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Alicia Castillo 
Instituto Nacional de Investigación
Agropecuaria (INIA), Canelones,
Uruguay.

Paul Chavarriaga 
Centro Internacional de
Agricultura Tropical (CIAT), Cali,
Colombia.

Correspondence

Virginia Martínez Alcántara,
vmalcan@gmail.com

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Bacterial endophytes diversity of tree legumes from Argentina

Diversidad de endófitos bacterianos aislados de leguminosas arbóreas de Argentina

Diversidade de endófitos bacterianos de leguminosas arbóreas da Argentina

Martínez Alcántara, V. ¹; Medina, R. ¹; Gauna, J. M. ¹; Balatti, P. A. ¹

¹Universidad Nacional de La Plata, Facultad de Ciencias Agrarias y Forestales, Cátedra de Microbiología Agrícola, La Plata, Argentina.



Abstract

The aim of this work was to isolate and characterize, based on microbiological as well as molecular markers, bacteria within nodules of three native trees species from South America: *Poecilanthe parviflora* Benth (Lapachillo), *Vachellia caven* (Molina) Seigler & Ebinger (Espinillo), and *Enterolobium contortisiliquum* (Vell.) Morong (Timbó). In line with this, we isolated and characterized 15 bacteria from *P. parviflora*, 6 from *V. caven* and 1 from *E. contortisiliquum*. Among them we identified representatives of *Bradyrhizobium* sp. from *E. contortisiliquum* and *P. parviflora*, as well as of *Ensifer* sp. (*Sinorhizobium*) and *Mesorhizobium* sp. from *V. caven*. Also, other bacteria were found, like representatives of *Bacillus* sp., *Microbacterium* sp. and *Curtobacterim* sp., as well as *Caulobacter* sp., *Dyella* sp., *Pseudomonas* sp. and *Enterobacter* sp. Some of these isolates inhibited the growth of two fungal plant pathogens. So, nodules of these native legumes not only are occupied by highly diverse rhizobia but also by other bacteria that might promote plant growth.

Keywords: biodiversity, legumes, rhizobia, symbiosis

Resumen

El objetivo de este trabajo fue aislar y caracterizar, con base en características microbiológicas y marcadores moleculares, bacterias de los nódulos de tres especies nativas de árboles de América del Sur: *Poecilanthe parviflora* Benth (Lapachillo), *Vachellia caven* (Molina) Seigler & Ebinger (Espinillo), y *Enterolobium contortisiliquum* (Vell.) Morong (Timbó). En línea con esto se aislaron y caracterizaron 15 bacterias de *P. parviflora*, 6 de *V. caven*, y 1 de *E. contortisiliquum*. Se identificaron representantes de *Bradyrhizobium* sp. de *E. contortisiliquum* y de *P. parviflora*, así como también representantes de *Ensifer* sp. (*Sinorhizobium*) y de *Mesorhizobium* sp. de *V. caven*. Además, se encontraron bacterias de los géneros *Bacillus* sp., *Microbacterium* sp. y *Curtobacterim* sp., así como también representantes de *Caulobacter* sp., *Dyella* sp., *Pseudomonas* sp. y *Enterobacter* sp. Algunos aislados inhibieron el crecimiento de dos hongos fitopatógenos. En síntesis, los nódulos de las leguminosas nativas no solo están ocupados por una población de rizobios diversa sino también con otras bacterias que podrían promover el crecimiento de las plantas.

Palabras clave: biodiversidad, leguminosas, rizobios, simbiosis

Resumo

O objetivo deste trabalho foi isolar e caracterizar, com base em características microbiológicas e marcadores moleculares, bactérias dos nódulos de três espécies arbóreas nativas da América do Sul: *Poecilanthe parviflora* Benth (Lapachillo), *Vachellia caven* (Molina) Seigler & Ebinger (Espinillo) e *Enterolobium contortisiliquum* (Vell.) Morong (Timbó). Em consonância com isso, foram isoladas e caracterizadas 15 bacterias de *P. parviflora*, 6 de *V. caven* e 1 de *E. contortisiliquum*. Foram identificados representantes de *Bradyrhizobium* sp. de *E. contortisiliquum* e de *P. parviflora*, bem como representantes de *Ensifer* sp. (*Sinorhizobium*) e *Mesorhizobium* sp. por *V. caven*. Os nódulos continham outras bacterias dos gêneros *Bacillus* sp., *Microbacterium* sp. e *Curtobacterim* sp. bem como representantes de *Caulobacter* sp., *Dyella* sp., *Pseudomonas* sp. e *Enterobacter* sp. Alguns isolados inibiram o crescimento de dois fungos fitopatogênicos. Em síntese, os nódulos das leguminosas nativas não são ocupados apenas por uma população diversa de rizobia, mas também por outras bactérias que podem promover o crescimento das plantas.

Palavras-chave: biodiversidade, leguminosas, rizobia, simbiose



1. Introduction

The Leguminosae (Fabaceae) is the third-largest plant family among angiosperms that comprises 20,000 species and 750 genera⁽¹⁾⁽²⁾⁽³⁾⁽⁴⁾. Legumes establish a symbiotic association with Gram-negative soil bacteria known as rhizobia⁽¹⁾⁽⁵⁾ that results in the development of nodules in roots and stems, where atmospheric nitrogen is reduced (fixed) to ammonia, that is assimilated by plants into organic compounds. The family of legumes is divided into three subfamilies that include mostly plants adapted to tropical regions, Cesalpinoidea, Papilinoidea and Mimosoidea; within the former one, only 11 genera form nitrogen-fixing nodules⁽¹⁾⁽⁶⁾. Rhizobia are clustered within the Alpha and Betaproteobacteria⁽⁷⁾, intermingled with non-symbiotic, photosynthetic and plant pathogenic genera and distributed in at least 14 genera⁽⁷⁾. Rhizobia vary in specificity as well as legumes, while some of them nodulate several hosts, others nodulate only one or two species. Furthermore, several hosts are nodulated by several rhizobia, including both Alpha and Betaproteobacteria. In other cases, the rhizobial strain-host genotype interaction is highly specific, to the point that two closely related species of plants are nodulated by different species of rhizobia⁽⁷⁾.

Legume nodules induced by rhizobia also might be co-inhabited by a set of various Gram-positive and Gram-negative bacteria, some of them unable to induce nodule development⁽¹⁾⁽⁶⁾⁽⁷⁾⁽⁸⁾. These endophytic bacteria can promote plant growth through many different mechanisms and they can do so in symbiosis or as free-living cells. Plant Growth-Promoting Bacteria (PGPB) may act directly by increasing the availability of nutrients such as P solubilization or N₂ fixation, by influencing plant hormone levels, or indirectly by attenuating the effects of pathogens⁽⁹⁾⁽¹⁰⁾, increasing in this way host fitness. In line with this, rhizobia also may act as PGPB in non-legume cash-crops such as rice or wheat, which turn out to be some of the best beneficial interaction between endophytic rhizobia and plants⁽¹¹⁾.

Both tree legumes and actinorrhic plants contribute to increasing N in soils, which is the main reason to consider such systems as a sustainable strategy to

improve the productive potential of many different agroecosystems⁽¹²⁾, to manage silvicultural systems or to prevent soil degradation and N depletion as well. Many legume trees contribute N to tropical wetlands and rainforests, and because of this, it is necessary to have a better understanding of such systems to improve the use and conservation of natural resources⁽¹³⁾. Additionally, such systems might be exploited in reforestation as well as in land restoration⁽¹⁴⁾. A good example of such systems is the *Acacia* sensu lato species, highly tolerant to drought, due to their large root systems that not only lead plants to explore more soil but also to access deeper layers of water. *Vachellia* sp. are fast-growing plant species pioneers in extreme environments due to their nitrogen-fixing ability, and also to other symbioses they establish with fungi (e.g. endo and/or ectomycorrhiza), which are critical for their role in poor and eroded soils⁽¹⁴⁾. Many legumes might be relevant tools to increase soil organic matter in such systems. Species of *Poecilanthe* grow in northeast Brazil, in arid areas where water is the main limiting factor. In such ecosystems, a subsistence agriculture mainly based in extensive livestock prevail, where pastures of native legumes are among the most important sources of cow food.

Considering the potential of tree legumes, several studies have been conducted in South America⁽¹²⁾⁽¹³⁾⁽¹⁴⁾⁽¹⁵⁾⁽¹⁶⁾⁽¹⁷⁾⁽¹⁸⁾⁽¹⁹⁾⁽²⁰⁾⁽²¹⁾⁽²²⁾⁽²³⁾⁽²⁴⁾⁽²⁵⁾⁽²⁶⁾⁽²⁷⁾⁽²⁸⁾⁽²⁹⁾⁽³⁰⁾. They consisted in the isolation of rhizobia and the study of nodule morphology, biochemical and genetic characteristics, systematics and phylogenetic relationships of bacterial symbionts, as well as their ability to nodulate and fix N. However, there are not very many reports regarding species like *P. parviflora* (Lapachillo), an endemic tree to South America, *Vachellia caven* (Espinillo) and *E. contorsiliquum* (Timbó). Regarding the genus *Poecilanthe*, there are only a few reports of bacteria isolated from nodulated plants growing in Brazil⁽¹³⁾⁽³⁰⁾. Thus, it is of great interest to study the rhizobia as well as other bacteria (nodule microbiome) associated with *Poecilanthe* sp., *Vachellia* sp., and *Enterolobium* sp. in South-American ecosystems, considering their role in nature, particularly regarding legumes growth and development⁽³¹⁾.



The aim of this work was to isolate and characterize, based on microbiological as well as molecular markers, bacteria within nodules of three native trees species from South America: *Poecilanthe parviflora* Benth, *Vachellia caven* (Molina) Seigler & Ebinger, and *Enterolobium contortisiliquum* (Vell.) Morong. Such information might lead to make a better use of the potential of these native legumes in complex environments.

2. Materials and methods

2.1 Source of bacterial endophytes and biological material

Composite samples of soil were collected from places located under the canopy of *P. parviflora* plants. Nodules were collected from roots of plants growing within the marginal rain forest of Martín García Island, a protected area located at 34°11'19"S, 58°15'00"W; 14 m.a.s.l (meters above sea level), Buenos Aires, Argentina, belonging to the Delta and Islas del Paraná ecoregion⁽³²⁾. Nodules were maintained in hermetic containers until they were processed, which occurred within 48 h. Collected plants were transferred and maintained in 3 L pots filled with soil from Martín García Island and vermiculite at a 1:1 ratio. Pots were watered every other day with distilled water.

Nodules collected from plant roots were surface sterilized by immersing them 2 min. in ethanol 50% followed by 2 min. in sodium hypochlorite 4 g L⁻¹. Then, they were rinsed at least 10 times with sterile distilled water. Sterilization of nodule surfaces was checked by rolling them on plates filled with: a) Yeast Extract Mannitol Agar (YMA)⁽³³⁾ K₂HPO₄ 0,5 g L⁻¹, MgSO₄. 7 H₂O 0,2 g L⁻¹, NaCl 0,1 g L⁻¹, CO₃Ca 1 g L⁻¹, yeast extract 1 g L⁻¹, mannitol 10 g L⁻¹, congo red (1/400) 10 ml, Agar 15 g L⁻¹, Ph 6.8; b) Cetrimide agar⁽³⁴⁾ (gelatin peptone 20 g L⁻¹, magnesium chloride 1.4 g L⁻¹, potassium sulfate 10 g L⁻¹, cetyltrimethylammonium bromide 0.3 g L⁻¹, Agar 15 g L⁻¹), and c) Nutrient agar⁽³⁵⁾ (peptone 5 g L⁻¹, meat extract 3 g L⁻¹, sodium chloride 8 g L⁻¹, Agar 15 g L⁻¹).

Isolations were made according to Vincent⁽³³⁾ using the described YMA and Nutrient agar media and inoculated plates were incubated at 28 °C. After 7-day-incubation period colonies were subcultured

until pure cultures of the isolates were obtained. Then, isolated bacteria were grown in YEM broth⁽³³⁾, until saturation and aliquots were mixed with glycerol to make a final concentration of 10% in stocks that were maintained at -80 °C.

V. caven and *E. contortisiliquum* nodule bacteria were obtained as described before, from nodules developed by inoculated plants. Seeds of these species were surface scarified and sterilized with sulfuric acid for 15 min., rinsed with sterile water and germinated in 10% water-agar in Petri dishes in the dark. Seedlings were transplanted into pots with sterile vermiculite and inoculated with 1 ml of soils decimal dilution in sterile water. Soils were collected at 24°48'52.3"S 65°29'59.6"W, 1.300 m.a.s.l. for *V. caven*. and at 24°35'18.7"S 65°02'50"W, 750 m.a.s.l. for *E. contortisiliquum*, from the dry Chaco ecoregion⁽³²⁾, Salta, Argentina. Stock cultures of isolated bacteria were made and kept as described before. Also, reference strains *B. japonicum* E109, SEMIA 5079, *B. elkanii* SEMIA 5019 and SEMIA 587, as well as the type strain *Mesorhizobium Chacoense* Pr5 (=LMG19008=CECT5336) isolated from *Prosopis alba*⁽³⁶⁾ at the dry Chaco ecoregion⁽³²⁾, Chancaní Reserva, Córdoba, Argentina, were included in this work.

2.2 Microbiological characterization

All isolates were observed with the light field optical microscope (1000X) after Gram staining⁽³⁷⁾. Alkalization or acidification of the culture medium was determined in non-buffered YEM broth supplemented with bromothymol blue, after 10 days of growth, at 28 °C in a rotary shaker at 250 rev. min⁻¹. Isolates were classified as developing a strong, moderately or slightly acid reaction based on the yellow colour developed, while neutral and basic reactions were associated with green and blue colours, respectively.

Bacterial isolates were grown for 10 days at 28 °C on NBRI-P (National Botanical Research Institute's phosphate)⁽³⁸⁾ medium (glucose 10g L⁻¹, Ca₃PO₄ 5 g L⁻¹, MgCl₂.6H₂O 5 g L⁻¹, KCl 0,2 g L⁻¹, (NH₄)₂SO₄ 0.1g L⁻¹, SO₄Mg.7 H₂O, Agar 20g L⁻¹) to evaluate their ability to solubilize inorganic phosphorous that was determined by the development of a transparent halo around bacterial colonies.



The antimicrobial activity of isolates was assayed by co-culturing them with two model plant pathogens, *Stemphylium lycopersici* CIDEFI 216, the causal agent of grey leaf spot⁽³⁹⁾, and *Fusarium graminearum* SP1, the etiological agent of head blight⁽⁴⁰⁾. These two pathogens have a wide host range, representatives of them have been reported to provoke diseases on legumes and are used as model systems to study plant-microbe interactions in our laboratory. Both fungi were grown on PDA (Potato Dextrose Agar, potato 250 g L⁻¹, dextrose 20 g L⁻¹, agar 20 g L⁻¹)⁽⁴¹⁾ medium, for 3 (*F. graminearum*) or 10 (*S. lycopersici*) days at 25 °C in the darkness. Antimicrobial activity was evaluated by placing bacteria on PDA medium in two equidistant parallel streaks. After an incubation period of approximately 7 days at 25 °C, a disc of the fungus was placed at the center of each Petri dish. Control treatment consisted of plates inoculated with fungi in the absence of bacteria. All plates were sealed with parafilm and incubated at 25 °C. The experiment finished within 3 (*F. graminearum*) or 10 (*S. lycopersici*) days.

The inhibitory activity of bacteria was estimated by measuring mycelial growth on the surface of the Petri dishes using the software Image tool 3.0 (Image Tool Software Copyright)⁽⁴²⁾. The percentage of inhibition (InC) was calculated with the formula: % InC = [(control mycelial growth area - treatment mycelial growth area)/control mycelial growth area]*100⁽⁴³⁾. Bacterial isolates ability to solubilize inorganic phosphorous and their antagonistic effect against *S. lycopersici* and *F. graminearum* were evaluated in three independent experiments. The collected data were subjected to a one-way analysis of variance (ANOVA), means were compared by the Tukey test. All statistical analyses were performed using Infostat version 2015⁽⁴⁴⁾.

2.3 Molecular analysis

2.3.1 DNA extraction and genetic diversity analysis

Isolates DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega)⁽⁴⁵⁾. Briefly, isolated bacteria were cultured in liquid media until a cell concentration of 1×10⁹ cells mL⁻¹ was obtained. Aliquots of 5 mL were used to extract DNA, whose quality and quantity was checked by electrophoresis in 0.7% agarose gels stained with ethidium bromide that included a molecular marker⁽⁴⁶⁾.

Endophytic bacterial isolates were fingerprinted using a BOX-PCR with the universal BOXA1R primer (5'-CTACGGCAAGGCGACGCTGACG-3'). PCR contained 1x amplification buffer (Inbio Highway), 2.5mM MgCl₂ (Inbio Highway), 50 pmol BOXA1R primer, 2 mM each dNTP (Inbio Highway), 50-100 ng of genomic DNA, and 0.6 U Taq DNA polymerase (Inbio Highway) in a 15 ul volume. Reactions were performed in a PTC-1152 Mini Cycler (MJ Research) programmed as follows: an initial step at 94 °C for 7 min, followed by 35 cycles at 94 °C for 1 min, 53 °C for 1 min, and a final step at 65 °C for 8 min, and a final cycle at 65 °C for 16 min⁽⁴⁷⁾. The PCR amplicons were separated on 1.5% agarose gels, stained with ethidium bromide.

The sizes of the fragments were normalized according to the MW of the DNA markers (λ HindIII marker, Invitrogen). Fingerprints were analyzed in a binary data matrix, scoring 0 for the absence and 1 for the presence of bands. A multivariate analysis was carried out using Past3 software⁽⁴⁸⁾. The Dice similarity index was used to create a similarity matrix that was used to generate a dendrogram using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm⁽³⁹⁾. All those bacterial cultures that had a unique fingerprint were selected for 16S rDNA analysis.

2.3.2 16S rDNA amplification and sequencing

Bacterial isolates were identified using a partial sequence of the 1500 bp 16SrDNA. Such fragments were amplified by PCR in a thermocycler (Minicycler™, MJ Research Inc., Waltham, MA, USA), using primers 27F (5'-AGAGTTT-GATCMTGGCTCAG-3') and 1492R (5'-ACGGTTACCTTGTTACGACTT-3') as described by Reysenbach and others⁽⁴⁹⁾. PCR products were purified as described by Sambrook and others⁽⁵⁰⁾, precipitated and sequenced at MACROGEN Inc. (Seoul, South Korea). Sequences were analysed and trimmed with Geneious R9 software version R9.0.5, Biomatters⁽⁵¹⁾. Sequences were analysed by Basic Local Alignment Search Tool (BLAST) against 16S rDNA sequences of type strains.

The phylogenetic analysis was conducted using Geneious R9 software⁽⁵¹⁾. Sequences were aligned using the default parameters of the ClustalW algorithm (gap opening penalty 15, gap extension penalty



6.66)⁽⁵²⁾. Phylogenetic analysis was performed using the genetic distance model as described by Tamura- Nei using the neighbor-joining method⁽⁵³⁾.

3. Results and discussion

3.1 Isolation of endophytic bacteria from nodules of tree legumes from Argentina

The isolates collected from nodules of native legume trees included in this work are presented in Table 1. All *P. parviflora* isolates were obtained from root nodules belonging to marginal rain forest plants, which was the only environment in the Martín García Island, among the three sampled sites (riparian forest, marginal rain forest and the coronillo forest). We found *P. parviflora* plants with functional indeterminate nodules whose interior were red, suggesting they contain leghemoglobin and are fixing nitrogen. In the riparian and the coronillo forests, samples of *P. parviflora* plants had no nodules or non-functional ones. This may be due to the different soil texture classes, frequent flooding of these soils and/or variations in legume species composing the flora at the different vegetative units, associated to the environmental gradient of the island⁽⁵⁴⁾⁽⁵⁵⁾, which might influence and/or determine the pools of resident bacteria in the soil.

Among isolated bacteria, 15 were obtained from *P. parviflora*, 6 from *V. caven* and 1 from *E. contortisiliquum*. Interestingly, the isolates from *P. parviflora* were recovered, some of them on one media and the other ones on other media, suggesting that nodules probably harbour a diverse array of bacteria.

3.2 Microbiological characteristics of the isolates

Four bacteria among those collected from *P. parviflora* and the *E. contortisiliquum* isolate alkalinize the culture medium, while 1 from *M. chacoense*, 7 from *P. parviflora* and all 6 isolated from nodules of *V. caven* acidified the media. However, while some of them generated a slight acid reaction, others generated a strong one on the same media. The other 4 bacteria isolated from *P. parviflora* did not alter the pH of the medium. The main microbiological characteristics of the isolates are presented in supplementary material (Table

S1). It is known that while *Bradyrhizobium* sp. alkalinize the YEM broth, *Ensifer* (*Sinhorhizobium*) acidified it and *Mesorhizobium* species activity on the culture broth is dependent on the carbon source of the media⁽³⁶⁾.

Table 1. Bacterial isolates obtained from nodulated plants and control strains used in our experiments. Host species and the media used to isolate bacteria as well as the site of material source (soil sample or nodule plant collected in situ) used for the bacterial isolation are presented. YMA: Yeast Extract Mannitol Agar supplemented with congo red; NA: Nutrient Agar. The isolates were named according to the host of origin E: *Enterolobium*, AC: *Acacia* (*Vachellia*) *caven* and LA: Lapachillo. Numbers before and after the letters indicate the number of the isolate, except for the denominations as an "S" followed by number (e.g. S43), which indicate different soil sites.

Isolates	Host	Isolation media	Site of material source
4ES6-1	<i>E. contorsiliquum</i>	YMA	Salta
ACS43	<i>V. caven</i>	YMA	Salta
9ACS43	<i>V. caven</i>	YMA	Salta
11AC3	<i>V. caven</i>	YMA	Salta
ACS41	<i>V. caven</i>	YMA	Salta
AC4S41	<i>V. caven</i>	YMA	Salta
5ACS4-2	<i>V. caven</i>	YMA	Salta
LA33A2	<i>P. parviflora</i>	YMA	Buenos Aires
LA11	<i>P. parviflora</i>	YMA	Buenos Aires
LA13	<i>P. parviflora</i>	YMA	Buenos Aires
LA2	<i>P. parviflora</i>	YMA	Buenos Aires
LA32B1	<i>P. parviflora</i>	YMA	Buenos Aires
LA32A2	<i>P. parviflora</i>	YMA	Buenos Aires
LA32A1	<i>P. parviflora</i>	YMA	Buenos Aires
LA35B	<i>P. parviflora</i>	YMA	Buenos Aires
LA33A1	<i>P. parviflora</i>	YMA	Buenos Aires
LA32B2	<i>P. parviflora</i>	YMA	Buenos Aires
LA26	<i>P. parviflora</i>	YMA	Buenos Aires
LA21	<i>P. parviflora</i>	NA	Buenos Aires
LA4	<i>P. parviflora</i>	NA	Buenos Aires
LA10	<i>P. parviflora</i>	NA	Buenos Aires
LA8	<i>P. parviflora</i>	YMA	Buenos Aires



We first screened the isolates to identify their capacity to inhibit *S. lycopersici* and *F. graminearum* growth (data not shown). *B. japonicum* SEMIA5079 and isolates 4ES6-1, 5ACS4-2, LA33A2 and LA8 inhibited fungal growth at values <10%. Based on these results we run additional experiments with selected isolates and reference strains that inhibited mycelial growth $\geq 10\%$ of any of the pathogenic fungi assayed. Among isolates of *P. parviflora*, *V. caven* and *E. contorsiliquum* analyzed (Figure 1), 11 of them inhibited the growth of *F. graminearum* and/or *S. lycopersici* within a range of 19% to 72% and 41% to 95%, respectively. Both fungal pathogens *F. graminearum* and *S. lycopersici* were inhibited by the following 5 isolates of *P. parviflora*: LA4, LA10, LA 11, LA21 and LA26. The former pathogen was inhibited in a 34%, 23%, 68%, 72% and 26%, and the latter one in an 82%, 80%, 91%, 95% and 43%, respectively. Also, isolate 9ACS43 obtained from *V. caven* inhibited *F. graminearum* and *S. lycopersici* in a 19% and 41%, respectively. The commercial strain *B. elkanii* 5019 behaved like the isolates described, inhibiting *F. graminearum* and *S. lycopersici* by a 23 and 49%, respectively (Figure 1).

Two isolates collected from *V. caven* (ACS43 and 11AC3) and three from *P. parviflora* (LA13, LA32B2, LA35B) as well as *M. chacoense* only inhibited the growth of *S. lycopersici* or *F. graminearum* (Figure 1) with values on *S. lycopersici* of 44% (LA13) and 64% (*M. chacoense*), and 28% (ACS43), 29% (LA32B2), 36% (LA35B) and 52% (11AC3) for *F. graminearum*. Among the isolates that proved to have a considerable capacity to inhibit fungal growth, only isolate 11AC3 that was collected from nodules of *V. caven* was able to solubilize phosphorus, suggesting that it might be a promising plant growth-promoting bacteria that not only control

plant pathogens but also might help plants to access more P, that most of the time is in unavailable forms in the soil.

3.3 Genetic studies: diversity and identification of endophytic bacteria.

Molecular biology provided researchers with powerful methods for the typing and genetic identification of organisms. Such information is crucial and is used to perform phylogenetic analysis, since universal conserved gene sequences such as the 16S rDNA are used. DNA-based methods have become increasingly important to identify and characterize rhizobia, in particular, phylogenetic analyses of sequences of the 16S ribosomal rDNA (*rRNA*) gene, a range of "housekeeping" genes and genes involved in symbiosis have been used and proposed as a "standard approach" for the final identification of organisms. Concomitantly, the 16S rDNA gene sequence on its own turn out to be a reliable tool mainly for the preliminary identification of microorganisms⁽⁵⁶⁾. However, several gene sequences like those of the *nif* and *nod* genes involved in N₂ fixation and legume host specificity are often carried on plasmids or symbiotic islands in many nitrogen-fixing bacteria, and therefore, might be horizontally transferred between different species within a genus and less frequently across genera⁽⁵⁷⁾⁽⁵⁸⁾; because of this their relevance or role in identification has to be cautiously considered.

Based on the reasons described we compared the genome fingerprints generated using the BOXAR1 primer of isolated bacteria with those of *B. diazoefficiens* (SEMIA 5080), *B. japonicum* (E109 and SEMIA 5079), *B. elkanii* (SEMIA 5019 and SEMIA 587), and *M. Chacoense* type strain using the BOXAR1 primer PCR (Figure 2).



Figure 1. Growth inhibition in vitro of fungal pathogens *F. graminearum* and *S. lycopersici* by 18 bacterial isolates. *B. elkanii* 5019 and 587, *B. diazoefficiens* SEMIA5080, *B. japonicum* E109 and *M. chacoense* were included as controls

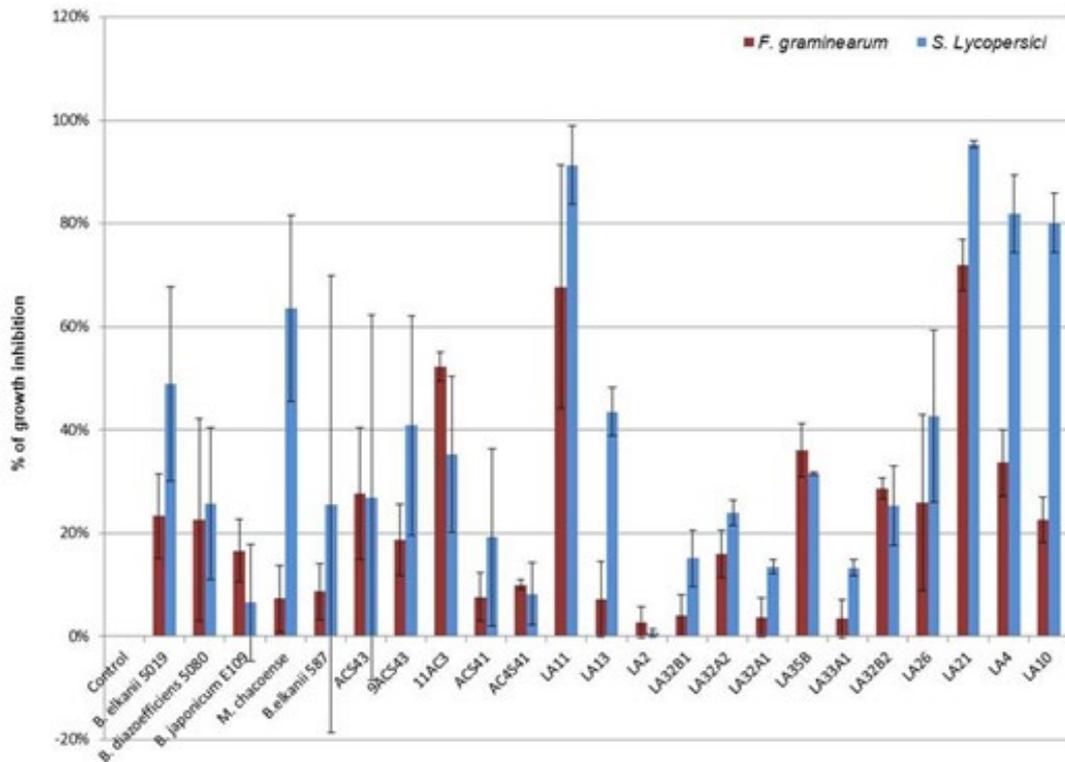
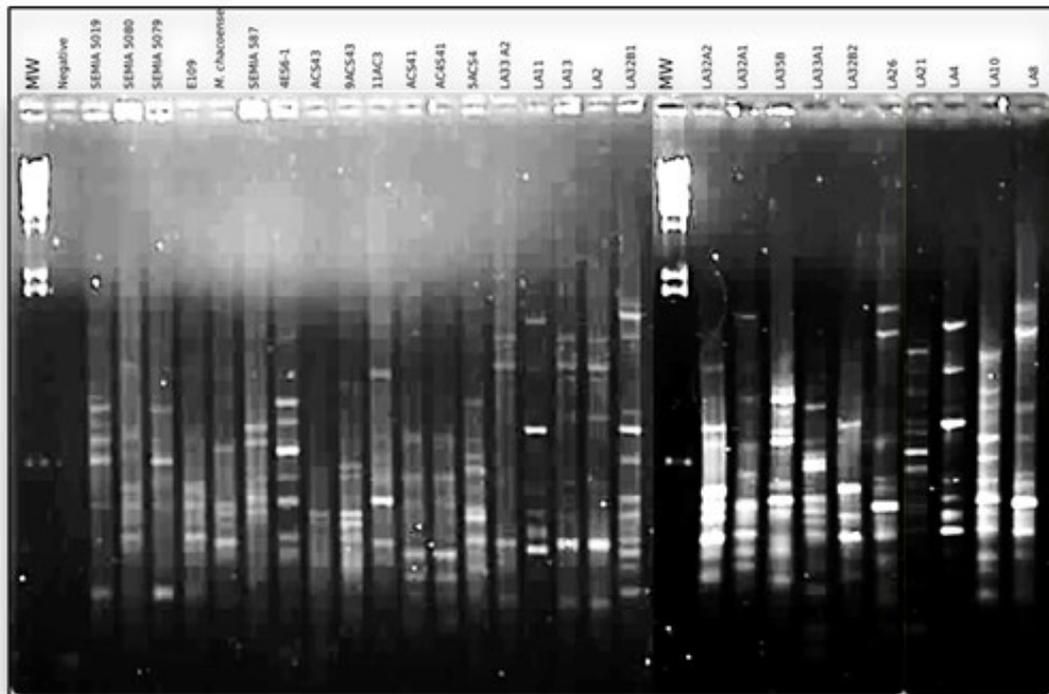


Figure 2. BOXPCR fingerprints of bacteria isolated from nodules of from *P. parviflora*, *V. caven* and *E. contorsiliquum*





Isolates and type strains were grouped into 4 major clusters (Figure 3) at a ~0.35 similarity level, two of them only containing isolates of *P. parviflora*. Cluster I was formed by isolates LA11, LA4 and LA21, and Cluster IV included isolates LA33A2, LA13 and LA2. Clearly, these two groups of bacteria were quite different. The other 16 isolates were grouped within cluster II and III. Cluster II included isolates of *P. parviflora* LA33A1, LA10 and LA32B1 that were distributed into 3 different subgroups, LA26 and LA8 were together and in a different subgroup than isolate 5ACS4-2 of *V. caven*, which also was different from *V. caven* 9ACS43 and ACS43, that were grouped at a ~0.7 similarity level. Cluster III included isolates of *V. caven*, *P. parviflora*, *E. contorsiliquum*, *M. Chacoense*, *B. elkanii*, *B. japonicum* and *B. diazoefficiens*, so most probably, as shown below, these bacteria were representatives of nitrogen-fixing ones. At a 0.6 similarity level, the isolate of *P. parviflora* LA32A1 was grouped with bacteria isolated from *E. contorsiliquum* in a different subgroup, apart from isolates LA35B, LA32A2 and LA32B. The isolate 11AC3 of *V. caven* differed from ACS41 or AC4S41, although additional genetic studies should be performed to accurately establish similarity between the isolates. Cluster IV included two isolates that had an identical fingerprint and therefore might be siblings of the same strain, while the other isolate proved to be quite diverse. In any case, organisms within this cluster are the more diverse compared to the other bacteria. It is evident that even though isolates were collected from the same hosts, they were genetically quite diverse, and even nitrogen fixing bacteria might belong to different rhizobial species as well.

Based on the BOX dendrogram we proceeded to identify the most diverse bacteria by amplifying and sequencing their 16S rDNA gene. The BLAST analysis of these sequences suggested that root nodule endophytic bacteria belonged to 10 genera that were either within the Alphaproteobacteria, Gammaproteobacteria, Firmicutes or Actinobacteria (Figure 4).

Within nodules we isolated bacteria that based on the 16S rDNA sequence were clustered in 4 main groups (Figure 4). Since they were isolated from nodules, they might either be nitrogen-fixing

bacteria, plant growth-promoting bacteria that live within nodules, or opportunistic saprophytic bacteria that alone or by interacting with the plant or other microbes, provide plants with a biological advantage for the ecological niche. One group of bacteria consisted in Gammaproteobacteria and included isolates LA8 and LA10 that were isolated from nodules of *P. parviflora*. These bacteria belonged to the families Rhodanobacteraceae (*Dyella* sp.) and Pseudomonadaceae (*Pseudomonas* sp.), with 99.86% and 100% of identity, respectively. This same cluster also included isolate 11AC3, that was collected from nodules of *A. caven* and belonged to Enterobacteraceae, being an *Enterobacter* sp. However, the sequence identity was too low, therefore, more sequences or a larger 16SrDNA sequence should be analyzed to accurately identify the isolate.

Figure 3. Dendrogram generated by UPGMA cluster analysis using the Dice similarity coefficient based on the BOXAR1 fingerprint of studied isolates

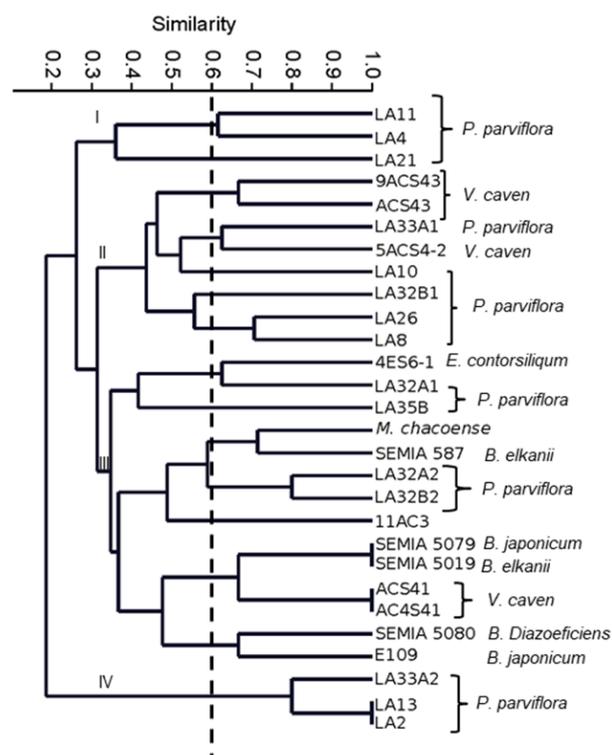
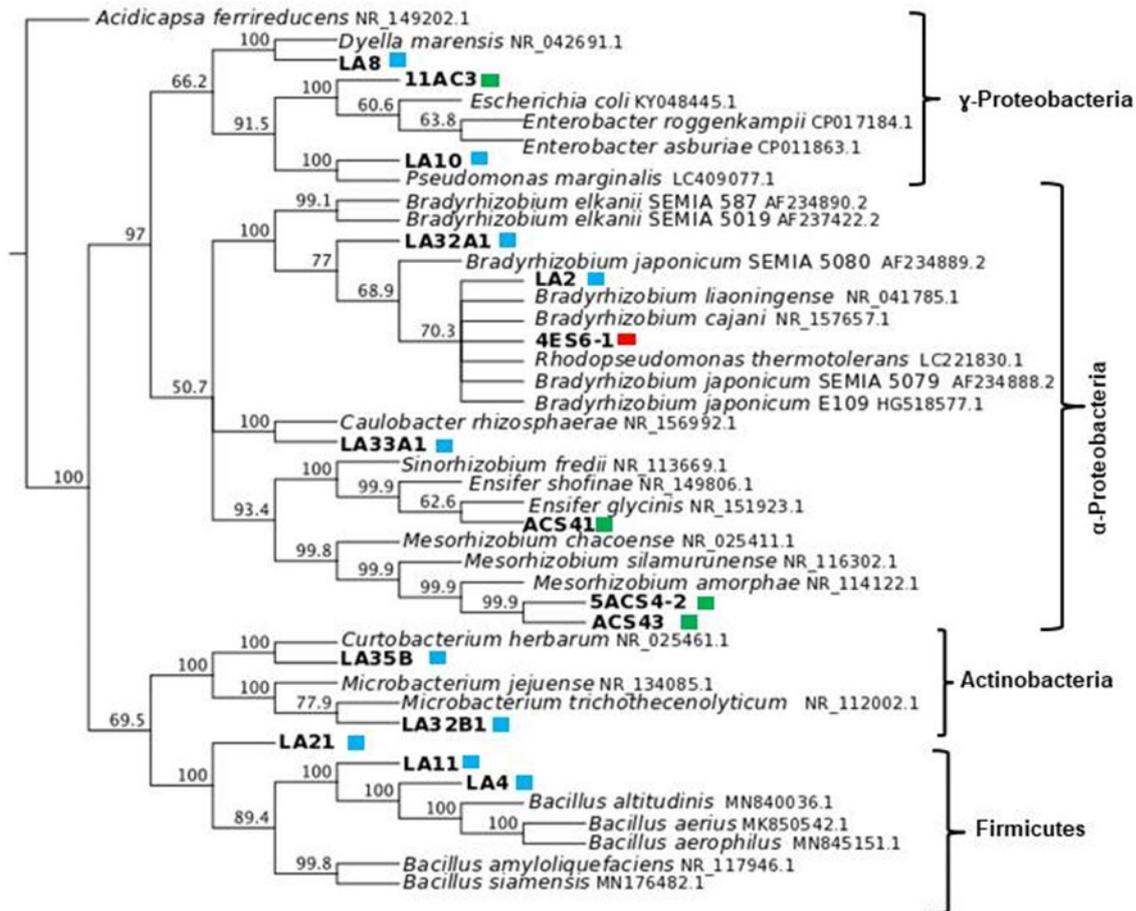




Figure 4. Phylogenetic relationship of the isolates based on aligned sequences of the 16S rDNA gene. Sequences accession number representing the genera is on the tree. Isolates from the same legume species are indicated as coloured squares: blue for *P. parviflora*, green for *V. caven*, and red for *E. contorsiliquum*. *Acidicapsa ferrireducens* was the outgroup



Among isolated bacteria, the blast analysis showed that at least 6 were nitrogen-fixing bacteria that belonged to Alphaproteobacteria. These were isolates LA2 from *P. parviflora*; 4-ES6-1 from *E. contorsiliquum*; isolates ACS41, 5ACS4-2, ACS43 that were collected from nodules of *A. caven*; and isolate LA32A1, whose 16SrDNA sequence, unfortunately, had a too low similarity level, indicating that we need to go back to more precisely identify the isolate. These bacteria, except for the latter one, were clustered separately with a high bootstrap value of 100% and 93.4%. Furthermore, they are most probably representatives of *Bradyrhizobium*, *Mesorhizobium* and *Ensifer* since their sequences shared

99.66 % and 100% identity with type strains of these genera. It has already been reported that nodules induced by rhizobia might contain other bacterial taxa such as representatives of the Gammaproteobacteria. *Pantoea agglomerans*, *Enterobacter kobei*, *Enterobacter cloacae*, *Leclercia adecarboxylata*, *Escherichia vulneris*, *Pseudomonas* sp., *Bacillus* sp., *Pseudomonas* sp., *Curtobacterium herbarum* and *Microbacterium* sp., among others, which were frequent bacteria within nodules of *Hedysarum* mediterranean species⁽⁵⁹⁾⁽⁶⁰⁾. This might be related to the nutrient level and/or particular environment of nodules that evidently harbour a wide variety of non-symbiotic bacteria,



which could be either necessary partners or opportunistic competitors.

Other isolates were clustered within the Actinobacteria group, like LA32B1 and LA35B organisms, that were isolated from *P. parviflora* and identified one as *Microbacterium* and the other as *Curtobacterium*. Representatives of the Firmicutes LA4, LA11 and LA21 were identified as *Bacillus* species, since their 16SrDNA sequence was 100% identical to that of *Bacillus* species. Bacteria recovered from native legume trees live endophytically within nitrogen-fixing nodules induced by rhizobia, but might be either cooperative microorganisms, plant growth promoting bacteria or nitrogen fixing ones. It has already been described that *Pseudomonas* (Gammaproteobacteria) developed N₂ fixing nodules on *Robinia pseudoacacia*⁽⁶¹⁾ and *Acacia confusa*⁽⁶²⁾. *Geobacillus* (Firmicutes), *Paenibacillus* (Firmicutes) and *Rhodococcus* (Actinobacteria) also were described as rhizobial symbionts of *Lotus corniculatus*⁽⁶³⁾. These bacterial species had similar *nodA* gene sequences to *Mesorhizobium* representatives isolated from the same plants; most probably these bacterial species received these genes by lateral gene transfer from *Mesorhizobium*. Although such process might not be so frequent, the analysis should be done to clarify this issue. As reported by Saïdi and others⁽⁶⁴⁾, *Pseudomonas*, *Enterobacter*, *Bacillus*, *Staphylococcus*, *Serratia*, *Stenotrophomonas* and *Xanthomonas* have already been found within *Vicia faba* root nodules, though these bacteria failed to nodulate it and did not have *nifH* or *nodC* genes. However, they also cited reports of Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria that were isolated from root nodules as nodulating bacteria or nodule associated bacteria in a wide array of legumes. All this together suggests that legume nodulation may not be exclusively triggered by Alphaproteobacteria⁽⁸⁾. Also, the other organisms living inside nodules provide plants with additional advantages that might be related to nutrition or plant health. This is not surprising considering that rhizobia free legumes like alfalfa can develop nodules even in axenic conditions⁽⁶⁵⁾, suggesting that legumes have preprogram sets of events that allow them to develop nodules. Probably, there are many more organisms than rhizobia alone that can trigger nodule formation in legumes⁽⁶⁶⁾.

Rhizobia isolates were clustered in three groups belonging to the families Bradyrhizobiaceae, Rhizobiaceae and Phyllobacteriaceae (Figure 4), among them, *Mesorhizobium* is closer to *Sinorhizobium* than to *Bradyrhizobium*, which is in agreement with previous reports⁽¹¹⁾. Isolates LA2, LA32A1 and 4ES61 that were isolated from *P. parviflora* and *E. contorsiliquum*, respectively, belonged to a cluster that included *B. japonicum* strains, suggesting that they most probably are *Bradyrhizobium* sp. Interestingly, *B. Elkanii* SEMIA 6403 (=BR 8205) has been recommended in Brazil to inoculate *P. parviflora*⁽⁶⁷⁾. Nodules of *E. contorsiliquum* contained a *Bradyrhizobium* sp. that was characterized by an extremely slow growth rate and very small cells, such that they were hardly visible in the light microscope. This and the 16S rDNA sequence homology with the 16SrDNA sequence of *B. liaoningense* type strain (data not shown) suggested that it is most probably a *B. liaoningense* representative. In line with this, cultures of such isolate alkalize the culture media. Isolates from *P. parviflora* belonged to *Bradyrhizobium* genus and, even though they were different, additional sequences should be examined to accurately establish their identity, particularly because there are other native legumes within the area suggesting that they might be promiscuous bacteria.

V. Caven was nodulated by *Mesorhizobium* strains since isolates 5ACS4-2 and ACS43 clustered with *Mesorhizobium* representatives with a high bootstrap value of 99.88 %. The 16S rDNA sequence of the isolated strains were 99.79 % and 100 % identical to that of *Mesorhizobium* strains. Unlike other genera of rhizobia, species within *Mesorhizobium* show relatively low sequence divergence at core loci, this is such that all *Mesorhizobium* type strains presented a high level of sequence homology⁽¹¹⁾⁽⁶⁸⁾. Laranjo and others⁽¹¹⁾ grouped 30 *Mesorhizobium* species in four clusters, which as in this work showed differences between them. Furthermore, MLSA phylogeny analysis divided isolates in three main groups that contained *M. plurifarium* and *M. chacoense*, suggesting, in a way, the low resolution of the 16SrDNA gene sequences⁽¹¹⁾. They stated that lateral gene transfer, as well as duplication, might bring additional problems for phylogenetic studies within the genus *Mesorhizobium*. Because of this, the phylogenetic analysis of *Mesorhizobium*



might disagree with broader phylogenies based on multiple core genes⁽⁶⁸⁾⁽⁶⁹⁾. However, Laranjo and others⁽¹¹⁾ found that there is no gene transfer of core loci between some specific groups of *Mesorhizobium*.

Vachellia is a genus that includes promiscuous species that might be nodulated by several species of rhizobia⁽⁷⁰⁾. Our results confirmed this since isolate ACS41 was clustered with *Sinorhizobium* sp. (*Ensifer* sp.) at 100% bootstrap value and a 99.85% of sequence identity, suggesting that it belongs to the genus *Ensifer*. Interestingly, *Ensifer* representatives have been found to have the widest host range so far studied⁽⁷¹⁾, and *Vachellia* species were nodulated by diverse and promiscuous *Ensifer* species⁽⁷²⁾⁽⁷³⁾.

The rhizobia isolated for the native trees included in our study are in line with previous findings that stated that 59 *Mesorhizobium* species nodulate a wide array of different legumes⁽⁷⁴⁾: *M. amorphae* from *Amorpha fruticosa*⁽⁷⁵⁾, *M. silamurunense* from *Astragalus membranaceus*⁽⁷⁶⁾, and *Mesorhizobium acaciae* from *Acacia melanoxylon* R. Br.⁽⁷⁷⁾, among others. Beukes and others⁽⁷⁰⁾ studied *Vachellia karroo* root nodules that contained the genera *Bradyrhizobium*, *Ensifer* (32 isolates), *Mesorhizobium* (27 isolates), and *Rhizobium*, as well as Betaproteobacteria; and more recently, Pereira-Gómez and others⁽²²⁾ reported 8 *V. caven* isolates, affiliated to the genus *Mesorhizobium*. Thus, this diversity may contribute to the ecological success of *Vachellia* sp. as pioneer species, as previously noted, for example, for *V. Karoo*⁽⁷⁰⁾.

Both genera *Ensifer* and *Mesorhizobium* include several species that nodulate *Acacia sensu lato* species; this is in agreement with previous data reported for other African Acacias (i.e., *Vachellia* sp. and *Senegalia* sp.), which also are predominantly nodulated by isolates of *Mesorhizobium*, *Rhizobium* and *Ensifer*⁽⁷⁰⁾. Also, Cordero and others⁽¹⁴⁾ found that *V. macracantha*, a legume tree distributed across neotropical regions, spreading from the Southern United States to Northern Argentina, was nodulated by *Ensifer numidicus*, *Ensifer kummerowiae*, *Ensifer fredii*, *Ensifer americanus* and *Ensifer sahelii*. A range of genera in the Proteobacteria (most commonly *Bradyrhizobium*, *Ensifer*

(*Sinorhizobium*), *Mesorhizobium* and *Rhizobium*) can form functional (N₂ fixing) nodules on specific legumes, and for the Mimoseae tribe genera *Acacia*, *Senegalia*, *Prosopis* and *Vachellia*, and a complete list of legume—rhizobia symbiosis—is available in Andrews and Andrews⁽⁵⁶⁾.

4. Conclusions

South American native tree legumes *V. caven* and *P. parviflora* harbour a great diversity of endophytic bacteria and alpha-rhizobia in their root nodules.

E. contorsiliquum may establish a nitrogen-fixing symbiosis with *Bradyrhizobium lianonginense*, and *P. parviflora* may develop nodules with *Bradyrhizobium* spp strains that need to be studied further to determine the species. *V. caven* within the environments sampled may be nodulated by both *Ensifer* spp and *Mesorhizobium* strains, suggesting that it is a promiscuous legume species that interacts mainly with broad host range rhizobia.

It is known that nodules are rich in nutrients providing a habitat for bacteria, not only for nitrogen-fixing rhizobia, but for other microorganisms (associated or endophytic) as well, and interestingly, the native legumes studied contained Gammaproteobacteria (*Pseudomonas*, *Dyella*, *Caulobacter*), Actinobacteria (*Curtobacterium*, *Micobacterium*) and Firmicutes (*Bacillus*), that in in-vitro studies showed they can control fungal pathogens or solubilize P. These non-rhizobia endophytes might play a role in nutrition, promoting plant growth and health. In this regard, more work is needed to understand the role of the endophytic bacteria in root nodules of these legumes in combination with rhizobia, as well as to establish their condition as micro-symbiont or endophytic bacteria.

These biological roles of symbiotic and endophytic microorganisms' interactions as well as the knowledge of nodule microbiomes might contribute to have a better understanding of the system, and a better management of natural resources.



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Author contribution statement

Virginia Martínez Alcántara and Rocío Medina wrote the manuscript and, in collaboration with Juan Gauna, made the data analysis, lab and field work. Pedro Balatti collaborated in planning the experiments, the analysis and writing the manuscript.

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Supplementary material

Table S1. Microbiological characterization of 22 isolates and 5 reference strains used in this study. Control treatment consisted in culture medium without bacteria. GN, Gram-negative; GP, Gram-positive; R, rods; A++, strong acid; A+, moderately acid; A, slightly acid; N, neutral; B, basic; ND, No Determined

Isolates	Gram staining	Change of medium pH
<i>B. elkanii</i> SEMIA 5019	GN, R	B
<i>B. diazoefficiens</i> SEMIA 5080	GN, R	B
<i>B. japonicum</i> SEMIA 5079	GN, R	B
<i>B. japonicum</i> E109	GN, R	N
<i>M. chacoense</i> Pr5	GN, R	A++
<i>B.elkanii</i> SEMIA 587	GN, R	B
4ES6-1	GN, R	B
ACS43	GN, R	A+
9ACS43	ND	A+
11AC3	GN, R	A++
ACS41	GN, R	A+
AC4S41	ND	A+
5ACS4-2	GN, R	A++
LA33 A2	ND	A++
LA11	GP, R	N
LA13	GN, R	A
LA2	GN, R	A
LA32B1	GP, R	N
LA32A2	GN, R	A++
LA32A1	ND	B
LA35B	ND	A
LA33A1	ND	B
LA32B2	GN, R	A++
LA26	GN, R	B
LA21	GP, R	A+
LA4	GP, R	N
LA10	ND	N
LA8	GN, R	B
control		N