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Selection of bean (*Phaseolus vulgaris* L.) rhizobial strains for the Brazilian Cerrados

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Abstract

The common bean crop (*Phaseolus vulgaris* L.) occupies 5.5 million hectares in Brazil and provides about 30% of the population's protein needs. Yield remains low mainly due to limited availability of N and P, and to the often acid soil conditions. Surprisingly, there have been only very limited studies in Brazil in which the isolation and evaluation of efficient N_2-fixing bean strains has been attempted. This paper reports the selection of bean rhizobia for the ''Cerrados'', the savannas that represent one-fourth of Brazilian land. Two-hundred strains were selected from large pink-colored nodules collected in the Cerrados region and biological nitrogen fixation was evaluated under optimal (27/21 °C, day/night) and high (37/21 °C) temperature conditions. Thirty-six strains were selected and their nitrogen-fixing capacity and competitiveness further evaluated with black-seeded ''Negro Argel'', and colored ''Carioca'' cultivars. One-fifth of the strains showed low genetic stability of nodulation genes, but some strains were as or more competitive than the strains currently recommended for use in commercial bean inoculants in Brazil. The superior performance of five strains was confirmed under field conditions and re-inoculation in the second year increased bean yield. The DNA fingerprintings obtained by the ERIC–REP-PCR analyses indicated a high level of genetic diversity, and among the 36 strains, six different patterns of RFLP-PCR of the 16S rRNA gene region were detected. The 16S rDNA sequences of the most efficient and competitive strains were genetically similar to *Rhizobium tropici*, suggesting that further studies on inoculant strains in the hot and acid soils of the Brazilian Cerrados and Africa should emphasize this species. © 2002 Published by Elsevier Science B.V.

Keywords: Common bean; Competitiveness; Nitrogen fixation; Rhizobium; Strain selection

1. Introduction

Modern agriculture aims to increase crop yields to satisfy the needs from a growing population, but to use sustainable approaches that should include the substitution of chemical inputs by a more effective use of natural resources. Biological nitrogen (N_2) fixation fits well in this model as it is a more environmentally clean way of satisfying plant N needs.

The common bean (*Phaseolus vulgaris* L.) crop, here referred to simply as bean, is very important in South America, especially in Brazil, where 5.5 million hectares are planted annually, supplying about one-third of population's protein needs. Yield is low, averaging only 710 kg ha^{-1} (IBGE, 1996), mainly

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due to poor cropping practices, and to the low availability of N (Hungria et al., 1997). Several restraints to the improved biological N_2 fixation rates in beans have been reported. They include poor nodulation capacity, competitive but inefficient indigenous rhizobia and lack of response to inoculation under field conditions (Graham, 1981; Pereira et al., 1984; Buttery et al., 1987; Hardarson, 1993). However, an approach not often considered is the isolation and assessment of efficient strains from local sites of bean production. A recent selection program in Brazil has identified an efficient and competitive *Rhizobium tropici* strain, PRF 81, now commercially recommended for this crop (Hungria et al., 2000); a more continuous program of strain selection could help to reverse the actual situation of low bean yield and depletion of soil N.

The ''Cerrados'', an edaphic type of savanna, covers about 25% of Brazilian land, occupying 207 million hectares in the central region of Brazil. Beans are grown over nearly 1.2 million hectares of Cerrados, with yields in the region averaging only 587 kg ha^{-1} (IBGE, 1996). Production is mainly by small farmers and uses minimal inputs (Hungria et al., 1997). The Cerrados are quite distinct from other areas of Brazil, being characterized by environmentally inhospitable conditions, especially long periods of water stress and high temperatures (>40 °C), as well as low pH (<5.0), aluminum toxicity and low soil P levels (Hungria and Vargas, 2000). These environmental conditions can limit N_2 fixation with bean and *Rhizobium* strain selection programs that emphasize tolerance to these specific constraints needed. In this paper, the selection of bean rhizobial strains for the Brazilian Cerrados is reported. The isolates were also genetically characterized in an attempt to define parameters that could assist future selection programs.

2. Material and methods

2.1. Bean rhizobia used as reference strains

Bean rhizobia reference strains in this study included: *R. tropici* IIA CFN 299 (=USDA 9039, =LMG 9517), IIB CIAT 899^T (=UMR 1899, =USDA 9030, =TAL 1797, =HAMBI 1163, =SEMIA 4077, =ATCC 49672) and *R. etli* CFN 42^T (=USDA 9032) were provided by Dr. E. Martínez, CFN, Cuernavaca, Mexico. *R. tropici* strains PRF 35, PRF 54 and PRF 81 (= SEMIA 4080) came from the Embrapa Soja germplasm bank. *R. leguminosarum* bv. phaseoli USDA 2671 (=RCR 3644) was provided by Dr. P. van Berkum, Beltsville, MD, USA. *R. giardinii* bv. giardinii strain $H152^T$ and *R. gallicum* bv. gallicum strain $R602^T$ were provided by Dr. N. Amarger, INRA, Dijon, France.

2.2. Isolation of bean rhizobial strains

A total of 200 isolates were obtained from the Brazilian Cerrados Region, in the state of Goiás (GO), and in the Distrito Federal (DF, Federal District). Strains were isolated from large pink-colored nodules of field-grown bean plants using standard microbiological methods (Vincent, 1970). The purity of the cultures was confirmed by repeated streaking on yeast extract–mannitol agar (YMA) medium (Vincent, 1970) with confirmation of single type of colony morphology, absorption of Congo red (25 μg ml^{-1}), Gram stain reaction and growth rate. Bacteria were maintained in YM mixed with glycerol (1/1, v/v) and stored at −80 °C; working cultures were maintained on YMA slants at 4 °C.

2.3. Nitrogen fixation capacity under optimal and high temperature conditions

The 200 isolates were evaluated for N_2 fixation capacity under greenhouse conditions. Seeds of cultivar Carioca were surface-sterilized (Vincent, 1970) and were inoculated with a single strain. Inoculants were prepared in YM at a concentration of 10^9 cells ml^{-1} and each seed was soaked for 20 min in 1 ml of inoculum. Five seeds were planted per sterilized Leonard jar containing sand and vermiculite (1/2, v/v) and thinned to two plants per pot after germination. Plants were grown at 27.5/21.3 °C, average day/night temperature, received N-free nutrient solution (Andrade and Hamakawa, 1994) and were harvested 5 weeks after emergence. Controls included non-inoculated plants with or without mineral N [30 mg of N (as KNO_3)/plant/week] and plants inoculated with strains CIAT 899 and PRF 81; the strains currently used in Brazilian commercial bean inoculants. Nodule number and dry weight, shoot dry

weight and total N (N-Kjeldahl) accumulated in shoots were analyzed.

N_2 fixation capacity under high temperature conditions was also evaluated in another experiment performed with the 200 strains grown under root controlled temperature conditions (37 °C for 8 h of day/21 °C, day/night) as described before (Hungria et al., 2000). Conditions other than root temperature were as described above.

2.4. Nitrogen fixation capacity and competitiveness of selected strains

Thirty-six strains were selected from the first two experiments, 25 from Goiás state and 11 from Federal District (see Tables 1 and 2). These strains showed best symbiotic performance under normal and high temperature conditions (data not included).

The N_2-fixing capacity was again evaluated in a greenhouse experiment with the bean cultivars Carioca (meso-American colored seed) and Negro Argel (meso-American black seed). The experiment was performed as described above at a mean temperature of 26.9/21.8 °C day/night. Non-inoculated controls, with and without mineral N, and controls inoculated with strains CIAT 899 and PRF 81 were included.

The experiment to evaluate competitiveness was also performed under greenhouse conditions, except that each strain (1 ml of 10^9 cells ml^{-1} for two seeds, soaking for half-an-hour) was inoculated in a proportion of 1/1 (v/v) with strain CIAT 899. The parameters evaluated were as described above, but, in addition, 60 nodules were randomly collected per treatment and analyzed for serological reaction using anti-serum of CIAT 899 (Somasegaran and Hoben, 1994).

All greenhouse experiments were performed using a randomized block design with three replicates, with the results statistically analyzed by Tukey's test at $p \leq 0.05$.

2.5. Field experiments

Two field experiments were performed in a Brazilian oxisol characterized by a low pH (4.8) and N content (0.13 g dm^{-3}). Lime was applied 3 months before planting, and at sowing pH was 5.3. Experimental plots measured $3.0 \times 2.0\,m^2$, with 0.5 m between rows, and plots separated by 2.0 m with terracing. Five days before sowing, plots received 84 kg P ha^{-1}, 60 kg K ha^{-1} and 40 kg ha^{-1} of micronutrients (containing (in %): Zn, 9.0; B, 1.8; Cu, 0.8; Fe, 3.0; Mn, 2.0; Mo, 0.10). The experiment was carried out in a soil that had not been cultivated with beans for 3 years, but nevertheless included significant bean rhizobia. Rhizobia were estimated, in the 0–20 cm depth layer, using the bean cultivar Carioca and the most probable number counting technique (MPN, Vincent, 1970) in jars containing nutrient solution at pH 5.3. The experiment was repeated in the following year in the same plots as before to verify the effects of re-inoculation. The pH was raised to 5.4 but N content was still 0.13 g cm^{-3}. The experiments were performed with the cultivar Carioca and five high-performing strains selected from those used in the greenhouse trials were evaluated. 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). Finally, controls inoculated with strains CIAT 899 and PRF 81 were also included. The inoculants were peat-based and prepared to a density of 10^9 cells g^{-1} of peat. Inoculants were added to the seeds at a rate of 500 g of inoculant for 50 kg of seeds with 300 ml of a 10% (w/v) sucrose solution to increase adherence. Each year, nodule number and dry weight, shoot dry weight and total N (Kjeldahl) accumulation in shoots were evaluated in 12 plants/plot at early flowering (35–38 days after emergence). Nodule occupancy was not evaluated because of cross-reaction with indigenous bean rhizobial strains. 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 analyzed by Duncan's test ($p \leq 0.05$).

2.6. Genetic characterization of the strains

2.6.1. Amplification with specific (ERIC and REP) primers

Total DNA was extracted from each of the 36 strains initially identified as having promising performance and 50 ng used in each amplification by PCR with ERIC and REP primers (de Bruijn, 1992) as described before (Santos et al., 1999). The reactions were carried

Table 1
Nodulation and nitrogen fixation capacity under greenhouse conditions when bean cultivar Carioca was singly inoculated with rhizobial strains from the Brazilian Cerrados, as well as with *R. tropici* strains CIAT 899 and PRF 81; also nodule occupancy by each strain when co-inoculated with CIAT 899[a]

Isolates	Nodulation		Shoot		Nodule occupancy (%)
	Number (No./plant)	Dry weight (g/plant)	Dry weight (g/plant)	Total N (mg N/plant)	
Isolates from Goiás state					
J 1	150	0.14	0.54	11.3	25
J 2	123	0.13	0.38	10.2	30
J 3	4	0.01	0.28	2.1	0
J 4	3	0.01	0.33	1.6	0
J 5	106	0.11	0.43	12.2	32
J 28	294	0.30	2.34	87.0	50
J 41	174	0.18	1.50	46.6	42
J 65	171	0.17	2.36	83.3	45
J 71	285	0.27	1.66	51.3	38
J 76	14	0.05	0.24	8.7	52
J 86	117	0.10	1.17	34.7	30
J 94	8	0.02	0.35	2.9	0
J 95	4	0.01	0.29	2.4	20
J 96	195	0.12	0.61	18.0	0
J 97	208	0.31	1.86	65.8	42
J 112	197	0.22	1.78	55.4	27
J 116	206	0.21	1.00	8.1	38
J 124	119	0.10	0.56	13.6	35
J 137	4	0.01	0.31	2.3	0
J 140	224	0.42	2.82	91.4	42
J 142	3	0.01	0.24	1.4	0
J 144	2	0.01	0.18	1.7	0
J 152	4	0.01	0.26	2.1	0
J 154	2	0.01	0.22	1.5	0
J 155	214	0.36	1.98	71.7	36
Isolates from Federal District					
H 12	175	0.14	2.15	100.4	55
H 14	167	0.14	2.42	84.9	53
H 15	247	0.25	2.78	69.9	45
H 20	370	0.32	3.58	132.2	40
H 48	105	0.18	2.15	77.2	55
H 49	130	0.17	2.14	89.1	49
H 51	168	0.16	2.25	93.2	52
H 52	101	0.12	1.96	80.2	50
H 53	171	0.16	2.20	90.1	50
H 54	179	0.17	1.98	82.8	51
H 57	112	0.16	1.92	77.2	47
Strains used as comparison					
CIAT 899	114	0.15	2.05	80.4	–
PFR 81	137	0.17	2.25	92.0	55
Non-inoculated controls					
Control − N	0	0	0.15	1.6	–
Control + N	0	0	2.91	90.2	–
CV (%)	51.4	45.2	15.1	16.2	41
LSD (5%, Tukey)	175	0.18	1.40	14.9	13.8

[a] Plants were harvested 5 weeks after emergence and data represent the mean of three replicates.

Table 2
Nodulation and nitrogen fixation capacity under greenhouse conditions when bean cultivar Negro Argel was singly inoculated with rhizobial strains from the Brazilian Cerrados, as well as with *R. tropici* strains CIAT 899 and PRF 81; also nodule occupancy by each strain when co-inoculated with CIAT 899[a]

Isolates	Nodulation		Shoot		Nodule occupancy (%)
	Number (No./plant)	Dry weight (g/plant)	Dry weight (g/plant)	Total N (mg N/plant)	
Isolates from Goiás state					
J 1	23	0.04	0.35	6.5	25
J 2	47	0.02	0.28	4.1	20
J 3	4	0.01	0.32	1.7	0
J 4	24	0.04	0.37	4.5	23
J 5	4	0.01	0.29	1.4	0
J 28	200	0.15	1.68	59.4	44
J 41	185	0.20	2.26	66.9	47
J 65	165	0.12	1.45	46.5	30
J 71	214	0.08	0.76	16.3	32
J 76	4	0.01	0.32	1.9	0
J 86	170	0.16	2.03	57.6	25
J 94	141	0.10	0.72	16.0	24
J 95	196	0.21	1.32	39.2	28
J 96	282	0.21	0.80	12.2	38
J 97	180	0.13	0.97	14.4	35
J 112	170	0.08	0.70	10.7	28
J 116	5	0.01	0.34	1.9	0
J 124	162	0.08	0.60	5.3	32
J 137	35	0.07	0.59	9.2	20
J 140	204	0.14	1.23	39.6	25
J 142	170	0.08	0.47	12.2	22
J 144	4	0.01	0.36	1.8	0
J 152	56	0.03	0.59	5.5	28
J 154	4	0.01	0.31	1.4	0
J 155	206	0.14	0.95	21.3	32
Isolates from Federal District					
H 12	220	0.11	2.08	72.2	51
H 14	210	0.13	1.62	60.1	48
H 15	220	0.15	1.23	48.1	50
H 20	196	0.14	1.99	77.8	45
H 48	137	0.14	1.71	65.8	45
H 49	142	0.16	1.98	74.0	53
H 51	188	0.17	2.29	85.6	40
H 52	189	0.15	1.80	71.4	48
H 53	176	0.14	2.16	80.3	51
H 54	192	0.16	2.24	82.8	50
H 57	186	0.25	2.63	100.2	52
Strains used as comparison					
CIAT 899	171	0.12	1.72	62.4	–
PFR 81	206	0.16	1.89	74.1	50
Controls					
Control − N	0	0	0.12	1.4	–
Control + N	0	0	2.71	80.1	–
CV (%)	39.6	42.1	14.31	14.9	35
LSD (5%, Tukey)	130	0.12	1.24	12.8	12.8

[a] Plants were harvested 5 weeks after emergence and data represent the mean of three replicates.

out in an MJ Research PT 100 thermocycler and amplified fragments were separated by horizontal electrophoresis on a 1.5% agarose (low EEO, type I-A) gel ($17 \times 18\,cm^2$) at 100 V for 6 h.

Cluster analyses were performed with the PCR products with the Bionumerics program (Applied Mathematics, Kortrijk, Belgium), using the algorithm UPGMA (unweighted pair-group method, with arithmetic mean) and the coefficient of Jaccard (*J*).

2.6.2. Restriction fragment length polymorphism (RFLP) analysis of PCR-amplified 16S rDNA genes

Five replicate quantities of total DNA were amplified by PCR with primers Y1 and Y3, which amplify almost the full-length of the region corresponding to the 16S rRNA as described before (Chen et al., 2001). All strains produced a single PCR product with the expected MW. The PCR products were then digested with five restriction endonucleases: *Cfo*I, *Msp*I, *Rsa*I, *Nde*II and *Hin*fI. For each enzyme, a mixture was prepared containing: 6 μl of the PCR product; 1 μl of the specific buffer for each enzyme (10×); 0.5 μl of the enzyme (5 U/reaction) and 2.5 μl of sterile milli-Q water. For *Nde*II, the mixture contained 1 μl of enzyme, 1 μl of DTT (10 mM), 6 μl of the PCR product, 1 μl of buffer and 1 μl of water. The mixtures were incubated overnight in the water bath at 37 °C. The fragments obtained were analyzed by horizontal electrophoresis in a gel ($17 \times 11\,cm^2$) with 3% of agarose, and carried out at 100 V for 4 h. Cluster analysis was performed as described in the previous item.

2.6.3. 16S rDNA sequence determination

The five efficient and competitive strains used in the field experiment were submitted to direct sequencing of PCR fragments obtained by the amplification with primers Y1 and Y3 and intermediate primers as previously described (Chen et al., 2001), and the sequences were determined in an ABI 377 (PE-Applied Biosystems) sequencer analyzer. The generated rDNA sequences were confirmed forward and backward.

2.6.4. GenBank accession numbers and phylogenetic analysis

The 16S rDNA sequences generated were submitted to the GenBank database (http://www.ncbi.nlm.nih.gov/blast) to seek significant 16S rRNA alignments. The sequences were then aligned pairwise and compared to those of the following organisms (accession No. of the GenBank data library in parentheses): *Rhizobium* sp. OR 191 (X91211); *R. galegae* HAMBI 540^T (Y12355); *Rhizobium* genomic species Q strain BDV 5102 (Z94806); *R. leguminosarum* bv. phaseoli ATCC 8002 (M55494); *R. tropici* IIB CIAT 899^T (U89832); *R. tropici* IIA LMG 9518 (X67233); *R. etli* CFN 42^T (U28916); *R. giardinii* bv. giardinii $H152^T$ (U86344); *R. gallicum* bv. gallicum $R602^T$ (U86343); and *R. tropici* strains PRF 81 (AF260274), PRF 35 (AF260298) and PRF 54 (AF260275). A phylogeny tree was inferred with the UPGMA algorithm using the Bionumerics program.

3. Results and discussion

Of the 200 isolates obtained from effective field-grown bean nodules in the Brazilian Cerrados, 36 were selected for good symbiotic performance under optimal (27.5/21.3 °C, day/night) and high temperature (37 °C for 8 h of day/21 °C, day/night) conditions (data not shown). Tolerance to high temperature is a major characteristic to be considered in rhizobial selection program for tropical regions, as in the Brazilian Cerrados, since soil temperatures regularly exceed 40 °C, and often drastically decrease nodulation and N_2 fixation rates (Hungria and Franco, 1993; Hungria et al., 1993, 2000; Hungria and Vargas, 2000).

Selected strains were then evaluated for N_2 fixation capacity and strain competitiveness, under optimal temperature conditions (26.9/21.8 °C, day/night), in comparison with the two strains currently recommended for Brazilian commercial bean inoculants, CIAT 899 and PRF 81. The symbiotic performance was evaluated using two-bean host plants, previously reported as good N_2-fixing hosts (Hungria and Neves, 1987), but of different seed color. It is known that bean seeds contain a variety of flavonoids related to the seed color and are responsible for the rhizobial *nod*-gene inducing activity (Hungria et al., 1991; Hungria and Phillips, 1993); therefore, the evaluation with two host plants with different seed colors aimed to verify possible effects of distinct seed flavonoids. Good nodulation and N_2 fixation rates were verified with both cultivars (Tables 1 and 2) with no apparent effect of seed color on nodulation.

When cultivar Carioca was inoculated and plants were grown under greenhouse conditions, several isolates fixed as much as or more N than the two *R. tropici* strains officially recommended for use in commercial bean inoculants (Table 1). Inoculation with 11 different strains resulted in plant N accumulation of more than 80 mg N/plant (Table 1). All strains identified as highly efficient were also competitive, occupying from 42 to 55% of nodules when co-inoculated with strain CIAT 899. Ten strains were genetically unstable, losing nodulation and N_2-fixation capacity.

Similar results were obtained for the black-seeded cultivar Negro Argel (Table 2). Most of the effective strains, accumulating more than 60 mg N/plant, were from the Federal District, with only one strain from Goiás state. Again the efficient strains were also competitive, occupying 40–53% of the nodules. In both the experiments, plant growth and total N accumulated in tissues of beans inoculated with selected strains did not differ statistically from plants receiving mineral N (Tables 1 and 2), and five strains were selected for the field experiments, H 12, H 20, H 53, H 54 and H 57. Although selected from effective nodules of field-grown plants, several strains from Goiás state showed a very poor nodulation or lost effectiveness when used as inoculants under greenhouse conditions (Tables 1 and 2).

MPN counts for the field experiment site were carried out using plants grown at the same pH as the field soil. The area had not been cultivated with beans for 3 years, but still contained 10^4 bean rhizobia/g soil. Despite the high population of indigenous bean rhizobia, inoculation in the first year with five selected strains resulted in statistically significant increases in nodule dry weight and yield (Table 3). In this trial, the non-nodulating bean line yielded poorly due to the low N conditions, and yield in the non-inoculated plots containing indigenous rhizobia was lower than in plots inoculated with selected strains. Re-inoculation in the following year resulted in a further increase in nodulation and higher yields (Table 3). In each year, the yield of treatments inoculated with the strains from Cerrados was not statistically different from that of non-inoculated plots receiving mineral N. These results are similar to previous reports for Brazilian soils (Mendes et al., 1994; Peres et al., 1994; Hungria et al., 1997, 2000), in which inoculation of the bean crop can result in yield increases at low cost to the

Table 3
Effects of bean inoculation and re-inoculation on nodule number (NN) and nodule dry weight (NDW) at flowering (38–42 DAE) and grain yield

Treatment	Inoculation in the first year			Re-inoculation in the second year		
	NN (No./plant)	NDW (mg/plant)	Yield[a] (kg/ha)	NN (No./plant)	NDW (mg/plant)	Yield[a] (kg/ha)
H 12	51[b]	68	2344	72	82	2581
H 20	48	79	2322	65	77	2488
H 53	40	75	2054	60	89	2220
H 54	52	82	2328	64	84	2502
H 57	45	71	2100	76	93	2302
CIAT 899	40	58	2095	72	90	2070
PRF 81	51	62	2228	55	65	2350
Control − N[c]	35	32	1521	38	35	1612
Control + N[c]	8[d]	1[d]	2498	15[d]	3[d]	2600
Non-nodulating[e]	0[d]	0[d]	383	0[d]	0[d]	320
CV (%)	22	17	11	25	21	9
LSD (Duncan, 5%)	13.6	19.3	325	16.7	23.1	342

[a] Yield corrected to 13% of moisture.

[b] 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, $p \leq 0.05$).

[c] Non-inoculated control with or without N fertilizer (30 kg/ha of N as urea at sowing and 30 kg of N at 35 days after sowing).

[d] Non-nodulating bean line NORH 54.

[e] Values not considered for the statistical analyses.

farmer. It is important to emphasize that under cerrado conditions re-inoculation was needed in soils that had quite high indigenous population levels. This approach needs to be considered in other tropical regions of South and Central America and Africa where beans are grown.

Isolates from this study were also genetically characterized. REP and ERIC consensus sequences have been extensively used in ecology, genetic and taxonomic studies, as well as for rhizobia strain identification (e.g., de Bruijn, 1992; Judd et al., 1993; Selenska-Pobell et al., 1995; Laguerre et al., 1997; Hungria et al., 1998, 2000; Santos et al., 1999). Amplification of DNA of the 36 strains by PCR with the specific primers ERIC and REP resulted in several products per strain, except for strains H 51, H 52, J 65, J 96 with REP primer and J 1 and J 28 with ERIC primer (Fig. 1). Amplification of those strains using different DNA extractions and lysis procedures was not successful, as has been described before for some *Bradyrhizobium* strains (Judd et al., 1993). For the remaining strains, a high level of diversity was observed, since each strain showed a unique combination of PCR products (Fig. 1). Clusters obtained by the analysis of PCR products confirmed the observation of Laguerre et al. (1997) that the technique is useful for strain identification, but not for phylogenetic characterization.

There are five described bean rhizobial species today: *R. 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 bean show distinct phylogenic positions and may well represent other species (Bromfield and Barran, 1990; Eardly et al., 1992, 1995). The analysis by the RFLP-PCR of the 16S rRNA gene region has proved to be useful for rhizobial species designation, with a good agreement with the 16S rRNA genes (e.g., Laguerre et al., 1994, 1996, 1997), although some species are closely related, as *R. tropici* and *Agrobacterium* cannot be distingui shed (Laguerre et al., 1997). In this study, six different profiles of RFLP-PCR of the 16S rRNA gene were obtained (Table 4). All strains from Federal District and eight from Goiás state had similar profiles to *R. tropici* IIB CIAT 899. Five strains from Goiás state had similar profiles to *R. leguminosarum*, four others differed only by the restriction with *Cfo*I and two

Table 4
Patterns of RFLP-PCR detected for the bean rhizobial strains with five restriction enzymes

Isolates	Restriction enzymes				
	*Cfo*I	*Msp*I	*Rsa*I	*Nde*II	*Hin*fI
Isolates from Goiás state					
J 1	A	A	A	A	A
J 2	A	A	A	A	A
J 3	F	F	F	F	F
J 4	D	D	D	D	D
J 5	F	F	F	F	F
J 28	A	A	A	A	A
J 41	D	D	D	D	D
J 65	A	A	A	A	A
J 71	F	D	D	D	D
J 76	F	F	F	F	D
J 86	D	D	D	D	D
J 94	F	D	D	D	D
J 95	D	D	D	D	D
J 96	F	D	D	D	D
J 97	A	A	A	A	A
J 112	A	A	A	A	A
J 116	F	D	D	D	D
J 124	F	D	D	D	D
J 137	F	F	F	F	D
J 140	A	A	A	A	A
J 142	D	D	D	D	E
J 144	D	D	D	D	E
J 152	F	F	F	F	D
J 154	F	F	F	F	D
J 155	A	A	A	A	A
Isolates from Federal District					
H 12	A	A	A	A	A
H 14	A	A	A	A	A
H 15	A	A	A	A	A
H 20	A	A	A	A	A
H 48	A	A	A	A	A
H 49	A	A	A	A	A
H 51	A	A	A	A	A
H 52	A	A	A	A	A
H 53	A	A	A	A	A
H 54	A	A	A	A	A
H 57	A	A	A	A	A
Strains used as comparison					
CIAT 899^T	A	A	A	A	A
PFR 81	A	A	A	A	A
CFN 299	B	C	C	C	C
U S D A 2671	F	D	D	D	D
CFN 42^T	B	E	E	E	E
R602^T	F	B	B	B	B
H152^T	F	F	F	F	F

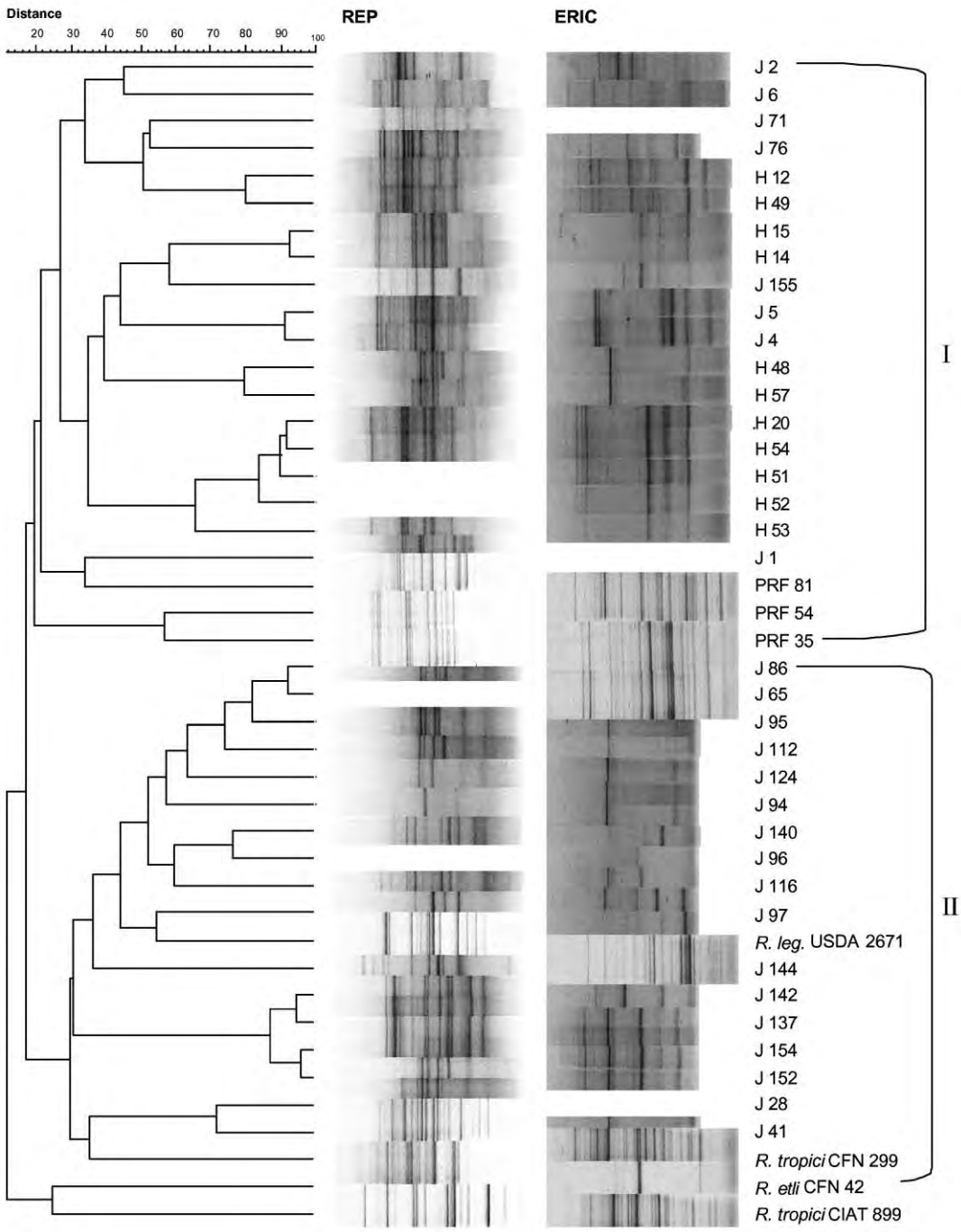

Fig. 1. Dendrogram of REP–ERIC-PCR products clustered with the UPGMA method and the Jaccard coefficient of 37 bean rhizobial strains from the Brazilian Cerrados and of reference strains of three bean rhizobial species.

others with *Cfo*I and *Hin*fI (Table 4); most of those strains showed a poor symbiotic performance (Tables 1 and 2). Two strains had identical profiles to *R. giardinii*, while four others differed only by the profile obtained with *Hin*fI (Table 4); that species is described as Fix$^-$ (Amarger et al., 1997), and the strains identified in this study were also ineffective (Tables 1 and 2). It appears therefore that we recovered three bean rhizobial species we tested among the Cerrados isolates.

A survey of bean rhizobia biodiversity was not the purpose of this study, therefore the genetic analysis aimed to relate symbiotic effectiveness of selected strains with rhizobial species. Qualitative evaluations of field populations were not performed, however, a recent study with all nodules isolated from bean plants grown in the same field site indicated that despite the acid conditions, the highest percentage was of *R. etli* (M. Hungria, I.C. Mendes, N. Amarger, M. Megías, unpublished data). This species was not detected in our study, suggesting that indigenous *R. etli* may be of limited efficiency, and were not selected. The selection of *R. giardinii* from effective nodules but subsequent poor performance indicate that this species is not necessarily Fix$^-$, but can easily undergo mutation affecting the symbiotic plasmids.

The most effective strains tested under field conditions, H 12, H 20, H 53, H 54 and H 57, were also submitted to the direct sequencing of the 16S rDNA fragment. Strains H 53 and H 54 had identical sequences. Strain H 12 showed a 99% similarity (1391/1403 bp) with *R. tropici* strain IAM 14206 (accession No. D12798), while with strains H 20, H 54 and H 57 the similarity was of 98% (1386/1403, 1377/1396 and 1213/1227 bp, respectively) with the same strain. The five strains showed mixed properties of *R. tropici* IIA and IIB, as has been described before for other bean rhizobia strains isolated from Brazilian soils (Hungria et al., 2000). All of them were able to grown at pH 4.0 and under high temperature (40 °C), but H 53, H 54 and H57 were able to grow in LB, while H12 and H 20 were not (data not shown).

The phylogenetic tree built with those strains and strains representative of bean rhizobial species confirmed a higher relatedness with *R. tropici* species (Fig. 2). Strain H 53 showed high relatedness with

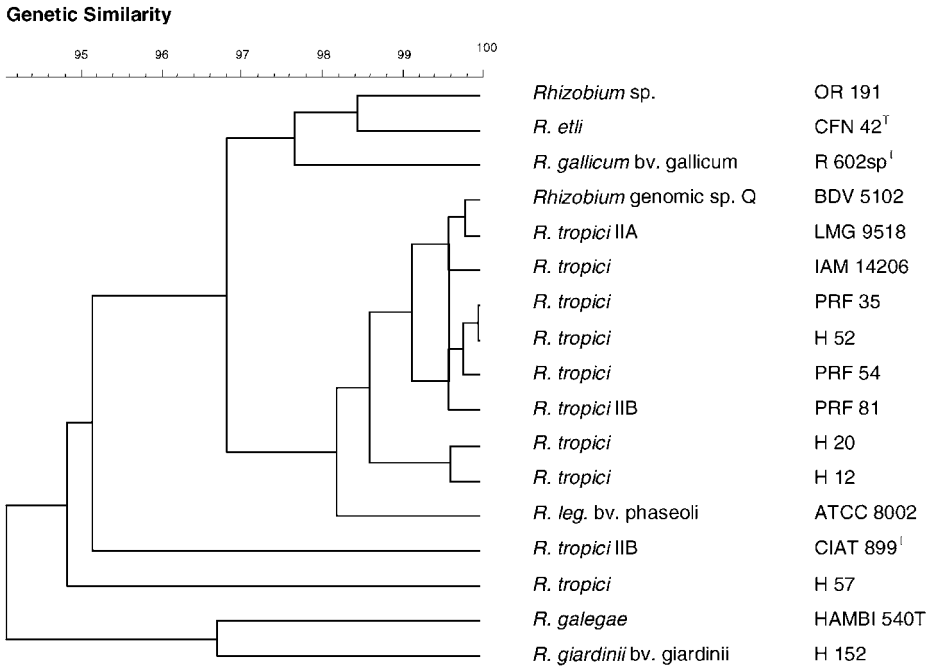

Fig. 2. Dendrogram built with the UPGMA algorithm with the aligned 16S rRNA sequences of five efficient and competitive bean rhizobial strains isolated from the Brazilian Cerrados and representative strains of bean rhizobial species. Accession No. of reference strains are listed in Section 2.

three other Brazilian strains isolated from Paraná state, one of them, PRF 81, today recommended for the use in commercial inoculants (Hungria et al., 2000); a lower genetic similarity was obtained with strain H 57. *R. tropici* seems to be native to tropical regions of South America (Martínez-Romero et al., 1991) and in the study of Mercante et al. (1998) represented most of a bean rhizobial population from the Cerrados Region that was trapped with *Leucaena* spp. *R. tropici* is known by a higher tolerance to high temperatures (Martínez-Romero et al., 1991; Hungria et al., 1993; Sá et al., 1993; Mercante et al., 1998; Hungria and Vargas, 2000), acid conditions (Graham et al., 1994), and a higher genetic stability than the other bean rhizobial species, maintaining its symbiotic properties under stressful conditions (Soberón-Chaves et al., 1986; Martínez-Romero et al., 1991; Hungria et al., 1993; Segovia et al., 1993; Hungria and Vargas, 2000). Those are important characteristics to be considered in commercial inoculants.

The most efficient and competitive strains from this study, also showing genetic stability, were classified as *R. tropici*, therefore adding evidence to the results of Hungria et al. (2000), that this species seems to be the most suitable for the recommendation for tropical regions, frequently submitted to environmental stressful conditions. Furthermore, the identification of efficient and competitive strains for the bean crop in the Cerrados region can also result in higher yields and help to reduce the soil N depletion.

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