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Nitric oxide synthase-like dependent NO production enhances heme oxygenase up-regulation in ultraviolet-B-irradiated soybean plants

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ABSTRACT

Heme oxygenase (HO) has antioxidant properties and is up-regulated by reactive oxygen species (ROS) in ultraviolet-B-irradiated soybean plants. This study shows that nitric oxide (NO) protects against oxidative damage and that nitric oxide synthase (NOS)-like activity is also required for *HO-1* induction under UV-B radiation. Pre-treatments with sodium nitroprussiate (SNP), a NO-donor, prevented chlorophyll loss, H_2O_2 and $O_2^{\cdot-}$ accumulation, and ion leakage in UV-B-treated plants. HO activity was significantly enhanced by NO and showed a positive correlation with *HO-1* transcript levels. In fact, *HO-1* mRNA levels were increased 2.1-fold in 0.8 mM SNP-treated plants, whereas subsequent UV-B irradiation augmented this expression up to 3.5-fold with respect to controls. This response was not observed using ferrocyanide, a SNP inactive analog, and was effectively blocked by 2-(4-carboxyphenil)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), a specific NO-scavenger. In addition, experiments carried out in the presence of N^G-nitro-L-arginine methyl ester (L-NAME) or tungsten, well-known inhibitors of NOS and nitrate reductase, showed that NOS is the endogenous source of NO that mediates *HO-1* expression. In summary, we found that NO is involved in the signaling pathway leading to *HO-1* up-regulation under UV-B, and that a balance between NO and ROS is important to trigger the antioxidant response against oxidative stress.

1. Introduction

Nitric oxide (NO) acts as a signaling molecule and mediates multiple physiological processes in plants (Leitner et al., 2009). In addition, it has been implicated in responses to biotic and abiotic stresses, such as disease resistance, salinity, drought, heat stress, among others (Beligni and Lamattina, 1999; Romero-Puertas et al., 2004; Zhao et al., 2007; Corpas et al., 2008). There are several sources of NO in plants, but mainly it can be enzymatically produced by nitrate reductase (NR) and nitric oxide synthase-like enzymes (NOS) (Wilson et al., 2008; Corpas et al., 2009). NO is a reactive nitrogen species and, depending on its concentration, it produces either protective or toxic effects. A low dose of NO modulates superoxide anion formation and inhibits lipid peroxidation, resulting in an antioxidant function during stress (Boveris et al., 2000). Moreover, microarray studies have shown that NO induces a large number of genes at transcriptional level, among them those of antioxidant enzymes (Parani et al., 2004). It has also been reported that NO gives rise to signaling pathways mediating responses of specific genes to ultraviolet-B (UV-B) radiation, such as chalcone synthase and phenylalanine ammonia lyase (A.-H.-Mackerness et al., 2001). However, information about the role that NO plays in regulation of antioxidant enzymes to counteract UV-B-induced oxidative stress is rather limited.

Ultraviolet-B radiation (280–320 nm) has gained a lot of interest in the past decades due to the depletion of the stratospheric ozone layer that increases the solar radiation reaching the earth's surface (Rowland, 2006). The deleterious effects of UV-B on plants include decreased biomass formation, reduced photosynthesis, impaired chloroplast function, damage to DNA, among others (Frohnmeyer and Staiger, 2003). UV-B exposure has also shown to increase reactive oxygen species (ROS) leading to oxidative stress (A.-H.-Mackerness et al., 2001). Heme oxygenase (HO, 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 (BV) and carbon monoxide (CO) (Kikuchi et al., 2005). We have previously shown that HO is induced in plant tissues as a result of cadmium treatment and confers protection against oxidative stress (Noriega et al., 2004; Balestrasse et al., 2005). Moreover, we have recently demonstrated that ROS are involved in *HO-1* up-regulation in soybean leaves subjected to UV-B radiation (Yannarelli et al., 2006). We hypothesized that NO may also participate in this process, as it regulates the oxidative status

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and mediates other UV-B responses. The aim of the present study was to investigate whether NO could protect soybean leaves against UV-B-induced oxidative stress through the modulation of HO activity. Soybean plants were subjected to UV-B radiation after pre-treatments with different concentrations of sodium nitroprussiate (SNP), a well-characterized NO-donor. Overall, our results indicate that NO is involved in the signaling pathway leading to *HO-1* up-regulation under UV-B, and that a balance between NO and ROS is important to trigger the antioxidant response against oxidative stress. In addition, experiments carried out using specific inhibitors under low UV-B doses showed that NOS, but not NR, is the endogenous source of NO required to mediate this response.

2. Results

2.1. Nitric oxide prevents chlorophyll loss in UV-B-treated plants

Chlorophyll can be bleached by oxidative stress, and it is particularly susceptible to UV-B radiation. Chlorophyll content significantly decreased (17%) in leaves of UV-B-treated plants with respect to controls (Fig. 1A). To examine whether NO could alleviate this damage, soybean plants were pretreated with different SNP concentrations. Experiments performed in the presence of 0.8 or 1.2 mM SNP indicated that these concentrations totally prevented chlorophyll loss against UV-B insult (Fig. 1A). Chlorophyll content decreased only 6% using 1.6 mM SNP, whereas 0.4 mM SNP did not show any protection (16% decrease compared with controls). Pre-treatments with FeCN, an inactive analog of SNP, failed to avoid chlorophyll loss indicating that NO released by SNP is involved (Fig. 1B). To further clarify the role of NO in preventing UV-B-induced oxidative damage, the NO-scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) was used. As it can be seen in Fig. 1B, the protective effect of 0.8 mM SNP could be negated by addition of cPTIO. Moreover, cPTIO treatment increased chlorophyll loss in UV-B-irradiated plants.

2.2. Nitric oxide protects soybean leaves from UV-B-induced ion leakage

Ion leakage is a marker of membrane injury as a consequence of an oxidative insult. Ultraviolet-B radiation increased ion leakage by 34% respect to controls (Fig. 2A). When 0.4 and 1.6 mM SNP doses were used, they had no effect or partially protected leaves with respect to UV-B-treated plants (32% and 23% enhancement compared to controls, respectively). Pre-treatments with 0.8 and 1.2 mM SNP fully protected soybean leaves against UV-B-induced ion leakage.

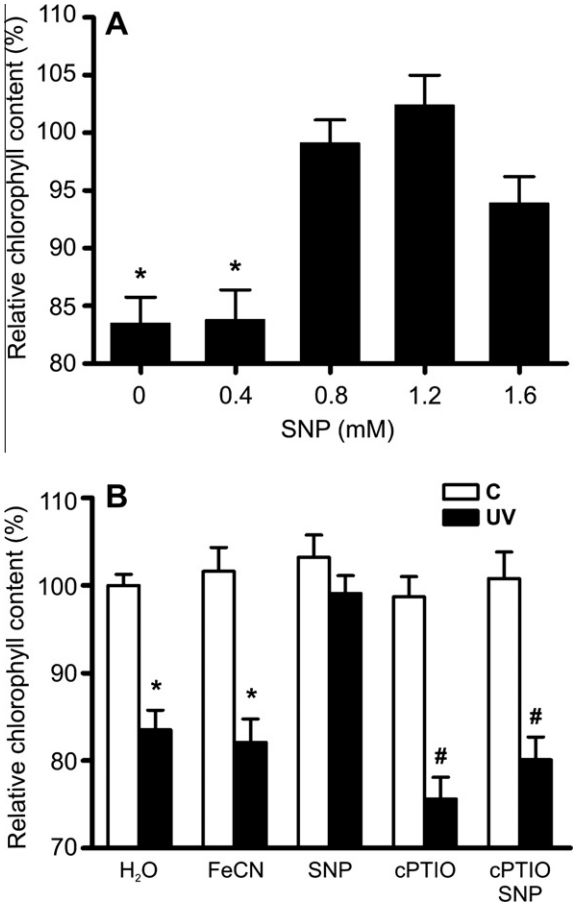

Fig. 1. Effect of NO on total chlorophyll content in soybean leaves subjected to 30 kJ m^{-2} UV-B. (A) Plants were pretreated with different SNP concentrations ranging from 0 to 1.6 mM prior to UV-B irradiation. Control was considered as 100% of chlorophyll content. (B) The NO-scavenger cPTIO (100 μM) negated the protection exerted by 0.8 mM SNP on chlorophyll loss and increased the damage in UV-B-treated plants. Potassium ferrocyanide (FeCN) 0.8 mM was used as a control for the SNP treatment. Values are the mean of four independent experiments and bars indicate SE. $^{*}P < 0.01$, $^{\#}P < 0.001$ compared to control according to Tukey's multiple range test.

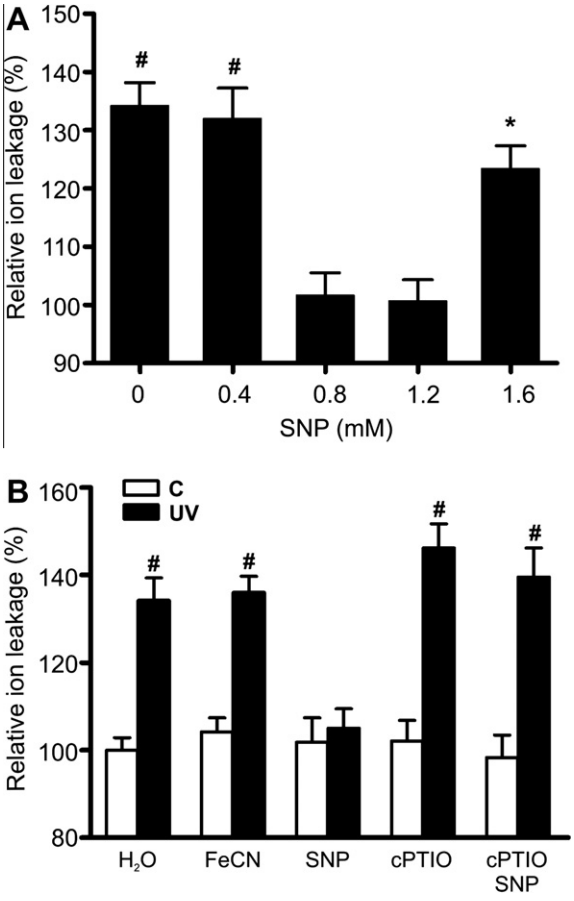

Fig. 2. Effect of NO on ion leakage in soybean leaves subjected to 30 kJ m^{-2} UV-B. (A) Plants were pretreated with different SNP concentrations ranging from 0 to 1.6 mM prior to UV-B irradiation. Control was considered as 100% of ion leakage. (B) cPTIO 100 μM negated the protection exerted by 0.8 mM SNP and augmented ion leakage in UV-B-treated plants. FeCN 0.8 mM was used as a control for the SNP treatment. Data are means of four independent experiments and bars indicate SE. $^{*}P < 0.001$ compared to control according to Tukey's multiple range test.

As shown in Fig. 2B, the effect of 0.8 mM SNP could be negated by the addition of cPTIO. Treatments with cPTIO + UV-B showed an increased ion leakage when compared with UV-B alone.

2.3. Effect of NO on H_2O_2 and $O_2^{\cdot-}$ content in UV-B-irradiated soybean leaves

To evaluate the effect of NO in UV-B-induced oxidative stress, H_2O_2 and $O_2^{\cdot-}$ contents were determined. As shown in Table 1, UV-B radiation significantly increased H_2O_2 and $O_2^{\cdot-}$ levels in soybean leaves (2.7 and 4.3-fold compared to controls, respectively). Pre-treatments with 0.4 mM SNP were not able to prevent ROS accumulation, whereas a mild protection was observed using 1.6 mM SNP (2-fold increase respect to controls). Hydrogen peroxide and $O_2^{\cdot-}$ concentrations were significantly diminished (49% and 57%, respectively) in 0.8 and 1.2 mM SNP pre-treatments with respect to UV-B-treated plants (Table 1). Nevertheless, these SNP doses only showed a partial protection as indicated by augmented ROS levels in comparison to controls. Pre-treatment with 0.8 mM FeCN had no effect and H_2O_2 and $O_2^{\cdot-}$ contents were similar to those found for UV-B-irradiated plants (data not shown).

2.4. Nitric oxide modulates HO-1 gene expression under UV-B radiation

We subsequently analyzed the effect of different SNP concentrations on *HO-1* gene expression under UV-B radiation. Realtime RT-PCR indicated that *HO-1* transcripts levels significantly decreased by 79.3% in leaves subjected to 30 kJ m^{-2} UV-B with respect to controls (Fig. 3). Pre-treatment with 0.4 mM SNP did not prevent *HO-1* down-regulation (75.4% decrease compared to control), whereas 1.6 mM SNP only showed an ~18.0% decrease compared to control. Interestingly, treatments with 8.0 and 1.2 mM SNP enhanced *HO-1* transcript levels by about 3.5-fold after UV-B irradiation. The expression of the constitutive gene (*18S*) was unaffected throughout all experiments (data not shown). To address whether NO alone could up-regulate *HO-1*, a set of experiments was carried out using the lower protective SNP dose (0.8 mM) in the presence or absence of UV-B radiation. We found that *HO-1* mRNA levels were increased 2.1-fold in SNP-treated plants, whereas subsequent UV-B irradiation augmented this expression up to 3.5-fold with respect to controls (Fig. 4). No changes in *HO-1* transcript levels nor protection against its down-regulation by UV-B were observed using FeCN. *HO-1* up-regulation could also be blocked by the addition of cPTIO, indicating that NO released by SNP mediates this phenomenon (Fig. 4).

2.5. Effect of NO on antioxidant enzyme activities

We also investigated whether NO can modulate the activities of classical antioxidant enzymes such as catalase (CAT) and ascorbate peroxidase (APX). These are the main H_2O_2-scavenging enzymes that control ROS-mediated responses under biotic and abiotic stresses (Mittler, 2002). CAT and APX activities were significantly affected by 30 kJ m^{-2} UV-B irradiation (Table 2). In contrast, they were increased by 90% and 107% in 0.8 mM SNP-treated plants compared to controls, respectively. Moreover, CAT activity significantly augmented up to 121% with respect to controls after irradiation of SNP-treated plants, whereas APX only showed a mild increase (from 107% to 114%, P = n.s.). Heme oxygenase behavior was similar to that found for CAT and showed a positive correlation with *HO-1* gene expression data (Table 2). The presence of cPTIO negated the protection conferred by SNP and produced a mild decrease on the antioxidant activity of UV-B-treated plants in comparison with UV-B alone (Table 2).

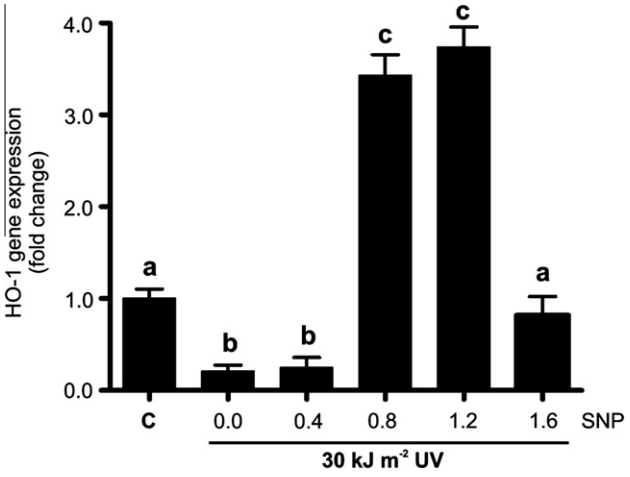

Fig. 3. Effect of different NO concentrations on *HO-1* gene expression in soybean leaves subjected to 30 kJ m^{-2} UV-B. Plants were pretreated with SNP doses ranging from 0 to 1.6 mM 12 h before UV-B irradiation. Quantitative *HO-1* expression was determined using realtime RT-PCR. Transcript level of untreated plants was normalized against *18S* and expressed as 1. 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.

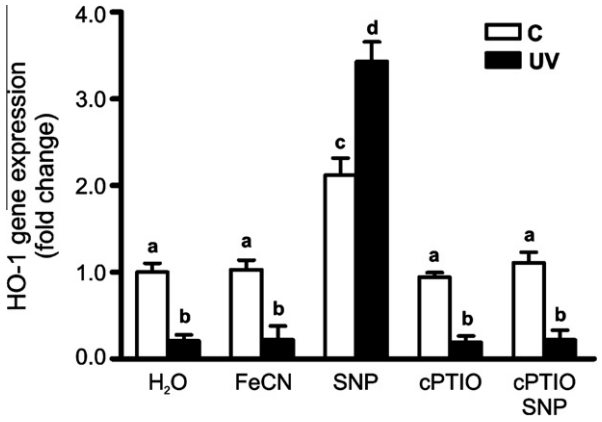

Fig. 4. NO enhances *HO-1* expression and protects against its down-regulation by 30 kJ m^{-2} UV-B-irradiation. Experiments were performed by spraying plants with 0.8 mM SNP 12 h before UV-B irradiation (UV) or control treatment (*C*). FeCN 0.8 mM was used as a control for the SNP treatment. cPTIO or the combined treatment with cPTIO + SNP was used to specifically block the response given by NO. Quantitative *HO-1* expression was determined by realtime RT-PCR. Transcript level of untreated plants was normalized against *18S* and expressed as 1. Data are means of four independent experiments and bars indicate SE. Different letters indicate significant differences ($P < 0.05$) according to Tukey's multiple range test.

Table 1
Effect of NO on H_2O_2 and $O_2^{\cdot-}$ contents in leaves of soybean plants subjected to 30 kJ m^{-2} UV-B.

Treatment	H_2O_2 (μM)	$O_2^{\cdot-}$ (μM)
Control	0.23 ± 0.05^a	0.11 ± 0.02^a
UV	0.61 ± 0.12^b	0.47 ± 0.06^b
0.4 mM SNP + UV	0.55 ± 0.10^b	0.45 ± 0.05^b
0.8 mM SNP + UV	0.34 ± 0.09^c	0.20 ± 0.04^c
1.2 mM SNP + UV	0.29 ± 0.07^c	0.19 ± 0.04^c
1.6 mM SNP + UV	0.43 ± 0.09^d	0.22 ± 0.05^c

Values are mean of three independent experiments ± SE. Plants were pretreated with different sodium nitroprussiate (SNP) concentrations 12 h before UV-B irradiation. Different letters indicate significant differences ($P < 0.05$) according to Tukey's multiple range test.

Table 2
Effect of NO on antioxidant enzyme activities in leaves of soybean plants subjected to 30 kJ m^{-2} UV-B.

Treatment	CAT (pmol mg^{-1} protein)	APX (U mg^{-1} protein)	HO (U mg^{-1} protein)
Control	0.70 ± 0.08[a]	139 ± 11[a]	0.148 ± 0.027[a]
SNP	1.33 ± 0.10[b]	288 ± 20[b]	0.219 ± 0.036[b]
cPTIO	0.68 ± 0.10[a]	144 ± 15[a]	0.135 ± 0.034[a]
UV	0.33 ± 0.09[c]	88 ± 10[c]	ND
SNP + UV	1.55 ± 0.14[d]	297 ± 22[b]	0.271 ± 0.031[c]
cPTIO + UV	0.24 ± 0.12[c]	80 ± 14[c]	ND
cPTIO + SNP + UV	0.27 ± 0.10[c]	85 ± 9[c]	ND

Values are mean of three independent experiments ± SE. Plants were treated with SNP (0.8 mM) and/or cPTIO (100 μM) 12 h before UV-B irradiation. One unit of APX forms 1 nmol of oxidized ascorbate in 30 min under assay conditions. One unit of HO forms 1 nmol of biliverdin in 30 min under assay conditions. Different letters indicate significant differences ($P < 0.05$) according to Tukey's multiple range test. Abbreviations: APX, ascorbate peroxidase; CAT, catalase; cPTIO, 2-(4-carboxyphenil)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; HO, heme oxygenase; ND, not detectable; SNP, sodium nitroprussiate.

2.6. NOS-mediated NO synthesis is involved in HO-1 up-regulation by a low UV-B dose

We have previously shown that ROS mediates *HO-1* up-regulation after irradiation with 7.5 kJ m^{-2} UV-B (Yannarelli et al., 2006). We now used this model to determine whether endogenous NO is also involved in this process. As shown in Fig. 5, *HO-1* mRNA levels were increased 2.74-fold in UV-B-irradiated plants with respect to controls. This response can be blocked by pre-treatment with cPTIO, demonstrating that endogenous NO production is required. To characterize the source of NO, experiments were carried out in the presence of N^G-nitro-L-arginine methyl ester (L-NAME) or tungsten, well-known inhibitors of NOS and NR, respectively. L-NAME, but not tungsten, significantly reduced UV-B-induced HO-1 up-regulation (Fig. 5). Moreover, an excess of L-arginine was able to reverse the blocking effect of L-NAME indicating that a NOS-like enzyme is implicated. We also assessed NR activity to confirm that a significant inhibition of this enzyme was achieved in our experimental conditions. We found that NR activity decreased 72.3% in plants subjected to 7 days of tungstate treatment with respect to controls (41.4 ± 3.6 vs. 149.4 ± 6.7 nmol g^{-1} PF min^{-1}; $P < 0.001$). These data demonstrate that NR-produced NO is unlikely to be involved, and suggest that a NOS-like mechanism is the endogenous source of NO required to mediate *HO-1* expression under low doses of UV-B radiation.

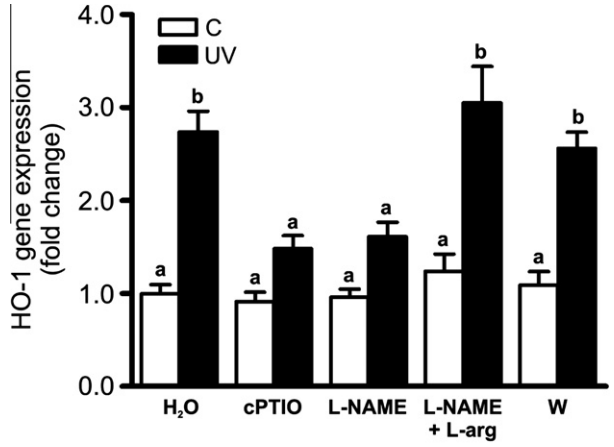

Fig. 5. NOS-generated NO mediates *HO-1* up-regulation after irradiation with a low UV-B dose (7.5 kJ m^{-2}). Experiments were performed by treating plants with different inhibitors before UV-B irradiation (UV) or control treatment (C). The NO-scavenger, cPTIO (100 μM), was able to block UV-B-induced *HO-1* up-regulation. The source of NO involved was determined by inhibition of NOS or NR using L-NAME (100 mM) and tungsten (W, 150 μM), respectively. L-Arginine (200 mM) was co-administered with L-NAME to specifically reverse the blocking effect of the latter. Quantitative *HO-1* expression was determined using realtime RT-PCR. Transcript level of untreated plants was normalized against *18S* and expressed as 1. Data are means of four independent experiments and bars indicate SE. Different letters indicate significant differences ($P < 0.05$) according to Tukey's multiple range test.

Table 3
Effect of ZnPPIX and BV on HO activity and TBARS content in leaf discs irradiated with a low dose of UV-B (7.5 kJ m^{-2}).

Treatment	HO (U mg^{-1} protein)	TBARS (nmol g^{-1} FW)
Control	0.144 ± 0.019[a]	24.2 ± 1.1[a]
UV	0.220 ± 0.012[b]	32.6 ± 1.4[b]
ZnPPIX	0.056 ± 0.008[c]	23.1 ± 1.3[a]
ZnPPIX + UV	0.061 ± 0.010[c]	45.0 ± 2.7[c]
BV	0.148 ± 0.014[a]	22.9 ± 1.3[a]
BV + UV	0.154 ± 0.015[a]	25.4 ± 1.5[a]

Values are mean of five independent experiments ± SE. Leaf discs were pretreated with 10 μM BV or 25 μM ZnPPIX for 4 h prior to UV-B irradiation (7.5 kJ m^{-2}). One unit of HO forms 1 nmol of biliverdin in 30 min under assay conditions. Different letters indicate significant differences ($P < 0.05$) according to Tukey's multiple range test. Abbreviations: BV, biliverdin IX; FW, fresh weight; HO, heme oxygenase; TBARS, thiobarbituric acid reactive substances; UV-B, ultraviolet-B; ZnPPIX, Zn-protoporphyrin IX.

To evaluate whether this response effectively protects against UV-B stress, HO activity was inhibited *in vitro* using Zn-protoporphyrin IX (ZnPPIX). As shown in Table 3, ZnPPIX pre-treatment produced a decrease of 60% in HO activity and negated HO enhancement after a low dose of UV-B (7.5 kJ m^{-2}). Thiobarbituric acid reactive substances (TBARS) formation is a reliable indicator of tissular free radical generation, and was assayed to assess UV-B-induced oxidative stress. TBARS content increased after UV-B irradiation (35%), but it was significantly higher when the HO response was inhibited in ZnPPIX-treated samples (86%) indicating that HO protects against oxidative stress. Moreover, incubation with the HO product biliverdin IXα (BV) prevented both the enhancement of HO activity and the increase of TBARS content under UV-B, demonstrating the antioxidant effect of this molecule (Table 3).

3. Discussion

In a previous work, we showed that ROS mediates HO up-regulation by low UV-B doses protecting soybean leaves, whereas irradiation with 30 kJ m^{-2} produce an oxidative insult that overwhelmed the antioxidant defence system (Yannarelli et al., 2006). More recently, we found that SNP pre-treatment ameliorates Cd-induced oxidative stress and modulates *HO-1* gene expression (Noriega et al., 2007). Taking into account the fact that NO is involved in various UV-B signaling pathways, in the present study we evaluated whether this molecule could enhance HO activity conferring a major protection against this physical stress.

Our data demonstrated that, depending on its concentration, NO can improve the plant antioxidant response against high levels of UV-B (30 kJ m^{-2}). Under this condition, the changes observed were probably related to the oxidative damage and general stress signals rather than UV-B-specific pathways (Brosche and Strid, 2003). However, this model was appropriate to determine the beneficial effect of exogenously added NO. While the lower dose of SNP did not reduce the oxidative damage, the application of 1.6 mM SNP only showed a mild protection suggesting a pro-oxidant effect of NO at this higher concentration. The pre-treatments with 0.8 and 1.2 mM SNP effectively ameliorated UV-B-induced oxidative stress, as indicated by the decrease in H_2O_2 and $O_2^{\cdot-}$ formation (Table 1), preventing chlorophyll loss (Fig. 1) and ion leak-

age (Fig. 2). The activities of classical antioxidant enzymes, such as CAT and APX, were also augmented by SNP treatment, instead of the drastically diminution observed with UV-B alone (Table 2). These data are in agreement with reports showing a protective effect of NO in plants subjected to UV-B and other stresses (Zhao et al., 2004; Laspina et al., 2005; Shi et al., 2005; Noriega et al., 2007). Nevertheless, the molecular mechanism that mediates NO enhancement of antioxidant enzyme activities is not completely understood. Interestingly, we found that HO and CAT activities had a similar behavior with respect to SNP pre-treatment under UV-B (Table 2). A recent study showed that the time-course of induction of those enzymes in soybean nodules subjected to Cd stress is related (Balestrasse et al., 2008). These results suggest a close relationship between the signal transduction pathways involved in the response of HO and CAT after oxidative stress generation and support the antioxidant role of HO. In addition, there was a positive correlation between *HO-1* transcript levels and enzyme activity (Fig. 4 and Table 2). Previous reports have also demonstrated that the enhancement of HO activity is associated with an increase in *HO-1* transcript levels and protein content (Yannarelli et al., 2006; Balestrasse et al., 2008; Han et al., 2008). Although this mechanism can account for the changes observed in HO activity, the incidence of post-translational modifications or different HO isoforms under stress conditions needs to be addressed. It is noteworthy that the protection observed using SNP was negated by the concomitant addition of the NO-scavenger cPTIO, indicating that SNP-derived NO is responsible for the beneficial effects. Moreover, treatment with FeCN, an analog of SNP which is unable to release NO but has a similar molecular structure, had minimal effects on the oxidative stress parameters evaluated (Figs. 1 and 2; Table 1) as well as in *HO-1* gene expression (Fig. 4).

Experiments carried out in plants treated with SNP in the absence of UV-B showed that NO itself can up-regulate *HO-1* mRNA expression, but to a lesser extent (Fig. 4). This observation indicates that a certain balance between NO and ROS is required to trigger the full response. Interestingly, a recent report found that the ROS–NO ratio is important to elicit ROS-activated stress responses and cell death regulation in plant leaves during ozone exposure (Ahlfors et al., 2009). Moreover, new evidence suggests that plastids and peroxisomes are important regulators of NO levels in plants (Corpas et al., 2009; Gas et al., 2009). It is worth mentioning that HO is a chloroplast-localized enzyme and that this organelle is one of the most sensitive targets of UV-B radiation (Jordan, 1996; Muramoto et al., 2002). Thus, HO could play a key role in protecting the chloroplast against UV-B-induced oxidative stress. Indeed, the specific inhibition of HO by Zn-protoporphyrin IX increased the oxidative stress parameters after irradiation with a low UV-B dose (Table 3). Moreover, we have previously shown similar results in leaves and nodules of Cd-treated plants (Noriega et al., 2004; Balestrasse et al., 2005). Biliverdin, one of the products of the HO, is an efficient scavenger of ROS and it can account for the antioxidant properties of this enzyme both in animals and plants (Otterbein et al., 2003; Noriega et al., 2004). More recently, it has been shown that CO released by HO is an important signal molecule for the tolerance mechanisms against cadmium and salt stress (Han et al., 2008; Xie et al., 2008). It would be interesting to determine whether CO has also a role in UV-B acclimation.

Taken together, these data suggest that NO may be involved in the UV-B-specific signaling pathway that mediates the HO response under low levels of radiation. Experiments using a low irradiation dose (7.5 kJ m^{-2}) demonstrated that scavenging NO with cPTIO can significantly decrease *HO-1* up-regulation (Fig. 5). Using a similar approach, previous reports have shown that NO is important for several UV-B responses, such as the induction of the gene encoding chalcone synthase for the synthesis of flavonoids, and the enhancement of the activity of antioxidant enzymes (A.-H.-Mackerness et al., 2001; Shi et al., 2005). We have also tested pharmacological inhibitors of NO-generating enzymes to evaluate the potential source implicated. We found that tungsten pre-treatment for 7 days was not able to block the *HO-1* response to UV-B irradiation, although a reduction of more than 70% in NR activity was achieved in our experimental condition. Comparable levels of inhibition have been previously shown (Deng et al., 1989), and the first evidence of a physiological role for the generation of NO by NR was in ABA-induced stomatal closure in *Arabidopsis* (Desikan et al., 2002). However, our data indicate that NR-produced NO is unlikely to be involved in *HO-1* up-regulation under UV-B. On the other hand, pre-treatments with L-NAME did not completely prevent *HO-1* induction by UV-B; nevertheless, mRNA transcript levels were similar to those found when cPTIO was used to negate that response (Fig. 5). These results confirmed that not only ROS but also NO is required to trigger a complete *HO-1* up-regulation, and suggest that a NOS-like mechanism is implicated. Consistent with this, L-arginine was able to suppress the inhibitory effect of L-NAME over *HO-1* supporting the involvement of a NOS-like enzyme, even though NOS activity was not assessed in our study. This observation is in agreement with a previous report demonstrating that L-arginine-dependent NOS activity is present in soybean chloroplasts (Jasid et al., 2006). More recently, Tossi et al. (2009) found that L-NAME partially blocked UV-B-induced NO accumulation in plants and their results suggest that a NOS-dependent pathway is required for obtaining a full UV-B response. In addition, NOS-produced NO has been associated with tolerance to other environmental factors, such as cadmium stress and salinity (Zhao et al., 2007; Rodríguez-Serrano et al., 2009). The NOS inhibitor L-NAME has been employed extensively and provided evidence that NOS-like enzymes are present in plants (Corpas et al., 2006, 2009; Crawford, 2006). However, there is a lot of debate in this field, as no direct homolog of any of the animal enzymes have been found and no other plant candidate for the role has yet been identified (Corpas et al., 2009; Gas et al., 2009).

3.1. Concluding remarks

The present study together with previous results (Yannarelli et al., 2006) support the model explaining the mechanisms involved in HO response in UV-B-treated soybean plants (Fig. 6). This model proposes that NO is implicated in the HO signaling pathway and, together with ROS, modulates the activity of this enzyme under UV-B radiation. Moreover, NO can enhance the antioxidant system allowing an improved plant defense to the subsequent oxidative insult. Interestingly, while NO may directly potentiate UV-B-induced *HO-1* transcription at the lower UV-B dose, the pre-treatment followed by exposure at 30 kJ m^{-2} may protect and enhance by inducing free radical scavenging enzymes that suppress the inhibitory effects of elevated ROS accumulation at this higher dose, resulting in an appropriate balance of ROS–NO to trigger the full HO response. Our results also suggest that NOS-like activity is the source of the NO required to mediate this process. In conclusion, the present study provides new insights into the molecular response of plants to UV-B radiation and also evidences that HO plays an important role during stress conditions.

4. Experimental

4.1. Plant material and treatments

Soybean (*Glycine max* L.) seeds were germinated and grown using Hoagland nutrient solution (Hoagland and Arnon, 1950) in a controlled climate room at 24 ± 2 °C and 50% relative humidity, with a photoperiod of 16 h and a light intensity of

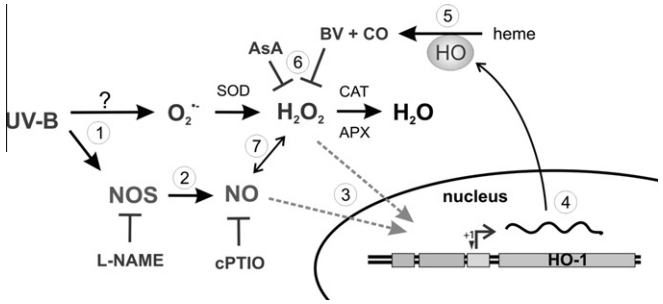

Fig. 6. Simplified scheme of the mechanisms involved in HO up-regulation under UV-B radiation as proposed in the present study. (1) Plant UV-B perception increases $O_2^{\cdot-}$ generation and enhances NO production mediated by NOS, although the signaling pathways involved are still unclear. (2) NOS-generated NO has an important role in various UV-B responses. NO and NOS activity can be efficiently blocked by cPTIO and L-NAME, respectively. (3) NO and H_2O_2 participate in the signaling required for HO-1 up-regulation. (4) An increase in *HO-1* transcript levels mediates the enhancement in HO activity. (5) Heme catabolism through HO produces BV and CO. (6) BV and ascorbic acid (AsA) play a crucial role controlling H_2O_2 levels in the chloroplast. (7) The balance between NO and ROS is critical to determine the cellular response to oxidative stress. Abbreviations: APX, ascorbate peroxidase; AsA, ascorbic acid; BV, biliverdin IXα; CAT, catalase; CO, carbon monoxide; cPTIO, 2-(4-carboxyphenil)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; HO, heme oxygenase; L-NAME, N^G-nitro-L-arginine methyl ester; NOS, nitric oxide synthase; SOD, superoxide dismutase; UV-B, ultraviolet-B.

175 μmol m^{-2} s^{-1}. After 3 weeks growth, leaves were treated with different sodium nitroprussiate (SNP; Sigma–Aldrich) concentrations (0, 0.4, 0.8, 1.2 and 1.6 mM) applied as a spray 12 h before irradiation. As a control, leaves were sprayed with 0.8 mM potassium ferrocyanide (FeCN; Fluka), which is an inactive analog of SNP. Afterward, plants were subjected to ultraviolet radiation during 100 min using a UV-B lamp (UVM-57 chromato-Vue, UVP, San Gabriel, CA, USA) (290–320 nm) at an irradiance of 5.2 W m^{-2} ultraviolet light at plant level. UV-B was filtered through 0.13 mm thick cellulose acetate filter (to avoid transmission below 290 nm) for UV-B treatments or through 0.13 mm thick Mylar Type S filter (absorbing radiation under 320 nm) for control treatments. According to Caldwell normalization (Caldwell, 1971), the UV-B dose employed was 30 kJ m^{-2}. This dose has shown to cause oxidative damage providing a suitable model to test the protective effect of exogenously added NO (Yannarelli et al., 2006). Immediately after completion of UV-B treatment, leaves were separated and used for determinations. When the potassium salt of 2-(4-carboxyphenil)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO; Sigma–Aldrich) was used as a NO-scavenger, it was added 6 h before irradiation or in combination with SNP.

To study whether NO is involved in the signaling pathway leading to HO response against UV-B, plants were irradiated with a low dose (7.5 kJ m^{-2}) that has previously shown to induce *HO-1* expression (Yannarelli et al., 2006). The endogenous source of NO was determined by inhibition of NOS or NR using N^G-nitro-L-arginine methyl ester (L-NAME; Sigma–Aldrich) and sodium tungstate (W; Sigma–Aldrich), respectively. L-NAME (100 mM) was applied as a spray 6 h before irradiation. L-arginine (200 mM; Sigma–Aldrich) was co-administered with L-NAME to demonstrate that the blocking effect of L-NAME can be specifically reversed. Tungstate (150 μM) was substituted for molybdate in the nutrient solution 7 days prior to UV-B treatment. Right after irradiation, leaves were separated and used for determinations. *In vitro* experiments were carried out to study the effect of exogenously added biliverdin IXα (BV), one of the HO products, or Zn-protoporphyrin IX (ZnPPIX), a strong inhibitor of HO, on HO activity and oxidative stress parameters. Leaf discs (12 mm diameter, 0.3 g) were floated abaxial side down in petri dishes containing 50 mM phosphate buffer (pH 7.4) supplemented with 10 μM BV or 25 μM ZnPPIX for 4 h before UV-B irradiation (7.5 kJ m^{-2}).

4.2. Chlorophyll content determination

Leaves (0.5 g of fresh weight) were homogenized with EtOH–H_2O (96:4, v/v) in a 1:30 w/v ratio. 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 (Wintermans and de Mots, 1965).

4.3. Ion leakage assay

Leaves were harvested and cut into 30 mm pieces. They were washed in deionized H_2O to remove surface added electrolytes and placed in Petri dishes with 15 ml of deionized H_2O at 25 °C for 3 h. Electrical conductivity in the bathing solution was determined (*C*1). The samples were heated at 80 °C for 2 h and the conductivity was read again in the bathing solution (*C*2). Relative ion leakage was expressed as a percentage of the total conductivity after heating at 80 °C (relative ion leakage % = *C*1/*C*2 × 100) (Zhao et al., 2004).

4.4. 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 (1968). Control and treated samples (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 EtOH 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}.

4.5. H_2O_2 and $O_2^{\cdot-}$ determination in leaf extracts

Leaves (0.5 g) were homogenized in 25 mM HCl (1.2 ml), and the crude extracts were filtered through two nylon layers. The pigments were removed by mixing with charcoal (15 mg) and separated by centrifugation at 5000*g* for 5 min. The supernatant pH was adjusted to 7.0 with 1 M NaOH and used for the assays. The H_2O_2 concentration was determined by spectrofluorometry using homovanillic acid and horse-radish peroxidase as described by Creissen et al. (1999). The concentration of $O_2^{\cdot-}$ was determined as the superoxide dismutase-sensitive rate of epinephrine–adrenochrome formation, measured in a Perkin–Elmer dual-wavelength spectrophotometer (Boveris, 1984).

4.6. Enzyme preparation and assay

Extracts for determination of catalase (CAT), ascorbate peroxidase (APX) and nitrate reductase (NR) activities were prepared from leaves (0.5 g) homogenized under ice-cold conditions in extraction buffer (5 ml), containing 50 mM phosphate buffer (pH 7.4), 1 mM EDTA, 1 g PVP, and 0.5% (v/v) Triton X-100. The homogenates were centrifuged at 10,000*g* for 30 min and the supernatant fraction was used for the assays. Catalase (EC 1.11.1.6) activity was determined measuring the pseudo-first order reaction constant ($k' = k$ [CAT]) of the decrease in H_2O_2 absorption and catalase content in pmol mg^{-1} protein was calculated using $k = 4.7 \times 10^7$ M^{-1} s^{-1} (Chance et al., 1979). Ascorbate peroxidase (EC 1.11.1.11) activity was measured in fresh extracts as described by Nakano and Asada (1981). The hydrogen peroxide-dependent oxidation of ascorbate was followed monitoring the absorbance

decrease at 290 nm (ε = 2.8 mM^{-1} cm^{-1}). Nitrate reductase activity was assayed according to Hageman and Flesher (1960). The NADH-dependant production of nitrite was developed using sulphanilamide and N-(1 napthyl) ethylene diamine hydrochloride. The absorbance of the sample and blank (without NADH) was measured at 540 nm and NR activity was expressed as nmol g^{-1} PF min^{-1}. Protein concentration was evaluated by the method of Bradford (1976), using bovine serum albumin as a standard.

4.7. Heme oxygenase preparation and assay

Leaves (0.3 g) were homogenized in a Potter–Elvehejm homogenizer using 1.2 ml 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 (Noriega et al., 2004).

4.8. Realtime quantitative RT-PCR

Total RNA was isolated using Trizol reagent (Invitrogen), treated with RNase-free DNase I (Promega), and reverse transcribed into cDNA using random hexamers and M-MLV Superscript II RT (Invitrogen). Quantitative RT-PCR reactions were carried out using *G. max HO-1* specific primers (Yannarelli et al., 2006) 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 min at 95 °C, 40 cycles with 95 °C for 15 s, 58 °C for 20 s and 60 °C for 40 s, 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 (Yannarelli et al., 2006). The data were calculated using the formula $2^{-\Delta\Delta C_t}$ and are presented as the fold change in gene expression normalized and relative to the untreated control.

4.9. Statistics

Continuous variables are expressed as mean ± SE. Differences among treatments were analyzed by one-way ANOVA, taking $P < 0.05$ as significant according to Tukey's multiple range test.

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References

A.-H.-Mackerness, S., Fred John, C., Jordan, B., Thomas, B., 2001. Early signaling components in ultraviolet-B responses: distinct roles for different reactive oxygen species and nitric oxide. FEBS Lett. 489, 237–242.

Ahlfors, R., Brosché, M., Kollist, H., Kangasjärvi, J., 2009. Nitric oxide modulates ozone-induced cell death, hormone biosynthesis and gene expression in *Arabidopsis thaliana*. Plant J. 58, 1–12.

Balestrasse, K.B., Noriega, G.O., Batlle, A., Tomaro, M.L., 2005. Involvement of heme oxygenase as antioxidant defense in soybean nodules. Free Radic. Res. 39, 145–151.

Balestrasse, K.B., Yannarelli, G.G., Noriega, G.O., Batlle, A., Tomaro, M.L., 2008. Heme oxygenase and catalase gene expression in nodules and roots of soybean plants subjected to cadmium stress. Biometals 21, 433–441.

Beligni, M.V., Lamattina, L., 1999. Nitric oxide protects against cellular damage produced by methylviologen herbicides in potato plants. Nitric Oxide 3, 199–208.

Boveris, A., 1984. Oxygen radicals in biological systems. Methods Enzymol. 105, 429–435.

Boveris, A.D., Galatro, A., Puntarulo, S., 2000. Effect of nitric oxide and plant antioxidants on microsomal content of lipid radicals. Biol. Res. 33, 159–165.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Brosche, M., Strid, A., 2003. Molecular events following perception of ultraviolet-B radiation by plants. Physiol. Plant. 117, 1–10.

Caldwell, M.M., 1971. In: Giese, A.C. (Ed.), Solar UV Irradiation and the Growth and Development of Higher Plants. Academic Press, NY, pp. 131–177.

Chance, B., Sies, H., Boveris, A., 1979. Hydroperoxide metabolism in mammalian organs. Physiol. Rev. 59, 527–605.

Corpas, F.J., Barroso, J.B., Carreras, A., Valderrama, R., Palma, J.M., León, A.M., Sandalio, L.M., del Río, L.A., 2006. Constitutive arginine-dependent nitric oxide synthase activity in different organs of pea seedlings during plant development. Planta 224, 246–254.

Corpas, F.J., Chaki, M., Fernández-Ocaña, A., Valderrama, R., Palma, J.M., Carreras, A., Begara-Morales, J.C., Airaki, M., del Río, L.A., Barroso, J.B., 2008. Metabolism of reactive nitrogen species in pea plants under abiotic stress conditions. Plant Cell Physiol. 49, 1711–1722.

Corpas, F.J., Palma, J.M., del Río, L.A., Barroso, J.B., 2009. Evidence supporting the existence of L-arginine-dependent nitric oxide synthase activity in plants. New Phytol. 184, 9–14.

Crawford, N.M., 2006. Mechanisms for nitric oxide synthesis in plants. J. Exp. Bot. 57, 471–478.

Creissen, G., Firmin, J., Fryer, M., 1999. Elevated glutathione biosynthesis capacity in the chloroplast of transgenic tobacco plants paradoxically causes oxidative stress. Plant Cell 11, 1277–1291.

Deng, M., Moureaux, T., Caboche, M., 1989. Tungstate, a molybdate analog inactivating nitrate reductase, deregulates the expression of the nitrate reductase structural gene. Plant Physiol. 91, 304–309.

Desikan, R., Griffiths, R., Hancock, J., Neill, S.J., 2002. A new role for an old enzyme: nitrate reductase-mediated nitric oxide generation is required for abscisic acid-induced stomatal closure in *Arabidopsis thaliana*. Proc. Natl Acad. Sci. USA 99, 16314–16318.

Frohnmeyer, H., Staiger, D., 2003. Ultraviolet-B radiation-mediated responses in plants. Balancing damage and protection. Plant Physiol. 133, 1420–1428.

Gas, E., Flores-Pérez, U., Sauret-Güeto, S., Rodríguez-Concepción, M., 2009. Hunting for plant nitric oxide synthase provides new evidence of a central role for plastids in nitric oxide metabolism. Plant Cell 21, 18–23.

Hageman, R.H., Flesher, D., 1960. Nitrate reductase activity in corn seedlings as affected by light and nitrate content of nutrient media. Plant Physiol. 35, 700–708.

Han, Y., Zhang, J., Chen, X., Gao, Z., Xuan, W., Xu, S., Ding, X., Shen, W., 2008. Carbon monoxide alleviates cadmium-induced oxidative damage by modulating glutathione metabolism in the roots of *Medicago sativa*. New Phytol. 177, 155–166.

Heath, R.L., Packer, L., 1968. Photoperoxidation in isolated chloroplasts I. Kineties and stoichiometry of fatty acid peroxidation. Arch. Biochem. Biophys. 125, 189–198.

Hoagland, D.R., Arnon, D.I., 1950. The water culture method for growing plants without soil. Calif. Agricultural Exp. Station Circular 347, 1–32.

Jasid, S., Simontacchi, M., Bartoli, C.G., Puntarulo, S., 2006. Chloroplasts as a nitric oxide cellular source. Effect of reactive nitrogen species on chloroplastic lipids and proteins. Plant Physiol. 142, 1246–1255.

Jordan, B.R., 1996. The effects of ultraviolet-B radiation on plants: a molecular perspective. Adv. Bot. Res. 22, 97–162.

Kikuchi, G., Yoshida, T., Noguchi, M., 2005. Heme oxygenase and heme degradation. Biochem. Biophys. Res. Commun. 338, 558–567.

Laspina, N.V., Groppa, M.D., Tomaro, M.L., Benavides, M.P., 2005. Nitric oxide protects sunflower leaves against Cd-induced oxidative stress. Plant Sci. 169, 323–330.

Leitner, M., Vandelle, E., Gaupels, F., Bellin, D., Delledonne, M., 2009. NO signals in the haze: nitric oxide signalling in plant defence. Curr. Opin. Plant Biol. 12, 451–458.

Mittler, R., 2002. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 7, 405–410.

Muramoto, T., Tsurui, N., Terry, M.J., Yokota, A., Kohchi, T., 2002. Expression and biochemical properties of a ferredoxin-dependent heme oxygenase required for phytochrome chromophore synthesis. Plant Physiol. 130, 1958–1966.

Nakano, Y., Asada, K., 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplast. Plant Cell Physiol. 22, 867–880.

Noriega, G.O., Balestrasse, K.B., Batlle, A., Tomaro, M.L., 2004. Heme oxygenase exerts a protective role against oxidative stress in soybean leaves. Biochem. Biophys. Res. Commun. 323, 1003–1008.

Noriega, G.O., Yannarelli, G.G., Balestrasse, K.B., Batlle, A., Tomaro, M.L., 2007. The effect of nitric oxide on heme oxygenase gene expression in soybean leaves. Planta 226, 1155–1163.

Otterbein, L.E., Soares, M.P., Yamashita, K., Bach, F.H., 2003. Heme oxygenase-1: unleashing the protective properties of heme. Trends Immunol. 24, 449–455.

Parani, M.R., Myers, R., Wireich, H., Smith, B., Leaman, D.W., Goldman, S.L., 2004. Microarray analysis of nitric oxide responsive transcripts in *Arabidopsis*. Plant Biotechnol. J. 2, 359–366.

Rodríguez-Serrano, M., Romero-Puertas, M.C., Pazmiño, D.M., Testillano, P.S., Risueño, M.C., Del Río, L.A., Sandalio, L.M., 2009. Cellular response of pea

plants to cadmium toxicity: cross talk between reactive oxygen species, nitric oxide, and calcium. Plant Physiol. 150, 229–243.
Romero-Puertas, M.C., Perazzolli, M., Zago, E., Delledonne, M., 2004. Nitric oxide signalling functions in plant pathogen interaction. Cell Microbiol. 6, 795–803.
Rowland, F.S., 2006. Stratospheric ozone depletion. Philos. Trans. R. Soc. Lond. B Biol. Sci. 361, 769–790.
Shi, S., Wang, G., Wang, Y., Zhang, L., Zhang, L., 2005. Protective effect of nitric oxide against oxidative stress under ultraviolet-B radiation. Nitric Oxide 13, 1–9.
Tossi, V., Lamattina, L., Cassia, R., 2009. An increase in the concentration of abscisic acid is critical for nitric oxide-mediated plant adaptive responses to UV-B irradiation. New Phytol. 181, 871–879.
Wilson, I.D., Neill, S.J., Hancock, J.T., 2008. Nitric oxide synthesis and signalling in plants. Plant Cell Environ. 31, 622–631.
Wintermans, J., de Mots, A., 1965. Spectrophotometric characteristics of chlorophylls a and b and their pheophitins in ethanol. Biochim. Biphys. Acta 109, 448–453.
Xie, Y., Ling, T., Han, Y., Liu, K., Zheng, Q., Huang, L., Yuan, X., He, Z., Hu, B., Fang, L., Shen, Z., Yang, Q., Shen, W., 2008. Carbon monoxide enhances salt tolerance by nitric oxide-mediated maintenance of ion homeostasis and up-regulation of antioxidant defence in wheat seedling roots. Plant Cell Environ. 31, 1864–1881.
Yannarelli, G.G., Noriega, G.O., Batlle, A., Tomaro, M.L., 2006. Heme oxygenase up-regulation in ultraviolet-B irradiated soybean plants involves reactive oxygen species. Planta 224, 1154–1162.
Zhao, L., Zhang, F., Guo, J., Yang, Y., Li, B., Zhang, L., 2004. Nitric oxide functions as a signal in salt resistance in the calluses from two ecotypes of reed (*Phragmites communis* Trin.). Plant Physiol. 134, 849–857.
Zhao, M.G., Tian, Q.Y., Zhang, W.H., 2007. Nitric oxide synthase-dependent nitric oxide production is associated with salt tolerance in *Arabidopsis*. Plant Physiol. 144, 206–217.