+ 1.1 g Na/L).

Note that cadmium nitrate [$Cd(NO_3)_2{\cdot}4H_2O$, CAS: 10325-94-7] and sodium chloride (NaCl, CAS: 7647-14-5), guaranteed reagents, were used in this study. A remarkable ($P< 0.05$) increase in IR was observed in rice seedlings from both Cd and Na treatments with different nominal initial concentrations (Supporting information M1). Accordingly, the EC was determined by IR (Lin et al., 2020). The EC_{20}, EC_{50} and EC_{75} of Cd were 0.14, 1.81 and 5.66 mg Cd/L, respectively, while the EC_{20}, EC_{50} and EC_{75} of Na were 0.2, 0.64 and 1.08 g Na/L, respectively. To minimize water loss and inhibit algae growth, all Erlenmeyer flasks were covered with aluminum foil, followed by incubation in a growth chamber (illumination intensity: 20,000 lux; relative humidity: 60 ± 2%). Four independent biological replicates were deployed at each Cd-/Na-/Cd+Na-treated concentration in the 3-d experiment. Each independent biological replicate contained ten rice seedlings of similar size. The selection criteria for exposure concentrations of Cd and Na are presented in Section 2.5 and Supporting information M1.

2.2 Effects of temperature and atmospheric CO_2 levels

A CO_2 controlled environment chamber (CCEC, LRH-325-GSIE-T) was obtained from Shaoguan Taihong Medical Appliance Co., Ltd. Three different levels of CO_2 at 360 (ambient), 450 (medium) and 700 ppm (high) were designed. The testing temperature was set at either 23°C or 27°C. The relative humidity in the CCEC was 60 ± 2%. Liquid CO_2 was stored in a tank connected to the computer-controlled commercial CO_2 injection system with a PID controller that served as the CO_2 source. The discharge of CO_2 in the CCEC was manually regulated by a needle valve that was connected to mechanical flow meters. The CO_2 concentration in the CCEC was a real-time detection and recorded by a computer. The resolution of CO_2 levels in this CCEC is 1 ppm. Four treatments and their abbreviations were as follows:

***Control A* (T1 + ambient CO_2)**: rice seedlings were exposed to Solution A, Solution B and Solution C for 3 days and incubated in CCEC with a level of CO_2 at 360 ppm (ambient CO_2) and a temperature of 23°C;

***Treatment 1A* (T1 + medium CO_2)**: rice seedlings were exposed to Solution A, Solution B and Solution C for 3 days and incubated in CCEC with a level of CO_2 at 450 ppm (medium CO_2) and a temperature of 23°C;

***Treatment 2A* (T1 + high CO_2)**: rice seedlings were exposed to Solution A, Solution B and Solution C for 3 days and incubated in CCEC with a level of CO_2 at 700 ppm (high CO_2) and a temperature of 23°C.

***Control B* (T2 + ambient CO_2)**: rice seedlings were exposed to Solution A, Solution B and Solution C for 3 days and incubated in CCEC with a level of CO_2 at 360 ppm (ambient CO_2) and temperature at 27°C;

***Treatment 1B* (T2 + medium CO_2)**: rice seedlings were exposed to Solution A, Solution B and Solution C for 3 days and incubated in CCEC with a level of CO_2 at 450 ppm (medium CO_2) and a temperature of 27°C;

***Treatment 2B* (T2 + high CO_2)**: rice seedlings were exposed to Solution A, Solution B and Solution C for 3 days and incubated in CCEC with a level of CO_2 at 700 ppm (high CO_2) and a temperature of 27°C.

2.3 Relative growth rate

The relative growth rate (RGR) is one of the most important parameters reflecting the overall performance of plant physiology under environmental abuse (Ling et al., 2021). The method for calculating the RGR was identical to our previous study (Lin et al., 2020) and was based on the following equation:

$$RGR = \frac{W_{(F)} - W_{(I)}}{W_{(I)}} \times 100\,\% \qquad (1)$$

where $W_{(I)}$ and $W_{(F)}$ are the initial and final fresh weights of rice seedlings, respectively.

2.4 Inhibition rate

The inhibition rate (IR, %) of RGR was calculated using the following equation:

$$IR_{(C,t)} = (1 - \frac{1/n\sum_{i-=1}^{n} RGR_{(C,t)}}{1/m\sum_{j=1}^{m} RGR_{(0,t)}}) \times 100 \qquad (2)$$

where C is the concentration of Cd and Na, t is the exposure period (d), RGR is the relative growth rate, i is replicate 1, 2,…, n and j is control 1, 2,…, m.

2.5 Determination of effective concentration

The effective concentration (EC) is the dose of pollutants that resulted in inhibitory effects on the possible parameters selected. In this study, the IR of RGR was used as a sensitive endpoint to estimate the EC value for Cd and Na treatments. The nominal concentrations of Cd treatments were 0, 0.25, 0.50, 1.00, 2.00, 4.00 and 8.00 mg Cd/L, while the nominal concentrations of Na treatments were 0, 0.1, 0.3, 0.5, 0.9, 1.2, and 1.6 g Na/L. For estimation of EC values, initial concentrations of Cd and Na were $\log_{10}$ transformed. Linear regression between $\log_{10}$Cd or Log_{10}Na and IR (%) was plotted to calculate the concentrations of EC_{20}, EC_{50} and EC_{75}, which refer to the inhibition of 20%, 50%, and 75% of the RGR at 360 ppm CO_2 and 25°C in comparison to the control, which were used as nominal initial concentrations for the tests with varying temperature and CO_2 concentrations. More detailed information is presented in Supporting information M1.

2.6 Measurement of Ca and Na in rice seedlings

After 3 d of exposure, rice seedlings were collected and rinsed with distilled water and placed in a pretreated solution containing MES-Tris buffer (pH=6.0) for 4 h to remove all additional ions from the root surface and from the apparent free space. Then, rice seedlings were divided into roots and shoots. The remaining procedure was identical to our previous work (Fan et al., 2020). Dried plant tissues were digested with HNO_3-$HClO_4$ (v/v: 4:1) solution. The content of total Cd and Na in rice tissues from different treatments was determined by using inductively coupled plasma-atomic emission spectrometry (ICP–AES, PerkinElmer Optima 700 DV).

2.7 Data analysis

All data obtained were subjected to analysis of variance (ANOVA) and Tukey's multiple range test at a significance level α of 0.01 or 0.05 between the treatments and control (Supporting information M1). The average of four independent biological replications ± the standard deviations (vertical lines) was used. Values within each graph followed by different letter(s) are significantly different ($P< 0.05$). The significance of correlations was judged using tabled values for critical r (significance level α was 0.01 or 0.05).

3 Results

3.1 Effects on plant growth

The measured RGRs for the Na treatments (0, 0.2, 0.6 and 1.1 g/L), Cd treatments (0, 0.2, 1.8, 5.4 mg/L), and Na + Cd treatments at T1 (23°C) and T2 (27°C) are shown in Figure 1. Under Na stress and T1 (T2), the RGRs of both ambient/medium/high CO_2-treated rice seedlings ranged between 6.69-21.07%, 6.85-23.27%, and 9.94-24.58% (8.91-25.29%, 11.35-31.68%, and 19.38-38.23%), respectively. Under Cd stress and T1 (T2), the RGRs of both ambient/medium/high CO_2-treated rice seedlings ranged between 5.43-21.07%, 6.97-23.27%, and 8.06-24.58% (7.80-25.29%, 11.03-31.68%, and 22.34-38.23%), respectively. Under Na+Cd stress and T1 (T2), the RGRs of both ambient/medium/high CO_2-treated rice seedlings ranged between 6.55-21.07%, 7.14-23.27%, and 8.09-24.58% (7.58-25.29%, 10.58-31.68%, and 16.26-38.23%), respectively. Apparently, for all treatments, the growth is positive, i.e., RGR is positive. In all cases, growth was significantly better at 27°C ($P< 0.05$) and at higher CO_2. Growth inhibition of (approximately) 25%, 50% and 75% was observed for salt treatments with Na at 0.2, 0.6 and 1.1 g/L, respectively, and with Cd at 0.2, 1.8, and 5.4 mg/L, respectively. At 23°C, the effect of Na and Cd applied simultaneously was not significantly different from that of individual treatments. At 27°C, a slight depression of growth can be noticed in the combined treatment in relation to the single treatment with Na or Cd only.

3.2 Measured concentration of Cd in rice at different temperatures and CO_2 concentrations

The measured Cd content in rice shoots and roots separated for T1 (23°C) and T2 (27°C) is shown in Figure 2. Under T1 and ambient CO_2 treatments, the concentrations of Cd in rice shoots (roots) with Cd stress ranged between 4.97-13.21 μg/g DW (95.31-1787.23 μg/g DW), while the concentrations of Cd in rice shoots (roots) with Na+Cd stress ranged between 3.80-25.93 μg/g DW (107.5-1411.33 μg/g DW). Under T1 and medium CO_2 treatments, the concentrations of Cd in rice shoots (roots) with Cd stress ranged between 7.31-27.66 μg/g DW (94.46-2652.8 μg/g DW), while the concentrations of Cd in rice shoots (roots) with Na+Cd stress ranged between 8.35-31.25 μg/g DW (126.5-2928.09 μg/g DW). Under the T1 and high CO_2 treatments, the concentrations of Cd in rice shoots (roots) with Cd stress ranged between 7.02-22.67 μg/g DW (95.15-1852.19 μg/g DW), while the concentrations of Cd in rice shoots (roots) with Na+Cd stress ranged between 6.14-22.50 μg/g DW (126.77-1574.02 μg/g DW).

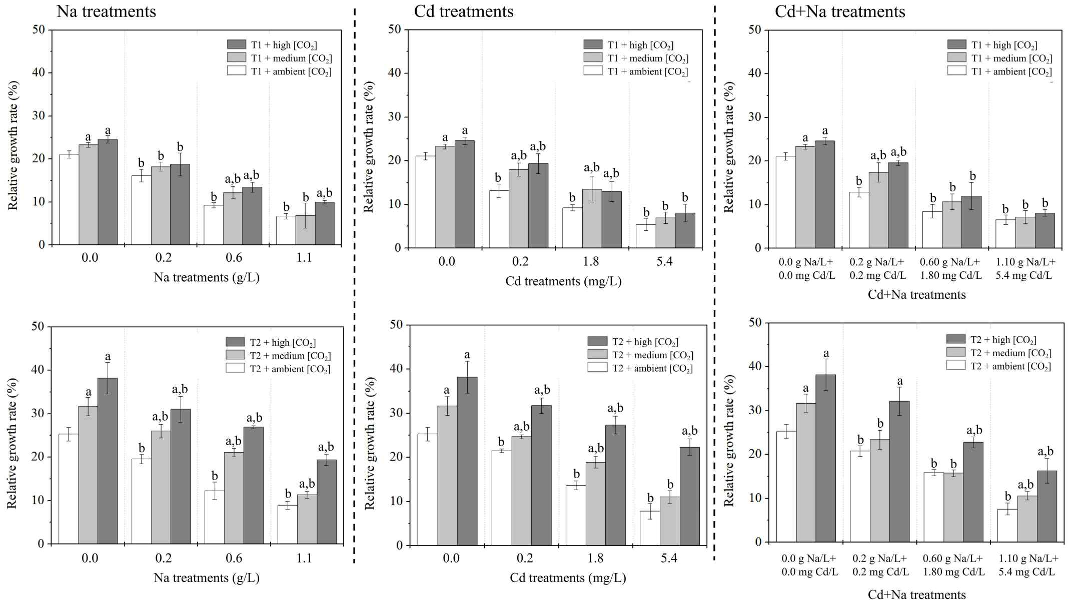

FIGURE 1
Relative growth rate (%) of rice seedlings. Left: Na treatments (0, 0.2, 0.6 and 1.1 g Na/L); middle: Cd treatments 0, 0.2, 1.8, 5.4 mg Cd/L); right; Na + Cd treatments. Top: T1 (23°C); bottom: T2 (27°C). The lowercase letter "a" indicates a significant difference between high/medium CO_2 and ambient CO_2 at the same temperature. The lowercase letter "b" indicates a significant difference between the Cd (i.e., 0.2, 1.8, and 5.4 mg Cd/L), Na (i.e., 0.2, 0.6, and 1.1 g Na/L), Cd+Na (i.e., 0.2 mg Cd/L+ 0.2 g Na/L, 1.8 mg Cd/L+ 0.6 g Na/L, and 5.4 mg Cd/L+ 1.1 g Na/L) treatments and the 0.0 mg/L treatment.

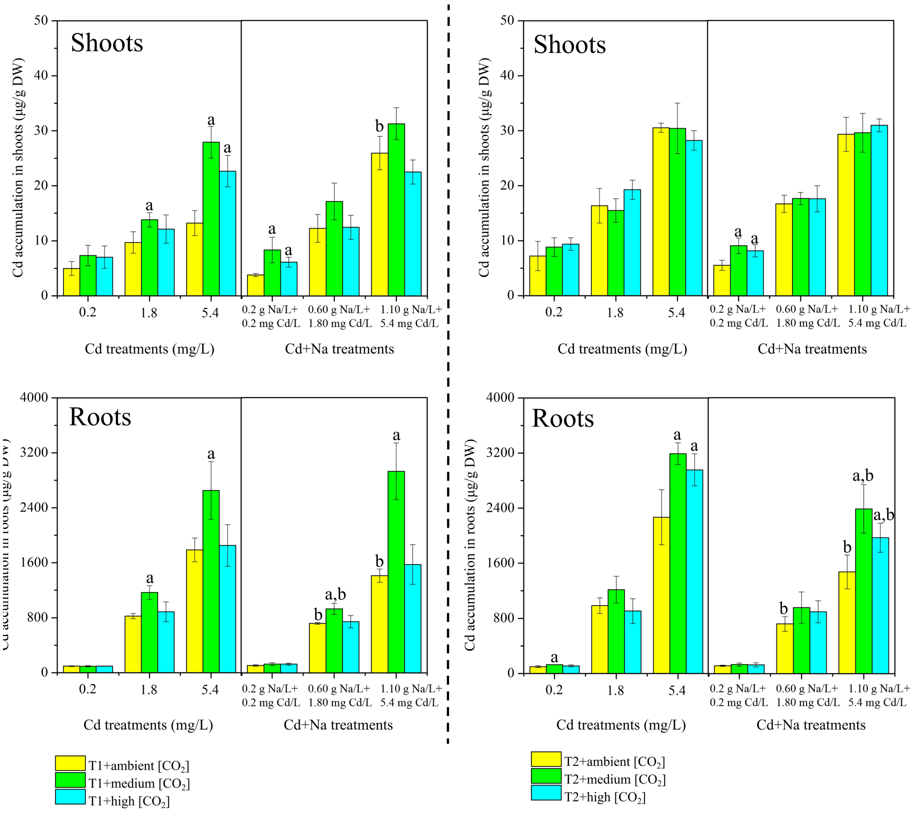

FIGURE 2
Measured concentrations of cadmium (Cd, μg/g dry weight) in rice plants. Top: shoots; bottom: roots. Left side: T1 (23°C); right side: T2 (27°C). The lowercase letter "a" indicates a significant difference between high/medium CO_2 and ambient CO_2 at the same temperature. The lowercase letter "b" indicates a significant difference between Cd treatments and Cd+Na treatments under the same temperature and CO_2 conditions.

Under T2 and ambient CO_2 treatments, the concentrations of Cd in rice shoots (roots) with Cd stress ranged between 7.21-30.55 μg/g DW (97.81-2267.69 μg/g DW), while the concentrations of Cd in rice shoots (roots) with Na+Cd stress ranged between 5.55-29.33 μg/g DW (113.65-1474.37 μg/g DW). Under T2 and medium CO_2 treatments, the concentrations of Cd in rice shoots (roots) with Cd stress ranged between 8.84-30.43 μg/g DW (127.72-3191.39 μg/g DW), while the concentrations of Cd in rice shoots (roots) with Na+Cd stress ranged between 9.09-29.61 μg/g DW (131.20-2387.40 μg/g DW). Under T2 and high CO_2 treatments, the concentrations of Cd in rice shoots (roots) with Cd stress ranged between 9.38-28.23 μg/g DW (110.39-2955.65 μg/g DW), while the concentrations of Cd in rice shoots (roots) with Na+Cd stress ranged between 8.18-30.95 μg/g DW (126.13-1970.16 μg/g DW).

Notably, the concentration of Cd in shoots was much lower than that in roots. In shoots, higher concentrations were observed only at 27°C ($P< 0.05$). At 23°C, there is a trend toward high Cd concentrations in shoots when coexposed to Na and at low and medium CO_2 (360 and 450 ppm), but this is not seen at 700 ppm CO_2 and not at 27°C. At 23°C, the highest Cd concentration is found at 450 ppm CO_2. Again, this trend is not apparent at 27°C, where concentrations of Cd are rather equal for all levels of CO_2. As growth is best at 700 ppm CO_2 and at 27°C, it can be concluded that the total uptake (mass of Cd) must be highest for these conditions.

Additionally, for roots, at 23°C, the highest concentrations of Cd were found with 450 ppm CO_2, and similar concentrations were found for 360 and 700 ppm CO_2. The trend is similar for 27°C but less pronounced, and concentrations are highest at 450 ppm CO_2 and lowest at 360 ppm CO_2. Concentrations of Cd in roots are higher when exposed solely to Cd than when exposed to Cd+Na stress.

3.3 Measured concentration of Na in rice at different temperatures and CO_2 concentrations

The measured concentrations of Na in rice shoots and roots separated for T1 (23°C) and T2 (27°C) are shown in Figure 3. Under T1 and ambient CO_2 treatments, the concentrations of Na in rice shoots (roots) with Na stress ranged between 3011.61-11655.06 μg/g DW (7569.71-12545.83 μg/g DW), while the concentrations of Na in rice shoots (roots) with Na+Cd stress ranged between 2813.5-11340.46 μg/g DW (7453.15-12805.63 μg/g DW). Under T1 and medium CO_2 treatments, the concentrations of Na in rice shoots (roots) with Na stress ranged between 2568.85-8641.81 μg/g DW (5500.33-10866.99 μg/g DW), while the concentrations of Na in rice shoots (roots) with Na+Cd stress ranged between 2220.49-8589.50

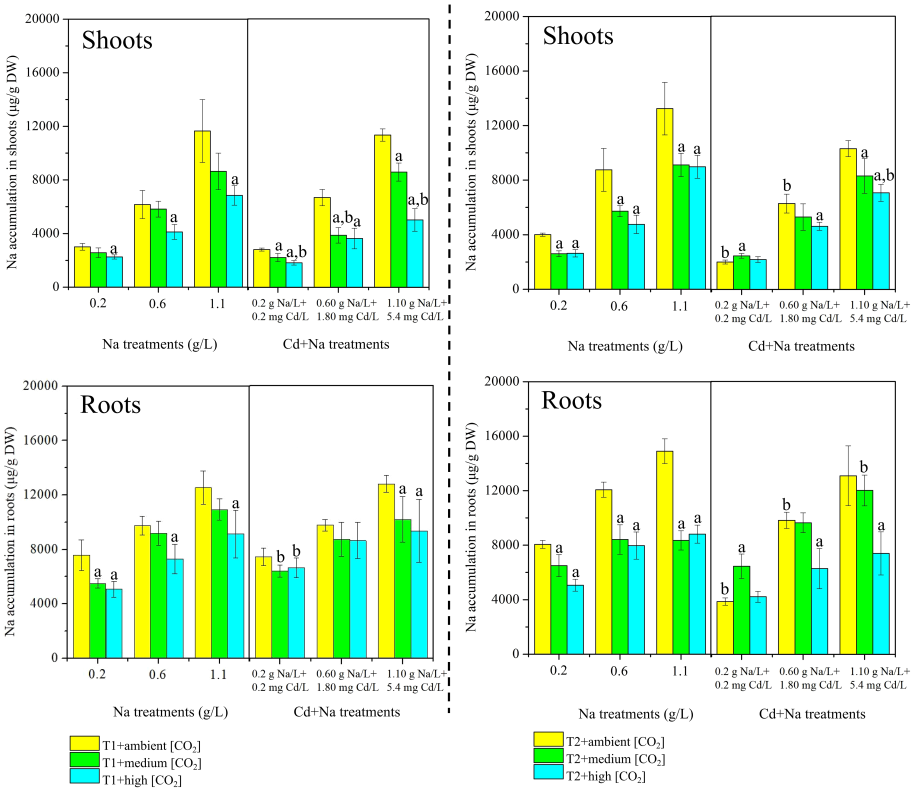

FIGURE 3
Measured concentrations of sodium (Na, μg/g dry weight) in rice plants. Top: shoots; bottom: roots. Left side: T1 (23°C); right side: T2 (27°C). The lowercase letter "a" indicates a significant difference between high/medium CO_2 and ambient CO_2 at the same temperature. The lowercase letter "b" indicates a significant difference between the Na treatments and Cd+Na treatments under the same temperature and CO_2 conditions.

μg/g DW (6425.30-10252.57 μg/g DW). Under T1 and high CO_2 treatments, the concentrations of Na in rice shoots (roots) with Na stress ranged between 2257.15-6848.62 μg/g DW (5067.89-9131.93 μg/g DW), while the concentrations of Na in rice shoots (roots) with Na+Cd stress ranged between 1825.34-5020.30 μg/g DW (6657.62-9354.65 μg/g DW).

Under T1 and ambient CO_2 treatments, the concentrations of Na in rice shoots (roots) with Na stress ranged between 4004.82-13243.01 μg/g DW (8057.37-14898.06 μg/g DW), while the concentrations of Na in rice shoots (roots) with Na+Cd stress ranged between 2006.02-10301.83 μg/g DW (3867.43-13083.43 μg/g DW). Under T1 and medium CO_2 treatments, the concentrations of Na in rice shoots (roots) with Na stress ranged between 2607.55-9115.69 μg/g DW (6500.54-8351.60 μg/g DW), while the concentrations of Na in rice shoots (roots) with Na+Cd stress ranged between 2457.76-8309.92 μg/g DW (6459.82-12013.72 μg/g DW). Under T1 and high CO_2 treatments, the concentrations of Na in rice shoots (roots) with Na stress ranged between 2635.73-8979.19 μg/g DW (5062.06-8811.56 μg/g DW), while the concentrations of Na in rice shoots (roots) with Na+Cd stress ranged between 2187.13-7068.07 μg/g DW (4223.16-7396.10 μg/g DW).

Overall, Na concentrations in roots are higher than in shoots, but the difference is small. The maximum concentrations in roots reached 15 mg/g DW, and those in shoots were >12 mg/g DW. There is little difference in concentrations with/without additional Cd. There is also little difference between concentrations at T1 (23°C) and T2 (27°C). However, for almost all variants, Na concentrations have a strong tendency to be lower at high CO_2 levels.

3.4 Translocation factors of Cd and Na in rice seedlings

The effect of temperature and CO_2 on the translocation factors (TFs) of Cd and Na in rice seedlings is shown in Figure 4. Apparently, the TFs of Cd in rice seedlings decrease with increasing Cd and Cd+Na concentrations. In contrast, the TFs of Na in rice seedlings decreased with increasing Cd and Cd+Na concentrations. Compared with T1 (23°C) at the same CO_2 concentrations, T2 (27°C) promotes the translocation of Cd and Na in rice seedlings.

In addition, at 23°C, medium and high CO_2 generally promoted Cd translocation in rice seedlings under 0.2 mg Cd/L stress, but no significant effect under 1.8 and 5.4 mg Cd/L stress was observed. While medium and high CO_2 have little effect on Na translocation in rice seedlings under 0.2 and 0.6 g Na/L stress, high CO_2 inhibits Na translocation under 1.1 g Na/L stress. At 27°C, high CO_2 had an apparent effect on Cd translocation in rice seedlings under 0.2, 0.6, and 1.1 g Na/L stress. While medium CO_2 inhibits Na translocation in rice seedlings under 0.2, 0.6, and 1.1 g Na/L stress, high CO_2 promotes Na translocation.

3.5 Coexposure to Na and Cd

The measured concentrations of Cd in plants exposed to Cd+Na versus those measured in plants exposed solely to Cd are shown in Figure 5. This underlines what can be seen in Figure 2, that is, the concentrations of Cd in shoots are generally rather similar. At 23°C, there is a tendency for higher Cd in shoots when additionally exposed to Na, but this trend almost vanishes at 27°C. In contrast, for roots, there was a tendency for higher Cd concentrations when solely exposed to Cd, and the deviation between the two exposure sets was stronger at 27°C. An influence of CO_2 is not apparent.

The measured concentrations of Na in plants exposed to Cd+Na versus those measured in plants exposed solely to Na are shown in Figure 6. There is a small tendency for higher concentrations of Na in shoots and roots at 27°C, while at T1, the difference vanishes. Concentrations are highest at low CO_2, but this is the case for both types of exposure (with/without Cd). Overall, both concentrations of Cd and Na in roots and shoots are surprisingly similar, almost independent from the single or combined exposure.

4 Discussion

4.1 The growth of stressed plants improved by higher CO_2 and temperature

Higher temperatures may lead to an increase in the agricultural productivity of plants when the plants are grown in a suboptimal temperature range (Berry and Bjorkman, 1980; IPCC, 2019), and elevated CO_2 in air increases water use efficiency and stimulates the growth of plants (Ainsworth and Rogers, 2007; IPCC, 2019). In general, increases in CO_2 can immediately stimulate photosynthesis and enhance the carbohydrate content, particularly in C_3 plants of rice (Wang et al., 2016). The interactive effects of temperature and CO_2 on the physiology, growth and development of various plant species have also been studied (Wang et al., 2012). However, very few studies control four parameters (i.e., temperature, CO_2, Cd, and Na) simultaneously; thus, the findings presented here are quite unique. Cd and Na (or salt) are two toxic chemicals affecting plant growth and development due to their toxicity (Das et al., 1997; Zhu, 2001; Singh et al., 2013; Haider et al., 2021). In the present study, we noticed that individual or combined treatments of Cd and Na significantly decreased the RGR of rice seedlings, but the synergistic or antagonistic effects of Cd and Na seem not significant, particularly at T1 (23°C) or high CO_2. In addition, the T2 (27°C) and medium/high CO_2 levels significantly increased the RGR of stressed plants compared to the T1 (23°C) and ambient CO_2 levels. Therefore, we believe that increasing temperature can stimulate the activities of enzymes in plants, such as RuBisCO (Suganami et al., 2021), and elevated CO_2 can improve the carboxylation capacity of RuBisCO in plants, which can be converted into nonstructural carbohydrates and some detoxicants under ionic stress conditions (Bazinet et al., 2022), thereby reducing phytotoxicity and promoting plant growth.

Several studies have examined the effect of elevated CO_2 on the toxicity of Na or salt to plants. These studies revealed that the salt tolerance of plants increases at elevated CO_2, while negative effects have not been observed (Pérez-López et al., 2009; Geissler et al., 2015; Yu et al., 2015). The effect of CO_2 on the uptake of Na by

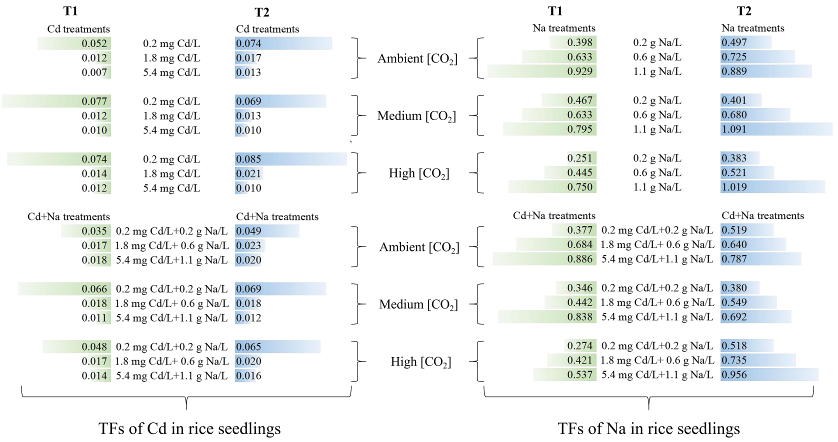

FIGURE 4
Translocation factors of Cd and Na under different temperature and CO_2 conditions.

plants occurs in addition to that of increased temperatures. Eller et al. (2014) studied the mutual effects of elevated temperature and CO_2 on the tolerance of plant species towards soil salinity and found that a temperature increase of 5°C and 700 ppm CO_2 alleviated salt stress in two phenotypes of *Phragmites*. The contribution of each factor was not investigated.

4.2 Effects of temperature and CO_2 on the distribution and translocation of Cd and Na

It is evident that exposed rice contained larger quantities of Cd in roots than in shoots (Figure 2), while the content of Na in rice shoots was rather similar to that in roots (Figure 3). Moreover, the

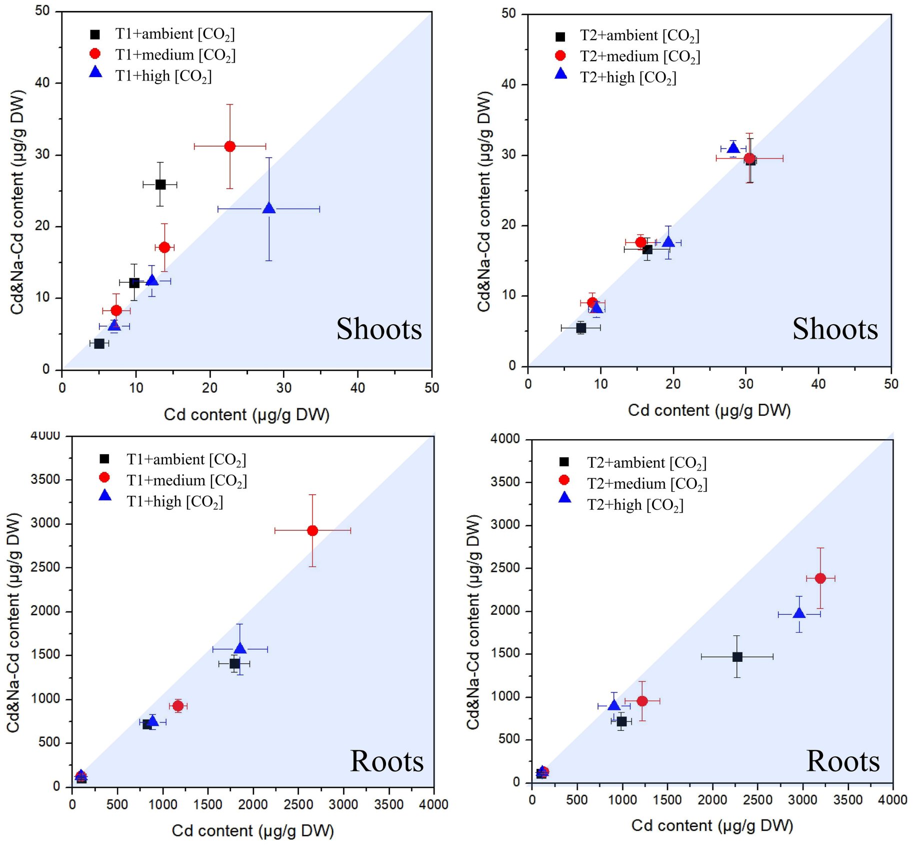

FIGURE 5
Plot of measured cadmium concentrations in plants exposed to Cd+Na (Y-axis) versus those measured in plants exposed solely to Cd (X-axis); top: shoots; bottom: roots; left side: T1 (23°C); right side: T2 (27°C).

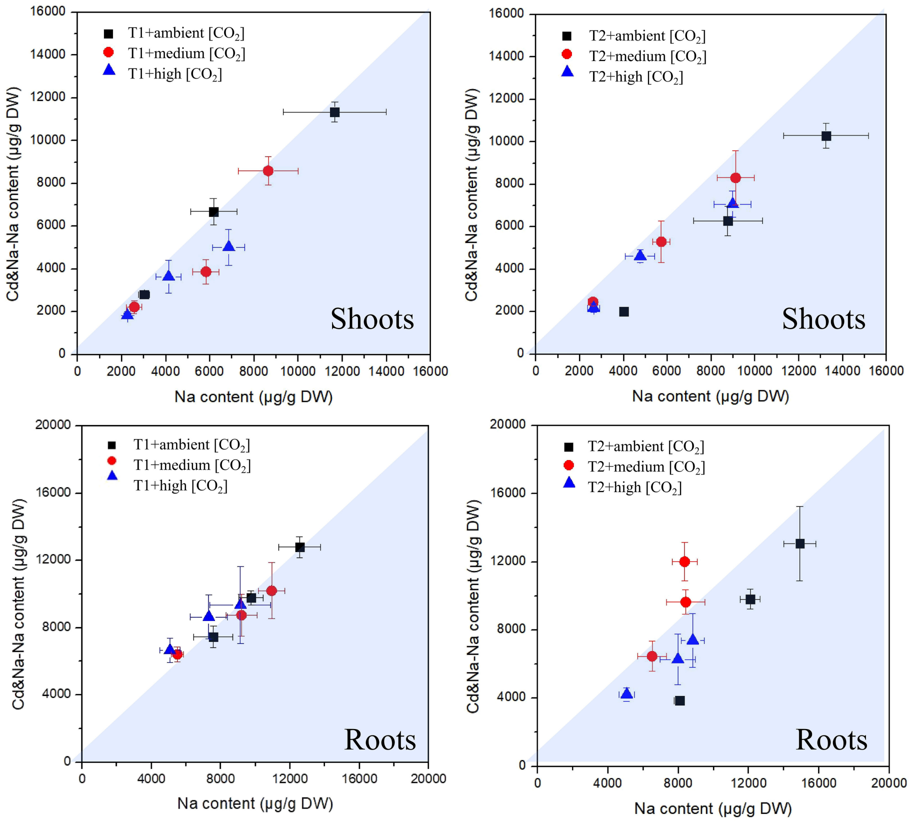

FIGURE 6
Plot of measured sodium concentrations in plants exposed to Cd+Na (Y-axis) versus those measured in plants exposed solely to Na (X-axis); top: shoots; bottom: roots; left side: T1 (23°C); right side: T2 (27°C).

individual and mutual effects of temperature and CO_2 on the distribution and translocation of Cd and Na in rice seedlings were not identical.

4.2.1 Temperature effects at the same CO_2 level

Similar to evaporation from soil, the transpiration of water from plant leaves increases with higher temperatures. This leads to a more rapid transport of solutes into and through plants. Additionally, enzymatic processes accelerate with temperature (Arrhenius Law). In this study, we noticed that the content of Cd in rice roots and shoots under Cd stress increased with increasing temperature in all CO_2 treatments. However, ample studies have shown that the temperature-mediated uptake and translocation of Cd and Na in plants are different. Herein, we noticed that increasing temperature promoted the uptake and accumulation of Na in rice tissues under Na stress at high CO_2 while inhibiting the uptake and accumulation of Na at medium/ambient CO_2. Unlike for Cd, the cell wall cannot act as a barrier to Na uptake, and uptake of Na from environmental media into roots is passive and follows the transpiration stream (Hussain et al., 2017), with subsequent active (enzymatic) exclusion (Trapp et al., 2008). In contrast, the degree to which temperature can affect Cd accumulation depends on the chemistry of the root zone, plant growth, and biomass (Fritioff et al., 2005; Peer et al., 2005). Root immobilization can elucidate substantial amounts of accumulated metals in plants and is affected by temperature, which can modify the structure and function of cell walls by inducing the synthesis of plant hormones (Liu et al., 2019; Cheng et al., 2020). In line with this, we found that the root-to-shoot TFs of Cd under T2 (27°C) were generally higher than those under T1 (23°C). Ge et al. (2016) also found an enhanced translocation of Cd from roots to shoots at elevated temperatures for rice.

4.2.2 CO_2 effects at the same temperature

The responses of Cd and Na in rice tissues to varying CO_2 concentrations in air were different. The concentration of Cd in rice tissues and, in particular, in roots increased at 450 ppm CO_2 but decreased at 700 ppm CO_2. The concentration of Na in rice tissues significantly decreased with increasing CO_2 concentration and was lowest at 700 ppm CO_2. This suggests that the uptake and translocation of Cd and Na are affected differently by the variation in CO_2. Indeed, elevated CO_2 resulting in higher Cd concentrations in plant tissues has been observed in different plant species (Li et al., 2010; Guo et al., 2011; Guo et al., 2015). Tang et al. (2003) and Wu et al. (2009) demonstrated that elevated CO_2 might lower the pH of the rhizospheric environment, which leads to a decline in the K_d of Cd and other heavy metals (Sheppard, 2011) and thereby promotes the plant uptake of heavy metals. In addition, the influence of elevated CO_2 on cell wall properties might explain this finding. Genes encoding expansins, esterase, and xyloglucan endotransglycosylase, which play important roles in cell wall loosening, are upregulated at elevated CO_2 and thus facilitate plant tissue expansion (Gamage et al., 2018). Plant hormones also play a major role in accumulating Cd in plants at elevated CO_2 (Wei et al., 2013) because plant hormones such as ethylene, ausins, gibberellic acids, and cytokinins can synergistically regulate cell division and expansion and control root and shoot elongation (Seneweera et al., 2003; Cato et al., 2013), thereby providing a larger zone for Cd storage in plants. The expression of metal tolerance protein genes in plants, such as the MTP family, may be activated by elevated CO_2, enhancing their metal tolerance (El-Sappah et al., 2021a; El-Sappah et al., 2021b). On the other hand, a CO_2-stimulated increase in plant biomass may lead to dilution and thus lower tissue concentrations of Cd and other heavy metals despite increasing uptake (Duval et al., 2011). Therefore, the mechanisms involved in CO_2-mediated Cd uptake and translocation in plants should be further investigated by using physio-biochemical analysis and multiomics approaches.

Transpiration and thus stomatal conductance are some of the most important factors affecting the uptake and translocation of Cd and Na in plants (Bauer-Gottwein et al., 2008; Trapp et al., 2008). Herein, we noticed that high CO_2 inhibited the translocation of Cd and Na in rice seedlings. Based on the reports by Ainsworth and Long (2005) and Leakey et al. (2009), the stomatal conductance of plant leaves decreased under elevated CO_2. Moreover, elevated CO_2 increases outwards rectifying K^+ channels relative to inwards rectifying K^+ channels, resulting in stomatal closure (Brearley et al., 1997). Apparently, stomatal closure directly reduced the transpiration stream of rice seedlings, eventually inhibiting Na uptake and translocation. Additionally, a previous study suggested that elevated CO_2 reduced transpiration by 30%, which not only affected root element acquisition by reducing mass flow into plants but also diminished the accumulation of elements in shoots through the translocation of sylem sap. Another study confirmed that elevated CO_2-mediated impairment of element translocation into the shoots is related to a specific effect on xylem morphogenesis (Cohen et al., 2019).

4.3 Effects of a mixture of Cd and Na on rice seedlings

To evaluate the effect of mixtures of Cd and Na on rice seedlings under different temperature and CO_2 conditions, we determined the Cd and Na contents in rice tissues under coexposure to Cd and Na. In general, the content of Cd in rice tissues under Cd+Na stress was higher than that under Cd stress under T1+medium CO_2 conditions, while the content of Cd in rice tissues under Cd+Na stress was lower than that under Cd stress under T2+ambient/medium/high CO_2 conditions. In addition, the content of Na in rice tissues under Cd +Na stress was generally lower than that under Na stress under T1 (T2)+ambient/medium/high CO_2 conditions. Interestingly, the interaction was not very pronounced but observable. These results implied that the temperature should be considered when CO_2 is used as the regulator in the phytoremediation of Cd/Na/Cd+Na pollution. In addition, we noticed that the concentration of Cd in roots was depressed by coexposure to Na and more at T2, while with T1, more Cd was translocated into shoots. These changes indicate that the uptake processes of Cd and Na in rice are mostly independent, and only minor interactive effects occurred during exposure to mixtures of Cd and Na. A plausible explanation is that both molecules have very different radii and different charges (Na^+ versus Cd^{2+}), and it is unlikely that the same transporter enzymes are involved. However, Cd and Na are both cations and might compete for electrically charged adsorption sites. This may be the mechanism behind the lower Cd concentration in roots when the Na concentration is high. The cytosolic ionic strength was 0.3 M, in xylem sap at 0.01 (Sitte et al., 1991). The highest measured concentrations of Na (15.0 g/L) correspond to a molality of 0.6 M. According to the Debye-Hückel theory (Debye and Hückel, 1923), the chemical activity of a bivariate ion decreases drastically at such an ionic strength, which can explain the elevated concentrations of Cd in roots and the stimulated transport of Cd into shoots in the presence of Na. Hence, the effects of Na on the uptake of Cd may be attributed to physico-chemical processes. This is less the case the other way round because molalities due to intracellular Cd concentrations are much smaller than those of Na. Generally, Cd shows a much higher concentration ratio of roots to external solution than Na and thus much higher adsorption. Overall, the effects of Cd in coexposure on Na uptake are also rather small and not very clear. The small changes in uptake when the mixture is applied may be among the reasons why the toxic effects are not more pronounced due to coexposure. It is likely that the modes of action of Na and Cd on rice seedlings are not the same and thus do not add (ECETOC, 2007).

5 Conclusions

This work clarifies the simultaneous effects of changing temperatures and CO_2 concentrations on the mutual uptake, translocation and effects of Na and Cd on rice seedlings. The hydroponic experimental results presented here showed the individual and mutual effects of temperature and CO_2 on Na and Cd tolerance of rice seedlings, judged by the biomass, Na/Cd distribution and translocation. The biomass response suggested that Cd and Na exposure significantly inhibited plant growth, but T2 and medium/high CO_2 levels could alleviate the toxicity exerted by Cd and Na. Interestingly, the synergistic or antagonistic effects of Cd and Na were not significant, particularly at T1 or high CO_2. The highest concentrations of Cd were observed at T2 and with 450 ppm CO_2. Na accumulation in rice seedlings increased with temperature but was reduced by higher CO_2. In summary, the optimum conditions to achieve low Cd concentrations in rice shoots are T1 and low or high CO_2, while with respect to the toxic effects of Na and Cd alone or in mixture, they are T2 and high CO_2. Another conclusion from the presented findings is that CO_2 concentrations in laboratory studies with terrestrial plants have a strong impact on the results and should be monitored and reported. Further comprehensive studies are needed to clarify the possible tolerance mechanisms involved in RNA (e.g., mRNA, lncRNA, small RNA, and circRNA), proteins, and metabolites.

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

Conceptualization, Methodology, Supervision, Writing-reviewing and editing, and Funding acquisition, ST and X-ZY. Writing-original draft preparation, and Visualization, Y-XF. Investigation, Data analysis, Visualization, PT, C-ZL, and QZ. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1161334/full#supplementary-material

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Interplay of silymarin and clove fruit extract effectively enhances cadmium stress tolerance in wheat (*Triticum aestivum*)

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Introduction: Osmoprotectant supplementation can be used as a useful approach to enhance plant stress tolerance. However, the effect of silymarin and clove fruit extract (CFE) on wheat plants grown under cadmium (Cd) stress has not been studied.

Methods: Wheat seeds were planted in plastic pots filled with ions-free sand. A ½-strength Hoagland's nutrient solution was used for irrigation. Pots were treated with eight treatments thirteen days after sowing: 1) Control, 2) 0.5 mM silymarin foliar application [silymarin], 3) 2% CFE foliar application [CFE], 4) CFE enriched with silymarin (0.24 g silymarin L-1 of CFE) [CFE-silymarin], 5) Watering wheat seedlings with a nutritious solution of 2 mM Cd [Cd]. 6) Cadmium + silymarin, 7) Cadmium + CFE, and 8) Cadmium + CFE-silymarin. The experimental design was a completely randomized design with nine replicates.

Results and discussion: The Cd stress decreased grain yield, shoot dry weight, leaf area, carotenoids, chlorophylls, stomatal conductance, net photosynthetic rate, transpiration rate, membrane stability index, nitrogen, phosphorus, and potassium content by 66.9, 60.6, 56.7, 23.8, 33.5, 48.1, 41.2, 48.7, 42.5, 24.1, 39.9, and 24.1%, respectively. On the other hand, Cd has an Application of CFE, silymarin, or CEF-silymarin for wheat plants grown under Cd stress, significantly improved all investigated biochemical, morphological, and physiological variables and enhanced the antioxidant enzyme activities. Applying CFE and/or silymarin enhanced plant tolerance to Cd stress more efficiently. Our findings suggest using CFE-silymarin as a meaningful biostimulator for wheat plants to increase wheat plants' tolerance to Cd stress *via* enhancing various metabolic and physiological processes.

KEYWORDS

antioxidant system, heavy metals, osmoprotectants, plant growth regulators, plant stress

1 Introduction

Wheat (*Triticum aestivum*) is one of the most important grain crops for food safety worldwide (Li et al., 2022). It is considered the most stable staple food resource, supplying the world population with a considerable proportion of the required calories (Desoky et al., 2021a). Annually, the world produces about 600 million tons grown on only 200 million hectares (Sowell and Swearingen, 2022). Since wheat is counted as the primary food crop in most regions of the world, advanced and effective methods must be followed to mitigate the harmful impacts of stressors on wheat production (Mansour et al., 2020). With urbanization and industrialization in the modern era, heavy metal (HM) pollution has been a prime obstacle to sustainable agricultural development (El-Sappah and Rather, 2022). Soil or water contamination with HM has expanded due to long-term waste recycling, industrial activities, sewage-based irrigation, and agrochemicals threatening human health and food security (Desoky et al., 2020a). Plants cultivated in HM-affected soil lead to the accumulation of such toxic metals in plant tissues, which causes morphological deformation and physio-biochemical disorders, resulting in severe damage during plant growth and development (Tiwari and Lata, 2018; El-Sappah et al., 2021b; Rady et al., 2023).

Cadmium (Cd) is not an essential nutrient for plants which was identified as a highly phytotoxic HM (Haider et al., 2021; Abbas et al., 2022.), as well as being a highly water-soluble element makes plants absorb it easily (Desoky et al., 2019a; 2019b). It replaces calcium (Ca) due to its similar chemical behavior, radius, and ionic charges. The toxicity of Cd aggravates the hyper-accumulation of the different reactive oxygen species (ROS;e.g., O^{2-}, H_2O_2, and OH^-), exacerbating cell redox homeostasis disruption and abnormalities to the plant organelles structures and cellular membranes (Yadav et al., 2012; Haider et al., 2022). Moreover, Cd could severely change many enzyme activities, including those involved in photosynthesis, carbon dioxide fixation, nitrogen (N), phosphorus (P) and carbohydrate metabolism, and rubisco (Tiwari and Lata, 2018), as well as interfere with gene expression, and signalling processes (Srivastava et al., 2017). Therefore, long-term exposure to Cd could adversely affect plants by decreasing the photosynthetic pigments contents and photosynthetic efficiency and inhibiting plant growth (Haider et al., 2021). Cd exposure reduces plant physiological activity by decreasing tissue water content, transpiration, and stomatal conductance (Gill and Tuteja, 2010). As a stress-counteract mechanism, the plant developed antioxidative machinery, including enzymatic and nonenzymatic antioxidants to scavenge oxidative stress biomarkers (Alzahrani and Rady, 2019; El-Sappah et al., 2021a; Kumari et al., 2021). Nevertheless, throughout most cases, internal defense systems were insufficient to protect plants from stress (Abd El-mageed et al., 2022). Therefore, several approaches are required for promoting Cd tolerance in crops, such as plant growth biostimulants. Exogenous support biostimulants/elicitors, such as natural plant extracts, may raise the plant's tolerance to Cd stress.

Silymarin is the primary bioactive substance extracted from all parts of the *S. marianum*; Milk thistle. plant (Ali et al., 2022). Silymarin comprises flavonolignans and a flavonoid mixture such as silibinin, silydianin, isosilychristin, isosilybin, silychristin, and taxifolin (Marmouzi et al., 2021). Accumulating such active substance (i.e., silymarin) in plant tissues highly interacts with environmental stresses, having antioxidant properties, directly detoxifying and preventing ROS formation *via* inhibiting ROS production (Surai, 2015). As a result, silymarin or silymarin-fortified biostimulants have been used recently to help plants withstand environmental stresses (Ali et al., 2022). Additionally, silymarin helps maintain mitochondrial integrity and cell redox homeostasis by activating nonenzymatic and enzymatic antioxidants (Ali et al., 2022). Under severe water stress, milk thistle (silymarin) accumulates more silymarin and related components, associated with increased enzymatic antioxidants activity and chalcone synthase gene expression, inducing drought stress tolerance (Elsayed et al., 2019). Thus, exogenous support biostimulants like silymarin can potentially increase plant tolerance to Cd stress, but this is still only speculative.

Plant biostimulants are foliar fertilizers that modify the plant's metabolic processes, significantly improving plant growth and production (European Biostimulants Industry Council, 2012; Mashamaite et al., 2022; Azzam et al., 2022). Some growth-promoting materials like antioxidants, osmoprotectants, phytohormones, and nutrients are found in plant extracts such as the seed extract of moringa (*Moringa oleifera* L.) and the root extract of licorice (*Glycyrrhiza glabra*) (Desoky et al. 2019a; Desoky et al. 2019b). These substances significantly strengthen the antioxidant defense systems in the plant to improve its ability to face different environmental stresses. Rady and Mohamed (2015) and Desoky et al. (2020b) reported that plant extracts significantly improve plant growth and productivity. Lately, Desoky et al. (2021b) have considered clove (*Syzygium aromaticum*) fruit extract (CFE) as one of these extracts. However, till now, scientists have not conducted any studies on using CFE as a bioactive stimulant to enhance plants' tolerance to Cd stress. Furthermore, the integrative effect of silymarin and CFE on wheat plants grown under Cd stress has not been thoroughly studied.

The primary goal of this study was to determine how CFE, when combined with silymarin, improved plant physio-biochemistry, nonenzymatic antioxidants, enzyme activities, and developmental characteristics in Cd-stressed wheat plants. Because they contain high levels of osmoprotectants and antioxidants such as phenolic compounds, glutathione, ascorbic acid, -tocopherol (-TOC), salicylic acid, soluble sugars, proline, amino acids, selenium, and vitamins, the chemical composition of these biostimulants has the potential to improve plant performance (i.e., E, B, and A). They also have a high concentration of phytohormones such as zeatin-type cytokinins, gibberellins, auxins, and others. Thus, we hypothesize that the co-addition of CFE and silymarin will improve the wheat plant's tolerance to Cd stress and ultimately increase wheat production. This hypothesis could be achieved by identifying a set of morpho-physio-biochemical attributes to study the effect of CFE and silymarin on wheat plants grown under Cd stress conditions.

2 Materials and methods

2.1 Materials, growth conditions, experimental treatments, and CFE preparation

Healthy grains of wheat (*Triticum aestivum* L., cv. Misr 2) were purchased from the Wheat Research Section, Agronomy Research

Institute, Agriculture Research Centre, Giza, Egypt. For one-minute, wheat grain surfaces were sterilized utilizing 0.1% $HgCl_2$. After that, seeds were swilled in deionized-sterilized water, air dried then implanted in plastic pots with a depth of 35 cm and diameter of 40 cm. The pots were filled with 10 kg and used as a growth medium. This sand was free from any cations or anions. Fifteen grains/pot were sown at equal distances and depths. After two weeks from sowing, seedlings were thinned to eight seedlings/pot. Under an open green-house condition, the experiments were conducted with 62.0–65.1% humidity and a day/night temperature of 19 ± 3/10 ± 2°C. Plants were irrigated using 0.5 strength Hoagland's nutrient solution (Hoagland and Arnon, 1938). After an interval of every two days, a nutrient solution free from any stress treatments was used to moisten the soil in all pots to the field capacity up to full emergence. The cadmium (Cd; Sigma-Aldrich, St. Louis, MO, USA) procedure was performed one month after sowing. The preliminary study used Cd sulfate (CdSO4) as a Cd source to make a solution concentrated with 2 mM of Cd (Alzahrani et al., 2018).

Silymarin (Sigma-Aldrich, St. Louis, MO, USA) and CFE, or silymarin-enriched CFE, was added as a foliar application and sprayed seven days after the first addition Cd solution. The addition rates of CFE, silymarin, and silymarin-enriched CFE were 2%, 0.5 mM, or 0.24 g Sm L^{-1} of CFE, respectively. Seven and fourteen days later, another two foliar sprays were done. This timeline is based on our previous study (Alharby et al., 2020). A few drops of Tween-20(1%) were used as a surfactant to improve the spray solution adherence performance. Continuous measurements were performed to keep the Cd concentration at 2 mM using the Optima 3300DV ICP-MS instrument (Perkin-Elmer, Inductively Coupled Plasma, Waltham, Mass Spectrometer, MA 02451, USA). Thirty days after the initial Cd addition, the trials were accomplished. A completely randomized design (CRD) with nine replicates was done to arrange the trial pots. The following eight treatments were performed:

1- Control: no stress and foliar spray.
2- Silymarin: Foliar spray with 0.5 mM silymarin.
3-CFE: Foliar spray with 2% CFE.
4-CFE-silymarin: Foliar spray with CFE enriched with silymarin (0.24 g silymarinL^{-1} of CFE).
5-Cd: Watering wheat seedlings with a nutritious solution of 2 mM Cd.
6- Cd+ silymarin: Watering wheat seedlings with a nutritious 2 mM Cd + foliar spray solution with 0.5 mM silymarin.
7-Cd+CFE: Watering wheat seedlings with a nutritious solution of 2 mMCd + foliar spraywith 2% CFE.
8- Cd+CFE-silymarin:Watering wheat seedlings with a nutritious solution of 2 mM Cd + foliar spraywith CFE enriched with silymarin (0.24 g silymarinL^{-1} of CFE).

2.2 Preparation and analysis of clove fruit extract

Clove fruit was extracted by weighing 10 g of clove fruits, drying, and soaking it in a liter of water at 50°C for 24 hours and then filtering and supplementing the final volume to a liter. The chemical analysis of the extract is represented in Table 1.

2.2.1 Determination of total phenolic compounds, total flavonoids of CFE

TPC content in CFE was estimated by a UV spectrophotometer (Jenway-UV–VIS Spectrophotometer 6705) because of a colorimetric reduction of the reagent by phenolic compounds utilizing the formation of a blue complex, as described by Škerget et al. (2005). The oxidative reagent used was the Folin-Ciocalteu reagent (AOAC, 1990). TPC quantity has been shown in Table 1 as mg GAE g^{-1} extract. TF content was determined according to Ordonez et al. (2006). Table 1 present the TF contents of the extract as mg quercetin equivalent/g extract (mg QE g^{-1}).

TABLE 1 Chemical constituents of clove fruit extract (CFE) (on a dry weight basis).

Component	Value
1. Total phenolic compounds (TPC; mg GAE/g CFE)	324
2. Total flavonoids (TF; mg QE/g CFE)	34.7
3. Phenolic compounds (mg/g CFE)	
3,4-Dihydroxybenzoic-acid	0.74
Ellagic-acid	0.62
Eugenol	105
Eugenyl-acetate	86.4
Gallic-acid	18.3
Naphthalene	0.21
Tannic acid	0.78
Vanillin	1.49
4. Antioxidants and osmoprotectants:	
Total free amino acid(g/kg)	70.2
Free proline (mg/kg)	19.0
Soluble sugars (mg/kg)	55.6
5. Mineral nutrients (g/kg)	
Mg	3.20
Ca	12.3
Fe	1.30
P	11.8
K	16.5
N	16.9
6. Vitamins (mg/kg)	
Vitamin A	25.6
Vitamin E	55.2
Vitamin D	32.4
Vitamin C	36.9

2.2.2 Antioxidant activity of CFE

1, 1-Diphenyl-2 picrylhydrazyl (DPPH$^{\cdot}$) *radical-antioxidant activity:* The electron donation ability of the extracts was measured by bleaching of the DPPH$^{\cdot}$ purple-colored solution Hanato et al. (1988). β-Carotene/linoleic acid bleaching: The capacity of CFE and synthetic antioxidants (gallic acid and TBHQ) for preventing the β-carotene bleaching was evaluated according to Dastmalchi et al. (2007). Ferric reducing antioxidant power (FRAP): The FRAP was measured following a methodology described by Gülçin et al. (2010). Tert-butyl hydroquinone (TBHQ) and gallic acid were regarded as positive control of all three methods. Three replicates were analyzed for all samples (Figure 1).

2.2.3 Determination of phenolic and flavonoid compounds by High-performance liquid chromatography

HPLC analysis was executed according to (Sati et al., 2020) with slight modifications using an Agilent Technologies 1100 series liquid chromatograph equipped with an autosampler (Table 1).

2.3 Wheat morphological traits

The plants were harvested after 35 days of Cd application. Samples were collected from each treatment to evaluate plant height (cm), leave area (cm^2), and shoot's dry weight (g plant^{-1}). At the harvest phase, samples were collected from ten randomly selected plants to estimate 1000 grain weight (g), the number of grains per spike, and grain yield (g plant^{-1}).

2.4 Determinations of chlorophyll content, PSII quantum yield, and CO_2 fixation rate

According to Fadeel (1962), pure acetone was used to extract total carotenoids and total chlorophyll from fresh leaves. Absorbance readings were recorded at 480 nm, 645 nm, and 663 nm using a spectrophotometer (Beckman 640D, USA) to calculate the contents of pigmentsin mg g–1 leaf fresh weight by utilizing a portable photosynthesis system (LF6400XTR, LI-COR, USA) stomatal conductance (gs), leaf net photosynthetic rate (Pn) and rate of transpiration (Tr), were estimated. Measurements were done between 09:00–11:00 a.m.

2.5 Determinations of relative water content, membrane stability index, inorganic ions leaked, malondialdehyde, leaf soluble sugars, and proline

The method by Barrs and Weatherley (1962) was used to estimate the relative water content (RWC). The fresh weight (FW) of the leaves was measured, and the leaves were left drenched in water for 3 hours. Then, the turgid weight (TW) of the leaves was calculated. The samples were then dried in an oven at 80°C for 24 hours and weighed (DW). The RWC was determined using the following formula: RWC = [(FW – DW)/(TW – DW)] × 100.

The membrane stability index (MSI) was determined using 200 mg of a fresh leaf (two sets) in test tubes containing 10 cm^3 of double-distilled water. One group of samples was heated at 40°C for 30 minutes. EC was recorded on a conductivity bridge (C1). The second group of samples was boiled at 100°C for ten minutes in a boiling water bath, and EC was measured (C2). As in the study by Premchandra et al. (1990), modified by Rady (2011), MSI was calculated using the following formula: MSI (%) = (1–[C1/C2]) × 100.

The method Sullivan and Ross (1979) described was used to measure the total ions seeped from leafy tissue. We measured the electrical conductivities (ECs) of the 20-leafy tissue disc solution three times, after 30 and 10 minutes of heating (45-55°C) and boiling (100° C), respectively. The following formula was utilized to compute EL:

$$EL(\%) = [(EC2 - EC1)/EC3] \times 100$$

Malondialdehyde (MDA) content (μmol g^{-1} FW) was measured in 0.1 g leaf homogenized in Na-phosphate buffer. The homogenate was centrifuged under cooling at 20,000 × g for 25 minutes. The supernatant was read at 532 nm and corrected for nonspecific turbidity at 600 nm, following the methods proposed by Heath and Packer (1968).

Total soluble sugars content was assessed: 0.2 g leaves were washed with 5 ml 70% ethanol and homogenized with 5 ml 96% ethanol. The extract was centrifuged at 3500 × g for 10 min. The supernatant was collected and stored at 4°C (Irigoyen et al., 1992). Freshly prepared anthrone (3 ml) was added to 0.1 ml supernatant. This mixture was incubated in a hot water bath for 10 min. The absorbance was recorded at 625 nm with a Bausch and Lomb- 2000 Spectronic Spectrophotometer

Bates et al. (1973) used the method to determine proline accumulation in bean leaves. Second fresh leaf material 0.1 g was ground with 10 ml of 3% (w/v) aqueous sulphosalicylic acid, and the homogenate was filtered through Whatman 2 filter paper. One milliliter of the filtrate was reacted with a one-milliliter acid ninhydrin reagent and one-milliliter glacial acetic acid in the test tube for one hour at 100°C. The reaction terminated in an ice bath. Two-milliliter Toluene was added to the mixture, and the upper toluene layer was measured absorbance at 520 nm using a UV spectrophotometer.

2.6 Evaluation of oxidative stress (hydrogen peroxide, and superoxide) and non-enzymatic antioxidant compounds

A 30 mM potassium phosphate buffer pH 7.4 + 8.4% H_2PO_4 + 2.5% TCA+ 0.3% $FeCl_3$ + 0.8% bipyridyl was used as a constituent to receive the extract to determine the extract's total content of Ascorbate AsA (μmol g^{-1} FW) (Kampfenkel and Van Montagu, 1995). The reaction was allowed to occur at 40oC and for only 30 minutes, and the readings were recorded at 525 nm absorbance. The method of Griffth (1980) was carried out to assess the content (μmol g^{-1} FW) of reduced and total glutathione (GSH). The content of GSH was estimated utilizing a unique reaction mixture consisting of leaf extract, sodium phosphate (7 mM) buffered at pH 6.8 with 5,5-dithiobis-(2-nitrobenzoic acid); DTNB (6 mM), and sodium phosphate (130 mM) buffered at pH 7.4. The reaction mixture

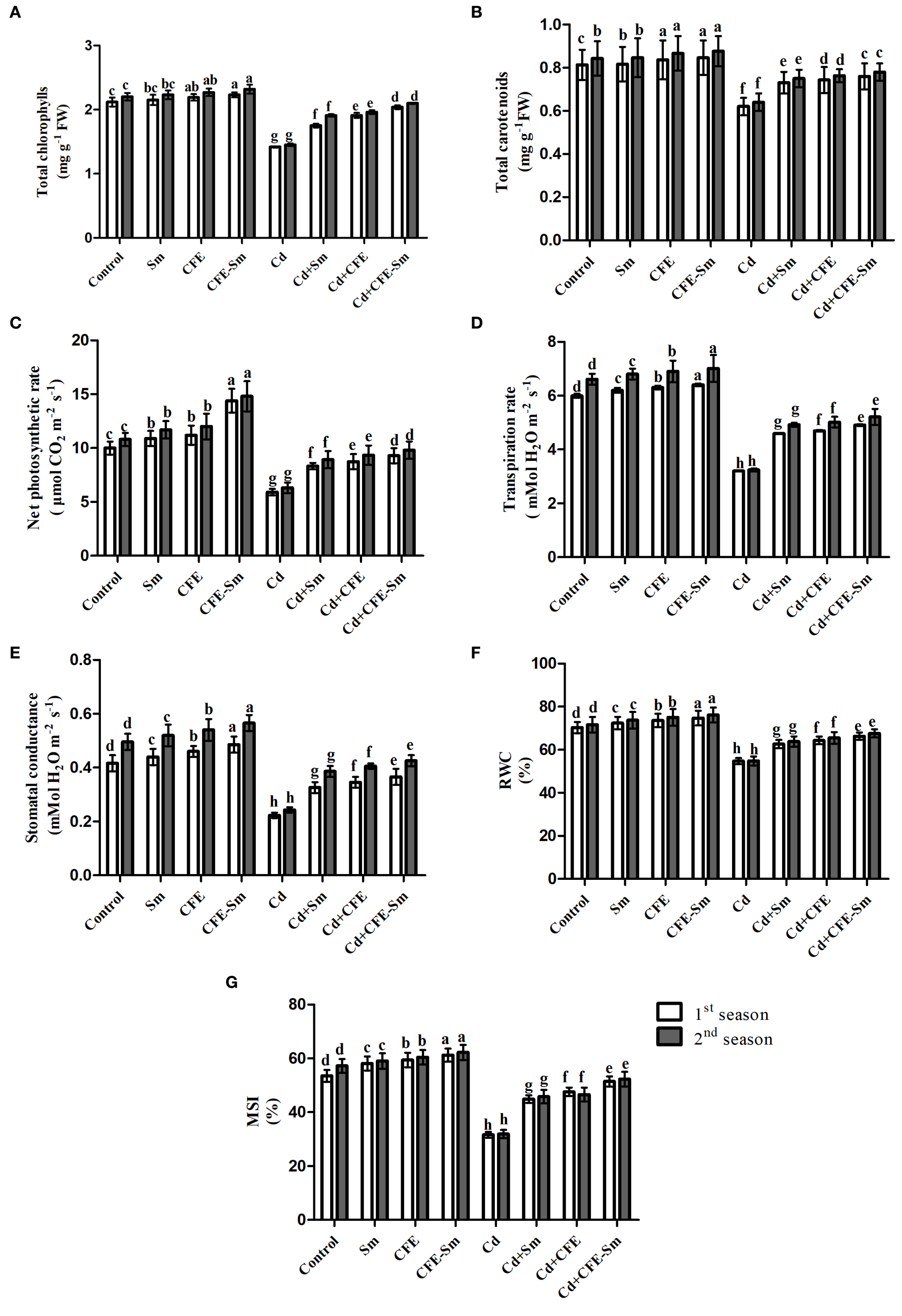

FIGURE 1
Physiological parameters of the wheat plant under Cd stress after foliar application of silymarin (Sm), clove fruit extract (CFE), or silymarin-enriched clove fruit extract (CFE-Sm). **(A)** total chlorophylls, **(B)** total carotenoids, **(C)** net photosynthetic rate, **(D)** Pn; transpiration rate (Tr); **(E)** Stomatal conductance (Gs), **(F)** relative water content (RWC), and **(G)** membrane stability index (MSI).

was left for only 10 minutes at 30°C, and the readings were recorded at 412 nm absorbance. The α-tocopherol content (α-TOC; μmol g^{-1} of dry leaf weight) was assessed (Konings et al., 1996 and Ching and Mohamed, 2001). 0.02 gm of butylated hydroxytoluene (BHT) was weighted to be dissolved in900 mL of extraction solvent consisting of 100 L ofCH3–COO–CH2–CH3, n-hexane and n-hexane-ethyl acetate. The standard solutions (0.02 – 0.2 mg mL^{-1}) were prepared using R-TOC with 0.05 gm 100 mL23er-=yh^{-1} n-hexane. Then, the content of α-TOC was evaluated utilizing HPLC systems with a mobile phase ratio of 94 methanol: 6 water(v/v) along with a flow rate of 1.5 mL/minute and setting the UV detector at 292 nm. H_2O_2 level (μmol g^{-1} leaf fresh weight) was estimated by homogenizing 0.25 gm of fresh leaves in 5mL of TCA (5%). At a temperature of 4°C and for only 15 minutes, the homogenate centrifugation at 12000 rpm was carried out. A standard solution made from H_2O_2 was used to calibrate the spectrophotometer to record the reading at a wavelength of 390 nm (Velikova et al., 2000).

The method explained by Kubis (2008) was followed to determine $O_2^{\bullet -}$, in which, into fragments with a size of 1 mm× 1 mm, a pea fully-expanded fresh leaf was divided. And then, at room temperature for only one hour, these fragments were submerged into 10 mM K-phosphate buffered at pH 7.8, 10 mM $NaN_{3,}$ and 0.05% NBT. At 85°C and for only 15 minutes, 2 mL of the solution was heated and rapidly cooled. Colorimetrically, the optical concentration was evaluated at the nm level of A580 nm.

Hydrogen peroxide (H_2O_2) content (μmol g^{-1} FW) was determined in the acetone extract. After adding titanium reagent and ammonium, the extract was dissolved in 1 M $H_2SO_{4;}$ the absorbance was measured at 415 nm (Mukherjee and Choudhari, 1983). The dried powdered spinach leaves were weighed to estimate their contents of heavy metals, which were determined using atomic absorption spectrophotometry according to AOAC (1984).

2.7 Evaluating the activities of antioxidant enzymes

0.5 gm of fresh leaves was extracted following the method described by Mukherjee and Choudhari (1983). The extracts were frozen in liquid nitrogen (N) and ground in a 100 mM phosphate buffer at a pH of 7.0. At a temperature of 4°C and for only 10 minutes, the homogenate centrifugation at 15,000 rpm was performed (Benchmark Scientific LC-8 Place Rotor; 1500xg, USA). Then, at a temperature of 4° C, the supernatants were kept in order to estimate the activity of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT). Using the method defined by Giannopolitis and Ries (1977), which called the nitro blue tetrazolium (NBT), SOD (EC1.15.1.1) activity was estimated. To evaluate CAT (EC1.11.1.6) activity, the method detailed by Aebi (1984) was carried out, while methods explained by Maehly and Chance (1954) and Klapheck et al. (1990) were performed to evaluate POD (EC1.11.1.7) activity.

2.8 Determinations of mineral content

We digested 0.1 g of dried-powdered leaf for 12 h using a mixture of 10 mL concentrated H_2SO_4 and 2 mL perchloric acid (80%) (Wolf, 1982). Using a flame photometer, the total concentration of potassium (K) and calcium (Ca) was estimated (Lachica et al., 1973), while a microkjeldahl method was carried out to assess total N (Chapman and Pratt, 1982). Using the ascorbic acid method, total phosphorus (P) was measured following the method of Watanabe and Olsen (1965). The method detailed by Chapman and Pratt (1962) was used to measure the content by Cd utilizing the atomic absorption spectrophotometer (Perkin-Elmer, Model 3300).

2.9 Anatomical studies

Fifty-five days after the seedling, the median portion of the main stem leaflet was used to perform the comparative microscopy analysis. The solution of formalin-acetic acid-alcohol was utilized to fix the samples Paolillo and Zobel (2002). The preserved stem and leaflet were divided into 5 mm segments cut horizontally into thin cross sections. Johansen's pigments were used to stain the cross sections Johansen, 1940. As a clearing solution, ethanol and xylene: methyl salicylate (1:2, v/v) (99% purity, Sigma-Aldrich, St. Louis, MO, USA) were used to clarify the samples for better images. Finally, brief observations were done under a microscope, and delicate images were obtained using the EVOS FL Cell Imaging System (Thermo Fisher Scientific).

2.10 Statistical analysis

Variance (ANOVA) was analyzed for randomized block design in three replicates. The mean differences between Silymarin and Clove Fruit Extract were compared using the least significant difference test at a $p \leq 0.01$ significance level.

3 Results

3.1 The response of wheat plant growth, yield components, photosynthetic pigments, photosynthetic efficiency, and leaf water status to CFE and/or silymarin

In the absence of stress, plant growth parameters (i.e., plant height, shoot dry weight, and leaf area), yield components (i.e., No grain spike^{-1}, 1000 grain weight, and grain yield plant^{-1}), photosynthetic pigments (i.e., total chlorophylls and total carotenoids), photosynthetic efficiency (i.e., Pn, Tr, and Gs), and leaf water status (i.e., RWC and MSI) were enhanced significantly ($p \leq 0.05$) under the treatment of 2% CFE or 0.5 mM silymarin. Compared to the standard control treatment, CFE enriched with silymarin was the most efficient treatment, enhancing plant height, shoot dry weight, leaf area, No grain spike, 1000 grain weight, grain yield, total chlorophylls, total carotenoids, Pn, Tr, Gs, RWC and MSI on average by 9.54, 13.74, 5.48, 8.25, 5.37, 18.66, 5.32, 3.98, 40.98, 6.35, 18.75, 6.27, and 11.56%, respectively (Table 2; Figure 1). Compared to the standard control, all of the above attributes markedly declined by 41.8, 60.6, 56.7, 55.9, 50.4, 66.3, 33.5, 23.8, 41.2, 48.7, 48.1, 22.7, and 42.5%, respectively the stressed control (0.5 mM Cd) treatment (Table 2; Figure 1). All of the above attributes were significantly ($p \leq 0.05$) enhanced with the treatment of CFE and/or silymarin compared to the stressed control, whereas CFE-silymarin treatment was the most efficient treatment, as all of the above attributes enhanced by 60.4, 97.1, 86.7, 94.1, 87.2, 160.9, 44.2, 22.2, 56.2, 57.1, 69.7, 22.2, and 63.3%, respectively. CFE-silymarin treatment improved the morphological parameters, yield component, photosynthetic pigments, leaf photosynthetic efficiency, and leaf water status of Cd-stressed plants. Still, it did not reach the same level as the typical control plants (Tables 2, S1; Figure 1).

TABLE 2 Wheat plant growth parameters and yield components after foliar application of silymarin (Sm), clove fruit extract (CFE), and silymarin-enriched clove fruit extract (CFE-Sm) under Cd stress.

Treatment	Growth parameters			Yield components		
	Plant height (cm)	Shoot dry weight (g)	Leaf area (cm^{2})	No grain spike^{-1}	1000 grain weight (g)	Grain yield plant^{-1}(g)
1st season						
Control	80.6 ± 2.3^{c}	4.94 ± 0.44^{d}	34.3 ± 1.6^{c}	60.0 ± 3.2^{c}	53.2 ± 3.5^{c}	12.1 ± 1.1^{d}
Sm	84.5 ± 4.1^{b}	4.98 ± 0.43^{c}	35.2 ± 2.1^{b}	61.6 ± 3.6^{b}	54.0 ± 3.6^{c}	13.4 ± 1.3^{c}
CFE	86.5 ± 4.9^{a}	5.25 ± 0.45^{b}	35.2 ± 2.2^{b}	63.7 ± 4.2^{a}	55.0 ± 3.1^{b}	13.8 ± 1.4^{b}
CFE-Sm	88.2 ± 4.6^{a}	5.54 ± 0.42^{a}	36.1 ± 1.6^{a}	64.9 ± 4.1^{a}	56.0 ± 3.2^{a}	14.4 ± 1.2^{a}
Cd	46.7 ± 1.3^{g}	1.91 ± 0.12^{h}	14.6 ± 1.1^{g}	26.2 ± 2.2^{g}	26.5 ± 3.3^{g}	3.96 ± 0.11^{h}
Cd+Sm	58.0 ± 2.5^{f}	2.70 ± 0.13^{g}	20.0 ± 1.2^{f}	37.0 ± 3.1^{f}	38.5 ± 2.5^{f}	5.61 ± .16^{g}
Cd+CFE	63.9 ± 3.6^{e}	3.07 ± 0.18^{f}	23.7 ± 1.3^{e}	41.8 ± 3.1^{e}	44.6 ± 3.8^{e}	7.95 ± 0.21^{f}
Cd+CFE-Sm	75.1 ± 3.9^{d}	3.73 ± 0.16^{e}	27.6 ± 1.5^{d}	51.3 ± 3.9^{d}	49.7 ± 3.4^{d}	10.4 ± 0.98^{e}
ANOVAdf Foliar 7	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
2nd season						
Control	81.8 ± 4.7^{c}	4.95 ± 0.32^{d}	35.0 ± 1.8^{c}	62.3 ± 4.8^{b}	54.7 ± 3.4c	13.1 ± 1.3^{d}
Sm	85.6 ± 4.3^{b}	5.11 ± 0.33^{c}	36.1 ± 2.5^{b}	63.9 ± 4.6^{b}	55.5 ± 4.1^{c}	14.1 ± 1.5^{c}
CFE	88.0 ± 3.9^{a}	5.40 ± 0.25^{b}	36.0 ± 2.1^{b}	66.2 ± 4.8^{a}	55.6 ± 4.2^{b}	14.8 ± 1.4^{b}
CFE-Sm	89.7 ± 4.2^{a}	5.71 ± 0.31^{a}	37.0 ± 2.2^{a}	67.5 ± 4.7^{a}	57.7 ± 3.9^{a}	15.5 ± 1.6^{a}
Cd	47.7 ± 1.8^{g}	1.98 ± 0.12^{h}	15.4 ± 1.6^{g}	27.7 ± 2.2^{f}	27.4 ± 2.6^{g}	4.36 ± 0.13^{h}
Cd+Sm	59.1 ± 1.7^{f}	3.16 ± 0.11^{g}	21.5 ± 1.7^{f}	38.6 ± 2.6^{e}	40.6 ± 3.1^{f}	6.20 ± 0.32^{g}
Cd+CFE	65.1 ± 2.3^{e}	3.50 ± 0.15^{f}	24.5 ± 1.2^{e}	43.7 ± 3.9^{d}	46.9 ± 3.9^{e}	9.35 ± 0.68^{f}
Cd+CFE-Sm	76.4 ± 2.8^{d}	3.94 ± 0.21^{e}	28.4 ± 1.3^{d}	53.3 ± 3.4^{c}	51.2 ± 2.9^{d}	11.3 ± 0.93^{e}
ANOVAdf Foliar	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Data are means (n = 9) ± SE. The same letters in each column indicate no significant differences according to the LSD test ($p \leq 0.05$).Control: There is no stress and no foliar applications, Sm: Foliar spray with 0.5 mM silymarin, CFE: Foliar spray with 2% clove fruit extract, CFE-Sm: Foliar spray with clove fruit extract enriched with silymarin (0.24 g Sm L^{-1} of CFE), Cd^{+}: Watering the wheat seedlings with a nourishing solution containing 2 mM Cd, Cd +Sm: Watering the wheat seedlings with a nourishing solution containing 2 mM Cd + foliar spray with 0.5 mM silymarin, Cd +CFE: Watering the wheat seedlings with a nourishing solution containing 2 mM Cd + foliar spray with 2% clove fruit extract, Cd+CFE-Sm: Watering the wheat seedlings with a nourishing solution containing 2 mM Cd + foliar spray with clove fruit extract enriched with silymarin (0.24 g Sm L^{-1} of CFE).

3.2 The response of free proline, soluble sugars, and oxidative stress markers to CFE and/or silymarin

Free proline and soluble sugars were increased while levels of EL, MDA, H_2O_2, and $O_2^{\bullet -}$ were slightly decreased with 2% CFE, 0.5 mM silymarin, or even with CFE-silymarin, which was the best treatment, compared to the standard control (Table S2; Figure 2). The free proline, soluble sugars, EL, MDA, H_2O_2 and $O_2^{\bullet -}$ were significantly ($p \leq 0.05$) stimulated under the treatment of 0.5 mM Cd compared to the standard control. These increases were 116, 78.1, 143, 209, 232 and 52.1%, respectively (Table S2; Figure 2). Compared to the stressed control treatment, EL, MDA, H_2O_2, and $O_2^{\bullet -}$ were significantly ($p \leq 0.05$) inhibited under the treatment of CFE or silymarin; however, CFE-silymarin was the best treatment, which decreased all of these parameters by 52.9, 53.9, 57.5 and 28.4%, respectively. However, proline and soluble sugars increased by 29.6 and 51.5%, respectively. In addition, El and MDA levels were significantly ($p \leq 0.05$) reduced in response to CFE-silymarin addition as a foliar spray compared to the stressed control treatment (Table S2; Figure 2).

3.3 The response of enzymes activities peroxidase, catalase, α-tocopherol, glutathione, nonenzymatic antioxidant compounds ascorbate, and superoxide dismutase

In the non-stress conditions, POX, CAT, SOD, AsA, α-TOC, and GSH were slightly elevated with 2% CFE, 0.5 mM silymarin, and also with CFE-silymarin, which had more efficiency, compared to the control (Table S3; Figure 3). Under Cd stress conditions, POX, CAT, SOD, AsA, α-TOC, and GSH were markedly elevated in

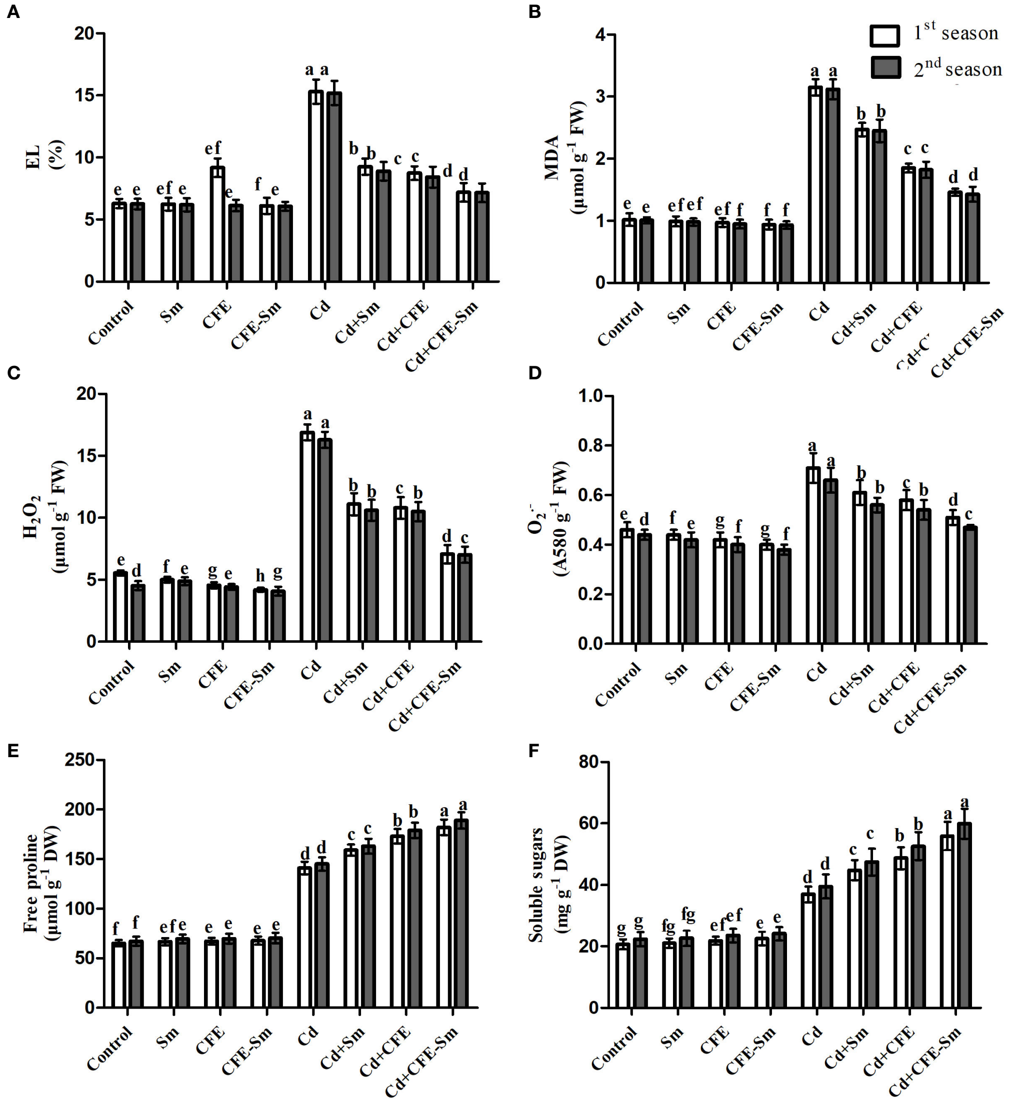

FIGURE 2
Impacts of silymarin (Sm), clove fruit extract (CFE), or silymarin-enriched clove fruit extract (CFE-Sm) foliar application on **(A)** Electrolyte leakage (EL), **(B)** malondialdehyde (MDA), **(C)** hydrogen peroxide (H_2O_2), **(D)** superoxide radical ($O_2^{\bullet-}$), **(E)** free proline and **(F)** soluble sugars.

average by 89.5, 79.8, 81.2, 92, 52.7 and 140% respectively when compared to the control. POX, CAT, SOD, AsA, α-TOC, and GSH were significantly ($p \leq 0.05$) improved with CFE or silymarin compared to control under Cd stress. However, CEF-silymarin had more efficiency, with all of the above parameters increasing on average by 9.22, 6.01, 9.26, 7.10, 3.35, and 9.59%, respectively. Wheat plants exposed to Cd stress could increase the activities of their enzymes and improve the activity of several antioxidants to cope with markers of oxidative stress when treated with CFE-silymarin as a foliar fertilizer (Table S3, Figure 3).

3.4 The response of nutrient content

The contents of N, P, K, and Ca significantly ($p \leq 0.05$) enhanced under the treatment of 2% CFE or 0.5 mM silymarin compared to the standard control, while Cd was not detected under normal conditions (Table 3). The concentration of N, P, K, and Ca ($p \leq 0.05$) decreased by 24.1, 39.9, 24.1, and 50.7 under Cd stress compared to control, while Cd concentration increased. On the other hand, applying % CFE, 0.5 mM silymarin, or even CFE-silymarin alleviated the nutrient content under the Cd stress condition, while CFE-silymarin gave the highest value.

3.5 Response of leaf feature to Cd stress and CFE and/or silymarin

Leaf features such as blade thickness, mesophyll tissue thickness, midvein length, midvein width, vascular bund midvein width, vascular bund midvein, and thickness and width of schalaranchyma tissue were slightly increased in non-stress conditions with the treatment of 2% CFE, 0.5 mM silymarin, and CFE-silymarin, which was more efficient than the control (Table 4;

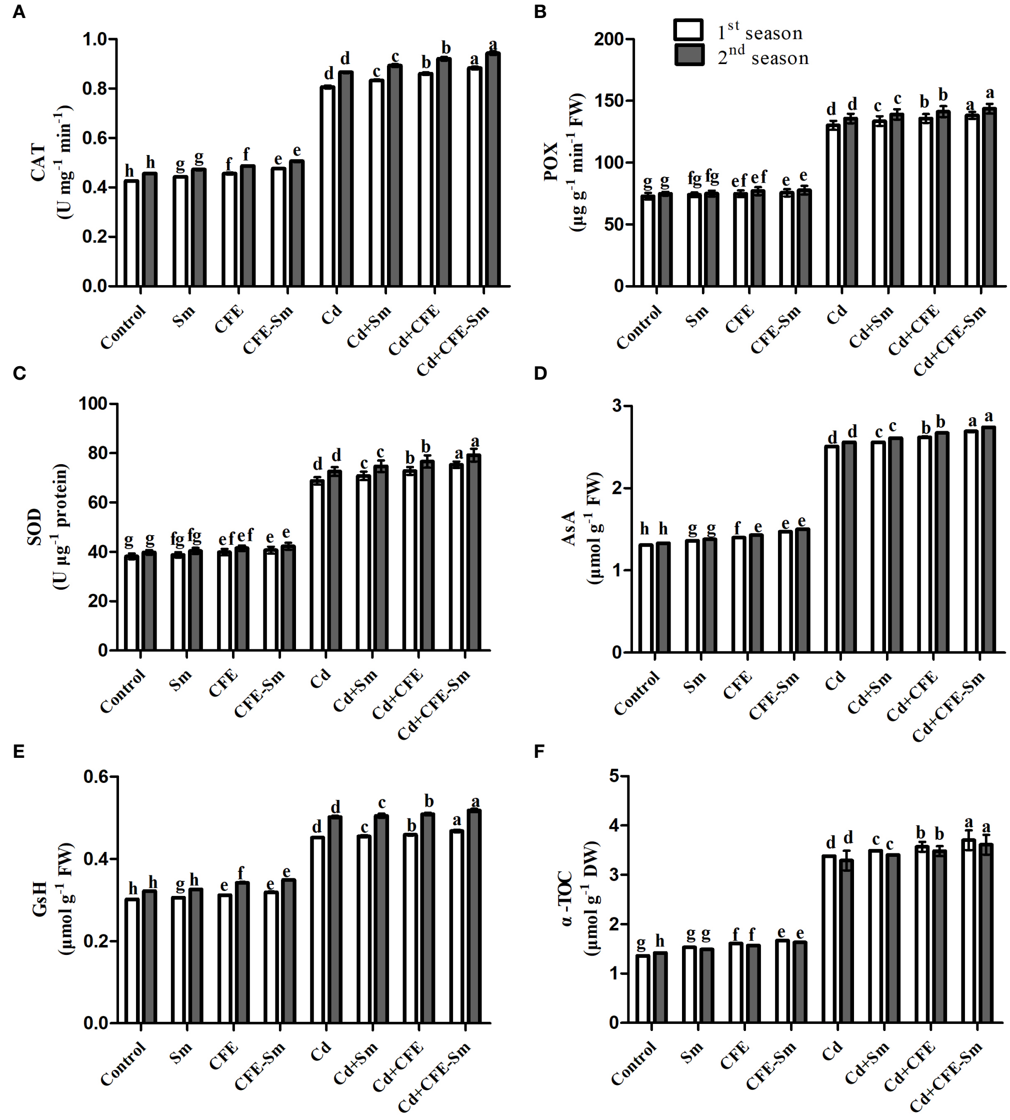

FIGURE 3
Impacts of silymarin (Sm), clove fruit extract (CFE), or silymarin-enriched clove fruit extract (CFE-Sm) foliar application on antioxidant enzymes **(A)** catalase; CAT, peroxidase; **(B)** POX, superoxide dismutase; **(C)** SOD and ascorbate peroxidase), **(D)** ascorbate (AsA), **(E)** glutathione (GSH), and **(F)** Tocopherol (-TOC) in wheat under Cd stress.

Figure 4). Under Cd stress conditions; leaf features were markedly decreased compared to the control. The leaf features of the plants treated with CFE or silymarin were improved significantly compared with the control under Cd stress. However, CEF-silymarin had more efficiency, with all of the above-studied parameters, which increased by 37.3, 55.5, 100, 140, 64.2, 50, 9.47, and 11.38%, respectively (Table 4; Figure 4).

4 Discussion

Cd is considered one of the most highly toxic and mobile heavy metals in soil and plant, owing to its obstruction of many physio-biochemical and molecular processes (Desoky et al., 2020a; Jung et al., 2021). In the present study, adding a Cd-containing nutrient solution to wheat plants hampered growth and productivity (Table 2) and reduced photosynthetic pigments and photosynthetic efficiency (Figure 1), along with increased oxidative stress inducers (H_2O_2 and $O_2^{\bullet -}$), lipid peroxidation (MDA) and EL (Figure 2). Cd degrades chlorophyll, restricts nutrient uptake, and reduces photosynthetic activity due to Cd toxicity-induced oxidative burst and over-ROS accumulation in plant cells, leading to reducing plant performance (Alzahrani et al., 2018; Haider et al., 2022). Furthermore, Cd toxicity may decrease dry matter accumulation due to the inhibition of root growth caused by Cd accumulation at a higher rate in roots than in shoots (Ali et al., 2022; Zeshan et al., 2022).

Nonetheless, our research demonstrated that applying CFE and silymarin mediated positive changes to relieve Cd toxicity in wheat plants. The highest positive effects were assigned to co-apply CFE-silymarin, yielding the best results. Exogenous application of CFE and silymarin improved the growth and yield of Cd-stressed wheat

TABLE 3 N, Nitrogen; P, phosphorus; P, potassium; Ca, calcium; and Cd, cadmium; response to foliar application of Sm, silymarin; CFE, clove fruit extract; or CFE-Sm, silymarin-enriched clove fruit extract; under Cd stress.

Treatment	N (%)	P (%)	K (%)	Ca (%)	Cd (mg kg^{-1} DW)	
1st season						
Control	2.20 ± 0.12^{b}	0.496 ± 0.02^{c}	1.86 ± 0.09^{c}	2.05 ± 0.13^{c}	Nd	
Sm	2.36 ± 0.16^{a}	0.580 ± 0.03^{b}	2.00 ± 0.11^{b}	2.15 ± 0.15^{b}	Nd	
CFE	2.39 ± 0.18^{a}	0.593 ± 0.03^{b}	2.02 ± 0.12^{b}	2.20 ± 0.21^{b}	Nd	
CFE-Sm	2.42 ± 0.11^{a}	0.613 ± 0.04^{a}	2.14 ± 0.14^{a}	2.29 ± 0.16^{a}	Nd	
Cd	1.68 ± 0.09^{e}	0.300 ± 0.01^{g}	1.37 ± 0.08^{f}	1.01 ± 0.09^{g}	43.9 ± 2.3^{a}	
Cd+Sm	1.99 ± 0.07^{d}	0.420 ± 0.02^{f}	1.62 ± 0.06^{e}	1.31 ± 0.06^{f}	12.6 ± 1.3^{b}	
Cd+CFE	2.10 ± 0.11^{c}	0.450 ± 0.03^{e}	1.64 ± 0.04^{e}	1.43 ± 0.04^{e}	7.33 ± 1.5^{c}	
Cd+CFE-Sm	2.16 ± 0.10^{b}	0.476 ± 0.03^{d}	1.70 ± 0.05^{d}	1.58 ± 0.05^{d}	3.69 ± 0.21^{d}	
ANOVAdf **Foliar 7**	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
2nd season						
Control	2.27 ± 0.13^{b}	0.520 ± 0.04^{c}	1.81 ± 0.06^{c}	2.11 ± 0.15^{c}	Nd	
Sm	2.41 ± 0.15^{a}	0.603 ± 0.05^{b}	2.08 ± 0.08^{b}	2.21 ± 0.12^{b}	Nd	
CFE	2.43 ± 0.13^{a}	0.606 ± 0.06^{ab}	2.10 ± 0.09^{b}	2.26 ± 0.16^{b}	Nd	
CFE-Sm	2.45 ± 0.16^{a}	0.633 ± 0.04^{a}	2.19 ± 0.13^{a}	2.35 ± 0.13^{a}	Nd	
Cd	1.71 ± 0.08^{c}	0.310 ± 0.02^{f}	1.41 ± 0.05^{f}	1.04 ± 0.03^{g}	43.1 ± 3.2^{a}	
Cd+Sm	2.03 ± 0.09^{c}	0.443 ± 0.03^{e}	1.66 ± 0.03^{e}	1.33 ± 0.02^{f}	12.0 ± 1.5^{b}	
Cd+CFE	2.20 ± 0.13^{b}	0.473 ± 0.03^{d}	1.68 ± 0.04^{de}	1.46 ± 0.04^{e}	6.84 ± 0.23^{c}	
Cd+CFE-Sm	2.21 ± 0.15^{b}	0.490 ± .02^{d}	1.73 ± 0.05^{d}	1.62 ± 0.05^{d}	3.26 ± 0.11^{d}	
ANOVAdf **Foliar 7**	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Data are means (n = 9) ± SE. The same letters in each column indicate no significant differences according to the LSD test ($p \leq 0.05$).Control: There is no stress and no foliar applications, Sm: Foliar spray with 0.5 mM silymarin, CFE: Foliar spray with 2% clove fruit extract, CFE-Sm: Foliar spray with clove fruit extract enriched with silymarin (0.24 g Sm L^{-1} of CFE), Cd^{+}: Watering the wheat seedlings with a nourishing solution containing 2 mM Cd, Cd +Sm: Watering the wheat seedlings with a nourishing solution containing 2 mM Cd + foliar spray with 0.5 mM silymarin, Cd +CFE: Watering the wheat seedlings with a nourishing solution containing 2 mM Cd + foliar spray with 2% clove fruit extract, Cd+CFE-Sm: Watering the wheat seedlings with a nourishing solution containing 2 mM Cd + foliar spray with clove fruit extract enriched with silymarin (0.24 g Sm L^{-1} of CFE).

plants (Table 2). These findings may be due to the fact that CFE and silymarin applications associated with decreased Cd contents (Figure 3), and H_2O_2 and $O_2^{\bullet -}$ (Figure 2), which are proposed to participate in maintaining membrane integrity, cell turgor status, reduced lipid peroxidation, and EL from the cells under Cd toxicity (Figures 1, 2) (Rady and Mohamed, 2015). CFE analysis ensured the existence of the flavonoids and phenolic components (i.e., Ellagic-acid, 3,4 Dihydroxybenzoic-acid, Eugenyl-acetate, Eugenol, Gallic-acid, tannic acid, and Naphthalene), osmoprotectants (i.e., proline, glutathione, and soluble sugars), nutrients (i.e., N, K, P, Ca, Mg, Fe), vitamins (i.e., A, E, D, C) and antioxidants (Table 1). As a result of its diverse composition, CFE was proposed as a plant biostimulator. The enhanced mobilization of inorganic metabolites solutes like ascorbic acid (Vitamin C), K, and Ca present in CFE (Table 1) to the growing plumule and/or the increase in amylase activity and reducing sugars, contributing to early vigor and increased plant growth, may explain the improved plant growth traits (i.e., area of leaves per plant, dry weight, and shoot fresh) caused by CFE application (Afzal et al., 2012; Desoky et al., 2021b). Also, the phenolic component is critical to decline the damaging effects of drought stress in the plant (Naikoo et al., 2019). The different physiological processes correlated to development and growth in plants, including the synthesis of photosynthetic pigments, cell division, and seed germination, were influenced by phenolic compounds (Tanase et al., 2019). Phenolics have been utilized for various applications, such as antioxidants as food additives, allelochemical bioremediation, and plant growth promotion (Bujor et al., 2015). The accumulation of phenolic compounds is generally a consistent feature of plants subjected to stress, which is considered a defensive mechanism to face various abiotic stresses (Cheynier et al., 2013). Phenolic compounds influence many physiological processes in plants, increasing the adaptability and tolerance of plants subjected to suboptimal conditions (Andersen, 2003). Flavonols' accumulation and biosynthesis were also catalyzed in plants subjected to water deficit conditions, accompanied by improved drought stress resistance (Ballizany et al., 2012). The

TABLE 4 Measurement in microns of certain light microscopically features of a transverse section through the leaf blade from the apex of a wheat plant (fully expanded leaf) as affected by foliar application of silymarin (Sm), clove fruit extract (CFE), or silymarin-enriched clove fruit extract (CFE-Sm) under Cd stress during the second season.

Treatment	Thickness of Blade	The thickness of mesophyll tissue	Length of midvein	Width of midvein	Length of vascular bund midvein	Width of vascular bund midvein	The thickness of schalaranchyma tissue	Width of schalaranchyma tissue
Control	202.1 ± 3.8^{d}	159.6 ± 1.8^{c}	476.8 ± 4.7^{d}	695.8 ± 5.9^{d}	120.9 ± 1.1^{d}	150.9 ± 3.5^{d}	121.5 ± 2.5^{d}	161.8 ± 4.5^{d}
Sm	205.2 ± 3.2^{c}	180.8 ± 2.4^{b}	574.5 ± 4.5^{c}	787.3 ± 6.9^{c}	123.7 ± 2.3^{c}	154.7 ± 3.8^{c}	122.3 ± 2.2^{c}	171.3 ± 4.8^{c}
CFE	223.4 ± 4.5^{b}	180.8 ± 2.7^{b}	627.7 ± 5.8^{b}	840.5 ± 7.2^{b}	130.9 ± 1.6^{b}	159.93.4 ± ^{b}	135.2 ± 3.1^{b}	199.8 ± 4.7^{b}
CFE-Sm	226.9 ± 3.7^{a}	191.5 ± 2.9^{a}	670.3 ± 6.2^{a}	904.4 ± 8.1^{a}	133.2 ± 1.8^{a}	164.2 ± 3.7^{a}	146.1 ± 3.4^{a}	204.8 ± 3.6^{a}
Cd	134.3 ± 2.6^{h}	95.7 ± 1.8^{g}	234.0 ± 2.6^{h}	234.1 ± 3.5^{h}	66.6 ± 1.5^{h}	92.8 ± 2.5^{h}	101.3 ± 3.8^{f}	158.1 ± 3.8^{g}
Cd+Sm	156.5 ± 2.4^{g}	112.4 ± 2.1^{f}	257.4 ± 3.4^{g}	385.6 ± 3.8^{g}	87.5 ± 1.4^{g}	110.6 ± 2.9^{g}	103.2 ± 3.8^{g}	158.9 ± 3.4^{g}
Cd+CFE	180.8 ± 2.7^{f}	138.3 ± 1.8^{e}	436.2 ± 4.1^{f}	559.6 ± 4.5^{f}	106.6 ± 2.4^{f}	137.5 ± 3.1^{f}	107.5 ± 1.6^{f}	168 ± 2.6^{f}
Cd+CFE-Sm	184.4 ± 2.9^{e}	148.9 ± 2.4^{d}	468.1 ± 4.8^{e}	563.9 ± 5.6^{e}	109.4 ± 2.9^{e}	139.2 ± 3.7^{e}	110.9 ± 5.2^{e}	176.1 ± 5.1^{e}
ANOVAdf Foliar	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Data are means (n = 9) ± SE. The same letters in each column indicate not significant differences according to theLSD test ($p \leq 0.05$).Control: There is no stress and no foliar applications, Sm: Foliar spray with 0.5 mM silymarin, CFE: Foliar spray with 2% clove fruit extract,CFE-Sm: Foliar spray with clove fruit extract enriched with silymarin (0.24 g Sm L^{-1} of CFE), Cd^{+}: Watering the wheat seedlings with a nourishing solution containing 2 mM Cd,Cd +Sm: Watering the wheat seedlings with a nourishing solution containing 2 mM Cd + foliar spray with 0.5 mM silymarin,Cd +CFE: Watering the wheat seedlings with a nourishing solution containing 2 mM Cd + foliar spray with 2% clove fruit extract, Cd+CFE-Sm: Watering the wheat seedlings with a nourishing solution containing 2 mM Cd + foliar spray with clove fruit extract enriched with silymarin (0.24 g Sm L^{-1} of CFE).

accumulation of flavonoids in cytoplasm plays a critical role in detoxifying and reducing the harmful effect of H_2O_2 molecules generated by the stress, and in the end, oxidation of flavonoids is followed by ascorbic acid-mediated re-conversion of flavonoids into primary metabolites (Hernandez et al., 2009). However, under stress, exogenous Gallic-acid (GLA) treatment resulted in the elevation of plant growth (Ozfidan-Konakci et al., 2015).In addition, the increased CFE contains macro elements that alleviate stress's reverse effect on growth (Yildirim et al., 2009). Erner et al. (2002) indicated that applying K mitigated the negative impact of stress on plant growth. Also, Martinez and Cerda (1999) reported that foliar spray with N alone ameliorated the stress effect on plant growth. On the other hand, CFE contains microelements that can enhance plant tolerance to drought by improving root growth and promoting nutrient uptake (Abou El-Nour, 2002). In addition,silymarin-fortified biostimulants improved crop growth and productivity by regulating adaptive mechanisms *via* modulation of physio-biochemical and molecular processes under Cd stress (Ali et al., 2022).

Cd uptake and accumulation in plants produce ROS by substituting cofactors for basic metal ions in metalloproteins *via* the Fenton reaction, which destroys membrane cells *via* lipid peroxidation and prevents the biosynthesis of cellular molecules necessary for plant growth (Jung et al., 2021). In this study, an increase in H_2O_2 and $O_2^{\bullet -}$ of Cd-stressed wheat plants was observed in tandem with an increase in EL and lipid peroxidation of membranes (determined as MDA). These unfavorable results were improved by foliage-applied CFE and silymarin, which reduced the concentrations of these free radicals, lipid peroxidation, and EL, indicating that co-applied CFE and silymarin improved Cd tolerance. CFE and silymarin co-application was a powerful catalyst for growing wheat against Cd stress, as it enhanced photosynthetic capacity (i.e., chlorophyll content and photosynthetic efficiency), providing wheat plants with more photo-assimilates needed to produce dry biomass and grain yield (Bel et al., 2003). These improvements in the photosynthetic efficacy due to the role of CFE and silymarin reduced inner Cd content in different wheat parts (roots, shoots, and grain). They elevated the antioxidative defense system for suppressing the oxidative stress markers. Bioactive compounds in silymarin, such as flavonoids and polyphenols, have the antioxidant capacity for scavenging ROS, decreasing free radicals levels, and protecting plant tissue from oxidative stress caused by Cd toxicity (de Oliveira et al., 2015; Ali et al., 2022).

Furthermore, the CFE application enhanced the photosynthetic efficiency of wheat plants, possibly due to increased growth for better water and nutrient uptake (Desoky et al., 2021b). The increase in the photosynthetic attributes of CFE under stress may be due to its effectiveness in reducing the MDA and H_2O_2 contents. Presence nutrients in CFE inhibit premature leaf senescence and

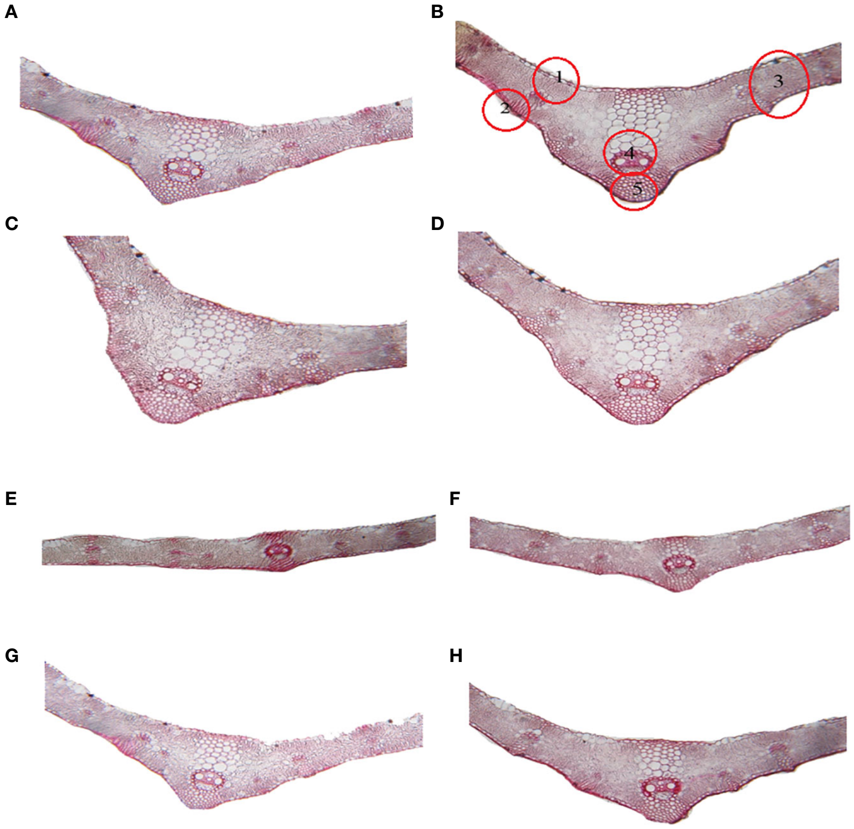

FIGURE 4
Transverse sections in the flag leaf blade on the main wheat stem as affected by foliar application of silymarin (Sm), clove fruit extract (CFE), or silymarin-enriched clove fruit extract (CFE-Sm) under Cd stress. **(A)** control, **(B)** treated with Sm, **(C)** treated with CFE, **(D)** treated with CFE-Sm, **(E)** treated with Cd, **(F)** treated with Sm + Cd, **(G)** treated with CFE + Cd, **(H)** treated with CFE-Sm + C1: Upper Epidermis, 2: Lower Epidermis, 3: Mesophyll tissue, 4: Vascular bundle of midvein, 5: Schalaranchyma tissue.

maintain higher leaf area, increasing photosynthetic pigments. In addition, Fe found in CFE may be available in plants after treatment to activate many enzymes involved in the chlorophyll biosynthesis pathway and some antioxidant enzymes such as APOX and GR that scavenge the ROS and protect chlorophyll from degradation (Zayed et al., 2011).

Plant defense mechanisms, such as the accumulation of antioxidative compounds, control the level of free radicals under normal conditions. Under Cd stress, the biosynthesis and accumulation of osmoadaptive compounds such as proline, soluble sugar, and antioxidant-related substances such as phenolic, AsA, GSH,α-TOC, and enzymatic antioxidants protect stressed plants by modulating osmotic pressure and suppressing Cd-induced oxidative stress (Gill and Tuteja, 2010). In the present study, we observed an increment in the proline, soluble sugar, AsA, α-TOC, and GSH contents in Cd-stressed wheat plants in response to the integrative application of CFE and silymarin. Proline accumulates in stressed plant tissues to act as an osmoprotectant for osmotic adjustment and is directly involved in ROS detoxification, cell structure maintenance, lipid membrane oxidation-reduction, and photoinhibition (Semida et al., 2020). This increased proline accumulation may be related to CFE and silymarin-induced significant modulation in proline metabolism *via* decreased P5CS anabolism and increased ProDH catabolism in order to balance the concentration of proline within plant cells (Alharby et al., 2020). The results presented here show an increase in AsA,α-TOC, and GSH levels in Cd-stressed wheat plants treated with CFE and silymarin, indicating an improvement in the AsA-GSH cycle with a decisive role in detoxifying ROS and wheat tolerance (Hasanuzzaman et al., 2019). Furthermore, increased GSH levels promote phytochelatin formation and Cd ion sequestration in the vacuole *via* complex phytochelatin formation (Elrys et al., 2021).

Kapoor et al. (2015) state that the antioxidative defense machinery usually regulates cellular redox homeostasis by controlling the ROS level in the cells. Therefore, and as evidenced herein, it is assumed that the activity of antioxidant enzymes will increase in Cd-stressed plants. Moreover, externally applied CFE and/or silymarin to Cd-stressed wheat plants further enhanced antioxidant enzymes (Table S3; Figure 3). CAT, POX, and SOD activity in this research was up-regulated against the free radicals induced by Cd toxicity. This elevated enzyme activity reached its maximum by co-applicating CFE and silymarin. Our findings are consistent with those of Alharby et al. (2021); Ali et al. (2022), and Desoky et al. (2021b), who reported that CFE and/or silymarin improved cell metabolism under Cd stress by up-regulating the activity of antioxidant enzymes such as SOD, CAT, and POX synchronized with reduced Cd content, boosting the plant's capacity to resist Cd stress.

Finally, the role of silymarin or CFE as a secondary metabolite in enhancing plant growth and production under Cd stress conditions was still unclear. Our findings evidenced that silymarin can increase the wheat quantity and quality under Cd stress conditions by improving wheat plant defenses. Our study is in line with the previous reports that considered silymarin as a powerful antioxidant, increasing plant resistance to stress (Ghavami and Ramin, 2008; Afshar et al., 2015). The CFE-silymarin results in our study align with the properties of the biostimulator reported by the European Biostimulant Industry Council (Colla and Rouphael, 2015) and with the findings of other work examining different stresses (Alharby et al., 2020). The bioactive compounds included in CFE-silymarin make it an effective biocatalyst and a unique environmentally friendly strategy. CFE-silymarin bioactive ingredients have different antioxidants, which possess high states of redox, making CFE-silymarin possess the ability to suppress ROS and lipid peroxidation. Accordingly, our study suggested that the interplaying of the bioactive ingredients of CFE-silymarin formed a robust defense system against Cd-induced oxidative damage in favor of wheat plants' performance.

5 Conclusions

Plant height, shoot dry weight, leaf area, No grain spike, 1000 grain weight, grain yield, total chlorophylls, total carotenoids, Pn, Tr, Gs, RWC, and MSI were all reduced by 41.8, 60.6, 56.7, 55.9, 50.4, 82.8, 33.5, 23.8, 41.2, 48.7, 48.1, 22.7, and 42.5%, respectively, under the stressed control (0.5 mM Cd) treatment. Compared to the stressed control, the above attributes were significantly enhanced with the treatment of CFE and/or silymarin. In contrast, CFE-silymarin treatment was the most efficient treatment, as all of the above attributes enhanced by 60.4, 97.1, 86.7, 94.1, 87.2, 160.9, 44.2, 22.2, 56.2, 57.1, 69.7, 22.2, and 63.3%, respectively. In contrast, the levels of free proline, soluble sugars, EL, MDA, H_2O_2, $O_2^{\bullet -}$ POX, CAT, SOD, AsA, α-TOC, and GSHwere significantly stimulated by 116, 78.1, 143, 209, 232, 52.1, 89.5, 79.8, 81.2, 92, 52.7 and 140%, respectively under the treatment of 0.5 mM Cd compared to the standard control. Compared to the stressed control treatment, EL, MDA, $H_2O_{2,}$ and $O_2^{\bullet -}$ were significantly inhibited under the treatment of CFE or silymarin; however, CFE-silymarin was the best treatment, which decreased these parameters by 52.9, 53.9, 57.5 and 28.4%, respectively, while increased POX, CAT, SOD, AsA, α-TOC, and GSH by 9.22, 6.01, 9.26, 7.10, 3.35, and 9.59%, respectively. The findings of our study indicate that CFE-silymarin spraying on wheat plants under Cd stress is an efficient method to enhance the buildup of biomass and plant development. In our investigation, the antioxidant components of CFE-silymarin served as a natural biostimulant that interacted with one another to benefit wheat plants exposed to Cd stress. Foliar spraying wheat plants with CFE-silymarin improved growth, photosynthetic efficiency, nonenzymatic and enzymatic antioxidants. This also reduced oxidative damage caused by ROS ($O_2^{\bullet -}$ and H_2O_2), ionic leakage, and lipid peroxidation. The limitation of Cd ion buildup and the activation of antioxidant defenses in wheat plants under Cd stress led to these adequate findings. The results of this work suggest using CFE-silymarin as a potent novel biostimulator for wheat to stimulate various physiological and metabolic processes and increase wheat plants' tolerance to Cd stress. In order to fully understand the mechanism of silymarin (in CFE-silymarin) for stress-tolerant plants, extensive 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 authors.

Author contributions

Conceptualization: E-SD, JL, HA, MR and AE-S. Methodology: E-SD. Software: E-SD, LW, and AE-S. Validation: HA and MI. Formal analysis, E-SD, LW, MM, and AE-S. Investigation: E-SD, AE-S, and MM. Resources: E-SD, MR, MM. Writing—original draft preparation: E-SD and MM. Writing—review and editing: XZ, HA, LW, MI, KY, MR, PM, E-SD and AE-S. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1144319/full#supplementary-material

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Characteristics of cadmium accumulation and tolerance in apple plants grown in different soils

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Cadmium (Cd) is a nonessential element and highly toxic to apple tree. However, Cd accumulation, translocation and tolerance in apple trees planted in different soils remain unknown. To investigate soil Cd bioavailability, plant Cd accumulation, physiological changes as well as gene expression patterns in apple trees grown in five different soils, 'Hanfu' apple seedlings were planted in orchard soils collected from Maliangou village (ML), Desheng village (DS), Xishan village (XS), Kaoshantun village (KS) and Qianertaizi village (QT), and subjected to 500 μM $CdCl_2$ for 70 d. Results showed that soils of ML and XS had higher content of organic matter (OM), clay and silt, and cation exchange capacity (CEC) but lower sand content than the other soils, thereby reduced Cd bioavailability, which could be reflected by lower concentrations and proportions of acid-soluble Cd but higher concentrations and proportions of reducible and oxidizable Cd. The plants grown in soils of ML and XS had relatively lower Cd accumulation levels and bio-concentration factors than those grown in the other soils. Excess Cd reduced plant biomass, root architecture, and chlorophyll content in all plants but to relatively lesser degree in those grown in soils of ML and XS. The plants grown in soils of ML, XS and QT had comparatively lower reactive oxygen species (ROS) content, less membrane lipid peroxidation, and higher antioxidant content and enzyme activity than those grown in soils of DS and KS. Transcript levels of genes regulating Cd uptake, transport and detoxification such as *HA11*, *VHA4*, *ZIP6*, *IRT1*, *NAS1*, *MT2*, *MHX*, *MTP1*, *ABCC1*, *HMA4* and *PCR2* displayed significant differences in roots of plants grown in different soils. These results indicate that soil types affect Cd accumulation and tolerance in apple plants, and plants grown in soils with higher OM content, CEC, clay and silt content and lower sand content suffer less Cd toxicity.

KEYWORDS

apple, cadmium, soil physicochemical property, oxidative stress, gene expression

1 Introduction

Extensive sewage irrigation and metal-based pesticide and chemical fertilizer application in recent years have contaminated fruit orchards with cadmium (Cd) to varying degrees (Li et al., 2006; Karimi Nezhad et al., 2011; Argüello et al., 2019). Cd is water-soluble and readily transported from the soil to roots (Luo et al., 2016; He et al., 2020). In plants, Cd induces reactive oxygen species (ROS) production, causes oxidative stress, destroys subcellular structure, reduces photosynthesis, inhibits growth, and may cause death (Liu and Kottke, 2004; Mobin and Khan, 2007; Podazza et al., 2012; Podazza et al., 2016). Moreover, Cd in agricultural products may be ingested by humans and cause various diseases such as hypertension, kidney dysfunction and lung cancers (Chen et al., 2015).

The Cd content in plant tissues depends largely upon the Cd bioavailability in the soil and the ability of the plants to absorb soil Cd. Therefore, it is important to understand the factors affecting Cd migration from the soil to roots. After Cd enters the soil, it undergoes physicochemical transformations such as dissolution, precipitation, complexation, and adsorption and assumes different forms (Qasim and Motelica-Heino, 2014). Cd bioavailability in plants is controlled by various soil physicochemical factors, such as pH value, organic matter (OM) content, soil texture, and cation exchange capacity (CEC) (Golia et al., 2008; Karimi Nezhad et al., 2011). This relationship has been established in some crops where soil Cd mobility and plant Cd uptake are decreased at high soil pH, OM content and CEC (Vega et al., 2010; Chen et al., 2015; Chen et al., 2016; Argüello et al., 2019). In addition, soil texture is also an important factor affecting heavy metal bioavailability. Finer soil particles have relatively larger surface areas, higher secondary mineral and OM content (Hardy and Cornu, 2006; Yu et al., 2017), and greater heavy metal adsorption capacity (Luo et al., 2011). Soils of fruit orchards differ greatly in terms of these key properties in different planting regions. However, little is known about Cd accumulation and toxicity in apple trees planted in different soils.

Excess Cd induces the production of ROS including superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) which cause cell membrane lipid peroxidation, nucleic acid and protein denaturation, abnormal cell metabolism, and even cell death (PÉRez-Chaca et al., 2014; Ahammed et al., 2021). Plants activate their antioxidant defense systems including antioxidant enzymes and non-enzymatic antioxidant metabolites to mitigate oxidative damage induced by Cd (Anjum et al., 2015; Zhou et al., 2017). The antioxidant capacity of soybean under heavy metal stress displayed significant differences when planted in different soils (Melo et al., 2011). In addition, application of organic amendments affected the antioxidant defense system and alleviated Cd toxicity in mung bean (Ramzani et al., 2017), possibly because they altered the pH, CEC, OM, and other physicochemical properties of the soil (Fellet et al., 2014; Cui et al., 2016). To our knowledge, however, little is known about how Cd stress affects the antioxidant defense system of apple trees grown in different soils.

Plant Cd absorption, transport, and detoxification are key processes controlled by several genes (He et al., 2020). Plasma membrane (PM) H^+-ATPase pumps protons extracellularly at the expense of ATP and creates an electrochemical gradient for transmembrane ion transport (Zhang et al., 2017). Upregulation of the gene encoding PM H^+-ATPase enhanced Cd^{2+} absorption in plants (He et al., 2015). ZRT-IRT-like protein 6.2 (ZIP6.2) and iron transporter 1 (IRT1) are plant cells membrane localized protein and regulate extracellular Cd^{2+} uptake (Migeon et al., 2010; Zhu et al., 2012). Upon entry into root cells, Cd may complex with Cd effective chelators including nicotianamine (NA; encoded by nicotianamine synthase 1 (NAS1)) and metallothionein (He et al., 2020). Metal tolerance protein 1 (MTP1), magnesium proton exchangers (MHX), and ATP-binding cassette transporter C1 (ABCC1) are localized to the tonoplasts. MTP1 and MHX transport Cd^{2+} and ABCC1 transports PC-Cd complexes into the vacuoles (Berezin et al., 2008; Gaillard et al., 2008; Migeon et al., 2010). Plant Cd resistance protein 2 (PCR2) and HM ATPase4 (HMA4) are localized to the PMs, pump Cd into the apoplast, and play critical roles in Cd translocation from the roots to the aerial parts (Hanikenne et al., 2008; Song et al., 2010). Up to now, little is known about the gene expression patterns related to Cd absorption, transport and tolerance in apple plants grown in different soils.

'Hanfu' apple (*Malus domestica* Borkh.) is a high quality variety with cold resistance and high yield, and has become the main variety grown in Liaoning Province. In this study, according to the different characteristics of the apple orchard soil in the Liaoning Province, orchards soils in five regions were collected. To explore the differences of Cd absorption, accumulation and tolerance of apple seedlings grown in different soils, 'Hanfu' apple grafted onto *M. baccata* Borkh. with relative higher Cd tolerance (Zhou et al., 2017) were planted in five soils with different physicochemical properties and subjected to 500 μM Cd for 70 d. We hypothesized that (i) there would be variations in Cd accumulation and tolerance in apple plants grown in different soils, and (ii) these variations are associated with physiological acclimation and transcriptional regulation. To examine these hypotheses, we compared and analyzed various soil Cd forms, growth characteristics of plants, Cd concentrations, ROS, antioxidants, and the transcription level of key genes related to Cd uptake, transport, and detoxification. The obtained results are of great significance for mitigating the risk of Cd phytotoxicity in apple as well as other fruit trees through the application of amendments to modify soil properties.

2 Materials and methods

2.1 Soil sources and physicochemical properties

Soils were sampled at 0–20 cm depth from 'Hanfu' apple (*Malus domestica* Borkh.) orchards in Maliangou village in Liaoyang city (ML), Desheng village in Panjin city (DS), Xishan village in Chaoyang city (XS), Kaoshantun village in Xinmin city (KS), and Qianertaizi village in Xinmin city (QT) in Liaoning Province, China. Visible residues were removed from the samples and they were then passed through a 2-mm mesh sieve and air-dried for the subsequent experiment.

The pH value was determined for each 1:2.5 (soil/water (w/v)) suspension by pH meter (PHS-3C; Shanghai Leici Equipment Factory, China). OM was analyzed according to Sparks et al. (1996). CEC was measured as described previously (Gaskin et al., 2008). The content of clay, silt, and sand were determined using a laser particle size analyzer (MS3000; Malvern PANanalytical, Malvern, UK). The initial soil Cd concentration was measured by the procedure described by Wu et al. (2014).

2.2 Plant material and Cd exposure

Full buds on branches of 'Hanfu' apple were grafted on annual seedlings of *M. baccata* Borkh., and planted in a plastic pots (one plant pot^{-1}) containing 2.5 kg soil in a greenhouse with natural photoperiod (day/night temperature, 25/17°C; relative air humidity, 50%–60%), and watered every dusk. When the plants grew to about 20 cm, 120 plants with similar heights were divided equally to ten groups, and transplanted them in different soils collected from five regions. After two weeks, 100 mL of 500 µM $CdCl_2$ or distilled water was irrigated to the plants of each group in every day, and the final soil Cd content was 156.8 mg kg^{-1}. A plastic tray was placed under each pot to collect the soil leachate which was poured back into the pot to prevent Cd leaching. The soil was regularly irrigated with distilled water, and the soil moisture of each pot was maintained at approximately 70% of field water capacity. The concentration of Cd treatment was used on the basis of previous study (Abdel Latef, 2013). Three replicates for each treatment were conducted, and each replicate contained 4 plants. Plants were harvested after Cd treatment for 70 d.

2.3 Soil collection and plant harvest

The rhizosphere soil of each treatment was collected by shaking off the roots in the air, and then air-dried and then passed through a 2-mm mesh sieve. The dust and soil on the surface of plants were washed with deionized water, and then the roots of each plant were rinsed in 50 mM $CaCl_2$ solution for 5 minutes to remove Cd from the root surface (He et al., 2013b). Subsequently, the roots were carefully washed with deionized water (He et al., 2013b). After separating into the roots, stems and leaves and recording their fresh weight, different tissues were immediately frozen in liquid nitrogen. The frozen samples were then ground to fine powder using a ball mill (MM400; Retsch, Haan, Germany) and subsequently stored at -80°C. The fresh material of each tissue per plant was dried at 60°C for 72 h to analyze the fresh-to-dry mass ratio.

2.4 Determination of Cd forms in soil

The content of Cd chemical forms including acid-soluble Cd, reducible Cd, oxidizable Cd and residual Cd in soil samples was measure by European Community Bureau of Reference (BCR) sequential extraction method (Sungur et al., 2014b). After digestion of residues with a mixture of acids [$HNO_3/H_2O_2/HF$ (3/2/1 (v/v/v)] using a microwave digestion system (MARS 6 CLASSIC; CEM Corporation, Matthews, NC, USA) as described by Wu et al. (2014), the Cd levels in the extract and digest were measured using a flame atomic absorbance spectrometry (FAAS; Hitachi 180-80; Hitachi Ltd., Tokyo, Japan).

2.5 Analysis of root characteristics and photosynthetic pigments

The WinRHIZO Root Analyzer System (WinRHIZO 2012b; Regent Instruments Canada Inc., Montreal, Canada) was used to measure the total root length, total root surface area and total root volume based on the method of Ma et al. (2014). Chlorophyll and carotenoids contents in the leaves of plants were determined using a spectrophotometer (UV-3802; Unico Instruments Co. Ltd, Shanghai, China) according to the method of He et al. (2011).

2.6 Analysis of Cd concentration, total Cd and bio-concentration factor (BCF)

Frozen samples (ca. 100 mg) of each tissue were digested at 170°C in a mixture containing $HNO_3/HClO_4$ (7/1 (v/v)) as previously described (Zhou et al., 2016). The content of Cd in each tissue was determined by flame atomic absorption spectrometry (Hitachi 180-80; Hitachi Ltd, Tokyo, Japan). The total Cd amounts of each tissue were calculated based on the concentration of Cd in each tissue multiplied by the dry mass of the corresponding tissue. BCF was calculated using Cd concentration (µg g^{-1} DW) in root or aerial parts tissues divided by the Cd concentration (µg g^{-1} DW) in the soil (He et al., 2015).

2.7 Measurement of $O_2^{\cdot-}$, H_2O_2 and MDA

The $O_2^{\cdot-}$ and H_2O_2 concentrations of each tissue were determined spectrophotometrically at 530 and 410 nm, respectively, as recommended by He et al. (2011). According to Lei et al. (2007), the MDA concentrations in each tissue of plants were analyzed at 450, 532 and 600 nm with a spectrophotometer.

2.8 Analysis of non-enzymatic and enzymatic antioxidants

The contents of non-enzymatic metabolites including free proline, ascorbate (ASC), T-SH and soluble phenolics in each tissue of plants were measured by the procedure described by He et al. (2013a).

The soluble protein contents in each sample were measured by the procedure described by Luo et al. (2008). The activities of SOD, CAT, POD and ascorbate peroxidase (APX) were measured as previously described by He et al. (2011), and the activities of glutathione reductase (GR) were determined as described by Wang et al. (2013).

2.9 Analysis of gene transcription levels

According to Zhou et al. (2016), the transcript level of genes was analyzed. Root total RNA was extracted and purified based on the manufacturer's instructions by a RNA extraction kit (R6827, Omega Bio-Tek, Norcross, GA, USA). Then a spectrophotometer (NanoDrop 2000; Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis was used to determine the concentration and quality of total RNA, respectively. Subsequently, 1 μg of total RNA was used to synthesize first-strand cDNA using a PrimeScript RT Reagent Kit with gDNA Eraser (DRR037A, Takara, Dalian, China) following the manufacturer's protocol. Quantitative PCR for each gene was run with 10 μL 2× SYBR Green Premix Ex Taq II (DRR820A, Takara), 0.5 μL cDNA and 0.2 μM each gene-specific primer (Table S1). *β-Actin* was used as a reference gene (Table S1). $2^{-\Delta\Delta CT}$ method was used to calculate the relative mRNA expression. For each gene in the roots, expression level was set to 1 in 'Hanfu' apple plants grown in soil of ML subjected to 0 μM $CdCl_2$. Corresponding fold changes in other treatments were then calculated accordingly. Gene expression heatmap was generated based on $\log_2$ average expression fold values.

2.10 Statistical analysis

All statistical analyses were processed using Statgraphics (STN, St Louis, MO, USA) after confirming their normality. For all physiological parameters, two-way ANOVA was employed using Cd treatment (Cd) and soil (S) as factors. To reduce the error of type I, Tukey-HSD method was used to correct the *P*-value obtained from multiple comparisons. When the *P*-value was < 0.05, differences between treatment means were considered significant. Pearson's correlation analysis was used to determine the relationships between soil Cd forms and soil properties as well as Cd content in plant tissues. For the principal component analysis (PCA), the data of plant growth related parameters, photosynthetic pigments, Cd concentrations, ROS and antioxidant levels in the roots, stems, and leaves were standardized and subsequently computed by the command prcomp () in R (http://www.r-project.org/), as suggested by He et al. (2015).

3 Results

3.1 Soil physicochemical properties

There were significant differences among the five soil types in terms of their physicochemical properties and initial Cd content (Table 1). The pH value of soil of KS was the lowest while those of soils of DS and QT were considerably higher than those of all others. Soils of DS, KS, and QT had relatively lower OM content, CEC, clay and silt content and relatively higher sand content than soils of ML and XS. Initial Cd content was higher in soil of XS than that in all others (Table 1).

3.2 Cd content and proportion in various soils

The acid-soluble Cd content was lower in soils of ML and XS than soils of DS and KS (Figure 1A). However, the reducible and oxidizable Cd contents in soils of ML and XS were dramatically higher than those in the other soils (Figures 1B, C). The residual Cd content was remarkably higher in soils of DS and QT than the other soils (Figure 1D).

The proportions of acid-soluble and reducible Cd were highest in all five soils followed by residual and oxidizable Cd (Figure 2). The proportions of acid-soluble Cd were significantly lower in soils of ML and XS than soils of DS and KS. By contrast, the proportions of reducible and oxidizable Cd were markedly higher in soils of ML and XS than the other soils. The percentages of residual Cd were highest in soils of DS and QT (Figure 2).

3.3 Plant Cd concentration, total Cd and BCF

Under Cd stress, the roots of the plants grown in soils of DS and KS accumulated more Cd than those of the plants grown in soils of ML, XS, and QT (Figure 3A). The Cd concentrations in the stems of

TABLE 1 Physicochemical properties of 0–20 cm soil samples collected from five apple orchards.

Soils	pH	OM (%)	CEC (cmol kg^{-1})	Clay (%)	Silt (%)	Sand (%)	Cd (μg g^{-1})
ML	5.82 ± 0.02 b	1.51 ± 0.09 a	21.81± 0.63 a	4.05 ± 0.53 b	56.05 ± 1.52 a	39.91 ± 1.10 c	0.20 ± 0.01 ab
DS	6.62 ± 0.03 a	1.08 ± 0.05 c	5.41 ± 0.11 c	0.95 ± 0.11 d	13.11 ± 0.69 d	85.94 ± 0.81 a	0.14 ± 0.01 b
XS	6.06 ± 0.17 b	1.61 ± 0.08 a	21.29± 0.92 a	22.96 ± 0.57 a	34.89 ± 0.65 b	42.15 ± 0.12 c	0.27 ± 0.01 a
KS	4.88 ± 0.10 c	1.36 ± 0.05 b	7.05 ± 0.43 bc	1.52 ± 0.13 c	17.62 ± 1.08 c	80.86 ± 1.22 b	0.20 ± 0.04 ab
QT	6.76 ± 0.05 a	1.32 ± 0.03 b	9.96 ± 0.35 b	0.95 ± 0.02 d	11.87 ± 0.74 d	87.19 ± 0.75 a	0.16 ± 0.02 b

Different letters beside values in same column indicate significant differences between soils. ML, Maliangou village in Liaoyang city. DS, Desheng village in Panjin city; XS, Xishan village in Chaoyang city; KS, Kaoshantun village in Xinmin city; QT, Qianertaizi village in Xinmin city; OM, organic matter; CEC, cation exchange capacity. Data are means ± SE (n = 3).

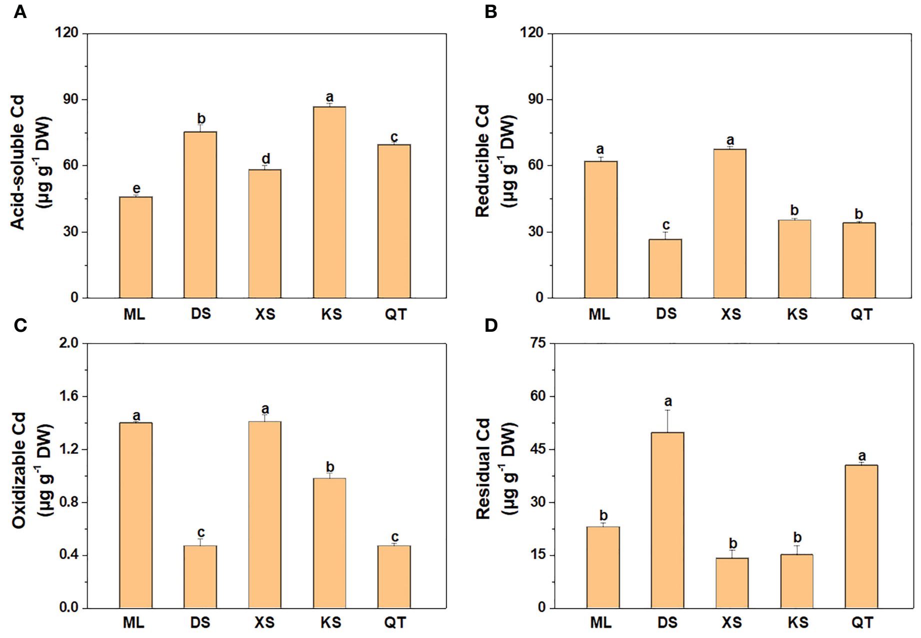

FIGURE 1
Cd concentrations of acid-soluble **(A)**, reducible **(B)**, oxidizable **(C)**, and residual **(D)** forms in soils sampled from five apple orchards subjected to 500 μM $CdCl_2$ for 70 d. Data are means ± SE (n = 3). Different letters on bars indicate significant differences between soils. ML, Maliangou village in Liaoyang city; DS, Desheng village in Panjin city; XS, Xishan village in Chaoyang city; KS, Kaoshantun village in Xinmin city; QT, Qianertaizi village in Xinmin city.

the plants grown in soils of ML and XS were significantly lower than those of the plants grown in the other three soils (Figure 3B). The Cd concentrations were 135.5–212.5% higher in the leaves of the plants grown in soil of DS than they were in those of the plants grown in the other four soils (Figure 3C).

Under Cd stress, the total Cd was highest in the roots of the plants grown in soil of KS and lowest in those of the plants grown in soil of XS. The total Cd was significantly lower in the aerial parts of the plants grown in soils of ML and XS than those of the plants grown in other soils (Figure 4A). After Cd exposure for 70 d, the BCFs were markedly higher in the roots and aerial parts of the plants grown in soils of DS and KS than they were in those of the plants grown in soils of ML, XS, and QT (Figure 4B).

3.4 Coefficients of correlation between content of different forms of Cd and soil physicochemical properties, and Cd content in various ‘Hanfu’ apple plant tissues

Acid-soluble Cd was significantly negatively correlated with CEC and silt content but significantly positively correlated with sand content (Table S2). Reducible and oxidizable Cd was significantly positively correlated with OM content, CEC, and clay and silt content but significantly negatively correlated with sand content (Table S2). Oxidizable Cd was significantly positively correlated with pH value (Table S2). Residual Cd was significantly

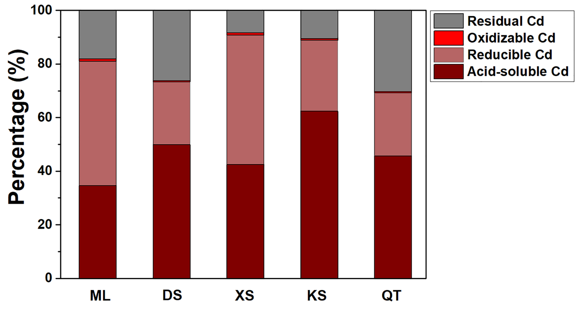

FIGURE 2
Proportions of various Cd fractions in soils sampled from five apple orchards subjected to 500 μM $CdCl_2$ for 70 d. Data are means ± SE (n = 3). ML, Maliangou village in Liaoyang city; DS, Desheng village in Panjin city; XS, Xishan village in Chaoyang city; KS, Kaoshantun village in Xinmin city; QT, Qianertaizi village in Xinmin city.

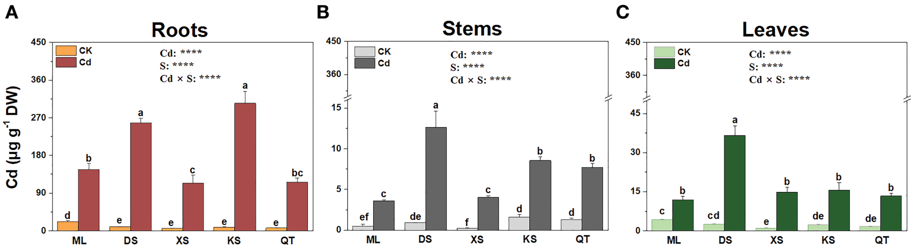

FIGURE 3
Cd concentrations in roots **(A)**, stems **(B)**, and leaves **(C)** of 'Hanfu' apple plants grown in soils sampled from five apple orchards subjected to 0 **(CK)** or 500 μM $CdCl_2$ **(Cd)** for 70 d. Data are means ± SE (n = 3). Different letters on bars indicate significant differences between treatments. *P*-values indicated for ANOVA of Cd treatment (Cd), soil (S), and their interaction (Cd × S). $^{****}P < 0.0001$. ML, Maliangou village in Liaoyang city; DS, Desheng village in Panjin city; XS, Xishan village in Chaoyang city; KS, Kaoshantun village in Xinmin city; QT, Qianertaizi village in Xinmin city.

positively correlated with pH value and sand content but significantly negatively correlated with OM content, CEC, and content of clay and silt (Table S2). In general, root, stem and leaf Cd concentrations of apple plants were significantly positively correlated with acid-soluble Cd but negatively correlated with reducible and oxidizable Cd (Table S3).

3.5 Influences of Cd stress on growth characteristics of apple plants grown in different soils

Relative to the untreated control, Cd stress markedly reduced the dry mass of root, stem, and leaf of the plants grown in all five soils (Table 2). Whereas the root dry mass of the plants grown in soils of ML and XS was only slightly inhibited, the root dry mass of the plants grown in soil of DS was inhibited to the greatest extent (Table 2). There were no marked differences in the inhibition degree of Cd stress on stem dry mass of plants grown in different soil types. After Cd exposure, foliar biomass was inhibited in all plants but to relatively lesser degree in those grown in soils of ML and KS (Table 2).

The total root length, total root surface area, and total root volume of the 'Hanfu' plants significantly decreased after Cd stress. However, these negative effects were relatively less pronounced in the plants grown in soil of ML and relatively more pronounced in those grown in soil of DS (Table S4). The chlorophyll content considerably differed among the plants grown in different soils (Table S4). Cd stress reduced chlorophylls a, b, (a+b), and carotenoid in all plants but to a greater extent in those grown in soil of KS than the others (Table S4).

3.6 Effects of Cd stress on $O_2^{\cdot-}$, H_2O_2, and MDA in plants grown under different soil conditions

Cd exposure significantly increased $O_2^{\cdot-}$ accumulation in the roots of the plants grown in soils of DS, XS and KS (Figure 5). Cd exposure induced a more pronounced increase in $O_2^{\cdot-}$ concentrations in the stems of plants grown in the soils of DS and QT (Figure 5). After Cd exposure, the $O_2^{\cdot-}$ concentrations were significantly increased in the leaves of the plants grown in all soils except for soil of ML (Figure 5). Cd exposure significantly

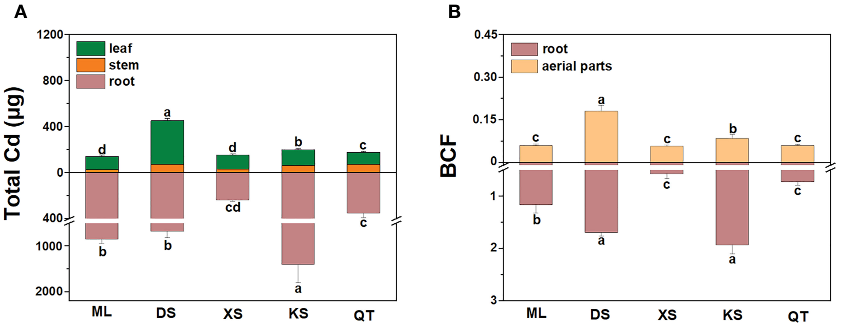

FIGURE 4
Total plant Cd content **(A)** and bio-concentration factor (BCF, **B)** of roots and aerial parts of 'Hanfu' apple plants grown in soils sampled from five apple orchards subjected to 500 μM $CdCl_2$ for 70 d. Data are means ± SE (n = 3). Different letters on bars indicate significant differences between soils. ML, Maliangou village in Liaoyang city. DS, Desheng village in Panjin city; XS, Xishan village in Chaoyang city; KS, Kaoshantun village in Xinmin city; QT, Qianertaizi village in Xinmin city.

TABLE 2 Dry mass (g) of roots, stems, and leaves and lengths of stems of ‘Hanfu’ apple plants grown in soils sampled from five apple orchards subjected to 0 or 500 μM $CdCl_2$ for 70 d.

Soils	Cd (μM)	Root (g DW)	Stem (g DW)	Leaf (g DW)	Stem (cm)
ML	0	5.97 ± 0.34 a	7.85 ± 0.17 b	9.66 ± 0.20 b	60.50 ± 1.59 bc
	500	4.58 ± 0.06 c	5.16 ± 0.21 d	8.60 ± 0.173 d	53.88 ± 0.07 d
DS	0	3.02 ± 0.26 ef	6.43 ± 0.11 c	9.48 ± 0.28 bc	55.13 ± 1.37 d
	500	1.27 ± 0.144 g	4.10 ± 0.245 e	7.41 ± 0.15 ef	45.13 ± 0.51 e
XS	0	3.32 ± 0.08 de	7.32 ± 0.193 b	9.14 ± 0.02 bcd	72.78 ± 0.66 a
	500	2.68 ± 0.14 f	4.50 ± 0.10 e	6.92 ± 0.32 f	61.50 ± 2.50 bc
KS	0	5.44 ± 0.10 b	7.21 ± 0.45 b	7.66 ± 0.23 e	64.50 ± 1.59 b
	500	3.59 ± 0.05 d	4.74 ± 0.08 de	6.94 ± 0.12 f	54.88 ± 0.07 d
QT	0	5.17 ± 0.19 b	8.96 ± 0.24 a	10.96 ± 0.42 a	63.50 ± 3.03 b
	500	3.07 ± 0.08 ef	6.12 ± 0.15 c	8.85 ± 0.31 cd	57.00 ± 0.87 cd
P-value	Cd	****	****	****	****
	S	****	****	****	****
	Cd×S	*	ns	*	ns

Data are means ± SE (n = 3). Different letters beside values indicate significant differences between treatments. *P*-values indicated for ANOVA of Cd treatment (Cd), soil (S), and their interaction (Cd × S). *$P < 0.05$. ****$P < 0.0001$. ns, not significant. ML, Maliangou village in Liaoyang city; DS, Desheng village in Panjin city; XS, Xishan village in Chaoyang city; KS, Kaoshantun village in Xinmin city; QT, Qianertaizi village in Xinmin city.

increased the H_2O_2 content in the roots of all plants except those grown in soil of XS, in the stems of all plants except those grown in soils of ML and XS, and in the leaves of all plants except those grown in soil of DS (Figure 5). Cd induced H_2O_2 accumulation to the greatest extent in the roots and leaves of the plants grown in soil of KS and in the stems of the plants grown in soil of DS (Figure 5). Compared with the control, Cd exposure had no effects on the MDA concentration in the roots, stems, or leaves of the plants

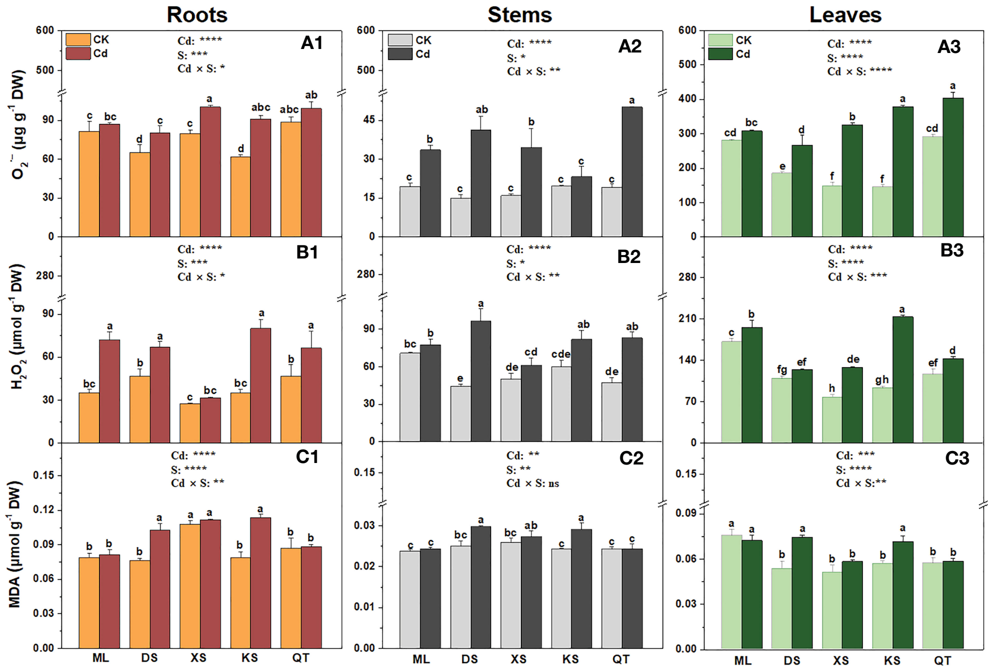

FIGURE 5
Superoxide anion ($O_2^{·-}$, **A1-A3**), hydrogen peroxide (H_2O_2, **B1-B3**) and malondialdehyde (MDA, **C1-C3**) in roots, stems and leaves of ‘Hanfu’ apple plants grown in soils sampled from five apple orchards subjected 0 (CK) or 500 μM $CdCl_2$ (Cd) for 70 d. Data are means ± SE (n = 3). Different letters on bars indicate significant differences between treatments. *P*-values indicated for ANOVA of Cd treatment (Cd), soil (S), and their interaction (Cd × S). *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$. ****$P < 0.0001$. ns, not significant. ML, Maliangou village in Liaoyang city; DS, Desheng village in Panjin city; XS, Xishan village in Chaoyang city; KS, Kaoshantun village in Xinmin city; QT, Qianertaizi village in Xinmin city.

grown in soils of ML, XS, and QT but significantly increased it in the same organs of the plants grown in soils of DS and KS (Figure 5).

3.7 Effects of Cd stress on the antioxidant systems of plants grown in different soils

Compared to the control, Cd stress significantly increased the free proline content in the roots of the plants grown in all soils except soil of ML (Figure 6A1). Cd exposure significantly lowered the free proline concentration in the stems of the plants grown in soils of DS and KS and in the leaves of the plants grown in soil of KS (Figures 6A2, A3). After Cd exposure, the ASC content increased in the roots and stems of the plants grown in soil of ML and the leaves of the plants grown in all soils except for soil of QT. However, it decreased in the roots of the plants grown in soils of DS and KS (Figures 6B1–B3). Under Cd stress, the T-SH content was highest in the roots of the plants grown in soil of ML, the stems of the plants grown in soil of XS, the leaves of the plants grown in soil of KS (Figures 6C1–C3). Cd exposure significantly decreased the soluble phenolic content in the roots of the plants grown in soils of DS and XS, but slightly increased the soluble phenolic content in the stems of the plants grown in soils of XS and KS and the leaves of the plants grown in soil of ML (Figures 6D1–D3).

After Cd exposure, the SOD was significantly enhanced by 98.7%, 344.5%, and 105.9% in the roots of the plants grown in soils of DS, XS, and KS, respectively (Figure S1A1). In general, Cd exposure increased SOD activity in the stems and leaves of all plants except the leaves of plants grown in soil of XS and QT (Figures S1A2, A3). Irrespective of Cd treatment, SOD activity was always higher in the stems of the plants grown in soil of ML than it was in those of the plants grown in all other soils (Figure S1A2). Generally, Cd exposure increased POD activity in all tissues of the plants grown in most soils compared to those under control conditions (Figures S1B1–B3). The highest POD activity was observed in the roots of the plants grown in soil of QT and the stems of the plants grown in soil of ML (Figures S1B1, B2). Cd exposure generally increased CAT in the roots and stems of most plants especially in the stems of the plants grown in soil of XS (Figures S1C1, C2). By contrast, CAT activity increased only in the leaves of the plants grown in soil of DS (Figure S1C3). Compared

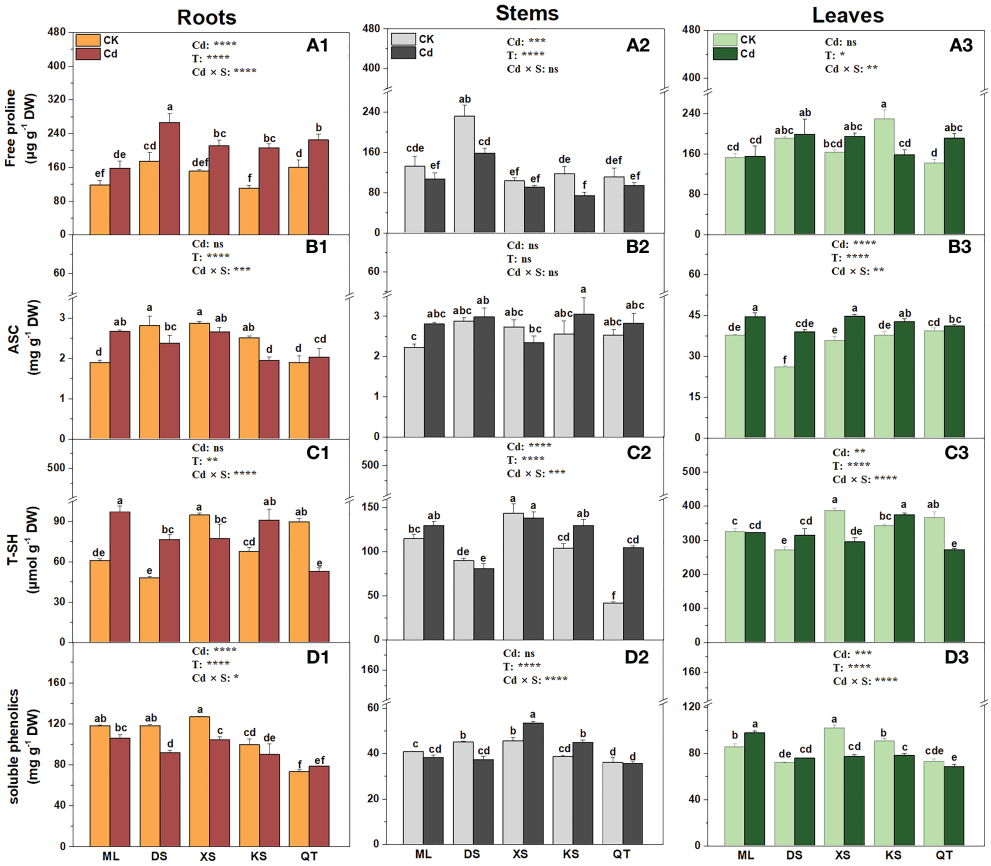

FIGURE 6
Free proline **(A1-A3)**, ascorbate (ASC) (**B1-B3**), total thiols (T-SH) **(C1-C3)** and soluble phenolics **(D1-D3)** in the roots **(A1-D1)**, stems **(A2-D2)** and leaves **(A3-D3)** of 'Hanfu' apple plants grown in the soils sampled from five apple orchards subjected to 0 (CK) or 500 μM $CdCl_2$ (Cd) for 70 d. Data indicate means ± SE (n = 3). Different letters on the bars indicate significant differences between the treatments. P-values indicated for ANOVA of Cd treatment (Cd), soil (S) and their interaction (Cd × S). *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$. ****$P < 0.0001$. ns, not significant. ML, Maliangou village in Liaoyang city; DS, Desheng village in Panjin city; XS, Xishan village in Chaoyang city; KS, Kaoshantun cillage in Xinmin city; QT, Qianertaizi village in Xinmin city.

with the control, APX activity was elevated in the roots of all plants except those grown in soil of KS (Figure S1D1). By contrast, APX activity was only increased in the stems of the plants grown in soils of DS and QT. The leaves of the plants grown in all five soil types did not differ in terms of APX activity following Cd exposure (Figures S1D2, D3). GR activity increased only in the roots of the plants grown in soil of QT, the stems of the plants grown in soils of ML and XS, and the leaves of the plants grown in soil of DS (Figures S1E1–E3).

3.8 PCA of physiological responses to Cd in apple plants grown under different soil conditions

To elucidate the response patterns of 'Hanfu' apple plants to Cd stress, a PCA was conducted using data associated with plant growth, photosynthetic pigments, Cd concentrations, and the ROS and antioxidant levels in the roots, stems, and leaves (Figure 7; Table S5). The effect of Cd treatment was separated by PC1 and its contribution rate was 31.4% (Figure 7). The dominant components in PC1 were root Cd concentration and free proline content, stem dry mass and Cd content, and leaf Cd content (Table S5). However, PC2 was affected by soil and its contribution rate was 15.2% (Figure 7). The dominant factors in PC2 were root POD and GR content, stem SOD content, and leaf H_2O_2 and SOD content (Table S5). The PCA showed that plants grown in various soils demonstrated different physiological responses to Cd exposure mainly because of the relative differences in their root POD and GR content, their stem SOD content, and their leaf H_2O_2 and SOD content. In the PCA plot, the shorter distances between CK and Cd treatment in soils of ML and XS indicated that the plants grown in these soils were relatively less affected by Cd exposure at the physiological level.

3.9 Transcriptional changes of the genes involved in Cd absorption, transport, and detoxification

PM H^+-ATPase pumps protons out of cells by consuming ATP, creates an electrochemical gradient for transmembrane ion transport (Zhang et al., 2017), and is encoded by several genes including *HA11* and *VHA4*. Cd exposure downregulated *HA11* in the roots of the plants grown in soils of ML, XS, and KS by 7.14-fold, 1.43-fold, and 1.58-fold, respectively (Figure 8). By contrast, Cd exposure upregulated *HA11* in the roots of the plants grown in soils of DS and QT, relative to their respective controls (Figure 8). Compared to untreated controls, the roots of the plants grown in soils of ML and QT presented with *VHA4* downregulation. However, the opposite trend was observed in the roots of the plants grown in all other soils. *ZIP* and *IRT1* transport Cd^{2+} intracellularly and regulate plant Cd absorption (Migeon et al., 2010; Wu et al., 2015). Compared with the control, Cd exposure upregulated *ZIP6* in the roots of the plants grown in soil of DS but downregulated it in the roots of the plants grown in all other soils and especially those of the plants grown in soil of XS. Cd exposure upregulated *IRT1* in the roots of the plants grown in all soils but especially in those of the plants grown in soil of DS (Figure 8).

NAS1 and *MT2* may regulate Cd detoxification in plants (Luo et al., 2016). Cd exposure did not affect *NAS1* expression in the roots of the plants grown in soil of ML but markedly downregulated *NAS1* in those of the plants grown in the other soils and especially soil of DS (Figure 8). Cd exposure downregulated *MT2* in the roots of the plants grown in all soils and especially in those of the plants grown in soil of DS (Figure 8). *MHX*, *MTP1* and *ABCC1* are localized to the tonoplast and transfer Cd^{2+} or the Cd-PC complex to the vacuole for storage (Berezin et al., 2008; Lin and Aarts, 2012; Park et al., 2012). Compared with the control, Cd exposure upregulated *MHX* only in the roots of the plants grown in

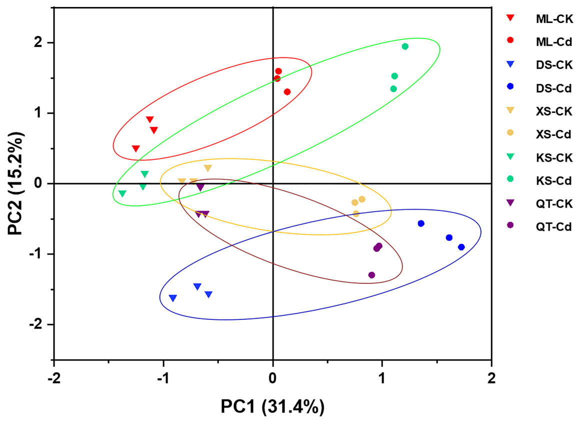

FIGURE 7
Principal component analysis (PCA) of plant growth related parameters, photosynthetic pigments, Cd concentrations, ROS and antioxidant levels in roots, stems, and leaves of apple plants grown in soils sampled from five apple orchards subjected to 0 (CK) or 500 μM $CdCl_2$ (Cd) for 70 d. Triangles and circles represent 0 (CK) and 500 μM $CdCl_2$ (Cd) treatments, respectively. Different colors represent different soils. Red, Maliangou village in Liaoyang city. Blue, Desheng village in Panjin city. Yellow, Xishan village in Chaoyang city. Green, Kaoshantun village in Xinmin city. Purple, Qianertaizi village in Xinmin city. PCA loadings presented in Table S5.

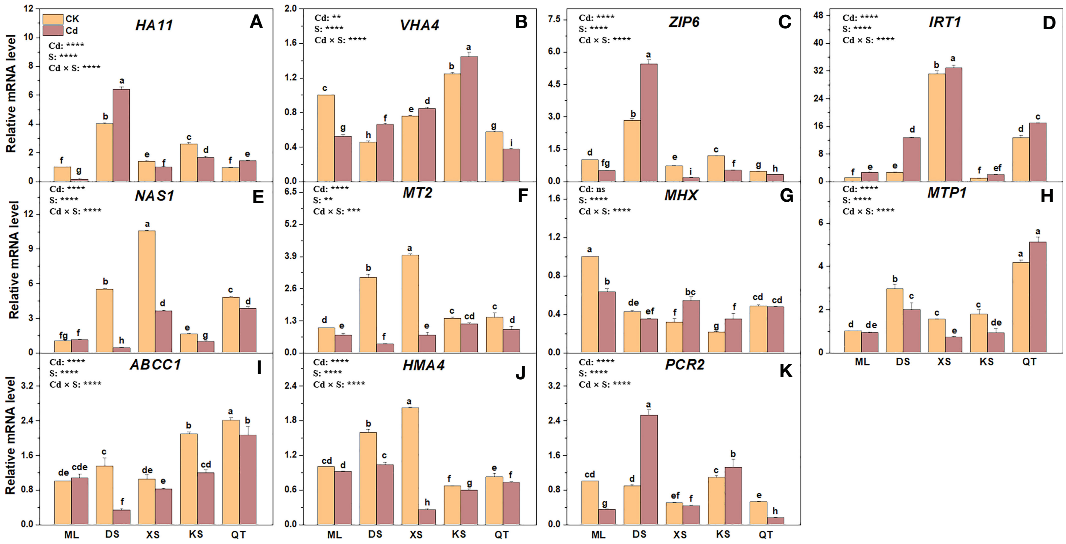

FIGURE 8
Relative expression levels of genes encoding proteins regulating Cd absorption **(A–D)** detoxification **(E–I)** and transport **(J, K)** in roots of 'Hanfu' apple plants grown in soils sampled from five apple orchards subjected to 0 (CK) or 500 µM $CdCl_2$ (Cd) for 70 d. Data are means ± SE (n = 3). Different letters on the bars indicate significant differences between the treatments. For each gene, expression level was normalized to the reference gene *β-Actin* and the expression level was set to 1 in the roots of 'Hanfu' apple plants grown in soil of ML subjected to 0 µM $CdCl_2$. Corresponding fold changes in other treatments were then calculated accordingly. *P*-values indicated for ANOVA of Cd treatment (Cd), soil (S) and their interaction (Cd × S). $^{**}P < 0.01$. $^{***}P < 0.001$. $^{****}P < 0.0001$. ns, not significant. ML, Maliangou village in Liaoyang city; DS, Desheng village in Panjin city; XS, Xishan village in Chaoyang city; KS, Kaoshantun village in Xinmin city; QT, Qianertaizi village in Xinmin city.

soils of XS and KS. Relative to the control, Cd exposure markedly downregulated *MTP1* in the roots of all plants except those grown in soils of ML and QT (Figure 8). Compared with the control, Cd exposure downregulated *ABCC1* in the roots of the plants grown in soils of DS and KS but not in the roots of the plants grown in soils of ML and XS (Figure 8).

PM-bound transporters such as HMA4 and PCR2 export Cd from the cytosol, facilitating Cd translocation into the central cylinder for xylem transport (Hanikenne et al., 2008; Song et al., 2010). Cd treatment caused downregulation of *HMA4* in the roots of the plants grown in all soils but to a greater extent in the roots of those grown in soil of XS (Figure 8). After Cd exposure, *PCR2* was downregulated in the roots of the plants grown in soils of ML and QT but upregulated in the roots of the plants grown in soils of DS and KS (Figure 8).

4 Discussion

4.1 Variations in Cd accumulation and tolerance in apple plants grown in different soils

Heavy metals in soils are compartmentalized into the acid-soluble, reducible, oxidizable, and residual fractions (Meng et al., 2018). Of these, the acid-soluble fraction has the highest mobility (Matong et al., 2016; Zhang et al., 2018b). Soil Cd fractions with different mobility are readily affected by the soil physicochemical properties (Mohamed et al., 2015; Filipović et al., 2018; Xiao et al., 2019). Elevated OM content, CEC, and silty clay loam content provide abundant exchange sites for Cd adsorption, reduce the soil available Cd content, and lower plant Cd uptake (Mohamed et al., 2015; Filipović et al., 2018). In contrast, high soil sand content decreases the surface area and the total negative charge and is not, therefore, conducive to metal cation retention in the soil (Sungur et al., 2014a). Consistent with these results, higher OM, CEC, silt content and lower sand content in soils of ML and XS led to relatively lower acid-soluble Cd and higher reducible and oxidizable Cd content and proportions. He et al. (2017) found that soil Cd availability decreased with the increase of soil pH value. Soil of KS had the lowest soil pH, which may be an important reason for the higher acid-soluble Cd content in soil of KS. Interestingly, the correlations between pH value and acid-soluble and reducible Cd were not significant, indicating that pH value played less important roles in regulating soil Cd forms than other soil characteristics in the present study.

Plant Cd content is usually positively correlated with soil bioavailable Cd (Geebelen et al., 2003). In this study, Cd accumulation varied greatly in apple plants grown in different soils. Plants grown in soils of ML and XS presented with lower root and stem Cd accumulation and BCF, which is attributed to relatively lower Cd bioavailability in these soils. Biomass, root architecture, and plant pigment content reflect heavy metal phytotoxicity (Shi and Cai, 2009). Plants grown in different soils with distinct physicochemical properties varied greatly in their growth parameters (Patel et al., 2015). Apple plants grown in soils ML and XS displayed lower inhibition of tissue biomass and root architecture as well as photosynthetic pigment, while the corresponding indexes of soils of DS, KS and QT showed opposite results. Thus, the plants grown in soils of ML and XS have a greater

Cd tolerance as these substrates had relatively higher OM content, CEC, and silt content, lower sand content, and, therefore, low Cd mobility. These results are consistent with those reported in cabbage and maize under Cd stress (Mohamed et al., 2015).

4.2 Physiological basis of the differences in Cd tolerance among plants grown under various soil conditions

Cd stress induces the accumulation of ROS and MDA, therefore, lead to oxidative stress in plants (Muradoglu et al., 2015). Here, the reductions in growth and photosynthetic pigment content of plant grown in five different soils under Cd stress are associated with Cd-induced oxidative stress. However, as the Cd accumulation was relatively lower in the plants grown in soils of ML and XS, there was relatively less oxidative stress in the plants grown in these two soils, indicating that these plants may adopt more effective coordinated regulatory mechanisms than the plants grown in the other soils.

Cd stress induces plants to produce non-enzymatic antioxidants, for example free proline, ASC, soluble phenolics, and T-SH to remove ROS and mitigate Cd phytotoxicity (He et al., 2020). Hussain et al. (2022) found that biochar application to wheat (*Triticum aestivum* L.) plants under Cd stress increased their foliar free proline, and total soluble phenolics content, which may be attributed to high pH and CEC of biochar (Cui et al., 2016; Moradi et al., 2019). Here, relative higher levels of non-enzymatic antioxidants in plants grown in soils of ML and XS are probably contributed to lower ROS and MDA accumulation. SOD, POD, CAT, APX, and GR are key antioxidant enzymes, playing pivotal roles in overcoming Cd-induced oxidative stress in plants. Biochar amendment significantly increased these enzyme activities in the leaves of pak choi (*Brassica chinensis* L.) under Cd stress possibly because biochar alters soil physicochemical properties (Kamran et al., 2019). Under Cd treatment, the lower ROS and MDA levels in the plants grown in soils of ML and XS were associated with increased antioxidant enzymes activities in tissues of plants grown in these two soils.

4.3 Variations in Cd accumulation and tolerance are related to different gene expression patterns in apple plants grown under various soil conditions

Our previous study showed that PM H^+-ATPase gene downregulation inhibited Cd uptake by apple rootstocks (He et al., 2020) and that PM H^+-ATPase activity might be affected by soil physicochemical properties (Zhang et al., 2018a; Olaetxea et al., 2019). Under Cd exposure conditions, the expression levels of *HA11* in the plants grown in soils of ML and XS and the expression level of *VHA4* in the plants grown in soil of ML were lower than those in the plants grown in soils of DS and KS. These observations were consistent with the fact that Cd concentrations were relatively lower in the roots of the plants grown in soils of ML and XS. *ZIP* and *IRT1* regulated Cd absorption in *Populus* × *canescens* and Chinese cabbage, respectively (Wu et al., 2015; Ding et al., 2017). Nevertheless, there is little empirical evidence about the expression of *ZIP* and *IRT1* regulating Cd uptake in plants grown under different soil conditions. Here, the downregulation of *ZIP6* in the plants grown in soils of ML and XS, and the upregulation of *ZIP* and *IRT1* in the plants grown in soil of DS were consistent with root Cd concentration in these plants. Therefore, variations in Cd accumulation are related to differential transcriptional regulation of genes related to Cd uptake in apple plants grown in different soils.

Arabidopsis thaliana harboring overexpressing *Brassica juncea* gene encoding MT2 had lower Cd sensitivity and higher Cd tolerance than its wild type counterpart (Zhigang et al., 2006). Under Cd stress, three *NAS* genes were strongly upregulated in the roots of durum wheat, thereby maintaining NA production and lowering Cd phytotoxicity (Aprile et al., 2018). Jaskulak et al. (2019) found that soil and metal cation type affected *MT* expression. In this study, the lowest mRNA levels of *MT2* and *NAS1* were found in the plants grown in soil of DS subjected to Cd stress. These findings were in line with the fact that Cd phytotoxicity was more severe in the plants grown in soil of DS. By contrast, the less repression of transcript levels of *MT2* in plants grown in soil of ML, and *NAS1* in plants grown in soils of ML and XS by Cd than in soil of DS corresponding well to its relative lower Cd toxicity than those grown in the soils of DS.

Transporters such as MHX, MTP1, and ABCC1 are located on the tonoplast, transport Cd or Cd complexes from the cytoplasm to vacuole, reduce cytoplasmic free Cd content (Berezin et al., 2008; Lin and Aarts, 2012), and mitigate Cd phytotoxicity. However, there is little empirical evidence that soil physicochemical properties affect transcription of the genes regulating Cd detoxification. Only Jaskulak et al. (2020) found that relative expression of *ABCC* in the shoots of *Sinapis alba* L. varied with soil CEC and total organic carbon content. We found that *MHX* was relatively upregulated in the roots of the plants grown in soil of XS subjected to Cd stress. Cd exposure downregulated *MTP1* in the roots of the plants grown in all soils except in soils of ML and QT and downregulated *ABCC1* in roots of the plants grown in soils of DS and KS. The foregoing results might indicate that the plants grown in soils of ML and XS had relatively lower Cd toxicity as they sequestered the Cd in vacuoles or formed relatively nontoxic Cd-containing complexes.

HMA4 and PCR2 are localized to the plasma membrane and transport Cd^{2+} extracellularly (Hanikenne et al., 2008; Song et al., 2010). Our previous study showed that exogenous melatonin restricted root-to-shoot Cd transport in apple rootstock by downregulating root *HMA4* and *PCR2* (He et al., 2020). In the present study, compared with soils of DS and KS, the *HMA4* levels were relatively lower in the roots of the plants grown in soil of XS and the *PCR2* levels were relatively lower in the roots of the plants grown in soils of ML, XS, and QT under Cd stress. For these reasons, these plants effectively inhibited Cd root-to-shoot transport and mitigated its shoot toxicity.

5 Conclusions

There were significant differences in Cd accumulation, translocation and tolerance in apple plants grown in soils with different physicochemical properties. Lower Cd accumulation and BCF were found in apple plants grown in soils of ML and XS, which could be ascribed to lower Cd bioavailability in these soils. The negative effects of Cd stress on the growth were less pronounced in the plants grown in soils of ML and XS. Moreover, the plants grown in these two soils had relatively lower ROS and MDA production, but higher antioxidant content and enzyme activity. The expression of genes related to Cd absorption and transport was lower in the roots of plants grown in the soils of ML and XS while the transcription levels of the key genes related to Cd detoxification were higher. Our data demonstrated that apple plants grown in soils of ML and XS with higher OM content, CEC, clay and silt content and lower sand content accumulated less Cd and had relative higher Cd tolerance, indicating that the soil types affect Cd accumulation and tolerance in apple plants. These results lay the foundation for mitigation strategies by application of amendments to modify soil properties to decrease soil Cd bioavailability.

Data availability statement

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

Author contributions

XZ and JH conceived and designed research. XZ, HXW and HYW performed the experiment. XZ and JH analyzed data and prepared the manuscript. JH, SQ and DL are responsible for conceptualization, funding acquisition, review and editing. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1188241/full#supplementary-material

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Foliar application of salicylic acid inhibits the cadmium uptake and accumulation in lettuce (*Lactuca sativa* L.)

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Introduction: Salicylic acid (SA) is a multi-functional endogenous phytohormone implicated in the growth, development, and metabolism of many plant species.

Methods: This study evaluated the effects of different concentrations of SA (0, 25, 100, 200, and 500 mg/L) on the growth and cadmium (Cd) content of lettuce (*Lactuca sativa* L.) under Cd stress. The different concentrations of SA treatments were administered through foliar application.

Results: Our results showed that 100-200 mg/L SA significantly increased the plant height and biomass of lettuce under Cd stress. When SA concentration was 200 mg/L, the plant height and root length of lettuce increased by 19.42% and 22.77%, respectively, compared with Cd treatment alone. Moreover, 200 mg/L and 500mg/L SA concentrations could reduce peroxidase (POD) and superoxide dismutase (SOD) activities caused by Cd stress. When the concentration of exogenous SA was 500 mg/L, the POD and SOD activities of lettuce leaves decreased by 15.51% and 19.91%, respectively, compared with Cd treatment. A certain concentration of SA reduced the uptake of Cd by the lettuce root system and the transport of Cd from the lettuce root system to shoots by down-regulating the expression of *Nramp5*, *HMA4*, and *SAMT*, thus reducing the Cd content of lettuce shoots. When the concentration of SA was 100 mg/L, 200 mg/L, and 500 mg/L, the Cd contents of lettuce shoots were 11.28%, 22.70%, and 18.16%, respectively, lower than that of Cd treatment alone. Furthermore, principal component and correlation analyses showed that the Cd content of lettuce shoots was correlated with plant height, root length, biomass, antioxidant enzymes, and the expression level of genes related to Cd uptake.

Discussion: In general, these results provide a reference for the mechanism by which SA reduces the Cd accumulation in vegetables and a theoretical basis for developing heavy metal blockers with SA components.

KEYWORDS

salicylic acid, lettuce, cadmium, ion transporter gene, antioxidant enzyme

1 Introduction

Cadmium (Cd) is a nonessential element for plants, and it causes plant poisoning when its toxicity threshold is exceeded (Saidi et al., 2014). Cd plant poisoning symptoms include yellowing and chlorosis of leaves, poor plant development, restricted root growth, photosynthesis inhibition, changes in chloroplast ultrastructure, lipid peroxidation, and nitrogen metabolism disorders (Ran et al., 2015; Haider et al., 2021). These symptoms often reduce crop yield (Aziz et al., 2015). Khan et al. showed that 50 μmol/L of Cd significantly reduced the biomass of *Brassica rapa ssp. chinensis* L. and reduced leaf photosynthetic parameters (photosynthetic rate, stomatal conductance, transpiration rate, and intercellular CO_2 concentration) (Khan et al., 2020). In addition, Cd can be transferred and accumulated in the edible part of crops, directly endangering human health through the food chain (Wang et al., 2019). Several studies showed that exogenous plant hormones and other antioxidant substances could activate the defense mechanism of plants to reduce Cd toxicity on plants (Chen et al., 2020; Waheed et al., 2021; Wang et al., 2021a). Moreover, spraying exogenous substances is an economical, effective, and easy measure of alleviating the Cd uptake and stress on crops. Salicylic acid (SA), a small molecular phenolic substance widely existing in plants, and participates in the growth and development and various physiological and biochemical activities in plants and can activate stress-related resistance metabolism in plants (Metwally et al., 2003; Sharma et al., 2020). Previous studies showed that SA could be a key signal molecule to mediate plant responses to biotic and abiotic stresses, including drought, salinity, cold, osmotic, and heavy metal stress (Szepesi et al., 2009; Khan et al., 2015). Several studies also showed that the exogenous application of SA can effectively alleviate the toxic effects and reduce the accumulation of Cd in plants under Cd stress (Metwally et al., 2003; Singh et al., 2015; Wang et al., 2021d). For example, Guo et al. (2007)showed that SA pretreatment could alleviate Cd-mediated inhibition on the growth of rice roots and enhance the antioxidant activity of rice under Cd stress. This reduced the toxicity induced by Cd and enhanced the rice tolerance to Cd. Similarly, Li et al. (2019) reported that spraying potato leaves with 600 μmol/L SA could alleviate the toxic effect of Cd (200 μmol/L Cd) by increasing the relative water content, chlorophyll, proline, and endogenous SA content of leaves and stimulating the antioxidant enzyme activity. Wang et al. also showed that spraying SA could reduce Cd accumulation in rice by regulating the expression level of genes related to Cd transport and absorption (*OsNramp1*, *OsNramp5*, *OsHMA2*, *OsHMA3*, and *OsHMA9*) (Wang et al., 2021c). However, there are relatively few reports on how SA changes the physiological mechanisms of Cd transport and accumulation in leafy vegetables.

Leafy vegetables are an indispensable part of a healthy diet. Leafy vegetables are more sensitive to Cd pollution than other crops and vegetables (such as Solanaceae, cabbage, root vegetables, onions, legumes, etc.) (Xiao et al., 2018; Wang et al., 2021e). Among them, lettuce (*Lactuca sativa* L.) is rich in various nutrients and highly edible. Though very sensitive to soil Cd pollution, lettuce is one of the leafy vegetables with the highest Cd accumulation ability, which greatly increases the risk of Cd entering the human body (Mehmood et al., 2013). Tang et al. found that 50 μmol//L cadmium significantly reduced the growth indicators of lettuce and significantly accumulated Cd content in the shoot, and caused stress but not death (Tang et al., 2022). However, there is insufficient information on the effects of different concentrations of SA on leafy vegetables under Cd stress conditions. Therefore, this study investigated the effects of exogenous spraying of the same concentration of SA on lettuce physiology, biochemistry, cadmium content, and gene expression related to SA synthesis under 50μmol/L Cd stress. To provide a good reference basis for improving the cadmium resistance of lettuce and ensuring the safe production of vegetables.

2 Materials and methods

2.1 Materials

The test species 'Glass lettuce' seeds were purchased from the Chengdu seed station (Chengdu, China). The Hoagland nutrient solution was used for cultivation, and the Cd compound was $CdCl_2{\cdot}2.5H_2O$ (analytical grade). SA was obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Experimental design

The study was conducted at the Chengdu Campus of the Sichuan Agricultural University from February to June 2022. Full and uniform-sized lettuce seeds were sterilized in 10% (v/v) hydrogen peroxide (H_2O_2) for 10 min, rinsed with ultrapure water, and evenly distributed in petri dishes containing moist filter papers. The plates were then placed in a 20 °C artificial incubator for germination. Thereafter, sprouted seeds were seeded in a seedling tray containing perlite and vermiculite in a 1:1 ratio and transferred to an artificial incubator. Half-strength Hoagland nutrient solution was added to the tray at an appropriate time daily, and the incubator was set to 23/18 °C (day/night) and a 14/10 h (day/night) photoperiod under a 200 $\mu mol/m^2/s$ light intensity. When four true leaves of the seedlings had fully unfolded, seedlings with vigorous and consistent growth were selected and transplanted into a 10×10 cm (depth × height) nutrient container with equal volumes of perlite and vermiculite. Each container contained one plant, and the containers were placed in a plastic dish (with a height of 8 cm) filled with full-strength Hoagland's nutrient solution, which was replaced every 3 days.

Three days after seedling transplantation, the lettuce leaves treated with CK (the control without Cd) and Cd treatments were sprayed with distilled water and different concentrations of SA solution. After 3 days of pretreatment, all treatment groups other than CK were treated with Hoagland nutrient solution containing 50 μmol/L Cd and different concentrations of SA solution. Similar to the pretreatment, the treatments were sprayed once every three days, for a total of three times. Throughout the experiment, different concentrations of SA solution were sprayed four times (pretreatment once and treatment thrice after adding Cd). When replacing the nutrient solution, we ensured that at least 30% of the nutrient solution flowed out to prevent Cd accumulation in the nutrient bowl. The six experimental treatments applied in the experiment included: CK (the control without Cd), Cd (50 μmol/L Cd), Cd + SA 25 (50 μmol/L Cd + 25 mg/L SA), Cd + SA 100 (50 μmol/L Cd +100 mg/L SA), Cd + SA 200 (50 μmol/L Cd + 200 mg/L SA), and Cd + SA 500 (50 μmol/L Cd + 500 mg/L SA). Throughout the experiment, the temperature was 23/18 °C (day/night), with a relative humidity of 75% – 80%, a light cycle of 14/10 h (day/night), and a light intensity of 300 μ mol/m^2/s in the artificial culture room. The pots were haphazardly rearranged regularly to weaken the impact of edge effects and promote the timely prevention and control of pests and diseases.

2.3 Sample analysis

Lettuce samples were collected 10 days after the last spraying of exogenous SA treatment, and the growth and morphological indicators of the whole plants were measured. Thereafter, fresh root and shoot samples were freeze-dried in liquid nitrogen and stored at -80 °C in an ultra-low temperature refrigerator for the subsequent determination of physiological and quality indicators. For the Cd content determination, shoot and root samples were fixed in an oven at 105 °C for 15 min and then dried at 75 °C to a constant weight.

2.3.1 Determination of plant growth and biomass

The plant height and root length of lettuce plants were measured to the nearest millimeter using a ruler. Thereafter, the lettuce shoots and roots were washed with tap water and rinsed with deionized water thrice, followed by oven-drying at 105 °C for 15 min and at 75 °C to constant weight for dry biomass determination.

2.3.2 Determination of photosynthetic pigment content

The second and third functional leaves from the shoot tip (n = 3) were used to determine the contents of photosynthetic pigments (chlorophyll a, chlorophyll b, and carotenoid) using the ethanol and acetone extraction methods described previously (Tang et al., 2020).

2.3.3 Determination of photosynthetic parameters

The same leaves were used to determine the net photosynthetic rate (Pn), stomatal conductance (Gs), transpiration rate (Tr), and intercellular CO_2 concentration (Ci) with a LI-6400XT portable photosynthetic system (LI-COR Inc., Lincoln, NE). The photosynthetic parameters were manually set at 25 °C, 1000 μmol/m^2/s light intensity, and a CO_2 concentration of 400 μmol/mol (Tang et al., 2022). Stomatal limitation (Ls) was 1-Ci/Ca, where Ca is the atmospheric CO_2 concentration.

2.3.4 Determination of membrane peroxidation

The proline content was assayed using the sulfosalicylic acid method, while the soluble protein and sugar contents were assayed using the Coomassie brilliant blue G-250 anthrone–ethyl acetate methods, respectively. The relative conductivity was assayed using a conductivity meter (Hi-Fidelity Technology Co. Ltd.; Beijing, China), and all the assays were conducted according to the methods described by Wang et al. (2021b).

2.3.5 Determination of antioxidant enzyme indexes

The antioxidant enzyme activity assays were conducted as previously described (Liang et al., 2022). Superoxide dismutase (SOD) activity was measured using the nitroblue tetrazolium method, peroxidase (POD) activity using the guaiacol method, and catalase (CAT) activity using the ultraviolet (UV) absorption method.

2.3.6 Determination of cadmium content

Plant samples (0.5 g) were treated with a 4:1 nitric acid: perchloric acid solution (v:v) for 12 h, digested to a clear solution, filtered, and diluted to a volume of 50 mL. The Cd content was then determined using an iCAP 6300 ICP spectrometer (Thermo Scientific, Waltham, MA, USA) (Tang et al., 2020). The translocation factor (TF) was calculated as the Cd content of shoots divided by the Cd content of roots (Rastmanesh et al., 2010).

2.3.7 Determination of the relative expression of genes related to cadmium uptake and SA synthesis in lettuce

The total RNA was extracted from all samples using a TIANGEN Biotech Co., Ltd. RNA prep pure plant kit (TIANGEN Biotech Co., Ltd., Beijing, China), according to the manufacturer's instructions. Real-time fluorescence quantitative PCR was conducted on a real-time quantitative PCR instrument (CFX Connect; Bio-Rad, Hercules, CA, USA) using 2X M5 HiPer SYBR Premix EsTaq (with Tli RNaseH) (Mei5 Biotechnology Co., Ltd.). All primers were synthesized by Beijing Tsingke Biotechnology Co., Ltd. (Beijing, China) and are listed in Table 1. The relative expression level of the genes was calculated by the $2^{-\Delta\Delta CT}$ method. Three independent biological replicates were set per sample (Pan et al., 2021).

2.4 Statistical analyses

Data were compiled and organized using Excel 2016 software (Microsoft Corp., Redmond, WA, USA), and statistical analyses were conducted using SPSS 25.0 statistical software (IBM, Armonk, NY, USA). The results were analyzed by one-way ANOVA with Duncan's multiple range test at the $P < 0.05$ significance level and presented as the means of three biological replicates ± standard error (SE). We conducted principal component and correlation analyses to study the relationship between various indicators. The figures were constructed using Origin Pro 2021 software (Electronic Arts Inc, USA).

TABLE 1 Primer information of quantitative real-time PCR.

Gene name	Gene symbol in NCBI	Forward primer(5′-3′)	Reverse Primer(5′-3′)
Nramp2	LOC111889112	CTCCGGTCGTCAACTCTTCC	ATCTTCGTCATCCGAGGTGC
Nramp5	LOC111901958	AGCTATGTGAAGCCACCAGC	GCATGCATCGTTGACACCAT
HMA3	LOC111887653	GGGGTGTTCACAAGTTCCCA	TGTGCGAAATTGGCTGCTTC
HMA4	LOC111881074	GCTTTGGAGTAGGAATGGAAGT	GTGGTGACACAATGGCACTTT
WRKY6	LOC111907870	TCGAGCAAGCTAATGACCCC	TGTGGGTGCCTTCATAGGTG
SAMT	LOC111890312	TTGTTCGCCGGAGAAACGTA	CGGTATGCCCTTGTGTCCAT
Actin		GTGAGTGAAGAAGGGCAATG	CACTTTCAACCCGATTCACC

3 Results

3.1 Plant growth and biomass

Cd stress significantly reduced the plant height, root length, shoot biomass, and root biomass of lettuce by 22.63%, 18.58%, 42.60%, and 42.53%, respectively, compared with CK (Figure 1). The plant height, root length, shoot biomass, and root biomass of lettuce treated with 200 and 500 mg/L SA were significantly higher and increased by 19.42%, 22.77%, 10.83%, and 6.58%, respectively compared to those treated with Cd treatment alone (Figures 1A, B). In addition, the shoot and shoot biomass of lettuce increased with the increase of SA concentration under Cd stress (Figures 1C, D). When SA concentration was 500 mg/L, the shoot and shoot biomass increased by 35.79% and 33.90%, respectively, compared with Cd treatment alone (Figures 1C, D).

3.2 Photosynthetic pigment content

Compared with CK, Cd stress significantly reduced the contents of chlorophyll and carotenoid in lettuce leaves (Figure 2). However,

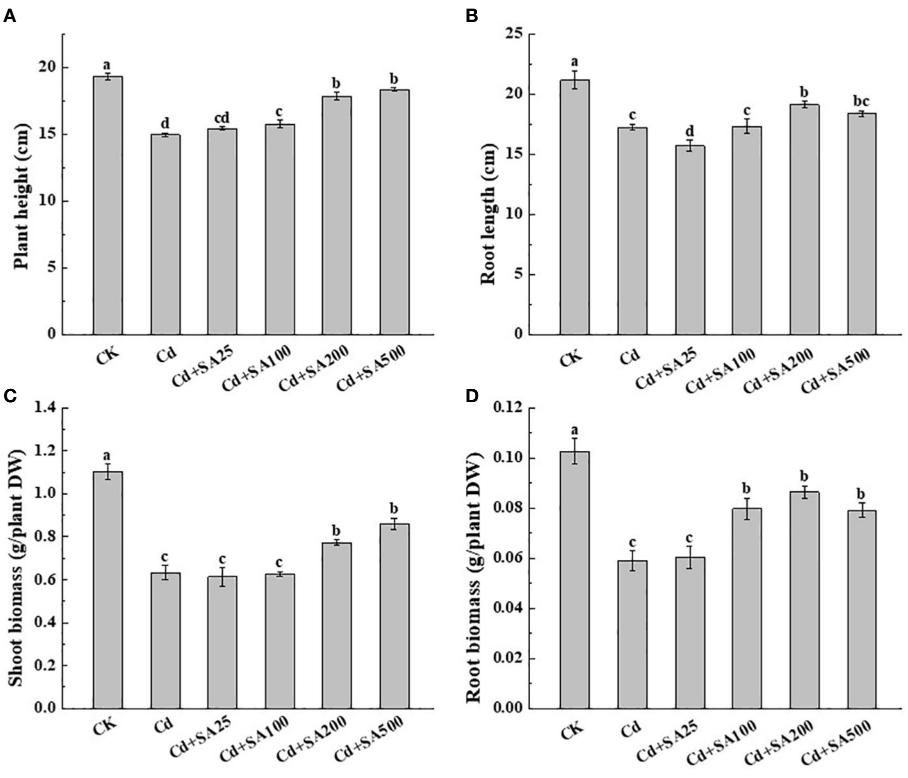

FIGURE 1
Effects of different concentrations of exogenous SA on lettuce growth and biomass under Cd stress. **(A)** The plant height. **(B)** The root length. **(C)** The shoot biomass of dry weight. **(D)** The root biomass of dry weight. CK: control; Cd: 50µmol/L Cd; Cd+SA25: 50µmol/L Cd+25mg/L SA; Cd+SA100: 50µmol/L Cd+100mg/L SA; Cd+SA200: 50µmol/L Cd+200mg/L SA; Cd+SA500: 50µmol/L Cd+500mg/L SA. Data are means ± SE of 3 replicate samples. Values with the different letters are significantly different ($P < 0.05$).

different concentrations of SA increased the content of chlorophyll a, chlorophyll b, and carotenoid in lettuce leaves compared with Cd stress (Figure 2). When SA concentration was 25, 100, 200, and 500 mg/L, the total chlorophyll content of leaves increased by 26.11%, 33.70%, 36.11%, and 19.54%, respectively compared with Cd treatment (Figure 2A). Similarly, when SA concentration was 100 mg/L and 200 mg/L, the chlorophyll a content of lettuce leaves increased by 37.49% and 38.55%, respectively, compared with Cd treatment alone (Figure 2A). The carotenoid content of lettuce increased with the increase of SA concentration, and when SA concentration was 200 mg/L, the carotenoid content of lettuce leaves increased by 34.80% compared with Cd treatment alone (Figure 2B).

3.3 Photosynthetic parameters

Cd stress significantly reduced the Pn and Ls of lettuce by 35.62% and 22.22% (Table 2) but significantly increased the Gs, Ci, and Tr of lettuce by 26.32%, 8.03%, and 60.49%, respectively, compared with CK. The different concentrations of exogenous SA could increase the Pn and Ls of lettuce; for example, when the concentration of SA was 200 mg/L, the Pn and Ls increased by 51.97% and 30.00%, respectively, compared with Cd treatment alone. However, exogenous SA reduced the Gs, Ci, and Tr of lettuce leaves to a certain extent, and when the concentration of SA was 100 mg/L, the Ci and Tr of lettuce leaves decreased by 9.70% and 29.23%, respectively, compared with Cd treatment alone (Table 2).

3.4 Membrane lipid peroxidation degree and osmotic regulating substances

Cd stress significantly increased the proline content, relative conductivity, and soluble protein content of lettuce leaves by 28.64%, 96.98%, and 32.96%, respectively, compared with CK (Table 3). With the increase of exogenous SA concentration, the soluble sugar content of lettuce leaves gradually increased; when SA concentration was 500 mg/L, the soluble sugar content increased by 27.97% compared with Cd treatment alone (Table 3). Conversely, exogenous SA significantly reduced the proline content of lettuce leaves under Cd stress; when the concentration of SA was 100 mg/L, the proline content decreased by 21.56% compared with Cd treatment alone. The exogenous SA concentration of 200 mg/L reduced the relative conductivity of lettuce leaves by 32.82% compared with the Cd treatment alone (Table 3).

3.5 Antioxidant enzyme activities

Cd stress significantly increased the POD and SOD activities of lettuce leaves by 33.23% and 41.19%, respectively, compared with CK (Figures 3A, B). However, Cd stress significantly reduced the CAT activity of lettuce leaves by 34.64% lower than that of CK (Figure 3C). Different concentrations of exogenous SA significantly reduced the POD and SOD activities of lettuce leaves compared with Cd treatment alone. The POD and SOD activities of lettuce leaves decreased by 17.85% and 15.87%, respectively, when the concentration of exogenous SA was 200 mg/L and by 15.51% and 19.91%, respectively, when the concentration of exogenous SA was 500 mg/L, compared with Cd treatment alone (Figures 3A, B). In addition, exogenous SA could significantly increase the CAT activity of lettuce leaves under Cd stress; when SA concentration was 200 mg/L, the CAT activity of lettuce leaves increased by 34.46% compared with Cd treatment alone (Figure 3C).

3.6 Cadmium content

Under Cd stress, the total Cd content of lettuce increased initially and then decreased with the increase of exogenous SA

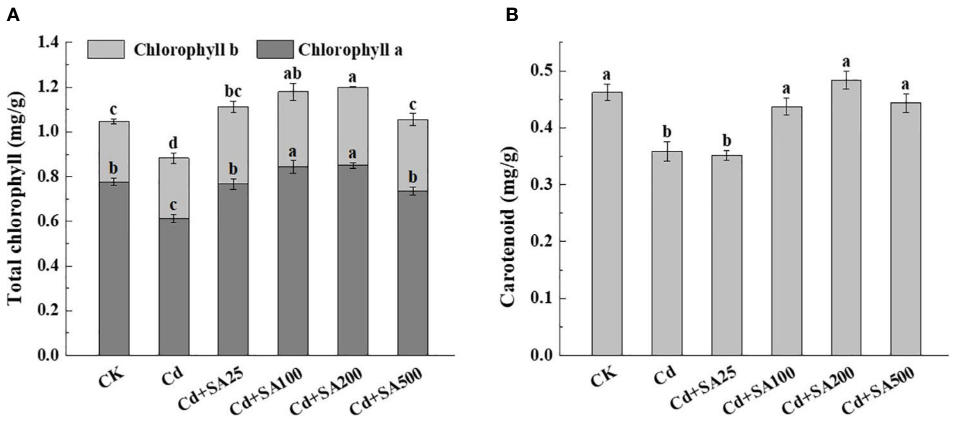

FIGURE 2
Effects of different concentrations of exogenous SA on the photosynthetic pigment content of lettuce under Cd stress. **(A)** The total chlorophyll content. **(B)** The carotenoid content. CK: control; Cd: 50μmol/L Cd; Cd+SA25: 50μmol/L Cd+25mg/L SA; Cd+SA100: 50μmol/L Cd+100mg/L SA; Cd+SA200: 50μmol/L Cd+200mg/L SA; Cd+SA500: 50μmol/L Cd+500mg/L SA. Data are means ± SE of 3 replicate samples. Values with the different letters are significantly different ($P < 0.05$).

TABLE 2 The effect of SA treatment on the photosynthesis of lettuce under Cd stress.

Treatments	Pn (μmolm^{-2} s^{-1})	Gs (mol m^{-2} s^{-1})	Ci (μmol mol^{-1})	Tr (mmol m^{-2} s^{-1})	Ls
CK	13.39 ± 0.692a	0.19 ± 0.003b	293.12 ± 2.167b	1.62 ± 0.026d	0.27 ± 0.005b
Cd	8.62 ± 0.447c	0.24 ± 0.009a	316.65 ± 4.498a	2.60 ± 0.016a	0.21 ± 0.011c
Cd+SA25	9.89 ± 0.070c	0.22 ± 0.006a	299.39 ± 4.119b	2.50 ± 0.017b	0.25 ± 0.010b
Cd+SA100	11.31 ± 0.349b	0.20 ± 0.004b	285.95 ± 6.695bc	1.84 ± 0.014c	0.29 ± 0.017ab
Cd+SA200	13.10 ± 0.364a	0.23 ± 0.006a	278.64 ± 3.133bc	1.62 ± 0.024d	0.30 ± 0.008a
Cd+SA500	12.02 ± 0.499ab	0.19 ± 0.010b	288.44 ± 3.606c	1.87 ± 0.028c	0.28 ± 0.009ab

Ci, intercellular CO_2 concentration; Gs, stomatal conductance; Pn, net photosynthetic rate; Tr, transpiration rate; Ls, stomatal limitation. CK: control; Cd: 50μmol/L Cd; Cd+SA25: 50μmol/L Cd +25mg/L SA; Cd+SA100: 50μmol/L Cd+100mg/L SA; Cd+SA200: 50μmol/L Cd+200mg/L SA; Cd+SA500: 50μmol/L Cd+500mg/L SA. Data are means ± SE of 3 replicate samples. Values with the different letters are significantly different ($P < 0.05$).

concentration. When SA concentration was 200 mg/L and 500 mg/L, the total Cd content of lettuce decreased by 7.29% and 9.22%, respectively, compared with Cd treatment alone (Figure 4A). Similarly, when the concentration of SA was 100 mg/L, 200 mg/L, and 500 mg/L, the Cd content in the lettuce shoots was 11.28%, 22.70%, and 18.16%, respectively, lower than that under Cd treatment alone (Figure 4A). The SA concentrations of 100 mg/L and 200 mg/L also reduced the translocation factor of Cd from lettuce roots to shoots by 22.80% and 24.96%, respectively, compared with Cd treatment alone (Figure 4B).

3.7 Expression of genes responsible for Cd uptake and transport and SA synthesis

Compared with CK, Cd stress significantly increased the relative expression levels of *Nramp2*, *Nramp5*, and *SAMT* by 42.00%, 302.84%, and 148.17%, respectively (Figures 5A, B, F). However, Cd stress significantly down-regulated the relative expression levels of *HMA3*, *HMA4*, and *WRKY6* compared to CK (Figures 5C, D, E). Under Cd stress, exogenous SA could up-regulate the relative expression of *HMA3* and *HMA4*, and when SA concentration was 200 mg/L, the relative expression of *HMA3* increased by 68.76% compared with Cd treatment alone (Figures 5F, E). When the concentration of SA was 25 mg/L, the relative expression of *SAMT* increased by 279.10% compared with CK and by 52.76% compared with Cd treatment alone (Figure 5F). These results showed that SA could alleviate Cd stress on lettuce by regulating the Cd absorption and transportation and the expression of genes related to SA synthesis in lettuce.

3.8 Principal component analysis

Principal component analysis (PCA) was performed to characterize the changes in various lettuce indicators under Cd stress conditions and different concentrations of SA treatment. The results showed that PCA could clearly separate lettuce samples from different treatments, and there were significant differences between the treatments (Figure 6A). The first component (PC1) and the second component (PC2) explained 62.0% and 15.8% of the variance, respectively, and the cumulative variance interpretation rate was 77.9%. PC1 was positively correlated with the shoot and root Cd contents, the translocation factor, and the relative expression of *Nramp2* in lettuce but negatively correlated with the shoot and root biomass and the relative expression of *HMA3* and *HMA4* in lettuce (Figure 6B). Moreover, total chlorophyll content, carotenoid content, and Pn were negatively correlated with PC2, indicating that exogenous Cd and SA significantly impacted the photosynthesis of lettuce.

3.9 Correlation analysis

To further intuitively display the relationship between the various lettuce indicators, we conducted a correlation analysis

TABLE 3 The effect of SA treatment on osmotic adjustment substances in lettuce under Cd stress.

Treatments	Proline (μg/g)	Relative Conductivity(%)	Soluble sugar Content (% FW)	Soluble protein Content (mg/g FW)
CK	33.03 ± 0.084d	6.96 ± 0.285c	1.35 ± 0.011a	13.50 ± 0.162e
Cd	42.49 ± 0.438a	13.71 ± 1.009a	0.85 ± 0.023e	17.95 ± 0.022b
Cd+SA25	33.44 ± 0.592d	10.22 ± 0.434b	0.96 ± 0.008d	18.93 ± 0.071a
Cd+SA100	33.33 ± 0.607d	8.69 ± 0.325bc	0.95 ± 0.016d	17.80 ± 0.086b
Cd+SA200	39.61 ± 0.356b	7.27 ± 0.435c	1.05 ± 0.024c	16.02 ± 0.121c
Cd+SA500	36.58 ± 0.172c	9.21 ± 0.492b	1.18 ± 0.009b	14.80 ± 0.078d

CK: control; Cd: 50μmol/L Cd; Cd+SA25: 50μmol/L Cd+25mg/L SA; Cd+SA100: 50μmol/L Cd+100mg/L SA; Cd+SA200: 50μmol/L Cd+200mg/L SA; Cd+SA500: 50μmol/L Cd+500mg/L SA. Data are means ± SE of 3 replicate samples. Values with the different letters are significantly different ($P < 0.05$).

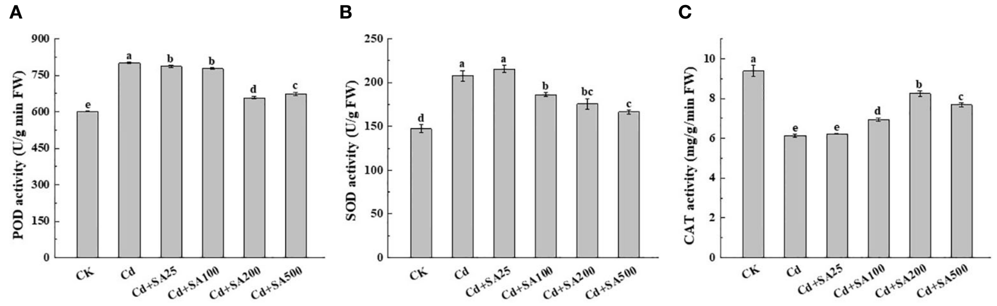

FIGURE 3
Effects of different exogenous SA on the activity of antioxidant enzymes in lettuce under Cd stress. **(A)** POD activity, **(B)** SOD activity, **(C)** CAT activity. CK: control; Cd: 50μmol/L Cd; Cd+SA25: 50μmol/L Cd+25mg/L SA; Cd+SA100: 50μmol/L Cd+100mg/L SA; Cd+SA200: 50μmol/L Cd+200mg/L SA; Cd+SA500: 50μmol/L Cd+500mg/L SA. Data are means ± SE of 3 replicate samples. Values with the different letters are significantly different ($P < 0.05$).

based on the heat map, as shown in Figure 7, the shoot and root Cd content and the Cd translocation factor in the lettuce had a significant positive correlation with the soluble protein, POD activity, and SOD activity but a negative correlation with the plant height (A), root length, shoot biomass, root biomass, soluble sugar, CAT activity and relative expression of *WRKY6*, and *HMA4* ($p \leq 0.01$) (Figure 6). At the same time, there was no significant correlation between Cd content in the shoot and root of lettuce, as well as Cd transport factors, and total chlorophyll content, Ci, and proline content in leaves (Figure 7). Moreover, carotenoid content had a significant positive correlation with plant height, root length, root biomass, soluble sugar, and CAT activity ($P \leq 0.01$), but not significantly correlated with the relative expression of *WRKY6*, *HMA3*, *HMA4* and *Nramp2* (Figure 5). The relative expression of *Nramp2* and *HMA3* were not significantly correlated with other indicators, while the relative expression of *HMA4* and *WRKY6* positively correlated with plant height, root length, shoot biomass, soluble sugar, and CAT activity. Moreover, the relative expression of *HMA4* and *WRKY6* was positively correlated with plant height, root length, shoot biomass, solve sugar, and CAT activity but negatively correlated with SOD activity, shoot and root Cd content, and Cd translocation factor of lettuce ($p \leq 0.01$) (Figure 6). Contrary to *HMA4* and *WRKY6*, the relative expression of *SAMT* had a significant negative correlation with plant height, root length, root biomass, carotenoid, and Pn, and a significant positive correlation with soluble protein, POD activity, SOD activity, and the relative expression of *Nramp5* ($p \leq 0.01$).

4 Discussion

Cd stress seriously affects the normal growth and development of most crops, resulting in symptoms such as chlorosis and yellowing of leaves, curling of leaves, delayed growth of stems, reduction of lateral roots, root tip necrosis, and plant dwarfing (Haider et al., 2021; Shaari et al., 2022). In this study, our results show that 50 μmol/L Cd significantly inhibited the growth of lettuce and reduced its biomass, consistent with the results of previous studies (Lavres et al., 2019; Huang et al., 2022b). Excessive concentration of salicylic acid can seriously damage the growth and development of plants. Research has shown that low concentration of salicylic acid can maintain a high electron transfer rate in the chloroplasts of tomato Guard cell,

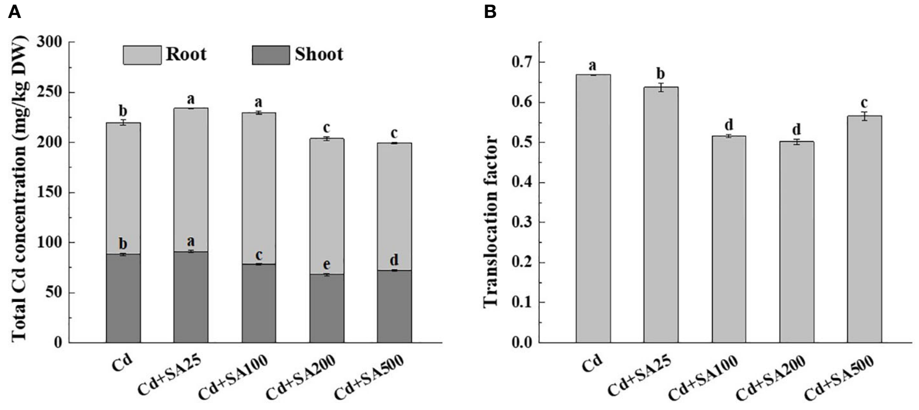

FIGURE 4
Effects of different exogenous SA on Cd content and Cd translocation factor of lettuce under stress. **(A)** Cd concentration, **(B)** Translocation factor. Cd: 50μmol/L Cd; Cd+SA25: 50μmol/L Cd+25mg/L SA; Cd+SA100: 50μmol/L Cd+100mg/L SA; Cd+SA200: 50μmol/L Cd+200mg/L SA; Cd+SA500: 50μmol/L Cd+500mg/L SA. Data are means ± SE of 3 replicate samples. Values with the different letters are significantly different ($P < 0.05$).

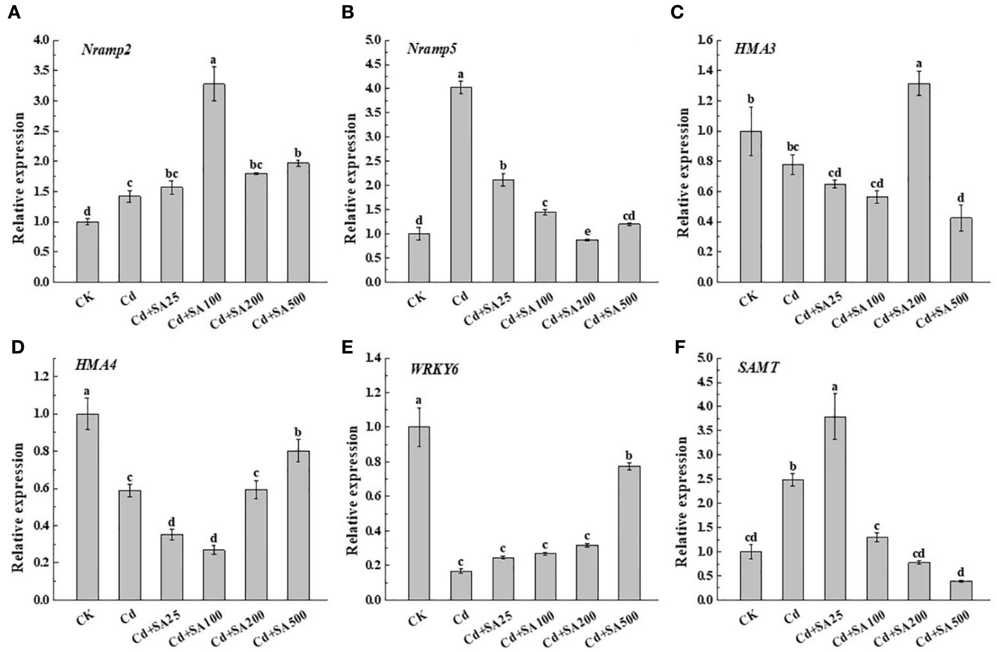

FIGURE 5
Effects of different exogenous SA on gene expression of *Nramp2* **(A)**, *Nramp5* **(B)**, *HMA3* **(C)**, *HMA4* **(D)**, *WRKY6* **(E)**, *SAMT* **(F)** in lettuce under Cd stress. Values are means ± SE from three independent experiments. Values with different letters above the bars are significantly different at $P<0.05$ according to LSD.

while high concentration of salicylic acid can destroy the photosynthetic electron transfer (Lawson, 2009). In our preliminary experiments, we found that high concentrations of SA would cause osmotic stress in leaves, leading to wilting. Therefore, we chose 25-500 mg/L concentrations of exogenous SA for lettuce treatment under Cd stress. Previous studies showed that 25 µmol/L of SA could alleviate Cd-mediated inhibition on rice growth and increase rice biomass (Majumdar et al., 2020). Moreover, Krantev et al. found that SA pretreatment of maize (*Zea mays* L.) seeds could reduce the negative impact of Cd stress on maize growth parameters (Krantev et al., 2008). Similarly, our results showed that the plant height, root length, and biomass of lettuce treated with 200 mg/L and 500 mg/L SA were significantly higher than those of Cd treatment alone (Figure 1). This indicated that exogenous SA could alleviate the growth inhibition of lettuce under Cd stress.

Cd stress can destroy the chloroplast ultrastructure of plant leaves and inhibit chlorophyll synthesis and leaf photosynthesis (Li et al., 2019b; Emanuil et al., 2020). The study showed that Cd stress significantly affected the photosynthetic pigment content, chlorophyll fluorescence (Fv/Fm), and photosynthetic parameters of the bean plant. When the bean was treated with 1.0 mmol/L SA, the Cd inhibition on the bean was significantly alleviated, and the photosynthetic parameters were significantly improved (Wael et al., 2015). Similarly, it was found that adding different concentrations of exogenous SA could effectively inhibit the reduction of photosynthetic pigment content (such as chlorophyll and

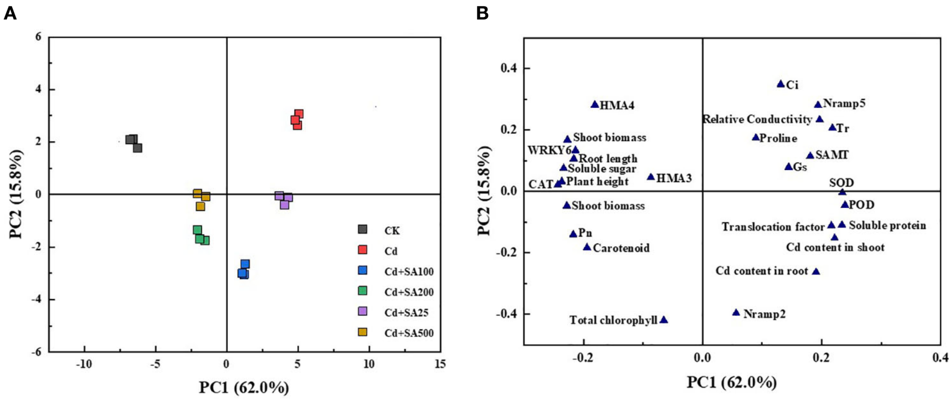

FIGURE 6
Principal component analysis of lettuce growth, physiological indexes and Cd content under Cd stress by different exogenous SA treatment. **(A)** PCA score plot; **(B)** PCA loading plot.

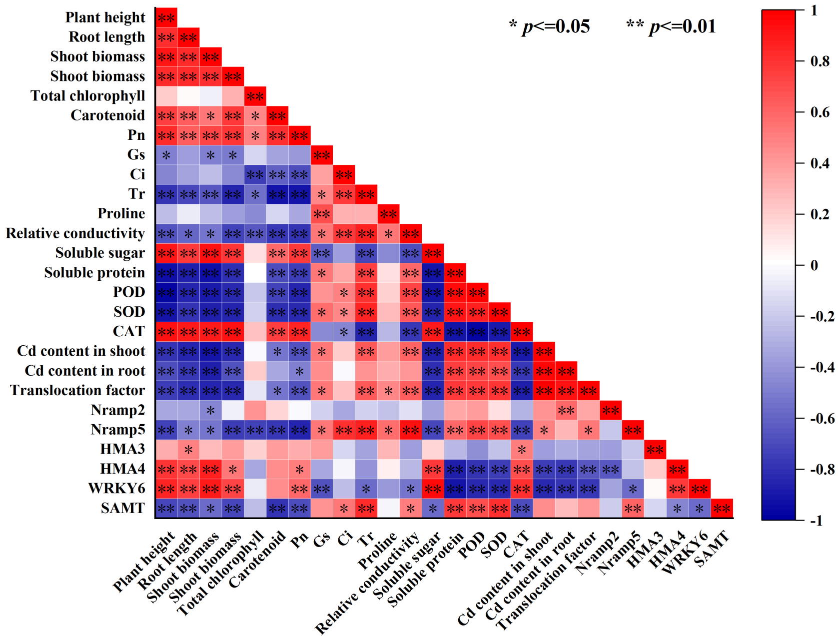

FIGURE 7
Correlation analysis of lettuce growth, physiological indexes and Cd content under Cd stress by different exogenous SA treatment.

carotenoid) caused by Cd stress and enhance the photosynthetic rate of leaves in *Iris hexagona* (Han et al., 2015) and *Lemna minor* (Lu et al., 2018). These findings are consistent with those reported in the present study. Under Cd stress, a certain concentration of SA increased the total chlorophyll and carotenoid contents of lettuce leaves, promoting photosynthesis (Figure 2, Table 2). The protective effect of SA on chlorophyll may be related to the alleviation of reduced chlorophyll enzyme activity caused by Cd stress and the enhancement of chlorophyll ester reductase activity related to chlorophyll synthesis (Kaur et al., 2017).

Many studies have shown that when plants are stressed by heavy metals, excessive production of related reactive oxygen species induces oxidative damage to plants (Su et al., 2020). An important mechanism by which SA alleviates Cd toxicity is strengthening the antioxidant defense system of the plant. This enables the plant to effectively prevent the excessive accumulation of ROS and slow down the oxidative damage caused by Cd stress (Li et al., 2019b; Liu et al., 2022). Furthermore, some studies showed that under Cd stress, SA pretreatment increased the content of antioxidant enzymes and non-enzyme antioxidants in maize, thereby reducing Cd-induced oxidative damage (Guo et al., 2009). Guo et al. reported that deleting SA in the SA-deficient mutant *sid2* of *Arabidopsis* aggravated the Cd-induced oxidative damage and growth inhibition (Guo et al., 2016). This study found that under Cd stress, a certain concentration of SA could reduce the relative conductivity, free proline content, and the POD and SOD activities of lettuce leaves (Table 3, Figure 3). Under Cd stress, SA treatment had varied effects on the activities of various antioxidant enzymes, possibly due to the different sensitivity levels of different plants or different tissues of the same plant to Cd and SA.

An important mechanism through which SA alleviates plant Cd toxicity is reducing plant uptake and transport of Cd (Singh et al., 2015; Gondor et al., 2016). Several studies showed that SA has a regulatory effect on the genes involved in Cd uptake and transport in plants, thus affecting the absorption of Cd ions (Jia et al., 2021). Singh et al. demonstrated that 100 μmol/L SA pretreatment could significantly increase the expression level of the natural resistance-associated macrophage protein 5 (*NRAMP5*) gene in rice seedling roots (Singh et al., 2015). At the same time, other studies have shown that ryegrass tolerance to Cd can be improved by increasing the expression of *Nramp2* in the stems and roots of ryegrasses (Li et al., 2019a). In addition to reducing the absorption and transport of Cd, other studies reported that SA could reduce Cd transportation from the aboveground parts to the grain, thus reducing Cd accumulation in the grain. Wang et al. reported that the Cd content in rice grains decreased from 0.29 mg · kg^{-1} to 0.12 mg · kg^{-1} after foliar application of 100 μmol/L SA (Wang et al., 2021d). The study also found that SA treatment could significantly reduce the Cd transfer from stem to leaf and from leaf to ear, possibly because Cd is mainly separated by the leaf cell vacuoles in leaves. This inhibits the expression of *OsLCT1* and *OsLCD* genes regulating Cd transport to rice grains, thus reducing Cd accumulation in grains (Wang et al., 2021d). The research results of Huang et al. showed that exogenous SA increased the expression of Cd stress tolerance genes (*OsHMA3* and *OsNRAMP5*) and Fe-transport-related genes (*OsIRT1*, *OsNRAMP1*, *OsNAS3*, and *OsYSL15*), thus enhancing the tolerance of rice to Cd and reducing the accumulation of Cd, Mn, and Fe (Huang et al., 2022a). Our research showed that under Cd stress, the total Cd content of lettuce increased initially and then decreased with the increase of exogenous SA concentration (Figure 4), similar to the research results of Wang et al. (2021c). In addition, our study also found that under Cd stress, a certain concentration of exogenous SA could up-regulate the relative expression of *HMA3*, *HMA4*, and

SAMT (Figure 5). This was inconsistent with the expression trend of *HMA* reported by Chen et al. (2021) when exogenous gibberellin (GA) was used to alleviate Cd stress in lettuce, probably due to the different parts of lettuce used for foliar application or differences in action mechanisms of SA and GA. In general, being low-cost and eco-friendly, foliar SA application is one of the most economical and effective methods for reducing Cd accumulation by plants from Cd-contaminated soils.

5 Conclusion

The results showed that exogenous SA could alleviate the inhibitory effect of Cd stress on lettuce growth and promote the photosynthetic pigment content and parameters under Cd stress. SA could also effectively protect plant cells from oxidative damage by reducing the activities of osmoregulation substances and antioxidant enzymes induced by Cd stress. Additionally, a certain concentration of SA could downregulate the relative expression of *Nramp5*, *HMA4*, and *SAMT* genes, thereby reducing the Cd content in lettuce shoots. In summary, applying 100-500 mg/L SA could effectively reduce the Cd toxicity in lettuce and the Cd accumulation in the edible parts of lettuce.

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

WT, investigation and writing-original draft. LL, YX, XL, LJL, ZH, BS, GS, and LT, investigation. HL, conceptualization. YT, conceptualization, writing-review, and editing. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher’s note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1200106/full#supplementary-material

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Research progress of the detection and analysis methods of heavy metals in plants

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Heavy metal (HM)-induced stress can lead to the enrichment of HMs in plants thereby threatening people's lives and health via the food chain. For this reason, there is an urgent need for some reliable and practical techniques to detect and analyze the absorption, distribution, accumulation, chemical form, and transport of HMs in plants for reducing or regulating HM content. Not only does it help to explore the mechanism of plant HM response, but it also holds significant importance for cultivating plants with low levels of HMs. Even though this field has garnered significant attention recently, only minority researchers have systematically summarized the different methods of analysis. This paper outlines the detection and analysis techniques applied in recent years for determining HM concentration in plants, such as inductively coupled plasma mass spectrometry (ICP-MS), atomic absorption spectrometry (AAS), atomic fluorescence spectrometry (AFS), X-ray absorption spectroscopy (XAS), X-ray fluorescence spectrometry (XRF), laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS), non-invasive micro-test technology (NMT) and omics and molecular biology approaches. They can detect the chemical forms, spatial distribution, uptake and transport of HMs in plants. For this paper, the principles behind these techniques are clarified, their advantages and disadvantages are highlighted, their applications are explored, and guidance for selecting the appropriate methods to study HMs in plants is provided for later research. It is also expected to promote the innovation and development of HM-detection technologies and offer ideas for future research concerning HM accumulation in plants.

KEYWORDS
heavy metals, plants, detection methods, analytical methods, technology

1 Introduction

In recent years, the HM contents of soil, water, and air have enhanced manifold due to rapid development and industrialization (Vareda et al., 2019). They are classified as essential nutrients like Fe, Zn, Cu, Ni, Mn, etc.; and non-essential nutrients like As, Hg, Cr, Pb, Cd, and Ag, based on the requirements of these elements by plants to fulfill its life cycle. HMs are mainly uptaken via the roots in the soil, while HMs in the atmosphere through the leaf surface which then accumulate in plants. *Cedrus deodara* and *Cupressus arizonica* absorb HMs through their leaves (Liang et al., 2017; Cesur et al., 2021) and those of *Nerium indicum*, *Pittosporum tobira*, and *Platanus acerifolia* through trichomes, stomata, or the mucous layer (Liang et al., 2017). The excessive accumulation of essential elements can negatively impact plants; that of Fe can cause the buildup of reactive oxygen species (ROS), damage to lipids and proteins, impairment of cell structure, leaf chlorosis, and other adverse effects (Kobayashi et al., 2019; Ahammed et al., 2020), while that of Zn can lead to nutritional imbalance, production of ethylene, stunted growth and chlorosis (Islam et al., 2014). Non-essential elements like As, Cd, Hg, and Pb, are called “toxic HMs” as they adversely affected plants when their concentrations exceeded certain levels (Wang et al., 2020; Rahman et al., 2022). These elements affected photosynthesis and respiration, damaged the structure and function of stomata, increased oxidative stress, modulated the uptake of mineral nutrients, caused DNA breaks and enzyme inactivation, and other serious adverse effects (Jaiswal and Verma A, 2018; Genchi et al., 2020; Guo et al., 2023). The toxicological impacts of common HMs on plants are summarized in Table 1. HMs are absorbed by the body via the food chain and are accumulated in vital organs like the liver, heart, brain, and kidneys, which disrupted their normal functions and interfered with various metabolic processes (Fu and Xi, 2020). HMs are absorbed by the body via the food chain and are accumulated in vital organs like the liver, heart, brain, and kidneys, which disrupted their normal functions and interfered with various metabolic processes (Kiran et al., 2022). Excessive accumulation of As can lead to skin diseases, diabetes, neurological complications, and impaired liver and kidney function (Kiran et al., 2022). The harmful repercussions of Hg are predominantly embodied as inattention, blurred vision, unstable walking, pneumonia, pulmonary edema, lung injury, and other symptoms. It can also be toxic to fetuses, resulting in fetal blindness, intellectual disability, speech impairment, and brain damage (Kiran et al., 2022).

TABLE 1 the toxicological impacts of common HMs on plants.

Heavy metals	Adverse impacts	References
Essential HMs		
Fe	Accumulation of reactive oxygen species, damage to lipids and proteins, damage to cell structure, leaf chlorosis	(Kobayashi et al., 2019; Ahammed et al., 2020)
Zn	Leaf fading, slow growth, nutrient imbalance, reduced photosynthesis, accumulation of reactive oxygen species, causing oxidative stress	(Islam et al., 2014; Rukhsar Ul et al., 2023)
Cu	Inhibition of plant growth, oxidative damage, nutrient imbalance, reactive oxygen species accumulation, reduced photosynthesis, leaf edge curling or greening	(Kohatsu et al., 2021; Yuan et al., 2023)
Ni	Disrupt cell metabolism, damage cells, break down chloroplast structure, decompose chlorophyll and cystoid membrane, disrupt water balance and nutrient relationship, accumulate reactive oxygen species	(Ahmed et al., 2023)
Mn	Inhibits plant growth, leaves crumpled and shriveled	(El-Jaoual and Cox, 1998)
Non-essential HMs		
Cd	Slow growth, oxidative damage, chlorophyll degradation, reduced photosynthesis, reactive oxygen species accumulation, stomatal opening and closing, nutrient distribution imbalance, altered water balance, senescence or death	(Tunçtürk et al., 2023; Yan et al., 2023)
Pd	Inhibits seed germination, slows plant growth, disturbs water balance and nutrient relationships, interferes with enzyme activity and stomatal closure, and delays carbon metabolism	(Zulfiqar et al., 2019)
Hg	Inhibits antioxidant enzyme activity and photosynthesis, inhibits plant growth, disrupts chlorophyll synthesis, and destroys chloroplast ultrastructure	(Tang et al., 2023)
As	Induces oxidative stress, damages cells and inhibits plant growth, decreases germination, reduces crop yield, and reduces carbon metabolism	(Souri et al., 2020; Banerjee et al., 2023)
Cr	Affects seed germination, slows root and tip growth, inhibits cell cycle and nitrogen assimilation, accumulates reactive oxygen species, inhibits photosynthesis, leaves chlorosis and necrosis	(Ali et al., 2023)
Aluminum (Al)	Nutritional imbalance, inhibition of photosynthesis, disruption of cellular organization, effects on protein metabolism, disruption of enzyme activity	(Zhang et al., 2014; Yang et al., 2015; Singh et al., 2017; Shetty et al., 2021)

In plants, the roots are the main organs that absorb various nutrients and non-essential elements. Roots act as the first barrier for the entry of HMs, and most plants retain these HMs in their roots to block their transfer into the aerial parts, thereby reducing the toxic effects. Therefore, root retention is significant for plant detoxification mechanisms (Ghuge et al., 2023). Additionally, certain plant species like *Arabis paniculata, Celosia argentea*, and *Corydalis davidii* accumulate HMs to a greater extent in their aboveground parts compared to the underground parts (Li et al., 2018). Due to the robust transport capacity of certain plants, the HM content in the aboveground parts is more than ten-fold higher than that in the underground parts, and the everyday life activities are not affected, making them hyperaccumulators (Shi et al., 2023). Hyperaccumulators can eliminate, reduce, or stabilize the contents of HM pollutants included in soils thus remediating HM-polluted soil. After entering its roots, the HMs are primarily localized in the vacuoles and cell walls (Cobbett, 2000; Yin et al., 2015). The cell wall is rich in pectin, hemicellulose, cellulose, lignin, and polysaccharides (Lao et al., 2023). These negatively charged biopolymers bind to HM ions; the enzymes associated with lignin biosynthesis promote cell wall synthesis and thickening, enriching HMs in the cell wall (Keyster et al., 2020; Chen et al., 2022; Lao et al., 2023). HMs that traverse the cell wall are internalized into the vacuole through ATP-dependent transporters, where they accumulate by chelating with phytochelatins (PCs) (Keyster et al., 2020; Khan et al., 2020). Therefore, compartmentalization in cell walls and vacuoles is crucial for plant detoxification mechanisms. Plants have not developed any carriers and channels specific for non-essential elements during the evolutionary process, so these elements mainly rely on the absorption pathway of mineral elements to enter plants (Andresen and Küpper, 2013; Shahid et al., 2017). HM ions enter the plant root with the assistance of nutrient element ion channels (such as Ca^{2+} and K^+ channels) and transport proteins present in the plasma membrane. Many transporters related to HMs, including Zn-regulated transporter, iron-regulated transporter-like proteins (ZIPs), HM-associated domain (HMA), iron-regulated transporter (IRT), natural resistance-associated macrophage protein (NRAMP), yellow stripe 1-like family (YSL), etc. have been identified (Li et al., 2017a; Leng et al., 2020; Kaur et al., 2021).

The food chain is the primary means through which humans are exposed to HMs, making the control of HM accumulation in food crops, pulses, vegetables, fruits, and medicinal plants particularly crucial. Understanding and investigating in plants the biological and physiological processing involving the transport, absorption, accumulation, and detoxification of HMs can facilitate the cultivation of edible and medicinal plants with low levels of HMs and enable the utilization of hyperaccumulators for remediation of HM-contaminated soils. To understand these mechanisms, specific instruments and technical methods are needed to obtain information regarding spatial and subcellular distribution, chemical morphology, uptake, and translocation pathways of HMs. The content of HMs can be analyzed using techniques for instance inductively coupled plasma mass spectrometry (ICP-MS) and atomic absorption spectrometry (AAS); chemical form by atomic fluorescence spectrometry (AFS) and X-ray absorption spectroscopy (XAS), respectively; spatial distribution through X-ray fluorescence spectrometry (XRF) and laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS); and the dynamics of uptake and transport by non-invasive micro-test technology (NMT). Moreover, “omics” and molecular biology approaches contribute to understanding the molecular mechanisms underlying plant-HM interactions, facilitating further exploration of HM-induced stress.

In recent years, the accumulation, chemical form, spatial distribution, and uptake and translocation of HMs by plants have received extensive attention. However, a complete overview of the research techniques and methods employed for gaining the information mentioned above is lacking. Therefore, this paper reviews recent advances in detecting and analyzing HMs in plants in three sections. The first section deals with the background of stress caused by HM pollution and introduces the distribution characteristics, forms of existence, and toxic effects of HMs. The second systematically discusses the principles, advantages, and applications of the frequently used detection and analytical techniques for different HMs. Finally, the current state and challenges of the various technologies are summarized, and prospects for development are projected. This review is expected to facilitate the selection of optimal research methods related to HMs in plants and provide ideas for developing and improving these technologies.

2 HM contamination in plants

2.1 Sources of HMs in plants

The three main origins of HMs in plants are soil, water, and air pollution (Nagajyoti et al., 2010; Rai et al., 2019). Inappropriate disposal of industrial waste, automobile exhaust emissions, mining, extraction and smelting, energy and fuel manufacturing, and other human activities can result in HM pollution (Wu et al., 2022). HM content is highest in water ecosystems and soils, with only a minuscule proportion in the atmosphere as vapor or particles. HMs are uptake by the roots via surface water and soil and then stored in the cell walls and vacuoles; additionally, they are absorbed and enriched in leaves from the atmosphere, which affects growth and development (Nadeem et al., 2010; Rai et al., 2019; Qin et al., 2021). Carrots, sweet potatoes, and other root vegetables are predominantly contaminated by HMs in the soil (Cobbett, 2000; Zhou et al., 2016; Cesur et al., 2021), while *C. arizonica, Pinus sylvestris L.*, and *Ficus elastica* primarily through atmospheric pollution (Zwolak et al., 2019; Alaqouri et al., 2020; Ghoma et al., 2022).

2.2 HMs in plants

HMs are mainly selectively absorbed or diffused from the soil into plants through roots, and H^+ coupled carrier proteins or transporters are critical for the entry of HMs into the roots (DalCorso et al., 2019). For example, plants can absorb Cd through Ca^{2+} and K^+ channels, while transporters, including ZIP, HMA, YSL, NRAMP, and IRT, are involved in the various processes of Cd absorption and transport (Andresen and Küpper, 2013; Yin et al., 2015; Shahid et al., 2017). After entry through the roots, HMs travel through the cell membrane into the cytoplasm or combine with the cell wall. Most of the absorbed HMs accumulate directly in the roots or are loaded into the xylem by transporter proteins and then, through the xylem, are translocated to the aerial part and accrue in foliage (Yang et al., 2022) (Figure 1). The accumulation levels of specific HMs are generally > 100 mg kg^{-1} for Cd; 1000 mg kg^{-1} for Pb, Co, Ni, etc.; and 10,000 mg kg^{-1} for Zn and Mn (Shi et al., 2023). For example, the hyperaccumulators of Pb include *A. paniculata*, *Isachne globosa*, and *Pogonatherum crinitum* (Kaur et al., 2021; Shi et al., 2023); of Mn include *C. argentea*, *Polygonum lapathifolium*, and *Schima superba* (Kaur et al., 2021;

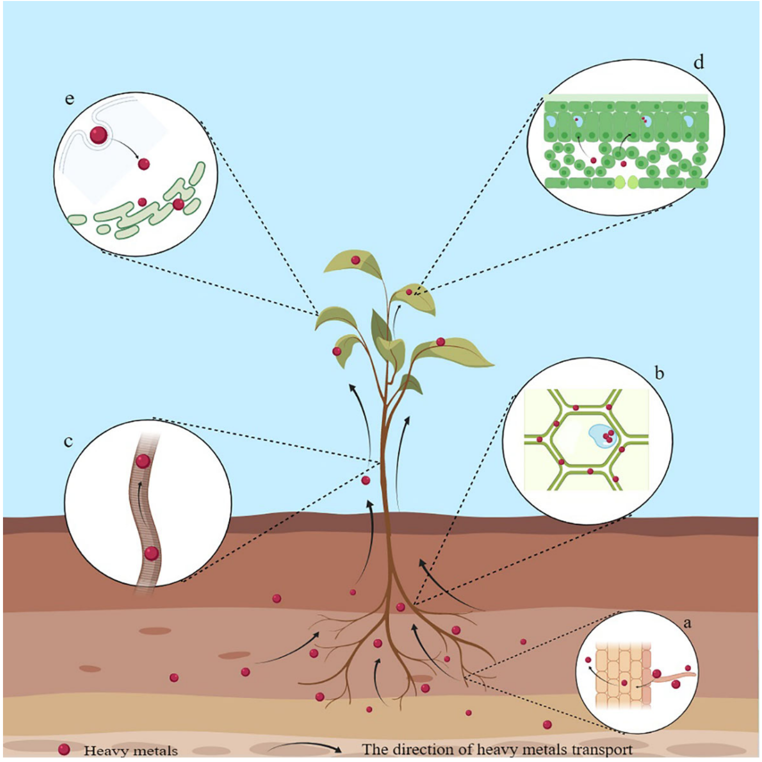

FIGURE 1
Schematic diagram of the mechanism of HM accumulation in plants. **(A)** Accumulation of HMs on the surface of the root system; **(B)** Accumulation of HMs in cell walls; **(C)** Root-to-shoot translocation; **(D)** Accumulation of HMs in leaves; **(E)** Endocytosis.

Shi et al., 2023); of Cd include *Sedum alfredii* Hance and *Solanum nigrum* L (Al-Huqail, 2023; Hu et al., 2023).; of As include *Pteris vittata* L (Zhang et al., 2022).; and of Zn include *C. davidii*, *Picris divaricata*, and *Viola baoshanensis* (in their aerial parts) (Kaur et al., 2021; Shi et al., 2023). At the subcellular level, HMs are primarily distributed in the cell walls and vacuoles, thereby compartmentalizing them, reducing their transfer to other parts, and minimizing toxicity (Corso, 2020; Yang et al., 2022). Vacuoles being non-physiologically active organelles, HMs, upon entry into the vacuoles, are isolated, passivated, and precipitated, thereby reducing their toxicity (Corso, 2020; Yang et al., 2022). Binding sites such as -COOH, -SH, and -OH provided by the cell wall can adsorb HM ions, better defining the role of the cell wall in accumulating metal to prevent their entry into plants (Ghori et al., 2019; Corso, 2020). The cell walls of *Boehmeria nivea* L. contain high levels of Cd (Chen et al., 2022). The cell walls and vesicles of the leaves in *Leersia hexandra* Swartz are the main sites of Cr(III) accumulation (Ma et al., 2022).

In plants, the chemical forms of HMs are strongly correlated with their mobility and biotoxicity (Duffus, 2002; Yang et al., 2022). As is found in distinct valence states, primarily in arsenite (AsIII) and arsenate (AsV) forms, of which AsIII is somewhat more toxic than AsV (Souri et al., 2017). Plant species, absorption mechanisms, As speciation, and soil type are critical factors that influence the speed of absorption and buildup of As through plants (Zhao et al., 2009). Moreover, the roots discriminatingly absorb particular As forms though different transporters and pathways (Gupta et al., 2011; Farooq et al., 2016). In root cells, in order to prevent As translocation to shoots, As is translocated into the vacuole in the form of AsIII or AsIII-glutathione/phytochelatin complexes or is turned into less toxic organic forms (Smita et al., 2015). In hyperaccumulator plants, AsV could be efficiently reduced to AsIII, and then mobilized from roots to aerial part (as the dominant form of As in xylem sap) (Raab et al., 2005; Su et al., 2008). Most angiosperms use a mechanism of Fe uptake based on reduction (Grillet and Schmidt, 2017). Roots can enzymatically reduce Fe by plasma membrane-bound ferric reductase/oxidase (FRO) or chemically reduce Fe by root secretion of organic compounds (Grillet and Schmidt, 2017). Using X-ray absorption near-edge spectroscopy (XANES), Cr was primarily detected as a trivalent form (less toxic than hexavalent Cr) in *L. hexandra* Swartz roots and leaves (Liu et al., 2014). High-performance liquid chromatography/inductively coupled plasma mass spectrometry (HPLC/ICP-MS) identified the various chemical forms of As, Hg, and Pb in lotus seeds including As(III), dimethyl arsenic acid (DMA), As (V), methyl arsenic acid (MMA), phosphorus mercury (PhHg), ethyl mercury (EtHg), methyl mercury (MeHg), Hg(II), TML, TEL, and Pb (II) (Zhang et al., 2020).

Under HM-induced stress, most metals will enter the root through general or specific pumps or ion channels, while the root also releases large amounts of secretions (e.g., malate, oxalate, citrate, etc.) that bind to the metal ions, concentrate them into the apoplast, or adsorb them to cell walls, stopping them reaching the cells (Ghori et al., 2019). When exposed to Al-induced stress, *Vigna umbellata* seedlings, in their late stages of growth, acclimatized to Al-related toxicity by releasing enormous amounts of citrate through their root systems (Liu et al., 2018). Using XANES, scanning electron microscopy (SEM), and synchrotron radiation (SR)-based technology, Zn in the root epidermis of *Helichrysum microphyllum* subsp. was identified to bind to organic molecules, like malate, histidine (His), and cysteine (Cys), thereby alleviating the toxicity related to excessive Zn, suggesting that *H. microphyllum* may be a potential candidate Zn-stress-tolerant plant (Boi et al., 2020). Pectin is an important constituent in cell walls and is also requisite to chelate metal ions (Ghori et al., 2019). Cd mainly existed in the pectin- and protein-bound forms in *S. nigrum* and *Koelreuteria paniculata* (Yang et al., 2018; Wang et al., 2021).

In addition, crucial ligands like PCs, glutathione (GSH), as well as metallothioneins (MTs) can chelate with HMs, activating the HM detoxification mechanisms in plants (Thakur et al., 2022). PCs are a family of proteins induced to synthesize Cys-polypeptides and are rich in them due to the presence of various metals (Ghori et al., 2019). They are common in plants, fungi, algae, and cyanobacteria (Gupta et al., 2013). PCs can utilize the thiol groups in the Cys residues to bind with Cd and sequester these complexes in vesicles through ABC transporter proteins (Ghori et al., 2019). Next, Cd can also form complexes with other proteins or compounds and be transported elsewhere through the xylem and phloem to mitigate Cd-related toxicity effectively (Ghori et al., 2019). *Arabidopsis* plants exhibited extreme sensitivity to Cd-induced toxicity when GSH and PC were deficient (Howden et al., 1995b; Howden et al., 1995a). The AtABCC1 and 2 transporter proteins from *Arabidopsis* are crucial for isolating the PC-Cd complexes within vacuoles (Park et al., 2012). Since the *OsPCS15* and *OsPCS5* genes may be associated with Cd reduction, in Park et al.'s study of rice subjected to both Cd and As stress, it was found that *OsPCS5* and *OsPCS15* were specific to Cd and As (Park et al., 2019). GSH exists in both oxidized and reduced forms, mediates the biosynthesis of PCs, its thiol groups form S-based bonds with HMs thereby sequestering them, and the complexes are transported to vacuoles for detoxification (DalCorso et al., 2008; Ghori et al., 2019). The OsCLT1 transporter protein in rice facilitated the efflux of GSH and γ-glutamyl-cysteine (γ-Glu-Cys) from plastids to the cytoplasm, mediating the biosynthesis of PCs; the mutants lacking *OsCLT1* exhibited hypersensitivity to Cd and other HMs (Yang et al., 2016). MTs are Cys-rich proteins that can bind to various HM ions such as Cd^{2+}, Cu^{2+}, and Zn^{2+}, maintaining metal homeostasis in plants and alleviating the undesirable toxic impacts of excessive metal ions (Saeed Ur et al., 2020; Ettiyagounder et al., 2021). *PaMT3-1* from *Phytolacca americana*, overexpressed in tobacco, encoded a protein that bound with Cd^{2+} and enhanced tolerance to Cd (Zhi et al., 2020). Results from studies on tobacco indicate that overexpression of *EhMT1* can enhance Cu buildup and tolerance in root cytoplasm as well as reduce hydrogen peroxide (H_2O_2) production (Xia et al., 2012).

2.3 Biomarkers of HMs in plants

Biomonitoring refers to using biomaterials or organisms to analyze specific characteristics of the biosphere (Decou et al., 2019). The application of biomarkers for environmental biomonitoring can detect the harmful effects of pollutants at an early stage and play the role of an early warning system. Morphology, physiology, and biochemistry alter when plants are subjected to HM-induced stress as well as can be used as biomarkers (Decou et al., 2019; Krayem et al., 2021). These alterations include suppression of root development, leaf discoloration and necrosis, suppression of photosynthesis and respiration, and induction of oxidative stress (Costa et al., 2018; Decou et al., 2019; Krayem et al., 2021). Substantial research has explored the biomarkers or bioindicators of HMs in plants. An analysis of biomarkers in *Myriophyllum alterniflorum* indicated a direct correlation between malondialdehyde (MDA), α-tocopherol, and glucose-6-phosphate dehydrogenase (G6PDH) with Cu and Cd, which suggested that these three biomarkers were effective for metal exposure analyses (Decou et al., 2019). A comparison of the selected biomarkers, MT and PC in *Cystoseria indica*, revealed that PC reacted to HMs to a lesser extent than MT, limiting its use in HM biomonitoring (Sinaei et al., 2018). Three types of markers (molecular markers, resistance markers, and damage markers) appeared at different frequencies when the HM accumulating plant *Tillandsia ionantha* Planch. was exposed to Cd (Zhang et al., 2023). Most of the markers mentioned above are really consequences of HM toxicity and are usually visible after severe toxicity, Only PCs and MTs are related to metals and the others are general stress markers. In conclusion, elucidating the mode of action and potential molecular mechanisms of biomarkers for detecting HMs in plants could improve their applicability in environmental quality assessment and facilitate the development of effective biomonitoring and phytoremediation technologies (Krayem et al., 2021; Saroop and Tamchos, 2021; Esmaeilzadeh et al., 2023).

3 Detection and analysis techniques of HMs in plants

Robust detection tools and analytical technologies enable a holistic understanding of the mechanisms involved in the uptake, transport, accumulation and detoxification of HMs in plants. In recent years, HM-detection technology has concentrated on precision, comprehensiveness, and automation, which are crucial for regulating and decreasing the levels of HMs in plants. ICP-MS, AAS, and AFS are used to detect the contents of HMs in plants. AFS and XAS can identify the chemical forms of HMs. XRF and LA-ICP-MS can elaborate the distribution of HMs in sub-cells and tissues. NMT enables measuring the flow rate of HM ions. Omics and molecular biology techniques can analyze the molecular mechanisms through which plants interact with HMs and discover more genes related to HM resistance. The principles, advantages, and applications of these analytical techniques for detecting different HMs in plants are systematically discussed subsequently.

3.1 Quantitative analysis

3.1.1 Inductively coupled plasma mass spectrometry

ICP spectroscopy is a method used to analyze trace metals by measuring light emission with characteristic wavelengths when the metal is exposed to an electric current. ICP-MS includes a sample introduction system, ion source, interface, ion lens, mass filter, and an ion detector (Yu et al., 2020). The details of how it works are as follows: First, the sample is drawn into the nebulizer by a peristaltic pump or self-priming to generate an aerosol of fine liquid droplets. Next, they are introduced into the central zone of the high-temperature plasma that was created by combining the energy supplied to the coil by the ICP radio frequency (RF) emitter and Ar gas. As their absorption increases, electrons are released, and cations are formed. The ion lens then focuses them through the interface, separates them based on their mass-to-nucleus ratio using a mass filter, and transports them to the detector for analysis resulting in a mass spectrum (Wilschefski and Baxter, 2019) (Figure 2). ICP-MS provides the advantages of being capable of identifying multiple elements, fast, sensitive, and capable of detecting minute levels. It also has a wide linear range, allows for easy control of interferences, and provides information on isotopes (Wilschefski and Baxter, 2019; Theiner et al., 2020; Penanes et al., 2022).

ICP-MS is a powerful technology with excellent capability for analyzing elements and detecting the concentration of HMs in plants at trace levels (Huang et al., 2006; Wilschefski and Baxter, 2019). Using ICP-MS, the content of Cd in the aromatic medicinal plant *Alkana trichophila* was detected to exceed the World Health Organization (WHO) standards; however, the content of other trace elements, like Al, V, and Cr, were within the acceptable range, suggesting that *A. trichophila* contains excessive amounts of Cd (Izol et al., 2023). It would depend on the concentration available in the soil (Emamverdian et al., 2018; Izol et al., 2023). The levels of four HMs: Hg, As, Cd, and Pb, in 50 medicine food homology plants were detected by inductively coupled plasma-tandem mass spectrometry (ICP-MS-MS) (Fu et al., 2018). Moreover, ICP-MS is considered the preferred way for detecting HM contents because of its advantages, like high accuracy, sensitivity, selectivity, and low detection limits. It is often used in combination with other analytical methods, for example, LA-ICP-MS, single-particle- inductively coupled plasma-mass spectrometry (SP-ICP-MS), liquid chromatography- inductively coupled plasma-mass spectrometry (LC-ICP-MS), etc., for the determination of the content and the form of multiple trace elements and isotope analysis in different types of samples and various matrix samples and simplifying the sample preparing process. Utilizing HPLC-ICP-MS, the As content in quinoa and rice was detected to exceed the food pollutant standards set by the European Commission, indicating a severe overaccumulation of As (D'Amore et al., 2023). LC-ICP-MS and ICP-MS were used to detect the As content of ten species of algae, and it was found that *Cystoseira species* and *Cystoseira fragile* had the highest total As content (Pell et al., 2013). I In addition to *Cystoseiraz*, inorganic As was dominant in the other nine algal species, indicating that ICP-MS and LC-ICP-MS provide reasonable technical support for further exploration of the As content in marine plants (Pell et al., 2013). LA-ICP-MS detected that, except *Ginkgo biloba*, Pb and Cd accumulated rapidly in four tree species including *Pinus densiflora*, *Chamaecyparis obtuse*, *Salix koreensis*, and *Platanus occidentalis*, while the distribution of Fe, Cr, Mn, and other elements exhibited changes in tree-ring patterns (Kim et al., 2020). This indicates that LA-ICP-MS can be employed to determine changes in levels of certain HMs in tree rings, providing valuable information and contributing to research in environmental pollution monitoring (Kim et al., 2020). The application of SP-ICP-MS offers a distinct opportunity to detect and study the concentration of metal particles, particle-particle and particle-matrix interactions, solubility, etc. Additionally, it allows for elemental analysis of single-celled organisms and provides isotope-related information over an extensive dynamic range of individual cells. SP-ICP-MS was utilized to detect the rapid absorption of Zn by *Lacuca sativa* L. plants grown in a medium containing ZnO nanoparticles (Wojcieszek et al., 2019). Nicotinamide formed a complex with 70% of the Zn in leaves as detected by ICP-MS and electrospray ionization-tandem mass spectrometry (ESI-MS-MS) (Wojcieszek et al., 2019). In summary, with the update and progress of technology and methods, ICP-MS has become a multifunctional and powerful platform that can determine the total elemental concentration and expand the scope of application with other technologies, providing a multidimensional perspective for solving problems related to HMs.

3.1.2 Atomic absorption spectrometry

AAS is one of the earliest commercially developed methods for the elemental analysis of HMs (Jin et al., 2020). AAS is an approach for quantitative analysis based on the absorption of characteristic

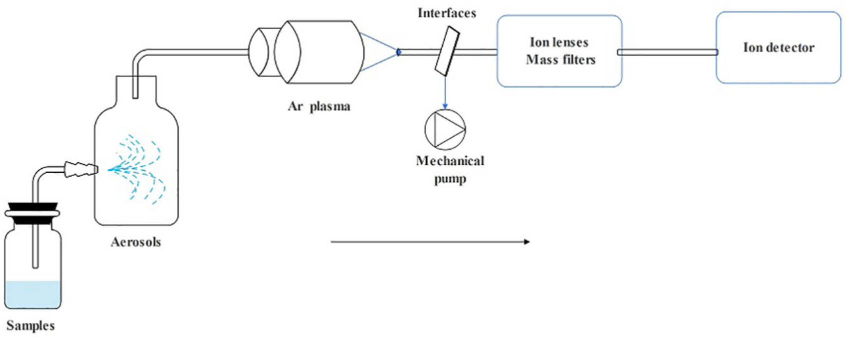

FIGURE 2
The operating principle schematic diagram of ICP-MS.

spectral lines by atomic vapor produced from a substance. It comprises five main components: a light source, an atomization system, a spectroscopy system, a detection system, and a display unit. The operating principle is as follows: First, an atomizer turns the sample to be measured into an atomic vapor under high temperature. Next, when the atomic vapor is irradiated with a light source, it can absorb light radiation of a certain wavelength. After this, the spectroscopic system distinguishes between different spectral lines. Finally, the content of the element to be measured in the sample is determined according to the degree of attenuation of the light when it is absorbed (Figure 3). AAS possesses the advantages of high selectivity, accuracy, sensitivity, and low interferences ability. The equipment is easy to operate, enables fast analysis, and provides an extensive range of analyses (Eskina et al., 2020). Depending on the atomization device used, AAS can be categorized into cold vapor generation atomic absorption spectrometry (CVAAS), hydride generation atomic absorption spectrometry (HGAAS), graphite furnace atomic absorption spectrometry (GFAAS), and flame atomic absorption spectrometry (FAAS) (Butler et al., 2006; Yuan et al., 2011; Guo et al., 2023).

AAS is one of the very popular methods for the qualitative and quantitative analysis of HMs in plants and can be employed to measure directly the content of metal elements in the sample (Adolfo et al., 2019). For instance, 12 elements, including Ag, Cr, Cu, Ca, and Fe, were detected in the leaves of *Andrographis paniculata* using AAS (Kartha. and PP, 2021). Similar results were observed with eight HMs (e.g., Cd, Cr, and Hg) in cereals, tubers, and vegetables, as detected by AAS (Omeje et al., 2021). Although AAS effectively determined the composition and quantification of elements, its efficacy can be influenced by the extraction technique, the elements to be determined, and the plant tissue (Elik et al., 2020). For example, AAS detected that the Mn content of five plant samples was highest when extracted using a nitric acid-perchloric acid solution, indicating that different digestion methods used for preparing plant samples impacted the detection of HM contents (Bankaji et al., 2023). Therefore, proper digestion methods are crucial for the maximal extraction of specific metals from various samples (Bankaji et al., 2023). Although new HM detection techniques have been developed, AAS remains one of the most potent tools in plant elemental metal analysis and trace analysis due to its cost-effective advantages, low price, low detection limit, and good repeatability (Vinogradova et al., 2023).

3.2 Chemical forms analysis

3.2.1 Atomic fluorescence spectrometry

AFS is a fast-growing technique for trace analysis and belongs to emission spectroscopy, an important branch of atomic spectroscopy. AFS consists of five parts: a light source, an atomization system, a spectroscopic system, a detection system, and a display device. The working principle of AFS is that after the sample to be analyzed is converted into atomic vapor by the atomizer, the atomic vapor absorbs light radiation at its characteristic wavelength, causing the atoms to transition from the ground state to the excited state for a duration of 8 – 10 seconds. During this process, fluorescence with either the same or different wavelength as absorption is emitted. This fluorescence is then converted into an electrical signal by a photoelectricity detector and processed into readable data by a data processing system (Costa Ferreira et al., 2019) (Figure 4). Atomic fluorescence is divided into three types: resonant fluorescence, non-resonant fluorescence, and sensitive light fluorescence. Among them, resonant fluorescence has the highest intensity and is the most commonly used. AFS by atomization can be further divided into cold vapor atomic fluorescence spectrometry (CVAFS) and hydride generation atomic fluorescence spectrometry (HGAFS). AFS offers

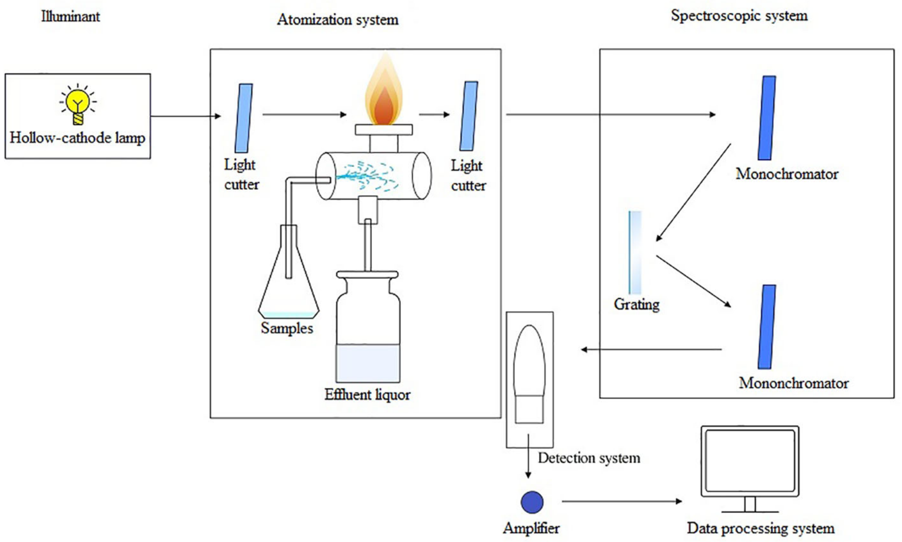

FIGURE 3
The operating principle schematic diagram of AAS.

advantages like high accuracy (similar to AAS), high sensitivity, simultaneous determination of multiple elements, low detection limits (including very low detection limits for Zn and Cd), simple spectral lines, minimal spectral interference, wide linear range, etc (Yogarajah and Tsai, 2015; Xia et al., 2019; Peng et al., 2022). It is a simple instrument with lower operating costs compared to AAS.

Studies have shown that AFS is often combined with chromatographic techniques to define trace and ultra-trace element levels in plants, as well as analyze their Chemical forms (Dai et al., 2019; Li et al., 2021; Guo et al., 2023). Cheng et al. used dual-frequency ultrasonic-assisted enzyme digestion (UDUE) combined with liquid chromatograph-hydride generation atomic fluorescence spectrometry (LC-HGAFS) technology to detect the content and form of As elements in *Paeoniae Raxix rubra, Angelica Sinensis, Codonopsis pilosula*, and *Salvia* (Cheng et al., 2020). The results indicate that inorganic As is present in both trivalent and pentavalent forms in four medicinal plants (Cheng et al., 2020). Deng et al. utilized the method of high-performance liquid chromatography and hydride generation atomic fluorescence spectrometry (HPLC-HG-AFS) to detect various forms of As in rice that include As(V), DMA, As(III), and MMA (Deng et al., 2021). Similarly, the As forms of the three aquatic plants were mainly As(III) and As(V), which were detected using liquid chromatograph-atomic fluorescence spectrophotometry (LC-AFS) by Wang et al (Wang et al., 2021). Besides, plenty of studies have shown demonstrated that As typically exists in the form of As(V) and As(III) within plants (Jedynak et al., 2010; Budzyńska et al., 2017; Budzyńska et al., 2021). Hu et al. determined the levels of inorganic Hg in various vegetable and fruit samples using hydride generation novel ultraviolet atomization atomic fluorescence spectrometry (HG-UV-AFS). They discovered that volatile methyl Hg^+ hydride (MeHgH) could be converted into elemental Hg vapor through UV-based atomization and KBH_4 treatment, and they also detected total Hg (Methyl Hg^+ (MeHg) and Hg^{2+}) (Hu et al., 2018). These results showed that HG-UV-AFS effectively detected gaseous organic Hg hydride, providing a reference for analyzing HMs in their gaseous state (Hu et al., 2018). Se in garlic was determined using reversed-phase high-performance liquid chromatography and hydride generation atomic fluorescence spectrometry (RP-HPLC-HG-AFS) revealing the four forms of Se: SeMeSeCys, SeMet, Se(VI), and Se(IV) (Castro Grijalba et al., 2017). Furthermore, RP-HPLC-HG-AFS method provides technical support for the Chemical forms analysis of Se in samples of complex plants by effectively separating organic and inorganic Se using different ionic liquids as mobile phases (Castro Grijalba et al., 2017). In conclusion, AFS is an effective technique for identifying the chemical forms of HMs.

3.2.2 X-ray absorption spectroscopy

XAS is a spectroscopic analysis approach that utilizes synchrotron X-ray sources and varies the photon energy to determine the absorption coefficient of a sample. The XAS consists of an absorption edge and a series of oscillating structures, mainly divided into two regions: the extended X-ray absorption fine structure (EXAFS) located in the 50 – 11000 eV region of the absorption edge, and the XANES located near the 50 eV region of the absorption edge (Sarret et al., 2013; Tang et al., 2023) (Figure 5). The XAS works by exciting the sample with X-rays, causing its core electrons to transition to either the continuum or empty orbitals (Figures 6, 7). This leads to the formation of waves that scatter with the surrounding atoms (Figure 7). EXAFS can identify local information structures, such as bond lengths, coordination numbers, and disorder of central and coordination atoms, with a spatial resolution ranging from 0.1 – 1 pm; moreover, it is sensitive to steric structures. XANES can provide a wealth of chemical information, including near-neighbor atomic positions and chemical valence states. Meng et al. discovered that methylmercury constituted the primary form of Hg in rice (Meng et al., 2014). EXAFS can also detect the Chemical forms information of metal elements by oscillating in the EXAFS region (Hu et al., 2020). Compared to EXAFS, XANES exhibits characteristics such as subtle structural changes, a shorter acquisition time, high sensitivity

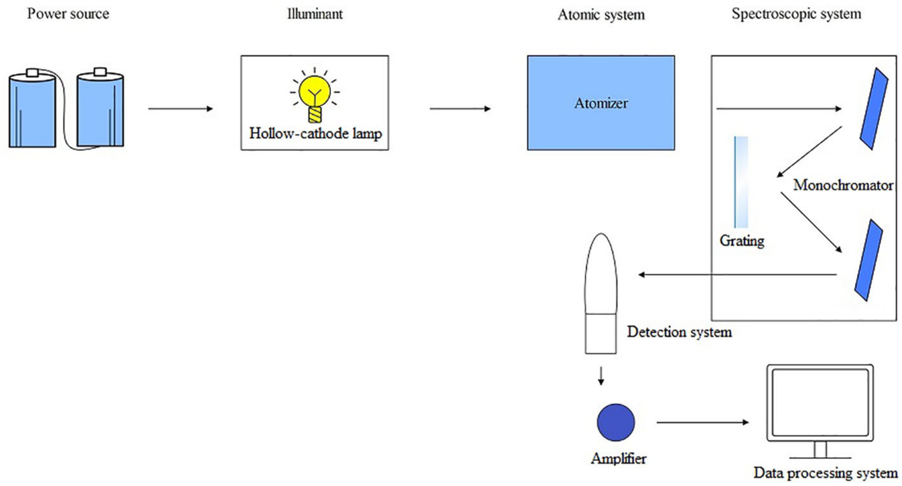

FIGURE 4
The operating principle schematic diagram of AFS.

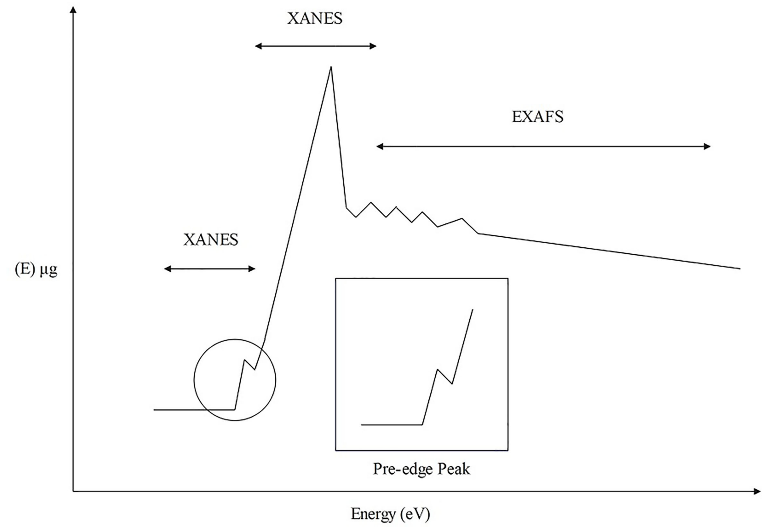

FIGURE 5
XAS structure composition diagram.

to information like valence states, and faster identification of chemical types and forms of elements (Hara et al., 2023; Ren et al., 2023). XAS requires low sample amounts, does not damage the sample, and provides information on coordination atom types, coordination numbers, and atomic spacing. This facilitates the study of dynamic processes in the *in situ* electrochemical reactions and enables the accurate establishment of conformational relationships (Newville, 2014; Mastelaro and Zanotto, 2018; Tang et al., 2023).

XAS is a local structure detection technique that can be utilized to determine the distribution and morphological structural information of metal elements in plants. Sikhumbuzo et al. detected that the photon energy position of Cd overlapped with CdO in rice crops through XANES spectroscopy, and EXAFS spectroscopy revealed that CdO formed clusters in rice with a CD-O coordination number of 4.2 and a bond distance of 2.83, indicating that the main form of Cd in rice was Cd(II)-O (Kunene et al., 2020). These results indicated that XAS could function as a fundamental tool for analyzing the local atomic structure and oxidation state of Cd in rice, which helps study the mechanism of the interactions between rice plants and Cd (Kunene et al., 2020). Huang et al. detected Cr in *Coptis chinensis* Franch. using XANES and observed that Cr(VI) was reverted to Cr(III) after treatment, suggesting that toxic hexavalent Cr can be transformed into non-toxic trivalent Cr in plants (Huang et al., 2018). XANES detected the main form of Pb in *Oenanthe javanica* as Pb-ferrihydrite, which was absorbed and transported as $Pb(Ac)_2$-$3H_2O$ (Liu and Luo, 2019). From the above studies, it can be observed that XAS enables the detection of HMs' forms in plants, facilitating the analysis of HMs' absorption and transport mechanisms in plants. This provides a direction for studying plant HM tolerance and offers possibilities for investigating the valency, ligand and dynamic metabolism of HMs in plants.

3.3 Spatial distribution

3.3.1 X-ray fluorescence spectrometry

XRF is an analytical technique based on the characteristic X-ray emission spectrum produced by the sample (Byers et al., 2019). It is

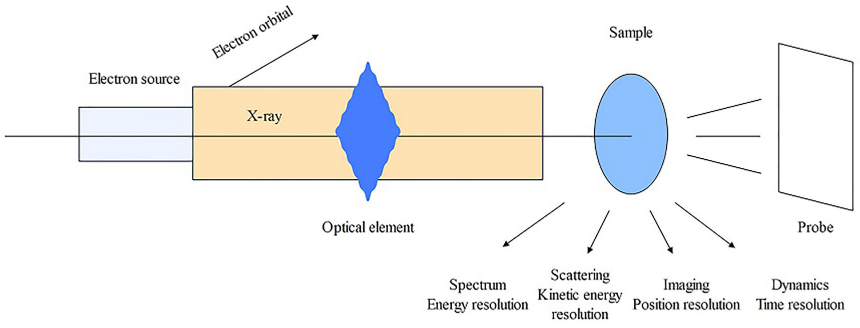

FIGURE 6
The operating principle schematic diagram of XAS.

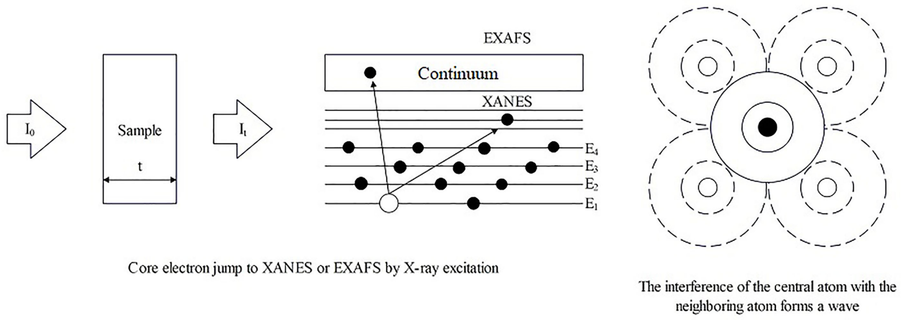

FIGURE 7
The operating principle schematic diagram of XAS.

primarily composed of an X-ray generator, a spectroscopic detection system, and a counting recording system. XRF is divided into energy-dispersive X-ray fluorescence spectroscopy (EDXRF/EDX) and wavelength-dispersive X-ray fluorescence spectroscopy (WDXRF/WDX) according to the description of X-rays (Figure 8) (Feng et al., 2021). XRF works by using an X-ray tube to excite and irradiate the sample, causing the elements in the sample to emit characteristic X-rays. These X-rays are then collimated by a filter and detected by a probe, such as a scintillation counter, which converts them into electrical signals. After signal processing, analytical results of the elemental composition of the samples are got (Figure 8). XRF is a physical analysis method, primarily used for surface scanning. XRF technology can be utilized to the direct analysis of solid samples and *in situ* analysis of plant samples (Marguí et al., 2009). To ground into a fine powder plant materials for high-pressure granulation, after is the preparation of plant samples by XRF technique carries on the analysis of the most common method (Rudman, 1999). It offers advantages such as high sensitivity, fast and accurate analysis, non-destructive sampling, and simple operability (Bamrah et al., 2019). SR is an advanced light source that emits pulsed light ranging from infrared to hard X-rays, with a pulse duration ≤ one nanosecond, which is many orders of magnitude higher than traditional light sources (Hu et al., 2020). Through the interaction of synchrotron radiation with matter, technologies such as XRF and X-ray photoelectron spectroscopy (XPS) have been developed (Li et al., 2015a; Li et al., 2015b). Synchrotron radiation X-ray fluorescence spectroscopy (SRXRF) can improve the sensitivity of Zn detection to about one part per billion (ppb) (Li et al., 2015a; Li et al., 2015b).

The XRF technique can generate a distinctive set of characteristics for each element in the sample, based on the concentration and intensity of the X-ray source (McGladdery et al., 2018). Fluorescence X-ray analysis is a reliable approach for both qualitative and quantitative assessment (McGladdery et al., 2018). Real-time analysis of XRF can be utilized to chart the distribution in plant tissues of HM elements (Jones et al., 2020; Ramakrishna, 2023). Studies have shown that XRF has become the preferred approach for studying the distribution of HMs in plants (Kopittke et al., 2018; van der Ent et al., 2022) The aboveground part of *Origanum sipyleum* L were prepared into powdered samples and aqueous extracts which were detected by XRF and found to be higher in nutrients such as K, Na, and Ca (Durmuşkahya et al., 2016). Sometimes, it can also be used in combination with other technologies to enhance the predictability of XRF (Feng et al., 2021). Vigani et al. utilized XRF and ICP-MS techniques to analyze cucumber leaves, revealing that Zn was primarily distributed in chloroplasts and mitochondria, and found that the Zn content significantly increased in chloroplasts and mitochondria when Fe was deficient, indicating that iron deficiency affected the

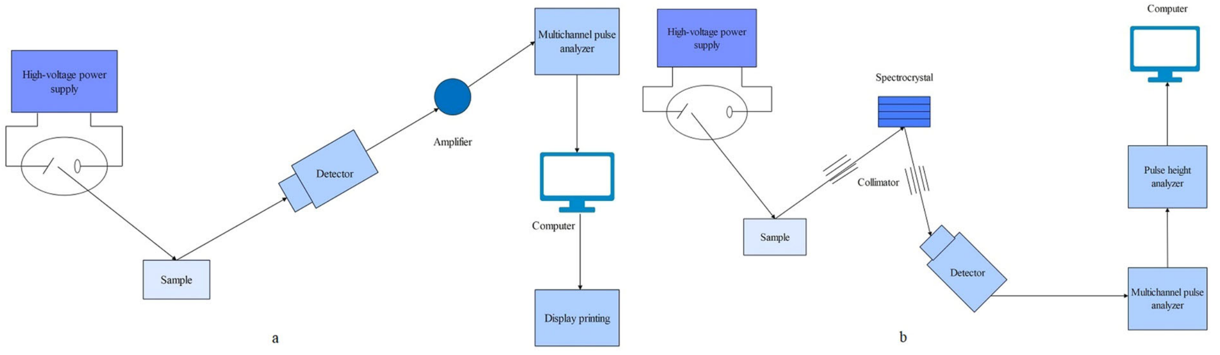

FIGURE 8
The operating principle schematic diagram of XRF. **(A)** Energy chromatograph **(B)** Wavelength chromatograph.

distribution of Zn in leaves (Vigani et al., 2018). SR technology offers significant advantages in terms of spatial resolution, distribution, and chemical state-related information. Microscopic X-ray fluorescence (μ-XRF) and XAS technologies, based on SR imaging technology, can be used to study the distribution of metals and metal-like elements in plants while providing valence states of elements with femtosecond sensitivity and micro/nano range spatial resolution (Vijayan et al., 2015). Zn and Cd in leaves of *Arabidopsis halleri* plants were analyzed by using μ-XRF, and the results shown that they were mainly distributed in leaf trichomes, while after treatment with 100 μM Zn and 20 μM Cd, they were primarily found in mesophyll tissues (Fukuda et al., 2020). At the cellular/tissue level, Zn is mainly distributed in mesophyll tissue, while Cd is primarily found in the vascular bundles of leaf veins (Fukuda et al., 2020). The results of XAS and synchrotron radiation microscopic X-ray fluorescence (SR-μXRF) showed that the main form of La in cucumber (*Cucumis sativus*) plants was phosphate or carboxyl complex, while the main form of Ce was CeO, and a small portion of CeO_2 was converted into a Ce(III)-carboxyl complex (Ma et al., 2015). The accumulation of Zn in leaf tips and vacuoles, as well as the dynamic complexation of histidine with Zn in edible plants, were discovered using μXRF and XAS techniques, providing new support for understanding the dynamic characteristics of Zn Chemical forms in plants (Mishra et al., 2020). *C. chinensis* was analyzed using SR-μXRF and LA-ICP-MS techniques to identify the distribution of Cr in root and the result revealed that Cr was largely accumulated in the vascular column, and part of the cortex (Huang et al., 2018). XRF is considered a powerful technical means for qualitative and quantitative detection of element distribution in single cells of plants or algae, making it highly suitable for detecting HMs in plants. It can aid in explaining the mechanisms behind the accumulation, absorption, and detoxification of HM elements in plants.

3.3.2 Laser ablation-inductively coupled plasma-mass spectrometry

LA-ICP-MS is a technique which enables rapid analysis of the distribution and content of elements in a sample at a micro-regional level, both for elemental and isotopic analysis (Pan et al., 2022). The LA-ICP-MS system is composed of three components: a laser exfoliation device (LXD), an ICP source, and a mass spectrometry detector. Among these, the LXD is composed of a laser, a beam delivery system, a sample cell, and an observation system. When a laser scans a sample, the excitation beam consisting of high-energy photons causes a photo-thermal effect that volatilizes atoms or molecules on the sample surface into particles and subsequently forms a small burrow hole, a process known as laser exfoliation (Trejos et al., 2003; Trejos and Almirall, 2005; López-Fernández et al., 2016). LA-ICP-MS works by melting and vaporizing the sample through laser ablation, and the carrier gas transports the ablated particles into the plasma (Figure 9). In the plasma, the particulates are ionized into positively charged ions, then analyzed by a mass spectrometry detector to identify the content and distribution of each element (Figure 9). During the laser scanning process, the changes in ionic strength of each element are recorded over time. This raw data is then reconstructed as an image, which is ultimately calibrated and visualized. The resulting image can be further processed using algorithms to visually represent the distribution of the elements. LA-ICP-MS can perform *in situ* analyses rapidly and in real-time on a micro-scale. It offers several advantages, including easy sample preparation, minimal contamination levels, high spatial resolution, sensitivity, low detection limits, provides relatively simple spectra while allowing for simultaneous determination of multiple elements and information about isotope ratio (Trejos and Almirall, 2005; López-Fernández et al., 2016; Nunes et al., 2016; Davison et al., 2023; Janovszky et al., 2023).

LA-ICP-MS is a robust technique that combines not only the high sensitivity and multi-element capability of ICP-MS but also good spatial resolution and laser radiation (Nunes et al., 2016). It is commonly used to directly analyze plant tissues and generate the multi-element bio-imaging of plant tissues to facilitate assess the ecological and toxicological risks of HMs to plants, and has been successfully used to analyze elemental distributions in samples such as leaves, roots, seeds, and bark (Narewski et al., 2000; Meharg et al., 2008; Shi et al., 2009; Wu et al., 2009; Yamaji and Ma, 2019). Using LA-ICP-MS imaging to detect Pb accumulation in wheat grain at maturation, it was identified the main distribution of Pb in wheat

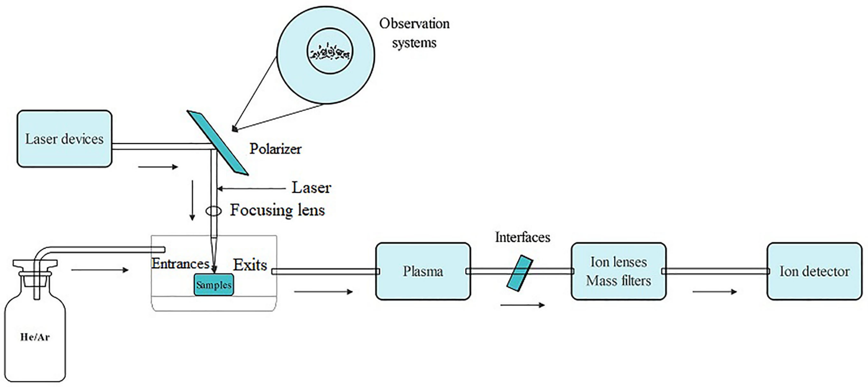

FIGURE 9
The operating principle schematic diagram of LA-ICP-MS.

peels and seed coating, while nutrients are primarily distributed in the inner seed coating region; it was hypothesized that removing bran may be an effective way to reduce Pb levels in wheat while retaining the nutrients (Zhang et al., 2022). LA-ICP-MS detected that Fe and Zn in *Phyllostachys edulis* seeds were mainly distributed and enriched in the aleurone layer and embryo and were significantly higher than rice, asserting their suitability as a natural food enhancer (Hu et al., 2023). Thus, LA-ICP-MS provided technical support for enhancing the preservation of nutrients in *P. edulis* seeds during food processing (Hu et al., 2023). Yamaji et al. utilized LA-ICP-MS technology to establish a bioimaging scheme for various elements at the cell level of rice nodes and identified the distribution characteristics of mineral elements between the wild-type and *OsHMA2* mutant rice plants (Yamaji and Ma, 2019). K, P, Mg, and other nutrients were mainly distributed in the phloem, while Zn, Fe, and other trace elements in the vascular tissues (Yamaji and Ma, 2019). The distribution of Zn in the nodal tissues of the *OsHMA2* mutant was not markedly distinct from that of the wild type. However, the contents of Zn were lower in the nodal vascular tissue I but elevated in III compared to the wild type (Yamaji and Ma, 2019). This suggests that LA-ICP-MS is a robust and powerful method for the qualitative and quantitative mapping of mineral elements in rice node tissues, especially when combined with the functional characterization of transporter proteins (Yamaji and Ma, 2019).

3.4 Absorption and transport of HMs by plants

3.4.1 Non-invasive micro-test technology

Non-invasive micro-test technology (NMT) is a microelectrode technology for the real-time, label-free detection of the flow rate and the influx or efflux of molecules and ions into the organisms (Yuan et al., 2021). NMT consists of a selective microelectrode system (SMS), signal amplification, microscopic imaging, 3D motion, and data processing systems. The SMS comprises a glass microsensor, Ag/AgCl electrode, electrolyte, and liquid ion exchanger (LIX). The selectivity is achieved through the specific recognition of ions by organic compounds in the large neutral molecular carriers within the LIX, this is essential for NMT. The selective microelectrode is moved back and forth between high and low electrochemical potentials carried by charged particles, measuring the potential value at each point obtaining the potential difference (AV) between the two points. The potential concentration calibration curve of the electrode was employed to measure the differences in the ion concentrations between these two points. The rate of ion movement was computed using Fick's First Law of Diffusion and Nernst's Equation (Figure 10). NMT can perform 3D measurements of the samples *in situ* with high sensitivity and spatial resolution without causing damage or requiring marker sampling. It is simple to operate and can detect the dynamic indicators of bioactivities of plants (Yuan et al., 2021; Wang et al., 2023). It can detect 50 types of metallic molecules and ions, including H^+, Ca^{2+}, Na^+, K^+, Cl^-, Cd^{2+}, Cu^{2+}, and NH_4^+ (Mugnai et al., 2012; Mardones et al., 2018; Wu et al., 2019; Lan et al., 2020; Zhang et al., 2020; Di et al., 2021; Hou et al., 2021). As the rate of NMT-based determination is robust, it enables the investigation of the flow characteristics of metal ions (mainly in roots); it can be used to screen species as low or hyperaccumulators and explore the enrichment characteristics of plants; and it provides technical support for hyperaccumulator-based remediation of contaminated soils, plant nutrition regulation, and plant physiology research (Han et al., 2022).

NMT is a handy tool for studying ion flow, signal transduction, and functional genes in plants under HM-induced stress. It can monitor the dynamic flow of HM ions in and out of plants in real-time, which is conducive to investigating the mechanism of tolerance to HMs, the toxic effects of HMs, and the mechanism by which hyperaccumulators absorb and transport HM ions (Liu et al., 2020). The scanning ion selective electrode technology (SIET) and microelectrode ion flux estimation technology (MIFE) are

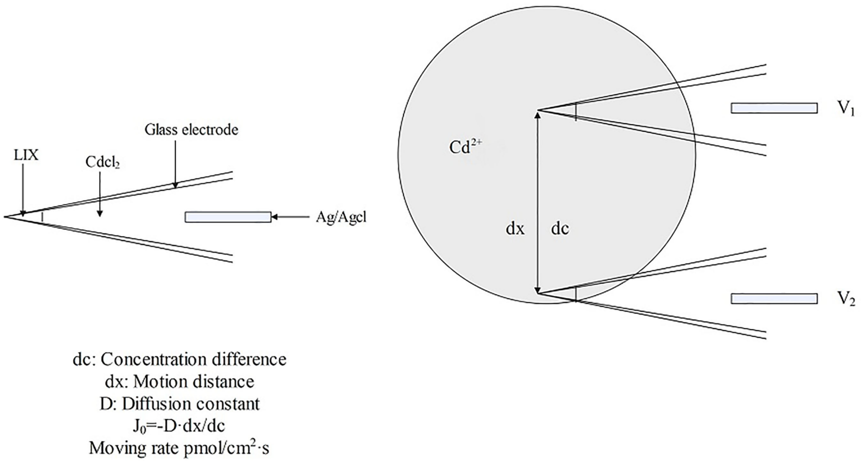

FIGURE 10
Schematic diagram of Cd^{2+} detection by NMT.

commonly used for measuring ion currents in plants (Mardones et al., 2018; Hou et al., 2021; Sun et al., 2021; Sun et al., 2021; Deng et al., 2022). The inflow of Cd^{2+} at 300 μm around the root tips of *Bidens pilosa* L. plants was reduced when treated with 16 mM Ca^{2+}, 8 mM Mg^{2+}, 8 mM SO_4^{2-}, or 18 mM K^+ as detected by NMT, indicating that treatment at a large concentration of nutrient ions inhibited the absorption of Cd^{2+} (Wang et al., 2023). Using the NMT method, a peak in the net Cd^{2+} flux was observed at a distance of 100 μm from the surface of the root tips of *Amaranthus hypochondriacus* L., a plant famous for its ability to accumulate large quantities of this metal ion (Han et al., 2023). Furthermore, fluorescent labeling revealed that Ca^{2+} channel blockers conspicuously suppressed Cd^{2+} influx compared to other blockers, suggesting that Ca^{2+} channels serve as primary pathways for Cd entry through the roots (Han et al., 2023). In a similar observation, Cd primarily entered *Sedum plumbizincicola* through Ca^{2+} channels; the net flux of Cd^{2+} altered from inflow to outflow as the concentration of Ca^{2+} increased (Li et al., 2017b). NMT detected a significant reduction in the flow of Cd^{2+} in the root hair area of rice plants when NH_4^+ was added, indicating that it reduced the influx of Cd and, therefore, is beneficial for cultivating rice with lower levels of pollutants (Wu et al., 2018). The influx and efflux of ions from plant cells were detected by NMT, providing the most intuitive and accurate data for studying plant stress resistance mechanisms and understanding the relationship between ion/molecular flow and specific cellular functions (Han et al., 2022).

3.5 Omics approaches for HM response and tolerance in plants

Plant reactions to HM toxicity are dependent on regulation by molecular factors (Raza et al., 2022). Deciphering HM toxicity and subsequent molecular, cellular and physiological responses is regarded to be a challenging, arduous and complex work (Mondal et al., 2022). A diverse range of biotechnologies are being applied to better understand and explore the pathways and mechanisms involved in plant responses and tolerance for HM toxicity (Raza et al., 2022). With the advancement of science and technology, the emergence of omics has provided a valuable terrace to investigate the physiological, biochemical, metabolical and molecular activities of HM-treated plants and cells. Well-established genomics technologies like genomics, transcriptomics, proteomics, metabolomics, ionomics, and other high-throughput technologies are valuable in elucidating plant stress responses and tolerance to HM. Through a comprehensive analysis of a large amount of omics data, the fundamental state of genes, mRNAs, proteins, metabolites, and elemental compositions in plants could be ascertained to fully explain the molecular mechanisms underlying the interaction between plants and HMs. The data gained will contribute to the enhanced research on plant stress tolerance and can be used in breeding and engineering schemes to breed plants with new and desirable agronomic traits and hyperaccumulators (Singh et al., 2016).

Analysis of genomics-based data aids in identifying genes, regulators, enzymes, signaling and other molecular factors involved in the HM uptake, transportation, resistance, and tolerance mechanisms of plants (Anjitha et al., 2023). DNA mismatch repair (MMR), targeted induced local lesions in genomes (TILLING), genome-wide association studies (GWAS), and clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) are some of the genomic methods that recognize genes are correlated with the interactions between plant and metal (Al-Khayri et al., 2023). GWAS study in *Medicago sativa* subjected to Cd stress reveals that response features are polygenic, involving several quantitative loci, and that stress-related UDP-glycosyltransferases, genes ATP-binding cassette transporters, *P*-type ATP transporters genes, and oxidative stress response genes are associated with Cd tolerance (Paape et al., 2022). In barley, under Cd stress, the genetic structure and genetic regulation study by QTL analysis revealed that antioxidant enzymes, stress signaling proteins and metal ion transporters showed different categories of InDels and SNPs in the QTL regions between the parents, which probably be critical for responding to Cd stress (Derakhshani et al., 2020). The ABC transporters detected are potential candidate genes that can be explored for raising of Cd-tolerant barley varieties (Derakhshani et al., 2020).

Transcriptomic profile can reflect the transcriptome differences among different cultivars, developmental stages, and tissues of plants under different environmental conditions, which provides us with further understanding and analysis of the responses under specific physiological states or stress conditions (Jamla et al., 2021). Transcriptomic analysis of *Calotropis gigantea* on exposure to Cd demonstrated that the leaves mitigated Cd stress by activating a variety of Cd detoxification processes such as cell wall remodeling, antioxidant systems, metal chelation, and the upregulation of genes related to the metal transporters (Yang et al., 2022). Using a gene co-expression network, the researchers was identified that *BnaCn.ABCC3* and *BnaC3.ABCC4* in Brassica are a pivotal factors participating in the storage of PC-Cd complexes in vacuolar (Zhang et al., 2019). The amount of genes participating in survival mechanisms and metabolic pathways would have been regulated by HM stress. Regulatory genes (e.g., TF) together with these functional genes are critical for the regulation of many stress-responsive genes, and will ultimately form gene networks (Jamla et al., 2021). In *Arabidopsis*, MYB4 TF modulates Cd tolerance by ameliorating the anti-oxidant defense system and enhancing the expression of the promoter regions of phytochelatin synthase 1 (*PCS1*) and metallothionein 1C (*MT1C*) (Agarwal et al., 2020).

The new insights provided by proteomics into stress-induced proteins and their involvement in mitigating HM toxicity is a powerful tool (Raza et al., 2022). It has also been used to compare changes in proteins at the organelle, cell, tissue and organ levels, and to provide an understanding of protein behavior under diverse stress situations, including HM stress (Singh et al., 2016). By 2-DE analysis, 18 proteins involved in Cd tolerance were discovered in the leaves of two tobacco genotypes, Gui Smoke 1 (Cd-sensitive) and Yunnan Smoke 2 (Cd-tolerant) (Ghatak et al., 2017). Proteomic analysis (LC-MS/MS) of Se-stressed pepper seedlings showed that 172 proteins were up-regulated and 28 proteins were down-regulated, and several heat shock proteins (HSPs), which help to cope with Se stress, were identified in the Se stress-induced differentially expressed proteins (DEPs) of pepper (Zhang et al., 2019).

Under stress conditions, plants become more focused on coping with adversity stress and survival, and regulate their growth and developmental processes. This leads to changes in the pattern of gene expression (or transcriptional reprogramming), which then causes changes in the concentration, type, and number of metabolites produced (Jamla et al., 2021). Metabolomics helps to understand the complex metabolic activities and differences in regulated metabolites produced by plants under different conditions including HM stress (Raza, 2022). In Cd-stressed tobacco plants, metabolomics investigation indicated that 76 metabolites in the leaves and 150 metabolites were in the roots individually. It is found that metabolites were in considerable abundance in the biosynthesis of amino acids, arginine and proline, flavone and flavonol, and nicotinate and nicotinamide (Zou et al., 2022). In *Calendula officinalis* plant exposed to Cd stress, the phenomena of the contest between specialized metabolites (triterpenoids) and general metabolites (sterols) were noted in the culture of plant roots and hairy root (Rogowska et al., 2022).

Plants need a suitable amount of minerals to maintain normal vital activities. The application of ionomics enables an in-depth understanding of the nutrient and trace element makeup of plants, explores the activities and mechanisms of plant absorption, storage, and assimilation, and provides new ideas for illuminating the response of plants to HMs (Raza et al., 2022). It has been shown that plants subjected to HM-induced stress can be adapted to unfavorable conditions via modulating elemental composition and content in the ionome (Mleczek et al., 2018). Researchers used transcriptomic and ionomic mapping to analyze the mechanisms of mitigating As(III) force in rice and found that ionome was significantly correlated with transcriptome in the aerial part of rice (Xiao et al., 2021). Upregulation of transporter genes associated with transmembrane transport and ion binding associated with nutrient elements was found, showing that plants tend to be more inclined to transport more nutrients, like ammonium, potassium and phosphate, in response to As(III) toxicity (Xiao et al., 2021).

The accurate selection of HM detection and analysis methods is crucial for explaining the stress caused by HMs in plants. By conducting a comprehensive analysis of the different, commonly used methods for HMs in plants, the application criteria and characteristics of each method were obtained, systematically compared, and summarized (Table 2).

4 Conclusions and prospects

With rapid urbanization and industrialization, HM pollution has become a major environmental problem. HMs released in the environment are absorbed and accumulated from plants, which are transmitted to humans along the food chain, causing grave adverse impacts on the life and health of plants and humans. Therefore, it is crucial to propose solutions that effectively control the content of HMs by studying the mechanisms of translocation, uptake, distribution, and storage of HMs in plants and identifying their chemical forms. This paper reviews the technical methods applied to study HMs in plants during recent years and summarizes the principles, advantages, disadvantages, and applications for these approaches.

ICP-MS and AAS are widely used to detect HM content in plants, both of which have the advantages of high sensitivity, a low detection limit, and high accuracy. Compared to AAS, ICP-MS has higher operating costs; however, it offers convenient and fast detection, lesser interference, simple sample preparation, and the ability to analyze multiple elements and their isotopes (Pei et al., 2023). These advantages meet the needs of large-scale detection, necessitating researchers with an expectation of obtaining additional information to choose ICP-MS more frequently. Therefore, designing and developing portable instruments based on ICP-MS and AAS-based multi-element analyses have become a priority. AFS and XAS are primarily used to study the chemical form of HMs in plants. AFS has a good selectivity, but its limited range of detecting elements severely restricts its application. XAS is preferred more because of its low maintenance cost and ability to provide more information, such as the type of coordinating atoms, coordination number, atomic spacing, which aligns with daily laboratory use. In recent years, XRF and LA-ICP-MS have become the primary methods often applied to the spatial distribution of HMs in plants. LA-ICP-MS, with its simple operability and instrumentation, has been widely used for studying the tissue distribution of HMs in plants. This technology can provide a basis for studying the mechanisms of HM transport and the function and localization of transport proteins by analyzing the distribution of HMs. Although SR-XRF requires large-scale instrumentation, the combined use of SR-XRF and XAS is often chosen for analyzing the chemical for and tissue distribution of HMs in plants (Bai et al., 2019; Li et al., 2020). NMT has demonstrated outstanding performance in monitoring the flow speed and direction of small molecules and ions in organisms and has rapidly developed in recent years. The difficulty in fabricating and preserving selective microelectrodes has limited their application to HM-related studies (Han et al., 2022). The rise and application of molecular biology and omics technologies have opened a new chapter in studying HMs in plants. Omics can determine the interactions between plants and HMs by obtaining metabolomics, transcriptomics, genomics, ionomics, and proteomics information on plants subjected to HM-induced stress. Molecular biology techniques, such as gene cloning and editing, investigate the function of proteins and genes and the response mechanisms of plants to HMs and provide theoretical guidance for improving the tolerance and resistance of plants to HMs.

In recent years, ICP-MS, XAS, LA-ICP-MS, NMT, omics, and molecular biology techniques have been increasingly applied in HM-related research in plants, providing technical support for studying the uptake, transport and distribution, accumulation and storage, and detoxification of HMs in plants. Using omics and molecular biology methods, the mechanisms of plant response to HM-induced stress were analyzed, and the plant varieties with HM resistance or low HM accumulation were screened. Furthermore,

TABLE 2 Comparison of HM detection and analysis methods commonly used in plants.

Method	Element detection range	linear range	sensitivity	Speed of analysis	Sample Preparation	flammable gas	isotopic analysis	Simultaneous multi-element or multi-ion analysis	operation	cost	LOD	major interference	reference
ICP-MS	wide	widest	Highest	Fast	simple	NO	YES	YES	simple	High	ppt	spectral interference	(Fu et al., 2018; Wilschefski and Baxter, 2019; Theiner et al., 2020; Penanes et al., 2022)
AAS	wide	narrow	High	slow	complex	YES	NO	NO	simple	Low	ppb	Matrix Interference	(Suquila et al., 2019; Eskina et al., 2020; Li et al., 2021; Zvěřina et al., 2023)
AFS	narrow	wide	Higher	slow	complex	NO	NO	YES	simple	Low	ppb	spectral interference	(Yogarajah and Tsai, 2015; Lancaster et al., 2019; Li et al., 2019; Xia et al., 2019; Liu et al., 2020; Peng et al., 2022)
XAS	wide	***	Highest	Fast	simple	NO	NO	YES	simple	High	***	spectral interference	(Newville, 2014; Mastelaro and Zanotto, 2018; Tang et al., 2023)
XRF	wide	wide	High	Fast	simple	NO	NO	YES	simple	Low	ppb	spectral interference	(Chuparina and Aisueva, 2011; McGladdery et al., 2018; Bamrah et al., 2019)
LA-ICP-MS	wide	widest	Highest	Fast	simple	NO	YES	YES	complex	High	ppt	spectral interference	(Davison et al., 2023; Janovszky et al., 2023)
NMT	wide	***	Highest	Fast	complex	NO	NO	YES	simple	High	***	sample status	Yuan et al., 2021; Wang et al., 2023)

*** means that no relevant information is given in the literature.

regarding environmental concerns, future research may focus on screening and breeding hyperaccumulating plant cultivars to remediate contaminated soils. The use of traditional methods for detecting HMs is maturing. Regarding HM detection technology, low-cost operability, and portability have become the focus of attention and application in laboratory research. Therefore, simplifying the operability of instruments, expanding their detection range, and reducing the costs of different detection technologies remain the main directions of progress. The detection technology of HMs in plants should be continuously refined and improved to develop it towards integration, efficiency, and high adaptability.

Author contributions

SH: Writing – original draft. YN: Writing – original draft. LX: Writing – original draft. ZL: Writing – review & editing. XS: Writing – review & editing. MD: Writing – review & editing. WH: Writing – review & editing.

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Conflict of interest

Author MD was employed by the company Xi’an Ande Pharmaceutical Co; Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher’s note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2024.1310328/full#supplementary-material

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Effects of arbuscular mycorrhizal fungi on the reduction of arsenic accumulation in plants: a meta-analysis

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Arsenic (As) accumulation in plants is a global concern. Although the application of arbuscular mycorrhizal fungi (AMF) has been suggested as a potential solution to decrease As concentration in plants, there is currently a gap in a comprehensive, quantitative assessment of the abiotic and biotic factors influencing As accumulation. A meta-analysis was performed to quantitatively investigate the findings of 76 publications on the impacts of AMF, plant properties, and soil on As accumulation in plants. Results showed a significant dose-dependent As reduction with higher mycorrhizal infection rates, leading to a 19.3% decrease in As concentration. AMF reduced As(V) by 19.4% but increased dimethylarsenic acid (DMA) by 50.8%. AMF significantly decreased grain As concentration by 34.1%. AMF also improved plant P concentration and dry biomass by 33.0% and 62.0%, respectively. The most significant reducing effects of As on AMF properties were seen in single inoculation and experiments with intermediate durations. Additionally, the benefits of AMF were significantly enhanced when soil texture, soil organic carbon (SOC), pH level, Olsen-P, and DTPA-As were sandy soil, 0.8%–1.5%, $\geq$7.5, $\geq$9.1 mg/kg, and 30–60 mg/kg, respectively. AMF increased easily extractable glomalin-related soil protein (EE-GRSP) and total glomalin-related soil protein (T-GRSP) by 23.0% and 28.0%, respectively. Overall, the investigated factors had significant implications in developing AMF-based methods for alleviating the negative effects of As stress on plants.

KEYWORDS

arbuscular mycorrhizal fungi, arsenic, reduction, factors, meta-analysis

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1 Introduction

Arsenic (As) contamination in agricultural soils is a common environmental issue that poses remarkable risks to human health and ecosystem sustainability (Wan et al., 2024). As a toxic metalloid, As can naturally occur in soils or be introduced through various human activities such as mining, industrial processes, and application of arsenical pesticides (Mawia et al., 2021; Gui et al., 2023). As contamination is a global issue in countries such as the United States, China, Argentina, Australia, Bangladesh, Chile, India, Mexico, Thailand, and Vietnam (Baruah et al., 2021; Wang et al., 2023; Jahandari and Abbasnejad, 2024). The As pollution in China is of grave concern, with the nation's farmland soil arsenic levels averaging a concerning 11.83 mg/kg, significantly surpassing the global average of 7.20 mg/kg (Ren et al., 2022). As uptake by plant roots and its subsequent translocation to edible parts of plants can result in potential health risks throughout the food chain. According to the U.S. Agency for Toxic Substances and Disease Registry (ATSDR), As was classified as a top hazardous substance in the United States (Escudero-Lourdes, 2016). As exposure has been found to contribute to different cancer types such as skin, lung, liver, kidney, and bladder cancers (Zakir et al., 2022). Worldwide, the threat of As poisoning affects over 230 million people, with 180 million in Asia being especially vulnerable (Shaji et al., 2021). Hence, there is a growing attention to research on soil As pollution issues among researchers.

Arbuscular mycorrhizal fungi (AMF) are beneficial soil microorganisms, which form mutualistic symbiotic associations with roots in most land plant species (Hawkins et al., 2023). These symbiotic relationships play crucial roles in enhancing plant nutrient uptake, stress tolerance, and overall growth and development. Recently, several research works have highlighted the key role of AMF in the absorption, translocation, and accumulation of As in plants (Li et al., 2016; Alam et al., 2020; Mitra et al., 2022). AMF involvement may be crucial in decreasing As phytoavailability, which achieves this by stabilizing As through mycelium and glomalin (Wu et al., 2015; Zhang et al., 2020; Riaz et al., 2021). AMF mycelium forms a network in the soil, effectively immobilizing and trapping As to prevent its uptake by plants. In addition, glomalin, a glycoprotein produced by AMF, binds to and sequesters As to further reduce its availability for plant uptake (Maldonado-Mendoza and Harrison, 2018). These mechanisms, which are adopted by AMF to stabilize As, significantly contribute to lowering its potential impact on plants, enhancing agricultural system sustainability, and mitigating the risks associated with As contamination.

The interaction of AMF colonization and As accumulation holds great promise for ensuring safe food production and implementing bioremediation programs (Gupta et al., 2022). AMF inoculation effect on As concentration in soil–plant systems has been extensively studied by scholars all over the world. Singh et al. (2023) found that applying 10 g/kg *Glomus mosseae* significantly reduced arsenic in wheat grains (Singh et al., 2023). Gupta et al. (2021) reported that *Rhizophagus intraradices* inoculation counteracted As-induced changes in sugar metabolism, affecting enzyme activities related to starch phosphorylase, α-amylase, β-amylase, acid invertase, sucrose synthase, and sucrose phosphate synthase in leaves (Gupta et al., 2021). Under As stress of 25 mg/kg, the synergistic effect of *Funneliformis mosseae* and iron can reduce the toxic effects of arsenic by enhancing phosphorus uptake and increasing the activity of antioxidant enzymes in rice (Zhou et al., 2023). However, a comprehensive review of previous experimental studies revealed significant inconsistencies in the published results. Some research works have reported that AMF inoculation resulted in significant As concentration reductions in both roots and shoots of maize (Yu et al., 2009), while others have found increases in As concentration in maize roots (Long et al., 2021). Similarly, conflicting results have been reported in rice, with some research works showing no significant effect of AMF inoculation on total As concentration in rice grains (Zhang, X. et al., 2016), while some others have reported increased total and inorganic As concentrations in rice grains (Li et al., 2016). Several qualitative literature reviews have focused on the complex interaction frameworks of environmental and biological factors influencing AMF-mediated As concentration (Cabral et al., 2015; Neidhardt, 2021; Mitra et al., 2022; Tan et al., 2023). However, no comprehensive meta-analysis has been performed to quantitatively determine and evaluate the relationship between AMF and As concentration in plants under various environmental and biological conditions. Meta-analysis is a powerful method aimed at deriving broad generalizations by pooling and analyzing outcomes from multiple studies. Its fundamental goal is to provide a more comprehensive understanding than what can be obtained from individual primary studies alone. However, it is important to note that meta-analysis has limitations. The quality of each study included and the potential publication bias can influence the results of the meta-analysis. Additionally, the inclusion of new studies in the future may lead to updates and modifications in the conclusions of the meta-analysis. It is a dynamic process that requires continuous evaluation and consideration of new evidence (Gurevitch et al., 2018). Therefore, this research aims to 1) verify the impact of AMF inoculation on As accumulation in plants grown in As-contaminated soils; 2) quantify the effects of AMF on plant As concentration, considering various biotic factors (e.g., AMF root colonization rate, AMF inoculum type, AMF species, and plant family) and abiotic factors [e.g., soil texture, soil pH, and soil organic carbon (SOC)]; and 3) investigate the mechanisms supported by data as proposed in the literature. Additionally, potential future research directions regarding the interaction between AMF and As will be discussed.

2 Materials and methods

2.1 Bibliometric analysis using CiteSpace

CiteSpace is a new statistical analysis software widely used for bibliometric analysis and visualization in the academic community (Chen and Song, 2019). A systematic literature search was conducted from the Web of Science (WoS) Core Collection database on the topic of arsenic and arbuscular mycorrhizal fungi.

A total of 227 publications from 1994 to 2024 were collected from the database and used CiteSpace software to visualize and analyze various aspects including annual publication volume, research countries, and keywords in this field (Supplementary Figure S1). The scientific knowledge mapping visualized by CiteSpace facilitated a better understanding of theoretical terms and provided some background knowledge for our meta-analysis work.

2.2 Literature collection

Peer-reviewed research papers on the subject were identified through the Web of Science (https://apps.webofknowledge.com) as of the end of December 2023. Search keywords included *(As or arsenic or arsenite or arsenate) and (plant or crop) and (Arbuscular mycorrhizal fungi or AM or AMF) and (soil)*. The selected research articles for meta-analysis needed to meet the following criteria: 1) the research must be original and focus on plant performance in As-contaminated soils in response to AMF treatments. 2) The experiment must include both a control group (i.e., without AMF application) and a treatment group (i.e., with AMF application). 3) The research paper must include the mean and standard deviation values of As concentration in different tissues of plants. If no standard deviation was reported, one-tenth of the mean value was used instead (Luo and Zhang, 2006). 4) Each treatment and the control must have at least three replicates. 5) The experiment was conducted using sterilized soil or sand mixture. 6) The effect of AMF was determined in the absence of rhizobia so that the effect of nitrogen-fixing bacteria was eliminated. The literature selection procedure for the meta-analysis is shown in Figure 1, including a total of 76 research articles with 1,362 observations from different parts of the world (Figure 2).

Data in numerical form were extracted directly from the original literature or tables, and data in graphical form were extracted using WebPlotDigitizer 4.5 (https://apps.automeris.io/wpd/index.zh_CN.html). The extracted data were used to establish a database using Microsoft Excel 2016, including the following categories: authors, titles, published journals, publication date, plant species, AMF species, soil physical and chemical properties, P fertilizer application and the application rate, As concentration, dry weight, and P concentration and its standard deviation in different plant tissues. In the case of reporting a standard error (SE), the standard deviation (SD) is calculated by $SD = SE * \sqrt{n}$.

2.3 Categorical independent variables

The effects of AMF on As concentration in different plant tissues were determined, including grain, husk, straw, shoot, and root tissues. To gain a better and more comprehensive understanding of how soil biotic and abiotic factors impact the response of selected variables to the experimental treatments, we classified those factors into 10 groups. These groups include both biotic factors (such as AMF inoculum type, AMF species, plant family, and experimental duration) and abiotic factors (such as soil texture, pH, SOC, soil DTPA-As, chemical forms of As, and soil Olsen-P levels).

The AMF inoculum type was classified into two categories: single and mixed AMF species. Single inoculation entailed using only one AMF species in the experiment, while mixed inoculation involved using multiple AMF species obtained from either field extraction or commercial suppliers. The species of AMF were classified into *Rhizophagus irregularis*, *R. intraradices*, *G. mosseae*, *Glomus intraradices*, *Glomus caledonium*, *Glomus* sp., *Glomus geosporum*, *Glomus versiforme*, *Glomus etunicatum*, and *F. mosseae*. Plant

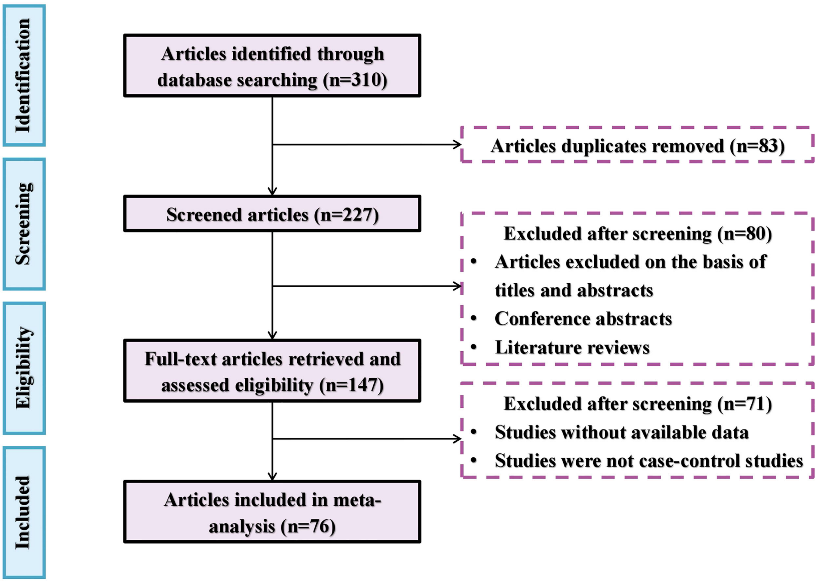

FIGURE 1
The flowchart of the literature selection procedure.

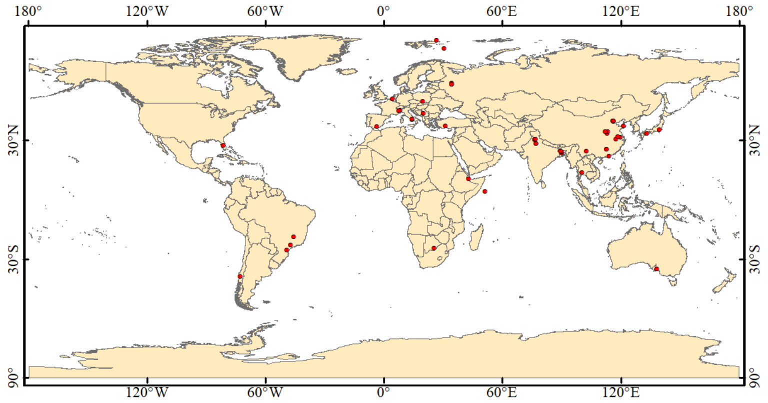

FIGURE 2
Geographical distribution of 76 published research articles in the world.

families were divided into Leguminosae, Pteridaceae, Compositae, Poaceae, Solanaceae, Plantaginaceae, and Melastomataceae. The experiment durations were categorized into three groups based on Lehmann and Rillig (2015): short with<56 days, medium with 56–112 days, and long with ≥112 days. According to the USDA Soil Classification (soils.USDA.gov), the soil texture included sandy and non-sandy soils. Sandy soil had a sand content of ≥50%. The soil pH had three levels: acid (pH< 6.5), neutral (pH 6.5–7.4), and alkaline (pH ≥ 7.4). The SOC also had three levels: low (SOC< 0.8%), medium (SOC 0.8–1.5%), and high (SOC ≥ 1.5%). Soil Olsen-P used as the background value was classified into two categories: deficiency (<9 mg/kg) and non-deficiency (≥9.1 mg/kg). P fertilizer application level was classified into three categories: low (<30 mg/kg), medium (30–60 mg/kg), and high (≥60 mg/kg). Soil DTPA-As used as the background value was divided into three categories: low (soil with final As concentration of<20 mg/kg or As added in irrigation water of<0.2 mg/L), medium (20–50 mg/kg or 0.2–1.0 mg/L), and high (≥50 mg/kg or ≥1.0 mg/L). The species of exogenous As were divided into As(III) and As(V). These categorization methods were used based on Lehmann and Rillig (2015) and Qiu et al. (2022).

2.4 Statistical analysis

The natural logarithm response ratio (ln*R*) method was used to calculate the effect size and variance of each set of data using MetaWin 2.1.

$$\ln R = \ln(X_E/X_C),$$

$$v = \frac{SD_E^2}{n_E X_E^2} + \frac{SD_C^2}{n_C X_C^2}$$

where ln*R* is the natural logarithm of the response ratio, defined as the effect size, X_E is the averaged plant As concentration of the treatment group, X_C is the averaged plant As concentration of the control group, and n_E and n_C are the number of replicates in the treatment group and the control group, respectively. SD_E and SD_C are the standard deviations of the treatment group and the control group, respectively.

To assess the overall variability of each variable, the Q_T statistic and the I^2 index were used (Supplementary Table S1). If the included studies showed significant heterogeneity ($p< 0.05$), either a random-effects model (REM) or a fixed-effects model (FEM) was applied for analysis. Since our dataset had a p-value below 0.05, the appropriate choice for analysis was the REM. The entire dataset was then divided into different subgroups to investigate the source of heterogeneity. Before the weighted analysis, the software Origin 2019 was used to generate a frequency distribution histogram of ln*R* and fit the Gaussian function to determine the normality of the data (Supplementary Figure S2). The bootstrap method was employed to establish the 95% confidence intervals (CIs) for effect sizes across all study variables and categories. The percentage change in response was calculated as (R − 1) × 100%. The positive effect size and positive percentage change indicated that AMF inoculation promoted plant As accumulation, and the negative effect size and negative percentage change indicated that AMF inhibited plant As accumulation. Publication bias is a common issue in meta-analyses due to the tendency to not publish non-significant results. This study aimed to address this problem by utilizing two methods to evaluate potential publication bias. One method used was the calculation of Rosenthal's fail-safe number using MetaWin 2.1. A fail-safe number equal to or greater than 5 * N + 10 (N representing the number of case studies) suggests a robust result. Another method employed was the use of funnel plots, where effect sizes were plotted against sample variances for ln*R* to assess publication bias. The data analysis results were then visually presented as color maps using Origin 2019 and as forest plots using GraphPad Prism 9.0.

3 Results

3.1 Effects of AMF on As concentrations, P/As ratio, and dry biomass in different tissues of plants

The effects of AMF on As concentrations in various tissues of plants grown on As-contaminated soil are illustrated in Figure 3. In comparison to non-mycorrhizal plants, those inoculated with AMF

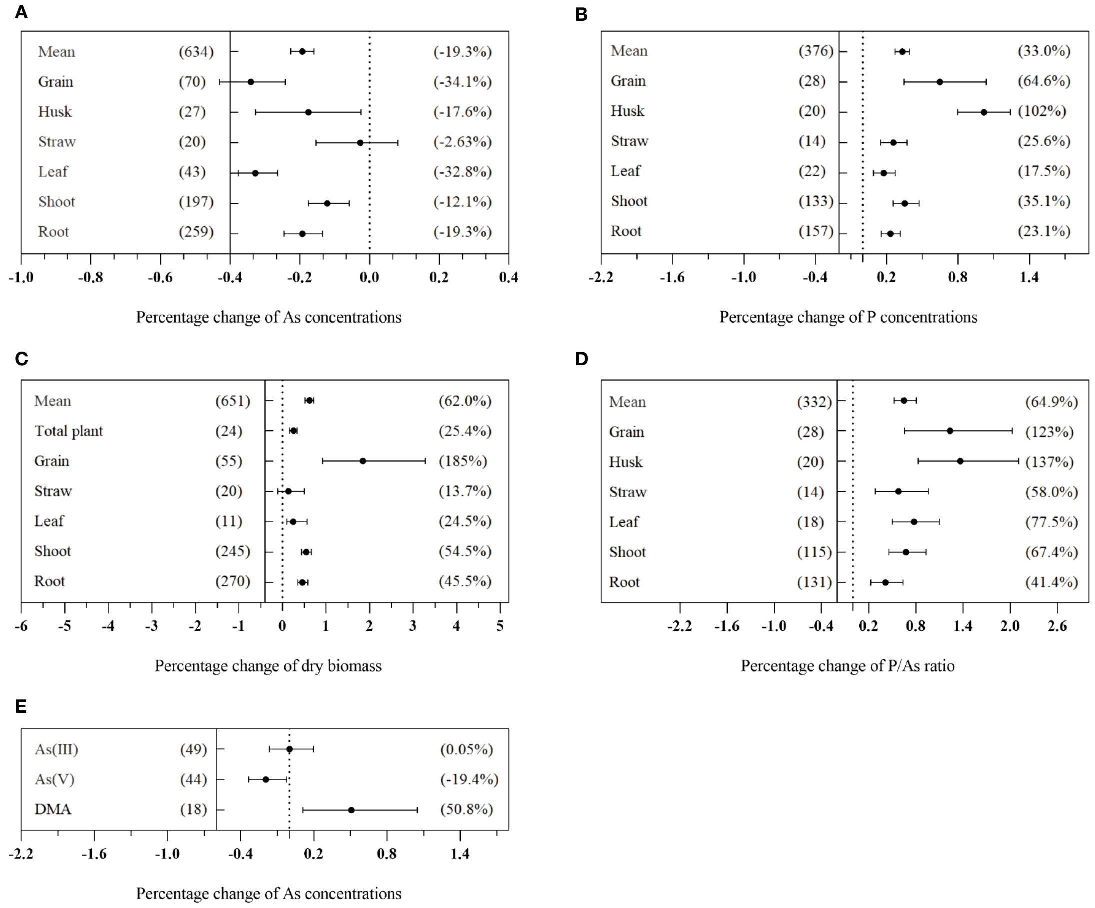

FIGURE 3
Effects of arbuscular mycorrhizal fungi (AMF) inoculation on total As concentration **(A)**, P concentration **(B)**, dry weight **(C)**, and P/As ratio **(D)** in different tissues of plants and different valence As concentration **(E)** in plants. The value in the left bracket is the number of tests included in the analysis, and the value in the right bracket represents the size of the effect value. When the mean value is regular, there is a positive effect, and when it is negative, there is a negative effect. When CI does not overlap with 0, there is no significant effect. The same as below.

exhibited a significant reduction in total As concentration, with an average decrease of 19.3% (CI: −22.6% to −16.0%) (Figure 3A). Additionally, AMF inoculation resulted in an improvement in the nutritional status of plants, as evidenced by a mean increase of 33.0% (CI: 27.0% to 39.2%) in P concentration, 62.0% (CI: 52.1% to 71.7%) in dry biomass, and 64.9% (CI: 53.3% to 80.5%) in the P/As ratio (Figures 3B–D). The impact of AMF on the concentration of As in plants varied significantly among different plant tissues ($Q_b = 246$, $p< 0.01$). There was a significant decrease in As concentration in grains, husks, leaves, shoots, and roots, with reduction rates of 34.1% (CI: −43.1% to −24.2%), 17.6% (CI: −32.8% to −2.5%), 32.8% (CI: −37.7% to −26.4%), 12.1% (CI: −17.6% to −5.8%), and 19.3% (CI: −24.5% to −13.5%), respectively. However, there was no significant effect on As concentration in straws (Figure 3A). It is worth noting that AMF inoculation significantly increased the grain dry biomass by 185% (CI: 91.9% to 328%) (Figure 3C). The application of AMF resulted in a significant reduction of As(V) concentration by 19.4% (CI: −33.4% to −2.34%), while no significant effect was observed on the concentration of As(III). Moreover, AMF application led to a significant increase in dimethylarsenic acid (DMA) concentration by 50.8% (CI: 11.0% to 115%) (Figure 3E).

3.2 Effects of abiotic factors on As concentration in plants treated with AMF

The impact of AMF on plant As concentration varied based on the soil properties (Figure 4). Medium SOC content (0.8%–1.5%) exhibited the most significant reduction in plant As concentration, with a decrease of 21.4% (CI: −27.7% to −14.2%) (Figure 4A). In terms of specific plant tissues, medium SOC levels (0.8%–1.5%) were found to significantly decrease As concentration in grains, shoots, and roots (Figures 4B–D). Sandy soils exhibited a higher reduction in plant As concentration compared to non-sandy soils, with reductions of 21.5% (CI: −27.4% to −15.0%) and 18.1% (CI: −21.8% to −14.1%), respectively (Figure 4A). The effect of varying soil pH levels on As concentration was determined to be significant ($Q_b = 31.5$, $p< 0.01$). Alkaline soil was identified as the most effective in reducing As concentration in different plant tissues mediated by AMF. Overall, there was a reduction in As concentration of 26.4% (CI: −30.1% to −21.9%) in total plants, 24.0% (CI: −33.7% to −11.8%) in grains, 29.2% (CI: −37.2% to −19.9%) in shoots, and 24.4% (CI: −38.4% to −9.64%) in roots (Figures 4A–D).

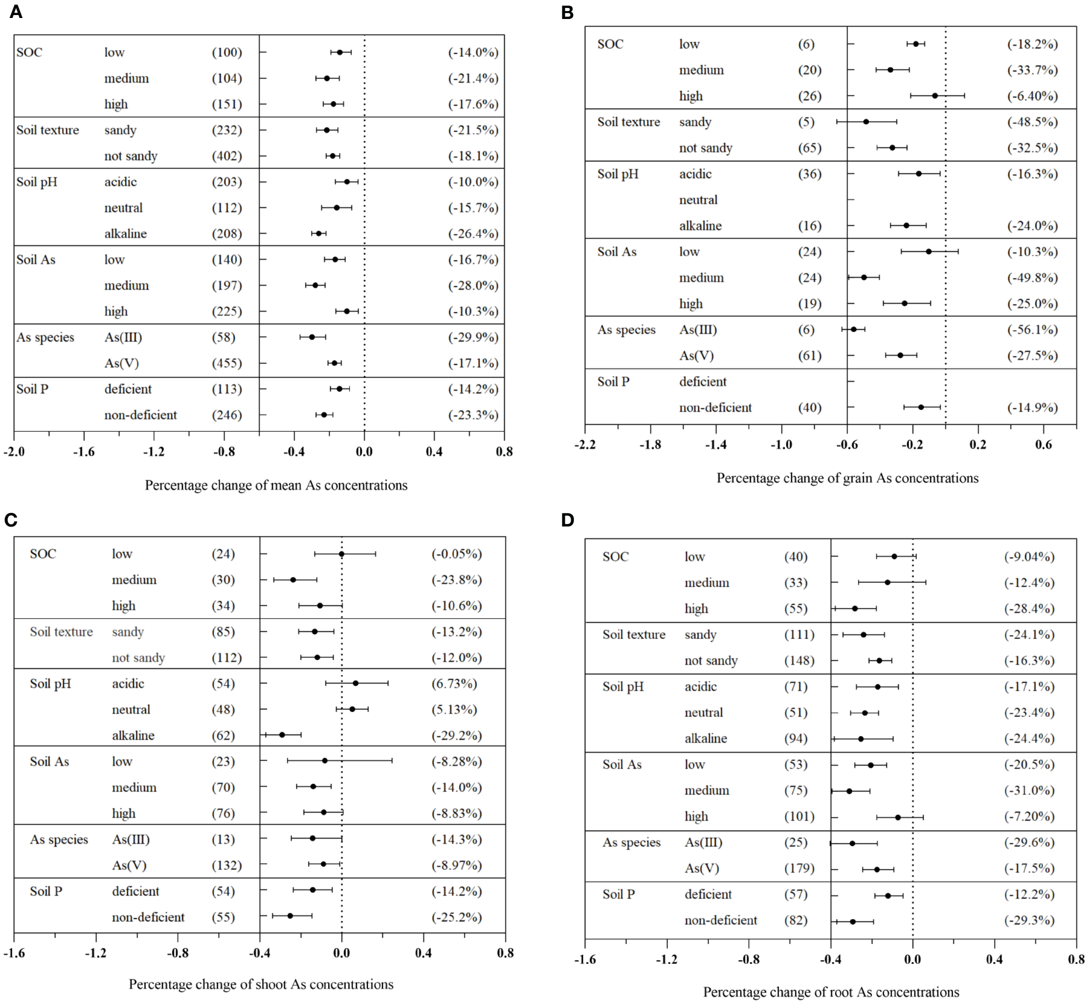

FIGURE 4
Effects of different soil properties on As concentration in the total plants **(A)**, grains **(B)**, shoots **(C)**, and roots **(D)** with arbuscular mycorrhizal fungi (AMF) inoculation.

Significant differences were observed among different levels of soil As concentration (Q_b = 95.1, $p<$ 0.01). Medium As concentration in the soil significantly inhibited As concentration in different plant tissues treated with AMF, resulting in reduction of 28.0% (CI: −33.5% to −22.4%) in total plants, 49.8% (CI: −59.1% to −40.3%) in grains, 14.0% (CI: −22.1% to −5.17%) in shoots, and 31.0% (CI: −39.5% to −21.0%) in roots (Figures 4A–D). Furthermore, significant inhibitory effects on As concentration were observed in soils containing deficient Olsen-P, with an average decrease of 23.3% (CI: −27.9% to −18.4%) (Figure 4A). Similarly, various abiotic factors influenced the impact of AMF on plant P concentration and dry biomass (Supplementary Figures S4, S6).

3.3 Effects of biotic factors on As concentration in plants inoculated with AMF

Significant differences were observed in the effects of different inoculation methods on plant As concentration (Q_b = 211, $p<$ 0.01). Single inoculation with AMF significantly decreased As concentration by 22.2% (CI: −25.3% to −18.8%). However, mixed inoculation showed a non-significant increase of 6.48% (CI: −5.33% to 19.4%) (Figure 5A). Moreover, there were significant differences in the effects of different AMF species on plant As concentration (Q_b = 226, $p<$ 0.01). Inoculation of *F. mosseae* and *R. intraradices* reduced As concentration by more than 30%, which was superior to other AMF species (Figure 6A). Additionally, the effects of AMF inoculation on As concentration in plants varied significantly among different families (Q_b = 537, $p<$ 0.01). In Leguminosae plants, the inoculation with *R. intraradices* resulted in the greatest reduction in As concentration (−44.9%, CI: −50.4% to −38.7%) (Figure 6B). The duration of the experiment did not have a significant effect on As concentration in plants (Q_b = 6.78, p = 0.568) (Figure 5A). However, intermediate experiment durations significantly decreased As concentration in different plant tissues treated with AMF. This result led to a reduction of 60.1% (CI: −68.3% to −49.8%) in grains, 13.0% (CI: −19.6% to −5.96%) in shoots, and 20.3% (CI: −26.6% to −13.4%) in roots (Figures 5B–D). Similarly, various biotic factors influenced the impact of AMF on plant P concentration and dry biomass (Supplementary Figures S5, S7).

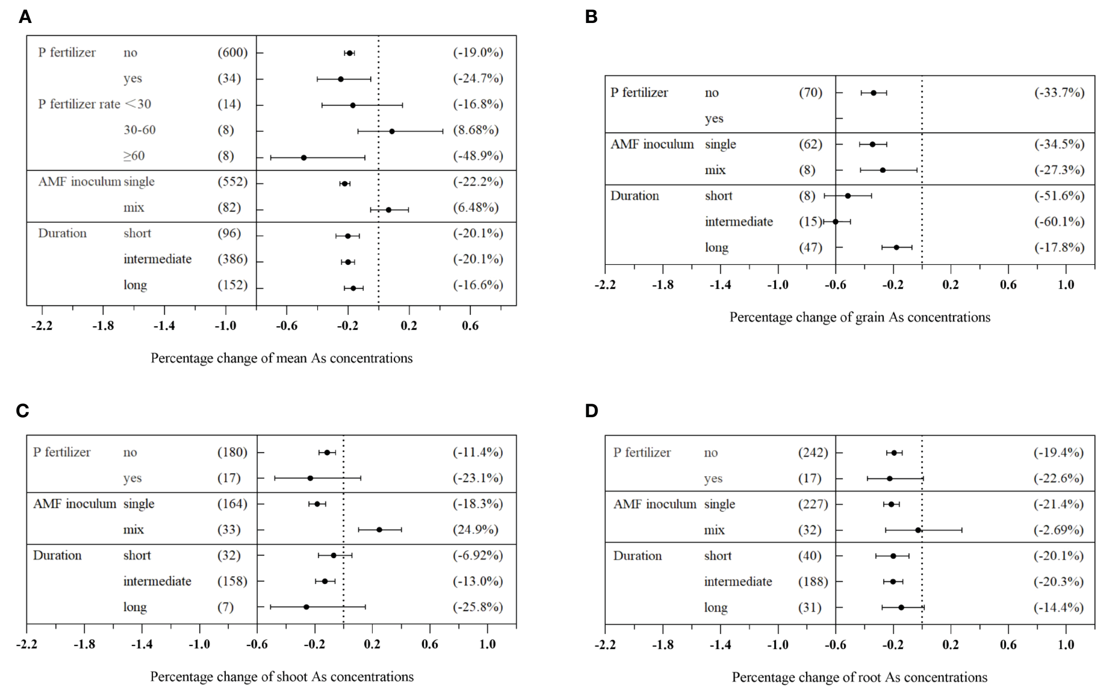

FIGURE 5
Effects of different external factors on As concentrations in the total plants **(A)**, grains **(B)**, shoots **(C)**, and roots **(D)** with arbuscular mycorrhizal fungi (AMF) inoculation.

The meta-regression model results for the full dataset revealed a dose-dependent decrease in As concentrations in plants as the mycorrhizal infection rate increased (Figure 7A). Specifically, at mycorrhizal infection rates of 34.2% and 80.9%, the total As concentration in the plants decreased by 15% and 20%, respectively. The analysis also indicated a significant decrease in As concentration in shoots and roots as the mycorrhizal infection rate increased (Figures 7C, D). However, there were no significant impacts observed on the As concentration in grains (Figure 7B). Furthermore, the analysis demonstrated a significant linear positive

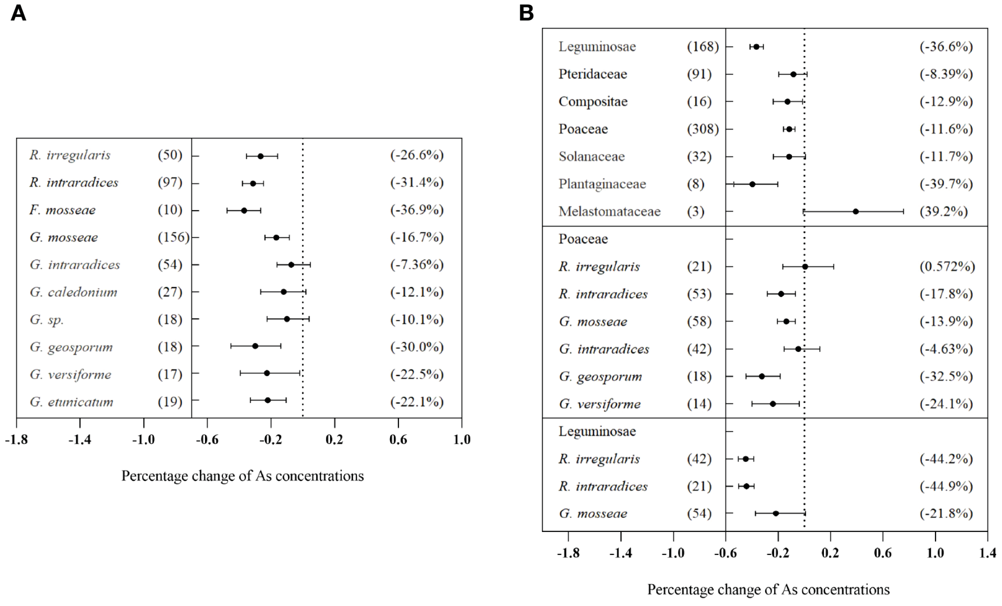

FIGURE 6
Effects of different arbuscular mycorrhizal fungi (AMF) species on As concentrations in total plants **(A)** and effects of AMF inoculation on As concentration in different plant families **(B)**.

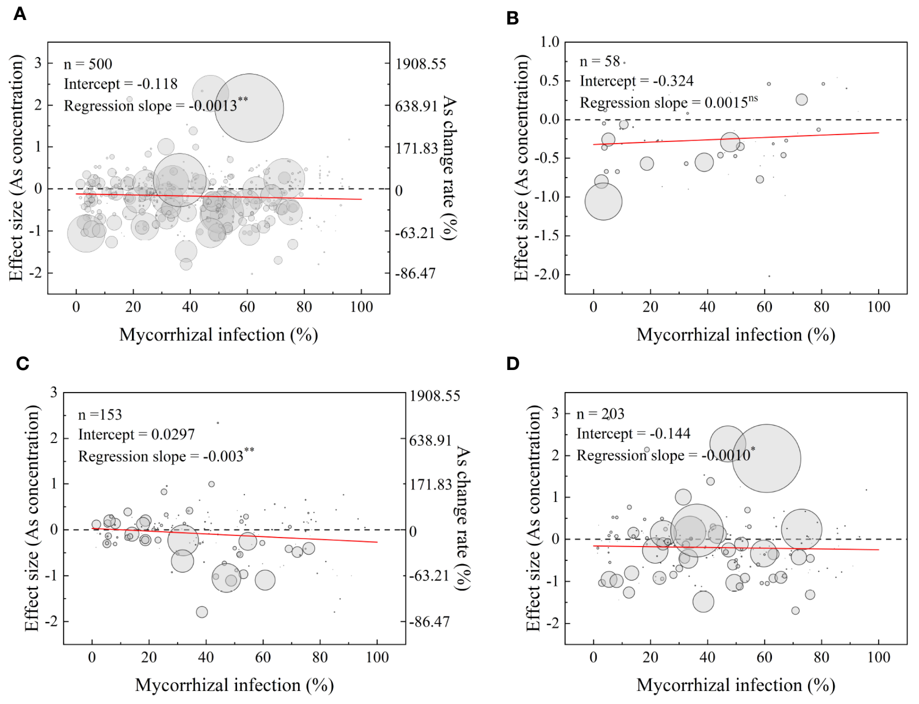

FIGURE 7
Relationships between mycorrhizal infection rate and effect size (As concentration) in plants **(A)**, grains **(B)**, shoots **(C)**, and roots **(D)**. The red lines represent the prediction of the meta-regression model. n: number of points. Significance of the model: ns, not significant; *significant at $p < 0.05$; **significant at $p < 0.01$. The right scales shown on **(A, C)** represent the values equivalent to the effect size in percentage of As concentrations change rates.

effect of mycorrhizal infection rate on the effect size of P concentration and dry biomass (Supplementary Figures S8, S9).

3.4 Effect of AMF treatment on As concentration in plants with P fertilizer

The study observed the influence of P fertilizer on the inhibitory effects of AMF on plant As accumulation. In the absence of P fertilizer, a significant reduction of 19.0% (CI: −22.3% to −15.8%) in plant As concentration was observed due to AMF. However, the presence of P fertilizer enhanced this reduction effect, resulting in a decrease of 24.7% (CI: −40.2% to −5.23%) in As concentration (Figure 5A). The effect of different P fertilizer conditions on plant As concentration showed a significant difference ($Q_b = 14.7$, $p < 0.01$). Under low P fertilizer conditions (<30 mg/kg), AMF decreased As concentration by 16.8% (CI: −37.0% to 15.3%), although the effect was not statistically significant. Interestingly, under high P fertilizer conditions (≥60 mg/kg), AMF significantly decreased As concentration by 48.9% (CI: −70.6% to −9.07%) (Figure 5A).

3.5 Effect of AMF treatment on As concentration and P/As ratio in plants under As exposure

The analysis of the fitting curve revealed that an increase in P concentration in plants resulted in a lower concentration of As in mycorrhizal plants compared to non-mycorrhizal plants (Figure 8A). Additionally, it was observed that the P/As ratio was higher in mycorrhizal plants compared to non-mycorrhizal plants (Figure 8B). These findings suggested that the AMF treatments enhanced the P accumulation and the P/As ratio in plants, consequently decreasing the As concentration. Furthermore, a significant negative correlation ($p < 0.05$) was observed between the P/As ratio and the As concentration in both total plants and grains (Figures 8C, D).

3.6 Effects of AMF on soil properties

To assess the changes in soil physical and chemical properties after AMF inoculation, we calculated the effect of soil pH, total As, easily extractable glomalin-related soil protein (EE-GRSP), and total glomalin-related soil protein (T-GRSP) in the AMF treatment group. The findings revealed that the AMF treatment had a significant effect on enhancing soil EE-GRSP and T-GRSP by 23.0% and 28.0%, respectively. However, there was no significant impact on the concentration of available As and soil pH (Figure 9).

3.7 Database and publication bias

From the extracted articles published by August 2023, a total of 76 articles met our selection criteria, and 1,362 data points were used for this meta-analysis. It was found that the fail-safe number was much higher than the threshold value (5 * N + 10, N is the number of case

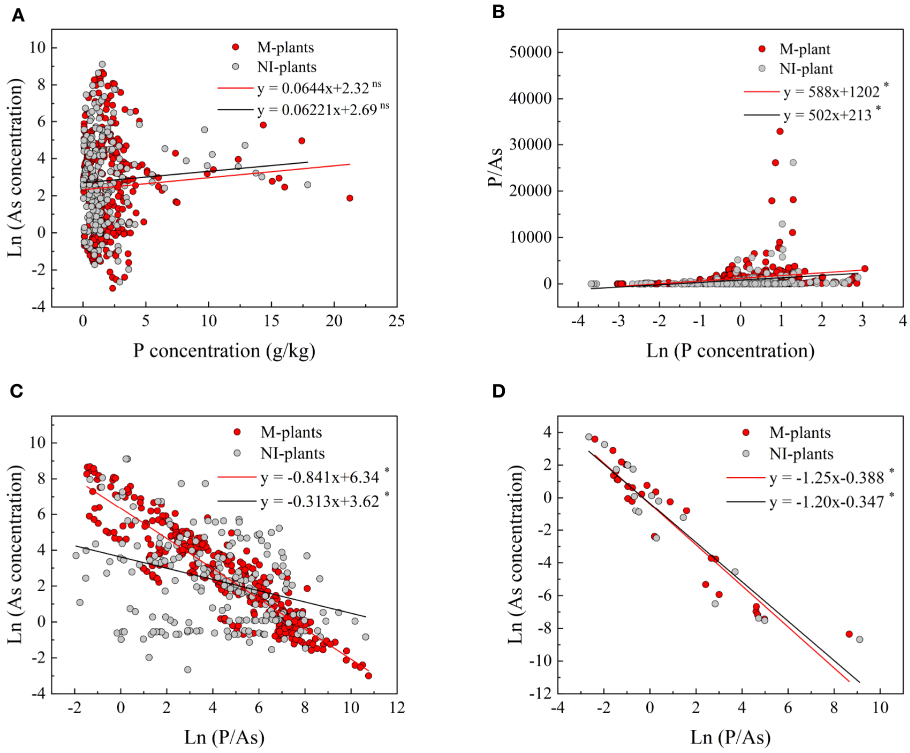

FIGURE 8
Relationships between P concentration and As concentration **(A)** and P/As ratio **(B)** in plants. Relationships between P/As ratio and As concentration in total plants **(C)** and grains **(D)**. Red dots represent mycorrhizal plants (M-plants), and gray dots represent non-mycorrhizal plants (NI-plants). ns, not significant; significant at $*p < 0.05$ and $*p < 0.01$.

studies), indicating that these results were relatively robust (Supplementary Table S1). Additionally, the sample data points in the funnel plots were evenly distributed on both sides of the funnel, indicating the absence of publication bias (Supplementary Figure S3).

4 Discussion

4.1 How much does AMF treatment decrease total As concentration in plants and grains?

The results of the meta-analyses performed on the entire dataset demonstrated significant and dose-dependent effects of AMF inoculation on decreasing As concentration in plants (Figure 7A). In addition, the analysis showed that AMF treatment significantly decreased the overall As concentration of plants by 19.3% and significantly reduced As concentration in grains by 34.1% (Figure 3A). These results revealed the potential of AMF inoculation as a promising strategy for the mitigation of As contamination in plants. A conceptual flow diagram outlining the AMF-induced reduction of As accumulation in plants has been specifically discussed in the last paragraph of the Discussion section (Figure 10). In fact, AMF reduced As accumulation while increasing P, N, K, Mg, Ca, Fe, Zn, Mn, Ni, and Se levels in grains, indicating that AMF may assist in overcoming mineral deficiencies in populations that consumed wheat-based diets, especially in As-contaminated areas (Gupta et al., 2022). AMF can play a key role in enhancing nutritional value and addressing mineral deficiencies in affected populations. Hence, AMF-based strategies were found to have the potential to provide multiple nutritional benefits in As-contaminated regions. Through the promotion of the availability and uptake of essential minerals, mycorrhizal colonization improved the nutritional quality of grains and potentially contributed to addressing mineral deficiencies for populations with wheat-based diets, especially in As-contaminated areas. However, AMF can be potentially utilized in a conventional biofortification strategy to provide appropriate levels of minerals in grains and, thereby, help to overcome mineral deficiencies for populations with grain-based diets, especially in As-contaminated areas.

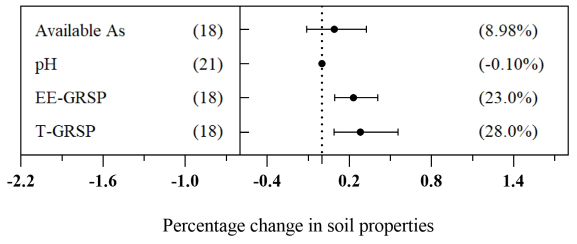

FIGURE 9
Change in soil properties induced by arbuscular mycorrhizal fungi (AMF) treatment.

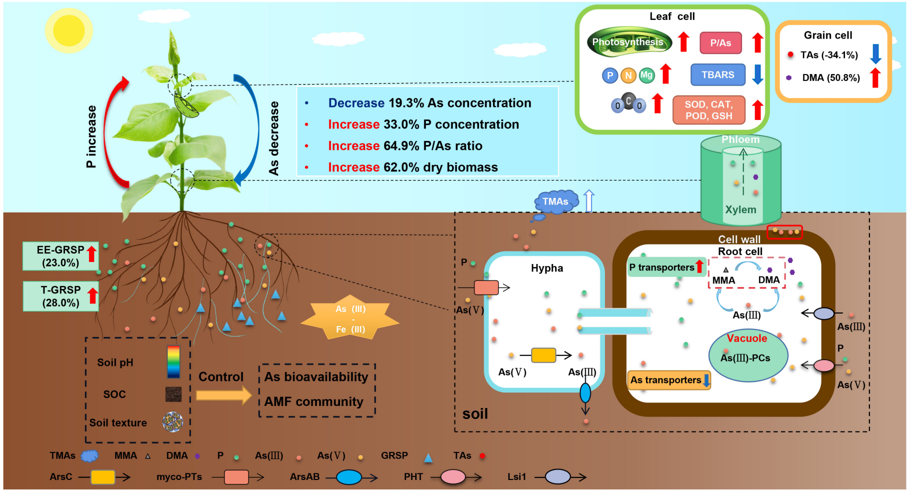

FIGURE 10
A conceptual flow diagram of the role of arbuscular mycorrhizal fungi (AMF) in regulating As bioavailability and accumulation in the plants. The red arrow represents an increase in content or effect, and the blue arrow represents a decrease.

This research revealed that AMF treatment significantly decreased As(V) concentration but presented no significant effect on As(III) concentration (Figure 3E). Research indicates that AMF inhibited As uptake through plant roots by specifically inhibiting the high-affinity transport system, which was responsible for P uptake. This inhibition ultimately decreased As(V) absorption (Chan et al., 2013; Anawar et al., 2018). Our findings were supported by previous research studies. For example, Cattani et al. (2015) reported a significant reduction in As(V) concentration in the shoots and roots of AMF-inoculated maize, while As(III) concentration remained unaffected. Similarly, Yu et al. (2009) concluded that AMF inhibited As(V) accumulation in maize shoots, significantly lowering its uptake compared to non-mycorrhizal plants. However, its effect on As(III) uptake was not statistically significant. Indeed, AMF inoculation suppressed the activities of peroxidase, superoxide dismutase, and As(V) reductase, indicating that AMF colonization can prevent As(V) reduction to As(III). Consequently, As toxicity to plants was alleviated due to the decreased conversion of As(V) to more toxic As(III). AMF inoculation was found to play a key role in mitigating As toxicity in plants (Yu et al., 2009). In addition, AMF facilitated As methylation and volatilization, increasing the concentration of DMA and other organic substances. These findings provided valuable insights into the potential of AMF in mitigating As contamination in plants and highlighted its contribution to As detoxification processes (Li et al., 2022). Organic As toxicity is generally considered to be lower than that of inorganic As (Bali and Sidhu, 2021). Inoculation of plants with AMF could also lead to a 50.8% increase in DMA concentration (Figure 3E). This finding was consistent with the results obtained by Li et al. (2016), who reported a significant DMA concentration increase in rice grains following AMF inoculation. Others have reported that AMF contributed to detoxifying microbial As through processes such as methylation and volatilization (Li et al., 2018b).

4.2 How do soil factors affect As concentration in AMF-inoculated plants?

Although our meta-analysis revealed a significant dose-dependent AMF vaccination effect on decreasing As concentration in plants (Figure 7A), relatively poor data fitting the model were explained by large differences in experimental conditions (different plant species and varieties, growing periods, soil conditions, etc.) (Figures 4–6). According to our previous research findings (Figure 4A), soil-mediated AMF with moderate SOC levels (ranging from 0.8% to 1.5%) presented the maximum ability to decrease As concentration in plants. It is noteworthy that the amount of SOC can greatly influence AMF community structure (Qin et al., 2015). Specifically, high SOC levels increased the germination of AMF spores and mycelium and ultimately impacted AMF community composition in rhizosphere soils or roots (Luo et al., 2019). However, excessive levels of soil nutrients decreased the diversity and mycorrhizal benefits of AMF, hindering mycorrhizal symbiosis and weakening its ability to inhibit As uptake (Qin et al., 2020; Ma et al., 2021).

AMF exhibited a maximum As concentration reduction effect on plants in sandy soils or soils containing ≥50% sand, accounting for a plant As concentration reduction of 21.5% (Figure 4A). In contrast to sandy soils, non-sandy soils possessed greater adsorption capacities for As, and As was stabilized in the soil, which resulted in the reduction of its fluidity and solubility (Suriyagoda et al., 2018). Therefore, AMF inhibitory effect on As uptake was much more apparent in non-sandy soils. By increasing soil pH, AMF significantly reduced As concentration in host plants, which became especially pronounced under weak alkaline conditions (Figure 4A). As uptake by plants mainly depends on As bioavailability in soil (Kumarathilaka et al., 2018). As solubility and availability were increased as soil pH increased, leading to the release of large amounts of As in weakly alkaline soils (Yao et al., 2022). However, soil pH was the main environmental factor affecting the composition of the AMF community (Qin et al., 2015). Acidic soils inhibited AMF growth and spore germination, hindering AMF function (Liu et al., 2020). Therefore, we can conclude that the mycorrhizal effect was more pronounced in alkaline soils.

AMF exhibited the maximum reduction effect on the As concentration in plants at a medium As level. However, at higher levels of As in the soil, this inhibitory effect was weakened (Figure 4A). High concentrations of heavy metals and metalloids in soils can be toxic to plants, bacteria, and fungi (Parvin et al., 2019). Although AMF inoculation improved the tolerance of plants to As exposure, excessive concentrations of As in soils decreased the AMF colonization rate, negatively affecting their physiological activities and decreasing the host plant's resistance to As symbiosis (Zhang et al., 2020). The physical and chemical properties of soils had significant effects on the AMF community, which in turn affected the germination of AMF spores and infection of hyphae, ultimately alternating As absorption and accumulation processes in plants (Tian et al., 2017; Xue et al., 2018). Strong interactions can occur between soil physicochemical properties and AMF. Therefore, further research is required to understand and optimize soil conditions under which AMF inoculation can have the most significant impact on the reduction of As concentration in plants and ecosystem protection. Meanwhile, further research is required to optimize AMF inoculation techniques, investigate genotype-specific responses, and evaluate long-term effects of AMF on As dynamics in different soil environments.

4.3 How do AMF inoculation methods modify As concentration in plants?

Our research revealed that several factors can influence the AMF effect on As concentration of plants. These factors included AMF inoculation species (Figure 6A), inoculation timing (Figure 5A), AMF colonization rate (Figure 7A), and host plants (Figure 6B). This was because various AMF species had distinct morphological properties, nutritional status, symbiotic efficiencies, and gene expression patterns during symbiotic interactions with plants. This research revealed a very interesting phenomenon: the interaction of AMF and crop plants affected As concentration. Maximum As reduction effects were obtained by *G. geosporum* and *R. intraradices* in Poaceae and Leguminosae (Figure 6B). From a food safety point of view, a significant reduction in As concentration of grains was highly desirable. This research also showed that certain varieties of Leguminosae exhibited significant suitability for AMF inoculation. Considering the significant importance of Leguminosae as a major crop plant, it was necessary to prioritize efforts aimed at decreasing As absorption and concentration in grains, specifically through AMF treatments, to improve plant health and decrease potential risks associated with As exposure.

Interestingly, the inhibitory effect of single AMF inoculation on total As levels in host plants was stronger than that of mixed AMF inoculation (Figure 5A). Chan et al. (2013) showed that rice grains inoculated with *G. mosseae* alone had remarkably lower total As concentrations than those inoculated with both *G. versiforme* and *G. mosseae*. Therefore, this observation suggested that fungi competed in roots and that one infection unit can prevent adjacent fungal infections, thereby avoiding secondary infections (Hepper et al., 1988; Buil et al., 2022). However, mixed inoculation presented a synergistic effect, which increased biomass and P concentration (Figures S4, S6). In fact, *Pteris vittata* co-colonized by both indigenous mycorrhizas and *G. mosseae* contained higher P concentrations than those colonized by either of the two AMFs (Leung et al., 2013).

Time plays a very important role in the biological process of AMF–plant symbiosis. Inhibitory effects of AMF on As concentration in host plants were gradually weakened with time (Figure 5A). This observation was consistent with the findings of Li et al. (2013). The colonization rates of the two rice cultivars exhibited significant decreases, with colonization rates ranging from 12% to 23% on day 7, from 7.3% to 11% on day 35, and from 1.3% to 4.9% on day 63. Correspondingly, shoot As concentrations appeared to be decreased on D63 when compared to D35 ($p < 0.05$) (Li et al., 2013). Initially, it was expected that longer experiments would promote the enhanced development of symbiosis, especially in situations where resources such as rooting space and nutrients were decreasing (Schroeder and Janos, 2004). However, contrary to these expectations, our findings showed that long duration levels (≥112 days) of the experiment did not result in higher mycorrhizal effects compared to short and intermediate experiments. Additionally, there was no significant difference among the three duration levels (Figure 5A). This is likely because As does not become limiting over time. Additionally, the results of the meta-analysis indicated a significant, dose-dependent effect of AMF infection rate on the reduction of As concentration in plants (Figure 7A). The duration of an experiment has a significant impact on the determining of AMF-mediated As tissue concentrations. Luo et al. (2017) found that the AMF colonization rates fluctuated with growth stages, reaching their peak at the jointing stage and then decreasing at flowering and ripening stages, but flowering and ripening stages were critical periods for AMF to limit grain Cd uptake. Wang et al. (2012) also observed a similar trend in alfalfa (*Medicago sativa* L.) that the root colonization rates by *R. intraradices* increased from 17% at day 25 to 69% at day 60 and then decreased to 43% after 80 days. This suggests that AMFs constitute an important functional component of the soil–plant system and the mechanisms cannot be explained by root colonization rates simply. Inoculation with *G. intraradices* at planting did not result in a higher root mycorrhizal colonization level than that found in non-inoculated control plants at the end of 18 months in the field. This highlights the presence of competitive processes between the natural AMF taxa and the introduced AMF strain that occurred over 18 months (Bissonnette et al., 2010). Based on the published literature, the AMF–plant symbiotic system generally exhibits ecological functions throughout the entire life span of a plant. Meanwhile, different symbiotic systems have varying time periods during which they exhibit maximum functionality. As the plant's life cycle comes to an end, the colonization rate of AMF significantly decreases. However, it should be noted that the time period during which AMF exerts maximum functionality does not necessarily coincide with the time of maximum colonization rate.

Although we have obtained the above general conclusion, we must acknowledge that the colonization rate alone cannot adequately explain the mechanism of AMF tolerance to As. Feddermann et al. (2010) suggested that AMF inoculated with different host plants may possess different nutritional status, symbiotic efficiency, and gene expression patterns leading to distinct colonization rates. Liu et al. (2005) found that the shoot As concentration of rice with AMF inoculation decreased at lower rates of As application to the soil (<50 mg/kg), with the opposite trend at higher soil As levels. There was a break point at the added As level of 50 mg/kg at which the lowest proportion of As was distributed in the shoots (Liu et al., 2005). Long et al. (2021) also found that when adding 10 g/kg iron tailings (IT), AMF significantly increased root As concentration ($p< 0.05$), while at 40 g/kg iron tailings (IT), AMF decreased As concentration in shoots by reducing As absorption efficiency. This suggests that soil arsenic levels also influence AMF-mediated uptake and translocation of As in host plants. In fact, the effect of As concentration in plants mediated by AMF symbiosis was influenced by many factors, including plant species (AbdElgawad et al., 2022), plant growth stage (Chen et al., 2006), AMF species (Bhalla and Garg, 2021), physical and chemical properties of soil (Coutinho et al., 2015), nutritional status (Hajiboland et al., 2009), and soil amendment Bissonnette. Our findings also supported this viewpoint (Figures 4–6). The relatively poor fits of the data to the model were explained by the large difference in experimental conditions (different plant species and varieties, AMF species, growing period, soils, etc.) of the 76 studies that matched the selection criteria (Figure 7A). The contradictions in the literature regarding the effects of AMF on plant As response can be attributed to the complex soil–AMF–plant mechanisms involved, which cannot be solely evaluated based on root colonization rates.

4.4 How do P fertilizers and AMF modify As concentration in plants?

Our meta-analysis presented compelling quantitative evidence demonstrating a significant decrease in As concentration with the increase of the P/As ratio in the plant. In addition, a significant negative correlation ($p< 0.05$) was observed between the P/As ratio and As concentration in total plants and grains (Figures 8C, D). This finding was important since P competed with As for plant uptake, limiting As absorption and translocation in plants (Li et al., 2016). The symbiotic relationship between AMF and plants can effectively reduce As influx into roots and enhance the accumulation of plant nutrients, resulting in a "dilution effect" on As in plant tissues. Consequently, As concentration in crops was effectively decreased, mitigating the risk of As exposure to humans through dietary intake (Cheema and Garg, 2022). In addition, AMF facilitated As translocation from the host plants to their hyphae, enabling the fungi to effectively remove As from plant systems and prevent its accumulation in host tissues. This translocation process further helped to decrease As concentration within plants (Li et al., 2018a). However, under low and medium P supply conditions (P levels of below 60 mg/kg), AMF inoculation did not significantly affect As levels in plants (Figure 5A). This finding was interesting because it suggests that the AMF effect on decreasing As concentration in plants depended on the availability of P in soil. In other words, when P was limited, AMF might be unable to fully exert its potential in decreasing As accumulation. However, under high P conditions (P levels equal to or above 60 mg/kg), AMF inoculation was found to significantly decrease As concentration in plants (Figure 5A). This result showed that when plants had sufficient P supply, AMF could effectively increase the uptake and utilization of P, improving plant growth and diluting As accumulation. This finding highlighted the importance of considering P availability in soil when implementing AMF as a strategy for mitigating As contamination. Optimizing soil P levels through fertilization or soil amendment can potentially improve AMF effectiveness in decreasing As accumulation in plants.

AMF inoculation alleviated As toxicity to plants through two mechanisms. First, it upregulated the levels of low-affinity P transporters, thereby improving P absorption efficiency and assisting host plants in acquiring more P (Paszkowski et al., 2002). Second, it downregulated the expression levels of high-affinity P transporters on the root surface and hair, decreasing As uptake (Gupta et al., 2022). Both of the above mechanisms contributed to As toxicity mitigation in plants. Overall, the shoot P/As ratio served as a valuable indicator for evaluating the beneficial effects of AMF species in enhancing As tolerance in various plant species (Mitra et al., 2022). In fact, the shoot P/As ratio was found to be a critical indicator for investigating As concentrations in both plants and grains (Figures 8C, D). In addition, the shoot P/As ratio can help identify plants or varieties exhibiting higher As tolerance or lower As accumulation, assisting in breeding programs and agricultural practices aimed at minimizing As uptake in crops.

4.5 What are the general mechanisms of AMF regulation on the bioavailability and accumulation of As in plants, as proposed in literature and presented data?

This research showed that inoculating AMF significantly enhanced the concentrations of EE-GRSP and T-GRSP in As-contamination soils (Figure 9), which was consistent with the findings of previous studies (Li et al., 2018a; Zhang et al., 2020). GRSP, a glycoprotein produced by AMF in soil, has been extensively recognized for its ability to bind to toxic metals, thus contributing to metal stabilization (Chen et al., 2022). Previous research studies have revealed a significant correlation between GRSP and mycorrhizal root volume (Bedini et al., 2009). Others have observed that higher glomalin contents corresponded to a greater expansion of AMF extraradical hyphae (Zhang, H. et al., 2016). This observation suggested that increased GRSP contents might contribute to longer AMF extraradical hyphae and faster turnover of those hyphae compared to no-inoculation treatment (Zhang et al., 2020). In fact, this consequence explained the potential reasons why AMF inoculation enhanced plant tolerance to toxic As in soils. However, further research is still required to determine whether glomalin can directly bind to As, decrease As bioavailability in soil, and subsequently decrease As uptake by plants.

Finally, by integrating previous analysis results and literature, we outlined a conceptual flow diagram depicting the role of AMF in regulating the bioavailability and accumulation of As in plants (Figure 10). Figure 10 shows the potential of AMF–plant symbiosis systems to enhance dry biomass, increase P concentration, and subsequently decrease As concentration in plants. Tolerance of host plants to toxic As mainly relies on As uptake reduction in AMF plants, which is accomplished through different mechanisms, including the following.

1) Downregulation of As transporters: As(III) is taken up by plants through the silicon transporter (Lsi1), and As(V) is taken up and transported by phosphate transporters (PhTs) (Ma et al., 2008; Zhao et al., 2009). Inoculation with AMF led to the downregulation of *Lsi1* and *Lsi2* in rice roots, resulting in reduced As(III) uptake and transport (Chen et al., 2012). In rice, inoculation with AMF downregulated the expression of several *OsPT* genes (*OsPT1–3*, *OsPT6*, and *OsPT9–10*) (Chen et al., 2013).
2) Upregulation of P transporters: Inoculation with AMF significantly enhanced P nutrition in plants and limited As uptake through the upregulation of the AM-induced *PhT* gene, *MsPT4* (Li et al., 2018a). Additionally, the activity of *Pht1;5* and *Pht1;6* increased and exhibited higher selectivity toward P than As, effectively reducing the absorption of As in AMF symbiosis (Christophersen et al., 2012).
3) Sequestration, methylation, and volatilization of As: Mycorrhizal association can lead to involvement in sequestration (storage), deposition (in external mycelium), chelation (form stable metal–organic complexes), excretion [As(III) excretion to external media], methylation (conversion to less toxic forms), and volatilization (release into the air) of As (Liu et al., 2005; Li et al., 2016; Li et al., 2018a, b; Xing et al., 2024). These processes helped in decreasing the overall As load in plants.
4) Improved physiological function: AMF can mitigate the oxidative stress induced by As, enhance the activity of the antioxidant enzyme system, and improve photosynthesis. By reducing the production of hydrogen peroxide (H_2O_2) and lipid peroxidation, AMF effectively countered oxidative damage in host plants (Shukla et al., 2023; Zhou et al., 2023). This symbiotic relationship significantly increased the activity of essential antioxidant enzymes, including catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) (Cheema and Garg, 2022, 2024; Mitra et al., 2022). AMF facilitated the restoration of pigment levels, including chlorophyll *a*, chlorophyll *b*, and carotenoids, and enhanced photosynthetic efficiency, transpiration rates, and water use efficiency under conditions of As stress (Zhang et al., 2022; Shukla et al., 2023).
5) Increased the accumulation of nutrients: AMF not only facilitated the enhanced uptake of vital macronutrients but also significantly boosted the accumulation of critical nutrients, including nitrogen (N), P, potassium (K), calcium (Ca), and magnesium (Mg), in the plant grains. By increasing the host plants' biomass, AMF also contributed to a reduction in the As concentration through a dilution effect, effectively mitigating the toxic effects of As exposure (Gupta et al., 2022).

To obtain a comprehensive understanding of the mechanisms applied by AMF to improve As tolerance in plants, further biochemical and physiological characterization is necessary. This knowledge will guide strategies to improve plant resilience to As contamination and contribute to developing sustainable and effective solutions for the management of As in agricultural systems.

5 Conclusions and prospects

Meta-analysis of 1,362 data records retrieved from 76 published studies evaluated the effects of various abiotic and biotic factors on AMF-related As accumulation in plants by subgroup and regression analyses. The findings of this research were as follows: AMF infection significantly lowered plant As levels in a dose-dependent manner ($p< 0.05$) and enhanced total P concentration and plant dry weight. Notably, As(V) levels decreased and DMA levels increased with AMF inoculation, but As(III) levels were unaffected. Single AMF inoculation was more effective at reducing As in plants than mixed inoculation. The optimal soil conditions for AMF are a SOC range of 0.8%–1.5% and a pH of ≥7.5 for maximum As reduction in plants. AMF inoculation increased the P/As ratio in plants, with a significant negative correlation ($p< 0.05$) between this ratio and As grain concentration. Among common AMF species, *R. intraradices* and *F. mosseae* were effective in reducing As in plants, with *R. intraradices* showing particular promise in Leguminosae plants, making them strong candidates for further research. AMF also increased EE-GRSP and T-GRSP concentrations in As-contaminated soils, offering a potential strategy to reduce As exposure and intake through the human diet, thereby enhancing human health.

For a further understanding of how AMF affects As accumulation in plants, future research should focus on 1) conducting field trials to optimize AMF use in biofortification, considering crop–AMF interactions, inoculation methods, soil properties, agricultural practices, and AMF's long-term impact on As dynamics; and 2) investigating the mechanisms of AMF–

plant synergy in As tolerance, including variability in AMF efficacy, environmental interactions, underlying metabolic pathways or signaling molecules, and key enzymes or proteins. Further in-depth research is needed to comprehensively understand the mechanisms by which AMF improves the As tolerance of plants.

Author contributions

SH: Data curation, Software, Visualization, Writing – original draft. YT: Data curation, Software, Visualization, Writing – original draft. ZL: Writing – review & editing. LX: Data curation, Software, Writing – original draft. XZ: Conceptualization, Visualization, Writing – review & editing. GB: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2024.1327649/full#supplementary-material

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The coexistence of arbuscular mycorrhizal fungi and dark septate endophytes synergistically enhanced the cadmium tolerance of maize

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Introduction: Arbuscular mycorrhizal fungi (AMF) and dark septate endophytic fungi (DSEs) generally coexist in the roots of plants. However, our understanding of the effects of their coexistence on plant growth and stress resistance is limited.

Methods: In the present study, the effects of single and dual inoculation of AMF and DSE on the growth, photosynthetic physiology, glutathione (GSH) metabolism, endogenous hormones, and cadmium (Cd) content of maize under 25 mg•kg^{-1} Cd stress were investigated.

Results: Compared with that after the non-inoculation treatment, AMF+DSE co-inoculation significantly increased the photosynthetic rate (Pn) of maize leaves; promoted root GSH metabolism; increased the root GSH concentration and activity of γ-glutamyl cysteine synthase (γ-GCS), ATP sulfatase (ATPS) and sulfite reductase (SIR) by 215%, 117%, 50%, and 36%, respectively; and increased the concentration of endogenous hormones in roots, with increases in zeatin (ZR), indole-3 acetic acid (IAA), and abscisic acid (ABA) by 81%, 209%, and 72%, respectively. AMF inoculation, DSE inoculation and AMF+DSE co-inoculation significantly increased maize biomass, and single inoculation with AMF or DSE increased the Cd concentration in roots by 104% or 120%, respectively. Moreover, significant or highly significant positive correlations were observed between the contents of ZR, IAA, and ABA and the activities of γ-GCS, ATPS, and SIR and the glutathione (GSH) content. There were significant or highly significant positive interactions between AMF and DSE on the Pn of leaves, root GSH metabolism, and endogenous hormone contents according to two-way analysis of variance. Therefore, the coexistence of AMF and DSE synergistically enhanced the Cd tolerance of maize.

KEYWORDS

endophytic fungi, cadmium stress, glutathione metabolism, endogenous hormone, synergistic effect

1 Introduction

Mining, metal smelting, chemical production, pesticide application, fertilizer application, and other production activities have aggravated soil heavy metal pollution in industrial and mining areas (Veltman et al., 2008). For example, soil cadmium (Cd) pollution is a serious problem in China according to the National Soil Pollution Survey Bulletin (Chen et al., 2019). Plants absorb and accumulate Cd in their organs, which affects their physiological and metabolic processes of plants, such as photosynthesis (Dias et al., 2013), glutathione metabolism, and endogenous hormones (Wang et al., 2012), and inhibits their plants.

Arbuscular mycorrhizal fungi (AMF) and dark septate endophytes (DSE) are widely distributed in natural ecosystems and colonize plant roots in Cd-polluted soils (Liang et al., 2007). The AMF forms a mycorrhizal association with approximately 80% of plants in terrestrial ecosystems (Aseel et al., 2019). and are beneficial to the growth of host plants via increasing nutrient absorption and improving host photosynthesis (Farzaneh et al., 2011). Under Cd stress, AMF increases the net photosynthetic rates of host plants (Zhang et al., 2017), promotes GSH metabolism in crops, alleviates the hormone imbalances caused by Cd stress (Zhan et al., 2017) and reduces the toxic effects of Cd stress on plants (AbdaAllah et al., 2015). AMF colonization in rice roots helped to improve rice tolerance to metals by stabilizing the Cd content in the rhizosphere and the Zn content in the root tissues where the fungal hyphal ramification was prominent (Janeeshma and Puthur, 2022). AMF promoted Cd binding to plant roots, inhibited Cd transport to leaves, and reduced Cd concentrations in leaves (Gu et al., 2017).

DSEs can also increase the nutrient acquisition of host plants, promote plant growth (Wang et al., 2016), resist Cd toxicity, and enhance metabolic activity against Cd stress (Selosse et al., 2004; Gadd, 2007). DSEs enhance plant adaptability to Cd by regulating GSH metabolism (Pan et al., 2016) and plant hormones concentrations in host plants (Singh et al., 2015). Additionally, the DSE promoted the accumulation of Cd in plant roots and reduced the migration of Cd from roots to leaves, which reduced the Cd content in leaves (Zhang et al., 2013).

Notably, AMF and DSEs colonize plant roots simultaneously (He et al., 2017). Some studies have investigated the effects of their co-inoculation on plant growth and physiology (Khaekhum et al., 2021). Single inoculation or co-inoculation of AMF and DSEs increased the maize biomass, mineral nutrient content, and growth under Cd stress (He et al., 2019). A single inoculation of AMF and co-inoculation of AMF+DSE significantly increased the biomass of maize, promoted Cd retention in roots, and reduced the Cd content in shoots (He et al., 2020). Thus, AMF and DSEs coexist in plant roots and promote plant resistance under adverse environmental stress (Bi et al., 2019). However, the physiological mechanism by which the co-inoculation of AMF and DSE enhances the Cd tolerance of plants is still unclear.

To determine whether AMF and DSE coexist in plant roots under Cd stress, the present study investigated the effects of single and double inoculation with AMF and DSE on maize growth, photosynthesis physiology, root GSH metabolism physiology, root endogenous hormone contents, and plant Cd content. The relationships between the growth physiology and Cd content in maize were analyzed. We hypothesized that the coexistence of AMF and DSEs in roots would synergistically enhance the Cd tolerance of the host plant by improving the photosynthetic physiology in leaves, endogenous hormone contents and GSH metabolism in roots.

2 Materials and methods

2.1 Materials tested

The soil type was mountain red soil, which was naturally dried indoors and then passed through a 2 mm sieve. The chemical properties of the soil were as follows: pH, 6.35; organic matter content, 13.87 $g\cdot kg^{-1}$; total nitrogen, phosphorus, and potassium contents, 0.112, 0.32, and 1.98 $g\cdot kg^{-1}$, respectively; alkali-hydrolyzable nitrogen, available phosphorus and available potassium contents, 26.18, 1.15 and 33.74 $mg\cdot kg^{-1}$, and soil Cd content, 0.275 $mg\cdot kg^{-1}$. Moreover, the sands were also screened through a 2-mm sieve after natural air drying. The soil and sand were fully mixed at a ratio of 3:1 (w/w) of sand to soil. Then, a mixture of sand and soil was added with $CdCl_2\cdot 2.5\ H_2O$ solution to achieve a Cd stress level of 25 $mg\cdot kg^{-1}$, which was balanced for 2 weeks before use in the pot experiment.

The AMF used in the experiment was *Funneliformis mosseae* (*F. mosseae*) BGC YN05 1511C0001BGCAM0013, which was provided by the Institute of Plant Nutrition and Resources, Beijing Academy of Agricultural and Forestry Sciences. The AMF inoculants (including spores, mycelia, sand mixtures and infected root segments, etc.) were obtained by the propagation of potted maize in the laboratory, and there were 30 spores per gram of inoculant. The DSE strain (*Exophiala pisciphila* (*E. pisciphila*) ACCC32496) was isolated from the root of *Arundinella bengalensis* (Spreng.) Druce naturally grows in the Huize lead-zinc mine area, in Yunnan Province. It was preserved at a low temperatures (4°C) in the laboratory. The DSE fungus was inoculated on the potato dextrose agar (PDA) medium, and cultured at 28°C for 2 weeks to activate the strain.

The trial maize (*Zea mays* L.) was Huidan No. 4. After surface disinfection with 10% hypochlorous acid (soaked for 2 min) and 75% ethanol (soaked for 1 min), the maize seeds were placed for 3 days in a constant temperature incubator at 28°C for germination. After the seeds germinated and sprouted, the unpolluted and healthy seedlings were selected for subsequent use.

2.2 Pot experiment

The establishment of the maize-DSE symbiont: A cylindrical glass bottle with 20 cm (height) and 6.5 cm (diameter) in height was used, in which 16.5 g of perlite and 20 mL of Hoagland nutrient solution were added. The device was autoclaved at 121°C for 30 min. For the DSE-inoculated treatment, 15 pieces of DSE colonies (2 mm diameter) from a potato dextrose agar (PDA) culture were added to each of the eight glass bottles. After mixing well with perlite, 2 maize seeds were added, covered with sterile sand to a thickness of 1 cm and sealed with sterile polyvinyl alcohol film (PVA). During the growth of maize plants, maize roots grew and attached to DSE colonies, therefore the DSE mycelium infested with maize roots. For the no-DSE-inoculated treatment, 15 autoclaved fungal disks were used, and the maize seedlings were placed in glass bottles. The maize seedlings were cultivated for 14 days at 25°C under light conditions of 1000–8000 lux for 10 hours per day. Some maize plants were sampled and examined under a the microscope to confirm the successful DSE colonization of DSE in the roots.

For the indoor pot experiments, plastic pots with a height of 20 cm, an upper diameter of 25 cm, and a bottom diameter of 21 cm were sterilized with 75% ethanol. Then, 5 kg of sterilized soil-sand mixture was added, and two maize seedlings were planted in each pot. The pot experiment was performed in a completely randomized design with the following variables: one Cd concentration (25 $mg \cdot kg^{-1}$), 4 treatments and 6 replicates in each treatment, for a total of 24 pots. The four treatments were as follows: the control treatment (CK) consisted of no AMF or DSE inoculation, AMF inoculation, DSE inoculation, and AMF+DSE co-inoculation. In the CK treatment, maize plants were not inoculated with DSE or AMF. For the AMF inoculation treatment, the maize plants without DSE colonization were inoculated with 50 g of AMF inoculants (approximately 1500 spores per pot) without DSE. For the DSE inoculation treatment, the maize plants were cultivated in glass bottles with DSE colonization, and an equal amount of sterilized AMF inoculants was added. For the AMF+DSE co-inoculation treatment, the maize plants with DSE colonization were added with 50 g of AMF. Each treatment had 6 pots, and one maize plant was planted per pot.

After planting, 150 mL of 50% Hoagland nutrient solution was added to the maize plants every 3 days. The soil moisture was kept constant at 55% by weighing and replenishing the water.

2.3 Determination of maize biomass and photosynthesis in maize leaves

At approximately 12:00 p.m., the photosynthetic rate (Pn), transpiration rate (Tr), stomatal conductance (Gs), and intercellular CO_2 concentration (Ci) in the maize leaves were measured using an LCA-4 photosynthetic tester (Analytical Development Co., Ltd., ADC, Hoddesdon, England). The maize was harvested after photosynthetic index measurement, and other indices were measured. For each treatment, the photosynthetic physiological parameters were collected from the fourth leaf from the top of maize plant.

After being planted for sixty days, the maize plants were harvested and divided into leaves and roots. The plants were washed with distilled water, dried in a 105°C oven (Model 101, Beijing Yongguangming Instrument Co., Ltd, Beijing, China) for 0.5 h and then dried in a 75°C oven for 48 h to a constant weight, after which their dry biomass was weighed.

2.4 Determination of AMF and DSE colonization in roots

The AMF spores in the maize rhizosphere soils were separated via wet sieve decantation-sucrose centrifugation, and the number of spores was observed and calculated under a microscope (Daniels and Skipper, 1982).

Some fresh roots were randomly sampled, washed with water, and then cut into fragments approximately 1 cm in length. First, these root fragments were placed into a centrifuge tube, with 10% KOH solution, incubated in a water bath at 90°C for 90 min, and then washed with water. Second, they were acidified and dissociated by lactic acid acidification at room temperature for 5 min, washed with water. Third, they were stained with 0.05% Tepan blue staining solution, at 90°C for 30 min and washed with water. Finally, the plants were decolorized with a decolorization solution at room temperature for 1 d. Ten root fragments were randomly selected 10 root segments were placed on slides, and sealed with 50% glycerol (Chen et al., 2021), and observed under a microscope. The colonization rates of AMF and DSE were calculated using the intersection method (McGonigle et al., 1990).

2.5 Determination of glutathione metabolism in maize roots

For sample preparation, fresh roots (0.1 g) were weighed 0.9% saline solution (2 mL) was added, and the roots were ground into a homogenate using a high-throughput tissue grinder (Scientitz-48, Shanghai Shengke Instrument Equipment Co., Ltd., Shanghai, China). These roots were transferred to a 2-mL centrifuge tube and placed in a high-speed freezing centrifuge (HC-3018R, Anhui Ustc Zonkia Scientific Instrument Co., Ltd., Anhui, China). Then, the samples were centrifuged at 12000 ×g for 30 min, and the supernatant was collected for analysis.

The glutathione (GSH) content was determined using the method described in the GSH test kit produced by Nanjing Jiancheng Bioengineering Institute, according to previous methods (Wang et al., 2023). The supernatant obtained by centrifugation was used for the determination of the GSH content by measuring the absorbance at 405 nm.

Glutathione reductase (GR) and γ-glutamylcysteine synthetase (γ-GCS) activities were determined using the methods described in the GR and γ-GCS test kits produced by Nanjing Jiancheng Bioengineering Institute, according to previous methods (Zhan et al., 2017). One unit of GR activity is equal to the oxidation of 1 nmol of NADPH per minute. The γ-GCS activity was defined as the amount of enzyme required to consume 1 μmol of NADH per minute.

ATPS (ATP sulfatase), OAS-TL (O-acetyl serine (thiol) lyase), and SIR (sulfite reductase) activities were measured using the plant ATPS kit, OAS-TL kit, and SIR kit, respectively, produced by Shanghai Jingkang Bioengineering Co., Ltd. Enzyme-linked immunosorbent assay (ELISA) with double antibodies was performed using kits. The specimen, standard, and HRP-labeled detection antibody were added to the coating micropores, which were coated with plant ATPS, OAS-TL, and SIR capture antibodies in advance, incubated, and thoroughly washed. The substrate 3, 3', 5,5'-tetramethylbenzidine (TMB) was used for color development. TMB is converted to blue under the catalysis of peroxidase and finally to yellow under the action of acid. The depth of color positively correlated with the plant ATPS, OAS-TL, and SIR in the samples. The absorbance (OD) was measured at 450 nm and the concentrations of ATPS, OAS-TL, and SIR in the samples were calculated from the standard curve.

2.6 Determination of endogenous hormones in maize roots

Maize roots (2.0 g) were sampled and cleaned thoroughly with water, put into a mortar with 5 mL of 80% ice methanol and ground to a homogenate for 10 min. The homogenate was transferred to a conical flask (100 mL) 20 mL of 80% ice methanol was added, and the mixture was shaken well and incubated at 0°C for 40 h. Then, the homogenate was filtered and concentrated to 10 mL under reduced pressure. The concentrations of zeatin (ZR), gibberellic acid (GA), indole acetic acid (IAA), and abscisic acid (ABA) in the homogenate were measured by high-performance liquid chromatography (HPLC, Thermo Fisher Scientific Ultimate 3000, Shanghai Lijing Scientific Instrument Co., Ltd.) A Diamonsil C18 column (4.6 mm×250 mm, 5 μm) was used as the chromatographic column, and methanol and water formed the mobile phase. The flow rate and temperature were set at 1.0 $mL\cdot min^{-1}$ and 25°C, respectively, and the injection volume was 10 μL. The detection wavelengths of ZR, GA, IAA, and ABA was at 254 nm (Wei et al., 2013). The retention times of ZR, GA, IAA, and ABA in the standard samples were 5.645, 4.366, 6.514, and 11.942 min, respectively.

2.7 Determination of Cd content in maize

The dried shoot and root samples (0.1 g) were weighed, ground into powder and digested using the nitric acid-perchloric acid (4:1) via the wet-digestion method. The digestion mixture was transferred to a volumetric flask and diluted to 50 mL with distilled water. The Cd concentration in solution was determined using flame atomic absorption spectrophotometry (TAS-990, Beijing Puxi Instrument Factory, Beijing, China). The appropriate quality control was determined using $CdCl_2$ as the standard solution. The Cd content ($mg\cdot kg^{-1}$) the plants was calculated according to the formula by Bao (Bao, 2000). The Cd accumulation in maize shoots or roots was equal to the Cd content in shoots or roots multiplied by their biomass.

2.8 Data analysis

The data are presented as the average of 6 replicates with the average standard deviation. The data figures were drawn using Origin Pro 9.0 and statistically analyzed using SPSS 25.0. The significance of the difference at 0.05 was detected using least significant difference (LSD). Two-way Analysis of Variance (ANOVA) was used to evaluate the effects of AMF and DSE inoculation on maize growth physiology and the interaction between AMF and DSE. The correlation coefficients between endogenous hormone content in maize roots and GSH metabolism and photosynthesis physiology in shoots of maize were analyzed by Pearson correlation.

3 Results

3.1 Colonization of maize roots by AMF and DSEs

In the single AMF or DSE inoculation and AMF+DSE co-inoculation treatments, AMF or DSE structures such as hyphae and spores were observed in the maize roots. However, these compounds were not detected in the roots of the maize plants in the non-inoculation treatment (CK).The number of AMF spores in the AMF inoculation treatment was greater than that in the AMF +DSE co-inoculation treatment. There was no significant difference in the AMF colonization rate between AMF inoculation and AMF +DSE co-inoculation treatments, and there was no significant difference in the DSE colonization rate between DSE inoculation and AMF+DSE co-inoculation treatments. These results indicated that AMF and DSE successfully established a symbiotic relationship with maize (Table 1).

3.2 Effects of AMF and DSEs on maize growth

Compared with the CK treatment, the DSE inoculation and AMF+DSE co-inoculation treatments significantly increased the

TABLE 1 Spore number of AMF and root colonization of AMF and DSE.

Treatment	Spore number of AMF (n/g)	AMF colonization rate (%)	DSE colonization rate (%)
CK	—	—	—
AMF	8.6 ± 0.44 a	49.82 ± 2.58 a	—
DSE	—	—	28.51 ± 4.85 a
AMF+DSE	7.1 ± 0.65 b	53.15 ± 2.21 a	25.48 ± 2.29 a

CK, the control of non-inoculation; AMF, *Funneliformis mosseae* inoculation; DSE, *Exophiala pisciphila* inoculation; AMF+DSE, co-inoculation of *F. mosseae* and *E. pisciphila*. "—" indicates the index of the material was not detected. The different lowercase letters in a column indicate significant differences among treatments at $p<0.05$ level.

biomass of maize leaves by 26% and 15%, respectively. The DSE-inoculation treatment also significantly increased the root biomass. According to the two-way ANOVA,DSE significantly increased maize biomass, but there was no interaction between AMF and DSE on maize biomass (Figure 1). These results indicated that the single inoculation with DSE and co-inoculation with AMF+DSE promoted maize growth.

3.3 Effects of AMF and DSEs on the photosynthesis of maize leaves

AMF+DSE co-inoculation enhanced the photosynthetic physiology of maize leaves. Compared with that after the CK treatment, AMF+DSE co-inoculation significantly increased the photosynthetic rate of leaves by 24.9%. AMF inoculation, DSE inoculation, and AMF+DSE co-inoculation significantly reduced the intercellular CO_2 concentration by 25.3%, 29.1%, and 24.5%, respectively. According to the two-way ANOVA,AMF significantly increased the transpiration rate of maize leaves. There was a very significant interaction effect between AMF and DSE on the photosynthetic rate (Figure 2).

3.4 Effects of AMF and DSEs on glutathione metabolism in maize roots

AMF inoculation and AMF+DSE co-inoculation enhanced GSH metabolism in maize roots. Compared with those in the CK group, the activities of γ-GCS and ATPS in the AMF+DSE co-inoculation group were significantly increased by 117.3% and 50.0%, respectively. According to the two-way ANOVA, AMF had significant effects on the activities of ATPS, SIR, γ-GCS and OAS-TL, and the GSH content in maize roots. There was a substantial interaction effect between AMF and DSE on the activities of OAS-TL, ATPS, and SIR (Figure 3).

3.5 Effects of AMF and DSEs on the endogenous hormone contents in maize roots

AMF inoculation and AMF+DSE co-inoculation promoted the production of endogenous hormones in maize roots. Compared with those following the CK treatment, AMF+DSE co-inoculation significantly increased the contents of ZR, IAA, and ABA by 81%, 209%, and 72%, respectively. AMF inoculation significantly increased the contents of ZR and GA by 27% and 21%, respectively. According to the two-way ANOVA results showed that AMF and DSE had significant effects on the contents of ZR, GA, IAA, and ABA. There was a very significant interaction effect between AMF and DSE on the contents of ZR and IAA (Figure 4).

3.6 Effects of AMF and DSEs on Cd accumulation in maize

AMF inoculation and DSE inoculation increased the Cd content of maize roots by 104% and 120%, respectively. Moreover, DSE inoculation significantly increased Cd accumulation in the shoots and roots of maize by 258% and 507%, respectively. According to the two-way ANOVA showed that DSE significantly increased Cd accumulation of maize. There was a very significant interaction effect between AMF and DSE on Cd content and accumulation in maize roots (Figure 5).

3.7 Correlation analysis

There were positive correlations between the ZR content and the GSH content and the activities of γ-GCS, ATPS, SIR, and OAS-TL. The IAA content was positively correlated with γ-GCS, ATPS, SIR, and OAS-TL. The ABA content was positively correlated with the GSH content and the activities of γ-GCS, GSH, ATPS, and SIR.

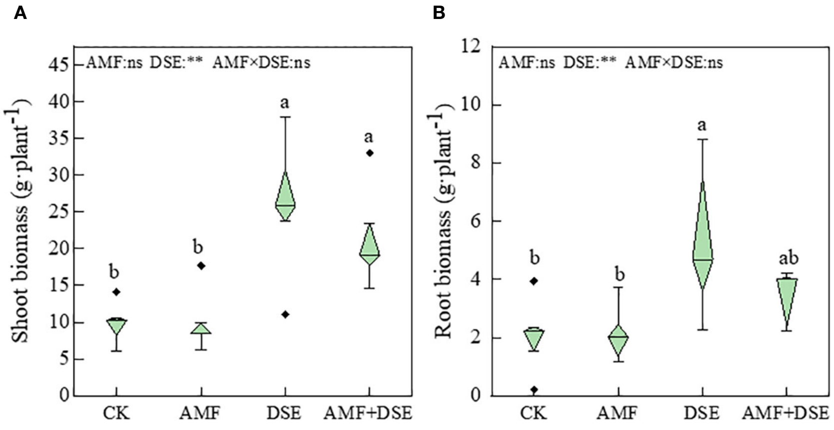

FIGURE 1
Effects of AMF and DSE on maize growth under Cd stress: **(A)** Shoot biomass; **(B)** Root biomass. The different lowercase letters and the same lowercase letters indicate significant and non-significant differences among treatments ($p < 0.05$), respectively. CK, the control of non-inoculation; AMF, *F. mosseae* inoculation; DSE, *E. pisciphila* inoculation; AMF+DSE, co-inoculation of *F. mosseae* and *E. pisciphila*. “ns”, “*” and “**” means no significant, and $p< 0.01$ according to two-way ANOVA, respectively.

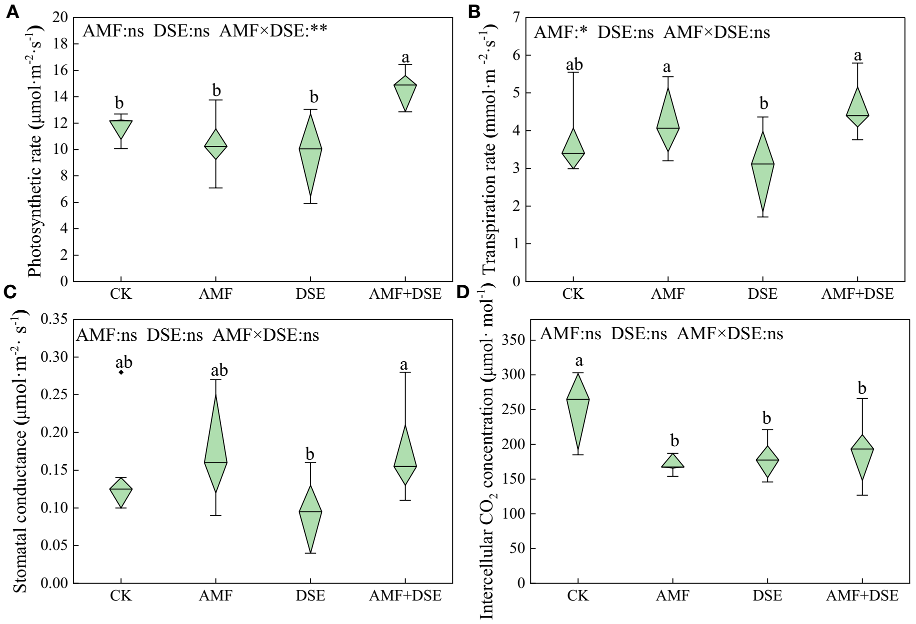

FIGURE 2
Effects of AMF and DSE on photosynthesis of maize leaves under Cd stress: **(A)** Photosynthetic rate; **(B)** Transpiration rate; **(C)** Stomatal conductance; **(D)** Intercellular CO$_2$ concentration. The and the same lowercase letters different lowercase letters indicate significant and non-significant differences among treatments ($p < 0.05$), respectively. CK, the control of non-inoculation; AMF, *F. mosseae* inoculation; DSE, *E. pisciphila* inoculation; AMF+DSE, co-inoculation of *F. mosseae* and *E. pisciphila*. "ns", "*" and "**" means no significant, and $p < 0.01$ according to two-way ANOVA, respectively.

Moreover, the ZR and IAA contents in roots positively correlated with the Tr and Pn in the leaves (Table 2). Thus, the endogenous hormone contents had a significant or highly significant positive correlations with GSH metabolism in maize roots and photosynthetic physiology in leaves, which plays a vital role in regulating the Cd tolerance of maize.

4 Discussion

Heavy metals inhibit plant growth and development, which can be alleviated by AMF and DSEs. AMF and DSEs can promote plant growth by improving plant photosynthesis and antioxidant physiology, and regulating plant hormone content, and enhancing host tolerance to adverse environmental conditions such as drought, cold, salinity, heavy metals, and other abiotic stresses (Ahammed et al., 2020). In the present study, inoculation with AMF and DSEs protected against Cd-induced growth inhibition and oxidative stress by regulating Cd accumulation in plants, endogenous hormone contents, and enzymes involved in GSH metabolism in maize.

Many studies have shown that AMF and DSEs regulate Cd uptake by host plants, inhibit Cd transfer from roots to leaves, and reduce the physiological toxicity of Cd to plants. For example, AMF increased the Cd concentration in the stems and roots of *Solanum nigrum* L. grown in Cd-polluted soils (Liu et al., 2015). AMF stabilizes Cd ions in soil and limits Cd absorption by plants, thus ensuring the normal metabolic function of plants, and improving the plant tolerance to Cd toxicity (Janeeshma et al., 2022). Similarly, DSE promoted the fixation of Cd in roots and limited the transfer of Cd from roots to shoots (Xiao et al., 2021). In the present study, the AMF inoculation, DSE inoculation, and AMF+DSE co-inoculation also increased the Cd content in maize roots and promoted Cd retention in the roots, which were both beneficial for enhancing plant tolerance to Cd stress. The increased Cd content in roots, which was promoted by AMF+DSE, was related to the Cd-binding capability of the fungal mycelia components, such as melanin (Ban et al., 2012). In addition, the extracellular secretion of endophytic fungi or exudates from inoculated roots and the intracellular sequestration of Cd ions are also important mechanisms for reducing Cd mobility in soil (Leonhardt et al., 2014).

Plant endogenous hormones play important roles in regulating plant growth and adaptability to abiotic stress. ZR, GA, and IAA are essential endogenous growth regulators (Gray, 2004), and ABA is a stress hormone that responds to abiotic stress (López-Climent et al., 2011). In many cases, AMF- or DSE-regulated hormones (such as ZR, GA, IAA, and ABA) contribute to improving plant growth under abiotic stress. For example, Yan and Zhong (2007) reported that AMF significantly increased the IAA content in the roots of *Cinnamomum camphora* L. and decreased the ABA content in the leaves and roots under aluminum stress. AMF increases the contents of IAA, GA, and ABA in the roots and leaves of tomato plants (He et al., 2010). Similarly, DSE colonization in roots resulted in a significant decrease in ABA levels in soybean (*Glycine max* L.)

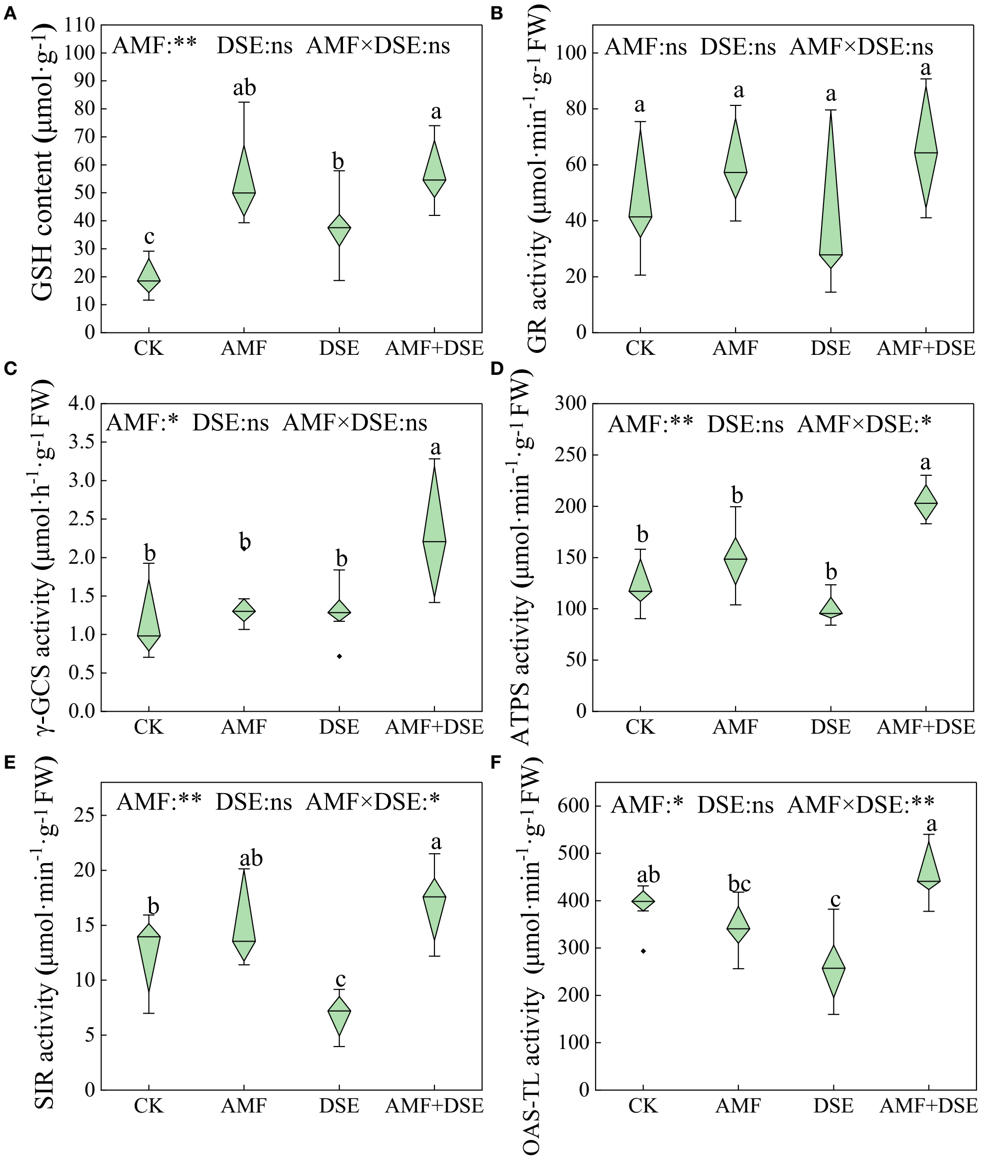

FIGURE 3
Effects of AMF and DSE on glutathione metabolism of maize roots under Cd stress: **(A)** GSH content; **(B)** GR activity; **(C)** γ-GCS activity; **(D)** ATPS activity; **(E)** SIR activity; **(F)** OAS-TL activity. The different lowercase letters and the same lowercase letters indicate significant and non-significant differences among treatments ($p < 0.05$), respectively. CK, the control of non-inoculation; AMF, *F. mosseae* inoculation; DSE, *E. pisciphila* inoculation; AMF+DSE, co-inoculation of *F. mosseae* and *E. pisciphila*. “ns”, “*” and “**” means no significant, and $p < 0.01$ according to two-way ANOVA, respectively.

under Cu stress (Khan and Lee et al., 2013), and a decrease in ABA levels and an increase in GA levels in cucumber (*Cucumis sativus* L.) under salt stress, thereby stimulating the growth of plant roots, improving nutrient absorption, and promoting plant growth (Khan et al., 2012; Sukumar et al., 2013). The DSE significantly affected the gene expression involved in phytohormone transport and the ABA and IAA contents in maize roots, which was considered as the main reason for promoting maize growth (Wang et al., 2023). Similarly, faba beans inoculated with endophytic fungi (*Aspergillus niger* and *Penicillium chrysosporium*) promoted plant growth and nutrient uptake by regulating endogenous plant hormones under Pb stress (El-Mahdy et al., 2021). In the present study, co-inoculation of AMF+DSE significantly increased the ZR and IAA contents in maize roots and promoted maize growth, suggesting that AMF and DSE inoculation might improve host growth by coordinating the balance of hormones and improving the ability of maize to cope with Cd stress.

Furthermore, glutathione (GSH), which is a tripeptide containing sulfhydryl (-SH) groups, participates in the complexation and detoxification of Cd ions in plants (Pearson and Cowan, 2021). GSH not only plays an important role in the antioxidant process though the glutathione-ascorbic acid cycle but also directly acts as a metal-chelating agent (Guo et al., 2012). There are many enzymes involved in GSH metabolism (Bano et al., 2022).

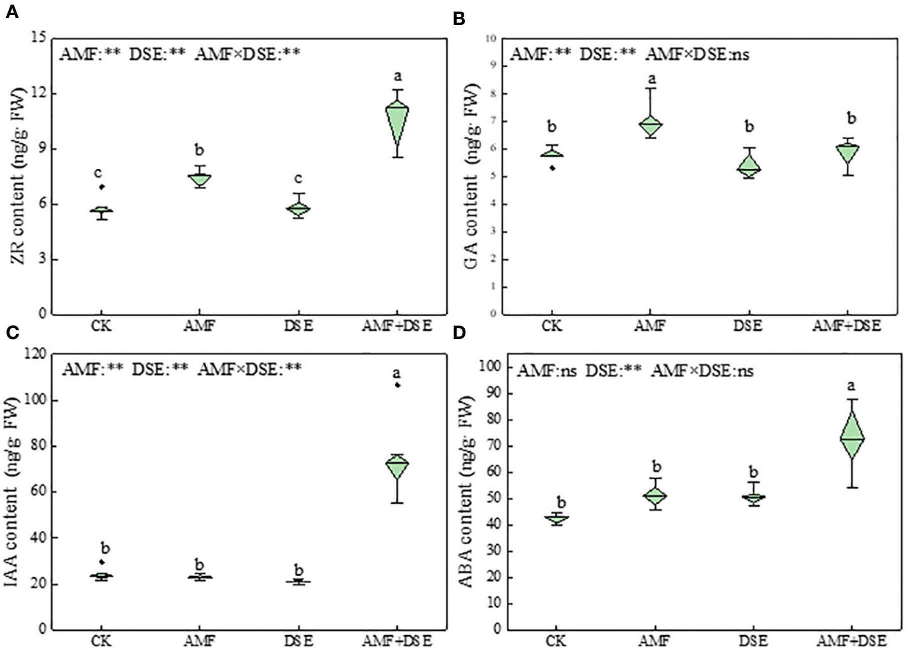

FIGURE 4
Effects of AMF and DSE on endogenous hormone content in maize roots under Cd stress: **(A)** ZR content; **(B)** GA content; **(C)** IAA content; **(D)** ABA content. The different lowercase letters and the same lowercase letters indicate significant and non-significant differences among treatments ($p < 0.05$), respectively. CK, the control of non-inoculation; AMF, *F. mosseae* inoculation; DSE, *E. pisciphila* inoculation; AMF+DSE, co-inoculation of *F. mosseae* and *E. pisciphila*. "ns", "*" and "**" means no significant, and $p < 0.01$ according to two-way ANOVA, respectively.

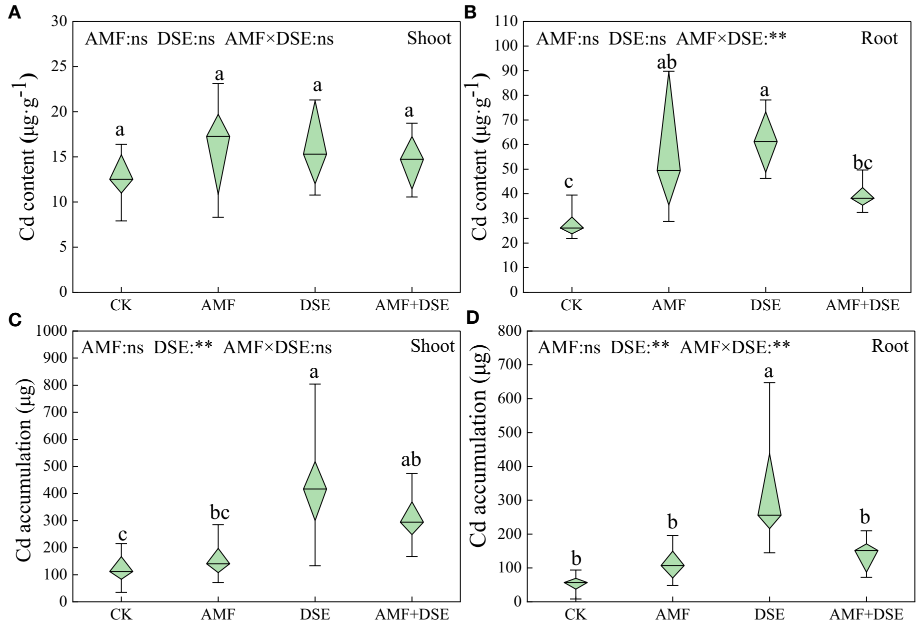

FIGURE 5
Effects of AMF and DSE on accumulation characteristics in maize under Cd stress: **(A)** Cd content in maize shoot; **(B)** Cd content in maize root; **(C)** Cd accumulation in maize shoot; **(D)** Cd accumulation in maize root. The and the same lowercase letters different lowercase letters indicate significant and non-significant differences among treatments ($p < 0.05$), respectively. CK, the control of non-inoculation; AMF, *F. mosseae* inoculation; DSE, *E. a pisciphila* inoculation; AMF+DSE, co-inoculation of *F. mosseae* and *E. pisciphila*. "ns", "*" and "**" means no significant, and $p < 0.01$ according to two-way ANOVA, respectively.

TABLE 2 Correlation analysis of endogenous hormone content with photosynthesis and GSH metabolism of maize.

	Pn	Tr	Gs	Ci	GSH	GR	γ-GCS	ATPS	SIR	OAS-TL
ZR	0.579**	0.433*	0.295	-0.212	0.549**	0.346	0.720**	0.559**	0.522**	0.521**
GA	0.035	0.183	0.178	-0.061	0.222	0.190	0.151	0.137	0.375	0.041
IAA	0.674**	0.429*	0.291	-0.075	0.366	0.239	0.515**	0.590**	0.519**	0.618**
ABA	0.561**	0.298	0.155	-0.286	0.482*	0.368	0.643**	0.501*	0.458*	0.383

"*" and "**" means $p < 0.05$ and $p < 0.01$ according to correlation analysis, respectively.

First, ATPS initiates GSH metabolism, which catalyzes sulfur assimilation, i.e., sulfate and ATP, to form adenosine phosphate sulfate (Anjum et al., 2015), and a low Cd concentration leads to an increase in ATPS activity in maize (Nazar et al., 2011). SIR is another key enzyme involved in sulfur assimilation to reduce sulfite to sulfide (Rather et al., 2020). In the last step of sulfur assimilation, OAS-TL catalyzes OAS and hydrogen sulfide to produce cysteine (Cys). Cys is the product of sulfur assimilation and the starting material for GSH synthesis. Additionally, γ-GCS is the rate-limiting enzyme for GSH synthesis, and the GSH content provides feedback to inhibit γ-GCS activity (Rennenberg et al., 2007). GR is important for GSH metabolism and can convert oxidized glutathione (GSSG) into GSH (Mishra et al., 2006). GSH and GR scavenge H_2O_2 (Anjum et al., 2012).

In the present study, AMF+DSE co-inoculation improved the activities of ATPS and SIR, indicating that AMF+DSE co-inoculation promoted sulfate reduction and assimilation in maize roots and met the Cys demand for GSH synthesis. In plants under Cd stress, inoculation with AMF and DSEs can enhance plant adaptability and alleviate Cd toxicity by promoting glutathione metabolism and thiol compound content in plants (Degola et al., 2015). In addition, a greater GSH content in the AMF-inoculated and DSE-inoculated plants led to feedback inhibition of γ-GCS activity, but AMF+DSE co-inoculation significantly increased γ-GCS activity in maize roots. These results indicated that GSH synthesis was enhanced by AMF and DSE, which promoted the Cd tolerance of maize. Similarly, maize inoculated with AMF (*Rhizophagus intraradices* and *Glomus versiforme*) significantly increased the GSH content, which helped to alleviate Cd toxicity and promoted maize growth (Zhang et al., 2019).

More importantly, there were positive correlations between the ZR and IAA contents and the photosynthetic physiology, the activities of γ-GCS, ATPS, SIR, and OAS-TL, and the GSH content in the present study. These correlations demonstrated the positive synergistic effects of AMF and DSE on photosynthesis, GSH metabolism, and endogenous hormone content in maize. These findings agreed with the results of Yang et al. (2015) who reported that AMF significantly enhanced the photosynthetic physiology of leaves by increasing the net Pn, Tr, and stomatal conductance. Similarly, AMF and DSEs improve photosynthesis and resistance physiology and enhance the ability of plants to resist the toxicity of heavy metals (Gadd, 2007; Ren et al., 2015).

However, several studies have shown that the co-inoculation of AMF and DSE has competitive effects on mycorrhizal colonization, maize growth, and root hydraulic conductivity under drought stress (Gong et al., 2023). Once AMF infests roots, mycelial development is inhibited by DSE fungal exudates (Scervino et al., 2009). These reports indicate the complexity of the interactions between AMF and DSEs that colonize plant roots and our lack of understanding of their working mechanisms at the physiological and molecular levels. Thus, more research on the ecological functions and interactions of AMF and DSEs is needed.

5 Conclusions

Under the stress of 25 $mg{\cdot}kg^{-1}$ Cd, AMF+DSE co-inoculation significantly enhanced the photosynthetic rate of maize leaves and increased the endogenous hormone contents and GSH metabolism in roots. Correlation analysis revealed that the endogenous hormone contents in maize roots was significantly related to photosynthesis and GSH metabolism. Moreover, there was a significant or highly significant positive interaction effect between AMF and DSEs on the photosynthetic rate, root GSH metabolism, and endogenous hormone content. Thus, the coexistence of AMF and DSEs had a synergistic effect on enhancing the Cd tolerance of maize.

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

ZW: Formal Analysis, Writing – original draft. LW: Data curation, Formal Analysis, Writing – original draft. XL: Data curation, Formal Analysis, Writing – original draft. GZ: Investigation, Methodology, Writing – original draft. ZL: Investigation, Methodology, Writing – original draft. ZY: Writing – review & editing. FZ: Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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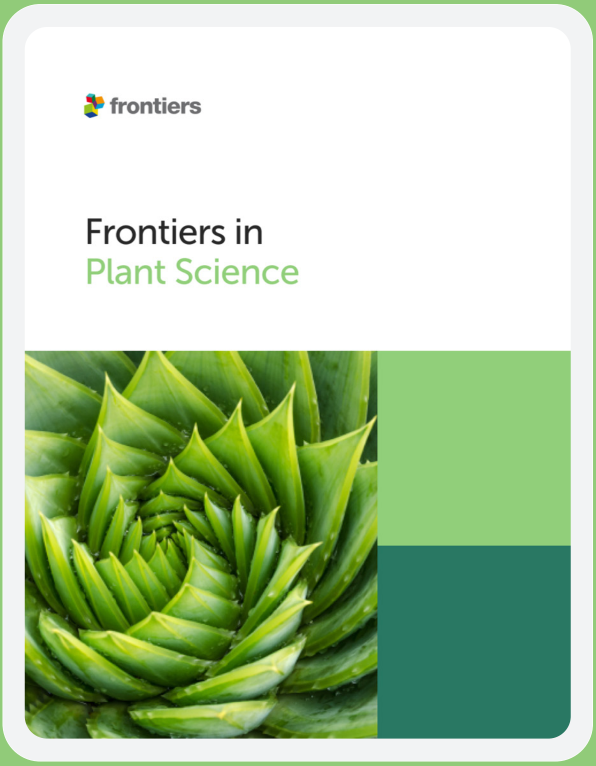

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