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LEAD TOLERANCE MECHANISMS IN *Robinia pseudoaccacia* L. – AN ATTEMPT TO A PRACTICAL APPROACH

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Abstract. *Robinia pseudoaccacia* plants grown hydroponically were treated $Pb(NO_3)_2$ with 15, 45 mg $Pb^{2+} \cdot dm^{-3}$. After 6, 12, 24, 72 hours of the metal treatment the plants were collected and dissected organs. The plants accumulated and transported to ground part 0.88% and 1.35% of total accumulated lead for the lower and higher dose of Pb^{2+} respectively. The level of GSH was differed and depended on organs, dose and time treatment of Pb^{2+}. We investigate (different pattern of expression) expression of *RpGSH1* and *RpPCS* genes in roots. The study showed that glutathione and genes encoded enzymes connected with synthesis of him, plays important role in the process of detoxification in plant.

Key words: *Robinia pseudoaccacia* (L), lead, glutathione, genes encoded glutathione and phytochelatins

INTRODUCTION

Over the past centuries, rapid growth of population, mining, industrialisation has significantly contributed to extensive soil contamination. Various physical, chemical and biological processes have been employed for effective remediation of contaminated soil. Several species of *Fabaceae* family were the first plants characterized as hyperaccumulators of heavy ions. Further research aim to find other species which could be used in phytoremediation and a special attention is paid to trees, since their advantage is a big biomass and thus also a high ion accumulation capacity [Mertens et al. 2004, Baycu et al. 2006].

Phytoremediation is a cost-effective and non-intrusive alternative to other technologies of remediation of heavy metal contaminated soils. Heavy metal ions, eg. Pb, are taken up by roots, bound and then transported to stems and leaves. Binding the heavy ions by glutathione (GSH) or its homologue homoglutathione (hGSH), is the first step it

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detoxification of heavy metals [Freeman et al. 2004], followed by transfer of metals to other ligands, like metallothioneins (MTs) and phytochelatins (PCs). Glutathione is also an effective scavenger of active oxygen species and free radicals released as a side effect of heavy ions binding and transport. Moreover, glutathine is a precursor for phytochelatins.

Glutathione is biosynthesized in two closely related ATP – dependant reaction: first, L-glutamic acid and L-cysteine are converted to γ-glutamylcysteine in reaction catalyzed by γ-ECS (encoded by *GSH1*), and then glutathione synthetase (encoded by *GSH2*) adds a glycine to γ-glutamylcysteine. GSH synthesis in the absence of heavy metals is probably regulated by availability of cysteine [Noctor et al. 1998].

In this research we aim to specify the lead tolerance mechanisms in false acacia *(Robinia pseudoaccacia)* in regard to and to its possible use in phytoremediation. The research included: the estimation of lead content in organs, studies on the glutathione level changes, as well as analyses of genes, which participate in the synthesis of glutathione, homo- and phytochelatins, metallothioneins and metal transporters.

MATERIAL AND METHODS

Plants were grown in hydroponic culture under controlled conditions during eight weeks. In the experiments macro-components were applied to obtain the following levels (mg· dm^{-3}): 350 N – $Ca(NO_3)_2$, NH_4NO_3, KNO_3, 60 P – KH_2PO_4, K 290 – KNO_3, KH_2PO_4, Mg 50 – $MgSO_4 \cdot 7H_2O$, Ca 200 – $Ca(NO_3)_2$. Micro-components were added in the amounts: 1.98 Mn – $MnSO_4 \cdot H_2O$, 0.3 B – H_3BO_3, 0.18 Cu – $CuCl_2 \cdot 2H_2O$, 0.1 Zn – $ZnSO_4 \cdot 7H_2O$, 0.2 Mo – $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$. Three concentration of $Pb(NO_3)_2$ were used 0, 15, 45 mg $Pb^{2+} \cdot dm^{-3}$. Plants were collected 6, 12, 24, 72 hours after treatment, dissected organs were weight and stored in -80°C for analysis.

The plant material was dried at 105°C, were wet digested in $HNO_3 \cdot HClO_4$ (4:1, v/v) [H_2O_2], and the content of lead was determined by atomic absorption spectrophotometer JCPS (Induced Coupled Plasma Spectrophotometer).

The concentration of glutathione was estimated by method of Akerboom and Sies, [1981].

Total RNA was extracted from 50 mg of each tissue grinded in liquid nitrogen according to the protocol from the TRIzol Reagent (Gibco BRL). Purity and quantity of isolated RNA was verified spectrophotometrically (BioPhotometer Eppendorf). RNA was stored at –80°C for further analyses. First – strand cDNA was synthesized with the use of Reverse Transcription System (Promega) at 42°C for 50 min and then the reaction was terminated by denaturing at 99°C for 5 min. Partial cDNA of genes encoding: synthetase γ-glutamylcysteine, synthetase glutathione, synthase phytochelatin, synthase homophytochelatin, metallothioneins and transporters of heavy metal, were amplified by PCR with the use of primers given in table 1, under conditions as follows: 30 cycles with annealing temperature as indicated in table 1, 30 sec denaturation, 30 sec of extension and a final 10 min elongation at 72°C. Primers were designed according to sequences these genes forward in literature in differed plants. The amplified DNA was resolved by agarose gel electrophoresis.

Table 1. Primers used in PCR and RT-PCR:
Tabela 1. Startery wykorzystane w technice PCR i RT-PCR

Name Nazwa	Sequence 5’→3’ Sekwencja 5’→3’	Annealing temp Temp. przyłączania		Mass of amplified fragment (bp) Wielkość produktu (pz)
		DNA	cDNA	
Primers used for amplified genes encoding metallothioneins Startery do powielenia fragmentów genów metalotionein				
AtMT2aF (1,a)*	TCTTGCTGTGGAGGAAACTG	37°C		246
tMT2aR (1,a)*	CAAGGATCACACACTTGCAGTC	37°C		246
AtMT2bF (1,a)*	ATGTCTTGCTGTGGTGGAAG	40°C		234
AtMT2bR (1,a)*	TTTGCAGGTACAAGGGTTGC	40°C		234
AtMT3 (2,a)	TGTGGATGTGGCTCCTCCT	45°C	37°C	250
AtMT4 (2,a)	CAAGAATTCCATCAACAGTTACAGTTTGAC	45°C		250
AtMT5 (2,a)	GCCTCATATGGCAGATTCTAACTGTGGATG	43°C		260
AtMT6 (2,a)	GGTTTGAGACCATGCTG	43°C		260
Primers used for amplified genes encoding synthetase γ-glutamylcysteine and synthetase glutathione Startery użyte do powielania fragmentów genów kodujących syntetazę γ-glutamylocysteinową oraz syntetazę glutationową				
Gsh1F (1,b)*	GAGTTACTCAACAGCATCGC	40°C		600
Gsh1R (1,b)*	CCGAAGCATCATATCAAGTC	40°C	37°C	600
Gsh2F (3,c)*	GCTGATTTTCGTTCCACT	48°C		617
Gsh2R (3,c)*	CCCAACCGTATTTCCTCT	48°C		617
Primers used for amplification of genes encoding: synthase phytochelatin and synthase homophytochelatin Startery użyte do powielenia fragmentów genów kodujących: syntazę fitochelatynową i syntazę homofitochelatynową				
AtPCS1 (1,a)*	CTATGGCGAGTTTATATCGG	43°C		424
AtPCS2 (1,a)*	CTCAACTTTTGCTCCTGAAC			424
AtPCS3 (1,a)*	AGTTGTCTGTTTGGCTCA	37°C		466
AtPCS4 (1,a)*	CTTCCTTCAAATACTTGGC			466
AtPCS5 (1,a)*	CTGCAAGGATGAAAGCTG	41/37°C	37°C	446
AtPCS6 (1,a)*	CAAGTTTCCCGACAACAA	41/43°C		446
AtPCS8 (1,a)*	GAGAGGATTTGGGACAAT	37°C		466
GmhPCS1F (4,d)	TGG AA(A/G) GG(A/C/G/T) (C/T)C(A/C/G/T) TGG (A/C)G(A/C/G/T) TGG	44°C	37˚C	330
GmhPCS1R (4,d)	G(A/G)TA(C/T)TT(A/G)AA(A/C/G/T)C(T/G)(A/C/G/T)GC(A/C/G/T)AC(A/G)	44°C		330
Primers used for amplification of genes encoding transporters Startery użyte do powielenia fragmentów genów kodujących białka transportowe				
ZntAF (1,e)*	GAGGTACCAAAGCAGGCTATTCCCTG			628
ZntAR (1,e)*	CTTCGAATTCTCTCCTGCGCAACAATC			628
HMA4F (1,a)*	GGCGTTACAAAACAAAGAAG			760
HMA4R (1,a)*	TCGTCTACCTCACAGTTTCC			760
HMA1F (1,a)*	TGGAACCTGCAACTCTTACT			760
HMA1R (1,a)*	ATATTTGGAACATTGCCATT	44˚C	37°C	760
AtNramp3F (1,a)*	AATGCCACAACTCGAGAACAACG			1248
AtNramp3R (1,a)*	GAAAGCTAAGAACTCATGATCCTAAGG			1248
LCT1F (1,f)*	TGGTCGCCTCCAAGTGCCTA			510
LCT1R (1,f)*	AACGCGAACGATGCTGCGAA			510
HMT1F (1,g)*	CGGTGATTTTGTCATAC			888
HMT1R (1,g)*	TTGTTGAAACCACATTT			888
ZNT1aF (1,h)*	GCTTCATCTCCACGAAA			1145
ZNT1aR (1,h)*	TAAGCCCAAATGGCGAGT			1145
ZNT1bF (1,h)*	AATGCTCATGGTACATTTCG	43°C	37˚C	578
ZNT1bR (1,h)*	GACATAAGTCCAGCTCCAA			578
ZNT1a` R (1,h)*	TGTACATGTCCGTGTGATTG			1145

*primers designed by Primer 3
*startety zaprojektowane z wykorzystaniem Programu Primer 3

Primers according to 1) Wińska-Krysiak 2000, 2) Zhou i Goldsbrough 1995, 3) Moran et al., 2000, 4) Oven et al., 2002
Startery według 1) Wińska-Krysiak 2000, 2) Zhou i Goldsbrough 1995, 3) Moran i in., 2000, 4) Oven i in., 2002

Primers designed on the basis of known nucleotide sequence of a) gene *A. thaliana,* b) gene *B. juncea,* c) gene *P. sativum,* d) gene *G. max,* e) gene *E. coli,* f) gene *T. aestivum,* g) gene *S. pombe,* h) gene *T. caerulescens*
Sekwencje starterów zaprojektowano na podstawie znanej sekwencji a) genu *A. thaliana,* b) genu *B. juncea,* c) genu *P. sativum,* d) genu *G. max,* e) genu *E. coli,* f) genu *T. aestivum,* g) genu *S. pombe,* h) genu *T. caerulescens*

The results achieved in the experiment were statistically evaluated with t-Tukey test at significance of 5%.

RESULTS AND DISCUSSIN

No significant differences were found in the dry weight of the treated and control plants (tab. 2). Treated plants have shown either visible signs of lead intoxication.

Table 2. Dry mass of *Robinia pseudoaccacia* (mg · plant^{-1}) grown in a hydroponic culture with different level of lead
Tabela 2. Sucha masa roślin *Robinia pseudoaccacia* (mg · roślina^{-1}) rosnących na pożywkach zawierających różne poziomy ołowiu

	Dose – Dawka Pb^{2+} mg · dm^{-3}			Mean – Średnio (A) NIR (A) = 384.60
	0	15	45	
Dry mass of *Robinia pseudoaccacia*	930.00 (A)	845.00 (A)	1030.67 (A)	935.22

In regard to the accumulation of lead (µg · g^{-1} s.m.) in organs of *Robinia pseudoaccacia,* plants treated with different dose of lead could be classified as separately statistically homogenous group. Almost 99% of total absorbed Pb was accumulated in roots of plants exposed to lead treatment, with the highest accumulated rates at 45 mg Pb^{2+} · dm^{-3}, the remaining 1.35% (dose 15 Pb^{2+} · dm^{-3}) and 0.88% (45 Pb^{2+} · dm^{-3}) of the total lead was transported to the over-ground parts (tab. 3). According to Cunningham and Ow [1996] as a good hiperaccumulators might be classified plants accumulating 1–3% ions of Pb in leaves and steams. As such, *Robinia pseudoaccacia* appears to be a promising tool for phytoremediation.

Table 3. The accumulation of lead (µg · g^{-1} s.m.) in organs of *Robinia pseudoaccacia*
Tabela 3. Akumulacja ołowiu (µg · g^{-1} s.m.) w zależności od organu u *Robinia pseudoaccacia*

	Dose Dawka Pb^{2+} mg · dm^{-3}	Roots Korzenie	Steams Łodygi	Leaves Liście	Mean – Średnio (B) NIR(B) = 0.50
Robinia pseudoaccacia	0	6.55	3.52	0.92	3.66 (B1)
	15	1297.52	12.60	3.55	437.89 (B2)
	45	24897.44	84.74	45.01	8342.40 (B3)
Mean Średnio (A) NIR (A) = 0.50		8733.84 (A1)	33.62 (A2)	16.49 (A3)	

Leaves of *Robinia pseudoaccacia* treated the highest dose of Pb^{2+} accumulated nearly two times more lead ions them stems (tab. 4). In contrary, similarly treated *Fabaceae* species usually contained more lead in stems than leaves.

Table 4. Accumulation of lead (μg Pb per for the total dry weight) in organs of *Robinia pseudoaccacia*
Tabela 4. Akumulacja ołowiu w μg na całkowitą suchą masę poszczególnych organów u *Robinia pseudoaccacia*

	Dose Dawka Pb^{2+} mg · dm^{-3}	Roots Korzenie	Steams Łodygi	Leaves Liście	Mean – Średnio (B) NIR (B) = 0.37
Robinia pseudoaccacia	0	0.97	0.60	0.65	0.74 (B1)
	15	299.45	2.33	1.76	101.18 (B2)
	45	6202.13	18.76	36.82	2085.90 (B3)
Mean Średnio (A) NIR (A) = 0.37		2167.52 (A1)	7.23 (A2)	13.08 (A2)	

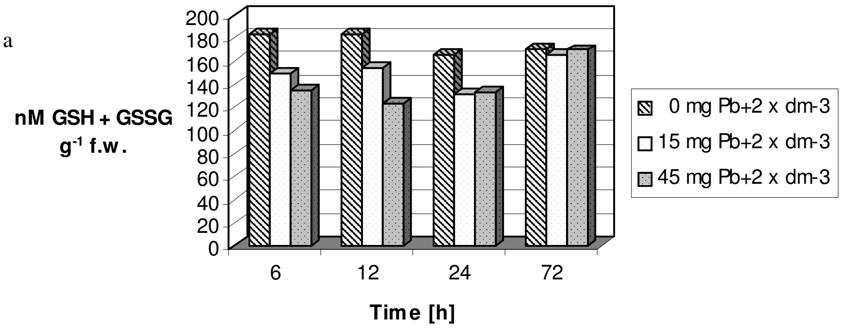
Level of glutathione (fig. 1), was different depending on the organ, and dose and time of exposure to Pb^{2+}. Glutathione content in untreated plants was determined at the level of 180 nM · g^{-1} f.w. in stems and roots and 75 nM · g^{-1} f.w. in leaves. In leaves and stems the highest glutathione level was detected after 3 days of lead treatment and the lowest after 12 hours. The mean content in roots was unaffected by time of treatment. Lead treatment has led to the decrease of GSH+GSSG content, except the leaves of plants treated for 24 and 72 hours with 45 mg Pb^{2+} · mg^{-1}, where it was almost doubled as compared to control plants. This can be attributed to high demand of GSH due to synthesis of phytochelations in response to high accumulation lead in this organs. Similar decrease of glutathione was also observed in roots of *Lupinus luteus* seedling [Tomaszewska and Piechalak 1997], *V. faba* and *P. vulgaris* [Piechalak et al. 2002] and *Sinapis alba* [Wińska-Krysiak 2000].

Expression of genes *RpGSH2, RpMT* and encoding heavy metal transporters in all organs of *Robinia pseudoaccacia* was not detected, possibly due to differences in the nucleotide sequences between genes of *Robinia* and species serving as a template for primers design.

Expression of *RpGSH1* and *RpPCS* was observed only in roots, however it the first observation of expression of gene encoding phytochelatin synthase in *Robinia pseudoaccacia*. Transcript level of *RpGSH1* has reflected the time and dose of lead treatment: the highest level was observed after 72 hours treatment with the highest Pb^{2+} dose applied. Surprisingly, 24 hours of treatment did not induce transcription of this gene (phot. 1).

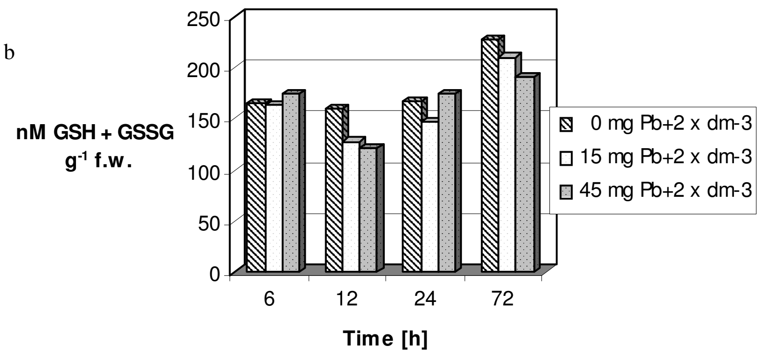
Studies of Bergman and Rennenberg [1993] and Rüegsegger et al. [1990] revealed that synthesis of phytochelatins in cadmium treated *Brassica juncea* is accompanied by increased activity of enzymes: γ-glutamylcysteine synthetase and glutathione synthase and requires glutathione as PC precursor. Increase of transcription of genes encoding these two enzymes was observed by Schäfler et al. [1997] in mustard plants treated with copper and zinc but not lead. In the previous study of Wińska-Krysiak [2000], expression of *gsh1* was shown in leaves and roots of *Brassica juncea* and roots of *Sinapis alba* in respose to Pb treatment. Tomaszewska et al. [1996] has observed a decrease of glutathione and γ-glutamylcysteine synthetase accompanied with increase of phytochelatins synthesis in Pb-treated *Lupinus*.

a



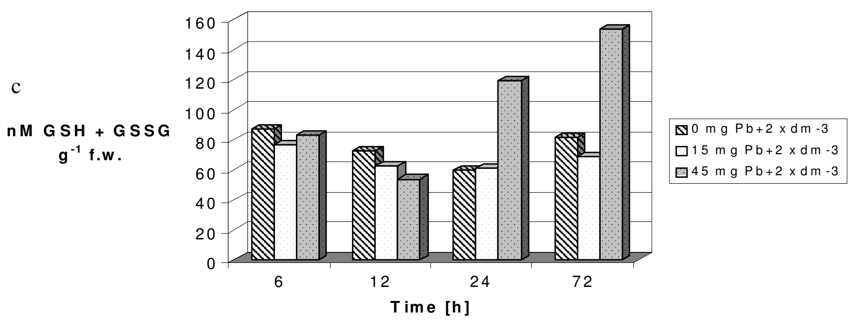
A – dose – dawka, NIR = 26.37, B – time – czas, NIR = 33.50

b



A – dose – dawka, NIR = 19.38, B – time – czas, NIR = 24.62

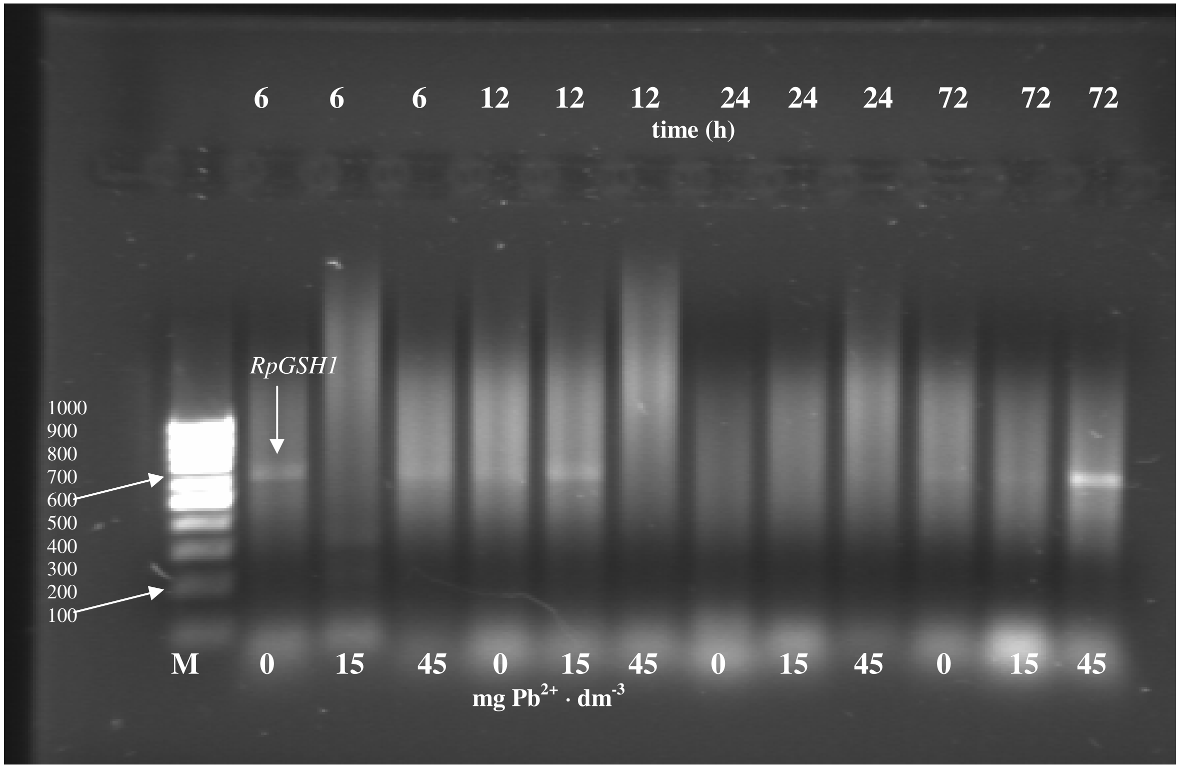
c



A – dose – dawka, NIR = 20.64, B – time – czas, NIR = 26.22

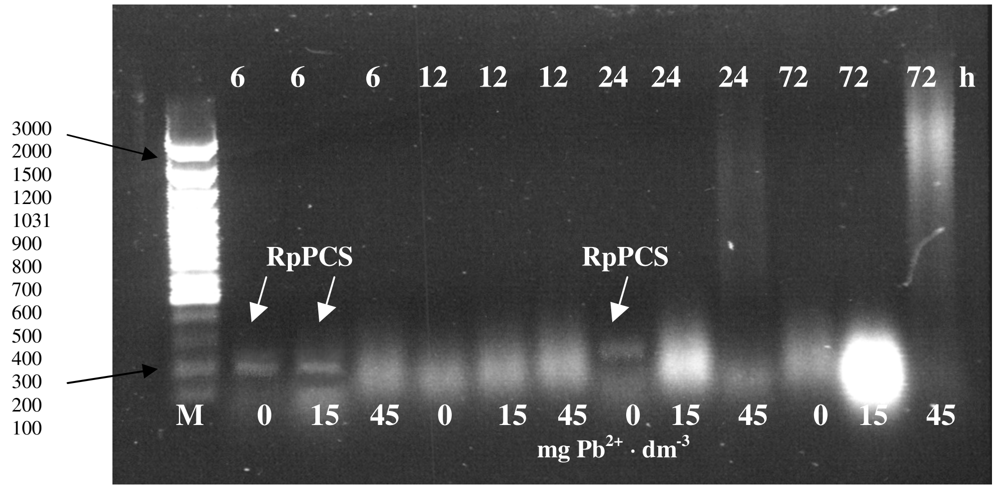
Fig. 1. Content of GSH+GSSG in organs (a – roots, b – steams, c – leaves) of *Robinia pseudoaccacia* after 6, 12, 24, 72 hours of treatment with different dose of lead

Rys. 1. Zawartość GSH + GSSG w organach (a – korzeniach, b – łodygach, c – liściach) *Robinia pseudoaccacia* po 6, 12, 24, 72 godzinach od traktowania różnymi dawkami ołowiu



Phot. 1. The electrophorogram of the amplified fragments of cDNA of genes encoding γ-glutamylcysteine synthetase (primers Gsh1F and Gsh1R) in *Robinia pseudoaccacia* roots, M – 100 bp DNA Ladder

Fot. 1. Elektroforegram rozdziału powielonych fragmentów cDNA genu kodującego syntetazę γ-glutamylcysteinową (startery Gsh1F i Gsh1R) w korzeniach *Robinia pseudoaccacia*, M – 100 bp DNA Ladder



Phot. 2. The electrophorogram of the amplified fragments of cDNA of genes encoding of γ-phytochelatin synthase (primers AtPCS 5 and AtPCS 6) in *Robinia pseudoaccacia* roots, M – 100 bp DNA Ladder

Fot. 2. Elektroforegram rozdziału powielonych fragmentów cDNA genu kodującego syntazę fitochelatynową (AtPCS 5 i AtPCS 6) w korzeniach *Robinia pseudoaccacia*, M – 100 bp DNA Ladder

Glutathione and its homologues are required as precursors for the synthesis of phytochelatins by phytochelatin synthase. However biosynthesis of PCs must be activated by heavy metal ions.

The expression of a gene encoding phytochelatin synthase was observed in roots of Robinia after 6 and 24 hours of lead treatment. Noteworthy, significant decrease of glutathione level has also occurred after 6 hours of treatment. These results suggest a link between accumulation of lead ions in *Robinia* tissues and synthesis of PCs.

Induction of phytochelatin synthase and biosynthesis of phytochelatins in response to Pb ions was found in *Lupinus luteus*, *Vicia faba* and *Phaseolus vulgaris* by Tomaszewska and Piechalak [1997] and Piechalak et al. [2002].

It should be emphasized that efficacy of heavy ions detoxication depends both on bounding them by phytochelatins and on transport of these complexes to vacuole. As such, the more interest should paid to mechanisms and pathways of transporters involved in this process. However, the high transporters specifity to certain heavy ions and little knowledge on nucleotide sequences of transporters encoding genes.

The received results confirm the existence of certain mechanism responsible for tolerance for Pb ions by *Robinia pseudoaccacia.* This tree, which commonly grows in polluted urban areas may be recommended for the techniques of phytoremediation.

CONCLUSIONS

1. The plants untreated and treated of lead did not shown important, relevant differences in dry mass.

2. Lead content in the studied organs were significantly higher in roots than steams and leaves.

3. The accumulation of Pb^{2+} in over-ground parts of *Robinia pseudoaccacia* treated of dose 15 $Pb^{2+} \cdot dm^{-3}$ and 45 $Pb^{2+} \cdot dm^{-3}$ were in 1.35% and 0.88% respectively. This plant is a good candidate for phytoremediation.

4. The level of glutathione in *Robinia pseudoaccacia* differed and depended on the organ, the dose and the time of exposure to Pb^{2+}.

5. Increase of level of glutathione in leaves plants after 72 hours of treated the highest dose of Pb can be connect with high accumulation of this element in this organ.

6. Expression of *RpGSH1* and *RpPCS* was detected in roots of *Robinia pseudoaccacia.*

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MECHANIZMY TOLERANCJI NA OŁÓW U *Robinia pseudoaccacia* L. I ICH ASPEKT PRAKTYCZNY

Streszczenie. Rośliny robinii akacjowej uprawianej w hydroponice traktowane były $Pb(NO_3)_2$ w ilościach: 0, 15, 45 mg $Pb^{2+} \cdot dm^{-3}$. Materiał roślinny zbierano po 6, 12, 24 i 72 godzinach od traktowania ołowiem i rozdzielono na organy. Procent pobranego ołowiu w przeliczeniu na suchą masę roślin przetransportowany do części nadziemnej wynosił 0,88 dla najwyższej dawki traktowania oraz 1,35 dla niższej. Poziom glutationu był zróżnicowany i zależał od badanego organu, dawki i czasu od potraktowania ołowiem. Odnotowano zróżnicowaną ekspresję *RpGSH1* i *RpPCS* w korzeniach roślin. Badania wykazały, że glutation i geny szlaku jego biosyntezy odgrywają ważną rolę w procesie detoksykacji ołowiu w roślinie.

Słowa kluczowe: *Robinia pseudoaccacia* (L), ołów, glutation, geny kodujące glutation i fitochelatyny

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