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Physiological responses and accumulation of heavy metals and arsenic of *Medicago sativa* L. growing on acidic copper mine tailings in arid lands

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ABSTRACT

Acidic copper mine tailings with high levels of heavy metals and arsenic pose a great risk to ecosystems and human beings. Physiological responses and phytoaccumulation of heavy metals and arsenic in *Medicago sativa* L. growing in soils with various proportions of acidic copper mine tailings were assessed in this study. Seed germination of *M. sativa* was enhanced by 5–10% tailings but inhibited at higher proportions (30% and 50%) of tailings. Seedling growth, cell membrane and photosynthesis were adversely affected when the plants were grown in soils with high proportions of tailings. The activities of antioxidant enzymes (catalase, superoxide dismutase and guaiocol peroxidase) increased with increasing proportions of tailings, indicating that the antioxidant system was not impaired and can tolerate the toxicity of mine tailings. The plants grew well in soil containing up to 50% of acidic copper mine tailings. Heavy metals and arsenic except Hg were overwhelmingly immobilized in roots. *Medicago sativa* is a promising plant species for revegetation and phytostabilization of acidic copper mine tailings in arid lands. However, the high mobility of Hg from root to shoot may pose risk to animals.

1. Introduction

Metalliferous mine tailings contain high contents of heavy metals and metalloids including Pb, Cu, Cd, Hg and As. As one of the major source of heavy metals in the environment, mine waste has caused serious environmental problems in many countries (Bes et al., 2014; Leiva and Morales, 2013; Navarro et al., 2008; Pan et al., 2014; Wilson et al., 2005). Recently, China has seen a number of major pollution accidents due to sudden dam break in mine dump ponds or metal leaching from mine refuse heaps in the open. Such pollution disaster has caused pollution of rivers, lakes and soils, and even death of animals and people. The small mine tailing particles and their associated toxic metal(loid)s are prone to wind-borne dispersion and water erosion and have been a great environmental and health risk (Allert et al., 2009; Antunes et al., 2008; Bae et al., 2010; Pyle et al., 2001; Wahl et al., 2012) subsequently requiring cost-effective remediation technologies. The most common way is to simply cap the sites with soil and sand. However, capping with soil and sand is not a permanent solution to this problem because the top layer of the cap is still susceptible to wind and water erosion. Revegetation is considered an inexpensive and ecological friendly strategy for long-term stability of surface layer of mine tailings (Pratas et al., 2005; Tordoff et al., 2000). However, high contents of heavy metals and metalloids, acidity, deficiency of organic nutrients and other harsh environmental factors inhibit plant growth and thus challenge successful application of revegetation. One of the important tasks to overcome these problems is to seek plants that can tolerate the toxicity and grow well in mine tails simply amended with soil. A variety of metal tolerant plant species have been screened out for revegetation (Bech et al., 2012; Lottermoser et al., 2011; Wanat et al., 2013).

The acidic copper mine tailings are most commonly found in northwest China. The tailings are rich in pyrite and heavy metals and metalloids including Pb, Cd, As and Hg. Moreover metal sulfides are prone to oxidation after exposure of the tailings to air and water (Chen and Morris, 1972), and this caused drastic decrease of pH as low as two and leaching out of the associated heavy metals. Owing to

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Abbreviations: CAT, Catalase; SOD, Superoxide dismutase; POD, Guaiocol peroxidase.

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the low pH and high levels of heavy metals, few plants can survive in such acidic condition.

In the present study, the potential of alfalfa (*Medicago sativa*) to stabilize acidic copper mine tailings was assessed. There are two reasons for selecting alfalfa as the test plant. One is that it can survive well under various stresses such as drought and low temperature and it can fix nitrogen efficiently, which is important for revegetation in nutrient deficient mine dumps. Seed germination, growth, physiological responses to heavy metals and arsenic of *M. sativa*, and the capacity of this species for phytostabilization of copper mine tailings amended with various proportions of farmland soils were investigated.

2. Materials and methods

2.1. Materials

The copper mine tailings were collected from one copper mine in Xinjiang, China. Farmland soil was collected from Toutunhe Farmland. The soil type is classified as silt loam with 56.5% silt, 25.5% clay and 18% sand. The soil pH, organic matter content and electric conductivity were 7.71, 19.3 g kg^{-1} and 0.2 mS m^{-1}. Seeds of *Medicago sativa* L.cv. Xinmu No.2 was kindly provided by Professor Weijun Li of College of Prataculture and Environment Science, Xinjiang Agricultural University.

2.2. Soil treatment

Farmland soils were mixed with different mass proportions (0%, 5%, 10%, 30% and 50%) of copper mine tailings and contents of heavy metals and pH of the mixed soils were measured, while the mixed soils were filled in pots or Petri dishes for further experiments.

2.3. Seed germination

One hundred alfalfa seeds were sowed in each Petri dish containing soil with various proportions of mine tailings and all tests run in triplicate. The Petri dishes were placed in a plant growth chamber with a day/night humidity of 80%/60%, a day/night temperature of 27/22 °C and a day/night light period of 12 h/12 h. The soils were watered daily with Milli-Q water and germination percentage was recorded daily. On the 10th day, at least ten seedlings for each treatment were randomly chosen to detect length of hypocotyls and radicle and current weight of seedlings and radicle were also measured.

2.4. Measurement of plant growth

The alfalfa seeds were germinated in Petri dishes at room temperature and were sowed in the soils with various proportions of copper mine tailings. The seedlings were watered with tap water, in which no heavy metals were detected. After three weeks of growth, seedling height was measured. Subsequently, three seedlings were randomly chosen and fresh weight of roots and shoots were measured.

2.5. Chlorophyll fluorescence test

On the third week, OJIP chlorophyll fluorescence of seedling leaves was measured with a pocket fluorometer (FP100, PSI, CZ) (Pan et al., 2011). All leaf samples were dark-adapted for 10 min in advance. Each leaf was tested five times and the mean value was used. F_o, F_J and F_m from the original measurements were used. $F_{300\mu s}$ are required for calculation of the initial slope (M_o) of the relative variable fluorescence (V) kinetics. The JIP-test parameters were calculated from the OJIP transients (Strasser et al., 2000). The formulae and terms and their illustration used in JIP-test parameters were illustrated in the Supplementary Table S1.

2.6. Measurement of chlorophyll content and enzymatic activities

Chlorophyll content of the leaves was measured by spectrophotometry (Lichtenthaler and Wellburn, 1983). Briefly, 0.5 g of fresh leaf was extracted in 10 ml of 80% acetone (v/v). The absorbance of the extracts at 663 and 645 for chlorophyll a and b were recorded by a UV-VIS spectrophotometer (UV-2800, Unico, Shanghai, China). Chlorophyll content was calculated according to the equations described by Lichtenthaler and Wellburn (1983).

For analysis of soluble protein content and enzymatic activities, fresh leaves were homogenized in chilled 50 mM potassium phosphate buffer (pH 7.0) containing 2.0% (w/v) insoluble polyvinylpolypyrrolidone, 0.5% (v/v) triton X-100 and 1.0 mM phenylmethylsulfonylfluoride in ice bath then the homogenate was filtered through two-fold muslin cloth and centrifuged at 20,000 × g for 10 min at 2 °C (Tewari et al., 2006). The supernatant was immediately used for enzyme assays and the soluble protein content was determined according to the method of Bradford (1976).

Superoxide dismutase (SOD) activity was assayed by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium (NBT) described by Giannopolitis and Ries (1977). The 4 mL of reaction mixture contained 50 mM K-phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM methionine, 60 μM riboflavin, 2.25 mM NBT and an appropriate aliquot of extract. The reaction was measured at 560 nm after 15 min of illumination with light intensity of 75 μmol m^2 s^{-1}.

Catalase (CAT) activity was analyzed according to the method described by Aebi (1984). The 3 ml of reaction media contained 30 mM H_2O_2 in a 50 mM phosphate buffer (pH 7.0) and 0.1 ml of enzyme. CAT activity was estimated by monitoring the decrease in absorbance of H_2O_2 at 240 nm using a UV-VIS spectrophotometer (UV-2800, Unico, Shanghai, China).

Peroxidase (POD) activity was measured by the method of Civello et al. (1995). The reaction mixture with a total volume of 3 mL contained 0.02 M Na_2HPO_4-0.08 M NaH_2PO_4 buffer with pH 6.0, 20 mM guaiacol, 4 mM H_2O_2 and 300 μL of enzymatic extract. The mixture reacted at 30 °C and the enzymatic activity was estimated by measuring the increase of OD (optical density) at 470 nm.

Measurement of proline content in leaves was performed according to the method of Bates et al. (1973). Briefly, 1.0 g of leaf sample was homogenized in 10 mL of 3% sulphosalycylic acid. This extract was used for measurement of proline content spectrophotometrically.

Malondialdehyde (MDA) content in the leaves was measured according to the method of Rajinder et al (1981). Fresh leaf sample (0.1 g) was homogenized in 5 ml of 0.1% trichloroacetic acid. After 5 min of centrifugation of the homogenate at 10 000×g at 4 °C, 0.3 mL of supernatant reacted with 1.2 mL of 0.5% thiobarbituric acid (in TCA 20%) and incubated for 30 min at 95 °C. The reaction was terminated in an ice bath for 5 min and the samples were centrifuged at 10 000×g for 10 min at 25 °C. The supernatant absorbance was measured at 532 nm using a UV-VIS spectrophotometer (UV-2800, Unico, Shanghai, China).

2.7. Measurement of heavy metals and arsenic content in soil/mine tailings and plant tissue

For measurement of contents of Cu, Zn, Pb and Cd in soil or/and mine tailings, the samples were ground with an agate mortar and pestle to pass a 100-mesh sieve. One gram of the finely ground sample was digested with HNO_3 and $HClO_4$ and filtered through 0.45 μm membrane. Contents of Cu, Zn, Pb and Cd in the filtrate were determined by an inductively coupled plasma mass spectroscope (ELANDRC II, PerkinElmer). Another one gram of the finely ground sample was digested with aqua regia and filtered through 0.45 μm membrane. Arsenic and mercury contents in the filtrate were measured by a HG-AFS (AFS-810, Jitan, China). Soil pH was determined at a ratio of soil to deionized water of 1:2.5 using a pH meter.

2.8. Statistical analysis

Each mine tailing treatment was at least triplicated. For biomass, seedling length and other plant growth measurements, at least ten plants were measured. For measurement of enzymatic activities, chlorophyll content and chlorophyll fluorescence, at least ten plants were measured. All the data used were the mean values. Student's *t*-test was employed for statistical analysis of experimental data. Significance was declared at $P < 0.05$.

3. Results

3.1. Content of heavy metals and arsenic in the mine tailings and the soils

Table 1 lists pH and the contents of heavy metals and As of the mine tailings and the farmland soils mixed with various proportions of mine tailings. The copper mine tailings is highly acidic with a pH of 2.8. Cu, Zn, Pb and As contents of mine tailings were several hundreds of mg kg^{-1}. Cadmium and Hg are also far higher than their background values (below 0.5 mg kg^{-1} for both heavy metals) of most soils (Pérez-Sirvent et al., 2009). The high levels of heavy metals and As in mine tailings imply its high ecological and environmental risk. The farmland soil pH was 7.7 and the content of heavy metals and As of farmland soil were far less than those of mine tailings. The soil pH decreased with increasing proportion of mine tailings mixed. When soil was mixed with 50% mine tailings, soil pH decreased to 6.8. Heavy metal contents in soil increased with increasing proportion of mine tailings.

3.2. Effect of copper mine tailings on seed germination and seedling growth

It can be seen from Fig. 1A that most of the seeds did not germinate during the first four days. The germination percentage for all treatments increased drastically from the fifth day. Most of the seeds germinated at the seventh day. Addition of low proportions (5% and 10%) of copper mine tailings increased the germination percentage by about 10% compared to the control. On 10th d, the maximum seed germination was found to be 83% for soil containing 10% mine tailings. In comparison with the control, treatment with 30% and 50% mine tailings reduced the germination percentage by about 5% and 10%, respectively. There was no significant difference in germination percentage between different treatments during 1–4 d ($p < 0.05$). Significant difference was found between the control and 50% tailing treatment on 5th and 6th d ($p < 0.05$). There was significant difference between the control and 30% and 50% tailing treatments on 7th d ($p < 0.05$). After 8–9 days of exposure to 30% tailing treatment, germination percentage was significantly different from the control ($p < 0.1$). On 10th d, there was significant difference in germination percentage between all treatments ($p < 0.1$). Generally, seed germination was inhibited by high proportions of mine tailings.

Fig. 1B shows length of radicles and hypocotyls of seeds germinated in soil with various proportions of mine tailings. Hypocotyl length for 10% tailing treatment was significantly greater than that for control, 30% and 50% tailings treatments while hypocotyl length for 30% tailing treatment was significantly less than 5% and 10% treatments ($p < 0.05$). Radicle length for 10% tailings treatment was significantly greater than that for 30% and 50% tailings treatments ($p < 0.05$) and radicle length for 50% treatment was significantly lower than for all other treatments.

The biomass of radicles and seedlings growing in soil containing various proportions of mine tailings is shown in Fig. 1C. Biomass of hypocotyl for 10% tailing treatment was significantly higher than that for 30% and 50% treatments, and biomass of hypocotyl for 50% treatment was significantly lower than that for control and 10% tailing treatment ($p < 0.05$). The radicle biomass for 5% tailing treatment was significantly higher than control, and the radicle biomass for both control and 5% tailing treatment was significantly higher than that for 50% tailings treatment ($p < 0.05$). There was no significant difference between 10%, 30% and 50% tailing treatments ($p < 0.05$).

Root biomass and total length of 31 d old seedlings growing in soil with various proportions of mine tailings are shown in Fig. 1D and E. Total seedling length decreased significantly with increasing proportion of tailings from 0 (control) to 30% and there was no significant difference between 30% and 50% tailings treatments ($p < 0.05$). Root biomass for control and 5% and 10% tailing treatments was not significantly different but was significantly higher than that for 30% and 50% tailings treatments ($p < 0.05$). No significant difference in shoot biomass was observed between control and 5% tailing treatment and shoot biomass significantly decreased with increasing proportion of tailings from 5% to 50% ($p < 0.05$), indicating the inhibitory effect of mine tailings on plant growth.

3.3. Effects on biochemical parameters

Soluble protein content of leaf decreased with increasing proportion of mine tailings in farmland soil (Fig. 2A), suggesting that heavy metals and As in the copper mine tailings inhibited synthesis of protein in alfalfa seedlings. 50% mine tailings resulted in a decrease of soluble protein content by 38.4%. The activities of SOD, POD and CAT generally increased with the increase of the proportion of mine tailings (Fig. 2B-D). Comparing to the control, when the seedlings were treated with 50% mine tailings, SOD, POD and CAT activities increased by 92.52%, 244.53% and 109.66%, respectively. In comparison with the control, malondialdehyde content in leaf decreased when the seedlings were treated with 5% and 10% mine tailings and increased significantly when the seedlings were grown in soils with higher proportions of mine tailings (Fig. 2E-F). Malondialdehyde content is an indicator of peroxidation of cell membrane lipid. This study indicates that low proportions of mine tailings have no effect on membrane lipid system and 30% and 50% mine tailings clearly have damage to the cell membrane lipid system. Proline content decreased when the plants were grown in soils containing 5–30% mine tailings but significantly increased by 30% compared to the control.

3.4. Effects on pigment synthesis and photosynthesis

Relative to the control, the chlorophyll *a* and *b* and carotenoid contents increased slightly when the plants were exposed to 5% mine tailings and decreased as the proportion of mine tailings increased (Fig. 2G-H), implying that addition of 5% copper mine tailings stimulates

Table 1
pH and heavy metal and As contents (mg kg^{-1}) of soil mixed with various proportions of copper mine tailings. All the data were presented the mean values ± S.D. of three measurements.

	Proportions of copper mine tailings mixed					
	0%	5%	10%	30%	50%	100%
pH	7.71 ± 0.02	7.68 ± 0.10	7.60 ± 0.02	7.26 ± 0.09	6.84 ± 0.05	2.80 ± 0.02
Cu	42.60 ± 0.63	126.00 ± 3.48	196.87 ± 5.32	320.17 ± 2.50	523.99 ± 14.49	979.41 ± 32.22
Zn	106.50 ± 0.92	141.19 ± 8.50	171.06 ± 6.27	323.25 ± 5.26	440.81 ± 15.06	527.63 ± 60.64
Pb	13.08 ± 0.26	21.81 ± 0.59	29.69 ± 0.29	68.40 ± 1.78	258.28 ± 4.82	290.12 ± 15.60
As	6.37 ± 0.88	21.15 ± 1.79	33.50 ± 4.14	70.70 ± 6.56	97.82 ± 6.84	234.43 ± 20.79
Cd	0.40 ± 0.00	0.67 ± 0.04	0.80 ± 0.04	2.01 ± 0.07	3.18 ± 0.04	4.41 ± 0.08
Hg	0.091 ± 0.012	0.144 ± 0.017	0.161 ± 0.012	0.289 ± 0.033	0.263 ± 0.051	1.339 ± 0.050

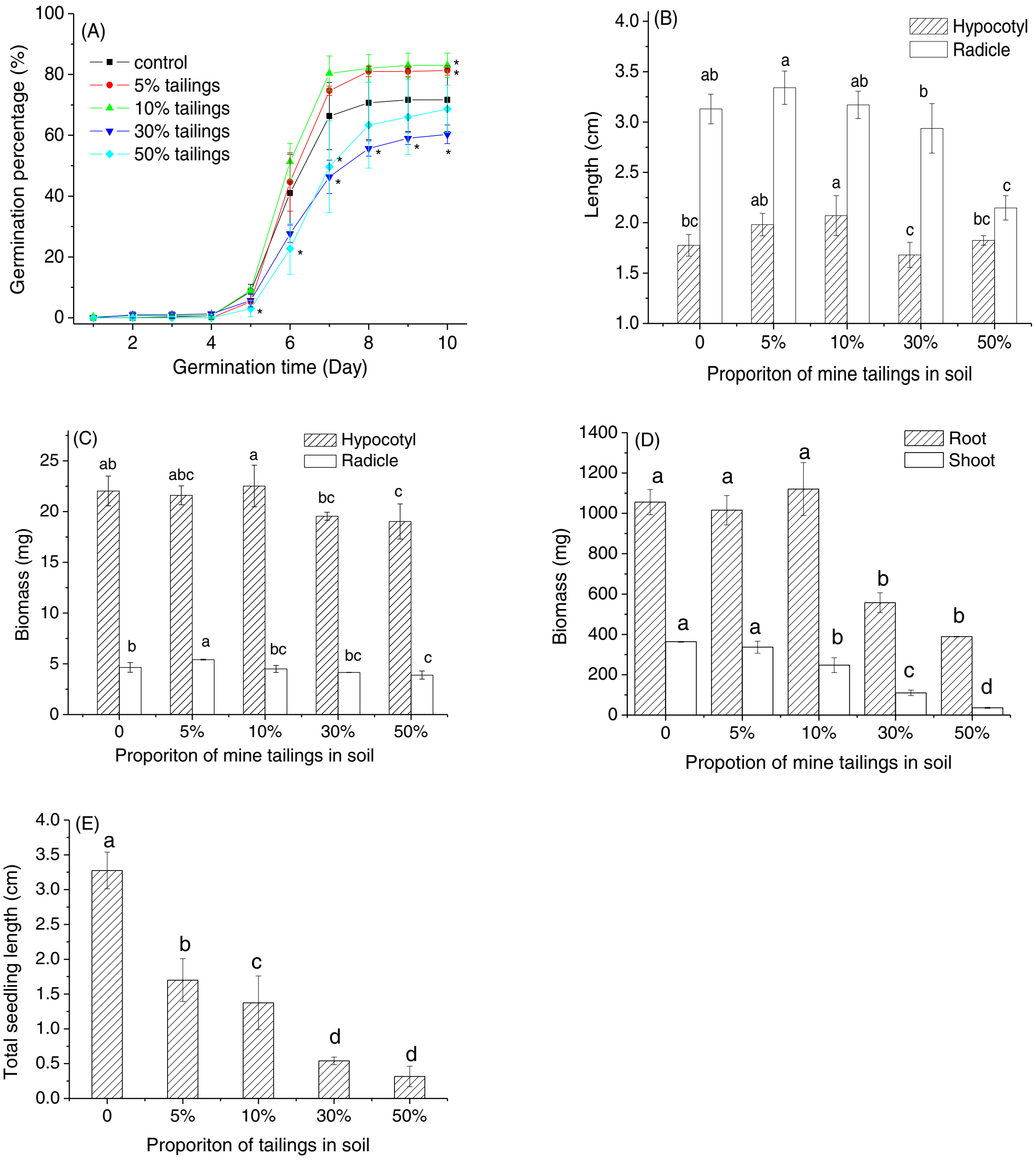

Fig. 1. (A) Alfalfa seed germination (%) in soil treated with various proportions of copper mine tailings as a function of time; (B) Length of hypocotyl and radicle of alfalfa seeds germinated in soils with various proportions of mine tailings; (C) Biomass of the seedlings and radicles grown in soils with various proportions of mine tailings; (D) Fresh biomass of the seedlings treated with various proportions of mine tailings. (E) Total length of the seedlings treated with various proportions of mine tailings in soil. * near the bars in Fig. 1A denotes significant difference at $p = 0.05$ or $p = 0.1$ (Duncan's New Multiple Range Test). Different letters above the bars in Fig. 1B-1E denote significant difference at $p = 0.05$ (Duncan's New Multiple Range Test).

synthesis of pigments while higher proportions of mine tailings inhibits pigment synthesis.

The fluorescence intensity at various steps of the OJIP curves changed with the proportion of mine tailings in soil (Fig. 3), implying that copper mine tailings influenced photosynthesis, especially the electron transport chain in photosystem II (PSII). Table 2 lists some selected JIP-test parameters. It was found that 5% and 10% mine tailings treatments had little effect on Fv/Fm (the maximum quantum yield for primary photochemistry) and other electron transport parameters such as ψo (probability that a trapped exciton moves an electron into the electron transport chain beyond Q_A) and φEo (quantum yield for electron transport). These parameters were slightly reduced when the plants were grown in soil with 30% or higher proportion of mine tailings, accompanied with increasing DIo/RC (dissipated energy flux per RC) and φDo (thermal dissipation quantum yield), TRo/RC (trapped energy flux per RC), ABS/RC (absorption flux per RC). This finally resulted in a significant decrease of PI_{ABS} (performance index on absorption basis), indicating that the copper mine tailings are toxic to photosynthetic apparatus. The results of chlorophyll fluorescence tests were in consistent with the results of pigments.

3.5. Accumulation of heavy metals in plant tissue

Heavy metals except Hg and As were dominantly accumulated in the roots (Fig. 4). The contents of heavy metals and As in plant tissues generally increased with increasing proportion of copper mine tailings. The contents of Cd, Cu, Zn, Pb and As in roots of plant growing in 50% tailings soil were 7.33, 372.44, 328.13, 5.59 and 9.08 mg kg^{-1}, respectively, which were much higher than those in stems and leaves. When the plants were grown in soil with 50% tailings, the transfer factor from root to leaf of Cd, Cu, Zn, Pb and As was 0.21, 0.084, 0.47, 0.026 and 0.081, respectively. The content of Hg in leaf was far higher than that in root and stem. When the soil was treated with 50% tailings, Hg content in leaf, stem and root was 1.49, 0.27 and 0.15 mg kg^{-1}, respectively.

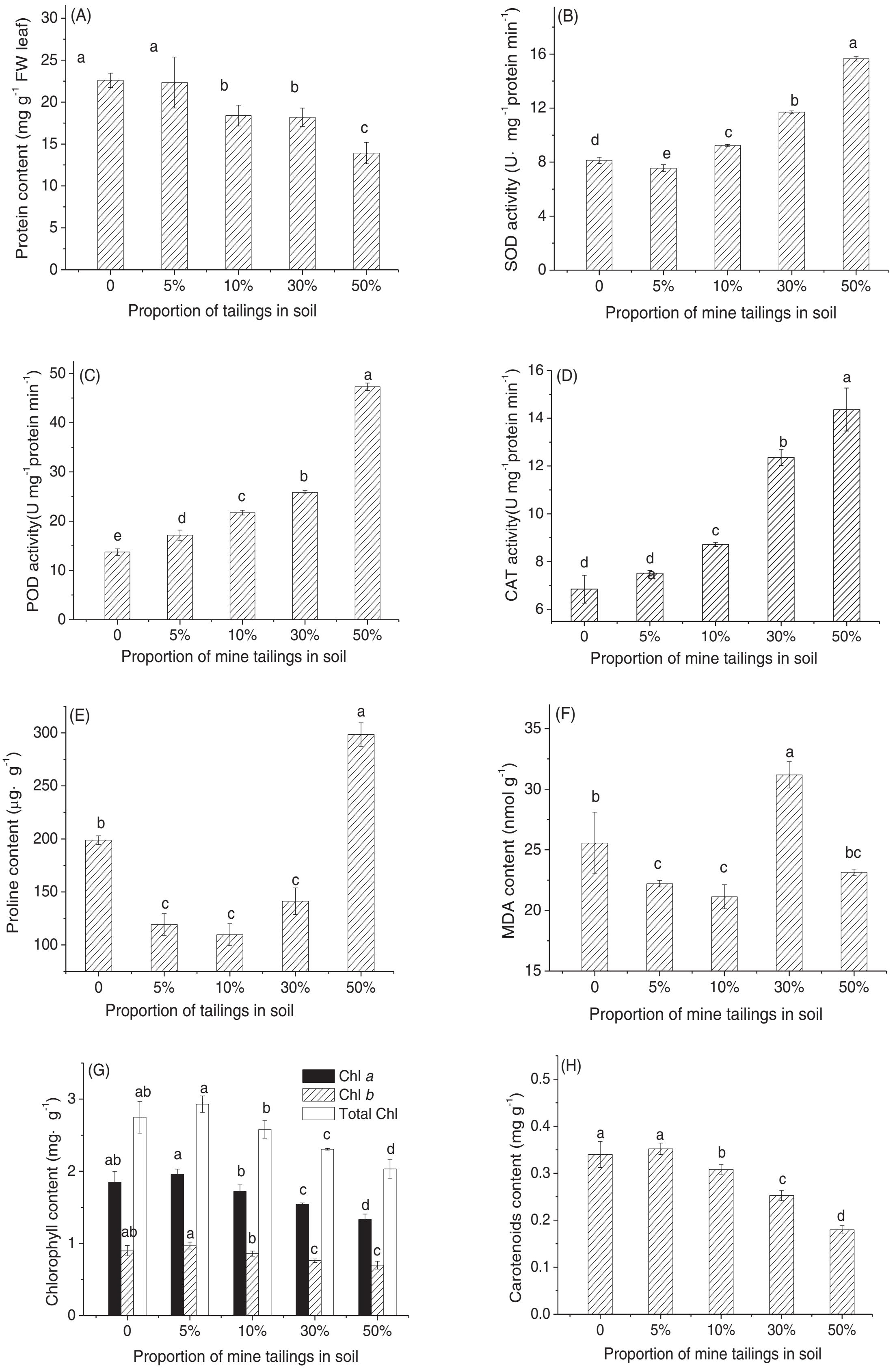

Fig. 2. Contents of soluble protein, proline and MDA, enzymatic activities and pigments of the seedlings growing on soils with various proportions of copper mine tailings. (A) the soluble protein content; (B) SOD; (C) POD; (D) CAT; (E) proline content; (F) MDA content; (G) chlorophyll content; and (H) carotenoid content. Different letters above the bars denote significant difference at $p = 0.05$ (Duncan's New Multiple Range Test).

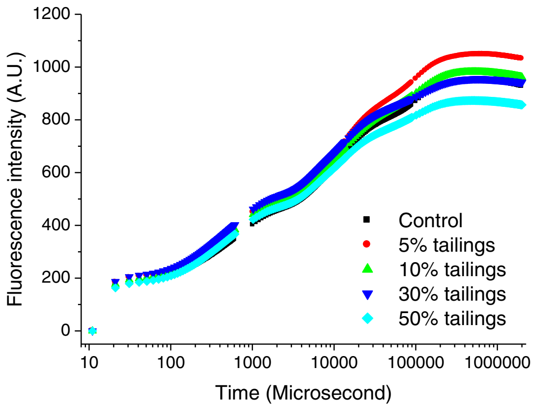

Fig. 3. OJIP transients of seedlings growing on soils with various proportions of copper mine tailings.

There was significant difference in accumulation of metal(loid)s in root, stem and leaf between different treatments except Pb accumulation in leaf (Fig. 4).

4. Discussion

This study showed that the acidic copper mine tailings contained high levels of Cu, Pb, Zn and As and showed some toxicity to alfalfa due to its high levels of potential toxic elements and low pH (2.8). Although high proportions of mine tailings inhibited seed germination and plant growth (Fig. 1), most plants grew well with high photosynthesis performance in soil mixed with up to 50% mine tailings. This indicates that alfalfa can tolerate the toxicity of multiple heavy metals and As in the mine tailings. Some other plants species can also grow well in various mine tailings or soil contaminated with mine tailings although their physiological indicators were inhibited (Craw et al., 2007; King et al., 2008; Shi et al., 2011). For example, growth of the herbaceous species *Sesbania cannabina* in Cu tailings an Pb/Zn tailings (Shi et al., 2011), eucalyptus species in arsenical, sulphidic gold mine tailings (King et al., 2008), and *Lygeum spartum* in acidic mine tailings ($\mathrm{pH} = 2.9$) untreated or treated with $CaCO_3$ or fertilizers (Conesa et al., 2007) was significantly retarded. However, they were still considered to be suitable for tailings revegetation due to their metal tolerance and high biomass. Similarly, in the present study, alfalfa can also be a potential candidate plant species for revegetation of acidic copper mine tailings.

The acidic copper tailings disturbed the physiological status including protein synthesis, antioxidant system and photosynthetic apparatus of alfalfa. Synthesis of protein was inhibited by tailings. The activities of SOD, POD and CAT increased as the proportion of mine tailings in soil increased (Fig. 2B-D). This implies that the reactive oxygen species (ROS) generated due to the toxicity of tailings (Devi and Prasad, 1998; Dietz et al., 1999; Pan et al., 2011), and the increased ROS production can cause inactivation of protein, membrane peroxidation, breakage of DNA strand, and even cell death (Luna et al., 1994). To eliminate the detrimental effects of ROS, a variety of antioxidant enzymes, such as POD, CAT and SOD, are involved in removing ROS (Winston, 1990). In the present study, the increase of enzymatic activity means that the antioxidant system was not destructed by the toxicity of up to 50% tailings in soil. The increased MDA content in the presence of high proportions of mine tailings showed that the membrane lipid system was adversely affected to some extent (Fig. 2C-D). However, treatment with low proportions of tailings reduced somewhat proline and MDA content, which at least means that the plant growth is not stressed by low proportions of tailings. Some studies showed similar response patterns of antioxidants system to mine tailings (Jiang et al., 2012; Wu et al., 2011). For example, the activities of SOD and CAT of *Paulownia fortunei* significantly increased as Cu accumulation increased. However, the seedlings of *P. fortunei* cultivated in Cu tailings grew poorly due to nutrient deficiency (Jiang et al., 2012). The leaf growth, dry matter accumulation, and photosynthesis of vetiver grass (*Vetiveria zizanioides* L.) growing in soils treated with high proportions of Pb and Zn tailings were remarkably reduced, whereas accumulation of proline and abscisic acid were enhanced and the activities of superoxide dismutase, peroxidase and catalase were stimulated (Pang et al., 2003).

The data on chlorophyll *a* and *b* and carotenoid content and JIP-test analysis showed that pigment synthesis and electron transport flow in PSII were inhibited in the presence of high proportion of tailings (Figs. 2G-H, 3), implying that the photosynthetic apparatus is the target site of potential toxic elements. Similarly, Jiang et al. (2012) showed that chlorophyll *a* and *b* and carotenoid content of *P. fortunei* significantly increased for 15.7–78.7 μM Cu treatment and significantly decreased for 157 μM Cu treatment. The JIP test provides valuable information about the fluxes of photons, excitons, electrons and further metabolic events of plants growing in soil with various proportions of mine tailings (Appenroth et al., 2001; Han et al., 2008). Fm value decreased and the shape of J-I-P phase became flat after exposure to high proportions of mine tailings (Fig. 3), implying that PSII reaction centers were partially inactivated and could not be closed and reduction of PQ (non-photochemical phase) was inhibited (Strasser et al., 2000; Zhang et al., 2010). The decrease in Fm associated with the increases of fluorescence intensity at J and I steps suggests that electron transport at the donor side of PSII was inhibited and this leads to accumulation of $P680^{*}$ (Govindjee, 1995). The slightly inhibited electron transport parameters and the increased energy dissipation parameters (DIo/RC, φDo and ABS/RC) for plants treated with high proportion of mine tailings could be attributed to the blocked excitation energy transfer among reaction centers, which finally caused some decrease of PI_{ABS}. A variety of pollutants and mine tailings have similar inhibitory effects on photosynthesis (Lange et al., 2012; Pan et al., 2011; Wang et al., 2012; Zhang et al., 2010, 2013). These results agree well with the results of seed germination, contents of pigments and the biochemical

Table 2
JIP-test parameters for seedlings growing in soil containing various proportions of copper mine tailings. Different superscript letters for values in the same row denote significant differences ($p < 0.05$). The significance decreases in the order of a > b > c.

	Proportion of copper mine tailings in soil				
	Control	5%	10%	30%	50%
F_v/F_m	0.822 ± 0.004^{a}	0.824 ± 0.006^{a}	0.820 ± 0.006^{a}	0.805 ± 0.012^{c}	0.813 ± 0.011^{b}
ψo	0.637 ± 0.012^{a}	0.636 ± 0.021^{a}	0.613 ± 0.019^{b}	0.581 ± 0.035^{c}	0.572 ± 0.022^{c}
ΦEo	0.524 ± 0.011^{a}	0.524 ± 0.019^{a}	0.502 ± 0.015^{b}	0.468 ± 0.034^{c}	0.465 ± 0.021^{c}
ΦDo	0.178 ± 0.004^{c}	0.176 ± 0.006^{c}	0.180 ± 0.006^{c}	0.195 ± 0.012^{a}	0.187 ± 0.011^{b}
PI_{ABS}	4.368 ± 0.416^{a}	4.171 ± 0.585^{a}	3.611 ± 0.461^{b}	2.883 ± 0.852^{c}	2.830 ± 0.524^{c}
ABS/RC	1.870 ± 0.073^{b}	1.985 ± 0.096^{a}	2.017 ± 0.144^{a}	2.082 ± 0.166^{a}	2.092 ± 0.143^{a}
TRo/RC	1.538 ± 0.060^{b}	1.635 ± 0.081^{a}	1.654 ± 0.123^{a}	1.675 ± 0.116^{a}	1.700 ± 0.108^{a}
ETo/RC	0.979 ± 0.027^{bc}	1.040 ± 0.049^{a}	1.012 ± 0.058^{ab}	0.971 ± 0.039^{c}	0.970 ± 0.047^{c}
DIo/RC	0.332 ± 0.015^{c}	0.350 ± 0.019^{bc}	0.363 ± 0.024^{b}	0.407 ± 0.054^{a}	0.393 ± 0.043^{a}

parameters, suggesting that addition of low proportions of tailings slightly enhanced plant physiological status but higher proportions of tailings would down-regulate plant physiological status somewhat.

Most of the studied chemical elements, except Hg, were accumulated in roots (Fig. 4), indicating that *Medicago sativa* L.cv. Xinmu No.2 is a potential phytostabilization plant for Cd, Pb, Cu, Zn and As. Similarly, a number of studies showed that most of heavy metals were accumulated in roots of a variety of plant species that grew in various types of mine tailings or heavy metals polluted soils (Abreu et al., 2012; Bech et al., 2012; Galende et al., 2014; Salazar and Pignata, 2014; Trigueros et al., 2012; Wanat et al., 2013). Table 3 listed some examples of accumulation of heavy metals or arsenic in root, stem and leaf of such plants. The plants with translocation factor of metal(loid)s from root to shoot/leaf lower than one may be used for phytostabilization. However, sometimes, even the same plant species may show different metal(loid) accumulation patterns when it grows at different sites. For example, Salazar and Pignata (2014) reported that the translocation factor of Pb from root to shoot of *Solanum argentinum* Bitter & Lillio growing in Pb contaminated soils at different sites changed from 0.32 to 1.87. This means that it is important to assess the suitability of plants for phytostabilization when they are applied to different sites.

The translocation of Hg from root to shoot might be due to the high levels of chlorides in the tailings. The tailings was recycled using 16 g L^{-1} $CuCl_2$ to recover Cu, Zn and Ag. In Cl^- rich solution, mercury mainly exists in the forms of anionic species such as $HgCl_4^{2-}$ and $HgCl_3^-$ owing to the favorable complexation of Hg^{2+} with Cl^- (Powell et al., 2005), which may be favorable for transfer of Hg from root to shoot. However, Hg content in shoots of *M. sativa* is much lower than in other reported plant species that were grown in solution or soils spiked with $HgCl_2$. Shoots of beard grass has been reported to accumulate Hg up to 13 μg g^{-1} growing in 1 mg Hg L^{-1} solution (De Souza et al., 1999) and <65 μg g^{-1} dry weight in shoots from plants growing in soils spiked with 50–1000 μg g^{-1} $HgCl_2$ (Su et al., 2007). Brake fern accumulated 540 μg g^{-1} and 1469 μg g^{-1} of Hg in shoots after 18 days of growing in soils treated with 500 mg kg^{-1} and 1000 mg kg^{-1} of $HgCl_2$ powder, respectively (Su et al., 2007). This implies that accumulation of Hg in shoots is not only dependent on plant species but also on Hg concentration in the media. In order to assess the potential of

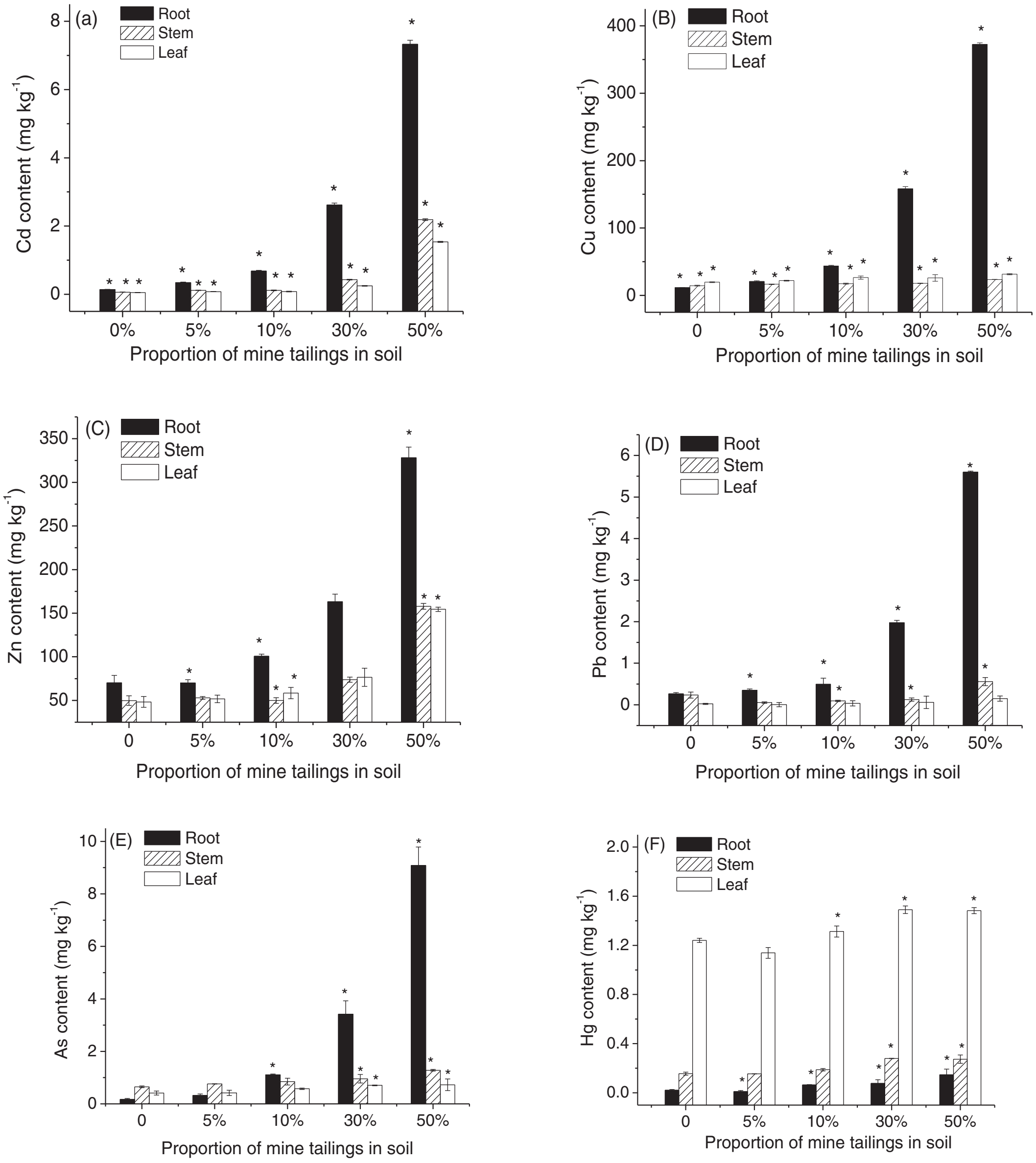

Fig. 4. Contents of Cd (A), Cu (B), Zn (C), Pb (D), As (E) and Hg (F) in roots, shoots and leaves of the alfalfa seedlings growing on soils with various proportions of copper mine tailings. The symbol asterisks (*)above the bars denote significant difference at $p = 0.05$ (Duncan's New Multiple Range Test).

Table 3
List of heavy metals or arsenic contents in root, stem and leaf of plants accumulate metal(loid)s mainly in root in the literature (mg kg^{-1} dry weight).

Plant species	Soil/tailings	Metal(loid)s	Root	Shoot/Stem	Leaf	References
Nero oleander	soil with 270 mg kg^{-1} Pb	Pb	13 (inner part) 26 (root bark)		2	Trigueros et al. (2012)
Miscanthus giganteus	gold mine soils	As	602.4–1284.5	3.6–5.4	4.3–17.1	Wanat et al. (2013)
		Sb	17.9–26.6	0.8–1.0	0.2–1.1	
		Pb	38.2–326.7	0.6–29.5	1.1–43.1	
Bidens triplinervia L.	polymetallic mine soil (mainly Ag, Pb and Cu)	Pb	5187	655		Bech et al. (2012)
		Zn	9900	1614		
Sorghum halepense (L.) Pers.	Pb contaminated soil	Pb	1406.8	3.1	5.2	Salazar and Pignata (2014)
Medicago sativa L.	soil with 50% acidic Cu mine tailings	Cd	7.3	2.2	1.5	This study
		Cu	372.4	23.6	31.5	
		Zn	328.1	157. 8	154.4	
		Pb	5.6	0.6	0.1	
		As	9.1	1.37	0.7	
		Hg	0.15	0.27	1.48	

phytoextracton of Hg by *M. sativa*, accumulation of Hg in leaves in the presence of a broad concentration range of Hg should be examined and the physiological status of plants should be also monitored in the future study.

In the present study, the good survival of *M. sativa* in soil with high proportions of tailings, overwhelming accumulation of heavy metals and As, except Hg, in root, N_2-fixing ability and its high tolerance to drought and low temperature stress make *M. sativa* a potential revegetation and phytostabilization plant of mine tailings in arid lands. In engineering application, the inhibitory effects of toxic mine tailings on plants can be alleviated by addition of fertilizers or organic waste such as biosolids (Santibáñez et al., 2008).

5. Conclusions

Seed germination of *M. sativa* was enhanced by low proportions of acidic copper mine tailings but inhibited by higher proportions (>30%) of tailings. Seedling growth, membrane and photosynthesis were adversely affected at high proportions of tailings in soil. The antioxidation enzymatic activities generally increased with increasing proportions of tailings and this indicates that the antioxidant system was not destructed and can deal with the stress from the toxicity of mine tailings. Regardless of the inhibitory effects of tailings, the plants showed high tolerance to multi-metal(loid) toxicity and grew well in soil mixed with various proportions of highly acidic copper mine tailings. The *M. sativa* can stabilize Cd, Cu and Zn in highly acidic copper mine tailings because of its strong accumulation of heavy metals in root. However, the high mobility of Hg from root to shoot may pose a risk to animals. The differences between the mechanism of translocation of Hg from root to leaf and high accumulation of other heavy metal(loid)s need further study.

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