Arch Environ Contam Toxicol (2010) 58:594–604
DOI 10.1007/s00244-009-9408-6

Changes in Growth, Biochemical Components, and Antioxidant Activity in Aquatic Plant *Wolffia arrhiza* (Lemnaceae) Exposed to Cadmium and Lead

Alicja Piotrowska · Andrzej Bajguz · Beata Godlewska-Żyłkiewicz · Elżbieta Zambrzycka

Received: 21 May 2009 / Accepted: 28 September 2009 / Published online: 16 October 2009

Abstract The present study investigated the biochemical response of aquatic plant *Wolffia arrhiza* (Lemnaceae) treated with lead (Pb) and cadmium (Cd) at a range of concentrations from 1 to 1000 μM. *W. arrhiza* has been identified as good scavenger of heavy metals from aqueous solution. Pb and Cd accumulation was found to be increased in a concentration- and duration-dependent manner. However, the highest biosorption of heavy metals was found in plants exposed to low levels (10 μM) of Cd and Pb in the nutrient medium. In observing the response to heavy-metal stress, we noted inhibited plant growth and decreased photosynthetic pigments, monosaccharides, and proteins. In addition, Cd was found to be more toxic to plants than Pb. Heavy metals also induced oxidative damage as evidenced by increased lipid peroxidation and hydrogen peroxide levels. In contrast, the deleterious effects resulting from the cellular oxidative state can be alleviated by enzymatic (catalase, ascorbate peroxidase, nicotinamide dinucleotide [NADH] peroxidase) and nonenzymatic (ascorbate, glutathione) antioxidant mechanisms activated in *W. arrhiza* plants exposed to Cd and Pb, especially at 10 μM. These results suggest that *W. arrhiza* is a promising bioindicator of heavy-metal toxicity.

Duckweed (Lemnaceae) has been reported to be a potential scavenger of heavy metals from aquatic environments and is being used in wastewater renovation systems (Prasad et al. 2001; Artetxe et al. 2002; Hou et al. 2007). *Wolffia arrhiza* from the Lemnaceae family is one of the smallest vascular plants, which seldom exceed 1 mm in size. The plant has neither leaves nor a stem and even lacks roots. The whole plant, called a "frond," may set flowers and seeds, although rapid multiplication is achieved by budding (Landolt 1986). *W. arrhiza* is also characterized by mixotrophic feeding, high rate of absorption of nutrients from polluted water, quick growth, fast multiplication, and resistance to numerous toxins (Fujita et al. 1999). In particular, *W. arrhiza* is reported to accumulate xenobiotics (Mical and Krotke 1999) and therefore is being used as experimental model system to investigate heavy-metal-induced responses.

Cadmium (Cd) and lead (Pb) are strongly phytotoxic partly because of the generation of reactive oxygen species (ROS), which react with lipids, proteins, photosynthetic pigments, and nucleic acids, causing lipid peroxidation, membrane damage, metabolite degradation, inactivation of enzymes, and cell death (Heath and Packer 1968; Hegedüs et al. 2001). In contrast, plants have evolved enzymatic (catalase, ascorbate peroxidase, nicotinamide dinucleotide [NADH] peroxidase) and nonenzymatic (ascorbate, glutathione) antioxidant mechanisms to prevent oxidative stress (Nakano and Asada 1981; Aeby 1984; Ishida et al. 1987; Xiang and Oliver 1998). Therefore, we studied the involvement of the antioxidant system in the biochemical detoxification of Cd and Pb in *W. arrhiza* culture in detail. The results of our study also demonstrate the effect of heavy-metal biosorption on the growth, lipid peroxidation, hydrogen peroxide level, and biochemical composition (proteins, monosaccharides, photosynthetic pigments) of this plant. The results of this study will be helpful in understanding the biochemical detoxification strategies that

A. Piotrowska (✉) · A. Bajguz
Department of Plant Biochemistry and Toxicology, Institute of Biology, University of Bialystok, Swierkowa 20 B, 15-950 Bialystok, Poland
e-mail: alicjap@uwb.edu.pl

B. Godlewska-Żyłkiewicz · E. Zambrzycka
Department of Analytic Chemistry, Institute of Chemistry, University of Bialystok, Hurtowa 1, 15-399 Bialystok, Poland

aquatic plant *W. arrhiza* adopts against stress induced by exposure to heavy metals.

Materials and Methods

Plant Material, Growth Conditions, and Treatment

Fronds of *W. arrhiza* (L.) Hork. ex. Wimm. (Lemnaceae) were grown in small sterile, plastic vessels (Phytatray, Sigma-Aldrich Co., USA) containing 100 mL culture solution under controlled conditions at 25°C ± 0.5°C, with a day-to-night cycle of 16:8 h (photon flux 50 μmol m^{-2} s^{-1}). A 1/50 dilution of Hutner's medium (Hutner 1953), pH 7.0, was used in this study. The plants of *W. arrhiza* (0.5 ± 0.005 g fresh weight) was treated with four different concentrations of Cd and Pb (1, 10, 100, 1000 μM) on day 1 of culture. Heavy-metal doses used in this work are environmentally relevant and were chosen appropriately to expose the plants to low to moderate levels of Cd and Pb. The different concentrations of Cd and Pb were prepared by diluting $Cd(NO_3)_2$ and $Pb(NO_3)_2$ (Sigma-Aldrich Co., USA) in sterile 1/50 Hutner's solution. Plants were harvested at days 7 and 14, at the middle, and at the end of the period of the plant developmental cycle. Biomass was used for growth estimation and determination of the biochemical parameters.

Heavy-Metal Determination

A Solaar M6 (Thermo Electron Corporation, UK) atomic absorption spectrometer with deuterium background correction system was used for Pb and Cd determination in biomass of *W. arrhiza* and in the medium. The absorbances of Pb and Cd were measured in air-acetylene flame with 0.5 nm spectral bandpass at $\lambda = 217.0$ nm and $\lambda = 228.8$ nm, respectively. The Pb and Cd hollow cathode lamps (CPI International, USA) were operated at 5 and 8 mA, respectively. A stock solution (0.1 mol L^{-1}) of Pb(II) and Cd were prepared by dissolving $Pb(NO_3)_2$ and $Cd(NO_3)_2$ (Sigma-Aldrich Co., USA) in 2 mL 2 mol L^{-1} HNO_3 and diluted with Milli-Q water. Standard solutions were prepared from stock solutions daily.

For metal determination in biomass, fronds were dried at 70°C for 12 h and ashed in a muffle furnace at 500°C ± 50°C for 6 h. For measurement, the ash was dissolved in 2 mL HNO_3 (65%; Sigma-Aldrich Co., USA). Moreover, the concentration of heavy metals in the medium was measured during 2 weeks of the culture. The calculated quantitation limit (LOQ) for Cd dissolved in nitric acid was 0.0273 mg L^{-1}, and the detection limit (LOD) was 0.00187 mg L^{-1}. LOQ = 0.0487 and LOD = 0.00609 mg L^{-1} were estimated for Cd present in the medium. The calculated LOQ for Pb dissolved in nitric acid was 0.228 mg L^{-1}, and the LOD was 0.0608 mg L^{-1}. The calculated LOQ and LOD for Pb in the medium were 0.176 and 0.039 mg L^{-1}, respectively.

Determination of Growth, Proteins, Monosaccharides, and Photosynthetic Pigments

For fresh-weight determination, the plants were filtered, washed three times with distilled water, kept on filter article for a few minutes to remove excess liquid, and then weighed. For chlorophyll *a* and carotenoid determination, the cultures were first collected by filtration, and then the pellets (0.1 g) were homogenized in methanol. The absorbance of the extract was measured at 652.4 and 665.2 nm for chlorophyll *a* and at 470.0 nm for carotenoids. The amounts of photosynthetic pigments present in the methanol extract were calculated according to the equations of Wellburn (1994). For sugar determination, the cultures were first collected by filtration, and then the pellets (0.1 g) were assessed using the Somogyi (1954) method. The measurement of the protein content was performed by the homogenization of biomass. The homogenate was centrifuged for 10 min at 12,000*g*, and an aliquot of the extract was used to determine protein content according to the Bradford (1976) method, using bovine serum albumin as standard.

Stress-Marker Determination

The level of hydrogen peroxide in *W. arrhiza* fronds was measured spectrophotometrically at 390 nm by reaction with potassium iodide (KI). The results were calculated using a standard curve prepared with fresh hydrogen peroxide solutions (Aliexieva et al. 2001).

Lipid peroxidation was estimated by measuring the formation of malondialdehyde (MDA) with thiobarbituric acid (TBA) reaction as described by Heath and Packer (1968). Filtered biomass (0.5 g) was homogenized in 0.1% trichloroacetic acid (TCA) and centrifuged at 10,000*g* for 5 min. Reaction mixture containing supernatant, 20% TCA, and 0.5% TBA was heated at 95°C for 30 min and then quickly cooled on ice. After centrifugation at 10,000*g* for 10 min, the absorbance at 532 nm was read, and the value for the nonspecific absorption at 600 nm was subtracted.

Estimation of Antioxidants

Extraction and determination of total ascorbate was carried out according to the method of Kampfenkel et al. (1995). Plant material (1 g) was harvested by filtration and quickly homogenized in liquid N_2 and thereafter extracted with 5% (w/v) TCA. The homogenate was centrifuged for 5 min at 15,600*g* (4°C). The supernatant was transferred to a new

reaction vessel and immediately assayed for the ascorbate content in a reaction mixture containing supernatant, 10 mM dithiothreitol, 0.2 M phosphate buffer (pH 7.4), 0.5% *N*-ethylmaleimide, 10% TCA, 42% H_3PO_4, 4% 2,2′-dipyridyl, and 3% $FeCl_3$. Glutathione extraction was performed by the method of De Kok et al. (1986). The content of glutathione was measured using a glutathione assay kit (Sigma-Aldrich Co., USA).

Extraction of Antioxidant Enzymes and Estimation of Their Activities

Enzymatic extracts were obtained from *W. arrhiza* (1 g fresh weight). The biomass was filtered and then homogenized in liquid N_2 and thereafter in 0.05 M phosphate buffer (pH 7.0) containing 0.1 M ethylenediaminetetraacetic acid and 1% polyvinylpyrrolidone at 4°C. The homogenate was centrifuged for 10 min at 15,000*g* (4°C), and the supernatant was dialyzed overnight in phosphate buffer. Estimation of the activity of the selected enzymes was performed as follows.

Catalase (EC 1.11.1.6) activity was measured spectrophotometrically as the rate of H_2O_2 decomposition at 240 nm (Aeby 1984). The reaction mixture consisted of 0.05 M phosphate buffer, 0.1 mM H_2O_2, and supernatant. One unit of catalase activity was assumed as the amount of enzyme that decomposed 1 μmol of H_2O_2/mg soluble protein/min at 30°C.

Total ascorbate peroxidase (EC 1.11.1.11) was determined according to the method of Nakano and Asada (1981). The reaction mixture consisted of 0.05 M phosphate buffer, 5 mM sodium ascorbate, 0.1 mM H_2O_2, and supernatant. Total ascorbate peroxidase activity was determined as the decrease in absorbance of ascorbate at 290 nm. The enzyme activity was calculated as the amount of the enzyme that oxidizes 1 μmol of ascorbate consumed/mg soluble protein/min at 30°C.

NADH peroxidase (EC 1.11.1.1) activity was determined according to Ishida et al. (1987). The reaction mixture consisted of 50 mM pH 6.0 sodium acetate buffer and 0.2 mM NADH. The reaction was initiated by adding the enzymatic extract and lasted for up to 5 min. The peroxidase activators *p*-cumaric acid and 5 nM $MnCl_2$ were used. One unit of NADH peroxidase activity was assumed to be the amount of the enzyme that oxidizes 1 μmol NADH/mg soluble protein/min at 30°C.

Replication and Statistical Analysis

Each treatment consisted of four replicates, and each experiment was carried out at least twice at different times. The data were analyzed by one-way analyses of variance, and the means were separated using Duncan's multiple-range test (Statistica 6, StatSoft Co., USA). The level of significance in all comparisons was $p < 0.05$.

Results

Heavy-Metal Uptake

W. arrhiza accumulated heavy metals in a dose- and exposure-dependent manner (Table 1). Plants exposed to 1000 μM Pb and Cd contained the highest amounts of these metals (0.549 mg Pb g^{-1} [fresh weight] and 0.107 mg Cd g^{-1} [fresh weight]) after 2 weeks of culture. Obtained data indicated that Pb, in the observed concentration ranges of 1 to 1000 μM, was accumulated more effectively compared with Cd. At the same time, the contents of both heavy metals decreased in the nutrient medium (Table 2). The highest biosorption of both metal ions was observed in plants exposed to 10 μM Cd and Pb. Decreased heavy-metal concentrations in the medium indicated that *W. arrhiza* is able to remove 64.6 and 36.0% amounts of Pb and Cd, respectively, from low-level polluted water during 2 weeks of culture.

The Effects of Heavy Metals on Plant Growth

The biomass of *W. arrhiza* decreased proportionally with increasing Pb and Cd doses, and the most phytotoxic effect of heavy metals was observed at a concentration of

Table 1 Accumulation of Cd and Pb (mg g^{-1} fresh weight) in *W. arrhiza* fronds at different concentrations on days 7 and 14 of culture[a]

Heavy metal	Day of culture	Heavy-metal concentration (μM)				
		0	1	10	100	1000
Cd	7	0	0.004 ± 0.001[a]	0.038 ± 0.008[b]	0.055 ± 0.004[b]	0.083 ± 0.012[b]
	14	0	0.004 ± 0.005[a]	0.046 ± 0.004[b]	0.076 ± 0.009[b]	0.107 ± 0.010[c]
Pb	7	0	0.002 ± 0.009[a]	0.152 ± 0.025[c]	0.198 ± 0.038[c]	0.269 ± 0.093[d]
	14	0	0.002 ± 0.004[a]	0.138 ± 0.081[c]	0.427 ± 0.062[e]	0.549 ± 0.031[f]

[a] Cd and Pb levels at the beginning of the experiment were 0 mg g^{-1} fresh weight. Data are the means of three independent experiments ± SD. Treatments with at least one letter the same are not significantly different according to Duncan's test

Table 2 The initial and final contents of Cd and Pb (mg 100 mL^{-1}) in the medium in which *W. arrhiza* fronds grew[a]

Heavy metal	Day of culture	Heavy-metal concentration (μM)				
		0	1	10	100	1000
Cd	0	0	0.014 ± 0.003[a]	0.139 ± 0.009[b]	1.399 ± 0.013[b]	13.995 ± 0.014[c]
	7	0	0.013 ± 0.003[a]	0.111 ± 0.017[b]	1.339 ± 0.055[b]	13.762 ± 0.828[c]
	14	0	0.009 ± 0.001[a]	0.050 ± 0.004[a]	1.196 ± 0.049[b]	13.844 ± 0.607[c]
Pb	0	0	0.021 ± 0.006[a]	0.212 ± 0.007[b]	2.072 ± 0.044[b]	20.720 ± 0.019[d]
	7	0	0.020 ± 0.002[a]	0.088 ± 0.008[a]	1.692 ± 0.088[b]	20.501 ± 0.825[d]
	14	0	0.019 ± 0.005[a]	0.075 ± 0.004[a]	1.145 ± 0.069[b]	19.942 ± 0.640[d]

[a] Data are the means of three independent experiments ± SD. Treatments with at least one letter the same are not significantly different according to Duncan's test

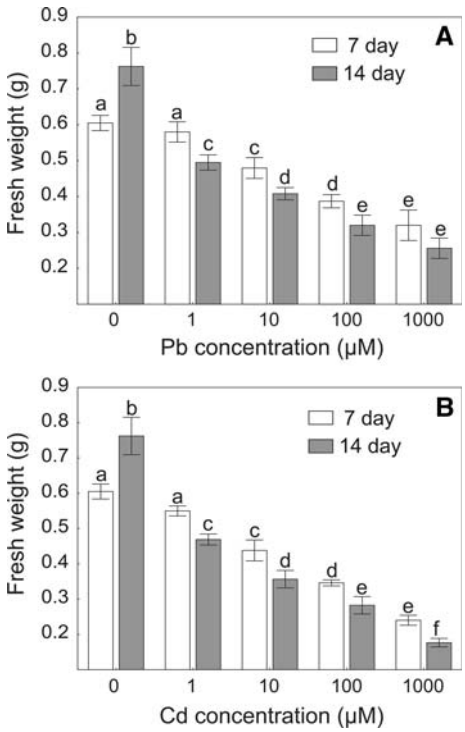

Fig. 1 Growth of *W. arrhiza* culture under the influence of Pb (**a**) and Cd (**b**) at a range of 1 to 1000 μM. Data are the means of four independent experiments ± SD. Treatments with at least one letter the same are not significantly different according to Duncan's test

1000 μM (Fig. 1a, b). However, the toxicities of Cd and Pb in *W. arrhiza* differed remarkably. Fresh weight was more intensively decreased in the presence of Cd in relation to Pb-treated cultures because 1000 μM Pb caused a 47.2% to 67.2% decrease in biomass, whereas 1000 μM Cd induced a 60.3% to 76.9% decrease in biomass.

The Effects of Heavy Metals on Photosynthetic Pigments

W. arrhiza fronds treated with Cd and Pb displayed chlorosis because a significant loss in chlorophyll *a* content was observed during 2 weeks of culture (Fig. 2a, b). The results showed also that Cd inhibited chlorophyll *a* content faster and stronger than did Pb. Therefore, the maximum decrease in chlorophyll *a* (75.4% and 72.7%) was obtained after application of 1000 μM Cd and Pb, respectively, on day 14 of cultivation. Heavy metals at lower concentrations did little harm to this chloroplast pigment in *W. arrhiza* plants.

In contrast, carotenoids were less sensitive than chlorophyll *a* toward both Pb and Cd, probably protecting the photosynthetic apparatus against heavy-metal stress (Fig. 2c, d). The external supply of both 1000 μM Pb and Cd (induced at 42.1 and 45.5%, respectively) decreased in content by day 14 of the experiment. By contrast, heavy metals at lower doses (1 μM) were characterized by a stimulating effect of 7.4 to 9.7% on carotenoid content compared with the control.

The Effects of Heavy Metals on Monosaccharides

Heavy metals were characterized by an inhibitory influence on monosaccharide content in a concentration-dependent manner (Fig. 2e, f). Therefore, the significant decrease (by 56.8% and 67.7%, respectively) in monosaccharide level noted on days 7 and 14 of *W. arrhiza* cultivation was obtained at the highest dose (1000 μM) of Cd. The application of Pb at 1000 μM was characterized by a lower toxic effect on this parameter because 49.5 and 61.4% inhibition in monosaccharide accumulation was observed on days 7 and 14 of culture, respectively. Both Cd and Pb, at a range of concentrations from 1 to 100 μM, possessed less destructive influence on the sugar level in fronds.

The Effects of Heavy Metals on Proteins

The protein content in *W. arrhiza* fronds decreased proportionally with increased Cd concentration at all of the exposure periods, and the maximum decreases in this biochemical parameter were recorded as 53.3% and 74.1% on days 7 and 14 of cultivation, respectively, under the

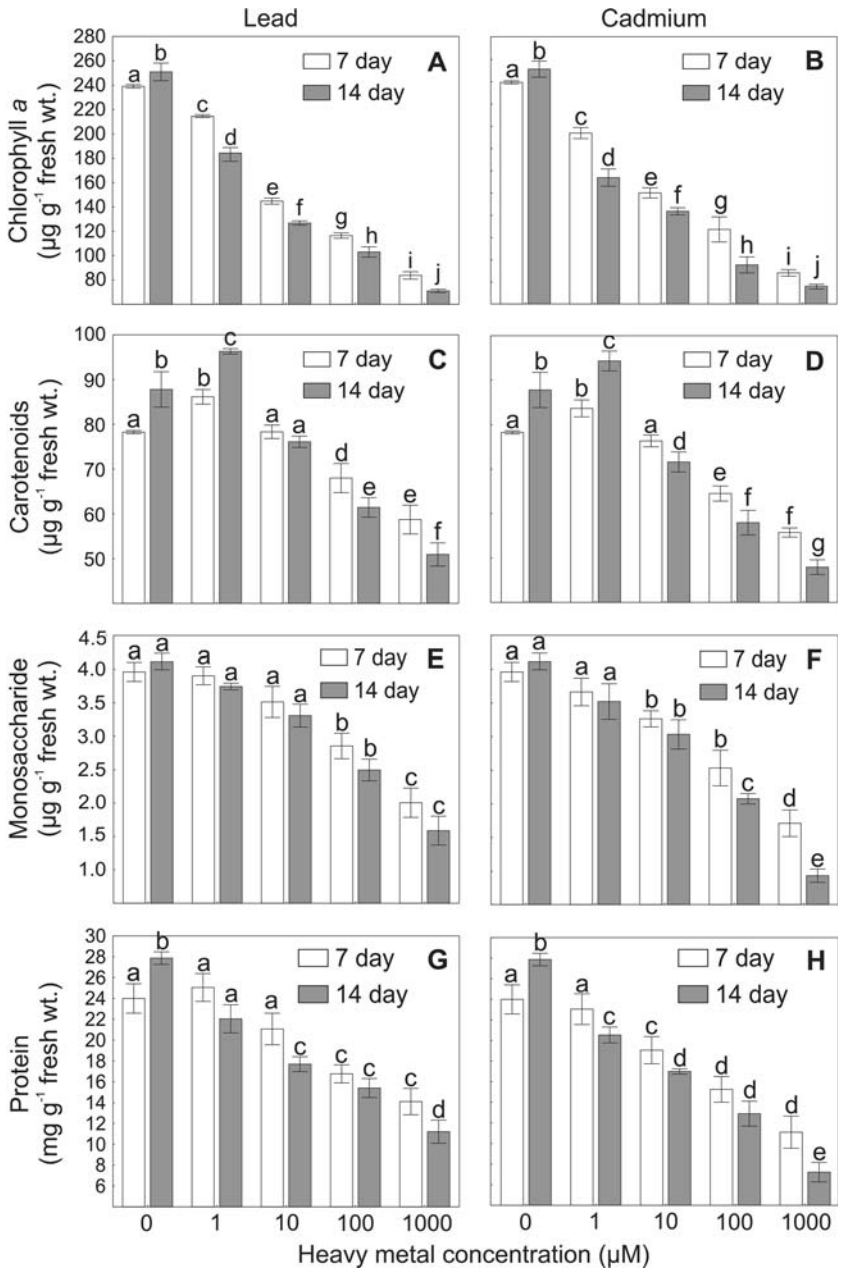

Fig. 2 The effects of Pb and Cd at a range of 1 to 1000 μM on chlorophyll *a* (**a**, **b**), carotenoid (**c**, **d**), monosaccharide (**e**, **f**), and protein (**g**, **h**) content in *W. arrhiza*. Data are the means of four independent experiments ± SD. Treatments with at least one letter the same are not significantly different according to Duncan's test

influence of 1000 μM Cd. In contrast, Pb provoked a weaker response because a 41.3% to 59.7% decrease in protein level was noted (Fig. 2g, h).

The Effects of Heavy Metals on H_2O_2 and Lipid Peroxidation

Compared with unstressed plants, a significant increase in hydrogen peroxide content was observed in the presence of Cd and Pb in *W. arrhiza* culture (Fig. 3a, b). H_2O_2 production was proportional with heavy-metal concentration. For example, increases in hydrogen peroxide level of 130.1% and 125.6%, respectively, were observed in plants treated with 1000 μM Cd on days 7 and 14. Pb at the same dose provoked a weaker response because fronds treated with 1000 μM Pb contained higher amounts (by 89% to 96%) of H_2O_2.

MDA content, a phytotoxic product of lipid peroxidation, increased gradually in proportion to increased concentrations of heavy metals (Fig. 3c, d). The highest stimulation by MDA level (105.9% and 76.1%, respectively) was recorded under the influence of 1000 μM Cd on days 7 and 14 of *W. arrhiza* culture. The application of Pb ions at 1000 μM provoked a weaker response, leading to a 63.1% to 86.9% increase in MDA accumulation during 2 weeks of the experiment.

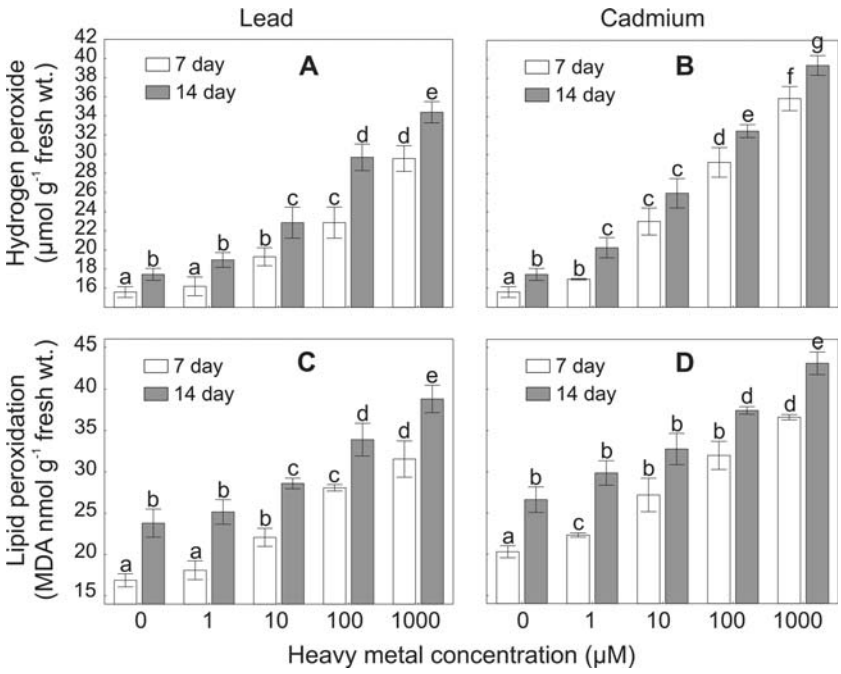

Fig. 3 The effects of Pb and Cd at a range of 1 to 1000 μM on hydrogen peroxide content (**a**, **b**) and lipid peroxidation (**c**, **d**) in *W. arrhiza*. Data are the means of four independent experiments ± SD. Treatment with at least one letter the same are not significantly different according to Duncan's test

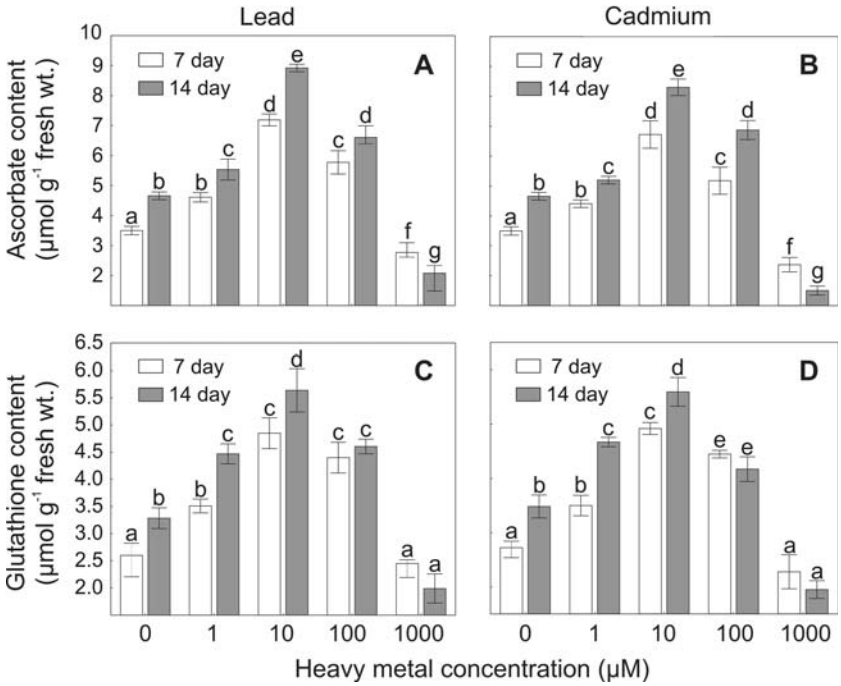

Fig. 4 The effect of Pb and Cd at range of 1 to 1000 μM on ascorbate (**a**, **b**) and glutathione (**c**, **d**) content in *W. arrhiza*. Data are the means of four independent experiments ± SD. Treatment with at least one letter the same are not significantly different according to Duncan's test

The Effects of Heavy Metals on Nonenzymatic Antioxidants

The highest enhancement in the ascorbate content (by 105.4%) was observed in fronds treated with 10 μM Pb on day 7 of cultivation. In contrast, Cd at 10 μM possessed a weaker effect on the ascorbate level because the content of this antioxidant increased by 92.0% on the day 7 of the experiment (Fig. 4a, b). In contrast, the inhibition of ascorbate accumulation (by 21–68%, respectively) was observed in fronds in the presence of Cd and Pb at 1000 μM.

Similarly, Pb at 10 μM was characterized by the highest stimulating influence (by 86.5%) on the glutathione level on day 7 of culture. Cd application at 10 μM resulted in a lower increase in glutathione level (by 57.9% to 76.1%) compared with the control (Fig. 4c, d). However, the 16–43% decrease in glutathione content was found in fronds treated with 1000 μM Pb and Cd, respectively.

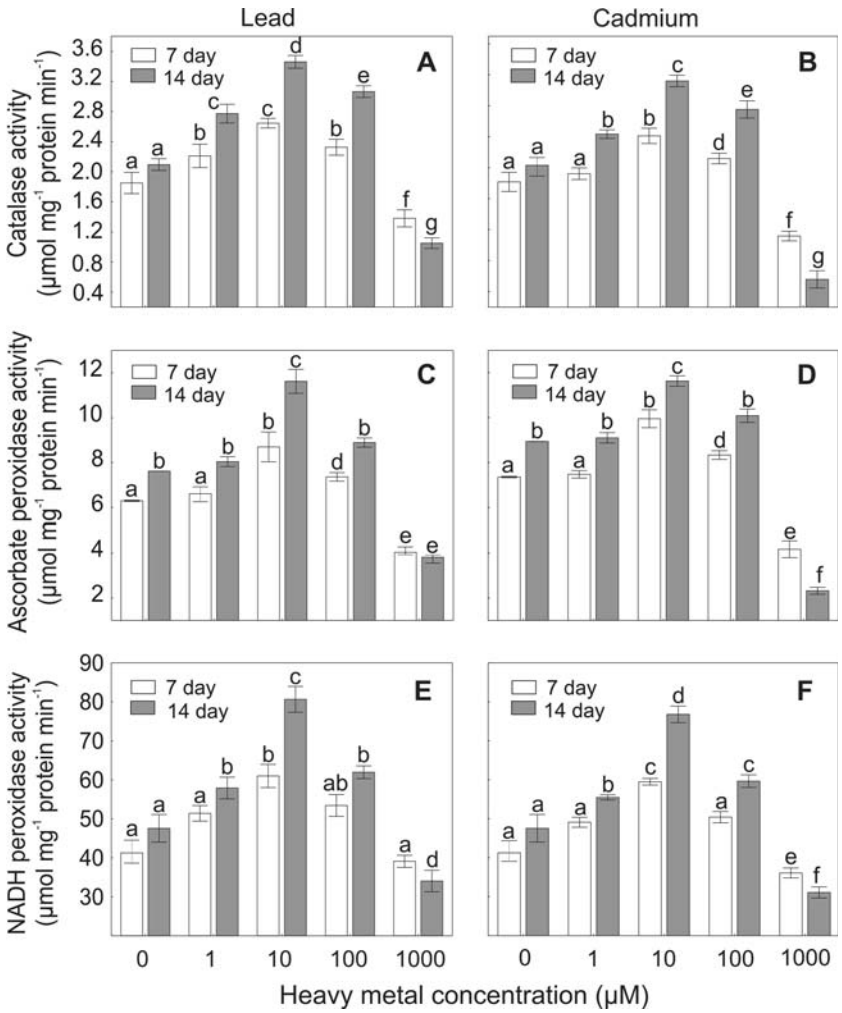

Fig. 5 The effects of Pb and Cd at range of 1 to 1000 μM on the activities of catalase (**a**, **b**), ascorbate peroxidase (**c**, **d**), and NADH peroxidase (**e**, **f**). Data are the means of four independent experiments ± SD. Treatment with at least one letter the same are not significantly different according to Duncan's test

The Effects of Heavy Metals on Antioxidant Enzymes

Apart from the effect of Cd and Pb on nonenzymatic antioxidants, heavy metals affected the activity of enzymes involved in H_2O_2 metabolism (Fig. 5a–f). It was found that the presence of 10 μM Cd and 10 μM Pb in the nutrient solution resulted in the highest catalase and peroxidases activities in *W. arrhiza* culture. In fronds treated with 10 μM Pb during 2 weeks of culture, ascorbate peroxidase activity increased by 38.1% to 52.5%; catalase activity increased by 42.7% to 65.5%; and NADH peroxidase activity increased by 47.8% to 69.8%. Our data indicate that Cd was characterized by a lower stimulating influence on the antioxidant enzymes activities involved in ROS scavenging in *W. arrhiza* fronds. Exogenously applied Cd (at 10 μM) stimulated ascorbate peroxidase activity by 34.3 and 29.7% on days 7 and 14t of culture, respectively. The significant 22.2 to 25.4% increase in NADH peroxidase activity was also observed when plants were treated with Cd at 10 μM during 2 weeks of the experiment. By analogy, catalase activity increased by 36.7% to 59.1% in response to exogenous 10 μM Cd. Both heavy metals at the highest concentration (1000 μM) inhibited the activity of all enzymes, which was probably caused by the harmful effect of H_2O_2 overproduction or its poisonous ROS derivatives.

Discussion

The results we obtained indicated that the biosorption of heavy metals by plants is accompanied by an induction of a variety of cellular changes, some of which directly contribute to the metal-tolerance capacity of the plant. The aquatic plant *W. arrhiza* accumulated heavy metals in a dose- and exposure-dependent manner. This is in agreement with earlier reports on aquatic plants, such as *Chlorella vulgaris*, *Nasturtium officinale*, *Mentha aquatica*, and *Bacopa monnieri* (Aslan et al. 2003; Bajguz and Godlewska-Żyłkiewicz 2004; Singh et al. 2006). In particular, members of the duckweed family (*Lemna minor*, *L. trisulca*) have been reported to bioaccumulate and bioconcentrate toxic

metals from polluted water (Samardakiewicz and Woźny 2000). Results after 2 weeks of exposure to low doses (10 μM) of Pb and Cd indicated that *W. arrhiza* achieved high Pb (64.6%) and Cd (36.0%) removal efficiency. These results correlate well with the observation that both heavy metals present at a concentration of 10 μM induced defense reactions in fronds, including antioxidant enzymes, ascorbate, and glutathione.

Literature data indicate that the most of Cd and Pb accumulated by the aquatic plants was retained by the root, leading to less translocation to the aerial parts. Roots of plants are a barrier against heavy-metal translocation, and this may be a potential tolerance mechanism operating in the roots (Singh et al. 2006). However, *W. arrhiza* does not possess roots, so heavy metals are absorbed by the fronds. Experiments performed by Samardakiewicz and Woźny (2000) showed that *L. minor* treated with Pb showed maximum concentrations of heavy metals in small vacuoles and the cell wall. The localization of Pb between vacuoles and the cell wall possibly results from redistribution of Pb, and it reflects increased apoplastic transport. The presence of heavy metals in small vesicles in Lemnaceae plants suggests that endocytosis plays an important role in metal uptake in these species. Moreover, the different accumulation of Cd and Pb ions further suggest different cellular mechanisms of the biosorption of these nonessential trace elements by *W. arrhiza* fronds.

Bioaccumulation of Cd and Pb has been reported to induce negative effects on some key metabolic processes coupled with plant development. The most dramatic symptom of Cd and Pb toxicity in *W. arrhiza* culture was the cessation of plant growth. Growth inhibition may be connected with the decrease in mitotic index observed in the case of Pb and Cd ion exposure (Vecchia et al. 2005). Experiments performed on synchronized soybean (*Glycine max*) cell suspension culture showed that Cd induced DNA damage, decreased the rate of DNA synthesis, and blocked cell division (Sobkowiak and Deckert 2004).

As a visible symptom, decreased chlorophyll *a* content can be used to monitor heavy-metal-induced damage in *W. arrhiza* fronds. Based on available data, it can be assumed that Cd and Pb may inhibit chlorophyll synthesis by causing impaired uptake of elements essential for photosynthetic pigments, such as magnesium, potassium, calcium, and iron (Burzyński 1987). Moreover, enhancement of chlorophyll damage occurs in plants growing in the presence of Pb ions due to increased chlorophyllase activity (Drążkiewicz 1994). The inhibition in photosynthetic pigment accumulation in response to heavy-metal stress may also be a consequence of peroxidation of chloroplast membranes by way of increased rate of ROS production. This observation is in good agreement with the increased rate of H_2O_2 and lipid peroxide formation in *W. arrhiza* exposed to Cd and Pb.

In contrast, carotenoids were less sensitive than chlorophyll *a* toward both Pb and Cd, probably protecting the photosynthetic apparatus against heavy-metal stress. The increased carotenoid level in *W. arrhiza* plants exposed to heavy metals is probably part of the strategy adopted by the plant to counteract the toxic effect of free radicals generated under Cd and Pb stress, which is agreement with other reports in aquatic plants, such as *L. minor* and *L. trisulca* (Prasad et al. 2001; Singh et al. 2006; Hou et al. 2007). Carotenoids, which play a part in guarding chlorophyll, also serve as antioxidants to quench or scavenge the free radicals and decrease damage to the cell, to the cell membrane, and to the plant's main genetic composition induced by heavy metal (Artetxe et al. 2002).

Monosaccharides are building substances for plants as well as a key source of energy necessary for inciting biochemical processes. The decrease in monosaccharide content noted in *W. arrhiza* growing in the presence of Cd and Pb may be caused by enhanced degradation of photosynthetic pigments, thus contributing to decreased photosynthesis and sugar accumulation. Significantly decreased activities of enzymes involved in CO_2 fixation in field-grown *Avena sativa* exposed to heavy metals was reported by Moustakas et al. (1994).

The protein content in *W. arrhiza* fronds decreased with increased heavy-metal concentrations at all of the exposure periods, and the maximum decrease in this biochemical parameter was obtained in response to 1000 μM Cd. Soluble-protein content in plants, which is an important indicator of reversible and irreversible changes in metabolism, is known to respond to a wide variety of stressors (Singh and Tewari 2003). The inability of *W. arrhiza* fronds to accumulate proteins after Cd and Pb application may be caused by acute oxidative stress induced by heavy-metal excess in plant cells. Protein degradation, as a consequence of metal exposure, has been observed in many aquatic plants, such as *L. minor*, *L. trisulca* and free-floating freshwater macrophyte *Ceratophyllum demersum* (Mohan and Hosetti 1997; Prasad et al. 2001; Aravind and Prasad 2003; Hou et al. 2007). This phytotoxic effect of Cd and Pb in plant culture may be explained by their influence on nucleic acid degradation. For example, Cd induces DNA damage in plant cells, such as single- and double-strand break, modified bases, abasic sites, DNA–protein cross-links, oxidized bases, 8-hydroxyguanine, and even bulky adducts (Liu et al. 2005).

Compared with unstressed plants, significantly increased hydrogen peroxide content was observed in the presence of Cd and Pb in *W. arrhiza* culture. The main sources of ROS are enzymes localized at the external surface of plant cells, plasma membrane NAD(P)H oxidases, and/or cell wall

peroxidases (Ishida et al. 1987). In apoplast, H_2O_2 may be produced by SOD for $^{\bullet}O_2^-$, resulting from the activity of oxidase/peroxidase NADH complex, and accumulate in a cell wall (Chaoui et al. 2004). Therefore, the present results suggest a correlation between peroxidase activity, for which NADH is a substrate (NADH peroxidase), and the generation of H_2O_2 in *W. arrhiza* fronds in response to exogenous Cd and Pb. MDA content also increased gradually in proportion to increased concentrations of heavy metals. MDA production in plants exposed to adverse environmental conditions is an indicator of free radical formation in biologic systems (Heath and Packer 1968). A concentration-dependent increase in the level of lipid peroxides occurred in spruce (*Picea abies*) needles (Radotic et al. 2000), barley (*Hordeum vulgare*) seedlings (Hegedüs et al. 2001), rice (*Oriza sativa*) shoots (Verma and Dubey 2003), water hyssop (*Bacopa monnieri*) plants (Singh et al. 2006), and duckweed (*L. minor*) fronds (Hou et al. 2007) growing in the presence of increased concentrations of heavy metals. However, the mechanism of oxidative stress in *W. arrhiza* generated by exposure to heavy metals is not clearly understood.

The stimulating influence of Cd and Pb on lipid peroxidation and H_2O_2 levels indicated that fronds encountered enhanced oxidative stress. Whenever ROS are produced, plants activate nonenzymatic antioxidant defense mechanisms comprising low molecular mass scavengers, such as ascorbate and glutathione, to remove these highly reactive molecules. Stimulation in the ascorbate level in response to heavy metals at a concentration of 10 μM suggests its role in ROS detoxification generated by stress. Ascorbate is known to operate as an antioxidant either in direct chemical interaction with free oxyradicals or during the reaction catalyzed by ascorbate peroxidase (Nakano and Asada 1981). Moreover, ascorbate oxidation affects the redox balance of other metabolites, such as glutathione, which are themselves involved in the perception of cellular redox unbalance (Kampfenkel et al. 1995). Similar results obtained by Artetxe et al. (2002), Rucińska-Sobkowiak and Pukacki (2006), and Singh et al. (2006) confirmed that ascorbate accumulation leads to enhancement of plant tolerance toward heavy-metal stress.

The adaptation of *W. arrhiza* to grow in the presence of 10 μM Cd and Pb was evidenced by the increment in ascorbate and glutathione, together with increased activities of antioxidant enzymes involved in H_2O_2 detoxification, such as ascorbate peroxidase, catalase, and NADH peroxidase. H_2O_2 is destroyed in chloroplasts through the action of a metabolic ascorbate–glutathione cycle involving successive oxidation and reduction of ascorbate, glutathione, and NAD(P)H. The following pathway has been proposed: ascorbate peroxidase reduces H_2O_2 into water using ascorbate as the electron donor; the resulting dehydroascorbate is cycled back to ascorbate using reduced glutathione as electron donor; and the oxidized glutathione formed is converted back to glutathione. The activation of this cycle on metal-stress situations has been well documented (Chaoui et al. 1997; Hegedüs et al. 2001). For each metal treatment at a concentration of 10 μM, enhanced antioxidant enzymes activities suggest an increased rate of ascorbate and glutathione turnover in *W. arrhiza* exposed to Cd and Pb. Despite the fact that neither reduced/oxidized ratio of ascorbate and glutathione nor glutathione reductase activity were assayed in this study, analysis of ascorbate peroxidase as well as ascorbate and glutathione level lends support to the hypothesis that the H_2O_2 scavenging ascorbate–glutathione cycle may be activated in *W. arrhiza* treated with both heavy metals, especially at 10 μM.

Increased glutathione levels have been also shown to correlate with plant adaptation to extreme heavy-metal stress, and decreased glutathione pool shows marked alterations in response to heavy-metal stress (Xiang and Olivier 1998; Jin et al. 2008). Moreover, glutathione is also a precursor of phytochelatins, which are low molecular mass peptides produced by plants to immobilize toxic heavy metals (Tsuji et al. 2005). Therefore, the increased glutathione level noted in *W. arrhiza* treated with Cd and Pb at a range of concentrations (1 to 100 μM) may precede phytochelatin accumulation. However, ascorbate and glutathione levels decreased in response to exposure to 1000 μM heavy metals. Reactive free oxygen radicals are assumed to be involved in the oxidation of ascorbic acid to dehydroascorbic acid, leading to decreased ascorbic acid content in *W. arrhiza*.

Apart from the effects of Cd and Pb on nonenzymatic antioxidants, heavy metals affected enzyme activities involved in H_2O_2 metabolism. Both heavy metals at the highest concentration (1000 μM) inhibited the activity of all enzymes, probably because of the harmful effect of H_2O_2 overproduction or its poisonous ROS derivatives. Varying responses to heavy-metal-induced oxidative stress might be related to the concentration of thiolic groups already present or induced by Cd and Pb exogenous application because they are consequently able to counteract oxidative stress (Sanita di Toppi and Gabbrielli 1999). However, heavy metals at lower concentrations (1 to 100 μM) were found to stimulate the activities of antioxidant enzymes.

Peroxidases can function as effective quenchers of reactive intermediary forms of oxygen and peroxy radicals induced by increased metal levels in plant cells (Radotic et al. 2000). They are considered to be heavy-metal stress-related enzymes and can be used as stress markers in metal (Cd, Pb) poisoning situations. Some acidic isoforms of cell wall peroxidases have been implicated in lignification by catalyzing oxidative polymerization of monolignols, e.g.,

coumaryl, in the presence of H_2O_2, which is produced by cell wall peroxidases having an NADH oxidase activity, such as NADH-peroxidase (Ishida et al. 1987). Peroxidase-catalyzed lignification represents a mechanical plant adaptation to metal contamination (Gaspar et al. 1985). Enhanced activity of ascorbate peroxidase in *W. arrhiza* is generally associated with an acclimation to increased amounts of active oxygen species generated by Cd and Pb. Catalase participates in the main defence system against accumulation and toxicity of active oxygen species, such as hydrogen peroxide, and can play a key role in controlling H_2O_2 levels in plant cells. Our findings correspond well with other literature data showing the positive effect of heavy-metal exposure on antioxidant enzymes activities (Hegedüs et al. 2001; Singh et al. 2006; Hou et al. 2007; Jin et al. 2008).

The enhanced activities of the enzymatic antioxidant system in *W. arrhiza* in response to exogenous metals, such as Cd and Pb at 1 to 100 μM, may account for its better growth and acclimation to metal pollution of the aquatic environment. Interpretation of the biochemical detoxification strategies of the plant against oxidative stress induced by metal accumulation is a key to optimize the phytoremediation of heavy-metal-affected biomes. Summarizing our results, it can be concluded that the antioxidant enzymes activities, such as catalase, ascorbate peroxidise, and NADH peroxidase, in *W. arrhiza* fronds were at their peak when fronds were treated with 10 μM Cd and Pb, whereas the highest dose (1000 μM) of heavy metals decreased these activities and contributed dramatically to inhibition of plant growth and development.

Conclusion

From the work presented here, the aquatic plant *W. arrhiza* can be effective as a biosorbent for the removal of low levels of Cd and Pb from nutrient medium. In the present study, the accumulation of metals resulted in considerable biochemical changes. The data confirm the inhibitory effect of heavy metals on the growth and development of *W. arrhiza*. Cd was found to be comparatively more toxic than Pb. The rate of proteins, photosynthetic pigments, and monosaccharide degradation was proportional to the increased dose of heavy metals, and the most phytotoxic influence of Cd and Pb was observed at the highest tested concentration (1000 μM). Treatment with Cd and Pb also caused oxidative damage as evidenced by increased lipid peroxide and H_2O_2 formation, indicating the presence of poisoning ROS. However, to cope with heavy-metal toxicity, *W. arrhiza* fronds are able to carry out a cellular strategy involving activation of various enzymatic (catalase, ascorbate peroxidase, NADH-peroxidase) and nonenzymatic (ascorbate, glutathione) antioxidants, which serve as important components of mechanisms that detoxifying heavy metals. The highest enhancement in antioxidant activity was observed in fronds exposed to 10 μM heavy metals, which may account for higher plant tolerance and acclimation to metal contamination. The results obtained with Cd and Pb, showing that Lemnaceae are much more sensitive than other experimental plants used so far, are in agreement with the use of *W. arrhiza* in bioassays for phytotoxicity.

Acknowledgments The authors are grateful to Marta Kaczorowska and Luba Siemianowicz for skilful technical assistance.

References

Aeby H (1984) Catalase in vitro. Meth Enzymol 105:125–212

Aliexieva V, Sergiev I, Mapelli S, Karanov E (2001) The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. Plant Cell Environ 24:1337–1344

Aravind P, Prasad MNV (2003) Zinc alleviates cadmium-induced oxidative stress in *Ceratophyllum demersum* L.: a free floating freshwater macrophyte. Plant Physiol Biochem 41:391–397

Artetxe U, García-Plazaola JI, Hernández A, Becerril JM (2002) Low light grown duckweed plants are more protected against the toxicity induced by Zn and Cd. Plant Physiol Biochem 40:859–863

Aslan M, Unlü MY, Türkmen N, Yilmaz YZ (2003) Sorption of cadmium and effects on growth, protein content, and photosynthetic pigment composition of *Nasturtium officinale* R Br. and *Mentha aquatica* L. Bull Environ Contam Toxicol 71:323–329

Bajguz A, Godlewska-Żyłkiewicz B (2004) Protective role of 20-hydroxyecdysone against lead stress in *Chlorella vulgaris* cultures. Phytochemistry 65:711–720

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 72:248–254

Burzyński M (1987) The influence of lead and cadmium on the absorption and distribution of potassium, calcium, magnesium and iron in cucumber seedlings. Acta Physiol Plant 9:229–238

Chaoui A, Mazhoudi S, Ghorbal MH, Ferjani EE (1997) Cadmium and zinc induction of lipid peroxidation and effects on antioxidant enzyme activities in bean (*Phaseolus vulgaris* L.) Plant Sci 127:139–147

Chaoui A, Jarrar B, El Ferjani E (2004) Effects of cadmium and copper on peroxidase, NADH oxidase and IAA oxidase activities in cell wall, soluble and microsomal membrane fractions of pea roots. J Plant Physiol 161:1225–1234

De Kok LJ, Maas FM, Godeke J, Haaksma AB, Kuiper PJC (1986) Glutathione, a tripeptide which may function as a temporary storage compound of excessive reduced sulphur in H_2S fumigated spinach plants. Plant Soil 91:349–352

Drążkiewicz M (1994) Chorophyll-occurrence, functions, mechanism of action, effects of internal and external factors. Photosynthetica 30:321–331

Fujita M, Mori K, Kodera T (1999) Nutrient removal and starch production through cultivation of *Wolffia arrhiza*. J Biosci Bioeng 87:194–198

Gaspar T, Penel C, Castillo FJ, Greppin H (1985) A two step control of basic and acidic peroxidases and its significance for growth and development. Physiol Plant 64:418–423

Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 125:189–198

Hegedüs A, Erdei S, Horvath G (2001) Comparative studies of H_2O_2 detoxifying enzymes in green and greening barley seedlings under cadmium stress. Plant Sci 160:1085–1093

Hou W, Chen X, Song G, Wang Q, Chang CC (2007) Effect of copper and cadmium on heavy metal polluter waterbody restoration by duckweed (*Lemna minor*). Plant Physiol Biochem 45:62–69

Hutner SH (1953) Comparative physiology of heterotrophic growth in plants. In: Loomis WE (ed) Growth and differentiation in plants. Iowa State College Press, Ames, IA, pp 417–446

Ishida A, Ookubu K, Ono K (1987) Formation of hydrogen peroxide by NAD(P)H oxidation with isolated cell wall-associated peroxidase from cultured liverwort cells, *Marchantia polymorpha* L. Plant Cell Physiol 28:723–726

Jin XF, Yang XE, Islam E, Liu D, Mahmood Q, Li H, Li J (2008) Ultrastructural changes, zinc hyperaccumulation and its relation with antioxidants in two ecotypes of *Sedum alfredii* Hance. Plant Physiol Biochem 46:997–1006

Kampfenkel K, Van Montagu M, Inzé D (1995) Extraction and determination of ascorbate and dehydroascorbate from plant tissue. Anal Biochem 225:165–167

Landolt E (1986) The family of Lemnaceae—a monographic study. Veröff Geobot Inst ETH, Stiftung Rübel, Zürich 71:1–563

Liu W, Li PJ, Qi XM, Zhou QX, Zheng L, Sun TH et al (2005) DNA changes in barley (*Hordeum vulgare*) seedlings induced by cadmium pollution using RAPD analysis. Chemosphere 61:158–167

Mical AH, Krotke A (1999) *Wolffia arrhiza* (L.)—small but strong. Acta Hydrobiol 41:165–170

Mohan BS, Hosetti BB (1997) Potential phytotoxicity of lead and cadmium to *Lemna minor* grown in sewage stabilization ponds. Environ Pollut 2:233–238

Moustakas M, Lanaras T, Symeonidis L, Kartaglis S (1994) Growth and some photosynthetic characteristics of field grown *Avena sativa* under copper and lead stress. Photosynthetica 30:389–396

Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol 22:867–880

Prasad MNV, Malec P, Waloszek A, Bojko M, Strzałka K (2001) Physiological responses of *Lemna trisulca* L (duckweed) to cadmium and copper bioaccumulation. Plant Sci 161:881–889

Radotic K, Ducic T, Mutavdzic D (2000) Changes in peroxidase activity and isoenzymes in spruce needles after exposure to different concentrations of cadmium. Environ Exp Bot 44: 105–113

Rucińska-Sobkowiak R, Pukacki PM (2006) Antioxidative defense system in lupin roots exposed to increasing concentrations of lead. Acta Physiol Plant 28:357–364

Samardakiewicz S, Woźny A (2000) The distribution of lead in duckweed (*Lemna minor* L.) root tip. Plant Soil 226:107–111

Sanita di Toppi L, Gabbrielli R (1999) Response to cadmium in higher plants. Environ Exp Bot 41:105–130

Singh VP, Tewari RK (2003) Cadmium toxicity induced changes in plant water relations and oxidative metabolism of *Brassica juncea* L plants. J Environ Biol 24:107–112

Singh S, Eapen S, D'Souza SF (2006) Cadmium accumulation and its influence on lipid peroxidation and antioxidative system in an aquatic plant, *Bacopa monnieri* L. Chemosphere 62:233–246

Sobkowiak R, Deckert J (2004) The effect of cadmium on cell cycle control in suspension culture cells of soybean. Acta Physiol Plant 26:335–344

Somogyi M (1954) Notes on sugar determination. J Biol Chem 195: 19–23

Tsuji N, Nishikori S, Ibawe O, Matsumoto S, Shiraki K, Miyasaka H et al (2005) Comparative analysis of the two-step reaction catalyzed by prokaryotic and eukaryotic phytochelatin synthase by an ion-pair liquid chromatography assay. Planta 222:181–191

Vecchia FD, La Rocca N, Moro I, De Faveri S, Andreoli C, Rascio N (2005) Morphogenetic, ultrastructural and physiological damages by submerged leaves of *Elodea canadensis* exposed to cadmium. Plant Sci 168:329–338

Verma S, Dubey RS (2003) Lead toxicity induces lipid peroxidation and alters the activities of antioxidant enzymes in growing rice plants. Plant Sci 164:645–655

Wellburn AR (1994) The spectral determination of chlorophylls *a* and *b*, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. J Plant Physiol 144: 307–313

Xiang C, Oliver DJ (1998) Glutathione metabolic genes coordinately respond to heavy metals and jasmonic acid in *Arabidopsis*. Plant Cell 10:1530–1550