

# Nitric Oxide Induces Stomatal Closure and Enhances the Adaptive Plant Responses against Drought Stress<sup>1</sup>

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Nitric oxide (NO) is a very active molecule involved in many and diverse biological pathways where it has proved to be protective against damages provoked by oxidative stress conditions. In this work, we studied the effect of two NO donors, sodium nitroprusside (SNP) and *S*-nitroso-*N*-acetylpenicillamine SNP-treated on the response of wheat (*Triticum aestivum*) to water stress conditions. After 2 and 3 h of drought, detached wheat leaves pretreated with 150  $\mu\text{M}$  SNP retained up to 15% more water than those pretreated with water or  $\text{NO}_2^-/\text{NO}_3^-$ . The effect of SNP treatment on water retention was also found in wheat seedlings after 7 d of drought. These results were consistent with a 20% decrease in the transpiration rate of SNP-treated detached wheat leaves for the same analyzed time. In parallel experiments, NO was also able to induce a 35%, 30%, and 65% of stomatal closure in three different species, *Tradescantia* sp. (monocotyledonous) and two dicotyledonous, *Salpichroa organifolia* and fava bean (*Vicia faba*), respectively. In SNP-treated leaves of *Tradescantia* sp., the stomatal closure was correlated with a 10% increase on RWC. Ion leakage, a cell injury index, was 25% lower in SNP-treated wheat leaves compared with control ones after the recovery period. Carboxy-PTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide), a specific NO scavenger, reverted SNP action by restoring the transpiration rate, stomatal aperture, and the ion leakage to the level found in untreated leaves. Northern-blot analysis showed that SNP-treated wheat leaves display a 2-fold accumulation of a group three late embryogenesis abundant transcript with respect to control leaves both after 2 and 4 h of drought periods. All together, these results suggest that the exogenous application of NO donors might confer an increased tolerance to severe drought stress conditions in plants.

Nitric oxide (NO) is a labile free radical that is produced from L-Arg by NO synthase (NOS) in various mammalian cells. NO was originally identified as an endothelium-derived relaxing factor in rabbits, but now it is recognized to be an intra- and inter-cellular mediator of several animal cell functions (Anbar, 1995; Moilanen and Vapaatalo, 1995). At the beginning, based in its ability to react with redox centers in proteins and membranes, NO was found to be toxic or injurious. Later, in animals, functional studies described NO as a molecular component of different signal transduction pathways. Depending on the concentration and the tissue where it is acting, NO can be considered either toxic or protective as well in animals as in plants (Wink et al., 1993; Beligni and Lamattina, 1999c, 2001).

Although plant NOS (gene, cDNA, or protein) has not been isolated yet, both NOS activity and NO accumulation has been reported in different plants species (Ninneman and Maier, 1996; Barroso et al.,

1999). Whereas some authors considered it as a stress-inducing agent (Leshem et al., 1997), others reported its protective role acting as a radical chain reaction breaker under oxidative stress conditions generated by pathogen attack or methylviologen herbicides (Laxalt et al., 1997; Beligni and Lamattina, 1999a, 1999b). New evidences involving NO in signal transduction pathways mediated by some key molecules such as cGMP, cADPR, and  $\text{Ca}^{2+}$  recently have been reported in plants (Durner et al., 1998; Durner and Klessig, 1999).

It is known that the most important factors limiting crop productivity are environmental stresses. The most serious among them is the lack of water. This lack of water occurs when the rate of transpiration exceeds water uptake and is a component of several different stresses including drought, salinity, and low temperatures (McCue and Hanson, 1990). Plants have different mechanisms to avoid water deficit. Among them, stomatal conductance is reduced as part of the systemic response triggered by a signal that originates in the root system. One of these responses is the production of abscisic acid (ABA) that, in turn, elevates cytosolic  $\text{Ca}^{2+}$  concentration in guard cells leading to stomatal closure. Increased concentrations of cytosolic  $\text{Ca}^{2+}$  may be produced either by  $\text{Ca}^{2+}$  influx from outside of the cell or by liberation from intracellular stores. This event involves different mechanisms such as voltage-dependant ion channels, inositol-1,4,5-triphosphate ( $\text{IP}_3$ ) cascade, and cADP-Rib (cADPR) (Muir and

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Sanders, 1996; Kopka et al., 1997; Wu et al., 1997; Blatt, 2000).

At the whole-plant level, the effect of stress is usually perceived as a decrease in photosynthesis and growth, and it is associated with alterations in C and N metabolism. At the molecular level, the negative effect is associated with damage produced by the oxidative stress to the cell, due to a deficiency in energy dissipation, as a consequence of the drought-induced photosynthesis limitation (Loggini et al., 1999). Thus, the oxidative damage of important molecules is produced as a result of the imbalance between production of reactive oxygen species (ROS) of reduced  $O_2$  (i.e. superoxide radical [ $O_2^-$ ], hydrogen peroxide [ $H_2O_2$ ], and the hydroxyl radical [ $OH$ ]) and antioxidant defenses (Iturbe-Ormaetxe et al., 1998).

During water deficit and other osmotic stresses, a plant strategy that may confer stress tolerance is the accumulation of compatible, low-molecular-weight osmolytes, such as sugar alcohols, special amino acids, and Gly-betaine. Another strategy is the activation of a large set of plant genes that leads to the accumulation of new proteins in vegetative tissues. That is the case of a gene family, which codifies for late embryogenesis abundant (LEA) proteins. In all angiosperms, LEA proteins are expressed to high levels during embryo maturation and often in dehydrated seedlings. These proteins are hydrophilic and, in some cases, it has been proposed to confer water stress tolerance. Whereas *lea* mRNAs and the corresponding proteins are not normally found in vegetative or immature seed tissue, the accumulation of some of these products can be induced in response to osmotic stress (Curry and Walker-Simmons, 1993; Ried and Walker-Simmons, 1993; Xu et al., 1996; Swire-Clark and Marcotte, 1999).

The aim of this work was to study, at the physiological and molecular level, the ability of NO to promote adaptive responses to cope with water deficit conditions. In this work, we show correlative evidence to explain why, when treated with NO donors, both wheat (*Triticum aestivum*) seedlings and detached leaves are more tolerant to drought-stress periods.

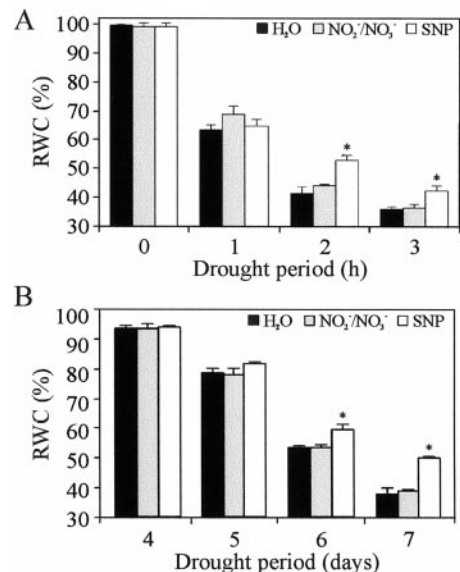
## RESULTS

### NO Diminished Water Loss Produced by Drought Stress

In previous works we demonstrate that NO has the capacity to counteract deleterious effects produced by methylviologen herbicides in plants (Beligni and Lamattina, 1999a, 1999b). We also discussed the dual behavior of NO (i.e. toxic or protective) depending on both the presence of large amount of ROS and the concentration of NO (Beligni and Lamattina, 1999c, 2001).

Since water deficit induces the generation of oxidative stress (Iturbe-Ormaetxe et al., 1998), we first

tested if NO had any protective effect on detached wheat leaves subjected to severe drought periods. As NO donor we used  $150 \mu M$  sodium nitroprusside (SNP), which releases  $0.5 \mu M$  NO, in solution, during the first 48 h (data not shown). With that purpose, we determined relative water content (RWC) of detached wheat leaves subjected to different periods of drought for no longer than 3 h. The experiments were not extended because after 3 h, desiccated leaves cannot regain full turgor when rehydrated. Figure 1A shows that just 1 h of drought is enough to produce a 30% reduction of the RWC. Differences between treatments were significant after 2 h of drought when SNP-treated leaves had an RWC of approximately 53%, whereas in control leaves, treated with either water or  $NO_2^-/NO_3^-$  solution (as control for NO decomposition), RWC was significantly lower (42%). To enlarge the results of NO effect toward whole plant system, we measured RWC of wheat seedlings treated with water or  $1 \mu M$   $NO_2^-/NO_3^-$  or  $150 \mu M$  SNP and subjected to a 10-d drought period. Figure 1B shows that SNP-treated seedlings tended to retain more water than control ones after 5 d without watering. This difference was statistically significant after 6 and 7 d of drought. Treatments with SNP did not affect the cycle of wheat, since all plants come into flowering and ear, normally (data not shown).

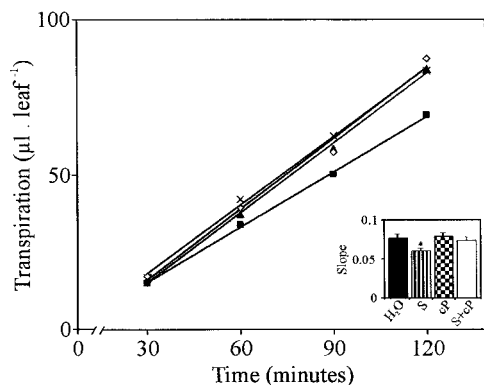


**Figure 1.** Effect of NO on RWC in wheat. A, RWC of detached leaves: fully expanded wheat leaves were detached and treated with water or  $1 \mu M$   $NO_2^-/NO_3^-$  or  $150 \mu M$  SNP and subjected to different drought periods (from 0–3 h). RWC values are expressed as percentages and represent the mean of 10 leaves per treatment repeated in three independent experiments. B, RWC of wheat seedlings: 10-d wheat seedlings watered once with Hoagland plus water or  $1 \mu M$   $NO_2^-/NO_3^-$  or  $150 \mu M$  SNP were kept for 10 d without watering. RWC values were determined after 4, 5, 6, and 7 d of drought and represent the mean of five seedlings per treatment. The values are the average of three independent experiments. Bars correspond to SE of each treatment. Asterisks show significant differences with a  $P < 0.05$  (non-parametric unpaired  $t$  test).

### Effect of NO on Transpiration and Stomatal Opening

A possible effect of NO on transpiration was tested in detached wheat leaves subjected to 3 h of drought (Fig. 2). Those leaves treated with 150  $\mu\text{M}$  SNP show a significant reduction ( $P < 0.05$ ) in the amount of water loss compared with those of control treatments (water). To attribute to NO a role in decreasing transpiration, a specific NO scavenger, carboxi-PTIO, was analyzed on the same experimental system. Carboxi-PTIO alone did not have any effect on transpiration compared with control leaves. However, when combined with SNP, c-PTIO was able to prevent the SNP-mediated decrease of the transpiration (Fig. 2). When transpiration rate (TR, represented by the slope of each straight) was statistically tested, results showed a significant reduction of the TR ( $P < 0.005$ ) in SNP-treated leaves compared with control ones (Fig. 2, inset).

Guard cells modulate leaf transpiration and  $\text{CO}_2$  uptake by changing stomatal aperture (Kopka et al., 1997). Since we could not find the way to detect stomatal opening in wheat leaves, we decided to use leaf peels of another monocotyledonous species (*Tradescantia* sp.), as well as two dicotyledonous species (*S. organifolia* and fava bean [*Vicia faba*]). Epidermis strips from the different species were treated with water, SNP, or SNP + c-PTIO and observed at 400 $\times$  under an optical microscope after 2 h of incubation. NO pretreated peels showed a significant reduction in the stomatal opening (35% for *Tradescantia* sp., 30% for *S. organifolia*, and 65% for fava bean) compared with control treatments, whereas c-PTIO treatments prevented again the SNP-mediated stomatal closure



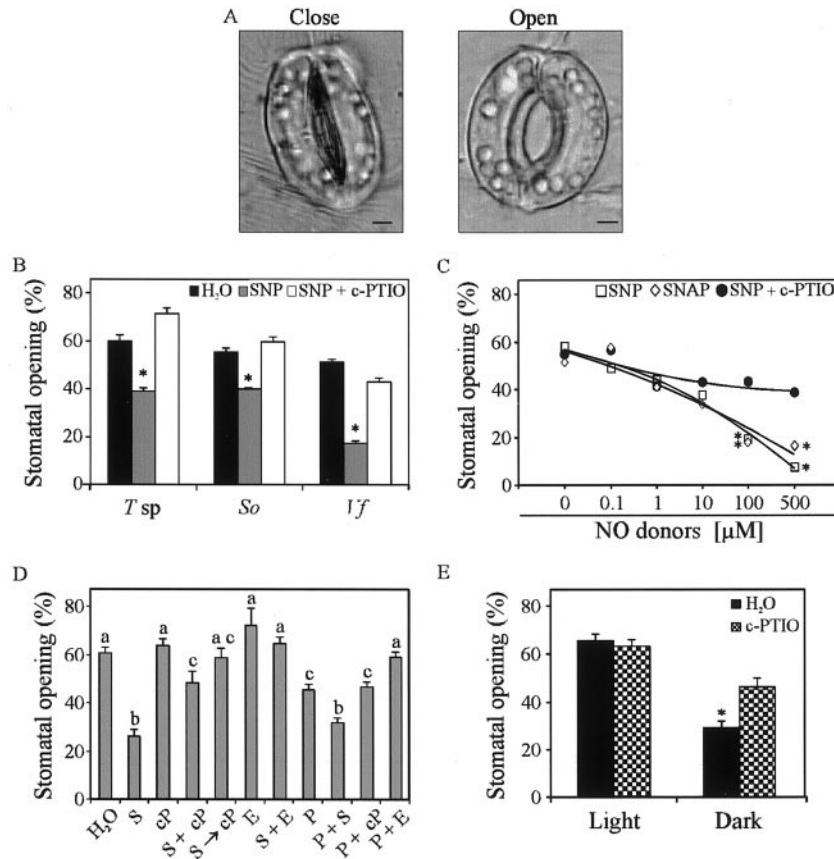
**Figure 2.** NO effect on the TR of detached wheat leaves. Fully expanded first leaves of 10-d-old wheat seedlings were detached and pretreated with water ( $\diamond$ ) or 150  $\mu\text{M}$  SNP ( $\blacksquare$ ), or 300  $\mu\text{M}$  c-PTIO ( $\blacktriangle$ ) or with 150  $\mu\text{M}$  SNP + 300  $\mu\text{M}$  c-PTIO ( $\times$ ) as described in "Materials and Methods." Detached leaves were then maintained on a white paper under light for 3 h (drought period). After that, leaves were transferred into tubes containing 200  $\mu\text{L}$  of water. Water loss was measured and expressed as  $\mu\text{L}$  leaf $^{-1}$ . The TR was represented by the slope of each straight as transpiration per hour. The values are the average of three independent sets of 10 leaves each. The values correspond to SE of each treatment. Asterisk means significant value with a  $P < 0.05$  (non-parametric unpaired  $t$  test).

in the three assayed plant species (Fig. 3B). This result confirmed that NO, and no others derived compounds, was responsible for the induction of stomatal closure. Moreover, complete reversibility of the closure after SNP-treatment was achieved in experiments where c-PTIO was added together with SNP treatment or 1 h after (Fig. 3D; S + cP or S $\rightarrow$ cP, respectively), whereas c-PTIO by itself did not induce stomatal closure (Fig. 3D; cP). When fava bean strips were treated with increasing concentrations of both NO donors, SNP or S-nitroso-N-acetylpenicillamine (SNAP), the percentage of open stomata decreased proportionally to increasing concentrations of the NO-donor, reaching significant differences at concentrations higher than 10  $\mu\text{M}$  of either SNP or SNAP (Fig. 3C). When c-PTIO was added together with SNP treatment (c-PTIO concentration at each point was twice the concentration of SNP), the percentage of open stomata remain constant, independently of SNP concentrations. It is of consequence that the reduction of the percentage of stomatal opening due to NO-treatment was dose-dependent and reversible. Stomatal closure is induced by many different factors, such as osmotic stress, darkness, high  $\text{CO}_2$  concentrations, and some mechanical stresses (Kearns and Assmann, 1993). Regardless of the stimulus, all responses are accompanied by an increase in intracellular  $\text{Ca}^{2+}$  concentrations in the guard cell. Increases of  $\text{Ca}^{2+}$  concentration precede stomatal closure (Grabov and Blatt, 1999). Figure 3D shows that when 2 mM EGTA was added to epidermal strips to chelate intracellular  $\text{Ca}^{2+}$ , NO-induced reduction of stomatal opening was reverted achieving values similar to control treatment. Also, when osmotic stress was imposed by incubating the epidermal strips in a 20% (w/v) polyethylene glycol (PEG) solution, the percentage of open stomata drops down from 60.6%  $\pm$  2.2% to 45.5%  $\pm$  1.8% (Fig. 3D; H $_2$ O and P). This reduction was more important (32.2%  $\pm$  2%) when 100  $\mu\text{M}$  SNP was added to the PEG solution (Fig. 3D; P + S). Even in this case, when  $\text{Ca}^{2+}$  was chelated, the percentage of stomatal opening reach the normal levels (Fig. 3D, P + E).

We also tested if a NO scavenger could be able to interfere with a natural stomatal closure response during a well described stimulus like darkness. Figure 3E provides evidence that, under darkness conditions, the percentage of stomatal closure was partially prevented in *V. faba* (50%,  $P < 0.05$ ) when endogenous NO was scavenged by 200  $\mu\text{M}$  c-PTIO compared with control treatments.

Since results on stomatal closure were not obtained from wheat leaves, we tested the effect of NO on drought stress tolerance in one of the species used for stomatal closure assays. With that aim, we measured RWC on *Tradescantia* sp. detached leaves treated with water or 1  $\mu\text{M}$   $\text{NO}_2^-/\text{NO}_3^-$  or 150  $\mu\text{M}$  SNP and subjected to different drought periods. *Tradescantia* detached leaves were more resistant to desiccation





**Figure 3.** Effect of NO on stomatal opening. A, Open and closed fava bean stomata observed under optical microscope ( $\times 400$ ) (bars =  $5 \mu\text{m}$ ). B, Epidermal strips of a monocotyledonous *Tradescantia sp.* (*T sp*) and two dicotyledonous *S. organifolia* (*So*) and fava bean (*Vf*) leaves were pre-incubated in opening solution and then treated with or without  $200 \mu\text{M}$  SNP and with  $200 \mu\text{M}$  SNP +  $400 \mu\text{M}$  c-PTIO for 2 h. C, Fava bean strips treated for 1 h with increasing concentrations (0, 0.1, 1, 10, 100, or  $500 \mu\text{M}$ ) of SNP ( $\square$ ) or SNAP ( $\diamond$ ) or with increasing concentrations of SNP (0, 0.1, 1, 10, 100, or  $500 \mu\text{M}$ ) plus increasing concentrations (0, 0.2, 2, 20, 200, or  $1,000 \mu\text{M}$ ) of c-PTIO ( $\bullet$ ; SE bars were excluded from the graph to make it clearer). D, Fava bean strips treated with: water,  $150 \mu\text{M}$  SNP (S),  $200 \mu\text{M}$  c-PTIO (cP),  $100 \mu\text{M}$  SNP +  $200 \mu\text{M}$  c-PTIO (S + cP),  $100 \mu\text{M}$  SNP for 1 h and then  $200 \mu\text{M}$  c-PTIO (S  $\rightarrow$  cP), 2 mM EGTA (E),  $150 \mu\text{M}$  SNP + 2 mM EGTA (S + E), 20% (w/v) PEG 8000 (P),  $100 \mu\text{M}$  SNP + 20% (w/v) PEG 8000 (S + P), and PEG 8000 +  $200 \mu\text{M}$  c-PTIO (P + cP) and 20% (w/v) PEG 8000 + 2 mM EGTA (P + E). Bars with different letters are significantly different at  $P < 0.05$ . E, Fava bean strips treated with or without  $200 \mu\text{M}$  c-PTIO under light or darkness. Each value represents the mean of at least 90 stomata taken from different leaves, bars (when present) represent the SE of each treatment. One way ANOVA was used for comparisons between the means. Asterisks indicates those treatments that were significantly different with respect to control treatments ( $P < 0.05$ ).

than wheat leaves. Consequently, the drought periods assayed for *Tradescantia sp.* were longer than those used for wheat. Figure 4 shows that after 18 h of drought, SNP-treated leaves retain approximately 12% more water than control ones.

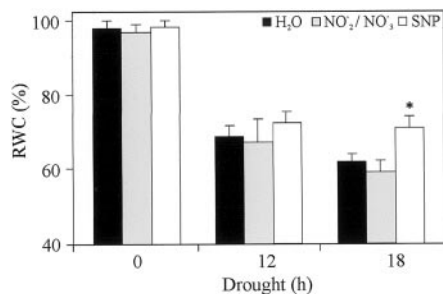
#### Effect of NO on Cell Membrane Injury

One of the described damages provoked by water-deficit stress is the membrane injury and the liberation of ions from the cell to the extracellular space (Halliwell and Gutteridge, 1984). This is a consequence of an oxidative burst leading to lipid peroxidation, membrane permeabilization and cell death (Scandalios, 1993). As shown in Figure 5, ion leakage expressed as a percentage of total conductivity

( $X_i/X_t$ ) increased from 30% at the starting point of the experiment to 75% after 3 h of drought. Pretreatment of leaves with  $150 \mu\text{M}$  SNP showed a slight decrease of the ion leakage after 3 and 4 h of drought, which was not statistically significant. However, ion leakage resulted 25% lower after 4 h of drought and 15 h of recovery in SNP-pretreated leaves compared with control ones (Fig. 5, Rec.). When c-PTIO was added together with SNP,  $X_i/X_t$  increased again up to levels of water treatment, arresting NO effect.

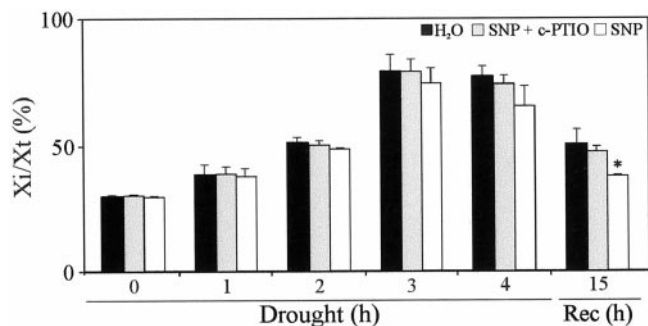
#### Changes in the Expression of LEA Transcript

Several molecules accumulated during water-deficit stress are believed to confer tolerance to des-

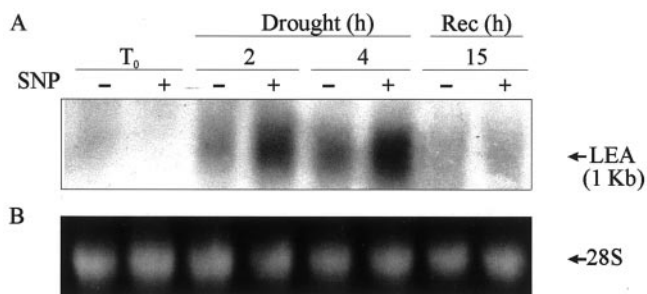


**Figure 4.** Effect of NO on the RWC of *Tradescantia* sp. leaves. Fully expanded detached leaves were floated overnight on water or 1 μM NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> or 150 μM SNP and subjected to different drought periods. RWC values are expressed as percentages and represent the mean of 10 leaves per treatment, repeated in three independent experiments. Bars correspond to SE of each treatment. Asterisks show significant differences with a *P* < 0.05 (non-parametric unpaired *t*-test).

iccation. Among them, the group 3 LEA proteins have been described in desiccation-tolerant seedlings of wheat (Ried and Walker-Simmons, 1993). Therefore, wheat seedlings were pretreated with SNP, and 24 h later the first leaves were assayed to analyze the accumulation level of the group 3 LEA transcript, as a molecular marker of the plant response to drought stress. One of the transcript of approximately 1.0 kb hybridize to pMA1949 cDNA (Fig. 6). After longer exposures (15 d) another transcript of 2.4 kb could also be detected (data not shown). The size of the 1.0-kb transcript is consistent with the molecular masses (27–30 kD) of the four mayor proteins detected with group 3 antibodies in wheat (Ried and Walker-Simmons, 1993). Figure 6 shows that the 1.0-kb transcript was not accumulated in leaves from SNP-treated seedlings just after it had been detached (T<sub>0</sub>). However, after 2 h of drought period, SNP-pretreated leaves exhibited a 2-fold accumulation of the LEA transcript compared with control ones. Fur-



**Figure 5.** Effect of NO on ion leakage in leaves subjected to drought stress. Ten-day-old wheat seedlings were treated with water or 300 μM c-PTIO + 150 μM SNP or 150 μM SNP. Twenty-four hours later, first leaves were detached and subjected to different drought periods (from 1–4 h) or to a 4-h drought period and 15 h of rehydration (Rec). Ion leakage was represented as the percentage of ions leaked at each time compared with the total ion leakage. Results are the average of three independent events. Asterisk shows significant differences with a *P* < 0.05 (non-parametric unpaired *t*-test).



**Figure 6.** Accumulation pattern of a group 3 LEA transcript in NO-treated wheat seedlings. Ten-day-old wheat seedlings were treated with 150 μM SNP or water. Twenty-four hours later, the first leaves were detached and subjected to drought and recovery as described in “Materials and Methods.” A, Total mRNA was isolated from detached leaves, fractionated by electrophoresis, transferred to nylon membranes, and hybridized with a [<sup>32</sup>P]-labeled cDNA probe of the pMA1949 clone (LEA). B, Ethidium bromide-stained 28S rRNA band is shown as loading control. Samples were collected before drought (T<sub>0</sub>), after 2 and 4 h of drought period (Drought), and after 4 h of drought plus 15 h of recovery (Rec).

thermore, this difference was even higher after a 4-h drought period (Fig. 6). It is interesting that after 4 h of drought and 15 h of rehydration by watering leaves to saturation, no differences in group 3 LEA transcripts were detected between leaves from SNP-pretreated seedlings and control ones, being the level similar to those of T<sub>0</sub> (Fig. 6, Rec).

**DISCUSSION**

In this work we demonstrate that NO, when applied exogenously, participates by conferring water-deficit tolerance to both detached wheat leaves and wheat seedlings subjected to drought stress conditions. As discussed by Bray (1997), resistance to water deficit occurs when a plant withstand the imposed stress, and may arise from either tolerance or a mechanism that permits avoidance of the situation. In this case, detached wheat leaves pretreated with the NO releaser SNP withstand the imposed stress by maintaining up to 15% more water than control ones pretreated with water or NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup>. In addition, at the whole plant level, the RWC of SNP-treated wheat seedlings was significantly higher than in control ones after 6 and 7 d of drought (Fig. 1, A and B).

During drought stress processes, stomata are induced to close as leaves sense water deficit, especially after the leaf water potential drops below some threshold level. The production of ABA triggers the increase of cytosolic Ca<sup>2+</sup> concentration in guard cells both via IP<sub>3</sub> signal transduction cascade and also via cADPR. These processes are correlated with a reduction in stomatal aperture (Muir and Sanders, 1996; Grill and Himmelbach, 1998; Leckie et al., 1998).

In animals, NO has been reported to increase intracellular Ca<sup>2+</sup> concentrations both in a cGMP-dependent or cGMP-independent way. These [Ca<sup>2+</sup>]<sub>i</sub>

increases were reported to be a consequence of an uptake from the extracellular space (via L-type membrane channels) or due to  $\text{Ca}^{2+}$  liberation from intracellular stores (via cADPR gated  $\text{Ca}^{2+}$  channels or  $\text{IP}_3$  signal transduction cascade) (Ishii et al., 1997; Volk et al., 1997; Berkels et al., 2000). In plants, some evidences show that NO increased cGMP levels, which in turn stimulates the expression of plant defense genes. Some of these genes were also induced by cADPR, which means that this molecule could be second messenger of NO. On the other hand, cADPR activation of two of these genes was inhibited by a  $\text{Ca}^{2+}$  channel blocker, which locates  $\text{Ca}^{2+}$  downstream of cADPR in the signal transduction pathway (Durner et al., 1998; Klessig et al., 2000). In this work we demonstrate that concentrations lower than 0.2  $\mu\text{M}$  NO, when applied exogenously through an NO donor, decreases the percentage of open stomata in a reversible manner. When  $\text{Ca}^{2+}$  was chelated with 2 mM EGTA, NO effect was completely reverted, suggesting that NO could be acting upstream of  $\text{Ca}^{2+}$ . As expected, when 20% (w/v) PEG was added, the percentage of open stomata was 20% lower than control. This difference was higher (45%) when PEG was added together with SNP. It cannot be concluded yet whether the effects of PEG and NO are acting in an additive way or if both responses can be achieved through different pathways.

In another experiment, c-PTIO alone was added to fava bean epidermal strips and put under darkness. An inhibition of stomatal closure was observed, suggesting that endogenous NO could be playing a physiological role in stomatal movement mechanisms. The overall pore aperture under any given set of environmental conditions is a complex function of the interactions of hormone levels and signal transduction pathways triggered by mechanical and biochemical stimuli. Moreover, new evidences propose that individual stomata behavior is not responding independently and similarly to stimuli (Mott and Buckley, 2000). Thus, a new theory called "patchy stomatal conductance" in which patches of stomata respond differently from those in adjacent regions of the leaf, could be closely related to endogenous production of NO which is a small, highly diffusible and short-lived molecule. NO rapidly crosses biological membranes and can trigger biological responses in a short period of time. Thus, NO might provide some possible explanation to assemble the complex network of cellular components involved in stomatal responses and dynamics. The results presented here bring strong evidences on the capability of NO to modulate stomatal opening by acting within  $\text{Ca}^{2+}$  signal transduction pathway.

Oxidative stress was proven to be generated as a consequence of drought in plants, among other environmental constraints. We have previously reported that NO alleviates several consequences of oxidative stress such as chlorosis, DNA fragmentation, and

apoptotic cell death (Laxalt et al., 1997; Beligni and Lamattina, 1999a, 1999b). One consequence of the oxidative stress is the ion leakage from the cell to the intercellular compartments (Halliwell and Gutteridge, 1984). In this matter, our results indicated that the percentage of ion leakage was significantly lower in those leaves treated with NO, after a drought period, and subsequent rehydration.

A number of water-deficit-induced gene products are predicted to protect cellular structures from the effects of water loss. These predictions are derived from the deduced amino acids sequences and mainly from the gene expression behavior. Some of these genes, called *lea*, were first identified as genes that are expressed during the maturation and desiccation phases of seed development (Close, 1996; Xu et al., 1996). However, it has been recognized that these genes are also expressed in vegetative tissues during periods of water loss resulting from water-deficit and low temperature stress (Curry and Walker-Simmons, 1993; Xu et al., 1996). In this work we report that in detached leaves from wheat seedlings treated with the NO releaser SNP, there is an accumulation of the LEA 3 transcript after different drought periods compared with control leaves and that this accumulation was parallel to an observed increase of water retention. These results are consistent with those reported by Xu et al. (1996) where a high level of the HVA1 protein (a barley group 3 LEA protein) in transgenic rice correlates with an increase in plant tolerance to water-deficit and salt stress. It remains to be demonstrated in our work if a proportional accumulation of LEA protein occurs in parallel to RNA increase. These results suggest that NO may also be acting somewhere in between the signal transduction pathway of LEA expression in response to drought stress.

In this paper, we expand upon our previous data concerning the putative physiological roles of NO in plants. Now, we are presenting results on the potential ability of NO to enhance plant fitness to withstand an environmental constraint like drought, probably through multiple factors that directly or indirectly result in a better housekeeping to make use of the available water.

## MATERIALS AND METHODS

### Plant Material

Wheat (*Triticum aestivum* L. var Oasis) seeds were obtained from EEA-INTA (Balcarce, Argentina). Seedlings were grown in pots containing vermiculite or soil:vermiculite (3:1, v/v). When wheat seedlings were growing in vermiculite, they were watered with Hoagland solution. Pots were kept at 25°C with a 14-h photoperiod, a photosynthetically active radiation (PAR) of 200  $\mu\text{E m}^{-2} \text{s}^{-1}$  and 40% to 50% relative humidity. Fava bean (*Vicia faba* [Leguminosae]), *Tradescantia* sp. (Commelinaceae), and *Salpichroa organifolia* (Solanaceae) plants were grown in soil:vermicu-



lite (3:1, v/v) at the same temperature and light conditions described above.

### Chemicals and Treatments

SNP (Merck, Darmstadt, Germany) and SNAP (Molecular Probes, Eugene, OR) were used as NO donors, and carboxy-PTIO (c-PTIO) (Molecular Probes) were used as NO scavenger. The amount of NO released from 100 and 200  $\mu\text{M}$  donor solutions in our experimental conditions was determined by the Griess reagent colorimetric kit (Cayman Chemical Company, Ann Arbor, MI) according to the manufacturer's instructions and resulted within the range described in the literature from nanomolar to low micromolar amounts (Ferrer and Ros Barceló, 1999).

Ethylene glycol-bis *N,N,N',N'*-tetraacetic acid (EGTA) and PEG 8000 were from Sigma (St. Louis).

### Whole Plant Assays

Wheat seedlings grown in soil:vermiculite (3:1) were watered to saturation with Hoagland solution or Hoagland plus 1  $\mu\text{M}$   $\text{NO}_2^-/\text{NO}_3^-$  or Hoagland plus 150  $\mu\text{M}$  SNP for 10 d at 25°C and 14-h photoperiod. Then, they were maintained for 10 d without watering under the same light and temperature conditions.

### Detached Leaf Assays

Experiments were done with the first, fully expanded leaves from 10-d-old wheat seedlings. Pretreatments were done in two different ways, obtaining similar results: (a) by putting detached leaves from untreated wheat seedlings in tubes containing water, 150  $\mu\text{M}$  SNP, 300  $\mu\text{M}$  c-PTIO, or 150  $\mu\text{M}$  SNP plus 300  $\mu\text{M}$  c-PTIO and submitting them to three pulses of 15 s of vacuum (with a gap of 1 min between each pulse). After vacuum pulses, leaves were transferred to water containing tubes for 2 h under light at 25°C; (b) 10-d-old wheat seedlings were treated with NO donors, and 24 h later the first leaves were detached and used for assays. Drought stress was performed by placing different sets of detached leaves over a white paper under light (200  $\mu\text{E m}^{-2} \text{s}^{-1}$ ) for 1 to 10 h depending on the experiment. After drought periods, different set of leaves were maintained under light in tubes containing distilled water to evaluate their rehydration capacity.

### *Tradescantia* sp. Detached Leaves

Fully expanded *Tradescantia* sp. leaves were detached and floated for 10 h in Petri dishes containing water or 1  $\mu\text{M}$   $\text{NO}_2^-/\text{NO}_3^-$  or 150  $\mu\text{M}$  SNP under light (200  $\mu\text{E m}^{-2} \text{s}^{-1}$ ). Drought treatment was done by putting leaves over a white paper under light at 25°C for different times of stress.

### RWC

RWC was determined for wheat seedlings, detached leaves, and for detached *Tradescantia* sp. leaves. For each

experiment, RWC measurements were determined after different periods of drought, according to the formula:

$$\text{RWC}(\%) = \frac{(\text{FW} - \text{DW})}{(\text{TW} - \text{DW})} \times 100$$

Fresh weight (FW) was measured at the end of the drought period, and dry weight (DW) was obtained after drying the samples at 75°C for at least 24 h. Turgor weight (TW) was determined by subjecting leaves to rehydration for 2 h, after drought treatments.

### Transpiration Assay

These assays were done according to the method described by Wilkinson et al. (1998) with some modifications. Fully expanded first leaf from 10-d wheat seedlings were cut and treated as described before for detached leaves. Later, leaves were kept for 2 h in water and then transferred to ependorff tubes containing 200  $\mu\text{L}$  of distilled water. Leaves were maintained under light (200  $\mu\text{E m}^{-2} \text{s}^{-1}$ ) at 25°C and weighed individually every 30 min for 2 h.

### Stomatal Aperture

Stomatal aperture experiments were performed with epidermis strips taken from fully developed leaves of one monocotyledonous plant, *Tradescantia* sp. (Commelinaceae), and two dicotyledonous plants, fava bean (Leguminaceae) and *S. organifolia* (Solanaceae).

Strips were cut with a scalpel and pre-incubated for 45 min with 10 mM KCl (fava bean) or 2 h with 50 mM PIPES, 50 mM KCl, 1 mM  $\text{MgCl}_2$  (*Tradescantia* sp. and *S. organifolia*) for promoting stomatal opening (at 25°C, under a PAR of 200  $\mu\text{E m}^{-2} \text{s}^{-1}$ ). After pre-incubation, strips were maintained in the opening buffer (control) or treated with different solutions according to the experiment. Chemicals were added to the opening solution in the same plastic pots on which the epidermal strips were floated. After 2 h (*Tradescantia* sp. and *S. organifolia*) or 1 h (fava bean) of incubation, stomatal opening was measured considering open those with a pore width  $>2.5 \mu\text{m}$  or close those with a pore width  $<2.5 \mu\text{m}$ . Measurements were done at 400 $\times$  with an optical microscope, and pore width was calculated using the Matrox Inspector 2.2 (Matrox Electronic System, Dorval, Canada) image analysis software. Each data value represents the mean of at least 90 stomata from at least three different epidermis sections of different leaves. Aperture values are presented as mean  $\pm$  SE.

### Ion Leakage

SNP-pretreated and control (water or SNP + c-PTIO) detached wheat leaves were subjected to different drought periods (from 1–4 h) and to 15 h of water recovery as was previously described. After treatments, leaves were cut into 25-mm<sup>2</sup> pieces and placed in Petri dishes with 12 mL of de-ionized water at 20°C to 25°C for 2 h. After the incubation, the conductivity in the bathing solution was determined (Xi) with an HI8733 conductivity meter (Hanna

Instruments, Sigma). Then, the samples were heated at 80°C for 2 h in their effusates, and conductivity was read again in the bathing solution (Xt). Electrolyte leakage was expressed as a percentage of the total conductivity after heating at 80°C  $[(X_i/X_t) \times 100]$  (Scotti Campos and Thu Pham Thi, 1997).

#### RNA Extraction and Northern-Blot Analysis

Total RNA was extracted with TRIZOL (RNA isolation reagent, Life Technologies/Gibco-BRL, Cleveland) according to the protocol suggested by the company. After the extraction, RNA was resuspended in 5 mM dithiothreitol, quantified, and stored at -80°C for further analysis. Extracted RNAs were separated and analyzed on denaturing 1% (w/v) agarose gels containing formaldehyde. After migration, RNAs were transferred onto a nylon membrane (Amersham, Buckinghamshire, UK) according to the manufacturer's directions and hybridized with the wheat LEA cDNA pMA1949 probe (Curry and Walker-Simmons, 1993). DNA probes were labeled by random priming (DuPont) using  $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ . Hybridization and washing conditions were as described by Laxalt et al. (1996).

#### Statistical Analysis

Each experiment was repeated at least three times. Values are expressed as means  $\pm$  SE. Data were analyzed using Statistica (Stat Soft Inc, Tulsa, OK) software. All mean comparisons were done using *t*-test for independent sample. For stomatal opening assays the different measurements were subjected to a one-way analysis of variance (ANOVA). In all cases the confidence coefficient was set at 0.05.

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