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Effect of *Azospirillum brasilense* coinoculated with *Rhizobium* on *Phaseolus vulgaris* flavonoids and Nod factor production under salt stress

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ABSTRACT

The effects of salt upon *Azospirillum brasilense* strain Cd on plant growth, nodulation, flavonoid and lipochitooligosaccharide (LCOs-Nod factor) production, were sequentially followed after 4, 7 and 14 days during a *Rhizobium-Phaseolus vulgaris* cv. Negro Jamapa interaction, in a hydroponics growth system. *Azospirillum brasilense* promoted root branching in bean seedling roots and increased secretion of *nod*-gene-inducing flavonoid species, as detected by high-performance liquid chromatography (HPLC). The results also support that *A. brasilense* allows a longer, more persistent exudation of flavonoids by bean roots. A general positive effect of *Azospirillum-Rhizobium* coinoculation on the expression of *nod*-genes by *Rhizobium tropici* CIAT899 and *Rhizobium etli* ISP42, and on nodulation factor patterns, was observed in the presence of root exudates. The negative effects obtained under salt stress on *nod*-gene expression and on Nod factors' appearance were relieved in coinoculated plants.

1. Introduction

Bacteria of the genus *Rhizobium* interact with leguminous plants in a host-specific manner and form N_2-fixing root nodules (Long, 1989). Successful establishment of the symbiotic interaction involves chemotaxis of the bacteria towards the roots, root colonization, root hair deformation, infection thread formation, and rapid division of root cortex cells (Long, 1989; van Rhijn and Vanderleyden, 1995; Spaink et al., 1998). Early events of nodule formation require expression of bacterial nodulation genes, including *nodABC*, which are induced by plant flavonoids, a large group of structurally related compounds with a chromane-type skeleton, with a phenyl substituent in the C2 or C3 position (de Rijke et al., 2006).

Rhizobia synthesize populations of lipochitooligosaccharides (LCOs) also called Nod factors that consist of between two and approximately 60 different individual structures (D'Haeze and Holsters, 2002).

Major Nod factor-triggered responses in root hairs include changes in ion fluxes and associated depolarization of the plasma membrane, intra- and extracellular alkalinization, calcium spiking, phosphatidic acid and diacylglycerol formation, accumulation of reactive oxygen species, root hair deformation involving changes in the actin cytoskeleton, and induction of early nodulin gene expression (Radutoiu et al., 2003; Riely et al., 2004; Mulder et al., 2006; Cooper, 2007). Nod factors allow rhizobia to enter the root and cortical cells, and induce nodulin gene expression and cell division, leading to nodule primordium formation (Radutoiu et al., 2003; Riely et al., 2004; Mulder et al., 2006; Cooper, 2007).

Common bean (*Phaseolus vulgaris* L.) is widely cultivated in the Central and South America and in Africa. However, bean yields are still very low (approximated average of 0.5 ton per ha), primarily due to poor cropping practices, such as an inefficient supply of nitrogen fertilizers (Hungría et al., 2000). This lack of efficiency can

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be attributed to poor nodulation and to the high sensitivity of the symbiosis to environmental stresses, such as high temperatures (Hungría et al., 2000).

Bacteria of the genus *Azospirillum* are free-living, surface colonizing, sometimes endophytic diazotroph and plant growth promoting rhizobacteria. They are capable of increasing the yield of important crops growing in various soils and climatic regions. The reviewed data from field inoculation experiments show statistically significant increases in yield in the order of 5–30% in 60–75% of the published reports (Okon and Labandera-Gonzalez, 1994; Fuentes-Ramirez and Caballero-Mellado, 2005; Castro-Sowinski et al., 2007). 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 responsible for this effect (Dobbelaere and Okon, 2007; Spaepen et al., 2007).

Dual inoculation with *Rhizobium* and *Azospirillum* and other plant growth promoting rhizobacteria was shown to significantly increase both upper and total nodule number of several legumes, acetylene reduction activities, faster ^{15}N dilution and the total N content of mineral macro- and micronutrients as compared to inoculation with *Rhizobium* alone (Sarig et al., 1986; Burdman et al., 1998; Rodelas et al., 1996, 1999). Inoculation of common bean or alfalfa (*Medicago sativa*) with *Azospirillum brasilense* in the absence of *Rhizobium* resulted in the production of plant root exudates in 6-day-old seedlings, with an increased capacity to induce *Rhizobium nod*-gene expression as compared to exudates of the non-inoculated controls (Burdman et al., 1998). This correlated with a change in the chemical composition of the root exudates and the quality of the flavonoids of inoculated plants (Burdman et al., 1996; Volpin et al., 1996).

The presence of *Azospirillum* in the rhizosphere was reported to elicit or activate the hydrolysis of conjugated phytohormones and flavonoids in the root tissue, thus bringing about the release of compounds in their active forms (Dobbelaere and Okon, 2007).

Nodulation and nitrogen fixation are inhibited by high levels of salt, with the consequent reduction in symbiotic effectiveness (Zahran, 1999). Salt stress, besides increasing the biosynthesis of Nod factors, alters their structure, with a great number of different new biologically active Nod factors being generated (Estévez, 2007). The study of the symbiotic features of rhizobia under salt stress is of great importance, and has agricultural relevance (Nogales et al., 2002; Mhadhbi et al., 2004; Bouhmouch et al., 2005). In a greenhouse experiment inoculation of chickpeas, *A. brasilense* was shown to significantly reduce the negative effects on plant growth caused by irrigation with saline water (Hamaoui et al., 2001). In this study, inoculation of chickpeas with *A. brasilense* increased nodulation, root and shoot development under both tap and saline water irrigation.

Most *Rhizobium-Azospirillum* coinoculation studies have focused on the final effects on plant growth and nodulation parameters; in contrast, few data are available on the simultaneous effect of double inoculation and stressful conditions on the early signaling exchange between the symbiotic partners. The objective of this research was to study the effects of single or combined inoculation with *A. brasilense* (strain Cd) and bean nodulating rhizobia on nodule formation, nitrogen fixation and plant growth of the common bean (*P. vulgaris*) cv. Negro Jamapa. Experiments were carried out in a hydroponic system with or without salt stress. In addition, we assessed secretion of *nod*-gene-inducing flavonoids by the roots, *nod*-gene expression and production of Nod factors in the presence of root exudates.

2. Materials and methods

2.1. Bacterial strains, medium and culture conditions

The bacterial strains used in this work were *Azospirillum brasilense* strain Cd (ATCC 29729), *Rhizobium tropici* CIAT899 (Martínez-Romero et al., 1985) and *Rhizobium etli* ISP42 (Rodríguez-Navarro et al., 2000). All rhizobial strains were grown in minimal B^- medium (van Brussel et al., 1977). *A. brasilense* was grown in minimal medium with fructose as a carbon source (Burdman et al., 1997). For all experiments, bacteria were grown at 28 °C and 150 rpm. Bacterial growth was evaluated by measuring absorbance at 600 nm. The number of viable cells was measured as CFU by the plating technique on TY agar (Beringer, 1974).

2.2. Growth of seedlings, preparation of bean root exudates and plant assays

Phaseolus vulgaris cv. Negro Jamapa (Mexico) seeds, germination, and growth on nitrogen-free medium have been described (Albareda et al., 2006). Ten pre-germinated seeds were aseptically transferred to stainless-steel lattice placed in a glass cylinder containing 130 ml of a modified nitrogen-free Rigaud–Puppo solution 0.25× pH 6.8 (Rigaud and Puppo, 1975). Each treatment was repeated seven times. Plants were maintained in a growth chamber at 24 °C (14 h) and 20 °C (10 h) for 14 days. Bean root exudates (BRE) were collected 4, 7 and 14 days after inoculation (d.a.i.), centrifuged at 4000 × *g*, 15 min, and sterilized by membrane filtration (8 and 0.2 μm pore size cellulose nitrate filters). For confirmation of sterile conditions, a sample of BRE (100 μl) was inoculated in TY and growth was assessed after overnight incubation at 28 °C. Sterile BRE were kept at 4 °C or −20 °C. The hydroponics systems were inoculated at the time of transferring the pre-germinated seeds with *A. brasilense* at 1×10^6 CFU ml^{-1} and *R. tropici* CIAT899 or *R. etli* ISP42 at 1×10^5 CFU ml^{-1}. Treatments consisted of BRE uninoculated, BRE inoculated with *A. brasilense*, BRE inoculated with *Rhizobium* alone and BRE coinoculated with *Rhizobium* and *Azospirillum*. The above treatments were also conducted in the presence or absence of NaCl at 50 mM. Bacterial numbers in the solutions were determined by plate count dilution method. Experiments were repeated three times.

Shoot and root dry weights were measured after 24 h of drying at 80 °C until constant weight. Nitrogenase activity was assessed by the acetylene reduction assay, on the entire root system of 14-day-old nodulated roots (six replicates per treatment). Ethylene production was analyzed in a Perkin Elmer 8600 gas chromatograph equipped with a Poropak column (Buendía-Clavería et al., 1986).

2.3. Analysis of flavonoid composition of BRE

One sixth of the evaporated solid BRE were resuspended in 50 ml of water and passed through a Resprep C18 solid phase extraction cartridge (Restek Corp., Bellefonte, USA). Flavonoids were released with 5 ml elution of 50%, 80% and 100% methanol. These fractions were combined and freeze-dried. For high-performance liquid chromatography (HPLC) analysis, samples were resuspended in 1 ml 50% methanol and 100 μl aliquots were injected in HPLC-ESI-MS/MS system (Ríos et al., 2005). Chromatographic separation was performed using a Perkin Elmer Series 200 HPLC system (Wellesley, USA) coupled to an Applied Biosystems QTRAP LC/MS/MS system (Foster City, USA) consisting of a hybrid triple quadrupole linear ion trap (QqQ_{lit}) mass spectrometer equipped with an electrospray ion source. HPLC analyses were performed on a 250 × 2.1 mm Tracer Spherisorb ODS2 C18 reversed-phase column with a particle size of 5 μm (Teknokroma, Barcelona, Spain). The flow rate was 0.3 ml min^{-1}. Chromatographic separation was performed using a binary gradient consisting of: (A) water; and (B) 50:50 (v/v) acenonitrile:methanol. Both components contained 0.1% formic acid (v/v). The elution profile was: isocratic for 5 min with 5% B, linear for 15 min up to 55% B, linear for 25 min up to 100% B, and isocratic for 5 min (100% B).

Multiple reaction monitoring (MRM) experiment was applied, where the parent ions and fragments ions were monitored at Q1 and Q3, respectively. Since both precursor (usually pseudomolecular ion) and fragment ion must be indicated before carrying out the analysis, the compound to identify must be known and have been well-characterized previously before this type of experiment is undertaken.

The standard flavonoids were purchased from SDS (Toulouse, France) and studied by HPLC-ESI-MS/MS and the MS/MS spectra obtained allowed us to set up the Q1/Q3 ions to monitoring. The MRM transitions chosen for the different standards are: 161.0/133.0 for umbelliferone, 253.0/133.0 for daidzein, 253.0/119.0 for chrysin, 253.0/117.0 for 4′,7-dihydroxyflavone, 255.0/171.0 for pinocembrin, 255.0/119.0 for isoliquiritigenin, 267.0/211.0 for coumestrol, 269.0/117.0 for apigenin, 269.0/133.0 for genistein, 271.0/119.0 for naringenin, 285.0/133.0 for luteolin, 285.0/135.0 for fisetin, 301.0/164.0 for hesperetin, 301.0/125.0 for morin, 301.0/151.0 for quercetin, and 303.0/125.0 for taxifolin. For HPLC-ESI-MS/MS analyses, the mass spectrometer was set to the following optimized tune parameters: curtain gas 35 psi, ion spray voltage −4500 V, source temperature 300 °C, source gas 20 psi, declustering potential −70 V, and entrance potential −10 V. MRM transitions were performed with the following parameters: collision cell exit potential −15 V, collision energy −35 V, and a dwell time of 40 ms for each transition. The retention time can be used to indicate the presence of glycosidated flavonoids. When standard glycosidated flavonoids were studied by HPLC-ESI-MS/MS, earlier HPLC retention times than their equivalent non-glycosidated flavonoids were obtained (data not shown). However, the fragmentation patterns of both glycosidated and non-glycosidated flavonoids are identical, as the sugar moiety is lost during the ionization (Rios et al., 2005). As a result, the MRM method allowed the identification of any flavonoid glycoside by using its aglycon Q1/Q3 ions.

2.4. Determination of β-galactosidase activity

Assays of β-galactosidase activity from *R. tropici* CIAT899 or *R. etli* ISP42 harboring pMP240 (*nodA*::*lacZ* transcriptional fusion) (de Maagd et al., 1988), were carried out as described by Zaat et al. (1987) to investigate the capacity of BRE to induce expression of nodulation genes. β-Galactosidase activities (Miller, 1972) were measured at least six times, each of which was in duplicate samples. For every experiment, we used 900 μl BRE and 100 μl rhizobial suspension (O.D $1.0_{600\ nm}$, about 10^8 CFU ml^{-1}). The strains were induced for a maximum period of 72 h at 28 °C on a rotary shaker (150 rpm) and bacterial growth was followed by measuring turbidity at 600 nm. Each experiment was repeated at least three times with three replicates each time, to determinate the reproducibility of the results.

2.5. Reverse phase thin layer chromatography (RP-TLC) analysis of lipochitooligosaccharides (LCOs)

BRE from five systems-replicates, each with 10 plants, were combined for analysis of LCOs. For every experiment, rhizobial strains were grown in 900 μl BRE and 100 μl rhizobial suspension (O.D $1.0_{600\ nm}$, about 10^8 CFU ml^{-1}) or mineral B^- medium only as control with or without flavonoids. The strains were induced for a maximum period of 72 h at 28 °C on a rotary shaker (150 rpm) and bacterial growth was followed by measuring turbidity at 600 nm. Each experiment was repeated at least three times with three replicates each time, to determinate the reproducibility of the results. Nodulation factors were labeled *in vivo* and analyzed by TLC using the procedure described by Spaink et al. (1992). For the radiolabeling of lipochitooligosaccharides 1 μCi of glucosamine hydrochloride ^{14}C (specific activity 52 mCi $mmol^{-1}$, Amersham®) was used. Cultures were grown to saturation and the supernatant was extracted with 0.5 ml of water-saturated *n*-butanol. The *n*-butanol was evaporated to dryness and the resulting powder resuspended in 40 μl of *n*-butanol. Ten microliters of this solution was applied to the TLC plate (Silica gel 60 RP-$18F_{254s}$, Merck®), where Nod factors were separated using 50% acetonitrile:water (vol/vol) as the mobile phase. The nature of radioactive bands produced by *Rhizobium* has been confirmed as LCOs in previous work in our laboratory (Folch-Mallol et al., 1996). TLC plates were exposed to a BAS-MS2040 imaging plate (Fuji Photo Film Co., Ltd., Kanagawa, Japan) for 48 h. The image was read by a BAS-2500 phosphoimager (Fuji Photo Film). Spot intensity was visually estimated.

2.6. Data analysis

All the experiments were carried out in triplicate. One-way ANOVA was used to analyze the results using Statistix 7.0. When the analysis of variance showed significant treatment effect, the least significant differences test (LSD, $p < 0.05$) was applied to make comparisons between the means.

3. Results

3.1. Effects of Azospirillum brasilense on root development

Inoculation with *A. brasilense* clearly increased root branching in Negro Jamapa beans (Fig. 1). At 14 d.a.i., in the different treatments did not significantly differ in dry weight of shoots and roots, but root dry weight averages were consistently and some times significantly higher in the presence of *A. brasilense* Cd (Table 1), with or without saline stress. In this day the pH of the plant growth solutions was reduced from pH 6.80 to about 4.59 in controls, 4.78 with *A. brasilense*, 4.47 with *R. tropici* CIAT899 and 4.93 in coinoculated treatments (Table 1). Similar growth promotion effects of *A. brasilense* were observed in presence of *R. etli* (Table 1). With 50 mM NaCl, the pH of the solutions was reduced with rhizobia or coinoculation treatments but not with *A. brasilense* alone (14 days) (Table 1). Acetylene reduction activities were significantly favored in plants coinoculated with *A. brasilense* and *R. tropici* (140 ± 10 nmoles ethylene h^{-1} g root dry $weight^{-1}$) as compared with inoculation with *R. tropici* alone (40 nmoles ± 9 ethylene h^{-1} g root dry $weight^{-1}$). The number of nodules did not increase significantly. In these hydroponic systems the number of nodules formed after 14 days, in common bean plants is generally low (about 10 nodules per plant) (Table 1).

3.2. Nodulation factors produced by Rhizobium tropici CIAT899 and nod-gene induction

The effect of *A. brasilense* on the variability of the production of LCOs structures (Nod factors) extracted from the bacteria and *nod*-gene induction, were investigated. *R. tropici* CIAT899 was induced for a maximum period of 72 h with exudates from plants inoculated with *R. tropici*, *A. brasilense*, coinoculated, or not inoculated. The controls were mineral B^- medium with or without flavonoids. Reverse phase thin layer chromatography (RP-TLC) analysis with radioactive detection was carried out.

The Nod factors of *R. tropici* CIAT899 obtained from the not inoculated control showed that the gene induction capacity was diminished as time elapsed for exudates collection. At collection Day 4, the lowest intensity of spots was registered with the exudates obtained in the presence of CIAT899 (Fig. 2, Lane 5). Findings at Days 4 and 7 revealed that *nod*-gene induction capacity was dependent on the time of exudates collection, with a higher intensity of bands obtained from exudates from CIAT899 and from

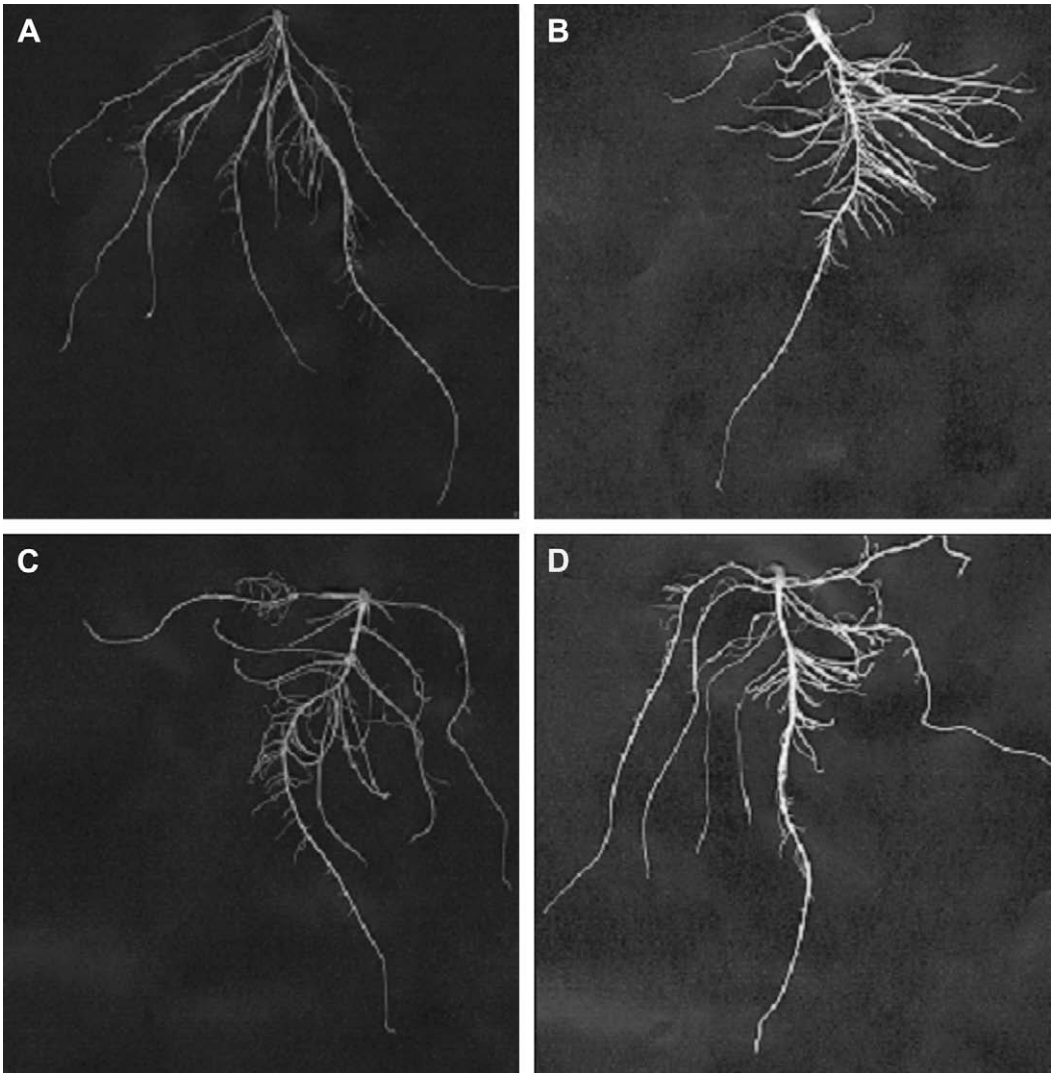

Fig. 1. Root of *Phaseolus vulgaris* cv. Negro Jamapa. **A**, Not inoculated; **B**, inoculated with *Azospirillum brasilense* Cd; **C**, inoculated with *Rhizobium tropici* CIAT899 and **D** coinoculated with *Azospirillum brasilense* Cd− *Rhizobium tropici* CIAT899. The experiment was repeated three times. Root photographs are from one representative experiment. Bar-10 cm.

coinoculated plants (Fig. 2A, Lanes 9 and 10) as compared to exudates of the uninoculated control and *Azospirillum* alone (Fig. 2A, Lanes 7 and 8). At Day 14, bands of Nod factors were obtained only from coinoculated plants (Fig. 2A, Lane 14), suggesting that the presence of *A. brasilense* is prolonging the signaling from the roots to *R. tropici* CIAT899. It can be also observed that the upper Nod factor band, possibly a sulphated LCO structure reported by Folch-Mallol et al. (1996), appeared only when *R. tropici* CIAT899 was present but not in the control or with *A. brasilense* alone (Fig. 2A, Lanes 9 and 10).

The effect of plant exudates on the expression of *nod*-genes was studied by utilizing the *nodA*::*lacZ* fusion of *R. tropici* CIAT899pMP240. The highest β-galactosidase specific activities obtained were with exudates from the control at Day 4 (Fig. 3A, column 3), correlating with the high number of flavonoids detected in this condition (Table 2). In the same manner that the intensity of bands diminished with time (Fig. 2A, Lanes 3, 7 and 11), β-galactosidase activity (*nod*-gene induction) diminished at Days 7 and 14 (Fig. 3A, columns 7 and 11). At Days 7 and 14 significant differences in reporter gene induction were obtained with coinoculated exudates (Fig. 3A, columns 10 and 14), thus corresponding to the production of Nod factors in the same conditions (Fig. 2A, Lanes 10 and 14).

Similar experiments were carried out with exudates of plants growing in 50 mM NaCl, thus simulating salt stress. The highest capacity of Nod factor production and *nod*-gene induction for *R. tropici* CIAT899 was again at Day 4 (Fig. 2B, Lanes 3–6). At 7 days the highest band intensity in the chromatographic profile was obtained when *A. brasilense* was present alone or coinoculated with CIAT899, suggesting that *A. brasilense* causes an improvement on the production of nodulation inducing molecules (Fig. 2B, Lanes 8 and 10). At Day 14, there was no detection of Nod factor bands (Fig. 2B, Lanes 11–14).

Expression of *nod*-genes as affected by exudates obtained from saline conditions was highest after 4 days, and diminished with time at Days 7 and 14 (Fig. 3B). At Day 14, the inducing activity of the BRE coinoculated with *Azospirillum brasilense* Cd− *Rhizobium tropici* CIAT899 (Fig. 3B, column 14) was higher than the obtained with others BRE ($p < 0.05$).

3.3. Nodulation factors produced by R. etli ISP42 and nod-gene induction

Further experiments were carried out to assess the effect of *Azospirillum* on the interaction between *P. vulgaris* and *R. etli* ISP42. In these experiments, the effects of inoculation with *A. brasilense*

Table 1
Growth parameters and in measurements in *Phaseolus vulgaris* cv. Negro Jamapa inoculated with *Azospirillum brasilense* Cd, *Rhizobium tropici* CIAT899

Treatment	Viability	pH days after inoculation			Nodulation (day 14)a		
	CFU/ml (day 7)	4	7	14	Shoot dry weight (mg plant^{-1} ± SD)	Root dry weight (mg plant^{-1} ± SD)	Nodule number for plant
Control							
Not inoculated	0	5.81	4.86	4.59	58.33 ± 18.82a	26.66 ± 1.52a	0
A. brasilense Cd	$<10^5$	5.90	4.88	4.78	69.66 ± 18.17a	31.66 ± 5.51a	0
R. tropici CIAT899	10^6	5.76	4.48	4.47	64.00 ± 14.00a	29.33 ± 5.86a	8 ± 3a
R. etli ISP42	10^6	5.80	4.52	4.50	63.02 ± 4.24a	25.75 ± 2.62a	9 ± 3a
A. brasilense Cd+	$<10^5$						
R. tropici CIAT899	10^6	5.93	5.37	4.93	80.00 ± 16.45ab	34.66 ± 2.88b	10 ± 1a
A. brasilense Cd+	10^5						
R. etli ISP42	10^6	5.94	4.81	4.56	81.75 ± 9.35b	35.00 ± 3.16b	10 ± 2a
50 mM NaCl							
Not inoculated	0	6.02	5.82	5.40	57.00 ± 6.16a	25.75 ± 4.03a	0
A. brasilense Cd	$<10^5$	5.92	5.75	5.40	71.02 ± 4.83b	39.75 ± 4.50b	0
R. tropici CIAT899	10^6	5.73	5.51	4.49	65.75 ± 8.86a	31.10 ± 2.81a	6 ± 2a
R. etli ISP42	$<10^6$	5.82	5.72	5.02	55.01 ± 6.21a	23.25 ± 2.50a	6 ± 1a
A. brasilense Cd+	$<10^5$						
R. tropici CIAT899	10^6	5.96	5.55	4.89	84.25 ± 12.42b	38.20 ± 3.40b	7 ± 2a
A. brasilense Cd+	$<10^5$						
R. etli ISP42	$<10^6$	5.95	5.60	5.16	99.50 ± 10.08b	39.75 ± 1.70b	7 ± 1a

Rhizobium etli ISP42 or coinoculated in presence or absence of 50 mM NaCl. Results represent the average of three experiments (seven replicates each) ± SD. Different letters represent significant differences ($p < 0.05$) between treatments at each column

and *R. etli* ISP42 (combined or alone) on plant growth promotion (Table 1) and on the production of *R. etli* Nod factors and *nod*-gene induction were studied. At Day 4, there were no differences in the induction capacity between all treatments (Fig. 4A, Lanes 3–6). Changes in the chromatographic profiles were obtained at Day 7 with the presence of radioactive LCOs in the lower part of the chromatography, indicating a non-polar nature of the molecules (Fig. 4A, Lanes 7–10). This was not observed either at Day 4 or at Day 14. In profiles obtained with *R. etli* under 50 mM NaCl, effects derived from *A. brasilense* were mainly at Day 7 (Fig. 4B, Lanes 7–10). With *A. brasilense* the bands observed were more intense (Fig. 4B, Lanes 8 and 10).

All exudates from plants inoculated or not, induced *nod*-gene transcription utilizing the *nodA*::*lacZ* fusion of *R. etli* ISP42pMP240 and showed a similar level of activity (Fig. 5A). For the non-inoculated control, the lowest induction was at Day 14 (Fig. 5A, column 11). The presence of *A. brasilense* alone (Fig. 5A, columns 4, 8 and 12) or in coinoculation (Fig. 5A, columns 6, 10 and 14) showed the highest induction capacity.

In contrast, under NaCl the induction was much lower at all collection times but the presence of *A. brasilense* resulted in a significant increase of nod-gene induction (Fig. 5B, columns 5 and 14).

3.4. Composition of nod-induced flavonoids in root exudates inoculated with A. brasilense Cd and R. tropici CIAT899

The flavonoids detected in exudates of roots of common bean grown in the presence of *R. tropici* CIAT899 and/or *A. brasilense* Cd or non-inoculated, 4, 7 and 14 d.a.i. are shown in Table 2. This particular common bean cultivar produces a wide variety of flavonoids and specially isoflavonoids. In the non-inoculated control, after 4 days, there were a high number of detected compounds, the majority of which was glycosilated (possibly with glucose or rhamnose (de Rijke et al., 2006)) (Table 2). In bean root exudates (BRE) of control plants, isoliquiritigenin, apigenin, pinocembrin, naringenin and taxifolin were present in different days. Pinocembrin, genistein, isoliquiritigenin and hesperetin were glycosilated but not taxifolin, apigenin, daidzein and naringenin.

At Day 14, the lowest amounts of flavonoids were obtained from BRE of non-inoculated plants and of plants inoculated with *Rhizobium* only. At Days 4, 7 and 14, genistein was detected in all treatments in the glycosilated form (with exception of *Rhizobium* only at Day 14). Isoliquiritigenin and naringenin were detected, with differences in glycosilation (Table 2). Interestingly isoliquiritigenin was never detected at Day 14.

Among the coinoculated plants the largest number of flavonoids was detected in exudates of Day 14, while the only one glycosilated was the isoflavone genistein. The flavonol quercetin was detected only from BRE of plants inoculated with *A. brasilense*. This flavonol was not detected in the exudates from control plants or treated with CIAT899 alone (Table 2). Naringenin was not identified in exudates of roots inoculated with CIAT899 but was present in those of roots inoculated with *A. brasilense* alone or coinoculated at 4, 7 and 14 d.a.i. (Table 2). This flavonone is likely to be bioavailable and mobile in soil (Shaw and Hooker, 2008) and is one of the main inducers of *nod*-genes in common bean rhizobial, by which can be converted to daidzein or genistein (Winkel-Shirley, 2001).

4. Discussion

In this work inoculation of *Phaseolus vulgaris* cv. Negro Jamapa with *Rhizobium tropici* strain CIAT899, or *Rhizobium etli* ISP42 and coinoculation with *Azospirillum brasilense* strain Cd clearly benefited the plant by enhancing root branching and acetylene reduction activities, thus confirming previous observations in other plants in general and legume plants in particular (reviewed by Dobbelaere and Okon, 2007). In this system we have inoculated with rhizobia and azospirilla using optimal concentrations for common beans as reported by Burdman et al. (1997). It has been reported that the root elongation rate, mineral N, P and K and microelements uptake are consequently improved after *Azospirillum* inoculation (reviewed by Dobbelaere and Okon, 2007). This could result in a general better mineral nutrition of the plant phosphorous, iron and molybdenum essential for rhizobia-nodule formation and nitrogen fixation activities (Burdman et al., 1998).

In the presence of *Azospirillum*, the pH of the growth solution was reduced from an initial pH of 6.80 to a lesser extent at Day 14 (pH 4.78), whereas in the control it was reduced to pH 4.59. A higher pH in the growth solution of beans, may favor rhizobia infection, nodule formation and fixation activity. For example, the

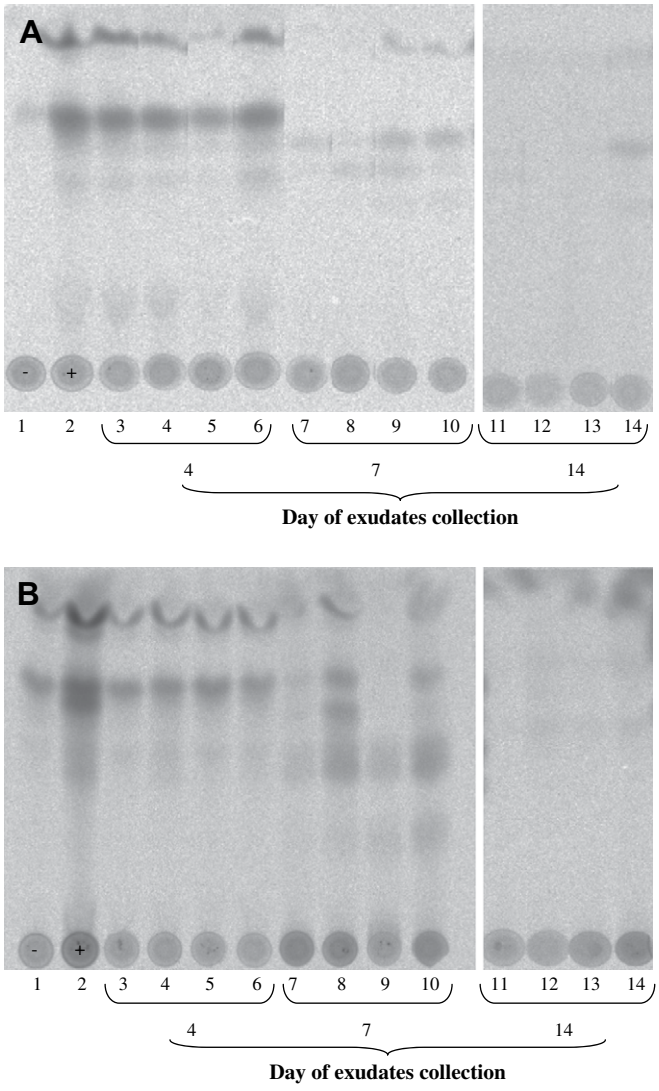

Fig. 2. RP-TLC analysis of Nod factors of *Rhizobium tropici* CIAT899 produced in the presence of ^{14}C labeled *N*-acetylglucosamine. Assay conditions: **A**, Lanes 1 and 2, mineral B^- medium, Lanes 3–14, bean root exudates (BRE). **B**, Lanes 1 and 2, mineral B^- medium NaCl 50 mM, Lanes 3–14, bean root exudates (BRE) NaCl 50 mM. Lanes 3, 7 and 11 BRE not inoculated; Lanes 4, 8 and 12 BRE inoculated with *Azospirillum brasilense* Cd; Lanes 5, 9 and 11 BRE inoculated with *Rhizobium tropici* CIAT899; Lanes 6, 10, 14 BRE coinoculated with *Azospirillum brasilense* Cd− *Rhizobium tropici* CIAT899. Lane 2 was induced by 1 μM of apigenin. The experiment was repeated three times. Results are from one representative experiment. Day of exudates collection: 4, 7 and 14.

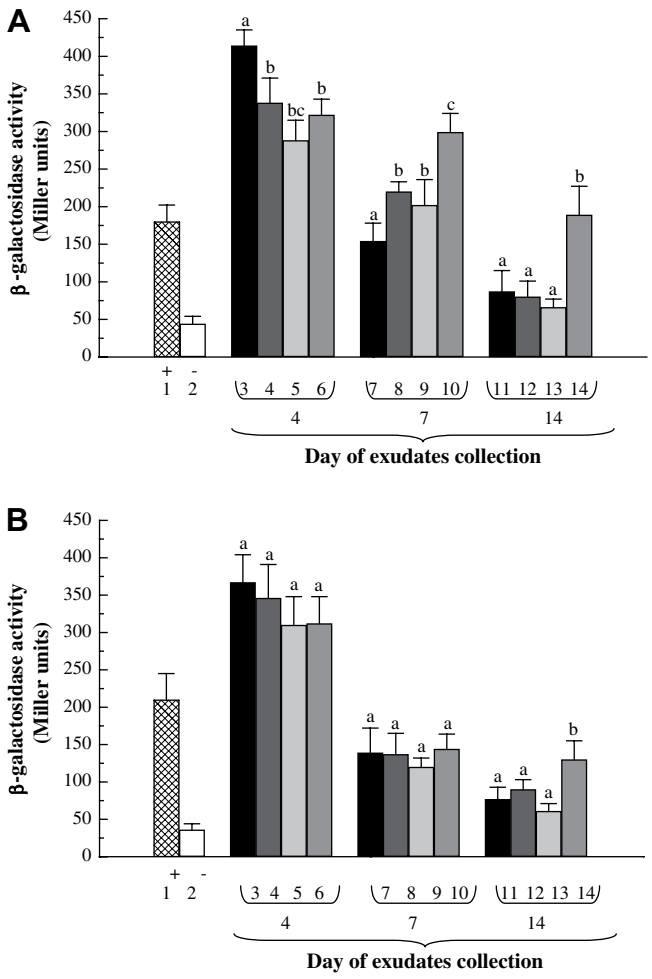

Fig. 3. β-galactosidase activity of *Rhizobium tropici* CIAT899pMP240 (*nodA R. leguminosarum*::*lacZ*). Assay conditions: **A**, columns 1 and 2, mineral B^- medium, columns 3–14, bean root exudates (BRE). **B**, columns 1 and 2, mineral B^- medium NaCl 50 mM, columns 3–14, bean root exudates (BRE) NaCl 50 mM. Columns 3, 7 and 11 BRE not inoculated; columns 4, 8 and 12 BRE inoculated with *Azospirillum brasilense* Cd; columns 5, 9 and 11 BRE inoculated with *Rhizobium tropici* CIAT899; columns 6, 10, 14 BRE coinoculated with *Azospirillum brasilense* Cd− *Rhizobium tropici* CIAT899. Column 1 was induced by 1 μM of apigenin. Bars represent ± SD from averages. Different letters represent significant differences ($p < 0.05$) between treatments at each Day (4, 7 and 14). Day of exudates collection: 4, 7 and 14.

release of *nod*-gene inducers by soybean and common bean roots was lower at pH 4.50 than at pH 5.80 (Hungría and Vargas, 2000). Differences in pH could be due to favorable root anion and proton exchanges as affected by IAA and other plant growth substances produced by *Azospirillum* (Bashan, 1991; Spaepen et al., 2007; Dobbelaere and Okon, 2007).

In agreement with previous studies by Volpin et al. (1996) on alfalfa and Burdman et al. (1996) on common bean, inoculation with *Azospirillum* favorably affected the production of more species of flavonoids in *P. vulgaris* cv. Negro Jamapa inoculated with *R. tropici* CIAT899 above those produced with *Rhizobium* alone at Day 14.

Although more flavonoids species were detected in non-inoculated controls at Day 4, the major effect of *Azospirillum* on flavonoid composition was observed at Day 14 after inoculation. Similarly, highest effect on different enzyme activities and IAA metabolism in maize roots were observed at Days 14–21 after inoculation with *Azospirillum* (Fallik et al., 1988, 1989).

In the previous study on *P. vulgaris* cv Bulgarian (Burdman et al., 1996), the effects of root flavonoids on expression of *nod*-genes were assessed by measuring of different HPLC-separated fractions derived from 6-day-old-seedlings, inoculated with *Azospirillum* only. In the present work, the effects were measured as affected by the whole BRE exudates. Additionally, coinoculation treatments of *Azospirillum* with *R. tropici* or *R. etli* were also assessed, and the effects were measured up to 14 days, when nodules were already developed. Activation of *nod*-genes by flavonoids involves subtle mechanisms (Long, 1989; van Rhijn and Vanderleyden, 1995; Spaink et al., 1998). In future studies, quantitative together with qualitative analysis of flavonoids would be helpful to better understanding the effects of the root exudates on *nod*-gene activation and other processes.

Given that flavonoid compounds are present in the rhizosphere we suggest the ability to exploit the flavonoid resource will have selective value in plant-microbe interactions (Shaw et al., 2006). Future experiments to examine the fate and impacts of flavonoids in the rhizosphere should attempt to mimic this diversity and continuity of rhizodeposition.

Table 2
Effect of the presence of *Rhizobium tropici* CIAT899 and *Azospirillum brasilense* Cd on flavonoid composition of composition of bean root exudates

Flavonoids	Root exudates											
	Experimental conditions: Control (days)			Cd (days)			CIAT899 (days)			Cd− CIAT899 (days)		
	4	7	14	4	7	14	4	7	14	4	7	14
Daidzein	+						+	+		+	+	
Pinocembrin	+g											+
Isoliquiritigenin	+g	+g		+g	+g		+g	+g		+g	+g	
Apigenin	+	+			+	+						+
Genistein	+g	+g	+g	+g	+g	+g	+g	+g		+g	+g	+g
Naringenin		+		+	+	+g				+	+	+
Hesperetin	+g			+		+						+
Morin							+g					+
Quercetin						+g					+g	+
Taxifolin		+					+					+
Total number	6	5	1	4	4	5	5	3	0	4	5	8
Glycosilated(g)	4	2	1	2	2	3	3	2	0	2	3	1

Experiments were repeated twice.

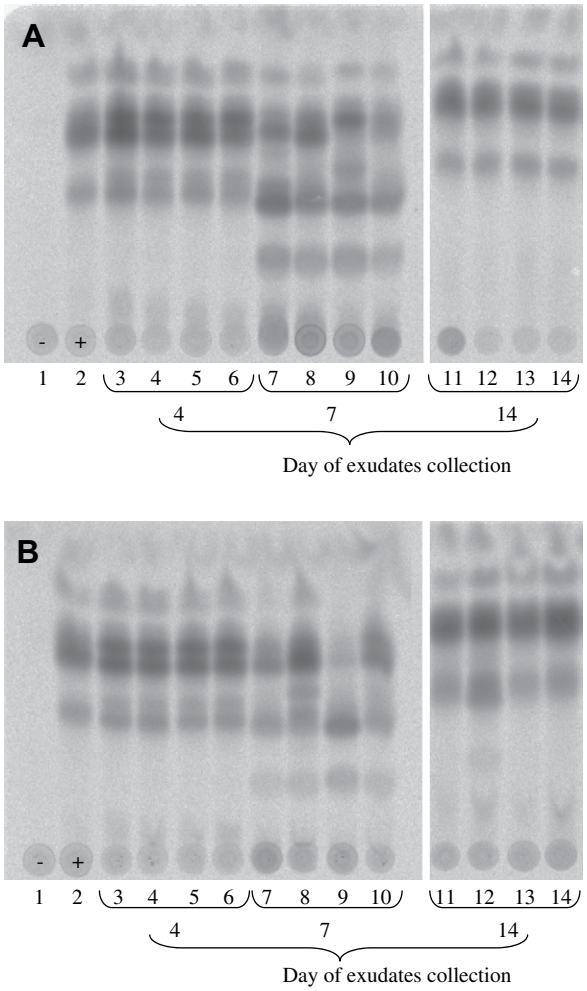

Fig. 4. RP-TLC analysis of Nod factors of *Rhizobium etli* ISP42 produced in the presence of ^{14}C labeled *N*-acetylglucosamine. Assay conditions: **A**, Lanes 1 and 2, mineral B^- medium, Lanes 3–14, bean root exudates (BRE). **B**, Lanes 1 and 2, mineral B^- medium NaCl 50 mM, Lanes 3–14, bean root exudates (BRE) NaCl 50 mM. Lanes 3, 7 and 11 BRE not inoculated; Lanes 4, 8 and 12 BRE inoculated with *Azospirillum brasilense* Cd; Lanes 5, 9 and 11 BRE inoculated with *Rhizobium etli* ISP42; Lanes 6, 10, 14 BRE coinoculated with *Azospirillum brasilense* Cd− *Rhizobium etli* ISP42. Lane 2 was induced by 1 μM of naringenin. The experiment was repeated three times. Results are from one representative experiment. Day of exudates collection: 4, 7 and 14.

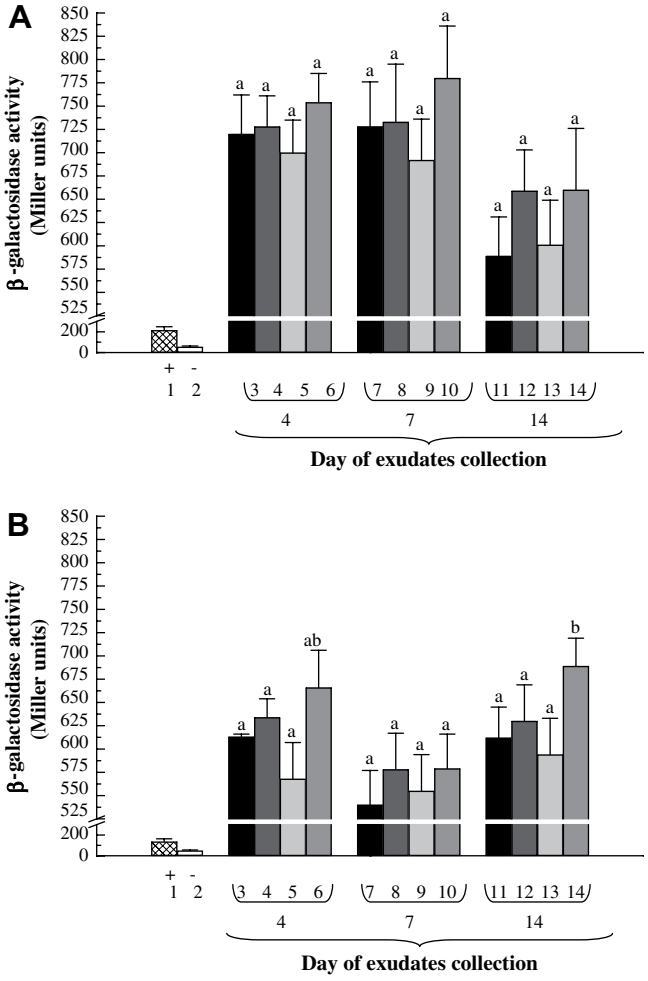

Fig. 5. β-galactosidase activity of *Rhizobium etli* ISP42pMP240 (*nodA Rhizobium leguminosarum*::*lacZ*). Assay conditions: **A**, columns 1 and 2, mineral B^- medium, columns 3–14, bean root exudates (BRE). **B**, columns 1 and 2, mineral B^- medium NaCl 50 mM, columns 3–14, bean root exudates (BRE) NaCl 50 mM. Columns 3, 7 and 11 BRE not inoculated; columns 4, 8 and 12 BRE inoculated with *Azospirillum brasilense* Cd; columns 5, 9 and 11 BRE inoculated with *Rhizobium etli* ISP42; columns 6, 10, 14 BRE coinoculated with *Azospirillum brasilense* Cd− *Rhizobium etli* ISP42. Column 1 was induced by 1 μM of naringenin. Results represent the average of three experiments. Bars represent ± SD from averages. Different letters represent significant differences ($p < 0.05$) between treatments at each Day (4, 7 and 14). Day of exudates collection: 4, 7 and 14.

An indole pyruvate decarboxylase (*ipdC*−) mutant of *A. brasilense* strain Sp245, producing only 10% IAA as compared to the wild type affected much less nodulation and nitrogen fixation in coinoculated common beans (J. Vanderleyden, personal communication), thus the positive effect on flavonoids production could be likely attributed to IAA and other plant growth substances produced by *Azospirillum* in the rhizosphere (Spaepen et al., 2007). Future studies of inoculation with *A. brasilense* should include an *ipdC*− mutant, to help elucidating the possible involvement of IAA in the process. In contrast, in legumes inoculated or coinoculated with *Pseudomonas putida* strain PML2 (Shaw et al., 2006) or *Chryseobacterium balustinum* strain AUR9 (M. Dardanelli, unpublished) the bacteria chemically converted and also utilized flavonoids as carbon sources. In the case of *Azospirillum* it is not apparent that the flavonoids were utilized or modified by the bacterium.

Under the conditions tested, plant exudates from 50 mM NaCl treatments inhibited the induction of *nod*-genes. Nevertheless exudates (flavonoids) produced in the presence of *Azospirillum* were capable of significantly induce *nod*-genes transcription by

R. tropici and *R. etli* in the presence or absence of NaCl. In both cases the effect was highest by exudates of coinoculated plants of Days 7 and 14 after inoculation. The results obtained indicate a positive, additive effect of both bacterial species in the initial nodulation processes and a clear relief of the negative effects of NaCl (see lower *nod*-genes transcription as expressed by lower Miller units compared to induction without NaCl in Figs. 3 and 5).

When comparing between the *P. vulgaris–R. etli* and the *P. vulgaris–R. tropici* systems, *R. etli* clearly produced higher amounts of Nod factors than *R. tropici* at all times assessed (Figs. 2 and 4). In agreement with these results, *nod*-gene expression by the *lacZ* expressing strains of each system, showed a stronger induction of *R. etli* than *R. tropici* (Figs. 3 and 5). These results strengthen previous observations suggesting that *R. etli* is more responsive than *R. tropici* to flavonoids present in root exudates of common bean (Dardanelli et al., 2008).

The effect of *Azospirillum* on nodulation factor patterns correlates with the number of flavonoids species (as detected in the *Azospirillum-R. tropici* combinations) produced mainly at Days 7 and 14. This observation was more evident in the presence of saline stress. For example at Day 7, in the presence of 50 mM NaCl, the intensity and number of bands (Nod factor species) was maintained with *Azospirillum* or coinoculated with *Rhizobium*, but not with *Rhizobium* alone (Figs. 2B and 4B, Lanes 8 and 10).

It is possible that the enhanced root branching and the improved root metabolism caused by *Azospirillum* as observed in chickpeas (Hamaoui et al., 2001), are important factors in relieving the stress caused by salinity, and positively affected induction of nodulation genes in *Rhizobium-P. vulgaris* interaction as observed in this work. These results are consistent with a better water status in *Azospirillum* inoculated wheat, maize and sorghum seedlings under water, osmotic and salt stress (Sarig et al., 1988; Alvarez et al., 1996; Creus et al., 1998).

The findings on relief of salinity stress in common bean investigated in hydroponic systems could be potentially relevant for bean and other legumes cultivation in many world regions since, in many cases, high salinity water is the only available source for supplementing the water needs by irrigation.

In conclusion, in the experimental system utilized in this work, the positive effect of *Azospirillum* coinoculation with *Rhizobium* was observed at the level of root development, nitrogen fixation, production of more flavonoid signals, *nod*-gene transcription, Nod factor patterns as detected by RP-TLC and relief of negative effects caused by NaCl on the above parameters. The results also suggest that *Azospirillum* allows a longer, more persistent exudation of flavonoids by bean roots. The positive trends obtained with *P. vulgaris* cv. Negro Jamapa are in agreement with the positive effects reported for many crops of agricultural interest (Dobbelaere and Okon, 2007) after *Azospirillum* inoculation.

Acknowledgments

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