MINI REVIEW

Nitric oxide in plants: the history is just beginning

M. V. BELIGNI & L. LAMATTINA

Instituto de Investigaciones Biológicas, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, CC 1245, 7600 Mar del Plata, Argentina

ABSTRACT

Nitric oxide (NO) is a bioactive molecule that exerts a number of diverse activities in phylogenetically distant species, as well as opposing effects in related biological systems. It was firstly described in mammals as a major messenger in the cardiovascular, immune and nervous system, in which it plays regulatory, signalling, cytoprotective and cytotoxic effects (Ignarro, Annual Review of Pharmacology and Toxicology 30, 535–560, 1990; Anbar, Experientia 51, 545–550, 1995). This versatility is mainly achieved through interactions with targets via either a redox or an additive chemistry (Stamler, Cell 78, 931–936, 1994). For this reason, metal- and thiol-containing proteins serve as major target sites for NO; these include signalling proteins, receptors, enzymes, transcription factors and DNA, among others. Furthermore, NO is a small, highly diffusible molecule. It rapidly crosses biological membranes and triggers various different processes in a short period of time. In this context, NO can co-ordinate and regulate cellular functions of microsomes and organelles such as mitochondria. The ubiquity of NO reactions, as well as the finding that the biochemical and molecular mechanisms underlying many physiological processes are well conserved between diverse species, have opened the exploration of NO chemistry in different organisms. Among these, plants were not the exception. The research in plants has been focused on three main fields: (i) the search for NO or any source of NO generation; (ii) the examination of the effects of NO upon exogenous treatments; and (iii) the search for the same molecules involved in NO-sensitive transduction pathways as in animals (e.g. cGMP, Ca^{2+}, calmodulin). As it is evident from this review, recent progress on NO functionality in plants has been impressive. With the use of biochemistry, molecular genetics and structural biology, together with classical physiological approaches, an explosion of new discoveries will surely begin. It is certainly a good time for plant biologists.

Key-words: germination; light-mediated responses; nitrate reductase; nitric oxide; nitric oxide synthase; oxidative stress; plant hormones; signal transduction; stress tolerance.

THE SEARCH FOR NITRIC OXIDE

Much of the research concerning the possible existence of nitric oxide (NO) in plants has taken into account the mammal counterpart. In these, the enzyme nitric oxide synthase (NOS; EC 1·14·23·29) is the main source that supports NO production. There are at least three NOS isoforms: two constitutive [neuronal NOS (nNOS) and endothelial NOS (eNOS)] and one inducible (iNOS). NOS catalyses NO and l-citrulline formation from one of the guanidine nitrogens of l-arginine requiring NADPH as an electron donor and O_2 as a cosubstrate (Griffith & Stuehr 1995). The cofactors FMN, FADH and tetrahydrobiopterin are also required. The constitutive NOS isoforms depend on Ca^{2+} and calmodulin, whereas the inducible NOS isoforms are calcium-independent. NOS proteins and/or activity have been detected in all vertebrate groups including the larval sea lampreys (Zielinski et al. 1994), in coelenterates such as the starfish Asterias rubens (Elphick & Melarange 1998), in several arthropods, among them Drosophila melanogaster (Regulski & Tully 1995), in the coelenterate Hydra (Colasanti, Lauro & Venturini 1995), in some fungi (Wernery-Felmayer et al. 1994; Ninnemann & Maier 1996), in the euglenozoan kinetoplastid Trypanozoma cruzi (Paveto et al. 1995), and in some bacteria (Chen & Rosazza 1995).

In plants, no gene or cDNA, nor any protein with high sequence similarity to known NOS have been found yet. All the information about putative plant NOS comes from the use of anti-NOS antibodies and the determination of NOS activity. Table 1 is a summary of the plant species, the methods used and the approximate levels found for each NOS activity that has been reported. Kuo et al. (1995) revealed positive immunoreactivity against proteins from yeast and wheat germ in Western blot analysis using mouse antibrain NOS. In pea embryonic axes, a single band of 105·4 kDa was found using rabbit antibrain NOS, whereas two distinct bands of 89·7 and 57·5 kDa were observed in wheat germ (Sen & Cheema 1995). NOS are bimodal enzymes with a carboxy-terminal domain with high sequence similarity to cytochrome P450 reductases (Cyt P450; Bredt et al. 1991), and this makes many anti-NOS cross-react with many oxidoreductases. This is the reason why much care should be taken before ascribing the name of NOS to any reactive band. A very distinguishable feature between NOS and Cyt P450, however, is their weight. In mammals, NOS are in the range of 130–170 kDa. In con-
Table 1. Putative nitric oxide synthase (NOS) in plant tissues

<table>
<thead>
<tr>
<th>Species</th>
<th>Method</th>
<th>Tissue, cellular compartment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td>I (antibrain NOS)</td>
<td>germ</td>
<td>Kuo et al. 1995</td>
</tr>
<tr>
<td>Pea</td>
<td>I (antibrain NOS)</td>
<td>embryonic axes</td>
<td>Sen &amp; Cheema 1995</td>
</tr>
<tr>
<td></td>
<td>I (murine anti-iNOS, C-terminus NOS)</td>
<td>leaf peroxisomes and chloroplasts</td>
<td>Barroso et al. 1999</td>
</tr>
<tr>
<td><em>Mucuna hassjoo</em></td>
<td>A [1·3–2·7 pmol (min mg prot)⁻¹]</td>
<td>total extract</td>
<td>NNinnemann &amp; Maier 1996</td>
</tr>
<tr>
<td><em>Lupinus albus</em></td>
<td>I (NADPH diaphorase), A [1·3 pmol (min mg prot)⁻¹]</td>
<td>roots and root nodules</td>
<td>Cueto et al. 1996</td>
</tr>
<tr>
<td>Tobacco</td>
<td>A [1–6 pmol (min mg prot)⁻¹]</td>
<td>leaves</td>
<td>Durner et al. 1998</td>
</tr>
<tr>
<td>Soybean</td>
<td>A (relative units)</td>
<td>cell suspensions</td>
<td>Delledonne et al. 1998</td>
</tr>
<tr>
<td>Maize</td>
<td>I (macrophage and rabbit antibrain NOS)</td>
<td>root tips and young leaves</td>
<td>Ribeiro et al. 1999</td>
</tr>
</tbody>
</table>

I, immunodetection; A, enzymatic activity.

The putative 166 kDa NOS-like protein has also been detected in the soluble fraction of root tips and young leaves of maize seedlings, based on Western blot analysis using mouse macrophage and rabbit brain anti-NOS (Ribeiro et al. 1999). NOS activity was also found in pea leaves, and four different approaches confirmed the subcellular localization of this protein in peroxisomes, as well as in chloroplasts (Barroso et al. 1999). However, the cellular localization of NOS with the antibody against murine iNOS showed no labelling inside the mitochondria. The activity found in peroxisomes was Ca²⁺-dependent and was also detected with a polyclonal antibody against 14 residues from the C-terminus of iNOS that are absent in the NADPH-cytochrome P450 reductases.

As a general remark, all this indirect evidence supporting NOS presence in plants must be finally confirmed with the finding of any protein with high sequence similarity to described NOS as well as any cDNA or DNA sequences coding for it. Nevertheless, it should be considered that, in plants, where redox chemistry is so rich, other pathways could be operating as the main sources for NO formation. Even in animals, where NOS presence has been thoroughly confirmed, there is some controversy about its protagonism in NO synthesis. Using purified NOS, Schmidt et al. (1996) demonstrated that NO detectable amounts were produced unless superoxide dismutase (SOD) was present. During the respiratory burst in phagocytic cells, SOD synthesizes NO, either directly or via a superoxide anion (O₂⁻) from NO⁻ or N-hydroxy-1-arginine (Schmidt et al. 1996; Modolell, Eichmann & Soler 1997). These results suggest a role for the respiratory burst in NO generation, probably by a two-step reaction in which both NOS and SOD participate.

Nitric oxide can be produced non-enzymatically from NO₂⁻ in the presence of a reductant, such as ascorbate. As this reaction requires the undissociated acid form of NO₂⁻ (HNO₂, pK = 3·2), chemical NO production is insignificant at physiological pH, but it might occur under acidic conditions in tissues and compartments (Weitzberg & Lundberg 1998). In contrast, the enzyme nitrate reductase (NR, EC
1-6.6-1) catalyzes the reduction of \( \text{NO}_2^- \) over a wide pH range. NO emission from NR activity has already been proved in legumes (Dean & Harper 1988; Klepper 1990). Recently, Yamasaki, Sakihama & Takahashi (1999), measured in vitro NO production by a maize NR incubated with \( \text{NO}_2^- \) and NADH. Lower rates were found when \( \text{NO}_3^- \) was used as a substrate and was increased by an excess of NADH. Although legumes have high constitutive NR levels, these results suggest that NR-dependent NO production could be extended to other plant species.

As an alternative to endogenous production, NO may enter the plant cell from the atmosphere or the soil. Soils globally release about 20 tonnes N year\(^{-1} \) as NO, and thus this is an important source for the atmospheric NO budget, which may also increase due to NO produced by fossil fuel combustion (Davidson & Kingerlee 1997). In soils, NO is simultaneously produced and consumed, being the balance regulated by soil conditions. Nitrification and denitrification are the most important NO-generating processes; consumption is due to the activity of various micro-organisms, such as denitrifying bacteria, methanotrophic bacteria and heterotrophic bacteria (Bollmann, Koschorreck & Meuser 1999). Once NO is produced within the atmosphere or the soil, it is available for plants and can have extreme effects on their physiology even if no endogenous source of NO exists. For instance, the exposure of spruce needles to gaseous NO leads to a rapid increase in the intracellular concentration of cGMP achieved through the activation of guanylyl cyclase (Peiffer et al. 1994). This idea implies that NO effects in plant cells could be a result of the endogenous NO formation plus exogenous NO concentration and, as a consequence, NO actions might be accomplished in a non-regulated fashion. Future work on the elucidation of NO origin/s in plants will have to take into account this and all possible sources of NO that may affect plant growth and development.

**NITRIC OXIDE ACTION IN PLANTS**

Plant metabolism is highly influenced by NO and, as a consequence, another field of research has focused on NO effects in plant physiology. This has been mainly accomplished by exogenous application of NO donors in a wide range of concentrations to a great variety of plant systems, although this has some disadvantages. First, as NO is a gas it is mostly applied in the form of donor compounds that release NO into solution. For this reason, the concentration of NO inside the plant tissue depends, among others, on some chemical features, such as the kinetics of release from the donor, the temperature and the reducing power (Tanno et al. 1996; Ramamurthi & Lewis 1997). This compels the researcher to further determine NO concentration in tissue, a task that is not always easy and reliable. Even in animals, there is still uncertainty about the physiological concentration of NO (T. Higenbottam, Symposium: The clinical use of inhaled nitric oxide, Stockholm, Sweden 1998). Second, with exogenous applications it is difficult to determine whether the effects really have physiological implications or are merely pharmacological. However, it may give a first approximation that can then be complemented with other studies. The main subjects under study up to date are NO’s possible role in plant growth and development, in signalling mechanisms and during plant stress situations.

**NO during the plant life cycle**

In rapidly growing pea foliage, a mixture of KNO\(_2\) and KI/H\(_2\)SO\(_4\) used to generate NO proved to have a dual behaviour (Leshem & Haramaty 1996). Low micromolar NO concentrations (5 \( \times 10^{-6} \) m) produced an increase in the rate of leaf expansion, whereas no promotive effect occurred at higher concentrations (12.5 \( \times 10^{-6} \) m). In the same direction, nanomolar to low micromolar amounts of the NO donors sodium nitroprusside (SNP), S-nitrosoglutathione and S-nitroso cysteine induced growth elongation in maize root segments, to the same extent or even more than the same concentration of indole acetic acid (IAA) (Gouvêa et al. 1997). The NO-mediated effect was reduced when methylene blue, an NO scavenger, was added together with any of the NO donors, but did not affect the elongation provoked by IAA. Although preliminary, the authors suggested that, if any possible link exists between NO and IAA-induced root elongation, it is not in steps that occur at the plasma membrane such as: (i) H\(^+\) extrusion for cell wall expansion; or (ii) Ca\(^{2+}\) entrance through specific channels. Related results were found by Ribeiro et al. (1999) when anti-NOS antibodies detected a protein from the cytosol of the active division zone of maize cells. Moreover, that protein was transferred to the nucleus in the elongation zone of maize root tips, suggesting a possible participation of NO in the activation of transcription factors involved in gene expression that regulates the rapid growth of the elongation zone. In animals, the transcription machinery is considered to be one of the main targets for NO action, especially for genes involved in long-term adaptative responses (Malyshiev et al. 1999).

Nitric oxide also seems to affect other events of the plant life cycle. In senescing pea foliage, NO has been shown to be simultaneously emitted with ethylene (Leshem & Haramaty 1996). On the other hand, NO concentration appeared to be lower in mature fruits than in green fruits and in senescing flowers compared with fresh ones (Leshem, Wills & Veng-Va Ku 1998). Taking into account the hypothesis that a decrease in NO production is associated with maturation and senescence, the authors applied exogenous NO to flowers, fruits and vegetables. In all cases, NO extended the post-harvest life and delayed senescence. The authors hypothesized that a stoichiometric relationship between the two gases, NO and ethylene, probably determined whether senescence took place or not.

The effect of NO on plant peroxidases, which are haem-containing enzymes and therefore, possible targets for NO action has also been studied. Ferrer & Ros Barceló (1999) found a differential effect of NO on lignification of xylem vessels. In their system, 5 mm SNP or 50 mm NO were able to inhibit guaiacol peroxidase activity in 42 and 62%,
respectively. Hydrogen peroxide production, which is used by guaiacol peroxidases for lignin biosynthesis, was not affected by NO.

Nitric oxide and its related nitrogen oxides have also been reported as stimulators of seed germination. Seeds of *Paulownia tomentosa* require long periods of light irradiation to germinate. However, a single red light (R) pulse was sufficient to induce germination if the seeds were supplied with 10 mM KNO₃ (Grubišić & Konjevic 1990). Sodium nitroprusside and some other inorganic and organic nitrates, stimulated germination with a similar efficacy, with optimal concentrations between 1 and 10 mM (Grubišić & Konjevic 1990; Grubišić, Giba & Konjevic 1992). Consequently, the authors suggested that the effect of nitrate compounds was due to their reduction to NO and that phytochrome-regulated dormancy breakage was closely connected with redox changes. Accordingly, in animals, various effects of organic nitrate esters had been attributed to their biological reduction by cytochrome P-450 (McDonald & Benett 1990).

In the same way, dormant seeds of a California chaparral were induced to germinate by smoke or vapours emitted from smoke-treated sand or paper (Keeley & Fotheringham 1997). Among the gaseous emissions, nitrogen oxides (NO + NOₓ and NO₂) induced 100% germination in a manner similar to smoke. Germination was not the result of changes in imbibition of the seeds. As already stated, organic matter combustion or post-fire biogenic nitrification generates considerable amounts of nitrogen oxides. These processes, during chaparral wildfires, occur in sufficient extent to trigger germination.

The stimulation of light-regulated germination by NO was also demonstrated for lettuce seeds (*Lactuca sativa* L. cv Grand Rapids) (Beligni & Lamattina 2000a). At temperatures between 26 and 32 °C, lettuce seeds need light to germinate (Bewley & Black 1982). In this system, dark-imposed dormancy of lettuce seeds could be broken by either 100 μM SNP or 100 μM S-nitroso-N-acetylpenicillamine (SNAP), two NO donors, in the same extent as 100 μM giberellic acid (GA₃). NO was even more potent than GA₃, since 50% germination was observed when the seeds were imbibed with 10 μM SNP, which releases nanomolar concentration of NO, whereas the same GA₃ concentration provoked only 2% of germination. Moreover, the SNP-mediated effect was arrested by the NO scavenger carboxy-PTIO. Dormancy of lettuce seeds can not be broken in the dark at temperatures above 34 °C. Under these conditions, NO was unable to induce the germination, suggesting that NO only participates in light-mediated germination, with no general action on all other types of dormancy. It would be interesting to study if the same signal transducers are involved in both NO- and GA-mediated stimulation of germination.

In the same work, NO also proved to increase chlorophyll levels of wheat seedlings grown in the dark. Short-term light pulses of white or red light produced a two- to three-fold increase in the chlorophyll content of NO-treated seedlings over H₂O-treated (control) ones, evidencing a putative role for NO in de-etiolation. The difference between NO-treated seedlings and control ones was even higher when seedlings were subjected to abiotic (wounding) or biotic (infection with the fungus *Septoria tritici*) action, suggesting a possible connection between NO, light and stress signalling pathways in plants (Beligni & Lamattina 2000a).

Another process during the plant life cycle that is affected by light is hypocotyl and internode elongation. Light causes an inhibition of both processes, by reverting the etiolated phenotype which consists of chlorotic and longer organs. In three different plant species (*Arabidopsis thaliana* L. cv Columbia, *Lactuca sativa* L. cv Grand Rapids and *Solanum tuberosum* L. cv Pampeana) grown in the dark or at low light intensity, nanomolar amounts of NO were able to significantly reduce hypocotyl and internode elongation (Beligni & Lamattina 2000a).

Table 2 shows the processes in which NO appears to be involved in plants. The overlapping between its actions and those of well-known plant hormones opens the question of whether NO could also present hormonal properties. Future work on the analysis of more functions of NO in plants, its possible mechanisms of action and its functional relation with plant hormone targets will certainly shed light on this question.

**NO and biotic stress**

The ability of NO to act as a stress-coping factor was studied for biotic stress in plants. Treatment of potato tuber tissues with the NO donor NOC-18 (1–10 mM) provoked an accumulation of the phytoalexin rishitin, an endogenous antibiotic compound. The effect of NOC-18 was counteracted by the specific NO-scavenger carboxy-PTIO and by the free radical scavenger Tiron (Noritake, Kawakita & Doke 1996). Hyphal wall components (HWC) also elicited the production of rishitin and had an additive effect with NOC-18. However, carboxy-PTIO did not affect HWC-induced rishitin increase, suggesting that NO and HWC signals are probably transduced through independent pathways.

Nitric oxide also proved to protect chlorophyll levels in potato leaves that were infected with the pathogen *Phytophthora infestans* (Beligni, Laxalt & Lamattina 1997; Laxalt, Beligni & Lamattina 1997). The effect was achieved with low NO concentrations (between 10 and 100 μM SNP). Lower concentrations were ineffective, whereas higher ones resulted in chlorophyll decay. Kunert (1995) has described the effect of NO donors on the survival of conidia, germination and growth of *Aspergillus fumigatus*, an opportunistic fungus that is a pathogen for animals. The report concluded that NO was probably not a major effector molecule in the killing of phagocytized fungal elements by the host’s immunocytes. Accordingly, NO did not affect *P. infestans* growth and infectiveness, suggesting that its protective effect in potato was related with plant defence mechanisms (Laxalt et al. 1997). This was also supported by an increase in the mRNA levels of phenylalanine-ammonia lyase (PAL), β-1,3-glucanase and glyceraldehyde-3-phos-
phate dehydrogenase upon treatment with NO donors (Beligni et al. 1997). These three enzymes are up-regulated and involved in plant defence mechanisms during plant pathogen interactions (Fritzemeier et al. 1987; Schröder, Hahlbrock & Kombrick 1992; Laxalt et al. 1996). Laxalt et al. (1997) hypothesized that NO-mediated chlorophyll maintenance could be directed either to chloroplast membrane integrity against the toxicity of reactive oxygen species or to any of the steps of chlorophyll turnover rate. As stated, the ability of NO to scavenge reactive oxygen species (ROS) had thoroughly been reported in animals (Stamler 1994). In plant–pathogen interactions, ROS are overproduced, and they have both signalling and toxic roles (Sutherland 1991; Levine et al. 1994). In accordance, NO proved to counteract some toxic processes that occur during potato–P. infestans interaction and that are attributed to ROS, such as DNA fragmentation, ion leakage to intercellular compartments and cell death (Beligni & Lamattina 1999b).

In the tobacco–Pseudomonas solanacearum pathosystem, NO donors were able to invoke a hypersensitive response (HR) (Huang & Knopp 1997). HR is a response characterized by rapid host cell death at the pathogen penetration site. As a result, the growth and development of the fungus is prevented and, thus, spreading to other parts of the plant is prevented (Schröder et al. 1992). This HR is triggered by a rapid and transient production of ROS, called the oxidative burst (Hammond-Kosack & Jones 1996). In a similar direction, an increase in NOS activity has been detected after infection of resistant, but not susceptible, tobacco with TMV (Durner et al. 1998). Furthermore, administration of NO donors or recombinant NOS protein to tobacco plants or cell suspensions, triggered expression of pathogenesis-related protein 1 (PR-1) and PAL and increased total salicylic acid (SA) levels. SA is a plant messenger molecule involved in responses to biotic stress. SA produces PR-1 accumulation but does not increase levels of PAL, which occurs through H$_2$O$_2$ (Levine et al. 1994). The ability of NO to up-regulate PAL mRNA levels (Beligni et al. 1997; Durner et al. 1998) means that this gas may be involved in plant defence mechanisms by both SA-dependent and -independent pathways (Van Camp, Van Montagu & Inzé 1998).

As explained above, H$_2$O$_2$ appeared to orchestrate the production of HR during plant infection with pathogens (Levine et al. 1994). However, the generation of O$_2^-$ within soybean cell suspensions proved to induce in some cases only a weak cell-death response (Delledonne et al. 1998). In this system, treatment with a relatively high NO concentration (500 μM of the NO donor, SNP) markedly potentiated the induction of cell death by H$_2$O$_2$ or O$_2^-$ or by an endogenous oxidative burst evoked by Pseudomonas syringae. The effect was arrested by carboxy-PTIO, by L-NNA (a NOS inhibitor) and by diphenylene iodonium (an inhibitor of NADPH oxidase-dependent oxidative burst). Inoculation of soybean cell suspension with P. syringae stimulated NO production and this was arrested by SOD or catalase, suggesting a link between NO and H$_2$O$_2$ during HR. Moreover, infiltration of NOS inhibitors to Arabidopsis leaves blocked HR, and induced the typical chlorosis and cell death spread observed during compatible interactions (Delledonne et al. 1998).

### NO and abiotic stress

Nitric oxide action has also been studied during abiotic stress conditions. An increase in NO production has been detected during both water and short-term heat stress (Leshem & Haramaty 1996; Leshem et al. 1998). Since many environmental stress responses are associated with ethylene upsurge, the authors extended their hypothesis about the relation of NO with ethylene for the establishment of stress responses within the plant. However, the treatment of pea leaves with high NO concentrations provoked symptoms that evidenced stress (Leshem et al. 1998). The NO releasing compound SNAP increased chlorophyll fluorescence, a factor often associated with impaired electron transport along photosystem II (PSII) caused by stress.

---

**Table 2.** Effects of NO on different plant developmental stages and its overlapping with the action of plant hormones

<table>
<thead>
<tr>
<th>Process</th>
<th>NO-mediated effect</th>
<th>Hormone involved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth and differentiation</td>
<td>Low: induction</td>
<td>Induction: auxins and cytokinins</td>
</tr>
<tr>
<td></td>
<td>[leaf expansion:</td>
<td>(Coenen &amp; Lomax 1997)</td>
</tr>
<tr>
<td></td>
<td>Leshem &amp; Haramaty</td>
<td></td>
</tr>
<tr>
<td></td>
<td>root growth:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gouveia et al. 1997</td>
<td></td>
</tr>
<tr>
<td></td>
<td>High: inhibition</td>
<td>Stimulation: ethylene</td>
</tr>
<tr>
<td></td>
<td>(Leshem &amp; Haramaty</td>
<td>(Orzaez &amp; Granell 1997)</td>
</tr>
<tr>
<td>Senescence</td>
<td>Delay (Leshem &amp;</td>
<td>Stimulation: giberellins</td>
</tr>
<tr>
<td></td>
<td>Haramaty 1996)</td>
<td>(Bewley 1997)</td>
</tr>
<tr>
<td>Seed germination</td>
<td>Stimulation</td>
<td>Stimulation: giberellins and</td>
</tr>
<tr>
<td></td>
<td>(Grubišić &amp;</td>
<td>cytokinins (Chory 1997)</td>
</tr>
<tr>
<td></td>
<td>Konjevic 1990;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Keeley &amp;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fotheringham 1997;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Beligni &amp;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lamattina 2000a</td>
<td></td>
</tr>
<tr>
<td>De- etiolation</td>
<td>Stimulation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Beligni &amp;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lamattina 2000a</td>
<td></td>
</tr>
<tr>
<td>Hypocotyl elongation</td>
<td>Inhibition</td>
<td>Inhibition: giberellins</td>
</tr>
<tr>
<td></td>
<td>(Beligni &amp;</td>
<td>(Sauter, Seagull &amp; Kende 1993)</td>
</tr>
<tr>
<td></td>
<td>Lamattina 2000a</td>
<td></td>
</tr>
</tbody>
</table>

* When data are available, the effects of low and high NO concentrations are presented.
SNAP also caused an increase in the surface tension of artificial monogalactolipid diacylglycerol monolayers, in a similar way to $O_2^-$. These lipids are very abundant within the curved sections of thylakoids and are known to confer microfluidity to the lipid bilayer matrix due to their unsaturated $C=C$ bonds (Carruthers & Melchior 1986). This, or an effect on any photosynthetic protein, could certainly alter the electron transport, resulting in higher chlorophyll fluorescence. Treatment of pea foliage with SNAP at a concentration equivalent to 1 mM NO significantly reduced lipoxygenase (LOX) activity. LOXs are a complex array of distinct enzymes playing regulatory roles in the production of defence-related compounds. Together, these results evidenced that, at high concentrations, NO can also be a stress-inducing factor in plants.

In another work (Beligni & Lamattina 1999c), ROS were artificially generated by treatments with diquat and paraquat, two bypyridinium herbicides that produce superoxide anion ($O_2^-$) inside the chloroplast (Dodge 1994). In this system, NO at low concentrations (100 nM to 1 μM) was able to reduce the extent of chlorophyll loss, ion leakage, necrosis and defoliation produced by the herbicides. NO-mediated protection against diquat, where unprogrammed cell death is expected to occur, was also extended to the photosynthetic machinery. Thus, diquat-triggered lipid peroxidation, ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) and D1 protein loss and mRNA breakdown were markedly reduced by NO (Beligni & Lamattina 2000b). Moreover, diquat caused an increase in the rate of photosynthetic electron transport in isolated chloroplasts, and NO restored it to control levels (Beligni & Lamattina 2000b). All these effects seem to come from an NO-mediated reduction in the free amount of ROS (Fig. 1). Consequently, a possible participation of NO in the antioxidant cellular system, as it does in animals, was strongly opened by these results. Moreover, as it is already known, adverse environmental conditions generate oxidative stress in plants. Nitric oxide was able to reduce the oxidative injury produced by drought on wheat seedlings and UV-B radiation on potato leaves, suggesting a putative role as an anti-stress molecule in plants (García-Mata, Beligni & Lamattina 2000).

**What are the real roles of NO and ROS during stress?**

There is an apparent contradiction between the situation in which NO appears to counteract ROS actions and those in which it acts in the same direction or synergistically with ROS. However, in a broad sense, there is no reason to think about contradictions. One of the most striking features of NO in animals is its ability to accomplish both cytotoxic and cytoprotective roles. NO is a reactive nitrogen species and therefore can start chain reactions that cause cell injury. This has been observed when NO is overproduced, for example in killing of bacteria and tumour cells by macrophages (Nathan 1995). One of the fastest reactions of NO within biological systems is the combination with ROS, a process that is well described as either toxic or protective. In animals, at high NO-donor concentrations (e.g. 0.5–1 mM sodium nitroprusside), NO is generally toxic. In these conditions, when combined with low amounts of superoxide anion ($O_2^-$), the formation of peroxynitrite (ONO$O_2^-$) was reported to be deleterious to lipids, proteins and DNA (Lipton *et al.* 1993). However, even under these conditions, when toxicity is incurred predominantly from ROS, NO may act as a chain breaker and thus limit damage. In these situations, peroxides are much more toxic than NO and ONOO$^-$, exerting NO, a protective function (Wink *et al.* 1993). NO has also been shown to play both of its actions by signalling mechanisms. For example, NO-mediated prevention of apoptosis in some mammal cells has been attributed to an inhibition of caspase-3-like activity (Kim, Talanian & Billiar 1997). As a concluding remark, the action of NO depends on its concentration and on the system analysed, and therefore, NO can be a friend or an enemy, causing some authors to introduce the term ‘NO paradox’.

<table>
<thead>
<tr>
<th></th>
<th>H$_2$O</th>
<th>SNP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diquat</strong></td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
</tbody>
</table>

Figure 1. Nitric oxide (NO) decreases ROS free amount. Potato leaflets were pretreated with 100 μM sodium nitroprusside (SNP) or $H_2$O (control) for 24 h and then treated with 0.4 mg ml$^{-1}$ diquat. Twenty-four hours after diquat application, the leaflets were harvested for diaminobenzidine (DAB) assays. Leaflets were submerged in a solution containing 1 mg ml$^{-1}$ DAB and kept for 18 h at 25 °C in the dark. (a) The $H_2O_2$-dependent oxidation of DAB forms a brown precipitate over the sections of the leaflets where hydrogen peroxide is present. (b) The leaflets were de-coloured with 100% ethanol for a better visualization.
Nitric oxide in plants

(Malyshev et al. 1999). As this is a consequence of its chemical reactivity, NO duality is expected to occur also in plants (Beligni & Lamattina 1999a).

Figure 2 summarizes the possible sources of protection and toxicity in plants in which NO may actively participate. The point is that neither of the two roles is exclusive or more important. Depending on the cell condition, NO can kill, but also let live if it results in an effective mechanism to cope with a stress condition. In fact, in plant–pathogen interaction, HR and cell death are recognized as part of the defence responses. However, these processes have also been proposed as an advantage for certain pathogens that live by killing host cells (McDowell & Dangl 2000). In this frame, NO resembles ROS, since they can act as signals for the activation of defence responses against stress (Levine et al. 1994) and, at the same time, higher amounts produced by uncontrolled ROS generation can cause severe injury to the cell. In addition, the role of HR in plant disease resistance is permanently challenged because there are remarkable differences in the death process for different pathosystems (Heath 1999). It is therefore essential to know very precisely the physiological situation under study to correctly interpret the results when dealing with free radicals and to assign definitive roles to NO. In plants, where very little data about endogenous NO production and variation levels between conditions are available, such physiological roles cannot be established yet.

**NO in legumes: a special case**

Legumes have always been a special target for the study of nitrogen metabolism, due mainly to their high protein content, their high levels of NR activity and the formation of nodules where symbiosis with *Rhizobium* takes place. In legume nodules, there occurs N₂ fixation by nitrogenase, which forms NH₄⁺ to be incorporated into amino acids. There, NO was first supposed to be generated from NO₃⁻, by either enzymatic or non-enzymatic production, as a

---

**Figure 2.** Schematic representation of nitric oxide (NO) effects in plants. The chemical reactions of NO for which more experimental evidence exists are shown. Stressing environmental clues trigger many responses within the plant cell (blue arrows). Among them, NO-generating systems are activated. In those situations, NO actions may be either cytotoxic (pink area and red arrows) or cytoprotective (green area and arrows). NO reaction with reactive oxygen species would account for both toxicity and protection. Indirect methods of protection would also come from interaction with or activation of the cellular antioxidant system by NO. On the other side, light or developmental signals (yellow arrows) could also activate NO production and modulate processes such as greening, growth, germination or senescence. This could be accomplished through cGMP, cADPR and Ca²⁺/calmodulin pathways (black arrows) and/or by direct modification of target molecules (e.g. S-nitrosylation, ADP-ribosylation). Protein nitration by ONOO⁻ is another putative mode for NO action. NO-mediated down regulation of ethylene-triggered responses could be a determining factor in delaying senescence (green-truncated arrow). R, non oxygen free radicals; RO, alcoxyl radicals; ROO, peroxyl radicals; OH, hydroxyl radical; ONOO⁻, peroxynitrite; GS-NO, nitrosoglutathione; cGMP, cyclic GMP; cADPR, cyclic ADP-ribose. Dashed lines correspond to NO-mediated effects whose chemical reaction has not been described in plants yet.
result of the strongly reducing conditions needed for N2 fixation (Kanayama & Yamamoto 1990). However, the great increase in the levels of arginine in Lupinus albus plants supplied with NO3- gave the idea that NOS could be present in nodules (Cueto et al. 1996). Since then, most of the studies on NOS activity and NO presence in plants were carried out in legumes, where special roles have been proposed. As an example, NOS activity in Lupinus albus (Cueto et al. 1996) appeared to be Ca2+-dependent in roots and independent in nodules. This fact, points at the presence of a putative constitutive isofrom in the roots and an inducible one in nodules. In both tissues, NOS was immunodetected in the vascular bundles. Since phloem vessels have been recently recognized as a signal transport system, this supports the idea of NO as a signalling molecule in legumes and playing a role during the symbiosis with Rhizobium.

Nitric oxide binds tightly to leghaemoglobin (Lb), by the same mechanism as it does with haemoglobin, forming nitrosoylleghaemoglobin complexes (NO-Lb) (Mathieu et al. 1998). The function of Lb is believed to involve the facilitation of O2 transport to the bacteroids. NO binding to Lb inhibits the O2-carrying activity of Lb, because NO-Lb is a very stable complex. Unlike NO-Lb, oxyLb undergoes facile auto-oxidation to form metLb, O2- and H2O2. These radicals initiate lipid peroxidation and an irreversible loss of the peribacteroid membrane, with a consequent loss of N2-fixing capacity of the nodules. This is also observed when nodules are subjected to external stress and may be favoured during natural senescence due to decreased levels of endogenous antioxidants. The NO-Lb complex was detected by electron paramagnetic resonance (EPR) in both young and mature nodules, whereas it was almost absent in senescent ones (Mathieu et al. 1998). Moreover, the treatment of young nodules with hydrogen peroxide changed the EPR spectra, resulting in NO-Lb loss and a spectra similar to those of senescent nodules or naturally stressed ones. This suggested that NO-Lb could react with O2- and/or H2O2, probably to avoid ROS damage to the nodules. Moreover, controlled levels of NO-Lb in active nodules could result in an equilibrium between oxy-Lb-mediated O2 transport and ROS formation.

In contrast with the apparent protection of the nodule against oxidative injury, NO appears to have a deleterious effect on the rate of nitrogen fixation. NO and also the NO-Lb complexes inhibit nitrogenase by reacting with Fe-S clusters (Nathan 1992). It has also been reported that there are heme-based sensors for NO in N2-fixing rhizobia that affect the regulatory cascade of gene expression involved in N2 fixation (Winkler et al. 1996). One member of this family, the FixL protein kinase, induces the regulatory cascade when present in its active form, deoxy-FixL. However, in response to oxygen, and possibly NO and CO, FixL shuts down nitrogen fixation. The significance of the effects of NO observed during the symbiosis is still far from being completely understood. However, this system may provide a useful tool for future study on NO biochemistry in plants.

HOW IS THE NO SIGNAL TRANSDUCED?

An immediate question, once an effect provoked by NO has been detected, points to the mechanism on which the effect relies. In this sense, the effect can be accomplished by a signal transduction pathway through the modification of transcription factors, receptors or enzymes that then activate or inactivate a signalling cascade. On the contrary, it can be a mere direct action of NO on a final target, such as the ending of chain-propagated reactions or enzymatic activation by direct modification. In mammals, many NO functions are accomplished through stimulation of soluble guanylate cyclase (GC) and consequent increase in cGMP levels. In addition, calcium and calmodulin have been described as alternative components in NO-mediated signalling (Stamler 1994). Furthermore, even some of the cytotoxic and cytoprotective effects of NO are accomplished through signal transduction, such as the prevention of apoptosis due to an inhibition of caspase-3 (Kim et al. 1997).

The search in plants has been directed to the targets of NO described in animals and to the molecules taking part in the processes affected by NO. High-performance liquid chromatography analysis of spruce tree needles and potato plants treated with low concentrations of NO revealed a 10-fold increase in the levels of cGMP (Pfeiffer et al. 1994, 1995). Moreover, an antibody raised against the α2-subunit of mammalian soluble GC detected a single band in spruce needles comigrating with its counterpart from rat brain. These results suggest that the NO/cGMP-dependent signalling pathway present in animals may also exist in plants.

A possible relationship between NO and cGMP in plants was later proposed to function during plant–pathogen interactions (Durner et al. 1998). NO-mediated stimulation of PAL expression in tobacco that was resistant to TMV was blocked by inhibitors of GC. One of them, however, produced only a partial inhibition, suggesting the existence of both cGMP-dependent and -independent pathways. The PAL gene was induced by cGMP and cyclic ADP-ribose, suggesting that these molecules could be second messengers for NO signals (Durner & Klessig 1999).

Treatment of tobacco cell suspensions with cantharidin, an inhibitor of type 2a protein phosphatases partially activated the signalling pathway mediated by H2O2 and NO in the absence of any pathogen avirulence factors (Delledonne et al. 1998). As cell death activated by an oxidative burst is known to involve a protein kinase cascade (Chandra, Martin & Low 1996), these results suggest that NO could share with H2O2 the same protein kinase pathway that leads to HR.

Complementary biochemical, molecular and genetic studies are being used to unravel the signalling mechanisms that modulate the immune and adaptive responses in plants. Strong evidence is being compiled showing the involvement and interaction of NO, ROS, Ca2+, SA, jasmonic acid (JA) and ethylene during responses in a very complex network. It seems, moreover, that the synergistic or antagonistic participation of each signal transducer relies on the characteristic of the different types of pathosystems.
However, in a wider sense, the existence of common defence systems to combat stress by activating overlapping signalling pathways are proposed for controlling the phenomenon of cross-tolerance in plants (Bowler & Flahour 2000).

In this respect, the potential role of NO in mediating cross tolerance should not be ignored. Although ROS are often invoked as being the most likely mediators of cross tolerance, the characteristics of the NO molecule (free radical, small size, no charge, short-lived and highly diffusible across biological membranes) makes it a very good candidate to simultaneously trigger different processes. Concomitantly, it appears that NO could possibly also be active as a systemic signal. In tobacco that was infected with TMV, NO triggered an increase in SA levels (Durner et al. 1998). SA, together with H₂O₂, have often been related to systemic acquired resistance (SAR), but there is still uncertainty as to whether either of these two molecules is the mobile signal responsible for it. NO chemical properties make it a good candidate in mediating SAR signals, and the exploration of this idea is actually a promising area of research.

In pea embryonic axes and wheat germ, where a putative NOS immunoreactive band was detected, simultaneous detection of anti-calmodulin reactive bands made the authors speculate about a possible relationship between both molecules (Sen & Cheema 1995). Additionally, the relationship of NO with cGMP and Ca²⁺/calmodulin pathways, as it occurs in animals was also suggested by experiments that show an involvement of NO in light-mediated processes (Beligni & Lamattina 2000a). Stimulation of many biological processes by the red/far-red absorbing photoreceptor phytochrome, is performed via heterotrimeric G proteins that are the initial steps of at least two main pathways: (i) via calcium and calmodulin; and (ii) mediated by increases in cGMP levels (Bowler et al. 1994). However, the complete molecular mechanisms by which light regulates development are largely unknown. In this sense, NO could affect developmental processes such as germination or hypocotyl elongation by the signalling pathway described above or by some way that is independent of light. A similar behaviour is displayed by plant hormones, which frequently regulate similar effects mediated by light in developing plants. These overlapping roles have also raised the interesting question of whether light and hormones act independently to affect developmental responses or whether plant hormones are involved in the sequence of events initiated by physiologically active photoreceptors (Chory et al. 1994). For NO, this question adds to an even more intriguing one: could NO function as a plant hormone?

CONCLUDING REMARKS AND PERSPECTIVES

In recent years, much evidence has been accumulated supporting the idea that NO versatility also exists in plants. Although some features of NO chemistry are probably different in plants and animals, due to their different tissue organization and complexities, some others seem to be sharply conserved. In both systems, NO appears to take part, either directly or otherwise, in regulating the cellular redox homeostasis, acting either as a powerful oxidant or as a potent antioxidant. This may not merely be a passive consequence of NO reactivity. NO is easily formed, highly diffusible and reactive. Therefore, biological systems could have taken advantage of these properties for their cellular regulatory mechanisms. Nitric oxide is believed to have been one of the first antioxidants during early life evolution (Feelisch & Martin 1995). It is speculated that, before specific enzymes such as superoxide dismutase, catalase and peroxidase were developed, those cells capable of generating NO were likely to have proved to be more resistant to aerobic oxidant living conditions than cells lacking such an activity. NO is thought to have originated during initial periods of aerobic evolution, when NO-producing cells were able to produce denitrification or nitrification. As biological systems developed more complex physiological processes, NO could further evolve to serve other useful functions beyond the mere guarantee of survival. In fact, NO might well have been one of the first biological signalling molecules, being involved not only in intra- and intercellular communication, but also in cell–cell adhesion mechanisms. If this is true, it is not surprising that NO plays such an important role in the regulation of cellular functions in evolutionary distant species.

The study of NO in plants is in fascinating progress, but most of the work has still to be done. Many effects may be reported, but a focus on the chemical and molecular mechanisms of its action will provide the field with more certainty. If NOS exists in plants, any strategy will have to allow the isolation of the corresponding gene/s and protein/s. Moreover, many other sources for NO production in plants probably exist. The integration of NO functions with plant metabolism, growth and development, especially with plant hormones will have necessarily to be examined in more detail. Furthermore, exogenous NO donor concentrations will have to be used consciously taking into account both the number of variables that can be affected by NO application and, especially, the duality of our protagonist: Dr Jekyll and Mr Hyde.

ACKNOWLEDGMENTS

The authors apologise for the omission of many relevant citations due to space limitations. They wish to thank Dr Raúl Cassia for comments on the manuscript and C. García-Mata for helpful discussions and technical assistance. The authors are supported by grants from UNMdP, CONICET and ANPCyT (L.L.) and PhD fellowships from CONICET, UNESCO and Fundación Antorchas (M.V.B.).

REFERENCES


Kanayama Y. & Yamamoto Y. (1990) Inhibition of nitrogen fixation in soybean plants supplied with nitrate. II. Accumulation
Nitric oxide in plants


Werner-Felmayer G., Golderer G., Werner R., Gröbner P. & Watcher H. (1994) Pteridine biosynthesis and nitric oxide syn-


Received 19 July 2000; received in revised form 13 October 2000; accepted for publication 13 October 2000