Nitric oxide metabolism and indole acetic acid biosynthesis cross-talk in Azospirillum brasilense SM

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Abstract

Production of nitric oxide (NO) and the presence of NO metabolism genes, nitrous oxide reductase (nosZ), nitrous oxide reductase regulator (nosR) and nitric oxide reductase (norB) were identified in the plant-associated bacterium (PAB) Azospirillum brasilense SM. NO presence was confirmed in all overexpressing strains, while improvement in the plant growth response of these strains was mediated by increased NO and indole-3-acetic acid (IAA) levels in the strains. Electron microscopy showed random distribution to biofilm, with surface colonization of pleiomorphic Azospirilla. Quantitative IAA estimation highlighted a crucial role of nosR and norBC in regulating IAA biosynthesis. The NO quencher and donor reduced/blocked IAA biosynthesis by all strains, indicating their common regulatory role in IAA biosynthesis. Tryptophan (Trp) and L-Arginine (Arg) showed higher expression of NO genes tested, while in the case of ipdC, only Trp and IAA increased expression, while Arg had no significant effect. The highest nosR expression in SMnosR in the presence of IAA and Trp, along with its 2-fold IAA level, confirmed the relationship of nosR overexpression with Trp in increasing IAA. These results indicate a strong correlation between IAA and NO in A. brasilense SM and suggest the existence of cross-talk or shared signaling mechanisms in these two growth regulators.

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Keywords: Azospirillum brasilense; nosR; nosZ; norBC; ipdC; Scanning electron microscopy

1. Introduction

Auxins are one of the most essential plant growth regulators, involved in functions ranging from apical dominance to root growth and development [1,2]. Different Azospirillum brasilense strains may be endophytic [3] or colonize the plant surface [4–6] for evading competition from the surrounding rhizosphere. In plants, various studies have elucidated the role of NO in growth and development, seed dormancy/germination [7], flowering [8], photosynthesis [9], stomatal movement [10,11] and programmed cell death [12], among many others. Nitrification-denitrification by Gram-negative bacteria has been established as an enzymatic process, with NO as an obligatory intermediate [13–15]. The genes involved in these processes are shown in Fig. 1 [16].

Nitrous oxide reductase, encoded by nosZ, is a copper-containing enzyme and a part of the gene cluster nosRZDFYL. In Pseudomonas stutzeri, transcription of nosZ is dependent on a membrane-bound iron—sulfur flavoprotein regulatory component, NosR [17,18]. Nitric oxide reductase (Nor) is a membrane-bound enzyme of the heme-copper oxidase superfamily and catalyzes the reduction of NO to nitrous oxide (N₂O), an obligatory step in bacterial denitrification [19,20]. Hino and colleagues established it to be an integral membrane protein consisting of small (NorC) and large
(NorB) subunits [21]. For a long time, nitrification-denitrification was considered the sole mechanism of biosynthesis of NO in microbes. However, Chen and Rosazza reported the presence of nitric oxide synthase (Nos) in *Nocardia* (1994), which converts L-arginine (Arg) to L-citrulline in the presence of oxygen, thereby releasing NO [22]. Sequence analysis of bacterial Nos has shown that it has high homology with the mammalian oxygenase domain, but lacks the reductase domain [14,23,24]. Creus et al. [13] established that Nos-like activity could be operative in *Azospirillum*, as the addition of Arg to growth media enhances NO production, though the *nos* gene (encoding a nitric oxide synthase) is absent in the *A. brasilense* Sp245 sequenced genome.

Previous studies have shown that NO mediates IAA-induced adventitious rooting in cucumber, as well as root hair formation and lateral branching in tomato [25–29]. Some intermediates of the signaling pathway play a pivotal role in the IAA–NO interaction, leading to improved root development in plants. Studies carried out by Pagnussat et al. [25–27] conclusively demonstrated cGMP-dependent and -independent pathways for IAA–NO–triggered rooting. NO could increase the levels of cGMP via guanylate cyclase and this mediates auxin-induced adventitious rooting in plants. Also, it was established that the mitogen-activated protein (MAP) kinase cascade is activated during IAA-dependant adventitious rooting [27].

Some of our earlier work demonstrated the functional and genetic characterization of IAA biosynthesis in *A. brasilense* SM mediated by means of the indole-3-pyruvate pathway involving indole-3-pyruvate decarboxylase, IpdC [30,31]. The absence of the strain Sp245 IAA regulator, *iaaC*, in strain SM was shown to be an important factor resulting in variations in IAA patterns between different strains [30]. In the present study, we reveal production of NO by strain SM and expression of NO metabolism genes, and we investigate the existence of a correlation/cross-talk between IAA and NO produced by this plant-associated bacterium (PAB).

2. Materials and methods

2.1. Bacterial strains and growth conditions

*A. brasilense* SM (MTCC 4037, India) and its derivatives were used in this study. The bacterial cultures were maintained on Luria–Bertani agar with antibiotics as per the requirement of the strain. Purity of the culture was checked on nitrogen-free basal (Nfb) medium as described [32]. For all experiments, an initial OD<sub>560</sub> of 0.1 was obtained by diluting overnight-grown cultures of *A. brasilense* cultured in 20 ml of buffered standard succinate medium (SSM). Cells were grown at 30 °C, 160 rpm for all experiments. All chemicals used were of analytical grade and purchased from Sigma–Aldrich (St. Louis, MO, USA). For details of bacterial strains used in this study, refer to Table 1.

2.2. DNA manipulations and in silico analysis

*A. brasilense* SM genomic DNA was isolated according to standard protocols [36]. Plasmids were transferred into *A. brasilense* SM by electroporation with the Gene-pulser (Bio-Rad Labs, Hercules, CA, USA) as mentioned earlier [32]. Plasmid isolation, restriction digestion, ligation and transformation of *Escherichia coli* strains were performed by standard techniques [33]. Restriction and other enzymes were purchased from New England Biolabs. DNA was extracted from agarose gel and eluted by the GenElute Gel Extraction kit (Sigma–Aldrich).

In order to confirm the presence of NO metabolism genes in strain SM (depicted in Fig. 1), evidence was sought using PCR with primers designed from the draft genome sequence of *A. brasilense* Sp245 available at http://img.jgi.doe.gov/cgi-bin/w/main.cgi?section=TaxonDetail&page=taxonDetail&taxon_oid=2511231222 [37]. The primers were thus designed to include the entire coding region, and were able to amplify *nosZ*, *nosR* and *norBC* from strain SM genomic DNA (for...
Table 1
Details of strains used and generated in this study.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XL1Blue</td>
<td>supE44 hsdR17 (m&lt;sup&gt;cr&lt;/sup&gt;) recA1 endA1 thi-1 gyrA96 relA1 lac- F&lt;sup&gt;proAB + lacIq lac Z&lt;/sup&gt; D(M15 Tn10(tec))</td>
<td>[33]</td>
</tr>
<tr>
<td>DH5&lt;sup&gt;+&lt;/sup&gt;</td>
<td>hsdR17 endA1 thi-1 gyrA96 relA1 recA1 supE44 ΔlacU169Φ80lacZΔM15</td>
<td>[33]</td>
</tr>
<tr>
<td>pBKS&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Cloning vector; ColE1 replicon; Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pME6000</td>
<td>7.20 kb cloning vector, pBRR1MCS derivative; Te&lt;sup&gt;C&lt;/sup&gt;, P&lt;sub&gt;lac&lt;/sub&gt;</td>
<td>Lab culture collection [34]</td>
</tr>
<tr>
<td>pMEK614</td>
<td>8.10 kb pME6000 derivative, carrying 0.90 kb SacI fragment encoding kan&lt;sup&gt;R&lt;/sup&gt;, Te&lt;sup&gt;C&lt;/sup&gt;</td>
<td>Lab culture collection, [30]</td>
</tr>
<tr>
<td>pBKSnosZ</td>
<td>pBKS + derivative carrying the ~1.5 kb A. brasilense SM nosZ gene in HindIII – XbaI sites</td>
<td>This study</td>
</tr>
<tr>
<td>pBKSnosR</td>
<td>pBKS + derivative carrying the ~2.2 kb A. brasilense SM nosR gene in XbaI – SacI sites</td>
<td>This study</td>
</tr>
<tr>
<td>pMEKnosZ</td>
<td>pMEK614 derivative carrying the ~1.5 kb A. brasilense SM nosZ gene, subcloned from pBKSnosZ</td>
<td>This study</td>
</tr>
<tr>
<td>pMEnosR</td>
<td>pMEK614 derivative carrying the ~2.2 kb A. brasilense SM nosR gene, subcloned from pBKSnosR</td>
<td>This study</td>
</tr>
<tr>
<td>pMeEnorBC</td>
<td>pMEK614 derivative carrying the ~1.83 kb A. brasilense SM norBC genes</td>
<td>This study</td>
</tr>
</tbody>
</table>

| **Azospirillum brasilense** |                          |           |
| SM                | Wild-type strain, isolated from Sorghum officinallis roots, Ap<sup>R</sup> | MTCC 4037, IMTECH, India; [32] |
| Sp245             | Wild-type strain, isolated from surface-sterilized wheat roots (Brazil), Ap<sup>R</sup> | [35] |
| SMnorBC          | Strain SM derivative carrying pMEEnorBC overexpressing A. brasilense SM norBC genes, Ap<sup>R</sup>, Te<sup>C</sup> | This study |
| SMnosZ           | Strain SM derivative carrying pMEKnosZ, overexpressing A. brasilense SM nosZ gene, Ap<sup>R</sup>, Km<sup>R</sup> | This study |
| SMnosR           | Strain SM derivative carrying pMEKnosR, overexpressing A. brasilense SM nosR gene, Ap<sup>R</sup>, Te<sup>C</sup> | This study |

Note: A. brasilense strain SM genomic DNA was isolated according to Ref. [36]. Plasmids were transferred into strain SM and its derivatives by electroporation as mentioned [32]. Plasmid isolation, restriction digestion, ligation and transformation of E. coli strains were performed by standard techniques [33].

All specific amplicons were sequenced and the sequences were analyzed for their homology with BLAST (megablast, NCBI), and their identity was confirmed. Strain SM sequences for nosR, nosZ, norC and norB were deposited in the NCBI Genbank under accession numbers FJ358640, FJ358638, KJ626308 and KJ626309. In addition, in silico analysis of the NO metabolism genes was performed to identify the conserved domains from the translated proteins using the Batch Web CD-Search Tool (NCBI).

The fragments were cloned using a two-step sequential strategy involving pBKS<sup>+</sup>, followed by subcloning into the modified broad host range cloning vector pME6000 and pMEK614, a variant of pME6000 [30] (Supplementary Fig. 1). Plasmid pBRR1MCS-based vectors had already been shown to be stable in A. brasilense [38] and suitable for cloning and gene expression studies in strain SM through our earlier studies [30–32]. The various PCR-amplified genomic fragments were cloned into the vectors to yield pMEKnosZ, pMEnorBC and pMeEnosR. All recombinant plasmids were introduced into strain SM to generate putative overexpressing strains SMnorBC, SMnosZ and SMnosR. Construction details of these clones are shown in Table 1.

2.3. Fluorescence assay for qualitative detection of NO

Detection of NO was assayed in A. brasilense cells by the specific fluorescence probe 4,5-diamino-fluorescein diacetate (DAF-2 DA; Sigma–Aldrich, USA). The SM wild type and derived strains were suspended in 20 mM HEPES-NaOH pH 7.8 in the presence or absence of 0.5 mM cPTIO(2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide: NO scavenger) for 30 min. Ten μM DAF-2 DA was added, incubated for 2 h and bacterial cells were examined by an LSM 710 Confocal laser scanning microscope (Carl Zeiss AG, Germany).

2.4. Quantification of NO in A. brasilense

A method previously described by Molina-Favero et al. [15], was used for measurements of NO production in the different treatments by monitoring changes in the fluorescence intensity of the NO-specific fluorescent probe DAF-2 DA [39]. Cultures (100 μl) of A. brasilense wild type and derived strains grown in SSM with or without the different treatments (200 μM sodium nitroprusside (SNP); 15 mM arg; 100 μM L-N<sup>3</sup>-nitroarginine methyl ester(-NAME); 100 μM N<sup>2</sup>-(1-iminoethyl)-L-ornithine, dihydrochloride(L-NIO); 100 μM 2,3,5-triodobenzoic acid (TIBA); 500 μM p-chlorophenoxysobutyric acid (PCIB); and 10 mM KNO<sub>3</sub>) were incubated at room temperature (approximately 25 °C) in an agitated 96-well plate containing 10 μM DAF-2 DA. Measurements of NO production kinetics induced by hydroxylamine were obtained by adding 0.1 mM NH<sub>2</sub>OH-HCl to the cultures. Non-inoculated media were used as controls. Fluorescence intensity was measured every hour for 14 h with an HIM microplate reader (Biotek, USA) at 480 nm excitation, 525 nm emission. Similar experiments were performed in the presence of 0.5 mM cPTIO with 30 min incubation, and a decrease in NO fluorescence was observed.

2.5. IAA detection, HPLC

For HPLC analysis, all strains were first grown in SSM with 1 mM Trp and different treatments [15 mM Arg, 200 μM SNP, 1 mM PCIB, 100 μM l-NAME, 0.5 mM cPTIO, 10 μM TIBA, 1M 3-indole acetic acid(LACa)] were added after 6 h of incubation. The amount of IAA was quantified by high performance liquid chromatography (HPLC) as described [31,32]. The culture filtrate samples were extracted with equal volumes of ethyl acetate, vacuum-dried (Eyela, Tokyo, Japan) and reconstituted in 1 ml of methanol; 20 μl aliquots were analyzed with a HPLC system (Shimadzu, Kyoto, Japan).
Samples were analyzed on the basis of standards (Sigma—Aldrich), in methanol: 1% acetic acid (40:60) at a flow rate of 1 ml/min at 280 nm with a UV detector. To account for variation caused by growth, IAA values were normalized to cell density in each set as described before [32].

2.6. Seed bacterization

Sorghum (var. Sudex chari; National Seed Corporation, IARI, New Delhi) seeds were surface-sterilized and bacterized as described previously [6,32]. Thirty seeds per treatment set were incubated with 20 ml cultures (SM wild type and derived strains) and different treatments (5 mM Trp, 200 μM SNP, 100 μM L-NAME and 500 μM PCIB) for two hours and the seeds were placed onto 0.8% agar. The seeds were allowed to germinate and grow for 7 and 14 days in a growth room with a 16 h photoperiod. For 14 days, the bacterized seeds were placed on sterile sand as described [32]. Shoot length, root length and morphology were observed and measured at 14 days post-bacterization.

2.7. Scanning electron microscopy (SEM)

For SEM, root samples from 7-day-old plantlets (1–1.5 cms) were fixed in 2.5% (v/v) glutaraldehyde solution buffered with 0.1 M phosphate buffer (pH 7.4) for 3 h at room temperature. Post-fixation was done for 30 min in 1% (v/v) osmium tetraoxide in the same buffer. Specimens were washed three times in milliQ water and treated with an aqueous solution of 2% (w/v) uranyl acetate for 40 min. After fixation, specimens were dehydrated through a graded ethanol series (30–100%) followed by acetone (100%). The treated samples were mounted on aluminum stubs, coated with gold—palladium and examined under a scanning electron microscope (EVO MA10, Carl Zeiss).

2.8. Real-time PCR

2.8.1. Total RNA extraction and cDNA preparation

The wild type SM and SM-derived strains were grown under four conditions: SSM, SSM containing 1 mM Trp, 15 mM Arg and 1 mM IAA, respectively. The bacterial cultures were treated with RNA Protect bacterial reagent (Qiagen Inc., Mississauga, Ontario, Canada) and subjected to total RNA isolation, followed by qRT-PCR. Total RNA was obtained using the RNeasy Mini kit (Qiagen Inc., Mississauga, Ontario, Canada). Isolated RNA (~1.5 μg) was treated with DNase I (Sigma—Aldrich, USA) and cDNA for each sample was prepared using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc.). Incubation conditions were as follows: 5 min at 25 °C, 30 min at 42 °C and finally 5 min at 85 °C. RNA and cDNA were quantified using the TAKE 3 plate for low volume measurements using the H1M microplate reader (Biotek, USA).

2.8.2. Gene expression: relative quantification

Gene expression quantification was performed on a CFX-96 real-time PCR detection system (Bio-Rad Labs, Inc.) with 50 ng of total cDNA template, 10 μl 2x Sso Fast Eva Green Supermix Dye (Bio-Rad Labs, Inc.), 100 nM forward and reverse primer in a final volume of 20 μl. Thermal cycling conditions were as follows: 30 s at 95 °C followed by 40 repeats of 10 s at 95 °C, 30 s at 58 °C and a melt curve from 65 °C to 95 °C. Data collection was performed during each cycle. Relative gene expression by qPCR was performed using 16S rRNA as the reference gene for normalization of expression of target genes nosZ, nosR, norB and ipdC (ΔΔCq). The primers designed for real-time PCR are mentioned in Supplementary Table 1. In each analysis, a No Template Control (NTC) was included and each sample was set up in duplicate. Each plate was repeated at least thrice.

2.9. Statistical analysis

All NO end-point measurements and qPCR reactions were carried out in triplicate and data is reported with standard error from the mean. The data for root and shoot lengths and the number of lateral roots of 12 plants per treatment set were subsequently analyzed to identify whether seed bacterization under different conditions influenced the subsequent growth of plants tested. For NO and IAA measurements in bacterial cells, 3 replicates were compared for each culture condition. The data in each of the 3 cases was analyzed using the non-parametric Kruskal—Wallis test. These were then subjected to pairwise comparison and Tukey’s post hoc analysis after Kruskal—Wallis analysis to compare the effect of the different treatments with each other and determine those treatment sets that were significantly different from each other. The significance level used for analysis was P ≤ 0.05. All statistical analyses were performed using SPSS version 22.0.

3. Results

3.1. Detection of NO in wild type A. brasilense SM and its effect on plant growth promotion

Qualitative evaluation of NO production by wild type A. brasilense SM showed that strain SM produces detectable amounts of NO, as observed by microscopy (Fig. 2A). To confirm that fluorescence was due to the presence of NO, the NO scavenger cPTIO was used to counteract the NO-mediated effect. Results in Fig. 2B show that fluorescence was drastically reduced in the presence of cPTIO, confirming the presence of NO in strain SM cultures. Fluorometric analysis was performed with wild type strain SM under various treatments, including the presence of the NO-donor-SNP, Arg, KNO3, nitric oxide synthase inhibitors L-NAME/L-NIO and auxin antagonists and inhibitors (PCIBand TIBA), along with Trp, IAA and unsupplemented SSM. Statistical analysis confirmed that treatment of A. brasilense cultures with Trp, IAA, TIBA, SNP, KNO3 and L-NIO significantly influenced NO production, while L-NAME only marginally influenced NO production. Similar levels of NO production compared to unsupplemented SSM were observed in the presence of Arg and KNO3, pointing to the fact that the nos and nitrification pathway may not be functioning.
under the conditions provided in strain SM. However, this needs to be further substantiated. The data also showed that the NO signal increased in case of L-NAME and remained similar to SSM levels in L-NIO treated cultures.

Results presented in Table 2 show that addition of SNP significantly improved lateral branching of sorghum roots, while cPTIO reduced them in the case of strain SM bacterized seeds. Addition of the auxin antagonist PCIB drastically reduced the root length, shoot length and number of lateral roots observed. Addition of SNP with PCIB resulted in masking of the effects of PCIB, thereby showing improved root development in comparison to PCIB treated seeds. Since similar bacterial loads were used for bacterization (~2$\times$10$^8$ cfu/ml) and similar number of bacteria were identified from treated roots after 14 days of bacterization (Table 2), the reduced ability of A. brasilense SM to promote lateral root formation (LRF) in cPTIO and PCIB treatments was attributed to reduced NO or IAA produced by the bacteria (Supplementary Fig. 2).

3.2. NO metabolism genes: cloning, sequencing and in silico analysis

A. brasilense SM nosZ, nosR and norBC genes were amplified from genomic DNA using PCR primers mentioned in Supplementary Table 1. Megablast results of strain SM nosZ gene showed 98% and 89% homology with the A. brasilense Sp245 plasmid AZOBR_p3 nosZ and the Azospirillum lipoferum 4B plasmid AZO_p2 nosZ gene, which is a part of the nosF-nosD-nosZ-nosR cluster. SM nosR was found to be 96% and 84% homologous with A. brasilense Sp245 plasmid AZOBR_p3 nosR and the A. lipoferum 4B plasmid AZO_p2 nosR gene, which is part of the gene cluster mentioned above. norBC genes amplified from strain SM showed 99% homology with A. brasilense Sp245 plasmid AZOBR_p3 norBC. Results of the in silico conserved domain search are shown in Supplementary Figs. 3–6.

3.3. Confocal microscopy and fluorometric analysis of NO detection in strain-SM-derived strains

SMnosZ, SMnorBC and SMnosR strains were analyzed for NO production as described before for wild type strain SM. The results shown in Fig. 2 and Table 3 proved that all over-expressing strains produced NO and fluorescence was quenched in the presence of the NO scavenger cPTIO. Interestingly, in strain SMnosZ, almost negligible fluorescence was observed even in the absence of cPTIO (Fig. 2E). Statistical analysis of the different strains studied showed that there was a
Fig. 3. Scanning electron microscopy of sorghum roots bacterized with *A. brasilense* SM and its derivatives to show the effect on roots: Untreated control (A), wild type *A. brasilense* SM (B and D), strain SM grown in Trp (C), strain SM grown in L-NAME and SNP (E and F), strain SMnosR grown in Trp and SNP (G and H) and strain SMnorBC grown in cPTIO, SNP, PCIB and Trp (I, J, K, L). Abbreviations: RH: root hair; R: rod-shaped; VS: vibroid-shaped; CF: cysted form; FM: fibrillar material; MM: mucilaginous masses; BC: bacterial clumps; BF: biofilm. Scale bars vary from 1 μm to 20 μm and are shown in each image.

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significant difference in NO production between wild type SM, SMnosZ and SMnosR under all conditions other than unsupplemented SSM, Arg, PCIB or t-NASE (Table 3). Pairwise comparisons carried out after the Kruskal–Wallis test confirmed that addition of Arg influenced NO production in SMnosR and SMnorBC, while t-NASE showed a significant difference from SMnosZ only in comparison with strain SM. L-NIO significantly reduced NO production in all strains tested, even though cultures were supplemented with media only set at P ≤ 0.05, as determined by the Kruskal–Wallis non-parametric test and subsequent post-hoc analysis and pairwise comparisons between all data sets as described in Materials and methods.

Effect of seed bacterization of sorghum with A. brasilense SM at 14 days post treatment.

<table>
<thead>
<tr>
<th>Treatment set</th>
<th>Root length (cm)</th>
<th>Shoot length (cm)</th>
<th>Lateral root number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>5.07 ± 0.45a,b,d,e</td>
<td>14.28 ± 0.578a,d,e,f</td>
<td>13.58 ± 0.784a,b,d,e,f</td>
</tr>
<tr>
<td>Strain SM + Trp</td>
<td>7.87 ± 0.386a,c,e</td>
<td>16.38 ± 0.922a,d,e</td>
<td>18.87 ± 0.452a,b,d,e,f</td>
</tr>
<tr>
<td>Strain SM + Trp + SNP</td>
<td>7.88 ± 0.522a,c,e</td>
<td>16.1 ± 0.484a,b,c,d</td>
<td>23.00 ± 0.926a,b,c,d,e,f</td>
</tr>
<tr>
<td>Strain SM + Trp + cPTIO</td>
<td>6.92 ± 0.280a,b,c,d,e</td>
<td>12.48 ± 0.458a,b,c,d,e</td>
<td>17.19 ± 0.593a,b,c,d,e,f</td>
</tr>
<tr>
<td>Strain SM + Trp + PCIB</td>
<td>4.80 ± 0.328ab,c,d,e,f</td>
<td>9.34 ± 0.945ab,c,d,e,f</td>
<td>10.53 ± 0.639a,b,c,d,e,f</td>
</tr>
<tr>
<td>Strain SM + Trp + SNP + PCIB</td>
<td>8.34 ± 0.423a,b,c,d,e</td>
<td>16.02 ± 0.795a,b,c,d,e,f</td>
<td>19.74 ± 1.26a,b,c,d,e,f</td>
</tr>
</tbody>
</table>

Note: Data shows average values ± standard error from the mean. Data from the untreated set and control sets treated with media only, SNP, and cPTIO were not significantly different from each other; hence, only the untreated data set is shown here. With the PCIB set, a marginal drop in root length and lateral root number was observed in comparison to the untreated set, as would be expected with inhibition of the endogenous auxin level.

4 Values significantly different from untreated set at P ≤ 0.05, as determined by the Kruskal–Wallis non-parametric test and subsequent post-hoc analysis and pairwise comparisons between all data sets as described in Materials and methods.
5 Values significantly different from strain SM + Trp set at P ≤ 0.05.
6 Values significantly different from strain SM + Trp + SNP set at P ≤ 0.05.
7 Values significantly different from strain SM + Trp + cPTIO set at P ≤ 0.05.
8 Values significantly different from strain SM + Trp + PCIB set at P ≤ 0.05.
9 Values significantly different from strain SM + Trp + SNP + PCIB set at P ≤ 0.05.

Pairwise comparisons confirmed that PCIB significantly affected NO production in SMnosZ and SMnorBC in comparison to wild type strain SM. The increase in NO in SMnorBC was corroborated, with a 2.7-fold-increase in IAA production by the strain, further affirming a role for norBC in regulation of both IAA and NO biosynthesis by A. brasilense strain SM.

3.4. Functional analysis of NO genes in Azospirillum: effect of NO overexpression on IAA levels

Statistical analysis of data represented in Table 4 clearly suggested that IAA biosynthesis was influenced under different conditions. The effect of Trp, Arg, SNP, t-NASE and PCIB varied across the panel of strains used in this study: they either did not show significant change or showed a drastic change in IAA levels produced. Strain SMnosZ produced 0.82-fold IAA, whereas SMnosR and SMnorBC produced 2.13- and 2.73-fold IAA compared to strain SM when cultures were grown with Trp.

HPLC analysis of strains SMnosZ and SMnosR also showed a similar reducing trend in IAA production, albeit to different levels: 0.32—0.51-fold in the presence of PCIB (Table 4). Interestingly, strain SMnorBC showed a 2.28-fold increase in its IAA level in the presence of PCIB in comparison to Trp supplemented cultures, suggesting that norB overexpression protects SMnorBC against PCIB action and is somehow able to overproduce IAA. This can be explained by the fact that, in SMnorBC, expression of ipDC was at least 5.08-fold higher than in strain SM in the presence of the IAA substrate Trp (see Fig. 4B).

The presence of the Nos substrate (Arg), the NO donor (SNP), the Nos inhibitor (t-NASE) and the NO quencher (cPTIO) resulted in a significant to drastic decrease in IAA for all strains tested, even though cultures were supplemented with reduced NO production in SMnosZ and SMnosR by 0.2—0.4-fold, while it did not affect SMnorBC (data not shown).

Table 3

Quantitative nitric oxide fluorescence observed under different conditions through fluorometric analysis for A. brasilense SM derivatives.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>SM #</th>
<th>SMnosZ #</th>
<th>SMnosR #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media only</td>
<td>6055 ± 31</td>
<td>5721 ± 98</td>
<td>6145 ± 130</td>
</tr>
<tr>
<td>Trp</td>
<td>7824 ± 34</td>
<td>6504 ± 118a</td>
<td>9264 ± 38b</td>
</tr>
<tr>
<td>IAA</td>
<td>7663 ± 23</td>
<td>6369 ± 25a</td>
<td>8109 ± 48b</td>
</tr>
<tr>
<td>Arg</td>
<td>6143 ± 29</td>
<td>6117 ± 127</td>
<td>4341 ± 119b</td>
</tr>
<tr>
<td>SNP</td>
<td>9118 ± 20</td>
<td>7385 ± 337a</td>
<td>5780 ± 290b</td>
</tr>
<tr>
<td>NAME</td>
<td>8208 ± 22</td>
<td>5501 ± 15a</td>
<td>5558 ± 40b</td>
</tr>
<tr>
<td>PCIB</td>
<td>8438 ± 30</td>
<td>6839 ± 148a</td>
<td>8431 ± 206a</td>
</tr>
<tr>
<td>TIBA</td>
<td>6382 ± 28a</td>
<td>3287 ± 59a</td>
<td>4871 ± 41b</td>
</tr>
<tr>
<td>KNO3</td>
<td>5755 ± 33</td>
<td>2949 ± 36a</td>
<td>3355 ± 91b</td>
</tr>
<tr>
<td>NIO</td>
<td>6127 ± 34</td>
<td>4643 ± 34a</td>
<td>4366 ± 138a</td>
</tr>
</tbody>
</table>

# NO was quantified as mentioned previously in Materials and methods. Values represent the average of net fluorescence units of three independent sets ± standard error from the mean. Statistical analysis was performed by non-parametric analysis and subsequent pairwise comparisons as described in Materials and methods.

* Growth (SSM) was supplemented with sodium nitroprusside (SNP), l-arginine (Arg), KNO3, L-NH2nitroarginine methyl ester (t-NASE), N2-(l- iminoethyl)-l-ornithine, dihydrochloride (L-NIO), p-chlorophenoxyl isobutyric acid (PCIB), 2,3,5-triiodo benzoic acid (TIBA), tryptophan (Trp) and indole acetic acid (IAA).

a Values significantly different from strain SM at P ≤ 0.05.

b Values significantly different between SMnosZ and SMnosR at P ≤ 0.05.

...
with Trp (Table 4). Interestingly, SMnosZ showed a 1.22-fold increase in IAA levels in the presence of L-NAME. Also, the levels of IAA produced in the NO overexpressing strains under NO-inducing conditions (Arg or SNP) were higher than for strain SM in all cases (varying between 2.84-fold and 20.2-fold). Since TIBA was confirmed to be involved in IAA biosynthesis in strain SM, and indole-3-acrylate (IAcA) is a known Trp competitor (by binding endogenous auxin), their effects on the IAA levels were studied. Within the strains (including wild type strain SM and the NO overexpressing strains) a drastic decrease in IAA or no IAA was detected. SMnorBC showed deviation from other strains as no significant change in IAA levels was noted in TIBA and IAcA.

It was observed that strain SM formed a biofilm in the presence of L-NAME (Fig. 3E). L-NAME was shown to increase NO (by 1.35-fold), but to drastically reduce IAA produced by strain SM. For firm anchorage of bacterial cells onto the root surface, production of fibrillar and exopolysaccharide material is essential. The A. brasilense SM-derived strains constructed in this study showed better root hair development than wild type SM (Fig. 3). The root hair zones were devoid of any colonization. The roots were heavily covered with mucilaginous masses (MM in Fig. 3D and H). Bacterial cells were found to be randomly dispersed in all cases, and rod-shaped, vibroid and cysted forms were observed along with clumps (Fig. 3C, D, G, H, I and L). When wild type strains SM and SMnosZ grown in Trp substituted media were used for bacterization, the entire root epidermal surface and lateral roots were heavily covered with mucilaginous material. Similar results were observed where thick fibrillar and mucilaginous material was found on the root surface treated with SM and SMnosR, both grown in the presence of SNP (Fig. 3F and H, respectively).

### 3.5. Effect of NO overexpression on plant growth

Most of the strains generated and used in this study showed improved root morphology in terms of root length and lateral branching of roots (Supplementary Fig. 2). In the absence of bacteria, when the seeds were treated with chemicals (Trp, SNP, NAME, cPTIO), the developed roots were observed to be similar to untreated seeds by electron microscopy. With PCIB, however, a marginal drop in root length and lateral root number was observed (data not shown). In most of the bacterial treatment sets, the Azospirillum cells were distributed randomly over the surface of the roots (Fig. 3). The cyst form was observed in treatment with wild type strain SM grown in the presence of SNP (Fig. 3F) and in SMnosZ grown in the presence of PCIB and L-NAME (data not shown). Also, dense bacterial aggregates or clumps (BCs) were formed by wild type strains SM, SMnosR and SMnorBC, all grown in the presence of Trp (Fig. 3D, G, 3L). In the case of SMnorBC, higher numbers of root hairs were observed in PCIB (Fig. 3K) than in the case of SNP treatment (Fig. 3J), confirming that higher IAA was produced in the presence of the former treatment than the latter (Table 4).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Action</th>
<th>SM</th>
<th>SMnosZ</th>
<th>SMnorBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRP (1 mM)</td>
<td>IAA substrate</td>
<td>15.68 ± 0.32</td>
<td>12.88 ± 0.28</td>
<td>33.46 ± 0.12&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>ARG (15 mM)</td>
<td>NOS substrate</td>
<td>0.97 ± 0.14</td>
<td>1.33 ± 0.12</td>
<td>3.11 ± 0.14&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>SNP (200 μM)</td>
<td>NO donor</td>
<td>0.64 ± 0.10</td>
<td>2.25 ± 0.16&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.82 ± 0.16</td>
</tr>
<tr>
<td>NAME (100 μM)</td>
<td>NOS inhibitor</td>
<td>2.49 ± 0.12</td>
<td>15.68 ± 0.22&lt;sup&gt;c&lt;/sup&gt;</td>
<td>11.24 ± 0.44&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>cPTIO (0.5 mM)</td>
<td>NO quencher</td>
<td>2.95 ± 0.10</td>
<td>Nd</td>
<td>Nd</td>
</tr>
<tr>
<td>PCIB (1 mM)</td>
<td>IAA antagonist</td>
<td>3.18 ± 0.22</td>
<td>6.55 ± 0.18&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10.68 ± 0.42&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>TIBA (10 μM)</td>
<td>IAA transport inhibitor</td>
<td>4.73 ± 0.32</td>
<td>Nd</td>
<td>Nd</td>
</tr>
<tr>
<td>IAcA (1M)</td>
<td>Binds to endogenous Trp</td>
<td>1.63 ± 0.22</td>
<td>Nd</td>
<td>Nd</td>
</tr>
</tbody>
</table>

Nd: not detected.

IAA was quantified as mentioned previously [32]. The values represent the average of three independent sets ± standard error from the mean.

Statistical analysis was performed as described in Materials and methods.

<sup>a</sup>, <sup>b</sup>, <sup>c</sup> Values significantly different from strain SM at P < 0.05.

<sup>d</sup> Values significantly different between SMnosZ and SMnorBC at P < 0.05.

<sup>e</sup>, <sup>f</sup> Values significantly different between SMnosR and SMnorBC, respectively, at P < 0.05.

<sup>g</sup> 1 mM Trp was added to each culture at the time of subculture and the respective substrates were added after 6 h of growth.

<sup>h</sup> IAA is represented as μg/OD560.

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Trp, thus indicating that Trp enhanced the expression of NO metabolism genes. On the other hand, in SMnorBC, Arg exposure enhanced norB expression 1414-fold in comparison with strain SM (Fig. 4A) and this was expected with the norB overexpressing strain. However, this did not seem to be an inducible property of SMnorBC, as expression of norB remained more or less unchanged.

In the case of nosZ, relative expression was higher in most strains and treatments studied, and varied between 0.26- and 80,628-fold (Fig. 4C). The only exceptions found were SMnosR grown in SSM and strain SM grown in the presence of IAA. nosR is known to be a regulator of nosZ, and the higher expression of nosR is involved in negatively regulating nosZ expression in the case of SMnosR. As for nosZ, the relative expression of nosR was higher in most of the cases tracked in the study, varying from 0.70 to 209,081-fold (Fig. 4D). In SMnorBC grown in Trp, the expression of norB and nosZ was high in comparison with strain SM (394.8- and 19.2-fold respectively), driving NO catabolism to N2; hence, expression of regulator nosR was low, as expected (0.7-fold, Fig. 4D). Since the highest nosR expression was observed in the presence of IAA and Trp, and the IAA produced was also 2-fold higher in SMnosR, this confirms the role of Trp in increasing IAA in the nosR overexpressing strain.

norB, nosZ and nosR were found to be regulated to varying extents by Trp supplementation of bacterial cultures. Expression of ipdC was studied in all NO overexpressing strains and compared with strain SM grown in SSM. All NO gene overexpressing strains showed reduced ipdC expression, just like the SSM sets. Trp increased ipdC expression in most cases, while Arg did not greatly impact ipdC expression in SMnosR, SMnosZ or SMnorBC (Fig. 4B). IAA addition increased ipdC expression in all strains, and varied from 1.8-fold in SMnosZ to 9.6-fold in SMnorBC (Fig. 4B).

4. Discussion

IAA and NO modulate expression of genes and accumulation of certain signaling molecules that are a part of essential transduction cascades. In plants, there appears to be a correlation between NO and IAA levels, as observed in the case of control explants, control explants containing the NO scavenger-cPTIO, auxin-depleted explants, explants with exogenous auxin addition and those with additional auxin and cPTIO [25,26]. A similar correlation between NO and IAA levels has not been reported from bacteria up until now. Hence, study of the bacterial genes influenced by these phytohormones and gasotransmitters will yield important information that may subsequently be applied to a variety of aspects of microbial biotechnology and agriculture, including biofertilizer sand biopesticides.

In this context, the interplay between IAA and NO production by A. brasilense SM was explored. The strain was found to produce NO, and Trp, IAA, TIBA, SNP, KNO3 and L-NIO supplementation were found to significantly influence NO production, while l-NAME only marginally influenced NO production. l-NAME is known to inhibit constitutive

![Figure 4](https://example.com/fig4.png)
NOS, thereby leading to inducible NOS expression; as is the case in mammalian systems [40]. This may account for increased NO release on l-NAME treatment [41]. l-NAME and L-NIO are weak and non-selective competitive inhibitors of NOS, respectively, and have shown conflicting results in the past [13,42]. This inconsistent inhibition was observed in our study with the wild type strain SM and other strains used in this study.

Following overexpression, only a marginally significant difference in NO production between wild type SM and its overexpressing derivatives was observed in conditions other than those of unsupplemented SSM, Arg and l-NAME. Negligible fluorescence was observed due to overexpression of nosZ, the nitrous oxide reductase responsible for conversion of N₂O to N₂ during catabolism of NO. Higher NosZ activity leads to breakdown of excess NO to N₂ via N₂O, and thus, negligible fluorescence is seen in the nosZ overexpressing strain. This study confirms the role of nosZ in NO catabolism in A. brasilense SM.

HPLC results point to the fact that nosR and norBC likely play an important role in regulating IAA biosynthesis in strain SM. The effect of supplementing Trp, Arg, SNP, cPTIO, l-NAME and PCIB varied among the SM-derived strains (SMnosZ, SMnosR and SMnorBC; carrying higher copy numbers of nosZ, nosR and norB); they either did not show significant change or showed a drastic change in IAA levels produced by them, strengthening our hypothesis that nosZ, nosR and norBC play an important role in regulation of IAA levels in A. brasilense SM.

PCIB inhibits the action of auxins. As IAA is self-inducible in strain SM [30], PCIB likely inhibits subsequent induction of IAA biosynthesis in strain SM. HPLC results obtained in this study confirmed that the varying responses in wild type strains SM, SMnosZ, SMnosR and SMnorBC; carrying higher copy numbers of nosZ, nosR and norB) likely inhibit subsequent induction of IAA in SMnosZ point to the involvement of l-NAME in regulation of IAA biosynthesis genes, directly or by allowing increased utilization of exogenously added Trp. Also, additional evidence is provided by the fact that NO production is negatively influenced by TIBA, thereby identifying nosZ and nosR as potential target genes interacting with IAA biosynthesis machinery in A. brasilense SM. To our knowledge, this is the first report of its kind that identifies any correlation between IAA and NO production by plant-associated beneficial bacteria.

The role of TIBA in relation to bacterial IAA biosynthesis or other plant growth regulators had not been determined up to now. Our study shows that TIBA not only reduces NO production, but also drastically decreases bacterial IAA biosynthesis. TIBA has been reported to induce formation of nodules-like structures resembling Rhizobium-induced nodules in alfalfa containing transcripts for early nodulin genes [43]. In a preliminary study, it was shown to inhibit transport of fungal IAA into the root and consequent LRF in a Spruce—Laccaria bicolor association [44].

LRF induced by Azospirillum inoculation is associated with NO production [11,13,15]. Keeping in mind that strain SM could produce NO, its contribution to the improvement of lateral root branching in sorghum was studied following bacterization of seeds. Results presented in this study confirm the role of IAA and NO in improving sorghum root development. They also highlight the fact that the contribution of NO to root development may be additive to IAA. The role of NO and IAA in LRF has also been reported from A. brasilense Sp245 and SM, respectively [13,15,30,31]. Zimmer et al. [45] reported that nitrite produced by Azospirillum could have hormonal effects in plants. However, a high nitrite concentration was required to obtain an optimum hormonal effect, ascribing this to a possible formation of NO, N₂O or N₂O₃ from nitrite by disproportionation [46]. PCIB showed a reduced stimulatory effect produced by Azospirillum on LRF, confirming that auxins are also involved in Azospirillum-induced effects, probably by triggering an increase in the NO concentration [13,25].

Azospirillum is a well-documented pleomorphic bacterium and changes its shape in response to various environmental conditions [4,47]. The colonization pattern on the root surface of the wild type as well as NO overexpressing strains varied from being randomly dispersed single cells to bacterial clumps and biofilms, in the elongation zone of the roots. The half-curved or vibroid and rod-shaped forms are highly motile, whereas, under stress conditions, the vibroid form transforms into a non-motile, round, cyst-like structure [47]. Biofilm formation by plant-associated bacteria has been linked to improved plant growth, as the dense population of bacteria can efficiently produce various phytohormones, antibiotics, beneficial secondary metabolites and exoenzymes [48–50].

Treatment of l-NAME does not allow wild type cells to produce sufficient amounts of IAA, but they overproduce NO and may associate closely in a biofilm so as to maintain successful surface colonization on sorghum roots and improve root development. Di Palma et al. [51] carried out mutational studies to establish that NO is the signaling molecule influencing biofilm formation in A. brasilense Sp245 in a dose-dependent manner.

Mucilaginous masses and mucigel layers are known to be excreted by plant roots to provide better anchorage to bacteria and a protected environment for their growth and nutrient uptake [3,6]. Plant mucigel is rich in pectic acid, glucose, galactose, arabinose, glucuronic acid and galacturonic acid [52]. Bashan et al. [47] studied colonization of A. brasilense Cd on non-cereal crops and found both vibroid and cysted forms attached to the root epidermal surface of cotton, tomato and pepper with thick fibrillar material. In the case of soya bean root colonization, cysted forms were predominant in the wilting soya bean roots. We had earlier seen similar patterns with A. brasilense SM and its over-expressing derivatives [6]. The A. brasilense SM strain has been established as a non-endophytic surface colonizer [31,53], just like A. brasilense Cd and Sp7, but unlike endophytic strain Sp245, which has been detected in the root xylem [3].

A recent study showed that NO may be required for auxin-dependent gene expression in Arabidopsis [53]. Seedlings treated with 10 nM of IAA showed poor GUS expression in
transgenic _Arabidopsis_ carrying GUS fused with auxin response elements. While treatment with SNP alone did not induce significant GUS activity, simultaneous application of IAA and SNP caused a substantial increase in GUS expression. When the NO scavenger cPTIO was used with 50 nM IAA, the seedlings showed GUS activity, but the application of cPTIO prior to IAA treatment prevented such induction. cPTIO inhibited auxin-dependent gene induction in a dose-dependent manner. Further studies with SNP showed that the expression of auxin-responsive genes _IAA1_ and _IAA5_ was increased in IAA-treated wild-type seedlings, highlighting the fact that NO plays a role in auxin-dependent gene expression in _Arabidopsis_ [54].

All NO metabolism target genes studied for expression, including _norB_, _nosZ_ and _nosR_, were found to be regulated to varying extents by the IAA substrate Trp. The interplay between IAA and NO metabolism genes was further confirmed by studying the expression of _ipdC_ in all NO overexpressing strains, and compared with strain SM. Trp and IAA increased _ipdC_ expression in most strains studied, while no significant impact of Arg was seen on _ipdC_ expression. The expression of _ipdC_ is Trp-dependent in strain SM, as determined earlier [30]. _ipdC_ expression has also been shown to be improved on exposure to nutrient limitations, temperature fluctuations and auxins like IAA, NAA, IBA and related compounds like ILA and Trp, as was shown by using an _ipdC-lacZ_ fusion in the same strain [30].

Growing evidence of the interaction of NO with other messengers like Ca^{2+} to mediate plant responses to biotic stress, and the fact that NO may be required for auxin-dependent gene expression in plants, coupled with the findings reported in this study, clearly indicate a strong correlation between IAA and NO in _A. brasilense_ SM and point to the existence of cross-talk and/or common signaling mechanisms which need to be elucidated in future studies using a variety of mutant strains.

Conflict of interest

Authors have no conflict of interest to disclose.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.resmic.2015.02.003.

References


