

# A Study on the Prevalence of Bacteria that Occupy Nodules within Single Peanut Plants

Jorge Angelini · Fernando Ibáñez · Tania Taurian ·  
María Laura Tonelli · Lucio Valetti ·  
Adriana Fabra

Received: 17 November 2010 / Accepted: 16 March 2011 / Published online: 27 March 2011  
© Springer Science+Business Media, LLC 2011

**Abstract** In this study, bacteria hosted in root nodules of single plants of legume *Arachis hypogaea* L. (peanut) cv Tegu Runner growing at field were isolated. The collection of nodule isolates included both fast and slow growing strains. Their genetic diversity was assessed in order to identify the more frequently rhizobial strain associated to nodules from single plants. Molecular fingerprinting of 213 nodular isolates indicated heterogeneity, absence of a dominant genotype and, therefore, of a unique strains highly competitive. Efficient nitrogen-fixing isolates were identified as *Bradyrhizobium* sp. by phylogenetic analysis of the sequences of their 16S rRNA genes. The genetic diversity of 68 peanut nodulating isolates from all the collected plants was also analyzed. Considering their ERIC-PCR profiles, they were grouped in eighteen different OTUs for 60% similarity cut-off. Results obtained in

this study indicate that the genetic diversity of rhizobia occupying nodules from single plant is very high, without the presence of a dominant strain. Therefore, the identification of useful peanut rhizobia for agricultural purposes requires strongly the selection, among the diverse population, of a very competitive genotype in combination with a high-symbiotic performance.

## Introduction

The nitrogen-fixing symbiosis established between legumes and prokaryotic microorganisms is characterized by the formation of nodules, being these organs subsequently colonized by the specific microsymbionts. The prokaryotic partners include members of the family Rhizobiaceae, collectively named rhizobia (genera *Bradyrhizobium*, *Rhizobium*, *Mesorhizobium*, *Ensifer*, or *Sinorhizobium*, *Azorhizobium*, *Allo-rhizobium*) as well as other taxa (*Burkholderia* [19], *Ralstonia* [5], *Methylobacterium* [26], and *Devosia* [21]).

Peanut (*Arachis hypogaea* L.) is a legume that has an important agronomic role in the economy of many countries. In Argentina, about 87% of peanut production takes place in the province of Córdoba. Currently, most identified rhizobia that nodulate peanut are slow-growing [22, 31, 32, 36]. These strains belong to the genus *Bradyrhizobium*. However, several authors found that, besides *Bradyrhizobium*, bacteria belonging to the genus *Rhizobium* were also associated with peanut nodules in Moroccan [8, 9] and Argentinean [28] soils. This last group includes isolates closely related to *R. huautlense*, *R. galegae* [9], and to *R. giardinii* and *R. tropici* [15, 28]. Furthermore, these studies on the diversity of the peanut rhizobia population in soils from the peanut growing area indicated that it is highly diverse [10, 28].

J. Angelini (✉) · F. Ibáñez · T. Taurian ·  
M. L. Tonelli · L. Valetti · A. Fabra  
Departamento de Ciencias Naturales, Facultad de Ciencias  
Exactas, Físico-Químicas y Naturales, Universidad Nacional de  
Río Cuarto, Enlace Rutas 8 y 36 km 601, 5800 Río Cuarto,  
Córdoba, Argentina  
e-mail: jangelini@exa.unrc.edu.ar

F. Ibáñez  
e-mail: fibanez@exa.unrc.edu.ar

T. Taurian  
e-mail: ttaurian@exa.unrc.edu.ar

M. L. Tonelli  
e-mail: mtonelli@exa.unrc.edu.ar

L. Valetti  
e-mail: lvaletti@exa.unrc.edu.ar

A. Fabra  
e-mail: afabra@exa.unrc.edu.ar

In the nodules, rhizobia are able to transform atmospheric nitrogen to ammonia, a form of nitrogen used by the plant. In this way, rhizobial inoculants may reduce the dependence on nitrogen fertilizers without affecting yields in the production of legumes. However, a very common problem is that efficient nitrogen-fixing laboratory strains are not competitive against native strains of the same species under field conditions. Therefore, a major barrier to the successful use of inoculants is the inability of the inoculated strains to out-compete the indigenous rhizobial strains in nodulation [3]. It is considered that a strain is a successful inoculant when, beside its high efficiency in nitrogen fixation, is able to establish in soil occupied by indigenous rhizobia and to colonize the greatest proportion of nodules.

In peanut, nodulation by native bacteria is usually assumed to be adequate. However, improved symbiotic efficiency can be achieved by increasing the soil cell number of a more effective and competitive strain than that naturally occupy peanut nodules. It was hypothesized that, at field, peanut nodules are occupied by a competitive strain, and that it is possible to select from soil other peanut symbionts more effective and competitive. Taking this into account, the objective of this study was to identify the more frequently *Bradyrhizobium* strain found inside nodules from single peanut plants growing under field conditions. The long-term objective is to select a more competitive rhizobial strain than that found occupying most of the nodules at field, able to provide better symbiotic benefits to legume, leading to an increase in peanut yield and quality. Furthermore, the genetic diversity of bacteria associated with nodules from all the collected plants was also analysed in this study.

## Materials and Methods

### Sampling Sites and Bacterial Isolates

Bacteria were isolated from nodules of peanut plants growing under field conditions. Two different sites were sampled (Charras and Bengolea, more than 36 km apart) located in the center and south region of the Argentinean province of Córdoba (latitude, 32°, 34'; longitude, 63°, 65'). As these sites have no history of *A. hypogaea* L. inoculation, it was assumed that isolates represent the native populations. Fourteen plants were collected from Charras and 13 plants from Bengolea. Each plant collected from Charras had an average of 19.5 nodules and each one collected from Bengolea had an average of 10 nodules, being 34 the maximum and 5 the minimal number of nodules per plant.

Bacteria were isolated from inside fresh superficially sterilized nodules formed in single plants by the method described by Vincent [34]. Sterilized nodules were individually crushed in a drop of sterile water and this suspension was streaked on YEM [34] plates supplemented with cycloheximide (200 mg ml<sup>-1</sup>) to suppress fungal growth. In order to check the efficiency of the sterilization method, a 100 µl aliquot of the last washing solution was incubated on YEM plates.

### Nodulation Test and Symbiotic Effectiveness

The nodulation ability of each isolate was verified by plant test using the method described by Taurian et al. [28]. Five seedlings *A. hypogaea* L. cv Tegua Runner (provided by El Carmen S.A, General Cabrera, Córdoba, Argentina), growing in plastic pots containing sterilized vermiculite, were inoculated with 3 ml of each bacterial broth culture (YEM) in stationary growth phase ( $4 \times 10^9$  cells ml<sup>-1</sup>). Seedlings uninoculated or inoculated with the strain recommended as peanut inoculant (*Bradyrhizobium* sp. SEMIA 6144, FEPAGRO collection, Porto Alegre, RS), and N-fertilized plants by adding 5 mmol l<sup>-1</sup> KNO<sub>3</sub> were included as control.

Plants were grown in a greenhouse under controlled environmental conditions (light intensity of 200 µ E m<sup>-2</sup> S<sup>-1</sup>, 16-h day/8-h night cycle, at a constant temperature of 28°C and relative humidity of 50%). They were harvested at 40 days after inoculation and number of root nodule was determined.

In order to make sure that nodules were formed by the inoculated isolates, the ERIC-PCR profiles obtained from the bacteria inside the nodules were compared with those from the original inoculants.

The ability of the peanut field isolates to fix nitrogen was assessed determining the shoot dry weights of inoculated peanut plants.

### DNA Extraction

Colonies grown on YEM plates were picked up by using a plastic disposable loop and suspended in 300 µl of 1 mol l<sup>-1</sup> NaCl, mixed thoroughly and centrifuged at 12,000 g for 5 min. The supernatant was discarded and the pellet was suspended in 300 µl bidistilled sterile water. After centrifugation at 12,000 g for 5 min, the pellet was suspended in 150 µl of 6% (aqueous suspension) resin Chelex 100<sup>®</sup> (Bio Rad<sup>®</sup>). This suspension was incubated at 56°C for 20 min, followed by mixing and further incubation at 99°C for 8 min [35]. DNA concentration of the samples was approximately 5 ng µl<sup>-1</sup>.

## ERIC-PCR Analysis

The enterobacterial repetitive intergeneric consensus (ERIC) primers used in this study (E1: 5'-ATGTAAGCTCCTGGGGATTAC-3' and E2: 5'-AAGTAAGTGACTGGGGTGAGCG-3') have been reported by Versalovic et al. [33]. Polymerase chain reaction (PCR) was performed in 12 µl reaction mixture containing 1× PCR buffer, 1.5 mM MgCl<sub>2</sub>, 200 µM each nucleotide (Promega), 0.3 µM each primer, 1 U of Taq DNA polymerase (Invitrogen) and 18 ng of template DNA solution. The temperature profile was as follows: initial denaturation at 95°C for 1 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, extension at 65°C for 8 min, and a final extension step at 68°C for 16 min. PCR-amplifications were performed in a thermal cycler (Master cycle, Eppendorf, Germany).

The ERIC amplification products in 6 µl sub-samples were separated by horizontal electrophoresis on 1.5% agarose gels and stained with ethidium bromide.

The band patterns of ERIC-PCR fingerprinting were converted into a binary matrix through a binary scoring system (one for the presence of band and zero for the absence). Computer-assisted analysis of the fingerprints was carried out using Cross Checker system software 2.91 [4]. With the assistance of the FAMD (Fingerprint Analysis with Missing Data) software package [23], a dendrogram was constructed from the distance matrix by the means of Unweighted Pair Group Method with Arithmetic mean (UPGMA) algorithm.

## Data Analysis and Evaluation of Diversity

The peanut nodulating isolates were grouped according to the degree of similarity of their ERIC-PCR banding patterns. A cut-off of 60% was considered, following criteria used to the study of diversity of tropical rhizobia [1, 13, 16].

The Shannon–Weaver index [24] was applied to determine the diversity index ( $H$ ) for each geographical site, and it was calculated by the following equation

$$H = - \sum_{i=1}^k p_i \ln p_i$$

where  $k$  is the number of operational taxonomic units (OTU defined by similar ERIC-PCR profiles) and  $p_i$  the relative abundance of isolates of each OTU [6].

## Amplification of the *nodC* Gene

In order to identify rhizobial strains among the peanut nodules isolates, *nodC* gene was amplified. The amplification of a 930 bp *nodC* gene fragment from the isolates

was assayed using primers *nodCF* (5'-AYGTHGTYGAYGACGGTTC-3')/*nodCR* (5'-CGYGACAGCGANTCKCTATTG-3') [17]. Genomic DNA from *R. tropici* NCHA22 was used as a positive control. PCR products were separated by horizontal electrophoresis on 1.5% (w v<sup>-1</sup>) agarose gels and were visualized by ethidium bromide staining.

## 16S Amplified rDNA Restriction Analysis (ARDRA)

Nearly full-length 16S rDNA gene was PCR amplified by using primers pA and pH [30]. PCR was performed in 20 µl reaction mixture containing 2 µl PCR buffer, 1.5 mmol<sup>-1</sup> MgCl<sub>2</sub>, 200 µmol<sup>-1</sup> each nucleotide (Promega), 1 µmol<sup>-1</sup> each primer, 1 U of Taq polymerase and 5 µl of template DNA solution (5 ng DNA µl<sup>-1</sup>).

The temperature profile was as follows: initial denaturation at 95°C for 3 min; 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min; and a final extension step consisting of 72°C for 10 min; 3 to 5 µl aliquots of PCR products was incubated, respectively, with endonuclease *CfoI* as it was described by Laguerre et al. [18]. Restricted fragments were separated by horizontal electrophoresis on 2.5% agarose gels and visualized by ethidium bromide staining.

## 16S rRNA Gene Sequencing and Data Analysis

The 16S rRNA gene was amplified as described above. PCR products were purified by QIAquick Spin columns (Qiagen), and sequenced by biomolecular analysis platform at University of Laval.

The sequences were aligned using the Clustal W Multiple Alignment program of BioEdit Sequence Alignment Editor Software package [29]. Aligned sequences were analysed using the Molecular Evolutionary Genetics Analysis 2 software, version 4.0, which was further used to produce bootstrap dendrogram reflecting the distance between isolates and reference strains by neighbor-joining method according to the model of Tamura and Nei [27].

## Nucleotide Sequence Accession Numbers

The nucleotide sequence of the 16S rRNA gene from isolates j72 and j237 have been deposited in GenBank data bank under accession N° GQ222233 and GQ222234, respectively.

## Statistical Analysis

Data were analysed by ANOVA and differences among treatment detected by LSD test ( $P < 0.05$ ).

**Results**

**Bacterial Diversity Inside Nodules from Single Plants**

The average number of isolates obtained from inside nodules of each plant was 9 in Charras and 5 in Bengolea, being in the two sites the minimal and the maximum numbers 5 and 19, respectively. In both localities analysed, almost the 41% of the isolates recovered from each plant were slow-growers.

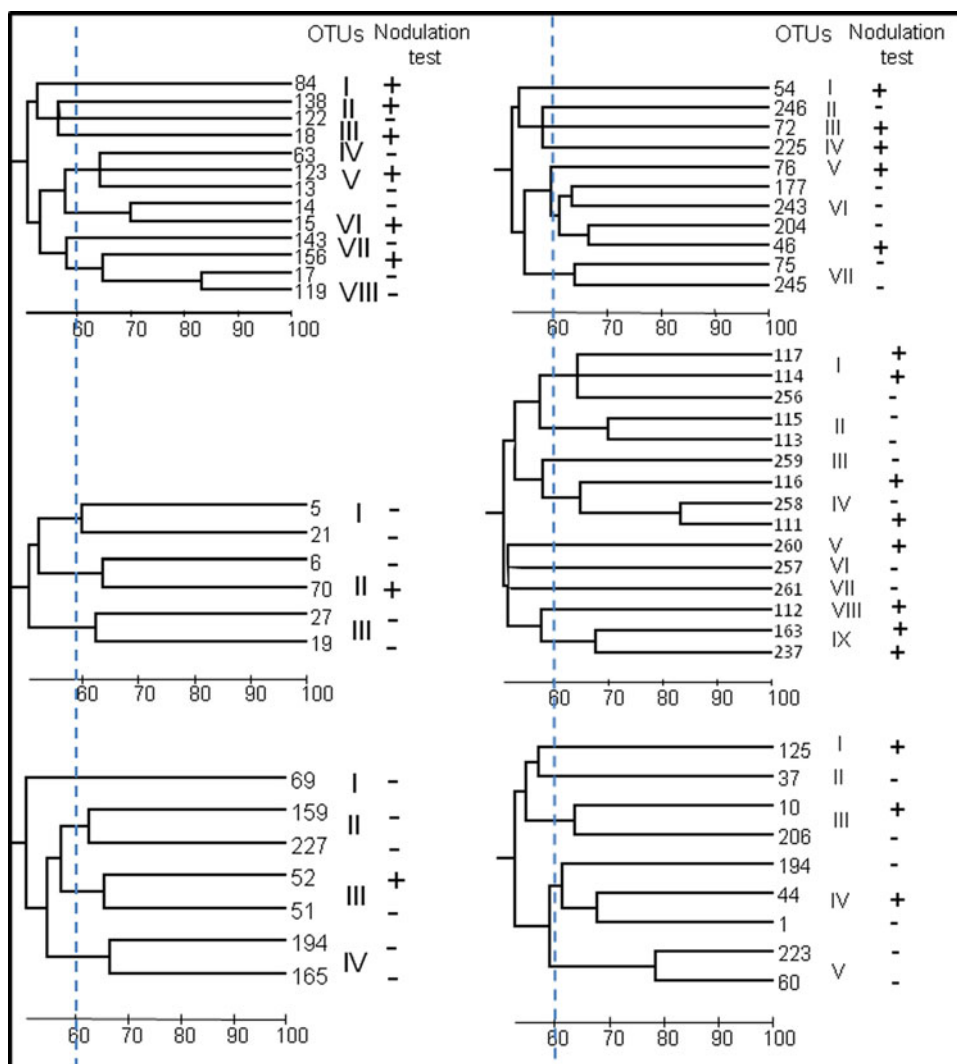
Dendrogram based on ERIC fingerprint patterns showed that the genetic diversity of isolates occupying nodules from each plant was high (Fig. 1) since only two isolates shared a same pattern. A predominant rhizobial genotype in nodules formed in single plants was not identified.

**Diversity of Bacteria Associated to Nodules in Peanut**

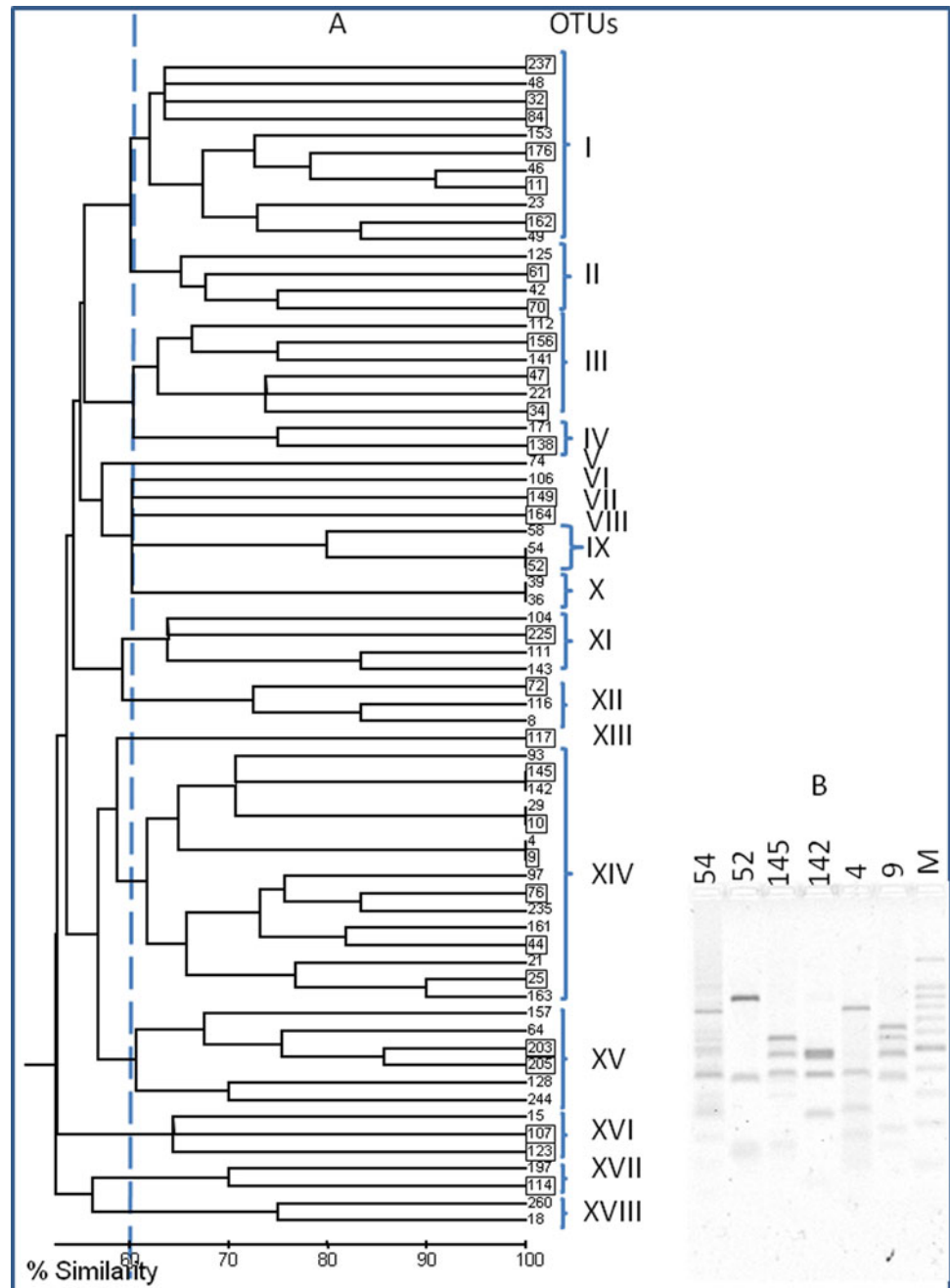
A total of 27 peanut plants and 402 nodules from both localities were evaluated. Bacterial isolates were recovered

from only the 52.9% of these nodules. Two hundred and thirteen bacterial isolates were obtained from inside the surface-sterilized nodules from these plants. Only 43% of these isolates were slow-growing bacteria. The ability to nodulate peanut was confirmed for 68 isolates (24 slow-growers and 44 fast-growers). The fingerprinting analysis from these isolates able to nodulate peanut yielded 58 different ERIC banding profiles, considering a similarity coefficient of 60%. By using this cut off value, the ERIC patterns could be divided into 18 different operational taxonomic units (OTUs) that included both fast and slow growers (Fig. 2a). Only isolates grouped in OTU II came from the same geographical origin (Charras). Differences between diversity indices obtained for the isolates from each location were not found ( $H' = 139.4$  for Charras and  $H' = 130.5$  for Bengolea). As a consequence of the low band number obtained for some isolates in the ERIC profiles, the image normalization process included in the same clusters the fast growing isolates 54, 4, and 142, and the slow growing isolates 52, 145, and 9. However, their

**Fig. 1** Dendrograms generated from the ERIC-PCR patterns of bacteria that occupy nodules within individual peanut plants, using the UPGMA algorithm and the Jaccard coefficient. These dendrograms are representative of those obtained for the totality of the plants analysed



**Fig. 2 a** Dendrogram generated from the ERIC-PCR patterns of the peanut nodule isolates grouped by UPGMA. Slow grower bacteria are marked with *boxes*. **b** Gel electrophoresis of 16S rRNA-RFLP from some fast and slow growing isolates. *M* molecular marker



ARDRA profiles clearly indicate the genetic variation among these fast and slow growers (Fig. 2b).

The presence of *nodC* was used in this study as a criterion to confirm that isolates are rhizobial strains, since this gene is found in almost all rhizobia. The *nodC* gene amplification with primers *nodCF/nodCR* yielded a product of the expected size (about 930 bp) for the reference strain *Bradyrhizobium* sp SEMIA 6144 and for 9 (j107, j225, j244, j153, j97, j125, j47, j260, j72) of the 213 peanut nodule isolates (data not shown). However, in the 37% of all analyzed strains, the DNA amplification resulted in more than one amplification product, higher or lower than

930 bp, probable because of some nucleotide mismatches between primers used and the 3' end region of *nodC* genes.

#### Symbiotic Behavior of the Isolates

The 9 strains which *nodC* gene was amplified were tested for their symbiotic behavior. The number of nodules formed varied greatly with the inoculated strain (Table 1).

Strains that showed more than one amplification product in the *nodC*-PCR assay were also evaluated for their symbiotic behavior. It was found that plants inoculated with the slow-growing strain j237, and with the fast-growing strain



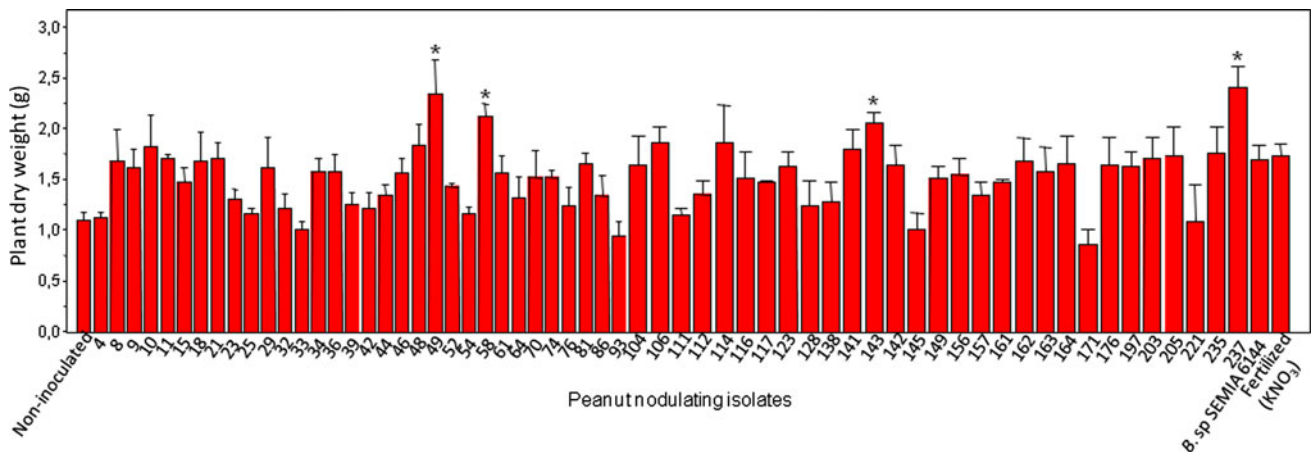
**Table 1** Response of peanut to inoculation with isolates whose *nodC* genes were amplified

Isolates	Percentage of nodulated plants	N° nodules/plant	Nodules dry weight (mg)	Shoot dry weight (g)
<i>Slow growing</i>				
j225	50	5 ± 1.2*	4 ± 0.5*	1.3 ± 0.1
j107	100	12 ± 6.3*	10 ± 1*	1.3 ± 0.1
j47	100	40 ± 14.8	47 ± 5*	1.6 ± 0.1
j72	100	85 ± 12*	70 ± 7.5	1.9 ± 0.2**
<i>Fast growing</i>				
j153	100	23 ± 11	23 ± 5	1.4 ± 0.1
j97	33	11 ± 4*	18 ± 6*	1.5 ± 0.3
j125	100	37 ± 9	50 ± 3	1.5 ± 0.2
j244	100	12 ± 5*	20 ± 3*	1.4 ± 0.3
j260	100	23 ± 7	60 ± 5.6	1.7 ± 0.1
<i>Bradyrhizobium</i> sp. SEMIA 6144	100	38 ± 4	60 ± 17	1.7 ± 0.1
Fertilized (KNO <sub>3</sub> )	-	-	-	1.7 ± 0.1
Non-inoculated	-	-	-	1.1 ± 0.1

Values are the mean ± SE of five replicates

\* Significantly different from plants inoculated with SEMIA 6144 ( $P \leq 0.05$ ) according to LSD multiple range test

\*\* Significantly different from plants fertilized ( $P \leq 0.05$ ) according to LSD multiple range test



**Fig. 3** Symbiotic effectiveness of peanut rhizobia values are the mean ± SE of five replicates. \* Significantly different from plants fertilized ( $P \leq 0.05$ ) according to LSD multiple range test

j58, j49, and j143 showed larger biomass than plants supplied with mineral N (Fig. 3). However, fast-growing strains lost their ability to nodulate peanut after storage. The fact that the ERIC-profiles from the strains when they were obtained before and after storage were identical (data not shown) is discarding the possibility that contamination events occur during the strains storage.

#### Sequence Analysis of 16S rRNA Gene

Isolate j237 and j72, which represent the OTU I and OUT XII, respectively, were selected to analyse their 16S rRNA gene because they were the slow growing isolates most efficient in the nitrogen fixation, considering the higher biomass of inoculated plants. The nucleotide sequences were compared to sequences of rhizobial species available

in data banks. The alignment of 1381 bp of the 16S rDNA sequence from isolate j237 revealed a high level of identity (99%) with the sequence of *Bradyrhizobium japonicum* SEMIA 6144, being eight the number of nucleotide differences. The alignment of 1383 bp of j72 16S rRNA gene showed a high level of identity (98%) with *Bradyrhizobium* sp SEMIA 5034.

#### Discussion

The traditional strategy used to investigate nodule associated microbial symbionts involves their isolation and cultivation from internal tissues of surface-sterilized nodules [34]. It is known that nodules can be colonized internally by several bacterial genera unrelated to rhizobial symbiotic

nitrogen fixation. Sturz et al. [25] reported the presence of not only rhizobial strains inside red clover nodules but also of non-rhizobial endophytes. Also Philipson and Blair [20] found diverse species, including Gram positive bacteria, in nodules of healthy red clover. Other authors reported the isolation of Gammaproteobacteria from surface-sterilized peanut nodules [14].

In this study, the fingerprinting analysis of isolates recovered from inside nodules formed in single peanut plants and whose capacity to nodulate peanut was further confirmed, revealed elevated genetic diversity without differences in the Shannon–Weaver index obtained from both analysed localities. The 58 different ERIC banding profiles obtained from the peanut nodulating isolates were grouped in 18 operational taxonomic units (OTUs). No relationship was found between the geographical origin of the isolates and the OTU they were grouped, except for OTU II which included only isolates from Charras.

In spite of the fact that nodulation ability of bacteria was confirmed directly after isolation, several months later some strains failed to nodulate peanut, probably due to the lost of symbiotic genes. This finding is confirming similar results that the authors have recently reported when another peanut nodule isolates collection from a different peanut growing area was analyzed [14], suggesting that this is a common phenomenon.

Products of the bacterial nodulation genes (*nod*) have been shown to play a role in the molecular dialog between legumes and rhizobia [11]. The presence of structural *nodABC* genes in almost all rhizobia (except *Bradyrhizobium* strains BTAi1 and ORS278; [12]) may be indicating the unique origin of these genes. The impossibility to amplify by PCR the *nodC* gene in some of the peanut nodule isolates could be associated with the previous finding that peanut nodules are colonized not only by rhizobial bacteria but also by non-rhizobial endophytes [14]. This may be related with the fact that less stringent requirements toward their micro-symbiotic partners have been proposed for legume that, as peanut, are infected by crack-entry [2]. Alternatively, the absence of amplification in some isolates capable to nodulate peanut could be related with the lack of primers specificity.

Host specificity is one of the important factors that affect the distribution of indigenous rhizobial population. Among the rhizobial isolates obtained in this study, it was identified both fast and slow growers. This was not unexpected since the authors obtained identical results when the diversity of peanut nodulating rhizobia in soils from Córdoba province was analyzed [28]. The slow-growing strains that proved to be more effective in the nitrogen fixation belong to different OTUs. Their 16S rRNA genes were sequenced and the alignments indicate that both sequences highly match the 16S rRNA from *Bradyrhizobium* species.

The fact that isolates were recovered from only the 52.9% of nodules is probably indicating that viable but uncultivable bacteria may also induce nodule formation in peanut. This study indicated that there is a high-genetic diversity in the population of cultivable rhizobia that forms nodules in single peanut plant, without the presence of a dominant strain. *A. hypogaea* L. can thus be considered to be promiscuous in nature since root nodules of a single plant are not induced by a predominant genetic variant enriched from the rhizosphere population. On the basis of this finding, peanut nodules may be considering a reservoir for different rhizobial lineages.

Even when there is information about peanut micro-symbiont diversity [28] this is the first report evaluating the genetic diversity among bacteria occupying peanut nodules from single plants. Findings here reported indicate that the identification of useful peanut rhizobia for agricultural purposes requires strongly the selection, among the diverse population, of a very competitive genotype in combination with a high-symbiotic performance.

**Acknowledgments** This study was supported by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Secretaría de Ciencia y Técnica de la Universidad Nacional de Río Cuarto (SECYT-UNRC), CONICET and Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT). M. Tonelli and F. Ibañez are fellowships from CONICET, T. Taurian, J. Angelini and A. Fabra are members of research career of CONICET, Argentina.

## References

1. Alberton O, Kaschuk G, Hungria M (2006) Sampling effects on the assessment of genetic diversity of rhizobia associated with soybean and common bean. *Soil Biol Biochem* 38:1298–1307
2. Alwi N, Wynne JC, Rawlings JO, Schneewies TJ, Elkan GH (1989) Symbiotic Relationship between *Bradyrhizobium* strains and peanut. *Crop Sci* 29:50–55
3. Beattie GA, Clayton MK, Handelsman J (1989) Quantitative comparison of the laboratory and field competitiveness of *Rhizobium leguminosarum phaseoli*. *Appl Environ Microbiol* 55: 2755–2761
4. Buntjer B (1999) Software Crosscheck, vol. 8. Developed in Wageningen University and Research Centre, Wageningen
5. Chen WM, Laevens S, Lee TM, Coenye T, De Vos P, Mergeay M, Vandamme P (2001) *Ralstonia taiwanensis* sp. nov., isolated from root nodules of Mimosa species and sputum of a cystic fibrosis patient. *Int J Syst Evol Micr* 51:1729–1735
6. Coutinho H, Olivera V, Lovato A, Maia A, Manfio G (1999) Evaluation of diversity of rhizobia in Brazilian agricultural soils cultivated with soybeans. *Appl Soil Ecol* 13:159–167
7. Dart PJ (1977) Infection and development of leguminous nodules. In: Hardy RWF, Silver WS (eds) *A treatise on dinitrogen fixation*. Section III Biology, 1st edn. John Wiley and Sons, New York, pp 367–472
8. El-Akhal M, Rincón A, Arenal F, Mercedes M, El Mourabit N, Barrijal S, Pueyo J (2008) Genetic diversity and symbiotic efficiency of rhizobial isolates obtained from nodules of *Arachis hypogaea* in northwestern Morocco. *Soil Biol Biochem* 40: 2911–2914

9. El-Akhal M, Rincón A, El Mourabit N, Pueyo J, Barrijal S (2009) Phenotypic and genotypic characterizations of rhizobia isolated from root nodules of peanut (*Arachis hypogaea* L.) grown in Moroccan soils. *J Basic Microb* 49:415–425
10. Fabra A, Castro S, Taurian T, Angelini J, Ibañez F, Dardanelli M, Tonelli M, Bianucci E, Valetti L (2010) Interaction among *Arachis hypogaea* L. (peanut) and beneficial soil microorganisms: how much is it known? *Crc Rev Microbiol* 36:179–194
11. Geetanjali NG (2007) Symbiotic nitrogen fixation in legume nodules: process and signaling. A review *Agron Sustain Dev* 27:59–68
12. Giraud E, Moulin L, Vallenet D, Barbe V, Cytryn E, Avarre JC, Jaubert M, Simon D, Cartieaux F, Prin Y, Bena G, Hannibal L, Fardoux J, Kojadinovic M, Vuillet L, Lajus A, Cruveiller S, Rouy Z, Mangenot S, Segurens B, Dossat C, Franck WL, Chang W, Saunders E, Bruce D, Richardson P, Normand P, Dreyfus B, Pignol D, Stacey G, Emerich D, Verméglio A, Médigue C, Sadowsky M (2007) Legumes symbioses: absence of *Nod* genes in photosynthetic Bradyrhizobia. *Science* 316:1307–1312
13. Grange L, Hungria M (2004) Genetic diversity of indigenous common bean (*Phaseolus vulgaris*) rhizobia in two Brazilian ecosystems. *Soil Biol Biochem* 36:1389–1398
14. Ibañez F, Angelini J, Taurian T, Tonelli L, Fabra A (2009) Endophytic occupation of peanut root nodules by opportunistic *Gammaproteobacteria*. *Syst Appl Microbiol* 32:49–55
15. Ibañez F, Taurian T, Angelini J, Tonelli ML, Fabra A (2008) Rhizobia phylogenetically related to common bean symbionts *Rhizobium giardinii* and *Rhizobium tropici* isolated from peanut nodules in Central Argentina. *Soil Biol Biochem* 40:537–539
16. Kaschuk G, Hungria M, Andrade DS, Campo RJ (2006) Genetic diversity of rhizobia associated with common bean (*Phaseolus vulgaris* L.) grown under no-tillage and conventional systems in Southern Brazil. *Appl Soil Ecol* 32:210–220
17. Laguerre G, Allard M, Revoy F, Amarger N (1994) Rapid identification of rhizobia by restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA genes. *Appl Environ Microb* 60:56–63
18. Laguerre G, Nour SM, Macheret V, Sanjuan J, Drouin P, Amarger N (2001) Classification of rhizobia based on *nodC* and *nifH* gene analysis reveals a close phylogenetic relationship among *Phaseolus vulgaris* symbionts. *Microbiology* 147:981–993
19. Moulin L, Munive A, Dreyfus B, Boivin-Masson C (2001) Nodulation of legumes by members of the beta-subclass of Proteobacteria. *Nature* 411:948–950
20. Philipson MN, Blair ID (1957) Bacteria in clover root tissue. *Can J Microbiol* 3:125–129
21. Rivas R, Velázquez E, Willems A, Vizcaíno N, Subba-Rao NS, Mateos PF, Gillis M, Dazzo FB, Martínez-Molina E (2002) A new species of *Devosia* that forms a unique nitrogen-fixing root-nodule symbiosis with the aquatic legume *Neptunia natans* (L.f.) Druce. *Appl Environ Microbiol* 68:5217–5222
22. Saleena LM, Loganathan P, Rangarajan S, Nair S (2001) Genetic diversity of *Bradyrhizobium* strains isolated from *Arachis hypogaea*. *Can J Microbiol* 47:118–122
23. Schlüter PM, Harris SA (2006) Analysis of multilocus fingerprinting data sets containing missing data. *Mol Ecol Notes* 6:569–572
24. Shannon CE, Weaver W (1949) The mathematical theory of communication. University of Illinois, Urbana
25. Sturz AV, Carter MR, Johnston HW (1997) A review of plant disease, pathogen interactions and microbial antagonism under conservation tillage in temperate humid agriculture. *Soil Till Res* 41:169–189
26. Sy A, Girud E, Jourand P, Garcia N, Willems A, De Lajudie P, Prin Y, Neyra M, Gills M, Catherine BM, Dreyfus B (2001) Methylophilic *Methylobacterium* bacteria nodulate and fix atmospheric nitrogen in symbiosis with legumes. *J Bacteriol* 183:214–220
27. Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol* 10:512–526
28. Taurian T, Ibanez F, Fabra A, Aguilar OM (2006) Genetic diversity of rhizobia nodulating *Arachis hypogaea* L. in Central Argentinean soils. *Plant Soil* 282:41–52
29. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tool. *Nucleic Acids Res* 24:4867–4882
30. Ulrike E, Rogall T, Blocker H, Emde M, Bottger EC (1989) Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Res* 17:7843–7853
31. Urtz BE, Elkan GH (1996) Genetic diversity among *Bradyrhizobium* isolates that effectively nodulate peanut (*Arachis hypogaea*). *Can J Microbiol* 42:1121–1130
32. van Rossum D, Schuurmans FP, Gillis M, Muyotcha A, Van Verseveld HW, Stouthamer AH, Boogerd FC (1995) Genetic and phenetic analyses of Bradyrhizobium strains nodulating peanut (*Arachis hypogaea* L.) roots. *Appl Environ Microbiol* 61:1599–1609
33. Versalovic J, Schneider M, de Bruijn FJ, Lupski JR (1994) Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods Mol Cell Biol* 5:25–40
34. Vincent JM (1970) A manual for the practical study of root nodule bacteria. Blackwell Scientific, Oxford
35. Walsh PS, Metzger DA, Higuchi R (1991) Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *BioTechniques* 10:506–513
36. Zhang X, Nick G, Kajjalainen S, Terefewirjm Z, Paulin L, Tighe SW, Graham PH, Lindstrom K (1999) Phylogeny and diversity of *Bradyrhizobium* strains isolated from the root nodules of peanut (*Arachis hypogaea*) in Sichuan, China. *Syst Appl Microbiol* 22:378–386