The addition of exogenous low-molecular-weight organic acids improved phytoremediation by Bidens Pilosa L. in Cd-contaminated soil

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Research Article

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Abstract

Enhancing the uptake and enrichment of heavy metals in plants is one of the important means to strengthen phytoremediation. In the present study, citric acid (CA), tartaric acid (TA), and malic acid (MA) were applied to enhance phytoremediation by *Bidens Pilosa* L. in Cd-contaminated soil. The results showed that by the addition of appropriate concentrations of CA, TA, and MA, the values of the bioaccumulation factor increased by 77.98%, 78.33%, and 64.49%, respectively, the transport factor values increased by 16.45%, 12.61%, and 5.73%, respectively, and the values of the phytoextraction rates increased by 169.21%, 71.28%, and 63.11%, respectively.

The minimum fluorescence values of leaves decreased by 31.62%, 0.28%, and 17.95%, while the potential efficiency of the PSII values of leaves increased 117.87%, 2.25%, and 13.18%, respectively, when CA, TA, and MA with suitable concentration were added. Redundancy analysis showed that CA and MA in plants were significantly positively correlated with plant growth, photosynthesis, and other indicators, whereas TA showed a negative correlation with most indicators. Moreover, CA addition could significantly increase the abundances of *Azotobacter*, *Pseudomonas*, and other growth-promoting bacteria, and the abundance values of *Actinophytocola* and *Ensifer* were improved in TA treatments. Therefore, our results demonstrated that low-molecular-weight organic acids could enhance phytoremediation, and exogenous CA could significantly improve the phytoremediation of Cd-contaminated soil by *Bidens Pilosa* L.

1. Introduction

Soil pollution by the metal heavy cadmium (Cd) has become more serious due to the rapid development of mining, metal smelting, waste treatment, and other industries (Dai *et al.*, 2021). Cadmium, as a permanent and non-biodegradable pollutant, can disturb the natural ecosystem balance and is toxic, carcinogenic, mutagenic, and teratogenic at higher concentrations. Additionally, plants can absorb and accumulate large amounts of Cd, posing a serious threat to human health through enrichment in the food chain (Si *et al.*, 2021). Among various methods used to remove Cd from the soil, phytoremediation is currently receiving increased attention (Niu *et al.*, 2021). However, the remediation efficiency can't be significantly improved since Cd hyper-accumulators such as *Thlaspi arvense* L., *Sedum sinense*, and *Brassica juncea* have a small biomass and poor adaptability (Gurajala *et al.*, 2019; Pence *et al.*, 2000; Pham *et al.*, 2013). *Bidens Pilosa L.*, as a Cd hyper-accumulator with the advantage of large biomass production and a short growth cycle, has gradually attracted the attention of Chinese researchers in recent years (Dai *et al.*, 2017; Wei *et al.*, 2008).

The form, migration, transformation, and bioavailability of Cd can affect the phytoremediation efficiency, which can be significantly enhanced by activating Cd difficult to be absorbed in the soil. Although artificial chelating agents can greatly activate soil Cd, the addition of ethylenediamine tetraacetic acid (EDTA) and diethylenetriaminepentaacetic acid (DTPA) not only destroys the original soil structure but also results in secondary contamination (Kanwal *et al.*, 2014). The application of EDTA could increase the absorption capacities of Cu and lead (Pb) by *Echinochloa Crusgallis*, but with the drawback of inhibiting plant stem and leaf growth (Kim *et al.*, 2010). To avoid these risks, it is recommended to use biodegradable chelating agents such as n,n-bis(carboxymethyl)-l-glutamic acid tetrasodium salt (GLDA) and ethylenediamine disuccinic acid (EDDS), but the high costs limit their application in restoration projects (Guo *et al.*, 2018).

Low-molecular-weight organic acids (LMWOAs), with low toxicity and biodegradability, can form chelates with heavy metals in the soil to increase metal mobility, thereby improving the phytoremediation efficiency (Almaroai, 2012; Han *et al.*, 2021; Kim and Lee, 2010). In a previous study, the application of citric acid (CA) could increase sunflower biomass and effectively improve the uptake and translocation of uranium (U) and Cd (Chen, 2020). Similarly, the addition of CA, oxalic acid (OA), and tartaric acid (TA) could increase the copper (Cu) concentration in the plant body of castor beans (Huang *et al.*, 2020), whereas gallic acid (GA) could increase the metal content of *Brassica juncea* and induce the removal of Cd, zinc (Zn), Cu, and nickel (Ni) from the soil without increasing the leaching risk (Nascimento *et al.*, 2006). These findings suggest that LMWOAs are safe, environmentally friendly, biodegradable, and can be used as reasonable metalchelating agents.

Although many studies suggest that exogenous LMWOAs can promote phytoremediation, there are few studies on whether LMWOAs can promote Cd absorption and accumulation by *Bidens Pilosa* L. In particular, most studies have neglected the role of microbial community characteristics and soil enzyme activities in phytoremediation, and the effects of LMWOA addition on plant photosynthesis have also been largely ignored. Moreover, the influences of LMWOA concentration in plants on the physiological characteristics (e.g., photosynthesis) and the correlation between the concentration and various indicators are not fully understood. In the present study, we enhanced the capacity of *Bidens Pilosa* L. to remediate Cd-contaminated soil by adding exogenous LMWOAs. The main objectives were as follows: (1) to investigate the effects of exogenous LMWOAs on various physiological and biochemical characteristics of *Bidens Pilosa* L. in Cd-contaminated soil and to clarify the correlation between the contents of LMWOAs in plants and various physiological indicators; (2) to evaluate whether the application of exogenous LMWOA can enhance the remediation ability of *Bidens Pilosa* L. in Cd-contaminated soil; (3) to explore the effects of exogenous LMWOA addition on the rhizosphere microbial composition and enzyme activities and to analyze the correlation between these factors and the contents of LMWOAs in the roots.

2. Materials And Methods

2.1 Soil sample preparation

The experimental soil was taken from the wheat field in Tongshan County, Xuzhou City, Jiangsu Province, China (N34.18, E117.17). The surface soil (0–20 cm) was sampled consistently, and rocks, plant residues, and other impurities were carefully removed. After drying, the soil was passed through a 2-mm nylon sieve and stored for subsequent experimental use. The physical and chemical properties of the soil were determined and were as follows: total Cd concentration, 0 mg/kg; pH value, 6.19; organic matter, 1.39%; available phosphorus and potassium, 15 and 20 mg/kg, respectively. Subsequently, $CdCl_{2*}2.5H_2O$ was added to the soil to simulate Cd contamination. Briefly, the water solution containing Cd was mixed thoroughly with the appropriate amount of soil, and the final Cd concentration of the soil was 32 mg/kg. The mixed and polluted soil was left for 60 days to balance the aging of soil contaminants. All reagents used in this study were of analytical grade and were purchased from Zhejiang Changqing Chemical Co., Ltd.

2.2 Experimental design

Bidens Pilosa L., an annual herb reaching a height of 30–100 cm, is a potential hyper-accumulator of Cd and widely used to remediate Cd-polluted soil in China. The soil prepared as above was used for the pot experiment. First, approximately 1000 g of dry contaminated soil was placed in a plastic pot with a diameter of 17 cm and a height of 8.5 cm, and a tray was placed at the bottom for leachate reflux to prevent the loss of Cd and nutrients. Subsequently, seeds of *Bidens Pilosa* L. were soaked and disinfected in 70% alcohol for 10 minutes, followed by washing with purified and sterile water. Eight seeds with the same shape and size were sown in each pot, and the surface layer was covered with 1.5 cm of soil. The plants were cultured in a growth chamber with fluorescent light with an intensity of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at a photoperiod of 12 hours of light and 12 hours of dark. The maximum and minimum culture temperatures were 25 and 20°C, respectively, at a relative air humidity of 65%. At 2 weeks after germination, three seedlings with similar size were kept in each pot. During cultivation, the soil moisture content was maintained at 60%, and water was added every 5 days (40 mL each time).

In the experimental group, CA, TA, and malic acid (MA) were used as the three exogenous LMWOAs at concentrations of 1, 5, and 15mmol/kg, respectively. The LMWOAs were added to the soil in the form of an aqueous solution. In the first sowing, organic acid solution with an appropriate concentration was mixed into the soil. During the entire growth cycle of the plant, a total of three applications of LMWOAs were conducted at a frequency of 15 days. Additionally, Cd-free (CKK) and Cd-contaminated (CK) soils were used as controls with water instead of LMWOAs. Our study involved a total of 11 sets of experiments, each containing five replicates, with a total of 55 pots. After 6 weeks of treatment, plants were harvested for further analysis.

2.3 Measurement of plant physiological characteristics

The content of chlorophyll in fresh leaves was determined via the anhydrous ethanol extraction method (Parker *et al.*, 2016). The activities of superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) levels of the plant were determined by NBT colorimetry, UV spectrophotometry, and the guaiacol method, respectively (Lu *et al.*, 2009). The content of malondialdehyde (MDA) was measured via the thiobarbituric acid (TBA) method (Heath, 1968). The glutathione (GSH) content was determined using 5,5-dithio-2,2-dinitrobenzoie acid (DTNB) (Foyer *et al.*, 1976), and the total soluble protein (Tsp) was measured by Coomassie Bright Blue (G-250) staining (Maehre *et al.*, 2018). The content of proline (Pro) was determined by the sulfosalicylic acid method (Banafsheh Noein, 2021), and the levels of CA, MA, and TA in plant leaves and roots were analyzed using high-performance liquid chromatography (Montiel-Rozas *et al.*, 2016).

2.4 Chlorophyll fluorescence parameter measurement

Chlorophyll fluorescence parameters and fast light response curves were obtained by using an imaging pulse amplitude-modulated fluorometer (IMAG-max/L; Walz, Germany). The samples of the experimental group were adapted to dark conditions for 20 minutes, and subsequently, three leaves were picked from the plants of each experimental group and placed in a pulse amplitude-modulated fluorometer for measurement. Chlorophyll fluorescence parameters of PSII, including minimum fluorescence (F0) and maximum fluorescence (Fm) were measured, and the potential efficiency of the PSII (Fv/F0) and the maximum quantum efficiency of PSII photochemistry (Fv/Fm) were determined (Liu *et al.*, 2018). The fast light response curves included the actual photochemical quantum yield (Y(II)), adjusting quantum yield for energy dissipation (Y (NPQ)), quantum yield of unregulated energy dissipation (Y (NO)), and the photosynthetic electron transfer rate (ETR)(Kim *et al.*, 2014; Lefebvre *et al.*, 2011); we used the following equations:

$$F_v = F_m - F_0$$

$$F_v / F_0 = (F_{\mathrm{m}} - F_0) / F_0$$

$$F_v / F_{\mathrm{m}} = (F_{\mathrm{m}} - F_0) / F_{\mathrm{m}}$$

$$\mathrm{Y(II)} = \Delta F_v / F_m^{'} = (F_m^{'} - F_{\mathrm{t}}) / F_m^{'}$$

$$\mathrm{ERT} = \mathrm{PFD} \times \mathrm{AF} \times \Delta\mathrm{F} / \mathrm{F}_{\mathrm{m}^{'}(\mathrm{MT})} \times F\mathrm{II}$$

$$\mathrm{Y(NPQ)} = 1 - \mathrm{Y(II)} - 1 / (\mathrm{NPQ} + 1 + \mathrm{qL}(\frac{F_{\mathrm{m}}}{F_0} - 1))$$

$$\mathrm{Y(NO)} = 1 / (\mathrm{NPQ} + 1 + \mathrm{qL}(\frac{F_{\mathrm{m}}}{F_0} - 1))$$

2.5 Determination of heavy metal contents

2.5.1 Cd determination in plants

The *Bidens Pilosa* L. plants were harvested in the 10th week of growth for Cd determination. We separated the aboveground from the belowground parts and washed, dried, and weighted the parts in preparation for the Cd determination. Subsequently, the dried tissue was placed in 10 mL 30% HNO_3 and digested on an electric heating plate at 180°C (Lu *et al.*, 2020). After digestion, the mixture was diluted with ultrapure water when the liquid was colorless and transparent. All glassware used in the experiments was soaked in 30% HNO_3 for 24 h and rinsed with ultrapure water before using. The Cd concentration in plants was determined by flame atomic absorption spectrometry with an FAAS instrument (iCE3300, Thermo Scientific).

2.5.2 Cd speciation distribution analysis

The form of Cd in soil was analyzed by the European Community Bureau of Reference (BCR) continuous extraction method (Wu *et al.*, 2016); 0.11 M CH_3COOH, 0.5 M $NH_2OH{\cdot}HCl$, 30% hydrogen peroxide (H_2O_2), and 1.0 M CH_3COONH_4 were used as acid, reducible, and oxidizable Cd extraction liquids, respectively. After digestion, the solution was diluted with ultrapure water to a constant volume and filtered through a 0.22 μm filter membrane. The concentration of Cd in the solution was determined by flame atomic absorption spectrometry with an FAAS instrument (iCE3300, Thermo Scientific).

2.6 Parameter calculation

The Cd bioaccumulation factor (BCF) is the ratio of the Cd concentration in *Bidens Pilosa* L. (mg kg^{-1} dry weight) to the Cd concentration in the soil (mg kg^{-1}). The transport factor (TF) is the ratio of the Cd concentration in the shoots to that in the roots (mg kg^{-1} dry weight). The phytoextraction rate (PR) is the ratio of the Cd content in the whole plant to that in the soil (Yang *et al.*, 2020).

2.7 Determination of soil enzyme activity

Soil urease, sucrase, protease, catalase, and phosphatase activities were analyzed by the sodium phenol colorimetric, 3,5-dinitrosalicylic acid colorimetric, ninhydrin colorimetric, and potassium permanganate titration and phenyl disodium phosphate colorimetry method, respectively (Ge *et al.*, 2009).

2.8 Soil DNA extraction, 16s RNA amplification, and high-throughput sequencing

Microbial DNA was extracted using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.), and concentration and purity were determined with a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA). The hypervariable region V3–V4 of the bacterial 16S rRNA gene was amplified with primer pairs 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') by an ABI GeneAmp® 9700 PCR thermocycler (ABI, CA, USA). Purified amplicons were pooled in equimolar ratios and paired-end sequenced on an Illumina MiSeq PE300 platform/NovaSeq PE250 platform (Illumina, San Diego, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

2.9 Statistical analysis

For one-way ANOVA, the IBM SPSS Statistics 20 was used and the significance of the treatments was evaluated by Student's t test ($n = 3$, $p \leq 0.05$). The correlation between various soil enzyme activities, plant physiological indicators, and the concentration of LMWOAs in the plant body was assessed using partial redundancy analysis (RDA) in CANOCO 5 ($n = 3$, $p \leq 0.05$). The Origin 2018 software was used for the drawing of graphs.

3. Results

3.1 Plant growth and biomass production

Plant leaf area increased with increasing CA concentration and decreased with increasing TA and MA concentrations (Fig. 1). Under Cd stress (CK), root, shoot, and plant biomass decreased by 64.66%, 24.86%, and 39.19%, respectively. After the application of CA at different concentrations, root, shoot, and plant biomass increased significantly compared with CK, namely by 153.96%, 38.39%, and 64.29%, respectively, with the addition of 15 mmol/kg CA. However, the addition of TA and MA at low concentrations significantly facilitated plant growth. Root, shoot, and plant biomass increased by 107.74%, 14.27%, and 31.86%, respectively, when 1 mmol/kg TA was added, and increased by 57.86%, 38.26%, and 45.03%, respectively, after the addition of 1 mmol/kg MA compared with CK. Low concentrations of CA, TA, and MA could also stimulate plant stem growth, which increased by 29.81%, 28.45%, and 49.81%, respectively, under the addition of 1 mmol/kg CA, TA, and MA.

3.2 Chlorophyll fluorescence parameters and fast light response curves

The addition of LMWOAs with higher concentration could significantly improve the plant chlorophyll content **(Fig. S1)**. The F0 and Fm values under Cd stress increased by 74.63% and 6.33%, respectively, whereas the Fv/Fm and Fv/F0 values decreased by 31.53% and 63.84%, respectively (Fig. 2a). The addition of CA could reduce the values of F0 and Fm and elevate those of Fv/Fm and Fv/F0. At a dose of 15 mmol/kg CA, the values of F0 and Fm decreased by 31.62% and 39.68%, whereas the Fv/Fm and Fv/F0 values were 1.36 and 2.18 times those of CK. The values of F0 and Fm increased continuously, and those of Fv/Fm and Fv/F0 decreased gradually with the application of TA and MA from low to high concentrations. Compared with CK, the F0 and Fm values at 15 mmol/kg TA increased by 13.68% and 65.78%, whereas the Fv/Fm and Fv/F0 values decreased by 13.04% and 23.14%, respectively. Meanwhile, the values of F0 and Fm increased by 25.64% and 55.57%, and that of Fv/Fm and Fv/F0 decreased by 6.52% and 12.31% with the addition of 15 mmol/kg MA. According to the Fv/Fm image (Fig. 2a), CKK was dark blue without Cd stress, and CK showed a light green color under Cd stress. With the application of CA, TA, and MA from low to high concentrations, the blue color deepened in the CA treatment, whereas the green color deepened in the treatments with TA and MA.

The fast light response curves were also measured, and the values of ETR, Y(II), and Y(NPQ) decreased by 39.61%, 35.60% and 30.70%, respectively, at the light intensity of 231 μmol $m^{-2}·s^{-1}$ under Cd stress (Fig. 2b, 2c, 2d, 2e). The addition of CA increased the values of ETR, Y(II), and (NPQ) by 53.50%, 51.42%, and 38.70%, respectively, at 15 mmol/kg CA. However, the application of TA and MA at low concentrations increased the values of ETR, Y(II), and Y(NPQ) by 4.35%, 9.29%, and 1.57%, respectively, when 1 mmol/kg TA was added and by 17.96%, 18.81%, and 1.59%, respectively, for 1 mmol/kg MA compared with CK. With increasing CA concentrations, the Y(NO) value decreased gradually by 41.06% at

15 mmol/kg CA. However, the value of Y(NO) increased with the increase of TA and MA concentrations by 21.64% and 15.91%, respectively, with the addition of 15 mmol/kg TA and MA.

3.3 Cd uptake by the plants

Metal accumulation was evaluated to reveal the effects of LMWOAs on Cd uptake in *Bidens pilosa* L. The Cd accumulated in roots and shoots increased gradually with the application of CA, TA, and MA from low to high concentrations. The Cd concentrations in roots and shoots were 75.22 and 109.49 mg/kg in the treatment of CK (Fig. 3a, 3b). The Cd concentrations in roots were 109.65, 152.07, and 108.07 mg/kg, and that in shoots were 186.38, 161.94, and 155.30 mg/kg, respectively, when 15 mmol/kg CA, TA, and MA were added. The transport, absorption, and enrichment of Cd by *Bidens pilosa* L. varied greatly with the application of LMWOAs. The TF value increased continuously with increasing CA concentrations, with an increase by 16.45% with the addition of 15 mmol/kg CA (Table 1). However, the TF value increased by 12.61% and 5.73%, respectively, with the addition of 1 mmol/kg TA and MA and decreased by 27.03% and 1.55%, respectively, after the application of 15 mmol/kg TA and MA. The PR value increased gradually with increasing CA concentrations and was 169.21% higher in the treatment with the addition of 15 mmol/kg CA. Higher PR values were found in the plants treated with 5 mmol/kg TA and MA, increasing by 71.28% and 63.11%, respectively. The BCF value increased continuously with increasing CA, TA, and MA concentrations, with 77.98%, 78.33%, and 64.49% higher levels, respectively, with the addition of 15 mmol/kg.

Table 1
Effects of exogenous LMWOAs addition on the bioaccumulation, transportation and extraction of heavy metal

	TF	**RE**	**BCF**
CK	1.460 ± 0.111a	0.100 ± 0.001a	2.808 ± 0.022a
CA1	1.555 ± 0.136acd	0.125 ± 0.006b	2.749 ± 0.091a
CA5	1.606 ± 0.103bcd	0.183 ± 0.014c	4.039 ± 0.081b
CA15	1.700 ± 0.005b	0.268 ± 0.006d	4.998 ± 0.143c
TA1	1.644 ± 0.086bc	0.142 ± 0.004e	3.219 ± 0.015d
TA5	1.481 ± 0.003ad	0.171 ± 0.001f	4.353 ± 0.115e
TA15	1.065 ± 0.014e	0.168 ± 0.007fg	5.007 ± 0.283c
MA1	1.544 ± 0.031acd	0.126 ± 0.006b	2.655 ± 0.080a
MA5	1.530 ± 0.071acd	0.163 ± 0.001g	3.997 ± 0.172b
MA15	1.437 ± 0.011a	0.141 ± 0.001e	4.619 ± 0.165f

3.4 Speciation distribution of Cd in the soil

The acid-soluble, reducible, oxidizable, and residual states of Cd accounted for 38.60%, 12.73%, 12.89%, and 35.79%, respectively, in CK (Fig. 4). The addition of LMWOAs significantly affected the speciation distribution of Cd in the soil and enhanced the proportions of the acid-soluble state. The proportions of acid-soluble Cd in the soil increased by 10.26%, 6.78%, and 6.58%, respectively, whereas those of the reducible state decreased by 3.00%, 2.97%, and 0.95%. The proportions of the residual state decreased by 8.00%, 4.07%, and 5.86%, respectively, when 15 mmol/kg CA, TA, and MA were added. However, no significant difference was found in the proportions of oxidizable Cd with the application of LMWOAs.

3.5 Soil bacterial community structure

To explore the influences of the three LMWOAs on the rhizosphere bacterial composition under Cd stress, bacterial 16Sr DNA was sequenced in the experiments treated with 15 mmol/kg CA, TA, and MA. The Chao, Ace, and Shannon index values of the rhizosphere bacterial communities decreased by 9.79%, 8.39%, and 3.16%, respectively, under Cd stress (Table 2). The application of 15 mmol/kg CA, TA, and MA reduced the diversity and abundance of rhizosphere bacteria, and the inhibitory effect of CA was the weakest. The values of the Chao, Ace, and Shannon indices decreased by 6.39%, 5.38%, and 4.28%, respectively with CA addition, and decreased by 21.85%, 21.59%, and 11.28%, respectively, with TA application. When MA was added, they decreased by 11.68%, 10.57%, and 5.15%, respectively. The percentages of Proteobacteria, Bacteroidoia, and Gemmatimonadota of rhizosphere bacteria decreased by 10.16%, 2.78%, and 2.21%, whereas those of Acidobacteriota and Chloroflexi increased by 8.96% and 4.84%, respectively, under Cd stress (Fig. 5a). With the application of LMWOAs, the proportions of Proteobacteria and Bacteroidota increased significantly, whereas those of Acidobacteriota, Chloroflexi, and Gemmatimonadota decreased compared to CK.

Table 2
Effects of exogenous LMWOAs addition on OTU abundance and diversity index of bacteria communities in Cd contaminated soil

	chao	ace	shannon	simpson	coverage
CKK	2413.21 ± 9.92b	2397.64 ± 3.95b	5.70 ± 0.09a	0.013 ± 0.002a	0.988 ± 0.002a
CK	2176.94 ± 125.95a	2196.52 ± 80.93a	5.52 ± 0.27a	0.016 ± 0.005a	0.986 ± 0.003a
CA	2037.79 ± 82.64c	2078.40 ± 54.09c	5.28 ± 0.04b	0.018 ± 0.001a	0.988 ± 0.001a
TA	1706.72 ± 0.54e	1716.66 ± 13.37e	4.90 ± 0.22c	0.032 ± 0.008b	0.989 ± 0.001a
MA	1922.53 ± 48.46d	1964.35 ± 34.29d	5.23 ± 0.14b	0.017 ± 0.002a	0.987 ± 0.001a

The proportions of the genera *Ensifer*, *Ramlibacter*, *Devosia*, and *Pseudoxanthomonas* in the rhizosphere soil were reduced due to Cd stress, whereas they were enhanced by the application of LMWOAs (Fig. 5b). Interestingly, the abundances of *Actinophytocola*, *Azotobacter*, *Pseudomonas*, and *Ramlibacter*, which were extremely low in the CKK and CK treatments, were high in the treatments with LMWOA addition. The three LMWOAs had different impacts on the bacterial genera in the rhizosphere. The addition of CA could significantly increase the abundances of *Azotobacter* and *Pseudomonas*, whereas the abundance levels of *Actinophytocola* and *Ensifer* were improved in the TA treatments.

3.6 Redundancy analysis

The CA, TA, and MA levels in *Bidens Pilosa* L. varied with the addition of exogenous LMWOAs, and the contents in the plant leaves were determined **(Fig. S2)**. The antioxidant levels (CAT, SOD, GSH), osmotic adjustment substance levels (Tsp, Pro), and oxidative damage index levels (MDA, H_2O_2, O^{2-}) of *Bidens Pilosa* L. were also measured after exogenous LMWOA addition **(Figs. S3, S4, S5)**. The correlation between LMWOAs in plants and the related parameters was described by redundancy analysis (Fig. 6a**)**. In the RDA diagram, axis 1 explained 58.10% of the variation of the physiological index, and axis 2 explained 16.48% of the variation. In the photosynthetic system, CA and MA addition was significantly negatively correlated with Fm and F0 and positively correlated with F0/Fm and Fv/F0. In the antioxidant system, CA and MA addition was significantly positively correlated with SOD and GSH and negatively correlated with MDA, H_2O_2, and O^{2-}. In the osmotic regulation system, CA and MA additions were significantly positively correlated with total soluble protein and proline levels.

The contents of CA, MA, and TA in plant roots and rhizosphere soil enzyme activity (sucrase activity, urease activity, protease activity, phosphatase activity, catalase activity) were also determined **(Fig. S6, S7)**, and there were correlations between LMWOAs in plant roots and soil enzyme activities and bacterial abundance **(**Fig. 6b**)**. In the RDA diagram, axis 1 explained 59.25% of the variation in the physiological index, and axis 2 explained 13.83% of this variation. The addition of CA and MA was positively correlated with protease, urease, and phosphatase levels, whereas TA addition was positively correlated with sucrose levels. The application of CA, TA, and MA could reduce catalase activity in the soil. Based on Pearson´s correlation heat map analysis, LMWOAs and soil enzymes had similar effects on bacterial phyla and genera **(**Fig. 6c, 6d**)**, and it can be concluded that CA, MA, and protease have similar effects on bacterial communities. The effect of TA on the bacterial community was similar to those of urease, phosphatase, and sucrase.

4. Discussion

4.1 Exogenous LMWOAs improve photosynthesis and antioxidant activity under Cd stress

Cadmium ions (Cd^{2+}) in the soil, when entering the plant body, can inhibit chlorophyll synthesis, weaken photosynthesis, produce a large amount of reactive oxygen species (ROS), and cause lipid peroxidation, cell inactivation, and other irreversible damage (Ashraf *et al.*, 2013). In previous studies, the levels of Chla, Chlb, and Caro were increased in *Phytolacca americana* (Liu *et al.*, 2015) and *Brassica juncea* (Kaur *et al.*, 2017a) with CA addition, which is in agreement with our finding, most likely because CA can inhibit the expression of the chlorophyll catabolism enzyme (CHLASE) and promotes the expression of phytoene synthase (PSY) (Chen *et al.*, 2020a). The lower value of Y(NO) illustrates the stronger ability of plants to dissipate too much light energy. In the present study, the increased F0, Fm, and Y(NO) values under Cd stress indicated that Cd caused irreversible damage to the PSII reaction center, whereas exogenous CA addition significantly reduced the F0, Fm, and Y(NO) values. This might explain why CA in plants can be used as a chelating agent to fix Cd in vacuoles and to limit the transport in the cytoplasm (Danijela *et al.*, 2019), which can protect the photosynthetic reaction center of the chloroplast from damage by ROS. Additionally, exogenous CA can also mitigate ROS damage in plants by increasing the content of antioxidants such as GSH, polyphenols, and total flavonoids(Kaur *et al.*, 2017b), and our results also showed that the addition of appropriate CA levels could significantly enhance SOD activity, GSH, as well as Tsp and Pro contents **(Fig. S3)**.

In our study, the MA content in the plant body was positively correlated with the values of Fv/Fm and Fv/F0 at a certain concentration, and MA addition could also significantly increase the plant chlorophyll content **(Fig. S1)**, most likely because the application of exogenous MA can promote the transport of more magnesium ions (Mg^{2+}) to the plant leaves (Feng *et al.*, 2012). According to a previous study, magnesium (Mg) is the central atom in chlorophyll and plays a crucial role in chlorophyll biosynthesis (Tränkner *et al.*, 2018). In addition, MA in the plant body could also produce carbon dioxide through malate dehydrogenation (MDH) decarboxylation, mediate carbon fixation, and then participate in photosynthesis (Chen *et al.*, 2020b). These mechanisms of action promote an increase in the chlorophyll content, consequently facilitating strengthen photosynthesis. Moreover, MA also had a significantly positive correlation with CAT, SOD, and other antioxidant enzyme activities since the addition of MA could promote the expression levels of *Cu/Zn-SOD*, *POD1*, *CAT1*, and other antioxidant genes in leaves (Guo *et al.*, 2017), which could increase the contents of antioxidant enzymes in plants.

In addition, low concentrations of TA could facilitate photosynthesis and antioxidant abilities, which is consistent with the findings that the chloroplast structure of *Salix Variegata* was intact, the number of chloroplasts was significantly increased, and the activity of antioxidant enzymes was stimulated after the application of TA under heavy metal stress (Chen *et al.*, 2020a). The antioxidant properties of plants are increased when cichoric acid, a derivative of TA with biological activity, forms complexes with metal cations (Świderski *et al.*, 2020). Exogenous TA can improve plant antioxidant activity by producing cichoric acid under heavy metal stress. Therefore, the addition of appropriate concentrations of CA, TA, and MA could increase plant chlorophyll content, reduce the damage to the photosynthetic reaction center, and enhance photosynthesis. This approach could also improve the activities of SOD, CAT, and POD and release a variety of antioxidant substances (GSH, Tsp, Pro), which interact with H_2O_2 to inhibit the production of O^{2-} and strengthen the plant's antioxidant capacity (Hawrylak-Nowak *et al.*, 2015).

4.2 Exogenous LMWOAs promote the absorption of Cd by plants

The Cd^{2+} absorbed by plant roots are gradually transported to the aboveground plant parts via transpiration and accumulate in the plant body (Naeem *et al.*, 2018; Schwalbert *et al.*, 2021). Our results showed that the CA, TA, and MA concentrations in plant leaves and roots were significantly increased with the addition of exogenous LMWOAs **(Fig. S2, S6)**. The application of LMWOAs at appropriate doses could increase the TF and BCF values and promote Cd accumulation in the plant body; this was obvious in the treatments with CA and MA addition. First, CA and MA in plants, combined with metal ions, can form negatively charged or uncharged chelates, and the negatively charged xylem of plants has a low adsorption of chelates, which can promote the transfer of metal ions to plant leaves (Wilfried, 1999). Therefore, adequate CA and MA addition could promote the transportation of metal ions from belowground to aboveground plant parts. Second, Cd^{2+} in the plant body can activate the antioxidant defense system, and cell wall fixation, cell membrane transport, cytoplasmic chelation, and vacuolar separation are performed (Gallego *et al.*, 2012). The Cd^{2+} and LMWOAs in the cytoplasm can form Cd-organic acid chelates, which can be moved to the vacuole through the transporter; regional separation of heavy metals is achieved and toxicity is reduced (Panchal *et al.*, 2021). In a previous study, exogenous CA addition could enhance detoxification by increasing the contents of metal chelate GSH and the metal-binding protein phytochelatin (Ehsan *et al.*, 2014). The expression level of the transporter plays a key role in the cell membrane transport of metals (Gallego *et al.*, 2012). In a similar study, exogenous CA could also increase the expression of Cd transport genes such as *NRAMP5*, *MTP1*, and *HMA1* by 6.96, 2.82, and 2.28 times, respectively, and MA could significantly promote the expression levels of *NRAMP5* and *PCS1* in leaves of *Salix variegata* Franch (Zhang *et al.*, 2020). Therefore, CA and MA addition could increase metal transportation by mediating the expression of transporters. However, the application of exogenous TA significantly reduced the gene expression levels of *MTP1*, *HMA3*, *MT2B*, and other transporter genes (Zhang *et al.*, 2020). Our results also showed that the TF value gradually decreased with the addition of high concentrations of TA, inhibiting the transport of Cd from plant roots to shoots and consequently leading to Cd accumulation in the roots.

In our redundancy analysis, a significantly positive correlation was found between PR and plant biomass. Moreover, PR increased by 169.21%, and root and shoot biomass increased by 153.96% and 38.39%, respectively, with the addition of 15 mmol/kg CA. However, plant biomass was decreased with the addition of high concentrations of TA and MA. Based on previous results, CA, TA, and MA around roots can promote plant growth by providing available ion compounds and depolymerizing high-molecular-weight humus (Chen *et al.*, 2020c). Also, CA can promote soil phosphorus solubilization, supply more carbon and phosphorus to plant roots, and improve N_2 fixation (Wang *et al.*, 2019). Additionally, CA in the plant can serve as a substrate for the synthesis of endogenous hormones, amino acids, fatty acids, and other metabolites that protect the plant from stress and promote plant growth (Tahjib-Ul-Arif *et al.*, 2021).

In the present study, CA, TA, and MA addition could promote the transformation of Cd in the soil and enhance the acid soluble state. The form of heavy metals in the soil determines the extent to which plants can absorb them, and for Cd, the weak acid extraction state represents the form with the strongest activity and bioavailability (Wang *et al.*, 2016). Regarding LMWOAs, the molecules themselves have one or more carboxyl (-COOH) negative charge and will be complexed with metal cations to form metal chelates (Yu *et al.*, 2020). These chelates are more unstable, more fluid, and have a higher bioavailability, increasing the concentration of Cd in the root (Diarra et al., 2021). The addition of exogenous LMWOAs can also dissolve and release metal ions co-precipitated with carbonate minerals, thereby increasing heavy metal mobility (Agnello *et al.*, 2014). Also, CA had the most significant effect on metal leaching because of its three -COOH groups and one -OH oxygen-containing functional group (Zhang *et al.*, 2019). Therefore, the addition of LMWOAs can increase the amount of absorbable Cd^{2+} ions in Cd-contaminated soil, promoting phytoextraction efficiency.

4.3 Exogenous LMWOAs increase soil enzyme activities and change microbial communities under Cd stress

Soil enzymes are sensitive biological indicators of environmental changes, and enzymes used to evaluate soil quality are divided into two categories (Deng *et al.*, 2015). The first category contains extracellular hydrolase, which is mainly involved in biological metabolism and nutrient cycling. Our results showed that the activities of protease, urease, sucrase, and phosphatase in the soil were enhanced after the application of LMWOAs **(Fig. S7)**, which was consistent with the results reported elsewhere (Huang *et al.*, 2020). In addition, the enhancement in soil enzyme activity also explained the increased plant growth. The second category directly participates in the detoxification of heavy metals and mainly includes catalase, polyphenol oxidase, and dehydrogenase (Du *et al.*, 2021). Catalase not only catalyzes the decomposition of hydrogen peroxide to reduce toxicity, but it also transforms metal ions from a high to a low toxic state and fixes them on the cell surface (Yang *et al.*, 2016). Based on our results, the catalase activity in soil was decreased with the addition of LMWOAs, since LMWOAs changed the availability of Cd ions and the microorganisms would consume more oxidoreductase to capture or precipitate metal ions (Tang *et al.*, 2020).

LMWOAs as plant root exudates play a key role in the composition of the rhizosphere microbial community (Sasse *et al.*, 2018). Our results showed that CA, TA, and MA addition could change soil diversity and abundance, and the application of CA significantly increased the levels of *Proteobacteria*, *Actinobacteria*, and *Bacteroidota*. Also, CA addition could effectively enhance the activity of soil protease **(Fig. S7)**, and a significant positive correlation was detected between protease activity and CA concentration in plant roots. Proteases in the soil can hydrolyze large proteins into bioavailable amino acids, which can be taken up by *Proteobacteria*, *Actinobacteria*, and *Bacteroidota*, increasing their abundance levels (Lin *et al.*, 2021). At the genus level, the addition of exogenous CA and TA could significantly increase the levels of *Azotobacter* and *Ensifer*, which were positively correlated with proteases and urease in the soil. Urease can promote the transfer of organic nitrogen to ammonia nitrogen in the soil (Lin *et al.*, 2021). Therefore, CA and TA addition can affect the form of nitrogen and provide more nitrogen sources for the growth of *Azotobacter* and *Ensifer* through increasing soil urease activity. Moreover, the abundance levels of *Pseudomonas* and *Pseudoxanthomonas*, which are also plant growth-promoting rhizobacteria (PGPR), were increased with the addition of exogenous CA. As reported previously, PGPRAs can synthesize phosphatase, siderophores, and antibiotics to enhance plant tolerance to Cd toxicity (Drehe *et al.*, 2018). Therefore, the abundance of PGPRs was greatly improved after the addition of exogenous LMWOAs, providing suitable conditions for plant growth and heavy metal speciation activation.

5. Conclusions

This study demonstrates the application potential of LMWOAs to enhance the phytoremediation of Cd-contaminated soil, and CA showed the highest efficiency when used with *Bidens Pilosa* L. The addition of LMWOAs, at reasonable doses, could significantly facilitate the growth and development of *Bidens Pilosa* L., enhance photosynthesis efficiency and reduce oxidative damage. The application of exogenous LMWOAs also improved soil enzyme activity, impacted the soil microbial community and the speciation distribution of Cd in the soil, and enhanced the absorption and accumulation of Cd by *Bidens Pilosa* L.

Declarations

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Compliance with Ethical Standards

The research was free of potential conflicts of interest and did not involve human participants and/or animals, and all investigators were informed.

Consent to Publish

I have not submitted my manuscript to a preprint server before submitting it to Environmental Science and Pollution Research. All the authors have read and agreed upon the contents of this manuscript, and the content of the manuscript didn't and won't be published by any other journals.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and data supervision were performed by Qing Yang, Junting Xie, Zhiguo Fang and Huijun Liu. The first draft of the manuscript was written by Qing Yang, and was reviewed and edited by Zhiguo Fang. The contributions of the authors are as follows:

Qing Yang: Investigation, Conceptualization, Methodology, Formal analysis, Original draft writing

Junting Xie : Data curation, Original draft preparation and revision

Huijun Liu：Chart making, Original draft revision, Data supervision

Zhiguo Fang: Supervision, Writing - Review & Editing, Project administration, Funding acquisition

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Availability of data and materials

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

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Tables

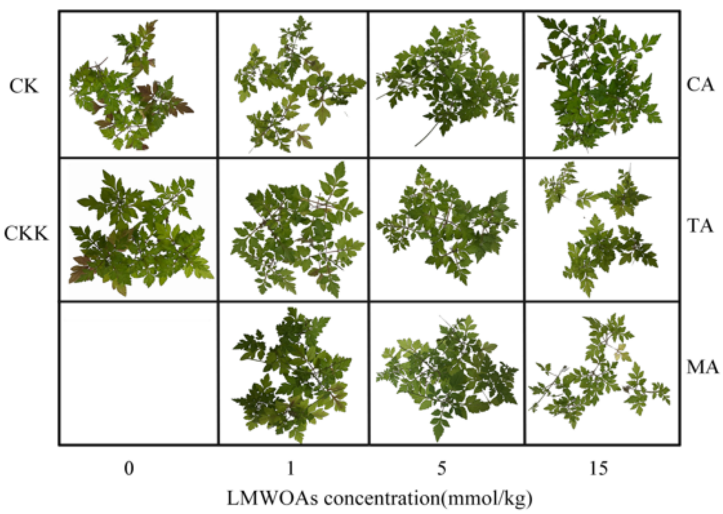
Table1 Effects of exogenous LMWOAs addition on the bioaccumulation, transportation and extraction of heavy metal

	TF	RE	BCF
CK	1.460 ± 0.111a	0.100 ± 0.001a	2.808 ± 0.022a
CA1	1.555 ± 0.136acd	0.125 ± 0.006b	2.749 ± 0.091a
CA5	1.606 ± 0.103bcd	0.183 ± 0.014c	4.039 ± 0.081b
CA15	1.700 ± 0.005b	0.268 ± 0.006d	4.998 ± 0.143c
TA1	1.644 ± 0.086bc	0.142 ± 0.004e	3.219 ± 0.015d
TA5	1.481 ± 0.003ad	0.171 ± 0.001f	4.353 ± 0.115e
TA15	1.065 ± 0.014e	0.168 ± 0.007fg	5.007 ± 0.283c
MA1	1.544 ± 0.031acd	0.126 ± 0.006b	2.655 ± 0.080a
MA5	1.530 ± 0.071acd	0.163 ± 0.001g	3.997 ± 0.172b
MA15	1.437 ± 0.011a	0.141 ± 0.001e	4.619 ± 0.165f

Table 2 Effects of exogenous LMWOAs addition on OTU abundance and diversity index of bacteria communities in Cd contaminated soil

	chao	ace	shannon	simpson	coverage
CKK	2413.21 ± 9.92b	2397.64 ± 3.95b	5.70 ± 0.09a	0.013 ± 0.002a	0.988 ± 0.002a
CK	2176.94 ± 125.95a	2196.52 ± 80.93a	5.52 ± 0.27a	0.016 ± 0.005a	0.986 ± 0.003a
CA	2037.79 ± 82.64c	2078.40 ± 54.09c	5.28 ± 0.04b	0.018 ± 0.001a	0.988 ± 0.001a
TA	1706.72 ± 0.54e	1716.66 ± 13.37e	4.90 ± 0.22c	0.032 ± 0.008b	0.989 ± 0.001a
MA	1922.53 ± 48.46d	1964.35 ± 34.29d	5.23 ± 0.14b	0.017 ± 0.002a	0.987 ± 0.001a

Figures



Treaments	Root dry weight (mg/plant)	Shoot dry weight (mg/plant)	Plant dry weight (mg/plant)	Plant stem length (cm)
CKK	123.23 ± 9.42g	254.37 ± 7.65cd	377.60 ± 16.30e	11.33 ± 0.15bc
CK	43.55 ± 11.55ab	191.13 ± 17.28a	229.6 ± 21.24a	10.30 ± 0.1a
CA1	78.85 ± 7.00d	242.60 ± 14.15c	329.35 ± 12.09cd	13.37 ± 0.42f
CA5	87.90 ± 11.44e	259.20 ± 18.95cd	347.70 ± 35.07d	12.53 ± 0.81de
CA15	110.60 ± 4.24f	264.50 ± 2.97d	377.20 ± 7.21e	11.73 ± 0.25bc
TA1	90.47 ± 10.95e	218.40 ± 13.01b	302.75 ± 9.12c	13.23 ± 0.51ef
TA5	53.375 ± 11.30b	213.70 ± 6.51b	264.15 ± 9.83b	11.00 ± 0.62ab
TA15	37.7 ± 5.46ab	195.05 ± 22.13a	230.70 ± 28.00a	10.47 ± 0.25a
MA1	68.75 ± 1.34cd	264.25 ± 3.61d	333.00 ± 4.95cd	15.43 ± 0.15g
MA5	60.57 ± 8.21c	224.65 ± 0.21b	278.10 ± 20.53b	12.07 ± 0.21cd
MA15	34.10 ± 3.96a	180.30 ± 5.66a	214.40 ± 9.62a	11.43 ± 0.55bc

Figure 1

Effects of exogenous LMWOAs addition on the plant growth and biomass of *Bidens Pilosa* L.

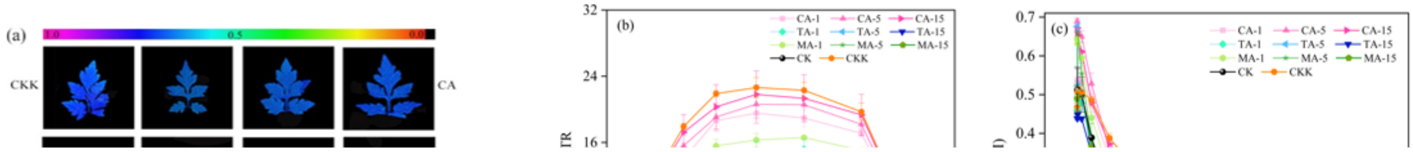


Figure 2

Effects of exogenous LMWOAs addition on chlorophyll fluorescence parameters of *Bidens Pilosa* L. (a) Fv/Fm image and fluorescence parameter (F0, Fm, Fv/Fm, Fv/F0); (b) Photosynthetic electron transfer rate (ETR); (c) Actual photochemical quantum yield (Y(II)); (d) Adjusting quantum yield for energy dissipation (Y(NPQ)); (e) Quantum yield of unregulated energy dissipation (Y(NO)).

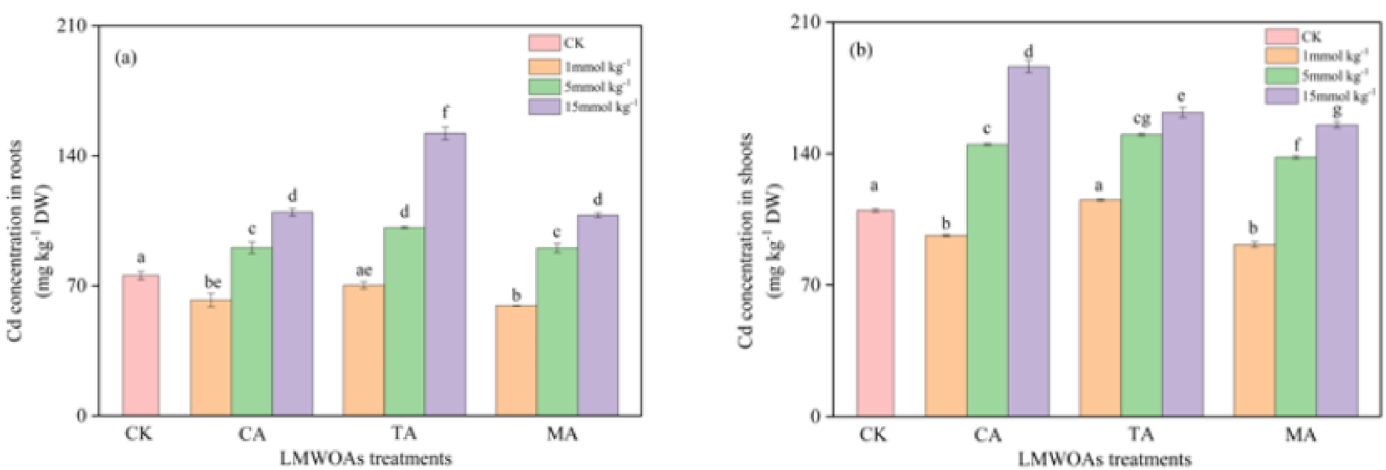


Figure 3

Effects of exogenous LMWOAs addition on Cd concentrations in the roots and shoots of *Bidens Pilosa* L.

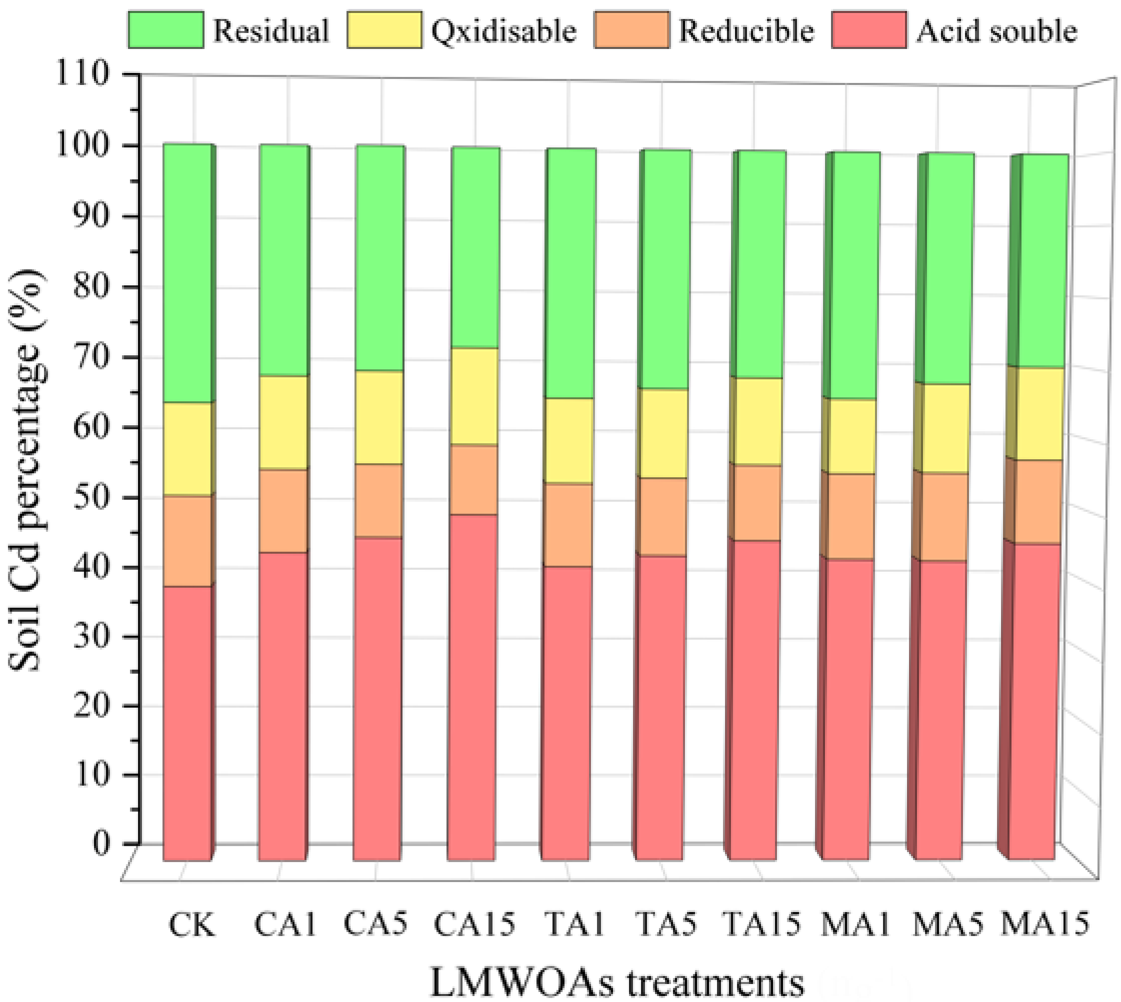


Figure 4

Effects of exogenous LMWOAs addition on the speciation distribution of Cd in the soil

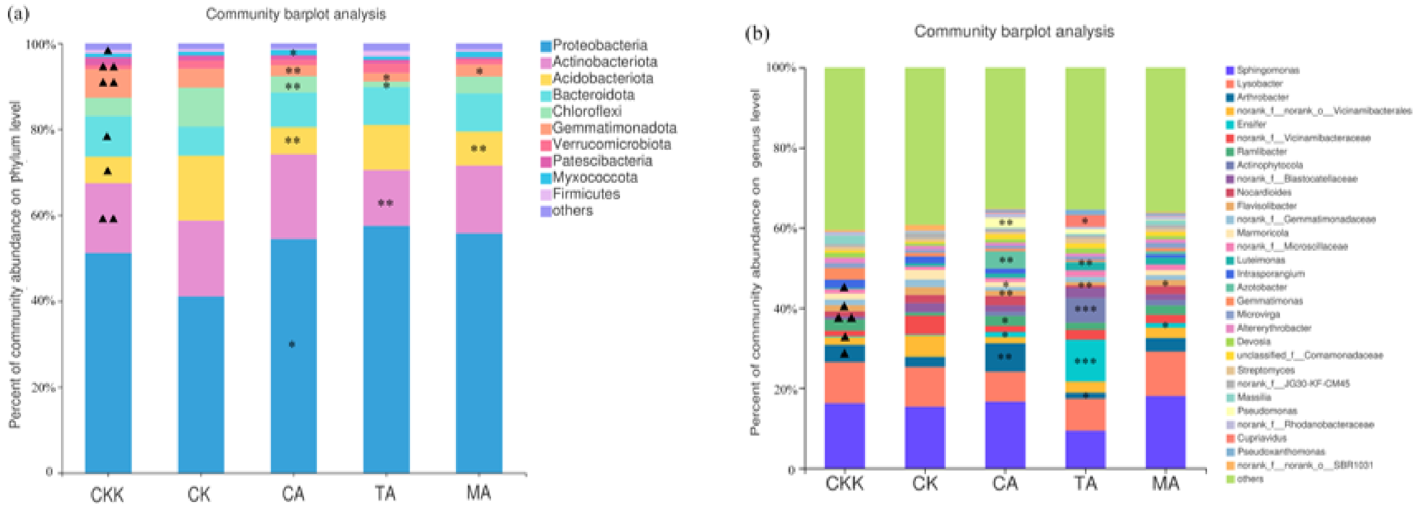


Figure 5

Effects of exogenous LMWOAs addition on the rhizosphere bacterial composition (a) at the phylum level, OTUs that account for less than 1% of the total reads are classified as "Other"; (b) at the genus level, OTUs that account for less than 1.2% of the total reads are classified as "Other". *indicates significant differences between CK and CA, TA, and MA treatment, respectively (* 0.01<p≤0.05, ** 0.001<p≤0.01, ***p≤ 0.01). ▲ indicates significant differences between five treatments (▲ 0.01<p≤0.05, ▲▲ 0.001<p≤0.01).

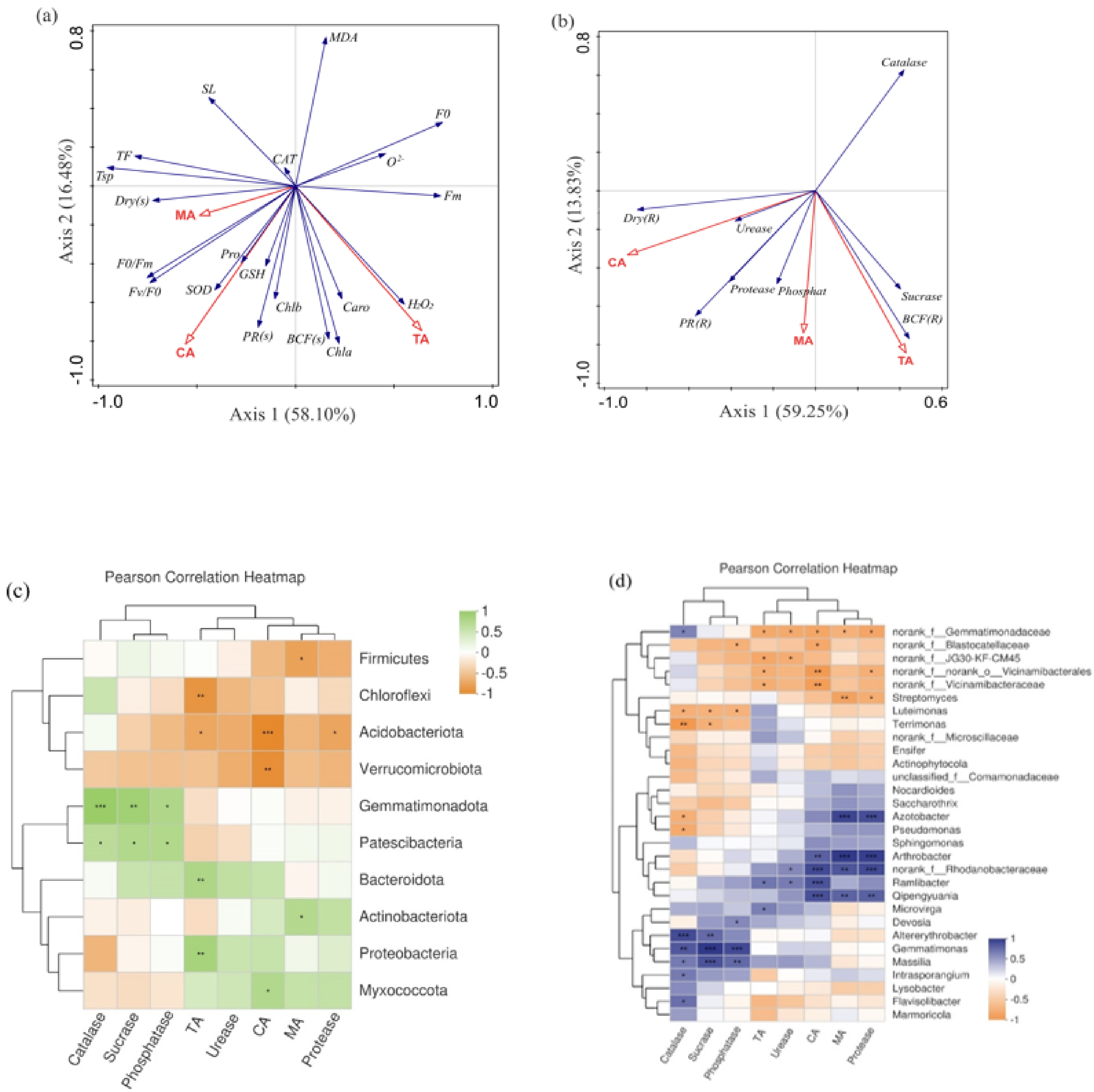


Figure 6

Correlation analysis (* 0.01<p≤0.05, ** 0.001<p≤0.01, ***p≤0.001). (a) the redundancy analysis between three LMWOAs in plants and various indicators, SL (shoot length), Tsp (total soluble protein), Pro (proline), Dry (s) (shoot dry weight); (b) the redundancy analysis between three LMWOAs in roots and soil indicators, Dry (r) (root dry weight); (c) Pearson correlation analysis of phylum bacteria and soil indicators, with OTU abundance ranked in the top 10; (d) Pearson correlation analysis of genus bacteria and soil indicators, with OTU abundance ranked in the top 30.

Supplementary Files

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- GraphicalAbstract2.jpg
- Supplementarymaterial.docx