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POLYPHASIC CHARACTERIZATION OF RHIZOBIAL ISOLATES OBTAINED FROM DIFFERENT COMMON BEAN-PRODUCING REGIONS

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Abstract

In Brazil, the common bean is a crop with significant social and economic importance. The prospecting of N_2 fixing bacteria is crucial since biological nitrogen fixation (BNF) is an eco-friendly technique. This work aimed to obtain and characterize rhizobium isolates based on morpho-physiological, molecular, and symbiotic efficiency parameters, using the strains SEMIA 4077, SEMIA 4080, and SEMIA 4088 as references. The characteristics of the isolates and colonies, their tolerance to salinity and temperature, as well as their utilization of carbon sources, served as the basis for the morpho-physiological characterization. BOX-PCR, REP-PCR, and ERIC-PCR markers were used for genotypic characterization. Assessment of the symbiotic efficiency was carried out in a greenhouse, determining the number of nodules (NN), nodule dry weight (NDW), shoot dry weight (SDW), and total-N (Total-N) accumulation in the shoot. Among the isolates, those exhibiting: neutral culture medium pH, fast growth, colony diameter <2 mm, opaque transparency, homogeneous appearance, and cream color were predominant. Compared to temperature, salinity was the most restrictive factor to the growth of the isolates. Most of the isolates grew on sucrose (88.43%) and mannitol (87.28%). Genotypic analysis revealed that 90% of the isolates clustered in the same group as the reference strain SEMIA 4080. The TaMsG2R1 and BaDeG4R2 isolates showed higher Total-N in the shoot than the reference strains and should be evaluated in future studies under field conditions.

Keywords: Biological Nitrogen Fixation. Characterization. Diversity. *Phaseolus vulgaris*.

1. Introduction

Common bean (*Phaseolus vulgaris* L.) is of tremendous economic and social importance worldwide, particularly in developing countries, owing to its capacity to supply the populace with proteins, carbohydrates, and other essential nutrients. Additionally, it is thought to be a more affordable source of protein than foods of animal origin and can be included in a variety of food formulations (Santiago-Ramos et al. 2018).

With an average export of 25 kg N per ton of grain, whose supply is accomplished by the application of N-fertilizers. The common bean is a crop with a high demand for nitrogen (N), one of the most absorbed nutrients by the crop (Soratto et al. 2013) and the one that most influences crop growth (Nascente et al. 2017). However, like other leguminous plants, the common bean can establish an association with

symbiotic diazotrophic bacteria known as rhizobia, which has the ability to fix atmospheric N2 through biological nitrogen fixation (BNF).

BNF can enhance the economic and environmental aspects related to N supply in common bean production. Several studies show the possibility of using this technology as a substitute for N-fertilizers (Chueire et al. 2013; Souza and Ferreira 2017) since a large proportion of the host plant's nitrogen needs can be achieved by BNF (Moreira et al. 2017).

However, the common bean belongs to the group of legumes that establish symbiosis with a wide range of bacteria species from the group of rhizobia. According to Dall'agnol et al. (2013), the common bean possesses the ability to associate with a variety of diazotrophic bacteria within the genus *Rhizobium*, including species effective in fixing N_2, the Fix^+ species (*R. leguminosarum* sv. phaseoli, *R. phaseoli*, *R. tropici*, *R. etli*, *R. leucaenae*, *R. giardinii* sv. phaseoli, *R. gallicum*, *R. lusitanum*, *R. pisi*, *R. freirei*, *R. mesoamericanum*), as well as less effective N_2-fixing species (Fix^-) such as *R. giardinii* sv. giardinii and *R. miluonense*. In addition to establishing symbiosis with different species of the genus *Rhizobium*, the common bean is also capable of forming nodules with species of other genera, mostly of the genus *Ensifer* (formerly *Sinorhizobium*) and *Pararhizobium* (formerly *Rhizobium*) and by a minority with species of the genus *Bradyrhizobium* (Shamseldin and Velázquez 2020).

This fact denotes the high promiscuity of the common bean with respect to the establishment of the symbiotic association and, therefore, its high capacity to nodulate with the native soil rhizobia population, which commonly presents low efficiency of the BNF (Argaw and Tsigie 2015). Thus, obtaining and selecting rhizobial isolates with high nitrogen-fixing capacity and adapted to different soil and climate regions are crucial to increase the efficiency of the BNF in common bean (Sampaio et al. 2016; Cardoso et al. 2017).

Accordingly, this work aimed to obtain and characterize, based on morpho-physiological, molecular, and symbiotic efficiency parameters, rhizobial isolates from nodules of six common bean genotypes cultivated in soil samples collected in different grain-producing areas of Brazil.

2. Material and Methods

Soil sampling and rhizobial isolation

Soil samples were collected at a depth of 0-20 cm in commercial production areas in the states of Bahia, Goiás, Minas Gerais, Paraná, and São Paulo. Details of the collection sites are outlined in Table 1. The physicochemical characteristics and granulometry of the samples were determined according to Embrapa (1997).

To obtain the rhizobial isolates, six common bean genotypes belonging to 3 different grain color groups, 1- carioca (BRS Aporé and Pérol), 2- black (Ouro Negro and BRS Grafite), and 3- mulatinho (BRS Agreste and Corrente) were used as trap plants. For each genotype, one 5 L pot containing the collected soil and sown with four seeds was used. The plantlets were thinned to two plants per pot five days post-emergence. Before sowing, the seeds were superficially disinfected (70% alcohol, 1 min; 2% NaClO, 30 s) and five times washed with sterile water. At 35 days after emergence (DAE), the nodules were collected, and five nodules from each pot were randomly selected to perform the isolation of the rhizobia.

The nodules were disinfected (70% alcohol, 3 min; 2% NaClO, 1 min), and ten times washed with sterile water. Then, in a laminar flow hood, each nodule was placed in an Eppendorf microtube containing 0.5 mL of sterile water and macerated using a sterile glass rod. The supernatant was inoculated in a Petri dish containing Yeast Mannitol Agar (YMA) with bromothymol blue (Fred and Waksman, 1928), according to the methodology described by Hungria (1994). The Petri dishes were incubated at 28 °C and checked daily for one week to track the appearance of colonies, resulting in a total of 479 rhizobial isolates.

Morphological characterization of rhizobial isolates

The 479 isolates obtained, and the type strains used as reference (SEMIA 4077 and SEMIA 4088 – *Rhizobium tropici*; SEMIA 4080 – *Rhizobium freirei*) were morphologically characterized based on six colony

characteristics: growth rate (fast – 24 h, normal – 48 h or slow – more than 48 h); culture medium pH (acidic, neutral or alkaline); colony appearance (homogeneous or heterogeneous); colony color (white, yellow or orange); colony size (>2 mm or <2 mm) and transparency (opaque or translucent), following the methodology described by Martins et al. (1997).

Table 1. Details of the soil sampling locations for obtention of new rhizobial isolates.

State	City	Farm	Acronym	Geographical coordinates	Altitude
Bahia	Barreiras	Morena	BaMo	11º46'23.21"S, 45º44'03.91"O	787
	Barreiras	Decisão	BaDe	11º47'00.69"S, 45º 47´43.40"O	792
	Luiz Eduardo Magalhães	Nossa Senhora de Fátima	LeNf	12º18´29.86"S, 45º 44´05.01"O	717
	Correntina	Xanxerê	CoXa	13º48`02.79"S, 46º05`42.67"O	940
Goiás	Ipameri	Vale Verde	IpVv	16º59`50.81"S, 47º47`37.17"O	869
	Cristalina	Cristal	CrCr	17º01`28.78"S, 47º16`29.11"O	877
	Silvânia	WB	SiWb	16º28`25.85"S, 48º19`01.55"O	1061
	Silvânia	Gaulanda	SiGa	16º25`11.21"S, 48º22`35.25"O	1033
Minas Gerais	Paracatu	Jucamaria	PaJu	17º18`08.36"S, 47º03` 32.22"O	649
	Paracatu	Poção	PaPo	17º03`34.55"S, 46º48`12.46"O	928
	Bonfinópolis de Minas	União	BoUn	16º25`05.16"S, 46º19`45.34"O	945
	Unaí	Canto	UnCa	16º21`57.03"S, 47º08´28.66"O	987
Paraná	Castro	Marujo	CaMa	24º47`53,70"S, 49º53`42.09"O	1022
	Castro	Piriquito	CaPi	24º48`02.11"S, 49º50`30.72"O	999
	Tibagi	Igreja Velha	TiIv	24º35`39.25"S, 50º21`24.68"O	906
	Tibagi	Fazendinha	TiFq	24º27`07.29"S, 50º25`52.71"O	805
São Paulo	Taquarituba	Miro Sai	TaMs	23º34`02.85"S, 49º 12` 29,25"O	630
	Taquarituba	Dois Irmãos	TaDi	23º38`59,36"S, 49º08`59.36"O	646
	Itaberá	Estância Seleções	ItEs	23º44`07.15"S, 49º08`46,38"O	634
	Itaberá	Campanina	ItCa	23º43`18.99"S, 49º14`32.44"O	666

Assessment of salinity and temperature tolerance

From the grouping of 479 isolates according to their morphological characteristics, 305 isolates most similar to the type strains (SEMIAs) were selected to determine their tolerance to salinity and temperature (TST).

The isolates and reference strains were assessed for TST on YMA culture medium on a 5×5 arrangement (five concentrations of NaCl: 0%, 1%, 2%, 4%, 6%, and five temperatures: 28 °C, 33 °C, 38 °C, 43 °C, 48 °C) incubated for a period of 48 h. After incubation, the presence of bacterial growth was checked.

Assessment of carbon sources use by rhizobial isolates

From the salinity and temperature tolerance assessment of the 305 isolates, 173 were selected for characterization based on the use of 12 different carbon sources: sucrose, glycerol, D-xylose, methyl-β-D-xylopyranoside, dextrose, D- mannose, L-sorbose, inositol, mannitol, D-sorbitol, methyl-α-glucopyranoside, and D-maltose monohydrate. The assessments were according to the method described by Cardoso et al. (2017).

Genotypic characterization of rhizobial isolates

Based on the assessment of salinity and temperature tolerance and carbon source use, 63 isolates were selected for genotypic characterization. Genomic DNA was extracted according to Ausubel et al. (1999). 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 A1R (5-ATGTAAGCTCCTGGGGATTCAC-3); REP-PCR was performed using the primers REP-1 (5-IIIICGICGICATCIGGC-3) and REP-2 (5-ICGICTTATCIGGCCTAC-3), while

ERIC-PCR was performed using the primers ERIC1 (5-CTACGGCAAGGCGACG-3) and ERIC2 (5-AAGTAAGTGACTGGGGTGAGCG-3), according to Versalovic et al. (1994).

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 Mg2+), 1.6 µL of primer BOXA1R (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 Mg2+), 1.25 µL of each primer REP1 and REP2 (10 pmol μL^{-1}) and 3 µL of DNA template (10 ng μL^{-1}). While the ERIC-PCR reaction was performed in a final volume of 10 µL, containing 2.75 µL of milli-Q water, 5 µL of 2× QIAGEN Multiplex PCR Master Mix (3 mM Mg2+), 0.25 µL of each primer ERIC1 and ERIC2 (50 pmol μL^{-1}) and 2 µL of DNA template (50 ng μL^{-1}). The amplification program was designed according to Kaschuk et al. (2006). 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, 44 °C; 1 min for ERIC-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 0.75x 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, 15 isolates were selected for symbiotic efficiency assessment under greenhouse conditions. In addition to the isolates, the treatments were composed of the reference strains (SEMIA 4077, SEMIA 4080, and SEMIA 4088), nitrogen fertilizer treatment (120 kg N ha^{-1}), and one control treatment (without inoculation and without N). A 1:1 mixture of autoclaved (1 h; 1.5 kg cm^{-2}; 127 °C) sand and vermiculite was placed in the upper part of the Leonard jar. Two seeds of common bean cv. Pérola were sown in autoclaved Leonard jars in a randomized block design with four 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 was added. To the nitrogen fertilizer treatment, 2 mL of a solution containing 106.68 mg mL^{-1} of urea was added. Plants were harvested at 35 DAE. Roots were carefully washed and dried in a paper towel, afterwards, the nodules were detached and counted to determine the number of nodules (NN). Shoots and nodules were dried (65 °C; 72 h) to determine the shoot dry mass (SDM) and nodule dry mass (NDM). Subsequently, shoots of plants were milled to determine the total N (N-Total) using the Kjedahl method, as described by Silva and Queiroz (2009).

Statistical analysis

The rhizobial isolates were grouped by their morphology, salinity and temperature tolerance, and carbon source use traits. The grouping analyses were performed separately for traits. Genotypic characterization data (BOX, ERIC, and REP-PCR) were transformed into a consensus binary matrix. The matrix was used for the construction of a similarity matrix using the Jaccard coefficient. The Unweighted pair-group method (UPGMA) was applied to transform the similarity matrix into a similarity dendrogram using NTSYSpc® software (Rohlf 1988). Data obtained from the greenhouse experiment were subjected to analysis of variance; when *F* was significant, the means were compared by Scott-Knott test ($p\leq0.05$) using the software SISVAR (Ferreira 2019).

3. Results

Morphological characterization of rhizobial isolates

In the morphological characterization, ten isolates (CaMaG1R5, CaPiG1R3, CaPiG5R5, PaJuG5R1, PaPoG1R4, SiGaG1R4, SiWbG6R1a, TaMsG4R5, TaMsG6R4, and TiFqG2R1) composed the same group of

SEMIA 4077, corresponding to 2.08% of the evaluated isolates. These isolates showed fast growth, acidic pH, colony larger than 2 mm, heterogeneous appearance, yellow color, and opaque transparency. The presence of four isolates (SiGaG4R1, SiGaG6R5, TiFqG2R5, and TiFqG4R1) in the same group of SEMIA 4080, representing 0.83% of the evaluated ones was also observed. The morphological characteristics observed were normal growth, acidic pH, colony larger than 2 mm, homogeneous appearance, cream color, and translucent transparency. The SEMIA 4088 group was composed of eight isolates (BoUnG6R3, CaPiG2R4, CaPiG4R1, IpVvG6R4, ItCaG2R1, ItCaG2R2, ItCaG3R3, and TiFqG3R3), corresponding to 1.67% of the evaluated isolates, whose morphological characteristics were normal growth, acidic pH, colony larger than 2mm, heterogeneous appearance, yellow color, and opaque transparency.

Salinity and temperature tolerance assessment

As seen in Figure 1, in the absence of salinity (0% NaCl), 100% of the isolates grew at temperatures of 28 °C and 33 °C, about 98% of the isolates grew at 38 °C, while 80% of the isolates grew at 48 °C. On the other hand, when the growth under high salinity (6% NaCl) was evaluated, it was observed that about 40%, 20%, and 5% of the isolates were able to grow at 28 °C, 33 °C, and 38 °C, respectively. Besides, no isolate growth was observed at 43 °C and 48 °C.

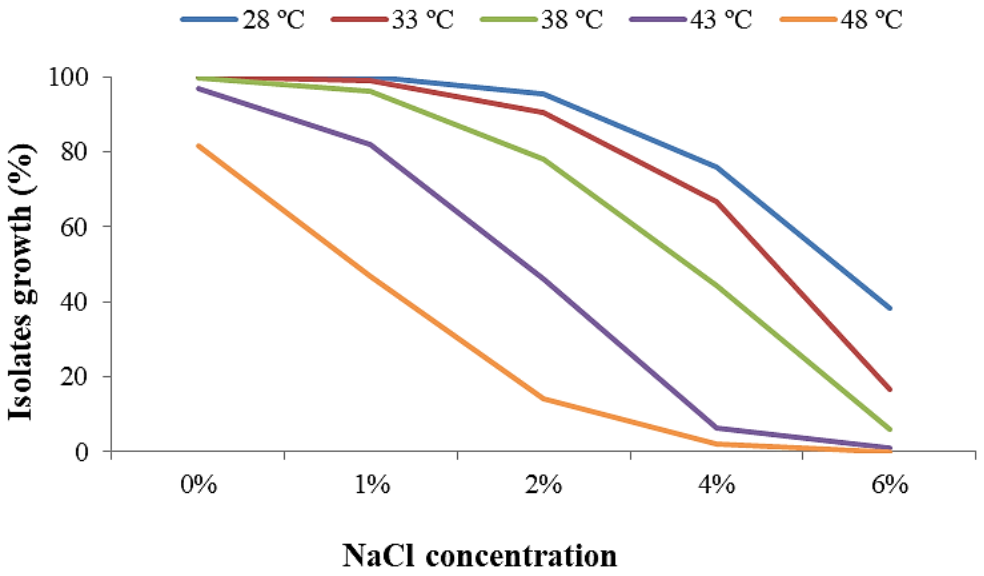

Figure 1. Growth percentage of rhizobial isolates under different salinity and temperature conditions.

The 305 isolates evaluated for salinity and temperature tolerance were distributed into 116 groups. Group 1 was composed of the isolate LeNfG5R1, showing growth from the least restrictive condition (28°C; 0% NaCl) to the most restrictive condition (43°C; 6% NaCl). In addition to the LeNfG5R1 isolate, another 195 isolates, distributed between groups 2 and 72, deserve special attention because they were more tolerant to salinity and temperature than the three standard strains. The isolate LeNfG5R1, from soil collected in the State of Bahia, showed greater resistance to stresses caused by changes in salinity and temperature.

Carbon source use assessment

The three standard strains and 173 rhizobial isolates, selected from the salinity and temperature tolerance assay, were evaluated for their ability to use 12 carbon sources. Based on the behavior of the rhizobial isolates in relation to the use of carbon sources, the reference strains and the isolates were divided into 102 groups. Group 1 was composed of SEMIA 4080 and 31 isolates, corresponding to 17.9% of the evaluated isolates, showing growth in all carbon sources.

Group 9, formed by the standard strains SEMIA 4077, SEMIA 4088, and 6 isolates, did not grow on L-Sorbose (CS7) as a carbon source. Besides, only 47.3% of isolates were able to grow using L-Sorbose (CC7) a carbon source.

On the other hand, sucrose (CS1) and mannitol (CS9) were the carbon sources in which the highest growth percentages were observed, with 88.43% and 87.28% of the total isolates, respectively, having the capacity to utilize them.

Genotypic characterization based on BOX, REP, and ERIC-PCR markers

BOX, ERIC, and REP-PCR markers were applied to determine the genetic diversity of 63 rhizobial isolates, selected from the carbon source use assay, using the three reference strains (SEMIAs 4077, SEMIA 4080, and SEMIA 4088) for the grouping of isolates.

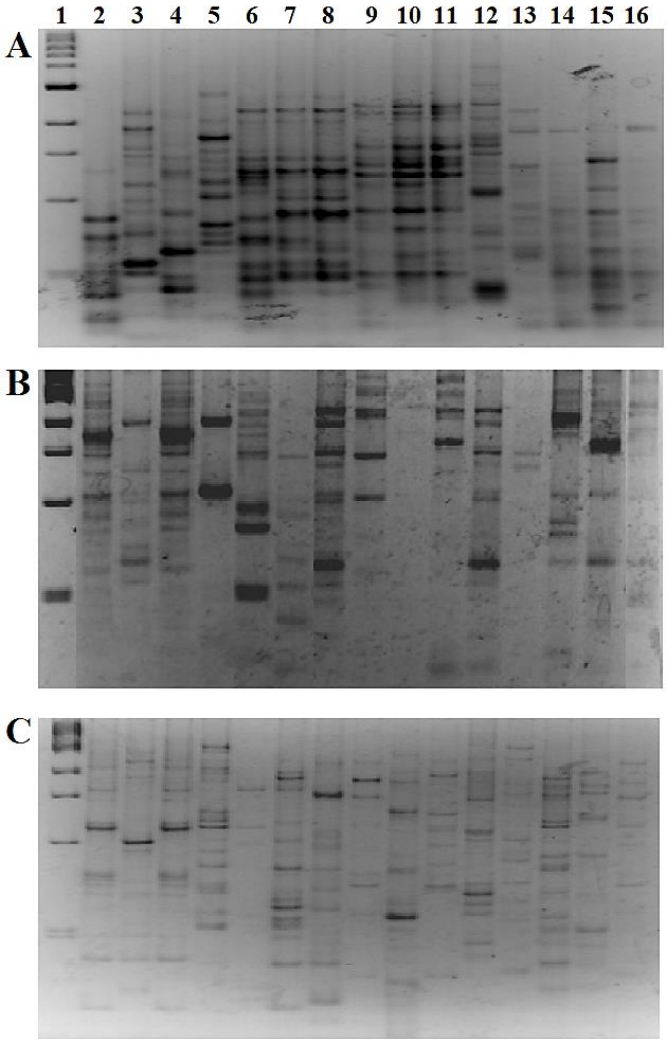

Figure 2. Electrophoretic fingerprinting of rhizobial isolates obtained from common bean nodules by BOX-PCR (A): Lines 1- ladder marker 1 Kb, 2- SEMIA 4077, 3- SEMIA 4080, 4- SEMIA 4088, 5- TaMsG2R1, 6- BaMoG5R3, 7- BaDeG4R2, 8- BaDeG4R3, 9- BaDeG5R1, 10- BaDeG5R5, 11- BaDeG6R3, 12- LeNfG1R4, 13- LeNfG2R1, 14- LeNfG2R3, 15- LeNfG3R3, 16- LeNfG4R1; REP-PCR (B): Lines 1- ladder marker 1 Kb, 2- SEMIA 4077, 3- SEMIA 4080, 4- SEMIA 4088, 5- PaPoG3R5, 6- PaPoG4R4, 7- UnCaG3R3, 8- UnCaG6R4, 9- SiWbG2R1, 10- SiWbG4R2, 11- SiWbG4R4, 12- SiWbG4R5, 13- SiWbG6R4, 14- SiGaG2R3, 15- SiGaG3R2, 16- SiGaG3R5; ERIC-PCR (C): lines 1- ladder marker 1 Kb, 2- SEMIA 4077, 3- SEMIA 4080, 4- SEMIA 4088, 5- TaDiG4R1, 6- ItEsG1R2, 7- ItEsG2R4, 8- ItEsG5R1, 9- ItCaG1R3, 10- ItCaG1R4, 11- ItCaG2R1, 12- ItCaG3R5, 13- CaMaG2R2, 14- CaMaG3R2, 15- CaMaG4R3, 16- CaPiG3R3.

The BOX-PCR fingerprinting revealed the formation of at least 4 and at most 20 bands with different molecular weights (Figure 2A), demonstrating a high polymorphism among the isolates. Similarity was

observed in the banding profile between BaDeG4R2 and BaDeG4R3 isolates; BaDeG5R5 and BaDeG6R3, LeNfG2R3 and LeNfG4R1. Despite the similarity found among some isolates, none of them presented an identical profile to the reference strains (SEMIAs 4077, SEMIA 4080, and SEMIA 4088). Similar results were observed by Cardoso et al. (2012).

With REP-PCR fingerprinting (Figure 2B) at least three and at most 12 bands were observed, demonstrating high polymorphism, represented by the predominance of single banding profiles. The reference strains SEMIA 4077 and SEMIA 4088 showed identical banding profiles. Although none of the isolates showed an identical profile to the reference strains, the SiGaG3R2 and SiGaG3R5 isolates showed a similar profile to each other.

By the ERIC-PCR fingerprinting (Figure 2C) a high polymorphism can be observed, represented by the predominance of single banding profiles. Among them, it was observed at least three and at most 15 bands, with the reference strains SEMIA 4077 and SEMIA 4088 presenting identical profiles.

The banding profiles from the BOX-PCR, REP-PCR, and ERIC-PCR markers were transformed into a consensus binary matrix, which was used to group the isolates using the UPGMA algorithm and the Jaccard coefficient (Figure 3). It was observed that the evaluated isolates, including the reference strains SEMIA 4077, SEMIA 4080 and SEMIA 4088, showed 70% similarity. Considering a similarity of 75%, seven groups were formed.

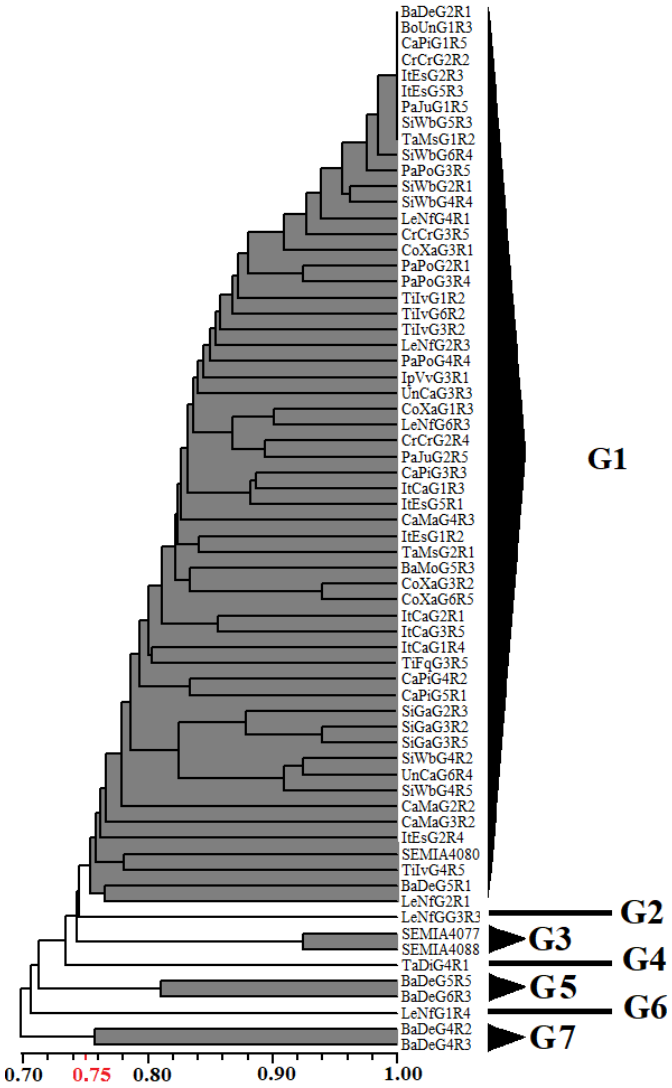

Figure 3. Consensus dendrogram produced by combining the BOX, REP and ERIC-PCR data of 63 isolates obtained from different soil origins and cultivars of common bean. The dendrogram was generated using the UPGMA algorithm, and the similarity matrix was determined using the Jaccard coefficient.

Group 1 (G1), composed of 57 isolates and the reference strain SEMIA 4080, was the largest group formed, comprising 90% of the total isolates evaluated, with 100% similarity between the isolates

BaDeG2R1, BoUnG1R3, CaPiG1R5, CrCrG2R2, ItEsG2R3, ItEsG5R3, PaJUG1R5, SiWbG5R3, and TaMsG1R2. The reference strains SEMIA 4080 and SEMIA 4088 (G3) showed approximately 93% of similarity between them (Figure 3).

Evaluation of symbiotic efficiency in a greenhouse

From the cluster analysis based on molecular markers (Figure 3), 15 isolates were selected, 13 belonging to the G1 group, the group with the highest number of isolates and where the reference strain SEMIA 4080 was found, and the two isolates from the G7 group, which showed the lowest similarity (70%) with the reference strains, to be evaluated for symbiotic efficiency. A significant difference ($p \leq 0.05$) was observed between treatments by the Scott-Knott test for the number of nodules (NN). Among the evaluated isolates, 11 of them presented NN statistically equal to the reference strains, SEMIA 4077, SEMIA 4080, and SEMIA 4088. The other 4 isolates (BaDeG4R3, BaDeG4R2, ItCaG1R4, and CaMaG4R3) presented NN statistically equal to the nitrogen (NfT) and absolute control (AC) treatments (Table 2).

Table 2. Nodule number (NN), nodule dry weight (NDW), shoot dry weight (SDW) and total nitrogen accumulation (Total-N) of the common bean inoculated with different rhizobial isolates.

Treatment	NN	NDW	SDW	Total-N
	unit plant^{-1}	mg plant^{-1}	g plant^{-1}	g Kg^{-1}
SEMIA 4080	190.66 a	34.94 b	0.84 b	22.36 b
SEMIA 4077	170.00 a	89.61 a	1.59 b	20.26 b
SEMIA 4088	119.33 a	51.52 a	0.74 b	19.43 b
PaPoG4R4	283.00 a	90.24 a	1.22 b	22.21 b
PaPoG2R1	187.66 a	70.69 a	1.56 b	24.17 b
TiIvG4R5	162.66 a	53.67 a	0.77 b	21.15 b
PaPoG3R4	162.33 a	67.47 a	1.58 b	26.39 b
TiIvG6R2	140.66 a	93.47 a	1.56 b	21.75 b
TaMsG2R1	121.00 a	51.32 a	0.96 b	34.85 a
SiGaG2R3	120.66 a	55.56 a	1.27 b	24.73 b
CrCrG2R4	117.33 a	83.48 a	1.32 b	27.41 b
ItCaG2R1	109.66 a	84.56 a	1.26 b	23.17 b
TiIvG1R2	103.00 a	101.01 a	1.36 b	24.12 b
PaJuG2R5	99.33 a	56.46 a	0.97 b	28.05 b
BaDeG4R3	68.00 b	55.87 a	0.95 b	22.86 b
BaDeG4R2	66.66 b	80.45 a	1.30 b	34.47 a
ItCaG1R4	64.66 b	57.15 a	1.27 b	20.24 b
CaMaG4R3	51.00 b	56.07 a	1.09 b	22.58 b
NfT	0.00 b	0.00 b	5.02 a	37.14 a
AC	0.00 b	0.00 b	1.44 b	22.57 b

NfT- N-fertilizer treatment, AC- absolute control. Means followed by different letters are statistically different by the Skott-Knott test ($p \leq 0.05$).

The NN ranged from 99.33 nodules plant^{-1} (PaJuG2R5) to 283 nodules plant^{-1} (PaPoG4R4), and the reference strains SEMIA 4077, SEMIA 4080 and SEMIA 4088 presented 170.00, 190.66 and 119.33 nodules plant^{-1}, respectively (Table 2).

For the nodule dry weight (NDW), all the evaluated isolates presented NDW significantly superior to the reference strain SEMIA 4080 and equal to the reference strains SEMIA 4077 and SEMIA 4088 (Table 2). The TiIvG1R2 isolate showed the highest NDW, with 101 mg plant^{-1}, while the UnCaG6R4 isolate presented an NDW of 5 mg plant^{-1}, which was the lowest value found (Table 2). In this work, we found a 20.2-fold variation in NDW among the isolates (5 to 101 mg plant^{-1}).

All isolates presented SDW values statistically equal to the values presented by the reference strains (SEMIAs), differing only with the NfT treatment (Table 2). The reference strains showed SDW values ranging from 0.74 (SEMIA 4088) to 1.59 g plant^{-1} (SEMIA 4077), while the isolates showed values ranging from 0.77 (TiIvG4R5) to 1.58 g plant^{-1} (PaPoG3R4).

By analyzing the Total-N, it was observed that the isolates TaMsG2R1 and BaDeG4R2 presented values higher than the reference strains, being statistically similar to the NfT treatment (Table 2).

4. Discussion

The morphological characterization of 479 isolates resulted in the formation of 62 morphological groups, representing a high diversity among the evaluated isolates. These results corroborate those of Sampaio et al. (2016), who used Cerrado soils and wild genotypes of the common bean as trap plants. Although there is a difference regarding the used trap plants, both studies indicate a high morphological diversity among rhizobial isolates obtained from Brazilian soils.

High salinity (6% NaCl) and temperature (43 °C and 48 °C) inhibited the bacterial growth. Sharma et al. (2013) also highlighted the effect of salinity on rhizobial growth, however, they also found isolates capable of growing in a culture medium with high salinity. However, the isolate LeNfG5R1, from soil collected in the State of Bahia, showed greater resistance to stresses caused by changes in salinity and temperature. This result is similar to that observed by Sharma et al. (2013), which showed that the rhizobia isolated from the desert soils are able to survive, grow and effectively nodulate their leguminous hosts even at high salt concentrations.

By the carbon source analysis the isolates were grouped into 102 groups. Among the isolates from G1, those from the state of Minas Gerais showed greater metabolic diversity, being able to use a greater number of carbon sources. This result corroborates the studies by Fernandes Júnior et al. (2012), Patel and Dubey (2014). The data obtained by these authors indicate that rhizobia have the ability to use several sources of carbon, including those used in this study.

Literature data suggest that carbon sources, such as xylose (CS3), can influence the composition and production of exopolysaccharides, commonly called mucus (Razika et al. 2012). This mucus is extremely important for soil bacteria because it protects against abiotic stresses such as pH, temperature, UV radiation, and salinity, among other factors. In this regard, the findings of this kind of research are important not only for understanding the symbiotic relationship but also for evaluating the biotechnological potential of these bacteria.

Sucrose (CS1) and mannitol (CS9) provided the highest growth percentages among the isolates. These results are in concurrence with those obtained by Sampaio et al. (2016), who found that 89% of the 76 isolates studied were able to utilize sucrose and mannitol as carbon sources.

YMA culture medium, which has mannitol in its composition, is the standard medium for the isolation of bacteria from legume nodules. However, studies of the metabolic behavior of bacteria regarding the use of carbon sources are important in order to allow the adoption of other carbohydrates for the preparation of the medium, replacing mannitol, favoring in a more specific way the conditions for microbial development (Fernandes Júnior et al. 2012).

The evaluated isolates are too close on the genotypic point of view, since only seven groups were formed at a similarity level of 75%. In a study carried out by Youseif et al. (2014) using these same molecular markers with 11 rhizobial isolates, these isolates grouped into three clusters at about 35% level of similarity.

Under greenhouse condition, the NN ranged from 99.33 to 283 nodules plant^{-1}, while for NDW was found a 20.2-fold variation among the isolates (5 to 101 mg plant^{-1}). A Similar variation in NDW (18.3-fold variation) was found by Cardoso et al. (2017), with values ranging from 21 to 384 mg plant^{-1}. Cardoso et al. (2012) found a much smaller variation between isolates (3.9-fold variation), with values ranging from 146 to 564 mg plant^{-1}.

Among the variables used in the evaluation of nodulation (NN and NDW), NDW was more useful for the selection of rhizobium isolates, due to the better correlation of NDW with symbiotic performance. Correlation analysis between NN and NDW with growth parameters, such as root length, root dry weight, plant height and shoot dry weight showed better adjustment for NDW over NN (Koskey et al. 2017; Samudin and Kuswantoro 2018). According to Yang et al. (2017), correlations were detected between fixed N_2 and NN. However, NN is not always positively correlated with fixed N_2 in plants, as they only found a significant correlation between fixed N_2 and NN after inoculation with certain rhizobium strains.

The SDW of the plants inoculated with the reference strains and with the isolates were so similar, indicating the effectiveness of the isolates in fixing N, as pointed out by Cardoso et al. (2017), resulting in high Total-N amounts accumulated in the aerial part of the plants.

The high value of Total-N in plant shoots inoculated with the isolate BaDeG4R2 may be associated with the large amount of NDW (80 mg plant^{-1}) as reported in the literature. The inoculation of common bean with the strains CIAT 899 promoted either a higher mass of viable nodules or higher nitrogen accumulation in the aerial part (Milcheski et al. 2022).

Although the TaMsG2R1 isolate showed low values for the other three parameters (NN, NDW and SDW), a high content of Total-N was observed, making it an interesting isolate for field studies and prospecting for use as an inoculant. Thus, both isolates TaMsG2R1 and BaDeG4R2 must be subjected to further field experiments for testing their agronomical performance in common bean.

5. Conclusions

Most of the isolates show neutral culture medium pH, fast growth, colony diameter <2 mm, opaque transparency, homogeneous appearance, and cream color.

Compared with temperature, salinity is the most restrictive factor to the growth of rhizobial isolates, and most of the evaluated isolates showed greater tolerance to salinity and temperature than the reference strains.

The isolates evaluated have a high capacity to use various carbon sources, and the highest percentages of growth of the isolates were observed in sucrose (88.43%) and mannitol (87.28%).

About 90% of the isolates cluster in the same group as the reference strain SEMIA 4080.

The TaMsG2R1 and BaDeG4R2 isolates presented higher Total-N accumulation than the reference strains and should be evaluated in further studies under field conditions.

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