Indole acetic acid is responsible for protection against oxidative stress caused by drought in soybean plants: The role of heme oxygenase induction

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Objectives: This study was focused on the role of indole acetic acid (IAA) in the defense against oxidative stress damage caused by drought in soybean plants and to elucidate whether heme oxygenase-1 (HO-1) and nitric oxide (NO) are involved in this mechanism. IAA is an auxin that participates in many plant processes including oxidative stress defense, but to the best of our knowledge no information is yet available about its possible action in drought stress.
Methods: To this end, soybean plants were treated with 8% polyethylene glycol (PEG) or 100 μM IAA. To evaluate the behavior of IAA, plants were pretreated with this compound previous to PEG addition. Lipid peroxidation levels (thiobarbituric acid reactive substances (TBARS)), glutathione (GSH) and ascorbate (AS) contents, catalase (CAT), superoxide dismutase (SOD), and guaiacol peroxidase (POD) activities were determined to evaluate oxidative damage.
Results: Drought treatment (8% PEG) caused a significant increase in TBARS levels as well as a marked decrease in the non-enzymatic (GSH and AS) and enzymatic (CAT, SOD, and POD) antioxidant defense systems. Pre-treatment with IAA prevented the alterations of stress parameters caused by drought, while treatment with IAA alone did not produce changes in TBARS levels, or GSH and AS contents. Moreover, the activities of the classical enzymes involved in the enzymatic defense system (SOD, CAT, and POD) remained similar to control values. Furthermore, this hormone could enhance HO-1 activity (75% with respect to controls), and this increase was positively correlated with protein content as well as gene expression. The direct participation of HO-1 as an antioxidant enzyme was established by performing experiments in the presence of Zn-protoporphyrin IX, a well-known irreversible inhibitor of this enzyme. It was also demonstrated that HO-1 is modulated by NO, as shown by experiments performed in the presence of an NO donor (sodium nitroprusside), an NO scavenger (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide), or an NO synthesis inhibitor (*N*-nitro-L-arginine methyl ester, NAME).
Discussion: It is concluded that IAA is responsible, at least in part, for the protection against oxidative stress caused by drought in soybean plants through the modulation of NO levels which, in turn, enhances HO-1 synthesis and activity.

Keywords: Drought stress, *Glycine max.* L, Heme oxygenase, Indole acetic acid, Nitric oxide

Introduction

Soybean (*Glycine max.* L) is an important crop in the world because it is used as a source of high-quality protein. Different environmental conditions may affect its yield. In this sense, drought is one of the major abiotic stresses that impair plant productivity.[1] Water deficit is characterized by a disturbance between the generation and quenching of reactive oxygen species (ROS).[2] In the absence of an effective protective system, ROS provokes lipid peroxidation, protein degradation, DNA breakage, and cell death.[3]

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Plant cells can overcome ROS deleterious effects by means of endogenous protective mechanisms involving non-enzymatic as well as enzymatic systems.[4]

Indole acetic acid (IAA) is one of the naturally occurring growth hormones that enhances cell division and elongation. The behavior of IAA in drought resistance is still rather contradictory. For a long time, it was generally assumed that water deficiency results in a decrease of IAA content.[5] However, it has become more evident that adaptation to drought is accompanied by an increase in the IAA levels.[6]

Several reports have demonstrated that heme oxygenase-1 (HO-1) participates in the response to different stress stimuli in plants,[7–10] but to the best of our knowledge no information is still available about its behavior against the oxidative stress damage caused by drought stress in soybean plants.

Heme oxygenase (HO; EC 1.14.99.3) catalyzes the degradation of heme into biliverdin IXa (BV), Fe^{2+}, and carbon monoxide (CO). This reaction requires molecular oxygen and electrons from NADPH.[11–13] The mechanism of heme degradation (Fig. 1) by HO is a common feature of all organisms and has been intensively investigated.[14] In plants, HO-1 is also associated with the biosynthetic pathway leading to phytochrome chromophore formation,[15–17] protection against[20] oxidative damage,[18,19] and root development.[20,21] BV generated by the HO-1 catalyzed reaction is an efficient scavenger of ROS.[22] In the soybean leaf, HO has been localized in chloroplast and mitochondria[23] and in the peribacteroid membrane of root nodules.[24] Moreover, expression of HO-1 in plants is regulated by ultraviolet-B (UV-B), H_2O_2, nitric oxide (NO), cytokinin, and heavy metals.[18,20,25–29] Heme catabolism also gives rise to CO production, and this molecule can participate in many physiological reactions,[30] including cell protection against oxidative stress.[31,32]

Recently, it has been demonstrated that HO-1 also performs a crucial role in NaHS-induced lateral root (LR) formation in tomato (*Solanum lycopersicum* L.) seedlings[33] and that HO-1 may be a central repeater for crosstalk among NO, CO, and hydrogen peroxide in plants.[34]

NO production is associated with many physiological situations in plants, and NO is a key signaling molecule throughout the lifespan of a plant. In fact, it plays many roles in biotic and abiotic stresses[3,35–37] and, depending on its concentration, it produces either protective or toxic effects. It has been established that low doses modulate superoxide anion formation and inhibit lipid peroxidation, acting in antioxidant defense during stress. Furthermore, microarray studies have shown that NO induces a large number of genes at the transcriptional level, among them those of antioxidant enzymes.[38] It also has been reported that NO gives rise to signaling pathways mediating responses of specific genes to UV-B radiation, such as chalcone synthase and phenylalanine ammonia lyase[39] and that NO is involved in auxin-induced LR formation.[40]

However, knowledge about the role that NO plays in regulating antioxidant enzymes to counteract drought-induced oxidative stress is rather scarce. Recently, a few studies have suggested that NO could act in protecting plants from oxidative damage;[40,41] therefore, NO-donor treatment would prevent plants from damage by increasing the activity of antioxidant enzymes.

These facts prompted us to investigate the role of IAA in the defense against oxidative stress damage caused by drought in soybean plants and to elucidate whether HO-1 and NO could be involved in this mechanism.

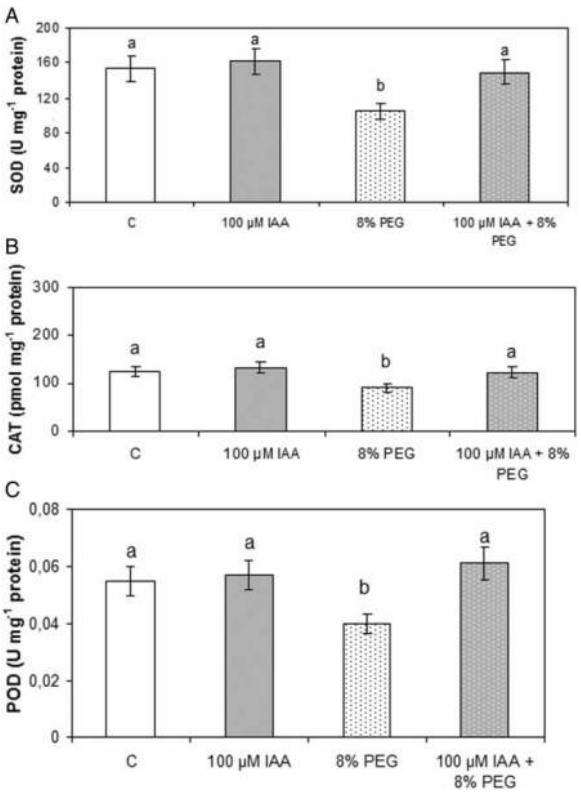

Figure 1 Effect of PEG and IAA pre-treatment on soybean roots SOD (A), CAT (B), and POD (C) activities. Experiments were performed according to the Material and methods section. Values are the mean of three independent experiments and bars indicate SE. Different letters indicate significant differences ($P < 0.05$) according to Tukey's multiple range test.

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 minutes and washed with distilled water four

times, and then they were pretreated with 100 μM IAA solution. Controls were performed in distilled water in the absence of the hormone. Control and treated seeds were maintained in the darkness for 48 hours. Afterwards, seeds were planted in vermiculite for 5 days in the presence of different IAA concentrations ranging from 0 to 100 μM. After 5 days of germination, plants were removed from pots; roots were gently washed and transferred to separated flasks containing PEG (MW20000) solutions ranging from 0 to 8% (w/v) for 72 hours in a controlled climate room at 24 ± 2°C and 50% relative humidity, with a photoperiod of 16 hours and a light intensity of 175 μmol/m^2/s. The hydroponics medium was Hoagland's nutrient solution. The medium was continuously aerated and replaced every 3 days. Roots were excised and used for determinations. To evaluate the effect of NO, seeds were pretreated with sodium nitroprusside (SNP), an NO donor, the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), or *N*-nitro-L-arginine methyl ester (L-NAME), an NO synthase (NOS) inhibitor, at 100, 100, and 200 μM final concentration, respectively, for 48 hours. When the effect of Zn-protoporphyrin IX (ZnPPIX, 20 μM) was investigated, it was added to the solutions in the presence or absence of IAA for 48 hours. Determinations were performed in triplicate in three different experiments employing six plants for each treatment.

Thiobarbituric acid reactive substances level

Lipid peroxidation was measured as the amount of thiobarbituric acid reactive substances (TBARS) determined by the thiobarbituric acid (TBA) reaction as described.[42] Fresh control and treated roots (0.3 g) were homogenized in 3 ml of 20% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 3500 *g* for 20 minutes. 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 in ethanol were added. The mixture was heated at 95°C for 30 minutes and then quickly cooled on ice. The contents were centrifuged at 10 000 *g* for 15 minutes and the absorbance was measured at 532 nm. Value for non-specific absorption at 600 nm was subtracted from the value at 532 nm. The concentration of TBARS was calculated using malondialdehyde extinction coefficient of 155 mM^{-1} cm^{-1}.

Glutathione determination

Non-protein thiols were extracted by homogenizing 0.3 g of roots in 3 ml of 0.1 N HCl (pH 2), 1 g polyvinylpyrrolidone (PVP).[43] After centrifugation at 10 000 *g* for 10 minutes at 4°C, the supernatants were used for analysis. Total glutathione content (GSH) was determined in the homogenates by spectrophotometry at 412 nm, using yeast-GR, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), and Nicotinamide adenine dinucleotide phosphate (NADPH). A calibration curve was performed using GSH (Sigma, Buenos Aires, Argentina) as a standard.

Ascorbate determination

Ascorbate (AS) was determined as described.[43] One gram of tissue was homogenized in 10% (w/v) TCA, and the supernatant was used for the assay. Sodium hydroxide (10 μl, 5 M) was added to 400 μl of extract, mixed, and the mixture was centrifuged for 2 minutes at 3500 *g*. To a 200 μl sample of the supernatant were added 200 μl of 150 mM NaH_2PO_4 buffer, pH 7.4, and 200 μl of water. To another 200 μl of supernatant, 200 μl of buffer and 100 μl of 10 mM dithiothreitol were added and, after thorough mixing and being left at room temperature for 15 minutes, 100 μl of 0.5% (w/v) *N*-ethylmaleimide was added. Samples were vortex-mixed and incubated at room temperature for 30 seconds. To each was then added 400 μl of 10% (w/v) TCA, 400 μl of 44% (v/v) H_3PO_4, 400 μl of 4% (w/v) bipyridyl in 70% (v/v) ethanol, and 200 μl of 3% (w/v) $FeCl_3$. After vortex mixing, samples were incubated at 37°C for 60 minutes and the absorbance at 525 was recorded. A standard curve of AS was used for calibration.

Antioxidant enzyme assay

Extracts for determination of superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), and guaiacol peroxidase (POD; EC 1.11.1.7) activities were prepared from 0.3 g of roots homogenized under ice-cold conditions in three of extraction buffers, 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 minutes 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 CAT activity in H_2O_2 pmol/mg protein was calculated using $k = 4.7 \times 10^7$ M^{-1} s^{-1}.[44] Total SOD activity was assayed by the inhibition of the photochemical reduction of Nitrotetrazolium Blue chloride (NBT), as described by Becana *et al.*[45] 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. Protein extracts were brought to a final volume of 0.3 ml with 50 mM potassium phosphate (pH 7.8) and 0.1 mM Na_2EDTA. Test tubes were shaken and placed 30 cm from light

bank consisting of six 15 W fluorescent lamps. The reaction was allowed to run for 10 minutes 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 the amount of enzyme which produced a 50% inhibition of NBT reduction under the assay conditions. POD activity was determined in the homogenates by measuring the increase in absorption at 470 nm due to the formation of tetraguaiacol (ε: 26.6 mM^{-1} cm^{-1}) in a reaction contained extract, 50 mM potassium phosphate buffer pH 7, 0.1 mM EDTA, 10 mM guaiacol, and 10 mM H_2O_2. One unit is defined as the amount of enzyme that catalyzes the formation of 1 μM of guaiacol per minute under the assay conditions.

HO preparation and assay

Roots (0.3 g) were homogenized in a Potter-Elvehjem homogenizer using four volumes 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 minutes and supernatant fractions were used for activity determination. HO activity was determined as previously described with minor modifications. The standard incubation mixture in a final volume of 500 ml contained 10 mM potassium phosphate buffer (pH 7.4), 60 nmol NADPH, 250 μl HO (0.5 mg protein), and 200 mM hemin. Incubations were carried out at 37°C during 60 minutes. The concentration of BV 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). One unit is defined as the amount of enzyme catalyzing the formation of 1 nmol of BV per 30 minutes under stander conditions.

DNA protein gel blot analysis for HO-1

Homogenates obtained for HO activity assay were also analyzed by DNA protein gel immunoblot technique. Forty milligrams of protein from root homogenates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis using a 12% acrylamide resolving gel (Mini Protean II System, BioRad, Hertz, UK), according to Laemmli (1970).[45] Separated proteins were then transferred to nitrocellulose membranes and non-specific binding of antibodies was blocked with 3% non-fat dried milk in phosphate-buffered saline, pH 7.4 for 1 hour at room temperature. Membranes were then incubated overnight at 4°C in primary antibodies raised against *Arabidopsis thaliana* HY-1[46] diluted 1:2000 in Tris-NaCl buffer plus 1% non-fat milk. Immune complexes were detected using alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G. The phosphatase-labeled antigens were visualized with the colorigenic substrate 5-bromo-4-chloro-3-indoleyl phosphate and nitroblue tetrazolium.

Real-time quantitative reverse transcription-polymerase chain reaction

Total RNA was isolated using Trizol reagent (Invitrogen, Buenos Aires, Argentina), treated with RNase-free DNase I (Promega, Buenos Aires, Argentina), and reverse-transcribed into cDNA using random hexamers and M-MLV Superscript II RT (Invitrogen). Quantitative reverse transcription-polymerase chain reaction (RT-PCR) reactions were carried out using *G. max HO-1* specific primers 5′-GATCCCCAAGCATTCAT TTG-3′ and 5′-TTTCTTCCAGACAGTGGTCC-3′, they were designed to amplify the 476–701 bp region of the *G. max* HO-1 cDNA (GenBank accession no. AF320024) and Power SYBR Green master mix (Applied Biosystems). Samples were assayed in triplicate on a 7900HT real-time PCR system (Applied Biosystems) with the following conditions: one cycle for 10 minutes at 95°C, 40 cycles with 95°C for 15 seconds, 58°C for 20 seconds, and 60°C for 40 seconds, followed by a melting curve analysis. Results were analyzed using the relative quantification ($\Delta\Delta C_t$) method. The threshold cycle (C_t) values were normalized against the reference gene *18S*, which has shown to be stable under several UV-B conditions.[48] The data were calculated using the formula $2^{-\Delta\Delta Ct}$ and are presented as the fold change in the relative steady-state level of specific mRNA normalized and relative to the untreated control.

Protein determination

Protein concentration was evaluated by the method of Bradford,[49] using bovine serum albumin as a standard.

Statistics

Values in the text, figures, and tables indicate mean values ± standard error (SE). Differences among treatments were analyzed by one-way analysis of variance, taking $P < 0.05$ as significant according to Tukey's multiple range test.

Table 1 Effect of PEG and IAA pre-treatments on GSH and AS content in soybean roots

Treatment	GSH (nmol/mg FW)	AS (μmol/mg FW)
Control	245 ± 20[a]	0.21 ± 0.03[a]
100 μM IAA	249 ± 18[a]	0.23 ± 0.02[a]
8% PEG	167 ± 12[b]	0.15 ± 0.02[b]
100 μM IAA + 8% PEG	242 ± 19[a]	0.24 ±0.04[a]

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

Results

TBARS level

Taking into account the fact that TBARS content is a good indicator of cell membrane damage, it was determined in plants subjected to different polyethylene glycol (PEG) concentrations ranging from 0 to 8% (w/v).

On one hand, PEG enhanced this parameter in a dose-depending manner. Fig. 2A shows that 2 and 4% PEG provoked an increase by about 40% while 8% PEG enhanced TBARS levels by 87%, with respect to controls. Considering these results, 8% PEG was chosen to perform further experiments. On the other hand, different IAA concentrations ranging from 0 to 100 μM did not modify TBARS levels with respect to controls (data not shown). Taking into account this fact, pre-treatments with different IAA concentrations were performed before 8% PEG application. Fig. 2B indicates that only 100 μM IAA was capable of preventing TBARS enhancement caused by 8% PEG. Based on these results, 8% PEG and 100 μM IAA were used to perform further experiments.

AS and GSH levels

It could therefore be expected that if drought induces ROS formation, it would also modify GSH levels. The data in Table 1 indicate that GSH concentration was not affected by IAA application. Plants subjected to drought showed a 32% decrease in GSH levels with respect to controls. Pre-treatment with IAA prevented the GSH decrease produced by 8% PEG. Similar results were obtained when AS content was assayed.

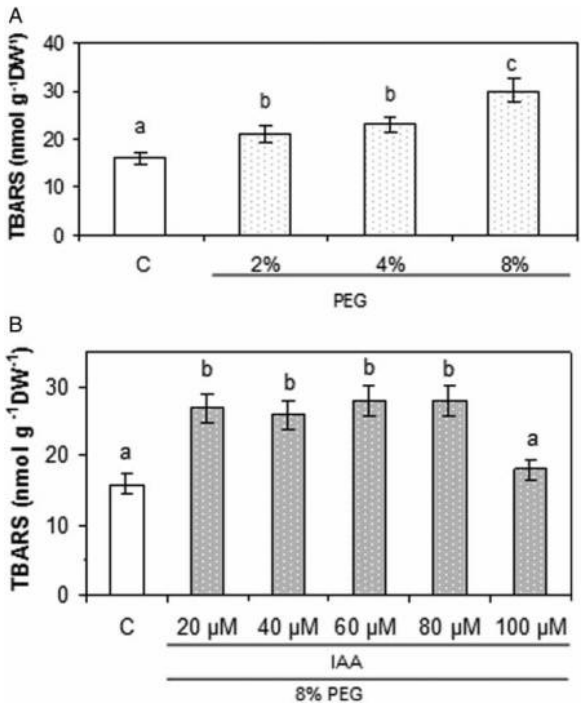

Figure 2 Lipid peroxidation evaluated as TBARS formation in soybean roots exposed to (A) different PEG concentrations and (B) pre-treatments with different IAA concentrations and addition of 8% PEG. Experiments were performed as described in the Materials and methods section. Values are the mean of three independent experiments and bars indicate SE. Different letters indicate significant differences ($P < 0.05$) according to Tukey's multiple range test.

Antioxidant enzyme system

As shown in Fig. 1A, IAA did not affect SOD activity while drought brought about a 33% inhibition with respect to controls. Pre-treatment with IAA totally prevented the SOD activity inhibition caused by PEG treatment.

Fig. 1B shows that on one hand, IAA did not affect CAT activity and, on the other hand, PEG caused a 26% inhibition with respect to controls. Moreover, pre-treatment with IAA before drought avoided the decrease previously observed in CAT activity. This behavior is similar to that obtained with SOD activity.

As can been see in Fig. 1C, IAA did not affect POD activity and PEG treatment provoked a 27% decrease in the enzyme activity. Once again, IAA pre-treatment counteracted this effect and the enzyme activity approached control levels.

HO activity, protein content, and RNA expression

The data indicate that drought did not affect HO-1 activity (Fig. 3A), nor the protein levels or relative steady-state level of specific mRNA (Fig. 3B and C). IAA treatment enhanced HO-1 activity with respect to controls (75%). Plants pretreated with IAA and then subjected to drought showed an enhancement of HO-1 activity (43%) with respect to plants treated with IAA alone. These results prompted us to investigate the behavior of HO-1 as a consequence of IAA treatment.

As already stated, HO-1 has been described as a feature of plant responses to stress conditions. To asses whether HO-1 is involved in the protection exerted by 100 μM IAA against drought, experiments were carried out in plants treated with ZnPPIX, a well-known irreversible HO-1 inhibitor, in the presence of 100 μM IAA alone and in plants pretreated with 100 μM IAA before 8% PEG addition. When HO-1 was inhibited, enhancement of TBARS levels (142%, with respect to controls) as well as a diminution of GSH (50%, with respect to controls) was observed (Table 2). In the presence of ZnPPIX, the above-mentioned protective role of IAA was not observed (Fig. 2, Tables 1 and 2). Similar results were obtained when the effects of HO-1 inhibition on SOD and CAT activities were assayed (Fig. 1, Table 3). Therefore, we can assume that protection exerted by 100 μM IAA may also be due to the increase of HO activity.

On the other hand, DNA protein gel blot analysis for HO-1 as well as RT-PCR analysis (Fig. 3B and

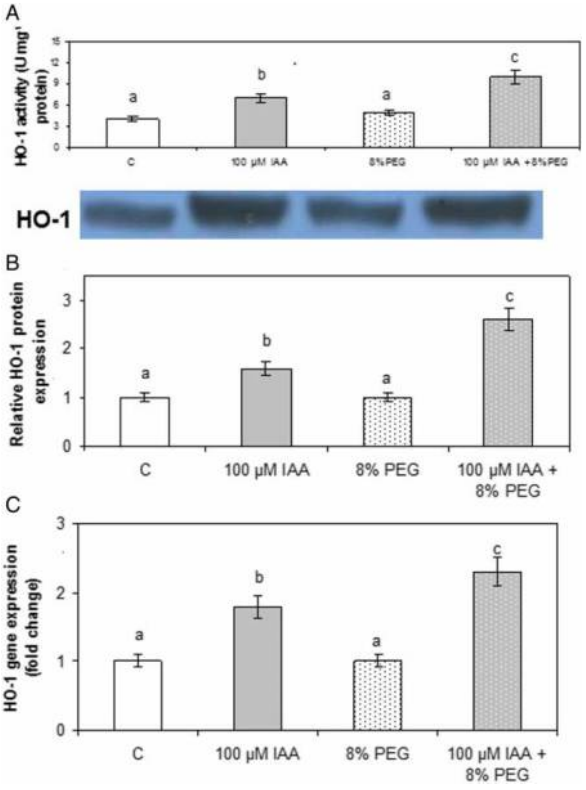

Figure 3 Effect of PEG and IAA pre-treatments on soybean roots HO activity (A), protein content (B), and gene expression (C). (A) Enzyme activity was assayed as described in the Materials and methods section. (B) HO-1 protein expression was analyzed by DNA protein gel blot as described in the Material and methods section. Densitometry was done by Gel-Pro® analyzer to quantify HO-1 protein expression. (C) HO-1 mRNA expression was analyzed by real-time RT-PCR as described in the Material and methods section. The 18S amplification band is shown to confirm equal loading of RNA and RT efficiency. Relative HO-1 transcript expression taking control as 1 U. Data are means of three independent experiments and bars indicate SE. Different letters indicate significant differences ($P < 0.05$) according to Tukey's multiple range test.

C) revealed a positive relationship between enzyme activity, protein content, and transcript levels. These results indicated that the enhancement of HO-1 activity was due to an increase in gene transcription.

Effect of NO on HO-1 activity

Plants were treated with water (C), IAA, or the NO-donor SNP either in the presence or the absence of cPTIO (100 µM) or L-NAME (200 µM), a specific NO scavenger and inhibitor of NO synthesis, respectively. Treatments with IAA in the presence of cPTIO or L-NAME reduced by 50% the HO-1 activity, indicating that NO is involved in the enhancement of HO-1 activity induced by IAA (Fig. 4). This result is confirmed by the fact that SNP alone also produced a 75% increase in HO-1 activity. In addition, plants subjected to SNP in the presence of cPTIO or L-NAME exhibited a significant reduction in HO-1 activity with respect to plants treated with SNP alone. On the other hand, cPTIO or L-NAME significantly decreased HO-1 activity (50%).

Discussion

It has been previously demonstrated that IAA action as well as HO-1 activity and gene expression are mediated by NO,[50,51] but to the best of our knowledge no information is available with respect to the role that this hormone could have in the defense system against drought in soybean plants. In this paper we present, for the first time, evidence that IAA contributes to the defense against oxidative stress generation in soybean plants subjected to a simulated drought, and that this effect is mediated by HO-1 activity, which in turn is modulated by NO.

Roots are very sensitive to IAA fluctuations and their response to increasing amounts of exogenous IAA has been described.[52] This is why this tissue has been chosen for this study. In this drought model, the non-enzymatic as well as the enzymatic defense systems are altered by drought and pre-treatment with IAA prevents these alterations.

Plants develop non-enzymatic and enzymatic antioxidants to reduce ROS-induced oxidative stress, which is indirectly induced by drought.[53] Taking into account the fact that the AS–GSH cycle plays an important role in the defense against ROS production, AS as well as GSH content were analyzed. GSH is a leading substrate for enzymatic antioxidant functions and it is also, together with AS, a known radical scavenger. In this work, drought treatment provokes a significant increase in TBARS levels (Fig. 2) and important decreases of soluble antioxidant compounds such as GSH and AS (Table 1). These alterations indicate that oxidative stress occurred.

Under unstressed conditions, the production as well as the removal of O_2^- are in balance. However, under stress ROS formation can be overwhelming. Within a

Table 2 Effect of ZnPPIX on GSH and TBARS content in soybean roots

Treatment	GSH (nmol/mg FW)		TBARS (nmol/mg DW)	
	Without ZnPPIX	With ZnPPIX	Without ZnPPIX	With ZnPPIX
100 µM IAA + 0% PEG	245 ± 20[a]	241 ± 12[a]	16.6 ± 0.9[a]	18.6 ± 0.3[a]
100 µM IAA + 8% PEG	242 ± 18[a]	121 ± 11[b]	17.5 ± 1.1[a]	45.1 ± 14.3[b]

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

Table 3 Effect of ZnPPIX on SOD and CAT activities in soybean roots

Treatment	SOD (U/mg protein)		CAT (pmol/mg protein)	
	Without ZnPPIX	With ZnPPIX	Without ZnPPIX	With ZnPPIX
100 μM IAA + 0% PEG	162 ± 20[a]	170 ± 12[a]	132.6 ± 10.8[a]	130.7 ± 11.3[a]
100 μM IAA + 8% PEG	160 ± 18[a]	95 ± 11[b]	122.5 ± 10.1[a]	105.1 ± 10.2[b]

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

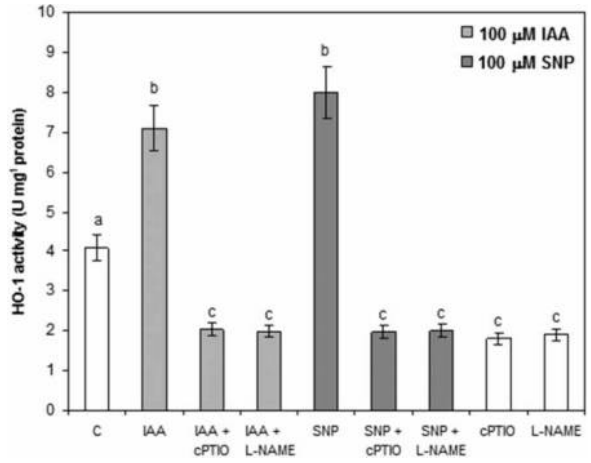

Figure 4 Effect of NO on HO-1 activity. Experiments were performed according to the Materials and methods section. Seeds were pretreated with IAA, SNP, NO donor, the NO scavenger cPTIO, or L-NAME, a NOS inhibitor, at 100, 100, or 200 μM final concentrations, respectively. Values are the mean of three independent experiments and bars indicate SE. Different letters indicate significant differences ($P < 0.05$) according to Tukey's multiple range test.

cell, the SODs constitute the first line of defense against O_2^-. Hydrogen peroxide is an important signal molecule involved in plant development and environmental responses. Changes in H_2O_2 availability can result from increased production or decreased metabolism. While plants contain several types of H_2O_2-metabolizing proteins, CATs are highly active enzymes that do not require cellular reductants as they directly catalyze a dismutase reaction. On the other hand, as an adaptive enzyme in the antioxidant system, POD has an important role in maintaining the redox state in the cells. Drought conditions diminished CAT, SOD, and POD activities with respect to non-stressed plants. These results strongly indicate that drought induced ROS production and oxidative stress. Nevertheless, pre-treatment with 100 μM IAA completely prevented these effects and kept the activities of SOD, CAT, and POD at control levels (Figs. 1 and 2, Table 1). These antioxidant enzymes might work cooperatively to eliminate the excess of ROS and to counteract drought-induced oxidative damage.

The protective role that HO-1 plays against oxidative stress caused by different stimuli is well documented.[18,19,53] When HO-1 was studied, it was observed that drought did not affect its activity, protein content, or relative steady-state level of specific mRNA. Moreover, 100 μM IAA increased HO-1 activity by 75% with respect to controls, and a significant increase (150%, with respect to controls) was observed in plants pretreated with 100 μM IAA and then subjected to drought stress. This enhancement was positively correlated with protein amount and gene expression (Fig. 3). Organisms tend to up-regulate the synthesis of heat shock proteins and to activate antioxidant enzymes under adverse environmental stresses. HO-1 induction enhances protection of normal cell functioning by yielding the potent antioxidant BV.[18] Therefore, it is assumed that as consequence of IAA treatment, an enhancement of HO-1 occurred to protect the plant against the oxidative damage. Experiments carried out in the presence of ZnPPIX clearly revealed the involvement of HO-1 in the protective action exerted by IAA (Tables 2 and 3).

It was demonstrated that NO is engaged in the signaling pathway of many phytohormones, for instance, the auxin response during adventitious root formation in cucumber[50] and soon after it was shown that a NO-mediated cGMP-dependent pathway is operating in that process.[51] Furthermore, accumulating evidence has shown that NO plays an important role in signaling plant responses to biotic and abiotic stresses[41,54] and it is also involved in the induction of HO-1 in soybean plants treated with UV-B.[50] However, it is worth pointing out that relevant information on the role of NO in dehydration/drought tolerance in soybean plants is relatively insufficient. In this study, we wanted to elucidate whether IAA action on HO-1 could be mediated by NO. Experiments performed in the presence of SNP or NO inhibitors, such as cPTIO or L-NAME, indicated that NO acts as a messenger in the cell response against drought. The enhancement of HO-1 activity observed in the presence of IAA, diminished when plants were pretreated with NO inhibitors. Moreover, treatment with SNP alone considerably increased HO-1 activity (Fig. 4). These data provide compelling evidence that IAA serves as an important molecule for signaling the dehydration/drought response in soybean, as has been found in other plants.[55]

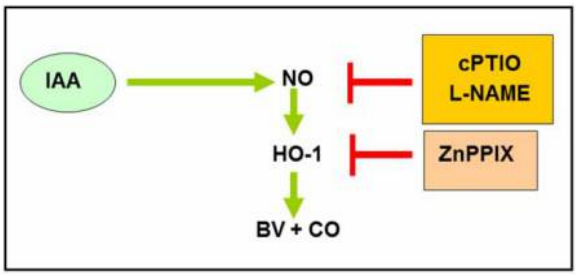

Figure 5 Possible mechanism of IAA action on HO-1. NO mediates HO-1 induction caused by IAA. IAA, indole acetic acid; L-NAME, NO synthesis inhibitor; cPTIO, NO scavenger; ZnPPIX, Zn-protoporphyrin IX; BV, biliverdin; NO, nitric oxide; CO, carbon monoxide.

Despite the fact that HO-1 is not affected by drought, an enhancement in its activity, protein amount, and gene expression was observed in IAA-treated plants. Surprisingly, an even more pronounced increase was obtained in plants pretreated with IAA prior to drought with respect to plants treated with IAA alone. The results obtained in the experiments performed with NO donors or inhibitors clearly indicate that, in this case, the enhancement of HO-1 activity is mediated by NO.

Summing up, a possible mechanism can be proposed to explain these effects (Fig. 5). IAA modules NO levels which, in turn, enhances HO-1 synthesis and activity. This increase in HO-1 activity raised BV and CO production. On one hand, BV is a potent antioxidant[16] that could be, at least in part, responsible for the observed protective role against drought. On the other hand, it also has been demonstrated that CO provides potent cytoprotective effects in plants.[56,57] Taken together, these results indicate that application of IAA to soybean seeds, previous to sowing, could be an interesting and novel strategy to improve soybean crop yield.

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