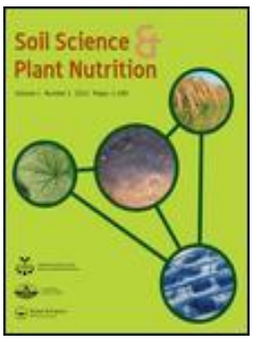

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ORIGINAL ARTICLE

Cadmium and lead affect the status of mineral nutrients in alfalfa grown on a calcareous soil

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ABSTRACT

Cadmium (Cd) and lead (Pb) are toxic trace elements which are not essential for plants but can be easily taken up by roots and accumulated in various organs, and cause irreversible damages to plants. A pot experiment was carried out to investigate the individual and combined effects of Cd (0, 10, 20 mg kg^{-1}) and Pb (0, 500, 1000 mg kg^{-1}) level in a calcareous soil on the status of mineral nutrients, including K, P, Ca, Mg, S, Fe, Mn, Cu, and Zn, in alfalfa (*Medicago sativa* L.) plants. Soil Pb level considerably ($P \leq 0.05$) affected the concentrations of more elements in plants than soil Cd level did, and there were combined effects of soil Cd level and Pb level on the concentrations of some nutrients (Ca, Mg, and Cu) in plants. The effects of soil Cd level and Pb level on plant nutrient concentrations varied among plant parts. Cd and Pb contamination did not considerably affect the exudation of carboxylates in the rhizosphere. An increase in rhizosphere pH and exudation of significant amounts of carboxylates (especially oxalate) in the rhizosphere might contribute to the exclusion and detoxification of Cd and Pb. Neither shoot dry mass nor root dry mass was significantly influenced by soil Cd level, but both of them were considerably reduced (by up to 25% and 45% on average for shoot dry mass and root dry mass, respectively) by increasing soil Pb level. The interaction between soil Cd level and Pb level was significant for root dry mass, but not significant for shoot dry mass. The results indicate that alfalfa is tolerant to Cd and Pb stress, and it is promising to grow alfalfa for phytostabilization of Cd and Pb on calcareous soils contaminated with Cd and Pb.

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Calcareous soil; carboxylates; mineral nutrition; plant growth; rhizosphere pH; soil contamination

1. Introduction

Cadmium (Cd) and lead (Pb) are toxic trace elements which have no biological functions and are naturally present in soils at low concentrations. However, they have become common and abundant toxic elements in soils and caused environmental and human problems in many parts of the world, due to the contamination of soils with Cd and Pb resulting from anthropogenic activities such as mining and processing of metals, and application of fertilizers (Clemens et al. 2013; Kushwaha et al. 2018). Although Cd and Pb are not essential for plants, they can be easily taken up by roots and accumulated in various organs, and cause irreversible damages to plants, including stunted plant growth and reproduction, altered uptake and distribution of mineral nutrients, etc. (Gielen, Vangronsveld, and Cuypers 2017; Gupta et al. 2017; Li et al. 2012; Zahedifar et al. 2016).

In the soil-plant system, many elements have similar chemical properties and interact with each other, and there is often a multi-element response in plants to an environmental stimulus, which usually creates a co-ordinated response of a series of physiological processes starting in the rhizosphere and ending with evapotranspiration and phloem recycling (Baxter 2015; Moosavi, Mansouri, and Zahedifar 2015; Moosavi, Zahedifar, and Mansouri 2018; alt, Baxter, and Lahner 2008). There are a few strategies plants employ to tolerate Cd and Pb, including exuding organic acids to sequester Cd and Pb (Kim et al. 2010; Pidatala et al. 2016), increasing rhizosphere pH to reduce the bioavailability of Cd and Pb (Kim et al. 2010), etc. Exudation of organic acids and alteration of the rhizosphere pH are likely to affect the bioavailability of multiple mineral nutrients in soils (Baxter 2015; Lambers, Pons, and Chapin 2008; Podar, Ramsey, and Hutchings 2004).

When rice (*Oryza sativa*) seedlings were treated with exogenous Cd, the uptake of calcium (Ca) and potassium (K) was impaired, Ca and K concentrations in both roots and shoots were significantly reduced (Li et al. 2012). Cadmium exposure has been found to induce copper (Cu) deficiency in plants such as *Arabidopsis thaliana* (Gielen, Vangronsveld, and Cuypers 2017). For *A. thaliana* and tobacco (*Nicotiana tabacum*) seedlings, Cd treatment increased iron (Fe) concentrations in roots but reduced Fe concentrations in shoots, and caused Fe-deficiency-like symptoms on plants; Cd increased Fe retention in roots and hampered its translocation to shoots (Xu, Lin, and Lai 2015; Yoshihara et al. 2006). The concentration of Fe in shoots of Indian mustard (*Brassica juncea*) was found to decrease proportionately with increase in Cd toxicity (Sharmila et al. 2017). With an increase in Pb supply, zinc (Zn) concentrations were increased in various parts of cabbage

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(*Brassica oleracea*) grown in refined sand with complete nutrient solution, whereas phosphorus (P), sulfur (S), Fe, manganese (Mn), and Cu concentrations were reduced (Sinha et al. 2006).

There are a number of studies on the individual effects of Cd and Pb on the status of mineral nutrients (Gielen, Vangronsveld, and Cuypers 2017; Li et al. 2012; Sinha et al. 2006). However, soil contamination is often a multi-element rather than a single-element problem, the effects of combined toxic trace elements contamination on mineral nutrients uptake and distribution by plants may be quite different from those of individual element contamination due to the interactions between elements in the soil-plant system (An et al. 2004; Luan, Cao, and Yan 2008). Therefore, it is necessary to study the combined effect of toxic trace elements on the status of mineral nutrients.

Alfalfa (*Medicago sativa* L.) is an important component of the agroecosystem on the Chinese Loess Plateau, where most soils are typically alkaline and calcareous, and it is often used for vegetation restoration and rehabilitation in this area because of its good growth performance (He et al. 2017). In this study, we carried out a pot experiment to investigate the individual and combined effects of Cd and Pb contamination on the status of mineral nutrients by alfalfa grown on a calcareous soil on the Loess Plateau, and to assess the potential of using alfalfa for vegetation rehabilitation on calcareous soils contaminated with Cd and Pb.

2. Materials and methods

2.1. Soil preparation

A calcareous soil was collected from the top 40-cm layer of an undisturbed site (39°47′35″N, 111°17′19″E) in the Jungar Banner of Inner Mongolia Autonomous Region, in the northern part of the Chinese Loess Plateau, and used for the pot experiment. The area has a semi-arid temperate continental climate, the mean annual precipitation is 404 mm, and the mean daily temperature is 7.2°C (Zhen et al. 2015). The soil was air-dried and screened to pass through a 2-mm sieve before physicochemical characterization. The distribution of soil particle size was analyzed on a Mastersizer 2000 laser diffraction particle size analyzer (Malvern Instruments, Malvern, UK) (Lamorski et al. 2014), and field capacity of the soil was determined by the indoor cutting-ring weighing method. Electrical conductivity (EC) and pH of the soil were measured in a 1:5 soil:water (w:v) suspension. Organic carbon (C) concentration in the soil was determined using the potassium dichromate titrimetric method (Mebius 1960). Ammonium-nitrogen (NH_4^+-N) and nitrate-nitrogen (NO_3^-N) concentrations were analyzed on a segmented-flow analyzer after extraction of the soil by a 2 M potassium chloride (KCl) solution [soil:solution = 1:10 (w:v)] (Kachurina et al. 2000). Concentrations total K, P, Ca, Mg, S, Fe, Mn, Cu, Zn, Cd, and Pb in the soil were analyzed on a inductively coupled plasma mass spectrometer (ICP-MS, ELAN 9000, PerkinElmer Instruments, Shelton, CT, USA) following a modified aqua regia digestion procedure (Reimann et al. 2015). The physicochemical properties of the soil are presented in Table 1.

The pot experiment was set in a two-factorial completely randomized design with three replications. Soil Cd level was designed as 0, 10, and 20 mg Cd kg^{-1} soil (hereafter referred to as Cd0, Cd10, and Cd20 treatment, respectively), and soil Pb level was designed as 0, 500, and 1000 mg Pb kg^{-1} soil (hereafter referred to as Pb0, Pb500, and Pb1000 treatment, respectively), to make a total of 16 (4 × 4) treatments, and there were six pots for each treatment. PVC tubes of 15-cm diameter and 20-cm height were used for the pots to grow plants. Each pot was first filled with 4 kg of the sieved soil, and then the soil was watered to 21% gravimetric soil water content (about 60% field capacity of the filled soil) with deionized (DI) water. To spike the soil with Cd and Pb at the designated levels, the total required amount of $Pb(NO_3)_2$ and $CdCl_2$ was weighed and dissolved in DI water separately with a known volume; then, the volume of the solution required for each pot was calculated according to the treatment, and the solution was applied to the soil at the calculated volume.

Table 1. Physicochemical properties of the loessial soil.

Parameter	Value	Parameter	Value
Sand (%)	69	Total P (mg g^{-1})	0.34
Silt (%)	18	Total Ca (mg g^{-1})	55.3
Clay (%)	13	Total Mg (mg g^{-1})	6.3
Field capacity (%)	35.0	Total S (mg g^{-1})	<0.2
EC (dS m^{-1})	0.17	Total Fe (mg g^{-1})	19.3
pH (H_2O, 1:5)	8.7	Total Mn (mg kg^{-1})	373
Organic C (mg g^{-1})	4.2	Total Cu (mg kg^{-1})	12.9
NH_4^+-N (mg kg^{-1})	57	Total Zn (mg kg^{-1})	34.4
NO_3^--N (mg kg^{-1})	1.2	Total Cd (mg kg^{-1})	0.08
Total K (mg g^{-1})	15.0	Total Pb (mg kg^{-1})	10.0

Values are means of three replicates. Data were modified after He et al. (2017).

To attain the quasi-equilibrium distribution of Cd and Pb, the spiked soil was subsequently placed at ambient temperature under a rain shelter and left undisturbed, except that the soil was watered to 21% gravimetric soil water content with DI water weekly during the spring, summer, and autumn, but the water was withheld during the winter. After 12-month equilibration, the soil in each pot was taken out and put in a plastic bag to be air-dried and sieved, and the sieved soil in the six pots of the same treatment was mixed thoroughly and split into six equal parts, with each part being 4 kg. To facilitate plant growth, phosphorus (P) was mixed with the soil at 20 mg P kg^{-1} soil in the form of calcium superphosphate, which was ground to a fine powder before mixing. The P required for each pot was mixed separately with the soil. Before filling, each pot was lined with a transparent plastic bag inside first and the bottom was covered with a layer of gravels; then, 4 kg of the thoroughly mixed soil was filled to the pot and watered to 21% gravimetric soil water content with DI water, and left undisturbed in a glasshouse for 1 week before seeds were sown.

2.2. Plant cultivation

Seeds of *Medicago sativa* L. cv Golden Empress were first sterilized for 5 min in 30% (v:v) hydrogen peroxide (H_2O_2) solution, then rinsed with cold sterile DI water three times and placed on moistened filter papers in petri dishes overnight (Kereszt et al. 2007). In early October 2016, three pots of each treatment were sown with 30 seeds per pot, and the remaining three pots of each treatment were not sown with seeds and served as blank pots. Seedlings in each pot were thinned to 20 plants 4 weeks

after sowing. The experiment was carried out from early October 2016 to late February 2017 for 100 days in a glasshouse in the Institute of Soil and Water Conservation, Yangling, Shaanxi Province, China. During the experiment, the soil in all pots was watered to 60% field capacity with DI water every 3 days.

2.3. Plant biomass measurement and rhizosphere carboxylates analysis

Plants were harvested 100 days after sowing. Shoots were cut at the stem base; then, the plastic bags were lifted out of the pots, and the soil was gently pressed to loosen the soil and separate the roots from the soil. Rhizosphere extracts of Cd0 and Cd10 in Pb0 and Pb500 were selected for the analysis of rhizosphere carboxylates. For each pot with plants, about 1.0 g fresh fine roots and the attached rhizosphere soil was soaked in about 20 mL of 0.2 mM $CaCl_2$ aqueous solution with known volume in a glass beaker for 20 min to extract the rhizosphere carboxylates (He et al. 2017). To ensure cell integrity and remove the rhizosphere soil, roots were gently stirred in the solution while soaked. A subsample of the rhizosphere extract was filtered through a 0.22-μm syringe filter into a 1-mL HPLC vial; then, the filtered extract was acidified by a drop of concentrated phosphoric acid and kept in a − 20°C freezer until analysis. The remaining unfiltered extract was measured using a pH meter to obtain the pH. Roots in the extract were collected and washed with tap water, then oven-dried at 60°C for 48 h and weighed to obtain the dry mass of soaked roots. The unfiltered extract was oven-dried together with the beaker at 105°C for 12 h and weighed to obtain the dry weight of the rhizosphere soil.

After harvesting, leaves were separated into young leaves and old leaves, with the top one-third of all leaves on the shoot taken as young leaves, and the remaining taken as old leaves. Roots not soaked for rhizosphere carboxylates extraction were washed thoroughly with tap water first to remove soil particles and then rinsed with DI water. Young leaves, old leaves, stems, and roots were oven-dried at 60°C for 72 h and weighed. Shoot dry mass was calculated as the sum of the dry mass of young leaves, old leaves, and stems; root dry mass was calculated as the sum of the dry mass of soaked roots and un-soaked roots. Root mass ratio was calculated as root dry mass divided by the sum of root and shoot dry mass.

The analysis of carboxylates was performed using a Waters E2695 HPLC equipped with a Waters 2998 detector and Waters X select columns T3 (Waters, Milford MA, USA), and the data were collected at 210 nm (He et al. 2017). The working standards for the analysis included acetic acid, oxalic acid, malic acid, malonic acid, citric acid, tartaric acid, and succinic acid. However, only oxalate, malate, malonate, and citrate were detected for all analyzed samples. The amounts of rhizosphere carboxylates were expressed on root dry mass basis and on rhizosphere soil dry mass basis as well.

2.4. Analyses of mineral nutrients in plant parts

Oven-dried young leaves, old leaves, stems, and un-soaked roots were finely ground using a ceramic mortar. To extract total K, P, Ca, magnesium (Mg), S, Fe, Mn, Cu, and Zn from the ground plant samples, about 0.1 g of each ground sample was digested with aqua regia and H_2O_2 (Lomonte et al. 2008; Sastre et al. 2002), and the digested solution was made to a final volume of 25 mL. Concentrations of the above-mentioned elements in the digested solution were analyzed using a NexION 300 Inductively Coupled Plasma Mass Spectrometer (ICP-MS) (PerkinElmer, Inc., Waltham, MA, USA).

2.5. Soil pH measurement

When plants were harvested, all bulk soil in each pot with plants and the soil in each blank pot was collected and air-dried, then passed through a 2-mm sieve. Subsamples of the sieved bulk soil and blank pot soil were taken to measure the pH in a 1:5 soil:water suspension using a pH meter (Little 1992).

2.6. Statistical analysis

The effects of soil Cd level, soil Pb level, and their interaction on shoot dry mass, root dry mass, root mass ratio, and the amounts of rhizosphere carboxylates were examined by performing a two-way (Cd × Pb) ANOVA. For concentrations of nutrients in plant parts, a three-way (Cd x Pb x plant part) ANOVA was performed to evaluate the influences of soil Cd level and soil Pb level, to show the variations among plant parts, and to investigate whether there was any interaction among the three factors. The variations in soil and rhizosphere pH were tested with a three-way ANOVA, including factors of soil Cd level, soil Pb level, and medium type (i.e., blank pot soil, bulk soil in pot with plants, and rhizosphere extract). All ANOVAs mentioned above were performed using the general linear model in the IBM SPSS Statistics 22.0 software package (IBM, Montauk, New York, USA). The differences were determined to be significant at $P \leq 0.05$, and the least significant difference (LSD) test for post-hoc means comparisons was performed when there were significant differences.

3. Results

3.1. Biomass accumulation and partitioning

Neither shoot dry mass (SDM), root dry mass (RDM), nor root mass ratio (RMR) was significantly influenced by soil Cd level, but all of them were considerably affected by soil Pb level (Figure 1, Table 2). The interaction between soil Cd level and Pb level was significant for RDM and RMR, but not significant for SDM. SDM significantly ($P = 0.002$ and $P < 0.001$, respectively) decreased in Pb500 and Pb1000, which had an average of 17% and 25% less SDM than Pb0 (Figure 1(a)). However, SDM did not differ considerably between Pb500 and Pb1000. In Pb0, RDM decreased by 9% in Cd10, but did not change in Cd20, compared with Cd0 (Figure 1(b)); in Pb500, it increased by 19% and 7% in Cd10 and Cd20, respectively, compared with Cd0; while in Pb1000, it decreased with increasing soil Cd level, being 22% and 68% less in Cd10 and Cd20, respectively, compared with Cd0. In Cd0, RDM in Pb500 and Pb1000 was 35% and 25% less than in Pb0; in both Cd10 and Cd20, it decreased with increasing soil Pb level, being 15% and 35% less in Pb500 and Pb1000 than in Cd10, and 31% and 76% less in Pb500 and Pb1000 than in

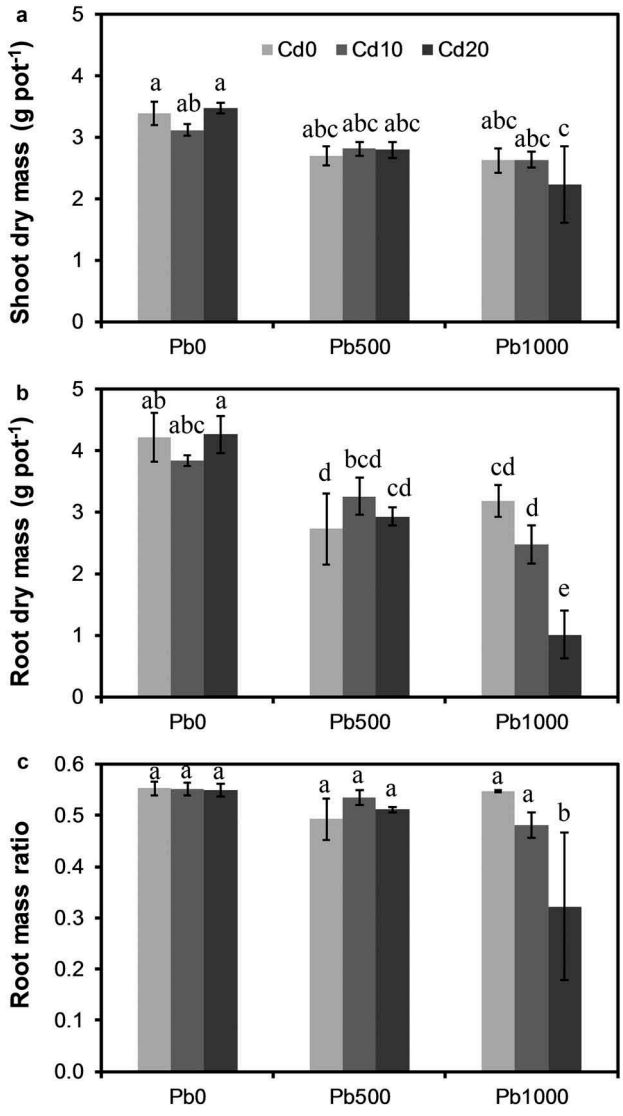

Figure 1. Biomass accumulation and partitioning of alfalfa plants grown on a calcareous soil spiked with different levels of cadmium (Cd) and lead (Pb). (a) Shoot dry mass, (b) Root dry mass, and (c) Root mass ratio. Cd0, Cd10, and Cd20 represent soil Cd level of 0, 10, and 20 mg kg^{-1}, respectively; Pb0, Pb500, and Pb1000 represents soil Pb level of 0, 500, and 1000 mg kg^{-1}, respectively. Data are presented as means ± SE (n = 3). Different lower-case letters above the bars indicate significant ($P \leq 0.05$) differences among treatments according to the results of LSD test of two-way (Cd × Pb) ANOVA.

Cd20, respectively. RMR did not change with soil Cd level in Pb0 (Figure 1(c)), but it rose by 9% and 4% in Cd10 and Cd20 than in Cd0 in Pb500, while it declined by 12% and 41% in Cd10 and Cd20 than in Cd0 in Pb1000. In Cd0, RMR declined by 11% in Pb500, but did not change in Pb1000, compared with Pb0; in Cd10, it was 3% and 13% lower in Pb500 and Pb1000, respectively, compared with Pb0; while in Cd20, it declined by 7% and 41% in Pb500 and Pb1000, respectively, compared with Pb0.

3.2. Concentrations of mineral nutrients in plant parts

Concentration of none of the nutrients measured was significantly influenced by soil Cd level, only S (P = 0.032) and Cu (P = 0.046) concentrations were markedly influenced by soil Pb level (Figures 2–4, Table 2). There were significant interactions between soil Cd and Pb level for Ca (P = 0.003), Mg (P = 0.024), and Cu (P = 0.004) concentrations. Concentrations of all nutrients measured varied considerably among plant parts (all $P < 0.001$). The interaction between soil Cd level and plant part was only significant for P concentration (P = 0.037). There were significant interactions between soil Pb level and plant part for all nutrients measured except P and S. The interactions among soil Cd level, soil Pb level, and plant part were significant for Ca (P = 0.007), Fe (P = 0.001), Cu (P = 0.001), and Zn (P = 0.011) concentrations.

Mean Ca concentration in stems was significantly lower than those in other parts ($P \leq 0.048$), while old leaves had considerably higher Ca concentrations than young leaves ($P < 0.001$) (Figure 3(a)); for Pb500 and Pb1000, Ca concentrations in young leaves in Cd20 were higher than those in Cd0 and Cd10; mean Ca concentration in old leaves decreased with increasing soil Pb level, as well as increasing soil Cd level; Ca concentration in roots increased with increasing soil Cd level in Pb500. Mean Fe concentration was considerably higher in roots than in other parts ($P < 0.001$), while young leaves and stems had similarly the lowest Fe concentrations among all parts (Figure 4(a)); Fe concentration in young leaves in Pb500 and Pb1000, in old leaves in Pb0, and in roots in Pb500 increased with increasing soil Cd level, but Fe concentration in old leaves in Pb1000 and in stems in Pb0 decreased with increasing soil Cd level; while Fe concentration in young leaves in Cd0, in old leaves in Cd10 and Cd20 decreased with increasing soil Pb level, but Fe concentration in stems in Cd10 increased with increasing

Table 2. Statistical level of significance (P value) of the ANOVAs for dry mass accumulation and partitioning (two-way ANOVA, Cd × Pb), and concentrations of various mineral nutrients in plant parts (three-way ANOVA, Cd × Pb × plant part).

	Source of variations						
Parameter	Cd	Pb	Plant part	Cd × Pb	Cd × plant part	Pb × plant part	Cd × Pb × plant part
Shoot dry mass	n.s.	<0.001		n.s.			
Root dry mass	n.s.	<0.001		0.017			
Root mass ratio	n.s.	0.010		0.030			
K concentration	n.s.	n.s.	<0.001	n.s.	n.s.	0.002	n.s.
P concentration	n.s.	n.s.	<0.001	n.s.	0.037	n.s.	n.s.
Ca concentration	n.s.	n.s.	<0.001	0.003	n.s.	0.001	0.007
Mg concentration	n.s.	n.s.	<0.001	0.024	n.s.	0.016	n.s.
S concentration	n.s.	0.032	<0.001	n.s.	n.s.	n.s.	n.s.
Fe concentration	n.s.	n.s.	<0.001	n.s.	n.s.	0.007	0.001
Mn concentration	n.s.	n.s.	<0.001	n.s.	n.s.	0.026	n.s.
Cu concentration	n.s.	0.046	<0.001	0.004	n.s.	0.026	0.001
Zn concentration	n.s.	n.s.	<0.001	n.s.	n.s.	0.029	0.011

n.s., not significant.

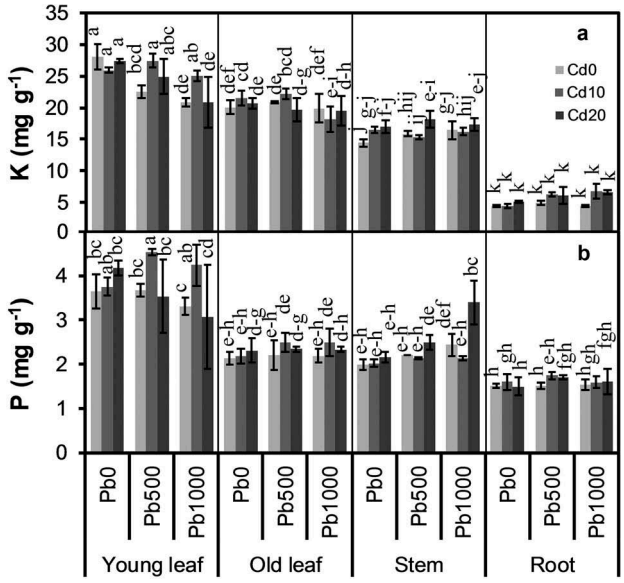

Figure 2. Concentrations of (a) potassium (K), and (b) phosphorus (P) in different parts of alfalfa plants grown on a calcareous soil spiked with different levels of cadmium (Cd) and lead (Pb). Cd0, Cd10, and Cd20 represent soil Cd level of 0, 10, and 20 mg kg^{-1}, respectively; Pb0, Pb500, and Pb1000 represents soil Pb level of 0, 500, and 1000 mg kg^{-1}, respectively. Data are presented as means ± SE ($n = 3$). Different lower-case letters above the bars indicate significant ($P \leq 0.05$) differences among combinations of Cd × Pb × Plant part according to the results of LSD test of three-way ANOVA.

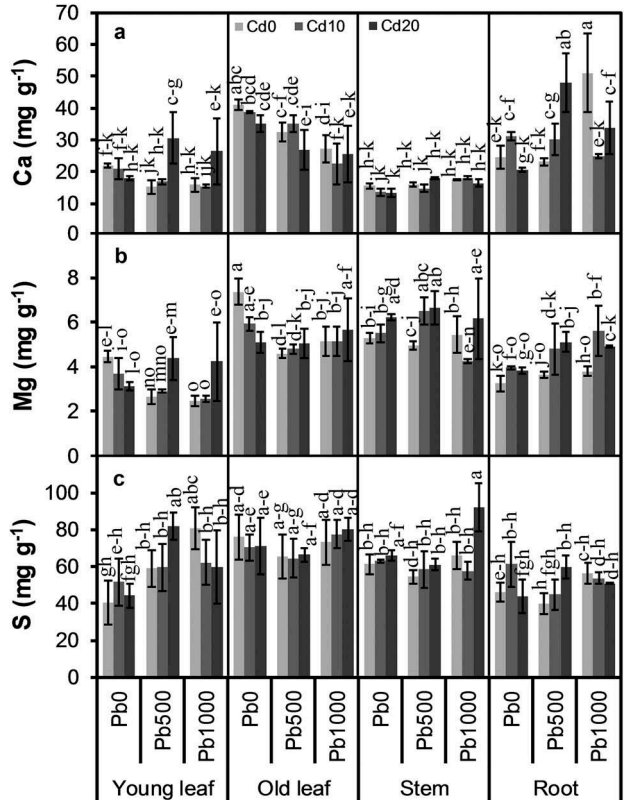

Figure 3. Concentrations of (a) calcium (Ca), (b) magnesium (Mg), and (c) sulfur (S) in different parts of alfalfa plants grown on a calcareous soil spiked with different levels of cadmium (Cd) and lead (Pb). Cd0, Cd10, and Cd20 represent soil Cd level of 0, 10, and 20 mg kg^{-1}, respectively; Pb0, Pb500, and Pb1000 represents soil Pb level of 0, 500, and 1000 mg kg^{-1}, respectively. Data are presented as means ± SE ($n = 3$). Different lower-case letters above the bars indicate significant ($P \leq 0.05$) differences among combinations of Cd × Pb × plant part according to the results of LSD test of three-way ANOVA.

soil Pb level. Mean Cu concentrations in young leaves and old leaves were similar and significantly higher than those in stems and roots ($P \leq 0.001$), which were also similar (Figure 4(c)); Cu concentration in young leaves in Pb0, in stems in Pb500, and in roots in Pb1000 decreased with increasing soil Cd level, but that in old leaves in Pb1000 increased with increasing soil Cd level. Mean Zn concentration in young leaves was significantly higher than those in other parts ($P < 0.001$), which were similar (Figure 4(d)); in most cases, Zn concentration did not vary considerably with soil Cd level or Pb level, only Zn concentration in stems increased with increasing soil Cd level in Pb1000.

Mean Mg concentrations in old leaves and stems were similar, and considerably higher than those in young leaves and roots ($P \leq 0.001$) (Figure 3(b)); Mg concentrations in young leaves and old leaves decreased with increasing soil Cd level in Pb0, but in most cases in Pb500 and Pb1000, Mg concentrations in Cd10 and Cd20 were higher than those in Cd0. Mean K concentration was the highest in young leaves, followed by old leaves, stems, and roots (Figure 2(a)); young leaves and old leaves had lower K concentrations in Pb1000 than in Pb0 and Pb500, but stems and roots had higher K concentrations in Pb500 and Pb1000 than in Pb0. Among all plant parts, young leaves had the highest P concentration on average, followed by old leaves and stems, which had similar P concentrations, and roots had the lowest P concentration (Figure 2(b)); Cd20 resulted in a higher P concentration in stems than Cd0 and Cd10, but not in other parts. Mean Mn concentration was similarly the highest in old leaves and roots, and the lowest in stems (Figure 4(b)); mean Mn concentration in old leaves decreased with increasing soil Pb level, but not in other parts. Mean S concentration was the lowest in roots, stems had a similar mean S concentration with both mature leaves and old leaves, while old leaves had a considerably higher mean S concentration than young leaves (Figure 3(c)); mean S concentration in Pb1000 was considerably higher than that in Pb0 and Pb500 ($P = 0.017$ and $P = 0.017$, respectively), which had similar S concentrations.

3.3. pH of the soil and rhizosphere extract

The difference among medium types was significant ($P < 0.001$) (Table 3); pH of the rhizosphere extract was markedly higher than that of the soil in blank pots ($P < 0.001$) and bulk soil ($P < 0.001$), but there was no significant difference between the soil in blank pots and the bulk soil in pots with plants. Neither soil Cd level nor Pb level significantly affected the pH of the soil and rhizosphere extract. There was no significant interaction between any two of the three factors, i.e., soil Cd level, soil Pb level, and medium type, and there was no significant interaction among the three factors.

3.4. Rhizosphere carboxylates

Plants mainly exuded oxalate, malate, citrate, and malonate into the rhizosphere, of which the average amount was 184, 55, 62, and 53 µmol g^{-1} root dry mass, respectively (Figure 5(a)), and 9.2, 2.8, 3.2, and 2.8 µmol g^{-1} rhizosphere soil, respectively (Figure 5(b)). Neither soil Cd level nor Pb level significantly influenced the amount of any of the four carboxylates

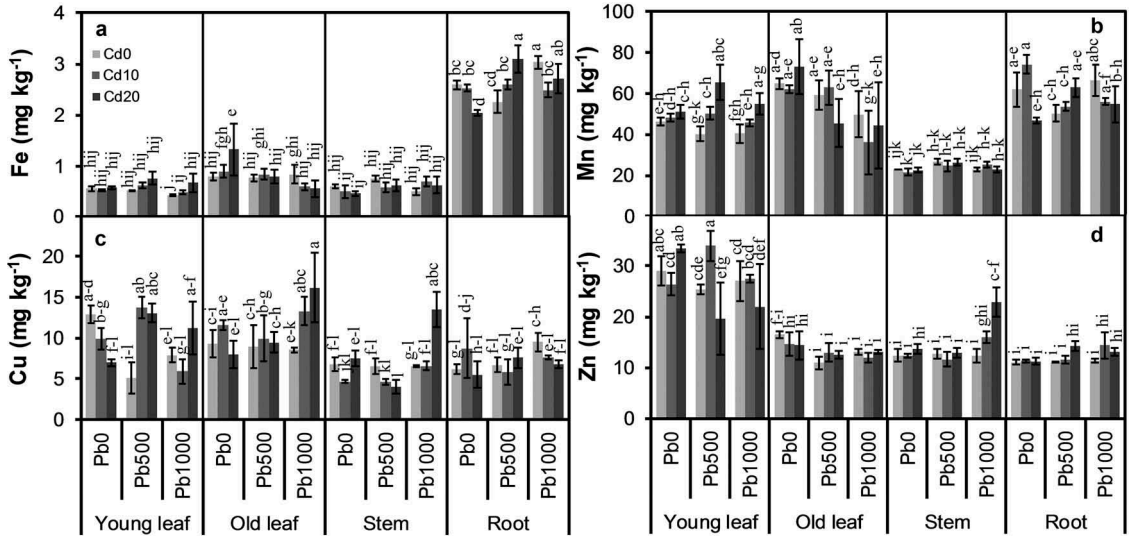

Figure 4. Concentrations of (a) iron (Fe), (b) manganese (Mn), (c) copper (Cu), and (d) zinc (Zn) in different parts of alfalfa plants grown on a calcareous soil spiked with different levels of cadmium (Cd) and lead (Pb). Cd0, Cd10, and Cd20 represent soil Cd level of 0, 10, and 20 mg kg^{-1}, respectively; Pb0, Pb500, and Pb1000 represents soil Pb level of 0, 500, and 1000 mg kg^{-1}, respectively. Data are presented as means ± SE (n = 3). Different lower-case letters above the bars indicate significant ($P \leq 0.05$) differences among combinations of Cd × Pb × plant part according to the results of LSD test of three-way ANOVA.

Table 3. pH of the blank pot soil, bulk soil, and rhizosphere extract. Data are presented as means ± SE (n = 3). Different lower-case letters after the SE indicate significant differences among combinations of Cd × Pb × medium type according to the results of three-way ANOVA.

Medium type	Treatment	Cd0	Cd10	Cd20
Blank pot soil	Pb0	8.32 ± 0.03 bcde	8.21 ± 0.10 cde	8.39 ± 0.04 abcde
	Pb500	8.39 ± 0.05 abcde	8.13 ± 0.17 de	8.13 ± 0.10 de
	Pb1000	8.09 ± 0.10 e	8.22 ± 0.01 cde	8.34 ± 0.18 bcde
Bulk soil	Pb0	8.49 ± 0.15 abcde	8.29 ± 0.13 cde	8.48 ± 0.09 abcde
	Pb500	8.37 ± 0.02 bcde	8.36 ± 0.01 bcde	8.22 ± 0.08 cde
	Pb1000	8.28 ± 0.02 cde	8.27 ± 0.03 cde	8.17 ± 0.05 cde
Rhizosphere extract	Pb0	8.17 ± 0.54 cde	8.51 ± 0.54 abcde	8.57 ± 0.47 abcde
	Pb500	8.75 ± 0.23 ab	8.86 ± 0.22 ab	8.89 ± 0.11 ab
	Pb1000	8.69 ± 0.22 abcde	8.98 ± 0.05 a	8.94 ± 0.09 ab

mentioned above in the rhizosphere, and there was no significant interaction between soil Cd level and Pb level for any of the four carboxylates, regardless of the calculation method.

4. Discussion

Cadmium and Pb often inhibit plant growth and result in decreased dry mass in a dose-dependent manner (Gupta et al. 2010; Kopittke et al. 2007; Perfus-Barbeoch et al. 2002). In our study, soil Cd level did not significantly affect plant growth, while soil Pb level affected root dry mass more significantly than shoot dry mass. The significant interaction between soil Cd level and Pb level for alfalfa root dry mass and root mass ratio suggests that the negative effect of Pb on plant growth was exacerbated in the presence of Cd. A number of studies have shown that plant growth suppression under the stress of toxic metals, including Cd and Pb, is linked to the negative impact of toxic metals on the ionic homeostasis in plants (Moosavi, Mansouri, and Zahedifar 2015; Moosavi, Zahedifar, and Mansouri 2018; Sharmila et al. 2017; Yoshihara et al. 2006), and the same might be applicable to the present study.

Disturbances in ionic homeostasis of some plant-essential elements in plants exposed to Cd and Pb are often reported, and such disturbances can be caused by ion competition between certain plant-essential element and Cd or Pb during their uptake and/or translocation (Lopes Júnior, Mazzafera, and Zezzi Arruda 2014; Sinha et al. 2006; Wu et al. 2012). The effects of soil Cd level on the concentrations of S and Cu in alfalfa plants were less significant than those of soil Pb level in the present study. The increased S concentrations in young leaves under higher soil Pb levels, and under the interactive effect of higher soil Cd level and soil Pb level were possibly related to the increased phytochelatins (PCs) and/or glutathione (GSH) levels (Ernst et al. 2008; Gielen, Vangronsveld, and Cuypers 2017; Gupta et al. 2010). It has been reported that Cd increased the levels of PCs that can complex both Cd and Cu, thus resulting in Cu deficiency in *A. thaliana* plants (Gielen, Vangronsveld, and Cuypers 2017). Our results showed that Cu concentration in young leaves decreased with increasing soil Cd level when no Pb was added. However, no significant reduction in Cu concentration was observed for other treatment, or for other plant parts; instead, Cu concentrations in young leaves in Pb500 and in old leaves in Pb1000 increased markedly when Cd was added to the soil, the increased Cu concentrations were likely due to the bioconcentration effect resulted from inhibited plant growth. For the alfalfa plants we studied, there was no observed Cd-induced Fe deficiency as that in tobacco seedlings reported by Yoshihara et al. (2006), and there was no observed Cd-induced Zn deficiency as that in *A. thaliana* reported by Weber, Trampczynska, and Clemens (2006). Although

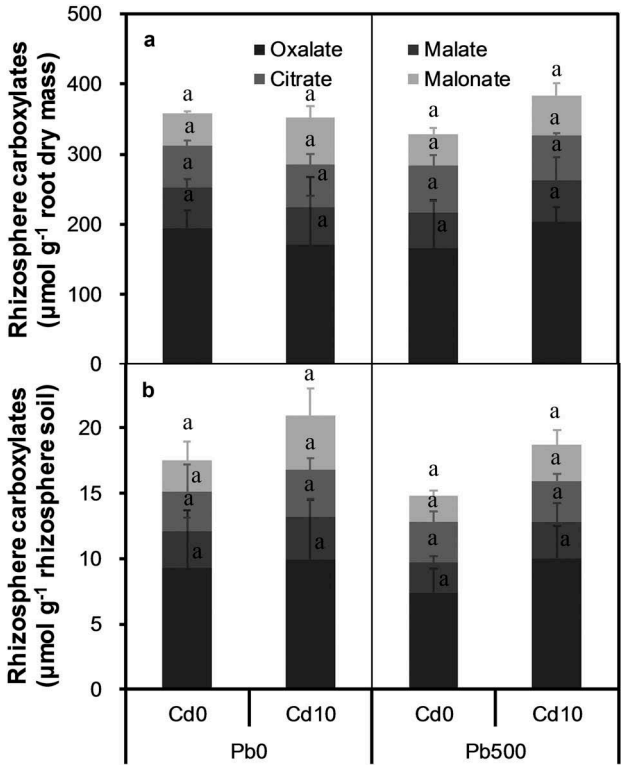

Figure 5. Rhizosphere carboxylates exuded by alfalfa plants grown on a calcareous soil spiked with different levels of cadmium (Cd) and lead (Pb). (a) Amounts of rhizosphere carboxylates calculated on root dry mass basis, and (b) Amounts of rhizosphere carboxylates calculated on rhizosphere soil dry mass basis. Cd0 and Cd10 represent soil Cd level of 0 and 10 mg kg^{-1}, respectively; Pb0 and Pb500 represent soil Pb level of 0 and 500 mg kg^{-1}, respectively. Data are presented as means + SE (n = 3). The same lower-case letters above the bars indicate no significant ($P \leq 0.05$) differences among treatments according to the results of LSD test of two-way (Cd × Pb) ANOVA.

Zn is the chemical analogue of Cd, Zn concentrations in alfalfa plants were relatively constant and not increased by increasing soil Cd level in most cases, very likely because Zn uptake is regulated by the physiological demand of plants (Smolders and Mertens 2013).

The results of our study suggest that the effects of soil Cd and Pb contamination on the concentrations of mineral nutrients are dependent on the plant parts, consistent with the results of a number of studies (Hart et al. 2005; Rauser 2003; Wu, Sato, and Ma 2015; Yoshihara et al. 2006). For alfalfa plants studied by López et al. (2007), Pb exposure reduced Ca concentrations in stems and leaves of alfalfa plants, Fe and Zn concentrations in roots, and Mn and Mo concentrations in roots and stems, but increased Cu concentrations in roots and stems. A few studies have reported reductions in plant P concentrations (Begonia et al. 1998; Geebelen et al. 2002; Sinha et al. 2006) and speculated that the formation of insoluble Pb-P complexes under Pb excess conditions contributed to P deficiency in plants (Begonia et al. 1998). We did not observe P deficiency or a reduction in P concentration in alfalfa plants in the present study, very likely because P was supplied at the optimal level in the studied soil for plant growth (He et al. 2017).

Rhizosphere processes such as changes in pH and exudation of organic acids can significantly affect the bioavailability of Cd, Pb, and some mineral nutrients (Kim et al. 2010; Lambers, Pons, and Chapin 2008; Podar, Ramsey, and Hutchings 2004; Wenzel 2009). Under Cd and Pb contamination, rhizosphere pH was significantly higher than bulk soil pH in the present study. However, our previous study on the same alfalfa cultivar growing in the same calcareous soil showed that rhizosphere pH was considerably lower than bulk soil pH under P deficiency (He et al. 2017). The differences in the pH change between the two studies suggest that rhizosphere pH is greatly affected by other soil chemical properties such as the levels of mineral nutrients and toxic metals. It is very likely that the higher rhizosphere pH, in comparison with bulk soil pH, reduced the bioavailability of Cd and Pb to some degree, thus mitigating the potential toxic effects of Cd and Pb to plants (Kim et al. 2010; Smolders and Mertens 2013; Steinnes 2013).

The amounts and compositions of rhizosphere carboxylates in the present study were different from those under P deficiency in our previous study (He et al. 2017), the total amounts of carboxylates exuded by plants in this study were much higher than those under P deficiency. In this study, oxalate accounted for the highest proportion (49–54%) of the total carboxylates measured, while malate, citrate, and malonate accounted for 16%, 18%, and 15%, on average, of the total carboxylates measured. However, malate accounted for the highest proportion (42% on average) of the total carboxylates measured at different soil P levels, followed by oxalate (34% on average) and malonate (23% on average) (He et al. 2017). The differences in the amounts and compositions of rhizosphere carboxylates between the two studies suggest that the exudation of carboxylates is strongly affected by soil chemistry, although the amounts and compositions of rhizosphere carboxylates did not vary considerably with soil Cd and Pb levels in this study. Furthermore, the exudation of carboxylates might also vary with the development stage and growth status of the plants.

The results of the present study showed that roots had considerably higher Cd and Pb concentrations than the aerial parts (Figure S1), and only small proportions of Cd and Pb were translocated to the aerial parts (Figure S2). We speculated that the secretion of oxalate played an important role in the exclusion and detoxification of Cd and Pb. The oxalate exuded by roots can immobilize free ions of Cd and Pb in the rhizosphere by producing oxalate-metal complexes, thus helping to exclude Cd and Pb from entering the roots (Wu et al. 2016; Zhu et al. 2011). Furthermore, Cd and Pb might be sequestered in calcium oxalate (CaOx) crystals in the vacuoles of root cells and thus showed reduced toxicity (Carvalho et al. 2015; He et al. 2014). It is very likely that the exudation of carboxylates was coincided with the release of cations other than protons (Roelofs et al. 2001), as the rhizosphere pH was higher than bulk soil pH, although it is reported that rhizosphere acidification often accompanies carboxylates exudation (Hinsinger et al. 2003). The co-release of cations other than protons and carboxylates might have advantage in exclusion and detoxification of toxic metals over the exudation of carboxylic acids, because it has been found that carboxylic acids such oxalic acid and citric acid facilitated the uptake and translocation of some toxic metals (Li et al. 2014).

5. Conclusions

Pb contamination considerably affected the concentrations of more mineral nutrients in plants than Cd contamination did, and there were combined effects of soil Cd level and Pb level on the

concentrations of some nutrients in plants. The effects of Cd and Pb contamination on plant nutrient concentrations varied among plant parts. Cd and Pb contamination did not considerably affect the exudation of carboxylates in the rhizosphere. An increase in rhizosphere pH and exudation of significant amounts of carboxylates (especially oxalate) in the rhizosphere might contribute to the exclusion and detoxification of Cd and Pb. Neither shoot dry mass nor root dry mass was significantly influenced by soil Cd level, but both of them were considerably reduced by increasing soil Pb level. The interaction between soil Cd level and Pb level was significant for root dry mass, but not significant for shoot dry mass. The results indicate that alfalfa is tolerant to Cd and Pb stress, and it is promising to grow alfalfa for phytostabilization of Cd and Pb on calcareous soils contaminated with Cd and Pb.

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