Symbiosis
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The benefits of foliar inoculation with *Azospirillum brasilense* in soybean are explained by an auxin signaling model

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Abstract

Azospirillum sp. is one of the most studied genera of plant growth-promoting rhizobacteria (PGPR). The ability of *Azospirillum* sp. to promote plant growth has been associated with its ability to produce several phytohormones, such as auxins, gibberellins and cytokinins, but mainly indole-3-acetic acid (IAA). It has been propoosed that the production of IAA explains the positive effects of co-inoculation with *Azospirillum* sp. on the rhizobia-legume symbiosis. In this study, we constructed an IAA-deficient mutant of *A. brasilense* Az39 (*ipdC*$^-$) by using a restriction-free cloning method. We inoculated soybean seeds with $1{\cdot}10^6$ cfu·seed^{-1} of *Bradyrhizobium japonicum* E109 and co-inoculating leaves at the V3 stage with $1{\cdot}10^8$ cfu.plant^{-1} of *A. brasilense* Az39 wt or *ipdC*$^-$ or inoculated leaves with 20 μg.plant^{-1} synthetic IAA. The results confirmed soybean growth promotion as there was increased total plant and root length, aerial and root dry weight, number of nodules on the primary root, and an increase in the symbiosis established with *B. japonicum* E109. Nodule weight also increased after foliar co-inoculation with the IAA- producer *A. brasilense* Az39. The exogenous application of IAA decreased aerial and root length, as well as the number of nodules on primary roots in comparison with the Az39 wt strain. These results allow us to propose a biological model of response to foliar co-inoculation of soybean with IAA-producing rhizobacteria. This model clearly shows that both the presence of microorganism as part of the colonization process and the production of IAA in situ are co-responsible, via plant signaling molecules, for the positive effects on plant growth and symbiosis establishment.

Keywords *Azospirillum* · Indole-3-acetic acid · Soybean · Foliar inoculation · *Bradyrhizobium*

1 Introduction

Soybean [*Glycine max* (L). Merr.] plants obtain nitrogen by absorbing it from the soil solution as nitrate or ammonium ions or via biological nitrogen fixation through the establishment of a mutualistic symbiosis with beneficial rhizobacteria belonging to the genus *Bradyrhizobium* (López-García et al. 2009). These soil bacteria induce nodule formation and nitrogen fixation in soybean roots. Formation of a nitrogen-fixing root nodule is a complex developmental event that depends on a chemical "dialog" between the microsymbiont and the plant. This "dialog", as well as the nitrogen fixation process and the soybean crop productivity under field conditions, can be modified by the presence of exogenous phytohormones (auxins, cytokinins, gibberellins) or the inoculation with beneficial rhizobacteria (Vicario et al. 2015; Hungria et al. 2015). These beneficial bacteria is known as plant growth-promoting rhizobacteria (PGPR) (Kloepper and Schroth 1978). The production of phytohormones by PGPR has been proposed as the most important mechanism in the rhizobia-legume symbiosis (Srinivasan et al. 1996; Frugier et al. 2008). The auxins produced by PGPR are thought to increase the number of root hairs and thus increase the interaction sites between the rhizobia and the root hairs, whereas cytokinins are considered as key differentiation signals for nodule organogenesis. The early

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work of Thimann (1936) suggested that auxins played an important role in the course development (ontogeny) and now we know that changes in the concentration of this phytohormone or in its balance with cytokinins are a pre-requisite for nodule organogenesis (Mathesius et al. 1998).

Azospirillum is one of the most studied PGPR over the last four decades because it improves the growth, development and yield of several crops, including some legumes (Bashan and de Bashan 2010). The positive effects of inoculation with these bacteria are related to their ability to produce phytohormones such as auxins (Crozier et al. 1988; Zimmer and Bothe 1988), cytokinins (Horemans et al. 1986; Cacciari et al. 1989), gibberellins (Bottini et al. 1989; Janzen et al. 1992; Piccoli and Bottini 1996), abscisic acid and ethylene (Perrig et al. 2007), as well as certain polyamines such as cadaverine (Cassán et al. 2009). The production of auxins, mainly indole-3-acetic acid (IAA), is considered the most important and significant mechanism that promotes plant growth (Cassán et al. 2014).

At least four metabolic pathways have been proposed for IAA biosynthesis in rhizobacteria: three using the amino acid L-tryptophan (Trp) as a precursor and one Trp-independent pathway (Prinsen et al. 1993; Carreño-Lopez et al. 2000). *Azospirillum* synthesizes IAA almost exclusively through the indole-3-pyruvic acid (IPyA) pathway (Patten and Glick 1996). The IPyA pathway was initially demonstrated in *A. brasilense* Sp245 by Costacurta et al. (1994) by cloning the *ipdC* gene, which encodes an indole-3-pyruvate decarboxylase (IPDC), a key enzyme in the pathway because it is the rate-limiting step. Prinsen et al. (1993) also showed that *ipdC*$^{-}$ knockout mutants are strongly reduced in IAA biosynthesis. The co-inoculation with an IAA-deficient strain of *Ensifer meliloti* (ex-*Sinorhizobium meliloti*) with an IAA-producing strain of *Azospirillum brasilense* on alfalfa seeds significantly increased the number of nodules on the primary root. By using an *ipdC* knockout mutant of *A. brasilense*, Remans et al. (2008) also found an increase in the nodule number by the co-inoculation with IAA-producing *A. brasilense* and *Rhizobium etli* in common bean. Other researchers, such as Yahalom et al. (1990), showed a beneficial response in biological nitrogen fixation after co-inoculation with *Rhizobium* and *Azospirillum* not only with regard to root morphology and nodule number, but also in relation to the functionality of the enzymatic machinery in nodules. Burdmann et al. (1997) also observed that common bean seedlings inoculated with IAA-producing *A. brasilense* showed increased production of flavonoids in the roots and a greater ability to induce the expression of *nod* genes in *Rhizobium*, compared with non-inoculated seedlings.

Despite the above findings, the value of employing a combination of *A. brasilense* and *B. japonicum* with soybean seeds is limited under agronomic conditions. This is because the use of a wide range of chemical pesticides on the seeds drastically reduces bacterial survival and plant growth promotion (Campo et al. 2009; Zilli et al. 2009). The aim of the present study was to evaluate the benefits of foliar inoculation with *A. brasilense* Az39 on soybean plants and its symbiosis with *B. japonicum* E109. Additionally, we aimed investigate whether any positive responses were related to the ability of *A. brasilense* Az39 to produce IAA.

2 Materials and methods

2.1 Biological materials

The bacteria used in this study were: (1) *B. japonicum* E109 (WDCM31) and (2) *A. brasilense* Az39 (strains deposited at the BPCV-IMYZA-INTA Culture Collection, Castelar, Buenos Aires, Argentina). Additionally, for the construction of the IAA-deficient mutant (4) *A. brasilense* Az39 *ipdC*$^{-}$, we used (3) *Escherichia coli* S17–1 (ATCC 47055). *Azospirillum brasilense* Az39 and *B. japonicum* E109 are two of the strains most currently used for inoculant formulations in Argentina and Brazil (Lodeiro 2015; Cassán and Díaz-Zorita 2016). Their complete genome sequences have been recently published by Rivera et al. (2014) and Torres et al. (2015). Seeds of soybean [*Glycine max* (L). Merr.] Nidera A 5009 RR were used.

2.2 Culture conditions

B. japonicum E109 was grown in Yeast Extract Mannitol (YEM) medium, as described by Vincent (1970), while *A. brasilense* was grown in liquid medium of Sadasivan and Neyra (1985). The liquid medium was complemented with 10 mg·mL^{-1} of L-tryptophan for IAA quantification and 50 μg·mL^{-1} of gentamicin in the case of the *ipdC*- strain (with exception of the cultures used for plant treatment). All rhizobacteria were incubated under stirring at 30 °C for 48–72 h (Az39) or 72–96 h (E109). To determine the typical phases of the growth curve of each microorganism in the used culture media, the number of viable cells (cfu·mL^{-1}) was quantified using the microdrop quantification method (Miles and Misra 1938). Plates containing: YEM medium (Vincent 1970) were used for *B. japonicum*, and Congo Red medium (Rodríguez Cáceres 1982) for Az39 wt, and Congo Red medium modified by the addition of 50 μg·mL^{-1} gentamicin for *ipdC*$^{-}$ Az39. The plates were analyzed after incubation at 30 °C for 7 days in the case of *B. japonicum* and for 6 days for *A. brasilense*, as previously described in Cassán et al. (2013), with modifications.

2.3 Construction of the *A. brasilense* Az39 *ipdC*$^{-}$ mutant

A restriction-free cloning method was used to obtain the *ipdC* mutant of *A. brasilense* Az39 deficient in IAA production, as described by Unger et al. (2010). This method uses a pair of

primers for each fragment cloned, corresponding to the 5′ and 3′ fragments of the target gene and the gentamicin-resistance cassette (GmR). The upstream and downstream fragments of the *ipdC* gene (target region) were amplified by PCR from genomic DNA using the primer pairs Az39–5′IPDC_FW/Az39–5′IPDC_RV and Az39–3′IPDC_FW/Az39–3′IPDC_RV respectively. A total of six primers were required to mutate a single gene (Table S1, supplementary material). The methodology consisted in amplifying each fragment with specific primers by using the Q5® HF DNA Polymerase (New England Biolabs, Ipswich, MA, USA), purifying each fragment, and combining them with the plasmid pK18mobsacB (ATCC® 87,097™, Manassas, USA) in an extension reaction. Then, the suicide plasmid carrying the mutagenesis cassette was treated with DpnI enzyme (Promega, Madison, WI, USA) to digest only methylated or hemimethylated DNA and subsequently transformed into *Escherichia coli* S17–1 *λpir* strain, a *λ-pir* lysogen of S17–1 [*thi pro hsd* R^-*hsd*M^+*recA* RP4 2-Tc::Mu-Km::Tn7 (Tp^R Sm^R)]. Finally, the construct was mobilized into *A. brasilense* Az39 by biparental mating, according to Simon et al. (1983). Double recombinant clones, resistant to gentamicin and sensitive to kanamycin, were recovered without the need of *sacB* counter selection. From the colonies recovered, those possessing the desired construction were selected by "colony PCR" and the sequences obtained were confirmed by sequencing and used in further experiments.

2.4 IAA quantification

The IAA from the wt and *ipdC*⁻ strains of *A. brasilense* Az39 and *B. japonicum* E109 was identified and quantified at the lag (2 h), exponential (4 h) and stationary (24 h for *Azospirillum* sp. and 30 h for *B. japonicum*) growth phases. The samples were analyzed by using High Performance Liquid Chromatography, according to Spaepen et al. (2008). Treatments from three independent experiments were performed in triplicate ($n = 9$). Values represent mean ± standard error. Data were analyzed for variance by ANOVA followed by Tukey's Test a posteriori analysis at $p \leq 0.05$. Analysis was performed using INFOSTAT version 2014 (Di Renzo et al. 2014) (Universidad Nacional de Córdoba, Argentina).

2.5 Biological assays

A set of biological assays were performed to analyze the soybean growth and *B. japonicum* E109 symbiosis, according with the following treatments: (1) soybean seeds inoculated with *B. japonicum* E109 (control); (2) soybean seeds inoculated with *B. japonicum* E109 and leaves co-inoculated with Az39 wt (IAA-producing strain); (3) soybean seeds inoculated with *B. japonicum* E109 and leaves co-inoculated with Az39 *ipdC*⁻ (IAA deficient); (4) soybean seeds inoculated with *B. japonicum* E109 and leaves exogenously treated with a pure solution of IAA (Note: we used a pure IAA solution with a concentration similar to that produced by Az39 at the stationary growth phase, as shown in Table 1). The seeds were inoculated with $1{\cdot}10^6$ cfu·seed^{-1} of *B. japonicum* and then sown in 330-cm^3 plastic pots containing sterile vermiculite as substrate. The foliar co-inoculation with *A. brasilense* and the exogenous application of IAA were performed when the plants reached the V3 stage (Fehr and Caviness 1977) of development or third-node stage. For the foliar application of *A. brasilense* Az39 and the IAA solution, independent manual atomizers were used. In both cases, the *A. brasilense* Az39 (wt and *ipdC*⁻ mutant) inoculum had a titer of $1{\cdot}10^9$ cfu·mL^{-1} at the moment of inoculation. The inoculation dose was established in a final volume of 2 mL.plant^{-1} containing 5.0% (*v*/v) of *A. brasilense* Az39 (wt or *ipdC*⁻) cultures ($1{\cdot}10^8$ cfu.plant^{-1}), centrifuged and resuspended in sterile physiological solution to prevent the possible introduction of exogenous IAA. In the case of the pure IAA solution, a dose of 2 mL.plant^{-1} containing 10 μg.mL^{-1} IAA was used. All doses and application times were standardized in previous experiments (data not shown). In the case of the foliar co-inoculation with *A. brasilense*, the application time at V3 showed the best results under environmentally controlled conditions. Additionally, the V3 stage is coincident with the time for application of chemical products in soybean crops under agronomic field conditions in South America. This coincidence was deliberately considered to extend this technology to field conditions in the future.

Plants were cultured in a plant growth chamber with a photoperiod of 16 h light (30 °C) and 8 h of dark (20 °C), 40–60% of relative humidity and irrigated with a nitrogen-deficient Hoagland's nutrient solution (Hoagland and Arnon 1950). After 28 days, the plants were uprooted and their nodules on primary and secondary roots counted. The root and shoot dry weight, as well as the root and shoot length, were determined. Additionally, the number of bacterial cells (wt or *ipdC*⁻) recovered from leaves of soybean seedlings was determined by the "most probable number" (Döbereiner et al. 1995) and "direct sowing" (Rodríguez Cáceres 1982) methods. Bacterial colonization was confirmed by the use of a FEI Quanta 250 Electron Microscope (Thermo Fischer, USA). A fully randomized block design with seven replicates per treatment was used for this experiment ($n = 42$). Data were analyzed using ANOVA and means were compared by Duncan's multiple range test ($p \leq 0.05$) using the software INFOSTAT version 2014 (Di Renzo et al. 2014) (Universidad Nacional de Córdoba, Argentina).

3 Results

An isogenic mutant of Az39 deficient in the expression of an indole pyruvate decarboxylase was successfully constructed in which a genomic fragment having the gene *ipdC* gene was

Table 1 Evaluation of bacterial growth (cfu·mL^{-1}) and indole-3-acetic acid production (μg·mL^{-1}) at different phases of the growth curve obtained from pure cultures of the wild type and *ipdC*⁻ mutant of *A. brasilense* Az39 and the wild type strain *B. japonicum* E109. *A. brasilense* was grown in a defined medium complemented with 10 mg·mL^{-1} of L-tryptophan and 50 μg·mL^{-1} of gentamicin (in the case of *ipdC*-). E109 was grown in YEM medium

Strain	Growth curve phase	Bacterial growth (log_{10} cfu·mL^{-1})	IAA concentration (μg·mL^{-1})
Az39 wt	lag	5.00 ± 0.08	3.34 ± 0.124[a]
	exponential	8.15 ± 0.03	5.26 ± 0.201[b]
	stationary	9.30 ± 0.05	10.84 ± 0.098[c]
Az39 *ipdC*⁻	lag	4.70 ± 0.02	*
	exponential	7.96 ± 0.04	*
	stationary	9.00 ± 0.06	*
E109	lag	5.48 ± 0.04	*
	exponential	8.43 ± 0.09	*
	stationary	9.72 ± 0.10	*

*: not identified

replaced with a gentamicin-resistant cassette by homologous recombination (Fig. 1). The genomic structures of the recovered gentamicin-resistant and kanamycin-sensitive clones were analyzed by PCR, using several combinations of primers. The analysis unambiguously confirmed the expected genomic array of the *ipdC*⁻ mutants, which differed from the Az39 wild type strain. Once the mutants were constructed, confirmatory experiments were carried out to analyze the bacterial growth and IAA production ability of the *ipdC*⁻ mutants in comparison with the Az39 wild type strain. The three clones were evaluated for IAA production on chemically defined medium (data not shown), but only *ipdC*⁻ 2 was used for inoculation assays.

Only the Az39 wt strain was able to produce and release IAA into the culture medium in the presence of L-Trp **(**Table 1**).** The highest IAA production (determined as IAA accumulation) was observed at the stationary growth phase (10.84 μg·mL^{-1}), coincident with the kinetics of a typical bacterial secondary metabolite. Neither the Az39 *ipdC*- mutant strain nor *B. japonicum* E109 were able to produce or

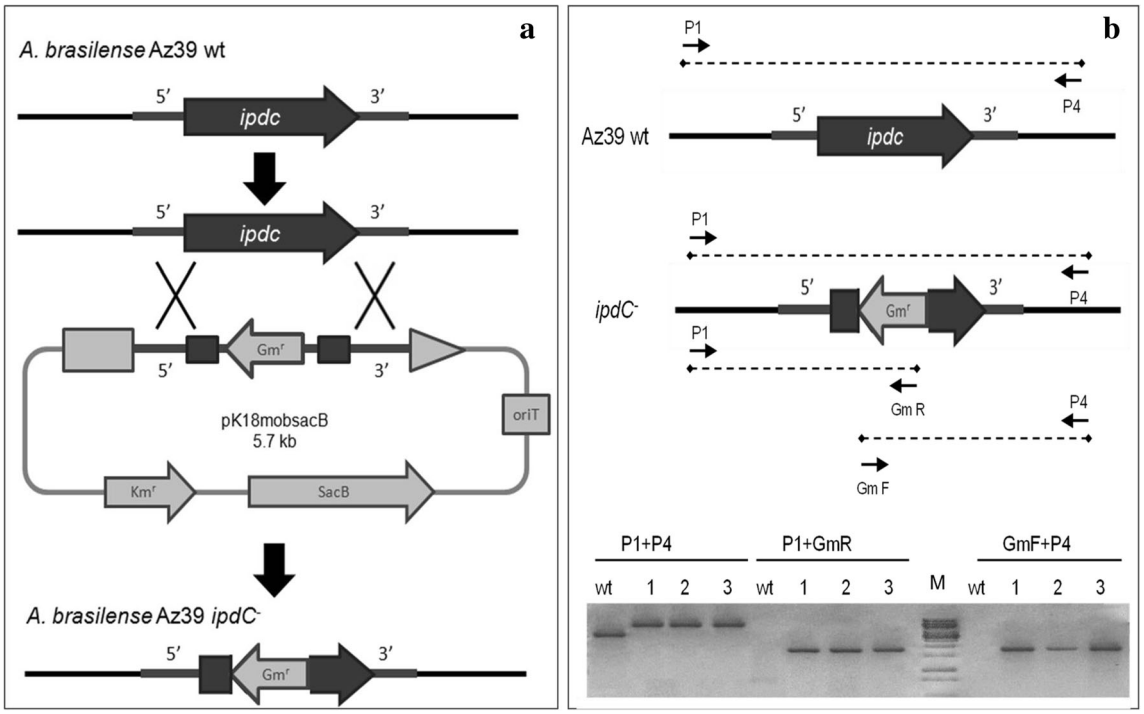

Fig. 1 Construction of the *A. brasilense* Az39 *ipdC*⁻ mutant. **a** The *A. brasilense* Az39 genomic region comprising the *ipdC* gene (in red) was replaced with a gentamicin resistance cassette (GmR, in black) by homologous recombination. The mutant allele in a suicide plasmid was introduced into Az39 by bi-parental mating, and double crossover mutants were recovered in selective medium. **b** The correct integration of the cassette into the genome was verified in three independent clones (*ipdC*⁻ 1, *ipdC*⁻ 2, and *ipdC*⁻ 3) by PCR, using different primer combinations (P1 + P4, P1 + GmR, and GmF+ P4; indicated in dotted lines). The reaction products were run on 1% agarose gel stained with ethidium bromide under standard conditions. The Az39 wild type (wt) strain was included in the analysis, as a control and GeneRuler™ (Thermo Scientific™, USA) 1 kb DNA Ladder used as molecular marker**.** The three clones were evaluated for IAA production on chemically defined medium (data not shown), but only *ipdC*⁻ 2 was used for inoculation assays

accumulate detectable amounts of this molecule throughout the growth curve, confirming the inability of the mutant and E109 to produce or accumulate IAA in the culture medium.

Azospirillum brasilense compatible colonies and cells were recovered from the inoculated plants (≈1E + 2 cfu.g^{-1}), 24 h after innoculation and this number increased at least three fold (≈3E + 2 cfu.g^{-1}) at 48 h post-inoculation and remained stable for another 96 h. There was no significant difference in the colonization of *A. brasilense* Az39 (wt and *ipdC*$^{-}$) and no compatible cells were obtained in the un-inoculated controls. Electron microscopy confirmed the presence of cells compatible with *A. brasilense* wt and *ipdC*$^{-}$ mutant on soybean leaves at 1, 24 (data not shown), and 48 h post-inoculation (Fig. S1).

The foliar co-inoculation of soybean with *A. brasilense* Az39 wt and *ipdC*$^{-}$ and the exogenous application of pure IAA solution were compared with the seed inoculation with *B. japonicum* E109, and the results are summarized in Figs. 2, 3 and 4.

The foliar co-inoculation with the IAA-producing Az39 wt strain showed significant differences in several parameters of plant growth, such as total plant and root length, aerial and root dry weight, number of nodules on the primary root and nodule weight. As summarized in Fig. 2, the foliar co-inoculation with wt Az39 led to an average increase of 12% in plant length as compared with the seed inoculation with *B. japonicum* E109 and co-inoculation with the mutant Az39 *ipdC*$^{-}$ (Fig. 2a). At the root length level (Fig. 2b), the co-inoculation with Az39 wt led to an average increase of 31%. The foliar treatment with the IAA-deficient mutant Az39 *ipdC*$^{-}$ induced a significant reduction in the plant and root length, but also in root weight and number and biomass of nodules when compared to the foliar co-inoculation with Az39 wt (Figs. 3 and 4). As summarized in Figs. 2 and 3, the foliar co-inoculation with Az39 *ipdC*$^{-}$ led to the lowest growth rate, with a reduction of 18.6% in plant height and 40% in root length, as compared with the foliar co-inoculation with Az39. The exogenous application of IAA decreased the root length (Fig. 2), but increased the number of nodules per plant in comparison with the control inoculated with *B. japonicum* E109 (Fig. 4), and decreased the aerial and root length, as well as the number of nodules on primary roots, in comparison with the Az39 wt strain. In addition, the foliar co-inoculation with Az39 wt led to significant differences compared to the foliar application of exogenous IAA in the roots (length and dry weight) and aerial parts (dry weight), as well as in the number of nodules on the primary root.

After inoculation with Az39 wt, aerial dry weight increased significantly (Fig. 3a); in contrast, root dry weight was statistically higher than that observed after inoculation with Az39 and E109 and application of IAA, with an increase of 17% (Fig. 3b). Foliar co-inoculation with Az39 *ipdC*$^{-}$ reduced aerial and root dry weight by 11% and 5.4% respectively, but these differences were statistically significant only in aerial dry weight.

Figure 4 summarizes the parameters related to the *Bradyrhizobium*-soybean symbiosis. Nodulation was positively affected by co-inoculation with Az39 wt. The number of nodules on the primary root showed an average increase of 20% compared with the other treatments (Fig. 4a). The number of nodules per plant had an average increase of 26%, compared with that observed after the single inoculation with E109 and co-inoculation with Az39 *ipdC*$^{-}$ (Fig. 4b). Although the nodule biomass showed no differences between treatments, an increase of 17% was observed after foliar co-inoculation with Az39 wt (Fig. 4c). The number of nodules in the secondary root was also affected only by the foliar application of IAA, with a significant increase of 29% compared to the other treatments (data not shown). Similarly to that observed in other parameters, the co-inoculation with *ipdC*$^{-}$ induced a decrease in nodulation parameters.

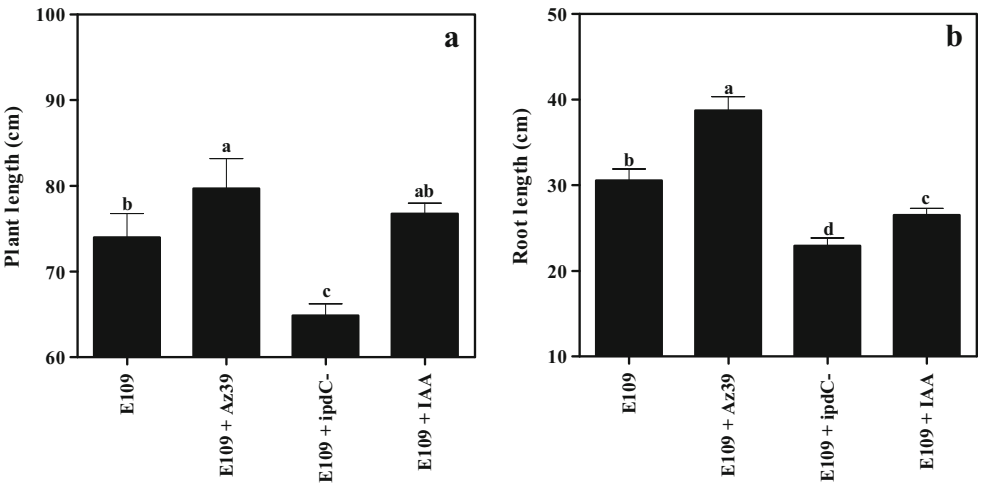

Fig. 2 **a** Aerial and **b** root length (cm) of soybean plants inoculated with *B. japonicum* E109 and leaves co-inoculated with *A. brasilense* Az39 wt (IAA producer) or Az39 *ipdC*$^{-}$ (IAA deficient), or treated exogenously with IAA pure solution. Different letters indicate significant differences ($p \leq 0.05$)

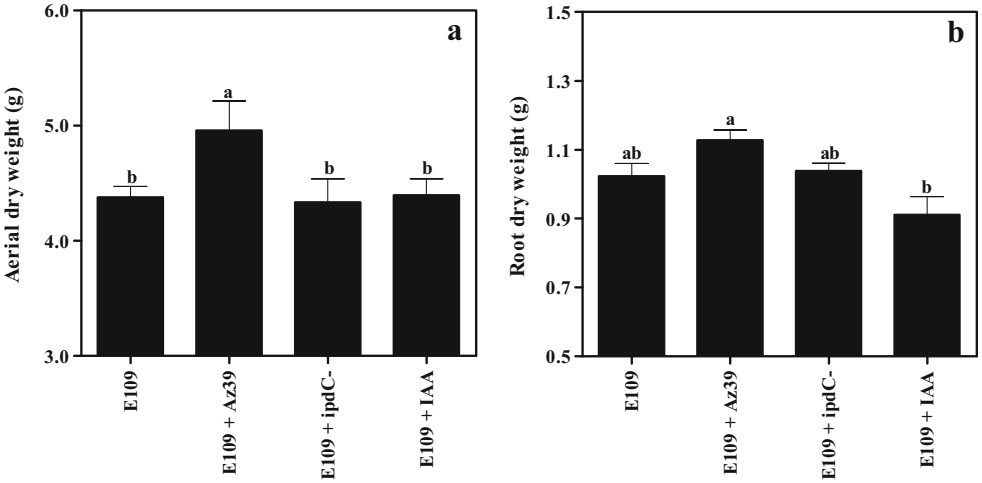

Fig. 3 **a** Aerial and **b** root dry weight (g) of soybean plants inoculated with *B. japonicum* E109 and leaves co-inoculated with *A. brasilense* Az39 wt (IAA producer) or Az39 *ipdC*⁻ (IAA deficient), or treated exogenously with IAA pure solution. Different letters indicate significant differences ($p \leq 0.05$)

4 Discussion

The use of phytohormones and PGPR is an extensive practice in agriculture to increase plant growth and crop yields around the world (Muniralzaman 2000). IAA is the best-characterized auxin known to be produced by many beneficial PGPR (Spaepen et al. 2007). Low concentrations of IAA can stimulate the elongation of taproots. High concentrations of IAA can stimulate lateral root formation, decrease the length of the primary root and increase the formation of root hairs with the concomitant increase in the root surface and water absorption volume (Dobbelaere et al. 1999; Patten and Glick 2002; Perrig et al. 2007; Spaepen et al. 2007; Remans et al. 2008). In legumes, the symbiosis with rhizobia and nodule ontogeny are controlled by the level of phytohormones produced at the different stages of the organ development (Dart 1977). Several authors have observed that the exogenous application of auxins (mainly IAA) in alfalfa and peanut seedlings promotes the formation of nodules and increases seedling weight (Gruodien and Zvironaite 1971; Srinivasan and Gopal 1977). Increases in crop dry weight compared with untreated controls were observed when IAA was applied in *Cassia absus* and common bean (*Phaseolus vulgaris* L.) (Hussain et al. 2011; El-Saeid et al. 2010). Furthermore, Remans et al. (2008) observed that a mutant of *A. brasilense* Sp245 *ipdC*⁻, deficient in IAA production, induced no increase in nodulation or nitrogen fixation in common bean (*Phaseolus vulgaris* L.). This indicated that bacterial production of this phytohormone is a key factor for the establishment of the rhizobium-legume symbiosis. In our experiments, the wild type strain of *A. brasilense* Az39 was the only strain able to produce IAA, reaching a maximum concentration at the stationary growth phase because of the accumulation of the metabolite in the culture medium. This production of IAA by *A. brasilense* Az39 wt was consistent with that observed by Perrig et al. (2007) and Cassán et al. (2009) in chemically defined media.

The production of plant hormones by *Azospirillum* sp. has been one of the most studied mechanisms of plant

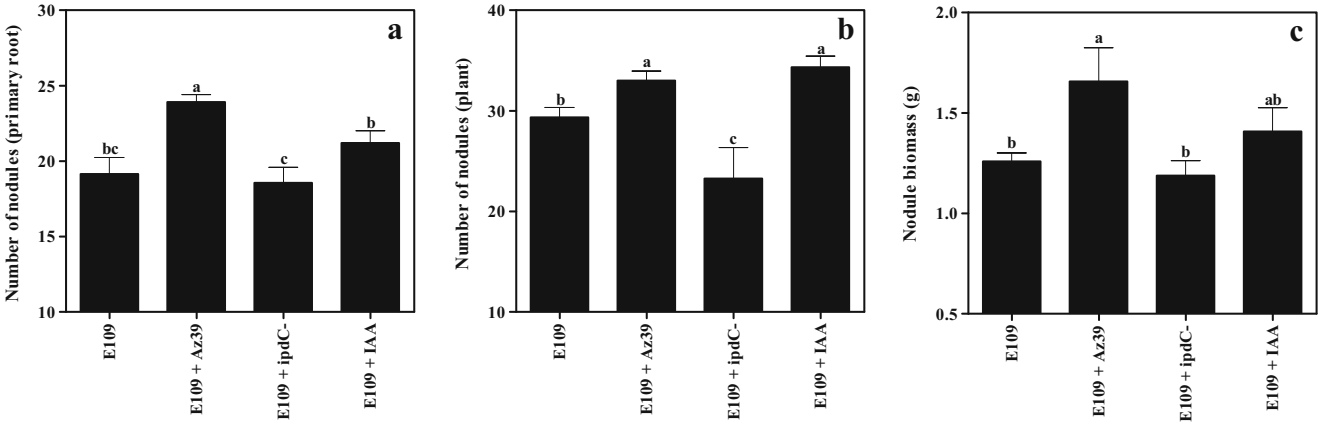

Fig. 4 **a** Number of nodules in primary root, **b** number of nodules per plant and **c** nodule biomass of soybean plants inoculated with *B. japonicum* E109 and leaves co-inoculated with *A. brasilense* Az39 wt (IAA producer) or Az39 *ipdC*⁻ (IAA deficient), or treated exogenously with IAA pure solution. Different letters indicate significant differences ($p \leq 0.05$)

growth promotion in the last decades (Spaepen et al. 2007; Cassán et al. 2014). Several authors have reported the beneficial role of IAA produced by *Azospirillum* sp. in plant growth (Tien et al. 1979; Hubbell et al. 1979; Dobbelaere et al. 1999; Spaepen et al. 2007). Tien et al. (1979). In addition, Hubbell et al. (1979) proved that the exogenous application of IAA, GA_3, and kinetin in pearl millet and sorghum induced changes in root morphology similar to those found in seedlings inoculated with *A. brasilense*. Later, Kolb and Martin (1985) showed that inoculation of *Beta vulgaris sp. and* wheat with *A. brasilense* increased the number of lateral roots compared to control plants. These authors correlated this effect with high levels of bacterial auxins in liquid culture and suggested that this effect could be mimicked by exogenous application of similar IAA concentrations. In the present study, the phytostimulatory role of IAA in foliar *Azospirillum* sp. inoculation was unequivocally demonstrated by comparing the plant-growth-promoting effects of the foliar inoculation with the wild-type strain, an auxin-impaired (*ipdC* knock-out) mutant, and the exogenous addition of auxins. Inoculation with the wild-type strain resulted in an increase in root growth, but inoculation with the *ipdC* mutant did not cause this effect. These results provided direct evidence for the role of the bacterial IAA during the bacteria-plant interaction.

Auxins increase the probability of interaction between the plant and the micro-symbiont (Hirsch et al. 1997). Furthermore, Schmidt et al. (1988) found that co-inoculation of alfalfa seeds with *Sinorhizobium meliloti* (inefficient IAA producer) and *A. brasilense* (efficient IAA producer) significantly increased the number of root nodules in the primary root. These authors correlated this increase with the inoculum size and suggested that this response could be mimicked by the addition of exogenous IAA. In the present study, the biosynthesis of *A. brasilense* Az39 IAA was modified at the IPyA pathway by knocking out the gene *ipdC*, which encodes a key enzyme in the IPyA pathway, to prevent the bacteria from producing IAA. This allowed us to evaluate the effects of the IAA produced by Az39 on the *Bradyrhizobium*-soybean symbiosis and soybean growth. The knock-out of the *ipdC* gene prevented Az39 from producing IAA and reduced the plant growth promotion and *Bradyrhizobium*-soybean symbiosis interaction compared to the Az39 wt (IAA producer) strain. These results allow us to propose a biological model of response to foliar co-inoculation of soybean with an IAA-producing rhizobacterium (Fig. 5). This model clearly shows that both the presence of the microorganism as part of the process of colonization and the production of IAA in situ are co-responsible for the positive effects on plant growth and symbiosis establishment, via plant signaling molecules. In contrast, pure IAA solution applied exogenously resulted only a response at the level of the symbiosis establishment. The best results with respect to growth parameters were only obtained in the presence of the bacteria. The construction of an *A. brasilense* Az39 strain deficient in IAA biosynthesis (*ipdC*$^{-}$) allowed us to demonstrate the importance of this molecule in the *Azospirillum*-soybean and *Bradyrhizobium*-soybean symbiosis.

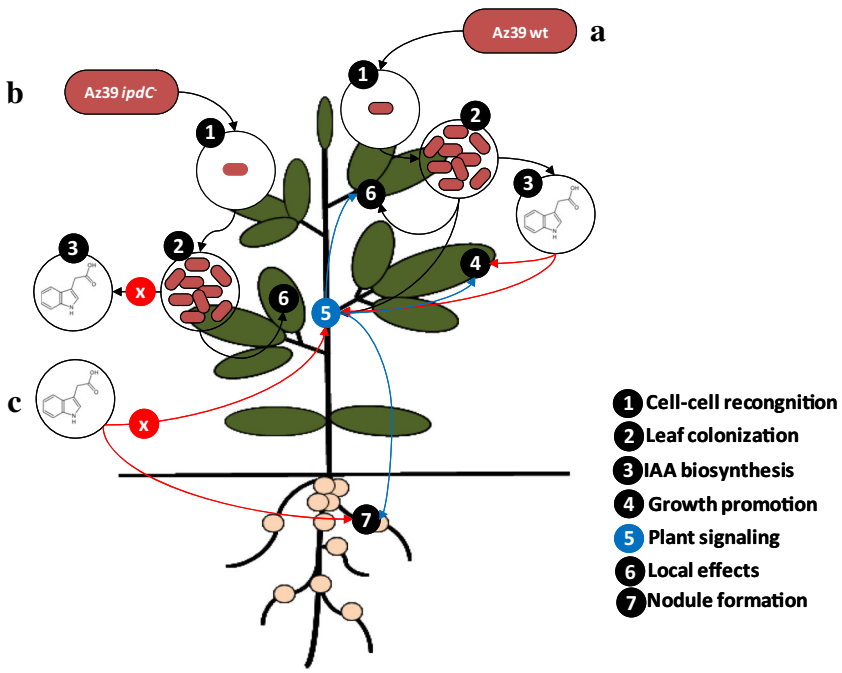

Fig. 5 Biological model explaining the foliar inoculation with *A. brasilense*, the IAA effects and plant signaling. The figure shows the plant response to inoculation with the IAA producer *A. brasilense* Az39 (A), the IAA-deficient mutant *ipdC*- (B), and the exogenous application of synthetic IAA (C). The presence of both bacteria and bacterial IAA on plant tissues induced a systemic signaling that positively affected aerial organs, roots and the symbiosis process with *B. japonicum* E109. The addition of exogenous IAA induced plant growth promotion independently of inoculation. References: black arrows (bacterial activity); blue arrows (plant signaling); red arrows (IAA activity)

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Compliance with ethical standards

Conflicts of interest The authors report no conflicts of interest.

References

Bashan Y, de Bashan L (2010) How the plant growth-promoting bacterium Azospirillum promotes plant growth. A critical assessment. Adv Agron 108:77–136. https://doi.org/10.1016/S0065-2113(10)08002-8

Bottini R, Fulchieri M, Pearce D, Pharis R (1989) Identification of gibberellins A1, A3, and Iso-A3 in cultures of a. Lipoferum. Plant Physiol 90(1):45–47. https://doi.org/10.1104/pp.90.1.45

Burdmann S, Kigel J, Okon Y (1997) Effects of *Azospirillum brasilense* on nodulation and growth of common bean (*Phaseolus vulgaris* L.) Soil Biol Biochem 29(5-6):923–929. https://doi.org/10.1016/S0038-0717(96)00222-2

Cacciari I, Lippi D, Pietrosanti T, Pietrosanti W (1989) Phytohormone-like substances produced by single and mixed diazotrophic cultures of *Azospirillum* and *Arthrobacter*. Plant Soil 115(1):151–153. https://doi.org/10.1007/BF02220706

Campo RJ, Araujo RS, Hungria M (2009) Nitrogen fixation with the soybean crop in Brazil: compatibility between seed treatment with fungicides and bradyrhizobial inoculants. Symbiosis 48(1):154–163. https://doi.org/10.1007/BF03179994

Carreño-Lopez R, Campos-Reales N, Elmerich C, Baca B (2000) Physiological evidence for differently regulated tryptophan-dependent pathways for indole-3-acetic acid synthesis in *Azospirillum brasilense*. Mol Gen Genet 2 64(4):521–530

Cassán F, Díaz-Zorita M (2016) *Azospirillum* sp. in current agriculture: From the laboratory to the field. Soil Biol Biochem 103:117–130. https://doi.org/10.1016/j.soilbio.2016.08.020

Cassán F, Maiale S, Masciarelli O, Vidal A, Luna V, Ruiz O (2009) Cadaverine production by *Azospirillum brasilense* and its possible role in plant growth promotion and osmotic stress mitigation. Eur J Soil Biol 45(1):12–19. https://doi.org/10.1016/j.ejsobi.2008.08.003

Cassán F, Penna C, Creus C, Radovancich D, Monteleone E. et al. (2013) Inoculantes forulados con *Azospirillum* sp. In: Albanesi, A; Benintende, S; Cassán F. y Perticari A. (Eds). Manual de procedimientos microbiológicos para la evaluación de inoculantes. pp 25–34. Editorial: Asociación Argentina de Microbiología. ISBN: 978-987-26716-4-8

Cassán F, Vanderleyden J, Spaepen S (2014) Physiological and agronomical aspects of phytohormone production by model plant-growth-promoting rhizobacteria (PGPR) belonging to the genus *Azospirillum*. Plant Growth Regul 33(2):440–459. https://doi.org/10.1007/s00344-013-9362-4

Costacurta A, Keijers V, Vanderleyden J (1994) Molecular cloning and sequence analysis of an *Azospirillum brasilense* indole-3-pyruvate deccarboxylase. Mol Gen Genet 243(4):463–472

Crozier A, Arruda P, Jasmim JM, Monteiro AM, Sandberg G (1988) Analysis of indole-3-acetic acid and related indoles in culture medium from *Azospirillum lipoferum* and *Azospirillum brasilense*. Appl Environ Microbiol 54(11):2833–2837

Dart P (1977) Infection and development of leguminous nodules. In: Hardy RWI, Silver WS (eds) A tratise of dinitrogen fixation section III-biology. Wiley, New York, pp 367–372

Di Rienzo J.A.; Casanoves, F; Balzarini, M.G; Gonzalez, L; Tablada, M; Robledo, C.W. InfoStat versión 2014. Córdoba: Grupo InfoStact, FCA, Universidad Nacional de Córdoba, Argentina. URL http://www.infostat.com.ar

Dobbelaere S, Croonenborghs A, Thys A, Broek AV et al (1999) Phytostimulatory effect of *Azospirillum brasilense* wild type and mutant strains altered in IAA production on wheat. Plant Soil 212(2):153–162. https://doi.org/10.1023/A:1004658000815

Döbereiner J, Baldani VLD, Baldani JI (1995) Como isolar e identificar bactérias diazotróficas de plantas não-leguminosas. Brasília: EMBRAPA-SPI: EMBRAPA-CNPAB, Itaguaí, RJ, 60p

El-Saeid H, Abou-Hussein S, El-Tohamy W (2010) Growth characters, yield and endogenous hormones of cowpea plants in response to IAA application. Res J Agric Biol Sci 6:27–31

Fehr, WR, Caviness CE (1977) Stages of soybean development. Cooperative Extension Service, Agriculture and Home Economics Experiment Station. Iowa State University, Ames, Iowa

Frugier F, Kosuta S, Murray JD, Crespi M, Szczyglowski K (2008) Cytokinin: secret agent of symbiosis. Trends Plant Sci 13(3):115–120. https://doi.org/10.1016/j.tplants.2008.01.003

Gruodien J, Zvironaite V (1971) Effect of IAA on growth and synthesis of N compounds in Lucerne. Luk TSR Aukstuju Mosklo Darbai Biologia 17:77–87

Hirsch A, Fang Y, Asad S, Kapulnik Y (1997) The role of phytohormones in plant-microbe symbioses. Plant Soil 194(1/2):171–184. https://doi.org/10.1023/A:1004292020902

Hoagland D, Arnon D (1950) The Water-Culture Method for Growing Plants without Soil. California Agricultural Experiment Station. Circular 347:1–32

Horemans S, Koninck K, Neuray J, Hermans R et al (1986) Production of plant growth substances by *Azospirillum* sp. and other rhizophere bacteria. Symbiosis 2:341–346

Hubbell D, Tien T, Gaskins M, Lee J (1979) Physiological interaction in the *Azospirillum*-grass root association. In: Vose P, Ruschel A (eds) Associative N2-fixation. CRC Press, Boca Raton, pp 1–6

Hungria M, Nogueira M, Araujo R (2015) Soybean seed co-inoculation with *Bradyrhizobium* spp. and *Azospirillum brasilense*: a new biotechnological tool to improve yield and sustainability. Am J Plant Sci 6(06):811–817. https://doi.org/10.4236/ajps.2015.66087

Hussain K, Hussain M, Nawaz K, Majeed A et al (2011) Morphochemical response of Chaksu (*Cassia absus* L.) to different concentrations of indole acetic acid (IAA). Pak J Bot 43:1491–1493

Janzen R, Rood S, Dormar J, McGill W (1992) *Azospirillum brasilense* produces gibberellins in pure culture and chemically-medium and in co-culture on straw. Soil Biol Biochem 24(10):1061–1064. https://doi.org/10.1016/0038-0717(92)90036-W

Kloepper J, Schroth M (1978) Plant growth-promoting rhizobacteria on radishes. In: Proceedings of the 4th international conference on plant pathogenic bacteria Vol, 2, pp 879–882

Kolb W, Martin P (1985) Response of plant roots to inoculation with *Azospirillum brasilense* and to application of indole acetic acid. In: *Azospirillum* III. Springer Berlin Heidelberg, pp 215–221. https://doi.org/10.1007/978-3-642-70791-9_20

Lodeiro AR (2015) Interrogantes en la tecnología de la inoculación de semillas de soja con *Bradyrhizobium* spp. Rev Argent Microbiol 47(3):261–273. https://doi.org/10.1016/j.ram.2015.06.006

López-García S, Perticari A, Piccinetti C, Ventimiglia L et al (2009) In-furrow inoculation and selection for higher motility enhances the efficacy of *Bradyrhizobium japonicum* nodulation. Agron J 101(2):357–363. https://doi.org/10.2134/agronj2008.0155x

Mathesius U, Schlaman H, Spaink H, Of Sautter C et al (1998) Auxin transport inhibition precedes root nodule formation in white clover roots and is regulated by flavonoids and derivatives of chitin

oligosaccharides. Plant J 14(1):23–34. https://doi.org/10.1046/j.1365-313X.1998.00090.x

Miles A, Misra S (1938) The estimation of the bactericidal power of blood. J Hyg 38(06):732–737. https://doi.org/10.1017/S002217240001158X

Muniralzaman M (2000) Effect of CyCoCel (CCC) on the growth and yield manipulations of vegetable soybean. ARG Traininy. Kasetsart university kamphaen sean. Nakhon phathom, Thailand, pp 1–16

Patten C, Glick B (1996) Bacterial biosynthesis of indole-3-acetic acid. Can J Microbiol 42(3):207–220. https://doi.org/10.1139/m96-032

Patten C, Glick B (2002) The role of *Pseudomonas putida* indoleacetic acid in the development of the host plant root system. Appl Environ Microbiol 68(8):3795–3801. https://doi.org/10.1128/AEM.68.8.3795-3801.2002

Perrig D, Boiero M, Masciarelli O, Penna C et al (2007) Plant-growth-promoting compounds produced by two agronomically important strains of *Azospirillum brasilense*, and implications for inoculant formulation. Appl Microbiol Biotechnol 75(5):1143–1150. https://doi.org/10.1007/s00253-007-0909-9

Piccoli P, Bottini R (1996) Gibberellins production in *A. lipoferum* cultures y enhanced by light. Biocell 20:185–190

Prinsen E, Costacurta A, Michiels K, Vanderleyden J et al (1993) *Azospirillum brasilense* indole-3-acetic acid biosynthesis: evidence for a non-tryptophan dependent pathway. Mol Plant-Microb Interact 6(5):609–615. https://doi.org/10.1094/MPMI-6-609

Remans R, Beebe S, Blair M, Manrique G, Tovar E, Rao I, Croonenborghs A, Torres-Gutierrez R, el Howeity M, Michiels J, Vanderleyden J (2008) Physiological and genetic analysis of root responsiveness to auxin-producing plant growth-promoting bacteria in common bean (*Phaseolus vulgaris* L.) Plant Soil 302(1-2):149–161. https://doi.org/10.1007/s11104-007-9462-7

Rivera D, Revale S, Molina R, Gualpa J et al (2014) Complete genome sequence of the model rhizosphere strain *Azospirillum brasilense* Az39, successfully applied in agriculture. Genome announcements 2(4):e00683–e00614

Rodríguez Cáceres E (1982) Improved medium for isolation of *Azospirillum* spp. Appl Environ Microbiol 44:990–991

Sadasivan L, Neyra C (1985) Floculation in *Azospirillum brasilense* and *Azospirillum lipoferum*: exopolysaccharides and cyst formation. J Bacteriol 163(2):716–723

Schmidt W, Martin P, Omay H, Bangerth F (1988) Influence of *Azospirillum brasilense* on nodulation of legumes. In: Kling-Müller W (ed) *Azospirillum* IV. Genetics, physiology, ecology. Springer, Heidelberg, pp 92–100

Simon R, Priefer U, Pühler A (1983) A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Biotechnology 1(9):784–790. https://doi.org/10.1038/nbt1183-784

Spaepen S, Vanderleyden J, Remans R (2007) Indole-3-acetic acid in microbial and microorganism-plant signaling. FEMS Microbiol 31(4):425–448. https://doi.org/10.1111/j.1574-6976.2007.00072.x

Spaepen S, Dobbelaere S, Croonenborghs A, Vanderleyden J (2008) Effects of *Azospirillum brasilense* indole-3-acetic acid production on inoculated wheat plants. Plant Soil 312(1–2):15–23. https://doi.org/10.1007/s11104-008-9560-1

Srinivasan P, Gopal K (1977) Effect of plantofix and NAA formulation on groundnut var TMU-7. Curr Sci 46:119–120

Srinivasan M, Holl F, Petersen D (1996) Influence of indoleacetic-acid-producing *Bacillus* isolates on the nodulation of *Phaseolus vulgaris* by *Rhizobium etli* under gnotobiotic conditions. Can J Microbiol 42(10):1006–1014. https://doi.org/10.1139/m96-129

Thimann K (1936) Auxins and the growth of roots. Am J Bot 23(8):561–569. https://doi.org/10.2307/2436087

Tien T, Gaskina M, Hubbell D (1979) Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of pearl millet (*Pennisetum Americanum* L). Appl Environ Microbiol 37(5):1016–1024

Torres D, Revale S, Obando M, Maroniche G et al (2015) Genome sequence of *Bradyrhizobium japonicum* E109, one of the most agronomically used nitrogen-fixing rhizobacteria in Argentina. Genome announcements 3(1):e01566–e01514

Unger T, Jacobovitch Y, Dantes A, Bernheim R (2010) Applications of the restriction free (RF) cloning procedure for molecular manipulations and protein expression. J Struct Biol 172(1):34–44. https://doi.org/10.1016/j.jsb.2010.06.016

Vicario J, Gallarato L, Paulucci N, Perrig D et al (2015) Co-inoculation of legumes with *Azospirillum* and symbiotic rhizobia. In: Cassán F, Okon Y, Creus C (eds) Handbook for *Azospirillum*. Springer, Cham

Vincent JM (1970) A manual for the practical study of the root-nodule bacteria. Blackwell Scientific Publications, Oxford

Yahalom E, Okon Y, Dovrat A (1990) Possible mode of action of *Azospirillum brasilense* strain cd on the root morphology and nodule formation in burr medic (*Medicago polymorpha*). Can J Microbiol 36(1):10–14. https://doi.org/10.1139/m90-003

Zilli JÉ, Ribeiro KG, Campo RJ, Hungria M (2009) Influence of fungicide seed treatment on soybean nodulation and grain yield. Revista Brasileira de Ciência do Solo 33(4):917–923. https://doi.org/10.1590/S0100-06832009000400016

Zimmer W, Bothe H (1988) The phytohormonal interactions between *Azospirillum* and wheat. Plant Soil 110(2):239–247. https://doi.org/10.1007/BF02226804