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Juscélio Donizete Cardoso, Mariangela Hungria & Diva S. Andrade

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Polyphasic approach for the characterization of rhizobial symbionts effective in fixing N_2 with common bean (*Phaseolus vulgaris* L.)

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Abstract Common bean (*Phaseolus vulgaris* L.) is a legume that has been reported as highly promiscuous in nodulating with a variety of rhizobial strains, often with low effectiveness in fixing nitrogen. The aim of this work was to assess the symbiotic efficiency of rhizobial strains isolated from common bean seeds, nodules of *Arachis hypogaea*, *Mucuna pruriens*, and soils from various Brazilian agroecosystems, followed by the characterization of elite strains identified in the first screening. Forty-five elite strains were analyzed for symbiotic properties (nodulation, plant-growth, and nitrogen-fixation parameters) under greenhouse conditions in pots containing non-sterile soil, and variation in symbiotic performance was observed. Elite strains were also characterized in relation to morpho-physiological properties, genetic profiles of rep-polymerase chain reaction (PCR; BOX), and restriction fragment length polymorphism (RFLP)-PCR of the 16S rRNA. Sequence analyses of the 16S rRNA were obtained for 17 strains representative of the main groups resulting from all previous analyses. One of the most effective strains, IPR-*Pv* 2604, was clustered with *Rhizobium tropici*, whereas strain IPR-*Pv 583*, showing lower effectiveness in fixing N_2, was clustered with *Herbaspirillum lusitanum*. Surprisingly, effective strains were clustered with unusual symbiotic genera/species, including *Leifsonia xyli*, *Stenotrophomonas maltophilia*, *Burkholderia*, and *Enterobacter*. Some strains recognized in this study were outstanding in their nitrogen-fixing capacity and therefore, show high biotechnological potential for use in commercial inoculants.

Keywords Biological nitrogen fixation · BOX-PCR · RFLP-PCR · 16S rRNA · *Phaseolus vulgaris* · Principal component analysis (PCA)

J. D. Cardoso
Department of Microbiology, Universidade Estadual de Londrina,
Cx. Postal 60001,
86051-990 Londrina, Paraná, Brazil
e-mail: juscelio.cardoso@yahoo.com.br

J. D. Cardoso · M. Hungria
Embrapa Soja,
Cx. Postal 231,
CEP 86001-970 Londrina, Paraná, Brazil

M. Hungria
e-mail: hungria@cnpso.embrapa.br

M. Hungria
e-mail: hungria@pq.cnpq.br

J. D. Cardoso · D. S. Andrade
Instituto Agronômico do Paraná,
Cx. Postal 481,
CEP 86047-902 Londrina, Paraná, Brazil

D. S. Andrade (✉)
IAPAR,
Cx. Postal 481,
CEP 86047-902 Londrina, Paraná, Brazil
e-mail: diva@iapar.br

Introduction

Phaseolus vulgaris L., known as common bean, is widely cultivated in Central and South America and in many countries of Africa and Asia, representing an important source of protein for human consumption. The crop can greatly benefit from its nitrogen (N_2)-fixing symbiosis with soil-borne bacteria belonging mainly to the genus *Rhizobium*. However, an important limitation to the nitrogen supplied by the root nodules has been attributed to the highly promiscuous nature of the legume in nodulating with

a variety of bacteria, often not symbiotically effective (e.g., Hungria et al. 2000, 2003; Michiels et al. 1998).

The efficacy of rhizobial strains in nodulating and fixing atmospheric nitrogen (N_2) with common bean is influenced by both the host and the bacterium genotypes, and broad variability in symbiotic performance has been reported (Aguilar et al. 1998; Michiels et al. 1998; Moawad et al. 1998; Caballero-Mellado and Martinez-Romero 1999). Although, nowadays, Brazil is the main producer and consumer of common bean worldwide and the legume has been cultivated in all Brazilian ecosystems for centuries, very few reports have described diversity of common-bean rhizobia (Andrade et al. 2002; Grange and Hungria 2004; Giongo et al. 2007; Stocco et al. 2008; Mostasso et al. 2002; Grange et al. 2007).

In this work, rhizobial strains isolated from common bean seeds, nodules of *Arachis hypogaea*, *Mucuna pruriens*, and from soils of various agroecosystems in Brazil were characterized in terms of phenotypic, genotypic, and symbiotic properties. Novelty was found in relation to unusual symbiotic genera/species associated with the legume. In addition, elite strains with biotechnological potential for use as inoculants were identified, and approaches towards speeding strain-selection programs are discussed.

Materials and methods

Bacterial strains

Approximately 1,500 isolates from different agroecosystems were analyzed in this study. They have been isolated from common bean seeds, nodules of nodules of *A. hypogaea* L, *M. pruriens* Linn, and soils from several Brazilian ecosystems. Strains were evaluated for symbiotic effectiveness (nodulation and shoot dry weight and total N) when used as inoculants for common bean plants grown under controlled greenhouse conditions in 500-mL glass jars containing sterile N-free nutrient solution as substrate. Two replicates were taken per site, and plants were grown for 3 weeks, when nodules were detached proceeding to the isolation of bacteria (data not shown). Forty-five strains were selected out of the 1,500 isolates and were used in this study (listed in Table 1 and Fig. 1). As some of the strains were kept for over 20 years, before starting the experiment, they were checked for the capacity of nodulating common bean under axenic conditions (Vincent 1970), with growth in 1,000-mL flasks filled with paper and sterile nitrogen-free nutrient solution.

All strains are kept cryopreserved at −80°C and lyophilized at the "Collection of Microorganisms of Interest of Agribusiness of the Laboratory of Soil Microbiology", IAPAR, Londrina, PR, Brazil, and are designed as IPR- and according to original hosts of nodule isolates: *A. hypogaea*, *M. pruriens*, and *P. vulgaris*. Three reference strains SEMIA 4077 (CIAT 899), SEMIA 4088 (H 12) (kindly supplied by FEPAGRO, Brazil), and SEMIA 4080 (PRF 81) that are used as commercial inoculants in Brazil for common bean were included in all assays (Table 1). For working samples, strains were maintained in tubes containing yeast mannitol agar medium (YMA) (Vincent 1970) with Congo red (0.00125%), at 4±2°C.

Symbiotic efficiency in greenhouse soil pot experiment

Experiment conduction and harvest

The symbiotic efficiency of the 45 elite rhizobial strains was verified in one greenhouse experiment using as host plant common bean cultivar IPR Colibri, characterized by an early maturing group (65 days to complete growth cycle) and colored seeds. The experiment was carried out at the experimental station of IAPAR, Londrina, Paraná, southern Brazil. Plastic pots were filled with 3.5 kg of soil collected from the surface layer (0–20 cm) of a soil covered with natural vegetation composed by grasses, with no history of common bean cultivation. Soil chemical properties at sowing were determined after Pavan et al. (1992), and the results obtained were: soil $pH_{(CaCl2\ 0.01M)}$ 4.40; exchangeable aluminum 0.60 $cmol_c\ kg^{-1}$; exchangeable acidity 7.20 $cmol_c\ kg^{-1}$; Ca^{2+} (KCl 1 N) 1.20 $cmol_c\ kg^{-1}$; Mg^{2+} (KCl 1 N) 0.53 $cmol_c\ kg^{-1}$; K^+ (Mehlich-1) 0.07 in $cmol_c\ kg^{-1}$; 5.40 mg dm^{-3} for P (Mehlich) and 13.84 for total C in mg dm^{-3}; base saturation of 20%, and effective aluminum saturation of 25%. Before sowing, soil acidity was corrected with dolomitic limestone (29% CaO, 20% MgO) to reach 80% of saturation of bases. Each pot received 4.72 g of triple superphosphate, 1.33 g of sulfate of potassium (K_2SO_4), and 100 mL of a micronutrient solution contained 18.54 mg L^{-1} of H_3BO_3, 13.08 mg L^{-1} of $ZnSO_4.7H_2O$, 0.79 mg L^{-1} of $CuSO_4.5H_2O$, 9.23 mg L^{-1} of $MnSO_4.H2O$, 0.18 mg L^{-1} of $(NH_4)_6Mo7O_{24}.4H_2O$, and 24.2 mg L^{-1} of $FeCl_3.6H_2O$.

At sowing, the number of rhizobia in the soil was assessed by the most probable number method and the statistical table of Andrade and Hamakawa (1994). Inoculation treatments included the 45 elite strains (IPR-*A. hypogaea*, *M. pruriens*, and *P. vulgaris*) identified in this study, and the three commercial strains *of R. tropici* (Table 1 and Fig. 1). The experimental design was completely randomized with three replicates. Before sowing, seeds were surface-sterilized by immersion in alcohol (95%) for 1 min, followed by sodium hypochlorite (3%) for 3 min, and washed seven times with sterile distilled water. Three plants were grown per pot. For inoculants production bacteria were grown for 3 days at 28°C in YM broth (Vincent 1970) and equalized to a concentration

Table 1 Geographical origin, geographic coordinates, and land use of sampling sites and collection code of bacteria used in this study

Municipality/state/collector(s)/source (day/month/year collected)	Geographic coordinates	Climate/ altitude	Land use	IPR- code of bacteria[a]	Reference
Wenceslau Bráz (Sitio Flor da Serra)/PR; DS Andrade (28/03/1989)	23°52′27″ S 49°49′14″ W	Cfa/850 m	*Pv*	*Pv* 3087[b] *Pv* 263[c]; 348[c]; 445[b]; 446[b]; 453[b]; 497[d]; 506[b]; 515[d]	This study
Wenceslau Bráz/PR, DS. Andrade (22/02/2001)	23°52′26″ S 49°48′10″ W	Cfa/841 m	*Pv*	*Pv* 517[d]; 524[d]	This study
Prado Ferreira/PR; AD Campos (18/02/2000)	23°02′20″ S 51°26′31″ W	Cfa/651 m	*C. arabica*	*Mp* 195[d]; *Pv 1*045[c]; *Ah* 367[d]	This study
Palmital/PR; WM Kranz (19/03/1990)	24°53′34″ S 52°12′10″ W	Cfb/840 m	*Z. mays*	*Pv* 680[b]	This study
Centenário do Sul/PR; DS Andrade (30/05/1990)	22°49′15″ S 51°35′42″ W	Cfa/560 m	*Pv*	*Pv* 685[b]	This study
Cerro Azul/PR; WM Kranz (29/03/1990)	24°49′26″ S 49°15′39″ W	Cfb/318 m	*Pv*	*Pv* 1097[b]	This study
Pitanga (Capoeira velha)/PR; WM Kranz (19/03/1990)	24°55′35″ S 51°45′39″ W	Cfb/952 m	*Z. mays*	*Pv* 1249[b]	This study
Pitanga (Boaventura)/PR; WM Kranz (19/03/1990)	24°45′25″ S 51°45′39″ W	Cfb/952 m	*Z. mays*	*Pv* 1326[b]	This study
Irati/PR; DS Andrade 28/05/1992	25°28′01″ S 50°39′03″ W	Cfb/812 m	*Pv*	*Pv* 1362[c]	This study
Rio Branco do Sul/PR; WM Kranz (28/03/1990)	25°11′24″ S 49°18′50″ W	Cfb/960 m	*Z. mays*	*Pv* 1252[b]	This study
Pato Branco/PR; DS Andrade (07/04/1989)	26°13′44″ S 52°40′15″ W	Cfa/760 m	*Pv*	*Pv* 1281[b]	This study
Tibagi/PR; DS Andrade (26/01/04)	24°30′32″ S 50°24′50″ W	Cfb/740 m	*Pv*	*Pv* 2604[d]; 2608[d]	This study
Cruz Machado/PR; WM Kranz (03/04/1990)	26°01′01″ S 51°20′49″ W	Cfb/950 m	*M. esculenta*/ *Oryza sativa*	*Pv* 3085[b]	This study
Londrina/PR; DS Andrade (26/04/2001)			Seeds	*Pv* 591[e]; 598[e]; 611[e]; 615[e]; 696[e]; 889[e]; 2230[e]	This study
Serrinha/MG; DS Andrade (20/04/2001)			Seeds	*Pv* 509[e]; 580[e]; 589[e]; 593[e]; 652[e] 689[e] 692[e]; 809[e]	This study
São Paulo/SP; DS Andrade (20/04/2001)			Seeds	*Pv* 583[e]; 2231[e]; 2234[e]; 2248[e]; 2303[e]	This study
Colombia				SEMIA4077 (CIAT899)	*R. tropici*[f]
Irati/PR; DS Andrade (28/05/1992)	25°28′01″ S 50°39′03″ W	Cfb/812 m	*Z. mays*	SEMIA4080 (PRF81=IPR- *Pv* 81[c])	*R. tropici*[g]
Planaltina/Go				SEMIA 4088 (H-12)	*R. tropici*[h]

PR Paraná, *SP* São Paulo, *MG* Minas Gerais, *Go* Goiás

[a] IPR-code of bacteria from Culture Collection of Agronomic Institute of Parana, Londrina, Paraná, Brazil

[b] Nodule from plants growing in soil in pot

[c] Nodule from plants inoculated with soil suspension

[d] Nodule from field-grown legumes

[e] Nodule from plants inoculated with seeds suspension

[f] Martinez-Romero et al. (1991)

[g] Hungria et al. (2000)

[h] Mostasso et al. (2002)

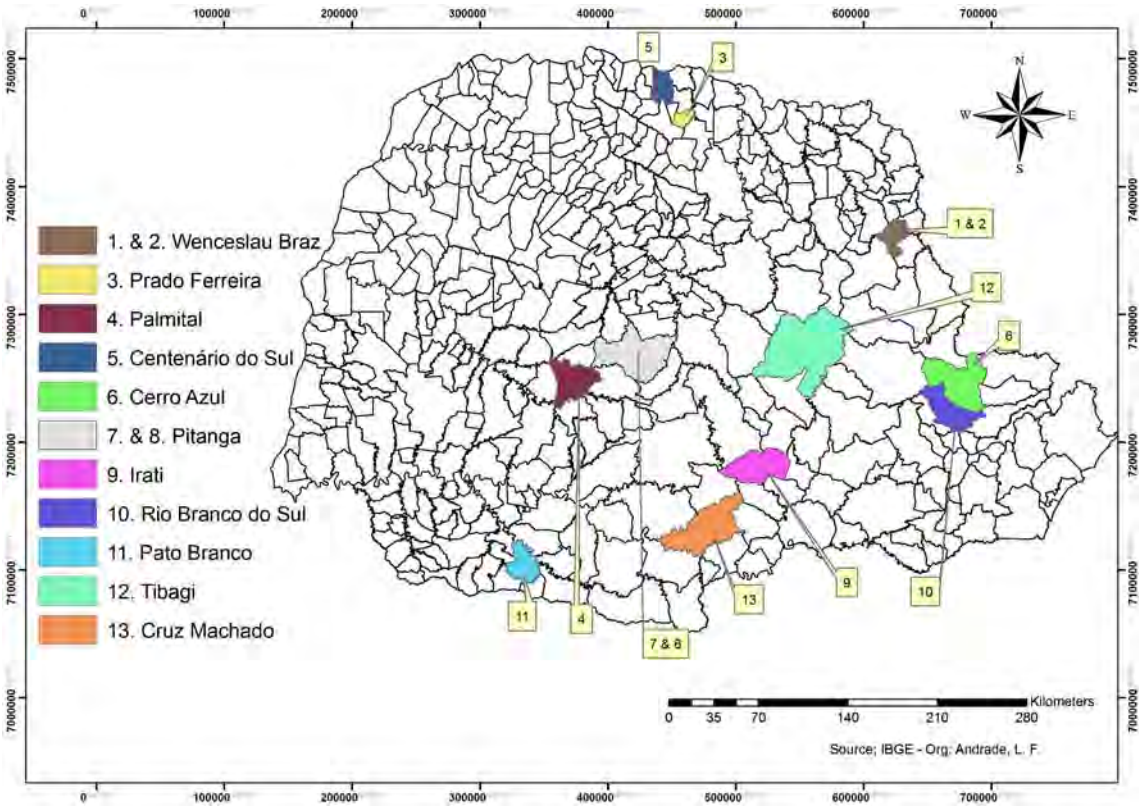

Fig. 1 Map of Paraná state (Brazil) showing the sampling sites indicated by *numbers* (For details see Table 1)

of 10^8 cells mL^{-1}. Surface sterilized seeds were immersed in the inoculants consisted of single strains. Two non-inoculated controls were included, without or with mineral N. Treatment with mineral N received 777 mg of N per pot containing three plants, split ten times, weekly, by using a solution of 2% NH_4NO_3.

During the experiment, soil moisture was kept around 60% to 70% of water hold capacity by irrigating daily with distilled sterile water. At 42 days after emergence (DAE)—flowering stage—plants were collected to evaluate the following parameters: shoot dry weight (leaves, stems, and petioles), nodulation (nodule number and dry weight), total N in shoots, total N, *N*-ureide (allantoin and allantoic acid), and *N*-nitrate in the xylem sap. Xylem sap was collected at 42 DAE from 6:00 AM to 8:00 AM. For sap collection, shoots were cut at the stem base close to the internodes, washed with sterile water, and then dried with sterile cotton. The sap, exuding from the three decapitated plants in each pot, was recovered with a sterile pipette after 10 to 15 min, bulked, immediately placed on ice, and then taken to the laboratory, where it was frozen till the analysis of total N, *N*-ureide, and *N*-nitrate.

Plant harvest and analyses

After sap collection, roots were removed, washed, and nodules were also removed. Plant material was placed in a forced-air dryer at 65°C till constant weight (approximately 72 h). Nodulation parameters evaluated included nodule number and dry weight.

Total N in shoots was evaluated in the dried (65 °C) and grinded tissues and in the xylem sap, by the Kjeldahl digestion method (Bremner and Keeney 1966). The determination of *N*-ureide in the xylem sap was based on the method of alkaline hydrolysis of allantoin and acid hydrolysis to determine the allantoic acid content (Herridge et al. 1990). Nitrate in the xylem sap was evaluated by the salicylic acid method as described elsewhere (Miyazawa et al. 1985). Percentage of *N*-ureide was calculated as described by (Herridge DF, Peoples MB 1990).

The relative efficiency index (Brockwell et al. 1966) that measures the accumulation of N fixed in relation to the controls was defined as REI=(T−C/N−C)×100, where T, C, and N = dry weight of shoots in each treatment, in control, and in nitrogen treatments, respectively.

Statistical analyses

Data obtained from the greenhouse experiment were subjected to the analysis of variance, and when *F* was significant, the Scott Knott test of means was applied with 5% of probability using the SAS statistical software (Statistical Analysis System), with the procedure "PRIN-

COMP" (SAS 1999). For the principal components analysis (PCA) the variables selected were based on the following data: shoot dry weight (grams per plant), total N in shoots (milligrams per plant), total N in the xylem sap (micrograms mole per milliliter), N-ureide (percent), dry weight of individual nodule (milligrams per nodule), and nodule dry weight per plant (milligrams per plant). From the data generated in the PCA the "PROC CLUSTER" and "COMPLETE", methods were applied to generate the dendrogram using the procedure "PROC TREE" from the matrix D2 of Euclidean distance (SAS 1999).

Morpho-physiological characterization

Colony morphology (size, shape, texture, gum production, elevation, borders, structure, brightness, and transparency) was assessed in bacteria grown in YMA medium containing Congo red (0.00125%) after 3 days of growth in the dark at 28°C. Acid/alkaline reaction was evaluated after 3 days of growth in YMA-containing bromothymol blue (0.00125%) as indicator, also after 3 days of growth in the dark at 28°C. Melanin production by strains was evaluated as previously described (Cubo et al. 1997). Briefly, each strain was grown for 6 days in plates containing tryptone agar medium supplemented with 600 μg mL^{-1} of L-tyrosine and 40 μg mL^{-1} of copper (III) sulphate. A solution of 10% (*w/v*) lauryl sulphate (SDS) sodium salt was sprayed over the rhizobia colonies, which were then kept at room temperature for at least 6 h. The data were record as absence (0) or presence (1) of melanin.

Data from morpho-physiological characterization were transformed into a binary matrix, and cluster analysis was performed by using NTSYS-pc (Numerical Taxonomic and Multivariate Analysis System, version 2.1, Exeter Software, USA).

Genetic characterization

DNA extraction

DNA of each strain was extracted according to the procedure of Kaschuk et al. (2006). Integrity of the DNA was confirmed by electrophoresis in 0.8% agarose gels, for 50 min, at 80 V, using as a comparison Low DNA Mass™ Ladder (Invitrogen™, Life Technologies). Gels were stained with ethidium bromide and visualized under UV light. Before all polymerase chain reaction (PCR) analyses, the extracted DNA was quantified using microspectrophotometer (Nanodrop® ND-1000 Spectrophotometer, Uniscience), with readings at a wavelength of 280 and 260 nm. When necessary, the DNA was diluted to perform PCR.

PCR fingerprinting with specific BOX A1R primer

The DNA of each bacterium was amplified by PCR with the BOX A1R (5′-CTACGGCAAGGCGACGCTGACG-3′, Invitrogen™, Life Technologies) primer (Versalovic et al. 1994). The PCR reaction and cycles were performed according to Kaschuk et al. (2006). The reactions were carried out in an MJ Research Inc. PTC-100TM thermocycler and amplified fragments were separated by horizontal electrophoresis on a 1.5% agarose gel at 100 V, for 6 h. Gels were stained with ethidium bromide, visualized under UV light, and photographed with a Kodak Digital Science 120 apparatus.

Cluster analysis was performed with the BOX-PCR-amplified products using the Bionumerics program (Applied Mathematics, Kortrijk, v.4.6), with the UPGMA algorithm (unweighted pair-group method with arithmetic mean) and the coefficient of Jaccard (*J*) (Sneath and Sokal 1973).

Restriction fragment length polymorphism analysis of PCR-amplified 16S rDNA genes

The DNA of each bacterium was amplified with primers fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rD1 (5′-AAGGAGGTGATCCAGCC-3′) (Weisburg et al. 1991). PCR products were digested with the restriction endonucleases *Msp*I (=*Hpa*II) (5′-C/CGG -3′), *Hin*fI (5′–G/ANTC–3′), *Rsa*I (5′–GT/AC–3′), and *Hae*III (5′–GG/CC–3′) (Invitrogen™, Life Technologies), following the manufacturer's specifications. Fragments obtained were analyzed by horizontal electrophoresis on a 3% agarose gel, at 120 V for 4 h and then photographed.

Sequence analysis of the DNA region coding for the 16S rRNA gene

For direct sequencing of the 16S rRNA gene, 17 strains were selected, representative of the main restriction fragment length polymorphism (RFLP)-PCR groups and of the cluster analysis based on the PCA of symbiotic characteristics. These strains were deposited at the "Collection of Rhizobium of the FEPAGRO", Porto Alegre, RS, Brazil, and are designed as SEMIA (Table 5). The DNA of each bacterium was amplified with the universal primers fD1, and rD1 and the sequencing was performed with internal primers, as described by Menna et al. (2006), in an ABI 3031xl (Applied Biosystems) sequencing analyzer.

The sequences obtained for each strain were assembled into contigs using the programs phred (Ewing et al. 1998; Ewing and Green 1998), phrap (www.phrap.org), and Consed (Gordon et al. 1998) and were submitted to the

Table 2 Variables associated with biological N_2 fixation with common bean cultivar IPR Colibri obtained in the experiment performed under greenhouse conditions in pots containing soil

IPR-	Shoot dry weight, g plant^{-1}	Shoot N, mg plant[1]	Total N (TN) of xylem sap, μg mol mL^{-1}	N-ureide [a], %	Nodule number[b]	Total nodule, mg dry weight plant^{-1}	Individual nodule, mg dry weight plant^{-1}	Relative efficiency index (REI)	
								TN	Nod
Mp 195	4.26 A	106 A	115.5 A	63.6 B	602.4 B	564 C	0.9384 B	20	200
Pv 263	6.01 A	145 B	66.8 A	82.7 C	485.6 B	406 C	0.8696 B	54	140
Pv 348	5.40 A	110 A	110.4 A	68.1 B	393.2 B	428 C	1.1724 B	23	148
Ah 367	7.51 B	198 B	94.9 A	62.3 B	664.9 B	494 C	0.7920 A	100	173
Pv 445	7.52 B	156 B	60.3A	74.9 C	497.6 B	433 C	0.8903 B	63	150
Pv 446	5.82 A	125 A	227.8 A	63.9 B	655.9 B	526 C	0.8080 A	37	185
Pv 453	4.87 A	111 A	115.9 A	73.2 C	454.9 B	443 C	0.9728 B	24	154
Pv 497	5.20 A	108 A	112.0 A	74.7 C	652.3 B	499 C	0.7655 A	22	175
Pv 506	4.81 A	107 A	88.7 A	78.0 C	465.2 B	465 C	1.0546 B	21	162
Pv 509	5.38 A	75 A	157.8 A	33.4 A	608.9 B	355 C	0.5848 A	−7	120
Pv 515	5.91 A	140 B	70.8 A	59.2 B	281.7 A	196 A	0.5126 A	50	60
Pv 517	4.53 A	115 A	136.1 A	61.3 B	342.0 A	235 B	0.6317 A	28	75
Pv 524	5.74 A	138 B	137.4 A	71.2 C	417.9 B	475 C	1.1330 B	48	166
Pv 580	7.02 B	162 B	60.0 A	79.2 C	229.2 A	146 A	0.5960 A	69	41
Pv 583	4.36 A	89 A	95.4 A	81.3 C	206.1 A	162 A	0.5086 A	5	47
Pv 589	6.75 B	110 A	89.6 A	74.1 C	501.4 B	375 C	0.7568 A	23	128
Pv 591	5.51 A	130 A	107.1 A	86.8 C	512.6 B	488 C	0.9779 B	41	171
Pv 593	6.57 B	175 B	85.8 A	73.4 C	343.4 A	243 B	0.7697 A	80	78
Pv 598	5.46 A	118 A	93.7 A	69.5 C	365.3 A	291 B	0.8214 A	30	96
Pv 611	5.49 A	119 A	188.0 A	78.9 C	423.4 B	428 C	1.0597 B	31	148
Pv 615	7.44 B	126 A	105.0 A	77.5 C	485.7 B	291 B	0.5897 A	37	96
Pv 652	6.81 B	169 B	50.0 A	77.2 C	593.2 B	458 C	0.8115 A	75	159
Pv 680	5.93 A	136 A	113.2 A	70.4 C	435.4 B	493 C	1.1656 B	46	173
Pv 685	5.46 A	123 A	163.2 A	57.9 B	452.8 B	468 C	1.0535 B	35	163
Pv 689	5.53 A	96 A	93.7 A	65.0 B	406.4 B	304 B	0.7480 A	11	101
Pv 692	7.10 B	103 A	96.1 A	63.1 B	589.5 B	404 C	0.6891 A	17	139
Pv 696	4.81 A	92 A	82.5 A	72.8 C	440.6 B	468 C	1.2564 B	8	163
Pv 809	5.05 A	89 A	102.6 A	68.0 B	412.3 B	425 C	1.0383 B	5	147
Pv 889	5.24 A	103 A	115.9 A	71.4 C	488.6 B	477 C	1.0649 B	17	167
Pv 1045	6.02 A	110 A	105.3A	72.8 C	561.6 B	520 C	0.9254 B	23	183
Pv 1097	5.70 A	134 A	115.9 A	67.9 B	330.4 A	246 B	0.7226 A	44	79
Pv 1249	5.67 A	111 A	109.2 A	65.8 B	518.1 B	507 C	0.9798 B	24	178
Pv 1252	4.90 A	120 A	101.3 A	64.6 B	537.0 B	484 C	0.9236 B	32	169
Pv 1281	5.69 A	128 A	127.5 A	63.0 B	478.6 B	472 C	1.0347 B	39	165
Pv 1326	7.13 B	169 B	87.1 A	74.4 C	495.8 B	472 C	1.0730 B	75	165
Pv 1362	4.69 A	103 A	119.7 A	56.6 B	543.4 B	500 C	0.9866 B	17	175
Pv 2230	4.80 A	95 A	138.9 A	71.7 C	424.8 B	344 B	0.8190 A	10	116
Pv 2231	5.02 A	114 A	152.5 A	65.8 B	492.9 B	372 C	0.7551 A	27	127
Pv 2234	5.92 A	115 A	125.8 A	72.5 C	403.4 B	416 C	1.0296 B	28	143
Pv 2248	5.94 A	118 A	131.8 A	54.3 B	459.2 B	366 C	0.7769 A	30	124
Pv 2303	4.63 A	87 A	101.2 A	78.5 C	491.0 B	416 C	0.8465 B	3	143
Pv 2604	4.21 A	62 A	110.9 A	57.7 B	417.8 B	379 C	0.9073 B	−18	129
Pv 2608	7.34 B	172 B	163.0 A	61.0 B	546.5 B	263 B	0.4809 A	77	85
Pv 3085	5.81 A	126 A	107.7 A	73.2 C	594.0 B	529 C	0.8891 B	37	186
Pv 3087	7.36 B	164 B	77.7 A	77.3 C	451.1 B	325 B	0.7163 A	70	109

Table 2 (continued)

IPR-	Shoot dry weight, g plant^{-1}	Shoot N, mg plant[1]	Total N (TN) of xylem sap, μgmolmL^{-1}	N-ureide [a], %	Nodule number[b]	Total nodule, mg dry weight plant^{-1}	Individual nodule, mg dry weight plant^{-1}	Relative efficiency index (REI)	
								TN	Nod
4077	7.60 B	131 A	102.6 A	77.5 C	673.3 B	302 B	0.4477 A	42	100
4080	6.68 B	114 A	99.4 A	75.6 C	545.3 B	365 C	0.6617 A	27	124
4088	7.44 B	99 A	98.3 A	66.4 B	749.8 B	303 B	0.4234 A	14	100
Control	5.53 A	83 A	83.2 A	63.2 B	208.0 A	39 A	0.2675 A	0	0
Nitrogen	11.23 C	198 B	99.9 A	57.0 B	510.7 B	202 A	0.4498 A	100	62
Pr > F	<0.0001	0.0003	0.0006	<0.0001	<0.0001	<0.0001	0.0002		
CV, %	16.65	28.17	33.98	9.26	14.37	25.70	30.71		

[a] Data transformed to arcsine and [b] square root, respectively. Means followed by the same letter are not statistically different at 5% level according to Scott Knott multiple range test. Relative efficiency index (REI)=(T−C/N−C)X 100, where *T*, *C*, and N = dry weight of shoots in each treatment, in control, and in nitrogen treatment, respectively

GenBank database (http://www.ncbi.nlm.nih.gov/blast) to seek for significant alignments.

The sequences obtained in this study and from the GenBank (Table 5) were aligned with the ClustalX version 1.83 (Thompson et al. 1997). The phylogenetic tree was generated using the MEGA program version 5 (Kumar et al. 2004) with the neighbor-joining algorithm (Saitou and Nei 1987). Statistical support for the tree nodes was evaluated by bootstrap analyses (Felsenstein 1985) with 2,000 samplings (Hedges 1992).

Results

Symbiotic efficiency

In the greenhouse experiment performed in pots containing soil, shoot dry weight ranged from 4.21 to 7.52 g plant^{-1}, referring to inoculation with strains IPR-*Pv* 2604 and *Pv*−445, respectively. Total N in shoots also was significantly affected by strains, ranging from 62 to 198 mg plant^{-1}. Based on shoot total N, two main groups were observed, one consisting of 11 very effective strains, accumulating from 138 to 198 mg N plant^{-1} and without statistical difference from the treatment receiving mineral N. The second group contained significantly less total N than the plants supplied with mineral N. The fraction of N-ureide ranged from 33% to 87% in inoculated plants, with the highest and lowest values obtained with strains IPR-*Pv* 509 and *Pv*−591, respectively (Table 2).

The indigenous population of rhizobia was estimated at 10^6 CFU (grams per dry soil), even though the soil had no history of common bean cultivation. Therefore, non-inoculated treatments plants were nodulated, and the ureide-N was estimated at 57% and 63% in the control treatments with and without mineral N, respectively. In general, common bean has small nodules; however, in some of the inoculated treatments, individual nodules were heavier than 1 mg of dry weight nodule^{-1}. The relative symbiotic efficiency index based on total N in the shoot and nodule mass revealed that some IPR- strains had high efficiencies, for example, IPR-*Ah* 367 and IPR-*Mp* 195, respectively, whereas others, such as strain IPI-*Pv* 2604, were poor N_2 fixers (Table 2).

Table 3 Pearson's correlation coefficients (N=50) among N_2-fixation variables and select multivariate variables

	Dry shoot, g	Shoot N, mg	Total N of xylem	N-ureide, %	Total nodule, mg	Individual nodule, mg	PC1 (Y1)	PC2 (Y2)	PC3 (Y3)
Dry shoot, g	1.000	0.718**	−0.187 ns	−0.178 ns	−0.269 ns	−0.423*	−0.784**	0.462**	0.199 ns
Shoot N, mg		1.000	−0.187 ns	−0.046 ns	−0.072 ns	−0.111 ns	−0.577**	0.728**	0.190 ns
Total N of xylem			1.000	0.266 ns	0.184 ns	0.079 ns	0.401*	−0.146 ns	0.682**
N-ureide, %				1.000	0.159 ns	0.191 ns	0.389*	0.094 ns	0.698**
Total nodule, mg					1.000	0.780**	0.720**	0.575**	−0.135 ns
Individual nodule, mg						1.000	0.772**	0.503**	−0.235 ns

PC1 (principal component axes 1, 2, and 3)

*p<0.001; **p<0.0001. Number of nodule was not included because of outliers

Six components of plant variables related to N_2 fixation were subjected to a multivariate analysis: shoot dry mass, total N in shoots, total N in the xylem of sap, percent of ureide-N, dry weight of individual nodules, and total nodule dry weight per plant. The association between multivariate analysis techniques (PCA) and cluster analysis was used to assist the selection of efficient strains, a procedure recommended when more than four variables are analyzed. Auto-values of the correlation matrix were chosen based on three principal components, explaining 81% of the variables. The auto-values were of 2.38 for PC1, 1.35 for PC2, and 1.10 for PC3, and the proportion of variance was of 40%, 22%, and 18%, respectively. The percentage of accumulated variance was of 40% for the first principal component (PC1), 62% for the second (PC2), and 81% for the third (PC3).

The coefficients of Pearson correlation and their probabilities are presented in Table 3. The variables of shoot dry

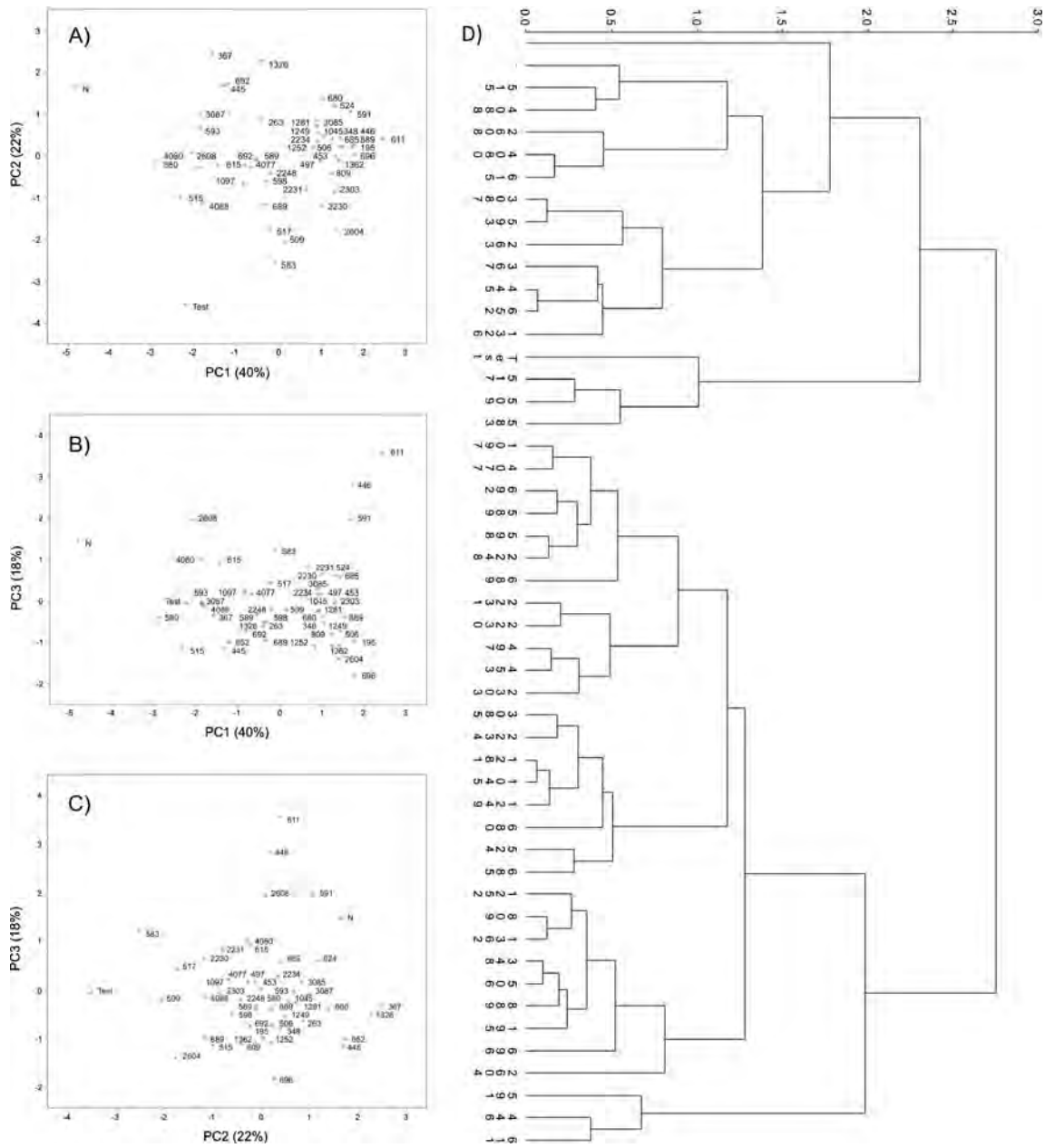

Fig. 2 Dispersion of rhizobial strains according to principal component analysis (PCA): **a** first and second; **b** first and third; and **c** second and third components. **d** Dendrogram showing clustering of the IPR-elite strains of and non-inoculated treatments (with (N) and without mineral N (test)) with PCA based on N_2-fixation variables (shoot dry weight, total N in shoot, total N, and %N-ureide in the xylem sap, dry weight of nodules, and individual nodule dry weight)

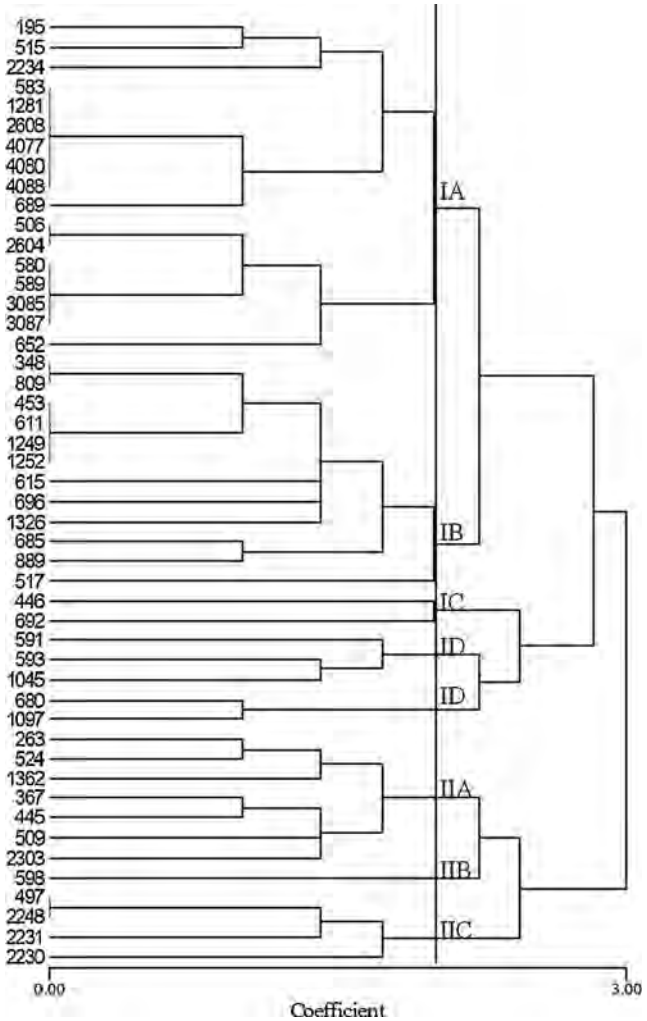

Fig. 3 Cluster analysis of 48 common-bean rhizobial strains isolated from nodules of field-grown plants, inoculated with soil or seed suspensions. Dendrogram was created based on binary matrix and Euclidean distance using 12 phenotypic traits

weight (r=−0.784, p<0.0001**), nodule dry weight (r=0.720, p<0.0001**), and dry weight of individual nodules (r=0.772, p<0.0001**) were highly correlated with the first component. On the other hand, the variable representing the total N in shoots (milligrams per plant) correlated significantly with the second component (r=0.782, p<0.001*), whereas the variables related to total N in the xylem sap (r=0.682, p<0.001*) and to the% of *N*-ureide-*N* (r=0.698, p<0.001*) showed significant correlation with the third component.

Figure 2a shows that strains IPR-*Pv* 583, 591, and 611 formed a group separate from the other treatments, with positive values for both the first (shoot dry weight and nodulation) and second component (PC2) (shoot N) indicators. When compared with the other treatments, mineral N treatment occupied an isolated position.

Table 4 Rhizobial phenotypic traits and profiles of RFLP-PCR of the 16S rRNA with the restriction enzymes *Rsa*I, *Hin*fI, *Hpa*II, *Hae*III

	Phenotype clusters							
	1A[a]	1B	1C	1D	1E	2A	2B	2C
Number of strains	17	12	2	3	2	7	1	4
Colony size (A)	>2 mm	>2 mm	>2 mm	>2 mm	>2 mm	<2 mm	>2 mm	>2 mm
Shape (B)	Circular	Circular	Not circular	Circular	Circular	Circular	Not circular	Circular
Texture (C)	Not viscous	Viscous	Not viscous	Not viscous	Viscous	Viscous	Viscous	Viscous
Absorbing dye (D)	Yes	Not	Yes	Yes	Yes	Yes	Yes	Yes
Gum production (E)	Yes	Yes	Yes	Not	Yes	Not	Not	Not
Colony elevation (F)	Convex	Convex	Flat	Convex	Convex	Flat	Convex	Flat
Colony borders (G)	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Structure (H)	Smooth	Smooth	Smooth	Corrugated	Corrugated	Smooth	Smooth	Corrugated
Brightness (I)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Transparency (J)	Opaque	Translucent	Opaque	Translucent	Translucent	Opaque	Translucent	Translucent
Acid/alkaline (L)	Acid	Acid	Acid	Acid	Acid	Acid	Acid	Acid
Melanin (M)	Mel$^-$	Mel$^-$	Mel$^-$	Mel$^-$	Mel$^-$	Mel$^-$	Mel$^+$	Mel$^+$
Genotype RFLP clusters	II; III; IV; V; VI; VII	I; II; III; IV; V; VI	II; VII	III; IV; VI	I; II	II; III; IV; IX	II	IV; VII

Evaluations were performed with three replicates

[a] Comprise the strains of *R. tropici* (SEMIAs 4077, 4080, and 4088) recommended for commercial inoculants in Brazil, except that these present texture viscous

Figure 2b shows that strains IPR-*Pv* 591 and 611, although showing high values in Y1 (first principal component), were not high on the axis Y3 (third component). On the scatter plot of the treatments, according to the third and second components (Y3= nitrogen fixation and Y2=plant growth), the strain IPR-*Ah* 367 stood out from the others, while strains IPR-*Pv* 652, 593, and 580 were in an intermediate position. The IPR-*Pv* 2604 strain occupied an isolated position, with a negative index for relative symbiotic efficiency (−18) and, accordingly, lower values for total N in shoots and shoot dry weight (Fig. 2c).

The cluster analysis split the strains in two main groups. Thirty-one IPR- strains, in addition to the commercial strain SEMIA 4080, originally isolated from a soil of Paraná state, fit into the first group. The second group was composed by 14 IPR- strains, in addition to the two remaining SEMIAs (4077 and 4088) and by the non-inoculated treatments, with and without mineral N (Fig. 2d).

Morpho-physiological characterization

When strains were grown in YMA, the predominant colony morphological characteristics were: larger than 2 mm, circular, non-viscous, with gum production, convex in elevation, flat, dull in transparency, and opaque. Similar features were observed in colonies of the reference *R. tropici* SEMIAs 4077, 4080, and 4088. Thirty-eight of the strains had an acid reaction in culture medium containing mannitol as C source, whereas the remainder had an alkaline reaction. Of the 45 strains analyzed, 14 produced melanin.

The cluster analysis based on the matrix and Euclidean distance using 20 phenotypic traits has clearly

Fig. 4 Cluster analysis (UPGMA with the coefficient of Jaccard index and 10% of tolerance in the program Bionumerics) of the PCR products obtained by BOX-PCR analysis of 45 IPR- strains and SEMIA4077 (CIAT899), 4080 (IPR *P. vulgaris* 81) e−4088 (H20)

shown the differentiation of the strains into phenetic clusters (Fig. 3 and Table 4).

BOXA1R-PCR genomic fingerprinting

When the forty-five strains in this study and the reference strains of *R. tropici* were amplified with the primer BOX1AR, two major clusters were obtained, with a very low final level of similarity (53%). Cluster I comprised eight strains and cluster II 37 strains, in addition to the three reference SEMIAs. Overall, 21 distinct BOX-PCR profiles were observed, but considering the similarity of 70% in the clustering analysis using the UPGMA algorithm and the Jaccard coefficient as in previous studies by our group, e.g., Kaschuk et al. (2006); Grange and Hungria (2004); and Stocco et al. (2008), the strains fit into four different clusters (Fig. 4).

Profiles of RFLP-PCR of the 16S rDNA region

In the analyses of RFLP-PCR of the 16S rDNA with four restriction enzymes, nine groups were observed, three of them comprising strains with unique profiles, for example, clusters V, VIII, and IX with IPR-*Pv* 3087, 445, and 524, respectively (Fig. 5). Strains in cluster I, comprising IPR-*Pv* 517, 348, and 680 isolated from nodules from field-grown plants, plants inoculated with soil suspension, and nodules from pot-grown plants, respectively. In cluster II, similar profiles were observed for 11 strains, which included IPR-*Mp* 195; *Ah* 367, and *Pv* 453, the first and the second isolated from nodules of *Mp* and *Ah* grown in the inter-rows of coffee (*Coffea arabica* L.) crop, and the third one from an area traditionally cropped to common bean. Cluster III included the reference strain of *R. tropici* (SEMIA

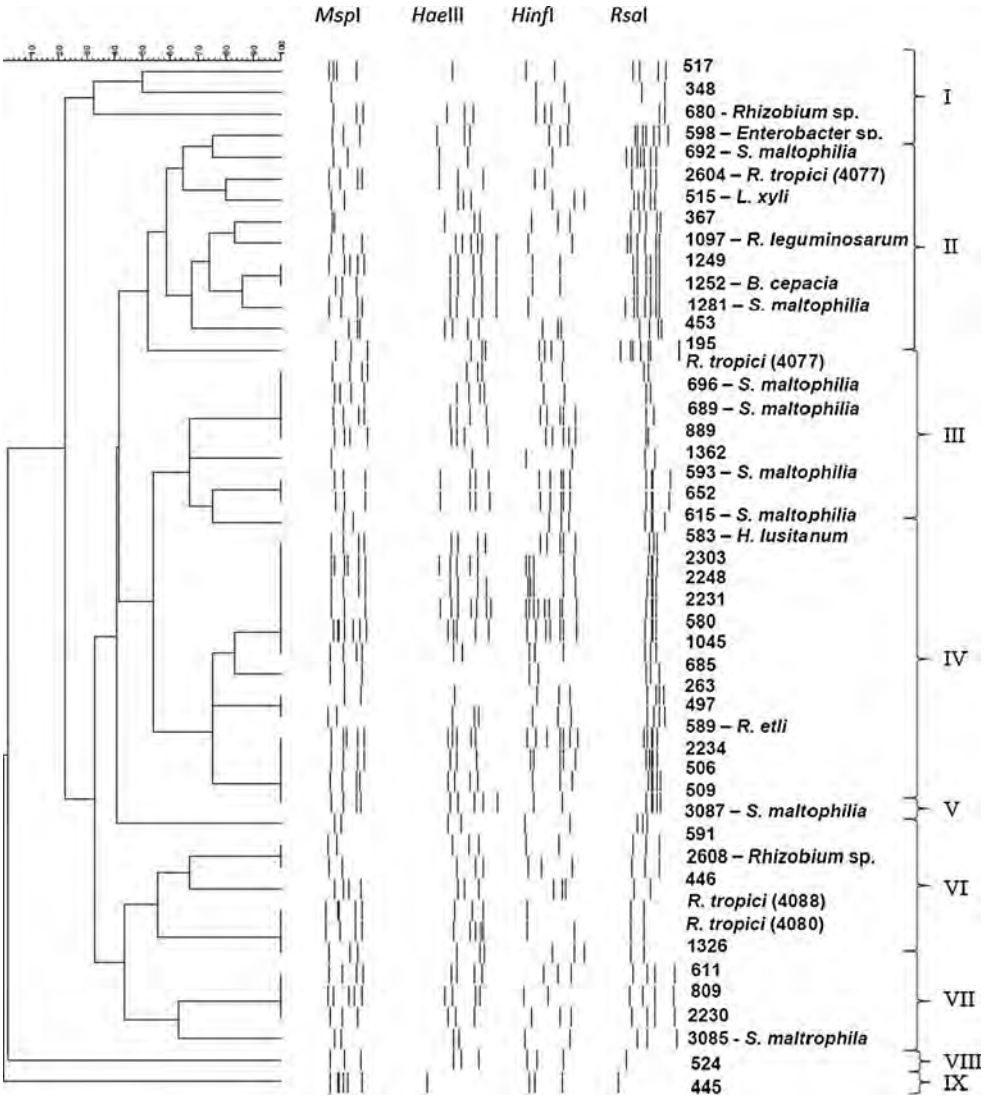

Fig. 5 Dendrogram showing the genetic diversity of 45 IPR-rhizobial strains based on profiles of RFLP-PCR of the 16S rRNA region analysis with the restriction enzymes *Rsa*I, *Hin*fI, *Hpa*II, *Hae*III. For cluster analysis UPGMA with the coefficient of Jaccard were used. The strains, SEMIA 4,077 (CIAT 899), 4080 (PRF 81), and 4088 (H20), recommended for commercial production of inoculants in Brazil, were included

4077, =CIAT 899) and seven IPR-*Pv* strains. Cluster IV was the largest group with 13 strains, only five (IPR-*Pv* 263, 497, 506, 1045, and 685) of which were isolated from nodules of common bean in Paraná State and the remaining from seeds harvested in the states of São Paulo and Minas Gerais. In this cluster, the strain IPR-*Pv* 583 isolated from seeds suspensions of similarity with IPR-*Pv* 2303, 2248, 2231, and 580 that were also isolated from seeds harvested in Minas Gerais and São Paulo and with strain −1045, isolated from a nodule of common bean plant inoculated with soil suspension from inter-row of coffee tree (Fig. 5).

Phylogenetic analysis based on 16S rRNA genes

Seventeen strains were sequenced for the 16S rRNA (Fig. 6 and Table 5). Considering the Blastn, five strains had highest similarity to the genus *Rhizobium*, eight to *Stenotrophomonas*, one to *Burkholderia* and *Leifsonia*, and one to *Herbaspirillum* and *Enterobacter* (Table 5). Of these 17 strains, IPR-*Pv* 589, 680, and 1097 had 100% similarity with *Rhizobium etli* (CIAT151), *Rhizobium* sp. (SEMIA 6435), and *Rhizobium leguminosarum* (RVS11), respectively. Strains IPR-*Pv* 692 and 696 showed similarity lower than 90% with the genus *Stenotrophomonas*. In terms of phenotypic characteristics (Fig. 3 and Table 4), strain IPR-*Pv* 1281 was clustered with the three SEMIAs and with five IPR-*Pv* strains in the genus *Rhizobium*.

Thirteen sequences with more than 1,385 bp were aligned and used to build the phylogenetic tree along with the reference strains, and major groups were observed and clustered at the genus level (Fig. 6).

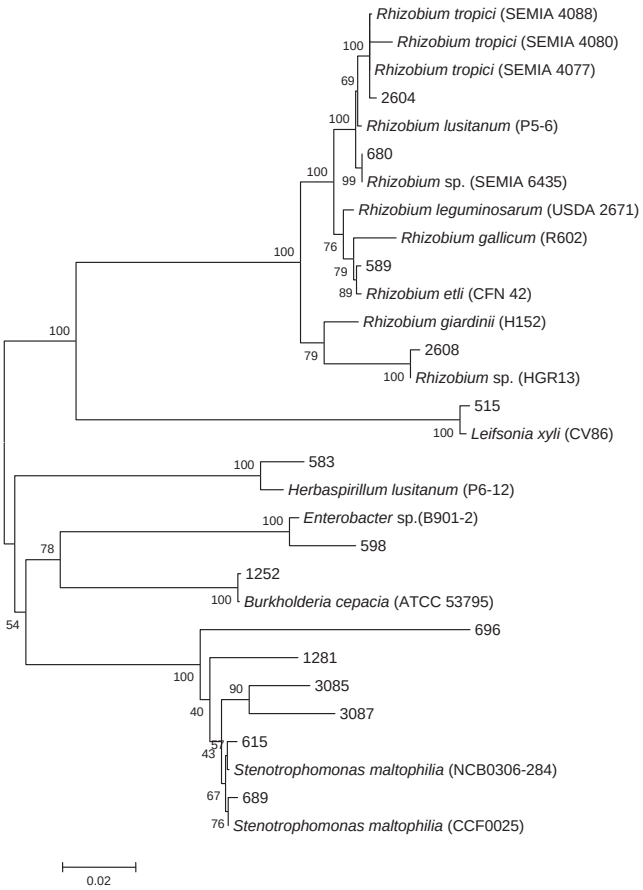

Fig. 6 Phylogenetic tree based on the 16S rRNA sequences of 14 strains (IPR-*Pv*). GeneBank accession numbers are available in Table 5. Phylogenetic trees were generated using MEGA version 5.0 with default parameters, K2P distance model, and the neighbor-joining algorithm

Discussion

Principal component analysis has been broadly used in several areas of research, and, in our study, its application to the data related to N_2-fixation parameters has been clearly effective in reducing the number of variables needed to evaluate the efficiency of the biological process. For example, it was important to realize that, for the common-bean symbiosis, the parameter of nodule number is not relevant for the selection of strains with higher N_2-fixing capacity.

The majority of the sites in our study had no history of common-bean inoculation, and many also had no history of common-bean cropping. For example, strains IPR-*Pv* 3085 and 1249 were isolated from soils cultivated with cassava (*Manihot esculenta* Crantz) and maize (*Zea mays* L.), respectively. Isolation of common-bean rhizobia from these soils could be attributed to the high promiscuity of the legume in nodulating with several rhizobial species, apparently resulting from the capacity of the host plant to perceive a variety of rhizobial molecular signals (Michiels et al. 1998). Unfortunately, in general, the promiscuous nodulation of common bean is not related to N_2-fixation efficiency, representing the main limitation to the agronomic exploitation of the symbiosis (e.g., Graham 1981; Hungria and Vargas 2000). However, within indigenous rhizobial populations, elite strains can be identified, and this approach has been successfully used in Brazil (Hungria et al. 2000, 2003). Also in our study, the relative efficiency index estimated taking into account the total N in the shoots revealed several elite strains with great biotechnological potential, such as IPR-*Ah* 367, surprisingly isolated from field-grown *Ah* L., which accumulated high amounts of N, comparable to the control receiving mineral N when inoculated in common bean.

The majority of the strains in our study fit into the long-recognized classification of fast-growers with acid

Table 5 Identification of strains based on the similarity of 16S rRNA sequences using sequences available in the GenBank (NCBI)

Strain (IPR-*Pv*)	SEMIA	Number of base pair	Most similar sequence found in GenBank (NCBI)			Accession number in GenBank of the strain
			Species	Similarity, %	Accession number	
515	4090	1385	*L. xyli* (CV86)	99	AJ717351.1	JN208893
583	4091	1391	*H. lusitanum* (P6-12)	99	NR028859.1	JN208894
589	4092	1425	*R. etli* (CIAT151)	100	AF313904.1	JN208895
593	4093	661	*S. maltophilia* (YHYJ-1)	94	FJ765513.1	JN208896
598	4094	1546	*Enterobacter* sp. (B901-2)	96	AB114268.1	JN208897
615	4095	1537	*S. maltophilia* (K279a)	99	AM743169.1	JN208898
680	4096	1417	*Rhizobium* sp. (SEMIA 6435)	100	FJ025130.1	JN208899
689	4097	1539	*S. maltophilia* (K279a)	99	AM743169.1	JN208900
692	4098	999	*S. maltophilia* (CCF0025)	89	GU391033.1	JN208901
696	4099	1526	*S. maltophilia* (ZZ7)	90	DQ113454.1	JN208902
1097	4100	950	*R. leguminosarum* (RVS11)	100	FJ595998.1	JN208903
1252	4101	1403	*Burkholderia cepacia* (ATCC 53795)	99	AY741354.1	JN208904
1281	4102	1524	*S. maltophilia* (IAM 12423)	96	AB294553.1	JN208905
2604	4103	1475	*R. tropici* (SEMIA 4088)	99	FJ025131.1	JN208906
2608	4104	1442	*Rhizobium* sp. (R-32539)	99	AM691584.1	JN208907
3085	4105	1533	*S. maltophilia* (pp5c)	96	GQ360071.1	JN208908
3087	4106	1475	*S. maltophilia* (PSM-1)	94	FJ888386.1	JN208909

reaction in medium containing mannitol as carbon source (Jordan 1984; Andrade et al. 2002). Melanin production by rhizobia-nodulating common bean has been described before (Cubo et al. 1997; Andrade et al. 2002); the polymer has been suggested by Geng et al. (2010) as a possible "DNA-sensing" checkpoint that protects the cell from propagation until the DNA damage is repaired prior to duplication, a feature that would be very important under tropical stress conditions. Interestingly, eight out of 14 melanin producers observed in our study (IPR-*Pv* 509, 580, 589, 598, 652, 2231, 2234, 2248) were isolated from common bean seeds under osmotic-stress conditions.

Both BOX-PCR and the RFLP-PCR of the 16S rRNA have been effectively used to determine rhizobial diversity (Laguerre et al. 1994; Kaschuk et al. 2006; Grange and Hungria 2004; Binde et al. 2009) and in our study revealed high diversity among the strains. Though valuable, BOX-PCR and RFLP-PCR of the 16S rRNA cannot determine the precise phylogenetic or taxonomic positions of the strains, therefore, sequencing analysis of the 16S rRNA of selected strains was performed.

The sequencing analysis of 17 IPR-*Pv* strains indicated genera in addition to the traditional *Rhizobium* species. *Herbaspirillum lusitanum* (Valverde et al. 2003) has been reported as a symbiont of common bean, and, in our study, strains belonging to this genus were identified. *Burkholderia* and *Enterobacter* have been described as bacteria with potential for both biological N_2 fixation and plant-growth promotion (James 2000; Dôbereiner 1992; Baldani et al. 1986); however, this is the first report of isolation of these genera from common-bean nodules. Strain IPR-*Pv* 515 was identified as *Leifsonia xyli*, a species reported to cause ratoon stunting of sugarcane (*Saccharum* spp.) (Gagliardi and Camargo 2009).

The identification of eight strains belonging to *Stenotrophomonas* in symbiosis with common bean was surprising. This genus has been found to be an endophyte of sugar cane and wheat (*Triticum aestivum* L.) and was related to a better growth of sunflowers under greenhouse conditions (Fages and Arsac 1991). It was also described as a growth promoter of maize, producing indole acetic acid in the presence of tryptophan and antifungal metabolites to several soil-borne pathogens (Mehnaz et al. 2010). *Stenotrophomonas maltophilia* is found in a broad range of environments and is usually referred as an agent of nosocomial infections, representing an opportunistic pathogen of humans. Isolates of *S. maltophilia* display intrinsic resistance to many commonly prescribed antimicrobials, particularly β-lactams and aminoglycosides and can develop broad-spectrum resistance to several drugs that have been used to treat infections (Denton and Kerr 1998). The species is also found as a plant-growth-promoting bacterium in the rhizosphere of several plant species (Hauben et al. 1999). The ability of *Stenotrophomonas* to fix atmospheric N_2 has been suggested by the acetylene reduction

assay and by the detection of *nifH* gene sequences (Reinhardt et al. 2008; Teixeira et al. 2007). However, the strains isolated in our study showed low similarity (less than 90%) to other *Stenotrophomonas* from the GenBank database and, in the phylogenetic tree, were clustered in a different group, therefore they might represent a new species. Noteworthy were the high values of percent ureide-N achieved with these strains when in symbiosis with common bean, closer to or greater than those with the SEMIAs used as commercial inoculants in Brazil.

In conclusion, remarkably high efficiency in N_2 fixation and broad diversity were found among elite isolates from common bean in our study. The strains compose an interesting collection with prospects for biotechnological potential and probable identification of new species.

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