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Diversity and symbiotic effectiveness of rhizobia isolated from field-grown soybean nodules in Paraguay

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Abstract Soybean was introduced in Paraguay in the 1920s and commercial crops have been grown since the 1970s. Root nodulation occurs at the majority of the producing sites, although inoculation has been practiced in only 15–20% of the cropping areas. The diversity and symbiotic effectiveness of soybean rhizobia was studied using 78 isolates obtained from root nodules of field-grown plants at 16 sites located in the two main producing states. The rhizobial isolates were characterized in relation to several parameters in vitro (colony morphology, tolerance to high temperature and salinity, intrinsic resistance to antibiotics, synthesis of indole acetic acid, profiles of proteins and lipopolysaccharides) and in vivo (nodulation, plant growth and total N accumulated in shoots). Fifty-eight isolates had slow growth rates and alkaline reaction in medium containing mannitol as the carbon source, whereas 20 had fast growth rates and an acid reaction. Most isolates did not tolerate acidity (pH 4.5) or high temperature (40°C). Very few isolates shared similar protein and lipopolysaccharide profiles; therefore a high level of diversity was detected, with most of the isolates representing unique strains. Some of the isolates with an outstanding symbiotic performance were identified, and will now be tested under field conditions in a search for efficient and competitive strains for use in commercial inoculants in Paraguay.

Keywords Bacterial diversity · Biological nitrogen fixation · *Bradyrhizobium* · *Glycine max* · *Rhizobium*

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Introduction

The economy of Paraguay is based predominantly on cattle and agriculture. Soybean [*Glycine max* L. (Merrill)] production is in excess of 3 million tonnes year^{-1}, with a mean yield of 2,500 kg ha^{-1} (CAPECO 1999); it is one of Paraguay's chief exports.

Soybean was introduced into Paraguay in the 1920s, with seeds from the United States, Argentina and Japan, but commercial expansion did not occur until the 1970s, resulting from increased international demand (Alvarez 1989). Also in the 1970s, importation of Brazilian cultivars adapted to the tropics began, which continues today (Oliveri et al. 1981). In Brazil, with the expansion of commercial production in the 1960s, inoculants containing rhizobia were imported mainly from the United States, but soon a programme of selection of strains adapted to local conditions was initiated. In Brazil, biological N_2 fixation fulfills most of the plant's need for N (Vargas and Hungria 1997). In contrast, Paraguay has had no program of strain selection, and inoculation is practiced with just 15–20% of the soybean crop. Furthermore, little is known of the history of inoculation and the locally produced and foreign inoculants that have been used for the past two decades are of dubious quality, since there is no quality-control program. However, nodulation occurs in the absence of inoculation in the majority of the areas of the States of Alto Paraná and Itapúa, responsible for 80% of the national production, although the symbiotic effectiveness varies from site to site (Figueredo 1998).

In the tropics, responses to inoculation of soybean are variable and depend especially on soil fertility, temperature and moisture content (Hungria and Vargas 2000). Inconsistent responses to inoculant application are frequently attributed to the rhizobial strains previously applied and naturalized, to the enrichment of indigenous populations due to prior cropping of legumes, or to a combination of both factors (e.g. Herridge et al. 1987; Sadowsky and Graham 1998). Therefore, it is important to characterize the indigenous population, to understand responses, or lack of them, to inoculation.

There have been few studies on N_2 fixation in Paraguay and almost nothing is known about the symbiosis with the soybean crop. Therefore in this study, the diversity of rhizobial isolates from soybean root nodules, collected under field conditions in the two main producing States of Paraguay, Alto Paraná and Itapúa, was assessed. Morphological and physiological properties of the isolates and protein and lipopolysaccharide profiles were determined, as well as their symbiotic effectiveness. Outstanding strains were selected for possible future use in commercial inoculants in Paraguay.

Materials and methods

Reference strains

Bradyrhizobium strains used for comparisons were: SEMIA 566 [same serogroup as SEMIA 5079 (= CPAC 15)] and SEMIA 5080 (= CPAC 7), classified as *B. japonicum* and SEMIA 587 and SEMIA 5019, classified as *B. elkanii* (Chueire et al. 2000), all of which are or were used in commercial inoculants in the South Region of Brazil, close to Paraguay. Strains USDA 205 (from USDA, Beltsville, Md.) and CCBAU114 (from Beijing University of Agriculture, Beijing, People's Republic of China) are representative of *Sinorhizobium fredii*, and USDA 110 and USDA 123 are *B. japonicum* strains, also from Beltsville, which were used in inoculants in Argentina.

Isolation of rhizobial strains from nodules of field-grown soybean in Paraguay

Isolates were obtained from nodules collected in situ of commercial cultivars of soybean in the States of Alto Paraná and Itapúa (Table 1). For each location, 20% of the nodules collected were randomly chosen for the isolation of the strains, in total 29 isolates from the State of Alto Paraná and 49 from the State of Itapúa. There is no information on whether the sites had been previously inoculated. The rhizobia were isolated from surface-sterilized nodules by streaking on yeast-extract mannitol-agar medium (YMA, Vincent 1970) containing cycloheximide (50 µg ml^{-1}). After purification by repeatedly streaking the bacteria in YMA medium, isolates were grown in YM broth (YMB) and glycerol was added (1:1, v:v); stocks were maintained at –80°C, and working cultures were maintained on slants of YMA at 4°C. Rhizobia were cultured routinely at 28°C in YMB on a rotary shaker operating at 65 cycles per minute.

Colony morphology

Colony morphology was evaluated after Vincent (1970), with bacteria grown on YMA with 0.005% Congo red at 28°C and evaluated after 3, 5 and 7 days of growth, on triplicate plates. Bacteria were also evaluated on YMA with 0.0025% bromothymol blue, after 7 days of growth, for the acid (yellow) or alkaline (blue) reaction (Vincent 1970).

Tolerance of acidity, alkalinity, salinity and high temperature

Bacteria (10^8 cells ml^{-1}) grown in YMB were used as initial inocula. Tolerance of acidity and alkalinity was evaluated with the transfer of aliquots (100 µl) of each initial inoculum to tubes containing 5 ml YMB with pH previously adjusted to 3.5 or 9.0. The pH was adjusted by adding sterile solutions of diluted HCl or NaOH to the autoclaved medium. To examine for high-temperature tolerance, the bacteria were incubated in YMB at 40 or 45°C. For salt-tolerance screening, the isolates were added to YMB supplemented with 0.1, 0.3 or 0.5 M NaCl. Controls consisted of bacteria grown in YMB, pH 6.8 at 28°C. All assays were performed in triplicate.

Synthesis of indole acetic acid

The isolates and strains SEMIA 587, SEMIA 5019, SEMIA 5080, USDA 205 and CCBAU 114 were grown in Tris-YMRT medium (containing, per liter: mannitol, 10 g; $CaCl_2.2H_2O$, 0.15 g; $MgSO_4.7H_2O$, 0.25 g; Tris-HCl, 1.21 g; yeast extract, 0.2 g; casamine acid, 1.0 g; pH adjusted to 6.8), enriched with tryptophan (0.3 mM of tryptophan sterilized by membrane filtering, 0.22 µm; Minamisawa and Fukai 1991). Indole acetic acid (IAA) produced was evaluated by the colorimetric assay, as described before (Boddey and Hungria 1997).

Table 1 Origin of the isolates obtained from nodules of field-grown soybean in Paraguay

State	Location	Isolate	Soil type[a]	Crop rotation[b]
Itapúa	Bella Vista	11, 27, 29, 30, 31, 37, 38, 41, 42, 60, 71, 72, 73, 78	Oxisol kandiudalfic	S/W/M
	Capitán Meza	7, 28, 40, 61, 74, 75, 76.	Ultisol rhodic	S/W
	Capitán Miranda	64, 65	Oxisol typic	S/W
	Edelira	5, 55	Ultisol rhodic	S/W/M
	Encarnación	33, 43, 44, 62	Oxisol typic	S/W
	General Delgado	8, 9, 45, 46, 47, 48, 63, 66	Ultisol typic	S/W/M
	Obligado	12, 17, 18	Ultisol rhodic	S/W
	Pirapó	6, 10, 19, 20, 32, 58, 59	Oxisol kandiudalfic	S/W
	San Pedro del Paraná	13, 14	Ultisol typic	S/W
Alto Paraná	Domingo M. Irala	21, 22, 23	Alfisol rhodic	S
	Hernandarias	25, 35, 36, 67	Oxisol rhodic	S/W/M
	Juan L. Mayorquín	15, 49	Alfisol rhodic	S/SC
	Mbaracayú	1, 2, 3, 4	Ultisol rhodic	S/W
	Santa Rita	53, 54, 56, 57, 70	Alfisol rhodic	S/W/M
	Santa Rosa	24, 26, 34, 39	Alfisol rhodic	S/W
	Tavapy	16, 50, 51, 52, 68, 69, 77	Alfisol rhodic	S/W

[a] US Soil Taxonomy System; *Oxisol*, degraded soil; *Ultisol*, mineral soil with ≤35% saturation of bases; *Alfisol*, mineral soil with ≥35% saturation of bases

[b] *S* soybean, *W* wheat, *M* maize, *SC* sugar cane

Intrinsic resistance to antibiotics

Isolates were tested for intrinsic resistance to antibiotics using YMB cultures at 10^4 cells ml^{-1} as inocula. Plates containing YMA medium, with or without antibiotics, were streaked with a loop of inoculum (approximately 3 µl), and the presence or absence of colonies was scored at 15 days. Antibiotics were added to the medium after sterilization by membrane filtering (0.22 µm). The following antibiotics were tested (in µg ml^{-1}), as follows. For group A (alkalizing and slow growers): erythromycin (100, 150, 200, 300, 400 and 500), chloramphenicol and rifampin (250, 350, 500, 600, 700, 800, 900 and 1,000), tetracycline (50, 75, 100, 125, 150, 150, 175 and 200) and nalidixic acid (10, 20, 50, 100, 150, 175 and 200). For group B (acidifying and fast growers): nalidixic acid, erythromycin, and spectinomycin (50 100, 150, 200 and 250), neomycin and chloramphenicol (10, 25, 50, 75, 90 and 100), rifampin (10, 30, 50, 80 and 100), tetracycline, kanamycin and gentamycin (5, 10, 20, 30 and 40). All assays were performed in triplicate. One or two of the most discriminating concentrations of each antibiotic were selected and the results were transformed into a binary matrix (1/0), in which the resistance to an antibiotic was considered as "1" and the sensitivity as "0". The clustering analysis was performed using the NTSYS-PC program (version 1.8, Exeter Software, New York) with the algorithm of UPGMA (unweighted pair-group method, with arithmetic mean; Sneath and Sokal 1973) and the Jaccard (J) coefficient.

Protein and lipopolysaccharide (LPS) profiles

Protein profiles were determined as described before (Ferreira et al. 2000) and LPS profiles as described by Hungria et al. (2001).

Symbiotic performance

Each isolate was grown in YMB, for 7 days, and adjusted to a concentration of 10^9 cells ml^{-1}. Soybean seeds of cultivar BR-16 (genealogy, D69-B10-M58 × Davis) were surface sterilized and planted in sterilized modified Leonard jars (Vincent 1970) containing a mixture of sand and vermiculite (2:1, v:v). Four seeds were planted per jar, individually treated with 1 ml inoculum, and thinned to two per jar 5 days after emergence (DAE). Plants were grown under greenhouse conditions, received N-free nutrient solution (Andrade and Hamakawa 1994) and were harvested 45 DAE, evaluating nodulation, plant dry weight and total N content, as described before (Santos et al. 1999). The experiment was performed in a completely randomized design, with three replicates. Univariate statistical analysis (one-way classification analysis of variance) was performed after Scott and Knott (1974) and, for the multivariate analysis, the principal components analysis (PCA) was used (Morrison 1990).

Results

Isolates obtained from nodules of field-grown soybeans

Seventy-eight isolates were obtained from nodules of field-grown plants collected at 16 sites: 49 from the Itapúa State and 29 from Alto Paraná (Table 1). Fifty-eight isolates (1–58), characterized by alkaline reaction on YMA containing bromothymol blue (group A), showed slow growth (colonies visible only after 5 days; except for isolate 58, which had an intermediate rate of growth); they were present in 15 of the 16 locations. The other 20 isolates (59–78) showed an acid reaction in YMA and were fast growers (visible growth at 3 days), with 75% isolated from Itapúa and 25% from Alto Paraná (Table 1).

Table 2 Tolerance to salinity, acidity and high temperature and synthesis of indole acetic acid (*IAA*) in vitro by 78 isolates from nodules of field-grown soybean plants in Paraguay

Isolate/strain	NaCl (M)		pH 3.5	Temperature (40°C)	IAA (µM)
	0.3	0.5			
Slow growers					
1	–[a]	–	–	–	26.19
2	–	–	w	–	15.30
3	+	w	w	w	11.29
4	+	w	–	+	9.18
5	+	w	–	–	12.66
6	+	–	–	+	9.64
7	–	–	–	+	13.24
8	–	–	–	–	74.69
9	–	–	–	–	3.69
10	–	–	–	–	3.69
11	–	–	–	+	47.74
12	–	–	w	w	46.98
13	–	–	w	+	4.93
14	–	–	–	w	130.32
15	–	–	–	+	6.65
16	–	–	–	–	14.20
17	–	–	w	+	7.72
18	+	+	–	–	9.18
19	–	–	–	–	1.02
20	–	–	–	–	22.77
21	–	–	–	+	11.65
22	+	w	–	+	8.22
23	–	–	–	+	11.29
24	–	–	–	–	9.18
25	–	–	w	+	13.99
26	–	–	–	–	30.54
27	–	–	–	–	32.10
28	–	–	–	–	2.39
29	–	–	–	w	11.65
30	–	–	–	–	26.56
31	–	–	w	w	54.47
32	–	–	w	+	8.71
33	w	–	w	–	5.52
34	–	–	–	w	4.32
35	+	+	+	–	11.29
36	–	–	–	–	15.93
37	–	–	w	w	17.99
38	–	–	w	w	7.19
39	–	–	–	–	101.55
40	–	–	–	–	49.95
41	+	+	+	–	10.90
42	+	+	+	–	1.71
43	+	+	–	+	4.93
44	+	+	–	+	1.71
45	+ [a]	+	–	+	4.32
46	+	w	–	w	1.02
47	+	+	–	w	3.05
48	–	–	–	–	14.58
49	w	–	–	–	1.71
50	+	+	–	–	40.00
51	–	–	–	–	47.11
52	–	–	–	+	49.50
53	–	–	–	–	15.51
54	–	–	w	–	16.34
55	–	–	+	–	37.22
56	–	–	–	–	54.17
57	–	–	–	–	15.93
58	–	–	–	–	10.08

Table 2 continued

Isolate/strain	NaCl (M)		pH 3.5	Temperature (40°C)	IAA (µM)
	0.3	0.5			
Fast growers					
59	–	–	+	–	45.80
60	+	–	w	–	17.17
61	–	–	–	–	16.55
62	+	–	w	w	37.85
63	w	–	–	–	8.22
64	+	+	–	w	31.23
65	+	–	–	–	12.66
66	–	–	–	–	32.78
67	–	–	–	+	31.93
68	–	–	–	–	12.96
69	–	–	–	+	54.47
70	–	–	+	+	34.63
71	–	–	–	–	6.10
72	w	–	w	–	48.15
73	–	–	+	–	57.19
74	+	+	–	–	77.65
75	+	+	–	–	35.61
76	+	+	–	–	45.80
77	–	–	+	–	11.65
78	w	–	–	–	13.99
Reference strains					
SEMIA 587	–	–	–	–	74.38
SEMIA 5019	–	–	–	–	80.77
SEMIA 5080	–	–	–	–	3.05
USDA 205	–	–	–	–	64.51
CCBAU 114	–	–	w	–	105.25

[a] – Absence of growth, + growth and *w* indicates weak growth, corresponding to 10–20% of the growth of the control treatment

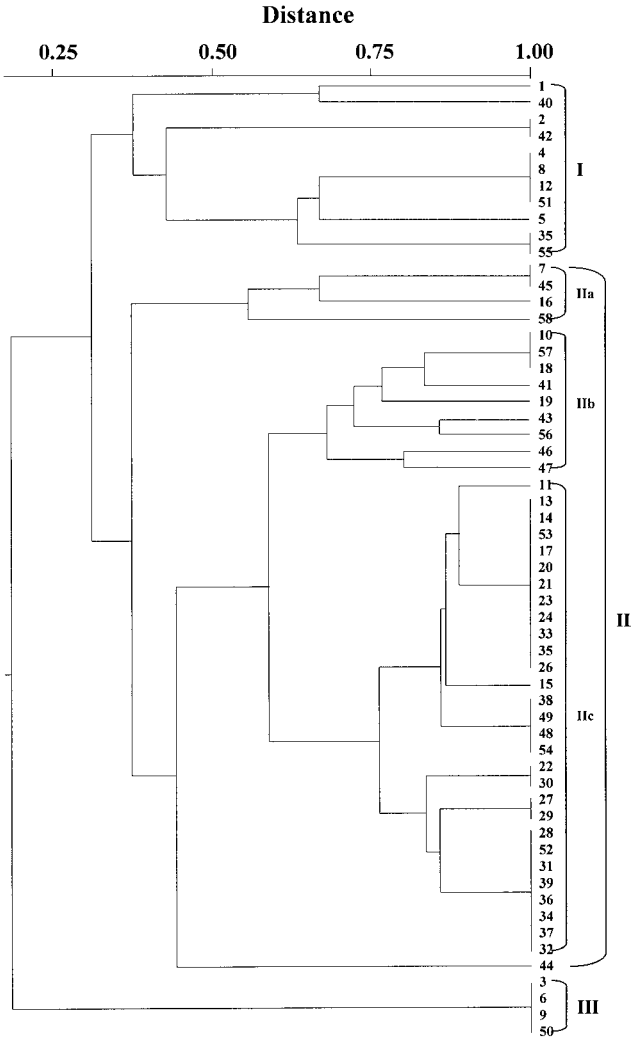

Fig. 1 Dendrogram of intrinsic resistance to 5 antibiotics of 58 isolates that alkalinized the yeast extract-mannitol (YM) medium (group A). Antibiotics considered for the analysis were (in µg ml^{-1}): chloramphenicol (500), erythromycin (200 and 400), nalidixic acid (100 and 200), rifampin (250 and 500) and tetracycline (50 and 200). The assays were performed in triplicate

Colony morphology

The isolates varied in morphology, i.e. 13% formed small colonies (≤0.5 mm), whereas 17% had diameters ≥2.5 mm. Colony size was not necessarily correlated with growth rate, although fast growers tended to form smaller colonies. Convex elevation was detected in 55% of the isolates, 82% were circular with regular borders, and 55% and 24% produced moderate and much mucous, respectively (data not shown). Some relationship was observed between morphology and site of isolation; for example, all isolates from Tavapy and Mbaracayú produced a moderate amount of mucus, while those of Santa Rosa, Juan L. Mayorquín, San P. del Paraná, Obligado, Gral. Delgado and Domingo M. Irala, had low to moderate mucoidy. Almost all of the isolates from Alto Paraná State, where there is a predominance of more-fertile Alfisols (US Soil Taxonomy System), with high saturation of cations (≥35%), produced less mucus than those from the State of Itapúa, with degraded Oxisols poor on nutrients, and Ultisols with low cation saturation (≤35%).

Physiological properties

All isolates grew in YM supplied with 0.1 M NaCl (data not shown), 22 grew at 0.3 M, and 13 were able to grow in the medium supplemented with 0.5 M NaCl (Table 2). All isolates grew in YM at pH values of 6.5 and 9.0 (data not shown). Although bacteria had been isolated from acid soils (pH 4.0–6.0), only 8 isolates were able to grow well at pH 3.5; 15 others and *S. fredii* CCBAU 114 showed weak growth at this pH (Table 2). Weak growth of 12 isolates occurred at 40°C, and 19 were able to grow well at this temperature (Table 2), but none of the isolates was able to grow at 45°C (data not shown). Three isolates, 43, 44 and 45, were able to grow well in medium supplied with 0.5 M NaCl and at 40°C, but did not tolerate high acidity; three others (35, 41, 42) tolerated 0.5 M NaCl and pH 3.5, but

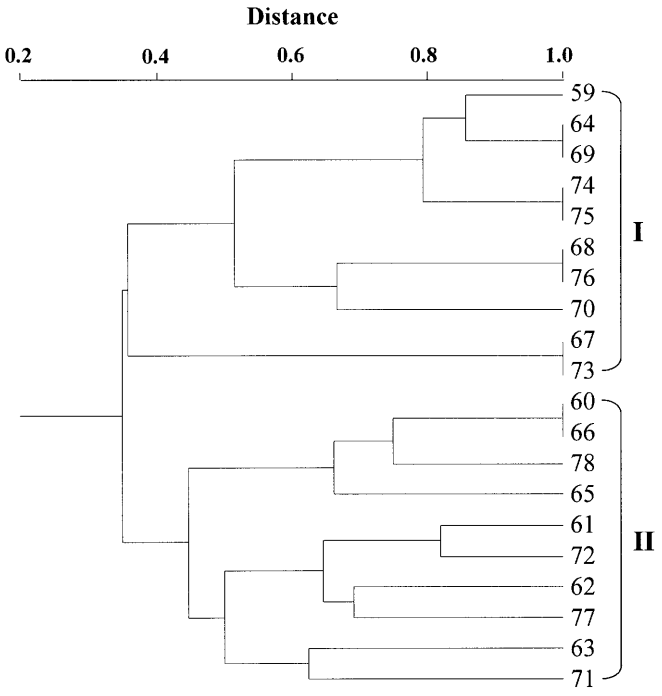

Fig. 2 Dendrogram of intrinsic resistance to 7 antibiotics of 20 isolates that acidified the YM medium (group B). Antibiotics considered for the analysis were (in µg ml^{-1}): erythromycin (150 and 250), gentamycin (10 and 30), kanamycin (5 and 40), nalidixic acid (50), rifampin (10 and 50), spectinomycin (100 and 250) and tetracycline (5 and 40). The assays were performed in triplicate

not high temperature, and one isolate, 70, tolerated acidity and high temperature, but not salinity (Table 2).

Forty-seven of the isolates, as well as reference strains SEMIA 566 (data not shown) and SEMIA 5080, synthesized low amounts of IAA (1.00–15.55 µM), 23 produced medium levels (16.6–50.0 µM) and 8 isolates, as wells as *B. elkanii* reference strains SEMIA 587 and SEMIA 5019 and *S. fredii* USDA 205 and CCBAU 114 produced relatively high concentrations (50–130 µM; Table 2).

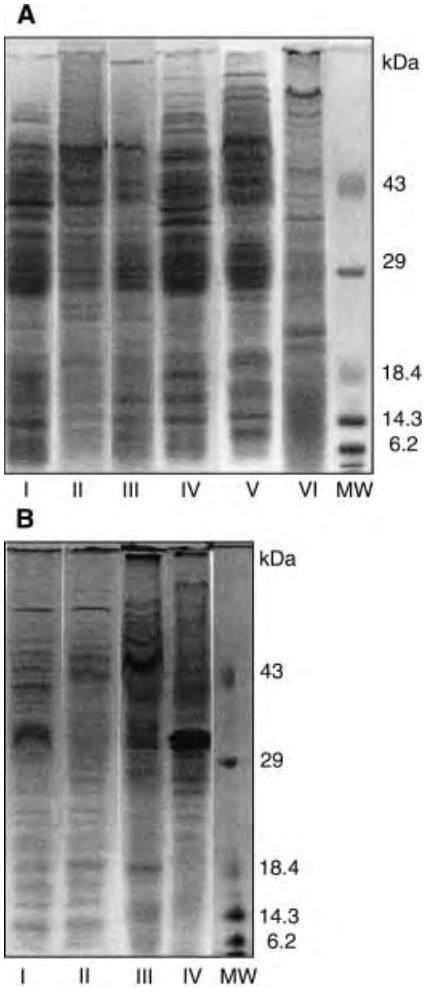

Fig. 3 Protein profiles of isolates from field-grown soybean in Paraguay showing alkaline (**A**) or acid reaction (**B**) in yeast-extract mannitol-broth (YMB) medium. In **A**, strains were grouped as follows: *Ia* (6, 7, 8, 9, 12, 16, 18, 19, 35, 40, 41, 42, 50, 51, 54, 55, 57); *IIa* (11, 13, 14, 25, 26, 28, 29, 30, 31, 32, 34, 36, 37, 38, 39, 48, 49, 52, 53); *IIIa* (10, 17, 20, 21, 22, 23, 27, 56); *IVa* (2, 4); *Va* (1, 3); *VIa* (43, 44). In **B**: *Ib* (60, 62); *IIb* (64, 69, 74, 77); *IIIb* (65, 78) and *IVb* (67, 73)

Intrinsic resistance to antibiotics

The cluster analysis of the results obtained for the alkalinizing group (A), considering one or two of the most discriminating concentrations of each antibiotic, resulted in three main groups, joined at a 0.2 relative level of similarity (Fig. 1). Group I included 19% of the isolates and group II, 74%, with several isolates within each group showing similar levels of resistance; group III had four isolates showing similar levels of resistance. Group I was characterized by sensitivity to erythromycin (≥200 µg ml^{-1}), to rifampin (≥250 µg ml^{-1}, except for isolate 1), and tolerance of up to 100 µg ml^{-1} nalidixic acid. Within this group, isolates 1, 40, 2 and 42 were also sensitive to 500 µg ml^{-1} chloramphenicol. In group II, most of the isolates tolerated at least 200 µg ml^{-1} erythromycin and 500 µg ml^{-1} chloramphenicol. Groups IIa and IIb were sensitive to 250 µg ml^{-1} rifampin, but differed because IIb tolerated 200 µg ml^{-1} nalidixic acid. Group IIc included isolates with higher tolerance to antibiotics and differed from IIa and IIb by tolerance to rifampin (at least 500 µg ml^{-1}) and also because several isolates tolerated up to 200 µg ml^{-1} tetracycline and nalidix acid. Isolates belonging to group III were the most sensitive to all antibiotics tested, except chloramphenicol. All isolates from Edelira and Mbaracayú (Ultisol) showed low resistance to the antibiotics used in this work. In contrast, all isolates from J. L. Mayorquín, D. M. Irala, Santa Rosa, Santa Rita (Alfisol), San P. del Paraná (Ultisol), Encarnación and Bella Vista (Oxisol; Table 1), showed moderate to high resistance to antibiotics (except for isolate 42).

Figure 2 shows the results obtained with the isolates with an acid reaction (group B), with 50% fitting within each group, joined at a relative similarity level of 0.35. Group I included isolates sensitive to 5 µg ml^{-1} kanamycin

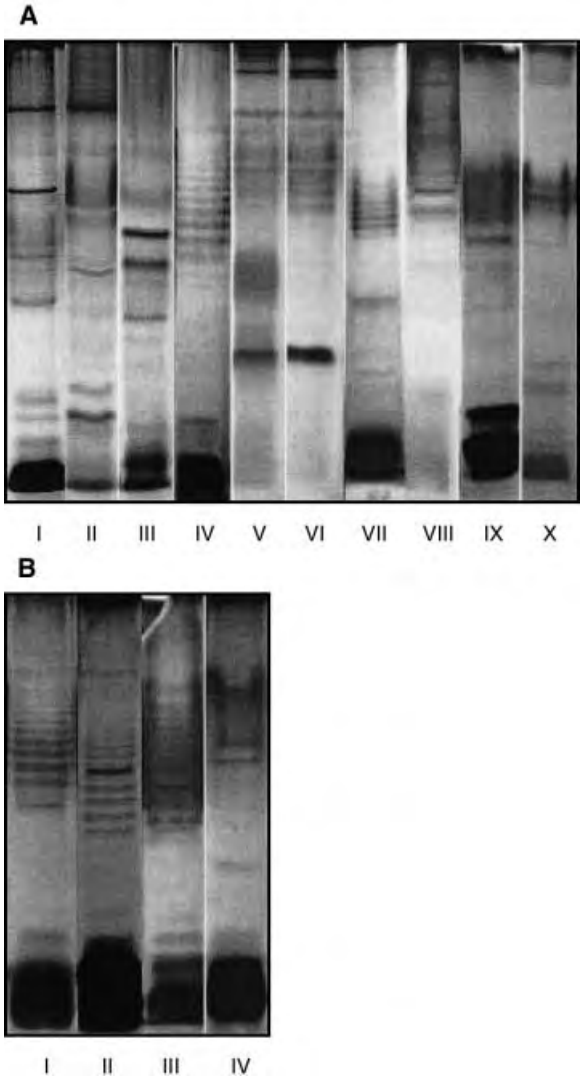

Fig. 4 Lipopolysaccharide profiles of isolates from field-grown soybean in Paraguay showing alkaline (**A**) or acid reaction (**B**) in YMB medium. In **A**, strains were grouped as follows: *Ia* (1, 10, 20), *IIa* (15, 17, 23); *IIIa* (45, 46, 47, 57); *IVa* (25, 39, 51, 52, 54, 56); *Va* (4, 18, 41); *VIa* (36, 53); *VIIa* (19, 40, 50); *VIIIa* (43, 44); *IXa* (35, 55); *Xa* (6, 7). In **B**: *Ib* (59, 65, 73, 74); *IIb* (64, 66, 67, 75, 76, 77); *IIIb* (62, 72); *IVb* (60, 70)

(except for isolates 67 and 73), to nalidixic acid (≥50 µg ml^{-1}) and to rifampin (≥10 µg ml^{-1}), but tolerant of 250 µg ml^{-1} erythromycin. The majority of the isolates in group II tolerated up to 50 µg ml^{-1} nalidixic acid and 40 µg ml^{-1} kanamycin; isolates 61, 72, 62, 77, 63 and 71 were the only ones to tolerate rifampin (at least 10 µg ml^{-1}). All isolates from Bella Vista (except 73), Encarnación and Gral. Delgado showed relatively high resistance to antibiotics.

Protein and LPS profiles

Fifty of the 58 isolates of group A (i.e. with alkaline reaction) fit into 6 protein profiles (Fig. 3A), and isolates 5, 15, 24, 33, 45, 46, 47 and 58 showed unique profiles (data not shown). In the group producing an acid reaction, 50% showed unique profiles (59, 61, 63, 66, 68, 70, 71, 72, 75 and 76, data not shown), and the others fit into 4 protein-profile groups (Fig. 3B). Some correlations were found between site of origin and protein profile for the alkalinizing bacteria, e.g. the 11 isolates belonging to protein profile (PP) group IIa came from Itapúa, 6 of which were from Bella Vista. All isolates from D.M. Irala had the same profile, as had the isolates from San P. del Panará. Finally, isolates 1 and 3 (PP Va), and 2 and 4 (PP IVa) came from Mbaracayú. There was no clear relationship between site of origin and the protein profile of the bacteria showing an acid reaction.

A higher level of polymorphism was detected in the LPS profiles, when compared with the protein profiles. Thirty isolates with alkaline reaction fit into 10 groups (Fig. 4A), whereas 28 (2, 3, 5, 8, 9, 11, 12, 13, 14, 16, 21, 22, 24, 26, 27, 28, 29, 30, 31, 32, 33, 34, 37, 38, 42, 48, 49 and 58) showed unique profiles (data not shown). The isolates with an acid reaction fit into 4 LPS groups (Fig. 4B) and isolates 61, 63, 68, 69, 71 and 78 showed unique profiles (data not shown). Some isolates showed similar protein and LPS profiles (6 and 7; 10 and 20; 17 and 23; 18 and 41; 19, 40, and 50; 25, 39 and 52; 35 and 55; 36 and 53; 43 and 44; 51 and 54; 64 and 77). Therefore just 12 isolates out of the 58 showing slow growth rate and 1 out of 20 with a fast growth rate showed similar protein and LPS profiles. Protein and LPS profiles of the isolates also differed from those obtained for reference strains SEMIA 566, SEMIA 5080, SEMIA 587, SEMIA 5019, USDA 110 and USDA 123 (data not shown).

Symbiotic performance

The best symbiotic performances, with plants showing higher nodule mass, shoot dry weight and accumulating more than 120 mg of N in shoots, were achieved by alkalinizing isolates 27, 40, 41, 42, 45, 47, 50 and 52, and acidifying isolates 60, 71, 73 and 77 (Table 3). However, several other isolates showed good symbiotic performance, accumulating more than 100 mg of N in shoots. The highest amount of N_2 fixation resulted from inoculation with isolate 47, with 158 mg N, 10%, 14% and 322% higher than the N accumulated in shoots of plants inoculated with SEMIA 587, SEMIA 5080 and USDA 205, respectively (Table 3). The multivariate analysis was assessed considering two factors that expressed 89% of the variations of all parameters. Each of these factors is a linear combination of all parameters and it shows that SEMIA 5080, SEMIA 587 and six isolates (47, 42, 40, 41, 45 and 71) had the best symbiotic performance with cv. BR-16 (left side of Fig. 5). The less effective isolates were those in the superior and inferior quadrants at the right side of Fig. 5. Within the group of five bradyrhizobia isolates with good symbiotic performance, four showed low (41, 42, 45 and 47) and one (40) an intermediate level of IAA and none was within

Table 3 Nodule number (ln[nodule number+1].jar^{-1}) and dry weight (g nodules jar^{-1}), shoot dry weight (g jar^{-1}) and total N accumulated in shoot (mg N jar^{-1}) of soybean cultivar BR-16[a] inoculated with 78 isolates from field-grown soybean plants in Paraguay and with reference strains SEMIA 587, SEMIA 5080 and USDA 205

Isolate/strain	Nodule number		Nodule dry weight		Shoot dry weight		Total N in shoots	
Slow-growers								
1	4.94	d[b]	0.229	b	3.75	c	90.65	c
2	4.91	d	0.204	b	3.45	c	93.34	c
3	4.82	c	0.210	b	3.27	c	88.30	c
4	4.72	c	0.219	b	3.45	c	91.69	c
5	4.99	d	0.397	d	3.79	c	111.47	c
6	4.80	c	0.330	c	2.90	b	81.60	b
7	5.11	d	0.606	e	4.24	d	95.74	c
8	4.84	c	0.375	c	3.11	b	67.25	b
9	4.96	d	0.579	e	4.17	d	102.89	c
10	4.98	d	0.403	d	3.68	c	101.38	c
11	4.79	c	0.335	c	3.39	c	68.66	b
12	5.10	d	0.440	d	4.01	d	96.58	c
13	5.15	d	0.525	e	3.98	d	112.21	c
14	4.66	c	0.388	d	3.67	c	104.58	c
15	5.01	d	0.528	e	4.49	d	114.17	c
16	4.88	c	0.339	c	4.19	d	110.61	c
17	4.97	d	0.274	b	2.66	b	70.77	b
18	4.82	c	0.355	c	3.25	c	87.30	c
19	4.68	c	0.247	b	2.88	b	65.72	b
20	4.79	c	0.278	b	2.74	b	69.66	b
21	4.49	b	0.233	b	2.44	b	61.59	b
22	4.99	d	0.380	c	3.56	c	84.60	b
23	4.59	b	0.294	b	2.63	b	59.71	b
24	4.74	c	0.251	b	2.28	b	68.08	b
25	5.04	d	0.541	e	3.67	c	103.63	c
26	5.11	d	0.466	d	3.75	c	82.47	b
27	5.03	d	0.642	e	4.44	d	125.75	d
28	4.88	c	0.303	c	2.75	b	73.72	b
29	4.76	c	0.346	c	3.30	c	76.77	b
30	4.84	c	0.392	d	3.97	d	90.80	c
31	4.90	c	0.393	d	3.66	c	96.07	c
32	4.86	c	0.393	d	3.79	c	95.45	c
33	4.73	c	0.346	c	3.88	c	107.00	c
34	4.90	c	0.424	d	3.38	c	85.58	b
35	4.72	c	0.436	d	4.10	d	94.07	c
36	4.68	c	0.266	b	2.83	b	66.32	b
37	4.68	c	0.255	b	2.61	b	61.86	b
38	4.73	c	0.292	b	3.42	c	104.08	c
39	4.71	c	0.346	c	3.17	b	99.22	c
40	4.99	d	0.546	e	4.31	d	147.33	d
41	4.89	c	0.518	e	4.11	d	130.51	d
42	4.97	d	0.567	e	4.54	d	154.41	d
43	4.84	c	0.405	d	3.35	c	89.14	c
44	5.10	d	0.407	d	4.34	d	110.26	c
45	4.90	c	0.665	e	4.75	d	146.02	d
46	4.89	c	0.373	c	3.43	c	86.58	c
47	4.91	c	0.506	e	4.67	d	158.16	d
48	4.73	c	0.348	c	3.59	c	100.41	c
49	4.82	c	0.400	d	3.89	c	109.04	c
50	5.03	d	0.627	e	4.47	d	125.46	d
51	4.82	c	0.149	a	1.67	a	39.77	a
52	5.10	d	0.549	e	3.94	c	122.87	d
53	4.75	c	0.318	c	3.09	b	83.49	b
54	5.14	d	0.515	e	3.98	d	95.07	c
55	5.01	d	0.450	d	3.92	c	109.02	c
56	4.88	c	0.372	c	3.54	c	89.74	c
57	5.03	d	0.583	e	4.49	d	104.68	c
58	5.05	d	0.269	b	2.63	b	52.25	b
Mean	4.88		0.39		3.58		95.10	

Table 3 continued

Isolate/strain	Nodule number		Nodule dry weight		Shoot dry weight		Total N in shoots	
Fast growers								
59	4.23	a	0.046	a	1.27	a	26.39	a
60	5.26	d	0.595	e	4.18	d	124.16	d
61	4.82	c	0.255	b	2.76	b	78.90	b
62	5.08	d	0.474	d	3.78	c	115.24	c
63	4.85	c	0.515	e	3.97	d	81.75	b
64	4.94	d	0.593	e	4.15	d	92.08	c
65	5.08	d	0.559	e	4.41	d	115.54	c
66	4.97	d	0.589	e	4.14	d	101.24	c
67	4.94	d	0.269	b	2.48	b	57.65	b
68	4.44	b	0.222	b	2.08	a	67.09	b
69	4.41	b	0.067	a	1.25	a	20.38	a
70	4.71	c	0.329	c	3.10	b	73.81	b
71	4.89	c	0.522	e	4.34	d	135.70	d
72	5.06	d	0.512	e	4.35	d	119.95	d
73	4.59	b	0.436	d	4.48	d	122.25	d
74	4.35	b	0.071	a	1.33	a	18.08	a
75	4.04	a	0.035	a	1.25	a	11.54	a
76	4.95	d	0.415	d	3.45	c	101.57	c
77	5.01	d	0.476	d	4.53	d	123.22	d
78	4.94	d	0.304	c	2.95	b	79.30	b
Mean	4.78		0.36		3.21		83.29	
Reference strains								
SEMIA 587	4.93	d	0.612	e	4.79	d	143.60	d
SEMIA 5080	4.97	d	0.454	d	4.54	d	138.42	d
USDA 205	4.95	d	0.269	b	1.86	a	37.50	a

[a] Plants grown under greenhouse conditions, in Leonard jars, with two plants per jar, supplied with N-free nutrient solution and harvested at 45 days after emergence

[b] Means of three replicates and values followed by the same letter did not show statistical difference by the Scott and Knott test ($P \leq 0.05$)

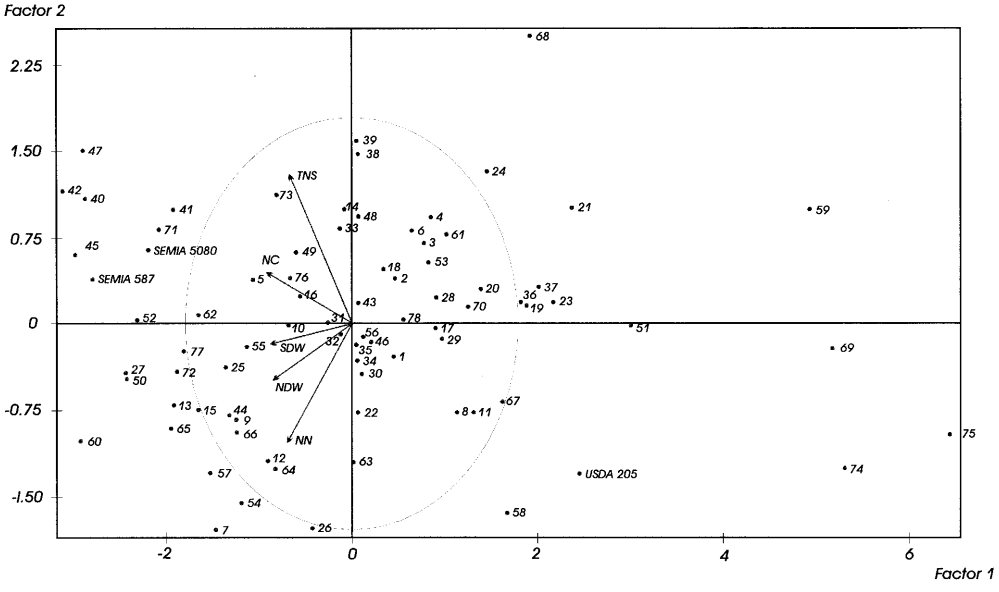

Fig. 5 Principal component analysis considering the parameters of nodule number (*NN*) and dry weight (*NDW*), shoot dry weight (*SDW*), N content (*NC*) and total N accumulated in the shoot (*TNS*) of soybean cv. BR-16 inoculated with 78 isolates from Paraguay and with reference strains SEMIA 587, SEMIA 5080 and USDA 205

the cluster of higher tolerance to antibiotics; therefore they were closer to the characteristics of *B. japonicum* species.

Discussion

In this paper 78 isolates from nodules of field-grown soybean from 16 sites located in the two main producing States were obtained and studied. Fifty-eight isolates were characterized by slow growth and alkaline reaction in YM, while 20 showed fast growth and acid reaction. Just 12 of the 58 bradyrhizobia and 1 of the 20 rhizobia isolates showed similar protein and LPS profiles, therefore a high level of diversity was detected among the Paraguayan isolates, with most of them representing unique strains. The protein and LPS profiles were also different from those obtained for reference strains carried in inoculants in the neighboring countries, thus the isolates might represent native strains. However, the hy-

potheses of transference of symbiotic genes to indigenous non-symbiotic bradyrhizobia (Sullivan et al. 1995, 1996) as well as of changes in bacteria properties due to stressing environmental conditions (Santos et al. 1999; Hungria and Vargas 2000) should also be investigated.

In 1992 *Bradyrhizobium* strains which nodulate soybean were split into two species, *B. japonicum* and *B. elkanii* (Kuykendall et al. 1992). Among the criteria for distinguishing the species it has been demonstrated that *B. elkanii* shows a higher intrinsic resistance to several antibiotics (Kuykendall et al. 1988), synthesis of rhizobitoxine (Minamisawa 1989, 1990), synthesis of IAA (Minamisawa and Fukai 1991; Minamisawa et al. 1992), absence of hydrogenase (Minamisawa 1990; Minamisawa et al. 1992), among others (Kuykendall et al. 1992). From those criteria, Minamisawa et al. (1992) and Boddey and Hungria (1997) suggested that a fast, easy and cheap method to distinguish among the species would be the analysis in vitro of IAA. In this study, 30 bradyrhizobia isolates showed intrinsic resistance to high levels of the antibiotics tested (Fig. 1, cluster IIc), a characteristic of *B. elkanii* species (Kuykendall et al. 1988, 1992); however, half of them synthesized low amounts of IAA (Table 2), as *B. japonicum* does (Minamisawa and Fukai 1991; Minamisawa et al. 1992; Boddey and Hungria 1997). Also within the group with lower resistance to antibiotics (Fig. 1, cluster III), isolate 50 accumulated high amounts of IAA (Table 2). Therefore many isolates from this study showed mixed characteristics of both bradyrhizobia species, as has been described before for several Brazilian isolates (Boddey and Hungria 1997).

Few studies have attempted to correlate soybean *Bradyrhizobium* species with N_2 fixation performance. Hypothetically, since Hup^+ phenotype and no production rhizobitoxine are restricted to *B. japonicum*, this species might be more efficient (Minamisawa 1989, 1990), a possibility indicated by some studies performed with few strains (Fuhrmann 1990; Teaney and Fuhrmann 1992; Vasilas and Fuhrmann 1993; Hungria et al. 1998). In this paper, none of the five most efficient bradyrhizobia isolates synthesized high amounts of IAA and none was within the group of higher resistance to antibiotics, therefore the properties are typical of *B. japonicum*. Indeed, two of those isolates, 40 and 42, were recently submitted to the sequencing of the 16S rRNA region and proved to be taxonomically related to *B. japonicum* (Chen et al. 2000). However, within the strains with similar properties, symbiotic performance was variable and thus the results from this study do not give strong support to the hypothesis of superiority of *B. japonicum* species.

Rhizobial soybean isolates characterized by a fast growth rate and acid reaction in vitro were first isolated in 1982, in the People's Republic of China (Keyser et al. 1982), and are today classified as *Sinorhizobium fredii* (Chen et al. 1988). Initially, those fast growers were thought to establish efficient symbioses only with primitive soybean cultivars, such as Peking (Keyser et al. 1982; Devine 1985), but later there were reports of *S. fredii* being able to nodulate several modern soybean cultivars (Balatti and Pueppke 1992; Chueire and Hungria 1997). In this study, 20 isolates exhibited fast growth rate and acid reaction in medium with mannitol as the C source and one of those isolates, 71, was very effective in fixing N_2 with soybean. However, 16S rRNA analyses recently performed with some of those isolates have shown that isolates 71 and 72 were genetically related to *Rhizobium* genomic species Q, while isolates 62 and 65 had higher similarity to *Agrobacteriu*m spp. (Chen et al. 2000). Therefore none of the four isolates was genetically related to *S. fredii* and genetic characterization of the other fast growers from this study is now under way.

This paper provides, for the first time, an indication of the diversity and symbiotic effectiveness of rhizobia able to nodulate soybean in Paraguay. A high level of diversity was detected, with most isolates representing unique strains. Within the diversity detected, some isolates with an outstanding symbiotic performance were identified, and will now be tested under field conditions in a search for efficient and competitive strains for use in commercial inoculants in Paraguay.

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