

RESEARCH PAPER

Exposure to nitric oxide protects against oxidative damage but increases the labile iron pool in sorghum embryonic axes

Sebastián Jasid, Marcela Simontacchi and Susana Puntarulo*

Physical Chemistry-PRALIB, School of Pharmacy and Biochemistry, University of Buenos Aires, Junín 956, Buenos Aires, C1113AAD, Argentina

Received 29 May 2008; Revised 19 August 2008; Accepted 26 August 2008

Abstract

Sodium nitroprusside (SNP) and diethylenetriamine NONOate (DETA NONOate), were used as the source of exogenous NO to study the effect of NO upon germination of sorghum (*Sorghum bicolor* (L.) Moench) seeds through its possible interaction with iron. Modulation of cellular Fe status could be an important factor for the establishment of oxidative stress and the regulation of plant physiology. Fresh and dry weights of the embryonic axes were significantly increased in the presence of 0.1 mM SNP, as compared to control. Spin trapping EPR was used to assess the NO content in axes from control seeds after 24 h of imbibition (2.4 ± 0.2 nmol NO g⁻¹ FW) and seeds exposed to 0.01, 0.1, and 1 mM SNP (3.1 ± 0.3 , 4.6 ± 0.2 , and 6.0 ± 0.9 nmol NO g⁻¹ FW, respectively) and 1 mM DETA NONOate (6.2 ± 0.6 nmol NO g⁻¹ FW). Incubation of seeds with 1 mM SNP protected against oxidative damage to lipids and maintained membrane integrity. The content of the deferoxamine-Fe (III) complex significantly increased in homogenates of axes excised from seeds incubated in the presence of 1 mM SNP or 1 mM DETA NONOate as compared to the control (19 ± 2 nmol Fe g⁻¹ FW, 15.2 ± 0.5 nmol Fe g⁻¹ FW, and 8 ± 1 nmol Fe g⁻¹ FW, respectively), whereas total Fe content in the axes was not affected by the NO donor exposure. Data presented here provide experimental evidence to support the hypothesis that increased availability of NO drives not only protective effects to biomacromolecules, but to increasing the Fe availability for promoting cellular development as well.

Key words: Iron, labile iron pool, nitric oxide, protein nitration, protein oxidation, sorghum.

Introduction

Plants can produce and release significant amounts of nitric oxide (NO), especially under stress or in certain physiological processes (Neill *et al.*, 2003), mainly in actively growing tissues such as embryonic axes (Caro and Puntarulo, 1999; Simontacchi *et al.*, 2004). *In vivo* generation of NO in plants is achieved through different pathways, both enzymatically employing either nitrite or arginine as substrates (Crawford, 2006), and non-enzymatically (Bethke *et al.*, 2004). Recently, Zhao *et al.* (2007) showed that an Arabidopsis mutant (*Atnoal*) with defective *in vivo* NOS activity, displayed lower endogenous NO levels than wild-type plants and was more sensitive to salt stress than wild-type plants, as indicated by a greater inhibition of root elongation and seed germination, lower survival rates, and a greater accumulation of hydrogen peroxide in the mutant plants than in wild-type plants when treated with moderate NaCl. Moreover, Sun *et al.* (2007) reported that SNP, an NO donor, partially reversed Fe deficiency-induced retardation of plant growth as well as chlorosis, suggesting both a physiological role for NO and a link between NO and Fe metabolism *in vivo*. Besides endogenous production, plants are in contact with atmospheric NO (Yamasaki, 2000), provided by various sources (Wildt *et al.*, 1997). It has been described that the emission rate of NO_x species (NO+NO₂+N₂O) to the atmosphere is about

* To whom correspondence should be addressed: E-mail: susanap@ffyb.uba.ar

Abbreviations: DETA NONOate, diethylenetriamine NONOate; DF, deferoxamine mesylate; EPR, electronic paramagnetic resonance; MGD, sodium-N-methyl-D-glucamine dithiocarbamate; NO, nitric oxide; SNP, sodium nitroprusside; TEMPOL, 4-hydroxy-2,2,6,6-tetramethyl piperidine N-oxyl.

$260 \times 10^9 \text{ kg year}^{-1}$, being the major greenhouse pollutant derived from both anthropogenic (such as the combustion of fossil fuels) and non-anthropogenic (such as lightening and many biological processes) sources (Elstner and Oswald, 1991). In addition, soils can provide environmental NO, and contribute to almost 20% of the global atmospheric NO budget (Conrad, 1995). NO_x are formed in soils as by-products of nitrification and denitrification processes (Colliver and Stephenson, 2000), and its emission strongly depends on the NH₄⁺ and NO₃⁻ concentration in the soil (Thornton and Valente, 1996).

It has been reported that exogenously applied NO can enhance germination or break seed dormancy (Beligni and Lamattina, 2000; Keeley and Fotheringham, 1997). Moreover, in *Lupinus luteus* seeds, in which germination is light independent and no dormancy breakage is required, higher germination rates have been observed by supplementation with an NO donor (Kopyra and Gwózdź, 2003). Once NO is endogenously generated or gets inside the cell from an exogenous source, it reacts with a wide range of targets, including protein and non-protein thiols, superoxide anion (O₂⁻), and Fe. The interaction between NO and thiols could be responsible for protein activity regulation, or play a role in cell signalling pathways, or drive the generation of an NO reservoir (Lindermayr and Durner, 2007). The reaction of NO with O₂⁻ is among the faster reactions known, and leads to the formation of peroxynitrite (ONOO⁻), which is a powerful oxidant species (Blough and Zafirou, 1985). In spite of the fact that there are few reports regarding the presence of nitrative modifications in plants, ONOO⁻ is specially known to cause nitration of phenolic rings, including tyrosine residues in proteins (Alamillo and Garcia-Olmedo, 2001). In this regard, Morot-Gaudry-Talarmain *et al.* (2002) reported that tyrosine nitration increased in leaves, following increases in endogenous NO production or exogenous ONOO⁻ addition. Moreover, nitration of proteins in animal systems was also described as being mediated by an enzymatic mechanism as well, namely a peroxidase-mediated reaction (Sakihama *et al.*, 2003).

A role for NO in Fe homeostasis in plants has been suggested, since a relationship between plant ferritin expression and NO supplementation was described (Murgia *et al.*, 2002). On the other hand, exogenously applied NO induces greening in Fe-deficient maize plants without changes in total Fe content per gram of fresh matter (Graziano *et al.*, 2002). Recently, a role for NO in Fe uptake (Graziano and Lamattina, 2007) has also been described.

Fe is a janus element, depending on whether it serves as a micronutrient or as a catalyst of the formation of reactive species. The unique ability of iron of changing its oxidation state and redox potential in response to

changes in the nature of the ligand makes this metal essential for almost all living organisms (Kruszewski, 2003). Very little information is presently available on intracellular Fe movement in plant cells, where plant vacuoles are likely to play an important role in handling excess Fe (Curie and Briat, 2003). Plastids contain ferritin, the main Fe storage protein (Briat *et al.*, 1999). It has been reported that Fe-ferritin represents more than 90% of the Fe found in a pea embryo axis (Marentes and Grusak, 1998). Fe-containing enzymes are the key components of many essential biological reactions. However, the same biochemical properties that make Fe beneficial in many biological processes might be a drawback in some particular conditions, when improperly shielded Fe can catalyze one-electron reductions of O₂ species that lead to the production of very reactive free radicals. The toxicity of Fe may be dependent on the Fenton reaction, which produces the hydroxyl radical (·OH) or an oxoiron compound (LFeO²⁺) (Lu and Koppenol, 2005) and from its reactions with lipid hydroperoxides (Lu and Koppenol, 2005). Thus, cells have evolved co-ordinated mechanisms to maintain labile Fe pool (LIP) within physiological values (Cairo *et al.*, 2002). LIP is defined biochemically as a pool of redox-active Fe complexes and operationally, as a cell chelatable pool that comprises both ionic forms of Fe (Fe²⁺ and Fe³⁺) associate with a diverse population of ligands. The broadest definition of LIP implies that it consists of chemical forms that can potentially participate in redox-cycling but can be scavenged by permeant chelators (Kakhlon and Cabantchik, 2002).

The hypothesis of this work is that exposure of sorghum seeds to exogenously generated NO could result in protection from oxidative alterations of biologically critical macromolecules. Moreover, possible NO effects on Fe homeostasis upon the early stages of germination are studied. On the basis of NO chemical reactivity the following biological targets were analysed: (i) membrane integrity, (ii) protein damage, and (iii) effect on Fe homeostasis.

Materials and methods

Plant material and treatments

Sorghum bicolor (L.) Moench seeds were grown in the dark at 26 °C over distilled water saturated filter paper either in the presence or the absence of the NO donors diethylenetriamine NONOate (DETA NONOate) or sodium nitroprusside (SNP) up to a concentration of 1 mM. To assess SNP effects, both photodegraded SNP and the SNP analogue K₄[Fe(CN)₆] were used when indicated. After 24 h of imbibition embryonic axes were excised from seeds, washed several times with distilled water, and used for further assays. The water used to prepare all solutions was passed through columns containing Chelex 100 resin (Sigma Chemical Co.) to remove metal contaminants.

Fresh (FW) and dry weight (DW) of intact recently harvested sorghum embryonic axes were obtained by measuring the weight

before or after exposure to 60 °C for 48–72 h, respectively. Relative water content (RWC) in embryonic axes was calculated according to equation 1

$$RWC (\%) = [(FW - DW) / FW \times 100] \quad (1)$$

Electrolyte leakage assay

Ten embryonic axes were excised from either treated or control seeds, and placed in vials containing 20 ml of distilled water. The conductivity of the medium was measured immediately (L_0) and after 3 h of incubation at room temperature (L_3), employing a multi-parameter analyser (Consort C831). To evaluate the maximal conductivity (L_m), the axes were boiled for 10 min and the conductivity was assayed. Electrolyte leakage was calculated according to equation 2 (Sairam and Srivastava, 2002)

$$\text{Electrolyte leakage } (\%) = [(L_3 - L_0) / L_m] \quad (2)$$

EPR detection of NO

Isolated sorghum embryonic axes (0.5 g FW) were excised from seeds, homogenized in 100 mM phosphate buffer, pH 7.4, and supplemented with the spin trap solution (10 mM MGD, 1 mM FeSO_4) (Komarov and Lai, 1995). The homogenates were immediately transferred to Pasteur pipettes for EPR spin trapping measurements. The spectra were recorded at room temperature (18 °C) with a Bruker ECS 106 EPR spectrometer, operating at 9.5 GHz. Instrument settings include 200 G field scan, 83.886 s scan time, 327.68 ms time constant, 5.983 G modulation amplitude, 50 kHz modulation frequency, and 20 mW microwave power. Quantification of the spin adduct ($\text{MGD}_2\text{-Fe}^{2+}\text{-NO}$) was performed using as standard an aqueous solution of TEMPOL, a stable free radical, introduced into the same sample cell used for spin trapping measurements. The TEMPOL solutions were standardized spectrophotometrically at 429 nm ($\epsilon = 13.4 \text{ M}^{-1} \text{ cm}^{-1}$), and the concentration of $\text{MGD}_2\text{-Fe}^{2+}\text{-NO}$ adduct was obtained by double integration of the spectra of the three lines and cross-checked with the TEMPOL spectra.

EPR detection of lipid radicals

Embryonic axes were homogenized in 40 mM potassium phosphate buffer, 120 mM KCl, pH 7.4 (300 mg ml^{-1}). Homogenates were centrifuged at 10 000 g for 10 min, and the supernatant obtained was centrifuged for 1 h at 100 000 g. The pellet obtained (microsomal fraction) was added to the spin trap POBN (50 mM final concentration), incubated for 20 min at 30 °C, and used for lipid radical detection. EPR spectra were obtained at room temperature using a Bruker spectrometer, ECS 106, operating at 9.81 GHz with 50 kHz modulation frequency. EPR instrument settings for the experiments were as follows: microwave power, 20 mW; modulation amplitude, 1.232 G; time constant, 81.92 ms; receiver gain, 2×10^4 (Jurkiewicz and Buettner, 1994). The content of the spin trap adduct POBN-LR was quantified as described above.

Western blot analysis of protein oxidation

Embryonic axes were homogenized in 100 mM phosphate buffer, pH 7.1, and centrifuged at 1500 g for 3 min. The supernatant was then used for Western blot determination of protein oxidation. Protein content in samples was measured according to Bradford (1976). Carbonyl groups in soluble proteins were derivatized as described by Levine *et al.* (1994), by mixing 1 vol. of sample with an equal volume of SDS (12% w/v) and then with 2 vols of 20 mM dinitrophenylhydrazine dissolved in 10% (v/v) trifluoroacetic acid.

This mixture was incubated for 25 min at room temperature, and the reaction was stopped by adding 1.5 vols of sample to 2 M TRIS-HCl 30% (v/v) glycerol. Proteins (1 μg per well) were loaded in 12% (w/v) acrylamide mini-gels and electrophoresis was performed at room temperature under a constant voltage (120 V) for 2 h. Afterwards, the proteins were electrotransferred to nitrocellulose membranes at 130 V for 1 h. Blots were blocked with 5% (w/v) non-fat dry milk dissolved in PBS-T [10 mM potassium phosphate buffer, pH 7.4, 150 mM NaCl, 0.1% (v/v) Tween 20], incubated overnight with primary antibody dissolved in blocking buffer (1/2500), and washed several times with PBS-T. For the detection of carbonyl groups, rabbit anti-DNP antibody (Santa Cruz) was used. Blots were then incubated for 2 h with the secondary antibody (goat anti-rabbit IgG conjugated to horseradish peroxidase) prepared 1/15 000 in PBS-T with 1% (w/v) non-fat dry milk and washed several times with PBS-T. The chemiluminescence detection kit (Bio-Rad) was used for developing. Band intensity was determined employing Scion Image for Windows.

Western blot analysis of nitrotyrosines

Embryonic axes were homogenized and centrifuged as described above, and the supernatant was mixed with an equal volume of sample buffer according to Laemmli (1970) and incubated for 10 min at 95 °C. Proteins (25–50 μg per well) were loaded in 10% (w/v) acrylamide mini-gels and electrophoresis was performed at room temperature under the conditions described above. After protein transference, membranes were blocked with 3% (w/v) BSA dissolved in PBS-T, incubated overnight with the primary antibody dissolved in blocking buffer (1/4000), and washed several times with PBS-T. Mouse anti-nitrotyrosine IgG (Chemicon International) was used as the primary monoclonal antibody. Membranes were then incubated for 2 h with the secondary antibody (goat anti-mouse IgG conjugated to horseradish peroxidase) prepared 1/10 000 in PBS-T with 1% (w/v) BSA and washed several times with PBS-T. Western blot assays were developed by using a chemiluminescence kit (Bio-Rad). Band intensity was assessed using Scion Image for Windows.

Total Fe content determination

Embryonic axes from sorghum seeds were washed three times with Chelex 100-treated water, and dried until constant weight in an oven at 60 °C. Then, dry axes were mineralized using $\text{HNO}_3/\text{HClO}_4$ (1:1) (Laurie *et al.*, 1991). The content of total Fe was determined spectrophotometrically after reduction of the samples with thioglycolic acid, measuring the absorbance at 535 nm in the presence of bathophenanthroline (Brumby and Massey, 1967).

EPR detection of the labile iron pool (LIP)

Embryonic axes were homogenized in 40 mM potassium phosphate buffer, pH 7.4, 120 mM KCl. Homogenates ($300 \text{ mg FW ml}^{-1}$) were centrifuged at 10 000 g for 10 min, and the supernatant obtained was re-centrifuged for 1 h at 100 000 g in order to obtain the cytosolic fraction. Either homogenates or cytosolic fractions were added with deferoxamine mesylate (DF, 1 mM final concentration), frozen and transferred to a fingertip Dewar flask containing liquid nitrogen for EPR examination at 77K (Woodmansee and Imlay, 2002). Measurements were performed using the following instrument settings: modulation frequency 50 kHz; microwave power 20 mW; microwave frequency 9.45 GHz; centred field 1600 G; time constant 81.92 ms; modulation amplitude 4.759 G; and sweep time with 800 G. The concentration of the DF-Fe complex [DF-Fe (III)] in the samples was obtained by comparison of the signal height to a standard curve where 1

mM DF was added to solutions of known concentrations of Fe (Yegorov *et al.*, 1993).

Statistical analyses

Data in the text, figures, and tables are expressed as means \pm SE of three to six independent experiments, with two replicates in each experiment. Effect of treatments on measured parameters was tested for significance using single-factor ANOVA. Significantly different means were evaluated using the Tukey post test (GraphPad InStat for Windows Version 3.0; GraphPad Software Inc.).

Results

NO from sodium nitroprusside increases fresh and dry weight in sorghum axes

To characterize the effect of NO on sorghum axes, sodium nitroprusside (SNP, $\text{Na}_2[\text{Fe}(\text{CN})_5\text{NO}]$) was selected as the NO donor. SNP is a suitable compound for long-term treatments (such as 24 h) (Floryszak-Wieczorek *et al.*, 2006) since its stability is higher than that of other known NO releasing compounds. EPR spin trapping in the presence of $\text{MGD}_2\text{-Fe}^{2+}$ was used in order to assess NO content in axes excised from control and SNP exposed seeds. Axes from seeds imbibed for 24 h showed the triplet EPR-signal that matches with the spectrum from a chemically generated $\text{MGD}_2\text{-Fe}^{2+}\text{-NO}$ complex, and represents the endogenous NO content (Fig. 1A). Axes from seeds incubated in the presence of either 100 μM or 1 mM SNP showed a significantly higher NO content compared with control axes (Fig. 1B), while EPR-signals were not detected when the spin trap solution was mixed with the imbibition medium at any SNP concentration in the absence of axes (data not shown). Moreover, when photodegraded SNP (1 mM) was used for imbibition of the seeds, NO content ($2.5 \pm 0.6 \text{ nmol g}^{-1} \text{ FW}$) was not significantly different from that recorded in control axes ($2.4 \pm 0.2 \text{ nmol g}^{-1} \text{ FW}$) (Fig. 1A).

The data in Table 1 show that the imbibition of the seeds for 24 h in the presence of SNP (100 μM and 1 mM) significantly increased both FW and DW in axes, compared with axes excised from seeds placed in distilled water for 24 h. However, there was no change in relative water content (RWC) or in the germination rate of seeds imbibed with SNP up to a concentration of 1 mM as compared to control seeds. It has been described that other compounds with some type of biological activity, besides NO, are generated from SNP. To evaluate if NO was the compound responsible for increasing FW and DW in sorghum axes, control experiments were carried out using potassium hexacyanoferrate (II), $\text{K}_4[\text{Fe}(\text{CN})_6]$, since it has a similar chemical structure to SNP but lacks the ability to produce NO. The parameters tested in axes from seeds imbibed for 24 h in the presence of $\text{K}_4[\text{Fe}(\text{CN})_6]$, were similar to those determined in axes from control seeds (Table 1).

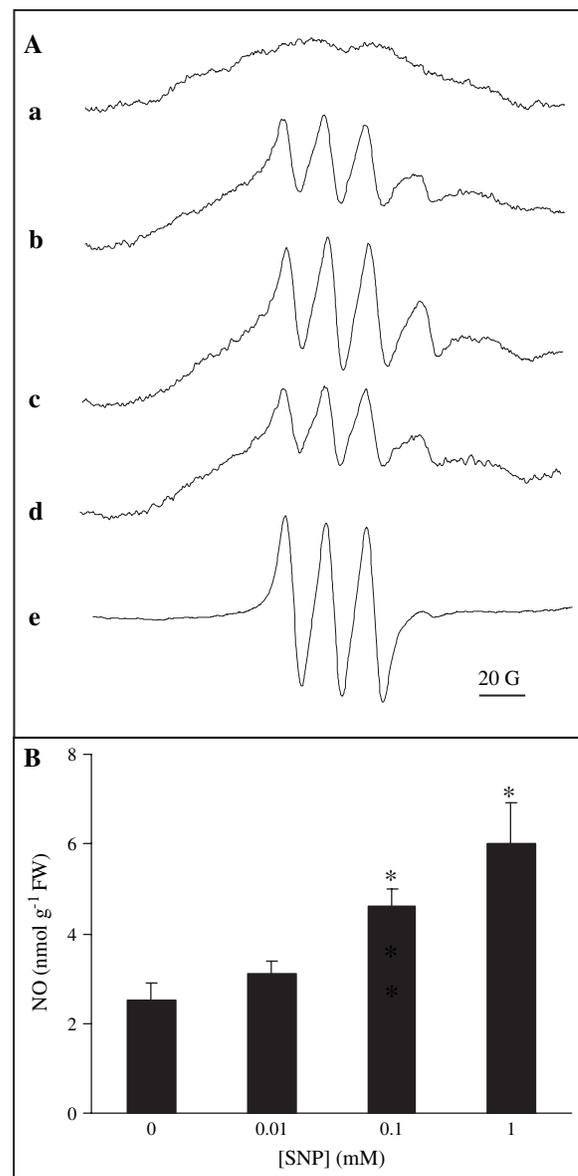


Fig. 1. EPR detection of NO in sorghum embryonic axes. (A) Characteristic EPR signal for the adduct $\text{MGD}_2\text{-Fe}^{2+}\text{-NO}$. Typical EPR spectra corresponding to: (a) solution of $\text{MGD}_2\text{-Fe}^{2+}$ itself, (b) control axes, (c) axes incubated with 1 mM SNP, (d) axes incubated with photodegraded 1 mM SNP, and (e) chemically synthesized adduct $\text{MGD}_2\text{-Fe}^{2+}\text{-NO}$ (250 μM GSNO mixed with the spin trap solution). Homogenates were mixed with the spin trap solution. (B) Quantification of $\text{MGD}_2\text{-Fe}^{2+}\text{-NO}$ adduct signals was carried out using as a stable standard the free radical solution of TEMPOL, setting the same instrument parameters. An asterisk indicates a significant difference from values for control embryonic axes (imbibed for 24 h in distilled water), at $P < 0.05$ (GraphPad InStat for Windows Version 3.0; GraphPad Software Inc.).

NO effect on nitrative and oxidative modifications in sorghum axes proteins

Nitration on the 3-position of tyrosine is a major product of peroxynitrite (ONOO^-) attack on proteins, after its generation from the reaction of NO with superoxide anion

Table 1. Physiological parameters of sorghum seeds imbibed with SNP solutions

Sorghum seeds were imbibed for 24 h in solutions of a NO donor (10 μ M to 1 mM SNP) or 1 mM $K_4[Fe(CN)_6]$. Relative water content (RWC) was calculated as indicated in the Materials and methods. Data are expressed as means \pm SE of four independent experiments.

| | Germination (%) | Fresh weight (mg axis ⁻¹) | Dry weight (mg axis ⁻¹) | RWC (%) |
|------------------------|-----------------|---------------------------------------|-------------------------------------|---------|
| Control | 96 \pm 1 | 6.8 \pm 0.3 | 0.88 \pm 0.06 | 87 |
| SNP (0.01 mM) | 95 \pm 2 | 8.4 \pm 0.6 | 1.05 \pm 0.09 | 88 |
| SNP (0.1 mM) | 97 \pm 1 | 10.0 \pm 0.5 ^a | 1.13 \pm 0.03 ^a | 89 |
| SNP (1 mM) | 96 \pm 1 | 10.8 \pm 0.6 ^a | 1.23 \pm 0.07 ^a | 89 |
| $K_4[Fe(CN)_6]$ (1 mM) | 96 \pm 2 | 6.4 \pm 0.3 | 0.80 \pm 0.06 | 88 |

^a Significantly different from values for control embryonic axes (corresponding to seeds imbibed for 24 h in the presence of distilled water), at $P < 0.05$ (GraphPad InStat for Windows Version 3.0; GraphPad Software Inc).

(O₂⁻) at near diffusion-limited rates (Huie and Padmaja, 1993). This protein modification was evaluated by Western blot techniques using monoclonal anti-nitrotyrosine antibodies. The formation of nitrotyrosine was detected in sorghum embryonic axes from control seeds, suggesting that reactive nitrogen species are physiologically produced in 24 h-imbibed embryonic axes developed in the absence of exogenous NO (Fig. 2A). The content of nitrotyrosine in proteins showed an increase in axes from SNP-exposed seeds in a dose-dependent manner (Fig. 2A), showing significant differences as compared to controls in the presence of 0.1 and 1 mM SNP (Fig. 2B). Oxidative modifications on total proteins of the embryonic axes were studied by Western blot assays using anti-dinitrophenyl (DNP) primary antibodies (Fig. 3A). Embryonic axes from seeds exposed for 24 h to 1 mM SNP showed a significant decrease in the content of carbonylated proteins, as compared to control samples (Fig. 3B).

NO effect on sorghum axes membranes

A spin-trapping EPR technique was used to assess the protective role of NO against lipid oxidative alterations. Microsomal fractions from sorghum embryonic axes were mixed with the spin trap POBN, and the carbon-centred POBN spin adducts were registered, indicating the presence of alkyl, alcoxyl, and peroxy radicals. Samples from axes incubated for 24 h in the presence of 1 mM SNP showed a significantly lower lipid radical content, as compared to control axes (Fig. 4).

In addition, the *in vivo* effect of an NO supplement on membrane integrity was evaluated as the electrolyte leakage from intact embryonic axes, since the loss of ions from intact tissues is considered a suitable parameter for the estimation of membrane injury (Beja-Tal and Borochoy, 1984). Intact axes were excised from seeds incubated for 24 h in the presence of different

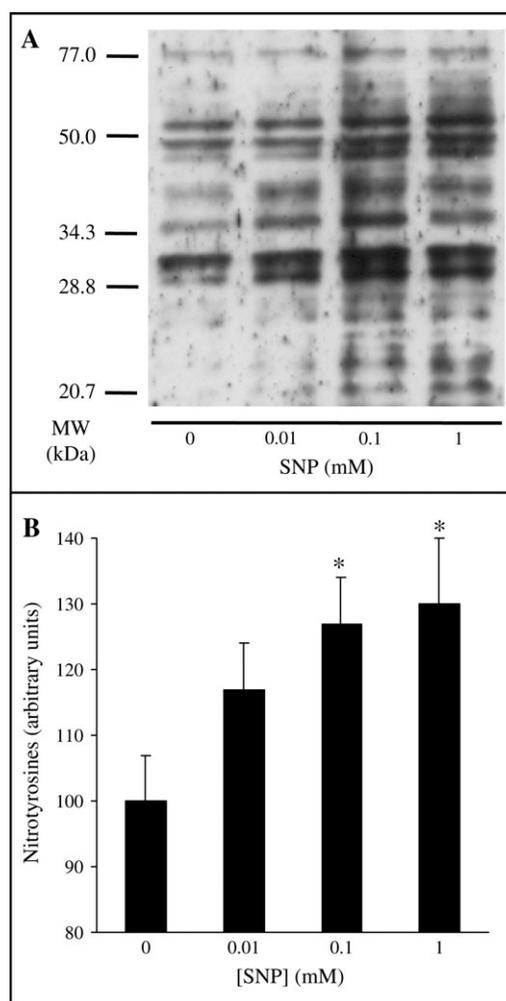


Fig. 2. Analysis of nitrotyrosines in sorghum embryonic axes from seeds incubated for 24 h in the presence of 10 μ M to 1 mM SNP. (A) Nitration of protein tyrosines in homogenates of embryonic axes was evaluated by semi-quantitative Western blotting, using a monoclonal anti-nitrotyrosine antibody. (B) Relative amount of protein nitrotyrosines considering control homogenates (no SNP added) as 100 au. Data are mean values of three independent experiments and bars indicate SE. An asterisk indicates a significant difference from values for homogenates of control embryonic axes, at $P < 0.05$ (GraphPad InStat for Windows Version 3.0; GraphPad Software Inc.).

concentrations of the NO donor, and placed in distilled water to determine the electrolyte leakage. Accordingly, electrolyte leakage in 1 mM SNP-treated axes was significantly lower than in control axes (Fig. 4).

NO increases redox active Fe in sorghum axes

The labile iron pool (LIP) was evaluated in homogenates, filtered homogenates (previously passed through 30 000 Da pore size filter), and cytosol from sorghum embryonic axes exposed for 24 h to SNP. Homogenates from sorghum embryonic axes were mixed with 1 mM DF and examined by low temperature EPR in the region of $g \sim 4.0$. A typical signal corresponding to the adduct

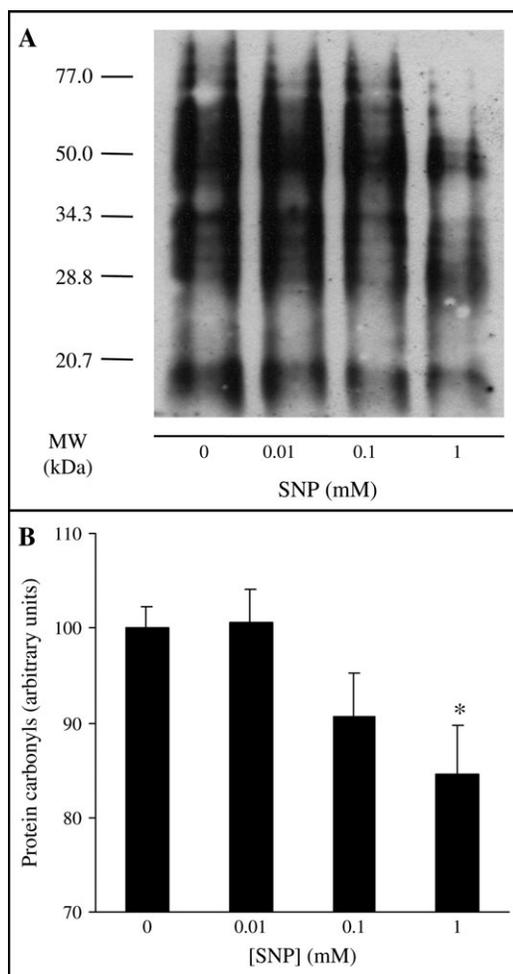


Fig. 3. Protein oxidation in sorghum embryonic axes. Seeds were incubated for 24 h in the presence of SNP (10 μ M to 1 mM). (A) The protein carbonyl content in homogenates of embryonic axes was evaluated by semi-quantitative Western blotting, using anti-DNP antibodies. (B) The relative amount of carbonylated proteins considering control homogenates (no SNP added) as 100 au. Data are mean values of three independent experiments and bars indicate SE. An asterisk indicates a significant difference from values for homogenates of control embryonic axes, at $P < 0.05$ (GraphPad InStat for Windows Version 3.0; GraphPad Software Inc.).

DF-Fe (III) was detected for control homogenates (8 ± 1 nmol g^{-1} FW), suggesting that there is a LIP available to participate in redox reactions under physiological conditions (Fig. 5). Imbibition of the seeds in the presence of 1 mM SNP led to an increase in the content of DF-Fe (III) adduct (19 ± 2 nmol g^{-1} FW), as compared to control homogenates (Fig. 5). Imbibition of the seeds in the presence of 1 mM photodegraded SNP did not affect the content of DF-Fe (III) adduct (9 ± 1 nmol g^{-1} FW) as compared to control homogenates (Fig. 5).

In addition, the same profile (NO exposure and increasing levels of LIP) was observed in filtered homogenates (data not shown), and in the cytosolic fraction of sorghum embryonic axes (Table 2). The exposure of seeds

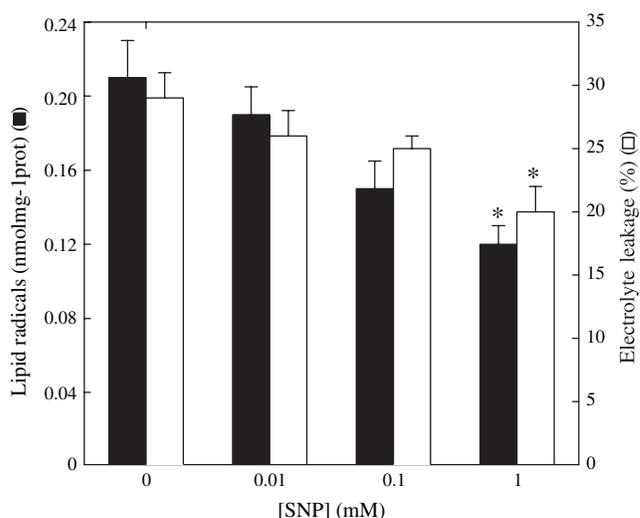


Fig. 4. Effect of the NO donor on axes membranes. Sorghum seeds were exposed to SNP (0 to 1 mM) for 24 h. The lipid radical content in the axes microsomal fraction was evaluated by EPR-spin trapping (filled squares), and ion leakage in intact axes was assessed by conductance measurements (open squares). Data are expressed as means \pm SE of three independent experiments. An asterisk indicates a significant difference from values for control samples (imbibed 24 h in distilled water), at $P < 0.05$ (GraphPad InStat for Windows Version 3.0; GraphPad Software Inc.).

to 1 mM SNP for 24 h significantly increased by 3.2-fold the cytosolic LIP (Table 2).

To make sure that increased levels of LIP were not due to a direct assimilation of the structural Fe present in the SNP molecule ($Na_2[Fe(CN)_5NO]$), the seeds were imbibed for 24 h in the presence of 1 mM potassium hexacyanoferrate (II) ($K_4[Fe(CN)_6]$). The axes excised from these seeds showed a DF-Fe (III) adduct content in the cytosol similar to that found in control axes, suggesting that NO is responsible for the observed effect in axes incubated in the presence of SNP. Moreover, the total iron content in sorghum embryonic axes was evaluated in order to make sure that Fe from SNP was not actively incorporated. As shown in Table 2, the total Fe content spectrophotometrically assessed in sorghum embryonic axes, was not affected either by treatments with SNP up to 1 mM, or by incubation with 1 mM hexacyanoferrate (II). The total Fe content assayed by atomic absorption spectroscopy confirmed the spectrophotometric measurements.

It is important to distinguish the effects of NO from those of the degradation products. In this regard, nitrite was reported to be able to cause protein nitration (Sakihama *et al.*, 2003) and multiple gene expression at nM concentrations (Wang *et al.*, 2007). Thus the effects of nitrite and nitrate on the LIP were tested. No significant differences in the LIP after treatment of the axes with either 1 μ M $NaNO_2$ or $NaNO_3$ was observed, as compared to controls.

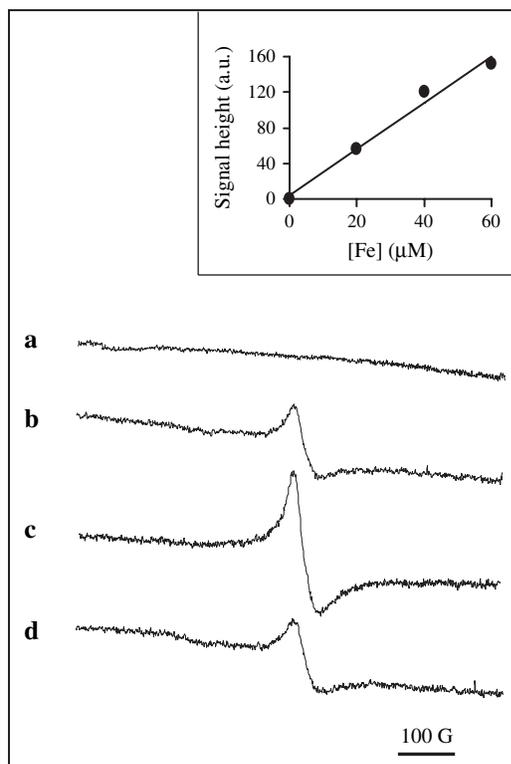


Fig. 5. The labile Fe pool (LIP) in homogenates from sorghum embryonic axes. The axes were homogenized in buffer supplemented with 1 mM DF and used for EPR measurement at 77K. Characteristic EPR signal for DF-Fe (III) adduct corresponding to: (a) buffer alone, (b) control axes, (c) axes exposed to 1 mM SNP, and (d) axes exposed to 1 mM photodegraded SNP. Data in the inset show the standard curve performed using solutions of Fe_2SO_4 (0 to 60 μM) added with 1 mM DF.

Effects of DETA NONOate as a NO donor

The effect of exposure to the NO donor DETA NONOate was assayed on critical parameters over the initial 24 h of imbibition of sorghum seeds. Data in Table 3 show that NO content in the axes was increased by 2.6-fold after exposure to 1 mM DETA NONOate. This effect on NO content was accompanied by a significant increase in the axis fresh weight (index of growth), a significant decrease in electrolyte leakage (index of membrane damage), and a significant increase in the LIP (Table 3).

Discussion

NO is a main character in plant metabolism that could either be generated endogenously or supplemented by the environment. Even though the germination percentage remained over 95% both after the exposure to NO and under physiological conditions, the results presented here indicated that, as the NO steady-state concentration inside the embryonic axes increased FW and DW of embryonic axes also increased, suggesting a beneficial effect of NO upon the early stages of imbibition. Moreover, *in vivo*

Table 2. Total Fe content and labile Fe pool (LIP) in sorghum embryonic axes imbibed with SNP solutions

Sorghum seeds were exposed for 24 h to solutions of the NO donor. Embryonic axes were excised from seeds, mineralized, and total Fe content was evaluated spectrophotometrically. The labile Fe pool (LIP) in the cytosol was assessed by EPR at 77 K using 1 mM DF as Fe chelator. The quantification of the adduct DF-Fe (III) signal was performed using standard solutions of Fe_2SO_4 . Data are expressed as means \pm SE of three independent experiments.

| | Total Fe ($\mu\text{mol Fe g}^{-1}$ FW) | LIP in cytosol (nmol DF-Fe (III) mg^{-1} protein) |
|---|---|--|
| Control | 0.36 ± 0.06 | 2.5 ± 0.2 |
| SNP (0.01 mM) | 0.36 ± 0.06 | 2.8 ± 0.3 |
| SNP (0.1 mM) | 0.39 ± 0.04 | 3.1 ± 0.2 |
| SNP (1 mM) | 0.37 ± 0.08 | 8.0 ± 0.4^a |
| $\text{K}_4[\text{Fe}(\text{CN})_6]$ (1 mM) | 0.36 ± 0.06 | 3.1 ± 0.2 |

^a Significantly different from values for control embryonic axes (corresponding to seeds imbibed 24 h in the presence of distilled water), at $P < 0.05$ (GraphPad InStat for Windows Version 3.0; GraphPad Software Inc).

Table 3. Effect of DETA NONOate on sorghum embryonic axes

Sorghum seeds were exposed for 24 h to distilled water (control) or 1 mM DETA NONOate. Intact embryonic axes were excised from seeds and employed for fresh weight (FW) and electrolyte leakage determinations. EPR measurement of NO content and the labile Fe pool (LIP) were performed in homogenates. Data are expressed as means \pm SE of three independent experiments.

| | Treatment | |
|--------------------------------------|---------------|------------------|
| | Control | DETA NONOate |
| NO content (nmol g^{-1} FW) | 2.4 ± 0.2 | 6.2 ± 0.6^a |
| Fresh weight (mg axis $^{-1}$) | 6.8 ± 0.3 | 9.7 ± 0.9^a |
| Electrolyte leakage (%) | 29 ± 2 | 18 ± 1^a |
| LIP (nmol Fe g^{-1} FW) | 8 ± 1 | 15.2 ± 0.5^a |

^a Significantly different from values for control embryonic axes, at $P < 0.05$ (GraphPad InStat for Windows Version 3.0; GraphPad Software Inc).

exposure of sorghum seeds to NO donors protected axis membranes from electrolyte leakage, that is understood to be an indication of membrane injury due to oxidative damage (Noriega *et al.*, 2007). A protective role of NO against lipid peroxidation was previously reported (Radi, 1998). In this regard a significantly lower lipid radical content was measured in sorghum axes incubated in the presence of 1 mM SNP as compared to the control. Protective effects of NO have also been proposed for protein oxidation since the addition of SNP to bean under UV-B radiation decreased the levels of thylakoidal carbonylated proteins, alleviating the oxidative damage suffered as a consequence of the UV exposure (Shi *et al.*, 2005). In agreement with this information, the data presented here showed a relationship between the increased NO steady-state concentration and the decrease in the level of carbonylated proteins in axes. On the other hand, germination in the

presence of the NO donor led to a significant increase in the content of protein nitrotyrosines in the axes. Associated with the increase in oxygen consumption after 24 h of imbibition of sorghum embryos, a higher steady-state concentration of reactive oxygen species was suggested as compared to the initial conditions (Simontacchi *et al.*, 2003). In this report, a significantly increased NO steady-state concentration was detected after 24 h of exposure to the NO donor as compared to non-treated values, thus ONOO⁻ formation from the reaction between NO and O₂⁻ could be significantly increased *in vivo*. Even though the presence of nitrotyrosines is often associated with pathological conditions (Valderrama *et al.*, 2007), a number of studies have also indicated that the nitration of proteins could significantly alter protein function, protein turn-over, and possibly be involved in signal transduction processes (Ischiropoulos, 2003; Radi, 2004). Thus, further analysis would be required to analyse the role of nitrative modifications in sorghum embryonic axes upon germination.

Surprisingly, in some stress situations, such as those induced by Fe overload, or ethanol in hepatocytes (Sergent *et al.*, 1997), it has been reported that NO protected cells from oxidative damage even though an increase in the labile iron pool (LIP) was reported. Previous data indicated that the LIP represents a minor fraction of the total Fe in a cell (3–5%) (Kakhlon and Cabantchik, 2002). The fraction LIP/total Fe in homogenates from embryonic axes was 2.1% for control axes and 5.2% after exposure of the axes to 1 mM SNP, suggesting that exogenous application of NO could be related to an increase on Fe availability. The steady-state concentration of the LIP could be understood as the addition of the contribution of several Fe adducts, where Fe is bound to each physiological available Fe chelator such as citrate, ATP, ADP, oxalate, and NO, among others.

NO could be bound to Fe-generating dinitrosyl-Fe, dinitrosyl-diglutathionyl-Fe, or dinitrosyl-glutathionyl Fe complexes among other nitrosyl-Fe complexes (Pedersen *et al.*, 2007).

Since DF binds Fe more tightly than do most intracellular metabolites and therefore extracts the Fe from them (Woodmansee and Imlay, 2002), it could be assumed that the EPR method used to evaluate LIP detects these forms of chelated Fe. After the exposure to 1 mM SNP even though total Fe content did not change, LIP was significantly increased, membranes were protected and protein oxidation was reduced compared with physiological conditions. These results could strike as a paradox. This fact could be interpreted by assuming that LIP was increased in the presence of supplemental NO by making Fe available by the allocation of Fe from other biological sources, such as ferritin, thus increasing the concentration of the nitrosyl-Fe complexes. These complexes would be unable to induce oxidative stress as suggested by Sergent *et al.* (1997) in hepatocytes. Also,

Lu and Koppenol (2005) demonstrated by using a chemical system, that NO can inhibit the Fenton reaction by reacting with Fe (II) to form a nitrosylferrate (II) complex. These authors suggested that in complex biological systems an excess of NO would bind to Fe (II) and slow down the Fenton reaction. This hypothesis may explain the beneficial effects of NO (increases in fresh and dry weight, protection of membranes, and decreased content of oxidized proteins), in spite of the increased LIP cellular content as shown here in sorghum embryonic axes.

Even though the mechanism is still not clear, it has previously been reported that, upon application of SNP to cells, NO release is mediated by reducing agents such as thiols, NADH or NADPH (Wang *et al.*, 2006), and that SNP decays to yield cyanide, NO, and nitrite (Sarath *et al.*, 2006). Thus, since compounds other than NO are generated from SNP that might have biological activity (Bethke *et al.*, 2006), DETA NONOate was included in the study to relate the observed effects clearly to NO. The data presented here showed that DETA NONOate-dependent NO release, that occurs by a completely different mechanism from that postulated for SNP, also produced similar changes in LIP after increasing NO content.

In summary, the data presented here showed that sorghum embryonic axes were able to take up NO from an exogenous source and keep it in the cells at a relative high steady-state concentration, as compared to physiological conditions. As a consequence of the NO exposure, membranes and proteins were preserved from oxidative damage during the initial steps of development. However, NO seems to exert a double effect in sorghum axes, increasing Fe availability but preventing its toxicity. In this regard, Graziano and Lamattina (2007) reported that NO supplementation to tomato roots improved plant growth under low Fe supply, suggesting that NO could be a key component of the regulatory mechanisms that control Fe uptake and homeostasis in plants. Further studies are required to understand the nature of the signalling mechanisms that lead to the complex response reported here in sorghum cells during the initial stages of germination.

Acknowledgements

This study was supported by grants from the University of Buenos Aires, Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) and CONICET. SP and MS are career investigators and SJ is a fellow from CONICET.

References

- Alamillo JM, Garcia-Olmedo F. 2001. Effects of urate, a natural inhibitor of peroxynitrite-mediated toxicity, in the response of *Arabidopsis thaliana* to the bacterial pathogen *Pseudomonas syringae*. *The Plant Journal* **25**, 529–540.

- Beja-Tal S, Borochoy A.** 1984. Age-related changes in biochemical and physical properties of carnation petal plasma membranes. *Journal of Plant Physiology* **143**, 195–199.
- Beligni MV, Lamattina L.** 2000. Nitric oxide stimulates seed germination and de-etiolation, and inhibits hypocotyl elongation, three light-inducible responses in plants. *Planta* **210**, 215–221.
- Bethke PC, Badger MR, Jones RL.** 2004. Apoplastic synthesis of nitric oxide by plant tissues. *The Plant Cell* **16**, 332–341.
- Bethke PC, Libourel IGL, Jones RL.** 2006. Nitric oxide reduces seed dormancy in *Arabidopsis*. *Journal of Experimental Botany* **57**, 517–526.
- Blough NV, Zafirou OC.** 1985. Reaction of superoxide with nitric oxide to form peroxynitrite in alkaline aqueous solution. *Inorganic Chemistry* **24**, 3502–3504.
- Bradford MM.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry* **72**, 248–254.
- Briat JF, Lobreaux S, Grignon N, Vansuyt G.** 1999. Regulation of plant ferritin synthesis: how and why. *Cellular and Molecular Life Sciences* **56**, 155–166.
- Brumby PE, Massey V.** 1967. Determination of nonheme iron, total iron and copper. *Methods in Enzymology* **10**, 463–474.
- Cairo G, Recalcati S, Pietrangelo A, Minotti G.** 2002. The iron regulatory proteins: targets and modulators of free radical reactions and oxidative damage. *Free Radicals in Biology and Medicine* **32**, 1237–1243.
- Caro A, Puntarulo S.** 1999. Nitric oxide generation by soybean embryonic axes. Possible effect on mitochondrial function. *Free Radical Research* **31**, S205–S212.
- Colliver BB, Stephenson T.** 2000. Production of nitrogen oxide and dinitrogen oxide by autotrophic nitrifiers. *Biotechnology Advances* **18**, 219–232.
- Conrad R.** 1995. Soil microbial processes involved in production and consumption of atmospheric trace gases. *Advances in Microbial Ecology* **14**, 207–250.
- Crawford NM.** 2006. Mechanisms for nitric oxide synthesis in plants. *Journal of Experimental Botany* **57**, 471–478.
- Curie C, Briat JF.** 2003. Iron transport and signaling in plants. *Annual Reviews in Plant Biology* **54**, 183–206.
- Elstner EF, Oswald W.** 1991. Air pollution: involvement of oxygen radicals (a mini review). *Free Radical Research Communications* **12–13**, 795–807.
- Floryszak-Wieczorek J, Milczarek G, Arasimowicz M, Ciszewski A.** 2006. Do nitric oxide donors mimic endogenous NO-related response in plants? *Planta* **224**, 1363–1372.
- Graziano M, Beligni MV, Lamattina L.** 2002. Nitric oxide improves internal iron availability in plants. *Plant Physiology* **130**, 1852–1859.
- Graziano M, Lamattina L.** 2007. Nitric oxide accumulation is required for molecular and physiological responses to iron deficiency in tomato roots. *The Plant Journal* **52**, 949–960.
- Huie RE, Padmaja S.** 1993. The reaction of NO with superoxide. *Free Radical Research Communications* **18**, 195–199.
- Ischiropoulos H.** 2003. Biological selectivity and functional aspects of protein tyrosine nitration. *Biochemical and Biophysical Research Communications* **305**, 776–783.
- Jurkiewicz BA, Buettner GR.** 1994. Ultraviolet light-induced free radical formation in skin: an electron paramagnetic resonance study. *Photochemistry and Photobiology* **59**, 1–4.
- Kakhlon O, Cabantchik ZI.** 2002. The labile iron pool: characterization, measurement, and participation in cellular processes(1). *Free Radicals in Biology and Medicine* **33**, 1037–1046.
- Keeley JE, Fotheringham CJ.** 1997. Trace gas emissions and smoke-induced seed germination. *Science* **276**, 1248–1250.
- Komarov AM, Lai CS.** 1995. Detection of nitric oxide production in mice by spin-trapping electron paramagnetic resonance spectroscopy. *Biochimica et Biophysica Acta* **1272**, 29–36.
- Kopyra M, Gwózdź EA.** 2003. Nitric oxide stimulates seed germination and counteracts the inhibitory effect of heavy metals and salinity on root growth of *Lupinus luteus*. *Plant Physiology and Biochemistry* **41**, 1011–1017.
- Kruszewski M.** 2003. Labile iron pool: the main determinant of cellular response to oxidative stress. *Mutation Research* **531**, 81–92.
- Laemmli UK.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Laurie SH, Tancock NP, McGrath SP, Sanders JR.** 1991. Influence of complexation on the uptake by plants of iron, manganese, copper, and zinc. I. Effect of EDTA in a multi-metal and computer simulation study. *Journal of Experimental Botany* **42**, 509–513.
- Levine RL, Williams JA, Stadtman ER, Shacter E.** 1994. Carbonyl assays for determination of oxidatively modified proteins. *Methods in Enzymology* **233**, 346–357.
- Lindermayr C, Durner J.** 2007. S-nitrosylation in plants: spectrum and selectivity. *Plant Cell Monographs* **6**, 53–71.
- Lu C, Koppenol WH.** 2005. Inhibition of the Fenton reaction by nitrogen monoxide. *Journal of Biological Inorganic Chemistry* **10**, 732–738.
- Marentes E, Grusak MA.** 1998. Iron transport and storage within the seed coat and embryo of developing seeds of pea (*Pisum sativum* L.). *Seed Science Research* **8**, 367–375.
- Morot-Gaudry-Talarnain Y, Rockel P, Moureaux T, Quillere I, Leydecker MT, Kaiser WM, Morot-Gaudry JF.** 2002. Nitrite accumulation and nitric oxide emission in relation to cellular signaling in nitrite reductase antisense tobacco. *Planta* **215**, 708–715.
- Murgia I, Delledonne M, Soave C.** 2002. Nitric oxide mediates iron-induced ferritin accumulation in *Arabidopsis*. *The Plant Journal* **30**, 521–528.
- Neill SJ, Desikan R, Hancock JT.** 2003. Nitric oxide signalling in plants. *New Phytologist* **159**, 11–35.
- Noriega GO, Yannarelli GG, Balestrasse KB, Batlle A, Tomaro ML.** 2007. The effect of nitric oxide on heme oxygenase gene expression in soybean leaves. *Planta* **226**, 1155–1163.
- Pedersen JZ, De Maria F, Turella P, Federici G, Mattei M, Fabrini R, Dawood K, Massimi M, Caccuri AM, Ricci G.** 2007. Glutathione transferases sequester toxic dinitrosyl-iron complexes in cells. *Journal of Biological Chemistry* **282**, 6364–6371.
- Radi R.** 1998. Peroxynitrite reactions and diffusion in biology. *Chemical Research in Toxicology* **11**, 720–721.
- Radi R.** 2004. Nitric oxide, oxidants, and protein tyrosine nitration. *Proceedings of the National Academy of Sciences, USA* **101**, 4003–4008.
- Sairam RK, Srivastava GC.** 2002. Changes in antioxidant activity in sub-cellular fractions of tolerant and susceptible wheat genotypes in response to long-term salt stress. *Plant Science* **162**, 897–904.
- Sakihama Y, Tamaki R, Shimoji H, Ichiba T, Fukushima Y, Tahara S, Yamasaki H.** 2003. Enzymatic nitration of phytonolins: evidence for peroxynitrite-independent nitration of plant secondary metabolites. *FEBS Letters* **553**, 377–380.
- Sarath G, Bethke PC, Jones R, Baird LM, Hou G, Mitchell RB.** 2006. Nitric oxide accelerates seed germination in warm-season grasses. *Planta* **223**, 1154–1164.
- Sergent O, Griffon B, Morel I, Chevanne M, Dubos MP, Cillard P, Cillard J.** 1997. Effect of nitric oxide on iron-mediated oxidative stress in primary rat hepatocyte culture. *Hepatology* **25**, 122–127.

- Shi S, Wang G, Wang Y, Zhang L.** 2005. Protective effect of nitric oxide against oxidative stress under ultraviolet-B radiation. *Nitric Oxide* **13**, 1–9.
- Simontacchi M, Jasid S, Puntarulo S.** 2004. Nitric oxide generation during early germination of sorghum seeds. *Plant Science* **167**, 839–847.
- Simontacchi M, Sadovsky L, Puntarulo S.** 2003. Profile of antioxidant content upon developing of *Sorghum bicolor* seeds. *Plant Science* **164**, 709–715.
- Sun B, Jing Y, Chen K, Song L, Chen F, Zhang L.** 2007. Protective effect of nitric oxide on iron deficiency-induced oxidative stress in maize (*Zea mays*). *Journal of Plant Physiology* **164**, 536–543.
- Thornton FC, Valente RJ.** 1996. Soil emissions of nitric oxide and nitrous oxide from no-till corn. *Soil Science Society of America Journal* **60**, 1127–1133.
- Valderrama R, Corpas F, Carreras A, Fernández-Ocaña A, Chaki M, Luque F, Gómez-Rodríguez M, Colmenero-Varea P, del Río L, Barroso J.** 2007. Nitrosative stress in plants. *FEBS Letters* **581**, 453–461.
- Wang J, Fillebeen C, Chen G, Andriopoulos B, Pantopoulos K.** 2006. Sodium nitroprusside promotes IRP2 degradation via an increase in intracellular iron and in the absence of S nitrosylation at C178. *Molecular and Cellular Biology* **26**, 1948–1954.
- Wang R, Xing X, Crawford N.** 2007. Nitrite acts as a transcriptome signal at micromolar concentrations in Arabidopsis roots. *Plant Physiology* **145**, 1735–1745.
- Wildt J, Kley D, Rockel A, Rockel P, Segschneider HJ.** 1997. Emission of NO from several higher plant species. *Journal of Geophysical Research* **102**, 5919–5927.
- Woodmansee AN, Imlay JA.** 2002. Quantitation of intracellular free iron by electron paramagnetic resonance spectroscopy. *Methods in Enzymology* **349**, 3–9.
- Yamasaki H.** 2000. Nitrite-dependent nitric oxide production pathway: implications for involvement of active nitrogen species in photoinhibition *in vivo*. *Philosophical Transactions of the Royal Society B Biological Sciences* **355**, 1477–1488.
- Yegorov DY, Koslov AV, Azizova OA, Vladimirov YA.** 1993. Simultaneous determination of Fe(III) and Fe(II) in water solutions and tissue homogenates using desferal and 1,10-phenanthroline. *Free Radicals in Biology and Medicine* **15**, 565–574.
- Zhao MG, Tian QY, Zhang WH.** 2007. Nitric oxide synthase-dependent nitric oxide production is associated with salt tolerance in Arabidopsis. *Plant Physiology* **144**, 206–217.