

MORPHOPHYSIOLOGICAL CHARACTERIZATION OF RHIZOBIA ISOLATED FROM WILD GENOTYPES OF THE COMMON BEAN

CARACTERIZAÇÃO MORFOFISIOLÓGICA DE RIZÓBIOS ISOLADOS DE GENÓTIPOS SILVESTRES DE FEIJOEIRO-COMUM

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ABSTRACT: This study aimed to characterize, based on the morphology and on the use of carbon sources, rhizobial isolates obtained from nodules of wild common bean genotypes cultivated on soil samples from the states of Goiás, Minas Gerais and Paraná. Based on morphological and carbon source use, similarity matrixes were generated to perform clustering analysis based on the Jaccard index. A total of 523 isolates were obtained with the predominance of isolates that acidify the culture medium and showed fast growth. Isolates from the soils of Araucária and Prudentópolis produced a greater amount of mucus than those from Jussara, Nova Veneza, Uberlândia and Unai soils. The rhizobia isolates obtained from soils of Goiás and Minas Gerais showed greater phenotypic diversity than those from the soils of Paraná. Analysis based on the use of different carbon sources revealed that rhizobia isolates obtained from soils of Goiás have higher metabolic diversity. Overall, the most part of isolates grouped with the reference strains, especially with the *Rhizobium tropici* and *R. freirei* strains used as inoculants for the common bean.

KEYWORDS: *Phaseolus vulgaris*. BNF. Morphological characterization. Carbon sources use.

INTRODUCTION

The common bean (*Phaseolus vulgaris* L.) shows great social and economic importance worldwide (BUILES et al., 2011), in addition to being the main source of protein for the poor population. Brazil is the third common bean producer of the world (FAO, 2015), with a harvested area of 2.94 million hectares and grain yield of 1070 kg ha⁻¹ (IBGE, 2015). This crop is able, when associated with soil bacterial known as rhizobia, to perform the biological nitrogen fixation (BNF), which takes place on specialized structures named nodules. The atmospheric nitrogen (N₂) is reduced to ammonium (NH₃), being catalyzed by the enzyme complex nitrogenase (HUNGRIA et al., 1997).

The obtaining and selection processes of efficient and well adapted rhizobia for the local conditions are essential to improve the efficiency of BNF on the common bean. According to Vargas and Hungria (1997), studies about the host-specificity of rhizobia strains obtained from the common bean, carried out under Cerrado conditions, provided good information about the amplitude of adaptation of local native rhizobia to a wide range of hosts. This same approach can be applied to other producing regions of the common bean.

On the other hand, few of the plant diversity available on the germplasm bank are exploited for the bacterial trapping. In this way, the use of the common bean wild genotypes as trapping plant can be an important factor to obtain high bacterial diversity, since different cultivars of a same plant species can promote a direct effect on microbial diversity of the rhizosphere (FERREIRA et al., 2008).

The selection process of N-fixing bacteria is compounded by many steps leading to the reduction of bacterial isolates to be tested under field conditions. Among these steps, the selection based on morphophysiological parameters allows us to select isolates very close to those used as commercial inoculants. Thus, this work aimed to obtain and to characterize based on morphophysiological parameters, rhizobia isolates from soils of Goiás, Minas Gerais and Paraná states cultivated with wild genotypes of the common bean.

MATERIAL AND METHODS

Origin of soil samples

Soil samples were collected at 0-0.20 m widths on commercial production areas in the municipalities of Jussara (15°51'54"S, 50°52'05"W) and Nova Veneza (16°22'15"S, 49°19'23"W) in the state of Goiás; in Unai

(16°21'27"S, 46°54'22"W) and Uberlândia (18°55'08"S, 48°16'37"W) in the state of Minas Gerais and; in Araucária (25°32'22"S, 49°30'44,9"W) and Prudentópolis (25°11'11,9"S,

51°00'23"W) in the state of Paraná. Soil chemical characteristics were determined according to Embrapa (1997) and are shown in Table 1.

Table 1. Chemical characteristics of the soils used to obtain rhizobia isolates.

Site	pH	O.M.	P	K	Ca	Mg	Al	(H+Al)	Cu	Zn	Fe	Mn	Clay	Silt	Sand
	In H ₂ O	g kg ⁻¹	mg dm ⁻³				mmol _c dm ⁻³			mg dm ⁻³				g kg ⁻¹	
Jussara	6.4	23.3	50	119	9	3	0	5	1.7	0.6	24	27	140	2	858
Nova Veneza	4.8	11.1	2.2	69	23	9	2	50	5.8	8.5	23	31	620	62	318
Unai	6.4	31.1	25.3	86	27	20	0	15	0.8	41.5	25	28	320	122	558
Uberlândia	6.7	10.6	30	56	17	11	0	5	2	21.3	26	14	220	62	718
Araucária	6.4	25.5	72	237	32	18	0	15	1	3.5	31	34	460	82	458
Prudentópolis	5.2	63.2	18.2	367	40	20	2	112	1.3	5.1	32	39	460	222	318

O.M.= organic matter

Rhizobia isolation

To obtain the rhizobia isolates, 11 wild genotypes of the common bean showing different morphological characteristics and origin, were chosen (Table 2).

The seeds of each wild genotype of the common bean were placed on moistened sterile germ test paper and incubated (25 °C, 24-36 h) for pre-germination. Two seedlings of each wild genotype were transplanted into 3 L pots containing soil from each state, and for each one, two pots were conducted under greenhouse condition. 30 days after the transplantation, the roots of the plants were carefully removed from the pots, washed and the nodules were removed and kept for a maximum period of one week, in sealed flasks containing silica gel.

The isolation of bacteria was performed using 10 nodules of the each common bean wild genotype. The nodules were randomly selected and rehydrated with autoclaved water. Then the nodules were surface disinfected (70% ethanol, 30 sec; sodium hypochlorite 5%, 3 min; 10 successive washes in autoclaved water). Then the nodules were macerated and scattered in Petri dishes containing Yeast Mannitol Agar (YMA) culture medium (Fred & Waksman 1928), according to Hungria (1994). Petri dishes were kept at 28 °C until the appearance of colonies. Simple colonies were transferred to other Petri dishes containing the same culture

medium and incubated again, to obtain pure cultures.

Morphological characterization of rhizobia isolates

Morphological characterization of isolates and the standard strains of *Rhizobium tropici* (SEMIA 4077 and SEMIA 4080), *R. freirei* (SEMIA 4088), *R. leguminosarum* bv. *phaseoli* (BR266, BR351 and BR281) and *R. multihospitium* (R82) was carried out in Petri dishes containing YMA medium with bromothymol blue as indicator (Fred & Waksman 1928). The characterization was performed taking into account the pH of the culture medium (acidic and neutral), colony size (1-2 mm > 2 mm), growth speed (fast= <2 days and slow= > two days) colony color (orange, yellow, white and cream), mucus quantity (few, regular and high) and colony appearance (heterogeneous and homogeneous).





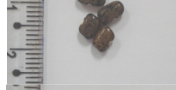
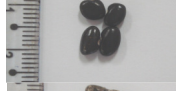





Carbon source use by rhizobia isolates

The characterization based on the use of different carbon sources by isolates and the standard strains of *Rhizobium tropici* (SEMIA 4077 and SEMIA 4088), *R. freirei* (SEMIA 4080), *R. leguminosarum* bv. *phaseoli* (BR266, BR351 and BR281) and *R. multihospitium* (R82) was carried out with 10 carbon sources: sucrose, glucose, malic acid, maleic acid, nicotinic acid, inositol, sorbitol, arabinose, fructose and glycerol. The isolates and

standard strains were incubated under stirring (150 rpm, 28 °C, 48 h) in penicillin tubes containing 5 mL of liquid YMA medium. After each isolate has grown, each one was transferred to a Petri dish

containing YMA medium prepared with the different carbon sources. Then they were incubated at 28 °C. After 48 h and, followed by a period of 96 h of growth, bacterial growth was checked out.

Table 2. Morphological characteristics and origin place of wild genotypes of the common bean used as trapping plants.

Genotype	Seed Morphology	¹ 100 seeds weight (g)	¹ Genotype origin	
			(District/Country)	(Latitude; Longitude)
G23499A		25.2	Mara/Peru	13°52'S; 72°04'W
G23490		40.0	Curpahuasi/Peru	13°28'S; 72°28'W
G23500A		24.0	Mara/Peru	13°52'S; 72°04'W
G12912		14.0	Cuerámaro/Mexico	20°36'N; 101°43'W
G23475		3.0	n.i/Mexico	n.i
G12858		8.0	San Ramón/Peru	11°13'S; 75°28'W
G23460		24.8	Mara/Peru	13°52'S; 72°04'W
G12904		16.0	Cuerámaro/Mexico	20°36'N; 101°43'W
PHA VUL 8169B		n.i	n.i	n.i
PHA VUL 8141		n.i	n.i	n.i
PHA VUL 8122		n.i	n.i	n.i

¹Source: CIAT (2015). n.i: no information.

Statistical analysis

Morphological and carbon source use data were, separately, converted into binary matrixes using 0 (zero) for the absence and one (1) for the presence of the characteristic. The matrixes were analyzed using NTSYS-pc software, version 1.8, to generate similarity dendrograms using Jaccard as the similarity coefficient and UPGMA

(Unweighted Pair Group Method with Arithmetic Mean) as the clustering method.

RESULTS AND DISCUSSION

Obtaining rhizobia isolates

A total of 523 rhizobia isolates were obtained of which 145, 208 and 170 were from

Goiás, Minas Gerais and Paraná states, respectively. About 40% of the isolates was obtained from Minas Gerais soils, while the states of Parana and Goiás contributed with 32 and 28% of the isolates, respectively. Among the wild genotypes of the common bean, the largest numbers of isolates (63, 63 and 57) were obtained using the genotypes G23500A, G23490 and G23460, respectively, as trapping plants while the lowest number of isolates (42, 36 and 26) were obtained using the genotypes PHA VUL 8169B, 8141 PHA VUL and VUL PHA 8122, respectively as trapping plants.

Morphological characterization of rhizobia isolates

Considering the morphological parameter, most of the isolates were able to acidify the culture medium and showed colony size >2 mm, fast growth, yellow color, regular mucus production and heterogeneous appearance (Table 2). All those characteristics are common among bacteria belonging to the genus *Rhizobium* (PINTO et al., 2007; MARQUINA et al. 2011; DALL'AGNOL et al. 2013).

Table 2. Number of rhizobia isolates obtained showing each morphological characteristic.

Parameter	Morphological characteristic	Number of isolates
pH	acid	415
	neutral	108
Colony size (mm)	>2	373
	1-2	150
Growth velocity	fast	491
	slow	32
Colony color	orange	20
	yellow	289
	white	7
	cream	207
Mucus quantity	high	157
	regular	234
	few	132
Colony appearance	heterogeneous	344
	homogeneous	179

Among the isolates obtained from soil samples from Goiás, most of those acidified the culture medium, formed colonies >2 mm, showed fast growth, yellow coloring, few quantity of mucus and heterogeneous appearance (Figure 1A).

Regarding the isolates obtained from soil samples from Minas Gerais, most acidified the culture medium, formed colonies >2 mm, showed fast growth, yellow color, regular amount of mucus and heterogeneous appearance (Figure 1B).

For Paraná soils most of the isolates showed acid pH, colonies with size >2 mm, fast growth, yellow color, produced high amount of mucus and heterogeneous appearance (Figure 1C).

The change in pH of YMA culture medium is a characteristic commonly used for the differentiation of genera of N-fixing bacteria. Bacteria belonging to the genus *Rhizobium* have the property to acidify the culture medium, while *Azorhizobium* and *Bradyrhizobium* alkalize it (COUTINHO et al. 1999). This indicates that most of isolates obtained in this study, regardless of soil origin, have characteristics similar to those of *Rhizobium* genus, due mainly to the acidity of the culture medium and displaying fast growth, as well as other features like colonies with a diameter >2 mm and high mucus production (PINTO et al., 2007; MARQUINA et al. 2011; DALL'AGNOL et al. 2013).

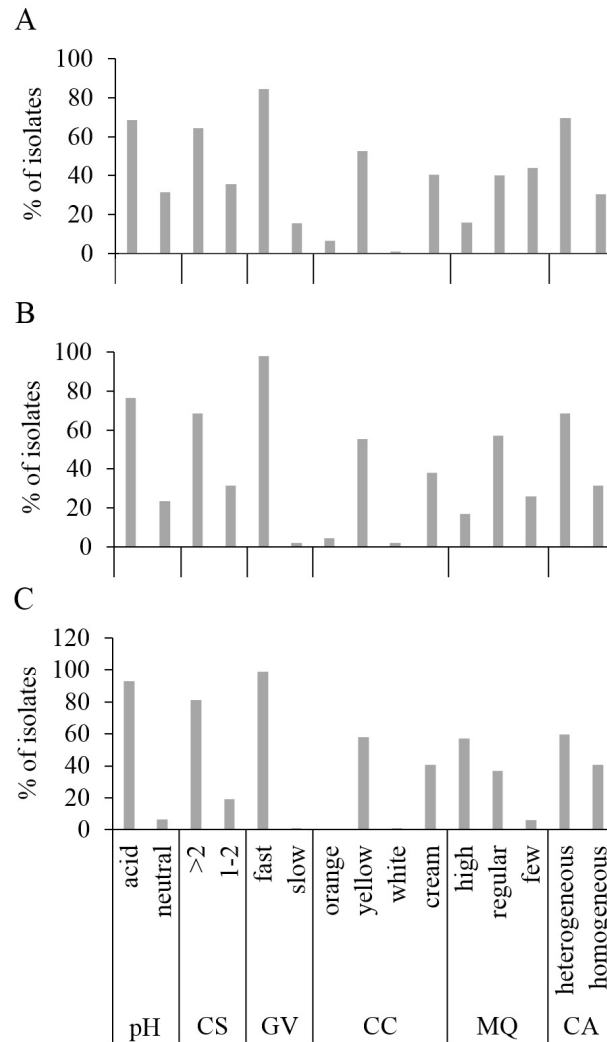


Figure 1. Percentage distribution of rhizobia isolates obtained from nodules of wild genotypes of the common bean cultivated in soil samples from Goiás (A), Minas Gerais (B) and Paraná (C) states. pH of culture medium (pH), colony size (CS), growth velocity (GV), colony color (CC), mucus quantity (MQ) and colony appearance (CA).

Nevertheless, high morphological diversity was observed among the obtained isolates, whereas the isolates showed unusual characteristics for the genus *Rhizobium*, such as neutral pH, slow growth and small production of mucus (Figure 1). High morphological diversity among the common bean rhizobia isolates has been reported in Brazil (HUNGRIA et al. 2000; MOSTASSO et al. 2002; OLIVEIRA et al. 2011), reinforcing reports about the promiscuity of the common bean to establish association with the rhizobia soil community (OLIVEIRA et al. 2011).

Use of carbon sources by rhizobia isolates

Based on morphological characteristics; 76, 99 and 55 isolates were selected from the soils of Goiás, Minas Gerais and Paraná, respectively, for evaluation regarding the use of carbon sources.

The cluster analysis based on carbon source use by the isolates obtained from soil samples from the municipalities in the state of Goiás and the standard strains, showed the formation of 10 different groups at 55% of similarity. The most numerous group (G1) was formed by 88% of the isolates and by the standard strains, except the standard strain BR281 of *R. leguminosarum* bv. *phaseoli* (Figure 2).

The isolates JPrG7A5, JPrG8A3 and JPrG8A6 showed a 100% similarity with the standard strains BR266 and BR351 of *R. leguminosarum* bv. *phaseoli* and with the standard strain R82 of *R. multihospitium*. On the other hand, isolates JPrG10A1, JPrG3A4, JPrG7A2, NVSG2A4, NVSG6A2, NVSG2A1 and NVSG7A9 showed a 100% similarity with the standard strains of *R. tropici* (SEMIA 4077) and *R. freirei* (SEMIA 4080).

The isolates JPrG10A8, JPrG1A1, JPrG5A7, JPrG6A8, NVSG11A3, NVSG1A1, NVSG2A6, NVSG3A4, NVSG4A5 and NVSG8A9 showed a 100% similarity with the standard strain SEMIA

4077 of *R. tropici*. Also, the isolate JPrG3A3 showed a 75% similarity with the standard strain BR281 of *R. leguminosarum* bv. *phaseoli* (Figure 2).

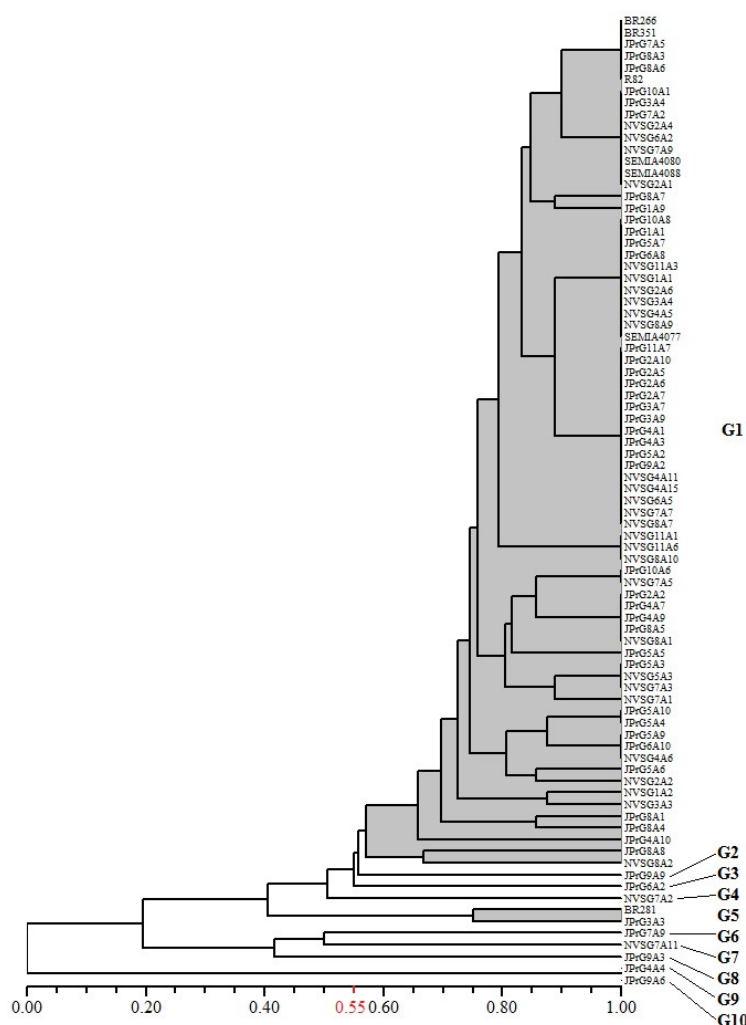


Figure 2. Similarity dendrogram constructed using Jaccard as similarity coefficient and UPGMA as grouping method, with basis on the use of different carbon sources by 76 rhizobia isolates obtained from soil samples of Goiás state.

Groups G2, G3 and G4 were formed by isolates with a 40 to 50% similarity with the standard strains, whereas G6 to G10 were formed by isolates with less than a 20% similarity with these standard strains (Figure 2).

For the isolates from Minas Gerais soils, cluster analysis revealed the formation of 7 groups with a similarity of up to 55% (Figure 3). The G5 group was formed with about 92% of the tested isolates and the standard strains, except for the BR281 strain of *R. leguminosarum* bv. *phaseoli*. In this group, 34 isolates had a 100% similarity with the standard strain SEMIA 4077 of *R. tropici* and 12 isolates were 100% similar to the standard strains SEMIA 4080 of *R. freirei* and SEMIA 4088 of *R. tropici* (Figure 3).

Also in the G5 group, the isolates UbAIG3A3, UbAIG6A3, UnPaG3A9 and UnPaG6A2 showed a 100% similarity with the standard strains BR266 and BR351 of *R. leguminosarum* bv. *phaseoli* and with the standard strain R82 of *R. multihospitium* (Figure 3).

The standard strain BR281 of *R. leguminosarum* bv. *phaseoli* grouped between G1 and G3 and G4, showing about 32% of similarity with the isolates of these groups. The isolates UnPaG6A9 and UnPaG4A6 showed the lowest similarity in relation to the standard strains, forming the groups G6 and G7 (Figure 3).

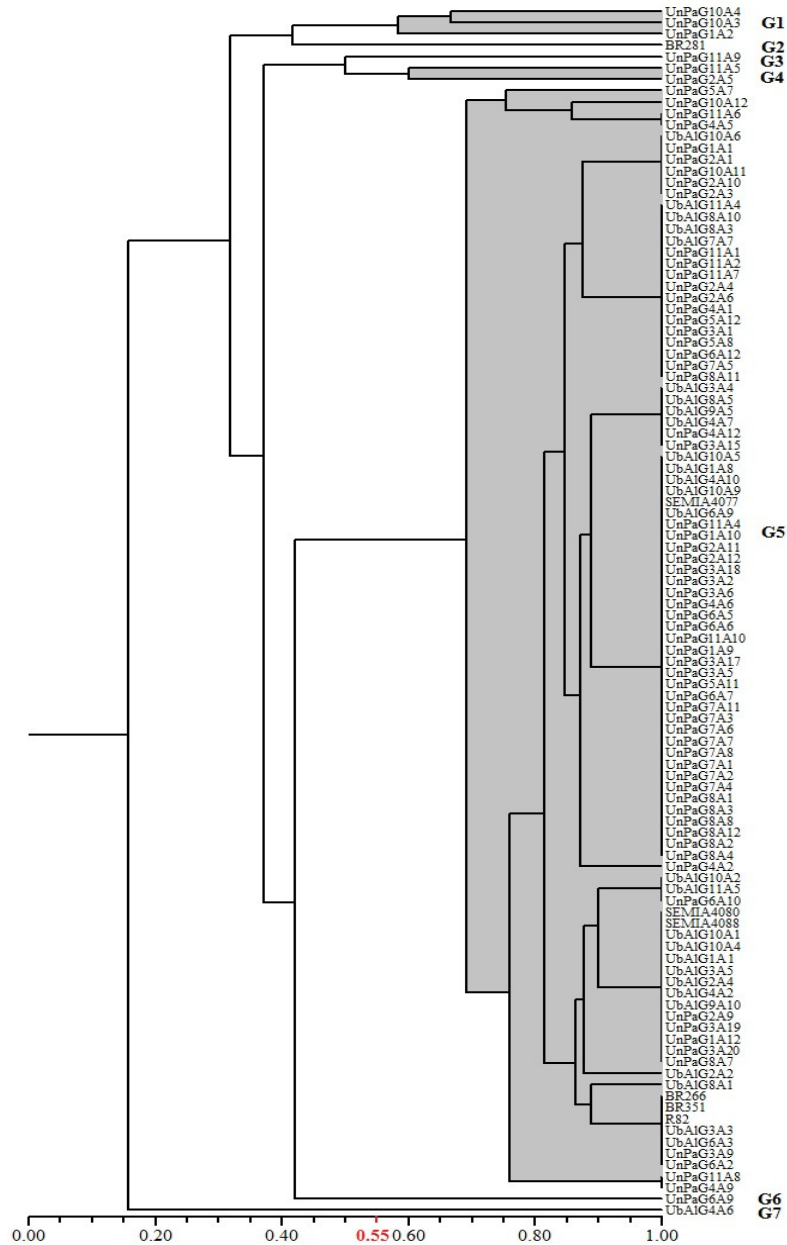


Figure 3. Similarity dendrogram constructed using Jaccard as similarity coefficient and UPGMA as grouping method, with basis on the use of different carbon sources by 99 rhizobia isolates obtained from soil samples of Minas Gerais state.

For isolates from Paraná soils, cluster analysis revealed the distribution of bacteria in five different groups, formed with about a 55% similarity (Figure 4).

The groups G1, G2 and G3 were formed by the isolates ALSG1A7, PCG8A2 and PCG10A6, the latter showed a 100% similarity with the standard strain BR281 of *R. leguminosarum* bv. *phaseoli*. The group G4 was formed by the other standard strains and 91% of tested isolates. Within the group G4, 27 isolates were 100% similar to the standard strains SEMIA 4080 of *R. freirei* and SEMIA 4088 of *R. tropici*. Also, 13 other isolates showed a 100% similarity with the standard strain SEMIA 4077 of

R. tropici. In addition, 7 isolates showed a 100% similarity with the standard strains BR266 and BR351 of *R. leguminosarum* bv. *phaseoli* and with the standard strain R82 of *R. multihospitium* (Figure 4).

According to Castellane and Lemos (2007), strains of *Rhizobium* genus are fast growing bacteria, and use a variety of carbohydrates as carbon source, such as pentoses, hexoses, polyphenols, organic acids, disaccharides and polysaccharides. The strains of the species *R. tropici* and *R. freirei* have a great ability to use carbon sources, the latter of which has relatively lower capacity (DALL'AGNOL et al. 2013). The results

presented in our study corroborate these authors and indicate that many of the tested isolates have characteristics very similar to those showed by the

standard strains of *R. tropici* (SEMIA 4077 and SEMIA 4088) and *R. freirei* (SEMIA 4080) used in Brazil as commercial inoculant for common bean.

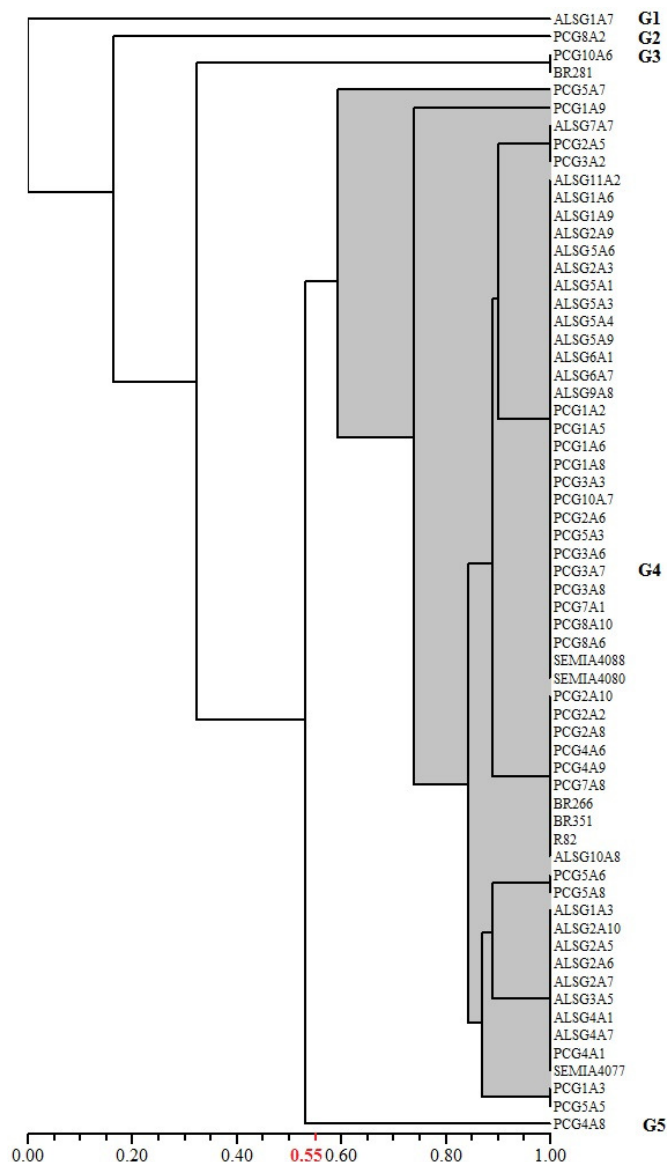


Figure 4. Similarity dendrogram constructed using Jaccard as similarity coefficient and UPGMA as grouping method, with basis on the use of different carbon sources by 55 rhizobia isolates obtained from soil samples of Paraná state.

In general, the largest number of isolates showed morphological characteristics similar to those of the standard strains of *R. tropici* (SEMIA 4077 and SEMIA 4088) and *R. freirei* (SEMIA 4080). Also, by the use of carbon source, most of the isolates grouped with the standard strains used as inoculant of the common bean (SEMIA 4080, SEMIA 4088 and SEMIA 4077), indicating that these isolates have metabolic characteristics similar to these strains, revealing great potential for use as inoculant for common bean. However, further studies must be carried out under field conditions to

confirm the potential of these isolates to promote gain in growth and grain production of the common bean.

CONCLUSIONS

About 75% of the isolates show typical morphologic characteristics of *Rhizobium* strains;

Most of the isolates group with the standard strains of *Rhizobium tropici* and *R. freirei*, used in Brazil as commercial inoculant for the common bean;

The isolates from Goiás and Minas Gerais soils show higher metabolic diversity compared to those from Paraná soils.

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RESUMO: O objetivo deste trabalho foi caracterizar, com base em características morfológicas e quanto ao uso de fontes de C, isolados de rizóbio obtidos de genótipos silvestres de feijoeiro-comum cultivados em amostras de solo dos Estados de Goiás, Minas Gerais e Paraná. A partir das informações foram geradas matrizes de similaridade, usadas para análise de agrupamento por dendrogramas de similaridade, com base no coeficiente de Jaccard. Foram obtidos 523 isolados com predominância de isolados que acidificaram o meio de cultivo e de crescimento rápido, sendo que os provenientes de solos de Araucária e Prudentópolis produziram maior quantidade de muco que os de Jussara, Nova Veneza, Uberlândia e Unaí. Os isolados de rizóbios obtidos de solos do estado de Goiás e Minas Gerais apresentaram maior diversidade fenotípica do que aqueles oriundos de solos do Paraná. A análise de uso de fontes de C revelou que os isolados de rizóbios obtidos de solos do estado de Goiás apresentaram maior diversidade metabólica. De forma geral, a maior parte dos isolados agrupou com as estirpes-padrão, especialmente com as estirpes de *Rhizobium tropici* e *R. freirei* usadas como inoculante do feijoeiro-comum.

PALAVRAS-CHAVE: *Phaseolus vulgaris*. FBN. Caracterização morfológica. Uso de fontes de C.

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