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Physiological responses and detoxific mechanisms to Pb, Zn, Cu and Cd in young seedlings of *Paulownia fortunei*

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Abstract

Paulownia fortunei has been successfully used in the phytoremediation of many Pb/Zn mine tailings. However, seed germination and young seedlings of *P. fortunei* rarely occurred in these mine tailings. The physiological responses and detoxific mechanisms of *P. fortunei* young seedling to Pb, Zn, Cu and Cd stress were investigated. The germinated rate, shoot length, chlorophyll and carotenoid contents in leaves of young seedlings had a great reduction under Zn and Cu treatments, but had little decrease under Pb and Cd treatments. The production rate of $O_2^{\bullet-}$, H_2O_2 and malondialdehyde (MDA) contents significantly increased in response to added Zn and Cu indicating great oxidative stress for young seedlings, but they had no significant change to added Pb and Cd. Young seedlings had effective detoxific mechanism to Pb and Cd, as antioxidant enzymes activities, phytochelatins (PCs-SH) and proline contents increased with increasing rates of added Pb and Cd. However, young seedlings had un-effective detoxific mechanisms to Zn and Cu stress. Results revealed the heavy metals (such as Cu) that present at low concentrations in mine tailings may be major constraint for the survival of young seedlings.

Key words: heavy metals; defense system; oxygen species; antioxidant enzyme; phytochelatins
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Introduction

China has numerous heavy metal mines, and the mining activities produce huge amounts of mine tailings (Wong, 2003). The Dragon Tree, *Paulownia fortunei*, is indigenous to China and is commonly regarded as a miracle tree because of its fast growth, tolerance to drought and universal adaptability for various uses (Carpenter, 1997). It has been introduced to other countries or regions such as North America, Australia, Europe, and Japan (Kumar et al., 1999). *P. fortunei* was used to phytoremediate 3000 acres of contaminated soil downwind of a Pb/Zn smelter in Guangdong Province, China (Wang et al., 2009). However, most of the phytoremediation works used transplanted adult plants (more than one year old) because seeds of *P. fortunei* rarely germinated and young seedlings (less than one month old) were also rarely found in field soils contaminated with heavy metals. Most of phytoremediation works with other plant species also had this problem.

The physiological responses of adult plants and young seedlings are rarely related due to differences in the mechanisms that control the performance of the various stages of the life cycle (Lloret et al., 2004). Moreover, the initial development of seedlings is sensitive to metal toxicity and acidity (Bell and Teramura, 1991). The ability of seeds germination and the initial growth in contaminated soil is important for plant regeneration. However, most studies conducted with adult plants (Mishra et al., 2006), and few studies exposed seeds to contaminants to study tolerance mechanisms in the early stages of plant development (Xiong, 1998).

Mine tailings often exhibit strong enrichments of one metal, but slight or moderate enrichments of other metals, and it is often difficult to estimate the precise toxicity thresholds (Arnetoli et al., 2008). Moreover, the tolerance of plants should only be confined to the metals that present at high toxic concentrations (McNeilly, 1968). However, the non-effective tolerance to metals that present at low concentrations may lead to the death of young seedlings. The studied tailing is predominantly enriched in Pb and Zn, but little enriched in Cu and Cd (Wang et al., 2009). However, Cu and Cd, even at low concentrations, will cause growth inhibition and even plant death (Kohen and Nyska, 2002).

Adult *P. fortunei* accumulated high Pb and Zn contents (Wang et al., 2009), indicating a high tolerance for Pb and Zn. In this study, antioxidative enzymes (superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX) and glutathione peroxidase (GPX)) activities, phytochelatins (PCs), photosynthetic

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pigment, lipid peroxidation and proline were determined to reflect the physiological responses and detoxific mechanisms of young seedlings of *P. fortunei* to Pb, Zn, Cu and Cd. We pose two hypotheses with regard to the tolerance of young seedlings to heavy metals: (1) the tolerance to heavy metals may differ between young seedlings and adult plants; (2) the heavy metals that present at low concentrations in mine tailings may be the major constraint for the survival of young seedlings.

1 Materials and methods

1.1 Plant growing conditions

The concentrations of heavy metals (Pb, Zn, Cu and Cd) were designed according to the contents of extractable fraction in Pb/Zn smelter contaminated area (Wang et al., 2009). Five heavy metal application rates (Pb: 0, 20, 40, 80 and 160 mg/L; Zn: 0, 20, 40, 80 and 160 mg/L; Cu: 0, 10, 20, 40 and 80 mg/L; Cd: 0, 2.5, 5, 10 and 20 mg/L) and ten replicates were used in a complete factorial design. Seeds of *P. fortunei*, obtained from a contaminated area, were germinated in separate 500 mL containers filled with washed silica sand. The containers were watered on alternate days using a modified Hoagland's solution (Hoagland and Arnon, 1950) containing (in mg/L): KNO_3 1020, $Ca(NO_3)_2{\cdot}4H_2O$ 700, $NH_4H_2PO_4$ 230, $MgSO_4{\cdot}7H_2O$ 490, 490, H_3BO_3 2.86, $MnCl_2{\cdot}4H_2O$ 1.81, $CuSO_4{\cdot}5H_2O$ 0.08, $ZnSO_4{\cdot}7H_2O$ 0.22 (the added Cu and Zn were regarded as background value), $Na_2MoO_4{\cdot}2H_2O$ 0.11, $FeSO_4{\cdot}7H_2O$ 3.00, and allowed to drain freely. The water-soluble sulfates of Pb, Zn, Cu and Cd were added to this nutrient solution to obtain the application rate concentrations. The control treatments (0 mg/L) had no added sulfates of Pb, Zn, Cu and Cd. The pH of culture solutions were controlled at 5.0 with H_2SO_4.

1.2 Germination rate and shoot length of early seedlings

Fifty seeds were put into each 500 mL container. The number of germinated seeds was counted to calculate germination rate. After four weeks culture, the shoot length of ten young seedlings in every container were measured and then all young seedlings were harvested for analysis of physiological parameters.

1.3 Pigment content

Five plants per replicate were used for pigment determination. Prior to extraction, fresh leaf samples were cleaned with deionized water to remove any surface contaminants. The pigments were extracted from leaves using 80% acetone. The chlorophyll and carotenoid concentrations were determined by spectrophotometry according to the procedures described by Lichtenthaler (1987). The following equations were used for calculating concentrations of chlorophyll and carotenoid in the leaves:

$$C_a = 12.25 \times A_{663} - 2.79 \times A_{645} \quad (1)$$

$$C_b = 21.50 \times A_{645} - 5.10 \times A_{663} \quad (2)$$

$$C_{a+b} = 7.15 \times A_{663} + 18.71 \times A_{645} \quad (3)$$

$$C_c = (1000 \times A_{470} - 1.82 \times C_a - 85.02 \times C_b)/198 \quad (4)$$

where, C_a, C_b and C_{a+b} are chlorophyll *a*, *b* and total concentrations, respectively; C_c is the carotenoid concentration; and A_{663}, A_{645}, and A_{470} represent absorbance values read at wavelengths of 663, 645 and 440 nm, respectively.

1.4 Antioxidant enzyme extraction and assays

Plant material (about 500 mg) was homogenized in 5 mL solutions containing: 100 mmol/L of potassium phosphate buffer (pH 7.0) containing 0.1 mmol/L of EDTA-Na_2 and 1% polyvinylpyrrolidone (*W/V*) at 4°C. The homogenate was filtered through four layers of cheesecloth and centrifuged at 15,000 ×*g* for 15 min at 4°C. Enzyme activity was measured in the supernatant solution. Protein contents were determined according to Bradford (1976), using bovine serum albumin as a standard. SOD, POD and CAT activities were determined using the method of Gajewska et al. (2006), and APX and GPX activities were determined using the methods of Mishra et al. (2006).

1.5 Assay of reactive oxygen species (ROS)

The level of $O_2^{\bullet-}$ was measured using a slightly modified method of Elstner and Heupel (1976) with the level of H_2O_2 determined as described by Jana and Choudhuri (1981).

1.6 Lipid peroxidation, free proline content, total non-protein SH and total GSH

Lipid peroxidation was determined by estimation of the melondialodehyde (MDA) content following Heath and Packer (1968). Free proline content was determined using the ninhydrin method of Bates et al. (1973). Total non-protein SH (TNP-SH) were extracted and assayed according to the method of De Vos et al. (1992). Total GSH was extracted and assayed according to the method reported by Hissin and Hilf (1976) and Gupta et al. (1998). Total PCs concentration was assessed by subtracting the amount of total GSH from the amount of total non-protein SH compounds (Hartley-Whitaker et al., 2001).

1.7 Statistical analysis

The statistical significance of the results was analyzed using one-way analysis of variance (ANOVA), and the LSD test to determine significant differences between treatments.

2 Results

2.1 Germination rate and shoot length

Germination rate and shoot length generally had a reduction as the concentration of Pb, Zn, Cu and Cd in the growing media increased (Table 1). However, the negative effects of Pb, Zn, Cu and Cd treatments had great difference. Cd treatments nearly had no negative effects on germination rate and shoot length, and Pb treatments also had little negative effects. However, Zn and Cu treatments

had great restrained effects on germination rate and shoot length. Particularly, no seeds can germinate at the highest concentration (80 mg/L) of Cu.

2.2 Pigment content

Pigment contents in the leaves of young seedlings, in general, showed consistent decrease as the concentration of added Pb, Zn, Cu and Cd increased (Fig. 1). The pigment contents slightly decreased under Pb and Cd treatments (Fig. 1). However, the pigment contents greatly decreased with the increase in Zn and Cu concentrations, particularly in Cu treatments (Fig. 1). Chlorosis was not observed under Cd treatments, and had little area when added Pb exceeded 80 mg/L, while the chlorosis existed when the added Zn and Cu exceeded 20 and 10 mg/L, respectively. Moreover, the chlorotic areas continually and greatly extended over the course of Zn and Cu treatments.

2.3 $O_2^{\cdot-}$ and H_2O_2

In *P. fortunei* young seedling, the production rate of $O_2^{\bullet-}$ and H_2O_2 contents had no significant increase with the increase of Cd concentrations (Table 2) indicating no oxidative stress to young seedlings, but greatly increased when added Pb exceeded 80 mg/L indicating an certain capacity of anti-oxidation to Pb. While, under Zn and Cu treatments, the production rate of $O_2^{\bullet-}$ and H_2O_2 contents greatly increased, which pronounced great oxidative stress to young seedlings.

2.4 MDA and proline

MDA contents had no significant change with the increase in Pb and Cd concentrations, but the proline contents greatly increased (Table 3). On the contrary, MDA contents significantly increased with the increase in Zn and Cu concentrations, while the proline contents had no significant change under Zn treatments, and even decreased under Cu treatments.

2.5 Antioxidant enzymes

High plants have defense system that constitutes various antioxidant enzymes to combat increased production of ROS caused by heavy metals. The enzyme activities of SOD, POD, CAT, APX and GPX were determined to investigate the tolerance of young seedlings to Pb, Zn, Cu and Cd stress. The enzyme activities of SOD, POD, CAT, APX and GPX generally increased with the increase of Pb and Cd concentrations, particularly under Cd treatments (Table 4). However, the enzyme activities of SOD, POD, CAT, APX and GPX reached the maximum at 20 mg/L of added Zn, and then significantly decreased. In addition, these enzyme activities were greatly restrained with the increase of Cu concentrations, indicating little tolerance of

Table 1 Effects of Pb, Zn, Cu and Cd treatments on germination rates and shoot length of young seedlings of *P. fortunei*

Growth parameter	Heavy metal				
	0 (control)	20 mg/L Pb	40 mg/L Pb	80 mg/L Pb	160 mg/L Pb
Germination rate (%)	80.2 ± 5.3 a	77.3 ± 6.4 a	75.7 ± 4.3 a	72.7 ± 1.7 ab	61.7 ± 3.1 b
Shoot length (cm)	35.6 ± 1.8 a	33.8 ± 1.1 a	32.2 ± 1.7 ab	30.2 ± 0.4 b	19.7 ± 1.4 c
	0 (control)	20 mg/L Zn	40 mg/L Zn	80 mg/L Zn	160 mg/L Zn
Germination rate (%)	80.2 ± 5.3 a	75.3 ± 4.7 b	64.7 ± 3.3 b	51.3 ± 3.7 c	48.6 ± 3.4 c
Shoot length (cm)	35.6 ± 1.8 a	36.9 ± 2.2 a	17.8 ± 2.0 b	11.9 ± 0.9 c	8.1 ± 0.6 d
	0 (control)	10 mg/L Cu	20 mg/L Cu	40 mg/L Cu	80 mg/L Cu
Germination rate (%)	80.2 ± 5.3 a	53.1 ± 4.2 b	12.4 ± 1.8 c	1.3 ± 0.3 d	0.0 ± 0.0 e
Shoot length (cm)	35.6 ± 1.8 a	13.2 ± 0.6 b	4.4 ± 0.6 c	2.8 ± 0.4 d	0.0 ± 0.0 e
	0 (control)	2.5 mg/L Cd	5 mg/L Cd	10 mg/L Cd	20 mg/L Cd
Germination rate (%)	80.2 ± 5.3 a	76.7 ± 5.1 a	75.3 ± 4.7 a	78.4 ± 4.3 a	73.4 ± 5.1 a
Shoot length (cm)	35.6 ± 1.8 a	33.2 ± 1.3 a	32.8 ± 1.4 a	31.9 ± 1.2 a	27.1 ± 1.3 b

Data are mean ± SE (standard error), $n = 10$ for germination rates, $n = 100$ for shoot height. ANOVA significant at $P < 0.05$. Means followed by the same letters within a row are not significantly different ($P > 0.05$).

Table 2 Effects of Pb, Zn, Cu and Cd treatments on the production rate of $O_2^{\cdot-}$ and H_2O_2 contents in leaves of *P. fortunei* young seedlings

Oxygen species	Heavy metal				
	0 (control)	20 mg/L Pb	40 mg/L Pb	80 mg/L Pb	160 mg/L Pb
$O_2^{\bullet-}$ (μmol/(min·mg protein))	1.25 ± 0.12 b	1.28 ± 0.10 b	1.29 ± 0.16 b	1.57 ± 0.17 a	1.58 ± 0.13 a
H_2O_2 (nmol/g fw)	0.52 ± 0.02 b	0.57 ± 0.02 b	0.58 ± 0.01 b	0.74 ± 0.02 a	0.76 ± 0.02 a
	0 (control)	20 mg/L Zn	40 mg/L Zn	80 mg/L Zn	160 mg/L Zn
$O_2^{\bullet-}$ (μmol/(min·mg protein))	1.25 ± 0.12 c	1.26 ± 0.09 c	1.67 ± 0.17 b	1.97 ± 0.13 a	2.08 ± 0.21 a
H_2O_2 (nmol/g fw)	0.52 ± 0.02 d	0.56 ± 0.01 d	0.78 ± 0.02 c	0.98 ± 0.02 b	1.12 ± 0.03 a
	0 (control)	10 mg/L Cu	20 mg/L Cu	40 mg/L Cu	80 mg/L Cu
$O_2^{\bullet-}$ (μmol/(min·mg protein))	1.25 ± 0.12 d	1.98 ± 0.21 c	2.63 ± 0.24 b	2.82 ± 0.31 a	–
H_2O_2 (nmol/g fw)	0.52 ± 0.02 d	0.92 ± 0.19 c	1.27 ± 0.12 b	1.92 ± 0.21 a	–
	0 (control)	2.5 mg/L Cd	5 mg/L Cd	10 mg/L Cd	20 mg/L Cd
$O_2^{\bullet-}$ (μmol/(min·mg protein))	1.25 ± 0.12 a	1.23 ± 0.18 a	1.19 ± 0.09 a	1.27 ± 0.17 a	1.19 ± 0.14 a
H_2O_2 (nmol/g fw)	0.52 ± 0.02 a	0.49 ± 0.09 a	0.54 ± 0.11 a	0.57 ± 0.15 a	0.57 ± 0.11 a

Data are mean ± SE, $n = 5$. ANOVA significant at $P < 0.05$. Means followed by the same letters within a row are not significantly different ($P > 0.05$).

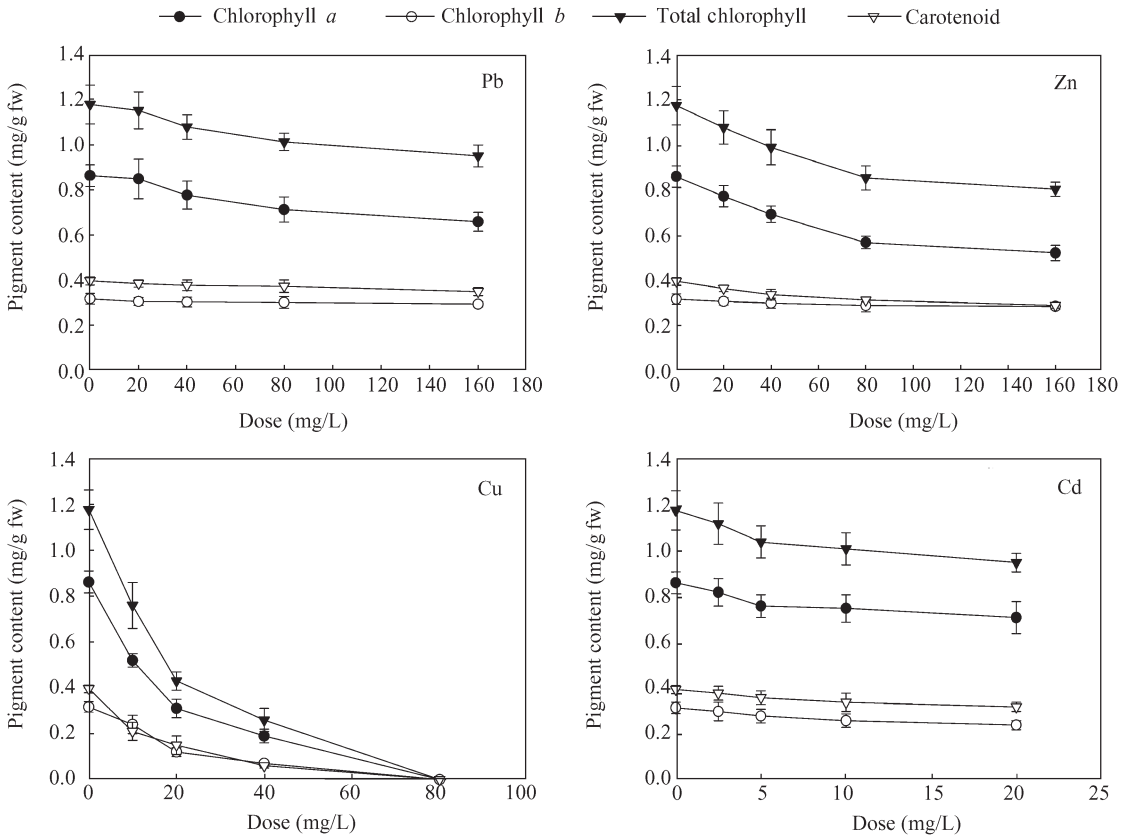

Fig. 1 Effects of Pb, Zn, Cu and Cd treatments on chlorophyll *a*, chlorophyll *b*, total chlorophyll (chlorophyll *a* + *b*) and carotenoid of leaves of *P. fortunei* young seedlings. All the values are mean of replicates ± SE.

Table 3 Effects of Pb, Zn, Cu and Cd treatments on malondialdehyde (MDA) and proline contents in leaves of *P. fortunei* young seedlings

	Heavy metal				
	0 (control)	20 mg/L Pb	40 mg/L Pb	80 mg/L Pb	160 mg/L Pb
MDA (μmol/g fw)	2.1 ± 0.4 a	2.1 ± 0.2 a	2.2 ± 0.3 a	2.2 ± 0.3 a	2.4 ± 0.2 a
Proline (μmol/g fw)	3.5 ± 0.3 d	5.5 ± 0.5 c	8.7 ± 0.5 b	10.7 ± 0.8 a	11.7 ± 0.5 a
	0 (control)	20 mg/L Zn	40 mg/L Zn	80 mg/L Zn	160 mg/L Zn
MDA (μmol/g fw)	2.1 ± 0.4 d	3.7 ± 0.3 c	5.3 ± 0.3 b	5.5 ± 0.3 b	7.3 ± 0.5 a
Proline (μmol/g fw)	3.5 ± 0.3 b	3.6 ± 0.2 b	3.9 ± 0.2 a	4.1 ± 0.3 a	4.3 ± 0.2 a
	0 (control)	10 mg/L Cu	20 mg/L Cu	40 mg/L Cu	80 mg/L Cu
MDA (μmol/g fw)	2.1 ± 0.4 d	5.2 ± 0.4 c	7.3 ± 0.3 b	9.1 ± 0.5 a	–
Proline (μmol/g fw)	3.5 ± 0.3 a	3.7 ± 0.2 a	3.1 ± 0.2 ab	2.5 ± 0.2 b	–
	0 (control)	2.5 mg/L Cd	5 mg/L Cd	10 mg/L Cd	20 mg/L Cd
MDA (μmol/g fw)	2.1 ± 0.4 a	2.1 ± 0.2 a	2.1 ± 0.3 a	2.1 ± 0.3 a	2.2 ± 0.2 a
Proline (μmol/g fw)	3.5 ± 0.3 e	4.5 ± 0.2 d	6.3 ± 0.3 c	8.1 ± 0.3 b	10.8 ± 0.6 a

Data are means ± SE, $n = 5$. ANOVA significant at $P < 0.05$. Means followed by the same letters within a row are not significantly different ($P > 0.05$).

young seedlings to Cu stress.

2.6 Total non-protein SH (TNP-SH) and total GSH

All the Pb, Zn, Cu and Cd treatments did not significantly induce the production of total GSH (Fig. 2). TNP-SH significantly increased with the increase of Pb and Cd concentrations, but had no change under Zn treatments, and even significantly decreased with the increase of Cu concentrations. The increase of TNP-SH under Pb and Cd treatments had close link with the production of PCs, approximately 80% of which was accounted for by PCs-SH.

3 Discussion

This study examined the effect of Pb, Zn, Cu and Cd stress on seed germination and physiological responses of *P. fortunei* young seedlings. Zn and Cu treatments had great restrained effects on germination rate and shoot length, while Pb and Cd treatments had little influence. The contents of chlorophyll *a*, *b*, total chlorophyll and carotenoid also had great reduction with the increase of added Zn and Cu, but had little change with the increase of added Pb and Cd. Moreover, chlorosis, a common symptom of heavy metal toxicity, occurred at low concentrations

Table 4 Enzyme activities of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX) and glutathione peroxidase (GPX) in leaves of *P. fortunei* young seedlings treated with Pb, Zn, Cu and Cd

Antioxidant enzyme	Heavy metal				
	0 (control)	20 mg/L Pb	40 mg/L Pb	80 mg/L Pb	160 mg/L Pb
SOD (unit/mg protein)	103.1 ± 6.0 c	115.1 ± 5.8 bc	122.2 ± 4.1 b	173.2 ± 7.2 a	175.5 ± 5.3 a
POD (unit/mg protein)	43.9 ± 1.2 c	42.8 ± 1.0 c	46.1 ± 1.3 bc	51.2 ± 2.0 b	62.1 ± 2.8 a
CAT (unit/mg protein)	16.6 ± 1.0 a	16.8 ± 1.0 a	17.2 ± 1.2 a	18.9 ± 2.1 a	18.2 ± 2.1 a
APX (unit/mg protein)	4.6 ± 0.5 c	4.8 ± 0.2 c	6.2 ± 0.8 b	9.1 ± 0.5 a	9.0 ± 0.7 a
GPX (unit/mg protein)	40.2 ± 1.3 c	41.9 ± 1.1 c	48.9 ± 1.7 b	58.3 ± 2.0 a	59.0 ± 1.1 a
	0 (control)	20 mg/L Zn	40 mg/L Zn	80 mg/L Zn	160 mg/L Zn
SOD (unit/mg protein)	103.1 ± 6.0 a	106.0 ± 6.0 a	95.8 ± 3.2 ab	79.1 ± 4.2 b	74.9 ± 4.1 b
POD (unit/mg protein)	43.9 ± 1.2 a	44.2 ± 1.0 a	40.8 ± 1.0 ab	37.5 ± 1.1 b	32.9 ± 0.9 c
CAT (unit/mg protein)	16.6 ± 1.0 a	17.1 ± 1.1 a	10.3 ± 0.7 b	10.0 ± 0.5 b	8.1 ± 0.7 c
APX (unit/mg protein)	4.6 ± 0.5 a	4.9 ± 0.4 a	4.2 ± 1.4 ab	4.1 ± 0.9 ab	4.0 ± 0.4 b
GPX (unit/mg protein)	40.2 ± 1.3 ab	46.3 ± 1.4 a	40.1 ± 1.0 ab	37.3 ± 1.0 b	30.1 ± 1.1 c
	0 (control)	10 mg/L Cu	20 mg/L Cu	40 mg/L Cu	80 mg/L Cu
SOD (unit/mg protein)	103.1 ± 6.0 a	61.0 ± 5.3 b	32.8 ± 3.2 c	21.1 ± 4.2 d	–
POD (unit/mg protein)	43.9 ± 1.2 a	30.2 ± 1.8 b	21.8 ± 1.0 c	7.5 ± 1.1 d	–
CAT (unit/mg protein)	16.6 ± 1.0 a	10.1 ± 1.1 b	5.3 ± 1.7 c	2.0 ± 0.4 d	–
APX (unit/mg protein)	4.6 ± 0.5 a	3.1 ± 0.4 b	1.2 ± 0.2 c	0.8 ± 0.2 d	–
GPX (unit/mg protein)	40.2 ± 1.3 a	30.3 ± 2.4 b	20.1 ± 2.0 c	6.3 ± 0.8 d	–
	0 (control)	2.5 mg/L Cd	5 mg/L Cd	10 mg/L Cd	20 mg/L Cd
SOD (unit/mg protein)	103.1 ± 6.0 b	105.1 ± 5.8 b	118.2 ± 4.1 ab	129.2 ± 7.2 a	124.5 ± 5.3 a
POD (unit/mg protein)	43.9 ± 1.2 c	44.8 ± 1.0 c	46.1 ± 1.3 bc	49.2 ± 2.0 b	58.9 ± 3.9 a
CAT (unit/mg protein)	16.6 ± 1.0 c	16.8 ± 1.1 c	19.2 ± 1.2 bc	26.2 ± 1.0 b	33.2 ± 1.1 a
APX (unit/mg protein)	4.6 ± 0.5 c	4.8 ± 0.2 c	5.2 ± 0.8 bc	7.1 ± 0.5 b	9.2 ± 0.8 a
GPX (unit/mg protein)	40.2 ± 1.3 c	41.9 ± 1.1 c	48.9 ± 1.7 bc	52.3 ± 3.0 b	60.1 ± 4.1 a

Data are means ± SE, $n = 5$. ANOVA significant at $P < 0.05$. Means followed by the same letters within a row are not significantly different ($P > 0.05$).

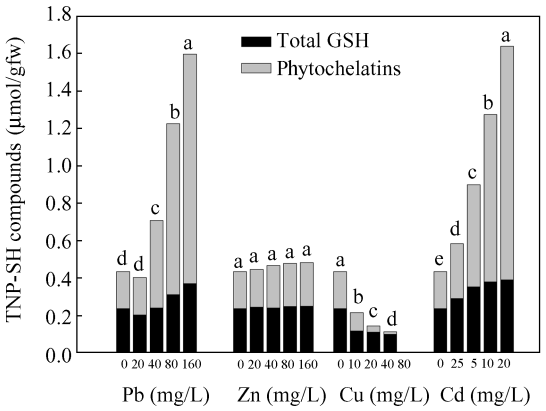

Fig. 2 Effects of Pb, Zn, Cu and Cd treatments on total non-protein SH compounds (TNP-SH), total GSH and phytochelatins (PCs) in leaves of *P. fortunei* young seedlings. Different letters indicate significant difference of PCs between different treated doses ($P < 0.05$).

of Zn and Cu, which indicating great restrained effects of Zn and Cu stress on chlorophyll formation (Somashekaraiah et al., 1992). Results demonstrated that Pb, Zn, Cu and Cd had great difference in the effect on seed germination and physiological responses of *P. fortunei* young seedlings.

In higher plants, heavy metals induce the generation of $O_2^{\bullet -}$, H_2O_2, and HO·, collectively termed ROS, and exert a variety of damaging effects, also called oxidative stress (Fang and Kao, 2000; Tewari et al., 2002). The greater increase in the production rate of $O_2^{\bullet -}$ and H_2O_2 contents with the increase of Zn and Cu concentrations showed great oxidative stress of Zn and Cu to young seedlings. However, Pb and Cd treatments adversely induced little generation of $O_2^{\bullet -}$ and H_2O_2 in young seedlings. The change of MDA contents (a cytotoxic product of lipid peroxidation and an indicator of free radical production and consequent tissue damage also confirmed the great oxidative stress (Dhir et al., 2004) to young seedlings under Zn and Cu treatments, but little oxidative damage to young seedlings under Pb and Cd treatments.

High plants defenses to heavy metal toxicity may constitute different detoxific mechanisms. First defense system constitutes various antioxidants (mainly antioxidant enzymes) to combat increased production of ROS caused by heavy metal (Zhang et al., 2007). The increase of added Pb and Cd caused great increases in the activities of SOD, POD, CAT, APX and GPX, indicating that the *P. fortunei* young seedlings had effective defense mechanisms to the stress of Pb and Cd. These tolerance mechanisms are also found in other species under stress conditions (Lee and Shin, 2003; Li et al., 2006; Lin et al., 2007). On the contrary, the activities of antioxidant enzymes significantly decreased with the increase of added Zn and Cu. The observed decreased activities of antioxidant enzymes may be due to the over-production of ROS induced by the added Zn and Cu, leading to damage to the antioxidant enzymes (Verma and Dubey, 2003; Dazy et al., 2008). Second defense system constitutes free proline, ligands, organic acid, GSH or PCs to render heavy metal harmless before entry to the cell (Mishra et al., 2006). PCs particularly play a important role to protect plants against heavy metals stress in plants (Kawakami et al., 2006). Heavy metals can be sequestered through forming complexes of with PCs and lead to their detoxification (Mishra et al., 2006). In this study, the increase of added Pb and Cd significantly induced the production of PCs, but PCs had no change

under Zn treatment and even significantly decreased under Cu treatments. Moreover, the great accumulation of proline in the Pb and Cd treatments may be another explanation for *P. fortunei* young seedlings having a more effective defense mechanism to Pb and Cd than to Zn and Cu. Accumulation of proline can not only enhance antioxidation but also remove the ROS in plant tissues (Jiang et al., 1997).

Unlike other studies of the tolerance mechanisms, the essential metal (Zn and Cu) exhibited a more toxic effect on the young seedlings of *P. fortunei* than did the unessential metal (Pb and Cd). This may be related to the great accumulation of Zn and Cu in young seedlings (data not shown). Zn is the second most abundant transition metal and is involved in various biological processes in plants (Broadley et al., 2007). Cu is also a micronutrient of plants and plays important roles in CO_2 assimilation, ATP synthesis and is a component of various proteins (Boojar and Goodarzi, 2007). Young seedlings are in the rapid growth stage of plants and consequently require considerable Zn and Cu. Adult *P. fortunei* accumulated high Pb and Zn contents indicating high tolerance for both metals (Wang et al., 2009), while young seedlings only had effective detoxific mechanism to Pb stress, but were very sensitive to Zn stress. Moreover, adult *P. fortunei* accumulated low Cu and Cd contents (Wang et al., 2009), while young seedlings preferred to uptake Cu and had uneffective detoxific mechanisms to Cu. Consequently, the tolerant mechanisms of plants to heavy metal stress should be studied in different growth stages for the mechanisms that control the performance of the various stages of plants will be different (Lloret et al., 2004), which will lead to different tolerant characteristics between young seedlings and adult plants.

Many mine tailings often exhibit strong enrichments of particular metals, but slight or moderate enrichments of others, and it is often difficult to estimate the precise toxicity thresholds (Arnetoli et al., 2008). In this study, Cu slightly enriches in Pb/Zn mine tailing, but is the major constraint for seed germination and young seedling growth of *P. fortunei*. Consequently, the heavy metals with a low enrichment in mine tailings should be pay more attention, which may be the major constraint for the survival at a certain growth stage of plants. This study showed that different tolerant characteristics in different growth stages of *P. fortunei* led to different constrained factors. Therefore, successful revegetation of mine tailings requires information of major constrains of different growth stages of revegetated plants. The information of this study indicated that we should take measures to decrease the bioavailability of Cu to increase the self-sustain ability of revegetated communities of *P. fortunei*.

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