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DETERMINATION OF THE TOLERANCE OF SUNFLOWER TO LEAD-INDUCED STRESS*

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Abstract

Six-week old sunflower seedlings, cv. Ogrodowy, were treated with 0, 15, 45 and 60 mg Pb dm^{-3}, and then the content of lead and selected physiological and biochemical parameters were measured. Photosynthesis efficiency, water relations (intensity of transpiration, relative water content (RWC)) and gene-encoding metallothionein were measured three times after 24, 48 and 72 hours of exposure to Pb. The content of glutathione and lead was analysed after 72 hours' exposure to Pb. Most of the lead uptake was accumulated in the roots, then in the stems and leaves, but when re-calculated per plant dry weight, the uptake of the metal did not depend on the lead dose applied. The highest 60 mg Pb dm^{-3} treatment was accompanied by a significant decrease in dry weight content. Moreover, most of the lead taken up in these plants was transported to the stems and leaves (23.6% of total lead uptake). The lead doses used in this study did not affect the intensity of photosynthesis, but a decrease in transpiration and relative water content was observed. The glutathione level in the plants varied depending on the organ examined and the Pb concentration in the treatment. The expression of the metallothionein gene *HaMT1* was observed in the stems only. These results indicate that the sunflower cultivar Ogrodowy is a promising plant for phytoremediation of lead-polluted soils.

Key words: Pb, phytoremediation, *Helianthus annuus,* glutathione, metallothionein genes.

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INTRODUCTION

Lead (Pb) is one of the most significant heavy metal contaminants in soil in industrialised countries. Phytoremediation has been proposed as a low-cost, environmental-friendly way of removing lead from soil. Among other things, the principles of phytoremediation require an easy harvest of hyperaccumulators grown in polluted soils, and so the accumulation of heavy metal ions in these plants must not be limited to their roots alone. Sunflower (*Helianthus annuus* L.), a plant species native to North America, is grown in many countries around the world owing to its high adaptability (relatively tolerant to drought, able to grow in quite a wide temperature regime, insensitive to photoperiod) and qualitative traits (high content of oil and proteins). Given its high biomass, sunflower has also a high ion accumulation capacity (Chen, Cutright 2001, Meers et al. 2005). Sunflower has been studied in hydroponics trials at laboratory and field scale for the decontamination of soil and water containing low-to-moderate levels of heavy artificial radionuclides and natural radionuclides.

Lead is taken up by roots, bound and then transported to stems and leaves. Binding heavy ions to glutathione (GSH) is the first step in the detoxification of heavy metals (Freeman et al. 2004), followed by the transfer of metals to other ligands, such as metallothioneins (MTs) and phytochelatins (PCs). Moreover, glutathione is a precursor for phytochelatins. Glutathione synthesis involves two ATP-dependent steps: the first reaction is catalysed by the γ-ECS encoded by *GSH1*, while the second reaction is catalysed by glutathione synthetase encoded by *GSH2*. GSH synthesis in the absence of heavy metals is probably regulated by the availability of cysteine (Noctor et al. 1996).

This project was carried out to specify the lead tolerance mechanisms and to verify a possible usage of the Polish sunflower cultivar called Ogrodowy for lead phytoextraction.

MATERIAL AND METHODS

The research was performed on the most popular commercial sunflower cultivar in Poland. The plants were grown in a greenhouse in a continuously aerated hydroponic culture with modified Hoagland's nutrient solution at 1200-1400 µmol m^{-2} s^{-1} of PAR. Six-week-old plants, in four replicates of four plants each, were exposed to the following doses of $Pb(NO_3)_2 \cdot H_2O$: 0, 15, 45, 60 mg Pb dm^{-3}. In ordere to evaluate the content of Pb in plant tissues, the plants were collected after 72 hours of treatment. The plant material was dried at 105°C, wet digested in $HNO_3 \cdot HClO_4$ (4:1, v/v), and then the lead content was determined in an atomic absorption spectrophotometer JCPS

(Induced Coupled Plasma Spectrophotometer) in an accredited laboratory (Analytical Centre SGGW). The accuracy of determinations was ensured by a control system based on the criteria contained in the standard PN-EN ISO/IEC/17025:2005.

Due to the visible signs of lead intoxication in the plants treated with the highest dose, which may have an impact on the reliability of measurements, physiological, biochemical and molecular analyses were performed on the plants treated with 0, 15, 45 mg Pb dm^{-3}. Three measurements were performed on each of the plants (after 24, 48 and 72 hours of treatment) for plant gas exchange (intensity of photosynthesis and transpiration, stomatal resistance) using LICOR 6200 (LI-COR, Nebraska, USA). Relative water content (RWC) was estimated at the end of the experiment.

For biochemical analysis, the plants were collected after 72 hours of treatment with 0, 15, and 45 mg Pb dm^{-3}. The dissected organs (stems, leaves and roots) were weighed, frozen in liquid nitrogen and stored at -80°C until analysis. The concentration of low molecular weight thiols (GSSG – oxidised glutathione, GSH+GSSG – total glutathione, and their precursor cysteine) was measured by HPLC (Waters Company, System Breeze) using a method developed in the Laboratory of Basic Sciences in Horticulture (Łata et al. 2005). The total concentration of GSH+GSSG was determined in the supernatant after reducing GSSG with DL-dithiothreitol and derivatisation with monobromobimane. Monobromobimane derivatives were detected fluorometrically at 480 nm by excitation at 380 nm. The determination of GSSG was based on the irreversible alkylation of the free thiol group of GSH with N-ethylmaleimid, and the subsequent reduction of GSSG with DTT. During the same analysis, the content of L-cysteine was also measured.

For molecular analysis, the plants were collected after 24, 48 and 72 hours of treatment with 0, 15, and 45 mg Pb dm^{-3}. Total RNA was isolated from organs using the TRIzol Reagent (Gibco BRL). First-strand cDNA was synthesised using the Reverse Transcription System (Promega). Partial cDNA sequences of gene-encoding metallothionein were obtained by PCR with primers: HaMt1 forward ATGTCTTGCTGTGGTGGAAG, HaMt1 reverse TTTGCAGGTACAAGGGTTGC. The number of cycles of PCR gene amplification was 28 and the temperature of the primers' hybridisation was 37°C. The amplified products were separated by agarose gel electrophoresis.

Dry mass gain and lead concentration in whole plants and the translocation factor were evaluated by one-way factorial analysis of variance (Anova I). Dry mass and lead concentration in organs, as well as gas exchange parameters, water-use efficiency parameters, and the distribution of cysteine and glutathione were estimated by two-way factorial analysis of variance (Anova II). The results of the lead content analysis were subjected to logarithmic transformation prior to statistical evaluation. The significance of differences between the means of main effect was evaluated using the Tukey test at $\alpha = 0.05$.

RESULTS AND DISCUSSIONS

Biomass and bioaccumulation of Pb

Significant differences in the dry weight of whole plants were found between those plants treated with 60 mg Pb dm^{-3}, showing a maximum decrease (up 47.1% less than control) in dry weight, and the other plants (Table 1).

When analysing the dry weight of dissected organs, the weight of the leaves was significantly higher than that of the stems and roots (Table 2). The dry mass of the leaves and roots increased by 25.6% and 17.9% respectively following the application of 15 mg Pb dm^{-3}, but the subsequent dose, 45 mg Pb dm^{-3}, did not affect the dry weight of leaves. That dose, however, *did* cause a decrease in the dry mass of roots and stems. At 60 mg Pb dm^{-3},

Table 1

Dry weight and the accumulation of lead in whole plants of *Helianthus annuus* L. after 72 hours of exposure to Pb ions

Specification	Dose Pb (mg dm^{-3})			
	0	15	45	60
Dry mass (g) of whole plants	10.97*b*	11.60*b*	10.17*b*	5.80*a*
mg Pb of whole plants	0.52*a*	12.83*c*	10.68*c*	6.06*b*
mg Pb kg^{-1} d.m. of whole plants	47.3*a*	1105.7*b*	1050.6*b*	1044.9*b*

The means are assigned letters to indicate homogeneous groups. The same letter (in rows) indicates that means are not statistically significant at $\alpha = 0.05$

Table 2

Dry weight of organs and the accumulation of lead in organs of *Helianthus annuus* L., plants after 72 hours of exposure to Pb ions

Organs (A)	0	Dose Pb (mg dm^{-3}) (B)			Mean (A)
		15	45	60	
Dry weight (g)					
Roots	2.40	2.83	1.57	1.20	2.01*a*
Stems	5.30	3.70	3.37	1.90	3.07*a*
Leaves	5.23	6.57	5.23	2.70	4.93*b*
Mean (B)	3.66*b*	4.37*b*	3.39*b*	1.93*a*	LSD(A/B)=2.20 LSD(B/A)=2.42
Accumulation of lead (µg) in organs					
Roots	391.0	12507.1	9480.9	4627.1	6751.5*c*
Stems	85.9	163.2	1029.9	1317.8	649.2*b*
Leaves	41.8	155.8	173.3	115.4	121.6*a*
Mean (B)	172.9*a*	4275.4*d*	3561.4*c*	2020.1*b*	LSD(A/B)=0.84 LSD(B/A)=0.93

The figures marked with the same letter in rows and columns do not differ significantly at $\alpha = 0.05$

all the organs tested showed a decrease in dry weight gain of between 48.4 and 64.2%.

The negative impact of lead on the plant growth and dry weight of *Sesamum indicum* and *Vigna radiata* (Singh et al. 1997), *Phaseolus vulgaris* (Geebelen et al. 2002), *Helianthus annuus* L., *Brassica juncea* L., *Medicago sativa* L., *Ricinus communis* L. (Niu et al. 2007, Zhixin et al. 2009) and *Sedum alfredii* H. (Gupta et al. 2010) was also noted. In the experiments carried out by Kumar et al. (1995), lead caused a decrease in mass in all organs, most significantly in the roots. In contrast, an increase in dry weight with a treatment of heavy ions was also noted. A stimulating effect of root growth and dry weight (hormesis) was observed in *Helianthus annuus* treated with 10^{-5} M Cu (Jiang et al. 2000) and also in *Vicia faba* (Wang et al. 2010).

Interestingly, the mean accumulation of lead in plants treated with different doses of Pb was very similar when re-calculated to mg Pb per kg d.m. of whole plants, ranging from 1044.9 to 1105.7 mg Pb per kg d.m. (Table 1).

The maximum content of Pb was recorded in roots (6.75 mg in the roots of each plant) and the minimum one was recorded in leaves (0.12 mg in the leaves of each plant) – Table 2. The lead content in stems was ten times lower than that in roots, whereas the lead content in leaves was more than five times below the values recorded in stems. The highest content of lead in the aerial parts (23.6% as compared to 76.4% in roots) was noted in plants exposed to 60 mg Pb dm^{-3}, whereas the 45 mg Pb dm^{-3} and 15 Pb dm^{-3} treatments caused an accumulation mostly in roots (88.7% and 97.5% respectively of total absorbed Pb, with the remaining 11.3% and 2.5% transported to the aerial parts) – Table 2. Cunningham and Ow (1996) suggest that good hyperaccumulators can accumulate 1-3% Pb ions in leaves and stems. In that context, *Helianthus annuus* cv. Ogrodowy would appear to be a promising tool for phytoremediation, since the current data showed the capability of *H. annuus* L. to accumulate and tolerate significant quantities of Pb and thus its suitability for phytoremediation. It is thought that plants resistant to metal ions induce oxidative stress *via* the synthesis of cysteine, GSH, thus securing a pool of GSH not only for PC synthesis, but probably also for the antioxidant function. These data are similar to that obtained by Seth et al. (2011).

Physiological parameters: including photosynthesis efficiency and water relations

The Pb dose did not significantly affect the intensity of photosynthesis, but the length of Pb treatment *did* have statistically important effects, expressed as a decrease during the first time period and an increase later on. The maximum decrease in photosynthesis intensity of up to 8.9% was observed 24 hours after the treatment with 45 mg Pb dm^{-3} and 7.2% with 15 mg Pb dm^{-3}, while an increase in the photosynthesis intensity observed later at its maximum (up to 17.0%) was observed in plants treated with

15 mg Pb dm^{-3} for 48 hours. After two and three days of lead treatment, the stimulating effect on the photosynthesis intensity was recorded (Table 3).

Stomatal resistance significantly increased following lead application. The greatest increase, up 95.4%, was recorded at 15 mg Pb dm^{-3} after 72 hours of treatment, and the lowest one appeared at 45 mg Pb dm^{-3} after the same period of exposure. However, the stomatal resistance in plants after two days of treatment was significantly lower than that in plants exposed to lead for one and three days (Table 3).

There are numerous studies reporting on the inhibitory effects of Pb ions on the intensity of photosynthesis and photosystem II (Xiong et al. 2006, Liu et al. 2008, Cenkci et al. 2010, Sing et al. 2010, Bharwana et al. 2013). This inhibition is thought to result from the secondary/indirect effects of lead rather than from the direct effect, *i.e.* the distortion of the chloroplast ultrastructure due to lead's affinity to protein N and S ligands (Weryszko-Chmielewska, Chwil 2005, Islam et al. 2007) and the inadequate concentration of

Table 3

Effect of different lead concentrations and time of treatment on the intensity of photosynthesis and transpiration, stomatal resistance and relative water content of *Helianthus annuus* L. plants

Time (A)	Dose Pb (mg dm^{-3}) (B)			Mean (A)
	0	15	45	
Photosynthesis (μmol CO_2 m^{-2} s^{-1})				
24 h	2.92	2.71	2.66	2.76*a*
48 h	3.52	4.12	3.63	3.75b
72 h	3.66	3.67	4.07	3.80*b*
Mean (B)	3.36*a*	3.50*a*	3.45*a*	LSD(A/B)=0.94 LSD(B/A)=0.94
Stomatal resistance (s cm^{-1})				
24 h	5.16	3.98	7.98	5.69*b*
48 h	3.12	5.48	4.85	4.48*a*
72 h	3.66	7.15	4.81	5.87*b*
Mean (B)	3.98*a*	5.54*b*	5.87*b*	LSD(A/B)=1.81 LSD(B/A)=1.81
Transpiration (μmol H_2O m^{-2} s^{-1})				
24 h	0.80	0.69	0.67	0.72*a*
48 h	0.91	0.86	0.75	0.84*b*
72 h	1.05	0.71	0.78	0.85*b*
Mean (B)	0.92*b*	0.75*a*	0.73*a*	LSD(A/B)=n.s. LSD(B/A)=n.s.
RWC (%)				
Leaves	87.8*c*	83.5*b*	76.1*a*	

The figures marked with the same letter in rows and columns do not differ significantly at $\alpha = 0.05$

carbon dioxide in stomatal closure (Romanowska et al. 2006). In the present research, the inhibition of photosynthesis was observed only 24 hours after Pb treatment. There was no apparent prolonged inhibition of photosynthesis, which may be related to efficient mechanisms of stress defence induced after the first period of exposure. However, it cannot be excluded that the period of exposure was too short or the applied doses were too low to cause permanent damage to the metabolic processes in plants. Nevertheless, the plants treated with 60 mg Pb dm^{-3} were so visibly affected that measurements of physiological parameters would probably not be reliable.

Both the dose and duration of treatment significantly affected transpiration. In Pb-treated plants, lower transpiration and simultaneous increase in stomatal resistance were noted. The decrease of transpiration varied from 5.5% (15 mg Pb dm^{-3}, 48 hours) to 25.7% (45 mg Pb dm^{-3}, 72 hours) and 32.4% (15 mg Pb dm^{-3}, 72 hours) compared with the control (Table 3).

Plants exposed to heavy metals respond by reducing transpiration and increasing stomatal resistance (Stancheva et al. 2014), especially in reaction to cadmium (Poschenreider et al. 1989) and lead (Marchiol et al. 2004, Bharwana et al. 2013). A transpiration decrease leads to a reduction of water uptake intensity, and thus also limits the uptake of metal ions from the substrate, which is a known stress avoidance mechanism. In the present work, the exposure to lead ions decreased the relative water content: in the control plants a very high RWC was observed accompanied by very good cell turgor. Plants treated with 15 and 45 mg Pb dm^{-3} showed a decrease in RWC by approximately 5 and 10% respectively, probably affecting plant turgor as well. Such disorders in plant water management may result from changes of cytoplasmatic membranes induced even by very low concentrations of metal ions (Poschenrieder et al. 1989), where the biological functions of cell membranes may be disabled as a result of a changed permeability to water and ions (Hall 2002).

Metal tolerance: cysteine, glutathione, GSH/GSSG ratio and metallothioneins

The results of the analysis of cysteine and glutathione content are presented in Table 4. The content of these molecules varied significantly in the organs analysed. The lowest mean concentration of cysteine and glutathione (GSSG and GSH+GSSG) was observed in stems, while the highest concentration was found in leaves. The mean content of cysteine did not depend on lead application, whereas the content of GSSG and GSH+GSSG was dependent on the lead pulse.

Generally, in the roots of lead-treated plants the content of cysteine and GSH+GSSG decreased in comparison to the control plants, whereas a reverse relationship was observed in the leaves of plants treated with 60 mg Pb dm^{-3}. The highest decrease of GSH+GSSG content was noted in the roots of plants treated with 45 mg Pb dm^{-3} (50% compared to the control). This

Table 4

Effect of different concentrations of lead treatment on the cysteine and glutathione content and GSH/GSSG ratio of *Helianthus annuus* L. plants

Organs (A)	Dose Pb (mg dm^{-3}) (B)			Mean (A)
	0	15	45	
Cysteine (nmol g^{-1} f.m.)				
Roots	6.70	3.20	0.57	3.49*b*
Stems	1.60	2.23	0.83	1.55*a*
Leaves	5.90	5.87	12.20	7.99*c*
Mean (B)	4.73*a*	3.77*a*	4.53*a*	LSD(A/B)=2.52 LSD(B/A)=2.52
GSSG (nmol g^{-1} f.m.)				
Roots	9.97	10.30	11.30	10.52*b*
Stems	7.27	6.20	5.00	6.16*a*
Leaves	19.70	35.53	98.80	51.34*c*
Mean (B)	12.31*a*	17.34*b*	38.37*c*	LSD(A/B)=7.08 LSD(B/A)=7.08
GSH+GSSG (nmol g^{-1} f.m.)				
Roots	52.8	36.3	26.4	38.5*b*
Stems	21.9	19.8	14.4	18.7*a*
Leaves	69.0	92.7	198.1	119.9*c*
Mean (B)	47.9*a*	49.6*a*	79.6*b*	LSD(A/B)=17.2 LSD(B/A)=17.2
GSH/GSSG				
Roots	4.31	2.54	1.33	2.73*b*
Stems	2.00	2.19	1.91	2.03*a*
Leaves	2.49	1.56	1.01	1.69*a*
Mean (B)	2.93*c*	2.10*b*	1.42*a*	LSD(A/B)=0.88 LSD(B/A)=0.88

The figures marked with the same letter in rows and columns do not differ significantly at $\alpha = 0.05$

could be attributed to the high demand for glutathione due to the increased synthesis of phytochelatins in response to the accumulation of lead in this organ. A similar decrease has also been observed in the roots of *V. faba* and *P. vulgaris* (Piechalak et al. 2002). It has also been reported that a shortage of glutathione and glutathione reductase (observed in response to Cd, Pb and Ni treatment) can limit the uptake of metal ions by roots, thus reducing the toxic effects within the plant (Alkorta et al. 2004, Freeman et al. 2004). The decrease in GSH content may also result from its decreased synthesis due to the lack of available precursors, *i.e.* cysteine, but the extent of the decrease may also be explained by the inactivation of free radicals released due to oxidative stress. When the latter happens, the increase in the oxidated GSSG

form is observed together with a decrease in the oxidative potential of GSH/GSSG (Penugonda, Ercal 2004).

The GSH/GSSG ratio varied from 1.01 (leaves from plants treated with 45 mg Pb dm^{-3}) to 4.31 (control plant roots). In the roots and leaves of lead-treated plants, the content of GSSG increased, whereas GSH/GSSG decreased compared to the control plants. This phenomenon strongly suggests stress conditions induced by the lead treatment.

An increase of cysteine, GSH+GSSG and GSSG was observed in leaves of Pb-treated plants. This increase was accompanied by a decline in GSH/GSSG, and both observations indicated that the plants were defending themselves against oxidative stress, producing antioxidants and the precursor to phytochelatins, GSH. Similar observations in cadmium-treated plants were reported by Schützendübel and Polle (2002) and Gupta et al. (2010). The increased ability of glutathione synthesis appears to be a key factor in heavy ion tolerance mechanisms in plants.

The results presented in this paper indicate that the intensity of biosynthesis and the pool of glutathione and phytochelatins in plant tissues are directly related to plant resistance to heavy ions.

The expression of the gene of metallothionein 1 (*HaMT1*) was observed in stems only (Photo. 1). After 24 and 48 hours of treatment, the expression of *HaMT1* was more pronounced in plants treated with 15 mg Pb dm^{-3} than in the controls, while after 72 hours the expression of this gene was observed in untreated plants only. This suggests that metallothioneins are only involved in Pb detoxification when the stress pulse is low or moderate.

It has already been reported that metallothionein expression is organ-dependent (Zhou, Goldsbrough 1995) and regulated at the transcription level, and may be induced by the presence of hormones, toxins and heavy ions (Mejare, Bulow 2001). In *A. thaliana*, the synthesis of mRNA *MT2* was strongly induced by the presence of Cu, Cd, Zn ions (Zhou, Golsbrough 1994). A family of genes encoding a protein similar to metallothionein (htMT2) was

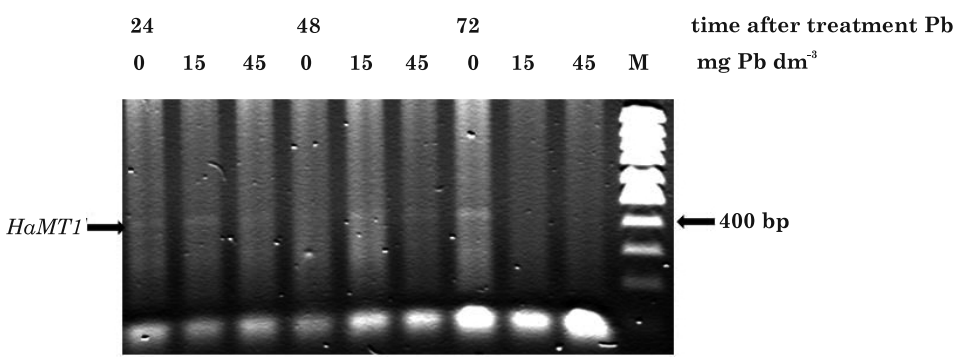

Photo. 1. Effect of Pb treatment on the expression of *HaMT 1* in stems of *Helianthus annuus* L. plants. Effects were evaluated after 24, 48 and 72 hours of exposure to lead (15 and 45 mg Pb dm^{-3}). M- Gene Ruler 100 bp DNA Ladder

identified in *Helianthus tuberosus*. The transcripts of these genes were detected in stems and – at low levels – in leaves, but not in roots, and declined after the plants' exposure to zinc and copper ions (Chang et al. 2004).

The mechanisms of Pb-detoxification include the sequestration of Pb in the vacuole, phytochelatin synthesis and binding to glutathione and amino-acids. Pb tolerance is associated with the capacity of plants to restrict Pb to the cell walls, the synthesis of osmolytes and the activation of an antioxidant defence system. The role of metallothionein is to bind metal ions by cysteine thiol groups (Gavanji et al. 2014).

CONCLUSIONS

1. Sunflower cv. Ogrodowy showed both high accumulation of lead in roots and an effective transport of this element to aerial parts. Sunflower has a high biomass, makes no particular demands on growers and is easy to grow.

2. The lead doses used in this study inhibited water uptake and transpiration. The decline in transpiration was accompanied by an increase in stomatal resistance.

3. Multiple mechanisms were involved in lead tolerance in sunflower cv. Ogrodowy.

4. Changes of cysteine and glutathione levels in plant organs indicated that these molecules played a significant role in lead detoxification and also suggest synthesis of phytochelatins.

5. The expression of metallothionein gene *HaMT1* was only observed in stems.

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