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Characterization of rhizobia isolates obtained from nodules of wild genotypes of common bean

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ABSTRACT

This study aimed to evaluate the tolerance to salinity and temperature, the genetic diversity and the symbiotic efficiency of rhizobia isolates obtained from wild genotypes of common bean cultivated in soil samples from the States of Goiás, Minas Gerais and Paraná. The isolates were subjected to different NaCl concentrations (0%, 1%, 2%, 4% and 6%) at different temperatures (28 °C, 33 °C, 38 °C, 43 °C and 48 °C). Genotypic characterization was performed based on BOX-PCR, REP-PCR markers and 16S rRNA sequencing. An evaluation of symbiotic efficiency was carried out under greenhouse conditions in autoclaved Leonard jars. Among 98 isolates about 45% of them and *Rhizobium freirei* PRF81 showed a high tolerance to temperature, while 24 isolates and *Rhizobium tropici* CIAT899 were able to use all of the carbon sources studied. Clustering analysis based on the ability to use carbon sources and on the tolerance to salinity and temperature grouped 49 isolates, *R. tropici* CIAT899 and *R. tropici* H12 with a similarity level of 76%. Based on genotypic characterization, 65% of the isolates showed an approximately 66% similarity with *R. tropici* CIAT899 and *R. tropici* H12. About 20% of the isolates showed symbiotic efficiency similar to or better than the best *Rhizobium* reference strain (*R. tropici* CIAT899). Phylogenetic analysis of the 16S rRNA revealed that two efficient isolates (ALSG5A1 and JPrG6A8) belong to the group of strains used as commercial inoculant for common bean in Brazil and must be assayed in field experiments.

Introduction

The common bean (*Phaseolus vulgaris* L.) is a leguminous plant of worldwide social and economic importance, providing most of the daily requirements of protein and carbohydrates for the poorest populations of South and Central America, Africa and India.[1] With respect to international agriculture, Brazil is the world's third largest producer of common bean, accounting for 12.7% of worldwide production.[2] In Brazil, the common bean is cultivated on a total area of 3.1 million hectares with a total grain production of approximately 2.8 million tons,[3] for which high amounts of nitrogen (N) are required.

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Despite its abundance in the atmosphere, N is scarce in tropical soils due to the fast mineralization of organic matter in tropical conditions. Although the decomposition of organic matter is an important source of N for crops, the adequate supply of N to crops depends largely on the use of nitrogen fertilizers.[4] However, biological nitrogen fixation (BNF) is considered a more sustainable approach for supplying N to the production system.

BNF is a key process for the conversion of nitrogen gas (N_2) into ammonia (NH_3) performed by bacteria belonging to the group of rhizobia. The reduction reaction of N_2 to NH_3 is carried out by N-fixing bacteria or diazotrophic microorganisms containing the enzymatic complex in which nitrogenase takes part.[5] Among N-fixing bacteria of the rhizobia group, a variety of *Rhizobium* and *Ensifer* species is able to colonize and establish a symbiotic partnership with common bean.[6,7]

To improve BNF efficiency, more efficient rhizobia strains are needed. Many isolating works have been performed using soil from different sites; however, as trap plant usually is used a commercial variety of common bean. The strategy used in our work was to collect soil in different sites and to use wild genotypes of common bean as trap plant looking for a better exploration of the rhizobial community, since wild genotypes show a broader genetic base. This work aimed to characterize and determine the symbiotic efficiency of rhizobia isolates obtained from the root nodules of wild genotypes of common bean.

Materials and methods

Bacterial strains and rhizobia isolates

The isolates evaluated in this work were obtained by Sampaio FB[8] and are available at the Collection of Microorganisms and Multifunctional Fungi of Embrapa Rice and Beans. Strains of *Rhizobium tropici* (CIAT899 and H12), *Rhizobium freirei* (PRF81) were used as reference strains in all analyses and, *Rhizobium etli* bv. *phaseoli* (CFN42) used in the BOX- and REP-PCR analyses.

Carbon source use (CSU) and tolerance to salinity and temperature (TST) assays

CSU was assayed for 98 isolates and for the *R. tropici* reference strains. Bacteria were kept for growth on modified YMA (Yeast Mannitol Agar) culture medium, without mannitol, added with individual carbon sources sucrose, glucose, malic acid, maleic acid, nicotinic acid, inositol, sorbitol, arabinose, fructose and glycerol. After incubation at 28 °C, bacterial growth was verified from 48 to 96 h at each 24 h.

The same isolates and reference strains were assessed for TST on YMA culture medium on a factorial (5 × 5) arrangement (concentrations of NaCl – 0%, 1%, 2%, 4%, 6% and temperature – 28 °C, 33 °C, 38 °C, 43 °C, 48 °C) incubated for a period of 48 h.

Genotypic characterization based on molecular markers

Based on CSU and TST, 55 isolates were selected for genotypic characterization. Genomic DNA was extracted according to Ausubel et al.[9] DNA quantity was estimated by spectrophotometry (NanoDrop®, Thermo scientific, Wilmington, USA), and DNA concentration was adjusted to 50 ng μL^{-1} for all samples. BOX-PCR was performed using the primer BOX A1R (5′-CTACGGCAAGGCGACG-3′), while REP-PCR was performed using the primers REP-1 (5′-IIIICGICGICATCIGGC-3′) and REP-2 (5′-ICGICTTATCIGGCCTAC-3′) according to Versalovic et al.[10]

The BOX-PCR reaction was performed in a final volume of 15 μL, containing 2.9 μL of milli-Q water, 7.5 μL of 2× QIAGEN Multiplex PCR Master Mix (3 mM Mg^{2+}), 1.6 μL of primer BOX A1R (50 pmol μL^{-1}) and 3 μL of DNA template (50 ng μL^{-1}). The REP-PCR reaction was performed in a final volume of 15 μL, containing 2.0 μL of milli-Q water, 7.5 μL of 2× QIAGEN Multiplex PCR Master Mix (3 mM Mg^{2+}), 1.25 μL of each primer REP 1 and REP 2 (10 pmol μL^{-1}) and 3 μL of DNA template (10 ng μL^{-1}).

The amplification program was designed according to Kaschuk et al.[11] PCR amplification consisted of an initial denaturing step (95 °C; 7 min); followed by 35 cycles of denaturation (94 °C; 1 min), annealing (55 °C; 1 min for BOX-PCR and 40 °C; 1 min for REP-PCR) and extension (65 °C; 8 min); followed by a final extension cycle (65 °C; 15 min). The PCR program was performed in a thermocycler Biocycler® (Applied Biosystems).

PCR products were subjected to electrophoresis on an agarose gel 1% (50 V; 7 h) in TAE buffer 0.75×[12] using 1 kb DNA Ladder® (Norgen) as a DNA band position marker. The agarose gel was stained with SYBR® green (Life Technologies) and visualized with a MultiDoc-it® system.

Symbiotic efficiency under greenhouse conditions

Based on genotypic characterization, 30 isolates were selected to evaluate their symbiotic efficiency. In addition to the isolates, the treatments were composed of two *R. tropici* strains (CIAT899 and H12), one *R. freirei* strain (PRF81), two nitrogen fertilized treatments (NT1 = 60 and NT2 = 120 kg ha^{-1} of N) and one control treatment (CT – without inoculation and without N).

Seeds of common bean cv. Pérola were sown in autoclaved Leonard jars in a random block design with three replicates. At five days after emergence (DAE), plantlets were inoculated with a cell suspension containing 1×10^9 cell mL^{-1} of each isolate and reference strain. Once a week, 200 mL of nutritive solution without N were added.[13] To the nitrogen fertilized treatments NT1 and NT2, 1 and 2 mL, respectively, of a solution containing 106.68 mg mL^{-1} of urea were added.

Plants were harvested at 35 DAE. Roots were carefully washed, dried in a paper towel, and the nodules were detached and counted to determine the number of nodules (NN). The leaves were detached from shoots to determine the leaf area (LA) using a leaf area meter LI-COR model 3100. Shoots and nodules were dried (65 °C; 72 h) to determine the shoot dry mass (SDM) and nodule dry mass (NDM). Subsequently, to determine SDM, shoot of plants were milled to determine the total N (N-Total) using the Kjedahl method, as described by Silva and Queiroz.[14]

16S rRNA sequencing analysis

Based on the symbiotic efficiency five isolates (ALSG5A1, JPrG1A1, JPrG6A8, JPrG8A7 and PCG4A2) were selected for

16S rRNA sequencing. The DNA of the isolates was obtained according to Laranjo et al.[15] The 16S rRNA region was amplified by PCR reaction using the primers Y1 and Y3.[15,16] The amplicons were purified and used on the sequencing reaction according to Laranjo et al.[15] Sequences coding for the partial 16S rRNA genes of the isolates ALSG5A1, JPrG1A1, JPrG6A8, JPrG8A7 and PCG4A2 were obtained and when submitted to the GenBank database (www.ncbi.nlm.nih.gov) received the accession numbers KU598665, KU598663, KU598662, KU598664 and KU598661, respectively.

Statistical analyses

CSU, TST and genotypic characterization data were transformed into a binary matrix. The binary matrices were used for the construction of a similarity matrix using the Jaccard coefficient. The UPGMA (Unweighted pair-group method) was applied to transform the similarity matrix into a similarity dendrogram using NTSYSpc® software.[17]

Data obtained from the greenhouse experiment were subjected to analysis of variance; when *F* was significant, the Scott Knott test of means was applied with a 5% probability using SISVAR statistical software.[18] Pearson correlation analyses were also performed for NDM and SDM, LA and N-Total using R statistical software.[19]

For the 16S rRNA-based phylogeny, the sequences obtained were submitted to NCBI BLAST against a non-redundant nucleotide database for getting homologous sequences.[20] Sequences showing degree of similarity were aligned using the CLUSTAL W program.[21] The evolutionary history was inferred using the Maximum-likelihood method,[22] with tree consensus being inferred from 500 replicates using bootstrap.[23] The evolutionary distances were computed using the Maximum Composite Likelihood method proposed by Tamura et al.[24] All positions containing gaps and missing data were eliminated. There were a total of 989 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.[25]

Results

CSU and TST clustering analysis

CSU evaluation revealed the distribution of 98 isolates among 28 groups, while for TST the isolates were distributed into 27 groups. Among the reference strains, *R. tropici* CIAT899 grew in all carbon sources, showing the same reference of CSU observed to the 24 isolates (Table S1). Regarding TST, *R. freirei* PRF81 showed greater tolerance among the reference strains; however, 18 isolates were able to grow under more stringent conditions as compared to *R. freirei* PRF81 (Table S2).

Similarity analysis based on the CSU and TST data resulted in similarities among isolates varying from 60% to 100%, and five physiological groups (PG) were formed considering a similarity level of 75% (Fig. 1). PG1 comprises the reference strain *R. freirei* PRF81 and three isolates, representing 3% of the total isolates. The reference strains *R. tropici* CIAT899 and *R. tropici* H12 clustered with 49 isolates on PG4, representing 48.5% of the evaluated isolates. Moreover, 38, 7 and 1 isolates formed PG2, PG3 and PG5 clusters, respectively (Fig. 1).

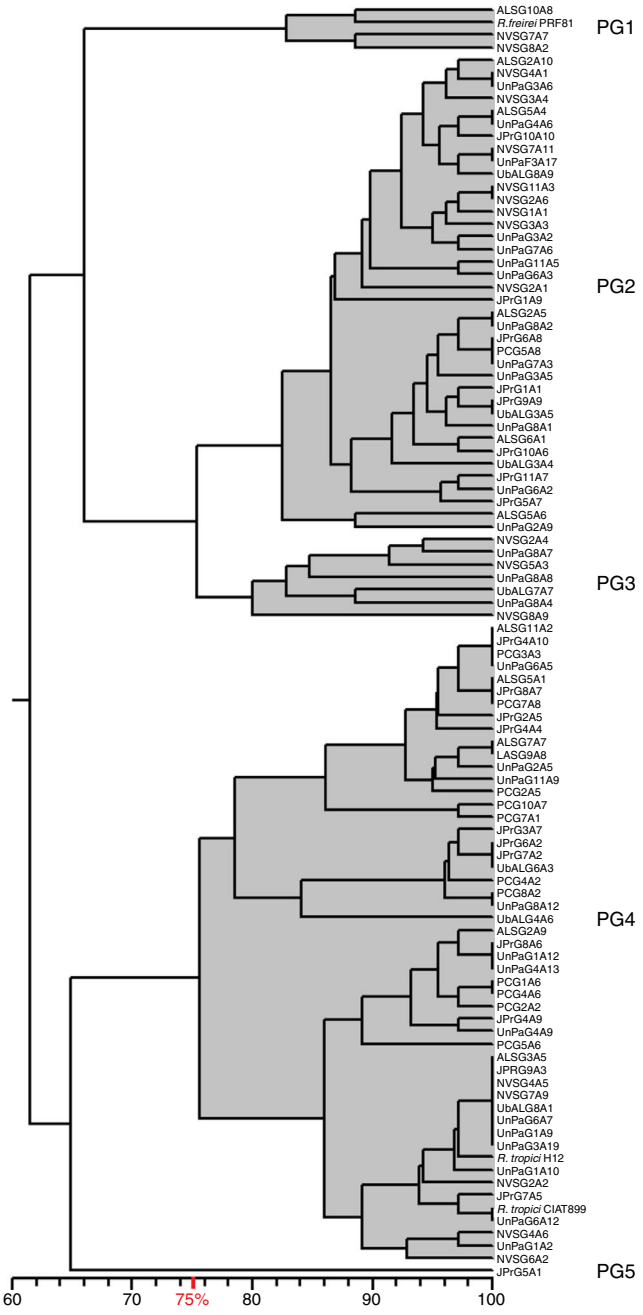

Fig. 1 – Consensus dendrogram obtained by combining CSU and TST data of 98 isolates from wild genotypes of common bean. The dendrogram was generated using the UPGMA algorithm, and the similarity matrix was determined using the Jaccard coefficient.

Genotypic characterization based on BOX and REP-PCR markers

Based on the results of physiological clustering (Fig. 1), 55 isolates were selected for genotypic characterization with the REP- and BOX-PCR markers. Using this approach, isolates were distributed among 11 different genotype groups (GG) when considering a 65% similarity (Fig. 2).

Fingerprinting analysis based on BOX- and REP-PCR markers showed that most of the isolates (65.45%) clustered with the *R. tropici* reference strains *R. tropici* CIAT899 and *R. tropici* H12, forming GG1 with an approximately 65% similarity. The isolates JPrG4A9, UbALG7A7 and PCG4A2 showed an approximately 69% similarity with the reference strain *R. freirei* PRF81 (GG3). Moreover, the isolate ALSG9A8 showed an approximately 72% similarity with the strain of *R. etli* bv. *phaseoli* CFN 42 (GG2). Even with a large fraction of isolates clustering with *R. tropici* reference strains, no instances of identical fingerprinting were observed among the bacteria studied, indicating high polymorphism among the isolates.

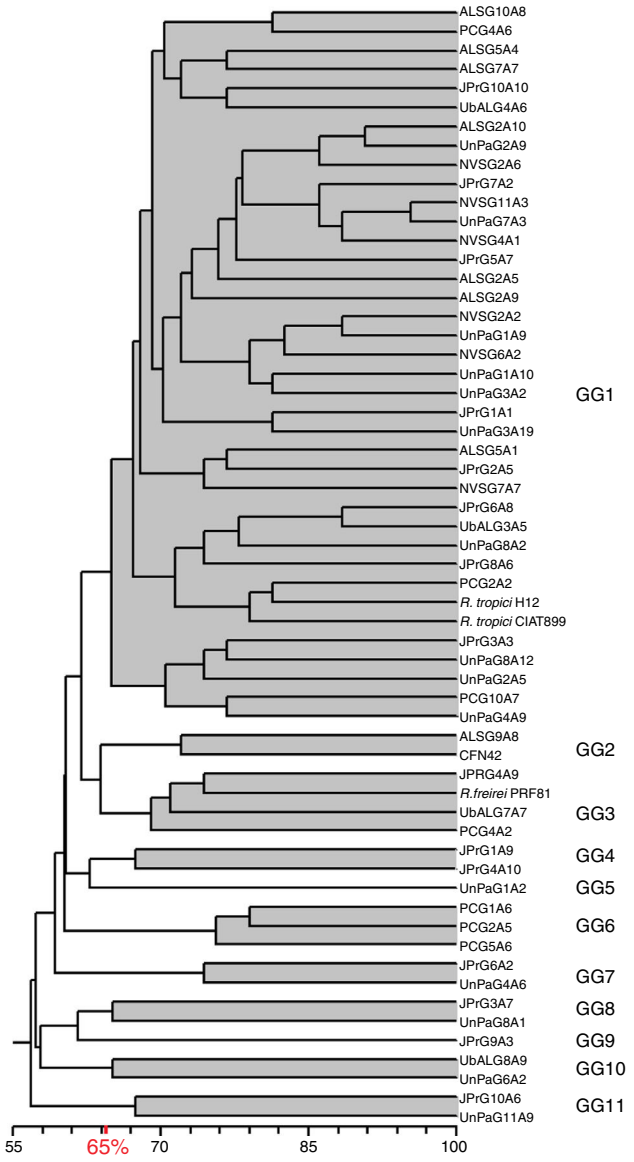

Fig. 2 – Consensus dendrogram obtained by combining the REP and BOX-PCR data of 55 isolates from wild genotypes of common bean. The dendrogram was generated using the UPGMA algorithm, and the similarity matrix was determined using the Jaccard coefficient.

Table 1 – Nodule number (NN – n° plant^{-1}), nodule dry mass (NDM – mg plant^{-1}), shoot dry mass (SDM – g plant^{-1}), leaf area (LA – cm^2 plant^{-1}) and total nitrogen (N-Total – g kg plant^{-1}) of common bean inoculated with different rhizobia isolates.

Treatments	NN	NDM	SDM	LA	N-Total
R. tropici CIAT899	110 b	260 a	1.5 b	261 b	21.6 b
R. freirei PRF81	52 c	95 b	0.5 c	86 c	12.3 d
R. tropici H12	96 b	150 a	1.2 b	184 b	13.0 d
ALSG5A1	85 b	131 b	0.5 c	100 c	11.4 d
ALSG5A4	133 a	279 a	0.9 c	150 c	12.9 d
ALSG7A7	88 b	150 a	0.3 c	61 c	16.0 c
JPrG1A1	39 c	23 c	0.4 c	72 c	15.7 c
JPrG2A5	63 b	243 a	0.9 c	135 c	16.7 c
JPrG3A3	55 b	73 b	0.7 c	123 c	11.0 d
JPrG5A7	42 c	101 b	0.5 c	83 c	8.6 d
JPrG6A8	174 a	372 a	1.0 b	154 c	14.6 c
JPrG8A7	60 b	331 a	0.9 c	154 c	9.2 d
JPrG9A3	11 d	22 c	0.5 c	83 c	10.8 d
NVSG11A3	170 a	341 a	1.3 b	220 b	14.6 c
NVSG2A2	61 b	303 a	1.3 b	247 b	22.0 b
NVSG2A6	75 b	196 a	0.6 c	220 b	16.0 c
NVSG7A7	81 b	271 a	1.4 b	206 b	11.0 d
PCG10A7	30 c	38 c	0.4 c	51 c	10.2 d
PCG1A6	75 b	53 c	0.5 c	77 c	12.0 d
PCG2A5	95 b	336 a	1.5 b	230 b	13.2 d
PCG4A2	96 b	292 a	0.7 c	126 c	18.1 c
PCG4A6	43 c	85 b	0.5 c	109 c	17.0 c
UbALG3A5	37 c	100 b	1.2 b	150 c	13.1 d
UbALG4A6	89 b	197 a	1.1 b	155 c	14.0 c
UnPaG11A9	154 a	334 a	0.8 c	197 b	12.7 d
UnPaG1A10	52 b	132 b	0.8 c	131 c	9.0 d
UnPaG2A5	13 d	21 c	0.4 c	54 c	9.8 d
UnPaG2A9	28 c	32 c	0.6 c	77 c	9.9 d
UnPaG3A19	32 c	101 b	0.8 c	108 c	10.1 d
UnPaG3A2	74 b	138 a	0.6 c	89 c	11.1 d
UnPaG4A9	59 b	174 a	0.9 c	138 c	14.9 c
UnPaG6A2	98 b	279 a	0.8 c	146 c	18.3 c
UnPaG8A12	169 a	384 a	1.8 b	285 b	19.9 b
NT1	0 d	0 c	1.6 b	242 b	37.5 a
NT2	0 d	0 c	4.6 a	486 a	32.9 a
CT	0 d	0 c	0.3 c	63 c	14.8 c

Values followed by the same letter in the same column were not significantly different, as determined by the Skott–Knott test ($p < 0.05$).

Symbiotic efficiency under greenhouse conditions

To evaluate symbiotic efficiency in greenhouse conditions, 30 isolates were used based on genotype clustering (Fig. 2). The inoculation significantly affected all of the evaluated parameters (Table 1).

The NN showed significant difference among the isolates. The Significant greater values varied from 133 to 174 nodules plant^{-1} and, it were found for the isolates ALSG5A4, JPrG6A8, NVSG11A3, UnPaG11A9 and UnPaG8A12. Among the reference strains, the best results were observed for *R. tropici* CIAT899 and *R. tropici* H12, with 110 and 96 nodules plant^{-1}, respectively. Those same isolates also showed high NDM values; however, there was no significant difference between the reference strains *R. tropici* CIAT899 and *R. tropici* H12. Moreover, 17 isolates showed greater NDM than the *R. freirei* PRF81 (Table 1).

Regarding SDM, greater values were observed for the treatment corresponding to 120 kg ha^{-1} of N (NT2). However, the isolates JPrG6A8, NVSG11A3, NVSG2A2, NVSG7A7, PCG2A5, UbALG3A5, UbALG4A6 and UnPaG8A12 showed values of SDM similar to those obtained by the reference strains *R. tropici* CIAT899 and *R. tropici* H12 and by the treatment corresponding to 60 kg ha^{-1} of N (NT1) (Table 1).

The effect of the treatments on LA was similar to those on SDM, except for the isolates JPrG6A8, UbALG3A5 and UbALG4A6, which had lower values of LA compared to the reference strains *R. tropici* CIAT899 and *R. tropici* H12. Additionally, the isolates NVSG2A6 and UnPaG11A9 showed values equal to those observed for the reference strains *R. tropici* CIAT899 and *R. tropici* H12 (Table 1).

Similar to SDM and LA, higher N-Total values were observed for NT1 and NT2 and between inoculated treatments. Among the inoculated treatments, the isolates NVSG2A2 and UnPaG8A12 had N-Total values that were statistically similar to that observed for the reference strain *R. tropici* CIAT899 (Table 1).

Correlation analysis of NDM with parameters of shoot growth (SDM, N-Total and LA) allowed for the identification of different classes of symbiotic efficiencies, such as few nodulation/few growth, few nodulation/high growth, high nodulation/few growth and high nodulation/high growth. The distribution of the isolates occurred in different quarters of the graphic defined in function of the general mean of the treatments (Fig. 3).

Correlation analyses between NDM × SDM, NDM × N-Total and NDM × LA showed highly significant results, with correlation coefficients (*r*) of 0.7, 0.48 and 0.78, respectively. This analysis showed that for the three correlations performed, the isolates UnPaG8A12, NVSG11A3, NVSG2A2, JPrG6A8 and UbALG4A6 and, the reference strain *R. tropici* CIAT899, were above the general mean (Fig. 3). Remarkably the isolates UbALG3A5 and UnPaG4A9 (Fig. 3A), JPrG1A1, PCG4A6, ALSG7A7 and UnPaG4A9 (Fig. 3B) and UbALG3A5 (Fig. 3C) also showed high values of SDW, N-Total and LA, respectively, even with low values NDM.

Regarding the correlation between NDM and SDM, 10 isolates and the reference strain *R. tropici* CIAT899 appeared on the upper right quarter, indicating that these bacteria show a high capacity for both nodulation and accumulation of SDM. In contrast, the isolates UbALG3A5 and UnPaG4A9, and the reference strain *R. tropici* H12, accumulated a high SDM, even with few nodulations (Fig. 3A). The isolates and the reference strain *R. freirei* PRF81 appearing in the lower left quarter had a symbiotic performance below the general mean, and for those in the lower right quarter, the vegetal growth was weak, even with high nodulation. For the NDM × N-Total correlation, nine isolates and the reference strain *R. tropici* CIAT899 showed a high symbiotic efficiency, promoting high values for both NDM and N-Total. In addition, the isolates JPrG1A1, PCG4A6, ALSG7A7 and UnPaG4A9 were able to promote high N-Total accumulation with low NDM (Fig. 3B).

Regarding the NDM and LA correlation, twelve isolates and the reference strain *R. tropici* CIAT899 appeared on the upper right quarter, indicating that these bacteria show high capacity for both nodulation and LA growth, and the isolate UbALG3A5 and the reference strain *R. tropici* H12 promoted high LA with low NDM (Fig. 3C).

Sequencing analysis revealed the grouping of three isolates (ALSG5A1, JPRG1A1 and JPrG6A8) into the *R. tropici* and *R. freirei* cluster, while the other two isolates grouped with *Rhizobium leguminosarum* bv. *viciae*, *R. leguminosarum* bv. *phaseoli*, *Rhizobium fabae*, *Rhizobium pisi* and *Rhizobium phaseoli* strains (Fig. 4).

Discussion

The clustering analysis based on CSU and TST showed that the *Rhizobium* reference strains clustered with 52 isolates, forming

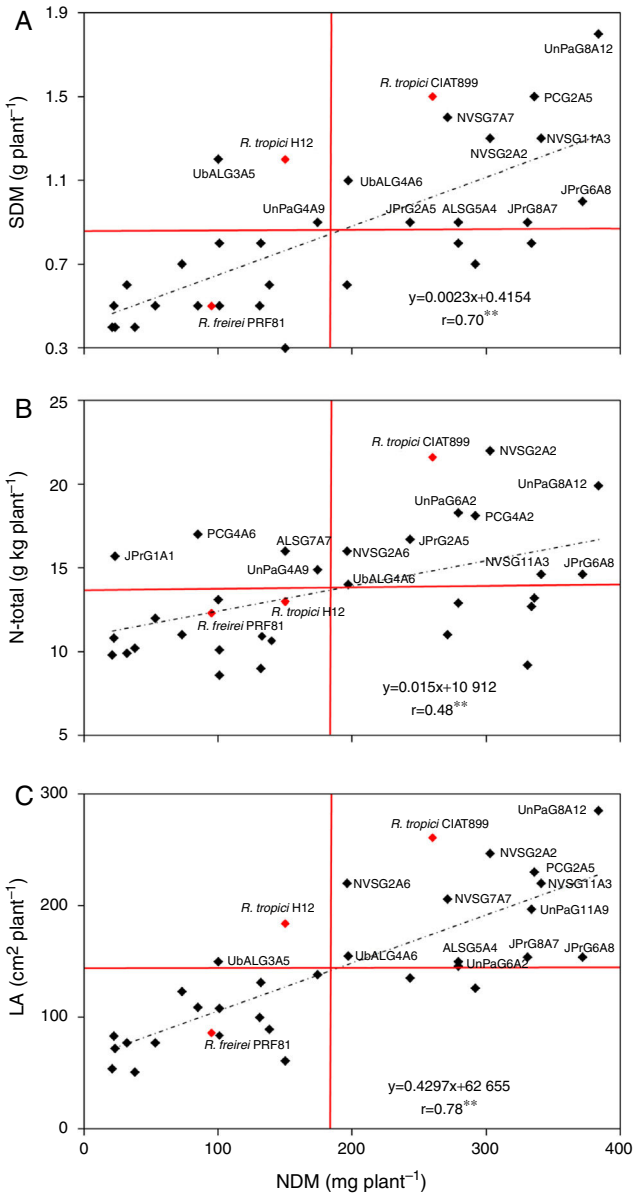

Fig. 3 – Pearson correlation analysis for (NDM) nodule dry mass and (SDM) shoot dry mass. (A) NDM and (N-Total) total nitrogen, (B) NDM and (LA) leaf area, and (C) common bean inoculated with different rhizobia isolates. Dashed lines represent the correlation tendency, and red lines represent the general mean for each parameter. ** $p < 0.01$.

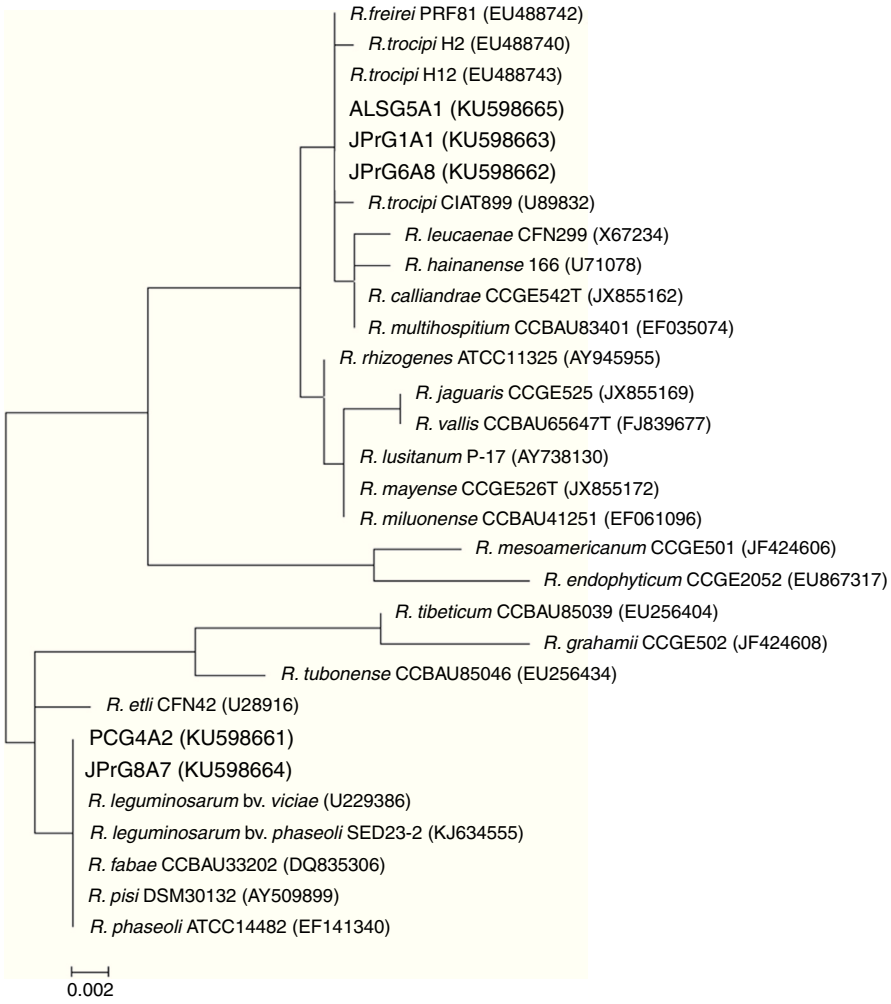

Fig. 4 – Maximum-likelihood phylogeny of the 16S rRNA gene showing the relationships between rhizobia isolates obtained from wild genotypes of common bean (in bold) and *Rhizobium* sp. reference strains. GenBank accession numbers are shown in parentheses. Bar, 2 nt substitutions per 1000 nt.

the groups PG1 and PG4. Isolates grouped in these two PGs accounted for more than 50% of the isolates evaluated (Fig. 1).

The reference strain *R. freirei* PRF81 and the isolates NVSG7A7, ALSG10A8 and NVSG8A2 were grouped in the PG1 cluster and showed a high TST. However, these bacteria showed weak CSU (Table S2). High TST may be associated with a specific genetic control, conferring such ability to these bacteria. Gomes et al.[26] showed that the PRF 81 (SEMIA 4080) strain is capable of differentially expressing some conserved heat-responsive proteins, such as *DnaK* and *GroEL*. They also reported the up-regulation of proteins involved in a variety of metabolic pathways, including translation factors and oxidative stress-responsive proteins, indicating that the responses of *R. freirei* strain PRF 81 to heat stress go beyond the induction of heat shock proteins.

Reference strains *R. tropici* CIAT899 and *R. tropici* H12 formed PG4 together with 48.5% of the evaluated isolates. These bacteria were not able to grow under temperatures of 43 and 48 °C; however, they grew until 2% NaCl content on the other tested temperatures. Moreover, these bacteria showed greater CSU than the bacteria of the PG1 (Table S1). These results corroborate those of Dall'Agnol et al.,[6] who reported a greater capacity of *R. tropici* CIAT899 to use C source as compared to *R. freirei* PRF81.

In our work, *R. tropici* CIAT899 and *R. tropici* H12 were able to grow on culture medium containing glucose and glycerol. According to Castellane and Lemos,[27] these C sources favor growth and exopolysaccharide (EPS) production in these strains. EPS production is related to the process of rhizobia adaptation to limiting environmental conditions,[28] allowing these bacteria to grow under conditions of high temperature and soil salinity. Thus, the evaluation of temperature and salinity tolerance is a key step in the selection process of rhizobia because such factors can inhibit their growth.[6,29,30]

On the fingerprinting analysis based on BOX- and REP-PCR markers the highest similarity (95.5%) was identified between the isolates NVSG11A3 and UnPaG7A3 (GG1). These results corroborate the work of Grange,[31] who reported high diversity among rhizobia isolates obtained from common bean nodules cultivated in Cerrado soils.

These markers are used to characterize and determine genotypic differences among bacterial strains to describe new

species[32–34] and identifying the rhizobia strains used to compose the Brazilian commercial inoculants for common bean.[35]

Among the 30 isolates evaluated under greenhouse conditions, the isolates JPrG6A8, NVSG11A3, UnPaG8A12, UnPaG11A9 and ALSG5A4 produced higher values of NN than the reference strains and, higher NDM than the reference strain *R. freirei* PRF81. Although NN is not a determining factor for the efficiency of BNF,[36] it may be indicative of the high nodulation efficiency of bacteria. Moreover, NN and NDM are measurements frequently used as indicators of nodulation.[37]

When NN and NDM were evaluated simultaneously, verified that 80% of the isolates had better or equal results compared to those of the *R. tropici* and *R. freirei* reference strains. Because NDM shows a better correlation with symbiotic performance,[36] our results demonstrated that 17 isolates produced similar amounts of NDM compared to the reference strains *R. tropici* CIAT899 and *R. tropici* H12, indicating the high symbiotic efficiency of these isolates.

Results of nodulation had a direct effect on the accumulation of dry matter on the shoots of the plants. The treatments with higher NN and NDM also showed high values of SDM and LA. Among the inoculated treatments, greater values of SDM and LA were found for the reference strains *R. tropici* CIAT899 and *R. tropici* H12 and for the isolates NVSG11A3, NVSG2A2, NVSG7A7, PCG2A5 and UnPaG8A12. Measurements of SDM and LA provide important information about plant growth[38] because these parameters are good indicators of plant nutritional status, which has a direct influence on crop production.[39,40] Moreover, this approach has been used for strain selection to compose bacterial inoculants.[40,41]

The nitrogen content of plants (N-Total) was also affected by inoculation; however, only two isolates, NVSG2A2 and UnPaG8A12, showed N-Total content similar to that of the reference strain *R. tropici* CIAT899. Greater values of N-Total were observed to the nitrogen treatments. Similar results reported by Gonzáles et al.[41] in a field experiment showed that nitrogen treatment resulted in greater N-Total compared to inoculation.

The use of correlation of NDM with parameters of shoot growth (SDM, N-Total and LA) allows to identify more efficient isolates, which are located at the upper left and upper right quarters (Fig. 3). Interestingly, our results revealed a high symbiotic efficiency of the isolates JPrG6A8, NVSG11A3, NVSG2A2, UbALG4A6 and UnPaG8A12, showing NDM, SDM, N-Total and LA values similar to the best reference strain, *R. tropici* CIAT899. Moreover, the isolate UnPaG4A9 also had promising results, with high values of SDM and N-Total even under low NDM. These are very interesting features for rhizobial isolates, since it can allow to provide better producing results under field conditions.[26,31,34,39]

Sequencing analysis of the 16S rRNA gene revealed that the isolates ALSG5A1, JPrG1A1 and JPrG6A8 are very closely related with the reference strains of *R. tropici* CIAT899, *R. tropici* H12 and *R. freirei* PRF81, which are used in Brazil as commercial inoculant for common bean[42]; however, the isolate JPrG1A1 showed low efficiency in the greenhouse experiment (Table 1).

Considering that many studies have discussed the importance of investigating the efficiency of biological nitrogen fixation in the selection of isolates for the development of inoculants,[43,44] the isolates ALSG5A1 and JPrG6A8 must be tested for their agronomic efficiency under field conditions, aiming to state their potential use as inoculant for common bean.

Conclusions

Forty-five percent of the isolates evaluated and the reference strain *R. freirei* PRF81 show high tolerance to temperature, while 24% of the isolates and the reference strain *R. tropici* CIAT899 are able to use all of the carbon sources studied. Clustering analysis based on physiological parameters group 50% of the isolates, *R. tropici* CIAT899 and *R. tropici* H12 with a similarity level of 76%. REP- and BOX-PCR markers group about 65% of the isolates, *R. tropici* CIAT899 and *R. tropici* H12 with a similarity level of 66%. About 20% of the isolates show symbiotic efficiency similar to or better than the best *Rhizobium* reference strain (CIAT899). By the phylogenetic analysis of the 16S rRNA the isolates ALSG5A1 and JPrG6A8 belong to the group of strains used as commercial inoculant for common bean in Brazil.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjm.2016.09.002.

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