

Nitric Oxide and Plant Growth Promoting Rhizobacteria: Common Features Influencing Root Growth and Development

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ABSTRACT

Nitric oxide (NO) is a gas produced by prokaryotes and eukaryotes as part of their N metabolism that profoundly influences the physiology of the cells. In plants, the biological implications of NO as a signal molecule modulating physiological responses have been elucidated in the last decade. The NO action as an intermediary in auxin-regulated signaling cascades influencing root growth and developmental processes is probably one of the most important functions in plant biology. Here we describe the signaling pathways and the cellular messengers involved in the NO induction of adventitious root formation, lateral root development, and root hair formation. We also review the first evidence supporting the NO role in the induction of adventitious and lateral root development by plant growth promoting rhizobacteria (PGPR). Finally, it is presented and discussed as an overview of the putative and potential biosynthetic pathways of NO and their close dependence on the different N sources in PGPR.

ABBREVIATIONS

AR, adventitious root; BNF, biological nitrogen fixation; CDPK, Ca²⁺-dependent protein kinase; cGMP, cyclic guanosine 3'/5'-monophosphate; CDK, cyclin-dependent kinase; Nas, cytoplasmic assimilatory nitrate reductase; [Ca²⁺]_{cyt}, cytosolic Ca²⁺ concentration; CPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; Nir, dissimilative nitrite reductase; DAF-2 DA, 4,5-diaminofluorescein diacetate; EPR, electron paramagnetic resonance; IAA, indole-3-acetic acid; LR, lateral root; LRD, lateral root development; Nar, membrane-bound nitrate reductase; MAPK, mitogen-activated protein kinase; NO, nitric oxide; Nor, nitric oxide reductase; Nos, nitrous oxide reductase; NOS, nitric oxide synthase; PCIB, *p*-chlorophenoxy isobutyric acid; Nap, periplasmic nitrate reductase; PGPR, plant growth promoting rhizobacteria; RHF, root hair formation; Trp, tryptophan.

I. INTRODUCTION

One important goal to improve agricultural performance and increase food production is to attain high yields, even at low soil fertility or without intensive fertilization. To achieve this goal, the control of processes that determine root architecture and physiology appears to be central. Roots are dynamic anchorages of plants. They not only support the whole plant architecture, but also its entire physiological activity. Greater adventitious rooting, increased number of lateral roots (LRs), and higher length and density of root hairs are targets of many research projects in plant biology.

Some of the most complex physical, chemical, and biological interactions experienced by plants are those that occur between roots and their surroundings. Signals derived from changes in the soil environment trigger selective root and shoot responses. In this scenario, the interrelationships established between roots and the biotic components of the rhizosphere would have a strong impact on plant growth. Undoubtedly, there are numerous processes

occurring in the rhizosphere and the signals that govern and orchestrate their dynamic are still hidden to our knowledge. The symbiotic and nonsymbiotic associations between organisms in the rhizosphere rely on interacting factors and chemical signals that operate on time and space scales. Among them, compounds of hormonal nature play major roles. To make the picture more complex, all these factors vary with water content, temperature, nutrients and soil structure, and others.

Root-colonizing bacteria are able to both suppress disease in host plants by the production of inhibitory compounds that inhibit soil pathogen growth and, at the same time, stimulate growth and defense responses in host plants. There are complex and multitargeted responses that are yet poorly understood since the knowledge of chemical signals involved in plant-microorganism association are largely unknown. In this chapter, we present a review of the available data that strongly support a central role for nitric oxide (NO) as a chemical signal involved in root growth and development and in the interaction of roots with the plant growth promoting rhizobacteria (PGPR) *Azospirillum*.

II. NO IS A REGULATOR OF ROOT GROWTH AND DEVELOPMENTAL PROCESSES

A. NO INDUCES ADVENTITIOUS ROOT FORMATION

Auxin is known to be involved in the process of adventitious root (AR) formation for a long time, mainly in promoting the initiation of root primordia (Haissig and Davis, 1994). Steffens *et al.* (2006) showed that the development and emergence of root primordia are positively controlled by ethylene. AR formation can also be induced by sugars, temperature, and light conditions (Takahashi *et al.*, 2003). By contrast, there are few reports regarding the inhibition of AR formation. Kuroha *et al.* (2002) showed that exogenous treatment with gibberellins, cytokinins, and abscisic acid (ABA) results in an inhibitory effect on AR formation in cucumber (*Cucumis sativus*) hypocotyls. Thus, data indicate that complex interactions between different phytohormones take place in determining the timing and intensity of the AR formation process.

The ability to form ARs is critical for plants that are propagated through vegetative cuttings and, as a consequence, problems associated with rooting of cuttings frequently result in significant economic losses (De Klerk *et al.*, 1999). While the physiology of AR formation is reasonably well known, the genetic and molecular mechanisms involved are still poorly understood. During the last years, several observations support a link between auxin- and NO-dependent signaling pathways during AR formation in cucumber explants

(Lanteri *et al.*, 2006a; Pagnussat *et al.*, 2002, 2003, 2004). The first evidence showed that auxin induces AR formation through an increase of the NO concentration at the base of cucumber hypocotyls (Pagnussat *et al.*, 2002). The maximum NO concentration was 60 nmol per gram of fresh weight after 24 h of auxin treatment, measured by electron paramagnetic resonance (EPR; Pagnussat *et al.*, 2002). Since this work, many reports have enlarged the knowledge of the NO actions in the network that controls root morphology and physiology (Lanteri *et al.*, 2006b). As a result, we now know that components that were described as cellular messengers for NO in animal cells are also involved in NO-regulated responses in plants (Lamattina and Polacco, 2007). It was demonstrated that auxin and NO trigger both cGMP-dependent and cGMP-independent pathways leading to AR formation (Lanteri *et al.*, 2006a; Pagnussat *et al.*, 2003, 2004). Cumulative evidence indicates that the NO-dependent activation of the guanylate cyclase-catalyzed synthesis of cGMP results in an increase in cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{cyt}}$) through the release of Ca^{2+} from intracellular stores regulated by cADPR. The entrance of Ca^{2+} from the extracellular space and from Ca^{2+} channels triggered by IP_3 would also contribute to this raise in $[\text{Ca}^{2+}]_{\text{cyt}}$. As a consequence, Ca^{2+} -dependent protein kinases (CDPKs) become activated (Lanteri *et al.*, 2006a). Another line of evidence suggests a role for NO in the activation of a cGMP-independent mitogen-activated protein kinase (MAPK) signaling cascade (Pagnussat *et al.*, 2004). Collectively, available data support the claim that, in cucumber, AR formation is controlled by a complex and intricate set of cellular messengers involving auxin, NO, cGMP, cADPR, IP_3 , Ca^{2+} , CDPKs, and MAPKs. Future analyses will have to be directed at the identification of the molecular mechanisms that characterize the interaction between the different components of the signaling cascade.

We have as yet no exact knowledge regarding the mechanism by which auxin increase NO level in cucumber hypocotyls and the specific NO source/s during AR formation. In a recent report, the histological distribution and the source of NO during AR formation in mung bean hypocotyls cuttings were investigated (She and Huang, 2004). It was concluded that the enzyme nitric oxide synthase (NOS) is responsible for the production of NO during this process analyzed by the NADPH-diaphorase activity assay, commonly employed as a marker for NOS (She and Huang, 2004). Authors showed that NADPH-diaphorase activity and the specific NO fluorescence detected by the probe 4,5-diaminofluorescein diacetate (DAF-2 DA) gradually increased during AR formation and were mainly distributed in the AR meristem (She and Huang, 2004). Taking into consideration that the activity of the *Arabidopsis* NOS1 gene (*AtNOS1*) is a matter of discussion since results from Zemojtel *et al.* (2006) raise critical questions regarding both the activity and function of *AtNOS1*, our understanding of the participation, occurrence, and

putative function of NOS in plants is not yet complete. Therefore, it will be interesting to explore the involvement of other enzymatic and nonenzymatic sources of NO (reviewed in Stöhr and Stremlau, 2006) during AR formation.

B. NO AND LATERAL ROOT DEVELOPMENT: NO IS DOWNSTREAM AUXIN IN TRIGGERING LRD

The process of LR formation has been extensively studied in many plants. Diverse signals regulate LR formation, including environmental and intrinsic factors (Malamy, 2005). Among environmental signals, nutrients are one of the major regulators of lateral root development (LRD). The concentration and patchy distribution of the nutrients nitrate, phosphate, and sulphate in soils have been shown to regulate the spatial distribution, density, and length of LRs (Kutz *et al.*, 2002; Linkohr *et al.*, 2002; Zhang and Forde, 2000). Furthermore, novel reports suggest an osmotic regulation during LRD (Deak and Malamy, 2005; van der Weele *et al.*, 2000). Among internal signals, even though many hormones have been involved in the regulation of LRD, auxin plays a major role in this process.

In accordance with the process of adventitious rooting (Pagnussat *et al.*, 2002, 2003), a link between auxin and NO was shown during LRD. The application of NO donors resulted in an increase of LR number in tomato (Correa-Aragunde *et al.*, 2004). The auxin-induced LR formation could be repressed by the addition of 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (CPTIO), a specific NO scavenger. In addition, the *Arabidopsis* mutant *noal*, in which NO production is impaired (Crawford *et al.*, 2006), failed to respond to auxin in LRD (Todd, C. D., Correa-Aragunde, N., Hoyos, M. E., Dhanoa, P. K., Santa-Catarina, C., Lamattina, L., Mullen, R.T., Segal Floh, E. I., and Polacco, J. C., unpublished results). The available data suggest that NO acts downstream of auxin leading to LRD.

1. NO acts at earlier stages of LR formation through the activation of cell division in pericycle

In spite of intensive studies on root growth and developmental processes, the control of LR initiation is a yet poorly understood mechanism. In *Arabidopsis*, LRs are formed from a subset of pericycle cells termed founder cells, which are adjacent to the two xylem poles. Once activated, founder cells undergo anticlinal divisions followed by radial expansion and subsequent periclinal division, giving rise to an LR primordium. The LR primordium grows up through the cortex and emerges from the parent root primarily by expansion of the preexisting cells rather than by cell division (Malamy and Benfey, 1997). The mechanism by which specific pericycle cells became founder cells is still unknown.

Several observations suggest an NO role in early stages of LR initiation. Microscopically, detection of NO during LRD in tomato reveals an accumulation of NO during the first stages of LR primordium development. In addition, NO depletion results in a severe reduction of LR formation (Correa-Aragunde *et al.*, 2004). Reports have presented data supporting a role of NO in the stimulation of cell division (Correa-Aragunde *et al.*, 2006; Otvos *et al.*, 2005). During LR initiation in tomato, NO induces the expression of the cell cycle regulatory genes *CDKA1*, *CYCD3;1*, and *CYCA2;1* while the gene encoding the cyclin-dependent kinase (CDK) inhibitor *KRP2* is repressed. Moreover, the regulation of these cell cycle regulatory genes by auxin is NO dependent (Correa-Aragunde *et al.*, 2006). In agreement, similar results were shown in a cell culture system. NO can stimulate the activation of cell division and embryogenic cell formation in leaf protoplast-derived cells of alfalfa (Otvos *et al.*, 2005). Even though the participation of NO in the control of cell cycle progression was already demonstrated, it still remains to be elucidated the NO source/s and the specific target molecules regulated by NO leading to the activation of cell division.

C. GENERAL FEATURES ASSOCIATED TO ROOT HAIR FORMATION

Root hairs are specialized cell types that function in root anchoring and for increasing the soil area exploitable by the plant (Peterson and Farquhar, 1996). By greatly increasing the total surface area of the root system, root hairs are believed to play an important role in the absorption of water and nutrients from the soil (Clarkson, 1985). In the root system of higher plants, the epidermis is composed of two cell types: (1) root hair cells or trichoblasts and (2) non-hair cells or atrichoblasts. Trichoblast and atrichoblast show different cellular characteristics in the meristematic root region, indicating that the cue of cellular specification must be operating during the first stage of root development (embryonic development). The identity of epidermal cells, as trichoblast or atrichoblast, when protodermic cells, is defined on entering the elongation phase. At this time, the fate of root epidermal cells is determined by their position with respect to the underlying cortical cells. Atrichoblasts are located over a periclinal (outer tangential) cell whereas trichoblasts are located over the clef of two cells formed by adjacent cortical cells (Dolan *et al.*, 1994; Galway *et al.*, 1994). The cortical cells might confer positional information to result in a precise pattern of cell fate (Gilroy and Jones, 2000). This patterning is characteristic in *Arabidopsis* roots, where files of trichoblasts alternate with files of atrichoblasts (Dolan *et al.*, 1993), suggesting a noticeable cell-to-cell communication soon after differentiation.

Root hair formation (RHF) can be analyzed in phases: cell fate specification, initiation, tip growth, and maturation. Although positional information is provided postembryonically, epidermic root cells are defined as atrichoblast or trichoblast due to genetic action. However, the last proportion of trichoblasts is determined by environmental factors and nutritional requirements. The environmental factors that influence RHF are temperature, pH, calcium, iron, and phosphorus availability, among others (Hofer, 1996). NO was reported to be involved in the regulation of RHF in *Arabidopsis* and lettuce (Lombardo *et al.*, 2006).

1. NO regulates RHF

As stated, NO affects the morphology and developmental pattern of roots in a noticeable manner. NO is involved in the promotion of lateral and AR initiation in several plant species (Correa-Aragunde *et al.*, 2004; Pagnussat *et al.*, 2002, 2004). NO was shown to be also involved at the initiation and the elongation processes of RHF (Lombardo *et al.*, 2006). In lettuce, NO is a critical molecule in determining root hair differentiation and elongation, mediating an auxin-triggered signaling cascade (Lombardo *et al.*, 2006). In *Arabidopsis*, NO and auxins are mainly involved in the regulation of mechanisms controlling the elongation process (Lombardo *et al.*, 2006; Pitts *et al.*, 1998). Indeed, several auxin response mutants of *Arabidopsis* display a phenotype similar to that generated by NO depletion in which root hair elongation is the main process affected during RHF (Pitts *et al.*, 1998). Auxin treatment stimulates NO production in *Arabidopsis* roots and this NO production is mainly located in the root hair cell files (Lombardo *et al.*, 2006). Figure 1 shows a detail of NO localization in different developmental stages of RHF in tomato roots.

After initiation of root hairs, elongation proceeds by polarized expansion. This expansion involves tip growth and requires biosynthesis of new wall

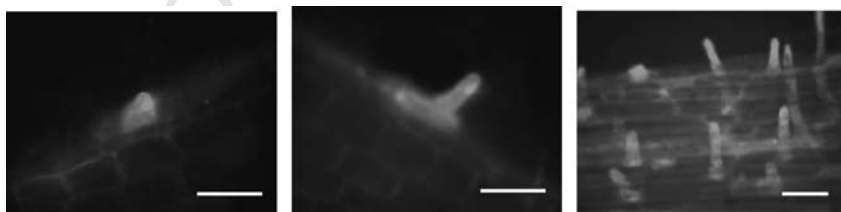


Fig. 1. Endogenous NO production during root hair development in tomato. Tomato roots (15-day-old) were loaded with $15 \mu\text{M}$ of the specific NO probe DAF-2 DA for 1 h. Photographs show the presence of NO in different stages of root hair development in tomato root. Bar = 0.1 mm. (See Color Plate Section in the back of the book.)

material, localized wall loosening, and the flux of vesicles from the endomembrane system to the growing tip. These processes are regulated by the activity of ion channels and by the cytoskeleton (Ryan *et al.*, 2001). The available data indicate that a signaling network including changes in reactive oxygen species (ROS), phospholipids, and $[Ca^{2+}]_{\text{cyt}}$ operates during root hair initiation and tip growth (Foreman *et al.*, 2003; Ohashi *et al.*, 2003).

A Ca^{2+} current enters the root hair cell exclusively at the apex (Jones *et al.*, 1995; Schiefelbein *et al.*, 1992; Wymer *et al.*, 1997). This Ca^{2+} current is confined to the apical 20–50 μm of the root hair and depends critically on external pH and $[Ca^{2+}]$. A parallel gradient in $[Ca^{2+}]_{\text{cyt}}$ is observed in this region (Felle and Hepler, 1997; Wymer *et al.*, 1997). The $[Ca^{2+}]_{\text{cyt}}$ at the apex is several-fold greater than $[Ca^{2+}]_{\text{cyt}}$ in the basal region. These phenomena appear to be specifically associated with root hair elongation (White, 1998). A very recent report have also established that extracellular ATP is also playing a role in root hair growth (Kim *et al.*, 2006).

On the other hand, NADPH oxidase/RHD2 (ROOT HAIR DEFECTIVE 2) is a key enzyme that produces ROS as second messengers involved in intracellular signaling. Foreman *et al.* (2003) demonstrated that ROS activate a specific type (hyperpolarization activated) of Ca^{2+} channel localized on root hair tips. Neither the apical Ca^{2+} current (Schiefelbein *et al.*, 1992) nor the gradient in $[Ca^{2+}]_{\text{cyt}}$ (Wymer *et al.*, 1997) are observed in mature, nongrowing root cells or in root hairs of *rhd2* Arabidopsis mutant. This mutant forms root hair bulges but no elongated root hairs (Foreman *et al.*, 2003). Interestingly, the requirement of a high $[Ca^{2+}]_{\text{cyt}}$ at the root tip for maintaining its growth rate fits with the already established action of NO in modulating Ca^{2+} level in guard cells (García-Mata *et al.*, 2003): (1) the elevation of $[Ca^{2+}]_{\text{cyt}}$ through the regulation of Ca^{2+} release from intracellular stores and (2) the regulation of Ca^{2+} -dependent ion channel activities. In guard cells, NO treatment increased $[Ca^{2+}]_{\text{cyt}}$ from 500 to 800 nM when cells were stimulated by -200 mV (García-Mata *et al.*, 2003). Additionally, as was previously stated, disruption in Ca^{2+} homeostasis was shown to severely affect NO-induced AR formation in cucumber (Lanteri *et al.*, 2006a). Two other reports showed that NO induces $[Ca^{2+}]_{\text{cyt}}$ in *Nicotiana* cells (Lamotte *et al.*, 2004, 2006). Authors demonstrated that cells challenged by cryptogein (Lamotte *et al.*, 2004) or hyperosmotic stress (Lamotte *et al.*, 2006) increased $[Ca^{2+}]_{\text{cyt}}$ in an NO-dependent pathway.

2. Cross talk between NO and other plant hormones during RHF

An interaction between NO and ethylene was reported during the maturation and senescence of plant tissues (Lamattina *et al.*, 2003; Leshem *et al.*, 1998), and an antagonistic action of both gases was suggested during senescence (Leshem *et al.*, 1998). Lindermayr *et al.* (2006) showed that NO might

influence ethylene production in plants by inhibiting methionine adenosyltransferase through *S*-nitrosylation. In roots, ethylene is another hormone involved in the regulation of RHF. Ethylene acts as a positive regulator of root hair differentiation. Antagonists that block either the synthesis or the perception of ethylene inhibit the differentiation of root hairs (Tanimoto *et al.*, 1995). The constitutive triple response (CTR1) gene encodes a Raf-like protein kinase that negatively regulates the ethylene signal transduction pathway (Kieber *et al.*, 1993).

The ethylene-dependent triple response Arabidopsis mutant *etr1* possesses ectopic root hairs on their atrichoblasts. Ethylene functions as a diffusible positive regulator and confers the “hair” character on cells overlying cortical, anticlinal cell walls. Ethylene may accumulate in the air spaces that are formed at the junction between trichoblasts and the underlying cortex. The location of trichoblast cells over these spaces may expose these cells to elevated levels of ethylene and thereby induce hairs preferentially in these cells (Dolan *et al.*, 1994). It is possible that NO could be regulating the action of this hormone during RHF. On the other hand, Zhu *et al.* (2006) have demonstrated that jasmonic acid (JA) and methyl jasmonate (MeJA) promote RHF. They also concluded that ethylene is a prerequisite for JAs’ function since the effect of JAs is abolished in the ethylene-insensitive Arabidopsis mutants *etr1-1* and *etr1-3*, or by inhibiting ethylene action (Ag^+) or biosynthesis (AVG). Furthermore, it was found that inhibitors of JA biosynthesis ibuprofen and SHAM (a known inhibitor of lipoxygenase in jasmonate biosynthesis) repressed ACC-driven or *eto1-1* (ethylene overproducing mutant)-induced RHF (Zhu *et al.*, 2006). Collectively, these data support a role for the interaction between JAs and ethylene in the regulation of RHF in Arabidopsis. It remains to be elucidated in which part of the signaling cascade, which regulates RHF, occurs the cross talk between ethylene, JA, and NO.

Finally, a role of microtubules during root hair initiation has been demonstrated (Samaj *et al.*, 2004). A putative linkage between microtubules and NO during root hair initiation deserves to be analyzed. It is already known that cortical microtubules become randomized during initiation of LR primordia in pericycle cells (Baluska *et al.*, 2000) as well as during root hair initiation in trichoblasts (Baluska *et al.*, 2000; Van Bruaene *et al.*, 2004). Since NO is involved in LRD (Correa-Aragunde *et al.*, 2004) and in RHF (Lombardo *et al.*, 2006), it is also possible that NO could be involved in the randomization of cortical microtubules which have been shown to precede the dramatic switch in cell polarity during the morphogenetic events described above. NO has already been involved in microtubule configuration in neurons (He *et al.*, 2002). Altogether, the advances of the knowledge concerning the NO functions in root growth and developmental processes

indicate that it is a central signal molecule in the auxin transduction pathways leading to the determination of root morphology and physiology.

D. THE EFFECTS OF PGPR ON ROOT ARCHITECTURE

Root is the organ through which the plant can sense and communicate with other living systems that inhabit the soil. It is accepted that root activity alters the habitat of microorganisms and these, in turn, could trigger changes in the overall plant behavior. Among microorganisms living in the rhizosphere, root colonizers that exert beneficial effects on plant growth and development are referred to as PGPR (Kloepper, 1992). As a primary target, root is the organ that shows the first stimulating bacterial effects. This was particularly remarkable in plants inoculated with *Azospirillum* spp. (Okon, 1985), the most studied rhizobacteria (Bashan *et al.*, 2004). Indeed, field experiments performed with azospirilla-inoculated crops have shown significantly increased yields accompanied by better water and mineral uptake and positive changes in the root morphology and growth (Creus *et al.*, 2004; Dobbelaere *et al.*, 2001; Okon and Labandera-González, 1994; Sarig *et al.*, 1988).

An increase in the branching degree of roots, an improvement in the root architecture, and its associated enhanced capacity to explore soil in the quest for water would contribute to a better hydrated status of plants exposed to water deficit. It was reported that *Azospirillum*-inoculated wheat seedlings subjected to osmotic stress developed a significant higher coleoptile and better water status than noninoculated seedlings (Alvarez *et al.*, 1996; Creus *et al.*, 1998). Taking into account that plant exposed to salt stress also suffers water deficit, when assayed, it was proved that *Azospirillum*-inoculated wheat seedlings were able to survive when exposed to up to 320-mM NaCl for 3 days (Creus *et al.*, 1997). In salty soils or in those lacking enough water, the success of inoculation will be dependent on the seed capability to germinate under these stressing conditions. In field assays, *Azospirillum*'s effects in mitigating water stress were observed in maize and wheat crops (Casanovas *et al.*, 2003; Creus *et al.*, 2004). Germination and growth under 80-mM NaCl could be greatly improved in lettuce seeds inoculated with *A. brasilense* Sp245 (Barassi *et al.*, 2006).

The beneficial effects that *Azospirillum* exerts on plants, whether they are achieved under normal or environmental stressing conditions, rely on molecular mechanisms that are poorly understood. Several mechanisms have been postulated to explain how PGPR enhances plant growth and development. These can be broadly distinguished as providing either direct or indirect growth stimulation (Glick, 1995). Direct mechanisms elicit growth promotion by bacterial determinants, while indirect ones outcome is the result of

skipping the plant from growth limitations imposed by pathogenic or non-pathogenic microorganisms (Ryu *et al.*, 2004). Whatever the type of ecological relationship occurring between plant and rhizobacteria, the mechanisms that enable roots to interpret the innumerable signals they receive from the rhizosphere, including those produced by PGPR, and how those signals elicit plant growth promotion, are largely unknown.

As mentioned above, the most studied PGPR is *Azospirillum* spp., included in the alpha subclass of Proteobacteria belonging to the IV rRNA superfamily (Xia *et al.*, 1994). This group of free-living microorganisms encompasses eight species, each one classified according to its particular biochemical and molecular characteristics (Bashan *et al.*, 2004; Peng *et al.*, 2006; Xie and Yokota, 2005). Since the genera can be found in a wide range of habitats associated to roots of both graminaceous as well as nongraminaceous species, it has been regarded as a general plant colonizer (Bashan and Holguin, 1997). *Azospirillum* can fix atmospheric N₂ through nitrogenase complex, when the availability of N compounds and oxygen tension are low (Döbereiner and Day, 1976; Steenhoudt and Vanderleyden, 2000). Even though this characteristic could be extremely valuable in agriculture, field studies including those in which isotopic dilution techniques were used, failed to demonstrate a significant biological nitrogen fixation (BNF) in *Azospirillum*-inoculated crops (Vande Broek *et al.*, 2000). Even at the organism level, the growth promotion induced by the inoculation of axenic seedlings could not be ascribed to BNF (Bashan *et al.*, 1989).

One of the first observations regarding plant growth promotion activity exerted by *Azospirillum* was on root morphology (Okon, 1985). On inoculation, the root displayed a significant increase in the number and the length of root hairs, the rate of appearance and number of LRs, the diameter and length of lateral and ARs, and the root surface area (Creus *et al.*, 2005; Dobbelaere *et al.*, 1999; Fallik *et al.*, 1994; Kapulnik *et al.*, 1985). Besides, Levanony and Bashan (1989) reported an increase in cell division in the root tips of inoculated wheat. Several reports showed that the inoculation of wheat or maize seedlings with *Azospirillum* cells resulted in an increased number of root hair showing a Y-shaped deformation (Jain and Patriquin, 1984; Kapulnik *et al.*, 1985; Patrikin *et al.*, 1983; Zamudio and Bastarrachea, 1994). All these effects are dependent on the plant species and cultivar inoculated and on the concentration of *Azospirillum* inoculum (Vande Broek *et al.*, 2000). Inoculation of many different plant species with *Azospirillum* in a range between 10⁶ and 10⁸ cells per seedling provoked root elongation (Creus *et al.*, 1996; Kapulnik *et al.*, 1985). However, higher concentrations of bacteria always result in an inhibition of root elongation (Harari *et al.*, 1988). Thus, there exists a bacterial concentration that results

optimum for triggering root elongation. The dose response of the root system to *Azospirillum* inoculation resembles the responses triggered by exogenous hormonal application.

The production of phytohormones, namely auxins, cytokinins, and gibberellins, is the most commonly invoked mechanism of plant growth promotion exerted by PGPR (García de Salamone *et al.*, 2001). Among them, auxins are thought to play the major role. Even though it was suggested more than 60 years ago that rhizobacteria could produce auxins (Roberts and Roberts, 1939), it was only in the seventies that this assumption was proved (Barea and Brown, 1974; Brown, 1972; Tien *et al.*, 1979). Nowadays it is well known that *Azospirillum* can synthesize indole-3-acetic acid (IAA) by at least three different pathways. By means of *in vivo* labeling experiments, Prinsen *et al.* (1993) demonstrated the existence of one tryptophan (Trp)-independent pathway and two Trp-dependent biosynthetic routes. The presence of Trp in culture medium strongly induces the Trp-dependent pathways, resulting in a tenfold increase of the IAA levels. Although Trp-independent IAA biosynthesis occurs in various plant species, *Azospirillum* is so far the only bacterium in which such an IAA biosynthetic pathway has been identified (Vande Broek *et al.*, 2000). Therefore, this ability could be of biochemical and ecological significance, since some root exudate like those produced by maize lack Trp (Guckert, 1985). Controlled experiments *in vitro* showed that IAA content increased in roots and shoots of *A. brasilense* FT326-inoculated tomato (Ribaudó *et al.*, 2006).

To evaluate the involvement of bacterial IAA in the promotion of root development, several investigations were conducted with mutant strains altered in IAA production. *A. brasilense* SpM7918, a very low-IAA producer, showed a reduced ability to promote root system development in terms of both number and length of LR's and distribution of root hairs compared to the wild-type (wt) strain Sp6 (Barbieri and Galli, 1993; Dobbelaere *et al.*, 1999). Another mutant of *A. brasilense* with low production of phytohormones but high nitrogenase activity did not enhance root growth over uninoculated controls (Kundu *et al.*, 1997). However, there are no reports showing to what extent IAA is produced in the rhizosphere by *Azospirillum* (Lambrecht *et al.*, 2000; Steenhoudt and Vanderleyden, 2000). On the other hand, several authors have shown evidence of a lack of correlation between the capacity for IAA synthesis of *Azospirillum* and the effects on root growth promotion (Bothe *et al.*, 1992; Harari *et al.*, 1988; Kapulnik *et al.*, 1985). Nevertheless, the possibility that *Azospirillum* could not only produce IAA but also to enhance the endogenous IAA produced by the plant should not be excluded. Most studies on the mechanisms for plant growth promotion by

PGPR have focused on bacterial traits without examining the host plant's physiological responses (Bloemberg and Lugtenberg, 2001). Moreover, the role of chemical signals in mediating rhizospheric interactions is beginning to be understood (Bais *et al.*, 2006).

If a positive effect of inoculation with *Azospirillum* sp. is expected, a successful colonization of roots followed by an appropriate bacterial cells location is needed. Using the green fluorescent protein to tag bacteria, Liu *et al.* (2003) confirmed that bacteria are established mainly on the root surface. Even though some strains of *A. lipoferum* and *A. brasilense* are capable of colonizing the inner part of the root, they always locate outside the plant cells in the apoplast and intercellular spaces. The fact that *Azospirillum* affects plant cell metabolism from outside, the plant cell suggests that the bacteria is capable of excreting and transmitting signals that are perceived by the plant cell wall and/or the plasma membrane. This interaction initiates a chain of events that results in the observed altered metabolism of inoculated plants. Since membranes are extremely sensitive to any change, they may serve as the precise indicator for *Azospirillum* activity at the cellular level (Bashan *et al.*, 1992).

1. *Azospirillum*-promoted root growth involves NO-mediated actions

Bloom *et al.* (2003) have reviewed the signals and molecules that are potentially involved in root development. Among them, nitrogen species as ammonium, nitrate, and NO were proposed to be implicated in root growth and proliferation. As stated above, it has been already demonstrated that NO functions as a signal molecule in the IAA-induced signaling cascade leading to AR formation, LRD, and RHF (Correa-Aragunde *et al.*, 2004; Lombardo *et al.*, 2006; Pagnussat *et al.*, 2002). It is largely known that *Azospirillum* can produce NO at low O₂ pressure by denitrification (Hartmann and Zimmer, 1994). Creus *et al.* (2005) have reported the NO production by *Azospirillum* growing under aerobic conditions. Figure 2 shows cells of *A. brasilense* cultured in liquid medium supplemented with 0.1% (w/v) NH₄Cl as N source. The green fluorescence is produced by the addition of the NO-specific fluorescent probe DAF-2 DA whereas the addition of CPTIO, an NO scavenger, diminished the fluorescence (Fig. 2). A concentration of 6.4 nmol of NO per gram of *A. brasilense* was quantified when bacterium reached the end of growing log phase (Creus *et al.*, 2005). The remarkable analogies found between the experimental data concerning *Azospirillum* stimulation of plant root development and the capability of NO to act as a nontraditional plant growth regulator (Beligni and Lamattina, 2001) promoting AR formation, LRD, and RHF led us to explore whether the *Azospirillum* ability to promote root growth and modify root architecture relies on NO.

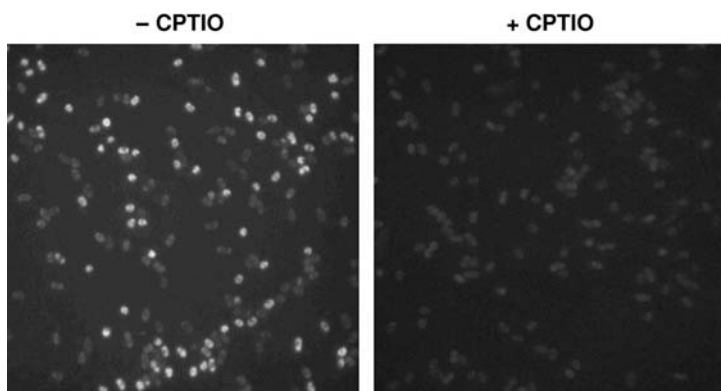


Fig. 2. *A. brasilense* produces NO. *A. brasilense* Sp245 was cultured in medium containing NH_4Cl as N source. At late exponential phase, cells were pelleted, washed, and resuspended in buffer HEPES–NaOH 20 mM pH 7.8 in the absence (left) or presence (right) of the NO scavenger CPTIO at 0.5 mM. After 30 min of incubation, 15 μM of the NO-specific probe DAF-2 DA was added, and samples were incubated for two more hours. Washed bacteria were examined by epifluorescence microscopy at 1000 \times magnification. (See Color Plate Section in the back of the book.)

Azospirillum-inoculated tomato roots incubated with the NO-specific fluorescent probe DAF-2 DA displayed higher fluorescence intensity compared to noninoculated roots. Fluorescence was mainly located at the vascular tissues and subepidermal cells of roots (Creus *et al.*, 2005). Moreover, the *Azospirillum*-mediated induction of LRD appears to be NO dependent since treatment of inoculated seedlings with the NO scavenger CPTIO completely blocked this effect (Creus *et al.*, 2005). In addition, two other strategies were carried out to validate this result: (1) the use of a mutant strain of *A. brasilense* with reduced ability to synthesize IAA, but the same capacity for NO production; and (2) the addition of the auxin antagonist *p*-chlorophenoxy isobutyric acid (PCIB), which competes with endogenous auxin. Inoculation of tomato seedlings with either the wt or the IAA-deficient mutant of *Azospirillum* increased LR number and percentage of seedlings displaying AR formation with respect to the noninoculated ones. The wt strain induced higher LRD than the mutant strain, in agreement with previous findings in wheat inoculated with different IAA-impaired *Azospirillum* mutants (Barbieri and Galli, 1993; Dobbelaere *et al.*, 1999). However, when NO was removed with the NO scavenger CPTIO, both lateral and AR formation were inhibited and attained to the noninoculated values, evidencing that NO is strongly involved in the *Azospirillum*-induced root branching (Molina-Favero, C., Creus, C. M., Simontacchi, M., Puntarulo, S., and Lamattina, L., unpublished results). Besides, the addition of PCIB to inoculated tomato seedlings decreased the percentage of seedlings with LRs (Creus *et al.*, 2005).

Altogether, these results suggest that auxins are involved but not exclusively in *Azospirillum*-mediated effects on roots. Indeed, data support that both auxins and NO have a role as cellular messengers in the interaction occurring in the rhizosphere between roots and PGPR. Whether the auxin synthesized by PGPR triggers an NO production in the bacterial cell and/or in the plant root remains to be elucidated.

2. NO sources in *Azospirillum* and other PGPR

NO is a central component in the nitrogen cycle. It is produced and released by almost all soils, particularly those well fertilized (Stöhr and Ullrich, 2002). Several biological and chemical pathways are involved in regulating the NO steady state levels in soils, including denitrification, nitrogen mineralization (i.e., conversion of organic N into inorganic forms), dinitrogen fixation, and nitrification. In the interaction between plants and PGPR, these pathways can be accomplished alternatively or simultaneously according to the nutrient availability, physical conditions, and the organisms involved. In associative or symbiotic relationships between roots and microorganisms, it is likely that both partners contribute to NO production. In addition, it could be expected that either bacteria or plant could influence NO synthesis in the partner in a synergistic, compensatory, and/or complementary way.

NO production in plants relies principally on nitrite reduction. This intermediary can be reduced enzymatically by a cytosolic nitrate reductase (Stöhr and Ullrich, 2002), the root mitochondria (Gupta *et al.*, 2005), and a plasma membrane-bound nitrite:NO reductase (Stöhr *et al.*, 2001); and nonenzymatically in the apoplast at acidic pH values (Bethke *et al.*, 2004) or by carotenoids in a reaction mediated by light (Cooney *et al.*, 1994). The presence of an NOS in plants has not been fully confirmed (Zemojtel *et al.*, 2006), but there is biochemical and immunological evidence supporting it (Barroso *et al.*, 1999; Jasid *et al.*, 2006). In bacteria, there are also several NO-producing pathways that share similar features to the plant pathways. Meyer and Stöhr (2002) suggested that NO might be one of the signals for the presence of nitrate in a given place. Likewise, the NO synthesis by PGPR from nitrate or ammonium may generate an NO gradient which could trigger specific signaling processes. All these considerations make NO a potential signal molecule in the bacterial plant root association.

a. Denitrification. Denitrification is the stepwise dissimilative reduction of nitrate (NO_3^-) to nitrite (NO_2^-), NO, nitrous oxide (N_2O), and dinitrogen (N_2) by the corresponding NO reductases. In this process, nitrate is used instead of oxygen as a final electron acceptor in respiration. This pathway allows denitrifiers to generate energy and to grow under low oxygen or

anaerobic conditions (Zumft, 1997). Denitrification has been known for a long time, although it has been more recently accepted that NO is an obligatory intermediary (Ye *et al.*, 1994). Several PGPR are able to denitrify, including species of genera such as *Pseudomonas* spp. and *Bacillus* spp. (Cutruzzolá, 1999). In the genus *Azospirillum*, most strains of *A. lipoferum* and *A. brasilense* are denitrifiers, but *A. amazonense*, *A. irakense*, and *A. oryzae* are unable to denitrify (Hartmann and Zimmer, 1994; Xie and Yokota, 2005). Anaerobic growth of *A. brasilense* in nitrate, nitrite, or nitrous oxide has been well established (Penteado Stephan *et al.*, 1984; Zimmer *et al.*, 1984). In contrast, *A. brasilense* cannot grow with NO as sole electron acceptor since its reduction does not generate a proton electrochemical gradient across the membrane (Voßwinkel *et al.*, 1991). Denitrifiers are predominantly heterotrophic microorganisms and facultative anaerobes (Wrage *et al.*, 2001). Thus, the easily decomposable matter provided by root exudates could increase the activity of denitrifiers in the rhizosphere. Besides, it has been reported that anoxic roots accumulate and excrete nitrite (Stoimenova *et al.*, 2003), which may be further reduced to NO by denitrifiers. Though rhizosphere is not always an anoxic place, *A. brasilense* and other PGPR may inhabit microaerobic or anaerobic microsites in which the conditions for denitrification are given.

The enzymes involved in denitrification are nitrate reductase, nitrite reductase, nitric oxide reductase (Nor), and nitrous oxide reductase (Nos). Three different nitrate reductase activities can be found in bacteria. *Azospirillum* and other PGPR can perform all of these activities. First, the cytoplasmic soluble assimilatory nitrate reductase (Nas) reduces nitrate to nitrite which is further reduced by a nitrite reductase to ammonia in order to be incorporated into amino acids (Hartmann and Zimmer, 1994; Steenhoudt *et al.*, 2001a). Second, the membrane-bound nitrate reductase (Nar) allows PGPR to generate energy through reduction of nitrate to nitrite (Steenhoudt *et al.*, 2001a). Finally, there is a periplasmic nitrate reductase (Nap), which is thought to function maintaining an optimal redox balance by dissipation of the reducing equivalent excess (Steenhoudt *et al.*, 2001a,b). Both, Nap and Nar are dissimilatory enzymes that reduce nitrate to nitrite in the first step of denitrification. The three enzymes bind the molybdenum cofactor (Steenhoudt *et al.*, 2001a). Dissimilative nitrite reductase (Nir) is considered the major known source of NO in bacteria. This is a periplasmic-located enzyme that catalyzes the reduction of nitrite mainly to NO (Cutruzzolá 1999) and, only in minor quantities, to N₂O (Ye *et al.*, 1994). Two distinct types of Nir have been found in denitrifiers: (1) a cytochrome *cd*₁-dNir, containing one heme *c* prosthetic group covalently linked to the polypeptide chain and one heme *d*₁ noncovalently associated with the protein; and (2)

a copper-containing protein called Cu-dNir, which is found in about one-fourth of the isolated denitrifiers (Ye *et al.*, 1994). These different Nir are never synthesized by the same organism (Zumft, 1997). Nor is a plasma membrane-bound enzyme that catalyzes the reduction of NO to N₂O (Ye *et al.*, 1994). Since the accumulation of NO can be lethal for bacteria (Ye *et al.*, 1994), expression of Nir and Nor are tightly regulated (Tosques *et al.*, 1996) and it has been suggested that both enzymes form a functional unit (Jetten *et al.*, 1997). Two Nor have been isolated. Both enzymes are cytochrome complexes containing heme *b* and heme *c* (Ye *et al.*, 1994). Finally, Nos is a periplasmic-located copper-containing enzyme that reduces N₂O to N₂ in the last step of denitrification (Jetten *et al.*, 1997).

Aerobic denitrification occurs when denitrification genes are activated at a high O₂ level (Zumft, 1997). Steenhoudt *et al.* (2001b) have identified and characterized a Nap in *A. brasilense* Sp245, which is neither repressed nor inactivated by oxygen. In aerobically grown cultures of *A. brasilense* Sp245 with nitrate as the sole N source, a production of ~120-nmol NO per gram of bacteria was determined at the end of log phase of growth by EPR. The NO concentration was 25-fold higher in NO₃⁻-than in NO₄⁺-grown cultures (Molina-Favero, C., Creus, C. M., Simontacchi, M., Puntarulo, S., and Lamattina, L., unpublished results). A Nap⁻ knockout mutant of *A. brasilense* Sp245 (strain Faj164; Steenhoudt *et al.*, 2001b) produced only 5% of NO with respect to the wt level indicating that aerobic denitrification can be an important source of NO in this bacterium (Molina-Favero, C., Creus, C. M., Simontacchi, M., Puntarulo, S., and Lamattina, L., unpublished results). Since the derived protein sequence of the *A. brasilense* Nap is highly homologous to the NapABC protein sequences of *Escherichia coli*, *Pseudomonas* sp. G-179, *Ralstonia eutropha*, *Rhodobacter sphaeroides*, and *Paracoccus denitrificans* (Steenhoudt *et al.*, 2001b), the possibility of an aerobic synthesis of NO by these microorganisms cannot be excluded.

NO production could be now considered as an advantage of the process of denitrification which was first described as undesirable in PGPR, since it may contribute to the loss of nitrogen available for plants (Paul and Clark, 1996). Supporting this suggestion, it has been reported that the root colonization ability by rhizobacteria and the plant growth-stimulatory effects are significantly diminished in the Sp245chl1 strain of *A. brasilense*, a mutant defective in both assimilatory and Nap activity (Boddey *et al.*, 1986; Jetten *et al.*, 1997). Before the finding of the signaling role of NO in plant development, it has been proposed that PGPR strains that can reduce nitrate to nitrite show a competitive advantage (Döbereiner and Pedrosa, 1987). During the nitrate respiration, part of the nitrite produced is excreted to the external medium (Bothe *et al.*, 1981; Neuer *et al.*, 1985; Zimmer *et al.*, 1984).

Moreover, classical tests for determining auxin effects show that nitrite, in concentrations similar to those produced by nitrate respiration, can mimic the IAA- and the *Azospirillum*-promoting effects (Zimmer and Bothe, 1988; Zimmer *et al.*, 1988). Authors also showed that the promoting effects of nitrite could be enhanced by adding ascorbate. Regarding NO chemistry, this observation can be explained by the nonenzymatic reduction of nitrite by ascorbate at acidic pH (Weitzberg and Lundberg, 1998). Furthermore, *A. brasilense* can increase the proton efflux by root cells, making the external pH more acidic (Bashan *et al.*, 1992) and therefore leading to NO formation in the apoplastic and intercellular space.

b. Heterotrophic nitrification. Nitrification is the biological oxidation of ammonium to nitrate. The first step of the general pathway is the oxidation of ammonium to hydroxylamine (NH₂OH), which is catalyzed by the enzyme ammonium monooxygenase. Next, hydroxylamine is oxidized to nitrite by hydroxylamine oxidoreductase. Finally, nitrite is oxidized to nitrate by nitrite oxidoreductase (Wrage *et al.*, 2001). In this pathway, NO and N₂O are produced in the reduction of to N₂ or by chemical decomposition of or NH₂OH (Anderson *et al.*, 1993; Wrage *et al.*, 2001).

Nitrification was first described in autotrophic bacteria belonging to genera such as *Nitrosomonas* and *Nitrobacter*. The complete nitrification is accomplished in two steps by two different groups of microorganisms, the NH₃ oxidizers and the NO₂ oxidizers (Wrage *et al.*, 2001). These oxidations allow autotrophic bacteria to generate energy for CO₂ fixation (Paul and Clark, 1996). Besides autotrophic nitrification, it has been recognized that heterotrophic nitrification is an important process in soils. This pathway is carried out by several fungi and heterotrophic bacteria (Paul and Clark, 1996). Autotrophic and heterotrophic nitrifications have the same substrates, intermediaries, and products, even though the enzymes involved may be different in each route. Other important differences are that the heterotrophic nitrification is accomplished by a single organism and that energy is not produced during the process (Wrage *et al.*, 2001). In addition to , some heterotrophic nitrifiers are capable of producing nitrate by oxidation of organic amines or amides (Papen *et al.*, 1989).

Heterotrophic nitrification can be a significant source of NO from bacteria living in aerobic and microaerobic soil and water (Anderson *et al.*, 1993; Papen *et al.*, 1989). This process is connected with denitrification through its products and and it has been demonstrated that both pathways could be performed simultaneously in some organisms (Wrage *et al.*, 2001). Moreover, it is frequent that the heterotrophic nitrifiers would also be aerobic denitrifiers (Anderson *et al.*, 1993; Steenhoudt *et al.*, 2001a; Wrage *et al.*, 2001).

Heterotrophic nitrification has been proved in some PGPR strains of *Pseudomonas* sp. (Castignetti and Hollocher, 1984; Papen *et al.*, 1989), *Arthrobacter* sp. (Verstraete and Alexander, 1972; Witzel and Overbeck, 1979), and *Bacillus* spp. (Lang and Jagnow, 1986). Aerobically grown cultures of *A. brasilense* are able to produce NO with ammonium as N source (Creus *et al.*, 2005). When these cultures were supplemented with hydroxylamine, a fourfold increase in the rate of NO production was observed. This increase was dose dependent, being highest at 5-mM hydroxylamine. Overall, these results suggest that *A. brasilense* possesses a heterotrophic nitrification-like pathway (Molina-Favero, C., Arruebarrena Di Palma, A., Barassi, C. A., Lamattina, L., and Creus, C. M., unpublished results).

c. Nitric Oxide synthase. In the past, attention was focused on bacterial NO production by nitrification–denitrification related processes. However, it has now been established that some bacteria can also synthesize NO in a reaction catalyzed by an NOS. This enzyme converts, in presence of oxygen, L-arginine to L-citrulline and NO in a mechanism similar to that of eukaryotes (Adak *et al.*, 2002a,b; Midha *et al.*, 2005; Sari *et al.*, 1998). Bacterial NOS can also oxidize *N*-hydroxy-L-arginine (NOHA), which is the intermediary in the reaction of mammalian NOS (Chen and Rosazza, 1995; Sari *et al.*, 1998). The first report on a bacterial NOS was published in 1994. In their work, Chen and Rosazza (1994, 1995) described an NOS activity in the genus *Nocardia*. Subsequently, NOS activity has been characterized in microorganisms such as *Deinococcus radiodurans* (Adak *et al.*, 2002b), *Rhodococcus* spp. (Cohen and Yamasaki, 2003; Sari *et al.*, 1998), *Bacillus subtilis* (Adak *et al.*, 2002a), *B. anthracis* (Midha *et al.*, 2005), *Physarum polycephalum* (Werner-Felmayer *et al.*, 1994), *Staphylococcus aureus* (Choi *et al.*, 1997; Hong *et al.*, 2003), *Salmonella typhimurium* (Choi *et al.*, 2000), *Geobacillus stearothermophilus* (Sudhamsu and Crane, 2006), and *Streptomyces turgidiscabies* (Kers *et al.*, 2004), among others.

The mammalian NOS is a dimeric protein formed by an N-terminal oxygenase domain (NOSoxy) that binds protoporphyrin IX (heme), 6*R*-tetrahydrobiopterin (H₄B), and the substrate L-arginine; and by a C-terminal reductase domain (NOSred) that binds FMN, FAD, and NADPH (Stuehr, 1997). Studies of the sequence of bacterial NOS reveal the lack of an NOSred domain but show high homology with the mammalian NOS oxygenase domain. Bacterial NOS also shows similarities in key structural features involving the conformation of the active site, the heme environment and its interaction with substrates, cofactors, and coenzymes. Generally, bacterial NOS lacks an N-terminal extension implicated in the dimer formation of mammalian isoforms, however alternative interactions

allow bacterial NOS to form a dimer (Adak *et al.*, 2002a,b; Bird *et al.*, 2002; Midha *et al.*, 2005).

Despite the numerous studies on the role of NO in plant physiology and the recognized existence of NOS in bacteria, little is known about NOS-mediated NO production and its function/s in PGPR. Some strains of *Rhodococcus* spp. and *Nocardia* spp. are able to nonpathogenically colonize the root apoplast and, the former, also the leaf apoplast in several plants. In these places, it is thought that they might benefit the plant by providing metabolites and/or outcompeting pathogens (Araujo *et al.*, 2002; Cohen and Yamasaki, 2003; Conn and Franco, 2004). It was suggested that the activity of NOS in *Rhodococcus* sp. R312 could be involved in the regulation of the enzyme nitrile hydratase (Sari *et al.*, 1998). In addition, Cohen and Yamasaki (2003) proposed that NOS could promote tolerance of *Rhodococcus* APG1 to oxidative stress. In *Nocardia* sp., NO produced by NOS could increase the levels of cyclic guanosine 3',5'-monophosphate (cGMP) by activation of guanylate cyclase. The function of cGMP remains to be determined in this bacterium (Son and Rosazza, 2000). As described before, cGMP mediates auxin responses leading to AR formation in plants (Pagnussat *et al.*, 2003, 2004). In *Bacillus* sp., the role of NOS-dependent NO production is unknown, neither in its own physiology nor in the interaction with plants. Nevertheless, a role for NOS could be hypothesized given that exogenously added NO can modify gene expression in *B. subtilis* (Moore *et al.*, 2004; Nakano, 2002). Recently, evidence for an NOS-like activity in *A. brasilense* has been found. Pure cultures of a wt strain or a Nap-deficient mutant (strain Faj164; Steenhoudt *et al.*, 2001b) showed a significant increase in NO production when culture mediums were supplemented with L-arginine (Creus *et al.*, 2005; Molina-Favero, C., Creus, C. M., Simontacchi, M., Puntarulo, S., and Lamattina, L., unpublished results). However, this stimulation was insensitive to mammalian NOS inhibitors (Creus *et al.*, 2005). Taking into account the location of PGPR in plant root tissues, the possibility that they could improve plant growth by an NOS-related NO synthesis is open and further research is needed to determine the importance of this activity in the interaction.

In addition to the occurrence of NOS in nonsymbiotic PGPR, an NOS-like activity was detected in *Lupinus albus* nodules (Cueto *et al.*, 1996). Moreover, working in the symbiosis between *Medicago truncatula* and *Sinorhizobium meliloti*, Baudouin *et al.* (2006) found that functional nodules synthesized NO by a mechanism that is neither related to denitrification nor nitrogen fixation. In both reports, mammalian NOS inhibitors were effective in the inhibition of NO synthesis suggesting that an NOS-like activity is the active pathway. It is still uncertain whether the plant or the bacteria carry out

the NOS activity. The role of NO in the interaction is also unclear. Considering the evidence that involves both the inhibition of polar auxin transport during the first steps of nodulation (Mulder *et al.*, 2005) and the requirement of NO in auxin-induced root developmental processes (Correa-Aragunde *et al.*, 2004; Lombardo *et al.*, 2006; Pagnussat *et al.*, 2002, 2003), it was hypothesized that NO could have a signaling role in the establishment of legume–rhizobia interactions (Baudouin *et al.*, 2007).

III. PERSPECTIVES

NO is a gas with a broad chemistry that involves several reactive forms that could explain its versatility as an extensive signal molecule in intra- and intercellular communication (Lamattina *et al.*, 2003). In 1992, NO was termed “Molecule of the year” by the magazine Science. Since then, a great amount of data is coming from studies on NO biology in plants (Lamattina and Polacco, 2007). In particular, the NO role in root growth and development is one of the most studied fields at the moment. We know that a close relationship and similarities exist between auxin’s actions and NO effects in root responses. It would be extremely interesting to find at what step of auxin signaling pathway NO is acting, and what molecular mechanism/s and NO form/s are involved.

It was demonstrated that when a plant senses a pathogenic bacteria it synthesizes microRNAs (miRNAs) that interfere with the production of specific proteins related with auxin signaling (Navarro *et al.*, 2006). It was shown that repression of auxin signaling restricts bacterial growth, implicating auxin in disease susceptibility (Navarro *et al.*, 2006). It would be interesting to know if a repression of auxin signaling occurs when nonpathogenic bacteria like PGPR associate with plants. We could probably speculate that plant cells would not restrict PGPR growth by repression of auxin signaling, since most of the effects in roots after PGPR colonization rely on auxin.

Figure 3 shows a schematic model with proposed pathways for NO and auxin synthesis in *Azospirillum* and their possible effects on roots. IAA is synthesized in these bacteria by different Trp-dependent and Trp-independent pathways (Prinsen *et al.*, 1993). Furthermore, NO is potentially produced by several reactions as a part of the nitrogen metabolism, including denitrification, heterotrophic nitrification, and NOS (Creus *et al.*, 2005; Hartmann and Zimmer, 1994; Molina-Favero, C., Creus, C. M., Simontacchi, M., Puntarulo, S., and Lamattina, L., unpublished results). During the PGPR–plant interaction, both IAA and NO of bacterial origin could reach plant cells and initiate the rooting processes. It is also possible that these

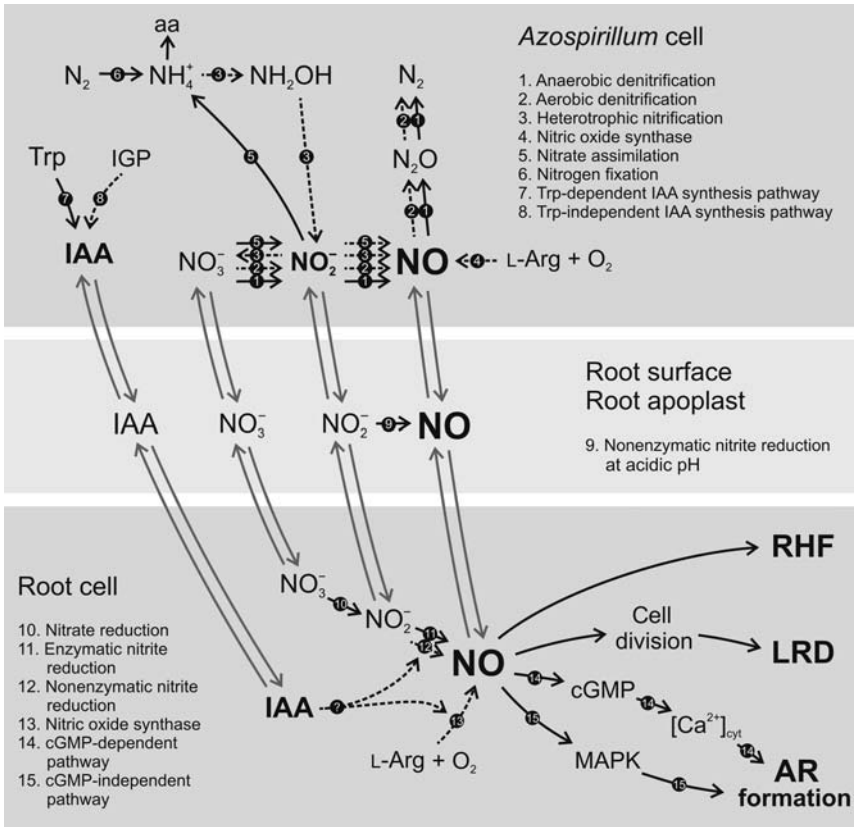


Fig. 3. Schematic model proposing NO synthesis and signaling pathways influencing root growth and developmental processes in the PGPR–root association. NO and IAA are produced by different metabolic pathways in both PGPR and root cells. In the PGPR–root interaction, these signaling compounds can be exchanged between both partners. In root cells, IAA induces NO production by one or more hypothetical mechanisms. Then, NO acts as a messenger triggering a complex signaling network that leads to root branching and growth. The role of other hormones and cellular messengers are presented for LRD, AR formation, and suggested for RHF. Solid arrows indicate established pathways. Dashed arrows indicate pathways with supporting experimental evidence but not completely proved. Double gray arrows indicate unknown transport and/or diffusion processes. Abbreviations: aa, amino acids; cGMP, cyclic GMP; IAA, indole-3-acetic acid; IGP, indole-3-glycerol phosphate; L-Arg, L-arginine; MAPK, mitogen-activated protein kinase; Trp, tryptophan.

signaling compounds, as well as nitrite and nitrate, could be freely interchanged between both partners of the association. In root cells, NO could be produced from nitrite, nitrate, and L-arginine in enzymatic and nonenzymatic pathways (Stöhr and Stremlau, 2006). Moreover, it is known that auxins increase NO production triggering RHF, LRD, and AR

formation (Correa-Aragunde *et al.*, 2004; Lombardo *et al.*, 2006; Pagnussat *et al.*, 2002). Several second messengers such as cGMP, Ca²⁺, and MAPK were reported to be involved in these developmental processes (Lanteri *et al.*, 2006a; Pagnussat *et al.*, 2003, 2004). Taking into account the similarities that *Azospirillum* and NO display on influencing root growth, developmental, and physiological processes, it would be interesting to know if these effects are exerted through the same second messengers.

Even though a general picture can be depicted, several questions raise from experiments involving NO in the PGPR–root interaction and in the root developmental processes. It would be also valuable to find a more precise explanation of the roles of NO, produced by both bacteria and root cells, in the establishment of the association and in root branching, in order to correlate it with plant fitness. The understanding of how auxin induces NO synthesis in root cells and how plant modulates NO production in the micro-organism would be major aims in the future research. Concerning this aspect, the use of genetic tools will be necessary to find PGPR strains and plants with lower and higher production of NO to study the mechanisms of NO synthesis and the ways by which NO modifies root architecture. The exploration of the tight link of NO in auxin-modulated processes like root growth and development will surely be a matter of intense research in the next future.

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