Environmental and Experimental Botany 75 (2012) 188–194

Contents lists available at SciVerse ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

Glutathione plays a role in protecting leaves of *Salvinia minima* from Pb^{2+} damage associated with changes in the expression of SmGS genes and increased activity of GS

Neyi E. Estrella-Gómez [a], Enrique Sauri-Duch [b], Omar Zapata-Pérez [c], Jorge M. Santamaría [a,*]

[a] Laboratorio de Fisiología Vegetal Molecular, Unidad de Biotecnología, Centro de Investigación Científica de Yucatán. Mérida, Yucatán, México
[b] Instituto Tecnológico de Mérida, Mérida Yucatán, México
[c] Departamento de Recursos del Mar, Cinvestav-Unidad Mérida, Mérida, Yucatán, México

ARTICLE INFO

Article history:
Received 20 August 2010
Received in revised form 30 August 2011
Accepted 4 September 2011

Keywords:
Aquatic fern
Lead accumulation
GSH
GS
SmGS gene
Heavy metal
Chlorophylls

ABSTRACT

The relationship between accumulation of Pb^{2+} and changes in GSH biosynthesis was analyzed in both leaves and roots of the Pb^{2+}-hyperaccumulator aquatic fern *Salvinia minima*, after exposure to 40 μM $Pb(NO_3)_2$. Lead accumulation in both tissues increased the accumulation of GSH, increased the enzymatic activity of glutathione synthase (GS), and caused changes in the expression levels of *Sm*GS genes in both tissues. The damage caused by Pb on plant performance, was evaluated by the changes in the content of pigments, particularly on the carotenoids content. Lead accumulation caused more damage in roots than in leaves as indicated by the decrease on their carotenoids content. It is interesting that in leaves, the concentration of GSH, the activity of GS and the expression levels of *Sm*GS gene were all higher than in roots. These results, together with our previous finding that roots accumulated more phytochelatins than did leaves of *S. minima* plants exposed to similar concentrations of lead (Estrella et al., 2009), suggest that the Pb-hyperaccumulator aquatic fern, *S. minima*, displays a coordinated and differential response to Pb^{2+} at leaves and roots, where GSH may play an important role in protecting leaves from the detrimental effects of lead, perhaps by counteracting the effect of free radicals.

1. Introduction

Gluthatione (γ-L-glutamyl-L-cysteine-L-glycine; GSH) is a tripeptide distributed extensively in the majority of cells; it is a low molecular weight non-protein compound with antioxidative properties. GSH is synthesized in two steps. The first step involves the formation of γ-L-glutamyl-L-cysteine (γ-EC) from L-glutamate and L-cysteine, this step is catalyzed by the enzyme γ-glutamylcysteine synthetase (γ-EC, EC 6.3.2.2.) while the second step is the conjugation of γ- L-glutamyl-L-cysteine with glycine; this step is catalyzed by the enzyme glutathione synthase (GS, EC 6.3.2.3.) (Meister, 1995). The biosynthesis of GSH involves sulfur assimilation in the form of sulphate (Leustek et al., 2000). Glutathione participates in a variety of activities such as signal transduction pathways and cellular defense (Noctor and Foyer, 1998), as the substrate of GSH-S-transferase, which catalyzes the conjugation of GSH with xenobiotic substances, such as weed killers (Marrs, 1996). In addition, GSH actively participates in the redox balance preventing the accumulation of free radicals and hence reducing oxidative stress. Evidence exists for the participation of GSH in stress caused by exposure to heavy metals. In cell cultures of tomato and tobacco, GSH concentration increased significantly in the presence of Cd^{2+} (Chen and Goldsborough, 1994); a similar behavior was observed in roots of maize (Rüegsegger and Brunold, 1992). On the other hand, changes in the expression of the gene glutathione synthase (GS) have been documented in *Arabidopsis thaliana* and *Schizosaccharomyces pombe* in response to Cd^{2+} (Harada et al., 2002; Kim et al., 2003). Glutathione is also the immediate precursor of phytochelatins (PC). Accumulation of PC is considered to be an important mechanism of heavy metal detoxification in terrestrial plants (Xiang et al., 2001) and aquatic plants such as *Salvinia minima*, where the presence of Pb^{2+} increases the production of this cysteine rich polypeptide (Estrella et al., 2009).

S. minima is an aquatic fern capable of removing and accumulating heavy metals which exhibits high growth rate and elevated biomass production and is easy to handle and collect from water body surfaces (Estrella et al., 2009). Each unit consists of three fronds, two floating fronds (leaf-like fronds) and a submerged modified frond (root-like frond). The abaxial surface of the floating fronds has abundant structures similar to absorbent hairs, which are in direct contact with water. Leaf-like fronds are referred to in this paper as "leaves", while root-like fronds are referred to in this paper as "roots". Under controlled culture conditions, *S. minima* removes 82% of Cd^{2+} (10.9 ppm) and 97% of Pb^{2+} (9.7 ppm)

* Corresponding author. Tel.: +52 01 999 9428330; fax: +52 01 999 9813900.
E-mail address: jorgesm@cicy.mx (J.M. Santamaría).

0098-8472/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.envexpbot.2011.09.001

added to the culture, and, in fact, it has been described as a hyperaccumulator for Pb^{2+} (Olguín et al., 2002).

Therefore, in the present paper, an attempt is made to establish whether a relationship exists between the capacity of *S. minima* plants to accumulate and tolerate Pb^{2+}, and their capacity to increase their GSH production, GS activity, and/or modify the expression of *S. minima* gene encoding GS (*SmGS*). This study should contribute to an increased understanding of the possible participation of GSH in a part of a lead detoxification mechanism.

2. Materials and methods

2.1. S. minima growing culture

Plants of the aquatic fern *S. minima Baker* (Salviniaceae) ecotype Yucatán, were cultivated in hydroponics conditions in a modified Hoagland's solution (Hoffmann et al., 2004), at 25 ± 2 °C, in a greenhouse with a photon flux density that varied from 25 to 120 μmol m^{-2} s^{-1}, a relative humidity of 70 ± 3.6%, and with a natural light photoperiod of 12 h.

2.2. Determination of internal Pb^{2+} concentrations in tissues

From our previous work, we were expecting early responses of *S. minima* to lead, so samples were taken every 3 h during the first 12 h (0, 3, 6, 9, 12), later we took samples at day 1 and 2 (24, 48 h) with the objective of evaluating later responses, while the last point was taken to define if the trends found at earlier times would remain after 5 days of exposure. At each sampling time, plants from each treatment were washed with 10 mM EDTA pH 8.0 followed by a rinse with de-ionized water, to remove external metal ions. Pb^{2+} was quantified according to Hoffmann et al. (2004).

2.3. Determination of Pb^{2+} removal

The quantification of Pb^{2+} in the solution was made on 3 mL of medium in which *S. minima* plants were exposed to 40 μM of $Pb(NO_3)_2$ for 0, 3, 6, 9, 12, 24, 48 and 120 h and subsequently analyzed in an atomic emission spectrometer inductively coupled to plasma (ICP-IOES 400 PERKIN ELMER). The detection of Pb^{2+} was carried out by readings at 220 and 353 nm using 1% HNO_3 as blank and spectrometer calibration standards of 100.0, 10.0, 1.0 and 0.1 ppm of Pb^{2+} (Merck) (Hoffmann et al., 2004).

2.4. Quantification of chlorophyll concentration

The chlorophyll content determination was performed according to the method described by Dere et al. (1998). One gram of fresh tissue was crushed in a mortar, adding 20 mL of acetone 99.9% (Sigma) for extracting pigments. Subsequently, in order to separate the cellular tissue of the pigments, the extraction solution was filtered using a Whatman # 1 filter. The filtrate was recovered and brought up to 50 mL with acetone. Finally, spectrophotometric readings were made at absorbances of 662, 645 and 470 nm with a visible light lamp using a spectrophotometer DU 650 (Beckman Coulter). The concentration of each pigment was reported as mg g dw^{-1} and calculated as:

$$Ca = 11.75\,(A662) - 2.350(A645).$$
$$Cb = 18.61\,(A645) - 3.960\,(A662).$$
$$Ct = 1000\,(A470) - 2.270\,(Ca) - 81.4\,(Cb)/227.$$

where *Ca*, Chlorophyll *a*; *Cb*, chlorophyll *b*; *Ct*, total carotenoids; A662, A645 and A470 nm, readings of absorbance's at these wavelengths.

2.5. Quantification of gluthatione (GSH)

Extraction of GSH was carried out by a modification of the method of Grill et al. (1991), using 250 mg of freeze-dried tissue (roots or leaves). Freeze-dried material was ground in a mortar in 1.5 mL of 50 mM Tris (Sigma) pH 7.0, 100 mM ascorbic acid (Sigma), 1 mM DTT (Sigma). This mixture was homogenized for 1 min. The samples were centrifuged at 15 000 × *g* for 30 min at 4 °C. An aliquot of supernatant (350 μL) was derivatized with 100 μL 0.5 M HEPES pH 8.0, 0.5 μL 0.5 M EGTA, pH 8.0 and 1 μL 100 mM monobromobimane (Fluka, St. Louis, MO, USA). The tubes were incubated at 30 °C for 12 h in the dark. Finally, the reaction was stopped by adding 50 μL of 30% trichloroacetic acid and shaking the tubes for 5 min. The samples were filtrated through Millex filters of 0.45 μm pore diameter (Millipore, Jaffrey, NH, USA) and injected (20 μL) into a high performance liquid chromatography apparatus (HPLC-200, USA) equipped with a fluorescence detector. The metabolites were separated on a C18 reverse phase column (Phenomenex 6 nm, 5 mm, and 3.9 mm × 100 mm) and using a linear gradient of methanol–acetic acid pH 3.9. The L-cysteine (Sigma, USA) and the glutathione (Sigma, USA) were used for identification and curve calibration.

2.6. Quantification of proteins

Total protein content was quantified using the method of Bradford (1976). A calibration curve was constructed using dilutions from an initial solution of 1 mg/mL of bovine serum albumin (BSA, Sigma).

2.7. Activity of glutathione synthetase (GS)

The activity of glutathione synthetase (GS) was determined through the modified method of Kim et al. (2003). The assay is based on measuring the formation of adenosine diphosphate (ADP) spectroscopically. The reaction mixture contained 100 mM Tris–HCl pH 7.8, 100 mM potassium chloride (KCl, Sigma), 5 mM adenosine triphosphate (ATP, Sigma), 50 mM magnesium chloride ($MgCl_2$, Sigma), 10 mM glycine, 5 mM γ-L-glutamyl-L-cysteine (γ-EC, Sigma), 0.4 mM phosphoenolpyruvate (Sigma), 0.2 mM nicotinamide adenine dinucleotide (NADH, Sigma), 5 μL of pyruvate kinase (5 U/μL, Sigma), 2.5 mM lactate rabbit muscle type II (Sigma). The reaction was initiated by adding 400 μg total protein and incubated for 2.5–30 min at 37 °C. The amount of ADP was calculated by the change in the readings obtained at an absorbance of 340 nm with 100 μL (1 U/μL) of lactate dehydrogenase.

2.8. Isolation of RNA and semi-quantitative PCR analysis of SmGS

Total RNA was isolated from roots and leaves using CTAB and PVP in the extraction buffer and LiCl for RNA precipitation, according to the method described by Gasic et al. (2004). The purity of RNA samples and their concentrations were determined spectrophotometrically, where the A260/A280 ratios obtained were between 1.8 and 2.0. Five micrograms of total RNA were reverse transcribed into cDNA by using 1 μL Superscript RT II (50 U/μL) (Invitrogen, CA, USA) and incubated for 90 min at 42 °C.

The analysis of the expression of glutathione synthetase was performed by PCR using the following amplification program: 95 °C for 2 min, 49 °C for 2 min, 72 °C for 1 min in 38 cycles. The primers utilized to carry out the amplification of the fragment of *SmGS* were 5′-CCAGACAGTTGCTGTGGTGT-3′ (Forward, Integrated DNA Technologies) and 5′-ACCATCACGGACCAGAAAAG-3′ (Reverse, Integrated DNA Technologies). The PCR contained 2 μg of ADNc 1.0 pmol of primers (forward and reverse), 1 μL of 40 mM $MgCl_2$ (Invitrogen) and 25 μL of PCR supermix High fidelity

Table 1
Concentration of lead (Pb^{2+}) found at various times, in the solution, and in leaves and roots from plants of *Salvinia minima* exposed or not, to aqueous solutions containing 40 μM of $Pb(NO_3)_2$.

Exposure time	Conc. Pb^{2+} in solution ($nmol\ Pb^{2+}\ (mL)^{-1}$)	Leaves without Pb^{2+}	Leaves with Pb^{2+}	Roots without Pb^{2+}	Roots with Pb^{2+}
			($nmol\ Pb^{2+}\ (mg\ dw)^{-1}$)		
0 h	4.560 ± 0.165	0	0	0	0
6 h	2.637 ± 0.120	0	0.120 ± 0.0077	0	1.71 ± 0.127
12 h	0.550 ± 0.176	0	1.01 ± 0.097	0	2.39 ± 0.320
24 h	0.395 ± 0.161	0	1.21 ± 0.021	0	3.019 ± 0.570
120 h	0.354 ± 0.197	0	1.431 ± 0.034	0	3.121 ± 0.619

Values are means ± S.D. of two independent experiments, each with 3 replicates.

(Invitrogen). Sterile water was used as a negative control reaction mixture. The PCR products were separated by electrophoresis using a 1% (w/v) agarose gel; the gels were stained with ethidium bromide and visualized under UV light. Once the total RNA with a high degree of purity was obtained, the first strand DNA was synthesized, and control reactions were performed in the absence of sample, showing absence of contamination bands.

Using the primers mentioned above, a fragment of 478 bp was obtained and amplified. The PCS fragment was purified by using the QIAEX II gel extraction kit (QIAGEN, CA, USA), and it was sequenced at Macrogen Inc. (Seoul, Korea). Virtual translations of the cDNA sequences were analyzed by the BLAST program (National Center for Biotechnology Information, Bethesda, MD, USA) (Altschul et al., 1990) to identify similarities in the GenBank database: *MsGS* 84% (*Medicago sativa* CAL69591), *ZmGS* 80% (*Zea mays* CAE18179), *HvGS* 80% (*Hordeum vulgare* ABC17624), *OsGS* 80% (*Oryza sativa* NP001066914) *TaGS* 79% (*Triticum aestivum* CAE18176) and *LoGS* 75% (*Lotus orniculatus* AF279703). A highly conserved sequence (DYPSEAEWSVRLL.—AIEKPLFVLKPQREGG) was observed in this search, that may correspond to the catalytic domains of the enzyme. The partial sequence of *SmGS* cDNA is available at the GenBank with the accession number EU247453.

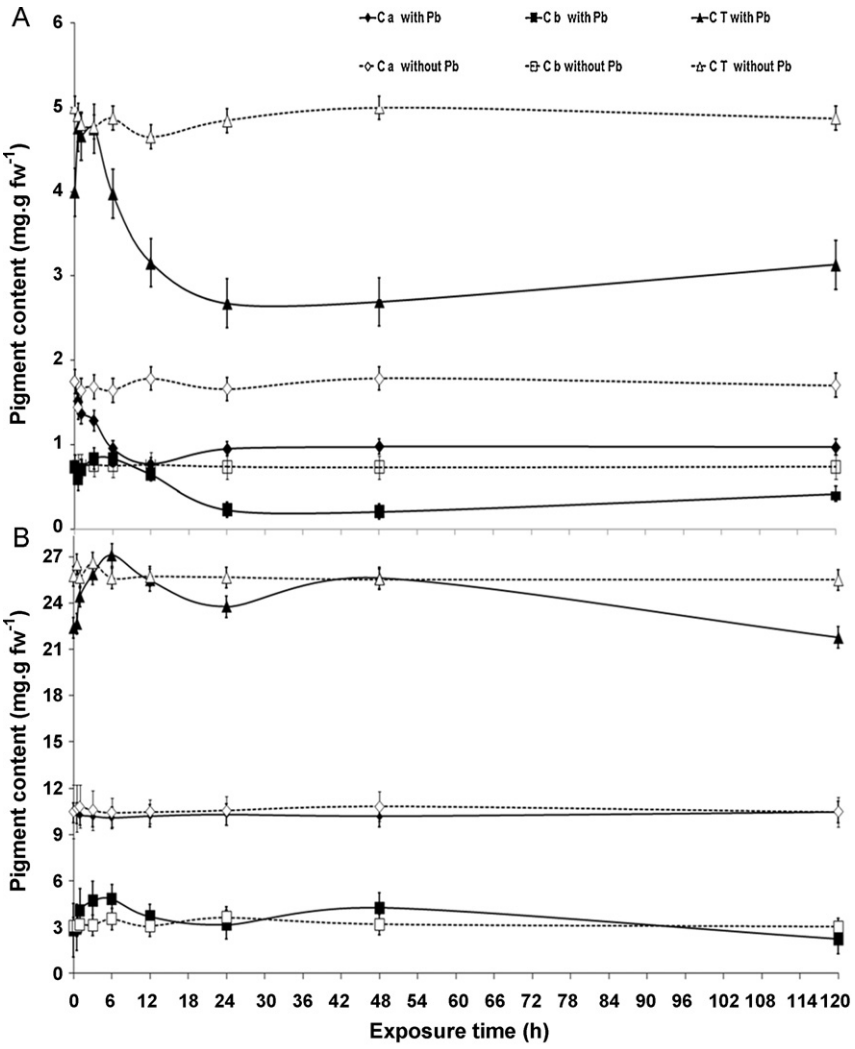

Fig. 1. Internal concentration of pigments found at various times in roots (A) and leaves (B) of *S. minima* Baker plants exposed to 40 μM $Pb(NO_3)_2$. *Ca*, chlorophyll *a*; *Cb*, chlorophyll *b*; and *Ct*, total carotenoids. Values are means ± S.D. of two independent experiments, each with three replicates.

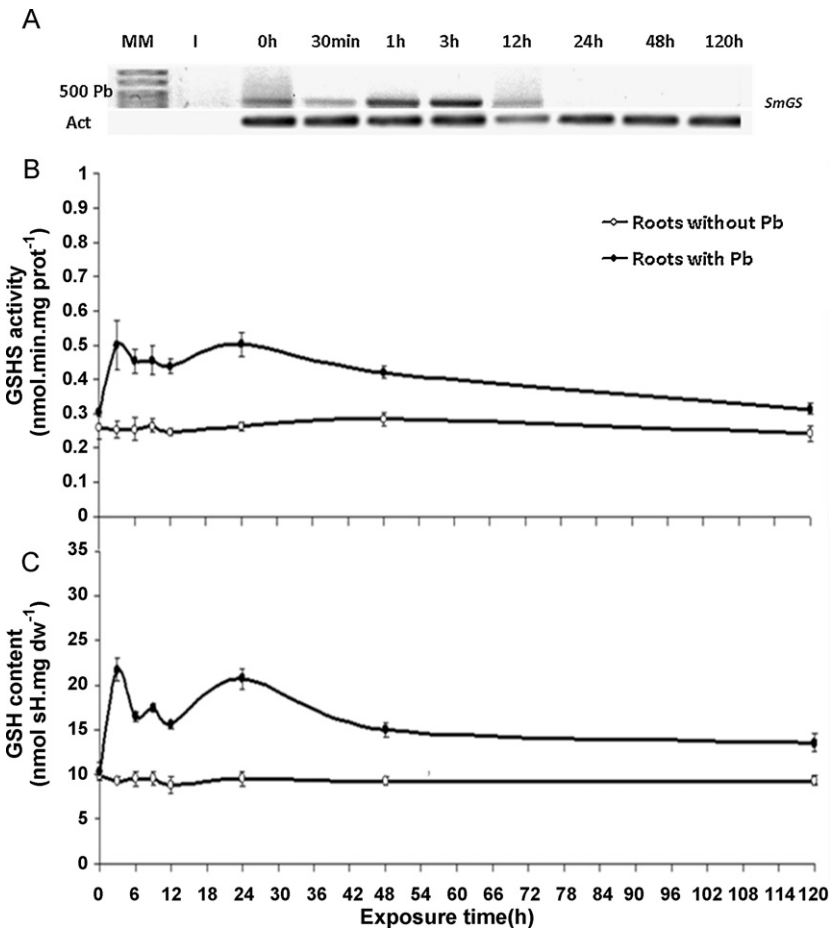

Fig. 2. RT-PCR gels of *Sm*GS; MM, 1 kb DNA marker; I, negative control (water); Act, actin (A). Glutathione synthetase activity (B) and concentration of glutathione (GSH) (C) in roots of plants exposed to 40 μM $Pb(NO_3)_2$ at various exposure times. Values in B and C are means ± S.D. of two independent experiments, each with three replicates.

3. Statistical analysis

Data shown are means from two independent experiments each one with three replicates. Data were subjected to multi-factorial analysis of variance ANOVA ($p < 0.05$) and Pearson correlation analysis by Statistical Package for the Social Sciences version 9 (SPSS Inc., Chicago, IL, USA) for Windows.

4. Results

4.1. Pb^{2+} removal from the aqueous solution

The capacity of *S. minima* to remove Pb^{2+} from the water solution was analyzed after being exposed to a concentration of 40 μM $Pb(NO_3)_2$ at different time intervals. At time 0, the concentration of lead in the solution was (4.55 nmol Pb^{2+} mL^{-1}). It was observed that as early as after the first 6 h, *S. minima* had removed 40%, and, after 12 h, it had removed 80% of the Pb^{2+} contained in the water solution. By day 5, *S. minima* plants had removed 90% of the lead contained in the water solution (Table 1).

4.2. Pb^{2+} concentration in leaves and roots

The accumulation pattern of metal into the plant showed that *S. minima* roots accumulated twice as much Pb than leaves. At the end of the experiment, after 5 days exposure, it was found that roots had a concentration of 3.21 nmol Pb^{2+} per mg, while there was only 1.25 nmol Pb^{2+} per mg in leaves (Table 1).

4.3. Photosynthetic pigments content in leaves and roots

The possible damage caused by Pb to *S. minima* tissue was analyzed by determining the content of chlorophyll *a* (C*a*), chlorophyll *b* (C*b*) and total carotenoids (C*t*), in both leaves and roots.

In roots, the content of photosynthetic pigments decreased substantially ($p < 0.05$) when plants were exposed to Pb^{2+} compared with control treatment. It was found that C*a* decreased 1.7 times, C*b* 1.8 times and C*t* decreased 1.54 times with regard to the roots of plants not exposed to Pb^{2+} (Fig. 1A).

However, in *S. minima* leaves of plant exposed to lead, the concentration of C*a* and C*b* did not present significant changes, and only the content of C*t* showed a slight decrease compared with control plants (Fig. 1B).

4.4. Time-course of SmGS mRNA in roots and leaves

In roots, at time 0 a basal expression of *SmGS* was found. After 30 min of exposure to Pb^{2+}, the expression level increased slightly while the maximal expression occurred after 1 h and 3 h exposure. Finally, at 12 h exposure, the expression level returned to basal level (Fig. 2A).

On the contrary, no basal expression of *Sm*GS was found in leaves at 0 h but the expression level changed dramatically after 1 and 3 h

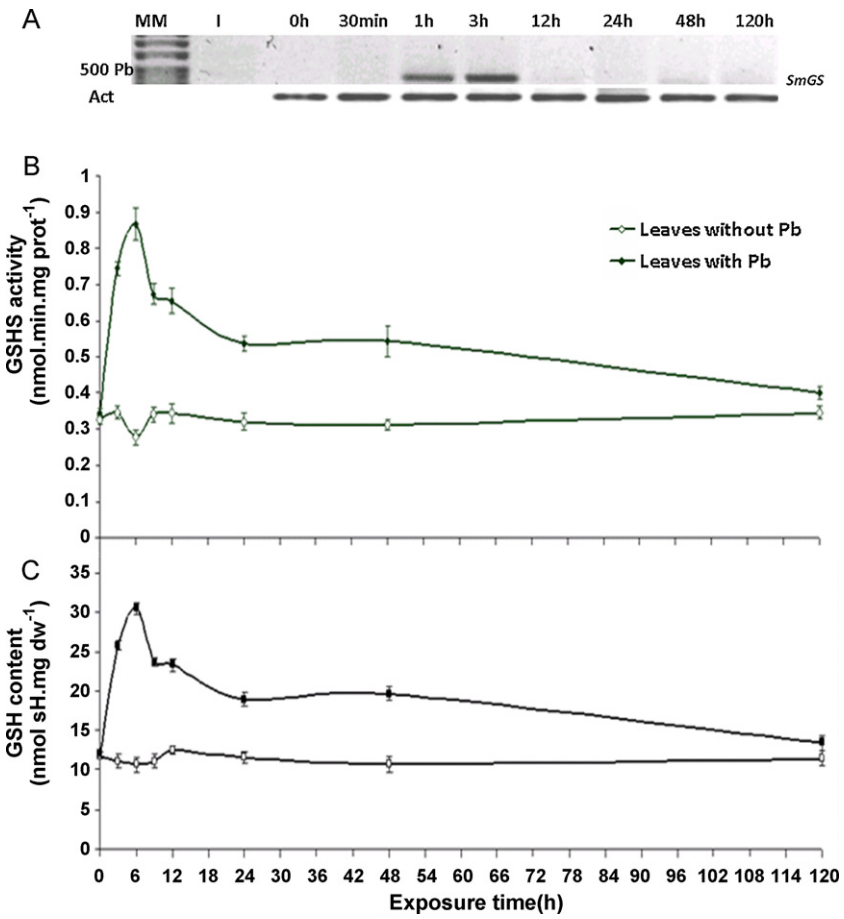

Fig. 3. RT-PCR gels of *Sm*GS; MM, 1 kb DNA marker; I, negative control (water); Act, actin (A); glutathione synthetase activity (B) and concentration of glutathione (GSH) (C) in leaves of plants exposed to 40 μM $Pb(NO_3)_2$ at various exposure times. Values in B and C are means ± S.D. of two independent experiments, each with three replicates.

of plant exposure to lead, to later disappear after 12 h exposure (Fig. 3A).

4.5. Glutathione synthetase activity in leaves and roots

The enzymatic activity of GS trials showed that exposing *S. minima* to a high concentration of lead, enzyme activity increased in both leaves and roots. At the roots, GS activity increased (2.0 times) at 3 h compared with the control, remaining without significant changes for 48 h, at which GS activity decreased very markedly until 120 h (Fig. 2B).

In the leaves on the other hand, the enzymatic activity of GS showed an increase of 2.8 times relative to control, during the first 3 h, declining after 9 h to its lowest point at 120 h (Fig. 3B).

4.6. GSH content in leaves and roots

Glutathione was assessed in *S. minima* plants exposed to 40 μM $Pb(NO_3)_2$ during 120 h through high-resolution liquid chromatography (HPLC). The results of this experiment showed that the presence of lead increases the accumulation of GSH in both leaves and roots of *S. minima*.

In roots, the maximum GSH concentration found was 21.74 nmol sH mg dw^{-1} after 3 h exposure to lead, then it showed a slight decline between 6 and 12 h, remaining at similar values from 48 h to the end of the experiment except at 24 h when it reached a second peak of 20.70 nmol sH mg dw^{-1} (Fig. 2C).

In leaves on the other hand, a maximum of 30.5 nmol sH mg dw^{-1}, was found after 6 h exposure to lead, which represents an increase of 2.7 times the content found in leaves of control plants (not exposed to lead). The content of GSH in leaves presented the following behavior: an initial increase at 3 h, reaching a peak at 6 h to later decrease to the levels found after 3 h exposure at 9 and 12 h to finally decline after 120 h of exposure to Pb^{2+} to values similar to those of control plants (Fig. 3C).

5. Discussion

Our results showed that *S. minima* is able to remove up to 90% of Pb^{2+} from aqueous solutions containing 40 μM $Pb(NO_3)_2$, which was accumulated first in roots (modified fronds) and later in leaves, as previously described (Estrella et al., 2009), confirming its Pb^{2+} removal capacity. We found Pb^{2+} in both tissues from exposed plants, although roots showed higher concentrations than leaves throughout the experiment.

Exposure of *S. minima* plants to 40 μM Pb $(NO_3)_2$ in the medium, resulted in root damage in terms of loss of pigment concentration, but leaves were much less affected than pigments roots. Interestingly, leaves accumulated more GSH, showed higher activity of Gs and also showed a clearer gene expression change in *Sm*Gs gene than roots did.

GSH is a metabolite that is significantly involved in the detoxification of free radicals, xenobiotics substances and heavy metals (Noctor and Foyer, 1998). It has been documented that GSH may be directly related to the stress caused by heavy metals in higher plants. It can serve as a metal chelator agent (Vögeli-Lange and Wagner, 1996). Likewise, GSH is recognized as an excellent antioxidant that plays a key role in the defense mechanism of

plants (Alscher, 1989). GSH is also essential for the synthesis of metal-binding peptides such as phytochelatins, which inactivate and sequester heavy metals such as Cd, Pb, and Hg by forming stable metal-complexes in the vacuole (Cobbett, 2000; Hall, 2002). In aquatic ferns, such as *Salvinia minima* which are accumulators of Pb^{2+} (Olguín et al., 2002; Estrella et al., 2009) it was not known however, whether GSH would play a role in the detoxification of Pb^{2+}.

Exposure to other heavy metals has resulted in changes in the concentration of GSH. For instance, increased GSH concentration as a result of Cd^{2+} exposure, have been reported in various species such as *Rauwolfia serpentina* (Grill et al., 1987), maize (Rüegsegger and Brunold, 1992), spinach (Tukendorf, 1993), tobacco (Vögeli-Lange and Wagner, 1996), carrot (Di Toppi et al., 1999) and pine (Schützendübel et al., 2001). When *Silene cucubalus* was exposed to high concentrations of Cu^{2+}, it also showed increased GSH concentrations (De Vos et al., 1992). However, no reports exist of the effect of lead resulted GSH metabolism.

In the present work, exposing *S. minima* plants to lead, resulted in a threefold increase in both GSH content and enzymatic activity of GS in leaves, compared with control plants. Also in leaves, there was no expression of *SmGS* gene before the plants were exposed to lead (0 h). However, its expression increased during the first hour of metal exposure remaining at similar levels at 3 h, and later decreased again.

In roots, on the other hand, the concentration of GSH and the activity of GS also increased in response to lead, although at much lower proportions that it did in leaves. A basal expression (even in the absence of Pb at time 0) of *SmGS* was found in roots. The presence of Pb did not affect its expression up to the first 12 h exposure. However, after 24 h exposure, somehow the presence of Pb caused a down regulation of *SmGS* expression in this tissue. This down-regulation might have resulted from cell damage after a prolonged exposure to the metal at the concentrations tested.

In both tissues the concentration of GSH increased in response to the metal during the first 6 h but declined later. However, this behavior has been reported in other species, including *Fontinalis antipirética*, *Lactica sativa* var. Longifolia, *Triticum aestivum* L. when they were exposed to high concentrations of Cd^{2+} (Bruns et al., 2001; Maier et al., 2003; Sun et al., 2005).

In terms of GS activity, it is important to note that an increase in the enzymatic activity of GS has only been reported in response to Cd^{2+} exposure (Rüegsegger et al., 1990; Rüegsegger and Brunold, 1992; Klapheck et al., 1995). In the present work, when *S. minima* was exposed to Pb^{2+}, the activity of GS increased 2.8–2.0 times (with respect to the control) in leaves and roots, respectively. Therefore, this is the first report of increased activity of GS in response to Pb^{2+}.

Molecular studies had shown that Cd^{2+} induced the expression of genes involved in glutathione synthesis. In *A. thaliana* exposed to Cd^{2+}, it was found that levels of the GS gene transcripts increased approximately 2.0-fold (Harada et al., 2002). Similarly, in the roots of *Brassica juncea*, exposure to Cd^{2+} increased the concentration of the mRNA for enzymes associated with the biosynthesis of glutathione (Schäfer et al., 1998). However, no reports exist of lead causing changes at the levels of gene expression of GS gene. In the present paper, we report the first isolation of a fragment of GS gene in an aquatic fern, which allowed us to observe that differences exist in both leaves and roots of *S. minima* in the *SmGS* gene expression changes occurring when exposed to Pb^{2+}.

In summary, it was shown that *Sm*GS expression, enzymatic activity of GS and production of GSH increased more in leaves than in roots of *S minima* plants exposed to lead, suggesting a fast and coordinated response between mRNA-protein-product and differential response between tissues. It is possible that the reduced damage (in terms of pigment concentrations) observed in leaves of *S. minima* plants exposed to Pb^{2+} resulted from the protective effects of GSH, which is known to minimize the effect caused by the oxidative stress of Pb^{2+} on chlorophylls as demonstrated in *Vigna unguiculata* (Bhattacharya et al., 1995), and *Vallisnaria spiralis* (Gupta et al., 1999).

Taken together, these findings and those reported in a previous study from our group (Estrella et al., 2009) showed that the accumulation and detoxification of Pb^{2+} in tissues of *S. minima* might be the result of a complex regulation between tissues, genes and simultaneous processes including enzymes involved in the biosynthesis of glutathione (GSH) and phytochelatins (PC) polymerization.

We propose that Pb^{2+} is first taken by roots of *Salvinia minima* from the aqueous solution and eventually is directed to the leaves. The accumulation of lead in the tissues has an effect on the metabolism of thiols. The increased concentration of internal Pb^{2+} promotes changes in the expression of glutathione synthetase (*Sm*GS; GeneBank: EU247453) and phytochelatin synthase (*Sm*PCS; GeneBank: EU048205) genes. Similarly, it causes an increased activity of the enzymes glutathione synthetase (GS) and phytochelatin synthase (PCS), that in turn, result in an increased content of GSH and PC (phytochelatin 2 (PC2), phytochelatin 3 (PC3) and phytochelatin 4, PC4).

However, it appears that GSH and PC have a differential role to play in both tissues, PC being relatively more important in roots, while GSH appears to be more important in leaves. This is most likely part of a complex and coordinated mechanism of *S. minima* to deal with Pb^{2+}.

In roots, Pb^{2+} accumulation was higher than in leaves. This is most likely because the roots are submerged in the solution, have greater area for exploration, and are the first tissue in direct contact with the lead solutions. In this tissue, the presence of Pb^{2+} increased the accumulation of PC and induced changes in the expression of the gene, *Sm*PCS. It is possible that the phytochelatin–Pb complexes are driven towards the interior of the vacuoles via an ABC-type transporter. Once in the vacuole, it is believed that a sulfur molecule is added to form stable complexes (Ortiz et al., 1992). The lower concentration of GSH found in roots might be the result of the fact that, in roots, GSH is metabolized as a precursor for PC ensuring the production of this tripeptide as the main mechanism of Pb-detoxification operating in roots.

In leaves, on the other hand, part of the Pb^{2+} might be translocated from the roots, although the possibility that Pb^{2+} could enter leaves directly from the medium cannot be ruled out, as leaves are floating over the medium and they have hair-like structures on the abaxial surface. The presence of Pb^{2+} in leaves had less effect on the metabolism of PC, but causes a greater effect on GSH. Therefore, in leaves, GSH might participate in the detoxification of Pb^{2+}, by operating more as an antioxidant agent involved in the ascorbate peroxidase system, to neutralize the effect of free radicals in this Pb-hyper-accumulator aquatic fern, which might explain the fact that leaves are less affected than roots when plants are exposed to lead.

Acknowledgements

The authors are particularly grateful to Dr. L.F. Cuevas (ITM-Mérida, México), Dr. N. Terry and Dr. D. LeDuc (University of California, Berkeley, USA). Dr. E. Olguín (INECOL, México) for donating early *S. minima* cultures. NE acknowledges a scholarship (173394) by CONACyT-México. The present work was partially financed by a 2005 UC-MEXUS CONACyT grant.

References

Alscher, R.G., 1989. Biosynthesis and antioxidant function of glutathione in plants. Physiol. Plant. 77, 457–464.

Altschul, S., Gish, W., Miller, W., Myers, E., Lipman, D., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.
Bhattacharya, M., Choudhury, M.A., Bhattacharya, M., 1995. Heavy metal (Pb^{2+} and Cd^{2+}) stress induced damages in Vigna seedings and possible involvement of phytochelatin like substances in mitigation of heavy metal stress. Indian J. Exp. Biol. 33 (3), 236–238.
Bradford, M.M., 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.
Bruns, I., Sutter, K., Menge, S., Neumann, D., Krauss, G., 2001. Cadmium lets increase the glutathione pool in bryophytes. J. Plant Physiol. 158, 79–89.
Chen, J., Goldsborough, P.B., 1994. Increased activity of γ-glutamylcysteine synthetase in tomato cells selected for cadmium tolerance. J. Plant Physiol. 106, 233–239.
Cobbett, C., 2000. Phytochelatins and their roles in heavy metals detoxification. Plant Physiol. 123, 825–832.
De Vos, C.H.R., Vonk, M.J., Schat, H., 1992. Glutathione depletion due to copper induced phytochelatin synthesis causes oxidative stress in *Silene cucubalus*. Plant Physiol. 98, 853–858.
Dere, S., Günes, T., Sivaci, R., 1998. Spectrophotometric determination of chlorophyll *a*, *b* and total carotenoid contents of some algae species using different solvents. Turk. J. Bot. 22, 13–17.
Di Toppi, L.S., Lambardi, M., Pazzagli, L., Cappugi, G., Durante, M., Gabbrielli, R., 1999. Response to cadmium in carrot in vitro plants and cell suspension cultures. Plant Sci. 137, 119–129.
Estrella, G.N., Mendoza, C.D., Moreno, S.R., González, M.D., Zapata, P.O., Martínez, H.A., Santamaría, J.M., 2009. The Pb-hyperaccumulator aquatic fern *Salvinia minima* Baker, responds to Pb^{2+} by increasing phytochelatins via changes in *SmPCS* expression and in phytochelatin synthase activity. Aquat. Toxicol. 91, 320–328.
Gasic, K., Hernández, A., Korban, S., 2004. RNA extraction from different apple tissues rich in polyphenols and polysaccharides for cDNA library construction. Plant Mol. Biol. Rep. 22, 427a–437a.
Grill, E., Winnacker, E., Zenk, M., 1987. Phytochelatins, a class of heavy heavy-metal-binding peptides from plant, are functionally analogous to metallothioneins. Proc. Natl. Acad. Sci. U.S.A. 84, 439–443.
Grill, E., Winnacker, E.L., Zenk, M.H., 1991. Phytochelatins. Methods Enzymol. 205, 333–341.
Gupta, M., Tripathi, R.D., Raj, U.N., Haq, W., 1999. Lead induced synthesis of metal binding peptides (phytochelatins) in the submerged macrophyte *Vallisneria spiralis* L. Physiol. Mol. Biol. Plant 5, 173–180.
Hall, J.L., 2002. Cellular mechanisms for heavy metal detoxification and tolerance. J. Exp. Bot. 53, 1–11.
Harada, E., Yamaguchi, Y., Koizumi, N., Sano, H., 2002. Cadmium stress induces production of thiol compounds and transcripts for enzymes involved in sulfur assimilation pathways in Arabidopsis. J. Plant Physiol. 159, 445–448.
Hoffmann, T., Kutter, C., Santamaría, J., 2004. Capacity of *Salvinia minima* Baker to tolerate and accumulate As and Pb. Eng. Life Sci. 4, 61–65.
Kim, S., Shin, Y., Kim, K., Park, E., Sa, J., Lim, C., 2003. Regulation of the gene encoding glutathione synthetase from the fission feast. J. Biochem. Mol. Biol. 36, 326–331.
Klapheck, S., Schlunz, S., Bergmann, L., 1995. Synthesis of phytochelatins and homophytochelatins in *Pisum sativum* L. Plant Physiol. 107, 515–521.
Leustek, T., Martin, M., Bick, J., Davies, J., 2000. Pathways and regulation of sulfur metabolism revealed through molecular and genetic studies. Annu. Rev. Plant Phys. 51, 141–165.
Maier, E.A., Matthews, R.D., McDowell, J.A., Walden, R., Ahner, B.A., 2003. Environmental cadmium levels increase phytochelatin and glutathione in lettuce grown in a chelator-buffered nutrient solution. J. Environ. Qual. 32, 1356–1364.
Marrs, K., 1996. The functions and regulation of glutathione S-transferases in plants. Annu. Rev. Plant Phys. 47, 127–158.
Meister, A., 1995. Glutathione. Methods Enzymol. 251, 3–13.
Noctor, G., Foyer, C., 1998. Ascorbate and glutathione: keeping active oxygen under control. Annu. Rev. Plant Phys. 49, 249–279.
Olguín, E.J., Hernández, E., Ramos, I., 2002. The effect of both different light conditions and the pH value on the capacity of *Salvinia minima* Baker for removing cadmium, lead and chromium. Acta Biotechnol. 22, 121–131.
Ortiz, D.F., Kreppel, L., Speiser, D.M., Scheel, G., McDonald, G., Ow, D.W., 1992. Heavy metal tolerance in the fission yeast requires an ATP-binding cassette-type vacuolar membrane transporter. EMBO J. 11, 3491–3499.
Rüegsegger, A., Brunold, C., 1992. Effect of cadmium on γ-glutamylcysteine synthesis in maize seedlings. Plant Physiol. 99, 428–433.
Rüegsegger, A., Schmutz, D., Brunold, C., 1990. Regulation of glutathione synthesis by cadmium in *Pisum sativum* L. Plant Physiol. 93, 1579–1584.
Schäfer, H.J., Haag-Kerwer, A., Rausch, T., 1998. cDNA cloning and expression analysis of genes encoding GSH synthesis in roots of the heavy metal accumulator *Brassica juncea* L. Evidence for Cd-induction of a putative mitochondrial γ-glutamylcysteine synthetase isoform. Plant Mol. Biol. 37, 87–97.
Schützendübel, A., Schwanz, P., Teichmann, T., Gross, K., Langenfeld-Heyser, R., Godbold, D.L., Polle, A., 2001. Cadmium-induced changes in antioxidative systems, H_2O_2 content and differentiation in pine (*Pinus sylvestris*) roots. J. Plant Physiol. 127, 887–892.
Sun, Q., Wang, X., Ding, S., Yuan, X., 2005. Effects of interaction between cadmium and plumbum on phytochelatins and glutathione production in wheat (*Triticum aestivum* L.). J. Integr. Plant Biol. 47, 435–442.
Tukendorf, A., 1993. The role of glutathione in detoxification of cadmium and excess copper in spinach plants. Acta Physiol. Plant 15, 175–183.
Vögeli-Lange, R., Wagner, G.W., 1996. Relationship between cadmium, glutathione and cadmium-binding peptides (phytochelatins) in leaves of intact tobacco seedlings. Plant Sci. 114, 11–18.
Xiang, C., Werner, B.L., Christensen, E.M., Oliver, D.J., 2001. The biological functions of glutathione revisited in Arabidopsis transgenic plants with altered glutathione levels. Plant Physiol. 126, 564–574.