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Metabolic responses of *Lemna minor* to lead ions
I. Growth, chlorophyll level and activity of fermentative enzymes

Małgorzata Garnczarska, Lech Ratajczak

Department of Plant Physiology, A. Mickiewicz University, Al. Niepodległości 14, 61-713 Poznań, Poland

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Abstract

Duckweed *Lemna minor* L. was grown on Wang culture medium supplemented with lead ions for 24 hours. Metal was tested at 1.5, 3 and 6 mg·dm^{-3} concentrations. The growth of *Lemna minor* was inhibited by lead ions, but the dry to fresh weight ratio increased as the concentration of Pb^{2+} in the medium increased. With increased concentrations of Pb ions, the contents of chlorophyll *a* and chlorophyll *b* in roots and fronds were correspondingly lower in comparision with the control. The effect of lead upon activities of some glycolitic and fermentative enzymes in roots of duckweed was examined. The activity of pyruvate kinase decreased with increasing lead concentrations, but cytosolic malate dehydrogenase behaved in an opposite manner. The lowest concentration of Pb stimulated alcohol dehydrogenase; phosphoenolopyruvate carboxylase activity was maintained at relatively constant values at all tested lead concentrations.

List of abbreviations: ADH, alcohol dehydrogenase; cMDH, cytosolic malate dehydrogenase; PEPC, phosphoenolopyruvate carboxylase; PK, pyruvate kinase

Introduction

In recent years biological systems for wastewater treatment based on aquatic plants received growing attention as they represent an alternative approach for pollutant removal. Bio-monitoring studies demonstrated the ability of certain aquatic plants to accumulate metals, so that they could be used as agents for removing metals from polluted water (Qian *et al.* 1999). They may also be regarded as bioindicators of ecological relevance for the detection and monitoring of metal pollution. Many widespread aquatic floating weeds, such as water hyacinth and duckweed, are commonly utilised for this purpose (Miranda and Ilangovan 1996, Zayed *et al.* 1998, Rahmani and Sternberg 1999, Zhu *et al.* 1999).

This work is focused on duckweeds whch are small and green vascular plants growing rapidly under favourable conditions. Easiness of culture and possibility of manipulation in aseptic laboratory conditions make them suitable organisms for studies on metals uptake capability as well as on phytotoxic effects of metals (Mohan and Hosetti 1997, Barber *et al.* 1999, Bengtsson *et al.* 1999). Since duckweeds exhibits very rapid growth, they trap very effectivly mineral components from water, including heavy metals, but the intensity of such uptake varies significantly depending upon environmental conditions, *Lemna* species. *Lemna minor*, the common duckweed, was recommended as a standard test species for toxicity evaluation of heavy metal ions (Dirilgen 1998). The typical test end points in re-

sponse to heavy metals are changes in the growth rate (expressed as fresh and dry weight) and changes in pigment content. One of the most frequently observed symptoms of metal toxicity is limited mitochondrial respiration (Koeppe and Miller 1970), but at low concentration, lead ions can induce the activity of fermentative enzymes (Mazurowa *et al.* 1993).

The purpose of this preliminary study was to investigate, under controlled laboratory conditions, the influence of Pb ions on growth and photosynthetic pigments of *Lemna minor* L. In addition, the response of duckweed roots to different lead concentrations was also followed using activity of some glycolytic and fermentative enzymes.

Material and Methods

Duckweed (*Lemna minor* L.) was used for all experiments. Plants were grown on Wang nutrient medium (Wang 1990) under sterile conditions. Before the medium was autoclaved, its pH was adjusted to 5.7. The stock and experimental cultures were placed in a controlled environment room under continuous illuminations of 50 μmol quanta·m^{-2}·s^{-1} at 23±2 °C. The stock cultures were subcultured once a week. Experimental cultures were started by picking out healthy colonies with 2-3 fronds from stock cultures and transfering them into the 500 ml crystallizing dishes containing 350 ml of 1/50-strength Wang medium supplemented with Pb ions. Analytical grade $Pb(NO_3)_2$ salt was used for studies. Metal was tested at 1.5, 3 and 6 mg·dm^{-3} concentrations. These tested concentrations were chosen on the basis of experiments of Borek *et al.* (1998), which showed that 3 mg·dm^{-3} Pb^{2+}caused 50% inhibition of roots growth (RG_{50} at pH 5.7). 1/50-strength Wang's solution with no metal served as the control. Test period was 24 hours.

For estimation of plant growth twenty healthy looking *Lemna minor* plants of about 50.0 to 60.0 mg total fresh weight were introduced into 200 ml of 1/50-strength Wang nutrient medium supplemented with Pb^{+2}. After 24 hours, the tested plants were harvested and rinsed three times with distilled water, kept on filter paper to remove excess liquid and weighed. Growth was estimated as a change in the fresh weight according to the formula:

$$\frac{\text{final fresh weight - initial fresh weight}}{\text{initial fresh weight}}$$

After determination of fresh weight, plants were dried at 80 °C to constant weight and their dry weight was measured. Dry to fresh weight ratios as percentage of control were calculated.

The chlorophylls were extracted separately from fronds and roots in 80 % acetone and their content was determined spectrophotometrically according to Arnon (1949).

For enzymatic estimations the roots were harvested and homogenized in a cold buffer composed of 50 mM Tris-HCl pH 7.5, 0.4 M sucrose, 10 mM DTE, 1mM EDTA, PVP (0.1 g/g FW), 5 mM $MgCl_2$ and 10 % glicerol. The homogenate was centrifuged at 12 000 x g for 10 min. and the supernatant was used for enzyme assays. The activity of the following enzymes was determined spectrofotometrically in root cytosol extracts: phosphoenolopyruvate carboxylase (PEPC, EC 4.1.1.31.), as described by Deroche and Carrayol (1989), pyruvate kinase (PK, EC 2.7.1.40) by modified method of Podesta and Plaxton (1992), alcohol dehydrogenase (ADH, EC 1.1.1.1) according to Cossins *et al.* (1968), and malate dehydrogenase (MDH, EC 1.1.1.37), as described by Reeves *et al.* (1971). Protein content in the root cytosol was determined by the method of Bradford (1976).

Results and Discussion

The effect of Pb ions on growth of *Lemna minor* is shown in Fig. 1. Growth rate showed a progressive decrease when external concentrations of Pb^{2+} increased, but net fresh weight loss did not occur even at the highest lead concentration. The media with 3 mg·dm^{-3} Pb^{2+} caused 55 % inhibition of plant growth as compared to the control. The observed decrease in fresh weight in response to lead ions suggests a change in the plant's water status, which may be the result of decreased water uptake or enhanced water loss, both of which may occur following membrane damage. Plant cell membranes are generally considered primary sites of metal injury (Barcelo and Poschenrieder 1990).

Table 1. Chlorophylls content and the chl*a*/ chl*b* ratio in roots of *Lemna minor* after 24 hours of cultivation in the nutrient media supplemented with lead ions. The value is the mean ± SD of three replicates. The results are expressed as $\mu g \cdot g_{FW}^{-1}$.

Tested solutions	Chlorophyll *a*	Chlorophyll *b*	Total chlorophyll	chl *a*/ chl *b*
Control	63.025±3.352	24.473±2.561	87.498±5.367	2.57
1.5 $mg \cdot dm^{-3}$ Pb^{2+}	54.628±4.109	20.989±2.987	75.756±6.060	2.60
3 $mg \cdot dm^{-3}$ Pb^{2+}	51.155±2.006	19.527±2.002	70.813±4.870	2.62
6 $mg \cdot dm^{-3}$ Pb^{2+}	48.487±3.450	17.949±1.153	66.070±2.931	2.70

Table 2. Chlorophylls content and the chl*a*/ chl*b* ratio in fronds of *Lemna minor* after 24 hours of cultivation in the nutrient media supplemented with lead ions. The value is the mean ± SD of three replicates. The results are expressed as $\mu g \cdot g_{FW}^{-1}$.

Tested solutions	Chlorophyll *a*	Chlorophyll *b*	Total chlorophyll	chl *a*/ chl *b*
Control	149.080±3.080	51.978±1.314	201.418±3.184	2.87
1.5 $mg \cdot dm^{-3}$ Pb^{2+}	147.128±1.926	49.873±2.081	196.775±4.021	2.95
3 $mg \cdot dm^{-3}$ Pb^{2+}	143.202±2.012	47.836±1.007	190.959±2.985	2.99
6 $mg \cdot dm^{-3}$ Pb^{2+}	138.487±2.170	45.292±1.113	183.870±3.001	3.06

All three tested concentrations of Pb^{+2} increased the dry to fresh weight ratio (Fig. 2). The dry to fresh weight ratio has often been used as a parameter in toxity bioassays for a number of substances. An increase in this ratio occurs when chloroplasts become loaded with starch grains under the different stress conditions (Hillman 1961). In our investigation, the increase of dry to fresh weight ratio after the treatment with Pb ions could be explained by the change of the plant's water status or by accumulation of starch grains. Our future experiments will towards the conformation of starch grain accumulation.

Lead ions caused the change of chlorophylls content in roots and fronds of *Lemna minor*. With increased concentrations of Pb, the concentration of chlorophyll *a*, chlorophyll *b* and total chlorophyll in roots was correspondingly lower in comparison with the control, but the ratio chl*a*/chl*b* increased (Table 1). Similar changes were observed in fronds but differences were not so significant (Table 2). It is known that heavy metals, like Pb, Hg and Cd, inhibit chlorophyll biosynthesis (Assche and Clijsters 1990). The increase in chl*a*/chl *b* ratio is in agreement with the results of Tkalec *et al.* (1998), who tested the effect of oil industry "high density brines" on photosynthetic pigmets in *Lemna minor*.

In this study the effect of lead upon activities of glycolytic and fermentative enzymes: PK, PEPC, cMDH and ADH in roots of duckweed was examined. Pyruvate kinase activity decreased as the concentration of pollutant in the medium increased (Table 3). The lowest concentration of Pb stimulated alcohol dehydrogenase activity, but at higher concentrations ADH activity sharply decreased to values significantly lower than those found in control plants. PEPC activity was maintained at relatively constant values but cMDH activity increased with increasing lead concentrations (Table 3). It is known that heavy metals reduce mitochondrial respiration, but the pyrimidine nucleotides reduced in glycolysis should be reoxidized to ensure the continuation of the glycolytic pathway producing ATP. In our experiment, the reoxidation of NADH to NAD can be accomplished by the reactions catalysed by alcohol dehydrogenase (in the case of the lowest lead concentration) or malate dehydrogenase. The activity of cMDH increased 39 % at the highest lead concentration, as compared to control. Malate fermentation is also the main anaplerotic route producing organic acids (Lambers 1985). Organic acids were thought to play a role as metal-binding compounds, eg. malic and citric acid for Zn and Ni tolerance (Ernst *et al.* 1992). This hypothesis is based on the observation that Zn-tolerant and Ni-tolerant plants often exhibit increased concentrations of these compounds. We do not estimate the

Table 3. PK, ADH, PEPC and cMDH activities in roots of *Lemna minor* after 24hours of exposure to the nutrient media supplemented with lead ions. The value is the mean ± SD of five replicates.

Tested solutions	Enzyme activity (μmole NADH/ min/ mg protein)			
	PK	ADH	PEPC	cMDH
Control	0.395±0.023	0.191±0.013	0.123±0.024	9.514±0.375
1.5 mg·dm^{-3} Pb^{2+}	0.342±0.021	0.223±0.020	0.127±0.016	10.487±0.297
3 mg·dm^{-3} Pb^{2+}	0.312±0.015	0.128±0.011	0.131±0.014	11.610±0.401
6 mg·dm^{-3} Pb^{2+}	0.287±0.028	0.114±0.017	0.119±0.026	13.225±0.312

level of malic acid in duckweed root, but higher activity of malate dehydrogenase in response to lead ions suggests that malic acid might form complexes with lead ions, detoxifying them.

The data presented in this study demonstrate the effects of lead ions on growth, chlorophylls content and respiratory metabolism of *Lemna minor*. From the results we suggest that *Lemna minor* can be useful in the monitoring of lead pollution in freshwater ecosystems and for rapid toxicity assessment of wastewaters. In our further research we plan to investigate the potential of *Lemna minor* for removing lead from polluted water.

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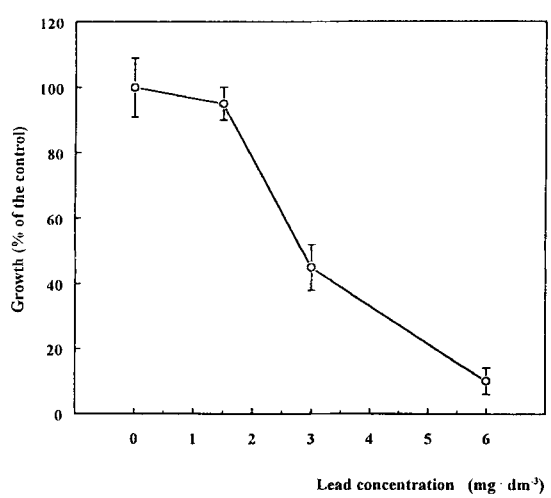

Fig. 1. Growth of *Lemna minor* cultivated 24 hours in the nutrient media supplemented with lead ions in comparision with the control represented as 100 %. Vertical bars indicate SD, n=4.

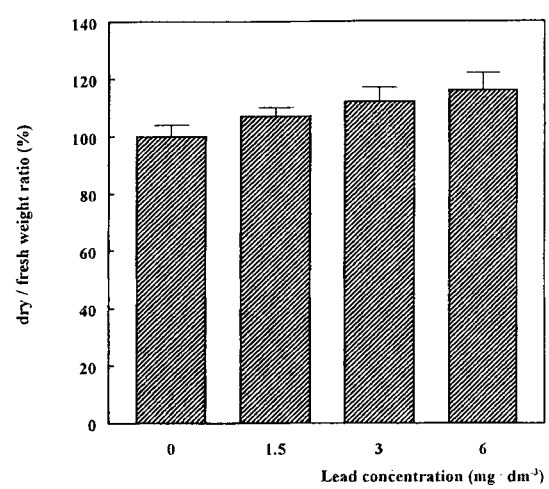

Fig. 2. The dry to fresh weight ratio of *Lemna minor* after 24 hours of exposure to the nutrient media supplemented with lead ions in comparison with the control represented as 100 %. Vertical bars indicate SD, n=4.

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