

EFFECTS OF CADMIUM IN PLANTS OF *Sphagneticola trilobata* (L.) Pruski

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ABSTRACT

Cadmium (Cd^{2+}) is a pollutant of great environmental concern due to its multiple origins (natural and anthropogenic), the ability to accumulate in organs and tissues, and the deleterious effects it can cause in organisms. In the present study, we investigated the effects of Cd^{2+} exposure on *Sphagneticola trilobata* plants. We evaluated the accumulation of Cd^{2+} , the plant biomass, the content of chlorophyll, soluble sugars, proteins and the production of malondialdehyde and thiols as markers of oxidative stress and tolerance, respectively. The Cd^{2+} content in the plant organs increased proportionally to the concentration of CdCl_2 in the environment, reaching accumulatively 1306, 193 and 52 $\text{mg}\cdot\text{kg}^{-1}$ in roots, stems and leaves, respectively. Some toxic effects such as a decrease in the root biomass, alterations in the polypeptide pattern of the leaves, reduction in the chlorophyll content, and an increase in the amount of malondialdehyde was observed at higher concentrations of CdCl_2 . An increase in the content of soluble sugars was also seen, which are markers of tolerance associated with a protection mechanism against the oxidative stress. In addition, an increase on heavy metals chelating thiols such as L-cysteine, glutathione and various phytochelatin was obtained in the plant roots. Our results demonstrated that *S. trilobata* is capable of accumulating Cd^{2+} and possesses tolerance mechanisms that make this plant an excellent option to be used for Cd^{2+} phytoremediation.

Additional key words: Glutathione, oxidative stress, phytochelatin, phytoremediation, tolerant plants, wedelia

RESUMEN

Efectos del cadmio en plantas de *Sphagneticola trilobata* (L.) Pruski

El cadmio (Cd^{2+}) es un contaminante que genera mucha preocupación a nivel ambiental debido a sus múltiples orígenes (naturales y antropogénicos), la capacidad de acumularse en órganos y tejidos y los efectos nocivos que puede causar en los organismos. En el presente estudio se investigó el efecto de la exposición a Cd en plantas de *Sphagneticola trilobata*. Se evaluó la capacidad de acumular Cd, la biomasa de las plantas, el contenido de clorofila, azúcares solubles, proteínas, patrón polipeptídico y la producción de malondialdehído y tioles como marcadores de estrés oxidativo y tolerancia, respectivamente. El contenido de Cd en los tejidos de las plantas se incrementó proporcionalmente a la concentración de CdCl_2 en el medio de cultivo, acumulando 1306, 193 y 52 $\text{mg}\cdot\text{kg}^{-1}$ en raíces, tallos y hojas, respectivamente. A altas concentraciones de Cd se observaron efectos tóxicos tales como reducción en la biomasa de las raíces, cambios en el patrón polipeptídico de las hojas, reducción en el contenido de clorofila y un incremento en el contenido de malondialdehído. Dentro de los marcadores de tolerancia se observó un incremento en el contenido de azúcares solubles asociados a protección contra el estrés oxidativo. También se observó un incremento de tioles quelantes de metales pesados en las raíces tales como: L-cisteína, glutatión, y varias fitoquelatinas. Se demostró que *S. trilobata* es capaz de acumular cadmio y presenta mecanismos de tolerancia que la convierten en una excelente opción para la fitorremediación de Cd.

Palabras claves adicionales: Estrés oxidativo, fitoquelatinas, fitorremediación, glutatión, margarita rastrera, plantas tolerantes

INTRODUCTION

Cadmium, one of the most toxic metals in the environment, is released from both natural and

anthropogenic sources such as mining, metal smelting, combustion of fossil fuels, use of phosphate fertilizers, and manufacture of batteries and other industrial goods such as cement,

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pigments and plastics (Sarwar et al., 2017).

Plants exposed to Cd have shown prejudicial effects including reduction in growth and biomass rate in both tolerant and non-tolerant plants (Fusconi et al., 2010; Son et al., 2012); decrease in chlorophyll and carotenoid content (Cherif et al., 2011); increased level of malondialdehyde (MDA), which is a lipid peroxidation product (Gratao et al., 2008; Fidalgo et al., 2011); changes in protein expression related to general defense; and production of detoxification pathways, namely reactive oxygen species (ROS) scavenging, chelation, and compartmentalization (Hossain and Komatsu, 2013).

Cd stress in plants is also capable of activating the sulphide assimilation pathway as a defense mechanism, by increasing transcription of genes involved in the biosynthesis of thiols, cysteine (Cys), reduced glutation (GSH) and phytochelatins (PCs) (Davidian and Kopriva, 2010). Cys is an amino acid that plays an essential role in the structure of proteins and in redox balance and it is also a precursor of GSH and PCs (Josefczak et al., 2012). The tripeptide GSH is one of the most abundant low molecular weight thiols that participate in a variety of cellular processes, including ROS defense. GSH is an antioxidant involved in redox-homeostatic buffering and as such, plays an essential role in plant metabolism and stress tolerance (Gallego et al., 2012).

It is also considered one of the main biomarkers of lipid peroxidation in response to oxidative stress (Pernía et al., 2008). PCs are polypeptides with molecular masses ranging from 10 to 14 kDa that bind metals; they are synthesized from GSH by a trans-peptide reaction, catalyzed by the phytochelatin synthase enzyme. The role of PCs is vital in heavy metal detoxification and maintenance of the ionic homeostasis given that PCs form complexes with metallic ions sequestering them at the vacuole (Castrillo et al., 2012). The induction of PCs upon exposure to Cd in numerous plant species has been reported (Mohamed et al., 2012). Inside plant cells, Cd binds to S-containing ligands such as those present in GSH and PCs.

Sphagneticola trilobata (L.) Pruski, a species belonging to the Asteraceae family, has a fast growth and a wide range of ecological tolerance given that: i) it grows in both sun and shade, and in all types of soil, and ii) it tolerates periods of

drought, flood and high salinity (Meyer, 2000). Its root system favors the creation of an extended rhizosphere capable of covering a larger area. It is an ornamental plant that helps to prevent soil erosion. Its agronomic requirements are widely known and it reproduces vegetatively by cuttings.

The aim of this study was to investigate the effects of Cd exposure on *S. trilobata* plants. Specifically, we evaluated Cd accumulation, biomass, content of chlorophyll, soluble sugars and proteins, oxidative stress, and the plant defense system involved in tolerance.

MATERIALS AND METHODS

Plant material and growth conditions. The experiments were carried out in the glasshouse at Universidad Simón Bolívar, Caracas, Venezuela (10°25' N, 66°50' W). Young rooted cuttings of 10 cm each from *S. trilobata* plants were grown in plastic pots containing nutritive solution (pH 5.7) according to Chaoui et al. (1997), containing 2 mM KNO₃, 2,5 mM Ca(NO₃)₂·4H₂O, 1 mM KH₂PO₄, 1 mM MgSO₄·6H₂O, 30 μM H₃BO₄, 50 μM Fe-K-EDTA, 10 μM MnSO₄·H₂O, 1 μM ZnSO₄·7H₂O, 1 μM CuSO₄·5H₂O and (NH₄)₆Mo₇O₂₄·4H₂O.

They were maintained for 10 days under greenhouse conditions for acclimatization: an average temperature (°C) of 20 ± 2.5 / 32 ± 2.8 (minimum/maximum); an average relative humidity (RH) of 54 ± 9 / 97 ± 3 % (minimum/maximum; a 12 h photoperiod with a maximum sun radiation of 659 μmolm⁻²·s⁻¹. Immediately, after the acclimatization period the plants were exposed to different concentrations of Cd. The concentration of CdCl₂ in the nutritive solution was 0, 0.5, 5, 25 and 50 μM; these values were selected according to those reported in contaminated soils (Sanita di Toppi and Gabbrielli, 1999). After 96 hours of exposure, plants were harvested, washed with distilled water and dried with paper towels. Trials were carried out in quadruplicate (n=4) with three repetitions.

Accumulation of Cd. On the day of harvest, at 96 h of exposure to Cd, the roots of the plants were washed with distilled water and dried with paper towel. Fresh weights were determined of the plants in an analytical balance. Subsequently, the plant material (roots, stems and leaves) was dehydrated in an oven at 80 °C for 72

h. Dried roots, stems and leaves were ground, digested with a mixture of $\text{HNO}_3/\text{H}_2\text{O}_2$ (3:1, v/v), heated at 60 °C on a hot plate and filtered using Whatman paper N° 4. Cd concentration was measured using a flame atomic absorption spectrophotometer (Perkin-Elmer 2380).

The bioconcentration factor (BCF) and translocation factor (TF) were calculated according to Ali et al. (2013). The ability to accumulate heavy metals in *S. trilobata* was determined using the BCF. This is the relationship between the Cd accumulated in the plant and the Cd present in the medium and was determined using the following formula: $\text{BCF} = \text{Concentration of Cd in the shoot or root} / \text{Concentration of Cd in the substrate}$.

The Cd Transfer Factor (TF) expresses the ability to transfer the heavy metal from the roots to the stem (Brooks 1998). It was determined using the following formula: $\text{TF} = \text{Concentration of Cd in the stem} / \text{Concentration of Cd in the root}$.

Determination of biomass and content of chlorophylls (Chl) and soluble sugars. Washed and paper towel dried plants were weighed in fresh weighed (FW), separated by organs which were also weighed FW and kept at -80 °C for analysis. The dry weight (DW) of the samples was determined after being kept in an oven at 80 °C for 48 hours. One gram of fresh leaf sample was cut and leaf extract were prepared following the method described by Chaoui et al. (1997). In leaf extracts, total Chl was determined according to Lichtenthaler (1987), and the sugar content was evaluated by the anthrone- H_2SO_4 method (Castrillo, 1999) using sucrose as standard (Sigma). Trials were carried out in quadruplicate (n=4) with three repetitions.

Polypeptide separation and detection by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). One gram of fresh leaf sample was cut and leaf extract were prepared following the method described by Chaoui et al. (1997). Protein gel electrophoresis was carried out in the presence of sodium dodecyl sulfate (SDS) on slabs containing 15 % (w/v) of polyacrylamide (Laemmli, 1970). Coomassie brilliant blue R-250 was used for protein staining (Palma et al., 1997). Trials were carried out in quadruplicate (n=4).

Measurements of lipid peroxidation as a

marker of oxidative stress. The level of lipid peroxidation was measured by determining the amount of MDA, as described by Heath and Packer (1968). The absorbance at 532 nm was evaluated by UV-visible spectrophotometer (Shimadzu UV-160A), and the level of MDA was estimated using its extinction coefficient ($155 \text{ mM}^{-1}\cdot\text{cm}^{-1}$), calculated by Heath and Packer (1968). Trials were carried out in quadruplicate (n=4) with three repetitions.

Determination of phytochelatin and other thiols by high performance liquid chromatography (HPLC). PCs and other low molecular weight thiols were extracted and analyzed by HPLC (Waters 1525 Binary HPLC Pump) according to Sneller et al. (2000). Thiols were separated using a Nova-Pak C18 column (Waters). A process of precolumn derivatization was implemented with monobromobimane (mBBR), and fractions were acquired following a 70-min gradient of 12- 100 % (v/v) methanol. Retention times were evaluated using commercial standards of GSH, L-Cys, Glu-Cys and PCs. N-acetylcystein (NAC) was included as an internal standard and individual PCs subtypes were quantified by using the peak area / concentration ratio of the standard GSH solution. All determinations were performed in triplicate (n=3). Corrections for differential derivatization efficiencies were carried out according to Sneller et al. (2000). The analytical data was integrated using the Waters Millennium software.

Statistical analysis. Data were tested using one way Anova followed by Dunnett test for mean separation, using the statistical pack Minitab 17.0.

RESULTS AND DISCUSSION

Cadmium accumulation in *S. trilobata* followed the order root > stem > leaves: 83-87 % in roots, 11-14 % in stems, and 2-3 % in leaves (Figure 1), similar to what was reported for *Echinochloa crus-galli* (Peng et al., 2017). The Cd concentration in the organs of *S. trilobata* increased proportionally to the concentration of the metal in the nutrient solution, with R^2 of 0.995, 0.9861 and 0.9134 for roots, stems and leaves, respectively. The greatest Cd accumulation was 1306, 193 y 52 $\text{mg}\cdot\text{kg}^{-1}$ on roots, stems, and leaves at a concentration of 50 μM (Table 1). Plants accumulated most of the Cd in the roots,

which has been considered a Cd tolerance strategy and has been attributed to adsorption of into the negatively charged surfaces of the root cell walls (Solís et al., 2007; Lux et al., 2011; Akhter et al., 2014).

The BCF was higher than 1 and the TF was lower than 1 at all Cd concentrations. BCF also increased with concentration reaching maximum values of 232.41 ± 30.03 , 34.28 ± 12.41 y 11.75 ± 0.00 on roots, stems, and leaves at a concentration of $50 \mu\text{M}$. According to Brooks (1998) the species are hyperaccumulator if they meet three assumptions: build a hundred times the concentration of the metal accumulated in a normal plant, for the case of Cd $100 \text{ mg}\cdot\text{kg}^{-1}$; to have a bioconcentration factor greater than 1, which means that the concentration of the element is higher on the plant than in the substrate; and that the Cd translocation factor is greater than 1, so that the metal concentration must be higher in the stem than at the root. Using the criterion of Brooks (1998) our species in study is not a

hyperaccumulator because its translocation factor is less than 1, but can be considered an accumulator species for collecting more than $100 \text{ mg}\cdot\text{kg}^{-1}$ and have a BCF greater than 1.

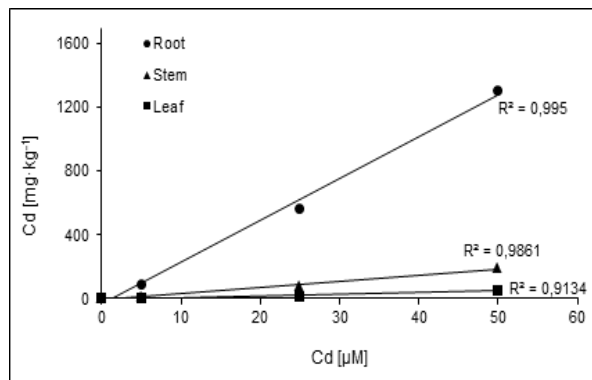


Figure 1. Cd accumulated by tissue (root, stem, leaf) of plants of *S. trilobata* exposed during 96 hours at different concentrations of Cd in the nutrient solution (0, 5, 25, 50 μM)

Table 1. Cd content in *S. trilobata* plant organs exposed to different Cd concentrations for 96 hours

[Cd] μM	Root [Cd] $\text{mg}\cdot\text{kg}^{-1}$ PS	Stem [Cd] $\text{mg}\cdot\text{kg}^{-1}$ PS	Leaf [Cd] $\text{mg}\cdot\text{kg}^{-1}$ PS	BCF Shoot	BCF Root	TF
0	0.00 a	0.00 c	0.00 a	0.00 a	0.00 a	0.00 a
5	87.18 b	14.93 bc	3.22 a	20.46 b	76.34 b	0.27 a
25	564.16 c	74.56 b	11.25 a	15.03 b	98.80 bc	0.16 a
50	1306.16 d	192.63 a	52.04 b	21.42 b	114.37 c	0.19 a
<i>P</i>	0.000	0.000	0.000	0.000	0.000	0.152

With respect to biomass, no significant differences between control (0 μM Cd) and Cd-treated samples were observed in leaves and stems, while a significant decrease was observed in roots at 50 μM CdCl₂ (Figure 2A), probably caused by the high accumulation of Cd in roots (Table 1); this reduction in roots has been reported in the Cd hyperaccumulator *Solanum nigrum* (Yan et al., 2015). Cd has been described as an inhibitor of growth for many species such as *Brassica pekinensis* (Su et al., 2010), *Hordeum vulgare* (Tamas et al., 2008), *Lemna gibba* and *Solanum melongena* (Sun et al., 2007), and some Cd accumulating species such as *Brassica juncea* (Ahmad et al., 2011), *Sedum alfredii* (Zhou and Qiu, 2005; Jin et al., 2008) and *Thlaspi caerulescens* (Wojcik et al., 2005).

At exposures of 25 and 50 μM CdCl₂, the low leaf Cd concentrations diminished the total

chlorophyll content (Figure 2B). Photosynthetic pigment reduction is considered a Cd toxic effect by Gill and Tuteja (2010). It has been widely reported a decreased content of chlorophylls in *Brassica juncea* (Mohammed et al., 2012) *Bacopa monnieri* (Mishra et al., 2006), *Phyllanthus amarus* (Rai et al., 2005) *Sagittaria sagittifolia* (Hu et al., 2009) and *Zea mays* (Lagriffoul et al., 1998) exposed to Cd.

On the other hand, a statistically significant increase in sugar content was observed in the leaves exposed to 50 μM CdCl₂ with a sustained tendency to increase as the level of Cd increased (Figure 2C) which coincides with the results of Anjum et al. (2016). This could be related to a protective function of sucrose against oxidative stress in Cd-stressed plants. It has been suggested that sucrose and SOS-derived radicals may play a role in oxidation reduction

reactions taking place in the vicinity of membranes (Van den Ende and Valluru, 2008). Furthermore, Filek et al. (2010) suggested that in rape plants, sucrose deposited in high amounts in Cd-stressed plants appeared to act as traps for free radicals produced under those conditions. An increase in total sugar content in *Oryza sativa* leaves under Cd exposure has also been reported (Verma and Demey, 2001).

Similarly, significant increases in the concentration of MDA at 0.5, 5, and 25 μM of CdCl_2 were observed (Figure 2D) suggesting that exposure to Cd resulted in an increase of ROS and

oxidative damage (Pallavi et al., 2016; Rizwan et al., 2016). However, at 50 μM of CdCl_2 there was a non-significant MDA concentration decrease (76 % from maximum observed) coinciding with a significant increase in sugar productions, which could neutralize the oxidative stress caused by Cd throughout the suggested role for sucrose and sucrosyl oligosaccharide in oxidative defense (Van den Ende and Valluru, 2008). The same increase in MDA concentration was reported on *Helianthus annuus* (Gallego et al., 2005), *Solanum nigrum* (Fidalgo et al., 2011) and *Oryza sativa* (Shah et al., 2001).

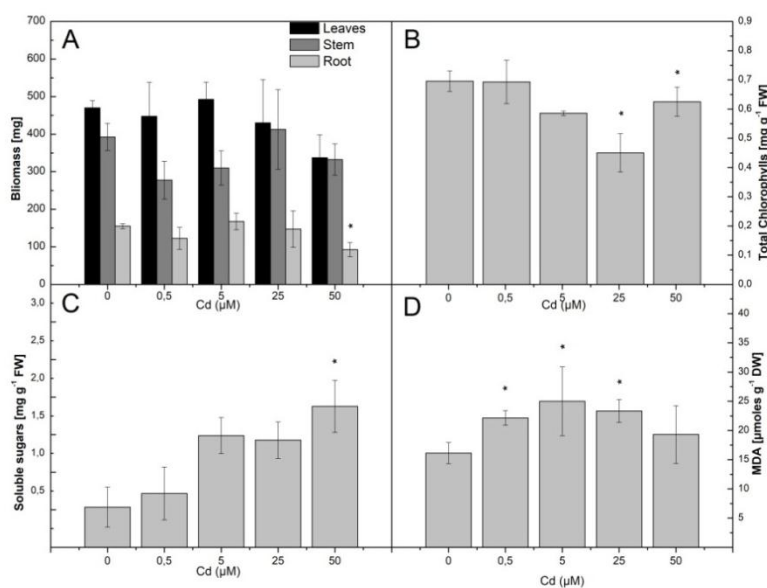


Figure 2. Effects of the exposure for 96 h to different concentrations of Cd on *S. trilobata* plants. A: Biomass; B: Chl: Chlorophyll and Car: carotenoids; C: Soluble sugars; D: Lipid peroxidation, measured as MDA production. Bars in each column means SE (n=4). * Significantly different from the control according to Dunnett test ($P \leq 0.05$)

At 25 μM higher toxicity was observed than at 50 μM in the content of chlorophyll and MDA, it is probably due to the expression of phytochelatins at 50 μM , which sequester Cd, decreasing its bioavailability in the cell and therefore its toxicity.

Another observed effect of cadmium on *S. trilobata* was the variation in its polypeptide composition. The polypeptide pattern in the plant leaves was modified at 50 μM CdCl_2 (Figure 3). The results obtained following SDS-PAGE showed an increase in bands with apparent molecular masses of 66, 91 and 107 kDa. These changes in the polypeptide pattern are probably

caused by Cd toxicity, which, could be demonstrated through changes in protein expression; such as in the cases of plant defense and detoxification pathways, namely ROS scavenging, chelation, compartmentalization, antioxidant activity and pathogenesis-related proteins (Fidalgo et al., 2011; Ovecka and Takac, 2014).

With respect to thiols, PCs were not detected in leaves at any Cd concentration and in roots up to 25 μM CdCl_2 . In plants treated with 50 μM , the values of thiols increased in the following manner: L-Cys (9 times), PC4 (6 times), GSH (3 times) and there was the appearance of PC1 (Figure 4A).

The Cys thiol group (-SH) is able to bind Cd, acting as a chelating agent, thereby avoiding the toxic effects of free Cd and as a precursor of other chelators such as GSH and PCs. Similarly, species *Vallisneria spiralis* (Singh et al., 2010), *Matricaria chamomilla* (Kováčik et al., 2009), and *Solanum nigrum* (Deng et al., 2010) have also shown an increase in Cys in the presence of Cd.

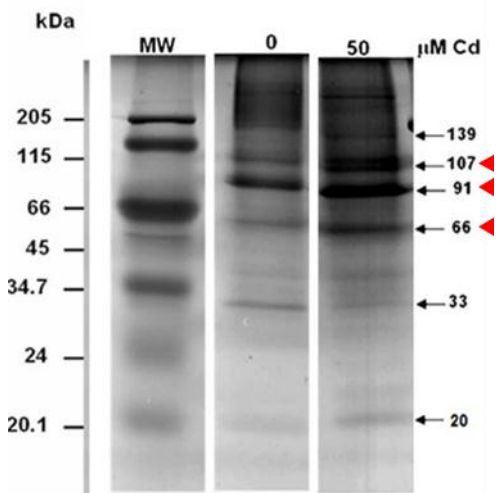


Figure 3. Polypeptidic pattern changes in *S. trilobata* leaves exposed to 0 and 50 μM Cd for 96 hours showing an increase in bands with apparent molecular masses of 66, 91 and 107 kDa. SDS-PAGE gel at 15 %. Protein molecular weight marker (MW) is located in the first column

The significant increase in the concentration of GSH showed by *S. trilobata* in the presence of Cd was probably due to an increase in sulphide uptake, synthesis of Cys and overexpression of GSH synthetase enzyme in the presence of this heavy metal as demonstrated by Harada et al. (2002). Some authors have reported this event on the species *Arabidopsis thaliana*, it was shown that the transcripts of the glutathione synthetase (Gsh2) were increased two fold in the presence of Cd (Harada et al., 2002). Similarly, an increase of GSH in the presence of Cd was found for the species *Arabidopsis thaliana* (Sadi et al., 2008), *Bacopa monnieri* (Mishra et al., 2006), *Brassica juncea* (Seth et al., 2008), *Phragmites australis* (Fediuc et al., 2005) and in the Asteraceae *Helianthus annuus* (Gallego et al., 2005).

Unlike the PCs that appear in the control

plants, a PC1 appeared in the plants treated with Cd and there was a significant increased in PC4, meaning that PC1 and PC4 play an important role in the processes of detoxification and tolerance to Cd in *S. trilobata*. On the other hand, small amounts of PC3, PC4 and PC5 are observed in the control samples which may suggest that they play a role in homeostasis of essential metals.

The chromatogram obtained from root samples exposed at 50 μM of CdCl_2 , with the retention times of several phytochelatin (PCs) is shown (Figure 4B). Six peaks of PCs can be observed, and were designated from PC1 to PC6 with the following retention times: 33 min (PC1), 34 min (PC2), 41 min (PC3), 42 min (PC4), 47 min (PC5) and 48 min (PC6). These PCs must play an important role in tolerance to cadmium by *S. trilobata*.

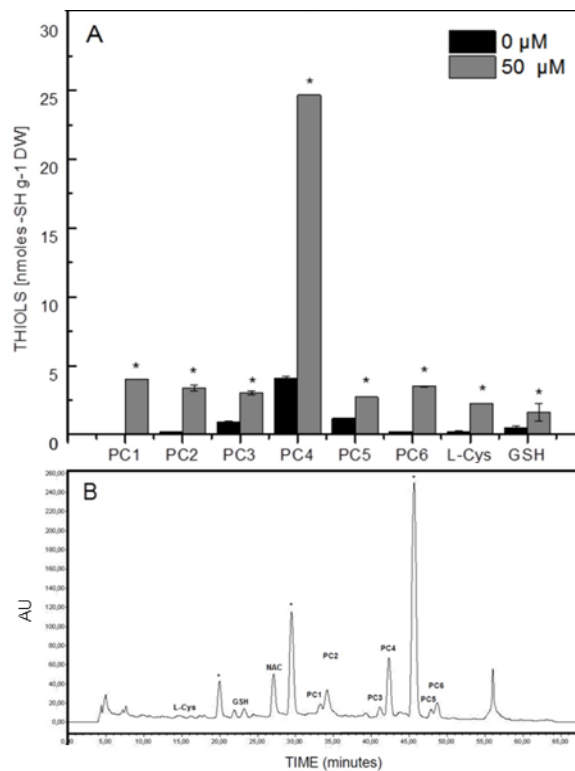


Figure 4. A: Comparison between concentration of L-Cys, GSH and PC in roots treated with 0 or 50 μM Cd. Bars in each column means SE (n=3). *Significant differences according to Dunnett test ($P \leq 0.05$). **B:** Chromatogram of HPLC in roots of *S. trilobata* exposed to 50 μM Cd for 96 hours. *Monobromobimane, NAC, L-Cys, GSH, and phytochelatin (PC, identified as PC 1 to 6). AU: arbitrary units.

Other authors have reported different types of PCs in tolerant species such as *Phragmites australis* (Fediuc et al., 2005), *Triticum aestivum* (Ranieri et al., 2005) and *Zea mays* (Keltjens and van Beusichem, 1998) where they found four types of PCs; *Helianthus annuus* (Gallego et al., 2005) and *Thlaspi caerulescens* (Wojcik et al., 2005) presented three types of PCs, and 2 types in *Bacopa monnieri* (Mishra et al., 2006), *Brassica juncea* (Gadapati and Macfie, 2006; Seth et al., 2008) and in *Silene vulgaris* (Sneller et al., 1999). Jia et al. (2011) also observed an increase in the concentration of high molecular weight PCs when exposing *Lolium perenne* plants to high concentrations of Cd.

Even though the *S. trilobata* is not a hyper-accumulating species, it shows many benefits for its potential use in phytoremediation as it tolerates high concentrations of Cd, generates fast growth, has a wide ecological range of tolerance, is mat-forming stoloniferous herb with a creeping, scrambling or climbing habit. They also develop adventitious roots at their nodes favoring the creation of an extended rhizosphere capable of embracing a bigger portion of heavy metals.

CONCLUSIONS

The concentration of Cd in the organs of *S. trilobata* increased proportionally to the concentration of the metal in the nutrient solution in order root>stem>leaves. This species accumulates more than 100 mg·kg⁻¹ Cd and have a bioconcentration factor greater than 1, but is not a hyperaccumulator plant because its translocation factor is less than 1, but can be considered an accumulator for phytoextraction of Cd polluted sites.

High concentrations of Cd generated inhibition of root growth, reduction in the chlorophyll content, alterations in the polypeptide pattern of the leaves and an increase in the amount of malondialdehyde. An increase in the content of soluble sugars was also seen, which are markers of tolerance associated with a protection mechanism against the oxidative stress. In addition, an increase on heavy metals chelating thiols such as L-cysteine, glutathione and various phytochelatins was obtained in the plant roots.

Unlike the PCs that appear in the control plants, in the plants treated with Cd PC1 appear,

and PC4 are significantly increased. By virtue of these results, the study of these two PCs as possible targets in detoxification processes and tolerance in *S. trilobata* is therefore proposed. PCs were only detected in roots, and some free Cd was translocated to the leaves producing injury such as chlorophylls and increase in lipid peroxidation.

S. trilobata shows benefits its for potential use in Cd phytoremediation as it tolerates high concentrations of this heavy metal.

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