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REGULAR ARTICLE

The *Vicia sativa* spp. *nigra* - *Rhizobium leguminosarum* bv. *viciae* symbiotic interaction is improved by *Azospirillum brasilense*

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Abstract This study aimed to examine the effects of inoculation with wild type (Sp7) and mutant strains of *Azospirillum brasilense* on the *Vicia sativa* spp. *nigra* (vetch)-*Rhizobium leguminosarum* bv. *viciae* (*Rlv*) symbiosis. The *A. brasilense* mutants were *ipdC*$^-$ and *napA*$^-$, impaired in indole pyruvate decarboxylase and periplasmic nitrate reductase, respectively; and *acdS*$^+$, carrying the ACC deaminase gene. Inoculations were done in pots, pouches and hydroponics and we measured shoot and root weight parameters as well as effects on root morphology and *nod* gene induction ability by roots. In pots, wild type Sp7 and the *acdS*$^+$ strain, but *ipdC*$^-$ and *napA*$^-$ mutants, lead to an increase in root hair density, 3–4 cm above the root tip. In pouches, combined inoculation with *Rlv* and strains Sp7, *acdS*$^+$ or *ipdC*$^-$, but *napA*$^-$, increased shoot dry matter and nodulation relative to *Rlv* alone. In a hydroponic system, co-inoculation with strains Sp7 or *acdS*$^+$, but with *ipdC*$^-$ and *napA*$^-$ mutants, enhanced root secretion of *nod* gene-inducing flavonoids in comparison with *Rlv*-inoculated plants. These results support that auxin production by *A. brasilense* has a positive effect on root secretion of *nod* gene-inducing flavonoids and auxin absorption activity by the plant.

Keywords Co-inoculation · Flavonoids · *nod* gene expression · Auxin · Nitric oxide

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Introduction

Bacteria of the genus *Rhizobium* symbiotically interact with leguminous plants in a host-specific manner to form N_2-fixing root nodules (van Rhijn and Vanderleyden 1995). Successful establishment of such symbiosis involves chemotaxis and movement of the bacteria towards the roots, root colonization, root hair deformation, formation of infection threads, and rapid division of root cortex cells (van Rhijn and

Vanderleyden 1995). The early events of nodule formation require expression of bacterial nodulation (*nod*) genes, including *nodABC*, which are induced by plant flavonoids (de Rijke et al. 2006).

The *Azospirillum* genus comprises free-living, N_2-fixing, plant growth promoting rhizobacteria (PGPR). Azospirilla are able to colonize the root surface, often possessing endophytic ability. These bacteria are capable of increasing the yield of important crops growing in various soils and climatic regions (Okon and Labandera-Gonzalez 1994; Fuentes-Ramirez and Caballero-Mellado 2005). Effects of *Azospirillum* inoculation are mainly attributed to improved root development and enhanced water and mineral uptake. Secretion of plant growth promoting substances, mainly indole-3-acetic acid (IAA), is strongly associated with the positive response by the plant (Dobbelaere and Okon 2007; Spaepen et al. 2007; 2009).

Dual inoculation of several legumes with *Rhizobium* and *Azospirillum*, as well as with other PGPR has been shown to significantly increase nodulation, N_2-fixation, accumulation of macro and microelements, and plant biomass as compared to inoculation with *Rhizobium* alone (Iruthayathas et al. 1983; Grimes and Mount 1984; Plazinski and Rolfe 1985; Sarig et al. 1986; Yahalom et al. 1991; De Freitas et al. 1993; Burdman et al. 1998; Rodelas et al. 1996; 1999; Dardanelli et al. 2008). Inoculation of both common bean and alfalfa seedlings with *Azospirillum brasilense* resulted in an increased production of plant root flavonoids and an enhanced capacity to induce *Rhizobium nod*-gene expression as compared to non-inoculated controls (Burdman et al. 1996; Volpin et al. 1996; Dardanelli et al. 2008). The presence of azospirilla in the rhizosphere was reported to activate the hydrolysis of conjugated phytohormones and flavonoids in the root tissue, thus leading to the release of compounds in their active forms (Dobbelaere and Okon 2007). In addition, secretion of plant growth promoting substances such as auxins, gibberellins and cytokinins by azospirilla plays an important role in plant growth promotion effects by the bacteria (Dobbelaere et al. 1999; Spaepen et al. 2007).

The objective of the present work was to study the effects of single or combined inoculation of *Vicia sativa* subsp. *nigra* (vetch) with *Rhizobium leguminosarum* bv. *viciae* and wild type (strain Sp7) or mutant strains of *A. brasilense*. Here we report experiments that were carried out in pots, pouches and in a hydroponic system.

Material and methods

Bacterial strains, culture conditions and plant material

All bacterial strains used in this study and the description of their characteristics are shown in Table 1. *Azospirillum brasilense* strains were cultivated in liquid or solid Luria Bertani (LB) medium, while solid or liquid yeast mannitol (YM) media (Vincent 1970) were used for *Rhizobium leguminosarum* bv. *viciae* (*Rlv*) strains. Antibiotics were added when appropriate (see Table 1) at the following concentrations: kanamycin (Km, 50 μg mL^{-1}); tetracycline (Tc, 2 μg mL^{-1}); and chloramphenicol (Cm, 10 μg mL^{-1}). For inoculation, bacteria were grown in liquid media with shaking (150 rpm) at 30°C. Bacteria were centrifuged (4,000*g*, 10 min, 4°C; twice) and resuspended with phosphate buffer-saline (PBS; 0.24 gL^{-1} KH_2PO_4, 1.44 gL^{-1} Na_2HPO_4, 0.2 gL^{-1} KCl, 8 gL^{-1} NaCl; pH 7.4). Bacterial concentrations were adjusted by measuring absorbance at 600 nm using a spectrophotometer (Genesys 10vis, Thermo Scientific), and the numbers of viable cells (colony forming units, CFU) were confirmed by dilution plating on solid media.

Inoculation experiments in different growth systems

Vetch (*Vicia sativa* spp. *nigra*; kindly supplied by F. Temprano, Sevilla, Spain) seeds were surface sterilized as described (van Brussel et al. 2002). Bacterial suspensions of 10^5 and 10^6 CFU mL^{-1}, for *Rlv* and *A. brasilense*, respectively, were used to inoculate seeds (1 mL per seed) at sowing. Non-inoculated control seeds were treated with PBS. Plants were grown in 400-mL plastic pots with sterilized vermiculite (size 1, Habomin) as substrate and a 3-cm layer of sterile perlite in the top that was added to avoid cross contamination. The pots were maintained in a greenhouse (22°C, 8 h-light) and were irrigated with 50% diluted sterile Jensen solution (Vincent 1970). The pots were maintained at field capacity throughout the experiment. These experiments were performed to assess the effects of inoculation with *A. brasilense*

Table 1 Bacterial strains used in this study

Strain	Characteristics[1]	Source/reference
Azospirillum brasilense		
Sp7	wild type strain (ATCC 29145)	Tarrand et al. (1978)
Cd 1843 (*acdS*$^+$)	wild type Cd (ATCC 29729; Eskew et al. 1977), carrying plasmid pRKTACC with the *Enterobacter cloacae* UW4 ACC deaminase (*acdS*) gene; Tc^R	B. Glick (Waterloo, Canada); Holguin and Glick 2001
FAJ009 (*ipdC*$^-$)	mutant of wild type Sp245 (Baldani et al. 1986), with a Tn5 insertion in the indole-3-pyruvate decarboxylase *(ipdC)* gene; produces 10% indole-acetic acid (IAA) relative to the wild type; Km^R	J. Vanderleyden (Heverlee, Belgium); Costacurta et al. 1994
FAJ164 (*napA*$^-$)	mutant of wild type Sp245, carrying a Km^R cassette in the open reading frame of *napA*, encoding the catalytic subunit of periplasmic nitrate reductase; does not produce nitric oxide (Molina-Favero et al. 2008).	J. Vanderleyden; Steendhoudt et al. 2001
Rhizobium leguminosarum bv. *viciae* (*Rlv*)		
VF39	wild type, isolated from nodules of *Vicia faba* cv. Kristall from a field in Germany	U. Priefer (Aachen, Germany); Prell et al. 2002
A76 (RBL 5280)	*Rlv* containing plasmids pMP154 (IncQ carrying pr. *nodA-lacZ*) and pMP280 (IncP carrying pr. *nodD-nodD*); Tc^R, Cm^R	A. van Brussel (Leiden, The Netherlands); Tak et al. 2004

[1] Antibiotics: Km, kanamycin; Tc, tetracycline; Cm, chloramphenicol

and/or *Rlv* on the morphology of root tips, following staining with methylene blue and observation in a binocular, at different times after inoculation. At each time, roots of 10 randomly selected plants from each treatment were observed and photographed. Inoculation experiments were also performed in pouches as described (Yahalom et al. 1987). Pouches were irrigated and maintained under similar conditions as described for pots. Growth of vetch in a hydroponic system was as described (Burdman et al. 1996). The plant growth solution was at 50% strength of a sterile Jensen solution (Vincent 1970) and 12 germinating seeds were placed in each hydroponic box, which were incubated in a growth chamber at 20°C, 8 h-light.

Collection of root exudates from the hydroponic system and assessment of *nod* gene induction and other parameters

Samples from the growth solution of hydroponic-grown vetch seedlings were collected 2, 4, 7 and 16 days after inoculation (d.a.i.). At each time, 20 mL of the growth solution were collected, and the same volume of fresh solution was added to the boxes. The samples were centrifuged (4,000*g*, 15 min) and sterilized by membrane filtration to remove bacteria. For confirmation of sterility, 100-μL samples were plated on nutrient agar (NA) and growth was assessed after incubation at 28°C. Five milliliters were lyophilized, resuspended in 500 μL PBS, and used to assess *nod* gene induction in *Rlv* A76 (harboring a *nodA*::*lacZ* transcriptional fusion) by β-galactosidase activity assays according to Miller (1972). β-galactosidase activities were measured from at least six independent replicates (boxes) per treatment, per d.a.i. In each replicate, 100 μL of each sample were mixed with 375 μL of a bacterial suspension (10^8 CFU mL^{-1} in PBS) of overnight-grown *Rlv* A76, and 500 μl PBS. *Rlv* cells were induced for a maximum period of 5 h at 28°C on a rotary shaker (150 rpm). The rest of the filtrated growth solution (about 15 mL samples) were lyophilized, resuspended in 1 mL ethanol, and used to determine indole content (mainly auxin) with the Salkowski reagent as described (Glickmann and Dessaux 1995).

Data analysis

All experiments were carried out at least twice in randomized layouts, unless otherwise stated. Statistical analyses were carried out using analysis of variance (ANOVA), and significance was determined by Tukey-Kramer HSD test. The analyses

were done with the JMP IN v 3.2.1 software (SAS Institute Inc.)

Results

Effects of co-inoculation of vetch with *Azospirillum brasilense* wild type or mutants and *Rhizobium leguminosarum* bv. *viciae* (*Rlv*) on root tip morphology

Three pot experiments were carried out to assess the effects of inoculation with various *A. brasilense* strains and *Rlv* VF39 (alone or in co-inoculation) on root tip morphology. No apparent differences among treatments were observed 2 and 4 days after inoculation (d.a.i.) on root tip morphology (not shown). However, 7 d.a.i., clear differences were observed among some of the treatments: at this time, root hair formation was observed in some of the tips of roots inoculated with *Rlv* alone; however, root hairs of *Rlv*-inoculated roots were shorter and thinner, and formed at a lower density than in roots inoculated with the wild type strain of *A. brasilense*. No root hairs were observed at this stage on roots of non-inoculated controls (Fig. 1).

At 7 d.a.i., clear differences were also observed between co-inoculation with the wild type strain of *A. brasilense* and the tested mutants. Co-inoculation with *Rlv* and the *acdS*$^+$ mutant lead to the formation of root hairs that were longer than root hairs from other treatments (Fig. 1). In contrast, co-inoculation with *Rlv* and the *ipdC*$^-$ mutant delayed root hair formation (Fig. 1). Co-inoculation with *Rlv* and the *napA*$^-$ mutant also reduced root hair formation in comparison with co-inoculation with *Rlv* and the wild type strain of *A. brasilense*; however, the differences were not as accentuated as with the *ipdC*$^-$ mutant (not shown).

Forty d.a.i., when the pot experiments were concluded, the variability of shoot and root weight as well as nodulation parameters among replicates of the different treatments, was too high and no significant differences could be detected in most cases. Therefore, for more precise analyses of these parameters we later used the pouch growth system (see below).

Effects of vetch inoculation with *A. brasilense* and *Rlv* in pouches

We used pouches to assess the effects of inoculation with wild type and mutant strains of *A. brasilense*, with or without *Rlv*, on the development of vetch

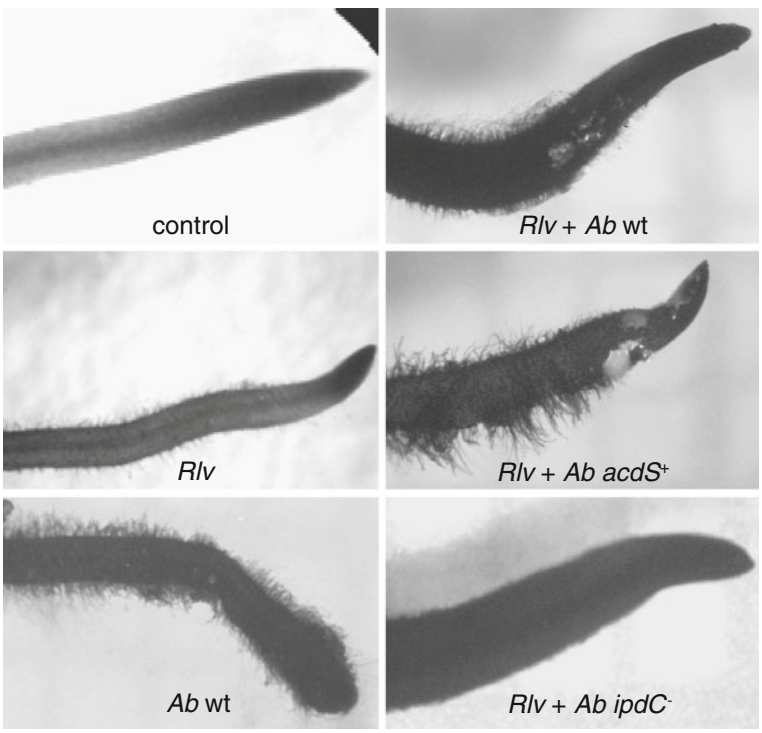

Fig. 1 Effect of inoculation with *Rhizobium leguminosarum* bv. *viciae* (*Rlv*) and/or different *Azospirillum brasilense* strains on root tip morphology of vetch. *A. brasilense* strains: *Ab* wt, wild type Sp7; *Ab acdS*$^+$, expressing ACC deaminase; *ipdC*$^-$, indole-3-pyruvate decarboxylase mutant. Controls were non-inoculated plants. Pictures were taken 7 days after inoculation (d.a.i.). Representative pictures from one experiment of two with similar results are shown

roots and shoots, 35 d.a.i. In this system, roots of plants inoculated with *Rlv* (with and without *A. brasilense* strains) showed reduced root dry weights relative to plants inoculated with the different *A. brasilense* strains alone and non-inoculated controls, although no significant differences were observed between most treatments (Table 2). Plants inoculated with the wild type strain of *A. brasilense* had a significantly ($P \leq 0.05$) lower number of lateral roots relative to plants inoculated with the *ipdC*$^-$ mutant. This result was unexpected given the demonstrated role of auxin production by *A. brasilense* in lateral root induction (Lambrecht et al. 2000). Nevertheless, differences between these treatments were small and no significant differences between these and most *A. brasilense* strains were observed for this parameter in co-inoculation with *Rlv* (Table 2).

In contrast to the little differences observed in root parameters, clear differences among treatments were observed in shoot development. Shoots of plants inoculated with *Rlv* alone or co-inoculated with *Rlv* and any of the *A. brasilense* strains were significantly ($P \leq 0.05$) longer than those of plants inoculated with *A. brasilense* strains alone or non-inoculated controls (Table 2). Accordingly, similar differences between these treatments were observed in shoot dry weight (Table 2). In agreement with these findings, the shoots of co-inoculated plants were greener and healthier than shoots of plants inoculated with *A. brasilense* alone or non-inoculated (not shown). In all experiments, a tendency was observed by which plants inoculated with *A. brasilense* strains had higher shoots than non-inoculated controls; however, these differences were not statistically significant (Table 2). Although no significant differences were observed for most parameters among different *A. brasilense* strains, it appeared that the *napA*$^-$ mutant had a weaker performance than the other strains in co-inoculation with *Rlv*.

Nodule appearance on plant roots started 2 weeks after inoculation. Combined inoculation of *Rlv* with most *A. brasilense* caused a significant ($P \leq 0.05$) increase in nodule dry weight relative to inoculation with *Rlv* alone (Table 2). The only exception was co-inoculation with the *napA*$^-$ mutant that did not significantly differ from inoculation with *Rlv* alone in this parameter. No nodules were observed on roots of each of *A. brasilense* alone-inoculated plants or untreated control (Table 2).

Effects of inoculation of vetch in a hydroponic system

Pouch experiments revealed that most *A. brasilense* strains were able to enhance nodulation by *Rlv* on vetch roots (Table 2). Therefore, we performed inoculation experiments in a hydroponic system, to collect root exudates of plants from different treatments and compare them for their ability to induce *Rlv nod* gene expression in β-galactosidase assays, using strain *Rlv* A76.

Table 2 Effect of inoculation with *Rlv* and/or *A. brasilense* (*Ab*) Sp7 (wt) and mutants on plant growth and nodulation of vetch in pouches. Controls were non-inoculated plants. Measurements were made 35 days after inoculation. Results represent averages of three experiments with similar results, with a total of 54 plants per treatment. Different letters represent significant differences ($P \leq 0.05$) between treatments.

Treatment	Root dry weight (mg/plant)	Lateral root number	Nodule dry weight (mg/plant)	Shoot dry weight (mg/plant)	Shoot length (cm)
Rlv	11 bc	18.2 bc	0.28 c	14 c	10.6 bc
Rlv + *Ab* wt	11 abc	18.4 bc	0.37 a	16 bc	11.0 abc
Rlv + *Ab acdS*$^+$	11 c	18.9 ab	0.35 ab	18 ab	11.2 ab
Rlv + *Ab ipdC*$^-$	12 bc	17.5 c	0.33 ab	19 a	11.8 a
Rlv + *Ab napA*$^-$	11 bc	17.9 c	0.31 bc	15 c	10.4 c
Ab wt	12 abc	19.8 c	0 d	5 d	6.8 d
Ab acdS$^+$	13 ab	21.5 a	0 d	5 d	7.3 d
Ab ipdC$^-$	13 a	20.1 ab	0 d	5 d	6.8 d
Ab napA$^-$	13 abc	20.2 abc	0 d	5 d	7.0 d
Control	12 abc	19.8 abc	0 d	5 d	4.5 d

Root exudates were collected and assayed 2, 4, 7 and 16 d.a.i. When comparing between exudates extracted 2 d.a.i., the highest *nod* gene induction abilities were observed for exudates from roots co-inoculated with *Rlv* and *A. brasilense* wild type or *acdS*$^+$ mutant (not shown). These differences were maintained 4 d.a.i., although significant ($P \leq 0.05$) differences between inoculation with *Rlv* alone and the co-inoculation treatment were observed only when the wild type strain of *A. brasilense* was used (Fig. 2). Also, co-inoculation with this strain and with the *acdS*$^+$ mutant significantly ($P \leq 0.05$) differed from co-inoculation with *ipdC*$^-$ and *napA*$^-$ mutants, and from non-inoculated controls at this time (Fig. 2). Similar patterns of *nod* gene induction by the different treatments were observed 7 (not shown) and 16 d.a.i. (Fig. 2), although at the latter time, an increase in *nod* gene induction was observed for all treatments, and co-inoculation with the wild type strain of *A. brasilense* but not with the *acdS*$^+$ mutant significantly ($P \leq 0.05$) differed from co-inoculation with the other mutants or inoculation with *Rlv* alone.

In these experiments, treatments in which plants were inoculated with *A. brasilense* strains alone were also included. In agreement with the above findings, similar trends were observed by which exudates of wild type- and *acdS*$^+$ mutant-inoculated roots possessed higher *nod* gene inducing abilities than exudates from roots inoculated with the *ipdC*$^-$ and *napA*$^-$ mutants (not shown).

We also assessed the concentration of indoles (mainly auxin) in the hydroponic growth solutions during the experiment. At 2 and 4 d.a.i., indole concentrations were too low to be detected. At 7 d.a.i., indole concentrations in growth solutions of plants inoculated with *Rlv* and *A. brasilense* wild type and *acdS*$^+$ mutant were significantly ($P \leq 0.05$) lower than those of the other co-inoculation treatments or inoculation with *Rlv* alone (Fig. 3). In contrast, inoculation treatments with the *ipdC*$^-$ and *napA*$^-$ mutants did not lead to significant decreases in indole content in the growth solution relative to inoculation with *Rlv* alone and non-inoculated controls (Fig. 3).

Following the results of those hydroponic experiments, we hypothesized that increased production of auxin by the wild type and the *acdS*$^+$ mutant of *A. brasilense* relative to the other mutants could lead to an increase in auxin absorption and possibly enhanced auxin metabolism by the plants. To further test this hypothesis, we performed additional experiments, in which plants were inoculated with *Rlv* alone or with

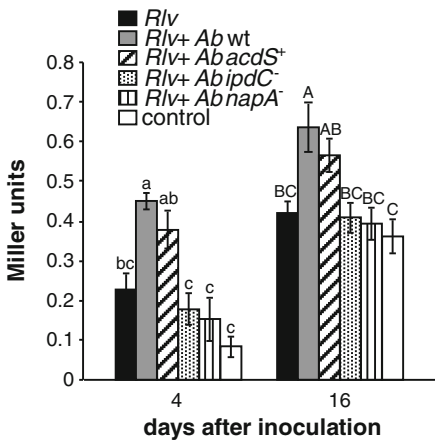

Fig. 2 Effect of inoculation of vetch with *Rlv* and/or different *A. brasilense* strains on *nod* gene activation by concentrated hydroponic growth solutions. *nod* gene expression values are expressed in Miller units, and were measured 4 and 16 d.a.i. *A. brasilense* strains: *Ab* wt, wild type Sp7; *Ab acdS*$^+$, expressing ACC deaminase; *ipdC*$^-$, indole-3-pyruvate decarboxylase mutant; *Ab napA*$^-$, periplasmic nitrate reductase mutant. Results represent averages and standard errors from 9 hydroponic boxes per treatment, each box containing 12 plants. Different letters represent significant differences ($P \leq 0.05$) between treatments for each time post inoculation. Data from one experiment of two with similar results are shown

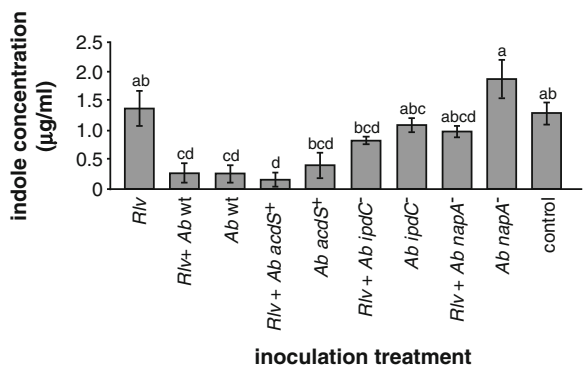

Fig. 3 Effect of inoculation of vetch with *Rhizobium leguminosarum* bv. *viciae* (*Rlv*) and/or different *Azospirillum brasilense* strains on the concentration of indole compounds in the hydroponic growth solutions. Indole concentrations were measured 7 d.a.i. *A. brasilense* strains: *Ab* wt, wild type Sp7; *Ab acdS*$^+$, expressing ACC deaminase; *ipdC*$^-$, indole-3-pyruvate decarboxylase mutant; *Ab napA*$^-$, periplasmic nitrate reductase mutant. Results represent averages and standard errors from 9 hydroponic boxes per treatment, each box containing 12 plants. Different letters represent significant differences ($P \leq 0.05$) between treatments. Data from one experiment of two with similar results are shown

Rlv in co-inoculation with the wild type strain or the mutant impaired in auxin production *ipdC*$^-$. Plants were grown for 7 or 14 days, after which indole-3-acetic acid (IAA) was added to the plant growth solutions at 200 μg mL^{-1}. Then auxin concentration in the growth solutions was assayed 12, 24 and 48 h after addition of IAA.

Clear differences between the inoculation treatments were already detected 12 h after IAA addition, when IAA was added both 7 and 14 d.a.i. In both cases, the indole concentration was significantly ($P \leq 0.05$) lower in the growth solution of plants co-inoculated with *Rlv* and the wild type strain of *A. brasilense*, relative to those of plants inoculated with *Rlv* alone or co-inoculated with *Rlv* and the *ipdC*$^-$. The differences in auxin concentration between the inoculation treatments became more accentuated 24 and 48 h after IAA addition (shown in Fig. 4 for addition of IAA 14 d.a.i.). In both cases (addition of IAA after 7 and 14 days), no significant differences were observed between inoculation with *Rlv* and co-inoculation with *Rlv* and the *ipdC*$^-$ mutant (Fig. 4). This experiment was repeated again, with similar results being obtained. In this second experiment we also assessed *A. brasilense* strains *acdS*$^+$ and *napA*$^-$. While the former behaved as similar as the wild type strain of *A. brasilense*, the latter behaved as similar as the *ipdC*$^-$ mutant (not shown).

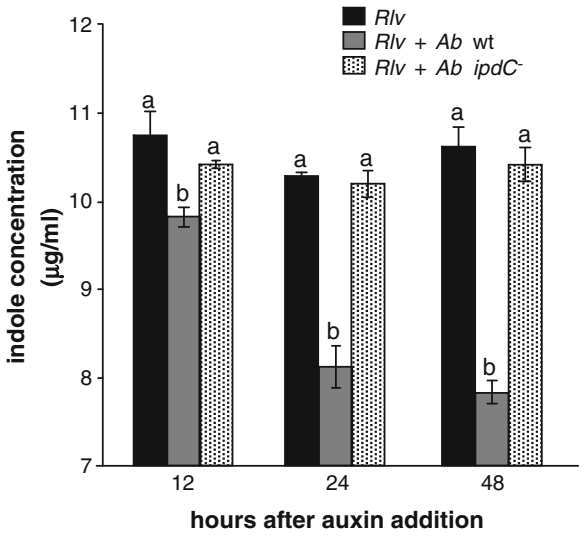

Fig. 4 Effect of inoculation of vetch with *Rhizobium leguminosarum* bv. *viciae* (*Rlv*) and/or *Azospirillum brasilense* wt or *ipdC*$^-$ mutant on the concentration of indole compounds in the hydroponic growth solutions. In this experiment, IAA was added at 200 μg/ml, 14 days after inoculation, and the indole concentration was determined 12, 24 and 48 h after IAA addition. Results represent averages and standard errors from 6 hydroponic boxes per each treatment, each box containing 12 plants. Different letters represent significant differences ($P \leq 0.05$) between treatments for each time post IAA addition. Data from one experiment of two with similar results are shown

Discussion

Here we demonstrate that inoculation of *Vicia sativa* subsp. *nigra* (vetch) with the wild type (Sp7) or the *acdS*$^+$ strain of *Azospirillum brasilense* benefit the plant by enhancing root hair formation, expression of nodulation genes and nodule formation by *Rhizobium leguminosarum* bv. *viciae* (*Rlv*), as well as plant growth. These findings are in agreement with previous reports on plant growth promotion effects of co-inoculation with rhizobia and *A. brasilense* in other legume plants (Burdman et al. 1998; Hamaoui et al. 2001; Dobbelaere and Okon 2007; Dardanelli et al. 2008; Cassan et al. 2009).

Earlier studies on alfalfa inoculated with *Sinorhizobium meliloti* (Volpin et al. 1996), on common bean inoculated with *Rhizobium tropici* (Burdman et al. 1996), and more recently, in common bean inoculated with both *R. tropici* or *R. etli* (Dardanelli et al. 2008), showed that inoculation with *A. brasilense* significantly enhances the production of various species of flavonoids. The enhanced production and secretion of root flavonoids is likely one of the mechanisms responsible for the increased nodulation observed following co-inoculation with *Rhizobium*, relative to inoculation with *Rhizobium* alone, as observed in this and other studies with legumes.

Here we compared the performance of *A. brasilense* Sp7 with some mutants affected in genes that form part of pathways that are believed to be important for the plant-*A. brasilense* interactions. One of the tested mutants was impaired in *ipdC* (indole pyruvate decarboxylase). This mutant was shown to produce only 10% of IAA relative to its parental strain, Sp245 (Costacurta et al. 1994). This mutant also possesses a reduced effect on nodulation and nitrogen fixation in co-inoculated common beans, in comparison with wild type Sp245 (Remans et al. 2008). Here, the *ipdC*$^-$ mutant did not differ significantly from the wild type strain for most growth parameters and nodulation in vetch grown in pouches. Nevertheless, and in agreement with the results

observed in common bean, we show that the *ipdC*$^-$ mutant is affected in induction of secretion of *nod* gene inducers by roots as well as root hair formation in vetch grown in hydroponics and pots, respectively. Moreover, the indole (mainly auxin) content in the hydroponic solution of vetch plants inoculated with this mutant was reduced at a significantly lower rate than that of plants inoculated with the wild type and the *acdS*$^+$ strains of *A. brasilense*.

Our findings with the *ipdC*$^-$ mutant strengthen the important role of auxin production by *A. brasilense* in the interaction between this bacterium and legume plants (Spaepen et al. 2007). In the past, roots of *A. brasilense*-inoculated maize seedlings were found to have higher amounts of free and bound IAA, as compared with non-inoculated plants (Fallik et al. 1989). Further studies should be conducted to elucidate the mechanisms by which auxin production by *A. brasilense* affects the symbiosis between legumes and rhizobia.

We also assessed the performance of an *A. brasilense* strain expressing the *acdS* (ACC deaminase) gene from *Enterobacter cloacae* (Holguin and Glick 2001; Glick et al. 2007). This strain did not significantly differ from wild type Sp7 in most tested parameters, including vetch growth parameters and nodulation in pouches, and *nod* gene induction ability and reduction in auxin content in the growth solution in hydroponics. The only clear difference observed between the *acdS*$^+$ strain and Sp7 was that on young root segments, root hairs of root tips inoculated with the former were longer than those of plants inoculated with the latter. It has been demonstrated that the production of ACC deaminase in the rhizosphere by various PGPR diminishes ethylene production, thus enhancing root elongation and plant growth (Holguin and Glick 2001). Under the experimental conditions of this work, such effect on vetch was less clear, with the exception mentioned above for root hair formation and elongation.

The other strain assessed in this study was a *napA*$^-$ mutant, impaired in periplasmic nitrate reductase, and thus being unable to produce nitric oxide (NO) (Molina-Favero et al. 2008). This mutant was shown to possess a reduced ability to induce root hair formation and nodulation by *Rlv* in vetch roots relative to wild type Sp7, in pots and pouches, respectively. In agreement with these results, in the hydroponic system, vetch roots inoculated with this mutant secreted less *nod* gene inducers than roots inoculated with strain Sp7, and the indole content of the growth solution of *napA*$^-$-inoculated plants was reduced at a lower rate than those of Sp7-inoculated plants.

It has been reported that NO produced by *A. brasilense* (Steendhoudt et al. 2001) is involved in the formation of lateral roots in tomato (Creus et al. 2005; Molina-Favero et al. 2008). NO is involved in several metabolic processes, defense mechanisms, development and signal transduction in the plant (Molina-Favero et al. 2008). NO was shown to participate in signal transduction for auxin synthesis, which affects adventitious roots formation in cucumber and lateral roots in tomato (Correa-Aragunde et al. 2006; Pagnussat et al. 2003). The involvement of NO has been recently studied in the *Sinorhizobium meliloti*-alfalfa interaction, where about 100 bacterial genes whose expression is upregulated in the presence of NO were identified. Interestingly, most of the identified genes are regulated by the two-component system FixLJ, which is known to control the majority of rhizobial genes expressed in mature nodules (Meilhoc et al. 2010). Results from our study strongly support that NO produced by *A. brasilense* is a critical compound affecting a successful symbiosis between legumes and rhizobia, possibly also by mediating auxin metabolism.

Last, it is important to refer to the fact that the mutants assessed in this study were generated in the background of wild type strains Cd and Sp245, while the wild type strain used here was Sp7. Although it would be more convenient to use mutants in the background of the same wild type strain, the comparisons from this study are valid since it has been shown many times that any of the wild type strains lead to similar positive effects on root morphology and plant growth of diverse plants when applied at optimal inoculum concentrations (Burdman et al. 1998; Dobbelaere et al. 1999; Dardanelli et al. 2008). This work also provides new insights into the performance of *A. brasilense* Sp7 in comparison with some mutants affected in genes that are important for the plant-*A. brasilense* interaction.

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