Journal of Coastal Life Medicine

journal homepage: www.jclmm.com

Document heading doi:10.12980/JCLM.2.2014JCLM-2014-0077

Cadmium-induced oxidative damage and antioxidant defense mechanisms in *Glycine max* L.

Emiliano Felici[1,2], Alicia Molina[2], Cesar Almeida[1], Martin Fernández Baldo[1], Fanny Zirulnik[2], María Roxana Gomez[1*]

[1]*Area Analytical Chemistry, INQUISAL-CONICET, Faculty of Chemistry, Biochemistry and Pharmacy, National University of San Luis, Chacabuco 917, San Luis, D5700HHW, Argentina*

[2]*Area Biological Chemistry, Faculty of Chemistry, Biochemistry and Pharmacy, National University of San Luis, Chacabuco 917, San Luis, D5700HHW, Argentina*

PEER REVIEW

Peer reviewer

Dr. Patricio Muñoz Torres, Fundación Científica y Cultural Biociencia, José Domingo Cañas 2280, Ñuñoa, Santiago, Chile.
Tel: +56 2 23432578
E-mail: pmunoz@bioscience.cl

Comments

This work is interesting as the authors contribute to the understanding of Cd-induced oxidative stress through the study of gene expression and antioxidant activity of some enzymes. Moreover, the use of capillary electrophoresis allowed the measure of reduced and oxidized form of glutathione.
Details on Page 797

ABSTRACT

Objective: To analyze the effect of Cadmium (Cd) on the metabolism of reduced glutathione (GSH) in roots and leaves of *Glycine max* L.
Methods: A capillary electrophoresis methodology was optimized to determinate simultaneously GSH and reduced and oxidized GSH in a precise and accurate way. The functional role of the genes involved in GSH cellular metabolism (γ-glutamylcysteine synthetase), GSH synthetase and glutathione reductase (GR) was evaluated. Finally, the activities of antioxidant enzymes as GR and superoxide dismutases were determinate.
Results: The studies of γ-glutamylcysteine synthetase and GSH synthetase gene expression showed an increase and GR showed a decrease in Cd-treated plants. GR and superoxide dismutases activities increased at 24 h and 6 h respectively in roots under Cd exposure. GSH content was higher and showed a significant increase in roots and leaves at 6 h and 24 h of treatment.
Conclusions: The results of the present study showed a better understanding in signaling pathway by alterations in antioxidant mechanisms by Cd in soybean seedlings.

KEYWORDS
Soybean, Cadmium toxicity, Oxidative stress, Antioxidant defense, Glutathione, Capillary electrophoresis

1. Introduction

The adverse effects of heavy metals on human health have been known for a long time. Heavy metals are toxic even at low concentrations; also, they are not biodegradable, and have the tendency to accumulate in the living organisms[1]. In spite of this, the exposure to heavy metals continues and is increasing in some areas due to anthropogenic activities[2].

Plants are frequently exposed to a variety of conditions that affect their growth, development and productivity. Many heavy metals such as copper (Cu), iron (Fe) and zinc (Zn) are essential for plants in low quantities, but cadmium (Cd) is toxic even in trace levels. Cadmium accumulation causes morphological and physiological alterations, reduction in the photosynthetic activities, diminution in the water and nutrients uptake and also results in visible symptoms of injury in plants such as chlorosis, growth inhibition, browning of root tips, and even death[3,4].

Although the average levels of Cd can be quite low, its inhibitory or toxic impacts on the metabolic activity of living systems or, in particular, on the natural microflora and microfauna in the soil can have a deleterious effect on the quality of the soil and, consequently, on nutrient

*Corresponding author: Dr. María Roxana Gomez, Department of Pharmacy, Faculty of Chemistry, Biochemistry and Pharmacy, National University of San Luis, Argentina.
Tel: +54 266 4446765
E-mail: roxanag@unsl.edu.ar

Foundation Project: Supported by National Scientific and Technical Research Council (CONICET), the National Agency of Scientific and Technologic Promotion (FONCYT) (PICT-BID) and National University of San Luis (Argentina) PROICO N° 2/1112, PROICO N° 2/0403 and PROICO N° 2/1512.

Article history:
Received 11 Jun 2014
Received in revised form 19 Jun, 2nd revised form 25 Jun, 3rd revised form 2 Jul 2014
Accepted 28 Jul 2014
Available online 18 Sep 2014

cycling.

At the cellular level, Cd causes membrane damage due to changes in lipid composition, disruption of electron transport, inhibition/activation of enzymes, interaction with nucleic acids and induction of oxidative stress process in plant cells[5,6]. The induction of oxidative stress and the replacement of essential cofactors of many enzymes by elements such as Zn, Fe and Mn are the possible mechanisms of some of these disorders[7].

Studies of the relationship between oxidative stress and heavy metal ion toxicity in plants showed that the damage was due to an enhanced production of reactive oxygen species (ROS) in different plant species and activation of enzymes[4], which probably contribute to general stress-induced morphological changes such as root thickening, ectopic root hair formation, root cell wall modifications and lignification.

Plants detoxify ROS via a combination of mechanisms include non-enzymatic and enzymatic defenses in plants. Non-enzymatic systems consist on high concentrations of antioxidants, such as ascorbic acid, reduced GSH, α-tocopherols, and carotenoids. Enzymatic defenses include cytosolic and mitochondrial superoxide dismutases (SOD; EC 1.15.1.1) and GSH reductase (GR; EC 1.8.1.7) among others[8,9].

In plants, GSH is the major soluble antioxidant in photosynthetic and non-photosynthetic tissues. It is able to detoxify ROS by direct scavenging or acting as a cofactor in the enzymatic reactions that are involved in the ascorbate-GSH cycle[10].

GSH is an attractive target for engineering stress tolerance in plants because of its multiple roles against both biotic and abiotic stresses. Stimulation of GSH biosynthesis is frequently observed under adverse conditions; this compound is also a precursor of phytochelatins (PCs), which enable the plant to resist supraoptimal concentrations of heavy metals such as Cd[10-12].

Reduced GSH is the major non-protein thiol present in high levels in living cells. Although the oxidized glutathione (GSSG) is detected at lower concentrations, the GSH/GSSG ratio influences are more deeply in the control of gene expression and protein function than in the GSH pool[10,12].

The synthesis of GSH (ATP-dependent steps) is initialized by γ-glutamyl-cysteine synthetase (γ-ECS; EC 6.3.2.2). First, γ-glutamylcysteine is synthesized from L-glutamate and L-cysteine by the enzyme γ-glutamylcysteine synthetase. This reaction is the rate-limiting step in GSH synthesis. Then, a glycine is added to the C-terminal of γ-glutamylcysteine via the enzyme glutathione synthetase (GS; EC 6.3.2.3).

GSH reductase has the ability to regenerate GSH by recycling it from the ascorbate-GSH cycle[10]. SODs are metal-containing enzymes which comprise several isoforms that can be distinguished on the basis of their metal co-factor. In general, plants contain a mitochondrial MnSOD, as well as a cytosolic and a chloroplastic Cu/Zn-SOD; also, many plants contain chloroplast Fe-SOD[5,9].

Plants can also take up heavy metals from fertilizers, pesticide treatment and other industrial and anthropogenic operations. After exposure to Cd, it is accumulated in different vegetal parts, preferentially in roots[13,14], and only a small portion is transported to the aerial organs.

Due to the great solubility of Cd in water and the high mobility in the soil-plant system, Cd is readily taken up by the roots and translocated to roots-stems-seeds, indicating that the accumulation of the roots is much larger than other parts of soybean plant, and it might cause deleterious effects to the root systems[15].

The tolerance of plants to Cd involves metal detoxification processes, such as complexation with PCs, that are specific chelating compounds[10,11,14,15]. PCs may transport metals from roots to shoots and leaves[12], and thus the quantification of metals in different plant parts may correlate PCs production with metal accumulation and its transport[16,17].

The aim of the present work was to analyze the role of Cd in the behaviour of GSH-GSSG levels, activity of antioxidant enzymes as GR and SOD, genes expression of interest in production of GSH and relative water content (RWC) on *Glycine max* L. (*G. max*).

2. Materials and methods

2.1. Plant material and growing conditions

Soybean seeds (*G. max*) were sterilized with 2.5% of sodium hypochlorite before they were potted and allowed to grow under hydroponics conditions in a controlled climate room [(24±2) °C, 50% of relative humidity and a photoperiod of 16 h with a photon flux density of 280 μmol/m^2/s]. The plants were watered with a Hoagland nutrient solution, and separated into two groups, control and Cd-treated seedlings. Then, they were exposed to 40 μmol/L Cd solution prepared from anhydrous $CdCl_2$ at Day 10. Roots and leaves of control and Cd-supplied plants were processed and tested at 4, 6, and 24 h. Samples of soybean were frozen at −80 °C until the time of analysis. For all determinations, mean values were obtained from five replicates (n=5). Each replicate was a pool composed at least by 3 different plants.

About 250 mg of roots and leaves were used for the determination of GR activity. The extracts were obtained by grinding the roots and leaves in liquid nitrogen using mortar and pestle, and 2 mL extraction buffer, composed by 50 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EDTA, 1 g PVP, 10 mmol/L $MgCl_2$ and 2 mmol/L DTT. The homogenates were centrifuged at 10 000 r/min for 20 min at 4 °C, and supernatants were used for enzyme assay.

To determine enzymatic activity of total SOD, 250 mg of roots and leaves were homogenized (at 4 °C) in 1 mL of extraction buffer containing 50 mmol/L phosphate (pH 7.4), 1 mmol/L EDTA, 1 g PVP and 0.5% (v/v) Triton X-100. Then, homogenates were centrifuged at 10 000 r/min for 20 min at 4 °C, and the supernatant fraction was used for assays.

To carry out the determinations by capillary electrophoresis, extracts from 250 mg of roots and leaves were homogenized (at 4 °C) in 1.5 mL of HCl 0.5 mol/L. The homogenates were centrifuged at 11 000 r/min for 10 min at 4 °C and the supernatants were used for the assays. To obtain a good electrophoretic performance, the supernatants were filtered through 0.45 μm filters, sonicated in an ultrasonic bath, and then aliquots of 100 μL were diluted up to 1 000 μL with the BGE solution[18].

For Cd determination, roots and leaves were washed with ultrapure water, oven-dried at 80 °C to constant weight.

Then samples were mineralized by a microwave-assisted digestion as follows: 100 mg of dried biomass were placed in a 100 mL reactor and after that, 5 mL of concentrated nitric acid, 2 mL of H_2O_2 and 1 mL hydrofluoric acid were added. Then the samples were digested applying different microwave powers, *i.e.* microwave power was held at 250 W (5 min), 500 W (10 min). The vessels were then removed from the oven and cooled at 20 °C.

2.2. Instrumentation

GSH and glutathione disulphide were determined by using a CE Beckman P/ACE MDQ instrument (Beckman Instruments, Fullerton, CA, USA) equipped with a diode array detector and a data handling system comprising an IBM PC and P/ACE System MDQ Software. Detection was performed at 198 and 203 nm. The fused-silica capillaries were obtained from MicroSolv Technology Corporation and had the following dimensions: 60 cm total length, 50 cm effective length, 75 μm ID, 375 μm OD. The temperature of the capillary and the samples were maintained at 25 °C.

The measurements of Cd content were performed with a model ICP 2070 sequential spectrometer (Baird, Bedford, MA, USA). The 1 m Czerny-Turner monochromator had a holographic grating with 1 800/mm groove. An inductively coupled plasma mass spectrometer, Perkin-Elmer Sciex, Elan DRC-e (Thornhill, Canada) was also used for the metal content determination. Microwave digestion was performed with a domestic microwave oven (Philco, Ushuaia, Argentina). A Beckman coulter UV-vis spectrophotometer model DU640 with 10 mm optical path cells was used to record the absorption spectra (Beckman Coulter Inc., Fullerton, CA, USA). A pH meter (Orion Expandable Ion Analyzer, Orion Research, Cambridge, MA, USA) model EA 940 with combined glass electrode was used for monitoring pH adjustments.

2.3. Reagents and solutions

All chemicals used in this work were of analytical reagent grade and were used without further purification. The water used in all studies was ultra-high-quality water with a maximum resistivity of 18.2 mol/LΩ/cm obtained from a Barnstead easy pure RF compact ultrapure water system. All the plastic and glassware materials were cleaned by soaking in diluted HNO_3 and were rinsed with distilled water prior to use. All solutions were de-gassed by ultrasonication (Testlab, Argentina).

2.4. Estimation of Cd content

The concentrations (μg/g dry weight) in the dried biomass of Cd were measured in roots and leaves by ICP-MS. Standard solutions were prepared by appropriate dilutions of a 1 000 mg/L stock solution using HNO_3 0.1 mol/L as diluent.

2.5. RWC determination

Dry material of roots and leaves were obtained after heat at 80 °C during 48 h. RWC was measured as:

RWC (%)=[Fw (Fresh weight)-Dw (Dry weight) /Fw]×100[19].

2.6. Enzyme assays

GSH reductase activity was measured by following the decrease in absorbance at 340 nm resulting from NADPH oxidation. This reaction mixture consisted of Tris-HCl 50 mmol/L (pH 7.8), NADPH 0.15 mmol/L, GSSG 0.5 mmol/L, $MgCl_2$ 3 mmol/L, and EDTA 1 mmol/L at 25 °C. One unit of GR activity was defined as the amount of enzyme that catalyses the oxidation of 1 μmol/L of NADPH per min[19].

The SOD activity was determined spectrophotometrically as the ability to inhibit the photoreduction of nitroblue tetrazolium by 50%[1]. The specific activity of SOD was expressed as unit/mg protein.

2.7. Protein determination

Protein concentration was determined using bovine serum albumin as the standard spectrophotometrically[20].

2.8. Quantification of GSH and glutathione disulphide

GSH and glutathione disulphide were separated and simultaneously quantified by capillary electrophoresis. The electrolyte solution (background electrolyte, BGE) was prepared daily and filtered through a 0.45 μm Titan Syringe filters (Sri Inc., Eaton Town, NJ, USA). At the beginning of the day, the capillary was conditioned with 0.1 mol/L NaOH for 10 min, followed by water for 10 min, and then with running electrolyte for 10 min before sample injection.

To achieve high reproducibility of migration times and to avoid solute adsorption, the capillary was washed between analyses with sodium hydroxide for 2 min, followed by water for 2 min, then equilibrated with the running buffer for 3 min. Samples were pressure-injected at the anodic side at 0.5 psi for 5 seconds. A constant voltage (25 kV) was used for all the experiments.Stock solutions of GSH (50 mmol/L) and GSSG (0.1 mmol/L) were prepared quantitatively in HCl and stored at 4 °C until analysis. The working standard solutions were obtained by appropriately diluting the stock solutions with the BGE containing 20% (v/v) HCl.

The amount of standard that could be detected with a signal-to-noise ratio $\geqslant$3 was considered to be the limit of detection (LOD). The limit of quantfication (LOQ) was calculated as the analyte concentration that can be accurately and reliably determined with a signal-to-noise ratio $\geqslant$10. LODs and LOQs were evaluated based on the signal background obtained with the analysis of a diluted standard solution (n=6) containing GSH and GSSG (Table 1).

Table 1
Oligonucleotide primers sequences for RT-PCR.

Primers	Primer forward (5′-3′)	Primer reverse (5′-3′)	Fragment size
γ-ECS	GGTGGCCGTAGAGTGATTGT	CTCTGCTTCCCCTGTTTGAG	290pb
GS	CCGAAATTGATCAGGAAGGA	TCTTCCAAACTCCACAACCC	285pb
GR	CTACGGCGCTTCTGTCGC	ATCAACCGTGTGAGGATCTATCATC	331pb
β-Actina	GTTGGTATGGGCCAGAAAGA	GAACAGCCAGAACAGCAACA	280pb

With the aim of monitoring GSH and GSSG levels after Cd adding, electrophoretic conditions were optimized; the parameters that affect the separation of these analytes (BGE, pH and concentration) were also tested in detail. The influence of type, concentration and pH of the BGE on the analytes resolution was studied. Also, separation temperature, sample storage temperature, voltage applied, injection mode and time of injection were investigated. BGEs of different composition were analyzed within the concentration range of 10-50 mmol/L; the effect of pH was examined within the range 7.0-9.5. The best results in terms of resolution and analysis time were obtained using the following conditions: BGE, 20 mmol/L sodium tetraborate buffer, pH 8.0; 25 °C separation temperature and 25 °C sample storage temperature. The samples were pressure-injected at the anodic side at 0.5 psi for 5 seconds. The applied voltage was 25 kV. GSH and GSSG were baseline separated in less than 6 min; the corresponding migration times were 4.97 and 5.41 min, respectively. Figure 1 shows the optimized separation by capillary electrophoresis.

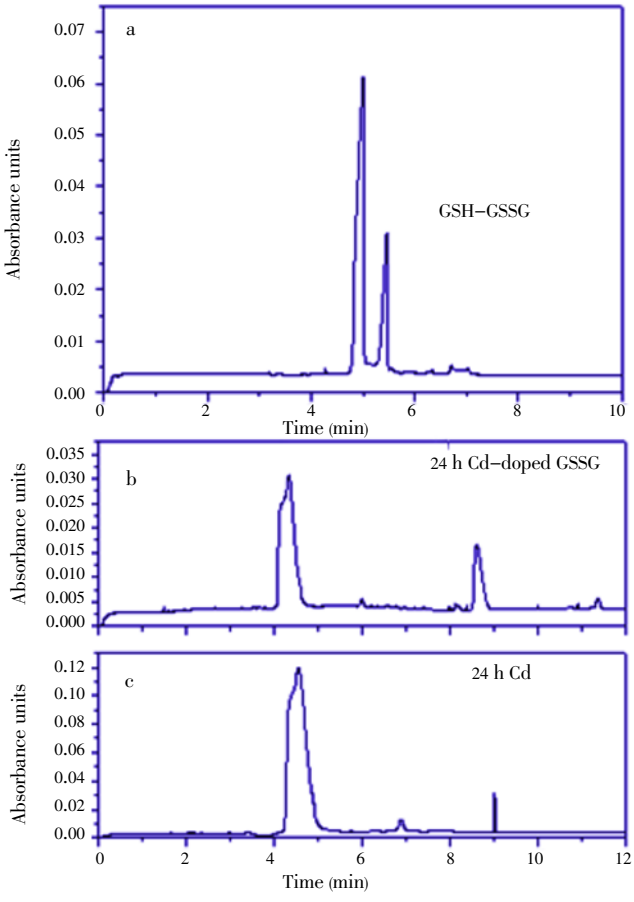

Figure 1. Simultaneous quantification of GSH and GSSG standards, GSH in Cd-treated samples at 24 h and Cd at 24 h.

a: The absorbance of GSH-GSSG; b: The absorbance of Cd-doped GSSG; c: The absorbance of Cd; BGE: Sodium tetraborate buffer 20 mmol/L, pH 8.0, 25 kV applied voltage, 25 °C capillary temperature, 25 °C sample temperature; hydrodynamic mode sample injection, 0.5 psi during 5 seconds; detection by DAD at 203 nm.

2.9. RT-PCR

The levels of γ-ECS, GS and GR mRNA were determined by RT-PCR. First, total RNA was isolated from samples of roots and leaves using Trizol® reagent (Invitrogen) and following the manufacturer indications. Second, 4 μg of total RNA were reversed transcribed using random primer hexamers (Biodynamics, SRL) and M-MLV reverse transcriptase (Promega), at 37 °C during 1 h. PCR amplification was carried out using specific oligonucleotide primers (Table 1). A cDNA aliquot (1/10 of the RT reaction product) was amplified with a PCR master mix, using *Taq* DNA polymerase (Invitrogen). PCR products were analysed on 2% agarose gels, containing GelRed (Genbiotech) to visualize the bands. Bands intensities were quantified using NIH ImageJ software (Image Processing and Analysis in Java from http://rsb.info.nih.gov/ij/). Relative amounts of mRNA were expressed as the ratio of band intensity for the target genes relative to that for β-actin.

2.10. Statistical analysis

Prior to statistical analysis, the normality of the data was tested by the Kolmogorov-Smirnov test. In all cases, the variances were homogeneous. Data were analysed by One-way ANOVA, and the treatment mean values were compared by the *post-hoc* Tukey-Kramer test at $P<0.05$. Statistical analysis was performed by InfoStat[21].

3. Results

3.1. Soybean plants under Cd exposure

A comparative assessment of Cd content in roots and leaves of Cd-treated seedlings showed that the accumulation of Cd was significantly higher in roots increasingly during the experiment. No significant differences in leaves under Cd exposure were observed (Table 2).

Table 2

Cd content (μg/g dry weight) in soybean seedlings subjected to 40.0 μmol/L Cd.

Time	Roots		Leaves	
(h)	Control	Treatment	Control	Treatment
4	0.021±0.009	9.87±0.98*	0.005±0.001	0.006±0.001
6	0.017±0.004	9.47±1.02*	0.006±0.001	0.006±0.001
24	0.021±0.002	15.76±1.23*	0.006±0.001	0.008±0.001
% RSD	0.31	0.32	0.31	0.30

Mean±SD, $n=5$, *$P<0.05$ according to Tukey test. The relative standard deviation (RSD) was calculated from the peak heights obtained.

3.2. Effect of Cd on oxidative stress parameters

Figure 2 depicts the variability of RWC in roots and leaves. No significant differences were observed in roots

exposure to Cd. However, a significant increase at 24 h was observed in Cd-treated leaves.

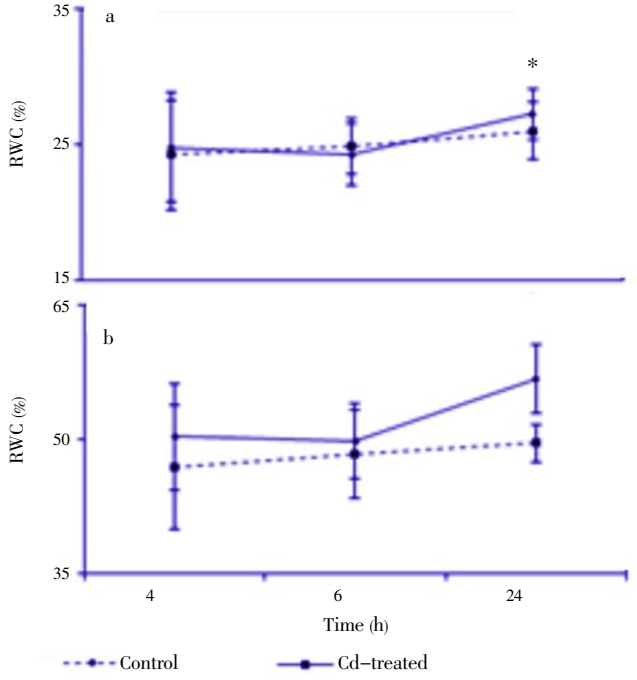

Figure 2. Relative water content levels in roots (a) and leaves (b) of soybean seedlings.

*$P<0.05$, according to Tukey test.

The analysis of total SOD activity (Figure 3) showed a significant decrease at 24 h in roots and leaves under Cd exposure. However, significant increase in SOD activity was observed at 6 h in treated leaves. The GR activity showed a significant increase in Cd-treated roots at 24 h, but differences were not significant in leaves (Figure 4).

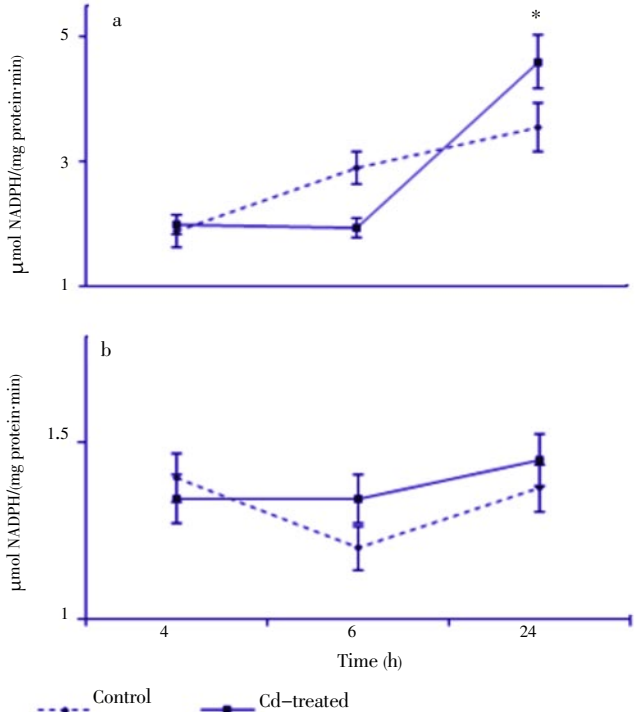

Figure 3. Enzyme activity of SOD in roots (a) and leaves (b) of soybean seedlings.

*$P<0.05$, according to Tukey test.

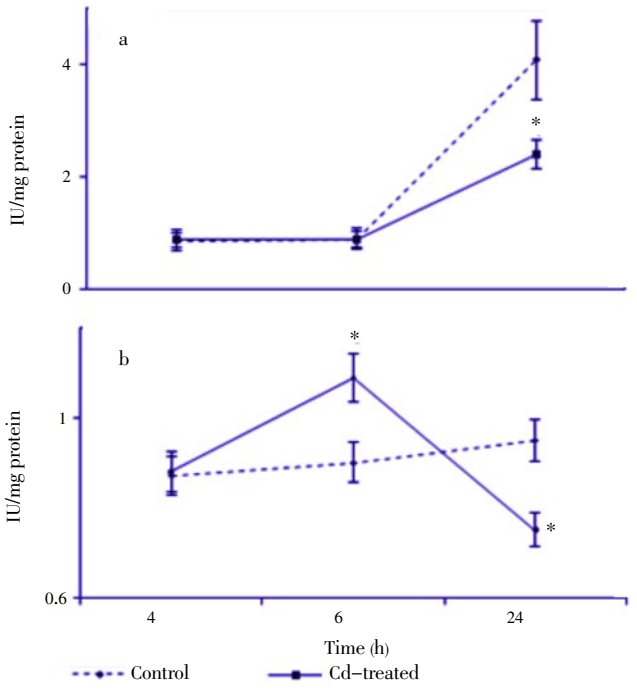

Figure 4. Enzyme activity of GR in roots (a) and leaves (b) of soybean seedlings.

*$P<0.05$, according to Tukey test.

Under the optimum experimental conditions, linear relationships were obtained between corrected peak areas and concentrations of the analytes. Linearity has been determined by a series of three replicates of five levels of standards in the expected concentration range for each analyte (10-50 mmol/L for GSH and 0.05-0.1 mmol/L for the GSSG). The LODs for GSH and GSSG were 0.005 mmol/L and 0.001 mmol/L respectively. The LOQs for GSH and GSSG were 0.015 mmol/L and 0.05 mmol/L, respectively.

The samples were analyzed fresh or stored at -80 °C, therefore the stability of stored samples was also checked by comparing the values obtained for GSH and GSSG in a fresh sample and in aliquots of the same soybean measured at 7, 14, 28 and 30 d. No differences were found in the results and therefore, liver samples can be stored for at least 1 month at -80 °C for GSH analysis.

The content of GSSG in the analyzed samples was very low making it impossible to quantify in some samples because GSSG levels escape the detection limits of the equipment, while GSH content was higher and showed an significant increase in roots and leaves at 6 h and 24 h of exposure to Cd. This difference was greater for the Cd-treated samples. For this reason, in Figure 5 only GSH data are shown. However, both compounds could be simultaneously separated and quantified without any additional dilution in a fast and accurate way. In addition, no interference of other sample constituents was observed.

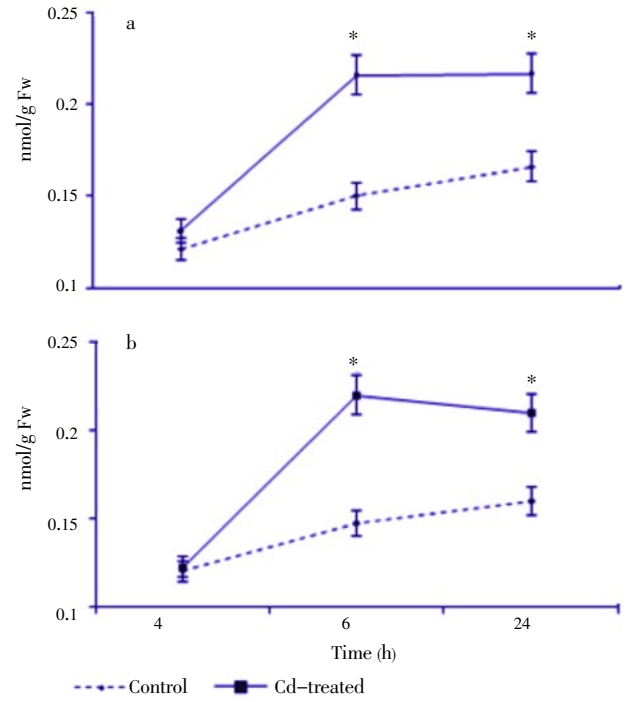

Figure 5. Quantification of GSH in roots (a) and leaves (b) of soybean seedlings by CE.
*$P<0.05$, according to Tukey test.

3.3. RNA isolation and semi-quantitative RT-PCR analysis

The RNA levels were determined as described previously using RNA extracted from the roots and leaves of 3 independent experiments. Gel analysis and relative mRNA levels showed that the expression levels were nearly constitutive in control and Cd-treated samples at 4 h, 6 h and 24 h respectively.

The results given in Figure 6 showed the behavior expression levels of γ-ECS, GS and GR in roots and leaves exposured to Cd. Interestingly, γ-ECS mRNA levels increased at 6 h and 24 h in Cd-treated roots and leaves. Increases in GS mRNA levels were observed at 6 h and 24 h of Cd exposure in roots and leaves in a similar way to γ-ECS. However, significant decreases in GR expression were observed in Cd-treated samples.

4. Discussion

Toxic actions of heavy metal ions cause many physiological, biochemical changes in growing plants and an imbalance between oxidant generation and antioxidant systems. Some metals are well-established oxidative stressors resulting in ROS production. The GSH

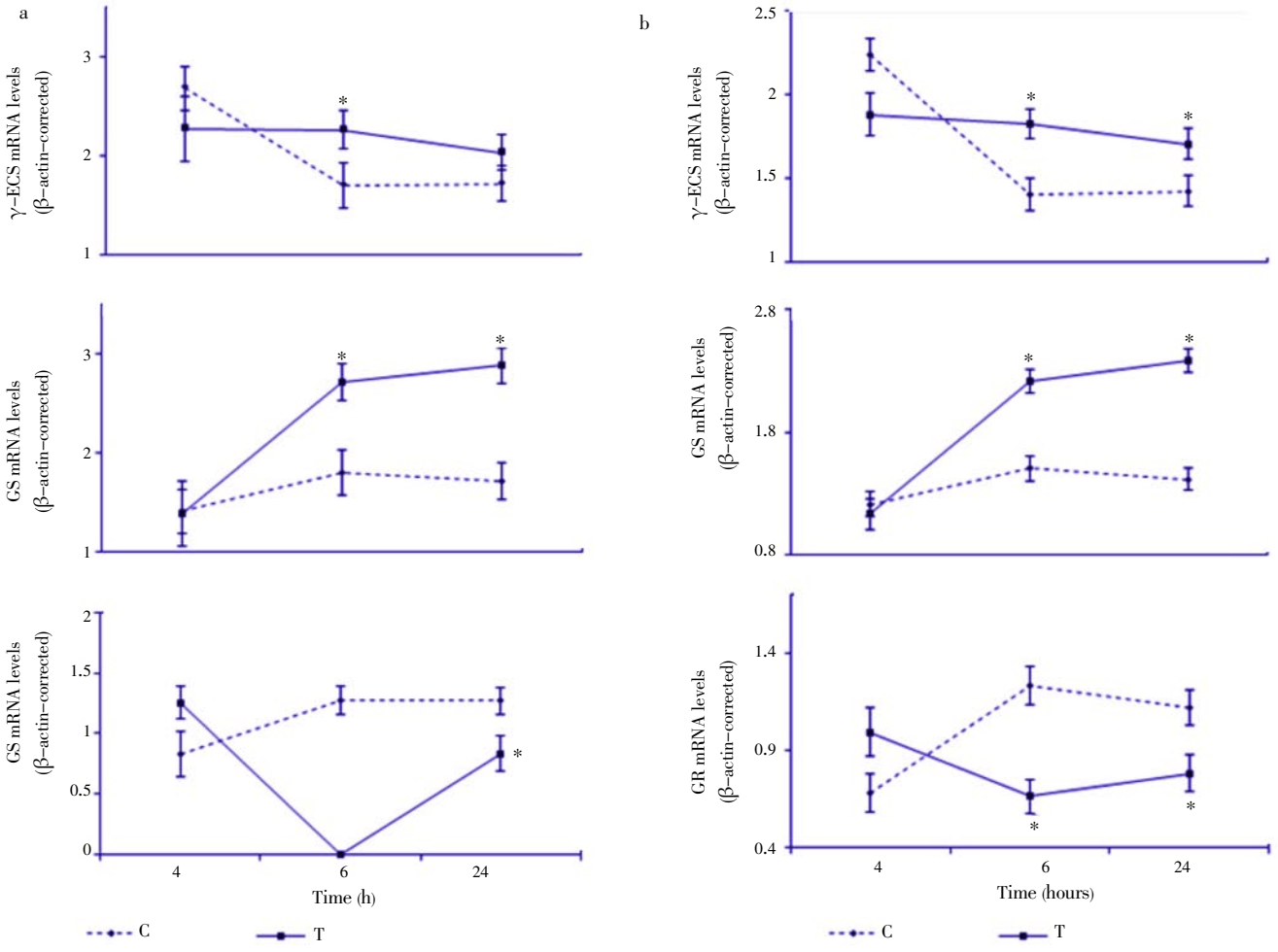

Figure 6. Antioxidant enzymes subunits expression in roots (a) and leaves (b) of soybean seedlings.
Antioxidant enzymes (γ-GSH GS and GR) subunits transcript levels were measured by RT-PCR and normalized against β-actin mRNA levels. *$P<0.05$, according to Tukey test.

accumulation in several plant species is triggered by ROS availability[10,12,17,22]. In some types of stress, increased synthesis occurs within a context of accumulating GSH, a response that has presumably evolved to offset changes in redox potential caused by increase of GSSG[10]. On the other hand, GS and GR, as well as GSH are important components of the ascorbate-GSH cycle responsible for the removal of H_2O_2 in different cellular compartments[10,19].

In this work, we observed that typical level of cadmium on agricultural practices of soybean seedlings, the enzymatic and no-enzymatic components of the antioxidant defense system showed significant increases by exposure to this metal. However, RWC increased at 24 h in treated leaves, demonstrating that the water uptake in soybean is inhibited by Cd.

Cd exposure triggers PCs biosynthesis, so there is an increased demand for GSH. We observed this behavior, where GSH increase responsed to Cd toxicity[11,12]; also this behavior was observed in previous work in *Vigna mungo* L. under Cd exposure[13].

The quantification of GSH and GSSG is very important because of its biological signification. The qualitative and quantitative analysis can supply useful information about the response of plants to high concentrations of heavy metals present in the environment[22,23]. The chromatographic methods associated to different detection systems are the most commonly used. The major disadvantages of these techniques are the elevated volumes of organic solvent required and the long-time of analysis. CE has emerged as an alternative to the established chromatographic methods because of its resolution power, short analysis time and the low consumption of reactive samples[24]. A rapid CE method was developed, making it possible to identify and quantify simultaneously GSH and GSSG in plant extracts. The reported procedure is simple in its preparation and analytical stages and it has a relatively short analysis time (10 min including capillary conditioning). The determinate GSH and GSSG in soybean leaves subjected to salt stress and demonstrated that GSSG-content was 10% compared with GSH-content[25]. Monostori *et al.*[26], showed in his review work, the determination of one GSH in cell and plant extracts, demonstrating that there are not many jobs in plants.

The GR activity showed an increase only at 24 h of Cd-treated in roots; this could account for changes in the GSH reserve, consumed in the early hours of Cd exposure with an increase from 6 h; these results were similar to those obtained in *Vigna unguiculata* and in *Arabidopsis*[27,28].

GSH levels increase in treated plants because it is necessary as antioxidant molecule at 6 h and 24 h, but no significant differences are showed at 4 h under Cd exposure. The limiting step in GSH synthesis is the activity of γ-ECS that suffers a negative feedback regulation caused by GSH[28,29]. This would be consistent with the increase in transcripts for γ-ECS and GS at 6 h and 24 h of Cd exposure in roots and leaves, restoring GSH synthesis by pre-existing enzymes and *de novo* synthesis of enzymes[28]. The higher GSH contents either in roots or leaves under Cd-stress in this experimental model could show that signaling mechanisms are activated at short times.

Cd is concentrated in the root and sends signals to the aerial part of the plant, thereby activates the expression of genes encoding enzymes of the *de novo* synthesis of GSH; such signals may be H_2O_2 or the same GSH. The reduced expression of GR indicates no formation of GSH from GSSG. Not always agree the enzymatic activity with gene expression, as observed with lower expression for GR and increased activity in the root at 24 h of treatment. It is remarkable antioxidant defense response of the plant in a short time model of Cd-treatment.

Moreover, stress by Cd increases transcriptional activation of antioxidant enzymes in soybean, resulting in a post-transcriptional regulation using transcription factors such as H_2O_2 or changes in the GSH/GSSG ratio[28].

The changes produced in the levels of reduced/oxidized GSH, inhibition/activation of enzymes and stress parameters constitute a good indicator of an early response to metal stress in developed plants exposing to Cd, and this showed a better understanding of the signaling pathway with alterations in antioxidant mechanisms by Cd in soybean seedlings.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

We gratefully acknowledge the financial support of National Scientific and Technical Research Council (CONICET), the National Agency of Scientific and Technologic Promotion (FONCYT) (PICT-BID) and National University of San Luis (Argentina) PROICO N° 2/1112, PROICO N° 2/0403 and PROICO N° 2/1512.

Comments

Background

Cd induces damaging and repair responses in which the cellular redox status plays a crucial role. Understanding the role of Cd in oxidative stress could improve the knowledge in antioxidant mechanism in *G. max*.

Research frontiers

This research depicts the antioxidant defense of *G. max* induced by Cd. It estimated the differences in gene transcription and activity of some antioxidant enzymes in response to a Cd treatment.

Related reports

Cd stress is an oxidative challenge. Cd does not generate ROS directly. However, Cd-induced oxidative stress is a phenomenon observed in different studies. The role of antioxidant enzyme in oxidative stress is crucial to avoid the death in different species.

Innovations and breakthroughs

The improvement is that capillary electrophoresis can determine the reduced and oxidized forms of glutathione. However, there are some works that study the effect of Cd in

G. max. Also, this work includes the analysis of some genes involved in oxidative defense.

Applications

The changes produced in the levels of reduced/oxidized glutathione, inhibition/activation of enzymes and stress parameters constitute a good indicator of an early response to metal stress in developed plants exposing to Cd.

Peer review

This work is interesting as the authors contribute to the understanding of Cd-induced oxidative stress through the study of gene expression and antioxidant activity of some enzymes. Moreover, the use of capillary electrophoresis allowed the measure of reduced and oxidized form of glutathione.

References

[1] Pavlova D, Karadjova I. Toxic element profiles in selected medicinal plants growing on serpentines in Bulgaria. *Biol Trace Elem Res* 2013; **156**(1-3): 288-297.

[2] Sarma H, Deka S, Deka H, Saikia RR. Accumulation of heavy metals in selected medicinal plants. *Rev Environ Contam Toxicol* 2011; **214**: 63-86.

[3] Finger-Teixeira A, Ferrarese Mde L, Soares AR, da Silva D, Ferrarese-Filho O. Cadmium-induced lignification restricts soybean root growth. *Ecotoxicol Environ Saf* 2010; **73**(8): 1959-1964.

[4] Gallego SM, Pena LB, Barcia RA, Azpilicueta CE, Iannone MF, Rosales EP, et al. Unravelling cadmium toxicity and tolerance in plants: insight into regulatory mechanisms. *Environ Exp Bot* 2012; **83**: 33-46.

[5] Liu Y, Lv Y. Oxidative damage induced in *Vicia faba* by coke plant wastewater. *Toxicol Ind Health* 2011; **27**(9): 787-792.

[6] Tkalec M, Štefanić PP, Cvjetko P, Šikić S, Pavlica M, Balen B. The effects of cadmium-zinc interactions on biochemical responses in tobacco seedlings and adult plants. *PLoS ONE* 2014; **9**(1): e87582.

[7] Gallego SM, Benavídes MP, Tomaro ML. Effect of heavy metal ion excess on sunflower leaves: evidence for involvement of oxidative stress. *Plant Sci* 1996; **121**(2): 151-159.

[8] Habib D, Chaudhary MF, Zia M. The study of ascorbate peroxidase, catalase and peroxidase during *in vitro* regeneration of *Argyrolobium roseum*. *Appl Biochem Biotechnol* 2013; **172**(2): 1070-1084.

[9] Ribera-Fonseca A, Inostroza-Blancheteau C, Cartes P, Rengel Z, Mora ML. Early induction of Fe-SOD gene expression is involved in tolerance to Mn toxicity in perennial ryegrass. *Plant Physiol Biochem* 2013; **73**: 77-82.

[10] Foyer CH, Noctor G. Ascorbate and glutathione: the heart of the redox hub. *Plant Physiol* 2011; **155**(1): 2-18.

[11] Fernández R, Fernández-Fuego D, Rodríguez-González P, Alonso JI, Bertrand A, González A. Cd-induced phytochelatin synthesis in *Dittrichia viscosa* (L.) Greuter is determined by the dilution of the culture medium. *Environ Sci Pollut Res Int* 2014; **21**(2): 1133-1145.

[12] Nakamura S, Suzui N, Nagasaka T, Komatsu F, Ishioka NS, Ito-Tanabata S, et al. Application of glutathione to roots selectively inhibits cadmium transport from roots to shoots in oilseed rape. *J Exp Bot* 2013; **64**(4): 1073-1081.

[13] Molina AS, Nievas C, Chaca MVP, Garibotto F, González U, Marsá SM, et al. Cadmium-induced oxidative damage and antioxidative defense mechanisms in *Vigna mungo* L. *Plant Growth Regul* 2008; **56**(3): 285-295.

[14] Guo J, Chi J. Effect of Cd-tolerant plant growth-promoting rhizobium on plant growth and Cd uptake by *Lolium multiflorum* Lam. and *Glycine max* (L.) Merr. in Cd-contaminated soil. *Plant Soil* 2014; **375**: 205-214.

[15] Benavides MP, Gallego SM, Tomaro ML. Cadmium toxicity in plants. *Braz J Plant Physiol* 2005; **17**(1): 21-34.

[16] Yadav SK. Heavy metals toxicity in plants: an overview on the role of glutathione and phytochelatins in heavy metal stress tolerance of plants. *S Afr J Bot* 2010; **76**(2): 167-179.

[17] Wojas S, Hennig J, Plaza S, Geisler M, Siemianowski O, Skłodowska A, et al. Ectopic expression of *Arabidopsis* ABC transporter MRP7 modifies cadmium root-to-shoot transport and accumulation. *Environ Pollut* 2009; **157**(10): 2781-2789.

[18] Maeso N, Garcia-Martinez D, Ruperez FJ, Cifuentes A, Barbas C. Capillary electrophoresis of glutathione to monitor oxidative stress and response to antioxidant treatments in an animal model. *J Chromatogr B Analyt Technol Biomed Life Sci* 2005; **822**: 61-69.

[19] Contreras-Porcia L, Thomas D, Flores V, Correa JA. Tolerance to oxidative stress induced by desiccation in *Porphyra columbina* (Bangiales, Rhodophyta). *J Exp Bot* 2011; **62**(6): 1815-1829.

[20] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**: 248-254.

[21] Di Rienzo JA, Casanoves F, Balzarini MG, Gonzalez L, Tablada M, Robledo CW. InfoStat version 2011. Argentina: Infostat group from the National University of Córdoba; 2011. [Online] Available from: http://infosta.com.ar [Access on 10 Jun 2014]

[22] Smith ZM, Terry JM, Barnett NW, Gray LJ, Wright DJ, Francis PS. Enhancing permanganate chemiluminescence detection for the determination of glutathione and glutathione disulfide in biological matrices. *Analyst* 2014; **139**: 2416-2422.

[23] Camera E, Picardo M. Analytical methods to investigate glutathione and related compounds in biological and pathological processes. *J Chromatogr B Analyt Technol Biomed Life Sci* 2002; **781**(1-2): 181-206.

[24] Herrero-Martínez JM, Simó-Alfonso EF, Ramis-Ramos G, Deltoro VI, Calatayud A, Barreno E. Simultaneous determination of L-ascorbic acid, glutathione, and their oxidized forms in ozone-exposed vascular plants by capillary zone electrophoresis. *Environ Sci Technol* 2000; **34**(7): 1331-1336.

[25] Balestrasse KB, Zilli CG, Tomaro ML. Signal transduction pathways and haem oxygenase induction in soybean leaves subjected to salt stress. *Redox Rep* 2008; **13**: 255-262.

[26] Monostori P, Wittmann G, Karg E, Túri S. Determination of glutathione and glutathione disulfide in biological samples: an in-depth review. *J Chromatogr B Analyt Technol Biomed Life Sci* 2009; **877**(28): 3331-3346.

[27] Gomez LD, Noctor G, Knight MR, Foyer CH. Regulation of calcium signalling and gene expression by glutathione. *J Exp Bot* 2004; **55**: 1851-1859.

[28] Xiang C, Oliver DJ. Glutathione metabolic genes coordinately respond to heavy metals and jasmonic acid in *Arabidopsis*. *Plant Cell* 1998; **10**(9): 1539-1550.

[29] May MJ, Vernoux T, Sánchez-Fernández R, Van Montagu M, Inzé D. Evidence for posttranscriptional activation of gamma-glutamylcysteine synthetase during plant stress responses. *Proc Natl Acad Sci U S A* 1998; **95**(20): 12049-12054.