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ORIGINAL PAPER

Diversity and stress tolerance in rhizobia from Parque Chaqueño region of Argentina nodulating *Prosopis alba*

Lucía Chávez Díaz · Pablo González · Esteban Rubio · Mariana Melchiorre

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Abstract The aim of this work was to investigate the genetic diversity, symbiotic effectiveness, drought tolerance, and indole acetic acid production of indigenous rhizobial populations in the Parque Chaqueño of Argentina able to nodulate *Prosopis alba*, the dominant forest tree of this region. The populations were sampled at five locations from the Arid, Semi-arid, and Humid Chaco in the Parque Chaqueño region. A set of rhizobial strains able to nodulate *P. alba* was obtained and selected based on their molecular diversity. Data obtained by BOX-PCR indicated that the highest molecular variability was observed in rhizobial isolates from Semi-arid Chaco. High level of indolic compound production and tolerance to osmotic treatment were significantly ($p \leq 0.05$) correlated with water restrictions of the environments where the strains belonged. A small set of rhizobial strains that stimulate *P. alba* growth was selected from a large group of strains. The strains were identified by 16S rDNA sequencing as belonging to the genera *Mesorhizobium*, *Bradyrhizobium*, and *Ensifer*. To our knowledge, this is the first report of *P. alba* nodulation by strains other than *Mesorhizobium chacoense*, which was already described for the Parque Chaqueño.

Keywords *Prosopis alba* · Symbiosis · Rhizobial diversity · Parque Chaqueño

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L. Chávez Díaz · P. González · M. Melchiorre (✉)
Instituto de Fisiología y Recursos Genéticos Vegetales (IFRGV), Centro de Investigaciones Agropecuarias–Instituto Nacional de Tecnología Agropecuaria (CIAP–INTA), Camino 60 Cuadras km 5 1/2, X5020ICA, Córdoba, Argentina
e-mail: mmelchiorre@correo.inta.gov.ar

M. Melchiorre
e-mail: mmelchio@hotmail.com

E. Rubio
Instituto de Zoología y Microbiología Agrícola (IMYZA), Instituto Nacional de Tecnología Agropecuaria (INTA), Buenos Aires, Argentina

M. Melchiorre
Facultad de Ciencias Exactas Físicas y Naturales, Universidad Nacional de Córdoba, Córdoba, Argentina

Introduction

The genus *Prosopis* (family Fabaceae) comprises trees and shrubs and occurs in arid and semi-arid regions worldwide. Argentina has been proposed as the main diversity center of the genus, with 11 endemic species (Burkart 1976). The phytogeographic region "Parque Chaqueño" covers the provinces of Formosa and Chaco, east of Salta, Jujuy, Catamarca, and Tucuman, north of Cordoba and Santa Fe, west of Corrientes, and south of La Rioja and San Juan. The climate is sub-tropical, with annual rainfall between 500 and 1,100 mm year^{-1}, decreasing from east to west. *Prosopis alba* (Grisebach) together with *Prosopis chilensis*, *Prosopis flexuosa*, and *Prosopis nigra* make up a complex of species known as "black and white algarrobos", with no strict reproductive isolation among species and with natural hybrids being rather common (Verzino et al. 2003). The genus *Prosopis* is a very important natural resource in dry areas and has multi-purpose nature, with the potential to provide a wide range of products. In addition, *Prosopis* plays a very important role against soil desertification and erosion, improving soil fertility by producing leaf litter. Because *Prosopis* can colonize and survive in restrictive environments, it has a high potential to be used in reforestation programs for restoration of degraded areas as well as for biological axis of new production systems (Mottura 2006).

As all legumes, *Prosopis* species are capable of fixing atmospheric nitrogen in symbiosis with root nodule bacteria, collectively named rhizobia, playing a major role in sustainability of

natural ecosystems. During the establishment of rhizobium–legume symbiosis, plants produce and release rhizosphere flavonoids, which are important secondary metabolites that induce the structural nod genes of rhizobia (Cesco et al. 2012). In *Prosopis* roots, soil rhizobia induce indeterminate nodules, which have a persistent meristem that often yields a cylindrical or branched structure. The nitrogen biologically fixed in nodules is transported to leaves as amides, as it was suggested for plants belonging to Mimosaceae subfamily (Sprent and James 2007).

Even though information about *Prosopis* nitrogen fixation in the field is scarce, mainly because of methodological problems, it has been suggested that *Prosopis* and *Acacia* trees growing in arid and semi-arid regions fix less nitrogen (6–34 kg N ha^{-1} $year^{-1}$) than corresponding woody legumes from more humid climates (110–800 kg N ha^{-1} $year^{-1}$); however, these data should be considered in an ecological context, where restrictive environmental condition reduces biological nitrogen fixation (BNF) (Räsänen et al. 2001).

Prosopis trees of the arid and semi-arid regions in Africa can be nodulated by *Mesorhizobium plurifarium*, *Rhizobium etli*, *Sinorhizobium saheli*, *Sinorhizobium kostiense*, and *Sinorhizobium arboris*, with all these strains having been isolated from *P. chilensis*. In North America, nodulation by *Rhizobium leguminosarum*, *Sinorhizobium meliloti*, and *Bradyrhizobium* sp. has been reported (Iglesias et al. 2007 and references therein). In South America, two *Ensifer* species (*Sinorhizobium* sp.) from Brazil and Mexico have been reported to nodulate *P. chilensis* (Haukka et al. 1998); in addition, a new species, *Mesorhizobium chacoense*, isolated from the Arid Chaco region in Argentina, was reported as a fast-growing rhizobium species able to develop root nodules in *P. alba* (Velázquez et al. 2001).

The Parque Chaqueño in Argentina is a very extensive region, subjected to abiotic stresses commonly limiting growth, nodulation, and biological nitrogen fixation (BNF) in plants and also modifying biodiversity of soil microorganisms and their capacity to establish symbiotic relationships. To our knowledge, there is no information about rhizobium diversity in areas of different moisture in the Parque Chaqueño region or about the physiological characteristics of rhizobia that might contribute to improve *Prosopis* nodulation, growth, and survival.

The aim of this work was to investigate the genetic diversity, symbiotic effectiveness, drought tolerance, and indole acetic acid (IAA) production of indigenous rhizobial populations able to nodulate *P. alba*. The populations were sampled at five locations from the Arid, Semi-arid, and Humid Chaco in the Parque Chaqueño region of Argentina. We hypothesize that rhizobia from Arid, Semi-arid, and Humid Chaco of the Parque Chaqueño region of Argentina are able to nodulate *P. alba*, have genetic polymorphism, and the strain behavior under osmotic stress and IAA production is correlated with the hydric characteristics of the environment where the strains come from.

Materials and methods

Reference strains and rhizobial isolates from Parque Chaqueño soil samples

Reference rhizobial strains used for comparison were *Bradyrhizobium japonicum* USDA138, *Mesorhizobium loti* MAFF303099, *Mesorhizobium chacoense* LMG19008, *Ensifer meliloti* 2011 (also called *Sinorhizobium meliloti* Sm2011), and *Rhizobium etli* SC15. Soil samples were collected from five locations in Parque Chaqueño region, Argentina: (1) San Miguel (SM) 31°45′59″ S, 65°25′00″ W, SW of Córdoba province, Arid Chaco; (2) Colonia Benítez (CB) 27°20′00″ S, 58°55′60″ W, Chaco province, Humid Chaco; (3) Padre Lozano (PL) 23°12′51″ S, 63°50′39″ W, NE of Salta province, Semi-arid Chaco; (4) Isla Cuba (IC) 24°17′31″ South, 61°51′10″ W, SW of Formosa province, Semi-arid Chaco; and (5) Bolsa Palomo (BP) 24°13′15″ S, 61°57′42″ W, Formosa province, Semi-arid Chaco.

Soil samples were collected at 20 cm depth under *Prosopis* treetop from sites separated by approximately 50 m from each other in September 2010. Samples from each location were pooled and kept at 4 °C until use. Soil samples from SM, CB, and PL sites are representative of their original districts, and IC and BP samples are alluvial soils on the banks of Bermejo River, even though they belong to Semi-arid Chaco.

Rhizobial isolates from *P. alba* trap plants

Rhizobial isolates were obtained from nodules developed in *P. alba* trap plants 120 days after sowing. Seeds were mechanically scarified with fine sandpaper, disinfected in 70 % (v/v) ethanol for 1 min, and immersed in a solution of 20 % (v/v) commercial hypochlorite (55 mg active chloride) and 0.1 % Tween 20 for 10 min. They were rinsed five times with sterile water and sown in 10-L pots filled with a mix of sterile sand and soil samples (1:1) from the locations described above. Plants were grown for 120 days in greenhouse with 14-h photoperiod (400 μmol m^{-2} s^{-1}) and at 27±2 °C.

Ten plants per location were individually harvested, and nodules of each plant were collected and pooled. Nodules were surface-sterilized with 70 % ethanol for 1 min and with 5 % (v/v) commercial hypochlorite (55 mg active chloride) for 10 min; then they were rinsed with water six times using a water jet pump. The nodules were crushed under sterile conditions and bacteria were isolated using standard procedures (Vincent 1970). Purity of cultures was confirmed by repeatedly streaking the bacteria on yeast extract–mannitol–

agar (YMA) medium and verifying a single type of colony morphology (color, mucosity, diameter, transparency, and border elevation), and absorption of Congo red after they were grown in the dark at 28 °C for 5 days.

P. alba seeds from a seed stand were gently provided by INTA Plaza Forest Station, Chaco province. The seed stand was established using seeds collected from selected *P. alba* trees in Bermejito city (Chaco province) on the banks of Bermejo River.

DNA extraction

Three to five single colonies from each nodule pool were individually transferred to 3 mL YM liquid broth (YMB), grown (3 days) on rotary shaker at 28 °C, 100 cycles min^{-1}, mixed with glycerol (50 %), and stored at −80 °C. Working cultures were maintained on YMA slants at 4 °C.

The modified CTAB method was used for DNA extraction (Ausubel et al. 1987). Briefly, bacteria cultures were grown in 3 mL of YMB at 28 °C for 3 days; then they were centrifuged at 12,000×*g* for 5 min. The supernatant was discarded and the pellet was washed with distilled water and centrifuged at 12,000×*g* for 5 min. The pellet was resuspended in 600 μL Lysis buffer (10 mM Tris–HCl pH 8, 1 mM EDTA, 20 % SDS, and 3 μL of 20 mg mL^{-1} proteinase K) and incubated at 37 °C for 1 h. After this incubation, 100 μL of 5 M NaCl and 80 μL of 65 °C pre-warming CTAB buffer [2 % w/v cetyltrimethylammonium bromide (CTAB), 1.4 M NaCl, 0.2 % v/v β-mercaptoethanol, 20 mM EDTA, and 100 mM Tris–HCl pH 8] were added. Samples were incubated at 65 °C for 15 min. DNA was extracted with 1 vol of chloroform/isoamyl alcohol (24:1 v/v), centrifuged at 12,000×*g* for 5 min, and the upper aqueous phase containing DNA was transferred to a clean tube, precipitated by adding 0.6 vol of cold isopropanol, and incubated at −80 °C for 10 min. The samples were centrifuged at 12,000×*g* for 5 min, the supernatant was discarded, and the precipitates were left to dry at room temperature. Each precipitate was resuspended in 30 μL of distilled water. Samples were then diluted to 50 ng μL^{-1} DNA and kept at −20 °C.

Rep-PCR fingerprinting with BOX-A1R primer

Supernatant containing DNA (5 μL) was used for rep-PCR fingerprinting (interspersed repetitive sequence; Versalovic et al. 1994) with BOX-A1R primer (5′-CTACGGCA AGGCGACGCTGACG-3). The final volume of the PCR reaction was 30 μL and contained dNTPs (1.5 mM of each), 5 μL; buffer 10× (500 mM KCl; 100 mM Tris–HCl, pH 8.3), 3 μL; $MgCl_2$ 50 mM, 1.5 μL; primer (50 pmol μL^{-1}), 1 μL; Taq DNA polymerase (5 U μL^{-1}), 0.2 μL; DNA template, 5 μL; and sterile milli-Q water to complete the volume. The following cycles were used: one cycle of denaturation at 95 °C for 7 min; 35 cycles of 1 min denaturation at 94 °C, annealing at 53 °C for 1 min, and extension at 65 °C for 8 min; one cycle of final extension at 65 °C for 16 min; and a final soak at 4 °C. The reactions were carried out in an Eppendorf® thermocycler and amplified fragments were separated by electrophoresis on 1.5 % agarose gel (20×25 cm), at 120 V, for 4 h in 0.5× TBE buffer; 1 kb plus (Life Technology) was included as ladder. Gels were stained with ethidium bromide, visualized under UV light, and photographed.

Analysis of electrophoretic pattern

Cluster analysis

Cluster analyses were performed with the BOX A1R-PCR results. Electrophoretic band patterns were analyzed using the Gel Compar II software version 4.6 (Applied Mathematics, Kortrijk, Belgium) http://www.applied-maths.com. For each location, similarity of band patterns was evaluated with cluster analysis using the unweighted pair-group method with arithmetic mean (UPGMA) clustering method and the coefficient of Dice as similarity index. The similarity matrix generated by UPGMA and Dice coefficient was used to perform the Principal coordinate analysis (PCO) to evaluate the relationship among strains. PCO is an ordination method that extracts successive components, which are functions of original data containing the greatest portion of information, from a multivariate similarity/dissimilarity matrix containing information about all pairwise profile comparisons. The components are used as new axes to graphically represent the observations. Main differences between observations are visualized on the axes, beginning on axis 1. According to the nature of the data, we selected the Dice distances based on Dice's similarity index (Balzarini et al. 2011). The analysis was performed with InfoStat software (Di Rienzo et al. 2012). Grouping for BOX A1R-PCR was obtained by considering a level of similarity of 60 % in the cluster analysis with the UPGMA algorithm.

Genetic diversity

A diversity index was estimated based on the number of isolates belonging to each group of profiles in BOX A1R. Diversity was calculated with InfoGen software (Balzarini and Di Rienzo 2011) by using the Shannon–Weaver index $H' = \Sigma S[(n1/n)\ln(n1/n)]$ (Shannon and Weaver 1949), where S is the total number of species or groups studied, $n1$ is the number of isolates in each group, and n is the number of isolates in all groups; therefore, $n1/n$ ratio is a measure of the relative abundance of each group.

Analysis of molecular variance

Analysis of molecular variance (AMOVA) was calculated with InfoGen software (Balzarini and Di Rienzo 2011) by using grouping for BOX A1R-PCR similarity matrix generated by UPGMA and Dice coefficient, within each location, within regions, and among Arid, Semi-arid, and Humid regions in Parque Chaqueño.

Water deficit treatment to rhizobial isolates

Considering the results obtained from cluster analysis, several isolates were selected to assay their tolerance to water deficit treatment in free-living state. Water potential was lowered by adding various amounts of polyethylene glycol (PEG) (molecular weight 8,000; Sigma, St Louis, MO, USA) to the YMA medium.

Solutions with 25 and 70 % w/v PEG were prepared with sterile water under flow chamber and were not autoclaved. PEG solution was poured on top of solidified YMA medium in a Petri plate, using a volume ratio of 1:1.5 (PEG/YMA). Petri dishes were incubated for 16 h; then the solution on top of the plate was poured off and the plate was used for experiments. During the 16-h period, the PEG diffused into the agar medium to reach an approximate equilibrium, and lowered the water potential to −0.6 and −2 MPa for 25 and 70 % PEG solutions, respectively. Water potential was measured by moistening filter paper on the top and bottom surfaces of the YMA medium and using isopiestic thermocouple psychrometry (Wescar® Point Microvoltmeter HR-337 Dew) (van der Weele et al. 2000). Two independent replicates were made. Tolerance of rhizobial strains to water deficit was tested by adding drops of YM liquid broth of each strain serially diluted (10^6 to 10^3 cells approximately) in YMA and each PEG/YMA treatment. Cultures were incubated at 28 °C during 5 days and growth was visually determined.

The criterion for assaying these water potentials was related to behaviors previously observed in *P. alba* plants under drought stress. Moderate and severe wilting responses have been determined in young plants when leaves reached water potential of −0.6 and −2 MPa (Lopez Lauenstein, personal communication).

Indolic compound production by free-living rhizobial isolates

Indolic compound production was evaluated by testing the group selected after cluster analyses. Bacteria was grown for 3 days in 3 mL yeast extract–mannitol (YM) medium supplemented with 100 μg mL^{-1} tryptophan in an orbital shaker at 28 °C up to 0.6 optical density at 600 nm (OD_{600}). As it was described by Glickman and Dessaux (1995), the PC colorimetric method used in this assay has high specificity reaction with indole acetic acid (IAA); however, it reacts with other indolic compounds such as indolpyruvic and indolacetamide (IPyA and IAM) to a lesser extent. Briefly, rhizobial culture was incubated in a 1:1 volumetric ratio with Salkowski reagent containing 12 g L^{-1} $FeCl_3$ in 7.9 M SO_4H_2 and incubated at 25 °C in the dark for 30 min. Optical density was determined at 530 nm (OD_{530}) as a function of the IAA concentration of samples, using IAA (Sigma) up to 20 μg mL^{-1} as standard. Data were obtained from two independent replicates, and comparisons were made using DGC test model with InfoStat software (Di Rienzo et al. 2012).

16S ribosomal DNA (rDNA) sequence analysis

Rhizobial isolates representing the main BOX-REP-PCR clusters were selected and sequenced by Macrogen (Korea) 16S rRNA sequencing service. A single colony was suspended in 500 μL of sterile water and centrifuged at 10,000×*g* for 10 min. After removal of the supernatant, the pellet was suspended in 500 μL of InstaGene Matrix (Bio-Rad, USA), incubated at 56 °C for 30 min, and then heated at 100 °C for 10 min. After heating, 1 μL supernatant was used for PCR reaction. 16S rRNA was amplified performing 35 cycles at 94 °C for 45 s, 55 °C for 60 s, and 72 °C for 60 s with 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGTTACCTTGTTACGACTT-3′) primers. PCR products of about 1,400 bp were purified by using Montage PCR Clean up kit (Millipore) and sequenced in a Applied Biosystems model 730XL automated DNA sequencing system (Applied BioSystems, USA) using 518F (5′-CCAGCAGCCGCGGTAATACG-3′) and 800R (5′-TACCAGGGTATCTAATCC-3′) as forward and reverse primers, respectively. SeqMan and EditSeq programs of DNASTAR (Lasergene, Inc., Madison, WI, USA) were used for assembling sequence data of 16S rDNA rhizobial isolates and for contig management. The obtained sequences were 1,300–1,400 bp in length. Sequence alignment was performed with ClustalW alignment tool of MEGA version 5 software. Percent identity was calculated by sequence pair distances using MegAlign ClustalW alignment software of DNASTAR. The phylogenetic tree of rhizobial isolates and reference strains was constructed with a sequence stretch of 1,163 bp, with ClustalW nucleotide alignments of 16S rRNA sequences by the UPGMA method with 1,000 bootstrap test and using MEGA 5 software (Tamura et al. 2011). The sequences were submitted to the NCBI database under accession numbers KC 759691 to KC 759699.

Inoculation of *P. alba* plantlets: nodulation and growth parameters

Rhizobial isolates selected after cluster analyses were used in a *P. alba* plantlets inoculation assay. Bacterial cultures were

grown in 5 mL YM medium up to 0.8 OD_{600} for 3 days and centrifuged at 12,000×*g* for 5 min. The supernatant was discarded and the pellet was washed with distilled water, centrifuged at 12,000×*g* for 5 min, suspended in 700 μL of distilled water, and used to inoculate 7-day-old *P. alba* plantlets.

P. alba seeds were scarified and disinfected as described above, and pre-germinated in moist chamber in the dark at 28 °C for 4 days. Five plantlets per treatment were individually transferred to 200-mL pots filled with sterile vermiculite and watered by sub-irrigation with B&D solution (Broughton and Dilworth 1971) with a low N (0.25 mM NH_4NO_3) content. Individual trays were used to avoid contamination. After 3 days, the plantlet root zone was inoculated with 100 μL of each rhizobial isolates, then plants were grown in greenhouse under a 14-h photoperiod (400 μmol m^{-2} s^{-1}) at 27±2 °C. Sixty-day-old plants were harvested and nodule number, aerial and root length, and root and aerial dry weight (after 72 h of incubation at 70 °C) were determined. These growth parameters were analyzed by principal component analysis (PCA). This multivariate method extracts successive components, which are functions of original data with the greatest portion of information, from a multivariate correlation matrix containing information about standardized original data. The components are used as new axes to graphically represent the observations. Main differences between observations are visualized on the axes, beginning on axis 1. PCA reduces a set of correlated variables to a small number of linear combinations of these variables (principal components), and the results can be visualized simultaneously by means of biplots, where observations and variables are represented in a common space (Balzarini et al. 2011).

Results

Rhizobial isolates from Parque Chaqueño region obtained using *P. alba* trap plants

To obtain rhizobial isolates, nodules developed in *P. alba* trap plants grown in soils from different locations of the

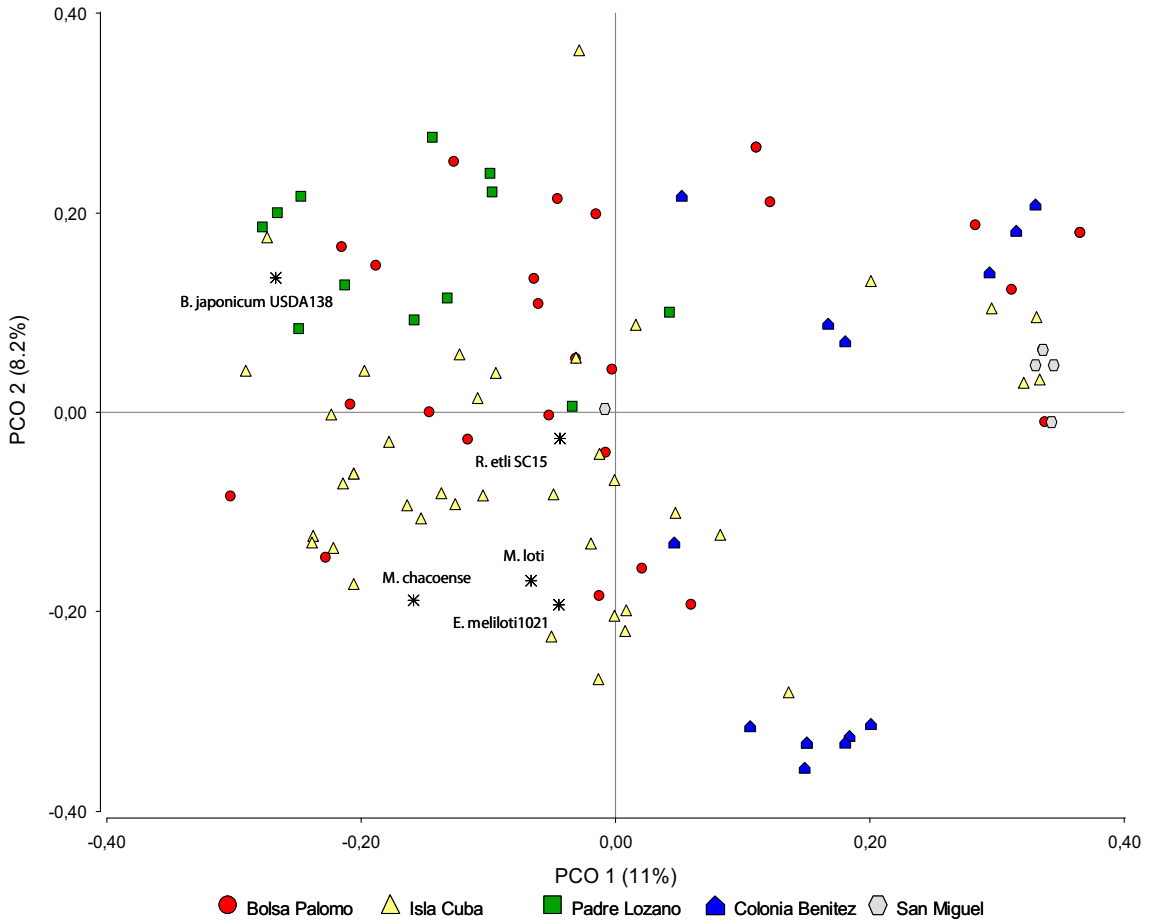
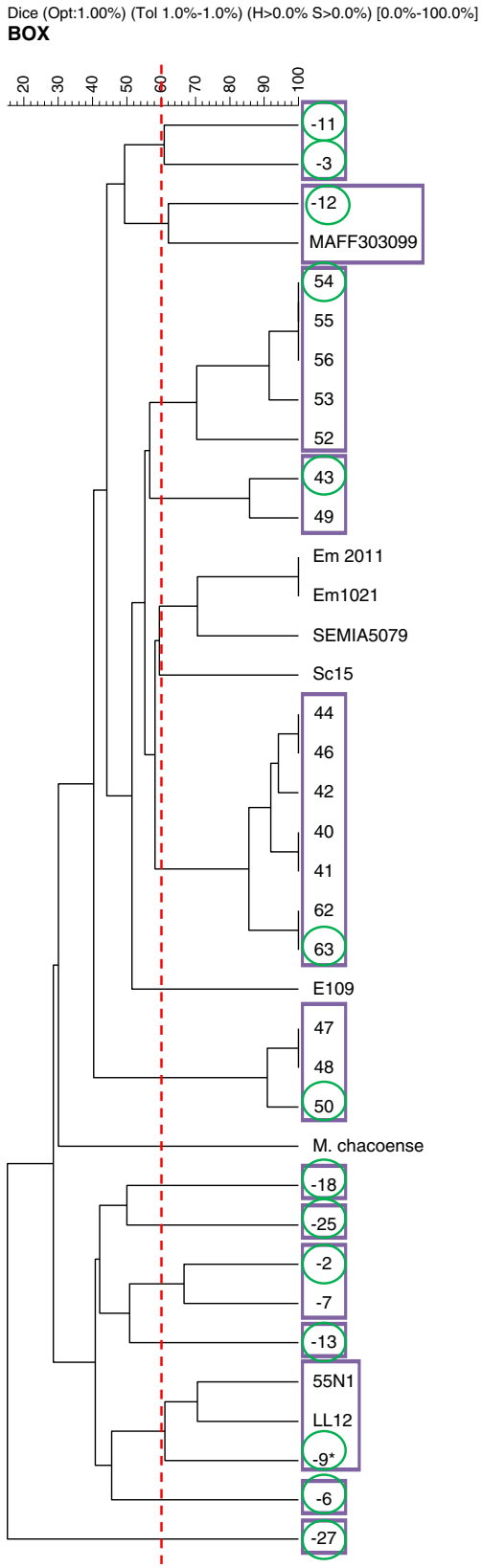


Fig. 1 Principal coordinate analysis (PCO) based on similarity matrix generated by UPGMA and Dice coefficient of the rep-PCR (primer BOX-A1R) products of DNA from rhizobial isolates obtained from locations in Parque Chaqueño region in Argentina. Semi-arid Chaco; Bolsa Palomo, Isla Cuba, and Padre Lozano, Arid Chaco; San Miguel, Humid Chaco; Colonia Benitez and reference strains



Parque Chaqueño region were collected 120 days after sowing. Indeterminate nodules were distributed both in the main and secondary roots and were between 3 and 5 mm in size. The highest percentages of nodulated plants were obtained using soils of Semi-arid Chaco locations; indeed, Bolsa Palomo, Isla Cuba, and Padre Lozano showed 72 %, 62.5 %, and 87.5 % of nodulated plants, respectively. By contrast, the lowest percentages were obtained in soils from Colonia Benitez, in the Humid Chaco (25 %), and from San Miguel, in the Arid Chaco (12.5 %). Both *P. alba* and *P. nigra* are naturally distributed in all sampling locations, with the exception of San Miguel, where *P. flexuosa* is the only species found (Verga, personal communication).

Diversity based on molecular analysis

One hundred strains were isolated from nodules from different locations in Parque Chaqueño region, most of which were fast growers in the YMA medium. Cluster analysis was performed with the UPGMA algorithm using the binary matrix based on DNA BOX A1R amplification electrophoretic patterns of isolates from each location and reference strains. Principal coordinate analysis (PCO) was performed within locations to evaluate the relationship among them.

The PCO showed that strains isolated from Bolsa Palomo had the highest variability, and these strains were almost homogeneously distributed along both PCO axes. The strains of Semi-arid Chaco, Padre Lozano and Isla Cuba, were distributed on the left of the orthogonal axis in PCO1, but were differentiated located along the PCO2 axis. The strains isolated from Padre Lozano were the most similar to *B. japonicum* USDA138 and the strains from Isla Cuba were similar to faster growing rhizobia, such as *Ensifer meliloti* 2011, *Mesorhizobium loti* and *M. chacoense*, and *Rhizobium etli* SC15. The strains isolated from Colonia Benitez (Humid Chaco) were distributed on the right of the orthogonal axis in PCO1 and were differentiated as two subgroups along the PCO2 axis, one of them with high similarity to strains from San Miguel (Arid Chaco), which showed the lowest variability (Fig. 1). The distribution of strain diversity was calculated with Shannon–Weaver index. The highest diversity was observed in Isla Cuba and Bolsa Palomo (H'=3.75 and 3.74, respectively) strains, intermediate values were obtained in Padre Lozano and Colonia Benitez (H'=3.2 and 3.3, respectively) strains, whereas San Miguel showed the lowest diversity values (H'=2.98).

◀ **Fig. 2** Dendrogram generated by UPGMA and Dice coefficient of the rep-PCR (primer BOX-A1R) products of rhizobial isolates from Bolsa Palomo in Semi-arid Chaco and reference strains. Isolates with 60 % similarity are highlighted in *boxes*; *circles* show strains selected for subsequent analysis

Table 1 Total number of isolates, number of clusters with less than 60 % similarity, and strains chosen for subsequent analysis

Location	Number of strains isolated	Number of clusters of <60 % similarity	Strains selected
Bolsa Palomo	28	13	11, 3, 12, 54, 43, 63, 50, 25, 2, 13, 9, 6, 27
Isla Cuba	40	11	35, 24, 18, 32, 1, 36, 29, 23, 14, 33, 38
Padre Lozano	13	5	43, 53, 58, 39, 40
San Miguel	5	1	57
Colonia Benitez	14	2	72, 66

Genetic diversity, based on UPGMA algorithm and Dice coefficient, was analyzed within isolates obtained from each location. A dendrogram using isolates collected from Bolsa Palomo in Semi-arid Chaco and reference strains is shown, as an example, in Fig. 2, where strains with 60 % similarity are highlighted in boxes, and circles show strains selected for further analysis. The numbers of strains isolated from each location, as well as the number of clusters with less than 60 % similarity from which one strain was chosen, are summarized in Table 1. The highest numbers of strains were obtained from Bolsa Palomo and Isla Cuba in Semi-arid Chaco soils, and the lowest number was in Arid and Humid Chaco soils. Of 100 rhizobial isolates originally obtained, 33 strains were selected and used in subsequent analysis.

Based on molecular patterns, average linkage distances among strains were analyzed using location as the selection criterion. The shortest distances were observed in strains from Semi-arid Chaco, and these strains had a high distance with respect to strains from Arid and Humid Chaco (Fig. 3).

An AMOVA was performed to compare variability in strains collected from each location, within and among regions. Significant differences ($p<0.01$) in molecular patterns were observed in strains within location and within regions; however, no significant differences were observed among regions. AMOVA results showed that the highest contribution to total variability, 81.87 %, was due to differences in strains within location, whereas the variability among locations within each region represented 7.84 %.

Responses of free-living rhizobial isolates to water deficit

The capacity of 33 free-living rhizobial isolates selected from cluster analysis to grow in hyperosmotic medium was evaluated under moderate and severe water deficit stress (−0.6 MPa and −2 MPa, respectively). Strains isolated from San Miguel (Arid Chaco) and Padre Lozano (Semi-arid Chaco), both environments being characterized by soil water shortage conditions, showed the highest survival under the highest stress treatment. By contrast, the rhizobial strain from Humid Chaco was only able to grow under moderate stress. The other strains isolated from Semi-arid Chaco locations had intermediate behaviors. All isolates from Isla Cuba grew under −0.6 MPa, and 36 % of them were able to grow under severe stress, whereas 71 % of strains from Bolsa Palomo grew under −0.6 MPa and 35 % tolerated the severe stress treatment (Table 2).

Indolic compound production by free-living rhizobial isolates

Indolic compound production was determined in the 33 rhizobial strains selected from cluster analysis (Table S1 in the supplemental material) and the mean was calculated per

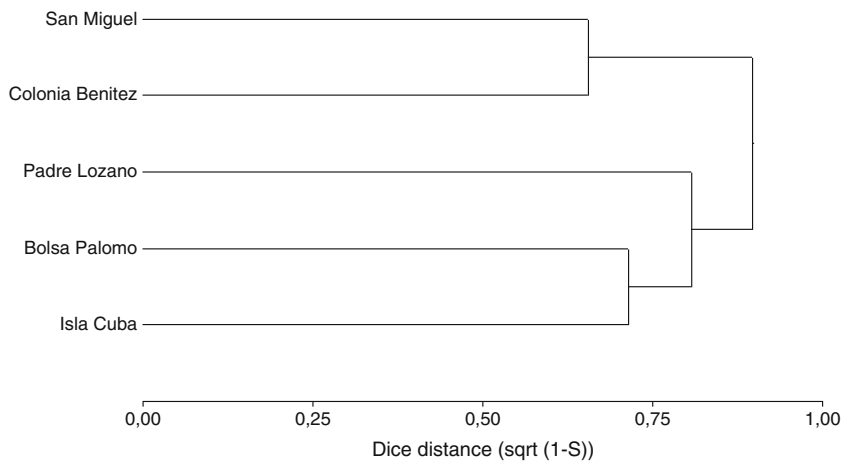


Fig. 3 Average linkage in distances among strains using location as a selection criterion

Table 2 Survival of free-living rhizobial strains under water deficit of −0.6 and −2 MPa

Location	Strains	Total	−0.6 MPa	Total	−2 MPa	Total
Bolsa Palomo	11, 3, 12, 54, 43, 63, 50, 18, 25, 2, 13, 9, 6, 27	14	11, 3, 12, 54, 43, 18, 2, 13, 9, 6	10	11, 12, 13, 43, 6	5
Isla Cuba	35, 24, 18, 32, 1, 36, 29, 23, 14, 33, 38	11	35, 24, 18, 32, 1, 36, 29, 23, 14, 33, 38	11	35, 18, 36, 33	4
Padre Lozano	43, 53, 58, 39, 40	5	43, 53, 58, 39, 40	5	43, 53, 39, 40	4
San Miguel	57	1	57	1	57	1
Colonia Benitez	72, 66	2	66, 72	2	–	0

location. The highest levels were correlated with water restrictions of the environments where the strains belonged. Isolates from Padre Lozano and San Miguel showed the highest values, whereas strains selected from Humid Chaco showed the lowest levels with significant differences ($p \leq 0.05$) (Fig. 4a). The capacity of strains to survive under severe hyperosmotic stress treatment was correlated with their indolic compound production. Strains producing high

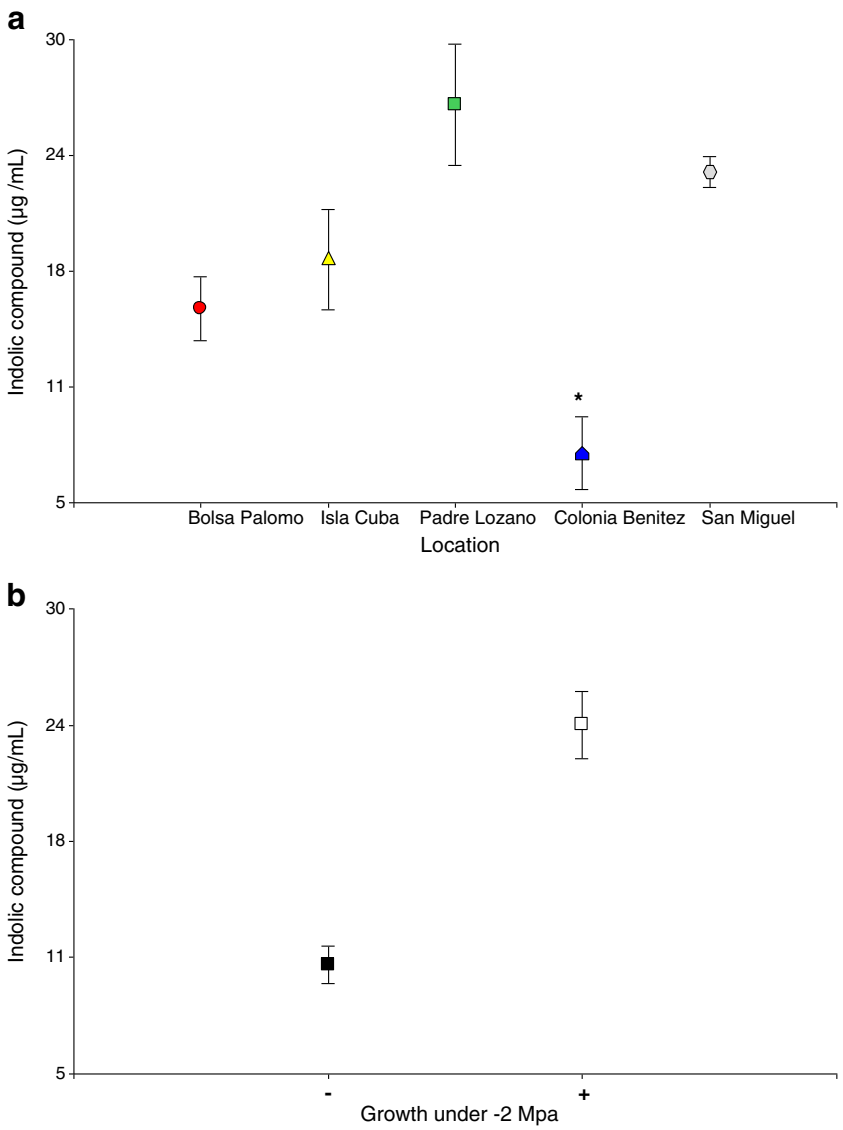


Fig. 4 **a** Indolic compound production by free-living rhizobial strains from locations of Parque Chaqueño region; *asterisk* (*) indicates significant differences from DGC test ($p \leq 0.05$). **b** Indolic compound levels and their capacity to tolerate (+) water deficit of −2 MPa

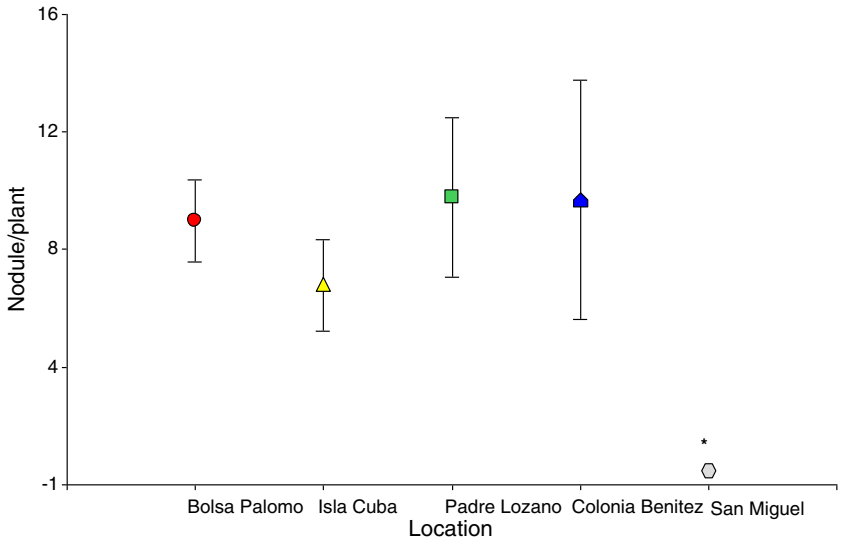


Fig. 5 Nodule development in *Prosopis alba* inoculated with rhizobial strains isolated from locations of Parque Chaqueño region

indolic compound levels were able to grow at −2 MPa (Fig. 4b).

Growth parameters in *P. alba* inoculated with rhizobial strains

Each rhizobial isolate selected from cluster analysis was used to inoculate *P. alba* plantlets with the aim of evaluating the effects on growth and nodulation. The within-location analysis showed high variability among strains and significant differences in nodule number (Table S1 in the supplemental material). The strain from San Miguel was not able to induce nodules in *P. alba* due to its limited ability to colonize *P. alba*, probably because *P. flexuosa* is the only *Prosopis* species present in the Arid Chaco. The rhizobial isolates from other regions did not show significant differences among locations (Fig. 5).

Nodule number as well as *P. alba* growth variables were analyzed with PCA. Results showed that PC1 explained 62.8 % of total variability, whereas PC2 explained 21.7 %. Aerial and total biomass and aerial length were explained by PC1, whereas variability in root length was mainly explained by PC2 (Fig. 6). Rhizobial isolates showed high variability, and this affected the correlation of *P. alba* growth parameters with locations where the isolates come from. Nevertheless, nine rhizobial isolates contributed to *P. alba* growth because they were on the right of the PC1 orthogonal axis. Five of them were from Bolsa Palomo,

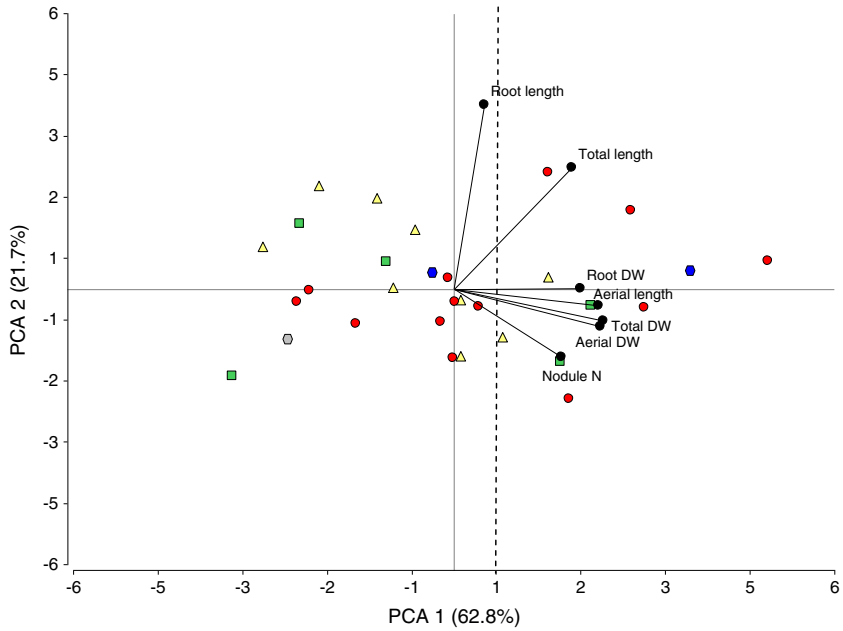


Fig. 6 Principal component analysis (PCA) of *Prosopis alba* growth parameters in plants nodulated with rhizobial strains isolated from locations of Parque Chaqueño region

Table 3 Summarized measures of growth parameters of *Prosopis alba* inoculated with rhizobial isolates selected according to PCA of Fig. 6 (isolates on the right of orthogonal axis, whose values on CP1 were greater than 1)

		Nodule no.	Aerial length	Root length	Total length	Aerial DW	Root DW	Total DW
2 Bolsa Palomo	Treatment mean	20.00	27.25	23.75	51.00	326.5	160.00	486.50
	Standardized mean	1.37	2.02	0.63	1.65	1.30	1.88	1.52
3 Bolsa Palomo	Treatment mean	31.00	18.30	14.00	32.30	283.00	82.00	365.00
	Standardized mean	2.64	0.48	−1.08	−0.22	0.93	0.11	0.75
12 Bolsa Palomo	Treatment mean	18.5	17.65	30.5	48.15	337.50	80.50	418.00
	Standardized mean	1.19	0.37	1.81	1.37	1.39	0.08	1.08
54 Bolsa Palomo	Treatment mean	8.50	16.00	31.50	47.50	209.00	102.00	311.00
	Standardized mean	0.03	0.09	1.99	1.30	0.31	0.57	0.41
63 Bolsa Palomo	Treatment mean	11.5	20.45	20.00	40.45	355.5	124.5	480.00
	Standardized mean	0.38	0.85	−0.03	0.60	1.54	1.07	1.48
66 Colonia Benitez	Treatment mean	14.00	21.5	23.75	45.25	367.50	130.00	497.50
	Standardized mean	0.67	1.03	0.63	1.08	1.64	0.20	1.59
40 Padre Lozano	Treatment mean	19.67	19.90	20.67	40.57	286.67	87.00	373.67
	Standardized mean	1.33	0.76	0.09	0.61	0.96	0.23	0.80
53 Padre Lozano	Treatment mean	16.50	19.00	15.00	34.00	271.00	107.50	378.50
	Standardized mean	0.96	0.60	−0.90	−0.05	0.83	0.69	0.83
36 Isla Cuba	Treatment mean	4.00	17.75	20.8	38.55	219.5	140.00	359.5
	Standardized mean	−0.49	0.39	0.11	0.41	0.40	1.42	359.50
Summarized mean		8.23	15.48	20.16	34.47	171.76	76.93	246.78
SD		8.61	5.82	5.71	9.9	119.33	44.28	157.95
Min		0.00	4.00	9.50	12.00	14.50	3.00	20.00
Max		31	28	33	54	498	212	679

General mean: mean values of growth parameter of all inoculation treatments. Treatment mean: mean values of growth parameter of each inoculation treatment. Standardized value: treatment mean−general mean/SD

two from Padre Lozano, one from Colonia Benitez, and one from Isla Cuba.

Based on these data, standardized values of growth in *P. alba* trees inoculated with each rhizobial isolate were obtained by the following relationship:

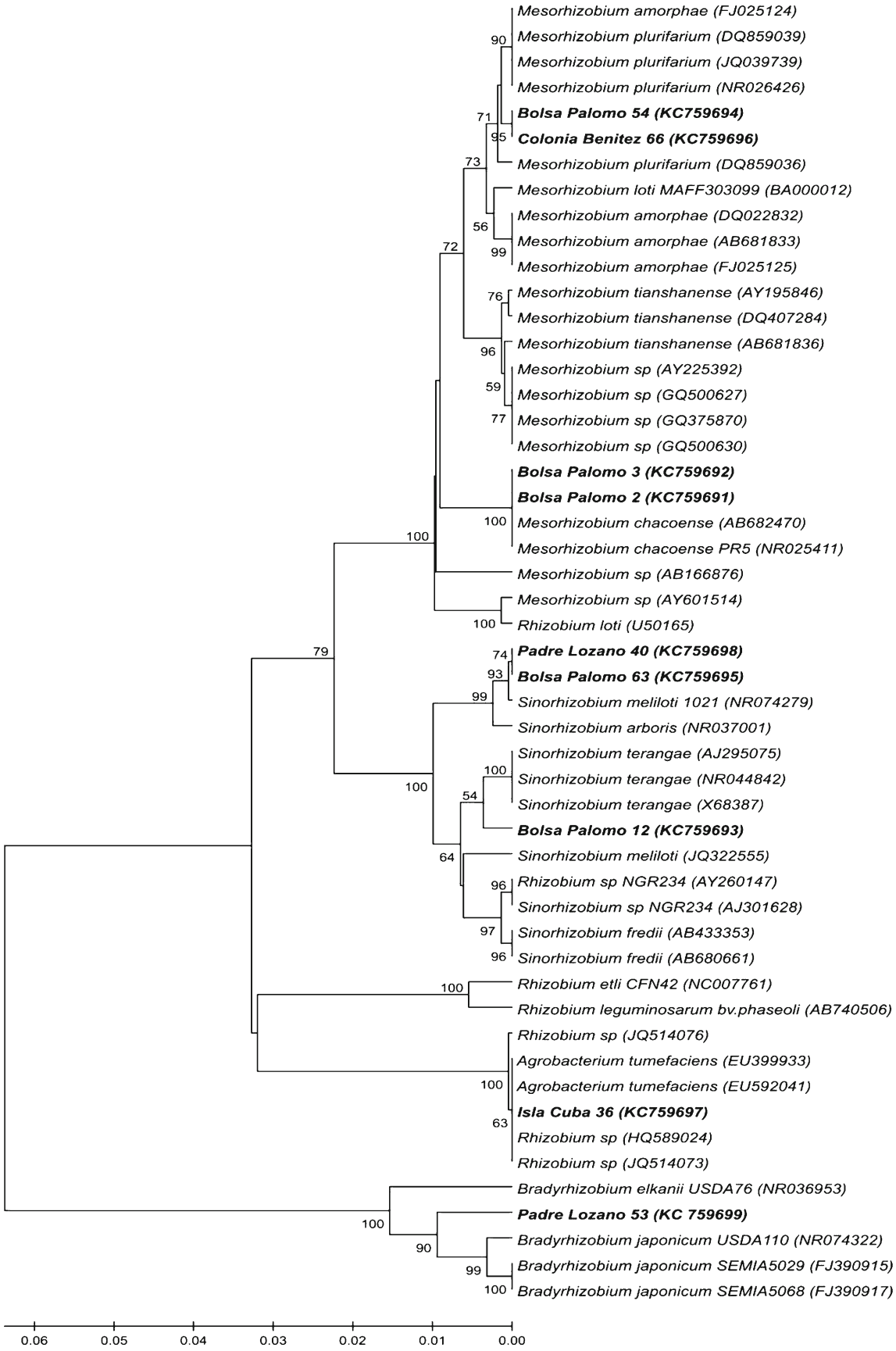
$$\text{Standardized value} = \text{growth value in inoculated treatment} - \text{general mean}/\text{SD}.$$

Where general mean is the mean of grow parameters considering all data and SD is the relative standard deviation. The standardized value can give an indication of the contribution each rhizobial strain can give to *P. alba* growth with respect to the total value (Table 3). The obtained values depended on rhizobial isolates identified as closely related to growth parameters and whose values on CP1 were greater than 1 (Fig. 6). Except the standardized value for the nodule number of the isolate from Isla Cuba, all standardized data were positive, indicating that inoculation with each specific strain contributed to growth of *P. alba*. All growth parameters of trees inoculated with each of the nine selected strains were analyzed by MANOVA (multivariate analysis of variance), and this analysis did not show significant differences ($p>0.001$) in the average profiles among strains related to the plant growth variables. This was expected because these strains are the most closely related to the growth variables, as shown by the PCA (Fig. 6).

16S ribosomal DNA (rDNA) sequence analysis

Comparison with 16S rDNA sequences from representative reference rhizobial strains showed that three of the five rhizobial isolates from Bolsa Palomo (2 KC 759691, 3 KC 759692, and 54 KC 759694) were species of the *Mesorhizobium* genus; isolate 54 showed a 99.7 % similarity with *M. amorphae* (strain SEMIA 6430), whereas isolates 3 and 2 showed the highest, 99.9 % and 100 %, similarity with *M. chacoense* (strain PR5). The other two isolates from Bolsa Palomo (12 KC 759693 and 63 KC 759695) were species of the *Sinorhizobium* (*Ensifer*) genus;

Fig. 7 16S rDNA gene phylogeny of Parque Chaqueño rhizobial isolates from *P. alba* and other. The tree was constructed from 1,163 bp aligned nucleotide sequence data by using the UPGMA algorithm. The *numbers* at branch points are the significant bootstrap values (expressed as percentages based on 1,000 replicates; only values greater than 50 % are shown). Accession numbers are written in *parentheses* after the species name


Mesorhizobium amorphae (FJ025124)
Mesorhizobium plurifarium (DQ859039)
Mesorhizobium plurifarium (JQ039739)
Mesorhizobium plurifarium (NR026426)
Bolsa Palomo 54 (KC759694)
Colonia Benitez 66 (KC759696)
Mesorhizobium plurifarium (DQ859036)
Mesorhizobium loti MAFF303099 (BA000012)
Mesorhizobium amorphae (DQ022832)
Mesorhizobium amorphae (AB681833)
Mesorhizobium amorphae (FJ025125)
Mesorhizobium tianshanense (AY195846)
Mesorhizobium tianshanense (DQ407284)
Mesorhizobium tianshanense (AB681836)
Mesorhizobium sp (AY225392)
Mesorhizobium sp (GQ500627)
Mesorhizobium sp (GQ375870)
Mesorhizobium sp (GQ500630)
Bolsa Palomo 3 (KC759692)
Bolsa Palomo 2 (KC759691)
Mesorhizobium chacoense (AB682470)
Mesorhizobium chacoense PR5 (NR025411)
Mesorhizobium sp (AB166876)
Mesorhizobium sp (AY601514)
Rhizobium loti (U50165)
Padre Lozano 40 (KC759698)
Bolsa Palomo 63 (KC759695)
Sinorhizobium meliloti 1021 (NR074279)
Sinorhizobium arboris (NR037001)
Sinorhizobium terangae (AJ295075)
Sinorhizobium terangae (NR044842)
Sinorhizobium terangae (X68387)
Bolsa Palomo 12 (KC759693)
Sinorhizobium meliloti (JQ322555)
Rhizobium sp NGR234 (AY260147)
Sinorhizobium sp NGR234 (AJ301628)
Sinorhizobium fredii (AB433353)
Sinorhizobium fredii (AB680661)
Rhizobium etli CFN42 (NC007761)
Rhizobium leguminosarum bv.phaseoli (AB740506)
Rhizobium sp (JQ514076)
Agrobacterium tumefaciens (EU399933)
Agrobacterium tumefaciens (EU592041)
Isla Cuba 36 (KC759697)
Rhizobium sp (HQ589024)
Rhizobium sp (JQ514073)
Bradyrhizobium elkanii USDA76 (NR036953)
Padre Lozano 53 (KC 759699)
Bradyrhizobium japonicum USDA110 (NR074322)
Bradyrhizobium japonicum SEMIA5029 (FJ390915)
Bradyrhizobium japonicum SEMIA5068 (FJ390917)
0.06
0.05
0.04
0.03
0.02
0.01
0.00

isolate 12 showed 99.3 % similarity with *Sinorhizobium* sp. (strain NGR234) and isolate 63 showed 99.9 % similarity with *S. meliloti* (strain 1021). Likewise the isolates from Padre Lozano (53 KC 759699 and 40 KC 759698) were species of *Bradyrhizobium* and *Sinorhizobium* genera, respectively; isolate 53 showed 98.4 % similarity with *B. japonicum* (strain SEMIA 5029) and isolate 40 showed 99.9 % similarity with *S. meliloti* (strain 1021). The isolates obtained from Colonia Benitez (66 KC 759696) and Isla Cuba (36 KC 759697) were species of *Mesorhizobium* and *Rhizobium* genera, respectively; isolate 66 showed 99.7 % similarity with *M. amorphae* (strain SEMIA 6430), and isolate 36 showed 100 % similarity with *Rhizobium* sp. (strain PRNB-4). Figure 7 shows the grouping of isolates by cluster analysis of 16S rDNA sequences, using the UPGMA method (1,000 bootstrap). On the basis of 16S rDNA sequences, isolates 54 and 66 from Bolsa Palomo (Semi-arid Chaco) and Colonia Benitez (Humid Chaco), respectively, clustered with *Mesorhizobium amorphae* (strain SEMIA 6430) in the phylogenetic tree. Isolates 40 from Padre Lozano and 63 from Bolsa Palomo clustered in a branch together with the sequence of *S. meliloti* strain 1021, and isolate 12 from Bolsa Palomo clustered with sequences of *Sinorhizobium terangae*. The isolate 36 from Isla Cuba was included in the cluster with *Rhizobium* sp. (R5_Betul and PRNB-4 strains) and *Agrobacterium tumefacies* (AFM2 and CAF 428 strains). On the other hand, isolate 53 from Padre Lozano clustered with sequences of *B. japonicum* (SEMIA 5029, 5068, and USDA 110).

Discussion

Rhizobia from wild legumes have been described as diverse, mainly in arid regions, and capable of establishing symbiotic association with several tree partners, showing low selectivity (Zahran 2001). The variability observed with PCO, molecular variance (AMOVA), and the Shannon–Weaver index showed a highly significant diversity of rhizobial isolates within and among locations. Bolsa Palomo and Isla Cuba showed the highest diversity index, probably because these two locations have similar environmental characteristics with Bermejito city, where the *P. alba* seed came from.

A critical aspect in survival of rhizobial strains in soil is their adaptation to changing environmental conditions, such as fluctuations in pH, nutrients, temperatures, and water availability, the latter being sometimes associated with salinity increases. Rhizobial strain tolerance to drought and saline excess is extremely diverse. *B. japonicum* is sensitive to osmotic stress (Chang et al. 2007; Melchiorre et al. 2011), whereas the free-living *Ensifer meliloti* and several *Mesorhizobium* species can tolerate salinity caused by 300 to 700 mM NaCl (Laranjo and Oliveira 2011).

From an inoculant perspective, it is important to identify and characterize rhizobial isolates with capacity to tolerate environmental stresses. Here we have observed a relationship between the behavior of isolates under water deficit and the location where they come from. Rhizobial isolates from San Miguel and Padre Lozano were able to grow under severe osmotic stress, whereas isolates from Humid Chaco (Colonia Benitez) were able to tolerate only moderate stress. The differential behavior of isolates demands a detailed characterization of the physiological responses expressed under stress.

Among rhizobial species, and other plant-growth-promoting microorganisms, production of phytohormones has been related to the stimulation of plant growth (Baca and Elmerich 2007). Auxins, particularly IAA, can stimulate root exudate production thus increasing bacterial colonization of root surface also favored by root growth (Steenhoudt and Vanderleyden 2000; Baca and Elmerich 2007). IAA production by bacteria is finely modulated by environmental stresses, such as acid pH, osmotic and matric stress, and C limitation (Spaepen et al. 2007). Here we have showed a close relationship between IAA production by rhizobial isolates and their capacity to survive under severe osmotic stress with higher indolic compound levels in strains from locations with water shortage such as San Miguel and Bolsa Palomo than strains with no water shortage from Colonia Benitez in Humid Chaco. However, we were not able to determine the relationship between indolic compound levels produced by rhizobial strains and their effect on *P. alba* growth probably because plant growth is due to a complex variety of processes. For example, other plant responses promoted by these isolates, such as early seedling growth and plant phytohormone induction, can improve mineral and water absorption and should be topics of future research.

Of the chosen rhizobia strains affecting *P. alba* growth, strains from Bolsa Palomo belonged to *Mesorhizobium* and *Sinorhizobium* genera, as determined by the analysis of nearly full-length PCR-amplified 16S rDNA sequences. The rhizobial strain from Colonia Benitez was also identified as *Mesorhizobium*, whereas the isolates from Padre Lozano were identified as *Bradyrhizobium* and *Sinorhizobium*. These genera have been previously isolated from other regions as *Prosopis* microsymbionts, whereas *M. chacoense* has been described as the rhizobial strain able to nodulate *P. alba* in Argentina. To our knowledge, this is the first report on *P. alba* nodulation by rhizobial strains other than *M. chacoense* in Parque Chaqueño region of Argentina. Additionally, this study on the behavior of these rhizobial isolates obtained from diverse environments and characterized under osmotic stress in the free-living state provide basic information for an effective inoculation of a *P. alba* plantation with productive purposes.

Conclusions

Parque Chaqueño region of Argentina showed very high diversity in rhizobial strains able to nodulate *P. alba*, and strains other than *M. chacoense*, which has been already described as *P. alba* symbionts, have been identified. Rhizobia isolates selected in this work showed that high level of indolic compound production and tolerance to osmotic stress depended on the environments where the strains belonged. This study can help to make effective inoculations of *P. alba*.

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