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Arbuscular mycorrhizal fungi influence the uptake of cadmium in industrial hemp (*Cannabis sativa* L.)

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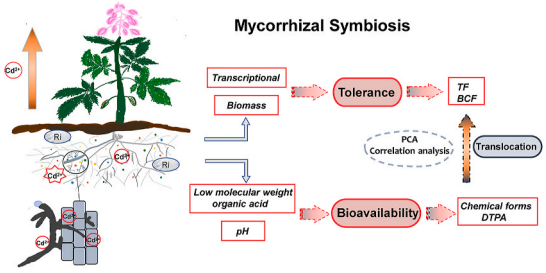
HIGHLIGHTS

- *R. irregularis* enhanced Cd compartmentation and reduces its transfer.
- *R. irregularis* increases the physiological tolerance of *C. sativa* to Cd-toxicity.
- ABC transporter may be the target of translocation regulation between *R. irregularis* and *C. sativa.*
- LMWOAs may play important roles in changing the rhizosphere environment of Cd stress.

GRAPHICAL ABSTRACT



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ABSTRACT

Phytoremediation is currently a more environmentally friendly and economical measure for the remediation of cadmium (Cd) contaminated soil. Heavy metal-resistant plant species, *Cannabis sativa* L. was inoculated with *Rhizophagus irregularis* to investigate the mechanisms of mycorrhizal in improving the Cd remediation ability of *C. sativa*. The results showed that after inoculation with *R. irregularis*, *C. sativa* root Cd contents increased significantly, and leaf Cd enrichment decreased significantly. At the transcriptional level, *R. irregularis* down-regulated the expression of the ABC transporter family but up-regulated differentially expressed genes regulating low molecular weight organic acids. The levels of malic acid, citric acid, and lactic acid were significantly increased in the rhizosphere soil, and they were significantly and strongly related to oxidizable Cd concentrations. Then citric acid levels were considerably and positively connected to exchangeable Cd concentrations. Our findings revealed that through regulating the movement of root molecules, arbuscular mycorrhizal fungus enhanced the heavy metal tolerance of *C. sativa* even more, meanwhile, they changed the Cd chemical forms by altering the composition of low molecular weight organic acids, which in turn affected soil Cd bioavailability.

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1. Introduction

The problem of metal toxicity in lands becomes more complicated. Cd is a soluble heavy metal ， has been listed as an environmental priority pollutant (Cai et al., 2020). According to the 2020 Bulletin on China's ecological environment, the main pollutants affecting the environmental quality of farmland soil around the country are heavy metals; among them, cadmium is the primary pollutant (Lu et al., 2022). In 2015, a soil research survey showed that 16.1% of China's soil and 19.4% of agricultural soil were polluted by heavy metals, of which cadmium pollution (7.0%) was the most serious (Zhao et al., 2015). The Cd content of soil and substrate surpassed the acceptable level of 37 mg/kg for habitat and agricultural soil quality in a certain place in Thailand (Meeinkuirt et al., 2019), some areas even reached 284 mg/kg (Auesukaree et al., 2021). This will not only damage the ecological environment but also endanger human life as Cd is transferred in the food chain after absorption by plants (Huang et al., 2017a, Ogunkunle et al., 2021). Currently, among various environmental remediation patterns, phytoremediation is one of the most promising bioremediation techniques and can be utilized to remediate large-scale areas that may be contaminated with harmful metals (Huang et al., 2016). Phytoremediation is predicated on plant species or genotypes that have a special ability to absorb and enrich one or more heavy metals, i.e., hyperaccumulators (Luo et al., 2006). However, most of the hyperaccumulators found mostly have shorter plants and lower biomass, which limit their efficiency in removing heavy metals (Liu et al., 2016). Finding a plant that can fast-growing plants that can tolerate high concentrations of heavy metal environments and have a certain ability to absorb heavy metals is thus a pressing issue.

As an important non-edible fiber crop with high resistance and a large root system, *Cannabis sativa* L. has a variety of industrial uses and wide application prospects, and high economic value (Abot et al., 2013). Besides, *C. sativa* is a pioneer remediation plant species for a variety of contaminated soils (Gao et al., 2018). Luyckx et al., 2021 found that the translocation factors of *C. sativa* were higher than 1, confirming the interest in hemp for phytoextraction purposes. The physiological and metabolic levels of *C. sativa* are found to have a strong tolerance to certain Cd levels and can grow normally (Huang et al., 2019; Stonehouse et al., 2020). Therefore, *C. sativa* is a reasonable material for the restoration of Cd-contaminated areas, but how to further enhance the remediation efficacy is still a challenge.

It has been shown that inoculation with Arbuscular mycorrhizal fungi (AMF, especially *Rhizophagus irregularis*) can reduce Cd toxicity in host plants (Kong et al., 2019; Fan et al., 2018), alter the rhizosphere microenvironment, and indirectly affect the Cd enrichment and translocation mechanisms (Dary et al., 2010; Wang et al., 2017). It's also generally known that increasing soil acidity can accelerate the dissolution of immobilized Cd, effectively promoting the bioaccumulation of the metal. AMF can secrete low molecular weight organic acid (LMWOAs) and affect the type and amount of LMWOAs secreted by plants (Parihar et al., 2019), improving the rhizosphere environment of the host plant, forming chelates with Cd^{2+} and thus enhancing the tolerance (Bali et al., 2020). Although it has been demonstrated that LMWOAs may improve metal dispersion by changing the soil microenvironment, its underlying process remains uncertain. Zhu et al. (2019) found that LMWOAs could bind metal ions to enhance their activities while increasing soil nutrients, which may once again stabilize some metal ions. However, it is not clear whether AMF inoculation of *C. sativa* will enhance rhizosphere soil Cd bioavailability by mediating LMWOAs.

It is integral to efficiently better the utilization of Cd-contaminated soils while achieving the objective of remediation while production. The impacts of AMF inoculation on the growth, Cd accumulation, root transcriptome, rhizosphere soil Cd chemical forms, and root LMWOAs composition of *C. sativa* under Cd stress were investigated to clarify the mechanisms of mycorrhizal biotechnology in remediating Cd-contaminated soils. We aimed to determine: (1) AMF inoculation will enhance Cd uptake by improving the *C. sativa* growth, (2) mycorrhizal symbiosis will influence Cd distribution pattern by regulating root transport genes, and (3) mycorrhizal symbiosis will affect soil Cd bioavailability by altering LMWOAs composition in *C. sativa* rhizosphere soil.

2. Materials and methods

2.1. Hemp pot experiment

The topsoil (0–20 cm) from a cornfield and no history of heavy metal or Cd contamination was sieved through a 2 mm mesh before being integrated with sand and vermiculite in a 5: 3: 2 ratio and sterilized. The substrate contained (on a dry weight basis): pH 7.69; 14.73 g/kg organic matter; 586 mg/kg total N; 11.52 mg/kg available P; 128.86 mg/kg soluble K. The 80 mg/kg $CdCl_2{\cdot}2.5H_2O$ solution was added to the soil and an equal sterile distilled water to the control treatment, then aging for one month (Zhang et al., 2010). Then determined the soil Cd concentration, which is 0.4 and 80.7 mg/kg respectively. AMF inoculum (*Rhizophagus irregularis*, Ri) was provided by Heilongjiang University, isolate number: CGMCC No.10607 (Sun et al., 2022). We mixed with 200 g of AMF inoculum (5%, w/w) was put just below the germinated seeds. The non-AMF treatment received 200 g autoclaved AMF inoculum, and microbial filtrate. Four hemp seeds (provided by Daqing Branch of Heilongjiang Academy of Sciences) were sown in each pot. There was a total of six replications of four treatments: NM (sterilized inoculum and filtrate), AM (Ri inoculum and distilled water), NM_{Cd} (sterilized inoculum with filtrate + Cd), and AM_{Cd} (Ri inoculum and distilled water + Cd). A pot experiment was conducted in a conservatory of Hulan Campus of Heilongjiang University. The experiment used a completely randomized factorial block design. All pots were maintained at room temperature under a natural illumination regimen throughout the experimental period: 35 °C in the daytime and 20 °C during the night. The pots were watered daily to 60% of the water-holding capacity of the soil, and tap water was supplemented accordingly. 40 mL of 0.25 × fresh Hoagland's nutrient solution was added to each pot every week to maintain an adequate soil nutrient level for plant growth.

All four plants from each pot were collected for analysis after growing for 60 days. Rinse the roots of plants repeatedly with tap water, absorb water with filter paper, and then weigh the roots, stems, and leaves respectively. Rhizosphere soil was collected by shaking and sieved (100 mesh); one part is placed in a cool place for drying and the other part is placed in a - 80 °C refrigerator for storage.

2.2. Determination of Cd concentration

About 0.30 g air-dried plant sample was weighed and 10 mL mixed solution of HNO_3 and $HClO_4$ (9: 1 v/v) was added (Gao et al., 2021).

Soil Cd concentration: four acid digestion methods, the air-dried sample of about 0.10 g was added to HCl–HNO_3–HF–$HClO_4$ (v/v/v/v = 5: 5: 4: 2) mixed solution. DTPA-Cd concentration: diethylenetriaminepentaacetic acid (DTPA) extract (Feng et al., 2005), 5.00 g of air-dried soil with 25 mL of DTPA extract (0.005 mol/L DTPA-0.1 mol/L TEA-0.01 mol/L $CaCl_2$). Then the samples were centrifugated successively at 180 r/min for 2 h and 2500 r/min for 5 min. Cd chemical form: BCR continuous extraction method (Rauret et al., 1999).

All reagents and containers were soaked in 4 mol/L HNO_3 solutions overnight and then rinsed repeatedly with deionized water. Microwave digestion was used for all digestion processes (temperature control range: 50–200 °C). The Cd concentrations in the digestion solution were determined by inductively coupled plasma mass spectrometry (ICP-MS) (X Series2, USA, 2–250 AMU).

The Translocation Factor (TF) and the Bio-concentration Factor (BCF) were calculated according to the formula (Zhou et al., 2020) (1-1, 1–2, and 1–3):

$$TF = Cd\ concentration\ in\ (shoot + leaf)\ /\ Cd\ concentration\ in\ \text{the}\ root \quad (1\text{-}1)$$

$$BCF_{root} = Cd\ concentration\ in\ \text{the}\ root / Cd\ concentration\ in\ \text{the}\ soil \quad (1\text{–}2)$$

$$BCF_{shoot+leaf} = Cd\ concentration\ in\ (shoot + leaf)\ /\ Cd\ concentration\ in\ \text{the}\ soil \quad (1\text{–}3)$$

The total Cd uptake per plant is calculated according to formula (1-4):

Total Cd accumulation per plant = Cd_n* $Biomass_n$, where n is a part of the plant (root, shoot, leaf) (1–4).

2.3. Rhizosphere soil pH and LMWOAs determination

Suzhou Panomick Biomedical Technology Co., Ltd. (https://www.panomix.com/) determined LMWOAs. A suitable sample volume was measured using a steel ball in a 2 mL EP tube, and 500 L of a 30% methanol aqueous solution (containing 0.1% formic acid) was then added. The samples were then ground in a high flux tissue grinder at 60 Hz for 120 s, followed by 10 min of centrifugation at 4 °C under 12000 r/min. A detection bottle was filled with the collected supernatant. Chromatographic analysis was performed using a 5 L injection volume and an ACQUITY UPLC® BEH C18 column (2.1100 mm, 1.7 m, Waters, USA). 40 °C was the column's temperature. Formic acid-containing water and B formic acid-containing water made up the mobile phase. Gradient elution conditions were as follows: 30% B for 0–3 min; 30% B for 3–5 min; 50% B for 5–7 min; 90% B for 7–9 min; and 30% B for 9–13 min. The ion source had a temperature of 500 °C and a voltage of 4500 V, respectively. The collision and curtain gases had pressures of 6 and 30 psi, while the atomizing and auxiliary gases had pressures of 50 psi. The samples were scanned utilizing multiple response monitoring (MRM). Using a pH meter, the pH of the soil/water (w/v = 1: 2.5) was determined.

2.4. Root transcriptome sequencing

RNA samples were extracted from the roots of NM, NM_{Cd}, and AM_{Cd} with 3 biological replicates of each treatment by the total RNA extraction kit purchased from Bao Biological Engineering (Dalian) Co. Before the extraction, the mortar, grinding rod, forceps, and spoon were wrapped in tin foil and sterilized in an oven at 180 °C for 2 h. The RNA samples were examined by 1% agarose gel electrophoresis to analyze the degradation and contamination. The qualified samples were placed in a foam box covered with dry ice, sealed firmly, and sent to Nanjing Paisenor Gene Technology Co for sequencing.

Oligo (dT) magnetic beads were used to enhance the mRNA with polyA structure in the total RNA, and ion interruption was used to fragment the RNA into fragments that were about 300 bp long. Using RNA as a template, reverse transcriptase, and 6-base random primers were used to create the first strand of cDNA. The first cDNA strand served as a template for the creation of the second strand. Following the creation of the library, PCR amplification was used to enrich the library fragments, and then the library was chosen based on fragment size. The Agilent 2100 Bioanalyzer (U.S.A.) assessed the library's quality for total and effective library concentrations, and libraries with various Index sequences were proportionally blended. The mixed libraries were base-denatured into single-stranded libraries after being uniformly diluted to 2 nM. Using Next-Generation Sequencing (NGS) technology based on the Illumina sequencing platform with double-end (PE) sequencing, these libraries are sequenced after RNA extraction, purification, and library creation (Xu et al., 2019). The high-quality sequences from the filtered raw downstream data (Raw Data) are then compared to the reference genome.

Each gene's read count values were statistically compared using HTSeq as the gene's raw expression. The genuine expression level of the gene, as well as its length and sequencing depth, were all positively linked with the read count. Genes with FPKM >1 were often regarded as being expressed. FPKM was used to normalize the expression (Normalization) to make the gene expression levels comparable across genes and samples. To identify differentially expressed genes (DEGs), DESeq was utilized to perform a differential analysis of gene expression. The following criteria were used to identify DEGs: expression difference fold $|\log2FoldChange| > 1$, significance P-value 0.05. For ease of use, DEGs were classified as "up-regulated" and those in opposition as "down-regulated" if their expression levels were higher at AM_{Cd} than at NM_{Cd} or higher at NM_{Cd} than at NM.

Using topGO, a Gene Ontology (GO) enrichment analysis was carried out. Using the DEGs annotated in the GO term, the list of genes and gene numbers for each term was calculated. The P-value (P-value 0.05 for significant enrichment) was then calculated using the hypergeometric distribution method to identify the GO terms with significant DEGs compared to the entire genomic background. The classification of GOs into the molecular function (MF), biological process (BP), and cellular component (CC) were done using the results of the GO enrichment analysis of DEGs. For each GO category, the top 6 GO term entries with the most gene enrichment were chosen to be displayed. The Kyoto Encyclopedia of Genes and Genomes was used to find pathways that were enriched for up-and down-regulated genes (KEGG, Japan). Based on the results of KEGG enrichment, as determined by a Rich factor, FDR value, and the number of genes enriched to the pathway, the top 20 KEGG pathways with the least FDR values, i.e., the most significant enrichment, were chosen for exhibition.

2.5. Validation of qRT-PCR for DEGs

Four DEGs from the ABC transporter family (*LOC115709820*, *LOC115696727*, *LOC115697113*, and *LOC115706838*) were selected in the NM_{Cd} and AM_{Cd} treatments for qRT-PCR validation, using *GAPDH* as the internal reference gene, based on the DEGs levels in the transcriptome data (Zhou M et al., 2019). Table S1 displays the primer sequences that were employed. SYBR Green dye was used for the qRT-PCR, which was carried out on an ABI 7500 fluorescent quantitative PCR (Beijing Dongsheng Innovation Biotechnology Co., Ltd., China) with three duplicates of each PCR reaction for all samples and one template-free control set for each primer pair. The 10 μL 2 × SYBR select master mix, 0.3 μL forward and reverse primers, 1 μL cDNA template and water make up the 20 μL qRT-PCR reaction system. 95 °C, 3 min for the hold stage, 95 °C 15 s, 60 °C 30 s for 40 cycles, and then 95 °C 5 s, 65 °C 5 s, and 90 °C 15 s made up the reaction procedure. Following the mixing of the samples, the initial amplification was done, and the PCR reaction was run for a total of 40 cycles, as determined by the $2^{-\Delta\Delta Ct}$ analysis method.

2.6. Data analysis

Values are presented as means of triplicate measurements ± standard error (SE). The SPSS 25 software was used to test whether the data conform to the normal distribution, if they do, we continue the analysis, if not, we need to test the homogeneity of the variance by the Levene test. ANOVA analysis with LSD's multiple comparisons was used to assess the significance of different treatments, with $P < 0.05$ being used as the significant level. The Origin 2021b, TBtools, and PowerPoint software were used to draw graphs. The mycorrhizal colonization percentage of both AM and AM_{Cd} treatments was above 30.0%, establishing a good symbiosis. Meanwhile, NM and NM_{Cd} treatments did not have AMF colonization (Table S2). Variation amplitude of biomass and plant Cd content selection independent sample *t*-test. The total fresh weight biomass of *C. sativa* (per plant) = $Biomass_{root}$ + $Biomass_{shoot}$ + $Biomass_{leaf}$. Rhizosphere soil differential metabolites under exogenous Cd stress using ROC curves to evaluate and screen potential biomarkers (https://www.genescloud.cn). The sensitivity and specificity of each

metabolite were calculated using the ideal threshold of the ROC curve. The Area Under Curve (AUC) was used to assess the sensitivity and specificity of the biomarkers: 0.5–0.7 generally has low predictive accuracy, 0.7–0.9 generally has moderate predictive accuracy, and over 0.9 generally has good predictive accuracy. At 0.5, the biomarker has no predictive value for events. PCA analysis of different forms of rhizosphere soil heavy metals under exogenous Cd stress with LMWOAs was performed using Canoco5. Linear regression analysis of different forms of rhizosphere soil heavy metals with LMWOAs under exogenous Cd stress was performed using https://www.genescloud.cn.

3. Results

3.1. Effect of AMF on C. sativa biomass under Cd stress

Ri inoculation significantly improved *C. sativa* fresh weight at both Cd levels ($P < 0.05$, Fig. 1a). Multiple interaction analysis revealed that Cd stress and Ri inoculation's interactions had significant effects on the fresh weight of each part ($P < 0.05$, Table S3). Comparing the AM treatment to the NM treatment, the weight of the root, stem, leaf, and total increased in the absence of Cd stress by 37.3%, 22.7%, 22.9%, and 25.7%, respectively. Comparing the AM_{Cd} treatment to the NM_{Cd} treatment, root, stem, leaf, and total fresh weight all improved by 12.4%, 17.9%, 36.7%, and 23.6%, respectively (Fig. 1c). The promoting impact of AMF on leaf and total fresh weight was strengthened under Cd stress. This shows that the harmful effects of Cd on *C. sativa* were lessened by AMF.

3.2. Effect of AMF on Cd concentration in C. sativa

The Cd content in AM_{Cd} treatment roots and leaves was 68.9% and 73.0% higher and lower than that of NM_{Cd} treatment, respectively ($P < 0.01$, Fig. 1b). Ri inoculation exhibited highly significant impacts on root and leaf Cd concentrations, according to multiple comparison investigations (Table S4). In both comparisons (NM vs. AM, NM_{Cd} vs. AM_{Cd}), compared to the AM treatment, the AM_{Cd} treatment significantly increased the root Cd concentration and decreased the leaf Cd concentration ($P < 0.05$, Fig. 1c).

The TF of the inoculated treatment was lower than that of the non-inoculated treatment at the same Cd level ($P < 0.05$, Table 1). The maximum $BCF_{root/soil}$ was discovered in the AM_{Cd} treatment, which was significantly higher compared to the NM_{Cd} treatment ($P < 0.01$). BCF did not differ between the AM_{Cd} and NM_{Cd} treatments. Compared to the NM_{Cd} treatment, the total Cd absorbed by roots and stems in the AM_{Cd} treatment increased by 81.5% and 32.7%, respectively, and the total Cd absorbed by leaves in the AM_{Cd} treatment decreased by 18.0% ($P < 0.05$). This further showed that Cd enrichment and translocation effects in the roots of *C. sativa* may be improved by AMF.

3.3. Comparative transcriptome analysis of C. sativa roots under Cd stress

379 (218 up-regulated, 161 down-regulated) and 877 (147 up-regulated, 730 down-regulated) DEGs were identified in NM/NM_{Cd} and NM_{Cd}/AM_{Cd}, respectively (Fig. S1a). DEGs in NM/NM_{Cd} and NM_{Cd}/AM_{Cd} were mainly enriched in carbohydrate metadata process, cell wall organization or biology, and cell wall organization in the BP process basing on GO analysis (Fig. S1b). In CC process, they mainly concentrated in intrinsic component of membrane, integral component of membrane and cell wall. In the MF process, they mainly concentrated on catalyst activity and hydrolase activity. Cell wall is a major component of plant cells, and GO terms associated with cell wall biosynthesis were present in both comparison groups. In cell wall organization or biogenesis, there were 12 DEGs in NM/NM_{Cd} and 22 DEGs in NM_{Cd}/AM_{Cd}; in cell wall organization there were11 DEGs in NM/NM_{Cd} and 19 DEGs in NM_{Cd}/AM_{Cd}; in the cell wall there were 9 DEGs in NM/NM_{Cd} and 16 DEGs in NM_{Cd}/AM_{Cd}. The DEGs of NM_{Cd}/AM_{Cd} were generally

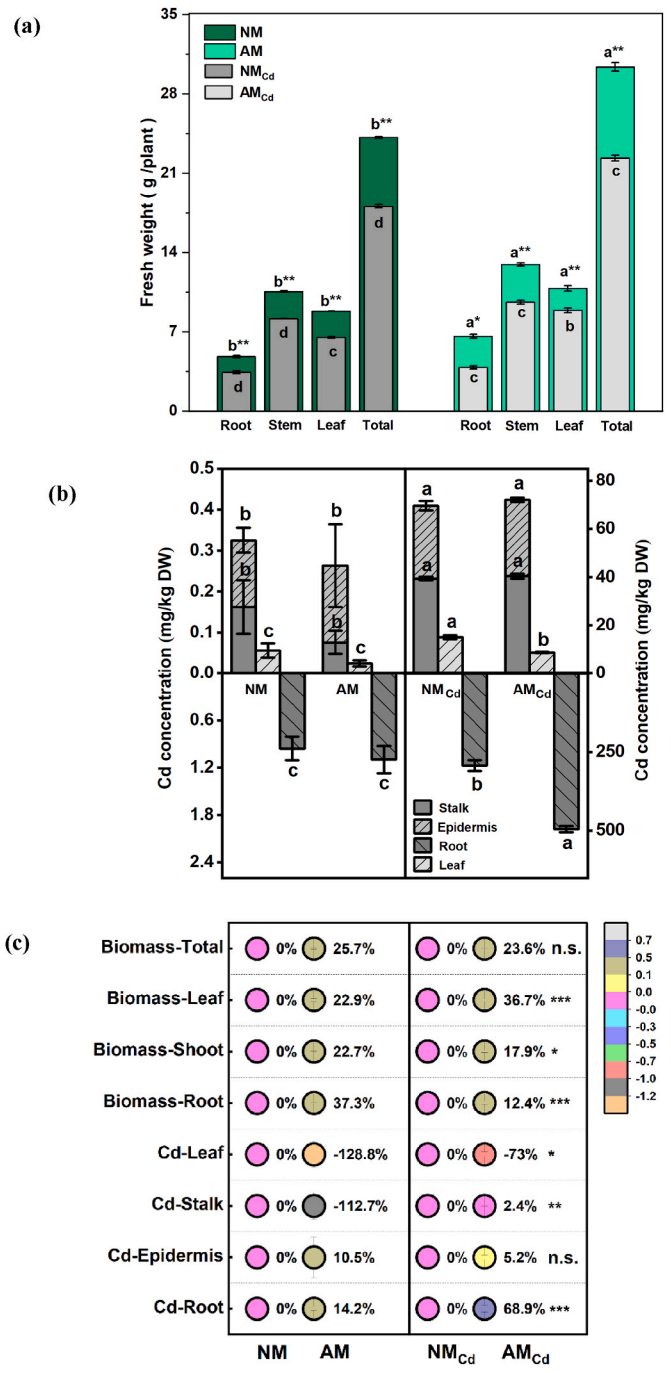

Fig. 1. Growth of *C. sativa* and Cd concentration in different treatments. Note: (a) Fresh weight, different letters indicate the difference between the same plant structure under the same inoculation conditions ($P < 0.05$); (b) Plant Cd concentration, different letters indicate statistically significant differences between treatments ($P < 0.05$); (c) The magnitude of change, significance comparison was performed in AM and AM_{Cd} treatments (means ± SE, n = 3), $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; n.s. No significant.

enriched in starch and sucrose metabolism as well as pentose and glucuronate interconversions, which are relevant to cell wall biosynthesis, according to KEGG analysis (Fig. S2).

In this study, four KEGG pathways were identified: calcium signaling pathway, MAPK signaling pathway-plant, phosphatidylinositol signaling system, and plant hormone signal transduction. Three candidate genes possibly involved in signal transduction and transmission proteins were identified in NM_{Cd}/AM_{Cd}: one DEG encoding calmodulin

Table 1
The TF, BCF, and Cd uptake of different treatments of *C. sativa*.

Treatment	$TF_{(stalk+\ epidermis+\ leaf)/root}$	$BCF_{(stalk+\ epidermis\ +leaf)/soil}$	$BCF_{root/soil}$	Total uptake root (μg/plant DW)	Total uptake (stalk + epidermis) (μg/plant DW)	Total uptake leaf (μg/plant DW)
NM	0.4 ± 0.07a	1.0 ± 0.27a	2.5 ± 0.52c	1.0 ± 0.17c	1.4 ± 0.42c	0.2 ± 0.05c
AM	0.3 ± 0.04b	0.6 ± 0.19b	2.5 ± 0.42c	1.4 ± 0.23c	1.4 ± 0.43c	0.1 ± 0.03c
NM_{Cd}	0.3 ± 0.01b	1.1 ± 0.06a	3.7 ± 0.28b	255.2 ± 14.59b	213.6 ± 0.84b	30.3 ± 1.54a
AM_{Cd}	0.2 ± 0.01c	1.0 ± 0.01a	6.4 ± 0.20a	463.1 ± 8.75a	283.5 ± 6.10a	24.9 ± 0.68b

Note: Different letters indicate statistically significant differences between treatments ($P < 0.05$) (means ± SE, n = 3). *$P < 0.05$; **$P < 0.01$; n.s. No significant.

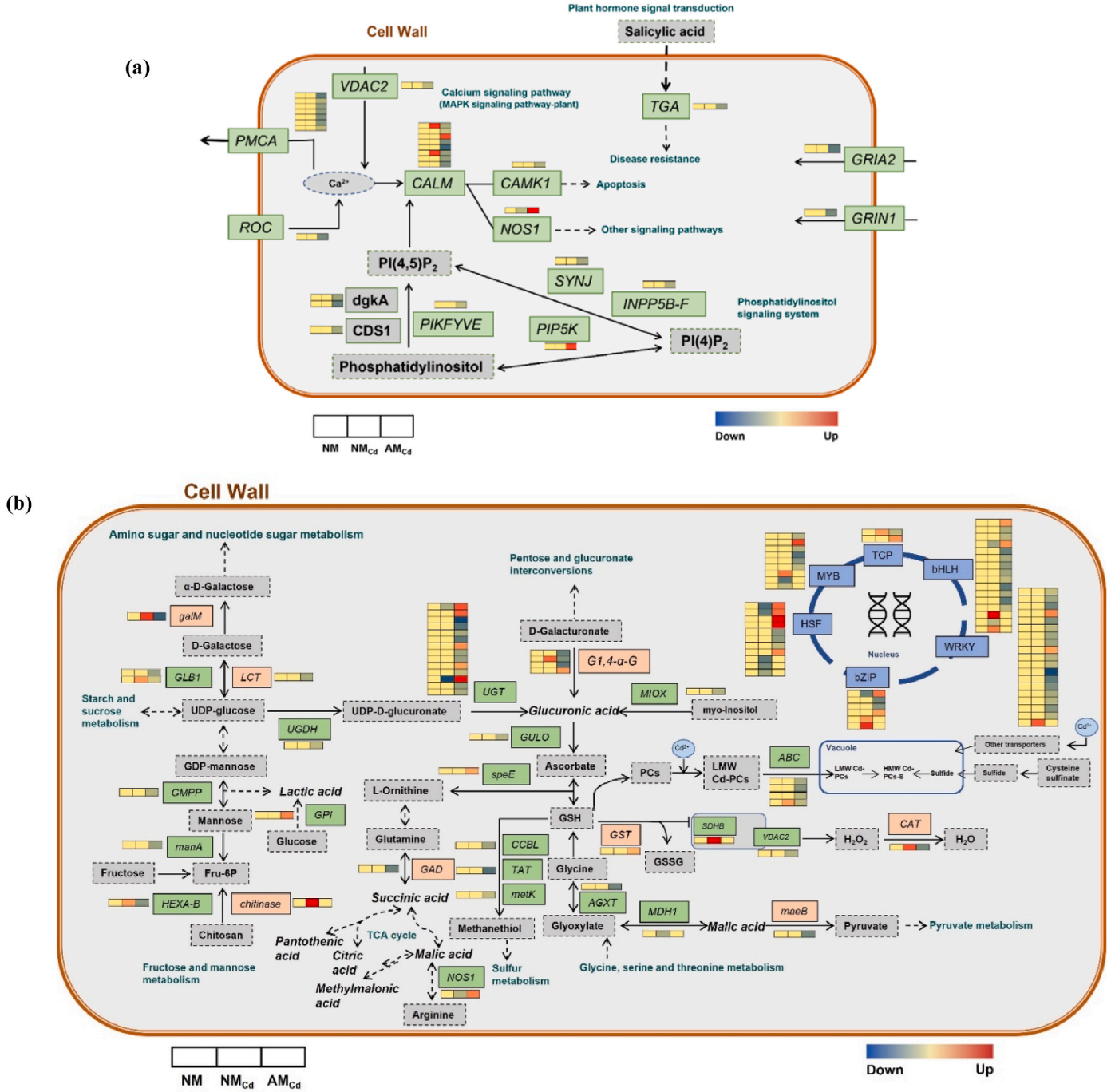

Fig. 2. Transcriptome analysis of NM, NM_{Cd}, and AM_{Cd} treatments.

(*CALM*): *LOC115702921*; one DEG encoding nitric-oxide synthase (*NOS1*): *LOC115697214*; one DEG encoding 1-phosphatidylinositol-4-phosphate 5-kinase (*PIP5K*): *LOC115698691*. They are significantly up-regulated (Fig. 2a).

Four significantly up-regulated DEGs identified were candidate genes involved in heavy metal stress-related TFs only in NM_{Cd}/AM_{Cd}, encoding NF-YB (*LOC115719530*, *LOC115711579*), LSD (*LOC115716485*), and HRT-like (*LOC115705874*), respectively (Table S5). TCP, bHLH, WRKY, bZIP, HSF, and MYB were identified in both comparison groups. In the present study, ASA-GSH-mediated Cd chelation was enhanced in NM_{Cd}/AM_{Cd}; *UGT* (*LOC115699562*, *LOC115719547*, *LOC115725407*, *LOC115722394*, *LOC115712299*), *GST* (*LOC115695824*) and *G1,4-α-G* (*LOC115708975*) are involved in GSH precursor synthesis (Fig. 2b).

The expression of some genes with segregation and transport functions can lead to differential Cd accumulation. In NM_{Cd}/AM_{Cd}, four DEGs encoding the ABC transporter family: *ABCA3* (*LOC115709820*), *ABCC2* (*LOC115696727*), *ABCG1* (*LOC115697113*), *ABCG2* (*LOC115706838*), were determined down-regulated in this study. Among them, *GPI* (*LOC115700647*) regulating lactic acid, and *NOS1* (*LOC115697214*) regulating malic acid metabolism were found to be significantly up-regulated in AM_{Cd} treatment. These four genes were subjected to qRT-PCR and showed consistent expression profiles, demonstrating the validity of the RNA-seq results (Fig. S1c).

Note: (a) Comparative transcriptomic network of signaling sensing and transduction proteins; (b) Metabolic analysis comparing transcriptomic networks. From left to right, NM, NM_{Cd}, and AM_{Cd} are indicated by green boxes for the genes, gray boxes for the metabolites, pink boxes for the enzymes, blue boxes for the transcription factors, and italics for the LMWOAs. The arrows between the two metabolites indicate the direction of the catalytic reaction, and the names and expression patterns of the genes encoding the corresponding enzymes in the three treatments are given near the arrows.

3.4. Effect of AMF on the chemical form of rhizosphere soil Cd

The effect of Ri inoculation on DTPA-Cd concentration was highly significant, and the interaction of the two on DTPA-Cd concentration was highly significant ($P < 0.001$, Table S6). Total Cd concentration was reduced by 2.5% in AM_{Cd} treatment compared to NM_{Cd} treatment, and they were significantly related ($P < 0.05$, Fig. S3); the DTPA-Cd concentrations of AM_{Cd} and NM_{Cd} treatments were highly significant ($P < 0.01$), accounting for 39.2% and 31.8% of the total Cd concentration, respectively.

The exchangeable Cd accounted for 19% and 16% of the total Cd content in AM_{Cd} and NM_{Cd} treatments ($P > 0.05$, Fig. S4). The reducible Cd in AM_{Cd} and NM_{Cd} treatment accounted for 47% and 51% of the total Cd content ($P < 0.05$). The oxidizable Cd content in AM_{Cd} and NM_{Cd} treatment accounted for 18% and 14% of the total Cd content showed high significant different ($P < 0.001$). The residual Cd content of AM_{Cd} and NM_{Cd} treatment accounted for 16% and 19% of the total Cd content, with a highly significant difference ($P < 0.01$). Multiple analyses showed that Ri inoculation had a highly significant impact on oxidizable and residual Cd contents, and their interaction had a highly significant impact on the concentrations of oxidizable and residual Cd (Table S6). These results suggested that AMF affected Cd bioavailability by changing Cd chemical form composition in rhizosphere soil.

3.5. Effect of AMF on rhizosphere soil pH and LMWOAs under Cd stress

Rhizosphere soil pH decreased in AM_{Cd} and NM_{Cd} treatments, and rhizosphere soil pH decreased by 0.11 units in AM_{Cd} treatment compared to NM_{Cd} treatment. In addition, rhizosphere soil pH was higher in inoculated than in non-inoculated treatment under the same Cd concentrations (Fig. 3a).

A total of eight LMWOAs were detected in the rhizosphere soil:

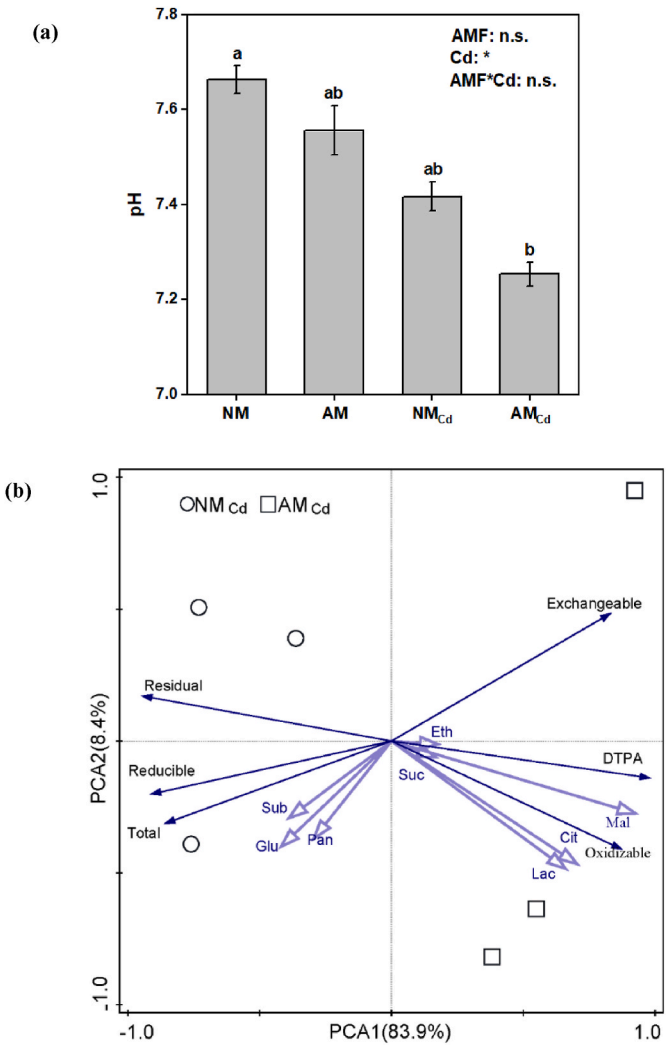

Fig. 3. Rhizosphere soil pH and LMWOAs composition of different treatments. Note: (a) pH; (b) PCA analysis, blue arrows indicate LMWOAs, black arrows indicate rhizosphere soil Cd chemical form, total Cd concentration, and DTPA-Cd concentration. Suc: succinic acid, Mal: malic acid, Cit: citric acid, Glu: glucuronic acid, Pan: pantothenic acid, Met: methylmalonic acid, Sub: suberic acid, Lac: lactic acid. Different letters indicate statistically significant differences between treatments ($P < 0.05$) (means ± SE, n = 3). *$P < 0.05$; **$P < 0.01$; n.s. No significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

succinic acid (Suc), malic acid (Mal), citric acid (Cit), glucuronic acid (Glu), pantothenic acid (Pan), methylmalonic acid (Met), suberic acid (Sub), and lactic acid (Lac). Eight LMWOAs were present in greater amounts in the AM treatment's rhizosphere soil than in the NM treatment. The concentration of Suc, Mal, Cit, Met, and Lac in the rhizosphere soil of AM_{Cd} treatment was larger than in the NM_{Cd} treatment, and Mal, Cit, and Lac reached significant levels ($P < 0.05$, Table S7).

PCA showed the two principal components explained 92.3% of the overall variation, with Mal having a higher load in PC1, indicating its higher correlation with PC1, and residual having a higher load in PC2, indicating its higher correlation with PC2. This indicates that inoculation with AMF affects the exchangeable, oxidizable, and DTPA-Cd of rhizosphere soil mainly through Mal, Cit, and Lac (Fig. 3b, Table S8).

Screening for potential biomarkers of LMWOAs showed high predictive accuracy for Mal, Cit, and Lac, and low predictive accuracy for Suc and Met (Fig. S5a). This suggests that Mal, Cit, and Lac may be potential biomarkers of rhizosphere heavy metal chemical form influenced by the mycorrhizal symbiosis of *C. sativa* under Cd stress.

Linear regression revealed a positive correlation between Mal and oxidizable and DTPA-Cd, and a negative correlation between Mal and reducible Cd ($P < 0.01$, Fig. S5b). Cit showed a positive correlation with the exchangeable and oxidizable Cd ($P < 0.05$). Lac showed a positive correlation with oxidizable and DTPA-Cd ($P < 0.05$). These also demonstrated that Mal, Cit, and Lac are key substances affecting the Cd bioavailability in the rhizosphere soil of mycorrhizal symbionts, thus indirectly affecting Cd accumulation in *C. sativa*.

4. Discussion

4.1. Effect of AMF on the growth and Cd accumulation of C. sativa under Cd stress

In this study, we discovered that AMF inoculation boosted the fresh weight of all hemp organs. AMF has been demonstrated to boost the effectiveness of host plants' use of water and mineral nutrients, while a specific concentration of Cd^{2+} has been proven to activate plant cytokinin production and speed up plant growth (Zhang et al., 2013a). AMF improved the photosynthetic capacity of a host plant, which might explain the significant increase in leaf fresh weight and inhibition of Cd translocation to the leaf in AM_{Cd} treatment (Huang et al., 2017b) (Fig. 1a and b). In addition, AMF exerted a protective effect on *C. sativa*, which has been reported on *Phragmites australis* (You et al., 2021). According to Chen et al. (2012), with comparable leaf Cd uptake in AM_{Cd} and NM_{Cd} treatments, a rise in biomass led to that drop in Cd content, which may have lessened Cd toxicity in plants.

Metals may be bound by the AMF symbiosis inside the mycelium as well as prevented from being absorbed by the host plants (Jiang et al., 2016). More research, nevertheless, data indicates that AMF might encourage the buildup of metals in host plants. At Cd concentrations of 25–50 mg/kg, Liu et al. (2015) discovered that *Glomus versiforme* considerably enhanced Cd level and absorption in roots of *Solanum nigrum* L. Therefore, *C. sativa* to be inoculated with AMF after 60 days of Cd exposure at 80 mg/kg, which can not only stabilize the host plants but also improve their metal extraction.

4.2. Effect of AMF on root transcriptome under Cd stress

When under significant metal stress, plants frequently utilize many resistance and detoxifying systems. These regulatory mechanisms mainly include the generation of signal sensing and transduction proteins, the regulation of relevant TFs, the activation of metal transporters, and the synthesis of biological chelates (Yaashikaa et al., 2022). The protein TFs, which has a unique structure, is essential for improving plant adaptation because it can accurately control the spatiotemporal specific expression of downstream genes. NF-YB, a transcription factor that contributes to plant tolerance to abiotic stress, was markedly up-regulated in NM_{Cd}/AM_{Cd} in response to and resistance to Cd stress, contributing to the improvement of symbiont resistance (Yan et al., 2013).

Plant cell walls can prevent heavy metal ions from penetrating protoplasts by activating various stress response signals and transduction proteins, which mainly include *CALM*, *NOS1*, and *PIP5K* (Leng et al., 2020). As the second messenger of signal transduction, Ca^{2+} is almost involved in all responses to environmental changes during plant growth. Ca^{2+} concentration in cytoplasm fluctuates when plants feel the external stimulus signal, and further downstream *CDPK* transfers with it (Marie et al., 2013). There is a correlation between signal pathways, as evidenced by studies showing that Ca^{2+} reduces the harmful effect of Cd in *Arabidopsis thaliana* through modulating auxin homeostasis (Li et al., 2016). *C. sativa* can sustain typical growth, development, and metabolism by being more tolerant to Cd stress owing to the up-regulated expression of *CALM* inside the NM_{Cd}/AM_{Cd} comparison group.

The transfer of toxic metals in plants was tied to the ABC transporter family. Under Cd stress, *ABCC* and *ABCG* were found involved in the regulation of Cd tolerance in *Lycopersicon esculentum* and *Arachis hypogaea*, respectively (Su et al., 2021; Yu et al., 2018). Under stress conditions, AMF-dependent plant genes that are related to heavy metal tolerance are down-regulated (Ouziad et al., 2005). The significant down-regulation of genes encoding the ABC transporter family in the NM_{Cd}/AM_{Cd} comparison group may be because Cd accumulation reached the trigger threshold of root homeostasis, leading to the down-regulation of genes encoding endogenous transporters and related synthesis (Banakar et al., 2017). This strategy may also provide a basis for the symbiont to enhance tolerance and establish a good growth state under Cd stress.

4.3. Effect of AMF on Cd chemical forms in rhizosphere soil under Cd stress

The proportion of contaminants capable of being absorbed by plants that are "bioavailable" to metals (Hu et al., 2013). The DTPA extraction method is used to evaluate potentially absorbable (effective) heavy metals in soil and was used to examine the relationship between chemical form and bioavailability of pollutants (Qiao et al., 2015). AMF inoculation increased the percentage of DTPA-Cd compared to the uninoculated treatment. It is most likely because secondary metabolites in the soil, such as tartaric acid, and Cit, have numerous carboxyl groups and can combine with Cd to create complexes with 5–6 rings, improving the absorption and mobility of Cd (Islam et al., 2021). Under 0–120 mg/kg Cd, exchangeable and DTPA-Cd had a highly significant linear correlation in the soil after inoculation of *S. nigrum* with AMF (Wang et al., 2019). According to PCA analyses, DTPA-Cd, exchangeable Cd, and oxidizable Cd have a positive correlation, and there is a high level of plant utilization of them. AMF inoculation enhanced the fraction of exchangeable and oxidizable Cd while decreasing the amount of reducible and residual Cd as compared to the NM_{Cd} treatment. Given that exchangeable Cd was more bioavailable (Zeng et al., 2018), AMF altered the Cd chemical forms in soil, making them more readily available to plants and decreasing the possibility for host plants to absorb Cd. Similarly, inoculation with *Funneliformis mosseae* and the addition of Cit co-treatment increased the conversion of vanadium (V) from the residual form to the exchangeable form, according to Qiu et al. (2021). Our results support the hypothesis that inoculation with AMF can increase Cd absorption and deposition in *C. sativa* by changing the chemical form and bioavailability of Cd.

4.4. Effect of AMF on rhizosphere soil LMWOAs under Cd stress

Root exudates are an essential tool for modifying the rhizosphere environment of plants to stimulate heavy metal uptake (Montiel-Rozas et al., 2016). Tao et al. (2020) demonstrated that tartaric acid could combine with Cd to form a soluble compound, encouraging *Sedum alfredii* to increase its Cd content (Tao et al., 2020). According to Huang et al. (2020), the fiber crop *Ricinus communis* L. had a greater concentration of oxalic acid and Suc in its rhizosphere soil and accumulated more Cu in its roots. The increase in the concentration of some LMWOAs and the acidification of rhizosphere soil, which increases the availability of heavy metals, may be responsible for the drop in soil pH. It has been demonstrated that a pH fall of just 0.2 units will result in a more than threefold increase in the amount of unstable Cd (Haoliang et al., 2007). The rise in the concentration of some LMWOAs and the acidification of rhizosphere soil, which increases the availability of heavy metals, might have been to blame for the drop in soil pH. Chen et al. (2022) found that in Cd-polluted farmland, AMF promoted the root to secrete Mal by 1.3 times, and the content of Cd absorbed by shoot was significantly positively correlated with LMWOAs concentration. Our findings demonstrated that the overall LMWOAs content in the AM_{Cd} treatment is larger than the NM_{Cd} treatment, which is consistent with the AM_{Cd} treatment's low rhizosphere soil pH. It may be that the generation of photosynthetic products increases due to the inoculation of AMF, and some of them are

converted into root exudates (Sharma et al., 2021), improving the bioavailability of Cd thus encouraging *C. sativa* may absorb more Cd. Additionally, LMWOAs in the AM_{Cd} treatment in this study were primarily Mal, Cit, and Lac, which may be connected to distinct plant species and soil habitats.

4.5. Effect of AMF on BCF and TF under Cd stress

BCF is a fundamental indicator of plant contaminant carrying capacity, while TF is a significant indicator for assessing plants' capacity to remove pollutants (Tang et al., 2020). Since none of the treatments in this investigation had TF values above 1.0, *C. sativa* was not a hyperaccumulator (Likar et al., 2010). At Cd contents of 60 and 90 mg/kg, respectively, *Siegesbeckia orientalis* L. was a Cd hyperaccumulator, and the $BCF_{root/soil}$ was 1.30 and 1.23 (Zhang et al., 2013b). In our study, $BCF_{root/soil}$ for AM_{Cd} treatments was higher than theirs, suggesting that the heavy metal extraction efficiency of the symbiosis may be higher. The differences in heavy metal species and host plant species induce different AMF regulation strategies (Aalipour et al., 2021). The study by Wu et al. (2016) emphasized the AMF exclusion strategy, while the study by Yizhu et al. (2020) emphasized the AMF accumulation strategy. The results of this experiment were like the latter, indicating that AMF has a protective effect on Cd transport and a facilitative effect on root Cd enrichment. This may be related to the release of some secondary metabolites in root exudates, e.g., Cit and Mal (Luo et al., 2014). Plants will modify the status of the rhizosphere, particularly when exposed to heavy metals, to adapt to their surroundings (Wang et al., 2018). This agrees with our results on the determination of LMWOAs in soil.

5. Conclusion

Our results showed that Ri inoculation stimulates *C. sativa* growth and alters the LMWOAs composition in rhizosphere soil, mainly by regulating Mal, Cit, and Lac to affect the bioavailability of Cd, thus increasing the ratio of exchangeable and oxidizable Cd in rhizosphere soil. At the same time, in the Ri- *C. sativa* symbiotic system, the root enrichment and transport of Cd were significantly different. Root transcriptome analysis showed that Ri further improved the heavy metal tolerance of *C. sativa* by altering the root molecular transport mechanism. As a result, *C. sativa*, a biotechnological cash crop (AMF - cash crop) that doesn't get into the food chain, it's crucial to achieve ecological and economic benefits in a heavy-metal-stressed environment.

Credit author statement

Simiao Sun: Conceptualization, Methodology, Data curation, Writing – original draft, Formal analysis, Visualization, Writing – review & editing. **Xiaoxu Fan**: Data curation, Writing – review & editing. **Yuhan Feng**: Supervision. **Xiaohui Wang**: Methodology. **Hongsheng Gao**: Project administration. **Fuqiang Song**: Resources, Supervision, Funding acquisition.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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