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Isolation and characterization of new efficient and competitive bean (*Phaseolus vulgaris* L.) rhizobia from Brazil

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Abstract

The common bean (*Phaseolus vulgaris* L.) is widely cultivated in South and Central America and Africa, but inoculation with rhizobia often does not lead to a response in field experiments. A selection program was started in the State of Paraná, Brazil, in which three promising strains, PRF 35, PRF 54 and PRF 81, showing high rates of N_2 fixation, were competitive and tolerated high temperatures. The performance of the strains was also verified in four field experiments, where inoculation with PRF 81 allowed yield increases of up to 906 kg ha^{-1}, compared with the non-inoculated (control) with a high population of native bean rhizobia. The high performance of PRF 81 was confirmed in several other field trials carried out in Brazil, leading to its recommendation for use in commercial Brazilian inoculants. PRF 34, PRF 54 and PRF 81 were further characterized and compared with four strains, representative of bean rhizobia species in an effort to define variables which could aid future selection programs. The Brazilian strains showed unique profiles of protein, lipopolysaccharide and PCR using specific (ERIC and REP) or arbitrary short primers. The DNA fingerprints obtained with specific or arbitrary primers showed that strains PRF 35 and PRF 54 were genetically very close, nevertheless, there were substantial differences between the strains in nodulation and N_2 fixation rates, as well as in the synthesis of Nod factors after induction with naringenin. The Brazilian strains showed Nod factor profiles similar to those of *R. tropici* type IIA CFN 299 and IIB CIAT 899 strains, and mixed characteristics of both types. That is, they were unable to grow in LB and PY minus Ca, as with type IIA, but were tolerant to high temperature, acidity, and had the same PCR product with Y1 and Y2 primers, as type IIB strain. The Brazilian strains showed mixed host range spectra between strain types IIA and IIB and, by the analysis of 17 fatty acids, strains PRF 35 and PRF 54 were grouped with CFN 299 and PRF 81 with CIAT 899. The performance of strain PRF 81 in field experiments indicates future potential for identification of new competitive and efficient *R. tropici* strains for tropical and subtropical areas.

Keywords: Nitrogen fixation; Inoculant; Rhizobium tropici; Competitiveness

1. Introduction

The common bean (*Phaseolus vulgaris* L.), simply referred to here as bean, is widely cultivated in the Central and South America and in Africa. In Brazil, the crop occupies 5.5 million ha of area and contributes to about 28% of the population's protein consumption but the yield is very low, on average 505 kg ha^{-1}, primarily due to poor cropping practices, such as an inefficient supply of N fertilizers (Hungria et al., 1997b). Since most Brazilian soils are N deficient, N_2-fixing *Rhizobium* bacteria could increase yield at a low

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cost and preserve water resources from pollution by nitrates. However, poor nodulation and the lack of response to inoculation in field experiments has frequently been reported worldwide, raising doubts about the efficiency of bean inoculation (Graham, 1981; Pereira et al., 1984; Buttery et al., 1987; Hardarson, 1993). The lack of response to inoculation can be attributed to intrinsic characteristics of both the host plant and the bacteria, as well as the great sensitivity of the symbiosis to environmental stresses, such as high temperatures, soil dryness and low soil fertility (Graham, 1981; Hungria et al., 1997b).

A broad range of *Rhizobium* species are able to nodulate and fix N_2 with beans, including *Rhizobium leguminosarum* bv. phaseoli (Jordan, 1984), *R. tropici* (Martínez-Romero et al., 1991), *R. etli* (Segovia et al., 1993), *R. gallicum* and *R. giardinii* (Amarger et al., 1997). Furthermore, other isolates able to nodulate beans show distinct phylogenic positions in relation to these described species (Bromfield and Barran, 1990; Eardly et al., 1992, 1995) and may well represent other species. *R. tropici* seems to be native to tropical regions of South America (Martínez-Romero et al., 1991) and represented most of the bean *Rhizobium* population in some Brazilian soils (F.M.[1] Mercante, 1993; Hungria and Stacey, 1997; Hungria et al., 1997a, 1997b). Some characteristics associated with *R. tropici* are the tolerance of high temperatures (Martínez-Romero et al., 1991; Hungria et al., 1993; Mercante, loc cit; Sá et al., 1993) and acid conditions (Graham et al., 1994), as well as a broad host range (Martínez-Romero et al., 1991; Hungria et al., 1993), being able to nodulate *Leucaena* sp. (Martínez-Romero et al., 1991). Most importantly, *R. tropici* shows greater genetic stability than the other bean *Rhizobium* species, maintaining its symbiotic properties under stressful conditions (Soberón-Chaves et al., 1986; Flores et al., 1988; Martínez-Romero et al., 1991; Segovia et al., 1993). There are conflicting results when N_2 fixation capacity and competitiveness of *R. tropici* are compared with those of the other bean rhizobia species (Martínez-Romero and Rosenblueth, 1990; Oliveira and Graham, 1990; Straliotto et al., 1991; Hungria et al., 1993), but a high degree of heterogeneity among *R. tropici* strains has been reported for these properties (Mercante, loc cit; Sá et al., 1993; Hungria et al., 1997a, 1997b), indicating that efficient and competitive strains can be obtained through a selection program.

The choice of genetically-stable rhizobia is essential for their recommendation as commercial inoculants, especially in countries like Brazil, where stressful environmental conditions are observed frequently. This was demonstrated in 1994, when it was reported that one of the two strains used in Brazilian commercial inoculants, SEMIA 4064 (= UMR 1135, from the University of Minnesota, USA), classified as *R. leguminosarum* bv. phaseoli, lost its ability to fix N_2 in several greenhouse and field experiments (Hungria and Araujo, 1995). Since the inoculants in Brazil must carry two strains, it became necessary to identify urgently at least one new efficient, competitive and genetically-stable strain.

In the first stage of this study, more than 400 isolates from soils of the State of Paraná, were obtained and tested for N_2 fixation capacity and for the ability to nodulate both bean and *Leucaena leucocephala*. Here we show the results from greenhouse and field experiments, comparing the three best isolates from Paraná soils with the remaining strain used in Brazilian commercial inoculants, CIAT 899, and with a promising strain from the State of São Paulo (CM 255). The isolates were further characterized in relation to several physiological and genetic properties, in an attempt to define those variables which could assist in future the selection of efficient and competitive strains for inoculants.

2. Materials and methods

2.1. Rhizobial strain selection

PRF strains were isolated in 1992/93 during a survey of 15 soils of the State of Paraná, Brazil. The soils had never been inoculated but had been cultivated with beans for several years. Soil dilutions were prepared as described (Andrade and Hamakawa, 1994) and used for the inoculation of bean seeds of cultivars Carioca and Negro Argel, both characterized as good N_2-fixing host plants (Hungria and Neves, 1987). Plants were grown in modified Leonard jars containing sand and vermiculite (1:2, v/v) and received N-free nutrient solution (Andrade and Hamakawa, 1994) until the pod filling stage, 45 days after emergence (DAE). Controls included non-inoculated plants with or without mineral N, supplied as KNO_3 (30 mg of N $plant^{-1}$ $week^{-1}$) and plants inoculated with strain CIAT 899 (provided by Dr. E. Martínez, CFN, Cuernavaca, Mexico; other designations for this strain are UMR 1899, USDA 9030, TAL 1797, HAMBI 1163, SEMIA 4077 and ATCC 49672). Plants characterized by high shoot dry weight and total N content (N-Kjeldahl) were selected, and bacteria were isolated from large (2–3 mm) pink nodules. Since the purpose was to select strains belonging to *R. tropici* species, all isolates were tested for the ability to form N_2-fixing nodules with *Leucaena leucocephala*, considered at the time of this selection as a typical characteristic of *R. tropici* species (Martínez-Romero et al., 1991). The strains able to nodulate this

[1] M.Sc thesis, UFRRJ, Itaguaí, p. 149.

host were reinoculated into beans of cultivar Carioca, and the three which gave largest plant N contents were selected and named PRF 35, PRF 54 and PRF 81.

2.2. N_2 fixation rates and nodule occupancy under greenhouse conditions

A greenhouse experiment (temperatures averaged 28.5/23.4°C, day/night) was performed using modified Leonard jars to evaluate the N_2 fixation rates of strains PRF 35, PRF 54, PRF 81 and of a promising strain isolated from a soil from the State of São Paulo, CM 255 (Dr S. M. Tsai, CENA, Piracicaba, SP, Brazil). The performance of the strains was compared with those of the following type strains: *R. tropici* type IIA CFN 299 (=USDA 9039, =LMG 9517, provided by Dr. E. Martínez) and type IIB CIAT 899 (Dr. E. Martínez), *R. etli* CFN 42 (=USDA 9032, Dr. E. Martínez) and *R. leguminosarum* bv. phaseoli USDA 2671 (=RCR 3644, provided by Dr. P. van Berkum, Beltsville, MD, USA). The cultivar Carioca was used in the experiments. Inoculant preparation (10^9cells ml^{-1}), seed inoculation and plant growth conditions were performed as described before for soyabeans (Hungria et al., 1996). Plants received N-free nutrient solution and the experiment also included non-inoculated controls with or without mineral N (30 mg of N as KNO_3 $plant^{-1}$ $week^{-1}$). Plants were harvested at 45 DAE and the variables evaluated were nodule number and dry weight, shoot and root dry weight and plant total N (N-Kjeldahl of N shoot + N root + N nodules − N seed). N_2 fixation rates under high temperature conditions were evaluated in another experiment performed under root controlled temperature conditions (37°C 8 h^{-1} day^{-1}), as described by Hungria et al. (1993). Temperatures surrounding the shoot were on average 30.1/23.2°C, day/night, and plants were harvested at 45 DAE.

The experiment to evaluate nodule occupancy was performed under greenhouse conditions as described above, except that each strain (10^9cells ml^{-1}, adjusted from earlier calibrated curves relating viable counts with optical density) was inoculated in a proportion of 1:1 (v/v) with strain CIAT 899. The non-inoculated control, supplied with mineral N, was not included in this experiment. Data were collected as described, but in addition, 60 nodules were randomly collected per treatment and assayed for serological agglutination reactions (Somasegaran and Hoben, 1994) against the anti-sera of each inoculated strain.

The greenhouse and growth chamber experiments were performed in a randomized block design with five replicates, and the data was subjected to analysis of variance.

2.3. Field experiments

Four field experiments were performed during the years 1994 and 1995 in Oxisols of the State of Paraná, in the districts of Londrina and Ponta Grossa. The main chemical characteristics of the soils for Londrina and Ponta Grossa, respectively were as follows: acid soils, with pH in $CaCl_2$ of 5.12 and 4.98 and low contents of N 0.15 and 0.12 g dm^{-3}, C 1.36 and 2.18 g dm^{-3} and P 7.5 and 3.6 mg dm^{-3}. The experimental plots measured 3.0 × 2.0 m, with 0.5 m between lines, and plots were separated by 2.0 m and small terraces. Five days before sowing, plots received 300 kg ha^{-1} of N–P–K (0–28–20) and 40 kg ha^{-1} of micronutrients (containing, in percentage: Zn, 9.0; B, 1.8; Cu, 0.8; Fe, 3.0; Mn, 2.0; Mo, 0.10). The cultivar Aporé was used in Londrina (seeds supplied by the germplasm bank of Embrapa-Arroz e Feijão, Goiânia, GO, Brazil) and IAPAR-14 (germplasm bank of Instituto Agronômico do Paraná, IAPAR, Londrina) was used in Ponta Grossa. In the first year, the experiments were performed in soils that had not been cultivated with beans for at least 3 years, but nevertheless showed large numbers of bean rhizobia, as evaluated by the most probable number counting technique (Andrade and Hamakawa, 1994) using plants of cultivar Carioca. The bean rhizobia population evaluated at the depth of 0–20 cm was approximately 10^5 and 10^4 cells g^{-1} of soil, for Londrina and Ponta Grossa, respectively. In the second year, the experiments were performed in the same plots as the previous year, to verify the effects of reinoculation. Strains evaluated in field experiments were PRF 35, PRF 54, PRF 81, CM 255 and SEMIA 4077 (=CIAT 899, but supplied by the Brazilian rhizobia germplasm bank, FEPAGRO, Porto Alegre, RS, Brazil). According to the Brazilian legislation for inoculants, SEMIA 4077 was used, because strains supplied to the inoculant industries must come from this source. The experiments also included non-inoculated controls with or without N fertilizers (30 kg of N ha^{-1} as urea at sowing and 30 kg of N at 35 days after sowing) as well as a non-nodulating bean line (NORH 54, originally from CIAT, Cali, Colombia, but seeds were multiplied and supplied by Embrapa-Arroz e Feijão). The inoculants were peat based and prepared by Dr. R. S. Araujo at Embrapa-Arroz e Feijão, at a concentration of 10^8 cells g^{-1} of peat and distributed to several Brazilian institutions which would perform the same experiment. Inoculant was added to the seeds with a 15% (w/v) sucrose solution to increase adherence. Nodulation (nodule number and dry weight) was verified in 12 plants at early flowering (38– 42 DAE). Yield was evaluated at the final harvest and values were corrected for 13% moisture. The experiments were performed in

a randomized block design with six replicates, and data were subjected to analysis of variance.

2.4. Characterization of strains

Colony morphology was evaluated in yeast mannitol agar (YMA) medium (Vincent, 1970), after 2 and 4 days of growth at 28°C. Other variables evaluated were: Growth in Luria broth (LB) and peptone yeast extract (PY) minus Ca media according to Martínez-Romero et al. (1991); growth in tryptone yeast extract (TY) (Somasegaran and Hoben, 1994) at 37 and 40°C; growth in TY at pH 4.0; synthesis of melanin in TY with tyrosine at 1.2 mg ml^{-1}, and $CuSO_4$ at 40 μg ml^{-1} (Rodriguez-Navarro et al., 1996).

Host range was verified in glass jars containing N-free nutrient solution (Andrade and Hamakawa, 1994) with surface disinfected seeds (Vincent, 1970) of the following legumes: *Calopogonium muconoides, Crotalaria juncea, Centrosema pubescens, Indigofera hirsuta, Leucaena leucocephala, L. esculenta, Lupinus albus, Macroptilium atropurpureum, Medicago sativa cv. Crioula, Pisum sativum* and *Vicia sativa*.

The lipopolysaccharide and protein profiles of the strains were obtained as described by Santos et al. (1999). Thin layer chromatographic analysis (TLC) of lipo-chitin oligosaccharide Nod signals (Nod factors) of bean strains was performed (Spaink et al., 1992). Initially, *nod* genes were induced with 2 μM naringenin. For strain PRF 35, the profile of Nod factors was also obtained after induction with bean seed exudates. Cells incubated in the absence of any *nod* gene inducer served as controls, and 2 μl of D-[^{14}C]-glucosamine HCl (50–60 mCi $mmol^{-1}$, Amersham) was used as label. TLC plates were exposed to Kodak Biomax MR-Kodak film for 15 days, then developed with Kodak reagents.

The phospholipid fatty acid (PLFA) analyses were carried out with duplicate washed plates containing bacteria that had been grown on YMA for 48 h at 28°C. To wash the plates and collect the bacteria, 3 ml of citrate buffer (0.15 M, pH 4.0) were added to the plate and the agar surface was gently scraped with a glass stick, recovering 1.5 ml of bacterial suspension. Lipids were extracted by the procedure of Frostegård et al. (1991). The lipid material was fractionated into neutral lipids, glycolipids and polar lipids on silicic acid (100–200 mesh, Unisil) columns by elution with chloroform, acetone and methanol, respectively. The fraction containing phospholipids was collected and dried at 40°C under N_2, and a nonadecanoic acid (19:0) was added as an internal standard. The samples were subjected to mild alkaline methanolysis (Dowling et al., 1986). The resulting fatty acid methyl esters were analyzed on a Hewlett Packard 6890 gas chromatograph equipped with a flame ionization detector and a 60 m HP 5 capillary column. All the solvents and chemicals used were of analytical grade (Scharlau), fatty acids standards were obtained from Sigma–Aldrich and glassware was washed with distilled water and heated at 400°C overnight. The percent of the PLFA values were $\log_{10}$ transformed before being subjected to principal component analysis (PCA) to elucidate major variation and covariation patterns. The multivariate calculations were performed using the program SYSTAT v 5.0, for Windows.

For genetic analyses, first a DNA fingerprint was obtained after PCR reaction with Y1 and Y2 primers, as described by Young et al. (1991). Another DNA fingerprint was obtained by the amplification by PCR with ERIC and REP primers (de Bruijn, 1992) (at a concentration of 50 pmol μl^{-1}), as described by Santos et al. (1999). DNA amplification was also obtained by the RAPD technique with 12 short primers (1, 3, 5, 6, 7, 9, 10, 12, 16, 17, 19, 20) from kit-S of Operon (Operon Technologies, 1000 Atlantic Avenue, Alameda, CA, 94501, USA). The RAPD was performed with DNA at 2 ng μl^{-1} and the basic procedure of Nishi et al. (1996). The reaction included 45 cycles as follows: at 94°C for 1 min, at 35°C for 1 min and at 72°C for 2 min. Each strain was analyzed with the repetitive or arbitrary primers at least twice, after DNA extraction from independent liquid cultures. After separation of the amplified fragments by electrophoresis in 1.5% agarose gel, the presence or absence of bands was transformed in a binary matrix of presence/absence (1/0). Cluster analysis was carried out with the NTSYS-PC program (Numerical Taxonomic and Multivariate Analysis System, version 1.70, Exeter Software, New York, USA), using the UPGMA (unweighted pair group arithmetic average clustering) and the SM (simple matching) and the J (Jaccard) coefficients.

3. Results and discussion

3.1. Rhizobial strain selection

After the analyses of more than 400 isolates from 15 soils of the State of Paraná, three promising strains were identified. The strains obtained, PRF 35, PRF 54 and PRF 81, were able to nodulate both bean and *Leucaena* plants and could grow in vitro at temperatures higher than 37°C. Consequently, they were assigned to *R. tropici*, according to the description of this species (Martínez-Romero et al., 1991). *R. etli* was found in Mesoamerica (Segovia et al., 1993) and in the Northwest region of Argentina, considered as one of the two main centres of origin of *Phaseolus vulgaris* (Aguilar et al., 1998), *R. leguminosarum* bv. phaseoli predominates in Europe (Segovia et al., 1993) and

most strains which have been isolated in Brazil belong to the *R. tropici* species (Mercante, loc. cit; Hungria et al., 1997a, 1997b). Predominance of a certain species seems to be a result of two factors: (1) introduction of the crop in that place, e.g., seeds carried from Brazil to some African countries in the 16th century; and (2) environmental factors, such as soil pH (Anyango et al., 1995). The three strains described in this paper were also isolated from acid soils (pHs 3.5–5.4).

3.2. N_2 fixation rates and nodule occupancy under greenhouse conditions

Under controlled greenhouse conditions, inoculation with strains PRF 81 and CIAT 899 enhanced nodulation, compared to other treatments, resulting in accumulation of more N in plant tissues (Table 1). An efficient symbiosis was achieved with strains PRF 81 and CIAT 899, since total N content of plants inoculated with these strains was similar to that of plants supplied with mineral N. Rates of N_2 fixation achieved with *R. tropici* type IIB CIAT 899 were 112% greater than with *R. leguminosarum* bv. phaseoli strain USDA 2671 and could reflect the adapted symbiosis of a Brazilian cultivar with *R. tropici* species. Strains PRF 54, PRF 81 and CIAT 899 were also able to nodulate and fix N_2 at high temperatures (37°C 8 h^{-1} day^{-1}, at the root system) under greenhouse conditions (Table 1), an important feature for their commercial use in inoculants for tropical and subtropical areas. These results also show that *R. tropici* is more tolerant than the other bean rhizobia species to high temperatures, as observed by Hungria et al. (1993), Mercante (1993) and Michiels et al. (1994).

Table 1
Response of bean cultivar Carioca to inoculation with eight *Rhizobium* strains[a]

Treatment	28/23°C			37/23°C
	Nodulation		Total N[b]	Total N[b]
	Number (no. $plant^{-1}$)	Dry weight (mg $plant^{-1}$)	(mg $plant^{-1}$)	(mg $plant^{-1}$)
PRF 35	67.5 c[c]	107 cd	47.9 de	29 c
PRF 54	85.4 bc	137 bc	74.2 bc	45 b
PRF 81	141.0 a	224 a	99.7 a	66 a
CM 255	101.2 b	152 b	75.2 bc	27 cd
CFN 299	81.0 bc	128 bcd	65.1 cd	25 cd
CIAT 899	128.0 a	191 a	85.4 ab	60 a
CFN 42	95.8 b	125 bcd	75.0 bc	20 d
USDA 2671	68.0 c	94 d	40.2 e	10 e
Control[d]−N	0[e]	0[e]	9.7[e]	3[e]
Control[d]+N	0[e]	0[e]	98.6 a	49 ab
CV (%)	21	18	14	25

[a] Plants were grown either at optimal temperatures (28/23°C, day/night) or stressful conditions (37/23°C), in modified Leonard jars containing N-free nutrient solution, and were harvested at 45 days after emergence.

[b] N-Kjeldahl (N roots + N shoot + N nodules − N seed).

[c] All values represent the mean of five replicates and when followed by the same letter, in the same column, did not show statistical difference (Tukey, $P \leq 0.05$).

[d] Non-inoculated controls supplied or not with mineral N (30 mg of N as KNO_3 $plant^{-1}$ $week^{-1}$).

[e] Values not considered for the statistical analyses.

In the experiment where the Brazilian cultivar Carioca received a mixed inoculum, *R. tropici* strains PRF 81, PRF 54 and PRF 35 showed higher nodule occupancy than *R. etli* strain CFN 42 and *R. leguminosarum* bv. phaseoli strain USDA 2671 (Table 2). These results confirm a report also with a Brazilian cultivar (Straliotto et al., 1991), and disagree with results obtained in other countries by Martinez-Romero and Rosenblueth (1990), Oliveira and Graham (1990) and Streit et al. (1992). This result could be attributed to the development of an efficient symbiosis of Brazilian cultivars with the native strains, most belonging to *R. tropici* species.

3.3. Field experiments

In the first year of field experiments, in soils of Londrina and Ponta Grossa which had not been cultivated with beans for at least 3 years, but with high number of bean rhizobia cells (10^5 and 10^4 cells g^{-1} of soil, for Londrina and Ponta Grossa, respectively), inoculation increased nodule number and nodule dry weight in relation to the naturalized bean rhizobia population (Table 3). However, this increase in nodulation did not result in statistically significant yield increases, although addition of mineral N fertilizer also did not result in a better performance. Reinoculation in the following year improved nodulation, with a mean increase of 22% in nodule number and of 12% in nodule dry weight. The reinoculation also improved yield and, for strain PRF 81, statistically significant increases of 800 and 906 kg ha^{-1} were obtained, in Londrina and Ponta Grossa respectively, with respect to the non-inoculated control. Therefore, reinoculation probably aided the establishment of the most efficient inoculated rhizobia, improving symbiotic performance and could be essential for obtaining a positive response to bean inoculation. Unfortunately, nodule occupancy by the inoculated strains could not be studied by serology in the field experiments, because of cross reactions among some of the strains used in this study and the native bean rhizobia population.

Poor nodulation and failure to respond to inoculation have often been reported for the bean crop and attributed to several factors, e.g., the lack of competitiveness against native rhizobia, bean genotypes with a low capacity to fix N_2 and the short growth cycle of the crop (Graham, 1981; Pereira et al.,

Table 2
Response of bean cv. Carioca when co-inoculated with eight *Rhizobium* strains in mixed ratios of 1:1 (v/v, 10^9 cells ml^{-1}) with strain CIAT 899[a]

Treatment	Nodule number ($plant^{-1}$)	Total N (mg $plant^{-1}$)[b]	Nodule occupancy (%)		
			Test strain	CIAT 899[c]	Double[c]
PRF 35	52.1 e[d]	35.2 d	40 bc	38	22
PRF 54	73.2 d	62.1 bc	46 ab	35	19
PRF 81	121.6 a	87.6 a	55 a	26	19
CM 255	97.3 bc	66.1 bc	38 bc	50	12
CFN 299	88.4 bcd	56.4 c	35 c	51	14
CIAT 899	105.3 ab	78.7 ab	–[c]	100	–
CFN 42	78.2 cd	65.2 bc	33 cd	52	15
USDA 2671	48.2 e	30.4 d	28 d	54	18
Control[e] − N	0[c]	9.1[c]	–[c]	–	–
CV (%)	18	14			

[a] Plants were grown under greenhouse conditions (mean temperatures of 28/23°C, day/night), in modified Leonard jars containing N-free nutrient solution and harvested at 45 days after emergence.
[b] N-Kjeldahl (N root + N shoot + N nodules − N seed).
[c] Values not considered for the statistical analyses.
[d] All values represent the mean of five replicates and when followed by the same letter, in the same column, did not show statistical difference (Tukey, $P \leq 0.05$).
[e] Non-inoculated control.

Table 3
Effects of inoculation and reinoculation with five *Rhizobium* strains on nodule number and dry weight at early flowering (38–42 DAE) and grain yield in four field experiments performed in oxisols of Londrina and Ponta Grossa, PR, Brazil

Treatment	Londrina			Ponta Grossa		
	Nodule number (no. $plant^{-1)}$	Nodule dry weight (mg $plant^{-1}$)	Yield (kg ha^{-1})[a]	Nodule number (no. $plant^{-1}$)	Nodule dry weight (mg $plant^{-1}$)	Yield (kg ha^{-1})[a]
First year						
PRF 35	25 de[e]	44 c	1344 bc	38 c	63 c	2838 b
PRF 54	32 cd	56 b	1561 ab	57 b	75 b	2937 ab
PRF 81	48 a	75 a	1571 ab	74 a	92 a	3177 ab
CM 255	37 bc	52 bc	1527 ab	60 ab	80 ab	2959 ab
SEMIA 4077[b]	45 ab	72 a	1423 bc	69 ab	85 ab	3163 ab
Control[c] − N	20 e	20 d	1356 bc	22 d	25 d	3092 ab
Control[c] + N	2[f]	1[f]	1954 a	4[f]	2[f]	3326 a
Non-nodulating[d]	0[f]	0[f]	977 c	0[f]	0[f]	1477 c
CV (%)	25	22	18	21	21	15
Second year						
PRF 35	39 c	57 c	2874 bc	45 c	68 b	2527 c
PRF 54	52 bc	76 b	2921 bc	67 ab	79 ab	2968 bc
PRF 81	68 a	92 a	3326 a	79 a	94 a	3425 a
CM 255	46 c	65 bc	2872 bc	60 b	80 ab	2922 bc
SEMIA 4077[b]	60 ab	80 ab	3120 ab	74 ab	87 a	3271 ab
Control[c] − N	22 d	21 d	2526 c	23 d	20 c	2519 c
Control[e] + N	4[f]	2[f]	3520 a	5[f]	3[f]	3519 a
Non-nodulating[d]	0[f]	0[f]	816 d	0[f]	0[f]	750 d
CV (%)	28	25	16	23	20	17

[a] Yield corrected to 13% of moisture.
[b] SEMIA 4077 = CIAT 899, but received from the Brazilian rhizobia germplasm bank.
[c] Non-inoculated control with or without N fertilizer (30 kg of N ha^{-1} as urea during sowing and 30 kg of N at 35 days after sowing).
[d] Non-nodulating bean line NORH 54.
[e] All values represent the mean of six replicates and when followed by the same letter, in the same column, during the same year, did not show statistical difference (Duncan, $P \leq 0.05$).
[f] Values not considered for the statistical analyses.

1984; Buttery et al., 1987). The lower N_2 fixation capacity of beans was also confirmed and compared with six other legumes in several countries, showing that beans had a lower contribution of N from N_2 fixation, i.e. around 40% (Hardarson, 1993). However, in the Central Region of Brazil, the Cerrados, where over 0.8 $\times 10^6$ ha are being cultivated with beans and soil N content is low, bean yield is generally increased by inoculation (Mendes et al., 1994; Peres et al., 1994; Hungria et al., 1997b). Thus, N_2 fixation with Brazilian cultivars can support yields of about 2500 kg ha^{-1} in soils poor in N, a value five-fold higher than the average Brazilian yield. It is possible that the available soil N was depleted in the second year, and therefore, a response to inoculation was found. However, it is also possible that the reinoculation may be important to help the establishment of new strains in the soil, resulting in increases in nodulation and yield.

As the naturalized bean rhizobia population of Brazilian soils, as well as of soils of other countries of the Central and South Americas and Africa are highly competitive but inefficient (Graham, 1981; Hardarson, 1993; Hungria et al., 1997b), the success of bean inoculants is directly related to the strain competitiveness. CIAT 899 has been used in N_2 fixing studies performed worldwide and, in some of these countries, is used in commercial inoculants. In Brazil, for the last 10 years none of the strains tested by several laboratories has shown symbiotic performance comparable to that of CIAT 899, considering the their N_2fixation rates, tolerance to high temperatures, nodule occupancy and genetic stability. Therefore, commercial inoculants were carrying exclusively CIAT 899 for the last 4 years, even though the Brazilian law requires the presence of two strains. The good performance of PRF 81 was confirmed in more than 25 field trials performed by other Brazilian institutions in several states and the strain has been officially recommended for the use in Brazilian commercial inoculants, together with CIAT 899, since June 1998. Promising strains, with better symbiotic performance than CIAT 899, were also found in Kenya (Anyango et al., 1995). The preliminary tests showed that PRF 81 fell into the *R. tropici* species, characterized by higher genetic stability than *R. etli* and *R. leguminosarum* bv. phaseoli (Flores et al., 1988; Martínez-Romero et al., 1991; Hernandez-Lucas et al., 1995). Consequently, *R. tropici* seems to be the most suitable species to be recommended for tropical and subtropical conditions, such as those found in Brazil. This idea is reinforced by experience with three other strains used in Brazilian inoculants, C-05-0, V-23 and SEMIA 4064, none being classified as *R. tropici*, which lost their capacity to fix N_2, discouraging the practice of inoculation of bean (Hungria and Araujo, 1995).

3.4. Characterization of strains

A programme of characterization of the new strains was initiated, in an attempt to define variables which could assist in selection of efficient, competitive and genetically-stable strains in future. For example, selection may be easier if it was known that such strains were generally as *R. tropici* type IIA or type IIB genotypes.

3.4.1. Characterization in vitro

Colony morphology of strains PRF 35, PRF 54 and PRF 81 was similar to that described for *R. tropici* strains (Martínez-Romero et al., 1991) (data not shown). Strains PRF 35 and PRF 54 were unable to grow in LB and in PY minus Ca media (Table 4), typical characteristics of *R. tropici* type IIA strains (Martínez-Romero et al., 1991). Strain PRF 81 grew poorly

Table 4
Growth in vitro of nine bean rhizobia strains in LB and PY without Ca media and in TY under different conditions of temperature and pH. Also, synthesis of melanin in TY medium supplied with tyrosine and copper sulphate

Characteristics	Bean rhizobia strains								
	PRF	PRF	PRF	CM	SEMIA	CFN	CIAT	CFN	USDA
	35	54	81	255	4077[a]	299	899	42	2671
LB	N[b]	N	P	Y	Y	N	Y	N	N
PY minus Ca	N	N	P	Y	Y	N	Y	N	N
37°C	Y	Y	Y	Y	Y	Y	Y	N	N
40°C	Y	Y	Y	Y	Y	P	Y	N	N
pH 4.0	Y	Y	Y	Y	Y	P	Y	N	N
Synthesis of melanin	N	N	P	Y	Y	N	Y	N	N

[a] SEMIA 4077 = CIAT 899 from the Brazilian rhizobia germplasm bank.
[b] All characteristics were confirmed in three replicates. Y, yes, normal growth; N, no growth; P, poor growth, of about 10% of that observed in the strains classified as Y.

on both media, giving colony sizes of only 10% of those observed for SEMIA 4077, CIAT 899 and CM 255. Although SEMIA 4077 and CIAT 899 are the same strain, both were characterized in this study because they evolved from different culture collections.

In relation to other characteristics evaluated in vitro, similarly to type IIB CIAT 899 and SEMIA 4077, strains PRF 35, PRF 54, PRF 81 and CM 255 were able to grow at 40°C and pH 4.0 (Table 4). Strains CM 255, SEMIA 4077 and CIAT 899 were character-

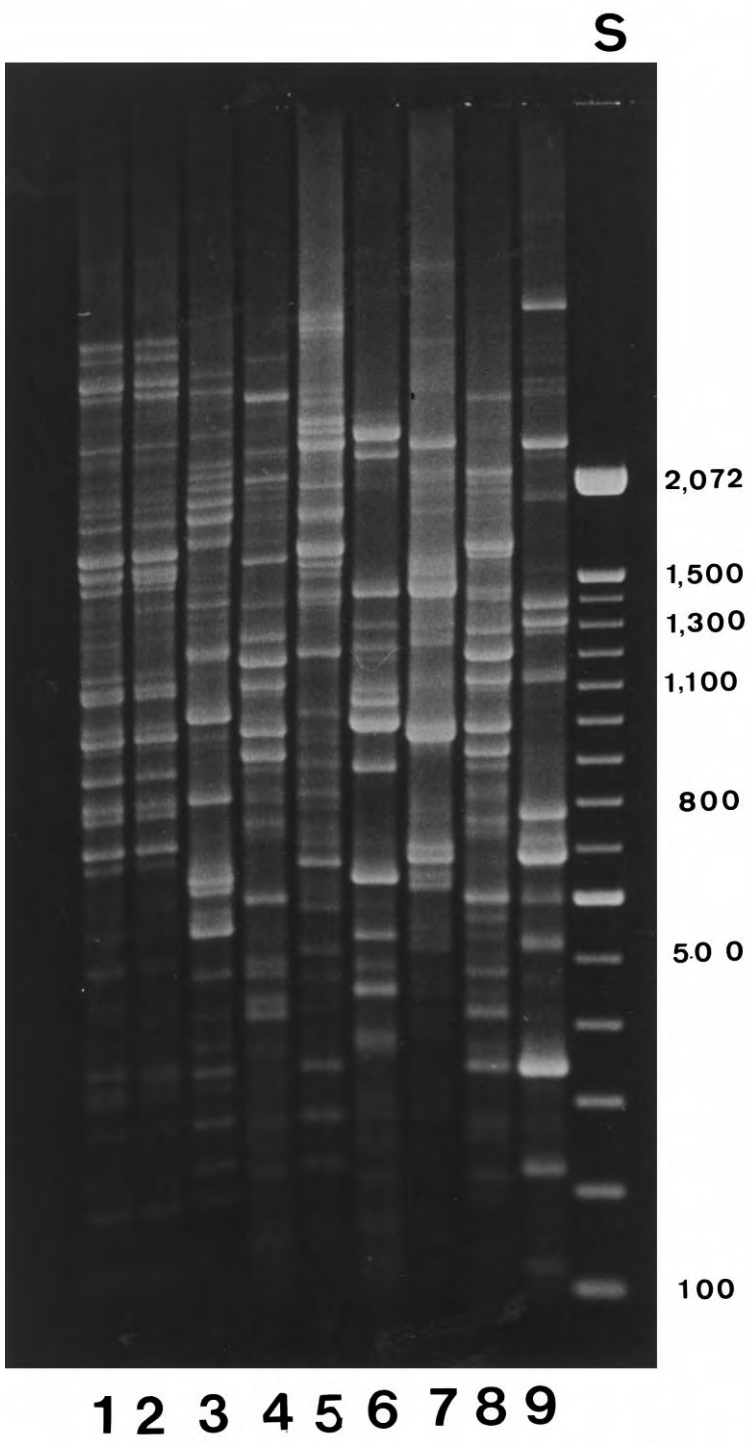

Fig. 1. REP-PCR fingerprint patterns obtained for bean rhizobia strains, (1) PRF 35, (2) PRF 54, (3) PRF 81, (4) CM 255, (5) SEMIA 4077, (6) USDA 2671, (7) CFN 421, (8) CFN 299, and (9) CIAT 899. The last lane shows the DNA molecular weight standard (S) and the size markers are indicated in base pairs on the right side.

ized as melanin producers in vitro, and poor production (of about 10%) of this pigment was verified for PRF 81 and none for PRF 35 and PRF 54.

3.4.2. Host range spectra

The three strains from the State of Paraná, as well as CM 255, SEMIA 4077, CIAT 899, CFN 299, CFN 42 and USDA 2671 were unable to nodulate *Centrosema pubescens, Lupinus albus, Medicago sativa* (as does strain Or 191, described in Eardly et al., 1995), *Pisum sativum* and *Vicia sativa*. All strains, except for USDA 2671, formed small, white pseudonodules on *Calopogonium muconoides*. Large (2–3 mm) pink nodules were verified on *Leucaena leucocephala* and *L. esculenta* when inoculated with all strains except for CFN 42 and USDA 2671. Although the ability to nodulate *Leucaena* seemed to be a characteristic of *R. tropici* (Martínez-Romero et al., 1991), and was therefore, used as a selection indicator at the beginning of this work, Hernandez-Lucas et al. (1995) later reported that *R. etli* CFN 42 formed nodules lacking leghaemoglobin and strain F 16 established an effective symbiosis with *Leucaena*. Moreover, the nodulation assays did not confirm nodulation of leucaena by CFN 42. Strains CM 255, SEMIA 4077, CIAT 899 and CFN 42 nodulated *Indigofera hirsulta*, but none of the strains from the State of Paraná did. Differences among the three strains from the State of Paraná were detected in relation to the nodulation of *Crotalaria juncea* and *Macroptilium atropurpureum*; both hosts were nodu-

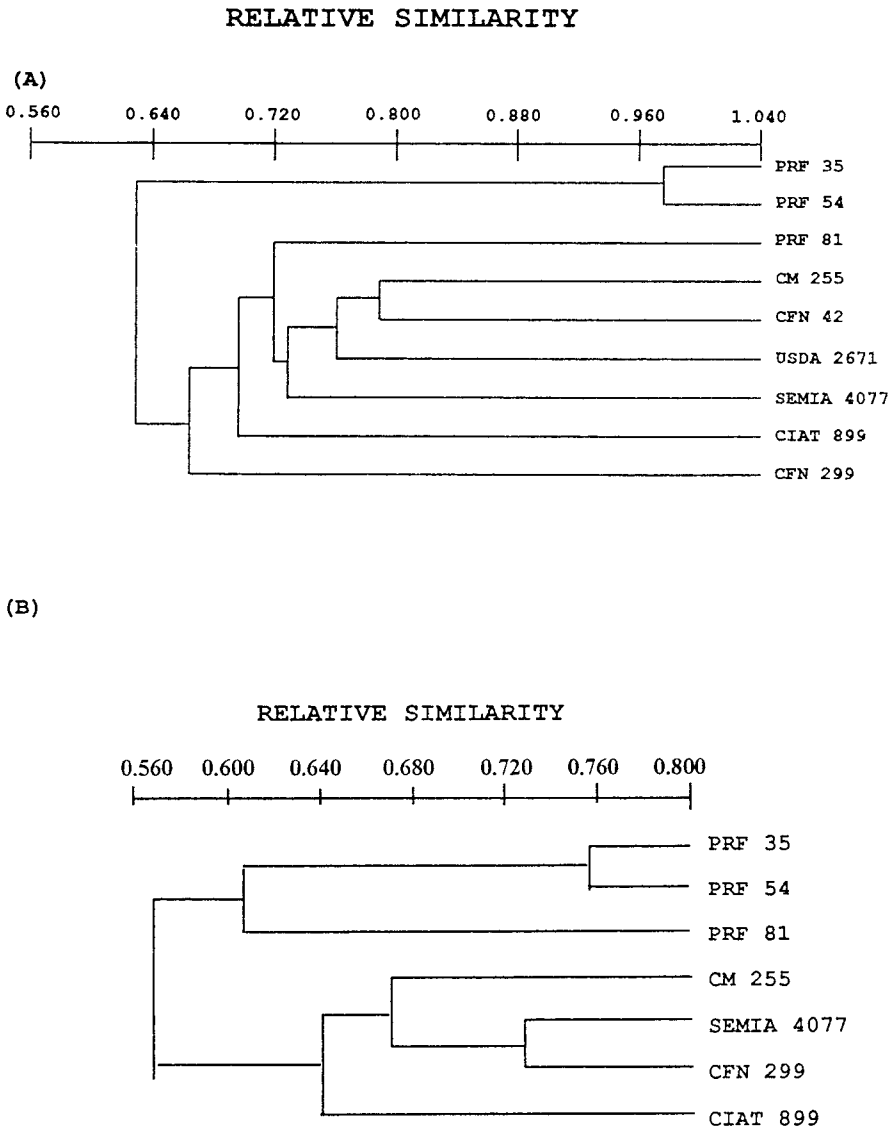

Fig. 2. Dendrogram showing the strains from Brazil and representative strains of bean rhizobia species after the cluster analysis of: (A) ERIC and REP-PCR products; and (B) RAPD products obtained with 12 short primers, both using the Simple Matching coefficient.

lated by PRF 54 and PRF 81 but not PRF 35. *C. juncea* was not nodulated by CM 255, CFN 299 and USDA 2671, and this last strain also did not nodulate *M. atropurpureum*.

3.4.3. Cellular lipopolysaccharide profiles

Strains PRF 35 and PRF 54 showed similar patterns, but differed from PRF 81 by two upper bands and contained a unique lower band (data not shown). Strains SEMIA 4077 and CIAT 899 showed similar profiles which were different from all other strains and CM 255 and CFN 299 also showed unique profiles (data not shown)

3.4.4. Protein profiles

Similar profiles were observed for strains PRF 35 and PRF 54, but they differed from those obtained with all other strains. Again, SEMIA 4077 showed a similar pattern to CIAT 899, but these were different from all other strains and CM 255 and CFN 299 showed distinct profiles (data not shown).

3.4.5. DNA fingerprint with specific (ERIC and REP) or arbitrary primers

Polymorphism was easily detected among the strains by the REP (Fig. 1) and ERIC-PCR analysis (Fig. 1). Strains PRF 35 and PRF 54 had patterns that were almost identical with the two sets of primers; each other strain produced a clearly distinct DNA fingerprint. The ERIC and REP-PCR products were combined to generate a dendrogram. The cluster analysis, using the simple matching (SM) coefficient showed that strains PRF 35 and PRF 54 were very similar, with a similarity level of 0.978 (Fig. 2A). The cluster formed by these two strains was linked to the cluster formed by the other strains, including PRF 81, at a level of 0.637. Using a more discriminating analysis, the Jaccard coefficient, similarities between PRF 35 and PRF 54 were confirmed, and PRF 81 was linked to this cluster at a level of 0.160. Three other clusters were formed by CM 255 and SEMIA 4077; *R. tropici* type IIA and type IIB; *R. leguminosarum* bv. phaseoli and *R. etli* (data not shown).

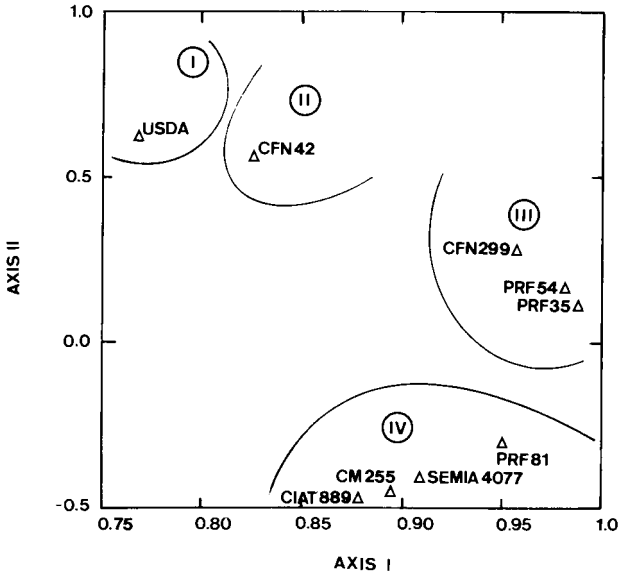

Fig. 3. Principal component analysis (two-dimensional plot) showing strains from Brazil and representative strains of rhizobia species and obtained with 17 fatty acids. Strains are grouped as follows: (I) USDA 2671; (II) CFN 42; (III) CFN 299, PRF 35 and PRF 54; (IV) CIAT 899, SEMIA 4077 and PRF 81.

The RAPD amplification with 12 arbitrary and short primers produced up to 14 products per strain. Some primers were very successful in generating polymorphisms, e.g., OPS 6, 9, 17, 19 (data not shown). As in ERIC and REP-PCR, slight differences were detected between strains PRF 35 and PRF 54. These two strains were grouped at a similarity level of 0.757 using the SM coefficient (Fig. 2B) and 0.917 with the Jaccard coefficient (not shown). The fingerprints obtained with both repetitive and arbitrary primers were highly reproducible.

Consequently, the analyses carried out in vitro added to the determination of lipopolysaccharide, protein and DNA profiles and showed that the Brazilian strains were different from the strains representative of *R. tropici* IIA and IIB, *R. etli* and *R. leguminosarum* bv. phaseoli species.

3.4.6. DNA amplification with Y1–Y2 primers

The PCR reaction with Y1 and Y2 primers following the methodology of Young et al. (1991) amplifies a region of 308–312 bp, that is useful to separate *R. tropici* type IIA and type IIB strains. Since type IIA has an insertion of 72 nucleotides in this fragment, this results in a larger product than type IIB strains (van Berkum et al., 1994). In this study, the amplification with Y1 and Y2 has shown that strain CM 255 had the same pattern as *R. tropici* type IIA, while the PCR products of the three strains from Paraná had the same molecular weight as type II B strains CIAT 899 and SEMIA 4077 (data not shown). *R. etli* and *R. leguminosarum* bv. phaseoli products obtained by the amplification with Y1 and Y2 also had the same molecular weight as CIAT 899.

3.4.7. Phospholipid fatty acids

In general the composition of phospholipids and fatty acids was quite similar to that found by Jarvis and Tighe (1994) for different rhizobia species (data not shown). However, some fatty acids were detected in higher amounts than those described earlier (e.g., 17:0, up to 4% vs. 0.2%, in this paper and in Jarvis and Tighe (1994), respectively; 18:1ω9, 1.15% vs. 0.2%) or lower amounts on other fractions, e.g., 17:0

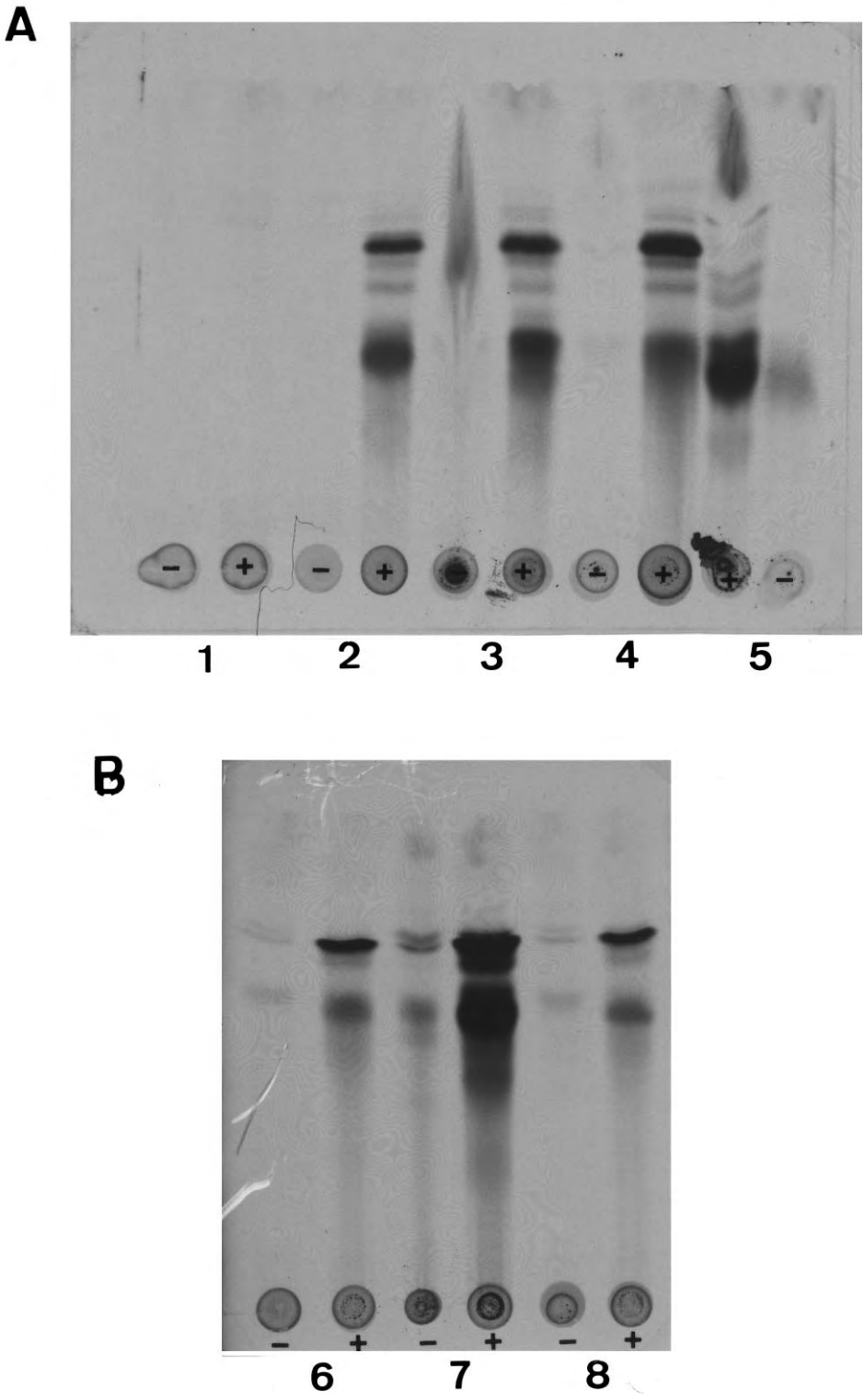

Fig. 4. Thin layer chromatography (TLC) of radiolabelled Nod metabolites showing (−) uninduced or (+) naringenin induced strains of: (A) Plate with (1) PRF 35(2) PRF 54(3) PRF 81(4) CIAT 899, (5) CFN 42; (B) Plate with (6) SEMIA 4077(7) CFN 299, and (8) PRF 35. (This last one is induced with seed exudates).

(0.43% vs up to 9%); 16:0 (0.16% vs 16%) or 20:3 (0.55% vs 3%). Fig. 3 shows a two-dimensional plot of principal component analysis carried out with 17 PLFAs considered for the nine strains studied. The two first axes explain 99.32% of the results (82.570% by axis I and 16.758% by axis II). *R. leguminosarum* bv. phaseoli strain USDA 2671 and *R. etli* CFN 42, appear clearly separated from each other and also from the other strains. The strains CFN 299, PRF 54 and PRF 35 appear closely related (at higher values of axis I). Finally, a fourth group of strains, composed by CM 255, PRF 81, and *R. tropici* IIB (SEMIA 4077 and CIAT 899) appear linked at negative values of axis II.

3.4.8. Synthesis of Nod factors

Strains PRF 54 and PRF 81 had the same profile as *R. tropici* IIB CIAT 899 (Fig. 4A), SEMIA 4077 and CFN 299 (Fig. 4B), after induction with naringenin. Strain PRF 35 was not induced by naringenin (Fig. 4A), but produced the same pattern as the strains cited above after induction with seed exudates (Fig. 4B). A different profile was obtained for *R. etli* CFN 42 (Fig. 4A). It is known that *R. tropici* CIAT 899 produces different Nod factors than *R. etli* CFN 42 (Poupot et al., 1995; Folch-Mallol et al., 1996), and the Brazilian strains had the same profile as CIAT 899. However, analysis of Nod factors has revealed differences between PRF 35 and PRF 54, since the former strain did not produce Nod factors in response to naringenin, a known *nod* gene inducer of strain CIAT 899 (van Rhijn et al., 1994).

Strains PRF 35 and PRF 54 have shown similarity in many properties analyzed in vitro and were found genetically closely related using both ERIC and REP-PCR and the RAPD techniques. However, the strains differed substantially in symbiotic properties, such as, nodulation and N_2 fixation capacity, as well as in the synthesis of Nod factors after induction with naringenin. Consequently, important symbiotic differences can be detected among strains showing genetic relatedness using DNA fingerprints obtained by PCR with these primers. Moreover, the differences detected between PRF 35 and PRF 54 in the induction of Nod factors and symbiotic effectiveness, when viewed in the light of a report on soybean (Hungria et al., 1996), seem to indicate that Nod factors may play an important role not only in nodulation but also in the efficiency of N_2 fixation and competitiveness. Although in our work we did not progress in understanding the genetic and biochemical basis for increased nodulation competitiveness, after several years we have finally identified a very competitive strain (PRF 81) which can be used in future studies to understand basic aspects of competitiveness. Such studies could take advantage of strains PRF 35 and PRF 54, showing genetically relatedness but with considerable differences on competitiveness.

Consequently, the evaluation of some characteristics of three Brazilian strains, such as morphological and physiological properties in vitro, host range spectrum, amplification with Y1–Y2 primers, composition of phospholips and fatty acids and Nod factors profiles have shown that they have mixed characteristics between *R. tropici* type IIA and type IIB strains. Therefore, although there is an increasing interest for creating new rhizobia species, at this moment, *R. tropici* should neither be subdivided into new species nor should the strains belonging to this species be necessarily classified as type IIA or type IIB. Another important conclusion of this study is that the response to inoculation to one of these strains, PRF 81, encourages the identification of new genetically stable, competitive and efficient bean rhizobia strains for each cropping area. A concerted program of strain selection for bean cropping areas of the South and Central America, and other parts of the tropics, could revert the current situation of poor yields and depletion of soil N.

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