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Cadmium induced oxidative stress in soybean plants also by the accumulation of δ-aminolevulinic acid

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Abstract Cadmium toxicity has been extensively studied in plants, however its biochemical mechanism of action has not yet been well established. To fulfil this objective, four-weeks-old soybean nodulated plants were treated with 200 μM Cd^{2+} for 48 h. δ-aminolevulinic acid dehydratase (ALA-D, E.C. 4.2.1.24) activity and protein expression, as well as δ-aminolevulinic acid (ALA) and porphobilinogen (PBG) concentrations were determined in nodules, roots and leaves. In vitro experiments carried out in leaves were performed using leaf discs to evaluate the oxidant and antioxidant properties of ALA and *S*-adenosyl-L-methinone (SAM), respectively. Oxidative stress parameters such as thiobarbituric acid reactive substances (TBARS) and GSH levels as well as superoxide dismutase (SOD, E.C. 1.15.1.1), and guaiacol peroxidase (GPOX, E.C. 1.11.1.7) were also determined. Cadmium treatment caused 100% inhibition of ALA-D activity in roots and leaves, and 72% inhibition in nodules whereas protein expression remained unaltered in the three studied tissues. Plants accumulated ALA in nodules (46%), roots (2.5-fold) and leaves (104%), respect to controls. From in vitro experiments using leaf discs, exposed to ALA or Cd^{2+}, it was found that TBARS levels were enhanced, while GSH content and SOD and GPOX activities and expressions were diminished. The protective role of SAM against oxidative stress generated by Cd^{2+} and ALA was also demonstrated. Data presented in this paper let us to suggest that accumulation of ALA in nodules, roots and leaves of soybean plants due to treatment with Cd^{2+} is highly responsible for oxidative stress generation in these tissues.

Keywords δ-Aminolevulinic acid · δ-Aminolevulinic acid dehydratase · Cadmium · Oxidative stress · Soybean

Abbreviations

ALA	δ-aminolevulinic acid
ALA-D	δ-aminolevulinic acid dehydratase
BHT	Butylated hydroxytoluene
DTNB	5,5′ dithio-bis-(2-nitrobenzoic acid)
DTT	Dithiotreitol
FW	Fresh weight
GPOX	Guaiacolperoxidase
GSH	Reduced glutathione
GSSG	Oxidized glutathione

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NBT	Nitroblue tetrazolium
PBG	Porphobilinogen
PVP	Polyvinylpyrrolidone
ROS	Reactive oxygen species
SAM	*S*-adenosyl-L-methionine
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid

Introduction

Plants synthesize a wide variety of tetrapyrroles including chlorophylls, hemes, siroheme, and bilins, which participate in many cellular processes (Beale and Weinstein 1991). Hemes are ubiquitous in living organisms and heme proteins are directly involved in oxidation-reduction, oxygenation, hydroxylation, binding of oxygen and other diatomic gases reactions.

Synthesis of chlorophyll and hemes from the tetrapyrrol precursor δ-aminolevulinic acid (ALA), share common enzymatic steps up to the formation of protoporphyrin IX from where they diverse to the heme or chlorophyll pathway.

ALA can be formed via two pathways: the enzymatic condensation of glycine and succinyl-CoA by ALA synthase (ALA-S, E.C. 2.3.1.37) (Zamman et al. 1973), and the C_5 pathway converting glutamate to ALA (Beale 1978). The C_5 pathway is prevalent in plants, algae and most bacteria, while ALA-S is mainly confined to animal mitochondria, some fungi and the α-group of purple eubacteria, to which the genera Rhizobium and Rhodobacter belong. ALA undergoes enolization and further metal-catalyzed aerobic oxidation at physiological pH to yield superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\cdot}$). Therefore, accumulation of ALA in the cell might endogenously enhance reactive oxygen species (ROS) levels leading to oxidative stress (Reyter and Tyrrell 2000 and references herein).

δ-aminolevulinic acid dehydratase (ALA-D, E.C. 4.2.1.24) is a metalloenzyme that catalyses the asymmetric condensation of two molecules of ALA to form porphobilinogen (PBG). This reaction is common to tetrapyrrol biosynthesis in all phyla and is essential for cellular life. Concerning its mechanism of action, it is known that there is a Schiff base formed between a conserved lysine and one of the two ALA molecules at the active site of the enzyme (Batlle and Stella 1978).

Plants are affected by different kind of stresses such as drought, salinity and metal toxicity among others. Cadmium is one of the most toxic pollutants found in the air, water and soil and is non-essential for plants. It is released into the environment by traffic, metal-working industries, mining, as a by-product of mineral fertilizers and from other sources. This ion induces complex changes in plants at the genetical, biochemical and physiological levels, leading to phytotoxicity (Benavides et al. 2005; Gratão et al. 2005); the most obvious symptoms of which are: reduction of tissue and organ growth, leaf chlorosis and leaf and root necroses (Hernandez and Cooke 1997). Among other effects, Cd^{2+} alters mineral nutrition (Moral et al. 1994; Ouzonidou et al. 1997), chlorophyll metabolism (Poschenrieder et al. 1989) and water balance (Valle and Ulmer 1972), thus these parameters may be used as indicators of its phytotoxicity (Ernst et al. 2000). Very recently, it has been proposed that Cd^{2+} binds competitively to the essential Ca^{2+} site in Photosystem II during photoactivation (Faller et al. 2005). Although toxicity has been extensively studied, its biochemical mechanism of action has not yet been well established.

On these grounds, it was our aim to evaluate the effect of Cd^{2+} on PBG synthesis in soybean nodules, leaves and roots in an attempt to elucidate a possible mechanism of action for Cd^{2+} toxicity in plant tissues.

Materials and methods

Chemicals

NADPH, GSH, GSSG, DTNB, NBT, and 2-vinylpyridine were from Sigma Chemical Company (St. Louis, MO). All other chemicals were of analytical grade.

Plant material and growing conditions

Seeds of soybean (*Glycine max* L.) were surface sterilized with 5% v/v sodium hypochlorite for 10 min and then washed four times with distilled water. The seeds were inoculated with 10^8 cell ml^{-1} of *Bradyrhizobium japonicum* (109, INTA Castelar) and were planted in vermiculite for five days. After germination, plants were removed from pots; roots were gently washed and transferred to separated containers for hydroponics. Plants were germinated and grown in a controlled climate room at 24 ± 2°C and 50% relative humidity, with a photoperiod of 16 h and a light intensity of 175 μmol m^{-2} s^{-1}. The hydroponics medium was Hoagland nutrient solution (Hoagland and Arnon 1957). The medium was continuously aerated and replaced every 3 days. After 4 weeks growth, plants were treated with nutrient solution devoid of Cd^{2+} (control) or containing 200 μM Cd^{2+}. After 48 h of treatment, roots, leaves and nodules were isolated and used for determinations. In vitro experiments carried out in leaves were performed using leaf discs (12 mm diameter, 0.3 g) from 30 days-old control plants. When discs were used, they were floated abaxial side down in the dark during 14 h in flasks containing 20 ml of the treatment solutions. Treatments were as follows: (a) Controls in phosphate buffer (pH 7.4), (b) 200 μM Cd^{2+}or 18 mM ALA in 50 mM phosphate buffer (pH 7.4). When 200 μM *S*-adenosyl methionine (SAM) was investigated, segments were pre-treated with this compound during 4 h before Cd^{2+} or ALA addition.

Thiobarbituric acid reactive substances (TBARS) determination

Lipid peroxidation was measured as the amount of TBARS determined by the thiobarbituric acid (TBA) reaction as described by Heath and Packer (1968). Fresh control and treated leaf discs (0.3 g) were homogenized in 3 ml of 20% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 3,500 × g for 20 min. To 1 ml of the aliquot of the supernatant, 1 ml of 20% TCA containing 0.5% (w/v) TBA and 100 μl 4% butylated hydroxytoluene (BHT) in ethanol were added. The mixture was heated at 95°C for 30 min and then quickly cooled on ice. The contents were centrifuged at 10,000 × g for 15 min and the absorbance was measured at 532 nm. Value for non-specific absorption at 600 nm was subtracted. The concentration of TBARS was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Glutathione determination

Non-protein thiols were extracted by homogenizing 0.3 g of leaf discs in 3.0 ml of 0.1 N HCl (pH 2.0), 1 g PVP. After centrifugation at 10,000 × *g* for 30 min at 4°C, the supernatants were used for analysis. Total glutathione (GSH plus GSSG) was determined in the homogenates spectrophotometrically at 412 nm, after precipitation with 0.1 N HCl, using yeast-glutathione reductase, 5,5′ dithio-bis-(2-nitrobenzoic acid) (DTNB) and NADPH. GSSG was determined by the same method in the presence of 2-vinylpyridine and GSH content was calculated from the difference between total glutathione and GSSG (Anderson 1985).

Antioxidant enzymes preparations and assays

Extracts for determination of superoxide dismutase (SOD) and guaiacol peroxidase (GPOX) were prepared from 0.3 g leaf discs, homogenized under ice-cold conditions in 2 ml of extraction buffer, containing 50 mM phosphate buffer (pH 7.4), 1 mM EDTA, 1 g PVP, and 0.5% (v/v) Triton X–100 at 4°C. The homogenates were centrifuged at 10,000 × g for 20 min and the supernatant fraction was used for the assays. Total SOD activity was assayed by the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) (Becana et al. 1986). The reaction mixture consisted of 50–150 μl of enzyme extract and 3.5 ml O_2^- generating solution which, contained 14.3 mM methionine, 82.5 μM NBT, and 2.2 μM riboflavin. Test tubes were shaken and placed 30 cm from a light bank consisting of six 15-W fluorescent lamps. The reaction was allowed to run for 10 min and stopped by switching the light off.

The reduction in NBT was followed by reading the absorbance at 560 nm. Blanks and controls were run in the same way but without illumination and enzyme, respectively. GPOX activity was determined in homogenates by measuring the increase in absorption at 470 nm due to formation of tetraguaiacol (ε: 2.8 mM^{-1} cm^{-1}) in a reaction containing extract, 50 mM K-phosphate buffer pH 7.0, 0.1 mM guaiacol and 10 mM H_2O_2.

Western-blot analysis for SOD, GPOX and ALA-D expressions

Homogenates obtained from leaf discs for SOD and GPOX activities assays were also analyzed by Western inmunoblot technique. An amount of protein (50 μg) from homogenates of control and treated leaf discs were run in sodium dodecyl sulfate (SDS)-poliacrylamide gel electrophoresis using a 12% acylamide resolving gel (Mini Protean II System, BioRad, Hertz, UK). Separated proteins were transferred to nitrocellulose membranes at 250 mA constant for 2 h in a Mini-Trans-Blot Electrophoretic System (Bio-Rad) according to the manufacturer's instructions, and non-specific binding of antibodies was blocked with 3% non-fat dried milk in PBS, pH 7.4 for 1 h at room temperature. Membranes were then treated with polyclonal rabbit anti peroxidase antibody (Sigma-Aldrich Chemical Co., St. Louis, MO), and polyclonal rabbit anti Cu/Zn SOD (Stress gene Biotechnologies Co., San Diego, CA), (1:300 dilution in Tris–buffered saline, pH 7.4) over night at 4°C. Immune complexes were detected using goat anti-rabbit horseradish peroxidase as secondary antibody (1:1500), (Dako, A/S, Denmark), and were visualized using ECL reagent (Amersham, Pharmacia). ALA dehydratase was detected in nodules, roots and leaves immunologically using antibodies raised against the spinach enzyme (Schaumburg et al. 1992) in slot blots. Cross-reactive protein on nitrocellulose filters was discerned visually using peroxidase-conjugated goat anti-rabbit IgG as described previously (Ausubel et al. 1987). Intensity of bands was analyzed with Gel-Pro® analyzer 3.1 version, Media Cybernetics.

ALA-D enzyme activity

The enzyme activity of ALA-D was measured as the amount of PBG formed from ALA as described previously (Sangwan and ÓBrian 1991). Extracts of soybean leaves, roots and nodules (1:10 w/v) were prepared in 25 mM buffer Tricine pH 8.0, containing 20 mM DDT, by using a Potter-Elvehjem glass homogenizer equipped with a motor driven Teflon pestle (30 sec.). After centrifugation at 24,000 × g for 20 min, the resulting supernatant was used as ALA-D source. Reactions were carried out for 1 h at 37°C in Tricine buffer (pH 8.0), 8 mM ALA, and 20 mM DTT. PBG was quantified spectophotometrically after reaction with Ehrlich reagent (Mauzerall and Granick 1956).

ALA and PBG content

ALA and PBG from nodules, roots and leaves of control and treated plants were separated and determined according to the method of Mauzerall and Granick (1956). Briefly, ALA and PBG extracts were purified on Dowex 50WX2 columns, and eluted with 1 M ammonium acetate or 1 M acetic acid, respectively. For ALA determination eluates were condensed with acetylacetone by boiling for 15 min in a water bath, treated with an equal volume of modified Ehrlich's reagent, and assayed spectrophotometrically at 553 nm. A molar extinction coefficient of 6.8×10^4 M^{-1} cm^{-1} was used for calculation of the ALA content. PBG was determined by adding an equal volume of Ehrlich's reagent to the eluate, and assayed spectrophotometrically at 555 nm.

Protein determination

Protein concentration was evaluated by the method of Bradford (1976), using bovine serum albumin as a standard.

Statistics

Values in the text and tables indicate mean values ± S.E. Differences among treatments were analyzed by one way ANOVA, taking $P < 0.05$ as significant according to Tukey's multiple range test.

Results

ALA-D activity and expression in nodules, roots and leaves of control and Cd^{2+} treated plants

ALA-D activities measured in various tissues were rather different. As shown in Fig. 1, the highest enzyme activity was found in the control nodules, while not nodulated roots exhibited the lowest activity (12% of that observed in nodules). Cadmium treatment inhibited nearly 100% ALA-D activity in roots and leaves, whereas inhibition of this enzyme in nodules was 72% respect to controls. In contrast, ALA-D protein expression was not affected by Cd^{2+} stress in the different analyzed tissues, as can be shown in Fig. 2.

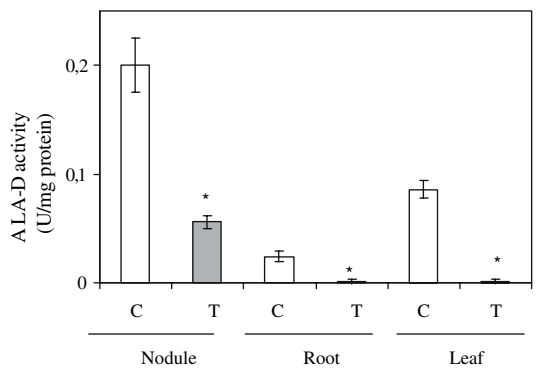

Fig. 1 Effect of Cd^{2+} on ALA-D activity in soybean nodules, roots and leaves. Control (C) and treated (T) plants were grown in the presence and absence of 200 μM Cd^{2+} as described in Materials and methods. One unit of ALA-D forms 1 nmol of PBG/h under the assay conditions. Values are the means of three different experiments with five replicated measurements (n = 15), and bars indicate S.E. *Significant differences ($P < 0.05$) according to Tukey's multiple range test

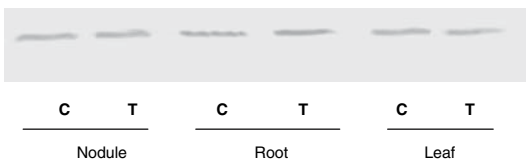

Fig. 2 Western blot analysis of ALA-D expression in nodules, roots and leaves of soybean plants. Controls (C) and 200 μM Cd^{2+} treatment (T). The blot is representative of three blots with a total of 4–5 samples/group between the three blots

Densitometry performed to quantify ALA-D protein expression corroborates this result (data not shown).

ALA and PBG content in nodules, roots and leaves of control and Cd^{2+} treated plants

Plants exposed to Cd^{2+} in the nutrient solution accumulated substantial amounts of ALA in the nodules (46%), roots (2.5-fold) and leaves (104%) respect to controls (Fig. 3). On the other hand, PBG content diminished drastically in the nodules (91%) and it was not found in roots or leaves (Fig. 4). These data clearly showed that as a consequence of ALA-D inhibition there was an accumulation of ALA and a severe diminution of PBG content in these tissues.

Protective effect of *S*-adenosyl-L-methionine (SAM) on ALA-D activity

In vitro experiments were carried out to assess the protective role of SAM against enzyme inhibition. Extracts of control nodules, roots and leaves were prepared as described in Materials and methods. Incubations were performed in the presence or absence of 200 μM Cd^{2+} with or without 200 μM SAM. As shown in Table 1, SAM totally protected against ALA-D inhibition caused by Cd^{2+}.

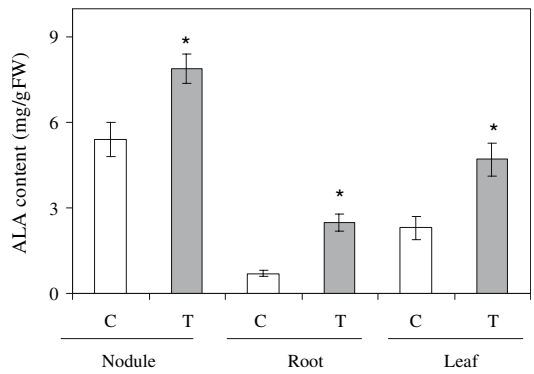

Fig. 3 Effect of Cd^{2+} on ALA content in soybean nodules, roots and leaves. Control (C) and treated (T) plants were grown in the presence and absence of 200 μM Cd^{2+} as described in Materials and methods. Values are the means of three different experiments with five replicated measurements (n = 15), and bars indicate S.E. *Significant differences ($P < 0.05$) according to Tukey's multiple range test

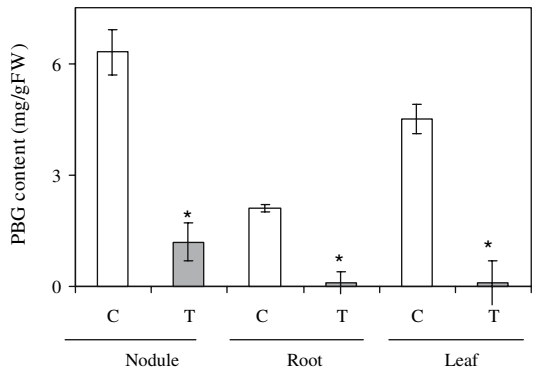

Fig. 4 Effect of Cd^{2+} on PBG content in soybean nodules, roots and leaves. Control (C) and treated (T) plants were grown in the presence and absence of 200 μM Cd^{2+} as described in Materials and methods. Values are the means of three different experiments with five replicated measurements ($n = 15$), and bars indicate S.E. *Significant differences ($P < 0.05$) according to Tukey's multiple range test

Effect of ALA and Cd^{2+} on TBARS formation, GSH level, total SOD and GPOX activities

Accumulation of ALA observed in Cd^{2+} treated plants, prompted us to evaluate the effect of this compound regarding its oxidative properties. To this end, in vitro experiments were performed. Leaf discs were incubated in the presence or absence of 18 mM ALA or 200 μM Cd^{2+} as described in Materials and methods. Afterwards, oxidative stress parameters were evaluated. Incubations were carried out in the presence of a concentration of ALA that resembled the amount accumulated in the tissues. Table 2 shows that TBARS levels were enhanced by about 60%, respect to the controls values, after ALA or Cd^{2+} treatment. GSH content as well as total SOD and GPOX activities were reduced (67%, 46% and 51%, respectively) after Cd^{2+} treatment. Similarly, ALA treatment produced a 53% decrease in GSH content and diminished SOD and GPOX activities (34% and 57%, respectively). Administration of SAM completely prevented the effects of both ALA and Cd^{2+}. It was also found that treatment with SAM alone did not modify oxidative stress parameters (data not shown).

Effect of ALA and Cd^{2+}on SOD and GPOX protein expression

In correlation with the results obtained with SOD and GPOX activities, Fig. 5A, and 5B show that SOD and GPOX protein expression was reduced after ALA (at concentrations comparable to those found in leaf discs) or Cd^{2+} treatment, and SAM pre-treatment totally prevented the effects of both inhibitors. This indicates that the loss of SOD and GPOX activity is correlated with a loss of protein contents rather than with an inhibitory effect of Cd or ALA on the enzyme activities.

Discussion

The goal of this work was to find out a possible explanation for Cd^{2+} toxicity in soybean plants. Up until now there is plenty of information about Cd^{2+} toxicity in plants, but to the best of our knowledge its possible mechanism of action remains unknown.

Cadmium is regarded as a non-essential metal without any known physiological function. It is extremely toxic to plants and animals, have a long

Table 1 Effect of Cd and SAM on ALA-D activity

Treatment	ALA-D activity (U/mg protein)		
	Nodules	Roots	Leaves
Control	0.201 ± 0.002[a]	0.0252 ± 0.0002[a]	0.0861 ± 0.0052[a]
200 μM Cd	0.061 ± 0.001[b]	0.0011 ± 0.0001[b]	0.0010 ± 0.0001[b]
200 μM SAM	0.200 ± 0.002[a]	0.0249 ± 0.0003[a]	0.0872 ± 0.0090[a]
200 μM SAM + 200 μM Cd	0.190 ± 0.005[a]	0.0240 ± 0.0005[a]	0.0821 ± 0.0071[a]

Enzymatic activity was assayed as described in Materials and methods. Data are the means ± S.E. of three different experiments with five replicated measurements. Different letters within columns indicate significant differences ($P < 0.05$) according to Tukey's multiple range test. One unit of ALA-D forms 1 nmol of PBG/h under the assay condition

Table 2 Effect of ALA and Cd on oxidative stress parameters

Treatment	TBARS (nmol/g FW)	GSH (nmol/g FW)	Total SOD[A] (U/mg protein)	GPOX[B] (U/mg protein)
Control	23.2 ± 0.2[a]	0.15 ± 0.02[a]	1.52 ± 0.01[a]	6.3 ± 0.2[a]
Cd	37.2 ± 0.3[b]	0.05 ± 0.01[b]	0.82 ± 0.01[b]	3.2 ± 0.1[b]
ALA	38.1 ± 0.3[b]	0.07 ± 0.01[b]	1.00 ± 0.01[c]	2.7 ± 0.1[c]
SAM + Cd	23.5 ± 0.1[a]	0.13 ± 0.02[a]	1.50 ± 0.01[a]	6.5 ± 0.4[a]
SAM + ALA	23.8 ± 0.2[a]	0.14 ± 0.01[a]	1.51 ± 0.01[a]	6.0 ± 0.1[a]

Experiments were performed using leaf discs. Enzymatic activities were assayed as described in Materials and methods. Data are the means ± S.E. of three different experiments with five replicated measurements. Different letters within columns indicate significant differences ($P < 0.05$) according to Tukey's multiple range test

[A] One unit of SOD is the amount of enzyme which produced a 50% inhibition of NBT reduction under the assay conditions

[B] One unit of GPOX produces 1 μmol of tetraguaiacol per min under the assay conditions

half-life and is extremely persistent in the environment. In the present work, an acute intoxication model was employed, using a high Cd^{2+} concentration (Wagner 1993).

Certain heavy metals, such as Cu or Fe, can be toxic through their participation in Fenton-type reactions producing ROS, which are known to be extremely harmful for living cells (Stochs and Bagchi 1995). However, Cd^{2+} is a non-redox metal unable to take part in these kind of reactions. Nevertheless, it has been clearly demonstrated that Cd^{2+} induces changes in the antioxidant status in plants (Balestrasse et al. 2001; 2006; Noriega et al. 2004; Benavides et al. 2005; Gratão et al. 2005). Moreover, in pea plants, long-term exposure to Cd^{2+} produces oxidative

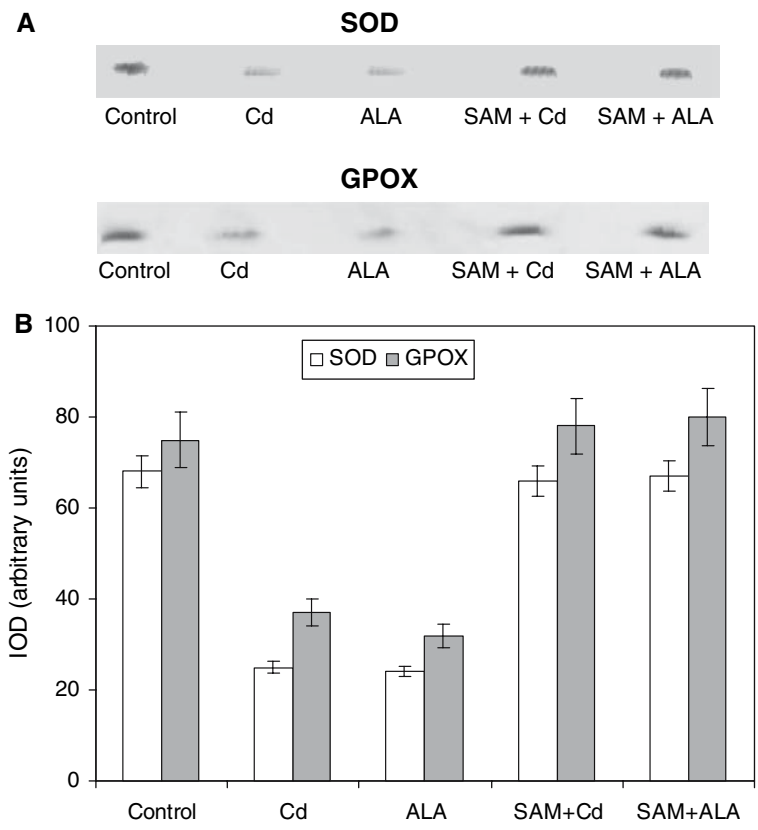

Fig. 5 Western blot analysis of SOD and GPOX expression in leaf discs of soybean plants (**A**). Treatments were as follow: Control, 200 μM Cd^{2+} (Cd^{2+}), 18 mM ALA, (ALA), pre-treatment with 200 μM SAM and then 200 μM Cd^{2+} (SAM + Cd^{2+}) or pre-treatment with 200 μM SAM and then 18 mM ALA (SAM + ALA). Densitometry was done to quantify SOD and GPOX protein expression (**B**). The blot is representative of three blots with a total of 4–5 samples/group between the three blots

stress in roots as a result of disturbances in enzymatic and no enzymatic antioxidant defenses, bringing about an increase in ROS accumulation and decrease in the NO level (Rodríguez-Serrano et al. 2006). Recently, Garnier et al. (2006) have demonstrated that Cd^{2+} induces within minutes a transient increase in cytosolic Ca^{2+} concentration, that appears to regulate the extracellular NADPH-oxidase depending generation of H_2O_2. In this way, transcriptome analysis of the antioxidative enzymes in leaves of pea plants grown with cadmium and treated with some modulators of the signal transduction cascade suggested that at least $Ca(2^{+})$ channels, phosphorylation/dephosphorylation processes, nitric oxide, cGMP, salicylic acid (SA) and H_2O_2 were involved in some steps between the cadmium signal and transcript expression of some antioxidant enzymes. This indicated the existence of cross-talk between these elements and reactive oxygen species (ROS) metabolism during cadmium stress (Romero-Puertas et al. 2006).

It has been established the participation of both oxidative stress and proteolytic degradation in the mechanism of Cd^{2+} toxicity in leaves of pea plants (Romero-Puertas et al. 2004). Moreover, in roots and leaves of pea plants Cd^{2+} produced a significant inhibition of growth as well as a reduction in the transpiration and photosynthesis rate, chlorophyll content of leaves, and an alteration in the nutrient status in both tissues (Sandalio et al. 2001). Previous studies have demonstrated that 200 μM Cd^{2+} produced increased concentrations and in situ accumulation of H_2O_2 and $O_2^{\cdot-}$ in soybean leaves (Balestrasse et al. 2006). Here, we clearly demonstrated that SAM protect against the deleterious effects of Cd^{2+}. It is well known that SAM, via transsulfuration reactions is a good precursor of GSH, and because GSH constitutes the bulk of available sulfydryl groups for binding electrophilic species, it will actively function in the detoxification of xenobiotics. Moreover, the presence of an increased GSH pool would avoid the action of Cd^{2+} on enzyme activity by binding to this peptide. Recently, Ortega-Villasante et al. (2005) have demonstrated that the inhibition of GSH/hGSH synthesis by L-buthionine sulphoximine increased the oxidative stress symptoms in alfalfa plantlets subjected to Cd or Hg stress. Taking into account the fact that oxidative stress is a condition referred to as an imbalance between oxidant generation and antioxidant defense systems, which GSH is a leading substrate for enzymatic antioxidant functions and it is also a known radical scavenger, its precursor SAM would be acting as an antioxidant. This explanation could also be extended to ALA toxicity, because ALA accumulation generates ROS (Princ et al. 1997; 1998; Reiter and Tyrrel 2000) and it has been shown that SAM was capable of counteracting this effect (Paredes et al. 1987). Our results clearly demonstrated that in all tissues of Cd^{2+} treated plants there is accumulation of ALA as well as diminution of PBG content as a result of ALA-D activity inhibition. Either Cd^{2+} or ALA treatments produced not only antioxidant enzyme activities inhibition, but also a significant decrease in SOD and GPOX protein expression. It is well known that Cd^{2+} leads to a strong diminution in antioxidant enzymes protein expression (Del Río et al. 2002 and references herein). Romero Puertas et al. (2004) described in pea leaves from plants grown in the presence of Cd^{2+} the rate of protein degradation and proteolytic activity, but the effect of ALA on SOD and GPOX expression had not been reported so far. Considering that ROS production affects the antioxidant enzyme activity and expression we could speculate that ALA decreased both parameters due to its capacity to generate ROS (Reiter and Tyrrel 2000). As a precursor of GSH, SAM can act as a ROS scavenger and would be able to restore enzyme activity and protein expression.

ALA-D is a zinc dependent enzyme in animals, yeasts, and some bacteria (Jordan 1990). Cysteine residues participate in Zn binding and these enzymes contain a cysteine rich domain that may be involved in metal binding. Plant dehydratases are localized in plastids and are needed for chlorophyll synthesis in addition to other cellular tetrapyrroles. They share 35 to 50% identity with non-plant enzymes, but activity requires Mg rather than Zn. The peptide region in the plant enzyme corresponding to the Zn domain of animals lacks the cysteines and histidines residues and contains aspartate, alanine, or threonine instead (Boese et al. 1991).

B. japonicum ALA-D is unusual in that it is a Mg-dependent enzyme even though it is no photosynthetic and it contains a metal-binding domain that has some residues often found in plants (Chauhan and ÓBrian 1993). It is not yet clear whether there is a physiological reason for the existence of an ALA-D with different metal requirements. The mechanism of action of heavy metals toxicity lies in their ability to form strong bonds with bases and phosphates from nucleic acids and with reactive groups from proteins, modifying both their structure and functions (Wang 1999). They compete with other divalent cations such as Zn^{2+} and Mg^{2+} replacing them in their physiological roles.

In animals, yeast and non-photosynthetic bacteria, which do not have the chlorophyll branch of the pathway, the major regulatory step is at the level of the synthesis of the initial precursor ALA. This step is regulated by heme feedback in bacteria and animals (Andrew 1990). In plants, ALA synthesis is also a pivotal control point and determines the total flux through the pathway. ALA-S was established to be the rate-limiting step in the tetrapyrrole pathway in early experiments demonstrating that the lag phase in chlorophyll accumulation in the light could be abolished by the addition of ALA (Castelfranco and Zeng 1991). Subsequently, it was shown in many systems, from cyanobacteria through green algae to higher plants, that heme inhibits the synthesis of ALA from glutamate in vitro, whereas other intermediates, including Mg-protoporphyrin, had much less effect (Weinstein and Beale 1985). In addition, artificial depletion of the heme pool in intact plastids using apoperoxidase resulted in a 32% stimulation of ALA synthesis in chloroplasts (Thomas and Weinstein 1992). These considerations are indicating that ALA accumulation could occur not only after ALA-D inhibition but also by deregulation of ALA synthesis.

Our results showed that Cd^{2+} is a potent inhibitor of ALA-D activity in different soybean tissues, although the enzymatic protein expression was not altered. This inhibition leads to ALA accumulation, which in turn exerts its toxic effect as a source of ROS (Noriega et al. 2003). This observation prompted us to evaluate to what extent this increased ALA content could be responsible for the alterations observed. So that, incubations using leaf discs were carried out in the presence of a concentration of ALA of an equal order as that found in vivo in the same tissue. According to data obtained we suggest that accumulation of ALA in the chlorophyll and heme pathways due to ALA-D inhibition and its consequent generation of ROS is indeed highly responsible for the deleterious action of Cd^{2+} in soybean.

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