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The role of salicylic acid in the prevention of oxidative stress elicited by cadmium in soybean plants

Guillermo Noriega · Ethel Caggiano · Manuel López Lecube · Diego Santa Cruz · Alcira Batlle · María Tomaro · Karina Beatriz Balestrasse

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Abstract The protective action of salicylic acid (SA) pre-treatment on soybean plants before cadmium (Cd) addition was tested. Oxidative stress parameters, such as TBARS formation, glutathione and chlorophyll content, were altered by Cd, instead no differences were observed in plants only pre-treated with SA. Antioxidant enzymes were affected by Cd treatment, while SA protected against these effects. These findings indicated that SA could act as a protector against oxidative stress induced by Cd. Taking into account the fact that heme-oxygenase-1 (HO-1) has been previously described as a novel antioxidant enzyme, experiments were carried out to determine whether it was involved in the protection exerted by SA. As expected, Cd brought about an enhancement of 57 % in HO-1 activity and 150 % in protein content (150 %), SA also increased both the enzyme activity and its protein content (28 and 75 %, respectively). Surprisingly, the observed rise of HO activity and protein content under SA treatment was lower than that produced by Cd alone. These lower values indicated, that HO-1 could not be directly involved in the protection of SA against Cd effects. In order to shed light in the mechanisms involved in SA effects, Cd content was determined in the tissues of Cd treated plants with and without SA pre-treatment. Results indicated that, in the presence of SA, Cd uptake was inhibited, thus avoiding its deleterious effects. Moreover, the observed HO-1 activity enhancement by SA indicates that this phytohormone could be engaged in the signalling pathway of heme degradation.

Keywords Cadmium · Heme oxygenase · Oxidative stress · Salicylic acid · Soybean plants

G. Noriega · A. Batlle · M. Tomaro
Centro de Investigaciones sobre Porfirinas y Porfirias (CIPYP), CONICET, Buenos Aires, Argentina

E. Caggiano · M. L. Lecube · D. S. Cruz · K. B. Balestrasse (✉)
Departamento de Química Biológica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, 1113 Buenos Aires, Argentina
e-mail: kbale@ffyb.uba.ar

D. S. Cruz · K. B. Balestrasse
Instituto de Investigaciones en Biociencias Agrícolas y Ambientales (INBA), CONICET, Buenos Aires, Argentina

Introduction

Cadmium (Cd) is a toxic trace pollutant for humans, animals and plants which enters the environment mainly from industrial processes and phosphate fertilizers and then is transferred to the food chain. This metal is easily taken up by plant roots and can be loaded into the xylem for its transport into leaves. A large number of studies have demonstrated its toxic effect on plant metabolism, such as decrease uptake of nutrient elements (Wagner 1993; Mc Laughlin et al. 1999; Järup and Åkesson 2009), changes in nitrogen

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metabolism (Popova et al. 2009), alterations in heme metabolism (Noriega et al. 2004), among others. The toxicity of Cd has been related with the increase of lipid peroxidation and alterations in the antioxidant system (Popova et al. 2009).

Cadmium promotes the accumulation of reactive oxygen species (ROS), causes severe damage to important cellular components, such as lipids, proteins, DNA, and RNA (Foyer et al. 1994), and leads to a decreased growth, roll and chlorosis of leaves, and root necroses (Schutzendubel et al. 2001). These ROS include superoxide radical, hydroxyl radical and hydrogen peroxide. Plants have evolved a complex antioxidant system (i.e., enzymatic and nonenzymatic detoxification mechanisms) for protecting potential cell injury against tissue dysfunction. The antioxidant enzymes, such as catalase (CAT), peroxidases (POD), superoxide dismutases (SOD), can scavenge different types of ROS.

Salicylic acid (SA) is an endogenous growth regulator of phenolic nature, which participates in physiological processes in plants, such as growth, photosynthesis, nitrate metabolism, ethylene production and flowering (Hayat et al. 2007). SA is also known to be involved in abiotic stress signalling, including plant response to heavy metals. SA pretreatment alleviates Cd toxicity; SA not only plays a key role in establishing and signalling a defense response against various pathogenic infections, but also plays an important role in mediating plant response to some abiotic stresses including salinity, temperature, UV radiation and heavy metal stress (Wang et al. 2011).

Salicylic acid is a phytohormone with ubiquitous distribution in plants; it plays an important role in the regulation of plant growth and development (Malamy et al. 1990; Durner et al. 1997; Horváth et al. 2007). Recent studies indicate that it also participates in the signalling of abiotic stresses. There is strong evidence that SA mediates the oxidative burst that precedes the hypersensitive response and the development of systemic acquired resistance. Previous studies have shown that SA conferred plant resistance to various abiotic stresses such as heat acclimation (Dat et al. 1998), chilling tolerance (Kang and Saltveit 2002), salinity stress (Gunes et al. 2007), and cadmium toxicity (Krantev et al. 2008). All these studies demonstrated that the majority of SA-regulated abiotic stresses in plants are involved in antioxidant responses, thus indicating that protection of plants from oxidative damage by SA is associated with an enhanced antioxidant system (Dat et al. 1998; Horváth et al. 2007).

Heme oxygenase (HO-1, EC 1.14.99.3) catalyzes the oxidative degradation of heme and has well-known antioxidant properties in mammals by mean of its products biliverdin IXα and carbon monoxide (Kikuchi et al. 2005). We have previously shown that HO-1 is induced in plant tissues as a result of Cd treatment and confers protection against oxidative stress (Noriega et al. 2004; Balestrasse et al. 2005).Recently, it has been demonstrated that carbon monoxide mitigates salt-induced inhibition of root growth and suppresses programmed cell death in wheat primary roots by inhibiting superoxide anion overproduction (Ling et al. 2009). Moreover, it has been reported that ROS are involved in HO-1 up-regulation in soybean leaves subjected to Cd treatment (Balestrasse et al. 2006). We hypothesized that SA may also participate in this process, as it regulates the oxidative status and mediates other responses.

The aim of this work was to study the influence of SA on the antioxidant system of soybean plants grown in the presence of Cd, and to find whether oxidative stress tolerance could be the result of HO-1 modulation by exogenous SA thus supplying information on the possible involvement of oxidative stress in the mechanism of damage by Cd.

Materials and methods

Plant material and growing conditions

Seeds of soybean (*Glycine max* L., A6445RG) were surface sterilized with 5 % (v/v) sodium hypochlorite for 10 min and then washed with distilled water four times and were planted in vermiculite for 5 days. After germination, plants were removed from pots; roots were gently washed and transferred to separated containers for hydroponics. Five liter pots were used, containing six plants each. 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 hydroponic medium consisted of Hoagland nutrient solution (Hoagland and Arnon 1950). The medium was continuously aerated (100 ml min^{-1}) and replaced every 3

days. Plants were pre-treated with different SA concentrations ranging from 0 to 500 mM. Afterwards, treatment with Cl_2Cd (50 μM) was performed for 5 days h. Leaves were separated and used for determinations. Meausurements were performed by triplicate in three different experiments employing six plants for each treatment.

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 leaves (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 1 ml 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 leaves in 3.0 ml of 0.1 N HCl (pH 2.0), and 1 g PVP. After centrifugation at 10,000×*g* for 30 min at 4 °C, the supernatants were used for analysis. Total glutathione content (GSH plus GSSG) was determined in the homogenates by spectrophotometry at 412 nm, using yeast-GR, 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 the total glutathione content and GSSG content (Anderson 1985).

Chlorophyll determination

Leaves (0.5 g, fresh weight) were homogenized with 96 % ethanol (1:30 w/v). Extracts were heated in a boiling bath until complete bleaching. After centrifugation, the absorbance was measured in the supernatant at 665, 649 and 654 nm as described by Wintermans and de Mots (1965).

Superoxide dismutase and catalase assays

Extracts for determination of SOD and CAT activities were prepared from 0.3 g of leaves homogenized under ice-cold conditions in 3 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.

CAT activity was determined in the homogenates by measuring the decrease in absorption at 240 nm in a reaction medium containing 50 mM potassium phosphate buffer (pH 7.2) and 2 mM H_2O_2. The pseudo-first order reaction constant ($k' = k[CAT]$) of the decrease in H_2O_2 absorption was determined and the catalase content in pmol mg^{-1} protein was calculated using $k = 4.7 \times 10^7$ M^{-1} s^{-1}.

Total SOD activity was assayed by the inhibition of the photochemical reduction of NBT, as described by 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. Extracts were brought to a final volume of 0.3 ml with 50 mM K-phosphate (pH 7.8) and 0.1 mM Na_2EDTA. 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 absorbance at 560 nm. Blanks and controls were run the same way but without illumination and enzyme, respectively. One unit of SOD was defined as amount of enzyme which produced a 50 % inhibition of NBT reduction under the described assay conditions.

GPOX activity was determined in the homogenates by measuring the increase in absorption at 470 nm due to the formation of tetraguaiacol (e: 26.6 m M^{-1} cm^{-1}) in a reaction containing extract, 50 mM K-phosphate buffer pH 7, 0.1 mM EDTA, 10 mM guaiacol and 10 mM H_2O_2. One unit of GPOX forms 1 μmol of oxidised guaiacol per minute under the assay conditions.

Histochemical analysis

To analyze H_2O_2 generation leaves were excised and immersed in a 1 % solution of 3,3'-diaminobenzidine

(DAB) in Tris–HCl buffer (pH 6.5), vacuum-infiltrated for 5 min and then incubated at room temperature for 16 h in the absence of light. Roots were illuminated until appearance of brown colors characteristic of the reaction of DAB with H_2O_2.

In the same way to show O_2^- production roots were excised and immersed in a 0.1 % solution of NBT in K-phosphate buffer (pH 6.4), containing 10 mM Na-azide, and were vacuum-infiltrated for 5 min and illuminated until appearance of dark spots, characteristic of blue formazan precipitate.

Heme oxygenase preparation and assay

Leaves (0.3 g) were homogenized in a Potter-Elvehejm homogenizer using 4 vol of ice-cold 0.25 M sucrose solution containing 1 mM phenylmethyl sulfonyl fluoride, 0.2 mM EDTA and 50 mM potassium phosphate buffer (pH 7.4). Homogenates were centrifuged at 20,000×*g* for 20 min and supernatant fractions were used for activity determination. Heme oxygenase activity was determined as previously described with minor modifications (Muramoto et al. 2002). The standard incubation mixture in a final volume of 500 ml contained 10 mmol potassium phosphate buffer (pH 7.4), 60 nmol NADPH, 250 ml HO (0.5 mg protein), and 200 nmol hemin. Incubations were carried out at 37 °C during 60 min. Activity was determined by measuring biliverdin formation, which was calculated using the absorbance change at 650 nm employing an 1 value of 6.25 mM^{-1} cm^{-1} (vis_{max} 650 nm).

Western-blots analysis for HO protein

Proteins from leaves were subjected to denaturing SDS-PAGE in a Mini-PROTEAN 3 System (Bio-Rad Laboratories, California). SDS-PAGE was performed in 12 % gels for HO (4 % stacking gels), respectively and run according to Laemmli (1970). The separated polypeptides were transferred to a nitrocellulose membrane at 25 V/300 mA for 2 h in a Mini-Trans-Blot Electrophoretic System (Bio-Rad Laboratories, California) according to the manufacturer's instructions. The membranes were washed in 25 mM Tris–HCl (pH 8.5), 192 mM glycine and 20 % (v/v) methanol. The blots were blocked by incubation for 2 h in 2 % (w/v) powdered non-fat dry milk dissolved in T-TBS (20 mM Tris–HCl at pH 7.6, 137 mM NaCl and 0.1 % (v/v) Tween 20 before reaction with an appropriate antibody. The membrane was incubated with rabbit antibody *A. thaliana* HO-1 (dilution 1:2,000) (Muramoto et al. 1999). The antibody for HO-1 was diluted in T-TBS with 2 % (w/v) powdered non-fat dry milk and were washed twice for 10 min each with T-TBS. Goat anti-rabbit horseradish peroxidase conjugate was used as a secondary antibody and incubated for 1 h. The blots were washed twice for 10 min before color development (ECL immunodetection system, ECL Western Blotting protocols, Dako). The intensity of bands was analyzed with Gel-Pro Analyzer 3.1 software (Media Cybernetics, MD, USA). The films were scanned (Fotodyne Incorporated, WI, USA) and analysed using Gel-Pro Analyzer 3.1 software (Media Cybernetics, MD, USA).

Cadmium determination

Segments from the second pair of fully expanded leaves upper the cotyledons with or without Cd addition were thoroughly rinsed to eliminate the metal that could be superficially adsorbed. Plant material was dried at 80 °C during 48 h, weighed and ground to a fine powder. Cd determinations were made digestion in HNO:HClO (3:1, v/v) by atomic absorption spectrophotometry (Perkin-Elmer, Analyst 300).

Protein determination

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

Results

Lipid peroxidation

Oxidative stress was assessed by measuring lipid peroxidation rate when soybean plants were exposed to 50 μM Cd and to SA (0–500 mM) (Fig. 1). On one hand, TBARS content was significantly enhanced in plants treated with Cd (100 % respect to controls). On the other hand, only 500 mM SA increased 43 %

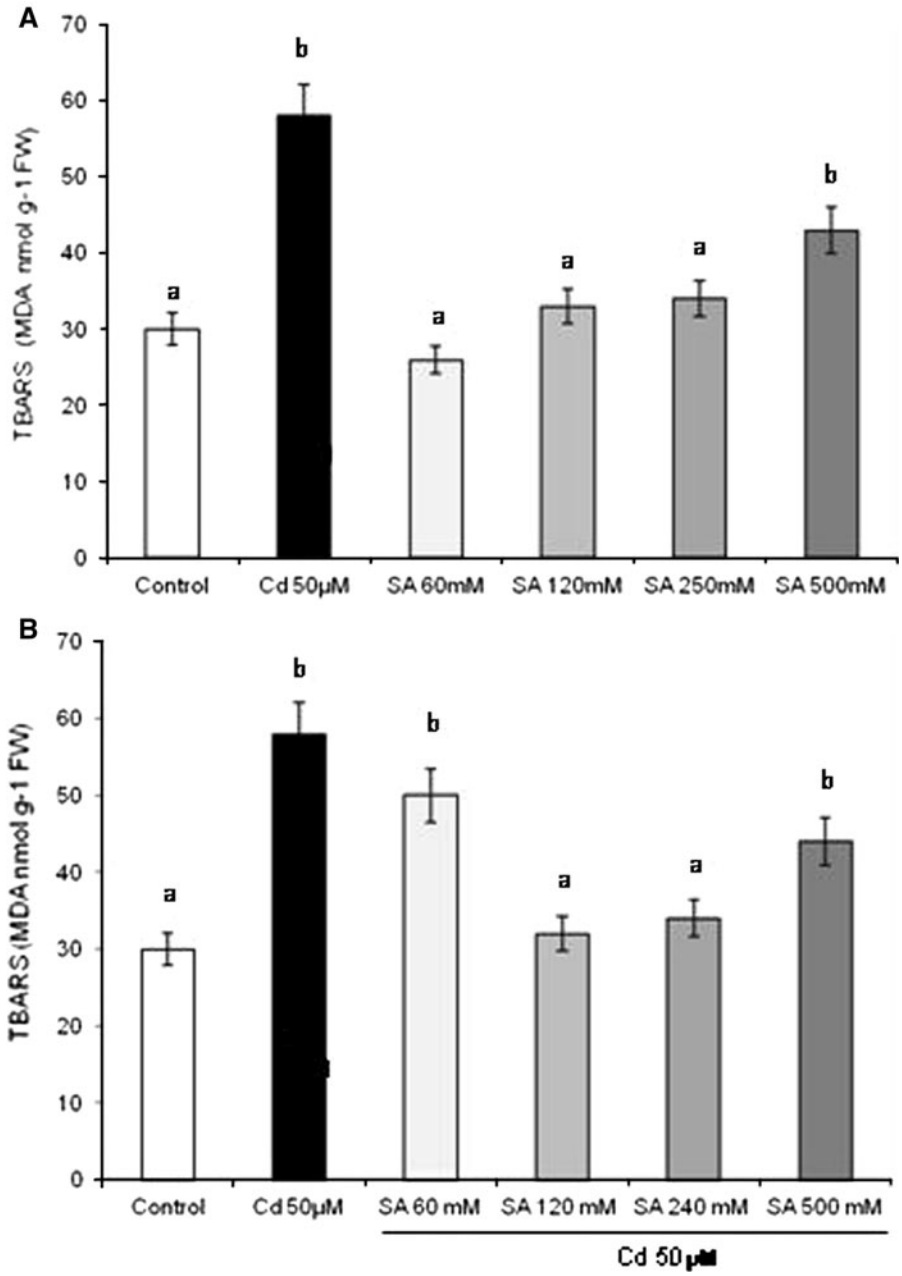

Fig. 1 **a** Effect of Cd and SA on soybean leaves TBARS formation. **b** Effect of SA pre-treatment before Cd addition on soybean leaves TBARS formation. Experiments were performed as described in "Materials and Methods" section. Different *letters* within columns indicate significant differences ($P < 0.05$)

RBARS content respect to controls. Lower concentrations did not produce any changes respect to controls. In order to know whether pre-treatment with SA could prevent lipid peroxidation caused by Cd, experiments were carried out employing different SA acid concentrations ranging from 0 to 500 mM. Figure 1b shows that 120 mM SA completely abolished TBARS enhancement brought about by Cd, but it did not alter any other parameter As a consequence 120 mM SA was chosen for further experiments.

Reactive oxidative species production

Cadmium application resulted in ROS accumulation as observed in Fig. 2. Salicylic acid pre-treatment partially abolished O_2^- as well as H_2O_2 formation.

Fig. 2 H_2O_2 and O_2^- localization in situ. Experiments were performed as described in "Materials and Methods" section

Table 1 Effect of Cd and SA pre-treatment on soybean leaves antioxidant enzyme activities

Treatment	SOD (U/mg protein)	CAT (pmol/mg protein)	GPOX (U/mg protein)
Control	19.5 ± 0.5^a	150 ± 8^a	0.043 ± 0.001^a
Cd 50 µM	10.4 ± 0.7^b	50 ± 15^b	0.024 ± 0.001^b
120 mM SA	18.9 ± 0.6^a	187 ± 6^c	0.045 ± 0.001^a
120 mM SA+Cd 50 µM	21.2 ± 0.5^a	140 ± 14^a	0.039 ± 0.001^a

Enzymatic activities were assayed as described in the experimental section. Different letters within columns indicate significant differences ($P < 0.05$) according to Tukey's multiple range test

Antioxidant enzymes

Cadmium inhibited 46 % in SOD activity respect to controls and SA had no effect on this parameter. On the other hand, plants subjected to SA previously to Cd addition did not show any changes respect to controls (Table 1).

Catalase activity was diminished by Cd (47 %) and an increase (25 %) was observed in SA-treated plants respect to controls. Moreover, pretreatment with SA before Cd addition avoided the inhibition previously observed in the presence of Cd (Table 1). When GPOX activity was analyzed, an inhibition was observed under Cd treatment (45 %) and SA alone did not cause any changes respect to controls. Salicylic acid pre-treatment totally avoided Cd inhibition.

Chlorophyll content

Chlorophyll can be bleached under oxidative stress. Figure 3 shows that chlorophyll content was markedly decreased under Cd treatment (71 % respects to controls). Experiments carried out in the presence of SA revealed that this hormone partially protects against chlorophyll loss against Cd insult (20 %). On the other hand, SA alone had no effect on this parameter.

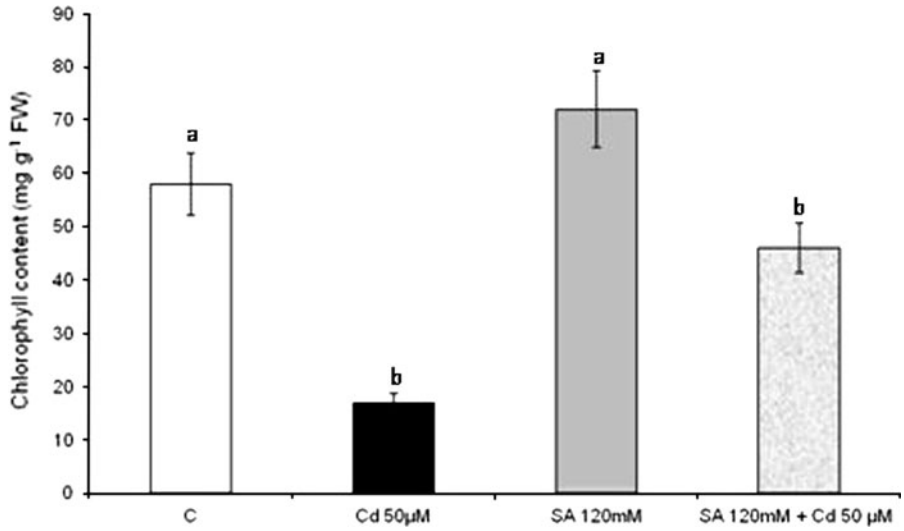

Fig. 3 Effect of Cd and SA pre-treatment on soybean leaves chlorophyll content. Experiments were performed as described in "Materials and Methods" section. Different *letters* within columns indicate significant differences ($P < 0.05$)

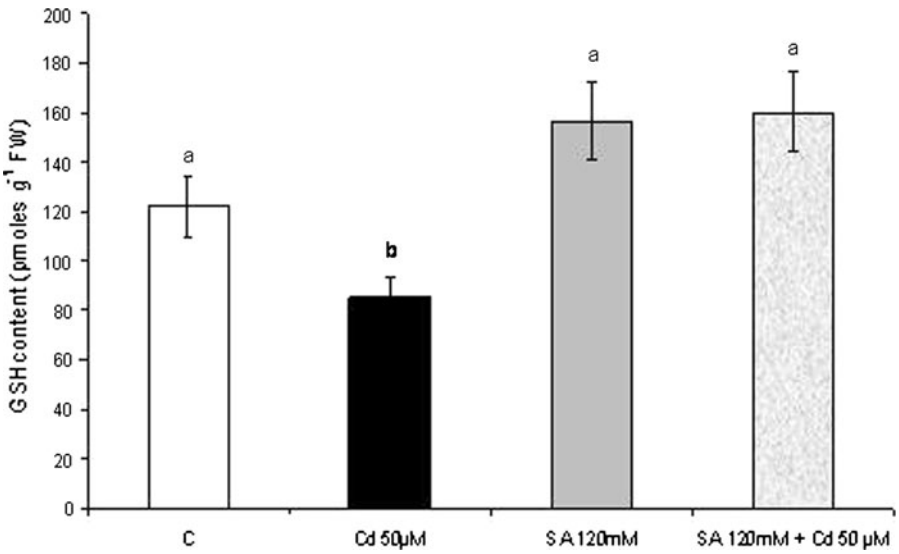

Fig. 4 Effect of Cd and SA pre-treatment on soybean leaves GSH content. Experiments were performed as described in "Materials and Methods" section. Different *letters* within columns indicate significant differences ($P < 0.05$)

Glutathione levels

Glutathione content was determined as a non enzymatic defense. Figure 4 shows a 30 % diminution in GSH content respect to controls in Cd treated plants. Experiments carried out in the presence of SA indicated that no changes have occurred respect to controls. On the other hand, a protection against GSH loss was also observed in plants pretreated with SA before Cd addition.

Heme oxygenase

We have previously reported that Cd treatment results in an increase of HO-1 activity in soybean roots nodules (Balestrasse et al. 2005). To further characterize this induction, here we analyze HO-1 activities in leaves in response to 50 μM Cd in the presence and absence of 120 μM SA. Figure 5a shows that in the presence of 50 μM Cd a 57 % increase in HO-1 activity occurred, respect to controls. Salicylic acid alone also increases,

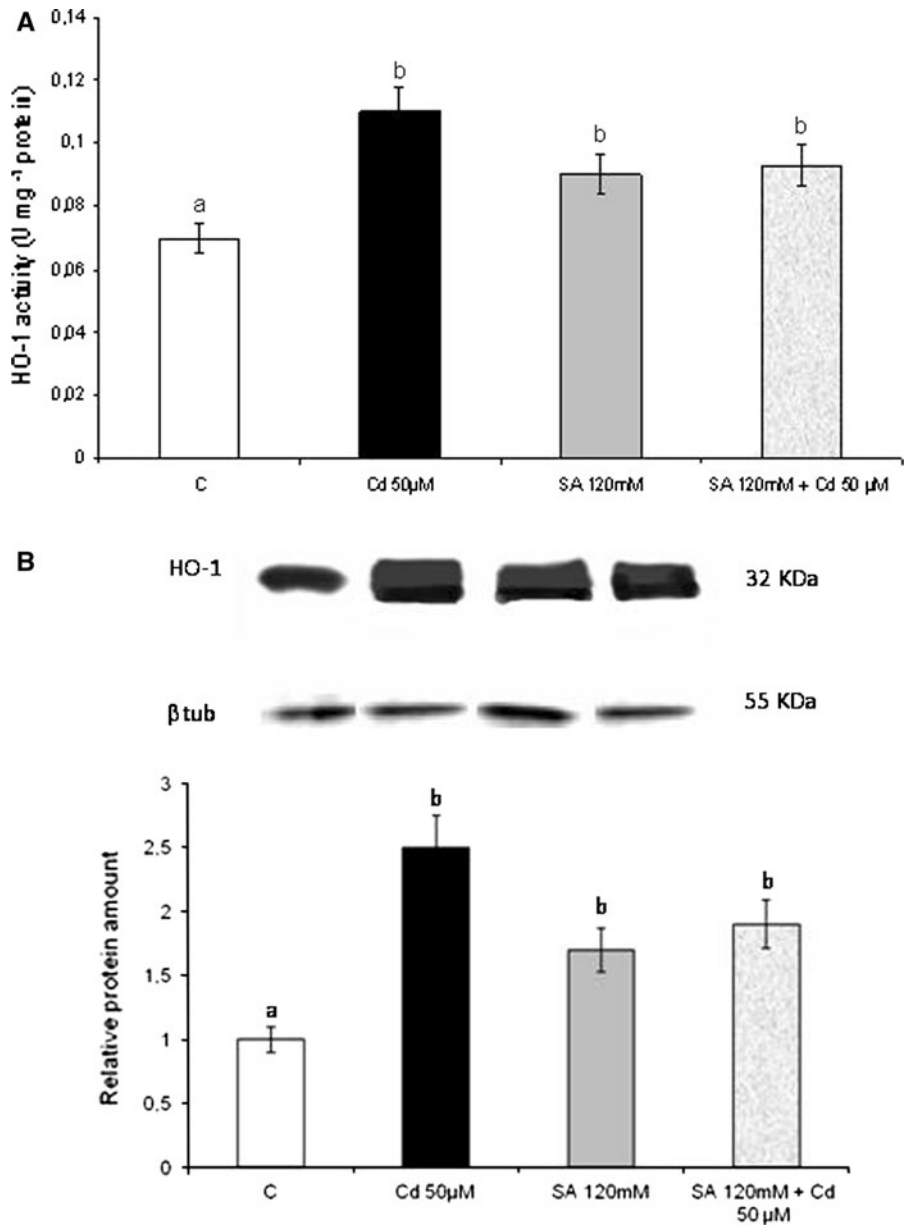

Fig. 5 Effect of Cd and SA pre-treatment on soybean leaves HO activity (**a**) and protein amount (**b**). Data are means of three independent experiments and *bars* indicate SE. Different *letters* within columns indicate significant differences ($P < 0.05$).Experiments were performed as described in "Materials and Methods" section

but only 28 % enzyme activity respect to controls. The same result was obtained in plants pre-treated with SA before Cd addition. On the other hand, western blott analysis Fig. 5b revealed a positive relationship between enzyme activity and protein content. Plants treated with SA also showed an increase in HO-1 protein content (75 %) respect to controls. When this analysis was carried out in plants treated with SA before Cd addition, once again an enhancement (by about 78 %) was detected.

Cadmium determination

Cadmium determinations were performed by atomic absorption spectrometry in an attempt to elucidate whether SA could have any effect on its uptake. Table 2 shows that in the presence of 120 µM SA Cd uptake was diminished compared with values obtained with Cd was added alone. Cd content was not detected in the presence of 120 µM SA alone.

Table 2 Effect of SA pre-treatment on Cd content in soybean leaves

Treatment	Cadmium content (mg/g FW)
Control	0.002 ± 0.001^{a}
Cd 50 μM	0.029 ± 0.008^{b}
120 mM SA	ND
120 mM SA+Cd 50 μM	0.008 ± 0.001^{c}

Enzymatic activities were assayed as described in the experimental section. Different letters within columns indicate significant differences ($P < 0.05$) according to Tukey's multiple range test

Discussion and conclusions

Elevated TBARS content, ROS accumulation and chlorophyll loss are regarded as sensitive indicators of heavy metals toxicity in plants and their measurements have been proposed as bioassays for identifying plants exposed to metals, including Cd (Popova et al. 2009).

Data here reported, indicate that Cd treatment induced oxidative stress and production of ROS (H_2O_2 and O_2^-) as well as enhanced TBRS content, decreased GSH levels and activity of antioxidant enzymes such as SOD, CAT and GPOX, and also enhanced HO-1 activity and its protein content.

To cope with Cd toxicity, plants can make use of several defense strategies, including phytochelation and sequestration as well as the induction of antioxidant machinery and stress proteins (Cobbett and Goldsbrough 2002; Vázquez et al. 2006). In the case of metals toxicity, in particular Cd, plants have developed other detoxification mechanisms related with some stress signalling molecules, such as SA and NO (Rodriguez-Serrano et al. 2006). Salicylic acid is considered as a hormone-like substance, which plays an important role in regulating a number of physiological processes and plant resistance to biotic and abiotic stresses (Mishra and Chudhuri 1999).

Data here reported, do indicate that SA protects against the deleterious effects produced by Cd.

This phytohorme, is also involved in ion uptake and transport (Ray 1986). There are evidence that SA ameliorates the damaging effects of heavy metals by broad but different effects (Drazic and Mihailovic 2005; Metwally et al. 2003; Mishra and Chudhuri 1999). Salicylic acid pre-treatment alleviated Cd toxicity in barley and maize plants (Metwally et al. 2003). The protective function of SA includes regulation of ROS and antioxidants, induction of gene expression (Shah 2003), and absorption and distribution of elements (Metwally et al. 2003, Mishra and Chudhuri 1999).

However, the role of exogenously applied SA under Cd stress in plants is not yet clear and needs further investigations.

Based on the above studies, our aim was to examine the possible interaction between SA and HO-1 in plants exposed to Cd.

Data here reported showed that SA enhanced both HO-1 activity and its protein content compared with controls, and that these increments were lower than those produced by Cd alone. Unexpectedly, this behaviour was also observed in plants treated with both SA and Cd. These results prompted us to evaluate Cd content in the leaves. Data obtained, indicated that Cd uptake is reduced in the presence of SA and let us conclude that its protective effect is not due to its action on HO-1 as it happens with another phytohormone tested such as jasmonic acid (Noriega et al. 2012), but as a result of a lower Cd content in the leaves.

It has been already reported that a moderate resistance to heavy metals can occur by selective Cd exclusion, diminished uptake, or active efflux from the roots, i.e. by mechanisms leading to lower cytoplasmic Cd content (Hall 2002). It is interesting to note, that on one hand, SA inhibits Cd uptake and on the other hand, SA alone enhanced both HO-1 activity and its protein content, as well as increased by 25 % CAT activity, but it did not alter any of the other oxidative stress parameters here assayed This particular behaviour could prepare the tissue to protect itself against a possible aggression and this is the reason why a concomitantly up-regulation of HO-1 and CAT gene expression was also found in soybean nodules subjected to high Cd stress (Balestrasse et al. 2008).

All together these findings are indicating that in the presence of another stressing factor, which could be transported into the cells, SA could exert its protective effect through HO-1. We have found that soybean plants treated with ZnPPIX, an irreversible HO-1 inhibitor, are prone to oxidative damage (Noriega et al. 2004; Balestrasse et al. 2005).

In this paper we have found good evidence that SA can exert a significant beneficial effect on Cd exposed soybean plants. Interestingly, increased antioxidant defense appears not to be involved in the alleviation of

Cd toxicity, because SA itself inhibited Cd accumulation in the leaves. These data contrast with our own previously reported results about jasmonic acid, another phytohormone, which protected soybean plants against Cd induced oxidative stress, by enhancing HO-1 activity and its expression. (Noriega et al. 2012).

Taken together, data reported herewith suggested that SA could prevent Cd-induced oxidative damage, which might be due to a diminished metal uptake. Nevertheless, it should be recalled that although SA inhibited Cd transport, it also enhanced per se HO-1 activity and its protein content as well as increased CAT activity, that could eventually prevent oxidative stress provoked by other stressing factors.

In other words, SA not only can inhibit Cd transport but it can also prevent oxidative stress by modifying the antioxidant defense system, whenever necessary.

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