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Nodulation and Delayed Nodule Senescence: Strategies of Two *Bradyrhizobium Japonicum* Isolates with High Capacity to Fix Nitrogen

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Abstract

The purpose of this work was to study further two *Bradyrhizobium japonicum* strains with high nitrogen-fixing capacity that were identified within a collection of approximately 200 isolates from the soils of Argentina. Nodulation and nitrogen-fixing capacity and the level of expression of regulatory as well as structural genes of nitrogen fixation and the 1-aminocyclopropane-1-carboxylate (ACC) deaminase gene of the isolates were compared with that of E109-inoculated plants. Both isolates of *B. japonicum*, 163 and 366, were highly efficient to fix nitrogen compared to commercial strain E109. Isolate 366 developed a higher number and larger biomass of nodules and because of this fixed more nitrogen. Isolate 163 developed the same number and nodule biomass than E109. However, nodules developed by isolate 163 had red interiors for a longer period, had a higher leghemoglobin content, and presented high levels of expression of *acdS* gene, that codes for an ACC deaminase. In conclusion, naturalized rhizobia of the soils of Argentina hold a diverse population that might be the source of highly active nitrogen-fixing rhizobia, a process that appears to be based on different strategies.

Introduction

Rhizobia–legume interactions lead to the development of nitrogen-fixing nodules where atmospheric N_2 is reduced to ammonia and assimilated by the plant. This process, known as Biological Nitrogen fixation, is one of the most important biotechnological tools in sustainable agriculture management [29, 48].

Soybean (*Glycine max* [L.] Merr) is nowadays the most important legume worldwide. The crop was introduced in Argentina and Brazil, in both countries its expansion started in the 1970 and nowadays conform the most important area of soybean production worldwide. In Argentina, 90% of the soybean-cultivated area is inoculated at seeding with bacteria of the genus *Bradyrhizobium*, mainly *B. japonicum* and occasionally with *B. elkanii*. Because of this, most of the soils, contain naturalized populations of bradyrhizobia [17, 28, 38, 43], which often outcompete selected strains provided by commercial inoculants [2, 25, 27, 30]. Soil bacteria populations are under continuous evolution due to genetic changes that might lead to the appearance of new isolates, which might be highly competitive and proficient saprophytic strains [2, 6, 25]. In line with this, bradyrhizobia isolated from the soils are often more competitive than the highly efficient nitrogen-fixing bacteria introduced by means of commercial inoculants [1, 25]. The genetic changes that occur in soil bacteria are frequently driven by a negative selection against nitrogen fixation, which is frequently impaired within naturalized populations of rhizobia [2, 6].

The amount of N fixed by a legume along its life cycle is a function of nodule number as well as nitrogen-fixing efficiency and the length of time nodules remain active, before senescence when a complex set of interactions occur. Nitrogen fixation in nodules increases 15 days after inoculation and remains at high levels within 2–4 weeks, decreasing thereafter due to nodule senescence [47].

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During senescence, nodules' interior shifts from a pink to green color because of the nitration of the heme group of leghemoglobin [36]. Furthermore, Vance et al. [57], Pfeiffer et al. [42], Pladys and Rigaud [44, 45], demonstrated that age-related senescence, in alfalfa, soybean, and French beans, respectively, was related to the activity of plant proteolytic enzymes. Pladys and Vance [46] also found that in nitrogen-fixing nodules of alfalfa proteolysis increased slowly within 21 days. The targets of these proteases are nodule cytosolic proteins, especially leghemoglobin, whose degradation affects the O_2 supply to bacteroides. Membranes and bacteroides also are digested by proteolytic enzymes; iron is released into the host cell cytoplasm and because of this nitrogen fixation decreases [10].

Ethylene is a plant hormone that plays a key role in plant and nodule senescence [16, 34]. Like other hormones, it plays many roles particularly in symbiosis; it affects nodule number and development as well as the location of nodule primordia, but it is also involved in nodule senescence [16, 18, 20, 39, 53]. 1-aminocyclopropane-1-carboxylate (ACC) deaminase is an enzyme that cleaves 1-aminocyclopropane-1-carboxylate (ACC) to ammonia and α-ketobutyrate, reducing in this way ethylene production [15, 49, 52]. Interestingly, some plant growth-promoting bacteria promote growth due to the synthesis and activity of ACC deaminases.

ACC deaminases coding genes *acdS* have been described in many different bacteria, fungi, and actinomycetes [35]. Among nitrogen-fixing organisms commonly known as rhizobia, *acdS* has also been described in *Bradyrhizobium*. In *Mesorhizobium loti, acdS* is under the transcriptional control of the *nifA* promoter, which regulates the transcription of *nif* genes [33, 37, 51]. The activity of the ACC deaminase has been associated with nitrogen fixation and to the delay of nodule senescence [53], in such a way that a decrease in fixation might lead to ethylene synthesis and nodule senescence [13, 47]. Thus, in this scenario, a reduction in ethylene synthesis by an increase in ACC deaminase activity might delay nodule senescence and increase the period of nitrogen fixation. Consequently, the production of ACC deaminase might be a biological strategy to improve nitrogen fixation [16, 53]; however, the mechanisms that govern all the events that lead to nodule senescence remain elusive.

In this work, we characterized the nodulation and nitrogen fixation ability of soybean plants inoculated with two naturalized strains of bradyrhizobia with high nitrogen-fixing capacity that were isolated from soybean fields with different histories of management in Argentina. We evaluated the expression level of genes related to nodulation and nitrogen fixation and the gene coding for the ACC deaminase, in both free-living bacteria and nodule-forming bacteroides, as well as the content of leghemoglobin and ammonia in nodules.

Materials and Methods

Bradyrhizobium Cultures

Two hundred representatives of *Bradyrhizobium japonicum* and *B. elkanii* were isolated from soybean (*Glycine max* [L]. Merr) plants growing in a field located at Runciman, Santa Fe, Argentina (located at latitude: -33.900, longitude: -61.250) [38]. They were all slow-growing bacteria that developed 1–3 cm diameter colonies on yeast extract mannitol (YEM) after an incubation period of 7 days at 28 °C. These organisms were identified by PCR amplification of the internal transcribed spacer (ITS) *23S-16S rDNA* [22] and RSα, a specific fragment that corresponds to repetitive DNA of *Bradyrhizobium* [26] and then were incorporated to the culture collection of the CIDEFI.

Bacteria were cultured on liquid YEM medium [58] at 28 °C in the darkness for 144 h. Stock cultures were prepared by adding 10% glycerol to a 1×10^9 cell ml^{-1} culture and then 1.5 ml aliquots were frozen in liquid nitrogen. Stock cultures were kept at -70 °C. *B. japonicum* E109 was included as reference strain in all the studies (Culture Collection of INTA - Argentina); this strain is a derivative of USDA138 that has been used to formulate commercial inoculants in Argentina for a long time.

Based on the outstanding nitrogen-fixing capacity compared to control strain E109, two isolates of *B. japonicum* were selected 163 and 366. Isolates as well as control strains were grown on YEM agar medium at 28 °C or in liquid YEM medium, at 150 rev min^{-1} [58]. Tubes containing agar slants grown on YEM agar were kept at 4 °C and were used as starter cultures.

Isolates of *B. japonicum* 163 and 366 together with a description of their properties were deposited in the culture collection WDCM31 at the Collection of Instituto de Microbiologia y Zoología Agrícola (IMYZA), Laboratorio de Bacterias Promotoras del Crecimiento Vegetal (BPCV), INTA Castelar Address: Las Cabañas y De los Reseros SN, CC 25 B1712WAA, Castelar, Buenos Aires, Argentina. The culture collection identification numbers were for isolates 163: E421 and 366: E422.

Plant Assays

Soybean (*Glycine max* [L] Merr) seeds cv DM4670 were surface sterilized by immersing them in 50% ethanol for 5 min and then 5 additional minutes in 5% v/v sodium hypochlorite, which was followed by ten washings with distilled water until no vapors of hypochlorite were perceived. Then, seeds were germinated in water agar at 28 °C in the darkness. Forty-eight hours later seedlings

were transplanted to 2 l plastic pots filled with vermiculite and were inoculated with 10^7 cell ml^{-1} of *B. japonicum* strain E109 (positive control), isolate 163 or 366. Plants were grown under controlled conditions in the greenhouse at 24 ± 2 °C, 50% RH, 16 h photoperiod, and were watered with a nitrogen-free nutrient solution [58]. Three different experiments were performed, with twenty-four replicates of each assayed combination. Plants were harvested 6 weeks after seeding. Cultivar DM4670 is produced by the company Don Mario and is a cultivar that belongs to group four (a long one that is why it is a 4.67) and it is a widely used cultivar within Argentina.

Nitrogen-fixing activity was estimated by means of the acetylene reduction assay [5, 50]. Detached nodulated roots were enclosed in 250 ml airtight bottles. Roots were supplied through rubber stoppers with C_2H_2 (10% v/v) at time zero and incubated for 10 min at room temperature (25 ± 2 °C). Samples (0.5 ml) taken from airtight bottles were analyzed in a Gas Chromatograph equipped with a flame ionization detector (FID, KONIX 3000).

Dry weight of nodules and plant shoots were determined. Nodules were detached from roots, counted, placed in paper bags, and desiccated in an oven at 70 °C until constant dry weight is achieved. Similarly, plant shoots also were placed in paper bags and dried at 70 °C in an oven until constant weight is achieved.

Gene Expression in Nodules and Free-Living Bacterial Cultures

RNA was extracted from both liquid cultures and nodules. In order to extract RNA, cultures were grown on Peptone-Salts-Yeast Extract-Arabinose medium at 28 °C in a rotary shaker at 150 rev min^{-1} [31].

Bradyrhizobium Japonicum cultures were grown to mid-exponential phase, which corresponded to an optical density at 600 nm of 0.4 to 0.5 in microoxic cultures (peptone-salts-yeast extract-arabinose medium). Cultures were rapidly transferred to cold tubes, centrifuged for 5 min (13,000 rev min^{-1}, 4 °C), the supernatant was decanted, and cells were frozen in liquid nitrogen and stored at −80 °C.

RNA of nodules was extracted from 1 g of nitrogen-fixing nodules that were harvested and ground in a mortar previously sterilized twice at 120 °C for 45 min and washed with DEPC-treated water. The powder was homogenized in 200 µl of deionized water and centrifuged at 2000 rev min^{-1} for 5 min to pellet bacteroides. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Netherlands) according to the manufacturer's instructions. 1–2.5 µg of DNA-free RNA was used with random hexamers for first-strand cDNA synthesis using the avian myeloblastosis Reverse Transcriptase for RT-PCR (Reverse Transcriptase, AMV-Roche®) according to the manufacturer's instruction. qPCR was conducted using a set of specific primers amplifying two regulatory nitrogen fixation genes *nifA* and *fixL*, the ACC deaminase coding gene (*acdS*), a constitutive and an inducible type III secretion system gene, *rhcC1* and *nopP*, respectively, and two transcriptional regulators *nodW* and *nodD1* (Table 1). Each reaction was performed in a final volume of 10 µl, containing 0.2 mM of the primers pair, one microliter of a 1:10 dilution of the previously synthesized cDNA and the corresponding volume of iQ™ SYBR® Green Supermix (Bio-Rad) using the iCycler iQ system (Bio-Rad). The reaction conditions were 95 °C for 3 min followed by 40 cycles of 95 °C for 15 s, 56 °C for 15 s, and 72 °C for 15 s and one cycle of final extension of 72 °C for 3 min. Melting curve analysis was performed. Data were analyzed using the iQ5 software. Gene expression of the above described genes was normalized according to the expression levels of *16S rRNA* gene and expressed as the relative fold change compared to control strain *B. japonicum* E109, following the formula $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = [(C_t$ gene of interest $- C_t$ *16S rRNA* gene) isolate 163 or 366 $- (C_t$ gene of interest $- C_t$ *16S rRNA* gene) control strain].

Leghemoglobin and Ammonia Content in Nodules

Fresh nodules in an amount of 0.5 g were homogenized in 3 ml of an extraction buffer containing 0.02% (w/v) potassium ferricyanide and 0.1% sodium bicarbonate. Homogenates were centrifuged at 13,000 rev min^{-1} for 30 min and

Table 1 Primers synthesized with DNA Synthesizer® 3400 (Applied Biosystem) at the Institute of Parasitology and Biomedicine "López-Neyra" (C.S.I.C., Granada)

Primer	Sequence 5′–3′	Gene
SL19	ATTGTCACCGCCATTGTA	*16S rADN*
SL20	CGTCCTTAGTTGCTACCA	
SL1	ACATCGTATGGCAAGACA	*acdS*
SL2	CATTCTCGCACCACTAAC	
SL5	CTGTACTACAACCGTTATCTTG	*nodW*
SL6	GCCATCAACTGTAACTTCG	
SL7	AATTTCCTGCTGGATTCTG	*nopP*
SL8	CTTCATCCGCTTCAACAG	
SL9	GTGCTGATCGAGGACTAC	*nodD1*
SL10	ATAGCCTTTCTGCGTGAG	
SL11	TGTGGACGAAGAATGAGAA	*fixJ*
SL12	AATAGACTGACGGATCATCTT	
SL13	AGAACAGGCAGTGATGAA	*fixX*
SL14	CGTCTCGCTCATTCTGAT	
SL15	GCGATGAACTCTTTACTATGA	*nifA*
SL16	CTGAGAGAATGACCCTGAA	
SL17	TGATGAACTGTTTGGAATGAG	*rhcC1*
SL18	TGTTACGAAGTCAGAAAGGA	

leghemoglobin was measured fluorometrically in the red supernatant obtained [24]. Bovine hemoglobin was used as a standard. Nodule samples were collected five, six, and seven 7 weeks after inoculation.

The ammonia content was determined by means of the phenol-hypochlorite method [4]. 0.5 g of nodules was homogenized in 2.5 ml of 0.3 mM H_2SO_4 and centrifuged at 13,000 rev min^{-1} for 15 min. A calibration curve with NH_4Cl was used as standard. Nodule samples were taken six and seven 7 weeks after inoculation.

Results

B. japonicum Isolates 163 and 366 Induce Nitrogen-Fixing Nodules in Soybean Plants

We estimated the nodulation capacity of the selected isolates by measuring nodule number and dry weight. In addition, we determined the nitrogen fixation capacity indirectly by measuring plant biomass and by estimating the activity of nitrogenase through the acetylene reduction assay. Regarding nodulation, soybean plants inoculated with E109 and those inoculated with isolate 163 developed a similar number of nodules 24 per plant that were of approximately the same size, the average weight per nodule was 12 mg (Table 2). Soybeans inoculated with isolate 366 developed a significantly higher number of nodules per plant that also were larger (Table 2).

It is well known that growth depends on available N, so plant dry weight can be used to estimate nitrogen fixation of legume–rhizobia interactions along plant growth. On the other hand, the acetylene reduction assay reflects nitrogenase activity at a moment (sampling time) in plant growth. Plant biomass of soybeans inoculated with isolate 366 or 163 was larger than that of plants inoculated with E109, which indicates that most probably isolates 366 and 163 provided more nitrogen than E109 (Table 2). Interestingly, nitrogenase activity estimated based on the acetylene reduction assay 6 weeks after inoculation was similar among treatments. While doing this analysis, we observed that some nodules induced by control strain E109 as well as 366 had interiors with a greenish/brownish appearance compared to those induced by isolate 163, which had interiors with red color. All this together suggested that nodules induced by these two isolates probably were at a different developmental stage. While those induced by E109 and isolate 366 were probably at the beginning of senescence, those induced by 163 appeared to be actively fixing nodules.

ACC Deaminase Expression in *B. japonicum* 163 Bacteroides Suggested a Delay in Nodule Senescence

The level of expression of genes coding for proteins involved in Type III secretion system, nodulation, and nitrogen fixation, was evaluated. The activity of these genes might be considered indexes of highly efficient nitrogen-fixing strains. We evaluated by means of RT-PCR the expression of (a) *nifA* and *fixL*, genes that encode transcriptional regulators of nitrogen fixation; (b) *rhcC1* and *nopP* that encode a constitutive and an inducible gene of a type III secretion system, respectively; (c) *nodW* and *nodD1*, two transcriptional regulators controlling the nodulation process; and (d) *acdS* (*blr0241*), a gene that encodes for ACC deaminase. This was done by using the sets of primers presented in Table 1.

In liquid cultures of isolates 163 and 366, genes were expressed at low levels compared to control strain E109 (Table 3). In nodules, expression of nodulation and nitrogen fixation genes was different in plants inoculated with isolate 163 or 366 than on nodules of plants inoculated with E109 (Fig. 1). Regulatory genes of nodulation had a similar transcriptional pattern in nodules induced either by isolates 163 or 366, though higher levels of expression were found in nodule samples induced by 163. Regulatory genes of nitrogen fixation were expressed at different levels in nodules induced by any of the three isolates. In nodules developed by *B. japonicum* 163, the level of expression of *fixL, acdS,* and *rhcC1* was higher than that of *nifA* and *nopP*. Interestingly, most of these genes, except *rhcC1*, were slightly

Table 2 Nodulation, nitrogen fixation, and plant growth of soybean plants inoculated with *B. japonicum* E109, 163, and 366

Strain	Number of nodules	Nodule dry weight (mg)	Shoot dry weight (mg)	Nitrogenase activity (nmol de ethylene. h^{-1} mg nodule dry $weight^{-1}$)
E109	24.67 ± 1.5^b	12.33 ± 0.52^b	223.17 ± 0.75^b	31.67 ± 0.58^a
163	24.00 ± 1.7^b	12.16 ± 0.52^b	230.00 ± 0.89^a	32.83 ± 0.70^a
366	34.50 ± 1.1^a	14.33 ± 0.68^a	231.00 ± 0.89^a	32.50 ± 0.50^a
Control	–	–	169.50 ± 1.05^c	–

The number of treatments was four and the design of the experiment was completely at random. Means were compared by Tukey Test $p < 0.05$. These results correspond to one and are representative of the three replicate experiments performed with many replicates per experiment under controlled conditions in the greenhouse

Table 3 Expression levels of nitrogen fixation constitutive and regulatory genes as well as ACC coding gene of *Bradyrhizobium japonicum* in liquid cultures of 163, 366, and E109

Gene symbol	Gene assignment	Fold change (soil isolate/control strain)*	
		Strain 163/E109	Strain 366/E109
nifA	*nif*-specific regulatory protein	-1.44 ± 0.568	-2.22 ± 0.03
fixL	Two-component oxygen sensor	-1.56 ± 0.17	-19.63 ± 0.21
acdS	1-aminocyclopropane-1-carboxylate deaminase	-0.76 ± 0.22	-20.83 ± 1.45
rhcC1	Type III secretion apparatus outer membrane protein	-1.32 ± 0.26	-1.27 ± 0.05
nopP	Nodulation protein NopP	-3.49 ± 0.08	5.89 ± 1.16
nodW	Two-component regulator	-1.58 ± 0.56	1.30 ± 0.18
nodD1	NodD1 nod-box dependent transcriptional activator	-1.21 ± 0.24	-7.10 ± 0.28

*The means $\pm$ SD of three biological replicates are shown

Fig. 1 Expression levels of constitutive and regulatory nitrogen fixation genes as well as ACC coding gene of *B. japonicum* in nitrogen-fixing nodules. Bars indicate the level of expression of *nifA, fixL, acdS, rhcC_1, nopP, nodW,* and *nodD_1* genes for nodules induced for isolates 163 and 366, with respect to expression on nodules of E109
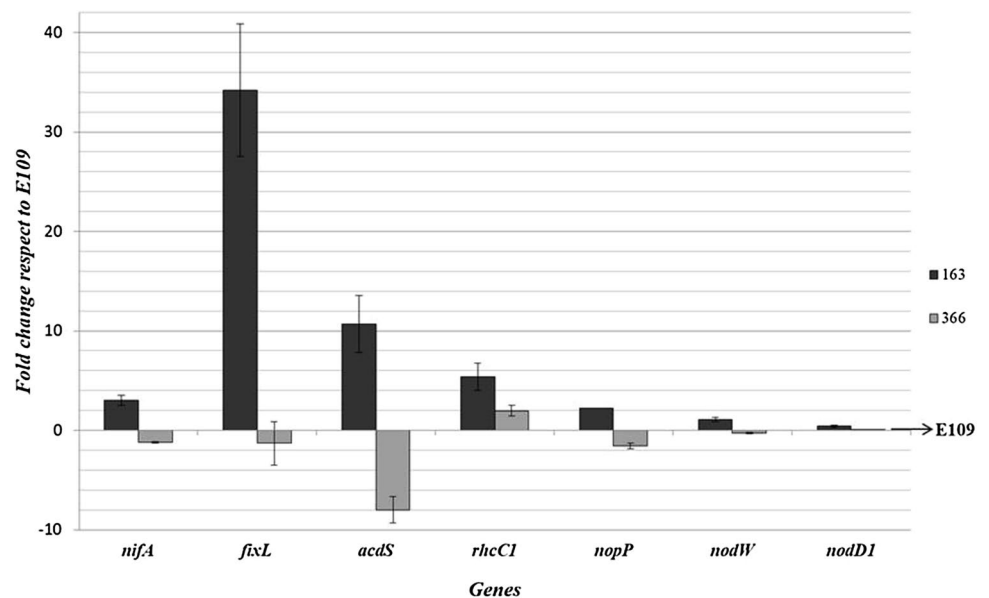
downregulated in nodules induced by *B. japonicum* 366. In addition, we observed that in nodules formed by 163 the *acdS*, gene that encodes an ACC deaminase, was expressed at much higher levels than in nodules induced by control strain E109 or isolate 366.

Leghemoglobin and Ammonia Content Indicates Different Rates of Nodule Senescence

In preliminary experiments nodules of soybean plants inoculated with isolates 163, 366, or control strain E109 differed in the color of their interior, which was confirmed in the experiments described above. Therefore, we cultivated soybeans inoculated with each of the isolates and determined the leghemoglobin and ammonia content in 5-, 6-, and 7-week-old nodules. Five weeks after inoculation, nodules of plants inoculated with *B. japonicum* isolates 163 and 366 had significantly higher levels of leghemoglobin than those induced by control strain E109. The content of leghemoglobin decreased substantially in nodules 6 weeks after inoculation. As shown in Fig. 2 , primarily the content of leghemoglobin decreased by 15.45, 13.40, and 15.95% in nodules induced by E109, 163, and 366, respectively. Such reduction in leghemoglobin continued and 7-week-old nodules had less protein; such reduction consisted in an additional loss of leghemoglobin of 6.00, 5.85, and 17.40% in nodules induced by E109, 163, and 366, respectively. As a whole, the reduction in leghemoglobin content in nodules along the period was of 20.50, 18.50, and 30.50% in nodules induced by E109, 163, and 366, respectively. The content of ammonium was indirectly related with that of leghemoglobin. The largest quantity of ammonium was detected in *B. japonicum* 366-induced nodules, then in nodules induced by E109 and the lower amount was detected in nodules induced by *B .japonicum* 163. Interestingly, the latter were those where senescence was delayed, as estimated based on the leghemoglobin content.

Fig. 2 Leghemoglobin and ammonia content of soybean nodules obtained from plants inoculated with E109, 163, or 366, respectively. Bars indicate the level of leghemoglobin in nodules induced by each isolate 5, 6, and 7 weeks after inoculation. Lines correspond to the ammonia content found in nodules of plants inoculated with the *B. japonicum* isolates under study 6 and 7 weeks after inoculation
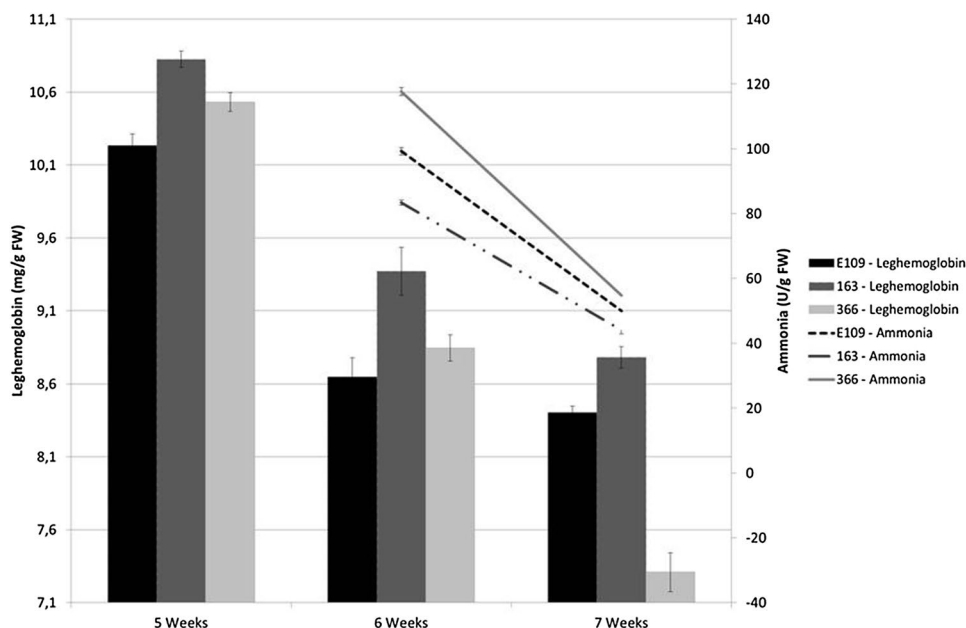
Discussion

The use of plant growth-promoting bacteria is crucial to produce crops and vegetables in a sustainable management system of production. Rhizobia are probably the most important and frequently used PGPR Gram (−) bacteria. They establish interactions either with legumes, a symbiotic one at the root level that fixes nitrogen, or with non-legumes. In such interactions, alternative mechanisms of growth promotion are phosphate solubilization and siderophore production [9], systemic resistance induction [40], and biosynthesis of hormones [3, 7, 8]. In both cases, interactions with legumes or non-legumes, bradyrhizobia might contribute to improve soil fertility and N availability [2].

The technology to produce soybean includes the addition of a bacterial suspension of N-fixing bacteria at seeding, a process known as inoculation. Soybean was introduced in Argentina in the early 1970s and since then, the cultivated area with it, along the country, has increased substantially. Because of this, the soils of Argentina nowadays contain naturalized populations of rhizobia. One of the first strains used as inoculant was USDA138 and a re-isolation of it, which is the most efficient strain used in Argentina, has been identified as E109 and has been and still is recommended by INTA to formulate commercial inoculants. It has an outstanding ability to fix nitrogen, compete, and survive [17, 43, 54]. Recently Torres et al. [54] sequenced the genome of E109, which has characteristic features already described in other bradyrhizobia.

In the fields of Argentina, even if you use high-quality inoculants, soybean develops nodules that are frequently occupied by naturalized isolates of rhizobia that are mostly adapted to the soil rather than fixing nitrogen efficiently [38]. Soils in Argentina were initially devoid of soybean rhizobia; all naturalized populations might have their origin in commercial inoculants. These populations quite often have an outstanding competitive ability but low nitrogen-fixing capacity [6, 12]. The soils of Argentina contain highly diverse populations of bradyrhizobia [38], which might be the result of natural mutations and/or horizontal gene transfer events that occur in nature, an adaptation process of the organisms to improve their biological fitness [12, 19]. However, despite considerable efforts, it has been and still is particularly difficult to identify more effective N_2-fixing strains of *B. japonicum* [30]. Regarding this, we identified, among two hundred isolates, two *B. japonicum* isolates, 163 and 366 that based on biomass production, an indirect index of N availability, fix more N than *B. japonicum* E109.

Nitrogenase activity remained the same whether the plants were inoculated with control strain E109 or any of the collected isolates. However, isolate 366 developed more and larger nodules and soybeans inoculated with this strain presented a larger biomass, probably due to a higher amount of available N. The other isolate 163 might have a different strategy, since nodulation was not different from that induced by E109 but still soybean plants have a larger biomass. Evidently, these two isolates 163 and 366 are potential resources to develop new inoculants since plants inoculated with any of the isolates performed better than E109. Interestingly, it appears that the N-fixing ability of each isolate relies on different mechanisms or strategy.

Young non-fixing nodules have white interiors that later evolve to mature nitrogen-fixing nodules with red interiors that after 20–30 days senesce developed a brownish color in

the interior [13, 47], typical changes that occur in senescing nodules. Senescence is a complex and programmed process that leads to a reduction in N_2-fixing activity and leghemoglobin content in aging nodules [4]. The interior of nodules induced by isolate 163, 366, and E109 6 weeks after inoculation differed in the color of their interiors suggesting that they might be in a different developmental stage. The amounts of leghemoglobin and ammonia in 6–7-week-old nodules formed by *B. japonicum* 163 also suggest that these nodules were at a different developmental stage than those induced by *B. japonicum* 366 or E109. While the latter ones are senescing, those induced by isolate 163, are still active. These changes in color of nodules interiors have been attributed to the activity of a plethora of proteases [46, 56]. Federova [14] found that a cluster of Cys-proteins are induced in nodules before N fixation commences activity that is extended throughout nodule senescence. So, the larger biomass of plants inoculated with isolate 163 might be due to the fact that more N was provided by nodules that remain actively fixing nitrogen for a longer period of time.

Ethylene is the plant hormone related with senescence that might inhibit nodulation but it might also play a role in nitrogen-fixing nodules and nodule senescence. It has been proposed that nodulation of soybean might be improved by inhibiting ACC synthase and because of this nitrogen fixation as well [16, 34]. Alternatively, a similar effect might be achieved by inoculating rhizobia with a highly active ACC deaminase activity, which might either increase nodulation capacity and/or delay senescence, since the enzyme prevents ethylene synthesis and accumulation [53]. We measured the expression of nodulation and nitrogen fixation genes in aerated liquid cultures of control strain *B. japonicum* E109 and *B. japonicum* 163 and 366. All the genes were hardly expressed in culture. Interestingly, it has been found that expression of *nifA* and *acdS* depended on the oxygen-level. *acdS* was upregulated during free-living microoxic (4.8-fold change) and also in anoxic conditions (12-fold change) as well as in symbiosis [21, 41]. In addition, Murset et al. [32] found that *acdS* was expressed at high levels in symbiosis compared to cells grown in aerobic cultures. Furthermore, they showed that *acdS* expression was dependent on the expression of transcription factor genes *nif*A and *rpoN*, as it was described for *Mesorhizobium* [37, 55]. In agreement with this we found that *nifA* and *fixL* as well as *acdS* were expressed at high levels in symbiosis but not under oxic environments. The dependence of *acdS* expression on the transcription factor NifA explains the relationship of the oxic conditions and *acdS* expression, and so there might not be a straight relationship between nitrogen fixation and *acdS* expression. Interestingly, a proteomic study of *B. japonicum* revealed that ACC deaminase is abundant in root-nodule bacteroides of soybean, siratro, and cowpea [11, 23]. Furthermore, Murset et al. [32] found high levels

of ACC activity in soybean nodules induced by ACC. In our view, in nodules induced by *B. japonicum* 163 the anoxic conditions lead to the expression of nitrogen-fixing regulatory genes, whose presence is crucial for the expression of *acdS*. The latter one is expressed at high levels in the nodules, and most probably because of this degradation of the enzymes involved in nitrogen fixation including all the necessary accessory proteins needed for the process is delayed.

Conclusions

B. japonicum isolates 163 and 366 were more efficient in fixing nitrogen than the highly proficient strain E109. While isolate 366 induced a larger nodule biomass, nodules induced by isolate 163 remained with red interiors due to leghemoglobin for a longer period. *acdS*, an ACC deaminase coding gene, was expressed only in nitrogen-fixing nodules and at higher levels in nodules induced by isolate 163, this probably reduced the amount of ethylene and delayed nodule senescence. We confirmed that ACC expression is dependent on *nifA*, whose expression is a function of the anoxic conditions. To our knowledge this is the first natural isolate described with a delayed senescence phenotype that leads to higher nitrogen fixation in plants. This study suggests that diverse naturalized rhizobia develop in the soil as a result of genetic changes that occur during adaptation. Along the process, changes generate alternative mechanisms that might make organisms more efficient nitrogen fixers that might lead to increase crop yield. So evidently, the soil is an enormous source of organisms that differ not only in their biological proficiency but also in the mechanisms that lead to their outstanding competitiveness and ability to fix nitrogen. The question arises regarding the fact that selected strains have been and still are on the market and have not been displaced by new isolates that establish more proficient symbiotic interactions.

References

1. Althabegoiti MJ, Covelli JM, Pérez-Giménez J, Quelas JI, Mongiardini EJ, López MF, López-García S, Lodeiro AR (2011) Analysis of the role of the two flagella of *Bradyrhizobium japonicum* in competition for nodulation of soybean. FEMS Microbiol Lett 319(2):133–139
2. Althabegoiti MJ, López-García SL, Piccinetti C, Mongiardini EJ, Pérez-Giménez J, Quelas JI, Perticari A, Lodeiro AR (2008) Strain selection for improvement of *Bradyrhizobium japonicum*

competitiveness for nodulation of soybean. FEMS Microbiol Lett 282(1):115–123

3. Antoun H, Beauchamp CJ, Goussard N, Chabot R, Lalande R (1998) Potential of *Rhizobium* and *Bradyrhizobium* species as plant growth promoting rhizobacteria on non-legumes: effect on radishes (*Raphanus sativus* L.). Plant Soil 204(1):57–67

4. Balestrasse KB, Gallego SM, Tomaro ML (2004) Cadmium-induced senescence in nodules of soybean (*Glycine* max *L.*) plants. Plant Soil 262(1–2):373–381

5. Bergersen FJ (1970) The quantitative relationship between nitrogen fixation and the acetylene-reduction assay. Aust J of Biol Sci 23(4):1015–1026

6. Bogino P, Banchio E, Bonfiglio C, Giordano W (2008) Competitiveness of a *Bradyrhizobium sp*. strain in soils containing indigenous rhizobia. Curr Microbiol 56(1):66–72

7. Boiero L, Perrig D, Masciarelli O, Penna C, Cassán F, Luna V (2007) Phytohormone production by three strains of *Bradyrhizobium japonicum* and possible physiological and technological implications. Appl Microbiol Biotechnol 74(4):874–880

8. Cassan F, Perrig D, Sgroy V, Masciarelli O, Penna C, Luna V (2009) *Azospirillum brasilense* Az39 and *Bradyrhizobium japonicum* E109, inoculated singly or in combination, promote seed germination and early seedling growth in corn (*Zea mays* L.) and soybean (*Glycine* max L.). Eur J Soil Biol 45(1):28–35

9. Chabot R, Antoun H, Cescas MP (1996) Growth promotion of maize and lettuce by phosphate-solubilizing *Rhizobium leguminosarum biovar. phaseoli*. Plant Soil 184(2):311–321

10. Chungopast S, Hirakawa H, Sato S, Handa Y, Saito K, Kawaguchi M, Tajima S, Nomura M (2014) Transcriptomic profiles of nodule senescence in *Lotus japonicus* and *Mesorhizobium loti* symbiosis. Plant Biotechnol 31(4):345–349

11. Delmotte N, Ahrens CH, Knief C, Qeli E, Koch M, Fischer HM, Pessi G (2010) An integrated proteomics and transcriptomics reference data set provides new insights into the *Bradyrhizobium japonicum* bacteroid metabolism in soybean root nodules. Proteomics 10(7):1391–1400

12. Dwivedi SL, Sahrawat KL, Upadhyaya HD, Mengoni A, Galardini M, Bazzicalupo M, Biondi EG, Hungría M, Kaschuk G, Blair W, Ortiz R (2015) Chapter one-advances in host plant and *Rhizobium* genomics to enhance symbiotic nitrogen fixation in grain legumes. Adv Agron 129:1–116

13. Evans PJ, Gallesi D, Mathieu C, Hernandez MJ, de Felipe M, Halliwell B, Puppo A (1999) Oxidative stress occurs during soybean nodule senescence. Plant 208(1):73–79

14. Fedorova M, Van De Mortel J, Matsumoto PA, Cho J, Town CD, VandenBosch KA, Gantt JS, Vance CP (2002) Genome-wide identification of nodule-specific transcripts in the model legume *Medicago truncatula*. Plant Physiol 130(2):519–537

15. Glick BR (2005) Modulation of plant ethylene levels by the bacterial enzyme ACC deaminase. FEMS Microbiol Lett 251(1):1–7

16. Glick BR (2014) Bacteria with ACC deaminase can promote plant growth and help to feed the world. Microbiol Res 169(1):30–39

17. González N (2007) Fijación de nitrógeno en soja. Inoculantes: situación actual y perspectivas en la Argentina. In: Thuar A, Cassan F, Olmedo C (eds) De la Biología del Suelo a la Agricultura. Universidad Nacional de Río Cuarto, Río Cuarto, pp 161–169

18. Goodlass G, Smith KA (1979) Effects of ethylene on root extension and nodulation of pea (*Pisum sativum* L.) and white clover (*Trifolium repens* L.). Plant Soil 51:387. https://doi.org/10.1007/BF02197785

19. Gordon BR, Klinger CR, Weese DJ, Lau JA, Burke PV, Dentinger B, Heath KD (2016) Decoupled genomic elements and the evolution of partner quality in nitrogen-fixing rhizobia. Ecol Evol. https://doi.org/10.1002/ece3.1953

20. Guinel FC (2015) Ethylene, a Hormone at the Center-Stage of Nodulation. Front Plant Sci 6:1121. https://doi.org/10.3389/fpls.2015.01121

21. Hauser F, Pessi G, Friberg M, Weber C, Rusca N, Lindemann A, Fischer HM, Hennecke H (2007) Dissection of the *Bradyrhizobium japonicum* NifA+ σ 54 regulon, and identification of a ferredoxin gene (*fdxN*) for symbiotic nitrogen fixation. Mol Genet Genom 278(3):255–271

22. Kwon SW, Park JY, Kim JS, Kang JW, Cho YH, Lim CK, Parker M, Lee GB (2005) Phylogenetic analysis of the genera Bradyrhizobium. *Mesorhizobium, Rhizobium* and *Sinorhizobium* on the basis of *16S rRNA* gene and internally transcribed spacer region sequences. Int J Syst Evol Microbiol 55(1):263–270

23. Koch M, Delmotte N, Rehrauer H, Vorholt JA, Pessi G, Hennecke H (2010) Rhizobial adaptation to hosts, a new facet in the legume root-nodule symbiosis. Mol Plant 23(6):784–790

24. La Rue TA, Child JJ (1979) Sensitive fluorometric assay for leghemoglobin. Anal Biochem 92(1):11–15

25. Lodeiro AR (2015) Interrogantes en la tecnología de la inoculación de semillas de soja con *Bradyrhizobium* spp. Rev Argent Microbiol 47(3):261–273

26. López SMY, Balatti PA (2012) Closely related strains of *Bradyrhizobium* contained in commercial inoculates of soybean are identified by a set of PCR reactions. Genet Eng Biotechnol J 2011:

27. López SMY, Pastorino GN, Martínez-Alcántara V, Salvucci D, Balatti PA (2013) Los rizobios que nodulan la soja en sitios con ambientes nativos y cultivados de la Argentina. In: Albanesi AS (ed) Microbiología agícola. Un aporte de la investigación en Argentina, 2ª edn. Tucumán, pp 237–252

28. López-García SL, Vázquez TE, Favelukes G, Lodeiro AR (2002) Rhizobial position as a main determinant in the problem of competition for nodulation in soybean. Environ Microbiol 4(4):216–224

29. Mano H, Morisaki H (2008) Endophytic bacteria in the rice plant. Microbes Environ 23(2):109–117

30. Melchiorre M, De Luca MJ, Anta GG, Suarez P, Lopez C, Lascano R, Racca RW (2011) Evaluation of bradyrhizobia strains isolated from field-grown soybean plants in Argentina as improved inoculants. Biol Fertil Soils 47(1):81–89

31. Mesa S, Hauser F, Friberg M, Malaguti E, Fischer HM, Hennecke H (2008) Comprehensive assessment of the regulons controlled by the FixLJ-FixK2-FixK1 cascade in *Bradyrhizobium japonicum*. J Bacteriol 190(20):6568–6579

32. Murset V, Hennecke H, Pessi G (2012) Disparate role of rhizobial ACC deaminase in root-nodule symbioses. Symbiosis 57(1):43–50

33. Nascimento FX, Brígido C, Glick BR, Oliveira S, Alho L (2012) *Mesorhizobium ciceri* LMS-1 expressing an exogenous 1-aminocyclopropane-1-carboxylate (ACC) deaminase increases its nodulation abilities and chickpea plant resistance to soil constraints. Lett Appl Microbiol 55(1):15–21

34. Nascimento FX, Brígido C, Glick BR, Rossi MJ (2016) The role of rhizobial ACC deaminase in the nodulation process of leguminous plants. Int J Agron 2016:1369472

35. Nascimento FX, Rossi MJ, Soares CR, McConkey BJ, Glick BR (2014) New insights into 1-aminocyclopropane-1-carboxylate (ACC) deaminase phylogeny, evolution and ecological significance. PLoS ONE 9(6):e99168

36. Navascués J, Pérez-Rontomé C, Gay M, Marcos M, Yang F, Walker F, Desbois A, Abián J, Becana M (2012) Leghemoglobin green derivatives with nitrated hemes evidence production of highly reactive nitrogen species during aging of legume nodules. Proc Natl Acad Sci USA 109(7):2660–2665

37. Nukui N, Minamisawa K, Ayabe SI, Aoki T (2006) Expression of the 1-aminocyclopropane-1-carboxylic acid deaminase

gene requires symbiotic nitrogen-fixing regulator gene *nifA2* in *Mesorhizobium loti* MAFF303099. Appl Environ Microbiol 72(7):4964–4969

38. Pastorino GN (2016) Diversidad de los rizobios que nodulan la soja en los suelos de la Pampa húmeda e identificación de cepas para la fabricación de inoculantes comerciales. Doctoral dissertation, Facultad de Ciencias Naturales y Museo. http://hdl.handle.net/10915/55778

39. Penmetsa RV, Frugoli JA, Smith LS, Long SR, Cook DR (2003) Dual genetic pathways controlling nodule number in *Medicago truncatula*. Plant Physiol 131(3):998–1008

40. Persello-Cartieaux F, Nussaume L, Robaglia C (2003) Tales from the underground: molecular. Plant Cell Environ 26(2):189–199

41. Pessi G, Ahrens CH, Rehrauer H, Lindemann A, Hauser F, Fischer HM, Hennecke H (2007) Genome-wide transcript analysis of *Bradyrhizobium japonicum* bacteroids in soybean root nodules. Mol Plant 20(11):1353–1363

42. Pfeiffer NE, Torres CM, Wagner FW (1983) Proteolytic activity in soybean root nodules. Plant Physiol 71(4):797–802

43. Piccinetti C, Arias N, Ventimiglia L, Díaz-Zorita M, Murua L, Sánchez H, Ferraris G, Mousegne F, Fontanetto H, Sá Pereira E, Capurro J, Enrico JM, López C, Carrizo AS, Salvagiotti F, Collino D, Perticari A (2013) Efectos positivos de la inoculación de soja sobre la nodulación, la FBN y en los parámetros de producción del cultivo. In: Albanesi AS (ed) Microbiología agícola. Un aporte de la investigación en Argentina, (2ª edn) Tucumán, Argentina, pp 283–297

44. Pladys D, Rigaud J (1985) Senescence in French-bean nodules: Occurrence of different proteolytic activities. Physiol Plant 63(1):43–48

45. Pladys D, Rigaud J (1988) Lysis of bacteroids in vitro and during the senescence in *Phaseolus vulgaris* nodules. Plant Physiol Biochem (France) 26:179–186

46. Pladys D, Vance CP (1993) Proteolysis during development and senescence of effective and plant gene-controlled ineffective alfalfa nodules. Plant Physiol 103(2):379–384

47. Puppo A, Groten K, Bastian F, Carzaniga R, Soussi M, Lucas M, de Felipe M, Harrison J, Vanacker H, Foyer C (2005) Legume nodule senescence: roles for redox and hormone signalling in the orchestration of the natural aging process. New Phytol 165(3):683–701

48. Rosenblueth M, Martínez-Romero E (2006) Bacterial endophytes and their interactions with hosts. Mol Plant-Microbe Interact 19(8):827–837

49. Santoyo G, Moreno-Hagelsieb G, Orozco-Mosqueda M, Glick BR (2016) Plant growth-promoting bacterial endophytes. Microbiol Res 183:92–99

50. Subramanian P, Kim K, Krishnamoorthy R, Sundaram S, Sa T (2015) Endophytic bacteria improve nodule function and plant nitrogen in soybean on co-inoculation with *Bradyrhizobium japonicum* MN110. Plant Growth Regul 76(3):327–332

51. Sullivan J, Trzebiatowski J, Cruickshank R, Gouzy J, Brown S, Elliot R, Fleetwood D, McCallum N, Rossbach U, Stuart G, Weaver J, Webby R, de Bruijn F, Weaver JE (2002) Comparative sequence analysis of the symbiosis island of *Mesorhizobium loti* strain R7A. J Bacteriol 184(11):3086–3095

52. Tittabutr P, Awaya JD, Li QX, Borthakur D (2008) The cloned 1-aminocyclopropane-1-carboxylate (ACC) deaminase gene from *Sinorhizobium sp*. strain BL3 in *Rhizobium sp*. strain TAL1145 promotes nodulation and growth of *Leucaena leucocephala*. Syst Appl Microbiol 31(2):141–150

53. Tittabutr P, Sripakdi S, Boonkerd N, Tanthanuch W, Minamisawa K, Teaumroong N (2015) Possible role of 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity of *Sinorhizobium sp*. BL3 on symbiosis with mung bean and determinate nodule senescence. Microbes Environ 30(4):310

54. Torres D, Revale S, Obando M, Maroniche G, Paris G, Perticari A, Vazquez M, Wisniewski-Dyé F, Martínez-Abarca F, Cassán F (2015) Genome sequence of *Bradyrhizobium japonicum* E109, one of the most agronomically used nitrogen-fixing rhizobacteria in Argentina. Genome Announc 3(1):e01566-14

55. Uchiumi T, Ohwada T, Itakura M, Mitsui H, Nukui N et al (2004) Expression islands clustered on the symbiosis island of the *Mesorhizobium loti* genome. J Bacteriol 186:2439–2448

56. Van de Velde W, Guerra JCP, De Keyser A, De Rycke R, Rombauts S, Maunoury N, Mergaert P, Kondorosi E, Holsters M, Goormachtig S (2006) Aging in legume symbiosis. A molecular view on nodule senescence in *Medicago truncatula*. Plant Physiol 141(2):711–720

57. Vance CP, Heichel GH, Barnes DK, Bryan JW, Johnson LE (1979) Nitrogen fixation, nodule development, and vegetative regrowth of alfalfa (*Medicago sativa* L.) following harvest. Plant Physiol 64(1):1–8

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