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OXIDATIVE STRESS ENZYME ACTIVITY IN *LEMNA MINOR* L. EXPOSED TO CADMIUM AND LEAD

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Duckweed (*Lemna minor*) is an aquatic plant used in phytotoxicity tests for xenobiotic substances. This study assessed whether ascorbate peroxidase (APX), guaiacol peroxidase (GPX) and superoxide dismutase (SOD) in *L. minor* may be used as bioindicators of stress caused by heavy metals. The plants were cultivated on Knopp's medium spiked with cadmium (0.001–1.0 mM) and lead (0.01–1.0 mM). Cadmium showed higher toxicity than lead in *L. minor*. At lower lead doses (0.01–0.1 mM) growth was even slightly stimulated. Both heavy metals (0.001–0.05 mM Cd, 0.01–0.5 mM Pb) brought about chlorosis and modified the enzymes of the antioxidative system. GPX showed the highest increase in response to increased cadmium content in the medium and cadmium bioaccumulation. APX activity was affected slightly, but SOD activity was not correlated with cadmium or lead accumulation. Of the antioxidative enzymes analyzed in *L. minor* test plants, only GPX proved useful as a biochemical stress indicator of heavy metal pollution.

Key words: *Lemna minor*, ascorbate peroxidase, cadmium, guaiacol peroxidase, lead, superoxide dismutase.

INTRODUCTION

In industrial areas, especially near steelworks, working mines and closed mine, the environment is polluted by toxic heavy metals. High concentrations of these elements are also found along roads and motorways. In water environments these elements accumulate in the organs of macrophytes, fatty tissues of fish species, and bottom sediments (Wilson and Bell, 1996; Karczewska, 2002).

Duckweed (*Lemna minor* L.) is an aquatic plant living in many types of water ecosystems, including lakes, streams and ponds. Because it floats on the water surface, it is exposed to both water and air contaminants (Mohan and Hosetti, 1999). In the past it was thought that duckweed is highly tolerant to toxic substances. Currently there are many suggestions that *L. minor* is sensitive to xenobiotic substances. To explain this contradiction it has been suggested that duckweed is highly adaptive to environmental toxicity (Gabrielson et al., 1990; Mohan and Hosetti, 1999).

Lemna minor can be used in phytotoxicity tests of contaminants, including heavy metals, phenolics and herbicides (Vujevic et al., 2000). Tests of heavy metal toxicity consist in measurements of growth parameters and physiological and biochemical indicators, including changes in carbohydrate, protein and chlorophyll content (Mohan and Hosetti, 1999). Experts from the U.S. Environmental Protection Agency (EPA) and the Organization for Economic Cooperation and Development (OECD) have classified this plant as a bioindicator (Kiss et al., 2003).

Symptoms of heavy metal toxicity are chlorosis, necrosis and root damage, as well as changes in biochemicals including antioxidant enzymes. The sensitivity of *L. minor* has been tested in terms of some metabolic indicators, in sewage ponds (Mohan and Hosetti, 1999) and under laboratory conditions (Garnczarska and Ratajczak, 2000a,b; Wang et al., 2002). Since the data are not conclusive, duckweed's potential as a bioindicator for aquatic systems needs further investigation. The present experiments addressed the effect of cadmium and lead on the

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Abbreviations: APX – ascorbate peroxidase; GPX – guaiacol peroxidase; SOD – superoxide dismutase.

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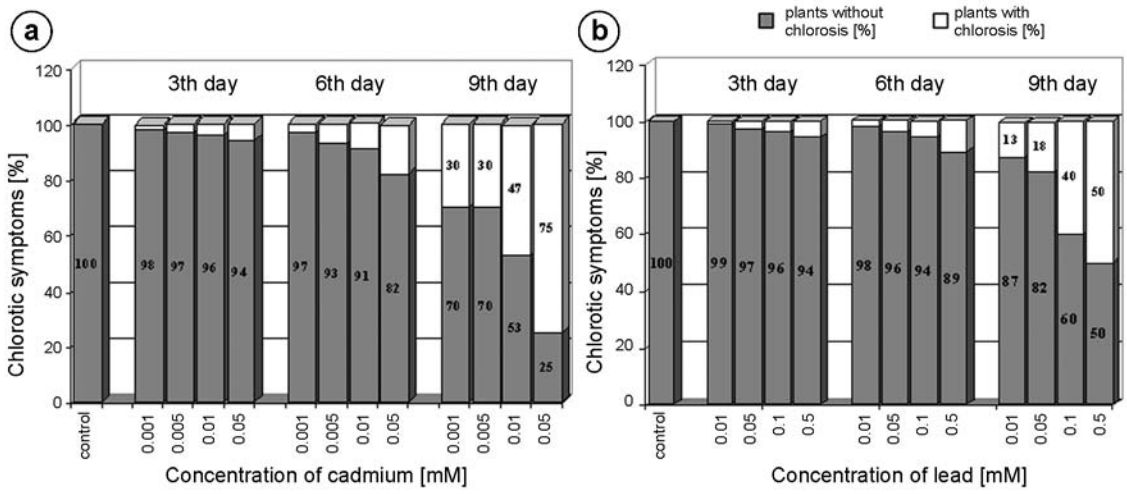

Fig. 1. Influence of cadmium (**a**) and lead (**b**) on the vitality of *L. minor*, on days 3, 6 and 9 of culture: percentage of plants with and without chlorosis (average of three experiments).

growth of *L. minor* and on the activity of the antioxidant enzymes ascorbate peroxidase (APX), guaiacol peroxidase (GPX) and superoxide dismutase (SOD).

MATERIALS AND METHODS

L. minor plants were collected from an uncontaminated pond and grown in a laboratory. For the experimental procedure, plants were introduced to glass ponds (500 cm^3) and cultivated on Knopp's medium (Strebeyko, 1967) spiked with cadmium and lead salts, 50 plants per 200 cm^3 medium. The applied concentrations were, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5 and 1.0 mM $CdCl_2$, and 0.01, 0.05, 0.1, 0.5 and 1.0 mM $Pb(NO_3)_2$. Controls without heavy metals were run simultaneously. The cultures were maintained in a growth chamber for 9 days under a 14 h photoperiod at photosynthetic photon flux density (PPFD) of 120 μmol m^{-2} s^{-1}. Each treatment was done in two replicates and the experiment was run three times. At days 3, 6 and 9 of culture the percentage of plants with chlorotic symptoms was estimated and fresh weight was measured. Antioxidant enzyme activity was measured at days 6 and 9 of the experiment. For these analyses the plants were rinsed with distilled water, portioned in samples weighing 200 mg, and frozen in liquid nitrogen.

ACTIVITY OF ASCORBATE AND GUAIACOL PEROXIDASE

Plant samples were homogenized for 30 sec in a chilled mortar with 50 mM potassium phosphate buffer (pH 7.0) with 2% Polyclar AT added. Homogenates were centrifuged at 15,000 g for 30 min at 4°C and the supernatant was used for enzyme assays.

The activity of APX was measured according to Nakano and Asada (1987). The reaction mixture contained 50 mM potassium-phosphate buffer, 0.5 mM L-ascorbate (AsA), 0.1 mM H_2O_2 and the enzyme extract. H_2O_2-dependent oxidation of AsA was followed by a decrease in absorbance at 290 nm. APX activity was expressed as the absorbance decrease (ΔE) min^{-1} g^{-1} fresh weight.

GPX activity was estimated according to Hammerschmidt et al. (1982). The reaction mixture contained 25 mM potassium phosphate buffer (pH 7.0), 0.2 mM guaiacol, 0.09 mM H_2O_2 and the enzyme extract. H_2O_2-dependent oxidation of guaiacol was followed by an increase of absorbance at 470 nm. Enzyme activity was calculated as the increase of absorbance (ΔE) min^{-1} g^{-1} fresh weight.

ACTIVITY OF SUPEROXIDE DISMUTASE

Plant samples were homogenized in a chilled mortar with 3 cm^3 buffer (50 mM sodium phosphate buffer containing 1 g 1% polyvinylpolypyrrolidone, 1.0 mM EDTA-Na and 0.5 M NaCl) and centrifuged at 15,000 g for 25 min at 4°C. Activity was determined according to Beauchamp and Fridovich (1971). The incubation mixture contained 50 mM sodium phosphate buffer (pH 7.8), 0.1 mM EDTA-Na, 4 mM methionine, 0.1 mM nitro blue tetrazolium (NBT) and the enzyme extract. Riboflavin (2.4 mM) was added last and the samples were placed under fluorescent lamps for 10 min. At the same time a blank without the enzyme extract was prepared. Absorbance was measured at 560 nm and the unit of activity was taken as the quantity of enzyme reducing absorbance to 50% of the blank.

TABLE 1. Correlation coefficients and ANOVA for linear regression between cadmium or lead accumulation and the activity of antioxidative enzymes at p = 0.05

Metal accumulation	Enzyme activity		
	APX	POD	SOD
Cadmium	-0.19	0.78	-0.35
	–	+	–
Lead	-0.01	0.10	-0.20
	–	–	–

+ linear dependence; – linear independence

HEAVY METAL CONTENT

Plant samples were mineralized in a microwave oven for 40 min at 100°C in 25 cm^3 HNO_3 and 5 cm^3 H_2O_2. Metal content was determined by atomic absorption spectrophotometry (AAS) with a Varian Spectra AA 200 Plus spectrometer.

STATISTICAL ANALYSIS

Antioxidative enzyme activity and cadmium and lead accumulation levels were analyzed statistically with MS Excel software. Correlation coefficients and single linear regressions between metal content in plants and enzyme activity were determined, and ANOVA for linear regressions was performed, with significance assumed at p = 0.05.

RESULTS

Both of the heavy metals – cadmium at 0.001–0.05 mM and lead at 0.01–0.5 mM – brought about chlorosis symptoms in the duckweed plants (Fig. 1). Cadmium-treated plants exhibited higher rates of damage than plants exposed to lead. Fresh weight was lower at the cadmium doses of 0.005 mM (by 22%), 0.01 mM (35%), and 0.05 mM (43%), and at the 0.5 mM lead dose by 65% (Fig. 2). At the lower lead concentrations of 0.01 mM, 0.05 mM and 0.1 mM, *L. minor* growth was even slightly stimulated. At 0.001 mM cadmium and 0.01 mM lead, the leaf blade area was enlarged.

APX activity in *L. minor* plants exposed to heavy metals did not differ significantly from the control levels (Fig. 3a). Only after 9 days of cadmium treatment at 0.05 mM was APX activity decreased by 43%. APX activity on the 9th day of the experiment was generally lower than on day 6 of culture.

The presence of cadmium ions in the growth medium had a stimulating effect on GPX activity in *L. minor* (Fig. 3b). After 6 days of exposure to 0.001–0.05 mM cadmium, GPX activity was 18.8, 23.1, 28.5 and 34.8 units at the increasing doses, while in control plants it was 16.2. Lead ions did not significantly change GPX activity. As for APX, GPX activity was lower in 9-day-old than in 6-day-old plants.

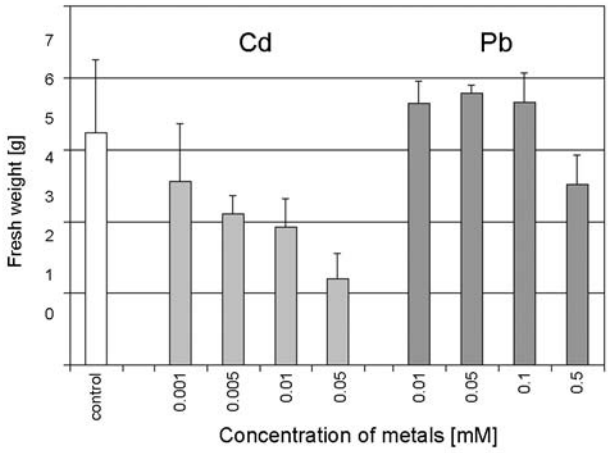

Fig. 2. Fresh weight of *L. minor* at day 9 of culture in the presence of cadmium and lead: bars indicate SD for plants from two glass ponds and three experiments.

SOD activity under the influence of cadmium and lead was similar to that in the control plants (Fig. 3c); there was no correlation between the day of culture and enzyme activity.

After 9-day exposure to heavy metals, cadmium and lead content was assayed in the duckweed plants (Fig. 4). Generally, metal content in *L. minor* increased with its increasing concentration in the medium. The cadmium concentration in plants was higher than in the medium, indicating high uptake of the element. The 0.05 mM $CdCl_{2\ dose}$ was an exception in this respect, as the cadmium level in plants was about half its content in the medium. In lead-treated plants the concentration of the metal was higher in the medium than in *L. minor*.

Statistical calculations (Tab. 1) confirmed that both cadmium and lead, which brought about chlorotic changes at 0.001–0.05 mM Cd and 0.01–0.5 mM Pb, modified the activity of antioxidative system enzymes only slightly. No dependence between APX and SOD activity and cadmium and lead accumulation was confirmed. GPX activity did increase with increased cadmium accumulation in plants (correlation ratio = 0.78).

DISCUSSION

Chlorotic changes and the fresh weight decrease showed that in duckweed cadmium was more toxic than lead, as also reported by Mohan and Hosetti

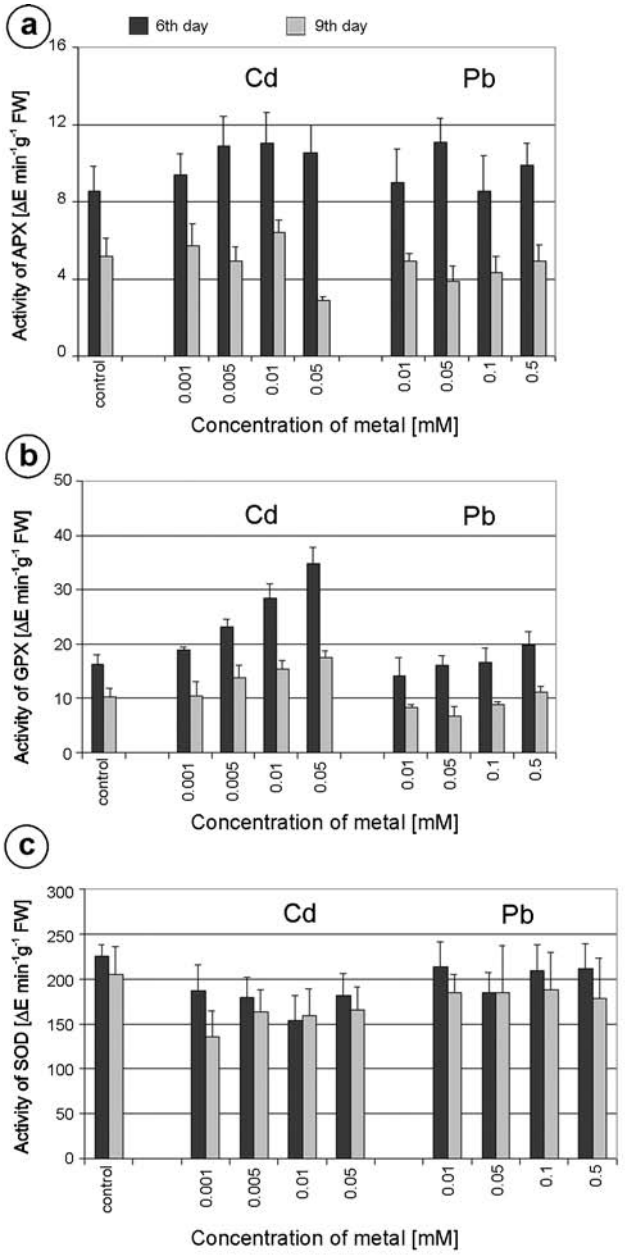

Fig. 3. Influence of cadmium and lead on antioxidant enzyme activity in *L. minor*. (**a**) Ascorbate peroxidase (APX), (**b**) Guaiacol peroxidase (GPX), (c) Superoxide dismutase (SOD); bars indicate SD for two separate tissue samples and three experiments.

(1997). The high toxicity of cadmium might result from its easy absorption by plants; lead shows low phytoavailability and restricted transport within the plant (Kabata-Pendias and Pendias, 1999; Sharma and Dubey, 2005). Wang et al. (2002) found high Cd accumulation and a high bioconcentration factor (BCF) in duckweed. In our model experiment, cadmium bioaccumulation reached the maximum level of 2–3 ppm (Fig. 4) and the concentration was several times higher than in the medium (at 0.001–0.01 mM). The highest Cd dose (0.05 mM) was toxic to *L. minor*: the plants were chlorotic and fresh weight decreased threefold; their accumulation capacity probably was exhausted, since their Cd level was lower than in the medium. This is in agreement with the observations of other authors studying cadmium toxicity (for review see Das et al., 1997). The lead concentration in *L. minor* was lower than in the medium, confirming its low mobility and the plant's low accumulation capacity as was reported by Wang et al. (2002) and also recorded in our experiments.

Cadmium treatment at concentrations exceeding 0.05 mM caused toxic effects – growth retardation and chlorotic changes – while concentrations below 0.01 mM were tolerated by *L. minor*. Lead became toxic at a much higher dose (0.5 mM), a finding probably connected with its low uptake from the medium.

Kabata-Pendias et al. (1999) referred to reports on the positive effect of lead (II) nitrate (V) on plant growth, although data showing a physiological justification for this phenomenon are lacking. In our study, lead at concentrations lower than 0.1 mM did not harm the growth of *L. minor*, and even stimulated it slightly. In experiments with duckweed, Mohan et al. (1997) found no negative symptoms up to day 4 of exposure to 0.25 mM Pb, but the presence of Pb as well as Cd drastically decreased catalase and protease activity and increased peroxidase activity.

In our model conditions the activity of guaiacol peroxidase (GPX) increased in duckweed plants exposed to cadmium, but there was no effect on the other two antioxidative stress enzymes, ascorbate peroxidase (APX) and superoxide dismutase (SOD). Mohan et al. (1997) suggested that the increase of peroxidase activity may be an effect of accelerated senescence, connected with enhanced formation of hydrogen peroxide (H_2O_2) or secondary metabolites such as phenolic compounds. We can add that this possibility does not apply to ascorbate peroxidase, an enzyme of the Halliwell-Asada pathway, but only to GPX activity. Garnczarska and Ratajczak (2000a,b) observed the stimulating effect of lead on peroxidase activation in duckweed, but only in roots, and similarly to our study, at concentrations which caused root growth inhibition.

Our results suggest that, of the antioxidative enzymes we analyzed in *L. minor* test plants, only GPX may serve as a biochemical stress indicator for heavy metal pollution.

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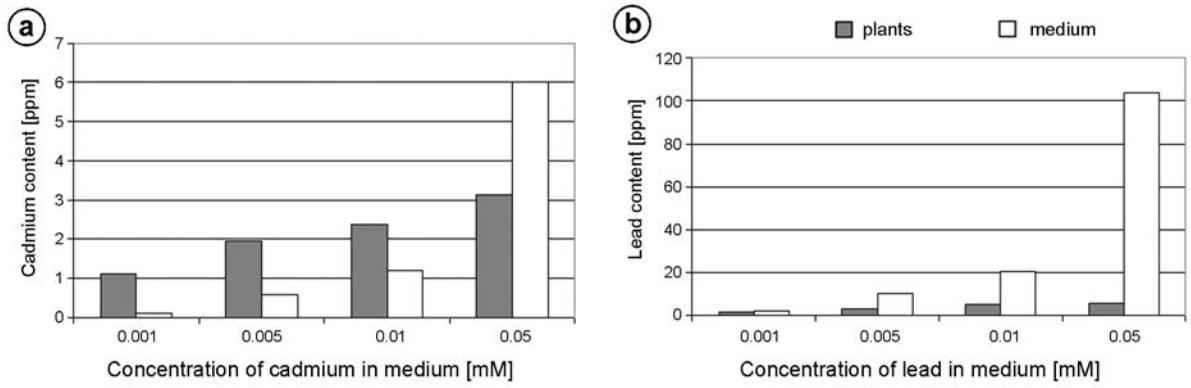

Fig. 4. Bioaccumulation of cadmium (**a**) and lead (**b**) in *L. minor*, and cadmium and lead content in medium, measured after 9 days of culture.

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