

Effects of Intercropping Patterns on Cadmium Tolerance of *Perilla Frutescens* and Soybean

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Research Article

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Abstract

In this study, we intercropped hyperaccumulator *Perilla frutescens* with soybean to explore the effects of intercropping under cadmium stress based on the physiological growth indexes of plant, cadmium absorption capacity, and soil cadmium forms. Experiments showed that compared with mono-cropping, intercropping soybean's biomass, catalase (CAT) activity, peroxidase (POD) activity, total chlorophyll and net photosynthetic rate were significantly increased by 1042%~10658%, 1578%~7381%, 1345%~2337%, 376%~1500% and 7121%~31278%, while *P. frutescens*'s significantly decreased by 9387%~9507%, 1562%~2572%, 12056%~15331%, 715%~756% and 3056%~5076%. Under 5 and 10 mg kg⁻¹ Cd treatments, the Cd content in roots, stems, leaves, pods and grains of intercropping soybean decreased significantly by 1072%~3174%, 1488%~2168%, 1995%~3413%, 2222%~5270% and 791%~1786% along with enrichment coefficient decreasing by 1486%~2787%, while those of *P. frutescens* significantly increased by 8700%~2141%, 948%~2155%, 2305%~3766% and 1818%~9412% along with enrichment coefficient significantly increasing by 2000%~7775%. Additionally, citric acid, succinic acid, oxalic acid and tartaric acid worked importantly in heavy metal detoxification in plants. Strikingly, the content of soil exchangeable Cd (EXC) under intercropping was the highest, even higher than that of mono-cropping soybean and *P. frutescens*, which accounted for 37%~42% under the same stress condition. Therefore, intercropping *Perilla frutescens* with soybean provide a potential strategy for Cd phytoremediation.

Introduction

At present, serious cadmium (Cd) pollution in farmland directly leads to excessive Cd content in crops, which is harmful to human health (Kim et al, 2018). Therefore, how to reduce Cd pollution and ensure the safe production of food crops has become a current research hotspot in the field of soil and environment (Bashir et al, 2018; Saqib et al, 2018). To solve this problem, plant intercropping remediation technology is regarded as one of the most economical and effective technologies (Huang et al, 2018). In particular, the use of intercropping of hyperaccumulators with crops are currently the most effective treatment of heavy metals in soil. The effective intercropping restoration mode can not only reduce the absorption of heavy metals by crops, but also achieve the purpose of production at the same time (Zou et al, 2016; Chen et al, 2019). For different absorption capacities of heavy metals between hyperaccumulators and crops, less heavy metals absorbed by crops can be achieved.

The intercropping of hyperaccumulators and crops is firstly reflected in the physiological and biochemical effects of intercropping on the two plants. According to reports, the photosynthetic pigment content of tomato leaves increased by 1113%~3558% during the intercropping of *Solanum* and tomato, and the antioxidant enzyme activity increased by 050%~4706% compared with mono-cropping (Hu et al, 2019). Wang et al (2019) found that the photosynthetic pigment content of lettuce leaves increased by 144%~1440% after two ecological types of *Solanum photeinocarpum* and its hybrid progeny were intercropped with lettuce, and antioxidant enzyme activity is significantly increased by 7330%~28095%. In terms of the intercropping mechanism involving hyperaccumulators and crops, it is generally believed that the intercropping will change the type of plant root exudates. Root exudates can change the rhizosphere microbial groups, soil enzyme activities and soil pH, leading to changes in the form of soil heavy metals. Eventually, changes in the form of heavy metals in the soil will affect the absorption of heavy metals by plants. Root exudates of plants include organic acids, amino acids, soluble sugars and polysaccharides. Organic acids as a ligand of heavy metal elements, participating in the absorption, transportation, and accumulation process of heavy metals. Organic acids can also combine with heavy metals entering plants to protect the activities of important proteins and enzymes in cells, thereby reducing the toxicity of heavy metals. Therefore, organic acids play an important role in the detoxification mechanism of hyperaccumulation plants that are resistant to heavy metal.

poisoning(Zhang et al, 1999)Sha et al (2019)found that under the intercropping of *Paspalumscrobiculatum* and *Macleaya cordata*soil enzyme activity was enhanced, and the proportion of tolerant bacteria in the soil also increasedThe changes in these intercropping soils eventually increased the amount of heavy metals enriched by hyperaccumulators Soil pH affects the bioavailability of heavy metals through adsorption and precipitation Under normal circumstances, the content of exchangeable heavy metals in the soil will decrease with the increase of pH(Liu et al, 2011), and there is a significant negative correlationIn the intercropping system of *Oryza sativa* and *Thalia dealbata*, the increasing of pH in rice rhizosphere soil can reduce the absorption of heavy metals by crops(Wang et al, 2020)Intercropping can change the form of heavy metals in the soil and enhance the bioavailability of heavy metals in the soil(Lu and Yan, 2007) For example, under the intercropping of *Dipsacus chrysanthemum* with broad bean, the Cd exchangeable content in the mature stage was significantly lower than that in the seedling stage by 1748% The possible reason is that under the intercropping mode, the roots secrete organic acids to complex with Cd to form a soluble complex, thereby increasing the activation efficiency of soil Cd, and then being absorbed by *Dipsacanthus*(Tan et al, 2016)

In fact, the selection of hyperaccumulators is the key to the restoration of heavy metal-contaminated soil by intercropping with hyperaccumulators and cropsAt present, there are still deficiencies in hyperaccumulators Field restoration experiments have shown that many kinds of hyperaccumulators, such as *Solanum nigrum*, *Sedum alfredii* and *Bidenspilosa*have slow growth, small biomass, and unsatisfactory restoration effects(Cao et al, 2014; Chen et al, 2014b; Lin et al, 2020)Choosing hyperaccumulators with suitable growth habits and higher biomass for better extraction of heavy metals, which is essential for the intercropping system*Perilla frutescens* is a Cd hyperaccumulator with the advantages of large biomass, short growth cycle and strong adaptability to the growth environment,which is an ideal Cd accumulating plant for intercropping research(Xiao et al, 2020)Currently, the post-treatment problem of hyperaccumulators has always limited the development of phytoremediation technology In fact, *P frutescens* still a kind of aromatic plant, rich in volatile oil(Zhong et al, 2021)Therefore, the cadmium-rich *P frutescens*can be separated by steam distillation to separate the volatile oil without Cd pollution In addition,*P frutescens*rich in heavy metal Cd can also be prepared into high-performance activated carbon, which shows a good removal effect on Pb^{2+} and Cd^{2+} in water, and effectively inactivates Cd in biomass(Zheng et al, 2019)These characteristics of *P frutescens*greatly improve the economic benefits of repairing hyperenrichment plants Soybean is an important oil crop and economic crop in china, and it is sensitive to Cd and can easily absorb heavy metals(Finger-Teixeira et al, 2010)According to reports, under the condition of intercropping between legumes and hyperaccumulators, legumes can provide nitrogen to hyperaccumulators through nitrogen fixation, thus promoting the accumulation of hyperaccumulators(Li et al, 2007)Studies have found that Cd stress seriously affects the growth and development of soybean, which in turn affects soybean yields(Huang et al, 2006)Therefore, research to improve the situation of soybean growth under Cd contamination is of great significanceAt present, our lab has conducted preliminary explorations on the Cd enrichment capacity of the hyperaccumulator*P frutescens*(Xie et al, 2011),and explained the Cd tolerance of *P frutescens*from the perspective of organic acids(Chen et al, 2018) and plant chelating peptides(Xiao et al, 2020)Nevertheless, whether the intercropping of *P frutescens*and crops can reduce the Cd content in the crops while repairing the soil, and the mechanism of Cd tolerance in plants has not been studied yet

In this paper, the effects of intercropping patterns on Cd tolerance of *P frutescens* and soybean will be explored from the aspects of plant growth indicators, physiological levels, the correlation between plant organic acids and Cd content, and soil Cd forms This study aims to provide a reference for the study of the mechanism of Cd tolerance in *P frutescens* and soybean

Materials And Methods

21 Plant materials

In this experiment, the soil was collected in the westernmost glass room (26°5'22" N, 119°13'49" E) of the College of Life Sciences of Fujian Agriculture and Forestry University on March 16, 2019. Firstly, the soil was placed in the laboratory to be air-dried naturally, and the stones and other debris in the soil were removed before being crushed. Secondly, the ground soil of the same weight was then weighed and placed in the culture basins (62 cm×35 cm×26 cm, length × width × height), with or without partitions. Finally, the chemical reagent CdCl₂·25H₂O was used to prepare the soil treated with 0, 5 and 10 mg kg⁻¹ Cd separately, and the soil was mixed thoroughly and equilibrated for 14 d. The pH of the collected soil was 5.72, organic matter was 43.12%, total nitrogen was 3090 mg kg⁻¹, total phosphorus was 374.90 mg kg⁻¹, total potassium was 45878 mg kg⁻¹, and available phosphorus was 12015 mg kg⁻¹, quick-acting potassium was 14986 mg kg⁻¹.

Perilla frutescens (L.) Britt was a Cd hyperaccumulator screened in the laboratory early, which was purchased from a plant farm in Huichang county, Jiangxi province. Soybean (*Glycine max* (L.) Merrill) is a dicotyledonous herb of the genus legume and is cultivated worldwide. The soybean variety "Ruidou No 1" was adopted in this experiment, purchased from Zhencheng Agricultural Materials Management Department in Muyang county, Jiangsu province.

22 Intercropping experiment

The experiments were set with Cd treatment conditions at concentrations of 0, 5 and 10 mg kg⁻¹ and three planting methods of *P. frutescens* monocropping, soybean monocropping and *P. frutescens* soybean intercropping in potted soil culture experiments. After the soil was aged, the soybean and *P. frutescens* seeds were soaked firstly and then put directly in the Cd-treated potting soil. The planting method of 5 cm×5 cm intervals was used in each treatment, and 30 *P. frutescens* and 30 soybeans were planted in each pot. Intercropping planting mode were set as 1:1. By externally adding with Hoagland nutrient solution, same nutrient supply and soil moisture content of soybeans and *P. frutescens* were ensured during cultivation. Samples of soybeans and *P. frutescens* of each treatment were collected during the soybean bulging period. The fresh plant samples were cleaned with distilled water, then quickly frozen with liquid nitrogen, and kept in a refrigerator at -20°C for the determination of physiological and biochemical index. Some plant samples were weighed on the electronic balance as fresh weight, then dried in the oven at 105°C to constant weight at 72°C for the measurement of the dry weight of plants and the determination of Cd content. After the plants were collected, the soil samples were collected immediately, then were mixed evenly, air-dried, and passed through a 30-mesh sieve for use.

23 Experiment methods for plant samples

The biomass of plant samples was measured by tape and balance. The determination of net photosynthetic rate: taking three function leaves from plants for measurement by the portable meter plant photosynthesis (LI-COR6400, LI-COR), and select 3 strains of each processing. Determination of chlorophyll content: referring to the method of Wang et al (2006). The activity of antioxidant enzymes was determined by ultraviolet spectrophotometry: 0.500 g fresh sample of plant leaves was taken and ground into a powder with liquid nitrogen. Plant sample powder were collected from the previous step into a 10 ml centrifuge tube, and added 8 ml of 50 mmol L⁻¹ phosphate buffer (pH 7.8) containing 1% PVP. After mixing, the obtained samples were centrifugated at 10000 r min⁻¹ and 4°C for 20 min in a high-speed cryogenic centrifuge. Malondialdehyde (MDA) content was determined by

the thiobarbituric acid method, catalase (CAT) activity was determined by the ultraviolet absorption method, and peroxidase (POD) activity was determined by the guaiacol method (Wang et al, 2006)

The determination of organic acid content was slightly modified according to Chen et al (2018) The plant roots, stems and leaves are dried at 60°C to a constant mass, then were crushed and passed through a 60-mesh sieve (0.5~1.2 mm), and stored under dry conditions for later use Accurately, 0.1 g of the plant sample were weighed, then 10 mL of 0.1% potassium dihydrogen phosphate solution were added to dissolve it, and were soaked for 4 hours, ultrasonically extracted for 30 minutes, filtered and centrifuged for 10 minutes (3500 r min⁻¹) The supernatant was filtered through a 0.45 µm organic filter membrane and sampled for analysis

Determination of Cd content in plant samples: The Cd determination method in the national food safety standard (GB5009.15-2014) was adopted (National Health Commission, 2015) The digested plant samples' Cd content was determined by atomic absorption spectrometry (WFX-130A, Beijing Beifen-Ruili) The determination of different Cd forms in soil was based on Bi et al (2006)

2.4 Data analysis

The ANOVA in Microsoft Office and IBM SPSS Statistics 21.0 software was used for data processing, analysis and mapping, and Duncan's new complex range method was used for significant difference test at a level of $P < 0.05$ All data in the table are mean $\pm$ standard deviation (n=3)

Bioconcentration factor (BCF) = total Plant Cd content/soil Cd content

Transfer coefficient (TF) = Aboveground Cd content/underground Cd content of plants

Result

3.1 *P. frutescens* and soybean biomass under intercropping

As shown in Table S1, under the three Cd treatments, the biomass of intercropped soybean increased by 104.2%~106.58% compared with mono-cropped soybean The results showed that with the increase of Cd concentration, the biomass of mono-cropped soybeans first decreased and then increased, the biomass of intercropped soybeans first increased and then decreased, and the biomass of intercropped soybeans was significantly higher than that of mono-cropped soybeans With the increase of Cd concentration, the biomass of *P. frutescens* decreased Interestingly, the biomass of intercropping *P. frutescens* was significantly lower than monocropping

3.2 Effects of intercropping on Cd content and Cd absorption capacity of soybean and *P. frutescens* organs under Cd stress

3.2.1 Cd content and absorption capacity of soybean

The Cd content of soybean organs is shown in Fig 1 Under 0 mg kg⁻¹ Cd treatment, the Cd content of intercropped soybean roots was significantly lower than that of monocropping by 70.27% In addition, no Cd was detected in other soybean organs Under 5 and 10 mg kg⁻¹ Cd treatments, compared with monocropping, the Cd content of intercropping soybean roots, stems, leaves, pods and grains decreased significantly by 10.72%~31.74%, 14.88%~21.68%, 19.95%~34.13%, 22.22%~52.70% and 7.91%~17.86% The BCF of intercropping soybean was

significantly decreased by 1486%~2787% Under 5 mg kg⁻¹ Cd treatment, the TF of intercropped soybean was significantly reduced by 1500%, while under 10 mg kg⁻¹ Cd treatment, it was significantly increased by 1200% (Table S2) These results confirmed that intercropping significantly reduced the Cd content and Cd absorption capacity of soybean

322 Cd content and absorption capacity of *P frutescens* treated

The content of Cd in all organs of *P frutescens* shown in Fig 1 Under 0 mg kg⁻¹ Cd treatment, the Cd content in the roots, stems and leaves of the intercropping *P frutescens* was significantly increased by 11898%, 3092% and 3689%, respectively There was no Cd detected in the spicas of the intercropping *P frutescens* Under 5 and 10 mg kg⁻¹ Cd treatments, in intercropping *P frutescens*, Cd content of roots, stems, leaves and spicas were significantly increased by 8700%~2141%, 948%~2155%, 2305%~3766% and 1818%~9412% Under 0, 5 and 10 mg kg⁻¹ Cd treatments, the BCF of intercropping *P frutescens* significantly increased by 2000%~7775% The TF of intercropped *P frutescens* decreased by 4043% and 2941% under 0 and 5 mg·kg⁻¹ Cd treatments, while there was no significant change under 10 mg·kg⁻¹ Cd treatment (Table 2) These results showed that intercropping significantly increased the Cd content and Cd absorption capacity of *P frutescens*

33 Effect of intercropping on physiological characteristics of soybean and *P frutescens*

331 Effect of intercropping on chlorophyll of soybean and *P frutescens*

The Chlorophyll A (Chla) and Total chlorophyll (ChIT) content of intercropped soybean leaves were significantly increased by 209% and 376%, and there was no significant difference in Chlorophyll B (Chlb) content under 0 mg kg⁻¹ Cd treatment Under 5 and 10 mg kg⁻¹ Cd treatments, the content of Chla, Chlb and ChIT of intercropped soybean leaves were obviously increased by 370%~904%, 1446%~3529%, and 546%~1500% (Fig 2) The content of Chla, Chlb and ChIT of intercropping *P frutescens* was significantly increased by 6214%, 7048% and 6217% under 0 mg·kg⁻¹ Cd treatment However, under 5 and 10 mg kg⁻¹ Cd treatments, the content of Chla, Chlb and ChIT in intercropping *P frutescens* was reduced by 785%~833%, 487%~1771% and 715%~756% The net photosynthetic rate (Pn) of intercropped soybean leaves was significantly increased by 7121%, 19429% and 31278% under different Cd treatments Interestingly, the Pn of intercropping *P frutescens* leaves significantly decreased by 3056%, 5076% and 4490% This study found that under the same Cd stress, intercropping significantly increased soybean chlorophyll content and Pn, while significantly reducing *P frutescens*'s (Fig 2)

332 Effects of intercropping on malondialdehyde content and antioxidant enzyme activities of soybean and *P frutescens* leaves

Under 0, 5, and 10 mg kg⁻¹ Cd treatments, the malondialdehyde (MDA) contents of intercropped soybean leaves not only caused a certain degree of reduction by 1692%~1152%, but also the activities of POD and CAT were significantly increased by 1345%~2337% and 1578%~7381% Similarly, *P frutescens* appeared the same results The MDA content of *P frutescens* leaves was significantly reduced by 5765%~7278%, and the activities of POD and CAT were increased by 1562%~2572% and 12056%~15331% under intercropping In summary, intercropping reduced the MDA content, and increased the CAT and POD activity of soybean and *P frutescens* leaves (Table S3)

34 Effect of intercropping on the organic acid content of soybean and *P frutescens*

341 Determination of organic acid content

Under the set chromatographic conditions, the mixed standards containing eight organic acids are completely separated within fourteen minutes. The standard chromatogram measured by HPLC is shown in Fig 3, and the linear regression equation is shown in Table 4. Chromatographic conditions: Chromatographic column is ZORBAX SB-C18 (250 mm×4.6 mm, 5 μm); mobile phase: methanol (A); 0.1% potassium dihydrogen phosphate solution (B); Isocratic elution: 5%A~95%B; flow rate 0.8 mL min⁻¹; injection volume 10 μL; detection wavelength 210 nm; column temperature 30°C.

342 Correlation between Cd content and organic acid in various organs of soybean and *P frutescens* under different planting methods

It can be seen from Table 1 that more organic acids were significantly positively correlated with Cd content in mono-cropped soybeans than in intercropped soybeans, and the organic acids with the largest correlation coefficient were citric acid, succinic acid, tartaric acid and oxalic acid. There was no significant difference between the organic acids of mono-cropped *P frutescens* and intercropped *P frutescens*. The largest correlation coefficients were citric acid, tartaric acid and oxalic acid of intercropped *P frutescens*.

The correlation coefficient of organic acids and Cd in the roots of intercropped soybean was higher than that of mono-cropped soybean except fumaric acid and tartaric acid, and the greatest correlation was citric acid. The number of species with a positive correlation between organic acid and Cd content in intercropped soybean stems was less than that in mono-cropped soybean. Among them, the greatest correlation was oxalic acid and tartaric acid. The number of species with a positive correlation between organic acid and Cd content in intercropped soybean leaves was less than that in mono-cropped soybean, and the greatest correlation was citric acid. Except that, the correlation coefficient of fumaric acid and Cd in stems of intercropped *P frutescens* was higher than mono-cropped. The correlation coefficient of organic acid and Cd in other organs of intercropped *P frutescens* was lower than mono-cropped.

In general, intercropping changed the correlation between Cd content and free organic acids in *P frutescens* and soybean. Citric acid, oxalic acid and tartaric acid were the most important in soybean, meantime, tartaric acid, citric acid and succinic acid was the most important in *P frutescens*.

Table 1 Correlation between Cd content and organic acids in roots, stems and leaves of soybean and *P frutescens*

Plant	Organs	Treatment	Malic acid	Citric acid	Succinic acid	Fumaric acid	Oxalic acid	Tartaric acid	Total organic acid
Soybean	Root	M	-0984**	0982**	-0887**	0933**	-0521	0721*	0982**
		I	0346	0984**	-0416	0514	0275	0347	0986**
	Stem	M	0578	0998**	0964**	0348	0339	0176	0998**
		I	-0805**	-0854**	0198	-0963**	0950**	0905**	-0819**
	Leaf	M	0485	0023	0990**	0675*	-0963**	-0428	0769*
		I	-0752*	0075	-0085	-0926**	-0347	-0045	-0259
<i>Perilla frutescen</i>	Root	M	-0118	0886**	-0016	-0249	0271	0955**	0861**
		I	-0403	0127	-0421	-0506	-0798**	0912**	006
	Stem	M	-0678*	0963**	-0940**	0677*	0821**	a	0965**
		I	-0931**	0881**	-0675*	0880**	-0288	a	0868**
	Leaf	M	0727*	0921**	0565	-0857**	0424	a	0858**
		I	0438	-0092	0560	-0905**	-0084	a	-0199

Note: "*" indicates significant correlation at 005 level; "**" indicates a significant correlation at 001 level "a" At least one variable is a constant, so it cannot be calculated

35 Effect of *P frutescens* and soybean intercropping on Cd forms in cadmium-contaminated soil

There are five forms of Cd in soil, namely, exchangeable form (EXC-Cd), carbonate binding form (Crab-Cd), iron and manganese oxidation form (FeMnO₂-Cd), organic form (OM-Cd) and residual form (RES-Cd) As shown in Fig 4, in the soil by monocropping soybean and monocropping *P frutescens*, the content of FeMnO₂-Cd was the largest, accounting for 34% and 36% under the condition of 0 mg kg⁻¹ Cd treatment However, under the conditions of 5 and 10 mg kg⁻¹ Cd treatments, the EXC-Cd content was the highest, accounting for 37% and 35% in monocropping soybean soil, and 35% and 32% in monocropping *P frutescens* soil Remarkably, EXC-Cd content in the soil of the intercropping planting mode is the largest, even higher than that of the monocropping soybean and *P frutescens*, accounting for 37% to 42% (Fig 4)

In conclusion, in intercropping mode, the proportion of EXC-Cd increased with increasing Cd concentration, and EXC-Cd content was significantly higher than that in monocropping mode, and it accounted for the largest proportion of chemical forms of Cd in soil

Discussion

41 Effects of the intercropping system on biomass and photosynthesis of *P frutescens* and soybean

Different plants possess different uses of soil nutrients, water, light, and heat, thereby brings differences to the growth, which depends on photosynthesis and antioxidant systems (Mnk et al, 2020) of plants in intercropping

system Chlorophyll plays a very keystone role in the process of photosynthesis (Paul et al, 2017) Intercropping can change the utilization of light energy by varying the chlorophyll content of plants, so the photosynthetic capacity and dry matter accumulation of leaves changed (Li et al, 2019) In this study, the biomass, chlorophyll content and net photosynthetic rate of intercropped soybean was significantly higher than that of mono-cropped, while those of intercropped *P frutescens* were significantly lower than mono-cropped *P frutescens*, which indicated that intercropping patterns could significantly promote the growth of soybean and inhibited the growth of *P frutescens* For one thing, it is speculated that soybean and *P frutescens* had the same needs or uses for soil resources, so interspecific competition was gradually formed during the growth process As more nitrogen, phosphorus and organic matter in soil utilized by soybean, a disadvantageous position of *P frutescens* had been resulted The other assumption suggested that *P frutescens* have a strong ability to absorb heavy metals in the soil, so the absorption of heavy metals by soybean reduced Certainly, excessive Cd would bring necrosis and withering of plant leaves, photosynthesis efficiency decline, biomass reduction and even death (Xie et al, 2011; Yang et al, 2020) Tang et al (2020) found that compared with monoculture, the contents of chlorophyll A, total chlorophyll and carotenoids were significantly increased in intercropped spinach, and this result is consistent with our experiment After intercropping of hyperaccumulator *Tagetes patula* with *Brassica chinensis*, the biomass of *Tagetes patula* increased significantly while the biomass and net photosynthetic rate of *Brassica chinensis* decreased significantly (Yan et al, 2020), which is contrary to the results of this experiment It is suggested that the competitive ability of *Tagetes patula* for soil nutrients and other resources was significantly stronger than that of *Brassica chinensis* Therefore, intercropping with suitable hyperaccumulators and crops can restore soil and maintain crop yield

4.2 Effects of the intercropping system on Cd absorption capacity of *P frutescens* and soybean

Plant absorption of heavy metal Cd in soil is mainly related to the form of Cd in soil Tessier et al (1979) divided forms of heavy metals in sediment or soil into five forms: exchangeable form (EXC-Cd), carbonate binding form (Crab-Cd), iron and manganese oxidation form (FeMnO₂-Cd), organic form (OM-Cd) and residual form (RES-Cd) Easy being transferred and transformed, these five forms can change with the environmental conditions (Giasson et al, 2005; Shang et al, 2016) They also have very different bioavailability Heavy metals in Crab and EXC forms have high bioavailability, which can be easily absorbed by plants, while the FeMnO₂ form and OM form are not easy to be absorbed directly, but can be transformed into EXC form and Crab form under certain redox conditions Lastly, the RES form is generally not bioavailable (Fei et al, 2012; Zu et al, 2014) In this work, 0 mg kg⁻¹ Cd intercropping significantly increased the EXC-Cd content in soil, accounting for 37.16% of the total, and significantly decreased the Crab-Cd, OM-Cd and RES-Cd content Under 5 and 10 mg kg⁻¹ Cd treatments, the intercropping mode significantly increased EXC-Cd in soil, separately at a proportion of 39.63% and 42.06%, and reduced FeMnO₂-Cd and OM-Cd content It is clear that intercropping could significantly increase the content of bioavailable Cd in soil under Cd stress These results indicated that soil EXC-Cd was converted from Crab-Cd, FeMnO₂-Cd and OM-Cd under intercropping conditions There are two possible reasons for this phenomenon Firstly, low-molecular-weight organic acids, such as oxalic, and citric et al could form soluble complexes with Cd, which would be released from the soil matrix then (Krishnamurti et al, 1997) In this study, the bioavailability of Cd in the soil enhanced may be resulted in the root exudates, mainly organic acids, released by soybean and *P frutescens* roots As is known to all, leguminous plants can release a large amount of H⁺ into the soil in the process of biological nitrogen fixation to acidify the soil (Chang et al, 2006) So the second reason is that the low pH value of the soil will increase the dissolution and release of heavy metals such as carbonate and Fe-Mn combination (Sappin-Didier et al, 2005; Yang et al, 2005) Furthermore, the Cd enrichment amount and enrichment coefficient of intercropped *P frutescens* were significantly higher than those of mono-cropped, confirming that most EXC-Cd released from soil was absorbed by *P frutescens* However, the

hyperaccumulator *P. frutescens* may have a similar "foraging" for heavy metals in the soil environment (Guo et al, 2021), which inhibits the absorption of heavy metals in soybeans. Interestingly, Tan et al (2016) found that EXC-Cd in soil was significantly lower at the maturity stage than seedling stage of crops, which was inconsistent with the results of this study. It was indicated that the intercropped *P. reticulum* grew faster, having greater biomass, and absorbed more Cd from the soil, which led to the decrease of exchangeable Cd content in the soil during the mature stage. Different from the *P. reticulum*, the biomass of *P. frutescens* significantly decreased in intercropping conditions, which could not completely absorb the EXC-Cd in the soil, increasing exchangeable Cd content in the soil. These results suggest that the accumulation of heavy metals in soil by plants depends on the bioavailability of heavy metals in soil and which species are under intercropping treatment. In this study, it was found that intercropping increased the bioavailability of Cd, and more EXC-Cd was absorbed by *P. frutescens*.

In this study, Cd in roots of soybean and roots, stems, and leaves of *P. frutescens* were detected under 0 mg kg⁻¹ Cd treatment. It is showed that *P. frutescens* could still absorb Cd from soil and transport it to stems and leaves from roots when the background concentration of Cd in soil is low. Apparently, *P. frutescens* has a stronger ability to deal with Cd stress problem than soybean. Under the same Cd stress condition, the Cd content and its enrichment coefficient of soybean under intercropping conditions were significantly lower than those of mono-cropped. Conversely, the Cd content and Cd enrichment coefficient of intercropped *P. frutescens* were significantly higher than those of mono-cropped. The transfer coefficient of intercropped soybean first decreased and then increased with increasing Cd content while that of intercropped *P. frutescens* decreased. The transfer coefficient of soybean in intercropping was significantly increased under 10 mg kg⁻¹ Cd treatment compared with that in monocropping. It is likely that the biomass of soybean in intercropping was significantly increased, and the Cd content was higher. In order to improve the Cd tolerance of soybean, the aboveground biomass provided more locations for heavy metals to accumulate. This situation would increase the transfer coefficient so as to avoid the negative impact on plants caused by the excessive accumulation of heavy metals in the ground (Zhang et al, 2020). Transfer coefficient of intercropped *P. frutescens* decreased with the increase of Cd content. The reason for this phenomenon is that the *P. frutescens* above-ground biomass decreased and enabled to provide more heavy metal accumulation locations. This change allows *P. frutescens* to retain more of the heavy metal Cd in its underground parts, which is consistent with the study of Xie et al (2011). In conclusion, under intercropping patterns, Cd enrichment of soybean mainly occurred in the roots, and then gradually transferred to the stems and leaves with the increase of biomass. On the contrary, *P. frutescens*'s uptake and tolerance of Cd is stronger, after the absorption of Cd, quickly from their roots transferred to the stems and leaves, which led the biomass and transfer coefficient decreases.

4.3 Resistance mechanism of antioxidant enzyme activity system and organic acids to Cd

Heavy metal stress can cause plants to produce a large amount of active oxygen free radicals. Excessive accumulation of reactive oxygen species will break the balance and lead to protein degeneration, DNA damage, cell enzyme inactivation and membrane lipid peroxidation damage, and even death (Wang et al, 2014; Jiang et al, 2016). The antioxidant enzyme system is an important mechanism of plant tolerance under heavy metal stress and plays a keystone role in plant adaptation to stress (Zhang et al, 2014). The changes in antioxidant enzyme activities and MDA content in plants were closely related to Cd stress (Yi and Kao, 2007). POD can regulate the level of indole-acetic acid (IAA) in plants and avoid the toxic effect of H₂O₂ in the organism. CAT converts H₂O₂ to H₂O and O₂ and participates in the glutathione-ascorbic acid cycle to remove H₂O₂ from cells, and the change of MDA content could reflect the damage degree of the plant cell membrane (Jamieson et al, 2012; Chen et al, 2014a; Guo et al, 2019). In this study, intercropping increased CAT activity and POD activity of soybean and *P. frutescens* leaves under Cd stress, and decreased MDA content under Cd stress. The finding of Tang et al (2017) also confirmed that the activity of

antioxidant enzymes increased under the intercropping of *Solanum nigrum* and *Solanum photeinocarpum*, which was consistent with this study. The resistance of antioxidant enzymes to heavy metal stress is a complex physiological process, which is affected by plant species, heavy metal concentration and properties. For example, the SOD activity of intercropped *Solanum nigrum* and *Lycopersicon esculentum* had no significant difference compared with that of monoculture, but the POD and CAT activities were significantly increased (Hu et al, 2019). In this study, the changes in the antioxidant enzyme activity system brought by inter-cropping could reduce the oxidative stress response of cells to a certain extent and improve the heavy metal tolerance of *P. frutescens* and soybean. However, the intercropping of hyperaccumulators and crops may produce opposite results in this study, such as the intercropping of hyperaccumulators *Pteris vittata* L and *Broussonetia papyrifera* L, which decreased POD activity and increased MDA content in leaves of *Broussonetia papyrifera* L (Peng et al, 2018). That's because *Pteris vittata* L is an As hyperaccumulator, with the increasing of root exudates, the absorption of As increased. On the contrary, *Broussonetia papyrifera* L is not a hyperaccumulator but inhibited by As. Therefore, only by selecting suitable plants for intercropping can the toxicity of reactive oxygen species to plants under heavy metal stress be alleviated to varying degrees by increasing antioxidant enzyme activity and reducing MDA content without damaging plant growth.

As the ligand of heavy metal elements, organic acids have multiple functions, including participating in the absorption, transportation, and accumulation of heavy metals. These functions can not only promote the accumulation of metals in plants, but also improve the tolerance of plants to heavy metals (Henry et al, 2007). When plants are stressed by heavy metals, many plants can actively respond to organic acid metabolism in order to improve their resistance. Organic acids in plants combine with heavy metal ions to form complexes, which are deposited in cell walls or vacuoles, reducing their free concentration in plants and thus the toxicity of heavy metals decreased (Polle and Schützendübel, 2003). As we all known, the molecular weight of each organic acid is different, and the coordination ability with Cd ion is also different. Therefore, it is of little significance to compare the absolute amount of each organic acid to measure the detoxification ability of organic acid in *P. frutescens* and soybean with the change of Cd concentration. For this reason, in intercropping system of *P. frutescens* and soybean, there were two explanations in regulation and detoxification mechanism of Cd in these two plants. It can be seen from Table 5 that in the intercropping soybean's roots and leaves, citric acid has the largest correlation coefficient with the Cd content. Meanwhile, in the intercropping soybean stems, oxalic acid has the largest correlation coefficient with the Cd content. This showed that under the intercropping system, the citric acid and oxalic acid in soybeans played a key role in detoxification of heavy metals. *P. frutescens* in the intercropping mode, the tartaric acid in the roots, citric acid in the stems, and succinic acid in the leaves have the largest correlation coefficient. According to the correlation coefficient, tartaric acid, citric acid and succinic acid in *P. frutescens* plays a greater role in the detoxification process. Different kinds of organic acids have different complexing and detoxification abilities with heavy metals in plants. For example, the stability coefficient of citric acid complexing with Cd (10^{315}) is significantly higher than that of malic acid (10^{134}). Thus, the low-toxic or non-toxic complex formed by citric acid and Cd in plants is more stable and plays a certain role in detoxification (Shi et al, 2003). At the same time, studies have shown that the extraction rate of succinic acid for heavy metals is similar to the extraction rate of citric acid (Wu et al, 2011), which indicates that succinic acid can also form non-toxic or low-toxic complex complexes with Cd. Oxalic acid plays an important biological role in plants and participates in the detoxification of metals (Klug and Horst, 2010). Oxalic acid is mainly involved in the transport of Cd in soybean stems, transporting heavy metals to various organs of soybean. Tartaric acid has an obvious detoxification effect on Cd and can reduce the toxicity of plants (Chen et al, 2000). Cd content in plants is closely related to the synthesis and accumulation of organic acids in plant species and organs of the same plant. Studies on exogenous addition of organic acids have proved that organic acids can reduce the toxicity of

heavy metals to plants (Guo and Zhuang, 2021). Therefore, organic acids can complex with heavy metals in plants, reducing the chance for heavy metals to bind to important proteins and enzymes in cells, thus reducing the toxicity of heavy metals in plants.

Thus, antioxidant enzymes can alleviate the toxicity of ROS to plants under heavy metal stress in varying degrees. Organic acids can also participate in the detoxification process of heavy metals, which also explained why *P. frutescens* could still grow after accumulating a large amount of heavy metals.

Conclusion

In this study, the intercropping of *P. frutescens* and soybean could not only enhance the tolerance of hyperaccumulator *P. frutescens* to Cd and improve its ability to absorb heavy metals, which is a "win-win" model. The intercropping system can strengthen the efficiency of phytoremediation, but not damage the economic benefits of farmers, and reduce the content of heavy metals in the soil, so the intercropping system of *P. frutescens* and soybean has important practical significance.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Competing interest

The authors declare that they have no known competing interests.

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Author contributions

All authors contributed to the study conception and design. Bolun Han: Conceptualization, Software, Writing-original draft; Wan Zhan: Resources, Investigation, Data curation; Rongrong Fan: Data curation, Software, Writing-review & editing; Rui Jing: Writing-review & editing; Ruiyu Lin: Writing-review & editing; Haibin He: Writing-review & editing; Xinyu Zheng: Methodology, Writing-review & editing, Supervision, Data curation. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

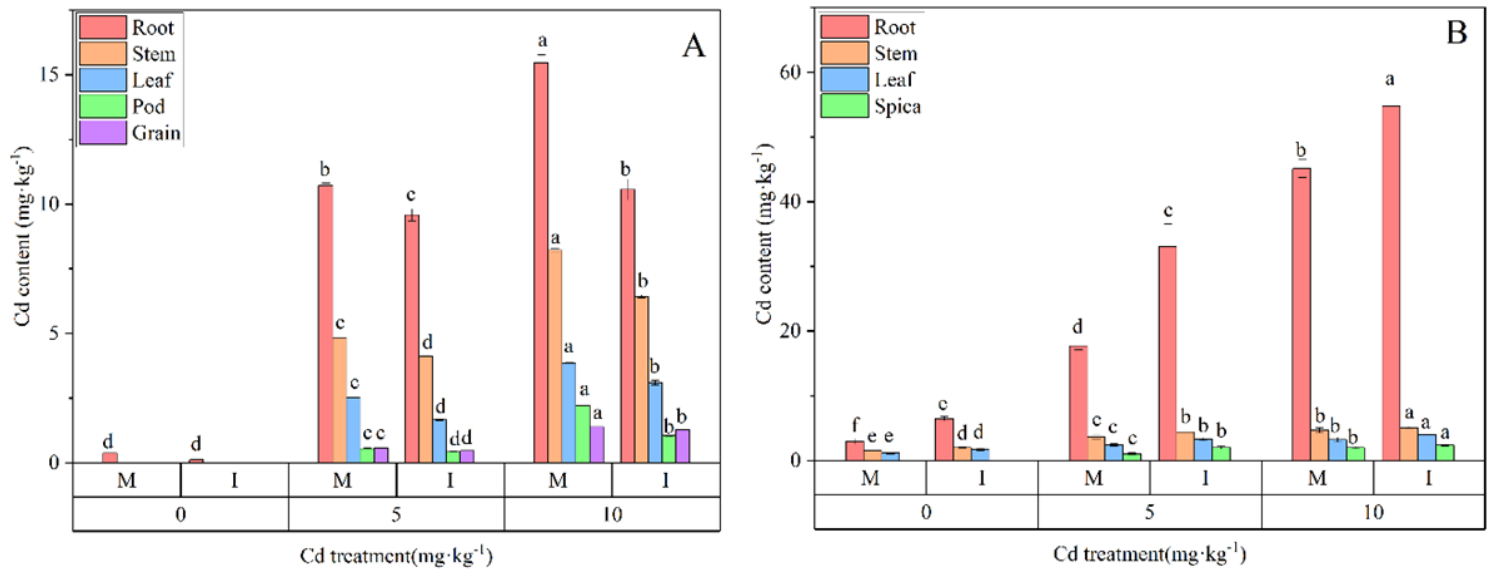


Figure 1

Cadmium content in various organs A) *Glycine max*, and B) *Perilla frutescens* under different planting patterns and cadmium stress (Different lowercase letters in the figure indicate the significant difference between treatments at 0.05 level)

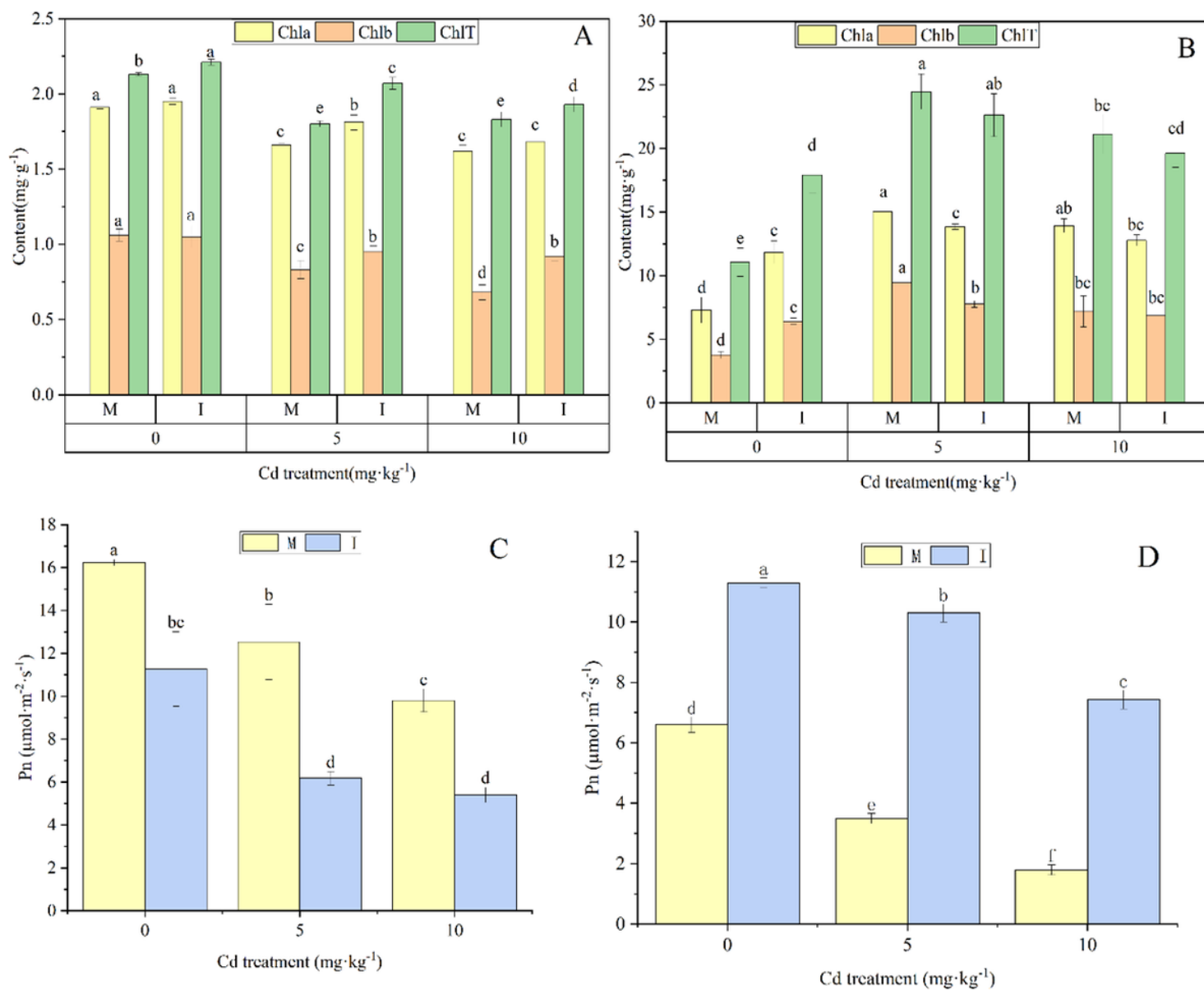


Figure 2

Chlorophyll content and Pn in leaves of plants under different Cd treatments: A) Soybean chlorophyll content, B) P frutescens chlorophyll content, C) P frutescens net photosynthetic rate, and D) Soybean net Photosynthetic Rate (Different lowercase letters in the figure indicate the significant difference between treatments at 0.05 level)

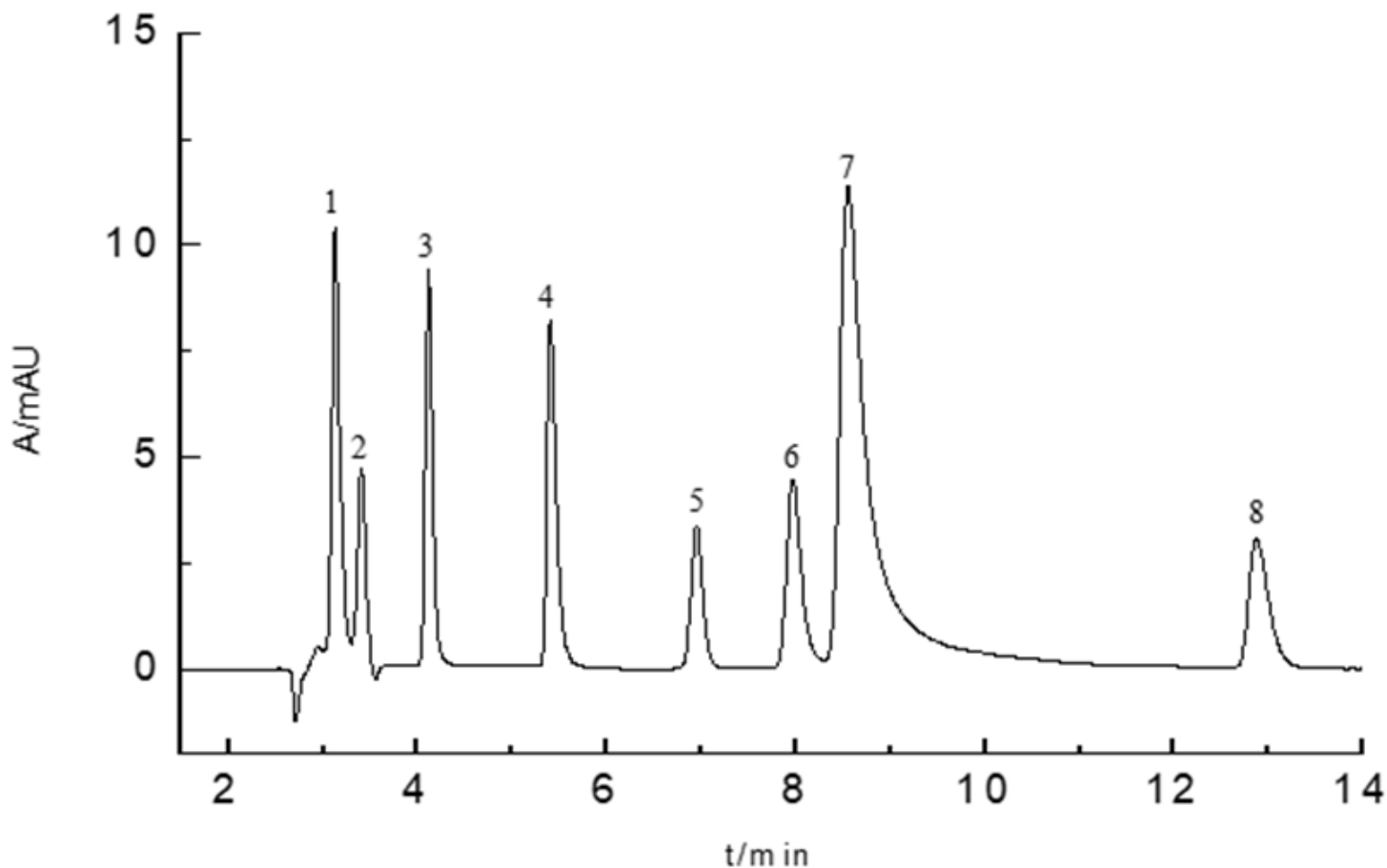


Figure 3

Chromatogram of organic acid mixed standard solution (1Oxalic acid, 2Tartaric acid, 3 Malic acid, 4 Glacial acetic acid, 5Citric acid, 6Butanedioic acid, 7Fumaric acid, 8Propionic acid)

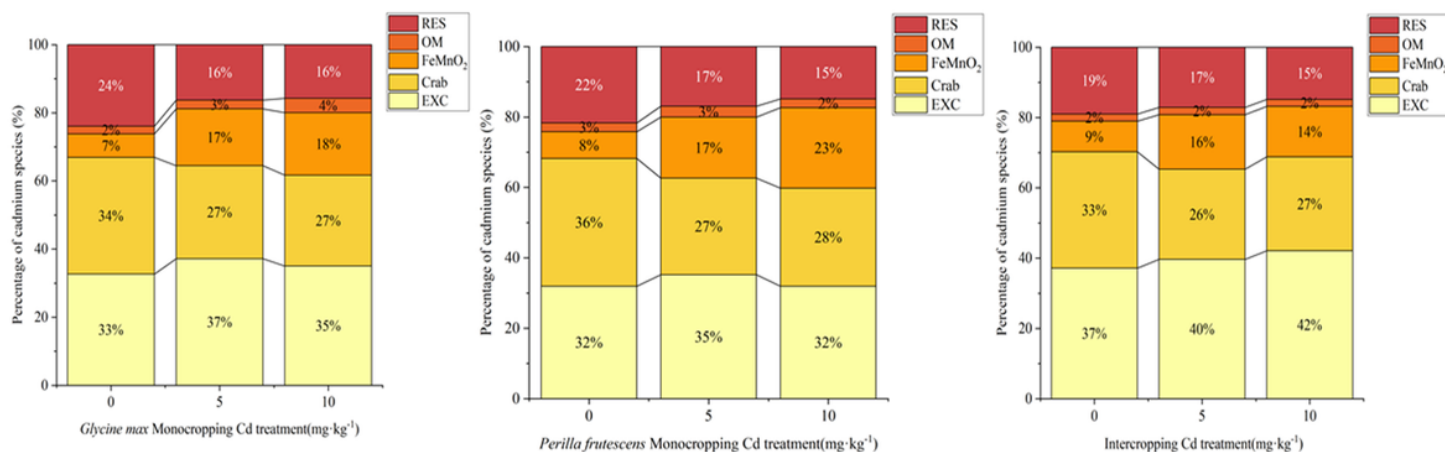


Figure 4

Cd forms in soil under different Cd treatments (Different lowercase letters in the figure indicate the significant difference between treatments at 005 level)

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