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Characterization of Nodulating Peanut Rhizobia Isolated from a Native Soil Population in Córdoba, Argentina

TANIA TAURIAN[1], O.MARIO AGUILAR[2], and ADRIANA FABRA[1*]
[1]*Departamento de Ciencias Naturales, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, 5800 Río Cuarto (Córdoba), Argentina, Tel. +54-358-4676438, Fax. +54-358-4676230, E-mail. afabra@exa.unrc.edu.ar;*
[2]*Instituto de Bioquímica y Biología Molecular, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Argentina*

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Abstract

A total of 39 root nodule bacterial isolates were obtained from soils or *Arachis hypogaea* L. growing in fifteen different locations in Córdoba, Argentina. After authentication on homologous host species, the presence of *nifD* gene was analyzed, and their cultural and physiological properties were studied. About 46% of the isolates were fast-growers and produced an acid reaction in YEM medium. It was possible to differentiate this group from the slow growing isolates by their tolerance to environmental stresses (elevated concentrations of salt, acid pH and high temperature). The use of different carbon sources showed no differences among the isolates. Almost all the isolates were symbiotically effective with peanut and their nodulation ability in other legumes was also studied.

Keywords: Peanut (*Arachis hypogaea* L.), fast and slow growing rhizobia, root nodule bacteria

*The author to whom correspondence should be sent.

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1. Introduction

Deficiency in the availability of nitrogenous compounds in soils may represent a limitation in plant growth. In the case of legumes, nitrogen-fixing root-nodule bacteria of the family Rhizobiaceae, could increase yields. It is generally accepted that peanut is native to the Americas; it was grown in Mexico, Central and South America and had been proposed that domestication took place in a region located between the north of Argentina and the south of Bolivia (Krapovickas, 1968). So far, bacteria that nodulate peanuts in natural environments from all over the world have been classified as *Bradyrhizobium* (*Arachis*) sp., but species names have not been defined. However, there are several reports describing the isolation and characterization of peanut nodulating *Bradyrhizobium* strains such as NC92 (Gillette and Elkan, 1996); MAR 411 (van Rossum et al., 1995); Spr 3-7 (Zhang et al., 1999; Tighe et al., 2000). The conclusion is that our current knowledge of peanut nodulating rhizobia restricts them to the genus *Bradyrhizobium*.

Arachis hypogaea is an important crop all over the world that provides direct subsistence food and several other food products. Argentina, one of the major peanut producers in the world, concentrates about 98% of its production in the province of Córdoba. The rhizobial populations associated with peanut in the Argentinian area of production, as well as the effects of land management practices on rhizobial diversity, have not yet been examined. Urtz and Elkan (1996) examined the symbiotic gene diversity of *Bradyrhizobium* isolates symbiotically effective with peanut. Sixteen of the thirty-three isolates they analyzed were obtained from South America, but only two isolates were from Argentina. The authors concluded that South America isolates accounted for most of the diversity observed and that more investigations are needed to complete the taxonomic status of rhizobia nodulating *Arachis hypogaea*.

We have isolated peanut rhizobial strains from soils or *A. hypogaea* nodules from the main peanut production region of Argentina and initiated phenotypic and molecular studies in order to characterize their biodiversity and taxonomy. In this paper we report the results of phenotypic analysis and symbiotic efficiency of the isolates.

2. Materials and Methods

Sampling sites and rhizobial isolates

Soils and peanut plants were collected from 15 different locations in the central and southern region of Córdoba, Argentina (latitude, 32° to 34°; longitude, 63° to 65°). No peanut inoculation had been made to these fields,

Table 1. Sources of peanut (*Arachis hypogaea* L.) rhizobia

Isolate	Geographical origin	
	Locality	Department
1. NLH24	Las Higueras	Río Cuarto
2. NLH25	Las Higueras	
3. NLH30	Las Higueras	
4. NLH22	Las Higueras	
5. NLH27	Las Higueras	
6. TT001	Río Cuarto	
7. TT002	Río Cuarto	
8. NOD31	Río Cuarto	
9. NCAR2B	Carnerillo	
10. NHOL2	Holmberg	
11. NHOL16	Holmberg	
12. NMAL12	Malena	
13. NDE11	General Deheza	Juárez Celman
14. NDEHE	General Deheza	
15. NCHAX	Charras	
16. NCHA30	Charras	
17. NCHA31	Charras	
18. NCHA22	Charras	
19. NCHA32	Charras	
20. NCHA33	Charras	
21. NCHA35	Charras	
22. NCHA42	Charras	
23. NALE	Alejandro	
24. NMAN6	Manfredi	Río Segundo
25. NMAN10	Manfredi	
26. NMAN11	Manfredi	
27. NMAN5	Manfredi	
28. NONC1	Oncativo	
29. NONC11	Oncativo	
30. NONC13	Oncativo	
31. NONC4	Oncativo	
32. NONC5	Oncativo	
33. NONC8	Oncativo	
34. NONC9	Oncativo	
35. NONC10	Oncativo	
36. NCHOO4	Chazón	San Martín
37. NVAM24	Villa María	
38. NTI31	Ticino	
39. NET30	Etruria	

Reference strains: *Bradyrhizobium* sp SEMIA 6144, Brazil (IPAGRO); *Bradyrhizobium japonicum* USDA 110, Lab. Dr. Aguilar. O.M.; *Rhizobium etli* CFN42, Lab. Dr. Aguilar, O.M.; *Sinorhizobium meliloti* 116, Brazil (IPAGRO).

therefore, the isolates were considered to be native. The rhizobia isolates used in this study are listed in Table 1. *Bradyrhizobium* sp. (*Arachis hypogaea*) SEMIA 6144 (recommended as peanut inoculant for Instituto de Pesquisas Agronómicas, IPAGRO) was used as reference strain.

Isolation of bacteria from nodules or soil

Bacteria were isolated directly from field peanut root nodules or from soil using peanut as trap host. The isolation from nodules was done by the method of Vincent (1970) and for the isolation from soil, seeds of *Arachis hypogaea* L. cultivar Tegua were surface-sterilized following the method described by Vincent (1970).

All the isolates (obtained from soil samples or nodules) were reinoculated onto plants to confirm their ability to nodulate *A. hypogaea* L. Surface sterilized seeds (Vincent, 1970) were transferred to plastic pots containing sterilized vermiculite. The seedlings were inoculated with 3–5 ml of the appropriate rhizobial broth culture (YEM) in stationary growth phase (1–4 × 10^9 cells/m). A negative control (uninoculated seedling) and a positive control (inoculated with the strain recommended as peanut inoculant) were included. Plants were placed in a growth chamber at 28°C (16 h) and 18°C (8 h) and watered regularly with sterilized top water and, twice a month, with Hoagland's medium. Plants were harvested 5 weeks after inoculation and their roots were observed for nodulation. Their ability to efficiently nodulate peanut was evaluated by observing the presence of leghaemoglobin in nodules.

Authenticated cultures were stored in 20% (v/v) glycerol at –80°C.

Amplification of nifD sequences

Total rhizobial DNA was obtained by using the procedure described by Meade et al. (1982). A primer pair to amplify a 390-bp DNA fragment of the *nifD* gene from different isolates was used. The sequences of the primers are as follows: primer O_1 (5'-TGGGGICCIRTIAARGAYATG-3'); primer O_2 (5'-TCRTTIGCIATRTGRTGNCC-3') (Stoltzfus et al., 1997).

PCR mixtures, in a final volume reaction of 15 µl, contained the following: a 0.4 µM concentration of each primer; 0.2 µM deoxynucleoside triphosphates; 7.5 µl of template DNA; 7.5 µl of *Taq* polimerase (Promega Corp.). Cycling conditions were as follows: 94°C for 1 min, followed by 29 cycles at 95°C for 30 s, at 55°C for 30 s and at 72°C for 1 min; 95°C for 30 s, 55°C for 30 s, and 72°C for 10 min. After the reaction, 5–7 µl of the PCR products was separated in 1.2% agarose gels containing 0.5 to 1 µg of ethidium bromide per ml and visualized under UV light.

Rate of growth and acid production of the isolates

Doubling times were calculated, according to Brock and Madigan (1991), from the exponential growth phase of cultures grown at 28°C in GTS medium (Howieson, 1985). Growth was determined by measuring the O.D. at 620 nm. Typical well-isolated colonies were restreaked on YEMA containing 25 mg/l (w/v) bromothymol blue as pH reaction indicator, in order to differentiate acid from alkali-producing isolates and to determine rate of appearance of colonies.

Carbon source utilization

This was tested by the method described by Missbah El Idrissi et al. (1996). Briefly, filter-sterilized solutions of the carbohydrates (80 µl of 10% w/v solutions) were added to 5 ml of YEM broth medium with yeast extract reduced to 50 mg/l. Growth was initiated by addition of 80 µl of an actively growing bacterial suspension containing about 10^8 cells/ml. After shaking at 100 rpm, 28°C during 7 d (for the slow growers) or 3 d (for the fast growers), the ability to use different carbon sources was assessed by determining the absorbance at 600 nm. Tests were performed in triplicate.

Tolerance to acidity

Tolerance to acidity was determined by inoculating strains from exponentially growing GTS broth cultures onto the GMS agar minimal media or to GMS broth medium, buffered to pH 5 by the addition of MES [2-(*N*-morpholino) ethanesulphonic acid] (3 g/l) (Howieson, 1985). Plates were incubated at 28°C during 7–14 d or until colonies appearance. Tests were performed in triplicate.

Sodium chloride tolerance

The method described by Missbah El Idrissi et al. (1996) was followed and YEMA plates containing 0, 0.17 M, 0.34 M and 0.68 M NaCl were used. The plates were inoculated with 10 µl of a culture containing about 10^8 cells/ml and the growth was scored after 7-14 d at 28°C. Tests were done in triplicate.

Growth at different temperatures

The ability to grow under conditions of high temperature was assessed in YEMA medium plates inoculated with 10 µl of a culture containing about 10^8 cells/ml. Plates were placed in temperature-controlled incubators at 28°C, 37°C,

or 42°C. Colony formation was observed during two weeks. Tests were done in triplicate.

Symbiotic effectiveness

The symbiotic effectiveness of isolates was assessed by applying the following procedure. Pots with single peanut plants each were inoculated as described above. Plants were harvested 60 d after inoculation and nitrogen content in shoots was determined following the procedure proposed by Nelson and Sommers (1973).

The ability of isolates to nodulate other legumes

Twelve isolates were assessed for their ability to nodulate the following legume hosts: *Glycine max* (soybean), *Phaseolus vulgaris* (common bean) and *Medicago sativa* (alfalfa). Seeds were surface-sterilized by immersion in 70% (v/v) ethanol for 30 sec, soaked with 20% (v/v) commercial bleach and washed seven times with sterile distilled water. Plants were grown in plastic pots containing vermiculite and they were regularly watered with sterilized tap water and, twice a month, with Hoagland medium. Each seed was inoculated with 4 ml of a stationary phase culture (10^8 cells/ml in YEM broth) and five replicates were done for each isolate. At 40 d post-inoculation, their ability to nodulate efficiently the legume host was evaluated by observing leghaemoglobin nodule presence. A negative control (uninoculated seedlings) and a positive control (inoculated with each of the recommended legume nodulating strains) were included.

Statistical analysis

The data were analyzed using the Student *t* test at the 0.05 significance level.

3. Results

Slow and fast growers in the native peanut nodulating rhizobial populations

The rhizobial collection used in this study was obtained from nodulated peanuts and soil samples collected in different sites in Córdoba, Argentina (Table 1). Isolates were found to be Gram-negative, non-spore-forming, rod-shaped bacteria. In addition, the nitrogen fixing potential of each bacterial

isolate was confirmed by detecting the presence of the *nif*D gene. This was performed by assessing for PCR amplification products of a highly conserved region of the gene *nif*D. Both in liquid medium (GTS) as in agar-solidified YEM medium, about half of the isolates were found to be fast growers, showing a generation time of about 3 h while the other isolates had a mean duplication time of 19 h (Table 2).

Table 2. Culture characteristics of peanut nodulating isolates

Isolates	Rate of growth in GTS (dt[a])	Reaction in YEMA + BTB[b] (%isolates analyzed)	% of isolates able to use the different carbon sources				
			Monosaccharides				
			Glucose	Lactose	Maltose	Xylose	Fructose
Slow-growing							
	19	90.5% alkaline 9.5% acid	95	90	95	100	95
Fast-growing							
	3	100% acid	94	100	83	100	94
			Disaccharides		**Polyol**	**Carboxylic acid**	
			Galactose	Sucrose	Glycerol	Fumarate	
Slow-growing							
	19	90.5% alkaline 9.5% acid	100	76	100	74	
Fast-growing							
	3	100% acid	100	100	100	100	

a: doubling time (h), b: Bromothymol Blue.

The isolation of fast-growing rhizobia from peanut nodules was unexpected to us since previous works had found *Bradyrhizobium* spp. as the natural

microsymbiont of peanut (Urtz and Elkan, 1996; Somasegaran and Hoben, 1994). Therefore, in order to confirm this finding, rhizobia from single colonies were suspended in 0.1% Tween 20 and appropriate dilutions of this suspension were used to inoculate YEM agar plate. Colonies appeared after 24 h, thus confirming the growth characteristics observed in our early isolation. Furthermore, all these fast-growing isolates were able to nodulate peanut in greenhouse inoculation experiments, and appearance of plants showed no obvious symptoms of nitrogen deficiency. Isolates from these plant nodules were found to be fast-growers. Therefore, we concluded that in the area we surveyed, peanut is able to establish efficient symbiosis with a native population of fast-growing rhizobia in addition to the already known slow-growing rhizobial population. No association was found between these two populations and the origin of the isolates. On the other hand, no relationship was found between the isolate growth rate and their geographical origin, since both fast- and slow- growers or only fast or only slow growers were recovered from the same localities.

The collection was assessed for their ability to acidify the growth medium YEMA and it was found that all the fast-growing isolates produced acid whereas slow growers alkalinized this growth medium. Thus it can be noted that there was a good correlation between the bacterial growth rate and alkali-production data obtained from the slow-grower isolates and those from the reference strain *Bradyrhizobium* SEMIA 6144. Exceptionally, the slow growing isolates NONC9 and NMAN6 were found to produce acid (Table 2). Slow-grower acid-producers were also reported among isolates from *Hedysarium* species and from *Leucaena leucocephala,* respectively (Kishinevsy et al., 1996; Kozusny-Andreani, 2000).

Carbon source utilization

Most of the isolates were able to utilize diverse sugars (such as monosaccharides and disaccharides), dicarboxylate fumaric acid and polyol glycerol. Only few slow grower isolates were unable to use disaccharide sucrose. In general, it was found that the final values of cell density reached by fast growing isolates were higher than those of the slow growers, indicating that fast growers would be more efficient in the utilization of the different carbon sources we have tested (Table 2).

Slow and fast grower peanut nodulating rhizobia diverge in their tolerance to environmental stresses

In order to assess acid tolerance, isolates were tested for the ability to initiate growth at pH 5.0. Although a different degree of tolerance was

observed, in general, fast-growers were more tolerant to acidic conditions than slow growers, since 13% of the latter grew at pH 5.0, whereas 76% of the fast-growers did so (Table 3). All the isolates were able to grow at pH 7.

It was also observed that fast and slow growers might be differentiated by their salt tolerance level. Five slow growers (24%) were able to grow at 0.17 M, and only two (9%) tolerated up to 0.68 M NaCl. By contrast, almost all the fast-growing isolates (76%) were found to be tolerant to high salt concentration (Table 3). Fast-growing, salt-tolerant and slow-growing, salt sensitive strains have also been isolated from *Acacia ampliceps* (Zou et al., 1995), *Acacia saligna* (Marsudi et al., 1999) and from other woody legumes (Odee et al., 1997).

The two groups also exhibited differences in temperature tolerance. About 38% and 9% of the slow growers were able to grow at 37°C and 42°C, respectively. By contrast, 89% and 71% of the fast grower isolates were able to grow at 37°C and 42°C, respectively (Table 3).

Taken these results altogether, we can conclude that the rhizobial population of fast growers is more tolerant to extreme environmental conditions.

Symbiotic effectiveness and host range of peanut nodulating rhizobia

Results from the inoculation assays done to determine the symbiotic effectiveness of the isolates are shown in Table 4. Shoot nitrogen content indicated that peanut inoculation rendered a significant increase ($p<0.05$) compared to the uninoculated plants.

In order to determine the host range, inoculation experiments with 12 isolates were performed with the host legumes alfalfa, soybean and common bean. Although nodules on soybean were not observed, five slow growers and all the fast growers analyzed were able to form nodules on bean. Interestingly, two fast growing isolates (NCHA32, TT001) were also able to nodulate alfalfa (Table 5). These results show that our collection includes rhizobial genotypes having a broad host range.

Table 3. Tolerance of isolates to NaCl, high temperatures and low pH

Isolates	% of tolerant isolates					
	NaCl (M)			pH	Temperature	
	0.17	0.34	0.68	5	37°C	42°C
Slow-growing	24	9	9	13	38	9
Fast-growing	94	76	76	76	89	71

Table 4. Symbiotic effectiveness of the isolates

Treatment	Peanut shoot nitrogen content (mg/d.w.)
Uninoculated	18.8
Uninoculated + NO_3K (0.05%)	29.5*
Bradyrhizobium sp SEMIA 6144	33.5*
Slow-growing isolates	
NLH25	39.3*
NLH30	29.6*
NDEHE	30.6*
NOD31	36.9*
NONC4	32.1*
NONC5	30.9*
NONC8	20.9
Fast-growing isolates	
NLH27	38.5*
NET30	34.4*
NCHA22	47.3*
NCHA32	28.8
NHOL2	31.8*
NMAL12	31.3*
NONC11	19.5
NONC13	34.7*

Data are means of 6 determinations, *$p<0.05$, d.w.: dry weight.

4. Discussion

In this work we examined a collection of peanut nodulating rhizobia that represents the native population in the producing area of Córdoba. It was found that the collection encompasses both slow (alkali-producing) and fast (acid-producing) growing rhizobia. Several phenotypic characteristics add further evidence supporting the grouping in the fast- and slow-growers. We found that almost all the fast-growing isolates able to nodulate *A. hypogaea* L. are salt-tolerant, most of them could grow at low pH and at high temperatures. By contrast, the slow growing isolates able to nodulate *A. hypogaea* L. are more sensitive to the environmental stresses assayed. Therefore we concluded that this phenotypic characterization clearly separates both groups.

Table 5. Host range of peanut rhizobia isolates

Isolates	Legumes tested		
	Glycine max	*Phaseolus vulgaris*	*Medicago sativa*
Slow- growing isolates			
NLH25	–	+	–
NDEHE	–	–	–
NOD31	–	+	–
NONC8	–	+	–
NAMN6	–	–	–
NMAN5	–	+	–
NCHA42	–	+	–
Fast-growing isolates			
NET30	–	+	–
NLH27	–	+	–
NALE	–	+	–
NCHA32	–	+	+
TT001	–	+	+
Reference strains			
B. japonicum USDA110	+	ND	ND
Rhizobium etli CFN42	ND	+	ND
S. meliloti 116	ND	ND	+

ND: not determined, +: with nodules, –: without nodules.

Our findings make it clear that *Arachis hypogeae* L. is able to establish symbiosis with fast and slow growing rhizobia. From this point of view, *Lotus* and *Acacia* are similar to *Arachis hypogaea* since all these legumes are promiscuous, being nodulated by both slow and fast rhizobia (Marsudi et al., 1999; Irisarri et al., 1996). The occurrence of a fast-growing rhizobia population able to nodulate *A. hypogaea* becomes more interesting if it is considered that, as there is no previous history of peanut inoculation in Argentina, rhizobia isolates used in this study are components of the indigenous rhizobial community.

Some of the peanut nodulating isolates analyzed were found to be able to nodulate other legumes. It is known that the specificity of the nodulation process plays a key role in the establishment of the symbiosis. While some rhizobia have a broad host range, other strains have a narrow one. On the other hand, the legumes can also be host to one symbiont or be nodulated by a large number of rhizobia. Initially, rhizobia were classified taking in

consideration the legume from which they were isolated. Then, it became obvious that rhizobia are not restricted to one or few hosts and that some of them are promiscuous since they can form effective associations not only with the legume from which they were isolated but also with many genera of legumes (Perret et al., 2000). Thus, it has been reported that *Rhizobium sp* strain NGR234 can nodulate many plants (Pueppke and Broughton, 1999), and this would be probably related with its ability to produce a large variety of Nod factors.

One characteristic of the host range is its scarce agreement with the rhizobial taxonomy. As it has been reported, some of the genes involved in the nodulation process could be acquired by different genera of rhizobia by lateral transfer (Pennisi, 1998). This gene exchange could allow the convergence between rhizobia taxonomically distant from each other but that display similar host ranges. This hypothesis is being tested in our collection by molecular studies.

The diversity of nodulating peanut rhizobia detected in this study was much greater than the one reported for other countries (Urtz and Elkan, 1996). As reported by Santamaria et al. (1997), the diversity of natural populations of rhizobia deserves more attention than received until now because they could be a source of valuable genotypes to improve strains of agricultural importance. Our results emphasize the importance of analyzing the soil area where the legume is believed to have originated. In order to gain insight into the characterization of this peanut nodulating collection, molecular analyses are being carried out.

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