Journal of Photochemistry and Photobiology B: Biology 141 (2014) 202–209

Contents lists available at ScienceDirect

Journal of Photochemistry and Photobiology B: Biology

journal homepage: www.elsevier.com/locate/jphotobiol

Nitric oxide induces specific isoforms of antioxidant enzymes in soybean leaves subjected to enhanced ultraviolet-B radiation

Diego M. Santa-Cruz [a,c], Natalia A. Pacienza [c], Carla G. Zilli [b], Maria L. Tomaro [a], Karina B. Balestrasse [a,b], Gustavo G. Yannarelli [a,c,*]

[a] Departamento de Química Biológica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina
[b] Instituto de Investigaciones en Biociencias Agrícolas y Ambientales (INBA), CONICET, Buenos Aires, Argentina
[c] Área de Investigación y Desarrollo, Universidad Favaloro/CONICET, Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 4 June 2014
Received in revised form 26 September 2014
Accepted 30 September 2014

ABSTRACT

Antioxidant enzymes play a key role in plant tolerance to different types of stress, including ultraviolet-B (UV-B) radiation. Here we report that nitric oxide (NO) enhances antioxidant enzymes gene expression and increases the activity of specific isoforms protecting against UV-B radiation. Pre-treatments with sodium nitroprussiate (SNP), a NO-donor, prevented lipid peroxidation, ion leakage and H_2O_2 and superoxide anion accumulation in leaves of UV-B-treated soybean plants. Transcripts levels of superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) were significantly induced by SNP. These data correlated with the enhancement of particular antioxidant enzyme isoforms, such as one CAT isoform and two APX isoforms. Moreover, SNP induced the expression of three new isoforms of SOD, identified as Mn-SOD subclass. Further results showed that total activities of SOD, CAT and APX significantly increased by 2.2-, 1.8- and 2.1-fold in SNP-treated plants compared to controls, respectively. The protective effect of SNP against UV-B radiation was negated by addition of the specific NO scavenger cPTIO, indicating that NO released by SNP mediates the enhancement of antioxidant enzymes activities. In conclusion, NO is involved in the signaling pathway that up-regulates specific isoforms of antioxidant enzymes protecting against UV-B-induced oxidative stress.

1. Introduction

The reduction of the stratospheric ozone layer has increased the influx of ultraviolet-B radiation (UV-B; 280–315 nm) reaching the earth's surface [48]. Augmented UV-B exposure has a negative impact on plant growth and development due to reduced photosynthesis, impaired chloroplast function and DNA damage [22]. UV-B radiation also increases reactive oxygen species (ROS) such as superoxide anion and H_2O_2, leading to oxidative stress [1]. To counteract this effect, an antioxidant enzyme system efficiently removes and regulates ROS levels in plant cells [24]. Superoxide dismutase (SOD) catalyzes the breakdown of superoxide anion [21]. Catalase (CAT), ascorbate peroxidase (APX) and general peroxidases (POD) constitute the main H_2O_2-scavenging enzymes [6,24,36]. In the last decade, it has been demonstrated that ROS act as signaling molecules which initiate specific responses including enzyme activation, gene expression, programmed cell death and cellular damage [41,37]. In addition, recent studies propose a model for cellular redox homeostasis in which the interaction between ROS and antioxidants acts as a metabolic interface for signals regulating plant development and acclimation processes. Cellular ascorbate and glutathione pools may be configured to react to environmental factors in a compensatory manner and both are involved in initiating and controlling redox signal transduction [19,20]. Consequently, the modulation of antioxidant enzymes plays a key role in mediating plant tolerance to different types of stress, including UV-B radiation [16,62]. Moreover, several studies emphasized the importance of changes in isoform pattern and kinetic properties of different antioxidant enzymes during plant stress [49,63,61].

Nitric oxide (NO) is a signaling molecule involved in plant adaptation to biotic and abiotic stresses [10,47,65,14,33]. Microarray studies have shown that NO induces a large number of genes at transcriptional level, among them those of antioxidant enzymes [44]. Moreover, NO has an antioxidant function during stress as it reduces superoxide anion formation and lipid peroxidation [11]. Under UV-B conditions, the enhancement of specific genes involved in plant tolerance to this stressor, such as chalcone synthase and phenylalanine ammonia lyase, requires NO [1].

* Corresponding author at: Área de Investigación y Desarrollo, Universidad Favaloro, Solis 453, Buenos Aires 1078, Argentina. Tel.: +54 11 4378 1143; fax: +54 11 4378 0323.
E-mail address: gyannarelli@favaloro.edu.ar (G.G. Yannarelli).

http://dx.doi.org/10.1016/j.jphotobiol.2014.09.019
1011-1344/

However, whether NO mediates the response of antioxidant enzymes in UV-B-induced oxidative stress has not been well determined. Shi et al. [52] reported that NO increases the activities of SOD, CAT and APX under UV-B radiation, but changes in their isoform pattern and gene expression levels were not studied. We hypothesized that NO induces a specific response of antioxidant enzymes to regulate the oxidative status and mediate plant UV-B acclimation. Thus, we investigated whether NO protects soybean leaves against UV-B-induced oxidative stress through the up-regulation of gene expression to modulate the activity and isoforms of antioxidant enzymes. Soybean plants were subjected to UV-B radiation after pre-treatment with sodium nitroprussiate (SNP), a well-characterized NO donor. Overall, our results indicate that NO participates in the signaling pathway leading to an enhancement of antioxidant enzymes under UV-B, and that changes in the isoform pattern represent an important protection mechanism against oxidative stress.

2. Materials and methods

2.1. Plant material and treatments

Soybean (*Glycine max.* L.) seeds were germinated and grown using Hoagland nutrient solution [28] in a controlled climate room at 24 ± 2 °C and 50% relative humidity, with a photoperiod of 16 h. After 3 weeks growth, leaves were treated with 0.8 mM sodium nitroprussiate (SNP; Sigma–Aldrich) applied as a spray 12 h before irradiation. The effective concentration of SNP was determined in a previous report by testing different SNP doses that ranged from 0.4 to 1.6 mM [50]. 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. The UV-B dose employed in this study was 30 kJ m^{-2} calculated according to Caldwell normalization [12]. This UV-B dose was chosen from previous results obtained using the same plant model [62,50]. 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 in combination with SNP. After completion of UV-B treatment, leaves were harvested and used for determinations.

2.2. Thiobarbituric acid reactive substances (TBARS) determination

Lipid peroxidation was measured as the amount of TBARS determined by the thiobarbituric acid reaction [26]. Briefly, fresh leaves (0.3 g) were homogenized in 3 ml of 20% (w/v) trichloroacetic acid and centrifuged at 3500*g* for 20 min. An aliquot (1 ml) of the supernatant was mixed with 1 ml of 20% trichloroacetic acid containing 0.5% (w/v) thiobarbituric acid and 100 μl 4% butylated hydroxytoluene in ethanol. The mixture was heated at 95 °C for 30 min, cooled on ice and centrifuged at 10,000*g* for 15 min. The reaction was measured at 532 nm and non-specific absorption at 600 nm was subtracted. The concentration of TBARS was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

2.3. Ion leakage assay

Leaf samples were cut into 30 mm pieces, washed in deionized water to remove surface electrolytes and incubated at 25 °C for 3 h in Petri dishes containing deionized water. Electrical conductivity in the bathing solution was determined (C1). Samples were then heated at 80 °C for 2 h and the conductivity was read again in the bathing solution (C2). Relative ion leakage was expressed as a percentage of the total conductivity after heating at 80 °C (relative ion leakage% = C1/C2 × 100) [64].

2.4. Histochemical detection of H_2O_2

Leaves from control and treated plants were excised and immersed in Tris-HCl buffer (pH 6.5) containing 1% 3,3'-diamino benzidine (DAB), vacuum-infiltrated for 5 min and then incubated at room temperature for 16 h. Leaves were observed until the appearance of brown spots, characteristic of the reaction of DAB with H_2O_2, and then bleached by immersion in boiling ethanol. H_2O_2 deposits were determined by scanning the spots from leaf pictures and the numbers of pixels were quantified using the public domain ImageJ software (developed at the US National Institutes of Health). The results were expressed as percentage of spot area versus total leaf area [(spot area/total leaf area) × 100] to compensate for possible differences in leaves size.

2.5. Histochemical detection of superoxide anion

Leaves from control and treated plants were excised and immersed in 50 mM phosphate buffer (pH 6.4) containing 0.1% nitroblue tetrazolium (NBT), vacuum-infiltrated for 5 min and illuminated until the appearance of dark spots, characteristic of blue formazan precipitate. Leaves were bleached by immersing in boiling ethanol to better visualize the dark spots. Superoxide deposits were quantified by scanning spots from leaf pictures as mentioned above.

2.6. Enzyme preparation and assay

Extracts for determination of superoxide dismutase (SOD), catalase (CAT) and peroxidases (POD) activities were prepared from 0.5 g of leaves homogenized under ice-cold conditions in 5 ml of extraction buffer, containing 50 mM phosphate buffer (pH 7.4), 1 mM EDTA, 1 g PVP, and 0.5% (v/v) Triton X-100. Samples for ascorbate peroxidase (APX) activity were extracted with the same buffer except that it also contained 5 mM ascorcorbic acid. The homogenates were centrifuged at 10,000*g* for 30 min and the supernatant fraction was used for the assays. Protein concentrations were determined using the Bradford micromethod assay (BioRad). Total SOD (EC 1.15.1.1) activity was assayed by the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT), as described by Becana et al. [9]. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 14.3 mM methionine, 82.5 μm NBT, and 2.2 μM riboflavin. The reduction of NBT under white light was followed by reading absorbance at 560 nm. One unit of SOD was defined as the amount of enzyme that produced a 50% inhibition of NBT reduction under the assay conditions. Catalase (EC 1.11.1.6) 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. Catalase content in pmol mg^{-1} protein was calculated using $k = 4.7 \times 10^7$ M^{-1} s^{-1} [13]. Ascorbate peroxidase (EC 1.11.1.11) activity was measured in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM H_2O_2, 0.5 mM ascorbate and 0.1 mM EDTA. The H_2O_2-dependent oxidation of ascorbate was followed monitoring the absorbance decrease at 290 nm ($\varepsilon = 2.8$ mM^{-1} cm^{-1}) [40]. Peroxidase (EC 1.11.1.7) activity was determined in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7), 0.1 mM EDTA, 10 mM guaiacol and 10 mM H_2O_2 by measuring the increase in absorption at 470 nm due to the formation of tetraguaiacol ($\varepsilon = 26.6$ mM^{-1} cm^{-1}).

2.7. Native PAGE and activity staining

Equal amounts of protein (50 μg) from leaf extracts were subjected to discontinuous polyacrylamide gel electrophoresis under non-denaturing and non-reducing conditions (native-PAGE) as described by Laemmli [30], except that SDS was omitted. Electrophoretic separation was performed in 10% polyacrylamide gels at 4 °C for 3 h with a constant current of 30 mA per gel using Mini-Protean 3 Electrophoresis System (BioRad). To detect SOD activity, gels were stained for 30 min in the dark with 50 mM phosphate buffer (pH 7.8) containing 2.5 mM NBT, and then developed for 20 min under fluorescent light using a mixture of 0.03 mM riboflavin and 28 mM TEMED in 50 mM phosphate buffer (pH 7.8). SOD activity was observed as a clear band on a purple background [8]. Gels were also pre-incubated with 5 mM KCN (which inhibits Cu/Zn-SOD) or 5 mM H_2O_2 (which inhibits both Cu/Zn-SOD and Fe-SOD) to identify the different activities. The ferricyanide staining for CAT isozymes was carried out using the method described by Woodbury et al. [60]. The gels were washed three times (15 min each) in distilled water to remove interfering buffer salts, and then they were incubated in 3.27 mM H_2O_2 solution for 25 min. The gels were rinsed quickly in H_2O and stained in a solution containing 1% (w/v) potassium ferricyanide and 1% (w/v) ferric chloride. As soon as a green color began to appear, they were washed three times with water. CAT activity was observed as a clear band on a dark green background. For detection of APX activity, samples were subjected to native PAGE as described above, except that the carrier buffer contained 2 mM ascorbate [38]. Following the electrophoretic separation, gels were equilibrated with 50 mM sodium phosphate buffer (pH 7) and 2 mM ascorbate for 30 min. Then, the gels were incubated with 50 mM sodium phosphate buffer (pH 7) containing 4 mM ascorbate and 2 mM H_2O_2 for 20 min. Finally, they were washed with buffer during 1 min and placed in a solution of 50 mM sodium phosphate buffer (pH 7.8) containing 28 mM N,N,N′,N′-tetramethylethylenediamine (TEMED) and 2.45 mM NBT with gentle agitation. The reaction was continued for 10–15 min and stopped by a brief wash in deionized water. APX activity was observed as a clear band on a purple background. For POD isozymes staining, the gels were equilibrated with sodium phosphate buffer (50 mM, pH 7) for 30 min, then they were incubated in the same buffer containing 5 mM α-naphthol and 0.33 mM H_2O_2 during 15 min [18]. All gels were photographed (G:BOX Chemi XX6, Syngene) and the activity of the different isoforms was quantitated by densiometric analysis using Gel-Pro Analyzer 3.1 software (Media Cybernetics).

2.8. Real-time 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 was performed using soybean specific primers for Cu/Zn SOD (NM_001248512.2; sense primer: 5′-GGTTG TGAGGATTCTCGGTTCA-3′, antisense primer: 5′-GTCTGCTCCAAAGC TTCAGTCA-3′); CAT isoforms 1, 2 and 3 (NM_001250627.1, AF_035253.1, NM_001249045.1; sense primer: 5′-GGAGCTAGCGCAAA GGGTTT-3′, antisense primer: 5′-CGGCACATGTGAGGTGAGAA-3′); and APX isoforms 1 and 2 (NM_001250856.1, NM_001248658.1; sense primer: 5′-TGGCTCACAGCGCTAACAAC-3′, antisense primer: 5′-CGCCTTGAGTGGCTCCAA-3′). Samples were assayed in triplicate using Power SYBR Green master mix on a StepOne real-time PCR system (Applied Biosystems). The threshold cycle (Ct) values were normalized against the reference gene 18S, which has shown to be stable under several UV-B settings [62]. Results were calculated using the Relative Quantification (ΔΔCt) method [34] and are presented as the fold change in gene expression normalized and relative to the untreated control.

2.9. Statistics

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

3. Results

3.1. Nitric oxide protects soybean leaves from UV-B-induced oxidative stress

The formation of TBARS is a reliable indicator of free radical generation and lipid peroxidation in biological systems. TBARS formation significantly increased (more than 2-fold) in leaves of UV-B irradiated soybean plants with respect to untreated controls

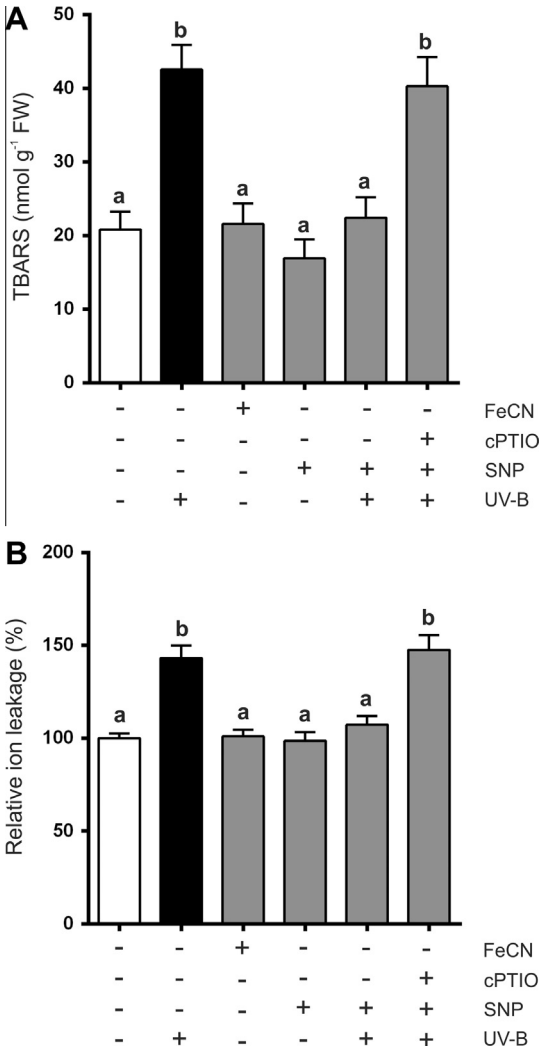

Fig. 1. Effect of NO pre-treatment on oxidative stress parameters in soybean leaves subjected to 30 kJ m^{-2} UV-B. Plants were treated with 0.8 mM SNP, a well-characterized NO donor, 12 h before UV-B irradiation. 100 μM cPTIO was used as a NO scavenger and it was added in combination with SNP to specifically block the response given by NO. (A) Lipid peroxidation evaluated as TBARS formation. Values are the mean of five independent experiments and bars indicate SD. Different letters indicate significant differences ($P < 0.05$) according to Tukey's multiple range test. (B) Ion leakage assay. Control was considered as 100% of ion leakage. Values are the mean of five independent experiments and bars indicate SD. Different letters indicate significant differences ($P < 0.05$) according to Tukey's multiple range test.

(Fig. 1A). Application of 0.8 mM SNP 12 h before irradiation could reverse the effect of UV-B on lipid peroxidation. Pre-treatment with 0.8 mM FeCN, an inactive analog of SNP, failed to protect against UV-B-induced TBARS formation. The protective effect of SNP could also be negated by the co-administration of 100 μM cPTIO, a selective NO scavenger (Fig. 1A). Oxidative stress also results in loss of membrane integrity and increased ion leakage. Ultraviolet-B radiation augmented ion leakage in leaves by 43% respect to controls (Fig. 1B). Pre-treatment with 0.8 mM SNP but not FeCN protected against UV-B-induced ion leakage. As shown in Fig. 1B, the effect of SNP could be reversed by the addition of cPTIO. Overall, these results demonstrate the role of NO in preventing UV-B-induced oxidative damage.

3.2. Nitric oxide prevents H_2O_2 and superoxide anion accumulation in leaves of UV-B-irradiated soybean plants

The accumulation of H_2O_2 under UV-B treatment was determined *in situ* using a histochemical method based on the formation of local brown spots by the reaction of H_2O_2 with DAB (Fig. 2). The H_2O_2 accumulation increased 9.3-fold in leaves of UV-B-irradiated plants with respect to untreated controls, and could be prevented by pre-treatment with 0.8 mM SNP (Fig. 2B).

The production of superoxide anion in leaves was studied using NBT which is specifically reduced and forms dark spots of blue formazan (Fig. 2). Results obtained were similar to those found for H_2O_2. The accumulation of superoxide anion was significantly higher (13-fold) in leaves of UV-B-treated plants (Fig. 2C). Pre-treatment with 0.8 mM SNP reduced superoxide anion accumulation induced by UV-B (Fig. 2C).

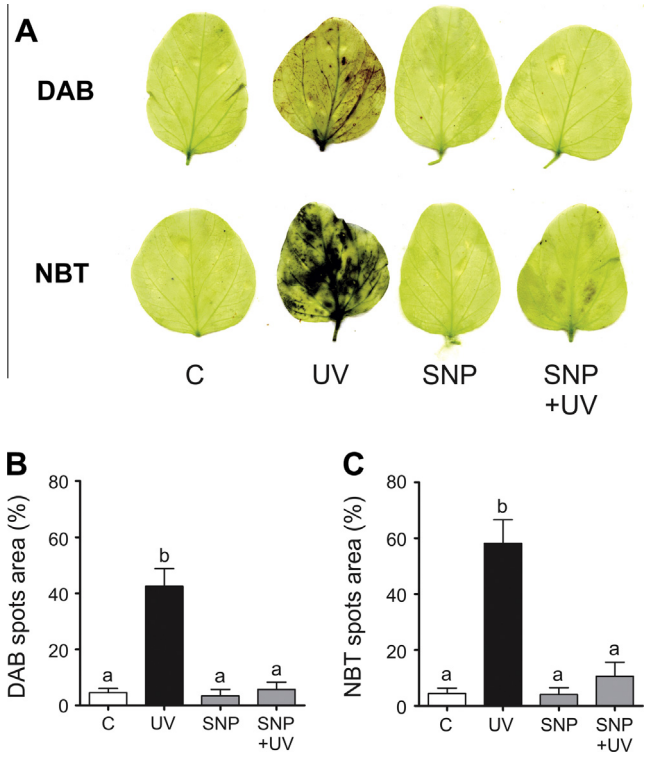

Fig. 2. Histochemical detection of H_2O_2 and superoxide anion in soybean leaves. Plants were pre-treated with 0.8 mM SNP prior to UV-B irradiation with a dose of 30 kJ m^{-2}. Leaves were infiltrated with 1% (w/v) DAB for H_2O_2 determination or with 0.1% (w/v) NBT for superoxide anion determination. (A) Leaves from control and UV-B-irradiated plants stained for H_2O_2 (DAB) and superoxide anion (NBT) content. The figure is representative of four independent experiments with duplicates for each treatment. Hydrogen peroxide (B) and superoxide anion deposits (C) were quantified by measuring the number of pixels of spots using the ImageJ software (National Institutes of Health, USA). Results are expressed as percentage of spot area versus total leaf area. Different letters indicate significant differences ($P < 0.05$) according to Tukey's multiple range test.

3.3. Effect of NO on antioxidant enzyme activities and isoforms in leaves of soybean plants subjected to UV-B radiation

We investigated changes in total activities of antioxidant enzymes responsible for scavenging of ROS, such as SOD, CAT, APX and POD. All these antioxidant enzymes were significantly affected by UV-B radiation (Table 1). Catalase and APX activities decreased by 51% and 32% in UV-B-treated plants compared to controls, respectively. In contrast, total SOD and POD activities increased 2.2-fold and 2.5-fold after UV-B irradiation. Treatments with 0.8 mM SNP significantly enhanced the activities of all the antioxidant enzymes assayed with respect to controls (Table 1). Pre-treatment with SNP protected against subsequent UV-B irradiation and interestingly, total activities of CAT and APX increased significantly with respect to controls and UV-B treatments (Table 1). No significant changes were observed with respect to controls when FeCN was substituted for SNP. Moreover, the positive effect of SNP pre-treatment was negated by the concomitant addition of the specific NO scavenger cPTIO (Table 1). These results indicate that NO released by SNP is responsible for the enhancement of antioxidant enzymes activities against UV-B.

The analysis of SOD isoforms by native-polyacrylamide gel electrophoresis (native-PAGE) showed four major bands of activity (I, II, III and IV) in leaves of control plants (Fig. 3A). After UV-B irradiation, the activities of all these isoforms increased significantly (Table 2). In addition, three SOD high mobility isoforms (V, VI and VII) were induced by UV-B. Inhibitors were used to identify the different isoforms of SODs (data not shown). The SOD isoforms I, II and III were identified as Mn-SOD. All of the remaining isozymes belonged to the Cu/Zn-SOD subclasses of SOD enzymes. Of note, the administration of SNP alone or followed by UV-B irradiation showed similar results to those found with UV-B, suggesting that NO is involved in the enhancement of SOD isoforms. Control as well as treated plants showed only one CAT isoform by native-PAGE (Fig. 3B), which in-gel activity was associated with changes observed in total CAT activity (Tables 1 and 2). Analysis of another major H_2O_2-scavening enzyme, APX, identified three bands that corresponded to different APX isozymes, with APX-I and APX-II contributing the greatest activities (Fig. 3C). These two isoforms were significantly enhanced after treatment with SNP alone or followed by UV-B irradiation (Table 2). Finally, three isoforms of POD were detected by native-PAGE in leaves of control plants (Fig. 3D). The activity of POD isoform I was not affected by the different treatments. Ultraviolet-B radiation significantly enhanced the activity of the POD isoform II, whereas plants treated with SNP showed a significant increase in the activity of the higher mobility isoform POD-III (Fig. 3D and Table 2).

3.4. Nitric oxide up-regulates the expression of antioxidant enzymes genes under UV-B radiation

To determine whether changes in the antioxidant enzymes isoform patterns are related to regulation of gene expression, we subsequently investigated the effect of SNP pre-treatment on SOD, CAT and APX transcripts levels under UV-B radiation. Considering that Cu/Zn subclasses of SOD enzymes were particularly induced by UV-B, primers were designed to specifically detect this type of isoforms. For CAT and APX isoforms, primers were designed to measure the expression of the main reference transcripts described for soybean (see Materials and Methods section). Ultraviolet-B-irradiated soybean plants showed no significant changes in SOD gene expression, whereas CAT and APX transcripts levels

Table 1
Effect of NO on antioxidant enzyme activities in leaves of UV-B irradiated soybean plants.

Treatment	SOD (U mg^{-1} protein)	CAT (pmol mg^{-1} protein)	APX (U mg^{-1} protein)	POD (U mg^{-1} protein)
Control	1.42 ± 0.07^{a}	0.78 ± 0.09^{a}	149 ± 16^{a}	28 ± 4^{a}
UV	1.67 ± 0.09^{b}	0.38 ± 0.10^{b}	102 ± 15^{b}	71 ± 10^{b}
FeCN	1.38 ± 0.04^{a}	0.81 ± 0.09^{a}	155 ± 11^{a}	33 ± 6^{a}
SNP	3.14 ± 0.13^{c}	1.40 ± 0.15^{c}	319 ± 26^{c}	47 ± 7^{c}
SNP + UV	2.97 ± 0.10^{c}	1.71 ± 0.18^{d}	331 ± 28^{c}	75 ± 9^{b}
cPTIO + SNP + UV	1.63 ± 0.11^{b}	0.36 ± 0.15^{b}	110 ± 20^{b}	66 ± 12^{b}

Values are mean of four independent experiments ± SE. Experiments were carried out as described in Materials and Methods section. One unit of SOD was defined as the amount of enzyme that produced a 50% inhibition of NBT reduction under the assay conditions. One unit of APX forms 1 nmol of oxidized ascorbate per min under assay conditions. One unit of POD forms 1 nmol of tetraguaiacol per min under the assay conditions. Different letters indicate significant differences ($P < 0.05$) according to Tukey's multiple range test.

significantly decreased by 56.4% and 75.7% compared to controls, respectively (Fig. 4). Interestingly, treatments with SNP alone significantly enhanced SOD and APX transcript levels by 2.2-fold and 2.6-fold respect to controls, respectively. Irradiation of SNP-pretreated plants did not further enhanced SOD transcripts levels respect to SNP alone. Of note, pre-treatment with SNP prevented CAT and APX down-regulation caused by UV-B radiation. In this condition, APX gene expression was similar to that found with SNP alone. In contrast, we found that CAT mRNA levels increased 1.6-fold in SNP-treated plants, whereas subsequent UV-B irradiation augmented this expression up to 3.0-fold with respect to controls (Fig. 4). The expression of the constitutive gene (18S) was unaffected throughout all experiments (not shown).

4. Discussion

Several studies have demonstrated that NO is involved in various UV-B signaling pathways leading to protective mechanisms such as accumulation of UV-B absorbing compounds [57], induction of pathogenesis-related proteins [56], and scavenging of ROS [10]. While it has also been shown that NO increases antioxidant enzymes activities under UV-B radiation [52], the molecular mechanisms involved are not completely understood. Here we show that NO mediates the up-regulation of SOD, CAT and APX transcript levels leading to changes in isoform patterns associated to a better antioxidant response against a subsequent UV-B stress.

In plants, UV-B exposure increases ROS production and generates oxidative stress [5,15]. In an earlier study, we found that irradiation of soybean plants with 30 kJ m^{-2} UV-B produces an oxidative insult that cannot be alleviated by the antioxidant defense system [62]. In that setting, our previous data demonstrated that, depending on its concentration, NO improves the plant antioxidant response against UV-B [50]. Moreover, pre-treatments with 0.8 mM SNP have shown to reduce oxidative stress by enhancing the expression of a novel antioxidant enzyme heme oxygenase-1 [50]. However, the effects of NO over classical antioxidant enzymes were not addressed in that study. Using the same experimental conditions, here we found that pre-treatments with SNP prevent lipid peroxidation and ion leakage, which are markers of oxidative stress (Fig. 1). In addition, SNP pre-treatment reduced H_2O_2 and superoxide anion accumulation in leaves of UV-B-irradiated soybean plants (Fig. 2). Of note, the protection observed using SNP was negated by the addition of cPTIO, a well-known NO-scavenger, indicating that SNP-derived NO is responsible for the beneficial effects. Other studies have also demonstrated a protective effect of NO in plants subjected to UV-B and other types of stress [64,31,52,42]. Shi et al. [52] found that NO alleviates UV-B-induced oxidative stress and suggested that this protection is probably mediated by increased activities of antioxidant enzymes. Our data showing a significant increase in SOD, CAT and APX activities in SNP-pretreated plants after UV-B irradiation are in agreement with this previous report. Interestingly, here we found that SNP treatment alone also enhances the activities of these enzymes (Table 1), a result that was not previously reported and infers that NO is required to trigger this response.

The mechanisms by which NO increases the activities of antioxidant enzymes remain unclear. A microarray study on *Arabidopsis thaliana* showed that NO induces the transcription of large number of genes involved in different processes such as basic metabolism, signal transduction, defense, and ROS degradation [44]. More recently, common transcription factor-binding sites in promoter regions of NO regulated genes were identified using a bioinformatics analysis of microarray data [43]. Neither the up-regulation of genes codifying for antioxidant enzymes nor the involvement of the NO identified motifs in the response of these enzymes were further validated. Our quantitative RT-PCR data indicated that NO significantly enhances the expression of SOD, CAT and APX in soybean plants (Fig. 4), inferring that NO itself participates in this response. Subsequent exposure to 30 kJ m^{-2} UV-B did not additionally induce the expression of these enzymes. Conversely, plants only exposed to UV-B radiation showed a significant decrease in transcripts levels codifying for antioxidant enzymes mainly related to hydrogen peroxide scavenging (Fig. 4). These data indicate that a balance between NO and ROS is important to trigger the antioxidant response. Thus, SNP pre-treatment may protect by inducing free radical scavenging enzymes that suppress the deleterious effects of excessive ROS accumulation during UV-B irradiation. In this regard, earlier studies have shown that both ROS and NO are required to elicit different UV-B protection mechanisms, including hemo oxygenase-1 up-regulation and the induction of chalcone synthase for the biosynthesis of flavonoids [1,62,50,57]. Moreover, it has been reported that a specific ROS-NO ratio produces ROS-activated stress responses and cell death regulation during other types of oxidative stress, such as ozone exposure [3].

We further investigated the effect of gene expression changes on antioxidant enzyme isoform patterns and activity. The up-regulation of SOD is implicated in defense against oxidative stress caused by abiotic stress, such as salinity, drought and UV-B [17,2,23,59]. Some reports [29,27] showed a discrepancy between SOD transcript level and enzyme activity that was attributed to post-translational regulation [35] or inactivation of the enzymes by H_2O_2 [51]. Here we found that the up-regulation of SOD transcripts by SNP and UV-B was associated with an enhancement of SOD isoforms and increased total enzyme activity. Moreover, we observed a significant change in the expression of Cu/Zn-SOD subclasses, which have been implicated in multiple stress tolerance [7,17,32]. Catalase directly dismutates H_2O_2 into H_2O and O_2 and provides ROS detoxification during stress conditions [24,36]. There are several CAT isoforms independently expressed and regulated in higher plants [53,45]. Previous reports have identified only one CAT isoform in soybean leaves [55,25]. Here we found that the activity of this unique CAT isoform is enhanced by NO. Ascorbate

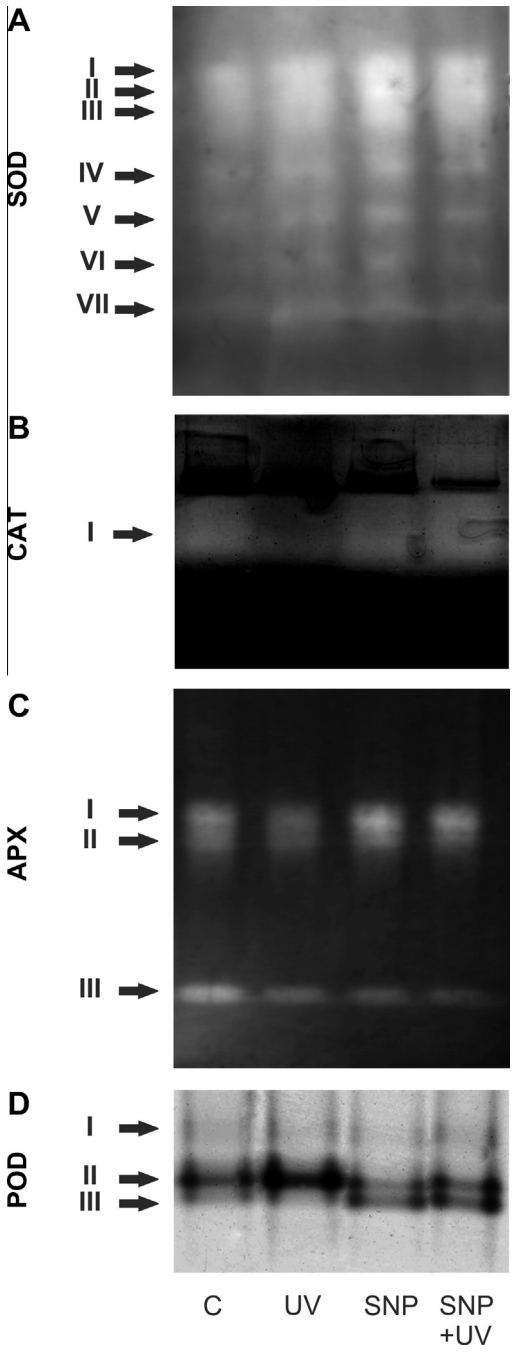

Fig. 3. Effect of NO pre-treatment on SOD, CAT, APX and POD isoforms in soybean leaves subjected to 30 kJ m^{-2} UV-B. Plants were treated with 0.8 mM SNP 12 h before UV-B irradiation. Proteins extracts (50 μg per well) were separated by native PAGE and stained for superoxide dismutase (A), catalase (B), ascorbate peroxidase (C), and peroxidase activities (D) as described in materials and methods (Section 2.7). Gels presented are representative of three independent experiments with two replicated measurements (see Table 2 for quantitative analysis).

peroxidase has a higher affinity for H_2O_2 than CAT and POD and it is essential for the management of ROS during stress. Several APX isoforms are distributed in different cell compartments, including the stroma, thylakoid membrane, glyoxisome membrane and cytosol [6]. Interestingly, NO-dependent increase of total APX activity under UV-B radiation was associated to a selective response of each isoform, presumable due to its location. Other biotic and abiotic stresses have shown to selectively increase different APX isoforms [54]. Taken together, changes in the isoform population, whose individual members have different characteristics, could

Table 2
Densitometric quantitation of different enzymes isoforms detected by in gel activity staining.

Isoforms	Experimental groups			
	C	UV	SNP	SNP + UV
SOD I	1.00 ± 0.00^{a}	1.49 ± 0.12^{b}	2.56 ± 0.30^{c}	1.63 ± 0.21^{b}
SOD II	1.00 ± 0.00^{a}	1.35 ± 0.17^{b}	2.49 ± 0.28^{c}	1.68 ± 0.27^{b}
SOD III	1.00 ± 0.00^{a}	1.44 ± 0.14^{b}	2.29 ± 0.38^{c}	1.54 ± 0.23^{b}
SOD IV	1.00 ± 0.00^{a}	1.25 ± 0.08^{b}	1.52 ± 0.23^{c}	1.18 ± 0.11^{ab}
SOD V	1.00 ± 0.00^{a}	1.41 ± 0.07^{b}	1.63 ± 0.16^{c}	1.27 ± 0.10^{d}
SOD VI	1.00 ± 0.00^{a}	1.54 ± 0.09^{b}	1.59 ± 0.18^{b}	1.06 ± 0.12^{a}
SOD VII	1.00 ± 0.00^{a}	1.39 ± 0.10^{b}	1.34 ± 0.14^{b}	0.96 ± 0.15^{a}
CAT I	1.00 ± 0.00^{a}	0.52 ± 0.19^{b}	1.62 ± 0.32^{c}	1.77 ± 0.29^{c}
APX I	1.00 ± 0.00^{a}	0.72 ± 0.12^{b}	1.99 ± 0.24^{c}	1.53 ± 0.21^{d}
APX II	1.00 ± 0.00^{a}	0.58 ± 0.16^{b}	1.96 ± 0.22^{c}	1.46 ± 0.29^{d}
APX III	1.00 ± 0.00^{a}	0.75 ± 0.19^{a}	0.90 ± 0.15^{a}	0.86 ± 0.21^{a}
POD I	1.00 ± 0.00^{a}	0.89 ± 0.12^{a}	0.93 ± 0.10^{a}	1.08 ± 0.09^{a}
POD II	1.00 ± 0.00^{a}	4.68 ± 1.02^{b}	0.51 ± 0.21^{a}	0.63 ± 0.26^{a}
POD III	1.00 ± 0.00^{a}	1.10 ± 0.17^{a}	2.31 ± 0.36^{b}	2.54 ± 0.29^{b}

The activity of each isoform was quantified by densitometric analysis and expressed as arbitrary units taking control as 1 (see Fig. 3 for representative gels). Data represent the mean ± S.D. of three independent experiments with two replicated measurements ($n = 6$). Different letters within a row (a–d) indicate significant differences ($P < 0.05$) according to Tukey's multiple range test.

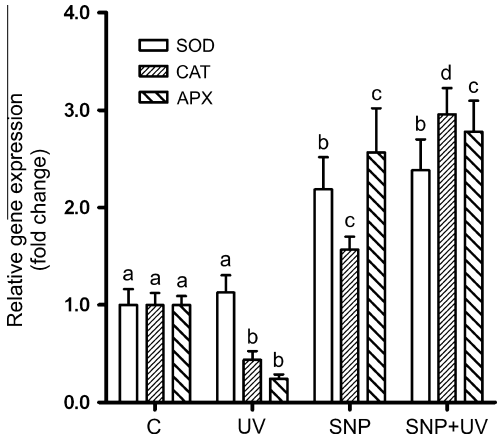

Fig. 4. Effect of NO pre-treatment on SOD, CAT and APX gene expression in soybean leaves subjected to 30 kJ m^{-2} UV-B. Experiments were performed by spraying plants with 0.8 mM SNP 12 h before UV-B irradiation. The combined treatment with cPTIO + SNP was used to specifically block the response given by NO. Quantitative gene expression was determined by realtime RT-PCR. Transcript levels of untreated plants were normalized against 18S and expressed as 1. Data are means of four independent experiments and bars indicate SD. Different letters for the expression of each gene indicate significant differences ($P < 0.01$) according to Tukey's multiple range test.

result in a significant improvement of the enzymatic properties. Moreover, the induction of antioxidant enzyme isoforms with a higher affinity for the enzyme substrates may result in an increased activity during periods of oxidative stress. This mechanism can explain the protective effect of NO against UV-B demonstrated in the present study. In this regard, previous reports support the major role that changes in the pattern of antioxidant isoforms plays in stress tolerance [49,63,61,24].

The notion that NO mediates different UV-B tolerance mechanisms in plants is widely accepted [10,1,52]. The present report, as many others, utilized the exogenous NO donor SNP to study NO participation in defense. While this represents an established experimental approach, an issue associated with the use of SNP is the release of active iron which can have a pro-oxidant effect. On one hand, it has been shown that SNP induces the up-regulation of ferritin transcripts in plants [39]. Ferritins are proteins highly and quickly produced in response to iron overload and such accumulation leads to a subsequent iron storage in a bioavailable

and non-toxic form [4]. On the other hand, results obtained employing the inactive SNP analog (FeCN) and the specific NO-scavenger (cPTIO) strongly suggest that exogenous NO, and not iron, mediates the enhancement of antioxidant enzymes in our experimental model. Other studies have inhibited specific NO-generating enzymes to confirm and identify the endogenous source of NO involved. Consistent with this, L-NAME partially blocked UV-B-induced NO accumulation and increased cell damage inferring that a nitric oxide synthase (NOS)-dependent pathway is required for a full plant response to UV-B [58]. In addition, we recently showed that NOS-like activity, but not nitrate reductase, is the source of the NO required for the up-regulation of the antioxidant enzyme heme oxygenase-1 in UV-B-irradiated soybean plants [50]. Moreover, NOS-like activity has also been associated with tolerance to other abiotic stress, such as heavy metals and salinity [65,46].

In conclusion, we found that NO is involved in the signaling pathway that up-regulates SOD, CAT and APX transcript levels. Moreover, NO enhances the antioxidant system by inducing specific isoforms of antioxidant enzymes that allows an improved plant response to a subsequent UV-B oxidative insult.

5. Abbreviations

APX	ascorbate peroxidase
CAT	catalase
cPTIO	2-(4-carboxyphenil)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
POD	non-specific peroxidase
SNP	sodium nitroprussiate
SOD	superoxide dismutase
UV-B	ultraviolet radiation-B

Acknowledgements

This work was supported by grants from the Universidad de Buenos Aires (UBA, Argentina) and from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina). NAP, CGZ, MLT, KBB and GGY are established investigators of CONICET.

References

[1] S. A.-H.-Mackerness, C. Fred John, B. Jordan, B. Thomas, Early signaling components in ultraviolet-B responses: distinct roles for different reactive oxygen species and nitric oxide, FEBS Lett. 489 (2001) 237–242.
[2] S. Agarwal, Increased antioxidant activity in Cassia seedlings under UV-B radiation, Biologia Plantarum 51 (2007) 157–160.
[3] R. Ahlfors, M. Brosché, H. Kollist, J. Kangasjärvi, Nitric oxide modulates ozone-induced cell death, hormone biosynthesis and gene expression in Arabidopsis thaliana, Plant J. 58 (2009) 1–12.
[4] N. Arnaud, I. Murgia, J. Boucherez, J.F. Briat, F. Cellier, F. Gaymard, An iron-induced nitric oxide burst precedes ubiquitin-dependent protein degradation for Arabidopsis AtFer1 ferritin gene expression, J. Biol. Chem. 18 (2006) 23579–23588.
[5] T. Arnott, T.M. Murphy, A comparison of the effects of a fungal elicitor and ultraviolet radiation on ion transport and hydrogen peroxide synthesis by rose cells, Environ. Exp. Botany 31 (1991) 209–216.
[6] K. Asada, Ascorbate peroxidase-a hydrogen peroxide-scavenging enzyme in plants, Physiologya Plantarum 85 (1992) 235–241.
[7] G.H. Badawi, Y. Yamauchi, E. Shimada, R. Sasaki, N. Kawano, K. Tanaka, Enhanced tolerance to salt stress and water deficit by overexpressing superoxide dismutase in tobacco (*Nicotiana tabacum*) chloroplasts, Plant Science 166 (2004) 919–928.
[8] C. Beauchamp, I. Fridovich, Superoxide dismutase: improved assays and an assay applicable to acrylamide gels, Anal. Biochem. 44 (1971) 276–287.
[9] M. Becana, P. Aparico-Tejo, J.J. Irigoyen, M. Sanchez-Diaz, Some enzymes of hydrogen peroxide metabolism in leaves and root nodules of *Medicago sativa*, Plant Phys. 82 (1986) 1169–1171.
[10] M.V. Beligni, L. Lamattina, Nitric oxide counteracts cytotoxic processes mediated by reactive oxygen species in plant tissues, Planta 28 (1999) 337–344.
[11] A.D. Boveris, A. Galatro, S. Puntarulo, Effect of nitric oxide and plant antioxidants on microsomal content of lipid radicals, Biol. Res. 33 (2000) 159–165.
[12] M.M. Caldwell, Solar UV irradiation and the growth and development of higher plants, in: A.C. Giese (Ed.), Photophysiology, Academic Press, NY, 1971, pp. 131–177.
[13] B. Chance, H. Sies, A. Boveris, Hydroperoxide metabolism in mammalian organs, Physiol. Rev. 59 (1979) 527–605.
[14] F.J. Corpas, M. Chaki, A. Fernández-Ocaña, R. Valderrama, J.M. Palma, A. Carreras, J.C. Begara-Morales, M. Airaki, L.A. del Río, J.B. Barroso, Metabolism of reactive nitrogen species in pea plants under abiotic stress conditions, Plant Cell Physiol. 49 (2008) 1711–1722.
[15] Q. Dai, B. Yan, S. Huang, X. Liu, S. Peng, M.L. Miranda, A.Q. Chavez, B.S. Vergara, D.M. Olszyk, Response of oxidative stress defense systems in rice (*Oryza sativa*) leaves with supplemental UV-B radiation, Physiologia Plantarum 101 (1997) 301–308.
[16] M.L. Dionisiosese, S. Tobita, Antioxidant response of rice seedlings to salinity stress, Plant Science 135 (1998) 1–9.
[17] F. Eyidogan, M.T. Oz, Effect of salinity on antioxidant responses of chickpea seedlings, Acta Physiologiae Plantarum 29 (2005) 485–493.
[18] M.A. Ferrer, A.A. Calderon, R. Munoz, Barcelo A. Ros, 4-Methoxy-α-naphtol as a specific substrate for kinetic, zymographic and cytochemical studies on plant peroxidase activities, Phytochem. Anal. 1 (1990) 63–69.
[19] C.H. Foyer, G. Noctor, Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses, Plant Cell 17 (2005) 1866–1875.
[20] C.H. Foyer, G. Noctor, Ascorbate and glutathione: the heart of the redox hub, Plant Physiol. 155 (2011) 2–18.
[21] I. Fridovich, Superoxide radical and superoxide dismutases, Annu. Rev. Biochem. 64 (1995) 97–112.
[22] H. Frohnmeyer, D. Staiger, Ultraviolet-B radiation-mediated responses in plants. Balancing damage and protection, Plant Physiol. 133 (2003) 1420–1428.
[23] M. Gapinska, M. Sklodowska, B. Gabara, Effect of short- and long-term salinity on the activities of antioxidative enzymes and lipid peroxidation in tomato roots, Acta Physiologiae Plantarum 30 (2008) 11–18.
[24] S.S. Gill, N. Tuteja, Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants, Plant Physiol. Biochem. 48 (2010) 909–930.
[25] M. Hamurcu, A.H. Sekmen, I. Turkan, S. Gezgin, T. Demiral, R.W. Bell, Induced anti-oxidant activity in soybean alleviates oxidative stress under moderate boron toxicity, Plant Growth Regulat. 70 (2013) 217–226.
[26] R.L. Heath, L. Packer, Photoperoxidation in isolated chloroplasts I Kineties and stoichiometry of fatty acid peroxidation, Arch. Biochem. Biophys. 125 (1968) 189–198.
[27] J.A. Hernandez, A. Jimenez, P. Mullineaux, F. Sevilla, Tolerance of pea (*Pisum sativum L.*) to long-term salt stress is associated with induction of antioxidant defenses, Plant, Cell Environ. 23 (2000) 853–862.
[28] D.R. Hoagland, D.I. Arnon, The water culture method for growing plants without soil, California Agric. Exp. Stat. Publ. 347 (1950) 1–32.
[29] D.J. Kliebenstein, R.A. Monde, R.L. Last, Superoxide dismutase in Arabidopsis: an eclectic enzyme family with disparate regulation and protein localization, Plant Physiol. 118 (1998) 637–650.
[30] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[31] N.V. Laspina, M.D. Groppa, M.L. Tomaro, M.P. Benavides, Nitric oxide protects sunflower leaves against Cd-induced oxidative stress, Plant Science 169 (2005) 323–330.
[32] S.H. Lee, N. Ahsan, K.W. Lee, D.H. Kim, D.G. Lee, S.S. Kwak, S.Y. Kwon, T.H. Kim, B.H. Lee, Simultaneous overexpression of both CuZn superoxide dismutase and ascorbate peroxidase in transgenic tall fescue plants confers increased tolerance to a wide range of abiotic stresses, J. Plant Physiol. 164 (2007) 1626–1638.
[33] M. Leitner, E. Vandelle, F. Gaupels, D. Bellin, M. Delledonne, NO signals in the haze: nitric oxide signalling in plant defence, Curr. Opin. Plant Biol. 12 (2009) 451–458.
[34] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)), Methods 25 (2001) 402–408.
[35] N.R. Madmanchi, J.L. Donahue, C.L. Cramer, R.G. Alscher, K. Pedersen, Differential response of Cu, Zn superoxide dismutases in two pea cultivars during a shortterm exposure to sulfur dioxide, Plant Mol. Biol. 26 (1994) 95–103.
[36] A. Mhamdi, G. Noctor, A. Baker, Plant catalases: peroxisomal redox guardians, Arch. Biochem. Biophys. 525 (2012) 181–194.
[37] R. Mittler, S. Vanderauwera, N. Suzuki, G. Miller, V.B. Tognetti, K. Vandepoele, M. Gollery, V. Shulaev, F. Van Breusegem, ROS signaling: the new wave?, Trends Plant Sci 16 (2011) 300–309.
[38] R. Mittler, B. Zilinskas, Detection of ascorbate peroxidase activity in native gels by inhibition of the ascorbate-dependent reduction of nitroblue tetrazolium, Anal. Biochem. 212 (1993) 540–546.
[39] I. Murgia, M.C. de Pinto, M. Delledonne, C. Soave, L. De Gara, Comparative effects of various nitric oxide donors on ferritin regulation, programmed cell death, and cell redox state in plant cells, J. Plant Physiol. 161 (2004) 777–783.
[40] Y. Nakano, K. Asada, Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplast, Plant Cell Physiol. 22 (1981) 867–880.

[41] S. Neill, R. Desikan, J. Hancock, Hydrogen peroxide signaling, Curr. Opin. Plant Biol. 5 (2002) 388–395.
[42] G.O. Noriega, G.G. Yannarelli, K.B. Balestrasse, A. Batlle, M.L. Tomaro, The effect of nitric oxide on heme oxygenase gene expression in soybean leaves, Planta 226 (2007) 1155–1163.
[43] M.C. Palmieri, S. Sell, X. Huang, M. Scherf, T. Werner, J. Durner, C. Lindermayr, Nitric oxide-responsive genes and promoters in Arabidopsis thaliana: a bioinformatics approach, J. Exp. Botany 59 (2008) 177–186.
[44] M. Parani, S. Rudrabhatla, R. Myers, H. Weirich, B. Smith, D.W. Leaman, S.L. Goldman, Microarray analysis of nitric oxide responsive transcripts in Arabidopsis, Plant Biotechnol. J. 2 (2004) 359–366.
[45] N.A. Polidoros, J.G. Scandalios, Role of hydrogen peroxide and different classes of antioxidants in the regulation of catalase and glutathione S-transferase gene expression in maize (*Zea mays L.*), Physiologia Plantarum 106 (1999) 112–120.
[46] M. Rodríguez-Serrano, M.C. Romero-Puertas, D.M. Pazmiño, P.S. Testillano, M.C. Risueño, L.A. Del Río, L.M. Sandalio, Cellular response of pea plants to cadmium toxicity: cross talk between reactive oxygen species, nitric oxide, and calcium, Plant Physiol. 150 (2009) 229–243.
[47] M.C. Romero-Puertas, M. Perazzolli, E. Zago, M. Delledonne, Nitric oxide signalling functions in plant pathogen interaction, Cell. Microbiol. 6 (2004) 795–803.
[48] F.S. Rowland, Stratospheric ozone depletion, Philos. Trans. Royal Soc. London. Series B, Biol. Sci. 361 (2006) 769–790.
[49] L.M. Sandalio, H.C. Dalurzo, M. Gómez, M.C. Romero-Puertas, L.A. del Río, Cadmium-induced changes in the growth and oxidative metabolism of pea plants, J. Exp. Botany 52 (2001) 2115–2126.
[50] D.M. Santa-Cruz, N.A. Pacienza, A.H. Polizio, K.B. Balestrasse, M.L. Tomaro, G.G. Yannarelli, Nitric oxide synthase-like dependent NO production enhances heme oxygenase up-regulation in ultraviolet-B-irradiated soybean plants, Phytochemistry 71 (2010) 1700–1707.
[51] J.G. Scandalios, Oxygen stress and superoxide dismutases, Plant Physiol. 101 (1993) 7–12.
[52] S. Shi, G. Wang, Y. Wang, L. Zhang, L. Zhang, Protective effect of nitric oxide against oxidative stress under ultraviolet-B radiation, Nitric Oxide 13 (2005) 1–9.
[53] R.W. Skadsen, P. Schulz-Lefert, J.M. Herb, Molecular cloning, characterization and expression analysis of two classes of catalase isozyme genes in barley, Plant Mol. Biol. 29 (1995) 1005–1014.
[54] N. Smirnoff, Ascorbic acid: metabolism and functions of a multifaceted molecule, Curr. Opin. Plant Biol. 3 (2000) 229–235.
[55] R. Sobkowiak, K. Rymer, R. Rucińska, J. Deckert, Cadmium-induced changes in antioxidant enzymes in suspension culture of soybean cells, Acta Biochim. Polonica 51 (2004) 219–222.
[56] S.L. Surplus, B.R. Jordan, A.M. Murphy, J.P. Carr, B. Thomas, S. A.-H. Mackerness, Ultraviolet-B-induced responses in Arabidopsis thaliana: role of salicylic acid and reactive oxygen species in the regulation of transcripts encoding photosynthetic and acidic pathogenesis-related proteins, Plant, Cell Environ. 21 (1998) 685–694.
[57] V. Tossi, C. Lombardo, R. Cassia, L. Lamattina, Nitric oxide and flavonoids are systemically induced by UV-B in maize leaves, Plant Science 193–194 (2012) 103–109.
[58] V. Tossi, L. Lamattina, R. Cassia, An increase in the concentration of abscisic acid is critical for nitric oxide-mediated plant adaptive responses to UV-B irradiation, New Phytologist 181 (2009) 871–879.
[59] C.Q. Wang, R.C. Li, Enhancement of superoxide dismutase activity in the leaves of white clover (*Trifolium repens L.*) in response to polyethylene glycol-induced water stress, Acta Physiologiae Plantarum 30 (2008) 841–847.
[60] W. Woodbury, A.K. Spencer, M.A. Stahmann, An improved procedure using ferricyanide for detecting catalase isozymes, Anal. Biochem. 44 (1971) 301–305.
[61] G.G. Yannarelli, A.J. Fernández-Alvarez, D.M. Santa-Cruz, M.L. Tomaro, Glutathione reductase activity and isoforms in leaves and roots of wheat plants subjected to cadmium stress, Phytochemistry 68 (2007) 505–512.
[62] G.G. Yannarelli, S.M. Gallego, M.L. Tomaro, Effect of UV-B radiation on the activity and isoforms of enzymes with peroxidase activity in sunflower cotyledons, Environ. Exp. Botany 56 (2006) 174–181.
[63] G.G. Yannarelli, G.O. Noriega, A. Batlle, M.L. Tomaro, Heme oxygenase up-regulation in ultraviolet-B irradiated soybean plants involves reactive oxygen species, Planta 224 (2006) 1154–1162.
[64] L. Zhao, F. Zhang, J. Guo, Y. Yang, B. Li, L. Zhang, Nitric oxide functions as a signal in salt resistance in the calluses from two ecotypes of reed (*Phragmites communis Trin.*), Plant Physiol. 134 (2004) 849–857.
[65] M.G. Zhao, Q.Y. Tian, W.H. Zhang, Nitric oxide synthase-dependent nitric oxide production is associated with salt tolerance in Arabidopsis, Plant Physiol. 144 (2007) 206–217.