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Symbiotic performance of common bean and soybean co-inoculated with rhizobia and *Chryseobacterium balustinum* Aur9 under moderate saline conditions

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Abstract

The effect of co-inoculating beans and soybeans with rhizobia and *Chryseobacterium*, a plant growth promoting bacteria (PGPR), was studied under conditions of mild saline stress. *Chryseobacterium balustinum* Aur9 was used with *Rhizobium tropici* CIAT899 or *R. etli* ISP42 to inoculate common bean (*Phaseolus vulgaris* L.), or jointly with *Ensifer* (*Sinorhizobium*) *fredii* SMH12 and HH103 to inoculate soybean (*Glycine max* (L.) Merrill). The effect of co-inoculation was studied by following nodule primordia initiation, nodulation kinetics and symbiotic performance in plants grown under moderate saline conditions (25 mM NaCl). In common bean, co-inoculation improved nodule primordia formation when compared with single inoculation (*R. tropici* CIAT899). However, co-inoculation did not provide benefits in the development of nodule primordia in soybean with *E. fredii* SMH12. The kinetic of nodulation in bean was also favored by double inocula resulting in a higher number of nodules. Long-term effects of co-inoculation on beans and soybeans depended on the rhizobial species used. In both, control and saline conditions, co-inoculation of *R. tropici* CIAT899 and *C. balustinum* Aur9 improved bean growth when compared with the single inoculation (CIAT899). However, the positive effect of double inocula on plant growth did not occur when using *R. etli* ISP42. Soybean plants receiving double inoculation (*E. fredii* SMH12 and *C. balustinum* Aur9) showed better symbiotic performance, mostly under saline stress, than with a single inoculation. The results indicate that co-inoculation with *C. balustinum* and rhizobia under mild saline conditions partially relieves the salt-stress effects, although do not always result advantageous for symbiotic N_2 fixation in legume plants.

Keywords: *Rhizobium*, *Ensifer*, PGPR, *Chryseobacterium balustinum, Phaseolus vulgaris*, *Glycine max*, saline stress

1. Introduction

Salinity is a major threat to agriculture mostly in arid and semi-arid climates where soluble salts in the soil or the irrigation water inhibit plant metabolism and growth (Munns, 2002). Salinity may affect soil microbial populations (Polonenko et al., 1986; Sardinha et al., 2003; Yuan et al., 2007) but in the particular case of the association legume-rhizobia, it affects more the plant and the symbiosis than the bacteria (Singleton, 1983; Zhou et al., 1992; Bordeleau and Prévost, 1994). Root hair development and the initial process of nodulation are affected under salinity (Tu, 1981; Singleton and Bohlool, 1984), and in the long term decreases plant biomass, nodule number and mass and nitrogen fixing capacity (Soussi et al., 1998; Elsheikh and Wood, 1990; Tejera et al., 2005; Khadri et al., 2006).

The plant rhizosphere is an important soil ecological environment for plant-microbe interactions. The colonization by a variety of microorganisms in and around the roots may have important effects on plant growth and development (Glick, 1995; Vessey, 2003; Welbaum et al., 2004). In fact, the use of rhizobial inoculants for legume crops is a well-known agricultural practice that has been successfully applied all over the world (Catroux et al., 2001).

However, there are environmental constrains, as salinity, that might preclude their use or limit the expected beneficial results of the rhizobial symbiosis on plant

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growth, as nodulation and nitrogen fixation processes could be impaired or absent (Zahran, 1999; Gentili and Jumpponen, 2006).

A variety of soil bacteria known as plant growth-promoting rhizobacteria (PGPR) may stimulate plant growth and development under stress conditions (Ashraf et al., 2004; Mayak et al., 2004 a,b; Barassi et al., 2006; Yue et al., 2007). This property of PGPR has prompted the use of double or mixed inoculants to overcome environmental limitations on nitrogen fixation and improved crop production (Dashti et al., 1998; O´Hara et al., 2002; Bai et al., 2003; Zaidi et al., 2003; Rudresha et al., 2005).

The aim of this research was to study the effect of co-inoculation of specific rhizobia and a PGPR (*C. balustinum* strain Aur9) on nodulation and symbiotic performance of bean and soybean under moderate saline conditions. To our knowledge this is one of few reports studying the effect of double-inocula on nodule primordia initiation and on nodulation kinetics of plants grown under salt stress.

2. Material and Methods

Bacterial strains and plant materials

Rhizobial strains used in this work were *R. tropici* CIAT899, a worldwide used bean-nodulating strain (Martínez-Romero et al., 1991), *Rhizobium etli* ISP42 isolated from nodules of *Phaseolus vulgaris* (Rodríguez-Navarro et al., 2000), and *Ensifer* (*Sinorhizobium*) *fredii* SMH12 and *E. fredii* HH103 selected for soybean and tested in our laboratory (Rodríguez-Navarro et al., 2003). The PGPR used for co-inoculation was *Chryseobacterium balustinum* Aur9 from the Spanish Culture Collection (CECT5399 Family Flavobacteriaceae), a Gram-negative rod isolated from the rhizosphere of *Lupinus albus* that has proved PGPR activity in several plants species (Gutierrez Mañero et al., 2003; Lucas García et al., 2003, 2004c).

Two different cultivars of common bean (*Phaseolus vulgaris*) were used: Negro Jamapa (for nodule primordia assay) and Bush Blue Lake (BBL) (for nodulation kinetic, greenhouse experiments). This later cultivar, of greater commercial importance, was adopted with the aim of testing the potential use of co-inoculation agronomically. Soybean (*Glycine max*) cv. Osumi was used for both, nodule primordia and greenhouse experiments.

Bacterial cultures

Rhizobial strains were routinely grown in yeast extract-mannitol (YMB) medium (Vincent, 1970) or B^- medium (van Brussel et al., 1977), *Chryseobacterium balustinum* strain Aur9 was grown in TY medium (Beringer, 1974). Bacteria were stored in 10% glycerol at -70ºC, and working cultures were maintained in slants at 4ºC.

Development of nodule primordia assays

These biological tests were performed according to López-Lara et al. (1995). Seeds were surface-sterilized with pure sodium hypochlorite (5%) and germinated in an incubator at 28ºC. Pre-germinated seeds with root length ranging 1.5–2.5 cm were nested on curled wires kept in test tubes (3 × 20 cm). The tubes with 25 ml of N-free plant nutrient solution (Fåhraeus, 1957) were set on special racks to keep roots in darkness. Two saline treatments were set: control (0 mM NaCl) and a mild saline stress (25 mM NaCl). Plants were grown in environmental growth chamber (day/night temperature: 28/20º C; 16 h light/8h dark period; relative humidity 40/60%) during 10 days (beans) and 14 days (soybean).

Previous to rhizobial inoculation, 20 µl of *C. balustinum* Aur9 (10^8 CFU/ml) per plant were added. The pre-incubation with *C. balustinum* lasted 15 h in bean or 48 h in soybean. Plants were then inoculated with rhizobia at 1 cm below the zone of emerging root hairs using 20 µl of bacterial cultures (10^8 CFU/ml) of *Rhizobium tropici* CIAT899 (bean) or *Ensifer* (*Sinorhizobium*) *fredii* SMH12 (soybean). Each assay was repeated at least three times and three replicates were set up for each treatment. At the end of the growth period, roots were detached, cleared with 5% sodium hypochlorite during 20 min, copiously washed with distilled water and stained with methylene blue (1%) to visualize primordia, nodule appearance and size by optical microscopy (Truchet et al., 1989).

Nodulation kinetic assay

Seeds of common bean cv. Bush Blue Lake (BBL) were surface-sterilized and inoculated at sowing time with 1 ml *C. balustinum* culture 1 day-old (10^5–10^6 CFU/ml). Fifteen hours after inoculation, seeds were re-inoculated with 1 ml (10^8–10^9 CFU/ml) of *R. tropici* CIAT899. The control consisted in single inoculation (rhizobia) at sowing time. Seedlings were grown in glass vessels (ca. 300 cc) filled up with sterile quartz-sand, and watered with diluted (1/2) N-free nutrient solution (Rigaud and Puppo, 1975). Three replicates were set up (two seeds per replicate) for each treatment/sampling time. Salt treatments were carried out in parallel, inoculated as described above, but the nutrient solution was supplemented with NaCl (25 mM). Salt-watering started one week after bacterial inoculation. All plants were watered every two days. Sampling time started 5 days after inoculation, and continued every two days up to 4 weeks (a total of 6 samplings were done). At each sampling time, three replicates per treatment were removed and the number of nodules counted.

Plant growth promotion experiments

All experiments were carried out under controlled conditions (25ºC/18ºC day/night, and 88% RH). Common bean cv. BBL and soybean cv. Osumi were grown in plastic pots (ca. 1.5 l), filled up with sterile quartz-sand and watered with diluted (1/2) Rigaud and Puppo N-free nutrient solution. Plant tests aimed at determining the effect of salinity were carried out using the same nutrient solution supplemented with NaCl (25 mM). Salt-watering started one week after bacterial application. All plants were watered with nutrient solutions every other day.

Surface sterilized and pre-germinated seeds of both legumes were transferred aseptically to pots (three per pot) and inoculated with 1 ml (10^5–10^6 CFU/ml) of one day-old bacterial culture of *C. balustinum* Aur9. After 15 h (beans) or 48 h (soybean) of *C. balustinum* inoculation, seedlings were inoculated with 1 ml (10^8–10^9 CFU/ml) of the specific rhizobial cultures. Uninoculated plants were also included in each plant test. A week after inoculation, seedlings were thinned down to uniformity to two per pot. Pots were arranged in a complete randomized block design with four replicates per bacterial and NaCl treatment (0 or 25 mM NaCl). Plants were harvested 4 weeks after inoculation and symbiotic effectiveness was estimated by comparing the shoot dry weights (dried at 70ºC) with those of uniinoculated control plants. Nodulation was evaluated by the number and dry nodule biomass. Root mass (dry weight) was recorded once the nodules were detached and roots dried in oven (70ºC, 48h).

Statistical analyses

Statistical analysis was performed by means of the Analysis of Variance (ANOVA) following a completely randomized design. Multiple comparisons of means were done by L.S.D. method. All hypotheses were tested at the 95% confidence level. The analysis was performed using Statistix 8.0 software (NH Analytical Software, USA).

3. Results

Effects of co-inoculation on development of nodule primordia

Short-term observations (10 days) of bacterial co-inoculation on nodule initiation and development in bean cv. Negro Jamapa and soybean cv. Osumi roots are presented in Fig. 1. Nodule primordia were completely absent in either uninoculated plants or *C. balustinum* inoculated plants (data not shown). Under control conditions (0 mM NaCl), bean plants only inoculated with *R. tropici* CIAT899 (Fig. 1) displayed similar nodule primordia development as those inoculated with the double inocula (CIAT899 + Aur9). In contrast, under saline conditions (25 mM NaCl), co-inoculation resulted in a significant improvement of nodule primordia development (Fig. 1). A reduction in nodule number was recorded in plants single-inoculated with CIAT899 growing under saline conditions (data not shown).

Soybean plants did not show significant differences in nodule initiation associated with single or double inoculation. In this species, co-inoculation did not promote the formation of nodule primordia in saline or non-saline treatments (Fig. 1). The appearance of nodule primordia was unaffected by the PGPR and a similar number of nodules was recorded in saline and non-saline conditions (Fig. 1).

Nodulation kinetic assays

The observations performed on nodule primordia initiation in Negro Jamapa beans suggested that the presence of Aur9 might positively affect bean nodulation by CIAT899 in co-inoculated plants. To investigate this possibility, the nodulation kinetic was assayed in a different bean cultivar (cv BBL) using single (CIAT899) and double inocula (CIAT899 + Aur9). Five days after sowing (DAS) there were no significant differences between treatments (Fig. 2). From 7 to 15 DAS there was a significant ($p<0.05$) positive effect of Aur9 on nodule formation under control conditions (0 mM NaCl). At 22 and 28 DAS, significant differences in the number of nodules were only recorded between plant-growing conditions (saline or control conditions) not between bacterial treatments (single rhizobia or co-inoculation). Salinity significantly delayed nodule formation and number of nodules in bean almost in all samplings, except at the first sampling (5 DAS). The reduction in number of nodules associated to the presence of salt ranged from 15 to 66% in CIAT899 inoculated plants, and from 17 to 84% in co-inoculated plants.

The effect of the PGPR on nodulation kinetics of soybean was not studied because Aur9 did not produce any effect on the development of nodule primordia.

Effects of co-inoculation on nodulation and plant growth

The inoculation of beans with rhizobia and *C. balustinum* Aur9 led to a significant increase in shoot growth in comparison with uninoculated plants (data not shown). Saline conditions reduced plant growth up to 50%, and bean plants inoculated either with ISP42 or with ISP42 + Aur9 did not show significant differences in growth (Table 2). Conversely, plants inoculated with Aur9 + CIAT899 showed greater growth than single-inoculated plants (CIAT899) in both control and salinity. In saline conditions, nodulation parameters were significantly higher in plants inoculated with the rhizobia alone either with strain ISP42 or CIAT899 (Table 2). When Aur9 was present, it led to fewer nodules and smaller nodule biomass.

The effects of co-inoculation on soybean nodulation and growth are presented in Table 3. Co-inoculation of Aur9

Table 1. Bacterial strains used in this work.

Strain	Host-plant	Origin	Source of reference
Rhizobium etli ISP42	*Phaseolus vulgaris*	Spain	Rodríguez-Navarro et al. (2000)
Rhizobium tropici CIAT899	*Phaseolus vulgaris*	Mexico	Martínez-Romero et al. (1991)
Ensifer fredii HH103	*Glycine max*	China	Dowdle and Bohlool (1985)
Ensifer fredii SMH12	*Glycine max*	Vietnam	Cleyet-Marel (1987)
Chryseobacterium balustinum Aur9	*Lupinus albus*	Spain	Lucas García et al. (2004b)

Table 2. Effect of co-inoculation with specific rhizobia and *C. balustinum* Aur9 on nodulation and plant growth of bean cv. BBL grown under controlled conditions.

Treatments	0 mM NaCl				25 mM NaCl			
	SDW	NNOD	NDW	RDW	SDW	NNOD	NDW	RDW
ISP42	650 a	704 a	210 a	380 a	312 a	207 a	34.5 a	188 a
ISP42+Aur9	660 a	423 b	171 a	317 a	279 a	87 b	18.4 b	172 a
CIAT899	760 b	242 b	139 b	343 a	311 b	253 a	98.0 a	170 b
CIAT899+Aur9	1030 a	327 a	191 a	378 a	434 a	119 b	24.4 b	278 a

Data represent mean values of four replicates. Significant differences ($p<0.05$) between values within a column, for each pair of rhizobial treatments, are indicated by different letters. SDW = shoot dry weight (mg/plant), NNOD = number of nodules (per plant), NDW = nodule dry weight (mg/plant), and RDW = root dry weight (mg/plant).

Table 3. Effect of co-inoculation with specific rhizobia and *C. balustinum* Aur9 on nodulation and plant growth of soybean cv. Osumi grown under controlled conditions.

Treatments	0 mM NaCl				25 mM NaCl			
	SDW	NNOD	NDW	RDW	SDW	NNOD	NDW	RDW
HH103	472.5 a	34.3 b	27.4 b	181 b	445 a	27 b	23.3 a	172 b
HH103+Aur9	510 a	77.7 a	57.2 a	319 a	427.5 a	37.5 a	27.2 a	288 a
SMH12	507.5 b	23.5 b	38.9 b	198 b	410 b	16.5 b	15.2 b	145 b
SMH12+Aur9	792.5 a	60.0 a	98.7 a	409 a	590 a	63.7 a	62.5 a	325 a

Data represent mean values of four replicates. Significant differences ($p<0.05$) between values within a column, for each pair of rhizobial treatments, are indicated by different letters. SDW = shoot dry weight (mg/plant), NNOD = number of nodules (per plant), NDW = nodule dry weight (mg/plant), and RDW = root dry weight (mg/plant).

and rhizobial strains HH103 and SMH12 led to a significant increase in the number of nodules and root growth (Table 3). The SMH12 + Aur9 treatment significantly increased growth of soybean shoots in plants under saline or non-saline conditions. The same treatment resulted in a remarkable increase of nodulation and root growth in saline conditions.

4. Discussion

Co-inoculation of common bean with *C. balustinum* Aur9 and rhizobia increased the formation of nodule primordia when compared with treatments with rhizobia alone. The effect of double inoculation strongly depended on rhizobial species. While *R. tropici* CIAT899 used jointly with Aur9 did improve plant growth and nodulation (nodule number and weight) in control and in salt-treated plants, the use of *R. etli* ISP42 did not provide any benefit to the symbiotic association. The situation was different in soybean, in which co-inoculation did not affect primary events in nodule initiation, but it had a long-term effect resulting in the improvement of shoot and root growth, nodule number and nodule mass, at both saline and non-saline conditions.

In common bean, pre-inoculation with the PGPR *C. balustinum* Aur9 might have improved rhizobial infection at initial stages by increasing root hair formation and infection sites. Several reports have shown that co-inoculation with PGPRs promotes root hair growth and

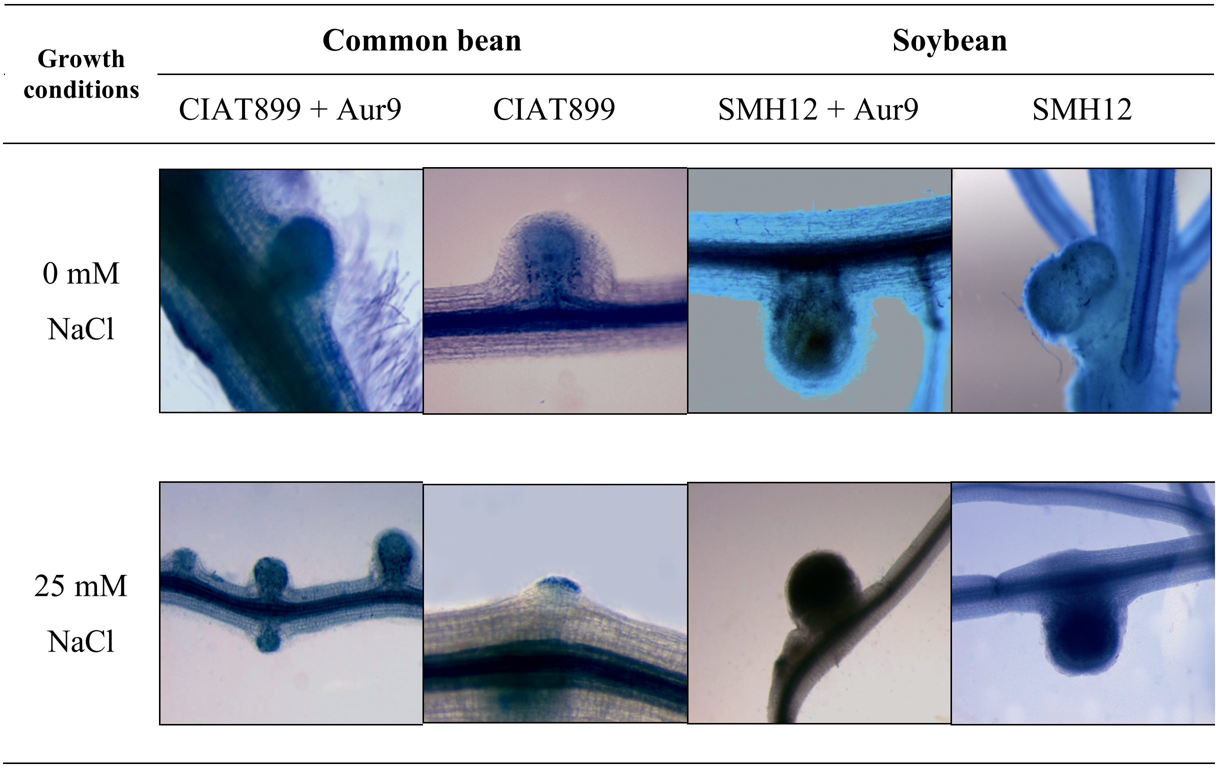

Figure 1. Nodule primordia of bean cv. Negro Jamapa plants inoculated either with *Rhizobium tropici* CIAT899 and *Chryseobacterium balustinum* Aur9 (left column) or with *R. tropici* CIAT899 (right column); and nodule primordia of soybean cv. Osumi plants inoculated either with *Ensifer fredii* SMH12 and *C. balustinum* Aur9 (left column) or with *E. fredii* SMH12 (right column). Row-1, plants grown under control conditions. Row-2, plants grown under salt conditions (25 mM NaCl). Pictures were taken 10 days after sowing.

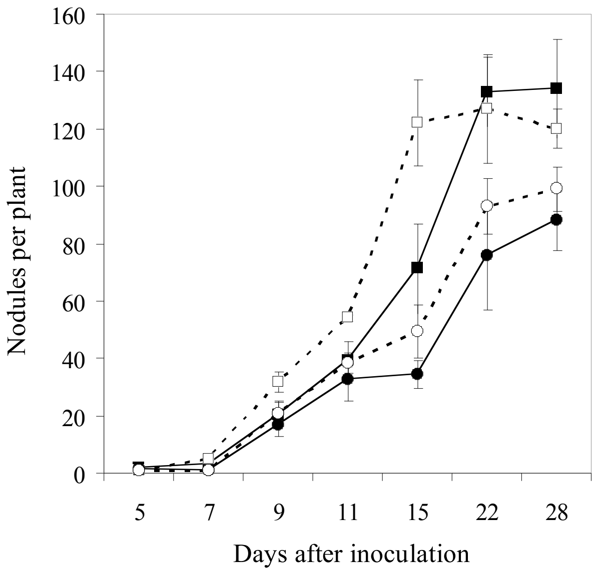

Figure 2. Nodulation kinetics of bean cv. BBL seedlings inoculated with *R. tropici* CIAT899 (black symbols) and *R. tropici* CIAT899 and *C. balustinum* Aur9 (white symbols), under control-growth condition (square) and salt-growth conditions (25 mM NaCl, circle). Data represent mean values ± standard deviation of three replicates.

enhances root flavonoid secretion (Ahmad et al., 2005; Spaepen et al., 2007; Estévez, 2007; Dardanelli et al., 2008). The promotion of infection sites favored by the PGPR may have contributed to increase the formation of nodule primordia and early nodule development. However, further development of nodules into active N_2-fixing organs mainly depends on plant growth and its capacity to export sugars into nodules and develop effective sinks for the N-rich compounds produced by N_2 fixation (Phillips, 1980; Hartwig et al., 1994). Photosynthate deprivation, nodule gas exchange and feedback regulation by N accumulation are among the main limiting factors of abiotic stress on symbiotic N_2 fixation (Zahran, 1999). Common bean and soybean show different sensitivity to salinity and are rated as sensitive and moderately tolerant, respectively (Maas, 1986). In fact, a substantial 50% growth inhibition occurred in bean at 25 mM NaCl while it just affected soybean in 5–25% (Tables 2 and 3). In the long-term, salinity-induced inhibition of shoot growth may have affected photosynthate supply to nodules and N sinks reducing nodule growth and N_2 fixing activity. The greater sensitivity of bean than soybean to 25 mM NaCl might be hampering further nodule development and N_2 fixation activity overturning the primary events induced by co-inoculation in this species.

Most scientific works dealing with co-inoculation in legumes have reported plant growth promotion and enhancement of symbiotic parameters (Dashti et al., 1998, 2000; Hamaoui et al., 2001; Kuklinsky-Sobral et al., 2004; Tilak et al., 2006). Nevertheless, other works have pointed out that co-inoculation might impair rhizobial colonization and interfere nodule formation and development (Camacho et al., 2001; Zaidi et al., 2003; Berggren et al., 2005). Recently, Albareda et al. (2006) have demonstrated that *C. balustinum* Aur9 did not affect rhizobia attachment to roots of bean and soybean. Under saline conditions, attachment of rhizobia to Aur9 pre-inoculated roots was greater than to roots without PGPR pre-treatment.

The co-inoculation pattern may also play a critical role in the outcome results. Lucas García et al. (2004a,b) have demonstrated a competition effect among several PGPRs and rhizobia in *E. fredii*-soybean and *Bradyrhizobium* sp.-lupine systems, when plants were at once co-inoculated compared with a delayed mode of inoculation, leading in the former case to no significant effects on plant growth. Thus, in this work we have followed a delayed mode of co-inoculation of PGPR and rhizobia.

In the present work it is shown that *C. balustinum* Aur9 did not interfere with *R. tropici* CIAT899 root infection and nodule initiation either under control or saline conditions. Likewise, co-inoculation partially overcame the negative effects of salinity on the number and size (data not shown) of nodules and the delay of nodule appearance.

Thus, pre-inoculation with strain Aur9 clearly increased the number of nodules by *R. tropici* CIAT899 under saline stress. It may be related to the increased secretion of root flavonoids in co-inoculated plants (Estévez, 2007) and/or to the PGPR production of IAA promoting root hair development and hence possible sites for rhizobial entry (Lucas García et al., 2004a; Ahmad et al., 2005; Spaepen et al., 2007).

Soybean seedlings did not show differences in the development of nodule primordia induced by co-inoculation either in saline or non-saline conditions. These results do not agree with a previous research performed using split-root systems where the PGPRs relieved nodule inhibition in soybean induced by salinity (Bellogín et al., 2006).

It seems rather clear that plant growth promotion in co-inoculations depends on the rhizobial species (or strains), growth conditions and/or the symbiotic associations. Remans et al. (2008) have described differences in the responsiveness to PGPR among cultivars of *P. vulgaris*, which suggest genetic variation for this trait within bean germplasm. Common bean plants cv. BBL co-inoculated with *R. etli* ISP42 and Aur9 did not show any advantage in comparison with the single inoculation with rhizobia. However, co-inoculation led to greater symbiotic performance when strain CIAT899 was used (Table 2). While strain ISP42 alone provided greater nodulation than CIAT899, nodulation by this later strain was clearly promoted by the PGPR in the absence of salt. In soybeans, plant growth was mainly dependent on the rhizobial strain. Double inoculation led to a significant increase in shoot mass only when strain SMH12 was used (Table 3). However, co-inoculation improved root growth independently from the rhizobia strain in both saline and non-saline conditions.

Some authors (Jebara et al., 2001; Remans et al*.,* 2008) have proposed that bacterial strain-plant genotype combination should be considered for selecting the most adapted microbe-plant combinations to environmental limitations like salinity. In accordance with our data, the main conclusion that may be drawn for co-inoculation with PGPRs is that each symbiotic association requires a careful preliminary assessment in order to optimise the efficiency of the system under particular environmental conditions. Co-inoculation might contribute to enhance N_2 fixation in soybean and beans in the absence of salt but requires a careful selection of appropriated partners. In moderate salinity levels, only soybean showed improvement of plant growth and symbiotic parameters when using the PGPR Aur9.

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