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A new PGPR co-inoculated with *Bradyrhizobium japonicum* enhances soybean nodulation

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ABSTRACT

A new PGPR (plant growth promoting rhizobacteria) strain was isolated from soybean seeds and the bacterial mechanisms related to plant growth promotion were evaluated and characterized. Isolates were genotypically compared and identified by amplification of partial sequences of 16S DNAr as *Bacillus amyloliquefaciens* strain LL2012. Isolates were grown until exponential growth phase to evaluate the atmospheric nitrogen fixation, enzymatic activities, phosphate solubilization, siderophores and phytohormones production. LL2012 strain was able to grow and to produce high levels of auxin, gibberellins and salicylic acid in chemically defined medium. Co-inoculation of soybean plants with LL2012 strain and the natural symbiont (*Bradyrhizobium japonicum*) altered plant growth parameters and significantly improved nodulation. Our results show that the association of LL2012 with *B. japonicum*, enhanced the capacity of the latter to colonize plant roots and increase the number of nodules, which make the co-inoculation technique attractive for use in commercial inoculant formulations following proper field evaluation.

1. Introduction

Food security is increasingly under threat in many developing countries of the tropics in which traditional agricultural systems are becoming unsustainable as a result of demographic pressures. The global population is expected to double by 2040, and much of that increase will occur in developing countries in which hunger is already a threat. Local needs to make arable land satisfy present food demands are resulting in over-exploitation that results in ever-less productivity per unit area. When soil goes into a phase of rapid decline in fertility, erosion often increases with far-reaching deleterious environmental consequences that may be impossible to reverse. In such circumstances, crop productivity may not be significantly improved by even heavy applications of synthetic fertilizers, in the rare situation where such are available to the subsistence farmer, because levels of organic matter that define fertility have reached critically low values (IAEA/FAO-TECDOC-1027 1998).

The use of beneficial microorganisms has been proven to be an environmentally sound option to increase crop yields. Plant growth-promoting bacteria (PGPR) may facilitate plant growth either indirectly or directly. There are several ways in which plant growth promoting bacteria can directly facilitate plant growth. They may: fix atmospheric nitrogen and supply it to plants, although this is usually a minor component of the benefit that the bacterium provides to the plant; synthesize siderophores which can sequester iron from the soil and provide it to plant cells as a siderophore–iron complex which can be taken up; synthesize phytohormones such as auxins, cytokinins and gibberellins which can act to enhance or regulate various stages of plant growth; solubilize minerals such as phosphorus, making them more readily available for plant growth (Glick 1995; Glick et al. 2007; Bashan and de-Bashan 2010). Bacteria may directly affect plant growth and development by using anyone or more than one of these mechanisms. Since many plant growth-promoting bacteria possess several of these traits simultaneously, different mechanisms at various times during the life cycle of the plant can be used. However, the exact modes by which plant growth-promoting bacteria (PGPR) promote plant growth at a specific step in life cycle are not fully understood. Inoculation with PGPR can be used also to promote plant biocontrol (Senthil-Kumar et al. 2009; Bhattacharyya and Jha 2012).

On the other hand, some of the nodule-forming legumes are important crop plants such as soybean, peanut, and common bean (Barea et al. 2005; Esitken et al. 2006) which sometimes have to grow in stressful environments. Identification and possible manipulation of their relationships with PGPR has been considered a basic strategy of modern agriculture in developing countries.

Grain legumes are a good source of proteins which content ranges from 17 to 42% together with significant concentrations

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of minerals (calcium, zinc, and iron) and vitamins (folic acid and vitamin B including riboflavin, thiamin and niacin).

Soybean (*Glycine max* L. (Merr.)) is a subtropical member of the Leguminosae Family, an erect bushy annual crop with considerable morphological diversity. Soybean is one of the most important oil seed crops in the world (18–22% oil) which proteins (40–42%) provide all eight essential amino acids in the amount needed for human health; hence it is the best source of protein and oil and truly claims the title of the meat/oil on plants (Ali 2010). Generally, soybean is used in the food industry for flour, oil, cookies, candy, milk, vegetable cheese, lecithin and many other products. Argentina is a competitive producer of oilseeds and has developed a world-class vegetable oil industry. Since the 1980s, this country has emerged as one of the main exporters of oilseeds and vegetable oil to the international market, at the top of the exporters ranking in soybean oil (Racca and Collino 2005).

The microsymbiont of soybean *B. japonicum* is a highly efficient N fixer forming symbiotic association with soybean. Notwithstanding, some host genotypes are superior to others in the ability to fix N_2, and, in turn, some rhizobial strains have similar superior capability. According to Unkovich and Pate (2000), the amounts of N_2-fixed (kg ha^{-1}) by soybean have been up to 450 kg N ha^{-1}. Thus soybean depends on its symbionts for a large part of its N requirements for effective growth and dry matter production. Additionally, this phosphate solubilizing bacteria (PSB) have the capacity to convert insoluble phosphates into soluble forms for plant growth. Linu et al. (2009) reported that inoculation with PSB improved nodulation, root and shoot biomass, straw and grain yield and P and N uptake by the crop.

Studies carried out by Dubey (1996) and Wasule et al. (2007) clearly revealed that co-inoculation of *Bradyrhizobium* and PGPR microorganisms significantly improved soybean growth and its yield components as compared with the sole application of *Bradyrhizobium*. Therefore considering the benefits to crop growth attributed to PGPR, co-inoculation with these microorganisms might improve plant's performance. This approach is in agreement with modern demands of agricultural, economic, social and environmental sustainability (Chaparro et al. 2012).

The aim of this study was the isolation and characterization of a new PGPR from soybean seeds and its evaluation for co-inoculation with *B. japonicum* on soybean plants. We evaluated the possibility of growth promotion and nodulation improvement in soybean through production of plant growth regulators by the bacteria.

2. Materials and methods

2.1. Bacteria isolation from soybean seeds

Seeds were randomly collected from Department of Colón, Province of Córdoba, Argentina (Campaign 2011/12). Seeds containing rhizospheric soil were washed with phosphate buffer during 15 min and macerated with mortar and pestle. One gram of macerated tissue was placed in a tube containing 9 ml sterile 0.9% NaCl. One milliliter of appropriate (10^{-1}–10^{-5}) dilution was plated on Luria Bertani (LB) medium (Döbereiner et al. 1995). Plates were incubated at 30 °C for 2 days and morphology and mobility of cultured bacteria were examined by optical microscopy. Isolated bacteria were tested for Gram coloration with a kit (Britania Laboratories, Argentina).

2.2. Determination of PGPR characteristics of the isolated bacteria

2.2.1. Phosphate solubilization

Phosphate solubilization was determined by the methods of Katznelson and Bose (1959). Plates containing trypticase soya agar medium supplemented with $Ca_5(PO_4)_3OH$ were inoculated with 1 μl LB pure bacterial culture. Plates were incubated at 30 °C and observed daily for 7 days until formation of transparent "halos" around each colony. Experiments were performed in triplicate. Positive control was made with *Pseudomonas fluorescens* strain P1 (provided by the Agriculture Collection Laboratory, IMYZA-INTA; Argentina) in similar culture conditions.

2.2.2. Siderophore production

Siderophore production was determined by the method of Schwyn and Neilands (1987); 1 μl pure bacterial culture grown in LB was inoculated in plates containing Chrome Azurol S (CAS) agar. Plates were incubated at 30 °C and observed daily for orange color formation around each colony for up to 4 days. Experiments were performed in triplicate. Positive control was made with *P. fluorescens* strain P1 (provided by the Agriculture Collection Laboratory, IMYZA-INTA; Argentina), in similar culture conditions.

2.2.3. Nitrogen fixation

Each isolated strain was inoculated in plates containing NFb medium with or without addition of NH_4Cl as a unique nitrogen source (Döbereiner et al. 1995). Plates were incubated at 28 °C for 7 days, and bacterial growth was observed as qualitative evidence of the atmospheric nitrogen fixation. As negative controls *P. fluorescens* R1 were used.

2.2.4. Protease production

Protease production was determined according to Abo-Aba et al. (2006) with modifications. Plates were inoculated with 1 μl LB pure bacterial culture in halfway points of a Petri dish containing 3% agar milk. Plates were incubated at 28 °C and daily for formation of transparent haloes around each colony was observed for up to 4 days. Experiments were performed in triplicate.

2.2.5. Cellulolytic activity

Cellulolytic activity was determined according to Karui and Kushner (1988). Isolates were inoculated with a 10 ml of basal medium liquid (K_2HPO_4 1 g; $NaNO_3$ 0.5 g; $MgSO_4$ 0.5 g; $FeSO_4$ 0.01 g; CMC 10 g; Distilled water 1000 ml) with the addition of carboxymethylcellulose (CMC) as a sole carbon source, incubated at 30 °C for 15 days; positive activity was determined by the presence of cell biomass.

2.2.6. Catalase and oxidase activity

Isolated bacteria were tested for catalase and oxidase activity with standard disks provided by Britania Laboratories (Argentina).

2.2.7. Production of phytohormones (ABA, SA, JA, GA and AIA)

Four 20 ml fractions of defined cultures of *Bacillus amyloliquefaciens* LL2012 strain were taken in exponential growth phase, and centrifuged separately at 8000 rpm, 4 °C, for 15 min. Supernatants were acidified at pH 2.5 with acetic acid solution (1%, v/v); 50 ng of deuterated hormones ($^2H_6$-ABA, $^2H_4$-SA, $^2H_2$-GA_3, $^2H_6$-JA and $^2H_5$-AIA) (OlChemIm Ltd., Olomouc, Czech Republic) were added as internal standards. Each sample was partitioned four times with the same volume of acetic acid-saturated ethyl acetate (1%, v/v). After the last partition, acidic ethyl acetate was evaporated to dryness at 36 °C. Dried samples were dissolved in 1500 μl methanol. Extracts were resuspended in 50 μl methanol (100%), and placed in vials. 10 μl of each sample were injected, and the phytohormones were determined by liquid chromatography with electrospray ionization (LC-ESI) (Waters Corp., New York, NY, USA).

2.2.8. Liquid chromatography and mass spectrometry

Analyses were performed using an Alliance 2695 (Separation Module, Waters, USA) quaternary pump equipped with

auto-sampler. A Restek C_{18} (Restek, USA) column (2.1 mm × 100 mm, 5 μm) was used at 28 °C, with injected volume 10 μl. The binary solvent system used for elution gradient consisted of 0.2% acetic acid in H_2O (solvent B), and MeOH (solvent A), at a constant flow-rate of 200 μl min^{-1}. A linear gradient profile with the following proportions (v/v) of solvent A was applied [*t* (min), % A]: (0, 40), (25, 80), with 7 min for re-equilibration.

MS/MS experiments were performed on a Micromass Quatro UltimaTM PT double quadrupole mass spectrometer (Micromass, Manchester City, UK). All analyses were performed using turbo ion spray source in negative ion mode with the following settings for each hormone: capillary voltage −3000 V, energy cone 35 V, RF Lens1 (20), RF Lens2 (0.3), source temperature 100 °C, solvation temperature 380 °C, gas cone 100 l h^{-1}, collision (50), and multiplier (650). MS/MS parameters were optimized in infusion experiments using individual standard solutions of each hormone at a concentration of 50 ng μl^{-1} diluted in mobile phase A/B (40:60, v/v). MS/MS product ions were produced by collision-activated dissociation of selected precursor ions in the collision cell of the double quadrupole mass spectrometer, and mass was analyzed using the second analyzer of the instrument. In negative mode, the spectrum for each hormone gave deprotonated molecule $[M-H]^-$. Quantitation was performed by injection of samples in multiple reaction monitoring (MRM) modes, since many compounds could present the same nominal molecular mass. The combination of parent mass and unique fragment ions was used to selectively monitor hormones in plants extracts. MRM acquisition was performed by monitoring the 137/93 and 141/97 transitions for SA and ($^2H_4$)-SA; 263/153 and 269/159 for ABA and ($^2H_6$)-ABA209/59 and 215/59 for JA and ($^2H_6$-JA) and 174/130 and 179/135 for AIA and ($^2H_5$)-AIA respectively, with dwell 1000 ms for each transition. Data were acquired and analyzed using MassLynxTM 4.1 and QuanLynxTM 4.1 (Micromass, Manchester, UK) software. For quantification, values were obtained from a calibration curve previously constructed using known amounts of each hormone and their pure standard/deuterated internal standard ratio (Sigma, St. Louis, MO, USA).

2.2.9. Genotypic characterization and identification

Genotypic identification was made by amplification and partial nucleotide sequences of the ribosomal 16S DNA (rDNA 16S). The partial nucleotide sequences of 16S rRNA gene were determined by direct sequencing of appropriate PCR products. A DNA region corresponding to nucleotides 20–338 of *Escherichia coli* 16S rDNA was amplified from each strain with the universal primers Y1 (59-TGG CTC AGA ACG AAC GCT GGC GGC-39) and Y2 (59-CCC ACT GCT GCC TCC CGT AGG AGT-39) as previously described for proteobacteria (Young et al. 1991). The nucleotide sequence of the PCR products was determined for both strands with an Automatic Laser Fluorescent DNA Sequencer (Pharmacia).

2.2.10. Sequence alignment and phylogenetic tree

Sequence identities and similarities were determined using the BLAST program and the GenBank database on the NCBI web server. Multiple sequence alignment of the sequence isolated was performed by ClustalX using a MEGA5 program. The phylogenetic tree was constructed from the ClustalX results and the Maximun Likehood test with Kimura 2-parameter model. Boostrap method was used as the phylogeny test.

2.3. Inoculation tests in plant growth chamber

2.3.1. Bacterial strains

B. japonicum E109 strain was provided by the Agriculture Collection Laboratory of the Instituto Nacional de Tecnología Agropecuaria (INTA), Castelar, Argentina.

2.3.2. Soybean seeds

Soybean (*G. max* L.) seeds (Don Mario 3810 cultivar) provided by Laboratorios López SRL (Jesus María, Province of Córdoba, Argentina) were used. Quality control parameters were established by the International Seed Test Association (ISTA) (http://www.ista.org).

2.3.3. Bacterial growth and seed inoculation

Seeds were inoculated with individual or combined *B. japonicum* strain E109 and *B. amyloliquefaciens* cultures. *B. japonicum* titer was adjusted to 10×10^8 CFU (colony-forming unit) ml^{-1}, obtained at exponential growth phase on YEM broth, while *Bacillus* was adjusted to 1×10^8 CFU ml^{-1}, obtained at exponential growth phase on LB medium.

Inoculation doses were adjusted to a final volume of 150 cm^3/50 kg for soybean seed; equal volumes of each strain were used for co-inoculation.

Four treatments were used: (1) non-inoculated seeds (control) treated with a final volume of buffer solution; (2) seeds inoculated with *B. japonicum*; (3) seeds inoculated with *Bacillus* sp. (4) seeds coinoculated with *B. japonicum* and *B. amyloliquefacien*.

2.3.4. Soybean early growth promotion test

Ten soybean seedlings were grown hydroponically in plastic pots (1 l volume) containing vermiculite as substrate and with nitrogen-deficient sterile Hoagland's solution (25%, v/v) provided by capillary watering. Seedlings were maintained for 14 days in a growth chamber with a photoperiod of 16 h light and 8 h darkness at 28 °C. The following parameters were measured as indicators of early growth promotion: (a) shoot and root dry weight; (b) shoot and root fresh weight (c) number of nodules in principal roots (d) number of nodules in secondary roots and (d) total nodules. Roots and shoots from control and inoculated plants were separated and their fresh weights (FW) were recorded. Samples were dried in an oven at 70 °C until constant dry weight (DW) was obtained. FW and DW were expressed as grams per plant (g $plant^{-1}$).

2.3.5. Statistical analysis

Data were analyzed using InfoStat (v. 2011 InfoStat program), National University of Córdoba, Argentina. One-way general linear model ANOVA was used to determine the effect of each treatment. Normality was verified with the Shapiro–Wilk test. Homogeneity of variance was verified with Levene test. When necessary, data were transformed to meet the assumptions of ANOVA. The Duncan test was used for post hoc analysis to determine differences between means. Differences were considered significant at $P < 0.05$.

3. Results

Identification of a putative plant growth-promoting bacterial strain isolated from soybean seeds through 16S rDNA sequence analysis indicates that the new strain has high homology (98%) with *B. amyloliquefaciens* subsp. *Plantarum*. The phylogenetic tree constructed using the partial 16S rDNA sequences of this strain and representative bacteria sequences of related taxa retrieved from GenBank database are shown in Fig. 1. The result of this phylogenetic analysis revealed clearly that the isolated strain belongs to the *B. amyloliquefaciens* species. There are three major clades of bacteria classified in this phylogenetic tree, the major clade is *Bacillus*, and had strong support (>70% bootstrap support). The new isolate determined by phylogenetic positioning was correctly classified in this group. The tree also shows the phylogenetic distance between the two bacteria used in this work which are classified in different clades.

As shown in Table 1, microscopic examination revealed that the new isolate was Gram positive, endospore-forming, with rod

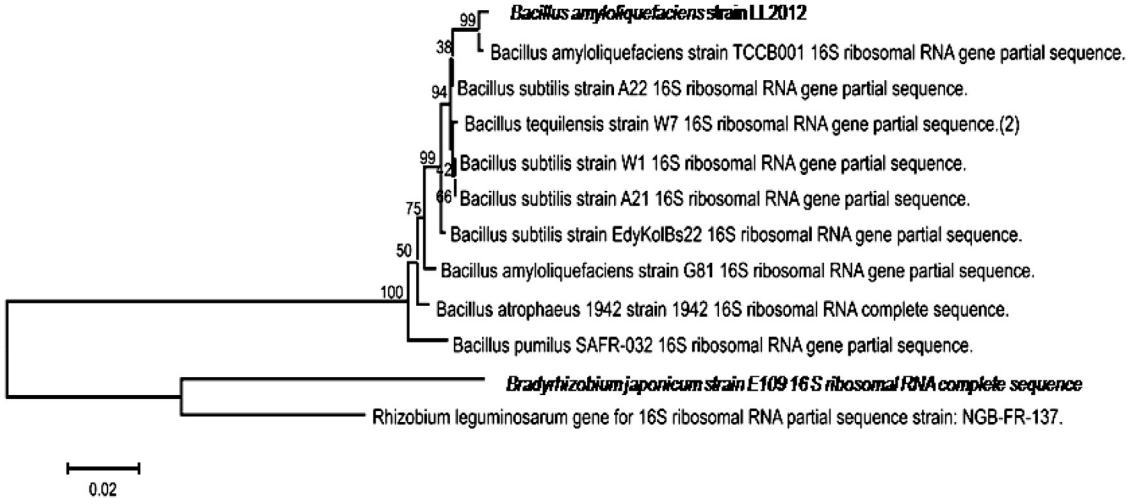

Fig. 1. Phylogenetic tree of partial 16S rDNA sequences of a putative plant-growth promoting isolates recovered from soybean seeds. *Bacillus amyloliquefaciens* LL2012 represents to new isolated used in this study.

Table 1
Morphology, biochemical characteristic and phytohormone production (SA, salicylic acid; ABA, abscisic acid; IAA, indole-3-acetic acid; JA, jasmonic acid; GA^3, gibberellins-3-acid) in exponential growth culture of *Bacillus amyloliquefaciens* LL2012 and *Bradyrhizobium japonicum* E109. Phyhormones are expressed in $\mu g\,ml^{-1}$ culture.

Strain	Biochemical characteristic of bacteria							
	Gram staining	Siderophore production	Phosphate solubilization	Nitrogen fixation	Protease activity	Catalase activity	Oxidase activity	Cellulolytic activity
B. amyloliquefaciens LL2012	+	–	–	+	+	+	+	+
B. japonicum E109	–	–	–	–	+	+	–	+

Strain	Phytohormone production in exponential growth culture of bacteria				
	SA ($\mu g\,ml^{-1}$)	ABA ($\mu g\,ml^{-1}$)	IAA ($\mu g\,ml^{-1}$)	JA ($\mu g\,ml^{-1}$)	GA^3 ($\mu g\,ml^{-1}$)
B. amyloliquefaciens LL2012	6.3	0.53	18.8	0.54	8.94
B. japonicum E109	0.3	0.01	6.6	0.1	0.93
Medium without inoculated (control)	0.43	0.15	0.12	0.014	0.37

shaped cells forming chains. Similarly to some species of *Bacillus* known for their catabolic properties and degradation of complex macromolecules, the isolate was positive for catalase, oxidase and protease activity and has ability to degrade cellulose. Additionally, this isolate was positive for nitrogen fixation. This strain did not have the ability to solubilize phosphate and was negative for siderophore production as discernible by the absence of halo formation.

On the other hand, microscopic examination of *B. japonicum* E109 showed that this strain was Gram negative. Similarly to *B. amyloliquefaciencs*, *B. japonicum* E109 was not able to produce siderophores neither to solubilize phosphate, and it was positive for protease, catalase and cellulolytic activity; contrarily to LL2012 strain, E109 was negative for oxidase activity (Table 1).

The effect of individual inoculation and co-inoculation with both strains on soybean seedling growth was evaluated. Fig. 2 shows early seedling growth in terms of root and shoot dry and fresh weight. Remarkably, no significant differences were observed in shoot fresh weight of controls and *B. amyloliquefaciencs* inoculated plants, while this parameter decreased in *B. japonicum* inoculated and co-inoculated plants ($P < 0.05$). However, shoot dry weight increased in all inoculated plants, the highest value being observed in *B. amyloliquefaciencs* inoculated plants.

Dry weight increased in roots of inoculated plants as compared to controls, but no significant differences were observed between them. Root fresh weight was not modified.

Symbiosis-related parameters were shown in Fig. 3 on seedlings inoculated with *B. japonicum*, *B. amyloliquefaciencs*, or both.

As expected, uninoculated plants did not show any nodule. In inoculated plants, the number of nodules was variable according with the strain used. In the main root, this parameter increased only in *B. japonicum* inoculated and co-inoculated plants and no significant differences were observed between them. The highest number of nodules was observed in secondary roots of co-inoculated plants, followed in descending order by *B. japonicum* and *B. amyloliquefaciencs*-inoculated plants. This behavior was reflected in total number of nodules.

Table 1 summarizes the production of phytohormones by cultures of *B. amyloliquefaciencs* LL2012 strain and *B. japonicum* E109 in exponential growth phase, determined by LC–MS–MS. Higher levels of IAA, GA_3 and SA were observed in cultures of LL2012 strain. Notably, the IAA content was three times higher than IAA production by *B. japonicum* (18.8 $\mu g\,ml^{-1}$ versus 6.6 $\mu g\,ml^{-1}$) and GA_3 and SA levels were greatly higher compared to *B. japonicum* (8.98 $\mu g\,ml^{-1}$ versus 0.95 $\mu g\,ml^{-1}$ and 6.3 $\mu g\,ml^{-1}$ versus 0.3 $\mu g\,ml^{-1}$, respectively). ABA and JA were detected but in lower levels than the other phytohormones.

4. Discussion

The results on phytohormones production by *B. japonicum* were correlated with previous studies from our laboratory which demonstrated that *B. japonicum* E109 has the capability to produce phytohormones such as IAA, GA_3, zeatin, ethylene, and ABA (Boiero et al. 2006). ABA production was detected in very low levels in this work, in coincidence with those results. Also JA and SA were

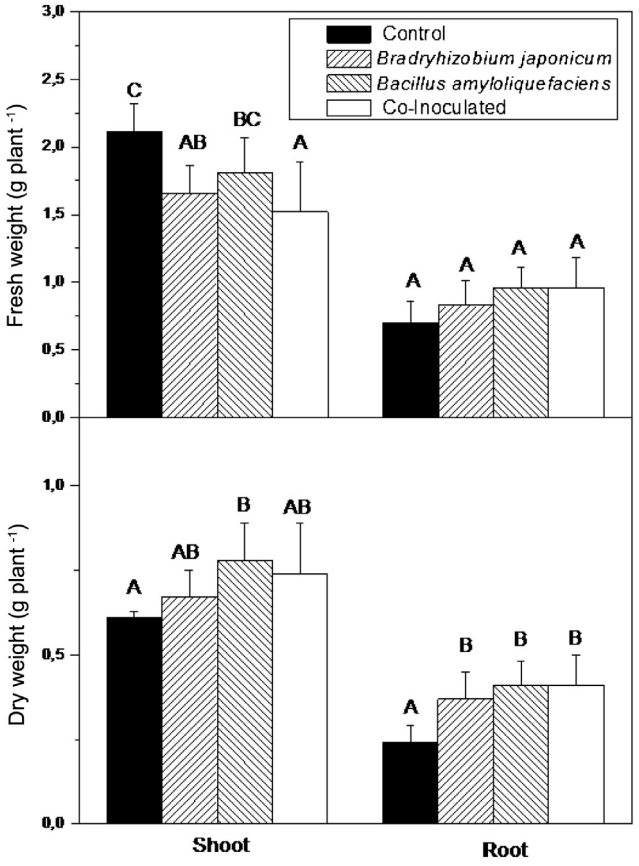

Fig. 2. Fresh weight of shoot and root of soybean seedling and dry weight of shoot and root at day 14 after sowing. Data represent mean ± SE. Different letters above data indicate significant differences among treatments ($P < 0.05$).

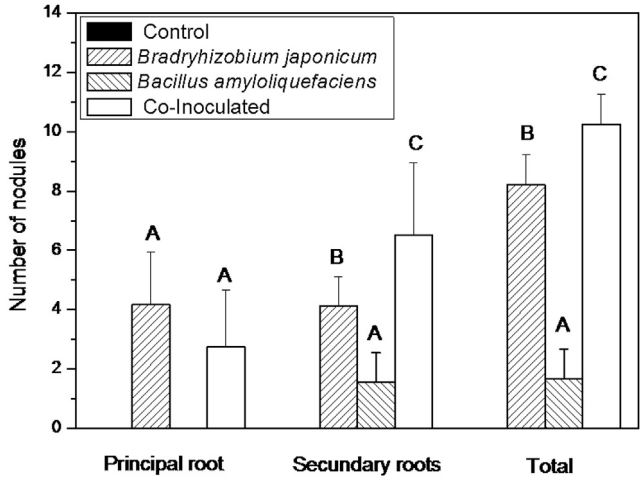

Fig. 3. Number of nodules on principal, secondary root and total of soybean seedling at day 14 after sowing. Data represent mean ± SE. Different letters above data indicate significant differences among treatments ($P < 0.05$).

detected in lower levels than IAA and GA_3 (Table 1); no previous reports of SA production by *B. japonicum* E109 have been found in the literature.

The notably higher production of IAA, GA_3, and SA by the new isolated strain shows that its plant-growth promoting activity includes not only symbiotic nitrogen fixation, but also additional properties such as high production of phytohormones and enhanced enzymatic activities.

Our results indicate that *B. amyloliquefaciencs* LL2012 and *B. japonicum* inoculated singly or in combination, have the capacity to promote early seedling growth in soybean; nevertheless, the main impact was detected in soybean nodulation when both strains are together in the medium. In those crops in which nitrogen nutrition depends mainly on biological nitrogen fixation, the number of nodules occupied by effective bacteria is important in order to obtain the maximum benefits from the symbiotic association (Wang and Martinez-Romero 2000). The catabolic properties for degradation of complex macromolecules by LL2012 strain, associated with biosynthesis of several plant growth regulators which are excreted into the culture medium could explain at least in part, the observed promotion of nodulation after co-inoculation on soybean. Indeed, phytohormones from the culture medium at a concentration sufficient to produce morphological and physiological changes in young seedling tissues would be the first contact between the bacterial formulation and the seed. Remarkably, this would not necessarily depend on bacterial cell presence as proposed recently in our previous work (Masciarelli et al. 2013) where it was demonstrated that the release of IAA into the culture medium by the bacteria appeared to be the main activator of the early growth promotion observed in inoculated maize seedlings. However, the presence of bacteria may also contribute to *in situ* phytohormone production, since it was shown, that e.g. *Azospirillum brasilense* induces the key gene of indole-acetic acid production (ipdC-gene) when colonizing the wheat root surface (Rothballer et al. 2005). Similarly, in our present study, an increase in shoot and root dry weight observed in soybean under co-inoculation, as well as in plants inoculated only with *B. amyloliquefaciens*, could be due in part to differential embryo development induced by bacterial growth regulators which penetrate the seed coat along with water, and accelerate root growth with concomitant increases in water and mineral uptake. Dobbelaere et al. (1999) showed that inhibition of root length together with increase of root weight is a typical response to bacterial IAA production; hence the increase of root weight in this work may be the result of the high levels of IAA produced by *B. amyloliquefaciencs*.

Several studies have suggested that auxins play essential roles in nodule development. Schmidt et al. (1988) showed that co-inoculation of *Medicago sativa* seeds with a *Rhyzobium melilotti* strain inefficient for IAA production and *A. brasilense* significantly increased the number of nodules on the main root, and Yahalom et al. (1990) reported that co-inoculation of *M. polimorpha* with *Rhizobium* sp. and *Azospirillum* sp. increased the number, weight, and nitrogenase activity of root nodules in comparison with single-inoculated plants. Considering previous studies, *B. amyloliquefaciencs* LL2012 strain produces really high IAA levels. For example, Ivanova et al. (2001) detected 0.03–100 μg/ml IAA in different obligate and facultative methylotropic bacteria cultured in liquid culture medium. Pedraza et al. (2004) found that *Azospirillum* strains produced the highest concentration of IAA (16.5–38 μg IAA/mg protein) whereas *Gluconacetobacter* and *Pseudomona stutzeri* strains produced lower concentration of IAA ranging from 1 to 2.9 μg IAA/mg protein in a culture medium supplemented with tryptophan.

On the other hand, the high levels of GA production observed by *B. amyloliquefaciencs* LL2012 adds an additional advantage to the PGPR characteristics of this strain.

Gibberellins (GAs) are a large group of tetracyclic diterpene acids that regulate diverse processes in plant growth and development, such as germination, stem elongation, flowering, and fruiting (Davies 1995). Evidence of gibberellin biosynthesis by *Bacillus* sp is rare; however, Gutiérrez-Mañero et al. (2001) provide evidence that four different forms of physiologically active GAs are produced by *Bacillus pumilus* and *B. licheniformis* isolated from the rhizosphere of alder (*Alnus glutinosa* [L.] Gaertn.): GA1 (0.13 μg ml^{-1}), GA3 (0.05 mg ml^{-1}), and GA4 as well as the precursor GA20 and the

isomers 3-epi-GA1 and iso-GA3. They demonstrated that the inoculation of alder (*A. glutinosa*) with these bacteria have the potential to elongate the stem and shoots of this plant, indicating that the GAs produced by the bacteria are probably physiologically active in the host plants. Recently, Sgroy et al. (2009) confirmed the GA_3 production in *Lysinibacillus fusiformis*, *Achromobacter xylosoxidans*, *Bacillus halotolerans*, *B. licheniformis*, *B. pumilus*, and *B. subtilis* strains isolated from roots of the halophytic legume *Prosopis strombulifera*, with a production of 36.5, 50.0, 80.5, 75.5, 21.3, and10.0 mg ml^{-1}, respectively. This is a strong evidence of GA production being a common mechanism of growth promotion by several PGPR. It is complex to hypothesize a physiological role for the GAs produced by rhizobacteria, but it seems logical to think that promoting the overall growth of colonized plants could be beneficial to bacteria because of the increased nutrient availability in the rhizosphere.

Although in smaller amounts than the other phytohormones, *B. amyloliquefaciencs* LL2012 had the ability to produce also JA under our experimental conditions. Mabood and Smith (2005) showed that JA and methyl-jasmonate (MeJA) strongly induced the expression of nodulation genes of *B. japonicum* and that preincubation with JAs enhanced nodulation, N fixation, and plant growth in soybean under controlled environmental conditions. Similarly, Mabood et al. (2006) reported that inoculation of soybean plants with bradyrhizobial cells incubated with MeJA alone or in combination with genistein (GE) increased nodule number, nodule dry matter (DM) per plant, and seasonal N_2 fixation, as compared to *B. japonicum* cells that were not incubated with those compounds.

In this work, both bacteria showed the capacity of phytohormone production and release into the culture medium. However, considering the greatly higher levels of these compounds (mainly IAA, GA3 and SA) produced by *B. amyloliquefaciens* it is much more feasible that this strain could regulate plant nutrient distribution and tissue growth and differentiation to form new nodules (thus converting nodulation in the main process consuming photosyntates and temporarily delaying plant growth) and to create a more suitable environment for *B. japonicum* to establish and fix nitrogen. Bashan et al. (2004) emphasized the importance of biological nitrogen fixation for biomass increase in later developmental stages, once a significant number of bacteria have become established in plant tissues. Thus, plant growth stimulation by PGPR bacteria would be crucial in early developmental stages such as germination and seedling growth. These results are consistent with previous studies in our laboratory, in which we found that co-inoculation with Az39 and E109 significantly increased the number of nodules per plant and percentage of nodulated plants, compared to single inoculation with E109. This finding could be explained by the capacity of Az39 to synthesize and release phytohormones into the culture medium applied to the seeds (Cassan et al. 2009).

Also, it should be kept in mind that the amount of new "cross-talk" interactions described in literature for phytohormone signaling pathways in thousands of plant species, gives new insights into the simple phytohormonal growth promotion-dependent model. In this regard, Ross and O'Neill (2001) suggested that auxin could promote, at least in part, stem elongation by increasing endogenous levels of 3β-hydroxylated gibberellins. There is some evidence of the interaction between the phytohormones produced by *Azospirillum* sp. and the hormonal background of inoculated plants, but a detailed analysis of this interaction may reveal specific interactions that could result in their PGP effect (Cassan et al. 2011).

Bashan and Holguin (1998) proposed dividing the PGPR group into bacteria which promote plant growth (a) through direct physiological or biochemical mechanisms (PGPR), and (b) indirectly through pathogen biocontrol or competition (biocontrol-PGPB). According to this classification and to our results, *B. amyloliquefaciens* strain LL2012 can be included in group (a).

On the other hand, by using the data from six independent micro array experiments, Fan et al. (2012) differentially transcribed genes of *B. amyloliquefaciens* strain FZB42. A large group of genes specifically expressed suggested that root exudates serve primarily as a source of carbon and energy for FZB42. Another group of genes significantly induced by plant root exudates encode the non-ribosomal synthesis of antimicrobial secondary metabolites. These authors proposed that it is possible that enhanced synthesis of antimicrobial compounds might suppress the competing phytopathogenic organisms growing within the plant rhizosphere. Additionally, our results show that *B. amyloliquefaciencs* LL2012 has the ability to produce also SA, proteolytic and cellulolytic activity; therefore, this strain could have an important role on the biological efficacy and preservation of a commercial formula as biocontrol agent, which would include *B. amyloliquefaciens* also as a PGPB. In this sense, Pieterse et al. (1996) reported that PGPR can induce systemic acquired resistance characterized by an accumulation of salicylic acid (SA) and pathogenesis-related proteins. There are several mechanisms for different biocontrol agents, and the ultimate aim of these biocontrol bacteria is to regulate ecological balance for controlling pathogen and promoting host plant growth, which also needs to cooperate with the bacteria in soil and plant (Zhang et al. 2011).

Thus, *B. amyloliquefaciencs* strain LL2012 possess PGPR and PGPB mechanisms that may influence positively root colonization and establishment of *B. japonicum*, which would make mixed commercial formulations more effective, thus reducing the doses of application and then, the costs. A recent study demonstrated that there was strong positive correlation ($r = 0.982$) between number of root nodules and crop yield (Dhami and Prasad 2009), which suggest that optimization of root nodulation by inoculating compatible PGPR/PGPB and effective *B. japonicum* strains could significantly increase the soybean crop yield decreasing the need to use exogenous mineral fertilizers.

In conclusion, the new PGPR strain isolated from soybean seeds in the present study can be utilized as bio-inoculant for improving plant growth and nodulation of different legumes. *B. amyloliquefaciencs* LL2012 strain could be further exploited in sustainable agriculture and reforestation mainly when it is associated with *B. japonicum*. Furthermore, alteration of crop hormonal status to decrease evolution of the growth-retarding and senescence-inducing hormone ethylene (or its precursor 1-aminocyclopropane-1-carboxylic acid), or to maintain source–sink relations, photosynthesis, and biomass production and allocation (by altering indole-3-acetic acid and cytokinin biosynthesis) seem to be promising target processes for soil biota-improved crop stress tolerance. Although, many of the underlying physiological and molecular mechanisms still need to be identified in order to optimize the agronomic applications of soil microorganisms (Dodd and Pérez-Alfocea 2012). Further study is required to understand the interactions between plant nodule – endophytic bacteria, symbiotic bacteria and host plants on plant growth, nodulation, and symbiotic genes.

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