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Insights into the mechanisms of Cd hyperaccumulation in *S. kali*, a desert plant species

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Abstract

Tumbleweed (*S. kali*), a desert plant worldwide distributed, has been proposed as a potential Cd-hyperaccumulator. X-ray studies showed that thiol and oxygen related compounds are involved in Cd sequestration within the plant. Thus, we have proposed that organic acids, cell wall, phytochelatins, and other glutathione related compounds might be involved in the mechanisms of Cd hyperaccumulation in tumbleweed. In this study, native plants were used to determine Cd content in phloem/xylem tissues and the related biochemical mechanisms of Cd uptake at the protein level. In addition, plant extracts were analyzed by high pressure liquid chromatography (HPLC) to identify and quantify organic acids. Plants were treated with 0, 20, 200, and 400 mg Cd L^{-1} for 48 h in hydroponic media. Cd incorporation was measured in roots, phloem/cortex, and xylem/pith, separately. It was found that in plants treated with Cd concentrations above 200 mg Cd L^{-1}, Cd content was higher in phloem than that in xylem. The protein profile in SDS-PAGE showed that in Cd-treated plants, two peptides were enhanced while a new peptide was expressed. After G25 gel filtration and Cd codetermination it was found that two proteins (of 29 and 14 kDa) are probably associated to Cd. The use of degenerated primers of the *Brassica* family allowed the identification of a possible phytochelatin synthase gene. Citric and oxalic were the main acids identified in plant extracts. No significant differences were found in the concentration of citric acid in control and Cd-treated plants. On the other hand, less oxalic acid was quantified in Cd-treated plants as compared to controls. These data indicate that cadmium may have precipitated as oxalate crystals. The results reported herein will be helpful to better understand the mechanisms of Cd hyperaccumulation in *S. kali.*

Keywords: *Salsola kali*, Cd, phytoremediation, mechanisms

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Resumen

Salsola kali, una planta desértica, ha sido propuesta como un potencial hiperacumulador de Cd. Estudios de espectroscopía de Rayos-X han mostrado que compuestos conteniendo oxígeno y grupos tiol están involucrados en la absorción de Cd en esta planta. Por ello se ha planteado que

algunos ácidos orgánicos, pared celular, fitoquelatinas y otros compuestos conteniendo glutatión, pueden estar relacionados con los mecanismos de hiperacumulación de Cd en *S. kali*. En esta investigación se expusieron plantas silvestres a diferentes concentraciones de Cd para determinar el contenido de este metal en el xilema y floema, así como en fracciones proteínicas. Adicionalmente, extractos de las plantas se analizaron mediante cromatografía líquida a alta presión para identificar y cuantificar ácidos orgánicos. Las plantas se trataron con 0, 20, 200 y 400 mg Cd L^{-1} por 48 h en hidroponia. La incorporación de Cd se midió por separado en raíces, floema y xilema. Se encontró que en plantas tratadas con concentraciones de Cd por encima de los 200 mg Cd L^{-1}, el contenido de Cd fue mayor en el floema que en el xilema. El perfil proteínico (SDS-PAGE) mostró que en plantas tratadas con Cd se incrementa la presencia de dos péptidos y se expresa uno nuevo. Después de una filtración en gel G25 y codeterminación de Cd, se encontró que es muy probable que dos proteínas (de 29 kDa y 14 kDa) estén asociadas al Cd. El uso de primers degenerados de la familia *Brassica* permitió la identificación de un posible gen de la fitoquelatin sintasa. Los ácidos cítrico y oxálico fueron identificados en los extractos de las plantas. No se encontraron diferencias significativas entre las concentraciones de ácido cítrico en plantas control y en aquellas tratadas con Cd. Por otro lado, la cantidad de ácido oxálico en plantas expuestas a Cd fue significativamente menor que en las plantas control. Estos datos pueden indicar que el Cd pudo haber precipitado como cristales de oxalato. Los resultados reportados aquí serán útiles para entender a mayor profundidad los mecanismos de hiperacumulación de Cd en *S. kali*.

Palabras Clave: *Salsola kali*, Cd, fitorremediación, mecanismos

Introduction

Recently, the interest in developing and applying phytoremediation techniques for the restoration of contaminated environments has substantially increased. A great deal of research is being performed with the purpose of making phytoremediation a commercially feasible technique (Barceló and Poschenrieder, 2003). Part of this research has been directed to identify hyperaccumulator plants, those able to tolerate and accumulate high concentrations of metals in their tissues (Brooks, 1998). Of paramount importance is also the elucidation of the mechanisms of metal hyperaccumulation as well as the understanding as to why plants hyperaccumulate metals. The understanding of those mechanisms will lead to the improvement of phytoremediation techniques for their successful application in real situations. Though several hypotheses have been suggested to clarify these issues, a full explanation is not yet complete (Noret et al., 2005).

Plants use different strategies for the absorption and accumulation of metals. At root level, some plants produce chelating agents including organic acids and other phytosiderophores that increase the bioavailability of metals and inorganic nutrients in the root-soil interface (Ma and Nomoto, 1996; Ma et al., 2001). The absorption of inorganic salts is mediated by two different mechanisms, passive and active, though the active seems to be the most important. Contrary to the passive mechanism, in the active absorption metabolic energy is required (Devlin and Withman, 1983). In the active transport of inorganic nutrients and metals, carriers are extremely important (Prasad, 2004). In addition, phytochelatins seem to play an important role in metal accumulation in plant tissues (Gong et al., 2003).

Metal tolerance in plants is usually energy consuming resulting in low biomass accumulation, phenomenon that has been called trade-off (Barceló and Poschenrieder, 2003). However, plants desirable for phytoremediation purposes should produce high amounts of biomass since this will be translated into a better extraction of the pollutant from the contaminated media.

It has been proposed that in some plants, Cd might be absorbed either via a high Fe-affinity system, or through low Ca-affinity pathways (Roosens et al., 2003). Preliminary results have shown that in tumbleweed plants Cd concentrations in the grow media below 45 µM significantly increased Ca absorption, while higher Cd concentrations caused a decreased in Ca

accumulation in the tissues. These data suggested that in this plant, Cd is uptaken via calcium channels. Furthermore, we have found that the uptake of Cd in *S. kali* is significantly correlated to the concentrations of non-proteinic low molecular weight thiols (De la Rosa, 2005).

Thlaspi caerulescens and *Arabidopsis halleri* are two plants species reported as Cd hyperaccumulators (Baker and Whiting, 2002). More recently tumbleweed (*S. kali*) has been identified as a potential Cd hyperaccumulator for desert areas (de la Rosa et al., 2004). This plant accumulated up to 2000 mg kg^{-1} Cd dry leaves when grown in agar. Furthermore, through X-ray absorption spectroscopy studies (XAS), we found that organic acids, cell wall, phytochelatins, and other thiol related compounds might be involved in the mechanisms of Cd hyperaccumulation in tumbleweed. Contrary to other Cd hyperaccumulators, tumbleweed (also known as *Russian thistle*) displays high biomass accumulation, reaching a weight of 1250 g per plant in some conditions (Schillinger and Young, 2000). Since it is also able to grow in very disturbed environments, it is a very promising plant for the phytoremediation of Cd contaminated soils where other plants are unable to survive.

The aim of this research was to obtain information about the nature of metabolites possibly involved in Cd transport and sequestration in tumbleweed. For this purpose, native tumbleweed plants were hydroponically exposed to different concentrations of Cd (supplied as $Cd(NO_3)_2 \cdot 4H_2O$) for 48 hours. The content of Cd in phloem and xylem tissues was determined and the protein profile of extracts obtained from treated and untreated plants was analyzed by SDS-PAGE (Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis). Protein bands were confirmed after G25 gel filtration and Cd codetermination. Since there is strong evidence indicating that phytochelatins may be involved in Cd binding in tumbleweed tissues, polymerase chain reaction (PCR) experiments were performed in an attempt to identify the gene that codes for the phytochelatin synthase. Additionally, high pressure liquid chromatography (HPLC) was used to identify and quantify citric acid and oxalic acid in tumbleweed plants exposed or not to Cd treatments. The results reported herein will be helpful in better understand the mechanisms of Cd hyperaccumulation in *S. kali*.

Methodology

Plant treatments

Wild tumbleweed plants were collected from areas around El Paso, TX. Soil samples were also collected to assess metal contamination. Plants were approximately 20 cm in length (root plus shoot). Upon arrival to the laboratory, plants were thoroughly rinsed with tap water and roots were immersed for 1 hour on a 2% captan solution in order to avoid fungal contamination. Subsequently, plants were rinsed with deionized (DI) water and placed on a modified Hoagland nutrient solution prepared as previously described by Peralta et al. (2001). After one day of adaptation, plants were transplanted to the Cd treatments (0, 20, 200, and 400 mg L^{-1} Cd) and allowed to stay for 48 hrs. These concentrations were selected based on seedlings tolerance and screenings on adult plants (data not shown).

Harvesting and tissue separation

Upon harvesting, roots were rinsed in 0.01M HNO_3 (Aldrich et al., 2003) and cut. A portion of the aerial part was used to separate the epidermis in order to obtain phloem and xylem tissues. Another portion of tumbleweed shoot was maintained complete. All the samples were frozen in liquid nitrogen for at least 45 min, and subsequently lyophilized for 2-3 days on a Labconco Freeze-drier at -40 °C and $80x10^{-3}$ Mbar pressure.

Hystochemical dyeing

Fresh thin cross sections of stems of tumbleweed plants were stained by immersion in an acidic solution of Ponceau S (0.1% in 5% acetic acid) for 10 min. Subsequently, sections were destained for 10 min by immersion on a methanol:acetic acid:water (45:10:45) solution (Bannur et al., 1999). Samples were then placed on slides and observed by light microscopy (60X) using an Olympus BX 51 microscope adapted with an Olympus DP 11 digital camera.

Sample digestion

Roots, phloem and xylem samples were treated according to EPA method 3051 (Kingston and Jassie, 1988) with minor modifications. Three to four mL trace pure HNO_3 were added to approximately 50 mg of dried tissue. Samples were digested on a microwave oven (CEM MarsX, CEM Corporation, Mathews, North Carolina). After digestion, samples were diluted to 15 mL

using DDI (double deionized water) and analyzed for Cd content using inductively coupled plasma-optical emission spectroscopy.

SDS-PAGE Analysis

Tumbleweed plants were hydroponically exposed to 0, 20, 200 and 400 mg L^{-1} Cd for 48 h and then lyophilized. Approximately 1 g of aerial part of tumbleweed plants of each treatment were pulverized to powder using a porcelain mortar. Subsequently, 0.1 g of sample powder was washed three times with 1 mL cold acetone in order to eliminate pigments. After acetone evaporation, biomass was added to 0.1 mL loading buffer (35 mM Tris pH 6.8, 1.4 mM EDTA, 0.7% w/v SDS, 100 mM 2-mercaptoethanol, 5% glycerol, 0.01% w/v bromophenol blue) and placed on a water bath for 5 min (T=100ºC). Ten µL of supernatant of each sample were loaded on a discontinuous 10% polyacrylamide following the standard methodology described elsewhere (Coligan et al., 2005). Gel was run on an 80 V current until the bromophenol blue reach the bottom of the gel (approx. 2 h). After electrophoresis, protein bands were developed by using the silver stain methodology (Coligan et al., 2005). After gel staining, photographs were taken (VersaDoc System BIO-RAD) and density normalization and analysis was done by using the PDQUEST software (BIO-RAD).

Fractionation of plant extracts and protein assay

Extracts of the complete aerial part of tumbleweed plants were obtained by boiling 1 gr of ground to powder freeze-dried tissue for 5 min in 15 mL DI water adjusted to pH 4.8 with trifluoroacetic acid (TFA). Extraction mixture was cool down and centrifuged for 5 min at 3000 rpm. The supernatant was then passed through a 0.2 µm filter. Two mL of filtered extract were passed through a 150 x 1 cm Sephadex G25 column, isocratically eluted with TFA (pH=4.8). Fourty five fractions (2.5 mL each) were collected at a flow rate of 0.5 mL min^{-1}. After collection, samples were analyzed for protein and metal content. Fractions were assayed for protein content using the Bradford assay. After Cd quantification to identify the fractions where

the metal and protein were present, 50 µL of fractions where mixed with 25 µL of 3x loading buffer and run in a discontinuous 10% polyacrilamide gel. Sigma M4038 molecular weight standards were used for calibration.

Polymerase chain reaction experiments

Genomic DNA and PCR experiments were done on seedlings of tumbleweed using the Sigma Extract-N-AmpTM kit (SIGMA), following the instructions of the provider (technical bulletin numbers MB-850 and MB-410). In summary, approximately 10 mg of tissue were incubated in 0.1 mL of REDExtract-N-AmpTM (SIGMA) at 95ºC for 10 min. Later, 0.1 mL of dilution solution was added to the extraction mixture. Samples were then centriged at low speed and supernatants were used as DNA source. In an attempt to initially identify the gene that codes for phytochelatin synthase, we performed a PCR experiment using primers of *Arabidopsis thaliana* (gi 3559804) and *Zea mays* (gi 6690553) (Invitrogen). On a second experiment, we designed degenerated primers using the information available for the Brassicca family (PEIF and PEIB, SIGMA). Description of the primers is given in Table 1.

Table 1. Primers used for initial identification of the phytochelatin synthase gene in tumbleweed plants*

Source	name	Sequence
Arabidopsis thaliana	gi 3559804	left 5'-GAGAAAGGTTGGGCTTTTCC-3' right 5'-CCGTAACCCTAAGCAAACCA-3'
Zea mays	gi 6690553	left 5'-CAAATGCTGCTTCCTCCTTC-3' right 5'-TTGGGAATATGTGCAGGTCA-3'
Degenerated Brassica family	PEIF PEIB	left-5'-GCCTCCCTGTCCATGGTGYTNAAYGCNYT-3' right-5'-TYGTYTGNCCNWGGCCGGTGAAGAGGGG-3'

* Y= C+T; W= A+T; N = A+T+G+C.

PCR conditions were: 10 µL of REDExtract-N-Amp PCR reaction mix, 1 µL of 10 µM primer, and 4 µL of DNA source. Reagents were mixed and diluted to fill 20 µL. Cycling

conditions were; 94ºC/3 min initial denaturation, 32 cycles of 94ºC/0.5 min denaturation, 64ºC/Annealing (65ºC for degenerated primers), 72ºC/1 min extention, and a final extention at 72ºC/10 min following by a holding step at 4ºC. Samples were analyzed on a 2% agarose gel and stained with ethidium bromide (Ausubel et al., 2003).

Identification and quantification of organic acids in extracts of tumbleweed plants.

We have previously reported that in tumbleweed plants Cd is bound to oxygen moieties, probably from organic acids (De la Rosa et al., 2004). With the aim of obtaining more information about the role of organic acids in Cd hyperaccumulation/tolerance in tumbleweed plants, HPLC experiments were performed using extracts of tumbleweed tissues. The method used was modified from Cawthray (2003). In summary, freeze-dried stems of control and Cd-treated plants (200 mg L^{-1}) were ground to powder. Subsequently, 10 mg of tissue were boiled for 20 min in 1 mL of 0.1 % v/v trifluoroacetic acid in double deionized water. After cooling, samples were centrifuged for 15 min at 10,0000 rpm (Beckman benchtop ultracentrifuge) and filtered using 0.2 µm syringe Millipore filters. Two hundred µL were injected for analysis on a GBC equiped with a LC150 pump and UV-Vis detector LC1205K. An Alltima C18 (250 mm X 4.6 mm, ID.) with 5 µm particle size (Alltech) columm was used. Data were analyzed with WinChrom 1.32 software (GBC). The mobile phase was a 0.1% v/v trifluoroacetic acid in MQ water, at a flow rate of 0.5 ml min^{-1}. Separation was performed at room temperature (25°C). Calibration curve covered from 200 to 2400 nmol of each acid (r^2 for citric acid = 0.998, r^2 for oxalic acid = 0.997), and all measurements were inside the calibration curve without doubt of inferior or superior detection limits. Standards used were citric, and oxalic acid (SIGMA). Organic acids were identified and quantified by comparing retention times (citric acid Rt = 4.433 ± 0.1146; oxalic acid Rt = 7.267 ± 0.0732) and peak areas.

Cadmium quantification

A Perkin-Elmer ICP/OES Optima 4300 DV (Perken Elmer Corporation, Shelton, CT) with a Perkin-Elmer AS-90 plus autosampler rack was used for cadmium quantification. For this purpose, a background equivalent concentration test was performed and the following parameters introduced: nebulizer flow, 0.7 L min^{-1}; radio frequency power, 1450 watts; sample introduction,

1.5 mL min^{-1}; flush time, 15 sec; delay time, 15 sec; read time, 10 sec; wash time, 90 sec; replicates, 3. Five standards for calibration curves were prepared from a 1000 mg Cd L^{-1} stock solution and diluted using a 2% HNO_3 solution, which also served as the blank. Three different wavelengths were read to obtain the calibration curves aiming at the selection of the best suitable wavelength to be used in the analysis. Correlation coefficients for the calibration curves were ≥ 0.9998. Wavelength was selected according to the best correlation coefficient and detection limit (214.44 nm).

Results

Cadmium content in tumbleweed tissues.

Figure 1 shows a cross section of wild tumbleweed stem indicating the location of different tissues as well as the section that was cut to perform the analyses of the xylem/pith and cortex/phloem. The stem shows a secondary xylem forming a continuous ring surrounding the pith. The same ring appears to be parenchyma tissue associated to the xylem. The pith is shown right at the center of the stem cross section. It is formed by large

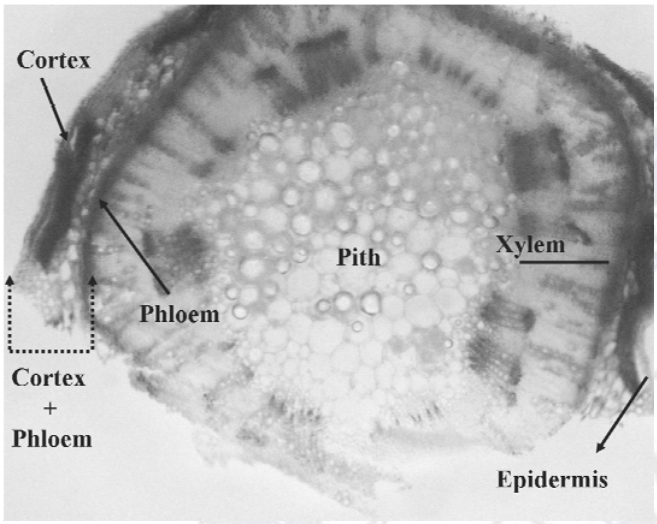


Figure 1. Cross section of native tumbleweed stem stained with Read Ponceau. Different tissues are indicated.

cells with very tiny walls. The phloem tissue is located around the external section of the xylem tissue. The epidermis is located on the most external part of the stem section. Right below the

epidermis, collenchyma tissue is also shown. In *Chenopodiaceous*, internal phloem is absent (Watson and Dallwitz, 1992).

Cadmium content in cortex/phloem and xylem/pith tissues of tumbleweed plants exposed for 48 hours to the Cd treatments used in this study are shown in Figure 2. This figure shows that at 20 mg Cd L^{-1}, no significant differences ($P<0.05$) were observed in Cd content between xylem and phloem tissues (170 ± 30, and 190 ± 40 mg Cd kg^{-1} d.wt., respectively) and the highest concentration was found in root tissues (1300 ± 80 mg Cd kg^{-1} d.wt). On the other hand, at Cd concentrations in the growing media of 200 and 400 mg L^{-1}, Cd content in xylem was lower than that in phloem and root tissues. Moreover, figure 2 shows that Cd content in xylem of plants grown in 200 mg L^{-1} Cd, and those treated with 400 mg L^{-1} Cd was of 3000 mg kg^{-1} d.wt for both of them, while in phloem, the concentration of Cd reached the 7500 ± 1000 mg kg^{-1} d.wt.

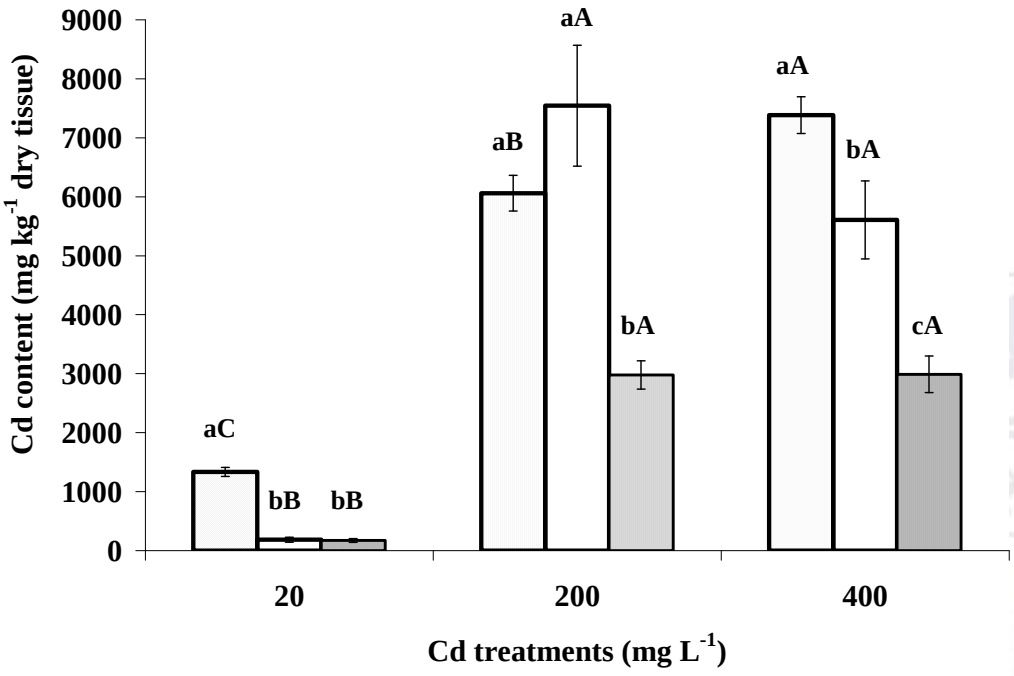


Figure 2. Cd content in ⬚ roots, ☐ phloem/cortex, and xylem/pith ▥ of tumbleweed plants cultivated in hydroponics for 48 h with different Cd concentrations. Error bars indicate ± S.E ($P<0.05$; n=5). Uppercase letters indicate significant differences in Cd content in the same tissue of different treatments. Lowercase letters indicate significant differences in Cd content in different tissues of the same treatment.

Given that Cd was quantified in the phloem, this means that this metal is passing through the stem reaching the leaves. Here, Cd is processed and subsequently transported through the phloem. This means that the processes involved in Cd uptake are not purely hydrostatic pressure gradient-driven, but instead, the plant is actively transporting the metal. There is strong evidence indicating Cd transport in the xylem of tumbleweed plants has low selectivity (unpublished data). The results presented herein show that Cd is moving from xylem to phloem and in consequence this might imply that tumbleweed possesses specific metabolic mechanisms to translocate this heavy metal.

Tumbleweed modifies its protein content after Cd exposure

Figure 3 shows the differential display of proteins in the whole extracts of tumbleweed plants exposed to increasing Cd concentrations. Black arrow indicates a protein that was constant between control plants and those exposed to Cd. White arrows show proteins that were enhanced after Cd exposure. The asterisk shows the position of a new protein that was expressed after treatment with Cd. In summary, the expression of two proteins increased while a new protein appeared in Cd treated plants. The purpose of this experiment was to perform a quick survey to determine if tumbleweed modifies its protein content after Cd exposure. Further experiments will provide more information about the nature of these proteins.

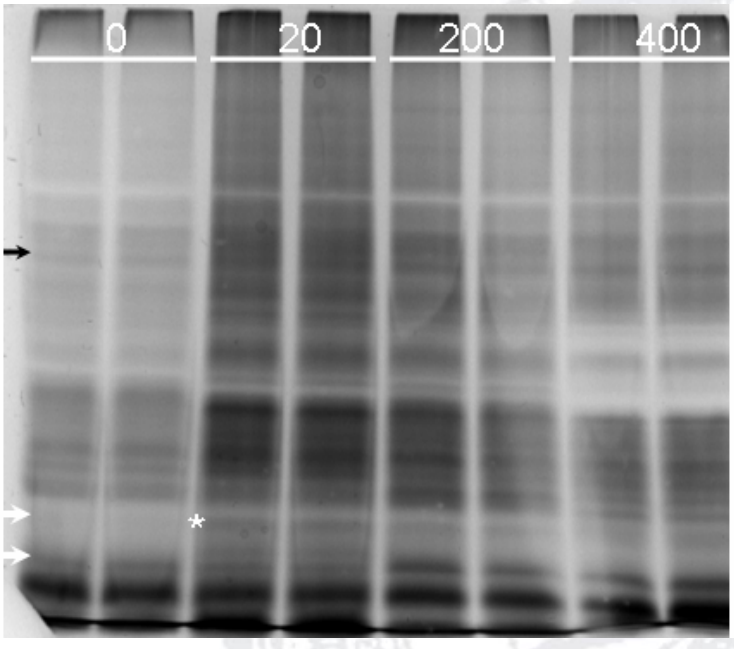
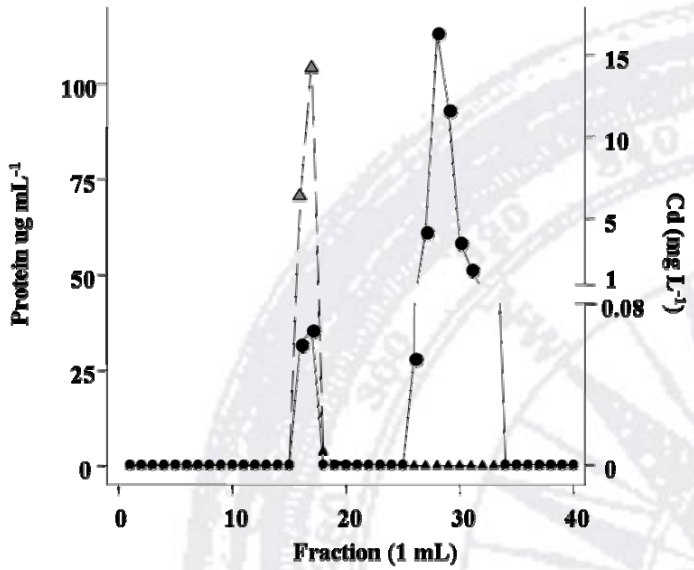


Figure 3. Protein differential display of whole extracts of tumbleweed exposed to increasing Cd concentrations. (a) Black arrow indicates a protein that was constant in control and Cd exposed plants; (b) white arrows indicate a protein that was enhanced after Cd exposure; asterisk indicates a new protein expressed after tumbleweed plants were exposed to Cd.

Low molecular weight, water soluble proteins are probably synthesized after Cd exposure

In order to obtain more information about the proteins expressed in tumbleweed plants exposed to Cd, aqueous extracts of treated plants were fractionated using a Sephadex G 25 column. Figure 4(a) shows the results for Cd and protein content in Sephadex G 25 fractions. Figure 4(a) shows that Cd was differentially eluted from the Sephadex G 25 column. Thus, peak concentrations of Cd where found in the 17, and 29 fractions, while protein content peaked in fraction 17 with a concentration of 100 µg mL^{-1}. No protein was detected in fractions 26-33. Figure 4(b) displays the polyacrilamide gel for fractions 15 to 20 after resolution. In this figure, arrows point to the main protein bands stained using the silver staining protocol as described in methods. The bands probably correspond to the proteins shown in Figure 4(a). Figure 4(b) clearly shows that fractions 15 and 18-20 did not contain protein. Besides, in fractions 16 and 17, the molecular weight of the proteins corresponds to 29 and 22 kDa.



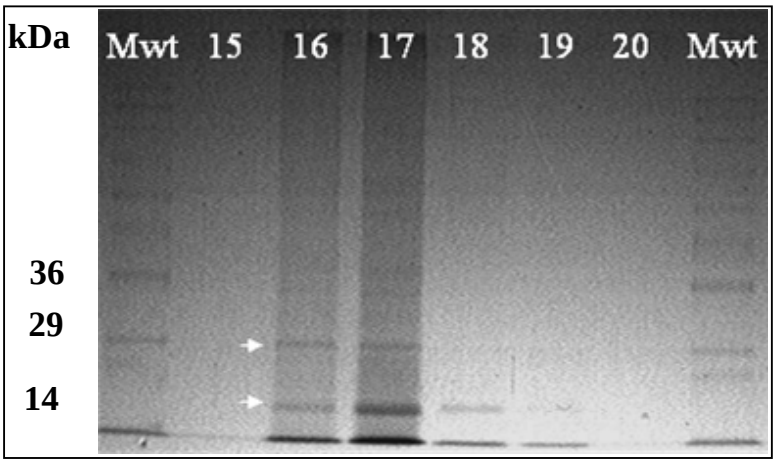


Figure 4 (a) Cd (●) and protein (▲) content in extracts of tumbleweed plants passed through a Sephadex G25 column. Extracts were isocratically eluted with water adjusted to pH 4.8 with TFA acid. 50 µL of every fraction were mixed with 25 µL of 3X loading buffer and run in a discontinuous 10% polyacridamide gel. Molecular weight standards were purchase from SIGMA (M 4038).

In this investigation, only aqueous extracts were analyzed. Thus, if some Cd was associated to non-soluble compounds, they were not identified in these experiments. However, we have identified two proteins apparently associated to a small fraction of the total soluble Cd present in tumbleweed plants. As the results showed, most of the eluted Cd was not associated to protein (Figure 4(a)). Previous experiments have demonstrated that low molecular weight thiols contribute up to ½ of the total Cd binding (De la Rosa et al., 2005). These thiols may be glutathione and glutathione-related compounds including phytochelatins. In that case, not protein was associated to the low molecular weight thiols extracted for their quantification. In the present research, no LMWT were detected in the protein fraction, which means that, in addition to the production of LMWT, we are possible identifying an additional mechanism for Cd hyperaccumulation/detoxification in tumbleweed plants. Several proteins have been identified as participating in tolerance and transport of metals in plants (Verret et al., 2004). In tumbleweed, previous data have indicated that heavy metal transporters may be induced after Cd exposure (De la Rosa et al., 2005). However, more study is needed in order to prove this hypothesis.

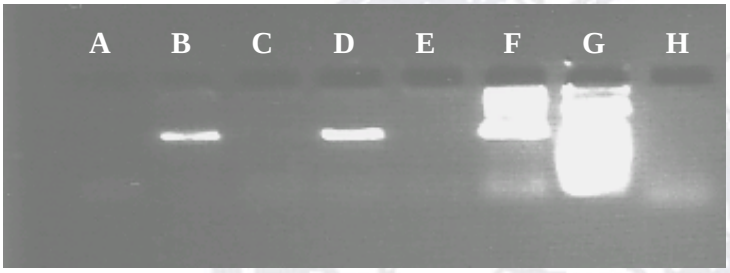
PCR experiments

Cd is a well known inducer of phytochelatins (PCs) biosynthesis in different plant species (Mejare and Bulow, 2001). In this process, phytochelatin synthase (PCS) is the enzyme that

catalyzes the PCs synthesis from glutathione. The enzyme is activated in the presence of heavy metals. Strong evidence supports the hypothesis that phytochelatins are involved in the mechanisms of Cd hyperaccumulation/tolerance in tumbleweed plants. As previously explained, in an attempt to initially identify the gene that codes for phytochelatin synthase, PCR experiments were performed using primers of *Arabidopsis thaliana* (gi 3559804), *Zea mays* (gi 6690553), and degenerated primers using the information available for the Brassicca family (PEIF and PEIB).

Figure 5(a) displays the PCR results obtained using *Arabidopsis thaliana* (expected product size equal to 211 pair bases) and *Zea maize* (expected product size equal to 215 pair bases) primers. Figure 5(b) shows the PCR results when degenerated primers of the Brassica family were used. As seen in Figure 5(a), *Arabidopsis thaliana* and *Zea mays* primers did not amplified any tumbleweed DNA (A and C). Also shown are the *Zea mays* and corn truffle amplicons (B and D). The positive PCR control (F) indicates that this is a true negative result for tumbleweed and alfalfa DNA amplification using MP1. In Figure 5(b), the arrow shows a potential amplicon similar to *Brassica* PCS (L), while asterisk indicates a primer-dimer artifact (I); In L, the tenuous bands at the bottom are the primers. PCR conditions are detailed under PCR experiments. According to these preliminary results, it is very possible that tumbleweed possesses the phytochelatin synthase gene. In addition, the gene has a sequence similar to those in the *Brassica* family. We propose the cloning, sequenciation and expression of this PCR fragment in a non Cd hyperaccumulator plant to determine if this gene has any role in the Cd tolerance and hyperaccumulation in tumbleweed plants.

(a)



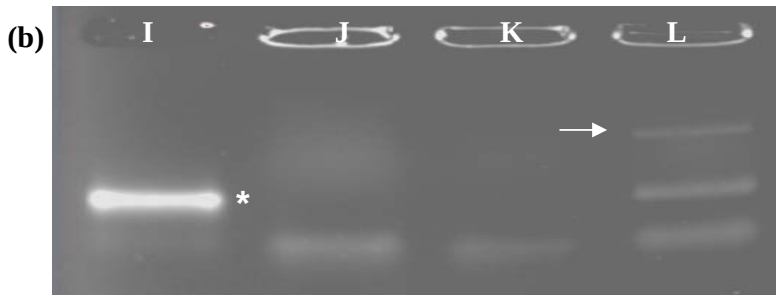


Figure 5. (a) PCR using primers of *Arabidopsis thaliana* and *Zea maize*. A, tumbleweed DNA + *Arabidopsis thaliana* primers; B, *Zea mays* DNA + MP1[£]; C, tumbleweed DNA + *Zea mays* primers; D, corn truffle DNA + MP1[£]; E, alfalfa DNA + MP1[£]; F, positive control; G, molecular weight marker; H, MP1. **(b)** PCR using degenerated primers of the *Brassica* family. I, MP2[§]; J, tumbleweed DNA + PEIF; K, tumbleweed DNA + PEIB; L, tumbleweed DNA + MP2[§]. [£]*Arabidopsis thaliana* + *Zea maize* primers; [§]PEIF + PEIB. Arrow shows a potential amplicon; Asterisk indicates primer/dimer artifact.

Identification and quantification of organic acids in tumbleweed plants

Citric acid and oxalic acid were identified as the main organic acids in tumbleweed extracts (retention times of 5.2 and 7 min approximately). Samples of control plants and those treated with 200 mg Cd L^{-1} were analyzed in order to determine if the presence of this heavy metal affected the concentration of organic acids in tumbleweed tissues. The results are shown in Figure 6. This figure shows that in Cd treated and untreated plants concentration of citric acid was of approximately 1800 mmol kg^{-1}. No difference was observed in citric acid content in both treatments. On the other hand, the concentration of oxalic acid in plants was different according to the treatment. Thus, 1386 and 593 mmol kg^{-1} were quantified in control and Cd-treated plants, respectively. The reduction in oxalic acid in Cd-treated plants does not necessarily means that its production was reduced. Instead, it is possible that oxalic acid bound Cd and its signal was not detected as it was already complexed, probably precipitated in the form of cadmium oxalate crystals.

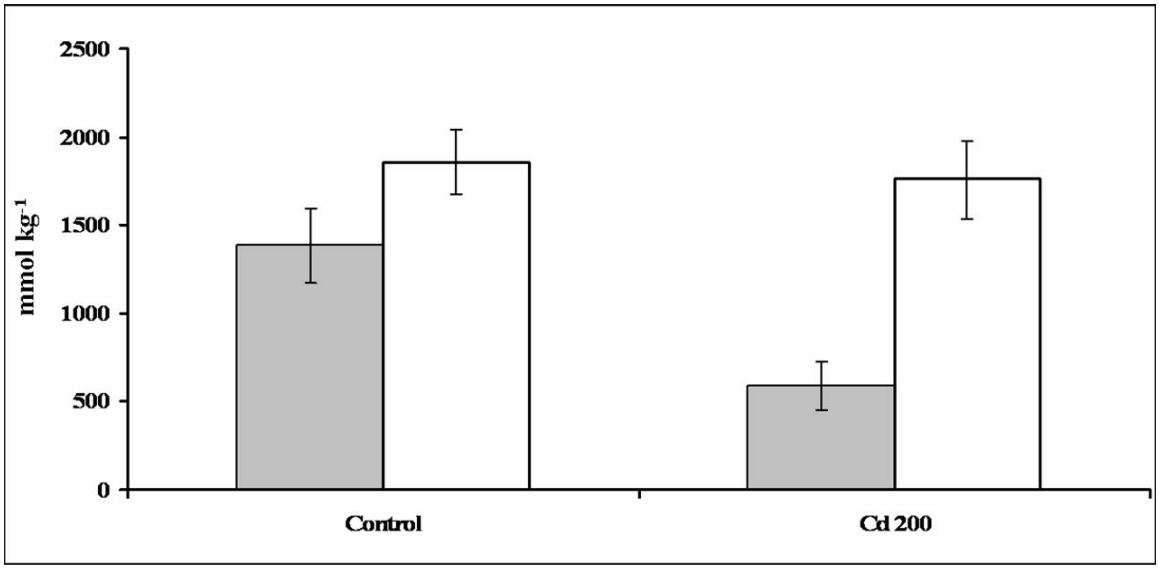


Figure 6. Content of oxalic acid ■ and citric acid □ in stems of tumbleweed plants exposed for 48 h to 0 and 200 mg Cd L^{-1}. Data are mean of 3-4 replicates ± S.E.

It is well known that a great number of plants accumulate calcium oxalate crystals in their tissues (Nakata, 2003). It has been proposed that oxalates play an important role in calcium regulation, plant protection, tissue support, and heavy metal detoxification, among others (Franceschi and Horner, 1980). Oxalic acid is a well know complexing agent of heavy metals leading to the precipitation of the oxalate salts. In experiments performed with the fungus *Beauveria caledonica*, Cd among other heavy metals, induced the production of oxalic acid subsequently forming cadmium oxalate crystals (Fomina et al., 2005). In addition, Lutts et al. (2004) suggested that the formation of Cd oxalate crystals in Mediterranean saltbush (*Atriplex halimus* L.) as an important mechanism for Cd detoxification. According to this, it is very possible that in tumbleweed oxalic acid concentration was reduced in Cd-treated plants a result of the precipitation of Cadmium oxalate crystals. To confirm this possiblitily, we propose for future research the microscopic observation of living tumbleweed plants exposed to Cd in search of the presence of those crystals.

Conclusions

The present study explored the possible participation of proteins and organic acids in Cd hyperaccumulation in tumbleweed plants. It was demonstrated that Cd is differentially

incorporated in plant tissues as more Cd was found in phloem/cortex than in xylem/pith. In addition, it was found that a new protein is expressed in Cd-treated plants. After fractionation it was determined that two low molecular weight proteins may be associated to Cd. In addition, it is very possible that oxalic acid acts as a Cd-complexing molecule, precipitating as cadmium oxalate crystals and decreasing the amount of oxalic acid quantified in plant extracts. In addition, an initial identification of the phytochelatin synthase gene was performed, and this was achieved when degenerated primers of the *Brassica* family were used. The results reported herein provide helpful insights into the mechanism of Cd hyperaccumulation in a desert plant species.

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