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Nitric Oxide is Involved in the *Azospirillum brasilense*-induced Lateral Root Formation in Tomato

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Abstract *Azospirillum* spp. is a well known plant-growth-promoting rhizobacterium. *Azospirillum*-inoculated plants have shown to display enhanced lateral root and root hair development. These promoting effects have been attributed mainly to the production of hormone-like substances. Nitric oxide (NO) has recently been described to act as a signal molecule in the hormonal cascade leading to root formation. However, data on the possible role of NO in free-living diazotrophs associated to plant roots, is unavailable. In this work, NO production by *Azospirillum brasilense* Sp245 was detected by electron paramagnetic resonance (6.4 nmol. g⁻¹ of bacteria) and confirmed by the NO-specific fluorescent probe 4,5-diaminofluorescein diacetate (DAF-2 DA). The observed green fluorescence was significantly diminished by the addition of the specific NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (cPTIO). *Azospirillum*-inoculated and noninoculated tomato (*Lycopersicon esculentum* L.) roots were incubated with DAF-2 DA and examined by epifluorescence microscopy. *Azospirillum*-inoculated roots displayed higher fluorescence intensity which was located mainly at the vascular tissues and subepidermal cells of roots. The *Azospirillum*-mediated induction of lateral root formation (LRF) appears to be NO-dependent since it was

completely blocked by treatment with cPTIO, whereas the addition of the NO donor sodium nitroprusside partially reverted the inhibitory effect of cPTIO. Overall, the results strongly support the participation of NO in the *Azospirillum*-promoted LRF in tomato seedlings.

Keywords *Azospirillum* · Lateral roots · Nitric oxide · Tomato

Abbreviations ACR: Agar Congo Red · ARF: adventitious root formation · cPTIO: 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide · DAF-2 DA: 4,5-diamino-fluorescein diacetate · EPR: electron paramagnetic resonance · IAA: indole acetic acid · L-NAME: N^G-Nitro-L-arginine methyl ester hydrochloride · L-NIO: L-N⁵-(1-iminoethyl)-ornithine dihydrochloride · LR: lateral root · LRF: lateral root formation · NO: nitric oxide · OAB: Okon-Albrecht-Burris · PCIB: α -(*p*-chlorophenoxy) isobutyric acid · SDW: sterile distilled water · SNP: sodium nitroprusside

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Introduction

Azospirillum represents one of the best characterized free-living diazotrophs among plant growth-promoting bacteria (Bashan and Holguin 1997). It can be found in a wide range of habitats associated with roots of various types of plant species. Under certain environmental and soil conditions, *Azospirillum* was shown to exert beneficial effects on plant growth, water status, N-content and crop yield (Okon and Labandera-González 1994; Casanovas et al. 2003; Creus et al. 2004).

Azospirillum induces remarkable changes in root morphology. Inoculation of wheat promotes lateral root and root hair formation (Kapulnik et al. 1985)

and increases the diameter and length of both lateral and adventitious roots (Okon and Labandera-González 1994). The production or modification of plant growth substances by *Azospirillum* has often been proposed as one of the key factors responsible for the observed plant growth promotion (Tien et al. 1979; Cassán et al. 2001). Some experiments with *Azospirillum* mutants altered in IAA production support this view (Dobbe-laere et al. 1999). Other reports have ascribed to the ion nitrite produced by *Azospirillum* metabolism to be responsible for the effects observed on plant growth and developmental processes upon root inoculation (Zimmer et al. 1988; Didonet and Magalhaes 1993). When oxygen is a limiting factor in cultures, *Azospirillum* can utilize nitrate, nitrite or nitrous oxide(s) as terminal respiratory electron acceptors. Even though nitric oxide (NO) is one of the nitrogen species produced by the nitrate dissimilation pathway in bacteria, no data about NO production under aerobic conditions in *Azospirillum* spp. is available. Moreover, no data were reported about the NO function in the association of free-living diazotrophs with plant roots. Nitric oxide is a small diffusible gas and a ubiquitous bioactive molecule, which participates in a broad spectrum of pathophysiological and developmental processes in living organisms (Lamattina et al. 2003). The ability of NO to act simultaneously on several unrelated biochemical nodes through the modulation of the cell redox status and Ca^{2+} cytosolic concentrations suggests NO as a molecule with interesting signaling and homeostatic properties for the coordination and synchronization of cell metabolism (Lamattina et al. 2003).

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 are clearly implicated in root growth and proliferation. In this regard, it has been already demonstrated that NO functions as a signal molecule in the IAA-induced signaling cascade leading to adventitious root development (ARD) (Pagnussat et al. 2002; 2003). More recently, it was also reported that NO plays a central role during lateral root formation (LRF) in tomato (Correa-Aragunde et al. 2004).

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 ARD and LRF led us to explore whether the *Azospirillum* ability to promote root growth relies on NO. Therefore, tomato seedlings represent an excellent model to study: (1) *Azospirillum*-stimulated LRF and (2) the NO involvement in the *Azospirillum*-promoted LRF. Thus, the aims of this work were to investigate if *Azospirillum* is able to synthesize NO itself and to study whether NO could be the inducer molecule involved in the *Azospirillum*-mediated changes in the tomato root system.

Materials and methods

Bacterial growth

Azospirillum brasilense Sp245 was grown on Agar Congo Red (ACR) medium (Rodríguez Cáceres 1982) during 4 days, transferred to OAB liquid medium (Okon et al. 1977) containing 0.1% NH_4Cl , incubated for 16 h at 30°C with orbital agitation (100 rpm), and cells harvested by centrifugation. The resulting pellet was washed twice with sterile phosphate buffer (pH 7) and subsequently used either as inoculum or frozen in liquid nitrogen to determine NO content. Inoculum was obtained by resuspending the bacterial pellet to 10^7 cells ml^{-1} in phosphate buffer (pH 7). For NO quantification assays in *Azospirillum* cultures, 1 or 100 nM indole acetic acid (IAA), 1 mM tungstate, 100 μM L-NIO, 100 μM L-NAME or 15 mM L-arginine were added to the OAB media.

Quantification of NO in *Azospirillum*

NO production by *Azospirillum* was quantified by electron paramagnetic resonance (EPR). Cells were harvested by centrifugation after 16 h of growth, at the end of the log phase. The bacterial pellets were frozen in liquid nitrogen and stored until they were processed. A spin trap solution of 10 mM sodium N-methyl-d-glucamine dithiocarbamate (MGD) in 1 mM FeSO_4 was added to the frozen pellet and NO content measured with the Bruker ECS 106 EPR spectrometer with a cavity ER 4102ST, operating at 9.75 GHz.

Seedling growth and quantification of lateral roots

Seeds of *Lycopersicon esculentum* (cv ACE 55) were surface-disinfected in 3% NaClO for 2 min and washed twice with sterile distilled water (SDW), then sequentially soaked for 48 h in SDW and 2 h in the inoculum (inoculated) or phosphate buffer (control, non-inoculated). They were transferred to paper and incubated in moist chamber at 25°C for 10 days in darkness. For NO treatments, filter papers were moistened with 100, 200, and 300 μM of the NO donor sodium nitroprusside (SNP). One mM of carboxy-PTIO (cPTIO) was used as a NO scavenger to prevent NO-mediated effect. Five mM of the auxin antagonist p-chlorophenoxy isobutyric acid (PCIB) was used to compete with endogenous auxin. Percentage of plants with lateral roots (%LR) was calculated by counting plants with at least one LR considered as such when its length was no less than 1 mm.

Fluorescence assays

Detection of NO by the specific fluorescent probe 4,5-diamino-fluorescein diacetate (DAF-2 DA; Calbiochem,

USA) was assayed in *A. brasilense* cells. The bacteria were resuspended in 20 mM HEPES-NaOH pH 7.8 in the presence or absence of 0.5 mM cPTIO for 30 min. Fifteen μM DAF-2 DA was added, incubated for 2 h, washed, and bacteria examined by epifluorescence microscopy at 1000 \times magnification. Nitric oxide detection in inoculated and noninoculated root segments were assayed as previously described (Correa-Aragunde et al. 2004). Briefly, root segments were taken at 5 mm from the hypocotyl-root junction, incubated with 15 μM DAF-2 DA for 2 h, and washed. Root segments were transversally cut with a razor blade or squashed, and observed by epifluorescence and bright-field microscopy.

Bacterial colonization assessment

A 0.5 g of root material was homogenized in a mortar with 4.5 mL of 66 mM phosphate (pH 7). Three 0.1 mL replicates from serial dilutions were cultured in semisolid NFB medium (Döbereiner and Day, 1976), and most probable numbers of bacteria per gram of root fresh weight (MPN g^{-1} FW) were estimated. Bacteria were then transferred to ACR medium to detect stained colonies identical to those formed by pure *A. brasilense* culture (Rodríguez Cáceres 1982).

Experimental design and statistical analysis of data

Three independent experiments were done with at least 60 seeds per treatment. For NO quantification by EPR duplicated samples were assayed in all cases. Photographs are representative of results obtained after the processing and analysis of ten seedlings for each condition in three independent experiments.

Results

Aerobically-grown *Azospirillum brasilense* produces nitric oxide

In order to evaluate NO production by *A. brasilense* growing in aerobic culture, different amounts of bacteria were prepared to measure NO concentration by EPR. Figure 1a shows that *A. brasilense* produces significant amounts of NO and this quantity is correlated with the amount of bacteria analyzed. It also shows that NO signal remained at the baseline in the control boiled sample of bacteria. Based on these measurements, a concentration of 6.4 nmol NO per gram of *A. brasilense* growing in aerated OAB medium during 16 h was calculated. Nitric oxide production by *A. brasilense* was further confirmed by the NO-specific fluorescent probe DAF-2 DA. Green fluorescence was detected at different intensities in individual bacterium, which was significantly diminished by the addition of the NO

scavenger cPTIO (Fig. 1b). Since nitrogen fixation was inhibited by aerobiosis, the sole source of nitrogen for *Azospirillum* growth was ammonium. The absence of nitrate in the medium and the aerobic conditions could probably not allow *Azospirillum* to use the dissimilation pathway to generate NO. Thus, it still remains possible that under our experimental conditions, NO synthesis

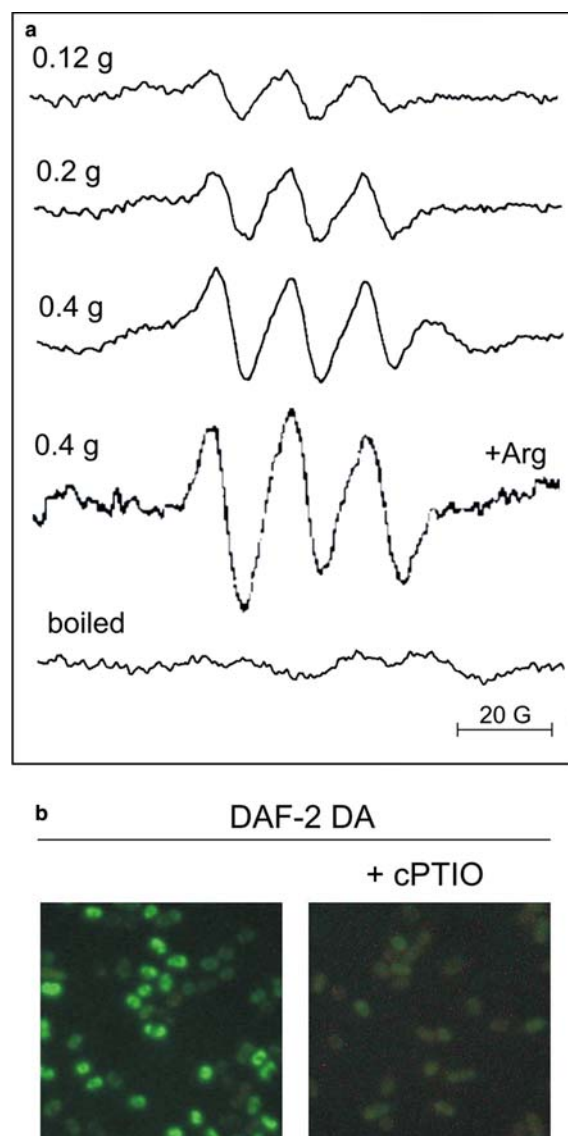


Fig. 1 Nitric oxide (NO) detection in *Azospirillum brasilense*. **a** Endogenous NO level was determined by Electron Paramagnetic resonance spectra of the NO-MGD-Fe adduct in the pellet of different amounts (0.12, 0.2 and 0.4 g) of *A. brasilense* Sp245 growing in OAB medium. EPR spectra were also performed with 0.4 g of bacteria boiled for 5 min and 0.4 g of bacteria growing in OAB medium supplemented with 15 mM Arginine (+Arg). The area above the baseline indicates the amount of NO for each homogenate. **b** NO detection by the fluorescent probe DAF-2 DA in *A. brasilense*. The bacteria was resuspended in 20 mM HEPES-NaOH pH 7.8 in the presence or absence of 0.5 mM cPTIO for 30 min. Fifteen μM DAF-2 DA was added, incubated for 2 h, washed, and bacteria examined by epifluorescence microscopy at 1000 \times magnification

could occur through an NO synthase (NOS)-like activity as described in other plant colonizing bacteria (Cohen and Yamasaki 2003). However, we were unable to inhibit the NO production with the specific NOS inhibitors L-NAME (100 μ M) or L-NIO (100 μ M) (not shown). The nitrate reductase (NR) inhibitor tungstate (1 mM) was also unable to reduce the NO production in *Azospirillum* (not shown). In addition, the NOS and NR inhibitors were also ineffective in combined treatments (not shown). In contrast, when the NOS substrate arginine (15 mM) was added to the culture media, the production of NO increased by 50% (Fig. 1a). This preliminary evidence supports the presumption that at least a NOS-like activity, which use arginine as substrate, could be operative in *Azospirillum* growing in aerobiosis with ammonium as nitrogen source.

Lateral root formation induced by *Azospirillum* inoculation is associated to NO production

Taking into account that *Azospirillum* could produce NO by itself, it was interesting to analyze the presence of NO in *Azospirillum*-inoculated roots of tomato seedlings. The sensitivity of the EPR method did not allow to measure NO in inoculated tomato roots. Therefore, NO accumulation was evaluated by the reaction with DAF-2 DA, a more sensitive method. *Azospirillum*-inoculated and control tomato roots grown in the presence or absence of 1 mM cPTIO were incubated with DAF-2 DA and examined by epifluorescence microscopy. Roots from inoculated seeds displayed higher fluorescence intensity compared to noninoculated ones. This fluorescence could be partially blocked by cPTIO (Fig. 2a, b). Fluorescence was located mainly at the vascular tissues and subepidermal cells (Fig. 2b).

The next step was to analyze the effect of *Azospirillum* inoculation on LRF in tomato roots and its dependence on NO. We have established experimental conditions in which noninoculated control seedlings do not develop lateral roots. Under these conditions, the treatment with the NO donor SNP induces LRF in a dose-dependent manner (Fig. 3a). When seeds were *Azospirillum*-inoculated, 28% of the seedlings were able to display LRF (Fig. 3a). No additive effect was evidenced in a combined treatment when SNP was added to inoculated seedlings. Treatment with cPTIO completely blocked *Azospirillum*-induced LRF, whereas the further addition of SNP partially reverted the inhibitory effect of cPTIO (Fig. 3a). Therefore, it can be concluded that *Azospirillum*-promoted LRF requires NO. On the other hand, Zimmer et al. (1988) reported that nitrite produced by *Azospirillum* could have hormonal effects in plants. However, a high nitrite concentration to obtain an optimum hormonal effect was required, ascribing this to a possible formation of NO, N₂O or N₂O₄ from nitrite by disproportionation (Jones 1973). Nevertheless, previous experiments performed in our lab have shown no

effect of nitrite in promoting LRF in tomato (unpublished results).

When analyzed, the number of LR primordia correlated with the number of emerged LRs (not shown), suggesting that the promotion effect of *Azospirillum* and NO is at earlier stages of LR development. Figure 3a also shows that the competitive auxin inhibitor PCIB was able to decrease the percentage of inoculated tomato seedlings displaying LRF, suggesting that auxins are partially involved in *Azospirillum*-induced LRF. Previous results showed that exogenous NO application could revert the action of the inhibitor of basipetal auxin efflux 1-naphthyl-phtalamic acid (NPA) on LRF in tomato (Correa-Aragunde et al. 2004). Therefore, we assayed whether NO was also able to restore the level of *Azospirillum*-promoted LRF when it is affected by PCIB. Figure 3a shows that the maximum promotion activity

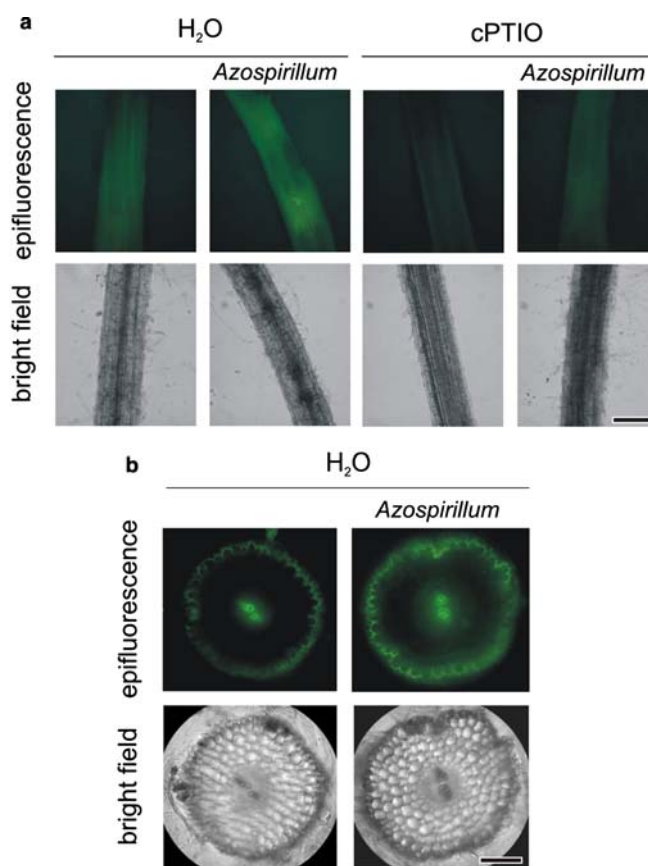


Fig. 2 Nitric oxide accumulation in *A. brasilense*-inoculated tomato seedlings. Tomato seeds were inoculated with *A. brasilense* and treated with H₂O or 1 mM cPTIO for 7 days. Control seeds were not inoculated (-). Root segments were taken at 5 mm from the hypocotyls-root junction, loaded with 15 μ M of the NO-specific fluorescent probe DAF-2 DA, incubated, washed and observed by epifluorescence (upper panels) and bright-field (lower panels) microscopy. **a** Longitudinal view of squashed roots. **Bar** = 0.3 mm. **b** Transversal sections of the primary root showing the green fluorescence representing NO. **Bar** = 0.05 mm. Photographs are representative of results obtained after the analysis of ten tomato seedlings for each condition in three independent experiments

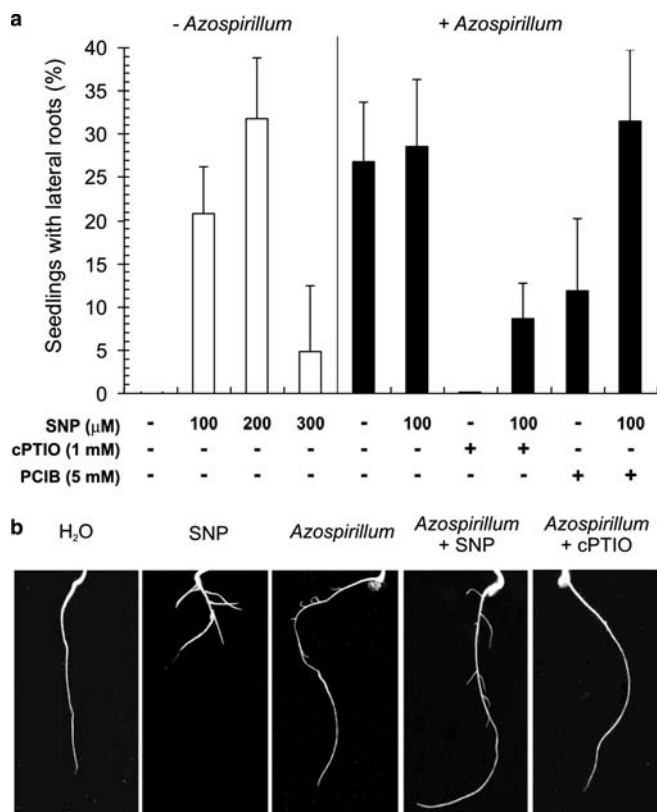


Fig. 3 Nitric oxide is required for *A. brasilense* induced LRF. Seeds were inoculated with *A. brasilense* Sp245 (10^7 cells ml^{-1} in phosphate buffer pH 7) as indicated. Noninoculated control seeds were imbibed in phosphate buffer and did not develop lateral roots. Sodium nitroprusside (SNP) was used as NO donor. cPTIO (1 mM) and PCIB (5 mM), were used as NO- and IAA-blockers, respectively. **a** Percentage of tomato seedlings displaying lateral roots. Values are expressed as percentage of plants with one or more lateral roots \pm SE ($n = 60$, $P < 0.05$). **b** Photographs of tomato roots representative of the different treatments

of *Azospirillum* on LRF was restored when 100 μM SNP was added together with PCIB. Figure 3b shows representative tomato seedlings displaying *Azospirillum*- and NO-induced LRF after 12 days of treatment. It should be pointed out that, while the exogenous NO application induces LRF and shortens the primary root, the endogenous NO generated in *Azospirillum*-inoculated seedlings induces LRF but it is not paralleled by a decreased primary root growth (Fig. 3b). This could be explained by the high NO concentration in roots when supplied exogenously, which was already shown to diminish the primary root growth (Correa-Aragunde et al. 2004).

Since the different treatments could affect the ability of *Azospirillum* to colonize tomato roots, bacterial number in inoculated and noninoculated roots was determined. In all cases, seedlings from inoculated seeds had an average of 10^6 bacteria per g FW (Table 1). Thus, the reduced ability of *Azospirillum* to promote LRF in cPTIO and PCIB treatments cannot be attributed to a lack or diminished colonization. In roots from noninoculated controls 10^3 bacteria were recovered

(Table 1). When transferred to ACR medium, only samples from inoculated plants developed scarlet colonies similar to those obtained with pure *A. brasilense* Sp245 cells (not shown). Bacterial mass per root was estimated from MPN g^{-1} of roots and MPN in a known pellet mass data. Table 1 shows that an average of 0.5 μg of bacteria per root could be estimated. Overall, these data remain still insufficient to distinguish if the association of *Azospirillum* with plant root cells could modify the NO production by the bacteria and/or the NO production by root cells. Therefore, further approaches should be designed to find answers to this question and to elucidate the origin of the increased NO production and the way it promotes LRF in inoculated tomato roots.

Discussion

One of the most accepted explanations on how *Azospirillum* could promote plant growth is the bacterial ability to produce phytohormones (Tien et al. 1979) and to transform plant inactive forms into active ones (Cassan et al. 2001). In this regard, even though auxin production has been thoroughly studied, it is still not clear to what extent *Azospirillum* produces IAA in the rhizosphere and how is it regulated (Steenhoudt and Vanderleyden 2000). The auxin competitive inhibitor PCIB reduced the stimulatory effect produced by *Azospirillum* on LRF suggesting that auxins are also involved in *Azospirillum*-induced effects, probably triggering an increase in NO concentration as was previously reported (Pagnussat et al. 2002). On the other hand, treatments with the NO scavenger cPTIO completely blocked *Azospirillum*-induced LRF and the subsequent addition of SNP partially reverted the inhibitory effects of cPTIO and PCIB. All together, these results support the hypothesis that NO is required for the *Azospirillum*-promoted LRF and that both compounds auxins and NO, are cellular messengers involved in the intimate association *Azospirillum*-root leading to LRF.

We show with two different experimental approaches that NO is produced by *Azospirillum* growing in aerobic conditions. Our preliminary results using NOS inhibitors as well as NOS substrate are not conclusive. Nitric oxide production in *Azospirillum* is probably accomplished through a NOS-like activity, at

Table 1 Most probable number (MPN) of *Azospirillum brasilense* in tomato roots. Values are expressed as MPN per gram of FW of roots (MPN g^{-1} FW) and micrograms of bacteria estimated per root ($\mu\text{g root}^{-1}$). Values are means of three independent measurements (\pm SE) representing any treatment in which tomato seeds were inoculated (Yes) or noninoculated (No)

<i>A. brasilense</i> -inoculation	MPN g^{-1} FW ($\times 10^5$)	$\mu\text{g root}^{-1}$
Yes	25 ± 1	0.5 ± 0.08
No	0.023 ± 10^{-3}	$0.5 \cdot 10^{-3} \pm 10^{-4}$

least in aerobiosis and with ammonium as nitrogen source. The apparent contradiction between the results obtained with NOS inhibitors and NOS substrate was already stated as the L-arginine paradox and could be explained by the presence of endogenous NOS inhibitors (Tsikas et al. 2000). Similar results were also described in two other reports in which known inhibitors of mammalian NOS were unable to inhibit NOS activity (Choi et al. 1998; Cohen and Yamasaki 2003). However, other NO sources in *Azospirillum* growing under different conditions, as well as complementary pathways cannot be discarded. Since NO formation would occur in the absence of denitrification, more experiments should be performed in order to discard a putative heterotrophic nitrification as was described in other gram-negative bacteria including *Pseudomonas putida* and *Alcaligenes faecalis* (Anderson et al. 1993). Additionally, the presence and expression of a NOS-like activity in *A. brasilense* should be demonstrated.

Increased NO accumulation in inoculated plants could be the consequence of some processes other than NO production by *Azospirillum* itself, among them: (a) Induced reduction of nitrite into NO in inoculated plant roots (Zimmer et al. 1998; Stöhr and Ullrich 2002); (b) Acidification of the root apoplast caused by *Azospirillum* (Bashan and Levanony 1991) that in turn could induce a non-enzymatic NO production in plant roots (Bethke et al. 2004). Genetically-modified plants or *Azospirillum* strains with diminished NO generation would be valuable tools for discriminating between these processes.

In short, the results presented here show that *A. brasilense* Sp245 cells growing in aerobiosis could produce NO using ammonia as the sole nitrogen nutrient. Also, NO produced directly or indirectly by *Azospirillum* when colonizing tomato roots is involved in the process of lateral root formation promoted by the bacterium. These results open the door to new research lines aimed to unravel the molecular mechanisms involved in the plant-growth-promoting properties of the bacteria when associated to plant roots.

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