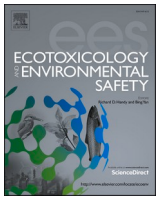
Ecotoxicology and Environmental Safety 241 (2022) 113739

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv



Physiological and rhizospheric response characteristics to cadmium of a newly identified cadmium accumulator *Coreopsis grandiflora* Hogg. (Asteraceae)

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ARTICLE INFO

Edited by Dr Yong Liang

Keywords:
Coreopsis grandiflora
Cd accumulator
Phytoextraction
Amino acid metabolism
Plant growth-promoting microorganism

ABSTRACT

Screening for superior cadmium (Cd) phytoremediation resources and uncovering the mechanisms of plant response to Cd are important for effective phytoremediation of Cd-polluted soils. In this study, the characteristics of *Coreopsis grandiflora* related to Cd tolerance and accumulation were analyzed to evaluate its Cd phytoremediation potential. The results revealed that *C. grandiflora* can tolerate up to 20 mg kg^{-1} of Cd in the soil. This species showed relatively high shoot bioconcentration factors (1.09–1.85) and translocation factors (0.46–0.97) when grown in soils spiked with 5–45 mg kg^{-1} Cd, suggesting that *C. grandiflora* is a Cd accumulator and can potentially be used for Cd phytoextraction. Physiological analysis indicated that antioxidant enzymes (i.e., superoxide dismutase, peroxidase, and catalase) and various free amino acids (e.g., proline, histidine, and methionine) participate in Cd detoxification in *C. grandiflora* grown in soil spiked with 20 mg kg^{-1} of Cd (Cd20). The overall microbial richness and diversity remained similar between the control (Cd0) and Cd20 soils. However, the abundance of multiple rhizospheric microbial taxa was altered in the Cd20 soil compared with that in the Cd0 soil. Interestingly, many plant growth-promoting microorganisms (e.g., *Nocardioides*, *Flavisolibacter*, *Rhizobium*, *Achromobacter*, and *Penicillium*) enriched in the Cd20 soil likely contributed to the growth and vitality of *C. grandiflora* under Cd stress. Among these, some microorganisms (e.g., *Rhizobium*, *Achromobacter*, and *Penicillium*) likely affected Cd uptake by *C. grandiflora*. These abundant plant growth-promoting microorganisms potentially interacted with soil pH and the concentrations of Cd and AK in soil. Notably, potassium-solubilizing microbes (e.g., *Rhizobium* and *Penicillium*) may effectively solubilize potassium to assist Cd uptake by *C. grandiflora*. This study provides a new plant resource for Cd phytoextraction and improves our understanding of rhizosphere-associated mechanisms of plant adaptation to Cd-contaminated soil.

1. Introduction

Cadmium (Cd) exposure is detrimental to human health. Unfortunately, this toxic metal is found in the environment worldwide (Cui et al., 2021; Ramlan et al., 2021). Cd in the soil is easily absorbed by plants and enters the human body via the food chain (Mao et al., 2022). Therefore, the remediation of Cd-contaminated soils is a significant challenge. Among various remediation tools, economically viable and eco-friendly phytoremediation techniques have attracted significant attention (Liu et al., 2022). Although phytostabilization is important for

Abbreviations: AK, available potassium; AP, available phosphorus; BCF, bioconcentration factor; CAT, catalase; Cd, cadmium; DW, dry weight; HN, hydrolysable nitrogen; ICP–MS, inductively coupled plasma–mass spectrometry; MDA, malondialdehyde; OTU, operational taxonomic unit; POD, peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; TF, translocation factor.

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https://doi.org/10.1016/j.ecoenv.2022.113739

Available online 14 June 2022

managing Cd-contaminated soils, phytoextraction, i.e., removing the accumulated Cd by harvesting the aerial parts of Cd accumulators or hyperaccumulators (Oladoye et al., 2022), is a safer and more sustainable remediation technique for disposing this toxic element. Several potential accumulators or hyperaccumulators of Cd have been screened from naturally or artificially Cd-polluted soils (Reeves et al., 2018).

However, Cd phytoextraction efficiency is still limited because: (1) many identified high-Cd-accumulating plants have slow growth rates and low biomass (Shen et al., 2021); (2) many plants have limited ability to transport and detoxify Cd, so they cannot absorb or bear high concentrations of Cd; and (3) the availability of nutrient elements and Cd in soil may be low, restricting plant growth and Cd absorption. To maximize the value of phytoextraction during the remediation of Cd-polluted soils, it is necessary to screen or breed high-Cd-accumulating plant resources with large biomass and fast growth rates and to optimize strategies to enhance Cd absorption and accumulation by such plants.

Uncovering the mechanisms of Cd tolerance and accumulation in plants is important for improving phytoextraction efficiency. Physiological detoxification strategies form the basis for plants to tolerate Cd exposure. The main mechanisms of Cd detoxification in plants include compartmentalization, chelation, antioxidant processes, and osmotic adjustment (Li et al., 2018). Antioxidant enzymes, such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), regulate the accumulation of reactive oxygen species (ROS) to prevent oxidative stress (Jan et al., 2018; Mahawar et al., 2021). Various amino acids participate in chelation, antioxidant processes, and osmotic regulation in response to Cd stress (Zhu et al., 2018). In addition to physiological Cd tolerance mechanisms in plants (Feki et al., 2021), rhizospheric environments (e.g., rhizospheric microorganisms) greatly affect Cd phytoextraction efficiency (Bian et al., 2021). Many rhizospheric microorganisms are beneficial for enhancing plant growth and mitigating the negative effects of Cd on plants (Khanna et al., 2019a; Manoj et al., 2020). Additionally, rhizospheric microorganisms influence the chemical forms and bioavailability of Cd (Yuan et al., 2021), which directly determine Cd accumulation levels in plants (Khanna et al., 2019a). Li et al. (2021b) determined that induced rhizobacteria associated with ABC transporters improved Cd tolerance and accumulation in *Salvia tiliifolia* plants in Cd-polluted soils. Yang et al. (2022) found that various plant growth-promoting bacteria enriched in the rhizosphere of rhubarbs likely contributed to the growth and vitality of plants under Cd stress and promoted Cd uptake by plants. These studies indicate that the dynamics of rhizospheric microorganisms under Cd stress vary considerably among different soil–plant systems. Therefore, a systematic and unified understanding of the effects and mechanisms of rhizospheric microorganisms on plant response to Cd is required.

Plants from Asteraceae, the largest angiosperm family, have pronounced heavy metal phytoremediation potential, and the family includes several heavy metal accumulators or hyperaccumulators (Nikolic and Stevovic, 2015; Reeves et al., 2018). The genus *Coreopsis* in the Asteraceae family comprises approximately 100 species worldwide (erect annual or perennial herbs). To date, the heavy metal accumulation characteristics have been determined for *C. basalis* and *C. lanceolata* (Lin et al., 2016; Xu et al., 2018). Both the shoot Cd bioconcentration factors (BCFs) and translocation factors (TFs) of the two *Coreopsis* species exceeded one (Lin et al., 2016; Xu et al., 2018), indicating that these *Coreopsis* species have relatively strong Cd absorption and transport capacities and can be used for Cd phytoextraction. *C. grandiflora* Hogg., a perennial herb native to America, can reach a height of up to 1 m. In China, *C. grandiflora* is often cultivated in various regions and is sometimes naturalized in the wild. The tall plants and wide distribution of *C. grandiflora* indicate its potential advantages in the phytoremediation of soils polluted with heavy metals. However, the characteristics of heavy metal accumulation in *C. grandiflora* remain unexplored. In this study, the Cd tolerance and accumulation characteristics of *C. grandiflora* were systematically analyzed to explore its phytoremediation potential in Cd-polluted soils. Moreover, the physiological responses and variations in the rhizospheric microenvironments (especially rhizospheric microorganisms) of *C. grandiflora* were analyzed to understand their contribution to Cd tolerance and accumulation characteristics. These findings are expected to provide novel plant resources for Cd phytoremediation and improve our understanding of the effects of rhizospheric microorganisms on interactions between Cd and plants.

2. Materials and Methods

2.1. Pre-experiment

To tentatively understand the Cd tolerance capacity of *C. grandiflora*, the seeds were randomly sown in Cd-free and Cd-contaminated (Cd concentration: 16.9 mg kg^{-1}) soils, similar to the experiment for *Salvia tiliifolia* (Li et al., 2021b). As no visible differences in seed germination and seedling growth of *C. grandiflora* were observed between the control and Cd-contaminated soils (Fig. 1A), further experiments were conducted.

2.2. Seed and soil preparation

Mature *C. grandiflora* seeds and experimental soils were purchased from the Dounan flower market in Kunming, China.

The soils were sieved to remove impurities and mixed to obtain homogeneous composite soil, as previously described (Li et al., 2021b). Basic parameters, including the pH (5.55), concentrations of organic matter (313 g kg^{-1}), total Cd (0.02 mg kg^{-1}), total nitrogen (9.60 g kg^{-1}), total phosphorus (1.24 g kg^{-1}), hydrolysable nitrogen (HN: 1.19 g kg^{-1}), and available phosphorus (AP: 38.2 mg kg^{-1}), of the homogenized soil were analyzed prior to the experiment (Li et al., 2016; Liu et al., 2019). Cd concentration gradients of 0 (Cd0), 5 (Cd5), 20 (Cd20), and 45 (Cd45) mg Cd kg^{-1} dry soil were set for the experiment using a previously described method (Li et al., 2021b). These Cd concentrations were chosen based on several previous studies (Wu et al., 2018; Dhaliwal et al., 2020) to understand the Cd tolerance threshold of plants and to determine whether they can be defined as Cd hyperaccumulators. The prepared soils were equilibrated for one month (Wu et al., 2018) in a greenhouse (light: 12–14 h, 23–25 °C; darkness: 10–12 h, 18–20 °C; humidity: 40–60%) at the Kunming Institute of Botany, Chinese Academy of Science in Kunming, China. The actual Cd concentrations in spiked Cd5 (6.84 mg kg^{-1}), Cd20 (20.10 mg kg^{-1}), and Cd45 (45.40 mg kg^{-1}) soils were detected to reflect the reliability of the experimental treatment.

2.3. Experimental design

Homogenized soils with different Cd concentrations were divided into 2.0 kg aliquots (wet weight) and loaded into flowerpots (17.5 cm in height and 18.5 cm in diameter). Soil was padded to a height of 17 cm in each pot to ensure the same soil density. *C. grandiflora* seeds were surface-sterilized and washed using a previously described method (Yang et al., 2022a). Sterilized seeds were sown in pots filled with soil (five seeds per pot), which were placed in the aforementioned greenhouse. After the seeds were unearthed, one seedling was placed in each pot. Three biological replicates were used for each experiment.

2.4. Sample collection and treatment

After five months of growth, the plant and soil samples were collected. Fresh young *C. grandiflora* leaves were collected for antioxidant enzyme activity analyses. Shoots and roots of the plants were harvested separately and dried at 80 °C for 48 h to measure biomass, free amino acids, and Cd concentrations. Cd^{2+} adsorbed on the roots was removed using a previously reported method (Wu et al., 2018). Soils that naturally adhered to the root systems after gentle shaking were collected as rhizospheric soils (Li et al., 2022) to analyze the soil physicochemical

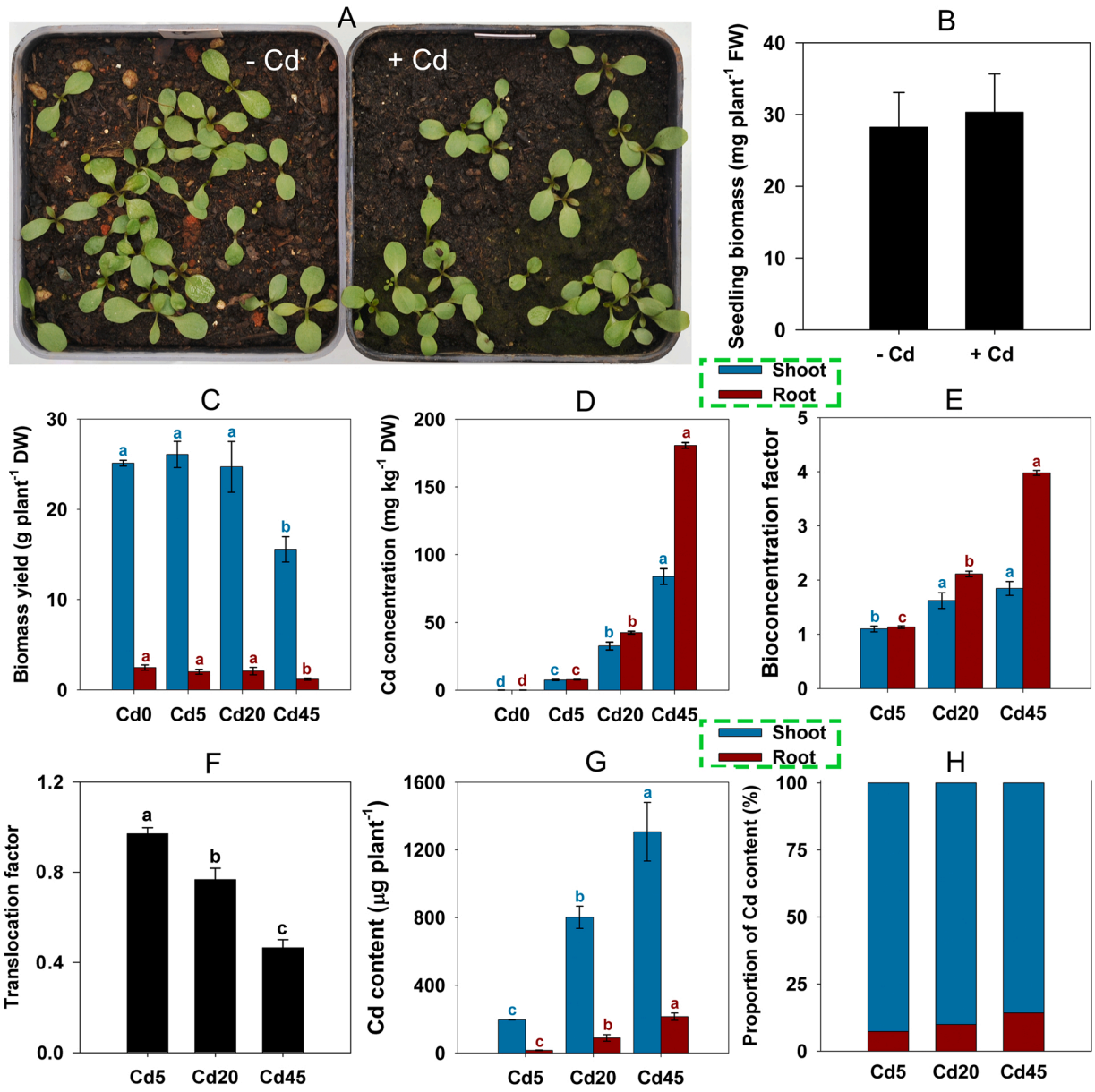

Fig. 1. Cd tolerance and accumulation characteristics of *Coreopsis grandiflora*. (A) Morphology of *C. grandiflora* seedlings sown in control and Cd-polluted (16.9 mg kg^{-1}) soils. (B) Plant height of *C. grandiflora* seedlings sown in control and Cd-polluted soils. Biomass yields (C), Cd concentrations (D), bioconcentration factors (E), translocation factors (F), Cd contents (G), and proportion of Cd contents (H) in shoots and/or roots of *C. grandiflora* in soils spiked with 0 (Cd0), 5 (Cd5), 20 (Cd20), and 45 mg kg^{-1} Cd (Cd45). Data represent means ± standard deviations (B–G: n = 3 or 10) or means (H: n = 3). The same-colored bars labeled with different letters indicate significant differences at the $P < 0.05$ level among different groups according to Tukey's test. DW: dry weight; FW: fresh weight.

indices and microbial community composition.

2.5. Determination of Cd concentration

Cd concentrations in plant shoots and roots were digested with HNO_3 and measured using inductively coupled plasma–mass spectrometry (ICP–MS), as reported previously (Li et al., 2017). The Cd detection limit of ICP–MS was 0.002 mg kg^{-1}, and the limit for Cd quantitative determination was 0.005 mg kg^{-1} in this study. The BCFs, TFs, and total Cd content accumulated in the plant shoots and roots were calculated as follows:

BCF = shoot (root) Cd concentration/soil Cd concentration (Acosta et al., 2018; Chen et al., 2021a),

TF = shoot Cd concentration/root Cd concentration (Acosta et al., 2018; Chen et al., 2021a),

Cd content in the shoot (root) = shoot (root) Cd concentration × shoot (root) biomass.

2.6. Antioxidant system assay

The activities of SOD, POD, and CAT in *C. grandiflora* leaves were measured using the corresponding assay kits (Solarbio, Beijing, China), as described previously (Li et al., 2021a, 2021c), which were detected at wavelengths of 560, 340, and 240 nm using an ultraviolet-visible spectrophotometer, respectively (Youke, Shanghai, China). One SOD activity unit was defined as the activity of the sample when the

inhibition rate of the xanthine oxidase coupling reaction system was 50%; one POD activity unit was defined as the change in absorbance by 0.01 in a 1 mL reaction system per minute per gram of sample, and one CAT activity unit was defined as the catalytic degradation of 1 nmol H_2O_2 in the reaction system per minute per gram of sample.

The malondialdehyde (MDA) concentration was measured using an MDA assay kit (BC0020; Solarbio, Beijing, China). In brief, approximately 0.1 g of plant sample was mixed for 30 min with 4 mL of extracting solution. After the mixture was centrifuged at 10,000 ×*g* for 15 min, 0.1 mL of the supernatant was mixed with 4 mL of the reaction solution. The mixture was heated in a water bath at 95 °C for 30 min, and then cooled to room temperature. Absorbance values were measured at wavelengths of 450, 532, and 600 nm.

2.7. Determination of free amino acids

Dry shoot samples were used to measure the concentration of 17 free amino acids (Li et al., 2018) in this study. Approximately 2 g of each sample was dissolved in 10 mL HCl (0.02 M). The C18 chromatographic columns were activated using 5 mL methanol and deionized water. Sample solutions (2.5 mL) added to 1.5 mL HCl (0.02 M) were flowed through the columns, and the volume was set to 5 mL using HCl (0.02 M). The sample solutions were filtered through 0.45 μm filtering membranes and then detected using an automatic amino acid analyzer (LA8080, EEM, Tokyo, Japan).

2.8. Determination of soil physicochemical indices

In this study, soil pH and the concentrations of Cd, HN, AP, and available potassium (AK) were determined using the methods introduced in the corresponding detection standards in China (Cha et al., 2020; Li et al., 2020; Zhang and Luo, 2020; Yang et al., 2022a). The methods and corresponding detection standards for these soil indices are listed in Supplementary Table S1.

2.9. Soil microbial community composition analysis

Soil microbial DNA extraction, 16S rDNA/ITS amplification, sequencing, and bioinformatics analyses were performed as previously described (Li et al., 2021b). For bacterial diversity analysis, the V3–V4 region of the 16S rDNA gene was amplified by PCR using primers 341 F-CCTACGGGNGGCWGCAG and 806R-GGACTACHVGGGTATCTAAT. For fungal diversity analysis, the ITS1 region of ribosomal DNA was amplified by PCR using the primers ITS1_F_KYO2-TAGAGGAAG TAAAAGTCGTAA and ITS86R-TTCAAAGATTCGATGATTCAC. The raw reads were deposited in the NCBI Sequence Read Archive (SRA) database (Accession Number: PRJNA799148), which should be released on July 31, 2022, or upon publication of this study.

2.10. Statistical analysis

The Statistical Package for the Social Sciences version 18.0 was used for statistical analysis. One-way analysis of variance with Tukey's test was used to determine significant differences ($P < 0.05$) among three or more groups, and an independent-sample *t*-test was used to determine significant differences ($0.01 < P < 0.05$, $0.001 < P < 0.01$, or $P < 0.001$) between the two groups. Correlation network analysis between soil indices and microorganisms was performed using the online Omicsmart platform (http://www.omicsmart.com).

3. Results

3.1. Cd tolerance and accumulation characteristics of C. grandiflora

In this study, a preliminary experiment was conducted to predict the Cd tolerance capacity of *C. grandiflora*, which is the basis for evaluating the suitability of plants for phytoremediation. As shown in Fig. 1A and B, the *C. grandiflora* seeds were able to germinate, and the seedlings grew normally in the Cd-polluted soil (16.9 mg Cd kg^{-1} soil), which exhibited little difference in plant size and biomass compared to those sown in control soil. The results indicated that *C. grandiflora* has some degree of Cd tolerance, and subsequent experiments were conducted. Although the *C. grandiflora* plants did not show symptoms of leaf chlorosis or injury under the 5–45 mg kg^{-1} Cd treatment for 5 months, the biomass yields of shoots and roots in Cd45 soil decreased by 38.0% and 52.0% ($P < 0.05$) compared with those in Cd0 soil, respectively (Fig. 1C). The average Cd concentrations in the *C. grandiflora* shoots were 0.01, 7.52, 32.60, and 83.80 mg kg^{-1}, whereas those in the roots were 0.02, 7.75, 42.47, and 180.67 mg kg^{-1} in the Cd0, Cd5, Cd20, and Cd45 soils, respectively (Fig. 1D).

Under Cd treatment, the average shoot Cd BCFs of *C. grandiflora* increased from 1.09 to 1.85, whereas the root Cd BCFs increased from 1.13 to 3.98 in soils spiked with different concentrations of Cd (Fig. 1E). However, the Cd TFs declined from 0.97 to 0.46 in *C. grandiflora* under 5–45 mg kg^{-1} Cd treatment (Fig. 1F). The total Cd contents accumulated in the individual plants are shown in Fig. 1G. The Cd contents in both the shoots (195.70–1,307.27 μg $plant^{-1}$) and roots (15.52–214.51 μg $plant^{-1}$) of *C. grandiflora* significantly increased ($P < 0.05$) with increasing soil Cd concentrations. Notably, approximately 85.7–92.7% of the total Cd accumulated in the *C. grandiflora* shoots under different Cd concentrations (Fig. 1H).

Because *C. grandiflora* could tolerate up to 20 mg kg^{-1} Cd treatment, the plant physiological responses and changes in rhizospheric microenvironments between the Cd0 and Cd20 groups were analyzed to understand the potential mechanisms of Cd tolerance and accumulation in *C. grandiflora*.

3.2. Changes in the antioxidant system of C. grandiflora leaves

In this study, the activities of three important antioxidant enzymes SOD, POD, and CAT in the *C. grandiflora* leaves in the Cd20 soil increased by 13.7%, 48.2%, and 24.6% ($0.01 < P < 0.05$ or $P < 0.001$) compared to those in the Cd0 soil (Fig. 2A–C), indicating that the antioxidant system of the *C. grandiflora* leaves was activated under Cd treatment. Interestingly, MDA concentrations in the leaves of *C. grandiflora* were not significantly different between the Cd0 and Cd20 groups (Fig. 2D).

3.3. Response of amino acid metabolism in C. grandiflora shoots

The concentrations of 17 major free amino acids in the *C. grandiflora* shoots are shown in Table 1, which exhibited various change trends between the Cd0 and Cd20 groups. In brief, the average concentrations of serine, valine, methionine, isoleucine, leucine, histidine, and proline in the Cd20 group increased by 22.9%, 15.8%, 125.1%, 41.9%, 29.7%, 28.1%, and 44.7% ($0.01 < P < 0.05$, $0.001 < P < 0.01$, or $P < 0.001$) compared to those in the Cd0 group, respectively, whereas the average concentration of glutamic acid in the Cd20 group decreased by 9.1% ($0.001 < P < 0.01$) (Table 1). The concentrations of the remaining amino acids (aspartic acid, threonine, glycine, alanine, cysteine, tyrosine, phenylalanine, lysine, and arginine) in *C. grandiflora* shoots were not significantly different between the Cd0 and Cd20 groups (Table 1). These results indicate that amino acid metabolism in *C. grandiflora* is regulated by Cd and that several amino acids are likely to play an important role in Cd detoxification.

3.4. Changes in physicochemical indices of rhizosphere soils of C. grandiflora

In this study, the changes in the rhizospheric microenvironments of *C. grandiflora* under control (Cd0) and Cd stress (Cd20) conditions were analyzed to understand how it copes with Cd stress. Table 2 presents the

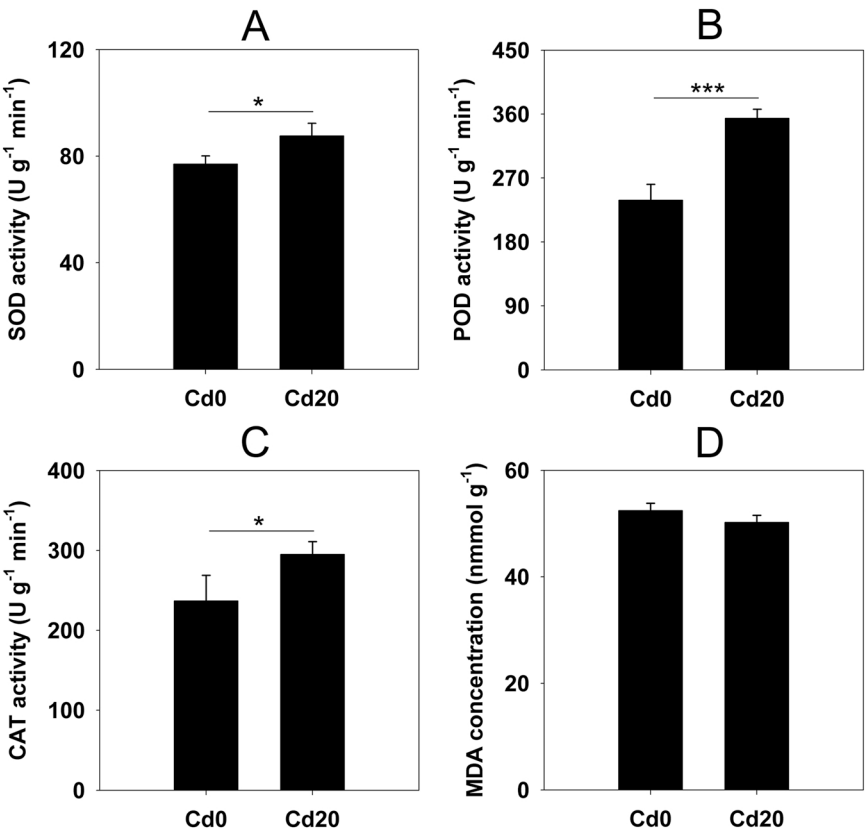

Fig. 2. Antioxidant enzyme activities and malondialdehyde (MDA) concentrations in leaves of *Coreopsis grandiflora* grown in soils spiked with 0 (Cd0) and 20 mg kg^{-1} Cd (Cd20). (A) Superoxide dismutase (SOD) activities. (B) Peroxidase (POD) activities. (C) Catalase (CAT) activities. (D) MDA concentrations. Data represent means ± standard deviations (n = 3). * and *** indicate significant differences at the $0.01 < P < 0.05$ and $P < 0.001$ levels, respectively, between two groups according to an independent-sample *t*-test.

Table 1
Concentrations (g 100 g^{-1} DW) of 17 free amino acids in *Coreopsis grandiflora* shoots grown in soils spiked with 0 (Cd0) and 20 mg kg^{-1} Cd (Cd20). Data represent means ± standard deviations (n = 3). *, **, and *** indicate significant differences at the $0.01 < P < 0.05$, $0.001 < P < 0.01$, and $P < 0.001$ levels, respectively, between two groups according to an independent sample *t*-test. DW: dry weight.

Amino acid	Concentrations (g 100 g^{-1} DW)	
	Cd0	Cd20
Aspartic acid	0.63 ± 0.01	0.66 ± 0.02
Threonine	1.65 ± 0.07	1.82 ± 0.06
Serine	0.77 ± 0.03	0.95 ± 0.03 **
Glutamic acid	0.95 ± 0.01	0.86 ± 0.02 **
Glycine	0.11 ± 0.00	0.12 ± 0.00
Alanine	2.23 ± 0.10	2.19 ± 0.06
Cysteine	0.12 ± 0.02	0.13 ± 0.01
Valine	1.37 ± 0.06	1.59 ± 0.06 **
Methionine	0.06 ± 0.05	0.14 ± 0.01 *
Isoleucine	0.86 ± 0.06	1.22 ± 0.06 ***
Leucine	0.93 ± 0.06	1.21 ± 0.09 **
Tyrosine	0.43 ± 0.03	0.43 ± 0.01
Phenylalanine	0.72 ± 0.06	0.80 ± 0.04
Lysine	0.48 ± 0.03	0.46 ± 0.02
Histidine	0.21 ± 0.00	0.27 ± 0.02 **
Arginine	1.02 ± 0.08	0.93 ± 0.08
Proline	6.49 ± 0.30	9.39 ± 0.32 ***

differences in physicochemical indices (i.e., total Cd, pH, HN, AK, and AP) between the Cd0 and Cd20 soils after 5 months of plant growth. The difference in soil Cd concentrations between the Cd0 and Cd20 soils was still large (Table 2). The average rhizosphere pH of the Cd20 soil (5.63) showed a decreasing trend ($0.01 < P < 0.05$) compared with that of the Cd0 soil (5.77) (Table 2). The average AK concentration significantly increased ($0.001 < P < 0.01$) in the Cd20 soil compared with that in the Cd0 soil (Table 2). However, the HN and AP concentrations were similar between the Cd0 and Cd20 soils (Table 2).

Table 2
Physicochemical properties in the rhizospheric soils (dry weight) of *Coreopsis grandiflora* grown in soils spiked with 0 (Cd0) and 20 mg kg^{-1} Cd (Cd20). Data represent means ± standard deviations (n = 3); *, **, and *** indicate $0.01 < P < 0.05$, $0.001 < P < 0.01$, $P < 0.001$, respectively, according to an independent sample *t*-test. EC: electrical conductivity; HN: hydrolyzable nitrogen; AK: available potassium; AP: available phosphorus.

Indices	Unit	Cd0	Cd20
Cd	mg kg^{-1}	0.02 ± 0.01	18.10 ± 0.57 ***
pH	/	5.77 ± 0.03	5.63 ± 0.04 *
HN	mg kg^{-1}	777 ± 8	762 ± 32
AK	mg kg^{-1}	156 ± 6	213 ± 1 **
AP	mg kg^{-1}	27.1 ± 1.5	30.4 ± 1.5

3.5. Dynamics of the microbial community in the rhizosphere of *C. grandiflora*

3.5.1. Richness, diversity, and composition of microbial communities in different soils

High-throughput sequencing was performed to analyze the dynamics of the microbial community in rhizospheric soils to understand the effects of rhizospheric microorganisms on plant growth and Cd accumulation in *C. grandiflora*. The results showed that 16S rDNA and ITS sequencing generated 120,446–137,455 and 121,979–137,581 raw sequencing paired-end reads in different samples, respectively, approximately 99.5–99.7% of which were effective reads (Supplementary Tables S2 and S3). These reads yielded 4,072–4,521 and 556–652 total operational taxonomic units (OTUs), respectively (Supplementary Tables S2 and S3). Approximately 99.8–99.9% and 83.0–86.7% of these OTUs were annotated to bacteria (4,067–4,511) and fungi (482–547) in different samples, respectively (Supplementary Tables S2 and S3).

Alpha indices (Sobs, Shannon, Simpson, and Chao1) for both bacterial and fungal communities exhibited no significant differences between the Cd0 and Cd20 soils (Supplementary Tables S4 and S5), indicating similar richness and diversity of the microbial community between the two rhizospheric soils.

Twenty-nine bacterial phyla were identified in this study, most (26) of which were shared by Cd0 and Cd20 soils (Fig. 3A). Only three specific bacterial phyla were identified in the Cd20 soil (Fig. 3A). Fourteen bacterial phyla (Supplementary Table S6) with a relative abundance of $> 0.1\%$ in at least one sample were identified in both soils. The top 10 bacterial phyla followed the order of relative abundance: Proteobacteria (41.7% and 44.6%) > Patescibacteria (10.2% and 8.9%) > Firmicutes (6.9% and 7.4%) > Acidobacteria (7.4% and 6.7%) > Actinobacteria (6.9% and 6.9%) > Bacteroidetes (6.8% and 5.9%) > Planctomycetes (5.8% and 5.1%) > Chloroflexi (4.8% and 4.1%) > Gemmatimonadetes (4.1% and 4.1%) > Verrucomicrobia (2.0% and 2.1%) (Fig. 3B; Supplementary Table S6), which accounted for approximately 95% of the total bacterial community (Fig. 3B; Supplementary Table S6). The abundance of 14 bacterial phyla in the two soils showed a complex correlation network (Supplementary Fig. S1). The phyla Proteobacteria and Firmicutes, which were highly abundant, showed significant negative correlations ($P < 0.05$) with several other phyla (Supplementary Fig. S1), indicating that they had a significant effect on these bacterial taxa. In addition, the phyla Patescibacteria, Actinobacteria, Bacteroidetes, Armatimonadetes, and Planctomycetes exhibited high connectivity (Supplementary Fig. S1), indicating the complex interactions among these bacterial taxa.

One hundred and fifty-three fungal genera were identified in this study, most (99) of which were shared by the Cd0 and Cd20 soils (Fig. 3C). Twenty-three and thirty-one specific fungal genera were identified in the Cd0 and Cd20 soils, respectively (Fig. 3C). Sixty-five fungal genera (Supplementary Table S7) with a relative abundance of $> 0.1\%$ in at least one sample were identified in both the soils. The top 20 fungal genera are shown in Fig. 3D and account for approximately 50% of the total fungal community (Fig. 3D; Supplementary Table S7). The abundance of 61 fungal genera in the two soils showed a complex correlation network (Supplementary Fig. S2). Unlike the bacterial community (Supplementary Fig. S1), the fungal genera with high abundance showed low connectivity (Supplementary Fig. S2), indicating that interactions among the different fungal genera were relatively weak.

3.5.2. *Variations in microbial taxa between Cd0 and Cd20 soils*

LEfSe analysis was used to identify the dominant microbial taxa (linear discriminant analysis score > 2) between the Cd0 and Cd20 soils (Supplementary Tables S8 and S9). As shown in Fig. 4A and B, the cladogram circles indicate the phylogenetic relationships between the dominant taxa from phylum to species. The numbers of dominant bacterial and fungal taxa in the different rhizospheric soils are shown in Fig. 4C and Fig. 4D, respectively. Generally, more bacterial taxa were enriched in the Cd20 soil than in the Cd0 soil, whereas more fungal taxa were enriched in the Cd0 soil than in the Cd20 soil (Fig. 4C and D). The dynamics of the rhizospheric microbial taxa may be attributed to their different sensitivities or tolerances to Cd and other soil factors. Interestingly, several microbial genera (bacteria or fungi) enriched in the Cd20 soil were plant growth-promoting microorganisms (Fig. 5), suggesting their key roles in regulating Cd tolerance and accumulation in *C. grandiflora* plants.

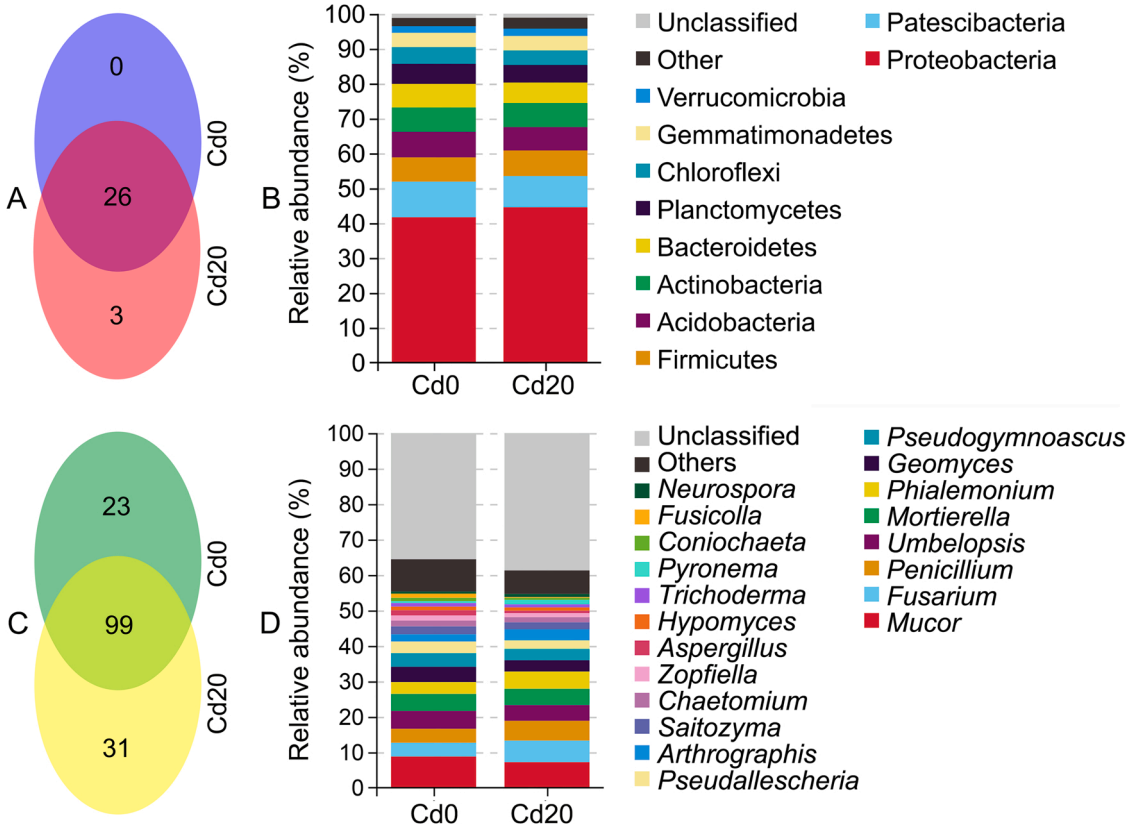

Fig. 3. Rhizospheric microbial community composition of *Coreopsis grandiflora* grown in soils spiked with 0 (Cd0) and 20 mg kg^{-1} Cd (Cd20). (A) Venn diagram of the identified bacterial phyla between the Cd0 and Cd20 soils. (B) Stacked diagram showing the relative abundance of the top 10 bacterial phyla identified in different soils. (C) Venn diagram of the identified fungal genera between the Cd0 and Cd20 soils. (D) Stacked diagram showing the relative abundance of the top 20 fungal genera identified in different soils.

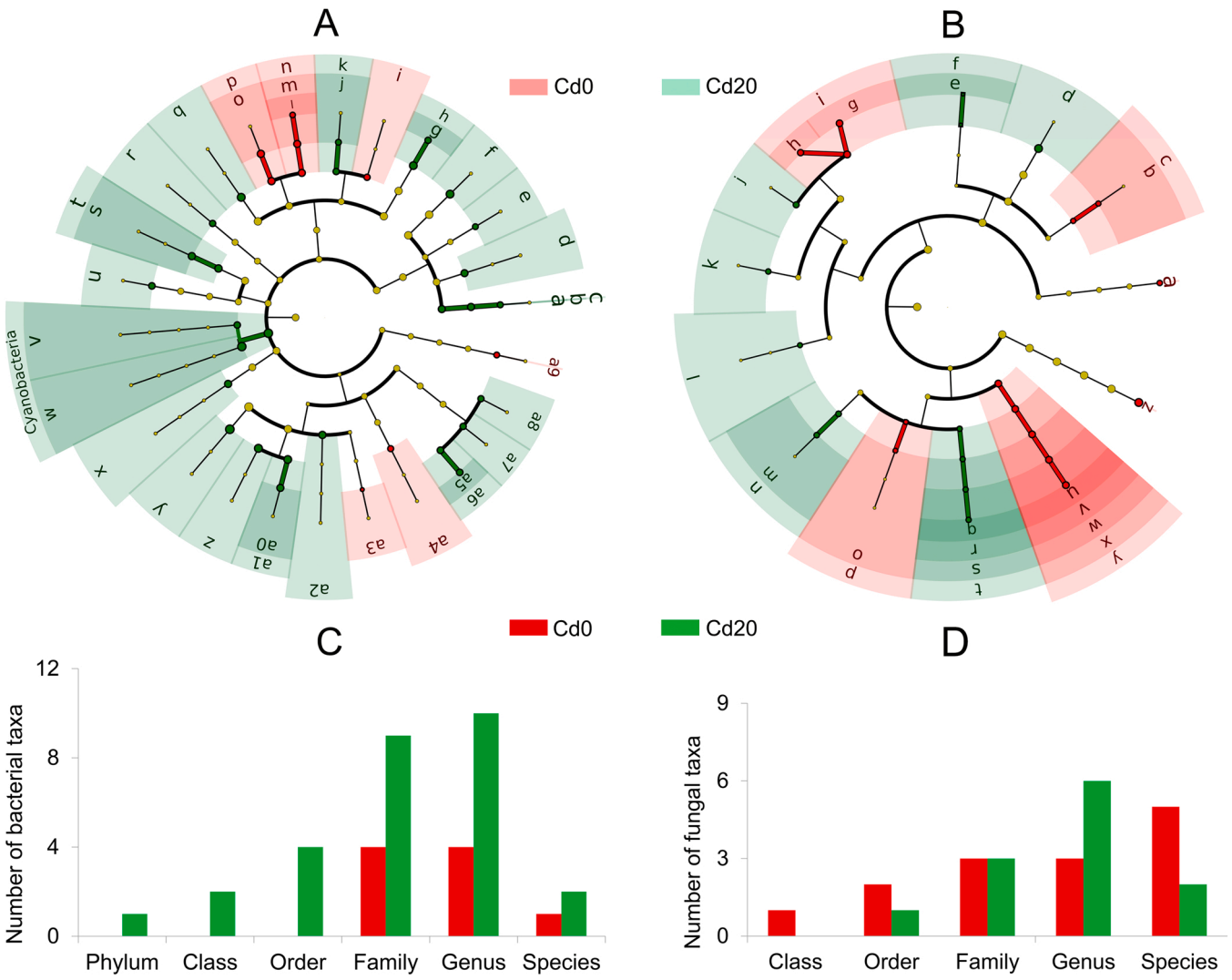

Fig. 4. LEfSe analysis results with linear discriminant analysis scores > 2 between the rhizosphere of *Coreopsis grandiflora* grown in soils spiked with 0 (Cd0) and 20 mg kg^{-1} Cd (Cd20). (A) Cladogram showing dominant bacteria between the Cd0 and Cd20 soils. Identifiers labeled on the cladogram correspond to those in Supplementary Table S8. (B) Cladogram showing dominant fungi between the Cd0 and Cd20 soils. Identifiers labeled on the cladogram correspond to those in Supplementary Table S9. Cladogram circles indicate phylogenetic taxa from phylum to species; the diameter of each circle is proportional to the abundance of the group; only the dominant groups are exhibited in the cladograms (A and B). (C) Numbers of dominant bacteria at different classification levels between the Cd0 and Cd20 soils. (D) Numbers of dominant fungi at different classification levels between the Cd0 and Cd20 soils.

3.5.3. Correlations between soil indices and rhizospheric microorganisms

Interactions between the altered soil indices (i.e., pH, AK, and Cd) (Table 2) and the dominant plant growth-promoting microorganisms with high abundance (Fig. 5) were further analyzed through a correlation network in this study. Several plant growth-promoting microorganisms were significantly correlated with each other (Fig. 6). Soil pH was significantly negatively correlated ($P < 0.05$) with *Flavisolibacter* and *Achromobacter*, whereas soil Cd and AK were significantly positively correlated ($P < 0.05$) with *Nocardioides*, *Flavisolibacter*, *Rhizobium*, and *Penicillium* (Fig. 6; Supplementary Table S10). These results indicate that soil Cd, pH, and AK may directly or indirectly affect changes in the abundance of different plant growth-promoting microorganisms. However, these microorganisms may also be a driver of changes in soil indices (e.g., pH and AK).

4. Discussion

4.1. Cd phytoextraction potential of C. grandiflora

The pot experiments in this study (Fig. 1) indicated that *C. grandiflora* has a relatively strong capacity to tolerate approximately 20 mg kg^{-1} Cd in soils. Although soil properties or Cd forms and bioavailability in different soils may lead to different tolerance limits of plants to Cd, the results still indicate that *C. grandiflora* can cover a wide range of actual Cd-contaminated soils in China according to the statutory limits of 0.3 mg kg^{-1} Cd (GB 15618—2018), suggesting that *C. grandiflora* has a broad application space if used for Cd phytoremediation.

Cd concentration in plant tissues is a direct indicator for understanding the strategies of plants to deal with Cd stress and determine whether they are suitable for phytoremediation. In this study, Cd concentrations in *C. grandiflora* increased considerably with increasing soil Cd concentrations (Fig. 1D), indicating that *C. grandiflora* can employ effective strategies for Cd detoxification until the defense system collapses at higher soil Cd concentrations (e.g., 45 mg kg^{-1}). BCFs and TFs are important indices that reflect Cd absorption and transport capacity in plants. In this study, the Cd BCFs in both the shoots and roots of *C. grandiflora* were greater than one (Fig. 1E), suggesting a strong Cd absorption capacity of *C. grandiflora*. These results are consistent with those of previous reports on other *Coreopsis* species (Lin et al., 2016; Xu et al., 2018). Notably, the increased Cd BCFs of *C. grandiflora* with

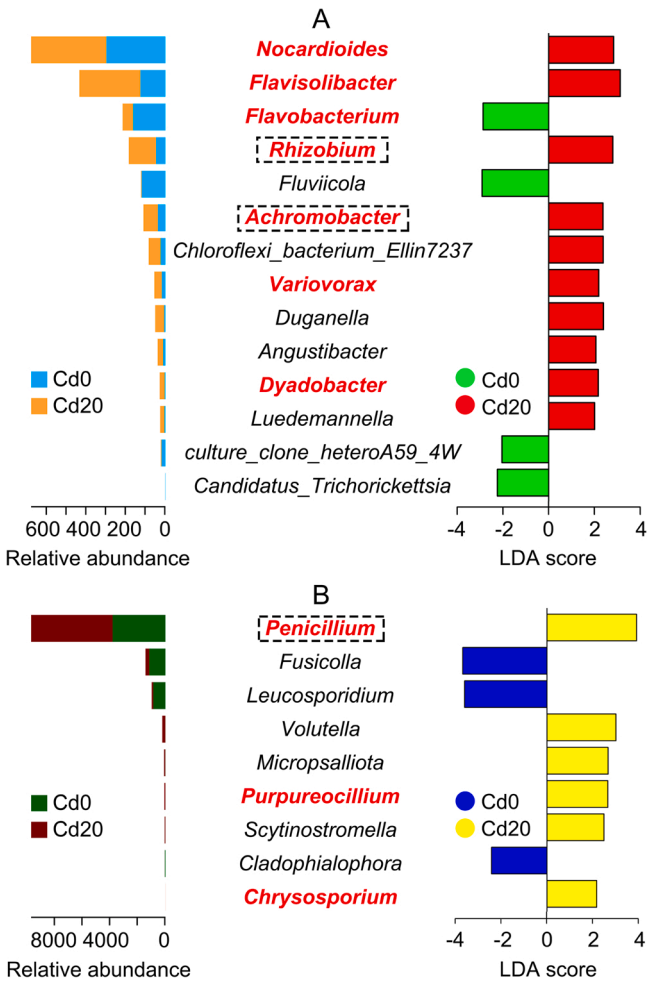

Fig. 5. Relative abundance of the dominant bacteria (A) and fungi (B) at genus level in rhizospheric soils of *Coreopsis grandiflora* grown in soils spiked with 0 (Cd0) and 20 mg kg^{-1} Cd (Cd20). Microbial genera in red bold font indicate potential plant growth-promoting microorganisms, and microbial genera labeled with black dashed frames indicate microorganisms involved in affecting Cd phytoextraction in plants and solubilizing potassium.

increasing soil Cd concentrations are contrary to those of several previous studies (Wang et al., 2012; Wu et al., 2018), indicating the special Cd accumulation characteristics of *C. grandiflora*, the mechanisms of which are worthy of future research. The Cd TFs of *C. grandiflora*, which were lower than one (Fig. 1F), were lower than those of other reported *Coreopsis* species (Lin et al., 2016; Xu et al., 2018). This may be because the shoot biomass of *C. grandiflora* was several times larger than the root biomass (Fig. 1C), leading to a greater dilution of Cd concentrations in the shoots. The decrease in Cd TFs with increasing soil Cd concentrations (Fig. 1F) suggests that Cd transport rates from roots to shoots in *C. grandiflora* plants may be inhibited by increasing Cd concentrations, which is consistent with previous studies (Eisazadeh et al., 2019; Li et al., 2021b).

Based on the values of shoot Cd concentrations, shoot BCFs, and TFs, *C. grandiflora* does not meet all the characteristics of Cd hyperaccumulators (shoot Cd concentration > 100 mg kg^{-1}, shoot BCF > 1, and TF > 1) (Li et al., 2018); however, this species can be designed as a Cd accumulator. The total Cd content and distribution, which depend on both the Cd concentration and biomass yield of plants, determines the phytoremediation type and efficiency of plants. In this study, more than 85% of the total Cd accumulated in *C. grandiflora* shoots at each soil Cd concentration (Fig. 1H). These results indicated that *C. grandiflora* can potentially be used for Cd phytoextraction from a suitable range of Cd-contaminated soils. However, actual soils are usually not as rich in organic matter and nutrients as the soils used in this study, and plant growth conditions in the field may be worse than those in the greenhouse. These differences indicate that the plant growth rate and Cd accumulation capacity of *C. grandiflora* in actual soils may be reduced. Therefore, the Cd phytoextraction potential of *C. grandiflora* should be further verified in actual Cd-contaminated soils. In addition, because polluted soils generally contain multiple heavy metals, the phytoextraction potential of *C. grandiflora* for other heavy metals should be evaluated.

4.2. Physiological Cd detoxification mechanism in *C. grandiflora* plants

Uncovering the mechanisms of Cd tolerance and accumulation in *C. grandiflora* is potentially useful for improving the phytoextraction efficiency. In plants, one of the main consequences of Cd toxicity is excessive generation of ROS, which can lead to membrane system damage and cell injury if they are not scavenged in time (Song et al., 2016). The induced antioxidant systems (i.e., antioxidant enzymes and non-enzymatic antioxidants) can effectively regulate ROS homeostasis under Cd stress (Li et al., 2021a, 2021c). For example, SOD catalyzes the dismutation of superoxide radicals (O_2^-) to decompose into O_2 and H_2O_2, and H_2O_2 can be further decomposed into H_2O and O_2 under CAT catalysis (Faizan et al., 2021; Mahawar et al., 2021). Moreover, POD can catalyze the oxidation of substrates (e.g., phenols and amines) using H_2O_2 as an electron acceptor (Chen et al., 2021b; Yue et al., 2021), leading to the elimination of H_2O_2. In this study, the higher activities of SOD, POD, and CAT ($0.01 < P < 0.05$ or $P < 0.001$) in the *C. grandiflora* leaves in the Cd20 soil compared with those in the Cd0 soil (Fig. 2A–C) suggest that they are induced to control ROS accumulation in the *C. grandiflora* plants under Cd stress. The unchanged concentrations of the lipid peroxidation product MDA, which acts as an indicator of the degree of oxidative stress (Hnilickova et al., 2021), between the Cd0 and Cd20 groups (Fig. 2D) further proved the effectiveness of the antioxidant system in *C. grandiflora* leaves.

Free amino acids in plants can contribute to the detoxification of toxic elements by regulating ion transport and chelation and by functioning as osmoregulators (Okunev, 2019; Lwalaba et al., 2020; Kocaman, 2022). In this study, the concentrations of serine, valine, methionine, isoleucine, leucine, histidine, and proline in *C. grandiflora* shoots significantly increased under Cd stress (Table 1), which is partially consistent with several previous studies (Li et al., 2018; Kato et al., 2020). Several of these amino acids play important roles in plant Cd tolerance. Proline participates in Cd detoxification through its direct function (e.g., adjusting osmotic potential and serving as an antioxidant and chelator) or through the biosynthesis of chelating peptides (Borgo et al., 2021; García de la Torre et al., 2022; Lwalaba et al., 2020). Our results showed that proline was the most abundant free amino acid detected in this study, and its abundance was significantly elevated ($P < 0.001$) under Cd stress (Table 1), indicating its key role in coping with Cd toxicity in *C. grandiflora*. Histidine and methionine also play important roles in plant responses and adaptations to Cd stress (Zemanova et al., 2014; Kato et al., 2020). Histidine participates in chelation and transport of metal ions (Xu et al., 2012a; Kato et al., 2020). Xu et al. (2012) found that a high accumulation of histidine promotes Cd uptake and root-to-shoot transport in *Solanum* species. Methionine, as the precursor of compounds associated with metal homeostasis (e.g., nicotinamide) and antioxidant defense (e.g., polyamines), is also likely to be involved in protecting plants against Cd toxicity (Kato et al., 2020). The increase in histidine and methionine concentrations ($0.001 < P < 0.01$ or $0.01 < P < 0.05$) in our study (Table 1) indicates their significance in the response of *C. grandiflora* to Cd. Additionally, increased concentrations of serine, valine, isoleucine, and leucine have also been found in other plants exposed to Cd (Li et al., 2018; Kocaman, 2022); however, the roles of these amino acids in plant responses to Cd stress are not yet clearly understood.

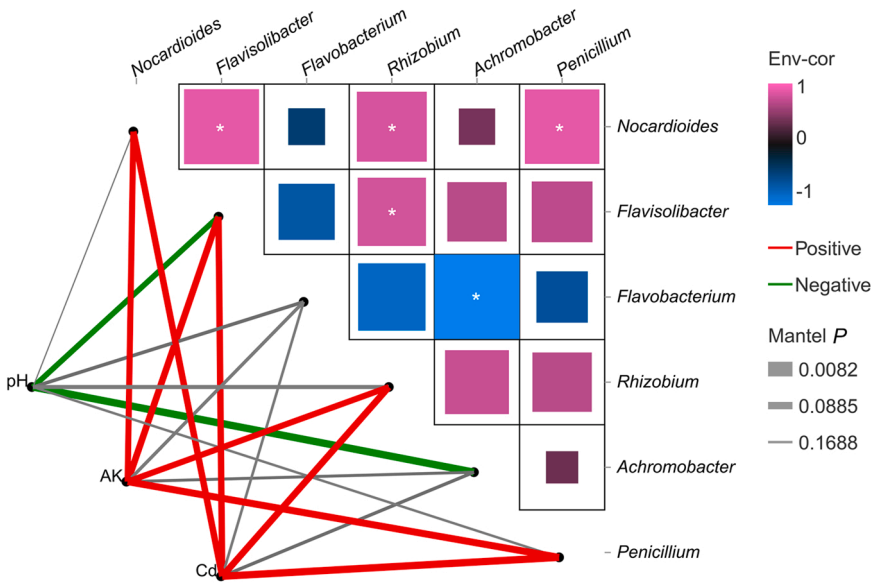

Fig. 6. Correlation network between soil indices and important plant growth-promoting microorganisms. The color block size in the correlation heatmap indicates the absolute value of the correlation coefficient. * represents $P < 0.05$. The red and green network lines indicate significantly positive correlations and significantly negative correlations ($P < 0.05$), respectively.

In summary, the antioxidant system and amino acid metabolism may play important roles in Cd detoxification in *C. grandiflora*, which conforms to the unchanged plant biomass observed under Cd stress.

4.3. *Effects of the rhizospheric microenvironment on the response of C. grandiflora to Cd*

The rhizosphere is the most dynamic area where roots interact with heavy metals and soils (York et al., 2016), which determines heavy metal tolerance and accumulation in plants. In this study, the rhizosphere pH of *C. grandiflora* significantly decreased ($P < 0.05$) under Cd stress (Table 2), which is consistent with the decreased pH of the rhizosphere of *Salvia tiliifolia* under Cd stress (Li et al., 2021b). The potential reason for the decrease in pH may be that Cd changes the ion balance in the rhizosphere or induces root and/or rhizosphere microorganisms to secrete more organic acids (Mo et al., 2022). With a decrease in pH, excess H^+ can be exchanged with other adsorbed metal cations, leading to an increase in the availability of some metals (Mo et al., 2022). This mechanism can also partially explain the increase in AK concentration in the Cd20 soil (Table 2). An increase in AK concentration can improve the nutrition and vitality of *C. grandiflora* plants under Cd stress, which enhances plant tolerance to Cd. Moreover, soil AK concentration is closely related to Cd migration and transportation in soil–plant systems (de Anicésio and Monteiro, 2019; He et al., 2021; Shi et al., 2020). For example, de Anicésio and Monteiro (2019) found that K fertilizers can increase NH_4NO_3-extractable Cd concentrations in soils and enhance Cd accumulation in plants. He et al. (2021) reported that enhanced K^+ concentrations induce the expression of Cd^{2+}-transporting proteins and inhibit the expression of Cd^{2+} efflux proteins, leading to an increase in Cd uptake by *Microcystis aeruginosa*. These studies suggest that an increase in AK likely contributes to Cd uptake by *C. grandiflora*. In this study, rhizospheric HN and AP concentrations did not differ between Cd0 and Cd20 soils (Table 2), indicating a dynamic equilibrium for nitrogen and phosphorus cycles.

Rhizosphere-associated microorganisms play an important role in promoting heavy metal tolerance and accumulation in plants (Hakim et al., 2021). In this study, the differences in the rhizospheric microbial communities between Cd0 and Cd20 soils were compared to understand their role in plant growth and Cd accumulation characteristics of *C. grandiflora* based on 16S/ITS high-throughput sequencing.

The similar alpha indices (Supplementary Tables S4 and S5) indicated that there was no significant difference in the richness and diversity of both bacterial and fungal communities between the two soils, which was consistent with a previous study (Li et al., 2021b). Most of the dominant bacterial phyla (e.g., Proteobacteria, Acidobacteria, Actinobacteria, Chloroflexi, Gemmatimonadetes, and Verrucomicrobia) in the rhizosphere of *C. grandiflora* (Fig. 3B) have often been identified in the rhizosphere of plants grown in heavy metal-polluted soils (Jiang et al., 2019; Li et al., 2021b). The dominant fungal genera identified in the rhizosphere of *C. grandiflora* (Fig. 3D) were distinct from those identified in the rhizospheres of other plants in heavy metal-polluted soils (Chen et al., 2021c; Solis-Hernandez et al., 2022), which may be attributed to multiple factors.

LEfSe analysis showed that the abundance of many microbial taxa differed between the Cd0 and Cd20 soils (Fig. 4), which may be related to Cd and the soil environments remolded by Cd (Li et al., 2021b). Interestingly, the abundance of many important plant growth-promoting microorganisms, including *Nocardioides*, *Flavisolibacter*, *Rhizobium*, *Achromobacter*, *Variovorax*, *Dyadobacter*, *Penicillium*, *Purpureocillium*, and *Chrysosporium* (Hamayun et al., 2009; Kumar et al., 2018; Baron et al., 2020; Efe, 2020; Chen et al., 2021d; Imran et al., 2021; Kumawat et al., 2022), was enriched in the Cd20 soil, whereas only one plant growth-promoting bacterial taxon, *Flavobacterium*, was enriched in Cd0 soil (Fig. 5). Plant growth-promoting microorganisms allow plants to resist extreme heavy metal concentrations through multiple mechanisms (Khanna et al., 2019a, 2019b; Yang et al., 2022b), indicating that the presence of these Cd-tolerant plant growth-promoting microorganisms, especially those with relatively high abundance (i.e., *Nocardioides*, *Flavisolibacter*, *Rhizobium*, and *Penicillium*) (Fig. 5), in the rhizosphere assists *C. grandiflora* in tolerating Cd stress. These results were similar to those reported for other plants (Wang et al., 2020; Li et al., 2021b; Yang et al., 2022a). However, the plant growth-promoting microorganisms recruited to the rhizosphere vary greatly in different soil–plant systems (Wang et al., 2020; Li et al., 2021b; Yang et al., 2022a). This difference may be partially attributed to the different soil types and/or specific root exudates of different plants.

Many plant growth-promoting microorganisms (e.g., *Rhizobium*, *Achromobacter*, and *Penicillium*) can improve metal solubility and ameliorate soil physicochemical conditions to maximize phytoremediation (Wang et al., 2021; He et al., 2022). *Rhizobium* can improve

the absorption of various heavy metals (e.g., Cd) by plants by releasing small organic molecules (e.g., phytosiderophores) to form complexes with them (Sahito et al., 2022). However, several *Achromobacter* species and strains can effectively adsorb and immobilize Cd (Zhang et al., 2019; Liang and Hu, 2021). *Penicillium* is a common Cd-tolerant fungus, and many species can adsorb Cd to remediate Cd-polluted soils (Xu et al., 2012b, 2015; Yuan et al., 2012). The good Cd adsorption characteristics of *Penicillium* species indicate that these fungi tend to stabilize soil Cd and reduce Cd uptake by plants. However, *Penicillium janthinellum* ZZ-2 promotes plant growth and Cd uptake by producing indole acetic acid or solubilizing Cd in soils (Xie et al., 2021). These studies indicate that *Rhizobium*, *Achromobacter*, and *Penicillium* enriched in Cd20 soil may facilitate Cd uptake by *C. grandiflora* plants. These potential plant growth-promoting microorganisms in the rhizosphere of *C. grandiflora* need to be screened and identified to explore their functions, which are helpful in assisting Cd phytoextraction by *C. grandiflora* and other plants.

Recruiting plant growth-promoting rhizosphere microorganisms is an important strategy for heavy metal accumulators in response to heavy metal stress (Wang et al., 2020; Chen et al., 2021d; Yang et al., 2022a). However, the mechanisms underlying this process are not clearly understood, as changes in rhizosphere microbial community composition are determined by multiple factors (Islam et al., 2022). In this study, the correlation analysis (Fig. 6) indicated that soil Cd concentration, pH, and AK concentration may have significant effects ($P < 0.05$) on different plant growth-promoting microorganisms (Fig. 6). For example, Cd stress had a selective effect on these Cd-tolerant plant growth-promoting microorganisms (e.g., *Nocardioides*, *Flavisolibacter*, *Rhizobium*, and *Penicillium*), leading to significantly positive correlations ($P < 0.05$) between them (Fig. 6). Significantly positive correlations ($P < 0.05$) between AK and *Rhizobium* and *Penicillium* abundance values indicate that these microorganisms may effectively solubilize potassium (Sattar et al., 2019). In addition, correlations between these microorganisms (Fig. 6; Supplementary Figs. S1 and S2) indicated that they interacted with each other. However, direct drivers of the dynamics of these plant growth-promoting microorganisms are difficult to determine and require definite evidence.

5. Conclusions

In this study, Cd tolerance and accumulation characteristics of *C. grandiflora* were investigated. *C. grandiflora* tolerated up to 20 mg kg^{-1} Cd in soils and was designed as a Cd accumulator based on appreciable Cd BCFs (shoot: 1.09–1.85, root: 1.13–3.98) and TFs (0.46–0.97) in soils spiked with different concentrations of Cd. Combined with its large biomass, *C. grandiflora* shows potential for Cd phytoextraction in Cd-polluted soils. The physiological response of *C. grandiflora* to Cd was also investigated. The increased activities of antioxidant enzymes (i.e., SOD, POD, and CAT) in *C. grandiflora* leaves grown in Cd20 soil indicated that the antioxidant system was activated to prevent oxidative stress, leading to the stabilization of the lipid membrane peroxidation product MDA. Moreover, various free amino acids (e.g., proline, histidine, and methionine) in *C. grandiflora* may play important roles in Cd detoxification. The 16S rDNA/ITS high-throughput sequencing results showed that the abundance of a few rhizospheric microorganisms was altered in the Cd20 soil compared with that in the Cd0 soil, although the overall bacterial and fungal richness and diversity remained similar between the two soils. Many plant growth-promoting microorganisms that were enriched in the Cd20 soil, such as *Nocardioides*, *Flavisolibacter*, *Dyadobacter*, *Rhizobium*, *Variovorax*, *Achromobacter*, *Penicillium*, *Purpureocillium*, and *Chrysosporium*, likely contributed to the plant growth and vitality of *C. grandiflora* under Cd stress. Additionally, some microorganisms (e.g., *Rhizobium*, *Achromobacter*, and *Penicillium*) may facilitate Cd uptake by *C. grandiflora*. Abundant plant growth-promoting microorganisms in the rhizosphere of *C. grandiflora* showed potential interactions with soil pH and concentrations of Cd and AK. Notably, potassium-solubilizing microbes (e.g., *Rhizobium* and *Penicillium*) effectively solubilize potassium to assist Cd uptake by *C. grandiflora*. In this study, we identified a new Cd accumulator, *C. grandiflora*, for Cd phytoextraction and revealed the potential Cd tolerance mechanism of *C. grandiflora* grown in Cd-polluted soils. However, some of the unclear problems derived from this study need to be explored further. For example, the phytoextraction potential of *C. grandiflora* in natural soils requires further verification. Moreover, the accumulation characteristics of other heavy metals in *C. grandiflora* are worth investigating. Additionally, potential rhizospheric plant growth-promoting microorganisms can be screened and identified to assist Cd phytoremediation.

Funding

This study was financially supported by the Youth Innovation Promotion Association CAS (2020387).

CRediT authorship contribution statement

Xiong Li: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization, Funding acquisition. **Boqun Li:** Investigation, Writing – review & editing. **Yan Zheng**: Investigation, Writing – review & editing. **Landi Luo**: Investigation, Writing – review & editing. **Xiangshi Qin**: Investigation. **Yongping Yang**: Writing – review & editing, Supervision. **Jianchu Xu**: Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.113739.

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