



So many rhizobial partners, so little nitrogen fixed: The intriguing symbiotic promiscuity of common bean (*Phaseolus vulgaris* L.)

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Abstract

Symbiotic diazotrophic bacteria collectively called “rhizobia” can associate with legumes and form specialized structures in the roots, called nodules, where the biological nitrogen fixation (BNF) process takes place. Common bean (*Phaseolus vulgaris* L.) is a major protein source in the diet of the population of many countries such as Brazil and can benefit from the BNF process, but there is still little knowledge about the diversity and effectiveness of indigenous microsymbionts. In this study, the diversity and the nitrogen fixation ability of bacterial isolates trapped by common bean nodules in 14 municipalities of Mato Grosso do Sul state, Central-Western region, comprising three Brazilian biomes were investigated. The DNA profiles (BOX-PCR) of 82 isolates indicated outstanding diversity, with 12 main clusters and 36 isolates occupying single positions, joined at a final level of similarity of less than 20%. The 16S rRNA phylogeny of 56 isolates representing the DNA profiles indicated ten genera, with 38 isolates identified as “classical rhizobia” and *Agrobacterium*, and the remaining 18 belonging to six other genera. The 38 isolates had their *glnII* gene sequenced and were evaluated for the capacity of nodulation and BNF with common bean, and only 12 formed effective nitrogen-fixing nodules, five positioned in the *R. etli* and six in the *R. tropici* clades, and one of *Agrobacterium*. These results highlight the promiscuity of common bean in capturing a variety of microbial species in their nodules, whose function has not been well elucidated yet. Only one-fifth of the isolates were effective in fixing nitrogen, which might explain the frequently reported low rates of contribution of the BNF with this legume, an intriguing paradigm in the evolution of the symbiosis.

Keywords Biological nitrogen fixation · Diazotrophic bacteria · Nodulation · BOX-PCR · 16S rRNA · *glnII* gene

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1 Introduction

Common bean (*Phaseolus vulgaris* L.) is one of the most important legumes cropped worldwide, and besides being of great social and economic importance, it represents the main source of protein in the diet of millions of people, mainly in developing countries (Myers and Kmiecik 2017; Shamseldin and Velázquez 2020). Common bean is also recognized for establishing promiscuous symbioses, being able to associate with a broad variety of rhizobial species; however, apparently several of them present low efficiency of biological nitrogen fixation (BNF) (Aserse et al. 2012; Dall’Agnol et al. 2013; Reinprecht et al. 2020; Shamseldin and Velázquez 2020).

Classification of rhizobia was initially based on phenotypic properties in vitro and on the capacity to nodulate host legumes, known as cross-inoculation groups (Peix et al. 2015; Velázquez et al. 2017). Based on this

concept, all common bean symbionts were first classified as *Rhizobium phaseoli* (Frank 1889; Peix et al. 2015; Kaschuk and Hungria 2017). With the development of methodologies, including molecular techniques, the polyphasic taxonomy was confirmed as the most appropriate approach to obtain the correct taxonomic position of a strain or a group of strains (Colwell 1970), and nowadays integrates phenotypic, genotypic, and phylogenetic information (Thompson et al. 2013; Fournier et al. 2015; Hugenholtz et al. 2021). Based on this approach, several rhizobia of environmental and economic interest, such as *Bradyrhizobium japonicum* (Jordan 1982), *Rhizobium tropici* (Martínez-Romero et al. 1991), *Bradyrhizobium elkanii* (Kuykendall et al. 1992), *Mesorhizobium ciceri* (Jarvis et al. 1997), *Rhizobium freirei* (Dall'Agnol et al., 2013), among others, have been reclassified or described.

Studies aiming to characterize rhizobia are of great importance for understanding the origin, evolution, diversity, symbiotic behavior, among other properties that indicate the richness and biotechnological potential of this important group of bacteria. Mato Grosso do Sul (MS) state, located in the Central-Western region of Brazil is recognized as a hotspot of diversity of plants and animals, and certainly also of microorganisms. It encompasses three important biomes, Pantanal, Cerrado, and Mata Atlântica (Myers et al. 2000; Eisenlohr et al. 2015), but rhizobial diversity is still poorly known. Based on this information, this study evaluated the diversity and BNF capacity of symbionts trapped by nodules of common bean plants grown in soils from 14 municipalities of Mato Grosso do Sul, comprising these three biomes.

2 Materials and methods

2.1 Bacterial isolation

The 82 isolates were previously isolated by Fabio Martins Mercante (born 1963-died 2016), researcher at Embrapa Agropecuária Oeste (Dourados, Mato Grosso do Sul-MS, Brazil). Soil samples from 14 municipalities from MS (Fig. 1, Table 1), comprising three out of the six Brazilian biomes, Pantanal, Cerrados, and Mata Atlântica were collected and used as the substrate for growth of common bean (*Phaseolus vulgaris* L.) as the trap host. After growth up to the end of vegetative stage, nodules were collected, surface-disinfested, crushed in sterile saline solution, and streaked on culture medium for isolation of single colonies, as described before (Hungria et al. 2016). For long-term storage, bacterial isolates were cryopreserved at $-80\text{ }^{\circ}\text{C}$ and $-150\text{ }^{\circ}\text{C}$ in modified-yeast extract-mannitol (YM) culture medium (Hungria et al. 2016) with 30% glycerol (v/v), and lyophilized, as described (Delamuta et al. 2017).

For the analyses, the strains were grown in modified-yeast extract-mannitol-agar (YMA) medium (Hungria et al. 2016), and incubated at $28\text{ }^{\circ}\text{C}$ for three to seven days, depending on the growth rate of each isolate. Regular bacterial maintenance was on modified-YMA medium at $4\text{ }^{\circ}\text{C}$.

All isolates are deposited at the “Diazotrophic and Plant Growth Promoting Bacteria Culture Collection of Embrapa Soja” (WFCC Collection # 1213, WDCM Collection # 1054), in Londrina, State of Paraná, Brazil.

Fig. 1 Brazilian map showing the biomes and the municipalities from which the isolates from this study were obtained

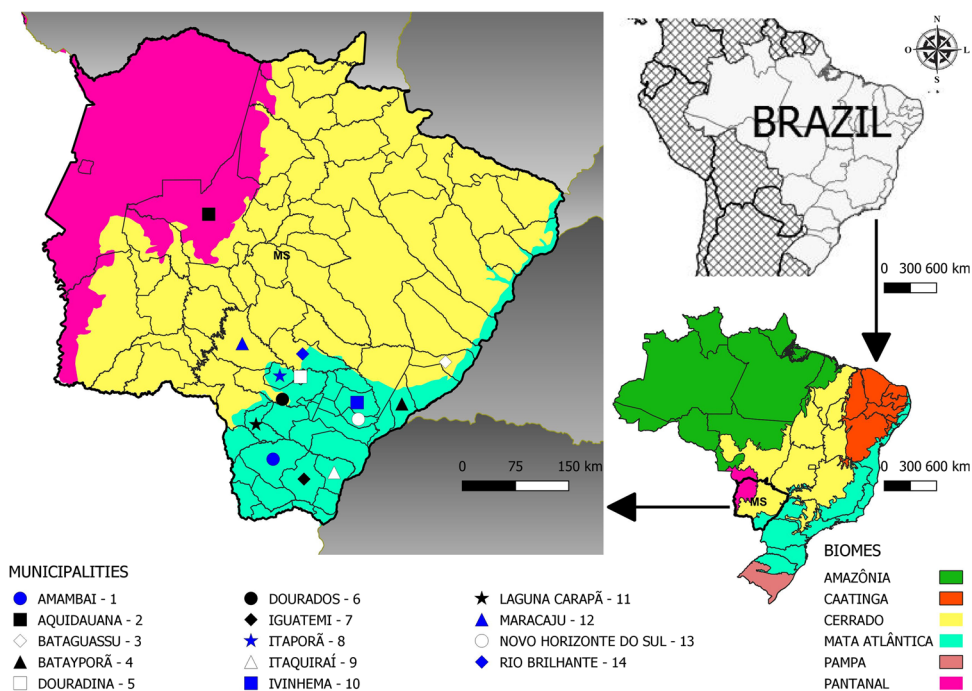


Table 1 Municipalities and biomes from where soil samples were taken in the state of Mato Grosso do Sul and the isolates (CNPSo—National Center for Soy Research) assessed in this study

Municipalities	Biomes	Identification of Isolates (CNPSo)
1 Amambaí	Mata Atlântica	3435/ 3631
2 Aquidauana	Cerrado and Pantanal	4038/ 4039
3 Bataguassu	Cerrado and Mata Atlântica	3436/ 3437/ 3457/ 3460/ 3462/ 3464
4 Batayporã	Cerrado and Mata Atlântica	3439
5 Douradina	Mata Atlântica	3529/ 3636
6 Dourados	Cerrado and Mata Atlântica	3966/ 3968/ 3969/ 3971/ 3972/ 3973 / 4053/ 4054/ 4055/ 4056/ 4057/ 4058/ 4061/ 4062/ 4063/ 4064
7 Iguatemi	Mata Atlântica	3497
8 Itaporã	Cerrado and Mata Atlântica	3974/ 3975/ 3976/ 3977/ 3978/ 3979/ 3981/ 3982/ 3988
9 Itaquiraí	Mata Atlântica	3440/ 3465/ 3490/ 3493/ 3494
10 Ivinhema	Mata Atlântica	3467/ 3468/ 3469/ 3470/ 3495/ 3496
11 Laguna Carapã	Mata Atlântica	3984/ 3985/ 3986/ 3987/ 3990/ 3991
12 Maracajú	Cerrado and Mata Atlântica	3993/ 3994/ 3995/ 3997/ 3998/ 3999/ 4000/ 4001/ 4007
13 Novo Horizonte do Sul	Mata Atlântica	3498/ 3499/ 3500/ 3501/ 3502/ 3633/ 3634
14 Rio Brilhante	Cerrado and Mata Atlântica	4002/ 4003/ 4005/ 4006/ 4031/ 4032/ 4033/ 4034/ 4035/ 4036

2.2 Genotypic characterization

2.2.1 DNA extraction and BOX-PCR fingerprinting

The genomic DNAs of 82 isolates were extracted using the DNeasy Blood & Tissue kit (Qiagen), following the manufacturer's instructions, and DNA quality was verified by electrophoresis on agarose gels (1%) stained with ethidium bromide and visualized under UV light. BOX-PCR fingerprinting fragments were obtained by DNA amplification using the BOX-A1R primer (5'-CTACGGCAAGGCGAC GCTGACG-3') (Versalovic et al. 1994) in a ProFlex PCR System Thermocycler (Applied Biosystems), following cycles as described before (Costa et al. 2018). The amplified fragments were separated by electrophoresis on 1.5% agarose gel, using 1 kb Plus Ladder (Invitrogen®) as molecular marker.

The fingerprinting profiles were used to build a dendrogram of similarity using the Bionumerics software (Applied Mathematics, Kortrijk, Belgium, v.7.6), applying the UPGMA (Unweighted Pair-Group Method with Arithmetic Mean) algorithm (Sneath and Sokal 1973), and the Jaccard coefficient (Jaccard 1912), with 2% tolerance.

2.2.2 Amplification and sequencing of 16S rRNA and *glnII* housekeeping genes

Based on the grouping according to the BOX-PCR fingerprinting dendrogram, 56 isolates were selected for sequencing of the 16S rRNA. The 16S rRNA genes were amplified using the pair of primers fD1 (5'-AGAGTTTGA TCCTGGCTCAG-3') and rD1 (5'-CTTAAGGAGGTG

ATC CAGCC-3'), according to Weisburg et al. (1991) and sequenced with the primers 362f (5'-CTCCTACGGGAG GCAGCAGTGGGG-3') and 786r (5'CGAAAGCGTGGG GAGCAAACAGG-3'), according to Menna et al. (2006). The PCR products were purified with the PureLink kit (Invitrogen®), following the manufacturer's recommendations, and sequenced on an ABI 3500xL (Applied Biosystems), as described by Menna et al. (2006), with modifications proposed by Delamuta et al. (2017). Nucleotide sequences obtained were analyzed and corrected manually using the Bionumerics software (v.7.6), and compared with sequences of the Genbank database using the Blastn tool. This step allowed the identification of the genera of each isolate.

Based on the 16S rRNA results, 38 isolates were selected for further sequencing of the *glnII* housekeeping gene. The amplification of *glnII* was performed with the pair of primers TSglnII_f (5'-AAGCTCGAGTACATCTGGCTCGAC GG-3') and TSglnII_r (5'-SGAGCCGTTCCAGTCGGTG TCG-3') and reaction cycles as described by Stepkowski et al. (2005). The purification, sequencing reactions, and correction of the sequences were performed as described for the 16S rRNA gene.

For the phylogenetic analysis, isolates from this study were analyzed along with the closest type strains, whose sequences were retrieved from the GenBank database, except for the 16S rRNA sequence of *Bradyrhizobium shewense* ERR11^T, which was retrieved from the Joint Genome Portal (JGI). Multiple sequence alignment for each gene was obtained with MUSCLE (Edgar 2004) and the best evolutionary distance model was inferred by the lowest Bayesian information criterion scores (Schwarz 1978) for maximum likelihood (ML) (Felsenstein 1981) phylogenetic

tree constructions in the MEGA software (Molecular Evolutionary Genetics Analysis, version 7.0) (Kumar et al., 2016). The statistical support of the phylogenetic trees was estimated by bootstrap analysis (Felsenstein 1985), with 1,000 replicates (Hedges 1992). The evolutionary models used to build the trees are described in the figure captions. For determination of nucleotide identity (NI) (Case et al. 2007) sequences were aligned in the Bioedit Sequence Alignment Editor software (v.7.2.5) (Hall 1999).

Sequences were deposited in the NCBI GenBank database and the access numbers are shown in the phylogenetic trees.

2.3 Morphophysiological characterization

The 38 isolates classified as “classical rhizobia” and *Agrobacterium* based on the 16S rRNA and *glnII* genes sequencing analyses were cultivated in Petri dishes on modified-YMA medium containing Congo red as indicator and pH adjusted to 6.8 to 7.0 (Hungria et al. 2016). Bacteria were also grown on modified-YMA medium with bromothymol blue used as indicator to assess the capacity of the strains to produce acid/alkaline reaction (Hungria et al. 2016). Growth was verified after 3 to 7 days of incubation at 28 °C, according to each bacteria. Tests were performed in triplicate. The following morphological properties were characterized: a) growth rate; b) colony diameter (mm); c) mucus production; d) colony shape; e) colony border; f) colony surface; g) colony elevation; h) consistency; i) optical details; j) acid or alkaline reaction; k) chromogenesis in both media, with bromothymol blue or Congo red indicators (Vincent 1970; Somasegaran and Hoben 1994; Hungria et al. 2016).

2.4 Nodulation capacity and nitrogen fixation efficiency

Evaluations were carried with *Phaseolus vulgaris* cultivar Esteio (black seeds). Seeds were surface-disinfested (Somasegaran and Hoben 1994; Hungria et al. 2016), pre-germinated on Germitest paper moistened with distilled water and kept at 25 °C for 72 h. After germination, one seedling was transplanted to each propylene bag containing a sheet of Germitest paper to serve as wick and 300 mL of sterilized N-free nutrient solution (Hungria et al. 2016). After transplanting, each seedling was inoculated with 1 mL of culture of each isolate, adjusted to approximately 10^8 cells mL^{-1} . The experiment was performed with five replicates, under aseptic conditions in a glasshouse for 30 days.

The nodulation capacity was evaluated by the presence or absence of nodules. Nodules were then removed from roots and cut to verify the internal color. Nitrogen fixation efficiency was confirmed if the internal color of the nodule

was red or pink, indicating functional leghemoglobin, and by the dark green color of the leaves.

3 Results

3.1 Genetic characterization

3.1.1 BOX-fingerprinting

DNA profiles were obtained in the BOX-PCR analyses for all 82 isolates from soils taken at 14 municipalities of the state of Mato Grosso do Sul, Brazil. Fragments within the range of 300 to 3,000 bp were considered in the analysis and a dendrogram of similarity was built (Fig. 2). Considering the similarity level of 70% as a cutoff, 48 distinct groups or single clades containing sole isolates were generated, indicating high genotypic diversity among the isolates. The dendrogram distributed 46 isolates in 12 groups and the other 36 occupied single positions joined at the final level of similarity of only 18.68%. Numbers presented in parentheses in the dendrogram (Fig. 2) refer to the municipalities from which the respective isolate was taken (Fig. 1, Table 1), showing high heterogeneity concerning the groups of isolates and their origin. The largest group (27) included 13 isolates belonging to the Cerrado and Mata Atlântica biomes, followed by group 3, with nine isolates from the Cerrado and Mata Atlântica biomes, and group 40, with four isolates belonging to the Cerrado, Mata Atlântica and Pantanal. The other groups had three (12 and 13) or two isolates (1, 7, 9, 17, 31, 32, 37) each, isolated from Mata Atlântica and Cerrado biomes.

It is worth mentioning that similarities of 100% were observed in only five groups, which included isolates CNPSO 3496, 3500, 3502, 3633, and 3634 (group 3); CNPSO 3494, 3499 and 3501 (group 3); CNPSO 3437 and 3457 (group 7); CNPSO 3493 and 3495 (group 13); CNPSO 4053, 4054 and 4055 (group 27); CNPSO 3971 and 3972 (group 27); and CNPSO 3460 and 3462 (group 40).

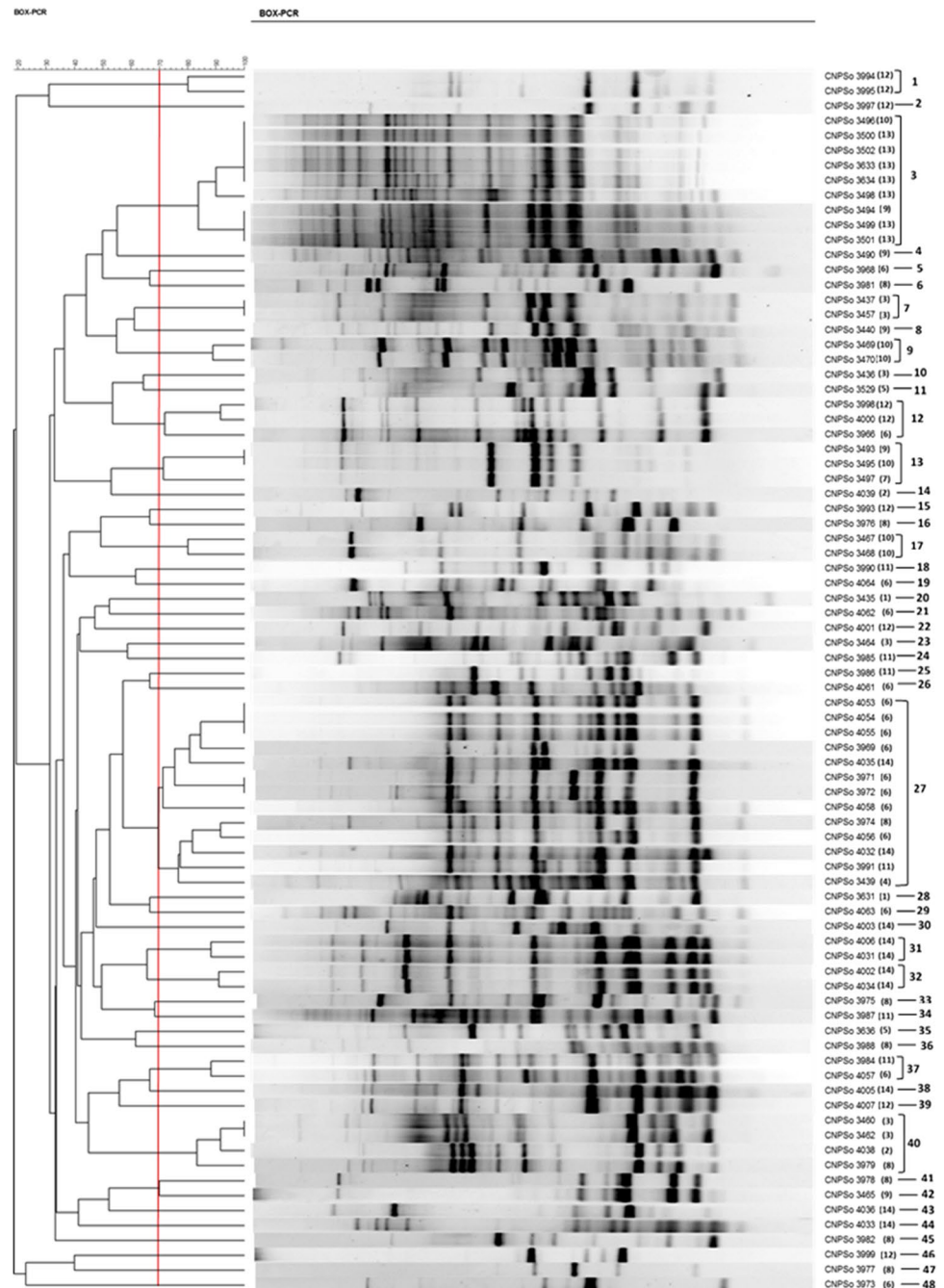
Based on the BOX-PCR dendrogram, 48 isolates were selected as representatives of the groups formed, in addition to other eight isolates showing great variability of the profiles, totalizing 56 isolates that were used for the following step of the polyphasic analysis.

3.2 Phylogenetic characterization

3.2.1 16S rRNA gene analysis

Sequences of 16S rRNA gene were obtained for the 56 isolates selected in the BOX-PCR analysis and were submitted to the Blastn tool, to identify their genera. Twenty-two out of 56 isolates were identified as representatives of three

Fig. 2 Fingerprinting dendrogram of similarity based on the BOX-PCR profiles of the isolates of this study, using the UPGMA algorithm and the Jaccard coefficient with 2% tolerance (software Bionumerics 7.6). The numbers in parentheses represent the municipalities in the state of Mato Grosso do Sul, Brazil, from which each isolate was obtained, according to Fig. 1 and Table 1



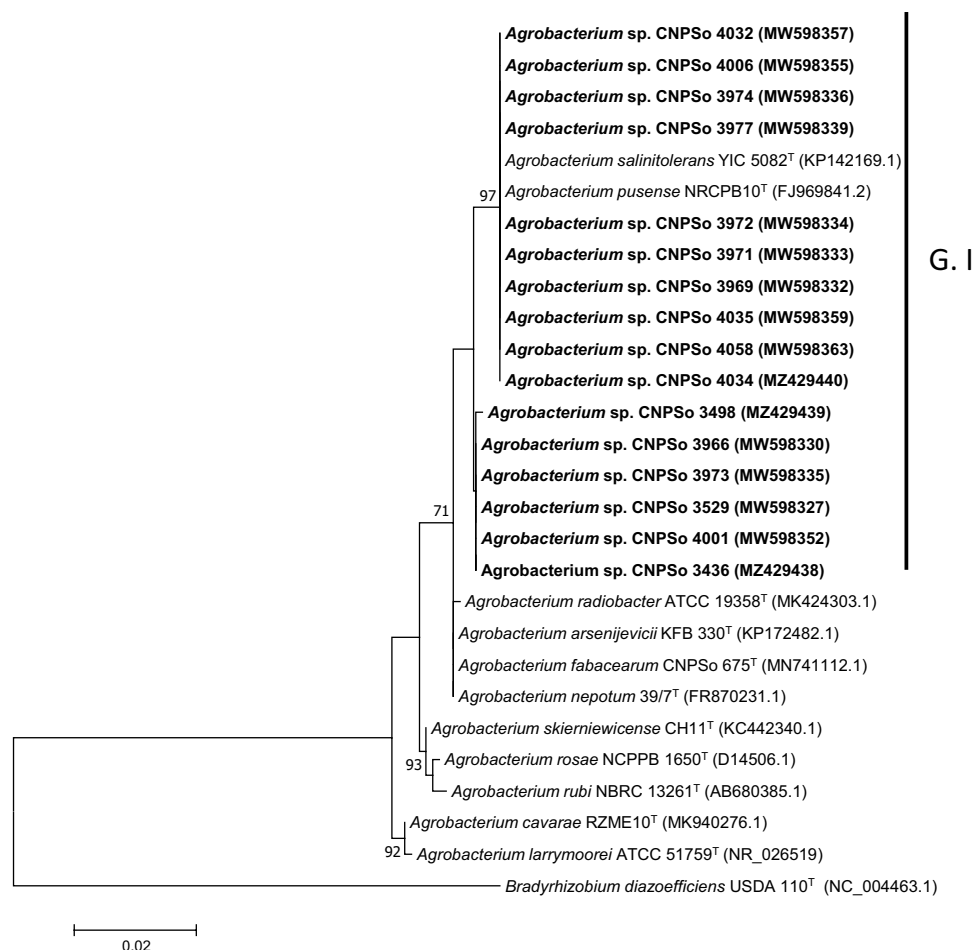
genera of the Alphaproteobacteria class known as “classical rhizobia”, including *Rhizobium* (20 isolates), *Bradyrhizobium* (1), and *Mesorhizobium* (1). The remaining 34 isolates were identified as members of the *Agrobacterium* (16), *Herbaspirillum* (11), *Pseudomonas* (3), *Achromobacter* (1), *Brevibacillus* (1), *Burkholderia* (1), and *Enterobacter* (1) genera, representing the Alpha-, Beta- and Gammaproteobacteria and Firmicutes. The distribution of the 56 isolates in ten genera confirmed high diversity among the isolates.

We proceeded with the characterization of the “classical rhizobia” and *Agrobacterium*, comprising 38 isolates. Four

phylogenetic trees were built with the *Agrobacterium*, *Rhizobium*, *Bradyrhizobium*, and *Mesorhizobium* genera. The phylogenetic tree based on 16S rRNA gene sequences with the isolates belonging to the *Agrobacterium* genus (Fig. 3) formed a large group (G.I) comprising the 16 isolates of this study with the species *A. salinitolerans* YIC 5082^T and *A. pusense* NRCPB10^T. Strains from G.I group share from 99.3 to 100% sequences similarity.

In the phylogenetic tree with isolates of the *Rhizobium* genus (Fig. 4), two large groups were formed. Group G.I clustered nine isolates with 16 species of the clade *R. etli/R.*

Fig. 3 Maximum likelihood phylogeny based on the 16S rRNA alignment of the genus *Agrobacterium* (942 bp), using the Kimura 2-Parameter + G model. Accession numbers are indicated in parentheses. Isolates from this study are shown in bold. Bootstrap values > 70% are indicated at the nodes. *Bradyrhizobium diazoefficiens* USDA 110^T was used as outgroup. Bar indicates two substitutions per 100 nucleotide positions



phaseoli/*R. leguminosarum*, called *R. etli* group, sharing NI from 98.4 to 100%. Group G.II clustered 11 isolates with 12 species belonging to the *R. tropici* group, with 94% bootstrap values, and sharing NI of 98 to 100%.

The phylogenetic tree with the genus *Bradyrhizobium* (supplementary Fig. S1) clustered isolate CNPSo 3435 within the large clade of *B. japonicum*, showing the highest NI with the species *B. japonicum* USDA 6^T (99.7%). The tree with the genus *Mesorhizobium* (Fig. S2) grouped the isolate CNPSo 3975 with the species *M. acaciae* RITF741^T, *M. atlanticum* CNPSo 3140^T and *M. plurifarum* LMG 11892^T, with 84% bootstrap values and sharing 100% NI.

3.2.2 *glnII* housekeeping gene analysis

To obtain a clearer taxonomic definition and better access to the diversity of the isolates, the phylogeny of the housekeeping gene *glnII* was analyzed. Again, four phylogenetic trees were constructed, with the genera *Agrobacterium*, *Rhizobium*, *Bradyrhizobium*, and *Mesorhizobium*.

In the phylogenetic tree of the *Agrobacterium* genus (Fig. 5), a large group (G.I) was formed with nine isolates from this study (CNPSo 4006, 4032, 3977, 3974, 3972,

3971, 4035, 4034, 3969), clustering again with the species *A. pusense* NRCPB10^T and *A. salinitolerans* YIC 5082^T, sharing 97.6 to 100% of NI (Table 2), with 72% of statistical support. The other seven isolates (CNPSo 4058, 3529, 3973, 3436, 3966, 4001, 3498) occupied sole positions and their NIs were compared with the G.I, CNPSo 4058 shared 97.6 to 99.1% of NI with group G.I, the isolates CNPSo 3529, 3973 and 3436 shared 95.8 to 96.7% of NI; CNPSo 3966 shared 94.6 to 95.5% of NI, CNPSo 4001 shared 93.1 to 94.3% of NI and CNPSo 3498 shared 88.7 to 89.6% of NI with group G.I (Table 2).

In the tree with the genus *Rhizobium* (Fig. 6), two large groups were formed. The large group G.I clustered nine isolates from this study with 15 species of the large clade *R. etli*. Two subgroups were formed, G.I.I clustered two isolates (CNPSo 3490 and 3982) sharing 96.5% of NI and showing 97.3 to 94.4% NI with the closest species *R. esperanzae* CNPSo 668^T. The second subgroup G.I.II included seven isolates (CNPSo 3462, 3995, 3993, 3997, 4005, 4057, 4007) and the species *R. phaseoli* ATCC14482^T, with 100% statistical support, sharing from 97.9 to 100% of NI (Table 3). The second large group (G.II) clustered 11 isolates from this study with seven species of the large clade *R. tropici*.

Fig. 4 Maximum likelihood phylogeny based on the 16S rRNA alignment of the genus *Rhizobium* (944 bp), using Tamura 3-Parameter + G + I model. Accession numbers are indicated in parentheses. Isolates from this study are shown in bold. Bootstrap values > 70% are indicated at the nodes. *Bradyrhizobium diazoefficiens* USDA 110^T was used as outgroup. Bar indicates two substitutions per 100 nucleotide positions

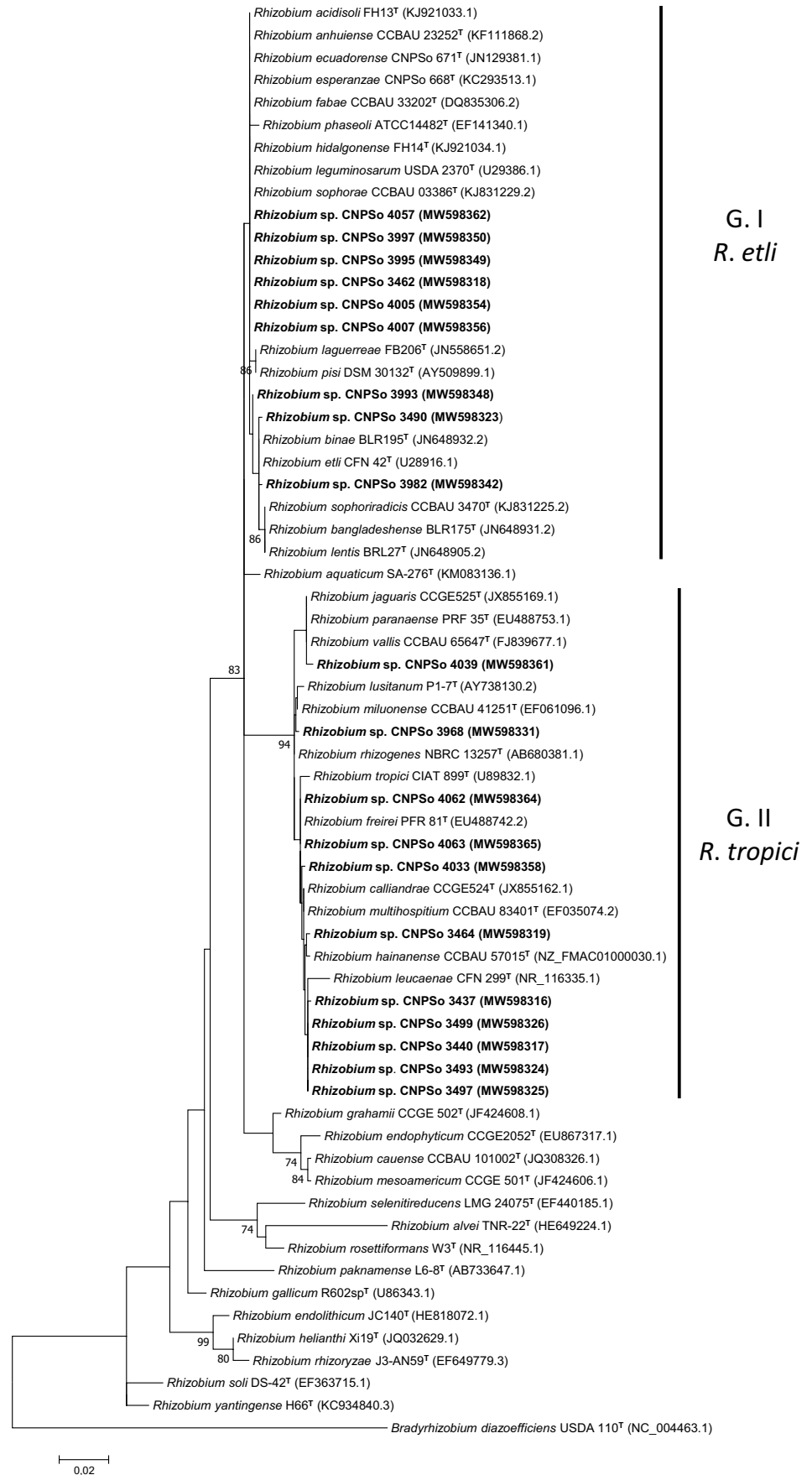
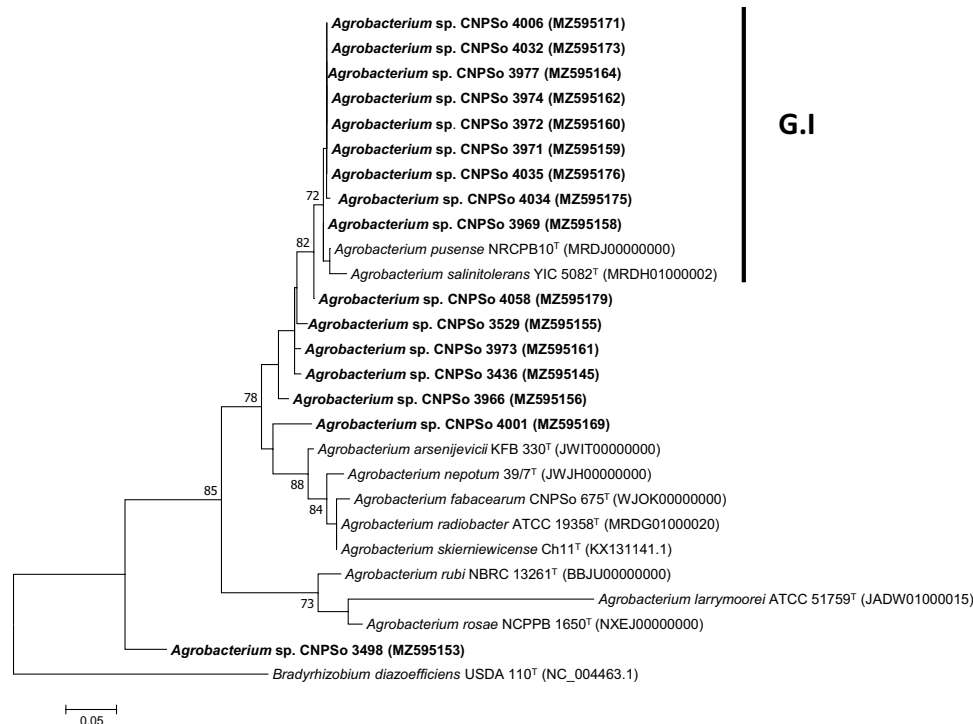


Fig. 5 Maximum likelihood phylogeny based on the *glnII* housekeeping gene alignment of the genus *Agrobacterium* (337 bp), using Tamura 3-Parameter+G model. Accession numbers are indicated in parentheses. Isolates from this study are shown in bold. Bootstrap values > 70% are indicated at the nodes. *Bradyrhizobium diazoefficiens* USDA 110^T was used as outgroup. Bar indicates five substitutions per 100 nucleotide positions



Four subgroups were formed, beginning with G.II.I, with five isolates (CNPSo 3440, 3499, 3493, 3497, 3437) and the species *R. leucaenae* CFN 299^T with statistical support of 100% and sharing 99.7 to 100% of NI. G.II.II was positioned close to the G.II.I subgroup, sharing 94.4 to 95% of NI, and grouped the *Rhizobium* sp. CNPSo 3464 with the species *R. paranaense* PRF 35^T, with 82% of statistical support, and sharing 96.8% NI. *Rhizobium* sp. CNPSo 3968 occupied an isolated position, presenting 94.7% of NI with the species *R. hainanense* CCBAU 57015^T, and from 95 to 95.6% with the closest subgroup G.II.IV. Subgroup G.II.III grouped isolates CNPSo 4033 and CNPSo 4039 with statistical support of 75% and sharing 97.3% of NI among them, and from 96.2 to 97.3% NI with the closest subgroup G.II.IV. The last subgroup (G.II.IV) clustered isolates CNPSo 4062 and CNPSo 4063 with statistical support of 89%, and 98.8% NI (Table 3).

Regarding the *Bradyrhizobium* genus (Fig. S3) phylogeny, as in the phylogenetic tree of the 16S rRNA gene, isolate CNPSo 3435 was clustered with *B. japonicum* USDA 6^T in the *glnII* housekeeping gene phylogeny sharing 100% of NI and with 100% statistical support.

Finally, in the tree of the genus *Mesorhizobium* (Fig. S4) the strain CNPSo 3975 was clustered in the same branch as the species *M. atlanticum* CNPSo 3140^T, *M. acaciae* RITF741^T, and *M. shonense* AC39a^T, sharing NI of 96.1, 95.3 and 94.7%, respectively.

3.3 Morphophysiological characterization

Morphophysiological characterization in vitro was performed with the 38 strains classified as “classical rhizobia” and *Agrobacterium* isolates according to the sequencing analysis. The results are listed in supplementary Table S1. Among the 38 isolates, 22 showed fast (3 days), 14 intermediate (4 days), and two slow (6 and 7 days) growth. Regarding the acidic/alkaline reaction in modified-YM medium containing bromothymol blue as pH indicator, 34 isolates attributed to the *Agrobacterium* and *Rhizobium* genera showed neutral reaction. Isolates CNPSo 3975, 3995 and 4057, belonging to the *Mesorhizobium* and *Rhizobium* genera, showed acid reaction, and *Bradyrhizobium* sp. CNPSo 3435 alkaline reaction.

Regarding the chromogenesis of the colonies in modified-YM medium with Congo red as indicator, colonies of 19 isolates were red, 11 pink, and eight white. In the presence of bromothymol blue as indicator, 22 isolates resulted in colonies with yellow color, and 16 presented cream color.

In the evaluation of the properties of border, shape and elevation of the colonies, all strains were similar, with smooth border and surface, with circular colonies and convex elevation. The diameter of the colonies ranged from 1.4 mm to 4.0 mm and the mucus production was considered moderate for 32 isolates, low for five isolates, and abundant for CNPSo 3977. For the optical details, 22 isolates were opaque and 16 translucent. The consistency of the growth

Table 2 Percentage of nucleotide identity (NI) among the strains in this study and related strains of the *Agrobacterium* genus based on phylogenetic analysis of the housekeeping gene *glnII*

Nucleotide Identity (%) <i>glnII</i>	
G.I (CNPSo 4006, 4032, 3977, 3974, 3972, 3971, 4035, 4034, 3969 <i>A. pusense</i> NRCPB10 ^T and <i>A. salinitolerans</i> YIC 5082 ^T)	
Among the group	97.6–100
Among the group and related strains	
<i>Agrobacterium</i> sp. CNPSo 4058	97.6–99.1
<i>Agrobacterium</i> sp. CNPSo 3529	95.8–96.7
<i>Agrobacterium</i> sp. CNPSo 3973	95.8–96.7
<i>Agrobacterium</i> sp. CNPSo 3436	95.8–96.7
CNPSo 4058	
Among CNPSo 4058 and related species/strains	
G.I	97.6–99.1
<i>Agrobacterium</i> sp. CNPSo 3529	97.6
<i>Agrobacterium</i> sp. CNPSo 3436	97.6
<i>Agrobacterium</i> sp. CNPSo 3973	97.6
CNPSo 3529	
Among CNPSo 3529 and related species/strains	
<i>Agrobacterium</i> sp. CNPSo 3973	98.2
<i>Agrobacterium</i> sp. CNPSo 3436	98.2
<i>Agrobacterium</i> sp. CNPSo 4058	97.6
G.I	95.8 – 96.7
CNPSo 3973	
Among CNPSo 3973 and related species/strains	
<i>Agrobacterium</i> sp. CNPSo 3436	98.8
<i>Agrobacterium</i> sp. CNPSo 3529	98.2
<i>Agrobacterium</i> sp. CNPSo 4058	97.6
G.I	95.8 – 96.7
CNPSo 3436	
Among CNPSo 3436 and related species/strains	
<i>Agrobacterium</i> sp. CNPSo 3973	98.8
<i>Agrobacterium</i> sp. CNPSo 3529	98.2
<i>Agrobacterium</i> sp. CNPSo 4058	97.6
G.I	95.8 – 96.7
CNPSo 3966	
Among CNPSo 3966 and related species/strains	
<i>Agrobacterium</i> sp. CNPSo 3973	97.0
<i>Agrobacterium</i> sp. CNPSo 3436	97.0
<i>Agrobacterium</i> sp. CNPSo 4058	96.4
G.I	94.9 – 95.5
CNPSo 4001	
Among CNPSo 4001 and related species/strains	
<i>Agrobacterium</i> sp. CNPSo 3973	94.3
<i>Agrobacterium</i> sp. CNPSo 3436	94.3
<i>Agrobacterium</i> sp. CNPSo 4058	94.3
G.I	93.1 – 94.3
CNPSo 3498	
Among CNPSo 3498 and related species/strains	
G.I	88.7 – 89.6

Table 2 (continued)

Nucleotide Identity (%) <i>glnII</i>	
<i>Agrobacterium nepotum</i> 3977 ^T	89.0
<i>Agrobacterium</i> sp. CNPSo 4058	88.7
<i>Agrobacterium</i> sp. CNPSo 3436	88.4

mass was considered gummy for all strains tested, except for the isolates CNPSo 4005 and 4057, which showed viscous consistency.

3.4 Nodulation and nitrogen fixation capacity

When the 38 isolates classified as “classical rhizobia” and *Agrobacterium* were evaluated for their capacity of nodulating and fixing nitrogen with *Phaseolus vulgaris*, only 13 (CNPSo 3437, 3440, 3462, 3464, 3490, 3493, 3497, 3498, 3499, 3982, 3993, 3997, 4005) were able to re-infect the host plant and form nodules. Verification of green color of the leaves and pink or red internal color of the nodules was performed as a first step to identify effective and ineffective strains (Fig. 7). All 13 isolates, except for *Rhizobium* sp. CNPSo 4005 formed nodules with internal color pink or red, indicating effective BNF.

4 Discussion

Particularly in the last decade, taxonomic studies of rhizobia symbionts of common bean have gained increasing notoriety worldwide and resulted in the description of several new species (e.g. Dall’Agnol et al. 2013, 2014; Ribeiro et al. 2013, 2015; Cordeiro et al. 2017; Huo et al. 2019; Helene et al. 2019). Although Brazil is not a genetic center of origin of common bean, the country embraces great diversity of rhizobia associated with this legume (e.g. Grange and Hungria 2004; Grange et al. 2007; Pinto et al. 2007; Stocco et al. 2008; Ribeiro et al. 2009, 2012; Dall’Agnol et al. 2013, 2014). The state of Mato Grosso do Sul is considered as a hotspot of biodiversity, encompassing three out of the six Brazilian biomes, Mata Atlântica, Cerrado, and Pantanal; however, although studies regarding rhizobial diversity point out high genetic richness (Pinto et al. 2007; Costa et al. 2018), they are still scarce. Additionally, indigenous rhizobial diversity in this Central-Western state of Brazil may represent an important source of strains with economic potential, as reported for common bean (Mercante et al. 2017).

The results obtained in this study highlight the outstanding rhizobial diversity in the Brazilian biomes. The DNA profiling by BOX-PCR of 82 isolates trapped in nodules of common bean grown in soils of 14 municipalities of MS

Fig. 6 Maximum likelihood phylogeny based on *glnII* housekeeping gene alignment of the genus *Rhizobium* (346 bp), using Tamura-Nei + G + I model. Accession numbers are indicated in parentheses. Isolates from this study are shown in bold. Bootstrap values > 70% are indicated at the nodes. *Bradyrhizobium diazoefficiens* USDA 110^T was used as outgroup. Bar indicates two substitutions per 100 nucleotide positions

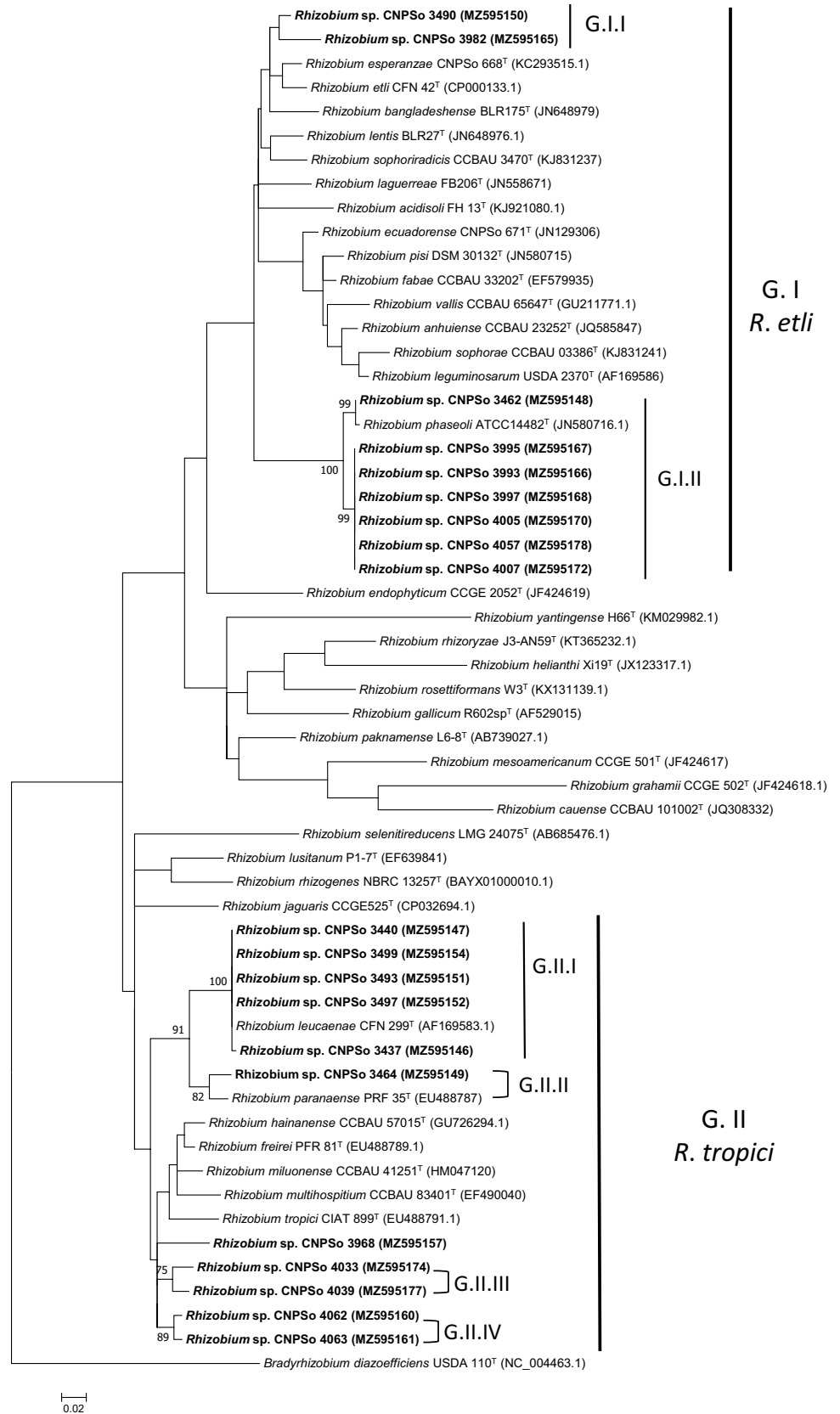


Table 3 Percentage of nucleotide identity (NI) among the strains in this study and related strains of the *Rhizobium* genus based on phylogenetic analysis of the housekeeping gene *glnII*

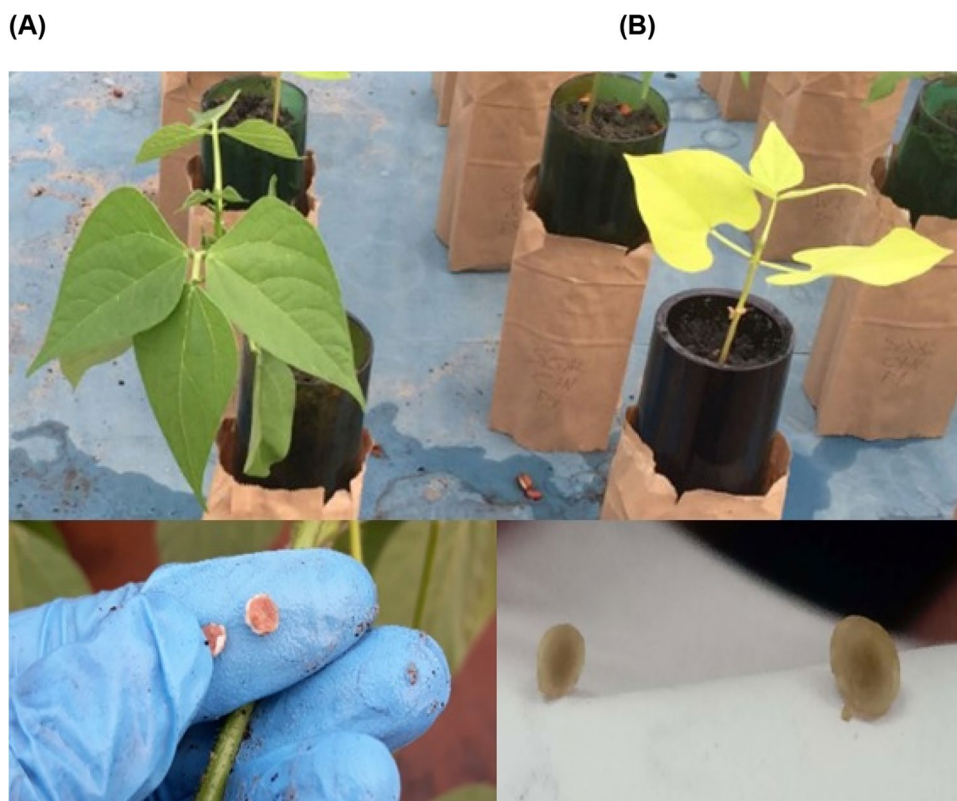
Nucleotide Identity (%)	
<i>glnII</i>	
G.I.I (CNPSo 3490 and CNPSo 3982)	
Among the group	96.5
Among the group and related species	
<i>Rhizobium esperanzae</i> CNPSo 668 ^T	94.4–97.3
<i>Rhizobium etli</i> CFN 42 ^T	94.4–96.2
<i>Rhizobium lentis</i> BLR27 ^T	94.7–95.9
G.I.II (CNPSo 3462, <i>R. phaseoli</i> ATCC14482 ^T , CNPSo 3995, CNPSo 3993, CNPSo 3997, CNPSo 4005, CNPSo 4057 and CNPSo 4007)	
Among the group	97.9–100
Among the group and related species	
<i>Rhizobium sophoriradicis</i> CCBAU 03470 ^T	92.4–92.7
<i>Rhizobium esperanzae</i> CNPSo 668 ^T	91.8–92.1
<i>Rhizobium lentis</i> BLR27 ^T	91.5–91.8
G.I.II (CNPSo 3440, CNPSo 3499, CNPSo 3493, CNPSo 3497, <i>R. leucaenae</i> CNF 299 ^T and CNPSo 3437)	
Among the group	99.7–100
Among the group and related species/strains	
G.II.II (CNPSo 3464 e <i>R. paranaense</i> PRF 35 ^T)	94.4–95.0
<i>Rhizobium freirei</i> PFR 81 ^T	93.0–93.3
<i>Rhizobium hainanense</i> CCBAU 57015 ^T	92.4–92.7
G.II.II (CNPSo 3464 and <i>R. paranaense</i> PRF 35 ^T)	
Among the group	96.8
Among the group and related species/strains	
G.II.I	94.4–95.0
<i>Rhizobium freirei</i> PFR 81 ^T	94.2–94.4
<i>Rhizobium miluonense</i> CCBAU 41251 ^T	93.6–94.4
CNPSo 3968	
Among CNPSo 3968 and related species/strains	
G.II.IV	95.0 – 95.6
G.II.III	94.7 – 95.0
<i>Rhizobium hainanense</i> CCBAU 57015 ^T	94.7
G.II.III (CNPSo 4033 and CNPSo 4039)	
Among the group	97.3
Among the group and related species/strains	
G.II.IV	96.2 – 97.3
<i>Rhizobium hainanense</i> CCBAU 57015 ^T	95.3
<i>Rhizobium freirei</i> PFR 81 ^T	95.0 – 95.3
G.II.IV (CNPSo 4062 and CNPSo 4063)	
Among the group	98.8
Among the group and related species/strains	
G.II.III	96.2 – 97.3
<i>Rhizobium hainanense</i> CCBAU 57015 ^T	95.9 – 96.5
CNPSo 3968	95.0 – 95.6

generated 48 distinct groups or isolates occupying single positions, with a final level of similarity lower than 20%.

Following, we selected 56 isolates representative of the BOX-PCR profiles chosen to continue with the 16S rRNA phylogenetic analysis. It is worth mentioning that

the 16S rRNA gene consists of about 1,500 bp, and for species definition the sequence of the whole gene is mandatory. However, in our study, the objective was to define the genera of the strains. Therefore, we used sequences of about 900 bp, because previous studies of diversity by

Fig. 7 A Green color of the leaves and internal pink color of the nodules indicating effectiveness of nitrogen fixation in common bean in comparison to (B) yellow leaves and ineffective nodules without the pink color that indicate an active leghemoglobin



our group have shown the viability of obtaining reliable classification at the genus level using shorter fragments, of about 1,000 bp (Costa et al. 2018; Chibeba et al. 2020; Klepa et al. 2021). In our study, one-third was composed by non-rhizobia, belonging to the genera *Achromobacter* (Yabuuchi and Yano 1981; Benata et al. 2008), *Brevibacillus* (Shida et al. 1996; Stajković et al. 2009), *Burkholderia* (Yabuuchi et al. 1992), *Enterobacter* (Hormaeche and Edwards 1960; Kan et al. 2007), *Herbaspirillum* (Baldani et al. 1986) and *Pseudomonas* (Yabuuchi et al. 1992). Noteworthy, some of these genera, such as *Achromobacter*, *Burkholderia*, *Enterobacter* and *Pseudomonas* may harbor severe human or plant pathogens. Following the Koch's postulates, the non-rhizobia strains were not able to form nodules in common bean and this reinforces reports of endophytic bacteria coexisting with rhizobia in nodules, e.g. in *Phaseolus lunatus* (Chibeba et al., 2020), soybean (*Glycine max* (L.) Merr.) (Li et al. 2008; Zhao et al. 2017; Delamuta et al. 2020), common bean (Wang et al. 2016; Yan et al. 2017; Delamuta et al. 2020), and other legumes (Aserse et al. 2013; Tariq et al. 2014). Endophytes may contribute to plant growth by means of a variety of microbial processes (White et al. 2019; Verma et al. 2021). In our study the endophytic bacteria might contribute with nitrogen fixation, as it could be the case of the *Herbaspirillum* sp., but also with other microbial processes, such as the synthesis of phytohormones, as with *Pseudomonas* and

Burkholderia, among others. The results confirm that there are far higher diversity of endophytes within the nodules as also shown in microbiome studies (Rocha et al. 2020), pointing out how far we are from understanding the role of this complex microbial community inhabiting legume nodules. Possible functions for these non-rhizobial nodule endophytes might include plant growth promotion by mechanisms such as synthesis of phytohormones, solubilization of phosphates, and tolerance to abiotic and biotic stresses (Schaedel et al. 2021).

Agrobacterium is another intriguing endophyte inhabiting nodules, reaching 28% of the isolates diversity based on the sequencing analyses. The genus includes several species commonly found in soil, generally pathogenic for several plants species (Singh and Prasad 2015; Barton et al. 2017). However, the genus includes as well non-pathogenic species, such as *Agrobacterium fabacearum*, also isolated from nodules, although, in general, they do not re-infect nodules in tests for nodulating capacity (Delamuta et al. 2020). Possible roles for endophytic *Agrobacterium* might include plant growth promotion, enhancement in nutrient acquisition, among other benefits (Chibeba et al. 2020; Delamuta et al. 2020; Dudeja et al. 2011).

The remaining 22 isolates whose 16S rRNA genes were sequenced were taxonomically positioned as "classical rhizobia", comprising 20 *Rhizobium*, one *Bradyrhizobium*, and one *Mesorhizobium*. The genus *Bradyrhizobium* can

associate symbiotically and endophytically with legumes such as *Glycine max* and *Phaseolus lunatus* (Durán et al. 2014; Chibeba et al. 2020), non-legumes as *Oryza* spp. and *Parasponia* spp. (Tan et al. 2001; Dupin et al. 2020), but is rarely reported as symbiont of common bean (Han et al. 2005; Cao et al., 2014; Wang et al. 2016). However, intriguingly, Da Conceição et al. (2018) reported that co-inoculation of common bean with *Bradyrhizobium* stimulated the symbiotic efficiency of *Rhizobium*. Therefore, a possible role for *Bradyrhizobium* in common bean nodules could rely on the improvement of the symbiotic performance of *Rhizobium*. The genus *Mesorhizobium* nodulates several legumes, such as chickpea (*Cicer arietinum* L.), another important legume for food security (Laranjo et al. 2014; Faridy et al. 2020), and although not usually reported, the genus has been isolated as an effective nitrogen-fixing symbiont of common bean (Helene et al. 2019).

The genus *Rhizobium* has a global distribution, being able to form symbiotic associations with a variety of legumes. They represent the main symbionts of *P. vulgaris*, and Brazilian soils are particularly rich in common bean *Rhizobium* diversity (e.g. Grange and Hungria 2004; Grange et al. 2007; Stocco et al. 2008; Ribeiro et al. 2009, 2012, 2013, 2015; Dall'Agnol et al. 2013, 2014; Gomes et al. 2015). In this study, about half of the *Rhizobium* isolates were positioned in the *R. etli*/*R. leguminosarum*/*R. phaseoli* clade, and the other half in the *R. tropici* clade. It has been suggested that the clade of *R. tropici* originated in the Andean region of South America, while species of the clade *R. etli* are from the Mesoamerican regions and Northern Argentina (Ribeiro et al. 2009, 2013; Gomes et al. 2015; Shamseldin and Velázquez 2020). In Brazil, bacteria belonging to both clades have been isolated from nodules of common bean all over the country, from the Northeast to the South (Grange and Hungria 2004; Grange et al. 2007; Pinto et al. 2007; Stocco et al. 2008).

As the 16S rRNA gene is highly conserved, the phylogenetic definition can be improved with the analysis of housekeeping genes, and the *glnII* (glutamine synthetase II) has proven to considerably help in taxonomic definition of rhizobia (e.g. Roma Neto et al. 2010). In our study, the main taxonomic position of rhizobia and *Agrobacterium* was confirmed and improved with the analysis of the *glnII* gene, except for the isolate CNPSo 3498 of *Agrobacterium* sp., indicating a putative event of horizontal transfer (HGT) of this gene.

Regarding nodulation and nitrogen fixation capacity, only 13 out of 38 “classical rhizobia” and *Agrobacterium* were able to re-infect the host plant. According to the analysis of the 16S rRNA and *glnII* genes, six were positioned in the *R. etli* clade, another six in the *R. tropici* clade, and one was classified as *Agrobacterium* sp. All isolates, except for CNPSo 4005, positioned in the *R. etli* clade, established

effective symbiosis, characterized by nodules with pink or red internal color and plant shoots with leaves of dark green color, indicating adequate biological N supply.

There are reports showing that in Brazilian soils, predominantly acid, strains of the *R. tropici* clade are more competitive, characterized by higher tolerance of abiotic stresses, genetically more stable, and with higher capacity of BNF; consequently, commercial strains recommended for this crop in Brazil carry only species belonging to this clade (Hungria et al. 2000, 2003; Gomes et al. 2015; Mercante et al. 2017). Unfortunately, in our study *R. tropici* represented only a small percentage of the isolates, what can explain in part the low efficiency of BNF in common bean in the field.

Our study confirms that the Brazilian biomes represent a rich repository of microbial species with importance for plant growth promotion and nutrition. It also confirms the promiscuous nature of the symbiosis with common bean, which establishes interactions with a variety of effective and non-effective nitrogen-fixing rhizobia. Intriguingly, apparently the non-effective strains overtake in number the effective ones. Due to the broad range of bacterial species identified, characterized by different mechanisms that could explain plant infection, it is feasible to conclude that the symbiotic promiscuity in common bean is controlled by the host plant and not by the bacteria. Understanding the genetic mechanisms in common bean that allow this promiscuity might represent the most promising way to increase the contribution of BNF in this crop. In addition, it might also pave ways to manipulate other plant species, facilitating plant–microbe interactions.

5 Conclusions

The results obtained in this study highlight outstanding genetic diversity in bacteria isolated from common bean nodules cultivated in soils of 14 municipalities in Mato Grosso do Sul, in the Central-Western region of Brazil. Estimates were that 32% of the isolates were non-rhizobia endophytes, which might play further roles in the symbiosis, such as plant growth promotion, and tolerance to abiotic and biotic stresses. The remaining isolates were classified as *Agrobacterium* and “classical rhizobia”, but only 31% were able to form effective nitrogen-fixing nodules when re-inoculated in common bean. The effective strains were positioned in the *R. etli* and *R. tropici* clades and one as *Agrobacterium*. These results emphasize the high promiscuity of common bean, which allows nodule colonization by a variety of bacterial species, most of them not efficient in nitrogen fixation, limiting the contribution of the BNF to the crop.

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Declarations

Competing or conflicting interests The authors declare that they have no competing or conflicting interests, or ethical conflicts.

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