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Heme oxygenase activity and oxidative stress signaling in soybean leaves

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Abstract

We have previously demonstrated that the induction of heme oxygenase-1 (EC 1.14.99.3) plays a protective role for soybean plants against oxidative stress. Here, we have investigated for the first time the possible signal transduction pathways involved in heme oxygenase-1 induction in leaves of soybean plants. Treatment with 200 μM Cd during 48 h increased 87% thiobarbituric acid reactive substances, whereas GSH decreased 70%, respect to controls. These effects were prevented by preincubation with diphenyleneiodonium (DPI, NADPH oxidase inhibitor), cantharidin (protein phosphatases inhibitor), $LaCl_3$ (calcium channel blocker) and [1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ, guanylate cyclase inhibitor). Two hundred micromoles Cd produced increased concentrations and in situ accumulation of H_2O_2 and $O_2^{\bullet -}$, which were again prevented by DPI, cantharidin, $LaCl_3$ and ODQ. Moreover, Cd-induced heme oxygenase-1 activity was also totally or partially abolished by pretreatment with the different inhibitors. These results clearly demonstrated that the signal transduction pathways involved in oxidative stress, triggered by cadmium ions were similar to those implicated in heme oxygenase-1 induction, and supported the proposal of a close relationship between oxidative stress generation and heme oxygenase induction in higher plants.
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Keywords: Cd toxicity; Heme oxygenase; Leaves; Signaling pathways; Soybean

1. Introduction

A wide range of environmental stresses, such as extreme temperatures, drought, salinity, UV or ozone and heavy metals, are potentially harmful to plants. A common aspect to all of these adverse conditions is the enhanced production of reactive oxygen species (ROS) within several subcellular compartments of the plant cell [1].

Cadmium is a toxic pollutant for living organisms, and enters the environment mainly from industrial processes. Cd is a non-redox metal unable to participate in Fenton-type reactions but capable of inducing oxidative stress in cells. There is strong evidence that oxidative stress is involved in Cd toxicity, by either inducing oxygen free radicals production, or by decreasing enzymatic and non-enzymatic antioxidants ([2] and references herein).

Studies carried out in different plant species have revealed that Cd is strongly phytotoxic and causes growth inhibition and even plant death [2,3], although the mechanisms involved in its toxicity are not yet completely understood. It has been demonstrated that Cd can induce oxidative stress in nodules, roots and leaves of soybean, characterized by accumulation of lipid peroxides and reduction of antioxidant soluble and enzymatic system [4–6]. In peroxisomes purified from pea leaves, Cd produced an increase of the H_2O_2 content and imbalances in the activity of antioxidant enzymes [7]. Moreover, cadmium induced subcellular accumulation of H_2O_2 in pea leaves and several enzymatic systems have been suggested as being responsible for $O_2^{\bullet -}$ and H_2O_2 production

Abbreviations: BHT, butylated hydroxytoluene; Canth, cantharidin; cGMP, cyclic GMP; DAB, 3,3′-diaminobenzidine; DPI, diphenyleneiodonium; DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid); GSH, reduced glutathione; GSSG, oxidized glutathione; HO-1, heme oxygenase-1; HO's, heme oxygenases; NBT, nitroblue tetrazolium; ODQ, [1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one; PVP, polyvinylpyrrolidone; ROS, reactive oxygen species; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; TCA, trichloroacetic acid

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on the surface of plant cell [8]. Romero-Puertas et al. [8] also studied the transduction pathways involved in ROS production in pea plants under Cd stress. Apparently, the expression of at least 1–2% of *Arabidopsis* genes is dependent on H_2O_2, some of them are antioxidant genes and others encode proteins involved in signaling such as calmodulin, protein kinases and transcription factors [9,10]. Ca^{2+} ions play a central role in different mechanisms such as activation of defense responses, mechanical stress and alleviation of mineral toxicities in plants [11]. Recently, evidence is emerging to support the existence in plants of a membrane-bound enzyme system similar to neutrophyl NADPH oxidase involved in production of $O_2^{\bullet -}$ radicals, which are then converted to H_2O_2 by superoxide dismutase during the oxidative burst [11,12].

Heme oxygenases (HO's) are ubiquitous enzymes, which catalyze the stereo specific cleavage of heme to biliverdin with the release of free iron and carbon monoxide [13]. HO has been widely studied in animal tissues, particularly in the liver ([14] and references herein). Its major role is associated with heme degradation and its participation in the antioxidant machinery of the cells by means of its product biliverdin, which in turn is subsequently converted to bilirubin through the action of biliverdin reductase. It has been demonstrated that one of the three known mammalian isoforms, heme oxygenase-1, is induced in animal tissues by many factors including its own substrate heme, several heme-proteins, heavy metals, UVA radiation, hypoxia, hyperoxia and others [15–18]. In our laboratory, it was demonstrated for the first time the presence, in soybean leaves and nodules, of one HO closely related to the HO-1 of mammalian cells, on the basis of its induction by prooxidants and its antioxidant behavior under Cd stress, indicating that HO plays a protective role against oxidative cell damage in soybean plants [5,6].

In view of these considerations, an attempt has been made to elucidate the mechanism of signal transduction involved in ROS generation and heme oxygenase induction during Cd stress in soybean leaves. The participation of H_2O_2 and $O_2^{\bullet -}$ in the damage induced by Cd toxicity was also investigated.

2. Materials and methods

2.1. Plant material and growing conditions

Seeds of soybean (*Glycine max* L., supplied by Nidera) were surface sterilized with 5% (v/v) sodium hypochlorite for 10 min and then washed with distilled water four times. The seeds were inoculated with 10^8 cell ml^{-1} of *Bradyrhizobium japonicum* (109, INTA Castelar) and were planted in vermiculite for five days. Ten replicates trays were prepared for each treatment, and 20 seeds/pot were sown. 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 with a 16 h photoperiod, a photosynthetically active radiation (PAR) of 175 $\mu mol\ m^{-2}\ s^{-1}$ and 50% relative humidity. The hydroponics medium was Hoagland nutrient solution [19]. The medium was continuously aerated and replaced every three days. After four weeks growth, leaf discs from the second pair of leaves upper the cotyledons (12 mm diameter, 0.3 g) were prepared and floated abaxial side down in the dark during 48 h in flasks containing 20 ml of 0 (Control) or 200 μM $CdCl_2$ in distilled water. When the effects of 0.25 mM diphenyleneiodonium (DPI, oxidase inhibitor), 0.01 mM cantharidin (Canth, protein phosphatases inhibitor), 0.15 mM $LaCl_3$ (calcium channel blocker), and 0.2 mM 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ, guanylate cyclase inhibitor) were investigated, leaf segments were pretreated with one of these compounds during 4 h before addition of Cd. Controls were incubated in distilled water. When the effects of inhibitors were examined for H_2O_2 and $O_2^{\bullet -}$ localization in situ and leaf concentrations, the second pair of leaves upper the cotyledons were selected to avoid the overlapping of senescence and Cd-induced effects that take place in the oldest leaves. In these experiments, leaves were pretreated with one of above-mentioned compounds during 4 h before addition of Cd. Controls were incubated in distilled water. Three different experiments were performed, with five replicated measurements for each parameter assayed.

2.2. Determination of Cd content

Segments from the second pair of fully expanded leaves upper the cotyledons without and with 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. Cadmium determinations were made on HNO_3:$HClO_4$ (3:1, v/v) digests by atomic absorption spectrophotometry (Perkin-Elmer, AAnalyst 300).

2.3. Relative water content (RWC) and osmotic potential

Relative water content (RWC), expressed as a percentage, was determined in soybean leaves according to the formula:

$$\mathrm{RWC}\,(\%) = (\mathrm{FW} - \mathrm{DW}) \times \frac{100}{\mathrm{DW}}$$

where FW and DW means fresh weight and dry weight, respectively. FW was measured just after collected the leaf discs at the end of the experiment and DW was measured after drying the leaves at 80 °C for 48 h. Osmotic potential was measured in the same leaves in a vapor pressure osmometer 5500 (Wescor, Logan, UT, USA).

2.4. 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 [20]. 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

3500 × *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}.

2.5. Glutathione determination

Non-protein thiols were extracted by homogenizing 0.3 g of leaf discs in 3.0 ml of 0.1N 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 0.1N 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 [21].

2.6. Heme oxygenase preparation and assay

Leaf discs (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 chloroplasts were used for activity determination. Heme oxygenase activity was assayed as previously described with minor modifications [22]. The assays (1-ml final volume unless otherwise indicated) contained, 250 μl HO (0.5 mg protein), 10 μM hemin, 0.15 mg/ml bovine serum albumin, 50 μg/ml (4.2 μM) spinach (*Spinacia oleracea*) ferredoxin (Sigma Chemical Co.), 0.025 U/ml spinach ferredoxin-$NADP^+$ reductase (Sigma Chemical Co.). The reaction was started by adding NADPH to a final concentration of 100 μM, samples were incubated at 37 °C during 30 min and biliverdin IXα formation was calculated by measuring the absorbance change at 650 nm after 30 min. The concentration of biliverdin IXα was estimated using a molar absorption coefficient at 650 nm of 6.25 mM^{-1} cm^{-1} in 0.1 M HEPES–NaOH buffer (pH 7.2).

2.7. H_2O_2 localization in situ

Leaves from control and Cd-treated plants were excised and immersed in a 1% solution of 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. Leaves were illuminated until appearance of brown spots characteristic of the reaction of DAB with H_2O_2. Leaves were bleached by immersing in boiling ethanol to visualize the brown spots. H_2O_2 deposits were quantified by scanning spots from leaf pictures and the number of pixels were quantified with the PHOTOSHOP 6.0 program (Adobe Systems, San Jose, CH, USA). The results were expressed as percentage of spots area versus total leaf area [(spot area/total leaf area) × 100] in order to compensate the differences in life size.

2.8. $O_2^{\bullet-}$ localization in situ

Leaves from control and Cd-treated plants 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. Leaves were bleached by immersing in boiling ethanol to visualize the dark spots. Superoxide deposits were quantified by scanning spots from leaf pictures as mentioned above.

2.9. H_2O_2 determination in leaf extracts

The H_2O_2 concentration of crude extracts from soybean leaves was determined by spectrofluorometry method as described by Creissen et al. [23]. Leaves (0.5 g) were homogenized in 1.2 ml of 25 mM HCl, and the crude extracts were filtered through two nylon layers, and the pigments were removed by mixing with 15 mg of charcoal. The pigment-containing charcoal was separated by centrifugation at 5000 × *g* for 5 min, and the supernatants were clarified by filtration through 0.20-μm filter unit. The pH of leaf disc extracts was adjusted to 7.0 with NaOH and these extracts were used to measure the H_2O_2 concentration. The reaction mixtures (3 ml) contained 50 mM HEPES buffer, pH 7.6, 5 mM homovanillic acid and 100 μl of sample. The reaction was started by adding 40 μM horse-radish peroxidase and the fluorescence produced was measured in a spectrofluorophotometer Shimadzu RF-540 (Kyoto, Japan), at excitation and emission wavelengths of 315 and 425 nm, respectively. The H_2O_2 concentration was determined from a calibration curve of H_2O_2 in the range 0.1–20 μM.

2.10. $O_2^{\bullet-}$ determination in leaf extracts

The $O_2^{\bullet-}$ concentration of crude extracts from soybean leaves was determined by spectrometry method as described by Boveris [24]. Leaves (0.5 g) were homogenized in 1.2 ml of 25 mM HCl, and the crude extracts were filtered through two nylon layers, and the pigments were removed by mixing with 15 mg of charcoal. The pigment-containing charcoal was separated by centrifugation at 5000 × *g* for 5 min, and the supernatants were clarified by filtration through 0.20-μm filter unit. The pH of leaf disc extracts was adjusted to 7.0 with NaOH and these extracts were used to measure the $O_2^{\bullet-}$ concentration. The reaction medium consisted of 40 mM potassium phosphate buffer (pH 7.4), 120 mM KCl, 1 mM EDTA, and 1 mM epinephrine. The rate of production of O_2^- was determined as the superoxide dismutase-sensitive rate of adrenochrome formation, measured at 485 to 575 nm ($\in$ = 2.97 mM^{-1} cm^{-1}) in a Perkin-Elmer dual-wavelength spectrophotometer.

Table 1
Cadmium concentrations in soybean leaves

Treatments	Cd concentration μg/g FW
Control	5.98 ± 0.52^a
200 μM Cd	45.52 ± 0.43^b

Segments from the second pair of fully expanded leaves upper the cotyledons, were used for the assays. Experiments were carried out as described in Section 2. Data are the means ± S.E.M. 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.

2.11. Protein determination

Protein concentration was evaluated by the method of Bradford [25], using bovine serum albumin as a standard.

2.12. Statistics

All data presented are the mean values of three independent set of experiments. Each value was presented as means ± standard errors of the mean (S.E.M.), with five replicates. Statistical assays were carried out by one-way ANOVA using the Tukey's test to evaluate whether the means were significantly different, taking $p < 0.05$ as significant.

3. Results

3.1. Cadmium concentration, water status and osmotic potential in soybean leaves

Cadmium accumulation was measured in order to evaluate the proportion of the metal that was translocated from the medium to the leaf discs. As can be shown in Table 1, Cd-treated segments showed a 6.5-fold increase in Cd concentration, compared to controls. However, this increase was not sufficient for altering neither the water status nor the osmotic potential of leaf discs (Table 2).

3.2. Oxidative stress generation

Oxidative stress is the result of excessive production of oxidant species and/or depletion of intracellular antioxidant defenses, leading to an imbalance in the redox status of the cell.

Table 2
Relative water content (RWC) and osmotic potential in soybean leaves

Treatments	RWC (%)	Osmotic potential (MPa)
Control	85.4 ± 0.7^a	-0.90 ± 0.02^a
200 μM Cd	84.9 ± 0.8^a	-0.89 ± 0.03^a

Segments from the second pair of fully expanded leaves upper the cotyledons, were used for the assays. Experiments were carried out as described in Section 2. Data are the means ± S.E.M. of three different experiments with five replicated measurements. Similar letters within columns indicate no significant differences ($p < 0.05$), according to Tukey's multiple range test.

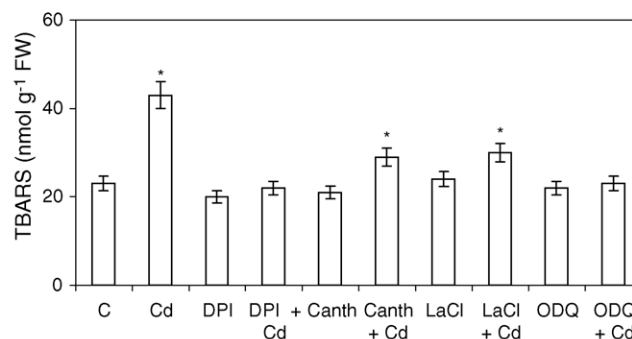

Fig. 1. Effect of preincubation with DPI, Canth, $LaCl_3$ or ODQ on the Cd-induced TBARS content in soybean leaf discs. Experiments were carried out as described in Section 2. Values are the means of three different experiments with five replicated measurements, bars indicate S.E.M. *Significant differences ($p < 0.05$), according to Tukey's multiple range test.

ROS are regarded to be initiators of peroxidative cell damage. TBARS formation in plants exposed to adverse environmental conditions is a reliable indicator of tissular free radical generation. An increase close to 87% in TBARS content was assessed in leaf discs in the presence of 200 μM Cd (Fig. 1). In order to get some insights into the signal transduction pathways involved in the plant response to Cd, the effect of different inhibitors of ROS accumulation in soybean leaves grown in the presence of Cd was examined. Preincubations with DPI, Canth, $LaCl_3$ or ODQ did not alter TBARS levels, compared to controls. These treatments prior to Cd addition prevented the increase in TBARS content at the different degree. While DPI and ODQ treated samples showed a total inhibition in TBARS enhancement, there was only 26 and 30% increase in the presence of Canth and $LaCl_3$, respectively (Fig. 1).

To verify the identity of ROS involved in Cd-induced oxidative stress, H_2O_2 and $O_2^{\bullet-}$ leaf concentrations were also determined. As can be shown in Table 3 Cd caused 6-fold

Table 3
Effect of Cd and DPI, Canth, $LaCl_3$ or ODQ pretreatments on H_2O_2 and $O_2^{\bullet-}$ accumulation in soybean leaves

Treatments	H_2O_2 (μM)	$O_2^{\bullet-}$ (μM)
Control	0.25 ± 0.02^a	0.12 ± 0.02^a
200 μM Cd	1.58 ± 0.16^b	1.12 ± 0.10^b
DPI	0.23 ± 0.02^a	0.11 ± 0.01^a
DPI + 200 μM Cd	0.24 ± 0.01^a	0.13 ± 0.01^a
Canth	0.21 ± 0.01^a	0.12 ± 0.01^a
Canth + 200 μM Cd	0.23 ± 0.02^a	0.62 ± 0.06^c
$LaCl_3$	0.24 ± 0.01^a	0.14 ± 0.02^a
$LaCl_3$ + 200 μM Cd	0.80 ± 0.07^c	0.11 ± 0.01^a
ODQ	0.22 ± 0.01^a	0.13 ± 0.01^a
ODQ + 200 μM Cd	0.24 ± 0.02^a	0.12 ± 0.01^a

Segments from the second pair of fully expanded leaves upper the cotyledons, were used for the assays. Experiments were carried out as described in Section 2. Data are the means ± S.E.M. 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.

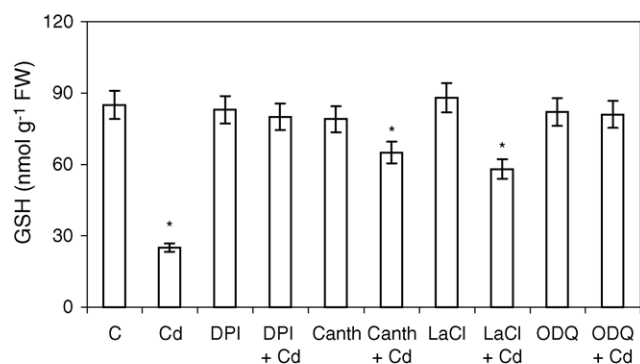

Fig. 2. Effect of preincubation with DPI, Canth, $LaCl_3$ or ODQ on the Cd-induced decrease in GSH levels in soybean leaf discs. Experiments were carried out as described in Section 2. Values are the means of three different experiments with five replicated measurements, bars indicate S.E.M. *Significant differences ($p < 0.05$), according to Tukey's multiple range test.

and 9-fold enhancement of H_2O_2 and $O_2^{\bullet-}$, respectively, compared to controls. Preincubations with each inhibitor alone, did not produce changes in H_2O_2 and $O_2^{\bullet-}$ leaf concentrations. Similar results were obtained when the inhibitors were administered before Cd addition, except $LaCl_3$ for H_2O_2 and Canth for $O_2^{\bullet-}$, which produced an increase close to half, respect to control values.

GSH is a leading substrate for enzymatic antioxidant functions and it is also a known radical scavenger. It could therefore be expected that if Cd induces the formation of oxidant species it would also affect GSH-leaf levels. Data in Fig. 2 show that GSH concentration in leaf discs treated with 200 μM Cd decreased 70% respect to controls. Preincubations with the inhibitors and further treatment with Cd reduced the decrease in GSH content caused by Cd. While DPI and ODQ totally prevented GSH decrease, Canth and $LaCl_3$ only caused 24 and 32% of GSH decrease, respectively. Preincubations carried out in the presence of each inhibitor alone produced no effect.

3.3. H_2O_2 and $O_2^{\bullet-}$ localization in situ.

Accumulation of H_2O_2 and $O_2^{\bullet-}$ were also performed in situ by histochemical methods. For H_2O_2 identification, DAB reaction was used. This method is based on the formation of local brown spots in leaves as a result of H_2O_2 presence (Fig. 3A). Production of $O_2^{\bullet-}$ was studied by infiltrating soybean leaves with nitroblue tetrazolium (NBT), which is reduced by $O_2^{\bullet-}$ giving rise to dark spots of blue formazan (Fig. 3B). The quantification of dark spots in both cases was made by counting the pixel number of spots. On the other hand, to shed light in the signal transduction pathways involved in the H_2O_2 and $O_2^{\bullet-}$accumulation in soybean leaves subjected to Cd stress, the effect of different inhibitors of H_2O_2 and $O_2^{\bullet-}$ production was studied.

As it is shown in Fig. 3A, Cd produced 10% H_2O_2 spots area versus total leaf area, while pretreatment with DPI and Canth prevented this effect and spot area was similar to controls. Preincubation with $LaCl_3$ partially inhibited the Cd-induced H_2O_2 accumulation (50%).

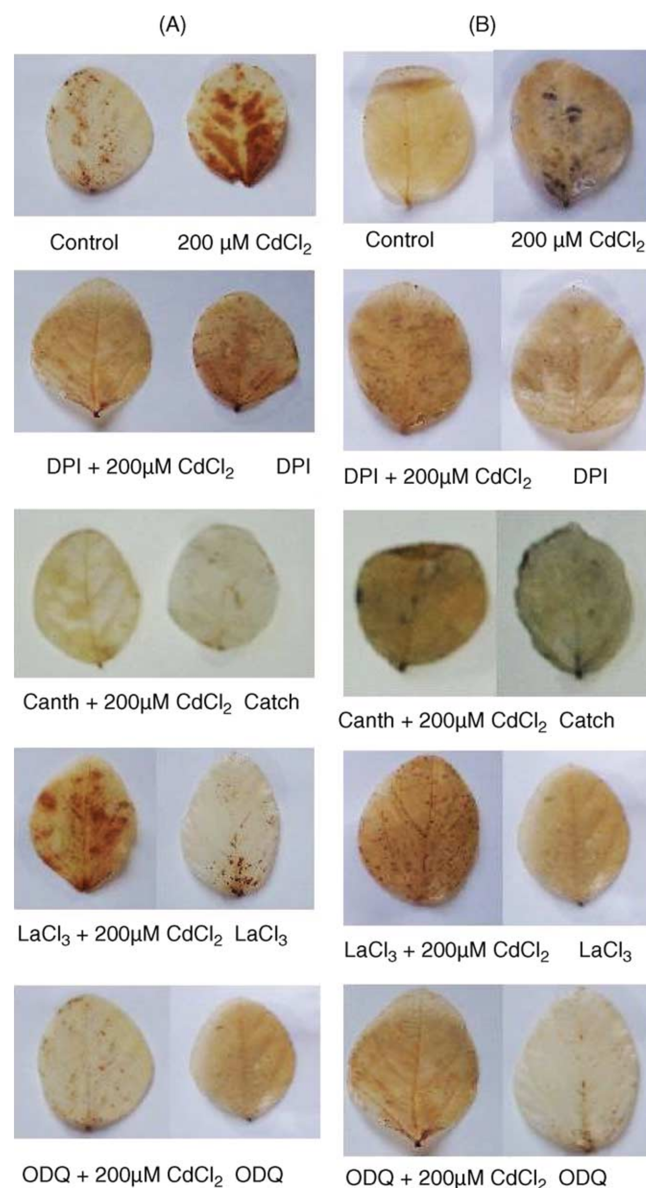

Fig. 3. Histochemical detection of H_2O_2 (A) and $O_2^{\bullet-}$ (B) in soybean leaves. The second pair of fully expanded leaves upper the cotyledons, were used for the assays. Experiments were performed as described in Section 2. Pictures are representative of three different experiments with five replicated measurements for each treatment.

Data in Fig. 3B showed that leaves treated with 200 μM Cd produced 6.4% $O_2^{\bullet-}$ spots area versus total leaf area. Pretreatments with DPI or $LaCl_3$ completely reverted the $O_2^{\bullet-}$ production induced by Cd, while Canth produced a 40% decrease in $O_2^{\bullet-}$ accumulation.

3.4. Heme oxygenase activity

Leaf discs exposed to 200 μM Cd showed a 2.8-fold enhancement in HO activity. When samples were preincubated with DPI, Canth, $LaCl_3$ or ODQ, induction of the enzyme activity was prevented (100, 58, 57 and 100%, respectively). On the other hand, these compounds alone had not any effect on HO activity (Fig. 4). These results corroborated the protection

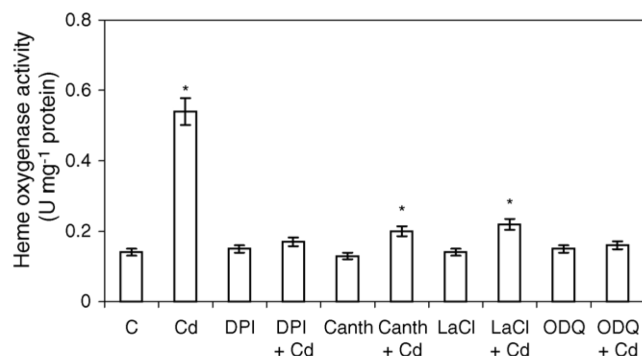

Fig. 4. Effect of preincubation with DPI, Canth, $LaCl_3$ or ODQ on the Cd-induced heme oxygenase activity in soybean leaf discs. Experiments were carried out as described in Section 2. Values are the means of three different experiments with five replicated measurements, bars indicate S.E.M. *Significant differences ($p < 0.05$), according to Tukey's multiple range test. One unit of the enzyme forms 1 nmol of biliverdin/30 min under the assay conditions.

action observed in the oxidative stress parameters in leaf discs pretreated with the different inhibitors.

4. Discussion

Oxidative stress is a condition referred to as an imbalance between oxidant generation and antioxidant systems. Although Cd is redox inactive, this metal is a well-established oxidative stressor as it depletes GSH and protein-bound sulphidryl groups, resulting in the production of ROS such as superoxide ion, hydroxyl radicals and hydrogen peroxide [8,26]. We have already reported oxidative damage induced by Cd in soybean plants [4–6], and an oxidative burst induced by Cd was found in sunflower discs [27], in pea plants [7,28] and in tobacco cell cultures [29].

In order to evaluate if Cd treatment produced an osmotic effect in leaf discs, relative water content and osmotic potential were measured. Because these parameters remain unaltered under Cd stress, we assumed that the oxidative stress found in the present study was Cd-specific and not induced by osmotic shock. These results were in accordance with those obtained by Milone et al. in wheat [30].

Since lipid peroxidation is one of the first consequences of oxidative damage, TBARS were measured as an indicator of Cd-induced ROS formation. Using different signal transduction pathways inhibitors, we found that Cd-induced TBARS content was prevented by inhibitor pretreatment.

In plants, GSH is the major soluble antioxidant in photosynthetic and non-photosynthetic tissues. It is able to detoxify ROS by direct scavenging or by acting as cofactor in the enzymatic reactions that are involved in the ascorbate-glutathione cycle. In fact, Cd forms stable complexes with thiol groups such as GSH and phytochelatins [31], and this could be explaining, at least in part, GSH decrease. The degree of Cd tolerance in plants has been correlated with inherent GSH levels and with cellular capacity to synthesize thiol compounds [32,33]. GSH is reduced by Cd in soybean leaves, but signal transduction process inhibitors were able to counteract efficiently GSH depletion. GSH decrease has also been reported by Balestrasse and co-workers in soybean nodules [4,6] and Noriega et al. in leaves [5] under Cd stress. All together, these results demonstrated the participation of NADPH oxidase, protein dephosphorylation, intracellular Ca^{2+}, and guanylate cyclase in Cd-induced oxidative stress.

The rapidly induced ROS generation under stress conditions (i.e. the oxidative burst) initially results in the formation of $O_2^{\bullet-}$, which then dismutate into H_2O_2 and O_2 either spontaneously or via superoxide dismutase. The involvement of H_2O_2 as a leading ROS in Cd-induced oxidative stress was reported in tobacco cell cultures [34], and H_2O_2 and $O_2^{\bullet-}$ were involved in Cd-induced oxidative stress generation in pea plants [8]. Both $O_2^{\bullet-}$ and H_2O_2 have been shown to act directly or indirectly in signal transduction [35]. Although H_2O_2 is generally considered to be a signaling molecule in defense responses, $O_2^{\bullet-}$ plays this role as well. Phytoalexin synthesis in soybean cells in response to pathogens or elicitors is blocked by DPI and superoxide dismutase but not by catalase [36]. The inhibition observed in $O_2^{\bullet-}$ and H_2O_2 production, and in TBARS content by pretreatment with DPI, let us speculate that a NAPH oxidase or NADPH oxidase-like enzyme is involved in ROS generation under Cd stress. Chemical inhibitors of NADPH oxidase, such as DPI, have been shown to block or severely reduce ROS production upon biotic and abiotic stresses [35]. Protein phosphatases have been implicated in a negative feedback loop that controls the wound-induced MAPK pathway in alfalfa [35]. The supply of Canth, a protein phosphatase inhibitor abolished the Cd-induced H_2O_2 and $O_2^{\bullet-}$. This fact suggested that the earliest control point in ROS regulation is at the level of phosphorylation/dephosphorylation of proteins. The reduction in ROS generation by blockage of Ca^{2+} channels using $LaCl_3$ suggested that changes in Ca^{2+} fluxes through the plasma membranes occurred. It has been known that $LaCl_3$ can enhance the adaptability to stress conditions or increase the tolerance to adversity such as low and high temperatures, drought and salt, and it was reported that $LaCl_3$ lightened the plant damage by lead [37]. Results obtained in soybean leaves with inhibitors of signal transduction pathways showed that ROS production induced by Cd can be regulated by diverse processes, some of which are common to the response to biotic stresses [35]. Because the inhibitor of guanylate cyclase, ODQ, also prevented TBARS increase, GSH depletion and H_2O_2 and $O_2^{\bullet-}$ accumulation, cGMP seems to be involved in Cd-induced ROS production. Cyclic GMP could induce ROS generation by producing an elevation of Ca^{2+} concentration [38]. But, it is also possible that carbon monoxide was implicated in the process mediate by guanylate cyclase, taking into account that it is activated when NO interacts with its heme component. Although it is conceivable that other agents capable of interacting with the heme group (other free-radicals) could produce changes in guanylate cyclase activity, it has been shown that NO is physiologically the most important and abundant of these agents [39]. However, carbon monoxide, one of the catalytic products of HO activity, seems to mimic many NO functions. CO derived from HO catalysis has shown to modulate the NO-induced cGMP signaling pathway in

neuronal tissue as well as hypoxia-induced HO-1 expression in cultured vascular cells [40,41]. Carbon monoxide activates purified soluble guanylate cyclase to increase cGMP formation, although its affinity for the heme moiety of guanylate cyclase is much lower than that associated with NO [40].

The results obtained using ODQ were supported by the fact that ODQ also prevented the Cd-induced HO activity. We have previously demonstrated a close relationship between oxidative stress generation and HO induction in soybean plants [5,6]. Cd produced a strong increase in HO activity, which was partially or totally abolished by preincubations with DPI, Canth, $LaCl_3$ or ODQ. In addition, all inhibitors of signaling pathways used in the present work showed similar behavior when ROS generation and HO activity were analyzed. It is noteworthy that, although the HO signal transduction pathways have been widely reported in animal tissues, to the best of our knowledge, they had not been studied yet in plant tissues.

Findings here reported clearly demonstrated, for the first time, that the signal transduction pathways involved in oxidative stress triggered by Cd ions were similar to those implicated in HO induction in leaves of soybean plants, and provide additional information suggesting that HO might play a key role in the anti-oxidative protection machinery of higher plants.

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