Antioxidant enzymes and proteins of wetland plants: Their relation to Pb tolerance and accumulation

Junxing Yang · Zhihong Ye

Received: 4 December 2013 / Accepted: 16 September 2014 / Published online: 2 October 2014
© Springer-Verlag Berlin Heidelberg 2014

Abstract Constructed wetlands used to clean up toxic metals such as lead (Pb) from contaminated wastewater are considered as an effective and low-cost technology. The effect of Pb on the biomass, tolerance, soluble protein, and antioxidant enzymes in 18 candidate wetland plant species grown in soils without (control) and spiked with 900 and 1800 mg Pb kg⁻¹ was studied in a pot trial. Our pot experiment showed that the biomass, tolerance, and leaf protein contents decreased with increasing concentrations of Pb in soil. There were significant differences between the plants in their Pb tolerance indices (29–82 % in the 900 mg Pb kg⁻¹ amended soil) and also Pb uptake (13–749 mg kg⁻¹ in shoots and 1112–4891 mg kg⁻¹ in roots, in the same treatments). Activities of superoxide dismutase (SOD) and peroxidase (POD) in leaves of most of the plants increased with increasing level of soil Pb concentration. Conversely, catalase (CAT) activity in leaves declined when plants were subjected to Pb stress. Lead accumulation by the 18 wetland plant species screened was strongly dependent on the species and Pb concentrations in the soil. However, Pb translocation from root to shoot was generally low in all species. Increases in SOD and POD activities suggest that the antioxidant system may play an important role in alleviating Pb toxicity in wetland plants. The data obtained should

help in future species selection for the use in designing wetlands in Pb-contaminated environments.

Keywords Accumulation · Antioxidant enzyme · Lead (Pb) · Tolerance index · Toxicity · Wetland plant

Introduction

Wastewater contaminated by heavy metals (e.g., Pb) is a serious environmental problem as a result of the mining industry in many countries (Singh et al. 2004; Sharma et al. 2007). In China, large areas of land (including paddy fields) in mining areas have been contaminated by Pb due to irrigation with Pb-contaminated wastewater arising from mineral processing plants (Li et al. 2009; Liu et al. 2013). High Pb concentrations in paddy soils (386 mg kg⁻¹ at the Dabaoshan and 2780 mg kg⁻¹ at Fankou mining areas) have been reported in South China (Zhuang et al. 2009; Li et al. 2012). There is an urgent need to develop cost-effective methods to control and remediate contaminated wastewaters in order to ensure safe crop production practices in areas such as these affected by Pb contamination.

Constructed wetlands have, for some time, been considered as an effective, low-cost, and practical approach for the clean up of metal-contaminated wastewaters (Sheoran and Sheoran 2006; Vymazal 2011). In a constructed wetland system, the plant is a key component in metal removal via filtration, adsorption, and cation exchange, and through plant-induced chemical changes in the rhizosphere (Wright and Otte 1999). Cattail (*Typha latifolia* L.) and common reed (*Phragmites australis* (Cav.) Trin. ex Steud.) have been used successfully for the phytoremediation of Pb/Zn mine tailings under waterlogged conditions (Ye et al. 2004).

The uptake and accumulation of Pb by wetland plants are strongly governed by substrate Pb concentrations and the

Responsible editor: Philippe Garrigues

J. Yang · Z. Ye (✉)
Key Laboratory of Biodiversity Dynamics and Conservation of
Guangdong Higher Education Institutes, College of Ecology and
Evolution, School of Life Sciences, Sun Yat-sen University,
Guangzhou 510006, People's Republic of China
e-mail: lssyzhh@mail.sysu.edu.cn

J. Yang
Center for Environmental Remediation, Institute of Geographic
Sciences and Natural Resources Research, Chinese Academy of
Sciences, Beijing 100101, People's Republic of China

plant species (Zhang et al. 1998; Deng et al. 2004). There is evidence that some emergent wetland plants such as cattail, common reed (Ye et al. 2001), yellow flag (*Iris pseudacorus* L.) (Han et al. 2008), and the aquatic macrophyte *Najas indica* (Willd.) Cham. (Singh et al. 2010) have a greater ability to tolerate Pb and to accumulate it to some degree in their tissues. However, there is considerable variation between species in their responses to Pb (Deng et al. 2006). Lead-tolerant plants tend to be root accumulators showing restricted Pb translocation from root to shoot (Baker and Walker 1990; Shu et al. 2012). Previous studies have suggested that some wetland plant species accumulate very low Pb concentrations in their shoots even when grown in highly Pb contaminated substrates (Ye et al. 1997a, b, 2001; Deng et al. 2004, 2006) and employing an ‘excluder’ strategy (sensu Baker 1981). Other species, however, accumulate higher Pb concentrations in their shoot tissues without any visible toxicity symptoms. These observations suggest that some innate mechanisms of metal detoxification (e.g., antioxidant enzyme activities) may exist in at least some wetland plant species (Fediuc and Erdei 2002; Adhikari et al. 2010; Singh et al. 2010).

Lead is a non-essential element and at high concentrations can lead to alterations in the morphology and physiology of plants thought to be caused by reactive oxygen species (ROS) stress from hydroxyl ($\text{OH}^\cdot$) and superoxide radicals ($\text{O}_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) which cause oxidative damage (Guo et al. 2004). For the protection against such damage to plant cells, increased activity of oxygen radical detoxifying enzymes, such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), may be induced in order to resist the oxidative stress caused by potentially toxic metal concentrations (Chaoui et al. 1997). SOD is a metalloenzyme that catalyzes the disproportionation of $\text{O}_2^{\cdot-}$ radicals to H_2O_2 and O_2 , and plays an important role in protecting cells against the toxic effects of superoxide radicals produced in different cellular compartments (Del Rio et al. 2002). POD catalyzes H_2O_2 -dependent substrate oxidation, and POD activity is also considered a useful biomarker for sublethal metal toxicity (Podazza et al. 2012). CAT is one of the most important enzymes for the regulation of intracellular H_2O_2 levels affected by SOD catalyzed reactions (Blokhina et al. 2003). Recently, studies have indicated that these antioxidant enzymes may play an important role in the defensive mechanism of wetland plants against Pb and other heavy metals. This has been suggested for *T. latifolia* (Fediuc and Erdei 2002), *P. australis* (Ederli et al. 2004), *Lemna minor* L. (Hou et al. 2007), *I. pseudacorus* (Zhou et al. 2010), and *Juncus effusus* L. (Najeeb et al. 2011). However, the existence of any specific relationships between Pb tolerance and antioxidant enzymes across a wider range of wetland plants remains unclear.

The aims of the current study were (1) to compare the Pb tolerance, uptake, and accumulation in a wide range of wetland plants and (2) to determine the effects of Pb on

antioxidant enzymes in these wetland plants. As Pb is one of the commonest heavy metals in industrial wastewater and mine processing wastes such as tailings lagoons (Ye et al. 2004), the results from this screening study should provide useful information for the selection of appropriate wetland plant species in constructed wetlands designed for the treatment of industrial run-off waters and in the phytoremediation of slurried mine tailings contaminated with Pb.

Materials and methods

Plant materials and Pb treatments

The 18 emergent-rooted wetland plants have been selected as they are (1) fast growing, (2) widely distributed, and (3) possess low nutrient requirement. The plant materials were collected from non-contaminated sites in Guangdong Province (Yang et al. 2014). Tillers of *Acorus tatarinowii* Schott, *Alternanthera philoxeroides* (Mart.) Griseb., *Ceratopteris thalictroides* (L.) Brongn., *Cyperus alternifolius* Rottb., *Hydrocotyle vulgaris* L., *Panicum repens* L., *Rotala rotundifolia* Buch.-Ham. ex Roxb., *Veronica serpyllifolia* L., and *Zantedeschia aethiopica* Spreng. were grown by vegetative propagation; the other nine species [*Alocasia cucullata* (Lour.) Schott, *Echinodorus amazonicus* Rataj, *Echinodorus osiris* var. *rubra*, *Eleocharis geniculata* (L.) Romer & Schult., *Fimbristylis monostachya* (L.) Hassk., *Jussiaea linifolia* Vahl., *Paspalum scrobiculatum* L., *Philydrum lanuginosum* Banks et Sol. ex Gaertner, and *Scirpus triquetus* L.] were germinated from seed. Because of the differences in growth rates between these, individuals of the same species with similar shoot heights and root lengths were selected for a pot trial. Plant cultivation was conducted in a glasshouse in a randomized block design. The glasshouse was illuminated with cool-white fluorescent tubes, supplying a photon flux density of $300 \mu\text{mol m}^{-2} \text{S}^{-1}$, a relative humidity of 85 %, and a light/dark cycle of 14 h day/10 h night. The day/night temperature regime was between 28 and 22 °C.

The soil used in the pot trial was collected from a ‘clean’ paddy field (0–20 cm) at South China Agricultural University campus, located in Guangzhou City, Guangdong Province, PR China. It was spiked with Pb (900 and 1800 mg kg^{-1} supplied as $\text{Pb}(\text{NO}_3)_2$), then mixed thoroughly and allowed to equilibrate for 2 months. These two levels of Pb treatment were selected on the basis of results of our pilot experiments and other previous studies (Adhikari et al. 2010). Unamended soil served as a control. During the equilibration, the soil water was maintained at 70 % of

maximum water holding capacity by weight. After 2 months, the bulk soil was air-dried and passed through a 2-mm sieve, and then 1 kg of air-dried soil was used in each pot. Physicochemical properties of the soil used for the experiment were pH 5.2 (2.5:1 distilled water: soil, v/w), organic matter content 17.54 g kg⁻¹ (K₂CrO₇-H₂SO₄), total N 0.836 g kg⁻¹ (semi-quantitative titration), Olsen-P 28.74 mg kg⁻¹ (0.5 M NaHCO₃), available K 63.95 mg kg⁻¹ (1.0 M NH₄OAc), and total Pb 36 mg kg⁻¹. To imitate wetland conditions, soils in the experimental units were maintained at 100 % water-holding capacity for 2 weeks.

The initial heights of the 18 plant species used in the pot trial ranged from 15 to 20 cm. For each species, two seedlings or tillers were transplanted into each pot and were grown under the same glasshouse conditions. There were three treatments employed in the pot trial (control, 900 mg Pb kg⁻¹ and 1800 mg Pb kg⁻¹) with four replicates for each treatment/plant species. The pots were arranged in a fully randomized design on the glasshouse bench and their positions rotated regularly to ensure uniform growth conditions. After 40 days, *P. lanuginosum* in the 1800 mg Pb kg⁻¹ treatments withered and died. At 120 days, plant height was measured for all other species and four leaves of each were removed, wrapped around with aluminium foil, and stored in liquid nitrogen for subsequent total soluble protein and antioxidant enzyme analysis. Plants were then carefully removed from the soil, separated into roots and shoots, thoroughly rinsed with deionized water, and then oven-dried at 70 °C to constant weight and used for the determination of biomass and Pb concentrations in root and shoot tissues.

Determination of total soluble proteins and antioxidant enzymes activities

Fresh leaf samples (0.5 g) were ground with liquid nitrogen and homogenized in 5 mL of 50 mmol sodium phosphate buffer (pH 7.0), including 0.5 mmol EDTA and 0.15 mol NaCl. The homogenate was centrifuged at 12,000g for 10 min at 4 °C, and the supernatant was used for protein determination and enzyme assays. Total soluble protein content was determined by the method of Bradford (1976) using BSA as a standard.

Superoxide dismutase (SOD) activity assay was based on the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) (Giannopolitis and Ries 1977). One unit of SOD activity was defined as the amount of protein inhibiting 50 % of the initial reduction of NBT under illumination, expressed as U mg⁻¹ protein. Catalase (CAT) activity was assayed by the consumption of H₂O₂ conversion by changes in absorbance at 240 nm (Aebi 1984) and was

expressed as U mg⁻¹ protein min⁻¹. POD activity was determined after H₂O₂-induced guaiacol oxidation by absorbance change at 470 nm (Chance and Maehly 1955), again expressed as U mg⁻¹ protein min⁻¹.

Analysis of Pb concentrations in plant tissues

Oven-dried root and shoot samples were homogenized using a Retsch grinder (Type: 2 mm, Retsch Company, Germany) and then digested in HNO₃ (Alexander et al. 2006). Sub-samples of plant tissue (0.5 g) were weighed into 200 mL block digestion tubes, concentrated nitric acid (10 mL) added and allowed to stand overnight. They were then heated for 3 h at 60 °C, followed by 6 h at 110 °C. After cooling, the digests were passed through pre-washed Whatman 540 filter papers (Whatman & Co., UK). The digestion tubes were rinsed four times, passing through the filter each time, and the combined filtrates made up to 50 mL volume using ultrapure water. Lead concentrations in the digests were determined by inductively-coupled plasma optical emission spectrometry (ICP-OES, Optima 2100, Perkin Elmer, USA). Blank and bush leaf material (BGW-07603) (China Standard Materials Research Center, Beijing, PR China) was used for quality control. The Pb recovery rates were 90±10 %.

Statistical analysis

Lead tolerance was quantified by a tolerance index (TI) calculated from a comparison between the growth of each plant in treatments with and without Pb additions (Wilkins 1978).

$$\text{Tolerance index(\%)} = \frac{\text{dry weight in soil with Pb}}{\text{dry weight in soil without Pb}} \times 100$$

Data on plant performance were tested for their normality and variance prior to a one-way analysis of variance (ANOVA). No data transformation was required. If the differences between plant species for each Pb treatment or between Pb treatments for each plant species were significant at the 5 % level, the least significant difference (LSD) was calculated as a post hoc test. All statistical analyses were performed using the SPSS 11.0 statistical package.

Results

Effects of Pb on plant growth and tolerance index

The height and biomass dry weight of most species tested in the Pb treatments were significantly lower than those in the control. Tolerance indices for the 18 plant species varied from 29 to 82 % in the 900 mg Pb kg⁻¹ and from 1 to 80 % in the 1800 mg Pb kg⁻¹ treatments (Table 1).

Table 1 Height (cm), biomass (g DW plant) and tolerance indices (TI, % of control) of 18 species of wetland plants grown in soils at different Pb concentrations: CK (control, without Pb), 900 and 1800 mg Pb kg⁻¹ (as Pb(NO₃)₂) treatments for 4 months (mean±S.E., n=4)

Species	Height (cm)			LSD _{0.05}	Biomass (shoot + root) (g plant)			LSD _{0.05}	Pb TI (%)	
	CK	Pb900	Pb1800		CK	Pb900	Pb1800		Pb900	Pb1800
<i>A. tatarinowii</i>	25±0.5a	26±1.1a	20±1.3b	2.99	2.85±0.19a	2.34±0.13b	2.29±0.09b	0.46	82	80
<i>A. cucullata</i>	22±0.6a	16±0.9b	13±0.7c	2.28	3.61±0.09a	2.83±0.13b	0.82±0.06c	0.32	78	23
<i>A. philoxeroides</i>	45±1.6a	40±0.6b	26±1.1c	3.52	1.22±0.08a	0.81±0.03b	0.66±0.02b	0.16	66	54
<i>C. thalictroides</i>	38±1.6a	36±1.1a	29±0.7b	3.64	1.78±0.04a	1.09±0.13b	0.57±0.04c	0.26	61	32
<i>C. alternifolius</i>	42±0.9a	38±0.7b	34±1.5c	3.28	3.40±0.24a	1.67±0.04b	1.20±0.03c	0.45	49	35
<i>E. amazonicus</i>	16±0.9a	10±0.7b	5±0.7c	2.22	2.54±0.20a	1.36±0.14b	0.29±0.04c	0.46	53	12
<i>E. osiris</i>	12±0.4a	8±0.5b	7±0.9b	2.16	0.93±0.07a	0.40±0.02b	0.28±0.05b	0.17	43	30
<i>E. geniculata</i>	29±1.1a	21±1.2b	12±0.7c	3.03	0.57±0.05a	0.24±0.01b	0.18±0.01b	0.10	42	31
<i>F. monostachya</i>	37±2.3a	23±1.2b	19±0.9b	4.74	1.11±0.05a	0.73±0.07b	0.51±0.08b	0.23	66	46
<i>H. vulgaris</i>	17±0.2a	13±0.7b	5±0.9c	2.04	3.97±0.47a	2.24±0.07b	0.53±0.07c	0.89	57	14
<i>J. linifolia</i>	47±2.1a	31±2.2b	19±2.1c	6.34	2.81±0.17a	1.53±0.23b	0.27±0.03c	0.45	55	1
<i>P. repens</i>	63±1.3a	58±2.3a	46±2b	5.72	2.73±0.26a	2.22±0.1a	0.53±0.05b	0.53	81	19
<i>P. scrobiculatum</i>	76±2.6a	62±2.3b	45±1.1c	6.40	0.67±0.04a	0.47±0.02b	0.40±0.02b	0.10	70	59
<i>P. lanuginosum</i>	50±2.9a	28±1.9b	–	7.73	1.80±0.09a	0.78±0.07b	–	0.28	43	–
<i>R. rotundifolia</i>	28±2.3a	10±0.7b	5±0.2c	4.16	2.83±0.07a	1.17±0.03b	0.12±0.01c	0.15	41	5
<i>S. triqueter</i>	63±2a	39±3.3b	22±1.6c	7.29	0.80±0.12a	0.52±0.06b	0.25±0.04c	0.14	64	31
<i>V. serpyllifolia</i>	11±0.5a	8±0.7b	6±0.4c	1.40	2.16±0.27a	0.62±0.10b	0.41±0.02b	0.53	29	9
<i>Z. aethiopica</i>	50±2.7a	46±1.2a	34±2.8b	6.99	6.50±0.11a	5.33±0.18b	4.02±0.20c	0.54	82	62

Different letters within the same row and the same index (height and biomass) indicate significant difference between the three treatments (CK, Pb900, and Pb1800) at the level of $P<0.05$. LSD value at 5 % is indicated by LSD_{0.05} (the same below)

Pb concentrations in plant tissues

Lead concentrations in shoots of the 18 wetland plants grown in the control, 900 and 1800 mg Pb kg⁻¹ treatments ranged from 4 to 36, 13 to 749, 49 to 18,650 mg kg⁻¹, respectively. The ranges in roots were 18 to 65, 1112 to 4891, 2477 to 17,620 mg kg⁻¹ (Table 2). The concentrations of Pb in root tissues increased significantly in the Pb treatments ($P<0.05$).

Effects of Pb on protein contents and antioxidant enzyme activities

The protein contents and CAT activities in leaves of the plants were significantly reduced in the Pb treatments (Table 3). The degrees of reduction (in terms of % of the control) of protein and CAT activities were significantly different ($P<0.01$) between the species tested, ranging from 3.2 to 69.3 % and 1.9 to 18.4 % in the 900 mg Pb kg⁻¹ treatment, respectively. The equivalent ranges in the 1800 mg Pb kg⁻¹ treatments were 6.6 to 92.1 % and 3.7 to 22.9 %. However, activities of SOD and POD in leaves significantly increased in the Pb treatments. The degrees of increase (in terms of % of the control) of SOD and POD activities were also significantly different ($P<0.01$), ranging from 4.8 to 152 % and 4.7 to 81.8 % in the 900 mg

Pb kg⁻¹ treatment, and from 13.1 to 104 % and 16.2 to 108 % in the 1800 mg Pb kg⁻¹ treatment, respectively.

Correlations between Pb tolerance indices, concentrations of Pb in roots and shoots, protein contents, and antioxidant enzyme activities

Significant correlations were apparent between protein contents; activities of SOD, POD, and CAT in Pb-treated plants; and their indices of Pb tolerance in the Pb treatments (Fig. 1). Negative correlations were suggested between Pb tolerance indices and the Pb concentrations in roots and shoots in Pb-treated plants in the Pb treatments (Fig. 2). Negative correlations were also found between protein contents and these Pb concentrations in the tissues of the 18 wetland species in the control and Pb treatments (Fig. 2).

Discussion

Pb tolerance and accumulation by the wetland plant species

Plant growth (biomass) is usually reduced when plants are challenged with high levels of Pb (Ye et al. 1997a, b; Deng

Table 2 Lead concentration (mg kg⁻¹ dry weight) in shoots and roots of 18 species of wetland plants grown in soils spiked without (control, CK) and with Pb (900 and 1800 mg Pb kg⁻¹ as Pb(NO₃)₂) for 4 months (mean±S.E., n=3)

Species	Shoot			LSD _{0.05}	Root			LSD _{0.05}
	CK	Pb900	Pb1800		CK	Pb900	Pb1800	
<i>A. tatarinowii</i>	6±0.6b	13±1b	107±10a	20.9	31±1.5c	1315±18b	8222±140a	281
<i>A. cucullata</i>	11±1.2c	43±4.8b	114±7a	16.8	36±2.2c	2092±145b	6979±119a	375
<i>A. philoxeroides</i>	22±1.2b	43±1.2b	295±16a	31.6	37±4.7c	2084±115b	7448±56a	256
<i>C. thalictroides</i>	18±1.8c	89±5.5b	312±17a	36.0	65±10.5c	3950±2.2b	8470±1207a	2445
<i>C. alternifolius</i>	3±0.9c	73±2.3b	146±6a	12.6	34±4.8c	1607±168b	3373±178a	489
<i>E. amazonicus</i>	10±0.6b	84±4.4b	2329±156a	312	22±0.9c	3164±115b	6261±412a	854
<i>E. osiris</i>	14±1.2c	738±34b	18650±596a	1193	25±2.9c	3148±395b	10,612±445a	1188
<i>E. geniculata</i>	36±5c	437±32b	1905±135a	277	45±2.2c	2769±170b	8236±249a	602
<i>F. monostachya</i>	9±0.9b	46±6.1b	177±23a	47.0	60±3.5c	1715±40b	4892±121a	254
<i>H. vulgaris</i>	6±0.9c	116±7.2b	535±27a	56.8	22±1.2c	1112±86b	3732±281a	588
<i>J. linifolia</i>	7±0.9b	234±32a	186±19a	74.2	18±4.3c	3962±545b	17,620±687a	1752
<i>P. repens</i>	4±0.3c	106±4b	308±42a	84.5	37±1.2c	1994±155b	5104±87a	355
<i>P. scrobiculatum</i>	33±0.9c	98±6b	220±4a	14.0	94±4.9c	1461±161b	2477±132a	416
<i>P. lanuginosum</i>	8±1.2b	384±80a	—	223	29±1.2b	6374±224a	—	621
<i>R. rotundifolia</i>	27±3.4c	749±29b	3581±312a	627	59±2.9c	4891±339b	13582±683a	1524
<i>S. triqueter</i>	6±0.9b	78±8b	2130±103a	207	61±1.9c	4590±296b	4891±319a	869
<i>V. serpyllifolia</i>	12±1.3b	216±56b	5286±431a	868	21±2c	2141±107b	8245±216a	482
<i>Z. aethiopica</i>	5±0.6c	25±2b	49±4a	8.42	62±5.5c	1884±69b	5330±214a	450

Note: Different letters within the same row and the same index (shoot and root) indicate significant difference between the three treatments (CK, Pb900, and Pb1800) at the level of $P<0.05$ level

et al. 2006). The present study clearly showed that dry weights of most of the wetland species tested were reduced in Pb treatments compared to the control, but the degrees of reduction differed significantly between plant species suggesting different intrinsic Pb tolerance between them. In addition, our data suggested that there are negative correlations between Pb concentrations in roots and shoots in Pb-treated plants and their Pb tolerance indices (Fig. 2). This indicates that the plants with higher Pb tolerance indices tend to accumulate lower concentrations of Pb in their roots and shoots when grown under Pb treatment stress.

Concentrations of Pb in shoots and roots also showed significant differences between the species in the same Pb treatment (Table 2), also demonstrating different abilities in Pb transport and accumulation. Concentrations of Pb in roots were higher than those in shoot tissues ($P<0.05$). This is due to the operation of an exclusion mechanism and so the concentrations of heavy metals in the shoots of plants are maintained at a constant low level when grown in metal-contaminated soils of variable Pb status (Baker 1981). The notably low Pb concentrations in shoots of *A. tatarinowii* and *Z. aethiopica* (49–107 mg kg⁻¹) also explains why plants grown in the 1800 mg Pb kg⁻¹ treatment did not show any visible Pb toxicity symptoms. However, even *P. scrobiculatum*, having the highest accumulation of Pb in

shoots (up to 220 mg Pb kg⁻¹) still did not show any severe symptoms of Pb toxicity. These findings indicate that internal mechanisms of Pb detoxification may also exist in some wetland plants, such as *P. scrobiculatum*, in addition to the exclusion mechanism. Baker (1981) also suggested that internal (genetically-determined) tolerance mechanisms can exist in plants when grown in heavy metal-contaminated soils.

Effects of Pb on protein contents and antioxidant enzyme activities

Metal stress can act at different cellular sites to inhibit enzymes having functional sulfhydryl groups. In the present study, both Pb treatments induced the decline in soluble protein contents and CAT activities in most of the species tested (Table 3). However, the species with higher Pb tolerance indices tended to have higher protein contents, SOD, and POD activities (Fig. 1), suggesting that there were indeed internal Pb detoxification mechanisms in the wetland species tested. Increased protein content is possibly due to the induction of stress proteins even under low metal exposure (Srivastava et al. 2005). However, in the present study, the reduction of protein contents in most species in the 900 and 1800 mg Pb kg⁻¹ treatments may be due to

Table 3 Concentrations of proteins and antioxidant enzymes in leaves of 18 species of wetland plants grown in soils spiked without (control, CK) and with Pb (900 and 1800 mg kg⁻¹ as Pb(NO₃)₂) for 4 months (mean±S.E., n=3)

Species	Protein (mg g ⁻¹)			LSD _{0.05}			SOD (U mg ⁻¹ protein)			LSD _{0.05}			POD (U mg ⁻¹ protein min ⁻¹)			LSD _{0.05}			CAT (U mg ⁻¹ protein min ⁻¹)			LSD _{0.05}		
	CK	Pb900	Pb1800	CK	Pb900	Pb1800	CK	Pb900	Pb1800	CK	Pb900	Pb1800	CK	Pb900	Pb1800	CK	Pb900	Pb1800	CK	Pb900	Pb1800	CK	Pb900	Pb1800
<i>A. tatarinowii</i>	4.40±0.18a	4.26±0.11a	4.11±0.07a	0.45	57±5b	85±4a	93±8a	20.4	308±11.3b	345±3.3a	358±5.8a	26.2	22±2.3a	24±3.7a	24±1.7a	8.66								
<i>A. cucullata</i>	3.39±0.11a	2.64±0.1b	0.78±0.06c	0.32	95±3b	136±4b	150±2a	11.0	289±14.1b	309±10b	355±6.3a	36.9	49±3.3a	40±4.1a	49±5.7a	15.5								
<i>A. philoxeroides</i>	1.99±0.13a	1.47±0.07b	0.99±0.08c	0.34	63±6b	81±4a	86±3a	15.7	153±8.2b	189±16ab	201±10a	41.0	42±3.3a	41±4.0a	39±2.3a	11.3								
<i>C. thalictroides</i>	2.44±0.18a	1.47±0.14b	0.72±0.05b	0.47	85±6b	82±9b	117±4a	22.2	120±15.8b	217±12.3a	250±17a	52.5	55±5.3a	48±3.9a	43±3.9a	15.3								
<i>C. alternifolius</i>	2.16±0.1a	1.21±0.07b	0.95±0.09c	0.31	27±3b	68±6a	55±7a	19.3	395±12.5c	448±13.5b	505±12.4a	44.3	53±6.5a	52±3.7a	45±3.4a	16.4								
<i>E. amazonicus</i>	3.57±0.19a	1.68±0.09b	0.36±0.1c	0.46	56±5b	87±4a	21±2c	13.9	412±7.2a	476±5.4a	163±20.6b	44.9	71±3.3a	61±4.3a	68±6.2a	16.6								
<i>E. osiris</i>	1.26±0.17a	0.52±0.07b	0.11±0.02c	0.37	45±5b	88±6a	9±2c	17.1	214±15.1b	389±18.6a	75±12.4c	53.8	80±2.5a	82±3.9a	77±4.6a	13.0								
<i>E. geniculata</i>	0.65±0.05a	0.35±0.04b	0.15±0.02c	0.14	36±4b	55±5a	47±5aab	15.7	176±17.4b	248±13.2a	271±13a	50.8	59±3.8a	65±6.7a	62±4.2a	17.6								
<i>F. monostachya</i>	0.37±0.03a	0.21±0.01b	0.18±0.01b	0.06	20±1b	38±3a	37±8a	16.5	249±18.6b	284±10ab	316±9.7a	46.5	41±2.6c	59±1.6a	50±2.4b	7.81								
<i>H. vulgaris</i>	2.48±0.1a	1.21±0.13b	0.24±0.04c	0.35	54±8a	75±7a	25±4b	23.4	299±12.4b	368±21.2a	92±21c	64.8	83±4.2a	83±4.1a	84±9.3a	22.0								
<i>J. linifolia</i>	0.59±0.05a	0.28±0.02b	0.08±0.02c	0.11	55±4b	97±6a	9±2c	15.2	214±19b	278±14a	57±16c	56.9	67±4.8a	72±4.9a	72±2.2a	14.4								
<i>P. repens</i>	3.22±0.12a	2.30±0.10b	0.61±0.05c	0.33	84±6a	88±6a	95±4a	19.7	446±18a	467±4a	188±7b	40.3	50±3.7a	52±5.4a	54±7.4a	19.8								
<i>P. scrobiculatum</i>	1.34±0.07a	0.75±0.08b	0.15±0.03c	0.22	52±4c	94±3a	73±5b	14.5	312±15b	371±9a	330±13ab	43.1	40±3.8a	49±5a	46±6.7a	18.3								
<i>P. lanuginosum</i>	2.30±0.11a	0.96±0.16b	—	0.53	53±5b	76±4a	—	14.9	194±13b	284±12a	—	49.1	64±6.8a	54±3.4a	—	21.2								
<i>R. rotundifolia</i>	1.14±0.13a	0.35±0.05b	0.09±0.01b	0.28	27±3ab	34±8a	11±3b	17.5	116±12b	192±10a	61±18c	47.3	48±3.9a	39±3.2ab	37±2.9b	11.6								
<i>S. triquetra</i>	1.55±0.10a	0.76±0.05b	0.46±0.04c	0.24	48±3b	59±3a	26±4c	10.9	186±16b	280±9a	293±21a	55.5	54±10.6a	63±13.4a	54±8.2a	37.8								
<i>V. serpyllifolia</i>	1.82±0.15a	0.61±0.09b	0.17±0.03c	0.36	51±4a	35±4b	22±3c	12.2	320±15a	342±22a	143±29b	78.2	58±8.4a	65±12.1a	55±5.3a	31.4								
<i>Z. aethiopica</i>	2.16±0.10a	1.84±0.12ab	1.49±0.10b	0.36	77±5b	90±5ab	102±8a	17.7	289±8b	327±23ab	369±23a	66.5	57±3.5a	58±10.2a	58±8a	26.8								

Different letters within the same row and the same index (protein, SOD, POD, and CAT) indicate significant differences between the three treatments (CK, Pb900, and Pb1800) at the levels of $P<0.05$

Fig. 1 Correlations between Pb tolerance index and protein contents (a), Superoxide dismutase (SOD) (b), peroxidase (POD) (c), and catalase CAT (d) activities of the 18 species of wetland plants grown in 900 and 1800 mg kg⁻¹ Pb treatments in pot culture

degradation by proteases as a result of high metal exposure (Romero-Puertas et al. 2002; Hou et al. 2007). Our results also showed significant negative correlations between Pb concentrations of roots and shoots in the Pb-treated plants and their protein contents (Fig. 2). Similar studies by other workers have shown that the protein content of plants is decreased by Pb accumulation (Mohan and Hosetti 1997; Singh et al. 2010), suggesting that the wetland species here with lower concentrations of Pb in their biomass tended to have higher protein contents and decreased degradation by proteases under Pb exposure.

Increased SOD activity with increased level of Pb in soils in most of the 18 species may be attributed to the increased production of active oxygen species, which results in an increase in expression of the gene encoding SOD (Bowler et al. 1992; Fatima and Ahmad 2005), suggesting that this increase provides increased protection against oxidative damage (Singh et al. 2010).

Previous studies with metal-tolerant plant species have reported that POD activity is found to be sufficiently high to enable the plants to protect themselves against oxidative stress

(Uraguchi et al. 2006; Kovacic and Klejdus 2008; Adhikari et al. 2010). In the present study, our results showed that those species with higher Pb tolerance were able to maintain higher levels of POD activity under higher Pb stress. POD also participates in lignin biosynthesis (Podazza et al. 2012), an important tolerance mechanism protecting the protoplast from toxic effects of heavy metals (Wojcik et al. 2005; Adhikari et al. 2010). It suggests that the wetland species with higher POD activity may be more efficient in avoiding damage from Pb.

For most species tested, a decline in CAT activity with increasing levels of Pb in soils may be attributed to enzyme inhibition, because Pb binds to the thiol group of this enzyme (Ouzounidou et al. 1997) or causes disruption of protein synthesis as well as direct damage to proteins (Srivastava and Dubey 2011). Some experimental data reported by other workers also suggest that the variations in POD and CAT activities under oxidative stress are contradictory: an increase, a decrease, or no change (Shaw 1995; Boscolo et al. 2003; Singh et al. 2010). This can perhaps be attributed to differences between species, plant tissues studied, or the duration and concentrations of metal exposure (Radic et al. 2010). Our data presented here, together with the previous reports,

Fig. 2 Correlations between total concentrations of Pb in roots (a, c) or shoots (b, d) and Pb tolerance index or protein contents of the 18 species of wetland plants grown in the control, 900 and 1800 mg kg⁻¹ Pb treatments in pot culture

suggest that antioxidant enzymes may have important roles in internal Pb detoxification in wetland plants.

In summary, our results showed that there are significant differences in Pb tolerance and accumulation between the wetland plants. The plants with higher Pb tolerances tended to have lower Pb accumulation but higher protein contents, SOD, and POD activities in their shoots when exposed to Pb. The results suggest that an ‘excluder’ strategy and an internal mechanism of Pb detoxification by antioxidant enzyme activities are both widely employed and play important roles in Pb tolerance in wetland plants. The existence of clear differences in the responses to Pb of the 18 species tested in this study will be helpful in the selection of species for use in constructed wetlands to limit the environmental impact of Pb contamination in effluents and drainage waters.

Acknowledgments This research was financially supported by the National Natural Science Foundation of China (No. 30570345, 41201312), Specialized Research Fund for the Doctoral Program of Higher Education of China (20558097), and Key Laboratory of Biodiversity Dynamics and Conservation of Guangdong Higher Education Institutes, Sun Yat-sen University. We thank Prof AJM Baker (Universities of Melbourne and Queensland, Australia, and Sheffield, UK) for his help in the early stages of the preparation on this paper.

References

- Adhikari T, Kumar A, Singh MV, Rao AS (2010) Phytoaccumulation of lead by selected wetland plant species. *Commun Soil Sci Pl Anal* 41: 2623–2632
- Aebi H (1984) Catalase in vitro. *Methods Enzymol* 105:121–126
- Alexander PD, Alloway BJ, Dourado AM (2006) Genotypic variations in the accumulation of Cd, Cu, Pb, and Zn exhibited by six common grown vegetables. *Environ Pollut* 144:736–745
- Baker AJM (1981) Accumulators and excluders-strategies in the response of plants to heavy metals. *J Plant Nutr* 3:643–654
- Baker AJM, Walker PL (1990) Ecophysiology of metal uptake by tolerant plants. In: Shaw AJ (ed) *Heavy metal tolerance in plants: Evolutionary aspects*. CRC Press, Boca Raton, pp 155–177
- Blokhina O, Virolainen E, Fagerstedt KV (2003) Antioxidants, oxidative damage and oxygen deprivation stress: a review. *Ann Bot* 91:179–194
- Boscolo PRS, Menossi M, Jorge RA (2003) Aluminum-induced oxidative stress in maize. *Phytochemistry* 62:181–189
- Bowler C, Vanmontagu M, Inze D (1992) Superoxide-dismutase and stress tolerance. *Annu Rev Plant Physiol Plant Mol Biol* 43:83–116
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248
- Chance B, Maehly A (1955) Assay of catalases and peroxidases. *Methods Enzymol* 2:764–775
- Chaoui A, Mazhoudi S, Ghorbal MH, El Ferjani E (1997) Cadmium and zinc induction of lipid peroxidation and effects on antioxidant enzyme activities in bean (*Phaseolus vulgaris* L.). *Plant Sci* 127: 139–147

- Del Rio LA, Corpas FJ, Sandalio LM, Palma JM, Gomez M, Barroso JB (2002) Reactive oxygen species, antioxidant systems and nitric oxide in peroxisomes. *J Exp Bot* 53:1255–1272
- Deng H, Ye ZH, Wong MH (2004) Accumulation of lead, zinc, copper and cadmium by 12 wetland plant species thriving in metal-contaminated sites in China. *Environ Pollut* 132:29–40
- Deng H, Ye ZH, Wong MH (2006) Lead and zinc accumulation and tolerance in populations of six wetland plants. *Environ Pollut* 141:69–80
- Ederli L, Reale L, Ferranti F, Pasqualini S (2004) Responses induced by high concentration of cadmium in *Phragmites australis* roots. *Physiol Plantarum* 121:66–74
- Fatima RA, Ahmad M (2005) Certain antioxidant enzymes of *Allium cepa* as biomarkers for the detection of toxic heavy metals in wastewater. *Sci Total Environ* 346:256–273
- Fediuc E, Erdei L (2002) Physiological and biochemical aspects of cadmium toxicity and protective mechanisms induced in *Phragmites australis* and *Typha latifolia*. *J Plant Physiol* 159:265–271
- Giannopolitis CN, Ries SK (1977) Superoxide dismutases I. Occurrence in higher plants. *Plant Physiol* 59:309–314
- Guo TR, Zhang GP, Zhou MX, Wu FB, Chen JX (2004) Effects of aluminum and cadmium toxicity on growth and antioxidant enzyme activities of two barley genotypes with different Al resistance. *Plant Soil* 258:241–248
- Han YL, Huang SZ, Gu JG, Qiu S, Chen JM (2008) Tolerance and accumulation of lead by species of *Iris* L. *Ecotoxicology* 17:853–859
- Hou WH, Chen X, Song GL, Wang QH, Chang CC (2007) Effects of copper and cadmium on heavy metal polluted water body restoration by duckweed (*Lemna minor*). *Plant Physiol Biochem* 45:62–69
- Kovacik J, Klejdus B (2008) Dynamics of phenolic acids and lignin accumulation in metal-treated *Matricaria chamomilla* roots. *Plant Cell Rep* 27:605–615
- Li PJ, Wang X, Wang MX, Allinson G, Li XJ, Xiong XZ (2009) Risk assessment of heavy metals in soil previously irrigated with industrial wastewater in Shenyang, China. *J Hazard Mater* 161:516–521
- Li B, Wang X, Qi XL, Huang L, Ye ZH (2012) Identification of rice cultivars with low brown rice mixed cadmium and lead contents and their interactions with the micronutrients iron, zinc, nickel and manganese. *J Environ Sci* 24(10):1790–1798
- Liu JG, Ma XM, Wang MX, Sun XW (2013) Genotypic differences among rice cultivars in lead accumulation and translocation and the relation with grain Pb levels. *Ecotox Environ Safe* 90:35–40
- Mohan BS, Hosetti BB (1997) Potential phytotoxicity of leaf and cadmium to *Lemna minor* grown in sewage stabilization ponds. *Environ Pollut* 98:233–238
- Najeeb U, Jilani G, Ali S, Sarwar M, Xu L, Zhou WJ (2011) Insights into cadmium induced physiological and ultra-structural disorders in *Juncus effusus* L. and its remediation through exogenous citric acid. *J Hazard Mater* 186:565–574
- Ouzounidou G, Moustakas M, Eleftheriou EP (1997) Physiological and ultrastructural effects of cadmium on wheat (*Triticum aestivum* L.) leaves. *Arch Environ Con Tox* 32:154–160
- Podazza G, Arias M, Prado FE (2012) Cadmium accumulation and strategies to avoid its toxicity in roots of the citrus rootstock Citrumelo. *J Hazard Mater* 215:83–89
- Radic S, Babic M, Skobic D, Roje V, Pevalek-Kozlina B (2010) Ecotoxicological effects of aluminum and zinc on growth and antioxidants in *Lemna minor* L. *Ecotox Environ Safe* 73:336–342
- Romero-Puertas MC, Palma JM, Del Rio A, Sandalio LM (2002) Cadmium causes the oxidative modification of proteins in pea plants. *Plant Cell Environ* 25:677–686
- Sharma RK, Agrawal M, Marshall F (2007) Heavy metal contamination of soil and vegetables in suburban areas of Varanasi, India. *Ecotox Environ Safe* 66:258–266
- Shaw BP (1995) Effects of mercury and cadmium on the activities of antioxidative enzymes in the seedling of *Phaseolus aureus*. *Biol Plantarum* 37:587–596
- Sheoran AS, Sheoran V (2006) Heavy metal removal mechanism of acid mine drainage in wetlands: a critical review. *Miner Eng* 19:105–116
- Shu X, Yin LY, Zhang QF, Wang WB (2012) Effect of Pb toxicity on leaf growth, antioxidant enzyme activities, and photosynthesis in cuttings and seedlings of *Jatropha curcas* L. *Environ Sci Pollut Res* 19: 893–902
- Singh KP, Mohon D, Sinha S, Dalwani R (2004) Impact assessment of treated/untreated wastewater toxicants discharge by sewage treatment plants on health, agricultural, and environmental quality in wastewater disposal area. *Chemos* 55:227–255
- Singh R, Tripathi RD, Dwivedi S, Kumar A, Trivedi PK, Chakrabarty (2010) Lead bioaccumulation potential of an aquatic macrophyte *Najas indica* are related to antioxidant system. *Bioresource Technol* 101:3025–3032
- Srivastava S, Dubey RS (2011) Manganese-excess induces oxidative stress, lowers the pool of antioxidants and elevates activities of key antioxidative enzymes in rice seedlings. *Plant Growth Regul* 64:1–16
- Srivastava M, Ma LQ, Singh N, Singh S (2005) Antioxidant responses of hyper-accumulator and sensitive fern species to arsenic. *J Exp Bot* 56:1335–1342
- Uraguchi S, Watanabe I, Yoshitomi A, Kiyono M, Kuno K (2006) Characteristics of cadmium accumulation and tolerance in novel Cd-accumulating crops, *Avena strigosa* and *Crotalaria juncea*. *J Exp Bot* 57:2955–2965
- Vymazal J (2011) Plant used in constructed wetlands with horizontal subsurface flow: a review. *Hydrobiologia* 674:133–156
- Wilkins DA (1978) The measurement of tolerance to edaphic factors by means of root growth. *New Phytol* 80:623–633
- Wojcik M, Vangronsveld J, D’Haen J, Tukiendorf A (2005) Cadmium tolerance in *Thlaspi caerulescens* - II. Localization of cadmium in *Thlaspi caerulescens*. *Environ Exp Bot* 53:163–171
- Wright DJ, Otte ML (1999) Wetland plant effects on the biogeochemistry of metals beyond the rhizosphere. *Biol Environ: Proceeding of the Royal Irish Academy* 99:3–10
- Yang JX, Tam NFY and Ye ZH (2014). Root porosity, radial oxygen loss and iron plaque of wetland plants in relation to zinc tolerance and accumulation. *Plant Soil* 374:815–828
- Ye ZH, Baker AJM, Wong MH, Willis AJ (1997a) Zinc, lead and cadmium tolerance, uptake and accumulation by *Typha latifolia*. *New Phytol* 136:469–480
- Ye ZH, Baker AJM, Wong MH, Willis AJ (1997b) Zinc, lead and cadmium tolerance, uptake and accumulation by the common reed, *Phragmites australis* (Cav.) Trin. ex Steudel. *Ann Bot* 80:363–370
- Ye ZH, Whiting SN, Lin ZQ, Lytle CM, Qian JH, Terry N (2001) Removal and distribution of iron, manganese, cobalt and nickel within a Pennsylvania constructed wetland treating coal combustion by-product leachate. *J Environ Qual* 30:1464–1473
- Ye ZH, Wong MH, Lan CY (2004) Use of a wetland system for treating Pb/Zn mine effluent: a case study in southern China from 1984 to 2002. In: Wong MH (ed) *Wetland ecosystems in Asia: Function and management*. Elsevier, Amsterdam, pp 413–434
- Zhang ZW, Takao W, Shinichiro S, Higashikawa K, Ikeda M (1998) Pb and cadmium contents in cereals and pulses in north-eastern China. *Sci Tot Environ* 220:137–145
- Zhou YQ, Huang SZ, Yu SL, Gu JG, Zhao JZ, Han YL, Fu JJ (2010) The physiological response and sub-cellular location of lead and cadmium in *Iris pseudacorus*. *Ecotoxicology* 19:69–76
- Zhuang P, McBride MB, Xia HP, Li NY, Li ZA (2009) Health risk from heavy metals via consumption of food crops in the vicinity of Dabaoshan mine, South China. *Sci Total Environ* 407:1551–1561